

QUALITATIVE AND QUANTITATIVE OF PHYTOCHEMICAL ANALYSIS OF MALAYSIAN *Euphorbia milii* (EUPHORBIACEAE) AND ITS ANTIOXIDANT ACTIVITIES

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ABSTRACT

Euphorbiaceae plants had been traditionally used in healing various diseases. This study was designed to determine the secondary metabolites that exist in Malaysian *Euphorbia milii* (Euphorbiaceae) and its potential in antioxidant activities. Dried leaves and flowers of *E. milii* were extracted using hexane, ethyl acetate, and methanol. The qualitative and quantitative phytochemical analysis was performed toward the crude extracts. The outcomes showed that alkaloids, flavonoids, quinones, carbohydrates, and reducing sugar were mainly present in all of the samples extracted by the three solvents. For quantitative analysis, methanolic leaves extract contained the highest amount of flavonoids, given as 0.0867 ± 0.005 mg QE/g of the sample compared to other extracts. Meanwhile, the determination of antioxidant activities of each extract showed that methanolic leaves extract consists of the highest antioxidant activity due to the presence of phytochemical compounds. This significant data provides preliminary findings that may lead to further development as a potential source of herbal medicine.

Key words: *Euphorbia milii*, Euphorbiaceae, phytochemical screening, total flavonoid content, antioxidant activities

INTRODUCTION

Antioxidant plays crucial role in combating oxidative stress (OS) that had become the reasons for varieties of chronic diseases such as cancer, cardiovascular disease, and Alzheimer's disease. However, this natural antioxidant mechanism can be inefficient due to bad dietary habits. This had been an important reason for the production of medicinal or dietary supplements that use synthetic antioxidants as their main ingredient. Unfortunately, in the recent past, some toxicological studies regarding the use of synthetic antioxidants have shown their unwanted or adverse effects (Anwar *et al.*, 2018). Hence, researchers need to go back to nature and identify or provide optional natural sources of antioxidants.

Euphorbia milii have been known as a rich source of bioactive constituents. *E. milii* is a decorative, cosmopolitan plant that belongs to the family of Euphorbiaceae. This plant is commonly known as the "Crown of Thorn" among Malaysians. The leaves of this plant used for medicinal uses including antiseptic, antibacterial, anticancer, anti-inflammatory effects, and mild hypersensitive properties (Okwu, 2004; Pradyutha *et al.*, 2015). According to the study carried out by Kamurthy *et al.* (2015), the obtained compounds of *E. milii* flowers are effective pharmaceutical compounds that will serve as a better alternative to chemical-based pharmaceuticals. Therefore, this study was carried out to provide data on the presence of the secondary metabolites in different parts of *E. milii* extracts and the consequence toward the antioxidant properties.

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MATERIALS AND METHODS

Plant materials

Two different parts (flowers and leaves) of the *Euphorbia milii* plant were collected from Kota Tinggi, Johor. The botanical identity of the plant specimen of *E. milii* was confirmed by Tuan Haji Razali Salam of the Biological Laboratory, Universiti Malaysia Terengganu.

Sample extraction

The materials were cleaned under running tap water and dried at room temperature for seven days before ground into powder form using a grinder. Then, the plant powders were extracted by soaking them with three different solvents with different polarities (Sigma-Aldrich, US) at room temperature for three days. Then, each extract was concentrated using a rotary evaporator (Büchi Rotavap R-200 CH-9230, Switzerland) under reduced pressure at 35-40°C for 15 min. The crude extracts obtained were weighed and stored in a chiller (4°C) for further analysis.

Qualitative phytochemical analysis

All crude extracts were subjected to eleven phytochemical tests which were alkaloids test, saponins test, tannin test (Braymer's test) (Morsi, 2014), triterpenoid and steroid test (Yelin & Kuntadi, 2019), flavonoids test (H₂SO₄ test), phlobatannins test (Coolborn *et al.*, 2015), quinones test (Amabye *et al.*, 2016), reducing sugar test (Fehling's test) (Karki, 2018), carbohydrate test (iodine test) (Karki, 2018), phenolic content test (ferric chloride test) (Pant *et al.*, 2017).

Quantitative phytochemical analysis

Determination of total flavonoid contents

The total flavonoid content was determined by using the aluminum chloride colorimetric method (Afify *et al.*, 2012; Khan *et al.*, 2018) using a UV- spectrophotometer (Shimadzu, UV-Vis 1800). Quercetin (Sigma – Aldrich, US) was used to make the calibration curve. The total flavonoid content was expressed in terms of quercetin equivalent (mg QE/g of the sample).

