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Research Article

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Antioxidant activities of extract and fractions from *Clerodendrum inerme*

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ABSTRACT

The antioxidant activities of ethanolic crude extract (ECE) and its five different solvent sub-fractions (namely, petroleum ether fraction (PEF), dichloromethane fraction (DMF) ethyl acetate fraction (EAF), n-butyl alcohol fraction (BAF) and the rest fraction (RF) from Clerodendrum inerme were investigated using several in vitro antioxidant assays. ECE and five sub-fractions possessed different antioxidant and radical-scavenging activities in different assays. BAF showed the most potent radical-scavenging activity on DPPH radicals with EC_{50} value of 0.28 mg/ml. EAF exhibited the highest ABTS radicals with EC_{50} value of 0.46 mg/mL. The total phenolics contents (TPC) and total flavonoid contents (TFC) were also determined. RF had the highest TPC (10.73 mg GAE/g DW), and BAF had the highest TF contents C (3.81 mg RT/g DW). Our work offers theoretical basis for C. inerme as a potential source of natural antioxidants.

Keywords: *Clerodendrum inerme*; Antioxidant activity; Free-radical scavenging activity; Phenolics; Flavonoids

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INTRODUCTION

Oxidative damage of biological molecules in the human body was implicated in degenerative or pathological processes, such as aging, cancer, atherosclerosis, gastric ulcer, and other conditions. Moreover, oxidation and the formation of free radicals were the major causes of deterioration of various foodstuffs [1]. In order to protect foods and human beings against oxidative damage caused by free radicals, synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) were created because of demand. However, consumers' concern has come to focus on the toxicity and potential health hazards of synthetic antioxidants [2, 3]. Therefore, in recent years interest in utilizing natural antioxidants has increased substantially, especially plant phenolics, flavonoids, are desired to protect the human body from oxidative stress and retard potential chronic diseases of aging. Considerable evidences had confirmed that medicinal plants are promising sources of natural antioxidant compounds, as many of the phytochemicals from plant extract have been identified to exhibit antioxidant activity [4]. Correlation studies have demonstrated the significant contribution which dietary intake of natural antioxidants such as flavonoids and other phenolic compounds, present in most plants, may act as potent candidates in preventing diseases related to oxidative stress, such as cancer, atherosclerosis, aging and rheumatoid arthritis [5]. The antioxidant activity of phenolic compounds depends on their structure, position and number of hydroxyl groups, polarity, and mostly the bond dissociation energy necessary to remove the hydrogen atom [6]. The mechanism of antioxidant activity of flavonoids involves the direct scavenging or quenching of oxygen free radicals or excited oxygen species, as well as the inhibition of oxidative enzymes that generate these reactive oxygen species [7].

As part of our efforts to find antioxidants from edible herbs, we have investigated the antioxidant potential of *Clerodendrum inerme*, a plant which belongs to the genus *Clerodendrum* of the family *Lamiaceae* (*Verbenaceae*). This genus is represented by 580 species of small trees, shrubs, lianas, or, occasionally, perennial herbs, most

growing in tropical and subtropical regions [8]. The species *C. inerme*(Linn.) Gaertn., semi-mangrove plant, were widely distributed in India, Southeast Asia and North Oceania, which could also been found in China intertidal estuarine zones, especially the Hainan island coast has the richest wild sources [9]. It was demonstrated to be an important and well-known traditional herbal on the treatment of various ailments, such as coughs, serofulous infection, buboes problem, venereal infections, skin diseases and as a vermifuge, febrifuge and also to treat Beriberi disease, also local people use it as an antidote of poisoning from fish, crabs and toads [10]. Among the various phytochemicals in *C. inerme* leaves, phenolic compounds, particularly flavonoids, are widely regarded as some of the major bioactive compounds which have been shown to possess various therapeutic properties [11-13]. Several studies has already been conducted and it was demonstrated that *C. inerme* may be an excellent source of antioxidants: its methanolic extract has strong free radical-scavenging activity [14]. In contrast, the present study was to investigate the antioxidative capacity of extract and fractions with different polarity that were derived from *C. inerme*, using the *in vitro* methods, such as the scavenging activity on 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline- 6-sulphonicacid) (ABTS) were evaluated. Furthermore, total phenolic content (TPC) and total flavonoid content (TFC) as the antioxidants, of all extract and fractions were also determined.

EXPERIMENTAL SECTION

Plant materials. Leaves of *C. inerme* were collected in from the lakeside of Hainan University in April 2012 and identified by Prof. Dr. Xiaobo Yang, College of Landscape and Horticulture, Hainan University, China. Leaves were selected, washed thoroughly in potable water, and then dried for 36 h using a hot air oven at 60℃. Dried leaves were then powdered using a herb disintegrator (118 Swing, Zhejiang, China) and subsequently sieved (20 mesh).

