EVALUATION OF ANTIOXIDANT PROPERTIES AND TOTAL PHENOLIC CONTENT OF LEAVES EXTRACTS OF *Christia vespertillionis*

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Abstract

Christia vespertilionis, often known as *Rerama* in Malaysia, has long been esteemed for its diverse medicinal uses across various cultures. Realising its importance for medicinal purposes, recent studies have rapidly increased to unlock the potential of *C. vespertilionis* as a source of new alternative natural drugs for medications. In this study, phytochemical screening, antioxidant properties, and the total phenolic content (TPC) were investigated. Three types of crude extracts of *C. vespertilionis* leaves were involved in the assessment: hexane, ethyl acetate (EA), and methanol crude extracts prepared from maceration, filtration, and evaporation. The evaluation of antioxidant properties of all types of extracts was held through semiquantitative dot blot assay and quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity studies. The quantification of TPC was conducted using Folin-Ciocalteu method. Preliminary phytochemicals study showed the presence of alkaloid, terpenoid, tannin, saponin, and phenolic in *C. vespertilionis* leaves. The semiquantitative dot blot assay study revealed methanol extract was the most antioxidative with the lowest concentration to scavenge DPPH radical (0.012 mg/). However, based on the quantitative DPPH radical scavenging activity study, the EA extract was estimated to be more antioxidative with the lowest value of IC_{50} (0.954 µg/mL). Folin-Ciocalteu method suggested that the EA extract of *C. vespertilionis* leaves contained higher TPC value (0.8711 mg GAE/g sample) compared to the other extracts. We conclude that the *C. vespertilionis* leaves have potential to act as an antioxidant agent, in which the findings could serve as basic scientific information for future investigation.

Keywords: Antioxidant activity, *Christia vespertilionis*, phytochemicals, total phenolic content

Introduction

Active compounds such as alkaloids, phenols, and secondary metabolites are recently gaining popularity as medicinal plant sources to function as therapeutic drugs. Bioactive compounds have been studied extensively since they are essential for human health to overcome diseases. Plant-derived drugs have grabbed worldwide attention, especially from pharmaceutical companies, because of their affordability, convenience, and safety (Apu et al., 2012; Burke et al., 2005).

Researchers revealed that the antioxidants of plant origin with free-radical scavenging properties could have enormous importance as therapeutic agents in diseases caused by oxidative stress (Ramchoun et al., 2009). *Christia vespertilionis*, also known as butterfly wing or *Rerama*, has a promising future as an antioxidant agent among the various medicinal plants worldwide. Previous study has shown that *C. vespertilionis* contains bioactive secondary compounds such as phenols, alkaloids, triterpenes, fatty acids, and long-chain alcohols. Additionally, *C. vespertilionis* roots contain flavonoids, coumarins, and quinones, leading to significant results for anti-breast cancer and antioxidants (Hofer et al., 2013).

Antioxidant activity is the ability of a substance to prevent or delay damage to cells caused by unstable molecules called free radicals, which are naturally formed in the body. However, too many free radicals can cause oxidative stress and cellular damage to DNA, proteins, and fats. Generally, antioxidants work by donating electrons to free radicals, neutralising them and finally stopping the chain reaction of damage. Antioxidant activity is often quantified using stable DPPH scavenging activity. This method is beneficial over others since it requires less time to reduce the DPPH form to the DPPH non-radical state through hydrogen atom donation (Dai and Mumper, 2021). The colour shifting from purple to yellow during DPPH assessment was caused by the scavenging of DPPH radical by polyphenols with hydroxyl groups (Dontha, 2016).

Flavonoids and phenolic acids are the most important groups of secondary metabolites. Bioactive compounds in plants serve as a magnificent source of natural antioxidants. They provide security implementations against diseases such as cancer, heart diseases, and gastric problems (Saxena et al., 2012). Therefore, the aim of this investigation is to provide basic scientific information on the antioxidant potential and total phenolic content (TPC) of *C. vespertilionis* leaves extracts.

Materials and Methods

Plant Collection and Extraction

The leaves of *C. vespertilionis* were collected from a local area in Bandar Jengka, Pahang, Malaysia. The leaves were air-dried at room temperature for five days and later were ground into small pieces. The ground leaves (100 g) were macerated consecutively for three weeks using organic solvents such as hexane, ethyl acetate (EA), and methanol in a ratio of 1:3 (sample: solvent) and followed by filtration using Whatman filter paper No. 1. All the filtrates were subjected to evaporation using a rotary evaporator at 40°C to yield three types of crude extracts (Shahrim et al., 2024).

