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PRELIMINARY PHYTOCHEMICAL SCREENING AND EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF AZOLLA PINNATA

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ABSTRACT

The aim of the present study was to evaluate the utilization of *Azolla pinnata* as potential source of antimicrobial compounds and investigate the antioxidant potential of the fern. Extracts of the fern was prepared using various solvents (ethyl acetate, methanol, chloroform, acetone and water) and preliminary screening was done. The phytochemical screening revealed the presence of various bioactive compounds especially phenols and flavonoids in the extracts. Further antimicrobial activity was carried out using different bacterial and fungal strains.Since the results of phytochemical analysis showed the presence of maximum phytochemicals in the methanolic extract, antioxidant activiy was evaluatd in the methanolic extractof Azolla. Antioxidant activity was evaluated by DPPH free radical scavenging assay and FRAP assay. Total phenolic and flavonoid content was estimated by Folin-Ciocalteau and aluminium chloride colorimetric method respectively. Extract of *Azolla pinnata* showed marked free radical scavenging and reducing potential. The significant antioxidant efficacy of *A.pinnata* could be attributed to the presence of high phenolic and flavonoid content. Hence the results of the present study suggests the potential of the aquatic pteridophyte as a rich source of antimicrobial compounds which could be exploited against pathogenic microbes and also a powerful antioxidant against oxidative stress.

INTRODUCTION

The aquatic fern *Azolla* has wide distribution in temperate and tropical freshwater ecosystems and paddy fields. It hosts the nitrogen fixing cyanobacterium *Anabena azollae* which is situated in special cavities of the dorsal leaf lobe. It is a pteridophyte belonging to the *Salviniaceae* family. Because of its potential to fix nitrogen traditionally it has been used as a biofertilizer for paddy crops(Lumpkin and Plucknett,1980).In addition to the biofertilizer potential it has many other uses such as animal feed, human food, medicine, hydrogen fuel, water purifier, production of biogas, weed control etc. Because of the multifaceted uses, Wagner (1997) aptly described it as "Green Gold Mine". However, in the recent times there had been some interest in the phytochemical properties of Azolla plants. There have been a few attempts to study the phytochemical composition of *Azolla* plants (Teixeeira *et al*, 2001, Sanchez Viveroset *et al*, 2011).The ability of the plants to produce an array of secondary metabolites is important from fact that they are used as antagonistic agents by the plants for self defence. The phytochemical properties of the plants have been exploited owing to their antimicrobial as well as pharmacological properties and the natural products of plant origin are reported to have diverse biological activities (Yamuna Devi *et al*, 2011). Although the fern is rich in phenolic contents no attempts have been made to correlate the phytochemical composition of the fern with antimicrobial activity. Phenolic compounds are one of the most diverse groups of secondary metabolites in plants and in particular, flavonoids, a group of phenolic compounds with the diverse chemical structure and characteristics have a broad spectrum of antioxidant properties (Chiang *et al*, 2004). The phenolic compounds have been reported to show antimicrobial activity (Chakraborty *et al*, 2007, Dalli *et al*, 2007).

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 production is one common feature of all aerobic organisms during their normal metabolic activities. ROS is defined as intermediate oxygen

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carrying molecules with or without unpaired electrons and include free radicals such as superoxide anions, hydroxyl radicals, peroxy radicalsand non-radical forms such as hydrogen peroxide, hypochlorous acid and singlet oxygen. These ROS are beneficial and play vital physiological roles in the living systems at required concentrations, however these ROS are detrimental in excess (Nordberg J, Arner ESJ , 2001, Conforti *et al*, 2008, Bouayed J , Bohn T, 2010). Oxidative damage of lipids, proteins and nucleic acids through the alteration of normal cellular metabolism is an impact of ROS (Mc Kersie *et al*, 1994, Imlay J.A, 2003). Stressors like drought, salt, uv-radiation, ozone, chilling, heat shock and pathogen attack increase the production of ROS in plants and depending on their natural and genetic capacity they have developed enzymatic and non – enzymatic defence systems. Osmotic and ionic stresses caused by salinity promote oxidative stress and plants with high constitutive and induced antioxidant levels have better resistance to damage (Dhindsa R.S *et al*,1981, Parida A.K. *et al*, 2005, Spychalla J.P *et al*,1990).