Antioxidant activity (AOA)

Total antioxidant activity

Antioxidant activity was measured by the paired diene method (Lingnert *et al.*, 1979; Alfatemi *et al.*, 2015) using a spectrophotometer (Shimadzu, UV-Vis 1800). α -tocopherol (Sigma – Aldrich, US) was

used as standard controls. The total antioxidant activity was calculated as follows:

$$= \left[\frac{(\Delta A_{234} \text{ of control} - \Delta A_{234} \text{ of sample})}{\Delta A_{234} \text{ of control}} \right] \times 100$$

Free radical scavenging assays

Scavenging effect on DPPH radical

The free radical scavenging activity of *E. milii* was determined by the 1, 1- diphenyl-2-picryl hydroxyl (DPPH) method (Shekhar *et al.*, 2014). Here, only those extracts that soluble in ethanol were used, and various concentrations were prepared by dilution method. The reduction of DPPH free radical was measured by reading the absorbance at 517 nm by using a UV- spectrophotometer (Shimadzu, UV-Vis 1800). The IC₅₀ value of extract, which is the concentration of the sample required to inhibit 50% of the DPPH free radical was calculated by the graphical method by plotting percent inhibition versus concentration. The DPPH radical scavenging effect (%) was calculated using the following formula:

$$\text{Scavenging effect (\%)} = \left[\frac{(A_0 - A_1)}{A} \right] \times 100\%$$

Where A₀ was the absorbance of the control, and A₁ was the absorbance of the test sample.

Non-enzymatic antioxidant assays

Determination of α -tocopherol

α -Tocopherol was determined according to the method proposed by Kanno and Yamauchi (1997) (Norhayati *et al.*, 2011). The absorbance was measured at 554 nm by using a UV-spectrophotometer (Shimadzu, UV-Vis 1800). A standard curve was prepared using α -tocopherol (Sigma – Aldrich, US) at various concentrations (0-1.4 μ g/mL). Then, the α -tocopherol in the extracts was calculated based on the standard curve.

Determination of carotenoids

Carotenoid content was extracted referring to the method proposed by Lichtenthaler (1987) (Norhayati *et al.*, 2011). Under dim light and over ice, 0.02 g of fresh samples were ground up with 3.0 mL of 80% (v/v) acetone (Sigma – Aldrich, US) in mortar and pestle. The homogenates were centrifuged at 10 000 rpm for 10 min. The absorbance of the supernatant obtains was measured at 663.2, 646.8, and 470 nm. As the blank, 80% acetone was used as blank. Carotenoids content was calculated using the formula:

$$C_a = 12.25 A_{663.2} - 2.79 A_{646.8}$$

$$C_b = 21.50 A_{646.8} - 5.1 A_{663.2}$$

$$C_{x+c} = \frac{1000 A_{470} - 1.82 C_a 85.02 C_b}{198}$$

Where C_a : chlorophyll a (mg/mL), C_b : chlorophyll b (mg/mL), C_{x+c} : carotenoids (mg/mL).

Statistical analysis

Data are reported as mean \pm standard error of six independent experiments. The data were analyzed using one-way ANOVA and Tukey's posttest. A difference was considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

In the present study, several phytochemical constituents were revealed in the *E. milii* plant. Alkaloids, flavonoids, quinones, carbohydrates, and reducing sugar were mainly present in all crude extracts. Previous research on leaves extracts (hexane, ethyl acetate, and methanol) of *E. milii* by Pradyutha *et al.* (2015) revealed the presence of alkaloids, flavonoids, carbohydrates, and reducing sugar in all of the crude extracts. Kamurthy *et al.* (2015) reported the findings of flavonoids, phenolic compounds, and triterpenoids extract in the flower extracts of *E. milii* and managed to isolate two triterpenoids, one flavone and two phenolic compounds from the red flowers of *E. milii*. In the part of that, saponin has an important role as a part of the plant's defense system. Yet, only ethyl acetate leaves extract produce stable bubble froth which indicated the presence of saponins. This findings

