Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(1):986-993

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Determination of the total phenolic and flavonoid contents in *Eupatoriurn odoraturn*, as well as their antioxidant activities

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ABSTRACT

Eupatoriurn odoratum aerial parts were extracted with ethanol using ultrasonic-assisted extraction (UAE). Total phenolic content (TPC) and total flavonoid content (TFC) were used to quantify antioxidant compounds with the values of 2.772±0.3 mg GAE (Gallic acid equivalents)/g DW and 7.085±0.2 mg of RT (Rutin)/ g DW. The activities of scavenging 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6 -sulphonicacid) (ABTS) radicals, hydroxyl radicals, ferric ion reducing power and ferrous ion-chelating activity were analyzed, with IC_{50} values of 3.00, 1.08, 1.18, 0.14 and 3.16 mg/ml, respectively. The study offers theoretic basis for pharmaceutical utilization of the medicinal plant E. odoratum.

Keywords: *Eupatoriurn odoratum*; Total phenolic content (TPC); Total flavonoid content (TFC); 2, 2'-diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS); Hydroxyl radicals, Ferric ion reducing power; Ferrous ion-chelating activity

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INTRODUCTION

Considerable evidences had confirmed that oxidative damage of biological molecules in the human body was involved in degenerative or pathological processes, such as aging, cancer, atherosclerosis, gastric ulcer, and other conditions [1]. Natural and synthetic antioxidants have a long history as preservatives in food, where they specifically retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals. Some synthetic antioxidants such as *n*-propyl gallate, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are commonly used to act against free radicals in food and biological systems, but their applications are restricted due to potential risks related to health [2, 3]. Therefore, there is a growing interest to identify antioxidants from natural sources.

Eupatorium odoratum Linn (*Asteraceae*: *Eupatoriae*), a perennial shrub, were historically used as folk medicinal plant for the treatment of various ailments, which were demonstrated to be an important and well-known traditional herbal. The aqueous and decoction of the leaves was used for the treatment of soft wounds, burn wounds and skin infections in Vietnam [4]. The juice of the aerial parts of this plant was used for cuts and wounds to arrest bleeding

and promote healing [5]. The macerated leaves were usually applied to swollen portion of the body to relieve inflammation amongst the rural populace in southern part of Nigeria [6]. Other medicinal uses include immunomodulator, antispasmodic, hepatoprotective, antiprotozoal, antidiabetic, antihypertensive, antipyretic [7-10]. Most of the antioxidant potentials in herbs and spices are due to the redox properties of phenolic compounds that allow them to act as reducing agents, hydrogen donators and free radicals quenchers. In several studies, phenolic acids, flavonoids, alkaloids, triterpenes and sterols have been isolated from various plant parts, phenolic acids and flavonoids comprise the major constituents of the plant [11-13].

Many researchers have studied the antioxidant activity of *E. odoratum*. Afolabi et al. reportec the antioxidant activity of *E. odoratum* in methanolic extract using DPPH scavenging activity [14]. Alisi and Onyeze showed nitric oxide scavenging ability of ethyl acetate fraction of methanolic leaf extracts of *E. odoratum* [15]. Rao et al. investigated the total phenolic content and antioxidant activities of chloroform extracts of Indian *E. odoratum* leaves through DPPH radical, hydroxyl radical, nitric oxide, ABTS radical scavenging [16]. Thang et al. studied the antioxidant effects of the extracts from the leaves of *E. odoratum* on human dermal fibroblasts and epidermal keratinocytes against hydrogen peroxide and hypoxanthine–xanthine oxidase induced damage [17].

To the best of our knowledge, however, no attempt has been made previously to explore the antioxidant of the ethanol extract of *E. odoratum*. Continuing our ongoing research into natural plant antioxidants [18-22], in the present study, we investigated the *in vitro* antioxidant activities of extracts from *E. odoratum*. Total phenolic content (TPC) and total flavonoid content (TFC) were used to quantify antioxidant compounds in the ethanol extract and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) radicals, hydroxyl radicals, ferric ion reducing power and ferrous ion-chelating models were adopted for the antioxidant activity study.

