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RESEARCH PAPER

Antioxidant and Free Radical Scavenging Activity of *Hygrophila schulli (Buch.-Ham.) Almeida and Almeida.* Seeds

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ABSTRACT

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human against infections and degenerative diseases. Current research is now directed towards natural antioxidants originated from plants due to safe therapeutics. Hygrophila schulli is used in Indian traditional medicine for a wide range of various ailments. To understand the mechanism of pharmacological actions, antioxidant properties of the Hygrophila schulli seed extract were tested using standard in vitro models. The alcoholic extract of Hygrophila schulli exhibited strong scavenging effect on 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radical, superoxide, nitric oxide radical and ABTS radical scavenging assay. The free radical scavenging effect of Hygrophila schulli extract was comparable with that of the reference antioxidants. The data obtained in the present study suggests that the extract of Hygrophila schulli seed have potent antioxidant activity against free radicals, prevent oxidative damage to major biomolecules and afford significant protection against oxidative damage.

***KEYWORDS**: Hygrophila schulli, Antioxidant, DPPH, FRAP, In vitro.*

INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals [1]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism [2]. The most common reactive oxygen species (ROS) include superoxide ($0_2^{\cdot}$) anion, hydrogen peroxide (H_20_2), peroxyl (ROO-) radicals, and reactive hydroxyl (OH.) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\cdot}$) and peroxynitrite anion ($ONOO^{\cdot}$). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome [3]. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers [4,5]. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability [6]. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic. etc. [7]. They were also suggested to be a potential iron chelator [8,9]. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties.

Hygrophila schulli (Buch.-Ham.) Almeida and Almeida seeds, *Hygrophila auriculata* (Schum) Heine (syn) *Asteracantha longifolia* Nees, Acanthaceae is described in ayurvedic literature as Ikshura, Ikshugandha, and Kokilasha "having eyes like the Kokila or Indian Cuckoo." The plant is widely distributed throughout India, Srilanka, Burma, Malaysia and Nepal. The whole plant, roots, seeds, and ashes of the plant are extensively used in traditional system of medicine for various ailments like rheumatism, inflammation, jaundice, hepatic obstruction, pain, urinary infections, oedema and gout. It is classified in ayurvedic system as seethaveeryam, mathuravipaka and used for the treatment of

premeham (Diabetes), athisaram (Dysentry) etc., [10,11]. The plant is known to possess antitumor [12,13], hypoglycaemic [14], antibacterial [15,16] and hepatoprotective [17] activities.
Therefore, the objective of the study was to investigate the phytochemical profile and *in vitro* antioxidant activity of *Hygrophila schulli (Buch.-Ham.) Almeida and Almeida seeds* (Gaertn) seeds.

MATERIAL AND METHODS

Chemicals

Trolox (6-hydroxy-2,5,7,S-tetramethylchromam-2-carboxylic acid) was purchased from Sigma Chemical Co. Ltd USA. . DPPH (1,1 – diphenyl – 1,2 – picryl hydrazyl), TPTZ(2,4,6,-tripyridy-s-triazine), potassium ferricyanide, trichloroacetic acid (TCA), $FeCl_3$, sodium nitroprusside, sulphanilamide, napthylethylenediamine dihydrochloride, TPTZ(2,4,6,-tripyridy-s-triazine), ascorbic acid, NBT (nitroblue tetrazolium), reduced NADH (nicotinamide adenine dinucleotide), PMS (phenazine methosulfate), sulphuric acid (H_2SO_4), ammonium molybdate, ascorbic acid/standard Vitamin C (Vit. C), quercetin and pyrocatechol was purchased from HiMedia, Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

Plant material

The seeds of *Hygrophila schulli (Buch.-Ham.) Almeida and Almeida seeds* were collected from Birla college campus, Kalyan, Thane district, Maharastra, India. The plant material was taxonomically identified by Blatter Herbarium St Xavier's College, Mumbai. A voucher specimen (No. 23218 of E. Blatter) has been preserved in a laboratory for further reference. The collected plant was dried under shade and powdered with a mechanical grinder and stored in an air tight container. The dried powder material of the seeds was soaked in ethanol for 10hrs, to get ethanolic extract (EEHS) after filtration through Whatman paper No. 42.

Preliminary phytochemical screening

Qualitative phytochemical analysis of EEHS was carried out as follows: Phenolics: 2ml of filtrate + 2ml $FeCl_3$, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5ml distilled water); frothing persistence indicated presence of saponins. Alkaloids: 2ml of filtrate +1%HCl+Dragendroff reagent, orange precipate indicate the presence of alkaloids. Flavonoids; 5ml dilute ammonia was added to a portion of filtrate +concentrated sulphuric acid; yellow colour indicates presence of flavonoids. Steroids (Liebermann-Burchard reaction: 2ml filtrate +2ml acetic anhydride +concentrated sulphuric acid; green color indicates the presence of steroids. Terpenoids: 4ml of filtrate +concentrated sulphuric acid 3ml was added to form a layer; reddish brown colouration interface indicates the presence of terpenoids. Cardiac glycosides (Keller-Kinliani test): 2ml filtrate + 1ml of glacial acetic acid + $FeCl_3$ +concentrated H_2SO_4; brown colour indicates the presence of cardiac glycosides (18).

