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Phytoconstituents based Antiradical Defense Response of *Hygrophila auriculata* Root

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A B S T R A C T

The accumulation of free radicals in the body leads to many diseases due to oxidative stress. Numerous studies are currently being conducted to prevent oxidative stress and neutralize the effects of free radicals by antioxidants primarily of plant origin rather than synthetic ones. *Hygrophila auriculata* belonging to the family Acanthaceae was used in this study to assess the phytochemical constituents, free radical scavenging ability, and the amount of enzymatic and non-enzymatic antioxidants. Four solvents, namely ethanol, ethyl acetate, chloroform, and water, were taken to extract *Hygrophila auriculata* root. Preliminary phytochemical analysis was carried out by adopting the standard protocol. Further, the free radical scavenging activity was assessed against two radicals, DPPH and FRAP, along with the reducing power ability. The enzymatic antioxidants include superoxide dismutase, catalase, peroxidase, polyphenol oxidase, glutathione-S-transferase, and non-enzymatic antioxidants such as ascorbic acid, α-tocopherol, and total phenols were assessed. The phytochemical screening revealed the presence of compounds such as alkaloids, flavonoids, sterols, phenols, saponins, tannins, proteins, carbohydrates, cardiac glycosides, and terpenoids firmly in the ethanol extract compared to all other solvents. The free radical scavenging activity showed a maximum scavenging power in the ethanol extract. The scavenging efficacy of DPPH and ABTS was 89.53 and 85.71%, respectively, at 100 µg/ml. Similarly, the reducing power was highest at the dose of 100 µg/ml. The ethanol extract possesses enzymatic antioxidants such as superoxide dismutase (111.3 ± 3.21 Units/mg), peroxidase (0.34 ± 2.11 Units/mg), catalase (4.53 ± 0.9 Units/mg), polyphenol oxidase (0.4 ± 0.01 µg/g), glutathione-S-transferase (2.3 ± 0.5 µg/g) and non-enzymatic antioxidants such as flavonoids (243.40 ± 1.23 mg/g), α – tocopherol (32.3 ± 0.18 µg/g), vitamin – C (2.93 ± 0.02). Thus, our findings suggest that roots of *Hygrophila auriculata* have the potential to scavenge the free radicals and prevent oxidative stress-related diseases, which pave the way for the plant to serve as a good phytotherapeutic agent against many diseases and disorders.

***Key words*:** *Hygrophila auriculata*, Antioxidants, Free radicals, Phytochemicals, DPPH

Traditionally used medicinal plants play a prominent role in human health as therapeutic remedies. Natural products have been discovered to be a repository of diverse biomolecular structures far beyond human knowledge [1]. Phytochemicals are bioactive plant compounds that alleviate many human physiological disorders and suppress synthetic antibiotics' consumption. Normal cellular metabolic reactions of the human body in a more exposed environment and higher levels of ingested xenobiotics lead to the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In distinct pathophysiological situations, ROS and RNS are responsible for causing oxidative stress. This further produces unstable molecules known as free radicals, enhancing many chronic and degenerative ailments [2]. Oxidative stress and free radical suppression could be achieved by efficiently neutralizing cellular responses in antioxidants [3].

The defense response against the reactive oxygen species could be activated by two antioxidants: enzymatic and non-enzymatic. The body safeguards itself from ROS by employing enzymatic antioxidant processes, which include enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase and glutathione-S-transferase (GST). These enzymes are essential in preventing the cells from lipid peroxidation and supporting cell membranes' stability and proper functioning. Non-enzymatic antioxidants disrupt the free radical chain reaction. Consequently, both antioxidants protect the body from DNA damage, tumor growth, cardiovascular diseases, neuroprotective diseases, etc. [4].

Phytoconstituents can scavenge free radicals by donating electrons or ions to those unpaired electrons. Several researchers have been exploring potent antioxidants to be

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extracted from medicinal plants because they are financially sustainable and have excellent antioxidant properties without adverse effects. *Hygrophila auriculata* (Buch. -Ham) (*H.auriculata*) is a thorny sub-shrub of the family Acanthaceae that grows widely throughout the moist places of India. The leaves and roots of this plant are medicinally utilized for treating many disorders, such as inflammation, jaundice, diabetes, etc. [5]. With the above context, the present investigation aimed to evaluate *H. auriculata* for its potential use as a natural source of phytochemicals and antioxidants.

