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Antioxidant Activity, Phenolic Compound Content and Phytochemical Constituents of *Eclipta prostrata* (Linn.) Linn.

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ABSTRACT

This study was carried out to investigate antioxidant activity, total phenolic content and phytochemical constituents of crude extract and fractions of *Eclipta prostrata*. Aqueous, ethanolic and various fractions of *E. prostrata* extracts were elucidated for antioxidant activity using DPPH assay and determined total phenolic content by Folin-Ciocalteau method. Moreover, the ability of plant extract on protein oxidation protection was also analysed. The results indicated that the EP07 fraction had the highest DPPH radical scavenging activity and total phenolic contents with GAE values 142.918 \pm 0.762 and 493.205 \pm 10.174 mg GAE/g extract, respectively. Moreover, crude ethanolic extract of *E. prostrata* could inhibit protein degradation induced by H₂O₂/Cu²⁺ catalyzed reaction by 51.94, 57.91, 62.69 and 63.23 %, after treatment with the crude extract at concentrations of 5, 10, 15 and 20 mg/ ml, respectively. In addition, flavonoids were found as major constituents in the EP07 fraction. Therefore, extracts of *E. prostrata* should be used as antioxidant and dietary supplement.

Keywords: Eclipta prostrata, antioxidant activity, phytochemical analyse

1. INTRODUCTION

Free radical especially reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by either normal physiological process in our body such as inflammation, the respiratory burst and many exogenous factors on environmental interaction including ultraviolet light, stress, pesticides, environmental pollutants, smoking and radiation. Moreover, free radical and relative oxygen species are caused by oxidation of biomolecule such as DNA, proteins, carbohydrates, and lipids. This may lead to cell death, cellular compound destruction, DNA breaks, mutagenesis, protein inactivation and membrane disruption. Thus, the effects of free radical were reported to be involved in aging process, cancer, diabetes, and cardiovascular diseases [1, 2]. In addition, protein oxidation resulted in a deleterious loss of protein or enzymatic activity function and protein signaling [3]. However, antioxidant supplement can help human body to reduce oxidative damage by free radical and oxygen species.

Eclipta prostrata or (syn. E. alba), plant is classified in family Asteraceae. It is widely distributed in tropical and subtropical area in the world and commonly known as Kra-meng in Thailand. The plant has been used in traditional medicine in Asia. Many reports showed that the stem of E. prostrata was useful in remedy of hypercholesterolemia, hepatic disease, hyperlipidemia and spleen enlargement [4]. The leaves of this plant have been used to treat skin diseases, while the stems have been used for treatment of anemia, asthma and tuberculosis. Moreover, the root has been used as hepatoprotectant and antibacterial agent [5, 6]. This plant has other activities such as antibacterial [7], anticancer [8], antifungal [9], antioxidant [10] and antiproliferative activities [11]. Thus, this study aimed to investigate antioxidant activity, total phenolic compound content and phytochemical constituents in crude plant extracts of E. prostrata and their fractions for an application as antioxidant and dietary supplement.

2. MATERIALS AND METHODS

2.1 Plant Materials

The ground plant material of *E. prostrata* was purchased from Lampang Herb Conservation, Thailand. The plant material was extracted with distilled water for 3 hours or macerated with 95% ethanol for 72 hours with the ratio of 1:10 (w/v). The extract was collected and filtered through Whatman[®] No. 1 filter paper. After that, the solvent was removed by rotary evaporator at 45°C under reduced pressure at 50 mbar in a rotary evaporator (Buchi[™]) and further lyophilized

to obtained crude extract powder. The percentage yields of aqueous and ethanolic extracts were 6.64 and 9.11 %, respectively.