Extraction procedure. The leaves (480 g) were extracted with ultrasonic wave assisted extraction method according to the protocol previously reported [15] using benign solvent ethanol and water under the condition of ethanol concentration 70%, solid/liquid ratio of 1:8, extraction time 60 min and extraction temperature at 60℃. Subsequently, the plant material was then filtered off and the extraction procedure was repeated three times. The combined filtrates were concentrated using rotary vacuum evaporator, to obtain dry extracts. Finally, from 480 g of the dry samples, the final yield of ECE was 57.34 g. Of the 57.34 g of dry extract, 5.0 g was redissolved in 60% ethanol to a concentration of 50 mg/ml and stored in the dark at 4℃ for further use. The rest of the dry extract was redissolved in distilled water (The solvent/water ratio was 1:2) and then sequentially extracted with petroleum ether (60–90℃), ethyl acetate and *n*-butanol, using liquid–liquid partition. After removal of the solvents, using a vacuum rotary evaporator, the concentrated solutions were lyophilised to get the dry form of respective fractions, the final yields of the PEF, DMF, EAF, BAF and RF were 3.23, 3.17, 8.55, 9.53 and 26.6 g, respectively. The four fractions were redissolved in their respective solvents accordingly, to a concentration of 50 mg/ml and stored in an amber vial at 4℃ until used to determine their antioxidant activities.

DPPH radical scavenging capacity measurement. The radical scavenging ability of 2,2'-diphenyl-b-picryl hydrazyl (DPPH) was estimated by a method adapted from Sharififar *et al* [16]. Thus, an aliquot of extract (0.1 mL) was added to 3.9 mL of ethanolic DPPH (60μM). The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark and absorbance was measured at 517 nm. The free radical scavenging activity was calculated as follows:

$$\%\mathrm{RSA} = \left[\left(A_{blank} - A_{sample}\right) / A_{blank}\right] \times 100\%$$

where A_{blank} was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} was the absorbance of the test compound.

ABTS radical scavenging capacity measurement. Free radical scavenging capacity using a stable ABTS radical was performed according to Deng *et al* [17]. The ABTS radical solution was produced by gently mixing 10 mL of 7 mM ABTS solution and 10 mL of 2.45 mM potassium persulfate solution. This was allowed to stand in the dark at room temperature for 12–16 h. The ABTS radical solution was adjusted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm before usage. Extract (100 μl) or ethanol (100 μl, control) was added to 3.9 mL ABTS radical solution and allowed to react for 30 min until a stable absorbance was obtained. The decrease in absorbance at 734 nm was measured against a blank (ethanol). Antioxidant activity of ABTS radical scavenging capacity was calculated as a scavenging percentage:

$$\%\mathrm{RSA} = \left[\left(A_{blank} - A_{sample}\right) / A_{blank}\right] \times 100\%$$

where A_{blank} was the absorbance of the control reaction(containing all reagents except the test compound), and A_{sample} was the absorbance of the test compound.

TPC measurement. TPC from leaf extracts was measured according to the Folin-Ciocalteu (FC) procedure [2] described previously. The FC phenol reagent was prepared according to King's method [18]. Thus, 10 g sodium tungstate and 2.5 g sodium molybdate were gently dissolved in 70 mL deionized water, 5 ml 85% phosphoric acid, and 10 mL concentrated hydrochloric acid were subsequently added and allowed to reflux for 10 hr. Then, 1.5 g lithium sulfate and 6 mL hydrogen peroxide were added and refluxed for another 15 min until the color changed to a glassy yellow. The volume of the reaction mixture obtained was increased to 100 ml (q.s., deionized water) before usage. Then, 2 mL of diluted extracts were mixed with 2 mL of FC reagent. After 3 min, 750 μL of sodium carbonate anhydrous solution (7.5%, w/v) was added and the sample was vortexed. The absorbance at 765 nm versus a blank control was measured with a UV light spectrophotometer (Shimadzu UV2754) after a 2-h incubation in the dark at room temperature. Measurements were calibrated to a standard curve of prepared gallic acid solution ranging from 0–100 μg/mL with $y = 0.0480x - 0.0071$ ($R^2 = 0.9991$) and TPC was then expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

TFC measurement. Estimation of TFC in extracts was performed according to colorimetric method [19] with some modifications. The reaction mixture contained 1.0 mL of extract, 4 mL of 60% ethanol and 0.3 mL of 5% sodium nitrite. Six minutes later, 0.3 mL of 10% aluminium nitrite was added. In the next six minutes, 4 mL of 1 M sodium hydroxide solution were added and the volume was increased to 10 mL (q.s. 60% ethanol). Immediately, the reaction mixture absorbance was measured by a spectrophotometer at 510 nm against a blank (control) and used to calculate TFC using rutin as a standard $y = 0.0116x - 0.0048$, ($R^2 = 0.9991$). The linear relationship between absorbance and flavonoids content ranged from 15–90 μg/mL. TFC was then expressed as rutin equivalents (RE), in mg RE per g DW.

