Cultivation, Processing and Analysis of Azolla Microphylla and Azolla Caroliniana as Potential Source for Nutraceutical Ingredients

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Abstract

Azolla fern is rich in micro-nutrients and non-enzymatic anti-oxidants. The nutritional profiling of Azolla revealed that the fern is rich in non-enzymatic antioxidants and micro-nutrients. Antioxidant properties of Azolla caroliniana eclipsed the properties of Azolla microphylla. However, β-carotene content of Azolla caroliniana overshadowed Azolla microphylla illustrating its better anti-cancerous and cardio protective properties. Broadly, the micro–nutrient profile of Azolla microphylla is higher than Azolla caroliniana. Azolla microphylla is rich in essential amino acids including Histidine, Lysine, Methionine and Valine. Quantitative analysis of B-vitamins showed that Azolla microphylla is rich in Vit B_{12} and Vit B_9 than Azolla caroliniana.

Key words

Azolla; Azolla microphylla; Azolla caroliniana; Vit B_{12}; β-carotene; amino acids

I. INTRODUCTION

Azolla are spore-producing (heterosporous), free- floating fresh water vascular plants that have neither seeds nor flowers. It is found in quiet waters, ponds, ditches, canals and paddy fields. Azolla live symbiotically with Anabaena Azolla, nitrogen-fixing blue-green algae. The endosymbiont, which is nitrogen-fixing, provides sufficient nitrogen for both itself and its host (Azolla). The Azolla fern, on the other hand, provides a protected environment for the alga and also supplies it with a fixed carbon source. Because of this symbiotic presence of nitrogen-fixing cyanobacterium with Azolla, and its tendency to block out light to prevent any competition from other plants, it is used in rice cultivation as companion plant.

Azolla grow or multiply through vegetative reproduction both under natural and laboratory conditions. However, under adverse conditions sexual reproduction also occurs. Azolla fronds(15cm) or more for A.nilotica (Ferentinos et al., 2002). Azolla contains many useful phytochemicals (secondary metabolites) such as flavonoids, steroids, alkaloids, phenols, triterpenoid compounds, varieties of amino acids and fatty acids (Selvaraj et al., 2014) These bioactive components contribute to many useful and medicinal properties like antioxidant, anticarcinogenic, antiinflammatory, antidiabetic, hepato and gastro-protective, antiviral, neuro-protective, cardio protective, anti-hypertensive properties (Hertog et al.,1992). Thus Azolla has got tremendous potential for development and production of functional foods and neutraceuticals. Besides these phytochemicals it contains all the essential amino acids making it favorable for fortification and enrichment of bakery products made from wheat flour which is deficient in essential amino acids like lysine (Preedy, 2011). Until now no Azolla recipes seem to have been published. However, several mentions of Azolla as food can be found in research papers. Researchers in China suggest that after sterilization by steam Azolla can be used in salad or as stuffing in Chinese spring rolls and dumplings (Sjödin et al., 2012). The aquatic nitrogen fixing pteridophyte Azolla is an excellent biofertilizer for rice paddy fields owing to its ability fix to atmospheric nitrogen. However, wider exploitation of Azolla is hampered due to increasing soil salinity. Understanding the physiological response of the cyanobiont to salinity is therefore important to unravel the salinity tolerance mechanism operating in this system. This will further pave way for the intervention of advanced molecular biological tools to work out the mechanisms operating under salinity stress conditions in the cyanobionts. Therefore in the present study the physiological and biochemical response of freshly separated cyanobionts from two different species of Azolla viz. A. microphylla and A. caroliniana exposed to salinity was studied. Freshly separated cyanobionts of Azolla microphylla and Azolla caroliniana plants exposed to salinity showed decline in the cellular constituents such as chlorophyll (23.1 and 38.9%) and protein (12.9 and 19.3%). However, an increase in the carotenoid and sugar content was observed. Exposure to salinity stress reduced the heterocyst frequency (35.4 and 57.2%) and nitrogenase activity (37.7 and 46.3%) of the

