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RESEARCH ARTICLE

FREE RADICAL SCAVENGING ACTIVITY OF *ECBOLIUM VIRIDE* (FORSSK) ALSTON

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ABSTRACT

The aim of the study was to perform *in vitro* phytochemical screening, Quantitative estimation and free radical scavenging activity of various extracts of the plant *Ecbolium viride* (Forssk).Alston belonging to the family Acanthaceae. It is a perennial woody shrub commonly known as Green Shrimp plant. Traditionally different parts of the plant like roots, leaves, stem and whole plant are used in folklore medicine for several medicinal purposes like cancer, jaundice and rheumatism.The preliminary phytochemical screening revealed the presence of various phytoconstituents, *in vitro* free radical scavenging activity of various crude extracts were determined using DPPH, Hydrogen peroxide, and reducing power assay. The present investigation was designed to evaluate the *in vitro* phytochemical screening, antioxidant activity of the extracts was compared with standard antioxidants and the dose dependent response was observed in reducing power of extracts. The findings suggest that *E. viride* exhibited potential *in vitro* free radical scavenging activity.

INTRODUCTION

Accumulation of free radicals and reactive oxygen species (ROS) in the body may lead to oxidative stress. Excess production of these free radicals leads to cause cellular damage by reacting with various bio molecules of body such as membrane lipids, nucleic acid, proteins and enzymes. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. Plants have antioxidants with strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis (Suhaj M, 2006). Therefore, the need exits for safe, economic, powerful, and natural antioxidants to replace these synthetic ones (Tadhani *et al*, 2007). *Ecbolium viride* (Forssk).Alston (Acanthaceae) is a perennial woody under shrub. It is commonly known as Green Shrimp plant. They can be easily identified by their intense green leaves and greenish blue flowers. The native range of *Ecbolium viride* extends from South and North eastern peninsular part of the country found occasionally in plains and forests of India. Traditionally different parts of the plant like roots, leaves, stem and whole plant which are used in folklore medicine for several medicinal purposes like cancer, jaundice and rheumatism (Chopra *et al*, 1956; The Wealth of India, 2006). It also possesses pharmacological properties such as anti-microbial, Cytotoxic, Antidiabetic, Analgesic, Anti-trypanosomal, Anti-inflammatory, Anti-Plasmodial, anti-diarrhoeal, hepatoprotective and antioxidant properties.

MATERIAL AND METHODS

Plant material

Fresh aerial parts of plant were collected from the vicinity of Tirumala hills, Chittor district of Andhra Pradesh, India in June 2013 and were authenticated by Dr. M. Venkaiah, Department of Botany. Voucher specimen has been kept in herbarium and preserved for future identification. (EVAU/2013/BGR).

Chemicals and reagents

All the reagents used were of analytical grade obtained from Sigma Chemical Co. St. Louis, USA and Fine Chemicals Ltd., Mumbai, India.

Preparation of the extracts

The plant materials were dried at room temperature, chopped into small pieces, ground into powder and was placed into the

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extractor of a Soxhlet. The extraction was carried out by using solvents of increasing polarity starting from hexane, ethyl acetate and methanol. Crude extract with methanol was also prepared. At the end of the extraction the respective solvents were concentrated by evaporation. The obtained extracts were stored in a refrigerator at 4^0 C until use.

Preliminary Phytochemical analysis

Qualitative phytochemical analysis was carried out (Harborne, 1984; Trease GE and Evans WC, 1989; Raaman N, 2006) and the results observed were based on the colour change or precipitate formation after the addition of specific reagents.

Quantitative analysis

Total Phenolic Content Determination

The total phenol contents were determined using a standard protocol with little modification (Hendra *et al*, 2011). Briefly, each 0.5 ml extracts were mixed with 2.5 ml Folin Ciocalteu reagent and 2 ml of 7.5% (w/v) Na_2CO_3. The mixtures were shaken and allowed to react for 30 minutes in the dark for 1 hour. Absorbance was measured at 765 nm, and gallic acid was used as standard. The total phenol content was determined as gallic acid equivalents (GAE) in µg/mg dry weight (DW) of sample.

