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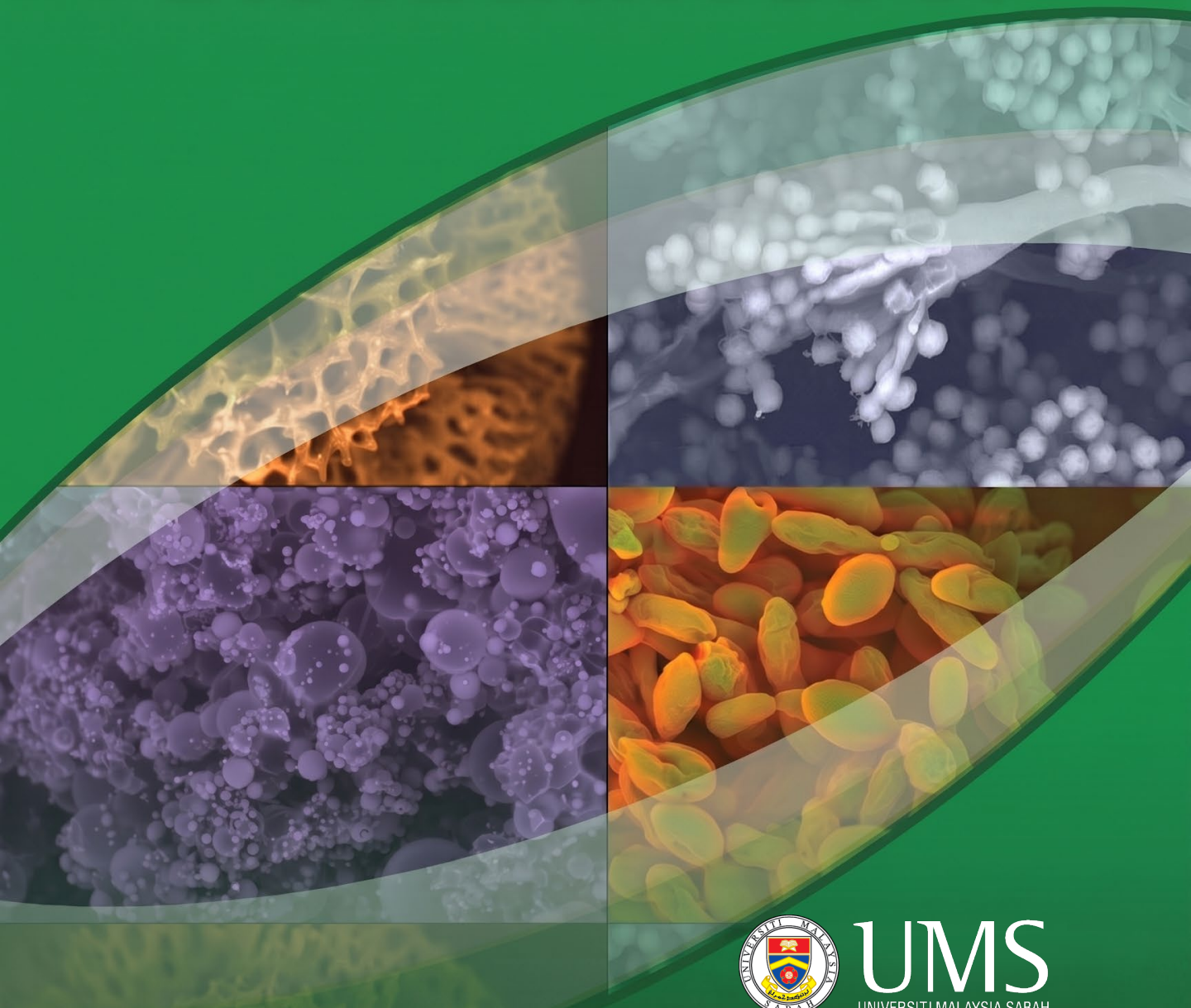

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Contents

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Borneo International Journal of Biotechnology (BIJB)
Vol. 4 (December 2024), 1 – 7

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Analyzing Cymbopogon sp methanolic extracts via liquid chromatography

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ABSTRACT

Cymbopogon species can be utilized in various ways. It is applied in cooking, and aromatherapy, among others. Essential oil is also used extensively in traditional medicine. From the literature, it possesses various classes of compounds, for example, terpenoids and flavonoids. In this study, an unidentified *Cymbopogon* methanolic was subjected to a Reversed Phased-High Performance Liquid Chromatography (RP-HPLC). The mobile phase was adapted from the national herbal monograph, comprising 0.3% formic acid and acetonitrile. The HPLC chromatograms were recorded, and two major peaks were observed. Both signals were apparent in the stepwise gradient when compared to the fast mode. In the absence of the standard monoterpenoids, geraniol, geranial and neral could be further investigated from this sample. In conclusion, a specimen of *C. citratus* could be involved in the experimental procedures.

Keywords: chromatography, *Cymbopogon*, serai makan, lemongrass, monograph

INTRODUCTION

The genus *Cymbopogon* encompasses approximately 55 species, native to tropical and semi-tropical regions of Asia. These species are also cultivated in South and Central America, Africa, and other tropical countries (Shah et al., 2011). *Cymbopogon* is commonly known as lemongrass or "serai" in Malay. Among its species, *C. citratus* is particularly popular, often referred to as "serai makan." The chemical composition of *Cymbopogon* species, including the essential oil and its citral content, can vary

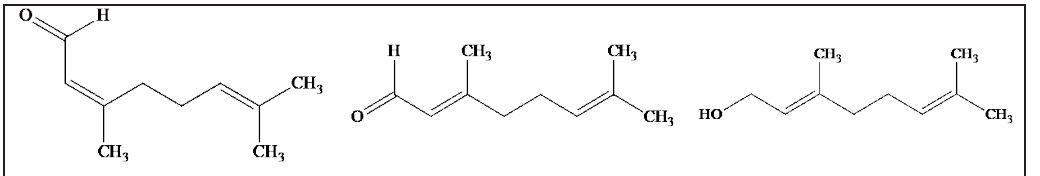
significantly depending on several factors, such as geographic origin, plant part used, maturity stage, genetic variations, extraction method, and harvest season (Méabed et al., 2018). Therefore, optimal collection at the correct stage of maturity is crucial for ensuring high-quality essential oil and minimizing production costs (Tajidin et al., 2012).

The natural bioactive compounds in *Cymbopogon* include terpenoids (Figure 1), saponins, tannins, alkaloids, phenols, flavonoids, and anthraquinones. Notable constituents include citral, geranial, geraniol, myrcene, limonene, citronellol, borneol, neral, nerol, α-terpineol, elemicin, kaempferol, apigenin, caffeic acid, quercetin, luteolin, geranyl acetate, and chlorogenic acid, along with other unidentified compounds (Shah et al., 2011).

Various analytical techniques have been employed to study *Cymbopogon* essential oils. For example, an assay was developed to quantify citral in *C. citratus* volatile oil using normal-phase High-Performance Liquid Chromatography (HPLC) combined with UV spectroscopic detection (Rauber et al., 2005). Additionally, gas chromatography-mass spectrometry (GC-MS) has been used to analyze these oils (Tajidin et al., 2012; Madivoli et al., 2012). For phenolic compound quantification, Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) has been successfully employed (Figueirinha et al., 2008; Costa et al., 2015; Méabed et al., 2018).

In urban areas of Sabah, *C. citratus* and *C. nardus* are commercially traded as medicinal plants (Foo et al., 2016a, 2016b), with Sabah Tea products available online in various forms, including pot bags and loose tea (Sabah Tea Resort, 2020). *C. schoenanthus* (camel grass) has also been used in Iranian traditional medicine for its purported ability to reduce fatigue (Alembagheri et al., 2023). Despite these uses, *Cymbopogon* species still lack comprehensive phytochemical characterization and standardization.

Recent studies have explored advanced techniques such as Nuclear Magnetic Resonance (NMR) to elucidate the flavonoid profiles of *C. giganteus*, a wild lemongrass species (Bationo et al., 2022). Additionally, a comprehensive NMR metabolic profiling study of five *Cymbopogon* species was conducted (Otify et al., 2022), where RP-HPLC was used to analyze an unidentified *Cymbopogon* species. This chromatographic approach may prove useful for identifying and distinguishing species based on the pattern of their alcoholic extract, especially given the current lack of studies focused on RP-HPLC analysis of *Cymbopogon* species.



Neral	Geranial	Geraniol
(*Z*)-3,7-dimethylocta-2,6-dienal	(*E*)-3,7-dimethylocta-2,6-dienal	(*E*)-3,7-dimethylocta-2,6-dien-1-ol
$C_{10}H_{16}O$	$C_{10}H_{16}O$	$C_{10}H_{18}O$

Figure 1 Structures of monoterpenes from the *Cymbopogon* extract.

MATERIALS AND METHODS

Sample preparation

Aerial portions of *Cymbopogon* species were procured from a local market in Puncak Alam, Selangor. The plant materials were mechanically fragmented using a mortar and pestle to enhance surface area (Tajidin *et al*., 2012). Dried and powdered samples were subjected to ultrasonic extraction using 100% methanol prior to chromatographic analysis. The extract was filtered through a 0.45 µm Millipore filter, into the vials. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) using an Agilent 1200 system was employed to profile the extract (Globinmed, 2021a; 2021b).

A 10 µL aliquot of the sample was injected into the HPLC system. The separation was achieved using a Thermo C-18 column (250 mm × 4.6 mm, 5 µm particle size) as the stationary phase. Two approaches were employed to compare the extract profiles between a rapid and a non-isocratic, stepwise gradient (Table 1, Figure 2) elutions in RP-HPLC methods. The mobile phase consisted of various compositions of 0.3% formic acid in water and acetonitrile, with a flow rate of 1 ml/min. A diode array detector (DAD) monitored the eluent at 254 and 360 nm and the column temperature was maintained at 36°C throughout the experiment. The running time was set at 30 and 50 minutes for the rapid and stepwise gradient modes, respectively. The data acquisition was performed by using ChemStation software.

Table 1 The solvents for the stepwise gradient elution.

Time (minutes)	Aceto-nitrile (%)	0.3% Formic Acid in Water (%)
0	10	90
5	10	90
20	40	60
22	40	60
30	70	30
32	70	30
40	90	10
45	90	10
47	10	90
50	10	90

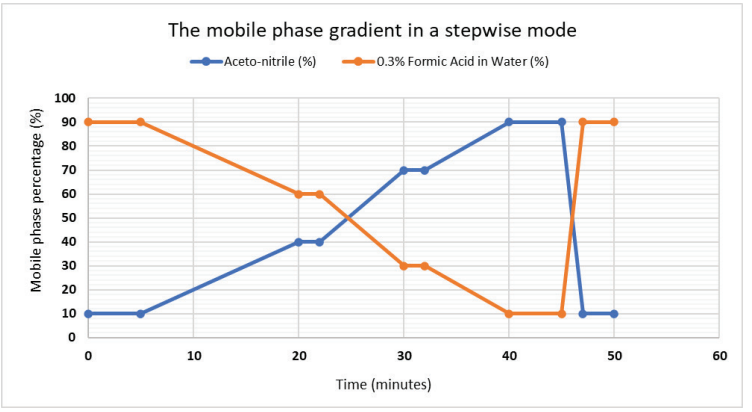


Figure 2 The composition of the mobile phase for the HPLC run (stepwise gradient mode).

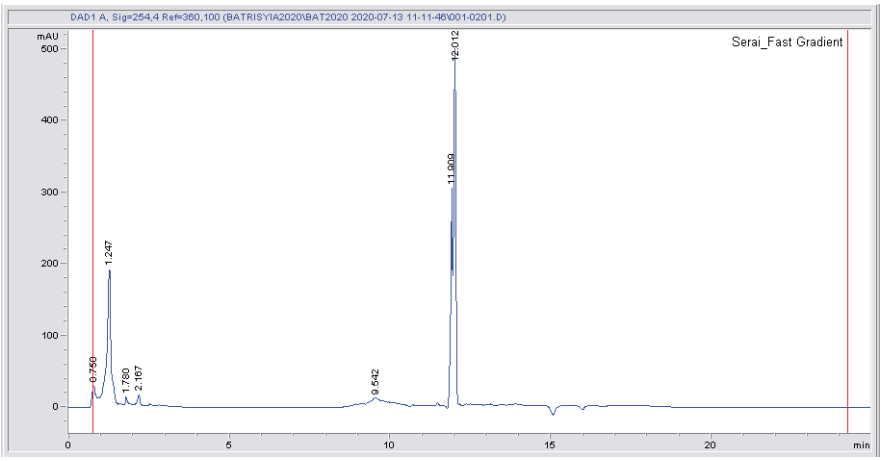


Figure 3 The chromatogram of *Cymbopogon* methanolic extract (fast gradient).

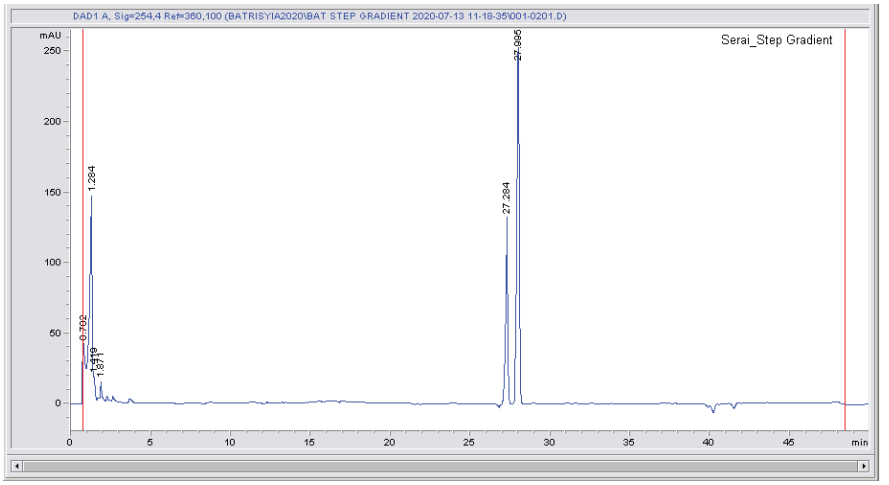


Figure 4 The chromatogram of *Cymbopogon* methanolic extract (stepwise gradient).

RESULTS AND DISCUSSION

The lipid- and essential oil-free infusion of *Cymbopogon* can be prepared by adding boiling water to the plant material (Figueirinha et al., 2008; Costa et al., 2015). In contrast, the essential oil can be extracted using methanol in this study. If a more nonpolar solvent, such as n-hexane, is employed for extraction (Rauber et al., 2005), normal-phase HPLC would be suitable for detecting compounds in the volatile oil. Figure 2 shows the chromatogram of the methanol extract under a fast gradient, while Figure 4 presents the chromatogram using a stepwise gradient. In the fast-gradient method, the compounds elute as two overlapping peaks at retention times (tR) of 11.909 and 12.012 minutes for peak 1 and peak 2, respectively (Figure 2). In the stepwise gradient mode, the peaks are better resolved, with tR values of 27.284 and 27.995 minutes for peak 1 and peak 2, respectively (Figure 3).

Baseline separation was achieved when the mobile phase composition approached a 50:50 ratio of acetonitrile and 0.3% formic acid. This setup is consistent with previously published data, which used an acetonitrile (70:30) system in isocratic flow at 1 mL/min for RP-HPLC. In that system, the order of elution was neral (tR = 7.276 minutes), followed by geranial (tR = 7.689 minutes). While *Cymbopogon* species can contain geraniol, it is typically present in trace amounts (Gaonkar et al., 2016).

In this study, the inclusion of formic acid increases the acidity of the acetonitrile mixture, which in turn lengthens the retention time. The protonated solvent interacts with the monoterpene aldehydes (Figure 2), helping to retain these molecules on the C-18 column. In the stepwise gradient mode (Figure 3), both compounds are retained longer in the column due to the gradual decrease in the mobile phase's polarity.

Optimization of the RP-HPLC mobile phase was conducted using acetonitrile and water, though the specific 50:50 acetonitrile
mixture was not previously reported (Gaonkar et al., 2016). As expected, both compounds eluted at retention times above 12 minutes, as shown in this experiment (Figures 2 and 3). It was found that acetonitrile (70:30) provided the best resolution for separating the neral and geranial peaks, along with other citral components (Gaonkar et al., 2016). Using a higher acetonitrile concentration (e.g., 90:10) resulted in faster elution and lower peak resolution (tR around 4.5 minutes), as seen in Figure 3. For *C. citratus*, a more complex RP-HPLC method has been described, using ammonium acetate and 0.1% formic acid in water as the first solvent, and methanol as the second mobile phase (Globinmed, 2021a). The elution order remained similar to that in Figures 3 and 4, with neral (tR = 16.850 minutes) eluting before geranial (tR = 19.006 minutes). A simpler RP-HPLC method for *C. nardus* has also been documented (Globinmed, 2021b), using a mobile phase of 0.1% formic acid in water and acetonitrile, with a major peak corresponding to geraniol (tR = 11.2 minutes).

In comparison, *C. citratus* has been found to be particularly enriched in neral and geranial, which are the primary characteristic metabolites of this species (Otify et al., 2022). This is in contrast to other *Cymbopogon* species such as *C. flexuosus*, *C. procerus*, *C. martini*, and *C. nardus* (locally known as "serai wangi"). Additionally, Kaur et al. (2021)

reported that the essential oil of *C. nardus* is primarily composed of citronellal, citronellol, and geraniol. The chemical composition of *Cymbopogon* essential oils can vary based on factors such as geographic origin, environmental conditions, developmental stages, harvest time, and genetic differences (Kaur et al., 2021). Furthermore, an HPLC study of *Cymbopogon* ethanolic extracts from Pakistan revealed additional phytochemicals, including flavonoids and phenolics (Lin et al., 2021). Given these variations, the *C. citratus* specimen selected for this study can be sourced from retail vendors (Globinmed, 2021a).

CONCLUSION

The identification of monoterpenoids was suggested by comparing the retention times and HPLC data of each compound of the peaks in this random *Cymbopogon* sample to those of the published chromatogram. In short, geraniol, geranial and neral could possibly be introduced in this chemical investigation due to the absence of any standard compounds.

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REFERENCES

Alembagheri, A., Hajimehdipoor, H., Choopani, R., Esmaeili, S. (2023). The role of selected medicinal plants from Iranian traditional medicine for the treatment of fatigue in metabolic syndrome. *Tradit Med Res*., *8*(4), 23. doi: 10.53388/TMR20220706001

Bationo, R. K., Dabiré, C. M., Hema, A., Nébié, R. H. Ch., Palé, E., & Nacro, M. (2022). HPTLC/HPLC-mass spectrometry identification and NMR characterization of major flavonoids of wild lemongrass (*Cymbopogon giganteus*) collected in Burkina Faso, *Heliyon*, *8*, e10103.

Costa, G., Nunes, F., Vitorino, C., Sousa, J. J., Figueredo, I. V. & Batista, M. T. (2015). Validation of a RP-HPLC Method for Quantitation of Phenolic Compounds in three Different Extracts from *Cymbopogon citratus*. *Research Journal of Medicinal Plant,* 1-9.

Figueirinha, A., Paranhos, A., Pérez-Alonso, J. J., Santos-Buelga, C. & Batista, M. T. (2008). *Cymbopogon citratus* leaves: Characterization of flavonoids by HPLC–PDA–ESI/MS/MS and an approach to their potential as a source of bioactive polyphenols, *Food Chem.*, *110*, 718–728.

Foo, J., Mohamad, A. L., Omar, M. & Amir, A. A. (2016a). Ethnobotanical Survey of Medicinal Plants Traded at Tamu in Sabah Urban Area. *International Journal of the Malay World and Civilisation (Iman)*, *4* (Special Issue 1), 79 – 87.

Foo, J., Mohamad, A. L., Omar, M. & Amir, A. A. (2016b). Utilitarian tumbuhan ubatan di Tamu Pantai Barat Sabah. *Geografia Online™, Malaysian Journal of Society and Space*, *12* (12), 99-112.

Gaonkar, R., Yallappa, S., Dhananjaya, B. L. & Hegde, G. (2016). Development and validation of reverse phase high performance liquid chromatography for citral analysis from essential oils. *J Chromatogr B*, *1036*, 50-56.

Globinmed (2021a). *Cymbopogon citratus* (DC.) Stapf, Accessed on 25th August 2022, https://www.globinmed.com/medicinal_herbs/cymbopogon-citratus-dc-stapf-105681/

Globinmed (2021b). *Cymbopogon nardus (L.)* Rendle, Accessed on 25th August 2022, https://www.globinmed.com/medicinal_herbs/cymbopogon-nardus-l-rendle-2/

Kaur et al. (2021). *Cymbopogon nardus* essential oil: a comprehensive review on its chemistry and bioactivity, *Journal of Essential Oil Research*, 33:3, 205-220, DOI: 10.1080/10412905.2021.1871976.

Lin, X., Rafique, A., Fayyaz, T., Bashir, W., Luqman, M., Zahid, F.M. and Zhou, K. (2021) Appraisal of *Cymbopogon Citratus* (Lemon grass) for antibacterial activity against Uropathogens. *Pak Vet J*, *41*(1): 122-126. http://dx.doi.org/10.29261/pakvetj/2020.040

Madi et al. (2022). Morphological and Anatomical Characterization of *Cymbopogon citratus* (DC.) Stapf Cultivated in Egypt," Bulletin of Faculty of Pharmacy Cairo University: Vol. 60: Issue 1, Article 6. Available at: https://doi.org/10.54634/2090-9101.1032

Madivoli, E. S., Gitu, L. & Gumba, E. (2012). Isolation and Identification of Essential Oils from *Cymbopogan Citratus* (Stapf) DC Using GC-MS and FT-IR. *Chemistry and Materials Research*, *2* (4), 13 – 22.

Méabed, E. M. H., Abou-Sreea, A. I. B. & Roby, M. H. H. (2018). Chemical analysis and giardicidal effectiveness of the aqueous extract of *Cymbopogon citratus* Stapf, *Parasitology Research*, *117* (6), 1745-1755.

Mohamad, T. A. S. T., Islahudin, F., Jasamai, M., Jamal, J. A. (2022). Traditional Practitioner's Knowledge of Malay Post-Partum Herbal Remedies in Malaysia. *Arch Pharm Pract*., *13* (2), 11-6. https://doi.org/10.51847/ani1usVKCvhttps://doi.org/

Otify, A. M., Serag, A., Porzel, A., Wessjohann, L. A. & Farag, M. A. (2022). NMR Metabolome-Based Classification of *Cymbopogon* Species: A Prospect for Phyto-equivalency of its Different Accessions Using Chemometric Tools. *Food Analytical Methods*, *15*, 2095–2106.

Rauber, C. da S., Guterres, S. S. & Schapoval, E. E. S. (2005). LC determination of citral in *Cymbopogon citratus* volatile oil. *J. of Pharm. and Biomed. Analysis*, *37*, 597–601.