MATERIALS AND METHODS

Plant collection and sample preparation

The roots of *H. auriculata* were collected from the areas of Coimbatore. The roots were washed entirely and let dry for 5-7 days at room temperature. The dried-out leaves were ground to powder and stored in screw-cap bottles until further analysis. Preparation of the extract A 50 g of sample was dissolved in 500 ml of various solvents (ethanol, ethyl acetate, water, and chloroform). It was then filtered and further concentrated by evaporation.

Phytochemical analysis

The extracts were subjected to preliminary phytochemical evaluation, which was done using standard color test methods [6].

Free radical scavenging activity

The radical scavenging activities of the extracts were determined *in vitro* against a battery of radicals, namely DPPH and ABTS, and FRAP.

DPPH radical scavenging activity [7]

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. About 3 ml of graded concentration (25 - 100µg/ml) of extracts were taken in different test tubes, and 1 ml of 0.3mM DPPH methanol solution was added to these test tubes and shaken vigorously. Methanol served as the blank, and DPPH in methanol, without the rhizome extracts, served as the positive control. After 30 min incubation of samples at 25 °C in the dark, the absorption was measured at 517 nm. The inhibition percentage of DPPH was calculated as follows:

Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control) × 100

Abs (control)- absorbance of DPPH radical with methanol

Abs (sample)- absorbance of DPPH radical with sample extract

ABTS radical scavenging activity [8]

ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 hours before use. Aliquots (5µl) of the different extracts were added to 0.3ml of ABTS solution, and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer, and the percent scavenging was calculated using the formula:

Scavenging activity (%) = Abs (control) – Abs (sample) / Abs (control) × 100

FRAP (Ferric reducing power assay) [9]

Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%), and varying concentrations of extracts (25 - 100µg/ml). After the reaction, mixtures were incubated at 50°C in a water bath for 30 min, allowed to cool at room temperature (28 °C), and 2.5 ml of 10% TCA (Trichloroacetic acid) was mixed into each reaction mixture, followed by the centrifugation at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in the test tube, added with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1.0%), and allowed to react for 10 min and absorbed at 700 nm.

Antioxidant activity

The antioxidant status of the roots of *H. auriculata* was estimated by analyzing various enzymic and non-enzymic parameters.

Estimation of catalase activity [10]

H_2O_2-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of enzyme extract (0.01 - 0.04), and mixed thoroughly. The time taken for a decrease in absorbance for 0.5 units is noted. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with a more concentrated sample solution.

Estimation of peroxidase activity [11]

Three milliliters of 0.05M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract were taken in a test tube. 0.5 ml of 1% hydrogen peroxide was added to the test cuvette. The spectrophotometer was adjusted to read '0' at 400nm. Changes in absorbance were recorded every 30 seconds up to 3 minutes.

Estimation of superoxide dismutase activity [12]

The incubation medium contained a 300µl of each reagent (50mM potassium phosphate buffer (pH 7.8), 45mM Methionine, 5.3mM Riboflavin, 84mM Nitro Blue Tetrazolium (NBT), and 20 mM potassium cyanide. 300µl of the sample was added to this mixture, and the final volume was made up to 3ml with water. The tubes were placed in an aluminum foil lined box maintained at 25 °C and equipped with 15W fluorescent lamps. The NBT reduction was measured at 600nm after 10 minutes of exposure to light. The maximum reduction was evaluated in the absence of an enzyme giving 50% inhibition of the reduction of NBT.

Estimation of polyphenol oxidase activity [13]

2.5 ml of 0.2M phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01 M) were taken into the cuvette and added the enzyme extract (0.2 ml). The spectrophotometer was set at 495nm and recorded the change in absorbance every 30 seconds up to 5 minutes.

Estimation of glutathione-s-transferase [14]

A total of 1.0 ml of buffer, 1.7 ml of water, and 0.1 ml of CDNB were added to the 0.1 ml of sample and incubated for 5 minutes at 37 °C. This was followed by the addition of 0.1 ml of glutathione s transferase was added. At 340 nm, the enzyme's optical density was calculated compared to a blank.

Non-enzymic antioxidants

Estimation of vitamin C or ascorbic acid [15]

The assay volumes were made up of 2.0ml with 4% TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate, respectively, were pipetted out into a clean, dry test tube, the volume of which was also made up to 2.0ml with 4% TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by two drops of 10% thiourea solution. The sample was incubated at 37 °C for 3 hours. The osazones formed were dissolved in 2.5ml of 85% sulphuric acid in cold, drop by drop, with no appreciable rise in temperature. The

DNPH reagent and thiourea were added to the blank alone after adding H_2SO_4. The tubes were incubated for 30 min at room temperature, and the absorbance was read spectrophotometrically at 540nm. The ascorbic acid content in the sample was calculated using the standard graph.

