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Research Article

Qualitative Phytochemical Analysis and Antioxidant Activity of Methonolic Extract of *Eichhornia crassipes* (Mart.) Solms and *Pistia stratiotes* L.

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ABSTRACT
Eichhornia crassipes (Mart.) solms and *Pistia stratiotes* (L.) are two invasive weed aquatic plants that have been traditionally known as “water hyacinth” and “Jalkumbhi” respectively. They are commonly used in Ayurvedic medicine which possesses diuretic, antidiabetic, antidermatophytic, antifungal, and antimicrobial properties. The present study was carried out to estimate the total phytochemicals such as phenolics, flavonoids, tannins, alkaloids, protein, carbohydrate, lipid, amino acids and antioxidant activity. The presence of various phytochemicals in the plants reveals that these plants may be good source for the production of new drugs for various ailments.

Keywords: *Eichhornia crassipes, Pistia stratiotes,* phytochemicals, antioxidant activity.

I
NTRODUCTION

Medicinal plant parts are commonly rich in phenolics, flavonoids, tannins, alkaloids, protein and amino acids. These compounds have multiple biological effects including antioxidant activity. The therapeutic potential of natural medicinal plants as an antioxidant in reducing free radical induced tissue injury, suggests many plants have antioxidant activities that can be therapeutically useful[1].The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties. Flavonoids are a group of polyphenolic substances exert antioxidant activity via radical scavenging, metal ion chelation, and membrane protective efficacy[2,3].

MATERIAL AND METHOD

Plant Collection and Identification
The *E. crassipes* and *P. stratiotes* were collected randomly and aseptically from different water bodies in and around Kota City, Rajasthan, India. The plant material was shade dried and different plant parts were collected separately, powdered and used as the experimental plant material for further analysis and experimentation.

Methanol extraction
Plant materials (leaves, petiole, root) extracts were prepared using soxhlet extraction unit, a quantity of 10gm plant materials (leaves, petiole, root) were weighed and suspended with 200 ml of solvent. The extraction for each plant material is carried out by using methanol solvent. The extracts were dried by using rotor evaporator and stored in a refrigerator at 4ºC for further analysis.

Quantitative Phytochemical Analysis
Various chemical tests were performed for the presence of bioactive constituents in each fraction of both plants by using standard procedures.

Quantification of Primary Metabolites

Determination of total Carbohydrates Content
A stock solution of glucose (1 mg/ml) was prepared in distilled water, out of which 0.1 to 0.8 ml were separately pipetted into the test tubes and the volume of each was raised to 1 ml with distilled water. 1 ml of 5% aqueous phenol was added and shaken gently. Later, 5 ml of conc. H_2SO_4 was added rapidly, accompanied with gentle agitation during the addition of the acid. These were allowed to stand in a water bath at 26-30°C for 20 min before taking the optical densities (ODs) of the yellow-orange colour thus developed at 490 nm in a spectrophotometer after setting for 100% transmission against the blank. Three replicates in each were run and their mean values were calculated.

Determination of total lipid Content
The test sample were dried, powdered and 100 mg was macerated with 1.5 ml NH_4OH and mix thoroughly. Add 3 drops of phenolphthalein indicator to help sharpen visual appearance of interface between ether and aqueous layers during extraction. Add 10 ml 95% alcohol and shake flask for 15 sec. For first extraction, add 25 ml ethyl ether and shake flask very vigorously for 1 min, releasing built-up pressure by loosening stopper as necessary. Add 25 ml petroleum ether and repeat vigorous shaking for 1 min. Centrifuge flasks at ca 600 rpm for ≥30 s to obtain clean separation of aqueous (bright pink) and ether phases.

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Decant ether solution into suitable weighing dish. When ether solution is decanted into dishes, be careful not to pour over any suspended solids or aqueous phase into weighing dish. Ether can be evaporated at ±100°C from dishes. For second extraction, add 5 ml 95% alcohol, and shake vigorously for 15 sec. Next, add 15 ml ethyl ether and shake flask vigorously for 1 min. Add 15 ml petroleum ether and repeat vigorous shaking for 1 min. Centrifuge flasks at ca 600 rpm for ≥30 s to obtain clean separation of aqueous (bright pink) and ether phases. If inter face is below neck of flask, add H_2O to bring level ca half way up neck. Add H_2O slowly down inside surface of flask so that there is minimum disturbance of separation. Decant ether solution for second extraction into same weighing dish used for first extraction. For third extraction, omit addition of 95% alcohol and repeat procedure used for second extraction. Completely evaporate solvents in hood on hot plate at ≤100°C (avoid spattering). Dry extracted lipid in weighing dish to constant weight in forced air oven at 100° ± 1°C (≥30 min) or in vacuum oven at 70° –75°C at >50.8 cm (20 in.) of vacuum for ≥7 min. Remove weighing dishes from oven and place in desiccator to cool to room temperature. Record weight of each weighing dish plus lipid.

