

COMPARATIVE STUDY OF ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF STEM BARK AND LEAVES FROM *Murraya koenigii* species

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Abstract

Natural products with medicinal value are gaining popularity because they have fewer side effects than synthetic drugs. Hence, this study aims to investigate the antioxidant potential and its total phenolic content in the leaves and stem bark of *Murraya Koenigii* species, which were collected from Taman Herba UiTM Jengka Pahang. The goal is to assess antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) through dot blot assay and radical scavenging assay. The total phenolic content (TPC) of leaves and stems of *M. koenigii* was measured using the Folin-Ciocalteu method. The crude extracts from the stem bark and leaves of *M. koenigii* were prepared through consecutive maceration using hexane, ethyl acetate, and methanol. The results revealed that the ethyl acetate extract from stem bark and the methanol extract of leaves had promising antioxidant activity, with IC₅₀ values of 77.67 µg/mL and 68.84 µg/mL, respectively. Meanwhile, the dot blot assay analysis indicated that hexane and methanol extracts from the leaves exhibited antioxidant activity at the lowest concentration of 0.048 mg/mL. The methanol extract from stem bark revealed its antioxidant properties through a dot blot assay at a concentration of 0.39 mg/mL. The highest total phenolic content (TPC) values belonged to the methanol extract of stem bark and leaves, with values of 114.25 mg GAE/g sample and 153.25 mg GAE/g sample, respectively. Therefore, the methanol extract from the leaves of *M. koenigii* is known to be the most promising source of the antioxidant agent, with a high TPC value compared to other extracts. This research's findings are applicable to the medical and pharmaceutical industries as they provide the fundamental scientific data essential for developing new natural drug sources.

Keywords: Antioxidant activity, total phenolic content, *Murraya koenigii*

Introduction

Antioxidants are substances that can neutralise free radicals, and their contribution is to prevent our body cells from being damaged, which allows them to counteract oxidative stress (Jain et al., 2019). Oxidative stress is a phenomenon that causes various diseases, including chronic inflammatory disorders, Alzheimer's, acute inflammatory complications, cataracts, cancer, and other age-related diseases (Balakrishnan et al., 2020). *Murraya koenigii* is a precious medicinal plant with vast concentrations of secondary metabolites (Kandimalla et al., 2020). Some of the common and important phytochemicals that were found in the leaves, roots, stem bark, seeds, and fruits of *M. koenigii* were alkaloids, flavonoids, terpenoids, and

polyphenols (Balakrishnan *et al.*, 2020). However, the main phytochemical substances that can be identified in *M. koenigii* belong to phenolic compounds. The hydroxyl group is the main functional group of phenolic compounds to react with free radicals, and this group is acting as a driving force behind the antioxidant activity of the substance (Aryal *et al.*, 2019).

Natural antioxidants are gaining popularity because the safety aspect is much more important, while artificial antioxidants can cause some adverse health effects on humans. Generally, different parts of plants may contain different amounts of phenolic compounds, which are recognised as powerful antioxidants (Lourenco *et al.*, 2019). Therefore, there is a need to conduct the study of antioxidant properties and total phenolic content (TPC) in both the leaves and stem bark of *M. koenigii*. The result may offer basic scientific information about the different total phenolic content between leaves and stem bark as well as its antioxidant properties.

Materials and Methods

Plant extraction

M. koenigii stem bark and leaves were collected from Taman Herba UiTM Jengka in Pahang, Malaysia. Each sample was air-dried at room temperature for five days and then finely powdered using a grinder. The fine powder was weighed, yielding 156.2 g for the stem bark and 169.5 g for the leaves. Each was macerated consecutively using three types of solvents, which are hexane, ethyl acetate (EA), and methanol, to obtain three types of extracts after filtration. All the filtrates were subjected to evaporation to yield three types of crude extracts.

Phytochemical Screening

All extracts from the stem bark and leaves of *M. koenigii* were subjected to TLC analysis and development using certain developing solvents to screen for phytochemicals such as alkaloids, phenolics, and terpenoids. After the development process, the TLC sheets were sprayed with Dragendorff's reagent, ferric chloride, and vanillin/H₂SO₄ reagent solely to screen the respective phytochemicals.