cyanobionts. Increase in the activity of antioxidant enzymes such as super oxide dismutase (50.6 and 11.5%), ascorbate peroxidase (63.7 and 57.9%), catalase (94.2 and 22.5%) as well as non-enzymatic antioxidant proline (18.8 and 13.3 %) was also observed in response to salinity. The cyanobionts exhibited significant increase in the intracellular Na^+ level and reduced intracellular K^+/Na^+ and Ca^{2+}/Na^+ ratio in response to salinity. The results demonstrated the adverse impact of salinity on the freshly separated cyanobionts as similar to free living cyanobacteria. Salinity stress response mechanisms in the freshly isolated cyanobionts Anabaena azollae was studied at the proteome level using two dimensional gel electrophoresis (2DE) followed by MALDI-TOF-MS/MS analysis. Thirty five (35) protein spots were significantly altered due to exposure to salinity. Among them, thirteen (13) spots were down regulated and twenty two (22) spots were up regulated. A total of twenty (20) new or hypothetical proteins have also been identified in the present study. These proteins are associated with a variety of functions including energy metabolism protein synthesis and folding, DNA damage and repair, stress and defence. The study might help in understanding the biological processes and stress proteins involved in salinity stress adaptation. Results show that the cyanobiont resorts to elaborate changes in the protein synthesis and cell signalling to survive under salinity induced stress conditions. These results may be helpful in the critical evaluation of salinity tolerance mechanism of the cyanobiont and its interaction with the host plants.

II. MATERIALS AND METHODS

A) Collection and culturing of Azolla

Azolla microphylla and Azolla caroliniana plants were maintained in a polyhouse at the Centre for Conservation and Utilization of Blue-Green Algae (CCUBGA), ICAR-Indian Agricultural Research Institute, New Delhi. Plant was grown in Espianase and Watanabe medium (Espianase and Watanabe, 1976) without nitrogen. The temperature was maintained at 30±2°C with a light intensity of 3-5 Klux and a light/dark cycle of 10/14 hours. The cultures were frequently sub-cultured to maintain steady availability of the cultures.

Surface sterilization of Azolla plants

The plants have been surface sterilized with a solution of mercuric chloride (0.1%) for thirty seconds and were dipped immediately in to a large volume of distilled water. Plants were then transferred to plastic trays containing double distilled water and washed thrice.

Dechlorophylisation of processed Azolla

The high chlorophyll content of Azolla would affect the nutritional analysis of Azolla Therefore, processed Azolla was dechlorophylized. Chlorophyll was extracted from dry Azolla fronds by using ethanol as solvent in Soxhlet apparatus at 60°C for 1hr.

B) Proximate & Biochemical analysis of Azolla microphylla and Azolla caroliniana

Moisture content determination (AOAC, 2000)

About 1.5 g of the sample was weighed in the petri dish, previously air dried in the oven and weighed. Then the dish was kept in a hot air oven at 105±1°C for 4 hours. The dish was removed and placed in desiccators for cooling and then weighed. The process of drying, cooling and weighing was repeated at 30 min intervals until the difference between the two consecutive weighing is less than 1 mg. The lowest weight was recorded.

Calculations:

Moisture, % by mass = $100 \times (W_1-W_2)/ (W_1-W)$

Where, W_1 = weight, in g, of the dish with the material before drying.

W_2 = weight, in g, of the dish with the material after drying.

W = weight, in g, of the empty dish.

Total ash content determination (AOAC, 2000):

Ash content is a measure of mineral content in the sample. Silica dishes were heat tarred. About 2 g of the sample was weighed in the silica dish. The dish was place muffle furnace at 500˚c for 4 hours till ash was obtained. The dish was removed, cooled in desiccators and then weighed.

Calculations:

Total ash, % by mass = $100 \times (W_2-W_1)/W$ where,

W_1 = weight, in g, of the empty crucible

W_2 = weight, in g, of the empty crucible with ash, and

W = weight, in g, of the test sample.

Crude fat determination By Soxhlet extraction method (AOAC, 2000)

Soxhlet extraction is a procedure to remove lipids (fats) from food. A solvent is used to wash the solid using a reflux apparatus. The sample is dried and ground and placed in a tube above the extraction solvent. When heated, the solvent evaporates into a gas, then cools into a liquid in a condenser. It then leaks into the sample tube. This continues several hours until the lipid is removed Crude fat was measured by the method of AOAC, (1990). Two

grams of dry bread sample was weighed into a thimble and plugged with cotton. The extraction was carried out in a Soxhlet apparatus for 16 hours with 150 ml of petroleum ether. The flask was removed and the solvent evaporated on water bath. The flask and its contents were dried in the hot air oven at 105°C for 5 hours, cooled in a desiccators and weighed. The weight of crude fat extracted was measured and the percentage fat calculated as:
Calculations:

Crude fat, % by mass = 100 × (M_1-M_2) /M where,

M_1 = mass, in g, of the Soxhlet flask with the extracted fat,

M_2 = mass, in g, of the empty Soxhlet flask, and

M = mass, in g, of the material taken for the test.