Free radicals and other ROS are effectively eliminated by an enzymatic system including superoxide dismutase, catalase, peroxidise and non-enzymatic factors such as Vitamin C, Vitamin E, thiols etc. Synthetic antioxidants have been used to reduce or retard oxidation process. However, they are volatile, decompose at high temperature and their use is restricted due to safety issues and potential health hazards. Hence there is a need for search of effective antioxidants from natural resources (Kim S *et al*,2006, Stoilova I *et al*,2007, Sheih I.C *et al*,2009, Junaid S *et al*, 2013).

Therefore, the objective of the present study was to screen *Azolla pinnata* for phytochemicals and to evaluate the antimicrobial and antioxidant activity of the fern .

MATERIALS AND METHODS

Plant Material

Azolla pinnata used in the present study were collected from Regional Station for Forage Production and Demonstration, Alamathi, Chennai. The plants were grown in mud pots outdoor. The whole plant material was shade dried, powdered and used for extraction.

Figure 1 *Azolla pinnata*

Test organisms

Bacterial strains of *Staphylococcus aureus, E.coli, Salmonella spp, Pseudomonas spp and Bacillus spp* and *Fungal strains of Candida albicans, Aspergillus niger, Trichoderma viridae, Rhizopus microspora* and *Penicillium chrysogenum* were used to study antimicrobial activity.

Preparation of the extracts

Fresh material was collected from the pots and were brought to the laboratory and cleaned of all debris. The fresh fern was then washed several times in tap water and subsequently with double distilled water and were shade dried for three weeks. The dried material was then ground to fine powder. For extraction, a known quantity (10g) of dried *A.pinnata* was added to known volume of different solvents (water, methanol, ethyl acetate, acetone and chloroform). The mixtures in respective solvents were subjected to soxhlet extraction, concentrated and dried in dessicator and used for the further experiments.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the extracts was carried out following the standard procedure (Raman, 2006).

Antimicrobial assay

The assay was performed by agar well disc diffusion method (Perez C, *et al*, 1990). Antibacterial activity of the extracts was determined by disc diffusion method on Muller Hinton agar (MHA) medium. Muller Hinton Agar (MHA) medium is poured in to the petriplate and after the medium solidified, the inoculums were spread on the solid plates with sterile swab moistened with the bacterial suspension. The filter discs were soaked with extracts at concentrations of 500- 1000 µg/ml and placed on petriplate in a circular fashion. The plates were incubated at 37ºC for 24 hrs. Then the antimicrobial activity was determined by measuring the diameter of zone of inhibition. In the similar procedure antifungal activity of the extracts was also determined by using Sabouraud Dextrose Agar (SDA) medium.

Estimation of total phenolic content of Azolla pinnata

The total phenolic content (TPC) of the extract was determined by spectrophotometric method (Zhishen J *et al*, 1999).1 ml of sample (1 mg ml^{-1}) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 minutes, 10 ml of 7% sodium carbonate solution was added to the mixture, followed by the addition of 13 ml of deionized distilled water and mixed thoroughly. The mixture was kept in dark for 90 minutes at 23°C, after which absorbance was read at 750 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing standard gallic acid solution. The TPC was expressed as milligrams of Gallic acid equivalents (GAE) per milligram of extract.

Estimation of total flavonoid content of Azolla pinnata

The total flavonoid content of the extract of the chosen sample was determined by spectrophotometric method (Wojdylo A *et al*, 2007).To 0.5 ml of extract, 3.4 ml of 30% methanol was added, followed by 0.15 ml of sodium nitrate (0.5M) and 0.15 ml of aluminium chloride (0.3M). After 5 minutes, 1 ml of

sodium hydroxide (1M) was added and the solution was mixed well and the absorbance was measured against reagent blank at 510 nm. The standard curve for total flavonoids was made using rutin as standard. The total flavonoids were expressed as milligrams of rutin equivalents (RU) per milligram of extract.