Sabah Tea Resort - Kota Kinabalu (2020). Accessed on 6th January 2023, via https://www.sabahtea-eshop.com/products/borneo-exotic-series-lemongrass

Shah, G., Shri, R., Panchal, V., Sharma, N., Singh, B., & Mann, A. S. (2011). Scientific basis for the therapeutic use of *Cymbopogon citratus*, Stapf (lemon grass). *J Adv Pharm Technol Res.*, *2* (1), 3–8.

Tajidin, N. E., Ahmad, S. H., Rosenani, A. B., Azimah, H. & Munirah, M. (2012). Chemical composition and citral content in lemongrass (*Cymbopogon citratus*) essential oil at three maturity stages. *African Journal of Biotechnology*, *11* (11), 2685-2693.

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Trends of Traditional Postpartum Practices Utilizing Local Medicinal Plants from Kota Belud, Sabah

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ABSTRACT

Ethnogynaecology is a field of study that focuses on managing gynaecological-related issues including postpartum care, through the utilization of traditional healers, tribal communities, and local practitioners. Traditional knowledge of Sabah medicinal plants is insufficiently documented, despite Sabah is home to more than 42 different ethnics and rich in flora diversity. This study aims to record medicinal plants from Kota Belud, that have been used as traditional remedies during postpartum care for mothers. This work also provides brief details of plant parts used as traditional remedies and the administrating methods. A total of nine plant species, namely *Aloe vera*, *Bambusa sp*., *Benincasa hispida*, *Cosmos caudatus*, *Curcuma longa*, *Curcuma xanthorrhiza*, *Ficus septica*, *Momordica charantia*, *Zingiber zerumbet*, were traditionally employed for postpartum care, a period spanning 40 to 90 days post-partum. The ethnopharmacological data gathered directly from these communities has the potential to serve as a substantial foundation for future research in natural product development and drug discovery.

Keywords: Medicinal plants, postpartum delivery, traditional practices.

INTRODUCTION

The state of Sabah in Malaysia is characterized by its tropical climate and annual rainfall of 4200 mm, which contributes to the preservation of its rich biodiversity. The forested area in Sabah covers approximately 37600 square kilometers, which is considered substantial compared to other regions in Southeast Asia (Reynolds et al., 2011). Sabah's diverse cultural landscape is home to over 40 indigenous ethnic and sub-ethnic groups, each with its own unique traditions and customs. Among these groups are five main ethnicities: Kadazandusun, Bajau, Murut, Chinese, and Brunei Malay (Madlan et al., 2014). With the exception of the Chinese, the other four main indigenous groups are collectively known as Bumiputra Sabah. Sabah's linguistic diversity is evident in its over 50 languages and 80 dialects (Muhammed & Muthu, 2015). Kota Belud, a developing district on Sabah's west coast, covers an area of 1385.6 square kilometers. The local people of Kota Belud are from various ethnic groups: Sama-Bajau, Bajau Ubian, Dusun, Irranun, Rungus, and Chinese. Sama-Bajau and Dusun communities in Kota Belud were selected due to their enthusiasm for sharing their traditional knowledge. Moreover, the district of Kota Belud has ecological diversity that ranges from coastal area, agricultural land, and the lower montane area around predominantly Dusun villages in Kadamaian. These varied ecological landscapes imply flora and fauna species richness could be present in Kota Belud.

Ethnogynaecology is a field of study, that focuses on the utilization of medicinal plants by indigenous communities, local healers, and traditional practitioners to address gynecological-related matters including postpartum care (Surendran et al., 2023; Jamal et al., 2011). The importance of plant-based medicine in the development of drugs has been well-established, as the use of plants for medicinal purposes dates back to ancient times. Plants contain secondary metabolites that serve as defense mechanisms, and these compounds have demonstrated both preventive and curative effects (Li et al., 2020). Traditional knowledge regarding the use of plants is often passed down through generations, and the pharmacological properties of these culturally significant plants form a link between drug discovery research and ethnopharmacology (Patwardhan, 2005). The ethnobotanical survey is among the most reliable approaches to initiate drug discovery (Surendran et al., 2023; Awang-Kanak et al., 2022). The main objective of this paper is to record medicinal plants used during traditional postpartum care in Sabah.

MATERIALS AND METHODS

Data collection and identification

Semi-structured interviews were conducted with villagers from three villages in Kota Belud district to gather information on the use of locally sourced vegetables as traditional medicine, particularly for postpartum care (Martin, 1995). Thirteen informants were selected using a

snowball sampling technique. Informants were chosen based on two criteria: (i) their willingness to share their knowledge about the utilization of freshly eaten vegetables as herbal medicine, specifically for postpartum care, and (ii) their ability to provide descriptions of how the herbal medicine is administered to women during their confinement period. Demographic information on the informants, as well as details on the specific plant parts used for postpartum remedies, were recorded. The identification of species was assisted by Sandakan Herbarium (SAN). Access to Sabah traditional knowledge and plant species is approved by Sabah Biodiversity Council (SaBC) (JKM/MBS.1000-2/2 JLD. 6 (24)) and by Sabah Forest Department (JPHTN/TKKH (PSH) 100-14/18/2 (47). All data obtained comply with Sabah state laws and regulations on biodiversity resources and access benefit sharing.

Ethnobotanical indices analysis

Frequency of Citation (FC) is a metric used to identify the most commonly used plants. FC is calculated by dividing the number of informants who mentioned a specific plant by the total number of informants interviewed. The Use Value (UV) was calculated using the following formula: UVis = ΣUis/ nis, where UVis is the use value of species s, ΣUis is the total number of uses reported for species s, and nis is the total number of informants. UVis is the use of value of the species s mentioned by informant i, ΣUis is the sum number of used of species mentioned by all informants i, nis is the total number of the informants (Silva et al., 2014).

RESULTS AND DISCUSSION

A total of thirteen informants from ethnics Sama-Bajau and Dusun were interviewed for their traditional knowledge. Informants ranged in age from 29 to 67 years old at the time of the interviews. Nine plant species were traditionally used for postpartum care, a period lasting 40 to 90 days. These nine plant species include *Aloe vera*, *Bambusa sp*., *Benincasa hispida*, *Cosmos caudatus*, *Curcuma longa*, *Curcuma xanthorrhiza*, *Ficus septica*, *Momordica charantia*, *Zingiber zerumbet* (Table 1).

Table 1: Selected medicinal plants used for postpartum care by local people in Kota Belud, Sabah.

Scientific name	Local name	Locality	Part used	Preparation
Aloe vera	Lidah buaya	TG	Leaves (flesh & bud)	Freshly eaten, mixed with drink or soup.
Bambusa sp.	Rebung buluh	M	Young shoot	Boiled before eaten.

Benincasa hispida	Kundur	TG	Fruit	Fruit soaked with water, use as bathing water for jaundice baby.
Cosmos caudatus	Ransa ransa (Bajau)	M	Leaves	Freshly eaten.
Curcuma longa	Kunyit	M, P	Tuber	Decoction, consume as drink for mother
Curcuma xanthorrhiza	Temulawak	TG	Young leaves	Freshly eaten, briefly boiled before eaten.
Ficus septica	Lintotobou (Dusun)	P	Root	Decoction, consume as drink for mother
Momordica charantia	Peria	M	Fruit	Fruit soaked with water, for bathing.
Zingiber zerumbet	Lempoyang	TG	Young leaves	Freshly eaten, briefly boiled before eaten.

Locality: TG = Taun Gusi, M = Menunggui, P = Pinolobu

Various parts of plants have been used in different preparations, including leaves, young shoots, tubers, roots, and fruits. These preparations were consumed orally and used as bathing mixtures for both mothers and newborn infants. The daily dietary intake for new mothers consists of boiled young shoots of *Bambusa sp*., fresh leaves of *Cosmos caudatus*, fresh cuts of *Aloe vera*, and a decoction made from the tuber of *Curcuma longa*. Consuming fresh leaves of *Cosmos caudatus* and *Aloe vera*, along with boiled *Bambusa sp*. shoots, will benefit as *Cosmos caudatus* leaves has antioxidative properties, bamboo shoots and *Aloe vera* also provide dietary fibre that may support easy digestion for mothers during postpartum recovery (Rafi et al., 2023). The utilization of *Curcuma longa* (tumeric) and other species from the Zingiberaceae family, such as *Zingiber aromaticum* and *Zingiber officinale* (common ginger), during postpartum care, is also a traditional practice among the Malay population in the states of Johor and Negeri Sembilan. Ginger species are ground and mixed with hot water, then consumed as a drink, this practice is believed able to improve blood circulation (Jamal et al., 2011). Traditionally drinking ginger decoction may help ease perineal pain (Khusniaty et al., 2023). The recent surge in the development and application of bioactive materials for skin wound healing has seen growing interest in incorporating natural anti-inflammatory and antioxidant components (Zhou et al., 2023). This innovative approach in biomedicine using natural anti-inflammatory elements like ginger extract aligns with traditional practices of consuming ginger decoction for perineal pain relief.

Curcuma spp., especially *Curcuma longa*, exhibits various phytochemical properties that are significant in the context of postpartum care. One particularly noteworthy property of the Curcuma genus is the presence of curcumin and its derivatives, which have the potential to act as the primary bioactive components responsible for enhancing blood circulation, and relieving pain (Chen et al., 2019). A decoction made from crushed Curcuma tuber also shows anti-inflammatory activity through the inhibition of cyclooxygenase and lipoxygenase and also has been shown to possess analgesic properties, which can help alleviate postpartum pain, including pain associated with uterine contractions and perineal tears (Tuntisayawadikul & Sripanidkulchai, 2022; Mutia et al., 2017). *C. longa* extracts contain curcuminoids, including curcumin, desmethoxycurcumin, and bisdemethoxycurcumin (Figure 1). These curcuminoids have shown scavenging activity against oxygen-free radicals, and also inhibit inflammatory mechanism of action (Tuntisayawadikul & Sripanidkulchai, 2022).

Ficus septica is a well-known medicinal plants among the Dusun people in Sabah, apart from being used as a medicinal plant for postpartum recovery, the decoction of *Ficus septica* is also consumed orally to relieve headache, stomach pain, and shivering (Awang-Kanak et al., 2022; Kulip 2014; Kulip 1997). Meanwhile, ethanol extract of *Ficus septica* has shown anti-parasitic activity, by promoting apoptosis to *Trichomonas vaginalis* (Vital et al., 2010). The parasitic protozoan *Trichomonas vaginalis* is the causative agent of trichomoniasis, a sexually transmitted infection (STI) primarily affecting the urogenital tract (Mehriardestani et al., 2017).

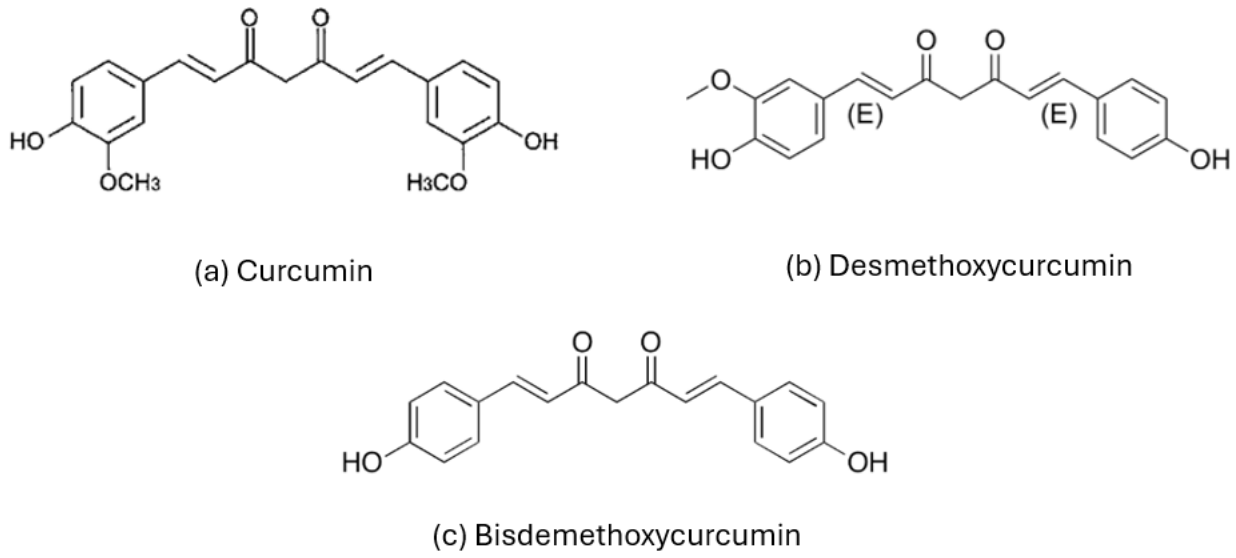

Figure 1: Chemical structures of curcuminoids; (a) Curcumin, (b) Desmethoxycurcumin, (c) Bisdemethoxycurcumin.

CONCLUSION

This paper reports data on medicinal plants that have been used for ethnogynaecological purposes by local people in Kota Belud, Sabah. It can be concluded that freshly prepared medicinal plants are the preferred traditional remedies for mothers during their confinement period. Meanwhile, species from the ginger family, Zingerberaceae, namely; *Curcuma longa*, *Curcuma xanthorrhiza*, and *Zingiber zerumbet* are the most useful for postpartum care in this district. The ethnobotanical index for frequency of citation (FC) for these three species of Zingiberaceae family is 0.17, meanwhile the use value (UV) is 0.33. This assertion is also supported by several studies on chemical properties and anti-inflammatory activity shown by Zingeberacae species extracts that promote the healing process of perineal tears. Ethnogynaecological data can provide a valuable foundation for pharmacology and drug discovery research in women's health. It offers a unique perspective on treatments, access to a diverse range of natural resources, and the potential to develop culturally relevant and sustainable solutions for gynaecological conditions. However, it's essential that such research is conducted ethically, respecting the knowledge and practices of local communities, and that rigorous scientific methods are employed to validate and refine traditional remedies into safe and effective pharmaceutical products.

REFERENCES

Awang-Kanak, F., Matawali, A., Jumat, N. R., & Bakri, S. N. S. (2021). A Preliminary Survey on Edibles and Medicinal Plants Used by Dusun of Kampung Pinolobu, Kadamaian, Kota Belud, Sabah, Malaysia. Journal of Tropical Biology & Conservation (JTBC), 18, 21-30.

Chen, Z., Quan, L., Zhou, H., Zhao, Y., Chen, P., Hu, L., ... & Cao, D. (2019). Screening of active fractions from Curcuma Longa Radix isolated by HPLC and GC-MS for promotion of blood circulation and relief of pain. Journal of Ethnopharmacology, 234, 68-75.

Jamal, J. A., Ghafar, Z. A., & Husain, K. (2011). Medicinal plants used for postnatal care in Malay traditional medicine in the Peninsular Malaysia. Pharmacognosy Journal, 3(24), 15-24.

Khusniyati, E., Purwati, H., & SB, E. M. (2023). Effectiveness of Non-Pharmacological intervention to reduce perineal pain in postpartum women. Journal of Scientific Research, Education, and Technology (JSRET), 2(2), 667-679.

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. (2014). The ethnobotany of Dusun people in Tikolod village, Tambunan district, Sabah, Malaysia. Reinwardtia, 14(1), 101-121.

Li, Y., Kong, D., Fu, Y., Sussman, M. R., & Wu, H. (2020). The effect of developmental and environmental factors on secondary metabolites in medicinal plants. Plant Physiology and Biochemistry, 148, 80-89.

Madlan, L. B., Seok, C. B., Mutang, J. A., Baharudin, S. A., & Joo, H. C. (2014). The prejudice of Bajau: from own and others ethnic perspective: A preliminary study in Sabah. International Journal of Information and Education Technology, 4(3), 244.

Martin, G. J. (1995). Ethnobotany: a methods manual, Chapman y Hall. Nowy Jork.

Mehriardestani, M., Aliahmadi, A., Toliat, T., & Rahimi, R. (2017). Medicinal plants and their isolated compounds showing anti-Trichomonas vaginalis-activity. Biomedicine & Pharmacotherapy, 88, 885-893.

Muhammed, N., & Muthu, T. A. (2015). Indigenous people and their traditional knowledge on tropical plant cultivation and utilization: A case study of Murut communities of Sabah, Borneo. Journal of Tropical Resources and Sustainable Science (JTRSS), 3(1), 117-128.

Mutia, W. O. N., Usman, A. N., Jaqin, N., Rahman, L., & Ahmad, M. (2021). Potency of complementary therapy to the healing process of the perineal wound; turmeric (Curcuma longa Linn) Infusa. Gaceta Sanitaria, 35, S322-S326.

Patwardhan, B. (2005). Ethnopharmacology and drug discovery. Journal of ethnopharmacology, 100(1-2), 50-52.

Rafi, M., Hayati, F., Umar, A. H., Septaningsih, D. A., & Rachmatiah, T. (2023). LC-HRMS-based metabolomics to evaluate the phytochemical profile and antioxidant capacity of Cosmos caudatus with different extraction methods and solvents. Arabian Journal of Chemistry, 16(9), 105065.

Reynolds, G., Payne, J., Sinun, W., Mosigil, G., & Walsh, R. P. (2011). Changes in forest land use and management in Sabah, Malaysian Borneo, 1990–2010, with a focus on the Danum Valley region. Philosophical Transactions of the Royal Society B: Biological Sciences, 366(1582), 3168-3176.

Silva, H. C. H., Caraciolo, R. L. F., Marangon, L. C., Ramos, M. A., Santos, L. L., & Albuquerque, U. P. (2014). Evaluating different methods used in ethnobotanical and ecological studies to record plant biodiversity. Journal of Ethnobiology and Ethnomedicine, 10(1), 48.

Surendran, S., Prasannan, P., Jeyaram, Y., Palanivel, V., Pandian, A., & Ramasubbu, R. (2022). Knowledge on ethnogynaecology of Indian Tribes-a comprehensive review. Journal of Ethnopharmacology, 115880.

Tuntiyasawasdikul, S., & Sripanidkulchai, B. (2022). Development and clinical trials on anti-inflammatory effect of transdermal patch containing a combination of Kaempferia parviflora and Curcuma longa extracts. Journal of Drug Delivery Science and Technology, 68, 103093.

Vital, P. G., Velasco, R. N., Demigillo, J. M., & Rivera, W. L. (2010). Antimicrobial activity, cytotoxicity and phytochemical screening of Ficus septica Burm and Sterculia foetida L. leaf extracts. Journal of Medicinal Plants Research, 4(1), 58-63.

Zhou, C., Zhao, K., You, X., Wang, Y., Chen, L., & Sun, L. (2023). Enhancement of Antioxidant/Anti-Inflammatory Activity, and Acceleration of Wound Healing Through the Combination of Ginger Extract and NAC: A Promising Approach. Journal of Biomaterials and Tissue Engineering, 13(11), 1072-1081.

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Molecular Detection of Schmallenberg Virus: An Initiative to Approach Sero-Positive Samples

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ABSTRACT

Schmallenberg virus (SBV) is a pathogen of veterinary importance that was first detected in the European ruminant population in 2011. Since then, the RNA virus has spread to various parts of the world, causing clinical manifestations such as abortions, stillbirths, and congenital malformations in ruminants. There are no molecular surveillance studies for SBV in Malaysia, and this study aimed to perform molecular detection of SBV using the positive SBV serum samples from small ruminants collected from selected farms in Terengganu and Negeri Sembilan in 2019. A molecular technique, reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze 78 serum samples in comparison to an in-house synthesized positive control of the SBV L segment sequence. None of the serum samples tested were positive for SBV RNA but this does not rule out the absence of SBV in animals. This was due to the fact that an undetectable viraemia period of SBV after a short duration of the active viral infection cycle or the presence of neutralizing antibodies could eliminate the viral particles from the bloodstream over time. It is therefore highly recommended to take samples from acutely infected animals.

Keywords: Schmallenberg virus, RNA virus, molecular detection, RT-PCR, viraemia, acute infection

INTRODUCTION

Schmallenberg virus (SBV) is an enveloped, segmented, negative-sense, single-stranded RNA virus that is classified in the Simbu serogroup of the genus Orthobunyavirus in the family Bunyaviridae (Wernike et al., 2015). According to the report of many epidemiological studies, SBV is known to infect ruminants but is not a zoonosis (OIE 2017). The origin of SBV remains controversial, but few reports of the discovery of SBV-like virus particles from equatorial Africa have been documented, even before the outbreak in Europe. The virus was first isolated in Germany and has currently been reported in many countries in Europe such as the UK, France, Italy, Luxembourg, Spain, Denmark, Estonia, and Switzerland (Lievaart-Peterson et al., 2015; Stavrou et al., 2017). Over time, the livestock trade has meant that SBV has also spread to the rest of the continent (Hoffmann et al., 2012; Zhai et al., 2018). In addition, recurring epidemics have been reported every few years in Germany, Belgium, Ireland and the United Kingdom (APHA, 2016; Collins et al., 2017; Dastjerdi & Steinbach, 2021; Delooz et al., 2017; Wernike & Beer, 2020; Wernike, Hoffmann, et al., 2015). The SBV outbreak from 2011 to 2012 resulted in estimated direct and indirect costs of approximately €150–€200 million for the European livestock industry. These costs include livestock losses, veterinary expenses, and reduced productivity (EFSA, 2014). In certain infected dairy herds, milk production dropped by as much as 25% in the weeks following infection, primarily due to fever and loss of appetite in the affected animals (Sudhakar et al., 2020). Additionally, studies have shown that SBV infection during pregnancy in sheep led to abortion and increases stillbirth rates within affected herds (Peperkamp et al., 2015).

First, SBV was found to infect a range of ruminant species, followed by wildlife species. It should be noted that only domesticated ruminants show overt clinical SBV symptoms, while other wild ruminants (buffalo, deer, alpaca, bison, mouflon) and other mammalian species (boar, horse, kudu, oryx, zebra) have been described exclusively with indirect application were serological proof (Wernike et al., 2013). Infection with SBV is associated with a short period of viraemia (2-6 days) that causes fever, diarrhea, hyperthermia, and reduced milk production (up to 50%) in cattle. On the other hand, similar clinical symptoms attributed to infected sheep and goats are mostly subclinical (Hoffmann et al., 2012). In addition, seroconversion of SBV antibodies has been reported approximately 1-3 weeks after infection (Hoffmann et al., 2012). In some extreme cases, miscarriages or congenital fetal malformations have been reported in pregnant ruminants in which SBV was transmitted vertically during gestation (Bayrou et al., 2014). Not to forget the detection of SBV RNA in the semen of infected bulls (Hoffman et al., 2013). SBV contains three core genomic segments: S (small), M (medium), and L (large) segments. The nucleoprotein N and nonstructural proteins are encoded by the S segment, while the envelope glycoproteins Gn and Gc are encoded by the M segment. In comparison, the L segment encodes RNA-dependent RNA polymerase (RdRp) (Chowdhary et al., 2012; Elliot 2014). All of these segments were used to detect

the presence of viruses by different PCR systems (Bilk et al., 2012; Fischer et al., 2013; Hoffmann et al., 2012). The assay based on S and L segments is considered to be superior in terms of specificity and sensitivity in detecting the viral RNA, while high genetic variations occur in the M segment, facilitating immune escape from the target host (Wernike et al., 2021).

