Antioxidant and Hepatoprotective Potential of *Hedyotis diffusa*: A Sabah Native Plant

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ABSTRACT

Hedyotis diffusa is a medicinal plant recognized for its potential as an alternative natural drug due to its rich polyphenol content. These compounds are known to possess antioxidant and hepatoprotective activities, which may be beneficial in treating liver diseases—a major cause of human mortality worldwide. This research was conducted to determine the phenolic compounds and their scavenging activity in *Hedyotis diffusa*. Additionally, *in vivo* experiments using Sprague-Dawley rats were performed to evaluate the hepatoprotective effects through serum and liver analysis based on CCl_4-induced model, a hepatotoxic agent (mimic free radical). The *Hedyotis diffusa* aqueous extract exhibited a phenolic content (90.22 ± 0.10 mg GAE/g) and scavenging activity (IC_{50} = 1540 µg/mL). In the animal study using Sprague-Dawley rats, treatment with *Hedyotis diffusa* at 150 mg/kg (HD150) and 300 mg/kg (HD300) significantly reduced AST (HD150 = 31.8%; HD300 = 49.4%), ALT (HD150 = 35.8%; HD300 = 48.6%), and MDA (HD150 = 10.6%; HD300 = 26.6%) levels, while increasing GSH levels (HD150 = 18.8%; HD300 = 36.4%) against CCl_4-induced oxidative stress. These findings demonstrate that *Hedyotis diffusa* possesses significant hepatoprotective effects against carbon tetrachloride-induced hepatotoxicity.

Keywords: *Hedyotis diffusa*, Antioxidant, Hepatoprotective, Polyphenol, AST, ALT, MDA, GSH

INTRODUCTION

Sabah is one of the states in Malaysia, located in the northern region of Borneo Island. It shares borders with Sarawak (Malaysia), Brunei, Kalimantan (Indonesia), and the Philippines. Borneo Island is endowed with rich biodiversity due to its extensive rainforests and tropical climate, with temperatures ranging from approximately 23–32°C. The indigenous people of Sabah traditionally use medicinal plants in their daily lives (Kulip, 2003). *Hedyotis diffusa* is one of medicinal plant, traditionally used for treating hepatitis, tonsillitis, appendicitis, pneumonia, mastitis, urethral infections, and tumours affecting the liver, lungs, and stomach (Z. Liang et al., 2008). *Hedyotis diffusa* belongs to Rubiaceae family and has been scientifically validated for its pharmacological properties, including anticancer (Chen et al., 2008), antifungal (Li et al., 2005), anti-inflammatory, antioxidant, immunoregulatory, and neuroprotective activities (Ahmad et al., 2005). However, the potential of *Hedyotis diffusa* as an antioxidant and hepatoprotective agent in Sabah remains unexplored. Therefore, investigating its bioactive properties could contribute significantly to scientific research and pharmaceutical drug development.

The liver is important organ that responsible for metabolism, secretion, and storage (Gutiérrez and Solís, 2009). According to (Shakya & Shukla, 2011), the liver diseases can be caused by toxic chemicals, excessive alcohol consumption, infections, and autoimmune disorders. Hepatotoxic chemicals such as carbon tetrachloride (CCl_4) induce liver cell damage through lipid peroxidation and oxidative stress (Fox et al., 1996). Alternative natural drugs with antioxidant and hepatoprotective properties is crucial in liver research through *in vitro* and *in vivo* experiments.

Polyphenols have garnered significant attention from researchers and the pharmaceutical industry due to their well-established antioxidant properties. They preventing diseases associated with oxidative stress, including cardiovascular and neurodegenerative diseases, as well as cancer (Manach et al., 2004; Rasmussen et al., 2005). Phenolic compounds are secondary metabolites characterized by aromatic rings with one or more

hydroxyl groups (Djeridane et al., 2006). Phenolic compounds in *Hedyotis diffusa* have demonstrated hepatoprotective activity through various mechanisms, including immunotherapeutic pathways (Zheng et al., 2025), enzymatic regulation involving superoxide dismutase and NADPH oxidase (Y. Liang et al., 2021) and studies using a zebrafish model (Wang et al., 2023).

Given the limited data on *Hedyotis diffusa* that integrates both *in vitro* and *in vivo* evaluation, a comprehensive investigation is necessary to provide complete pharmaceutical data, especially on its efficacy against free radicals. Research information will provide essential data for identifying a novel antioxidant source, offering a comprehensive assessment through both chemical reactions and animal models.

