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TOTAL PHENOLIC AND FLAVONOID CONTENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *HUGONIA MYSTAX* LINN LEAF (LINACEAE)

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ABSTRACT

In vitro antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol, and ethanol extracts of *Hugonia mystax* leaf have been tested using various antioxidant model system viz., DPPH, hydroxyl, superoxide, ABTS and reducing power. Methanol extracts of leaf of *H. mystax* is found to possess higher hydroxyl and ABTS radical scavenging activity. Petroleum ether and ethanol extracts of *H. mystax* leaf exhibited highest DPPH and superoxide radical scavenging activity respectively. Ethanol extract of leaf of *H. mystax* showed the highest reducing ability. This study indicates significant free radical scavenging potential of *H. mystax* leaf which can be exploited for the treatment of various free radical mediated ailments.

Key Words: *Hugonia mystax*, Antioxidant activity, Methanol, ABTS.

INTRODUCTION

Oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vitro* and *in vivo*. There ROS creates oxidative stress which results in numerous disease and disorders such as cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease; alcohol induced liver disease, ulcerative colitis, ageing and atherosclerosis. The compounds from natural sources are capable of providing protection against free radicals. This has attracted a great deal of research interest in natural antioxidants. It is necessary to screen out the medicinal plants for their antioxidant potential [1,2].

The genus *Hugonia* L. of family Linaceae comprise about 40 species in the world; of which *Hugonia mystax* L. was reported from India [3,4]. This plant *Hugonia mystax* is locally known as Modirakanni. Ethnobotanically, the fruits are used by the tribals of Kalakad Mundanthurai for the treatment of Rheumatism [5]. Roots were used as anthelmintic, astringent and also used for dysentery, snake bite, fever, inflammation and rheumatism. Biological activities such as analgesic, anti-inflammatory and ulcerogenic were also reported [6-9]. Roots of *Hugonia mystax* were evaluated for preliminary phytochemical screening and antimicrobial activity. Preliminary phytochemical screening showed the presence of various classes of secondary metabolites such as flavonoids, phenols, saponins, steroids, tannins and terpenoids. Antimicrobial activity of petroleum ether, chloroform, ethanol and aqueous extracts of root extracts showed significant activity against various human pathogens [10].

Taking into consideration of medicinal value and utility, the present study was planned to explore the antioxidant activity of the medicinal plant named *H. mystax.*

MATERIALS AND METHODS

The leaf of *H. mystax* L. were collected from Kodagiri, Nilagiri Biosphere Reserve, Western Ghats, Tamilnadu and identified by the Botanical Survey of India, Coimbatore. A voucher specimen was retained in Ethnopharmacology Unit, Research Department of Botany,

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V. O. Chidambaram College, Tuticorin for further reference. They were shade dried at room temperature for 10-15 days. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender, and sieved to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Preparation of Extracts

Freshly collected leaf samples of *H. mystax* were dried in shade, and then coarsely powdered separately in a Wiley mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No.41 filter papers. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of Total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu method [11]. Samples (100µl) were mixed thoroughly with 2 ml of 2% Na_2CO_3. After 2 min. 100 µl of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against a blank. Total phenolic content was expressed as gram of gallic equivalents per 100 gram of dry weight ($g100g^{-1}DW$) of the plant samples.

Estimation of Flavonoids

The flavonoids content was determined according to Eom *et al* [12]. An aliquot of 0.5ml of sample (1mg/ml) was mixed with 0.1ml of 10% aluminium chloride and 0.1ml of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the nonradical form DPPH-H [13].

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1ml of this solution was added to 3 ml of the solution of all extracts at different concentration (50,100,200,400 & 800µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbances were measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10s UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition)

$$= \{(A_0 - A_1)/A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell, *et al* [14]. Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1ml EDTA, 0.01ml of $FeCl_3$**,**0.1ml H_2O_2, 0.36ml of deoxyribose, 1.0ml of the extract of different concentration (50,100,200,400 & 800µg/ml) dissolved in distilled water,0.33ml of phosphate buffer (50mM , pH 7.9), 0.1ml of ascorbic acid in sequence . The mixture was then incubated at 37^0c for 1 hour. 1.0ml portion of the incubated mixture was mixed with 1.0ml of 10%TCA and 1.0ml of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

Hydroxyl radical scavenging activity

$$= \{(A_0 - A_1)/A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al* [15].The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, P^H 8.0), containing 0.5 ml of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 ml extract of different concentration (50,100,200,400 & 800µg/ml), and 0.5 ml Tris – HCl buffer (16mM, P^H 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12mM) to the mixture, incubated at 25^oC for 5 min and the absorbance was measured at 560 nm against a blank

sample, ascorbic acid. The percentage inhibition was calculated by using the following equation.