aligned with the data obtained by Pradyutha *et al.* (2015). Some research suggested that the distribution and composition of saponins in plants might be a reflection of varying needs for plant protection (Kregiel *et al.*, 2017). For the phenolic content test and tannin test, only ethyl acetate and methanol extracts of flowers and leaves gave a positive result. These obtained results were supported by the study done by Maneesha and Sudha (2018). The hexane crude extracts and ethyl acetate crude extracts of flowers showed changes of color to a redness which indicated the presence of triterpenoid meanwhile the other crude extracts showed green or greenish-blue coloration indicated the presence of steroids. The presence of steroids in ethyl acetate leaves and all methanol extracts indicated the presence of highly polarities of steroids. Meanwhile, the triterpenoids exist as low polarities compounds. In referring to the study done by Rauf *et al.* (2014) and Salehi *et al.* (2019), steroids and triterpenoids can be found in most of the Euphorbiaceae species including *E. milii*. However, no detection for phlobatannins test and in agreement with the data obtained from Rauf *et al.* (2014). The overall results of qualitative phytochemical analysis tests is presented in Table 1.

Quantitative phytochemical analysis

Determination of total flavonoids content

Among all the crude extracts, methanol leaves extract has the highest flavonoid content (0.0837 ± 0.005 mg QE/g of the sample) followed by ethyl acetate leaves (0.0585 ± 0.001 mg QE/g of the sample), methanol flowers extract (0.0484 ± 0.003 mg QE/g of the sample) and hexane leaves extract (0.0412 ± 0.002 mg QE/g of the sample). Nonetheless, the lowest flavonoid content was discovered from the flower extracts; ethyl acetate ($0.0402 \pm$

Table 1. Summary of qualitative phytochemical analysis tests on crude extracts of *E. milii*

Phytochemical test	Hexane		Ethyl acetate		Methanol	
	Flower	Leaves	Flower	Leaves	Flower	Leaves
Alkaloid Test	+	+	+	+	+	+
Triterpenoid Test	+	+	+	-	-	-
Steroid Test	-	-	-	+	+	+
aFlavonoid Test	+	+	+	+	+	+
Quinones Test	+	+	+	+	+	+
Phlobatannins Test	-	-	-	-	-	-
Saponins Test	-	-	-	+	-	-
Reducing Sugar Test	+	+	+	+	+	+
Carbohydrate Test	+	+	+	+	+	+
Phenolic Content Test	-	-	+	+	+	+
Tannin test	-	-	+	+	+	+

* - : Negative result. + : Positive result.

0.004 mg QE/g of the sample) and hexane (0.0296 \pm 0.006 mg QE/g of the sample). Referring to the parts of the plant, the highest flavonoid content was contributed by leaves part with 0.1835 mg QE/g of sample and followed by flowers, which was 0.1182 mg QE/g of the sample. Figure 1 summarizes the estimation of flavonoid contents in different crude extracts of *E. milii*.

Based on the result, we observed that the methanol solvent could provide higher flavonoids compounds. Different polarities of the extractions solvent will provide different types of compounds because of their variable chemical nature and sensitivity toward extraction or hydrolysis (Isfahlan *et al.*, 2010). This is also supported by the study *E. hirta* done by Ismail *et al.*, (2019). Most flavonoid compounds were found in leaves extract due to the photosynthesis in leaves (Saboonchian *et al.*, 2014) in which parallel with the findings of this study.

Antioxidant activity (AOA)

Total antioxidant activity

From this test, methanolic leaves extract shown the highest percentage of total antioxidant activity with 73 \pm 0.5% followed by methanol flowers extract (71 \pm 2.0%), ethyl acetate leaves extract

(45 \pm 4.4%) and lastly ethyl acetate flowers extract (35 \pm 0.8%). For the standard α -tocopherol, the percentage of antioxidant activity was 86 \pm 0.0%. The highest inhibition of linoleic acid-lipid radical in methanolic leaves extract may be due to the high presence of flavonoids compounds that consist of the hydroxyl group and carbonyl group as obtained in the quantitative phytochemical analysis. This finding was in agreement by Loganayaki *et al.* (2013) where the inhibition of lipid peroxidation might be due to the hydrogen ability of phenolic derivatives and subsequent radical stabilization. Figure 2 shows the percentage of total antioxidant activity toward *E. milii* extracts.