MATERIALS AND METHODS

Chemicals

Plant Collection and Extraction

Hedyotis diffusa samples were obtained from local herb suppliers in Kota Kinabalu, Sabah. The whole plants were washed and air-dried at 20°C ± 0.5°C of room temperature for one day, followed by oven drying at 37°C ± 0.5°C for three days (Thermo Scientific, Waltham, MA, USA). Heavy duty blender (Waring Commercial, PA, USA) was performed to ground sample into fine powder using a. Extraction was carried out based on the method by Maghrani et al. (2003), with modifications. In brief, 100 g powdered sample was mixed with 1000 mL boiled water on a hot plate with magnetic stirring for 10 minutes. The solids were removed from the mixture using Whatman filter paper and a chimney flask. The extract was kept for further analysis under temperature of -20°C ± 0.5°C.

Total Phenolic Content (TPC) Determination

Total phenolic content conducted based on Ringgit et al. (2024;2025) with some modifications. A 1 mL of 1:10 (v/v) Folin and Ciocalteu’s phenol

reagent (R&M Chemicals, Kumpulan Saintifik F.E. Sdn. Bhd., Selangor, Malaysia) mixed with 0.3 mL sample for 10 minutes dark incubation. Next, 1.2 mL of 7.5% (w/v) sodium carbonate (CNa_2O_3 (HmbG Chemicals, Hamburg, Germany) mixed with mixture for 30 minutes further dark incubation. The spectrophotometer (SP-3000 nano, Optima, Tokyo, Japan) performed to evaluate the activity under 743 nm and standard is gallic acid, $C_6H_2(OH)_3COOH•H_2O$ (Nacalai Tesque, Kyoto, Japan). The result expresses as milligrams of gallic acid equivalent per gram (mg GAE/g).

DPPH Radical Scavenging Activity

Antioxidant activity following Hatano et al. (1988) method with modifications. A 0.1M DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate; $C_{18}H_{12}N_5O_6$) (TCI, Tokyo, Japan) was dissolved in methanol. Then, 2.7 mL of the DPPH solution was mixed with 300 µL of the sample extract. Ascorbic acid (HmbG Chemicals, Hamburg, Germany) was used as the standard. The mixture was kept in the dark for one hour. Absorbance was measured at 517 nm using a spectrophotometer, and antioxidant activity was expressed as the percentage of radical scavenging activity (%RSA) using the following equation:

$$\%RSA = [(A_{standard} - A_{sample})/A_{standard}] \times 100\%$$

$A_{standard}$ and A_{sample} are absorbance readings of ascorbic acid and sample with DPPH, respectively. The %RSA graph was constructed and the IC_{50} was obtained.

Animal Experiment

The animal experiments were carried out in strict adherence to ethical guidelines, university standards, and federal legislation pertaining to animal experimentation (UMS/IP7.5/M3/4-2012). Sixteen adult male Sprague-Dawley rats (4–8 weeks old), weighing 150–200 g, were obtained from in-house breeding at the Animal Facility, Faculty of Food Science and Nutrition,

Universiti Malaysia Sabah. The rats were acclimatized for one week under controlled conditions: room temperature (20°C ± 0.5°C), humidity (50% ± 5%), and a 12-hour light/dark cycle. The animals were randomly divided into four groups. The control group I (CG; n = 4) received normal saline. The group II (CT; n = 4) was administered carbon tetrachloride at 1.2 mL/kg body weight by gavage on days 13 and 14. Groups III and IV received Hedyotis diffusa at 150 mg/kg (HD150; n = 4) and 300 mg/kg (HD300; n = 4), respectively, for 14 days by gavage, in addition to carbon tetrachloride at 1.2 mL/kg body weight on days 13 and 14. Hedyotis diffusa was dissolved in distilled water daily prior to oral administration. Carbon tetrachloride was dissolved in 1:1 corn oil. All rats had free access to water. After 24 hr of administration of last dose of carbon tetrachloride, the animals were killed by cervical dislocation, blood and liver samples were collected and stored in ice-cold saline (0.85% w/v sodium chloride) for biochemical and hematological analysis.