Total Flavonoid Content Determination

The total flavonoid contents were determined by the $AlCl_3$ method (Ismail *et al*, 2010). An aliquot (0.1ml) of extract was added to 0.3 ml 5% (w/v) $NaNO_2$ and incubated for 5 minutes. 0.3 ml $AlCl_3$ (10% w/v) and 2 ml 1 N NaOH was added, and the total volume was made up to 5 ml with distilled water. After 10 min of incubation at ambient temperature the absorbance was measured at 510 nm by using UV-visible spectrophotometer. Three replicates were made for each test sample. The total flavonoid contents were expressed as Quercetin equivalence (QE) in µg/mg DW of sample.

Total Alkaloid Content Determination

The plant extracts (1mg) were dissolved in dimethyl sulphoxide (DMSO) added 1ml of 2 N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4ml chloroform by vigorous shaking and collected in a 10ml volumetric flask and diluted to the volume with chloroform. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract (Fazel *et al*, 2008). The results are expressed as atropine equivalents (mg of A/ g dry extract).

Assessment of antioxidant activity

Determination of free radical scavenging activity by DPPH method

This test was measured as described by Blois (Blois MS, 1958). One milliliter of the fraction solutions (25,50, 100, 200,300,400 and 500 µg/ml in ethanol) was added to 1ml of a DPPH solution (0.2mM in ethanol). After a 30 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was calculated using the following equation:

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

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Ascorbic acid was used as a standard. The % inhibition was plotted against the sample extract concentration and a logarithmic regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50 µg/ml)

Determination of free radical scavenging activity by Reducing Power Assay

Reducing power was determined by the method (Oyaizu *et al*¸1986). The sample in 1ml of methanol at various concentrations was mixed with a phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%), and the mixture was incubated at 50°C for 20 min. Next, 5ml of trichloroaceticacid (10%) were added to the reaction mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5ml) and ferric chloride (1 ml, 1%), and the absorbance was measured at 700 nm. A stronger absorbance will indicate increased reducing power.

Determination of free radical scavenging activity by Hydrogen Peroxide assay

The hydrogen peroxide scavenging assay was carried out following the procedure (Ruch *et al*, 1989). A solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). The extract at different concentrations in 3.4 ml phosphate buffer was added to 0.6 ml of H_2O_2 solution (0.6 ml, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm.

H_2O_2 scavenging activity (%) = $[(A_0-A_1)/A_1] \times 100$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

Statistical Analysis

All experiments were carried out in triplicate, and data were expressed as mean ± standard deviation (SD) or standard error of mean (SEM). The plots were prepared using Microsoft excel and Graph pad Prism 6 software. Data were analyzed using one-way ANOVA.

Table 1 Preliminary Phytochemical Screening

Chemical Constituent	Tests	Extracts						
		Hexane	Pet. Ether ($60\text{-}80^0$)	DCM	$CHCl_3$	EA	CH_3OH	Aq.
Phenols & Tannins	1. $FeCl_3$ test	+	+	+	+	+	+	-
	2. Lead acetate test	+	+	+	+	+	+	+
Saponins	Foam test	+	+	+	+	+	+	+
Phytosterols & Triterpenes	Salkowski test	+	+	+	+	-	+	+
	Libermann-Burchard's test	+	-	+	+	-	+	-
Flavanoids	1. Shinoda test	+	+	-	-	+	+	-
	2. Zn-HCl reduction test	+	+	-	-	+	+	-
Glycosides	1. Borntragers test	-	+	-	-	+	-	-
	2. Legal's test	-	+	-	-	+	+	-
	3. Shinoda test	-	+	-	-	+	+	-
Alkaloids	1. Mayer's test	-	+	-	+	-	+	+
	2. Wagner's test	+	+	+	+	+	+	+
	3. Hager's test	+	+	+	+	+	+	+
	4.Dragendroff's test	+	-	+	+	+	+	+
Carbohydrates	1. Molisch's test	-	-	-	-	+	+	+
	2. Fehling's test	-	-	-	-	+	+	+
	3. Barfoed's test	-	-	-	-	+	+	+
	4. Benedict's test	-	-	-	-	+	+	+