$$\text{Fat \%} = \frac{[(\text{weight dish+ fat}) - (\text{weight dish})] - \text{average weight blank residue}}{\text{Weight test portion}} \times 100$$

Maximum recommended difference between duplicates is <0.03% fat[4].

Determination of total Protein Content

0.5-2 gm of each sample,with 2 gm of digestion mixture (potassium sulfate/copper sulphate/selenium dioxide: 5/2/1, w/w) and 20 ml of concentrated sulphuric acid were added into individual digestion tubes. The digestion tubes were heated up to 420 °C gradually in 7-10 hrs using heating block until the sample becomes bluish green. The cooled digested samples were made up to 50/100 ml. Five ml of each digested sample, along with 15 ml of 40% NaOH solution was distilled in a glass distillation unit passing steam. Ammonia gas liberated was condensed and trapped in 2% boric acid in a conical flask. After 15-20 min the conical flask was removed and rinsed with water along the walls to ensure all the ammonia has been collected. Ammonium sulphate was used as a standard. The ammonia trapped boric acid was titrated against '1/70' N hydrochloric acid. About 2-3 drops of mixed indicator (5 parts of bromocressol green and 2 parts of methyl red) was added to decide the end point. The titration was stopped when the colour of the solution in the conical flask turns from bluish green to orange red.

$$\text{Nitrogen (\%)} = \frac{(A-B)\times C\times E}{D(F\times G)}\times 100$$

Calculation of nitrogen

Where,

A = Titer value for digested sample in ml

B = Titer value for blank in ml

C = Nitrogen equivalent of ammonium sulphate in mg

D = Titer value for ammonium sulphate in ml

E = Volume of digested sample in ml

F = Volume of sample taken for distillation in ml

G = Sample weight in mg

Protein content was determined using a nitrogen-to-protein conversion factor of 6.25. The values were suitably corrected by accounting for nitrogen present as caffeine in the samples[5].

Determination of total Amino acid Content

To 0.2 ml of the sample 3.8 ml of ninhydrin-citrate glycerol mixture was added. The reaction mixture was prepared by mixing 1.0 ml 1% ninhydrin solution in 0.5 M citrate buffer (pH 5.5), 2.4 ml glycerol and 0.4 ml of 0.5 M citrate buffer. After shaking well, the whole mixture was heated in a boiling water bath for 12 minutes and cooled to room temperature, by keeping in tap water. The optical density of the solution was measured at 570 nm using spectrophotometer. Glycine was used as the standard.

Quantification of Secondary Metabolites

Determination of Total Phenolic Content

Total phenolic compound contents were determined by the Folin- Ciocalteau method[6-9]. The extract samples (0.5 ml; 1:10 diluted) were mixed with Folin Ciocalteu reagent (1.5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 ml, 1M) were then added. The mixture was allowed to stand for 30 min and the total phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using the standard solution of Gallic acid in methanol in the range 0.2-1mg/ml (R^2=0.987). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound. Total phenolic content can be calculated from the formula:

$$T = \frac{C.V}{M}$$

Where, T = Total Phenolic concentration, C = Concentration of gallic acid from caliberation curve (µg/ml), V = Volume of extract (ml), M = Wt. of methanol plant extract

Determination of Total Flavonoid Content

Total flavonoid content was determined by using aluminium chloride colorimetric method ($AlCl_3$) according to the known method [10,11] with slight modifications using quercetin as standard. 0.5ml of test material was added to 50ml volumetric flask containing 3ml of methanol. To above mixture, 2ml of 10% $AlCl_3$ was added. After 5min, the total volume was made up to 5ml with methanol. Then the solutions were mixed well and absorbance was measured against blank at 420nm. The standard curve was prepared using the standard solution of Quercetin in methanol in the range 0.2- 1mg/ml (R^2=0.991). Total flavonoid content of the extracts was expressed in milligram of quercetin equivalents/gdw. Total flavonoid content can be calculated from the formula:

$$T = \frac{C.V}{M}$$

Where, T = Total flavonoid concentration, C = Concentration of quercetin from caliberation curve (mg/ml), V = Volume of extract (ml), M = Wt of methanol plant extract.

