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Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta* L

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ABSTRACT

Objective: To assess antioxidant activities of different parts of *Euphorbia hirta (E. hirta)*, and to search for new sources of safe and inexpensive antioxidants. **Methods:** Samples of leaves, stems, flowers and roots from *E. hirta* were tested for total phenolic content, and flavonoids content and *in vitro* antioxidant activity by diphenyl-1-picrylhydrazyl (DPPH) assay and reducing power was measured using cyanoferrate method. **Results:** The leaves extract exhibited a maximum DPPH scavenging activity of (72.96±0.78)% followed by the flowers, roots and stems whose scavenging activities were (52.45±0.66)%, (48.59±0.97)%, and (44.42±0.94)%, respectively. The standard butylated hydroxytoluene (BHT) was (75.13±0.75)%. The IC_{50} for leaves, flowers, roots, stems and BHT were 0.803, 0.972, 0.989, 1.358 and 0.794 mg/mL, respectively. The reducing power of the leaves extract was comparable with that of ascorbic acid and found to be dose dependent. Leaves extract had the highest total phenolic content [(206.17±1.95) mg GAE/g], followed by flowers, roots and stems extracts which were (117.08±3.10) mg GAE/g, (83.15±1.19) mg GAE/g, and (65.70±1.72) mg GAE/g, respectively. On the other hand, total flavonoids content also from leave had the highest value [(37.970±0.003) mg CEQ/g], followed by flowers, roots and stems extracts which were (35.200±0.002) mg CEQ/g, (24.350±0.006) mg CEQ/g, and (24.120±0.004) mg CEQ/g, respectively. HPTLC bioautography analysis of phenolic and antioxidant substance revealed phenolic compounds. Phytochemical screening of *E. hirta* leaf extract revealed the presence of reducing sugars, terpenoids, alkaloids, steroids, tannins, flavanoids and phenolic compounds. **Conclusions:** These results suggeste that *E. hirta* have strong antioxidant potential. Further study is necessary for isolation and characterization of the active antioxidant agents, which can be used to treat various oxidative stress-related diseases.

1. Introduction

Free radicals have been claimed to play an important role in affecting human health by causing several chronic diseases, such as cancer, diabetes, aging, atherosclerosis, hypertension, heart attack and other degenerative diseases[1]. These free radicals are generated during body metabolism. Exogenous intake of antioxidants can help the body scavenge free radicals effectively. Nowadays, there is a noticeable interest in antioxidants, especially in those which can prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources[2]. At present, most of the antioxidants are manufactured synthetically. The main disadvantage with the synthetic antioxidants is the side effects *in vivo*[3]. Previous studies reported that butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) accumulate in the body and result in liver damage and carcinogenesis[4]. Therefore, strict governmental rules regarding the safety of the food have necessitated the search for safer alternatives as food preservatives[5].

*Euphorbia hirta (E. hirta)*L., belongs to the family Euphorbiaceae. It is a small annual herb common to tropical countries. It is usually erect, slender-stemmed, spreading up to 80 cm tall, though sometimes it can be seen lying down. The plant is an annual broad-leaved herb that

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has a hairy stem with many branches from the base to the top. The leaves are opposite, elliptical, oblong or oblong-lanceolate, with a faintly toothed margin and darker on the upper surface. The flowers are small, numerous and crowded together in dense cymes (dense clusters in upper axils) about 1 cm in diameter. The stem and leaves produce a white or milky juice when cut. It is frequently seen occupying open waste spaces, banks of watercourses, grasslands, road sides, and pathways[6,7].

E. hirta is a very popular herb amongst practitioners of traditional medicine, widely used as a decoction or infusion to treat various ailments including intestinal parasites, diarrhoea, peptic ulcers, heartburn, vomiting, amoebic dysentery, asthma, bronchitis, hay fever, laryngeal spasms, emphysema, coughs, colds, kidney stones, menstrual problems, sterility and venereal diseases. Moreover, the plant is also used to treat affections of the skin and mucous membranes, including warts, scabies, tinea, thrush, aphthae, fungal afflictions, measles, Guinea-worm and as an antiseptic to treat wounds, sores and conjunctivitis. The plant has a reputation as an analgesic to treat severe headache, toothache, rheumatism, colic and pains during pregnancy. It is used as an antidote and pain relief of scorpion stings and snakebites. The use of the latex to facilitate removal of thorns from the skin is common[8].

The present study was to assess the phytochemical content and the antioxidant activity of the methanol extract of different parts of *E. hirta*. The findings from this work may add to the overall value of the medicinal potential of the herb.