Estimation of α-tocopherol [16]

Into three stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5ml of extract, 1.5ml of standard (10mg of α-tocopherol was dissolved in 10ml of absolute alcohol), and 1.5ml of water, respectively. To the test and blank, 1.5 ml of ethanol was added, and to the standard, 1.5 ml of water was added. Added 1.5ml xylene to all the test tubes, stoppered, mixed well, and centrifuged. From this, 1.0ml of the xylene layer was transferred into another stoppered tube. Added 1.0ml of 2, 2'- dipyridyl reagent to each tube, stoppered, and mixed well. 1.5ml of this mixture was pipetted into colorimeter cuvettes, and the test's extinction and standard against the blank were noted at 460nm. 0.33 ml of ferric chloride solution was added to all the test tubes, including the blank. The amount of vitamin E can be calculated using the formula:

Amount of tocopherols in µg = reading at 520 nm- reading at 460 nm/reading of standard at 520 nm × 0.24 × 15

Estimation of flavonoids [17]

An aliquot of the extract was pipetted out and evaporated to dryness. Different volumes of standard catechin (0.2 to 1.0ml) were taken and made up to 1.0ml with distilled water. An aliquot of 4.0ml of vanillin reagent was added, and the tubes were heated for 15 minutes in a boiling water bath and cooled. The optical density of the solution was read at 340 nm. The standard curve was constructed in an electronic calculator set to the linear regression mode, and the concentration of flavonoids was calculated. The values are expressed as mg flavonoids/g tissue.

RESULTS AND DISCUSSION

The preliminary phytochemical screening was carried out on various solvents and revealed the presence of a wide range of phytoconstituents, including alkaloids, flavonoids, sterols, phenols, saponins, tannins, proteins, carbohydrates, cardiac glycosides and terpenoids which showed better result on ethanolic extract among the other three solvents (Table 1). This indicates that ethanol is highly capable of extracting secondary metabolites of *H. auriculata* compared with all other solvents since the high polarity of the solvent accounts for the extraction of a wide range of compounds.

Table 1 Qualitative phytochemical analysis of the extracts of *Hygrophila auriculata*

Constituents	Solvents			
	Chloroform	Ethanol	Ethyl acetate	Aqueous
Alkaloids	+	+	+	-
Flavonoids	+	+	+	+
Sterols	-	-	-	+
Phenols	-	+	+	-
Saponins	-	-	+	-
Tannins	+	+	+	-
Quinones	-	-	-	-
Proteins	+	+	-	+
Carbohydrates	-	+	-	+
Cardiac glycosides	-	+	-	+
Terpenoids	-	+	-	+

+ Present; - Absent

The capacity of the plant extracts to scavenge the free radicals such as DPPH (2,2- di (4-test-octyl phenyl) -1-picrylhydrazyl radical) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation followed by the ability to reduce ferric (III) iron to ferrous (II) iron were assessed by performing the *in vitro* antioxidant assays. Comparative analysis was done using various solvents on the antioxidant activity of *H. auriculata* roots (Table 2). In the present study, the results revealed that ethanol extract of *H. auriculata* has the highest antioxidant capacity against both DPPH (89.53%) and ABTS radicals (85.71%) and also exhibited the potential reducing power (86.24%). This was then followed by ethyl acetate (81.73%), aqueous (77.41%), and chloroform (59.45%). The solvent polarity strongly impacted the presence of secondary metabolites and their antioxidant potential [18]. The radical scavenging ability ranged from 38.76% to 89.53%, which was nearer to the standard (89.89%).

In the DPPH assay, the antioxidant was able to reduce and scavenge the violet-colored radical DPPH to the yellow-colored 1, 1-diphenyl-1, 2-picryl hydrazine stable compound [1]. The antioxidant in the *H. auriculata* root donates an H-atom to DPPH radical making it to DPPH-H. Consequently, as DPPH loses its reactivity, this reaction is distinguished by a decrease in absorbance [19]. Similar interactions occur in the ABTS assay, where the antioxidants act with the generated ABTS radical and decolorize its blue color. Our results showed that the order of ABTS radical scavenging activity of all the extracts was similar to that observed for DPPH. The FRAP assay is generally associated with the presence of reductones which have been shown to exert antioxidant action by donating a hydrogen atom and breaking the free radical chain. During the reducing power assay, reductants (antioxidants) in the *H. auriculata* root would reduce the Fe_3^+/ ferricyanide complex to the ferrous form (Fe_2^+). The amount of Fe_2^+ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Here occurs the color change of yellow to multiple shades of green and blue, which is proportional to its antioxidant ability.