Pet. Ether ($60\text{-}80^0$) – Petroleum ether ($60\text{-}80^0$); Hexane, DCM – Dichloromethane; $CHCl_3$ – Chloroform; EA – Ethylacetate; CH_3OH – Methanol; Aq. – Aqueous. '+' indicates presence; '-' indicates absence

Table 2 Total Phenolic, Flavonoid and alkaloid content of different extracts

Extract	TPC (mg GAE/g dried extract)	TFC (mg QE/g dried extract)	TAC (mg /g dried extract)
Hexane	34.63 ± 0.25	10.32 ± 0.23	12 ± 0.12
Ethyl acetate	128 ± 0.28	25.54 ± 0.18	15.60 ± 0.18
Methanol	360 ± 0.30	15.83 ± 0.15	18.98 ± 0.20
Crude methanol	402 ± 0.25	34.32 ± 0.36	26.18 ± 0.25

Values represent Mean ± SEM (n=3). Means with different values within a column are significantly different (P < 0.05)

The presence of Phytosterols and Triterpenes is slightly varying but present in almost all extracts. Extracts were conspicuous by the presence of Alkaloids in all extracts. Flavonoids are present in Polar and Non-Polar extracts. None of the extracts showed presence of all the phytoconstituents tested except for saponins.

Table3 Concentration dependent percentage inhibition of different extracts of *Ecbolium viride* on DPPH radical.

Conc (µg/ml)	Hexane Extract	Ethylacetate Extract	Methanol Extract	Crude Extract	Ascorbic acid
25	7.23 ± 0.04 **	20.36 ± 0.23**	25.32 ± 0.15**	26.65 ± 0.10**	27.35 ± 0.16
50	10.07 ± 0.02***	29.97 ± 0.27***	38.65 ± 0.19***	39.35 ± 0.18***	47.12 ± 0.13
100	22.11 ± 0.07***	38.63 ± 0.35***	50.12 ± 0.22***	51.89 ± 0.16***	68.32 ± 0.18
200	36.23 ± 0.05***	55.26 ± 0.41***	65.36 ± 0.34***	68.49 ± 0.21***	81.52 ± 0.20
300	43.28 ± 0.09***	63.86 ± 0.52***	72.57 ± 0.38***	75.45 ± 0.24***	87.96 ± 0.18
400	50.19 ± 0.10***	69.98 ± 0.65***	79.25 ± 0.58***	80.12 ± 0.22***	91.32 ± 0.15
500	58.31 ± 0.07***	80.12 ± 0.11***	89.78 ± 0.66***	90.11 ± 0.35***	99.19 ± 0.13

Results are expressed as Mean ± SEM., n=3. Comparisions are made between Ascorbic acid vs Hexane, ethylacetate, methanol and Crude extracts; statistically significant **P<0.01 ***P<0.001

Table 4 Concentration dependent percentage inhibition of different extracts of *Ecbolium viride* by Reducing power assay

Conc (µg/ml)	Hexane Extract	Ethylacetate Extract	Methanol Extract	Crude Extract	Ascorbic acid
25	2.59 ± 0.22**	13.08 ± 0.09**	14.21 ± 0.17**	20.38 ± 0.13**	27.35 ± 1.6
50	9.48 ± 0.21***	25.13 ± 0.18***	29.23 ± 0.28***	30.48 ± 0.16***	47.12 ± 1.2
100	20.41 ± 0.21***	32.79 ± 0.16***	35.73 ± 0.25***	40.22 ± 0.11***	68.32 ± 2.1
200	28.12 ± 0.12***	41.22 ± 0.06***	44.22 ± 0.34***	52.18 ± 0.32***	81.52 ± 1.4
300	35.23 ± 0.11***	49.19 ± 0.25***	54.26 ± 0.43***	68.36 ± 0.23***	87.96 ± 1.3
400	42.28 ± 0.25***	56.22 ± 0.08***	63.13 ± 0.19***	72.63 ± 0.36***	91.32 ± 1.5
500	56.23 ± 0.11***	63.23 ± 0.17***	74.08 ± 0.10***	78.13 ± 0.44***	99.19 ± 1.8

Results are expressed as Mean ± SEM., n=3. Comparisions are made between Ascorbic acid vs Hexane, ethylacetate, methanol and Crude extracts; statistically significant **P<0.01 ***P<0.001

RESULTS

Phytochemical Screening

E. viride exhibited differential distribution of phytoconstituents in seven extracts (Table 1). Phenols, Tannins and Saponins are present in all extracts.