Bunyaviruses consist largely of viruses transmitted by arthropod vectors, with the exception of hantaviruses, which are transmitted by rodents. SBV, like other members of its serogroup, is transmitted by haematophagus insect vectors, Culicoides or mosquitoes (Pawaiya & Gupta, 2013), which are common in Malaysia. Interestingly, several species of *Culicoides* namely *C. obsoletus*, *C. dewulfi* and *C. chiopterus* were found to be SBV positive by RT-PCR (de regge et al., 2012). However, studies on transmission of SBV by local *Culicoides* species have yet to be conducted in Malaysia. The year-round availability of mosquitoes in the country's tropical climate and the increasing number of livestock in Malaysia could influence the spread of SBV.

Small ruminant production in Malaysia is significantly impacted by high rates of morbidity and mortality, as well as the costs associated with treatment, all of which are exacerbated by the prevalence of diseases (Jesse et al., 2022). The global emergence of SBV expands our understanding of diseases that cause reproductive failure in ruminant livestock production, making it an important differential to consider in the clinical diagnosis of infertility. While SBV has become a significant economic disease in European countries, its potential to cross boundaries raises concerns, especially given Malaysia's heavy reliance on imported breeding stock for its livestock industry. Considering the conditions that may facilitate the spread of SBV in Malaysia, this study aimed to detect the presence of SBV nucleic acid using SBV serum positive samples as part of an early molecular surveillance initiative. Considering the fact that there is a lack of specific knowledge on SBV epidemiology in Malaysia, this study was initiated to detect SBV RNA by RT-PCR in serum from SBV-positive small ruminant flocks for the first time in the states of Negeri Sembilan and Terengganu, Malaysia.

MATERIALS AND METHODS

Ethics statement

The Department of Veterinary Services (DVS), Malaysia and The Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-013/2018) granted the required permission to conduct this study. Blood samples were collected by trained veterinarians by taking animal welfare into consideration and we performed the molecular assay at virology laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Sample Collection

In total, 362 individual sheep (n=132) and goats (n=230) were randomly selected from seven farms located at Negeri Sembilan and Terengganu states of Malaysia. Initial screening on SBV antibodies were conducted by Jesse et al. (2022) by using commercial ID vet® SBV multispecies ELISA test kit (produced by ID vet, Montpellier, France). Out of 362, only 78 samples were shown to be sero-positive against SBV, thus all the sero-positive samples were subjected for molecular detection subsequently in this study. All the SBV positive serum samples were stored in a -20°C freezer prior to nucleic acid extraction.

RNA extraction and RT-PCR assay

The RNA from the serum samples were purified and extracted using the GENEzol™ TriRNA Pure Kit according to the manufacturer's instructions. The concentration of extracted RNA was measured by spectrophotometry using the Nanodrop 1000 spectrophotometer (Thermo Scientific™). For a biosafety reason, a standard positive control in the molecular diagnosis of SBV was designed based on published SBV genomic L segment sequence available in the NCBI GeneBank (Accession number: KX384874.1) with the length of 1273 base pairs. The respective sequence manufactured by Integrated DNA Technologies, Inc., Malaysia and was delivered as gBlocks® Gene Fragments, in the form of lyophilized double-stranded DNA.

Prior to RT-PCR, the primers were designed based on published SBV genomic sequence available in NCBI GeneBank (Accession number: KY828195.1). To confirm the primer specificity, the primers were tested against the sequence of positive control gBlocks® Gene Fragments which was synthesized previously. Due to positive amplification (data not shown), the primer set was selected for this experiment. The forward sequence is 5'TGA CAT TCC ATG AGT CTA TG 3' and reverse is 5'GTC GGA TTG TCT CCT GCA AAC 3' targeting a specific region on the SBV L-segment sequence. Then, the extracted RNA was subjected to RT-PCR using MyTaq™ One-Step RT-PCR Kit, following the standard protocol. The following thermal program was applied: 1 cycle of 45°C for 20 min and 95°C for 1 min, followed by 40 cycles of 95°C for 10s, 53°C for 10s, and 72°C for 30s The analytical sensitivity of the assays was determined with 6 serials of 10-fold dilutions using a synthetic positive control of SBV with an initial concentration of 1000 ng/µL down to 0.01 ng/µL.

Analysis of the PCR Products

The PCR products were separated by electrophoresis using 1.5% of agarose gel in 1X TAE buffer solution. For gel analysis, 15µl of the products (samples and positive control) were loaded in each gel slot. To determine the fragment sizes, 1000 bp plus DNA Ladders (Qiagen, Germany, GmbH) was utilized followed by gel visualization using GeneSnap (Synoptics Ltd.), an image acquisition software to capture the electrophoretic gel images. A comparison was then made between the bands produced by the positive control, the negative control, and the samples to determine the presence of the virus DNA.

RESULTS AND DISCUSSION

The analytical sensitivity of PCR in this study was evaluated using 10-fold serial dilution of a synthetic positive control DNA sequence, with concentrations starting from 1000 ng/µL in lane 1 and decreasing sequentially to 0.01 ng/µL in lane 6. Clear PCR amplification bands are visible in lanes 1 through 5, which correspond to concentrations ranging from 1000 ng/µL down to 0.1 ng/µL (Figure 1). However, lane 6, containing 0.01 ng/µL, shows no visible band, indicating that the PCR assay was unable to detect the target sequence at this lowest concentration. Based on these observations, the limit of detection (LOD) for this assay can be defined as 0.1 ng/µL, the lowest concentration at which amplification was reliably observed.A total of 78 individual animal samples of selected farms from Negeri Sembilan and Terengganu were used to screen for SBV in serum. Of the 78 samples collected between March and September 2019, no SBV RNA was detected in any SBV positive serum samples as shown in Figure 2.

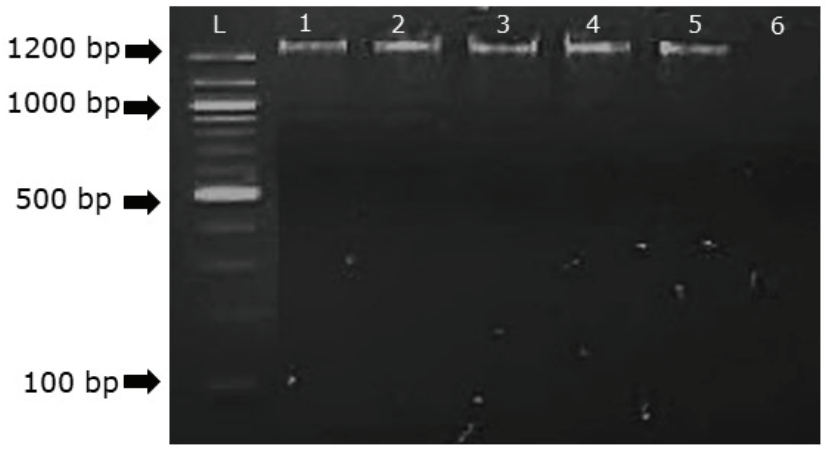

Figure 1 A 1.5% agarose gel electrophoresis of SBV positive control. Bands of PCR product were visualized by ethidium bromide staining. Lane L: 1000 DNA marker. Lanes from right to left corresponded to concentration of positive control from 1000 ng/µL to 0.01 ng/µL. The detection limit was 0.1 ng/µL.

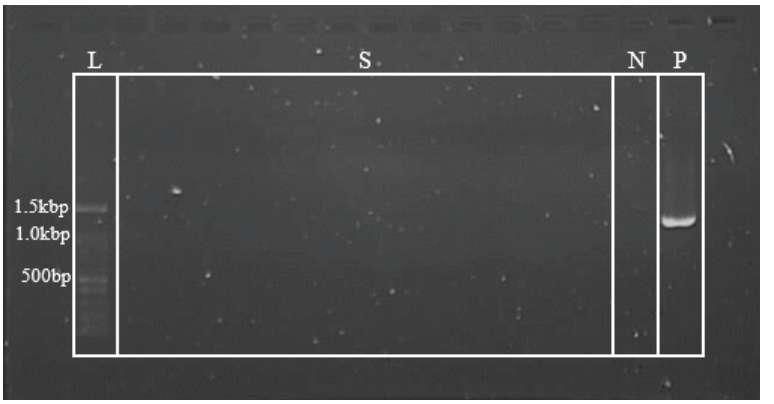

Figure 2 Agarose Gel Electrophoresis Illustration. The figure depicts the RT-PCR products of SBV L-segment with size of 1273 bp amplified using SBV primers. Column L represents the ladder (1.5 kbp), column S represents the RT-PCR products of the samples, whereas columns N and P show the negative and positive controls, respectively.

Based on the current literature, there are no studies on the molecular detection of SBV in Malaysia. Focusing on SBV, lower exposure in the country does not make a reliable laboratory diagnosis available in the country. The preliminary study on the detection of SBV antibodies in small ruminants was carried out by Jesse et al. (2022), while the current study is an ongoing attempt to detect the nucleic acids of SBV in positive SBV serum samples from Negeri Sembilan and Terengganu states in Malaysia. The results obtained from the molecular test showed negative results for SBV in all 78 serum samples. However, this result does not completely rule out the presence of SBV RNA in the animals, as all serum samples tested in this study were previously serologically positive for SBV by ELISA, suggesting that the animal was infected at some point in its life (Jesse et al., 2022). The number of positive cases detected by RT-PCR in the literature is consistent with this study in the sense that a small number of positive cases are usually detected by RT-PCR in serum samples. A study conducted by Chaintoutis et al. (2014) showed that out of 147 samples that tested positive by ELISA, no sample was positive for RT-PCR. The study by Yilmaz et al. (2014) reveals a similar relationship between the serological and genomic prevalence of SBV exposure. Among 116 aborted foetuses from small and large ruminants, they tested 46 blood samples from lambs and 20 organ samples from sheep using PCR. Of these, only three samples tested positive for RT-PCR: one from an aborted calf and two from aborted lambs. The inability of this study to detect the presence of SBV by RT-PCR assay could be explained by a few possible factors.

First, the duration of viremia in ruminant SBV infection is relatively short, lasting only a few days. In a study of experimentally infecting goats and buck with SBV, viral RNA was detected within three to four days post infection (pi), beginning on days one to three pi, as detected by RT-PCR. In particular, SBV RNA was no longer detectable from day six pi. Furthermore, this viremic phase was not accompanied by clinical symptoms (Laloy et al., 2015). In an experimental challenge in sheep, viral RNA was detectable for

three to five days, starting on days one, two and four after inoculation. Similar to the goat study, the majority of sheep with RNAemia showed no clinical signs including fever (Wernike et al., 2013). This proves that viral RNA could be successfully detected in serum samples when obtained during an acute infection. In the current study, the failure to detect viral RNA can be attributed to long-term infection, with the concentration of viral particles in the goats and sheep being low and undetectable by the RT-PCR threshold

Second, however, determining the acute phase of infection in adult animals is difficult in ruminants, especially goats and sheep, which rarely show clinical signs even during the viraemia phase (Wernike et al., 2013; Lievaart-Peterson et al., 2015). Therefore, other samples that have shown a higher concentration of viral RNA can be selected instead of serum samples for more successful molecular detection of SBV. This includes lungs, liver, outer placental fluid, and umbilical cord from aborted, stillborn, or malformed offspring (Bilk et al., 2012; Yilmaz et al., 2014). In addition, spleens and lymph nodes of infected adults proved to be a suitable target region, as viral RNA was detectable up to day 44 post-inoculation (Wernike et al., 2013). Furthermore, seropositive flocks for SBV sampling should be closely monitored for clinical signs such as abortions or congenital malformations that are strongly suggestive of SBV infection.

Next, the absence of viral RNA in the serum is considered to be the cause of elimination of viral particles by neutralizing antibodies in the bloodstream. Experimental infection of goats showed that seroconversion occurred between day seven and day 14 pi and the viral RNA was later undetectable after seroconversion by RT-PCR (Laloy et al., 2015) and similar findings were also demonstrated in sheep (Wernike et al., 2013). This was further supported by the virus neutralization test (VNT) performed concurrently in their studies along with ELISA. In comparison, the presence of antibodies against SBV in this study may have resulted in the virus being neutralized and subsequently the SBV RNA becoming undetectable in the RT-PCR assay. Finally, most ELISAs are unable to accurately discriminate between members of the Simbu serogroup of viruses, so claiming the samples as SBV seropositive required more validation. Because of the similar pattern of transmission, multiple viruses of the same serogroup could be circulating in certain regions, and the virus neutralization test (VNT) is more specific and can discriminate between them. Apart from that, it has been suggested that fetal body fluids would be better suited to test the antibody response of SBV infection.

CONCLUSION

In conclusion, this study could not detect SBV nucleic acid content in all SBV seropositive samples. Thus, no SBV viremia was detected in the analyzed samples. Collecting serum samples from acutely infected animals is strongly recommended since the duration of SBV viraemia is relatively short. Future studies should focus on collecting other infected tissue samples of placenta or umbilical cord of affected foetusinstead of serum samples. However, the successful design and synthesis of an innocuous SBV L segment can be used as a standard positive control in the diagnosis of SBV in Malaysia in the future.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

KNB, JAB, FFAJ and MLMA postulated the experimental design. KNB, AR, BTP, KMI and JMN performed work associated with this study. KNB, JAB and MLMA prepared the manuscript. All authors reviewed the manuscript upon submission.

REFERENCES

APHA. (2016). Disease surveillance report: Disease surveillance in England and Wales, November 2016.*Veterinary Record*, *179*, 565–568.

Azkur, A. K., Albayrak, H., Risvanli, A., Pestil, Z., Ozan, E., Yilmaz, O., & Bulut, H. (2013). Antibodies to Schmallenberg virus in domestic livestock in Turkey. *Tropical Animal Health Production*, *45*(8), 1825–1828.

Bayrou, C., Garigliany, MM., Sarlet, M., Sartelet, A., Cassart, D., & Desmecht, D. (2014). Natural Intrauterine Infection with Schmallenberg Virus in Malformed Newborn Calves. *Emerging Infectious Diseases*, *20*, 1327-1330.

Beer, M., Conraths, F. J., & Van Der Poel, W. H. M. (2013). "Schmallenberg virus" - A novel Orthobunyavirus emerging in Europe. *Epidemiology Infection, 141*(1), 1–8.

Bilk, S., Schulze, C., Fischer, M., Beer, M., Hlinak, A., & Hoffmann, B. (2012). Organ distribution of Schmallenberg virus RNA in malformed newborns. *Veterinary Microbiology*, *159*(1–2), 236–238.

Chaintoutis S.C., Kiossis E., Giadinis N.D., Brozos C.N., Sailleau C., Viarouge C., Bréard E., Papanastassopoulou M., Zientara S., Papadopoulos O., & Dovaet C.I., (2014). Evidence of Schmallenberg virus circulation in ruminants in Greece. *Tropical Animal Health and Production, 46*, 251-255.

Chowdhary, R., Street, C., Da Rosa, A. T., Nunes, M. R., Tee, K. K., Hutchison, S. K., & Briese, T. (2012). Genetic characterization of the Wyeomyia group of Orthobunyaviruses and their phylogenetic relationships. *Journal of General Virology*, *93*(5), 1023-1034.

Claine, F., Coupeau, D., Wiggers, L., Muylkens, B., & Kirschvink, N. (2013). Schmallenberg virus among female lambs, Belgium, 2012. *Emerging Infectious Diseases*, *19*(7), 1115–1117.

Claine, F., Coupeau, D., Wiggers, L., Muylkens, B., & Kirschvink, N. (2015). Schmallenberg virus infection of ruminants: Challenges and opportunities for veterinarians. *Veterinary Medicine: Research and Reports,* 261-272.

Collins, A. B., Barrett, D. J., Doherty, M. L., McDonnell, M., & Mee, J. F. (2017). Significant re-emergence and recirculation of Schmallenberg virus in previously exposed dairy herds in Ireland in 2016. *Transboundary and Emerging Diseases, 64*(5), 1359–1363.

Dastjerdi, A., & Steinbach, F. (2021). Schmallenberg virus found in England in autumn 2020. *Veterinary Record*, *188*(5), 192–192.

Delooz, L., Saegerman, C., Quinet, C., Petitjean, T., De Regge, N., & Cay, B. (2017). Resurgence of Schmallenberg virus in Belgium after 3 years of epidemiological silence. *Transboundary and Emerging Diseases, 64*(5), 1641–1642.

De Regge, N., Deblauwe, I., De Deken, R., Vantieghem, P., Madder, M., Geysen, D., & Cay, A. B. (2012). Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR. *Transboundary and emerging diseases, 59*(6), 471–475.

Doceul, V., Lara, E., Sailleau, C., Belbis, G., Richardson, J., Bréard, E., & Zientara, S. (2013). Epidemiology, molecular virology and diagnostics of Schmallenberg virus, an emerging orthobunyavirus in Europe. *Veterinary Research, 44*(1), 1–13.

Elbers, A. R. W., Stockhofe-Zurwieden, N., Van Der Poel, & W. H. M. (2014). Schmallenberg virus antibody persistence in adult cattle after natural infection and decay of maternal antibodies in calves. *BMC Veterinary Research, 10*, 2–5.

Elliott, R. M. (2014). Orthobunyaviruses: Recent genetic and structural insights. *Nature Reviews Microbiology*, *12*(10), 673-685.

European Food Safety Authority. (2014). Schmallenberg virus: State of art. *EFSA Journal, 12*(5), 3681.

Fischer, M., Schirrmeier, H., Wernike, K., Wegelt, A., Beer, M., & Hoffmann, B. (2013). Development of a pan-Simbu real-time reverse transcriptase PCR for the detection of Simbu serogroup viruses and comparison with SBV diagnostic PCR systems. *Virology Journal, 10*(1), 327-336.

Ganter, M., Eibach, R., & Helmer, C. (2014). Update on Schmallenberg virus infections in small ruminants. *Small Ruminant Research*, *118*(1–3), 63–68.

García-Bocanegra, I., Cano-Terriza, D., Vidal, G., Rosell, R., Paniagua, J., Jiménez-Ruiz, S., & Pujols, J. (2017). Monitoring of Schmallenberg virus in Spanish wild artiodactyls, 2006–2015. *Plos One, 12*(8), 1-12.

Garigliany, M. M., Hoffmann, B., Dive, M., Sartelet, A., Bayrou, C., Cassart, D., & Desmecht, D. (2012). Schmallenberg virus in calf born at term with porencephaly, Belgium. *Emerging Infectious Diseases, 18*(6), 1005–1006.

Goller, K. V., Höper, D., Schirrmeier, H., Mettenleiter, T. C., & Beer, M. (2012). Schmallenberg virus as possible ancestor of shamonda virus. *Emerging Infectious Diseases*, *18*(10), 1644–1646.

Helmer, Carina, Eibach, R., Humann-Ziehank, E., Tegtmeyer, P. C., Bürstel, D., Mayer, K., & Ganter, M. (2016). Seroprevalence of Schmallenberg virus infection in sheep and goats flocks in Germany, 2012–2013. *Veterinary Medicine and Science, 2*(1), 10–22.

Herder, V., Hansmann, F., Wohlsein, P., Peters, M., Varela, M., Palmarini, M., & Baumgärtner, W. (2013). Immunophenotyping of inflammatory cells associated with Schmallenberg virus infection of the central nervous system of ruminants. *Plos One*, *8*(5), 1–11.

Hoffmann, B., Scheuch, M., Hoper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., & Beer, M. (2012). Novel orthobunyavirus in cattle, Europe, 2011. *Emerging Infectious Diseases, 18*(3), 469–472.

Hoffmann, B., Schulz, C., & Beer, M. (2013). First detection of Schmallenberg virus RNA in bovine semen, Germany, 2012. *Veterinary Microbiology*, *167*(3-4), 289–295.

Jack, C., Anstaett, O., Adams, J., Noad, R., Brownlie, J., & Mertens, P. (2012). Evidence of seroconversion to SBV in camelids. *Vet Rec, 170*(23), p603.

Jesse, F. F. A., Paul, B. T., Hashi, H. A., Chung, E. L. T., Sedeka, N. I. H. B. M., & Lila, M. A. M. (2022). Schmallenberg virus (SBV) infection among small ruminants in selected states of Peninsular Malaysia. J. *Advance Veterinary Research*, *12*(1), 62-67.

Kęsik-Maliszewska, J., Larska, M. (2016). Detection of Schmallenberg Virus RNA in Bull Semen in Poland. *Poland Journal of Veterinary Science, 19*(3), 655–657.

Laloy, E., Riou, M., Barc, C., Belbis, G., Bréard, E., Breton, S., & Ponsart, C. (2015). Schmallenberg virus: Experimental infection in goats and bucks. *BMC Veterinary Research*, *11*(1), 9–13.

Larska, M., Krzysiak, M., Smreczak, M., Polak, M. P., & Zmudziński, J. F. (2013). First detection of Schmallenberg virus in elk (Alces alces) indicating infection of wildlife in Białowieża National Park in Poland. *The Veterinary Journal*, *198*(1), 279–281.

Lievaart-Peterson, K., Luttikholt, S., Peperkamp, K., Van Den Brom, R., & Vellema, P. (2015). Schmallenberg disease in sheep or goats: Past, present and future. *Veterinary Microbiology*, *181*(1–2), 147–153.

Luttikholt, S., Veldhuis, A., Van Den Brom, R., Moll, L., Lievaart-Peterson, K., Peperkamp, K., & Vellema, P. (2014). Risk factors for malformations and impact on reproductive performance and mortality rates of Schmallenberg virus in sheep flocks in the Netherlands. *Plos One*, *9*(6), 1–8.

Martinelle, L., Poskin, A., Dal Pozzo, F., Mostin, L., Van Campe, W., Cay, A. B., & Saegerman, C. (2017). Three different routes of inoculation for experimental infection with Schmallenberg virus in Sheep. *Transboundary and Emerging Diseases, 64*(1), 305–308.

Martinelle, Ludovic, Poskin, A., Dal Pozzo, F., De Regge, N., Cay, B., & Saegerman, C. (2015). Experimental infection of sheep at 45 and 60 days of gestation with Schmallenberg virus readily led to placental colonization without causing congenital malformations. *Plos One*, *10*(9), 1–13.

Meroc, E., De Regge, N., Riocreux, F., Caij, A. B., Van Den Berg, T., & Van Der Stede, Y. (2014). Distribution of Schmallenberg virus and seroprevalence in Belgian sheep and goats. *Transboundary and Emerging Diseases, 61*(5), 425–431.

OIE. (2017). Viraemia and incubation period. (November 2011), 1–5.