2. Materials and methods

2.1. Plant collection

The fresh plant was harvested from various areas in Universiti Sains Malaysia, in June 2009. The taxonomic identity of the plant was confirmed by the Botanist of School of Biological Sciences at Universiti Sains Malaysia. The plant materials were washed under tap water and separated into leaves, flowers, stems and roots. The separated parts were air dried in shade for ten days and then in oven at 60 ℃ for one to two days, then grinded to fine powder by using electric blender and stored in clean labelled airtight bottles.

2.2. Preparation of the plant extract

A hundred grams of each part powder was extracted by maceration in 400 mL of methanol for 14 days with frequent agitation. The mixture was filtered through clean muslin cloth followed by double filtration with Whatman No. 1 filter paper and the filtrate was concentrated by rotary evaporator with vacuum at 50 ℃, poured in glass Petri dishes and brought to dryness at 60 ℃ oven. The percentage yield of the crude extract was determined for each part and was 11.1%, 7.3%, 4.7% and 4.1% for leaves, stems, flowers and roots, respectively.

2.3. Diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging activity of *E. hirta* L was measured by using DPPH assay. The quantitative estimation of radical scavenging activity was determined according to the methods described by Torey *et al*[9]. Five millilitres of 0.004% DPPH radical solution was added to the plant extract solutions ranging from 0.031 to 2 mg/mL. The mixtures were vortex-mixed and kept at room temperature under dark conditions for 30 min. The optical density (OD) was measured at 517 nm (Shimadzu UV-Mini1240, UV/Vis spectrophotometers). Methanol was used as a blank, the methanol and DPPH solution as a baseline control (A_0) and BHT as positive control. The DPPH radical concentration was calculated using the following equation: Scavenging effect (%): $(A_0 - A_1) \times 100\%/A_0$, where Ao is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample of the tested extracts. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs the corresponding scavenging effect.

2.4. Reducing power determination

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action[10]. The reducing power of extracts was determined according to Dharmishtha *et al*[11]. One millilitre of the leaf extract (31-1 000 μg/mL) in water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium hexacyanoferrate [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 ℃ for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3 000 rpm for 10 min (Hettich Zentrifugen, Universal 320R). The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Sample free mixture was used as a blank while Ascorbic acid was used as positive control. All tests were performed in triplicate.

2.5. Determination of total phenolic content

The total phenolic content of *E. hirta* parts extracts was determined by the Folin-Ciocalteu reagent according to Singleton and Rossi procedure[12]. 200 μL plant extract (three replicates of 1.0 mg/mL) were introduced into test tubes; 500 μL of 10% Folin-Ciocalteu's reagent, 500 μL of distilled water and 800 μL of 7.5% saturated aqueous sodium carbonate (Na_2CO_3) were added. The tubes were mixed thoroughly and allowed to stand in dark condition at ambient temperature for 30 min. Absorption was measured at 765 nm using spectrophotometer (Hitachi U-1900, Tokyo, Japan). Distilled water was used as a blank and gallic acid (0-250 mg/L) was used to produce standard

calibration curve. The total phenolic content was expressed as gallic acid equivalent per gram of dry weight (mg GAE/g) of extracts. Total content of phenolic compounds in the plant extract was calculated using this formula: Total phenolic content = GAE×V/m. Where GAE is the gallic acid equivalence (mg/mL) or concentration of gallic acid established from the calibration curve (Y=0.0073X+ 0.1003; r^2= 0.987); V is the volume of extract (mL) and m is the weight (g) of the pure plant extract[13].

2.6. Determination of total flavonoid content

The total flavonoid content of *E. hirta* L methanol extracts was determined by using aluminium chloride colorimetric method. Briefly, 250 μL of each extract (1 mg/mL) was mixed with 1 mL of distilled water and subsequently with 75 μL of sodium nitrite solution [5% (w/w) $NaNO_2$]. After 6 min of incubation, 75 μL of Aluminium trichloride solution (10% $AlCl_3$) was added and then allowed to stand for 6 min, followed by addition of 1 mL sodium hydroxide solution (4% NaOH) to the mixture. Immediately, water was added to bring the final volume to 2.5 mL, and then the mixture was thoroughly mixed and allowed to stand for another 15 min at room temperature. Absorbance of the pinkish color mixture was measured at 510 nm versus reagent blank containing water instead of the sample[14]. Catechol was used as a standard compound for the quantification of total flavonoid. Results were expressed as milligrams of catechol equivalent per gram of dry weight of extracts (mg CE/g). Total content of flavonoid compounds in the plant extract was calculated using this formula:

Total flavonoid content = CE×V/m. Where CE is the catechol equivalence (mg/mL) or concentration of catechin solution established from the calibration curve; V is the volume of extract (mL) and m is the weight (g) of the pure plant extract[13]. Data was recorded as mean±SD for three replications.