Semi-Quantitative Dot Blot Assay

In the dot blot assay, the DPPH staining method was used to measure antioxidant activity in a sample by observing the lowest concentration of *M. koenigii* to reduce the purple colour of DPPH to yellow. Each extract, which was twofold serially diluted with concentrations ranging from 100 mg/ mL to 0.024 mg/ mL, was dropped onto TLC that contained a series of square blocks with a dimension of 1.5 x 1.5 cm. After the dropped sample was dried, it was sprayed with a solution of 0.05% DPPH in methanol. A yellow spot against a purple background was observed to assess the plant's antioxidant potential (Harun *et al.*, 2016). The test was conducted in triplicate.

DPPH radical Scavenging activity

The DPPH radical scavenging assay was utilised to evaluate the antioxidant activity level in each crude extract. Generally, when the DPPH reacts with the antioxidant in the crude extract, the purple colour is reduced. A series of concentrations ranging from 400 µg/mL to 12.5 µg/mL were prepared, and about 3 mL of 0.004% DPPH solution was added. Each

mixture was left in the dark for 30 minutes to initiate the scavenging reaction. After 30 minutes, the absorbance of all samples at 517 nm was recorded using a spectrophotometer. The experiment repeated the standard ascorbic acid reaction in triplicate.

The radical scavenging activity of the sample was measured as a percentage of the sample's ability to inhibit free radicals. It was determined by applying the given equation to the data (Daud *et al.*, 2022).

$$\text{Scavenging activity (\%)} = A_0 - A_1 / A_0 \times 100$$

where A_0 represents the DPPH control absorbance and A_1 represents the extracted/standard sample absorbance.

Determination of total phenolic content

The total phenolic contents of *M. koenigii* crude extracts from stem bark and leaves were determined using the Folin-Ciocalteu method, which utilises gallic acid as a reference and the Folin-Ciocalteu (FC) reagent as an oxidising agent. A measurement of 0.5 mL of 1,000 ppm of each extract was added along with 2.5 mL of FC reagent (5 mL of FC mixed with 45 mL of distilled water) and 2 mL of 7.5% Na_2CO_3 solution. All mixtures were kept in a dark place for 30 minutes for incubation. After incubation time, the samples were subjected to spectroscopic analysis using a UV spectrophotometer at 765 nm.

The method was repeated for the standard gallic acid solution with different concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 ppm for standard curve development. The total phenolic content was expressed in milligrams of gallic acid equivalent per gram of sample (mg GAE/g sample) (Daud *et al.*, 2022).

Results and Discussion

Phytochemical Screening

Table 1 Phytochemicals from the extracts of stem bark and leaves part of *M. koenigii*

Type of extract	Phytochemicals		
	Phenolic	Terpenoid	Alkaloid
Stem Bark			
Hexane	√	√	x
Ethyl acetate	√	x	x
Methanol*	√	x	x
Leaves			
Hexane*	√	√	√
Ethyl acetate*	√	√	x
Methanol*	√	x	x

√ = present; x = not present; * = antioxidative (yellow color) when sprayed with 0.6% DPPH

Screening for some of most important phytochemicals, such as alkaloids, terpenoids, phenols, and saponins is vital because they might contribute to antioxidant activity. Research from Lorenzo and Munekata (2016) reported that 23 green tea phenolics might assist in treating neurological disorders caused by oxidative stress, such as Parkinson's and Alzheimer's. Low-molecular, lipophilic terpenoids are an appealing group of secondary plant

metabolites that may prevent and treat Alzheimer's (Wojtunik-Kulesza *et al.*, 2017). **Table 1** revealed that the extracts from the leaves were screened to be more antioxidative compared to the extracts from the stem bark, and the antioxidant properties of the leaves was contributed by the phenolic and terpenoid compounds.

