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ORIGINAL ARTICLE

Euphorbia hirta Methanolic Extract Displays Potential Antioxidant Activity for the Development of Local Natural Products

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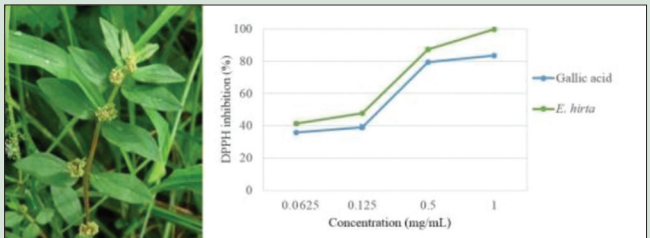
ABSTRACT

Background: The level of free radicals, which counteract the capability of the antioxidant system in plant products, is often measured in advance for further promising antidisease effect. **Objective:** In this study, we sought to evaluate the antioxidant activity of local medicinal plants (*Angelica keiskei*, *Annona muricata*, *Chromolaena odorata*, *Clinacanthus nutans*, *Euphorbia hirta*, and *Leea indica*) for their potential of use as distinctive local natural nutraceutical products. **Materials and Methods:** To recover active compounds, including yield and composition of the plants, the solvent extraction method, the Folin–Ciocalteu method, the aluminum chloride approach, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay were first performed to evaluate the antioxidant level and capacity of the plant extracts. **Results:** The aqueous extracts presented the highest yield for all plants, with the highest yield observed in *C. nutans*. However, the highest total phenolic and flavonoid contents were observed in the methanolic extract of *E. hirta* rather than in the aqueous extract. The methanolic extract of *E. hirta* also exhibited the most promising antioxidant activity, with the 50% inhibition concentration (IC_{50}) value of DPPH inhibition at 0.013 mg/mL. **Conclusion:** High total phenolic and flavonoid contents, as well as low IC_{50} value, suggested that *E. hirta* methanolic extract is a potential antioxidant agent for the development of local natural products for disease treatment.

Key words: Antioxidant activity, free radical scavenging activity, natural product, total flavonoid content, total phenolic content

SUMMARY

- The methanolic extract of *Euphorbia hirta* contained the highest total phenolic and flavonoid contents
- The extract also exhibited the most promising antioxidant activity with the 50% inhibition concentration value of 2,2-diphenyl-1-picrylhydrazyl inhibition in *E. hirta* at 0.013 mg/mL
- This phenomenon suggests that *E. hirta* methanolic extract is a potential antioxidant agent.



Euphorbia hirta DPPH inhibition of *Euphorbia hirta* and gallic acid

Abbreviations Used: DPPH: 2,2-diphenyl-1-picrylhydrazyl; DMSO: Dimethyl sulfoxide; RPMI: Roswell Park Memorial Institute; DMEM: Dulbecco's Modified Eagle Medium; GAE: Gallic acid equivalents; QE: quercetin equivalent; SEM: Standard error of the mean; IC_{50}: 50% inhibition concentration; MTT: 3-(4,5-Dimethylthial-2-yl)-2, 5-diphenyltetrazalium bromide.

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INTRODUCTION

From as early as 3000 B.C., humans have used plants as herbal medicines to treat ailments.[1] The use of herbal medicine is extensively quoted throughout history in numerous sacred texts, including the Quran and the Bible. The Bible tells us that herbs are placed on earth for the healing of humans. For example, *Punica granatum* or pomegranate has long been used in herbal medicine to treat a variety of diseases, including inflammation and rheumatism.[2] In Ayurvedic medicine, pomegranate is considered "a pharmacy unto itself," where the whole plant can be used to cure diseases.[3] Despite a plethora of claims about the therapeutic capabilities in plants, only recently have researchers seen the importance of plants as pharmaceuticals agents, leading to the isolation of active compounds and ingredients from plants. For example, isolation of morphine from *Papaver somniferum* (the opium poppy) in the early 19th century indicates the importance of identifying active compounds and ingredients from natural products for pharmaceutical purposes and industries. The contributions of natural products toward the development of novel pharmaceutical drugs have led many pharmaceutical companies to put forth effort to screen more plants to be used locally in treating diseases. Nearly 60% of anticancer products are derived from natural products, including vinblastine and vincristine, which are vinca alkaloids derived from *Catharanthus roseus*; etoposide, which is a semisynthetic derivative of mandrake plant substance podophyllotoxin; paclitaxel, which is derived from the bark of Pacific yew tree (*Taxus brevifolia*); docetaxel, which is derived from the needles of yew plants; topotecan,

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which is semisynthetically manufactured from the plant-derived alkaloid camptothecin; and irinotecan, which is a plant alkaloid.[4-6] These plant products have been identified as having the antioxidant effect and contain high levels of polyphenols and flavonoids, and their synthetic analogs dominate the list of promising anticancer agents in the treatment of various human cancers, including ovarian, breast, pancreatic, and lung cancers.[7-10] For screening purposes, the level of free radicals, which counteract the defense capability of the antioxidant system in a plant product, should be investigated before that plant product is subjected to the next steps for showing promise as an antidisease agent.