Post-mitochondrial Supernatant Preparation

A 1 g liver tissue was homogenized in 10 mL of chilled phosphate buffer (0.1 M, pH 7.4) containing 1.17% (w/v) potassium chloride (KCl) using a homogenizer (Polytron PT 1200E, Switzerland). The homogenate was first centrifuged at 2000 rpm for 10 minutes at 4°C ± 0.5°C using a refrigerated centrifuge (Avanti J-E, Brea, California, USA) to remove nuclear debris. The resulting supernatant was then centrifuged at 10,000 rpm for 30 minutes at 4°C ± 0.5°C to obtain the post-mitochondrial supernatant, which was used to determine malondialdehyde (MDA) and reduced glutathione levels.

Serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Determination

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the method of Reitman and Frankel (1957), with slight modifications. Briefly, 0.5 mL of substrate—

containing 2 mM α-ketoglutarate and 200 mM D,L-aspartate for ALT, or 2 mM α-ketoglutarate and 200 mM D,L-alanine for AST-was mixed with 0.05 mL of serum and 1.55 mL of phosphate buffer (pH 7.5). The mixture was incubated in a water bath at 37°C ± 0.5°C for 15 minutes. To stop the reaction, 0.5 mL of 1 mM 2,4-dinitrophenylhydrazine (DNPH) was added. After 20 minutes, 5 mL of 0.4 N NaOH was added, and absorbance was measured at 510 nm after 30 minutes using a blank as reference.

Lipid Peroxidation (MDA) Determination

Lipid peroxidation in the post-mitochondrial supernatant was evaluated following the method of Buege and Aust (1978), with modifications by Iqbal et al. (1999). One volume of the supernatant was mixed with 0.5 volumes of 10% (w/v) trichloroacetic acid and centrifuged at 8000 × g for 30 minutes at 4°C ± 0.5°C. The resulting supernatant (1 mL) was combined with 1 mL of 0.67% (w/v) thiobarbituric acid and incubated in a boiling water bath for 30 minutes. After cooling in an ice bath, the mixture was centrifuged at 14,000 × g using a refrigerated centrifuge. Absorbance was measured at 535 nm using a spectrophotometer at 37°C ± 0.5°C. Results were expressed as the amount of malondialdehyde (MDA) formed.

Glutathione Reduced (GSH) Determination

Reduced glutathione levels in the liver were determined following the method of Jollow et al. (1974), with modifications. Briefly, 0.1 mL of hepatic post-mitochondrial supernatant (10% w/v) was mixed with 1 mL of 4% (w/v) sulfosalicylic acid and kept at 4°C ± 0.5°C for one hour. The mixture was then centrifuged at 3000 × g for 30 minutes. For analysis, 0.1 mL of the clear supernatant was added to 2.7 mL of phosphate buffer (0.1 M, pH 7.4) and 0.2 mL of DTNB solution (4 mg/mL in 0.1 M phosphate buffer, pH 7.4). Absorbance was measured at 412 nm, and results were expressed as micromoles of reduced glutathione per gram of tissue.

Statistical analysis

All data were obtained from expression of standard deviation (± S.D) from triplicate of number of sample (n = 3). Graph was plotted to determine the the IC_{50} value from standard calibration curve (y= mx + C) and regression (r^2 = 0.999±0.1) from Microsoft excel software.

RESULTS AND DISCUSSION

Total Phenolic Content (TPC)

The phenolic content of *Hedyotis diffusa* was determined from its aqueous extract using a standard calibration curve (y = 0.0057x – 0.0026, r^2 = 0.9999) based on gallic acid. According to the standard curve, 100 g of *Hedyotis diffusa* in aqueous extract contained 90.22 ± 0.10 mg GAE/g (see Table 1).

Table 1 Total phenolic content of *Hedyotis diffusa*

Sample	TPC (mg GAE/g)
Hedyotis diffusa	90.22 ± 0.10

Milligrams of gallic acid weight, mg GAE/g.

Antioxidant Activity (DPPH)

Antioxidant activity of *Hedyotis diffusa* extract evaluated using DPPH assay, which measures the extract's ability to capture free radicals with IC_{50} value of 1540 μg/mL obtained. In this study, the concentration of 2400 μg/mL was prepared and subsequently diluted in a serial manner to a final concentration of 25 μg/mL. Based on this serial dilution, seven concentrations were obtained: 2400, 1200, 600, 300, 150, 75, and 25 μg/mL. Each of these concentrations from the extraction yield of *Hedyotis diffusa* and was dissolved in the presence of DPPH radicals. The same DPPH concentration was used in all treatments of scavenging activity with respective extract concentrations (see Table 2). The relationship between

the plant extract and DPPH radicals was evaluated by measuring absorbance using a spectrophotometer. Fig. 1 illustrates the plant extract effects in the presence of DPPH radicals. The highest point on the graph corresponds to the highest scavenging activity, while the lowest point indicates minimal activity (y-axis). Based on the graph, the scavenging activity of *Hedyotis diffusa* extract increasing with increasing concentration treatment. The maximum activity could reach up to 80% scavenging activity with maximum concentration of 2400 µg/mL than the lowest concentration of 25 µg/mL (5% scavenging activity).