Total Phenolic Content

The total phenolic content (TPC) was expressed as gallic acid equivalents (Table 2). Significant differences were observed for TPC among the four extracts. TPC was in the range of 34.63-402 mg GAE/g dried extract. Highest TPC was observed in crude methanolic extract followed by methanolic extract, ethyl acetate and hexane extracts. Generally, the phenolic

content of all the extracts were considerably hight, which could be a major contributing factor to the strong antioxidant activity.

Total Flavonoid Content

Total flavonoid contents were determined by the $AlCl_3$ method is presented in (Table 2). The content of flavonoids ranged between 10.32 and 34.32 mg QE/g showing differential distribution in the extracts. Comparatively higher amount of total flavonoid contents was found in crude methanolic extract followed by ethylacetate and methanol. Hexane extract was observed with least flavonoid content comparitively.

Total Alkaloid Content

Total alkaloid contents were determined by the Fazel *et al* method is presented in (Table 2). The content of alkaloids ranged between 12 and 26.18 mg/ g dried extract showing differential distribution in the extracts. Comparatively higher amount of total alkaloid contents was found in crude methanolic extract followed by ethylacetate and methanol. Hexane extract was observed with least alkaloid content comparitively.

DPPH radical scavenging method

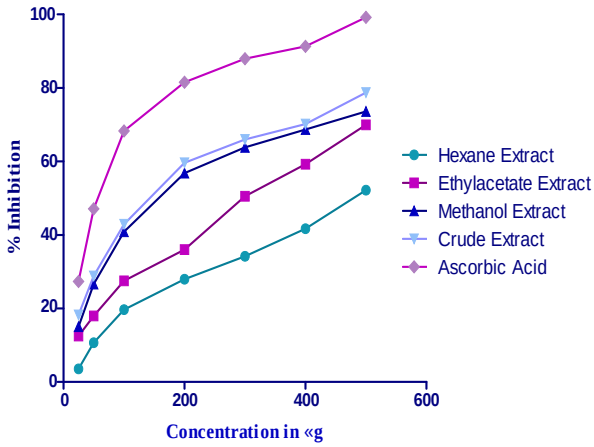

Fig.1 Concentration dependent percentage inhibition of different extracts of *Ecbolium viride* on DPPH radical.

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. Therefore each extract of *E.viride* is tested with DPPH to determine their free radical scavenging ability. The result of the DPPH scavenging assay is shown in Figure 1. It is found that antioxidant compounds in Crude and methanolic extracts have exhibited effective free radical scavenging activity. The IC_{50} values were calculated from the logarithmic regression curve. The highest free radical (DPPH) scavenging activity was observed in Crude extract with IC50 value 91µg/ml followed by Methanol, ethyl acetate and hexane extracts (99.7 µg/ml, 166 µg/ml and 394 µg/ml respectively), may due to the presence of flavonoids, tannins and phenolic compounds that act as primary antioxidants or free radical scavengers.

Reducing power assay

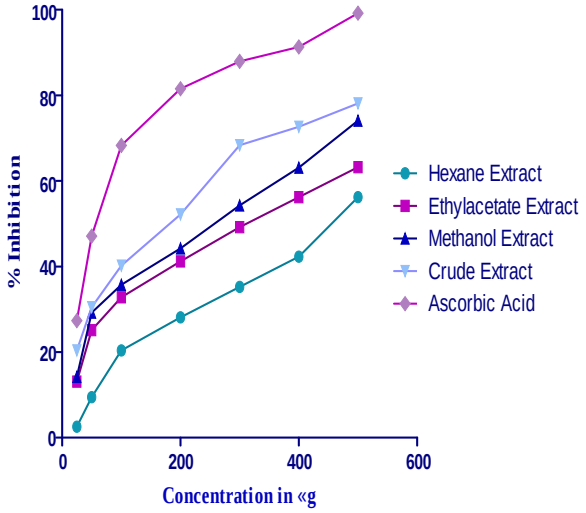

Fig 2 Concentration dependent percentage inhibition of different extracts of *Ecbolium viride* by Reducing power assay