Antioxidant activity of A.pinnata by DPPH free radical scavenging assay

DPPH radical scavenging assay was employed to determine radical scavenging potential of the methanolic extract of A.pinnata(Courh N *et al*, 2007). In this assay, 1ml of different concentrations of extract was taken and BHT was used as reference standard. 200µl of DPPH reagent was added to all the test tubes including blank. Incubate all test tubes at room temperature in dark condition for 30 minutes. The absorbance of all samples was read at 517nm.

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance of control}) - (\text{Absorbance of extract})}{(\text{Absorbance at control})} \times 100$$

FRAP assay of A.pinnata

In this assay , various concentrations of methanol extract of Azolla pinnata (5 - 100µg/ml) in 1ml of methanol were mixed in separate tubes with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of potassium ferricyanide (1%). The tubes were incubated for 20 minutes at 50 degree Celsius in water bath,cooled rapidly and mixed with 2.5ml of trichloroacetic acid (10%) and 0.5ml 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 700nm after 10 minutes. The increase in absorbance of the reaction mixtures indicates increased reducing power. Ascorbic acid was used as reference standard (Yuan Y.V *et al*, 2005).

RESULTS AND DISCUSSION

The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordan D M, 2001). The medicinal actions of plants unique to particular plant species or groups are consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Wink DA *et al*, 1999). Many naturally -occurring compounds found in plants have been shown to possess antimicrobial functions and could thus serve as a source of both traditional and orthodox medicine (Akinyemi KO *et al*, 2007, Yushau M *et al*, 2008).

Preliminary phytochemical screening of the extracts revealed the presence of several compounds (Table 1). Ramashankar and Khare (1992) reported several species of pteritophytes with medicinal properties. Recently Mithraja *et al* (2011) reported the presence of several bioactive compounds from *A.pinnata.* However, no attempts have been made to correlate the bioactive compounds of Azolla pinnata with respect to antimicrobial activity. The presence or absence of phyto-constituents depends on the type of solvent medium used for extraction. Tannins are known to possess general antimicrobial and antioxidant activities (Rievere C *et al*, 2009). Recent reports show that tannins may have potential value as cytotoxic and antineoplastic agents (Aguinaldo AM, 2005). Other compounds like saponins also have antifungal properties (Aboada OO *et al*, 2001, Mohanta TK *et al*, 2007). Saponins are a mild detergent used in intracellular histochemistry staining to allow antibody access to intracellular proteins. In medicine, it is used in hypercholesterolemia, hyperglycemia, antioxidant, anticancer, antiinflammatory and weight loss, etc. It is also known to have anti -fungal properties (De Lucca A *et al*, 2005). Saponins have been implicated as a bioactive antibacterial agent of plants (Mandal P *et al*, 2005, Manjunatha BK, 2006). Steroids are known to be important for their cardiotonic activities and possess insecticidal and anti-microbial properties. Plant derived natural products such as flavonoids, terpenoids and steroids, etc have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and antitumor activity. Phenolics have anti-oxidative, anti-diabetic, anti -carcinogenic, anti -microbial, anti- allergic, anti-mutagenic and anti-inflammtory activities (Arts IC *et al*, 2008, Scalbert A *et al*, 2005).

The extracts of *A.pinnata* showed diverse phytochemical profile with reference to the solvents used. The methanol extract of *A.pinnata* showed maximum occurrence of phytochemicals (5/9) followed by chloroform (4/9), ethyl acetate (4/9),water (4/9) and acetone (3/9).Phenol was present in all tested extracts of *A.pinnata*. Tannins and flavonoids showed its presence in aqueous, methanol and ethyl acetate extract. Presence of proteins was observed in methnol extract. Carbohydrates were present in aqueous, chloroform and acetone extract. The presence of saponins was seen in ethyl acetate, methanol and chloroform extract and steroids was present in ethyl acetate, acetone and chloroform extract. Anthroquinones and alkaloids was found to be absent in all the extracts.