Table 2 Antioxidant activity of *Hedyotis diffusa*

Parameter	Sample	IC_{50} (µg/mL)
DPPH	*Hedyotis diffusa*	1540
	Ascorbic acid	75

Half inhibition concentration, IC_{50}.

Fig. 1 Scavenging activity of *Hedyotis diffusa* extract against DPPH radicals (n = 3).

In vivo Hepatoprotective Activity

Serum Analysis (AST and ALT)

In this study, CCl_4 treatment performed to evaluate the hepatoprotective effects of *Hedyotis diffusa* extract in mitigating liver inflammation, as indicated by changes in ALT and AST levels. Elevated ALT (1.7 μmol/min/dL10^{-3}) and AST (1.5 μmol/min/dL10^{-3}) levels in the animal model are indicative of liver inflammation (*p≤0.05). To address this condition, *Hedyotis diffusa* extract was administered with the objective of reducing ALT and AST levels. Fig. 2 illustrates the effect of *Hedyotis diffusa* extract on serum AST and ALT levels in rats subjected to CCl_4 treatment. The control group exhibited significantly lower serum AST (0.5 μmol/min/dL10^{-3}) and ALT (0.7 μmol/min/dL10^{-3}) levels compared to the CCl_4-treated group, as well as the groups receiving *Hedyotis diffusa* extract at doses of 150 mg/kg (HD150 group) (ALT = 1.1 μmol/min/dL10^{-3} and AST = 1.08 μmol/min/dL$10^{-3;}$ **p≤0.05), and 300 mg/kg (HD300 group) (ALT = 0.9 μmol/min/dL10^{-3} and AST = 0.8 μmol/min/dL$10^{-3;}$ **p≤0.05).