A free radical is an atom or molecule with unpaired electrons that cause oxidative damage by stealing electrons from a nearby compound or molecule. The body generates free radicals as by-products of cells using oxygen to generate energy.[11] Elevated free radical production may lead to oxidative stress, damaging cells, leading to the development of chronic and degenerative diseases, including cardiovascular diseases, neurodegenerative diseases, and cancers.[12] Antioxidants, a group of defenders against free radicals, act by donating electrons to free radicals without turning into electron-scavenging molecules themselves. Antioxidants act as free radical scavengers, thus preventing free radical damage to cells and minimizing the risk of contracting diseases. Antioxidants, including phenols and flavonoids, are found abundantly in plants. The body can produce antioxidants (endogenous antioxidants), while antioxidants from the diet (exogenous antioxidants) are important helpers in neutralizing oxidative stress. Plants are commonly a good source of natural antioxidants. Epidemiological studies have shown that consumption of plants rich in antioxidants is beneficial to health because it lowers the risk of chronic diseases, especially cancers.[13] Hence, we sought to evaluate the antioxidant activity in local medicinal plants (*Angelica keiskei, Annona muricata, Chromolaena odorata, Clinacanthus nutans, Euphorbia hirta,* and *Leea indica*) in this study for their potential to be used as distinctive natural nutraceutical products for cancer prevention and treatment. This evaluation will help in the development of local industries for natural products. To obtain active compounds from plants, an extraction procedure is required to separate medicinally active portions of plants from the inactive or inert components using specific solvents. There are many techniques available to recover active compounds from plants, including Soxhlet extraction, maceration, and ultrasound-assisted extraction. The solvent used for the extraction also influences extraction yield and composition of active compounds. In the current study, the solvent extraction method, the Folin–Ciocalteu method, with an aluminum chloride approach, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay were performed to evaluate the antioxidant level and capacity of the plant extracts. The DPPH assay is widely used to evaluate the antioxidant capacity of the plant crude extracts. The DPPH assay provides information on the capacity of the active compounds in the extracts to reduce the stable free radical DPPH. The study provides appropriate assays that are simple, specific, and rapid to screen for the presence of active compounds in plants, which is valuable for the development of natural products for local industries.

MATERIALS AND METHODS

Plant materials

A total of six plants were included in this study. *C. odorata* and *E. hirta* were collected from the state of Kelantan, Malaysia, whereas the additional plants namely *A. keiskei*, *A. muricata*, *C. nutans*, and *L. indica* were generously contributed by Fukang Herbs Sdn. Bhd. All study plants were identified by the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia.

Preparation of plant extracts

The collected plants were subjected to extraction. Briefly, the plant materials were washed and placed in a drying oven at 42°C overnight. The dried plant materials were then ground to small particles using a domestic blender. Then, the solvent (water or methanol) was added in the proportion of 10 g in 100 mL solvent to the flasks. The mixture was left on a shaker set at 100 rpm and ran for 16 h at 30°C to macerate. The mixture was decanted through Whatman filter paper, and the filtrate was collected and concentrated by a vacuum rotary evaporator (Heidolph Rotavac, Germany). The stock solution of plant extracts (50 mg/mL) and cisplatin (100 mg/mL; Sigma-Aldrich, USA) was prepared by dissolving the dried substances in dimethyl sulfoxide (DMSO, ≥99.9% pure solution; Sigma-Aldrich, USA). The solution was filtered through a 0.22-μm polyethersulfone filter membrane (Millipore, USA) and serially diluted into several working solution concentrations in culture medium. Both stock and working solutions of plant crude extracts and cisplatin were stored at −20°C until further use. The percentage (%) yield of each plant crude extract prepared using different solvents was calculated.

Total phenolic content determination

The total phenolic content of the plant extracts was determined using the Folin–Ciocalteu method described by a previous study.[14] Briefly, the Folin–Ciocalteu reagent (Merck, USA) was diluted 10 times with distilled water. Then, 50 μL of 1.0 mg/mL extract or standard solution of gallic acid at various concentrations was added to 50 μL of distilled water. In a 96-well plate, 50 μL of diluted Folin–Ciocalteu reagent and 50 μL of 1.0 M sodium carbonate (Sigma-Aldrich, USA) were added to each well. The reactions were incubated for 1 h at room temperature in the dark. The absorbance was then measured at 750 nm with a SpectraMax M5 microplate reader (Molecular Devices, USA). A standard curve was prepared with gallic acid (r^2 = 0.999). The results were expressed as mg gallic acid equivalents (GAEs) per gram of dried plant material.

Total flavonoid content determination

The total flavonoid content was determined using a method described by the previous study.[14] In a 96-well plate, 50 μL of 1.0 mg/mL extract or a standard solution of quercetin in 80% ethanol was added to 10 μL of 10% aluminum chloride solution. Then, 150 μL of 95% ethanol and 10 μL of 1.0 M sodium acetate (Sigma-Aldrich, USA) were added to the mixture. The reaction was incubated for 40 min at room temperature in the dark, and then, the absorbance was measured at 415 nm. A standard curve of the quercetin was prepared (r^2 = 0.993). The total flavonoid content in the plant extract was expressed as quercetin equivalent (QE) per gram of dried plant material.

Free radical scavenging activity determination

The DPPH free radical scavenging activity determination was performed to determine the scavenging activity of the plant extracts. Briefly, 10 μL of crude extract at various concentrations (0.125–1.000 mg/mL) was added to a 96-well plate. Then, 20 μL of 0.5 mM DPPH was added to each well in the plate. The reaction was incubated for 30 min at room temperature in the dark, and then, the absorbance was measured at 517 nm. The reduction in absorbance is reflective of the radical scavenging capacity of the extract. The degree of color change is proportional to the concentration and potency of the antioxidant capacity in scavenging free radicals. The percentage (%) of DPPH free radical scavenging activity in the plant extracts was calculated by comparing with the % of DPPH free radical scavenging activity of gallic acid as below. The Pearson correlation analysis was also performed between DPPH scavenging activity with total phenolic and flavonoid contents.

$$\% \text{ scavenging activity} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

