

Phytochemical screening, Antibacterial and Antioxidant activity of *Azolla pinnata*

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Received: February 12, 2019

Accepted: March 27, 2019

ABSTRACT: *Azolla pinnata* is an aquatic nitrogen fixing pteridophyte commonly found in aquatic habitat. In the present study, the phytochemical analysis showed that Alkaloids, phenol, terpenoids and oxalate are present in four solvent extracts except water. Carbohydrate is absent in benzene but present in all other extracts. Flavonoids present in acetone and water extract, saponin is present in methanol and water extract. Ethanol and water extract exhibited the presence of anthocyanin, except benzene and methanol other extracts contain Coumarin. The phytochemicals tannins, Phlobatanins and glycosides are absent in all the solvent extracts of *A. pinnata*. This study was carried out against the various extracts namely Acetone, Benzene, Ethanol, Methanol and Water of *Azolla pinnata* for its antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The zone of inhibition *S. aureus* (13 mm), *P. aeruginosa* (10 mm) for Acetone extract, *S. aureus* (12 mm), *P. aeruginosa* (8 mm) for Benzene Extract, *P. aeruginosa* (12 mm), *S. aureus* (9 mm) for Ethanol extract, *S. aureus* (12 mm), *P. aeruginosa* (11 mm) for Methanol extract are observed and there is no activity for the Water extract of *A. pinnata*. The antioxidant activity of *A. pinnata* solvents extracts was more to the Ethanol followed by Methanol, Acetone, Benzene and Water sample. The results of the study indicate that the *Azolla pinnata* possesses phyto-constituents having antibacterial activity and thus it can be utilized as a natural plant based antimicrobial.

Key Words: *Azolla pinnata*, phytochemical analysis, Extracts, Acetone, Benzene, Ethanol, Methanol.

1. INTRODUCTION

Plants are mainly multicellular organisms. The plants from clade viridiplantae, group that includes flowering plants, ferns, conifer gymnosperms and green algae. There are about 320 thousands species of plants, and 260-290 thousands are seeded plants. Green plants provide gradual proportion of world molecular oxygen. Plants produce grains, fruits, flowers and vegetables for human basic foods. Plants have many cultural uses as ornaments, building materials, writing materials and in great variety that have source of medicines and psychoactive drug. Most of plants obtain the energy through photosynthesis using sun light, water and carbon dioxide to synthesize food. Plants synthesize lot of bioactive compounds for functions including defense against Bacteria, Virus, insects, fungi, diseases. Many phytochemicals with potential or established biological activity have been identified. Further the phytochemical contents and pharmacological actions, of many plants are offering medicinal potential for scientific research. The Food and Agriculture Organization estimated in 2002 that over 50,000 medicinal plants are used across world. The Royal Botanic Gardens, estimated in 2016 that 17,810 plant species have a medicinal use, out of some 30,000 plants is documented. In India, where Ayurveda has been practiced for centuries, herbal remedies are the responsibility of government department AYUSH under the Ministry of Health & Family Welfare. *Azolla* is classified under pteridophytes, *Azolla pinnata* is species of fern known as several common name such as mosquito fern, feather mosquito fern and water velvet. It is mostly grow in Africa, Asia (India, China, Japan, Korea and Philippines) and some parts of Australia. It is aquatic habitat plant floating on the surface of the water. It grows in quiet and slow moving water but not in the running water. It is small fern with triangular measuring up to 2.5 cm in length. They are green, blue green or dark red colour and coated with tiny hairs. The tiny hairs of the plant are responsible for the velvet appearance and make the top surface of the leaf as water repellent. The hair like roots extended into the water. The leaves contain *Cynobacterium anabaena azollae* which is symbiotic that fixes nitrogen from the atmosphere then the fern can use. This gives the fern ability to grow in habitats that are low in nitrogen. Sometimes rice farmers keep this plant in paddy field because it generates valuable nitrogen via its symbiotic Cyanobacteria. The plant can grow in wet soil generating a good amount of nitrogen rich fertilizer. It has ability to absorb a certain amount of heavy metal pollutant such as lead from contaminated water. It contains 25-30% of protein and also used as food for chicken. In recent studies, *Azolla pinnata* can clean up the environmental pollutants. *Azolla pinnata* are small free-floating aquatic Pteridophytes of Azollaceae, with a distribution on tropical,