2.2 Plant Fractionation

The crude ethanolic extract of E. prostrata was separated by partition method. Four solvents including hexane, ethyl acetate, n-butanol and water were selected to separate active constituents based on their polarity. The property of hexane, ethyl acetate, n-butanol and water were used to extract constituents from low to high polarity. The crude ethanolic extract (25 g) was initially dissolved in 50 ml of water and then partitioned with 50 ml of hexane four times to obtained hexane extract. After that, the water layer was partitioned with 50 ml of ethyl acetate four times to obtained ethyl acetate extract. Next, the water layer was partitioned with 50 ml of n-butanol four times to obtained n-butanol and water extracts. Supernatants from each partition were collected and evaporated to obtain the hexane, ethyl acetate, n-butanol and water extracts. The extracts were subsequently tested for their antioxidant activity.

The fraction with the highest antioxidant activity was further separated using liquid column chromatography on siliga gel (Merk Ltd., Lutterworth, UK) using gradient solvent system composed of choroform/acetone/methanol (85:5:5), chloroform/acetone/methanol (85:5:10), chloroform/ethanol (50:50) and finally eluted with ethanol. Fractions were collected and combined on the basis of their TLC chromatogram after spraying with anisaldehyde sulfuric acid reagent and visualization with ultraviolet light (254 and 366 nm). The eluted samples were collected in 16 fractions, dried under evaporator and then antioxidant activity was tested. The sample with high antioxidant activity was further determined phytochemical constituents.

2.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

Antioxidant activity of plant extracts was performed by DPPH radical scavenging assay, which was modified from previously described methods [12, 13]. Briefly, 0.5 ml of plant extracts at various concentrations were incubated with 1.5 ml of 0.1 mM DPPH in methanol solution. The reaction mixture was left in the dark for 20 minutes at room temperature and the absorbance was determined at 517 nm. All measurements were performed three times. The percentage of DPPH radical inhibition was calculated using this following equation:

% Inhibition = $[(A_0 - A_1)/A_0] \times 100$

 A_0 is the absorbance of control and A_1 is the absorbance of the tested sample after treatment with extracts. The half maximal (50%) inhibitory concentration (IC₅₀) was calculated from the regression line between % inhibition (y) and sample concentration (x) in the graph. The free radical scavenging ability was expressed as mg gallic acid equivalent, GAE/g extract.

2.4 Total Phenolic Compound

Total phenolic content in the extract was determined by the modified Folin-Ciocalteau method [14]. 250 μ l of each extract was mixed with 250 μ l of Folin-Ciocalteau reagent, 250 μ l of ethanol and 1.25 ml of distilled water, respectively. Then, the mixture was incubated for 5 minutes at room temperature. After that, 250 μ l of Na₂CO₃ was added to the mixture and incubated for 1 hour in the dark at room temperature. The absorbance was measured at 725 nm. The experiment was performed in three replicates and gallic acid was used as a standard for the calibration curve. Total phenolic compounds content was expressed as gallic acid equivalent.

2.5 Protective Oxidative Protein Damage

The modified hydroxyl radical mediated oxidation experiment was conducted using metal catalyzed reaction [15, 16]. Briefly, BSA was dissolved in 150 mM of phosphate buffer saline (pH 7.3) to a final concentration of 5 mg/ml. After that, BSA was incubated with or without 0.5 mM Cu²⁺ and 12.5 mM H_2O_2 in the presence or absence of crude plant extracts. The reactions were performed at 37°C for 30 minutes. Dimethyl sulfoxide (DMSO) and glutathione (GSH) were used as solvent control and positive control, respectively.

The protein quantification was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The treated and untreated BSA were mixed with loading dye (0.125 M Tris-Cl, 8% Sodium dodecyl sulfate (SDS), 20% (v/v) Glycerol, 0.2 % bromophenol blue, pH 6.8) and heated at 100°C for 2 minutes. The protein sample was loaded in 10 % SDS gel electrophoresis. After 2 hours, the gel was stained with commassie brilliant blue for 1 hour and destained until the background was clear. The protein intensity was measured using Gene Tool Match (LAB Focus, Co.ltd).