Antiproliferative effect determination

The antiproliferative effect of the selected extract on thyroid cells was determined using 3-(4,5-dimethylthial-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. For the process, Nthy-ori 3-1, FTC-133, and Hth-74 cells were seeded at a density of 5×10^4 cells per/mL in 100 µL culture medium: Roswell Park Memorial Institute-1640 (Nacalai Tesque, Japan), Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Japan), or DMEM/Ham's F12 (Nacalai Tesque, Japan) based on the needs of the respective cell line in 96-well culture plates overnight in a humidified CO_2 incubator at 37°C. The culture medium was supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 units/mL; Nacalai Tesque, Japan), and streptomycin (100 µg/mL; Nacalai Tesque, Japan). The extract was then added at various concentrations (0–250 µg/mL) to all cell lines, and the cells were incubated in the incubator at 37°C for 72 h. Ten microliters of freshly prepared MTT solution (Amresco, USA) was added to all wells 3 h before the end of incubation time. The media were aspirated from the wells without disturbing the formazan crystals formation and 100 µL of DMSO was added to the wells. The color intensity of the formazan solution, which reflects the number of cells under the specific growth conditions, was measured at 570 nm using the microplate reader SpectraMax M5 (Molecular Devices, USA). The proliferation curve of the extract in respective cells was plotted using nonlinear regression by GraphPad Prism 7 is GraphPad Software Inc., USA. The % of surviving cells in treated cells relative to untreated cells (control) and 50% inhibition of cells (IC_{50} value) were calculated from the curves.

Statistical analysis

All experiments were performed in triplicate. Data were expressed as the standard error of the mean of triplicates in three independent experiments. Statistically significant differences were determined with one-way analysis of variance, and differences were considered significant at $P < 0.05$.

RESULTS

Percentage yield of plant crude extracts

The % yield of *A. keiskei, A. muricata, C. odorata, C. nutans, E. hirta,* and *L. indica* crude extracts as prepared using different solvents (water and methanol) is shown in Table 1. Among the solvents used in the study, the extraction method using water possesses a higher recovery yield over the extraction with methanol. In general, the aqueous extracts presented the highest yield for all plants, with the highest yield observed for *C. nutans* at 11.45%. The % yield of the extracts from water extraction was found to be in the order of *C. odorata* (1.69%) < *L. indica* (6.29%) < *E. hirta* (6.85%) < *A. keiskei* (8.10%) < *A. muricata* (10.47%) < *C. nutans* (11.45%). For methanolic extracts, *C. odorata* provided the least % yield (0.80%), while *C. nutans* produced the highest yield (7.49%). The % yield of the extracts for methanol extraction was found to be in the order of *C. odorata* (0.80%) < *L. indica* (4.44%) < *E. hirta* (4.59%) < *A. keiskei* (4.86%) < *A. muricata* (5.35%) < *C. nutans* (7.49%).

Table 1: The percentage yield of plant crude extracts prepared using different solvents

Plant	Solvent	Yield (%)
Angelica keiskei	Methanol	4.856
	Water	8.097
Annona muricata	Methanol	5.346
	Water	10.473
Chromolaena odorata	Methanol	0.802
	Water	1.687
Clinacanthus nutans	Methanol	7.489
	Water	11.448
Euphorbia hirta	Methanol	4.593
	Water	6.846
Leea indica	Methanol	4.444
	Water	6.287

Data are expressed as the mean (SEM) of triplicates in three independent experiments. SEM: Standard error of the mean

Total phenolic content in plant crude extracts

The total phenolic content of the plant aqueous and methanolic crude extracts was calculated from the calibration curve of gallic acid [Supplementary Figure 1]. The highest total phenolic content among aqueous extracts was obtained in *L. indica* (235.56 ± 1.37 mg GAE/g dw), followed by *A. muricata* (74.35 ± 1.14 mg GAE/g dw), *C. odorata* (66.61 ± 1.22 mg GAE/g dw), *E. hirta* (60.84 ± 1.19 mg GAE/g dw), *A. keiskei* (46.67 ± 0.66 mg GAE/g dw), and *C. nutans* (31.21 ± 0.70 mg GAE/g dw), as shown in Figure 1a. *L. indica* aqueous extract is significantly higher compared to the aqueous extracts of *A. keiskei* ($P < 0.05$), *C. nutans* ($P < 0.01$), *C. odorata* ($P < 0.05$), and *E. hirta* ($P < 0.05$). In general, the total phenolic content of aqueous extracts is lower when compared to methanolic extracts despite the high % yield that was obtained during the extraction. As shown in Figure 1b, among the methanolic extracts, *E. hirta* showed the highest total phenolic content at 307.59 ± 3.57 mg GAE/g dw, followed by *L. indica* (243.67 ± 1.68 mg GAE/g dw), *A. muricata* (145.36 ± 0.68 mg GAE/g dw), *C. odorata* (134.26 ± 0.26 mg GAE/g dw), and *C. nutans* (98.24 ± 0.30 mg GAE/g dw), while *A. keiskei* exhibited the lowest total phenolic content at 70.49 ± 0.34 mg GAE/g dw. The total phenolic content of the *E. hirta* methanolic extract is significantly higher than the methanolic extracts of *A. keiskei* ($P < 0.001$), *C. nutans* ($P < 0.01$), and *C. odorata* ($P < 0.05$).

