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Research Article

Trends of Attacks on Humans by Protected Crocodiles Along Rivers and Associated Habitats in Borneo Island

Nurul Athirah Ruslan[1], John Madin[1,2]*, Slyvester Saimin[3]

[1] Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia.
[2] Small Island Research Centre, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia.
[3] Sabah Wildlife Department, Wisma Muis, 5th Floor, Block B, Kota Kinabalu 88100, Sabah, Malaysia.

*Corresponding author: jonmadin@ums.edu.my

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ABSTRACT

In the human-crocodile conflict, crocodile attacks on humans pose a serious threat and are becoming a complex conservation challenge in many countries. This study investigates the status and trend of crocodile attacks on humans in Malaysian Borneo (i.e., Sabah and Sarawak) where rivers and estuaries are inhabited by protected crocodile populations. Results show that between 2001 and 2020, a total of 205 attacks were reported which equals an average of nearly 11 cases per year. The number of attacks in Sarawak is estimated to be between 135 to 164 cases which is twice ($p < 0.05$) as high as in Sabah (70 cases), indicating a more serious human-crocodile conflict in the former. In Sabah, most of the attacks (81% or 57 cases) ($p < 0.05$) were in the districts of Lahad Datu, Kinabatangan, Tawau and Sandakan while in Sarawak (82% or 60 cases) ($p < 0.05$) were in Miri, Sri Aman, Kuching and Betong. Almost 80% (173 cases) of the victims were men between the ages of 30 and 39 who were fishing (42%) ($p < 0.05$) or bathing (27%) before the attack suggesting that such activities are risky and should be avoided in areas inhabited by crocodiles. Fatality rates of crocodile attacks have been increasing in recent years (i.e., 2015 – 2020) with an average of 10 cases per year. Therefore, control over the number of crocodile populations, especially the large-sized and dangerous individuals, needs to be implemented in high-risk areas. More research is needed to understand the capacity of their habitats to support optimal populations density and minimize conflicts with humans.

Keywords: Crocodile attacks; Protected crocodile populations; Rivers of Borneo Island

Introduction

The human-crocodile conflict is critical, especially in countries with naturally high crocodile populations (Amarasinghe et al., 2015; Patro & Padhi, 2019). In the past, crocodiles were hunted for their valuable hides, mainly in the production of leather goods that generate a lucrative income in addition to meat and eggs as by-products (Stubbs, 1998; Thorbjarnarson, 1999; Tisdell & Nantha, 2007). The increased demand for crocodile products prompted intensive hunting, which resulted in the population declining drastically in most countries, especially in the late 1980s (Webb et al., 1984; Saalfeld et al., 2015; Patro & Padhi, 2019). This is further exacerbated by the loss of their natural habitat due to competition with the ever-growing human population (Amarasinghe et al., 2015; Saalfeld et al., 2015; Grigg & Kirshner, 2015; Walker, 2016). In Sulawesi, for example, saltwater crocodiles were once widely distributed abundantly throughout the province. However, they were significantly depleted due to hunting and loss of habitat (Platt et al., 2007; Sideleau, 2016a). As a result of uncontrolled human activity, coupled with low survival rates mainly due to aggressive predation among juveniles, crocodiles are at risk of species extinction (Lang, 1987; Grigg & Kirshner, 2015; Walker, 2016). Concerns over the extinction of the species led to the idea of conserving crocodiles and their natural habitats around the world (Read et al., 2004; Mazzotti et al., 2007; Fukuda et al., 2015; Walker, 2016).

In many countries, conservation programmes have successfully restored crocodile populations to at least their optimal numbers, while in some countries, they are becoming increasingly dense and uncontrollable. As a result, cases of attacks on humans by crocodiles, which are considered an endangered species and need to be protected, are increasing (Fukuda et al., 2011; Sideleau & Britton, 2012; Fukuda et al., 2015). Crocodile attacks pose a serious threat and often result in serious injury to the victim, or even death (Gruen, 2009; Wamisho et al., 2009; Aust et al., 2019). Indeed, this conflict has made all crocodile species, including those that are less dangerous to humans, viewed with fear and usually with very negative perceptions (Treves et al., 2006; Fukuda et al., 2015). To date, crocodile attack on humans has been identified as a potential threat to its conservation approaches and alternatives to address this conflict are through prudent management strategy (Grajales & Silva, 2018; Murray et al., 2020). Certainly, this requires an in-depth study, especially the status and trend of crocodile attacks on humans in the areas concerned.

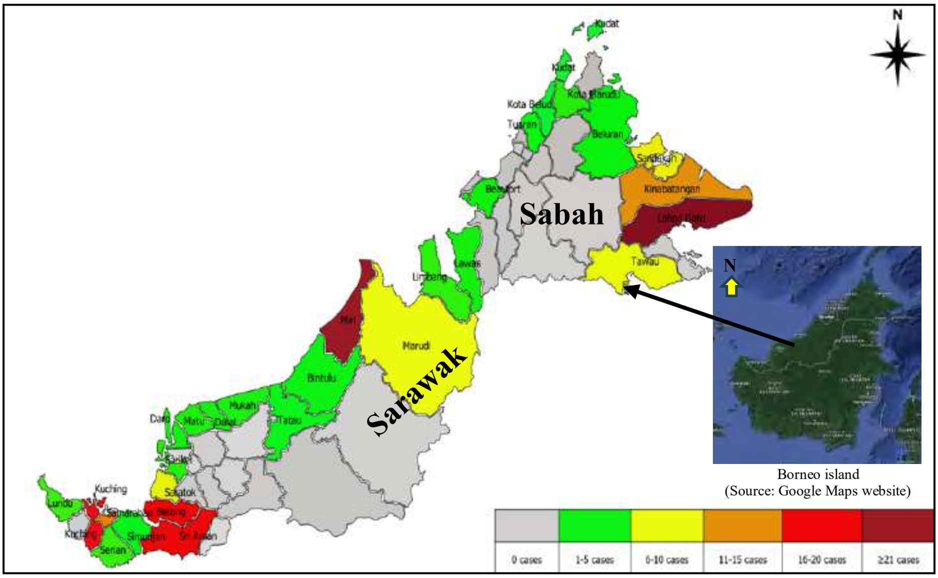
Malaysian Borneo, comprising the states of Sabah and Sarawak, is located on the island of Borneo, bordering the Indonesian province of Kalimantan. It has a large ecosystem of rivers and estuaries and is a natural habitat for crocodiles (Cox & Gombek, 1985; Tisen et al., 2013). These two states have embarked on crocodile conservation programmes by enforcing legal protections against this threatened species since the mid-1980s. The programme has generally been successful in restoring their populations. However, at the same time, it has led to human-crocodile conflicts, in particular attacks on communities living along rivers and estuaries that use these ecosystems in their daily life (Tisen et al., 2013). Information on crocodile attacks in Malaysian Borneo, especially in the state of Sabah is lacking. On the other hand, existing literature is insufficient to understand and further manage human-crocodile conflicts in these developing states with a growing human population (e.g., Abdul Gani et al., 2022). This study evaluates the status and trend of attack cases on humans by crocodiles, a threatened and protected species in Malaysian Borneo. It determined the geographical distribution, time of occurrence, yearly frequencies, the severity of the attack and personal identity of the victim including gender, age as well as activities performed during the incident, for the years 2001 to 2020 using secondary data generated from various sources. Such a study is imperative to understand the severity of human-crocodile conflicts and in turn, assist relevant agencies in establishing effective management strategies.

Materials and Methods

Data collection and methods

This study uses secondary data on crocodile attacks that have been reported to occur in several types of water ecosystems including rivers, estuaries, and several unspecified water systems including natural and man made ponds in both the states of Sabah and Sarawak (Figure 1). These water systems are usually connected to the coastal areas and serve as a natural habitat of crocodiles (Abd. Shukor, 2004; Tangah, 2006), and are particularly critical for their breeding as well as nursery grounds (Nagelkerken et al., 2008). At the same time, these water systems are also vital for the local community, especially for traditional fishing activities and other daily uses.

The secondary data on crocodile attack cases in Sabah was obtained from the Sabah Wildlife Department. Apart from this, data and other related information are also generated and extracted from other sources including local newspapers and interviews with relevant authorities. For Sarawak, data and information is mostly obtained from CrocBITE, the global crocodile attack database which can be found at http://www.crocodile-attack.info. Other relevant information is obtained from various sources including communication with relevant local authorities, newspapers and local residents through social media (i.e., Facebook). The detailed information on crocodile attacks, such as the location (i.e., state, district, and river), date (month and year), victim status (i.e., age, gender, and victim's activity before being attacked), and severity of the attacks (i.e., fatal and non-fatal), were collected and compiled.



Borneo island
(Source: Google Maps website)

Figure 1. Location of Sabah and Sarawak on the island of Borneo (inset map). The geographic distribution of crocodile attacks by the district for both states is shown. The colour indicators in silver, yellow-green, yellow, brown, red and dark red shows the range in the number of reported cases from 2001 to 2020.

Computation and statistical analysis

The data were subjected to a logarithmic transformation [log10 (x + 1)] to meet statistical analysis requirements. A Shapiro-Wilk test indicated that the data were not normally distributed. Therefore, the Wilcoxon Signed Rank Test and Friedman Test (non-parametric tests) were performed to determine the significant difference between the distribution data of cases within each designated category namely between state (Sabah & Sarawak), district (multiple districts), river (multiple rivers), year (2001, 2002,…2020) months (January, February,…December), gender (male & female), age group (0y–79y) and activities during the attacks (fishing, bathing…etc.). The significant level was set at $p < 0.05$. All statistical tests were performed using SPSS for Windows version 23 software (IBM, Armonk, NY, USA). The mapping of crocodile attack locations, including the cumulative number of cases for each district and river for both Sabah and Sarawak was presented in simplified heatmap.

Results and Discussion

The magnitude of crocodile attacks in Malaysian Borneo

A total of 205 cases of crocodile attacks were reported to occur over 20 years (2001–2020) in Malaysian Borneo which is equivalent to an average of 10.78 cases per year (Table 1). In the neighbouring country of Indonesia, number of attack cases were much higher, for example, a total of 279 attacks were reported in 27 of its 34 provinces over 7 years (2007–2014) which is equivalent to an average of 39.85 cases per year (Sideleau & Britton, 2014). This is nearly three times higher than the average number reported in this study, indicating that human-crocodile conflict in Malaysian Borneo is clearly at a staggering early stage compared to Indonesia. The relatively limited area of natural habitat with only two crocodile species existing may contribute to the low number of attacks in Malaysian Borneo compared to Indonesia. To date, there are two species of crocodiles found in Malaysian Borneo namely saltwater crocodile and Malayan gharial (tomistoma) (Cox & Gombek, 1985). In contrast, at least four species are known to inhabit extensive and diverse natural habitats in Indonesia including the saltwater crocodile, tomistoma, Siamese crocodile and New Guinean freshwater crocodile (Sideleau & Britton, 2014). Nevertheless, the actual number of crocodile attacks is difficult to infer since there are unreported cases, particularly in remote areas (Sideleau & Britton, 2013; Sideleau, 2016a; Sideleau, 2016b; Anonymous, 2017).

Table 1. Key points of the magnitude of crocodile attack cases in Malaysian Borneo from 2001 to 2020 shows the total number and percentage (in parentheses) for both the states of Sabah and Sarawak.

Key Points	Sabah	Sarawak	Total
Total attacks	70 (34%)	135 (66%)	205
Fatal attacks	52 (39%)	83 (61%)	135
Non-fatal attacks	18 (26%)	52 (74%)	70
Male victims	58 (36%)	115 (64%)	173
Female victims	9 (33%)	18 (67%)	27
Group age of victims (30 - 39 y)	19 (39%)	30 (61%)	49
Fishing activities	39 (41%)	56 (59%)	95

In the present study, the total number of fatal cases (135) was significantly higher ($p < 0.05$) than the number of non-fatal (70 cases), equivalent to an average of 6.75 deaths annually (Table 1). This is far less than the fatal attacks in Indonesia, where a total of 139 deaths between 2007 and 2014 or equal to an average of 19.85 annually (Sideleau & Britton, 2014). In Indonesia, the high number of fatal attacks is closely linked to the high population of saltwater crocodiles (Kaiser et al., 2009; Sideleau & Britton, 2012; Sideleau & Britton, 2014; Sideleau, 2016a), similarly, this species has been responsible for the most fatal attacks in Australia (Caldicott et al., 2005) and in India (Patro & Padhi, 2019). Although saltwater crocodiles are known to contribute to most attack cases in Malaysian Borneo (e.g. Tisen et al., 2011; Sabah Wildlife Department, 1992), other species of crocodiles may also be involved. The high number of fatal cases due to saltwater crocodile attacks is attributed to their aggressive nature, especially large-sized individuals with strong jaws and teeth, usually causing severe injury to victims (Caldicott et al., 2005; Kelly, 2006; Webb et al., 2010; Erickson et al., 2012). This is coupled with the ability to open its mouth underwater and perform a "death roll" making it a very effective predator (Hutchins et al., 2003; Doody, 2009).

In Malaysian Borneo, the total number of male victims (173 cases) is nearly seven times higher ($p < 0.05$) than the number of female victims (27 cases) (Table 1). A high number of attacks in males than females have also been reported in other countries such as India (Anonymous, 2017), Indonesia (Sideleau et al., 2021), Mexico (Grajales & Silva, 2018), and Australia (Fukuda et al., 2015). This shows that men are more at risk of being attacked by crocodiles than women and this is closely related to male-dominated activities such as fishing, especially those between the ages of 30 and 39.

The total number of attack cases was almost double ($p < 0.05$) in Sarawak (135 cases) compared to Sabah (70 cases) (Table 1). In terms of fatalities, the number was significantly higher ($p < 0.05$) in Sarawak (83 cases) compared to 52 in Sabah, while for non-fatal, it was 52 and 18, respectively for Sarawak and Sabah. The number of attacks on males is 58 which is significantly higher ($p < 0.05$) than females (19 cases) in Sabah while these are 115 ($p < 0.05$) and 18 respectively in Sarawak. Overall, the number of crocodile attacks is much higher in Sarawak, indicating that human-crocodile conflict is more critical in this state than in Sabah. In another study, Abdul Gani et al., (2022) reported at least 164 cases of crocodile attacks in Sarawak from year 2000 to 2020, suggesting that many cases are not widely reported especially in remote areas of the state. The relatively high number of attacks in Sarawak may be related to the high population density of crocodiles inhabiting its long and extensive river ecosystem (e.g., Landong & Zaini, 2010; Ngadan, 2015). Based on a separate population survey by the Sarawak Forestry Corporation and the

Sarawak Forest Department repectively in 2012 - 2014, there are approximately 12,000 - 13,507 crocodiles mainly adults (i.e., 34.9 %) in the state with a density of 1.06 individuals per km inhabiting more than 22 main rivers as reported in Anonymous, (2015).

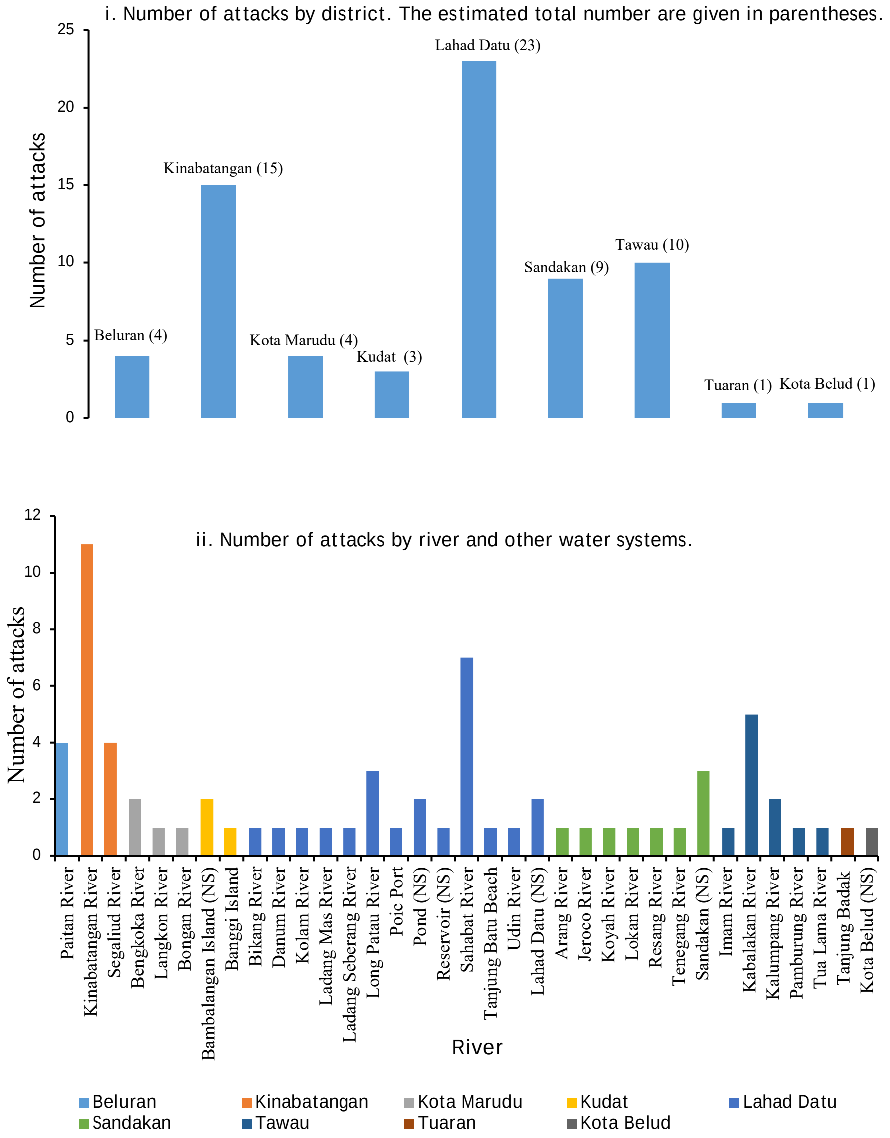
Geographical distribution of crocodile attacks

The geographical distribution of crocodile attacks by district in Sabah (i.e., 9 districts) and Sarawak (i.e., 19 districts) from 2001 until 2020 are shown in Figure 1. In Sabah, the district with a significantly ($p < 0.05$) high number of cases is Lahad Datu with more than 21 cases. This is followed by Kinabatangan with 11 - 16 cases, while both Sandakan and Tawau have between 6 -10 cases each. These three districts are known to have extensive river and wetland ecosystems, which account for the majority of the mangrove areas in Sabah (Abd. Shukor, 2004). Beluran, Kota Marudu, Kudat, Kota Belud, Tuaran and Beaufort reported between 1 - 5 cases whereas Kuala Penyu, Pitas, and Papar had very few. In Sarawak, the district with a significantly ($p < 0.05$) high number of attacks is Miri with more than 21 cases (see Figure 1). This is followed by Betong, Sri Aman and Kucing with the number of cases between 16 and 20, while Marudi and Saratok recorded between 6 - 10 cases. According to Abd. Shukor (2004), Kuching, Betong, Sri Aman, Miri and Marudi have extensive wetland ecosystems which account for the majority of mangrove areas in the state. The number of cases in Bintulu, Tatau, Mukah, Lundu and other districts near Kuching and Betong is relatively lower (i.e., less than 5 cases). The highest crocodile densities in Borneo are usually found in mid-river areas (Stuebing et. al., 1993), however, they are known to traverse across and utilize different habitats as a result of social dynamics (Web et al., 2010; Anonymous, 2015). This explains the widespread occurrence of crocodile attacks throughout Borneo which has a long network of rivers connecting each district.

There are 32 rivers and several natural water ponds that have recorded crocodile attacks in Sabah (Figure 2a). The number of attacks was significantly high ($p < 0.05$) in the Kinabatangan River which is situated in the Kinabatangan district with a total of 11 cases. The relatively high number of attacks in this area may have coincided with the high number of crocodile populations. According to Evans et al. (2016), the aquatic and semi-aquatic ecosystems found along the Kinabatangan River are very suitable as a natural habitat for crocodiles. The crocodile population has likely undergone rapid recovery following the state-wide protection of the species which was initiated in 1982 (Sabah Wildlife Department, 2010). A high number of attacks were also recorded in the Sahabat River of Lahad Datu with seven cases, followed by the Kalabakan River in Tawau with five cases, and four cases each for the Paitan River (Beluran) and the Segaliud River (Kinabatangan). Other rivers such as the Long Patau River in the district of Tawau and a few other locations in Sandakan with at least three cases. Numerous other rivers, including Balambangan and Bangi River in Kudat, Bengkoka, Langkon and Bongan River in Kota Marudu, Tanjung Badak River in Tuaran and a few rivers in Kota Belud reported at least 1 or 2 cases. Most of the rivers and wetland areas are known as the natural habitat of crocodiles in Sabah (CITES Malaysia Authorities, 2015; Nordkvist, 2015; Silla Datu, 2015). However, a large portion of these habitats have been developed for extensive oil palm plantations such as in Kinabatangan, Lahad Datu and Sandakan where the number of attacks is the highest. According to Stuebing et al., (2002) agricultural areas located adjacent to rivers will usually be covered by secondary vegetations suitable for nesting crocodiles and potentially attacking humans in the vicinity. This further proves a critical habitat conflict between humans and protected crocodile populations (e.g. Hassan & Abdul Gani, 2013; Abdul Gani et al., 2022).

In Sarawak, there are at least 72 rivers and several natural water ponds that have recorded crocodile attacks (Figure 2b). The number of attacks is significantly high ($p < 0.05$) in Batang Lupar in the district of Sri Aman with a total of six cases. This is followed by the Seblak River and Semarahan River with 5 cases each. The Niah and Sibuti River in Miri, Baram River in Marudi, Seribas River in Betong and Skrang River in Sri Aman recorded at least 4 cases, while the Oya River, Bako River, Karap River, Suai River and Sabang River recorded at least 3 cases. Crocodiles are known to live in these rivers and associated wetland areas (CITES Malaysia Authorities, 2015; Nordkvist, 2015; Silla Datu, 2015). The number of rivers that recorded crocodile attacks is relatively high in Sarawak and this is likely the result of conservation programmes. This is particularly noticeable with the presence of many hatchlings and yearlings, which is a clear indication of the population recovery in most of its rivers (Engkamat, 2007; Sullivan et al., 2010; Hassan & Abdul Gani, 2013). Crocodile attacks usually occur when protecting nests (Manolis & Webb, 2013), misidentifying humans as prey, and establishing self-defence mechanisms (Caldicott et al., 2005; Saragih et al., 2020).

a). Number of crocodile attacks by district (i) and river (ii) in Sabah.


i. Number of attacks by district. The estimated total number are given in parentheses.

ii. Number of attacks by river and other water systems.

b. Number of attack by district (i) and river (ii) in Sarawak.

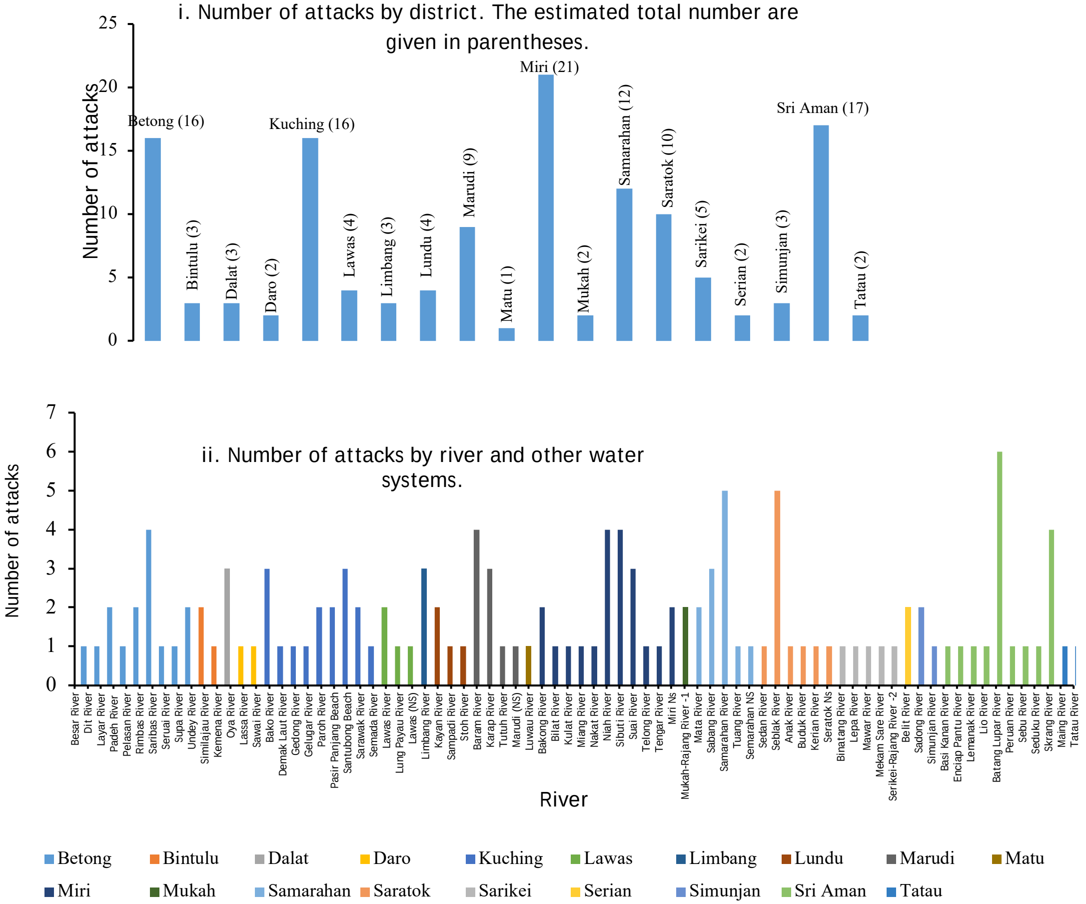


Figure 2. The estimated number of attacks by district and river in Sabah with 9 districts and 32 rivers (a), whereas in Sarawak with 19 districts and 72 rivers (b), recorded from 2001 to 2020.

The yearly trend of crocodile attacks

In both the states of Sabah (Figure 3a) and Sarawak (Figure 3b), the number of crocodile attacks from 2001 to 2006 was relatively low, i.e., less than two cases. However, the numbers show a linear positive trend ($r^2 = 0.7987$) with a marked increase thereafter. In Sabah, the fatal attacks reached four cases in 2011 and two non-fatal cases in 2008 and 2012, respectively (Figure 3a). The drastic increase in the number of attacks occurred between 2015 and 2020 when fatal cases increased significantly ($p < 0.05$) to nine cases while non-fatal cases reached six cases. In Sarawak, the number of attacks increased significantly ($p < 0.05$) from 2007 to 2020, reaching nearly triple the number reported in previous years (Figure 3b). Fatal attacks rose to ten cases in 2015 and 2020 respectively, while non-fatal attacks increased to nine cases in 2012. The results show that crocodile attacks including the fatal cases in Malaysian Borneo increased drastically in particular thereafter 2012 suggesting that the human-crocodile conflict has becomemore critical over the years.

In this study, the yearly rate of increase in the number of crocodile attacks was higher in Sarawak than in Sabah, for example, in 2012 there were a total of 12 cases in the former compared to 3 in Sabah. Relatively high number of attacks in Sarawak however is not a new trend, there were many fatal cases between the 1980s and early 1990s prompting the state government to launch an operation to remove large-sized crocodiles to minimize the risk of attacks (Cox & Gombek, 1985). For the record, crocodiles are considered a special animal by the Iban community of Sarawak practicing traditional beliefs (Abdul Gani et al., 2022) and killing or injuring the animal is prohibited. This traditional belief along with the long period of crocodile conservation, i.e., more than 40 years in the state has led to a significant increase in population density (e.g., Anonymous, 2015) and thus likely contributed to the recent high number of attacks. In contrast, Sabah had a low population of crocodiles until the mid 1980s (e.g., Web et al., 2010), however, the numbers have seen a recovery following statewide protection of the species that was initiated in 1982 (Sabah Wildlife Department, 2010). There is no current official estimate of the crocodile population in the state, however according to Sabah Wildlife Department, there were around 13,000 to 15,000 individuals across the state in 2010 as reported by Kan (2019). This indicates that the population increased slowly only after conservation and thus may explain the low yearly rate of crocodile attacks in the state.

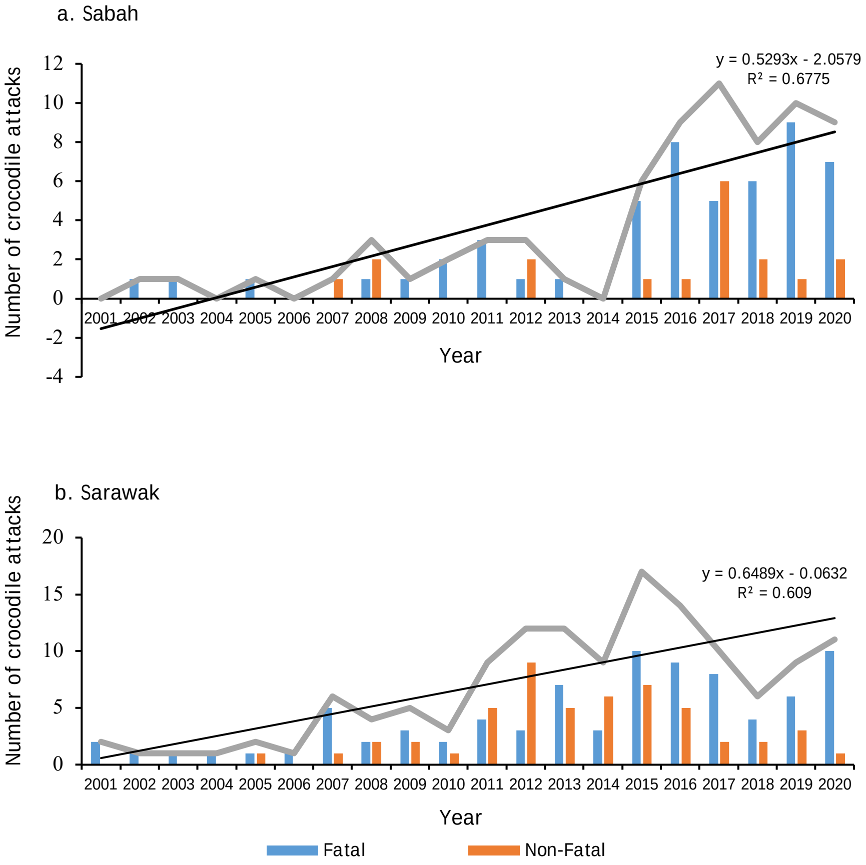


Figure 3. The estimated yearly number of crocodile attacks in Sabah (a) and Sarawak (b) from 2001 to 2020 includes the number of fatal, non-fatal, total and trends (i.e., linear) of attacks.

The increasing number of crocodile attacks due to growing population in the wild after conservation has been widely reported in many countries including India (Patro & Padhi, 2019), Australia (Fukuda et al., 2014) and Indonesia (Saragih et al., 2020). According to Webb et al., (2000), the recovery of crocodile populations after long-term protection will simultaneously increase the number of large-sized and aggressive individuals. This is also the case in Malaysian Borneo, for example, the ratio of large-sized adults (i.e. 34.9%) is high relative to juveniles and hatchling crocodiles throughout the state of Sarawak after long-term protection (Anonymous, 2015). It is believed that large-sized crocodiles may require larger prey and humans are well within the size range of prey that can be taken (Webb et al., 1982). The increasing number of large-sized adult crocodiles over time may also have increased the fatality rates of attack victims. According to Manolis & Webb, (2013), fatal cases are strongly related to the increasing proportion of larger crocodiles that can attack and overpower humans. In Australia, most attacks by large-sized crocodiles, i.e., over 4 m in length have resulted in the death of victims (Manolis & Webb, 2013). Recent information on the relative body size of the crocodile population in Malaysian Borneo is lacking. However, fatal attacks among large-sized crocodiles have been frequently reported where efforts to identify and hunt down the crocodiles are usually made after the attack.

Direct human competition with crocodiles for increasingly limited resources also contributed to the increase in crocodile attacks (Woodroffe, 2000; Treves & Karanth, 2003; Garcia-Grajales, 2013; Grajales & Silva, 2018). In Sarawak, many rivers are used daily for various purposes, especially by the local community and although their awareness level is high, safety issues among children are particularly a serious concern (Hassan & Abdul Gani, 2013). In addition, intensive use of mangrove areas and surrounding peat swamps for agricultural activities is likely to contribute to an increase in the number of fatal attacks, especially by saltwater crocodiles that naturally inhabit such ecosystems. The fatality rate due to saltwater crocodile attacks in Sarawak is nearly 61% (Tisen & Ahmad, 2010; Tisen et al., 2011; Ambu, 2011) while it is 36% in Sabah (Sabah Wildlife Department, 1992; Caldicott et al., 2005). These are much higher than in Australia (28.4%) (Manolis & Webb, 2013) and Sri Lanka (23.7%) (De Silva, 2010).

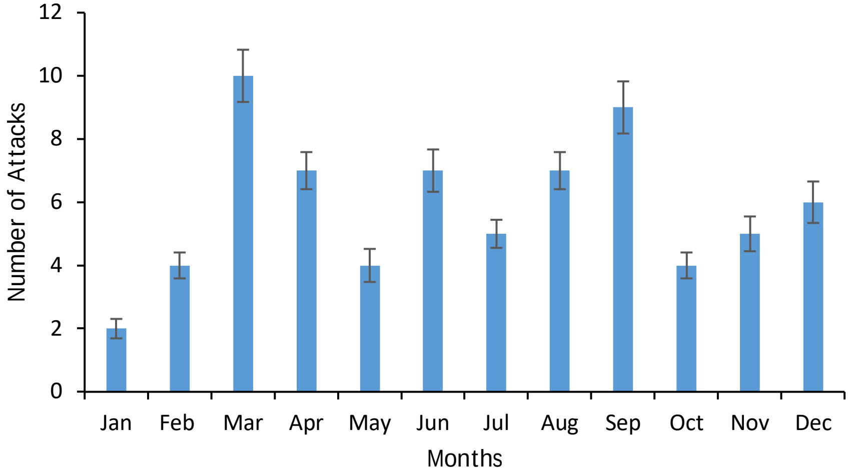
Monthly patterns of crocodile attacks

The monthly trend of crocodile attacks in the two states is different, especially the month that recorded the highest or lowest number of cases. In Sabah, the number of crocodile attacks was significantly high ($p < 0.05$) during March and April which range from 6 to 10 cases, followed by August and September with 7 to 9 cases (Figure 4a). The number of attacks is usually low in January, which is less than two cases compared to the other months. For the state of Sarawak, the number of attacks was significantly high ($p < 0.05$) during March and July with 20 and 17 cases, respectively (Figure 4b). The lowest number of attacks was recorded in December with 6 cases, while in other months, it varied from 8 to 11 cases. The results suggest that crocodile attacks occur all year round in Malaysian Borneo, however, a peak number is likely to occur during a particular time of year. In most countries, crocodile attacks take place throughout the year (Sideleau & Britton, 2014) with more attacks tending to occur during a particular period of the year which could have been associated with the reproductive cycle of the animals as well as environmental conditions that may influence their behaviour due to changes in the availability of food and habitat (Manolis et al., 2010; Grajales & Silva, 2018; Patro & Padhi, 2019).

Information on the reproduction cycle in particular the breeding season of crocodiles in Malaysia is rather limited, however, it has been reported that an increasing number of attacks are related to the aggression of the mother in nearby rivers when protecting hatchlings and yearlings (Hassan & Abdul Gani, 2013). In the Northern Territory of Australia, the peak season of crocodile attacks was from September to December coinciding with annual courtship and breeding (Manolis & Webb, 2013), however, fatal attacks occurred all year round (Sideleau & Britton, 2014; Fukuda et al., 2014; Fukuda et al., 2015). In both Sabah and Sarawak, the high number of attacks from March to July may coincide with the reproduction cycle during the dry season, however, further studies are needed to understand the breeding season of crocodiles.

In both Sabah and Sarawak, heavy rainfall during the monsoon season may contribute to the temporal variations of crocodile attacks due to changes in environmental conditions. The increase in food resources may coincide with the flooding of rivers following heavy rains during the monsoon, reducing the number of attacks on humans from October to December. On the other hand, during the driest inter-monsoon from January to March, the number of attacks may increase as crocodiles are more aggressive for limited food resources. Other factors, such as the seasonal peak of human activity in the nearby crocodile habitat, may also contribute to the high rate of attacks. In both states, artisanal fishing along the river usually occurs at certain times of the year when fishery resources such as shrimp are plentiful and are also expected to attract crocodiles. In Australia, the seasonal arrival of tourists along with other outdoor recreational activities during a particular time of year has been suggested to contribute to potential crocodile attacks (Caldicott et al., 2005).

a. Sabah



b. Sarawak

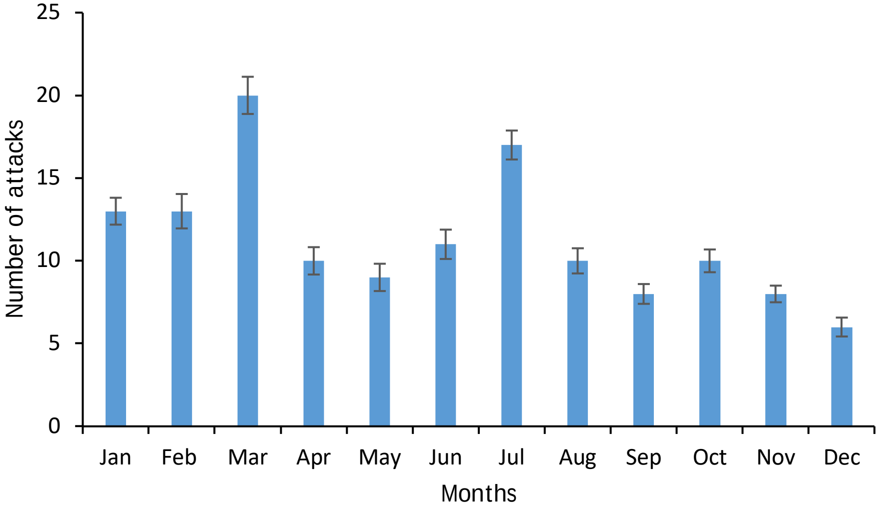
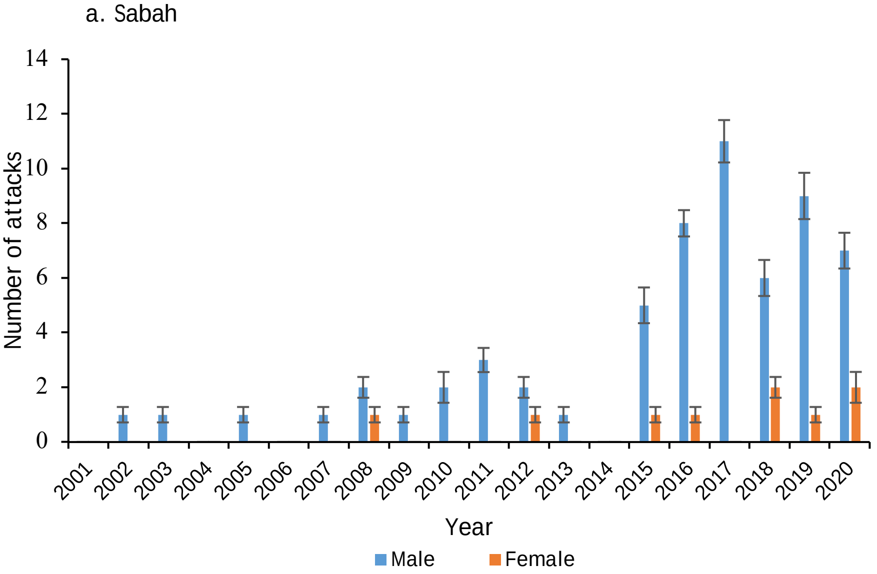


Figure 4. The monthly (January – December) number (mean ± SD) of crocodile attacks in Sabah (a) and Sarawak (b) from 2001 to 2020.

Gender and age group of attack victims

In Sabah, the yearly cases of crocodile attacks are significantly high among males than females (Figure 5a). Between 2001 and 2006, male victimss dominated the number of attacks, while females were only reported from 2008 to 2013 with a relatively small number (i.e., 1 case). However, the number of females increased to two cases from 2015 to 2020 in tandem with the increase in male victims which reached 11 cases in 2017. Similarly, in Sarawak, the number of attacks is dominated by male victims; from 2001 to 2010 only one female compared to seven males (Figure 5b). The significant increase in the number of female victims was from 2011 to 2018 with the highest being five in 2015 and is likely to be in tandem with the significant increase in male victims (i.e., 12 cases). The trend of an increase in the number of female victims that goes in tandem with the number of males has also been reported in other countries such as Australia (Caldicott et al., 2005; Manolis & Webb, 2013) and Indonesia (Sideleau & Britton, 2014; Sideleau et al., 2021). This shows that human-crocodile conflict can involve anyone regardless of gender when in a location at risk of a crocodile attack.

In Malaysian Borneo, the number of victims in the age group between 30 – 39 years was significantly ($p < 0.05$) higher than in the other age groups. In Sabah, the number of victims within this age group was the highest with 19 cases, followed by the age between 40 – 49 years with 14 cases and ages 20 – 29 with 9 cases (Figure 6a). Two victims aged between 0 and 9 years, and 4 victims aged 10 to 19 years were reported in the state, while those over 50 years old were also victims of attacks with 3 – 5 cases reported. In Sarawak, the number of victims between the ages of 30 and 39 years was 30 cases, this is followed by victims aged 40 to 49 and 20 to 29 years, with 24 cases each (Figure 6b). Unlike the trend of attacks in Sabah, the number of victims among teenagers and children is much higher in Sarawak. There are about 19 victims between the ages of 10 to 19 years, and three aged between 0 and 10 years have been recorded. In addition, the number of older people being attacked by crocodiles is also high in this state. There were a total of 14 victims in the age of 50 to 59 years, while 7 and 5 cases, respectively, for the ages of 60 to 69 years and 70 to 79 years. In general, the trend of crocodile attacks by age of the victim is similar to the trend reported elsewhere. Most common victims of crocodile attacks are between 30 to 39 years old and are likely to be carrying out their work activities (i.e., fishing) (Manolis & Webb, 2013) whereas children and senior individuals may be involved in water-related activities such as swimming (Fukuda et al., 2015).



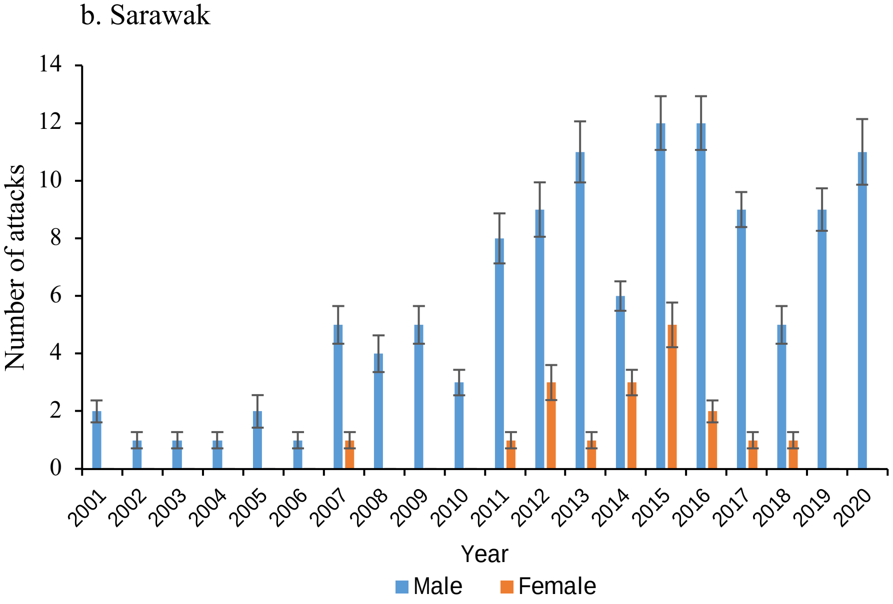
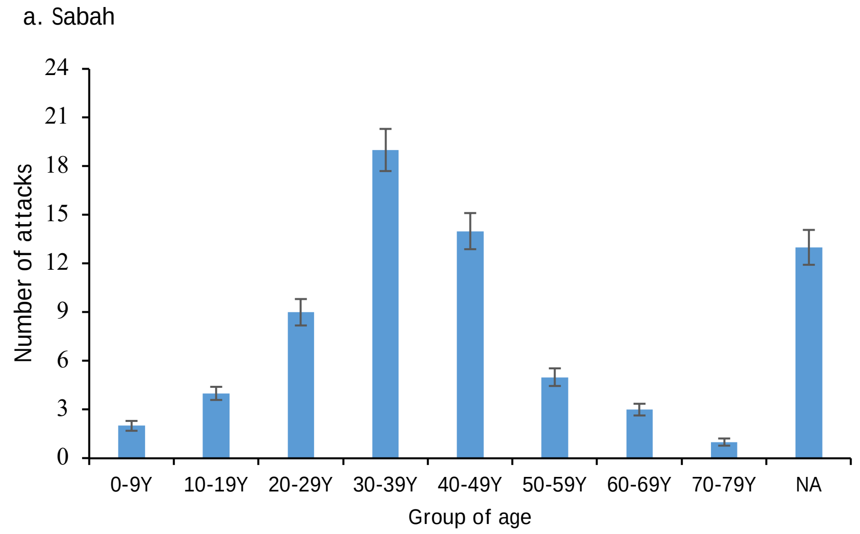


Figure 5. The yearly number (mean ± SD) of crocodile attack victims by gender in Sabah (a) and Sarawak (b) from 2001 to 2020.



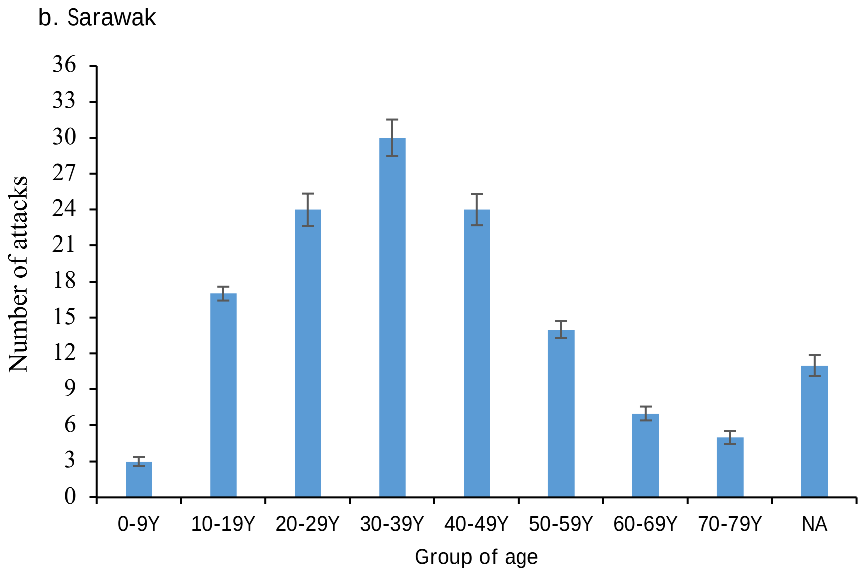
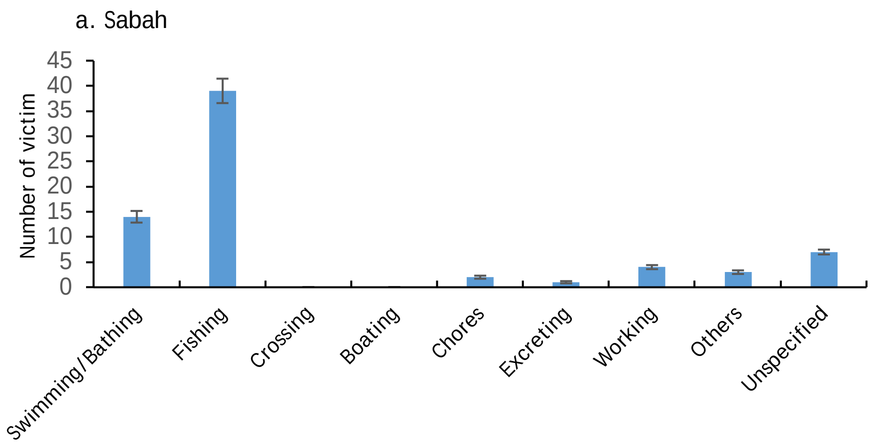


Figure 6. Total number (mean ± SD) of attacks by group age of victims in Sabah (a) and Sarawak (b) from 2001 to 2020.

Victim's activity during the attack

In Sabah, fishing activities before or during the crocodile attack involved 39 victims, which is significantly ($p < 0.05$) higher than other reported activities (Figure 7a). This is followed by swimming or bathing activities with a total of 14 victims. Other activities such as doing chores and even excreting, especially those carried out near the habitat of crocodiles, also make people vulnerable to attacks. The trend of victim activities before or during attacka in Sarawak is almost the same as in Sabah. In Sarawak, the number of victims who were involved in fishing was 56 cases, which is significantly ($p < 0.05$) higher than other activities (Figure 7b). This is followed by swimming or bathing activities with a total of 42 cases. Around the world, fishing and swimming are activities that involve a high number of crocodile attacks (e.g., Caldicott et al., 2005; Sideleau et al., 2017; Patro & Padhi, 2019; Khan et al., 2020) showing that such activities are risky to be carried out in areas inhabited by crocodiles. In contrast to the list of activities before the attack in Sabah, cases involving victims who were crossing the river and boarding a boat also occurred in Sarawak with at least 2 and 5 cases, respectively. This further indicates the severity of the human-crocodile conflict in Sarawak which relies heavily on the river system for various uses.

Several reasons have been suggested for the high number of attacks associated with water activities, in particular fishing and swimming. However, the crocodile's ability to be in the water without being noticed by the victim makes it a very efficient predator, especially saltwater crocodiles (Fukuda et al., 2015). According to Caldicott et al. (2005), victims were usually unaware of their presence in the water before the attack and took advantage of unbalanced prey in the water. Apart from these, the crocodile is capable of quiet, surreptitious movement before galloping and making a rapid movement toward its prey as an ambush predator (Grigg & Gans, 1993; Manolis & Webb, 2013). Surprisingly, activities such as boating as well as fishing from land are also at risk of being attacked by crocodiles. Such attacks may be subject to their body position before the attack and possibly the size of the boat used (Manolis & Webb, 2013; Fukuda et al., 2015) where small boats are at higher risk if attacked by a large size crocodile (Fukuda et al., 2015). Drowning is another cause of death for a crocodile attack victim based on the victim's body being relatively intact with no major trauma, thus the presence of an accompanying persons increases the survival of a victim (Cavanagh, 2004; Fukuda et al., 2015).



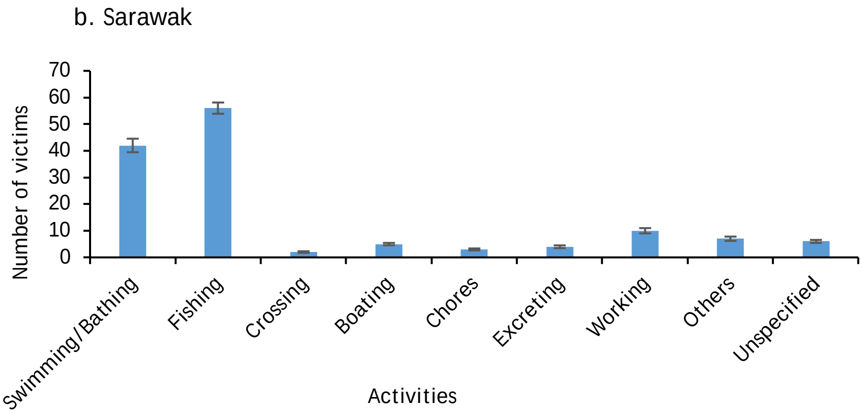


Figure 7. The number of victims and activities (mean ± SD) before the crocodile attacks in Sabah (a) and Sarawak (b) from 2001 to 2020.

Conclusion

The case of crocodile attacks in Malaysian Borneo shows an increasingly critical trend with an average of nearly 11 cases reported every year from 2001 until 2020. The number is higher in Sarawak than in Sabah, indicating a more worrying situation and this may be closely related to the higher population density of crocodiles inhabiting the river and estuary ecosystems which are also a source of livelihood for the local community. The fatality rate due to attacks continues to rise, indicating that the number of large size and dangerous crocodiles is increasing under conservation programmes that protect these animals in both states. The number of attacks is usually high in the natural habitat of crocodiles with most of the victims being men between the ages of 30 and 39 years old and involved in fishing activities. The findings show that the human-crocodile conflict in both states of Malaysian Borneo requires an immediate solution that includes systematic conservation strategies such as controlling population numbers in selected areas to reduce the risk of attacks among the local community. More studies should be conducted to understand the current population status of crocodiles in risky areas to minimize conflicts with humans.

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Authors' contributions: JM initiated the study and drafted the manuscript. NAR & SS collected and conducted the data analysis.
Competing interests & Consent for publication: All authors have no conflict of interests and agreed to publish the paper.

References

Abd. Shukor AH. 2004. The use of mangroves in Malaysia. In *Promotion of mangrove-friendly shrimp aquaculture in Southeast Asia Tigbauan, Iloilo, Philippines:* Aquaculture Department, Southeast Asian Fisheries Development Center. pp136–144.

Abdul Gani MIZ, Hassan R, Tisen OB, Ahmad R. 2022. Human-crocodile conflicts in Sarawak, Malaysian Borneo: An analysis of crocodile attacks from 2000 until 2020. *International Journal of Biology and Biomedical Engineering, 16*: 186–195. DOI: 10.46300/91011.2022.16.25.

Amarasinghe AAT, Madawala MB, Karunarathna DMSS, Manolis SC, de Silva A, Sommerlad R. 2015. Human-crocodile conflict and conservation implications of saltwater crocodiles Crocodylus porosus (reptilia: crocodylia: crocodylidae) in Sri Lanka. *Journal of Threatened Taxa, 7* (5): 7111–7130. https://doi.org/10.11609/jott.o4159.7111-30.

Ambu L. 2011. Current status of crocodiles in Sabah. In *Proceedings of the international crocodile conference, Kuching, Sarawak, Malaysia,* 19–21 October 2011.

Anonymous. 2015. *Convention on international trade in endangered species of wild fauna and flora 2015. Proposal for the transfer from appendices i to ii of Crocodylus porosus in malaysia. Interpretation and implementation of the convention species trade and conservation. Twenty-eighth meeting of the Animals Committee Tel Aviv (Israel), 30 August–3 September 2015*. Proposals for possible consideration at CoP17.

Anonymous. 2017. Crocodile specialist group newsletter, *36* (4) October 2017–December 2017.http://www.iucncsg.org/365_docs/attachments/protarea/0add0eef2e93114c16d6db73f1a9aab3.pdf.

Aust P, Boyle B, Fergusson R, Coulson T. 2009. The impact of Nile crocodiles on rural livelihoods in northeastern Namibia. *South African Journal of Wildlife Research, 39*: 57–69 doi: 10.3957/056.039.0107.

Caldicott DGE, Britton A, Webb GJW, Croser D, Manolis C. 2005. Crocodile attack in Australia: An analysis of its incidence and review of the pathology and management of crocodilian attacks in general. *Wilderness and Environmental Medicine 16* (3): 172–173 https://doi.org/10.1580/1080-6032(2005)16[172:AMSVCF]2.0.CO;2.

Cavanagh G. 2004. *Inquest into the death of Isobel Von Jordan NTMC 09. Coroner's Court Darwin*. Available at http://www.nt.gov.au/justice/courtsupp/coroner/findings/2004/von_jordan.pdf.

CITES Malaysia Authorities. 2015. *Proposal for the transfer from appendices I to II of Crocodylus porosus in Malaysia* pp 1–4.

Cox J, Gombek F. 1985. *A Preliminary survey of the crocodile resource in Sarawak, East Malaysia*.World Wildlife Fund Project No. MAL 74/85, Kuala Lumpur.

De Silva A. 2010. *Crocodiles of Sri Lanka: Preliminary assessment of their status and the human crocodile conflict situation.* Project No. 0905271, Mohamed Zayed species conservation fund.

Doody JS. 2009. Eyes bigger than stomach: Prey caching and retrieval in the saltwater crocodile, Crocodylus porosus. *Herpetological Review, 40* (1): 26–29.

Engkamat L. 2007. *Corporate management plan for crocodile in Sarawak.* Sarawak Forestry Corporation Sdn. Bhd., Kuching, Sarawak (unpublished).

Erickson GM, Gignac PM, Steppan SJ, Lappin AK, Vliet KA, Brueggen JD, Inouye BD, Kledzik D, Webb GJW. 2012. Insights into the ecology and evolutionary success of crocodilians revealed through bite-force and tooth-pressure experimentation. PLoS ONE, *7* (3): https://doi.org/10.1371/journal.pone.0031781.

Evans LJ, Hefin Jones T, Pang K, Saimin S, Goossens B. 2016. Spatial ecology of estuarine crocodile (*Crocodylus porosus*) nesting in a fragmented landscape. *Sensors,* 16: 1527 doi:10.3390/s16091527.

Fukuda Y, Manolis C, Appel K. 2014. Management of human-crocodile conflict in the Northern Territory, Australia : Review of crocodile attacks and removal of problem crocodiles. *Journal of Wildlife Management*, *78* (7): 1239–1249 https://doi.org/10.1002/jwmg.767.

Fukuda Y, Manolis C, Saalfeld K, Zuur A. 2015. Dead or alive? Factors affecting the survival of victims during attacks by saltwater crocodile (*Crocodylus* *porosus*) in Australia. *PLoS ONE, 10* (5): e0126778 doi:10.1371/journal.pone.0126778.

Fukuda Y, Webb G, Manolis C, Delaney R, Letnic M, Lindner G, et al. 2011. Recovery of saltwater crocodiles following unregulated hunting in tidal rivers of the Northern Territory, Australia. *Journal of Wildlife Management, 75:* 1253–1266 doi: 10.1002/jwmg.191.

Garcia-Grajales J. 2013. El conflicto hombre-cocodrilo en Mexico: Causas e implicaciones. *Interciencia*, *38*, (12): 881–884.

Grajales JG, Silva AB. 2018. Crocodile attacks in Oaxaca, Mexico: An update of its incidences and consequences for management and conservation. *Acta Universitatis, 28* (Online First): 1–8. doi: 10.15174/au.2018.1924.

Grigg G, Gans C. 1993. Morphology & physiology of the crocodylia. In: *Fauna of Australia Vol. 2A amphibia and reptilia*. pp 326–336.

Grigg G, Kirshner D. 2015. Biology and evolution of crocodylians. Cornell University Press, Ithaca, New York.

Gruen RL. 2009. Crocodile attacks in Australia: challenges for injury prevention and trauma care. *World Journal of Surgical Procedures, 33:* 1554–1561 doi: 10.1007/s00268-009-0103-6 PMID: 19543941.

Hassan R, Abdul Gani MIZ. 2013. *Crocodiles in western of Sarawak, Malaysia. In Crocodile specialist group. World crocodile conference.* Proceedings of the 22nd working meeting of the IUCN-SSC crocodile specialist group. IUCN: Gland, Switzerland. pp 90–95

Hutchins M, Murphy JB, Schlager N. 2003. *Grzimek's animal life encyclopedia Vol. 7: Reptiles (2nd ed.). Gale.* https://opentextbc.ca/meatcutting/.

Kan YC. 2019. Survey on crocodile population in Sabah. Daily Express Online. Wednesday, October 30, 2019. https://www.dailyexpress.com.my/news/142484/survey-on-crocodile-population-in-sabah/:

Kaiser H, Lopes Carvalho V, Freed P, O'Shea M. 2009. Status report on Crocodylus porosus and human-crocodile interactions in Timor-Leste. *Crocodile Specialist Group Newsletter, 28* (3): 12–14.

Kelly L. 2006. *Evolution's greatest survivor Crocodile.*

Khan W, Hore U, Mukherjee S, Mallapur G. 2020. Human-crocodile conflict and attitude of local communities toward crocodile conservation in Bhitarkanika Wildlife Sanctuary, Odisha, India. *Marine Policy, 121* (August): 104135. https://doi.org/10.1016/j.marpol.2020.104135.

Landong WL, Zaini MK. 2010. *Human crocodile conflict in Sarawak: Problems and potential solutions.* Paper presented during the IUCN-SSC Crocodile specialist group (CSG) Workshop on human-crocodile conflict, 23rd–25th June 2010, Shangri-la Rasa Ria Resort Hotel, Tuaran, Sabah, Malaysia.

Lang JW. 1987. Crocodilian behaviour: Implications for management. In: Webb GJ, Manolis CS, Whitehead PJ (Eds). *Wildlife management: crocodiles and alligators.* Surrey Beatty & Sons Pty Limited.

Manolis SC, Webb GJW. 2013. *Assessment of saltwater crocodile (Crocodylus porosus) attacks in Australia (1971–2013): Implications for management crocodiles.* Proceedings of the 22nd working meeting of the IUCN-SSC crocodile specialist group (Gland: Switzerland/IUCN). pp 97–104.

Manolis SC, Webb GJW, Brien ML. 2010. Saltwater crocodile *Crocodylus porosus*. *Crocodiles, 3:* 99–113.

Mazzotti FJ, Brandt LA, Moler P, Cherkiss MS. 2007. American crocodile (*Crocodylus acutus*) in Florida: Recommendations for endangered species recovery and ecosystem restoration. *Journal of Herpetology, 41:* 122–132 doi: 10.1670/0022-1511(2007)41[122:ACCAIF]2.0.CO;2.

Murray CM, Crother BI, Doody JS. 2020. The evolution of crocodilian nesting ecology and behavior. *Ecology and Evolution, 10* (1): 131–149 https://doi.org/10.1002/ece3.5859.

Nagelkerken I, Blaber SJM, Bouillon S, Green P, Haywood M, Kirton LG, Meynecke JO, Pawlik J, Penrose HM, Sasekumar A, Somerfield PJ. 2008. The habitat function of mangroves for terrestrial and marine fauna: A review. *Aquatic Botany, 89* (2):155–185 https://doi.org/10.1016/j.aquabot.2007.12.007.

Ngadan SD. 2015. *Local communities' attitudes toward crocodiles and human-crocodile conflicts in Sarawak.* Proceedings of the 17th Malaysian forestry conference Kota Kinabalu. pp 515–523.

Nordkvist M. 2015. Anthropogenic disturbance along the Kinabatangan River in Borneo, Malaysia and the distribution and abundance of the saltwater crocodile (*Crocodylus porosus*). http://files.webb.uu.se/uploader/858/MFS-195nordkvistliten.pdf.

Patro S, Padhi SK. 2019. Saltwater crocodile and human conflict around Bhitarkanika National Park, India: A raising concern for determining conservation limits. *Ocean and Coastal Management, 182* (1): 104923. https://doi.org/10.1016/j.ocecoaman.2019.104923.

Platt SG, Tasirin JS, Hunowu I, Siwu S, Rainwater TR. 2007. Recent distribution records of estuarine crocodiles (Crocodylus porosus) in northern Sulawesi, Indonesia. *Herpetological Bulletin, 100:* 13–17.

Read MA, Miller JD, Bell IP, Felton A. 2004. The distribution and abundance of the estuarine crocodile, *Crocodylus porosus*, in Queensland. *Wildlife Research 31:* 527–534.

Saalfeld K, Fukuda Y, Duldig T, Fisher A. 2015. Wildlife trade management plan for the saltwater crocodile (*Crocodylus porosus*) in the Northern Territory of Australia, 2016 – 2020. Northern Territory Department of Land Resource Management, Darwin.

Sabah Wildlife Department. 1992. *Crocodile management in Sabah. Kota Kinabalu, Malaysia:* Sabah Wildlife Department. pp 11 – 29.

Sabah Wildlife Department. 2010. *Crocodile management plan; Sabah state government:* Kota Kinabalu, Malaysia. pp 1–67.

Saragih GS, Kayat, Hidayatullah M, Hadi DS. 2020. A preliminary study on the population and habitat of saltwater crocodile (*Crocodylus porosus*) in Timor Island, East Nusa Tenggara. The 7th Symposium of JAPAN-ASEAN science technology innovation platform (JASTIP). *IOP Conference Series: Earth and Environmental Science, 591:* 012044 doi:10.1088/1755-1315/591/1/012044.

Sideleau B, Britton A. 2013. *An analysis of crocodilian attacks worldwide for the period of 2008 – July 2013.* Proceedings of the 22nd Working meeting of the IUCN-SSC crocodile specialist group, IUCN: Gland, Switzerland. pp110–113.

Sideleau BM, Britton ARC. 2014. *An analysis of recent crocodile attacks in the Republic of Indonesia - a Case study on the utility of the CrocBITE Database.* In Proceedings of the 23rd Working Meeting of the Crocodile Specialist Group – IUCN – The World Conservation Union, Gland, Switzerland

and Cambridge UK. pp 332–335.

Sideleau BM, Edyvane KS, Britton, ARC. 2017. An analysis of recent saltwater crocodile (*Crocodylus porosus*) attacks in Timor-Leste and consequences for management and conservation. *Marine and Freshwater Research*, *68* (5): 801–809. https://doi.org/10.1071/MF15354 http://www.iucncsg.org/

Sideleau B, Britton ARC. 2012. *A preliminary analysis of worldwide crocodilian attacks*. Crocodiles proceedings of the 21st working meeting of the IUCN-SSC crocodile specialist group. Gland, Switzerland IUCN 2012. pp 111–114.

Sideleau B, Sitorus T, Suryana D, Britton A. 2021. Saltwater crocodile (Crocodylus porosus) attacks in East Nusa Tenggara, Indonesia. *Marine and Freshwater Research*, *72* (7): 978–986 https://doi.org/10.1071/MF20237.

Sideleau B. 2016a. Recent records of crocodiles on the Island of Sulawesi, Indonesia East and Southeast Asia. *Crocodile Specialist Group Newsletter, April.* Pp. 17–19.

Sideleau B. 2016b. Summary of worldwide crocodilian attacks for 2015. *Crocodile Specialist Group Newsletter*, *35 (May):* 3.

Silla Datu N. 2015. Local communities' attitudes toward crocodiles and human-crocodile conflicts in Sarawak. In: *Proceedings of the 17th Malaysian forestry conference. A century of forest management: Lessons learnt & the way forward.* pp 515–523.

Stubbs A. 1998. *Information systems for new animal industries. RIRDC (Rural Industries Research and Development Corporation)* – Publication No. 98/139. Canberra: RIRDC. Available from: http://www.rirdc.gov.au/reports/NAP/PTP-1A.doc.

Stuebing RB, Andau M, Ambu L, Jensen SM. 2002. Crocodile recovery in Sabah. *Crocodile Specialist Group Newsletter, 21* (3): 6–9.

Stuebing RB, Mohd Sah SM, Andau M, Ambu L. 1993. *Conservation, management and farming of crocodiles in Sabah.* Working paper, Proceedings of the 2nd. Regional Meeting of the Crocodile Specialist Group, 12–19 March, 1993. Darwin, NT, Australia.

Sullivan S, Holden J, Williams C. 2010. Report on the distribution and abundance of the estuarine crocodile, *Crocodylus porosus,* in Queensland. Department of Environment and Resource Management, Queensland.

Tangah J. 2006. Notes on mangroves of Sabah.

Thorbjarnarson J. 1999. Crocodile tears and skins: International trade, economic constraints, and limits to the sustainable use of crocodilians. *Conservation Biology 13* (3): 465–470. https://doi.org/10.1046/j.1523-1739.1999.00011.x.

Tisdell C, Nantha HS. 2007. Management, conservation and farming of saltwater crocodiles: An Australian case study of sustainable commercial use. In: *Perspectives in animal ecology and reproduction 4,* Gupta VK, Anil KV. (Eds.). pp 233–264.

Tisen OB, Ahmad R. 2010. *Crocodylus porosus in Sarawak: Status management.* Paper presented during the IUCN-SSC crocodile specialist group (CSG) Workshop on human-crocodile conflict, 23rd–25th June 2010, Shangri-la Rasa Ria Resort Hotel, Tuaran, Sabah, Malaysia.

Tisen OB, Ahmad R, Kwan S, Robi N, Osaka V. 2011. Promoting awareness on human-crocodile conflict in Sarawak. In *Proceedings of the international crocodile conference,* Kuching, Sarawak, Malaysia, 19–21 October 2011.

Tisen OB, Gombek F, Ahmad R, Kri C. 2013. Human crocodile issues: Sarawak Report. In *World Crocodile Conference. Proceedings of the 22nd Working Meeting of the IUCN-SSC Crocodile Specialist Group. IUCN:* Gland, Switzerland. pp. 115.

Treves A, Karanth KU. 2003. Human-carnivore conflict and perspectives on carnivore management worldwide. *Conservation Biology, 17* (6): 1491–1499.

Treves A, Wallace RB, Naughton-Treves L, Morales A. 2006. Co-managing human-wildlife conflicts: A review. *Human Dimensions of Wildlife, 11:* 383–396 doi: 10.1080/10871200600984265.

Walker EM. 2016. *The historical ecology of Queensland's Australian saltwater crocodile (Crocodylus porosus).* Honors Thesis. P. 815 https://digitalcommons.colby.edu/honorstheses/815.

Wamisho BL, Bates J, Tompkins M, Islam R, Nyamulani N, Ngulube C, et al. 2009. Ward round crocodile bites in Malawi: microbiology and surgical management. *Malawi Medical Journal, 21:* 29–31 doi: 10.4314/mmj.v21i1.10986 PMID: 19780476.

Webb GJ, Manolis SC, Brien ML. 2010. *Saltwater crocodile Crocodylus porosus in Crocodiles. Status Survey and Conservation Action Plan. 3rd edition.* Manolis SC, Stevenson C (Eds). Crocodile Specialist Group. Darwin. Australia. pp. 99–113.

Webb GJW, Manolis SC, Whitehead PJ, Letts GA. 1984. A proposal for the transfer of the Australian population of *Crocodylus porosus* Schneider (1801), from Appendix I to Appendix II of C.I.T.E.S. Conservation commission of the northern territory, Tech. Report No. 21.

Webb GJW, Britton ARC, Manolis SC, Ottley B, Stirrat S. 2000. *The recovery of Crocodylus porosus in the Northern Territory of Australia: 1971–1998. Crocodiles:* Proceedings of the 15th Working Meeting of the IUCN-SSC Crocodile Specialist Group. January 17–20, 2000; Varadero, Cuba. Gland, Switzerland: IUCN; 2000: pp 196–235.

Webb GJW, Manolis SC, Buckworth R. 1982. *Crocodylus johnstoni* in the McKinlay River area, N.T.I: Variation in the diet and a new method of assessing the relative importance of prey. *Australian Journal of Zoology, 30:* 877–899.

Woodroffe R. 2000. Predators and people: using human densities to interpret declines of large carnivores. *Animal Conservation, 3:* 165–173.

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Research Article

Defining *Xerocomus s.str.* (Boletaceae) in Malesia

Lee SML.[1*], Helfer S.[2], Watling R.[3]

[1]*Singapore Herbarium, Singapore Botanic Gardens, National Parks Board, Singapore.*
[2,3]*Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, Scotland, United Kingdom.*

*Corresponding author: Serena_Lee@Nparks.gov.sg
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ABSTRACT

Spores from Corner's (1972, 1974) type specimens of *Xerocomus* (sensu Horak, 2011) housed at Edinburgh herbarium (E) were imaged for the first time, using Scanning Electron Microscopy (SEM) to ascertain the presence of *Xerocomus* sensu Šutara (2008) in Malesia. The following taxa *Boletus chlamydosporus, B. chrysops*, *B. lubricus*, *B. microcarpus, Xerocomus microcarpoides* and *Phylloporus rufoflavipes* were found to have bacillate ornamentations. New combinations and name changes have been proposed.

Keywords: Boletaceae; Singapore; macrofungi; diversity

Introduction

In the Boletales, *Xerocomus* Quél. is a genus of fungi found throughout the world but is poorly collected and studied in Malesia.

Xerocomus was first described by Quélet (1887). The type species is *Xerocomus subtomentosus* (L.) Quél. (Quélet, 1888). For many years, the classification of basidiomycetes was based on the fungal fruiting body, or basidiocarp, and *Xerocomus* has been no exception. Based on European collections, Oolbekkink (1991) pointed out that although *Xerocomus* could indeed be distinguished from a few taxa in *Boletus* on the structure the hymenophoral trama, as suggested by Singer (1951), other species, especially non-European ones remained ambiguous. For this reason, *Xerocomus* has not had universal acceptance at generic level (Smith & Thiers 1971; Corner, 1974; Oolbekkink, 1991; Watling, 2001; Halling, 2007). When molecular techniques became available, Binder & Hibbert (2006) showed that *Xerocomus* was not monophyletic. Šutara (2008) observed that *Xerocomus* was highly variable. Through the study of spore ornamentation among other anatomical characters, he re-delimited *Xerocomus.* The study by Wu et al. (2014), using DNA sequences of four genes (nrLSU, *tef1-a*, *rpb1*, and *rpb2*), confirmed that *Xerocomoid boletes* harbours six lineages (*Xerocomus s.str., Hemileccinum* Šutara, *Xerocomellus* Šutara, and 3 other clades). The latter paper had only taken into account the European *Xerocomus,* but not the ones found elsewhere.

Unlike the European taxa of *Xerocomus s.l.*, pre-Šutara (2008) the species described from Malaya and other parts of Malesia are mostly known only from the type specimen. In a revision of Corner's works (1972, 1974), Horak (2011) neither included molecular work nor spore SEM studies despite the latter having been shown by Šutara (2008) to be a key character in defining *Xerocomus s.str.* and relied entirely on hymenophoral trama and cystidial charcters

In Malesia, Corner (1972) placed 48 *Boletus* taxa under subgenus *Xerocomus*, as they differed from subgenus *Boletus* only by their tube-trama, which were phylloporoid rather than boletoid, although Corner himself pointed out that there were intermediate states and no sharp distinctions. The tube-trama character is illustrated in detail in Šutara (2005) for European taxa.

Šutara (2008) examined the spore ornamentation across *Xerocomus s.l.* and delimited the European species on the basis of the bacillate spore ornamentation, among other characters such as the size of the fruit bodies, the hymenophoral trama, the shape and size of the spore, etc. The bacillate spore ornamentationis is only found in the genera *Xerocomus s.str.* and *Phylloporus*, which finds itself in *Xerocomus* in the latest classification. Where *Xerocomus* are poroid, *Phylloporus* are gilled.

Horak (2011) reassessed the classification of Corner's Malaysian boletes and raised his subgenus *Xerocomus* to generic level. Taxa with lost or compromised type material, or with protologue descriptions inadequate or contradicting, were placed in a list of excluded or rejected species.

While Šutara (2008) had used the bacillate spore ornamentation as the defining character that placed poroid bolete taxa in *Xerocomus* Quél. *s.str.,*Horak did not discuss this spore *ornamentation in his work*.

The Malesian species of *Xerocomus* have been neglected almost entirely by all authors. In this study, we imaged with a SEM, 23 *Xerocomus* species upgraded by Horak (2011) in his revision of the Malesian species of Boletales *s.l.*, in addition to 13 of the taxa of *Boletus* (Xerocomus) *sensu* Corner (1972) which were subsequently excluded from his studies. The remaining 12 types from Corner's 48 taxa (1972) were not imaged as they were either too small for destructive sampling or not available during the time of study.

Materials and methods

In the present study, 36 taxa extracted from the type specimens of Corner's species were analysed. All the specimens were deposited at the Royal Botanic Garden Edinburgh (E).

Xerocomus as defined by Horak (2011). Collection details of examined taxa in appendix I.

1. *Xerocomus albipurpureus* (Corner) E. Horak
2. *Xerocomus asperipes* (Corner) E. Horak
3. *Xerocomus calocystides* (Corner) E. Horak
4. *Xerocomus calvus* (Corner) E. Horak
5. *Xerocomus catervatus* (Corner) E. Horak
6. *Xerocomus cuticulatus* (Corner) E. Horak
7. *Xerocomus cyaneirufescens* (Corner) E. Horak
8. *Xerocomus destitutus* (Corner) E. Horak
9. *Xerocomus dispersus* var. *dispersus* (Corner) E. Horak
10. *Xerocomus dispersus* var. *tembelingensis* (Corner) E. Horak
11. *Xerocomus ferruginosporus* (Corner) E. Horak
12. *Xerocomus gyrodontoides* (Corner) E. Horak
13. *Xerocomus lucescens* (Corner) E. Horak
14. *Xerocomus microcarpoides* (Corner) E. Horak
15. *Xerocomus prebadius* (Corner) E. Horak
16. *Xerocomus pseudochrysenteron* (Corner) E. Horak
17. *Xerocomus ranunculus* (Corner) E. Horak
18. *Xerocomus raphanolens* (Corner) E. Horak
19. *Xerocomus rectus* (Corner) E. Horak
20. *Xerocomus rufoflavipes* (Corner) E. Horak
21. *Xerocomus satisfactus* (Corner) E. Horak
22. *Xerocomus solitarius* (Corner) E. Horak
23. *Xerocomus tentabundus* (Corner) E. Horak

Boletus subgen. *Xerocomus* sensu Corner (1972). Collection details of examined taxa in appendix.

1. *Boletus aculifer* Corner
2. *Boletus blanditus* Corner
3. *Boletus chlamydosporus* Corner
4. *Boletus chrysops* Corner
5. *Boletus hastulifera* Corner
6. *Boletus honestus* Corner
7. *Boletus intentus* Corner
8. *Boletus lubricus* Corner
9. *Boletus microcarpus* Corner
10. *Boletus nugatorius* Corner
11. *Boletus polychrous* Corner
12. *Boletus sartor* Corner
13. *Boletus variisporus* Corner

Morphological (SEM) characters

Spores were examined with a LEO Supra 55vp Scanning Electron Microscope (Zeiss) at the Royal Botanic Garden, Edinburgh (RBGE), United Kingdom.

Dried herbarium material was mounted on aluminium SEM stubs with double-sided adhesive carbon stickers without treatment. Spirit material was taken through a series of chemical washes (70% ethanol (15min), 95% ethanol (10min), 100 % ethanol (5min), 100% acetone (5 min), 100% acetone (5 min)), and brought through the K850 Critical Point Dryer (Quorum Technologies) prior to being mounted on the SEM stubs. Mounted stubs were sputter coated with platinum for 1.5 min in the K575X Sputter Coater (Quorum Technologies,), at a 25mA current. Samples were scanned at magnifications of 1000X, 3000X and 10,000X and imaged at a working distance of 10mm and EHT 5kV.

Results

Scanning Electron Microscopy imaging of Malesian Xerocomoid boletes revealed the following taxa, *Boletus* (*Xerocomus*) *chlamydosporus* Corner, *Boletus* (*Xerocomus*) *chrysops* Corner*, Boletus* (*Xerocomus*) *lubricus* Corner and *Boletus* (*Xerocomus*) *microcarpus, Xerocomus microcarpoides* (Corner) E. Horak and *Xerocomus rufoflavipes* (Corner) E. Horak with bacillate ornamentations at 10000X magnification.

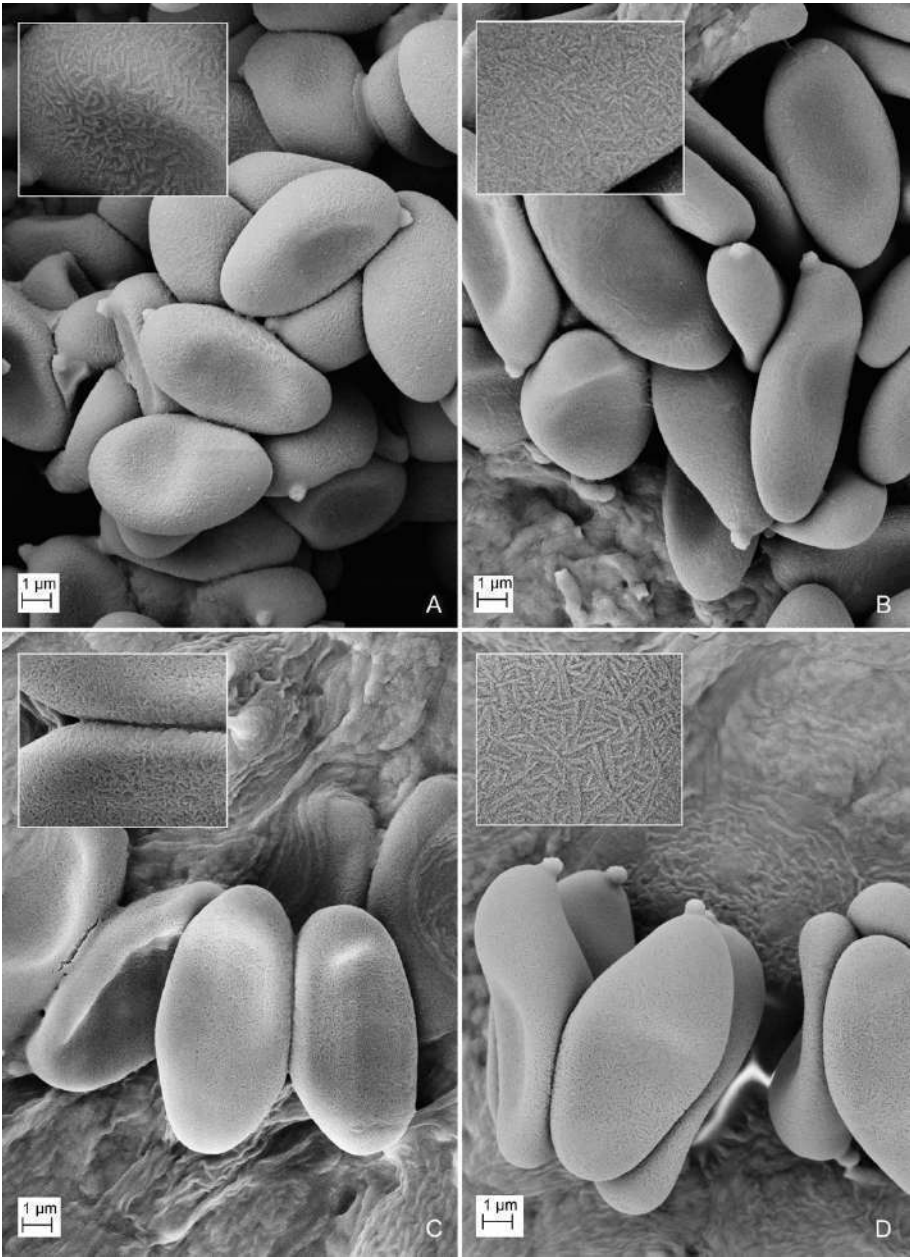

Figure 1.1. A. SEM spore images. Spores with bacillate ornamentations. A. *Boletus chlamydosporus* (sheet), B. *B. chrysops* (sheet), C. *B. lubricus* (sheet) and D. *B. microcarpus* (sheet). All holotype material.

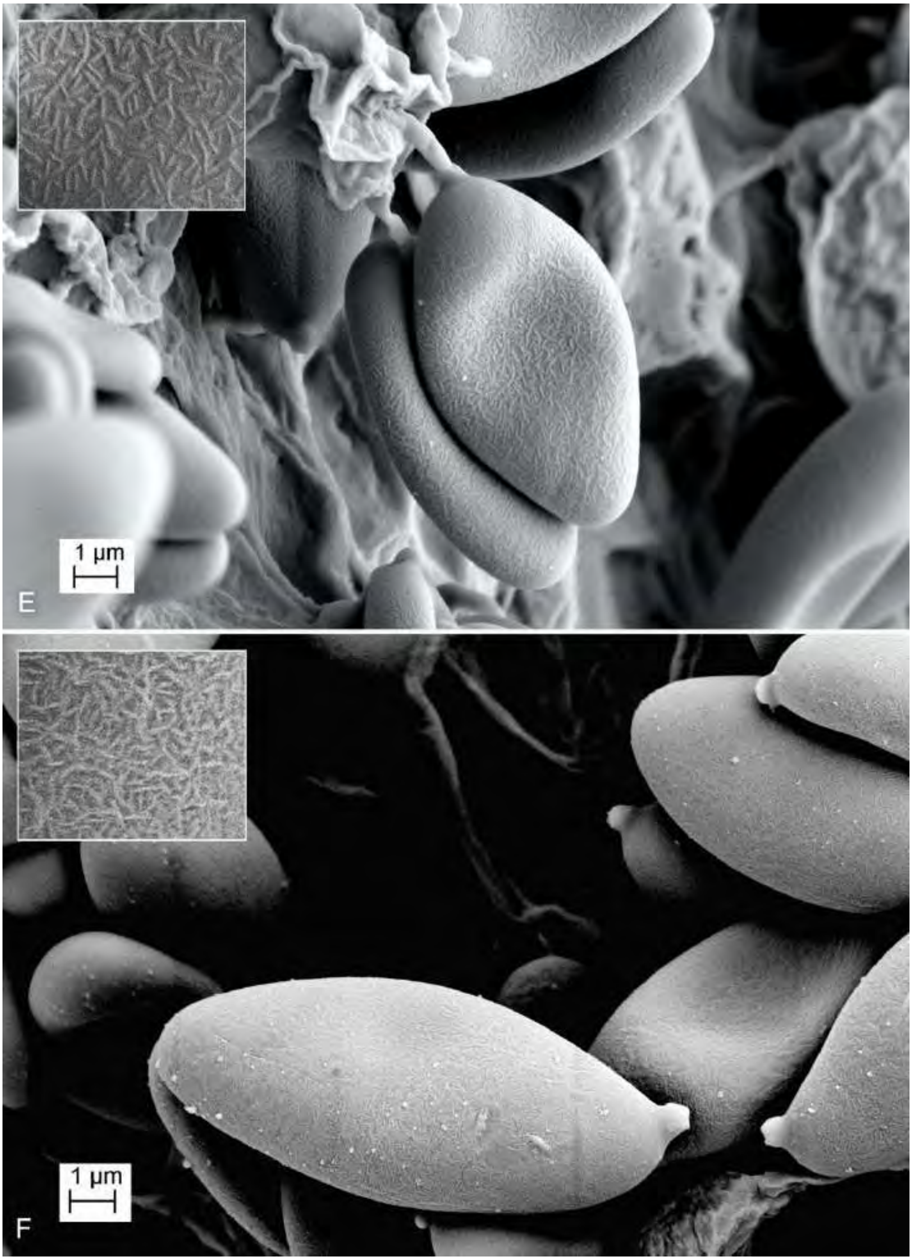

Figure 1.2. SEM spore images. Spores with bacillate ornamentations. E. *Xerocomus microcarpoides* (sheet). F. *X. rufoflavipes* (sheet). All holotype material.

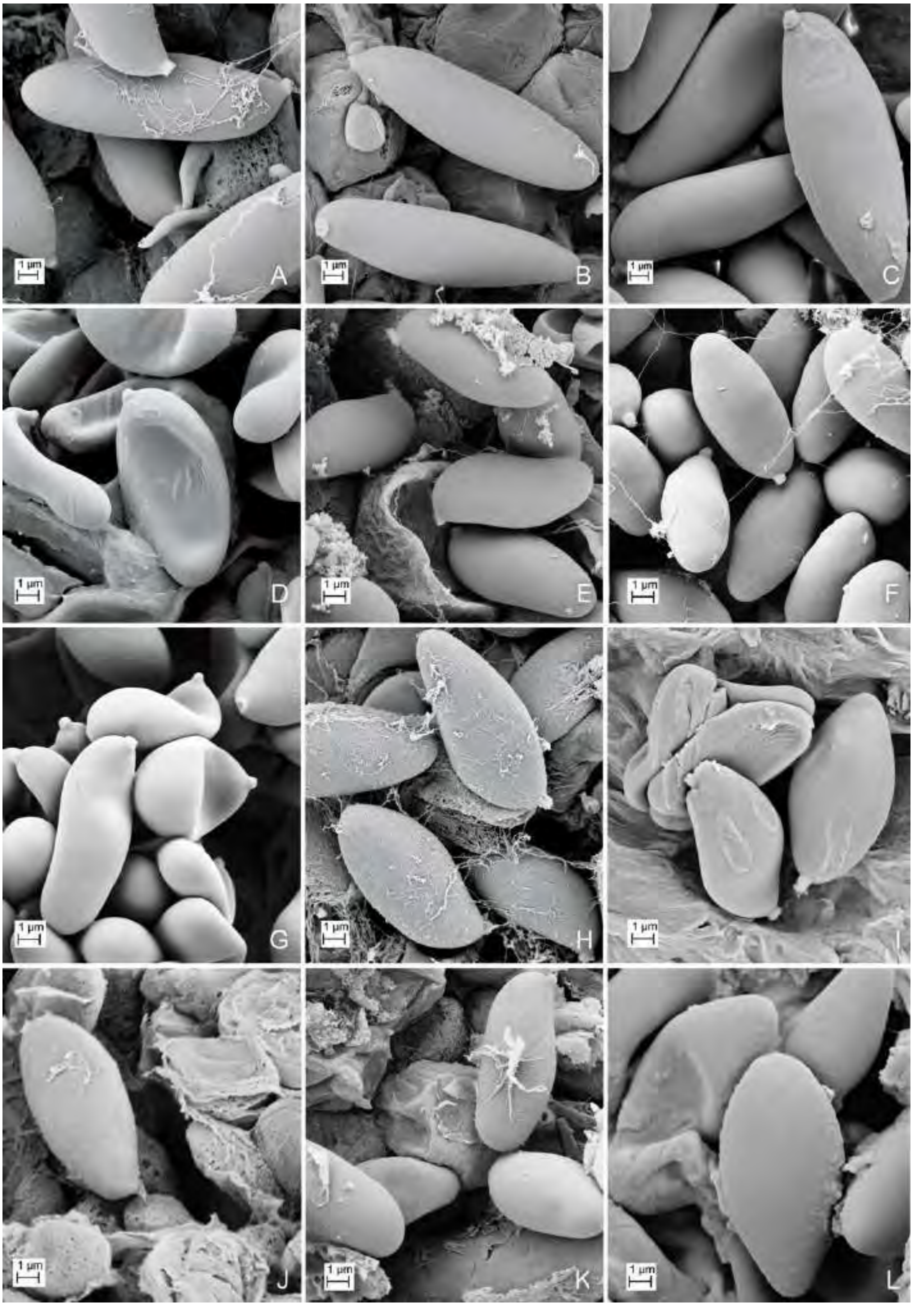

Figure 2.1. SEM images. A. *Xerocomus albipurpureus* (spirit). B. *X. ferruginosporus* (spirit). C. *X. raphanolens* (sheet). D. *X. asperipes* (sheet). E. *X. calvus* (spirit). F. *X. catervatus* (spirit). G. *X. cyaneirufescens* (sheet). H. *X. dispersus* var. *dispersus* (spirit) I. *X. dispersus var. tembelingensis* (sheet). J. *X. gyrodontoides* (spirit). K. *X. prebadius* (spirit). L. *X. ranunculus* (sheet). All holotype material except for *X. calvus* (holotype not found, but material (E00458467) cited by Corner, 1972).

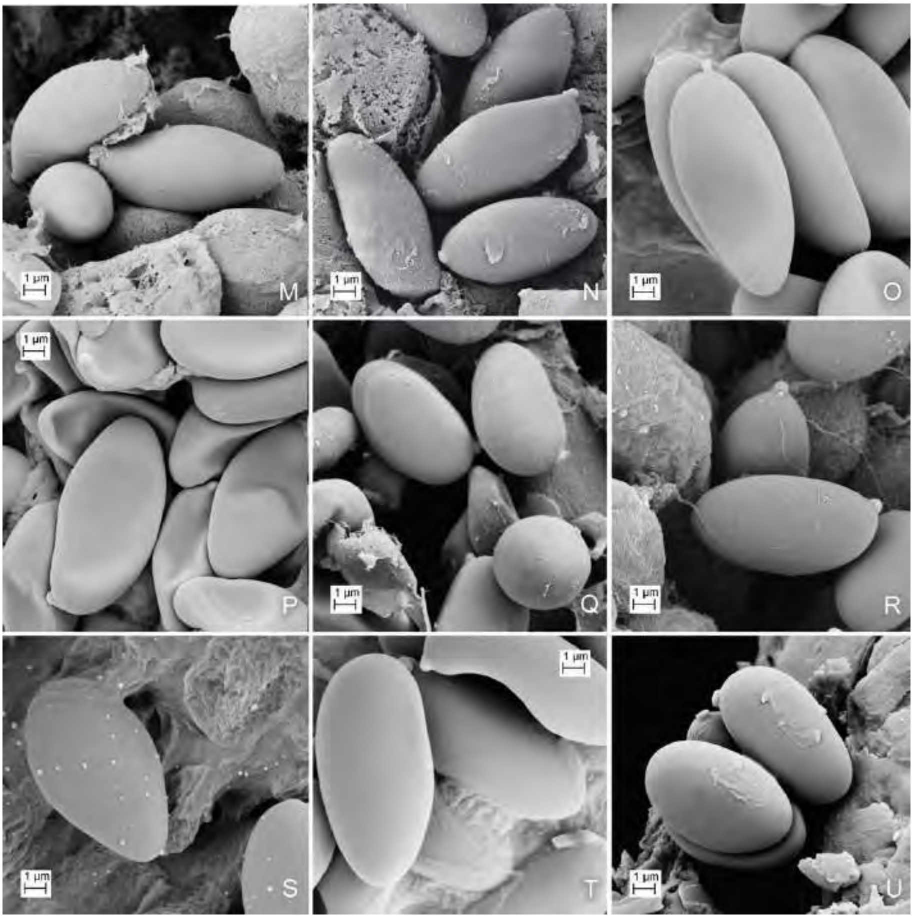

Figure 2.2.: SEM images. M. *Xerocomus rectus* (spirit). N. *X. satisfactus* (spirit). O. *X. solitarius* (sheet). P. *X. tentabundus* (sheet). Q. *X. calocystides* (spirit). R. *X. cuticulatus* (spirit). S. *X. lucescens* (sheet). T. *X. pseudochrysenteron* (sheet). U. *X. destitutus* (sheet). All holotype material.

