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Evaluation of phytochemical and antioxidant activities of the different fractions of *Hybanthus enneaspermus* (Linn.) F. Muell. (Violaceae).

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ABSTRACT

Objective: To investigate the naturally occurring antioxidant for the first time from the different solvent fractions of *Hybanthus enneaspermus (H. enneaspermus)* Linn F. Muell. family (Violaceae). **Methods:** Different fractions of *H. enneaspermus* were tested for total phenolic content, and *in vitro* antioxidant activity was measured by total antioxidant assay, DPPH assay, reducing power, nitric oxide (NO), hydrogen peroxide (H_2O_2) scavenging assays. **Results:** The ethyl acetate (EA) fraction was found to have high levels of phenolic content [(212.15±0.79) mg GAE/g]. The EA fraction exhibited higher total antioxidant capacity, higher percentage of DPPH radical scavenging activity [(127.07±2.29) μg/mL], nitric oxide [(245.16±1.44) μg/mL], hydrogen peroxide [(227.38 ±7.18) μg/mL], deoxyribose [(270.61±8.72) μg/mL] and higher reducing power. There was a significant correlation between total phenolic content and total antioxidant activity (r^2=0.972). **Conclusions:** These results reveal that EA fraction of *H. enneaspermus* has strong antioxidant potential compared with other fractions. Our further study has been extended to the isolation of the possible compound that is responsible for having antioxidant property.

1. Introduction

Human body has multiple mechanisms particularly enzymatic and nonenzymatic antioxidant systems to protect the cellular molecules against reactive oxygen species (ROS) induced damage[1]. The ROS include superoxide anions (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$)[2]. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is associated with several types of biological damage. Due to high side effects and toxic properties of many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), several food industries have drawn their attention from synthetic to natural antioxidants[3, 4].

Polyphenols are common constituents of the human diet, with fruits and vegetables being the major dietary source of these bioactive compounds. The possible health benefits of polyphenol consumption have been suggested deriving from their antioxidant properties. Evidence for their role in the prevention of degenerative diseases is emerging. Therefore, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks[5]. Many of these antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry, because they retard oxidative degradation of lipids and thereby improve the quality and nutritive value of food[2].

Hybanthus enneaspermus (H. enneaspermus) Linn F. Muell. (Violaceae) is a herb or a shrub distributed in the tropical and subtropical regions of world and occurs mostly in the warmer parts of India. The plant is popularly known

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as Ratanpurus (Hindi). Traditionally the plant is used as an aphrodisiac, demulcent, tonic, diuretic, in urinary infections, diarrhea, cholera, leucorrhoea, gonorrhea, dysuria, inflammation and sterility[6]. The plant has also been reported to have anti-inflammatory, antitussive, antiplasmodial, antimicrobial, and anticonvulsant activity [7-10]. Thus, the aim of the present study was to evaluate a potential new source of natural antioxidants from different fraction of *H. enneaspermus* which will prove beneficial for maintenance of optimal health and may increase the demand of these bioactive substances by food, cosmetic and pharmaceutical industries.

2. Materials and methods

2.1. Chemicals and instruments

1, 1-diphenyl-2-picryl-hydrazil (DPPH) free radicals were obtained from Sigma Aldrich. Other chemicals, sodium carbonate, sodium phosphate, potassium ferricyanide, ammonium molybdate, standard rutin, ascorbic acid and gallic acid, 2-thiobarbituric acid (TBA), butylated hydroxy anisole (BHA), Follin-Ciocalteu, 2- deoxyribose and H_2O_2 (30%, v/v) from Merck India Ltd and from Qualigens Fine Chemical Co. (India). All other chemicals and solvents used were of analytical grade. The absorbance measurements were recorded using the ultraviolet-visible spectrophotometer (Shimadzu, Pharmaspec-1700).

2.2. Preparation of plant extract, fractions and its phytochemical analysis

The plant material was procured from market (herbal vendors) from Chennai, and identified by the chief botanist TAMPCOL Anna Hospital Chennai and the voucher specimen (Cog/HE/01/08) were kept for further reference at our Laboratory Herbarium, Department of Pharmaceutics, IT-BHU, Vanarasi. The air-dried whole plant parts of *H. enneaspermus* (1 kg) were extracted with 95% ethanol in a Soxhlet apparatus for 12 hour. After filtration of the solvent, the organic phases were independently concentrated under a vacuum by evaporating to dryness. The ethanol extracts were then fractionated under solvents of varying polarity as shown in the flowchart (Figure 1). The preliminary phytochemical analysis of the various fractions was carried out as per the methods of Khandelwal, Wallis and Harborne[11-13].