Pawaiya, R. V. S., & Gupta, V. K. (2013). A review on Schmallenberg virus infection: A newly emerging disease of cattle, sheep and goats. *Veterinary Medicine, 58*(10), 516–526.

Peperkamp, K., Dijkman, R., Van Maanen, C., Vos, J., Wouda, W., Holzhauer, M., & Roumen, M. (2012). Polioencephalomyelitis in a calf due to infection with Schmallenberg virus. *Vet Rec*, *170*(22), 570.

Peperkamp, N. H., Luttikholt, S. J., Dijkman, R., Vos, J. H., Junker, K., Greijdanus, S., & Wouda, W. (2015). Ovine and bovine congenital abnormalities associated with intrauterine infection with Schmallenberg virus. *Veterinary Pathology, 52*(6), 1057–1066.

Ponsart, C., Pozzi, N., Bréard, E., Catinot, V., Viard, G., Sailleau, C., & Vitour, D. (2014). Evidence of excretion of Schmallenberg virus in bull semen. *Veterinary Research*, *45*(1), 1–6.

Rasekh, M., Sarani, A., & Hashemi, S. H. (2018). Detection of Schmallenberg virus antibody in equine population of Northern and Northeast of Iran. *Veterinary World*, *11*(1), 30–33.

Rodríguez-Prieto, V., Kukielka, D., Mouriño, M., Paradell, H., Plaja, L., Urniza, A., & Sánchez-Vizcaíno, J. M. (2016). Natural immunity of sheep and lambs against the Schmallenberg virus infection. *Transboundary and Emerging Diseases*, *63*(2), e220–e228.

Stavrou, A., Daly, JM., Maddison, B., Gough, K., & Tarlinton R. (2017). How is Europe positioned for a re-emergence of Schmallenberg virus? *The Veterinary Journal, 230*, 45–51.

Steukers, L., Bertels, G., Cay, A. B., & Nauwynck, H. J. (2012). Schmallenberg virus: Emergence of an Orthobunyavirus among ruminants in Western Europe. *Vlaams Diergeneeskundig Tijdschrift, 81*(3), 119–127.

Sudhakar, S. B., Gandhale, P. N., Singh, F., Raut, A. A., Mishra, A., Kulkarni, D. D., & Singh, V. P. (2020). Schmallenberg virus. *Emerging and Transboundary Animal Viruses*, 239-253.

Van Der Poel, W. H. M., Parlevliet, J. M., Verstraten, E. R. A. M., Kooi, E. A., Hakze-Van Der Honing, R., & Stockhofe, N. (2014). Schmallenberg virus detection in bovine semen after experimental infection of bulls. *Epidemiology & Infection, 142*(7), 1495–1500.

Varela, M., Schnettler, E., Caporale, M., Murgia, C., Barry, G., Mcfarlane, M., & Palmarini, M. (2013). Schmallenberg virus pathogenesis, tropism and interaction with the innate immune system of the host. *Plos Pathogens*, *9*(1).

Walter, C. T., & Barr, J. N. (2011). Recent advances in the molecular and cellular biology of bunyaviruses. *Journal of General Virology*, *92*(11), 2467–2484.

Wensman, J. J., Blomqvist, G., Hjort, M., & Holst, B. S. (2013). Presence of antibodies to Schmallenberg virus in a dog in Sweden. *Journal of Clinical Microbiology*, *51*(8), 2802–2803.

Wernike, K., Elbers, A., & Beer, M. (2015). Schmallenberg virus infection. *OIE Revue Scientifique et Technique, 34*(2), 363–373.

Wernike, K., & Beer, M. (2020). Re-circulation of Schmallenberg virus, Germany, 2019. *Transbound and Emerging Disease*, *67*(6), 2290–2295.

Wernike, Kerstin, Eschbaumer, M., Breithaupt, A., Hoffmann, B., & Beer, M. (2012). Schmallenberg virus challenge models in cattle: Infectious serum or culture-grown virus? *Veterinary Research*, *43*(84), 1-4.

Wernike, Kerstin, Hoffmann, B., Bréard, E., Bøtner, A., Ponsart, C., Zientara, S., & Beer, M. (2013). Schmallenberg virus experimental infection of sheep. *Veterinary Microbiology*, *166*(3–4), 461–466.

Wernike, K., Hoffmann, B., Conraths, F. J., & Beer, M. (2015). Schmallenberg virus recurrence, Germany, 2014. *Emerging Infectious Diseases, 21*, 1202–1204.

Wernike, Kerstin, Nikolin, V. M., Hechinger, S., Hoffmann, B., & Beer, M. (2013). Inactivated Schmallenberg virus prototype vaccines. *Vaccine*, *31*(35), 3558–3563.

Wernike, K., Reimann, I., Banyard, A. C., Kraatz, F., La Rocca, S. A., Hoffmann, B., & Beer, M. (2021). High genetic variability of Schmallenberg virus M-segment leads to efficient immune escape from neutralizing antibodies. *Plos Pathogens*, *17*(1), e1009247.

Yanase, T., Kato, T., Aizawa, M., Shuto, Y., Shirafuji, H., Yamakawa, M., & Tsuda, T. (2012). Genetic reassortment between Sathuperi and Shamonda viruses of the genus Orthobunyavirus in nature: Implications for their genetic relationship to Schmallenberg virus. *Archives of Virology*, *157*(8), 1611–1616.

Yilmaz, H., Hoffmann, B., Turan, N., Cizmecigil, U. Y., Richt, J. A., Van Der Poel, W. H. M. (2014). Detection and partial sequencing of Schmallenberg virus in cattle and sheep in Turkey. *Vector-Borne Zoonotic Diseases, 14*(3), 223–225.

Zhai, S. L., Lv, D. H., Wen, X. H., Zhu, X. L., Yang, Y. Q., Chen, Q. L., & Wei, W. K. (2018). Preliminary serological evidence for Schmallenberg virus infection in China. *Tropical Animal Health and Production, 50*(2), 449–453.

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The Antihyperglycemic Activity of Bruguiera gymnorrhiza Roots Extract in STZ-induced Diabetic Mice

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ABSTRACT

Mangrove plants are believed to possess a wide range of bioactive compounds due to their ability to thrive in harsh conditions enduring high salinity, low air humidity and high temperature. *Bruguiera gymnorrhiza* is one of the mangrove species that is commonly found in Sabah and its root has been discovered to have antidiabetic activity. However, previous investigations focused solely on ethanolic extract of *Bruguiera gymnorrhiza* root (BGR). Thus, this study aimed to assess antihyperglycemic activity of BGR methanolic extract in various fractions (aqueous, butanol, chloroform) using streptozocin-induced diabetic mice as an experimental model. The diabetic mice were administered different fractions of BGR extract at dose of 250 mg/kg with metformin (200 mg/kg) serves as standard drug. Blood glucose levels were measured on day 0th, 7th and 14th following the oral administration of BGR fractions in a fasting state mice. After 14 days treatment period, the results showed that the aqueous fraction of BGR extract significantly reduced the blood glucose levels in diabetic mice compared to other BGR fractions. The significant antihyperglycemic effect observed in aqueous fraction of BGR extract strongly indicates the presence of major potent antidiabetic components for decreasing the elevated blood glucose levels in diabetic mice.

Keywords: *Mangrove; diabetes; Bruguiera gymnorrhiza*

INTRODUCTION

Diabetes mellitus (DM) or diabetes is a group of metabolic conditions that associated with hyperglycemia due to partial or total insulin efficiency (Egan & Dinneen, 2019). Diabetes is one of the five leading causes of death worldwide that associated with many health complications such as long-term damage and failure of various organs (Chandramohan *et al*., 2008). International Diabetes Federation (2021) reported that 537 million adults diagnosed with diabetes in the range of 20 to 79 years old with approximately 6.7 million deaths estimated. Ministry of Health Malaysia conducted latest National Health and Morbidity Survey (NHMS) (2019) reported that about one in five Malaysians adult (18.3%) has diabetes.

Currently, diabetes is managed by different types of oral hypoglycaemic drugs such as sulfonylureas, biguanides, and alpha-glucosidase inhibitors that is available in market (Bader *et al*., 2017). The use of plants for medicinal purposes are being made toward its improvement. Some plant-drugs have been used for centuries, therefore, there are growing interest in medicinal plants and their bioactive molecules and central point of research. Traditional medicine from plants has been used to treat several diseases including diabetes since there are safe and available (Sathasivampillai *et al*., 2017). Insulin therapy required the patients to regularly monitor their blood glucose level and it will develop several harmful side effects (Bowker *et al*., 2006; Monami *et al*., 2009). Oral antidiabetic drugs also possess acute side effects such as liver toxicity, nephrotoxicity, heart diseases, hypoglycemic conditions, or common side effects, such as stomach-related problem and nausea (Ivemeyer *et al*., 2012). Herbal remedies are one of the approaches that could treat diabetes with less side effects. It has been reported that no side effects associated with herbal drugs (Saravana & Pari, 2006).

Mangrove is a small tree that grows in coastal brackish or saline waters in muddy or rocky soils. It is highly tolerant to salts, halophytes and quickly adapt in harsh conditions (Kathiresan & Bingham, 2001). The diversity of mangrove flora worldwide includes around 81 tree and shrub species of 30 genera from 17 families (Sachithanandam *et al*., 2019). Asia holds the biggest number of mangrove area which approximately 6.8 million ha out of 14-15 million ha (1990-2020 data) of the world's total mangrove area(Food and Agriculture Organization, 2020; Kauffman *et al*., 2011). Mangrove in Malaysia constitutes approximately 537,686 ha in area, and more than half of the total area was covered by Sabah with 364,100 ha (Olaniyi *et al*., 2012). Sabah covers the greatest mangrove area in Malaysia, yet limited amount of study has been done especially for *Bruguiera gymnorrhiza* species. Oral glucose tolerance test (OGTT) has been studied in diabetic rats with the treatment of aqueous and butanol extracts of *Bruguiera gymnorrhiza* root resulting significantly lower blood glucose level by 30% and 48.6% respectively (Singh *et al*., 2010). In this study, *Bruguiera gymnorrhiza* mangrove plant will be conducted to determine its anti-diabetic activity and its mechanisms in lowering blood glucose level.

MATERIALS & METHODS

Animals

Male Balb/c mice weighing 20g-30g were used in present study. Mice were housed in a clean cage at room temperature, 12-h light/12-h dark cycle and relative air humidity 40-60%. Mice were maintained in regularly monitored setting in quarantine room of Animal BSL-3 in Biotechnology Research Institute, Universiti Malaysia Sabah. All experiment protocols were reviewed and approved by Animal Ethics Committee, UMS with ethical code AEC 0019 / 2022. The mice used in the present study were kept in the full accordance of Researcher's Guidelines on Code of Practice for the Care and Use of Animal for Scientific Purposes, as well as standards and recommendations adhered by ethical committee.

Plant extract preparation and fractionation

The root of *Bruguiera gymnorrhiza* was air-dried and powdered using heavy duty blender. The powdered root weighed 10g and extracted via Soxhlet extraction in 90% methanol for 3 cycles. The concentrated extract was diluted in 90% methanol and fractionated with butanol and chloroform to make aqueous, butanol, and chloroform fraction extract of *Bruguiera gymnorrhiza* root.

Brine Shrimp Lethality Assay

The eggs of brine shrimp, and sea water were collected from UMS prawn hatchery. Air pump, separating funnel, eggs of brine shrimp and sea water were needed to be prepared for hatching process. Approximately 0.5g of brine shrimp eggs were added into separating funnel filled with 500 mL of sea water. The mixture was mixed well with a spatula. Hatching process took about 24 hours and to ensure a successful hatching and a production of mature brine shrimp, the lights in the setup area must be switched on throughout the process and maintain a proper aeration by placing the air pump into separating funnel. Once the brine shrimps mature, it was then called nauplii.

The aqueous, butanol and chloroform fraction extracts were dissolved in sea water and were added into 24-well plate. Each well was filled with each fraction extracts with different concentrations ranging from 50 µg/mL to 1500 µg/mL in triplicates. The final volume of each well was adjusted into 2 mL final volume of seawater and 10 living nauplii were introduced in each well. A volume of 2mL seawater and 70% ethanol were served as positive and negative control respectively with 10 living nauplii introduced. The surviving nauplii were counted after 24 hours. LC_{50} value was calculated using probit analysis in SPSS and will find the potential toxicity in plant extract.

Induction of STZ

Diabetes was induced using single high dose STZ at 200 mg/kg body weight of Balb/c strain mice via intraperitoneal injection. The concentration of the final solution is 20 mg/mL that makes 20 mg of STZ was dissolved in 1 mL of solvent. Once STZ dissolved, it must be used as soon as possible since STZ will lose its diabetogenic function in solvent and the injection process must be done within 10 minutes after dissolution. After one week of injection, the fasting blood glucose level of the mice was measured to confirm diabetes. The fasting blood glucose level was measured using OneTouch glucose meter and the mice that reached hyperglycemia (upper 11 mmol/L) were chosen for animal study.

Antihyperglycemic activity of *Bruguiera gymnorrhiza* roots fraction extracts in STZ-induced diabetic mice

The mice were isolated into six groups (Group I, II, III, IV, V and VI) of five mice each as follows, Group I served as normal control that received normal saline which is the only group that did not received any injection of STZ. Group II also received normal saline but served as diabetic control. Group III treated with metformin that act as standard drug at a dose of 200 mg/kg. The remaining groups (Group IV, V, and VI) were treated with *Bruguiera gymnorrhiza* root aqueous, butanol and chloroform fraction respectively at 250 mg/kg dosage. The treatment continued for 14 days with free access to food and water. Blood glucose levels were measured on day 0, day 7, and day 14 using OneTouch Ultra Plus machine. The mice were fasted for six hours prior to measurement of blood glucose level by housing the mice with new cage and clean bedding and removed their food access and water. After six hours of fasting, a drop of blood was drawn from the mice through tip of the tail to measure its blood glucose levels. After continuous treatment of extracts, the mice were further tested to Oral Glucose Tolerance Test (OGTT).

Oral Glucose Tolerance Test (OGTT)

The experimental animals from the previous assay were then fasted for six hours and given administration orally in their respective treatment. Group I and Group II were administered with normal saline, while metformin was treated to Group III in 200 mg/kg dosage. Group IV, V and VI were treated with aqueous, butanol, and chloroform fraction extract respectively in 250 mg/kg body weight. After 30 minutes of treatment administration, the fasting blood glucose level of each mouse was measured using OneTouch Ultra Plus Glucose Meter machine by collecting the blood through the tail tips of mice. This blood glucose level measurement indicated as 0 minutes prior to glucose (2 g/kg body weight) administration to all groups. Blood glucose levels were measured at 30 minutes, 60 minutes, and 120 minutes after glucose administration. The blood glucose level was expressed in millimole per liter (mmol/L).

Statistical Analysis

All data are presented as mean ± SEM. Differences between groups were evaluated by one-way ANOVA followed by Tukey's multiple comparisons tests, and differences were considered significant at $p < 0.05$.

RESULTS & DISCUSSION

Brine Shrimp Lethality Assay

In brine shrimp lethality assay, LC_{50} or 50% mortality rate of brine shrimp calculated and shown on Table 1. Through the LC50 value of each fraction, both aqueous and butanol fraction, is non-toxic while chloroform fraction is toxic, or medium toxic. The toxicity category was referred to according to Meyer et al., (1982), and Clarkson et al., (2004). However, the chloroform fraction of BGR that was administered to mice was in low concentration. This is supported by the positive correlation of brine shrimp lethality assay and mice acute toxicity test by Lagarto Parra et al., (2001).

Table 1 The LC_{50} value of fraction extracts of BGR

Concentration	LC_{50}
Aqueous fraction	2927.05 µg/mL
Butanol fraction	1217.76 µg/mL
Chloroform fraction	480.69 µg/mL

Antihyperglycemic activity of *Bruguiera gymnorrhiza* roots fraction extracts in STZ-induced diabetic mice

A significant reduction on fasting blood glucose levels demonstrated by diabetic mice that were treated with metformin at 200 mg/kg and aqueous fraction extract of *Bruguiera gymnorrhiza* root at 250 mg/kg. After 14 days of treatment, present data (Figure 1) showed that there is significant reduction in elevated fasting blood glucose levels by day 7 of treatment. Meanwhile, 250 mg/kg of butanol and chloroform fraction extracts of BGR showed no significant reduction in fasting blood glucose levels after 14 days of treatment. The antihyperglycemic activity shown by aqueous fraction of BGR may be due to the abundance of bioactive compounds.

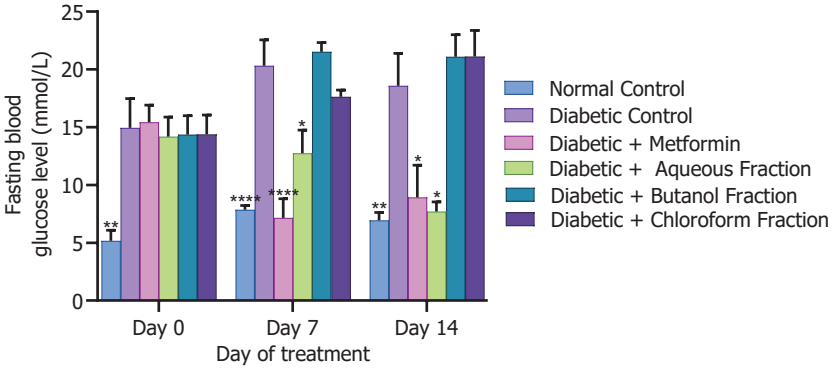

Figure 1 The antihyperglycemic effects of *Bruguiera gymnorrhiza* root fraction extracts in STZ-induced diabetic mice

Oral Glucose Tolerance Test (OGTT)

Metformin was used as a standard drug in present study, and it significantly lowered the fasting blood glucose levels of diabetic mice at 30 min, 60 min and 90 min compared to diabetic control group. Diabetic mice that were treated with aqueous fraction extract of BGR showed significant reduction at 120 min post glucose loading compared to diabetic control group. The butanol and chloroform fraction extract of BGR did not show any significant reduction in fasting blood glucose levels. This indicates the enhancement of glucose tolerance in aqueous fraction of BGR suggests that there is potent anti-diabetic properties.

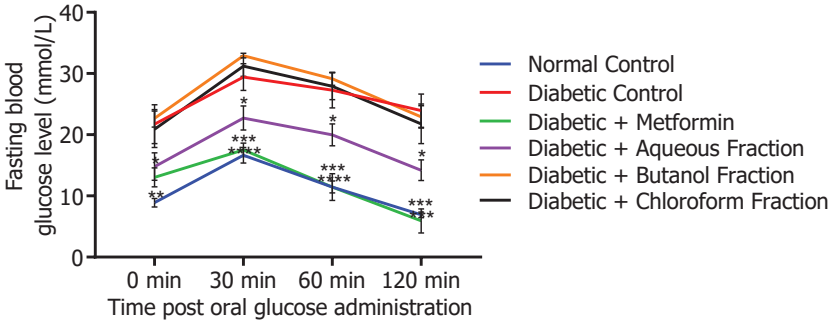

Figure 2 Effects on oral glucose tolerance test after treating *Bruguiera gymnorrhiza* root fraction extracts on STZ-induced diabetic mice

CONCLUSION

The present study has suggested that the aqueous fraction of BGR possesses anti-hyperglycemic activity. The compound isolated in aqueous fraction of BGR needs further investigations to discover its mechanism of action and to identify its active compound responsible in anti-hyperglycemic activity.

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REFERENCES

Bader, G. N., Mir, P. A., & Ali, S. (2017). Evaluation of Anti Inflammatory and Analgesic Activity of Rhizome of Swertia Petiolata. Am. J. Pharm. Tech. Res, *7*, 332-343.

Bowker, S. L., Majumdar, S. R., Veugelers, P., & Johnson, J. A. (2006). Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. Diabetes Care, *29*(2). https://doi.org/10.2337/diacare.29.02.06.dc05-1558

Chandramohan, G., Ignacimuthu, S., & Pugalendi, K. V. (2008). A novel compound from Casearia esculenta (Roxb.) root and its effect on carbohydrate metabolism in streptozotocin-diabetic rats. European Journal of Pharmacology, *590*(1–3). https://doi.org/10.1016/j.ejphar.2008.02.082

Clarkson, C., Maharaj, V. J., Crouch, N. R., Grace, O. M., Pillay, P., Matsabisa, M. G., Bhagwandin, N., Smith, P. J., & Folb, P. I. (2004). In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa. Journal of Ethnopharmacology, *92*(2–3). https://doi.org/10.1016/j.jep.2004.02.011

Egan, A. M., & Dinneen, S. F. (2019). What is diabetes? In Medicine (United Kingdom) (Vol. 47, Issue 1). https://doi.org/10.1016/j.mpmed.2018.10.002

Food and Agriculture Organization. (2020). Global Forest Resources Assessment 2020: Main report. In Reforming China's Healthcare System.

International Diabetes Federation. (2021). IDF Diabetes Atlas Tenth edition 2021. International Diabetes Federation.

IPH, I. for P. H., NIH, N. I. of H., & Malaysia, M. of H. (2019). National Health and Morbidity Survey (NHMS) 2019: NCDs - Non-Communicable Diseases: Risk Factors and other Health Problems. In Institute for Public Health, National Institutes of Health (NIH), Ministry of Health Malaysia (Vol. 1).

Ivemeyer, S., Smolders, G., Brinkmann, J., Gratzer, E., Hansen, B., Henriksen, B. I. F., Huber, J., Leeb, C., March, S., Mejdell, C., Nicholas, P., Roderick, S., Stöger, E., Vaarst, M., Whistance, L. K., Winckler, C., & Walkenhorst, M. (2012). Impact of animal health and welfare planning on medicine use, herd health and production in European organic dairy farms. Livestock Science, *145*(1–3). https://doi.org/10.1016/j.livsci.2011.12.023

Kathiresan, K., & Bingham, B. L. (2001). Biology of mangroves and mangrove ecosystems. In Advances in Marine Biology (Vol. 40). https://doi.org/10.1016/S0065-2881(01)40003-4

Kauffman, J. B., Heider, C., Cole, T. G., Dwire, K. A., & Donato, D. C. (2011). Ecosystem carbon stocks of micronesian mangrove forests. Wetlands, *31*(2). https://doi.org/10.1007/s13157-011-0148-9

Lagarto Parra, A., Silva Yhebra, R., Guerra Sardiñas, I., & Iglesias Buela, L. (2001). Comparative study of the assay of Artemia salina L. And the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine, *8*(5). https://doi.org/10.1078/0944-7113-00044

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica, *45*(1). https://doi.org/10.1055/s-2007-971236

Monami, M., Lamanna, C., Balzi, D., Marchionni, N., & Mannucci, E. (2009). Sulphonylureas and cancer: A case-control study. Acta Diabetologica, *46*(4). https://doi.org/10.1007/s00592-008-0083-2

Olaniyi, A. O., Abdullah, A. M., Ramli, M. F., & Alias, M. S. (2012). Assessment of drivers of coastal land use change in Malaysia. Ocean and Coastal Management, 67. https://doi.org/10.1016/j.ocecoaman.2012.05.029

Sachithanandam, V., Lalitha, P., Parthiban, A., Mageswaran, T., Manmadhan, K., & Sridhar, R. (2019). A Review on Antidiabetic Properties of Indian Mangrove Plants with Reference to Island Ecosystem. In Evidence-based Complementary and Alternative Medicine (Vol. 2019). https://doi.org/10.1155/2019/4305148

Saravana, G., & Pari, L. (2006). Effects of Syzygium cumini bark on blood glucose, plasme insulin and C-peptide in streptozotocin-induced diabetic rats. International Journal of Endocrinology Metabolism, 4.