All other *Xerocomus sensu* Horak which have smooth spores. See figures 2.1 and 2.2. Remaining species of *Boletus* (*Xerocomus*) *sensu* Corner (1972, 1974) which have smooth spores. See figure 3.

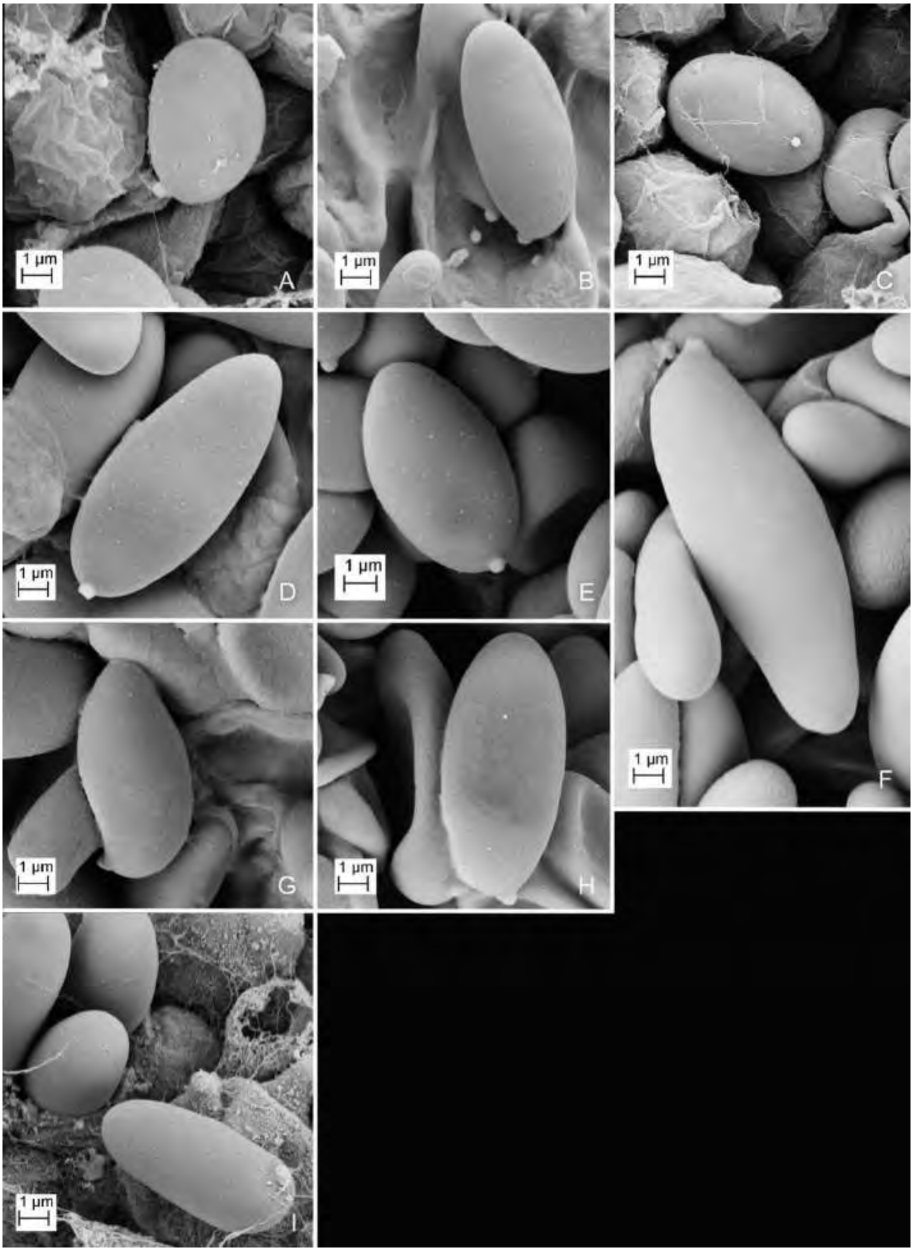

Figure 3. SEM images at 10000X magnification. Species from *Boletus* (Xerocomus) sensu Corner having smooth spores. A. *Boletus aculifer* (spirit), B. *B. blanditus* (sheet), C. *B. hastulifera* (spirit), D. *B. honestus* (sheet), E. *B. intentus* (sheet), F. *B. nugatorius* (sheet), G. *B. polychrous* (sheet), H. *B. sartor* (sheet) and I. *B. variisporus* (spirit). All holotype material.

Discussion

Assessment on the generic concept of Xerocomus in Malesia

Šutara's (2008) morphological investigation and the molecular evidence given by Binder and Hibett (2006) have resulted in a much better defined delimitation of the genus. However, their conclusions were derived from the study of the European species of *Xerocomus*, which often have much larger basidiocarps than the Malesian ones. Many of these other features are not clearly visible in the specimens available for this study, as the majority of the Malesian species of *Xerocomus s.str.* are diminutive and shorter than 50 mm, with the exception of *Boletus* (Xerocomus) *chlamydosporus* Corner. From our study of the taxa that Horak (2011) upgraded to *Xerocomus* in Malesia, we found that only *Xerocomus microcarpoides* has the bacillate spore ornamentation that Šutara (2008) described as one of the features differentiating *Xerocomus* from *Boletus*. All the other samples of *Xerocomus* (Horak, 2011) examined have smooth spores. The only other genus to have bacillate spore ornamentation is *Phylloporus*, of which we have studied the species *Phylloporus rufoflavipes* (*Xerocomus rufoflavipes*). *Phylloporus* has been separated from *Xerocomus* based on the lamellate hymenophore and clusters within the *Xerocomus subtomentosus* group (Binder & Hibett, 2006). Spore ornamentation seems to be the most crucial and defining *Xerocomus*, together with the absence of gills in most taxa. Of the other characters listed by Šutara (2008), the pore size did not quite match our putative *Xerocomus.* The other characters have to be redefined as more *Xerocomus* species are found in Malesia, starting with the knowledge of these five species.

Placement of our study taxa

On the basis of spore ornamentation alone, it can be concluded that *Xerocomus microcarpoides* is the only Malaysian species which should remain in *Xerocomus* whereas all remaining taxa included in our study, with the exception of *Xerocomus rufoflavipes* (Corner) E. Horak now placed in *Phylloporus* (Horak, 2011), should be retained meantime under *Boletus* until molecular sequences from novel DNA isolation methods for old types shed new light on generic delimitations.

In this work, an attempt was also made to sequence material available to us and samples follow general classical degradation as DNA yield gets less with time and from this exercise we can see that specimens from 1930 gave limited and low concentrations of genomic DNA in Qubit readings of 0.0265–0.082ng/µl, compared to those in 1964 and beyond with 0.156–37ng/µl, many of which when sequences yield very fragmented DNA.

There should also be an attempt to gather material from type sites where possible.

A preliminary SEM spore scan of other species described by Corner under *Boletus* subgen. *Xerocomus*, but treated by Horak (2011) as doubtful, rejected or excluded species, yields four other *Xerocomus s.str.* species: *Boletus* (*Xerocomus*) *chlamydosporus* Corner, *Boletus* (*Xerocomus*) *chrysops,Boletus* (*Xerocomus*) *lubricus* Corner and *Boletus* (*Xerocomus*) *microcarpus* Corner.

Taxonomy and nomenclature

As a result of our morphological investigation, we recommend the following nomenclatural changes from Horak's (2011) circumscription of *Xerocomus*:

Xerocomus as defined by Horak (2011) be retained as such:

1. *Xerocomus microcarpoides* (Corner) E. Horak

Xerocomus as defined by Horak (2011) to revert to back to Corner's names:

1. *Boletus albipurpureus* Corner
   Syn.: *Xerocomus albipurpureus* (Corner) E. Horak

2. *Boletus asperipes* Corner
   Syn.: *Xerocomus asperipes* (Corner) E. Horak

3. *Boletus calocystides* Corner
   Syn.: *Xerocomus calocystides* (Corner) E. Horak

4. *Boletus calvus* Corner
   Syn.: *Xerocomus calvus* (Corner) E. Horak

5. *Boletus catervatus* Corner
   Syn.: *Xerocomus catervatus* (Corner) E. Horak

6. *Boletus cuticulatus* Corner
   Syn.: *Xerocomus cuticulatus* (Corner) E. Horak

7. *Boletus cyaneirufescens* Corner
   Syn.: *Xerocomus cyaneirufescens* (Corner) E. Horak

8. *Boletus destitutus* Corner
   Syn.: *Xerocomus destitutus* (Corner) E. Horak

9. *Boletus dispersus* var. *dispersus* Corner
   Syn.: *Xerocomus dispersus* var. *dispersus* (Corner) E. Horak

10. *Boletus dispersus* var*. tembelingensis* Corner
    Syn.: *Xerocomus dispersus* var. *tembelingensis* (Corner) E. Horak

11. *Boletus ferruginosporus* Corner

Syn.: *Xerocomus ferruginosporus* (Corner) E. Horak

12. *Boletus gyrodontoides* Corner
    Syn.: *Xerocomus gyrodontoides* (Corner) E. Horak

13. *Boletus lucescens* Corner
    Syn.: *Xerocomus lucescens* (Corner) E. Horak

14. *Boletus prebadius* Corner
    Syn.: *Xerocomus prebadius* (Corner) E. Horak

15. *Boletus ranunculus* Corner
    Syn.: *Xerocomus ranunculus* (Corner) E. Horak

16. *Boletus raphanolens* Corner
    Syn.: *Xerocomus raphanolens* (Corner) E. Horak

17. *Boletus rectus* Corner
    Syn.: *Xerocomus rectus* (Corner) E. Horak

18. *Boletus satisfactus* Corner
    Syn.: *Xerocomus satisfactus* (Corner) E. Horak

19. *Boletus solitarius* Corner
    Syn.: *Xerocomus solitarius* (Corner) E. Horak

20. *Boletus tentabundus* Corner
    Syn.: *Xerocomus tentabundus* (Corner) E. Horak

Species classified as *Boletus* subgen. *Xerocomus* sensu Corner (1972), but rejected or excluded by Horak (2011), whose spores have bacillate ornamentations, the following new generic upgrades proposed for the following species.

1. *Xerocomus chlamydosporus* (Corner) S.M.L. Lee
   Syn.: *Boletus chlamydosporus* Corner [listed as doubtful species by Horak (2011)]

2. *Xerocomus chrysops* (Corner) S.M.L. Lee
   Syn.: *Boletus chrysops* Corner [listed in the excluded list by Horak (2011)]

3. *Xerocomus lubricus* (Corner) S.M.L. Lee
   Syn.: *Boletus lubricus* Corner [listed in the excluded list by Horak (2011)]

Boletus (*Xerocomus*) *microcarpus* has already been made *Hourangia microcarpa* (Corner) G. Wu, Xue T. Zhu & Zhu L. Yang (Zhu et al., 2015) as it was found to be monophyletic with *Xerocomus s.str.* and *Phylloporus*, differing from the other two genera by a hymenium 3–7 times thicker than the pileal context found in *Xerocomus s.str.* Hence *Xerocomus microcarpoides* which similarly follows the above character could potentially be a *Hourangia*. DNA sequence data from a new collection would be useful to test generic delimitations in the wider group. *Boletus* (*Xerocomus*) *pseudochrysenteron* spores which also has no bacillate ornamentations is currently accepted as *Xerocomellus corneri* Xue T. Zhu & Zhu L. Yang (Wu et al., 2016).

Conclusions

With the evidence of spore ornamentations, it can be concluded that *Xerocomus* s.l. has its presence in Malesia where *Xerocomus chlamydosporus, X. chrysops*, *X. lubricus*, *X. microcarpus and X. microcarpoides* occur. The delimitation based on other aspects of *Xerocomus* besides spores need to be addressed with newer collections as they do not concur with Šutara (2008).

References

Corner EJH. 1972. *Boletus in Malaysia*. Government Printing Office, Singapore, Malaya.

Corner EJH. 1974. *Boletus* and *Phylloporus* in Malaysia: further notes and descriptions. *Gardens' Bulletin, Singapore, 27:* 1–16.

Binder M., Hibbett DS. 2006. Molecular systematics and biological diversification of Boletales. *Mycologia, 98* (6): 971–981. doi:10.3852/mycologia.98.6.971.

Halling RE., Chan HT, Lee SS. 2007. *Basidiomycota: Boletaceae*. In: EBG. Jones, KD. Hyde, S. Vikineswary (eds.) Malaysian Fungal Diversity, Mushroom Research Centre, University of Malaya and Ministry of Natural Resources and Environment, pp. 41–53.

Horak E. 2011. Revision of Malaysian species of Boletales s.l. (Basidiomycota) described by EJH Corner (1972, 1974). *Malayan Forest Records,* 51: 1–283.

Oolbekkink GT. 1991. The taxonomic value of the ornamentation of spores in 'the *Xerocomus*-group' of Boletus. *Persoonia-Molecular Phylogeny and Evolution of Fungi 14,* (3): 245–273.

Quélet L. 1887. Xerocomus. In: Mougeot, A., Ferry, R. & Roumeguère, G. (eds.) La flore des Vosges, Champignons, Algues: In: Louis, L. Le Département des Vosges, Description – Histoire – Statistique. Tome II. La flore des Vosges. Épinal: E. Busy. Pp. 477–478. [in French].

Quélet L. 1888. Flore mycologique de la France. Paris, France. [in French].

Šutara J. 2005. Central European genera of the Boletaceae and Suillaceae, with notes on their anatomical characters. *Czech Mycology, 57*: 1–50.

Šutara J. 2008. *Xerocomus s.l.* in the light of the present state of knowledge*. Czech Mycology, 60*: 29–62.

Watling R. 2001. The relationships and possible distributional patterns of boletes in south-east Asia. *Mycological Research, 105* (12): 1440–1448.

Wu G, Feng B, Xu J, Zhu XT, Li YC, Zeng NK, Hosen MI, Yang ZL. 2014. Molecular phylogenetic analyses redefine seven major clades and reveal 22 new generic clades in the fungal family Boletaceae. *Fungal Diversity*, *69* (1): 93–115. doi:10.1007/s13.

Wu G, Li YC, Zhu XT, Zhao K, Han LH, Cui YY, Li F, Xu JP, Yang ZL. 2016. One hundred noteworthy boletes from China. *Fungal Diversity*, *81:* 25–188.

Zhu XT, Wu G, Zhao K, Halling RE, Yang ZL. 2015. *Hourangia*, a new genus of Boletaceae to accommodate *Xerocomus cheoi* and its allied species. *Mycological Progress, 14* (6): 1–10.

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Appendix

Appendix-I Specimens studied

Barcode	EDNA	Working labels	Collection type	Type	Genus (Corner / Horak)	Taxa	Collector	Prefix	no.	Collection date (dd/mm/yyyy)			Country	Gazetteer
E00458479		SL74	wet	Holo	Boletus /Gyroporus	aculifer	Corner, E.J.H.		s.n.	22	9	1940	Peninsular Malaysia	Pahang, Fraser's Hill
E00083993	EDNA16-0045143	SL01	dry	Holo	Boletus /Xerocomus	albipurpureus	Corner, E.J.H.	P	155	6	8	1972	Borneo	Sarawak, Kuching, Bako NP
E00458457		SL31	wet	Holo	Boletus /Xerocomus	albipurpureus	Corner, E.J.H.	P	155	6	8	1972	Borneo	Sarawak, Kuching, Bako NP
E00084006		SL02	dry	Holo	Boletus /Xerocomus	asperipes	Corner, E.J.H.		s.n.	24	9	1939	Peninsular Malaysia	Johor, Johor Bahru, Tebrau
E00458466		SL40	wet	Iso	Boletus /Xerocomus	asperipes	Corner, E.J.H.		s.n.	24	9	1939	Peninsular Malaysia	Johor, Johor Bahru, Tebrau
E00084008		SL53	dry	Holo	Boletus /[excluded]	blanditus	Corner, E.J.H.		s.n.	25	5	1930	Peninsular Malaysia	Pahang, Fraser's Hill
E00458463		SL37	wet	Holo	Boletus /Xerocomus	calocystides	Corner, E.J.H.		s.n.	26	6	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00458467		SL41	wet		Boletus /Xerocomus	calvus	Corner, E.J.H.		s.n.	6	9	1942	Singapore	Bukit Timah NR
E00458468		SL42	wet	Holo	Boletus /Xerocomus	catervatus	Corner, E.J.H.		s.n.	28	11	1940	Singapore	MacRitchie, Reservoir Jungle
E00084026		SL54	dry	Holo	Boletus /[excluded]	chlamydosporus	Holttum, R.E.		s.n.	27	3	1930	Peninsular Malaysia	Johor, Kota Tinggi, Gunung Panti
E00084025		SL55	dry	Holo	Boletus /[excluded]	chrysops	Corner, E.J.H.		s.n.	18	2	1959	Borneo	Brunei, Temburong, Amo, Ulu Belalong
E00084030	EDNA16-0044928	SL04	dry	Holo	Boletus /Xerocomus	cuticulatus	Corner, E.J.H.	RSNB	5759	12	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458458		SL32	wet	Holo	Boletus /Xerocomus	cuticulatus	Corner, E.J.H.	RSNB	5759	12	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458406	EDNA16-0044929	SL26	dry		Boletus /Xerocomus	cuticulatus	Ana		26748	9	2	1996	Peninsular Malaysia	Negeri Sembilan, Jelebu, Pasoh Forest Reserve
E00084029	EDNA16-0045144	SL03	dry	Holo	Boletus /Xerocomus	cyaneirufescens	Corner, E.J.H.		s.n.	27	12	1930	Singapore	Reservoir Jungle
E00458469		SL43	wet		Boletus /Xerocomus	cyaneirufescens	Corner, E.J.H.		s.n.	8	11	1940	Singapore	Seletar
E00084557	EDNA16-0044931	SL05	dry	Holo	Boletus /Xerocomus	destitutus	Corner, E.J.H.		s.n.	16	11	1930	Peninsular Malaysia	Pahang, Tembeling
E00084558	EDNA16-0044932	SL06	dry		Boletus /Xerocomus	destitutus	Corner, E.J.H.	P	68	0	0	1972	Peninsular Malaysia	Penang, Penang Hill
E00458470		SL44	wet		Boletus /Xerocomus	destitutus	Corner, E.J.H.	P	68	0	0	1972	Peninsular Malaysia	Penang, Penang Hill
E00458454		SL28	dry		Boletus /Xerocomus	destitutus	Watling, R.		24785	23	2	1992	Peninsular Malaysia	Selangor, Gombak, FRIM, Kepong
E00458455		SL29	dry		Boletus /Xerocomus	destitutus	Sims, K.		25593	29	6	1993	Philippines	Luzon, Quezon Province, Quezon NP
E00458407	EDNA16-0044930	SL27	dry		Boletus /Xerocomus	destitutus	Watling, R.		25800	24	2	1994	Peninsular Malaysia	Selangor, Gombak, FRIM, Kepong
E00084567		SL09	dry		Boletus /Xerocomus	dispersus var. dispersus	Corner, E.J.H.		s.n.	20	5	1930	Peninsular Malaysia	Pahang, Fraser's Hill
E00084565		SL07	dry	Holo	Boletus /Xerocomus	dispersus var. dispersus	Corner, E.J.H.	RSNB	8172	8	4	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458459		SL33	wet	Holo	Boletus /Xerocomus	dispersus var. dispersus	Corner, E.J.H.	RSNB	8172	8	4	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458456		SL30	dry		Boletus /Xerocomus	dispersus var. dispersus	Conveny, R.		78	14	4	1984	Australia	New South Wales
E00084566		SL08	dry		Boletus /Xerocomus	dispersus var. dispersus	Corner, E.J.H.		s.n.	1	7	1930	Peninsular Malaysia	Negeri Sembilan, Gunung Angsi

Appendix

Appendix-I Specimens studied

Barcode	EDNA	Working labels	Collection type	Type	Genus (Corner / Horak)	Taxa	Collector	Prefix	no.	Collection date (dd/mm/yyyy)			Country	Gazetteer
E00084568		SL10	dry	Holo	Boletus /Xerocomus	dispersus var. tembelingensis	Corner, E.J.H.		s.n.	26	5	1931	Peninsular Malaysia	Pahang, Tembeling
E00458471		SL45	wet	Holo	Boletus /Xerocomus	ferruginosporus	Corner, E.J.H.		s.n.	3	3	1940	Singapore	Bukit Timah NR
E00458464		SL38	wet	Holo	Boletus /Xerocomus	gyrodontoides	Corner, E.J.H.		s.n.	18	12	1939	Singapore	Bukit Timah NR
E00458480		SL75	wet	Holo	Boletus /Boletochaetae	hastulifera	Corner, E.J.H.		8286	14	4	1964	Borneo	Sabah, Kinabalu, Mesilau
E00084975		SL56	dry	Holo	Boletus /[excluded]	honestus	Corner, E.J.H.		s.n.	29	11	1930	Peninsular Malaysia	Pahang, Fraser's Hill
E00084979		SL57	dry	Holo	Boletus /[excluded]	intentus	Corner, E.J.H.		s.n.	21	3	1931	Singapore	MacRitchie, Reservoir Jungle
E00084980		SL58	dry		Boletus /[excluded]	intentus	Corner, E.J.H.		s.n.	19	8	1939	Singapore	MacRitchie, Reservoir Jungle
E00458481		SL76	wet		Boletus /[excluded]	intentus	Corner, E.J.H.		s.n.	28	11	1940	Singapore	MacRitchie, Reservoir Jungle
E00458490		SL59	dry		Boletus /[excluded]	intentus	Watling, R.	Wat.	25803	27	2	1994	Peninsular Malaysia	Selangor, Gombak, FRIM, Kepong
E00085031		SL61	dry	Holo	Boletus /[excluded]	lubricus	Corner, E.J.H.	RSBN	5612	3	3	1964	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00458482		SL77	wet	Holo	Boletus /[excluded]	lubricus	Corner, E.J.H.	RSBN	5612	3	3	1964	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00458473		SL47	wet		Boletus /Xerocomus	lucescens	no label			0	0	0		
E00085005	EDNA16-0044935	SL13	dry		Boletus /Xerocomus	lucescens	Corner, E.J.H.	RSNB	1866	19	8	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00085004	EDNA16-0044934	SL12	dry		Boletus /Xerocomus	lucescens	Corner, E.J.H.	RSNB	1867	19	8	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00458472		SL46	wet		Boletus /Xerocomus	lucescens	Corner, E.J.H.	RSNB	1867	19	8	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00085003	EDNA16-0044933	SL11	dry	Holo	Boletus /Xerocomus	lucescens	Corner, E.J.H	RSNB	1882	19	8	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00458465		SL39	wet	Holo	Boletus /Xerocomus	lucescens	Corner, E.J.H.	RSNB	1882	19	8	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu, Bembangan river
E00085029	EDNA16-0045763	SL14	dry	Holo	Boletus /Xerocomus	microcarpoides	Corner, E.J.H.		s.n.	23	5	1930	Peninsular Malaysia	Pahang, Fraser's Hill
E00085030		SL62	dry	Holo	Boletus /[excluded]	microcarpus	Corner, E.J.H.	RSBN	8073	2	4	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458483		SL78	wet	Holo	Boletus /[excluded]	microcarpus	Corner, E.J.H.	RSBN	8073	2	4	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458489		SL63	dry		Boletus /[excluded]	microcarpus	Watling, R.	Wat.	25437	22	3	1993	Peninsular Malaysia	Selangor, Gombak, FRIM, Kepong
E00085043		SL64	dry	Holo	Boletus /[excluded]	nugatorius	Corner, E.J.H.		94327	8	8	1960	Borneo	Sabah, Kinabalu, Ranau
E00154954		SL66	dry		Boletus /[excluded]	nugatorius	Woods, P.J.B.		6	30	5	1962	Peninsular Malaysia	
E00085044		SL65	dry		Boletus /[excluded]	nugatorius	Corner, E.J.H.	J	6	11	4	1972	Indonesia	Java, Cibodas
E00458484		SL79	wet		Boletus /[excluded]	nugatorius	Corner, E.J.H.	J	6	11	4	1972	Indonesia	Java, Cibodas
E00085070		SL68	dry	Holo	Boletus /[excluded]	polychrous	Corner, E.J.H.			21	8	1939	Singapore	Bukit Timah NR
E00085069		SL69	dry	Holo	Boletus /[excluded]	polychrous	Corner, E.J.H.			21	8	1939	Singapore	Bukit Timah NR

Appendix

Appendix-I Specimens studied

Barcode	EDNA	Working labels	Collection type	Type	Genus (Corner / Horak)	Taxa	Collector	Prefix	no.	Collection date (dd/mm/yyyy)			Country	Gazetteer
E00458485		SL80	wet	Holo	Boletus /[excluded]	polychrous	Corner, E.J.H.			21	8	1939	Singapore	Bukit Timah NR
E00458413		SL70	dry		Boletus /[excluded]	polychrous	Watling, R.		25808	2	3	1994	Peninsular Malaysia	Negeri Sembilan, Jelebu, Pasoh Forest Reserve
E00458474		SL48	wet	Holo	Boletus /Xerocomus	prebadius	Corner, E.J.H.		s.n.	28	11	1940	Singapore	MacRitchie, Reservoir Jungle
E00085074	EDNA16-0045145	SL15	dry	Holo	Boletus /Xerocomus	pseudochrysenteron	Corner, E.J.H.	RSNB	1565	30	9	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00085075		SL16	dry	Holo	Boletus /Xerocomus	pseudochrysenteron	Corner, E.J.H.	RSNB	1565	30	9	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00458460		SL34	wet	Holo	Boletus /Xerocomus	pseudochrysenteron	Corner, E.J.H.	RSNB	1565	30	9	1961	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00086541	EDNA16-0045146	SL17	dry	Holo	Boletus /Xerocomus	ranunculus	Corner, E.J.H.	s.n.		1	12	1930	Peninsular Malaysia	Pahang, Tembeling
E00086542	EDNA16-0045147	SL18	dry	Holo	Boletus /Xerocomus	raphanolens	Corner, E.J.H.	RSNB	8668	5	5	1964	Borneo	Sabah, Kinabalu, Mesilau
E00086549	EDNA16-0045148	SL19	dry	Holo	Boletus /Xerocomus	rectus	Corner, E.J.H.	RSNB	1869	19	8	1961	Borneo	Sabah, Kinabalu, Fl. Benbangan
E00458461		SL35	wet	Holo	Boletus /Xerocomus	rectus	Corner, E.J.H.	RSNB	1869	19	8	1961	Borneo	Sabah, Kinabalu, Fl. Benbangan
E00086569		SL21	dry		Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5756	12	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00086570		SL22	dry		Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5756	17	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00086571	EDNA16-0045765	SL23	dry		Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5756	13	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458475		SL49	wet		Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5756	13	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00086568	EDNA16-0045764	SL20	dry	Holo	Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5810	16	3	1964	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00458462		SL36	wet	Holo	Boletus /Phylloporus	rufoflavipes	Corner, E.J.H.	RSNB	5810	16	3	1964	Borneo	Sabah, Kinabalu, Mt. Kinabalu
E00088001		SL71	dry	Holo	Boletus /[excluded]	sartor	Corner, E.J.H.	RSBN	5861	18	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458486		SL81	wet	Holo	Boletus /[excluded]	sartor	Corner, E.J.H.	RSBN	5861	18	3	1964	Borneo	Sabah, Kinabalu, Mesilau
E00458476		SL50	wet	Holo	Boletus /Xerocomus	satisfactus	Corner, E.J.H.		s.n.	8	11	1940	Singapore	
E00088005	EDNA16-0045149	SL24	dry	Holo	Boletus /Xerocomus	solitarius	Corner, E.J.H.		s.n.	16	5	1930	Peninsular Malaysia	Pahang, Fraser's Hill
E00458477		SL51	wet		Boletus /Xerocomus	solitarius	Corner, E.J.H.		s.n.	30	6	1940	Singapore	Bukit Timah NR
E00088015		SL25	dry	Holo	Boletus /Xerocomus	tentabundus	Corner, E.J.H.		s.n.	24	9	1939	Peninsular Malaysia	Johor, Johor Bahru, Tebrau
E00458478		SL52	wet	Holo	Boletus /Xerocomus	tentabundus	Corner, E.J.H.		s.n.	24	9	1939	Peninsular Malaysia	Johor, Johor Bahru, Tebrau
E00088033		SL73	dry	Holo	Boletus /[excluded]	variisporus	Corner, E.J.H.	RSBN	2726	3	9	1961	Borneo	Sabah, Kinabalu, Fl. Liwag
E00458487		SL82	wet	Holo	Boletus /[excluded]	variisporus	Corner, E.J.H.	RSBN	2726	3	9	1961	Borneo	Sabah, Kinabalu, Fl. Liwag

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Research Article

Obligate Ant-Associated *Macaranga bancana* is Better Protected from Herbivory Than Facultative Ant-Associated *Macaranga tanarius*

Sze Huei Yek[1,3], Deniece Yin Chia Yeo[2,3], Suyee Sophia Tai[3], Zhi Hoong Wong[4*]

[1] *Institute for Tropical Biology & Conservation, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia.*
[2] *Education Excellence, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia.*
[3] *School of Science, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia.*
[4] *Malaysian Immersion & Pathways, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia.*

*Corresponding author: wong.zhihoong@monash.edu

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ABSTRACT

Protective mutualism between ant and *Macaranga* plants are complex between-species interactions found only in the tropical environment. In such interactions, plants provide housing structures (in the form of domatia) and food (in the form of food bodies) to their ant symbionts. In return, the ants protect their *Macaranga* plant hosts against herbivore attacks. *Macaranga* ant protective mutualism is manifested in a wide range of interactions, from facultative to obligate. In facultative interactions, *Macaranga* plants attract predatory ants to the plant *via* food rewards. In return, foraging ants may opportunistically provide protection from insect herbivores. In obligate interactions, plants provide shelter and food rewards to permanent ant partners. We hypothesize that in obligate *Macaranga,* the host is better protected because of the permanent presence of its resident ant partners, whereas in facultative *Macaranga*, the defense against insect herbivores may be less efficient depending on the attractiveness of the food rewards and the aggressiveness of the ant species in the plant's vicinity. In this study, we compare herbivory damage and phytophagous insect herbivore types between a facultative ant-plant *M. tanarius* and an obligate anti-plant *M. bancana*. This study further highlights that co-evolved ant partners are more efficient in defending their host plants against phytophagous insect herbivores than facultative ant-plant interactions.

Keywords
Facultative; Obligate; Phytophagous Insects; Herbivores; *M. bancana*; *M. tanarius*

Introduction

Phytophagous insects negatively impact a plant’s growth and reproductive success (Marquis, 1984; Marquis & Braker, 1994). In tropical forests, an estimated 11% of the annual leaf area produced is consumed by phytophagous insects and pathogens (Coley & Kursor, 1996) which is equivalent to the plant's investments in reproduction (Bazzaz et al., 1987). Over the course of the plant's defensive evolution, various strategies have been developed (Coley & Barone, 1996), a chief strategy being various kinds of protective mutualism with ant partners (Rosumek et al., 2009).

Protective mutualism between ants and plants is a common strategy adopted by the pioneer tree genus *Macaranga* (Euphorbiaceae), which is found throughout pristine and disturbed forests in Southeast Asia (Whitmore, 2008). Ant interactions within the *Macaranga* genus ranges in specificity from facultative (myrmecophilous) to obligate (myrmecophytic) (Fiala et Al., 1999). Facultative *Macaranga* plants are characterized by deriving their defense against herbivores from free-ranging opportunistic or predatory ants that forage on the plant. In exchange, ants receive direct or indirect food rewards from plants in the form of food bodies (FB) that are scattered widely on the dorsal surfaces of their leaves (Fiala & Maschwitz, 1991). These food bodies are rich in sugars, amino acids, lipids, phenols, alkaloids, and volatile compounds (González-Teuber & Heil, 2009), and have been linked to increase in colony growth and survivorship (Byk & Del-Claro, 2011). On the other end of the mutualism scale, obligate *Macaranga* species are characterized by the presence of FB that are contained within specialized structures known as stipules as well as nesting chambers called domatia that are structuralized by the plants for their specific ant partners (Fiala & Maschwitz, 1991; Linsenmair et al., 2001; Feldhaar & Fiala, 2021).

As pioneer trees typically make up the dominant species in their habitat, they are prone to suffering from proportionately larger amounts of damage from herbivores present. However, ant-associated *Macaranga* species are generally well-defended against all kinds of insect herbivores, such as lepidopteran larvae, beetles and grasshoppers (Fiala et al., 1989; 1994). Nevertheless, herbivore damage is most severe in seedlings and was observed to decrease as the trees grow (Itino & Itioka, 2001). As seedlings have a higher proportion of young, expanded leaves and shoots, the ants' defense is concentrated on these plant parts (Heil et al., 2004) which is consistent with higher secretion of FB and EFN in these plant parts as well (Heil et al., 2000). Although all ant-associated *Macaranga* uses food rewards to elicit ant-defense strategies, there is evidence that indicates defense resulting from facultative interactions are less efficient than defense provided by obligate ones (Fiala et al. 1994, 1989; Linsenmair et al. 2001).

Unlike the opportunistic ant partners of facultative *Macaranga* species, the ant partners of obligate *Macaranga* species are wholly dependent on their host. Each tree is host to only one ant colony (Fiala & Maschwitz, 1990), that are confined within the domatia. Additionally, these obligate ant partners sustain themselves primarily on FBs produced by the plant. Because of this dependency, we hypothesize that obligate ant partners are more invested in the protection of their host plant than the opportunistic ants in facultative relationships. As such we aim to test the following hypothesis; that the obligate *M. bancana* is better protected against insect herbivores than the facultative *M. tanarius*, and also attempt to provide insights on whether the different level of effectiveness is due to the kinds of herbivores that feed on two *Macaranga species*. This study is the first of a series that seeks to examine the protective mutualism of pioneer *Macaranga* species in human-disturbed forest edges.

Methods

Study sites

The sampling areas consisted of three forest edge sites in the State of Selangor, Malaysia (Figure 1). The first site is located at Bukit Nanas (BN - 3°9’N, 101°42’N), the second site at Ayer Hitam Forest Reserve (AH - 3°1’N, 101°37N), and the third site at Ulu Gombak (GB - 3°19’N, 101°45’E). Sampling was carried out between the weekends of June to August 2020. These three sites were composed of secondary growth vegetation, predominantly pioneer tree species such as *Macaranga*. The sites were relatively open and exposed to sunlight with lush plant undergrowth. From June to August, selected trails BN, AH and GB were surveyed by SST, DYCY and SHY for *Macaranga* spp. populations, and the two most abundant species of *Macaranga* were selected for this study, i.e., *Macaranga bancana* and *Macaranga tanarius*.

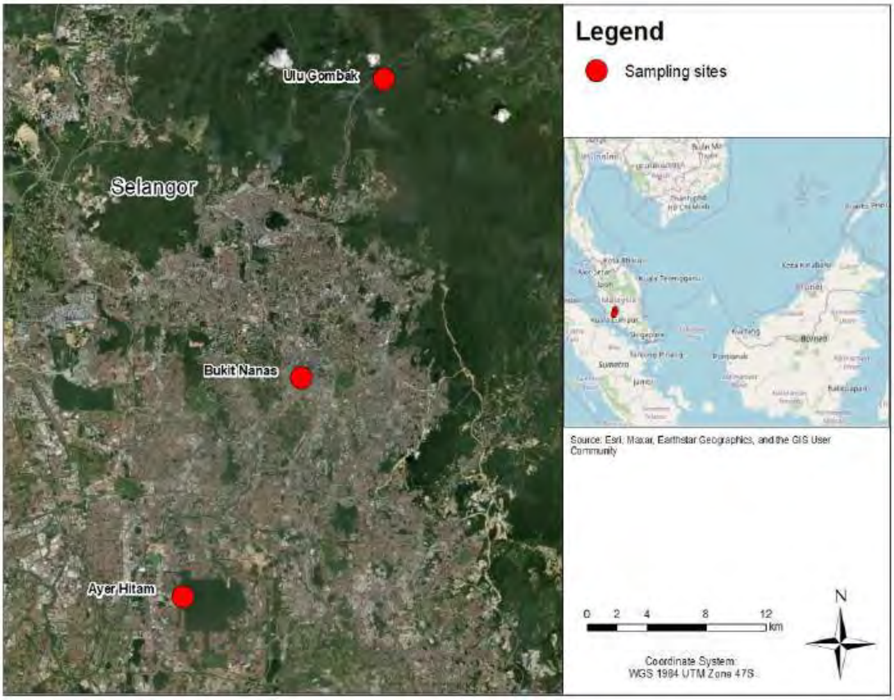

Figure 1. Three sampling sites (black circles) of human-disturbed forest edges in Selangor. The sampling sites are either hiking trails (Bukit Nanas - BN and Ayer Hitam Forest Reserve - AH) or along the road (Ulu Gombak - GB) of the forest tracts.

Macaranga species

Macaranga bancana is a myrmecophytic plant that has an obligate interaction with the *Crematogaster borneensis* ant group (Fiala et al., 1989). Conversely, *Macaranga tanarius* is a myrmecophilous plant that maintains facultative interactions with a wide range of generalist forager and predatory ants (Calixto et al., 2018) such as *Anoplolepis gracilipes* and *Tetraponera sp.* that can be commonly found in the same habitat. In *M. bancana*, nesting space in the form of domatia is provided to the *C. borneensis* ant partner that is not present in *M. tanarius*. Instead, *M. tanarius* possesses 5 extrafloral nectaries on the adaxial edge of its leaves (Fiala & Maschwitz, 1991) and food bodies scattered over leaf surfaces, petioles and stems (Heil et al., 2000) to attract patrolling ants to defend them against herbivores. Extrafloral nectaries are absent in *M. bancana* and while it does produce food bodies, these are protected within specialized structures known as stipules that prevent access to any insect other than its ant partners. In both M. bancana and M. tanarius, development of food bodies substantially reduces when the trees achieve a height of > 3–4m. Additionally in

M. tanarius stops producing extrafloral nectaries altogether once the plant has achieved a height of > 4m (Fiala & Maschwitz, 1991). This suggests that there are growth-stage dependent trade-offs herbivory protection derived from both facultative and obligate ant partners (Fiala et al., 1989; Heil et al., 2000).

Herbivory damage survey

In general, young leaves of obligate and facultative *Macaranga* species rely strongly on biotic (*i.e.,* ants) defense against insect herbivory, whereas more mature leaves employchemical and physical defenses to repel herbivores (Folgarait & Davidson 1995). Because biotic ant defenses tend to be focused on young leaves, and ae no longer prioritized by plants > 3m, we chose the top three leaves of *M. bancana* and *M. tanarius* < 2 m to quantify leaf herbivory. *M. bancana* saplings were visually inspected for the presence of domatia. Plants with no symbiont ant entrance were excluded from the survey as these young saplings cannot house ants' partners until the domatia are structuralized (Frederickson et al., 2012).

Images of the top three leaves were captured by SST using a mobile phone camera and the images were transferred to a desktop computer for processing using ImageJ software (Abràmoff et al., 2004). We excluded leaf area loss during the image processing due to mechanical damage, and only quantified leaf area loss from herbivore damage by identifying the presence of insect feeding marks of external chewers, as well as the track-like patterns left behind by leaf miners along the edges of missing portions on the leaf. The mean herbivory damage of the three leaves was computed, and this value was taken to the herbivory damage of each *Macaranga* tree.

Herbivore type survey

Lepidopteran larvae, beetles (both larvae and adults), as well as grasshoppers, are some of the more common phytophagous insects that can be found on *Macaranga* (Fiala et al., 1989; 1994). However, we were not always able to observe the actual insects on the sampled plants. As such, we attempted to identify the herbivores based on the characteristics of recorded feeding marks (e.g., Shimizu-Kaya et al., 2021). ZHW scored the herbivory types blindly based on the images captured by SST and grouped them into the following categories: (i) large phytophagous insects (BI) that left large sized bite marks; (ii) small phytophagous insects (SI) that left small sized bite marks (Fiala et al., 1989, 1994); and (iii) leaf miners (LM) who make track-like feeding patterns. We also noted down fungal infection on the leaves that were typically present as discoloured spots. For leaf damage that SST and WZH could not confidently assess as being caused by either herbivores or mechanical damage, a category of unknown was assigned. The classification of the herbivory damage from external chewers based on size was done to investigate whether the size of herbivore invaders had any impact on the efficacy of the biotic defenses of *M. bancana* due to the small size of *Crematogaster borneensis* workers (0.36 – 0.43 mm).

Figure 2. Examples of feeding marks left by small phytophagous insects (left) and large phytophagous insects (right) on *M. tanarius* leaves that were sampled from the GB field site.

We observed that one leaf could have more than one type of herbivore damage. We included the type of herbivore damage from all three replicate leaves for one tree in our computation of the herbivory damage data. As such, we found that one tree could have only one type of herbivore damage, or a maximum of five damage types (*i.e.,* large phytophagous insects, small phytophagous insects, leaf miners, fungal infection and unknown).

Statistical analysis

To test the effect of facultative or obligate interactions with ants on the amount of herbivory damage incurred across the two sampled *Macaranga* species, we combined the data from all three sites. We used two independent sample tests to determine whether there was a significant difference in herbivory damage (calculated as leaf-loss percentage) between the two *Macaranga* species. Herbivory data did not violate the assumption of homogeneity of variances (Levene test). However, the herbivory data violated the normality assumption

(Shapiro-Wild test). Q-Q plot demonstrated that the herbivory data had skewed distributed residuals, which precluded the use of frequentist parametric tests.

Since we did not sample *M. tanarius* at AH (Figure 3), we could not perform statistical tests to look at the effect of facultative interactions at this site. As such, this site was excluded in the two-sample comparison tests we conducted. We chose a frequentist non-parametric Mann-Whitney test since the herbivory damage data violated the normality assumption, and we rejected the null hypothesis when $P < 0.0001$ (Table 1). Simultaneously, we also performed Bayesian Mann-Whitney U Test (BayesFactor – BF; Morey & Rounder, 2015) because the Bayesian framework could supplement the frequentist p-value (Rounder et al. 2012). Typically, BF10>1 is used to quantify evidence in favour of the alternative hypothesis. Ultimately, we based our conclusions on the inference of both frequentist (*p*-value) and Bayesian (BF10) tests. All statistical tests were performed using JASP software (version 0.16.4; JASP Team 2022).

To examine the relation between herbivore types and *Macaranga* species, we performed a chi-square test of independence. Chi-square tests of independence were conducted using the statistical software R ver. 4.2.1. (R Core Team, 2022)

Results

Macaranga species at study sites

Altogether, we found 112 *Macaranga* trees across all three sites that fit into our survey criteria, which are trees < 2m in height. At Ayer Hitam Forest Reserve (AH), all *M. tanarius* trees were > 2 m in height and therefore only *M. bancana* were sampled from that site. At both Bukit Nanas (BN) and Ulu Gombak (GB), both *Macaranga* species f similar sizes occurred sympatrically, although the proportion of similarly sized *M. bancana* and *M. tanarius* was uneven. At BN, around 65% of *Macaranga* trees < 2 m were *M. bancana*, whereas at GB, *M. bancana < 2m composed* only 25% of *Macaranga* trees at this field site (Figure 3). In total, 53 *M. bancana* species (AH=24, BN=11, GB=18) and 59 *M. tanarius* (BN=6, GB=53) were surveyed for leaf herbivory damage and scored for herbivory types.

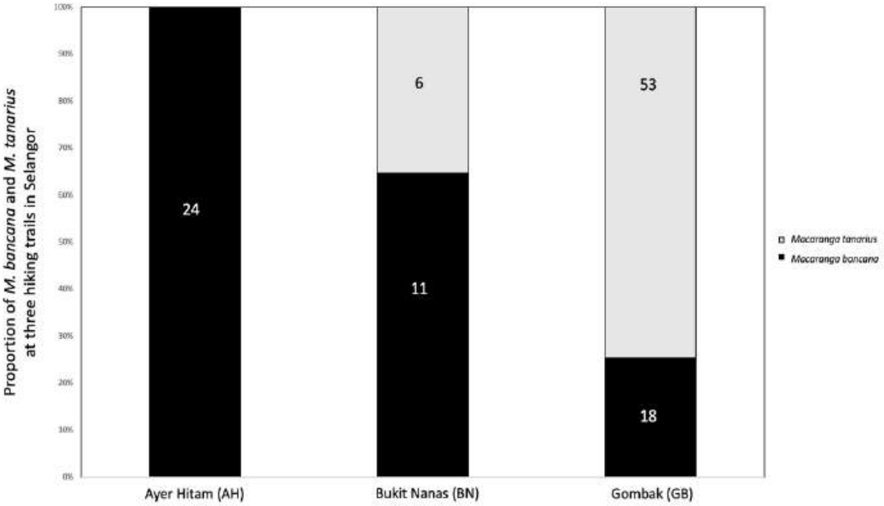

Figure 3. The proportion of *Macaranga bancana* (myrmecophyte) and *Macaranga tanarius* (myrmecophile) sampled at three sampling sites. At Ayer Hitam Forest Reserve (AH), only *M. bancana* were sampled as *M. tanarius* at AH were > 2 m in height, hence excluded from our selection criteria. At BN, *M. bancana* dominates (65%) and at GB, *M. tanarius* dominates (75%).

Table 1. Null hypothesis of no *Macaranga* species effect on herbivory were rejected based on Frequentist (F=765.000, P > 0.001) and Bayesian (BF10 = 84.168) tests.

	Frequentist Test	Statistic	*p*
Herbivory	Mann-Whitney	765.000	< 0.001
	Bayesian Test		BF_{10}
Herbivory	Mann-Whitney U		84.168

	Df	Sum sq	Mean sq	*F*-value	*p*
Macaranga Species	1	128.6	128.58	5.68	0.019*
Site	2	119.5	59.74	2.64	0.076
Species x Location	1	0.7	0.67	0.03	0.864
Residuals	107	2423.5	22.65		

Leaf herbivory damage

The herbivory damage ranged from 0.31% to 24.72%. Two independent tests (Mann-Whitney frequentist and Bayesian; Table 1) revealed that facultative and obligate interactions in *Macaranga* species have a statistically significant effect on leaf herbivory damage. We found that *M. bancana* across all sites experienced less herbivory damage (mean = 3.61%, SD-4.93) than *M. tanarius* (mean=5.76%) (Figure 4).

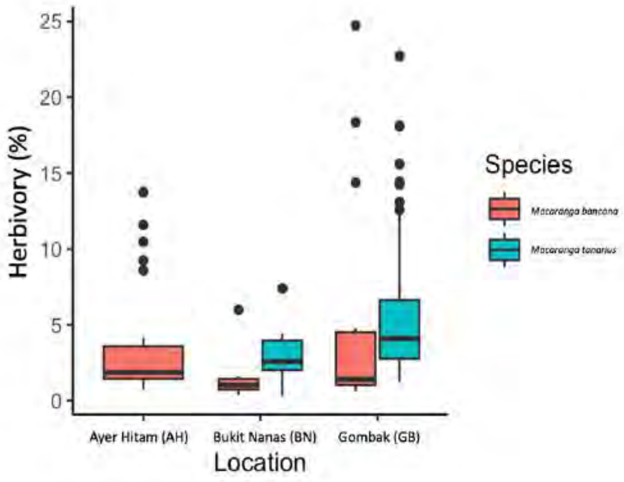

Figure 4. Boxplots displaying the percentage of leaf loss due to phytophagous insect herbivores on myrmecophyte *Macaranga bancana* (red) and myrmecophilous *Macaranga tanarius* (green) across three sampling sites - Ayer Hitam (AH), Bukit Nanas (BN) and Gombak (GB). Only *M. bancana* were surveyed at AH. Leaf loss due to herbivores varies across sites with *M. tanarius* suffering significantly more leaf loss due to herbivores damage compared to *M. bancana*.

Types and numbers of herbivores damage

We observed no significant difference between the categories of 5 herbivore types and the two *Macaranga* species ($\chi 2$=8.85, *p*=0.07). The dominant herbivores types across sites and *Macaranga* species were small phytophagous insects such as lepidopteran larvae (Figure 4). In *M. bancana,* large phytophagous insects caused up to 15.5% leaf herbivory damage, followed by leaf-miners (8.5%). On *M. tanarius*, although the dominant leaf herbivores are small phytophagous insects (48.4%), leaf-miner and large phytophagous insects were observed to be responsible for similar amounts of leaf-herbivory damage as *M. bancana* (21.3% and 20.2% respectively) (Figure 5). The proportion of herbivore type damage appears to vary slightly between sites, although not significantly (Supplementary Figure 1), likely due to slight variations in the composition of insect herbivores at the respective locations. Fungal damage was detected at some sites (AH and GB) but absent from saplings in BN. At AH, fungal infections were detected on *M. bancana* whereas at GB, fungal infections were only detected on *M. tanarius*, albeit both at low infection percentage (6.9% and 3.8% respectively)(supplementary Figure 1).

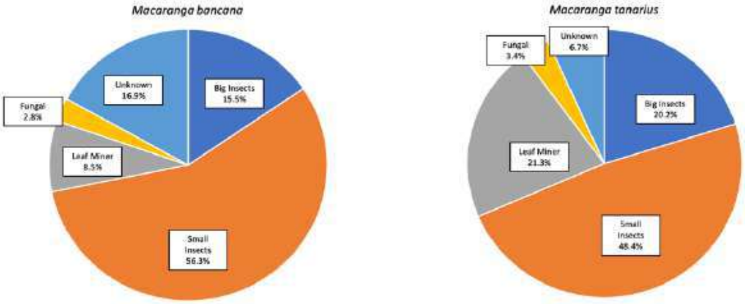

Figure 5. Pie charts of proportion of leaf loss found on *Macaranga bancana* and *Macaranga tanarius* from different herbivore types. The dominant herbivore types are small phytophagous insects (orange), followed by large phytophagous insects (dark blue). In *M. tanarius*, leaf-miner (grey) and large phytophagous insects (dark blue) cause a similar proportion of herbivore damage. Fungal infection (yellow) were observed from both *M. bancana* and *M. tanarius*.

Discussion

This study characterizes the herbivory damage and herbivore types experienced by two pioneer *Macaranga* species that often occur sympatrically in hiking trails and human-disturbed forest edges. The proportion of similar aged *M. bancana* and *M. tanarius* were unevenly distributed ross the three study sites. For example, at AH, only *M. bancana* was sampled as it dominated the landscape and we could not locate any *M. tanarius* (< 2m height) that fit our selection criteria. *M. bancana* also dominated the population at BN (around 65%) whereas *M. tanarius* dominated at GB (around75%) (Figure 3). The herbivory damage ranged from 0.31% to 24.72% with *M. bancana* across all sites experiencing less herbivory damage (mean=3.61%) than *M. tanarius* (mean=5.76%) (Figure 4). The dominant herbivore types across sites and *Macaranga* species are small phytophagous insects, followed by large phytophagous insects and leaf miners (Figure 4). The proportion of herbivore type damage appears to vary between sites, although not significantly (Supplementary Figure 1).

Macaranga distribution at secondary forest sites

Both *Macaranga* species are light-demanding pioneer trees that naturally grow in secondary forest, along riverbanks or in forest gaps (Feldhaar & Fiala, 2021). In our survey, both species occurred sympatrically. These forest sites are all remnants of once larger tracts of primary forests, which have undergone land encroachment from different types of human activities (Omar et al., 2013; Nurul-Shida et al., 2014; Salleh et al., 2017). There are only two common *Macaranga* species that persist at the forest edges and hiking trails at these sites. The composition of these two *Macaranga* species varies with *M. bancana* saplings dominating at BN and *M. tanarius* dominating at GB (Figure 2) possibly due the interplay of abiotic (*e.g.,* light, soil, drainage) and biotic (ants availability, herbivory, competition from other plants) factors. Future studies can benefit from surveying *Macaranga* species at different growth stages, and examining the trade-offs of protective strategies employed by *Macaranga* species at different growth stages.

Ants role in reducing leaf herbivory damage in Macaranga species

We found that the obligate *M. bancana* species experienced less herbivory damage than the facultative *M. tanarius* (Figure 4). These findings are consistent with evidence from past ant-exclusion experiments that have revealed similar results (Fiala et al., 1994; 1989; Linsenmair et al., 2001). One of the reasons obligate plants are better protected could be due to the different ant-defense strategies. In obligate *Macaranga* species, ants association is established early on in the plants' ontogenetic stages (ants were observed colonizing 5.5 cm tall seedlings). The relationship between the ant symbionts and their plant hosts remains stable throughout the ontogenetic development of the host plant. Hence, protection against phytophagous insects remains consistent across plants of similar sizes that are able to provide sufficient shelter for the ants in the form of domatia, and sustenance in the form of food bodies (Itino & Itioka, 2001). In contrast, facultative *Macaranga* species have adopted an induced biotic defense strategy *via* regulating FBs and EFNs secretion based on herbivory damage level (Lange et al., 2017) as well as the plants' sizes (Heil et al., 2000; 2004; Itino et al., 2001; Murase et al., 2003). The effectiveness of employing opportunistic and predatory ant species as a form of herbivory defense in facultative *Macaranga*, thus, depends on the composition and aggressiveness of ants available in the vicinity (Del-Claro & Marquis, 2015; Fagundes et al., 2017).

Ants partners' time investment in removing phytophagous insects could play an important role in minimizing herbivore damage on host plants. In obligate *Macaranga* species, symbiont ant partners rely solely on the FBs provided by their host plant (Fiala & Maschwitz, 1991) for sustenance. Herbivorous insects that are encountered, are therefore, not hunted and consumed, but were observed to have been dragged away by the ants, and then thrown off the plant surface (Linsenmair et al., 2001)(Supplementary Figure 2). In contrast, myrmecophilous *Macaranga* ants feed on EFNs and various other types of resources, for example, hemiptera exudates (‘honeydew’), captured phytophagous insects, and dead arthropods (Hölldobler & Wilson 1990; Blüthgen & Feldhaar 2010; Cerdá & Dejean 2011) while they are on the host plant, reducing the time investment into phytophagous insects removal. Future studies should record the ants composition, aggressiveness and time spent performing different foraging activities on their respective *Macaranga* host plants.

Herbivore types and numbers on Macaranga species

We employed an indirect quantification method in our classification of the phytophagous insects types. From the bite sizes and patterns of feeding marks, we found that the dominant herbivory types for *M. bancana* and *M. tanarius* are small phytophagous insects (Figure 5), which is consistent with Fiala et al. (1994) findings. Large phytophagous insects were the second dominant herbivory type (Figure 5) although the *Crematogaster borneensis* ant symbionts that dwell on the obligate *M. bancana* was found to be ineffective in removing these types of phytophagous insects (Fiala et al., 1989). The effectiveness of ant partners in removing large phytophagous insects on *M. tanarius* would vary with different ant associations (Itino et al., 2001; Heil et al., 2004; Murase et al., 2003). Hence, the variation in the proportion of herbivore types between sites (Supplementary Figure 1) could be due to the relative aggressiveness of the attracted ant species on the *M. tanarius plants which we sampled* (Supplementary Figure 1b) and/or spatial distribution and diversity of phytophagous insect assemblages at different sites (*e.g.* Oliveira & Del-Claro 2005). Future studies should aim to determine the particular phytophagous insect species specific to *M. bancana* or *M. tanarius* at different sites. A methodology that combines direct observations, rearing (Ødegaard et al., 2005; Weiblen et al., 2006) and recovery of environmental DNA from the external foliar feeding marks (Kudoh et al., 2020) can be employed for such purposes.

Acknowledgments

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References

Abràmoff MD, Magalhães PJ, Ram SJ. 2004. Image processing with ImageJ. *Biophotonics Int.*, *11* (7): 36–42.

Bazzaz FA, Chiariello NR, Coley PD, Pitelka LF. 1987. Allocating resources to reproduction and defense. *BioScience, 37* (1): 58–67.

Blüthgen N, Feldhaar H. 2010. Food and shelter: how resources influence ant ecology. In: Lach L, Parr C, Abbott K, editors. *Ant ecology*. Oxford: Oxford Academic; pp.115–136.

Byk J, Del-Claro K. 2011. Ant–plant interaction in the Neotropical savanna: direct beneficial effects of extrafloral nectar on ant colony fitness. *Popul. Ecol.*, *53* (2): 327–332.

Calixto ES, Lange D, Del-Claro K. 2018. Protection mutualism: an overview of ant-plant interactions mediated by extrafloral nectaries. *Oecologia Aust., 22* (4): 410–425.

Cerdá X, Dejean A. 2011. Predation by ants on arthropods and other animals. In: Polidori C, editor. *Predation in the Hymenoptera: an evolutionary perspective.* Kerala (India): Transworld Research Network; pp. 39–78.

Coley PD, Barone J. 1996. Herbivory and plant defenses in tropical forests. *Annual review of ecology and systematics, 27:* 305–335.

Coley PD, Kursor TA. 1996. Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological trade-offs. In: Mulkey SS, Chazdon RL, Smith AP, editors. *Tropical forest plant ecophysiology.* Boston (MA): Springer; pp. 305–336.

Del-Claro K, Marquis RJ. 2015. Ant species identity has a greater effect than fire on the outcome of an ant protection system in Brazilian Cerrado. *Biotropica, 47* (4): 459–467.

Fagundes R, Dáttilo W, Ribeiro S, Rico-Gray V, Jordano P, Del-Claro K. 2017. Differences among ant species in plant protection are related to production of extrafloral nectar and degree of leaf herbivory. *Biol. J. Linn. Soc., 122* (1): 71–83.

Feldhaar H, Fiala B. 2021. Ant Plants: Macaranga. In: Starr CK, editor. *Encyclopedia of Social Insects.* Cham (Switzerland): Springer; pp. 41–45.

Fiala B, Grunsky H, Maschwitz U, Linsenmair KE. 1994. Diversity of ant-plant interactions: protective efficacy in Macaranga species with different degrees of ant association. *Oecologia, 97* (2): 186–192.

Fiala B, Jakob A, Maschwitz U, Linsenmair KE. 1999. Diversity, evolutionary specialization and geographic distribution of a mutualistic ant-plant complex: Macaranga and Crematogaster in South East Asia. *Biol. J. Linn. Soc.*, *66* (3): 305–331.

Fiala B, Maschwitz U. 1991. Extrafloral nectaries in the genus Macaranga (Euphorbiaceae) in Malaysia: comparative studies of their possible significance as predispositions for myrmecophytism. *Biol. J. Linn. Soc., 44* (4): 287–305.

Fiala B, Maschwitz, U. 1990. Studies on the South East Asian ant-plant association *Crematogaster borneensis/Macaranga:* Adaptations of the ant partner. *Insectes Sociaux, 37:* 212–231.

Fiala B, Maschwitz U, Pong TY, Helbig AJ. 1989. Studies of a South East Asian ant-plant association: protection of Macaranga trees by *Crematogaster borneensis*. *Oecologia, 79* (4): 463–470.

Folgarait PJ, Davidson DW. 1995. Myrmecophytic Cecropia: antiherbivore defenses under different nutrient treatments. *Oecologia, 104* (2): 189–206.

Frederickson ME, Ravenscraft A, Miller GA, Arcila Hernández LM, Booth G, Pierce NE. 2012. The direct and ecological costs of an ant-plant symbiosis. *Am. Nat., 179* (6): 768–778.

González-Teuber M, Heil M. 2009. Nectar chemistry is tailored for both attraction of mutualists and protection from exploiters. *Plant Signal. Behav.., 4* (9): 809–813.

Heil M, Feil D, Hilpert A, Linsenmair KE. 2004. Spatiotemporal patterns in indirect defence of a South-East Asian ant-plant support the optimal defence hypothesis. *J. Trop. Ecol.,* 20 (5): 573–580.

Heil M, Fiala B, Baumann B, Linsenmair K. 2000. Temporal, spatial and biotic variations in extrafloral nectar secretion by *Macaranga tanarius*. *Funct. Ecol., 14* (6): 749–757.

Hölldobler B, Wilson EO. 1990. *The ants.* Cambridge (MA): Belknap Press of Harvard University Press.

Itino T, Itioka T. 2001. Interspecific variation and ontogenetic change in antiherbivore defense in myrmecophytic Macaranga species. *Ecol. Res., 16* (4): 765–774.

Kudoh A, Minamoto T, Yamamoto S. 2020. Detection of herbivory: eDNA detection from feeding marks on leaves. *Environ. DNA.*, *2* (4): 627–634.

Lange D, Calixto ES, Del-Claro K. 2017. Variation in extrafloral nectary productivity influences the ant foraging. *PloS one, 12* (1): e0169492.

Linsenmair KE, Heil M, Kaiser WM, Fiala B, Koch T, Boland W. 2001. Adaptations to biotic and abiotic stress: Macaranga-ant plants optimize investment in biotic defence. *J. Exp. Bot., 52* (363): 2057–2065.

Marquis RJ. 1984. Leaf herbivores decrease fitness of a tropical plant. *Science, 226* (4674): 537–539.

Marquis RJ, Braker HE. 1994. Plant-herbivore interactions: diversity, specificity, and impact. In: McDade LA, Hartshorn GS, Hespenheide HA, Bawa KS, editors. La Selva: *ecology and natural history of a neotropical rainforest.* Chicago: University of Chicago Press; pp. 261–281.

Murase K, Itioka T, Nomura M, Yamane S. 2003. Intraspecific variation in the status of ant symbiosis on a myrmecophyte, Macaranga bancana, between primary and secondary forests in Borneo. *Popul. ecol.*, *45* (3): 221–226.

Nurul-Shida S, Faridah-Hanum I, Wan Razali W, Kamziah K. 2014. Community structure of trees in Ayer Hitam Forest Reserve, Puchong, Selangor, Malaysia. *Malaysian For., 77* (1): 73–86.

Ødegaard F, Diserud OH, Østbye K. 2005. The importance of plant relatedness for host utilization among phytophagous insects. *Ecol. Lett., 8* (6): 612–617.

Oliveira P, Del-Claro K. 2005. Multitrophic interactions in a neotropical savanna: ant-hemipteran systems, associated insect herbivores and a host plant. In: Burslem DFRP, Pinard MA, Hartley SE, editors. *Biotic interactions in the tropics: their role in the maintenance of species diversity.* Cambridge (UK): Cambridge University Press; pp. 414–438.

Omar H, Hashim R, Bhassu S, Ruedi M. 2013. Morphological and genetic relationships of the *Crocidura monticola* species complex (Soricidae: Crocidurinae) in Sundaland. *Mamm. Biol., 78* (6): 446–454.

R Core Team. 2022. R: A language and environment for statistical computing. v4.2.1. Available at https://www.R-project.org/ (accessed 16 August 2022)

Rico-Gray V, Oliveira PS. 2008. *The ecology and evolution of ant-plant interactions.* Chicago: University of Chicago Press.

Rosumek FB, Silveira FA, de S Neves F, de U Barbosa NP, Diniz L, Oki Y, Pezzini F, Fernandes GW, Cornelissen T. 2009. Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. *Oecologia, 160* (3): 537–549.

Salleh N, Azeman S, Kiew R, Kamin I, Kong RC. 2017. Plant checklist of the Bukit Nanas Forest reserve, Kuala Lumpur, Malaysia. *One Ecosyst., 2:* e13708.

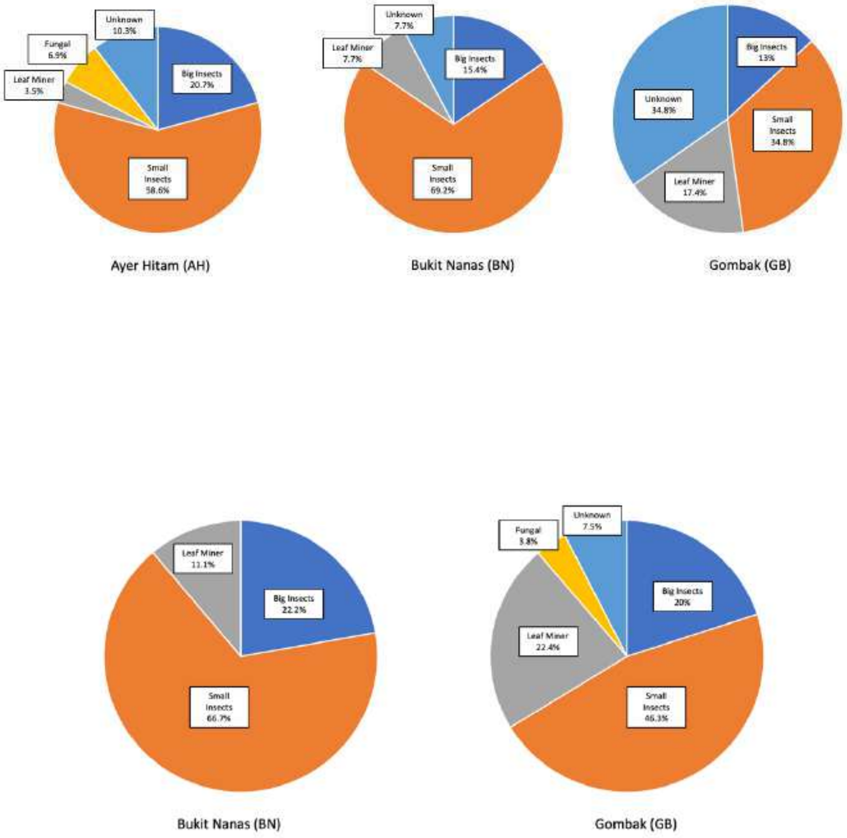
Shimizu-Kaya U, Itioka T, Meleng P. 2021. Difference in leaf herbivory between two plant-ant taxa associating with a myrmecophytic species, *Macaranga lamellata*. *Asian Myrmecol., 14:* e014003.

Weiblen GD, Webb CO, Novotny V, Basset Y, Miller SE. 2006. Phylogenetic dispersion of host use in a tropical insect herbivore community. *Ecology, 87* (sp7): S62–S75.

Whitmore TC. 2008. *The genus Macaranga: a prodromus.* Richmond (UK): Royal Botanic Gardens.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.



Supplementary Figure 1a & 1b. Pie charts of proportion of leaf loss found on (1a) *Macaranga bancana* and (1b) *Macaranga tanarius* across sites from different herbivore types. The proportion of herbivore type damage appears to vary between sites, although not significantly. Fungal damage was detected on some sites (AH and GB) but absent from saplings in BN. At AH, fungal infections were detected on *M. bancana* (a) whereas at GB, fungal infections were detected on *M. tanarius*, albeit both at low infection percentage.

Supplementary Figure 2. photo of obligate ant-partners from the *Crematogaster borneensis* group removing an insect larva from the leaf of *Macaranga bancana*.

[Raw data] Phytophagous insects scoring (sheet 1) from three sampling sites (AH, BN and GB). The top three leaves of each tree were scored for the phytophagous herbivore types according to feeding marks. Small Insects (SI) denotes leaf loss due to small phytophagous insects, such as lepidopteran larvae. Large Insects (BI) denotes leaf loss due to large phytophagous insects, such as phasmid and grasshopper. Leaf miner (LM) makes characteristic track-like marks on the *Macaranga* leaves. Fungal infections were noted as well. If the feeding marks from herbivores could not be determined, we scored the damage as ‘unknown’. Sheet 2 is the processed data of herbivory leaf loss from three sampling sites (AH, BN and GB). Leaf-loss percentages were computed from ImageJ analyses. Leaf loss area due to mechanical damage were excluded in the calculation of leaf loss area. We took the mean of the three top leaves as the mean leaf-loss from herbivore damage for each tree.

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Research Article

Diversity and Aboveground Carbon Stocks of Trees and Understorey Plant Species in Matalom, Leyte, Philippines

Carollina S. Gumela, Jay Ann G. Lantajo, Pearl Aphrodite Bobon-Carnice*

Department of Natural Sciences, Eastern Visayas State University, Arch. Lino R. Gonzaga Avenue, Tacloban City, Leyte, Philippines.

*Corresponding authors: pearl.carnice@evsu.edu.ph

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ABSTRACT

The biggest threats to plant species degradation must be the cutting of trees, conversion into croplands, and natural circumstances. Degradation of land cover has negatively affected plant diversity before the carbon stocks. This study utilized a stratified random sampling technique and allometric equation to determine the plant species, diversity level, and aboveground carbon stocks of trees and understorey found in Matalom, Leyte, Philippines. Results showed thirteen (13) trees and forty-nine (49) understorey species were present in the sampling area. The abundance of species or the total number of individuals per species of trees and understorey is 98 and 2814 respectively. For the diversity index, the result was led by tree species, then understorey species, respectively 1.76, interpreted as very low, and 2.83, which is moderate based on the Modified Fernando Biodiversity Scale. The species evenness, 0.69 and 0.73, was led by understorey plant species. Both signify unequal distribution of plant species in the study area. Moreover, tree species have a higher carbon stock of 34.15 t C ha^{-1} compared to understorey, contributing about 0.03 t C ha^{-1}. There are significant differences in the diversity and c-stocks between trees and understorey plant species based on independent t-test results.

Keywords: Species diversity; richness; evenness; abundance; aboveground carbon stocks

Introduction

Plant species diversity is strongly linked to the various functions of our ecosystems. Plants serve as an ecological buffer against undesirable events such as drought (Li et al., 2017). Plants are the primary source of food, medicine, livestock feed, and provide other essential products and services, including fibers, timber and clean water, and they also help prevent soil erosion (Corlett, 2016). However, as forests are destroyed, plants are also destroyed, affecting the ecosystem's cycle and societal function (Sorecha and Deriba, 2017).

On the other hand, above- and below-ground carbon stores make up terrestrial carbon stocks. The aboveground carbon stocks comprise plant parts and aboveground biomass, such as stems, twigs, leaves, vines, epiphytes, and understorey. The amount of carbon in a pool or a plant's body system is referred to as carbon stock. It is necessary to protect and manage our plant species for future ecosystem restoration to maintain and manage our diverse plant species to restore ecosystems for future uses (Li et al., 2017). Hence, increased carbon stocks sequestered from the atmosphere could be achieved by improving woody vegetation and expanding forest growth to reduce carbon emissions and global warming (Rahayu et al., 2016).

Recent research exposes Jubilee Cross Hill's situation considering its trees, understorey plant species diversity, and aboveground carbon stocks. According to natives, Jubilee Cross Hill contained many tall-huge trees and cogon grass. However, as an unintended fire occurred, a few trees grew and were brought back to life. With that, people near the area utilize the land by converting some parts of the hill into agricultural lands, such as croplands, as a source of food for the people living near Jubilee Cross Hill, along with other primary sources of income for the residents in Barangay San Juan, Matalom such as farming, sinamay weaving, and different types of labour. One of the agricultural processes is slash-and-burn (kaingin) and cutting some trees.

The person in charge of Matalom LGU information said that no government agency bureau focuses on forest management in the municipality of Matalom. Therefore, when it comes to issuing permits for converting some parts of land into cropland, the process will occur first in the barangay. However, people who transform the land into croplands do not ask permission from the barangay since the land is privately owned. Conjointly, only 10 m x 15 m on the top part of the hill is donated, according to the caretaker in charge of the Jubilee Cross.

Further, Jubilee Cross Hill also has the quality of becoming a tourist spot since the top of the hill offers a relaxing view of the seas, Canigao Island, and is a perfect spot for sunset viewing. It is also often visited by the people of Matalom and from nearby towns. However, some parts are relatively degraded, trees are cut, and the land is converted into croplands, which may end in the worst situation if ignored. It is in this regard this study is designed to identify the plant species and diversity level and quantify of the aboveground carbon stocks in Jubilee Cross Hill.

Additionally, investigating plant species diversity and aboveground carbon stocks in trees and understorey is a crucial area of research in the Philippines. It provides vital information for assessing plant species diversity levels and aboveground carbon stocks significantly influenced by anthropogenic activities along with climatic, topographic, and grazing pressures (Rahman et al., 2021). It is critical for policymaking and recommendations for sustainable environmental practices. However, despite its significance, this area remains understudied.

Considering this research gap, this study determines the plant species, diversity levels, and aboveground carbon stocks in Jubilee Cross Hill. The study employed scientific and local names to identify the plant species accurately. Additionally, the richness, abundance, species evenness, and the Shannon-Wiener diversity index were used as crucial indices to identify the diversity levels accurately. The findings of this study have implications for future research and environmental management in the Philippines. This underscores the need for more comprehensive studies that examine the possible relationships between plant species diversity and aboveground carbon stocks, and the effects of human activities on these variables. Such studies will better understand the ecological processes that underlie plant species diversity and carbon stocks and inform more effective policies and recommendations for sustainable environmental practices.

Methodology

Study Site

The study site (Figure 1) is in Jubilee Cross Hill, Matalom, Leyte, with a geographical position of 10°15'43.2" North latitude and 124°49'45.2 East longitude. The site was selected based on observable threats of degradation due to land conversion into croplands which is believed to be a dipterocarp forest as tree species of such forest can be easily seen in the area. Matalom is located on the southwest coast of Leyte, approximately 22 km north of Maasin, and has a type IV climate category (Pomeroy, 1987; Bendimerad et al., 2014).

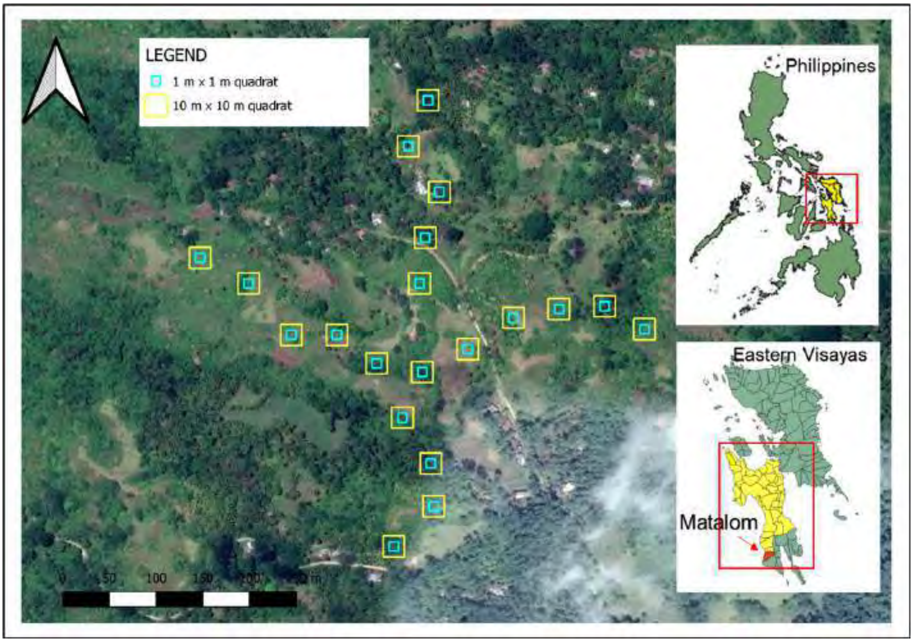

Figure 1. Study sites in Jubilee Cross Hill, Matalom, Leyte, Philippines.

Ethics Statement

The researcher asked for consent and informed the owner of the land and officials of the barangay before the study was conducted. Personal information remains anonymous and confidential.

Data Collection

A stratified random sampling technique was used in this study to establish 20 plots of 10 m x 10 m for trees and 20 subplots of 1 m x 1 m for understorey to determine plant species diversity and aboveground carbon stocks. A 250 metre transect line on each side (North, South, East, West) was established using a tie straw, and for every 50 metres, plots were allocated randomly in the study area. In the established 10 m x 10 m representative sampling plots, all trees with a diameter at breast height (1.3 m aboveground) of at least 10 cm were identified, measured using a diameter- tape (D-tape), and recorded. Photos were also taken from each tree species (Bobon-Carnice & Lina 2017; 2021). The diameters were measured separately above the swelling for tree species that branched at breast height, and the average measurements were recorded. For trees that forked below 1.3 m, the individual stem was separately measured and treated as two trees (Sintayehu et al., 2020).

All understorey plant species within 1 m x 1 m sub-plots were recorded, counted, photographed, and clipped to 1 cm stubble height using scissors. The clipped understorey was put in the paper bags and brought to the washing area. The understorey plant species were thoroughly washed with tap water and distilled water. Then, it was air-dried until there was no more water dripping. Fresh weight was recorded using a digital weighing scale. The collected understorey materials were put into the oven and dried at 65 degrees Celsius for approximately 24 hours until constant weight. Finally, the dried materials of understorey plant species were weighed and recorded (Sintayehu et al., 2020; Bobon-Carnice & Lina, 2017; 2021). Sampling was done between September and October 2021 for tree species and from January 22 to 31, 2022, for understorey plant species, after Typhoon Odette.

Books on Weeds in Irrigated and Rainfed Lowland Ricefields in the Philippines (Donayre et al., 2018), Weeds of Vegetables and other Cash Crops in the Philippines (Donayre et al.,2019), Ecosystems Research and Development Bureau. Common Weed Species with Medicinal Uses (2012), Forest Management Bureau-Department of Environment and Natural Resources (2007) Greenbook 2: Procedures and Technique in Planting, Forest Tree Species with Medicinal Uses (2002), and website www.phytoimages.siu.edu were utilized and serve as instruments for the initial identification of plant species scientific names. We also consulted with experts in plant taxonomy from DENR Region 8, Tacloban, City, to validate the initial identification of plant species.

Data and Statistical Analysis

Plant species richness was obtained as the number of species in the study site. Species abundance was obtained as the number of individuals per species in the study area (Locey and White, 2013). Plant species diversity was calculated using the Shannon - Wiener Diversity Index (H') (Whittaker, 1960; Sintayehu et al., 2020).

$$H' = -\sum_{i=1}^{S} pi\, ln\, pi$$

Where *pi* is the proportion of species *i*, *In* is the natural log, and *S* is the number of species in a specified area. A greater value of the Shannon-Weiner Diversity index (H') indicates a higher species diversity. A computed result of the Shannon - Wiener Diversity Index (H') was interpreted using the modified Fernando Biodiversity Scaling System (Fernando 1998) as cited by Aureo et al. (2020) where diversity levels 3.50 – 4.00, 3.00 – 3.49, 2.50 – 2.99, 2.00 – 2.49, and 1.99 and below was interpreted as very high, high, moderate, low, and very low respectively. Plant species evenness was estimated using Pielou's index (Pielou, 1966; Sintayehu et al., 2020):

$$J' = \frac{H'}{ln\, S}$$

Where: H' is the Shannon-Weiner Diversity index, and S is the total number of species. The interval of this index ranges from 0 to 1, where 0 is degenerate distribution, and 1 is complete evenness (uniform) (Pielou, 1966; Sintayehu et al., 2020; Kvålseth, 2015):

The relative abundance of plant species in the study site was calculated using the equation described by Garg et al. (2020):

$$\text{Relative abundance (\%)} = \frac{\text{Abundance of indidual species}}{\text{Total abundance of all species}} \text{ x } 100\%$$

Aboveground carbon assessment of trees

The allometric equation was used to estimate carbon stocks in each tree species within 10 m x 10 m representative sampling plots. The aboveground biomass of all trees with DBH ≥ 10 cm was calculated with the following equation by Brown (1997) (as cited by Cairns et al., 2003):

$$Y = \exp [-1.996 + 2.32*\ln (dbh)]$$

Where Y is aboveground per tree biomass (kg), dbh is the diameter at breast height (cm). And the results were converted to tons per hectare (1 ton =1,000 kg, 1 ha = 10,000 $\mathrm{m^2}$) (Sintayehu et. al., 2020). And the carbon content was calculated through the following the equation also (Bobon-Carnice, 2017):

$$\text{C storage (t } \mathrm{ha^{-1}}\text{) = Total biomass/ha x 0.45}$$

Understorey plant species aboveground carbon assessment

The carbon content was calculated as 50% on the dry biomass of understorey species. The carbon stock of dry biomass of understorey species was calculated with the following formula:

$$W_c = W_o \text{ x } 0.5$$

Where W_c is the weight of carbon in understorey biomass (g), W_o is the oven-dry weight of aboveground biomass, and 0.5 is the estimated carbon percentage in dry plant biomass. Moreover, the results of the samples were converted to tons per hectare (1 ton = 1,000,000 g) (Bobon-Carnice, 2017; Sintayehu et al., 2020).

For the statistical analysis, an independent t-test was used to determine if there were significant differences in the diversity and C-stocks between trees and understorey plant species since the two samples (trees and understoreys) under comparison are independent of each other. The non-directional (two-tailed) deviation was used as the null hypothesis does not specify in which the two samples differ. The alpha level used was 0.05. Wherein if the t-test result in the statistical analysis of data is larger than 0.05, the null hypothesis will be accepted, and if the t-test result is less than 0.05, then the null hypothesis will be rejected (Kim, 2015).

Results

Plant Species Diversity

Table 1 shows the different tree species found in the study sites and their relative abundance. The total number of individuals in all species of trees is 98. The most abundant species is *Swietenia macrophylla* (Mahogany), which has 44 individuals and has 44.90% relative abundance from the total number of individuals in all species of trees. While the least abundant species of trees found in all quadrats with the lowest relative abundance were also recorded for *Parishia insignis* (Badbad), *Mangifera indica* (Mangga), *Artocarpus blancoi* (Tipo), and *Melicope triphylla* (Tonggamos) with only one (1) total number of individuals found in each species and 1.02% relative abundance.

Aside from that, Table 2 also presents the different species of understoreys in the study area and their relative abundance. The total number of individuals in all species is 2814. The most abundant understorey plant species with the highest relative abundance was also recorded for *Urochloa mutica* (Miligoy), with 590 individuals and 20.97% relative abundance. In comparison, the least abundant species of understorey with the lowest relative abundance were recorded for *Phaius tankervilleae* (Banti), *Convolvulus arvensis* (Bagon-Bagon), *Colocasia esculenta* (Gabi-Gabi), *Mimosa pudica* (Hibi-Hibi), *Alternanthera sessilis* (Banwa), and *Ambrosia psilostachya* (Korhito), both with one (1) total number of individuals and with the relative abundance of 0.04%.

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Table 1. Tree species and relative abundance in the study area.

Species Name		Total Number of Individuals per species (Abundance)	Relative Abundance (%)
Local Name	Scientific Name		
Mahogany (Mahogany)	*Swietenia macrophylla*	44	44.9
Hambabawd (Leichhard Tree)	*Nauclea orientalis*	19	19.39
Lagnob (Hauili)	*Ficus septica*	10	10.2
Gemelina (White teak)	*Gmelina arborea*	9	9.18
Abgaw (Fragrant premna)	*Premna odorata*	4	4.08
Nangka (Jackfruit)	*Artocarpus heterophyllus*	3	3.06
Baganga (Bagalunga)	*Melia dubia*	2	2.04
Tugas (Molave)	*Vitex parviflora*	2	2.04
Badbad “Layang-layang (Red Dhup)	*Parishia insignis*	1	1.02
Lubi (Coconut)	*Cocos nucifera*	1	1.02
Mangga (Manggo)	*Mangifera indica*	1	1.02
Tipo (Atipolo)	*Artocarpus blancoi*	1	1.02
Tonggamos (Philippine box-orange)	*Melicope triphylla*	1	1.02
Total		98	100

Table 2. Understorey plant species and relative abundance.

Species Name		Total Number of Individuals per species (Abundance)	Relative Abundance (%)
Local Name	Scientific Name		
Miligoy (6) (Buffalo grass)	*Urochloa mutica*	590	20.97
Lukdo-lukdo (1) (Fern)	*Gleichenia japonica*	310	11.02
Cogon (Cogon grass)	*Imperata cylindrica*	267	9.49
Kunsinsi (Tick clover)	*Desmodium triflorum*	250	8.88
Miligoy (1) (Bristle basketgrass)	Oplismenus hertillus	241	8.56
Morio-morio (Bitter bush)	*Chromolaena odorata*	177	6.29
Lukdo-lukdo (2) (Giant sword fern)	*Nephrolepis biserrata*	131	4.66
Goob (Spike Moss)	*Sellaginella delicatula*	103	3.66
Miligoy (5) (African Bermuda-grass)	*Cynodon nlemfuensis*	78	2.77
Miligoy (7) (Indian crabgrass)	*Digitaria longiflora*	67	2.38
Miligoy (2) (Hilo grass)	Paspalum conjugatum	65	2.31
Sagbot (4) (Milk weed)	*Veronica perigrina*	65	2.31

Bogang (Wild sugarcane)	*Saccharum spontaneum*	51	1.81
Onod-onod (Bearded flatsedge)	*Cyperus squarrosus*	48	1.71
Disaplina (Blue porter weed)	*Stachytarpheta jamaicensis*	43	1.53
Miligoy (8) (Eastern African couchgrass)	*Digitaria abyssinica*	39	1.39
Kudsot (Blue wiss)	*Teramnus labialis*	35	1.24
Dawpang (Caesar weed)	*Urena lobata*	26	0.92
Kampisaw (Yellow star-thistle)	*Centaurea solstitialis*	24	0.85
Miligoy (3) (Southern crabgrass)	*Digitaria ciliaris*	24	0.85
Hagupit (Sal leaved desmodium)	*Desmodium gagenticum*	19	0.68
Bosikad (Purple leaved button weed)	*Borreria ocymoides*	16	0.57
Hakopaw (Ogiera)	*Eleutheranthera ruderalis*	16	0.57
Talikod (Chamber bitter)	*Phyllantus urinaria*	16	0.57
Tagbak (Garden ginger)	*Zingiber phumiangense*	15	0.53
Eping-eping (Round-leaved tick trefoil)	*Desmodium rotundifolium*	13	0.46
Sagbot (2) (Tilo)	*Justicia pectoralis*	13	0.46
Kokog banog (Dog's-tongue)	*Pseudelephantopus spicatus*	11	0.39
Hilba-hilba (Tall fleabane)	*Erigeron sumatrensis*	8	0.28
Kotocola (Lawn marshpennwort)	*Hydrocotyle sibthorpioides*	6	0.21
Rolio (Blood lily)	*Scandoxus multiflorus*	6	0.21
Lukdo-lukdo (3) (Interrupted Fern)	Osmunda claytoniana	5	0.18
Alibhaka (Pignut)	*Hyptis suaveolens*	4	0.14
Sagbot (3) (Red clover)	*Trifolium pratense*	4	0.14
Bagon-bagon (Black bindweed)	*Fallopia convolvulus*	3	0.11
Hakopaw (Shaggy soldier)	*Galinsoga quadrira*	3	0.11
Nito (Climbing Fern)	*Lygodium circinnatum*	3	0.11
Toway-toway (Beggar's Tick)	*Bidens pilosa*	3	0.11
Vietnam (Bitter vine)	*Mikania micrantha*	3	0.11
Kanaka (Milkweed)	*Euphorbia heterophylla*	2	0.07
Miligoy (4) (Kikuyu grass)	*Pennisetum clandestinum*	2	0.07
Tawa-tawa (Garden spurge)	*Euphorbia hirta*	2	0.07
Bagon-bagon (Bindweed)	*Convolvulus arvensis*	1	0.04
Banti (Greater swamp-orchid)	*Phaius tankervilleae*	1	0.04
Gabi-gabi (Taro)	*Colocasia esculenta*	1	0.04

Hibi-hibi (Sensitive plant)	*Mimosa pudica*	1	0.04
Korhito (Cuman ragweed)	*Ambrosia psilostachya*	1	0.04
Sagbot (1) (Sessile joyweed)	*Alternanthera sessilis*	1	0.04
Talampay (Black nightshade)	*Solanum nigrum*	1	0.04
	Total	2814	100

Table 3. Diversity of Trees and Understorey Plant Species in Jubilee Cross Hill.

Plant Species	Species Richness	Species Abundance	Species Abundance Density	Species Evenness	H'
Trees	13	98	4,900 / ha	0.69	1.76
Understorey	49	2814	1,407,00 / ha	0.73	2.83

The variation of species richness of trees and understorey is 13 and 49, respectively. Additionally, the abundance of species or the total number of individuals per species of trees and understorey is 98 and 2814. The variation of diversity index in trees and understorey plant species is 1.76 and 2.83. Trees and understorey plant species' evenness also are 0.69 and 0.73, respectively.

Aboveground Carbon Stocks

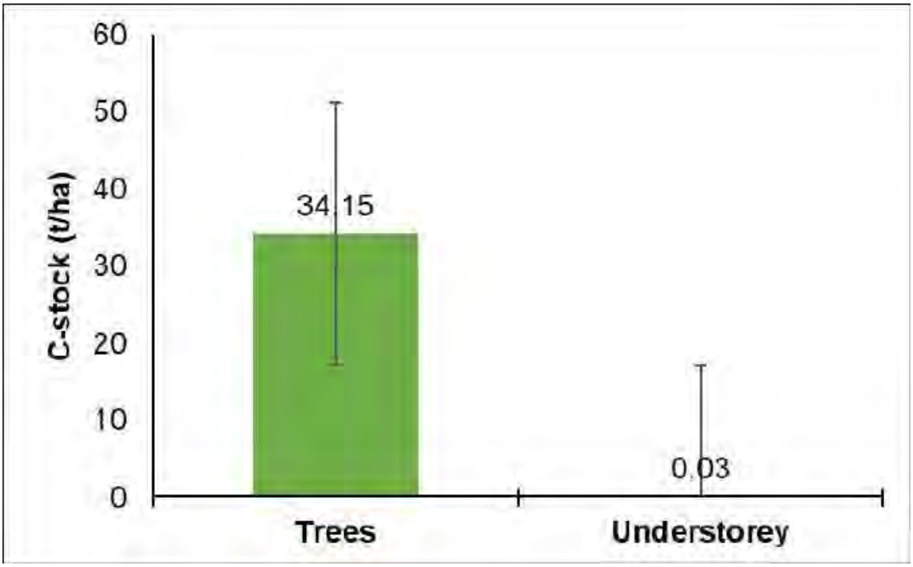

Figure 2. Total Aboveground Carbon Stocks of Trees and Understorey.

Aboveground trees have a total mean of 34.15 t C ha^{-1} of aboveground carbon stocks, whereas understorey plant species only have a total mean of 0.03 t C ha^{-1}. In determining if there are significant differences in the diversity and C-stocks between trees and understorey plant species, an independent t-test was used to analyze the data since trees and understorey plant species were independent. The computed t-test in the diversity of trees and understorey resulted in 0.0043. Furthermore, the significant difference in C-stocks between trees and understorey plant species t-test also resulted in 0.0064.

Discussion

This study shows the importance of plants in our ecosystem as they play a significant role in ecosystem diversity and the carbon cycle. Trees and understorey plant species are just one of the critical elements of our ecosystem. In this study, understorey plant species have a higher species richness and abundance. Trees also have a low diversity value in the diversity index, whereas understorey plant species have a moderate diversity value based on the Modified Fernando Biodiversity Scale (Fernando, 1998), as cited by Aureo et al. (2020). Both trees and understorey plant species also have unequal distribution of plant species in the study area based on the interval, according to Kvålseth, 2015. However, despite having a smaller species richness, abundance, and diversity value, trees could contribute higher carbon stocks than understorey plant species.

The study revealed that species richness is higher for understorey plant species than tree species, which is 49 and 13, respectively. The abundance of species or the total number of individuals per species of trees and understorey is 98 and 2814. For the diversity index, the result was 1.76, interpreted as very low, and 2.83, which is moderate based on the Modified Fernando Biodiversity Scale (MBFS) led by understorey species than tree species. Similarly, the species evenness, which is 0.69 and 0.73, led by understorey plant species, both signify unequal distribution of plant species in the study area. The implications of a moderate biodiversity index of trees and understorey based on the MFBS can be significant for ecosystem functioning, carbon storage, and wildlife habitat. Firstly, a moderate biodiversity index of trees and understorey can affect ecosystem functioning. Studies have shown that increasing tree and understorey biodiversity can increase the productivity and stability of ecosystem processes such as carbon sequestration and nutrient cycling (Watson et al., 2019; Jucker et al., 2020). Therefore, a moderate biodiversity index may limit the potential for these ecosystem functions to function optimally.