2.3. Antioxidant assays

2.3.1. Free radical scavenging activity

DPPH method[14] was used to determine the free radical scavenging activity of *H. enneaspermus* fractions, where 5 mL of DPPH (100 μM/mL) solution was added to 1 mL of different fractions as well as standard (25-200 μg/mL). The absorbance was then measured 30 min later at 517 nm and the free radical scavenging activity was calculated in the form of IC_{50} value according to the standard equation using ascorbic acid as control which is mentioned here. The IC_{50} value is the concentration where 50% inhibition occurs.

Percentage inhibition = $[(1-A_1/A_0)] \times 100$ ····· (i)

Where, A_0: Absorbance of the blank, A_1: Absorbance test sample.

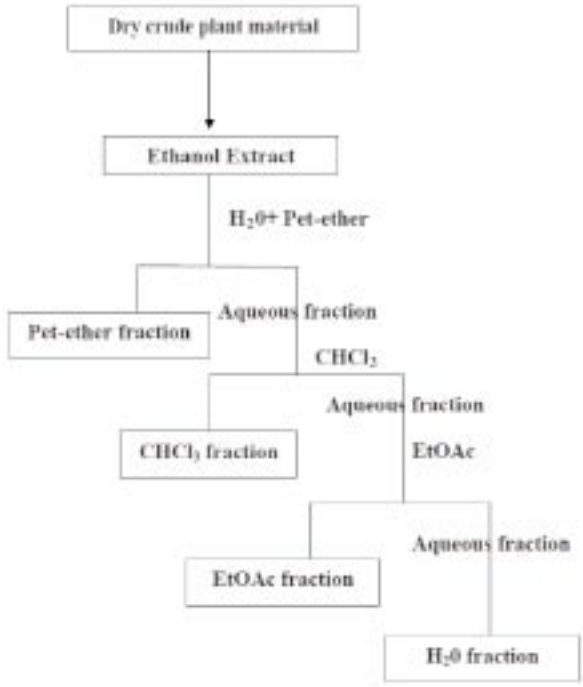

Figure 1. Schematic diagram for fractionation of *H. enneaspermus* extract.

2.3.2. Nitric oxide scavenging assay

Griess Illosvoy reaction is generally used to determine the nitrite ions, which is produced by sodium nitroprusside in aqueous solution at physiological pH by interaction with nitric oxide, and oxygen[15]. Two mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract/fraction at different concentrations and was then incubated at 250 ℃ for 150 min. 0.5 mL of this solution was added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance was measured at 546 nm. The nitric oxide radical scavenging activity was calculated using this equation (i) and IC_{50} was calculated by using rutin as control.

2.3.3. Scavenging of hydrogen peroxide

The scavenging activity of different fractions was evaluated by method described by [16]. One mL of methanolic solution of different fraction and standards were mixed with 2 mL (20 mM) of hydrogen peroxide prepared in phosphate

buffered saline (PBS, pH 7.4). After 10 minutes absorbance was measured at 230 nm. All readings were performed in triplicates and the percentage inhibition ware calculated using above equation (i).

2.3.4. Scavenging of hydroxyl radical by deoxyribose method

The hydroxyl radical scavenging of different fractions of the *H. enneaspermus* was evaluated by method described by Srinivasan *et al*[17]. One mL of the reaction solution consisted of aliquots (500 μL) of various concentrations of the fractions, 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). This was then incubated for 1 h at 37 ℃, after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2-thiobarbituric acid. Further it was heated in a boiling water-bath for 15 min and absorbance was measured at 532 nm against a blank.

2.3.5. Assay of reducing power

Potassium ferricynide method was used to determine the assay of reducing power of various fractions of *H. enneaspermus* (25-200 μg/mL)[18]. In this method 1 mL of test sample were added to 2.5 mL phosphate buffer (0.2 M, pH 6.6) followed by addition of 2.5 mL of potassium ferricyanide (10g/L) and 2.5 mL of trichloroacetic acid (100g/L). This was then incubated at 50 ℃ for 20 minutes after centrifugation for 10 min at 3 000 rpm, Further 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (1 g/L) and absorbance was measured at 700 nm, higher absorbance indicates stronger reducing power.

