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Antioxidant Property and Free Radical Scavenging Activity of *Hedyotis scandens* (Roxb). Rubiaceae

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Abstract

Hedyotis scandens Roxb is a common medicinal plant locally utilised for the treatment of diseases in many communities, especially in Mizoram. However, scientific evidence for its therapeutic properties is quite poor and no scientific validation is available of its antioxidant capacity, as per our knowledge. Therefore, the present study aims to investigate the phytochemical constituents and therapeutic potential of the aqueous leaf extract of *Hedyotis scandens* Roxb. Free radical scavenging activity was determined in a cell free system. The aqueous leaf extract of *Hedyotis scandens* inhibited the generation of ABTS 2, 2'- azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), DPPH (1, 1-diphenyl-2-picrylhydrazyl) and nitric oxide in a concentration dependent manner. The IC_{50} for DPPH was found to be 68.03 ± 0.15 µg/ml, IC_{50} for ABTS was 53.165 ± 0.3 µg/ml and the IC_{50} for Nitric oxide scavenging activity was found to be 173.15 ± 0.25µg/ml. Phytochemical analysis of the plant extract revealed the presence of 23.67 ± 0.003 GAE mg/g of Total Phenolic Content and 27.44 ± 0.28 QE mg/g of Total Flavonoid Content. The total antioxidant activity of the plant extract when compared to standard Ascorbic acid was found to be 51.11 ± 1.03 AE mg/g. The reducing power assay showed concentration dependent activity against potassium ferricyanide.

Keywords: *Hedyotis scandens*, Free radical scavenging, Antioxidants, Total Flavonoid and Total Phenol Content.

Medicinal plants are the basis of traditional medicine in various countries and the investigation of pharmacological properties of these plants has revealed numerous novel phytochemicals which can be used to treat diseases (1). Plant materials are considered one of the most important resources in curing serious diseases and pharmacognostic investigation of plants are carried out to discover novel drugs (2). Although more than 2,50,000 species of higher plants have been studied, only 5 to 10 percent are chemically investigated (3). The WHO (World Health Organization) suggested basic procedures for confirmation of drugs from plants in developing countries. The antioxidants from natural sources are considered safe, bioactive and are the only alternative to synthetic antioxidants in the free radicals associated disease (4).

Reactive oxygen species (ROS) or free radicals are highly reactive species created by cells during cell mediated immune functions and respiration (5). Free radicals can even be produced via other sources like automobile exhaust, pesticides, radiation etc (6). Low to moderate concentration of ROS helps in physiological functions like cellular growth, gene expression, defense against infection,

etc (7). However, if the ROS present within the organism exceeds the flexibility of the organism to provide adequate antioxidant for protection against them, it ends up in oxidative stress. Oxidative stress can cause many diseases including coronary heart diseases, diabetes, arthritis, inflammation, cancer and lung damage (8).

Normally cells are armed with antioxidant enzymes and antioxidant molecules, which can take care of the conventional ROS production however, these agents aren't sufficient to normalize the redox status during oxidative stress (9). Therefore, so as to revive the redox homeostasis in cells exogenous supply of antioxidants may be required. Like ROS, Reactive nitrogen species (RNS) are also produced by normal cellular metabolism (10). Nitric oxide (NO) reacts with certain types of proteins and free radicals like superoxide; it is classified as a free radical because of its unpaired electron. Chronic exposure to nitric oxide can cause various carcinomas, juvenile diabetes, multiple sclerosis and arthritis (11). *In Vitro* quenching of NO radical is one of the methods that can be used to determine antioxidant activity (12). Scavengers of nitric oxide reduce the production of nitrite ions by competing with oxygen (13). Butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert- butyl hydroquinone, the most commonly used synthetic antioxidants is suspected to cause liver damage and act as carcinogens in laboratory animals (14). Antioxidants offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent the disease progression (15). The risk of chronic diseases can be reduced and disease progression can be prevented by enhancing the body's natural antioxidant defences and also by supplementing with dietary antioxidants (16). Products with antioxidative properties and fewer side effects is an active domain of research. Therefore, the development and utilization of more effective antioxidants of natural origin is desirable (17). In the past few years, natural antioxidants from some plants have been studied for their antioxidant and radical scavenging property (18).