subtropical and temperate areas. This fern, although considered an invasive species, can be used as biofertilizer, in the phytoremediation of domestic and industrial wastewater and as animal or human food (Carrapico 2010). Despite the scarce ethno pharmacological data on the symbiosis of *A. azollae*, it has been indicated as having medicinal potential. In the 16th century, the Chinese Li Shi-Zhen described Azolla as having medicinal properties but with no specification to which disease. In addition the use of Azolla in New Zealand to cure sore throat when chewed and this fern as a traditional cough medicine in Tanzania. However, the potential use of Azolla in medicine or pharmacology and possible pathogenic targets remains obscure due to a lack of bioactivity data. The extracts of *A. filiculoides* had activity against *Penicillium expansum*, hydrophilic extract against *Agrobacterium vitis* and against the crustacean *Artemia salinaw* as reported by (Piccardi et al. 2000). Recently, the methanol extract of *A. microphylla* showed high antimicrobial activity against *Xanthomonas* spp., also a plant pathogen (Abraham 2013). The aim of the present research work is to evaluate the biological activities of organic and aqueous extracts of *Azolla pinnata* species against bacteria, to evaluate the phytochemical contents and study the antioxidant potentials.

2. MATERIALS AND METHODS

2.1. COLLECTION OF AZOLLA:

The *Azolla pinnata* is collected from Islamiah college campus (Fig.1) and it is identified and authenticated by Dr.N.P.M Mohamed Tariq, Assistant professor of Biotechnology, Islamiah College (Autonomous), Vaniyambadi. After identification, the azolla material was processed for extraction.

Fig.1 *Azolla pinnata* powder



2.2 PREPARATION OF AZOLLA EXTRACT

The *A. pinnata* was thoroughly washed with distilled water to remove dust particles and shadow dried at room temperature and reduced to coarse powder using a mechanical mixer (Fig 2). The powder was subjected to extraction by maceration using various solvents like Acetone, Benzene, Ethanol, Methanol and Water to obtain their respective extracts. To 5gm of the powder in 100ml solvent (Acetone, Benzene, Ethanol, Methanol and Water) were added and stirred occasionally in orbital shaker as recommended by Mangesh Kumar et al., 2016. The mixture was filtered (Fig.3) on the 2nd day and the solvent was evaporated at room temperature for 18-24 hours to obtain a solid mass, and stored in refrigerator (4°C) for further use.

Fig.2A. *Pinnata* powder

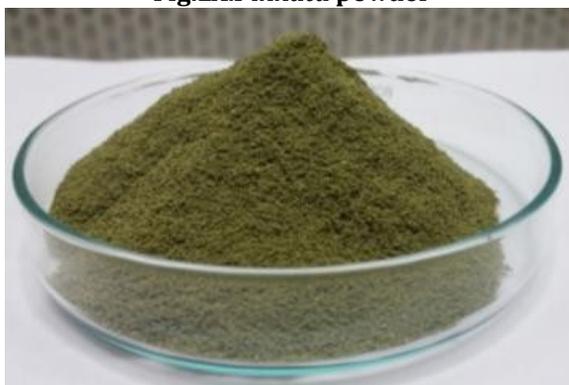


Fig.3A.Pinnatasolvent extracts

2.3. PHYTOCHEMICAL SCREENING:

2.3.1 Alkaloids:

Wagner's test:

1 ml of extract and 1ml of Wagner's reagent are added. Presence of reddish brown precipitate indicates the presence of Alkaloids.

2.3.2 Amino Acid:

Xanthoprotein test:

1 ml of extract and 1 ml of Concentrated Nitric Acid are added (white precipitate is formed) it is heated for 2-3 minutes and cooled. Then 1 ml of 20% NaOH is added. Appearance of orange colour indicates the presence of Aromatic Amino Acid.