Table 1 Preliminary phytochemical analysis of *Azolla pinnata*

S.No	Compound	Ethyl acetate Extract	Methanol Extract	Chloroform Extract	Acetone Extract	Aqueous Extract
1.	Alkaloids	-	-	-	-	-
2.	Phenols	+	+	+	+	+
3.	Flavonoids	+	+	-	-	+
4.	Tannins	-	+	-	-	+
5.	Saponins	+	+	+	-	-
6.	Steriods	+	-	+	+	-
7.	Anthroquinones	-	-	-	-	-
8.	Proteins	-	+	-	-	-
9.	Carbohydrates	-	-	+	+	+
	Total	4	5	4	3	4

Antimicrobial activity of the different extracts of *A.pinnata* was analysed using disc diffusion method and visually recorded after 24hrs of incubation. The results showed that the methanolic extract exhibit significant antimicrobial activity against both bacterial and fungal strains used in the study. The other extracts showed significant activity against fungi than bacteria. (Fig 2 – 3)

Flavonoids have been reported to inhibit microbial growth (Mbuh *et al*, 2007). Many plant flavonoids have also been reported to have significant antimicrobial activity (Cushnie and Lamb, 2005). High level of antifungal activity and antibacterial activity due to phenolic compounds isolated from the leaf extracts of Pistacia and Schinus sp was observed by Rhouma *et al* (2009). Thus the invitro antimicrobial activity of *Azolla*

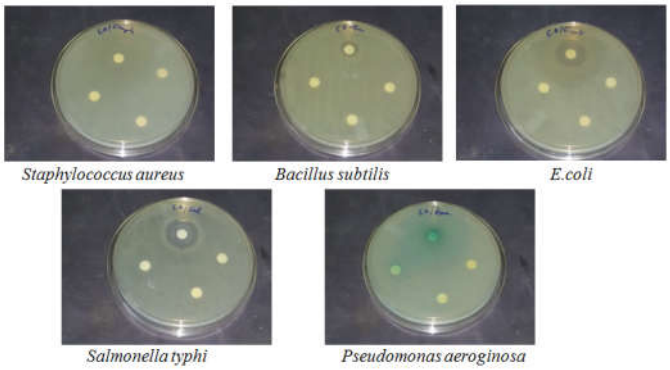
extracts against test fungi and bacteria could be contributed to the presence of polyphenolic compounds such as phenols and flavonoids. Hence this study reports the preliminary phytochemical screening and a positive relationship between the phytochemicals namely flavonoids and phenol and the antimicrobial activity of *azolla* extract.

However further studies on the total phenolic and flavonoid content and antioxidant activity of Azolla pinnata was carried out in the methanolic extract as it showed the presence of maximum phytochemicals. The content of total phenolics and flavonoids in the extract is shown in Table 2. The results show that Azolla pinnata was found to have a good amount of total phenolic and flavonoids.

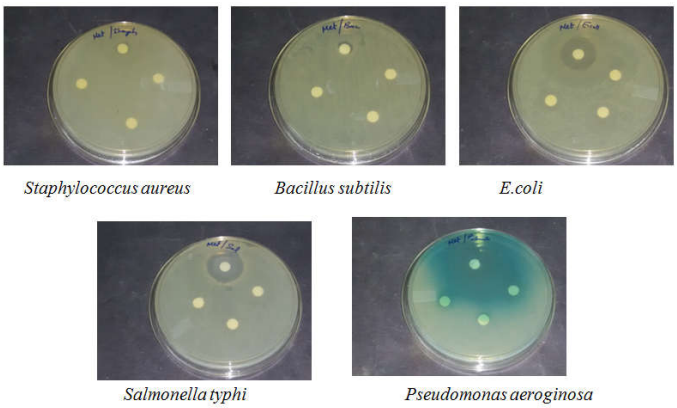
The antioxidant potential of Azolla pinnata to scavenge free radicals was determined by DPPH free radical scavenging assay and the result is shown in figure 4. The extract have shown significant free radical scavenging activity and also showed that the activity depends upon the concentration of the extract. Scavenging potential of BHT was higher than the extract.