EXPERIMENTAL SECTION

Plant materials. *E. odoratum* aerial parts were collected in from the campus of Hainan University. The voucher specimen of the plant was deposited in the herbarium of College of Landscape and Horticulture, Hainan University, China.

Extraction of plant material. The aerial parts of the plant (50 g) were extracted with ultrasonic wave assisted extraction (UAE) method according to the protocol previously reported [18] using benign solvent ethanol and water to determine the extraction efficiency under the condition of ethanol concentration 70%, solid/liquid ratio of 1:8, extraction time 2 h and extraction temperature at 60 ℃. The crude extracts were then filtered through filter paper and the filtrate was prepared with constant volume at ethanol concentration 60% to 100 mL for estimation of phenolics and antioxidant measurements through various chemical assays. Each extraction was performed in duplicate and all analyses were performed in triplicate.

TPC measurement. TPC from leaf extracts was measured according to the Folin-Ciocalteu (FC) procedure [23] as described with some modifications. The FC phenol reagent was prepared according to King's method [24]. 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 ·g/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 [25] 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 = 0.0118x + 0.0023, (R^2 = 0. 9995). The linear relationship between absorbance and flavonoids content ranged from 15–75 □g/mL. TFC was then expressed as rutin equivalents (RE), in mg RE per g DW.

DPPH radical scavenging capacity measurement. The radical scavenging ability of 2,2'-diphenyl-b-picrylhydrazyl (DPPH) was estimated by a method adapted from Sharififar *et al* [26]. Thus, an aliquot of extract (100 □l) was added to 3.9 mL of ethanolic DPPH (60 □M). 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:

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

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

ABTS radical scavenging capacity measurement. Free radical scavenging capacity using a stable ABTS radical was performed according to Thoo *et al* [27] with some modifications. 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 □l) or ethanol (100 □l, 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:

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

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

Hydroxyl radical-scavenging activity. Hydroxyl radical-scavenging activities of *E. odoratum* extract were determined according to the method described by Smirnoff and Cumbes [28] with some modifications. The following reagents were put into a reaction tube in the following order: 0.3 ml of 20 mM sodium salicylate, 2.0 ml of 1.5 mM $FeSO_4$, 1.0 ml of various concentrations of sample solution, 1 ml of 6mM H_2O_2. They were mixed immediately, and then the reaction tubes were put in the 37 □ water bath for 1 h, the absorbance of the mixture was recorded at 510 nm against a blank. Ascorbic acid was used as the positive control. The hydroxyl radical-scavenging ability was calculated as follows:

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

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

Reducing power assay. The Fe^{3+} reducing power of the extracts was determined by the method of Tan [29]. The extract (2.5 ml) at various concentrations were mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (w/v 1%), followed by incubating at 50 □ in a water bath for 20 min. The reaction was stopped by adding 2.5 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Ferrous ion-chelating activity. Ferrous ion-chelating activities of *E. odoratum* extract was evaluated by the method of Tan [29] with slight modification in the volume of sample and reagents. Briefly, an aliquot of each sample (1 ml), with different concentrations, was mixed with 100 □l of $FeCl_2 \bullet 4H_2O$ (2.0 mM) and 3.7 ml of distilled water. The

reaction was initiated by adding 200 □l of ferrozine (5.0 mM). After 20 min of incubation at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower spectrophotometrical absorbance means a higher ferrous ion-chelating activity. EDTA was used as the positive control. The ferrous ion-chelating ability was calculated as follows:

$$\%\text{Chelating activity} = \left[\left(A_{blank} - A_{sample}\right) / A_{blank}\right] \times 100\%$$

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

RESULTS AND DISCUSSION

Phenolic and flavonoid contents. TPC (2.772±0.3 mg GAE/g DW) of EOE was determined using the standard curve of gallic acid (R^2 = 0.9991). Using the standard curve generated by rutin (R^2 = 0. 9995), the TFC of EOE (7.085±0.2 mg of RT/100 g) as shown in Table 1. More flavonoids were recovered than phenols. This could be because flavonoids comprise a majority of the total phenols. The remainder of the plant's metabolic flavonoids are glycosides and derivatives with non-phenolic hydroxyl groups.