Sathasivampillai, S. V., Rajamanoharan, P. R. S., Munday, M., & Heinrich, M. (2017). Plants used to treat diabetes in Sri Lankan Siddha Medicine – An ethnopharmacological review of historical and modern sources. In Journal of Ethnopharmacology (Vol. 198). https://doi.org/10.1016/j.jep.2016.07.053

Singh, N., Vikram Patel, A., Alok, S., Kannojia, P., Garud, N., & Mehta, S. C. (2010). ANTI-DIABETIC ACTIVITY OF BRUGUIERA GYMNORHIZA ROOT. IJPSR, *1*(5). www.ijpsr.com

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Screening of coumarin derivatives as a potential Alzheimer's Disease treatment drugs on *Drosophila melanogaster*

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ABSTRACT

Alzheimer's disease (AD) stands as the most prevalent form of neurodegenerative ailment worldwide, characterized by the accumulation of amyloid beta (Aβ) plaques. Unfortunately, there is currently no effective cure for this condition. To investigate potential treatment options, researchers have turned to *Drosophila melanogaster* as an ideal animal model for studying AD. In this context, coumarin, a naturally occurring phytochemical initially discovered in tonka bean, and its derivatives have garnered significant attention for their diverse beneficial biological properties. The present study aimed to explore the efficacy of coumarin derivatives in mitigating the adverse effects of Aβ aggregation. Using the *Drosophila* model expressing human Aβ42, researchers observed a rough eye phenotype (REP) and decreased lifespan. To evaluate the neuroprotective effects of coumarin derivatives, the treated groups' eye morphology was compared with both positive and negative control groups. Encouragingly, the group treated with S5-44 exhibited the most favourable eye morphology, closely resembling that of the positive control group compared to other coumarin derivatives. Moreover, the group treated with S3-18 displayed a longer lifespan in comparison to the negative

control group. In summary, most coumarin derivatives utilized in this study partially restored the REP, while one derivative even extended the lifespan of *Drosophila*. These promising findings suggest that coumarin derivatives have the potential to serve as neuroprotective drugs for the treatment of AD. Further research and development in this area may open new avenues for combating this debilitating disease.

Keywords: Alzheimer's disease, amyloid beta, *Drosophila melanogaster*

INTRODUCTION

Alzheimer's disease (AD) was initially documented by Alois Alzheimer in 1907. It is a progressive and irreversible neurodegenerative disorder characterized by the gradual loss of synapses and neuronal cells in the brain. As the most prevalent form of dementia, AD accounts for 50 to 70% of all neurodegenerative dementia cases globally (Winblad et al., 2016). Disturbingly, it stands as the seventh leading cause of death globally (Collaborators, 2021). Remarkable advancements in medical science and technology have undoubtedly improved the quality of life and increased life expectancy. However, this increased longevity comes with a simultaneous rise in age-related diseases, including AD. As our population ages, it becomes crucial to address the challenges posed by Alzheimer's disease and actively seek ways to prevent, manage, and treat this condition to alleviate its impact on individuals and their families.

AD is characterized by the presence of amyloid beta (Aβ) plaques and neurofibrillary hyperphosphorylated tau tangles. Abnormal processing and accumulation of Aβ peptides, derived from the amyloid precursor protein (APP) through sequential cleavage by β- and γ-secretase, represent a significant hallmark of the disease (Wang et al., 2017; Selkoe & Hardy, 2016). The aggregation of misfolded Aβ42 polypeptides gives rise to amyloid clumps, triggering neurodegeneration through the activation of abnormal signalling pathways (Fernandez-Funez et al., 2013; Sarkar et al., 2016; Selkoe & Hardy, 2016; Shankar et al., 2008). Ultimately, these plaques disrupt neuron transmission at synapses, leading to a failure in information transfer and culminating in neuronal cell death (Hardy & Selkoe, 2002; Tanzi & Bertram, 2005; Blennow, 2006).

D. melanogaster has emerged as a widely preferred model for AD research, as evidenced by studies conducted by Iijima et al. (2004), and Wittmann et al. (2001). A notable advantage of using *Drosophila* as a model is that approximately 75% of human disease genes have orthologs in their genome (Rubin, 2000). Employing *Drosophila* as a model organism offers several benefits, including modest dietary and spatial requirements, easy observation and manipulation at various developmental stages, high reproductive capacity, and resilience against environmental challenges such as plagues and pathogens (Stocker & Gallant, 2008). The availability of fully known genome sequences, the simplicity of genetic manipulation, and an extensive collection of available mutants contribute to *Drosophila's* well-established status as a system that facilitates a deeper understanding of human diseases at the molecular level (Botas, 2007).

As projected by the U.S. Alzheimer's Association, the number of affected individuals worldwide is expected to surpass 22 million by 2025. Regrettably, a cure for AD remains elusive. Addressing this critical need, there is a pressing demand for more effective drugs to combat AD, which necessitates the exploration of compound screening using animal models to unlock potential cures. Adopting alternative approaches, such as compound screening, offers a promising avenue for discovering neuroprotective drugs to treat AD. The objective of this study was 1) to investigate the capability of coumarin derivatives in mitigating Aβ aggregation within the *D. melanogaster* model and 2) to assess and compare the neuroprotective effects of various coumarin derivatives for treating AD in the *D. melanogaster* model.

MATERIALS AND METHODS

Preparation of solid fly food

The ingredients for preparing fly food in solid form are shown in Table 1. A pot containing 1 litre of water was brought to a boil. Polenta and yeast were added to the boiling water while ensuring thorough mixing to prevent clumping. In a separate container, corn flour was dissolved in 100 mL of water. Pieces of agar were introduced into the boiling pot and mixed until they dissolved completely. Subsequently, the corn starch solution was added to the mixture. If the mixture appeared too thick, 100 mL of tap water was gradually incorporated into the mixture to achieve the desired consistency. Slowly and while stirring, brown sugar was added to the mixture. Afterwards, the mixture was allowed to cool at room temperature for a few minutes. Finally, nipagin and propionic acid were added to complete the preparation process.

Table 1 The ingredients for preparing fly food in solid form

Ingredients	Volume/Weight
Water	1L
Corn flour	40 g
Polenta	50 g
Brown sugar	100 g
Agar	7 g
Yeast	50 g
Propionic acid	5 mL
Nipagin	30 mL

The coumarin derivatives used in this study were sourced from the School of Chemical Sciences, USM. An amount of 100 mg of the compound was dissolved in 100 µL of 0.3% Dimethyl sulfoxide (DMSO) by thorough mixing. Subsequently, the resulting mixture was combined with 1 mL of solid fly food (Table 2).

Table 2 Preparation of 10 % of compound in the solid fly food

Ingredients	Volume/Weight
Fly food	1 mL
Compound	100 mg
0.3 % DMSO	100 µL

Fly stock and maintenance

The flies (as shown in Table 3) were raised and maintained at a temperature of 25 °C. After the parents were crossed, they were removed from the vial after 6 days, leaving only the larvae and pupae. After 10 days, the virgin female flies were collected for further experiments. Before conducting the experiments, the flies were anesthetized and placed on a CO_2 pad (specifically, the FlyStuff Flypad from Genesee Scientific), where a continuous supply of carbon dioxide was provided. To distinguish between virgin female flies and non-virgin female flies, the researchers relied on differences in their body coloration (lighter color for virgin flies and tan color for non-virgin flies). Additionally, the presence of meconium, a greenish spot visible at the abdomen of virgin flies, served as another key characteristic to differentiate them during the sorting process.

Table 3 Drosophila melanogaster strains

Source	Stock number	Genotype	Description
Kyoto Stock Center	107294	Oregon-R-P2	Wild-type
Bloomington *Drosophila* Stock Center	1104	w[*];P{w[+mC]=GAL4-ninaE.GMR}12	Express GAL4 in the eye under the control of the glass enhancer. Provides strong expression in all cells behind the morphogenetic furrow.
Kyoto Stock Center	107727	y[1] w[*]; P{w[+mC]=Act5C-GAL4}25FO1 / CyO, y[+]	Actin5C-GAL4 driver
Bloomington *Drosophila* Stock Center	33769	w[1118];P{w[+mC]=UAS-APP.Abeta42.B}m26a	Expresses the human Aβ42 fragment of APP under the control of UAS.

Compound screening using Rough Eye Phenotype (REP) assay

The male fly line was crossed with the virgin female fly line, and this cross was carried out in the solid fly food containing the compound (or without the compound). The vials were left undisturbed for 6 days to allow for the completion of the crossing process. After 10 days, the first generation (F1) of flies was collected for further analysis. The eyes of the F1 flies were carefully observed under a motorized stereo microscope (as described in Table 4). Subsequently, images of the fly eyes were captured to document and analyze any observable differences or effects resulting from the cross and the presence or absence of the compound in the solid food.

Table 4 The model of light microscope – Stereo Motorized (cellSens Dimension)

Model	Camera	Software
Olympus SZX16	Olympus DP72	cellSens Dimension version 1.5
(Olympus Optical Co. Ltd. Tokyo, Japan)	(Olympus Corporation, Japan)	(© Olympus Soft Imaging Solutions GmbH 2011)

The images of the F1 flies, obtained after observation under the motorized stereo microscope, were processed using CombineZP software (available at https://combinezp.software.informer.com/). The stacked images allowed for a comprehensive comparison of the eye structures of the treated F1 flies with those in both the positive control and negative control groups. By conducting this comparative analysis, any potential effects resulting from the treatment and the presence or absence of the compound in the solid food could be discerned and evaluated.

Table 5 Parent flies were crossed in three groups: positive control, treated, and negative control, for the REP assay.

Group	Female fly line	Male fly line	Condition
Positive control	Oregon R	GMR-GAL4	Food + DMSO
Treated	UAS-Aβ42	GMR-GAL4	Food + DMSO + compound
Negative control (Untreated)	UAS-Aβ42	GMR-GAL4	Food + DMSO

Capillary Feeder (CAFE) assay

The Capillary Feeder (CAFE) assay, as originally described by William et al. in 2007, was modified in this study by excluding cornmeal and agar and incorporating tryptone. To prepare the modified liquid food (refer to Table 6), 5 g each of yeast extract and glucose were mixed in a 100 mL Schott Duran Bottle. Sterile water was then added up to a volume of 50 mL. Additionally, 1.7 g of tryptone was placed in another 100 mL Schott Duran Bottle, and sterile water was added up to 50 mL. Both bottles were subjected to autoclaving for sterilization. In the bottle containing yeast extract and glucose, 1.5 mL

of nipagin and 0.25 mL of propionic acid were added as preservatives. To create 50 mL of the liquid food, 25 mL of the content from each bottle was carefully extracted and mixed in a new bottle. This resulting liquid food was then utilized for the CAFE assay in the experimental procedure.

Table 6 Preparation of liquid food for CAFE assay

Ingredient	Volume/Weight
Yeast extract	5 g
Glucose	5 g
Tryptone	1.7 g
Propionic acid	0.25 mL
Nipagin	1.5 mL

50 mg of the compound was dissolved in 50 µL of 0.3% Dimethyl sulfoxide (DMSO) through thorough mixing. The resulting mixture was then combined with 450 µL of liquid fly food to achieve a total volume of 500 µL of liquid fly food with the compound (refer to Table 7). During the experimental period, the flies were fed daily with 1 to 2 µL of the prepared liquid food, depending on the number of flies present in each tube. This feeding regimen was followed consistently to ensure that the flies received an appropriate and consistent amount of the liquid fly food with the compound throughout the study.

Table 7 Preparation of 10 % of compound in the liquid food

Ingredient	Volume
Liquid food	450 µL
Compound + 0.3 % DMSO	50 µL
Total	500 µL

Longevity assay

The male fly line was mated with virgin female flies, and this cross was performed in two different conditions: one group with the compound added to the food and another group without the compound. The vials containing the flies were then left undisturbed for 6 days to allow for proper mating and egg-laying. After 10 days, the first generation (F1) flies were collected and utilized in the CAFE assay for further investigation. During the assay, the F1 flies were provided with daily feeding and their housing tubes were changed every two days. Throughout this process, the flies' lifespans were carefully recorded and plotted into graphs for analysis. To ensure robust results, each group was replicated twice, providing two independent replicates for both the positive control

and the treated group. The initial number of flies used in the positive control was 26, while the treated group had 22 flies, and the negative control included 5 flies (Table 8). These numbers represent the total number of flies used at the beginning of the experiment in each respective group.

Table 8 Parent flies were crossed in three groups, namely positive control, treated, and negative control, for the longevity assay

Group	Female fly line	Male fly line	Condition
Positive control	Oregon R	Actin5C-GAL4	Food + DMSO
Treated	Actin5C-GAL4	UAS-Aβ42	Food + DMSO + compound
Negative control (Untreated)	Actin5C-GAL4	UAS-Aβ42	Food + DMSO

RESULT

Rough Eye Phenotype (REP) assay

In studies related to neurodegeneration, the REP (Rough Eye Phenotype) assay is utilized as a method to assess neurotoxicity in *Drosophila*. The REP can be observed in the first generation (F1) flies affected by the expression of the AD gene from their parents, facilitated through the GAL4/UAS system. Flies exhibiting REP display a disrupted hexagonal arrangement of the ommatidia, differing from the organized ommatidia arrangement in the wild-type flies.

To evaluate the neuroprotective effects of coumarin derivatives, the eye morphology of the treated groups receiving various coumarin derivatives (S3-18, S5-52, S3-30, S5-44, S2-12, and S4-34) was compared with both the positive control and negative control groups. To make a comparative analysis, the treated groups were also ranked based on the severity of their REP. The severity of REP in each treated group was determined by comparing their eye morphology with that of the positive control and negative control groups. The coumarin derivative that resulted in an eye phenotype closest to that of the positive control received a higher ranking, while those closer to the negative control had a lower ranking.

The positive control group (GMR-GAL4 × Oregon R) displayed a hexagonal array of organized ommatidia, similar to the characteristics of the wild-type fly eye (Figure 1) In contrast, the treated groups (GMR-GAL4 × UAS-Aβ42) showed a wide range of recovery degrees in their eye morphology (Figure 2 – 7). Visual assessments were made, and the eye phenotype condition was manually ranked from 1 to 6 based on the degree of severity, with rank 1 indicating the closest morphology to that of the wild type and rank 6 for the most severe eye phenotype (Table 9).

Most coumarin derivatives used in the treated groups demonstrated a less severe REP compared to the negative control (GMR-GAL4 × UAS-Aβ42), which displayed defects in the eye phenotype, such as more fused and disorganized ommatidia. This indicates that the majority of the coumarin derivatives used in the study partially restored the REP in the flies affected by AD.

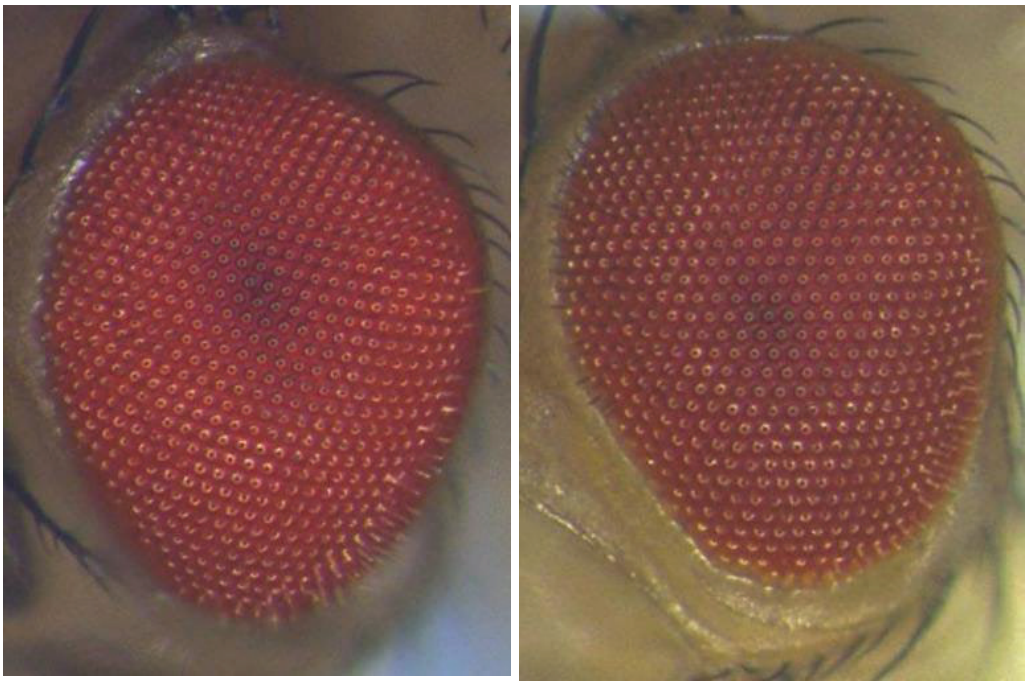

Figure 1 Eye phenotypes are shown for (A) the wild type or Oregon R and (B) the positive control group (GMR-GAL4 × Oregon R)

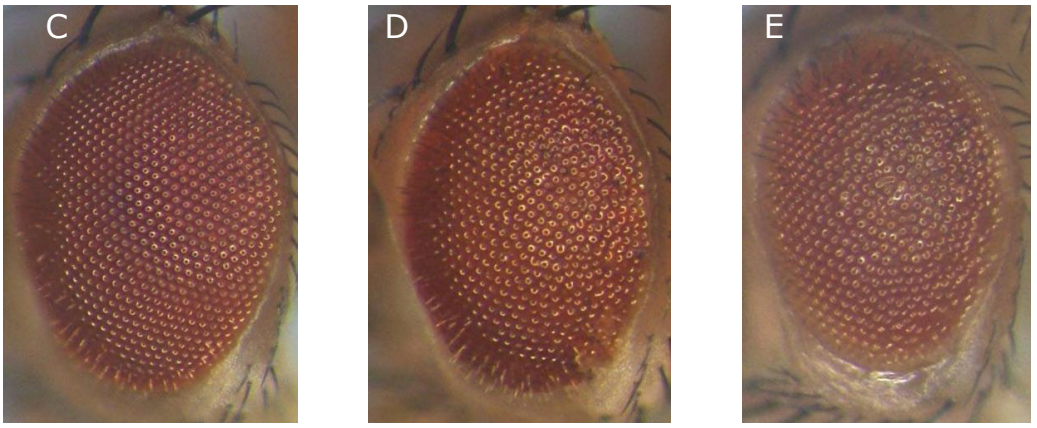

Figure 2 The eye phenotypes are as follows: (C) positive control, (D) treated group with S3-18, and (E) negative control

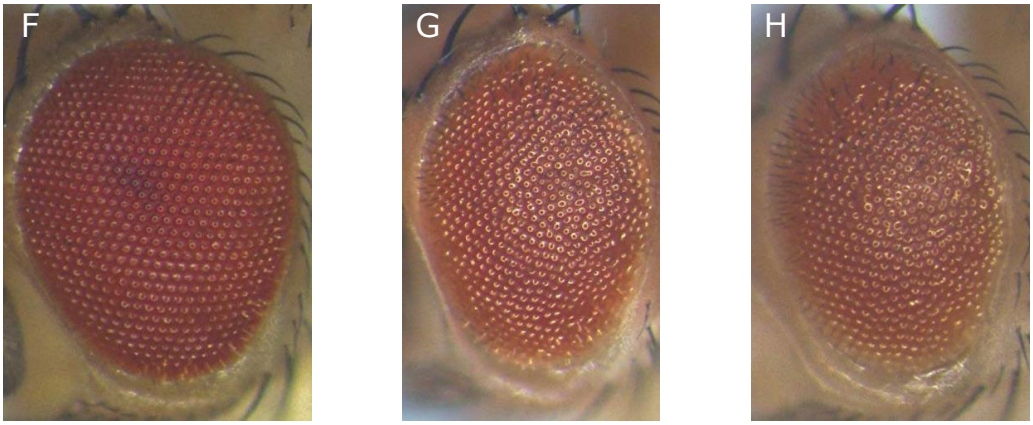

Figure 3 The eye phenotypes observed are as follows:
(F) positive control, (G) treated group with S5-52, and (H) negative control

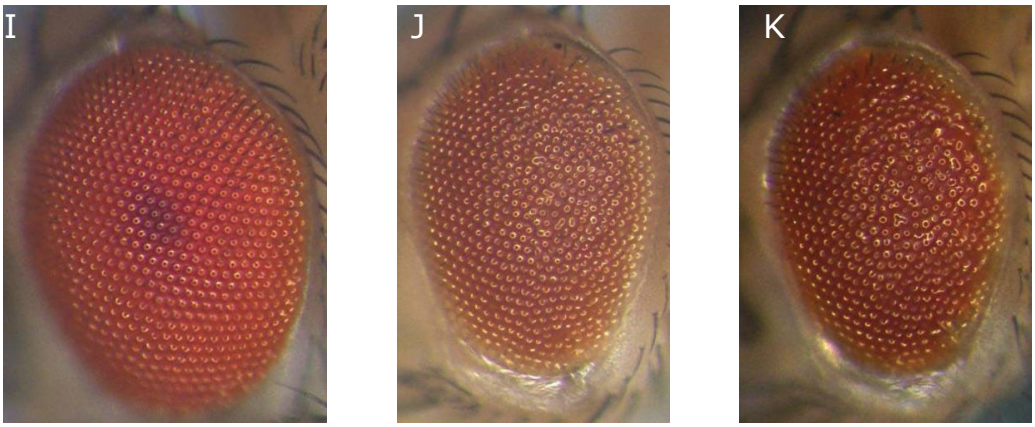

Figure 4 The eye phenotypes are recorded as follows:
(I) positive control, (J) treated group with S3-30, and (K) negative control