Secondly, a moderate biodiversity index of trees and understorey can also impact carbon storage. Trees and understorey vegetation play an important role in carbon sequestration, and studies have found that higher tree and understorey biodiversity can enhance carbon storage (Cavender-Bares et al., 2019; Xu et al., 2019). Therefore, a moderate biodiversity index may limit the potential for forests to store carbon effectively. Lastly, a moderate biodiversity index of trees and understorey can also impact wildlife habitats. A high biodiversity of trees and understorey vegetation can provide a habitat for a diverse range of wildlife species (Gehring et al., 2019; Walters et al., 2020). Therefore, a moderate biodiversity index may limit the potential for forests to support a diverse range of wildlife species.

It further implies that the growth of trees in Jubilee Cross Hill was significantly affected by the unintended fire and converting some areas into croplands. It is also supported by the study that the occurrence of a single fire event significantly affects the diversity and regeneration of trees (Verma et al., 2017). Anthropogenic activities have shaped and influenced species diversity (Giliba et al., 2011). Fire reduces the covering of trees, slows the advance of trees, and maintains the dominance of understorey species (Limb et al., 2011). One of the disadvantages of understorey dominance is that it may expose the hill to erosion. Given that understorey plant species can also yield or hasten the growth of trees if it dominates the hill entirely, this may result in a low abundance of trees. Hence it will cause damage to the environment and the population underneath the hill (Bruelheide et al., 2018).

The result also expressed that the area may have an unstable and unproductive ecosystem due to very low diversity of trees and moderate diversity of understorey plant species which contrasts with the study of Lillo et al. (2019), where an area with high species diversity results in a more stable and productive ecosystem. The result of the study was also supported by the statement that low species richness and unequal distribution of trees or a high relative abundance of a single species in the area resulted in low diversity which may be due to natural processes or human disturbances (Gadow et, al., 2012).

Research shows that the main driver of the richness of plants in the said biodiversity in Jubilee Cross Hill, Matalom, Leyte is the resource availability in which an estimated 50-90 per cent of life in the rainforest exists in the trees above the shaded forest floor. The primary tropical rainforest is divided into at least five layers: the overstorey, the canopy, the understorey, the shrub, and the forest floor. Each layer has unique plant and animal species interacting with the ecosystem (Royo & Carson, 2022). Hence, it is crucial to promote and protect biodiversity in forest ecosystems to maintain the health and sustainability of these ecosystems.

On the other hand, comparing trees and understorey plant species with the accumulation of the carbon stocks shows that their contribution to overall aboveground carbon (AGC) storage was due to species richness and unequal distribution of plant species. Cutting trees and converting them into croplands potentially reduce aboveground carbon stocks and affect plant species diversity. Trees were the most likely affected by the disturbances regarding diversity level. It was further found that large trees play a significant role in the accumulated carbon stock of the forests in Jubilee Cross Hill, Matalom, and Leyte. It is because the large-diameter trees store massive amounts of carbon and are a significant driver of carbon cycle dynamics in the forest.

Figure 2 further indicates that tree species resulted in a higher carbon stock with a mean of 34.15 t C ha^{-1} compared to understorey plant species, which only contributed a mean of 0.03 t C ha^{-1}. It was also presented that there are significant differences in the diversity of trees and understorey plant species based on an independent t-test result of 0.0043. The computed t-test in the C-stocks between trees and understorey plant species also resulted in 0.0064, which shows a significant difference. Comparing the carbon stocks of trees and understorey plant species indicates that tree species resulted in higher carbon stocks than understorey plant species. Since understorey has a higher species richness than trees, trees have fewer relative abundances than the understorey. It indicates a higher value of C-stocks of trees, which implies that trees play a vital role in the global carbon cycle and has a great potential to increase carbon stocks, as it means that trees need more conservation strategies. (Henry et al., 2011; Gebrewahid et al., 2018; Coritico et al., 2020).

Several studies have investigated the comparative analysis of carbon stocks in trees and understorey vegetation. For example, Bobon-Carnice and Lina (2017) found in a study on carbon storage and nutrient stocks distribution in three adjacent land use patterns in Lake Danao National Park, Ormoc, Leyte, Philippines, that the carbon stocks of trees in the old-growth forest were the highest, followed by the agroforestry area and the grassland as the lowest. In a subsequent study by the same authors in 2021, Bobon-Carnice and Lina found that carbon stocks in trees were significantly higher in the old-growth forest compared to the secondary forest.

Additionally, Yang et al. (2019) conducted a study in a subtropical forest in China and found that carbon stocks in understorey vegetation were significantly lower than in trees. However, the study also found that carbon stocks in understorey vegetation were highest in forests with diverse understorey communities. Similarly, Fuentes et al. (2020) found in a Peru tropical forest study that carbon stocks in trees were significantly higher than in understorey vegetation. Still, the contribution of understorey vegetation to total ecosystem carbon stocks increased with forest disturbance.

In contrast to this study's result, in temperate forests in China by Zhang et al. (2015), trees had significantly higher carbon stocks than understorey vegetation, with tree carbon stocks averaging 101.6 Mg/ha and understorey vegetation carbon stocks averaging 5.8 Mg/ha. The literature suggests that trees generally have significantly higher carbon stocks than understorey vegetation. However, the contribution of understorey vegetation to total ecosystem carbon stocks can vary depending on forest disturbance, understorey diversity, and other factors.

To determine if there are significant differences in the diversity and C-stocks between trees and understorey plant species, independent t-test was used to analyze the data. The computed result shows a significant difference in the diversity of trees and understorey plant species. Correspondingly, the computed t-test result of C-stocks between trees and understorey plant species shows a significant difference since trees and understorey plant species are independent.

This study shows that trees should be given further attention since they play a crucial role in the global carbon cycle (Salunkhe et al., 2017), despite their very low diversity index. Trees also stand an indispensable role in environmental protection, particularly soil erosion, since the dominance of understorey plant species exposes the hill to erosion (Bruelheide et al., 2018). Hence, there should be conservation and enhancement of trees in Jubilee Cross Hill to protect the area and maintain its beauty for tourist attractions. It is highly suggested that strong coordination from local communities, local government units, and concerned government agencies should arise to create programs to enhance and protect the study area.

Conclusion

The diversity levels of trees and understorey plant species in terms of species richness and abundance were led by understorey plant species. For the diversity index, understorey plant species still have a higher value, interpreted as moderate, and trees have a very low value, based on the Modified Fernando Biodiversity Scale. Both trees and understorey plant species signify unequal distribution in the study area. Despite trees' deficient diversity level, they still contribute to higher carbon stocks as trees tend to contribute more carbon to the ecosystem due to their larger diameter at breast height (DBH) compared to other plants, which have great potential for the global carbon cycle. Comparatively, there are significant differences in the diversity and c-stocks between trees and understorey based on the independent t-test. Furthermore, more conservation strategies are needed to protect and manage plant species in the study site, particularly trees.

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References

Aureo WA, Reyes TD, Mutia FCU, Jose RP, Sarnowski MB. 2020. Diversity and composition of plant species in the forest over limestone of Rajah Sikatuna Protected Landscape, Bohol, Philippines. *Biodiversity Data Journal, 8.*

Bendimerad, Fouad & Rizal, Ma & Bergonia, Ma & Borinaga, Moses & Beroya-Eitner, Mary Antonette & Tirkey, Nancy & Barba, Marivic & Padao, Ishtar & Padilla, Michael & Perez, Ma & Zayas, Jerome & Villa, Paolo & Dakis, Kristoffer & Valera, Annie. 2014. The Mw7.2 October 15, 2013, Bohol, Philippines Earthquake. 10.13140/RG.2.2.23010.76485/1.

Bobon-Carnice PA, Lına S. 2017. Carbon Storage and Nutrient Stocks Distribution of Three Adjacent Land Use Patterns in Lake Danao National Park, Ormoc, Leyte, Philippines. *Journal of Science, Engineering and Technology, 5:* 1–14.

Bobon-Carnice PA, Lina SB. 2021. Changes in Carbon and Nutrient Stocks of Secondary Forest Transformations Under Ultisol in Leyte Island, Philippines. *Mindanao Journal of Science and Technology, 19* (1).

Bruelheide H, Dengler J, Purschke O, Lenoir J, Jiménez-Alfaro B, Hennekens SM, Botta-Dukát Z, Chytrý M, Field R, Jansen F, Kattge J. 2018. Global trait-environment relationships of plant communities. *Nature ecology & evolution, 2* (12): 1906–1917.

Cairns MA, Olmsted I, Granados, J, Argaez J. 2003. Composition and aboveground tree biomass of a dry semi-evergreen forest on Mexico's Yucatan Peninsula. *Forest Ecology and Management, 186* (1–3): 125–132.

Cavender-Bares J, Keen A, Miles B, Quesada T. 2019. Biodiversity and ecosystem services in a climate context. *Frontiers in Ecology and Evolution, 7*: 464.

Coritico FP, Lagunday NE, Galindon JMM, Tandang DN, Amoroso VB. 2020. Diversity of trees and structure of forest habitat types in Mt. Tago Range, Mindanao, Philippines. *Philippine Journal of Systematic Biology, 14* (3): 1–11.

Corlett R. 2016. Plant Diversity in a Changing World: Status, Trends, and Conservation Needs. *Plant Diversity, 38:* 10–16.10.1016/j.pld.2016.01.001.

Donayre DKM, EC Martin, SE Santiago, JT Lee. 2018. *Weeds in Irrigated and Rainfed Lowland Ricefields in the Philippines. 2nd Edition.* Philippine Rice Research Institute, Maligaya, Science City of Muñoz, Nueva Ecija, Philippines. 197 p.

Donayre DKM, Santiago SE, Martin EC, Lee JT, Corales RG, Janiya JD, Kumar V. 2019. *Weeds of Vegetables and other Cash Crops in the Philippines.* Philippine Rice Research Institute, Maligaya, Science City of Muñoz, Nueva Ecija, Philippines. 141p.

Ecosystems Research and Development Bureau. Common Weed Species with Medicinal Uses Vol. 24 No.1, January-April 2012 Research Information Series on Ecosystems ERDB, College 4031, Laguna. Retrieved from: http://erdb.denr.gov.ph/files/publications/rise/r_v24n1.pdf

Forest tree species with medicinal uses. Ecosystems Research and Development Bureau, Department of Environment and Natural Resources, 2002. Retrieved from: https://erdb.denr.gov.ph/wp-content/uploads/2015/06/denr_v11.pdf

Forest Management Bureau-Department of Environment and Natural Resources. 2007. Greenbook 2: Procedures and Technique in Planting. Visayas Avenue, Diliman Quezon City 1100 Philippines. 33 pp. Retrieved from: https://forestry.denr.gov.ph/pdf/ref/greenbook1.pdf

Fuentes M, Malhi Y, Lloyd J, Vega RM. 2020. Above- and below-ground carbon stocks in lowland Amazonian and Andean forests: Implications of deforestation and forest degradation. *Global Change Biology, 26* (10): 5422–5437.

Gadow KV, Zhang CY, Wehenkel C, Pommerening A, Corral-Rivas J, Korol M, Myklush S, Hui GY, Kiviste A, Zhao XH. 2012. Forest structure and diversity. *Continuous cover forestry*: 29–83.

Gebrewahid Y, Gebre-Egziabhier TB, Teka K, Birhane E. 2018. Carbon stock potential of scattered trees on farmland along an altitudinal gradient in Tigray, Northern Ethiopia. *Ecological processes, 7* (1): 1–8.

Gehring TM, Swihart RK, Dey DC. 2019. Understorey plant communities influence habitat use by small mammals in managed forests. *Forest Ecology and Management, 432:* 633–641.

Henry M, Picard N, Trotta C, Manlay R, Valentini R, Bernoux M, Saint-André L. 2011. Estimating tree biomass of sub-Saharan African forests: a review of available allometric equations. *Silva Fennica, 45* (3): 477–569.

Jucker T, Bongalov B, Burslem DF, Nilus R, Dalponte M, Lewis SL, Phillips OL, Qie L, Coomes DA. 2020. Topography shapes the structure, composition and function of tropical forest landscapes. *Ecology Letters, 23* (3): 459–469.

Kim TK. 2015. T test as a parametric statistic. *Korean journal of anesthesiology, 68* (6): 540–546.

Kvålseth TO. 2015. Evenness indices once again: critical analysis of properties. *Springer Plus, 4* (1): 1–12.

Locey KJ, White EP. 2013. How species richness and total abundance constrain the distribution of abundance. *Ecology letters, 16* (9): 1177–1185.

Li W, Li J, Liu S, Zhang R, Qi W, Zhang R, Knops JM, Lu J. 2017. Magnitude of species diversity effect on aboveground plant biomass increases through successional time of abandoned farmlands on the eastern Tibetan Plateau of China. *Land Degradation & Development*, *28* (1): 370–378.

Lillo EP, Fernando ES, Lillo MJR. 2019. Plant diversity and structure of forest habitat types on Dinagat Island, Philippines. *Journal of Asia-Pacific Biodiversity, 12* (1): 83–105.

Limb RF, Fuhlendorf SD, Engle DM, Kerby J D. 2011. Growing-season disturbance in tallgrass prairie: evaluating fire and grazing on *Schizachyrium scoparium*. *Rangeland Ecology & Management, 64* (1): 28–36.

Pielou EC. 1966. The measurement of diversity in different types of biological collections. *Journal of theoretical biology, 13:* 131–144.

Pomeroy RS. 1987. The role of women and children in small scale fishing households: a case study in Matalom, Leyte, Philippines. *Philippine Quarterly of Culture and Society*: 353–360.

Salunkhe O, Khare PK, Kumari R, Khan ML. 2018. A systematic review on the aboveground biomass and carbon stocks of Indian forest ecosystems. *Ecological processes, 7* (1): 1–12.

Sintayehu DW, Belayneh A, Dechassa N. 2020. Aboveground carbon stock is related to land cover and woody species diversity in tropical ecosystems of Eastern Ethiopia. *Ecological Processes, 9* (1): 1–10.

Sorecha EM, Deriba L. 2017. Assessment of Plant Species Diversity, Relative Abundances and Distribution in Haramaya University, Ethiopia. *Journal of Physical Science and Environmental Studies, 3* (3): 30–35.

Rahayu S, Lusiana B, van Noordwijk M. 2005. Above ground carbon stock assessment for various land use systems in Nunukan, East Kalimantan. *Carbon Stock Monitoring in Nunukan, East Kalimantan: A Spatial and Modelling Approach. World Agroforestry Centre, SE Asia, Bogor, Indonesia*: 21–34.

Rahman B, Karmilah M, Kautsary J, Ridlo MA. 2021. The Tidal Flooding Causes in the North Coast of Central Java: A Systematic Literature Review. *Journal of Southwest Jiaotong University, 56* (6).

Royo AA, Carson WP. 2022. Stasis in forest regeneration following deer exclusion and understory gap creation: A 10-year experiment. *Ecological Applications*, *32* (4): p.e2569.

Walters JR, Derrickson SR, Michael Fry D, Haig SM, Marzluff JM, Wunderle Jr JM. 2020. Status of the California condor (Gymnogyps californianus) and efforts to achieve its recovery. *The Auk: Ornithological Advances, 137* (1): 1–18.

Watson R, Baste I, Larigauderie A, Leadley P, Pascual U, Baptiste B, Demissew S, Dziba L, Erpul G, Fazel A, Fischer M. 2019. Summary for policymakers of the global assessment report on biodiversity and ecosystem services of the

Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services. *IPBES Secretariat: Bonn, Germany:* 22–47.

Whittaker RH. 1960. Vegetation of the Siskiyou mountains, Oregon and California. *Ecological monographs, 30* (3): 279–338.

Xu X, Medvigy D, Powers JS. 2019. Diversity and structural complexity of understory vegetation are key predictors of carbon stocks in human-modified tropical forests. *Environmental Research Letters, 14* (10): 105008.

Yang Y, Zhang W, Li Y, Yu S, Ding Y, Wang H. 2019. Biomass and carbon stocks of understory vegetation in subtropical forests of China. *Forest Ecology and Management, 446:* 149–156.

Zhang W, Yang Y, Yu S, Li Y, Ding Y, Wang H. 2015. Biomass and carbon stocks of understory vegetation in temperate forests in China. *Forest Ecology and Management, 346*: 16–22.

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Research Article

Phenolic Content and Antioxidant Activity of Leaves and Rhizomes of *Etlingera coccinea* (Blume) S.Sakai and Nagam. (Zingiberaceae)

Noe P. Mendez*

Plant Biology Division, Institute of Biological Science, College of Arts and Sciences, Central Mindanao University, University Town, Musuan, Maramag, 8714 Bukidnon, Philippines.

*Corresponding author: npolomendez@gmail.com

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ABSTRACT

Most of the Zingiberaceae species in the Philippines have been used as ethnomedicinal plants due to the benefits they possess. One of these species is *Etlingera coccinea* (Blume) S.Sakai and Nagam. (Zingiberaceae), a species with a variety of uses in Malaysia, Borneo, and Philippines. In this study, ethanolic extracts of the dry weight leaves and rhizomes of *E. coccinea* were used to determine its phenolic content and antioxidant activity. The total phenolic content and total antioxidant activity were determined using Folin-Ciocalteu and phosphomolybdenum methods, respectively. Data revealed that the total phenolic content of dry weight, expressed as milligram gallic acid equivalent per gram sample (mg GAE/g sample) recorded that the leaves (11.69 ± 0.47 mg GAE/g sample) have greater amount of phenolics than the rhizomes (0.58 ± 0.06 mg GAE/g sample). The total antioxidant activity (TAA), expressed as milligram ascorbic acid equivalent per gram sample (mg AAE/g sample), obtained higher activity in the leaves (12.76 ± 0.31 mg AAE/g sample) than the rhizomes (0.85 ± 0.12 mg AAE/g sample), and the reducing power, expressed as milligram gallic acid reducing power equivalent per gram sample (mg GRPE/g sample) also revealed higher activity for the leaves (9.37 ± 1.88 mg GRPE/g sample) compared to rhizomes (0.28 ± 0.07 mg GRPE/g sample). Based on the correlation analysis, a perfect positive linear relationship was observed among the TPC, TAA, and RP ($r=1$, $p<0.001$), which means that phenolic compounds significantly contribute to the antioxidant activities of the extracts of *E. coccinea*. These data imply that *E. coccinea* could be potentially used as a new source of natural antioxidant. Furthermore, this paper adds information on the habitat and ecology, phenology, and coloured photographs of this species for future related studies and conservation initiatives.

Keywords: ginger species; native species; total antioxidant activity; total phenolic content; reducing power

Introduction

Zingiberaceae is one the eight families in Order Zingiberales and is known as the ginger family. In the Philippines, members of this family have recently been the subject of scientific investigations from field expeditions (e.g., inventory, diversity, and assessment, etc.) to laboratory analyses (e.g., biochemical analyses). Determination of phenolic content and antioxidant activities of some ginger species, such as the works of Mabini & Barbosa (2018), Barbosa & Nueva (2019), Mendez et al. (2022, 2023) have been conducted in response to the perceived natural benefits the ginger species offer to humans as reported by local people living in the Mindanao Mountain ecosystems. It is imperative that several studies are conducted on this family in the country to further explore its potential and future use in medicines, condiments, and food (Mendez et al., 2023).

Phenolic compounds have recently received considerable attention because of their physiological functions including as antioxidants and free radical scavenging activities that are affected by quality and nutritional value (Govindarajah et al., 2017). The compounds stimulate the synthesis of endogenous antioxidant molecules in the cell (Côté et al., 2010). Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity (Soobarattee et al., 2005). Plant antioxidant molecules are mostly secondary metabolites (Maulana et al., 2019) and these plants produce these compounds as adaptive responses (Wang, 2012). Antioxidants stabilize or deactivate free radicals, often before that attack targets in biological cells (Nunes et al., 2012).

In Marilog District, the fruits of *E. coccinea* are eaten as a forest candy especially by children, and its rhizome and leaves are boiled to cure fever, cough, and muscle fatigue which were also done to other gingers in the area. In Sabah, Malaysia, *E. coccinea* has been used by various indigenous communities as a remedy for stomachache, food poisoning, and gastric problems (Poulsen, 2006). Its fruits are edible and the pith of the leafy shoot is used as a condiment (Lamb et al., 2013). The indigenous people especially the Kadazan-Dusun use this species to flavour local dishes and the *E. coccinea*-based products, such as 'sambal tuhau' (paste), 'jeruk tuhau' (pickles) and 'serunding tuhau' (floss) have been commercially produced for the local market (Jualang et al., 2015). However, the Philippine materials of *E. coccinea* have not been studied in any laboratory research in the country, and thus, this paper makes it the first study to evaluate its phenolic content and antioxidant activities in the Philippines.

Methodology

Entry Protocol

As a courtesy to the Barangay officials and local people in Brgy. Datu Salumay in Marilog District, Davao, Southern Philippines, a prior informed consent and a formal letter were personally submitted by the author at the Barangay Captain's office before the collection of samples at Mt. Malambo. The gratuitous permit of Dr. Victor B. Amoroso issued by the Department of Environment and Natural Resources (DENR) – Region XI was used as the collection permit in this study.

Place and Duration of the Study

The collection of specimens was carried out at the base of Mt. Malambo (7°22'40'' N 125°19'38" E) in Marilog District, Davao City in September 2021 – January 2022 at an elevationof 1090 masl.. Samples and voucher specimens were collected on September 25, 2021 and were brought to Central Mindanao University, Musuan, Bukidnon for processing. The determination of total phenolic content (TPC), total antioxidant activity (TAA), and reducing power (RP) of dry weight leaves and rhizomes of *E. coccinea* was conducted at the Natural Science Laboratory of Natural Science Research Center (NSRC) in Central Mindanao University after necessary permits were obtained from the authorities concerned.

Sample Collection, Preparation and Extraction

Prior to the collection of samples for laboratory analyses, ecological notes were obtained in the field by taking notes of the habitat of the populations and its habit following the method of Mendez et al. (2017), Acma & Mendez (2018), Mendez & Acma (2018). Plant associates near the populations of *E. coccinea* were also taken using field notebooks and pencil.

The leaves and rhizomes of *E. coccinea* were collected and placed separately inside labelled plastic bags with wet tissue paper to prevent dehydration (Figure 1). These samples were brought to Central Mindanao University for further processing. Leaf and rhizome samples were washed, and the earthy matters were removed prior to air-drying. Dried samples were powdered and stored until these were used. On the other hand, the vegetative and reproductive parts of *E. coccinea* were collected for voucher purposes. Flowers and fruits were preserved in 70% ethanol as spirit/pickled collection, and the herbarium specimens were dried. The herbarium specimens were then deposited at the Central Mindanao University Herbarium (CMUH).

Figure 1. Specimens of *Etlingera coccinea* (Blume) S.Sakai and Nagam. A) Fresh rhizome sample; B) Fresh leaf sample. Scale bar: A & B = 3 cm. *Photographs: N.P. Mendez (A&B).*

Extracts were prepared following the method of Padda & Picha (2008) with some modifications. The dried leaf and rhizome specimens were homogenized using a household blender and weighed in analytical balance (Mettler). The dried leaf and rhizome powder were extracted with absolute ethanol with a ratio of 1 g: 25.0 mL at room temperature (24°C). The mixtures were shaken for 1 hour in an orbital shaker (Premiere, USA) at 300 rpm and centrifuged (Lab Kits) at 5,000 rpm for 5 minutes. The resulting supernatants were collected in separate 15 mL conical tubes. Extracts were stored at 2–8°C and used in the succeeding analyses. The ethanolic leaf and rhizome extracts of *E. coccinea* were subjected for the total phenolic content, total antioxidant analysis, and reducing power determination in 96-well plate format colorimetric assays.

Total Phenolic Content

The TPC of the extracts was determined using the method described by Ainsworth & Gillespie (2007) with some modifications. Briefly, 200 µL of the extracts and 200 µL of 10% Folin-Ciocalteu reagent were transferred in a 2-mL centrifuge tube. The reaction mixture was set aside for 5 minutes and added with 800 µL of 10% sodium carbonate. The mixture was set aside at room temperature for 30 minutes and centrifuged at 11,000 rpm for 3 minutes. A 200 µL of the resulting solution was then transferred to the assigned microplate wells. The absorbance was determined at 750 nm against a blank (ethanol) using a microplate reader (Molecular Devices Spectramax® 250). Gallic acid (100 ppm) was used as the standard solution with concentration range of 0–200 ppm. A calibration curve was constructed by using the absorbance values obtained at various concentration of gallic acid. The TPC was determined and expressed as milligram gallic acid equivalent per gram sample (mg GAE/g sample) by interpolating sample absorbance against the standard calibration curve using the formula below:

$$\text{Total Phenolic Content} = \left(\frac{\text{mg}}{\text{g sample}}\right) = \frac{\text{A}}{\text{B}}$$

where:
A = gallic acid concentration of the sample solution determined from the calibration curve (mg GAE/L)
B = the concentration of test solution (g/L, gram dried sample per L solution)

Total Antioxidant Activity

The TAA was determined using phosphomolybdenum method described by Prieto et al. (1999) with slight modifications. Briefly, 50 µL of extracts were placed in centrifuge tubes and diluted with 200 µL (1:1 ethanol: water). The solution was added with 600 µL of reagent solution (prepared by mixing equal amounts of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 minutes. The samples were allowed to cool down at room temperature (24°C) and centrifuged at 11,000 rpm for 3 minutes. The absorbance of the supernatant was measured at 695 nm against ethanol using a microplate reader (Molecular Devices Spectramax® 250). Calibration curve was also prepared using 0–300 ppm ascorbic acid as standard. TAA was determined and expressed as milligram ascorbic acid equivalent per gram sample (mg AAE/g sample) by interpolating sample absorbance against the standard curve. The TAA was calculated using the following equation:

$$\text{Total Antioxidant Content} = \left(\frac{\text{mg}}{\text{g sample}}\right) = \frac{\text{A}}{\text{B}}$$

Where:
A = ascorbic acid concentration of the solution determined from the calibration curve (mg AAE/L)
B = concentration of the test solution (g/L, gram dried sample per L solution)

Reducing Power

The reducing power was determined by adapting the method described by Murugan & Iyer (2012) with some modifications. In a centrifuge tube containing 1 mL of extracts, 200 µL of 0.2 M phosphate buffer (pH 6.6) and 200 µL of 1% (w/v) solution of potassium ferricyanide were added. The mixture was incubated at 50°C for 30 minutes. After cooling to room temperature (24°C), 200 µL of 1% (w/v) trichloroacetic acid was added. The mixture was centrifuged for 3 minutes at 11,000 rpm. An aliquot of 200 µL of the supernatant was transferred to a 96-well plate and 20 µL of 1% (w/v) solution of ferric chloride was added. The absorbance was measured at 620 nm against a blank (ethanol) using a microplate spectrophotometer (Molecular Devices Spectramax® 250). Standard gallic acid with concentrations ranging from 0–300 ppm was used to establish a calibration curve. Sample concentration was determined by interpolating sample absorbance against the standard curve. The reducing power, expressed as milligram gallic acid reducing power equivalent per gram sample (mg GRPE/g sample) was calculated as follows:

$$\text{Reducing Power} = \left(\frac{\text{mg}}{\text{g sample}}\right) = \frac{\text{A}}{\text{B}}$$

where:
A = gallic acid concentration of the test solution determined from the calibration curve (mg GRPE/L)
B = concentration of the test solution (g/L, gram dried sample per L solution)

Statistical Analysis

The TPC, TAA, and RP analyses were done in triplicates, and the determination for each assay was carried out in three trials per replicate. Correlations among the TPC, TAA, and RP were obtained using Pearson's correlation coeeficient (*r*) at a 0.001 level of significance. The results of absorption measurement were separately put into Microsoft Excel to obtain a calibration curve of standard gallic acid solution (TPC and RP) and ascorbic acid solution (TAA) in the form of a graph of concentration versus absorption curve. All results obtained in the study were expressed as mean values ± SD (standard deviation).

Results and Discussion

Etlingera coccinea (Blume) S. Sakai & Nagam. (Figure 2)

Habitat and Ecology: On 16 July 2017, three populations of *E. coccinea* were found along the roadside at the foot of Mt. Malambo fronting the Bemwa farm in Marilog District, Davao City, Philippines. During a revisit to the area on 21 September 2021 to collect samples for biochemical analyses and collection of voucher specimens, seven populations were recorded and most of the individuals were fertile. The populations of *E. coccinea* naturally grow in a partial shade canopy in wet, slope lowland area, along the roadside where there is minimal contact with sunlight at an elevation of 1090 masl. Inflorescence of *E. coccinea*

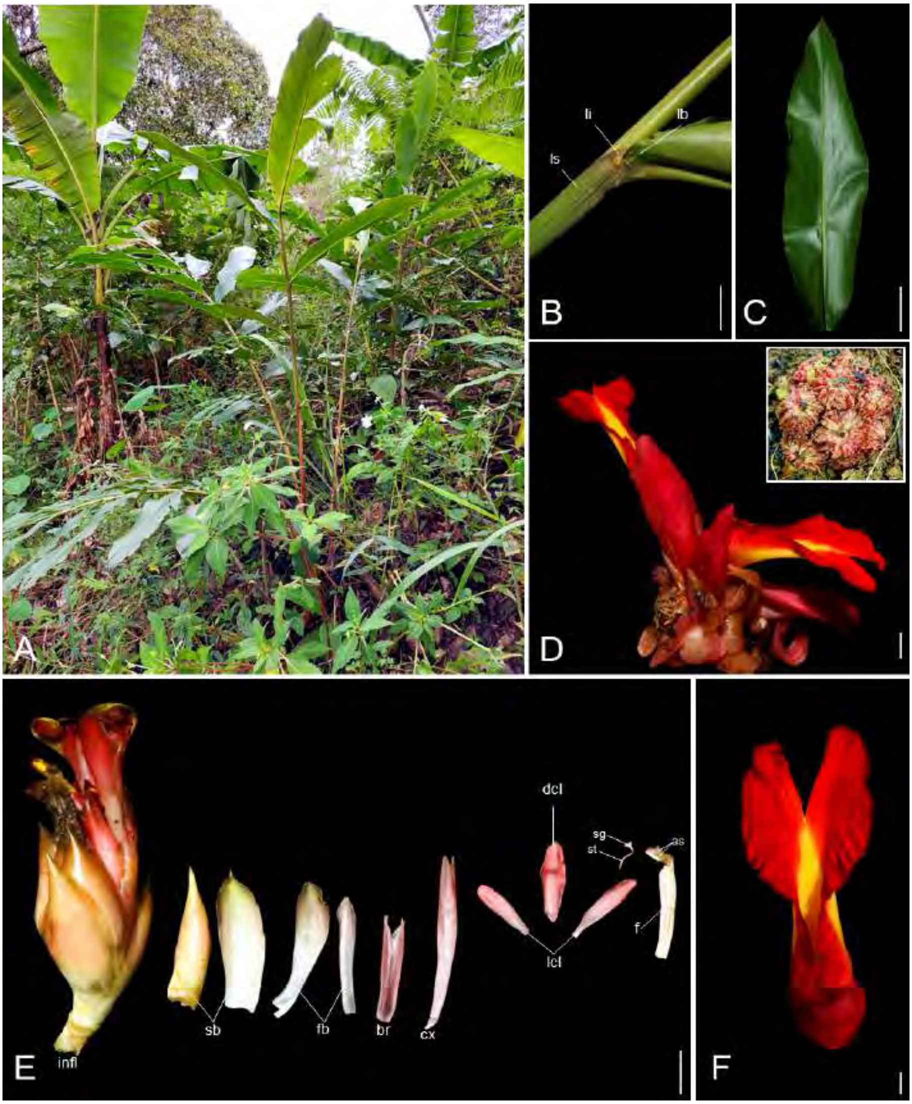

Figure 2. *Etlingera coccinea* (Blume) S.Sakai and Nagam. A) Habit; B) Leaf sheath (ls), ligule (li), and leaf base (lb); C) Lamina (adaxial surface); D) Inflorescence (inset: infructescence); E) Floral parts (infl – inflorescence, sb – sterile bracts, fb – fertile bracts, br – bracteole, cx – calyx, dcl –dorsal corolla lobe, lcl – lateral corolla lobes, sg – stigma, st – style, as – anther sacs, f – filament); F) Labellum (anterior view). Scale bars: 3 cm – B & C; 1 cm – D; 2 cm – E; 3 cm – F. *Photographs: N.P. Mendez (A–C and E), R.V.A. Docot (D & F).*

appeared at the ground level and showed four floral morphs during its anthesis. The species was found growing along with other Zingiberaceae species, such as *E. pubimarginata* (Elmer) A.D.Poulsen and *Zingiber* sp. (Zingiberaceae) and associated with *Alocasia heterophylla* (C.Presl) Merr. (Araceae), *Musa textilis* Nées (Musaceae), *Mimosa pudica* L. (Fabaceae), *Impatiens platypetala* Lindl. (Balsaminaceae), and *Angiopteris evecta* (G.Forst.) Hoffm. (Marattiaceae).

Phenology: Flowering usually occurs from July to September, fruits not observed during the collection of samples.

Total Phenolic Content

The results of the TPC of the extracts were derived from the regression equation of a calibration curve (y = 0.0546 x 0.0658, R^2 = 0.9946) of gallic acid (0–200 mg/mL). The ethanolic extracts of *E. coccinea* revealed that the leaves (11.69 ± 0.47 mg GAE/g sample) exhibited higher phenolic content than the rhizomes (0.58 ± 0.06 mg GAE/g sample) (Table 1).

Table 1. Mean total phenolic content extraction yield of leaves and rhizomes of *E. coccinea*.

Plant parts	mg GAE/g sample
Leaves	11.69 ± 0.47
Rhizomes	0.58 ± 0.06

The values of phenolic content in this study varied slightly compared to the studies on other ginger species in the Philippines, *viz.,* Mabini & Barbosa (2018), Barbosa & Nueva (2019), Mendez et al. (2002, 2003) and previous study on phenolic content and antioxidant activity of *E. coccinea* collected from Brunei Darussalam by Shahid-Ud-Daula et al. (2015). The mean value of TPC in leaves (11.69 ± 0.47 mg GAE/g sample) in this study is relatively higher than the studies of Mabini & Barbosa (2018) with 0.55 mg GAE/g sample on methanolic extracts of *Etlingera philippinensis* (Ridl.) R.M.Sm., Barbosa & Nueva (2019) with 1.67 mg GAE/g sample on methanolic extracts of *Hornstedtia conoidea* Ridl., and Mendez et al. (2023) with 7.21 ± 0.33 mg GAE/g sample on ethanolic extracts of *E. philippinensis*. However, it is lower than the studies of Shahid-Ud-Daula et al. (2015) 13.49 ± 0.26 mg GAE/g sample on methanolic extracts of *E. coccinea*, Mendez et al. (2022) with 27.25 ± 0.72 mg GAE/g sample on ethanolic extracts of *E. pubimarginata* (Elmer) A.D.Poulsen and Mendez et al. (2023) with 13.20 ± 0.35 mg GAE/g sample on ethanolic extracts of *E. fimbriobracteata* (K.Schum.) R.M.Sm.

The mean value of TPC in rhizomes (0.58 ± 0.06mg GAE/g sample) of *E. coccinea* is higher than the studies of Mabini & Barbosa (2018) and Mendez et al. (2023) on *E. philippinensis* with 0.35 mg GAE/g sample and 0.46 ± 0.30 mg GAE/g sample. But this value is lower than the studies of Shahid-Ud-Daula et al. (2015)

with 7.94 ± 0.01 mg GAE/g sample, Barbosa & Nueva (2019) with 1.28 mg GAE/g sample on *H. conoidea*, Mendez et al. (2022) with 0.76 ± 0.11 mg GAE/g sample on *E. pubimarginata*, and Mendez et al. (2023) with 1.44 ± 0.04 mg GAE/g sample on *E. fimbriobracteata*. These slight variations in the mean values of TPC might be due to the presence of different amounts of sugars, carotenoids, ascorbic acid, duration, geographical variation or methods of extraction, which may alter the amount of phenolics (Burri et al., 2017).

Phenolic compounds contribute to antioxidant activity due to the arrangement of functional groups (hydroxyl) about its nuclear structure for hydrogen donation in order to stabilize radical molecules (Soobarattee et al., 2008; Alam et al., 2018). The total phenolic compounds also play an effective role in stabilizing lipid peroxidation (Yen et al., 1993). It is worth to note that the result of TPC depends on the type of solvent used (Cesoniene et al., 2012), degree of polymeration of phenolics, interaction of phenolics with other food constituents and formation of insoluble complex (Galvez et al., 2005).

Ethanol was used in this study since phenolic compounds are more soluble in polar organic solvents due to the presence of a hydroxyl group (Wang & Weller, 2005). The quantification of TPC in ethanolic extracts of leaves and rhizomes of *E. coccinea* which was determined by employing Folin-Ciocalteu method, is convenient, simple, and reproducible (Cirilo & Lemma, 2012; Danciu et al., 2015). The mechanism involved in this method is electron transfer in alkaline medium from phenolic compound to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically (Singleton et al., 1999).

Total Antioxidant Activity and Reducing Power

The results for the TAA of the ethanolic extracts of leaves and rhizomes of *E. coccinea* were calculated from a calibration graph which were linear over the calibration range with R^2 value of 0.9963 ($y = 0.0149 \times 0.0465$) of L-ascorbic acid (0–100 mg/mL). The ethanolic extracts of *E. coccinea* revealed that the leaf extract (12.76 ± 0.31 mg AAE/g sample) had a higher activity compared to the rhizome extract (0.85 ± 0.12 mg AAE/g sample).

For the RP, results of ethanolic extracts of leaves and rhizomes of *E. coccinea* were derived from a calibration curve ($y = 0.0505 \times 0.5037$, $R^2 = 0.9975$) of gallic acid (0–1000 mg/mL). The ethanolic extracts of *E. coccinea* revealed that the leaves (9.37 ± 1.88 mg GRPE/g sample) had a higher activity compared to the rhizomes (0.28 ± 0.07 mg GRPE/g sample) (Table 2).

Table 2. Mean total antioxidant activity and reducing power extraction yield of leaves and rhizomes of *E. coccinea*.

Plant parts	TAA (mg AAE/g dried sample)	Reducing Power (mg GRPE/g dried sample)
Leaves	12.76 ± 0.31	9.37 ± 1.88
Rhizomes	0.85 ± 0.12	0.28 ± 0.07

The mean value of TAA of the leaves (12.76 ± 0.31 mg AAE/g sample) of *E. coccinea* had a higher activity compared to the studies of Mabini & Barbosa (2018) with 0.79 mg AAE/g sample on *E. philippinensis*, Barbosa & Nueva (2019) with 4.67 mg AAE/g sample on *H. conoidea*, and Mendez et al. (2023) with 12.69 ± 0.36 on *E. fimbriobracteata*, but lower in the studies of Mendez et al. (2022) with 34.83 ± 0.49 mg AAE/g sample on *E. fimbriobracteata* and Mendez et al. (2023) with 12.69 ± 0.36 mg AAE /g sample on *E. philippinensis.* For the mean value of TAA of the rhizomes (0.85 ± 0.12 mg AAE /g sample), this study obtained higher activity than the studies of Mabini & Barbosa (2018) with 0.55 mg AAE /g sample on *E. philippinensis*, and Mendez et al. (2022) with 1.82 ± 0.09 mg AAE/g sample on *E. pubimarginata*, but lower than the study of Barbosa & Nueva (2019) with 2.03 mg AAE /g sample on *H. conoidea*, Mendez et al. (2023) with 1.82 ± 0.01 mg AAE /g sample and 1.38 ± 0.07 mg AAE /g sample on *E. fimbriobracteata* and *E. philippinensis*, respectively.

The reducing power of the leaves of *E. coccinea* (9.37 ± 1.88 mg GRPE/g sample) revealed to have a higher activity than the study of Mendez et al. (2022) with 24.83 ± 2.99 mg GRPE/g sample in *E. pubimarginata* and Mendez et al. (2023) with 7.53 ±0.80 mg GRPE/g sample in *E. philippinensis*, but lower in *E. fimbriobracteata* with 10.16 ± 2.18 mg GRPE/g sample in *E. fimbriobracteata*. In terms of the rhizome ethanolic extracts, *E. coccinea* (0.28 ± 0.07 mg GRPE/g sample) is lower compared to the study of Mendez et al. (2022) with 0.33 ± 0.10 mg GRPE/g sample on *E. pubimarginata* and Mendez et al. (2023) with 0.97 ± 0.18 mg GRPE/g sample on *E. fimbriobracteata*, but lower in *E. philippinensis* with 0.09 ± 0.09 in the study of Mendez et al. (2023).

The last several decades have seen increased research attention of potential phytochemicals from plants for therapeutic uses because many phytochemicals have been demonstrated to have antioxidant activities (Kairupan et al., 2019). The TAA of plant extracts might be due to the presence of polyphenols, which act as reductones by donating electrons and reacting with free radicals converting them to a more stable product and subsequently terminating free radical chain reaction (Gordon, 1990). Populations of *E. coccinea* were found at the montane forests of Mt. Malambo in partially open forest, which supported Frankel & Berenbaum (1999) that foliage of tropical forest plants produced more antioxidants when exposed to elevated light conditions than the other plant parts. This answers why the leaves obtained the higher phenolic content and antioxidant activities than the rhizomes.

Correlation Analyses

The contribution of the phenolic compounds in the ethanolic extracts of the *E. coccinea* to the antioxidant activity was determined by Pearson's correlation coefficient. A perfect positive linear relationship was observed between TPC and TAA, TPC and RP, and TAA and RP ($r = 1$, $p < 0.001$) (Table 3). By comparing the correlation coefficients (R value), it is possible that phenolic compounds significantly contribute to the antioxidant activities of the plant's extracts.

Table 3. Pearson's correlation coefficients between TPC and TAA, TPC and RP, and TAA and RP.

Plant parts	Total phenolic content	Total antioxidant activity	Reducing Power
Total phenolic content	1	1**	1**
Total antioxidant activity	1**	1	1**
Reducing Power	1**	1**	1

**Correlation is significant at 0.001 level

The strong correlation between the results using the two methods of measuring TPC and antioxidant activity showed that phenol compounds largely contribute to the antioxidant activities of *E. coccinea* and, therefore, could play an important role in the beneficial effects of the plant. Several reports also showed a close relationship between TPC and antioxidant activity, since phenolic compounds serve as hydrogen-donating agents (Li and Jiang, 2007; Prasad et al., 2005; Yang et al., 2014). With the data of this paper, this study supported the claim of Stanković (2011) that the higher the phenolic content, the higher the antioxidant activity.

Conclusions and Recommendations

Higher TPC value was recorded higher in leaves (11.69 ± 0.47 mg GAE/g sample) than the rhizomes (0.58 ± 0.06 mg GAE/g sample). For the TAA, the leaves (12.76 ± 0.31 mg AAE/g sample) had a higher activity than rhizomes (0.85 ± 0.12 mg AAE/g sample) and the RP had a higher activity in the leaves (9.37 ± 1.88 mg GRPE/g sample) than the rhizomes (0.28 ± 0.07 mg GRPE/g sample). Overall, the leaves contributed higher phenolic content and antioxidant activity compared to rhizomes. Based on the correlation analysis, a perfect positive linear relationship was observed among TPC, TAA, and RP. These imply that the high content of phenolic compounds contributes to the antioxidant activity of extracts of *E. coccinea*. As this is the first report of the phenolic content and antioxidant activity of *E. coccinea* using Philippine materials, this calls for thorough phytochemical analyses to be done to identify the active phenolic and antioxidant components of this ginger species.

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References

Acma FM, Mendez NP, Amoroso VB. 2020. Zingiberaceae of Marilog Forest Reserve, Southern Philippines: Its Richness and Endemism. *Asian Journal of Biodiversity, 11* (1) : 76–90. doi: 10.7828/ajob.v11i1.1388.

Acma FM, Mendez NP. 2018. Pollen Morphology and Pollen Elemental Composition of selected Philippine Native Gingers in Tribe Alpinieae (Alpinioideae: Zingiberaceae). *Biological Forum - An International Journal, 10* (1): 1–10.

Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in pant tissues using Folin-Ciocalteu reagent. *Nature Publishing Group,* 2: 875–877. doi: 10.1038/nprot.2007.102.

Alam MA, Syazwanie NF, Mahmod NH, Badaluddin NA, Mustafa KA, Alias N, Aslani F, Prodhan MA. 2018. Evaluation of antioxidant compounds, antioxidant activities and capsaicinoid compounds of Chili (*Capsicum* sp.) germplasms available in Malaysia. *Journal of Applied Research on Medicinal and Aromatic Plants,* 9: 46–54. doi: 10.1016/j.jarmap.2018.02.001.

Barbosa GB, Nueva MCY. 2019. Antioxidant Activity and Phenolic Content of *Hornstedtia conoidea* (Zingiberaceae). *Asian Journal of Biological and Life Sciences, 8* (1): 1–7. doi: 10.5530/ajbls.2019.8.1.

Burri SCM, Ekholm A, Hákansson **Ā**, Tornberg E, Rumpunen K. 2017. Antioxidant capacity and major phenol compounds of horticultural plant materials not usually used. *Journal of Functional Foods, 38:* 119–127. doi: 10106/j.jff.2017.09.003.

Cirillo G, Lemma F. 2012. *Antioxidant Polymers: Synthesis, Properties, and Applications.* Calabria: John Wiley & Sons. 510.

Côté J, Caillet S, Doyon G, Sylvain J-F, Lacroix M. 2010. Bioactive compounds in cranberries and their biological properties. *Crit. Rev. Food Sci. Nutr.,* 50: 666–679. doi: 10.1080/10408390903044107.

Cesoniene L, Daubaras R, Viskelis P, Sarkinas A. (2012). Determination of the total phenolic and anthocyanin contents and antimicrobial activity of Viburnum opulus Fruit juice. *Plant Foods for Human Nutrition* 67: 256–261.

Danciu C, Vlaia L, Fetea F, Hancianu M, Coricovac DE, Ciurlea S A, Şoica CM, Marincu I, Vlaia V, Dehelean CA, Trandafirescu C. 2015. Evaluation of phenolic profile, antioxidant and anticancer potential of two main representants of Zingiberaceae family against B164A5 murine melanoma cells. *Biological Research, 48* (1): 1–9. doi: 10.1186/0717-6287-48-1.

Frankel S, Berenbaum M. 1999. Effects of light regime on antioxidant content of foliage in a tropical forest community. *Biotropica, 31* (3): 422–429. DOI: 10.1111/j.1744-7429.1999.tb00384.x.

Galvez CJ, Martin-Cordero P, Houghton AM. 2005. Antioxidant activity of methanol extracts obtained from plantago species. *Journal of Agricultural and Food Chemistry, 53:* 1927–1933.

Gordon M. 1990. The mechanism of antioxidant action *in Vitro*. In Hudson BJF, ed. *Food. Antioxidants*, Springer, Berlin. 1–18. doi: 10.1007/978-94-009-0753-9_1.

Govindarajan R, Singh DP, Rawat AKS. 2007. High-performance liquid chromatographic method for the quantification of phenolics in „Chyavanprash" a potent Ayurvedic drug. *Journal of Pharmaceutical Biomedical Analysis, 43:* 527–532.

Jualang AG, Nurul Humairah TA, Devina D, Hartinie M. 2015. *In vitro* shoot regeneration from rhizome bud of native ginger in Borneo, *Etlingera coccinea*. *Journal of Tropical Plant Physiology, 7:* 36–46.

Kairupan CF, Mantiri FR, Rumende RRH. 2019. Phytochemical Screening and Antioxidant Activity of Ethanol Extract of Leilem (*Clerodendrum minahassae* Teijsm. & Binn) as an Antihyperlipidemic and Antiatherosclerotic Agent. *IOP Conf. Series: Earth and Environmental Science, 217:* 1–7. doi:10.1088/1755-1315/217/1/012016.

Lamb A, Goblik J, Ardiyani M, Poulsen AD. 2013. *A Guide to Gingers of Borneo.* Natural History Publications (Borneo), 144.

Li J, Jiang Y. (2007). Litchi flavonoids: isolation, identification, and biological activity. *Molecule, 12* (4): 745–758. DOI: 10.3390/12040745.

Mabini JM, Barbosa GB. 2018. Antioxidant activity and phenolic content of the leaves and rhizomes of *Etlingera philippinensis* (Zingiberaceae). *Bulletin of Environment, Pharmacology and Life Sciences, 7* (9): 39–44.

Maulana, TI, Falah S,Andrianto D. 2019. Total phenolic content, total flavonoid content, and antioxidant activity of water and ethanol extract from Surian (*Toona sinensis*) leaves. In *IOP Conf. Series: Earth and Environmental Science* 299 (2019) 012021. doi:10.1088/1755-1315/299/1/012021.

Mendez NP, Mendez RA, Villafranca-Tuba ARA, Tubongbanua Jr. RM, Acma FM. 2023. Morphology, Phenolic Content, and Antioxidant Activity of *Etlingera fimbriobracteata* (K.Schum.) R.M.Sm. and *E. philippinensis* (Ridl.) R.M.Sm. (Zingiberaceae). *Philippine Journal of Science, 152* (2): 653–664.

Mendez NP, Lumista HP, Lituañas CRM, Mendez RA, Villafranca-Tuba ARA, Acma FM. 2022. Phenolic Content and Antioxidant Activity of ethanol extracts of *Etlingera pubimarginata* (Zingiberaceae), including notes on its

Morphology. *Journal of Tropical Biology and Conservation, 19:* 77–92. doi: https://doi.org/10.51200/jtbc.v19i.

Mendez NP, Acma FM. 2018. *In vitro* Studies on Pollen Viability, Pollen Germination and Pollen Tube Growth of *Hornstedtia conoidea* Ridl. – a Philippine endemic ginger species. *Journal of Tropical Life Science, 8* (3): 279–288. doi: http://dx.doi.org/10.11594/jtls.08.03.10.

Mendez NP, Porquis HC, Sinamban EB, Acma FM. 2017. Comparative Pollen Viability and Pollen Tube Growth of two endemic Philippine *Etlingera* (Zingiberaceae, Alpinioideae). *Philippine Journal of Systematic Biology, 11 (2):* 1–9.

Murugan K, Iyer W. 2012. Antioxidant and antiproliferative activities of marine algae, *Gracilaria edulis* and *Enteromorpha lingulata* from Chenai coast. *International Journal of Cancer Research, 8* (1): 15–26. doi: 10.3923/ijcr.2012.15.26.

Padda MS, Picah DH. 2008. Quantification of phenolic acids and antioxidant activity in sweetpotato genotypes. *Scientia Horticulturae,* 119: 17–20. doi: 10.1016/j.scientia.2008.07.008.

Pelser PB, Barcelona JF, Nickrent DL. (Eds.). 2011 onwards. *Co's Digital Flora of the Philippines.* Accessed at: www.philippineplants.org.

Poulsen AD. 2006. *Etlingera of Borneo.* Natural History Publications. Kota Kinabalu, Sabah. http://www.nhpborneo.com/book.

Prasad NK, Divakar S, Shivamurthy GR, Aradhya S. 2005. Isolation of a free radical scav¬enging antioxidant from water spinach (*Ipomoea aquatica* Forsk.) *Journal of the Science of Food and Agriculture, 85* (9): 1461–1468. DOI: 10.1002/jsfa.2125

Prieto P, Pineda M, Aguilar M. 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry, 269* (2): 337–341. doi: 10.1006/abio.1999.4019.

Shahid-Ud-Daula AFM, Kamariah AS, Lim LBL, Ahmad N. 2015. Phytochemical Screening, Antioxidant, and Antimicrobial Activities of Leaves, Stems, and Rhizomes of *Etlingera coccinea* (Blume) S.Sakai & Nagam. *International Journal of Pharmacognosy and Phytochemical Research*, *7* (5): 873–883.

Singleton VL, Orthofer R, Lamuela-Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299:* 152–178. doi: 10.1016/S0076-6879(99)99017-1.

Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorum OT. 2005. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutation Research - Fundamental and Molecular Mechanisms, 579* (1-2): 200–213. doi: 10.1016/j.mrfmmm.2005.03.023.

Soobrattee MA, Bahorun T, Neergheen VS, Googoolye K, Aruoma OI. 2008. Assessment of the content of phenolics and antioxidant actions of the Rubiaceae, Ebenaceae, Celastraceae, Erythroxylaceae and Sterculaceae families of Mauritian endemic plants. *Toxicology in Vitro, 22* (1): 45–56. doi: 10.1016/j.tiv.2007.07.012.

Stanković MS. 2011. Total Phenolic Content, Flavonoid Concentration and Antioxidant Activity of *Marrubium peregrinum* L. extracts. *Kragujevac Journal of Science, 33:* 63–72.

Wang DS. 2012. Secondary Metabolites from Plants. 1–52 pp.

Wang L, Weller CL. 2006. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol., 17:* 300–312. doi: 10.1016/j.tifs.2005.12.004.

Yang X, Yan F, Huang S, Fu C. 2014. Antioxidant activities of fractions from longan pericarps. *Food Science Technology, 34* (2): 341–345. DOI: 10.1590/S0101-20612014005000034.

Yen GC, Duh PD, Tsai CL. 1993. Relationship between antioxidant activity and maturity of peanut hull. *Journal of Agricultural and Food Chemistry, 41:* 67–70. doi: 10.1021/jf00025a015.

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Research Article

Diversity, Abundance and Distribution of Gastropoda in a Tropical Agricultural Village in Kadavoor, Kerala, India

Brigitt Baby, Aleena Elizabeth Cyril, Gigi K. Joseph*

Post Graduate and Research Department of Zoology, Nirmala College, Muvattupuzha, Kerala, India, 686661.

*Corresponding author: malacolabnirmala@gmail.com

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ABSTRACT

Gastropods are an ecologically significant taxon which take part in crucial ecosystem services like nutrient cycling, food web, calcium cycle, pollination and seed dispersal. The gastropod diversity of Kadavoor village was studied for eight months in monoculture plantations of pineapple, rubber, banana, mixed crop agroecosystems, paddy fields and freshwater bodies. Random sampling was employed monthly in five quadrats of 1 * 1 square metre. A total of 14 species belonging to two subclasses, four orders and nine families were recorded. These include land snails, freshwater snails, slugs and semislugs. Six out of 14 species recorded are endemic to the Indian subcontinent. Two invasive species namely *Laevicaulis alte* and *Allopeas gracile* were recorded. It is notable that *Achatina fulica* was not recorded during our study. Ariophantidae was the most abundant family followed by Subulinidae. The mixed crop agroecosystem was found to be more species-rich than monoculture plantations which might be due to an increase in microhabitats and leaf litter layer. The Simpson's diversity index values are highest for the rubber plantation and lowest for the banana plantation since the species are more evenly distributed in the rubber plantation. *Mariaella dussumieri* is a constant species in all three monoculture plantations since its constancy value is greater than 50%. Sorenson's similarity index for species composition between monoculture ecosystems is high but variable. Microhabitats of snails in all these ecosystems were also recorded. The study is a first attempt at the gastropod diversity of agroecosystems in Kerala and would help in the conservation and management of the gastropods.

Keywords: Snail; slug; semislug; agroecosystem; malacofauna; monoculture

Introduction

Gastropods form a lesser-known taxon despite the multitude of ecological roles played by them (Sen et al., 2012). They are found to inhabit various marine, freshwater and terrestrial ecosystems across the globe (Haszprunar & Wanninger, 2012). In terrestrial ecosystems, snails, slugs and semislugs play significant roles in nutrient cycling, food webs, calcium cycles, pollination, seed dispersal and even altering plant community structure by herbivory (Cyril & Joseph, 2023). Due to their limited dispersal ability and heat-sensitive nature, they are also one of those invertebrate taxa prone to extinction (Régnier et al., 2009).

Globally, around 40,000 terrestrial gastropod species have been recorded to date (Mordan & Wade, 2008). Indian land snail fauna accounts to more than 1109 species out of which 270 species have been recorded from the Western Ghats (Ramakrishna et al., 2010). About 76% of land gastropods of the Western Ghats are endemic (Aravind et al., 2005). The freshwater mollusc species identified from all over the world account for around 4800 in number out of which around 217 have been reported from India (Mukhopadhyay & Tripathy, 2017; Bohm et al., 2020).

Gastropod diversity in agroecosystems is seldom studied. While gastropods are considered mostly as pests in agricultural lands, they can also act as disease control agents for various crops and accelerate the decomposition of the leaf litter (Hajian-Forooshani et al., 2020). Our present study deals with land gastropod fauna in the state of Kerala. Kerala has a high diversity of land gastropods with about 119 species (Mavinkurve et al., 2005; Vijayan et al, 2021). Most parts of the state of Kerala is also facing serious threats from the invasion of exotic gastropods especially the Giant African Snail (Vijayan et al., 2021; Mathai, 2014; Vijayan, 2016). In Kerala, most of the studies on terrestrial gastropod fauna have been done around invasive snail species. Only a handful of studies have been done on the native gastropods of Kerala (Cyril & Joseph, 2021). Hence, there is a negative trend to salt out any snails and slugs in Kerala on the misconception that they may be invasive, pestiferous and pathogenic. In the present study, the terrestrial as well as freshwater gastropods of Kadavoor village in Ernakulam district of the state of Kerala were studied. It is a first-of-a-kind attempt to explore gastropod diversity in agroecosystems in Kerala. The study would act as baseline data for further studies and pace up the land malacofaunal conservation and invasive species management efforts in Kerala.

Materials and Methods

Study area

The study area Kadavoor is an agricultural village in Paingottoor Gram panchayat in Kothamangalam Taluk at the eastern border of the Ernakulam district, state

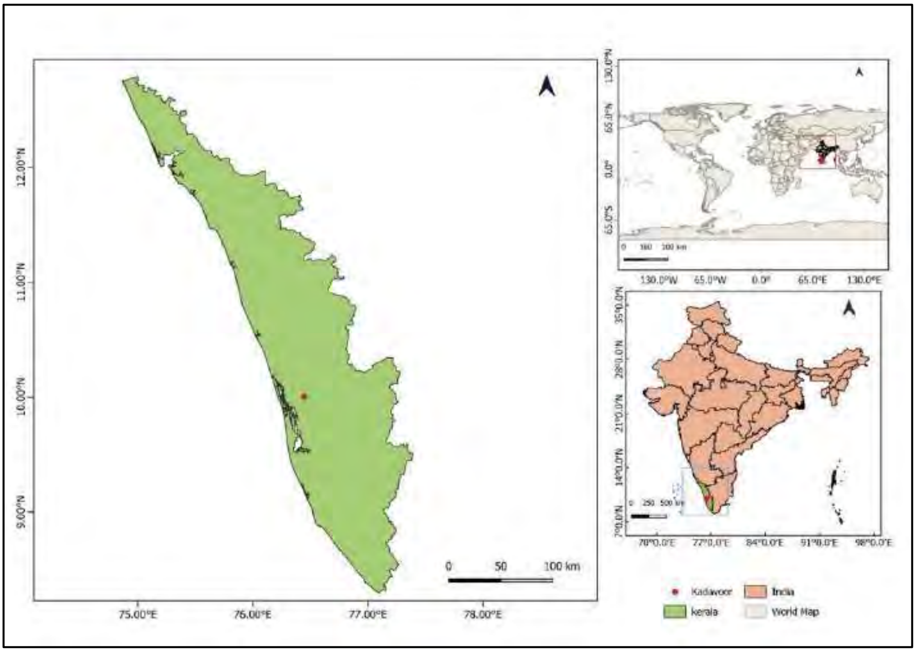

Figure 1. Map showing location of the study area in Kerala state of India.

of Kerala, India (Figure 1). It lies at 10° 0′ 0″ N, 76° 44′ 22″ E. It is located about twenty kilometres away from the nearby towns Muvattupuzha, Kothamangalam and Thodupuzha. The land area consists of various agroecosystems, hilly areas and human settlements. A part of the Kaliyar river locally known as Parithapuzhayar borders Kadavoor village on one side.

Data Collection

Gastropods of Kadavoor village were studied for a period of eight months from December 2021 to July 2022 across various ecosystems. For the feasibility of the study, three monoculture plantations namely, pineapple, banana and rubber plantations were sampled systematically throughout the study period. Random sampling was employed in five quadrats of 1 * 1 square metre in all the sites. Field visits were conducted in the early morning hours on a monthly basis. Occasional sampling was done in other ecosystems like mixed crop agroecosystems, paddy fields and freshwater bodies.

Manual searches for gastropods were conducted on plants, among leaf litter and in the soil at all sites. Photographs of gastropods and their microhabitats were taken in the field. The live gastropods as well as dead shells were collected by hand. The live specimens were killed by keeping them in a container filled with water, with no air inside the container and leaving the setup undisturbed for approximately 12 hours. After draining the water, they were then preserved in 70% isopropyl alcohol and stored in well-labelled sample bottles. Identification was done using standard textbooks like (Raheem et al., 2014; Blanford,1908; Gude, 1921; Gude, 1914).

Statistical Analysis

Several ecological indices were used to assess the composition of gastropod fauna among the study sites. Constancy index (C) is calculated with the formula: $C = (Pa * 100)/P$; Pa is the number of samples containing a particular species and P is the total number of samples. Value of C can be classified into three categories where ($C \geq 50\%$) means the species is constant in the area, ($25\% < C < 50\%$) means it is an accessory species in the area and ($C \leq 25\%$) means it is an accidental species (Dajoz R., 1975).

Relative Abundance (A) is estimated as the number of a particular species to the total number of all gastropod species in a particular area. The value of A can be used to classify species into common ($A > 50\%$), rare ($25\% \leq C \leq 50\%$) and very rare ($C < 25\%$) (Dajoz R., 1975).

Specific richness (S) quantifies the total number of species in a particular site (Magurran, 2004). Simpson's Diversity Index (D) which gives more weight to evenness than richness was also calculated (Magurran, 2004). The similarity in species composition between the two stations was calculated using Sorenson's similarity index (S) and represented in a proximity matrix (Magurran, 2004). All the statistical analysis and graphical representations were made using MS Excel. The map of the study area was created in QGIS software.

Results and Discussion

Inventory and Structure of Terrestrial Gastropod Communities

The study of gastropod diversity in Kadavoor village led to the inventory of 14 species out of which, eight are land snails, three are freshwater snails, two are slugs and one is a semislug. All these three groups of land gastropods namely, land snails, slugs and semislugs were recorded in previous malacofaunal inventories in agricultural lands across the world (Maheshini et al., 2019). The recorded species belong to two subclasses, four orders and nine families (Table 1). The species endemic to the Indian subcontinent in the study area accounts for 43% of the total species observed (Raheem et al., 2014). The six endemic species recorded are *Succinea baconi, Satiella* sp., *Ariophanta bistrialis, Mariaella dussumieri, Euplecta indica* and *Glessula* sp.

Two invasive species namely *Laevicaulis alte* and *Allopeas gracile* were recorded. The invasive *Achatina fulica* has been previously reported to be found in all except one of the districts of Kerala (Vijayan et al., 2021). It is notable that *Achatina fulica* was not recorded from our study area. This underlines the fact that various patches of ecosystems are still free from the grasp *Achatina fulica* and it is not late to take ample steps to control the spread of this invasive species.

Table 1. Systematic checklist of gastropod species in Kadavoor village, Ernakulam, Kerala.

Order	Family	Genus	Species
CLASS GASTROPODA			
SUBCLASS: PROSOBRANCHIA			
Mesogastropoda	Paludomidae	*Paludomus*	*tanschuaricus* (Gmelin, 1771)
Mesogastropoda	Ampullariidae	*Pila*	*globosa* (Swainson, 1822)
SUBCLASS: PULMONATA			
Bassommatophora	Planorbidae	*Indoplanorbis*	*exustus* (Deshayes, 1833)
Systellommatophora	Veronicellidae	*Laevicaulis*	*alte* (Férussac, 1822)
Stylommatophora	Succineidae	*Succinea*	*baconi* (Pfeiffer, 1855)
Stylommatophora	Helicarionidae	*Satiella*	sp.
Stylommatophora	Ariophantidae	*Ariophanta*	*bistrialis* (Beck, 1837)
Stylommatophora	Ariophantidae	*Macrochlamys*	*indica* (Godwin-Austen,1883)
Stylommatophora	Ariophantidae	*Mariaella*	*dussumieri* (Gray, 1855)
Stylommatophora	Ariophantidae	*Euplecta*	*indica* (Pfeiffer, 1846)
Stylommatophora	Subulinidae	*Allopeas*	*gracile* (Hutton,1834)
Stylommatophora	Subulinidae	*Zootecus*	*insularis* (Ehrenburg, 1831)
Stylommatophora	Subulinidae	*Glessula*	sp.
Stylommatophora	Streptaxidae	*Gulella*	*bicolor* (Hutton,1834)

The collected gastropods come under the following nine families namely, Ariophantidae, Subulinidae, Streptaxidae, Succineidae, Planorbidae, Paludomidae, Ampullariidae, Veronicellidae and Helicarionidae (Figure 2).

Family Ariophantidae is the most represented family with four species which are *Ariophanta bistrialis, Macrochlamys indica, Euplecta indica* and *Mariaella dussumieri*. Ariophanta was reported as the most abundant family in several gastropod surveys done in agroecosystems across the Indian subcontinent (Altaf et al., 2017; Altaf et al., 2016; Murtaza et al., 2020). Subulinidae is the second most represented family with three species namely, *Allopeas gracile*, *Zootecus insularis* and *Glessula* sp*.* The rest of the seven families were represented by one species each.

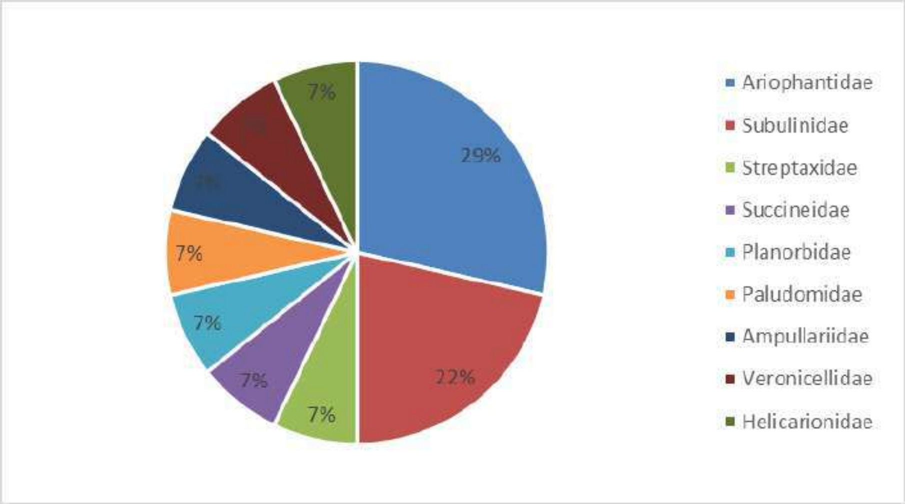

Figure 2. Composition of gastropod families in Kadavoor village.

Patterns in Gastropod Species Distribution Across Different Ecosystems

The mixed crop agroecosystem had a varied gastropod community with 11 species whereas the gastropod communities in monoculture plantations were less diverse (Figure 3). This can be attributed to the habitat heterogeneity hypothesis wherein varied microhabitats present in mixed crop ecosystems support more biodiversity (Gheoca, 2023).

Most of the snails reported only from mixed crop ecosystems in this study are leaf litter dwellers. An increase in the leaf litter layer and the presence of decaying stems or woods in the mixed crop ecosystem might be a reason for the increase in species richness in the mixed crop agroecosystem. The same trend for gastropod diversity and leaf litter layer association was observed in previous studies (Gheoca, 2023; Gheoca, 2021).

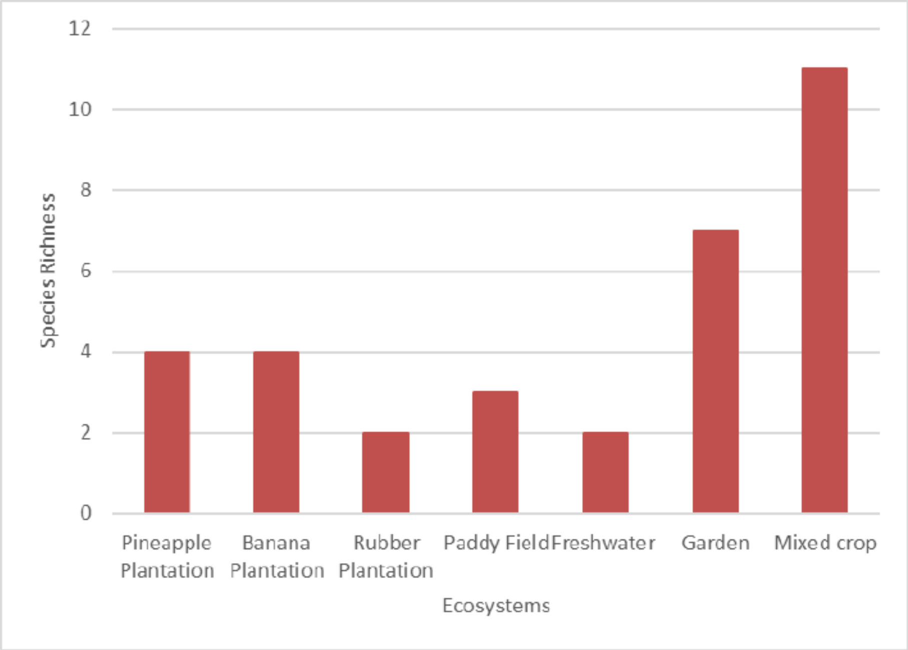

Figure 3. Species richness of land gastropods across different ecosystems.

Among the terrestrial gastropods, *Mariaella dussumieri* and *Satiella* sp. were seen in monoculture as well as mixed crop agroecosystems. *Laevicaulis alte* was seen only in mixed crop agroecosystem. Similarly, this slug was reported from polyculture agroecosystems in Indonesia (Atini & Rusae, 2022). Among the freshwater gastropods, *Indoplanorbis exustus* was observed only in paddy fields whereas *Paludomus tanschuaricus* and *Pila globosa* were observed in paddy as well as other freshwater ecosystems. The presence of different species of gastropods in various ecosystems is visualised in Table 2.

Table 2. Patterns of species distribution in various ecosystems of Kadavoor village.

Species	Pineapple Plantation	Banana Plantation	Rubber Plantation	Paddy Field	Freshwater ecosystems	Mixed crop agroecosystem
Ariophanta bistrialis	✓	✓				✓
Macrochlamys indica	✓	✓				✓
Allopeas gracile						✓
Gulella bicolor						✓
Succinea baconi						✓
Zootecus insularis						✓
Euplecta indica						✓
Glessula sp.						✓
Indoplanorbis exustus				✓		
Paludomus tanschuaricus				✓	✓	
Pila globosa				✓	✓	
Laevicaulis alte						✓
Mariaella dussumieri	✓	✓	✓			✓
Satiella sp..	✓	✓	✓			✓

The distribution of gastropod species in different ecosystems is given in Table 3. The relative abundance of gastropod species in all three monoculture plantations shows a similar trend with *Mariaella dussumieri* being the most abundant and *Satiella* sp. being the least abundant species in each of these ecosystems. The abundance of Mariaella dussumieri may be due to the fact that it is a slug, the most successful gastropod group in agroecosystems (South, 2012).

Mariaella dussumieri is a constant species in all three monoculture plantations since its constancy value is greater than 50%. Slugs are found to thrive well even in lands that grow crops with high physical disturbance (Port & Ester, 2002). Their limacization makes them less calcium dependent and helps them to get into moist cracks and crevices easily to protect them from adverse conditions (South, 2012; Hausdorf, 2001). *Satiella* sp. is a constant species in banana plantation but an accessory species in rubber plantation (25% < C < 50%) and an accidental species in pineapple plantation (C<25%). *Ariophanta bistrialis* and *Machrochlamys indica* are constant species in pineapple plantation.

Table 3. Constancy and relative abundance of gastropod species in monoculture plantations.

Species	Pineapple Plantation		Banana Plantation		Rubber Plantation	
	Constancy %	Relative Abundance%	Constancy%	Relative Abundance%	Constancy%	Relative Abundance%
Ariophanta bistrialis	50.00	8	25.00	3.27	-	-
Macrochlamys indica	87.50	26	87.50	19.67	-	-
Mariaella dussumieri	100.00	64	62.50	60.65	100.00	88.57
Satiella sp.	12.50	2	100.00	3.27	37.50	11.42

The specific richness for pineapple and banana plantations are same, while but is comparatively low for the rubber plantation (Table 4). The gastropod species richness in rubber plantations was found to be low compared to other croplands in a study in Nigeria (Oke & Chokor, 2011). The Simpson's diversity index values are highest for the rubber plantation and lowest for the banana plantation (Table 4). In rubber plantation, though the number of species recorded is low, they are evenly distributed. Hence, the high value of D. Previous studies on soil fauna has also revealed that rubber plantations harbour a lower biodiversity when compared to other ecosystems due to soil degradation and low resource heterogeneity of the rubber plantation (Sing et al., 2019; Hidayat et al., 2018; Miao et al., 2022). The evenness might be due to the ambient conditions like canopy and presence of moist, shady microhabitats (rubber shades, latex groves, damaged trunks) in the rubber plantations that equally favour the survival of both *Mariaella dussumieri* and *Satiella* sp.

Table 4. Simpson's Diversity Index value (D) and Specific richness (S) for study sites.

Sites	D	S
Pineapple plantation	0.526	4
Banana plantation	0.425	4
Rubber plantation	0.791	2

The similarity in species composition between monoculture ecosystems is high but variable. The taxonomic composition between mixed crop agroecosystem and monoculture crops is low. The gastropod community structure of the rubber plantation and mixed agroecosystem was the least similar (Table 5).

Table 5. Sorenson's similarity index values for similarity between taxonomic composition of ecosystems.

	Pineapple	Banana	Rubber	Mixed
Pineapple	100	-	-	-
Banana	100	100	-	-
Rubber	67	67	100	-
Mixed	53	53	29	100

Observations on Microhabitats of Terrestrial Gastropods

The study also recorded the various microhabitats from which the land gastropods were observed in different ecosystems (Table 6).The terrestrial gastropods were found from the leaf surfaces and leaf bases of pineapple plant. Some were also found from the soil in pineapple plantation. In banana plantation, they were found on leaf and stem surfaces, leaf bases, in between sheaths of stem, in curled portions of dry leaves, among leaf litter and on soil. In rubber plantation, most slugs were found in between the plastic tapping sheets and tapping marks. Snails were found near roots, on leaf litter and on tree trunks. In a mixed agroecosystem, most snails and slugs were found in the soil and leaf litter layer as well as on plants. The microhabitats are crucial because they provide adequate moisture and shelter for snail species which have limited dispersal ability (Nandy, 2022; Locasciulli & Boag, 1987).

Table 6. Microhabitats of land molluscs in different ecosystems.

Pineapple Plantation	Banana Plantation	Rubber Plantation	Mixed crop Agroecosystem
At the base of leaves	Among the litter near the base of the plant	On the tapping groove near the base of the latex cup	Under the grass cover
On the ground, near the base of the plant	Among the dried sheaths of the main stem of banana plant	On the plastic shade	On the soil
Near the flowers	Along the mid vein of leaf, near to the leaf base	In the trunk crevices	On the plant parts
Among the grass	At the base of leaves	Among the root curves that is seen above ground	In the crevices of rocks that border the land
	On the litter in the ground	On the base of branches	Beneath fallen logs and stones
	Inside the curls of dried leaves	Among the leaf litter	Among the leaf litter

Conclusion

The study shows that Kadavoor village has rich gastropod fauna across its varied ecosystems. Monoculture plantations have small gastropod communities while mixed crop agroecosystems support more species. The study also reveals that there still remain Giant African Snail uninfested patches in Kerala and it is high time to control the spread of this species. The study can be considered a stepping stone to discover more gastropod fauna in the agroecosystems of Kerala. Malacofaunal inventories of such kind would act as a first step in the conservation of the lesser-known molluscan fauna that are crucial to the survival of the ecosystem.

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References

Altaf J, Qureshi NA, Raza SH, Iqbal MJ. 2016. Assessment of diversity and distribution of Snails (Mollusca: Gastropoda) in the Agroecosystem of Faisalabad, Pakistan. *Journal of Biodiversity and Environmental Sciences*, *8* (1): 17–33.

Altaf J, Qureshi NA, Siddiqui MJI. 2017. Taxonomic studies on the occurrence of the snails (Mollusca : Gastropoda) in the agroecosystem. *Journal of Biodiversity and Environmental Science*, *10* (1): 240–252.

Aravind NA, Rajashekhar KP, Madhyastha NA. 2005. Species diversity, endemism and distribution of land snails of the Western Ghats, India. *Records of the Western Australian Museum, Supplement*, *68* (31): 031–038.

Atini B, Rusae A. 2022. Gastropoda Diversity in Polyculture Agricultural Ecosystem of Nansean Village , Insana District , North Central Timor Regency- East Nusa Tenggara Province (NTT). *BIOEDUSCIENCE, 6* (1): 8–13.

Blanford WT, Godwin-Austen HH. 1908. *The Fauna of British India.* Today & Tomorrow's Printers & Publishers. New Delhi; 1908.

Böhm M, Dewhurst-Richman NI, Seddon M, Ledger SE, Albrecht C, Allen D, Bogan AE, Cordeiro J, Cummings KS, Cuttelod A, Darrigran G. 2021. The conservation status of the world's freshwater molluscs. *Hydrobiologia*, *848* (12): 3231–3254.

Cyril AE, Joseph GK. 2021. Bibliometric Analysis Of Terrestrial Gastropod Studies in Kerala. In: *Biodiversity Conservation and Ecosystem Restoration.* p. 50.

Cyril AE, Joseph GK. 2023. An overview of terrestrial gastropods in India. Indian Forester, *149* (1): 76–82.

Dajoz R. 1975. *Precis d' ecologie.* Gauthier Villars, editor. Paris; 1975.

Gheoca V, Benedek AM, Schneider E. 2021. Exploring land snails' response to habitat characteristics and their potential as bioindicators of riparian forest quality. *Ecological Indicators*, *132*. p.108289.

Gheoca V, Benedek AM, Schneider E. 2023. Taxonomic and functional diversity of land snails reflects habitat complexity in riparian forests. *Scientific Reports*, *13* (1). p9808.

Gude GK. 1914. *The-Fauna-Of-British-India-Mollusca II.* London: Taylor and Francis.

Gude GK. 1921. T*he fauna of British India including Ceylon and Burma Mollusca III.*

Hajian-Forooshani Z, Vandermeer J, Perfecto I. 2020. Insights from excrement. *Ecology*, *101* (5): 1–4.

Haszprunar G, Wanninger A. 2012. Molluscs. *Current Biology, 22* (13): 510–514.

Hausdorf B. 2001. Macroevolution in progress: competition between semislugs and slugs resulting in ecological displacement and ecological release. *Biological Journal of the Linnean Society*, *74* (3): 387–395.

Hidayat MR, Endris WM, Dwiyanti Y. 2018. Effect of a rubber plantation on termite diversity in Melawi, West Kalimantan, Indonesia. *Agriculture and Natural Resources*, *52* (5): 439–444.

Locasciulli O, Boag DA. 1987. Microdistribution of Terrestrial Snails (Stylommatophora) in Forest Litter. *Canadian field-naturalist, 101* (1): 76–81.

Magurran AE. 2004. *Measuring Biological Diversity*. Blackwell publishing: Malden, MA, USA.

Maheshini PWDB, Thilakarathne KGDD, Hirimuthugoda GN, Ranawana KB, Kumburegama S. 2019. The distribution of terrestrial pest gastropods and their damage to agricultural crops in Kandy and Nuwara Eliya districts in Sri Lanka. *Ceylon Journal of Science*, *48* (2): 177–184.

Mathai RS. 2014. The Snail Spurt-An Issue of Concern. *Internafional Research Journal of Environment*, *3* (6): 88–91.

Mavinkurve G, Shanbhag PP, Madhyastha NA. 2005. Checklist of terrestrial gastropods of Tamil Nadu, India. *Records of the Zoological Survey of India*, *105* (1–2): 83–95.

Miao BG, Peng YQ, Yang DR, Guénard B, Liu C. 2022. Diversity begets diversity: Low resource heterogeneity reduces the diversity of nut-nesting ants in rubber plantations. *Insect Science*, *29* (3): 932–941.

Mordan P, Wade C. 2008. Heterobranchia II: The Pulmonata. In: *Phylogeny and Evolution of the Mollusca* : 409–426.

Mukhopadhyay A, Tripathy B. 2017. Mollusca. In: Current status of freshwater faunal diversity in India.

Murtaza G, Ullah N, Ramzan M, Razzaq F, Munawar N, Perven A, Ullah A, Ali A, Altaf J, Beanish R. 2020. Abundance and Diversity of Snails in Various Agroecosystems in District Multan, Pakistan. *Egyptian Academic Journal of Biological Sciences, B. Zoology*, *12* (2): 155–160.

Nandy G, Barman H, Pramanik S, Banerjee S, Aditya G. 2022. Land snail assemblages and microhabitat preferences in the urban areas of Kolkata, India. *Journal of Urban Ecology*, *8* (1). p.juac004.

Oke CO, Chokor UJ. 2011. Land-snail species richness in a rubber plantation in Iyanomo, Edo State, Nigeria. *Bioscience Research Journal*, *23* (1): 63–72.

Port G, Ester A. 2002. Gastropods as pests in vegetable and ornamental crops in Western Europe. In: Barker GM, editor. *Molluscs as Crop Pests.* New York: CABI Publishing; 2002.

Raheem DC, Taylo, H, Ablett J, Preece RC, Aravind, NA, Naggs, F. 2014. A systematic revision of the land snails of the Western Ghats of India. *Tropical*

Natural History, *4*: 1–294.

Ramakrishna, Mitra SC, Dey A. 2010. Annotated Checklist of Indian Land Molluscs. *Zoological Survey of India;*

Régnier C, Fontaine B, Bouchet P. 2009. Not knowing , not recording , not listing : Numerous unnoticed mollusk extinctions. *Conservation Biology*, *23* (5): 1214–1221.

Sen S, Ravikanth G, Aravind NA. 2012. Land snails (Mollusca: Gastropoda) of India: status, threats and conservation strategies. *Journal of Threatened Taxa*, *4* (11): 3029-3037.

Singh D, Slik JF, Jeon YS, Tomlinson KW, Yang X, Wang J, Kerfahi D, Porazinska DL, Adams JM. 2019. Tropical forest conversion to rubber plantation affects soil micro-& mesofaunal community & diversity. *Scientific reports*, *9* (1). p.5893.

South A. 2012. *Terrestrial slugs: Biology, ecology and control.* Springer Science & Business Media.

Vijayan K, Suganthasakthivel R, Sajeev TV, Naggs F. 2021. Report of *Lissachatina fulica* (Bowdich, 1822)(Stylommatophora: Achatinidae) in rubber plantations of Western Ghats, Kerala. *ENTOMON*, *46* (4): 333–336.

Vijayan K. 2016. Tracking the invasion of *Achatina fulica* (Bowdich , 1822) and its role in spreading the Rat lung worm. Thrissur.

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Research Article

Checklist and Assessment of Pteridophytes in Amai Manabilang, Lanao Del Sur, Philippines

Jay Ann L. Bada*, David John D. Bierneza, Fulgent P. Coritico, Victor B. Amoroso

Central Mindanao University, Musuan, Bukidnon, Philippines, Musuan, Maramag, Bukidnon 8710, Philippines.

*Corresponding author: jayannbada2@gmail.com

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ABSTRACT

The species of ferns and lycophytes in Municipality of Amai Manabilang are documented here in a checklist, along with information on their morphological characteristics, and conservation status. A series of field surveys in two barangays in Amai Manabilang determined the lycophytes and ferns, namely Frankfort and Sumugot. A total of 56 species were recorded, belonging to 18 families and 36 genera. Of these species, 50 are ferns and 6 are lycophytes. The highest number of species was observed in Sumugot (42 species) followed by Frankfort (26 species). Thirty-one species are terrestrial, 19 are epiphytes, 1 tree fern and 5 species have more than one growth form. The number of species is approximately about 5% and 9% of the total number of pteridophytes species in the Philippines and Mindanao, respectively.

Keywords: Amai Manabilang; Frankfort; Sumugot; transect walk; pteridophytes

Introduction

Ferns and fern allies are collectively called ‘pteridophytes’ (Smith et al., 2006) because of their shared life cycle as spore-dispersing vascular plants (Amoroso et al., 2016) dating back to 360 million years. The Philippine archipelago has an estimated 1,100 ferns and lycophytes spread across 154 genera and 34 families (Coritico et al., 2020; Amoroso et al., 2016). Furthermore, 202 species are reported as threatened (Fernando et al., 2008) and 266 species are endemic (Pelser et al., 2011).

With coordinates of 7°47′07″N 124°40′56″E, Frankfort has the highest elevation of 900 masl, and 7° 45' 12” N, 124° 38' 24” E, Sumugot has the highest elevation of 1,200 masl and is one of the remaining forest ecosystems in Lanao del Sur. So far, there are no studies about the flora and fauna in Amai Manabilang. The absence of research is of significant value to the current study as it impedes a comprehensive understanding of the biodiversity and ecological dynamics of the area.

This study seeks to provide foundational information for the area by conducting an inventory of ferns and lycophytes found in the municipality of Amai Manabilang through a checklist, classifying and identifying their diagnostic morphological characters, and assessing the conservation status of these ferns and lycophytes found in the area. This is the first documented study of ferns and lycophytes in Lanao del Sur. Providing essential baseline data for future research and conservation efforts in the area.

Materials and Methods

Species inventory

An inventory of ferns and lycophytes was conducted through a series of transect walks (1200–1900 m) from base to the highest point of Sumugot, (1200 masl) and Frankfort, (900 masl) from October to November 2021. Another field inventory was conducted from March to April 2022.

Collection, Processing, and Identification of Specimens

A minimum of two fertile fronds of each species were collected by using pruning shears. Small ferns were collected by uprooting the whole plant, removing the soil, and pressing the plant intact. For the tree ferns, each entire frond was collected and cut into five parts: the leaf apex, middle pinna, lower pinna, basal pinna, and stipe (Amoroso et al., 2016).

All specimens were processed using the wet method (Hodge 1947) "wet method" is a technique used to preserve plant specimens by storing them in a liquid solution (denature alcohol or formalin) to maintain their physical features and enable accurate study and documentation of their characteristics in botanical research. The specimens are the first collections of ferns and lycophytes in the area as no collections for herbarium vouchers have been done in the past.

Species identification was performed by referring to the following monographs, floras, and other publications: the book of Smith (2006), Co’s Digital Flora of the Philippines (2013), and Copeland’s Fern Flora of the Philippines (1958–1961).

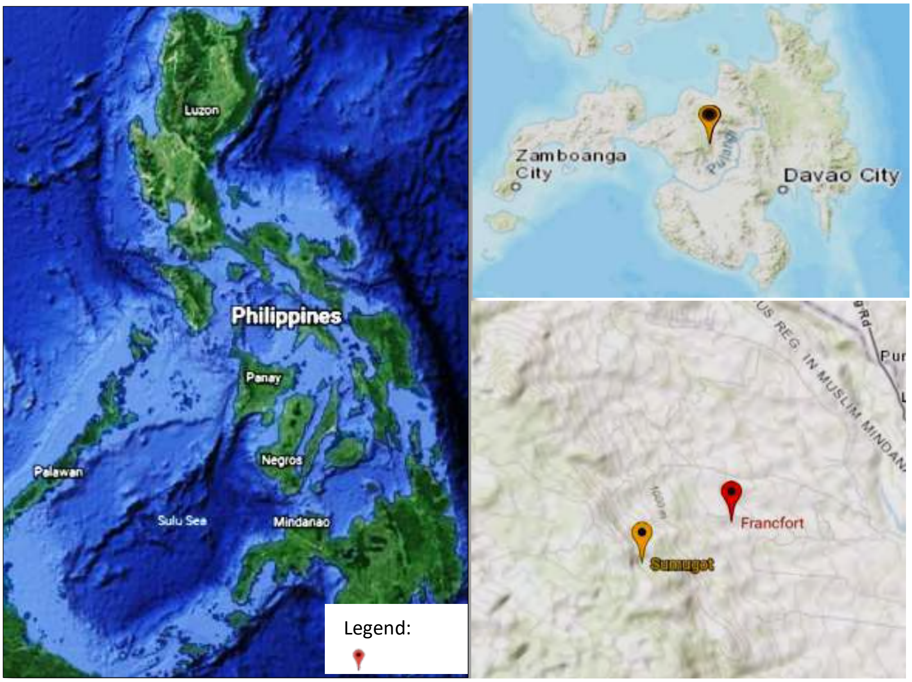

Figure 1. Location Map showing the two selected areas in Amai Manabilang, Lanao del Sur, Philippines.

Assessment of Conservation Status

The conservation status of the species is recorded based on the National List of Threatened Plants of the Philippines (DENRDAO 2017), and using the criteria of the International Union for Conservation of Nature (IUCN 2021). This information can form the basis for government agencies that set the environmental policies of DENR and local government units (LGUs) to monitor and protect threatened and endemic species, both in the forest area and its surroundings.

Results and Discussion

Species Richness

Fifty-six species, belonging to 36 genera and 18 families, were documented. Of these, 50 are ferns and 6 are lycophytes. The families with the highest number of species are Polypodiaceae & Pteridaceae (10 species), Thelypteridaceae (6), Aspleniaceae (4), and Nephrolepidaceae & Tectariaceae (3), the rest have less than 2 families. The lycophytes were represented by Lycopodiaceae and Selaginellaceae with 1 and 5 species, respectively (Table 1 & 2). These are also the families which have the largest number of species in the country. (Salgado 1990). This family is also common and widely distributed in Mindanao (Amoroso et al. 2009, 2012, and 2015).

Table 1. A total number of genera and species of ferns and lycophytes were recorded from Amai Manabilang.

Family	Number. of Genera	Number of Species
Aspleniaceae	2	4
Athyriaceae	2	2
Blechnaceae	2	2
Cyatheaceae	1	1
Dryopteridaceae	2	2
Gleicheniaceae	1	1
Hymenophyllaceae	1	1
Hypodematiaceae	1	1
Lindsaeaceae	1	1
Lycopodiaceae	1	1
Lygodiaceae	1	1
Marattiaceae	2	2
Nephrolepidaceae	1	3
Polypodiaceae	7	10
Pteridaceae	4	10
Selaginellaceae	1	5
Tectariaceae	1	3
Thelypteridaceae	5	6
TOTAL	36	56

Table 2. Checklist and conservation status of ferns and lycophytes found in Amai Manabilang, Lanao del Sur.

Family/Species	Collection number	Growth form
Aspleniaceae		
Asplenium affine Sw.	60/63	TE
Asplenium persicifolium J.Sm. ex Mett	37 / 35	EP
Asplenium sp.	29	EP
Hymenasplenium excisum (C.Presl) S.Linds.	17	EP
Athyriaceae		
Diplazium sp.	24	TE
Deparia petersenii (Kunze) M.Kato	71/86	TE
Blechnaceae		
Austroblechnum patersonii (R.Br.) Gasper & V.A.O.	46	TE
Dittrich	48	TE
Blechnopsis orientalis (L.) C.Presl		
Cyatheaceae		
Sphaeropteris glauca (Blume) R.M.Tryon	08/106	AR
Dryopteridaceae		
Bolbitis heteroclita (C.Presl)	19/27	TE/LI
Dryopteris sp.	40	TE
Gleicheniaceae		
Dicranopteris linearis (Burm.) Underw.	21/49	TE
Hymenophyllaceae		
Vandenboschia auriculata (Blume)	35/50	EP

Hypodematiaceae		
Leucostegia immersa (Wall.) C.Presl	30/10	TE
Lindsaeaceae		
Odontosoria chinensis (L.) J.Sm.	09	TE
Lycopodiaceae		
Palhinhaea cernua (L.) Vasc. & Franco	45	TE
Lygodiaceae		
Lygodium japonicum (Thunb.) Sw.	03	TE
Marattiaceae		
Angiopteris evecta (G.Forst.) Hoffm.	18	TE
Ptisana sylvatica (Blume) Murdock	16	TE
Nephrolepidaceae		
Nephrolepis cordifolia (L.) C.Presl	05/42	TE/EP
Nephrolepis biserrata (Sw.) Schott	23/97	TE
Nephrolepis falcata (Cav.) C.Chr.	44	EP
Polypodiaceae		
Drynaria quercifolia (L.) J.Sm.	20	EP
Drynaria rigidula (Sw.) Bedd.	43	EP
Microsorum punctatum (L.) Copel.	41/61	EP
Lepisorus zippelii (Blume) C.F.Zhao	28	EP
Goniophlebium persicifolium (Desv.) Bedd	62	EP
Goniophlebium pseudoconnatum (Copel.) Copel.	15	EP
Phymatosorus scolopendria (Burm.f.) Pic.Serm.	38/70	EP
Pyrrosia longifolia (Burm.f.) C.V.Morton	47	EP
Pyrrosia piloselloides (L.) M.G.Price	67	EP
Prosaptia sp.	34	EP
Pteridaceae		
Adiantum philippense L.	94	TE
Antrophyum callifolium Blume	39	EP
Antrophyum plantagineum (Cav.) Kaulf.	32	EP
Antrophyum sp.	95	EP
Pityrogramma calomelanos (L.) Link	02	TE
Pteris biaurita L.	11	TE
Pteris ensiformis Burm.f.	90	EP
Pteris mertensioides Willd.	99	TE
Pteris tripartita Sw.	64/89	TE
Pteris sp.	51	TE
Selaginellaceae		
Selaginella biformis A.Braun ex Kuhn	04	TE/LI
Selaginella delicatula (Desv.) Alston	06	TE
Selaginella involvens (Sw.) Spring	31	TE/LI
Selaginella sp. 1	07	TE
Selaginella sp. 2	26	TE/LI
Tectariaceae		
Tectaria melanocaulos (Blume) Copel.	72	TE
Tectaria sp. 1	36	TE
Tectaria sp. 2	22	TE
Thelypteridaceae		
Abacopteris nitida (Holttum) S.E.Fawc. & A.R.Sm.	25/87	TE
Christella dentata (Forssk.) Brownsey & Jermy	01/14	TE
Cyclosorus terminans (J. Sm. ex Hook.) KH Shing	12	TE
Cyclosorus unitus (L.) Ching	13	TE
Macrothelypteris polypodioides (Hook.) Holttum	38/74	TE
Strophocaulon unitum (L.) S.E.Fawc. & A.R.Sm.	78	TE

(LEGEND: Growth forms: AR – arborescent; EP – epiphytic; LI – lithophytic; TE – terrestrial)

The species richness estimate in Amai Manabilang is comparable to that of the Mount Agad-Agad, Lanao del Norte, and Kampo Uno, Katipunan, Davao-Arakan Valley Road, North Cotabato. It is higher than that of the gold-mine areas in Tumpagon, Cagayan De Oro City, Adams in the area of Northern Luzon and University of Mindanao, Matina Campus of Davao City. However, it is evidently lower than Mt. Hamiguitan, and (CEDAR) at Impalutao, Impasug-ong, Bukidnon Province (Table 3). Several factors may affect species richness in the Philippines such as the size of the area sampled, climatic conditions, soil type, and geographic location (Amoroso et al. 2009; 2016). Human activities such as the conversion of forests to agricultural or industrial lands, as well as pollution, have an impact on species richness. With the increase in land use and natural resources, many of these threatened taxa are expected to become even rarer, more susceptible, and endangered, and in some cases, local extinction, as a result of disturbance or imbalance in their tightly constrained ecosystems (Chandra et al., 2008).