Scavenging effect on DPPH free radical. One of the mechanisms to investigate antioxidant activity is to study the scavenging effect on proton radicals [30]. In the present study, investigation of total antioxidant capacity was measured as the cumulative capacity of the compounds present in *E. odoratum* to scavenge free radicals, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) reaction. The presence of antioxidant in the sample leads to the disappearance of DPPH radical chromogens which can be detected spectrophotometrically at 517 nm. The DPPH radical scavenging capacity of EOE was found to increase in dose dependent manner with increasing concentration in the range of 0.2-8 mg/ml (Fig. 1). The EOE displayed potential effect of DPPH activity with IC_{50} values of 3.0 mg/ml. VC and VE were used as positive controls with IC_{50} values of 0.1 and 0.32 mg/ml, respectively (Table 1).

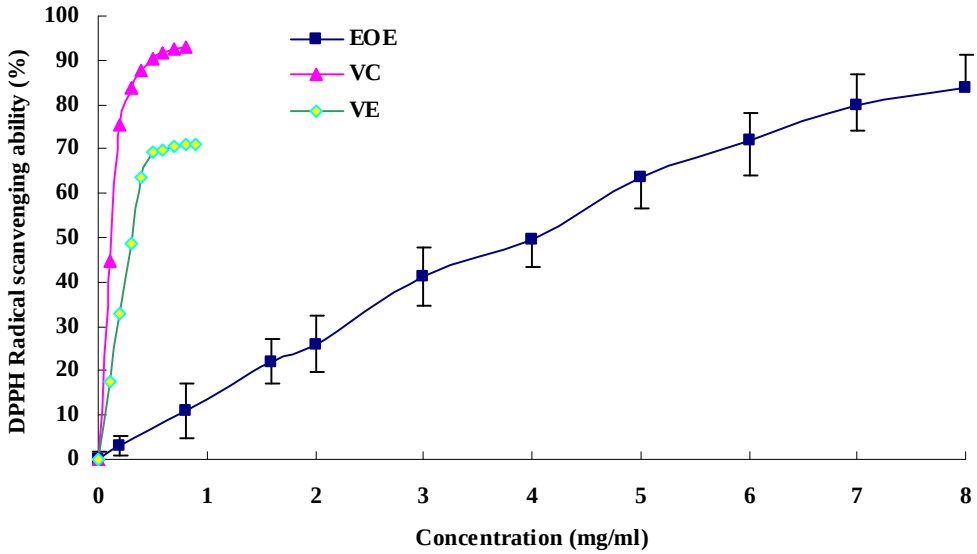

Fig. 1. Free radical (1,1-diphenyl-2-picrylhydrazyl (DPPH)) scavenging activities of extract from *E. odoratum* (EOE) at different concentrations.

Each value represents means ±SD (n = 3).

Scavenging effect on ABTS free radical. ABTS radical assay is an excellent tool for determining the antioxidative activity, in which the radical is quenched to form ABTS radical complex. Meanwhile, it is more sensitive to determine antioxidative capacities of plant extracts, because it can determine their capacities at lower inhibition concentrations. With increasing concentration, EOE showed increased ABTS radical scavenging activities**.** When a dose-response curve was plotted, EOE could inhibit over 80% of the ABTS radicals when the concentration is

higher than 5 mg/ml used. As shown in Fig. 2, EOE exhibited potential inhibitory effect on ABTS radicals with IC_{50} values of 1.08 mg/ml (Table 1).