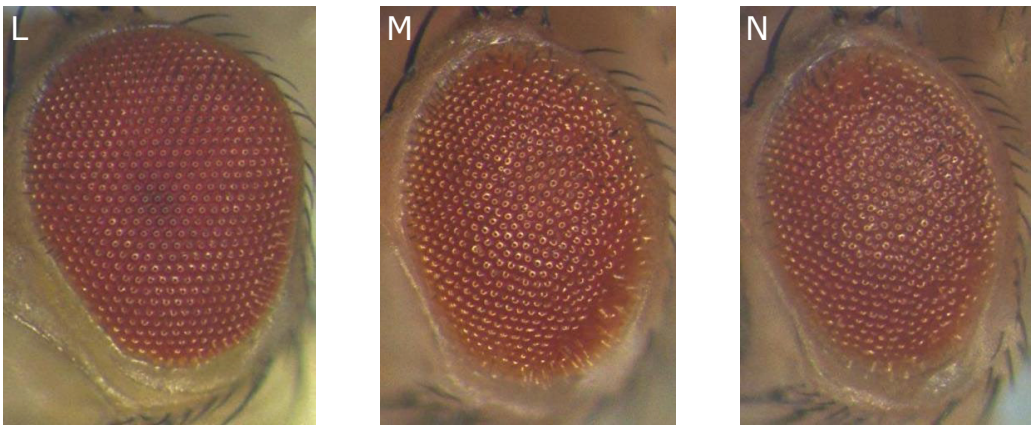

Figure 5 The eye phenotypes observed are as follows:
(L) positive control, (M) treated group with S5-44, and (N) negative control

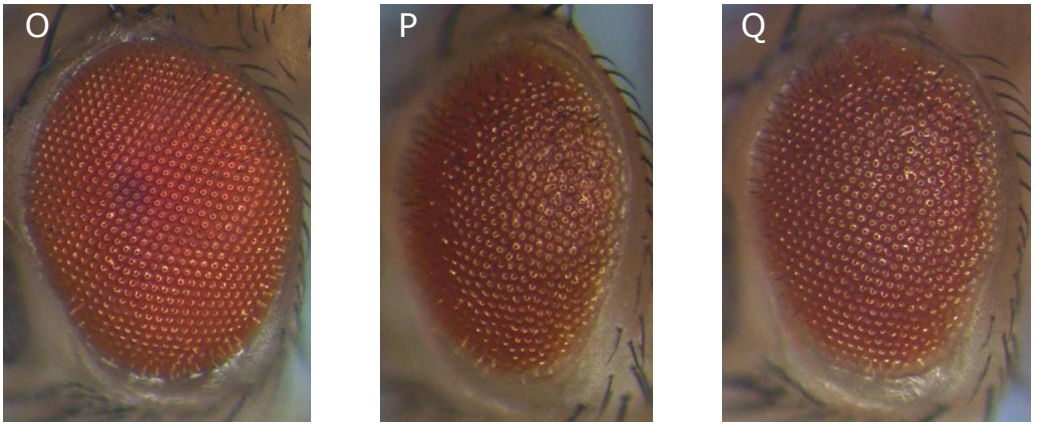

Figure 6 The eye phenotypes are recorded as follows:
(O) positive control, (P) treated group with S2-12, and (Q) negative control

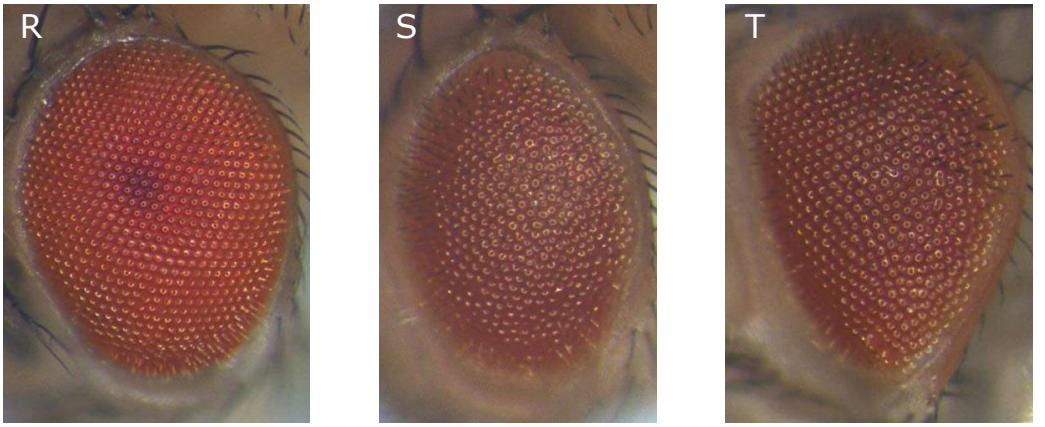

Figure 7 The eye phenotypes observed are as follows:
(R) positive control, (S) treated group with S4-34, and (T) negative control

Table 9 Rank order of the treated groups that treated with coumarin derivatives (S3-18, S5-52, S3-30, S5-44, S2-12 and S4-34)

Coumarin derivatives	Rank order
S3-18	3
S5-52	2
S3-30	4
S5-44	1
S2-12	6
S4-34	5

The group treated with S5-44 exhibited the most similar eye phenotype to that in the positive control, displaying partially organized and hexagonally arranged ommatidia (Figure 5). For S5-52, the eye phenotype showed partially organized ommatidia with only a few fused ommatidia (Figure 3). Flies fed with S3-18 had a moderate severity in eye phenotype, as the ommatidia were neatly arranged, but some fused ommatidia were observed (Figure 2). For S3-30, the eye morphology was almost the same as that in the S3-18 group, but it had more fused ommatidia (Figure 4).

The group treated with S4-34 had disorganized ommatidia, making it challenging to detect a hexagonal arrangement, and the size of the ommatidia was inconsistent (Figure 7). Flies treated with S2-12 had the most severe phenotype, as there was no observable hexagonal arrangement of the ommatidia. Additionally, unequal sizes and fused ommatidia were evident in this treated group (Figure 6).

Lifespan analysis

The lifespans of adult F1 flies, which were raised and kept at a constant temperature of 25 °C, were assessed in this study. The parents were crossed in solid food, with some groups containing the compound and others without it. After 6 days, the first generation (F1) flies emerged from the eggs, and they immediately started consuming the food. A substantial amount of food was consumed during their larval stage, leading to their development into pupae. Once fully developed, the enclosed flies were collected and sorted by sex.

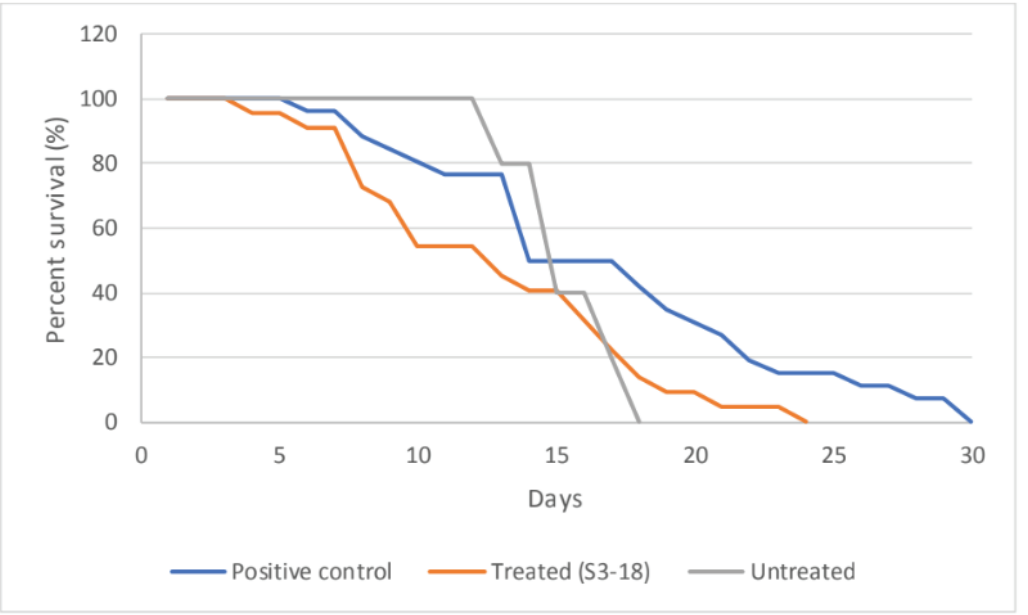

Figure 8 The survival curve shows the results for the positive control (initially 26 flies), the treated group with S3-18 (initially 22 flies), and the negative control (initially 5 flies), with each group having two replicates.

The flies were then maintained using the CAFE method, where they were fed with liquid food. In this method, the viability of the flies was recorded daily to monitor their longevity. The data obtained from the three groups, including the positive control, the treated group, and the negative control, were plotted and compared in a graph (Figure 8). This graph allowed for a visual comparison of the longevity of flies from the different groups over time.

Based on the graph, the positive control group (Actin5C-GAL4 × Oregon R) exhibited the longest lifespan, reaching 30 days. In contrast, the flies treated with S3-18 (Actin5C-GAL4 × UAS-Aβ42) had a lifespan of 24 days, and the negative control group (Actin5C-GAL4 × UAS-Aβ42) lived for 18 days.

Throughout the 30-day period, the survivability of the positive control group gradually declined. Similarly, the survivability of the flies treated with S3-18 also decreased slowly, reaching a significant drop on the 24th day. In contrast, the negative control group maintained its survivability at 100% until the 13th day, after which it experienced a sharp decrease in survival within five days. Comparing the treated group (S3-18) with the negative control, it becomes evident that the treated group exhibited a longer lifespan. This indicates that S3-18 had a positive effect on improving the longevity of flies expressing Aβ42.

DISCUSSION

Rough Eye Phenotype (REP) assay

Drosophila treated with S3-18, S5-52, S3-30, and S5-44 displayed improved eye morphology compared to the negative control group. These findings suggested that these compounds had neuroprotective effects on the flies expressing Aβ42. On the other hand, the eye morphology of flies treated with S4-34 and S2-12, ranked 5th and 6th respectively, closely resembled that of the negative control. This indicated that these two coumarin derivatives exhibited either weak or no neuroprotective effects against Aβ42 expression.

The severity of the eye phenotype correlated with the extent of neuronal cell loss (Lenz et al., 2013). Coumarin derivatives that showed stronger neuroprotective effects were able to rescue more neuronal cells from death, resulting in a lower severity of the observed eye phenotype (REP) in the flies. This was particularly relevant in this case, as neuronal death was triggered by the accumulation of Aβ42 plaques, a significant hallmark of Alzheimer's disease (AD). The accumulation of Aβ42 led to neurodegeneration, causing stress in the neurons and ultimately resulting in their death (Crews & Masliah, 2010; Hardy, 2009; Pandey & Nichols, 2011; Sarkar et al., 2016; Tare et al., 2011).

As a result, S5-44 was identified as having the most potent inhibition of Aβ42 aggregation, as flies treated with this derivative displayed the best eye morphology among all the tested compounds. Considering the critical role of Aβ42 peptide accumulation in AD pathology, inhibiting the assembly of Aβ monomers into aggregate structures has emerged as a promising strategy for AD treatment.

In recent years, coumarins, which are found in numerous plant species, have garnered significant attention due to their diverse biological properties associated with neurological diseases, particularly Alzheimer's disease (AD) (Hamulakova et al., 2017). Their potential has led to the consideration of coumarin as a valuable scaffold in designing drugs for AD treatment. Notably, coumarin derivatives have shown promise in preventing misfolded Aβ aggregation. Specific studies have highlighted the potency of certain coumarin derivatives, such as compound 13 and 15, in inhibiting Aβ aggregation (Huang et al., 2015). Interestingly, it was suggested that incorporating a hydroxyl group into the para position of the side chain-phenyl ring may enhance inhibitory activity against Aβ42 self-induced aggregation. Conversely, a reduction in the carbon nitrogen double bond appeared to diminish the inhibition of Aβ42 self-aggregation (Huang et al., 2015). This variation in structural modifications could account for the wide range of neuroprotective effects demonstrated by the coumarin derivatives investigated in this study.

In addition to these findings, other studies have also revealed the active inhibition of Aβ self-aggregation by certain coumarin derivatives. For example, compounds 4y and 4w exhibited the ability to actively inhibit Aβ self-aggregation. Furthermore, the conjugates of 7-hydroxycoumarin linked in position 4 to tacrine demonstrated Aβ aggregation inhibition as well (Hamulakova et al., 2017).

Another study investigated coumarin-pargyline hybrids, particularly compound 4x, and found that they exhibited remarkable inhibitory activities against Aβ42 aggregation (Yang et al., 2009). The research suggested that the stability of the 4x-Aβ complex resulted from π-π stacking interaction and hydrogen bond interactions (Yang et al., 2009). The coumarin derivatives used in this study may undergo similar interactions with Aβ, thereby contributing to their inhibition of Aβ aggregation.

In this particular study, the assessment of the reduced eye phenotype (REP) in flies was conducted qualitatively, relying on visual inspection of eye phenotypes and manual scoring. However, this method was limited by its lack of high sensitivity and accuracy. To address this limitation, a more robust approach could involve the use of a quantitative assessment of eye morphology in flies, such as the Flynotyper software. This software offers a quantitative measure that can effectively detect and differentiate between various levels of defects in the fly eye. By employing this more advanced quantitative method, researchers can obtain more precise and reliable data, enhancing the accuracy of their findings and enabling a more comprehensive analysis of the effects of coumarin derivatives on the flies' eye morphology.

To further investigate the ability of coumarin derivatives to inhibit Aβ aggregation, various methods can be employed, such as the Thioflavin T (ThT) fluorescence assay, 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence assay, turbidity assay, and native gel electrophoresis. These techniques provide valuable insights into the interactions between coumarin derivatives and Aβ peptides, helping to elucidate the mechanisms of inhibition.

Additionally, molecular docking studies can be conducted to gain a deeper understanding of how coumarin derivatives interact with Aβ peptides at the molecular level. These computational studies can provide valuable information about the binding modes and affinity of the coumarin derivatives to Aβ, complementing the experimental findings.

Furthermore, to validate the neuroprotective effects of the coumarin derivatives used in this study, it would be beneficial to apply these derivatives in treatment on mammalian models of Alzheimer's disease. By using mammalian AD models, researchers can observe the effects of the coumarin derivatives in a more clinically relevant context and assess their potential as therapeutic agents for neurodegenerative diseases.

Overall, employing these various methods and approaches would offer a comprehensive and multi-faceted investigation into the neuroprotective properties of coumarin derivatives and their potential as a treatment strategy for Alzheimer's disease.

Lifespan analysis

Among the various coumarin derivatives, S3-18 was selected to assess its effectiveness in improving the longevity of *Drosophila*. The positive control group displayed the longest lifespan, which was 30 days. In contrast, the lifespan of flies fed with S3-18 and the negative control was 24 days and 18 days, respectively. Previous studies had shown that the lifespan of Aβ42 flies was significantly reduced (Finelli et al., 2004; Iijima et al., 2004). However, flies treated with S3-18 demonstrated a longer lifespan compared to the negative control group. These findings strongly suggested that S3-18 had a positive impact on the longevity of flies expressing Aβ42, potentially exerting a beneficial effect on the health and lifespan of these Drosophila models.

D. melanogaster serves as a model organism extensively used in the study of age-related diseases, and its typical mean lifespan ranges from 2 to 3 months (Helfand & Rogina, 2003). In this specific study, the positive control group exhibited the longest lifespan, which was recorded as 30 days. This reduced lifespan in the positive control group was consistent with a previous study that indicated flies fed using the CAFE (Capillary Feeder) method had a shorter lifespan (Lee et al., 2008). The shorter lifespan observed in the CAFE method could be attributed to environmental stress and the nature of the diet provided.

In the CAFE assay, evaporation of liquid food might occur, leading to condensed liquid food forming at the tips of the pipette tips. This could result in a lower accessibility of food by the flies when compared to solid food. The specific type of food used can also have a substantial impact on the flies' lifespan. For instance, some commonly used fly food, such as yeast extract instead of lyophilized whole brewer's yeast, can significantly shorten the flies' lifespan (Bass et al., 2007).

In this study, S3-18 demonstrated the ability to extend the lifespan of *Drosophila*. However, it's worth noting that another study reported that coumarin, the parent compound of S3-18, was unable to extend the lifespan of *Drosophila*. The difference in outcomes between these studies could potentially be attributed to variations in

the chemical structures of coumarin and coumarin derivatives like S3-18. The study suggesting that coumarin was not able to extend lifespan also noted that coumarin might have potential toxicity (Abraham et al., 2010; Cohen, 1979).

Additionally, it's essential to consider that the *Drosophila* strains used in the two studies differed. The previous study utilized w 1118 and JIV strains, which are distinct from the UAS-Aβ42 line used in this current study. Genetic variations and specific characteristics of each *Drosophila* strain could contribute to differing responses to coumarin derivatives, potentially explaining the contrasting results.

Indeed, the reliability of the results obtained from the longevity assay with S3-18 may have been compromised due to the insufficient number of flies used at the beginning of the experiment. To ensure more robust and dependable data, it is recommended to use a larger sample size. Specifically, employing at least 100 flies in each group would enhance the statistical power and accuracy of the study. Additionally, to account for potential variations and increase confidence in the findings, conducting three replicates of the experiment would be beneficial.

CONCLUSION

The use of natural products holds promise as a potential alternative for treating neurodegenerative diseases like Alzheimer's disease (AD). These products offer the advantage of alleviating AD symptoms with potentially fewer or no side effects compared to synthetic drugs. Coumarin, being a versatile compound, allows for various chemical substitutions at different sites in its structure, making it a valuable tool for designing different derivatives, which could have implications for drug discovery.

However, further studies are necessary to assess the effectiveness of coumarin derivatives in other vertebrate model organisms to better understand their neuroprotective functions. Exploring their effects in more complex biological systems will provide crucial insights for potential translational applications.

One of the intriguing possibilities is the development of coumarin derivatives as potential drugs for targeting Aβ42-mediated neurodegeneration observed in AD. If successful, these derivatives could hold significant therapeutic potential, offering hope for the treatment of neurodegenerative diseases and improving the lives of those affected by these conditions. Continued research in this area could bring us closer to discovering novel and effective treatments for AD and other related disorders.

REFERENCES

Bass, T. M., Grandison, R. C., Wong, R., Martinez, P., Partridge, L., & Piper, M. D. (2007). Optimization of dietary restriction protocols in Drosophila. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*(10), 1071 – 1081.

Botas, J. (2007). Drosophila researchers focus on human disease. Nat Genet, *39*(5), 589-591.

Blennow, K., de Leon, M. J., & Zetterberg, H. (2006). Alzheimer's disease. Lancet (London, England), *368*(9533), 387–403.

Fernandez-Funez, P., Sanchez-Garcia, J. & Rincon-Limas, D. (2013) Unraveling the basis of neurodegeneration using the Drosophila eye. In: Molecular genetics of axial patterning, growth and disease in the Drosophila eye (A. Singh & M. Kango-Singh, eds.), pp 271 – 293. New York: Springer.

Hamulakova, S., Janovec, L., Soukup, O., Jun, D., & Kuca, K. (2017). Synthesis, in vitro acetylcholinesterase inhibitory activity and molecular docking of new acridine-coumarin hybrids. Int J Biol Macromol, *104*(Pt A), 333-338.

Hardy, J. (2009). The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *Journal of Neurochemistry*, *110*(4), 1129 – 1134.

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science, *297*(5580), 353 – 356.

Huang, M., Xie, S. S., Jiang, N., Lan, J. S., Kong, L. Y., & Wang, X. B. (2015). Multifunctional coumarin derivatives: monoamine oxidase B (MAO-B) inhibition, anti-beta-amyloid (Abeta) aggregation and metal chelation properties against Alzheimer's disease. Bioorg Med Chem Lett, *25*(3), 508 – 513.

Iijima, K., Liu, H. P., Chiang, A. S., Hearn, S. A., Konsolaki, M., & Zhong, Y. (2004). Dissecting the pathological effects of human Abeta40 and Abeta42 in Drosophila: a potential model for Alzheimer's disease. Proc Natl Acad Sci U S A, *101*(17), 6623 – 6628.

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W. O., Taylor, P. W., Raubenheimer, D. (2008). Lifespan and reproduction in Drosophila: new insights from nutritional geometry. Proc Natl Acad Sci USA, *105*(7), 2498 – 2503.

Lenz, S., Karsten, P., Schulz, J. B., & Voigt, A. (2013). Drosophila as a screening tool to study human neurodegenerative diseases. J Neurochem, *127*(4), 453 – 460.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, *443*(7113), 787 – 795.

Pandey, U. B., & Nichols, C. D. (2011). Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. Pharmacol Rev, *63*(2), 411 – 436.

Rubin, G. M. (2000). Biological annotation of the Drosophila genome sequence. Paper presented at the Novartis Foundation symposium.

Sarkar, A., Irwin, M., Singh, A., Riccetti, M., & Singh, A. (2016). Alzheimer's disease: the silver tsunami of the 21(st) century. Neural Regeneration Research, *11*(5), 693 – 697.

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Mol Med, *8*(6), 595 – 608.

Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in Alzheimer disease. Cold Spring Harb Perspect Med, *1*(1), a006189.

Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Selkoe, D. J. (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med, *14*(8), 837 – 842.

Stocker, H., & Gallant, P. (2008). Getting started: an overview on raising and handling Drosophila. Methods Mol Biol, *420*, 27 – 44.

Tanzi, R. E., & Bertram, L. (2005). Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective. Cell, *120*(4), 545 – 555.

Tare, M., Modi, R. M., Nainaparampil, J. J., Puli, O. R., Bedi, S., Fernandez-Funez, P., Singh, A. (2011). Activation of JNK signaling mediates amyloid-ss-dependent cell death. PLoS One, *6*(9), e24361.

Wang, J., Gu, B. J., Masters, C. L., & Wang, Y. J. (2017). A systemic view of Alzheimer disease - insights from amyloid-beta metabolism beyond the brain. Nat Rev Neurol, *13*(10), 612 – 623.

William, W. J., Carvalho, G. B., Mak, E. M., Noelle, N., Fang, A. Y., Liong, J. C., Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. Proceedings of the National Academy of Sciences, *104*(20), 8253-8256.

Winblad, B., Amouyel, P., Andrieu, S., Ballard, C., Brayne, C., Brodaty, H., Zetterberg, H. (2016). Defeating Alzheimer's disease and other dementias: a priority for European science and society. Lancet Neurol, *15*(5), 455 – 532.

Wittmann, C. W., Wszolek, M. F., Shulman, J. M., Salvaterra, P. M., Lewis, J., Hutton, M., & Feany, M. B. (2001). Tauopathy in Drosophila: neurodegeneration without neurofibrillary tangles. Science, *293*(5530), 711 – 714.

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Evaluation of Total Phenolic Content and Antioxidant Activity in Aqueous and Ethanolic Extracts of *Leucosyke capitellata*

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ABSTRACT

Leucosyke capitellata is rich in tannins, steroids, and flavonoids and has been traditionally used in the Philippines and Sabah, Malaysia, for treating inflammation, hypertension, and diabetes. However, scientific research on *L. capitellata* remains limited. This study aims to investigate the total phenolic content (TPC) and antioxidant potential of the aqueous and ethanolic extracts from the leaves, roots, and stems of *L. capitellata*. The TPC was measured using the Folin-Ciocalteu method, and the antioxidant activity was assessed through DPPH scavenging. Among the extracts, the ethanolic root extract exhibited the highest TPC (135.655 ± 0.045 mg GAE/g). All extracts demonstrated significant antioxidant activity (IC_{50} < 50 µg/mL), with the leaves aqueous extract showing the highest antioxidant activity (IC_{50} = 5.274 µg/mL) despite having a relatively low TPC (37.737 ± 0.013 mg GAE/g). This discrepancy between TPC and antioxidant activity highlights the complex nature of plant extracts and suggests that factors beyond TPC contribute to their antioxidant mechanisms. These findings indicate that *L. capitellata* is a promising source of natural antioxidants.