During the 2000s and up to the present, a significant portion of the region's land has been subjected to a traditional farming practice known as 'kaingin.' This practice involves the clearing of land through vegetation cutting, burning, and cultivation. However, it has raised environmental concerns due to deforestation and soil degradation. The rapid conversion of forest lands into agricultural areas has further resulted in the disappearance of the lowland forest.A combination of factors such as population growth, agricultural activities, developing tourism, and potential forest conversions pose a significant threat not only to the flora but also to the entire biodiversity of the area (Chandra et al., 2008).

Table 3. Total number of ferns and lycophytes in different locations in the Philippines.

Location	Total Number of Species	References
Gold-mined areas in Tumpagon, Cagayan De Oro City	36	Ascaño II et al., 2016
Adams, Northern Luzon	47	Magtoto & Austria, 2017
University of Mindanao, Matina Campus, Davao City	28	Morales, 2018
Kampo Uno, Katipunan, Davao-Arakan Valley Road, North Cotabato	60	Abas, 2017a
Mount Agad-Agad, Lanao del Norte	56	Coritico et al., 2020
Mt. Hamiguitan Range Wildlife Sanctuary, Davao Oriental	74	Amoroso et al., 2018
(CEDAR) at Impalutao, Impasug-ong, Bukidnon	87	Abas 2017b

Figure 2. Some ferns and lycophytes: *A.) Antrophyum callifolium* Blume, *B.) Tectaria sp. C.) Abacopteris nitida* (Holttum) S.E.Fawc. & A.R.Sm., *D.) Asplenium persicifolium* J.Sm. ex Mett., *E.) Bolbitis heteroclita* (C.Presl) Ching, *F.) Vandenboschia auriculata* (Blume) Copel., *G.) Leucostegia immersa* (Wall.) C.Presl, *H.) Selaginella involvens* (Sw.) Spring, *I.) Antrophyum plantagineum* (Cav.) Kaulf.

Growth form and Assessment

Majority of the ferns and lycophytes are terrestrial (31 species, 55.36 %), followed by epiphytes (19 species, 33.93 %), and arborscent (1 species, 1.79 %) (Tree fern) and other species were observed to have more than one growth form (5 species, 8.92 %). The growth form showed that the ferns and lycophytes in the area are mostly terrestrial and epiphytes. Some species like *Bolbitis heteroclita* (C.Presl), *Nephrolepis cordifolia* (L.) C.Presl can be terrestrial and epiphytes on trees, while *Selaginella biformis* A.Braunm, *Selaginella* sp. and *Selaginella involvens* (Sw.) Spring can also grow terrestrially and lithophytes. The outcome was predictable given that two-thirds of the pteridophytes were terrestrial and one-third were epiphytes (Schuettpelz and Pryer, 2009). *Sphaeropteris glauca* (Blume) R.M.Tryon is the only species recorded from Sumugot as endangered and *Angiopteris evecta* (G.Forst.) Hoffm. is the only species found in Frankfort and is considered a threatened species. (Table 2). All documented species of ferns and lycophytes are broadly distributed in different countries.

Conclusion

Municipality of Amai Manabilang is home to 56 species of ferns and lycophytes belonging to 36 genera and 18 families. Species richness of ferns and lycophytes in the area is estimated to be 5% of the total number of pteridophyte species in the Philippines and nearly 9% of the total number in Mindanao Island. The preferred habitats of the ferns and lycophytes are recorded, with 31 species being terrestrial, followed by 19 species which are epiphytes, and 1 arborescent. There is presence of endangered species, such as Sphaeropteris glauca, in Sumugot and Frankfort. Other threatened species, Angiopteris evecta, found in Frankfort reinforces the urgency for local governments to take immediate measures to preserve and protect the remaining forest fragments, ensuring the long-term conservation of these invaluable species.

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References

Abas CGS. 2017a. Preliminary Reporton Ferns and Lycophytes at Kampo Uno, Katipunan, Davao-Arakan Valley Road, North Cotabato. *International Journal of Engineering, Science and Mathematics, 6* (6): 33–41.

Abas CGS. 2017b. Inventory of Ferns and Lycophytes in the Center for Ecological Development and Recreation (CEDAR) at Impalutao, Impasug-ong, Bukidnon *Province. Bull. Env. Pharmacol. Life Sci., Vol 6* (5): 40–47.

Amoroso VB, Amoroso CB, Coritico FP. 2012. Diversity and status of plants in three mountain ecosystems in southern Mindanao, Philippines. *Asian Journal of Biodiversity, 3* (1): 50–73.

Amoroso VB, Acma FM, Coritico FP, Gorme FS, Lagunday NE, Salolog MCS, Colong RD. 2018. Floral diversity assessment of the buffer zones and vicinity of the Mt. Hamiguitan Range Wildlife Sanctuary (MHRWS), Davao Oriental: basis for inclusion to protected area zone. *Philippine Journal of Systematic Biology, 12* (2): 36–51.

Amoroso VB, Acma FM, Dela Cruz RY, Coritico FP, Nietes AD, Hamo GB, Lumista HP. 2015. Diversity of herbaceous pteridophytes in four long term ecological research sites in Mindanao, Philippines. *Asia Life Sciences-The Asian International Journal of Life Sciences*, *24*(1): 69–85.

Amoroso VB, Coritico FP, Fritsch PW. 2016. Species richness and conservation status of ferns and lycophytes in Mt. Hamiguitan Range Wildlife Sanctuary, Davao Oriental, Philippines. *Philippine Journal of Science*, *145* (2): 127–137.

Amoroso VB, Obsioma LD, Arlalejo JB, Aspiras RA, Capili DP, Polizon JJA, Sumile EB. 2009. Inventory and conservation of endangered, endemic and economically important flora of Hamiguitan Range, Southern Philippines. *Blumea-Biodiversity, Evolution and Biogeography of Plants*, *54* (1-2): 71–76.

Ascano CP, Albutra QB, Ansigbat VV, Mugot DA, Paz SL, Demayo CG. 2016. An Inventory of Pteridophytes in and Around Gold-Mined Areas in Tumpagon, Cagayan De Oro City, Philippines. *Journal of Scientific Research and Development, 3* (5): 236–241.

Barcelona JF, Nickrent DL, LaFrankie JV, Callado JRC, Pelser PB. 2013. Co's Digital Flora of the Philippines: plant identification and conservation through cybertaxonomy. *Philippine Journal of Science*, *142*: 57–67.

Chandra S, Fraser-Jenkins CR, Kumari A, Srivastava A. 2008. A summary of the status of threatened pteridophytes of India. *Taiwania*, *53* (2), pp.170–209.

Copeland EB. 1958-1961. *Fern Flora of the Philippines,* Vol. 3. Manila: Manila Bureau of Printing. 555 p.

DENR Administrative Order No. 2017-11, 2017. http://server2.denr.gov.ph/uploads/rmdd/dao-2017-11.pdf.

Fernando ES, Co LL, Lagunzad DA, Gruèzo WS, Barcelona JF, Madulid DA, Lapis AB, Texon GI, Manila AC, Zamora PM. 2008. Threatened plants of the Philippines: a preliminary assessment.

Hodge WH. 1947. The use of alcohol in plant collecting. *Rhodora, 49* (584): 207–210.

[IUCN] International Union for Conservation of Nature. 2021. The IUCN Red List of Threatened Species. Version 2021 -1. http://www.iucnredlist.org.

Magtoto LM, Austria CM, 2017. The pteridophytes of Adams, Northern Luzon, Philippines and their ecosystem services. *Philippine Journal of Systematic Biology*, *11* (2): 43–51.

Morales NE. 2018. Pteridophytes and Lycophytes Assessment in the University of Mindanao, Matina Campus, Davao City Philippines. *Univ. of Min. Intl. Mult. Disc. Res. Jour.*, *Vol. 3* (1).

Pelser PB, Barcelona JF, Nickrent DL (ed.). 2011 onwards. Co's Digital Flora of the Philippines. www.philippineplants.org.

Salgado EA. 1990. A checklist of Philippine ferns. *Philippine Journal of Science, 119:* 107–148.

Schuettpelz E, Pryer K. 2009. Evidence for a Cenozoic radiation of ferns in an angiosperm-dominated canopy. *Proceedings of the National Academy of Sciences USA*, *106* (27): 11200–11205.

Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG. 2006. A classification for extant ferns. *Taxon, 55*: 705–731.

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Research Article

Cryopreservation of the North Borneo *Phalaenopsis gigantea* J.J.Sm. Using a Vitrification Approach

Zaleha A. Aziz[1*], Mariani Ahmad[1], Low Wan Chin[1], Rimi Repin[3], Simun Limbawan[3], Ramlan Maidin[3], Rosmah Murdad[2]

[1] *Faculty of Science and Natural Resource, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[2] *Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[3] *Sabah Parks, P. O. Box 10626, 88806 Kota Kinabalu, Sabah, Malaysia.*

*Corresponding author: zalehaaz@ums.edu.my
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ABSTRACT

Phalaenopsis gigantea is an endangered orchid found in Borneo and Kalimantan. A cryopreservation protocol using vitrification was developed using seeds as explants. Seeds from green unburst capsules were precultured on New Dogashima Medium supplemented with 0–0.5 M sucrose prior to treatment with loading solution and vitrification with PVS2. Loading durations from 0–60 min and PVS2 dehydration time from 0–7 hours were tested. Following vitrification, seeds were stored in liquid nitrogen for one week before being recovered and subsequently cultured on a germination medium. The viability of the seeds post cryopreservation was evaluated based on 2,3,5-triphenyltetrazlium chloride reduction assay (TTC) by the seeds and germination. Seeds survived the cryopreservation treatments and germinated. Seeds precultured on the medium containing 0.3 M sucrose exhibited the highest germination (8.3% ± 2.3), while a 10-min loading time yielded the maximum germination (13.4% ± 2.9). Seeds dehydrated in PVS2 for 7 hours had the highest germination percentage (13.9% ± 2) after liquid nitrogen storage. The TTC and the germination test did not give similar results for the viability of seeds. Cryopreserved seeds developed into seedlings and showed normal morphology. Given that the seeds of *Phalaenopsis* lost viability at room temperature very fast, this protocol can potentially be used for its long-term storage which can assist in the conservation programme of the species.

Keywords: *Phalaenopsis gigantea*; cryopreservation; vitrification; sucrose preculture; loading time; PVS2 dehydration

Introduction

Phalaenopsis gigantea J.J.Sm., commonly known as the Elephant Ear Orchid, is endemic to Borneo (Malaysia – Sabah and Sarawak; Brunei and Indonesia -

Kalimantan). Previously, the species could be found in abundance in the lowland forest on the east coast of North Borneo. However, due to illegal collection activity and clearing of forests for agriculture and development, it is now challenging to find them in the wild habitat. Although its population in the wild is dwindling, it is still currently listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). Under this category, the trading of a species must be controlled to avoid utilization incompatible with their survival (CITES, 2013). Hence, it is essential to preserve the species before it disappears completely. Current conservation programmes for the species include growing it in gardens and nurseries managed by government agencies as well as maintaining plant materials *in vitro*. These methods have limitations - in gardens and greenhouses, the species is exposed to climatic and disease threats, whereas the *in vitro* materials are exposed to contamination and genetic alteration. Storing the seeds of *P. gigantea* is a potential approach to the preservation of the species. But, using the conventional seed banking method, the longevity of orchid seeds is poor (Schofeld et al., 2018). A storage method that can maintain seed viability would be an advantage for the species conservation programme.

Cryopreservation is a method of storing cells, tissues, and organs at a super-low temperature (-196 °C) which causes very minimal loss of viability when the conditions involved are optimal. The method has been used to conserve plant materials of endangered species; compared to conventional practices of conservation, cryopreservation offers more benefits which include requiring little space, being free from climatic and disease threats, and effective for long-term storage (Niino and Valle Arizaga, 2015; Streczynski et al., 2019). The method has been used successfully to preserve the seeds of several orchids such as *Dendrobium* hybrid (Galdiano et al*.,* 2014), *Cattleya tigrine* (Vettorazzi et al., 2019), *Paphiopedilum dayanum* (Petrus et al., 2019) and *Paphiopedilum bellatulum* (Rchb.f.) Stein (Rodpradit et al., 2022).

Cryopreservation of orchid seeds can be done in several ways, one of which is vitrification. Vitrification is a process where cryoprotectant solution solidifies into a metastable amorphous solid known as glass (Roque-Borda et al., 2021). The method uses concentrated cryoprotectants that can penetrate and dehydrates the cell, hence preventing the formation of intra- and extracellular ice crystals in cells and tissues that can cause damage to the cells (Londe et al., 2018). The procedure does not require the use of an expensive programmable freezer (Jiroutová & Sedlák, 2020) making it cheaper than the other methods of cryopreservation of plant materials. Examples of orchids that had been cryopreserved using vitrification are *Rhynchostylis gigantea* (Lindl.) Ridl (Jariyajirawattana et al., 2020), *Aranda* Broga Blue orchid (Khor et al., 2020), *Dendrobium cruentum* Rchb. f. (Prasongsom et al., 2019), *Caladenia latifolia* (Bustam et al., 2016) and *Coelogyne dayanum* (Hakim et al., 2015). These previous works reported that the conditions for vitrification of orchids vary depending on the species and the types of explants used (Seol, 2020). The success of cryopreservation by vitrification requires the optimization of critical variables such as the conditions of preculture, the time of exposure to loading solution, and the time of dehydration in cryoprotectant solutions (Zamecnik et al., 2021). One of the goals in optimizing these conditions is to reduce the water content in the explants.

At present, there is no report on the cryopreservation of *P. gigantea*; therefore, this study aimed to develop an effective vitrification protocol for the cryopreservation of *P. gigantea* seeds by optimizing the conditions involved in the method.

Materials and Methods

Plant Materials

Phalaenopsis gigantea growing in the Orchid Garden at Tawau Hill National Park were self-pollinated. Green, unburst capsules were harvested five months after pollination and sterilized by immersion in 25% (v/v) Chlorox containing one drop of Tween 20 for 20 mins, followed by three rinses with sterilized distilled water. Each capsule was longitudinally dissected, and the seeds were cultured on a germination medium (Murdad et al., 2006) for 10 days. This culture medium was New Dogashima Medium (NDM) (Tokuhara & Mii, 1993) supplemented with 20 gL^{-1} sucrose and 1 gL^{-1} activated charcoal. After this, the seeds were transferred to preculture media, which were NDM, with different concentrations of sucrose.

Vitrification

i) Preculture in sucrose

The seeds were precultured on semi-solid NDM media containing 1 gL^{-1} activated charcoal supplemented with different concentrations of sucrose (0.1 M, 0.2 M, 0.3 M, 0.4 M, or 0.5 M) for five days followed by exposure to loading solution. The loading solution consisted of 2 M glycerol and 0.4 M sucrose (Matsumoto et al., 1995) in an NDM medium at 25 °C for 20 mins. After the loading stage, the seeds were treated with PVS2 solution (Sakai et al., 1990) at 0 °C for 5 hours and subsequently stored in liquid nitrogen (LN) for 1–7 days. The PVS2 solution was made of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide (DMSO), and 0.4 M sucrose in NDM medium (pH 5.7). The control consisted of seeds that were precultured on sucrose-free media.

ii) Loading time

The seeds were precultured on the medium with 0.3 M sucrose for five days followed by incubation in loading solution for 10, 20, 30, 40, 50, or 60 mins at 25 °C followed by treatment in PVS2 as above and stored in liquid nitrogen (LN) for 1–7 days. For the control, the seeds were treated with loading solution but the solution was immediately removed and replaced with PVS2. Because the loading solution was not allowed time to take effect on the seeds, this was referred to as 0 min loading time.

iii) *PVS2 dehydration time*

The seeds were precultured on 0.3 M sucrose for five days and treated with loading solution for 20 mins at 25 °C before being exposed to PVS2 solution for 1, 2, 3, 4, 5, 6, or 7 hours at 0 °C. The vitrified seeds were then stored in LN for 1–7 days. The control consisted of seeds that were immediately transferred into the LN tank after the PVS2 solution was added into the cryotubes. Because the PVS2 was not allowed time to take effect, this was referred to as PVS2 for 0 h. The seeds that were cultured on the germination medium with no vitrification treatment and no exposure to LN were used as the negative control (Non-Cryo). Each treatment was replicated 3–4 times, and 100–300 seeds were tested in each replicate.

Thawing

After storage in LN, cryopreserved seeds were rapidly re-warmed in a water bath at 37 °C for 1 min, after which the PVS2 solution was removed and replaced with 1 ml of unloading solution which contained 1.2 M sucrose (Matsumoto et al., 1995) in NDM medium for 15 mins at room temperature. Finally, the seeds were rinsed five times with a washing solution (NDM medium containing 2% w/v sucrose) (Hakim et al., 2015).

Viability test

Viability was accessed by 2, 3, 5-triphenyltetrazlium chloride (TTC) reduction assay (Hu et al., 2013) and germination of seeds. For the TTC reduction, seeds were incubated in a 1% (w/v) TTC solution for 24 hours at 30 °C in the dark. The number of seeds with TTC-stained embryos were counted, and the viability was defined as the percentage of seeds showing staining. At least 100 seeds were counted.

Seed germination and regrowth

Thawed seeds were cultured on the germination medium which consisted of NDM containing 20 gL^{-1} sucrose and 1 gL^{-1} charcoal (Murdad et al., 2006) in Petri dishes and incubated at 25 °C ± 2 in the dark. Seeds were considered to have germinated when the seed coats cracked, and the protocorms emerged. Germination percentages were determined every 10 days for 210 days. The results were interpreted as germination percentage after 210 days. After 210 days, the germinated seeds were transferred to light conditions so that the protocorms turned green.

Seedling growth and acclimatisation

Green protocorms were transferred to a development medium, which consisted of Experimental Ernst Robert medium (XER) (Ernst, 1994) containing fructose. Protocorms were monitored for shoot and root development.

Data collection and analysis

This study was carried out in a Completely Randomized Design (CRD). Three replicates were prepared for each type of treatment. The germination rates of the seeds were analyzed using One-WayANOVA, means were compared using Turkey Range Test at $p < 0.05$. While data for seed viability based on TCC and seed germination were subjected to Correlation Coefficient Analysis to measure the strength of the relationship between two variables using IBM SPSS Version 21.0 (IBM Corp., Chicago, IL, USA). Pearson Correlation Test was employed to determine a significant difference at $p < 0.05$.

Results and Discussion

This study evaluates the viability of the seeds of *P. gigantea* post cryopreservation based on TTC and germination; hence results for both tests are presented. The embryos of the seeds that were taken out of liquid nitrogen storage slowly swelled up (Figure 1B) and broke out of the testea, forming protocorm (Figure 1C) before developing into roots and leaves (Figure 1D). Generally, cryopreserved seeds of *P. gigantea* exhibited lower germination as compared to the seeds that did not undergo cryopreservation treatment and were not stored in LN (Non-Cryo). These results suggest that damage had occurred in the seeds. This reduction in germination could have been caused by injury due to the formation of intracellular ice crystals during freezing (Chang & Zhao, 2021). Similarly, a reduction in the germination of the seeds of *Vanda tricolor* Lindl. post-cryopreservation was also reported by Jitsopakul et al. (2012).

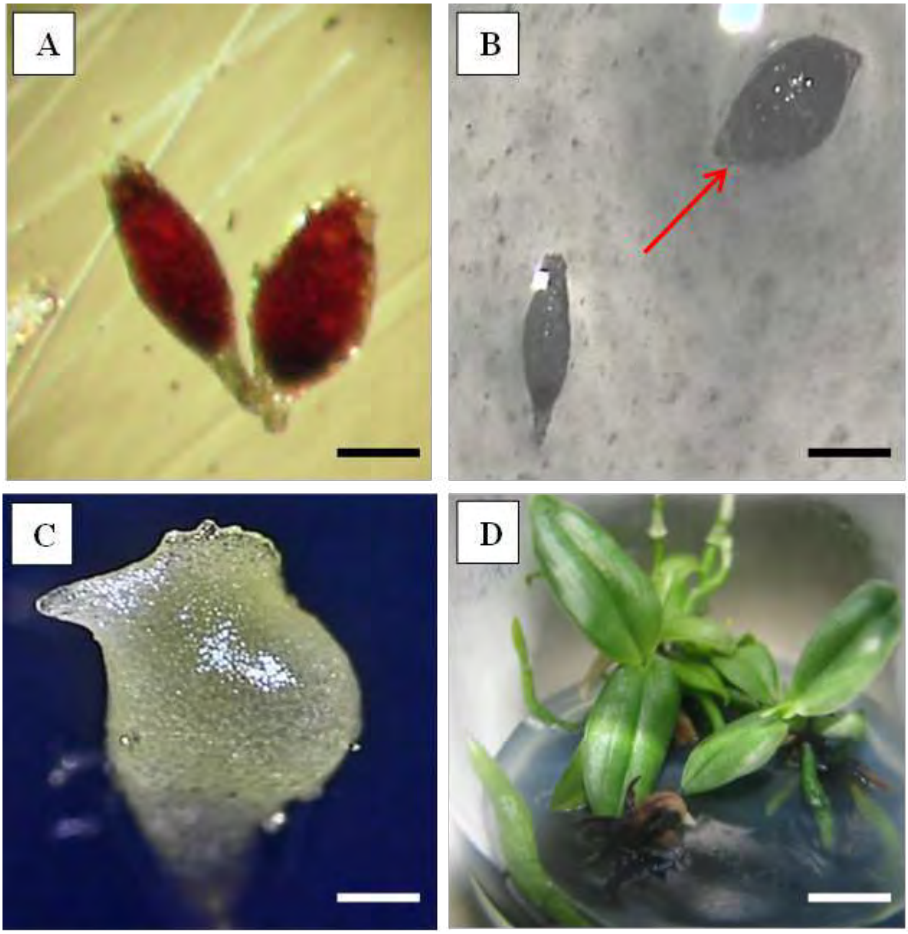

Figure 1. Development of cryopreserved seeds of *Phalaenopsis gigantea* after cryopreservation. (A) Seeds-stained red following TTC test, magnification x 100; (B) A swollen seed (arrow) on germination medium, following recovery from liquid nitrogen, Bar = 1.1×10^{-4} mm; (C) Germinated seed showing leaf primordium, Bar = 1.3 mm; (D) Seedlings from cryopreserved seeds, Bar = 8.3 mm.

Effect of preculture in sucrose on TTC test and the germination

Following recovery from liquid nitrogen, the viability of the seeds based on TTC test showed that precultured on 0.5 M sucrose gave the highest viability (41.8%), followed by those precultured on 0.2 M sucrose (34.3%) (Figure 2). However, the viability based on the germination test revealed that the germination of cryopreserved seeds was the highest (8.3% ± 2.3) when they were precultured on the medium containing 0.3 M sucrose, this is only 1.5-fold increment in germination as compared to the seeds that were pre-treated on the medium without sucrose 5.7% ± 1.5) (Control). The seeds precultured on the medium containing 0.25M sucrose showed the lowest germination (1.3% ± 0.6), this was a 97% reduction in germination as compared to the Non-Cryo seeds (42.0% ± 12.5) (Figure 2). Observation over 210 days revealed that germination declined when the sucrose concentration was further increased to 0.4 or 0.5 M (Figure 2). Our results indicated that preculturing *P. gigantea* seeds in sucrose improved their germination after storage in liquid nitrogen, however, there was no significant (*p* < 0.05) difference in the germination of seeds precultured in different sucrose concentrations.

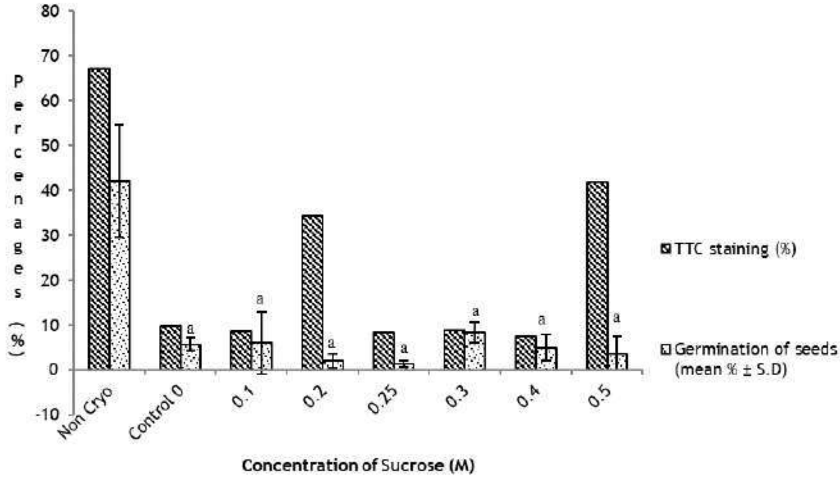

Figure 2. Survival of *Phalaenopsis gigantea* seeds precultured on sucrose after cryopreservation (LN), based on TTC and germination tests.

The beneficial effect of sucrose preculture is due to sucrose enhancing the physiological tolerance of plant materials that are to be cryopreserved by vitrification (Zhang et al., 2023). Enhancement of freeze tolerance because of preculture with sucrose is attributed to the sugar's ability to stabilize membranes by forming hydrogen bonds with membrane phospholipids (Crowe et al., 1987). In our work, 0.3 M sucrose is the best concentration for the preculture of *P. gigantea* seeds. But for other species such Jewel Orchid, *Ludisia discolor* sucrose at 0.2 M for 24 h was the best sucrose preculture treatment that enhanced germination (Burkhan et al., 2022). While *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer required 0.5 M sucrose preculture for the best recovery post-cryopreservation (Diengdoh et al., 2019). On the other hand, there are other orchid species that did not require the sucrose preculture step for successful cryopreservation, this was reported for *Bletilla formosana* (Hayata) Schltr. by Hu et al. (2013).

Effect of loading time on TTC test and germination

The viability of cryopreserved seeds based on TTC analysis was the highest (14.8%) when the seeds were incubated with loading solution for 30 mins (Figure 3). But evaluation of growth based on seed germination showed that the highest germination (13.4% ± 2.9) occurred when seeds were incubated in the loading solution for only 10 min (Figure 3). Germination percentages decreased when the loading period exceeded 10 mins, and the seeds which were treated with the loading solution for 0 min (Positive) had the lowest germination (4.3% ± 1.5). A significant ($P < 0.05$) increase in germination was observed when the seeds were treated for 10 mins in loading solution as compared with 0 min of loading time.

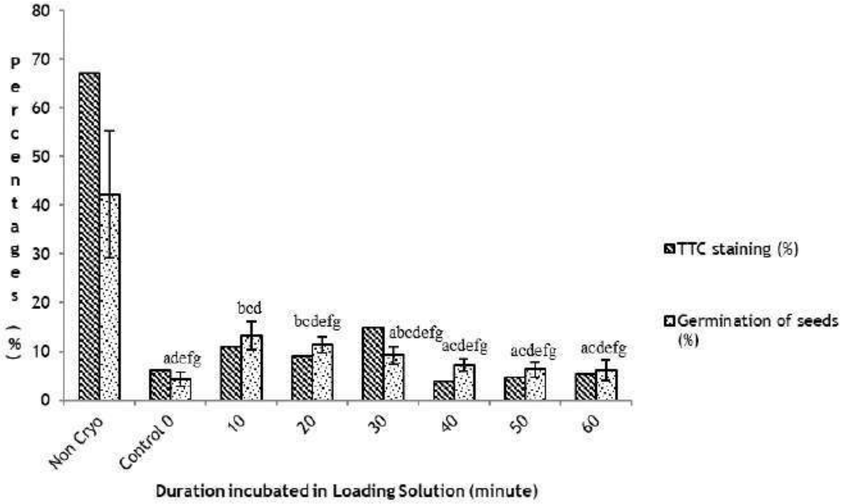

Figure 3. Survival of *Phalaenopsis gigantea* seeds incubated in loading solution for different times after cryopreservation (LN), based on TTC and germination tests.

Our studies showed that the loading treatment is an important step in enhancing the germination of *P. gigantea* seeds post-cryopreservation. When cells are incubated in concentrated PVS2 prior to storage in liquid nitrogen, the cells experience severe osmotic differences which can cause injury due to excessive dehydration, and chemical toxicity (Kaviani & Kulus, 2022). To reduce this osmotic shock when samples are exposed to the concentrated PVS2, treatment of samples with a loading solution having less concentrated osmoticum prior to dehydration is necessary (Matsumoto, 2017). According to Kim et al. (2009), loading treatment acts as an osmotic neutralizer and/or induces a physiological adaptation of tissues, cells, and membranes before the dehydration and freezing steps. In our present study, the protective effect of loading treatment for 10 mins improved germination post-storage by three-fold in LN. The benefit of loading treatment was also reported in the cryopreservation of *B. formosana*, a Taiwanese terrestrial orchid; it was postulated that treating *B. formosana* at different times of loading solution reduces the injuries on the membrane due to severe dehydration in PVS2 (Hu et al., 2013).

Effect of PVS2 dehydration time on the TTC test and germination

The germination test revealed that germination increased as the dehydration time in PVS2 increased; germination was maximal (13.9% ± 2) with 7 h incubation and minimal (3.4% ± 0.6) with 0 h (Control) incubation in PVS2 (Figure 4). As much as a 4-fold increase in germination was observed when the seeds were dehydrated in PVS2 for 7 h as compared with 0 h of incubation. A significant ($p < 0.05$) improvement in germination was observed when the dehydration time in PVS2 was longer than 3 h. In the vitrification procedure, explants must be dehydrated sufficiently to avoid cell damage due to the formation of ice crystals during storage (Rohmah et al., 2022). It is also known that PVS2 solution not only facilitates the survival of cells that are to be cryopreserved but is also toxic to cells when in contact with them (Zamecnik et al., 2021). Hence, it is vital to identify the optimal time of exposure to the vitrification solution so that cells are not damaged, but sufficiently dehydrated (Nakkanong & Nualsri, 2018). This present study indicated that *P. gigantea* seeds showed high tolerance towards desiccation, a desiccation time of 7 h resulted in the highest germination. The longer dehydration time required by *P. gigantea* seeds might be due to the high-water content of the seeds as the seeds were taken from green unburst capsules (immature). According to Wu et al. (2018), the drying time directly affects the water content of orchid seeds, hence affects the formation of intracellular ice nuclei. Desiccation time is species dependent, orchids such as *B. formosana* required 24 h for optimum germination (Wu et al., 2018), on the other hand, seeds of *Coelogyne dayanum* 3 h in PVS2 to yield 80.9% germination (Hakim et al., 2015), while *Coelogyne nitida* (Wall. ex D.Don) Lindl. seeds needed only 40-min incubation in PVS2 to enhance regrowth (Chaudhury et al., 2021). In contrast to the germination test, the viability test using TTC analysis showed that 2 h of incubation in PVS2 gave the highest viability (Figure 4).

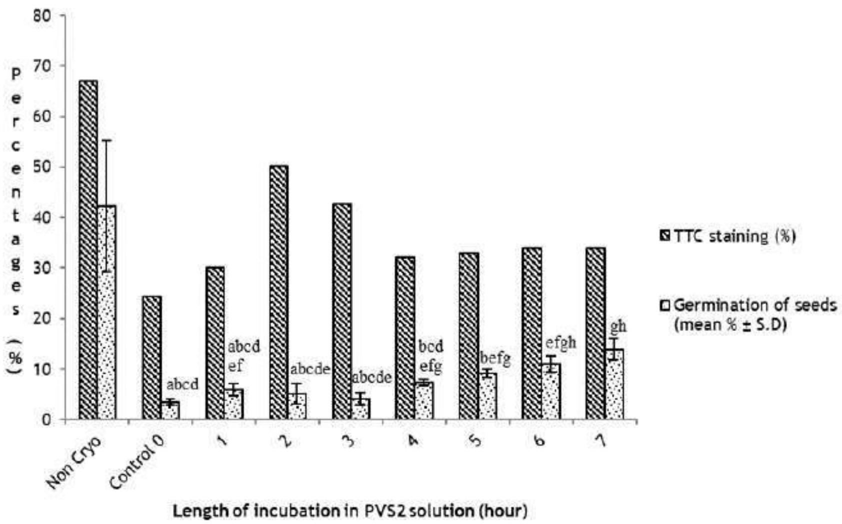

Figure 4. Survival of *P. gigantea* seeds incubated in PVS2 solution for different times after cryopreservation (LN), based on TTC and germination tests.

Throughout the study, the TTC test and the germination test did not give similar results for the viability of seeds, and correlation coefficient analysis revealed that there is no significant ($p < 0.05$) correlation between TTC and germination tests. The TTC test depends on the dehydrogenase enzymes, the enzymes present in living tissues reduce the colorless TTC salt into red-colored triphenylformazan (França-Neto & Krzyzanowski, 2019), hence viable seeds will be stained red (Figure 1A). The contradictory results between the TTC and germination tests may be due to the TTC test giving false positives when the enzymes present in the cell even after the cell death resulted in a viable score (Pradhan et al., 2022). A similar result was reported by Kolomeitseva et al. (2022) in *Dendrobium nobile* Lindl. and *in Rhynchostylis gigantea* (Lindl.) Ridl. (Jariyajirawattana et al., 2020). This study showed that TTC is not an accurate indicator of viability for the species, hence, for *P. gigantea* seeds, it is essential to confirm the survival of cryopreserved seeds through germination tests.

Seedling growth and acclimatization

Plantlets with developed roots were acclimatized and showed normal morphology like that of the parents (Figure 1D). When using cryopreservation technique in any conservation programme, it is very important that the cryopreserved materials retain their genetic stability (Engelmenn, 2011). The genetic integrity of plants surviving cryopreservation can be assessed using several methods, one of which is through the phenotypic level (Harding, 2004). Previous studies that compared the vegetative growth and morphology of seedlings from cryopreserved seeds to that of non-cryopreserved seeds and the parents reported that there were no significant differences in vegetative characteristics. The works included that of *Vanilla planifolia* (González-Arnao et al., 2022), *B. formosana* (Hu et al., 2013), and *Vanda coerulea* Griff. ex Lindl. (Thammasiri & Soamkul, 2007). This present study had shown that no morphological variation was observed as a result of using our method of cryopreservation by vitrification.

Conclusions and Recommendations

This study showed that *P. gigantea* seeds survived cryopreservation by vitrification and germinated successfully. Optimizing the vitrification condition, namely, preculture, loading time, and incubation time in PVS2 improved the survival of cryopreserved *P. gigantea* seeds. Because *Phalaenopsis* seeds lose their viability when stored at ambient temperatures, cryopreservation using this procedure can potentially be applied to conserve *P. gigantea* seeds for long-term storage.

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References

Burkhan H, Rajan KS, Appalasamy S, Poobathy R, Chew BL, Mariappan V, Subramaniam S. 2022. Effect of Cryopreservation Method Supported with Biochemical Analyses in the Axillary Bud of Jewel Orchid, *Ludisia discolor*. *Plants*, *11*: 879. DOI: org/10.3390/ plants11070879.

Bustam BM, Dixon K, Bunn E. 2016. A cryopreservation protocol for ex-situ conservation of terrestrial orchids using asymbiotic primary and secondary (adventitious) protocorms. *In Vitro Cellular Developmental Biology – Plant*, *52*: 185–195. DOI: 10.1007/s11627-015-9732-7.

Chang T, Zhao G. 2021. Ice inhibition for cryopreservation: materials, strategies, and challenges. *Advanced Science*, *8* (6): 2002425. DOI:10.1002/ advs.202002425.

Chaudhury R, Shankar M, Rampal, Awasthi M, Thongam B, Malik SK, Pritchard H 2021. Seed cryopreservation of orchid *Coelogyne nitida* (Wall. Ex don) Lindl. using air desiccation and vitrification techniques. *Indian Journal of*

Plant Genetic Resources, 33 (2): 146–153. DOI: 10.5958/0976-1926.2020.00022.4.

Crowe JH, Crowe LM, Carpenter JF, Wistrom CA. 1987. Stabilization of dry phospholipid bilayers and proteins by sugar. *Biochemical Journal*, *242* (1): 1-10. DOI: 10.1042/bj2420001

Convention on International Trade in Endangered Species (CITES). 2013. Accessed at www.cites.org/eng/disc/how.php

Diengdoh RV, Kumaria S, Tandon P, Das MC. 2017. Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. *South African Journal of Botany*, *112:* 215–224. DOI: 10.1016/j.sajb.2017.05.028.

Engelmann F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology-Plant 47*:5–16. DOI: 10.1007/s11627-010-9327-2.

França-Neto JB, Krzyzanowski FC. 2019. Tetrazolium: an important test for physiological seed quality evaluation. *Journal of Seed Science, 41* (3): 359–366. DOI: 10.1590/2317-1545v41n3223104.

Galdiano Jr RF, de Macedo Lemos EG, de Faria RT, Vendrame WA. 2014. Seedling development and evaluation of genetic stability of cryopreserved *Dendrobium* hybrid mature seeds. *Applied Biochemistry and Biotechnology, 172*: 2521–2529. DOI: 10.1007/s12010-013-0699-8.

González-Arnao MT, Cruz-Cruz CA, Hernández-Ramírez F, Alejandre-Rosas JA, Hernández-Romero AC. 2022. Assessment of vegetative growth and genetic integrity of *Vanilla planifolia* regenerants after cryopreservation. *Plant*, *11*(13): 1630. DOI: 10.3390/plants11131630.

Hakim MH, Elwon CAM, Norzahan MN, Ripin R, Aziz ZA. 2015. Cryopreservation of C*oelogyne dayanum seeds by vitrification. Acta Horticulturae, 1078*: 53–60. DOI:10.17660/ActaHortic.2015.1078.6.

Harding K. 2004. Genetic integrity of cryopreserved plant cells: A review. *CryoLetters 25*: 3–22.

Hirano T, Isikawa K, Mii M. 2005. Cryopreservation of immature seeds of *Ponerorchis graminifolia* var. *suzukiana* by vitrification. *CryoLetter*, *26* (3): 139–146.

Hu WH, Yang YH, Liaw SL, Chang C. 2013. Cryopreservation of the seeds of a Taiwanese terrestrial orchid, *Bletilla Formosa* (Hayata) Schltr. by vitrification. *Botanical Studies, 54*: 33–39. DOI: 10.17660/ActaHortic.2019.1234.24.

Jariyajirawattana D, Bunnag S. 2020. The effects of vitrification method on the seed survival and genetic stability of *Rhynchostylis gigantea* (Lindl.) Ridl. *Asia-Pacific Journal of Science and Technology, 25* (3): 1–9. Article ID.: APST-25-03-05.

Jitsopakul N, Thammasiri K, Yukawa T, Ishikawa K. 2012. Effect of cryopreservation on seed germination and protocorm development of *Vanda tricolor*. *ScienceAsia, 38*: 244–249. DOI: 10.2306/scienceasia1513-1874.2012.38.244.

Jiroutová P, Sedlák J. 2020. Cryobiotechnology of Plants: A Hot Topic Not Only for Gene Banks. *Applied Science, 10* (13): 4677. DOI: 10.3390/app10134677.

Kaviani B, Kulus D. 2022. Cryopreservation of Endangered Ornamental Plants and Fruit Crops from Tropical and Subtropical Regions. *Biology, 11* (6): 847. DOI: 10.3390/biology11060847.

Kim HH, Lee YG, Park SU, Lee SC, Baek HJ, Cho EG, Engelmann F. 2009. Development of alternative loading solution in droplet-vitrification procedures. *CryoLetters, 30* (3): 291–299.

Khor SP, Yeow LC, Poobathy R, Zakaria R, Chew BL, Subramaniam S. 2020. Droplet-vitrification of *Aranda* Broga Blue orchid: Role of ascorbic acid on the antioxidant system and genetic fidelity assessments via RAPD and SCoT markers. *Biotechnology Report*, *26*: e00448. DOI: 10.1016/j.btre.2020.e00448.

Kolomeitseva GL, Nikishina TV, Babosha AV. 2022. Morphophysiology and cryopreservation of seeds of *Dendrobium nobile* Lindl. (Orchidaceae) at different stages of development. *Acta Physiologiae Plantarum, 44*: 36 DOI: 10.1007/s11738-022-03372-z.

Londe LC, Vendrame WA, Sanafi M, De Olivera AB. 2018. Cryopreservation of banana's cv Grand Naine *in vitro* rhizomes. *Anais da Academia Brasileira de Ciências, 90* (1): 495–507. DOI: 10.1590/0001-3765201720170056.

Matsumoto T. 2017. Cryopreservation of plant genetic resources: Conventional and new methods. *Reviews in Agricultural Science, 5*: 13–20. DOI: 10.7831/ras5.13.

Matsumoto T, Sakai A, Yamada K. 1995. Cryopreservation of *in vitro*-grown apical meristems of lily by vitrification. *Plant Cell Tissue Organ Culture, 41*: 237–241.

Murdad R, Kuik SH, Choo KS, Abdul Latip M, Aziz ZA, Ripin R. 2006. High-frequency multiplication of *Phalaenopsis gigantea* using trimmed bases protocorms technique. *Science Horticulturae, 111*: 73–79.

Nakkanong, K. & Nualsri, C. 2018. Cryopreservation of *Hevea brasiliensis* zygotic embryos by vitrification and encapsulation-dehydration. *Journal of Plant Biotechnology, 45*: 333–339. DOI: 10.5010/JPB.2018.45.4.333

Niino T, Valle Arizaga M. 2015. Cryopreservation for preservation of potato genetic resources. *Breeding Science*, *65*: 41–52. DOI:10.1270/jsbbs.65.41.

Petrus E, Ripin R, Molidin D, Aziz ZA. 2019. Cryopreservation of *Paphiopedilum javanicum* seeds by encapsulation-dehydration. *Acta Horticulturae, 1234*: 181–188. DOI:10.17660/ActaHortic.2019.1234.24.

Prasongsom S, Thammasiri K, Narangajavana J, Thitamadee S, Chuenboonngarm N, Panvisavas N. 2019. Vitrification-based cryopreservation of *Dendrobium cruentum* Rchb.f. seeds. *Acta Horticulturae, 1234*: 157–166. DOI: 10.17660/ActaHortic.2019.1234.21.

Pradhan N, Fan X, Martini F, Chen H, Liu H, Gao J, Goodale UM. 2022. Seed viability testing for research and conservation of epiphytic and terrestrial orchids. *Botanical Studies, 63* (3): 14.DOI10.1186/s40529-022-00333-0.

Rodpradit S, Wangpakapattanawond P, Wongsawas P, Thammasiri, K. 2022. Capsule harvesting times and various dehydration methods for cryopreservation of *Paphiopedilum bellatulum* (Rchb.f.) Stein seeds. *Acta Horticulturae, 1339*: 101–110. DOI:10.17660/ActaHortic.2022.1339.14.

Rohmah LA, Latifah D, Wardani FF, Widjaya AH, Dewi K. 2022. Effect of Cryoprotectans and Cryopreservation on Physiological and Some Biochemical Changes of *Hopea odorata* Roxb. Seed. *Journal of Tropical Biodiversity & Biotechnology, 7* (1): jtbb67360. DOI: 10.22146/jtbb.67360.

Roque-Borda CA, Kulus D, Vacaro de Souza A, Kaviani B, Vicente EF. 2021. Cryopreservation of Agronomic Plant Germplasm Using Vitrification-Based

Methods: An Overview of Selected Case Studies. *International Journal of Molecular Science, 22*: 6157. DOI: 10.3390/ ijms22116157.

Sakai A, Kobayashi S, Oiyama I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) cooled to –196ºC. *Plant Cell Report, 9*: 30–33. DOI: 10.1007/BF00232130.

Schofeld E, Jones EP, Sarasan V. 2018. Cryopreservation without vitrifcation suitable for large scale cryopreservation of orchid seeds. *Botanical Studies, 59*: 13. DOI: 10.1186/s40529-018-0229-7.

Seol Y, Yong SH, Choi E, Jeong M J, Suh GU, Lee CH, Choi MS. 2020. Optimization Conditions for Cryopreservation of *Deutzia paniculata* Nakai, Endangered Plant. *Journal for Environmental Science, 36* (4): 274–280. DOI: 10.7747/JFES.2020.36.4.274.

Streczynski R, Clark H, Whelehan LM, Ang ST, Hardstaff LK, Funnekotter B, Bunn E, Offord CA, Sommerville KD, Mancera RL. 2019. Current issues in plant cryopreservation and importance for *ex situ* conservation of threatened Australian native species. *Australian Journal of Botany*, *67*: 1–15. DOI: org/10.1071/BT18147.

Thammasiri K, Soamkul L. 2007. Cryopreservation of *Vanda coerulea* Griff. ex Lindl. seeds by vitrification. *ScienceAsia 33*: 223–227. DOI: 10.2306/scienceasia1513-1874.2007.33.223.

Tokuhara K, Mii M. 1993. Micropropagation of Phalaenopsis and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Report, 13*: 7–11. DOI: 10.1007/BF00232306.

Vettorazzi RG, Carvalho VS, Teixeira MC, Campostrini E, Da Cunha M, de Matos EM, Viccin LV. 2019. Cryopreservation of immature and mature seeds of Brazilian orchids of the genus *Cattleya. Science Horticulturae, 256* (2019): 108603. DOI: 10.1016/j.scienta.2019.108603.

Wu RY, Chuang KC, Hsieh TF. 2018. Effect of capsule maturity and desiccation time on viability of Taiwan native orchid, *Bletilla formosana* seeds (Orchidaceae) after cryopreservation. *Taiwania*, *63* (4): 345–350. DOI: 10.6165/tai.2018.63.345.

Zamecnik J, Faltus M, Bilavcik A. 2021. Vitrification Solutions for Plant Cryopreservation: Modification and Properties. *Plants, 10*: 2623. DOI: 10.3390/plants10122623.

Zhang AL, Wang MR, Li Z, Panis B, Bettoni JC, Vollmer R, Xu L, Wang QC. 2023. Overcoming Challenges for Shoot Tip Cryopreservation of Root and Tuber Crops. *Agronomy, 13*: 219. DOI: 10.3390/agronomy 13010219.

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Research Article

Diversity and Conservation Significance of Angiosperm Climbers in Bhadrak District of Odisha, India

Taranisen Panda[1]*, Nirlipta Mishra[2], Shaik Rahimuddin[2], Bikram K. Pradhan[1], Master Apollo[3], Manoj K. Kar[4], Raj B. Mohanty[5]

[1] *Department of Botany, Chandbali College, Chandbali, Bhadrak 756133, Odisha, India.ORCID-0000-0003-1200-2532.*
[2] *Department of Zoology, Chandbali College, Chandbali, Bhadrak 756133, Odisha, India.*
[3] *Department of Botany, Simulia College, Markona, Balasore 756126, Odisha, India.*
[4] *Department of Botany, Dhamnagar college, Dhamnagar, Bhadrak 756117, Odisha, India.*
[5]*Ex-Reader in Botany, Plot No. 1311/7628, Satya Bihar, Rasulgarh, Bhubaneswar 751 010, Odisha, India.*

*Corresponding author: taranisenpanda@yahoo.co.in

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ABSTRACT

The present study seeks to investigate the distribution patterns of angiosperm climbers within the Bhadrak district of Odisha, India, along with an assessment of the ecosystem services they provide. The ongoing inquiry into climbing plant species within the Bhadrak district reflects the diversity of 103 species distributed across 71 genera and 33 families. The three most diverse families are Convolvulaceae (22), Cucurbitaceae (15), and Fabaceae (14). Out of 103 climbing plant species, 72 species are herbaceous vines and 31 species are woody vines representing 69.9%, and 30.1%, respectively. The most common climbing method in the studied sites is stem twining, accounting for 70 species or 68% of the total, the second most common is tendril climbing (22 species, 21.4%), and the least, root climber (2 species, 1.9%). Local communities use these climbers for various purposes. It has been observed that out of 103 species, 61 species have medicinal properties (59.2%), 16 species have ornamental value (15.5%), 17 species are edible (16.5%), and the remaining 8 species have other uses (8.8%). These results indicate the importance of climber communities to plant diversity of Bhadrak district, enhancing the regional diversity and the conservation value of these forest remnants. Habitat degradation because of rapid development activities with limitation of the supporting tree species is found to be a serious threat to climbing plants. Employing a multifaceted strategy such as securing their habitats through protection, restoration, managing invasive species and promoting collaboration among local stakeholders and organizations, ensures the preservation of these vital plants, sustaining biodiversity and ecosystem health for the future.

Keywords: lianas; medicinal plants; ornamental climbers; stem twiner; vines

Introduction

Various plant growth forms like herbs, shrubs, trees and climbers are structured by abiotic and biotic processes operating at multiple scales and contributed to the diversification of tropical forests (Givnish et al., 2014). It is well known that abundance and species richness of climbers are highest in tropical forests and decrease toward higher latitudes and drier ecosystems and represent about 25% of plant biodiversity in tropical and subtropical forests around the world (Gentry, 1991). Climbing plants are considered as all plants with flexible, thin, and quickly growing axes that germinate on the ground, and after a certain point of their growth, need to attach themselves to an external support—typically neighbouring plants—in order to grow vertically to a significant extent and enhance light acquisition (Moffett, 2000). Trellis availability influences climber diversity in forests (Garbin et al., 2012), and climbers that fail to encounter a trellis often show reduced growth and/or reproduction (Gonza lez-Teuber & Gianoli, 2008). Support finding involves enhanced fitness and triggers changes in growth form, biomass allocation, morphology and physiology (Gianoli, 2003). Therefore, the location (and colonization) of a suitable support is a key process in the life history of climbing plants (Hegarty, 1991). Darwin's observations on the oscillatory movements of exploring stems and tendrils (circumnutation) somehow founded the field of climbing plant behaviour (Darwin, 1875). Climbing mechanisms involve behavioural and structural modification of various organs such as roots, stems, leaves or inflorescence in order to climb. They are pretty much taxon-specific (Hegarty, 1991) and therefore are useful for identifying families, genera and even species. Climbers are taxonomically diverse, Gentry (1991) estimated the representation of climbers in at least 131 plant families before three decades. Gianoli (2015) documented climbers' representation in 171 plant families, including gymnosperms, pteridophytes, and angiosperms. Recently, (Vivek et al., 2022) reported a total of 194 flowering plant families roughly constitute 50% of the globally known angiosperm families. In families like Smilacaceae, Menispermaceae, Passifloraceae, Cucurbitaceae, Convolvulaceae Hippocrateaceae and Vitaceae nearly all the species are composed of or dominated by a climbing habit (Hegarty & Caballe, 1991). Four major characteristics i.e., high growth rates, roots lateral growth, propagation through seed and production of branches play vital roles in the colonization of climbers in the forest. Climbers are classified broadly into vines and lianas based on their stem type (Caballe, 1998). A vine is an herbaceous, thin-stemmed climber that prefers disturbed or high-light exposed habitats. A liana is a woody climber with roots that are usually found on the forest floor. Lianas are especially common in tropical forests (Rai et al., 2016). The climbers are further subdivided into five major types: twiners, tendril climbers, root climbers, hook climbers, and scramblers, based on their climbing strategies (Putz, 1984; Bongers et al., 2005). Tendrils are vegetative organs generated from leaves or stems that can only climb narrow supports (Santos et al., 2009). Twiners' spiraling around the host tree stem necessitates a relatively significant energy expenditure in their growth. They have a larger maximum diameter than tendril climbers (about 10 cm) (Schnitzer et al., 2015). Hook and scrambler climbers cling to supporting trees with hooks and thorns. Understorey root climbers cling to a host trunk with the help of adventitious roots (Schnitzer et al., 2015). Similarly, environmental gradients that affect the distribution of other plant species, such as soil moisture, seasonality, light, topography, rainfall, and soil fertility, also affect the growth of climbing species (Reddy & Parthasarathy, 2006; Swaine & Grace, 2007).

It has also been shown that liana diversity increases with host abundance and diversity (Garbin et al., 2012) indicating that host-climber interactions can influence the composition and structure of climbing plant communities. In particular, host structural characteristics and parasite climbing mechanisms may play a critical role in community assembly (Leicht-Young, 2010). Additionally, young lianas can grow over old and large lianas (Campanello et al., 2007), suggesting that climber-climber interactions may also play a role in the assembly of liana communities. It has been reported that the structure of the host tree is important in the determination of climber association (Putz, 1984; Muthuramkumar & Parthasarathy, 2001). Some of the host trees lack sites for attachment of climber species; for example, smooth-barked trees will evade climber plant infestation (Putz, 1984). The distribution and abundance of climbers (lianas) are also apparently influenced by the architecture of hosts than by climate or soil factors (Balfour & Bond, 1993). For instance, a previous study concluded that tall palms had fewer climber species rising into their crowns than shorter palms (Rich et al., 1987). Gardette (1998) reported that the major factors that contributed to a high species richness or great abundance of climbers were the presence of many supports of different height classes and the proximity of climber parents. Climbing or veining has an impact on the magnificent economy of nature. It permits plants to reach full disclosure to sunlight, nutrients and water with the least expenditure in vegetation support. They add sustainability to cover shutting after the tree falls and help equalize the micro-climate beneath. Forest plant diversity specifically added by Lianas give a valuable niche and contacts amongst tree covers which allow arboreal animal to cross tree tops. A climber plant species is vital in the forest ecosystem as it provides habitat and food in the form of nectar, pollen, fruits, leaves, or sap to many animal species (Schnitzer & Bongers, 2002; Sarvalingam et al., 2015). Climber distribution is influenced by type and forest locality as well (Balfour & Bond, 1993). Climbers play a major role in renewing forests' ecosystems and biodiversity, carbon sequestration, entire-forest transpiration and controlling soil erosion (Schnitzer & Bongers, 2002). A climber reduces the surrounding tree damage and reduces 50% of the post-harvest canopy gaps (Appanah & Putz, 1984). Climbers are essential resources used mainly by local communities, especially those living in nearby areas (Muthumperumal & Parthasarathy, 2013). Climbers provide medicine, food, artisan work, building materials for traditional houses, hunting tools for the local communities and constitute a large and important ornamental horticulture sector (Parthasarathy et al, 2015; Arroyo-Rodriguez & al., 2015). The evidence of climber dominance in certain forest ecosystems i.e., temperate (Allen et al., 2007) and tropical (Swaine & Grace, 2007;) are also attributed to climate change (Malhi & Wright, 2004). The higher frequency of lianas is not only caused by declining rainfall (Swaine & Grace, 2007) but also several other factors are known to favour them such as an increase in disturbance (Londre & Schnitzer, 2006), or especially high responsiveness to elevated CO_2 (Zotz et al., 2006). However, floristic data from 69 tropical forests worldwide found a negative correlation between mean annual precipitation and liana abundance (Schnitzer, 2005).

Various anthropogenic mechanisms such as cattle grazing, intentional or accidental fires, urbanization, agricultural expansion and invasion have caused significant effects on the forest structure, leading to habitat fragmentation with altered plant species composition and functions of forest dynamics (Zeballos et al., 2014; Newbold et al., 2015). Further, previous studies indicate that light availability, soil moisture, soil nutrients and vertical structure conditions are different among these fragmented vegetation patches (Zeballos et al., 2014). This environmental variability may promote a particular floristic and functional composition of the climbing community in each type of degraded forest. The majority of previous studies focused heavily on trees and shrubs and little consideration was given to climber plants in spite of various roles they play in ecosystems (Bongers et al., 2005). Over the past two decades, climber research has gained traction, scientists have discovered patterns in liana abundance and distribution across a variety of habitats and have been investigating the underlying causes of these patterns (Gentry, 1991; Schnitzer & Bongers, 2002; da Cunha Vargas et al., 2021). A number of inventories are available from different states of India addressing climber diversity and dynamics (Girish & Abdul, 2019; Bandyopadhyay & Mitra, 2021; Vivek et al., 2022; Vivek, 2023). However, such type of study is limited in Odisha (Jena et al. 2018) while reports from Bhadrak district are nil. The current study aims to explore the distribution of angiosperm climbers in Bhadrak district of Odisha, India and their ecosystem services.

Materials and Methods

The state of Odisha (81º 43' and 87 º 29' east longitudes and 17º 49' and 22 º 34' north latitude), India, consisting of 30 districts and geographically situated at the head of the Bay of Bengal, has a coastal stretch of around 482 km. It extends over an area of 155,707 sq. km accounting for about 4.87% of the total area of the country. Based on physico-geographical characteristics, the state has been divided into 5 major regions i.e., the coastal plain in the east, the middle mountainous and highlands region, the central plateaus, the western rolling uplands and the major flood plains. The varying climatic condition provides suitable habitats for supporting rich flora and fauna in the region (Patnaik, 1996). Additionally, a number of perennial rivers such as Mahanadi, Brahmani, Baitarani, Rushikulya, Birupa, Budhabalanga and Subarnarekha, and their tributaries pass through Odisha, making the state prone to flooding. Furthermore, the Eastern Ghat range of hills runs through the heart of Odisha i.e., it starts from north of Similipal and runs through Malkangiri crossing 17 districts of the state harbouring primarily moist deciduous vegetation (Champion & Seth, 1968). The state encounters a hot and humid climate round the year with short winters.

Bhadrak district (20° 43′–21° 13′N and 86° 6′–87° E) is located in northeast Odisha. It spreads over 2505 km^2 with 1.507 million inhabitants (2011 Census). It borders the Balasore district in the north, Jajpur in the south, the Bay of Bengal and Kendrapara district in the east and Koenjhar in the west (Figure 1). The district contributes 1.61% and 3.62% of the state’s territory and population respectively. Rice (*Oryza sativa* L.) is the major cereal crop cultivated by most of the people of the district. The district is located in the deltaic region close to the Bay of Bengal. Obviously, it has all the features of a coastal climate, i.e., saline weather, the influence of coastal wind, thunderstorms during monsoons, dust storms in summer and cyclone proneness.

Data collection

To assess the diversity of angiosperm climbers, field surveys were conducted monthly in different seasons (rainy, winter and summer) from July 2016 to July 2020. During field visits, plant samples were collected and photographs of plant species were taken from agricultural lands, wastelands, roadsides, railway tracks, parks, lawns, ponds, river banks and other relevant localities to cover almost all the district in a systematic manner. Information was collected from respondents, especially the local farmers, elderly people, and local healers through interviews following standard procedures (Martin, 1995; Huntington, 2000).

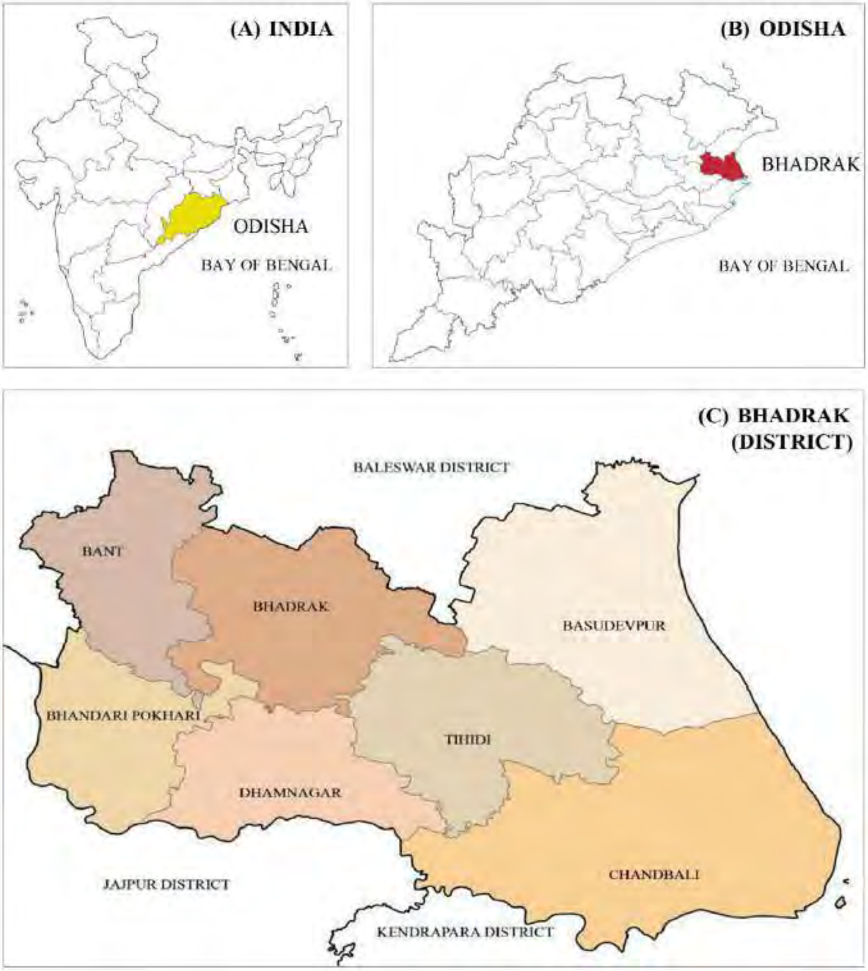

Figure 1. (A) Location of Odisha state in the eastern region of India (B) map of the Odisha state showing Bhadrak district and (C) study area showing different blocks of the Bhadrak district.

Two-hundred-and-seventy-nine (226 men and 53 women) persons were interviewed. Among the interviewees, 10% were aged 21–40, 40% were 61 or older, and 50% were aged 41–60. The questionnaire was semi-structured, followed by free interviews and informal conversations. Plant species were identified with the help of previous scientific literature (Haines, 1925; Saxena & Brahmam, 1996) and with live specimens on the field itself. However, plant samples were identified in the laboratory when it was not found. During the survey, important taxonomic parameters such as vernacular names, and flowering time were recorded from the respondents. The ecological parameters noted were the habits and habitat of the species. The economic uses of these species, if any, were discussed with the local people. The plant list was categorized according to their systematic positions following the APG IV (2016) classification system. The current nomenclature of each species was determined by referring to database POWO (2022).

Results and Discussion

Our study provides important information about the diversity of climbing plant species in the Bhadrak district of Odisha, India. This research is, to our knowledge, one of the few studies that address climbing plant diversity from the Bhadrak district of Odisha state. We found climbers are spreading in all the habitats such as forests, wasteland, village hedges, near canals, river and pond banks, rice fields, railway tracks, roadside, educational and public institutions, and horticultural crop fields. Climbing plants account for a significant component of diversity and abundance and play a major role in tropical forest communities and ecosystems (Schnitzer & Bongers, 2002). The present study documented a total of 103 climber plant species belonging to 71 genera and 33 families (Table 1 & Figures 2–5), contributing to the district's total floristic diversity of 524 plant species (Panda et al. 2020, 2023), with climbers constituting 19.65% of the recorded species. The number of species reported in the current investigation is consistent with floristic surveys from the Koch Bihar district of West Bengal, (98 spp.; Bandyopadhyay & Mukherjee, 2010; 116 spp. Saharanpur District, Uttar Pradesh (116 spp. Saini et al., 2021). However, compared to a few other districts in India, our numbers seem to be very high. Those are Dibrugarh district, Assam, Northeast India and Doaba region of Punjab, India which have 59 species and 53 species, respectively (Kaur et al., 2017; Gogoi & Nath, 2021).

Table 1. List of climbers in Bhadrak district, Odisha, India.

Scientific name with family	Local name	Life form	Climber type	Climbing mode	Frequency of occurrence	Economic uses
Abrus precatorius L. (Fabaceae)	Kaincha	P	Woody climber	Stem Twiner	+++	Medicinal
Allamanda blanchetii A. DC. (Apocynaceae)		P	Herbaceous climber	Stem Twiner	+++	Ornamental

Allamanda cathartica L. (Apocynaceae)		P	Herbaceous climber	Stem Twiner	+++	Ornamental
Antigonon leptopus Hook. & Arn. (Polygonaceae)		P	Herbaceous climber	Tendril climber	+++	Medicinal
Argyreia cymosa Roxb. ex Sweet (Convolvulaceae)		P	Woody climber	Stem Twiner	+++	Medicinal
Argyreia nervosa (Burm. f.) Bojer (Convolvulaceae)	Mundanoi	A	Herbaceous climber	Stem Twiner	+++	Medicinal
Aristolochia indica L. (Aristolochiaceae)	Panairi	P	Woody climber	Stem Twiner	+	Medicinal
Artabotrys hexapetalous (L.f.) Bhandari. (Annonaceae)	Chinichampa	P	Woody climber	Hook climber	+	Medicinal
Asparagus racemosus Willd. (Asparagaceae)	Satabari	P	Herbaceous climber	Scrambler armed	+	Medicinal
Azima tetracantha Lam. (Salvadoraceae)		P	Woody climber	Stem Twiner	++	Medicinal
Basella alba L. (Basellaceae)	Poi	A	Herbaceous climber	Stem Twiner	+++	Edible/ Medicinal
Benincasa hispida (Thunb.) Cogn. (Cucurbitaceae)	Panikakharu	A	Herbaceous climber	Stem Tendril	+++	Edible/ Medicinal
Bougainvillea spectabilis Willd. (Nyctaginaceae)	Kagajaphula	P	Woody climber	Hook climber	+++	Ornamental
Boerhavia diffusa L. (Nyctaginaceae)	Goudapuruni	P	Herbaceous climber	Hook Climber	+++	Edible/ Medicinal
Caesalpinia bonduc (L.) Roxb. (Fabaceae)	Gila	P	Woody climber	Stem twiner	+++	Medicinal
Caesalpinia crista L. (Fabaceae)	Nantei	P	Woody climber	Stem twiner	++	Medicinal
Campsis grandiflora (Thunb.) K.Schum. (Bignoniaceae)		P	Woody climber	Scrambler unarmed	+	Ornamental
Canavalia gladiata (Jacq.) DC. (Fabaceae)	Maharada	P	Woody climber	Stem Twiner	++	Edible
Capparis zeylanica L. (Capparaceae)	Asadua	P	Woody climber	Scrambler armed	++	Medicinal
Cardiospermum halicacabum L. (Sapindaceae)	Kanphuta	P	Herbaceous climber	Tendril climber	+++	Medicinal
Carissa spinarum L. (Apocynaceae)	Anku koli	P	Woody climber	Scrambler armed	++	Edible/ Medicinal
Cayratia pedata (Lam.) Gagnep. (Vitaceae)	Pitapotala	P	Woody climber	Tendril climber	+++	Medicinal
Cayratia trifolia (L.) Domin (Vitaceae)	Amla lata	P	Woody climber	Tendril climber	+++	Medicinal
Cissampelos pareira L. (Menispermaceae)	Akanbindi	P	Herbaceous climber	Stem Twiner	+++	Medicinal
Cissus quadrangularis L. (Vitaceae)	Hadabhanga	P	Woody climber	Tendril climber	++	Medicinal
Citrullus lanatus (Thunb.) Matsum. & Nakai (Cucurbitaceae)	Tarbhuja	A	Herbaceous climber	Stem Twiner	+++	Edible
Clerodendrum thomsoniae Balf.f. (Lamiaceae)		P	Woody climber	Stem Twiner	++	Ornamental
Clitoria ternatea L. (Fabaceae)	Aparajita	P	Woody climber	Stem Twiner	+++	Medicinal

Coccinia grandis (L.) Voigt (Cucurbitaceae)	Kunduri	A	Herbaceous climber	Tendril climber	+++	Edible
Combretum indicum (L.) DeFilipps (Combrataceae)	Madhumalati	P	Woody climber	Stem Twiner	+++	Ornamental
Cucumis melo L. (Cucurbitaceae)	Banakakudi	A	Herbaceous climber	Tendril climber	+++	Edible
Cucumis sativus L. (Cucurbitaceae)	Kakudi	A	Herbaceous climber	Tendril climber	+++	Edible
Cucurbita maxima Duchesne (Cucurbitaceae)	Boitikakharu	A	Herbaceous climber	Tendril climber	+++	Edible
Cuscuta reflexa Roxb. (Convolvulaceae)	Nirmuli	P	Herbaceous climber	Stem Twiner	+++	Medicinal
Dioscorea alata L. (Dioscoreaceae)	Khamba alu	P	Herbaceous climber	Stem Twiner	+++	Edible
Dioscorea bulbifera L. (Dioscoreaceae)	Pitaalu	P	Herbaceous climber	Stem Twiner	++	Edible
Dioscorea glabra Roxb. (Dioscoreaceae)	Kanta-alu	P	Herbaceous climber	Stem Twiner	+++	Edible
Dioscorea pentaphylla L. (Dioscoreaceae)	Tungialu	P	Herbaceous climber	Stem Twiner	++	Edible
Diplocyclos palmatus (L.) C.Jeffrey (Cucurbitaceae)	Kundia	A	Herbaceous climber	Tendril climber	++	Medicinal
Epipremnum aureum (Linden & André) G.S.Bunting (Araceae)		P	Herbaceous climber	Root climber	+++	Ornamental
Evolvulus alsinoides (L.) L. (Convolvulaceae)	Bichhamali	P	Herbaceous climber	Stem Twiner	+++	Medicinal
Evolvulus nummularius (L.) L. (Convolvulaceae)		P	Herbaceous climber	Stem Twiner	+++	Medicinal
Gloriosa superba L. (Liliaceae)	Ognisikha	A	Herbaceous climber	Tendril climber	++	Medicinal
Gmelina philippinensis Cham. (Lamiaceae)		P	Woody climber	Stem Twiner	+	Medicinal
Gymnema sylvestre (Retz.) R.Br. ex Sm. (Apocynaceae)	Gurmari	P	Herbaceous climber	Stem Twiner	++	Medicinal
Hemidesmus indicus (L.) R.Br. (Apocynaceae)		P	Herbaceous climber	Stem Twiner	+++	Medicinal
Hewittia malabarica (L.) Suresh (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	+	Not known
Ichnocarpus frutescens (L.) W.T. Aiton (Apocynaceae)	Madhobi	P	Woody climber	Stem Twiner	+++	Medicinal
Ipomoea aquatica Forssk. (Convolvulaceae)	Kalamasaga	A	Herbaceous climber	Stem Twiner	+++	Edible/ Medicinal
I. batatas (L.) Lam. (Convolvulaceae)	Kandamula	A	Herbaceous climber	Stem Twiner	++	Edible/ Medicinal
I. hederifolia L. (Convolvulaceae)	Panikoda	A	Herbaceous climber	Stem Twiner	++	Medicinal
I. indica (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	++	Medicinal
I. marginata (Desr.) Verdcourt (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	+++	Medicinal
I. mauritiana Jacq. (Convolvulaceae)	Bhuinkakharu	A	Herbaceous climber	Stem Twiner	+	Medicinal

I. nil (L.) Roth (Convolvulaceae)	Khami khondo	A	Herbaceous climber	Stem Twiner	++	Medicinal
I. obscura (L.) Ker Gawl.(Convolvulaceae)		A	Herbaceous climber	Stem Twiner	+++	Medicinal
I. pes-caprae (L.) R.Br. (Convolvulaceae)	Kansari nata	A	Herbaceous climber	Stem Twiner	+++	Medicinal
I. purpurea (L.) Roth (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	+	Medicinal
I. quamoclit L. (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	++	Ornamental
I. sepiaria Koenig ex Roxb. (Convolvulaceae)	Mushkani	A	Herbaceous climber	Stem Twiner	++	Medicinal
I. triloba L. (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	++	Medicinal
Jasminum sambac (L.) Aiton (Oleaceae)	Malli	P	Herbaceous climber	Stem Twiner	+++	Ornamental
Jasminum auriculatum Vahl		P	Herbaceous climber	Stem twiner	+++	Ornamental
Lablab purpureus (L.) Sweet (Fabaceae)	Shimba	A	Herbaceous climber	Stem Twiner	+++	Edible
Lagenaria siceraria (Molina) Standl. (Cucurbitaceae)	Laoo	A	Herbaceous climber	Tendril climber	+++	Edible
Lantana camara L. (Verbenaceae)	Nagaauri	P	Woody climber	Scrambler armed	+++	Medicinal
Luffa cylindrica M.Roem. (Cucurbitaceae)	Pitataradi	A	Herbaceous climber	Tendril climber	+++	Medicinal
Luffa acutangula (L.) Roxb. (Cucurbitaceae)	Janhi	A	Herbaceous climber	Tendril climber	+++	Edible
Merremia hederacea (Burm. fil.) Hall. fil. (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	+++	Medicinal
M. tridentata (L.) Hallier f. (Convolvulaceae)		A	Herbaceous climber	Stem Twiner	++	Medicinal
Mikania micrantha Kunth (Asteraceae)		A	Herbaceous climber	Stem Twiner	+++	Not Known
Mimosa pudica L. (Fabaceae)	Lajkuli	P	Herbaceous climber	Stem twiner	+++	Medicinal
Momordica charantia L. (Cucurbitaceae)	Kalara	A	Herbaceous climber	Tendril climber	+++	Medicinal
Mucuna monosperma Wight (Fabaceae)	Baidanka	P	Woody climber	Stem Twiner	+	Medicinal
M. pruriens (L.) DC. (Fabaceae)	Baidanka	P	Woody climber	Stem Twiner	+++	Medicinal
Mukia maderaspatana (L.) M.Roem. (Cucurbitaceae)	Pahari kakharu	A	Herbaceous climber	Tendril climber	++	Medicinal
Operculina turpethum (L.) Silva Manso (Convolvulaceae)	Dudholomo	P	Herbaceous climber	Stem twiner	++	Medicinal
Paederia foetida L. (Rubiaceae)	Pasaruni	A	Herbaceous climber	Stem Twiner	+	Medicinal
Passiflora foetida L. (Passifloraceae)	Jhumkalata	A	Herbaceous climber	Tendril climber	++	Medicinal
P. incarnata L. (Passifloraceae)		A	Herbaceous climber	Tendril climber	+	Ornamental
Pergularia daemia (Forssk.) Chiov. (Apocynaceae)	Uturudi	P	Herbaceous climber	Stem Twiner	+++	Medicinal

Psophocarpus tetragonolobus (L.) D.C. (Fabaceae)	Asanasimbo	A	Herbaceous climber	Stem Twiner	+	Medicinal
Piper betle L. (Piperaceae)	Pana	P	Woody climber	Stem Twiner	++	Medicinal
P. longum L. (Piperaceae)	Pipal	P	Woody climber	Stem Twiner	++	Medicinal
Pyrostegia venusta (Ker Gawl.) Miers (Bignoniaceae)		P	Herbaceous climber	Tendril climber	++	Ornamental
Rivina humilis L. (Petiveriaceae)		A	Herbaceous climber	Stem Twiner	++	Medicinal
Syngonium podophyllum Schott (Araceae)		P	Herbaceous climber	Root climber	+++	Ornamental
Tragia involucrata L. (Euphorbiaceae)	Bichuati	A	Herbaceous climber	Stem Twiner	+++	Medicinal
Telosma pallida (Roxb.) Craib (Apocynaceae)	Tokeikundhei	A	Herbaceous climber	Stem Twiner	+	Medicinal
Thunbergia erecta (Benth.) T.Anderson (Acanthaceae)		P	Herbaceous climber	Stem twiner	+++	Ornamental
T. fragrans Roxb. (Acanthaceae)	Chakrakedar	P	Herbaceous climber	Stem twiner	++	Ornamental
T. grandiflora (Roxb. ex Rottl.) Roxb. (Acanthaceae)	Chota-ganti	P	Woody climber	Stem twiner	++	Ornamental
Tiliacora racemosa Colebr. (Menispermaceae)	Kalajati noi	P	Woody climber	Stem twiner	+	Medicinal
Tinospora cordifolia (Willd.) Miers. (Menispermaceae)	Guluchilata	P	Woody climber	Stem twiner	++	Medicinal
Trichosanthes cucumerina L. (Cucurbitaceae)	Sallara	A	Herbaceous climber	Tendril climber	+++	Edible
Trichosanthes dioica Roxb. (Cucurbitaceae)	Potala	A	Herbaceous climber	Tendril climber	++	Edible
Trichosanthes tricuspidata Lour. (Cucurbitaceae)	Mahakal	A	Herbaceous climber	Tendril climber	+	Medicinal
Vigna pilosa Baker. (Fabaceae)	Jhikrai	A	Herbaceous climber	Stem Twiner	++	Medicinal
V. trilobata (L.) Verdc. (Fabaceae)	Sanmungo	A	Herbaceous climber	Stem Twiner	++	Medicinal
V. unguiculata (L.) Walp. (Fabaceae)	Judanga	A	Herbaceous climber	Stem Twiner	+++	Edible
Wisteria sinensis (Sims) DC.(Fabaceae)		P	Woody climber	Stem Twiner	++	Not known
Zanthoxylum armatum DC. (Rutaceae)	Tudapoda	P	Woody climber	Scrambler armed	+++	Medicinal
Ziziphus oenoplia (L.) Mill. (Rhamnaceae)	Kankoli	P	Woody climber	Scrambler armed	+++	Edible

Abbreviations: P=Perennial, A= Annual, + rare, ++ common, +++ frequent.

Figure 2. a. *Abrus precatorius* L. b. *Argyreia cymosa* Roxb. ex Sweet c. *Argyreia nervosa* (Burm. f.) Bojer d. *Aristolochia indica* L. e. *Asparagus racemosus* Willd. f. *Azima tetracantha* Lam. g. *Basella alba* L. h. *Boerhavia diffusa* L. i. *Caesalpinia bonduc* (L.) Roxb. j. *Canavalia gladiata* (Jacq.) DC. k. *Capparis zeylanica* L. l. *Cayratia pedata* (Lam.) Gagnep.

Figure 3. a. *Cissampelos pareira* L. b. *Cissus quadrangularis* L. c. *Dioscorea alata* L. d. *Diplocyclos palmatus* (L.) C. Jeffrey e. *Evolvulus alsinoides* (L.) L. f. *Evolvulus nummularius* (L.) L. g. *Gloriosa superba* L. h. *Gmelina philippinensis* Cham. i. *Gymnema sylvestre* (Retz.) R.Br. ex Sm. j. *Ichnocarpus frutescens* (L.) W.T. Aiton k. *Ipomoea aquatica* Forssk. l. *I. obscura* (L.) Ker Gawl.

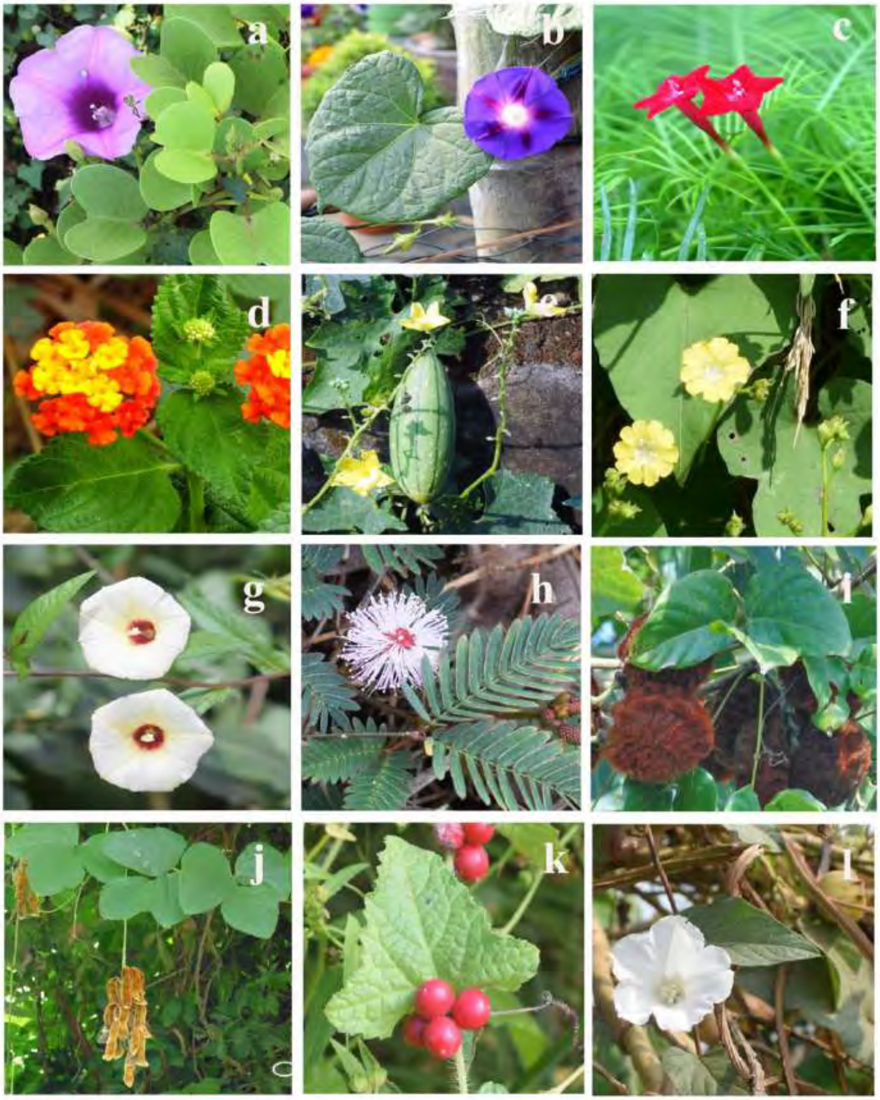

Figure 4. a. *Ipomoea. pes-caprae* (L.) R.Br. b. *I. purpurea* (L.) Roth c. *I. quamoclit* L. d. *Lantana camara* L. e. *Luffa cylindrica* M.Roem. f. *Merremia hederacea* (Burm. fil.) Hall. fil. g. *M. tridentata* (L.) Hallier f. h. *Mimosa pudica* L. i. *Mucuna monosperma* Wight j. *M. pruriens* (L.) DC. k. *Mukia maderaspatana* (L.) M. Roem. l. *Operculina turpethum* (L.) Silva Manso

Figure 5. a. *Paederia foetida* L. b. *Passiflora foetida* L. c. *P. incarnata* L. d. *Psophocarpus tetragonolobus* (L.) D.C. e. *Pyrostegia venusta* (Ker Gawl.) Miers f. *Rivina humilis* L. g. *Telosma pallida* (Roxb.) Craib h. *Tiliacora racemosa* Colebr. i. *Tinospora cordifolia* (Willd.) Miers. j. *Trichosanthes tricuspidate* Lour. k. *Zanthoxylum armatum* DC. l. *Ziziphus oenoplia* (L.) Mill.

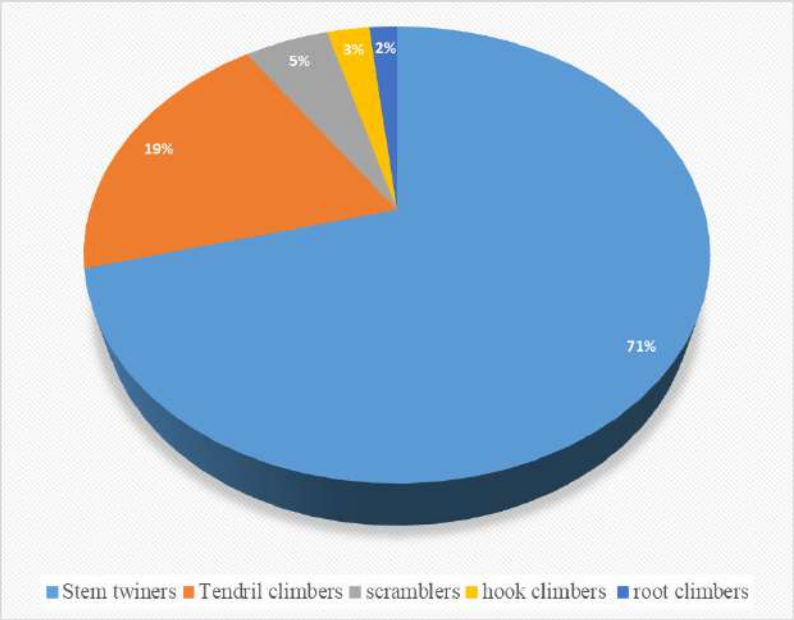

Figure 6. Different climbing mechanisms of climbers in Bhadrak district.

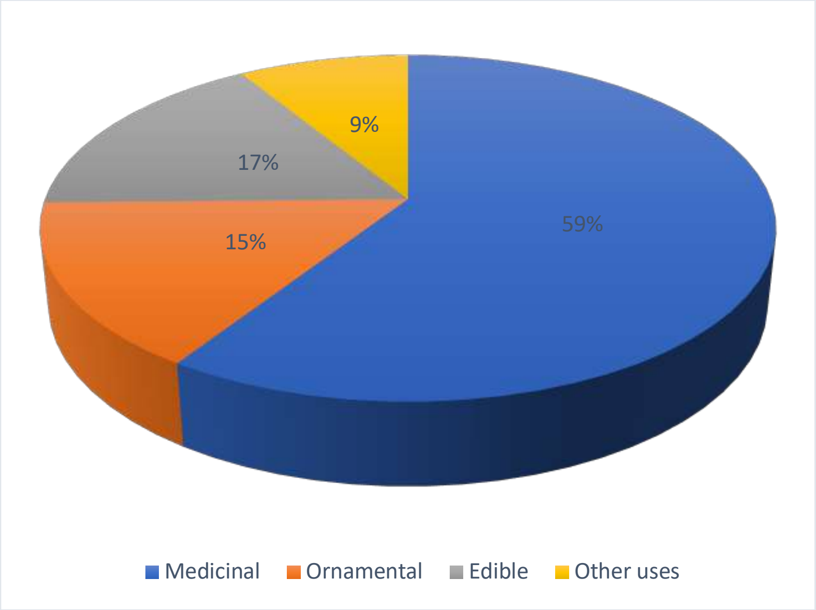

Figure 7. Traditional uses of climbers in Bhadrak district.

Moreover, a higher number of species is also reported from Papum Pare District of Arunachal Pradesh (187 sp. Kashung et al., 2021). Among the families, the most speciose families were Convolvulaceae (22 species), followed by Cucurbitaceae (15 species) and Fabaceae (14 species). Naidu et al. (2014), reported the most specious families, Convolvulaceae (23 spp.), Leguminosae (22 spp.), and Cucurbitaceae (9 spp.) in northern Eastern Ghats of India which is in accordance with the current study. Similarly, the dominant genera were *Ipomoea* possessing the highest number of species (13 species). Out of 103 climbing plant species, 72 species were herbaceous vines and 31 species were woody vines representing 69.9%, and 30.1%, respectively. Representation of perennials was higher (57 sp.) than the annuals (46 sp.). The enumerated climbing plants showed 5 different climbing mechanisms which were stem twiners (68 %), tendril climbers (21.4%), hook climbers (2.9%), scramblers (5.8%) and root climbers (1.9%) (Figure 6). The varied climbing mechanisms adopted by the climbers were similar to those studied in tropical forests by Jayakumar & Nair (2013) and Seger & Hartz (2014). According to several studies, stem twiners were the most frequent climbing plants in tropical forests (Gollasimood et al., 2012; Anbarashan & Parthasarathy, 2013). The most frequent climber species include *Boerhavia diffusa* L*., Bougainvillea spectabilis* Willd., *Caesalpinia bonduc* (L.) Roxb., *Cardiospermum halicacabum* L*., Cissampelos pareira* L., *Clitoria ternatea* L., *Coccinia grandis* (L.) Voigt, *Combretum indicum* (L.) DeFilipps, *Cuscuta reflexa* Roxb., *Evolvulus alsinoides* (L,) L., *Ipomoea aquatica* Forssk., *Lantana camara* L., *Luffa cylindrica, Mimosa pudica* L., *Mucuna pruriens* (L.) DC., *Pergularia daemia* (Forssk.) Chiov., *Tragia involucrata* L. and *Ziziphus oenoplia* (L.) Mill. Similarly, *Capparis zeylanica* L., *Carissa spinarum* L., *Cissus quadrangularis* L., *Gymnema sylvestre* (Retz.) R.Br. ex Sm. and *Mukia maderaspatana* (L.) M.Roem. were commonly found in the study site. Some species like *Aristolochia indica* L., *Asparagus racemosus* Willd., *Gmelina philippinensis* Cham., *I. mauritiana* Jacq., *Mucuna monosperma* Wight, *Psophocarpus tetragonolobus* (L.) D.C., and *Tiliacora racemosa* Colebr. were found restricted to only a few areas of the study site. Local communities were using these climbers for various purposes. It had been observed that out of 103 species, 61 species had medicinal properties (59.2%), 16 species had ornamental value (15.5%), 17 species were edible (16.5%), and the remaining 8 species had other uses (8.8%) (Figure 7). The most prominent species used for medicinal purposes include *Abrus precatorius* L., *Aristolochia indica* L., *Asparagus racemosus* Willd., *Capparis zeylanica* L., *Cissampelos pareira* L., *Cissus quadrangularis* L., *Gloriosa superba* L., *Gymnema sylvestre* (Retz.) R.Br. ex Sm., *Lantana camara* L., *Luffa cylindrica* M. Roem., *Momordica charantia* L., and *Zanthoxylum armatum* DC.