The reducing potential of Azolla was determined by FRAP assay in which the reduction of ferric ions to ferrous ions was determined in the presence of different concentrations of the extract. The absorbance at 700nm was found to increase with the increase in concentration of the extract which proved the reducing nature of Azolla. A.pinnata extract showed higher reducing potential than standard ascorbic acid at higher concentrations. (Figure5).

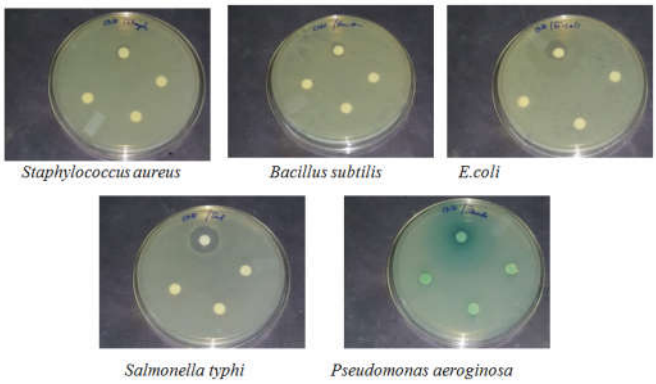
A. Ethyl Acetate extract



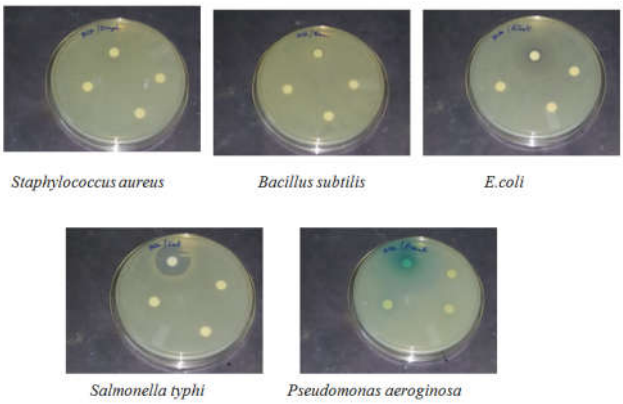
B. Methanol extract



C. Chloroform extract



D. Acetone extract



D. Acetone extract

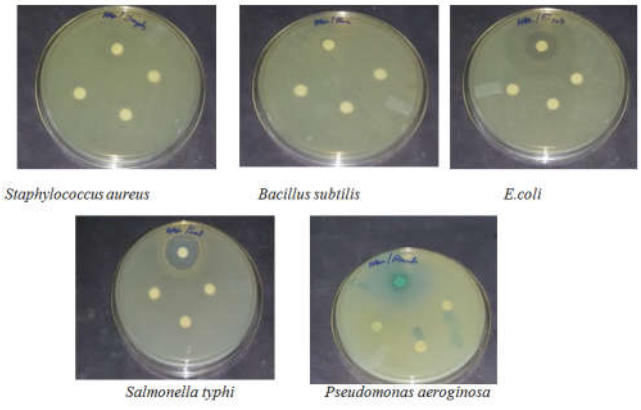
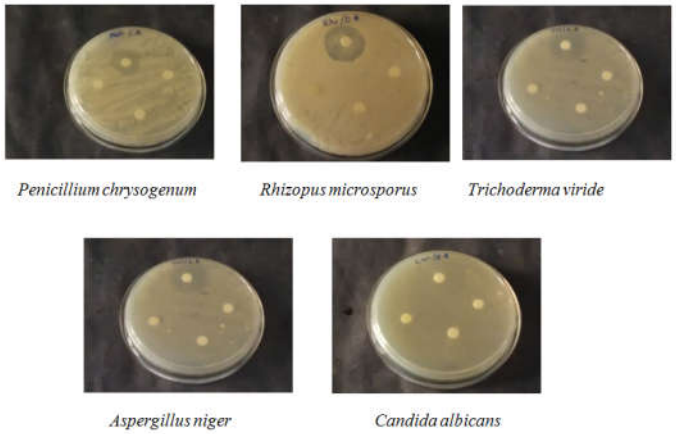
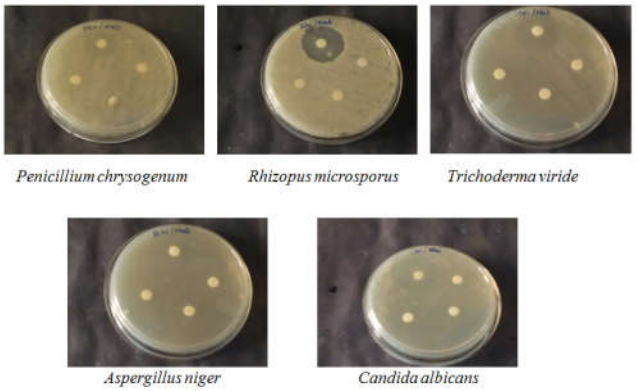