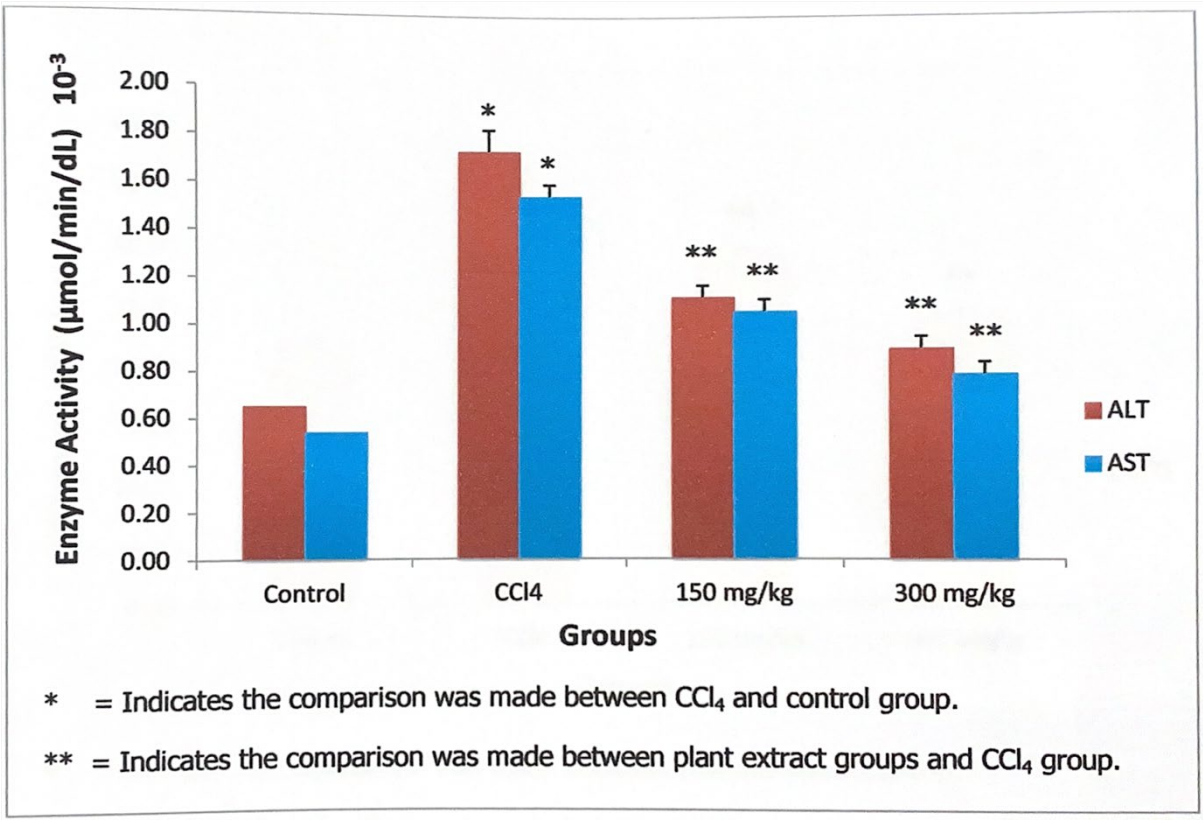

Fig. 2 Effects of Hedyotis diffusa on serum ALT and AST levels in the presence of CCl_4 radicals (n = 4).
* Indicates a statistically significant difference between CCl_4 and control group ($p \leq 0.05$).
** Indicates a statistically significant difference between plant extract groups and CCl_4 group ($p \leq 0.05$).

Liver Analysis (MDA and GSH)

The presence of CCl_4 radicals in rats resulted a significant elevated levels of lipid peroxidation (LOP) and malondialdehyde (MDA) (*$p \leq 0.05$) as shown in figure 3. However, these levels decreased significantly following treatment with *Hedyotis diffusa* extract at doses of 150 mg/kg and 300 mg/kg in the animal model (**$p \leq 0.05$). A higher extract concentration (300 mg/kg) led to a greater reduction in LOP and MDA levels compared to the 150 mg/kg treatment (**$p \leq 0.05$). As shown in the figure 4, hepatic reduced glutathione (GSH) levels were markedly decreased in the CCl_4-treated group compared with the control group (*$p \leq 0.05$), indicating oxidative stress induced by CCl_4 administration. Pretreatment with the *Hedyotis diffusa* extract significantly restored GSH levels in a dose-dependent manner. Animals receiving 150 mg/kg of the *Hedyotis diffusa* extract showed a significant increase in GSH levels compared with the CCl_4 group (**$p \leq 0.05$), while the 300 mg/kg dose produced a more pronounced elevation, bringing GSH levels close to those of the control group (**$p \leq 0.05$ vs. CCl_4). These findings suggest that the *Hedyotis diffusa* extract effectively attenuates CCl_4-induced depletion of GSH, with greater protection observed at the higher dose.