Total phenolic content

The total phenolic content of different extracts was measured using colorimetric Folin –Ciocalteu method. The reaction mixture consisted 5ml of diluted sample to which 3 ml of distilled water and 0.5 ml Folin –Ciocalteu reagent was added. After 3minutes, add 2ml of 20% Na_2CO_3 solution and place the tubes in boiling water bath for one min, cooled and the absorbance was measured at 760 nm. Standard graph was prepared by using different concentration of pyrocatechol [19].

Total flavonoid content

The flavonoid content of different extracts was measured using a modified colorimetric method. 0.5ml of sample was mixed with 0.5 ml of 2% $AlCl_3$ and incubated for 10mins. and the absorbance was measured at 415 nm. The measurement was compared to a standard graph for quercetin [20].

Antioxidant Activity

Determination of reducing power (Fe^{3+} - Fe^{2+} transformation ability)

The reducing power of a compound serves as significant indicator of its potential antioxidant activity. Increased absorbance of the reaction mixture indicates increased reducing power.
Various conc. of the extracts in 1ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50ºC for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged al 3000g for 10 min. upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5ml, 0.1%). The absorbance was measured at 700nm [21].

Super oxide anion scavenging activity

1ml of NBT solution (144µM in 100mM phosphate buffer , pH 7.4), 1ml of reduced NADH (677µM in100mM phosphate buffer, pH 7.4) and 0.5 ml of sample extract was mixed and the reaction was started with adding 100µl of PMS solution (60µM PMS in100mM phosphate buffer, pH 7.4) . The reaction mixture was incubated at 25°C for 5 min, and the absorbance 560 was measured against blank [22].

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} \text{ X } 100$$

Nitric oxide radical scavenging activity

Sodium nitroprusside (5mM, 1ml) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different conc. of the extract and incubated at 25°C for 150 min. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). Measure the absorbance at 546 nm [23].

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} \text{ X } 100$$

DPPH radical scavenging activity

The assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical DPPH. DPPH radical react with suitable reagent, the electrons become paired off and the solution looses color stoichiometrically depending on number of electrons taken up.A volume of 2ml of sample was added to 2ml of phosphate buffer (0.02M, pH 6) and 2ml of 0.2mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min. at R.T. and the absorbance was measured at 517 nm [24].

The capability to scavenge the DPPH radical was calculated using following equation:

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{Acon} \text{ X } 100$$

ABTS radical scavenging assay

For ABTS assay, the method of Re *et al*., 1999 was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate/ ammonium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml $ABTS^{\cdot+}$ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS.+ scavenging capacity of the extract was calculated as ABTS radical scavenging activity (%) = [(Abscontrol - Abssample)]/(Abscontrol)] × 100 where Abscontrol is the absorbance of ABTS radical + methanol; Abssample is the absorbance of ABTS radical + sample extract/standard [25].

FRAP assay

The stock solutions of 10mM TPTZ in 40 mM HCl, 20mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml of acetate buffer . It was prepared freshly and warmed at 37°C. 900µl of FRAP reagent was mixed with 90 µl of distilled water and 30µl of sample solution. The reaction mixture was then incubated at 37°C for 30 min and absorbance was recorded at 595 nm. The concentration of $FeSO_4$ was in turn plotted against concentrations of the standard antioxidants (L-ascorbic acid and Trolox) (26).

Total antioxidant capacity

0.1ml of extract was combined in eppendorf tube with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank [27].

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance i.e. one way ANOVA and student't' test using GraphPad QuickCalcs.. The two-tailed P <0.05 the difference is considered to be statistically significant and if P <0.0001 the difference is considered to be extremely statistically significant The IC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The EEHS was found to contain alkaloids, glycosides, tannins, flavonoids, reducing sugars, proteins, sterols through preliminary photochemical screening.

Total phenolic and flavonoid content

Total phenolic compounds are reported as pyrocatechol equivalents. The total phenolic contents of phenolics content of EEHS were 6.84±0.005 mg pyrocatechol equivalent/g of sample, respectively. The total flavonoid contents of EEHS were 2.71±0.01 mg quercetin equivalent/g of sample. It has been reported that green leafy vegetables, soft fruits and medicinal plants exhibited higher levels of flavonoids [28]. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [29].

Antioxidant activity

Antioxidant activity of the extracts of varying concentrations ranging form 10- 10000 µg/ml was evaluated by various in vitro models. It was observed that the test compounds scavenged free radicals in concentration dependent manner in all the models.