Figure 2 shows the reducing power of four different extracts as a function of their concentration. The presence of reductants causes the reduction of the Fe 3+ /ferricyanide complex to the ferrous form. Therefore, measuring the colour change at 700 nm can monitor the Fe 2+ concentration. The reducing power of the various extracts of *E.viride* increased with increase in the concentration. The highest reducing power was observed in Crude extract with IC50 value 185.2µg/ml followed by Methanol, ethyl acetate and hexane extracts (255 µg/ml, 317 µg/ml and 458 µg/ml respectively), which remained lower than that of ascorbic acid. In general, extracts produced lower activity at lower concentrations.

Hydrogen peroxide assay

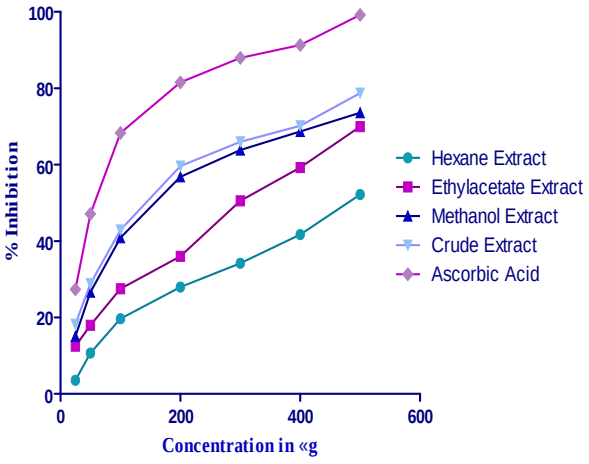

Fig 3 Concentration dependent percentage inhibition of different extracts of *Ecbolium viride* by Hydrogen Peroxide scavenging assay

The result of the Hydrogen peroxide scavenging assay is shown in Figure 3. It is found that antioxidant compounds in Crude and methanolic extracts have exhibited effective free radical scavenging activity. The IC_{50} values were calculated from the logarithmic regression curve. The highest free radical

(DPPH) scavenging activity was observed in Crude extract with IC50 value 133.23µg/ml followed by Methanol, ethyl acetate and hexane extracts (154.18 µg/ml, 300.25 µg/ml and 481.10 µg/ml respectively)

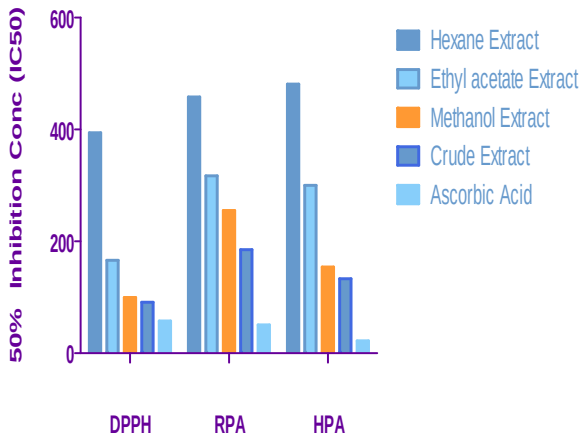

Fig 4 50% Inhibition concentrations (IC_{50}) of different extracts of *Ecbolium viride* against DPPH, Reducing Power and Hydrogen Peroxide scavenging assay

DPPH- DPPH Assay; RPA – Reducing Power Assay; HPA – Hydrogen Peroxide Assay

DISCUSSION

Flavonoids are a group of phenolic compounds having antioxidant potential and play an important role in protection against oxidative stress. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals. Further detailed work on isolation and characterization of specific chemical moieties from the *E.viride* aerial parts extracts and their biological testing can provide us with an effective nontoxic antibacterial, antioxidant, and antitumor agents in future.

CONCLUSION

The plant screened for phytochemical constituents seemed to have the potential to act as a source of useful drugs and also to improve the health status of the consumers as a result of the presence of various compounds that are vital for good health. The present findings of the study suggest *Ecbolium viride* could be a potential source of natural antioxidant that could have great importance as therapeutic agent.

Conflict of Interests

The authors declare that they do not have any conflict of interests.

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