The local people of the study area use *Aristolochia indica* for the treatment of snake bites. The root of *Aristolochia indica* with long peppers was ground to make a paste. The paste was given as an antidote for snake bites. The present result concomitates with Bhattacharjee and Bhattacharyya's results (2013). Similarly, the decoction obtained from the root tuber of *Asparagus racemosus* was used to cure diarrhoea, cough, bronchitis, fever and jaundice. The root boiled with cow's milk was used to increase milk secretion during lactation. Root tuber was eaten raw to remove kidney stones. The present result corroborates the finding of Kohli et al. (2022). The fruit of *Capparis zeylanica* was mildly burnt and the whole content was taken for diabetes. The present report draws support from the studies of Amit et al. (2010). The treatment of diabetic rats with *Capparis zeylanica* fruit methanolic extract leads to improved body weight, blood glucose and insulin levels in comparison with the diabetic control group. Improved body weight in diabetic animals specifies the role of extract in protecting the body tissues from hyperglycemic damage (Gireesh et al., 2009) by enhancing glycemic control and structural protein synthesis (Eliza et al., 2009). Likewise, the leaves of *Gymnema sylvestre* were consumed orally for the treatment of diabetes. This plant's most widely known effect is anti-diabetic activity (Laha & Paul, 2019). Ethanol extract of this plant is reported to reduce glucose level by 46% whereas the water extracts reduced glucose level by 26% and methanol extract by 12% (Tiwari et al., 2014). Similarly, leaves and fruits of *Momordica charantia* were used to control diabetes. The present result draws support from the studies of Liu et al. (2021). In the studied region, gently warmed leaf of *Cissampelos pareira* was kept on wounds to draw out purulent matter and help in healing wounds. The present result draws support from the studies of Kumari et al. (2021). Paste of the whole plant of *Cissus quadrangularis* with banana leaf was bandaged over the affected area to enhance bone fracture healing (Naveen Joshi, 2020; Ramachandran et al., 2021). Six species had both medicinal and nutritional values: *Basella alba* L., *Benincasa hispida* (Thunb.) Cogn., *Boerhavia diffusa* L., *Carissa spinarum* L., *Ipomoea aquatica* Forsk. and *I. batatas* (L.) Lam. In the current investigation, leaves of *Basella alba* are rubbed over the affected part to cure irritation and swellings due to caterpillar. In the present study, tender leafy shoots of *Boerhavia diffusa* are eaten fried or cooked. Root paste of the plant is taken orally for a week for the treatment of jaundice. Aqueous and powdered extracts of roots of *Boerhavia diffusa* L. have shown hepatoprotective properties against Thioacetamide induced hepatotoxicity in Wister albino rats (Rawat et al., 1997). Ursolic acid, a common triterpenic acid found in *Boerhavia diffusa* L. has been reported to have hepatoprotective activity against carbon tetrachloride, ethanol, thioacetamide and galactosamine damaged liver in rats (Negi et al., 2007). The most widely used hepatoprotective plant species in India was *Boerhavia diffusa* L., which is noted to be used in different regions of India (Alagesaboopathii, 2009; Janghel et al., 2019; Raghuvanshi et al., 2021; Sharma, 2022). Devaki et al (2005) studied the effect of ethanolic extract of *Boerhavia diffusa* on the tissue defense system against ethanol-induced hepatic injury in rats. The administration of *Boerhavia diffusa* extract (150 mg/kg/day for 30 days, orally) reversed the increase in the levels of lipid peroxides and increased the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase and reduced glutathione levels. Rawat et al. (1997) studied the effect of various factors on the hepatoprotection by *Boerhavia diffusa* extract and found that aqueous extract (2 mL/Kg) of 1–3 cm diameter roots from May displayed significant protection for serum parameters, like GOT (glutamate oxaloacetate transaminase), GPT (glutamate pyruvate transaminase), ACP (acid phosphatase), and ALP (alkaline phosphatase) but not GLDH and bilirubin in thioacetamide-induced liver toxicity in rat. It has been noted in this study that the roots, which were thin, showed maximum protection of serum parameters. Likewise, tender leafy shoots of *Ipomoea aquatica* are eaten fried or cooked. *Ipomoea aquatica* is a common food eaten by all social groups throughout tropical Asia (Burkill, 1966). A Literature survey revealed the presence of fiber (11.67%), carbohydrates (54.2%), lipid (11.0%), protein (6.3%), sodium (135mg/100g), phosphorous (109mg/100g), calcium (416mg/100g), iron (210mg/100g), zinc (2.47mg/100g) and magnesium (301 mg/100g) in the leaves of the plant (Vishwakarma & Dubey, 2009).The species also contains several vitamins, including A, B, C, E, and “U” (S-methyl-methionine), flavonoids, phenolic compounds, ß-carotene and ascorbic acid (Sundar Rao, 1990). Traditionally, *Ipomoea aquatica* is effectively used against nosebleeds, high blood pressure, leukoderma, liver complaints, worm infection, fever and so forth in humans (Alkiyumi et al., 2012; Srikanth et al., 2018). The fruits of *Coccinia grandis* (L.) Voigt*, Cucumis melo* L. and roots tubers of *Dioscorea pentaphylla* L. were widely collected for human consumption. It was observed that wild fruits of *Zanthoxylum armatum* DC. and *Zizyphus oenoplia* were consumed by children on the way to school, or when tending livestock. Some people still pick them on walks to relive the flavours of their childhood. Several workers have also reported such types of observation (Campbell, 1987; Balemie & Kebebew, 2006).

A sizeable number of ornamental climbers (16 sp.) were reported from the district. Prominent among them include. *Allamanda blanchetii* A. DC., Bougainvillea spectabilis Willd., *Campsis grandiflora* (Thunb.), *Clerodendrum thomsoniae* Balf.f., *Epipremnum aureum* (Linden & André) G.S.Bunting, *Pyrostegia venusta* (Ker Gawl.) Miers and *Syngonium podophyllum* Schott. Presently in the era of stressful lifestyles and anthropogenic climate change, a large number of people throughout the globe are enriching their surroundings with different types of ornamental plants. The ornamental plants play a significant role in preparing and modifying urban and rural landscapes, fallow land development, afforestation and managing open-air and indoor spaces (Nassary et al., 2022). These plants are essentially grown in our homes, educational and public institutions, and workplaces for beautification, amusement, and enlightenment (Ciftcioglu et al., 2019). The climbers such as *Cuscuta reflexa* Roxb.*, Lantana camara* L. and *Mikania micrantha* Kunth were the most abundant and were found across the study region. These invasive plants are quick to colonize damaged areas, causing significant ecological damage to natural ecosystems. The *Lantana camara* is a significant scrambler that has grown naturally all over the world. *L. camara* is one of the major invasive species in Indian forests and an aggressive colonizer practically at forest edges and disturbed and denuded areas (Sahu & Singh, 2008). The perennial *Mikania micrantha* which is a fast-growing species, is covering the habitats of the district and suppressing the growth of agricultural crops as well as natural vegetation through competition and allelopathic effects (Sankaran & Srinivasan, 2001; Huang et al., 2009). Moreover, the relative abundance of woody climbers is increasing in tropical forests (Phillips et al., 2002; Schnitzer & Bongers, 2011) and several of the most aggressive invasive plants worldwide are vines (Holm et al., 1991).

Conclusion

Documentation of diversity of plants especially climbers of angiosperms in Bhadrak district, Odisha, India will be of great significance to assess the present status of floristic diversity in the area. The present study revealed that the climbing plant diversity of Bhadrak district not only contributes to the overall plant biodiversity significantly, but also maintains ecological balance of the whole ecosystem. The results of the present study showed that climber resources of the district continue to play a crucial role in daily livelihood as well as the health care system. Presently, many anthropogenic factors pose a threat to the abundance and distribution of the climbing species. Moreover, invasive alien plant species like *Lantana camara* and *Mikania micrantha* are considered a major threat to the local biodiversity. It is also noticed that the abundance of important climbers of the region like *Aristolochia indica*, *Asparagus racemosus*, *Gloriosa superba*, *Gymnema sylvestre* and *Telosma pallida* are declining day by day. Therefore, there is a need to create awareness among local people to conserve these plants to ensure their continued existence in the long run. In addition, a multi-pronged approach such as securing their habitats through protection, restoration, managing invasive species, and promoting collaboration among local stakeholders and organizations, safeguards the preservation of these vital plants, sustaining biodiversity and ecosystem health for the future. It is also necessary to continue the study of the climbing plant covering all the ecosystems, as a way to detect the influence of different biotic and abiotic factors in this group of plants.

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References

Addo-Fordjour P, El Duah P, Agbesi DK. 2013. Factors influencing liana species richness and structure following anthropogenic disturbance in a tropical forest, Ghana. *ISRN Forestry*, 2013: 1–11.

Alagesaboopathii C. 2009. Ethnomedicinal plants and their utilization by villagers in Kumaragiri hills of Salem district of Tamilnadu, India. *African Journal of Traditional Complementary and Alternative Medicine*, *6* (3): 222–227.

Alkiyumi SS, Abdullah MA, Alrashdi AS, Salama SM, Abdelwahab SI, Hadi AH. 2012. *Ipomoea aquatica* extract shows protective action against thioacetamide-induced hepatotoxicity. *Molecules*, *17* (5): 6146–6155.

Allen BP, Sharitz RR, Goebel PC. 2007. Are lianas increasing in importance in temperate floodplain forests in the southeastern United States? *Forest Ecology and Management*, *242* (1): 17–23.

Amit L, Chaudary AK, Vikas G, Bansal P, Renu B. 2010. Phytochemistry and Pharmacological Activities of Capparis zeylanica: An Overview. *International Journal of Research in Ayurveda & Pharmacy*, 1: 384–389.

Anbarashan M, Parthasarathy N. 2013. Diversity and ecology of lianas in tropical dry evergreen forests on the Coromandel Coast of India under various disturbance regimes. *Flora*, *208*: 22–32.

APG IV. 2016. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants. *Botanical Journal of the Linnean Society*, *181*(1): 1–20.

Appanah S, Putz FE. 1984. Climber abundance in virgin dipterocarp forest and the effect of pre-felling climber cutting on logging damage [Peninsular Malaysia]. *AGRIS*, *47* (3): 335–342.

Arroyo-Rodriguez, V, Asensio N, Dunn JC, Cristobal-Azkarate J, Gonzalez-Zamora A. 2015. Use of lianas by primates: more than a food resource. In: Schnitzer SA, Bongers F, Burnham RJ, Putz FE. (ed.). *The ecology of lianas.* Oxford: Wiley-Blackwell.

Balfour DA, Bond WJ. 1993. Factors limiting climber distribution and abundance in a southern African forest. *Journal of Ecology*, *81* (1): 93–99.

Bandyopadhyay S, Mitra S. 2021. Diversity of the monocot climbers of West Bengal, India. *Plant Archives, 2* (2): 734–739.

Bandyopadhyay S, Mukherjee SK. 2010. Diversity of climbing plants in the Koch Bihar district of West Bengal, India. *Pleione*, *4* (1): 82–89.

Bhattacharjee P, Bhattacharyya D.2013. Characterization of the aqueous extract of the root of Aristolochia indica: evaluation of its traditional use as an antidote for snake bites. *Journal of Ethnopharmacology*, *145* (1): 220–226.

Balemie K, Kebebew F. 2006. Ethnobotanical study of wild edible plants in Derashe and Kucha Districts, South Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, *2*: 53–61.

Bongers F, Parren MP, D. Traoré D. 2005. *Forest climbing plants of West Africa: diversity, ecology and management.* Oxford: CABI Publishing.

Burkill IH. 1996. *A Dictionary of the Economic Products of the Malay Peninsula*. Kuala Lumpur: Ministry of Agriculture and Cooperatives.

Caballe G. 1998. The self-supporting part of the tropical lianas: A synthesis of the strategies of growth. *Canadian Journal of Botany*, 76:1703–1716.

Campanello PI, Garibaldi JF, Gatti MG, Goldstein G. 2007. Lianas in a subtropical Atlantic Forest: host preference and tree growth. *Forest Ecology and Management*, *242* (2–3): 250–9.

Campbell BM.1987. The use of wild fruits in Zimbabwe. *Economic Botany*, *41:* 375–385.

Champion HG, Seth SK. 1968. *A revised survey of the forest types of India*. New Delhi: Manager Publications.

Ciftcioglu GC, Ebedi S, Abak K. 2019. Evaluation of the relationship between ornamental plants–based ecosystem services and human wellbeing: A case study from Lefke Region of North Cyprus. *Ecological Indicators*, 102: 278–288.

da Cunha Vargas B, Grombone-Guaratini MT, Morellato LPC. 2021. Liana's research in the Neotropics: overview, interaction with trees, and future perspectives. *Trees*, *35* (2): 333–345.

Darwin C. 1875*. The movements and habits of climbing plants*. London: John Murray.

Devaki T, Shivashangari K, Ravikumar S, Govindraju V. 2005. Effect of *Boerhaavia diffusa* on tissue antioxidant defense system during ethanol induced hepatotoxicity in rats. *Journal of Natural Remedies*, *5* (2):102–107.

Eliza J, Daisy P, Ignacimuthu S, Duraipandiyan V. 2009. Antidiabetic and Antilipidemic Effect of Eremanthin from Costus speciosus (Koen.) Sm., in STZ-Induced Diabetic Rats. *Chemico-Biological Interactions*, *182*: 67–72.

Garbin ML, Carrijo TT, Sansevero JBB, Sa´nchez-Tapia A, Scarano FR. 2012. Subordinate, not dominant, woody species promote the diversity of climbing plants. *Perspectives in Plant Ecology, Evolution and Systematics*, *14*: 257–265.

Gardette E. 1998. The effect of selective timber logging on the diversity of woody climbers at Pasoh. Conservation, management and development of forest resources. *Forestry Research Institute of Malaysia, Kepong*, *31*: 115–226.

Gentry AH. 1991. The distribution and evolution of climbing plants. In: Putz FE, Mooney HA (ed.). *The Biology of Vines*. Cambridge: Cambridge University Press.

Gianoli E. 2003. Phenotypic responses of the twining vine *Ipomoea purpurea* (Convolvulaceae) to physical support availability in sun and shade. *Plant Ecology, 165*: 21–26.

Gianoli E. 2015. Evolutionary implications of the climbing habit in plants. In: Schnitzer S, Bongers F, Burnham RJ, et al. (ed.). *Ecology of lianas*. UK: Wiley.

Gireesh G, Thomas SK, Joseph B, Paulose CS. 2009. Antihyperglycemic and insulin secretory activity of *Costus pictus* leaf extract in streptozotocin induced diabetic rats and in in vitro pancreatic islet culture. *Journal of Ethnopharmacology*, 123: 470–474.

Girish KE, Abdul JV. 2019. A reconnaissance on diversity of angiosperm climbers from riparian flora of Kuppam River, Kannur, Kerala, India. *Eco Chronicle*, *14* (2): 109–115.

Givnish TJ, Barfuss MH, Van Ee B, Riina R, Schulte K, Horres R, Gonsiska PA, Jabaily RS, Crayn DM, Smith JA, Winter K, Brown GK, Evans TM, Holst BK, Luther H, Till W, Zizka G, Berry PE, Sytsma KJ. 2014. Adaptive radiation, correlated and contingent evolution, and net species diversification in Bromeliaceae. *Molecular Phylogenetics and Evolution*, *71*: 55–78.

Gogoi P, Nath N.2021. Diversity and inventorization of angiospermic flora in Dibrugarh district, Assam, Northeast India. *Plant Science Today*, *8* (3): 621–628.

Gollasimood S, Faridah-Hanum I, Nazre M, Kudus Kamziah ABD 2012. Abundance and distribution of climbers in a coastal hills forest in Perak, Malaysia. *Journal of Agricultural Science*, *4*: 245–254.

Gonzalez-Teuber M, Gianoli E. 2008. Damage and shade enhance climbing and promote associational resistance in a climbing plant. *Journal of Ecology*, *96*:122–126.

Haines HH. 1925.*The Botany of Bihar and Orissa*. London: Adland and Son, West Newman Ltd.

Hegarty EE. 1991. Vine–host interactions. In: Putz FE, Mooney HA. (ed.). *The biology of vines.* Cambridge: Cambridge University Press.

Hegarty EE, Caballe G. 1991. Distribution and abundance of vines in forest communities. In: Putz FE, Mooney HA. (ed.). *The biology of vines.* Cambridge: Cambridge University Press.

Holm LG, Pancho JV, Herberger JP, Plucknett DL. 1991. *A geographic Atlas of world weeds*. Malabar: Krieger Publishing Company.

Huang QQ, Wu JM, Bai YY, Zhou L, Wang GX. 2009. Identifying the most noxious invasive plants in China: role of geographical origin, life form and means of introduction. *Biodiversity Conservation*, *18*: 305–316.

Huntington HP. 2000. Using traditional ecological knowledge in science: Methods and applications. *Ecological Applications*, *10*: 1270–1274.

Janghel V, Patel P, Chande SS. 2019. Plants used for the treatment of icterus(jaundice) in Central India: A review. *Annals of Hepatology, 18*: 658–672.

Jayakumar R, Nair KKN. 2013. Diversity and distribution of vines in the tropical forests of Nilgiri Biosphere Reserve, India. *Current science*, *105*: 470–479.

Jena GSJP, Mishra R, Satapathy KB. 2018. An inventory of four new angiospermic climbers record from coastal districts of Odisha. *International Journal of Advanced Scientific Research and Management*, *3* (10): 294–303.

Kashung S, Gajurel PR, Singh B. 2021. Distribution and diversity of climbing species in Papum Pare District of Arunachal Pradesh, India. *Journal of Threatened Taxa*, *13* (3): 17972–17983.

Kaur K, Sidhu MC, Ahluwalia AS. 2017. Angiosperm diversity in Doaba region of Punjab, India. *Journal of Threatened Taxa,* 9 *(8):* 10551–10564.

Kohli D, Champawat PS, Mudgal VD. 2022. *Asparagus* (*Asparagus racemosus* L.) roots: nutritional profile, medicinal profile, preservation, and value addition. *Journal of the Science of Food and Agriculture*. doi.org/10.1002/jsfa.12358.

Kumari S, Anmol, Bhatt V, Suresh PS, Sharma U. 2021. *Cissampelos pareira* L.: A review of its traditional uses, phytochemistry, and pharmacology. *Journal of Ethnopharmacology*, 274: 113850.

Laha S, Paul S. 2019. *Gymnema sylvestre* (Gurmar): A Potent herb with anti-diabeticand antioxidant potential. *Pharmacognosy Journal*, *11* (2): 201–206.

Leicht-Young SA, Pavlovic NB, Frohnapple KJ, Grundel R. 2010. Liana habitat and host preferences in northern temperate forests. *Forest Ecology and Management*, *260* (9): 1467–1477.

Liu Z, Gong J, Huang W, Lu F, Dong H. 2021. The Effect of Momordica charantia in the Treatment of Diabetes Mellitus: A Review. *Evidence-Based Complementary and Alternative Medicine, 2021*: 3796265.

Londre RA, Schnitzer SA. 2006. The distribution of lianas and their change in abundance in temperate forests over the past 45 years. *Ecology, 87*: 2973–2978.

Malhi Y, Wright J. 2004. Spatial patterns and recent trends in the climate of tropical rainforest regions. *Philosophical Transactions of the Royal Society London B Biological Sciences*, *359*: 311–329.

Martin GJ. 1995. *Ethnobotany: A methods manual*. London: Chapman and Hall.

Moffett MW. 2000. What's "Up"? A critical look at the basic terms of canopy biology. *Biotropica*, *32*: 569–596.

Muthumperumal C, Parthasarathy N. 2001. Tree-liana relationships in a tropical evergreen forest at Varagalaiar, Anamalais, Western Ghats, India. *Journal of Tropical Ecology*, *17* (3): 395–409.

Muthumperumal C, Parthasarathy N. 2013. Diversity, distribution and resource values of woody climbers in tropical forests of southern Eastern Ghats, India. *Journal of Forestry Research*, *24* (2): 365–374.

Naveen KL, Joshi D. 2020. A case report on the effect of Cissus quadrangularis on fracture union in 26 years aged man. *International Journal of Research in Orthopaedics*, *6* (1): 224–226.

Naidu MT, Kumar OA, Venkaiah M. 2014. Taxonomic diversity of lianas in tropical forests of Northern Eastern Ghats of Andhra Pradesh, India. *Notulae Scientia Biologicae*, *6* (1): 59–65.

Nassary EK, Msomba BH, Masele WE, Ndaki PM, Kahangwa CA. 2022. Exploring urban green packages as part of Nature-based Solutions for climate change adaptation measures in rapidly growing cities of the Global South. *Journal of Environmental Management*, *310*: 114786.

Negi AS, Kumar JK, Luqman S, Shanker K, Gupta MM, Khanuja SPS. 2007. Recent advances in plant hepatoprotectives: a chemical and biological profile of some important leads. *Medicinal Research Review*, *28*: 746–772.

Newbold T, Hudson LN, Hill SL, L., et al. 2015. Global effects of land use on local terrestrial biodiversity. *Nature*, *520*: 45–50.

Panda T, Pradhan BK, Mishra RK, Rout SD, Mohanty RB. 2020. Angiosperm diversity in Bhadrak region of Odisha, India. *Journal of Threatened Taxa*, *12* (3): 15326–15354.

Panda T, M, Apollo M, Kar MK. 2023. Additions to angiosperm diversity in Bhadrak region of Odisha, India. *Flora and Fauna, 29* (1): 29–48.

Parthasarathy N, Vivek P, Muthumperumal C, Muthuramkumar S, Ayyappan N. 2015. Biodiversity of lianas and their functional traits in tropical forests of Peninsular India. In: Parthasarathy N. (ed.). *Biodiversity of Lianas. Sustainable Development and Biodiversity*. Edinburgh: Springer, Cham.

Patnaik SR. 1996. *Orissa Today. An annual survey* (Eds.). Bhubaneswar:Sun-Times,.

Phillips OL, Martı´nez RV, Arroyo L, Baker TR, Killeen T, Lewis SL, Malhi Y, Monteagudo-Mendoza A, Neill D, Nu´n˜ ez Vargas P, Alexiades M, Cero´n C, Di Fiore A, Erwin T, Jardim A, Palacios W, Saldı´as M, Vinceti B. 2002. Increasing dominance of large lianas in Amazonian forests. *Nature, 418*: 770–774.

POWO. 2022. *Plants of the World Online*. Facilitated by the Royal Botanic Gardens.

Putz FE. 1984. The natural history of lianas on Barro Colorado Island, Panama. *Ecology*, *65* (6): 1713–1724.

Raghuvanshi D, Dhalaria R, Sharma A, Kumar D, Kumar H, Valis M, Kuča K, Verma R, Puri S. 2021. Ethnomedicinal plants traditionally used for the treatment of jaundice (icterus) in Himachal Pradesh in Western Himalaya—a review. *Plants*, *10*: 232.

Rai A, Chettri A, Pradhan A, Rai SK, Rai AK, Lepcha NT. 2016. Diversity of climbing plants in ‘Gadi’sacred grove of Central Pendam in East Sikkim, India. *Pleione*, *10* (1): 97–107.

Ramachandran S, Fadhil L, Gopi C. et al. 2021. Evaluation of bone healing activity of Cissus quadrangularis (Linn), Cryptolepis buchanani, and Sardinella longiceps in Wistar rats. *Beni-Suef University Journal of Basic and Applied Science*, *10*: 30.

Rawat AKS, Mehrotra S, Tripathi SC, Shome U. 1997. Hepatoprotective activity of Boerhaavia diffusa L. roots-a popular Indian ethno medicine. *Journal of Ethnopharmacology*, *56*: 61–66.

Reddy MS, Parthasarathy N. 2006. Liana diversity and distribution on host trees in four inland tropical dry evergreen forests of peninsular India. *Tropical Ecology*, *47*: 109–123.

Rich PM, Lum S, Munoz LEDA, Quesada MAURICIO. 1987. Shedding of vines by the palms *Welfia georgii* and *Iriartea gigantea*. *Principes, 31* (1): 31–34.

Sahu PK, Singh JS. 2008. Structural attributes of lantana-invaded forest plots in Achanakmar–Amarkantak Biosphere Reserve, Central India. *Current Science*, *94* (4): 494–500.

Saini L, Tyagi A, Mohammad I, Malik V. 2021. Glimpse of climber diversity in Saharanpur District, Utar Pradesh, India. *Journal of Threatened Taxa*, *13* (5): 18390–18397.

Sankaran KV, Srinivasan MA. 2001. Status of Mikania infestation in the Western Ghats. In: Sankaran KV, Murphy ST, Evans HC. (ed.). *Alien weeds in moist tropical zones: banes and benefits.* Kerala: KFRI/CABI Bioscience.

Santos KD, Kinoshita LS, Rezende AA. 2009. Species composition of climbers in seasonal semi-deciduous forest fragments of Southeastern Brazil. *Biota Neotropica*, *9*: 175–188.

Sarvalingam A, Rajendran A, Sivalingam R. 2015. Wild edible plant resources used by the Irulas of the Maruthamalai Hills, Southern Western Ghats, Coimbatore, Tamil Nadu. *Indian Journal of Natural Products and Resources*, *5* (2): 198–201.

Saxena HO, Brahmam M. 1996. *The Flora of Orissa. Vol. I-IV.* Bhubaneswar: Orissa Forest Development Corporation.

Schnitzer SA. 2005. A mechanistic explanation for global patterns of liana abundance and distribution. *American Naturalist*, *166*: 262–276.

Schnitze SA, Bongers F. 2002. The ecology of lianas and their role in forests. *Trends in Ecology & Evolution*, *17* (5): 223–230.

Schnitzer SA, Bongers F. 2011. Increasing liana abundance and biomass in tropical forests: emerging patterns and putative mechanisms. *Ecology Letters*, *14*: 397–406.

Schnitzer SA, Putz FE, Bongers F, Kroening K. 2015. The past, present, and potential future of liana ecology. In: Schnitzer SA, Bongers F, Burnham RJ, Putz FE. (ed.). *Ecology of Lianas*. Hoboken, NJ, USA: John Wiley & Sons, Ltd.

Seger GDDS, Hartz SM. 2014. Checklist of climbing plants in an Araucaria Forest of Rio Grande do Sul State, Brazil. *Biota Neotropica*, *14* (4): 1–12.

Sharma J, Singh K, Gairola S. 2022. Plants used for magicoreligious purposes by the indigenous communities of sub-Himalayan Tract, Uttarakhand. *Ethnobotany Research and Applications*, *23*: 36. http://dx.doi.org/10.32859/era.23.36.1-19.

Srikanth I, Kiran KV, Krishna SK, Sunitha M, Ramanjaneyulu K, Himabindhu J. 2018. In vitro anthelmintic activity of *Ipomoea aquatica*. *International Journal of Current Pharmaceutical Research*, *10* (3): 52–54.

Sundar Rao K, Dominic R, Singh K, Kaluwin C, Rivett DE, Jones GP. 1990. Lipid, fatty acid, amino acid, and mineral composition of five edible plant leaves. *Journal of Agriculture and Food Chemistry*, *38*: 2137–2139.

Swaine MD, Grace J. 2007. Lianas may be favoured by low rainfall: evidence from Ghana. *Plant Ecology, 192*: 271–276.

Tiwari P, Mishra BN, Sangwan NS. 2014. Phytochemical and pharmacological properties of *Gymnema sylvestre*: An important medicinal plant. *Biomed Research International*, *2014*: 1–18.

Vishwakarma K, Dubey D. 2009. Nutritional potential of Ipomoea aquatica Forssk: an ethnobotanicaly important plant. *Journal of Indian Botanical Society*, *88*: 163–169.

Vivek P, Babu KN, Anbarashan M, et al. 2022. Taxonomic estimates of climbing plants in India: how many species are out there? *Écoscience*, *29* (4): 325–343.

Vivek P. 2023. Taxonomic Diversity of Climbing Flora in India: A Compendium. In: Taxonomy and Ecology of Climbers: Climbing Plants of India. Singapore: Springer.

Zeballos SR, Tecco PA, Cabido M, et al. 2014. Composición de especies leñosas en comunidades invadidas en montañas del centro de Argentina: su relación con factores ambientales locales. *Revista de Biología Tropical*, *62*: 1673–1681.

Zotz G, Cueni N, Korner C. 2006. In situ growth stimulation of a temperate zone liana (Hedera helix) in elevated CO_2. *Functional Ecology, 20*: 763–769.

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Research Article

Species Composition and Assessment of Zingiberaceae in the Forest Patches of Mt. Musuan, Bukidnon, Southern Philippines

Noe P. Mendez[1,2*], Florfe M. Acma[1,2], Maria Melanie M. Guiang[1,2], Fulgent P. Coritico[1,2], Victor B. Amoroso[1,2]

[1] *Plant Biology Division, Institute of Biological Science, College of Arts and Sciences, Central Mindanao University, University Town, Maramag, 8714 Bukidnon, Philippines.*
[2] *Center for Biodiversity Research and Extension in Mindanao (CEBREM), Central Mindanao University, University Town, Maramag, 8714 Bukidnon, Philippines.*

*Corresponding author: npolomendez@gmail.com
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ABSTRACT

This study documented 11 species of gingers belonging to nine genera distributed in three tribes and two subfamilies from Mt. Musuan and its vicinity in Bukidnon, Southern Philippines. Data revealed that Zingiberaceae species in these sites constitute *ca.* 8% of the total number of Philippine Zingiberaceae. *Alpinia haenkei* C.Presl., *A. purpurata* (Vieill.) K.Schum., *Curcuma zedoaria* (Christm.) Roscoe, *Etlingera philippinensis* (Ridl.) R.M.Sm., *Hornstedtia conoidea* Ridl., *Meistera muricarpa* (Elmer) Škorničk. & M.F.Newman, and *Zingiber zerumbet* (L.) Smith. were recorded in Mt. Musuan, while *Amomum dealbatum* Roxb., *Etlingera elatior* (Jack) R.M.Sm., *Hedychium coronarium* Koenig, *Hornstedtia conoidea* Ridl., and *Wurfbainia elegans* (Ridl.) Škorničk. & A.D.Poulsen. were collected in CMU View Deck in Kibulawan. Of the collected species, five of these (3.52% endemicity compared to the total number of Philippine Zingiberaceae) are endemic to the Philippines, while the other six species are introduced. These species are mostly associated with fern species, such *Dicranopteris linearis* (Burm.f.) Underw. and *Lygodium circinnatum* (Burm.f.) Sw., and angiosperms *Musa textilis* and under the shades of dipterocarpaceae species. This paper is the first taxonomic report on Zingiberaceae in Mt. Musuan and vicinity which was not documented in the previous studies conducted at Philippine Long Term Ecological Research (LTER) Sites. Since Mt. Musuan is not a protected area and an open site for ecotourism which is constantly visited by daily hikers, these species might be prone to depletion in their wild habitats. There is an urgent call for *in situ* conservation efforts that should be done by the concerned authorities of Central Mindanao University to help preserve and protect these ginger species.

Keywords: Alpinieae; Gingers; Invasive species; Mt. Kalayo; Philippine endemic

Introduction

The Zingiberaceae family is comprises of over 1,500 species distributed in at least 53 genera (Kress et al., 2002; Lamb et al., 2013; Christenhusz and Bying, 2016). Nineteen genera and 142 species of Zingiberaceae have been recorded so far in the Philippines (Pelser et al., 2011 onwards). Members of this family played a significant role in daily needs of human beings since the time of Linnaeus because of the benefits they offered, such as for food, medicine, spices, cosmetics and ornaments (Van Balgooy, 2001; Newman et al., 2004; Prabhukumar et al., 2015). The Philippine Zingiberaceae is an interesting family to study in terms of its taxonomic status, because since 2017, there were several reports of new species an new species records added to this family.

Mt. Musuan, also known as Mt. Kalayo, is one of the five Long Term Ecological Research (LTER) Sites in Mindanao. The established 2-ha LTER permanent plot is located at the lowland mixed dipterocarp/agroecosystem of Mt. Musuan at 388 masl. The establishment of the Mindanao LTER Sites is in response to the global campaign to move towards a more sustainable world in which the health of the ecosystem and human well-being are improved (Acma et al., 2018). During the previous LTER studies conducted on inventory of flowering plants in the permanent plot of Mt. Musuan, Zingiberaceae species were not included, and thus, no species of this group were recorded inside the 2-ha permanent plot and no species of gingers were reported to occur in its nearby forest patches, including the CMU view deck. Thus, this study was commenced to revisit the LTER Site as well as the forest patches of Mt. Musuan to conduct an inventory and assessment of Zingiberaceae species.

Materials and Methods

Study Sites and Description

This study was conducted in the forest patches of Mt. Musuan from January – March 2023 (Figure 1). Mt. Musuan (7° 52' 56.58" N 125° 3' 55.38" E) is also known as Mt. Kalayo and has deciduous tree species, which means that their leaves shed off at the same time leaving the tree temporarily bare for a certain time of the year (Acma et al., 2018). This mountain is composed of fragmented secondary forests and is dominated by some trees, such as dipterocarp species (Dipterocarpaceae), *Senna spectabilis* (DC.) H.S.Irwin & Barneby (Fabaceae), *Alstonia scholaris* (L.) R.Br. (Apocynaceae), and *Artocarpus blancoi* (Elmer) Merr. (Moraceae). The area is currently afflicted by the presence of some invasive species, such as *Chromolaena odorata* (L.) R.M.King & H.Rob. and *Lantana camara* L. (Asteraceae), *Donax canniformis* (G.Forst.) K.Schum.

(Marantaceae), *Spathodea campanulata* P.Beauv. (Bignoniaceae), and Piper aduncum L. (Piperaceae).

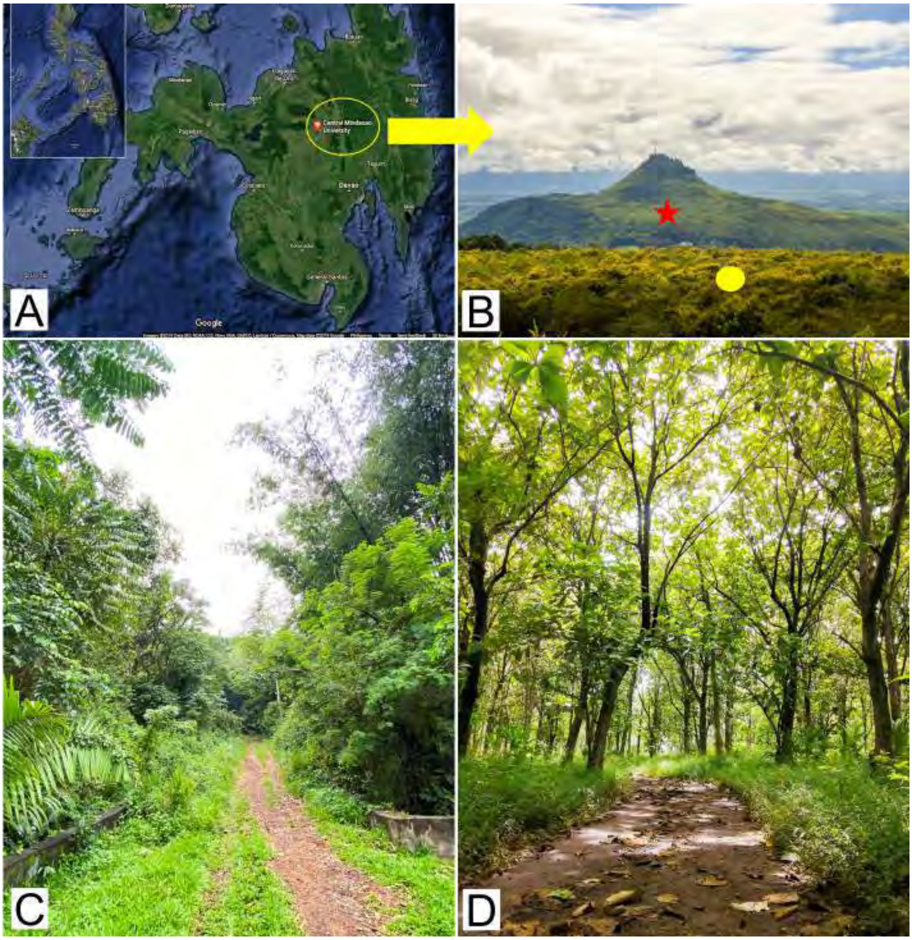

Figure 1. Study site. A) Map of Mindanao Island (inset: Philippine map), B) study sites (red star – Mt. Musuan; yellow circle – CMU view deck), C) trail to Mt. Musuan, D) trail to CMU View Deck. *Photographs: A – Google map ©2023; B&D – Rodel Tubongbanua; C – Noe P. Mendez.*

On the other hand, the CMU View Deck, located in Kibulawan (7^{o} 89' 11.82" N 125^{o} 5' 31.30" E) was just recently opened for ecotourism activities in Bukidnon due to its scenic view. The CMU View Deck is accessible since the peak can be reached by walking for approximately 40 minutes from the highway and by using 4-wheel-drive vehicles from the entrance of Sayre Highway to the peak. CMU View Deck is also known as a Station of the Cross site during Holy Week because the stations are located along the trail, adding to its popularity and accessibility to hikers/mountaineers. Some planted crops were observed to occur along the trail, such as *Cocos nucifera* L. (Arecaceae), *Musa textilis* Née (Musaceae), Hevea brasiliensis (Willd. ex A.Juss.) Müll.Arg. (Euphorbiaceae), and *Falcataria falcata* (L.) Greuter & R.Rankin (Fabaceae).

Sampling Techniques

Floristic surveys were carried out in Mt. Musuan and its vicinity to inventory the Zingiberaceae species present in these areas. These were done through repeated transect walks and opportunistic samplings in the two sites from Sayre Highway to the peak. Inventory inside the established 2-ha permanent plots was also done. Sterile species that were identifiable up to genera and species levels were also recorded.

Collection, Processing and Identification of Specimens

Collection of species was limited to only three pieces of plant parts per species per site as stipulated in the gratuitous permit. Representative vegetative plant parts from the terminal part, middle, and basal portions of the plant body were prepared. Collected specimens were numbered and documented as to the collector, date of collection, locality, common name, habitat, height and inflorescence. Species were collected for voucher purposes and the specimens were processed following the wet method, dried using a mechanical dryer, and the corresponding labels were affixed and herbarium specimens were deposited at the Central Mindanao University Herbarium (CMUH). Likewise, pickled collections of the floral parts were placed inside small plastic containers and preserved using 70% ethanol and pure glycerine which comprised about 10% of the total preservation mixture.

Field guides, online database (e.g. JSTOR), online e-Flora (e.g., Co's Digital Flora of the Philippines by Pelser et al., 2011 onwards), protologues (e.g., Ridley, 1909; Elmer 1915; Elmer, 1919), and published literature were used to identify the collected specimens. The assessment of endemism was based from Pelser et al. (2011 onwards).

Assessment of Conservation Status and Endemism

The conservation status was based on Fernando et al. (2022) and online websites of CITES (2023) and IUCN (2023). The assessment of endemism of each species was based on Pelser et al. (2011 onwards).

Results and Discussion

Species Composition

This study revealed a total of 11 species of Zingiberaceae belonging to 2 subfamilies, 3 tribes, and 9 genera (Table 1; Figure 2). The collected species is similar to the findings of Jayme et al. (2020) with 11 species, but lower than the studies of Naive (2017) with 12 species, Dalisay et al. (2018) with 23 species, Acero et al. (2019) with 14 species, and Acma et al. (2020) with 27 species. This study is the 6th report on taxonomic inventory of Philippine Zingiberaceae in a mountain ecosystem. This study is also consistent to the earlier reports of Naive (2017), Dalisay et al. (2018), Acero et al. (2019), Jayme et al. (2020), and Acma (2020) which collected the majority of species belonging to Tribe Alpinieae. Based on the repeated fieldwork conducted by the first author from 2017 to present in different mountain ecosystems and forest patches in Mindanao, it was observed that species of Tribe Alpinieae frequently occur and dominated the forest patches compared to other tribes. This claim is supported by a new genus record in the Philippines – *Sulettaria* A.D.Poulsen and Mathisen; new species reported in the Philippines – *Alpinia*, *Etlingera*, and *Hornstedtia*; and new species and a new record of *Plagiostachys*, in which all of these species are under the Tribe Alpinieae.

Table 1. Subfamilies, tribes and genera of the collected Zingiberaceae species.

No.	Subfamily	Tribe	Genus	No. of Species
1	Alpinioideae	Alpinieae	*Alpinia*	2
2			*Amomum*	1
3			*Etlingera*	2
4			*Hornstedtia*	1
5			*Meistera*	1
6			*Wurfbainia*	1
7	Zingiberoideae	Hedychieae	*Hedychium*	1
8		Zingibereae	*Curcuma*	1
9			*Zingiber*	1
			Total:	11

The genera *Alpinia* and *Etlingera* constitute the highest number of collected species with 2 species for each genus and the rest of the genera, such as *Amomum*, *Hornstedtia*, *Meistera*, *Wurfbainia*, *Hedychium*, *Curcuma*, and *Zingiber* are represented with only one species. Based on the list of Philippine Zingiberaceae by Pelser et al. (2011 onwards), the collected species constitute *ca.* 8% of the total number of Philippine Zingiberaceae. Eight species have been recorded in Mt. Musuan, while 5 species were collected in CMU view deck (*A. dealbatum* and *H. conoidea* occur in two sites) (Table 2). The collected species are few, but considering the presence of five Philippine endemic and noteworthy species out of 11 collected species, this paper is significant and warrants further studies. It is also interesting that eventhough Mt. Musuan has a secondary forest, these species are already in the sites and await discovery. This is because members of this family are neglected since vegetative morphologies of the species closely resemble each other and local people and researchers often mistakenly treat them as the same species.

Table 2. Occurrence of Zingiberaceae in Mt. Musuan and CMU View Deck, Bukidnon.

No.	Species	Distribution	
		Mt. Musuan (main mountain)	View Deck (base of Mt. Musuan in Kibulawan)
1	*Alpinia haenkei* C.Presl.	/	
2	*Alpinia purpurata* (Vieill.) K.Schum.	/	
3	*Amomum dealbatum* Roxb.	/	/
4	*Curcuma zedoaria* (Christm.) Roscoe	/	
5	*Etlingera elatior* (Jack) R.M.Sm.		/
6	*Etlingera philippinensis* (Ridl.) R.M.Sm.	/	
7	*Hedychium coronarium* Koenig		/
8	*Hornstedtia conoidea* Ridl.	/	/
9	*Meistera muricarpa* (Elmer) Škorničk. & M.F.Newman	/	
10	*Wurfbainia elegans* (Ridl.) Škorničk. & A.D.Poulsen		/
11	*Zingiber zerumbet* (L.) Smith	/	

According to Smith et al. (1988), the classification of Zingiberaceae had been recognized widely based on morphological characters and molecular phylogeny. As for this study, morphological characters were used for plant identification and species delineation. Variation on the inflorescence of each species is important, since it is the main characteristic in distinguishing specific genera. Based on their reproductive parts, inflorescence and infructescence emerge terminally on leafy shoots, such as *A. haenkei*, *A. purpurata*, and *H. coronarium* or arise from the rhizome, such as *A. dealbatum*, *C. zedoaria*, *E. elatior*, *E. philippinensis*, *H. conoidea*, *M. muricarpa*, *W. elegans*, and *Z. zerumbet*.

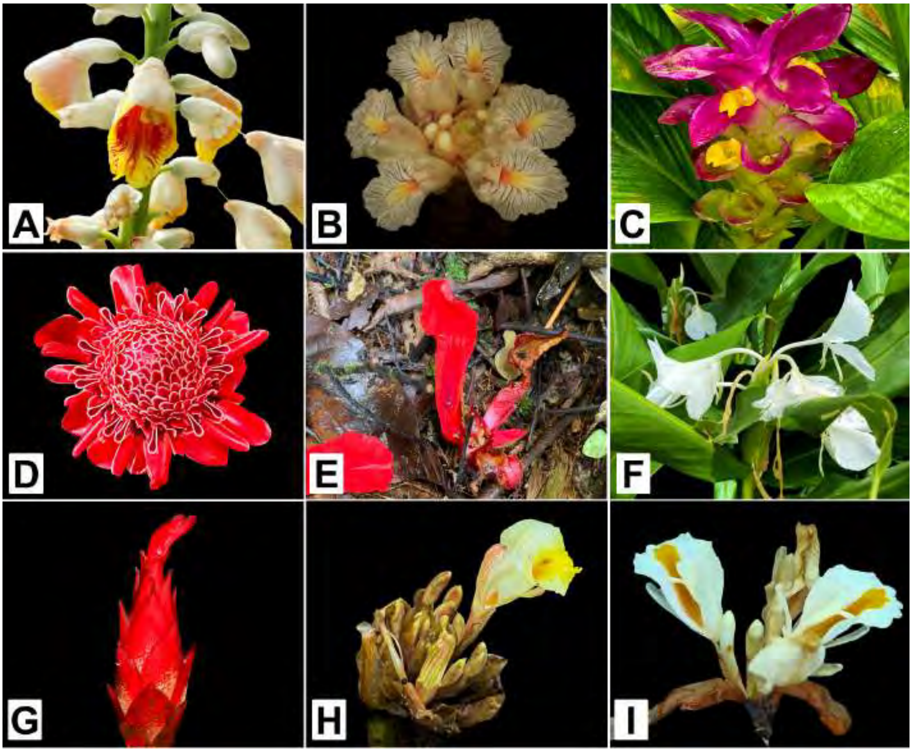

Figure 2. Representative ginger species in Mt. Musuan and its vicinity in Bukidnon, Southern Philippines. A) *Alpinia haenkei* C.Presl., B) *Amomum dealbatum* Roxb., C) *Curcuma zedoaria* (Christm.) Roscoe, D) *Etlingera elatior* (Jack) R.M.Sm., E) *E. philippinensis* (Ridl.) R.M.Sm., F) *Hedychium coronarium* Koenig, G) *Hornstedtia conoidea* Ridl., H) *Meistera muricarpa* (Elmer) Škornička & M.F.Newman, I) *Wurfbainia elegans* (Ridl.) Škornička & A.D.Poulsen. *Photographs by N.P.Mendez.*

Assessment of Conservation Status and Endemism of the Species

Among the 11 collected species, no species of gingers were considered threatened; however, five of these are endemic to the Philippines, *viz., A. haenkei*, *E. philippinensis*, *H. conoidea*, *M. muricarpa*, and *W. elegans*, while the rest of the species are introduced to the Philippines (Table 3). These five Philippine endemic species are rare in Mt. Musuan, but most of these, such as *A. haenkei*, *E. philippinensis*, *H. conoidea*, and *M. muricarpa* were frequently collected by the authors during the botanical expeditions in other Mindanao mountain ecosystems. It is also noted *E. elatior* and *Z. zerumbet* are naturalized in the Philippines (Steiner, 1959), *A. purpurata* and *C. zedoaria* are known for cultivation and not naturalized (Pelser et al., 2011 onwards), and *H. coronarium* is an invasive species (de Castro et al. 2016; CABI 2022). There were no threatened species recorded in the area compared to other mountain ecosystems.

The *C. zedoaria* is a species known as “luyang dilaw” aside from its sister species – *C. longa* L. by the local people in Mindanao and is produced to make a lot of products, such as tea, condiments and medicine. This species is planted outside the entrance of CMU view deck in addition to the garden decoration. The *A. purpurata* and *E. elatior* are usually propagated in Bukidnon because of their giant habits and very colourful inflorescences making the plant lovers cultivate them and their inflorescences were usually used for special occasions in church for decorative purposes. Upon the collection of the specimens, the former has only sterile population. Because of its variegated leaves, Z. zerumbet has also paid importance in terms of cultivation to the local people. This plant can be seen at the entrance of Mt. Musuan, and is usually seen outside houses nearby the study sites.

Table 3. Assessment and Ecological Status of Zingiberaceae in Mt. Musuan and CMU View Deck, Bukidnon

No.	Species	Assessment of Ecological Status
1	*Alpinia haenkei*	Philippine Endemic
2	*Alpinia purpurata*	Introduced
3	*Amomum dealbatum*	Introduced
4	*Curcuma zedoaria*	Introduced; Known for Cultivation
5	*Etlingera elatior*	Introduced, Known for Cultivation
6	*Etlingera philippinensis*	Philippine Endemic
7	*Hedychium coronarium*	Introduced; Invasive Species
8	*Hornstedtia conoidea*	Philippine Endemic
9	*Meistera muricarpa*	Philippine Endemic
10	*Wurfbainia elegans*	Philippine Endemic
11	*Zingiber zerumbet*	Introduced; Known for Cultivation

In Mt. Hamiguitan, 11 endemic species of gingers were collected including the *Etlingera* sp. which was later on identified as *Etlingera pilosa* A.D.Poulsen & Docot (Acero et al., 2019). In Marilog Forest Reserve, Southern Philippines, only one species (*Hedychium philippinense* K.Schum.) was recorded as threatened species under the Endangered category and 14 endemic species were recorded (Acma et al., 2020). In Cinchona Forest Reserve at the foot of Mt. Kitanglad, *H. philippinense* was also recorded as the only threatened species and nine species of gingers were recorded as endemic to the Philippines (Jayme et al., 2020). With the 11 recorded ginger species in Mt. Musuan, it is lower than the studies of Acero et al. (2018) and Acma et al. (2020), but has the same number of species collected by Jayme et al. (2020).

Conclusions and Recommendations

A total of 11 species of gingers belonging to nine genera in three tribes and two subfamilies were recorded from Mt. Musuan and its vicinity in Bukidnon, Southern Philippines. The collected species constitute *ca.* 8% of the total number of Philippine Zingiberaceae and 5% of the collected species are endemic to the Philippines. *A. haenkei*, *A. purpurata*, *C. zedoaria*, *E. philippinensis*, *H. conoidea*, *M. muricarpa*, and *Z. zerumbet*. were recorded in Mt. Musuan, while *A. dealbatum*, *E. elatior*, *H. coronarium*, *H. conoidea*, and *W. elegans* were collected from CMU View Deck. Of the collected species, five of these are endemic to the Philippines, while the other six species are introduced. These species are mostly associated with ferns, understorey flowering plants, and Dipterocarpaceae species.

It is recommended that an urgent call for *in situ* conservation efforts to be done by the concerned authorities of Central Mindanao University to help preserve and protect these species since 3.52% of these species are endemics in the Philippines. Also, as ecotourism sites, there is a need to regulate receiving visitors in these sites, and if possible, tree planting activities be considered as an activity. Botanists from CMU should also be consulted prior to road widening in the area, since this could threaten the species as most of them are found along the trail.

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References

Acero KML, Amoroso VB, Lumista HP, Mendez NP, Acma FM. 2019. Species Composition and Distribution of Zingiberaceae in Mt. Hamiguitan expansion site, Davao Oriental, Philippines. *Journal of Tropical Biology and Conservation, 16*: 121–136.

Acma FM, Mendez NP, Amoroso VB. 2020. Zingiberaceae of Marilog Forest Reserve, Southern Philippines: Its Richness and Endemism. *Asian Journal of Biodiversity, 11*: 73–87. doi: http://dx.doi.org/10.7828/ajob.v11i1.1388.

Acma FM, Quimpang VT, Amoroso VB, Mendez NP. 2018. Litterfall Production and Turnover in the Three Long-Term Ecological Research Sites in Southern Philippines. *CMU Journal of Science, 22* (2): 24–36.

CABI. (2022). Invasive Species Compendium online data sheet. *Hedychium flavescens*. In: *Invasive Species Compendium*. Wallingford, UK: CAB International. https://www.cabi.org/isc.

Christenhusz MJM, Bying JW. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa, 261* (3): 201. doi: 10.11646/phytotaxa.261.3.1

Dalisay JAGP, Bangcaya PS, Naive MAK. 2018. Taxonomic Studies and Ethnomedicinal uses of Zingiberaceae in the Mountain Ranges of Northern Antique, Philippines. *Biological Forum, 10* (2): 68–73.

De Castro WAC, Almeida RV, Leite MB, Marrs RH, Silva Matos DM. 2016. Invasion strategies of the white ginger lily *Hedychium coronarium* J. König (Zingiberaceae) under different competitive and environmental conditions. *Environmental and Experimental Botany, 127:* 55–62.

Elmer ADE. 1915. Notes and Descriptions of Zingiberaceae. *Leaflets of Philippine Botany, 8* (116): 2885–2919.

Elmer ADE. 1919. Zingiberaceae of the Sorsogon Peninsula. *Leaflets of Philippine Botany, 8* (119): 2963–2995.

Fernando ES, Amoroso VB, Tandang DN, Banag CI, Lapis AB, Gruezo WS, Coritico FP, Pollisco FA Jr., Palijon AM, Ching MD, Lansigan FP, Garcia-Francisco C, Lita NM, Tagtag AM, de Leon JL. 2022. *Threatened Plants of the Philippines.* Department of Environment and Natural Resources. Biodiversity Management Bureau. 149p.

Jayme JALF, Mendez NP, Mendez RA, Somera DF, Mohagan AB. 2020. Species Richness and Endemism of Zingiberaceae in Cinchona Forest Reserve, Lantapan, Bukidnon, Philippines. *Journal of Tropical Life Science, 10* (2): 175–180. http://dx.doi.org/10.11594/jtls.10.02.10.

Kress W, Prince L, Williams K. 2002. The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *American Journal Botany*, *89* (11): 1682–1696.

Lamb A, Goblik J, Ardiyani M, Poulsen AD. 2013. *A Guide to Gingers of Borneo.* Natural History Publications (Borneo): 144 pp.

Mendez NP, Acma FM, Villafranca-Tuba ARA, Mendez RA. 2023. Phenolic Content and Antioxidant Activity of *Etlingera dostseiana* Naive, Demayo & Alejandro, 2020 (Zingiberaceae). *Kragujevac Journal of Science, 45*: 277–286. doi: 10.5937/KgJSci2345277M.

Naive MAK. 2017. Zingiberaceae of Kalatungan Mountain Range, Bukidnon, Philippines. *Bioscience Discovery, 8* (3): 311–319.

Newman M, Lhuillier A, Poulsen AD. 2004. J. Blumea Supplement 92: 167–178.

Pelser PB, Barcelona JF, Nickrent DL. 2011 onwards. [accessed 2023 March 28]. Co’s Digital Flora of the Philippines. Accessed from: http://www.philippineplants.org.

Prabhukumar KM, Thomas VP, Sabu M, Prasanth AP, Mohana KV. 2015. *Journal of Horticulture, Forestry and Biotechnology, 19* (2): 18–27.

Ridley HN. 1909. New Philippine Zingiberaceae. *Leaflets of Philippine Botany, 2* (28): 569–572.

Smith RM. 1988. A Review of Bornean Zingiberaceae: IV. (Globbeae). *Notes from the Royal Botanic Garden Edinburgh, 45* (1): 1–19.

Steiner M. 1959. A new and illustrated flora of Manila. I Zingiberaeae. *Philippine Journal of Science, 88* (1): 1–247.

Van Balgooy, MMJ. 2001. *Portraits non-tree families*. (Leiden-Universiteit Leiden branch).

[CITES] Conservation on International Trade in Endangered Species of Wild Flora and Fauna. 2023. Checklist of CITES species. Accessed fro: http://checklist.cites.org/#/en/search/output_layout=alphabetical&level_of_listing=0&show_synonyms=1&show_author=1&show_english=1&show_spanish=1&show_french=1&scientific_name=Hedychium&page=1&per_page=20. Accessed on: 2023 May 29].

[IUCN] International Union for Conservation of Nature. 2023. The IUCN Red List of Threatened Species. Version 2023. Accessed from: http://www.iucnredlist.org.

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Research Article

A Brief Description of Avian Communities in Sungai Tongod Forest Reserve, Tongod, Sabah, Malaysia

Bernadette D. Joeman, George Hubert Petol*, Loraiti Lolin

Forest Research Centre, P.O. Box 1407, 90715, Sandakan, Sabah.

*Corresponding author: hubert.petol@sabah.gov.my

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Abstract

A rapid assessment of the avifauna of Sungai Tongod Forest Reserve (STFR), a logged-over forest, was conducted in Tongod district in central Sabah, Malaysia. A modified MacKinnon List method was used to assess species diversity. The four-day survey recorded a total of 15 MacKinnon lists, with 342 individuals detected. A total of 85 species from 38 families were recorded, with $H=4.07$ and $E_H=0.70$. True species richness was estimated (using SuperDuplicates® online calculator) to be approximately 114 species, with about 30 species not detected. There were 10 Bornean endemics, half of which were listed as Least Concerned, two as Near Threatened, and one as Vulnerable, in the IUCN Red List of Threatened Species. The Pellorneidae, Pycnonotidae and Nectariniidae were represented by eight, seven and six species respectively. The family Pycnonotidae had the highest number of individuals at 40 followed by Pellorneidae with 35. The five most detected species comprised 42.4 % of all individuals. The most detected species were the Bold-striped Tit-babbler (17 individuals), Green Iora (15), Pink-necked Green Pigeon (15), Black-and-yellow Broadbill (12), and Black-headed Bulbul (12). Most of the species detected (77) were forest-dependent, of which 62 were strictly forest birds. Insectivores comprised the most dominant dietary guild, i.e., 25 species (from 22 families). Frugivores ranked second with 25 species from 10 families.

Keywords: avifaunal survey; MacKinnon List method; Sungai Tongod Forest Reserve; Tongod district; Sabah, Malaysia; feeding guilds; rapid assessment

Introduction

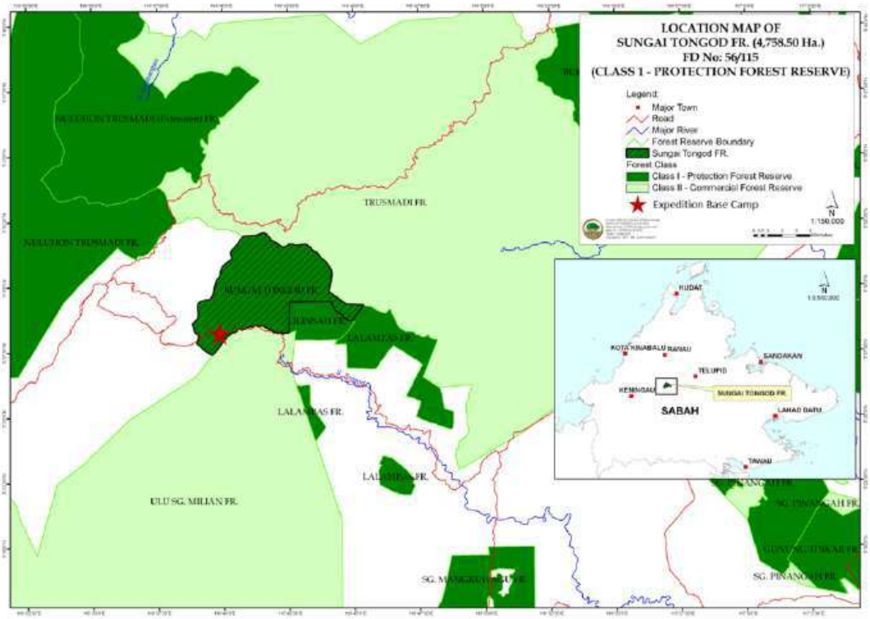
Since 1997 when the Sabah state government committed to implement sustainable forest management in all forest reserves, the Sabah Forestry Department (SFD) geared its management policies and practices towards multiple-use of forests. Much emphasis has been committed to timber certification and sustainability, forest conservation, rehabilitation and stakeholder participation. Over the past two decades, the Forest Research Centre (FRC) which serves as the R&D division of SFD, has contributed a significant amount of research-generated information to support such efforts, either for the department or for the private sector. FRC has realigned its research programmes to be more oriented towards supporting conservation efforts. Understanding that birds play important functional roles in ecosystems as pollinators, seed dispersers, and predators, FRC established an avifaunal research team in 2017. The team aims to develop a rapid assessment methodology based on a modified MacKinnon List method (MacKinnon & Phillipps, 1993) to allow researchers and field staff to collect reliable avifaunal data within a span of a few days. The Sungai Tongod Forest Reserve (STFR) survey was part of these ongoing field trials.

This paper documents the outcome of the said avifaunal survey conducted during the STFR scientific expedition from 10th to 16th October 2022. FRC organised the expedition with funds from the Heart of Borneo Initiative project. The main objective of this survey was to describe the avian community and ecology within the forest reserve for future forest management initiatives. Surveys were conducted along the main road within the forest reserve.

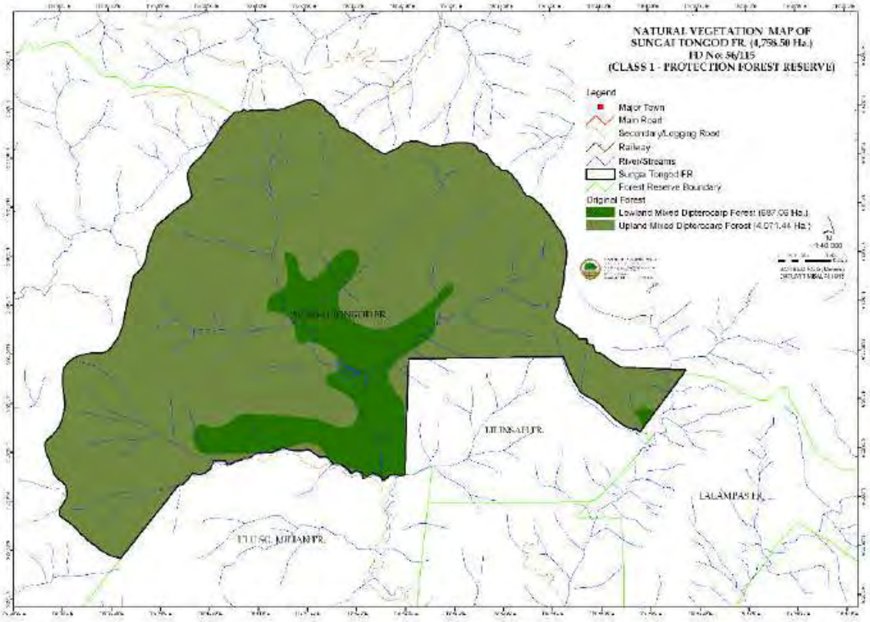
Site description

In 2010, STFR was gazetted as a Class I Protection Forest Reserve, approximately 4,758.5 ha in size, with elevation between 390 m (base camp) to 900 m (ridge close to western boundary). Prior to gazettement, it was state land and logged in most parts. It is located in central Sabah and is accessible by road from the Telupid-Pinangah main road. The expedition base camp was situated in the southwest portion of the reserve (5.472740 N, 116.684111 E), along a tributary of Sungai Tongod, where heavy road construction works were being carried out. The survey was conducted along this road which runs through STFR.

The documentation of historical forest logging has remained elusive. However, through the utilization of Google Earth's Historical Imagery feature, a conspicuous logging trail was discernible in the imagery from December 1984 (subsequent images were consistently captured each December). The expansion of a network of roads within the reserve became evident by 1990, serving as a clear indicator of pronounced logging activities that persisted until 1995–1996. Subsequent manifestations of logging road presence resurfaced between 2003 and 2007, suggestive of a potential resurgence in logging operations. Nevertheless, from 2008 to 2023, a gradual proliferation of vegetation obscured the visibility of these roadways.



Map 1. Location of STFR.



Map 2. Natural vegetation map of STFR.

The natural vegetation of STFR consisted of upland mixed dipterocarp forest over the Crocker soil association consisting of predominantly sandstone and mudstone-based soils. Its topography is typical of the Crocker association, with hills having amplitudes over 300 m and slopes typically greater than 25°. Ridge crests and valley bottoms are narrow, and landslips are common (Bower et al., 1975). As mentioned earlier, STFR was previously logged and much of its dipterocarp composition was lost. There were patches of relatively intact natural forest along the southwest and western boundaries, close to the expedition base camp. We suspect the hilly terrain and steep slopes prohibited logging in those areas.

Survey methods

The MacKinnon Lists (ML) method is a time-efficient, cost-effective sampling approach developed for studying avian tropical biodiversity, where lists of recorded species are collected from a single survey site (MacKinnon & Phillipps, 1993). The straightforward sampling method employed in this study proves to be well-suited for research endeavours characterized by constraints in time, resources, and personnel availability. It is particularly advantageous for entities like government agencies, non-governmental organizations, citizen scientists, and forest concessionaires, who often operate within limited capacities when conducting surveys. The simplicity of the approach facilitates its implementation in such contexts, enabling efficient data collection and analysis. This method thus emerges as a valuable tool for avifaunal research in Sabah, Malaysia, and holds the potential to contribute significantly to our understanding of the region's avian biodiversity. It also accounts for differences in effort, observer experience and knowledge, and weather (Poulsen et al., 1997). As the method relates species richness to the number of observations rather than to time, area, or walking speed, it allows the comparison of data obtained by different observers or under varying field conditions (Herzog et al., 2002). The ML method has gained popularity since the 1990s, in avian surveys and in biodiversity assessments of mammal and fish species (Bach et al., 2020).

To apply the ML method, we compiled lists of consecutive bird species recorded aurally and visually. Each list comprised 15 species. A species accumulation curve was generated by adding species not recorded on any of the previous lists to the total species number, which was then plotted as a function of the list number. However, in contrast to the traditional ML method, we also recorded the number of individuals for each species observed within each list. This provided more accurate species abundance ranks and decreased the chances of double-counting individuals.

Observation methods

Every observer had a pair of Nikon binoculars (8 x 42s). The reference field guide of choice was *Phillipps' Field Guide to the Birds of Borneo*', *3rd Ed*. (Phillipps and Phillipps, 2014). The latest taxonomic changes were determined from online sources (www.worldbirdnames.com and https://birdsoftheworld.org) and published papers. A Nikon P1000 mega-zoom camera (3000 mm equivalent) was used to photograph birds to confirm their identities.

Surveys were conducted over four days (11-14 October 2022), beginning at 7:30 am and ending after 4 hours. Two surveys were conducted along the road and another two along the temporary forest trails made by other expedition teams. Night surveys to detect nocturnal birds were conducted close to the base camp as rainy conditions during the evenings made the steep, bare-earth roads extremely slippery.

A designated person recorded all observations. Care was taken to prevent intra-list and inter-list double counts of individuals. As about half of the individuals were detected by their calls/vocalizations, individual birds were listed only if and when observers were confident that they were different, especially when inputting abundance data within the same 15-species list. Criteria for determining distinct individuals of the same species were: a) their calls originated from different directions; b) there were two or more calls heard subsequently from a similar direction of a previously recorded individual; c) the distance from a previously recorded individual was deemed sufficient for a call to be considered as from a separate individual. Photographs were taken and immediately viewed for species flying in flocks to estimate the number of individuals. Care was taken not to double-count the same flock. As the trails were not looped, only bird species not recorded earlier were recorded on the return leg.

Analyses

From survey observations, basic diversity information was extracted, including species richness, a diversity index (H), relative abundance (E_H), species family, endemicity, etc. A species accumulation curve was generated by adding species not recorded on any of the previous lists to the total species number, which was then plotted as a function of the list number. To estimate the area's species richness, we used the SuperDuplicates® online calculator developed by Chao et al. (2017), which requires only the total number of species observed and the number of species observed only once (uniques/singletons). The relative abundance indices of the species observed were calculated. The most common families and species, and the number of Bornean endemics, were also determined.

Analyses of feeding guilds provided information on how communities of species use specific forest resources (fruits, insects, arthropods, seeds, etc.) and may indicate the condition or health of the forest ecosystem. Thus, the species were categorised according to 6 feeding guilds based on their preferred diet; carnivores (Car), frugivores (Fru), insectivores (Ins), nectarivores (Nec), granivores (Gra) and omnivores (Omn). Species were considered omnivores if they were known to consume roughly similar amounts of animal- and plant-based food resources. Guild information was determined mainly from Phillipps and Phillipps (2014) and Wells (1999 & 2007). Feeding guilds were then described according to habitat type (e.g., forest, forest edge and open areas) to examine the importance of various habitats to different guilds.

Results

Avifaunal Composition and Species Richness

The four survey days yielded 15 lists of 15 individuals and a total of 342 individuals, of which 142 (41.5%) were detected by their calls/vocalisations. Table 1 shows a quick comparison of STFR with other forest reserves from previous surveys using the same methodology but in different forest types, conditions and elevations. The total species count of 85 in STFR was lower than the mean of 90.78 species.

Table 1. Comparison of STFR to other selected forest sites.

Forest site	Total species	Total families	Total individuals	Shannon Index, H’	Evenness, E_H
Sungai Tongod	85	38	342	4.07	0.70
Mt. Mandalom[1]	92	38	242	4.25	0.77
Sg. Tindikon & Sg. Tikolod[2]	79	36	328	3.67	0.63
Balingkadus[3]	86	37	388	3.89	0.65
Bukit Hampuan[4]	71	33	408	3.44	0.57
Sapagaya[5]	115	43	624	4.17	0.65
Mensalong[6]	101	37	566	4.18	0.75
Mengilan[7]	75	33	486	3.86	0.62
Sg. Rawog[8]	113	42	465	4.29	0.66
Average	90.78	37.44	427.67	3.98	0.67

Compiled from [1]Joeman *et al.* 2023, [2]Petol *et al.* 2022a, [3]Petol *et al*. 2022b, [4]Petol *et al.* 2021b, [5]Joeman *et al*., 2020a, [6]Joeman *et al.,* 2020b, [7]Petol *et al*., 2021a., [8]Petol & Rudolf 2019.

Although the STFR forest was relatively intact, its totals of species and families were similar to that of Balingkadus FR, a small, fragmented forest reserve with high levels of forest disturbance. In terms of forest conditions, STFR is very similar to Mensalong FR and Sg. Rawog Conservation Area, but both of these sites had significantly more species.

The survey also yielded 10 Bornean endemics: Bornean Wren-Babbler listed as Vulnerable (VU); Bornean Crested Fireback and Black-throated Wren-Babbler, Near Threatened (NT); and the rest, Least Concern (LC) (IUCN 2023; see Table 2. Only the wren-babblers and the Bornean Crested Fireback are uncommon forest birds.

Table 2. Species endemic to Borneo and their respective categories in the IUCN Red List (2023) of threatened species.

No.	Species	Family	Category
1	Black-throated Wren-Babbler	Pellorneidae	NT
2	Bornean Black-capped Babbler	Pellorneidae	LC
3	Bornean Brown Barbet	Megalaimidae	LC
4	Bornean Wren-babbler	Pellorneidae	VU
5	Chestnut-crested Yuhina	Zosteropidae	LC
6	Cream-eyed Bulbul	Pycnonotidae	LC
7	Bornean Crested Fireback	Phasianidae	NT
8	Dusky Munia	Estrildidae	LC
9	Golden-naped Barbet	Megalaimidae	LC
10	White-crowned Shama	Muscicapidae	LC

Table 3 lists species according to IUCN (2023) Red List categories. All species categorised as NT are lowland mixed dipterocarp forest species. The five hornbill species were listed as VU, EN or CR.

Table 3. Species listed as Near Threatened (NT), Vulnerable (VU), Endangered (EN) dan Critically Endangered (CR) by the IUCN (2023).

Species	IUCN Red List Category
Black-and-yellow Broadbill	NT
Black-throated Babbler	NT
Black-throated Wren-Babbler	NT
Bornean Crested Fireback	NT
Brown Fulvetta	NT
Chestnut-rumped Babbler	NT
Dark-throated Oriole	NT
Diard's Trogon	NT
Great Argus	NT
Green Iora	NT
Lesser Green Leafbird	NT
Rufous-crowned Babbler	NT

Scaly-breasted Bulbul	NT
Short-tailed Babbler	NT
Sooty-capped Babbler	NT
White-chested Babbler	NT
Yellow-crowned Barbet	NT
Asian Black Hornbill	VU
Bornean Wren-Babbler	VU
Rhinoceros Hornbill	VU
Wallace's Hawk-eagle	VU
Wreathed Hornbill	VU
White-crowned Hornbill	EN
Helmeted Hornbill	CR

Table 4 shows that Pellorneidae and Pycnonotidae were the most speciose families, with eight and seven species respectively. The seven families in the table contributed 41 species or approximately 48.2% of the total number of species observed.

Table 4. Top four most speciose families (with shared rankings).

Rank	Family	No. of species
1	Pellorneidae	8
2	Pycnonotidae	7
3	Nectariniidae	6
4	Bucerotidae	5
4	Megalaimidae	5
4	Muscicapidae	5
4	Timaliidae	5

As shown in Table 5, the Pycnonotidae (bulbuls) was the most commonly detected family, with 40 individuals. Of the seven species of bulbuls, the Black-headed Bulbul (12 individuals), Red-eyed Bulbul (11 individuals) and Spectacled Bulbul (11 individuals) were the most commonly detected.

Among the Pellorneidae, the White-chested, Sooty-capped, and Puff-throated Babblers claimed second place in rank, exhibiting 11, 9, and 7 individuals respectively. Virtually all babblers were identified through their vocalizations. Notably, the Puff-throated Babbler, though unlisted in Borneo, was distinguished by its persistent vocalizations. Its proximity to dense roadside vegetation facilitated comparison of its call with online databases (xenocanto.org). Owing to post-survey identification (no mobile reception at expedition basecamp or survey sites), a subsequent visit to confirm its true identity is warranted. For present publication purposes, we classify it as 'Puff-throated Babbler?' based on its call's closest resemblance to that of the Puff-throated Babbler.

The Timaliidae family featured the Bold-striped Tit-Babbler as the most frequently detected species, accounting for 17 individuals distinguished by their calls. Similarly, the Red-headed Tailorbird and Yellow-bellied Prinia of the Cisticolidae family, each with 9 individuals, were also discerned by their vocalizations. Among the Nectariniidae, the Purple-naped Sunbird was identified on 6 occasions, marking the highest count. In the fifth-ranking Columbidae family, the Pink-necked Green Pigeon prevailed as the most frequently detected species, numbering 15 individuals.

Table 5. The five families with the highest percentage of detected individuals (note shared rankings).

Rank	Family	No. of individuals	% of detected individuals
1	Pycnonotidae	40	11.7
2	Pellorneidae	35	10.2
3	Timaliidae	27	7.9
4	Cisticolidae	23	6.7
4	Nectariniidae	20	5.8
5	Columbidae	19	5.5

Table 6 shows the list of species detected 10 or more times (with shared rankings). These species made up approximately 33.3% of all individuals detected. Being aurally conspicuous, most individuals were first detected aurally. Some were later identified visually.

Table 6. List of species detected 10 or more times (note shared rankings)

Rank	Species	Family	No. of individuals detected	Relative abundance Index
1	Bold-striped Tit-Babbler	Timaliidae	17	0.050
2	Green Iora	Aegithinidae	15	0.044
2	Pink-necked Green Pigeon	Columbidae	15	0.044
3	Black-and-yellow Broadbill	Eurylaimidae	12	0.035
3	Black-headed Bulbul	Pycnonotidae	12	0.035
4	White-chested Babbler	Pellorneidae	11	0.032
4	Red-eyed Bulbul	Pycnonotidae	11	0.032
4	Spectacled Bulbul	Pycnonotidae	11	0.032
5	Chestnut-crested Yuhina	Zosteropidae	10	0.029

As expected for the ML rapid assessment method and only a 4-day survey, the species accumulation curve (Figure 1) did not achieve an asymptote. To estimate the species richness, the SuperDuplicates® online calculator was used (Chao et al., 2017). Only the total number of species detected and the number of singletons (species detected only once) were needed by the calculator (Table 7). It estimated Chao1 (species richness using abundance data) to be approximately 114 species, with an upper and lower threshold of approximately 138 and 101 species, respectively, in the 95% confidence interval. The number of doubletons (species detected only twice) was estimated to be 14, slightly more than the actual number (13) obtained from the survey. The calculator also estimated that approximately 30 species were undetected, i.e., the survey detected about 74.3% of the total species in the area. Based on the linear regression line in Figure. 1, it estimated that another nine lists, or an extra two-survey days, were needed to detect the estimated 144 species of birds in STFR.

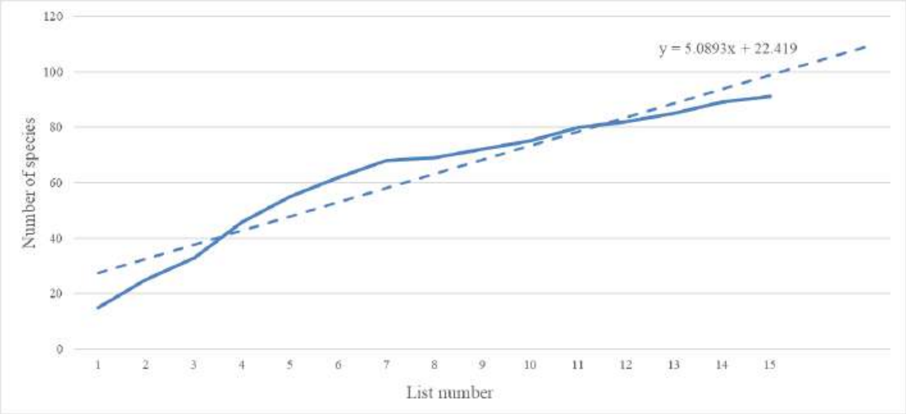


Figure 1. Species accumulation curve and linear regression line of birds in STFR.

Table 7. Results from SuperDuplicates®

Estimated number of doubletons	Estimated species richness	Stand ard error	95% C.I. lower	95% C.I. upper	Number of undetected species	Undetected percentage (%)
14.27	114.44	9.07	101.32	138.11	29.44	25.72

Habitat types and feeding guilds

Species were categorised according to their preferred habitats (e.g., forest, forest edge, open areas) and feeding guilds (Figure 2). Of the 77 forest-dependent species, 62 were strictly forest birds. Most of the common families in Bornean lowland rain forests were present. These included raptors, hornbills, woodpeckers, pigeons/doves, cuckoos, trogons, barbets, broadbills, leafbirds, tailorbirds, and bulbuls. Ground birds, such as pheasants and wren-babblers, were also detected. Pittas were not detected but this may be because they are quiet when not breeding. The commonly heard Plaintive Cuckoo, Indian Cuckoo, Banded Bay Drongo-cuckoo and Square-tailed Drongo-cuckoo were also not detected in STFR.

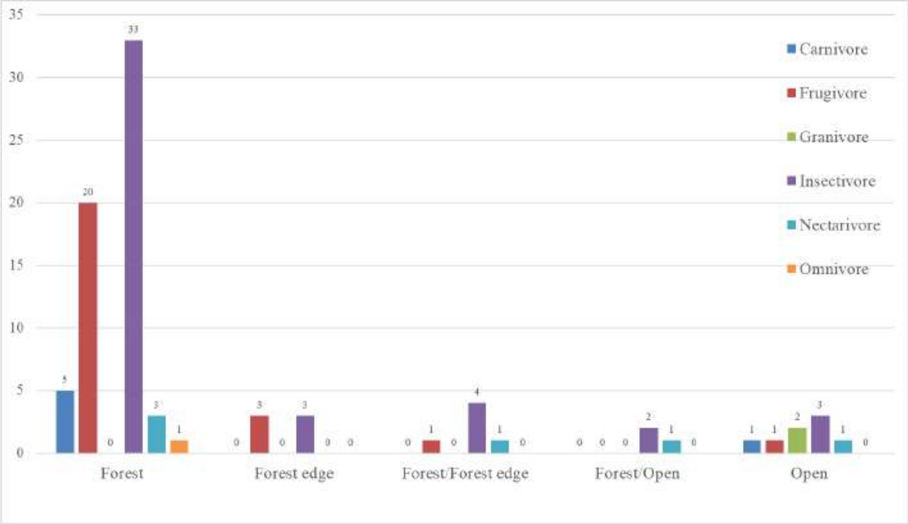


Figure 2: Number of species according to habitat types and feeding guilds in STFR.

In terms of feeding guilds, insectivores and frugivores comprised 89.5% of the total individuals detected, with the former being the most abundant at 188 individuals. Of the 45 species (representing 21 families) of insectivores, 33 were strictly forest species. The Black-and-yellow Broadbill, Green Iora, Bold-striped Tit-Babbler and White-chested Babbler were the most common insectivores.

The second most dominant guild was the frugivores, with 118 individuals detected (25 species, 10 families). With 40 individuals from seven species, the bulbuls (Pycnonotidae) were the most detected family amongst the frugivores. From their conspicuous calls, 15 individuals of barbets from five species were detected. As in our previous surveys, the total number of insectivorous and frugivorous species (33 & 20, respectively) were significantly higher in the 'Forest' habitat as compared to other habitats (Petol & Rudolf, 2019; Petol et al., 2021a; Petol et al., 2021b; Joeman et al., 2020a, Petol et al., 2022 & Joeman et al., 2020b).

Table 8 compares insectivores and frugivores figures between STFR and other selected FRs that were surveyed using similar methods. At 82.3%, the percentage of insectivorous-frugivorous species was similar to that in other forest reserves. In STFR, insectivores made up 55.0% of the total individuals detected, slightly above the average of 53.1% for all sites. The number of insectivorous species was slightly above average while the number of insectivorous species was about average. For frugivores, the percentage of total individuals (34.5%) was higher than the other forest reserves except for Mensalong FR. The number of frugivorous species was slightly below the average of 27 species. The number of frugivorous species was about average.

Table 8. Comparison of insectivores/frugivores in STFR and in other selected forest reserves.

Forest reserve	% of insectivorous + frugivorous species	Insectivores			Frugivores		
		% of total individuals	Number of species	Number of families	% of total individuals	Number of species	Number of families
Sg. Tongod	82.3	55.0	45	21	34.5	25	10
Mt Mandalom[1]	71.9	43.0	38	21	28.9	27	10
Sg. Tindikon & Sg. Tikolod[2]	83.8	58.1	40	22	25.7	25	10
Balingkadus[3]	82.5	52.8	41	22	29.6	29	10
Bukit Hampuan[4]	77.2	52.9	35	19	24.2	22	9
Sapagaya[5]	82.4	61.4	62	27	25.8	32	11
Mensalong[6]	80.4	43.1	48	22	45.0	34	12
Menghilan[7]	84.0	59.0	41	19	25.1	22	9
Average	80.1	53.1	43.8	21.6	29.9	27.0	10.1

Compiled using survey data from [1]Joeman et al. 2023, [2]Petol et al. 2022a, [3]Petol et al. 2022b, [4]Petol et al. 2021b, [5]Joeman et al., 2020a, [6]Joeman et al., 2020b, [7]Petol et al., 2021a.

Discussion

The findings of this survey underscore the capacity of heavily logged forest ecosystems to maintain a noteworthy diversity of avian species. Regeneration processes post-logging have demonstrated the potential to enhance species richness over extended time periods, particularly when regrowth remains undisturbed for durations ranging from two to four decades (Dent & Wright, 2009). The proximity of STFR to neighboring areas, specifically Trus Madi FR and Ulu Sungai Milian FR, raises the prospect of recolonisation, contributing to the gradual restoration of species within the logged forest. These contiguous Class II Production Forest Reserves offer the potential to serve as repositories for reinstating STFR's species composition. Moreover, observable indicators point to ongoing avian recolonisation within the surveyed habitat. Supported by diversity indices, H’ and E_H, signifying robust species diversity and even distribution, the presence of diverse taxa, including five hornbill species and 62 forest specialists, accentuates conducive conditions for avian niche expansion. The identification of ground-dwelling species such as the Great Argus and the Bornean Crested Fireback, along with flourishing forest specialists, bolsters the notion of abundant food resources within STFR. This deduction finds support in data from Table 8, where both insectivore and frugivore ratios exceed those of other forest contexts.

Similar investigations have provided insights into post-logging avian dynamics. Research in West Malaysian rainforests by Husin and Rajpar (2015) delineated temporal patterns post-logging, with an initial phase of species susceptibility followed by heightened diversity approximately three decades after disturbance. Although the pre-logging species inventory of STFR was not available, the diversity observed within the 15–16 years following logging aligns with regenerative trends. Similarly in Kalimantan, where Cleary et al. (2007) showed shifts in avian dietary preferences and sizes due to logging. Likewise, Edmunds et al. (2013) examined avian responses to logging-induced changes, highlighting the vulnerability of dietary specialists to localised extirpation, with persisting species displaying no strict alignment with trophic positions. These insights illuminate the complexities of avian dynamics post-logging, underscoring the interactions between ecological succession, trophic adaptation, and taxonomic persistence. The concealed intricacies of trophic recalibration, stemming from forest degradation, highlights the need to shift from exclusive feeding guild associations to a more holistic understanding of functional intricacies within logged-over forest ecosystems.

Conversely, the plausibility of STFR retaining a substantial portion of its initial avian community arises from the survey site's relative ecological integrity, distinct from the extensively degraded forest in other parts of STFR. This specific forest area might have functioned as a refugium, offering specialised habitats that safeguard and support avian populations. The presence of two distinct Bornean gibbon groups within the surveyed area serves to corroborate the notion put forth by Davies and Tan (2019), suggesting a resurgence in gibbon densities to levels akin to the pre-logging era. This alignment with the post-logging 20-year recovery timeline emphasizes the restorative potential of the ecosystem after logging activities. However, it is important to exercise caution in interpreting these survey findings.

Conclusion

While the rest of STFR was mainly degraded forest, the survey provided a window to understanding the avian community within the reserve. Its relatively high H' and E_H values indicate a diverse bird assemblage. The presence of hornbills and ground-dwelling species also indicated a moderately healthy bird community. However, while still in its early phases, the road construction project through the southern border of the reserve already showed signs of having a destructive impact on the few remaining tracts of intact forests in STFR. The extent of preservation of STFR in the years ahead remains uncertain. It would be interesting to survey this area a few years after the completion of the road to access the avian community's response to (potentially) further disturbance.

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References

Anne Chao, Chun-Huo Chiu, Robert K. Colwell, Luiz Fernando S. Magnago, Robin L. Chazdon and Nicholas J. Gotelli, 2017. Deciphering the enigma of undetected species, phylogenetic, and functional diversity based on Good-Turing theory. *Ecology*, *98,* (11): 2914–2929.

Bach LL, Bailey DM, Harvey ES, MacLeod R. 2020. The MacKinnon Lists Technique: An efficient new method for rapidly assessing biodiversity and species abundance ranks in the marine environment. *PLOS ONE 5* (4): e0231820. https://doi.org/10.1371/journal.pone.0231820.

Bower RP, Burrough PA, Kalsi MS, Thomas, P. 1975. The soils of Sabah. *Land Resource Study 20*, Ministry of Overseas Development, Surrey, England, pp 76–77.

Cai T, Cibois A, Alström P, Moyle R, Kennedy J, Shao S, Zhang R, Irestedt M, Ericson P, Gelang M, Qu Y, Lei F, Fjeldså J. 2018. Near-complete phylogeny and taxonomic revision of the world's babbler (Aves: Passeriformes). *Molecular Phylogenetics and Evolution*, BI 346–356. 10.1016/j.ympev.2018.10.010.

Chao A, Colwell, RK, Chiu C-H, Townsend D. 2017. Seen once or more than once: applying Good-Turing theory to estimate species richness using only unique observations and a species list. *Methods in Ecology and Evolution, British Ecological Society*. http://chao.stat.nthu.edu.tw/wordpress/paper/122.pdf

Cleary, D.F., Boyle, T.J., Setyawati, T., Anggraeni, C.D., Loon, E.E.V. and Menken, S.B., 2007. Bird species and traits associated with logged and unlogged forest in Borneo. *Ecological Applications*, *17* (4): 1184-1197. doi: 10.1890/05-0878.

Davies G, Tan HJ. 2019. Tabin revisited: 1981–2018*. Sabah Society Journal, 36:*1–38.

de Iongh HH, van Weerd M. 2006. The use of avian guilds for the monitoring of tropical forest disturbance by logging. *Tropenbos Documents, 17.* Wageningen, The Netherlands.

Dent DH, Wright SJ. 2009. The future of tropical species in secondary forests: a quantitative review. *Biological Conservation*, 142: 2833–2843.

Edwards DP, Woodcock P, Newton RJ, Edwards FA, Andrews DJ, Docherty TD, Mitchell SL, Ota T, Benedick S, Bottrell SH, Hamer KC. 2013. Trophic flexibility and the persistence of understory birds in intensively logged rainforest. *Conservation Biology*, *27* (5): 1079–1086.

Gill F, Donsker D, Rasmussen P. (Eds). 2022. IOC *World Bird List* (*v12.1).* doi: 10.14344/IOC.ML.12.1.

Herzog SK, Kessler M, Cahill TM., 2002. Estimating species richness of tropical bird communities from rapid assessment data. *Auk*, *119:* 749–769.

Husin MZ, Rajpar, M.N., 2015. Effects of logging and recovery process on avian richness and diversity in hill dipterocarp tropical rainforest-Malaysia. *Journal of Environmental Biology*, 36 (1): 121.

IUCN 2023. The IUCN Red List of Threatened Species. Version 2021-3. https://www.iucnredlist.org

Joeman BJ, Petol GH, Lolin L, Rudolf V, Tingkoi, L., 2020a. Avifaunal survey of Mt Silam and surrounding areas in the district of Lahad Datu, Sabah, Malaysia. *Compilation of papers – Mount Silam Scientific Expedition, Lahad Datu, Sabah: 87–95.* Sabah Forestry Department, Sandakan, Sabah.

Joeman BJ, Petol GH, Lolin L, Rudolf V., 2020b. Avifaunal survey of Mensalong Forest Reserve, Ranau, Sabah, Malaysia. *Sepilok Bulletin, Vol. 29:* 1–10.

Joeman BJ, Petol GH, Lolin L. 2023. Avifaunal survey of Mandalom Forest Reserve, Tenom, Sabah, Malaysia. Unpublished expedition report for Sabah Forestry Department.

Karr JR, Robinson S, Blake JG, Bierregaard Jr. RO. 1990. Birds of four neotropical forests. In A.H. Gentry (ed.) *Four tropical rainforests*. New Haven, Yale University Press, 69–237.

MacKinnon S, Phillipps K. 1993. *A Field Guide to the Birds of Borneo, Sumatra, Java and Bali,* Oxford University Press.

Naish D. 2014. The fossil record of bird behaviour, *Journal of Zoology*, *vol 292* (4): 268–280.

Poulsen BO, Krabbe N, Frølander A, Hinojosa BM, Quiroga OC. 1997. A rapid assessment of Bolivian and Ecuadorian montane avifaunas using 20-species lists: efficiency, biases and data gathered. *Bird Conservation International*, *7:* 53–67.

Petol GH, Ong RC. 2013. *A photographic guide to the lowland birds of Sepilok*. Sabah Forestry Department publication, Sabah, Malaysia.

Petol GH, Rudolf V. 2019. Avifaunal survey of Sungai Rawog, Conservation Area, Segaliud Lokan Forest Reserve. In *Proceedings of the Seminar of Sungai Rawog Conservation Area, Scientific Expedition, Segaliud Lokan Forest*

Reserve (FMU 19B), Sabah Forestry Department & KTS Plantation Sdn. Bhd., 2019, pp:191–202.

Petol GH, Lolin L, Rudolf V, Ardianshah, YBB. 2021a. Avifaunal survey of Mengilan Forest Reserve and its surrounding areas in Pensiangan, Sabah, Malaysia. *Journal of Tropical Biology & Conservation (JTBC)*, Vol. 18/2021: 215–228.

Petol GH, Lolin L, Rudolf V, Chin TT. 2021b. Avifaunal survey of the lower montane forest of Bukit Hampuan Forest Reserve, Ranau, Sabah, Malaysia. *Sepilok Bulletin, Vol. 30:* 19–31.

Petol GH, L Lolin, V Rudolf, MN Petol, F Joel, FR Juvilis, AR Khairul. 2022a. An avifaunal survey of Sg. Tindikon & Sg. Tikolod Forest Reserves in Tambunan, Sabah, Malaysia. *Sepilok Bulletin, Vol. 31:* 50–63.

Petol GH, Lolin L, Rudolf V, Joel F, Petol MN. 2022b. Avifaunal Survey of Bukit Balingkadus, A Small Fragmented Forest in Ranau, Sabah, Malaysia. *Journal of Tropical Biology & Conservation (JTBC)*, 19, 109–123. https://doi.org/10.51200/jtbc.v19i.3943.

Phillipps Q, Phillipps K. 2014. *Phillipps' Field Guide to the Birds of Borneo, 3rd Edition.* John Beaufoy Publishing Ltd., Oxford, England.

Wells DR. 1999. *Birds of the Thai-Malay Peninsula: Volume I: Non-Passerines.* Academic Press London, UK.

Wells DR. 2007. *The birds of the Thai-Malay Peninsula. Volume II: Passerines. Christopher Helm*, London.

APPENDIX I

List of bird species detected at Sungai Tongod Forest Reserve (Class I), sorted by families. Common names in bold denote Bornean endemics. Bird names are according to the classification in Gill, Donsker and Rasmussen (2023).

Common name	Species	Family
Crested Serpent Eagle	*Spilornis cheela*	Accipitridae
Wallace's Hawk-eagle	*Spizaetus nanus*	Accipitridae
Common Iora	*Aegithina tiphia*	Aegithinidae
Green Iora	*Aegithina viridissima*	Aegithinidae
Rufous-backed Kingfisher	*Ceyx rufidorsa motleyi*	Alcedinidae
Brown Fulvetta	*Alcippe brunneicauda*	Alcippeidae
Asian Palm Swift	*Cypsiurus balasiensis*	Apodidae
Little Egret	*Egretta garzetta*	Ardeidae
White-crowned Hornbill	*Aceros comatus*	Bucerotidae
Asian Black Hornbill	*Anthracoceros malayanus*	Bucerotidae
Rhinoceros Hornbill	*Buceros rhinoceros*	Bucerotidae
Helmeted Hornbill	*Rhinoplex vigil*	Bucerotidae
Wreathed Hornbill	*Rhyticeros undulatus*	Bucerotidae
Lesser Cuckoo-shrike	*Coracina fimbriata*	Campephagidae
Malaysian Eared Nightjar	*Lyncornis temminckii*	Caprimulgidae
Yellow-bellied warbler	*Abroscopus superciliaris*	Cettiidae
Lesser Green Leafbird	*Chloropsis cyanopogon*	Chloropseidae
Red-headed Tailorbird	*Orthotomus ruficeps*	Cisticolidae
Rufous-tailed Tailorbird	*Orthotomus sericeus*	Cisticolidae
Yellow-bellied Prinia	*Prinia flaviventris*	Cisticolidae
Emerald Dove	*Chalcophaps indica*	Columbidae
Pink-necked Green Pigeon	*Treron vernans*	Columbidae
Banded Bay Cuckoo	*Cacomantis sonneratii*	Cuculidae
Violet Cuckoo	*Chrysococcyx xanthorhynchus*	Cuculidae
Raffles's Malkoha	*Phaenicophaeus chlorophaeus*	Cuculidae
Scarlet-backed Flowerpecker	*Dicaeum cruentatum nigrimentum*	Dicaeidae
Orange-bellied Flowerpecker	*Dicaeum trigonostigma*	Dicaeidae

APPENDIX I (cont.)