Free radical scavenging assays

Scavenging effect on DPPH radical

The results obtained revealed that all of the plant samples shown positive results toward this test. The methanolic leaves sample seen to have a higher percentage of scavenging effect (70.76 \pm 0.4%) followed by flowers (67.77 \pm 0.0%) meanwhile the standard ascorbic acid was (99.94 \pm 0.0%) at the concentration of 25 μ g/mL. Besides, after performing IC₅₀, the value obtained was as 0.0346, 1.4483, and 2.2781 μ g/mL for ascorbic acid,

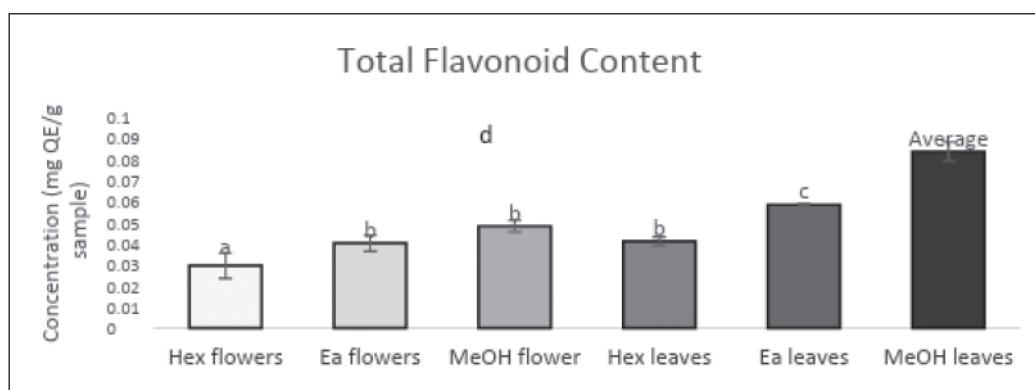


Fig. 1. Determination of total flavonoids content in different crude extracts of *E. milii* (n=6).

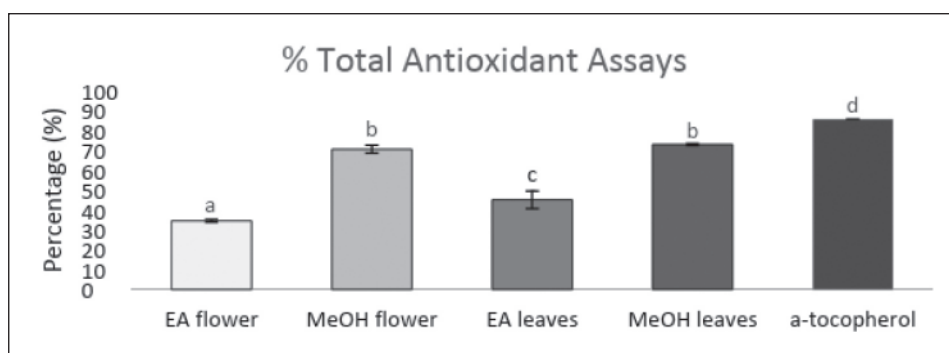


Fig. 2. Percentage of total antioxidant activity toward *E. milii* extracts (n=5).

methanol leaves extract, and methanol flowers extract, respectively. It means the methanolic extracts of *E. milii* at a higher concentration able to inhibit more free radicals formed by DPPH radical resulting in a decreasing of absorbance against the concentration and an increase in IC₅₀ value. The effects of antioxidants on DPPH radical scavenging were due to their hydrogen-donating ability of the polyphenolic compounds in the extracts to act as inhibitors or scavengers (Khan *et al.*, 2013). Figure 3 shows the percentage of scavenging effect on DPPH Radical.

Non-enzymatic antioxidant assays

Determination of α -tocopherol

The leaves part shows the higher amount of α -tocopherol ($0.4907 \pm 0.01 \mu\text{g/mL}$) followed by flowers ($0.3101 \pm 0.01 \mu\text{g/mL}$). This data related closely with the findings of research done by Inoue *et al.* (2017) toward 81 plants including *E. stenocardia* Baill and *Jatropha curcus* L. (Euphorbiaceae). In addition, the most abundant type of tocopherol present in leaves is α -tocopherol (Shintani *et al.*, 1998; Grusak *et al.*, 1999; Fritsche *et al.*, 2017) except for crops including wild Euphorbia species that produce seeds (Bruni *et al.*, 2004; Fritsche *et al.*, 2017).