Total flavonoid content in plant crude extracts

The total flavonoid content for all the plant crude extracts was determined by the aluminum chloride approach using a standard curve of quercetin [Supplementary Figure 2]. A similar trend was observed in the total flavonoid content, where the aqueous extract of *L. indica* and methanolic extract of *E. hirta* exhibited the highest amount of total flavonoid content. The methanolic extracts also showed higher flavonoid content when compared to aqueous extracts, except for *L. indica*, where the methanolic extract (26.12 ± 4.21 mg QE/g dw) was lower than the aqueous extract (40.22 ± 5.76 mg QE/g dw). Among the aqueous extracts, *L. indica* exhibited the highest flavonoid content, followed by *A. muricata* (20.14 ± 4.12 mg QE/g dw), *A. keiskei* (12.49 ± 3.45 mg QE/g dw), *E. hirta* (9.26 ± 3.50 mg QE/g dw), *C. nutans* (8.76 ± 2.58 mg QE/g dw), and *C. odorata* (8.16 ± 2.59 mg QE/g dw), as shown in Figure 2a. *L. indica* aqueous extract is significantly higher than the aqueous extracts of *A. keiskei* ($P < 0.01$), *C. odorata* ($P < 0.001$), *C. nutans* ($P < 0.001$), and *E. hirta* ($P < 0.01$). However, the *E. hirta* methanolic extract was observed at 76.43 ± 4.34 mg QE/g dw, followed by *C. nutans* (64.59 ± 3.54 mg QE/g dw), *A. muricata* (57.75 ± 7.04 mg QE/g dw), *C. odorata* (46.54 ± 3.37 mg QE/g dw), *L. indica* (26.16 ± 4.21 mg QE/g dw) and *A. keiskei* (22.82 ± 0.89 mg QE/g dw), as shown in Figure 2b. *E. hirta* methanolic extract is significantly higher than the methanolic extracts of *A. keiskei* ($P < 0.001$), *C. odorata* ($P < 0.01$), and *L. indica* ($P < 0.001$).

Free radical scavenging activity of plant crude extracts

The antioxidant activity of plant crude extracts was determined using the DPPH free radical scavenging assay and gallic acid as the control for the assay. All extracts inhibited DPPH, indicating the presence of antioxidant activity of the extracts. In general, the % inhibition

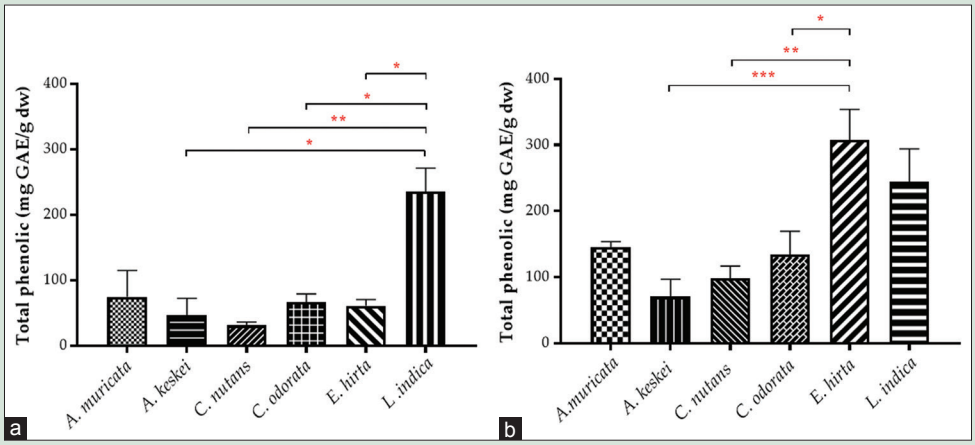

Figure 1: The total phenolic content of (a) aqueous and (b) methanolic extracts of medicinal plants (mg GAE/g dw). Data are presented as the mean (standard error of the mean) of triplicates in three independent experiments; GAE: Gallic acid equivalent; dw: Dry weight; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. *A. keiskei: Angelica keiskei*; *A. muricata: Annona muricata*; *C. odorata: Chromolaena odorata*; *C. nutans: Clinacanthus nutans*; *E. hirta: Euphorbia hirta*; *L. indica: Leea indica*

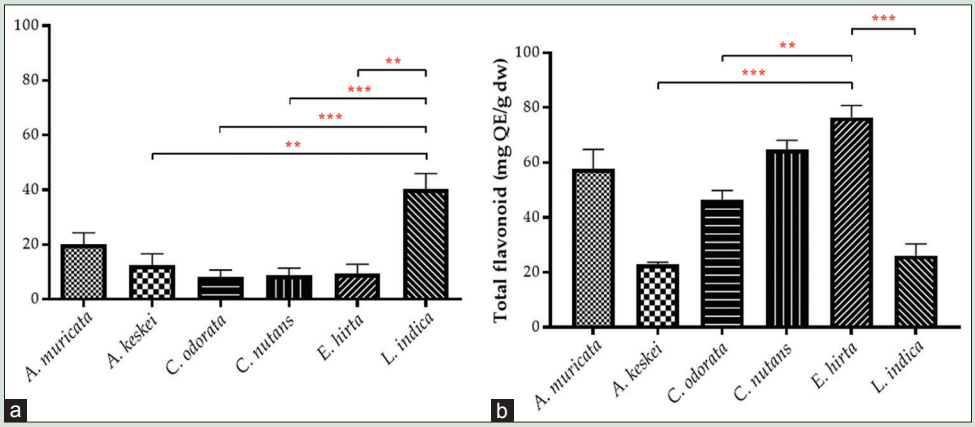

Figure 2: Total flavonoid content of (a) aqueous and (b) methanolic extracts of medicinal plants (mg QE/g dw). Data are presented as the mean (standard error of the mean) of triplicates in three independent experiments; QE: Quercetin equivalent; dw: Dry weight; **$P < 0.01$ and ***$P < 0.001$. *A. keiskei: Angelica keiskei*; *A. muricata: Annona muricata*; *C. odorata: Chromolaena odorata*; *C. nutans: Clinacanthus nutans*; *E. hirta: Euphorbia hirta*; *L. indica: Leea indica*

ranged from 16.08% to 99.77% within the concentrations used for all the tested plant extracts, whereby the methanolic extract of *E. hirta* exhibited the most promising antioxidant activity compared to gallic acid [Table 2]. At the concentration of 1.0 mg/mL, the methanolic extract of *E. hirta* showed 99.77% ± 0.16% ($P < 0.05$) of DPPH inhibition, which was significantly higher than the corresponding values of gallic acid. It is worth mentioning that at 0.5 mg/mL, the methanolic extract of *E. hirta*, which exhibited 87.21% ± 0.12% of DPPH inhibition, was not statistically significant in comparison to the scavenging activity shown by the standard antioxidant. This may be due to the experimental error. A better reduction of scavenging activity was observed when 0.25 mg/mL of methanolic extract of *E. hirta* was used, whereby the inhibition of DPPH was 89.17% ± 0.35% ($P < 0.01$).