Determination of Total Tannin Content

The total tannin content was determined using tannic acid colorimetric method according to the known method [12]

Table 1: Isolated Primary metabolite contents (mg/gdw) from different plant parts *Eichhornia crassipes.*

Primary Metabolite	Leaves[a]	Petiole[a]	Root[a]
Carbohydrate	57.26 ± 0.065	56.33 ± 0.094	38.16 ± 0.102
Lipid/Fat	5.22 ± 0.110	2.35 ± 0.187	2.79 ± 0.131
Protein	15.08 ± 0.084	5.53 ± 0.214	8.48 ± 0.200
Amino Acid	1.67 ± 0.122	1.53 ± 0.082	1.14 ± 0.126

mg/gdw: miligram / gram dry weight
[a]Results are mean value from at least 3 experiments.

Table 2: Isolated Primary metabolite contents (mg/gdw) from different plant parts *Pistia stratiotes.*

Primary Metabolite	Leaves[a]	Root[a]
Carbohydrate	53.46 ± 0.122	45.26 ± 0.122
Lipid/Fat	2.72 ± 0.149	4.21 ± 0.082
Protein	14.76 ± 0.096	10.82 ± 0.082
Amino Acid	1.21 ± 0.086	0.89 ± 0.124

mg/gdw :milligram /gram dry weight
[a]Results are mean value from at least 3 experiments.

Table 3: Total Phenolic content in different plant parts of *Eichhornia crassipes* and *Pistia stratiotes*

Plant Parts	Total Phenolic Content (mg GAE/gdw)
Eichhornia Leaves	0.217±0.032
Eichhornia Petiole	0.180±0.031
Eichhornia Root	0.187±0.014
Pistia Leaves	0.596±0.015
Pistia Root	0.173±0.022

TPC was expressed in mg gallic acid equivalents/g dry weight

Table 4: Total Flavonoid content in different plant parts of *Eichhornia crassipes* and *Pistia stratiotes*

Plant Parts	Total Flavonoidal Content (mg QE/gdw)
Eichhornia Leaves	0.481±0.023
Eichhornia Petiole	0.270±0.22
Eichhornia Root	0.389±0.025
Pistia Leaves	0.519±0.020
Pistia Root	0.418±0.040

TFC was expressed in mg quercetin equivalent/g dry weight

Table 5: Total Tannin content in different plant parts of *Eichhornia crassipes* and *Pistia stratiotes.*

Plant Parts	Total Tannin Content (mg tannin/gdw)
Eichhornia Leaves	0.223±0.023
Eichhornia Petiole	0.325±0.016
Eichhornia Root	0.341±0.033
Pistia Leaves	0.337±0.053
Pistia Root	0.204±0.026

TTC was expressed in mg Tannic acid equivalent/g dry weight

with slight modifications using tannic acid as standard. 1 ml of the extract was transferred to a 50mL volumetric flask containing 2mL of distilled water. To the mixture, 0.5mL of Folin-Denis reagent followed by 1mL of sodium carbonate solution was added and diluted to 10mL with distilled water. The mixture was shaken well and kept for 30 min at room temperature. The blue colour developed was read at 765nm using UV/visible spectrophotometer (Perkin Elmer, USA). The total tannin content was calculated using standard graph of tannic acid 0.1 – 0.5mg/ml (R^2=0.991) and the results were expressed as tannic acid equivalent (mg/g). Total tannin content of the extracts was expressed in milligram of tannic acid equivalents/gdw. Total tannin content can be calculated from the formula:

$$T = \frac{C.V}{M}$$

Where, T = Total tannin concentration, C = Concentration of tannic acid from caliberation curve (mg/ml), V = Volume of extract (ml), M = Wt of water plant extract.

Determination of Total Alkaloid Content

Estimation of alkaloids in the extract was done by the procedure[13]. 10 mg of plant material was homogenized in a motor and pestle. Added around 20 ml of methanol:ammonia (68:2). Decanted the ammonicial solution and after 24 hrs added fresh methanolic ammonia. Repeated the procedure thrice and pooled the extracts. The extracts were evaporated using a flash evaporator. Treated the residue with 1 N HCl and kept it overnight. Extracted the acidic solution with 20 ml of chloroform thrice, pooled the organic layers and evaporated to dryness, basic fraction. Basified the acidic layer with concentrated sodium hydroxide to pH 12 and extracted with chloroform (20 ml) thrice, Pooled the chloroform layers, dry over absorbent cotton and evaporated to dryness. Weigh the fraction that contains alkaloids expressed as mg/100 g.