Figure 2 Antibacterial activity of *Azolla pinnata* extracts

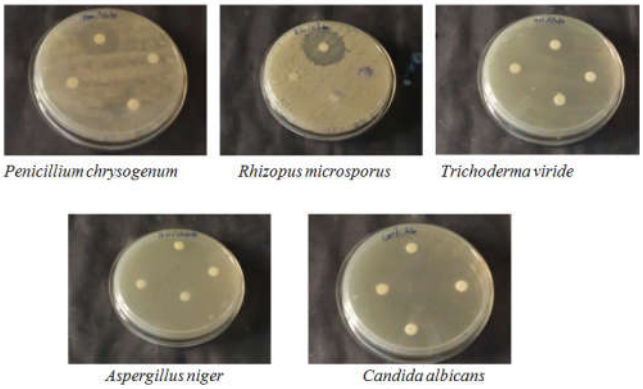
A. Ethyl acetate extract



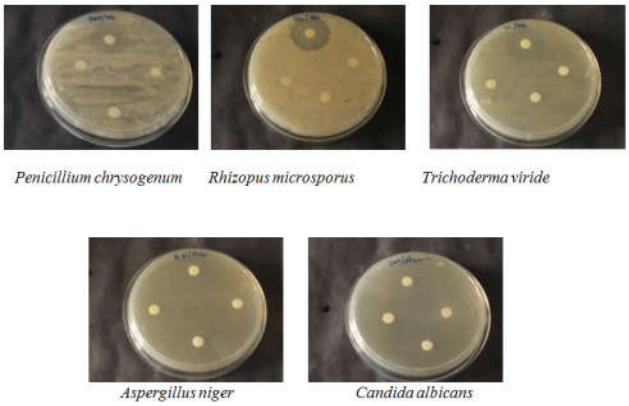
B. Methanol extract



C. Chloroform extract



D. Acetone extract



E. Aqueous extract

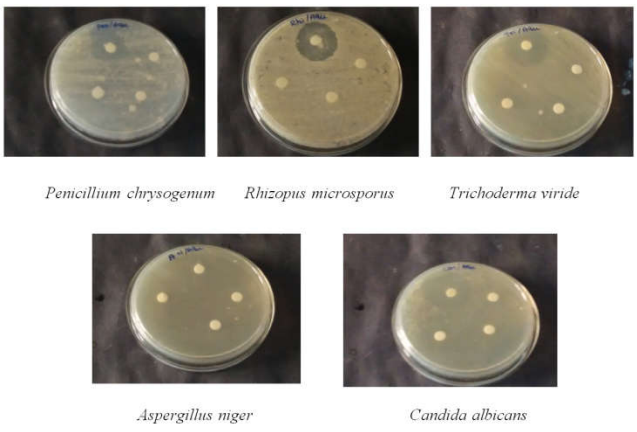


Figure 3 Antifungal activity of *Azolla pinnata* extracts

Table 2 Total phenolic and flavonoid content of the methonolic extract ofAzolla pinnata

Extract	Total phenolic content (µg GAE/mg extract)	Total flavonoid content (µg RU/mg extract)
A.pinnata	87.35± 0.06	56.27± 0.08