2.3.6. Antioxidant capacity by phosphomolybdenum method

Phosphomolybdenum method as described by Kannan *et al* was used to determine the total antioxidant capacity of the *H. enneaspermus* fractions[19]. Here 0.3 mL of methanolic extract (1 mg/mL) and standard ascorbic acid (50-300 μg/mL) were added with 3 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After incubation in a boiling water bath at 95 ℃ for 90 min, absorbance was measured at 695 nm against the blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid.

2.4. Determination of total phenolic content

Folin-Ciocalteu (FC) assay as described by Zovko *et al* was used to determination of the total phenolic (TP) content of the various fractions of *H. enneaspermus*[20]. Eight mL of water was added into 1 mL of extract in a 10 mL volumetric flask. 0.5 mL of FC reagent was added and mixed for 15 min followed by addition of 1.5 mL of 20% sodium carbonate solution. After 2 hours at ambient temperature the absorbance of the colored reaction product was measured at 765 nm, where different concentrations of standard gallic acid solutions were used for calibration curve and results were expressed as mg of Gallic Acid Equivalent per gram (mg GAE/g) of dried extract.

2.5. Statistical analysis

Results are expressed as mean value±standard error mean (SEM) of three independent determinations. Linear regression analysis was performed, quoting the correlation coefficient r^2.

3. Results

3.1. Preliminary phytochemical screening

Table 1 represents the preliminary phytochemical screening of different fractions of *H. enneaspermus* where alkaloids and polyphenolics were found to be dominant in all the fractions.

3.2. Anti-oxidant assay

3.2.1. Free radical scavenging activity

On reaction with a hydrogen donor the purple color of DPPH fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The more decrease in absorption in presence of scavengers in the fractions the more effective is its anti oxidant activity. Different fraction exhibited considerable free radical scavenging activity as indicated by their IC_{50} values which is shown in the Table 2. IC_{50} indicated the potency of scavenging activity. IC_{50} of standard ascorbic acid was (77.92±1.93) μg/mL. In comparison to ascorbic acid, IC_{50} of ethyl acetate fraction was (127.07±2.29) μg/mL.

3.2.2. Nitric oxide scavenging assay

Nitric oxide or reactive nitrogen species generally formed when their reaction occurs with oxygen or super oxides such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- which are highly reactive. Table 2 showed that ethyl acetate fraction possessed potent nitric oxide scavenging activity [IC_{50} value (245.16±1.44) μg/mL] and petroleum ether fraction has showed the least nitric oxide scavengers [IC_{50} value (646.12±11.41) μg/mL].

3.2.3. Scavenging of hydrogen peroxide

The ability of various *H. enneaspermus* fractions to scavenge H_2O_2 is presented in Table 2 using rutin as control. The ethyl acetate fraction as compared to standard showed a good activity in depleting H_2O_2, with an IC_{50} value of (227.38± 7.18) and (87.07±0.52) μg/mL, respectively.

3.2.4. Scavenging of hydroxyl radical by deoxyribose method

The results in the Table 2 showed that the scavenging capacity of hydroxyl radical by ethyl acetate fraction of *H. enneaspermus* was found to have an IC_{50} of 270.61±8.72. This depicts that ethyl acetate have more power (in fact lesser than the standard) to eliminate the hydroxyl radical as compared to the other tested fractions.

3.2.5. Determination of total antioxidant capacity

The assay of total antioxidant capacity is based on reduction mechanism of Mo(VI) to Mo(V) by the antioxidant

agents and the subsequent formation of a green phosphate/Mo(V) complex at 695 nm (35). The total antioxidant capacity in all the fractions was determined using the linear regression equation of the calibration curve (y= 0.005x−0.042, r^2= 0.996) and was expressed as the number of equivalent of ascorbic acid (μg/mL plant extract and their fractions). The results showed that ethyl acetate fraction had the highest antioxidant capacity [(58.7±0.84) μg/mL ascorbic acid equivalent](Figure 2).