Antioxidant activity using DPPH method

Antioxidant activity of the plant extracts and standard was assessed on the basis of the radical scavenging effect of the stable DPPH free radical. The diluted working solutions of the test extracts were prepared in methanol. Gallic acid was used as standard in solutions ranging from 0.5 to 4.0 µg/ml. 0.135mM DPPH solution in methanol was prepared. Then 2 ml of this solution was mixed with 2 ml of sample solutions ranging for *E. crassipes* leaves extract 0.265 to 1.06 mg/ml, *E. crassipes* petiole extract 2.2 to 8.8 mg/ml, *E. crassipes* root extract 1.464 to 4.392, *P. stratiotes* leaves extract 0.8 to 3.4 mg/ml and *P. stratiotes* root extract 1.15 to 4.6 mg/ml and the standard solution to be tested separately. These solution mixtures were kept in the dark for 30 min and optical density was measured at 517 nm using a UV-Vis spectrophotometer against methanol as blank. The control was used is 2 ml of methanol with 2 ml of DPPH solution. The optical density

Table 6: Total Alkaloids in different plant parts of *Eichhornia crassipes* and *Pistia stratiotes.*

Plant Parts	Total Alkaloid Content (mg alkaloid/gdw)
Eichhornia Leaves	0.546 ±0.020
Eichhornia Petiole	0.253 ±0.028
Eichhornia Root	0.548± 0.033
Pistia Leaves	0.163±0.041
Pistia Root	0.096±0.041

mg/gdw : miligram / per gram dry weight
Results are mean value SEM from at least 3 experiments (n=3)

Table 7: The IC_{50} values of different plant parts of *Eichhornia crassipes* of DPPH radical scavenging assay (mg/ml)

Plant Parts	IC_{50} values (mg/ml)
Leaves	0.742±0.02
Petiole	6.411±0.46
Root	4.324±0.54

Each value is expressed as mean ± SEM (Standard Error Mean) (n=3)

Table 8: The IC_{50} values of different plant parts of *Pistia stratiotes* of DPPH radical scavenging assay (mg/ml).

Plant Parts	IC_{50} values (mg/ml)
Leaves	2.463±0.018
Root	4.098±0.03

Each value is expressed as mean ± SEM (Standard Error Mean) (n=3)

was recorded and percentage of inhibition was calculated using the formula given below:

$$\% \text{ of inhibition of DPPH activity} = \frac{A\text{-}B}{A} \times 100$$

Where, A is optical density of the control and B is optical density of the sample.

Linear graph of concentration Vs percentage inhibition was prepared and IC_{50} values were calculated. The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), calculated from the inhibition curve [14].

RESULTS

Table 1 shows the content of primary metabolites from different plant parts of *E. crassipes*. In present study, carbohydrate content was maximum in leaves and petiole while minimum in roots (leaves; 57.26 ± 0.065 mg/gdw > petiole; 56.33 ± 0.094 mg/gdw > root; 38.16 ± 0.102 mg/gdw). Highest value of lipids was observed in leaves and minimum in roots (leaves; 5.22 ± 0.110 mg/gdw >petiole; 2.35 ± 0.187 mg/gdw >root; 2.79 ± 0.131 mg/gdw), Protein content was recorded maximum in leaves and minimum in petiole (leaves; 15.08 ± 0.084 mg/gdw > root; 8.48 ± 0.200 mg/gdw > petiole; 5.53 ± 0.214 mg/gdw), amino acid content was maximum in leaves and minimum in roots (leaves; 1.67 ± 0.122 mg/gdw >petiole; 1.53 ± 0.082 mg/gdw > root; 1.14 ± 0.126 mg/gdw).

Table 2 shows the content of primary metabolites from different plant parts of *P. stratiotes*. In present study, carbohydrate content was observed higher in leaves than roots (leaves; 53.46 ± 0.122mg/gdw> root; 45.26 ± 0.122 mg/gdw). Highest value of lipids was observed in roots than leaves (root; 4.21 ± 0.082 mg/gdw >leaves; 2.72 ± 0.149 mg/gdw), Protein content was recorded higher in leaves than roots (leaves; 14.76 ± 0.096 mg/gdw >root; 10.82 ± 0.082 mg/gdw), amino acid content was more in leaves than roots (leaves; 1.21 ± 0.086 mg/gdw > root; 0.89 ± 0.124 mg/gdw).