Superoxide radical scavenging activity

$$= \{(A_0 - A_1)/A_0)*100\}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the test were performed in triplicates and the results were averaged

Antioxidant Activity by Radical Cation (ABTS. +)

ABTS assay was based on the slightly modified method of *Huang et al* [16]. ABTS radical cation (ABTS.+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS.+ solution, absorbance was measured at 734 nm by Genesys 10s UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Reducing Power

The reducing power of the extract was determined by the method of Kumar and Hemalatha[17] with minor modification to Oyaizu [18]. 1.0ml of solution containing 50,100,200,400 & 800µg/ml of extract was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH6.6) and potassium ferricyanide (5.0ml, 1.0%): The mixture was incubated at 50^{o}C for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980gm (10 minutes at 5^{o}C) in a refrigerator centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0ml of distilled water and ferric chloride and absorbance read at 700nm. The experiment was performed thrice and results were averaged.

RESULT

Total phenolic content and total flavonoid content

The total phenolic content and total flavonoid content of the methanol extract of *H. mistax* leaf were found to be 0.37 and 0.96 respectively. (Fig1)

DPPH radical scavenging activity

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *H. mystax* leaf is shown in fig.2. The scavenging effect increase with the concentration of standard and samples. Among the solvent tested, petroleum ether extract exhibited highest DPPH radical scavenging activity. At 800 µg/ml concentration petroleum ether extract of *H. mystax* leaf possessed 113.66% scavenging activity on DPPH.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of petroleum ether, Benzene, ethyl acetate, methanol and ethanol extracts of *H. mystax* leaf is shown in figure 3. Methanol extracts showed very potent activity. Al 800 µg/ml concentration, methanol extract of *H. mystax* leaf possessed 99.04 % scavenging activity on hydroxyl radical.

Superoxide radical scavenging activity

The *H. mystax* leaf extract were subjected to the superoxide scavenging activity and the results were shown in fig. 4. It indicated that ethanol extract of *H. mystax* leaf (800µg/ml) exhibited the maximum superoxide scavenging activity of 114.2% which is higher than the standard ascorbic acid whose scavenging effect is 72.69%.

ABTS radical cation scavenging activity

The *H. mystax* leaf extracts were subjected to the ABTS radical cation scavenging activity and the results were presented in figure 5. The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration depended manner. At 800 µg/ml concentration, *H. mystax* leaf possessed 90.23% scavenging activity of ABTS which is higher than the standard trolox whose scavenging activity is 74.39%

Reducing Power

Figure 6 showed the reducing ability of different extracts of *H. mystax* leaf compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A highest absorbance indicated a higher reducing power. Among the solvent tested, ethanol extract exhibited higher reducing activity (0.792 OD). Which was higher than the standard ascorbic acid whose reducing ability is 0.493 OD.

IC_{50} value

IC_{50} value of petroleum ether extract of *H. mystax* leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 34.23 µg/ml and 20.14 µg/ml; 31.50 µg/ml and 23.55 µg/ml; 21.54 µg/ml, 22.93 µg/ml and 21.55 µg/ml and 25.09 µg/ml respectively. IC_{50} values of benzene extract of *H. mystax* leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 25.51 µg/ml and 20.14 µg/ml; 27.26 µg/ml and 23.55 µg/ml; 23.91 µg/ml and 22.93 µg/ml and 24.18 µg/ml and 25.09 µg/ml respectively. IC_{50} values of ethyl acetate of *H. mystax* leaf and standard ascorbic acid