On the other hand, the % inhibition of DPPH shown by *C. nutans* extracts was significantly lower than the corresponding values for gallic acid. At the concentration of 1.0 mg/mL, the methanolic extract of *C. nutans* showed 31.71% ± 1.88% ($P < 0.01$) of DPPH inhibition. At 0.5 mg/mL, the methanolic extract of *C. nutans* exhibited 26.01% ± 0.16% ($P < 0.01$) of DPPH inhibition. The inhibition of DPPH at both concentrations of methanol is significantly lower in comparison to the scavenging activity shown by the standard antioxidant. The lowest % inhibition of DPPH at 0.25 mg/mL *C. nutans* was not observed in the methanolic extract but in the aqueous extract. The inhibition of DPPH was 16.08% ± 1.12% ($P < 0.01$) in the aqueous extract. Almost all extracts showed concentration-dependent free radical inhibition in the range of the tested concentrations except for aqueous extracts of *A. muricata, C. odorata,* and *E. hirta* and methanolic extracts of *A. keiskei* and *C. nutans*, where the % of DPPH inhibition remains similar despite the differences in the extract concentrations.

The IC_{50} of the extracts is also shown in Table 2, whereby the lower IC_{50} value indicated higher antioxidant activity. Based on the data collected, the IC_{50} values of DPPH inhibition could be determined for several crude extracts, including aqueous extracts of *A. keiskei* (0.76 mg/mL), *A. muricata* (0.15 mg/mL), *C. odorata* (1.00 mg/mL), and *L. indica* (0.27 mg/mL) and methanolic extracts of *A. muricata* (0.34 mg/mL), *C. odorata* (0.35 mg/mL), *E. hirta* (0.013 mg/mL), and *L. indica* (0.28 mg/mL). The IC_{50} value of DPPH inhibition showed that methanolic extract of *E. hirta* possesses the highest antioxidant activity.

Correlations of antioxidant activity and total phenolic and flavonoid contents among plant crude extracts

Correlation coefficient analysis was performed by comparing the DPPH IC_{50} values, total phenolic content, and total flavonoid content of the plant crude extracts. The Pearson correlation coefficients between the variables are presented in Table 3. The current data suggest an inverse correlation ($r = -0.690$, $P > 0.05$) between the amount of phenolics and the value of IC_{50} in the DPPH assay [Figure 3a]. However, there was a strong negative significant correlation between DPPH radical scavenging and total flavonoid content ($r = -0.708$, $P < 0.05$), as shown in Figure 3b. High flavonoid content and low IC_{50} suggest that a small amount of flavonoid is required to scavenge DPPH, as observed in *E. hirta* methanolic extract. Therefore, the methanolic extract of *E. hirta* is selected for the subsequent study.

Table 2: Percentage inhibition of DPPH plant aqueous and methanolic crude extracts. All values were acquired from Supplementary Figure 3

Sample/standard	DPPH inhibition (%)			DPPH IC_{50} (mg/mL)
	1.0 mg/mL	0.5 mg/mL	0.25 mg/mL	
Gallic acid	83.45±0.17	79.45±0.13	36.84±0.09	~0.28
Angelica keiskei				
Water	58.04±5.77	44.22±13.63	28.04±1.45	~0.76
Methanol	49.74±1.06	44.90±2.05	46.63±1.40	>1.0
Annona muricata				
Water	61.83±1.67	72.97±0.11	62.60±5.24	~0.15
Methanol	78.20±0.19	56.51±0.27	46.37±0.32	~0.34
Chromolaena odorata				
Water	53.17±2.15	63.28±4.31	63.24±1.43	~1.00
Methanol	92.03±0.27	55.45±0.22	46.37±0.23	~0.35
Clinacanthus nutans				
Water	37.69±1.89	33.22±1.21	16.08±1.12*	>1.0
Methanol	31.71±1.88**	26.01±0.16**	31.16±1.69	>1.0
Euphorbia hirta				
Water	42.11±3.86	65.24±5.42	41.78±1.46	>1.0
Methanol	99.77±0.16*	87.21±0.12	89.17±0.35**	~0.013
Leea indica				
Water	94.74±1.47	81.03±1.33	67.22±0.85	~0.27
Methanol	68.07±0.14	58.37±0.09	47.48±0.13	~0.28

The total phenolic content of the plant aqueous and methanolic crude extracts was calculated from the calibration curve of gallic acid [Supplementary Figure 1]. All values were acquired from Supplementary Figure 3. Data are expressed as the mean (SEM) of triplicate in three independent experiments. The value indicates significant difference compared to gallic acid; *P<0.05, **P<0.01. SEM: Standard error of the mean; DPPH: 2,2-diphenyl-1-picrylhydrazyl

Table 3: Pearson correlation coefficients between the variables of the plant crude extracts

	Total phenolic	Total flavonoid
Total phenolic	-	-
Total flavonoid	0.670	-
DPPH IC_{50}	−0.690	−0.708*