Qualitative analysis: Preliminary phytochemical screening test

The screening tests were conducted to confirm the presence of phytochemicals in *C. vespertilionis* leaves, such as alkaloids, tannins, saponins, steroids, terpenoids, flavonoids, and phenolics. The standard method follows the method from Zambari et al. (2023) with slight modifications.

Alkaloid test

Each extract (3.0 mL) was added with 1.0 mL 1% (v/v) hydrochloric acid (HCl). The mixture was heated for 20 mins, and it was cooled and filtered. After that, two drops of Mayer's reagent were added. The sign of a creamy precipitate indicated the presence of alkaloids.

Tannin test

Each extract (1.0 mL) was added with 1.0 mL of 10% ethanolic potassium hydroxide (KOH). The presence of tannins was confirmed by the appearance of a dirty white precipitate.

Saponin test

Each extract (2.0 mL) was mixed with 2.0 mL of distilled water. The mixture was shaken vigorously and left for 5 mins. Stable frothing indicated the presence of saponins.

Steroid test

Each extract (1.0 mL) was added with five drops of concentrated sulfuric acid (H_2SO_4). The formation of red colouration revealed the presence of steroids.

Terpenoid test

Each extract (1.0 mL) was added with 0.5 mL chloroform and two drops of H_2SO_4. The observation of red brown precipitate gave the sign of terpenoids.

Flavonoid test

Each extract (3.0 mL) was added with 1.0 mL of 10% (w/v) sodium hydroxide (NaOH). The presence of flavonoids was detected from the yellow colouration of the mixture.

Phenolic test

Each extract (1.0 mL) was added with two drops of 5% (w/v) iron chloride ($FeCl_3$). The formation of greenish precipitation indicated the presence of phenolics.

Fourier Transform Infrared (FTIR) Analysis

FTIR analysis is the most useful method for identifying the types of functional groups that are present in the compounds. The analysis is conducted by using an attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Spectrum 100). A small amount of the extract sample was placed in a sample mold. It is necessary to scan the background before the analysis starts. The expected functional groups of *C. vespertilionis* leaves extracts obtained are OH, C=O, C-O, C=C, and C-H groups.

Antioxidant activity assay

Semiquantitative Dot Blot Assay

The dot blot assay technique is one of the easiest methods to evaluate antioxidant properties. In this method, all types of extracts of *C. vespertilionis* were subjected to two-fold serial dilution ranging from 400 mg/mL to 0.012 mg/mL. Each sample was consistently dropped (approximately 20 µL) on a square block of TLC plate with a 1.5 × 1.5 cm dimension. After the drying process, the TLC plates were sprayed with 0.05% (w/v) DPPH in methanol. The appearance of yellow colour on the square block of the TLC plate against a purple background indicated the presence of antioxidant properties of the extracts. The first yellow colour that appeared on the block of the TLC plate indicates the lowest concentration of that extract scavenged the DPPH radical. The method was repeated in triplicate.

Quantitative DPPH Radical Scavenging Assay

All types of extracts of *C. vespertilionis*'s leaves were subjected to a dilution process ranging from 400 µg/mL to 12.5 µg/mL. About 1.0 mL of each concentration was mixed with 3.0 mL of 0.004% DPPH solution. The mixture was gently stirred and stored in a dark place for 30 mins.

After 30 mins, the absorbances of all mixtures and standard ascorbic acid were recorded at 517 nm using a UV/Vis spectrophotometer. The percentages of radical scavenging activity of the extracts were determined as Eq. (1) follows:

$$\%\, radical\ scavenging = \frac{A_0 - A_1}{A_0} \times 100\% \quad (1)$$

where

A_0 = absorbance of the control reaction; A_1 = absorbance in the presence of samples

A bar chart (% radical scavenging activity vs concentration) was plotted to see the trend of antioxidant properties among the extracts. The inhibitory concentration (IC_{50}) values for each extract and the standard solution were calculated based on the linear equation ($y = mx + c$) obtained from the bar chart. This linear equation is used to estimate the inhibition concentration (IC_{50}), as DPPH scavenging data typically follow a non-linear dose–response trend. IC_{50} is the concentration of extract or standard to scavenge 50% of DPPH radical. The lower the IC_{50} value, the stronger the extract ability as an antioxidant agent (Shahrim et al., 2024).

