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Research Article

Antioxidant Activity of *Azolla Pinnata* and *Azolla Rubra*- A Comparative Study

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Abstract: *Azolla* is a free floating aquatic fern having agronomic importance worldwide. The present study was carried out to investigate antioxidant potential of two *Azolla* species namely *A. pinnata* and *A. rubra*. Antioxidant activity was evaluated by DPPH free radical scavenging assay and Ferric reducing assay. Total phenolic and flavonoid content was estimated by Folin-Ciocalteau reagent and Aluminium chloride colorimetric estimation method respectively. Extract of *A. pinnata* displayed marked radical scavenging and reducing potential when compared to *A. rubra*. The content of total phenolics and flavonoids were higher in *A. pinnata*. The higher antioxidant efficacy of *A. pinnata* could be ascribed to the presence of high phenolic and flavonoid content. These *Azolla* species can be used against oxidative stress.
Keywords: *Azolla*, Antioxidant, Total phenolics, Flavonoids.

INTRODUCTION

Reactive oxygen species (ROS) are formed in cells of all living organisms during normal metabolism and exposure to various environmental stress conditions such as UV radiations, microbes, allergens, pollutants etc. ROS are defined as intermediate oxygen carrying molecules with or without unpaired electron and include free radicals such as superoxide anions, hydroxyl radicals, peroxylradicals and non-radical forms such as hydrogen peroxide, hypochlorous acid and singlet oxygen. These ROS are beneficial and play vital physiological roles in the body at required concentrations, however, these ROS are detrimental in excess [1-3]. Free radicals and other ROS are effectively eliminated by an enzymatic system including Superoxide dismutase, Catalase, Peroxidase, Glutathione peroxidase and non-enzymatic factors such as vitamin C, vitamin E, thiols etc. Synthetic antioxidants have been used to decelerate or retard the oxidation process. However, they are volatile, decompose at high temperature and their use is restricted due to doubtful safety and potential health hazards. Hence, there is need for search of effective antioxidants from natural sources [4-7].

Azolla (Salviniaceae) is a small pteridophyte (aquatic fern) having agronomic importance in developing as well as developed countries. It is native to the tropics, subtropics, and warm temperate regions of Africa, Asia, and the America. It is one of the fastest growing aquatic macrophytes producing maximum biomass in relatively shorter period of time and is a classic example for symbiotic relationship between an eukaryotic partner *Azolla* and a prokaryotic partner *Anabena*. *Anabena* is an endosymbiont in the leaf cavities of *Azolla* and is associated with all stages of fern's development. *Azolla* provides carbon sources to Anabena and in turn obtain its nitrogen requirements. *Azolla* is used as biofertilizer of crops and is often employed in paddy fields because of its ability to fix dinitrogen at high rates and low cost. In addition, *Azolla* is used as animal feed, human food and medicine, water purifier, green manure, hydrogen fuel, biogas producer, weed and insect controller, and reduces ammonia volatilization after chemical nitrogen application. It improves the water quality by removing excess quantity of nitrates and phosphorus [8-11]. Experimentally, *Azolla* species have been shown to exhibit antioxidant [12, 13], bioremediation [14], plant growth promontory [15], hepatoprotective [16], and antimicrobial activity [17]. In the present study, we have determined antioxidant activity of two *Azolla* species namely *A. pinnata* and *A. rubra*.

MATERIALS AND METHODS

Collection of plant materials

A. pinnata and *A. rubra* were collected from Department of Agricultural Microbiology, UAS, GKVK, Bangalore. The whole plant materials were shade dried, powdered and used for extraction.

Extraction

For extraction, a known quantity (10g) of powdered *A. pinnata* and *A. rubra* was added to 100ml of methanol (HiMedia, Mumbai). The mixtures were left

at room temperature for two days with occasional stirring. The solvent extracts were filtered using Whatman No. 1 filter paper, concentrated by evaporating the solvent and dried in the desiccators [18].

Estimation of total phenolic content of *A. pinnata* and *A. rubra*

The total phenolic contents of methanol extract of both *Azolla* species was estimated by Folin-Ciocalteau reagent (FCR) method. Here, a dilute concentration of extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 4 ml of sodium carbonate (1M) and left for 15 minutes. The optical density was measured colorimetrically at 765nm. A standard curve was plotted using different concentrations of reference standard (Gallic acid, 0-1000 µg/ml) and the total phenolic content of extracts was expressed as µg Gallic acid equivalents (GAE) from the graph [7].

Estimation of total flavonoid content of *A. pinnata* and *A. rubra*

The content of total flavonoids was estimated by Aluminium chloride colorimetric method. Here, a dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of $NaNO_2$ (5%) and incubated for 5 minutes at room temperature. Following incubation, 0.3ml of $AlCl_3$ (10%) was added and again incubated at room temperature for 6 minutes. 2ml of NaOH (1M) and 2.4ml of distilled water were added and the absorbance was measured against blank (without extract) at 510nm using UV-Vis spectrophotometer. A calibration curve was constructed using different concentrations of reference standard (Catechin, 0-120 µg/ml) and the total flavonoid content of extracts was expressed as µg Catechin equivalents (CE) from the graph [19].