Hedyotis scandens Roxb (family: Rubiaceae) is a perennial climbing plant with stems that are more or less woody. Climbing stems can be several metres long, scrambling over the ground or clambering over other plants nearby. It is endemic to south-east Asia including India, Bangladesh, Nepal and Bhutan. *Hedyotis scandens* has been used as traditional medicine in many communities. People of Assam use the whole plant to treat gastric ulcers, heartburn and wound healing (19). In Nagaland, crushed leaves of the plant are applied on skin to cure wart like diseases (20). The people of Mizoram use a decoction of *Hedyotis scandens* against swelling and kidney problem whereas ethnic communities of Jorhat use a decoction of leaves to cure urinary and kidney problems (21) (22). The present study aims to investigate the Total Phenol and Flavonoid content and *in vitro* antioxidant activity of the aqueous leaf extract of *Hedyotis scandens*.

Materials and methods:

Preparation of plant extract

Hedyotis scandens was collected from Tanhril, Mizoram (23º44'20.13"N and (92º40'35.78"E) in July 2019. The plant was authenticated by Botanical Survey of India, Shillong. Leaves of the plant were thoroughly cleaned under running tap water and dried at room temperature. The dried plant material was then ground to make a coarse powder and stored in a closed container for further use.

Preparation of samples for extraction

The ground plant material was weighed and extracted using a soxhlet apparatus. Extraction was carried out for 72 hours and the temperature was adjusted just below the boiling point of the solvent. The plant extract was concentrated and the semi-solid extract

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was stored at 4°C for further use. Henceforth, aqueous leaf extract of *Hedyotis scandens* will be known as HSAE.

Total Phenol content (TPC)

The total phenol content of the plant was estimated by using the modified method of Folin - Ciocalteu assay (23)(24). 1ml of methanolic solution of gallic acid with concentration of 10, 20, 40, 60, 80 and 100µg/ml was mixed with 5ml of tenfold diluted Folin - Ciocalteu reagent. 4ml of sodium carbonate solution (0.7M) was added after 3 minutes and the mixture was allowed to stand at room temperature for 1 hr. Absorbance was measured at 765nm. The same procedure was carried out for the plant extract. A standard calibration curve was prepared from the absorbance readings at different concentrations of Gallic acid. The amount of total phenolic compounds was calculated from the calibration curve. The result was expressed as milligrams of Gallic acid equivalent (GAE) per gram of the dried extract.

Total flavonoid content (TFC)

Total flavonoid content was determined by Aluminium chloride method with slight modification using quercetin as a standard (25). 1ml of extract was mixed with 2ml of distilled water. 3ml of 5 % sodium nitrite ($NaNO_2$) and 0.3 ml of 10% aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1ml of 1M Sodium hydroxide was added to the reaction mixture and the final volume was made up to 10 ml with distilled water. Absorbance of sample was measured against the blank at 510nm. All the experiment was repeated three times for precision and values were expressed in terms of flavonoid content (Quercetin equivalent, QE) per g of dry weight.

DPPH radical scavenging activity:

Estimation of the antioxidant activity followed the method of Leong and Shui (26). Briefly, a stable free radical DPPH (2, 2- diphenyl-1-picrylhydrazyl) was used as a substrate. Antioxidant activity was shown by the scavenging of DPPH. The plant extract was prepared in different concentrations (*viz.* 10, 20, 30, 40, 50, 80, and 100 µg/ml) after that 1ml of 0.1mM solution of DPPH in methanol and 3 ml of different concentration of the extract were incubated at 37 ± 1 °C for 30 min. Butylated hydroxytoluene (BHT) was used as standard reference. Absorbance was measured at 517nm against control in a UV-Vis spectrophotometer. The percentage of inhibition was calculated by comparing the absorbance values of the test samples with those of the controls. The inhibition percentage (I) was calculated using the formula:

$$\% \text{ Inhibition} = (Abs_{control} - Abs_{sample}) / Abs_{control} \times 100$$