Common name	Species	Family
Bronzed Drongo	*Dicrurus aeneus*	Dicruridae
Greater Racquet-tailed Drongo	*Dicrurus paradiseus*	Dicruridae
Chestnut Munia	*Lonchura atricapilla*	Estrildidae
Dusky Munia	*Lonchura fuscans*	Estrildidae
Black-and-yellow Broadbill	*Eurylaimus ochromalus*	Eurylaimidae
Whiskered Treeswift	*Hemiprocne comata*	Hemiprocnidae
Grey-rumped Treeswift	*Hemiprocne longipennis*	Hemiprocnidae
Barn Swallow	*Hirundo rustica*	Hirundinidae
Asian Fairy-bluebird	*Irena puella*	Irenidae
Bornean Brown Barbet	*Calorhamphus fuliginosus*	Megalaimidae
Gold-whiskered Barbet	*Psilopogon chrysopogon*	Megalaimidae
Blue-eared Barbet	*Psilopogon cyanotis*	Megalaimidae
Yellow-crowned Barbet	*Psilopogon henricii*	Megalaimidae
Golden-naped Barbet	Psilopogon pulcherrimus	Megalaimidae
Red-bearded Bee-eater	*Nyctyornis amictus*	Meropidae
Blyth's Paradise Flycatcher	*Terpsiphone affinis borneensis*	Monarchidae
Grey Wagtail	*Motacilla citreola*	Motaciliidae
Oriental Magpie Robin	*Copsychus saularis adamsi*	Muscicapidae
White-crowned Shama	*Copsychus stricklandi*	Muscicapidae
Hill Blue Flycatcher	*Cyornis whitei*	Muscicapidae
White-crowned Forktail	*Enicurus leshenaulti*	Muscicapidae
Dark-sided Flycatcher	Muscicapa sibirica	Muscicapidae
Eastern Crimson Sunbird	*Aethopyga siparaja*	Nectariniidae
Brown-throated Sunbird	*Anthreptes malacensis bornensis*	Nectariniidae
Plain Sunbird	*Anthreptes simplex*	Nectariniidae
Thick-billed Spiderhunter	*Arachnothera crassirostris*	Nectariniidae
Little Spiderhunter	*Arachnothera longirostra*	Nectariniidae
Purple-naped Sunbird	*Kurochkinegramma hypogrammicum*	Nectariniidae
Dark-throated Oriole	*Oriolus xanthonotus*	Oriolidae
Sooty-capped Babbler	*Malacopteron affine*	Pellorneidae
Rufous-crowned Babbler	*Malacopteron magnum*	Pellorneidae

APPENDIX I (cont.)

Common name	Species	Family
Black-throated Wren-Babbler	*Napothera atrigularis*	Pellorneidae
Bornean Black-capped Babbler	*Pellorneum capistratoides*	Pellorneidae
Short-tailed Babbler	*Pellorneum malaccense*	Pellorneidae
White-chested Babbler	*Pellorneum rostratum*	Pellorneidae
Puff-throated Babbler?	*Pellorneum ruficeps*	Pellorneidae
Bornean Wren-Babbler	Ptilocichla leucogrammica	Pellorneidae
Great Argus	*Argusianus argus*	Phasianidae
Bornean Crested Fireback	*Lophura ignita ignita*	Phasianidae
Crimson-winged Woodpecker	*Picus puniceus*	Picidae
Rufous Piculet	*Sasia abnormis*	Picidae
Blue-crowned Hanging Parrot	*Loriculus galgulus*	Psittaculidae
Black-headed Bulbul	*Brachypodius melanocephalos*	Pycnonotidae
Spectacled Bulbul	*Ixodia erythropthalmos*	Pycnonotidae
Scaly-breasted Bulbul	*Ixodia squamata*	Pycnonotidae
Red-eyed Bulbul	*Pycnonotus brunneus*	Pycnonotidae
Yellow-vented Bulbul	*Pycnonotus goiavier*	Pycnonotidae
Olive-winged Bulbul	*Pycnonotus plumosus*	Pycnonotidae
Cream-eyed Bulbul	*Pycnonotus pseudosimplex*	Pycnonotidae
Boobook	*Ninox scutulata borneensis*	Strigidae
Bold-striped Tit-babbler	*Mixornis bornensis*	Timaliidae
Chestnut-backed Scimitar Babbler	*Pomatorhinus mantanus*	Timaliidae
Chestnut-rumped Babbler	*Stachyris maculata*	Timaliidae
Black-throated Babbler	*Stachyris nigricollis*	Timaliidae
Grey-headed Babbler	Stachyris rufifrons	Timaliidae
Diard's Trogon	*Harpactes diardii*	Trogonidae
Rufous-winged Philentoma	*Philentoma pyrhopterum*	Vangidae
Chestnut-crested Yuhina	*Staphida everetti*	Zosteropidae

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Research Article

Establishment of An in Vitro Mass Propagation System for *Dendrocalamus asper.*

Anis Adilah Mustafa[1], Wilson Thau Lym Yong[1], Julius Kulip[2], Kenneth Francis Rodrigues[1*]

[1]*Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[2]*Botanicals Sabah Sdn. Bhd., 4th Floor, Lot 12, Warisan Square, P. O. Box 10018, 88800 Kota Kinabalu, Sabah, Malaysia.*

*Corresponding author: kennethr@ums.edu.my
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ABSTRACT

Dendrocalamus asper is a species of bamboo that has high commercial value and is the bamboo of choice for large scale agro-forestry plantations in the tropical regions of the world. Micropropagation using tissue culture is essential to generate uniform clones that are amenable to establishment in industrial agro-forestry projects for bamboo biomass, habitat restoration or in carbon sequestration. This paper reports on the establishment of *D. asper* invitro using commercially available seeds. The seeds were surface sterilized using three different chemical agents which were Sodium Hypochlorite (20%), Mercuric Chloride (0.1%) and Ethanol (70%) followed by shoot initiation on Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) with a concentration ranging from 1.0 – 5.0 mg/L. Propagules were multiplied on MS media supplemented with different concentration of IBA Indole-3-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA), and finally rooted and hardened in peat moss. The findings of our study indicate that the sterilization protocol eliminated all the plant pathogens, resulting in an axenic culture. Full strength MS medium supplemented with 5 mg/L BAP yielded the highest number of shoots (11.46 per explant) after four weeks of inoculation. The highest multiplication rate (3.95 shoots per explant) was obtained on MS medium supplemented with 3 mg/L BAP. The time required from initiation to hardening was 70 to 90 days, following which the plantlets were ready for field trials. The findings of this study will facilitate the establishment of plant tissue culture programmes dedicated to the production of *D. asper* locally, thus eliminating the need for imports and the possible entry of plant pathogens that can be detrimental to the local agro-forestry industry.

Keywords: *Dendrocalamus asper;* bamboo; micropropagation; 6 Benzyl aminopurine; Indole-3-Butyric Acid; Naphthalene Acetic Acid; Murashige and Skoog medium

Introduction

Micropropagation of bamboos is accepted as one of the standard methods for the large-scale multiplication of bamboo clones with desirable characteristics. This is achieved by germination of seeds in vitro (Lv et al., 2021) to overcome the low germination rate of bamboo seeds using conventional approaches, by the in vitro establishment of axillary shoots (Sandhu et al., 2018), the induction of callus tissue (Obsuwan et al., 2019; Zang et al., 2016) and organogenesis (Dey et al., 2020). Methods developed for the micropropagation of bamboo must be optimized based on the variety and the type of the tissue (Ara et al., 2020) and this is achieved by the fine tuning of the components of the culture medium as well as the selection of the appropriate concentrations of the carbon sources (Yasodha et al., 2008), the concentration and the ratio of the phytohormones (Venkatachalam et al., 2015), and the mitigation of the activity of polyphenol oxidases in vitro that can contribute to abscission of growing tissue (Huang et al., 2002). Micro propagated bamboo is a downstream product of the biotechnology industry that can be genetically modified for further improvement to improve its growth characteristics (Ranjan, 2021) and contribute to food security (Bhardwaj et al., 2023) in alignment with the United Nations Sustainable Development Goals. One of the major challenges associated with the propagation of bamboo is the lack of seeds. Bamboo stands undergo flowering followed by mass mortality and most of the seeds are not viable. This has led to micropropagation of viable seeds being the method of choice for mass propagation of bamboo. This study set forth to establish a protocol for the large-scale multiplication of a commercial variety of *Dendrocalamus asper* within the context of a limited time frame to ensure that it can be upscaled for industrial production of bamboo clones locally to support the rural economy and contribute to food security. *Dendrocalamus asper* is a fast-growing species of bamboo that is cultivated both for industrial application and consumption. Bamboos are monocarpic and flower only once in 30 to 120 years, thus making it extremely difficult to source seeds for establishment of commercial plantations, per hectare is 9000 kgs per year and the most widely adopted method for propagation is via cuttings or to a lesser extent micropropagation. Previous attempts to establish *Dendrocalamus asper* in vitro relied on the use of a single concentration of MS medium with variable concentrations of 6 BAP (Arya et al., 2008) and this protocol was tested in order to establish the commercial variety of *Dendrocalamus asper* in vitro prior to optimizing the culture conditions.

Material and methods

The micropropagation experiments for the *Dendrocalamus asper* bamboo species were conducted at the plant invitro laboratory located at the Biotechnology Research Institute, Universiti Malaysia Sabah from March to November 2022. The methods of growing bamboos through tissue culture were conducted following a protocol using seeds as a source of explants. The seeds were dehusked and washed under running tap water for 15 min. Surface disinfection was done with 0.1% Mercuric Chloride ($HgCl_2$) for 3 min, 70% Ethanol for 15 min, and followed by 20 % Sodium Hypochlorite (NaOCl) for 10 min with the addition of a 0.1% V/v of the surfactant Tween 20. The seeds were rinsed four times with sterile distilled water and placed on an oscillating shaker to ensure an even complete disinfection of seeds. Sterile filter paper was used to remove the extra moisture on the seed surface. The disinfected seeds were germinated in 100 mL tissue culture flasks containing 35 mL of germination medium [MS medium supplemented with 100 mg/l myo-inositol, 30 g/l sucrose, 5.6 g/L Gelrite (Duchefa Biochemie), and 1.0–5.0 mg/L 6-BAP for a duration of 28 days. Propagules were separated carefully and were transferred on to two types of multiplication media (MS and half-strength MS) on day 22, and sub cultured on fresh media on days 14,21, 35, 46, and 60. Optimization of media for rooting in vitro was done by transferring single shoots to semi solid half-strength MS medium supplemented with different concentration of IBA Indole-3-Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) for 28 days. After rooting in vitro, the shoots with roots were taken out of the culture bottle carefully without damaging the roots. The roots were washed gently under running tap water to eliminate the culture medium. The plantlets with roots were acclimatized in peat moss (Kekkila, Finland) for hardening and maintained at 25°C ± 2°C under continuous light with an intensity of 30 µmol/ µmol/m2/s provided by cool-white 40 W fluorescent tubes (Philips) for 28 days. The pH of all culture media was adjusted to 5.8 ± 0.1 with 0.1 N NaOH or 0.1 N HCl prior to the addition of Gelrite, followed by autoclaving at 121°C for 20 min at 15 PSI. The experimental design for plant tissue culture was completely randomized and composed of 30 replicates per treatment for shoot initiation and proliferation, while 20 replicates were for rooting. The shoot formation, leaf formation, root formation, and the number of shoots, leaves, or roots were recorded visually and measured using a vernier calliper during each subculture. All the data were subjected to analysis of variance (ANOVA) performed in IBM SPSS statistical software Version 28.0. Significance differences between the means were assessed by Tukey's post hoc test at $p < 0.05$.

Results and Discussion

The process of disinfection of seeds using the three chemical agents in combination with continuous washing in sterile distilled water and de-husking of the seeds to remove the fibrous seed coat resulted in the elimination of microbial contaminant in the culture media. The establishment of an axenic culture of bamboo is critical to the reproducibility of the protocol as the microbes (Banik et al., 2018; Collinge et al., 2021; Djami-Tchatchou et al., 2022) that are cocultured with the target plant can produce a wide range of bioactive compounds that can serve as growth promoters or inhibitors and confound the interpretation of the results. Mercuric chloride at a concentration of 0.1% is ideal for the elimination of microbes and has been reported in other studies (Hypochlorite et al., 2021; Josephine U Agogbua & Bosa E Okoli, 2022), however caution should be exercised when working with Mercuric Chloride as it is a harmful chemical agent that must be eliminated completely from the culture medium by repeated washing followed by proper disposal. The seed husk serves as a matrix for fungal spores that may resist disinfection by chemical agents, and the removal of this outer layer of husk also contributed to the successful establishment of seeds during the first stage.

MS medium was used at two different concentrations in accordance with the experimental design. The full-strength MS medium (1X) was found to be ideal for initiation of shoots during germination, however, continued use of this concentration led to browning of tissue (Figure 1) that can be attributed to abscission as has been reported in other studies (My Ngan et al., 2020). This study did not revert to the usage of additional antioxidants such as Salicylic acid (Ali, 2021), because these would add to the complexity of the culture medium.

Figure 1. Evidence of abscission in *Dendrocalamus asper* plantlets cultivated on full-strength MS medium 10 days after subculture.

The problem of tissue necrosis in long-term culture was reduced significantly by using half-strength MS medium. The reason for the reduction in necrosis and abscission in lower strength of MS media is the presence of high concentration of micronutrients. One of the possible alternatives will be to optimize a novel medium for bamboo using response surface methodology.

The results of the germination of seeds followed by the development of seedlings using the cytokinin BAP after four weeks of culture are documented in Table 1. The number of shoots were determined to be independent of the concentration of 6-BAP. A similar observation was made with reference to the number of leaves at the tested concentration of 6 BAP.

Table 1. Effect of BAP concentrations in MS medium on development of shoot and leaves from single seed. Data recorded after 4 weeks of culture.

6-BAP concentration (mg/l)	Shoot length (cm)	Number of shoots	Number of leaves
0.0	2.93±1.11a	1.0±0.0d	5.37±1.94a
1.0	2.47±0.85a	2.06±0.77d	4.65±1.51a
2.0	1.52±0.72b	4.89±0.57c	2.62±0.91b
3.0	1.48±0.41b	7.2±1.44b	2.29±0.82b
4.0	1.08±0.20bc	10.86±0.85a	1.70±0.55bc
5.0	0.96±0.23c	11.46±0.47a	1.49±0.50c

Values are means ± SE (Standard Error) of 30 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c) do not differ significantly by Tukey's test ($p < 0.05$)

The objective of the first stage of tissue culture was to increase the number of shoots, without compromising the number of leaves, and it was concluded that the optimum concentration of BAP was 4 mg/l that yielded a lower number of shoots (10.86) with an increased number of leaves. Studies in other species of bamboo have revealed that 6 BAP is the ideal for mass propagation of bamboo and can be used in combination with the auxin Kinetin (Khare et al., 2021), however for the purpose of the development of this protocol, Kinetin was eliminated due to the high cost, making it not suitable for commercial projects. The previous study (Arya et al., 2008) reported that full strength MS medium could be utilized throughout the process of micropropagation, but based on the observations made during this study, the use of half strength MS medium resulted in a decrease in the formation of necrotic tissue. One of the possible explanations for this is that seeds derived from different locations are likely to have genetic and epigenetic variations that can influence the process of invitro establishment and propagation. Thus, future research work must consider the geographic origin of the germplasm prior to establishment in vitro.

The new shoots were separated into individual propagules comprising one shoot and its associated root from the in vitro-developed shoots for subculture into different concentrations of BAP for 14 days. The highest multiplication rate of 3.95 shoots per propagule was recorded in MS medium with 3 mg/L (Table 2).

Table 2. Effect of BAP concentration in MS medium on shoot multiplication. Propagules consisting of one shoot and its associated root were cultured and data recorded after 4 weeks of inoculation (± SE). Three propagules were inoculated in a single tissue culture jar.

6-BAP concentration (mg/l)	Shoot length (cm)	Number of shoots
0.0	3.37±0.86^{a}	1.0±0.0^{e}
1.0	2.86±0.51^{b}	6.68±0.89^{d}
2.0	1.75±0.61^{c}	18.37±1.012^{d}
3.0	1.55±0.35^{cd}	28.46±0.45^{a}
4.0	1.23±0.24^{de}	25.9±0.97^{b}
5.0	1.11±0.22^{e}	24.23±1.12^{b}

Green multiple shoots with 3 propagules were further sub-cultured into different concentrations of 6 -BAP for 14 days. Values are means ± SE of 30 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c) do not differ significantly by Tukey's test ($p < 0.05$)

Therefore, 3 mg/L BAP was selected for further proliferation. Subculturing was initiated every 2 weeks. The increase in shoot number was observed until the fourth subsequent subcultures, after which it declined in the fifth subculture (as shown in Table 3). In the present study, BAP was found to be effectual for shoot multiplication. These findings are consistent with those reported by (Suwal et al., 2020) and (Hiswan et al., 2020). To conclude, the use of a single auxin at lower concentration was determined to be ideal for the *D. asper* variety selected for this study.

Table 3. Effect of subsequent subculture on multiple shoot proliferation in MS basal medium supplemented with 3.0 mg/L BAP.

Subcultures	Number of shoots	Length of shoots (cm)	Multiplication fold
1st subculture (14d)	9.98±0.28e	1.55±0.82e	3
2nd subculture (21d)	20.91±0.098d	2.12±0.43d	2.09
3rd subculture (35d)	26.58±0.19c	3.81±0.51c	1.27
4th subculture (46d)	35.33±0.13a	6.61±0.29a	1.16
5th subculture (60d)	30.3±0.11b	5.01±0.74b	1.13

Means followed by the same alphabet (a, b, c, d, e) do not differ significantly by Tukey's test ($p < 0.05$)

The rooting stage is crucial for the establishment of plants in the field and the induction of a large network of roots can be promoted by the usage of auxins. Two auxins, 1-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA) were used in various combinations (Table 4) (Figure 2) and it was determined that the optimum concentration was 0.5 mg/l of IBA based on the rooting percentage (90%), the average number of roots in a clump (16.65) and the length of the roots (4.15 cm).

Table 4. Effect of different concentration of IBA and NAA on rooting of in vitro raised shoots *D. asper*. Data recorded after 4 weeks of inoculation (± SE).

Plant growth regulator (mg/ l)		Rooting (%)	No. roots in clump (Mean ± SE)	Root length (cm)
IBA	NAA			
0	0	0	0.00^{h}	0.00^{i}
0.5	-	96	16.65 ± 0.52^{a}	4.15 ± 0.75^{a}
1.0	-	81	9.9 ± 0.30^{bc}	3.35 ± 0.27^{b}
3.0	-	82	7.7 ± 0.45^{e}	2.45 ± 0.51^{c}
5.0	-	75	3.83 ± 0.24^{f}	2.0 ± 0.49^{cd}
7.0	-	63	2.82 ± 0.36^{fg}	1.25 ± 0.31^{de}
10.0	-	54	1.75 ± 0.61^{g}	0.62 ± 0.37^{gh}
-	0.5	60	3.75 ± 0.53^{f}	0.45 ± 0.30^{hi}
-	1.0	68	8.05 ± 0.44^{de}	1.0 ± 0.29^{fg}
-	3.0	80	10.85 ± 0.58^{b}	2.37 ± 0.49^{c}
-	5.0	76	9.3 ± 0.69^{cd}	1.37 ± 0.50^{ef}

Values are means ± SE of 20 replicates. Data scored after 4 weeks in culture.
Means followed by the same alphabet (a, b, c, d, e, f, g, h, i) do not differ significantly by Tukey's test ($p < 0.05$).

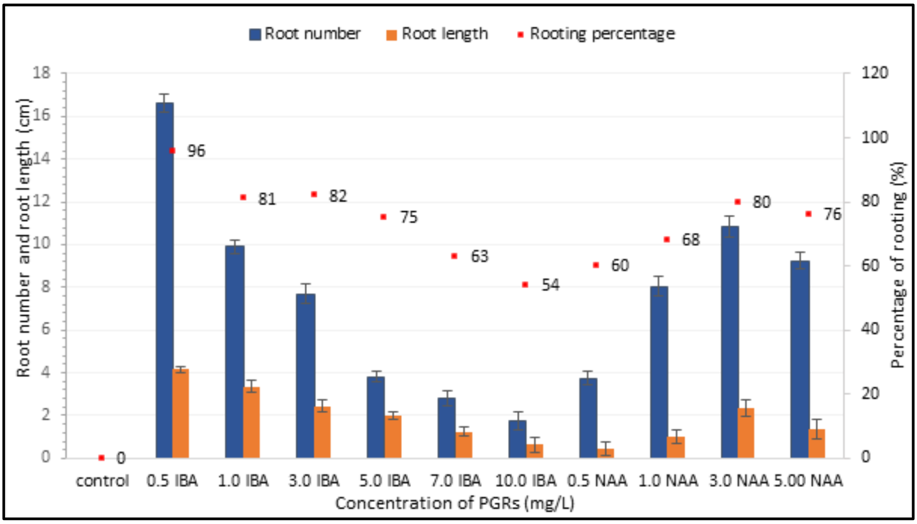

Figure 2. Effect of different concentrations of IBA and NAA on rooting of in vitro raised shoots *D. asper*. for 4 weeks. Values are Mean ±SE of 20 replicates. Bars denote the standard error (SE).

We concluded that IBA is more effective than NAA in establishing a high number of roots per clump. Studies involving other species of bamboo are indicative that the concentration of auxin varies from species to species (Yasodha et al., 2008)

(Sharothi et al., 2022) and this may be dependent on the tissue type as well as the genetic factors that are likely to differ based on the species.

The final stage involved hardening of the tissue culture plantlets in a climate-controlled greenhouse and finally transferring the propagated plantlets for field testing. The complete process from the establishment of seeds to the final stage of hardening is summarised in Figure 3. The methods established in this study, can be applied for the mass propagation of *D. asper* in Sabah and can be extended to other species of bamboo.

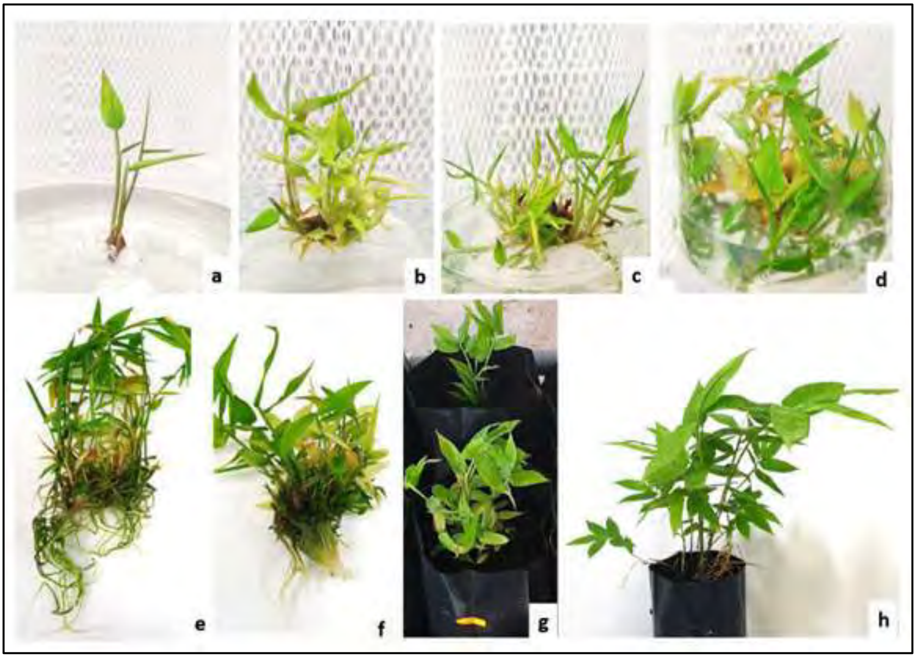

Figure 3. (a) Seedlings growth in MS medium (no Plant Growth Regulators). (b) Initiation of multiple shoots on MS + 3 mg/L BAP. (c) Proliferation of numerous shoots after 2 months on MS + 3.5 mg/l BAP. (d) Clusters of adventitious shoots after 3 months on MS + 3.5 mg/l BAP. (e) Root induction on MS + 0.5 mg/L IBA. (f) Root induction on MS + 3.0 mg/L NAA. (g) 1 month old, hardened plant in greenhouse. (h) 4 months old, hardened plant.

Conclusion

This study successfully developed a protocol for the large-scale production of *D., asper* from commercial seeds that were reproducible and yielded clones within a period of two months. Future research will focus on field trials and the selection of field grown plants with desirable phenotypes for the establishment of elite lines of plants.

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References

Ali B. 2021. Salicylic acid: An efficient elicitor of secondary metabolite production in plants. In *Biocatalysis and Agricultural Biotechnology, (Vol. 31)*. https://doi.org/10.1016/j.bcab.2020.101884.

Ara MT, Nomura T, Kato Y, Ogita S. 2020. A Versatile Liquid Culture Method to Control the <i>in Vitro</i> Development of Shoot and Root Apical Meristems of Bamboo Plants. *American Journal of Plant Sciences*, *11* (02). https://doi.org/10.4236/ajps.2020.112020.

Arya S, Satsangi R, Arya ID. 2008. Direct regeneration of shoots from immature inflorescences in Dendrocalamus asper (edible bamboo) leading to mass propagation. *The Journal of the American Bamboo Society*, *21* (1).

Banik A, Pandya P, Patel B, Rathod C, Dangar M. 2018. Characterization of halotolerant, pigmented, plant growth promoting bacteria of groundnut rhizosphere and its in-vitro evaluation of plant-microbe protocooperation to withstand salinity and metal stress. *Science of the Total Environment*, *630*. https://doi.org/10.1016/j.scitotenv.2018.02.227.

Bhardwaj DR, Rana S, Kumar D, Sharma P. 2023. Nutritive value of tender shoots of different bamboo species in relation to harvesting height in mid-hills of north-western Himalayas. *Applied Food Research*, *3* (1). https://doi.org/10.1016/j.afres.2022.100244.

Collinge DB, Gregersen PL, Thordal-Christensen H. 2021. The Induction of Gene Expression in Response to Pathogenic Microbes. In *Mechanisms of Plant Growth and Improved Productivity*. https://doi.org/10.1201/9781003210252-11.

Dey B, Verma A, Tewari S, Dubey A. 2020. Organogenesis optimization in Dendrocalamus asper. *Journal of Non Timber Forest Products*, *27* (2). https://doi.org/10.54207/bsmps2000-2020-zd4xvn.

Djami-Tchatchou AT, Li ZA, Stodghill P, Filiatrault MJ, Kunkel BN. 2022. Identification of Indole-3-Acetic Acid-Regulated Genes in Pseudomonas syringae pv. tomato Strain DC3000. *Journal of Bacteriology*, *204* (1). https://doi.org/10.1128/JB.00380-21.

Hiswan J, Larekeng SH, Gusmiaty W, Putri SD. 2020. In vitro shoot induction of batik bamboo using BAP and NAA. *Plant Archives*, *20* (2): 2343–2349.

Huang LC, Lee YL, Huang BL, Kuo CI, Shaw JF. 2002. High polyphenol oxidase activity and low titratable acidity in browning bamboo tissue culture. *In Vitro Cellular and Developmental Biology - Plant*, *38* (4). https://doi.org/10.1079/IVP2002298.

Hypochlorite S, Singh BK, Ali MN, Samanta S, Mandal N. 2021. A Comparative Analysis among Different Surface Sterilisation Methods for Rice Invitro

Culture. *International Journal of Plant & Soil Science*. https://doi.org/10.9734/ijpss/2021/v33i1730559.

Josephine U Agogbua, Bosa E Okoli. 2022. Procedure for in vitro seed sterilization, germination and aseptic seedling establishment of Zehneria capillacea (Schumach) C. jeffrey. *GSC Biological and Pharmaceutical Sciences*, *19* (1). https://doi.org/10.30574/gscbps.2022.19.1.0135

Khare SR, Kharate PS, Sahu RK, Jha Z. 2021. Rapid in-vitro micropropagation of Bamboo (*Dendrocalamus strictus*) and its genetic fidelity testing using ISSR markers. *Environment Conservation Journal, 22* (3): 69–77. https://doi.org/10.36953/ecj.2021.22308.

Lv Z, Zhu F, Jin D, Wu Y, Wang S. 2021. Seed Germination and Seedling Growth of Dendrocalumus brandisii in vitro, and the Inhibitory Mechanism of Colchicine. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.784581

Ngan HTM, Tung HT, Van Le B, Nhut DT. 2020. Abnormal symptoms in micropropagation and strategies to overcome. *Vietnam Journal of Biotechnology*, *18* (1): 23–39. https://doi.org/10.15625/1811-4989/18/1/15258.

Obsuwan K, Duangmanee A, Thepsithar C. 2019. In vitro propagation of a useful tropical bamboo, Thyrsostachys siamensis Gamble, through shoot-derived callus. *Horticulture Environment and Biotechnology*, *60* (2). https://doi.org/10.1007/s13580-018-00119-z

Ranjan A. 2021. Bamboo Tissue Culture - Commercial Applications and Environment. *Acta Scientific Agriculture*, *5* (10). https://doi.org/10.31080/asag.2021.05.1061

Sandhu M, Wani SH, Jiménez VM. 2018. In vitro propagation of bamboo species through axillary shoot proliferation: a review. In *Plant Cell, Tissue and Organ Culture, (Vol. 132, Issue 1)*. https://doi.org/10.1007/s11240-017-1325-1.

Sharothi P, Raju RI, Hossain MT. 2022. In vitro Propagation of an Ornamental Bamboo (Bambusa tuldoides Munro). *Plant Tissue Culture and Biotechnology*, *32* (2). https://doi.org/10.3329/ptcb.v32i2.63550.

Suwal MM, Lamichhane J, Gauchan DP. 2020. Regeneration Technique of Bamboo Species through Nodal Segments: A Review. *Nepal Journal of Biotechnology*, *8* (1). https://doi.org/10.3126/njb.v8i1.30209.

Venkatachalam P, Kalaiarasi K, Sreeramanan S. 2015. Influence of plant growth regulators (PGRs) and various additives on in vitro plant propagation of Bambusa arundinacea (Retz.) Wild: A recalcitrant bamboo species. *Journal of Genetic Engineering and Biotechnology*, *13* (2). https://doi.org/10.1016/j.jgeb.2015.09.006.

Yasodha R, Kamala S, Kumar SPA, Kumar PD, Kalaiarasi K. 2008. Effect of glucose on in vitro rooting of mature plants of Bambusa nutans. *Scientia Horticulturae*, *116* (1). https://doi.org/10.1016/j.scienta.2007.10.025.

Zang Q, Zhou L, Zhuge F, Yang H, Wang X, Lin X. 2016. Callus induction and regeneration via shoot tips of *Dendrocalamus hamiltonii*. *SpringerPlus*, *5* (1). https://doi.org/10.1186/s40064-016-3520-7

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Research Article

Phenolic Content, Antioxidant and Hepatoprotective Activities of Sabah *Hoya coronaria* Blume

Muhammad Dawood Shah[1], Senty Vun-Sang[2], Mohammad Iqbal[2*]

[1] Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Jalan UMS 88450, Sabah, Malaysia; dawoodshah@ums.edu.my
[2] Biotechnology Research Institute, Universiti Malaysia Sabah, Kota Kinabalu, Jalan UMS 88450, Sabah, Malaysia; sentyvunsang2707@gmail.com; miqbal@ums.edu.my

*Corresponding author: miqbal@ums.edu.my

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ABSTRACT

A common challenge that regularly results from oxidative stress is hepatic damage. This condition is characterised by a gradual progression from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. The study proposed to assess the antioxidant activity and efficacy of *Hoya coronaria* aqueous extract in preventing CCl_4-induced hepatic damage in rats. The DPPH technique was used in the study to assess the extract's antioxidant properties. The rats received dosages of 125 and 250 mg/kg body weight of *H. coronaria* extract for 14 days, followed by CCl_4 exposure. After two weeks, the rats were euthanised for analysis. The results indicated that the extract showed significant antioxidant potential and decreased the impact of CCl_4 on hepatic damage markers such as serum aspartate transaminase and alanine transaminase. Moreover, it increased hepatic reduced glutathione and various antioxidant enzymes while reducing malondialdehyde formation induced by CCl_4. Additionally, the histopathological analysis demonstrated that *H. coronaria* extract protected the liver against fatty degeneration and necrosis induced by CCl_4 toxicity. These outcomes suggest that *H. coronaria* extract could be used to prevent ROS-related hepatic damage.

Keywords: Antioxidant enzymes; Hepatoprotection; *Hoya coronaria*; Medicinal plants; oxidative stress

Intoduction

Liver injury is a common condition that, in the majority of cases, is caused by oxidative stress and is defined by a gradual development from steatosis to chronic hepatitis, fibrosis, cirrhosis, and cancer (Kodavanti et al., 1989). Many factors can cause liver damage, such as pollutants, radiation, chemicals, alcohol, medicines, malnutrition, and infection (Meng et al., 2020). Around 2 million people die from liver diseases each year around the globe, including one million from cirrhosis complications and one million from viral hepatitis and hepatocellular cancer, and developing countries carry the highest burden (Cainelli, 2012; Asrani et al., 2019). Many currently available drugs used for liver disease treatments suffer various limitations, such as side effects, low bioavailability, stability, and selectivity. It is vital to look for novel natural medications with the best possible effectiveness, stability, selectivity, and safety (Singh et al., 2016; Ma et al., 2019; Alnuqaydan et al., 2022).

Medicinal plants attract attention in being introduced in therapeutics for treating liver damage due to various antioxidant compounds (Shah et al., 2015a; 2017; Singh et al., 2016; Venmathi Maran et al., 2022). The natural antioxidant compounds modulate oxidative stress by reducing lipid peroxidation and elevating antioxidant enzymes (Singh et al., 2016; Alnuqaydan et al., 2022; Venmathi Maran et al., 2022). *Hoya coronaria* is a medicinal plant and belongs to the family Asclepiadaceae. It is a beautiful evergreen plant with a star-shaped large flower arising from its green stem. The plant is found in the moist habitats of Malaysia, Indonesia, the Philippines and other Southeast Asian countries (Milow et al., 2017; Rahayu & Fakhurrozi, 2020). In Malay, it is known as 'Akar Setebal' (MyBIS, 2023), while in Kadazan/Dusun language (Sabah Native), it is known as 'Wida' (Kulip, 1997). The leaves of the plants are crushed and applied for the treatment of wounds and cuts by the local people, the Orang Asli, in Kampung Bawong, Perak, West Malaysia (Samuel et al., 2010) and for pancreatitis treatment by the local people in Sabah, East Malaysia (Kulip, 1997). However, no scientific investigations have been conducted on the antioxidant activity and hepatoprotective properties of *H. coronaria*.

This study aims to evaluate the potential of *H. coronaria* as a hepatoprotective agent by investigating its antioxidant activity and chemopreventive effects against CCl_4-induced oxidative stress and hepatic dysfunction in rats.

Materials and Methods

Chemicals

Oxidizied glutathione, thiobarbituric acid, tricholoacetic acid, dithionitrobenzoic acid, 1-chloro 2,4 dinitrobenzene, hydrogen peroxide, folin-Ciocalteu reagent (FCR), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium nitrate ($NaNO_3$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), 2, 2-diphenyl-2-picrylhadrazyl (DPPH), gallic acid, catechol, and ferric chloride (FeCl3) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alcohol, acid alcohol, blue buffer, eosin, haema-toxylin, xylene and DPX mounting medium for histological assessment were purchased from Leica Biosystem (Wetzlar, Germany).

Plant Leaves Preparation

H. coronaria leaves were harvested from the Papar district (5.48943°N, 115.80992°E) of Sabah, Malaysia. Institute for Tropical Biology and Conservation (IBTP), Universiti Malaysia Sabah experts identified the collected leaves. After cleaning with distilled water, the leaves were dried in an oven at 37°C and ground into powder. To prepare the extract, 100 g of the powder was boiled in distilled water (1:10 ratio) on a stirring hot plate for 10 minutes. After cooling for an hour at room temperature, the decoctions were filtered with a strainer to remove significant residues and then filtered once more with Whatman No. 1 filter paper. The resulting filtrate was freeze-dried and stored in a tube at -80°C for further investigation, as Venmathi Maran et al., (2021) described.

Total Phenolic Content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu method. In this method, 200 ml of plant extract was mixed with 1.5 ml of Folin-Ciocalteu's reagent (1:10) and left at room temperature for 5 minutes. Next, 1.5 ml of sodium carbonate (60 g/l) was added, and the mixture was incubated in the dark at room temperature for 90 minutes. The absorbance at 725 nm was measured against a blank using a spectrophotometer. Gallic acid was used as a standard to determine the phenol content, expressed as gallic acid equivalents (GAE) per gram of material. The test was repeated thrice, and the obtained absorbance was averaged (Velioglu et al., 1998).

2, 2-Diphenyl-2-picrylhydrazyl (DPPH) Assay

The antioxidant activity of the extract was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Hatano et al., (1988) method. The extract was dissolved in dimethyl sulfoxide (DMSO) at a 5 mg/ml concentration and combined with DPPH radicals (6×10^{-5} mol/L) in an ethanol solution. The experiment was carried out in triplicate, with ascorbic acid as the control. The following formula was used to compute the percentage of radical scavenging activity: % RSA = [(Control absorbance – Sample absorbance)/Control absorbance] × 100.

Experimental Protocol

The animal experiment was treated ethically following the university's standards and federal legislation for animal experimentation (Animal Ethics Committee (AEC): UMS/IP7.5/M3/4-2012). Male Sprague-Dawley rats weighing 120-150 g and aged 8-12 weeks were obtained from the Animal House Health Campus at Universiti Sains Malaysia (USM). Animals were acclimatised for one week in an animal room on a 12-hour light-dark cycle and had ad libitum access to food and water. CCl_4 was mixed with corn oil (1:1) to prepare a 1.2 ml/kg body weight dose. In comparison, the *H. coronaria* extract was suspended in distilled water and administered to the rats through gastric gavage needles at 125 and 250 mg/kg body weight dosages. Twenty-four adult male rats were randomly distributed into four groups of six rats each: The first group was given saline; the second group was given CCl_4 (1.2 ml/kg b.w.); the third group was given *H. coronaria* (125 mg/kg b.w.) with CCl_4 (1.2 ml/kg b.w.); and the fourth group was given *H. coronaria* (250 mg/kg b.w.) with CCl_4 (1.2 ml/kg b.w.). CCl_4 treatment was given on the 13th and 14th days of the experimental period.

All of the rats were sacrificed 24 hours after the last CCl_4 treatment. Blood was taken through cardiac puncture with sterile disposable needles, and serum was recovered by centrifuging at 2000xg for 15 minutes. The animals' livers were quickly removed and cleansed with cold saline (0.85% w/v) to remove any unwanted debris. A small percentage of the liver tissues were maintained in a 10% neutral buffered formalin solution for histopathological studies, while the rest of the tissues were stored at -80°C for biochemical study.

Determination of Serum Biochemistry

The enzyme activities of serum transaminases (ALT and AST) were investigated using the Reflotron1 technology.

Preparation of Post-Mitochondrial Supernatant (PMS) from Liver

Sample livers (10% w/v) were homogenised in phosphate buffer (0.1 M, pH 7.4) and centrifuged at 3000 rpm for 20 minutes at 4°C to remove debris from the nuclei. The resulting supernatant was centrifuged at 10,000 rpm for 30 minutes at 4°C, and the resulting PMS was utilised to measure various biochemical parameters.

Biochemicals Assay

Lipid peroxidation in the liver was done following the method of Buege and Aust by measuring the rate production of thiobarbituric acid reactive substances (TBARS) and expressed as MDA equivalents (Buege & Aust, 1978). Reduced GSH was determined according to the method of Jollow *et al.* (1974). Glutathione peroxidase (GPx) activity was observed according to the procedure of Mohandas et al., (1984). Catalase (CAT) activity was conducted using the method of Claiborne (1985). Glutathione reductase (GR) activity was performed following the method of Carlberg and Mannervik (1975). Glutathione S-transferase (GST) activity using CDNB as a substrate was estimated following the method of Habiq et al., (1974), while quinone reductase (QR) activity was assessed following the method of Benson et al., (1980) as modified by Iqbal et al., (1999).

Histopathological Examination

Liver tissues were fixed in a 10% phosphate-buffered formaldehyde solution, embedded in paraffin wax, and sliced into 4 µm sections. The sections were stained with haematoxylin and eosin (H&E) and mounted on permanent slides. Photomicrographs were taken while inspecting the slides under a microscope equipped with photography tools.

Determination of Protein

Bicinchoninic acid and a protein assay kit (BCA1) were used to measure the total protein level, with bovine serum albumin as the reference.

Statistical Analysis

The mean ± SD was used to express the results, and statistical comparisons were performed using ANOVA (one-way analysis of variance). Levene's test was used to determine variance homogeneity, and data analysis was carried out using the computer program SPSS (Release 17.0, SPSS). A significant difference was considered when the p-value was less than 0.05.

Results

Total Phenolic

The total phenolic content of *H. coronaria* aqueous extract was 78.75±2.28 mg/g, expressed in gallic acid equivalent (GAE)/mg of extract.

Effect of H. coronaria on DPPH Radical Scavenging

Figure 1 illustrates the concentration-dependent increase in the DPPH scavenging capacity of the *H. coronaria* extract, ranging from 10 to 2400 µg/ml. The extract's EC_{50} value was calculated to be 2240 µg/mL.

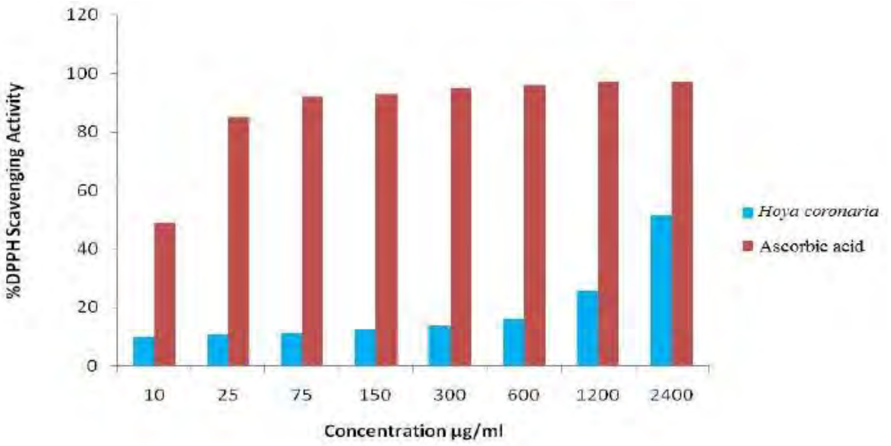

Figure 1. Free radical protection activity (DPPH) of *H. coronaria* at different concentrations. Each bar represents the mean ± SD (n=3).

Effects of H. coronaria on Lipid Peroxidation and GSH

Table 1 shows the impact of *H. coronaria* on malondialdehyde (MDA) and GSH. The level of hepatic MDA in CCl_4-intoxicated rats increased significantly ($p>0.05$) by 56.3% compared to the control group. However, pre-treatment with *H. coronaria* extract ($p < 0.05$) reduced liver MDA levels significantly by 19.3% and 30.6% in a dose-dependent manner. In addition, administration of CCl_4 alone significantly ($p < 0.05$) reduced GSH levels in the liver by 64.6%. The pre-treatment of *H. coronaria* extract caused a significant ($p<0.05$) increase in the level of hepatic GSH by 42.2% and 98.1% in a dose-dependent manner.

Table 1. Effect of *H. coronaria* on CCl_4-induced changes in hepatic lipid peroxidation and reduced glutathione.

Groups	LPO (nmol/g tissue)	GSH (µmol/g tissue)
Control	14.90 ± 0.6	3.08 ± 0.18
CCl_4	23.32 ± 0.8*	1.09 ± 0.05*
H. coronaria 125 mg + CCl_4	18.80 ± 0.4**	1.55 ± 0.09**
H. coronaria 250 mg + CCl_4	16.15 ± 0.4**	2.16 ± 0.06**

Each value represents the mean ± SD of six animals (n = 6).
* Indicate a significant difference from the control group ($p < 0.05$).
** Indicate significant difference from CCl_4 alone treated group ($p < 0.05$).

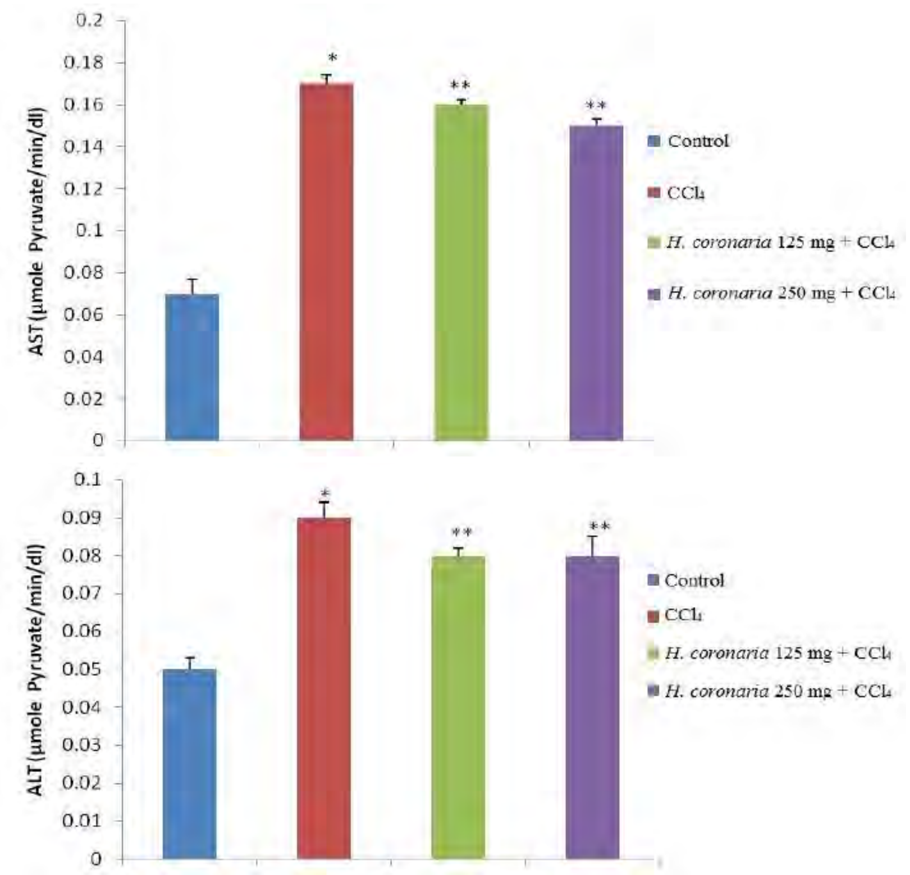

Figure 2. Hepatoprotective effect of *H. coronaria* on CCl_4-induced hepatotoxicity: serum transaminases. Each bar represents the mean ± SD of six animals (n = 6).
* Indicate a significant difference from the control group ($p < 0.05$).
**Indicate significant difference from CCl_4 alone treated group ($p < 0.05$).

Effects of H. coronaria on CCl_4-Induced Hepatotoxicity

The levels of serum transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) significantly ($p < 0.05$) increased in the CCl_4-treated group compared to the control group. However, pre-treatment with an extract of *H. coronaria* significantly ($p > 0.05$) reduced the elevation of serum transaminases, indicating its potential to inhibit the elevated levels of these enzymes in rats intoxicated with CCl_4 (Figure 2).

Effects of H. coronaria on Antioxidant Enzymes

Table 2 presents the effects of *H. coronaria* extract on antioxidant enzyme activities. The activities of CAT, GPx, GR, QR, and GST were significantly reduced by exposure to CCl_4 (37%, 31%, 43%, 75%, and 42%, respectively). However, pre-treatment with *H. coronaria* extract dose-dependently increased the activities of these enzymes and resulted in significant elevation ($p< 0.05$) of CAT (12% and 41%), GPx (7% and 24%), GR (22% and 47%), QR (47% and 88%), and GST (13% and 55%).

Table 2. Hepatoprotective effects of *H. coronaria* on the hepatic antioxidant profile of rats intoxicated with CCl_4.

Groups	CAT	GPx	GR	QR	GST
Control	328.61 ± 13.1	34.77 ± 1.9	35.09 ± 0.24	8.59 ± 0.1	35.09 ± 0.66
CCl_4	207.34 ± 16.9*	23.92 ± 0.6*	20.32 ± 0.21*	2.07 ± 0.1 *	20.32 ± 0.55*
H. Coronaria 125 mg + CCl_4	231.17 ± 13.7*	25.52 ± 0.5**	22.98 ± 0.23*	3.60 ± 0.2**	22.98 ± 0.54**
H. Coronaria 250 mg + CCl_4	292.97 ± 9.1**	29.67 ± 0.6**	31.67 ± 0.38*	8.04 ± 0.3**	31.67 ± 0.44**

The antioxidant enzyme activities are presented in Table 2 as follows: catalase (CAT) (nmol H_2O_2 consumed/min/mg protein), glutathione peroxidase (GPx) (nmol NADPH oxidized/min/mg protein), glutathione reductase (GR) (nmol NADPH oxidized/min/mg protein), quinone reductase (QR) (nmol dichloroindophenol reduced/min/mg protein), and glutathione-S-transferase (GST) (nmol CDNB conjugate formed/min/mg protein). Each bar represents the mean ± SD of six animals ($n = 6$).

* Indicate a significant difference from the control group ($p < 0.05$).

** Indicate significant difference from CCl_4 alone treated group ($p < 0.05$).

Effects of H. coronaria on Histopathology

The CCl_4-treated rats exhibited histological changes such as increased necrosis, blood vessel congestion, fatty degeneration and derangement of hepatocytes, as shown in Figure 3. However, the administration of aqueous extracts of *H. coronaria* significantly improved these changes, indicating that *H. coronaria* exposure reduced the hepatic injury induced by CCl_4.

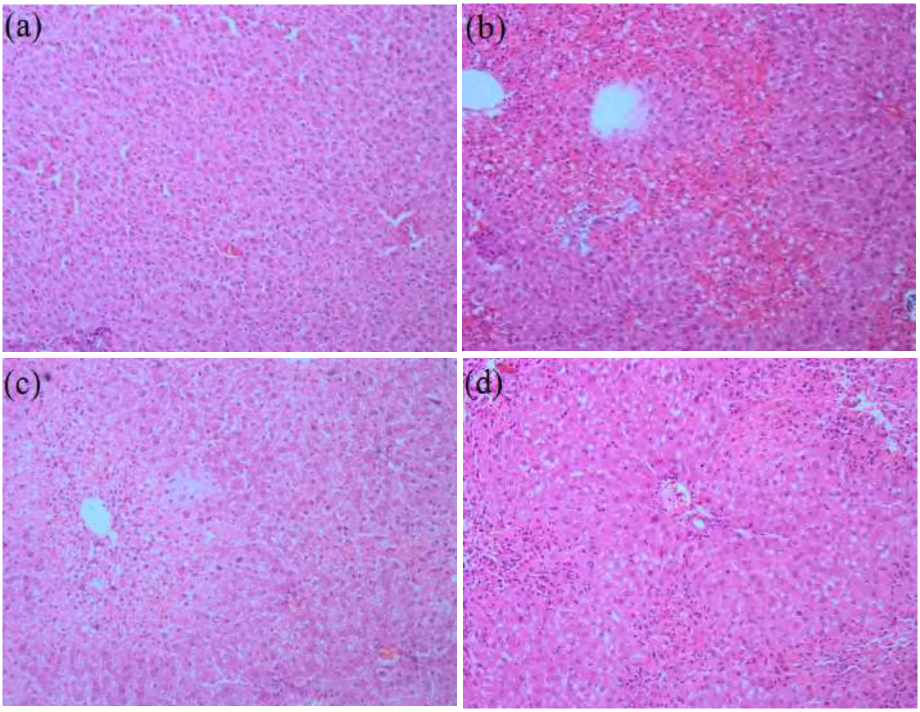

Figure 3. Haematoxylin and eosin (H&E) photomicrographs of histopathological changes in rat livers. The images show (a) a control liver, (b) CCl_4 intoxicated, (c) *H. coronaria* (125 mg/kg b.w.+ CCl_4) and (d) *H. coronaria* (250 mg/kg b.w. + CCl_4). All images are shown at a magnification of X20.

Discussion

The findings of this study suggest that the water extracts derived from *H. coronaria* leaves can protect the liver against CCl_4-induced damage in rats by regulating oxidative stress. Our studies have revealed that *H. coronaria* extract exhibits significant antioxidant activity, as demonstrated by its ability to scavenge the stable, accessible radical DPPH dose-dependent manner. The extract contains 78.75 ± 2.28 mg/g of total phenolics expressed as gallic acid equivalent (GAE, mg/g of extract), suggesting that phenolics are essential components of the extract. Literature suggests that the antioxidant activity of plant extracts is often attributed to their phenolic content, which can act as free radical scavengers, hydrogen donors, singlet oxygen quenchers, and metal ion chelators (Dillard & German, 2000; Yao et al., 2010; Hossain & Nagooru, 2011; Aryal et al., 2019).

The liver is responsible for detoxifying the body, and damage from toxic chemicals can lead to increased levels of serum transaminases, enzymes that are released into the bloodstream. Serum transaminases are commonly utilised as indicators of liver disease due to their sensitivity and reliability (Li et al., 2015; Vun-Sang et al., 2022). Consistent with previous studies, the activities of serum ALT and AST were elevated in the CCl_4 intoxicated group (Group 2) compared to the normal saline-treated group (Group 1) (Li et al., 2015; Vun-Sang et al., 2022). Following CCl_4 administration, liver damage can lead to increased membrane permeability and changes in transport function, resulting in the release of cytoplasmic hepatic enzymes like ALT and AST into the bloodstream (Li et al., 2015; Vun-Sang et al., 2022). In groups 3 and 4, where rats were pre-treated with *H. coronaria* extract and then treated with CCl_4, the activities of ALT and AST were dose-dependently reduced compared to group 2, suggesting the potential of the extract in mitigating liver damage. This effect could be attributed to the plant's anti-inflammatory and antioxidant properties (Singh et al., 2016) and possible hepatic damage repair (Vun-Sang et al., 2022).

The P450 system biotransforms CCl_4, specifically cytochromes CYP2E1, CYP2B2, and perhaps CYP3A, to generate the highly reactive metabolites CCl3• and CCl3OO• (Recknagel et al., 1989; Weber et al., 2003). These metabolites attack polyunsaturated fatty acids, leading to lipid peroxidation and the production of malondialdehyde (MDA) in liver cells, which is a significant factor in the development of diseases (Ohkawa et al., 1979; Vaca et al., 1988; Recknagel et al., 1989; Weber et al., 2003). Our results indicate that the levels of MDA produced in the CCl_4 intoxicated group (Group 2) were higher than the normal group (Group 1). However, treatment with *H. coronaria* extract at doses of 125 and 250 mg/kg body weight in groups 3 and 4, respectively, led to a reduction in MDA levels compared to group 2, indicating the potential of the extract to protect cell membranes from free radicals. The extract was found to reduce lipid peroxidation in a dose-dependent manner, consistent with previous studies (Shah et al., 2015b; Gnanaraj et al., 2016; Amzar & Iqbal, 2017).

Treatment with CCl_4 reduced the level of reduced glutathione (GSH), which is a crucial non-enzymatic antioxidant in the body that protects against oxidative damage. This finding is consistent with previous studies (Shah et al., 2015b; Gnanaraj et al., 2016; Amzar & Iqbal, 2017), which suggest that the decline in GSH level may be attributed to increased utilisation of cells to scavenge the free radicals caused by CCl_4. However, we observed that *H. coronaria* extract could significantly increase the levels of GSH in the liver, possibly by scavenging reactive oxygen species and preventing oxidative damage.

Antioxidant enzymes, including glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and quinone reductase (QR), are vital for safeguarding cells against oxidative damage (Li et al., 2015; Vun-Sang et al., 2022) CCl_4 metabolism into trimethyl free radicals through cytochrome P450 leads to increased oxidative stress (Recknagel et al., 1989; Weber et al., 2003), resulting in a reduction in the activity of antioxidant enzymes in the CCl_4-intoxicated group compared to the normal saline-treated group 1. Our research demonstrates that the *H. coronaria* extract can increase the activity of various antioxidant enzymes, including GR, CAT, GPx, GST, and QR, relative to the CCl_4-intoxicated group 2. This improvement in antioxidant enzyme activity is most likely due to the extract's ability to scavenge reactive oxygen species and protect against oxidative damage, as also seen in previous studies (Shah et al., 2015b; Gnanaraj et al., 2016; 2017; Amzar & Iqbal, 2017).

Histopathological studies involve examining morphological changes in liver tissue under microscopic examination to assess the effect of *H. coronaria* extract. In rats treated with CCl_4, the liver section exhibited sinusoidal dilation, inflammatory infiltration, necrosis of hepatocytes, and steatosis (fatty acid formation). Most of these alterations were significantly decreased when animals were pre-treated with *H. coronaria* extract. The histopathological findings confirmed the biochemical results and indicated that *H. coronaria* extract prevented liver cell necrosis, fatty infiltration, and fibrosis, all of which are markers of liver damage. These histopathological changes are consistent with previous studies that have demonstrated the protective effects of phytochemicals in plant extracts against liver damage caused by CCl_4 (Shah et al., 2015b; Gnanaraj et al., 2016; 2017; Amzar & Iqbal, 2017).

Conclusions

According to the study, *H. coronaria* aqueous extract exhibited significant antioxidant activity and protection against CCl_4-induced hepatic damage in rats. The extract improved hepatic GSH and other antioxidant enzyme levels. The histopathological study showed that the liver was protected by *H. coronaria* extract from fatty degeneration and necrosis induced by CCl_4 toxicity. The results suggest that *H. coronaria* extract may be helpful in minimising liver damage caused by ROS. More study is required to examine the extract's safety and effectiveness in humans and identify the potential bioactive compounds responsible for the extract's protective effects.

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Conflict of interest statement

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

References

Alnuqaydan AM, Almutary AG, A Alsahli M, Alnasser S, Rah B. 2022. *Tamarix articulata* induced prevention of hepatotoxicity effects of in vivo carbon tetrachloride by modulating pro-inflammatory serum and antioxidant enzymes to reverse the liver fibrosis. *Antioxidants (Basel, Switzerland)*, *11* (9): 1824. DOI: 10.3390/antiox11091824.

Amzar N, Iqbal M. 2017. The hepatoprotective effect of *Clidemia hirta* against carbon tetrachloride (CCl_4)-induced oxidative stress and hepatic damage in mice. *Journal of Environmental Pathology, Toxicology and Oncology*, *36* (4): 293–307. DOI: 10.1615/JEnvironPatholToxicolOncol.2017019824.

Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. 2019. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from western Nepal. *Plants (Basel,Switzerland)*, *8* (4): 96. DOI: 10.3390/plants8040096.

Asrani SK, Devarbhavi H, Eaton J, Kamath PS. 2019. Burden of liver diseases in the world. *Journal of Hepatology*, *70* (1): 151–171. DOI: 10.1016/j.jhep.2018.09.014.

Benson AM, Hunkeler MJ, Talalay P. 1980. Increase of NAD(P)H:quinone reductase by dietary antioxidants: Possible role in protection against carcinogenesis and toxicity. *Procedings of the National Academy of Sciences of the United States of America*, *77* (9): 5216–5220. DOI:10.1073/pnas.77.9.5216.

Buege JA, Aust SD. 1978. Microsomal lipid peroxidation. *Methods in Enzymology*, *52*: 302–310. DOI: 10.1016/s0076-6879(78)52032-6.

Cainelli F. 2012. Liver diseases in developing countries. *World Journal of Hepatology*, *4* (3): 66–67. DOI: 10.4254/wjh.v4.i3.66.

Carlberg I, Mannervik B. 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *The Journal of Biological Chemistry*, *250* (14): 5475–5480. DOI: 10.1016/S0021-9258(19)41206-4.

Claiborne A. 1985. Catalase activity. In: Greenwald, R.A., Ed., CRC *Handbook of Methods for Oxygen Radical Research.* Boca Raton: CRC Press, 283–284.

Dillard CJ, German JB. 2000. Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture*, *80* (12): 1744–1756. DOI: 10.1002/1097-0010(20000915)80:12%3C1744::AID-JSFA725%3E3.0.CO;2-W.

Gnanaraj C, Shah MD, Haque ATME, Makki JS, Iqbal M. 2016. Hepatoprotective and immunosuppressive effect of *Synedrella nodiflora* L. on carbon tetrachloride (CCl_4)-intoxicated rats. *Journal of Environmental Pathology, Toxicology and Oncology*, *35* (1): 29–42. DOI: 10.1615/JEnvironPatholToxicolOncol.2016013802.

Gnanaraj C, Shah MD, Song TT, Iqbal M. 2017. Hepatoprotective mechanism of *Lygodium microphyllum* (Cav.) R.Br. through ultrastructural signaling prevention against carbon tetrachloride (CCl_4)-mediated oxidative stress. *Biomedicine & Pharmacotherapy*, *92:* 1010–1022. DOI: 10.1016/j.biopha.2017.06.014.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249* (22): 7130–7139. DOI: 10.1016/S0021-9258(19)42083-8.

Hatano T, Kagawa H, Yasuhara T, Okuda T. 1988. Two new flavonoids and other constituents in licorice root. Their relative astringency and radical scavenging effects. *Chemical & Pharmceutical Bulletin (Tokyo)*, *36* (6): 2090–2097. DOI:10.1248/cpb.36.2090.

Hossain MA, Nagooru MR. 2011. Biochemical profiling and total flavonoids contents of leaves crude extract of endemic medicinal plant *Corydyline terminalis* L. Kunth. *Pharmacognosy Journal*, *3* (24): 25–30. DOI: 10.5530/pj.2011.24.5.

Iqbal M, Sharma SD, Rahman A, Trikha P, Athar M. 1999. Evidence that ferric nitrilotriacetate mediates oxidative stress by down-regulating DT-diaphorase activity: implications for carcinogenesis. *Cancer Letters*, *141* (1-2): 151–157. DOI: 10.1016/s0304-3835(99)00100-7.

Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology*, *11* (3): 151–169. DOI: 10.1159/000136485.

Kodavanti PR, Joshi UM, Young RA, Meydrech EF, Mehendale HM. 1989. Protection of hepatotoxic and lethal effects of CCl_4 by partial hepatectomy. *Toxicologic Pathology*, *17* (3): 494–505. DOI: 10.1177/019262338901700304.

Kulip J. 1997. A Preliminary survey of traditional medicinal plants in the west coast and interior of Sabah. *Journal of Tropical Forest Science*, *10* (2): 271–274.

Li S, Tan HY, Wang N, Zhang ZJ, Lao L, Wong CW, Feng Y. 2015. The role of oxidative stress and antioxidants in liver diseases. *International Journal of Molecular Sciences*, *16* (11): 26087–26124. DOI: 10.3390/ijms161125942.

Ma Z, Zhang B, Fan Y, Wang M, Kebebe D, Li J, Liu Z. 2019. Traditional Chinese medicine combined with hepatic targeted drug delivery systems: A new strategy for the treatment of liver diseases. *Biomedicine & Pharmacotherapy*, *117:* 109128. DOI: 10.1016/j.biopha.2019.109128.

Meng X, Tang GY, Liu PH, Zhao CJ, Liu Q, Li HB. 2020. Antioxidant activity and hepatoprotective effect of 10 medicinal herbs on CCl_4-induced liver injury in mice. *World Journal of Gastroenterology*, *26* (37): 5629–5645. DOI:10.3748/wjg.v26.i37.5629.

Milow P, Malek S, Ramli RM. 2017. Medicinal plants of the indigenous tribes in Peninsular Malaysia: Current and future perspectives. Active Ingredients from Aromatic and Medicinal Plants. *Rijeka: IntechOpen*. DOI: 10.5772/66658.

Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. *Biochemical Pharmacology*, *33* (11): 1801–1807. DOI: 10.1016/0006-2952(84)90353-8.

MyBIS. 2023. Malaysia Biodiversity Information System. Published on the Internet https://www.mybis.gov.my/, Ministry of Natural Resources, Environment and Climate Change, Malaysia Biodiversity Centre & Forest Research Institute Malaysia. [Retrieved 12 May 2023].

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95* (2): 351–358. DOI: 10.1016/0003-2697(79)90738-3.

Rahayu S, Fakhurrozi Y. 2020. High corolla color variation of *Hoya coronaria* Blume in Belitung Island: Potential use and conservation. *In Proceedings of the IOP Conference Series: Earth and Environmental Science*, *591:* 012043. DOI 10.1088/1755-1315/591/1/012043.

Recknagel RO, Glende EA, Dolak JA, Waller RL. 1989. Mechanisms of carbon tetrachloride toxicity. *Pharmacology & Theraputics*, *43* (1): 139–154. DOI: 10.1016/0163-7258(89)90050-8.

Samuel AJSJ, Kalusalingam A, Chellappan DK, Gopinath R, Radhamani S, Husain HA, Muruganandham V, Promwichit P. 2010. Ethnomedical survey of plants used by the Orang Asli in Kampung Bawong, Perak, West Malaysia. *Journal of Ethnobiology and Ethnomedicine*, *6:* 5. DOI: 10.1186/1746-4269-6-5.

Shah MD, Gnanaraj C, Khan MS, Iqbal M. 2015a. *Dillenia suffruticosa* L. impedes carbon tetrachloride–induced hepatic damage by modulating oxidative stress and inflammatory markers in rats. *Journal of Environmental Pathology, Toxicology and Oncology*, *34* (2): 133–152. DOI: 10.1615/jenvironpatholtoxicoloncol.2015013306.

Shah MD, Gnanaraj C, Haque ATME, Iqbal M. 2015b. Antioxidative and chemopreventive effects of *Nephrolepis biserrata* against carbon tetrachloride (CCl_4)-induced oxidative stress and hepatic dysfunction in rats. *Pharmceutical Biology*, *53* (1): 31–39. DOI: 10.3109/13880209.2014.909502.

Shah MD, D'Souza UJA, Iqbal M. 2017. The potential protective effect of *Commelina nudiflora* L. against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats, mediated by suppression of oxidative stress and inflammation. *Environmental Health and Preventive Medicine*, *22* (1): 66. DOI: 10.1186/s12199-017-0673-0.

Singh D, Cho WC, Upadhyay G. 2016. Drug-induced liver toxicity and prevention by herbal antioxidants: An overview. *Frontiers in Physiology*, *6:* 363. DOI:10.3389/fphys.2015.00363.

Vaca CE, Wilhelm J, Harms-Ringdahl M. 1988. Interaction of lipid peroxidation products with DNA. A review. *Mutation Research*, *195* (2): 137–149. DOI: 10.1016/0165-1110(88)90022-x.

Velioglu YS, Mazza G, Gao L. Oomah BD. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, *46* (10): 4113–4117. DOI: 10.1021/jf9801973.

Venmathi Maran BA, Josmeh D, Tan JK, Yong YS, Shah MD. 2021. Efficacy of the aqueous extract of *Azadirachta indica* against the marine parasitic leech and its phytochemical profiling. *Molecules*, *26* (7): 1908. DOI: 10.3390/molecules26071908.

Venmathi Maran BA, Iqbal M, Gangadaran P, Ahn BC, Rao PV, Shah MD. 2022. Hepatoprotective potential of Malaysian medicinal plants: A review on phytochemicals, oxidative Stress, and antioxidant mechanisms. *Molecules*, *27* (5): 1533. DOI: 10.3390/molecules27051533.

Vun-Sang S, Rodrigues KF, Dsouza UJA, Iqbal M. 2022. Suppression of oxidative stress and proinflammatory cytokines is a potential therapeutic action of *Ficus lepicarpa* B. (Moraceae) against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. *Molecules*, *27* (8): 2593. DOI: 10.3390/molecules27082593.

Weber LWD, Boll M, Stampfl A. 2003. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Critical Reviews in Toxicology*, *33* (2): 105–136. DOI: 10.1080/713611034.

Yao Y, Sang W, Zhou M, Ren G. 2010. Phenolic composition and antioxidant activities of 11 celery cultivars. *Journal of Food Science*, *75* (1): C9–13. DOI:10.1111/j.1750-3841.2009.01392.x.

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Research Article

Empty Forest Syndrome: Are we there yet? A preliminary Analysis of the Hunting and Poaching Activities in Selected Areas in Sabah, Malaysia.

Fiffy Hanisdah Saikim[1*], Nurin Shahira Mohd. Yunus[2], Azniza Mahyudin[3], Senthilvel K.S.S. Nathan[4], Hussien Muin[4], Arnie Hamid[1], Nor Akmar Abdul Aziz[5], Nordiana Mohd. Nordin[6], Norhuda Salleh[7], Maximus Livon Lo Ka Fu[1], Julia George Kunai[1], Petherine Anak Jimbau[1], Mohd. Afifi Mohd. Nasir[1], Cornelius Peter[1], Julianah Awang @ Joseph[1], Mahadimenakbar Mohamed Dawood[1]

[1]*Institute for Tropical Biology & Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[2]*Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[3]*Faculty of Tropical Forestry, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*
[4]*Sabah Wildlife Department, Ibu Pejabat Tingkat 5, Blok B, Wisma MUIS, 88100, Kota Kinabalu, Sabah, Malaysia.*
[5]*Department of Recreation and Ecotourism, Faculty of Forestry and Environment, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.*
[6]*Faculty of Information Management, UiTM Cawangan Selangor Kampus Puncak Perdana, Seksyen U10, 40150, Shah Alam, Selangor, Malaysia.*
[7]*Faculty of Social Sciences and Humanities, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.*

*Corresponding author: fiffy@ums.edu.my

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ABSTRACT

Hunting poses a longstanding threat to tropical wildlife conservation, now reaching critical levels. In Borneo, Malaysia, around six million animals were hunted yearly in the 1990s, about 36 per sq. km. High demand for game meat, facilitated by improved transportation, drives unsustainable consumption across the tropics. Depletion of animal populations leads hunters to new areas. Roughly 18% of rainforests are protected, yet pressures persist, endangering species due to weak regulations, limited resources, and external threats. This study examines wildlife hunting trends in Sabah, uncovering insights from community interviews regarding hunting impact and resource dependence. A total of 45 people were interviewed in six study areas: Sandakan, Tawau, Kota Belud, Tambunan, Keningau and Tenom. It was found that local communities hunt for food and that hunting is a common way of life for them. Based on the arrests of hunters by the Wildlife Rescue Unit, a report on animal hunting was compiled by the Sabah Wildlife Department and analysed. The most commonly hunted animal is the bearded pig. Parts of bearded pigs were found in the cars of 76.97% of hunters arrested, followed by parts of sambar deer (13.7%). Throughout Sabah, the empty forest syndrome (EFS) is becoming a dangerous silent disaster. While most "simple" forest features are attractive to the eye, they are gradually losing their ecological function. Although this study is not exhaustive, the preliminary findings point to a serious EFS scenario in Sabah if everyone continues to neglect the syndrome and do nothing about it.

Keywords: Empty Forest Syndrome (EFS); Hunting; Poaching; Community; Bush Meat; Sabah; Borneo

Introduction

Hunting behaviour is more strongly related to the current number of species in tropical forests than forest type, habitat size or conservation status (Woodroffe and Ginsberg, 1998; Peres, 2009). For example, tiny patches of forest near fishing settlements in Borneo may have abundant wildlife, while large, isolated protected areas may have declining numbers due to over-exploitation by local people (Bennett et al., 2000; McConkey and Chivers, 2004). Peres and Palacios (2007) observed that untamed populations in the Amazon Basin indicate the availability of an area to trackers rather than its conservation status, although Brashares et al. (2004) found that the extent of bushmeat hunting in West Africa depends largely on the accessibility of selected protein sources. At the point when the respective regulators had a viable interest in advancing non-hunting or hunting governance in their concessions, both logging (Clark et al., 2009; Berry et al., 2010) and oil palm (Laurance et al., 2008) concessions became important conservation areas for wildlife.

There is no doubt that the authorities of protected areas do not tolerate violations and that eradications occasionally occur in protected areas. It is therefore difficult to get a clear picture of how wildlife is faring in most protected areas. However, most tropical protected areas can be classified as empty forests, based on hunting literature. From southern China (Fellowes et al., 2004) to Laos (Nooren & Claridge, 2001), Myanmar (Rao et al., 2010), Cambodia (Loucks et al., 2009), Thailand (Brodie et al., 2009), Malaysia (Bennett et al., 2000; Wong, 2012; Kurz et al., 2021) and Indonesia (Corlett, 2007), there have been recent widespread decline in vertebrate populations in protected areas throughout Southeast Asia (Lee, 2000). Other tropical populations (Dunham et al., 2008; Golden, 2009), West and Central Africa (Fa & Brown, 2009), the Brazilian Atlantic Forest (Galetti et al., 2009) and Oceania appear to be in a similar situation (McConkey & Drake, 2006).

The inaccessibility of forests in parts of Amazonia, Congo and New Guinea certainly provides some protection for wildlife (e.g. Peres, 2009). However, given the current extent of mining, wildlife populations in these regions are expected to decline as contact expands (Levi et al., 2009). In Sabah, for example, accessibility has become another dilemma between people and wildlife. In 2017, there was an outcry among conservationists over the RM79 million Kinabatangan Bridge proposed by a member of the legislative assembly to provide access to about 2,000 people from Sukau to Litang Tomanggong (Chan, 2017). The project would also require the paving of an unpaved road, which would lead to more traffic in the area. The situation would endanger wildlife in Kinabatangan as traffic would increase. The project has been stopped, but it may only be a matter of time before it is revived under a different guise.

Only about 35%, 9% and 1% of sites in the Neotropical, Afrotropical and Indo-Malay zones, respectively, have combined megafauna populations (over 20 kilogrammes) (Morrison et al., 2007). With the exception of a few hunting-tolerant animals, virtually all species larger than two kilogrammes are extirpated or live at densities well below historical population ratios outside these regions (Corlett, 2007; Peres, 2009). Indeed, the focus on megafauna has made it difficult to understand the extent of hunting. Previous studies of defaunation in tropical Asia, for example, focused on reserves with megafauna ecosystems that were still fully or almost fully conserved, but with reduced densities (e.g. Datta et al., 2008). Therefore, the study seeks to address the challenge of understanding the dynamics of wildlife conservation in the face of hunting pressures, especially in protected areas. By analysing communities' perspectives, the study aims to shed light on the widespread decline of vertebrate populations due to hunting. Ultimately, the study intends to contribute to the broader understanding of conservation efforts and the management of hunting-related threats to tropical wildlife.

The Role of Wildlife in Livelihoods

Forest products and services play a crucial role in supporting rural employment in sub-Saharan Africa, Asia, and Latin America (Angelsen et al., 2014). Low-income families, in particular, rely significantly on forest resources for their annual income, in contrast to high-income families (Angelsen and Dokken, 2015). This dependence on forests implies that these families might encounter additional challenges if factors such as protective measures, access to, or utilization of forests become restricted. The significance of this reliance becomes even more apparent when considering the various ways in which forests contribute to their well-being. The yield from forests serves a multifaceted purpose. It contributes to essential needs such as energy, food, medicines, and building materials, functioning as a vital network of wealth for these families

(Angelsen et al., 2014; UN, 2020). For instance, game meat, derived from wild animals and often referred to as hedgerow meat, holds particular significance. Low-income families frequently turn to game meat not only for sustenance but also as a means to bridge gaps in their resources (De Merode et al., 2004; Schulte-Herbruggen et al., 2013; Nielsen et al., 2018). This reliance on forest resources, including game meat, serves as a crucial buffer for rural families, helping them withstand unexpected shocks and preventing them from falling into destitution (Angelsen and Wunder, 2003; Babulo et al., 2009; UN, 2020).

A global comparative survey conducted by Nielsen et al. in 2017 revealed that hunting was a prevalent practice, with participation from 39% of the surveyed households. This statistic suggests that approximately 150 million families residing in forested regions engage in the collection of wild meat from the forests, highlighting its importance as a source of sustenance for a significant portion of the population. The extent of reliance on wild meat in rural areas varies widely, depending on factors such as local fauna populations and the availability of payment methods. This variability is explored in studies by Angelsen et al. (2014), Tieguhong (2009), and Kuempel et al. (2010). Despite its limited contribution to cash wages, wild meat serves as a vital source of protein, fat, and essential micronutrients in many regions, as indicated by research conducted by Sarti et al. (2015) and Rowland et al. (2017). Animals are hunted for personal subsistence needs, but there is also a thriving market for game meat and other animal products at local, national, and international levels. These markets function as commodities traded between countries. The global wildlife trade is a substantial industry, estimated to be worth between USD4-20 billion annually, encompassing both regulated and unregulated, legal and illegal trade (Morton et al., 2021). A study by Scheffers et al. (2019), published in Science, delves into the scale of the global wildlife trade. It reveals that vertebrate species constitute a significant portion, accounting for 24% of globally traded species (see Figure 1). While wildlife meat consumption may have traditional and subsistence aspects, the global wildlife trade demonstrates a growing commercial demand for wildlife meat and products, often driven by economic, cultural, and other factors. This increased demand can have complex implications for both wildlife conservation and the global economy. Therefore, efforts to regulate and manage wildlife trade are essential to address these challenges and ensure the sustainability of ecosystems and wildlife populations.

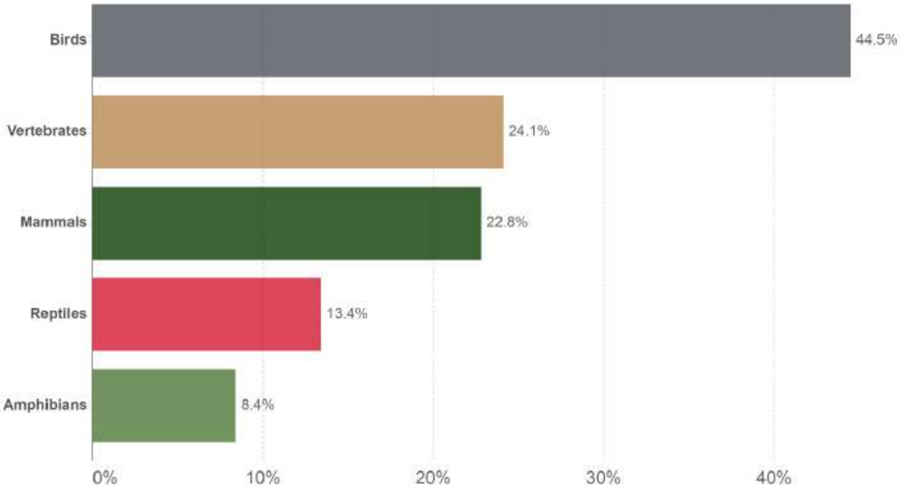


Figure 1. Share of species that are traded (Scheffers et al., 2019).

Emptying the Forest

While concerns about the direct impact of hunting on specific species are often at the forefront of conservation discussions, research into the broader effects of hunting, often referred to as de-hunting, has consistently demonstrated the significant disturbance of seriously hunted forests. One notable consequence is the selective targeting of creatures that feed on tree-based organic products, a practice that has been extensively documented (Harrison, 2011). This targeted hunting has had severe repercussions, particularly for larger frugivorous vertebrates and birds. These species play a pivotal role as seed dispersers within the ecosystem due to their ability to consume larger seeds and transport them over substantial distances. Consequently, in forests subjected to extensive hunting pressures, the recovery of plant species that rely on larger seeds for propagation is significantly impeded. Many of these plant species are slow-growing canopy species, which contrasts with species dispersing smaller seeds or seeds distributed abiotically (McConkey & Drake, 2006; Nuñez-Iturri & Howe, 2007; Wang et al., 2007; Terborgh et al., 2008; Brodie et al., 2009; Holbrook & Loiselle, 2009; Sethi & Howe, 2009).

In addition to the impacts on seed dispersal, hunting has been linked to a range of other disruptions to natural ecological processes. These include alterations in seed predation (Roldán & Simonetti, 2001; Beckman & Muller-Landau, 2007; Dirzo et al., 2007; Wright et al., 2007b), increased seedling mortality rates (Roldán & Simonetti, 2001; Nuñez-Iturri et al., 2008), changes in home range predation dynamics (Posa et al., 2007), and shifts in food availability for larger predators within the ecosystem.

When populations of wildlife species in tropical protected areas are not effectively safeguarded from hunting pressures, restoration efforts are often necessary to mitigate the ecological damage. It is increasingly clear that even supposedly "secured" or "protected" forests are unlikely to maintain their unique ecological structure and functions in the face of ongoing and intensive hunting pressures. Thus, this research not only enhances clarity but also provides a more comprehensive understanding of the ecological consequences of hunting on forest ecosystems, emphasizing the importance of conservation efforts to preserve these fragile environments based on insights gained through community interviews. The research findings are informed by community interviews, making it clear that community perspectives contribute to the understanding of the ecological consequences of hunting and the need for conservation.

Materials and Method

Study Areas

The study was conducted in six selected districts in Sabah, Malaysia, namely Sandakan, Kota Belud, Tambunan, Keningau, Tawau and Tenom (Figure 2). The demographic profiles of the respondents are shown in Figure 4. A seven-days visit was conducted in Sandakan district in June 2020; Tambunan, Keningau and Tenom in July 2020; Tawau in September 2020; and Kota Belud in October 2021.

Survey Methodology

In this study, a comprehensive multi-method approach was employed for data collection, encompassing both qualitative and quantitative methods. The questionnaire featured a combination of open and closed-ended questions, administered during semi-structured interviews with residents from the six selected districts. The interviews were conducted exclusively in the local language, Malay, chosen for it being used comfortably by participants, and for their convenience.

A total of 45 responses were gathered, reaching data saturation after the 34th interview. A snowball sampling method was applied to overcome challenges in identifying potential participants, particularly hunters. This approach relied on research participants' assistance in recruiting others to participate in the study.

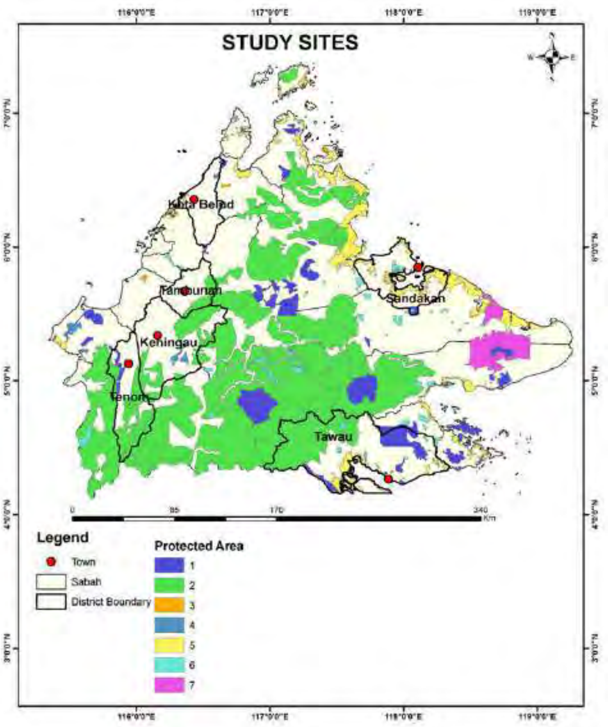


Figure 2. Map showing the selected districts for the study.

Transcriptions of the interviews were carried out verbatim, and the resulting transcripts underwent content analysis using Leximancer analysis software. However, it's important to acknowledge a limitation of the snowball sampling method. This approach hinges on referrals, with the initial participants recruiting subsequent ones. As a result, participants often share similar characteristics and connections, potentially introducing sampling bias, which must be considered. All participants in the interviews possess substantial experience in wildlife meat consumption, with some actively engaged in wildlife hunting activities in Sabah.

Leximancer is a programme that analyses material from interviews from large amounts of qualitative data, extracts information and presents the results visually in the form of a concept map (Leximancer, 2010). For key concepts, the programme extracts a thesaurus of terms and uses these to create a coding scheme, which in turn shows the frequency and co-occurrence of concepts. Two (2) languages are used in this programme, namely "concept" and "topics". The analysis of the frequency, occurrence and context of words shapes the system. The "concepts" were created from the semantic and rational associations of the terms, while the "themes" were derived from interrelated concepts combined from a higher-level concept. The "themes" are represented by coloured bubbles, and the linkage points within the bubbles are also referred to as "concepts'," which are text extracts from the collected data. Haynes et al. (2019) and Ho et al. (2011) found that the use of text mining was coherent with other conventional analyses of qualitative content, so recognition of coherence could justify the use of this software as an analytical tool.

The data analysis consisted of several important steps. The first step was formatting the transcripts. All responses were translated from Malay into English without affecting the meaning of the sentence. Each transcript was entered using Microsoft Word. Leximancer software processed the standard programmed concepts and thesauri. The result is displayed in the conceptual map and spider network configuration (Leximancer, 2010).

The document analysis was also carried out using the same qualitative method of coding the content into themes, similar to the analysis of focus groups or interview transcripts. Due to the increase in cases of Covid-19 in Sabah, reports on hunting and poaching in Sabah were obtained from the Sabah Wildlife Department.

Results

Figure 3 shows female and male respondents in the sample population are balanced: 22 or 48.89% are female and 51.11% or 23 are male. In terms of age group, the majority of the respondents are young professionals, 20 persons (44.44%). In terms of educational background, majority of the respondents have completed at least primary school (44.44%). The majority of the respondents are farmers (46.67%) and most of them belong to the B40 income group (86.67 %).

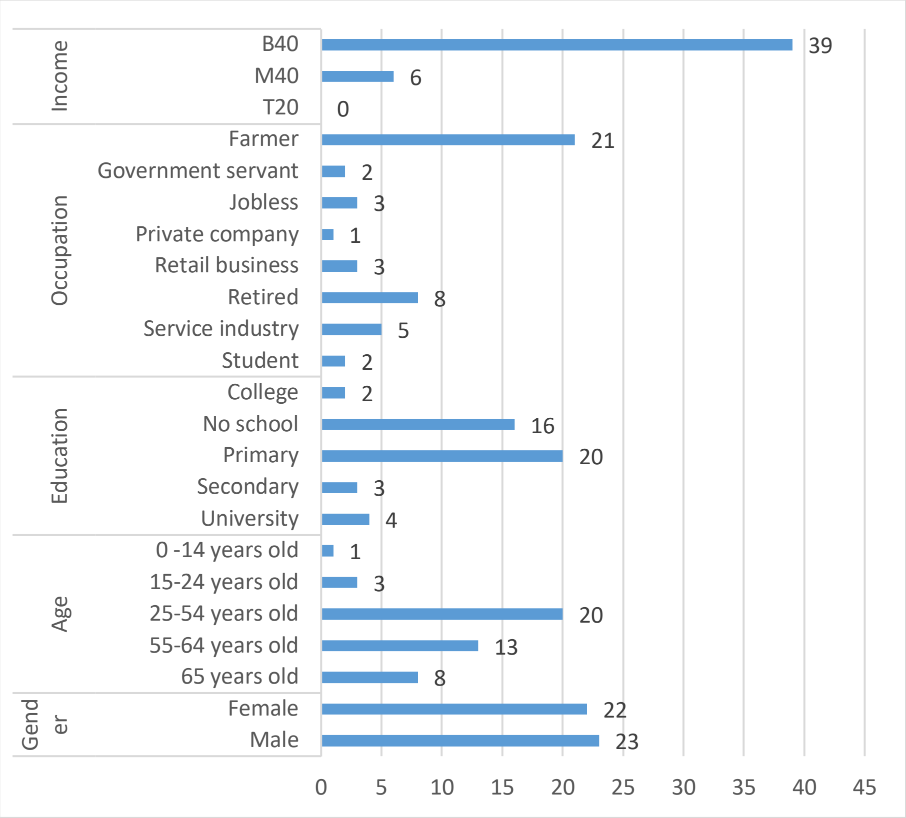


Figure 3. Demographic profile of the respondents.

Age distribution

A total of 44.44% of the respondents were in the 25–54 years age group, another 28.89% were between 55 and 64 years and 17.78% were 65 years and above. In addition, the age distribution for the study was found to be left skewed to cover the 25 to over 65 age group as this is the age group that has sufficient personal knowledge and experience of wildlife hunting in the villages (Figure 4).

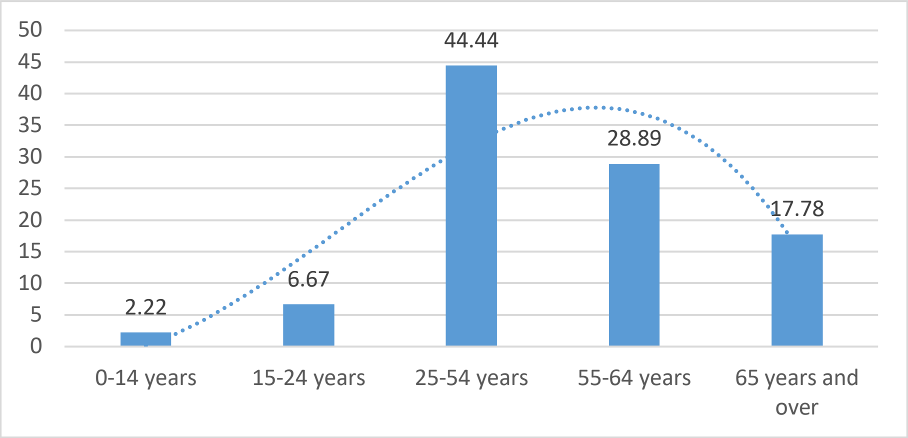


Figure 4. Distribution of age groups in this study which percentage based on the Malaysia Age Structure Index.

Wildlife Hunting Hotspots in Sabah

Wildlife hunting hotspots in Sabah were mapped based on Sabah Wildlife Department reports (Figure 5). It shows that hunters frequently hunt on the east coast of Sabah.

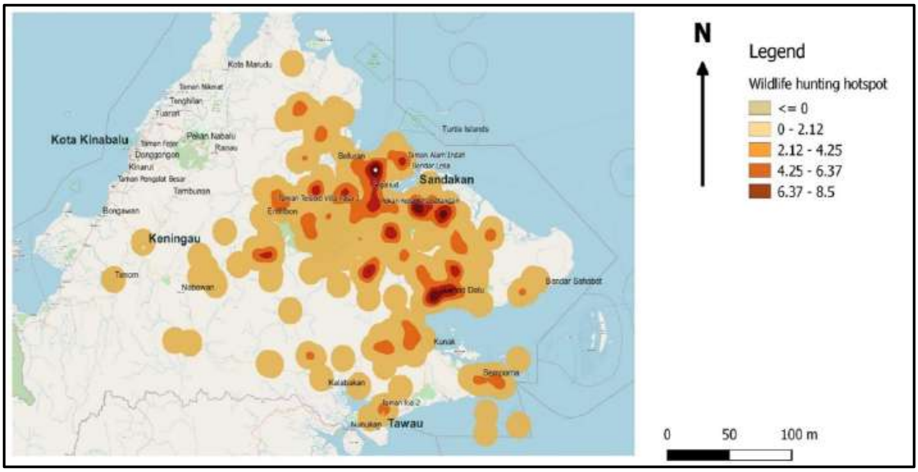


Figure 5. The hotspot of the hunting area in Sabah.

Types of Wildlife Hunted

Based on the document analysis of Sabah Wildlife Department's reports, it shows that bearded pigs are the most frequently hunted animals (Figure 6). For example, 76.97% of hunters were arrested for possessing meat and parts of bearded pigs in their vehicles, followed by sambar deer at 13.7 % and green turtles at 3.5% (Table 1).

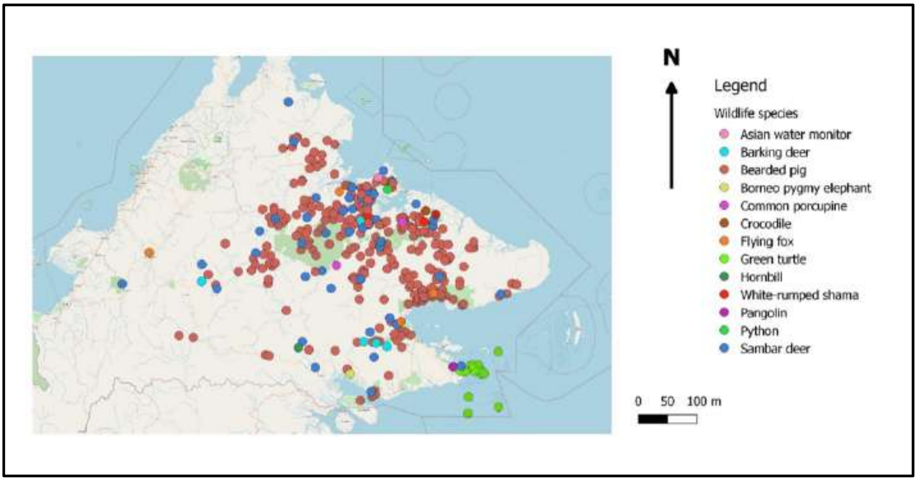


Figure 6. Types of wildlife hunted based on locations.

Table 1. Types of wildlife confiscated by the Sabah Wildlife Department.