2.7. High performance thin layer chromatography (HPTLC) study of phenol and DPPH

HPTLC was performed using silica gel aluminium sheet (20 cm × 20 cm, Silica gel 60 F254, Merck, Germany). The leaf extract was dissolved in methanol (10 mg/mL) and applied on aluminium silica gel sheet (3 cm × 7cm). A baseline was drawn on the TLC plate. A spot of the plant extract was placed on the baseline with use of a yellow tip and allowed to air dry. TLC plates were developed with ethyl acetate/benzene (1:1) solvent mixture as mobile solvent. The solvent front was drawn. The developed plates were examined under the UV/Vis lamp and the specks were circled with a pencil. Developed plates were stained with Folin-Ciocalteu's reagent and air dried. Spots with phenolic content appear as blue spots on yellow background. The antioxidant activity was determined using DPPH reagent. The developed silica gel sheets were dried and sprayed with 0.04% solution of DPPH in 80% methanol. The antioxidant activity spots appear as yellow spots on purple background.

2.9. Phytochemical screening

Standard procedures for phytochemical screening described by Sofowara[15], Trease and Evans[16] and Harborne[17] were adopted to screen the methanolic extract of *E. hirta* L leaves for secondary metabolites

3. Results

3.1. DPPH assay

The methanolic extract of *E. hirta* leaves exhibited a maximum DPPH scavenging activity of (72.96±0.78)% at 1 mg/mL followed by the flowers, roots and stems whose scavenging activities were (52.45±0.66)%, (48.59±0.97)%, and (44.42±0.94)%, respectively. Whereas for BHT (standard) was found to be (75.13±0.75)% at the same concentration. The IC_{50} of the methanol extract of *E. hirta* leaves, flowers, roots, stems and BHT values were found to be 0.803, 0.972, 0.989, 1.358 and 0.794 mg/mL, respectively.

3.2. Reducing power

Figure 1 shows the dose- response curves for the reducing powers of the extract. It was found that the reducing power of the leaf extract also increased with increasing the concentrations. The reducing power of the extract was comparable with that of ascorbic acid (the positive control).

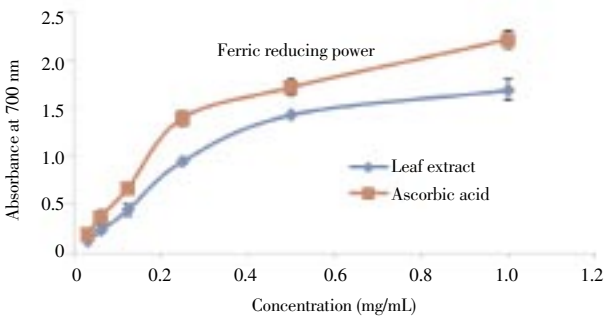

Figure 1 Reducing power of methanolic extract of *E. hirta* L leaves and ascorbic acid.

3.3. Total phenolics and flavonoids contents

The total phenol content is reported as gallic acid equivalents by reference to standard curve (Y= 0.0073X + 0.1003 and r^2 =0.987). Leaves extract had the highest total phenolic content [(206.17±1.95) mg GAE/g], followed by flowers, roots and stems extracts which were (117.08±3.10) mg GAE/g, (83.15±1.19) mg GAE/g, and (65.70±1.72) mg GAE/g, respectively.

The total flavonoids content is reported as catechol equivalents by reference to standard curve (Y= 0.0059X +

0.029 and r^2 = 0.9932). It was found that leaves also had the highest flavonoid contents which was (37.970±0.003) mg CEQ/g, followed by flowers, roots and stems extracts, which were (35.200±0.002) mg CEQ/g, (24.350±0.006) mg CEQ /g, and (24.120±0.004) mg CEQ/g, respectively.