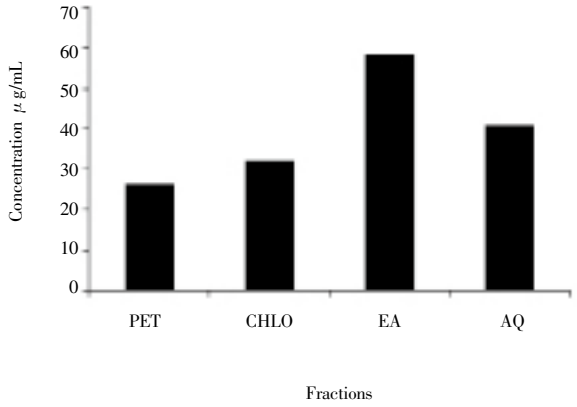

Figure 2. Total antioxidant capacity of different fraction of *H. enneaspermus* extract.

3.2.6. Assay of reducing power

The polar fractions *viz* ethyl acetate and aqueous part was found to exhibit a significant reducing power as compared to that of the non polar ones which can be arranged in the order as: Ascorbic acid > alcoholic extract > ethyl acetate fraction > aqueous fraction > Chloroform fraction > petroleum ether fraction (Figure 3).

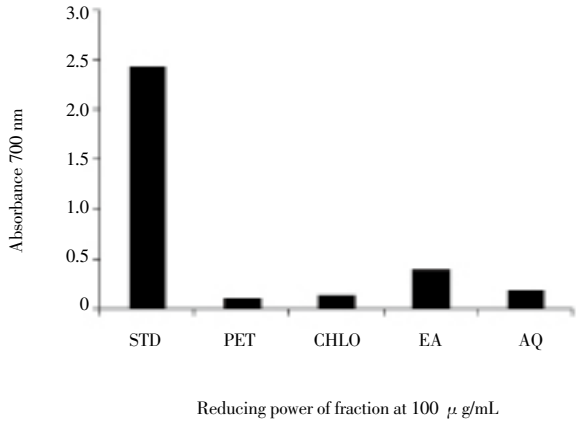

Figure 3. Reducing power of different fraction of *H. enneaspermus* extract.

3.3. Estimation of total phenolic content of various fractions of H. enneaspermus

In our present investigation, it was found that ethyl acetate fraction had the highest phenolic content followed by aqueous fraction and petroleum ether fraction showed negligible quantity of phenolics. The content of the total phenolics in extracts and sub-fractions was determined using the linear regression equation of the calibration curve (y=0.008x+0.092, r^2= 0.995) and was expressed as gallic acid equivalent (Table 2).

3.4. Co-relationship between the total antioxidant capacity and the total phenolic content

Since it is clear that the antioxidant activity of the plants

Table 1
Phytochemical analysis of various fraction of *H. enneaspermus* extract.

Extract / fractions	% yield	Components									
		Alkaloid	Steroid	Triterpenes	Glycoside	Sugars	Phenolic	Flavonoid	Saponins	Tannin	Amino acids
Petroleum ether	4.5	+	+	+	−	−	−	−	−	−	−
Chloroform	3.6	+	+	+	−	−	−	−	−	−	−
Ethyl acetate	2.8	+	−	−	+	+	+	+	+	+	+
Aqueous fraction	5.1	+	−	−	+	+	+	+	+	+	+

+ Present, − absent.

Table 2
Antioxidant activities and total phenolic content of the different fraction of *H. enneaspermus*.

Fraction	IC_{50} (μg/mL)				Total phenolic content
	DPPH	Nitric oxide	H_2O_2	Deoxyribose	
PET	414.76±9.82	646.12±11.41	446.47±10.19	658.68±9.13	36.68±0.43
CHLO	348.90±5.93	601.57±3.22	380.83±6.52	452.42±13.37	68.56±0.51
EA	127.07±2.29	245.16±1.44	227.38±7.18	270.61±8.72	212.15±0.79
AQ	187.00±3.12	300.23±6.21	189.64±3.26	311.36±7.01	140.62±0.57
Standards					
Rutin	NA	98.06±6.10	87.07±0.52	NA	NA
BHA	NA	NA	NA	123.55±1.62	
Ascorbic acid	77.92± 1.93	NA	NA	NA	NA

NA: Not analyzed, PET: Petroleum ether, CHLO: Chloroform , EA: Ethyl acetate, AQ:Water residue.

might be due to the presence of polyphenolics, hence it is necessary to correlate its total phenolics along with its antioxidant properties for further confirmation which is the best criteria to support the antioxidant activity of the plants[21]. The correlation was done simply by comparing the r^2 obtained by plotting a graph between the total antioxidant activities of various fractions versus its total phenolic content with that of the total antioxidant capacity (Figure 4). Our investigation results showed that the r^2 obtained between the total antioxidant capacity and the total phenolic contents of all the fractions was found to have a linear property with a value of 0.972 (r^2).