C) Estimation of Crude fiber (AOAC, 2000)

Crude fiber is mainly composed of cellulose and lignin (97%) and some mineral matter and quantity (Association of Analytical Chemists, 2000) (IS: 11062). Crude fiber is a rough indicator on how much energy is in the feed. The acid and subsequent alkali digestion, results in oxidative hydrolytic degradation of the native cellulose and considerable degradation of lignin. The residue obtained after final filtration is weighed, incinerated, cooled and weighed again. The loss in weight gives the crude fiber content.

Two gram of defatted sample was boiled with 200mL of 0.255N sulphuric acid for 20min. The extract was filtered through muslin cloth (4 -5 folding) followed by washing with boiling water. The residue was boiled with 200mL of 0.255N sodium hydroxide solution for 20min and again filtered through muslin cloth (4-5 folding's). The residue was then washed with cold water followed by washing with 10% potassium Sulphate, boiling hot water and finally with alcohol. The left out residue was dried in hot air oven at 110˚C for 1 hour and cooled indesiccator and weighed. The dried contents were ignited in muffle furnace for 20 min. cooled in a desiccator and Weighed. The loss in weight represents crude fiber.
Calculation:
% crude fiber = W_1/W ×100
W_1= loss in weight.

W= weight of sample used.

D) Protein Determination by BCA Method: (Smith et al., 1985)

The bicinchoninic acid (BCA) assay is used for the quantification of total protein in the sample. It is a spectrophotometric assay based on the alkaline reduction of the cupric ion Cu^{2+} to the cuprous ion Cu^{1+} by the protein, followed by chelating and color development by the BCA reagent. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments. Thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.

A stock solution of 1mg/ml Bovine Serum Albumin BSA was prepared by dissolving 1.5mg of BSA in 1.5ml of Phosphate buffer. The resulting solution was diluted with phosphate buffer to obtain different concentration of BSA solution .To each microplate well 200µl of BSA standard and 100µl each of working BCA reagent and 100µl buffer was pipetted out. The tubes were covered with aluminum foil and incubated at 30°C for 30 mins. The tubes were then kept at room temperature for 10 min before measurement. Absorbance was measured at 562nm.

The preparation of sample was similar to that of standard. About 5g of ground sample was taken and 10ml of phosphate buffer solution was added. The mixture was then homogenized in mortar pestle and filtered. Equal volume of hexane was added to the mixture in falcon tubes and then centrifuged at 3000rpm for 10 min. Supernatant was taken which contains proteins. The supernatant was diluted in phosphate buffer. From the dilution 200µl of sample was taken and 100µl each of reagent was added to each well. The tubes were covered and incubated at 37°C for 30 mins. The tubes were then kept at room temperature for 10mins before measurement. Absorbance was measured at 562nm.

E) Determination of Vit B_{12} and Folic acid in Azolla (Perveen et al., 2012)

Dried azolla powder (5 g) was accurately weighed and transferred into a 250 mL round bottom flask. Initially about 5mL of 0.1 N HCl and 40 mL water was added and then refluxed on boiling water bath for 15 min. After completion of refluxing period the flask was cooled and volume made up to 50 mL in a volumetric flask. The content was centrifuged (2500 rpm) for 15 min to remove suspended material. The supernatant solution was filtered through a Whatman No.1 filter paper.

The standards of B_{12} and folic acid of conc. 500µg and 5mg resp. were purchased from Schemadzu, Germany. Standards were accurately weighed and triturated in mortar and pestle. The stock solutions were prepared in the concentration of 1mg/ml and were bath sonicated for 30 mins under dark conditions. The standards were filtered through Whattman filter paper No.1 and the filtrate was used for UV- spectrophometry analysis. Different dilutions of stock solution were prepared and spectrum was taken from 190 to 400nm.