3.4. HPTLC bioautography analysis of phenolic and antioxidant substance

Seven different spots were detected using the visible and ultraviolet lights in the HPTLC bioautography analysis with the retention factors (R_f value) values of 0.1, 0.2, 0.4, 0.75, 0.88, 0.92 and 0.96, respectively. Plates examined for DPPH active spots showed pale-yellow coloured spots on purple background, thus indicating the presence of antioxidant substances. First three spots close to the baseline spot (R_f values: 0.1, 0.2, 0.4) appeared as a yellow smear instead of separated spots. Three faint yellow spots also appeared on the top (R_f = 0.75, 0.92, 0.96). On the other hand, plates stained with Folin-Ciocalteu reagent exhibited several phenolic active blue colour spots with yellow background. Interestingly, the same spots that gave blue color with Folin-Ciocalteu reagent appeared yellow when sprayed with DPPH.

3.5. Phytochemical screening

The qualitative screening of phytochemical compounds in *E. hirta* revealed the presence of reducing sugars, terpenoids, alkaloids, steroids, tannins, flavanoids and phenolic compounds.

4. Discussion

Renewed interest in plant antioxidant has emerged during the recent years, probably due to the appearance of undesirable side effects of certain commercial antioxidant. In medicinal plants world, there are a huge number of different types of bioactive compounds with antioxidant activity that play an significant role in terminating the generation of free radical chain reactions. Hence in this study we evaluate *E. hirta* as a new antioxidant agent by various *in vitro* antioxidant tests.

DPPH radical is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. DPPH was reduced to a pale yellow color due to the abstraction of hydrogen atom from antioxidant compound. The more antioxidants occurred in the extract, the more the DPPH reduction will occur. High reduction of DPPH is related to the high scavenging activity performed by particular sample[18]. IC_{50} was calculated as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity. *E. hirta* leaves leaf extract showed the lowest IC_{50} value with highest antioxidant activity. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability[10].

Phenolics present in edible plants have received considerable attention because of their potential antioxidant activity. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phospho- molybdic acids present in the Folin-Ciocalteu reagent[19]. Good correlation was found between phenolic contents of the different parts extract (r^2=0.989) and their IC_{50} DPPH values. The result suggests that 98.89% of the plant antioxidant activity results from the activity of phenolic compounds. Also, it can be concluded that the antioxidant activity of the plant is not limited to phenolics. Activity may come from the presence of other antioxidant secondary metabolites such as vitamins, volatile oils and carotenoids which in this case contribute to about 1.1%. This significant correlation was in agreement with the findings of Yan and Asmah[20] and Oktay *et al*[21] who also found a strong relationship between the antioxidant capacity and the phenolic content. Therefore, our findings indicate that the antioxidant activity of the plant extracts might be exerted by the phenolic compounds in the plant. The phenolic compounds were found to play an important role in stabilizing lipid peroxidation and direct antioxidative activity because of their scavenging ability due to their hydroxyl groups[22-27]. Previous studies have reported that consumption of foods high in phenolic content can reduce the risk of heart disease by slowing the progression of atherosclerosis, since they act as antioxidants[28].

On the other hand, moderate correlation was found between flavonoids content of the different parts extract (r^2= 0.696) and their IC_{50} DPPH values. This indicates that flavonoids which are subgroups of phenolic compounds had a lesser antioxidant activity than phenolic compounds which are the dominant contributors to the antioxidant activity of the plant. Therefore, the antioxidant activity of *E. hirta* might be exerted by phenolic compounds other than flavonoids. HPTLC bioautography method was used to qualitatively detect the presence of phenolic and antioxidant substances in *E. hirta* leaf extract. This method provides reliable, rapid detection and easy localization of the active compounds in a plant extract[29]. Interestingly, the same spots that gave blue color with Folin-Ciocalteu reagent appeared yellow when sprayed with DPPH. This probably suggests that the antioxidant activity of the plant extract may be due to phenolic substances. Moreover, screening of phytochemical compounds in *E. hirta* revealed the presence of reducing sugars, terpenoids, alkaloids, steroids, tannins, flavanoids and phenolic may relate the antioxidant activities of *E. hirta* extract to the presence of these phytochemicals and thus the plant could serve as potential source in herbal medicine drugs.

In the present study, the antioxidant activity from four Parts of *E. hirta* (leaves, stems, roots and flowers) was evaluated spectrophotometricaly. *E. hirta* exhibited high phenolic and flavonoid content and significant antioxidant activity by DPPH scavenging assay and ferric reducing power

assay. The use of *E. hirta* as a natural antioxidant source appears to be an alternative to synthetic antioxidants. This study was the first report about the antioxidant activities of different parts of *E. hirta*. Further investigation to determine antioxidant activity by *in-vivo* methods could be considered.

Conflict of interest statement

We declare that we have no conflict of interest.

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