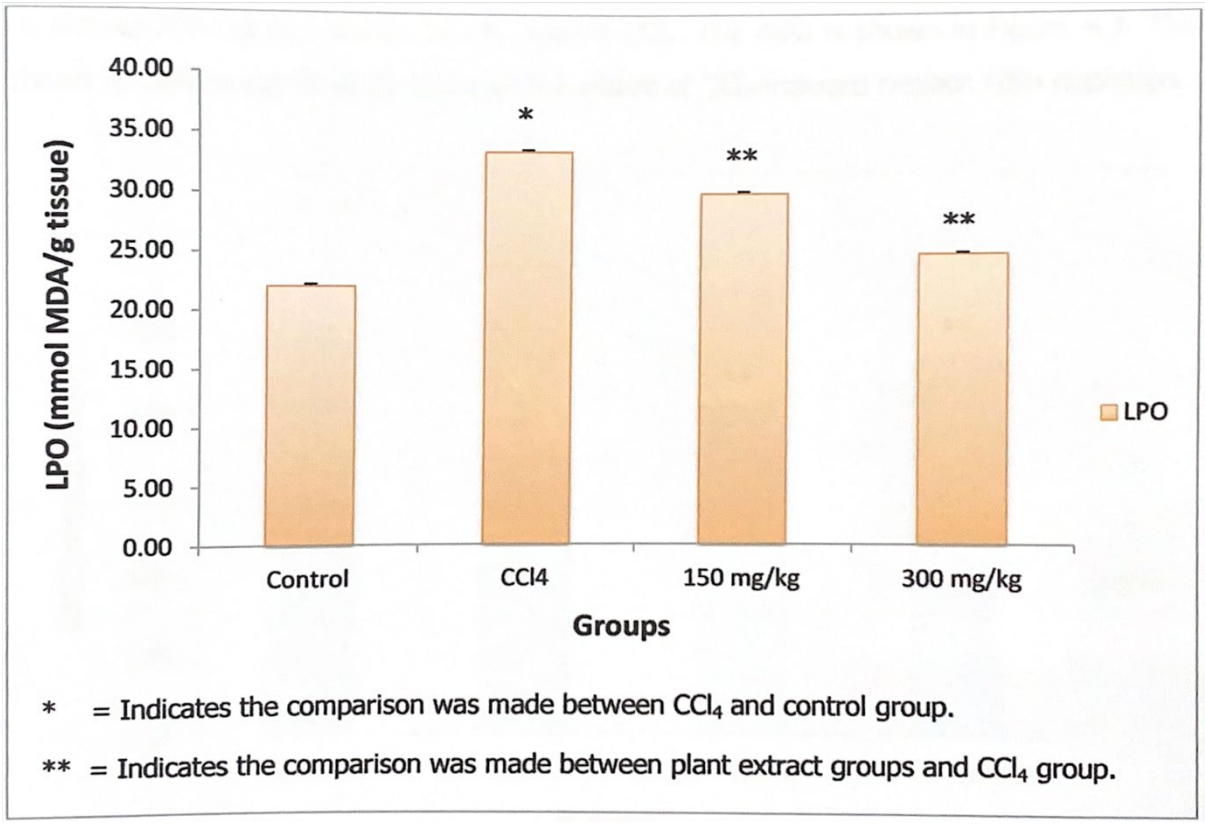

Fig. 3 Effects of *Hedyotis diffusa* on MDA levels in the presence of CCl_4 radicals (n = 4).
* Indicates a statistically significant difference between CCl_4 and control group ($p \leq 0.05$).
** Indicates a statistically significant difference between plant extract groups and CCl_4 group ($p \leq 0.05$).