2.6 Phytochemical Analysis

A preliminary phytochemical analysis was carried out using standard procedure [17] to detect the presence of alkaloids, flavonoids, coumarins, saponins, cardiac glycosides, anthraquinone glycosides and tannins.

2.7 Statistical Analysis

All experiments were performed in triplicate and the experimental results were recorded as mean \pm SD of three measurements. The experimental data were analyzed using one-way analysis of variance (ANOVA). Significant differences between means were determined by Duncan's new multiple-range test using SPSS version 17.0 (p < 0.05 was regarded as significant)

3. RESULTS AND DISCUSSION

3.1 DPPH Radical Scavenging Activity

DPPH is stable nitrogen free radicals and has been widely used to determine the free radical scavenging ability of various samples. The effect of antioxidant on DPPH radical was through their hydrogen or electron donor ability. The color of reaction mixture was changed from purple to yellow when DPPH radical was scavenged with decrease of an absorbance at 517 nm [18]. The antioxidant ability of plant extracts was expressed as IC550 and gallic acid equivalent (GAE). The DPPH radical scavenging activities of crude extract and fractions of this plant were presented in table 1. It was founded that IC₅₀ of crude extract and their fractions ranging from 0.053-0.891 mg/ml. The ethanolic extract showed strong DPPH radical scavenging activity of 23.462 ± 0.806 mg GAE/g extract, which was more than the scavenging activity of aqueous extract $(15.355 \pm 0.263 \text{ mg GAE/g extract}).$ Therefore, ethanolic extract of this plant was selected to fractionate by partition method. The results showed that n-butanol fraction demonstrated the highest antioxidant activities with 94.769 \pm 1.800 mg GAE/g extract followed by ethyl acetate, water

and hexane fractions by 67.210 \pm 0.056, 13.387 \pm 0.262 and 5.614 \pm 0.151 mg GAE/g extract, respectively. After that, n-butanol fraction was further fractionated by column chromatography. From table 2, IC₅₀ of various fractions ranged from 0.035-0.328 mg/ml. Subfraction EP07 showed significantly (p < 0.05) high antioxidant activity compared to n-butanol fraction with 142.918 \pm 0.762 mg GAE/g extract.

These results revealed that the ethanolic extract of *E. prostrata* had higher ability to scavenge DPPH radical more than aqueous extract. After fractionation, the ability of plant extract to scavenge DPPH radical has been significant increasing. Other study was demonstrated that crude ethanolic extracts from the leaves of Eclipta alba showed strong antioxidant activity by DPPH assay [19]. In addition, ethanolic extract of E. alba had high antioxidant activities, which analyzed by DPPH, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays and scavenging of nitric oxide radical with IC₅₀ 21.35, 41.8, 17.55 mg/ml, respectively [20]. Moreover, numerous studies have demonstrated antioxidant activity from various plant species. Ethanolic extract of Vitex negundo leaves showed high antioxidant activity after testing with DPPH assay and also showed high total phenolic and flavonoid content [21]. Additionally, the essential oil from Mesua ferrea leaves demonstrated high DPPH radical scavenging ability [22].

	DPPH		Total phenolic
Extracts/Fractions			compound
	IC_{50} (mg/ml)	mg GAE /g	mgotGtAtEt/g
	30 1 0 7	$extract^* \pm SD$	$extract^* \pm SD$
Crude extracts			
ethanolic extract	$0.213 \pm 0.007^{\circ}$	23.462 ± 0.806^{d}	$36.731 \pm 0.485^{d}A$
aqueous extract	$0.326\pm0.006^{\rm d}$	$15.355 \pm 0.263^{\circ}$	$29.337 \pm 0.962^{\circ}$
Fractions			
hexane	$0.891\pm0.024^{\rm f}$	5.614 ± 0.151^{a}	16.627 ± 0.366^{a}
ethyl acetate	0.074 ± 0.000^{b}	$67.210 \pm 0.056^{\circ}$	74.820 ± 0.905^{e}
n-butanol	0.053 ± 0.001^{a}	$94.769\pm1.800^{\rm f}$	$90.288 \pm 0.240^{\rm f}$
aqueous	0.374 ± 0.007^{e}	13.387 ± 0.262^{b}	$18.945 \pm 0.749^{\mathrm{b}}$

Table 1. Antioxidant activity and total phenolic compound content of crude *E. prostrata* extracts and fractions.