Bioactive compounds such as natural phenols and flavonoids quench the reactive oxygen species, which could defend against oxidative stress and inhibits lipid peroxidation [20-21]. In this study, phyto compounds such as flavonoids, phenolic acids, and phenolic diterpenes in the *H. auriculata* root extract naturally elevated the radical scavenging activities. These phenolic components possess many hydroxyl groups, including the o-dihydroxy group, which have a powerful radical scavenging effect and antioxidant power. Hydroxyl groups play a vital role in hydrogen bond donation that aids the scavenging of free radicals, reducing metal ions, and interacting with biomolecules [22]. A previous study regarding the free radical scavenging ability of *H. auriculata* root reported that petroleum ether extract indicated 93.91±6.57% DPPH radical scavenging ability at 120 µg/mL concentration, which was followed by 62.07±4.34 % of ferric reducing power at 120 (µg/mL) [23].

Toxic free radicals are effectively squelched by the cellular antioxidant defence mechanism endogenously, which shields the biomolecules from oxidative alteration. The endogenous compounds in cells are classified into enzymatic and non-enzymatic antioxidants. In addition to cellular antioxidants, exogenous antioxidants from herbal plants are proved to improve the body's natural defences against disorders and stress. The phytocompounds from the plant accelerate the scavenging and interruption of free radical chain in the body by generating enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase, glutathione-S-transferase (GST) and also the exogenous non-enzymatic antioxidants such as flavonoids, α – tocopherol and

Vitamin – C. These enzymes activate the plant's antioxidant system, which together alleviates the toxic effects of oxidative damage [24].

Table 2 Free radical scavenging activity of *Hygrophila auriculata*

Drug	Concentration	Scavenging ability (%)		
		DPPH	ABTS	FRAP
Chloroform	25	38.76	40.22	33.22
	50	41.09	43.21	47.32
	100	49.80	51.20	59.45
Ethanol	25	77.87	73.33	61.05
	50	81.22	80.07	73.37
	100	89.53	85.71	86.24
Ethyl acetate	25	73.23	74.88	70.33
	50	78.66	77.63	75.88
	100	81.73	79.55	79.38
Aqueous	25	59.21	60.22	61.88
	50	64.35	64.33	67.29
	100	68.20	67.30	77.41
	25	67.89	72.14	67.44
Standard	50	75.23	78.09	71.21
	100	89.89	84.77	87.37

In the present study, the activities of enzymatic and non-enzymatic antioxidants were assessed using ethanol extract of *H. auriculata* due to its high potential free radical scavenging ability. The accumulation of enzymatic antioxidants such as superoxide dismutase (111.3 ± 3.21 Units/mg), peroxidase (0.34 ± 2.11 Units/mg), catalase (4.53 ± 0.9 Units/mg), polyphenol oxidase (0.4 ± 0.01 µg/g), glutathione-S-transferase (2.3 ± 0.5 µg/g) and non-enzymatic antioxidants such as flavonoids (243.40 ± 1.23 mg/g), α – tocopherol (32.3 ± 0.18 µg/g), vitamin – C (2.93 ± 0.02) in the ethanolic extracts of *H. auriculata* showed significant results (Table 3). All the enzymatic antioxidants which involve in the act of initial defense mechanisms converts reactive oxygen species (ROS) and superoxide anion to lipid hydrogen peroxide (H_2O_2) and hydroperoxide. Further, the enzyme catalase facilitates the degradation of hydrogen peroxide (H_2O_2) and hydroperoxide as water and oxygen. The above process is accomplished in the presence of zinc (Zn), copper (Cu), and manganese (mn) metal ions. Thus, SOD, POD, and CAT are the most potent antioxidants that prevent the body from lipid peroxidation, toxification of the cells, and excessive oxygen radicals and maintain the cell structure and growth [4].