Antioxidant activity

a. Dot blot assay

The antioxidant activity of *M. koenigii* crudes was measured semi-quantitatively using a dot blot assay, and the analysis was performed only for crude extracts that were qualitatively screened to have antioxidative properties (refer to **Table 1**). As depicted in **Table 1**, all three types of crude extracts from the leaves and only the methanol extract of the stem bark revealed antioxidant properties. The absence of any yellow colour on TLC of ethyl acetate and hexane extracts of stem bark indicates that they are not antioxidative.

As illustrated in **Figure 1**, the antioxidant activity of each of the four crude extracts, including the standard ascorbic acid, was assessed by observing the appearance of yellow colour against the purple background on TLC, where radical-scavenger capacity was present. Any extract that gives the lowest concentration to inhibit DPPH was known as a good scavenger since it scavenged DPPH radicals at low concentration.

Table 2 Antioxidant activity of the extracts of *M. koenigii* through Dot Blot Assay

Extract/standard	Lowest concentration of DPPH Inhibition, mg/ mL
Methanol (stem bark)	0.390
Hexane (leaves)	0.048
Ethyl acetate (leaves)	0.097
Methanol (leaves)	0.048
Standard ascorbic acid	0.024

According to **Table 2** and **Figure 1**, the lowest concentration to scavenge DPPH radicals was 0.048 mg/ mL, which was obtained from hexane and methanol extracts of the leaves. It is noticeable that the leaf part is more antioxidant than stem bark. The result is consistent with a previous investigation, which reported that the hexane extract of *M. koenigii* leaves contained terpenoids, a nonpolar secondary metabolite; since hexane is a nonpolar solvent, secondary metabolites extracted by hexane should also be nonpolar, as there should be no alkaloids or phenol in the hexane extract (Amna *et al.*, 2019). The ethyl acetate extract from the leaves has also shown a lower concentration of 0.097 mg/ mL compared to the methanol extract from the stem bark. The presence of phytochemicals such as phenolic and terpenoid compounds in the leaves are responsible for its antioxidant activity.