The result of FRAP and TAC assay were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) and Ascorbic acid Equivalent Antioxidant Capacity as described. TEAC is the concentration of Trolox (µmol/L) required to give the same antioxidant capacity as 1% (w/v) test substance. AEAC is the same for Ascorbic acid [30].

Determination of reducing power (Fe^{3+} - Fe^{2+} transformation ability)

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700nm [31]. Increasing absorbance at 700 nm indicates an increase in reductive ability. Table 1 shows that the reducing powers of EEHS and standards also increased with the increase of their concentrations. There is a extremely significant difference ($p< 0.001$) among the EEHS and the standard.

Table 1. Reducing power ((Fe3+ - Fe2+ transformation ability) of EEHS, Vit C and Trolox

conc.(µg/ml)	Absorbance at 700nm		
	EEHS	Vit C	Trolox
5	0.02±0.00	0.20±0.01	0.11±0.01
10	0.02±0.01	0.23±0.02	0.13± 0.01
50	0.04±0.01	0.37±0.01	0.16± 0.02
100	0.06±0.02	0.48±0.02	0.2±0.001
500	0.12±0.01	0.88±0.03	0.58±0.02

Values are mean ± S.D (n=4)

Super oxide anion scavenging activity

Superoxides are produced from molecular oxygen due to oxidative enzymes [32] of body as well as via nonenzymatic reaction such as autoxidation by catecholamines [33]. In the present study,

superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm [34]. Table 2 shows the superoxide scavenging effect of EEHS on the PMS/NADH-NBT system. The increase of percentage scavenging activity thus indicates the consumption of superoxide anion in the reaction mixture by the plant extracts. Maximum percentage scavenging activity showed by EEHS is 200.17 ± 4.10 at 1000 µg/ml. The IC_{50} value of EEHS and Trolox 90.54±3.32 and 2857.14± 1.01 µg/ml respectively . There was a significant difference ($p<0.05$) between activities of EEHS and Trolox.

Nitric oxide radical scavenging activity

The extract effectively reduced the generation of nitric oxide from sodium nitroprusside (Table 2). *In vitro* inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent [35]. EEHS decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* which may be due to the presence of antioxidant principles in the extract. The percentage scavenging activity increased with increasing concentration of the extract. Lower the IC_{50} value of better is the scavenging abitity of the sample. The IC_{50} value of EEHS was found to be 130.42±3.32 µg/ml, ($p < 0.05$).

Table -2 % Radical scavenging activity of EEHS in *In vitro* Assays at various concentrations

conc.(µg/ml)	% scavenging activity			
	Superoxide anion radical	Nitric Oxide radical	DPPH radical	ABTS radical
	EEHS	EEHS	EEHS	EEHS
10	0.90±0.01	61.11±1.28	17.01±1.21	15.12±1.11
50	7.12±0.71	66.66±2.72	34.51±1.00	26.18±0.81
100	12.01±1.78	72.71±1.87	56.78±0.12	38.12±1.30
500	28.17±1.01	77.71±2.81	64.05±1.86	52.91±1.21
1000	38.09±1.91	77.74±2.12	74.28±1.82	63.94±1.43

Values are mean ± S.D (n=4)

DPPH radical scavenging activity

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts [36]. DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. All the concentration of EEHS demonstrated H-donor activity. Lower the IC_{50} value of better is the scavenging ability of the sample. The IC_{50} values of EEHS and Trolox was 667.64±5.12µg/ml and 17.42±1.21µg/ml respectively. (Table 2). There was significant difference ($p < 0.05$).

ABTS assay

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals (37). The EEHS were fast and effective scavengers of the ABTS radical and this activity was comparable to that of Vit. C. Higher concentrations of the extracts were more effective in quenching free radicals in the system. Trolox has shown higher antioxidant activity (% inhibition) as compared to EEHS in ABTS (2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) radical scavenging assay with IC_{50} values 5.42±0.27 and781.25±3.03 µg/ml respectively (Table 2)**.** There was significant difference ($p< 0.05$) between Trolox and EEHS

FRAP assay

In FRAP assay the ability of plant extract to reduce ferric ions was determined. FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe^{+2}- tripyridyltriiazine compound from the colourless oxidized Fe^{+3} form by the action of electron donating antioxidants [38]. The FRAP value of EEHS is presented in Table 3. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present it can be reported that EEHS may

act as free radical scavenger, capable of transforming reactive free radical species into stable nonradical products.

Total antioxidant capacity

Total Antioxidant capacity of EEHS is shown in Table 3. The phosphomolybdenum method was based on reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH [39]. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transformating reactive free radical species into stable non reactive products [40]

Table 3 FRAP and TAC value of EEHS

1% (w/v) extracts used	FRAP Values (µmol/L)	TAC Values (µmol/L)
EEHS (TEAC)	0.74±0.01	1.63±0.03

Values are mean ± S.D (n=4)

CONCLUSION

On the basis of the results obtained in the present study, it is concluded that a ethanolic extract of *Hygrophila schulli* seed exhibits high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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