*The correlation is significant at P<0.05. DPPH: 2,2-diphenyl-1-picrylhydrazyl

Anti-proliferative effect of methanolic plant extract on thyroid cells

In vitro MTT assay for methanolic extract of *E. hirta* in thyroid cells showed that the extract was not entirely cytotoxic to the cells, whereby the cells treated with the extract showed significant growth inhibition ($P < 0.05$) only after 72 h of treatment. Figure 4 shows the dose- and time-dependent growth inhibition of Nthy-ori 3-1 and FTC-133 cells treated with *E. hirta* methanolic extract for 72 h. At 72 h of treatment, the IC_{50} values for methanolic extract of *E. hirta* and cisplatin on Nthy-ori 3-1 cells were 62.7 and 145.5 μg/mL, respectively, whereas the IC_{50} values for the extract and cisplatin on FTC-133 cells were 119.3 and 145.8 μg/mL, respectively. The analysis of the *E. hirta* methanolic extract demonstrated that the extract could suppress cancerous cells from multiplying at a high concentration after 72 h of treatment. On the other hand, the cells treated with the extract (62.0 μg/mL) and cisplatin (123.6 μg/mL) showed dose-dependent growth inhibition of Hth-74 cells at 72 h of treatment. However, the IC_{50} value of *E. hirta* methanolic extract was determined as >50 μg/mL in Hth-74 cells at this treatment time point, indicating that the *E. hirta* methanolic extract is not a selective cytotoxic agent.

DISCUSSION

Many medicinal plants contain large amounts of antioxidant agents, including polyphenols. The presence of active phytochemicals with antioxidant activity contributes to the medicinal properties of plants. Polyphenols, including flavonoids, play an important role in reducing the risk of disease in humans, including cardiovascular diseases, inflammation, and cancers.[15-19] Increased evidence shows that free radicals give rise to oxidative stress, which leads to disease development.[20,21] Polyphenols are well-recognized antioxidant agents that can act as free radical terminators. The redox properties of polyphenols allow these compounds to act as potential antioxidant agents. Phenolic compounds are able to neutralize free radicals due to their redox properties, which allow the phenolic compounds to act as reducing agents or free radical inhibitors.[22] Quercetin is naturally found in plants and serves important roles in numerous biological activities, including antioxidant activity and

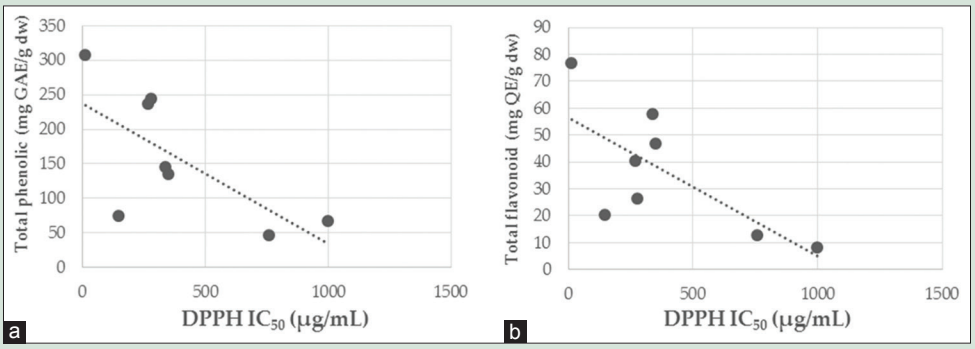

Figure 3: Linear correlation for DPPH radical scavenging versus (a) total phenolic content (Pearson correlation coefficient, $r = -0.690$; $P > 0.05$; $n = 8$) and (b) total flavonoid content of the extracts (Pearson correlation coefficient, $r = -0.708$; $P < 0.05$; $n = 8$). DPPH: 2,2-diphenyl-1-picrylhydrazyl