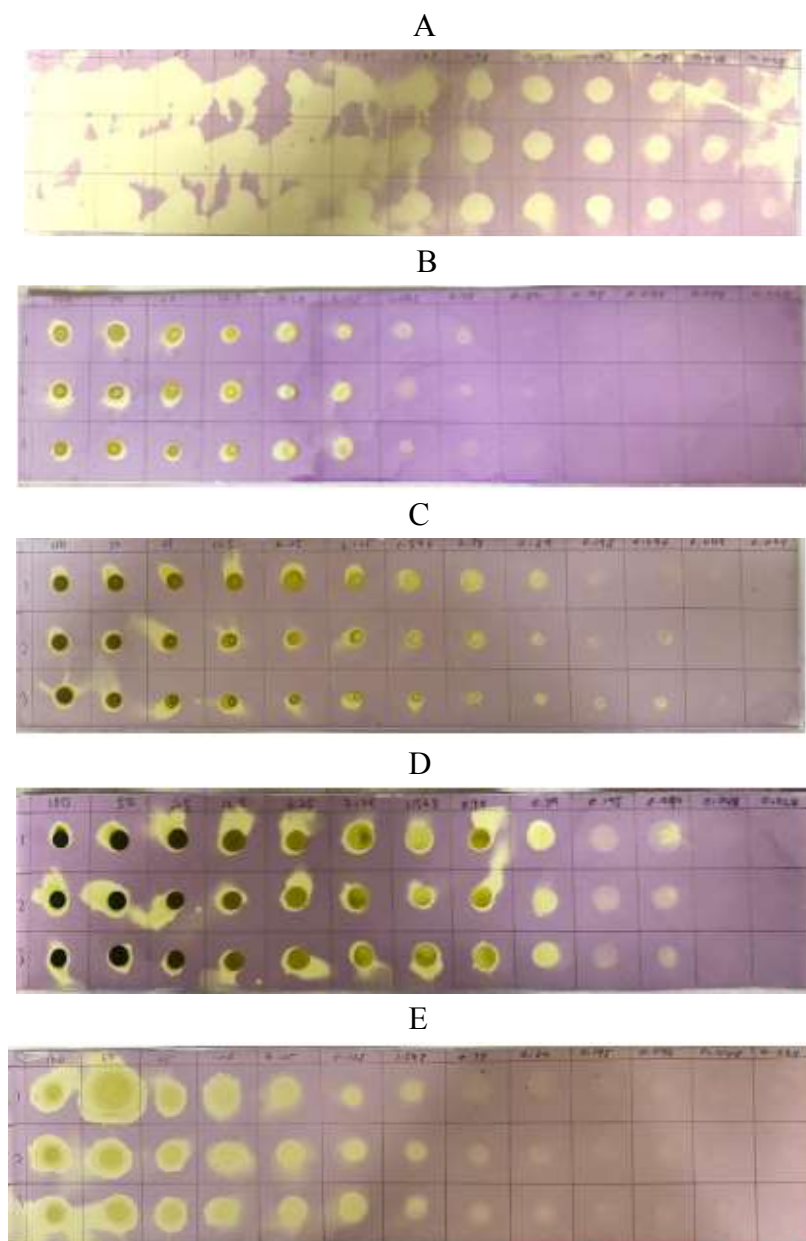


Figure 1 Chromatograms of dot blot assay.

- A: Chromatogram using standard ascorbic acid
- B: Chromatogram using the methanol extract from the stem bark of *M. koenigii*
- C: Chromatogram using the hexane extract from the leaves of *M. koenigii*
- D: Chromatogram using the ethyl acetate extract from the leaves of *M. koenigii*
- E: Chromatogram using the methanol extract from the leaves of *M. koenigii*

b. Quantitative DPPH Radical Scavenging activity

The DPPH radical scavenging assay is based on the ability of antioxidants to decolourise DPPH, and each crude extract's absorbance was measured using a UV/Vis spectrophotometer. The graph of the percentage of scavenging against concentration was used to calculate the IC₅₀ values. Conceptually, the DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm, where the changes in absorbance can be quantitatively

measured when antioxidants donate an electron to DPPH and decolourise it. **Table 3** shows the IC₅₀ values of radical scavenging activity for all six crude extracts and standard ascorbic acid. **Figures 2** and **3** depict the trend of scavenging activity with different concentrations of extract.

Table 3 Quantitative antioxidant activity of extracts of *M. koenigii* through DPPH radical scavenging activity

Extracts/standard	IC ₅₀ , µg/ mL	
	Stem bark	Leaves
Hexane	350.22	328.00
Ethyl acetate	77.67	246.97
Methanol	191.81	68.84
Ascorbic acid	7.07	7.07

The lower the IC₅₀ value, the better the extract's ability to scavenge 50% of the DPPH radical, indicating that it has stronger antioxidant activity, and vice versa (Daud *et al.*, 2022). In this assay, the extract was compared to standard ascorbic acid, as it is a potent antioxidant and free radical scavenger with the lowest IC₅₀ value. (Daud *et al.*, 2022; Ahmad, 2014). The trend of IC₅₀ for both parts of *M. koenigii* is not the same. According to its stem bark, the ethyl acetate extract is the most antioxidative with IC₅₀ values of 77.67 µg/mL, while the methanol extract from the leaves is the most antioxidative with IC₅₀ values of 68.84 µg/mL, but when compared between the two parts, the methanol extract from the leaves scavenged 50% more DPPH radicals than the stem bark.

Stem bark ethyl acetate extract was previously screened not to be antioxidative when its TLC was sprayed with 0.6% DPPH, but interestingly, it showed its antioxidant properties from quantitative radical scavenging activity. This result could be due to the presence of phenolic compounds, which gives a positive result in TLC screening when sprayed with FeCl₃ reagent.

The result of IC₅₀ for the leaf methanol extract is consistent with the preliminary analysis on TLC screening, and it is confirmed to have remarkable antioxidant activity due to the presence of phenolic compounds. Abeysinghe *et al.*, (2021) discovered that leaves of *M. koenigii* methanolic extract are rich in polyphenol content, which contribute to higher antioxidant activity, and other phytochemicals, such as terpenes, are also known to be the major component in *M. koenigii*. Another previous investigation reported methanolic extracts of *M. koenigii* had the highest yield (1.65%), 38.60 mg TAE/100 g fresh weight of total phenolic content, and 70.60% antioxidant activity using the FTC model when compared to all other local plant methanolic extracts such as *Cosmos caudatus*, *Polygonum minus*, *Oenanthe javanica*, and *Centella asiatica* (Huda-Faujan *et al.*, 2009).