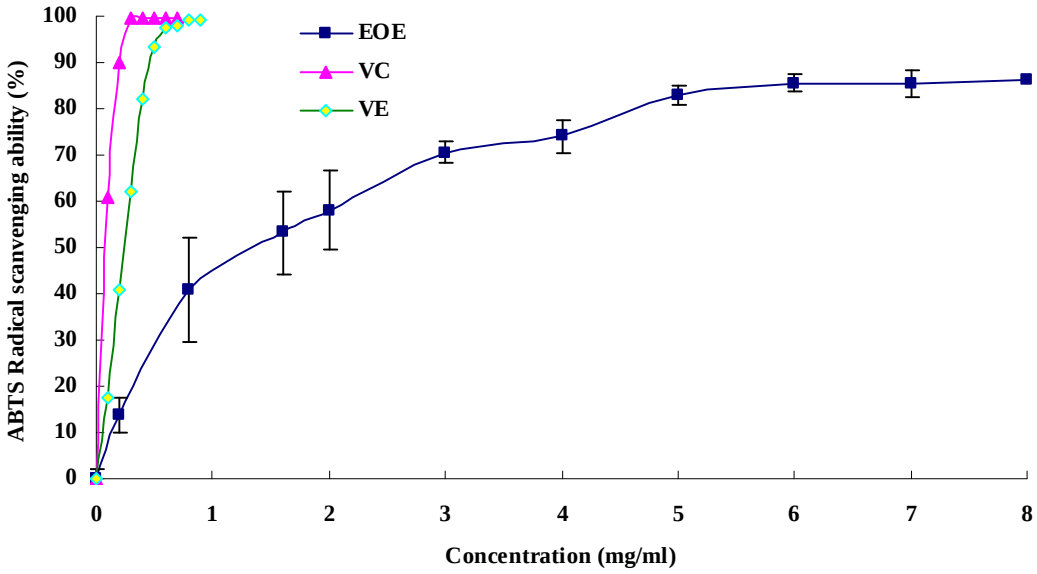

Fig. 2. Free radical (2,2'-azino-bis (3-ethylbenzothiazoline- 6-sulphonicacid)(ABTS)) scavenging activities of extract from *E. odoratum* (EOE) at different concentrations.
Each value represents means ± SD (n = 3).

Scavenging effect on hydroxyl radical. The hydroxyl radical is known to be the most reactive oxygen radical and it severely damages adjacent bio-molecules in the body, such as protein and DNA, resulting in cell damage. This damage causes ageing, cancer and several other diseases [29]. Fig. 3 showed that both EOE exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The IC_{50} of EOE is at 1.18 mg/ml (Table 1).

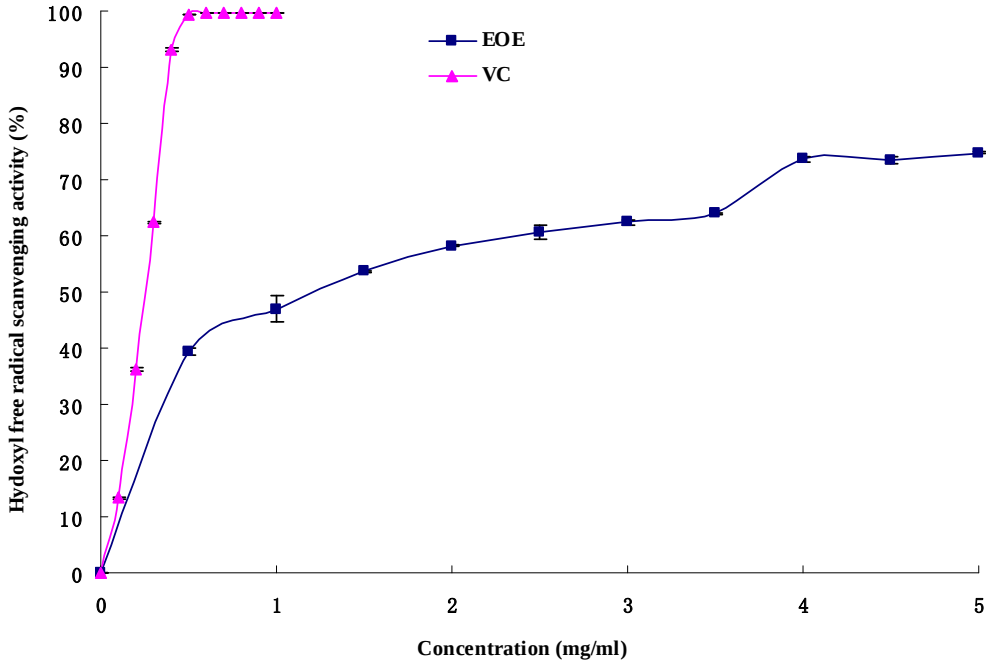

Fig. 3. Hydroxyl free radical scavenging activities of extract from *E. odoratum* (EOE) at different concentrations.
Each value represents means ± SD (n = 3).