Statistical analysis. Results were expressed as mean ± standard deviation of replicate solvent extractions and triplicate of assays and analyzed by Statistical Analysis System (EXCEL 2007). Data were analyzed by ANOVA ($p < 0.05$).

RESULTS AND DISCUSSION

Scavenging effect on DPPH free radical. The ability of fractions from *C. inerme* to quench reactive species by hydrogen donation was measured through the DPPH radical scavenging activity assay. As a kind of stable free radical, DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule, which is widely used to investigate radical scavenging activity. The antioxidants can react with DPPH, a deep-violet coloured stable free radical, converting it into a yellow coloured a,a-diphenyl-b-picrylhydrazine. The discolouration of the reaction mixture can be quantified by measuring the absorbance at 517 nm, which indicates the radical-scavenging ability of the antioxidant [20]. Fig. 1 illustrates a significant decrease in the concentration of DPPH due to the scavenging activities of the samples. The DPPH radical scavenging capacity of the samples was found to increase in dose dependent manner with increasing concentration in the range of 0.4-4 mg/ml. With regard to EC_{50}, as shown in Table 1, amongst all the extracts examined, the BAF, with the lowest EC_{50} (0.28 mg/ml), exhibited the highest DPPH radical-scavenging activity, followed by EAF (EC_{50} was 0.66 mg/ml), whilst the PEF with the highest EC_{50} value (11.71 mg/ml) exhibited the lowest DPPH radical-scavenging activity. VC and VE were used as positive controls with EC_{50} values of 0.09 and 0.32 mg/ml, respectively. The DPPH radical-scavenging activity was found to be in the order of: VC> VE> EAF > BAF > ECE >DMF >RF > PEF.

Scavenging effect on ABTS free radical. ABTS radical assay is one of the most commonly employed methods for measuring antioxidant capacity. It is recommended for use in plant extracts because the long wavelength absorption maximum at 734 nm eliminates colour interference in plant extracts [21]. As shown in Fig. 2, it was generally observed that the ABTS radical-scavenging effect increased as the concentration of the solvent extract increased. At the concentration of 0.8 mg/ml, ABTS radical-scavenging activities were ranked in the order: EAF (62.9%) > BAF (58.3%) > ECE (33.1%) > DMF (26.9%) > RF (13.7%) > PEF (6.6%). At the concentration of 1.6 mg/ml, the order is: ECE (87.6%) >BAF (85.2%) > DMF (81.3%) > EAF (78.9%) > RF (28.5%) > PEF (11.3%). Even with further increases in the concentration, ABTS radical-scavenging activities of ECE, DMP, EAF and BAF to a certain extent, and then levelled off. At the highest dosage level of 4.0 mg/ml, RF showed a radical-scavenging activity of 73.58% more than PEF (32.6%). EC_{50} values of ECE and each sub-fractions, shown in Table 1, clearly indicate that the EAF fraction exhibited the highest ABTS radical-scavenging activity with the lowest EC_{50} of 0.46 mg/ml. Meanwhile, VC and VE, serving as the positive controls, exhibited EC_{50} values of 0.07 mg/ml and 0.21 mg/ml, respectively.