Radical scavenging activity of *M. koenigii* stem bark extracts

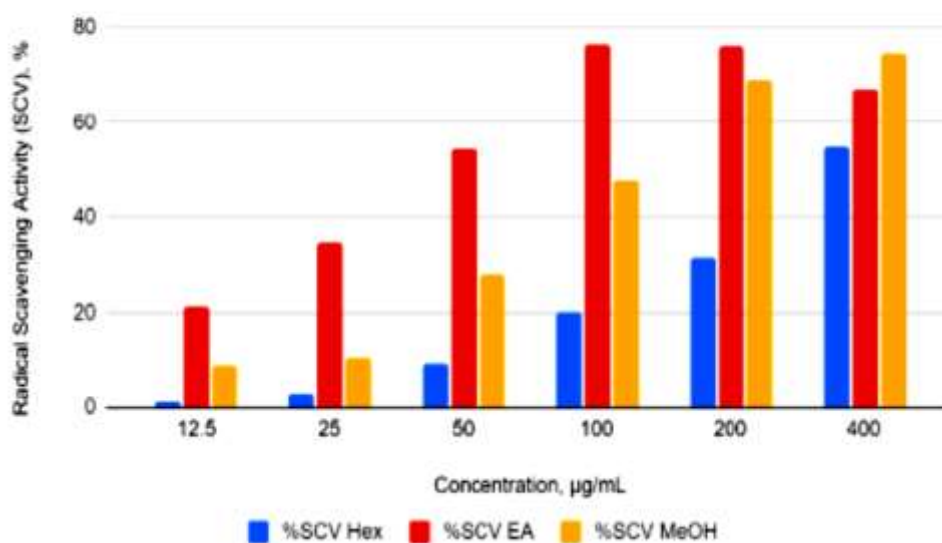


Figure 2 Radical scavenging activity of *M. koenigii* stem bark extracts

Figure 2 illustrates the radical scavenging activity of all stem bark extracts, which react in a concentration-dependent manner except for the ethyl acetate extract, in which its activity reduces slightly at 400 µg/mL. The higher the concentration of each extract, the greater the radical scavenging activity. Higher concentrations correspond to the extract's greater ability to donate hydrogen, which increased the conversion of the DPPH solution from its typical deep purple colour to yellow diphenylpicryl hydrazine (Daud *et al.*, 2022). According to **Figure 2**, the ethyl acetate extract is more active for each concentration used until 200 µg/mL but at 400 µg/mL, the hexane and methanol extracts dominate the radical scavenging activity with 54.6% and 74.3%, respectively. The best scavenging activity for the leaf extract is 76% at 200 µg/mL. The ethyl acetate extract dominates in the percentage radical scavenging activity from 12.5 µg/mL to 200 µg/mL, followed by methanol and hexane extracts, indicating that the ethyl acetate extract is the best antioxidant scavenger. Previous research has reported that the stem bark ethyl acetate extract has more DPPH radical scavenging effects than stem bark hexane (Ng *et al.*, 2018). Methanol extract has the highest percentage inhibition at 400 µg/mL concentration. Ng *et al.*, (2016) found that, using the DPPH assay, the methanol extract of *M. koenigii* stem bark yielded promising results with values of 432 mg TE/g, followed by chloroform, ethyl acetate, and hexane extract. Therefore, the current results are consistent with the previous investigation.