The chelating activity for ferrous ion. Transition metals such as ion can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction and accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, therefore drive the chain reaction of lipid peroxidation [31]. The chelating activities for ferrous ion of the extracts were assayed by the inhibition of formation of red-colored ferrozine and ferrous complex. As shown in Fig. 4, the formation of the red-colored complex was inhibited in the presence of EOE, indicating chelating activity. The light absorbance of the red-colored complex was decreased linearly in an EOE concentration dependent manner with an IC_{50} of 0.14 mg/ml (Table 1). At the concentration of 0.5 mg/ml, EOE chelated 71% of ferrous ions.

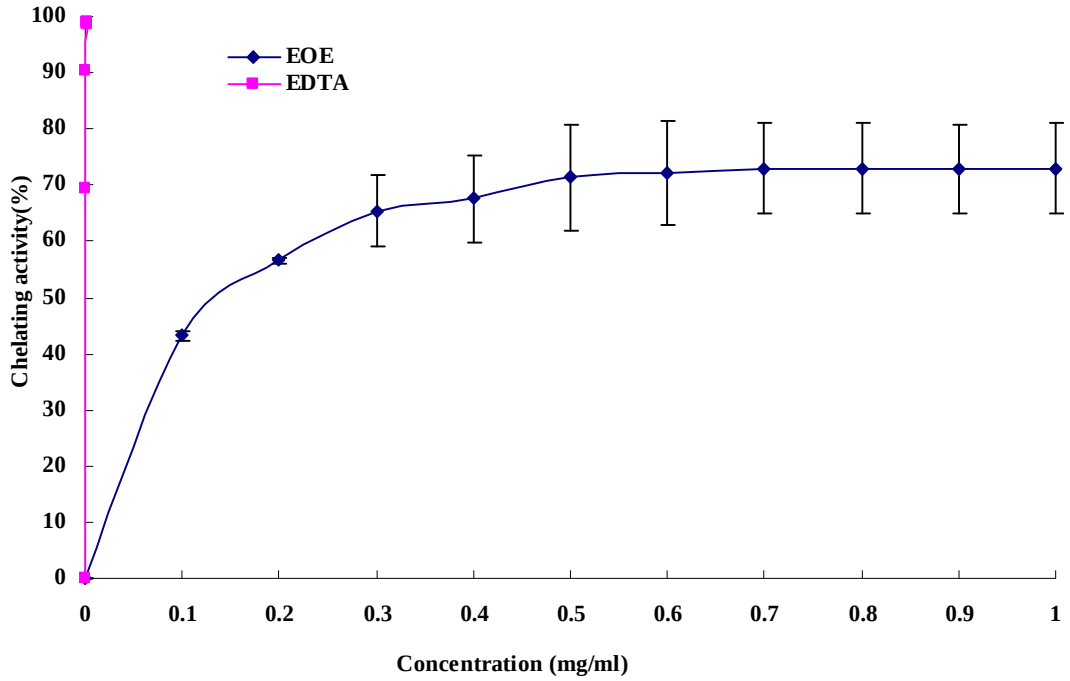

Fig. 4. Ferrous ion chelating activities of extracts from *E. odoratum* (EOE) at different concentrations.
Each value represents means ± SD (n = 3).