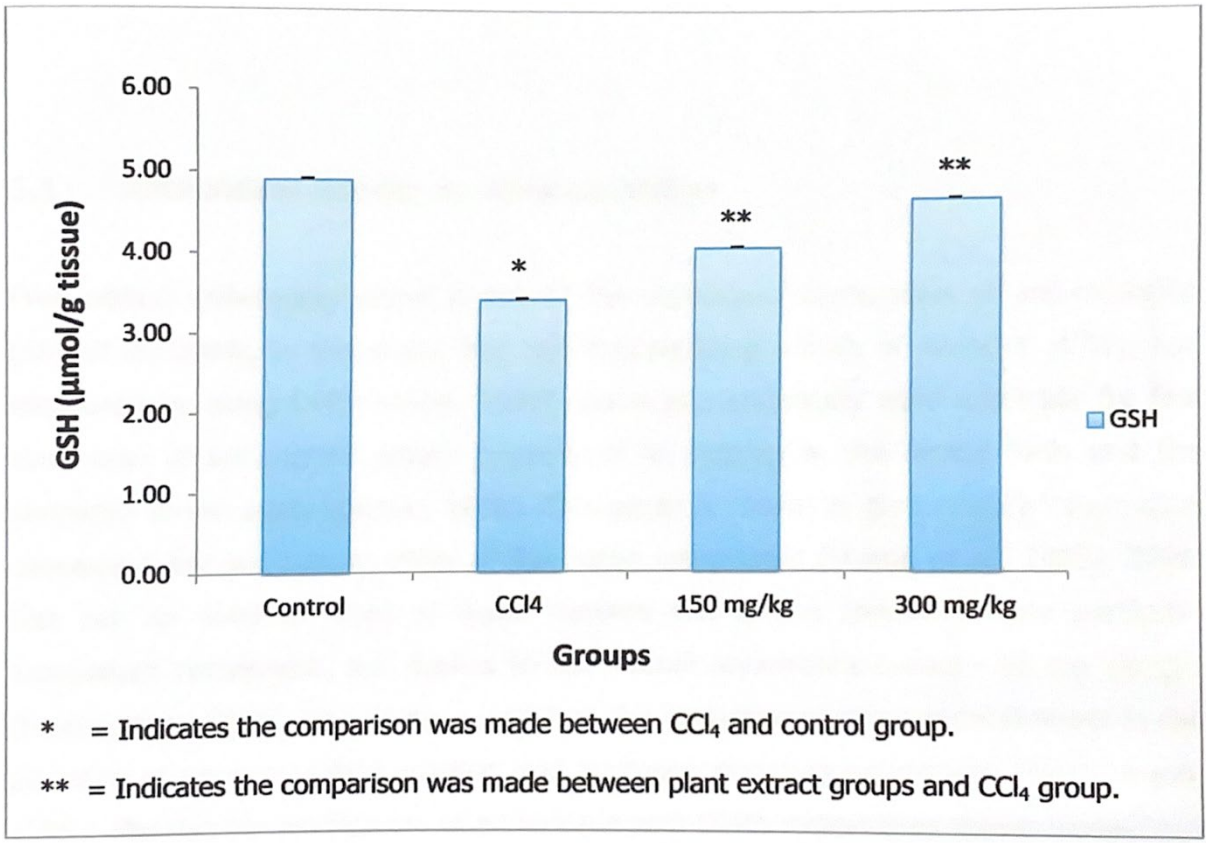

Fig. 4 Effects of *Hedyotis diffusa* on GSH levels in the presence of CCl_4 radicals (n = 4).
* Indicates a statistically significant difference between CCl_4 and control group ($p \leq 0.05$).
** Indicates a statistically significant difference between plant extract groups and CCl_4 group ($p \leq 0.05$).

In this study, the extraction process yielded approximately 0.09% of the initial weight (100 g) using aqueous extraction. The findings suggest that *Hedyotis diffusa* comprises approximately 0.09% phenolic compounds when extracted with a polar solvent such as water. Although the phenolic content appears relatively low, it is higher compared to previous findings. A prior study by Zheng et al. (2024) reported that *Hedyotis diffusa* aqueous extract contained a higher phenolic content than that extracted using 30% ethanol (6.47 ± 1.16 mg GAE/g). The phenolic content reported was lower a recent study by Gull et al. (2024), which used 80% ethanol (52.423 mg GAE/10 mg or 5242.3 mg GAE/g). These comparative studies indicate that solvent polarity and concentration significantly influence the extraction efficiency of phenolic compounds from *Hedyotis diffusa*. Highly polar solvents tend to extract higher amounts of phenolic content, as demonstrated in the present study. Since water is a more polar solvent

than ethanol, reducing the ethanol concentration from 80% to 30% resulted in a decrease in phenolic content. This observation suggests that *Hedyotis diffusa* aqueous extract may contain a high proportion of phenolic hydroxyl groups. The principal detection mechanism is attributed to the reducing power of phenolic hydroxyl groups and their interaction with the phosphotungstic-phosphomolybdic complex (Yu et al., 2000; Singleton et al., 1965).

The DPPH trend observed in the graph suggests a positive correlation between extract concentration and scavenging activity (%). The scavenging activity increased with increasing extract concentration, reaching approximately 80% at 2400 µg/mL of *Hedyotis diffusa* aqueous extract. From these data, the half-maximal inhibitory concentration (IC_{50}) was determined by interpolating the scavenging activity (%) to identify the specific extract concentration (µg/mL) required to inhibit 50% of radical activity. At 50% scavenging activity (y-axis), the IC_{50} value of *Hedyotis diffusa* aqueous extract was found to be approximately 1540 µg/mL, with significantly higher than standard antioxidant, ascorbic acid. As shown in Table 2, ascorbic acid exhibited radical scavenging activity at a minimum concentration as low as 75 µg/mL. In terms of effectiveness, a lower IC_{50} value indicates a more potent radical-scavenging ability. For instance, ascorbic acid required only 75 µg/mL to achieve significant radical scavenging, whereas *Hedyotis diffusa* aqueous extract required over 20-fold higher concentration (1540 µg/mL) for a comparable effect. Based on these results, it was estimated that *Hedyotis diffusa* aqueous extract contained approximately 4.87% essential antioxidant compounds at the IC_{50} value, in comparison to ascorbic acid (assuming 75 µg/mL corresponds to 100% scavenging activity). The IC_{50} value obtained in this study (1540 µg/mL) was higher than that reported in a previous study (268.1 µg/mL) by Zheng et al. (2024), suggesting that *Hedyotis diffusa* in aqueous extract contains phenolic hydroxyl groups with potent scavenging activity. The primary detection mechanism is attributed to the reducing power of phenolic hydroxyl groups and their interaction with the phosphotungstic-phosphomolybdic complex (Yu et al., 2000; Singleton et al., 1965).