Total Phenolic Content of C. vespertilionis's leaves

The Folin-Ciocalteu method was applied to measure the TPC for all tested extracts of *C. vespertilionis*'s leaves. About 0.5 mL of 1,000 ppm of each extract was measured and reacted with 2.5 mL of Folin-Ciocalteu reagent, which was previously diluted 10 times. After leaving for 2 mins, the reaction mixtures were mixed with 2.0 mL of sodium carbonate (Na_2CO_3) and incubated for 30 mins. Then, their absorbance was determined at 765 nm using the UV/Vis spectrophotometer. A blank sample without an extract was also prepared using the same procedure.

The same procedure was repeated for a set of gallic acid concentrations ranging from 1,000 to 31.25 ppm from a two-fold serial dilution process. A graph (gallic acid concentration (ppm) vs absorbance) was plotted to determine the TPC value using the linear equation. The value of TPC was recorded as mg GAE/g sample (Shahrim et al., 2024).

Results and Discussion

Phytochemical Screening

The phytochemical screening of *C. vespertilionis* has identified a wide variety of bioactive substances that support its characteristics. The main types of phytochemicals were alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds. According to Table 1, a wide range of phytochemicals were efficiently extracted from the EA and methanol extracts. Interestingly, terpenoids were found in the hexane (non-polar) and EA (moderate polar) extracts, but not in the methanol extract (polar). This result suggests that their backbone of terpene structure is non-polar but lacks oxygen-containing functional groups such as hydroxyls (OH), which can increase polarity and water solubility. However, many complex terpenoids with numerous carbon atoms and fewer polar groups remain non-polar and are insoluble in water. This finding would explain why terpenoids are found negative in the methanol extract. Previous study recorded that the polar extract of *C. vespertilionis* leaves showed the presence of alkaloids, flavonoids, glycosides, tannins, diterpenes, coumarin, and quinine (Sumitha & Jain, 2019; Zambari et al., 2023; Idris et al., 2024) and our findings seem consistent with their studies.

Table 1 Phytochemical screening of leaves extracts of *C. vespertilionis*

Phytochemicals	Hexane extract	Ethyl acetate extract	Methanol extract
Alkaloid	Negative	Positive	Positive
Phenolic	Negative	Positive	Positive
Steroid	Negative	Negative	Negative
Terpenoid	Positive	Positive	Negative
Saponin	Negative	Negative	Negative
Tannin	Positive	Positive	Positive
Flavonoid	Negative	Positive	Positive

FTIR Analysis

The FTIR analysis was conducted to investigate the presence of functional groups such as OH, C=C, C-H and C-O in the extracts. The presence of the groups will support the phytochemicals found in the extracts of *C. vespertilionis*. As tabulated in Table 2, the presence of alkaloid was supported by the appearance of C-N stretching at 1376 cm^{-1} and N-H stretching at 3374 cm^{-1}. The absorption peaks of OH stretching (3306–3378 cm^{-1}) proved the presence of phenolic and flavonoid in the extract, as well as the absorption of C=C stretching (1509–1652 cm^{-1}). Therefore, the FTIR analysis supported the phytochemical screening results by showing functional groups corresponding to the detected classes of compounds in the extracts.

Table 2 The functional groups from the leaves of *C. vespertilionis*

Absorption frequency (cm^{-1})	Functional group
3306–3378	OH stretching
1713–1735	C=O stretching
1165–1241	C-O stretching
1507–1652	C=C stretching
3374	N-H stretching
1376	C-N stretching

Antioxidant Activity

Semiquantitative Dot blot assay

A dot blot assay is a simple and rapid technique in chemistry, especially in natural products, to detect the presence of specific molecules, such as antioxidant sources in a sample with the aid of specific reagents, such as DPPH solution. Conceptually, when a solution of DPPH is mixed with any chemical substance that can donate a hydrogen atom, then this gives rise to the reduced form of DPPH-H nonradical species, which gives the colour changes from deep purple to yellow (Dontha, 2016). The decolourisation to yellow colour indicates the reduction of DPPH to DPPH-H nonradical by the antioxidant substance.