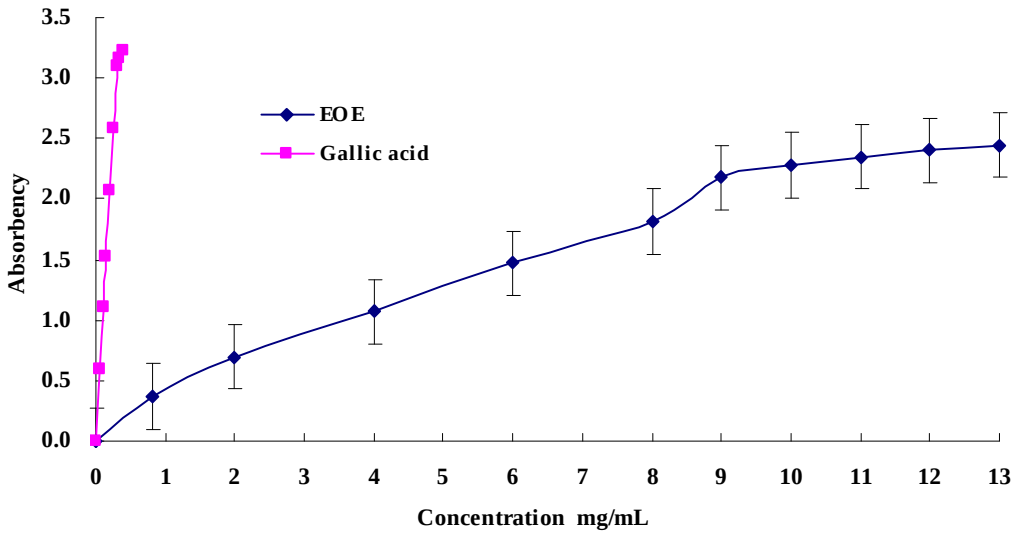

Fig. 5. Reducing power of extract from *E. odoratum* (EOE) at different concentrations by spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformation.
Each value represents means ± SD (n = 3).

The reducing power. The reducing ability to convert Fe^{3+} to Fe^{2+} is also an indirect evidence for the antioxidant activity of an extract or a compound. In the ferric reducing antioxidant power assay, the antioxidants i.e. the reducing species present in the extract causes the reduction of the Fe^{3+}/ ferricyanide complex to form Fe^{2+} ions; high absorbance at 700 nm indicates high reducing power [32]. As showed in Fig. 5, ESM exhibited strong reducing power for Fe^{3+} in a dose dependent manner with an IC_{50} value of 3.16 mg/ml (Table 1).

In this study we demonstrated the antioxidant properties of extract of *Eupatoriurn odoratum* using various in vitro testing systems, including DPPH and ABTS radicals, hydroxyl radicals, ferric ion reducing power and ferrous ion-chelating activity. Total phenolic content (TPC) and total flavonoid content (TFC) assays were used to quantify antioxidant compounds. Our data suggested that EOE possess direct and potent radicals scavenging activities through multiple mechanisms. It has been suggested that their antioxidant properties likely contributed to partly their pharmacologically uses. Further investigations are needed to identify the antioxidant compounds present in *E. odoratum* with better understanding of the antioxidant mechanisms.

Table 1. Antioxidant activity of *E. odoratum* ethanol extract

Concentration (mg/ml)	Mean inhibition (%) ±SD[a]											
	DPPH radical scavenging ability			ABTS radical scavenging ability			hydroxyl radical scavenging ability		ferrous ion-chelating activity		ferric ion reducing power	
	E. odoratum	VC	VE	*E. odoratum*	VC	VE	*E. odoratum*	VC	*E. odoratum*	EDTA	*E. odoratum*	Ascorbic acid
IC_{50}	3.00	0.10	0.32	1.08	0.06	0.22	1.18	0.23	0.14	0.06×10^{-3}	3.16	0.06

Acknowledgements
Co-Financed by grants of National Natural Science Foundation of China for Young Scholar (No. 81202456), Hainan Provincial Natural Science Foundation (No. 814285), Special Social Service Fund of Hainan University (No. HDSF201301), Special Foundation for Modernization of Traditional Chinese Medicine of Hainan (ZY201429) and the programs for New Century Excellent Talents in University (NCET-13-0760) are gratefully acknowledged.

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