ABTS Radical Scavenging Activity:

ABTS assay was performed according to the method of Re *et al* with slight modification (27). 7mM ABTS and 2.45mM potassium per sulphate were mixed in equal volumes to prepare a stock solution. The stock solution was then incubated for 12 h at room temperature in the dark to yield a dark coloured solution containing $ABTS^{\cdot+}$ radicals. A working solution was prepared freshly before each assay by diluting stock solution with 50% methanol for an initial absorbance of 0.70 (±0.02) at 745nm. Free radical scavenging activity was then assessed by mixing 500µl of different fractions of various extracts of *Hedyotis scandens* (10 - 100 µg/ml, dissolved in distilled water) with 1 ml of ABTS working solution. The decrease in absorbance was measured up to 3 min after mixing of the solutions. Ascorbic acid was used as standard reference and data was recorded in triplicate. The scavenging activity was estimated using the formula:

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Nitric oxide scavenging activity:

Nitric oxide scavenging assay was performed according to the method of Moon *et al* (28). 2ml of different concentrations (10,

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20, 40, 60, 80, 100, 150 and 200 µg/ml) of extract and standard were mixed separately with 2ml of 10mM sodium nitroprusside and incubated for 2 hours. The mixture was then reacted with 0.5 ml of greiss reagent and the absorbance was measured at 550nm using UV-Vis Spectrophotometer. The percent inhibition was calculated using the formula:

% Inhibition = $(Abs_{control} – Abs_{sample}) / Abs_{control} \times 100$

Determination of reducing power:

The reducing power was estimated following the modified methods of Oyaizu using ascorbic acid as standard (29). 1ml each of the plant extract and ascorbic acid of varying concentrations such as 10, 20, 40, 60, 80 and 100 µg/ml was taken. To this solution 2.5ml of phosphate buffer (6.6 pH) and 2.5 ml of 1% potassium ferricyanide were added. After 20 minutes of incubation at 50 °C, 2.5 ml of 10% tri-chloroacetic acid was added to determinate the reaction. The samples were centrifuged for 10 minutes at 3000 rpm after which 2.5 ml of the supernatant was taken and mixed with 2.5 ml of distilled water and then it was vortexed with 0.5 ml of freshly prepared 0.1% ferric chloride solution. The absorbance was measured at 700 nm. Higher absorbance indicated the increase in reducing power.

Determination of Total Antioxidant Activity:

Phosphomolybdate estimation was carried out by the method of Prieto *et al* with slight modification (30). Standard ascorbic acid was prepared in a series of concentration, *viz.* 10, 20, 40, 60, 80 and 100 µg/ml from which 0.1 ml was taken and mixed with 3ml of a reagent made up of 0.6M sulphuric acid, 4mM ammonium molybdate and 28mM sodium phosphate. The mixture was incubated for 90 minutes at 95°C. The absorbance was measured using UV-Vis spectrophotometer at 695nm to plot a standard curve. The same method was followed for the plant extract from a stock solution of 1mg/ml respectively. Total antioxidant activity was presented as milligrams of ascorbic acid equivalent to per gram of the dried extract.

Results and Disscussion

Total phenolic content (TPC) and total flavonoid content (TFC): TPC was calculated from the standard graph of gallic acid and expressed as milligrams of gallic acid equivalent of the dried extract. The total phenolic content of the plant extract was found to be 23.67 ± 0.003 GAE mg/g. TFC of the plant was estimated using quercetin as standard. The value is expressed as milligrams of quercetin per gram of the dried extract. From the standard graph it was calculated that the plant extract contained 27.44 **±** 0.28 QE mg/g.