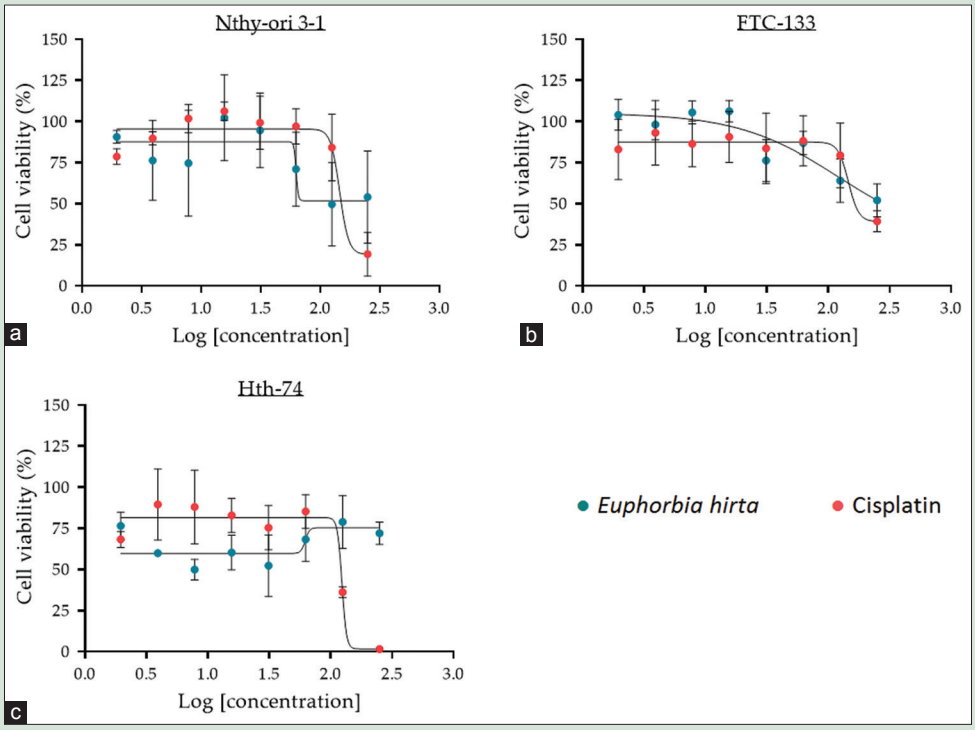

Figure 4: The viability of thyroid cells treated with methanolic crude *Euphorbia hirta* extracts and cisplatin for 72 h. The 50% inhibition concentration graphs correspond to the treatments at 72 hours in (a) Nthy-ori 3-1, (b) FTC-133, and (c) Hth-74 cells. Data are shown as percentage of viable cells calculated by comparing with untreated control group. All results are expressed in mean (standard error of the mean); $n = 3$

antitumorigenesis.[23,24] Phytochemicals present in plants protect against oxidative stress and help maintain the balance between free radicals and antioxidants. Nonetheless, biologically active phytochemicals that contribute to the medicinal properties usually occur in low concentration in plants.[25] Therefore, the extraction procedure of the antioxidants is a crucial process in studying the medicinal properties of the plants.

In the present study, six local medicinal plants: *A. keiskei, A. muricata, C. odorata, C. nutans, E. hirta,* and *L. indica* were selected for extraction using water and methanol as solvents. There are many methods to obtain phytochemicals from the plant, including extraction, homogenization, and grinding. Extraction is the most common method for recovering phytochemicals from plant materials. In addition, the type of extraction solvent also has a significant impact on the antioxidant activity of the extracts and the % yield of the plant materials.[26] The results obtained with the extracts demonstrate that the solvent of choice could influence the composition of the extracts and their antioxidant activity, due to the presence of compounds of varied chemical properties and polarities.[27] Polar solvents, such as water and methanol, are frequently used for recovering polyphenols from plants; therefore, water and methanol were used in the extraction process. Water has a polarity index of 10.2 while methanol has a polarity index of 5.1. In our current study, the % yield for water was higher than the % yield for methanol, showing that extraction yield increases when the polarity of the solvent increases. Water is commonly used for extraction because it is safe to use and is frequently used for the preparation of infusions and decoctions by herbal medicine practitioners, whereas methanol is commonly used because it is relatively inexpensive, able to dissolve various compounds, and easily evaporates, which is especially useful when the bioactive compounds need to be concentrated using a rotary evaporator. To further evaluate the efficiency of the extraction, total phenolic contents and flavonoid contents were determined using the Folin–Ciocalteu method and aluminum chloride method, respectively.

In general, the total phenolic content in the aqueous crude extracts was lower when compared to the methanolic extracts, possibly attributable to the content, where more nonphenol compounds, such as carbohydrates and terpenes, are accumulated in the aqueous extracts.[28] Furthermore, methanol is found to be more efficient in the extraction of lower molecular weight polyphenols,[29] causing more phenolic compounds to accumulate in the methanolic extracts. In addition, the increased temperature during the concentration step using rotary evaporation possibly could affect the total phenolic activity in the extracts. Plant phenolics are easily degraded when exposed to high temperature as heat may accelerate their oxidation. The effect of solvents on total flavonoid content in the crude extracts is similar to the effect on total phenolic content.

In accordance with the current study, other studies have found high phenolic content in methanolic extract of *E. hirta*. A study of Basma *et al.* showed total phenolic content in methanolic extract of *E. hirta* leaves at 206.17 ± 1.95 mg GAE/g dw, when compared to other parts of the plant.[30] Unlike the current study, Asha *et al.*'s study found the presence of high phenolic content in the methanolic extract (285.41 ± 3.00 GAE/g dw) and in the aqueous extract (275.64 ± 2.45 mg GAE/g dw) of *E. hirta* leaves.[31] The variation in the content of the extracts could be a consequence of several factors, including the differences in the plant matrix and the method, as well as the conditions of extraction, such as temperature and duration of extraction.[32,33] Several studies have reported the presence of polyphenols and antioxidant activity in the extracts of *L. indica*,[34-39] *C. nutans*,[40-43] *C. odorata*,[44-48] and *A. muricata*.[49-53] A study found variation in the polyphenol content in the extracts of *A. keiskei*, when the plant was extracted with different compositions of water/ethanol and stored at different temperatures.[54]

The DPPH scavenging activity of the *E. hirta* methanolic extract is higher than the scavenging activity of the standard drug showing proton-donating ability and thus could serve as the free radical inhibitor.

The potent antioxidant activity of the *E. hirta* methanolic extract was also reported by Rajeh *et al.*'s study,[55] showing the highest content in the leaf extract. The observed free radical scavenging activity may be attributed to the presence of polyphenols in the extracts.[56]

The correlation analysis was performed on total phenolic content, total flavonoid content, and DPPH IC_{50} of the *E. hirta* crude extracts in this study. The significant negative correlation between DPPH IC_{50} and total flavonoid content compared to total phenolic content, indicating a pronounced influence of flavonoids on the antioxidant activity of the crude extracts in terms of DPPH radical scavenging. Kiselova *et al.*'s study found a strong linear correlation between the polyphenol content of the aqueous extracts from Bulgarian herbs and the *in vitro* antioxidant activities.[57] Our data are in agreement with those reported by Borkataky *et al.*'s study[58] in *Eclipta alba* and the study by Rebaya *et al.*[59] in *Halimium halimifolium*, where the polyphenols in the medicinal plants exhibited strong linear relationships with the antioxidant activities. However, a study reported that the strong linear relationship could not be established between total polyphenols and antioxidant activity against β-carotene and linoleic acid of several medicinal plant extracts.[60] Ghasemi *et al.*'s study did not find a good correlation between the radical scavenging activity and phenolic content in 13 citrus species peels and tissue,[61] possibly due to the differences in structural features of polyphenols that determine their antioxidant properties, where the number of radicals that are reduced seems to be correlated with the number of the available hydroxyl groups.[62]

CONCLUSION

E. hirta methanolic extract is a potential antioxidant agent for the development of local industries for natural products as the *E. hirta* methanolic extract displays high total phenolic and flavonoid contents, as well as a low IC_{50} value. The study also provides appropriate assays that are simple, specific, and rapid to screen for the presence of active compounds in plants for the development of local industries for natural products.