2.3.3 Carbohydrate:

Molish test

2 ml of extract, 2 ml of Molish reagent and 2 ml of Conc.H₂SO₄ are added. Presence of reddish ring indicates the presence of Carbohydrate.

2.3.4 Phenol:

FeCl₃ test:

1 ml of the extract and 1 ml of 5% ferric chloride are added. Appearance of dark green colour / reddish brown / blue / violet / purple indicates the presence of Phenol.

Potassium dichromate test:

2 ml of extract and 1 ml of 10% of potassium dichromate are added. Appearance of red colour indicates presence of Phenol.

2.3.5 Flavonoids:

Alkaline reagent test:

1 ml of the extract and 1 ml of the 10% of sodium hydroxide are added. Presence of yellow fluorescence colour indicates the presence of Flavonoids.

Ammonia test:

1ml of extract, 2ml of 10% of ammonia solution and 1ml of concentrated Sulphuric acid are added. The yellow colour indicates the presence of flavonoids.

2.3.6 Tannins:

FeCl₃ test:

2 ml of the extract and 2 ml of the 5% ferric chloride are added. Appearance of green colour indicates the presence of Tannins.

2.3.7 Saponin:

Foam test:

2 ml of the extract and 2 ml of the Dis.H₂O are added and shaken vigorously. Formation of stable foam indicates the presence of Saponins.

2.3.8 Terpenoids:

Liebermann-Burchard test:

2 ml of the extract, 2 ml of the chloroform and 2 ml of the acetic acid, 1 ml of the conc.H₂SO₄ are added. Appearance of blue green colour/reddish ring indicates the presence of Terpenoids.

2.3.9 Phlobatanins:**1% Hydrochloric Acid test:**

2 ml of the extract, 2 ml of the 1% HCL is added and heated in boiling water bath. Appearance of red colour indicates the presence of Phlobatanins.

2.3.10 Quinones:-**Hydrochloric acid test:-**

1 ml of the extract and 1 ml of the conc.HCL are added. Appearance of yellow colour indicates the presence of Quinone's.

2.3.11 Coumarin:**Sodium hydroxide test:**

1 ml of the extract and 1 ml of 10% sodium hydroxide are added. Appearance of yellow colour indicates the presence of Coumarin.

2.3.12 Glycoside:**Keller-Killiani test:**

2 ml of the extract, 2 ml of the glacial acetic acid and few drops of the 5% FeCl₃ and conc.H₂SO₄ are added. Presence of reddish brown/blue green colour indicates the presence of Glycoside's.

Test for glycoside:

2 ml of extract, 3ml of chloroform and 1ml of 10% ammonia solution are added. Appearance of pink colour indicates the presence of glycoside.

2.3.13 Oxalate:**Glacial acetic acid test:**

3ml of extract and 1ml of glacial acetic acid are added. Appearance of green colour indicates the presence of oxalate.

2.3.14 Anthocyanin:**Sulphuric acid test:**

1 ml of the extract and 1 ml of concentrated Sulphuric acid are added. Appearance of yellowish orange colour indicates the presence of Anthocyanin.

2.4 Antibacterial Activity:

The Disc Diffusion Method of Antimicrobial Susceptibility test was used to evaluate the presence of Antibacterial Activities of the different solvent extracts of the plants. The Microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*) are used. It is collected from Department of Microbiology, Shanmuga Industries of Arts and Science College, Tiruvannamalai. The bacteria cultured overnight at 37°C in Nutrient broth. The sterile Muller Hinton Agar (Hi-media) plates were prepared. 100 mg / ml and 200 mg/ ml of the various solvent extracts were prepared by using solvents (acetone, benzene, ethanol, methanol and water). Then 0.2 ml of the bacterial suspension was introduced into the sterile MHA plates and spreading the bacteria using L-Rod to get an even culture all over the plates. 6mm Discs was prepared from Whatman No.1 filter paper and autoclaved. A plate comprises of four discs, one is positive control, one is negative control and two for the two different concentrations (100, 200 mg / ml) of the same plant extract, where ciproflaxin is used as positive control and respective solvents in which the sample is dissolved was used as negative control. The disc are placed with positive control, negative control, two different concentration of the plant extract was prepared and placed on the prepared Muller Hinton Agar plates. Then all the plates are kept for incubation at 37°C for 12-18 hours.