DPPH Radical Scavenging Activity: *In-vitro* antioxidant assay of HSAE revealed the presence of antioxidant potential. The extract showed a concentration dependent increase in the scavenging of DPPH radicals which was indicated by the increasing discoloration of DPPH. The highest scavenging activity was demonstrated by 100µg/ml with 72.3 % scavenging activity and the lowest scavenging activity at 10µg/ml was 14.57%. For the standard BHT the highest scavenging activity was 97.6 % and the lowest was 42.03%. The IC_{50} of the standard BHT was found to be 17.29 ± 0.13 µg/ml whereas the IC_{50} of the extract was 68.03 ± 0.15 µg/ml.

ABTS Radical Scavenging Activity: $ABTS^{\cdot+}$ radical scavenging activity of HSAE increased in a concentration dependent manner as indicated by discoloration of the $ABTS^{\cdot+}$ with the rise in concentration which was measured spectrophotometrically at 745 nm. The IC_{50} of the plant extract was 53.165 ± 0.3 µg/ml whereas the IC_{50} of the standard BHT was calculated to be 8.37 ± 0.21 µg/ml. Concentrations 10µg/ml and 100µg/ml showed the highest and lowest scavenging activity respectively. The lowest scavenging activity of the plant extract was 28.71 % and the highest scavenging activity was 75.39% whereas that of standard BHT was 46.4% and 95.84%.

Nitric oxide scavenging activity: Nitric oxide scavenging activity of the plant showed a concentration dependent activity. However, the extract at all concentrations showed a lower scavenging activity than the standard Ascorbic acid. Concentrations 10µg/ml and 200µg/ml showed the highest and lowest scavenging activity respectively. The lowest scavenging activity of the plant extract was 24.36 % and the highest scavenging activity was 50.12% whereas that of standard Ascorbic acid was 24.21% and 80.35%. The IC_{50} for the standard, calculated from the graph was found to be 65.05

Fig. 1: *Hedyotis scandens* (Roxb)

Table 1: IC_{50} (µg/ml) of HSAE, standard Ascorbic acid (ASA) and BHT. Values are expressed as Mean ± SEM, n= 3

DPPH	ABTS	NITRIC OXIDE
68.03 ± 0.15	53.165 ± 0.3	173.15 ± 0.25
BHT	BHT	ASA
17.29 ± 0.13	8.37 ± 0.21	173.15 ± 0.25

± 0.10 µg/ml and the IC_{50} for the plant extract was 173.15 ± 0.25 µg/ml.

Reducing power: HSAE showed positive reducing power against potassium ferricyanide which increases with concentration. However, standard Ascorbic acid showed high activity while *Hedyotis scandens* extract showed only minimal activity.

Total Antioxidant Activity: The total antioxidant activity of HSAE was estimated by the phosphomolybdenum assay. The value was expressed as ascorbic acid equivalent (AE) per gram of the dried extract. The plant extract showed 51.11 ± 1.03 AE mg/g of antioxidant activity.

Phytochemicals like carotenoids, ascorbic acid, phenolic compounds, etc found in plants possess significant benefits to human health. Phytochemical analysis conducted on HSAE revealed the presence of a significant amount of phenolic and flavonoid compounds. In compliance with this study, five new phenolic glycosides were isolated from *Hedyotis scandens* (31). Phenolic compounds are known to function as antioxidants by scavenging the free radicals involved in oxidative processess through hydrogenation or complexing with oxidizing species (32). Flavonoids and Phenols are also known to inhibit the initiation, progression and promotion of tumors and also reduce coronary heart diseases (33).