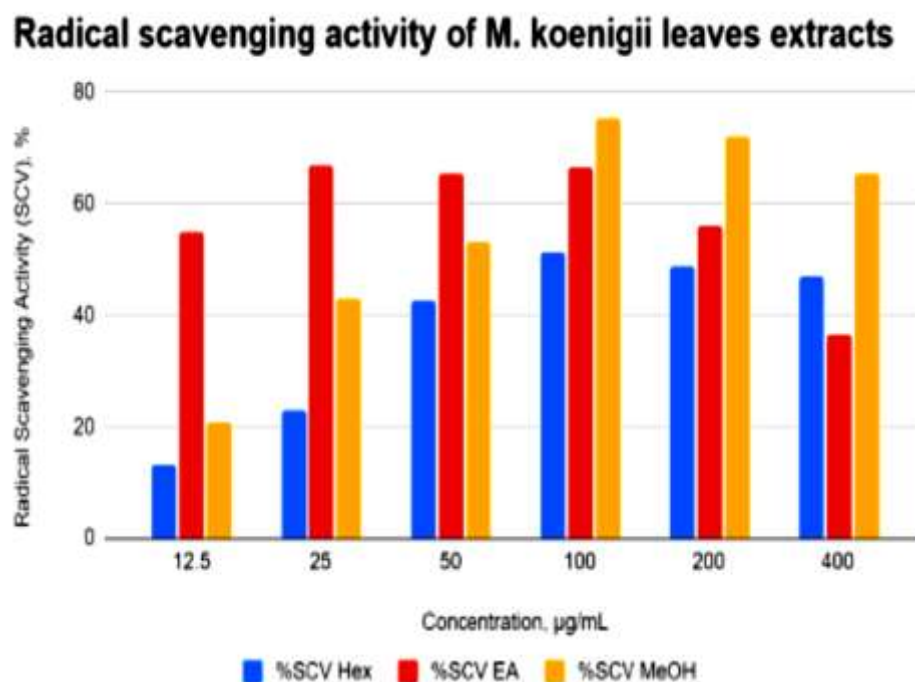


Figure 3 Radical scavenging activity of *M. koenigii* leaves extract

The trend of radical scavenging activity of leaf extracts was unique, as shown in **Figure 3**. Generally, the radical scavenging activity of all extracts increases as concentration increases up to 100 µg/mL but slightly decreases for hexane extract until 400 µg/mL and moderately decreases for ethyl acetate and methanol extracts. Ethyl acetate extract seemed to dominate the activity at all concentrations except at 400 µg/mL.

In a previous study, the percentage of scavenging activity of an ethyl acetate extract of *M. koenigii* leaves was around 56% at 50 µg/mL concentration, which is consistent with our findings at 54.6% (Zahin *et al.*, 2013). However, at 400 µg/mL, the activity of the ethyl acetate extract decreases, and the methanol extract was found to be the best scavenger. It was reported that among all extracts (methanol, ethanol, and essential oil), the methanol extract at 400 µg/mL had the highest free radical scavenging potential of 67.8% (Rajnikant *et al.*, 2015). However, when compared to the BHT standard (EC₅₀-0.59 mg), crude methanolic extract demonstrated the highest DPPH scavenging activity (EC₅₀-0.187 mg) (Mishra *et al.*, 2009).

Total Phenolic Content (TPC)

The Folin-Ciocalteu (FC) reaction is an antioxidant assay based on electron transfer that measures an antioxidant's reductive capacity (Lamuela-Raventós, 2017). The formation of complex blue compounds can be seen in the presence of phenols, which absorb at 765 nm (Everette *et al.*, 2010). Folin-Ciocalteu reagent oxidises phenol groups to reduce heteropoly acid contained in FC reagent into a molybdenum-tungsten complex, and Na₂CO₃ was added to support it in an alkaline atmosphere, causing protons in phenolic compounds to dissociate into phenolic ions (Martono *et al.*, 2019). The TPC value was calculated using the gallic acid standard curve, as illustrated in **Figure 4**.