for DPPH, Hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 21.66 µg/ml and 20.14 µg/ml; 25.99 µg/ml;25.99 µg/ml and 25.09 µg/ml respectively. IC_{50} values of methanol extract of *H. mystax* leaf and standard ascorbic acid for DPPH, Hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 32.54 µg/ml and 20.14 µg/ml; 33.58 µg/ml and 23.55 µg/ml; 36.59 and 22.93 µg/ml and1.64 µg/ml and 25.09 µg/ml respectively. IC_{50} values of ethanol extracts of *H. mystax* leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging and trolox for ABTS radical cation scavenging were found to be 26.08 µg/ml and 20.14 µg/ml; 29.22 µg/ml and 23.55 µg/ml; 38.54 µg/ml and 22.93 µg/ml and 23.54 µg/ml and 25.09 µg/ml respectively (figure 7).

Fig 1: Total phenolics and flavonoids content of *Hugonia mystax*

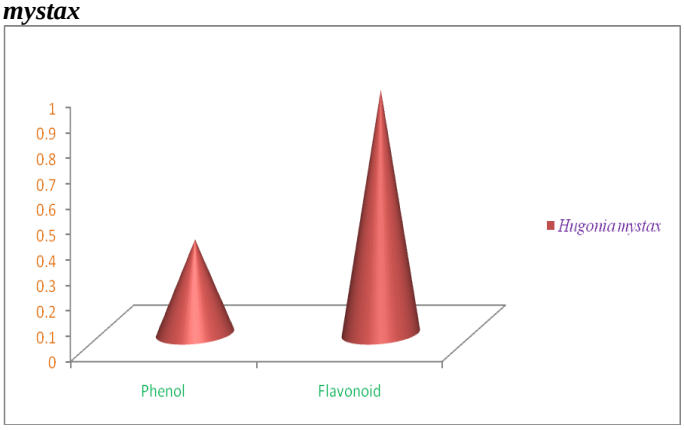


Fig 2: DPPH radical scavenging activity of different extracts of *Hugonia mystax* leaf

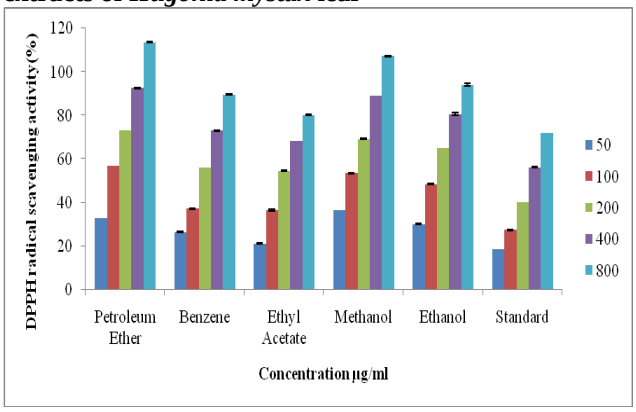


Fig 3: Hydroxyl radical scavenging activity of different extracts of *Hugonia mystax* leaf

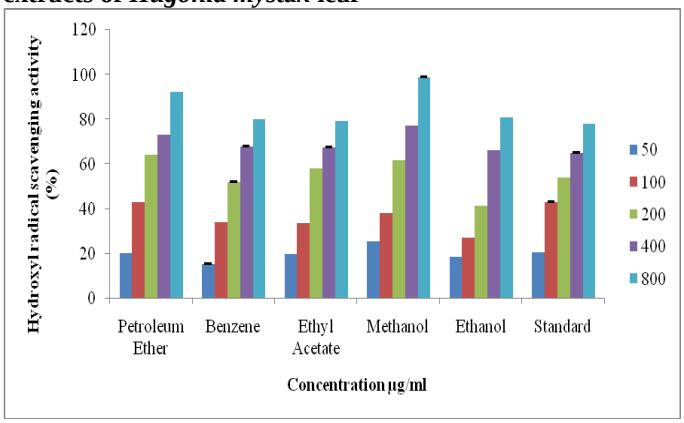


Fig 4: Superoxide radical scavenging activity of different extracts of *Hugonia mystax* leaf