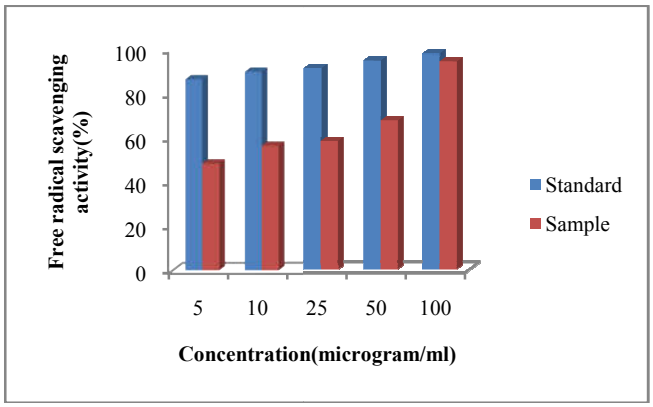


Figure 4 DPPH Free Radical Activity of Methanolic Extract of A.pinnata

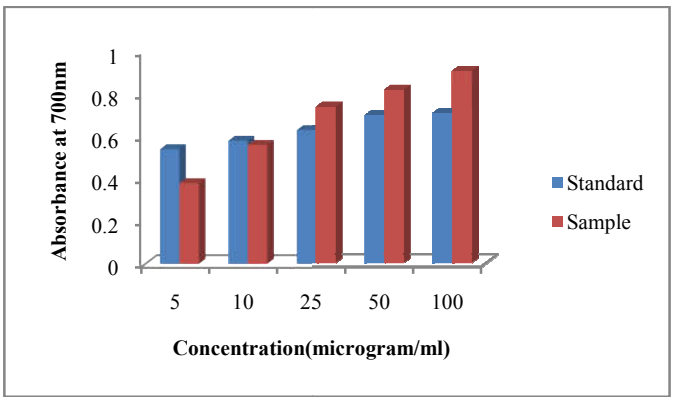


Figure 5 Ferric Reducing Activity of Methanolic Extract of A.pinnata

DPPH is a stable, organic nitrogen centred free radical which possess an absorption maximum band around 515 – 528 nm (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 potential of various types of samples including plant extracts (Elmastas M *et al*, 2006, Chung Y *et al*, 2006, Kaviarasan S *et al*, 2007, Rekha C *et al*, 2012, Poornima G *et al*, 2012) . In this study, the antioxidant potential of methanolic extract of A.pinnata was determined and various concentrations of the extract. Azolla pinnata extract showed marked free radical scavenging activity. Although the scavenging activity of A.pinnata xtract was lesser than the reference standard, it is evident that the extract showed hydrogen donating ability and therefore the extract of A.pinnata could serve ass free radical scavenger (Bondent V *et al*, 1997). It has been shown experimentally that azolla species exhibit scavenging of free radicals(Dai L *et al*, 2012, Selvaraj K *et al*, 2014).

The ferric ion reducing potential of A.pinnata extract was determined by FRAP assay. Ferric reducing assay is employed by several researchers in order to evaluate the antioxidant activity of a variety of compounds. In this assay, the presence of reductants in the sample would result in the reduction of

ferric ion to ferrous ion by donating an electron(Dai L *et al*, 2012). In the present study, the reducing activity of extract increased with increasing the concentration. Extract of A.pinnata showed a higher reducing potential than the reference standard. It is clearly evident from the study that A.pinnata extract possess reductive potential and could serve as electron donors, terminating the radical reaction (Bondent V *et al*, 1997).

Polyphenoic compounds including flavonoids of plant kingdom have been reported to possess multiple biological effects, including antioxidant activity. The antioxidant efficiency of these phenolic compounds is due to radical scavenging effect, inhibition of lipid peroxidation and chelation of metal ions. In this study a positive correlation between the content of phenolics and flavonoids and the antioxidant potential of A.pinnata extract was observed. Hence, the antioxidant activity of A.pinnata extract could be due to the presence of high phenolic and flavonoid content.

CONCLUSION

In the present study an attempt was made to screen A.pinnata for phytochemicals and also to evaluate the antimicrobial activity and antioxidant activity. The results of the study indicate the possibility of exploiting the fern as an effective biocontrol against pathogenic microbes in addition to its traditional use as a biofertilizer and also as a potential agent against oxidative stress.

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