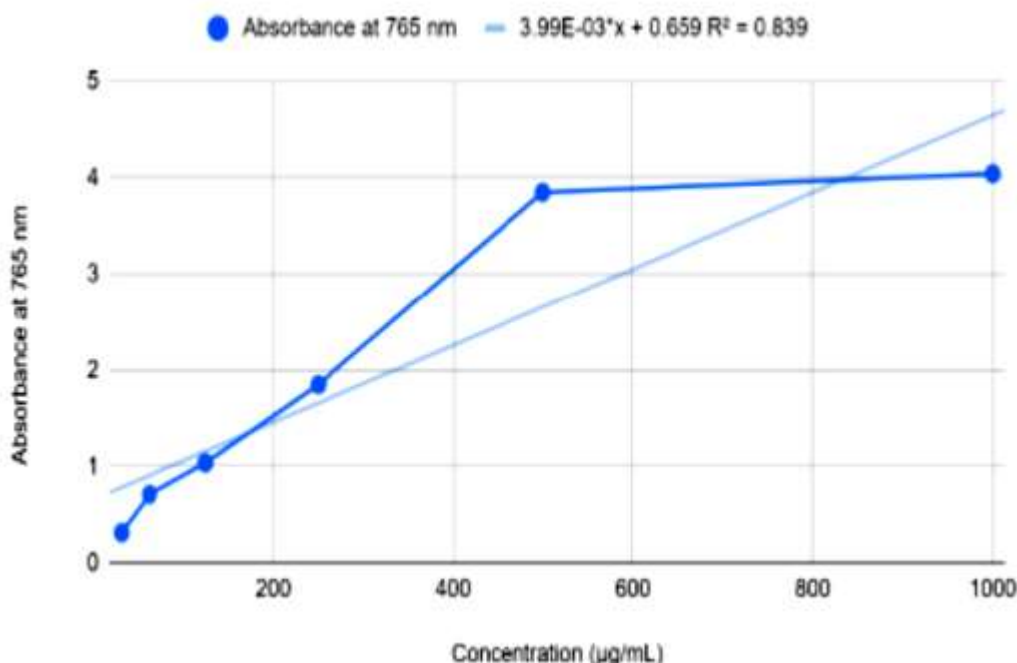


Figure 4 Gallic acid standard curve

Table 3 depicts the TPC value of all crude extracts of *M. koenigii* stem bark and leaves. Methanol extract was calculated to have a higher TPC value for both stem bark and leaves, which are 114.25 mg GAE/g sample and 153.25 mg GAE/g sample, respectively. When comparing the TPC values between both parts, the leaves' are richer in phenolic compounds compared to the stem bark. Therefore, the higher the value of TPC in leaves, the higher the antioxidant properties of the leaves. The value of TPC found in the methanol extract is comparable with the previous study, in which the TPC value was 101 mg GAE/g of methanolic leaves extract of *M. koenigii* (Abeyasinghe *et al.*, 2017).

Despite phenolic screening yielding a positive result indicating the presence of phenolic with only a small dark blue colour spot, hexane extract for both stem bark and leaves was not detected. As a result, it was assumed that the phenolic content was too low, resulting in an undetected TPC value reading, meaning neither extract's antioxidant activity was due to phenolic. Based on theory, since phenol is a polar compound and hexane is a nonpolar solvent, there should be no phenol in the extract, which correlates with the TPC data in this study.

Table 3 Total phenolic content (TPC) of stem bark and leaves extracts of *M. koenigii*

Extracts/standard	TPC,mg GAE/mg sample	
	Stem bark	Leaves
Hexane	Not detected	Not detected
Ethyl acetate	17.00	75.25
Methanol	114.25	153.25

Conclusion

The antioxidant activity from the dot blot assay of the four extracts from stem bark and leaves of *M. koenigii* was highest in the hexane and methanol leaves extract, with the lowest DPPH inhibition concentration of 0.048 mg/mL. Radical scavenging activity has quantitatively showed that the methanol extract from the leaves exhibited the highest antioxidant activity with the lowest IC₅₀ value of 68.84 ug/ mL. Methanol extracts from stem bark and leaves exhibited the highest TPC, with values of 114.25 mg GAE/g sample and 153.25 mg GAE/g sample, respectively. Therefore, the phenolic content in both parts is purposely the main reason for the antioxidant activity of *M. koenigii* species.

Ethics Statement

The research does not require research ethics approval.

Authors Contribution

Writing – Original draft preparation, Ilias Fazna, N.F; Literature Review, Daud, S; Methodology, Abdul Aziz, N; Writing – Review and editing, Harun, A.”

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Conflict of Interest

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

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