(*) The experiment data are given as mean \pm standard deviation (SD). Values with different alphabets within each column are significantly different (p < 0.05) by one way ANOVA.

Table 2. Antioxidant activity and total phenolic compound content of n- butanol fraction of *E. prostrata*.

	DPPH		Total phenolic compound content
Fractions	IC50 (mg/ml)	(mg GAE /g extract)*±SD	(mg GAE /g extract)*±SD
EP01	$0.158\pm0.002^{\rm f}$	31.633 ± 0.392°	$135.891 \pm 2.544^{\circ}$
EP02	-	-	-
EP03	-	-	-
EP04	-	-	-
EP05	-	-	-
EP06	$0.042 \pm 0.000^{\rm b}$	$119.887 \pm 0.607^{\rm i}$	377.698 ± 3.391^{j}
EP07	$0.035\pm0.000^{\text{a}}$	$142.918 \pm 0.762^{\rm j}$	$493.205 \pm 10.174^{\rm k}$
EP08	$0.069\pm0.000^{\rm d}$	$71.965 \pm 0.322^{\rm f}$	$258.193 \pm 2.544^{\rm g}$
EP09	$0.092 \pm 0.000^{\circ}$	54.381 ± 0.170^{d}	$205.835 \pm 5.087^{\mathrm{f}}$
EP10	$0.071\pm0.000^{\mathrm{d}}$	70.475 ± 0.237^{e}	$276.978 \pm 0.848^{\rm h}$
EP11	$0.162\pm0.003^{\rm f}$	$30.902 \pm 0.627^{\circ}$	151.479 ± 2.544^{d}
EP12	$0.214\pm0.003^{\rm g}$	23.414 ± 0.273^{b}	$116.307 \pm 1.696^{\mathrm{b}}$
EP13	$0.328\pm0.009^{\rm h}$	15.241 ± 0.411^{a}	89.528 ± 4.239^{a}
EP14	$0.092 \pm 0.001^{\circ}$	$54.140\pm0.428^{\rm d}$	$180.256 \pm 11.022^{\circ}$
EP15	$0.046 \pm 0.000^{ m b}$	$107.542 \pm 0.417^{\rm h}$	310.152 ± 4.239^{i}
EP16	-	-	-
n-butanol	$0.053\pm0.001^{\circ}$	$94.769\pm1.800^{\mathrm{g}}$	90.288 ± 0.240^{a}

(*) The experiment data are given as mean \pm standard deviation (SD). Values with different alphabets within each

(-) not determined

column are significantly different (p<0.05) by one way ANOVA.

3.2 Total Phenolic Compound Content

Phenolic acids are one of the major groups of compounds acting antioxidant activity by donating hydrogen or electron [23]. The results from table 1 and 2 showed that crude ethanolic extract of E. prostrata had high total phenolic content of $36.731 \pm$ 0.485 mg GAE/g extract compared to aqueous extract $(29.337 \pm 0.962 \text{ mg GAE}/$ g extract). After fractionation, n- butanol fraction demonstrated the highest total phenolic compound of 90.288 \pm 0.240 mg GAE/g extract, followed by ethyl acetate, aqueous and hexane fractions, which showed phenolic contents of 74.820 ± 0.905, 18.945 \pm 0.749 and 16.627 \pm 0.366 mg GAE/g extract, respectively. After that, n-butanol fraction was further purified using column chromatography and subfraction EP07 showed significantly the highest total phenolic contents of 493.205 \pm 10.174 mg GAE/g extract compared to other fractions.