Determination of carotenoids

Based on the results obtained, leaves part showed the highest carotenoid content ($0.7605 \pm 0.2 \mu\text{g/mL}$) as compared to flowers (0.3814 ± 0.04

$\mu\text{g/mL}$). According to Puzon *et al.* (2015), carotenoids were highly contained in young and matured leaves of *E. hirta* L. compared to the flowers in which aligned with the result obtained. Table 2 showed the summary of non-enzymatic antioxidant assays toward *E. milii*.

In this study, different polarities of solvents have been used to extracts different classes of compounds from the *E. milii* plant. The outcome showed that the methanolic leaves extract of *E. milii* was revealed to have the highest antioxidants activity due to the highest flavonoid content compared to other crude extracts. This is also proven by the ethanolic extracts of *E. hirta* that possess the highest antioxidant activities that also recorded to have the highest flavonoid content (Venkatachalam *et al.*, 2018). However, there is an obvious difference between the results of the extracts and the standard control used (α -tocopherol and ascorbic acid). This might be due to the low concentration of bioactive compounds that exist in the extracts compared to the pure standard control. Although phenolic compounds are responsible for their antioxidant behavior, however other factors may also caused the increasing and decreasing of the antioxidant activity such as the presence of electron-withdrawing or releasing group in the aromatic ring (Rosalind *et al.*, 2013; Devi *et al.*, 2016). In referring to the part of plants, the leaves of *E. milii* showed to possess the better activities compared to flowers due to the presence of phytochemical compounds parallel with research carried out by Abu Arra Basma *et al.* (2011) toward *E. hirta* L.

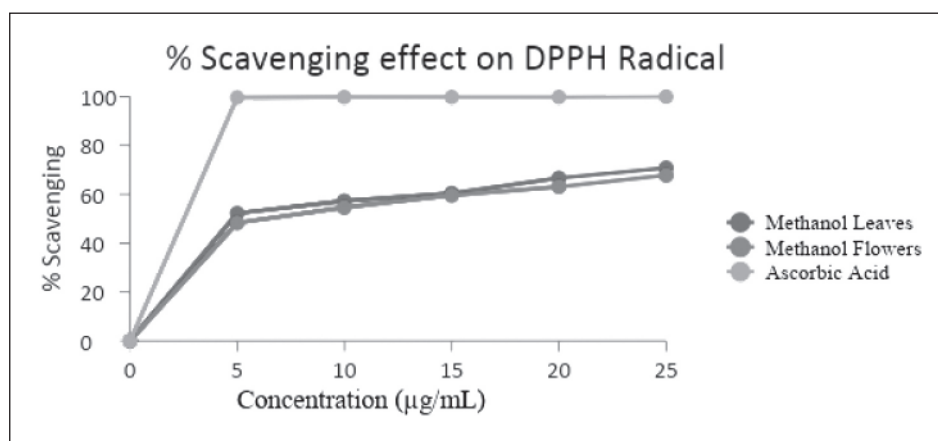


Fig. 3. Percentage of scavenging effect on DPPH Radical ($n=3$).

Table 2. Summary of non-enzymatic antioxidant assays toward *E. milii*

Non- enzymatic antioxidant assays	Leaves	Flowers
α -tocopherol	$0.4907 \pm 0.01 \mu\text{g/mL}$	$0.3101 \pm 0.01 \mu\text{g/mL}$
Carotenoids	$0.7605 \pm 0.2 \mu\text{g/mL}$	$0.3814 \pm 0.04 \mu\text{g/mL}$

Similar observations were obtained by the study done by Gapuz and Besagas (2018) where *E. milii* showed to have the highest antioxidants activity compared to *E. trigona* and *E. antiquorum* due to the highest amount of flavonoids and phenolic compounds exist in the methanolic leaves extract. The production, composition, and distribution of phytochemical compounds were caused by the chemical interaction between plant and environmental factors such as humidity, nutrition in the soil, and temperature changes (Sampaio *et al.*, 2016). Therefore, we observed the difference in classes, structure, and amount of phytochemical compounds that have been identified and recorded in other previous research that used the same species, *E. milii* as their samples. Thus, our findings provide significant data on the strong correlation between flavonoids content and the antioxidant activity presence in *E. milii* that might be useful in discovering new potential drugs.

CONCLUSION

This study discovered that *E. milii* has a wide variety of bioactive metabolite make this plant to possess antioxidant properties. However, further investigation needs to be done focusing on isolation and identification of bioactive compounds that present in *E. milii* to discovered other potential applications on healing diseases.

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