F) Total Carotenoid Estimation (Singh et al., 2003)

Total carotenoids were extracted from the fern Azolla microphylla and Azolla caroliniana by using solvent 85% acetone. To the accurately weighed 5g of fresh leaves 20ml of acetone was added in 50ml falcon tube. The mixture was ground in a mortar pestle and centrifuged at 3000rpm for 10 min to separate two phases. Supernatant containing pigment was collected and stored at 4°C. The extraction was repeated with acetone till supernatant became colourless. All the fractions of supernatant were collected and made into a final known volume. Optical density was taken at 450nm using 85% acetone as blank.

Calculations:-

Content of carotenoids (µg/g) = $A \times V(ml) \times 10^4 / A^{1\%} \times W(g)$ (Oliverial et al., 2017)

Where: A= absorbance measured

V= total extract volume

W= sample weight

2500= β-carotene absorption coefficient in acetone.

Spectrum was taken in the range of λ_{max} 400 to 500 nm

Determination of Carotene, Xanthophyll and Chlorophyll (Singh et al., 2001)

Carotene and chlorophyll was extracted by using ice- cooled acetone. 5g of fresh sample was accurately weighed and 40ml of acetone was added in the dark. The mixture was stored at -20°C for 18hrs and then triturated in mortar pestle. The supernatant was filtered and collected in 15ml falcon tube. Carotene, xanthophyll and chlorophylls a and b (µg/g) were determined spectrophotometrically at 470, 645 and 662 nm by means of equation proposed by Lichten thaler and Well-burn with slight modifications, Calculations was done as per Khuantrairong et al., 2012.

Chlorophyll a $= 11.75A_{662} - 2.35A_{645}$

Chlorophyll b $= 18.61A_{645} - 3.960A_{662}$

Carotene $= (1000A_{450} - 2.270C_a - 81.4C_b)/227$ (C_a = is chlorophyll a, C_b = is chlorophyll b)

Xanthophyll = Total carotenoid – Carotene.

G) Analysis of β-carotene by high performance thin layer chromatography (Mostafa et al., 2012)

Analysis of β-carotene by high performance thin layer chromatography (HPTLC) was carried out as per the method given by Mostafa et al., 2012. A stock solution of 1mg/ml of β-carotene of different dilutions was prepared in methanol. β-carotene was extracted from the Azolla fern using 99.8% ethanol as solvent. To the accurately weighed 1.6g of sample, 1.6ml of 100% potassium hydroxide and 6.4ml of 99.8% ethanol was added (1:4 v/v) and homogenized for 3 minutes using a blender. The mixture was saponified by using refluxing apparatus, and heated using heating mantle for 30 min and then cooled to room temperature. During heating mixture was frequently agitated to avoid any aggregation. For the extraction step, the mixture was transferred into a separation funnel and 10ml of n-hexane was added. The funnel was inverted, vented and then shaken vigorously for a few seconds, and the layers were allowed to separate. The upper layer (hexane extract) was pipetted out, and the aqueous layer was re-extracted with 10ml n-hexane. The extract was then filtered through anhydrous sodium sulphate to remove any water residue. β- carotene content was analysed using HPTLC. The filtrates (10µl of each) and the standards (10µl each at the concentration of 1mg/ml) were coated on a precoated TLC aluminum silica gel (Merck,Germany (20cm × 10cm). The TLC plates were developed with a solvent system consisting of petroleum ether/ diethyl ether/acetone (15:3:2). The developed plates were heated at 110°C for 10 min. These plates were scanned digitize analysed by using CAMAG software. UV detection was performed densitometrically at maximum absorbance wavelength, 450nm

H) Analysis of Amino acids present in Azolla protein by HPTLC

All the twenty two standard amino acids were purchased from Sisco Research Laboratories (SRL) and were prepared as 1mg/ml solutions in 70% ethanol. Accurately weighed azolla powder was taken and homogenized in water. The mixture was centrifuged at 3000 rpm for 10 mins. Supernatant was taken and dissolved in 70% ethanol (1:4). Total volume of mixture was measured and centrifuged again at 3000 rpm for 10 mins. Supernatant was discarded and pellet formed was taken. The pellet obtained was hydrolysed in 6N HCl at 100°C for 2hr. Red color was produced and 6N NaOH was added for neutralization. The mixture was filtered through whattman filter paper No.1 and the filtrate was used for HPTLC analysis. The filtrates (5µl of each) and the standards (5µl each at the concentration of 1mg/ml) were coated on a precoated TLC aluminum silica gel (Merck, Germany (20cm × 10cm). The TLC plates were developed with a solvent system consisting of n-butanol/ acetic acid/water (3:1:1). The developed

plates were stained using 0.3% ninhydrin in n-butanol as spraying reagent and the plates were heated at 110°C for 10 min. These plates were scanned digitize analysed by using CAMAG software. UV detection was performed densitometrically at maximum absorbance wavelength, 490nm.