Table 3 depicts the lowest concentration of each extract to scavenge the DPPH radical. Antioxidants are known as chemical sources that have the ability to stop oxidation reactions in body cells by removing free radicals and decreasing oxidative stress. The reaction of free radicals in body cells may lead to cell damage and homeostatic disruption. Therefore, enough antioxidants can remove harmful free radicals that cause the body to be exposed to harmful diseases. Natural sources such as *C. vespertilionis* can be utilised as an alternative antioxidant source.

According to Table 3, the choice of solvent used for extraction significantly affects the types of phytochemicals that can be extracted, particularly those with antioxidant properties. Among the three extracts, the methanol extract has become the most antioxidative extract with the lowest concentration of 0.012 mg/mL to scavenge DPPH radical, followed by the EA extract, and the least antioxidative is the hexane extract. The presence of phenolic and flavonoid in the methanol extract might possibly be responsible for enhancing its antioxidant properties. The result is consistent with the previous dot blot assay result, which revealed the methanol extract was more antioxidative (Harun et al., 2022; Harun et al., 2023).

Figure 1 depicts the TLC chromatogram dot blot assay of all extracts and standard ascorbic acid, in which the emergence of yellow colour against a purple background indicates the antioxidant properties.

Table 3 The lowest concentration of leaves extracts of *C. vespertilionis* to scavenge DPPH radical in dot blot assay

Extract/standard	Concentration, mg/mL
Hexane	0.390
Ethyl acetate	0.190
Methanol	0.012
Ascorbic acid (standard)	< 0.012