2.5 Antioxidant Activity:

The Antioxidant activity of the solvents extracts was performed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. A solution of 0.135mM DPPH was freshly prepared by dissolving 4 mg of DPPH in 100 ml of Methanol. The 2 ml of the different concentrations of extracts (20 µg, 40 µg, 60 µg, 80 µg, 100 µg, 150 µg, 200 µg, 250 µg /ml of acetone,benzene,ethanol,methanol and water) is taken and 2 ml of the DPPH solution (0.135mM) was added. The control sample was prepared by 2 ml of DPPH solution alone without sample mixed with 2 ml of methanol. Then all the test tubes were incubated in dark room for 30 minutes at room temperature.The changes in the absorbance were measured at 517 nm in UV-Spectrophotometer. Methanol is used as blank. The antioxidant activity was calculated in inhibition percentage by using following formula,

$$\text{Inhibition percentage} = \frac{[\text{Control O.D} - \text{Sample O.D}]}{\text{Control O.D}} \times 100$$

3. RESULT AND DISCUSSION

3.1 Phytochemical Screening

The phytochemical analysis of the various solvents extract is tested for 14 phytochemicals. The procedure for the phytochemical analysis is carried out using standardized protocols (Johnson Marimuthu et al., 2011). The acetone, benzene, ethanol, methanol and water extracts of *A. pinnata* are tested for the presence of phytochemicals. Alkaloids, phenol, terpenoids and oxalate are present in four solvent extracts except water. Amino acid is absent in acetone extract, carbohydrate is absent in benzene but present in all other extracts. Flavonoids present in acetone and water extract, saponin is present in methanol and water extract. Ethanol and water extract exhibited the presence of anthocyanin. Except benzene and methanol other extracts contain Coumarin. Whereas the phytochemicals tannins, Phlobatanins and glycosides are absent in all the solvent extracts of *A. pinnata* as reported by scalbert a et al., (2005) and Sathammaipriya. N et al., (2018) (Table.1).

3.2 Antibacterial Activity:

The Disc Diffusion Method for Antimicrobial Susceptibility test was used to evaluate the presence of Antibacterial Activities of the different solvent extracts of the *Azolla pinnata*. The *pseudomonas aeruginosa* and *staphylococcus aureus* used for the testing. The bacteria cultured overnight at 37°C in Nutrient broth. The sterile Muller Hinton Agar (Hi-media) plates were prepared. The 100 mg / ml and 200 mg/ ml of the Acetone, Benzene, Ethanol, Methanol, Water solvent extracts were prepared by using the respective solvents. Then 200µl of the bacterial suspension was introduced into the sterile plates and spreading the bacteria using L-Rod to get an even culture all over the plates. The 6mm Discs was prepared from Whatman No.1 filter paper and it also autoclaved. A plate comprises of four discs, one is positive control, one is negative control and two for the two different concentrations (100mg/ml, 200 mg / ml) of the same plant extract. The sterile discs are placed above the media and the samples are loaded gently by the following quantity given below. Where 10µl of Ampicillin (10 mg/ml) is loaded as positive control in the disc and 10µl of the respective solvents in which the sample is dissolved was used as negative control in the disc. Then 20µl of the 100 mg/ ml and 200 mg/ ml prepared concentration of the extract is loaded gently in the following discs. Then all the plates are kept for incubation at 37°C for 24 hours. The higher concentration of Acetone, Benzene, Ethanol, Methanol and Water extract of *A. pinnata* shows activity against the tested pathogens by the following order from the highest zone of inhibition *S. aureus* (13 mm), *P. aeruginosa* (10 mm) for Acetone Extract of *A. pinnata*, *S. aureus* (12 mm), *P. aeruginosa* (8 mm) for Benzene Extract of *A. pinnata*, *P. aeruginosa* (12 mm), *S. aureus* (9 mm) for Ethanol Extract of *A. pinnata*, *S. aureus* (12 mm), *P. aeruginosa* (11 mm) for Methanol Extract of *A. pinnata* and there is no activity for the Water Extract of *A. pinnata* as observed by Mangesh kumar et al., 2018 and Sathammaipriya et al., 2018 by assessing *in vitro* antibacterial activity of the crude extracts of *A. pinnata* on the basis of the inhibition zone (Fig. 4 and Table 2).