Antioxidant activity of *A. pinnata* and *A. rubra*

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay

DPPH radical scavenging assay was employed to determine radical scavenging potential of methanol extract of two *Azolla* species. In this assay, 1 ml of different concentrations of extract (5-100µg/ml of methanol) was mixed with 3 ml of DPPH solution (0.004% in methanol) in separate tubes. The tubes were incubated in dark at room temperature for 30 minutes. The optical density was measured at 517 nm using UV-Vis spectrophotometer. The absorbance of the DPPH control was also noted. Ascorbic acid was used as reference standard. The radical scavenging activity of extracts and ascorbic acid was calculated using the formula:

Scavenging activity (%) = [(Ao – Ae) / Ao] x 100, where Ao is absorbance of DPPH control and Ae is absorbance of DPPH and extract/standard combination [20].

Ferric reducing assay

In this assay, various concentrations of methanol extract of both *Azolla* species (5-100µg/ml) in 1 ml of methanol were mixed in separate tubes with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of Potassium ferricyanide (1%). The tubes were incubated for 20 minutes at 50^{o}C in water bath, cooled rapidly and mixed with 2.5 ml of Trichloroacetic acid (10%) and 0.5 ml of Ferric chloride (0.1%). The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 minutes. The increase in absorbance of the reaction mixtures indicates increased reducing power. Ascorbic acid was used as reference standard [7].

RESULTS

The content of total phenolics and flavonoids in the extracts is shown in Table 1. It has been found that the phenolic as well as flavonoid content were higher in the extract of *A. pinnata* when compared with *A. rubra*.

Table 1: Total phenolic and flavonoid content of *A. pinnata* and *A. rubra*

Extract	Total phenolic content (µg GAE/mg extract)	Total flavonoid content (µg CE/mg extract)
A. pinnata	95.25	41.13
A. rubra	92.16	39.66

The efficacy of extract of *A. pinnata* and *A. rubra* to scavenge free radicals was assessed by DPPH free radical scavenging assay and the result is shown in Figure 1. The extracts have shown marked radical scavenging activity and the activity was dose dependent. Among *Azolla* species, *A. pinnata* displayed stronger scavenging potential (IC_{50} value 7.32 µg/ml) than that of *A. rubra* (IC_{50} value 14.47 µg/ml). Scavenging potential of ascorbic acid was higher (IC_{50} value 1.39 µg/ml) than that of extracts of *Azolla* species.

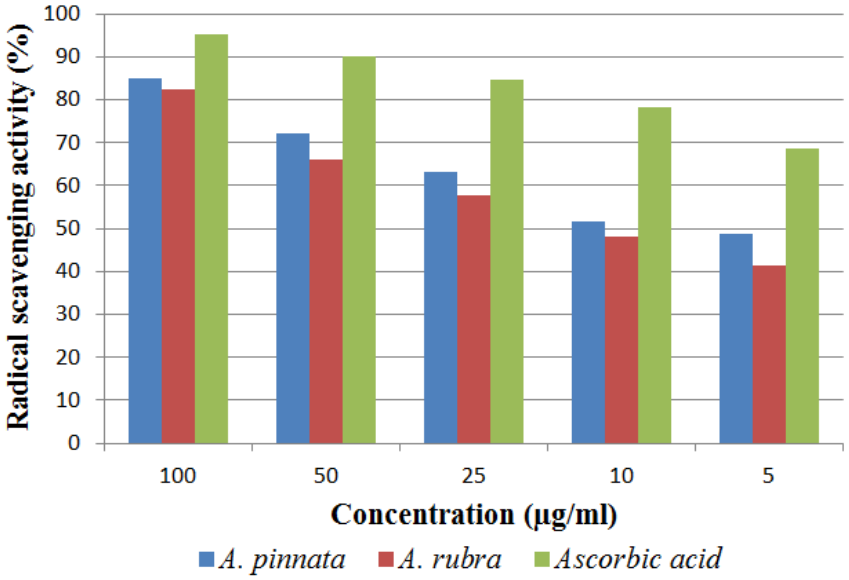

Figure 1: DPPH free radical activity of extract of *A. pinnata* and *A. rubra*

The reducing effect of extracts of *A. pinnata* and *A. rubra* was determined by ferric reducing assay in which the reduction of Fe^{3+} to Fe^{2+} was investigated in the presence of different concentrations of extracts. The absorbance at 700nm was found to increase with the increase in concentration of extracts which indicated the reducing nature of extracts. The reducing potential of *A. pinnata* was higher than that of *A. rubra*. Both extracts displayed higher reducing potential than that of ascorbic acid at higher concentrations (Figure 2).