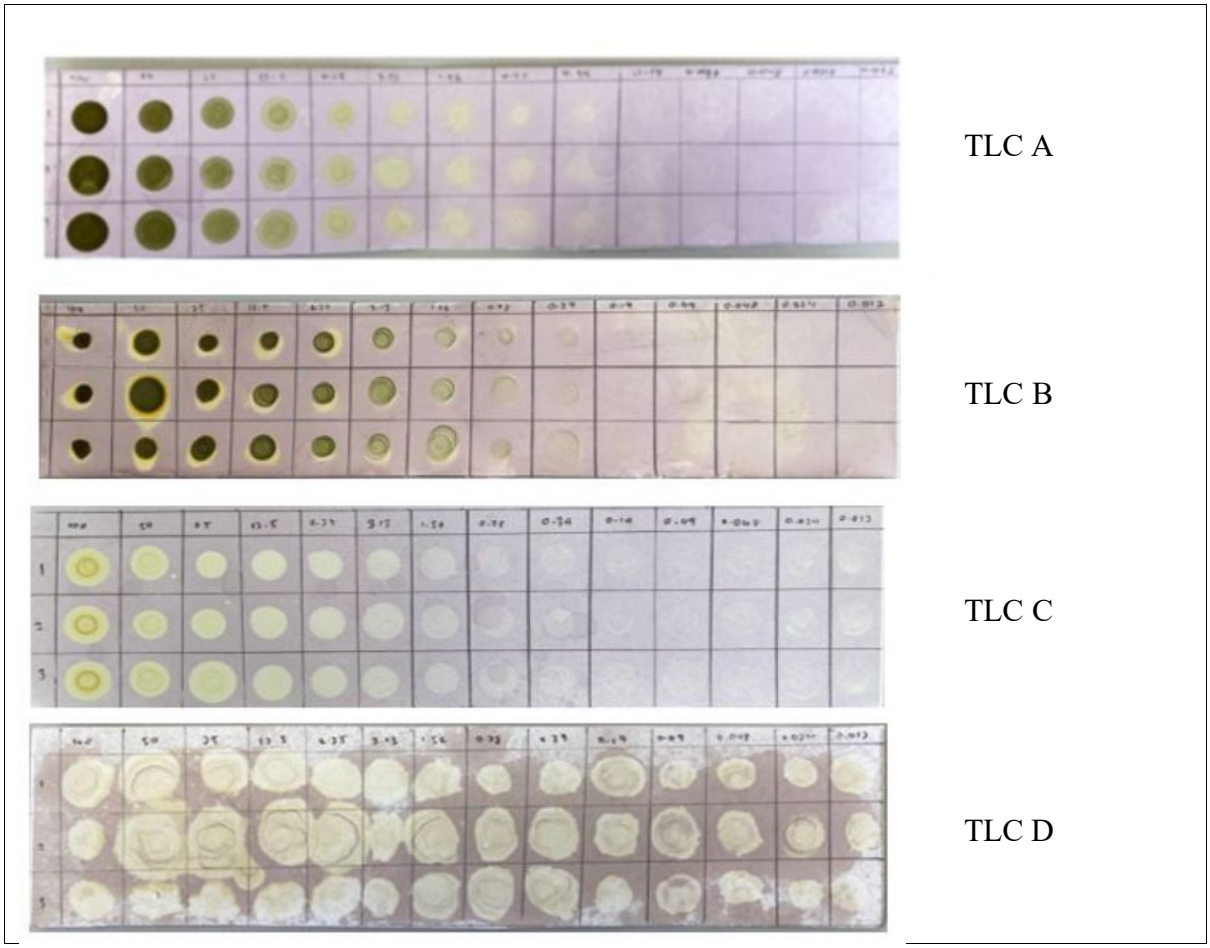

Figure 1 TLC chromatogram dot blot assay of leaves extracts of *C. vespertilionis.* TLC A: Chromatogram dot blot assay of hexane extract; TLC B: Chromatogram dot blot assay of ethyl acetate extract; TLC C: Chromatogram dot blot assay of methanol extract; TLC D: Chromatogram dot blot assay of standard ascorbic acid

Quantitative DPPH Radical Scavenging activity

The quantitative antioxidant activity is generally represented as the inhibition concentration (IC_{50}). The IC_{50} value indicates the concentration of samples scavenged 50% of DPPH radicals. The lower the IC_{50} value of a certain sample or extract, the stronger the antioxidant activity of the radical scavenger and vice versa (Daud et al., 2022). Based on Table 4, the most antioxidative extract is the EA extract, as it possesses the lowest IC_{50} value (0.954 µg/mL). The values of IC_{50} of all extracts is not polarity dependent since the methanol extract has shown a higher IC_{50} value, although it is more polar than the other extracts. Our result, which reveals that the EA extract is more antioxidative, is consistent with a previous result that reported the EA extract of *C. vespertilionis*'s root has the greatest level of radical scavenging activity (Lee et al., 2020). According to Table 1, the phytochemicals such as phenolic and flavonoid may be responsible for the antioxidant properties of the EA extract, as well as terpenoid and tannin in the hexane extract.

Figure 2 shows the trend of the percentage of scavenging activity in a concentration-dependent manner for all extracts. The greater the concentration, the higher the percentage of DPPH scavenging. Generally, concentrated form reflects to a higher concentration of phytochemicals and shows more effect on scavenging activity compared with lower concentration.

Conceptually, when a DPPH solution was mixed with a solution of an antioxidant substance, the DPPH radical will be reduced by the antioxidant (H-A) to form DPPH-H, in which the decolourisation from purple to yellow colour will occur after reduction. The simple reduction equation is as Eq. (2) follows:

$$\underset{\text{(purple)}}{\text{DPPH}} + \text{H-A} \rightarrow \underset{\text{(yellow)}}{\text{DPPH-H}} + \text{A} \qquad (2)$$

According to Eq. (2), when DPPH radical reacts with antioxidant molecules (H-A), the antioxidant molecule donates its proton to DPPH molecules to become DPPH-H molecules (nonradical) and as a result, the purple colour decolourises to yellow. The more the decolourisation, the more the reducing ability (Dontha et al., 2016). In our case, the phenolic and flavonoid found in the extracts may act as reducing agents and are antioxidant sources as well as DPPH radical scavengers. Regarding their molecular structures, the hydroxyl (OH) groups that covalently bonded to the base structure of phenolics and flavonoids would possibly be the reason for the reduction activity towards DPPH radical. It was reported that the position of hydroxyl groups and other chemical features affects the antioxidant and free radical scavenging activities (Saxena et al., 2012).

Table 4 Quantitative antioxidant activity of extracts of *C. vespertilionis* through DPPH radical scavenging method

Extracts/standard	IC_{50} (µg/mL)
Hexane	1.626
Ethyl acetate	0.954
Methanol	2.134
Standard ascorbic acid	1.019