3.3 Antioxidant activity:

The antioxidant activity of the *A. pinnata* Acetone, Benzene, ethanol, methanol and Water solvent extract's tested for antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method and it is compared with standard ascorbic acid. Antioxidant activity is very important in counteracting the deleterious role of free radical in food biological system. The DPPH alcohol solution is a deep purple colour with an absorption peak at 517 nm which disappears in the presence of radical scavengers in the reactive system. The scavenging capacity of biological reagents on DPPH free radical can be expressed as its antioxidant capability. Evidence collected in recent year suggest the involvement of free radicals and other oxidants as the major cause of oxidative stress that lead to a variety of diseases and disorders. This led to an increasing interest in natural products having antioxidant properties. Plants have been considered as richer sources of antioxidants. In our study the antioxidant activity of the *A. pinnata* solvents extracts was high to the Ethanol followed by Methanol, Acetone, Benzene and Water as reported by Amjad Masood et al., (2005) The data of Percentage of Inhibition and Standard Error was expressed as graphical representation is presented in Table.3.

4. CONCLUSION

Plants are the basic and traditional source of knowledge for modern life sciences especially in medicine. The relatively lower incidence of adverse reactions to plant preparations, compared to modern conventional pharmaceuticals, coupled with their reduced cost is encouraging both the consuming public and national health care institutions to consider plant medicines as alternative to synthetic drugs. Nowadays herbal drugs are prescribed widely even when their biologically active compounds are still unexplored, because of their effectiveness and minimal side effect in clinical applications and experiences.

The species *A. pinnata*, which was predominantly found in and around college campus were collected and various preliminary phytochemical screening and antibacterial activities were investigated and analyzed. *A. pinnata* was found to be rich in alkaloids and phenolic compounds which shows there will be a high medicinal value. Various extracts shows the presence of high concentration of terpenoids compounds which are the main sources of steroids. And the DPPH antioxidant activity of the various extracts shows good results where it shows the presence of rich antioxidants which can serve as a good source for development of health supplements for animal health. A good antibacterial activity of *A. pinnata*, were found against disease-causing organisms *pseudomonas aeruginosa* and *staphylococcus aureus* which shows a positive response against the infections caused by them. With this preliminary research studies, we conclude that the species *A. pinnata* found to be a good source of various bioactive compounds as it has good nutritional, antioxidant and antibacterial activities.