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Conflicts of interest

There are no conflicts of interest.

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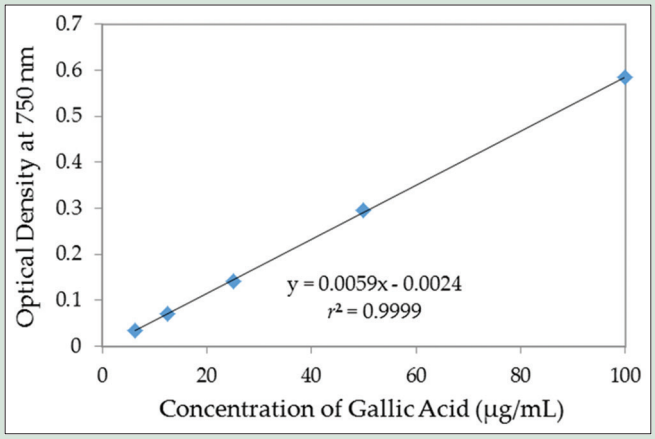
SUPPLEMENTARY MATERIALS

The absorbance of the standard compound (gallic acid) at λ_{max}=750 nm

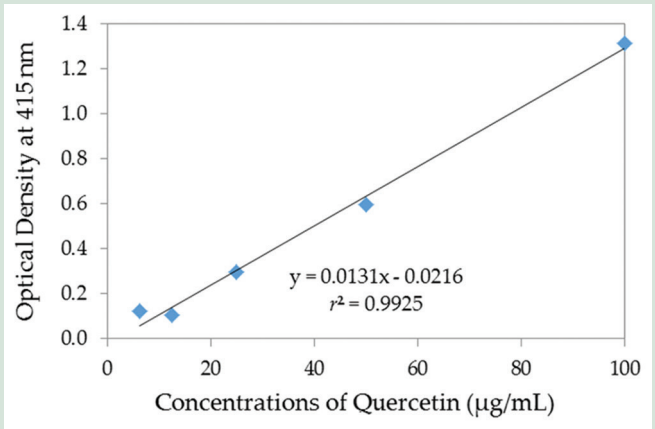
Concentration (µg/mL)	Absorbance (mean)
6.25	0.0353
12.5	0.0704
25	0.1422
50	0.2949
100	0.5848



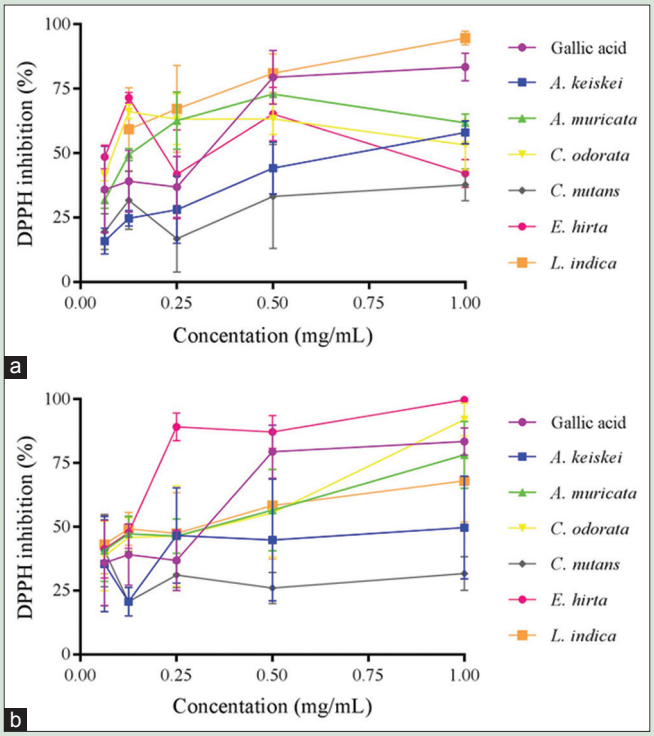
Supplementary Figure 1: The standard curve of gallic acid for quantification of total phenolic content in plant crude extracts. The linearity of the calibration curve was achieved with different concentrations of gallic acid

The absorbance of the standard compound (quercetin) at λ_{max}=415 nm

Concentration (µg/mL)	Absorbance (mean)
6.25	0.1238
12.5	0.1048
25	0.2960
50	0.5954
100	1.3117



Supplementary Figure 2: The standard curve of quercetin for determination of total flavonoid content in plant crude extracts. The linearity of the calibration curve was achieved with different concentrations of quercetin



Supplementary Figure 3-: Percentage scavenging activity of DPPH free radicals in response to (a) aqueous and (b) methanolic plant crude extracts. Data are expressed as the mean (SEM) of triplicates in three independent experiments. DPPH: 2,2-diphenyl-1-picrylhydrazyl; SEM: Standard error of the mean; A. keiskei: Angelica keiskei; A. muricata: Annona muricata; C. odorata: Chromolaena odorata; C. nutans: Clinacanthus nutans; E. hirta: Euphorbia hirta; L. indica: Leea indica