This result indicated that the ethanolic extract had total phenolic content higher than aqueous extract, and the partial purified fraction of these plants showed antioxidant activity higher than crude extract. Phenolic compounds such as phenolic acids, tannins and flavonoids are widely found from plant and they have been shown to possess various biological activities including antiinflammatory, anti-carcinogenic and antiatherosclerotic activities and they might be related to antioxidant activity [24, 25]. Previous study founded that the hydroalcoholic of this plant could inhibit the cell proliferation and also down regulated the expression level of matrix metalloproteinases (MMP-2 and 9) and NF-kB in HepG2, C6 glioma and A498 cell lines [8]. Furthermore, the 30% ethanol fraction and eclalbasaponin I inhibited the

proliferation of hepatoma cell in dose dependent manner [26].

3.3 Protection of Oxidative Protein Damage by Medicinal Plant Extracts

The oxidation of proteins are usually caused by ROS and RNS that may lead to protein structure modification such as fragmentation, polymerization, conformation change, loss of activity and loss of amino acids [27]. Hydroxyl radical (OH) is one of the most radicals found in human body reacting with almost all molecules in living cell and can damage to DNA, lipids and proteins [28]. In this study, oxidative protein damage was induced by the metal catalyzed reaction of BSA by H₂O₂ and Cu²⁺. Band intensity and percentage of prevention of oxidative protein damage by ethanolic extract of *E. prostrata* was shown in figure 1. Glutathione at the concentration of 5 mg/ml was used as a positive antioxidant compound. Dimethyl sulfoxide slightly decreased the band intensity of BSA as compared to the untreated control. The result revealed that the treated BSA with H_2O_2 /Cu²⁺ showed protein intensity by 29.59%. After treatment with crude E. prostrata extract at concentration of 5, 10, 15 and 20 mg/ml, the protein intensity was increased by dose dependent manner by 51.94, 57.91, 62.69 and 63.23 %, respectively. Moreover, glutathione which was a positive control showed oxidative protein damage prevention ability by 89.09 %. Similarly, the previous studies demonstrated that culinary plants, celery showed the highest inhibition of metal catatlyzed of BSA whereas basil, Thai copper pod, star fruit and red sorrel extract did not protect oxidative protein damage [16].



Figure 1. PAGE profile of BSA protein (a) and percent of BSA protein protection (b) after treatment with ethanolic extract of *E. prostrata* M = marker, U= untreated BSA, T = Cu²⁺ and H₂O₂ treated BSA, C = control DMSO, G = Glutathione treated BSA, 5= 5 mg/ml of extract treated BSA, 10= 10 mg/ml of extract treated BSA, 15= 15mg/ml of extract treated BSA, 20= 20 mg/ml of extract treated BSA.

3.4 Phytochemical Screening Analysis

For phytochemical analysis, flavonoids and hydrolysable tannins were found as major constituents in ethanolic extract of *E. prostrata* while flavonoids and phenolics were found in fraction EP07. Similar results were previously reported that triterpenoids, flavonoids and phenolic acids were found as major constituents in methanolic extract of *E. prostrata* [29]. Tannins, flavonoids, coumestans, saponin and alkaloids were also presented in methanolic extract of the plant, *E. prostrata* [30]. Thus, the presence of flavonoids in *E. prostrata* was likely to be responsible for the free radical scavenging activity in this study.

4. CONCLUSION

Aqueous, ethanolic extracts and fractions of *E. prostrata* extracts were investigated for anti-free radical activity by DPPH assay. Moreover, total phenolic contents and the prevention of oxidative protein damage ability by *E. prostrata* extracts were determined. From the present finding demonstrated that the partial purified EP07 fraction of *E. prostrata* showed effective antioxidant activity and total phenolic content more than crude extract. Moreover, crude ethanolic extract of this plant could inhibit the oxidation of BSA protein. In addition, flavonoids were found as major constituents in the EP07 fraction. Further study on determination of toxicity of the *E. prostrata* extracts should be carried on in order to use the plant extracts as antioxidant and dietary supplement.

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