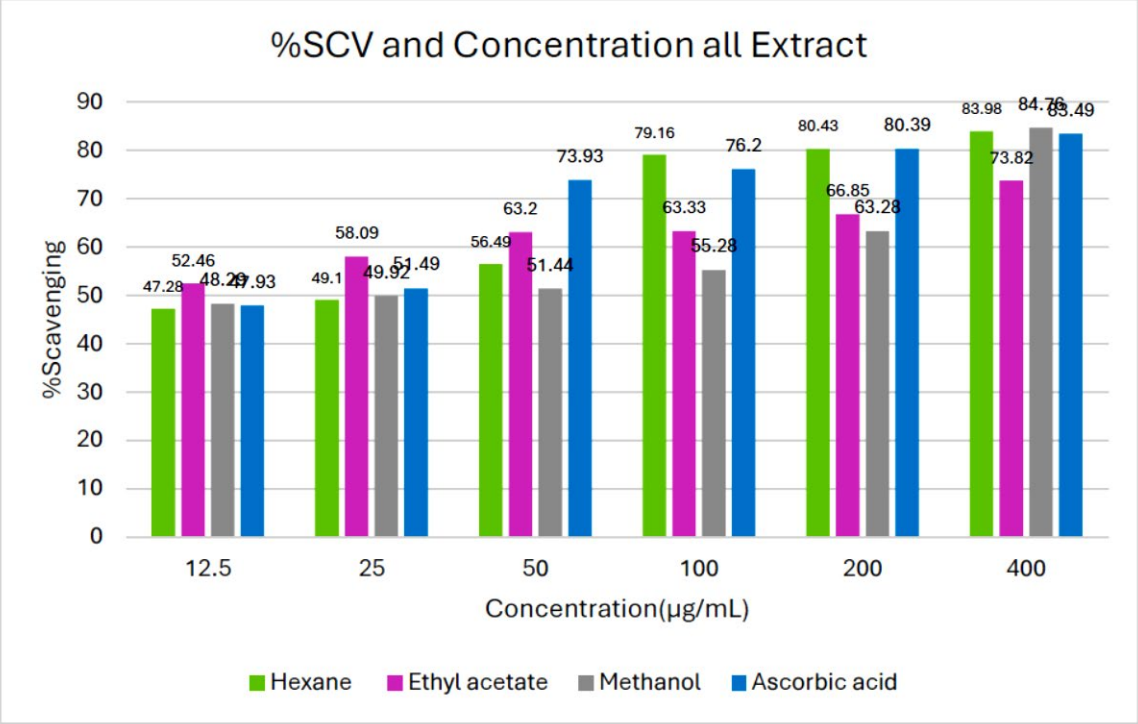

Figure 2 Radial scavenging activity of leaves extracts of *C. vespertilionis*

Total Phenolic Content (TPC)

Generally, TPC indicates the total amount of phenolic compounds in a sample extract using a specific reagent such as Folin-Ciocalteu, in which this reagent reacts with phenolic compounds in a sample to form a blue-coloured complex that can be measured by a spectrophotometer. Qualitatively, the more intense of the blue colour of the solution, the higher the content of the phenol in the sample. Based on Table 5, the medium polar extracts exhibited the highest TPC (0.8711 mg GAE/g sample) compared to the hexane extract (non-polar). However, the values of TPC between the EA and methanol extracts are not significant. Therefore, it is suggested that the value of TPC becomes higher in medium and polar extracts. This result is consistent with a previous study by Zambari et al. (2023) who reported that the polar extract of green *C. vespertilionis* has a higher TPC value of 29.25 mg GAE/mL sample.

Table 5 Total Phenolic Content (TPC) of leaves extracts of *C. vespertilionis*

Extracts	TPC, mg GAE/g sample
Hexane	0.4088
Ethyl acetate	0.8711
Methanol	0.7344

Conclusion

The qualitative analysis confirmed that the *C. vespertilionis* leaves extracts contain alkaloids, tannins, phenolics, terpenoids, and flavonoids. The expected functional groups present in the extracts' phytochemicals are OH, C=O, C-H, C=C, C-O, N-H, and C-N stretchings. Based on the dot blot assay, the methanol extract shows the lowest concentration to scavenge DPPH radical (0.012 mg/mL). the radical scavenging activity revealed that the EA extract was the most antioxidative with an IC_{50} value of 0.954 µg/mL. The EA extract exhibited the highest TPC value (0.8711 mg GAE/g sample). This result suggested that there is a strong relationship between the antioxidant activity and its TPC.

Ethics Statement

The research does not require research ethics approval.

Authors' Contribution

"Writing – Original draft preparation, Rosli, M.H.I; Literature Review, Daud, S; Methodology, Abdul Aziz, N; Writing – Review and editing, Harun, A."

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Conflict of interests

The authors declare that there is no conflict of interest regarding publication of this paper.

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