Keywords: *Leucosyke capitellata; total phenolic content; antioxidant activity*

INTRODUCTION

Living organisms naturally produce reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxy radicals as part of their metabolism (Indirayati et al., 2020). However, excessive ROS production can lead to biomolecule oxidation and cellular damage, ultimately contributing to various disease such as diabetes, cancer, cirrhosis, obesity, and cardiovascular disorders (Jafri et al., 2023; Jafri et al., 2022). Several factors, including exposure to ultraviolet rays, cigarette smoke, environmental pollutants, and toxic chemicals, can trigger the overproduction of ROS (Jafri et al., 2023). Therefore, enzymatic antioxidant barriers like superoxide dismutase (SOD) can act as defense mechanisms to neutralize the harmful effects of free radicals, which can damage cells in living organisms (Aryal et al., 2019; Jafri et al., 2023). However, the levels of these endogenous defenders produced in the body may be insufficient, particularly during the generation of free radicals is heightened (Ulewicz-Magulska & Wesolowski, 2018). Therefore, dietary antioxidants may be necessary to maintain optimal cellular functions under increased oxidative stress (Indirayati et al., 2020).

There are two types of antioxidants: synthetic and natural. Synthetic antioxidants like propyl gallate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) are commonly used (Jafri et al., 2022; Ulewicz-Magulska & Wesolowski, 2018), but their safety has been questioned due to potential health risks and toxicity (Indirayati et al., 2020; Molole et al., 2022). On the other hand, natural antioxidants extracted from plants are highly recommended for food applications due to their safety, nutritional potential, and therapeutic effects (Indirayati et al., 2020; Ulewicz-Magulska & Wesolowski, 2018). Medicinal plants with documented traditional use in folk medicines for treating oxidative and related diseases such as hypertension and diabetes are promising sources for novel and effective antioxidants (Molole et al., 2022). For instance, natural flavonoids are secondary metabolites found in plants, characterized by an aromatic ring containing at least one hydroxyl group (Aryal et al., 2019). Flavonoids offer potential health advantages owing to their antioxidant properties, primarily driven by phenolic hydroxyl groups (Vo et al., 2019).

Leucosyke capitellata, a plant species belonging to the family Urticaceae, has been found to contain elevated levels of tannins, steroids, and flavonoids, along with a moderate quantity of saponins (Gawat & Eupeña, 2022). Moreover, this plant has a history where the roots decoction have been traditionally used among local healers in the Philippines for treating inflammation on wound (Gawat & Eupeña, 2022). Furthermore, in Sabah, Malaysia, the leaves decoction of *L. capitellata* has been utilized by locals to manage hypertension and diabetes (Fasihuddin & Holdsworth, 2003). Hence, the aim of this study is to quantify the total phenolic content (TPC) in the ethanolic and aqueous extracts of various parts of *L. capitellata*, including stems, roots, and leaves, using the Folin-Ciocalteu method with slight modification, as well as to determine their free radical scavenging activities using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method.

MATERIALS & METHODS

Plant Material

The plant samples utilized in this study were the stem, root, and leaf of *L. capitellata*. Distilled water and 70% ethanol were the solvents used in preparing the plant extracts. For the TPC assay, the standard sample, chemicals, and reagents included gallic acid (Sigma-Aldrich, US), Folin-Ciocalteu reagent (Sigma-Aldrich, US), and sodium carbonate (Bendosen, Malaysia). As for the DPPH radical scavenging assay, the standard sample and reagents consisted of ascorbic acid (R&M Chemicals, Malaysia) and DPPH reagent from Sigma-Aldrich, US.

Instrument

Rotary evaporator (Buchi R-210, Switzerland) and freeze dryer (Brand?) machine were used for the extract preparation. Furthermore, quartz cuvette and UV-VIS Spectrophotometer (Genesys 20, Thermo scientific, model 4001/4) were used for quantitative analysis for both TPC and DPPH radical scavenging assay.

Sample Preparation

The fresh *L. capitellata* roots, stems, and leaves were collected from Jalan Kiulu, Tamparuli, Sabah, Malaysia. The samples of roots, stems, and leaves were cleaned and sent to the Herbarium of Institute for Tropical Biology and Conservation (ITBC), University Malaysia Sabah (UMS) for taxonomy and morphological confirmation with the assistance of botanist Mr. Johny Gilsil from the institute. After collection, the samples were cut into pieces and dried in an oven at 40°C. The dried samples were then ground into powder, stored in a zip lock bag, and preserved at 4°C until further solvent extraction.

Preparation of Aqueous and Ethanolic Extract

The oven-dried powdered plant samples underwent extraction using distilled water and 70% ethanol via sonication. The sample-to-solvent ratio was 1:20 (w/v), and they were sonicated for 25 minutes at room temperature. The extraction method was adapted from Ranjha et al. (2021) with slight modification. After extraction, different steps were followed for each solvent. For the aqueous extract, the filtrate was transferred to a 50 mL falcon tube and frozen at -80°C for two days before being placed in a freeze dryer to obtain the raw aqueous extract. The process in the freeze dryer took about three days, and the resulting concentrated extracts were stored at -80°C for further analysis.

For the ethanolic extract, after filtration, the resulting filtrate was concentrated under reduced pressure for one to two hours at 60°C using a rotary evaporator (Buchi R-210, Switzerland). The resulting filtrate was then frozen at -80°C for two days before being placed in the freeze dryer to remove the 30% aqueous in the 70% ethanol solvent. This process also took about three days. Afterward, the extracts were stored at -80°C for further analysis.

Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Kupina et al., 2018) with slight modification. Solutions of 1 mg/mL of both sample and gallic acid were prepared, with concentrations ranging from 50 to 250 µg/µL. The solvent used for gallic acid was methanol, while the solvent for plant extracts varied depending on their extraction method. Each concentration mixture was prepared with a final volume of 200 µL. After preparing the mixtures, 200 µL of each was dispersed into test tubes. Then, 1.5 mL of Folin-Ciocalteu reagent (diluted 1:9 with distilled water) was added to the test tubes, followed by incubation for 5 minutes at room temperature. Subsequently, 1.5 mL of sodium carbonate solution (0.06 g/mL) was added to each tube, and the mixtures were allowed to stand for 90 minutes at room temperature. The TPC was measured in a quartz cuvette using a spectrophotometer (Genesys 20, Thermo Scientific, model 4001/4) at 750 nm. A standard calibration curve of the gallic acid (standard sample) was prepared using concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 mg/mL of gallic acid. Finally, the TPC was expressed in milligrams of gallic acid equivalents per gram of plant extracts (mg GAE/g) and were calculated as follows:

$$TPC = (C \times V)/M$$

Where, C is the concentration of gallic acid determined from the standard curve (mg/mL), V is the volume of the extract (mL), and M is the mass of the plant extract (g). All samples were done in triplicate.

Determination of Antioxidant DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of the extracts was assessed following the method outlined by Brand-Williams and Berset (1995) and Indirayati et al. (2020) with a slight modification. To prepare the stock solution of DPPH reagent, a concentration of 300 µM was used. The extract sample and ascorbic acid (standard) were prepared at five concentrations (5, 10, 20, 40, 80 µg/mL), with a stock concentration of 1 mg/mL for both. Each concentration mixture was prepared with a final volume of 10 mL. After preparing the mixtures, 2.5 mL of each was dispersed in triplicate, followed by adding 1 mL of

DPPH solution into the sample. The mixture was then resuspended and allowed to stand for 30 minutes. The entire procedure was conducted in the dark. The absorbance of the mixture was measured using a quartz cuvette by using UV-VIS spectrophotometer (Genesys 20, Thermo Scientific, model 4001/4) at 517 nm. The percentage activity of the samples to scavenge the DPPH radical was calculated using the formula:

$$\text{Inhibition (\%)} = [(A_B - A_S)] / A_B] \times 100$$

Where A_B is the absorption of blank samples (mixture of solvent with DPPH) and A_S is the absorption of extract/standard solution. The assays were carried out in triplicate. The Inhibitory Concentration (IC_{50}) value, which represents the concentration of extract required to inhibit 50% of DPPH activity, was determined using regression analysis from a graph plotting of scavenging activity of sample/standard against concentration of sample/standard where the equation from the graph will be generated as the relationship between the concentration of the sample/standard and the scavenging activity. IC_{50} value was then estimated using the linear regression equation as follows:

$$Y = ax + b,$$
$$IC_{50} = (50 - b)/a$$

Where "a" represents the slope of the line, and "b" represents the y-intercept.

RESULTS & DISCUSSION

Total phenolic content (TPC)

The total phenolic content (TPC) in both aqueous and ethanolic extracts of *L. capitellata* leaves, roots, and stems was determined using the Folin-Ciocalteu method. The results for aqueous extracts were obtained from a calibration curve ($y = 6.4217x + 0.017$, $R^2 = 0.9971$) utilizing gallic acid concentrations ranging from 0.05 to 0.25 µg/mL. Similarly, for ethanolic extracts, the results were obtained from a calibration curve ($y = 5.1771x + 0.0027$, $R^2 = 0.9987$) of gallic acid. Both measurements were expressed in gallic acid equivalents (GAE) per gram of dry extract weight.

Table 1 Total phenolic content of the aqueous and ethanolic extracts of *L. capitellata* leaves, roots, and stems.

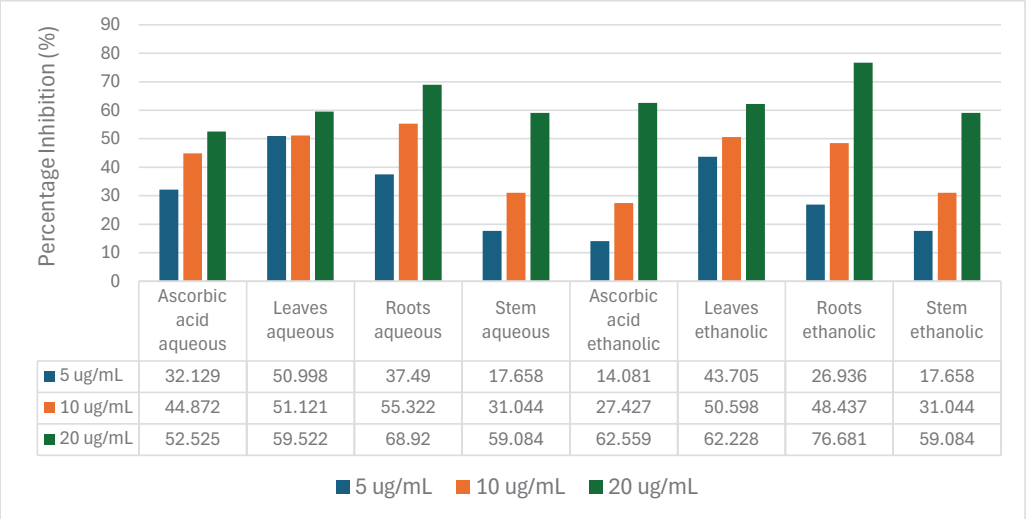
Leucosyke capitellata	Total phenolic content (mg GAE/g)	
	Aqueous extracts	Ethanolic extracts
Leaf	37.737 ± 0.013 [b]	73.657 ± 0.006 [a,b,c]
Root	117.563 ± 0.008 [a]	135.655 ± 0.045 [b]
Stem	34.762 ± 0.005 [a,c]	118.786 ± 0.004 [a,b,c]

All measurements were performed in triplicate, and the results are reported as the mean ± standard error mean (mean ± SEM). Different superscript letters indicate significant differences at $p < 0.05$, as analyzed by Tukey's multiple comparison test.

According to the findings in Table 1, the TPC in the aqueous extracts ranged from 34.762 mg GAE/g to 117.563 mg GAE/g, while for ethanolic extracts, it ranged from 73.657 mg GAE/g to 135.655 mg GAE/g. These results indicate that the ethanolic extracts of *L. capitellata* exhibited higher TPC compared to the aqueous extracts. In particular, the ethanolic extracts of root (135.655 mg GAE/g ± 0.045) showed the highest TPC, followed by stem ethanolic extracts (118.786 mg GAE/g ± 0.004) and leaf ethanolic extracts (73.657 mg GAE/g ± 0.006). The lowest TPC was observed in the aqueous extracts of stem (34.762 mg GAE/g ± 0.005). The extraction methods and solvents are responsible for dissolving the natural compounds present in the plants (Siddhuraju & Becker, 2003). Additionally, plant constituents can exhibit either polar or non-polar characteristics (Aryal et al., 2019). Phenolic compounds, containing hydroxyl groups which have higher solubility in polar organic solvents (Wang & Weller, 2006). Therefore, aqueous and 70% ethanol were chosen as the extracting solvents.

Antioxidant DPPH Radical Scavenging Assay

The DPPH scavenging assay operates on the principle of antioxidants interacting with DPPH, a free radical, through either electron transfer or hydrogen radical transfer. This interaction results in the neutralization of the DPPH radical (Indirayati et al., 2020). The quantification of DPPH scavenging was conducted after a 30 minute incubation period to allow for the reaction between DPPH as a free radical and the samples (Indirayati et al., 2020). The percentage inhibition (PI%) activity was presented in Figure 1, where all the extracts showed concentration-dependent increases in radical scavenging capacity.



	Ascorbic acid aqueous	Leaves aqueous	Roots aqueous	Stem aqueous	Ascorbic acid ethanolic	Leaves ethanolic	Roots ethanolic	Stem ethanolic
5 ug/mL	32.129	50.998	37.49	17.658	14.081	43.705	26.936	17.658
10 ug/mL	44.872	51.121	55.322	31.044	27.427	50.598	48.437	31.044
20 ug/mL	52.525	59.522	68.92	59.084	62.559	62.228	76.681	59.084

Figure 1 Comparison of percentage inhibition activity of ascorbic acid and L. capitellata leaves, roots, and stems aqueous and ethanolic extracts.

From Table 2, the highest DPPH radical scavenging potency with a minimum IC_{50} value was observed in the leaves aqueous extract *of L. capitellata* (5.274 µg/mL), followed by the aqueous extract of root (9.702 µg/mL), the ethanolic extract of leaf (9.889 µg/mL), the ethanolic extract of root (11.456 µg/mL), the ethanolic extract of stem (19.588 µg/mL), and finally the aqueous extract of stem (40.859 µg/mL).

Table 2 Comparison of antioxidant activity of the aqueous and ethanolic extracts of *L. capitellata* leafs, roots, and stems with ascorbic acid. The results are reported as the mean ± standard error mean (mean ± SEM).

Sample	IC_{50} value (µg/mL)	
	Aqueous extract IC_{50}	Ethanolic extract IC_{50}
Ascorbic acid	5.337 ± 0.006	4.885 ± 0.021
Leaf	5.274 ± 0.019	9.889 ± 0.025
Root	9.702 ± 0.006	11.456 ± 0.006
Stem	40.859 ± 0.003	19.588 ± 0.015

All data were compared with the IC_{50} value of standard ascorbic acid for both aqueous and ethanolic extracts. The utilization of IC_{50} in biological assays is extensively employed in pharmacological investigations (Benjamin et al., 2024). The classification of antioxidant activity based on IC_{50} is divided into five categories: highly active (<50 µg/mL), active (50 – 100 µg/mL), moderate (101 – 250 µg/mL), weak (250 – 500 µg/mL), and inactive (>500 µg/mL) (Indirayati et al., 2020).

The present study also reveals that both aqueous and ethanolic extracts of *L. capitellata* leaves, roots, and stems exhibit potent antioxidant properties. Remarkably, the aqueous extracts of the leaf show the highest antioxidant activity, with an IC_{50} value of 5.274 µg/mL ± 0.019, surpassing even the ascorbic acid standard (5.337 µg/mL ± 0.006). Despite this impressive antioxidant activity, the aqueous leaf extracts have one of the lowest total phenolic contents (37.737 ± 0.013 mg GAE/g). This paradox underscores the complexity of plant extracts and their antioxidant mechanisms, suggesting that factors beyond total phenolic content, such as other bioactive compounds, likely contribute to their observed antioxidant effects.

CONCLUSION

The total phenolic content (TPC) and antioxidant potential of the aqueous and ethanolic extracts from the leaves, roots, and stems of *L. capitellata* were investigated. The highest TPC was observed from root ethanolic extracts (135.655 mg GAE/g ± 0.045), while the lowest TPC was found from stem aqueous extract (34.762 mg GAE/g ± 0.005). All extracts in this study have a highly active antioxidant activity (IC_{50} <50 ug/mL). However, extracts that possess the highest antioxidant properties, as indicated by their DPPH scavenging activity, are from the leaf aqueous extract with the IC_{50} value of 5.274 ug/mL ± 0.019 and the lowest antioxidant properties was observed from the stem aqueous extract with the IC_{50} value of 40.859 µg/mL ± 0.003. Notably, the leaf aqueous extract had the second

lowest TPC (37.737 mg GAE/g ± 0.013). The discrepancy between TPC and antioxidant activity underscores the complexity of plant extracts and their antioxidant mechanisms, suggesting that factors beyond TPC may contribute to their antioxidant effects. A deeper investigation into the phenolic compositions and antioxidant properties is crucial for a thorough understanding of a plant extracts' antioxidant capacity.

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REFERENCES

Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal. *Plants 2019*, *8*(96), 1 – 12. https://doi.org/10.3390/plants8040096

Benjamin, M. A. Z., Mohd Mokhtar, R. A., Iqbal, M., Abdullah, A., Azizah, R., Sulistyorini, L., Zakaria, Z. A. (2024). Medicinal plants of Southeast Asia with anti-α-glucosidase activity as potential source for Type-2 diabetes mellitus treatment. *Journal of Ethnopharmacology*, *330*, 118239. https://doi.org/10.1016/j.jep.2024.118239

Brand-Williams, W., & Berset, M. E. C. a. C. (1995). Use of a Free Radical Method to Evaluate Antioxidant Activity *Lebensmittel-Wissenschaft und-Technologie*, *28*(1), 1 – 30. https://doi.org/https://doi.org/10.1016/S0023-6438(95)80008-5

Fasihuddin, A., & Holdsworth, D. K. (2003). Medicinal Plants of Sabah, East Malaysia – Part I. *Pharmaceutical Biology*, *41*(5), 340 – 346.

Gawat, P. N. U., & Eupeña, R. G. (2022). Phytochemical Screening of *Leucosyke capitellata* POIR and *Dendrocnide densiflora* C. B. ROB Plant in Preference of its Utilization in the Municipality of San Miguel, Surigao del Sur, Philippines. *International Journal of Biosciences*, *20*(3), 295 – 300. https://doi.org/http://dx.doi.org/10.12692/ijb/20.4.295-300

Indirayati, N., Nisa, K., Kurang, R. Y., Tarmo, N. C., & Adang, K. T. P. (2020). Radical Scavenging Activity and Total Phenolic Content of Seven Tropical Plants *IOP Conference Series: Earth and Environmental Science*, *462*(2020), 1 – 7. https://doi.org/10.1088/1755-1315/462/1/012043

Ipandi, I., Triyasmono, L., & Prayitno, B. (2016). Penentuan Kadar Flavonoid Total dan Aktivitas Antioksidan Ekstrak Etanol Daun Kajajahi (*Leucosyke capitellata* Wedd.). *Jurnal Pharmascience*, *3*(1), 93 – 100.

Jafri, S. A. A., Khalid, Z. M., Khan, M. R., Ashraf, S., Ahmad, N., Karami, A. M., . . . Aslam, S. (2023). Evaluation of Some Essential Traditional Medicinal Plants for their Potential Free Scavenging and Antioxidant Properties. *Journal of King Saud University – Science*, *35*(2023), 1 – 13. https://doi.org/https://doi.org/10.1016/j.jksus.2023.102562

Jafri, S. A. A., Khalid, Z. M., Khan, M. Z., & Jogezai, N. (2022). Evaluation of Phytochemical and Antioxidantpotential of Various Extracts from Traditionally Used Medicinal Plants of Pakistan. *Open Chemistry*, *20*(2022), 1337 – 1356. https://doi.org/https://doi.org/10.1515/chem-2022-0242

Kupina, S., Fields, C., Roman, M. C., & Brunelle, S. L. (2018). Determination of Total Phenolic Content Using the Folin-C Assay: Single-Laboratory Validation, First Action 2017.13. *Journal of AOAC International*, *101*(5), 1 – 7. https://doi.org/https://doi.org/10.5740/jaoacint.18-0031

Kusuma, I. W., Murdiyanto, Arung, E. T., Syafrizal, & Kim, Y.-u. (2014). Antimicrobial and Antioxidant Properties of Medicinal Plants used by The Bentian Tribe From Indonesia. *Food Science and Human Wellness*, *3*(2014), 191 – 196. https://doi.org/http://dx.doi.org/10.1016/j.fshw.2014.12.004

Molole, G. J., Gure, A., & Abdissa, N. (2022). Determination of Total Phenolic Content and Antioxidant Activity of *Commiphora mollis* (Oliv.) Engl. Resin. *BMC Chemistry*, *16*(48), 1 – 11. https://doi.org/https://doi.org/10.1186/s13065-022-00841-x

Ranjha, M. M. A. N., Irfan, S., Lorenzo, J. M., Shafique, B., Kanwal, R., Pateiro, M., . . . Aadil, R. M. (2021). Sonication, a Potential Technique for Extraction of Phytoconstituents: A Systematic Review. *Processes*, *9*(1406), 1 – 21. https://doi.org/https://doi.org/10.3390/pr9081406

Siddhuraju, P., & Becker, K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves. *Journal of Agricultural and Food Chemistry*, *51*(8), 2144 – 2155. https://doi.org/10.1021/jf020444+

Sundang, M., Nasir, S. N. S., Sipaut, C. S., & Othman, H. (2012). Antioxidant Activity, Phenolic, Flavonoid and Tannin Content of *Piper betle* and *Leucosyke capitella*. *Malaysian Journal of Fundamental & Applied Sciences*, *8*(1), 1 – 6. https://doi.org/http://dx.doi.org/10.11113/mjfas.v8n1.115

Ulewicz-Magulska, B., & Wesolowski, M. (2018). Total Phenolic Contents and Antioxidant Potential of Herbs Used for Medical and Culinary Purposes. *Plant Foods for Human Nutrition*, *74*(2019), 61 – 67. https://doi.org/https://doi.org/10.1007/s11130-018-0699-5

Vo, Q. V., Nam, P. C., Thong, N. M., Trung, N. T., Phan, C.-T. D., & Mechler, A. (2019). Antioxidant Motifs in Flavonoids: O–H versus C–H Bond Dissociation. *ACS Omega*, *4*(2019), 8935 – 8942. https://doi.org/10.1021/acsomega.9b00677

Wang, L., & Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology*, *17*(6), 300 – 312. https://doi.org/https://doi.org/10.1016/j.tifs.2005.12.004

Wulansari, D., & Chairul. (2011). Antioxidant Screening Activity of Several Indonesian Medicinal Plants Using 2,2-Diphenyl-1-picrylhydrazyl (DPPH). *Majalah Obat Tradisional*, *16*(1), 22 – 25.