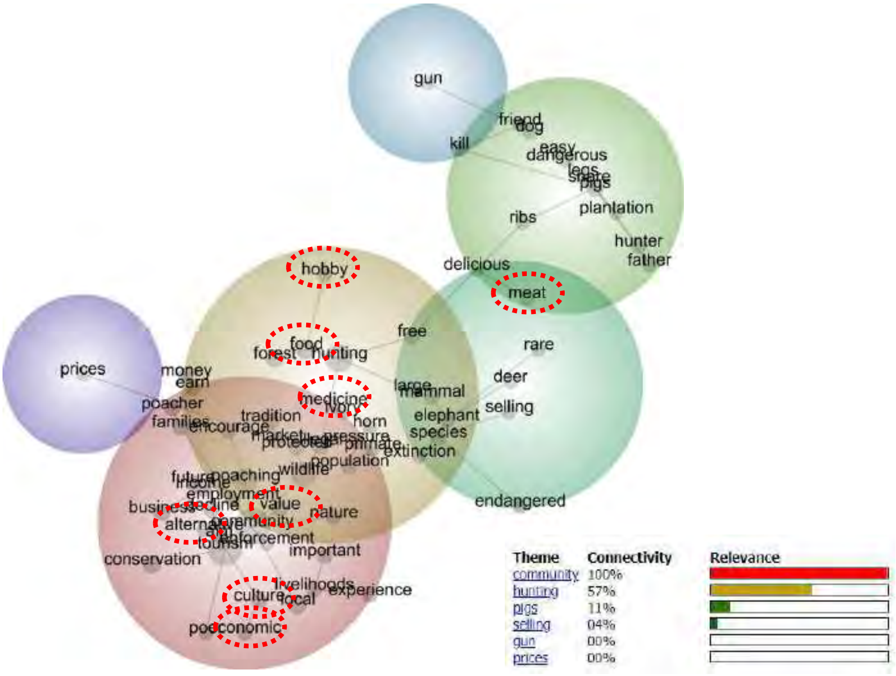
Class	Scientific name	Common name	Percentage of respondent consumed wildlife, %	Hunting cases reported
Mammalia	*Sus barbatus*	Bearded pig	76.97	264
	Rusa unicolour	Sambar deer	13.7	47
	Muntiacus	Barking deer	1.46	5
	Pteropus vampyrus,	Flying fox	1.17	4
	Manis javanica	Pangolin	0.29	1
	Hystrix brachyura	Common porcupine	0.58	2
	Elephas maximus borneensis	Borneo pygmy elephant	0.29	1
Reptilia	*Chelonia mydas*	Green turtle	3.5	12
	Malayopython reticulatus	Pythons	0.29	1
	Crocodylinae	Crocodile	0.29	1
	Varanus salvator	Asian water monitor	0.29	1
Aves	*Copsychus malabaricus*	White-rumped shama	0.87	3
	Bucerotidae	Hornbill	0.29	1

Reasons to Hunt Wildlife

The Leximancer analysis indicates that the themes of "community" and "hunting" were identified as the most prominent and interconnected concepts in the concept map. These themes were found to be highly related and could not be separated or expanded upon, highlighting the strong connection between them.

In the Paths mode of Leximancer, the frequency and weight of individual words in a sentence are displayed. In this context, a block of two sentences represents an influence on the evidence collected for the concept. In our specific scenario, the theme of "community" contributed 100% and the theme of "hunting" contributed 57% to the reasons for hunting.

Figure 7 of the analysis displays the results of interviews, where ten words (represented by red circles) signify the reasons for hunting within the community. These reasons include elements such as local culture and tradition, market demand, alternative livelihood, substitute meat, medicinal purposes, food purposes, free food, and hunting as a hobby. This finding aligns with the expectation that hunting plays a significant economic role for indigenous ethnic groups and contributes to the preservation of their cultural identity.



Theme	Connectivity	Relevance
community	100%	
hunting	57%	
pigs	11%	
selling	04%	
gun	00%	
prices	00%	

Figure 7. Conceptual map based on reasons to hunt.

Food procurement was the most frequently mentioned hunting motivation, with 125 occurrences of the word in the data pool; other important hunting motivations were market demand (98 occurrences of the word) and culture (71 times) (see Figure 8). Thirty-eight respondents emphasized the significant role of game meat as a primary dietary resource. Examples of statements made by respondents in relation to the hunting motivation include:

Respondent 24	*It is the most important source of food for people in the city. We ate it pretty much at every dinner.*
Respondent 1	*It is our life. We know how to hunt since we were 4 years old. If we do not hunt, we do not have food! The wild animals in our forests are our food.*
Respondent 25	*We cannot just stop eating them. They are our main source of food.*

Market demand for game meat was mentioned by respondents as a secondary motivation for hunting, with the word occurring 98 times in the data set, followed by mention of culture, which occurred 71 times in the data pool. For some respondents who regularly or occasionally sold wildlife meat, the sale was an important source of income.

Hunting has a fundamental impact on human development and is firmly linked to social aspects, including virtues, use and appropriation of assets, and political elements (van Vliet 2018). Hunting is thus one of the most important and enduring links between people and wildlife. Respondents were positive about the culture of wildlife hunting and when asked why, some of them emphasised their cultural attachment to nature and wildlife. The forest is important to fulfil their sense of belonging to their homeland and to evoke their childhood memories and experiences. Examples of statements made by respondents in relation to the linkages of hunting to their social aspects include:

Respondent 12	*I was born in a forested village where my house is surrounded by lush trees and there are wild animals everywhere. We give and take from each other. The forest gave us food - meat - and in return we took care of it. And after generations, it became our culture to protect our food in the forest.*
Respondent 42	*We have been hunting for a long time. The skill has been passed on from one generation to the next. This ensures that the tradition is preserved and not forgotten.*

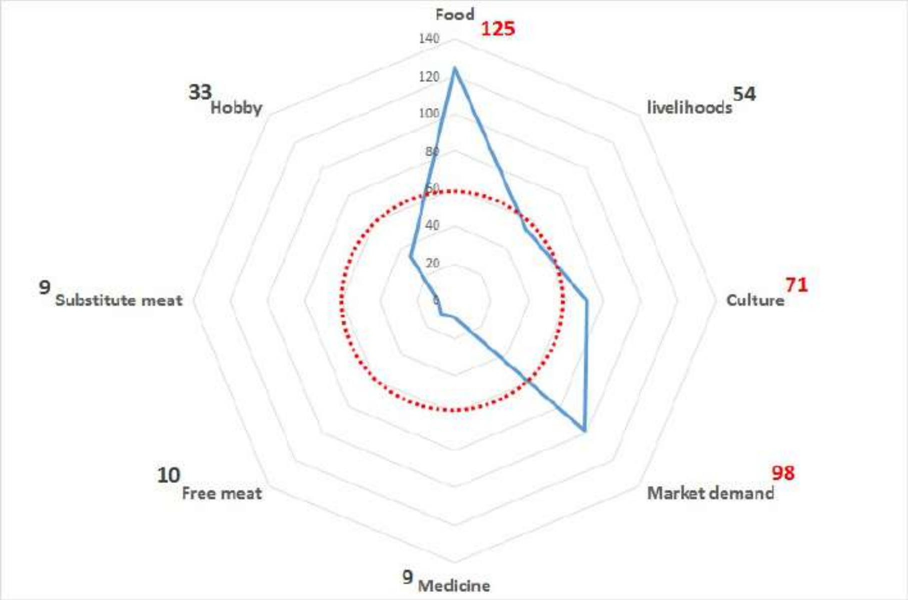


Figure 8. The rationales behind hunting activity among the respondents based on frequency on words occurrence in the interview data set.

A total of 76.97% of the wild animals killed were bearded pigs, 13.70% sambar deer, 3.50% green turtles, 1.46% barking deer and 1.17% fruit bats (Figure 9). Only 0.29% of the respondents reported hunting pangolins, hornbills, crocodiles and pygmy elephants, and 0.58% porcupines for their body parts (fur and ivory). The threat status of the Red List of reported hunted species included one Critically Endangered, one Endangered, two Vulnerable, two Threatened, and one Near Threatened species were among the reported hunted species on the Red List. The remaining five (5) were Least Concerned. The Bornean pygmy elephant (*Elephas maximus borneensis*) was the endemic species reported as hunted.

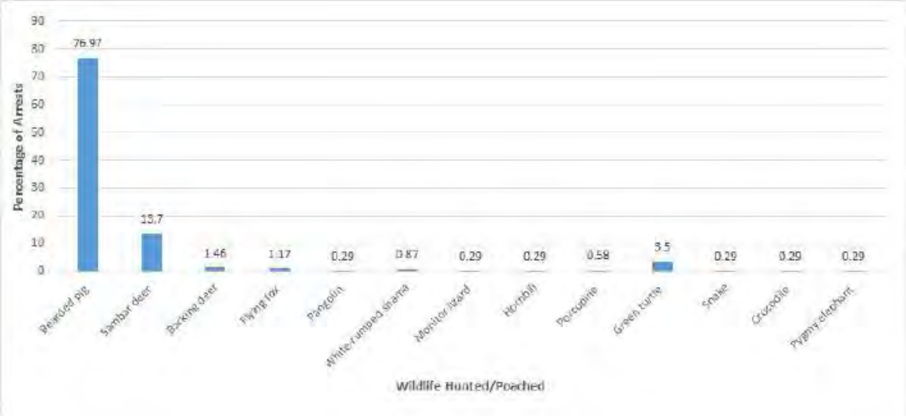


Figure 9. Wildlife species hunted in Sabah.

Hunting Areas in Sabah

The conceptual map shows that there are three areas where the respondents hunt (areas marked in dashed red). These are their own land or garden, the forest and the oil palm plantation (Figure 10). The analysis shows that the theme "areas" has 10 hits in the data sets, indicating that the respondents mainly hunt in the garden areas or their own land and the oil palm plantation. The theme "border" also has 10 hits, indicating that hunting wildlife, especially bearded pigs, is easy in the border areas between the forest and the oil palm plantation. The majority of respondents indicated that there are many footprints and tracks of bearded pigs in the border areas compared to other places, with the theme "footprint" recording 3 hits. The theme "protected" and "animals" with 11 and 3 hits respectively means that the respondents hunt in a protected area.

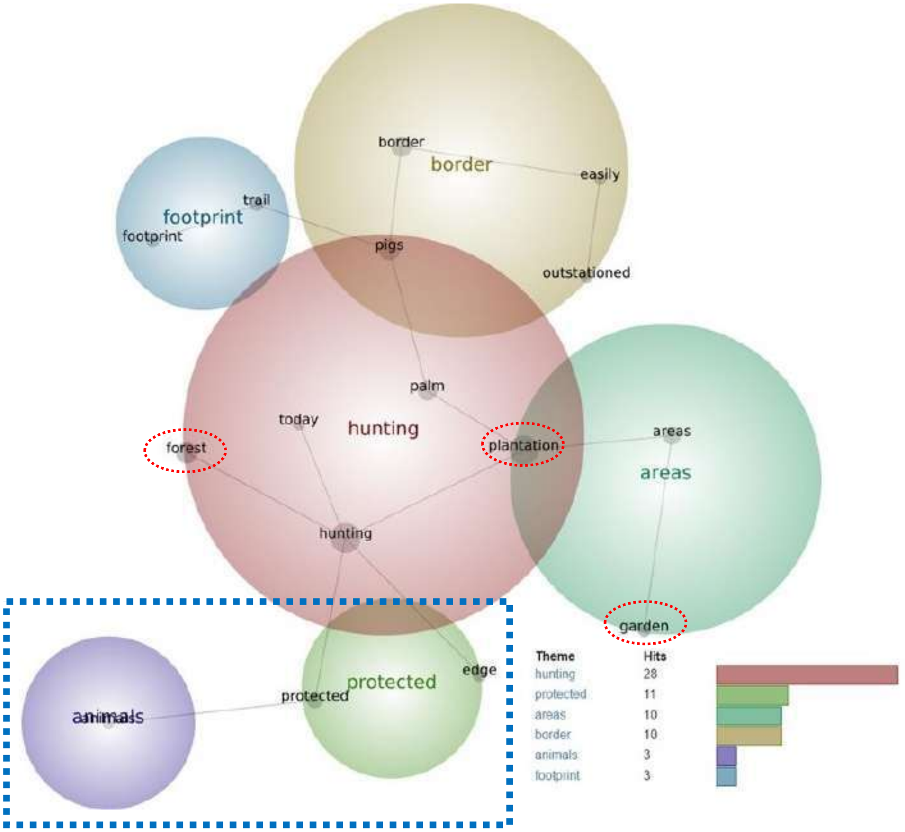


Figure 10. Conceptual map on hunting preferences.

Discussion

Sabah's communities engage in hunting activities in the vicinity of their settlements. This behaviour can be attributed to factors such as easy accessibility resulting from past logging activities or the presence of diverse wildlife. Predominantly, wildlife is targeted in plantation areas, where the conversion of land into agricultural estates has created favourable conditions for hunters. Notably, many oil palm plantations are strategically situated in proximity to wildlife sanctuaries, nature reserves, protected areas, and forest reserves, especially when compared to regions on the west coast.

An intriguing demographic trend emerges when considering the age groups of hunters, with individuals aged between 25 and 54 exhibiting the highest concentration (as depicted in Figure 8). This observation suggests that age plays a pivotal role in hunting effectiveness and resource knowledge. Surprisingly, age exhibits a statistically significant but negative correlation in this context, indicating that individuals under 24 tend to name more species than those over 54—a pattern not consistently validated by other research. Conversely, another statistically significant variable displays a positive correlation with the number of reported species, namely, the age of the hunter. This finding aligns with existing literature, which asserts that older hunters tend to be more effective, possess a deeper understanding of taxonomic diversity, and master a broader range of hunting techniques (Barbosa et al., 2020; Nunes et al., 2020; Silva Neto et al., 2017; Batista Santos et al., 2020; Francesconi et al., 2018). However, it is worth noting that alternative viewpoints are presented by Barbosa de Lima et al. (2021) and da Silva Nogueira Santos et al. (2020), both of whom found no statistical correlation between age and the number of species hunted.

Memory, a distinctive process within socioecological systems, sheds light on the negative correlation between age and the number of species referenced. Often overlooked, memory plays a pivotal role in shaping human interactions with nature, preserving vital information crucial for survival and reproduction. Within this context, memory profoundly influences an individual's comprehension of their local ecology, peaking during adulthood and middle age while either remaining stable or diminishing in old age (Albuquerque et al., 2020).

Research by Oliviera et al. (2019) elucidates that when individuals recall knowledge-related memories, they tend to prioritize recent experiences. This perspective aligns seamlessly with the study's context, as older participants engage in hunting less frequently compared to their more active counterparts, consequently recalling fewer game species. Consequently, a positive relationship emerges between the variable of hunting frequency and the number of mentioned species, corroborating the theory that individuals are more likely to lose their environmental knowledge if they are not actively engaged in it (Silva Neto et al., 2017).

Drawing from data provided by the Sabah Wildlife Department, it appears that the bearded pig is the most commonly encountered animal, with individuals often found in possession of its products. Nevertheless, it's imperative to acknowledge that the available data may not directly reflect the frequency of bearded pig hunting, rendering it challenging to definitively assert that they are indeed the most frequently targeted species. The introduction of the concept of EFS adds a new layer of awareness concerning hunting and poaching among Sabah's local communities. Embracing a more holistic perspective could prompt stakeholders to prioritize wildlife conservation efforts within Sabah's forests.

In village settings, a notable trend emerged where a greater number of respondents consumed bearded pig meat on a weekly basis compared to other sources of animal protein, with domestic chicken being the sole exception. Additionally, hunting bearded pigs was recognized as a vital means of pest control to mitigate disruptions caused by bearded pigs in oil palm plantations, encompassing both industrial and smallholder operations, as well as in subsistence crops like cassava and durian.

While subsistence hunting is extensively documented, instances where market demand serves as the primary motivation have been highlighted (Silva Santos et al., 2019). Within the scope of this study, hunters identified the primary reason for engaging in wildlife hunting as sustenance, followed by market demand and cultural factors (see Figure 8). The sustenance aspect pertains to the pivotal role of wildlife as a source of protein and nourishment for the communities involved. Market demand, on the other hand, denotes the commercial markets' desire for wildlife products, including restaurants and the exotic meat trade, where certain wildlife species are esteemed for their flavour or perceived medicinal attributes. In the realm of hunting, provisioning benefits serve as potent incentives, as they directly contribute to the sustenance and well-being of individuals or communities (Gill et al., 2012; Luz et al., 2015; Wilkie et al., 2005; Fa et al., 2009; Godoy et al., 2010; Brashares et al., 2011; Luz et al., 2015).

The hunting motives observed among local communities in select areas of Sabah closely align with the findings of Bennett et al. (2000) in Sabah and Sarawak, as well as Kurz et al. (2021), where meat procurement emerged as the primary motivation for wildlife hunting, particularly in the case of bearded pigs. It is probable that meat procurement has historically been the chief driving force for indigenous communities that have engaged in hunting across Borneo for millennia. This supposition is supported by archaeological discoveries featuring bearded pig bones at consumption sites (Medway, 1964). Confirming this perspective, Kurz et al. (2021) revealed that trackers widely indicated the current high cost of bearded pork, typically ranging from MYR 10-15/kg, in stark contrast to the advertised cost of approximately MYR 3-5/kg a decade ago (and significantly lower when adjusted for inflation). Monthly earnings from pig

hunting are reported to reach as high as MYR 5,000 (~USD 1,194) in favourable months, surpassing remuneration levels in the oil palm plantation sector.
Table 2 provides a comparative analysis of the market value of wildlife in the black market as opposed to the monthly income of Sabah's rural communities.

Table 2. Global illegal wildlife trade estimates versus rural income of Sabah.

Wildlife	Black Market Value USD, million, per year	Rural Income USD, annually
Live Animals		8,160
Primates	132	
Birds of prey	5	
Cage birds	11	
Reptiles	38	
Ornamental fish	358	
Animal Products		
Mammal furs	6,623	
Reptile skins	371	
Corals and shells	144	
Natural pearls	76	
Game meat	674	
Reptile meat	4	
Edible snails	102	

Source: van Uhm, 2016 and Department of Statistics Malaysia, 2020.

This phenomenon can be attributed to the findings of Bennett et al. (2000) and Mojiol et al. (2013), who highlight the widespread practice of hunting bearded pigs in various rural areas of Sabah, with bearded pig meat remaining a significant dietary resource for specific communities in the region. This observation is congruent with the research conducted by Kurz et al. (2021), which underscores food acquisition as the most commonly cited motivation for bearded pig hunting, particularly within the Kadazan-Dusun-Murut (KDM) ethnic group in Sabah. Additionally, Kurz et al. (2021) identified other motivational factors driving bearded pig hunting among KDM communities, including for pest control, gift-giving, and recreational pursuits.

The inclusion of market demand as a motivation for hunting in this study implies a market-driven component within hunting practices in Sabah. It suggests that certain hunters engage not only in subsistence hunting but also seek to fulfil the demand for wildlife products in commercial markets (Silva Santos et al., 2019). Furthermore, culture emerges as a motivating factor for hunting in this study. Cultural influences can significantly shape hunting practices, as specific wildlife species may hold cultural significance or be integral to traditional customs and rituals (Kurz et al., 2021). The act of hunting for cultural reasons underscores the importance of preserving cultural heritage and identity within the community. It is essential to recognize that motivations for hunting can vary significantly from individual to individual and from one community to another, influenced by a complex interplay of socio-economic, cultural, and ecological factors. The motivations elucidated in this study provide valuable insight into the multifaceted nature of hunting practices in Sabah, where the pursuit of sustenance, market demand, and cultural value collectively drive the hunting of bearded pigs and other wildlife species.

The primary areas where respondents engage in hunting—namely, their own land or gardens, the forest, and oil palm plantations—are closely connected to the concept of the "empty forest syndrome" (as depicted in Figure 10). In their own land or gardens, respondents target wildlife species that either inhabit or pass through these areas. The motive of pest control emerged prominently among our respondents (Kurz et al., 2021), emphasizing the significant influence of garden cultivation on hunting behaviour (Peres & Palacios, 2007). A notable majority of respondents cited pest control as a key reason for hunting wildlife. Depending on the intensity of hunting pressure and the targeted species, this practice can lead to a decline in wildlife populations within the immediate vicinity. Such localized reductions in wildlife populations contribute to the overall emptiness of the forest ecosystem.

However, hunting within forests can also exert more extensive consequences on wildlife populations. Forests typically harbour diverse species, including mammals, birds, reptiles, and insects. When hunting remains unsustainable or unregulated, it can result in predation and the depletion of wildlife populations (Joppa et al., 2008; Rhett, 2011). This disruption of the ecological balance diminishes biodiversity and further exacerbates the empty forest syndrome. Oil palm plantations, vast agricultural landscapes often replacing natural forests, introduce a unique dimension to this issue. While these plantations do not provide the same level of biodiversity and ecological services as natural forests, they can still support certain adaptable wildlife species (Shah et al., 2019).

Nonetheless, hunting within oil palm plantations can intensify the depletion of already diminished wildlife populations, pushing them closer to extinction in these areas and exacerbating the empty forest syndrome. The act of hunting within these three key domains—own land or gardens, the forest, and oil palm plantations—collectively contributes to the empty forest syndrome by directly diminishing wildlife populations and disrupting the ecological equilibrium within forest ecosystems.

Conclusion

The relationship between hunting for sustenance, market demand, cultural factors, and the emergence of the empty forest syndrome is intricate and interconnected. When hunting primarily serves as a means for securing food, it can inadvertently lead to the depletion of wildlife populations. If hunting remains unregulated and lacks effective enforcement measures, this can result in the overexploitation of target species. Such actions disrupt the delicate ecological equilibrium within the forest ecosystem, potentially culminating in the empty forest syndrome.

Conversely, a robust market demand for wildlife products, such as bushmeat, exotic animal parts, or live animals for the pet trade, can intensify hunting pressure on vulnerable species. This heightened demand can stem from cultural beliefs, traditional medicinal practices, or the desire for luxury and novelty items. Unsustainable hunting practices driven by market demand can yield severe repercussions for wildlife populations, ultimately contributing to the onset of the empty forest syndrome.

Cultural influences also play a pivotal role, as cultural practices and traditions can significantly mould hunting behaviours. When a particular species holds cultural significance or forms an integral part of traditional rituals and ceremonies, it often leads to increased hunting pressure. Cultural norms and beliefs do not always align with conservation principles or sustainable hunting practices. In such scenarios, cultural motivations for hunting can exacerbate the depletion of wildlife populations, potentially paving the way for the empty forest syndrome.

It is essential to emphasize that not all hunting practices or cultural traditions result in the empty forest syndrome. Sustainable hunting practices, guided by stringent regulations and quotas, can enable the responsible utilization of wildlife resources while safeguarding their long-term conservation. Furthermore, communities deeply attached to their natural environment and possessing a strong conservation ethic can actively contribute to the protection and sustainable management of their forests, thereby helping to prevent or mitigate the empty forest syndrome.

Furthermore, it's crucial to acknowledge that hunting within oil palm plantations can exert particularly damaging effects on wildlife populations. Given that these plantations often replace natural forests, they already contribute to the empty forest syndrome by reducing available habitat for numerous species. When hunting occurs within these oil palm plantations, it can further decimate already depleted wildlife populations, potentially pushing certain species in these areas closer to the brink of extinction. This amplifies the gravity of the empty forest syndrome and underscores the necessity for conservation measures addressing both hunting practices and habitat loss associated with activities like oil palm cultivation.

In summary, the adoption of sustainable hunting practices, effective regulation of wildlife trade, community engagement, and educational initiatives are paramount in combating the empty forest syndrome. Additionally, addressing the ecological impacts of industrial activities, notably oil palm plantations, is vital to mitigate further wildlife population declines and promote habitat conservation, ultimately safeguarding both wildlife populations and the integrity of forest ecosystems.

Acknowledgement

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Declarations

Ethical Approval

Informed consent was obtained from all subjects involved in this study, and all subjects participating in the study gave their consent to the use of all information and data analysed for the purpose of publication.

Competing interests

The authors declare no potential conflicts of interest concerning the research, authorship, and publication of this article.

Authors' contributions

The authors confirm their contribution to the paper as follows: study conception and design: Fiffy H. S., Nurin S. M. Y., Azniza M. Senthilvel K. S. S. N. and Hussien M.; data collection: Fiffy H. S., Nurin S. M. Y., Arnie H., Maximus L. L. K. F., Julia G. K., Petherine A. J., Mohd. Afifi M. N., Cornelius P., Juliana, A. and Mahadimenakbar M. D.; analysis and interpretation of results: Fiffy H. S., Nurin S. M. Y., Nordiana M. N., Nor Akmar A. A. and Norhuda S.; draft manuscript preparation: Fiffy H. S. and Nurin S. M. Y. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT in order to help in generating new ideas and expand on existing ones. The ChatGPT had provide fresh perspectives on a topic and helped overcome the author's block. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

References

Albuquerque UP, Ludwig D, Feitosa IS, de Moura JMB, de Medeiros PM, Gonçalves PHS. et al. 2020. Addressing social-ecological systems across temporal and spatial scales: a conceptual synthesis for ethnobiology. *Hum Ecol.*, *48* (5): 557–71.

Wong A, Huaimei Y, Wong C, Shukor JA. 2012. A study on hunting activity of Sambar deer bearded pig in Paitan Forest Reserve, Pitas, Sabah, Malaysia. *Journal of Tropical Biology and Conservation, 9* (1): 35–47.

Angelsen A, Dokken T. 2018. Climate exposure, vulnerability and environmental reliance: A cross-section analysis of structural and stochastic poverty. *Environment and Development Economics, 23* (3): 257–278.

Angelsen A, Jagger P, Babigumira R, Belcher B, Hogarth NJ, Bauch S, Börner J, Smith-Hall C, Wunder S. 2014. Environmental Income and Rural Livelihoods: A Global-Comparative Analysis. *World Development, 64* (S1): S12–S28.

Barbosa de Lima, JR, Oliveira Rebouças PL, Batista Santos CA. 2021. Hunting and use of wildlife species in the semi-arid region of Brazil. *Rev Amaz Investig.*, *9* (36): 9–21.

Barbosa, JAA, Aguiar JO, Alves RRN. 2020. Hunting strategies used in protected areas in the Atlantic rainforest of North-eastern Brazil. *Indian J Tradit Knowl.*, *9* (3): 509–18.

Batista Santos CA. 2020. Hunting practices among the indigenous "Truká" in the semiarid region of Brazil. Rev Amaz Investig. *9* (31):127–47.

Beckman NG, Muller-Landau HC. 2007. Differential effects of hunting on pre-dispersal seed predation and primary and secondary seed removal of two Neotropical tree species. *Biotropica, 39:* 328–339.

Bennett EL, Nyaoi AJ, Sompud J. 2000. Saving Borneo's bacon: The sustainability of hunting in Sarawak and Sabah. Pages 305–324. In Robinson, J. G. and Bennett, E. L. (Eds). *Hunting for Sustainability in Tropical Forests*. Columbia University Press.

Berry NJ. 2010. The high value of logged tropical forests: Lessons from northern Borneo. *Biodiversity and Conservation, 19:* 985–997.

Brashares JS, Arcese P, Sam MK, Coppolillo, P. B., Sinclair, A. R. E. and Balmford, A. 2004. Bushmeat hunting, wildlife declines, and fish supply in West Africa. *Science, 306:* 1180–1183.

Brodie JF, Helmy OE, Brockelman, W. Y. and Maron, J. L. 2009. Bushmeat poaching reduces the seed dispersal and population growth rate of a mammal-dispersed tree. *Ecological Applications, 19:* 854–863.

Brooks TM, Wright SJ, Sheil D. 2009. Evaluating the success of conservation actions in safeguarding tropical forest biodiversity. *Conservation Biology, 23:* 1448–1457.

Bruner AG, Gullison RE, Rice Rea, da Fonseca GAB. 2001. Effectiveness of parks in protecting tropical biodiversity. *Science, 291:* 125–128.

Chan J. 2017, January 25. *In RM79m Kinabatangan bridge, another human-wildlife dilemma*. Retrieved from https://www.malaymail.com/news/malaysia/2017/01/25/in-rm79m-kinabatangan-bridge-another-human-wildlife-dilemma/1300647.

Clark CJ, Poulsen JR, Malonga R, Elkan PW. Jr. 2009. Logging concessions can extend the conservation estate for Central African tropical forests. *Conservation Biology, 23:* 1281–1293.

da Silva Nogueira Santos S, Martins CSG, de Campos Martins F. 2020. Is the knowledge about the wild birds influenced by the socioeconomic conditions of the human populations? *Ethnobiol Conserv*., *9:* 1–19.

Department of Statistics Malaysia. 2020. Household income and basic amenities survey report by State and Administrative District. Putrajaya: Department of Statistics Malaysia.

Dirzo R, Mendoza E, Ortíz P. 2007. Size-related differential seed predation in a heavily defaunated neotropical rain forest. *Biotropica, 39:* 355–362.

Francesconi W, Bax V, Blundo-Canto G, Willcock S, Cuadros S, Vanegas M. et al. 2018. Hunters and hunting across indigenous and colonist communities at the forest-agriculture interface: an ethnozoological study from the Peruvian Amazon. *J Ethnobiol Ethnomed*., *14* (1): 1–11.

Harrison RD. 2011. Emptying the forest: hunting and the extirpation of wildlife from tropical nature reserves. *Bioscience, 61:* 919–924.

Haynes E, Garside R, Green J, Kelly MP, Thomas J, Guell C. 2019. Semiautomated text analytics for qualitative data synthesis. *Research Synthesis Methods*, *10* (3): 452–64.

Ho YC, Jannasch-Pennell A, DiGangi S. 2011. Compatibility between text mining and qualitative research in the perspectives of grounded theory, content analysis and reliability. *Qual Rep*, *16* (3): 730–44.

Holbrook KM, Loiselle BA. 2009. Dispersal in a Neotropical tree, *Virola flexuosa* (Myristicaceae): Does hunting of large vertebrates limit seed removal? *Ecology, 90:* 1449–1455.

Isaac G. 1978. The food-sharing behavior of protohuman hominids. *Scientific American*, *238* (4): 90–108.

Joppa LN, Loarie SR, Pimm SL. 2008. On the protection of "protected areas." *Proceedings of the National Academy of Sciences, 105:* 6673–6678.

Kuempel CD, Jones KR, Watson JEM, Possingham HP. 2019. Quantifying biases in marine-protected-area placement relative to abatable threats. *Conservation Biology, 33* (6): 1350–1359.

Kurz DJ, Saikim FH, Justine VT, Bloem J, Libassi M, Luskin MS, Withey LS, Goossens B, Brashares JS, Potts MD. 2021. Transformation and endurance of Indigenous hunting: Kadazandusun-Murut bearded pig hunting practices amidst oil palm expansion and urbanization in Sabah, Malaysia. *People and Nature*, *3* (5): 1078–1092.

Lancaster JB. 1978. Carrying and sharing in human evolution. *Human Nature Magazine*, 82–89.

Laurance WF, Croes BM, Guissouegou N, Buij R, Dethier M, Alonso A. 2008. Impacts of roads, hunting, and habitat alteration on nocturnal mammals in African rainforests. *Conservation Biology*, 22: 721–732.

Leximancer. 2010. *Leximancer manual version 3.5*. Brisbane, Australia: Leximancer Pty. Ltd. Retrieved from https://info.leximancer.com/v35.

Lovejoy C. 1981. The origin of man. *Science*, *211:* 341–50.

McConkey KR, Chivers DJ. 2004. Low mammal and hornbill abundance in the forests of Barito Ulu, Central Kalimantan, Indonesia. *Oryx, 38:* 439–447.

Mojiol AR, Ganang GM, Fatt BS. 2013. Study of birds composition at the burned and unburned forests in Klias Forest Reserve, Sabah, Malaysia. *Tigerpaper*, *40* (1): 21–29.

Morton O, Scheffers BR, Haugaasen T, Edwards DP. 2021. Impacts of wildlife trade on terrestrial biodiversity. *Nat. Ecol. Evol., 5:* 540–548.

Nielsen J, Shrestha A, Neupane D, Kallestrup P. 2017. Non-adherence to anti-hypertensive medication in low- and middle-income countries: a systematic review and meta-analysis of 92443 subjects. *J Hum Hypertens, 31:* 14–21.

Nuez-Iturri G, Olsson O, Howe HF. 2008. Hunting reduces recruitment of primate-dispersed trees in Amazonian Peru. *Biological Conservation, 141:* 1536–1546.

Nunes AV, Oliveira-Santos LGR, Santos BA, Peres CA, Fischer E. 2020. Socioeconomic drivers of hunting efficiency and use of space by traditional amazonians. *Hum Ecol*., *48* (3): 307–15.

Oliveira ES, Albuquerque UP, Alves AGC, Ramos MA. 2019. Is local ecological knowledge altered after changes on the way people obtain natural resources? *J Arid Environ*., *167:* 74–8.

Peres C. 2009. Overexploitation. In NS. Sodhi, & PR. Ehrlich (Eds.), *Conservation Biology for All* (pp. 107–130). Oxford University Press.

Peres CA. 2009. Overexploitation. Pages 107-130 in Sodhi, N. S. and Ehrlich. P. R. (eds). *Conservation Biology for All*. Oxford University Press.

Peres CA, Palacios E. 2007. Basin-wide effects of game harvest on vertebrate population densities in Amazonian forests: Implications for animal-mediated seed dispersal. *Biotropica, 39:* 304–315.

Rhett DH. 2011. Emptying the Forest: Hunting and the Extirpation of Wildlife from Tropical Nature Reserves. *BioScience*, *61* (11): 919–924.

Scheffers BR, Oliveira BF, Lamb I, Edwards DP. 2019. Global wildlife trade across the tree of life. *Science*, *366* (6461): 71–76.

Sethi P, Howe, H. F. 2009. Recruitment of hornbill-dispersed trees in hunted and logged forests of the Indian Eastern Himalaya. *Conservation Biology, 23:* 710–718.

Shah HA, Huxley P, Elmes J, Murray KA. 2019. Agricultural land-uses consistently exacerbate infectious disease risks in Southeast Asia. *Nature Communications*, *10:* 4299.

Silva Neto BC, Nascimento ALB, Schiel N, Alves RRN, Souto A, Albuquerque UP. 2017. Assessment of the hunting of mammals using local ecological knowledge: an example from the Brazilian semiarid region. *Environ Dev Sustain*., 19(5): 1795–813.

Silva Santos S, Lucena RFP, Lucena Soares HK, Santos Soares VM, Sales NS, Mendonça LET. 2019. Use of mammals in a semi-arid region of Brazil: an approach to the use value and data analysis for conservation. *J Ethnobiol Ethnomed*., *15* (1): 1–14.

Swift HJ, Abrams D, Drury L, Lamont R. 2018. Categorization by Age. In: Shackelford T., Weekes-Shackelford V. (eds) *Encyclopedia of Evolutionary Psychological Science.* Springer, Cham. https://doi.org/10.1007/978-3-319-16999-6_2431-1.

Terborgh J, Nuez-Iturri G, Pitman NCA, Cornejo Valverde FH, Alvarez P, Swamy V, Pringle EG, Paine CET. 2008. Tree recruitment in an empty forest. *Ecology, 89:* 1757–1768.

Tieguhong JC, Zwoliniii J. 2009. Supplies of Bushmeat for livelihoods in logging towns in the Congo Basin. *Journal of Horticulture and Forestry*, *1* (5).

Van Uhm, D. 2016. *The Illegal Wildlife Trade: Inside the World of Poachers, Smugglers and Traders. Chapter 6: "Studies of Organized Crime."* New York: Springer.

Wang BC, Sork VL, Leong MT, Smith TB. 2007. Hunting of mammals reduces seed removal and dispersal of the afrotropical tree *Antrocaryon klaineanum* (Anacardiaceae). *Biotropica, 39:* 340–347.

Washburn S, Lancaster C. 1968. The evolution of hunting. In R. B., Lee, and I., Devore, (eds.): *Man the Hunter*. Chicago: Aldine, pp. 293–303.

Woodroffe R, Ginsberg, J. R. 1998. Edge effects and the extinction of populations inside protected areas. *Science, 280:* 2126–2128.

Wright SJ, Hernandz A, Condit, R. 2007. The bushmeat harvest alters seedling banks by favoring lianas, large seeds, and seeds dispersed by bats, birds, and wind. *Biotropica, 39:* 363–371.

Yu DW, Levi T, Shepard GH. 2010. Conservation in Low-governance Environments. *Conservation Biology, 42:* 569–571.

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Research Article

Butterfly Diversity in the Campus Area of University of North Bengal, West Bengal, India

Abhirup Saha, Subhajit Das, Prapti Das, Debayan Raha, Dhiraj Saha*

Insect Biochemistry and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, P.O. NBU, District: Darjeeling, West Bengal, Pin: 734013, India.

*Corresponding author: dhirajsaha@nbu.ac.in
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ABSTRACT

Butterflies play a crucial role as bio-indicators, signaling the health of ecosystems and biodiversity, making their conservation vital. The northern region of West Bengal in eastern India is renowned for its remarkable butterfly diversity. The University of North Bengal campus, spanning 315.99 acres at the Eastern Himalayan foothills (26°42′34.03″N; 88°21′14.96″E), is home to diverse flora and fauna. The surrounding area offers a variety of habitats, including forests, grasslands, tea gardens, and wetlands, which support numerous animal species. This study aimed to assess butterfly diversity in relation to their habitats and conservation needs within the university campus. Three distinct geographical sites within the university campus were selected, namely the tea garden area (dominated by tea and rubber plants), Salkunja (a forested area with a semi-perennial stream dominated by Sal trees), and Magurmari (an area with semi-perennial streams, ponds, ephemeral water bodies, paddy fields, and grasslands). Butterfly observations were conducted monthly from December 2021 to January 2023. The study documented 55 butterfly species from six families (Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, Pieridae, and Riodinidae), with Nymphalidae being the most prevalent and Riodinidae the least. Notably, the ecotone area of Magurmari exhibited the highest butterfly species diversity. Four species protected under the Wildlife (Protection) Act (WPA), 1972, were also identified. The study contributed a concise checklist and underscores the significant butterfly diversity found in the campus, emphasizing the urgent need for focused conservation actions.

Keywords: Butterfly; Diversity; University of North Bengal; Checklist; Lepidoptera

Introduction

Butterflies play a crucial role in maintaining the structure and function of almost all terrestrial ecosystems as they are commonly involved in pollination, herbivory and preyed upon by several predators. The aesthetic beauty of butterflies, along with their role as a key pollinator and an indicator of ecosystem health, makes them an integral part of both the natural and human worlds. Globally, more than 19 thousand species of butterflies are reported (Westgate et al., 2016). There are more than 1500 species of butterflies reported from the Indian subcontinent (Tiple, 2011). Butterflies are crucial pollinators to support the world's food supply (Lindström et al., 2018). Since they are extremely sensitive to even minor environmental changes and shifts in the makeup of the forest, butterflies are thought to be excellent markers of the health of an ecosystem (Pollard, 1977). Numerous butterfly species are facing threat due to habitat loss, climate change and other anthropogenic activities (Brereton et al., 2011). To conserve and manage butterfly species effectively, it is crucial to have a comprehensive understanding of their distribution and diversity.

The northern part of West-Bengal encompasses a vast range of ecosystems, including forests, grasslands and wetlands, which support a variety of flora and fauna. Few systematic studies on butterflies were carried out in the Darjeeling hills (Pradhan & Khaling, 2020). The University of North Bengal (NBU) is located on a tract of land covering roughly 315.99 acres at the base of Eastern Himalaya, occupying the geographic coordinates of 26°42'34.03"N and 88°21'14.96"E (Figure 1). It is situated within the Siliguri sub-division of the Darjeeling district. The climate of this region is subtropical. The campus of the university is located in a forested region characterized by a diverse mixture of deciduous and evergreen trees. Additionally, the surrounding area comprises various habitats such as grasslands, forests and wetlands, which create an ideal environment for a variety of faunal species. Within the confines of the campus, two semi-perennial streams are present. Both of the streams are formed on an ecotone area among grasslands and riverside. During the monsoon season, a significant portion of the campus remains submerged. Previous studies have documented the presence of a diverse array of avian species as well as damselflies, dragonflies, and other taxa on the campus (Mukhopadhyay et al., 2015; Pal, 2017). This study sought to fill the knowledge gap on the diversity of butterflies and their pollinator plants in the campus of University of North Bengal. The goal of this study is to examine the diversity of butterflies and to make a checklist of their host and pollinator plants at the university campus. The knowledge obtained from the study is required to effectively and sustainably protect butterflies and their preferred host and pollinator plants. Although the current work is merely a first investigation, it offers a strong framework for future investigations that are required to fully comprehend the importance of butterflies and plant interaction.

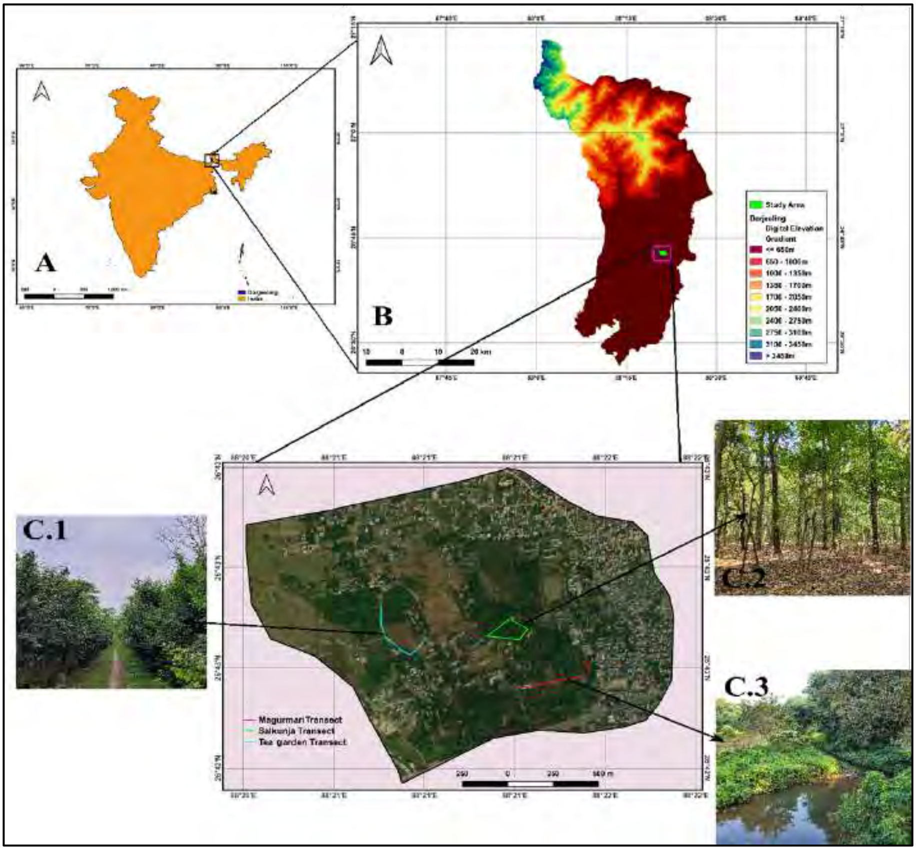

Figure 1. A) Map showing the location of Darjeeling in respect to India B) The elevation gradient of Darjeeling district and study area within C) the map of the University of North Bengal and the study area showing C.1) Tea garden area, C.2) Salkunja, C.3) Magurmari (This map was created using QGIS software version 3.22. Shapefiles were downloaded from: https://www.naturalearthdata.com/, https://sedac.ciesin.columbia.edu/data/set/india-india-village-level-geospatial-socio-econ-1991-2001/data-download and DEM file was downloaded from https://bhuvan-app3.nrsc.gov.in/data/download/index.php.).

Material and Methods

The current study was conducted to understand the butterfly diversity from three geographically different study sites namely tea garden (TG), Salkunja (SK) and Magurmari (MM) of the campus. The tea garden area is mostly comprised of grassy plains where tea and rubber plants are the main vegetation. In contrast, Salkunja is a forested area with a semi-perennial stream, pre-dominated by Sal trees. The Magurmari area contains various features such as a semi-perennial stream, ponds, temporary waterbodies and grasslands. The Pollard walk method (Pollard, 1977) was used to conduct this study between December 2021 to January 2023. Each study area was examined once a month. Observation of butterflies were conducted within a distance of 2.5 meters on either side of the transect line and 5 meters ahead of it. All three belt transects from Magurmari, Salkunja and tea garden were selected for the study and each transect was visited from 8am to 11am (Figure 1).

The study relied on photographs of the butterflies taken in their natural habitat using a DSLR camera in each study area. Common butterflies were identified immediately on the sampling sites by examining their colour patterns, size and wing designs. However, for most of the study, field guide books of butterflies (Smetacek, 2017), published literature (Mukherjee & Mondal 2020), relevant websites (https://www.knowyourinsects.org/.) and guides by entomological specialists were consulted to make a conclusive identification. Another survey was conducted to document larval host plants and nectar plants within the university campus for various butterfly families using previous published literature from this area (Saha & Das 2012).

Results and Discussion

This preliminary study reveals a total of 55 species of butterflies belonging to 6 families in the campus of the University of North Bengal (Figure 2). All the butterfly names with their family are in the Table 1. Among all the families recorded, Nymphalidae was the most dominant one, whereas Riodinidae was the least found. Other families recorded during the study were Hesperiidae, Lycaenidae, Papilionidae and Pieridae. Figure 3 illustrates the distribution of these families among all three study sites. Twenty-three (23) species from family Nymphalidae (making up 42% of the total), 10 species from Lycaenidae (18%), 9 species from Pieridae (16%), 7 species from Hesperiidae (13%), 5 species from Papilionidae (9%) and 1 species from Riodinidae (2%) were recorded during the study (Figure 3 and 4).

Additionally, the highest variety of butterflies was recorded from the Magurmari area followed by tea garden and Salkunja. Out of all the butterflies observed, there were 41 species that were present in all three study sites. The butterflies *Zemeros flegyas* and *Chliaria othona* were exclusively observed in the Salkunja area, while the butterflies *Delias descombesi* and *Ampittia dioscorides* were exclusively recorded in the Magurmari area. Among all the recorded species, four species were found to be protected under the Wildlife (Protection) Act (WPA), 1972.

Table 1. Checklist of the butterflies reported from North Bengal university campus.

SL. No	Family	Common name	Scientific name	Habitat	Occurrence	Wildlife (Protection) Act, 1972
1	Riodinidae	Punchinello	*Zemeros flegyas*	SK	+	—
2	Papilionidae	Common Jay	*Graphium doson*	TG, SK, MM	++	—
		Tailed Jay	*Graphium agamemnon*	TG, SK, MM	++	—
		Common Mormon	*Papilio polytes*	TG, SK, MM	+++	—
		Lime Butterfly	*Papilio demoleus*	SK, MM	+	—
		Common Rose	*Pachliopta aristolochiae*	TG, SK, MM	++	—
3	Hesperiidae	Fulvous Pied Flat	*Pseudocoladenia dan*	TG, SK, MM	+++	—
		Dark Palm Dart	*Telicota bambusae*	TG, MM	++	—
		Grass Demon	*Udaspes folus*	TG, SK, MM	++	—
		Straight Swift	*Parnara guttatus*	TG, SK, MM	++	—
		Rice Swift	*Borbo cinnara*	TG, SK, MM	++	—
		Chestnut Bob	*Iambrix salsala*	TG, SK, MM	++	—
		Common Bush Hopper	*Ampittia dioscorides*	MM	++	—
4	Pieridae	The Common Jezebel	*Delias eucharis*	TG, MM	+	—
		Red-Spot Jezebel	*Delias descombesi*	MM	+	—
		Striped Albatross	*Appias libythea*	TG, SK, MM	++	Schedule IV
		Common Emigrant	*Catopsilia pomona*	TG, SK, MM	++	—
		Mottled Emigrant	*Catopsilia pyranthe*	TG, SK, MM	+++	—
		Psyche	*Leptosia nina*	TG, SK, MM	+++	—
		Asian Cabbage White	*Pieris canidia*	TG, SK, MM	++	—
		Common Grass Yellow	*Eurema hecabe*	TG, SK, MM	+++	—
		Tree Yellow	*Gandaca harina*	TG, SK, MM	++	—
5	Lycaenidae	Common Imperial	*Cheritra freja*	TG, SK, MM	++	—

		Plains Cupid	*Chilades pandava*	TG, SK, MM	++	—
		Common Pierrot	*Castalius rosimon*	TG, SK, MM	++	—
		Red Pierrot	*Talicada nyseus*	TG, SK, MM	+++	—
		Apefly	*Spalgis epius*	TG, MM	+	—
		Slate Flash	*Rapala manea*	TG, SK, MM	++	—
		Pale Grass-Blue	*Pseudozizeeria maha*	TG, SK, MM	+++	—
		Common Hedge Blue	*Acytolepis puspa*	TG, MM	+	—
		Common Cerulean	*Jamides celeno*	TG, SK, MM	++	—
		Orchid Tit	*Chliaria othona*	SK	+	Schedule I (Part IV)
		Common Four-ring	*Ypthima huebneri*	TG, SK, MM	+++	—
6	Nymphalidae	Common Five-ring	*Ypthima baldus*	TG, SK, MM	++	—
		Striped Tiger	*Danaus genutia*	TG, SK, MM	++	—
		Plain Tiger	*Danaus chrysippus*	TG, SK, MM	++	—
		Blue Tiger	*Tirumala limniace*	TG, SK, MM	++	—
		Tawny Coster	*Acraea terpsicore*	TG, MM	++	—
		Painted Lady	*Vanessa cardui*	SK, MM	+	—
		Commander	*Moduza procris*	TG, MM	++	—
		Danaid Eggfly	*Hypolimnas misippus*	TG, SK, MM	++	Schedule II (Part II)
		Great Eggfly	*Hypolimnas bolina*	TG, SK, MM	++	—
		Common Sailor	*Neptis hylas*	TG, SK, MM	+++	—
		Extra Lascar	*Pantoporia sandaka*	TG, SK, MM	++	—
		Lemon Pansy	*Junonia lemonias*	TG, SK, MM	+++	—
		Grey Pansy	*Junonia atlites*	TG, SK, MM	+++	—
		Peacock Pansy	*Junonia almana*	TG, SK, MM	+++	—
		Common Indian Crow	*Euploea core*	TG, SK, MM	++	Schedule IV
		Common Baron	*Euthalia aconthea*	SK, MM	++	—
		Common Bushbrown	*Mycalesis perseus*	TG, SK, MM	+++	—
		Long-brand Bushbrown	*Mycalesis visala*	TG, SK, MM	++	—
		Common Castor	*Ariadne merione*	TG, MM	+	—
		Common Evening Brown	*Melanitis leda*	TG, SK, MM	+++	—

	Common Palmfly	*Elymnias hypermnestra*	TG, SK, MM	++	—
	Common Leopard	*Phalanta phalantha*	TG, SK, MM	++	—

(+++) = Very Common; (++) = Moderate; (+) = Rare
TG: Tea garden area, SK: Salkunja, MM: Magurmari

According to this list, *Appias libythea* is listed under Schedule IV, *Chliaria othona* is listed under Schedule I (Part IV), *Hypolimnas misippus* is listed under Schedule II (Part II), and *Euploea core* is listed under Schedule IV. Species richness was highest in the Magurmari area. During the study, the number of recorded genera varied among different butterfly families. Nymphalidae had the highest number with 17 genera, followed by Lycaenidae (10), Hesperidae and Pieridae (7), Papilionidae (3) and Riodinidae (1) had only one genus. In total, 45 genera were recorded throughout the study.

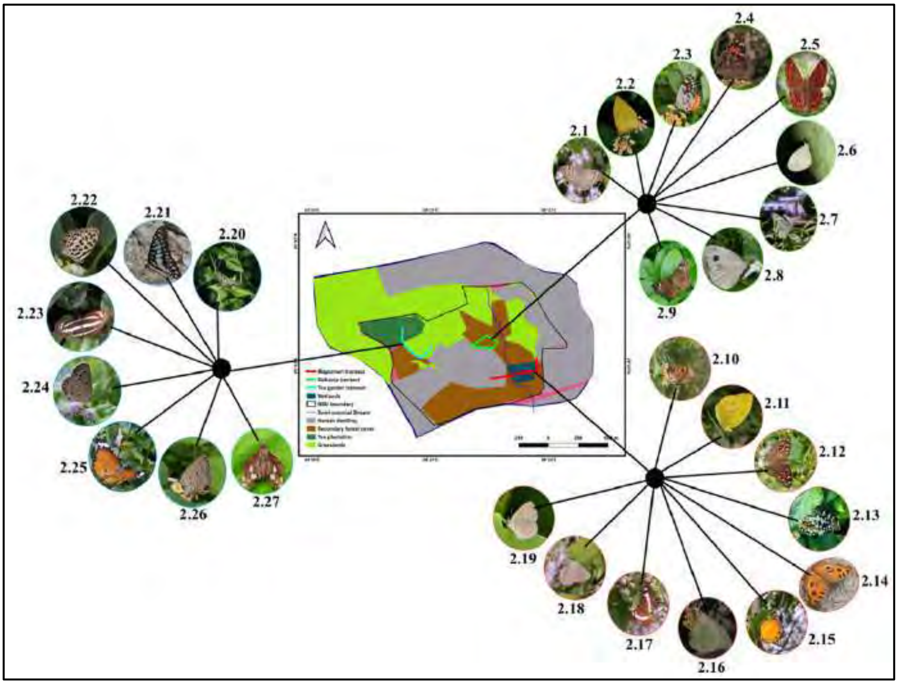

Figure 2. Different butterfly species recorded from the university campus. 2.1: *Junonia atlites*. 2.2: *Catopsilia pomona*. 2.3: *Talicada nyseus*. 2.4: *Vanessa cardui*. 2.5: *Zemeros flegyas*. 2.6: *Leptosia nina*. 2.7: *Appias libythea*. 2.8: *Pieris canidia*. 2.9: *Pseudocoladenia dan*. 2.10: *Acraea terpsicore*. 2.11: *Eurema hecabe*. 2.12: *Junonia lemonias*. 2.13: *Papilio demoleus*. 2.14: *Junonia almana*. 2.15: *Delias descombesi*. 2.16: *Catopsilia pyranthe*. 2.17: *Moduza procris*. 2.18: *Rapala manea*. 2.19: *Spalgis epius*. 2.20: *Graphium agamemnon*. 2.21: *Graphium doson*. 2.22: *Castalius rosimon*. 2.23: *Neptis hylas*. 2.24: *Pseudozizeeria maha*. 2.25: *Danaus chrysippus*. 2.26: *Chilades pandava*. 2.27: *Udaspes folus*.

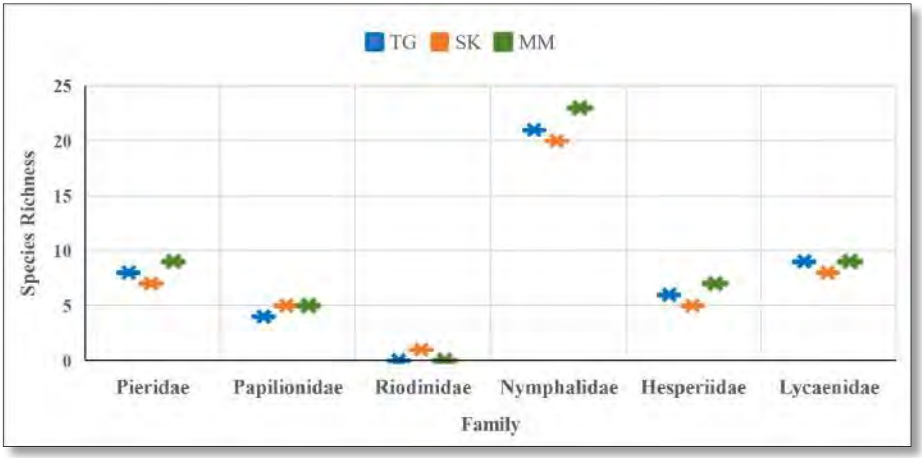

Figure 3. Comparative abundance of recorded butterfly species among different families from three study areas. (TG: Tea garden area, SK: Salkunja, MM: Magurmari).

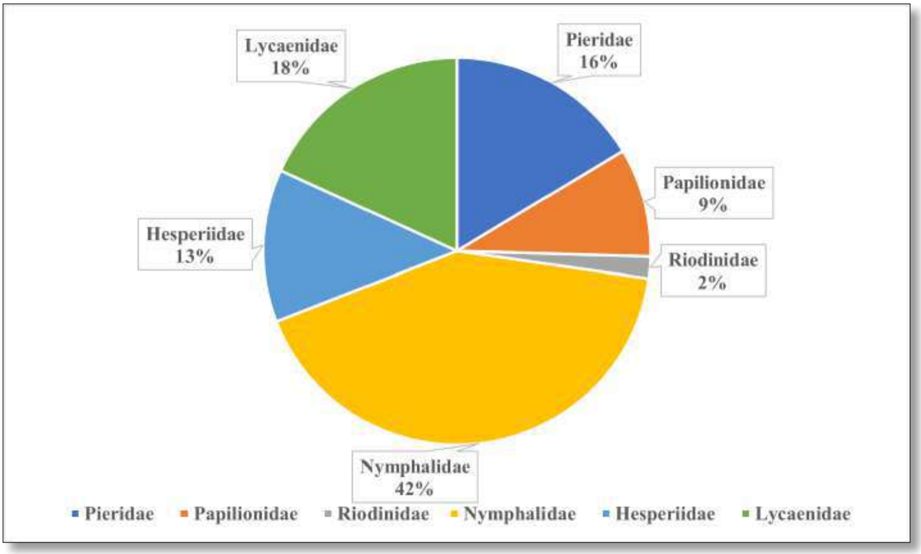

Figure 4. Family-wise percent distribution of butterflies from the university campus.

The university campus was found to be a significant habitat for butterflies as 55 different species of butterflies were observed during the preliminary study. The area contains a variety of cultivated and wild plant species that provide suitable hosts for egg laying, as well as nectar-producing plants that serve as a source of nectar. Previously, few studies regarding butterfly diversity were carried out in this district (Pradhan & Khaling, 2020). This study also reported similar types of butterfly species from this region.

Additionally, the butterfly families found in this study are consistent with previous research on the diversity of butterflies in West Bengal's diverse areas with comparable landscape patterns (Ghorai & Sengupta, 2014; Roy et al., 2022). Among all the families, Nymphalidae bore the highest number of species, maybe because the university campus provides a large number of their larval host plants (Table 2).

Table 2. Checklist of the larval host plant and nectar plant sources utilized by different families of butterfly reported from the University campus.

SL No.	Family	Larval Host Plants	Nectar Plants
01.	Riodinidae	*Maesa* spp.	-
02.	Papilionidae	*Polyalthia* sp., *Michelia* sp., *Annona* sp., *Citrus* spp., *Glycosmis* sp., *Aegle* sp., *Aristolochia* sp.	*Lantana* spp., *Ixora* sp., *Mussaenda* sp., *Jasminum* spp., *Cosmos* sp., *Zinnia* spp.
03.	Hesperiidae	*Achyranthes* sp., *Cocos* sp., *Oryza* sp., *Saccharum* sp., *Curcuma* spp., *Andropogon* sp.	*Lantana* spp., *Tridax* spp., *Vinca* sp., *Phaulopsis* sp., *Sida* sp.
04.	Pieridae	*Cleome* sp., *Bombax* sp. *Cassia* spp., *Cleome* sp., *Capparis* sp.	*Lantana* spp., *Ixora* sp., *Abutilon* sp., *Caesalpinia* sp., *Tridex* sp., *Bidens* sp., *Impatiens* spp., *Leucas* sp., *Crepis* spp.
05.	Lycaenidae	*Cinnamomum* sp., *Xylia* sp., *Cycas* sp., *Acacia* spp., *Ziziphus* sp., *Antidesma* sp., *Oxalis* sp., *Paracalyx* sp., *Saraca* sp., *Butes* sp., *Abrus* sp.	*Justicia* spp., *Sida* sp., *Tridax* sp., *Lantana* spp., *Glycosmis* sp.
06.	Nymphalidae	*Cynodon* sp., *Asclepias* sp., *Ceropegia* sp., *Cynanchum* spp., *Calotropis* sp., *Wattakaka* sp., *Passiflora* sp., *Artemisia* sp., *Blumea* sp., *Gnaphalium* sp., *Neolamarckia* sp., *Portulaca* sp., *Abutilon* sp., *Hibiscus* spp., *Moulluva* sp., *Dalbergia* sp., *Corchorus* sp., *Nerium* sp., *Ficus* sp., *Mangifera* sp., *Streblus* sp., *Oryza* sp., *Ricinus* sp., *Tragia* sp., *Panicum* sp., *Areca* sp., *Cocos* sp., *Calamus* spp., *Flacourtia* sp.	*Adelocaryum* sp., *Cipadessa* sp., *Zinnia* sp., *Tagetes* sp., *Lantana* sp., *Cosmos* spp., *Chromolaena* sp.

On the other hand, Riodinidae had the least number of butterfly species because of the lower number of larval host plants. Table 2 describes the checklist of family-wise larval host plants and nectar plant sources reported from the campus. The area with the highest species richness was found to be Magurmari, which may be attributed to its ecotone characteristics and the presence of wetlands and ponds. Another contributing factor could be the presence of a medicinal garden, which provides a significant number of host and nectar plants. Conversely, Salkunja exhibited the lowest species richness, which may be linked to the dominance of Sal trees over other host plants. Notably, the majority of the recorded butterflies were observed during the nectar collection of *Lantana* plants. These findings support that the university campus is conducive for butterfly diversity. As previously mentioned, four butterfly species have been identified as protected under the Wildlife (Protection) Act (WPA) of 1972 and efforts should be made for proper conservation. To ensure their conservation, steps must be taken to safeguard their habitats and the plants they rely on for nourishment. Human activities such as deforestation and pollution may have a negative impact on their behaviour and should be addressed accordingly.

Conclusion

It is vital to comprehend how habitat, climate and other biogeographic factors impact the diversity of butterflies in order to conserve these significant pollinators and safeguard the ecosystem in which they reside. As this was a preliminary study, more research should be done to compile a compact check list from this region. Butterfly species diversity may vary to some extent compared to other parts of this region. This study not only assisted in the making a checklist of butterflies from this district, it also helps to identify the host and nectar producing plants for conservation of butterflies.

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References

Brereton T, Roy DB, Middlebrook I, Botham M, Warren M. 2010. The development of butterfly indicators in the United Kingdom and assessments in 2010. *Journal of Insect Conservation, 15:* 139–151.

Ghorai N, Sengupta P. 2014. Altitudinal Distribution of Papilionidae Butterflies along with Their Larval Food Plants in the East Himalayan Landscape of West Bengal, India. *Journal of Biosciences and Medicines, 2* (1): 1–8.

Insect Identification Key: A Guide to the Insects. https://www.knowyourinsects.org/. (Electronic version accessed 21 June 2022).

Lindström SAM, Klatt BK, Smith HG, Bommarco R. 2018. Crop management affects pollinator attractiveness and visitation in oilseed rape. *Basic and Applied Ecology, 26:* 82–88.

Mukherjee K, Mondal A. 2020. Butterfly diversity in heterogeneous habitat of Bankura, West Bengal, India. *Journal of Threatened Taxa, 12* (8): 15804–15816.

Mukhopadhyay A, Biswa R, Khewa S, Prasad A, Basnet K. 2015. *Guide to the Birds of North Bengal University Campus*. Published by the Registrar: University of North Bengal, Darjeeling, West Bengal.

Pal A. 2017. Dragonflies and damselflies of University of North Bengal campus, West Bengal, India with new distribution record of *Agriocnemis kalinga Nair & Subramanian*, 2014. *Journal of Threatened Taxa, 9* (12): 11067–11073

Pollard E. 1977. A method for assessing changes in the abundance of butterflies. *Biological Conservation, 12* (2): 115–134.

Pradhan A, Khaling S. 2020. Butterfly diversity in an organic tea estate of Darjeeling Hills, eastern Himalaya, India. *Journal of Threatened Taxa, 12* (11): 16521–16530.

QGIS.org, %Y. QGIS Geographic Information System. QGIS Association. http://www.qgis.org

Roy GC, Miah A, Roy S, Roy D, Kar D, Banerjee S, Gupta M. 2022. Diversity and abundance of butterfly as an environmental indicator at Dinhata Subdivision, Cooch Behar, West Bengal, India. *Notulae Scientia Biologicae, 14* (1): 11156.

Saha GK, Das RP. 2012. Diversity, distribution, and ecology of butterfly communities of West Bengal. West Bengal Biodiversity Board, Department of Environment, Govt. of West Bengal.

Smetacek P. 2017. *A naturalist's guide to the butterflies of India, Pakistan, Nepal, Bhutan, Bangladesh and Sri Lanka*. John Beaufoy Publishing Limited

Tiple AD. 2011. Butterflies of Vidarbha region, Maharashtra State, central India. *Journal of Threatened Taxa, 3* (1): 1469–1477.

Westgate MJ, Tulloch AIT, Barton PS, Pierson JC, Lindenmayer DB. 2016. Optimal taxonomic groups for biodiversity assessment: a meta-analytic approach. *Ecography 40:* 539–548.

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Research Article

Influence of Water Depth on the Morphology Structure of Seagrass from the Southern of Peninsular Malaysia

Ng Kah Choon*

Department of Geography, Universiti Malaya, 50603 Kuala Lumpur, Wilayah Persekutuan Kuala Lumpur Kuala Lumpur Kuala Lumpur 50603, Malaysia.

*Corresponding author: kahchoon.15@gmail.com

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ABSTRACT

Globally, seagrass meadows have declined due to environmental factors and human activities, particularly by limiting light to seagrass in turbid coastal waters. Furthermore, publications of seagrass research findings from the Southeast Asia region are scarce, making understanding these habitats difficult despite their ecological and economic importance. This research aimed to provide mean and standard deviation of seagrass morphology, as well as to examine the morphology structures in response to water depth. Samples of two species of seagrass, *Halodule uninervis* and *Halophila ovalis*, were collected by using random sampling in a line transect at Pulau Besar and Pulau Tinggi in Johor, southern Malaysia, in September 2013 and April 2014. Six morphological features of each seagrass species were measured physically using CPCe software and the relationship of water depth to seagrass were evaluated using Pearson's Correlation. The result highlights that the leaf and root morphology is larger in Pulau Tinggi because it is nearer to the Johor mainland, where the introduction of nutrients from economic activity positively influence seagrass growth. The overall morphology structures of both species in both islands are greater in 2013 than 2014. For the relationship with water depth, it had greater positive relationship to H. univervis leaf width ($r = 0.7532$), internode width ($r = 0.6722$), leaf length ($r = 0.5739$); whereas for H. ovalis, water depth was correlated strongly with leaf width ($r = 0.6697$) and leaf surface area ($r = 0.6313$). The morphology of seagrass species varies depending on habitat conditions, this study can fill knowledge gaps, but more fundamental research on seagrass meadows is required particularly for the seagrasses in the Southeast Asia marine region.

Keywords: Halodule; Halophila; Morphology; Seagrass; Water depth

Introduction

Seagrass is a productive ecosystem in the world (Lin et al., 2018). They provide food for herbivores (Inoue et al., 2021), habitat for juvenile and aquatic ecosystems (Nakaoka & Supanwanich, 2000, Unsworth et al., 2019; Johan et al, 2020), and stabilize sediment of seabed (Christianen et al., 2013). Furthermore, seagrass offers nutritional and pharmaceutical values to local communities (Wisespongpand et al., 2022), as well as support local economic development (Praisankul & Nabangchang-Srisawalak, 2016).

Many previous studies indicated that the seagrass morphology is subject to the environmental parameters of different water depth (Short et al., 2001; van der Heide et al., 2010). Water depth and sunlight penetration are related to seagrass growth in terms of photosynthetic function (Duarte, 1991; Kenworthy & Fonseca, 1996; Enríquez et al., 2019). The maximum depth at which seagrass can grow was determined by the growth compensation irradiance (Hemminga & Duarte, 2000) and low amount of light penetration can also limit the efficiency of photosynthesis efficiency of seagrass (Short et al., 2001). For example, tropical seagrasses are adaptable to water depth of less than 10 meters (Short et al., 2001). In Malaysia, Ooi et al. (2011a) recorded seagrass meadows below 10 meters of water depth. In northeastern Queensland, there are 13 seagrass species under 6 meters and four seagrass species above 20 meters (Lee Long et al., 1996).

Morphological variation of seagrass, including leaf area, length and width and number of leaves per shoot, associated to differences of water depths have been observed (Short, 1983, Collier et al., 2007). A recent study of *Halophila stipulacea* shows that significantly larger (11 %) rhizome internodes and longer (19 %) and wider (15 %) leaves were observed in deeper plants compared to those inhabiting shallow meadows. In contrast, shoot and internode formation rates in shallow plants were markedly higher than in deep-adapted plants (Azcárate-García et al., 2020). Additionally, smaller leaves and lower leaf area index of *Halophila stipulacea* were found at shallower (1.8–2.0m depth) than deeper depth (3.0–4.0m) in Palinuro harbour, Mediterranean Sea (Di Genio et al., 2021).

In addition, the amount of light a seagrass species can tolerate varies greatly among species. For examples, *Halophila ovalis* has a low tolerance to light deprivation (Longstaff et al., 1999) whereas *Thalassia testudinum* can survive more than 5 months without sufficient sunlight (e.g., Lee & Dunton, 1997).

In Malaysia, there are 16 seagrass species with distinctive morphological features in leaves, rhizomes, and roots (Bujang et al., 2018). Loss of approximately 50.7 kg seagrass from 2009–2013 was reported in Merambong, Straits of Johor (Misbari & Hashim, 2016). Due to continuing decline of seagrass meadows, fundamental research on seagrass biology, ecology and conservation is needed for future assessment of marine community and restoration planning (Sievers et al., 2019). However, research is few in seagrass and their environment interaction, especially the relationship between morphology and environment parameters (Fortes et al., 2018).

Along the Johor offshore of southern Malaysia, two seagrass species, *Halodule uninervis* and *Halophila ovalis* are relatively common. Currently, the seagrass bed in this area has been greatly affected by coastal reclamation and garbage dumping (Unswoth et al., 2018; Huang et al., 2020). Accordingly, the habitat changes could potentially affect the morphology and ecology of seagrass. This present study aims to observe the relationship between water depth and morphology of *H. uninervis* and *H. ovalis*, which could provide fundamental information for future assessment and restoration programmes of Malaysian seagrass bed.

Materials and Methods

Study area

The present study was conducted in the seagrass meadows of Pulau Besar (Besar Island) (2°25’25”–2°26’00”N; 103°58’22”–103°59’16”E) and Pulau Tinggi (Tinggi Island) (2°16’55”–2°17’32”N; 104°06’10”–104°07’22”E), Johor, Malaysia (Figure 1), known as part of the Sultan Iskandar Marine Park. These islands are annually affected by monsoon seasons. Pulau Besar is about 16.49 km north off Pulau Tinggi. Pulau Tinggi is located 12 km off the southeast coast of Peninsula Malaysia and around 30 km southeast of Mersing (Azman et al., 2008). The distribution of seagrass in Pulau Tinggi is limited to depths of less than 10 m and the mean wave height around this island is 0.9 ± 0.2 m (Ooi et al., 2011a). The water in the study area is relatively shallow, with a mean depth of 5.0 m. Both islands receive a lot of rain each year (an average of 2500 mm), and the climate and seagrass meadows are both influenced by two tropical monsoons: the wet Northeast monsoon from November to March, and the dry Southwest monsoon from May to September (monthly average 120 mm) (Malaysian Meteorological Department 2016). It can be assumed that the water conditions for both islands are similar to other nearby islands within the marine park. Both islands have relatively high turbidity probably due to proximity to the mainland (Mohamed et al., 2015). The water parameters of Johor Marine Parks were within the standard range of the Malaysian Marine Quality Criteria and Standards. Although Pulau Tinggi and Pulau Besar are located about 8.0 km away from each other, the water temperature of this area was constantly between 28.0–31.6°C. The pH value was between 8.0–8.2 and salinity was between 26.12–33.10 ppt

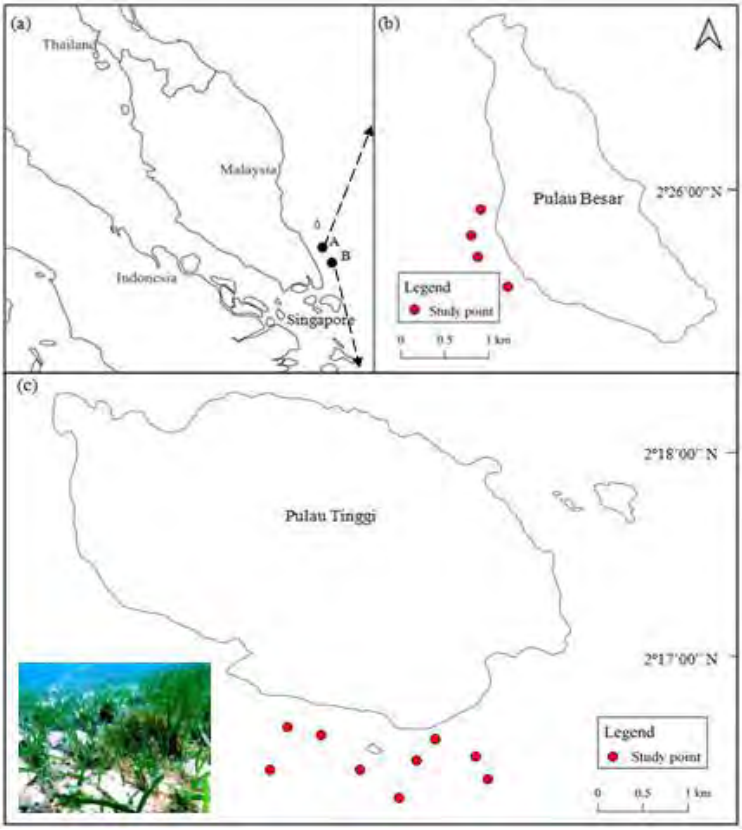

Figure 1. (a) Approximate location of Pulau Besar (A) and Pulau Tinggi (B) in Peninsular Malaysia, location of study points at Pulau Besar (b) and Pulau Tinggi (c), and the image of seagrass meadows in the study areas.

(Mohamed et al., 2015). The Marine Water Quality Status for Island (MDQI) value for Pulau Tinggi and Pulau Besar were 89.51 and 87.65 respectively (MDQI: 0-100; the study areas are in relatively good condition) (Department of Environment, 2015).

Pulau Tinggi and Pulau Besar are mainly tourist attractions and have less than 400 inhabitants and Mohamed et al. (2015), suggested that domestic waste and sewage contributed to the issue of heavy metals pollution in the marine waters, and the high turbidity of the study sites. From personal observation, the seabed is characterized as sandy and muddy. The sandy seabed of both islands has a high variation of seagrass species. Pulau Tinggi was reported to have high seagrass diversity between 3 and 6 m, covering of two dominant species, *H. uninervis* and *H. ovalis* (Ooi et al., 2011a).

Field sampling

The samples of *H. uninervis* and *H. ovalis* were collected by hand on 8–14 September 2013 and on 6–12 April 2014. Seagrass samples were collected at 4 sampling points at Pulau Besar and 9 sampling points at Pulau Tinggi, Johor. Seagrasses were collected by snorkeling during low tide or by SCUBA diving during high tide. Water depth was recorded. The sampling method was random sampling in a line transect perpendicular to shoreline for each study point. One line transect was placed in each sampling point. Five samples were collected for *H. uninervis* and *H. ovalis* in every transect. Each seagrass sample consisted of five shoots linked on a rhizome, beginning with the youngest shoots. The sediment and epiphytes attached to the samples were removed *in situ* with sea water prior to the physical measurement (Rattanachot et al., 2020).

Seagrass measurement

For every five-linked shoots seagrass, seagrass morphology was measured on the fourth shoot using vernier caliper. The morphology included in this study: 1. leaf length, 2. leaf width, 3. root length, 4. internodes length, 5. internodes width, and 6. leaf surface area (Figure 2). For leaf surface, the complete leaf without being grazed or broken was taken into account, the average value of leaf surface area for both species was taken if there were two or more leaves on the third shoot of seagrass. For both species, leaf length was measured from the tip to the end of the leaf blade without taking petiole into account, while leaf width was measured from the left to the right of the widest part of the leaf horizontally. The longest seagrass root was measured for each sample. Internode length was measured from the connecting point of shoot, rhizome and root of one shoot to the next shoot. Rhizome and internode are interchangeable for the present study. Internode diameter was measured on the middle part of each internode. The average values of both third and fourth internode length and rhizome width were calculated. The leaf images of the third shoot on samples were recorded digitally using camera. The leaf surface area was generated by using the digitized sample pictures in the CPCe (Coral Point Count with Excel extension) software (Kohler & Gill, 2006). The measurements were saved for further analyses to explore the relationship between water depths and the morphology of the studied seagrass. The water depths of sampling points were corrected according to the Tide Tables Malaysia 2014 (Nichols & Williams, 2009). Readings of water depth was obtained in situ by using a dive computer with recorded time and time for each sampling point. Water depths of sampling points were corrected according to the Tide Tables Malaysia. These tables provide information on heights and times of maximum and minimum water levels (Nichols, 2009).

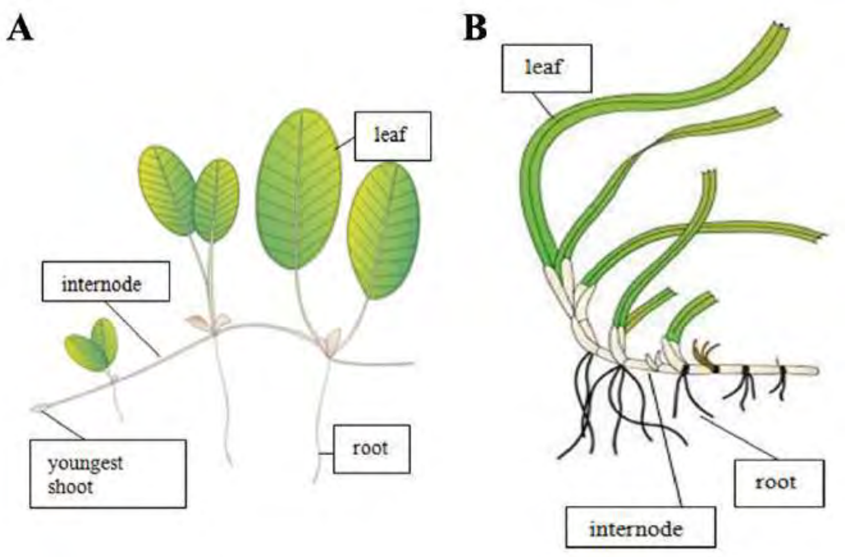

Figure 2. Measurement of seagrass (A) *Halophila ovalis* and (B) *Halodule uninervis* (adapted from Lelian et al., 2008).

Data analysis

The data were analyzed statistically using Pearson Correlation analysis in SAS/PC 9.3 software (Wang et al., 2017) to examine the relationship between water depth and morphological characteristics of seagrass samples. The tests were considered significant at p-value < 0.05.

Table 1. Interpretation of correlation coefficient and relationship level for this study (adapted from Wahyuni & Purwanto, 2020).

Correlation Coefficient (r)	Relationship Level
0.00-0.19	Negligible correlation
0.20-0.39	Weak correlation
0.40-0.59	Moderate correlation
0.60-0.79	Strong correlation
0.80-1.00	Very strong correlation

The correlation coefficient was interpreted using the standard shown in Table 1. The level of relationship between water depth and seagrass morphology has been classified into five categories: negligible correlation, weak correlation, moderate correlation, strong correlation, and very strong correlation for positive relationship and vice versa.

Results and Discussions

The physical characteristics of seagrass is presented in Table 2, the overall leaf and root structures of *H. uninervis* and *H. ovalis* were greater in Pulau Tinggi than Pulau Besar, whereas the internode structures of seagrass were higher in Pulau Besar.

Table 2 Mean and standard deviation of morphology of *H. uninervis* (*Hu*) and *H. ovalis* (*Ho*) from Pulau Tinggi and Pulau Besar between 2013 and 2014 (The greater value of morphology is highlighted in grey by comparing both island in the same year).

Morphological features (mean value)	2013				2014			
	Pulau Tinggi		Pulau Besar		Pulau Tinggi		Pulau Besar	
	Hu (n=35)	*Ho* (n=35)	*Hu* (n=35)	*Ho* (n=35)	*Hu* (n=35)	*Ho* (n=35)	*Hu* (n=35)	*Ho* (n=35)
Leaf length (mm)	68.86 ± 8.16	18.69 ± 1.56	47.98 ± 3.14	14.70 ± 0.76	59.68 ± 8.56	18.27 ± 1.55	41.78 ± 4.12	14.30 ± 0.42
Leaf width (mm)	3.98 ± 0.14	12.93 ± 0.51	2.67 ± 0.31	9.70 ± 0.69	3.58 ± 0.13	12.58 ± 0.48	2.27 ± 0.27	9.50 ± 0.52
Leaf surface area (mm^2)	4.45 ± 0.39	1.54 ± 0.16	3.36 ± 0.26	1.37 ± 0.15	4.05 ± 0.27	1.30 ± 0.10	2.76 ± 0.37	1.17 ± 0.12
Internode length (mm)	27.50 ± 2.73	28.93 ± 7.02	28.77 ± 4.25	31.44 ± 5.36	26.50 ± 2.23	27.91 ± 5.06	26.57 ± 4.14	31.04 ± 5.36
Internode width (mm)	1.58 ± 0.20	1.24 ± 0.14	1.63 ± 0.16	1.36 ± 0.15	1.18 ± 0.20	1.14 ± 0.14	1.33 ± 0.16	1.16 ± 0.15
Root length (mm)	50.99 ± 1.60	26.59 ± 3.48	48.08 ± 5.59	24.97 ± 2.78	47.85 ± 1.60	26.15 ± 3.48	46.95 ± 5.59	24.77 ± 2.78

Seagrass meadows were found in the water depth from 3.0 to 8.3m, which is within the range of <1.00–10.72m (Ooi et al., 2011a). Both species in Pulau Tinggi had greater leaf structures and root length than Pulau Besar. Pulau Tinggi is nearer to the Mersing mainland compared to Pulau Besar. The larger leaf and root of seagrass maybe due to the anthropogenic discharges of the industrial and domestic wastewater that causes high nutrition values for the plant growth in Pulau Tinggi marine area. Most internode structures of both species in Pulau Besar were greater than those collated in Pulau Tinggi except for internode width for *H. uninvervis*.

Relationship of water depth and seagrass morphology

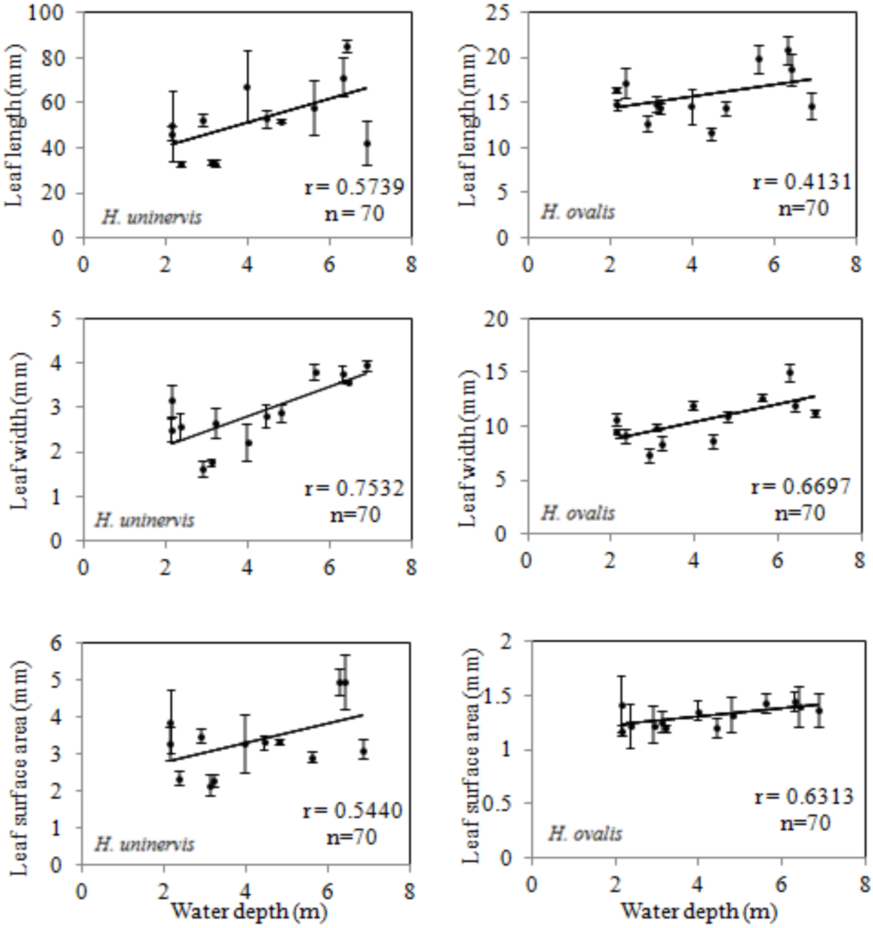

Figure 3 Pearson correlation of water depth and leaf morphology for *Halodule uninervis* and *Halophila ovalis*. The tests were considered significant at p-value < 0.05.

Internode morphology

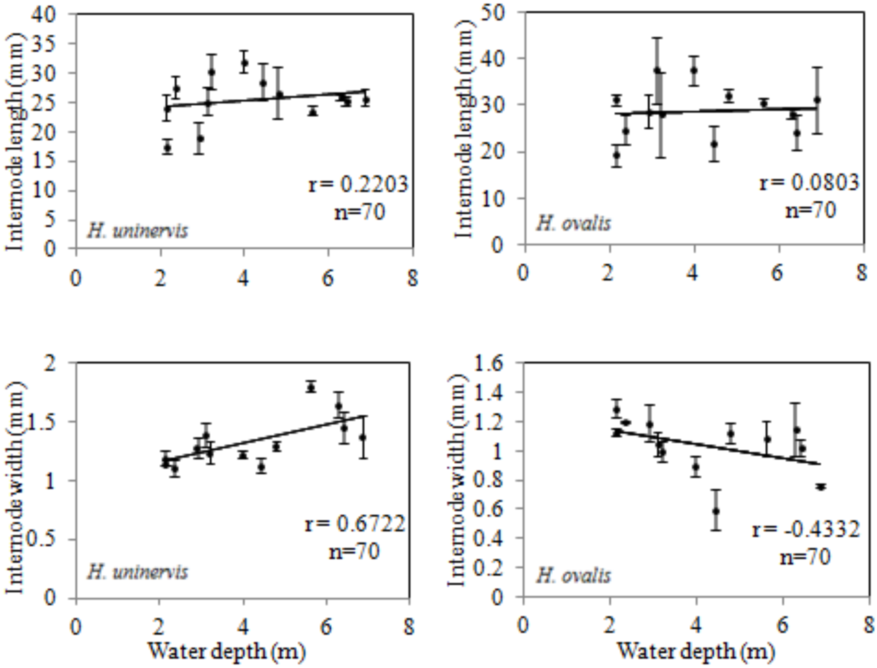

Figure 4. Pearson correlation of internode morphology for *Halodule uninervis* and *Halophila ovalis*. The tests were considered significant at p-value < 0.05.

Root morphology

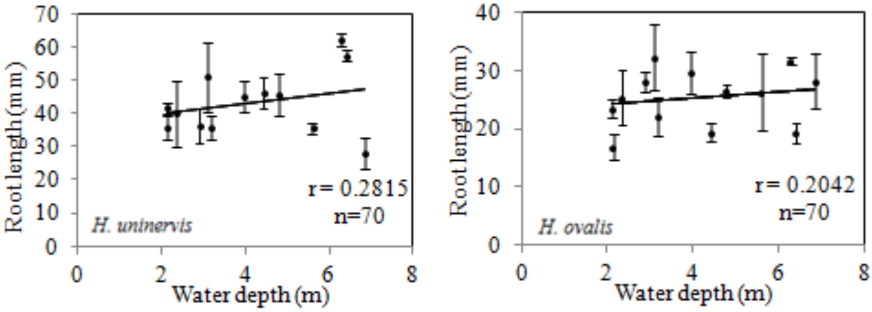

Figure 5. Pearson correlation of root morphology for *Halodule uninervis* and *Halophila ovalis*. The tests were considered significant at p-value < 0.05.

For *H. uninervis*, water depth had positive relationship to leaf length (r = 0.5739) and leaf width (r = 0.7532) (Figure 3), internode width (r = 0.6722) (Figure 4) and leaf surface area (r = 0.5440) (Figure 3); but it was less correlated to root length (0.2815) (Figure 5) and internode length (0.2203) (Figure 4). There was a negative correlation between water depth and seagrass decline in earlier research, but there were few fundamental research on the relationship between water depth and seagrass morphology, especially in Malaysia (Zhang et al., 2020).

For *H. ovalis*, water depth had strong relationship with leaf width (r = 0.6697) and leaf surface area (r = 0.6313) (Figure 3). Water depth had moderate relationship with leaf length (r = 0.4131) (Figure 3) and internode width (r = -0.4332) (Figure 4). Water depth had weak correlation with root length (r = 0.2042) (Figure 5) and no correlation with internode length (r = 0.0803) (Figure 4).

In the study area, Ooi et al. (2011) reported that H. *ovalis* (mean shoot density 1454.6 ± 145.1 m^{-2}) and *H. uninervis* (mean shoot density 861.7 ± 372.0 m^{-2}) growing in relatively low light conditions at 3 meter water depth. Water depth had a positive relationship with all leaf structures (leaf length, leaf width, leaf surface area) and internode width, but a weak relationship with internode length and root length in *H. uninervis*. For *H. ovalis*, water depth had a strong relationship with leaf width and surface area; a moderate relationship with leaf length and internode width (negative relationship); a weak relationship with root length; and no correlation with internode length. Water depth had a moderate relationship with leaf surface area for both *H. uninervis* and *H. ovalis*.

In the present study, seagrass morphology varied to water depth. The growth of seagrass required light for photosynthesis. This process was also affected by many environmental factors that were not included in this study, such as light intensity, surrounding temperature, salinity, and nutrient quality (Longstaff & Dennison, 1999). Previous research suggested that habitat conditions and seasons greatly impacted the seagrass morphological variation (Vermaat & Verhagen, 1996), but a recent study on *H. ovalis* shows reduction of about 32% in leaf surface area under high shading (68%) treatment (Kong et al., 2019). For example, the width of the leaves of *Thalassia testudinum* which decreased 26.6 % as depth increases in response to 41 % light reduction (Lee & Dunton, 1997). The average leaf length, rhizome elongation and photosynthetic rate of *H. ovalis* were higher in seawater with higher salinity level (S30) compared to river water with lower salinity level (S20) (Lamit & Tanaka, 2021). The morphological characteristics of seagrass are also greatly affected by human activities. Increasing turbidity level of 87.6% caused increased leaves length of 46.1%, leaf width of 27.5%, rhizome diameter of 36.8 % and root length of 12.6% for H. ovalis in Pulau Bintan, Indonesia. The concentration of nutrient such as nitrate and phosphate is higher near to the shore areas where domestic dumping is relatively higher than in the deeper water area which is less disturbed by anthropogenic factors. The increasing nutrient levels in the water caused by human activities lead to higher turbidity levels, high suspended sediment concentration and decreasing dissolved oxygen levels in the water. This is likely to cause regression of seagrass (Nugraha et al., 2020). Preliminary laboratory studies suggest that currents between 2 and 50 cm s^{-1} affect leaf production of *Zostera marina L.* under light-saturated conditions (Fonseca & Kenworthy, 1987).

Significant variations of leaf width of *H. ovalis* were found at different seasons and locations (Hedge et al., 2009). Environmental parameters such as salinity, disturbance, intertidal conditions, and water depth were also affected seagrass morphology. Furthermore, the study of seagrass leaf morphology revealed significant differences among populations (Bujang et al., 2006; Vy et al., 2013), but they are in the range of morphological parameters concluded by den Hartog (1970).

For *H. uninervis*, there was high correlation between water depth with leaf width and internode width, which showed that larger leaf surface was beneficial to obtain more sunlight in the deeper water. This result is in contrast to the hypothesis that the energy and nutritional costs of producing leaves are lower along a continuum of water depth. In this instance, the leaf surface area was therefore used as an indicator of the distribution of nutrients and energy as it grows larger in deeper sea beds (Sinden-Hempstead & Killingbeck, 1996).

In addition, water depth was weakly correlated to root length and internode length shows that these parts are less affected by light attenuation. However, the growth and morphology of these below ground structures are more affected by sediment composition and sedimental burial (Ooi et al., 2011b). Seagrass meadows receive organic inputs of various origins, and this organic matter forms a complicated mix of labile and more refractory compounds (Hemminga & Duarte, 2000). Therefore, the high complexity of sediment compounds leads to a weak relationship between water depth and internode length, which is often buried under the sand.