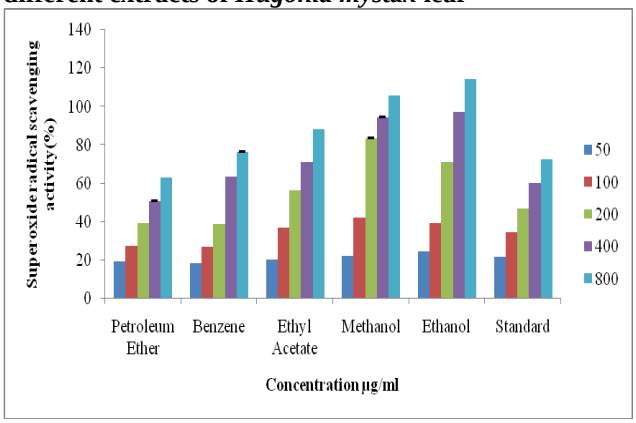


Fig 5: ABTS radical cation scavenging activity of different extracts of *Hugonia mystax* leaf

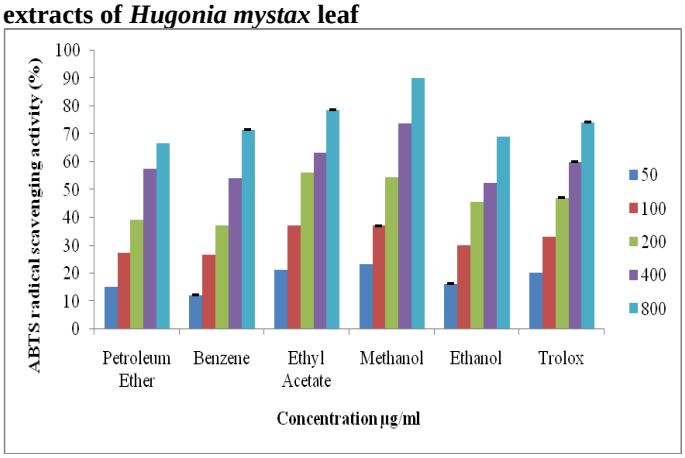


Fig 6: Reducing power ability of different extracts of *Hugonia mystax* leaf

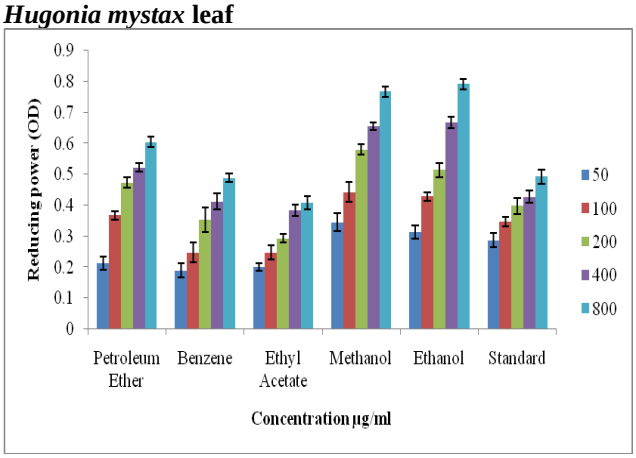
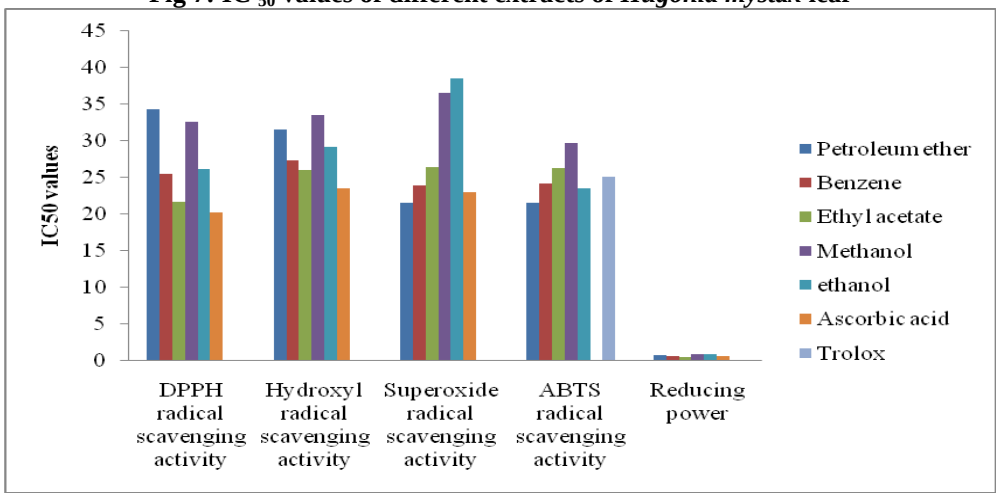