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for Extraction and Isolation of Natural Products: A Comprehensive Review. *Chinese Medicine*, *13*(20), 1 – 26. https://doi.org/https://doi.org/10.1186/s13020-018-0177-x

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Investigation of bacterial antigenic fragments OMPs as a potential vaccine candidate against vibriosis in TGGG

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ABSTRACT

Aquaculture holds a crucial position in the economies of developing nations, making significant contribution to both food security and income. Despite its importance, aquaculture encounters challenges including bacterial infections threats like vibriosis that caused by *Vibrio* spp. Concerns regarding antibiotic resistance have prompted a shift towards usage of vaccine as a sustainable alternative treatment method. This study focused on the development of a fusion fragment protein GADPH-OmpK as a potential vaccine candidate against vibriosis in hybrid TGGG Grouper (*Epinephelus fuscoguttatus x E. lanceolatus*) and the impact it has on the expression of immune genes. The fusion fragment protein was successfully overexpressed and used to intraperitoneally immunize hybrid TGGG grouper. The immunogenetic expression of Interleukin-2, Interleukin-6 and Interferon-ϒ in the results indicated that the vaccine elicits a considerable immune response in the fish, demonstrating its potential in enhancing aquaculture sustainability. Overall, this research presents a promising avenue for advancing aquaculture practices and mitigating antibiotic resistance issues in fish farming since recombinant protein vaccine offer various advantages.

Keywords: aquaculture, TGGG hybrid grouper, vaccine, immune response

INTRODUCTION

Aquaculture is an essential source of aquatic food and revenue in developing countries, as this sector offers financial benefits and also valuable species like farmed shrimp, marine fish, and freshwater fish for the export market (Ina-Salwany et al., 2018). Cole et al. (2009) stated that the marine culture industry has produced about 40% of the world's seafood, resulting this sector supplanting conventional marine capture commodities (Kurniawan et al., 2021). Nevertheless, high-density species environments employed in the aquaculture industry accelerate parasite and disease transmission, necessitating monitoring quality, safety, and microbiological indicators for a sustainable future. Some chemicals including antibiotics, insecticides, herbicides and hormones have been used to treat and control disease outbreaks however, studies found that they can cause environmental contamination and antibiotic resistance, potentially leading to more unintended species and diseases emergence (Okeke et al., 2022).

One of the common bacterial infections is vibriosis caused by *Vibrio* spp, a prevalent pathogenic bacterium such as *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* has been known to negatively affect various shellfish and marine fish, causing hindrance for sustainable growth for marine vertebrates (Tall et al., 2013; Baker-Austin et al., 2018). Vibriosis has been reported to infect groupers (*Epinephelus* spp.) at all growth stages with about 66.7% of cases and be the cause of death rates of up to 50% (Liao and Leao., 2008; Chong et al., 2011; El-Galil and Mohamed, 2012). Therefore, this study delves into the development and evaluation of a fusion fragment protein as a potential vaccine candidate targeting vibriosis in hybrid groupers.

The immune system in fish is composed of adaptive and innate components that respond to exogenous or endogenous stimuli as well as defend against foreign substances like malignant cells, microorganisms, or toxins. The natural killer cells (granulocytes, monocytes, macrophages), the complement system, the antimicrobial enzymes, the interleukins, and the interferon are all examples of organic defense mechanisms (Biller-Takahashi & Urbinati, 2014). Previous research done by Monir et al. (2022) demonstrated that monovalent vaccine has a limited immunogenicity profile while bivalent vaccine shows a promising spectrum in achieving strong immunocompetent effects. Hence, this study looked at the efficacy of fusion fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and outer membrane protein K (OmpK) as a potential recombinant vaccine candidate and their immunogenicity was evaluated by analyzing the quantitative expression of gene encoding Interleukin-2 (IL-2), Interleukin-6 (IL- 6) and Interferon-gamma (IFN-ϒ).

The objective of this study was 1) to overexpress bacterial antigenic fusion fragment of OMP as a vaccine candidate, 2) to immunize the hybrid TGGG grouper (*Epinephelus fuscoguttatus x E. lanceolatus*) with the vaccine candidate and 3) to evaluate the immune response of unvaccinated and vaccinated fish via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

MATERIALS AND METHODS

Vaccine Candidate Development

Overexpression of a Fusion Fragment Protein

In this study, the plasmid system harboring the recombinant protein of GAPDH-OmpK employed was attained from previous research done by Budiman et al. (2022). An overexpression was conducted following the protocol established by Zhang et al. (2007). Briefly, 3mL of pre-cultured *Escherichia coli* Rosetta carrying GAPDH-OmpK was inoculated into 100mL Terrific Broth (TB) containing 100 µL of kanamycin antibiotic at a ratio of 1:100 (v/v). The culture flask was vigorously shaken and incubated at 37°C until an optical density (OD600) of 0.6 was reached, later induced with 100 µL of isopropyl – D – thiohalactopyranocide (IPTG). The culture further incubated overnight (12 – 16 hours) at 18°C. The cell was harvested by centrifugation for 10 min at 8000 rpm at 4°C and the supernatant was discarded. The pellet was washed and resuspended with 10 mM Tris-HCl buffer (pH 7.6) containing 1mM EDTA and proceeded with sonication. The soluble fraction was then separated from the cell debris by centrifugation at 35,000x g for 30 min at 4°C. The expressed protein was analyzed on 15% SDS–polyacrylamide gel electrophoresis.

Vaccination of Hybrid TGGG Grouper

TGGG Acclimatization and Vaccination

This research involving animal experimentation of hybrid TGGG grouper was approved by the University of Malaysia Sabah Animal Ethics Committee with reference number AEC 0018/2023.

Groups of 5-inch length of hybrid grouper weighing 40 ± 6 g were bought and acclimatized for 1 week at the UMS Fish Hatchery in a controlled environment (28 ppt salinity, 6.0 mg/L dissolved oxygen, 30°C and pH of 7.9). The fish resided in 100 L fiberglass-reinforced plastic (FRP) tanks filled with aerated recirculating saltwater. TGGG fish daily diet was fed twice with commercial dry pellets containing crude protein (50%), crude fat (>8.4%), crude fiber (<2.9%), ash content (<16%) and moisture (<10%). Daily assessments of fish health and regular monitoring of water quality in each tank were conducted.

2 groups of 5-inch hybrid TGGG grouper (n=42) were selected for the vaccination procedure. The first group (n=21) (vaccinated) was immunized with pure recombinant protein vaccine, GADPH-OmpK at a dosage of 50 µL/100 g while another group (n=21) (unvaccinated) served as a control injected with 50 mM Tris HCl (pH 7.5) at a dosage of 50µL/100g through intraperitoneal injection (IP) using 23G needle. This procedure was performed at the UMS Fish Hatchery and guided by the trained research assistant

from Borneo Marine Research Institute (BMRI). Fish sampling was done at day 7 and day 14 post-vaccination using 250 mg/L l–1 of MS-222 (Tricaine®- S, Western Chemical) for euthanizing.

Immune-related Gene Expression Analysis by qRT-PCR

Extraction of RNA

The spleen of the fish was harvested following the protocol used by Zhang et al. (2007) with slight modifications. A fish from each tank was lethally anesthetized with 250 mg/L l–1 of MS-222 (Tricaine®-S, Western Chemical) for 20 minutes. Using a scalpel, fish spleen was excised under RNase-free conditions. The sample was then homogenized using mortar and pestle on ice. Approximately 3 volumes of RNAlater ™ solution (Invitrogen by Thermo Fisher Scientific) was subsequently added to 1 volume of the homogenized sample for storage at -80ºC. This method was done for day 0, day 7 and day 14 post-vaccination.

The RNA extraction method was done according to the RNeasy® Plus Mini Kit protocols with some modifications. Sample pooling was performed from unvaccinated and vaccinated groups of treatments, accordingly. The RNA integrity and concentration were determined using the Nanodrop spectrophotometer. The total RNA synthesized samples were preserved at –80°C until further use.

qRT-PCR Analysis of Immune-related Genes

A quantitative real time–polymerase chain reaction (qRT-PCR) procedure was performed using the ViPRimePLUS One Step AtTaq RT-qPCR Green Master Mix I (SYBR® Green Dye) kit. A standard total of 20 µL reaction mix was prepared for all samples (10 µL AtTaq one Step RT-qPCR Green Master Mix I, 1 µL forward primer, 1 µL reverse primer followed by 5 µL of RNA sample and 3 µL of distilled water. Amplification was performed using the C100™ Thermal Cycler (Bio-Rad) with the following program conditions: reverse transcription cycle at 42°C for 10 minutes followed by 95°C for 8 minutes (enzyme activation), 95°C for 10 seconds (denaturation) and 60°C for 60 seconds (data collection). All data were normalized to the reference gene, β-actin (Yang et al., 2022). A non-template control sample (NTC) was included in the qPCR test to confirm the master mix has no contamination.

Oligonucleotide Primers

The oligonucleotide primers used in this study are Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interferon-gamma (IFN-g) and ^{2}-actin as a housekeeping gene. The genes are listed in Table 1 below.

Table 1 Primers used in qPCR

No.	Genes	Sequence (5'-3')	Reference
1	β-actin	F: TGCGTGACATCAAGGAGAAGC R: TCTGGGCAACGGAACCTCT	Yang et al., 2022
2	Interleukin-2 (IL-2)	F: GCCGACCTGGTTGTAATCCTCA R: ATCTCAAAGCCTGTCTCATTGGT	He et al., 2021
3	Interleukin-6 (IL-6)	F: AGGAAGGTCTGGCTGTCAGGA R: GCCCTGAGGCCTTCAAGATT	He et al., 2021
4	Interferon-γ (IFN-γ)	F: CCACCAACATGGAGGCTAAC R: CTGCCACCTCACCATTGCT	He et al., 2021

RESULTS

Vaccine Candidate Development

Overexpression of a Fusion Fragment Protein

The expression of Escherichia coli Rosetta containing the fusion fragment protein GADPH-OmpK was assessed on 15% SDS-PAGE. The results of after and before induction by IPTG were visualized in Lanes 1 and 2, respectively, as depicted in Figure 1. The solubility of the protein was also observed by examining the appearance of bands for supernatant in Lane 3 and pellet in Lane 4 of the protein after ultracentrifugation. The observed band size was ~23kDa which corresponds to the study done by Budiman et al. (2022).

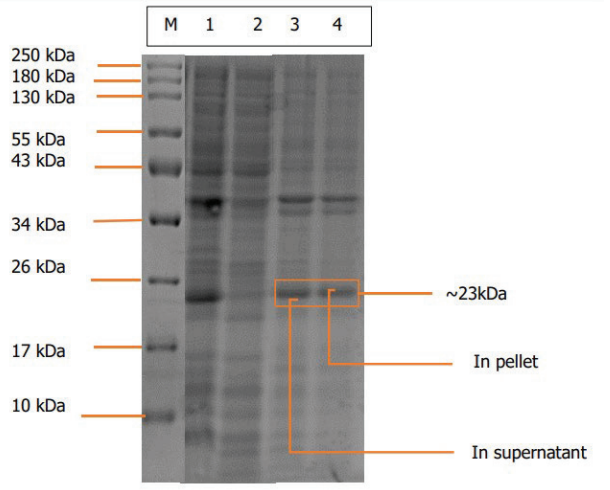

Figure 1 SDS-PAGE of the recombinant protein GADPH-OmpK expression in Escherichia coli Rosetta. The M lane represent marker, band in Lanes 1 and 2 represent the pre and post IPTG induction. Lanes 3 and 4 represent the soluble and insoluble fusion fragment protein obtained after sonication.

Immune-related Gene Response Analysis

As seen in Figure 2, the expressions of IL-2 and IL-6 were observed to be downregulated both at day 7 and day 14 post-vaccination. In contrast to IFN-ϒ where the gene was seen to be upregulated at day 7 and day 14 post-vaccination. The fold changes were calculated according to the Livak method (Livak & Schmittgen, 2001).

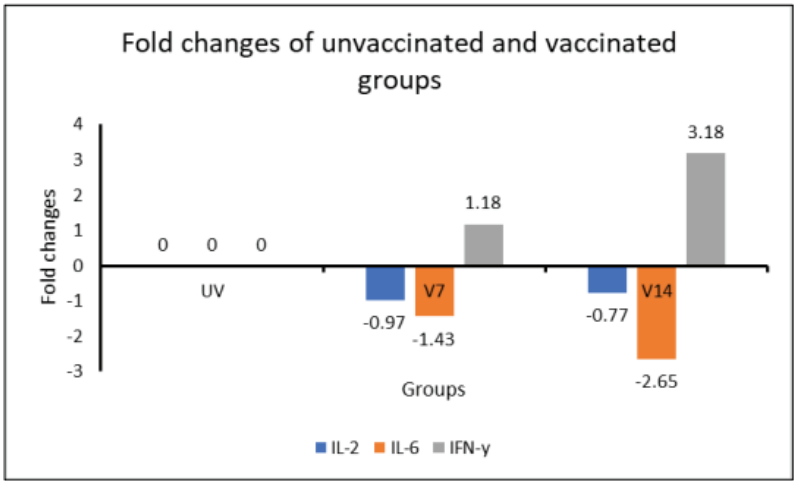

Figure 2 Fold changes of different genes with unvaccinated and vaccinated treatments. The data above showed downregulation for gene expressions of IL-2 and IL-6 at day 7 and day 14, but displayed upregulation for gene expression of IFN-ϒ at day 7 and day 14, respectively.

DISCUSSION

This study reported the possibility of GAPDH-OmpK as a recombinant protein vaccine candidate that can induce immunoprotection in hybrid TGGG grouper (Epinephelus fuscoguttatus x E. lanceolatus). This research focused mainly on 1) overexpression of bacterial antigenic fusion fragment of GAPDH and OmpK as a vaccine candidate, 2) immunization of the hybrid TGGG grouper with the vaccine candidate and 3) the immune response of unvaccinated and vaccinated fish to indicate regulation of fish immunity.

As seen in Figure 1 the overexpression of fusion fragment protein vaccine candidate was successfully performed under the induction of 1mM IPTG at 37°C. The protein size was approximated at ~23kDa however, according to Budiman et al. (2022) the size is slightly larger than the theoretical fusion size which is 20.29 kDa due to the presence of His-tag at the N–terminal fusion. This, nevertheless, confirmed that the band is a product of the expression of the GADPH–OmpK gene. The vaccination procedure of TGGG grouper was performed following animal ethics and guided by a trained person. Fish vaccination was done through intraperitoneal injection (IP) via the abdominal cavity since this method is believed to be the most successful (Vinitantharat et al., 1999).

In this research, the unvaccinated group served as control hence, the fold changes (ΔΔCq) were zero for all IL-2, IL-6 and IFN-ϒ genes as shown in Figure 2. Figure 2 displayed fold changes for both unvaccinated and vaccinated groups for day 7 and day 14. In quantitative gene expression analysis, fold changes of > 2 are accepted as significant expressions. Based on the results obtained, the IFN-ϒ gene showed a significant upregulation by 8 fold in the vaccinated day 14 group as compared to the unvaccinated group. In contrast, the IL-6 gene was significantly downregulated by -2.65 in the vaccinated day 14 group as compared to the unvaccinated group. Downregulation expression of the IL-2 and IL-6 genes in the vaccinated day 7 group occurred but were not significantly different with -0.97 and -1.43 fold respectively as compared to the unvaccinated group. The IL-2 gene exhibited insignificant downregulation expression in the vaccinated day 14 group as compared to the unvaccinated group.

According to the study done by Chen et al. (2017), cytokines modulate the immune response to infection or inflammation and regulate inflammation itself via a complex network of interactions. This could be because the immune system modifies both IL-2 and IL-6 expression after the initial activation to prevent excessive inflammation or immune dysregulation. In other words, downregulation after vaccination could indicates a regulatory mechanism to prevent excessive inflammation. Vaccination may induce an early immune response, leading to a slight upregulation at day 7 and a more pronounced and significant upregulation at day 14. It concurred with Makesh et al. (2023) who observed that substantially higher antibody levels were generated by the recombinant vaccine at 50 and 100 µg/fish, and an immunological response was shown as soon as one week after immunization. This implies that the vaccination is successful in promoting IFN-ϒ expression.

In summary, vaccination with the recombinant protein has successfully evokes varied fold changes in hybrid grouper immunegenetic response, thus indicating the immunoprotective potential of GAPDH-OmpK as a fish vaccine.

CONCLUSION

In conclusion, this study lays the foundation for the development of a potential bivalent vaccine against vibriosis in hybrid grouper fish as the GAPDH-OmpK vaccine candidate showed varied expression patterns of the fish immune-related genes, demonstrating a dynamic response to immunization. Further investigations and improvements in the experimental design would contribute more to the advancement of fish farming practices, addressing challenges related to bacterial infections as well as promoting sustainable aquaculture developments.

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REFERENCES

Baker-Austin, C., Oliver, J. D., Alam, M., Ali, A., Waldor, M. K., Qadri, F., & Martínez-Urtaza, J. (2018). Vibrio spp. infections. *Nature Reviews Disease Primers, 4*(1), 1 – 19.

Biller-Takahashi, J. D., & Urbinati, E. C. (2014). Fish Immunology. The modification and manipulation of the innate immune system: Brazilian studies. *Anais Da Academia Brasileira De Ciencias, 86*(3), 1484 – 1506.

Budiman, C., Rahim@Roslam, R. H., Nik Asri, N. A. A., & Amin, Z. (2022). In silico Analysis and Preliminary Expression of Antigenic Fragments of OmpK and GAPDH of Vibrio species. *Malaysia Journal of Microbiology*. [Manuscript submitted for publication]

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204 – 7218.

Chong, R., Bousfield, B., & Brown, R. (2011). Fish Disease Management. In http://www.afcd.gov.hk/english/quarantine/qua_vb/qua_vb.html. Agriculture, Fisheries and Conservation Department, Hong Kong.

Cole, D., Cole, R., Gaydos, S., Gray, J. R., Hyland, G., Jacques, M. L., Powell-Dunford, N., Sawhney, C., & Au, W. W. (2009). Aquaculture: Environmental, toxicological, and health issues. *International Journal of Hygiene and Environmental Health, 212*(4), 369 – 377.

El-Galil, M. A., & Mohamed, M. (2012). First Isolation of Vibrio alginolyticus from Ornamental Bird Wrasse Fish (Gomphosus caeruleus) of the Red Sea in Egypt. *Journal of Fisheries and Aquatic Science, 7*(6), 461 – 467.

He, Y., Guo, X., Tan, B., Dong, X., Liu, H., Zhang, S., & Chi, S. (2021). Replacing fish meal with fermented rice protein in diets for hybrid groupers (Epinephelus fuscoguttatus♀× Epinephelus lanceolatus♂): Effects on growth, digestive and absorption capacities, inflammatory-related gene expression, and intestinal microbiota. *Aquaculture Reports*, 19, 100603.

Ina-Salwany, M. Y., Al-saari, N., Mohamad, A., Mursidi, F., Mohd-Aris, A., Amal, M. N. A., Kasai, H., Mino, S., Sawabe, T., & Zamri-Saad, M. (2018). Vibriosis in Fish: A review on Disease Development and Prevention. *Journal of Aquatic Animal Health, 31*(1), 3 – 22.

Kurniawan, S. B., Ahmad, A., Rahim, N. F. M., Said, N. S. M., Alnawajha, M. M., Imron, M. F., Abdullah, S. R. S., Othman, A. R., Ismail, N. I., & Hasan, H. A. (2021). Aquaculture in Malaysia: Water-related environmental challenges and opportunities for cleaner production. *Environmental Technology and Innovation*, 24, 101913.

Liao, I. C. and Leaño, E. M. (Eds.). (2008). *The aquaculture of groupers*. Asian Fisheries Society.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using Real-Time Quantitative PCR and the 2−ΔΔCT method. *Methods, 25*(4), 402 – 408.

Makesh, M., Satyanarayana, N. V., Muddukrishnaiah, K., Kumar, S., Thiagarajan, G., Jangam, A. K., Subburaj, R., Kailasam, M., & Vijayan, K. (2023). Humoral immune response in Asian seabass vaccinated with inactivated and recombinant viral nervous necrosis vaccine. *Aquaculture*, 569, 739384.

Monir, M. S., Yusoff, M. S., Zamri-Saad, M., Amal, M. N. A., Mohamad, A., Azzam-Sayuti, M., & Yasin, I. S. M. (2022). Effect of an Oral Bivalent Vaccine on Immune Response and Immune Gene Profiling in Vaccinated Red Tilapia (Oreochromis spp.) during Infections with Streptococcus iniae and Aeromonas hydrophila. *Biology, 11*(9), 1268.

Okeke, E. S., Chukwudozie, K. I., Nyaruaba, R., Ita, R. E., Oladipo, A., Ejeromedoghene, O., Atakpa, E. O., Agu, C. V., & Okoye, C. O. (2022). Antibiotic resistance in aquaculture and aquatic organisms: a review of current nanotechnology applications for sustainable management. *Environmental Science and Pollution Research, 29*(46), 69241 – 69274.

Tall, A., Hervio-Heath, D., Teillon, A., Boisset-Helbert, C., Delesmont, R., Bodilis, J., & Touron-Bodilis, A. (2013 Diversity of Vibrio spp. isolated at ambient environmental temperature in the Eastern English Channel as determined by pyrH sequencing. *Journal of Applied Microbiology, 114*(6), 1713 – 1724.

Vinitnantharat, S., Gravningen, K., & Greger, E. (1999). Fish vaccines. *In Advances in Veterinary Medicine* (pp. 539–550).

Yang, X., Zhao, X., Wang, G., Dong, X., Yang, Q., Liu, H., Zhang, S., Tan, B., & Chi, S. (2022). Improvement of hybrid grouper (Epinephelus fuscoguttatus ♀ × E. lanceolatus ♂) by enzyme-digested poultry by-product: Growth performance, amino acid and peptide transport capacity, and intestinal morphology. *Frontiers in Nutrition*, 9.

Zhang, C., Yu, L., & Qian, R. (2007). Characterization of OmpK, GAPDH and their fusion OmpK-GAPDH derived from Vibrio harveyi outer membrane proteins: their immunoprotective ability against vibriosis in large yellow croaker (Pseudosciaena crocea). *Journal of Applied Microbiology, 103*(5), 1587 – 1599.