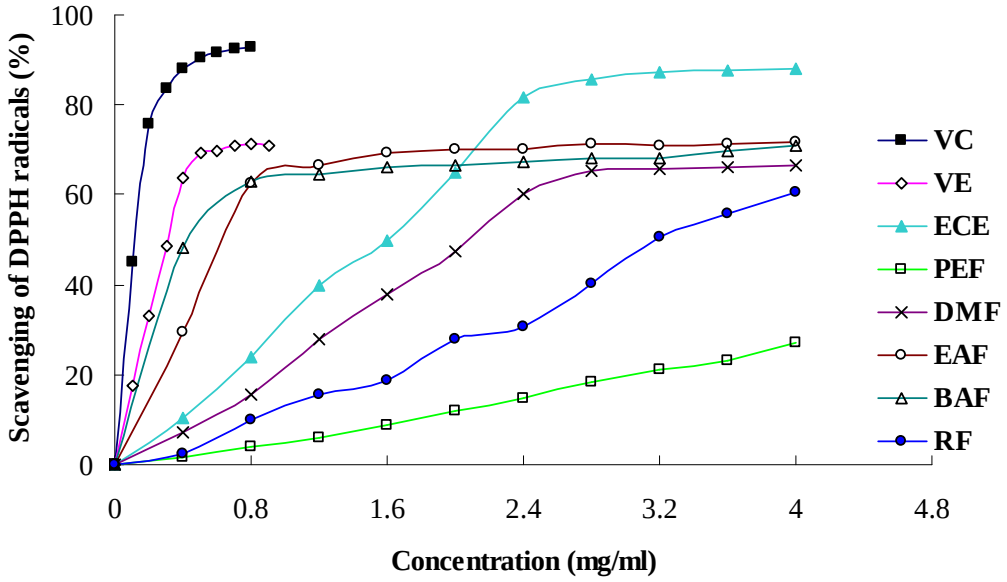

Fig. 1. Free radical (1,1-diphenyl-2-picrylhydrazyl (DPPH)) scavenging activities of ECE and various sub-fractions at different concentrations

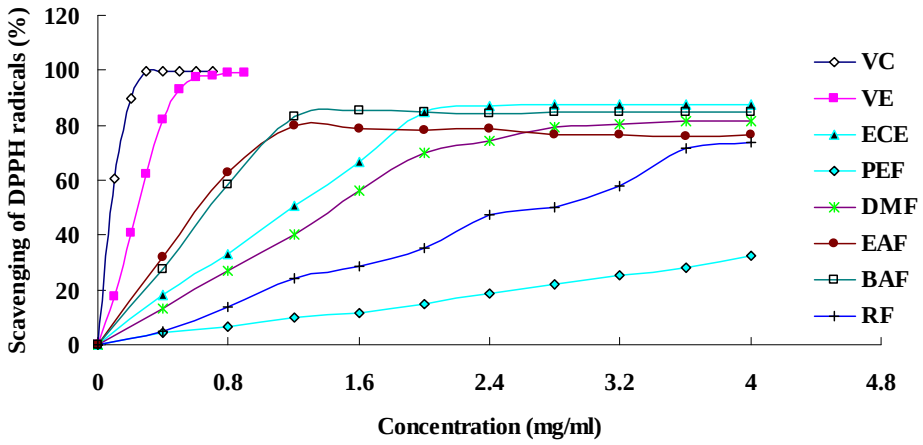

Fig. 2. Free radical (2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulphonicacid)(ABTS)) scavenging activities of ECE and various sub-fractions at different concentrations

Antioxidant components. Many studies have found that plant extracts possess potent antioxidants, such as phenolics and flavonoids [7, 20]. In the present study, the total phenolic (TP) and total flavonoid (TF) contents of ECE and various sub-fractions of *C. inerme* were determined. The total phenolics contents of sub-fractions were in the order of RF > BAF > EAF > DMF > PEF, the total flavonoids in the order of EAF >BAF > RF >DMF > PEF (Table 2).

Table 1. EC_{50} values obtained in the antioxidant activity assays and contents of total phenolics and total flavonoids of ethanolic extracts and sub-fractions from *C. inerme*

EC_{50} value (mg extract/ml)	ECE	PEF	DMF	EAF	BAF	RF	VC	VE
DPPH radicals	1.34	11.71	2.14	0.66	0.28	3.45	0.09	0.32
ABTS radicals	1.02	11.20	1.35	0.46	0.55	2.48	0.07	0.21

Table 2. Contents of total phenolics and total flavonoids of ethanolic extracts and sub-fractions from *C. inerme*

Fraction	ECE	PEF	DMF	EAF	BAF	RF
TPC(mg RT/g DW)	8.61	0.43	0.44	1.22	1.33	3.81
TFC(mg GAE/g DW)	25.71	0.23	0.25	10.73	7.89	1.85

CONCLUSION

In conclusion, the antioxidant capacity of the extract from *C. inerme* and its five different solvent sub-fractions have antioxidant activity, as seen in the DPPH and ABTS free radical assay. Total phenolic content (TPC) and total flavonoid content (TFC) assays were used to quantify antioxidant compounds. Our data suggested that *C. inerme* possess direct and potent radicals scavenging activities through multiple mechanisms. Of all the four fractions, BAF and EAF showed the most potent antioxidant properties. Further work should be done to isolate and identify the specific compounds in BAF and EAF that are responsible for the antioxidant capability.

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