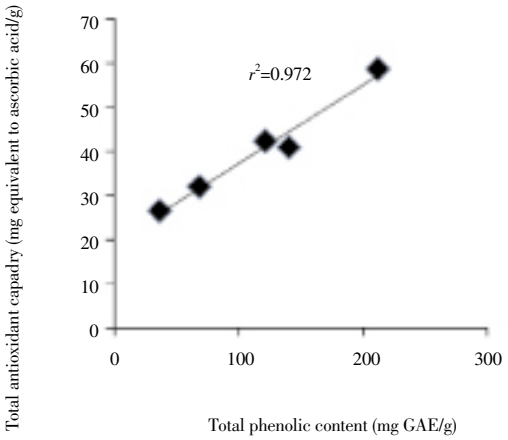

Figure 4. Correlations between the total antioxidant capacity and total phenolic content of different fraction of *H. enneaspermus* extract.

4. Discussion

The result reveals that ethyl acetate is regarded as the most effective solvent for extracting phenolics from *H. enneaspermus*. The total phenolic content of the ethyl acetate was higher than those of the petroleum ether, ethanol and aqueous extract. This could be explained by the possible formation of complexes by certain part of the phenolic compounds with other components, which are more extractable in ethyl acetate than those of other fractions[22, 23]. Due to the vast diversity in the mechanism of protective effects and the complex nature of the phytoconstituents present in the plant extracts, the antioxidant evaluation using any single method seems to be rather impractical[24].

Reducing power of the plant is one mechanism for the possible antioxidant activity and may serve as a significant indicator of potential antioxidants[25]. In this assay, ethyl acetate fraction of *H. enneaspermus* showed good concentration-dependent manner with a perfect reducing power, thus indicating a good electron donors and ability to terminate radical chain reaction[20].

Hydrogen peroxide itself is not very reactive in nature, but sometimes it is harmful to the cell due to the fact that it may give rise to hydroxyl radical present in the cells. Therefore, eradication of H_2O_2 is the superior steps for antioxidant defence in cell or food systems. Dietary polyphenols have also been shown to protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide. Therefore, the phenolic compounds of the *H. enneaspermus* extracts may probably be involved in removing the H_2O_2. It is observed that scavenging of nitric oxide by the extract is concentration dependent. It is also observed that all the extracts are likely to have the nitric oxide scavenging activity. The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to the human health. Nitric oxide is also implicated for other disorders like inflammation, cancer and other pathological conditions[26].

Hydroxyl radical is highly reactive in nature and can cause serious oxidative damage to the DNA, lipids and proteins[27-31]. Hydroxyl radical, like many other free radicals can be neutralized provided if hydrogen atoms is supplied to it. Our result on the scavenging of hydroxyl radical shows that ethyl acetate has significant effect in neutralizing the harmful hydroxyl radical.

In general, the ethyl acetate extracts of *H. enneaspermus* showed strong antioxidant activity, reducing power, DPPH radical, H_2O_2 scavenging, nitric oxide scavenging and scavengers of hydroxyl radical when comparing with standards such as BHT and ascorbic acid. The antioxidative effect of *H. enneaspermus* extract may be likely due to the phenolic components. Thus, the DPPH radical scavenging activity of *H. enneaspermus* extracts may be mostly related to their phenolic hydroxyl group. Our data demonstrate the high total phenolic contents from ethyl acetate extracts of *H. enneaspermus* for the first time. The positive correlation between phenolic content and antioxidant potential of various plant extracts have been well demonstrated in prior reports[32]. Our data on the reducing power of the tested extracts suggest that it is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity of antioxidants has been attributed by various mechanisms, among which some of them are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[33]. Phenolic compounds such as flavonoids, phenolic acids and tannins are widely distributed in plants which have gained much attention, due to their antioxidant activities and free radical-scavenging abilities, which potentially have beneficial implications for human health[34].

The ethyl acetate fraction of *H. enneaspermus* have showed impressive antioxidant activity and free radical scavenging activity. The mechanism behind such criterion could be due to presence of high content of phenolics. So further investigation are under progress by isolating the pure phenolics compounds from then ethyl acetate fractions and to rescreen their *in vivo* antioxidant activity on animal models.

Conflict of interest statement

We declare that we have no conflict of interest.

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