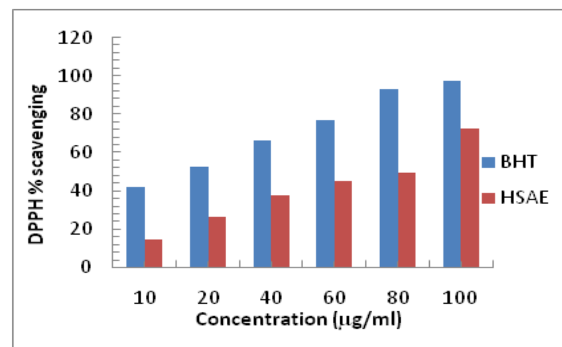

FIG. 2: DPPH scavenging activity of HSAE and Standard BHT. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3

The reduction of methanolic DPPH to a non radical DPPH-H is extensively used to evaluate the antioxidant properties of certain compounds. HSAE effectively reduced the stable radical

DPPH to the yellow coloured diphenyl – pycrylhydrazine as shown in Fig: 2. Compounds like glutathione, ascorbic acid, tocopherol and polyhydroxyl aromatic compounds have been reported to reduce DPPH owing to their hydrogen donating ability (34). The scavenging activity of HSAE increased with increase in concentration. However, standard Ascorbic acid showed higher scavenging activity than the extract. Studies have shown that other *Hedyotis*

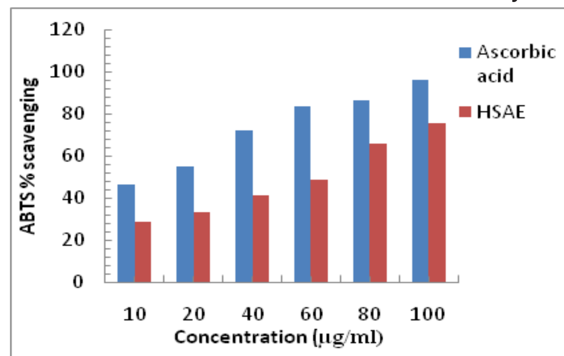

FIG. 3: ABTS scavenging activity of HSAE and Standard Ascorbic acid. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3

species had effectively scavenged DPPH free radicals *in vitro* (35).

The interaction of ABTS and potassium fericyanide produces a blue coloured ABTS·+. The scavenging of ABTS·+ by HSAE took place in a concentration dependent manner, indicated by the discolouration of ABTS·+. This study shows that HSAE has potent antioxidant activity and the ABTS·+ scavenging property can be attributed to the phenolic and flavonoid compounds present in the plant (Fig: 3). A study had shown that methanolic extract of *Hedyotis corymbosa* possessed appreciable ABTS·+ scavenging activity (36). The scavenging of ABTS·+ can be attributed to the abundance of aromatic rings, types of hydroxyl group substitution, molecular weight of phenolic compounds (37).

HSAE showed good NO scavenging activity and reduced the nitrite concentration in the assay medium. Mammalian cells produces nitric oxide which is a free radical, this nitric oxide is involved in a variety of physiological processes. However, excess of nitric oxide is associated with a number of diseases (38). Species of *Hedyotis* like ethanolic extract of *Hedyotis diffusa* and methanolic extract of *Hedyotis corymbosa* has been reported to successfully scavenge nitric oxide (39)(36). In the present study, HSAE inhibited the nitric oxide radical generated from sodium nitroprusside

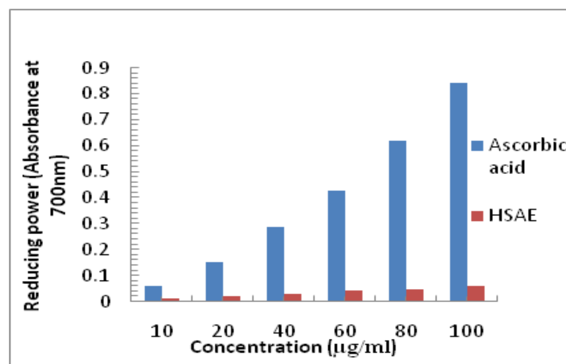

FIG. 4: Nitric oxide scavenging activity of HSAE and Standard Ascorbic Acid. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3.