For *H. ovalis*, there was also high correlation between water depth and leaf width; water depth and leaf surface area whereas root length was weak correlated to the water depth. Water depth showed the variability best for leaf width, followed by leaf surface area, internode width, leaf length, root length, and internode length. This partially agreed with the findings on *Halodule wrightii* in Rio de Janeiro, where each seagrass morphological parameter varied depending on location and water depth. Similar to the reported study, seagrass variation in some characteristics was high within a single population (Creed, 1997).

Only the rhizome width of this species had a negative relationship with water depth. The complexity of nutrient contents in soil composition and wave movement at the study sites are factors that explain the negative relationship between water depth and internode width of *H. ovalis*. The leaf morphology of small and fast growing Halophila species varies greatly between study sites and is highly dependent on various environmental conditions (Emmclan et al., 2022), with substrate conditions significantly affected the growth of seagrass compared to other habitat variables (Zabarte-Maeztu et al., 2020).

Additionally, there was a moderate correlation between leaf surface area and water depth because the amount and quality of light available for photosynthesis determines how much seagrass can grow (Ziemen & Wetzel, 1980). Therefore, decreases in underwater lighting in the deep sea frequently cause widespread seagrass die-off (Short & Wyllie-Echeverria, 1996). As a result, the leaves of this species grew bigger at deeper water depths to catch more light for photosynthesis. The biggest leaf size of *H. ovalis* was noticed in the lower intertidal zone among three zones of upper intertidal, lower intertidal, and subtidal zones (Kaewsrikhaw et al., 2016). Masini and Manning (1997) found that certain plants had higher photosynthesis rate and lower light requirements in deeper water.

Seagrasses were also attached with epiphytes and the roots were tangled in the soil, the seagrass roots for these small species are fragile and easily broken when sampling and cleaning, so the removal of epiphytes and soils on the samples had to be done gently to prevent broken samples. Overgrazing activities are popular for the Johor Marine Park Islands due to high density of seagrass and coral habitats. Seagrasses of these study sites are popular for overgrazing other aquatic herbivores, so many samples were observed to have broken leaves when sampling which agreed with the previous observations (Bujang et al., 2006).

The result of this study is important for understanding the environmental factors that influence the growth of marine plants. For this study, only water depth was selected to study its relationship with the different morphology of two dominant seagrass species at Johor Marine Park islands. This study also aims to close the knowledge gaps in seagrass research and conservation in Southeast Asian countries, which are important for many tropical marine animals and which also provide significant economic value to island residents.

This study has several limitations. The sample is small and not representative of the broader seagrass population in the study area. Additionally, parameters of sunlight and wave movement aren't included in the study due to limited facilities and funding at the time of field survey. We are currently developing a more comprehensive and long-term study on the seagrass meadows of the study area. Additionally, the parameters of other variables will also be taken into account in the future study to provide an ongoing seagrass survey for the study area. As a result, future research on variations in seagrass morphology is required to fill knowledge gaps in the study area.

Conclusion

The overall leaf structures of *H. uninervis* and *H. ovalis* were greater in Pulau Tinggi than Pulau Besar in the same year. In terms of morphological structures, water depth had positive relationship to the morphological properties of *H. uninervis* (e.g., leaf length, leaf width, internode length and leaf surface area) whereas other structures had weak relationships with different water depth (e.g., root length and internode length). The ecological and biological functions of seagrass meadows are often underrated in Malaysia, therefore, understanding how seagrass morphology responds to water depth is essential for providing fundamental information in effective restoration and management of seagrass meadows.

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References

Azcárate-García T, Beca-Carretero P, Villamayor B, Stengel DB, Winters G. 2020. Responses of the seagrass Halophila stipulacea to depth and spatial gradients in its native region (Red Sea): morphology, in situ growth and biomass production. *Aquatic Botany*, *165:* 103252. DOI: 10.1016/j.aquabot.2020.103252.

Azman BAR, Ramlan O, Wan-Lotdi WM, Zaidi CC, Othman BHR. 2008. Seagrass biodiversity of Pulau Tinggi, Johor. In: *Malaysia Marine Ecosystem: The Studies of Johor Darul Takzim East Coast* (eds. C.A.R. Mohamed, F.K. Sahrani, M. Mohd. Ali, Z.C. Cob and N. Ahmad), pp. 53–57. Marine Ecosystem Research Centre (EKOMAR), Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia.

Bujang JS, Zakaria MH, Arshad A. 2006. Distribution and significance of seagrass ecosystems in Malaysia. *Aquatic Ecosystem Health and Management*, *9* (2): 203–214. DOI: 10.1080/14634980600705576.

Bujang JS, Zakaria MH, Short FT. 2018. Seagrass in Malaysia: Issues and challenges ahead. *The Wetland Book II: Distribution, description, and conservation, 1* (3): 1875–1883.

Carruthers TJB, Dennison WC, Kendrick GA, Waycott M, Walker DI and Cambridge ML. 2007. Seagrasses of south–west Australia: A conceptual synthesis of the world's most diverse and extensive seagrass meadows. *Journal of Experimental Marine Biology and Ecology*, *350* (1–2): 21–45.

Christianen MJ, van Belzen J, Herman PM, van Katwijk MM, Lamers LP, van Leent PJ, Bouma TJ. 2013. Low-canopy seagrass beds still provide important coastal protection services. *PloS ONE, 8* (5): e62413. DOI: 10.1371/journal.pone.0062413.

Collier CJ, Lavery PS, Masini RJ, Ralph PJ. 2007. Morphological, growth and meadow characteristics of the seagrass Posidonia sinuosa along a depth-related gradient of light availability. *Marine Ecology Progress Series, 337:* 103–115. DOI: 10.3354/meps337103.

Creed JC. 1997. Morphological variation in the seagrass Halodule wrightii near its southern distributional limit. *Aquatic Botany, 59* (1–2): 163–172. DOI: 10.1016/S0304-3770(97)00059-4.

den Hartog, C. 1970. *The Sea-Grasses of the World.* North-Holland Publishing Company, Amsterdam, Netherlands. 275 pp.

Department of Environment. 2015. *Malaysian environmental quality report: Chapter 5: Marine and island marine water quality, marine water quality status for island.* https://enviro2.doe.gov.my/ekmc/wp-content/uploads/2016/09/6-EQR-2015-Bab-5-1.pdf. Cited 02 Nov 2022.

Di Genio S, Gaglioti M, Meneghesso C, Barbieri F, Cerrano C, Gambi MC. 2021. Phenology and ecology of the alien seagrass Halophila stipulacea in its northern range limit in the Mediterranean Sea. *Aquatic Botany, 168:* 103304. DOI: 10.1016/j.aquabot.2020.103304.

Duarte CM. 1991. Seagrass depth limits. *Aquatic Botany*, *40:* 363–377. DOI: 10.1016/0304-3770(91)90081-F.

Emmclan LSH, Zakaria MH, Ramaiya SD, Natrah, Bujang JS. 2022. Morphological and biochemical responses of tropical seagrasses (Family: Hydrocharitaceae) under colonization of the macroalgae Ulva reticulata Forsskål. *PeerJ*, *10:* p.e12821. DOI: 10.7717/peerj.12821.

Enríquez S, Olivé I, Cayabyab N, Hedley JD. 2019. Structural complexity governs seagrass acclimatization to depth with relevant consequences for meadow production, macrophyte diversity and habitat carbon storage capacity. *Scientific Reports, 9:* 14657. DOI: 10.1038/s41598-019-51248-z.

Fonseca MS, Kenworthy WJ. 1987. Effects of current on photosynthesis and distribution of seagrasses. *Aquatic Botany*, *27* (1): 59–78. DOI: 10.1016/0304-3770(87)90086-6.

Fortes MD, Ooi JLS, Tan YM, Prathep A, Bujang JS, Yaakub SM. 2018. Seagrass in Southeast Asia: a review of status and knowledge gaps, and a road map for conservation. *Botanica Marina*, *61* (3): 269–288. DOI: 10.1515/bot-2018-0008.

Hayes MA, McClure EC, York PH, Jinks KI, Rasheed MA, Sheaves M, Connolly RM. 2020. The differential importance of deep and shallow seagrass to nekton assemblages of the Great Barrier Reef. *Diversity, 12* (8): 292. DOI: 10.3390/d12080292.

Hemminga MA, Duarte CM. 2000. *Seagrass Ecology.* Cambridge University Press, Cambridge, UK. 298 pp.

Huang Y, Xiao X, Xu C, Perianen YD, Hu J, Holmer M. 2020. Seagrass beds acting as a trap of microplastics-Emerging hotspot in the coastal region? *Environmental Pollution, 257:* 113450. DOI: 10.1016/j.envpol.2019.113450.

Inoue H, Mizutani A, Nanjo K, Tsutsumi K, Kohno H. 2021. Fish assemblage structure response to seagrass bed degradation due to overgrazing by the green sea turtle Chelonia mydas at Iriomote Island, southern Japan. *Ichthyological Research, 68* (1): 111–125. DOI: 10.1007/s10228-020-00775-1.

Johan I, Abu Hena MK, Idris MH, Amin SMN, Denil NA, Kumar U, Karim NU. 2020. Species composition and diversity of fishes from the seagrass habitat of Lawas, Sarawak, Malaysia. *Journal of Environmental Biology*, *42:* 1382–1389. DOI: 10.22438/jeb/41/5(SI)/MS_32.

Kaewsrikhaw R, Ritchie RJ, Prathep A. 2016. Variations of tidal exposures and seasons on growth, morphology, anatomy and physiology of the seagrass Halophila ovalis (R. Br.) Hook. f. in a seagrass bed in Trang Province, Southern Thailand. *Aquatic Botany, 130:* 11–20. DOI: 10.1016/j.aquabot.2015.12.006

Kenworthy W, Fonseca M. 1996. Light requirements of seagrasses *Halodule wrightii* and Syringodium filiforme derived from the relationship between diffuse light attenuation and maximum depth distribution. *Oceanographic Literature Review, 4* (44): 374–375.

Kohler KE, Gill SM. 2006. Coral Point Count with Excel extensions (CPCe): A Visual Basic program for the determination of coral and substrate coverage using random point count methodology. *Computers and Geosciences*, *32* (9): 1259–1269. DOI: 10.1016/j.cageo.2005.11.009.

Kong E, Ow YX, Lai S, Yaakub SM, Todd P. 2019. Effects of shading on seagrass morphology and thermal optimal of productivity. *Marine and Freshwater Research*, *71* (8): 913–921. DOI: 10.1071/MF19173.

Lamit N, Tanaka Y. 2021. Effects of river water inflow on the growth, photosynthesis, and respiration of the tropical seagrass *Halophila ovalis. Botanica Marina, 64* (2): 93–100. DOI: 10.1515/bot-2020-0079

Lee K, Dunton KH. 1997. Effects of in situ light reduction on the maintenance, growth and partitioning of carbon resources in Thalassia testudinum Banks ex Konig. *Journal of Experimental Marine Biology and Ecology, 234:* 1–27. DOI: 10.1016/S0022-0981(96)02720-7.

Lee Long WJ, Coles RG, McKenzie LJ. 1996. Deepwater seagrasses in northeastern Australia-how deep, how meaningful? In: *Seagrass Biology: Proceedings of an International Workshop held on 25-29 January 1996 in Rottnest Island, Western Australia* (eds. J. Kuo, R.C. Philips, D.I. Walker and H. Kirkman), pp. 41–50. University of Western Australia, Perth,Australia.

Lelian E, Fazrullah Rizally AR, Ang ANF. 2008. *Rumput laut perairan Sabah.* Dewan Bahasa dan Pustaka Kuala Lumpur, Malaysia. 106 pp.

Longstaff BJ, Loneragan NR, O'Donohue MJ, Dennison WC. 1999. Effects of light deprivation on the survival and recovery of the seagrass *Halophila ovalis* (R.Br.) Hook. *Journal of Experimental Marine Biology and Ecology*, *234* (1): 1–27. DOI: 10.1016/S0022-0981(98)00137-3.

Longstaff BJ, Dennison WC. 1999. Seagrass survival during pulsed turbidity events: the effects of light deprivation on the seagrasses Halodule pinifolia and Halophila ovalis. *Aquatic Botany*, *65:* 105–121. DOI: 10.1016/S0304-3770(99)00035-2.

Malaysian Meteorological Department. 2016. Buletin Cuaca Bulanan. http://www.met.gov.my. Cited 22 October 2022.

Masini RJ, Manning CR. 1997. The photosynthetic responses to irradiance and temperature of four meadow-forming seagrasses. *Aquatic Botany, 58:* 21–36. DOI: 10.1016/S0304-3770(97)00008-9.

Mazlan H, Dalhatu AS, Nurul NY. 2022. Appraisal of seagrass aboveground biomass changes using satellite data within the tropical coastline of Peninsular Malaysia. *Geocarto International*, *37* (18): 5453–5478. DOI: 10.1080/10106049.2021.1917007.

Misbari S, Hashim M. 2016. Change detection of submerged seagrass biomass in shallow coastal water. *Remote Sensing*, *8* (3): 200. DOI: 10.3390/rs8030200.

Mohamed KN, May MSY, Zainuddin N. 2015. Water quality assessment of marine park islands in Johor, Malaysia. *Bulletin of Environmental Science and Management*, *3* (2): 19–27.

Nichols CR, Williams RG. 2009. *Encyclopedia of Marine Science.* Facts On File, Incorporation, USA. 624 pp.

Nakaoka M, Supanwanich C. 2000. Quantitative estimation of the distribution and biomass of seagrasses at Haad Chao Mai National Park, Trang Province, Thailand. *Journal of Fisheries and Environment*, *22:* 10–22.

Nugraha AH, Hazrul H, Susiana S, Febrianto T. 2020. Morphology characteristic and growth of *Halophila ovalis* at some coastal area in Bintan Island. (Karakteristik morfologi dan pertumbuhan lamun *Halophila ovalis* pada beberapa kawasan pesisir Pulau Bintan.) *Depik-Jurnal Ilmu Ilmu Perairan, Pesisir, dan Perikanan, 9* (3): 471–477. DOI: 10.13170/depik.9.3.17781

Ooi JLS, Kendrick GA, van Niel KP, Affendi YA. 2011a. Knowledge gaps in tropical Southeast Asian seagrass systems. *Estuarine, Coastal and Shelf Science*, *92* (1): 118–131. DOI: 10.1016/j.ecss.2010.12.021.

Ooi JLS, Kendrick GA, van Niel KP. 2011b. Effects of sediment burial on tropical ruderal seagrasses are moderated by clonal integration. *Continental Shelf Research*, *31* (19–20): 1945–1954. DOI: 10.1016/j.csr.2011.09.005

Praisankul S, Nabangchang-Srisawalak O. 2016. The Economic Value of Seagrass Ecosystem in Trang Province, Thailand. *Journal of Fisheries and Environment, 40* (3): 138–155.

Rattanachot E, Stankovic M, Tuntiprapas P, Prempree S, Prathep A. 2020. Monitoring of seagrass along southern Andaman coast of Thailand. *Ecological Research, 35* (5): 773–779. DOI: 10.1111/1440-1703.12123.

Short FT. 1983. The seagrass, Zostera marina L.: plant morphology and bed structure in relation to sediment ammonium in Izembek Lagoon, Alaska. *Aquatic Botany*, *16* (2): 149–161. DOI: 10.1016/0304-3770(83)90090-6

Short FT, Coles RG, Pergent-Martini C. 2001. Global seagrass distribution. In: *Global Seagrass Research Methods* (eds. F.T. Short and R.G. Coles), pp. 5–30. Elsevier, Amsterdam, Netherlands.

Short FT, Wyllie-Echeverria S. 1996. Natural and human-induced disturbance of seagrasses. *Environmental Conservation, 23* (1):17–27.

Sinden-Hempstead M, Killingbeck KT. 1996. Influences of water depth and substrate nitrogen on leaf surface area and maximum bed extension in Nymphaea odorata. *Aquatic Botany*, *53:* 151–162. DOI: 10.1016/0304-3770(96)01020-0.

Sievers M, Brown CJ, Tulloch VJ, Pearson RM, Haig JA, Turschwell MP, Connolly RM. 2019. The role of vegetated coastal wetlands for marine megafauna conservation. *Trends in Ecology and Evolution*, *34* (9): 807–817. DOI: 10.1016/j.tree.2019.04.004.

Unsworth RK, Ambo-Rappe R, Jones BL, La Nafie YA, Irawan A, Hernawan UE, Moore AM, Cullen-Unsworth LC. 2018. Indonesia's globally significant seagrass meadows are under widespread threat. *Science of the Total Environment, 634:* 279–286. DOI: 10.1016/j.scitotenv.2018.03.315.

Unsworth RK, Nordlund LM, Cullen-Unsworth LC. 2019. Seagrass meadows support global fisheries production. *Conservation Letters*, *12* (1): e12566. DOI: 10.1111/conl.12566.

Valentine JF, Duffy JE. 2006. The central role of grazing in seagrass ecology. In: *Seagrasses: Biology, Ecology and Conservation* (eds. A.W.D. Larkum, R.J. Orth and C.M. Duarte), pp. 1–24. Springer, Amsterdam, Netherlands.

Van Der Heide T, Bouma TJ, Van Nes EH, Van De Koppel J, Scheffer M, Roelofs JG, Van Katwijk MM, Smolders AJ. 2010. Spatial self-organized patterning in seagrasses along a depth gradient of an intertidal ecosystem. *Ecology*, *91:* 362–369. DOI: 10.1890/08-1567.1.

Vermaat JE, Verhagen FC. 1996. Seasonal variation in the intertidal seagrass Zostera noltii Hornem.: coupling demographic and physiological patterns. *Aquatic Botany*, *52* (4): 259–281. DOI: 10.1016/0304-3770(95)00510-2.

Vy NX, Holzmeyer L, Papenbrock J. 2013. New record of the seagrass species Halophila major (Zoll.) Miquel in Vietnam: evidence from leaf morphology and ITS analysis. *Botanica Marina*, *56* (4): 313–321. DOI: 10.1515/bot-2012-0188.

Wahyuni TS, Purwanto KK. 2020. Student’s conceptual understanding on acid-based titration and its relationship with drawing skills on a titration curve. *Journal of Physics Conference Series*, *1440* (1): 012018. DOI: 10.1088/1742-6596/1440/1/012018.

Wang W, Ndungu AW, Li Z, Wang J. 2017. Microplastics pollution in inland freshwaters of China: a case study in urban surface waters of Wuhan, China. *Science of the Total Environment*, *575:* 1369–1374. DOI: 10.1016/j.scitotenv.2016.09.213.

Wisespongpand P, Khantavong A, Phothong P, Wanghom W. 2022. Antimicrobial, antioxidant, and antifouling activity from extracts of aboveground and belowground parts of seagrasses Cymodocea rotundata and Cymodocea serrulata. *Journal of Fisheries and Environment*, *46* (1): 37–53.

Zabarte-Maeztu I, Matheson FE, Manley-Harris M, Davies-Colley RJ, Oliver M, Hawes I. 2020. Effects of fine sediment on seagrass meadows: a case study of *Zostera muelleri* in Pāuatahanui Inlet, New Zealand. *Journal of Marine Science and Engineering*, *8* (9): 645. DOI: 10.3390/jmse8090645.

Zhang X, Zhou Y, Adams MP, Wang F, Xu S, Wang P, Liu P, Liu X, Yue S. 2020. Plant morphology and seed germination responses of seagrass (*Zostera japonica*) to water depth and light availability in Ailian Bay, northern China. *Marine Environmental Research*, *162:* 105082. DOI: 10.1016/j.marenvres.2020.105082.

Zieman JC, Wetzel RG. 1980. Productivity in seagrasses: methods and rates. In: *Handbook of Seagrass Biology: An Ecosystem Perspective* (eds. R.C. Phillips and C.P. McRoy), pp 87–116. Garland STPM Press, New York, US.

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Research Article

Effects of *Andrographis paniculata* on Carbon Tetrachloride (CCl_4)-Mediated Renal Oxidative Damage in Rats

Mohammad Iqbal*, Senty Vun-Sang, Pei-Hoon Koh

Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.

*Corresponding author: miqbal@ums.edu.my or driqbalmohammad@gmail.com
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ABSTRACT

A herbal medicinal plant known as *Andrographis paniculata*, or "*hempedu bumi*," is recognised for its numerous medicinal properties and role in promoting community health. Despite its widespread use, the potential nephroprotective effects and underlying mechanism of action of *Andrographis paniculata* remain unexplored. To address this gap, the present study aimed to investigate the nephroprotective effects of *Andrographis paniculata* against renal oxidative damage induced by carbon tetrachloride (CCl_4) in rats. Sprague-Dawley rats were pre-treated with *Andrographis paniculata* extract via gavage (100, 200, and 300 mg/kg b.w., respectively) once daily for 14 days, followed by two doses of CCl_4 (1.2 ml/kg b.w.) on the 13th and 14th days. After two weeks, rats were sacrificed, and a nephroprotective analysis was performed. CCl_4 administration at a dose of 1.2 ml/kg body weight resulted in oxidative stress in the renal system, as evidenced by elevated lipid peroxidation levels (TBARS). This oxidative stress was accompanied by a significant decrease in the activities of antioxidant enzymes and a depletion in the levels of reduced glutathione ($p<0.05$). Histopathological examination confirmed the impairment of renal function. *Andrographis paniculata* significantly mitigated the majority of these alterations. Based on our research, the nephroprotective advantages of *Andrographis paniculata* can be attributed to its ability to act as an antioxidant and scavenge free radicals.

Keywords: Oxidative stress; *A. paniculate*; nephrotoxicity; carbon tetrachloride

Introduction

Reactive oxygen species (ROS), including O2•-, H2O2, and •OH, are continuously produced within the human body due to exposure to exogenous environmental chemicals and various endogenous metabolic processes involving redox enzymes and bioenergetics electron transfer. Under normal circumstances, the body's antioxidant defence system efficiently detoxifies these ROS. However, when there is excessive production of ROS or an inadequate antioxidant defence, oxidative stress occurs. Oxidative stress is a condition that occurs when there is an imbalance between the production of free radicals and the ability of the body to neutralize them with antioxidants. Free radicals are unstable molecules that can cause damage to the cells and tissues of the body (Aranda-Rivera et al., 2022). This oxidative stress leads to the attack of ROS on various biomolecules such as proteins, lipids, mitochondria, lipoproteins, and DNA, ultimately resulting in oxidative damage (Hajam et al., 2022). Oxidative damage is a term that refers to the harmful effects of free radicals on the cells and tissues of the body. Oxidative damage can affect various components of the cell, such as DNA, proteins, lipids, and membranes. Oxidative damage can lead to mutations, inflammation, cell death, and tissue dysfunction. Oxidative damage can be prevented or reduced by antioxidants, which are substances that can donate an electron to free radicals and neutralize them. The body can produce antioxidants or obtain them from dietary sources, such as fruits and vegetables. Antioxidants can also repair some of the damage caused by free radicals (Sies, 2020; Aranda-Rivera et al., 2022). Oxidative stress contributes to the development of several chronic human diseases, including diabetes mellitus (Zhang et al., 2020; Darenskaya et al., 2021), cancer (Klaunig, 2018; Jelic et al., 2021), atherosclerosis (Poznyak et al., 2020; Batty et al., 2022), arthritis (Phull et al., 2018; Kaur et al., 2021), neurodegenerative diseases (Singh et al., 2019; Teleanu et al., 2022), and the ageing process (Romano et al., 2010; Martemucci et al., 2022).

Carbon tetrachloride (CCl_4) is a well-established hepatotoxicity model that has been extensively studied (Clemens et al., 2019). However, it is important to note that CCl_4 can also induce toxicity in other organs, such as the kidney, heart, and brain (Unsal et al., 2020). Due to its toxicity, CCl_4 is commonly employed by researchers as a screening model to evaluate the protective effects of natural products or compounds against tissue damage caused by CCl_4. In the liver, CCl_4 is metabolised by the P450 enzyme system, forming a highly reactive trichloromethyl radical (CCl3•). This radical initiate lipid peroxidation of the cytoplasmic membrane phospholipids leads to physiological and morphological changes in hepatocytes, ultimately causing liver injury (Arroyave-Ospina et al., 2021; Vun-Sang et al., 2022).

Andrographis paniculata, a member of the Acanthaceae family, is commonly referred to as the "King of the Bitters" or "*hempedu bumi*." It has been traditionally used to treat various ailments, including respiratory infections, fever, digestive disorders, and is a general immune booster. It is often used in traditional medicine systems like Ayurveda and Traditional Chinese Medicine (TCM). The active compounds in *A. paniculata* are diterpene lactones known as andrographolides (Akowuah et al., 2008, 2009). This medicinal herb possesses substantial therapeutic potential for managing a range of illnesses and promoting general well-being, as indicated by multiple studies (Zaridah et al., 2001; Singha et al., 2003, 2007; Reyes et al., 2006; Sheeja et al., 2007; Hossain et al., 2007; Neogy et al., 2008; Akowuah et al., 2008, 2009). In traditional medicine, it is used for treating infectious fevers, colic pain, and liver diseases (Singha et al., 2007; Akowuah et al., 2008, 2009). Numerous studies have explored the biological and pharmacological effects of *A. paniculata*, including antimicrobial properties (Singha et al., 2003), anti-filarial activity (Zaridah et al., 2001), antiangiogenic effects (Sheeja et al., 2007), antidiabetic potential (Reyes et al., 2006; Hossain et al., 2007; Dandu & Inamdar, 2009), antioxidant properties (Neogy et al., 2008; Akowuah et al., 2008, 2009), as well as anticancer and antitumor activities (Trivedi & Rawal, 1998; Kumar et al., 2004; Verma & Vinayak, 2007). However, to date, no investigations have been conducted to explore the nephroprotective activity of *A. paniculata* and its underlying mechanism of action. Therefore, this study aimed to assess the nephroprotective effects of *A. paniculata* and elucidate its mechanism of action against CCl_4-induced oxidative renal damage.

Materials and Methods

Chemicals and Reagents

Oxidizied glutathione, thiobarbituric acid, tricholoacetic acid, dithionitrobenzoic acid, 1-chloro 2,4 dinitrobenzene, hydrogen peroxide, folin-Ciocalteu reagent (FCR), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium nitrate ($NaNO_3$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), 2, 2-diphenyl-2-picrylhadrazyl (DPPH), gallic acid, catechol, and ferric chloride (FeCl3) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alcohol, acid alcohol, blue buffer, eosin, haema-toxylin, xylene and DPX mounting medium for histological assessment were purchased from Leica Biosystem (Wetzlar, Germany).

Plant Material

A. paniculata was obtained from Papar (5.48943°N, 115.80992°E), Sabah, and its identification and authentication were conducted by Mr Jonny Gisil, a botanist from the Institute for Tropical Biology and Conservation (ITBC) at Universiti Malaysia Sabah (UMS). The plant sample (voucher no: KPH003) was deposited at ITBC Herbarium, UMS, for future reference.

Preparation of Plant Extract

The aerial parts of *A. paniculata* were thoroughly rinsed with tap water before being dried in an oven at 35°C to 40°C. Once dried, the plants were finely ground using a blender to obtain a powdered form. For the extraction process, 5.0 g of the dried powder was mixed with 100 ml of 80% ethanol and allowed to extract for 4 hours at 40°C. The resulting mixture was then centrifugated and filtered through Whatman paper No. 1 (Whatman, Maidstone, Kent, UK). The filtrate was concentrated using Rotavapour® and then lyophilised. To ensure preservation, the lyophilised powder was stored in a dark bottle at a temperature of -80°C until it was ready for use.

Total Phenolic Content

The Folin-Ciocalteau method (Velioglu et al., 1998) was used to determine total phenolic content. A mixture consisting of Folin-Ciocalteau's reagent, 1.5 ml (1:10), and plant extract 200 µl was prepared. The mixture was thoroughly mixed and wait for 5 minutes at room temperature. Next, sodium carbonate (Na_2CO_3) 1.5 ml (60 g/l) was added to the mixture. Following an incubation period of 90 minutes in the dark at room temperature, the absorbance was measured at 725 nm against a blank using a spectrophotometer. Gallic acid served as the standard for determining the phenol content via the Folin-Ciocalteu method (Velioglu et al., 1998). The results were expressed in gallic acid equivalents (GAE) per gram of the sample. All measurements were performed in triplicate, and the mean values and standard deviations were calculated.

2, 2-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Assay

The ability to scavenge the stable free radical, DPPH was assessed (Hatano et al., 1988). All extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml. Different concentrations of the plant extracts (0.3mL) were mixed with 2.7 mL of an ethanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was vigorously shaken and allowed to stand in the dark for 60 minutes. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm using a spectrophotometer (model 4001/4). The radical scavenging activity (RSA) was expressed as a percentage of DPPH decolourisation using the equation: % RSA = ((Acontrol × Asample)/Acontrol) × 100, where Acontrol is the absorbance of the solution without the extract and Asample is the absorbance of the solution with extract at different concentrations. The extract concentration that caused 50% inhibition (EC50) was calculated from the plot of RSA percentage versus extract concentration. Ascorbic acid was used as the standard, and triplicate measurements were conducted.

Animals

Animal experiments were conducted in compliance with standard ethical principles, adhering to university regulations and federal laws regarding animal experimentation (Animal Ethics Committee (AEC): UMS/IP7.5/M3/4-2012). Adult male Sprague-Dawley rats, aged 8-12 weeks and weighing 120-150 g were obtained from the Animal House facility at the Health Campus of Universiti Sains Malaysia. The rats were housed in a controlled environment with a temperature of 25±2°C, a 12-hour light-dark cycle, and allowed to acclimatise for one week before the experiments. Food and water were provided *ad libitum* throughout the study. The freeze-dried extract was dissolved in distilled water daily before oral administration using a gavage method.

Experimental Protocol

To investigate the effects of *A. paniculata* extract on CCl_4-induced oxidative renal damage, antioxidant enzymes, and histopathological changes, a total of thirty adult male Sprague-Dawley rats (8-12 weeks old) weighing 120-150 g were randomly divided into five groups, with six rats in each group. Group I served as the control and received saline. Group II was administered two doses of CCl_4 (1.2 ml/kg b.w.) dissolved in a 1:1 corn oil solution via oral gavage on the 13th and 14th days. Groups III, IV, and V were pre-treated with *A. paniculata* extract orally via gavage at selected doses of 100 mg/kg b.w., 200 mg/kg b.w., and 300 mg/kg b.w., respectively, for 14 days, followed by two doses of CCl_4 (1.2 ml/kg b.w.) on the 13th and 14th days. All animals were euthanised 24 hours after the last dose of CCl_4 or saline within one hour. Renal tissues were promptly collected, carefully cleaned to remove extraneous material, and immediately perfused with ice-cold saline solution (0.85% w/v sodium chloride). These samples were then used for biochemical and histopathological analyses to evaluate renal function impairment. Doses of *A. paniculata* were selected based on previously published data (Koh et al., 2011).

Preparation of Renal Post-Mitochondrial Supernatant (PMS)

We followed the standard procedure described by Mohandas et al. (1984) for all biochemical estimations to prepare tissue fractionations.

Determination of Renal Lipid Peroxidation

To assess lipid peroxidation in renal PMS, we employed a modified version of the method described by Buege & Aust (1978), wherein the rate of TBARS (malondialdehyde equivalents) production was measured. The absorbance at 535 nm was measured using a spectrophotometer. The results were expressed as nmol MDA formed per gram of tissue, employing a molar extinction coefficient of 1.56×10^5 M-1cm-1.

Determination of Renal Glutathione Reduced

The method described by Jollow et al. (1974) was utilised to determine the concentration of reduced glutathione in the kidney. The development of yellow colour was observed, and the absorbance was immediately read at 412 nm using a spectrophotometer. The results were expressed as µmol of reduced GSH per gram of tissue, employing a molar extinction coefficient of 13.6×10^3 M-1cm-1.

Assays of Renal Antioxidant Enzymes

a) Determination of Catalase (CAT) activity

The method of Claiborne (1985) was used to measure the catalase (CAT) activity. The reaction mixture consisted of 0.01 ml PMS (10% w/v), 0.99 ml phosphate buffer (0.1 M, pH 7.4), and 1.0 ml H_2O_2 (0.019 M). The absorbance change was monitored at 240 nm every 30 seconds for 3 minutes. The result was expressed as the amount of H_2O_2 consumed per minute per mg protein, using the molar extinction coefficient of 6.4 × 10/M/cm.

b) Determination of glutathione peroxidase (GPx) activity

The activity of glutathione peroxidase (GPx) was determined by following the method of Mohandas et al. (1984). The reaction solution consisted of 0.01 ml PMS (10% w/v), 1.58 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (0.5 mM, pH 8.0), 0.1 ml sodium azide (1.0 mM), 0.1 ml GSH (1.0 mM), 0.1 ml NADPH (0.1 mM), and 0.01 ml hydrogen peroxide (30%) in a total volume of 2.0 ml. The reduction of NADPH at 340 nm was measured for 3 minutes at 30 seconds interval. Enzyme activity was calculated as the amount of NADPH consumed per minute per mg protein, using the molar extinction coefficient 6.22×10^3 /M/cm.

c) Determination of glutathione reductase (GR) activity

The method of Carlberg & Mannervik (1975) was used to determine the activity of glutathione reductase (GR). The assay mixture contained 0.025 ml PMS (10% w/v), 1.725 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1.0 mM), and 0.1 ml NADPH (0.1 mM). The reduction of NADPH at 340 nm was measured for 3 minutes at 30 seconds interval using a spectrophotometer (model 4001/4) and calculated as the amount of NADPH consumed per minute per mg protein, using the molar extinction coefficient 6.22×10^3 /M/cm.

d) Determination of glutathione S-transferase (GST) activity

The activity of glutathione S-transferase (GST) was measured following the method of Habig et al. (1974) using 1-chloro 2,4 dinitrobenzene as a substrate. The reaction mixture consisted of 0.1 ml PMS (10% w/v), 1.75 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml GSH (1.0 mM), and 0.05 ml 1-chloro-2,4-dinitrobenzene (CDNB) (1.0 mM) in a total volume of 2.0 ml. The absorbance change was monitored at 340 nm for 3 minutes at 30 second intervals. The enzyme activity was calculated as the amount of CDNB conjugate formed per minute per mg protein, using the molar extinction coefficient of 9.6×10^3 /M/cm.

e) Determination of NAD(P)H: quinone oxidoreductase (QR) activity

The activity of NAD(P)H: quinone oxidoreductase (QR) was determined by following the method of Benson et al. (1980) as described by Iqbal et al. (1999). The reaction solution contained 0.01 ml PMS (10% w/v), 1.1 ml Tris-HCl buffer (25 mM, pH 7.4), 0.7 ml bovine serum albumin (1 mg/ml), 0.02 ml Tween-20 (1% w/v), 0.1 ml FAD (0.15 mM), 0.02 ml NADPH (30 mM), and 0.05 ml 2,6-dichlorophenolindophenol (2.4 mM) in a total volume of 2.0 ml. The reduction of 2,6-dichlorophenolindophenol was measured at 600 nm for 3 minutes at 30 second intervals. The activity was expressed as the amount of 2,6-dichlorophenolindophenol consumed per minute per mg protein, using the molar extinction coefficient of 2.1×10^4 /M/cm.

f) Determination of glucose-6-phosphate dehydrogenase (G6PD) activity

The method of Zaheer et al. (1965) was used to measure the activity of glucose-6 phosphate dehydrogenase (G6PD). The total volume of 3.0 ml contained 0.1 ml PMS (10% w/v), 0.5 ml Tris-HCl buffer (0.2 M, pH 7.6), 0.05 ml NADP (0.1 mM), 0.05 ml glucose-6- phosphatase (0.8 mM), 0.25 ml MgCl2 (8 mM), and 2.05 ml distilled water. The absorbance change was monitored at 340 nm for 3 minutes at 30 second intervals. The activity was expressed as the amount of NADP consumed per minute per mg protein, using the molar extinction coefficient of 6.22×10^3 /M/cm.

g) Determination of gamma-glutamyl transpeptidase (γ-GGT) activity

The method of Orlowski & Meister (1973) was used to determine the activity of gamma-Glutamyl transpeptidase (γ-GGT). A total of 0.2 ml PMS (10% w/v) was added to 0.8 ml of substrate mixture containing glycyl glycine (40 mM), magnesium chloride (11 mM), and L-gamma-glutamyl-p-nitroanilide (4 mM) in

Tris-HCl buffer (185 mM, pH 8.25). The mixture was incubated for 10 minutes at 37°C in water bath. Then, 1.0 ml of trichloro acetic acid (25% w/v) was added to each tube and centrifuged at 8000 rpm for 20 minutes. The absorbance was measured at 405 nm by a spectrophotometer (model 4001/4). The activity was expressed as the amount of p-nitroanaline released per minute per mg protein, using the molar extinction coefficient of 1.74×10^3 / M/cm.

Renal Histopathological Examination

A section of the kidney was excised and immersed in a 10% phosphate-buffered formaldehyde solution for fixation. After fixation, the kidney tissues were embedded in paraffin wax, and thin sections measuring 4 µm thick were obtained. These sections were then stained with haematoxylin-eosin (H&E) using conventional methods. The stained kidney sections were converted into permanent slides and mounted. The slides were examined using a microscope equipped with photographic capabilities, and photomicrographs were captured.

Determination of Protein

Total protein content was determined using a protein assay kit (BCA1) based on the bicinchoninic acid method. Bovine serum albumin was employed as the standard for calibration purposes.

Statistical Analysis

The results were presented as mean ± SD. Statistical comparisons were conducted using one-way analysis of variance (ANOVA), and the homogeneity of variance was assessed using Levene's test. Data were analysed using the SPSS software (Release 17.0, SPSS). A significance level of $p < 0.05$ indicated a significant difference. The EC_{50} values were determined by employing non-linear regression analysis of the dose-response curve using GraphPad Prism 5.

Results

Total Phenolic

The total phenolic content of *A. paniculata* ethanolic extract was 72.39 ± 1.75 mg/g, expressed in gallic acid equivalent (GAE)/mg of extract.

Effect of A. paniculata on DPPH Radical Scavenging

As shown in Table 1, the 50 % scavenge of the DPPH radical (EC_{50} values) were as follows: ascorbic acid (10.06 ± 1.42) and *A. paniculata* (583.60 ± 4.25).

Table 1. DPPH free radical scavenging activity of ascorbic acid and *A. paniculata*.

Plant	EC_{50} (µg/ml)
Ascorbic acid	9.06 ± 1.89
A. paniculata	683.40 ± 4.55

Effects of A. paniculata Extract on Body Weight and Renal Index

The rats' body weight and renal index did not exhibit any notable alteration (Data not presented).

Effects of A. paniculata Extract on Renal Lipid Peroxidation and GSH

According to Table 2, renal malondialdehyde (MDA) levels showed a significant increase ($p< 0.05$) of 37% in CCl_4 challenge rats (39.85 ± 1.10 nmol MDA/g tissue) compared to control rats (29.15 ± 1.30 nmol MDA/g tissue). Additionally, renal GSH levels exhibited a significant decrease ($p< 0.05$) of 10% in CCl_4 challenge rats (7.32 ± 0.26 µmol reduced GSH/g tissue) compared to control rats (8.10 ± 0.34 µmol reduced GSH/g tissue). Pre-administration of *A. paniculata* extract showed a significant improvement in renal MDA and GSH levels.

Table 2. Effects of *Andrographis paniculata* extract on renal glutathione and lipid peroxidation of rats intoxicated with carbon tetrachloride.

Groups (n=6)	GSH (µmol reduced GSH/g tissue)	LPO (nmol MDA/g tissue)
Control	8.10 ± 0.34	29.15 ± 1.30
Carbon tetrachloride (CCl_4)	7.32 ± 0.26 *	39.85 ± 1.10 *
A. paniculata 100mg + CCl_4	7.46 ± 0.29	39.75 ± 1.77
A. paniculata 200mg + CCl_4	7.57 ± 0.22	39.34 ± 1.25
A. paniculata 300mg + CCl_4	7.54 ± 0.17	38.84 ± 1.30

Results are expressed as mean ± SD (n = 6).
* indicates significance at $p < 0.05$ compared to control group.
Abbreviation: CCl_4 (Carbon tetrachloride), GSH (Glutathione reduced), LPO (Lipid peroxidation), MDA (Malondialdehyde).

Effects of A. paniculata Extract on Renal Antioxidant Enzyme Activities

Table 3 displays the impact of *A. paniculata* extract on renal antioxidant enzyme activities. The administration of CCl_4 resulted in a significant decrease in the renal activities of CAT (31%), GPx (25%), QR (36%), GST (35%), GR (23%), and G6PD (33%). Notably, a significant increase in renal antioxidant enzyme activities was observed following a 14-day pretreatment of animals with *A. paniculata* extract. However, no significant changes in renal γ-GGT activity were observed with the administration of CCl_4 and pretreatment with *A. paniculata* extract.

Table 3. Effects of *Andrographis paniculata* extract on renal antioxidant profile of rats intoxicated with carbon tetrachloride.

Groups	CAT (µmol H_2O_2/min/mg protein)	GPx (nmol NADPH oxidised/min/mg protein)	QR (nmol 2,6-DCP reduced/min/mg protein)	GST (nmol CDNB conjugate formed/min/mg protein)	GR (nmol NADPH oxidised/min/mg protein)	G6PD (nmol NADP reduced/min/mg protein)	γ-GGT (nmol p-nitroanaline released/min/mg protein)
Control	1.40 ± 0.04	94.93 ± 5.97	39.32 ± 2.95	56.46 ± 1.62	112.18 ± 4.30	4.67 ± 0.17	15.67 ± 0.70
Carbon tetrachloride (CCl_4)	0.97 ± 0.05 *	71.28 ± 1.74 *	25.30 ± 1.28 *	36.66 ± 1.85 *	85.82 ± 3.36 *	3.11 ± 0.15 *	16.00 ± 0.41
A. paniculata 100mg + CCl_4	0.97 ± 0.07	72.48 ± 4.15	25.69 ± 1.86	37.58 ± 1.34	86.76 ± 4.06	3.14 ± 0.11	15.47 ± 0.50
A. paniculata 200mg + CCl_4	1.00 ± 0.03	72.93 ± 3.87	27.58 ± 3.84	39.20 ± 1.22	87.87 ± 6.22	3.26 ± 0.08	15.52 ± 0.34
A. paniculata 300mg + CCl_4	1.03 ± 0.03	74.79 ± 6.23	27.80 ± 3.09	39.33 ± 2.27	91.87 ± 4.57	3.33 ± 0.21	16.10 ± 0.35

Results are expressed as mean ± SD (n = 6).
*indicates significance at $p < 0.05$ compared to control group.
Abbreviations: CAT (Catalase), GPx (Glutathione peroxidase), QR (Quinone oxidoreductase), GST (Glutathione S-transferase (GST), GR (Glutathione reductase), G6PD (Glucose-6 phosphate dehydrogenase), γ-GGT (Gamma-Glutamyl transpeptidase).

Effects of A. paniculata Extract on Renal Histopathological Changes

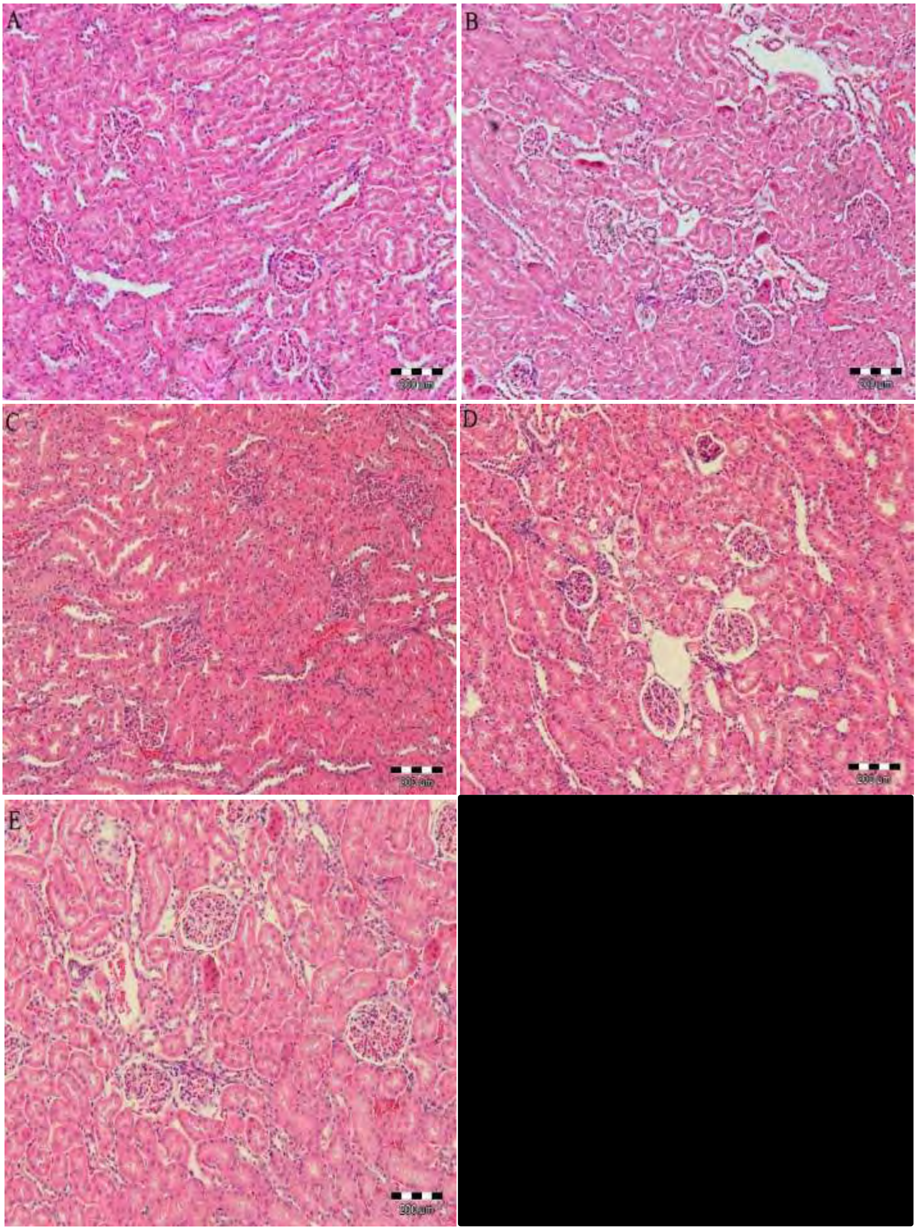

Figure 1. Histopathological sections of kidney. (A) Control; (B) Carbon tetrachloride, CCl_4; (C) *A. paniculata* 100 mg/kg b.w. and CCl_4; (D) *A. paniculata* 200 mg/kg b.w. and CCl_4; (E) *A. paniculata* 300 mg/kg b.w. and CCl_4. Sections stained with Hematoxylin and eosin. Magnifications 100X.

The renal section showed normal architecture of the kidney section with a normal tubular brush border, glomerulus and Bowman's capsule (Figure 1A). In contrast, marked histological changes were observed in the CCl_4-treated group (Figure 1B). Some glomeruli showed dilatation of Bowman's space and glomerular atrophy. In addition, the tubular was dilated, and brush borders were lost in proximal convoluted tubules. Capillary congestion was also observed in the peritubular vessels. However, pretreatment with *A. paniculata* extract markedly ameliorated the kidney morphology and architecture (Figures 1C, D and E). The kidney lesions such as necrosis, glomerular atrophy, tubular dilatation, loss of brush border and congestion were reduced in the pretreatment of rats with *A. paniculata* extract.

Discussion

Our present study was conducted to evaluate the *in vivo* protection of *A. paniculata* against CCl_4- induced nephrotoxicity. Our study has revealed that CCl_4 caused toxicity in renal tissues. CCl_4-induced toxicity is a well-known experimental model. In addition to hepatotoxicity (Clemens et al., 2019; Baig & Ahmad Khan, 2023), it was also reported to cause toxicity in other tissues such as the kidney, heart, brain, and testis (Adewole et al., 2007; Jayakumar et al., 2008; Khan & Ahmed, 2009; Khan et al., 2009, 2010). Previous studies have reported abnormal renal function with increased blood urea nitrogen and creatinine (Adewole et al., 2007; Khan et al., 2009, 2010). The elevation of blood urea nitrogen and creatinine indicates the possible indicator of kidney damage. However, the creatinine level is not necessarily elevated until at least half of the nephrons are damaged (Khan et al., 2009). We failed to observe the elevation of blood urea nitrogen and creatinine in injecting CCl_4 rats (Data not shown). This observation is in good harmony with other studies where there were no alterations in blood urea nitrogen and creatinine levels (Tirkey et al., 2005; Ogeturk et al., 2005; Manna et al., 2006). Ogeturk et al. (2005) reported no significant increase in blood urea nitrogen and creatinine, while damage to renal cortical and subcortical areas was observed in the histopathological examination. This might be due to insufficient exposure to CCl_4. Eventually, it demonstrated no elevation of blood urea nitrogen and creatinine levels. Khan et al. (2009) reported the elevation of blood urea nitrogen and creatinine after exposure to CCl_4 for four months.

CCl_4 undergoes biotransformation by P450 enzymes such as cytochrome CYP2E1, CYP2B2, and possibly CYP3A, resulting in the formation of highly reactive metabolites, namely CCl3• and CCl3OO• (Recknagel et al., 1989; Weber et al., 2003). These metabolites attack polyunsaturated fatty acids, triggering lipid peroxidation and the generation of malondialdehyde (MDA) within liver cells (Recknagel et al., 1989; Weber et al., 2003). MDA is known to play a significant role in the development of various diseases (Recknagel et al., 1989; Weber et al., 2003). It indicates tissue damage and is considered the end product of lipid peroxidation (Ohkawa et al., 1979; Vaca et al., 1988). Our study showed a significant increase in renal MDA levels in rats treated with CCl_4.

Additionally, GSH, an essential non-enzymatic antioxidant involved in detoxifying reactive, toxic metabolites within cells, plays a crucial role. Depletion of GSH can initiate necrosis (Yang et al., 2008). Renal GSH level was decreased in CCl_4-treated rats in the current study, parallel to previous studies (Tirkey et al., 2005; Ogeturk et al., 2005; Manna et al., 2006; Adewole et al., 2007; Jayakumar et al., 2008; Khan et al., 2009, 2010). The decline of renal GSH level might be due to the increased utilisation of cells to scavenge the free radicals caused by CCl_4 (Jollow et al., 1973). Renal toxicity was observed by the elevation of MDA and reduced GSH in the present study. Pretreatment of rats with *A. paniculata* extract revealed significant amelioration of renal MDA and reduced GSH levels, possibly due to its antioxidative activity (Neogy et al., 2008; Akowuah et al., 2008, 2009). The mechanism behind the antioxidative property involves the action of antioxidants, which are molecules capable of neutralizing free radicals and preventing or minimizing the damage they can cause.

Cellular antioxidant enzymes play a crucial role in defending against oxidative stress. CAT and GPx are responsible for breaking down harmful hydrogen peroxide (H_2O_2) into water. The catalytic action of GPx leads to the oxidation of GSH to GSSG, which is then reduced back to GSH by GR. As the primary phase II enzyme, GST plays a vital role in detoxification (Srivastava & Shivanandappa, 2010). Its function involves facilitating the conjugation of GSH with xenobiotics and their reactive metabolites, producing water-soluble compounds (Yang et al., 2008). G6PD, on the other hand, is an enzyme bound to the cell membrane and is involved in eliminating lipid peroxides and toxic oxygen radicals. It initiates the initial steps of the pentose phosphate metabolic pathway (Shreve & Levy, 1977). QR, a flavoprotein, utilises either NADH or NADPH as a reducing cofactor and plays a vital role in detoxifying reactive quinones into less toxic hydroquinones, as described by Iqbal et al. (1999). The reducing process by QR prevents the interaction between semiquinone and oxygen molecules, thus bypassing the production of semiquinone. Additionally, the formation of endogenous catechol estrogen quinones leads to the generation of O2•- and their covalent binding to DNA, resulting in the formation of depurination adducts and the induction of cancer (Ohkawa et al., 1979). Our study observed a decrease in renal antioxidant enzyme levels (CAT, GPx, GR, GST, and G6PD) in the group administered with CCl_4. The findings of our study align with previous studies, which demonstrated a depletion of antioxidant enzymes in CCl_4-induced oxidative renal damage (Güven & Yilmaz, 2005; Tirkey et al., 2005; Ogeturk et al., 2005; Manna et al., 2006; Adewole et al., 2007; Khan et al., 2009, 2010). We found that rats pre-treated with *A. paniculata* extract for 14 days exhibited a significant elevation in antioxidant enzyme levels. Moreover, there were no notable changes in the activity of renal γ-GGT in animals treated with CCl_4 or pre-treated with *A. paniculata* extract, similar to previous studies (Idéo et al., 1972).

Furthermore, the biochemistry results were complemented by histopathological examination. CCl_4-treated rats showed abnormality in renal tissues such as atrophy of glomerulus, dilatation of Bowman's capsule, dilatation of tubule, loss of brush border and tubular necrosis. It is in good agreement with previous studies (Tirkey et al., 2005; Ogeturk et al., 2005; Manna et al., 2006; Adewole et al., 2007; Khan et al., 2009, 2010; Xu et al., 2010). Pretreatment with *A. paniculata* extract showed amelioration in renal histopathological changes. Histopathological examination revealed the same finding as the biochemistry results.

Overall, the nephroprotective effect of *A. paniculata* extract was observed in the present study. This might be due to the biodistribution of the active antioxidative compounds. Affinity to specific proteins in tissue and the amount and interaction of the antioxidant affect the compound biodistribution. Renal tissues might have a relatively low intake of active compounds compared to other tissue, like the liver, where the hepatoprotective effect has been reported. The lower retention of active compounds eventually causes the lesser absorption of active compounds into cells and acts as an antioxidant. Thus, the transient interaction with kidney cells disallows the active compounds to be taken by the cells (Stahl et al., 2002; Manach et al., 2004; Porrini & Riso, 2008; Xu et al., 2010). The study conducted by Kwon et al. 2001 highlighted an intriguing finding regarding the accumulation of polyphenol isoflavone aglycones in the prostate gland compared to other tissues. Polyphenol isoflavones are a subgroup of phytochemicals found in various plant-based foods, particularly in legumes like soybeans. They have garnered considerable attention for their potential health benefits, including their role in reducing the risk of certain chronic diseases (Kwon et al., 2001).

Bioavailability is also playing an important factor in affecting the mechanism of action of active compounds. Phytochemicals like andrographolide, which is the primary active compound in A. paniculata, significantly contribute to its bioactivity. A deeper exploration of the specific mechanisms by which these phytochemicals exert their medicinal effects, such as their anti-inflammatory and immunomodulatory properties, can provide a more comprehensive understanding of A. paniculata's bioactivity. The bioavailability of active compounds through the intravenous route is typically very high but not 100%, as there is usually a small, albeit possibly insignificant, amount of absorption involved. For the oral route, the bioavailability is generally lower, unless the bioavailability (BA) of these active compounds is known. By considering both bioavailability and biodistribution, the higher concentration of liver accumulation and retention of antioxidants in the liver through the oral route causes the effective protection of antioxidants in the liver. On the other hand, the lower active compound accumulation and retention in the kidney subsequently cause lesser protection. Furthermore, the plasma concentration of active compounds is much higher throughout the intravenous route than the oral route. Thus, the active compounds are transported by systemic circulation to the kidneys and excreted through urine. Given this, the intravenous routes transport the active compounds to the kidney without passing through the liver; hence it might be more effective than the oral route in protecting the kidney against CCl_4-induced oxidative damage (Xu et al., 2010).

Conclusions

In viewing all the observations, a significant nephroprotective effect was found in the present study. Further investigations of biodistribution and bioavailability of active compounds are essential to further evaluate the nephroprotective effects of *A. paniculata* extract. In addition, other studies on different administration routes are also necessary to be conducted.

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Authors Contributions

Senty Vun Sang and Pei Hoon Koh experimented. Dr Mohammad Iqbal helped design the experiments and prepare the manuscript.

Conflict of Interests

The authors declare no conflict of interest.

References

Adewole SO, Salako AA, Doherty OW, Naicker T. 2007. Effect of melatonin on carbon tetrachloride-induced kidney injury in wistar rats. *African Journal of Biomedical Research*, *10* (2): 153–164. DOI: 10.4314/ajbr.v10i2.50619.

Akowuah GA, Zhari I, Mariam A. 2008. Analysis of urinary andrographolides and antioxidant status after oral administration of *Andrographis paniculata* leaf extract in rats. *Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association*, *46* (12): 3616–3620. DOI: 10.1016/j.fct.2008.09.008.

Akowuah GA, Zhari I, Mariam A, Yam MF. 2009. Absorption of andrographolides from *Andrographis paniculata* and its effect on CCl_4-induced oxidative stress in rats. *Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association*, *47* (9): 2321–2326. DOI: 10.1016/j.fct.2009.06.022.

Aranda-Rivera AK, Cruz-Gregorio A, Arancibia-Hernández YL, Hernández-Cruz EY, Pedraza-Chaverri J. 2022. RONS and Oxidative Stress: An Overview of Basic Concepts. *Oxygen*, *2* (4):437–478. DOI: 10.3390/oxygen2040030.

Arroyave-Ospina JC, Wu Z, Geng Y, Moshage H. 2021. Role of Oxidative Stress in the Pathogenesis of Non-Alcoholic Fatty Liver Disease: Implications for Prevention and Therapy. *Antioxidants*, *10* (2):174. DOI: 10.3390/antiox10020174.

Baig MF, Ahmad Khan MK. 2023. Renal Toxicity Induced by Carbon Tetrachloride in Experimental Model: Renal Toxicity Induced by CCl4 in Experimental Model. *Pakistan BioMedical Journal, 6* (3). DOI: 10.54393/pbmj.v6i3.897.

Batty M, Bennett MR, Yu E. 2022. The Role of Oxidative Stress in Atherosclerosis. *Cells*, *11* (23):3843. DOI: 10.3390/cells11233843.

Benson AM, Hunkeler MJ, Talalay P. 1980. Increase of NAD(P)H:quinone reductase by dietary antioxidants: Possible role in protection against carcinogenesis and toxicity. *Procedings of the National Academy of Sciences of the United States of America*, *77* (9): 5216–5220. DOI:10.1073/pnas.77.9.5216.

Buege JA. Aust SD. 1978. Microsomal lipid peroxidation. *Methods in Enzymology*, *52:* 302–310. DOI: 10.1016/s0076-6879(78)52032-6.

Clemens MM, McGill MR, Apte U. 2019. Mechanisms and biomarkers of liver regeneration after drug-induced liver injury. *Advances in Pharmacology*, *85:* 241–262. DOI: 10.1016/bs.apha.2019.03.001.

Carlberg I, Mannervik B. 1975. Purification and characterisation of the flavoenzyme glutathione reductase from rat liver. *The Journal of Biological Chemistry*, *250* (14): 5475–5480. DOI: 10.1016/S0021-9258(19)41206-4.

Claiborne A. 1985. Catalase activity. In: Greenwald, R.A., Ed., CRC *Handbook of Methods for Oxygen Radical Research*. Boca Raton: CRC Press, 283–284.

Dandu AM, Inamdar NM. 2009. Evaluation of beneficial effects of antioxidant properties of aqueous leaf extract of *Andrographis paniculata* in STZ-induced diabetes. *Pakistan Journal of Pharmaceutical Sciences*, *22* (1): 49–52.

Darenskaya MA, Kolesnikova LI, Kolesnikov SI. 2021. Oxidative Stress: Pathogenetic Role in Diabetes Mellitus and Its Complications and Therapeutic Approaches to Correction. *Bulletin of Experimental Biology and Medicine*, *171* (2):179–189. DOI: 10.1007/s10517-021-05191-7.

Güven A, Yilmaz S. 2005. The effect of carbon tetrachloride (CCl_4) and ethanol (C_2H_5OH) on the determination of levels glutathione peroxidase, catalase, glucose-6-phosphate dehydrogenase and lipid peroxidation liver and kidney in the goose. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, *11* (2): 113–117.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249* (22): 7130–7139. DOI: 10.1016/S0021-9258(19)42083-8.

Hajam YA, Rani R, Ganie SY, Sheikh TA, Javaid D, Qadri SS, Pramodh S, Alsulimani A, Alkhanani MF, Harakeh S, Hussain A, Haque S, Reshi MS. 2022. Oxidative Stress in Human Pathology and Aging: Molecular Mechanisms and Perspectives. *Cells*, *11* (3): 552. https://doi.org/10.3390/cells11030552.

Hatano T, Kagawa H, Yasuhara T, Okuda T. 1988. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chemical & Pharmceutical Bulletin (Tokyo)*, *36* (6): 2090–2097. DOI:10.1248/cpb.36.2090.

Hossain MA, Roy BK, Ahmed K, Chowdhury AMS, Rashid MA. 2007. Antidiabetic Activity of *Andrographis paniculata*. *Dhaka University Journal of Pharmaceutical Sciences*, *6* (1): 15–20.

Idéo G, Morganti A, Dioguardi N. 1972. γ-Glutamyl Transpeptidase: a Clinical and Experimental Study. *Digestion*, *5* (6): 326–336. DOI: 10.1159/000197206.

Iqbal M, Sharma SD, Rahman A, Trikha P, Athar M. 1999. Evidence that ferric nitrilotriacetate mediates oxidative stress by down-regulating DT-diaphorase activity: implications for carcinogenesis. *Cancer Letters*, *141* (1-2): 151–157. DOI: 10.1016/s0304-3835(99)00100-7.

Jelic MD, Mandic AD, Maricic SM, Srdjenovic BU. 2021. Oxidative stress and its role in cancer. *Journal of Cancer Research and Therapeutics*, *17* (1): 22-28. DOI: 10.4103/jcrt.JCRT_862_16.

Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB. 1973. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. *The Journal of Pharmacology and Experimental Therapeutics*, *187* (1): 195–202.

Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. 1974. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology*, *11* (3): 151–169. DOI: 10.1159/000136485.

Kaur G, Sharma A, Bhatnagar A. 2021. Role of oxidative stress in pathophysiology of rheumatoid arthritis: insights into NRF2-KEAP1 signalling. *Autoimmunity*, *54* (7): 385–397. DOI: 10.1080/08916934.2021.1963959.

Koh PH, Mokhtar RAM, Iqbal M.Iqbal, 2011. Andrographis paniculata ameliorates carbon tetrachloride (CCl_4)-dependent hepatic damage and toxicity: Diminution of oxidative stress, *Redox Report, 16* (3): 134–143.

Khan MR, Ahmed D. 2009. Protective effects of *Digera muricata* (L.) Mart. on testis against oxidative stress of carbon tetrachloride in rat. *Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association*, *47* (6): 1393-1399. DOI: 10.1016/j.fct.2009.03.020.

Khan MR, Rizvi W, Khan GN, Khan RA, Shaheen S. 2009. Carbon tetrachloride-induced nephrotoxicity in rats: Protective role of *Digera muricata*. *Journal of Ethnopharmacology*, 122(1): 91-99. DOI: 10.1016/j.jep.2008.12.006.

Khan RA, Khan MR, Sahreen S. 2010. Evaluation of *Launea procumbens* use in renal disorders: A rat model. *Journal of Ethnopharmacology*, *128* (2): 452–461. DOI: 10.1016/j.jep.2010.01.026.

Klaunig JE. 2018. Oxidative Stress and Cancer. *Current Pharmaceutical Design*, *24* (40): 4771–4778. DOI: 10.2174/1381612825666190215121712.

Kumar RA, Sridevi K, Vijaya Kumar N, Nanduri S, Rajagopal S. 2004. Anticancer and immunostimulatory compounds from *Andrographis paniculata*. *Journal of Ethnopharmacology*, *92*(2–3): 291–295. DOI: 10.1016/j.jep.2004.03.004.

Kwon SM, Kim SI, Chun DC, Cho NH, Chung BC, Park BW, Hong SJ. 2001. Development of rat prostatitis model by oral administration of isoflavone and its characteristics. *Yonsei Medical Journal*, *42* (4): 395–404. DOI: 10.3349/ymj.2001.42.4.395.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79* (5): 727–747. DOI: 10.1093/ajcn/79.5.727.

Manna P, Sinha M, Sil PC. 2006. Aqueous extract of *Terminalia arjuna* prevents carbon tetrachloride induced hepatic and renal disorders. *BMC Complementary and Alternative Medicine*, *6:* 33. DOI: 10.1186/1472-6882-6-33.

Martemucci G, Portincasa P, Ciaula AD, Mariano M, Centonze V, D'Alessandro AG. 2022. Oxidative stress, ageing, antioxidant supplementation and their impact on human health: An overview. *Mechanisms of Ageing and Development*, *206:* 111707. DOI: 10.1016/j.mad.2022.111707.

Mohandas J, Marshall JJ, Duggin GG, Horvath JS. Tiller DJ. 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. *Biochemical Pharmacology*, *33* (11): 1801–1807. DOI: 10.1016/0006-2952(84)90353-8.

Neogy S, Das S, Mahapatra SK, Mandal N, Roy S. 2008. Amelioratory effect of *Andrographis paniculata* Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. *Environmental Toxicology and Pharmacology*, *25* (3): 321–328. DOI: 10.1016/j.etap.2007.10.034.

Ogeturk M, Kus I, Colakoglu N, Zararsiz I, Ilhan N, Sarsilmaz M. 2005. Caffeic acid phenethyl ester protects kidneys against carbon tetrachloride toxicity in rats. *Journal of Ethnopharmacology*, *97* (2): 273–280. DOI: 10.1016/j.jep.2004.11.019.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95* (2): 351–358. DOI: 10.1016/0003-2697(79)90738-3.

Orlowski M, Meister A. 1973. γ-Glutamyl cyclotransferase distribution, isozymic forms and specificity, *The Journal of Biological Chemistry*, *248* (8): 2836–2844. DOI: 10.1016/S0021-9258(19)44083-0.

Phull AR, Nasir B, Haq IU, Kim SJ. 2018. Oxidative stress, consequences and ROS mediated cellular signalling in rheumatoid arthritis. *Chemico-Biological Interactions*, *281:* 121–136. DOI: 10.1016/j.cbi.2017.12.024.

Porrini M, Riso P. 2008. Factors influencing the bioavailability of antioxidants in foods: a critical appraisal. *Nutrition, Metabolism and Cardiovascular Disease: NMCD*, *18* (10): 647–650. DOI: 10.1016/j.numecd.2008.08.004.

Poznyak AV, Grechko AV, Orekhova VA, Chegodaev YS, Wu WK, Orekhov AN. 2020. Oxidative Stress and Antioxidants in Atherosclerosis Development and Treatment. *Biology (Basel)*, *9* (3): 60. DOI: 10.3390/biology9030060.

Reyes BAS, Bautista ND, Tanquilut NC, Anunciado RV, Leung AB, Sanchez GC, Magtoto RL, Castronuevo P, Tsukamura H, Maeda KI. 2006. Antidiabetic potentials of *Momordica charantia* and *Andrographis paniculata* and their effects on estrous cyclicity of alloxan-induced diabetic rats. *Journal of Ethnopharmacology*, *105* (1-2): 196–200. DOI: 10.1016/j.jep.2005.10.018.

Romano AD, Serviddio G, de Matthaeis A, Bellanti F, Vendemiale G. 2010. Oxidative stress and ageing. *Journal of Nephrology*, *23* (Suppl 15): S29–36.

Sheeja K, Guruvayoorappan C, Kuttan G. 2007. Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. *International Immunopharmacology*, *7* (2): 211–221. DOI: 10.1016/j.intimp.2006.10.002.

Shreve DS, Levy HR. 1977. On the molecular weight of human glucose-6-phosphate dehydrogenase. *Biochemical and Biophysical Research Communications*, *78* (4): 1369–1375. DOI: 10.1016/0006-291x(77)91443-7.

Sies H. 2020. Oxidative Stress: Concept and Some Practical Aspects. *Antioxidants (Basel)*, *9* (9): 852. DOI: 10.3390/antiox9090852.

Singh A, Kukreti R, Saso L, Kukreti S. 2019. Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. *Molecules*, *24* (8): 1583. DOI: 10.3390/molecules24081583.

Singha PK, Roy S, Dey S. 2003. Antimicrobial activity of *Andrographis paniculata*. *Fitoterapia*, *74* (7-8): 692–694. DOI: 10.1016/s0367-326x(03)00159-x.

Singha PK, Roy S, Dey S. 2007. Protective activity of andrographolide and arabinogalactan proteins from *Andrographis paniculata* Nees. against ethanol-induced toxicity in mice. *Journal of Ethnopharmacology*, *111* (1): 13–21. DOI: 10.1016/j.jep.2006.10.026.

Srivastava A, Shivanandappa T. 2010. Hepatoprotective effect of the root extract of *Decalepis hamiltonii* against carbon tetrachloride-induced oxidative stress in rats. *Food Chemistry*, *118* (2): 411–417. DOI: 10.1016/j.foodchem.2009.05.014.

Stahl W, van den Berg H, Arthur J, Bast A, Dainty J, Faulks RM, Gärtner C, Haenen G, Hollman P, Holst B, Kelly FJ, Polidori MC, Rice-Evans C, Southon S, van Vliet T, Vina-Ribes J, Williamson G, Astley SB. 2002. Bioavailability and metabolism. *Molecular Aspects of Medicine*, *23* (1-3): 39–100. DOI: 10.1016/s0098-2997(02)00016-x.

Teleanu DM, Niculescu AG, Lungu II, Radu CI, Vladâcenco O, Roza E, Costăchescu B, Grumezescu AM, Teleanu RI. 2022. An overview of oxidative stress, neuroinflammation, and neurodegenerative diseases. *International Journal of Molecular Sciences*, *23* (11): 5938. DOI: 10.3390/ijms23115938.

Tirkey N, Pilkhwal S, Kuhad A, Chopa K. 2005. Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. *BMC Pharmacology*, *5:* 2. DOI: 10.1186/1471-2210-5-2.

Trivedi N, Rawal UM. 1998. Effect of aqueous extract of *Andrographis paniculata* on liver tumor. *Indian Journal of Pharmacology*, *30* (5): 318–322.

Unsal V, Cicek M, Sabancilar İ. 2020. Toxicity of carbon tetrachloride, free radicals and role of antioxidants. *Reviews on Environmental Health*, *36* (2): 279–295. DOI: 10.1515/reveh-2020-0048.

Vaca CE, Wilhelm J, Harms-Ringdahl M. 1988. Interaction of lipid peroxidation products with DNA. A review. *Mutation Research*, *195* (2): 137–149. DOI: 10.1016/0165-1110(88)90022-x.

Velioglu YS, Mazza G, Gao L, Omah BD. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry*, *46* (10): 4113–4117. DOI: 10.1021/jf9801973.

Verma N, Vinayak M. 2007. Antioxidant action of *Andrographis paniculata* on lymphoma. *Molecular Biology Reports*, *35* (4): 535–540. DOI: 10.1007/s11033-007-9119-x.

Vun-Sang S, Rodrigues KF, Dsouza UJA, Iqbal M. 2022. Suppression of Oxidative Stress and Proinflammatory Cytokines Is a Potential Therapeutic Action of *Ficus lepicarpa* B. (Moraceae) against Carbon Tetrachloride (CCl_4)-Induced Hepatotoxicity in Rats. *Molecules*, *27* (8): 2593. DOI: 10.3390/molecules27082593.

Xu JY, Su YY, Cheng JS, Li SX, Liu R, Li WX, Xu GT, Li QN. 2010. Protective effects of fullerenol on carbon tetrachloride-induced acute hepatotoxicity and nephrotoxicity in rats. Carbon, *48* (5): 1388–1396. DOI: 10.1016/j.carbon.2009.12.029.

Yang YS, Ahn TW, Lee JC, Moon CJ, Kim SH, Jun W, Park SC, Kim HC, Kim JC. 2008. Protective effects of pycnogenol on carbon tetrachloride-induced hepatotoxicity in Sprague-dawley rats. *Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association*, *46* (1): 380–387. DOI: 10.1016/j.fct.2007.08.016.

Zaheer N, Tiwari KK, Krishnan PS. 1965. Exposure and solubilisation of hepatic mitochondrial shunt dehydrogenases. *Archives of Biochemistry and Biophysics*, *109:* 646–648. DOI: 10.1016/0003-9861(65)90412-1.

Zaridah MZ, Idid SZ, Omar AW, Khozirah S. 2001. In vitro antifilarial effects of three plant species against adult worms of subperiodic *Brugia malayi*. *Journal of Ethnopharmacology*, *78* (1): 79–84. DOI: 10.1016/s0378-8741(01)00286-0.

Zhang P, Li T, Wu X, Nice EC, Huang C, Zhang Y. 2020. Oxidative stress and diabetes: antioxidative strategies. *Frontiers Medicine*, *14* (5): 583–600. DOI: 10.1007/s11684-019-0729-1.

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Review Article

A Review of *Etlingera coccinea* (Blume) S. Sakai and Nagam (Zingiberaceae) on Achievement of Producing An Essential Oil and Medicinal Properties in Sabah, East Malaysia.

Emmeldah Joseph, Elia Godoong*

Faculty of Tropical Forestry, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.

*Corresponding author: elia@ums.edu.my

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ABSTRACT

The wild ginger, Tuhau, *E. coccinea* is a plant species from *Etlingera* genus and *Zingiberaceae* family. This study is focused on a reviewed paper reported on the extraction method used to derive essential oils and the overview of the published data regarding medicinal properties of *Etlingera coccinea* spp. The search was performed in several databases such as *ScienceDirect*, *Scopus*, *SpringerLink*, and *Researchgate* and also Google Scholar. The following keywords were used individually and or in combination "*E. coccinea*" and "Tuhau". This article highlights the extraction techniques used to derive the essential oil of *E. coccinea* and also provide briefly overview of the medicinal properties from the data in recent literature.

Keywords: *Etlingera coccinea;* Tuhau; medicinal properties

Introduction

Sabah is one of the areas where tropical rainforests predominate; these rainforests are well-known among researchers for their vast diversity of flora as well as their special geological, topographical, and climatic circumstances (Dee-luxe Journeys, 2020). There are up to 4,500 species of flora and fauna; one of these is the wild ginger species, *Etlingera coccinea*.

Etlingera coccinea is a plant species from the *Etlingera* genus and *Zingiberaceae* family (Shahid-Ud-Daula et al. 2015). This plant is distributed throughout Asia to the Pacific islands including Borneo, Peninsular Malaysia, Philippines, Singapore, Sumatra, Thailand and Vietnam (Devanathan & Mustaqim, 2020; Ardiyani & Poulsen, 2019). As described by Poulsen (2006), this species was primarily found in lowland environments, mature primary forest, shaded areas, secondary logged woods, young or old fields, grazing areas and grazed woodlands, orchards, paddy fields, and also close to streams.

It is locally known as Tuhau and used by the native people of Sabah (Kulip, 2007) and grows to an average of 2 m (Daniel-Jambun et al. 2017), 5-8 m (Devanathan & Mustaqim, 2020) and grows wildly to an average of 40 to 1,650 m elevation (Devanathan & Mustaqim, 2020). As mentioned by Shahid-Ud-Daula & Basher (2019), *Etlingera* sp., grows as clumps of leafy shoots, while others have long creeping rhizomes with each of their leafy shoots being be more than a metre apart.

E. coccinea is known for its distinctive inflorescence, which is highly prized as an ornament. Bright red inflorescences that resemble petals are present on the surface of the ground (see Figure 1). Its fruit matures below the ground (see Figure 2). This plant has a strong fragrant content because of the high concentration of essential oils in the leaves, stems, flowers, fruits and rhizomes (Jems et al. 2021; Vairappan et al. 2012; Nagappan et al. 2017). We discovered that the colour of the stems can distinguish between two types of *E. coccinea,* the red stems (dark reddish stem) are more pungently scented than white stems. While the scent of white Tuhau (greenish stem) is less pungent than red Tuhau and supported by the results of a survey we conducted among forest rangers in the DaMal Conservation Areas (Danum Valley, Maliau Basin and Imbak Canyon), Tuhau sellers in the market and consumers who come from various areas such as Ranau, Kadamaian, Kiulu, Tambunan, Tamparuli and Telupid.

Most research on this native ginger species focuses on how it can be used as a spice or as a medicine. *E. coccinea* has been commercially cultivated for the local market and is used by indigenous ethnic groups to flavour foods such as "sambal tuhau" (paste), "jeruk tuhau" (pickles), and "serunding tuhau" (floss) (Jualang et al. 2015). According to Naïve et al. 2018, the pith of the leafy shoot is used as a condiment in Borneo and Java and is also eaten as a vegetable. It is traditionally used to treat stomach aches, urine cleanser, food poisoning and gastric problems (Poulsen, 2006; Vairappan et al. 2012; Shahid-Ud-Daula et al. 2015; Devanathan & Mustaqim, 2020). The local also utilized it as a wound healer and removing body odour during the postpartum period (Lee, 2017; Geraldine, 2017).

Figure 1. The yellowish-red flowers of *E. coccinea* are shown. (Naïve et al. 2018) (Photo by Emmeldah Joseph)

Figure 2. The fruits of *E. coccinea*. (Naïve et al. 2018) (Photo by Emmeldah Joseph)

Methods

The aim of this paper is to review studies of Tuhau that has been reported by previous research studies database from reviewed literature platforms between 2008 to 2021. This study consists of collecting and selecting journal articles, which were then analysed based on objectives stated from the selected articles. This research will be presented in extracted information in table and figures and performed with descriptive analysis. The research framework is illustrated in Figure 2.1.

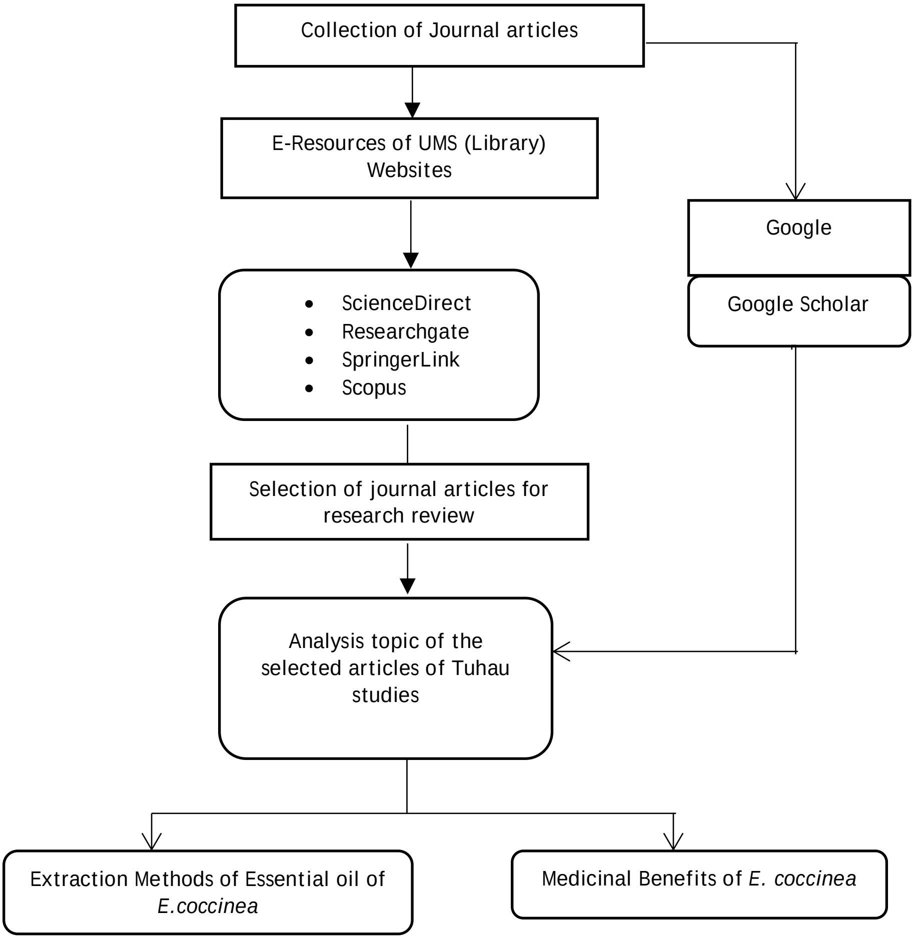

Figure 2.1 Research Framework for Review of Tuhau.

Literature Searching and Data Collection

A systematic search of peer-reviewed literature from e-Resources under the official website, Universiti Malaysia Sabah (subscribe database: Scopus, ScienceDirect, Researchgate and SpringerLink). The search for relevant research papers for data collection were also obtained from Google Scholar. The following search strings are used such as "*E. coccinea*" and "Tuhau".

Analysis topic of the selected articles of Tuhau studies on Extraction Methods of Essential Oils

The selected articles for this study were documented between 2008 to 2021. Various extraction methods are performed to acquire essential oils. For instance, hydro-distillation, solvent extraction, effleurage, aqueous infusion, cold or hot pressing, supercritical fluid extraction, solvent free microwave extraction and phytonic process (Shahid Ud-Daula & Basher, 2019). Table 1 shows the methods used to extract essential oil of *Etlingera coccinea* as documented in previous research studies.

Table 1 Extraction Method Used to Achieve Essential oil of *Etlingera coccinea* spp.

Article Title	Extraction Method Used	References
Allelopathic Potential of *Etlingera coccinea* (B.) Sakai & Nagam on Seed Germination and Growth of Mung Bean and Siam Weed.	10 g of powdered stems and leaves were soaked 48 hours in 100mL of 80% of three different solvents (hexane, ethyl acetate and methanol) using cold maceration method.	Mohamad et al. (2021)
The Anti-Candida Studies on Edible Gingers of Sabah.	500 g stalks of tuhau were soaked 96 hours in 100 mL of analytical grade (AR) methanol.	Hafiz et al. (2008)
Diversity in Volatile Chemical and Antibacterial Activity Among Selected Genus of *Cinnamomum*, *Etlingera* and *Schizostachyum* From Sabah.	Hydro-distillation: 200g freshly chopped rhizome were hydro-distilled for 8 hours via Clevenger-type apparatus.	Nagappan et al. (2017)
Essential Oil Composition, Cytotoxic and Antibacterial Activities of Five Etlingera Species from Borneo.	Hydro-distillation: 200 g freshly chopped rhizomes were hydro-distilled for 8 hours via Clevenger-type apparatus.	Vairappan et al. (2012)
Investigation on the antimicrobial activities of gingers (*Etlingera coccinea* (Blume) S.Sakai & Nagam and *Etlingera sessilanthera* R.M. Sm) endemic to Borneo.	Leaves and rhizomes were crushed and then extracted using hexane, dichloromethane, ethyl acetate, methanol and distilled water for 15 minutes followed by filtration respectively.	Daniel-Jambun et al. (2017)
The Protective Effect of *etlingera coccinea* (TUHAU) against Autoxidation -induced Ox Brain Homogenate	Aqueous Extraction: 1g of powdered leaves mixed with 100 ml distilled water was boiled for 10 minutes and then cooled for 15 minutes.	Anuar et al. (2018)
Chemical Composition of Essential Oil from *Etlingera coccinea.*	Hydro-distillation: 250-300g leaves chopped and hydro-distilled at 100°C for 7 to 8 hours.	Jems et al. (2021)

As shown in Table 1, most of the previous studies use hydro-distillation as extraction method of essential oils of *E. coccinea* spp. As mentioned by Shahid Ud-Daula & Basher (2019), hydro-distillation and steam distillation are extensively used for extracting essential oil from plants nowadays. This is because the volatile components can be distilled at lower temperature than their individual boiling points and can also be separated easily from condensed water.

Medicinal Uses of Etlingera coccinea

Past studies found that essential oil extracted from *E. coccinea* spp., have volatile compounds which reveal the potential of selected plants used by indigenous communities of Borneo as antimicrobials in food, cosmetics and pharmaceutical industries. Many pharmacological activities have been reported on *Etlingera* genus especially, including their benefits as antioxidants, antibacterials, cholesterol-lowering and anti-cancer (Wahyuni et al. 2021).

Table 2. Medicinal Benefits of *E.coccinea* (Tuhau).

Parts of Plant and Preparation	Medicinal Benefits	References
Young shoots	Treat stomach ache Food poisoning Gastric problems	(Kulip, 1997; Poulsen, 2006) (Subramaniam et al. 2010; Vairappan et al. 2012) Shahid-Ul-Daula et al. (2015) Devanathan & Mustaqim (2020)
Essential Oil extracted from rhizome of *Etlingera coccinea* spp.,	Antimicrobial Inhibit the four strains of clinical bacteria (*Staphylococcus aureus, Staphylococcus* sp., *Streptococcus pyrogenes* and *Salmonella enteriditis*) with MIC values less than 10 µg/mL. Display best inhibition against *Listeria monocytogenes* (MIC: 4.60 ± 0.5 µg mL-1)	Vairappan et al. (2012) Nagappan et al, (2017)
Essential Oil extracted from leaves of *Etlingera coccinea* spp.,	Anti-inflammatory Anti-depressant agent	Rivas da Silva et al. (2012) Guzman-Gutierrez et al. (2012)
Young shoots immersed into hot ash (squeeze the extract before consuming)	Cure cough & wounds	Handayani (2015)
Young shoots	Wound healer and helps to eliminate body odour during postpartum period	Lee (2017); Geraldine (2017)

Table 3. Shows the comparison between past research reported on Microbial activities of *E.coccinea* essential oils against microorganisms.

References	Method Used	Microorganism	Volatile Compounds
Vairappan et al. (2012)	Agar Dilution Method	*Staphylococcus aureus*, *Staphylococcus* sp., *Streptococcus pyrogenes*, (*Salmonella enteritidis*	3-Thujanone, Borneol (25.8%), Camphor (3.8%), Cedr-9-ene, L-Calamenene, Carophyllene oxide, α-Bisabol, α-Epi-muurolol and Cycloartanyl acetate.
Nagappan et al. (2017)	Agar Dilution Method	*S. aureus* (ATCC 29213), *L. monocytogenes* (ATCC 7644), *S. typhimurium* (ATCC 25922) and *S. enteritidis* (ATCC 29213).	Borneol (28.2%), Aromadendrene oxide (10.9%), Elemicin (9.7%), Lauryl aldehyde (5.9%), 1-dodecanol (3%), Camphor (2.8%) and 5-Decen-1-ol (1.3%)

Conclusion

According to data collected, the extraction method often used to derive essential oils of *E. coccinea* is hydro-distillation. The volatile compounds found in *E. coccinea* proved its ability in the medicinal industry hence increasing its value in the market. However, the rising demand from local use and start-ups of industries around tuhau-based food poses a real risk of overharvesting especially from the wild. Therefore, further studies such as understanding its distribution, environmental aspects and sociology of this species need to be done to provide alternative sources for these natural plants to reduce the pressure especially on the wild population.

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References

Anuar NNM, Mohamed J, Hanipah ENA, Yahya NJ, Ajik EM, & Taib IS. 2018. The protective effect of Etlingera coccinea (TUHAU) against autoxidation-induced ox brain homogenate. *Malaysian Journal of Health Science, 16:* 35–39.

Ardiyani M, & Poulsen AD. 2019. An Update of the Genus *Etlingera* (Zingiberaceae) in Sulawesi Including the Description of A new Species. *Reinwardtia, 18* (1): 1–50.

Daniel-Jambun D, Dwiyanto, Lim YY, Tan JBL, Muhamad A, Yap SW, & Lee SM. 2017. Investigation on the antimicrobial activities of gingers (*Etlingera coccinea* (Blume) S. Sakai & Nagam and *Etlingera sessilanthera* R.M.Sm.) endemic to Borneo. *Journal of Applied Microbiology, 123:* 810–818.

Dee-luxe Journey. 2020. Sabah Borneo Facts: Everything you want to know about the land below the wind. Retrieved from: https://www.dee-journeys.com.au/borneo/sabah/ Accessed 30 July 2022.

Devanathan K. & Mustaqim WA. 2020. *Etlingera coccinea* (Blume) S. Sakai & Nagam. Zingiberaceae. *SpingerLink*:1–7.

Geraldine A. 2017. Borneon "tuhau" plant rises to prominence, thanks to Kadazandusun cousins. New Straits Times. 2017. Retrieved from: https://www.nst.com.my/news/2017/03/222474/borneon-tuhau-plant-rises-prominence-thanks-kadazandusun-cousins. Accessed 20 June 2022.

Guzman-Gutierrez SL, Gomez-Cansino R, Garcia-Zebadua JC, Jimenez-Perez NC, & Reyes-Chilpa R. 2012. Anti-depressant activity of *Litsea glaucescens* essential oil: Identification of ß-pinene and linalool as active principles. *Journal of ethnopharmacology, 143* (2): 673–679.

Hafiz MAT, Siti SAM, Indim D, & Julenah AN, 2008. The Anti-Candida Studies on Edible Gingers of Sabah. In: Proceedings Biodiversity-Biotechnology: Gateway to Discoveries, Sustainable utilization & Creation of Wealth. At: Kuching, Sarawak. *ResearchGate* :1–4.

Handayani A. 2015. Utilization of medicinal plants by people around Gunung Simpang Nature Reserve, West Java. *Pros Semin Nas Masy Biodiv Indon, 1* (6): 1425–32.

Jems NS, Mus AA, Rusdi NA, & Godoong E. 2021. Chemical Composition of Essential Oil from *Etlingera coccinea* (Blume) S. Sakai & Nagam in Kadamaian, Kota Belud, Sabah. *Journal of Biology and Conservation, 18:* 91–105.

Jualang AG, Nurul Humaira TA, Devina D, Hartinie M. 2015. In Vitro Shoot Regeneration from Rhizome Bud of Native Ginger in Borneo, *Etlingera coccinea*. *Journal of Tropical Plant Physiology, 7*: 36–46.

Kulip J. 1997. A Preliminary Survey of Traditional Medicinal Plants In The West Coast and Interior of Sabah. *Journal of Tropical Forest Science, 10* (2): 271–274.

Kulip J. 2007. Gingers in Sabah and their traditional uses. *Sepilok Bulletin, 7* (1): 23–44.

Lee S. 2017. Plant-based beauty product gains ground in Sabah. The Star. Retrieved from:https://www.thestar.com.my/metro/community/2017/03/11/plant-based-beauty-product-gains-ground-in-sabah. Accessed by 20 June 2022.

Mohamad NMZ, Nurfitri H, Farnidah J, & Chai WL. 2021. Allelopathic Potential of *Etlingera coccinea* (B.) Sakai & Nagam on Seed Germination and Growth of Mung Bean and Siam Weed. *Science Letters, 15* (2): 79–89.

Nagappan T, Yatau MH, Salim JM, & Vairappan CS. 2017. Diversity in volatile chemicals and antibacterial activity among selected genus of *Cinnamomum,* *Etlingera* and *Schizostachyum* from Sabah. *Journal of Sustainability Science and Management, 12* (2): 26–33.

Naïve MAK, Pabillaran RO, & Escrupulo IG. 2018. *Etlingera coccinea* (Blume) S. Sakai and Nagam. (Zingibearaceae - Alpineae): An addition to the Flora of the Philippines, with notes on its distribution, phenology and ecology. *Bioscience Discovery, 9* (1): 107–110.

Poulsen AD. 2006. *Etlingera* of Borneo. Kota Kinabalu, Sabah: *Natural History Publication (Borneo)* :1–263.

Rivas da Silva AC, Lopez PM, Barros de Azevedo MM, Costa DC, Alviano CS, & Alviano DS. 2012. Biological activities of α-pinene and ß-pinene enantiomers. *Molecules (Basel, Switzerland), 17* (6): 6305–6316.

Shahid-Ud-Daula AFM & Basher MA. 2019. Genus *Etlingera* -A Review on Chemical Composition and Antimicrobial Activity of Essential Oils. *Journal of Medicine Plants Research, 13* (7): 135–156.

Shahid-Ud-Daula AFM, Kamariah AS & Ahmad N. 2015. Phytochemical Screening, Antioxidant, and Antimicrobial Activities of Leaves, Stems, and Rhizomes of *Etlingera coccinea* (Blume) S. Sakai & Nagam. *International Journal of Pharmacognosy and Phytochemical Research, 7* (5): 873–883.

Subramaniam JTL, Sreenivasan S, Vello S & Zakaria Z. 2010. Pharmacological Activity, Phytochemical Analysis and Toxicity of Methanol Extract of *Etlingera elatior* (torch ginger) flowers. *Asian Pacific Journal of Tropical Medicine, 1:* 769–774.

Vairappan CS, Nagappan T & Palaniveloo K. 2012. Essential Oil Composition, Cytotoxic and Antibacterial Activities of Five *Etlingera* Species from Borneo. Natural Product Communications, *7* (2): 239–242.

Wahyuni, Diantini A, Ghozali M & Sahidin 2021. *Etlingera* Genus: Phytochemical Screening and Anticancer Activity. *Jurnal Farmasi Sains dan Praktis*, *7* (3): 343–355.

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Managing Editors: Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, MALAYSIA. Tel: +60-88-320000 ext. 103134; Fax: +6088-329246
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Institute for Tropical Biology and Conservation
Universiti Malaysia Sabah
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