Table 3 Quantitative estimation of enzymatic and non-enzymatic antioxidants

Enzymatic antioxidants	Level
SOD (Units/mg)	111.3 ± 3.21
POD (Units/mg)	0.34 2.11
CAT (Units/mg)	4.53 ± 0.9
Polyphenol oxidase (µg/g)	0.4 ± 0.01
GST (µg/g)	2.3 ± 0.5
Non-enzymatic antioxidants	
Flavonoids (mg/g)	243.40 ± 1.23
α – tocopherol (µg/g)	32.3 ± 0.18
Vitamin – C (µg/g)	2.93 ± 0.02

Similarly, the reduction of glutathione in the body promotes defense against oxidants and neutralizes the hydrogen peroxide in the cell. Glutathione S-transferases (GSTs) is a detoxification enzyme that helps reduce and conjugates glutathione with various electrophilic compounds, making the macromolecule more soluble [25-26].

Polyphenol oxidase is a vital copper enzyme known as catechol oxidase, tyrosinase, etc. [27]. Polyphenol oxidase is a potent antioxidant that scavenges H_2O_2 thereby neutralizing oxidative stress and regulating the other oxidases in the body [28-29]. This is because the electrons in the phenolic hydroxyl structure have a conjugation effect, which weakens the binding ability of hydrogen ions and raises their risk of dissociation. As a result, the active hydrogen ion suppresses the reactive oxygen species and other oxidants, stabilizing themselves [30].

Following this, the second line defense mechanism inhibits the production of damaged cell species and the progressing of harmless free radicals, thereby reducing the impact of oxidative reaction. This was worked by some of the non-enzymatic antioxidants such as flavonoids, α-tocopherol, and Vitamin – C [31].

The presence of flavonoids in the qualitative phytochemical analysis of ethanol extract of *H. auriculata* root is comparable to the accumulation of 243.40 ± 1.23 mg/g of flavonoids in the quantitative analysis. In the phenolics group, flavonoids constitute polyphenols, highly associated with antioxidant potential [32]. Flavonoids play a vital role in protecting DNA from damage caused by hydroxyl radicals. The chelation reaction involving the metal ions like copper and iron elucidates the preventive effect of flavonoids on DNA damage. The flavonoids suppress the production of free radicals by complexing with the chelating metal ions, thereby neutralizing it [33-34].

Subsequently, vitamin E is a lipid-soluble vitamin with high antioxidant potency. α -tocopherol, a stereoisomer of vitamin E, is the most bioactive form in humans. As fat-soluble, α -tocopherol safeguards cell membranes from damage by free radicals. Its direct antioxidant action is to prevent lipid peroxidation. It interrupts lipid peroxyl radicals (LOOc) and stops the lipid peroxidation reactions. Tocopheroxyl radical is generated when α -tocopherol reacts with lipid peroxyl radicals, transferring the phenolic hydrogen ion. Although relatively stable, this tocopheroxyl radical can neither trigger nor initiate additional lipid peroxidation, which is a crucial characteristic of a powerful antioxidant [35-37].

The antioxidant Vitamin C, otherwise known as ascorbic acid, retains the α-tocopherol radical to its original form by reducing the generated vitamin E radicals. Therefore, vitamin C can function as an antioxidant by contributing electrons to various enzymatic and non-enzymatic activities [38-39]. The protection of the macromolecules against biological oxidation is achieved through the reduction of transition metal ions of numerous biosynthetic enzymes by vitamin C. This also aids in the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) by behaving as a substrate for the enzyme ascorbate peroxidase [40].

Like vitamin E, vitamin C also inhibits the lipid peroxidation chain reaction by contributing an electron to lipid radical and changing itself to an ascorbate radical. The rapid interaction between ascorbate radicals leads to the generation of one molecule of ascorbate and one molecule of dehydroascorbate, where the dehydroascorbate doesn't have the antioxidant ability. The addition of two electrons to the radical converts it into ascorbate, which has been proposed to carry out by oxidoreductase [4]. Hence the synergistic activity of vitamin C and E suppress the formation of hydroperoxide and other radicals. Thus, the aforementioned function of all enzymatic and non-enzymatic antioxidants demonstrates their potent role

in the antiradical defense mechanism. Hence the observed elevation in SOD, CAT, POD, polyphenol oxidase, and GST indicates that *H. auriculata* can inhibit the effect of oxidative stress.

CONCLUSION

According to the findings of the current study, it was observed that the root of *H. auriculata* contains certain active phytochemical constituents that improve the antioxidant status. It was clearly demonstrated that in each investigation such as free radical scavenging assay and quantitative estimation of the antioxidants significant percentage of scavenging capacity was determined with potent antioxidants. This experimental evidence suggests the candidate plant's use to treat human pathologies in which free radicals play a significant role. However, further investigation is required on the isolation and characterization of the antioxidant constituents.

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