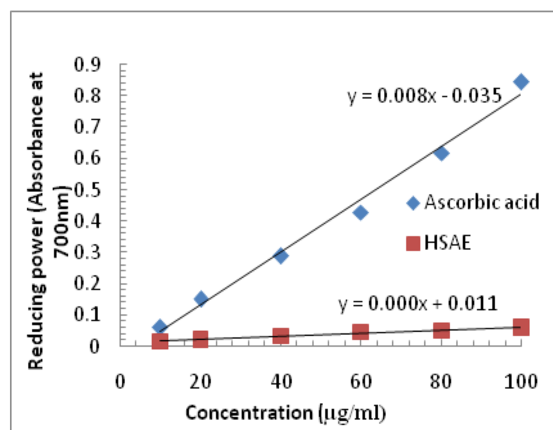

FIG. 5: Reducing power of HSAE and Standard Ascorbic Acid. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3.

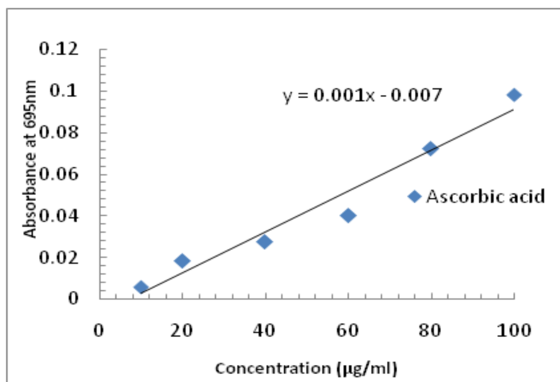

FIG. 6: Standard curve of Ascorbic acid for Total Antioxidant Assay. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3.

in a concentration dependent manner at physiological pH as seen in Fig: 4. Studies have reported the nitric oxide scavenging property of flavonoids and phenolic compounds (40)(41)(42)(43)(44). Therefore, it can be speculated that these constituents might be responsible for the scavenging of nitric oxide.

As shown in Fig: 5, the reducing power of HSAE was evaluated by measuring the transformation of Fe^{3+} to Fe^{2+} using Ascorbic acid as standard. Fe^{3+} reduction is employed as an indicator of electron donating activity, which is an important means of phenolic antioxidant action (45). Reductones (antioxidants) are generally responsible for the reducing ability of a compound, these reductones donate a hydrogen atom which breaks the free radical chain and thereby exerts antioxidant activity (46). The reduction of the Fe^{3+}/Ferricyanide complex to the Fe^{2+}/Ferrous form is caused by antioxidants present in the plant (47).The reducing power of HSAE suggests that it is likely to add considerably towards the overall antioxidant effect. However, the standard Ascorbic acid indicated high activity in comparison to the plant extract which showed only minimal activity.

The total antioxidant capacity of HSAE was evaluated using the phosphomolybdenum assay using Ascorbic acid as standard as shown in Fig: 6. This assay relies on the reduction of molybdenum (IV) to molybdenum (V) by the plant sample and the production of phosphate/molybdenum (V) complex at acidic conditions, which is green in colour and has maximum absorbance at 695nm (48). The phosphomolybdate scavenging activity of medicinal plants has been related to the flavonoid and polyphenols present in the plant (49)(50). Therefore, the total antioxidant capacity of HSAE could be contributed to the presence of phenolics and flavonoids in the plant extract.

Conclusion

Although this plant is widely used in Mizoram for different medicinal purposes, there is very limited scientific proof of its medicinal value. Our study shows that the aqueous extract of *Hedyotis scandens* leaves contain appreciable quantity of bioactive compounds like flavonoid and phenol. The scavenging potential, total antioxidant capacity and reducing properties of the plant observed in this study indicates that it could serve as an alternative for synthetic antioxidants. However this study is not exhaustive and further analysis will be required to confirm the potential effectiveness of the plant extract.

Conflict of Interest

The authors declare no conflict of interest.

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