III. RESULTS AND DISCUSSION

A) Proximate analysis of Azolla

Nutritional analysis of Azolla microphylla & Azolla caroliniana was carried out. Both the species of Azolla were subjected to the standardized tests for different nutritional analysis. Protein estimation was done by bicinchoninic acid (BCA) micro assay protein estimation kit. Different concentrations (Table. 1) standard solution was prepared in phosphate buffer and absorbance was measured at 562 nm to make a standard plot (Figure1).Linearity of the standard BSA was plot by regration equation R^2 of 0.94. The maximum total protein content was better Azolla microphylla then A. caroliniana but total moister and total ash content was more in A. caroliniana shows that Azolla caroliniana has more dead tissues or thick cell wall in the cells,which was observed by the microscopy earlier reported total lipids content was not much difference in both the species having lipid content of 1.33% and 1.30% in Azolla caroliniana and Azolla microphylla respectively (Table 2). The protein content in Azolla microphylla was found to be16.70 mg/g and 11.57 mg/g in Azolla caroliniana.

Table 1: Concentration of standard BSA with absorbance

	Concentration (µg/ml)	Absorbance
	7.812	0.29
	15.625	0.30
	31.25	0.44
	62.5	0.60
	125	1.00
	250	1.39
	500	2.56
	1000	3.31

Table 2: Proximate Analysis of Azolla samples

Sample	Total Protein (%)	Total Lipid (%)	Total fiber (%)	Total moisture (%)	Total ash (%)
Azolla microphylla	1.67	1.30	18.07	93.8	9.5
Azolla caroliniana	1.15	1.33	13.44	95.28	12

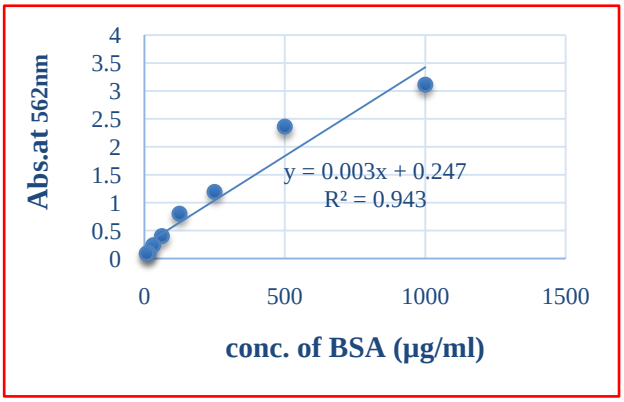

Fig. 1 Standard curve for protein (BSA)

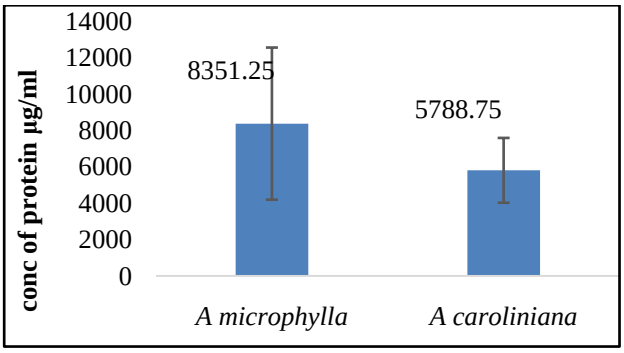

Fig. 2: Variation of protein content in Azolla species

B) Micro-nutrient analysis of Azolla

Vitamin B_{12}, Vitamin B_9, Carotene, Xanthophyll, Total carotenoids and β-carotene content of Azolla microphylla and Azolla caroliniana was estimated. For standard plot preparation, different concentrations of methylocobalamine (Table. 3) and folic acid (Table 4) were prepared in distilled water and absorbance was measured at 350nm and 311nm respectively. the linearity of vitamin B_{12} was derived for the concentration 15.6 ug/ml till 250 ug/ml with R^2 =0.99. Similarly for folic acid, R^2 was found to be 0.98.