Additionally, *Hedyotis diffusa* extract demonstrated the ability to scavenge free radicals and terminate reactions generated by radical chain processes (Duh et al., 1997). According to the mechanism proposed by Larson (1988), antioxidants (AH) donate a hydrogen atom to DPPH radicals, forming a stable, non-radical DPPH-H, as illustrated below.

DPPH + AH → DPPH-H + A

Previously, the potential antioxidants capable of donating a hydrogen atom include cysteine, glutathione, ascorbic acid, tocopherol, and polyhydroxyl aromatic compounds such as pyrogallol and gallic acid (Sini and Devi, 2004).

In the CCl_4-treated group, radical exposure led to an increase in AST and ALT levels above 1.60 μM/min/d (*p≤0.05). However, the administration of *Hedyotis diffusa* extract at 150 mg/kg and 300 mg/kg resulted in a dose-dependent reduction in AST and ALT activity (**p≤0.05).The underlying mechanism involves the formation of trichloromethyl free radicals ($\bullet CCl_3$), which react with oxygen to generate trichloromethyl peroxy radicals ($\bullet CCl_3O_2$). These radicals attack membrane polyunsaturated fatty acids, triggering lipid peroxidation, which in turn leads to liver injury and impaired membrane function (Krishna et al., 2009; Yang et al., 2010). Based on the results, treatment with *Hedyotis diffusa* extract at 150 mg/kg and 300 mg/kg resulted in a reduction of serum AST by 31.8% and 49.4%, respectively (**p≤0.05). while serum ALT levels decreased by 35.8% and 48.6%, respectively (**p≤0.05). The observed decrease in ALT and AST levels with increasing extract concentration suggests that *Hedyotis diffusa* extract exerts a protective effect against liver damage.

Fig. 3 illustrates the inhibitory effect of *Hedyotis diffusa* extract on MDA formation, an indicator of lipid peroxidation induced by CCl_4 radicals (Yang et al., 2010). The results indicate that 150 mg/kg treatment *Hedyotis*

diffusa significantly reduced MDA levels by 10.6%, while the 300 mg/kg *Hedyotis diffusa* treatment achieved a significantly greater reduction of MDA 26.6% in liver tissue (**p≤0.05).. The treated groups exhibited significantly lower MDA levels compared to the CCl_4 group (**p≤0.05)., suggesting that *Hedyotis diffusa* possesses antioxidant properties that mitigate lipid peroxidation by scavenging free radicals. Fig. 4 presents the effect of CCl_4 treatment on glutathione (GSH) levels, along with the impact of *Hedyotis diffusa* extract at 150 mg/kg and 300 mg/kg. The results indicate that a higher dose of 300 mg/kg increased GSH levels by 36.4% in response to CCl_4 exposure, whereas the 150 mg/kg dose led to an 18.8% increase (**p≤0.05). GSH levels serve as a critical biomarker for liver health, as oxidative stress depletes GSH due to its consumption by glutathione-related enzymes, leading to cellular intoxication (Sen et al., 1996). The significant elevation of GSH (**p≤0.05) levels following *Hedyotis diffusa* extract treatment suggests that this plant exhibits hepatoprotective properties by counteracting free radical-induced oxidative damage.

CONCLUSION

Hedyotis diffusa is rich in phenolic compounds with potent antioxidant activity. Polyphenols derived from this plant mitigate the elevated levels of AST and ALT and restore the levels of MDA and GSH, which are associated with hepatoprotective effects. Notably, *Hedyotis diffusa* sourced locally exhibits high scavenging activity (IC_{50} = 1540 µg/mL) and effectively inhibits lipid peroxidation, as demonstrated through both in vitro and in vivo assays. This study provides a valuable reference for the potential discovery of novel hepatoprotective compounds derived from *Hedyotis diffusa* with minimal side effects. Future studies should focus on the bioassay-guided isolation of the specific phenolic compounds responsible for this activity and evaluation of the extract's efficacy in chronic liver disease models.

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AUTHOR CONTRIBUTIONS

Gilbert Ringgit prepared the manuscript. Robin Jacobson Laurduraj conducted the experiment. Associate Professor Dr. Mohammad Iqbal helped in designing the experiment and proofread the manuscript.

DECLARATION OF COMPETING INTEREST

The authors ensure that the work presented in this article is free of financial interests or personal relationships that may have influenced its outcome.

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