Fig 7: IC $_{50}$ values of different extracts of *Hugonia mystax* leaf



DISCUSSION

The systematic literature collection, pertaining this investigation indicates that the plant phenolics constitute one of the major groups of compounds using as primary antioxidants or free radical scavengers. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity.

Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that, pharmacological effects of flavonoids is correlating with their antioxidant activity [19]. Phenolic compounds are considered to be the most important antioxidants of plant materials. They contribute one of the major groups and compounds acting as primary antioxidants or free radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties [20]. The presence of these compounds such as phenolics and flavonoids in the leaf extracts of *H. mystax* may give credence to its local usage for the management of oxidative stress induced ailments.

Free radicals and other reactive species are thought to play an important role in many human diseases. Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. Many secondary metabolites which include flavonoids, phenolic compounds, etc. serve as sources of antioxidants and do scavenging activity [21,22]. In this study, it is evident that the extracts of the study species, *H. mystax* possess effective antioxidant activity. This feature is perhaps due to the presence of respective phytochemicals like flavonoids, phenolics, etc. in this species [23].

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf of *H. mystax* were investigated in the present study by DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activities. These methods have proven the effectiveness of the extracts in comparison to that of the reference standard antioxidants, ascorbic acid and trolox.

DPPH assay is the most widely reported method for screening antioxidant activity of many plant drugs, based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It was reduced to a yellow colored product, diphenylpicrylhydrazine, with the addition of extracts of *H. mystax* in a concentration-dependent manner [24]. Among the solvent tested, Petroleum ether extracts of leaf of *H. mystax* exhibited more DPPH radical scavenging activity.

Hydroxyl radicals are major active oxygen species causing lipid peroxidation and enormous biological damage. Hydroxyl radical scavenging capacity of extracts of *H. mystax is* directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_2O_2 system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid [25]. When *H. mystax* leaf extracts were added to the reaction mixture, it removed the hyxdroxyl radicals from the sugar and prevented the reaction. Among the solvent tested, methanol extract of leaf possessed more hydroxyl radical

scavenging activity when compared with standard ascorbic acid.

Superoxide anion is also very harmful to cellular components and produced from molecular oxygen due to oxidative enzyme of body as well as via non-enzymatic reaction such as auto oxidation by catecocholamines [26]. The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. The decrease in absorbance at 560nm, *H. mystax* leaf extracts indicated the ability to quench superoxide radicals in the reaction mixture. The present study showed potent superoxide radical scavenging activity for *H. mystax* leaf extracts. The ethanol extracts of leaf showed potent superoxide radical scavenging activity with IC_{50} values 38.94µg/mL compared to ascorbic acid 22.93µg/mL respectively.

ABTS radical cation scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of $ABTS^{+}$ for the estimation of antioxidant activity [27]. In the present study, leaf methanol extracts of *H. mystax* were fast and effective scavengers of ABTS radical and this activity was higher than that of trolox standard. Proton radical scavenging is an important attribute of antioxidants. ABTS a protonated radical has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals [28].

Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity [29]. Therefore, it is necessary to determine the reducing power of phenolic constituents contained in the plant extracts to elucidate the relationship between their antioxidant effect and their reducing power. In the present study, increase in absorbance of the reaction mixture indicates the reductive capabilities of ethanol extracts of *H. mystax* leaf in concentration dependent manner when compared to the standard ascorbic acid.

The present study reveals that the leaf extracts of *H. mystax* exhibited satisfactory scavenging effect in all the radical scavenging assays. This is the first report on the antioxidant property of this plant. It is reported that phenolics and flavonoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms [30]. Thus in the present study, the antioxidant potential of *H. mystax* may be attributed to the presence of flavonoids, phenolics and other constituents present in them.

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