Table 3 shows the isolated phenolic content in *E. crassipes and P. stratiotes*. Maximum amount of total bound form of phenols was observed in leaves and minimum in petiole (leaves; 0.217 ± 0.032 mg/gdw>root; 0.187 ±0.014 mg/gdw> petiole; 0.180 ±0.031 mg/gdw) of *E. crassipes.* The total phenolic content was observed more in leaves than roots (leaves; 0.596±0.015mg/gdw>root; 0.173±0.022 mg/gdw) of *P. stratiotes*.

Table 4 shows the isolated flavonoid content in *E. crassipes and P. stratiotes*. Maximum amount of total bound form of flavonoids was observed in leaves and minimum in petiole (leaves; 0.481 ± 0.023 mg/gdw>root; 0.389 ±0.025 mg/gdw> petiole; 0.270 ±0.22 mg/gdw) of *E. crassipes.* The total flavonoid content was observed more in leaves than roots (leaves; 0.519±0.020mg/gdw>root; 0.418±0.040 mg/gdw) of *P. stratiotes*.

Table 5 shows the isolated Tannin content in *E. crassipes and P. stratiotes*. Maximum amount of total bound form of Tannin was observed in roots and minimum in leaves (root; 0.341± 0.033 mg/gdw>petiole; 0.325 ±0.016 mg/gdw> leaves; 0.223 ±0.023 mg/gdw) in *E. crassipes.* The total Tannin content was observed more in leaves than roots (leaves; 0.337±0.053mg/ gdw>root; 0.204±0.026 mg/gdw) of *P. stratiotes*.

Table 6 shows the isolated Alkaloid content in *E. crassipes and P. stratiotes*. Maximum amount of total bound form of Alkaloid was observed in roots minimum in petiole (root; 0.548± 0.033 mg/gdw>leaves; 0.546 ±0.020 mg/gdw> petiole; 0.253 ±0.028 mg/gdw) of *E. crassipes.* The total Alkaloid content was observed more in leaves than roots (leaves; 0.163±0.041mg/gdw> root; 0.096±0.041 mg/gdw) in *P. stratiotes*.

The IC_{50} values of methanolic extracts of different plant parts of *E. crassipes* of DPPH free radical scavenging assay are reported in Table 7, which shows petiole have highest antioxidant activity whereas leaves show minimum activity(petiole; 6.411±0.46mg/ml> root; 4.324±0.54 mg/ml> leaves; 0.742±0.02 mg/ml).

The IC_{50} values of methanolic extracts of different plant parts of *P. stratiotes* of DPPH free radical scavenging assay are reported in Table 8, which shows roots have highest antioxidant activity whereas leaves show minimum activity (root; 4.098±0.03 mg/ml> leaves; 2.463±0.018 mg/ml).

DISCUSSION

The amount of phytochemicals was quantified as per the methods described and the values are expressed. It is evident from the results both the plant extract has good sources of metabolites. Phenolic compounds have therapeutic potential against different diseases because of their antioxidant property. They are known to possess antispasmodic, antiviral, anti-inflammatory, antisecretory, antiulcer, antidiarrheal and antitumor activities. Flavonoids are polyphenolic compounds and widely reported for vasoprotective, anti-inflammatory as well as antioxidants properties. Alkaloids provide defence mechanism and acts as phytoprotective agent, hypoglycemic activities, anti-inflammatory effects[15,16].

Antioxidant values and total polyphenol and flavonoids concentration can be correlated on the basis of concentration of IC_{50} values. Leaves of *E. crassipes* have less IC_{50} value which shows increased concentration of polyphenol and flavonoids which act as good antioxidant while petiole and root of *E. crassipes* show high IC_{50} values which signify less potent antioxidant and has good concentration of polyphenol and flavonoids. The antioxidants plays significant role in maintaining integrity of the cell membrane by prevention of lipid peroxidation and DNA damage caused by a cascade of free radical reaction[17].

CONCLUSIONS

In the present study *E. crassipes* and *P. stratiotes* showed the presence of primary and secondary metabolite. The presence of phenolics and flavonoids appear to be responsible for the antioxidant activity in the plant extracts. This study leads to further research in the isolation and identification of active compounds using spectroscopic techniques.

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