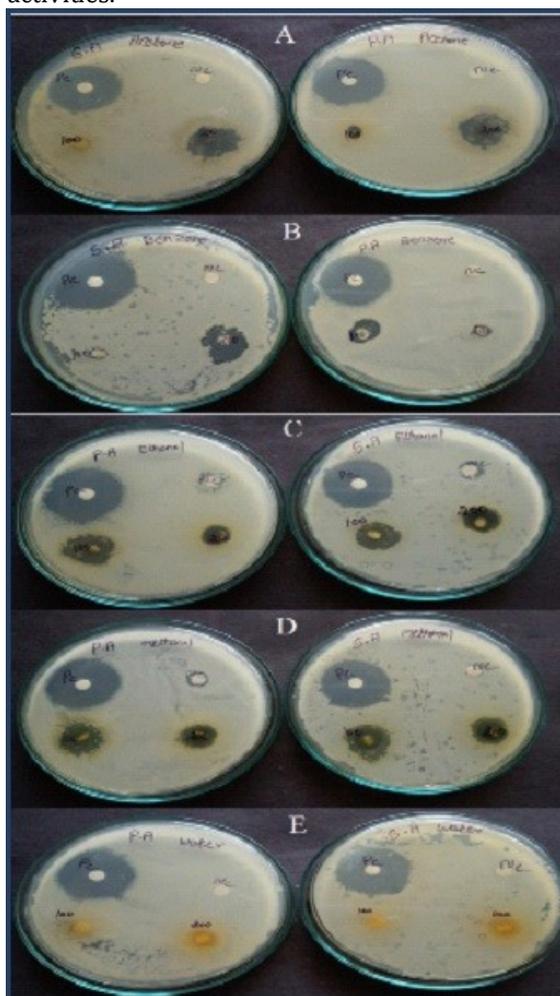


Fig.4. Antibacterial activity of *Azolla pinnata* extracts using various concentrations at various solvents. A - Acetone extract, B - Benzene extract, C - Ethanolic extract, D - Methanolic extract and E - Water extract.

Table.1 Phytochemical analysis for various organic extract of *Azolla pinnata*

S.No	Tests	Acetone	Benzene	Ethanol	Methanol	Water
1	Alkaloids: <i>Wagners test</i>	+	+	+	+	-
2	Aminoacid: <i>Xanthoprotein test</i>	-	+	+	+	+
3	Carbohydrate: <i>Molish test</i>	+	-	+	+	+

	<u>Phenol:</u>					
4	a) Ferric chloride test	+	+	+	+	-
	b) Potassium dichromate test	+	+	+	+	-
	<u>Flavonoid:</u>					
5	a) Alkaline reagent test	+	-	-	-	+
	b) Ammonia test	+	-	-	-	+
	<u>Tannins:</u>					
6	Ferric chloride test	-	-	-	-	-
	<u>Saponin:</u>					
7	Foam test	-	-	-	+	+
	<u>Terphenoids:</u>					
8	Liebermann-burchard test	+	+	+	+	-
	<u>Anthocyanins:</u>					
9	Sulphuric acid test	-	-	+	-	+
	<u>phlobatanins:</u>					
10	1% Hydrochloric acid test	-	-	-	-	-
	<u>Coumarin:</u>					
11	Sodium hydroxide test	+	-	+	-	+
	<u>Oxalate:</u>					
12	Glacial acetic acid test	+	+	+	+	-
	<u>Glycoside:</u>					
13	a) Keller killiani test	-	-	-	-	-
	b) Test for glycoside	-	-	-	-	-
	<u>Quinones:</u>					
14	Hydrochloric acid test	-	-	-	-	+

(Presence+)

(Absence-)

Table 2 Antibacterial activity of *Azolla pinnata*

Name of the Microorganism	Solvents	Zone of Inhibition in mm			
		*P.C	*N.C	100 mg/ml	200 mg/ml
<i>P. aeruginosa</i>	Acetone	14	-	-	10
	Benzene	14	-	4	8
	Ethanol	14	-	5	12
	Methanol	14	-	10	11
	Water	14	-	-	-
<i>S. aureus</i>	Acetone	13	-	-	13
	Benzene	15	-	-	12
	Ethanol	13	-	8	9
	Methanol	14	-	9	12
	Water	13	-	-	-

PC=Positive control (Ciproflaxin)

NC=Negative control (Respective solvents)

Table 3 Optical Density value of *A. pinnata* extracts at 517nm

Concentration	Ascorbic Acid	Acetone	Benzene	Ethanol	Methanol	Water
20 µg / ml	0.0159	0.4727	0.4905	0.4688	0.3727	0.4866
40 µg / ml	0.0143	0.4578	0.4880	0.4206	0.3114	0.4843

60 µg / ml	0.0125	0.4363	0.4745	0.3929	0.2350	0.4695
80 µg / ml	0.0108	0.3469	0.4473	0.3599	0.2091	0.4606
100 µg / ml	0.0077	0.3062	0.4283	0.3112	0.1468	0.4756
150 µg / ml	0.0070	0.1838	0.3196	0.0635	0.1243	0.3933
200 µg / ml	0.0059	0.1418	0.2882	0.0530	0.1020	0.3731
250 µg / ml	0.0049	0.1154	0.2739	0.0528	0.0877	0.3658

Acknowledgements

The authors thank the management of Islamiah College (Autonomous) for providing the facilities to carry out this research work.

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