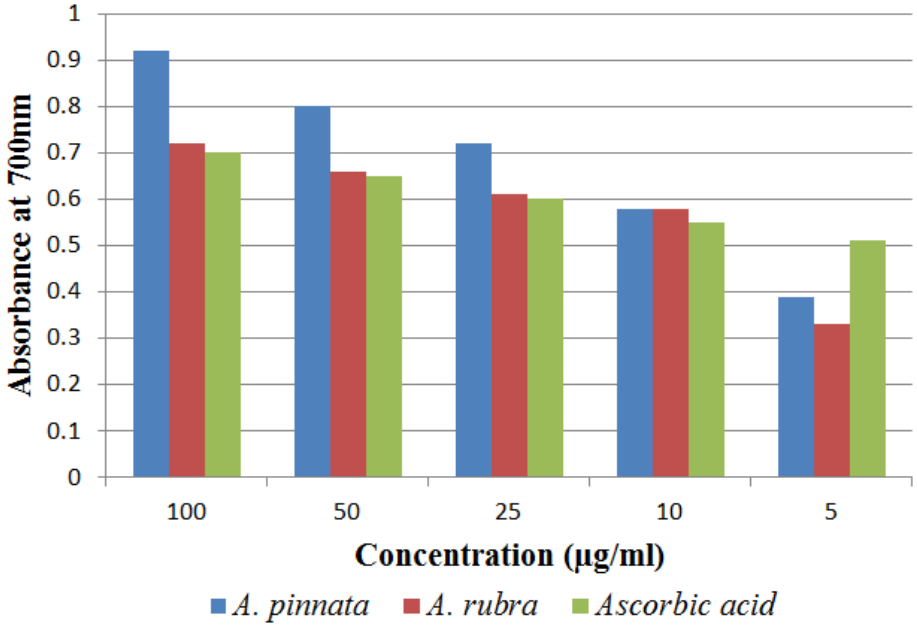

Figure 2: Ferric reducing activity of extract of *A. pinnata* and *A. rubra*

DISCUSSION

DPPH is a stable, organic, nitrogen centred free radical which possess an absorption maximum band around 515-528nm (517nm) in alcoholic solution. On accepting an electron or hydrogen atom, it becomes a stable diamagnetic molecule. The effect of antioxidants on scavenging DPPH radical is due to their hydrogen donating ability. The DPPH free radical scavenging assay is one of the widely used *in vitro* assays for evaluation of free radical scavenging efficacy of various types of samples including plant extracts [20-24]. The antioxidants reduce the purple colored DPPH radical to a yellow colored compound diphenylpicrylhydrazine, and the extent of reaction will depend on the hydrogen donating ability of the antioxidants [25]. In this study, we measured the absorption of DPPH solution in the presence of various concentrations of extract of *A. pinnata* and *A. rubra* at 517nm. It was observed that the radical scavenging activities of both the extracts increased on increasing the concentration of extracts. The scavenging effect on DPPH free radicals was

higher in *A. pinnata* than *A. rubra*. Although the scavenging effect of both extracts were lesser than that of reference standard, it is evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants [21]. It has been experimentally shown that *Azolla* species exhibit scavenging of free radicals. The anthocyanins of *A. imbricata* exhibited dose dependent scavenging of DPPH free radicals with an EC_{50} value of 19.08 µg/ml [12]. The rutin and quercetin isolated from *A. microphylla* were shown to exhibit dose dependent scavenging activity [13].

The reduction of $Fe^{+3}(CN)_6$ to $Fe^{+2}(CN)_6$ was measured in order to evaluate the reducing potential of extract of *A. pinnata* and *A. rubra*. The reducing potential of compounds can be determined by measuring the absorbance resulting from the formation of Perl's Prussian blue complex on addition of excess of ferric ions (Fe^{+3}). An increase in absorbance at 700nm on increase in extract concentrations indicates reducing capacity of a compound. The reducing properties of compounds (antioxidants) are associated with the presence of reductones. Ferric reducing assay is employed by several researchers in order to evaluate antioxidant activity of a variety of compounds [4, 7, 21, 23, 24, 26, 27]. In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron [21]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [28]. In this study, the reducing powers of extracts increased with the increase of their concentrations. Extract of *A. pinnata* displayed higher reducing power than extract of *A. rubra*. It is evident from the study that the extracts possess reductive potential and could serve as electron donors, terminating the radical chain reactions [21].

Polyphenolic compounds including flavonoids of plant kingdom have been reported to possess multiple biological effects, including antioxidant activity. Antioxidant efficacies of these phenolic compounds are due to radical scavenging effect, inhibition of lipid peroxidation and chelation of metal ions [26, 29, 30]. A positive correlation between the content of phenolics and flavonoids and the antioxidant potential of extracts was observed in this study. Hence, the higher antioxidant activity of extract A. pinnata could be due to the presence of high phenolic and flavonoid contents. Such positive correlations have been observed in earlier studies [31-33].

CONCLUSION

A marked antioxidant activity of *A.pinnata* and *A.rubra* was observed in this study. Extract of *A.pinnata* showed marked antioxidant activity when compared to extract of *A.rubra*. These *Azolla* species could be used as potential candidates for the development of agents active against oxidative stress.

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