Table 3: Concentration of standard VitB_{12} with absorbance

Sr. No.	Concentration	Absorbance at 350nm
1	15.625	0.305
2	31.25	0.369
3	62.5	0.688
4	125	1.342
5	250	2.247

Table 4: Concentration of standard Folic acid with absorbance

Sr. No.	Concentration (µg/ml)	Absorbance at 311nm
1	15.625	0.134
2	31.25	0.239
3	62.5	0.489
4	125	0.917
5	250	1.514

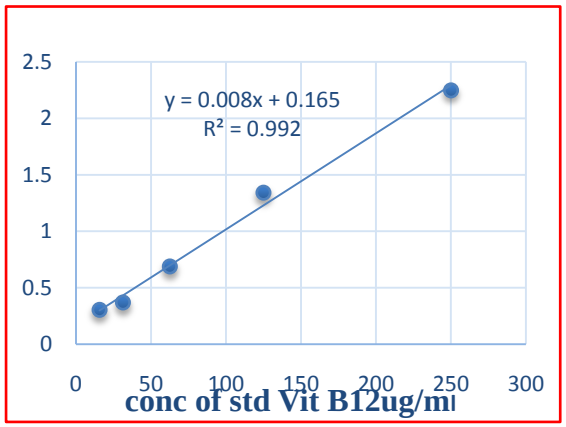

Fig. 3 Standard curve for VitB$_{12}$

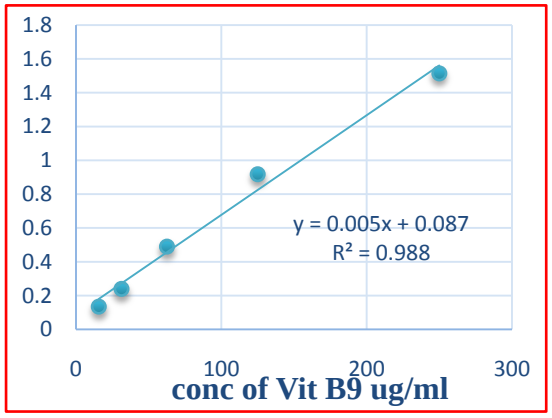

Fig. 4 Standard curve for VitB$_9$

Vitamin B12 was also higher in a Azolla

microphylla biomass. Total carotenoid content in Azolla caroliniana was higher but ß carotene and carotene concentration was lower in comparison to Azolla microphylla. Azolla microphylla has less xanthophyll and chlorophyll A, concentration in comparison A. caroliniana hence Azolla microphylla has ß carotene majorly as the carotenoid in it which establish it as potential ß carotene source (Table 5). While checking the spectra for total carotenoid the analysis the ƛ max was found to be 435.65nm and 432nm for Azolla microphylla and Azolla caroliniana resp.

Table 5: Vitamin B$_{12}$ and folic acid content in Azolla dried powder

Sample	Vit B$_{12}$ (mg/g)	Folic acid (mg/g)
Azolla microphylla	9.16	22.62
Azolla caroliniana	3.43	10.67

It was analysed that VitB$_{12}$ and Folic acid was 2.26% in Azolla microphylla and 1.06% in Azolla caroliniana respectively.

It was observed that total carotenoids, carotene, β-carotene, and xanthophyll was in the range of 19.81µg/g, 13.06 µg/g, 471.73mg/g & 6.75µg/g respectively in Azolla microphylla while50.75µg/g,12.76 µg/g,354.57mg/g & 38.74 µg/g respectively in Azolla caroliniana. Azolla microphylla contains 33.48µg/g of chlorophyll 'a' & 12.76 µg/g of chlorophyll 'b' and 43.12µg/g of chlorophyll 'a' & 17.09 µg/g of Chlorophyll 'b' in Azolla caroliniana (Table 6).

Table 6: Micro-nutrient analysis of non-enzymatic antioxidants in Azolla Species

Sample	Total carotenoids (µg/g)	Carotene (µg/g)	β carotene (mg/g)	Xanthophyll (µg/g)	Chl a (µg/g)	Chl b (µg/g)
Azolla microphylla	19.81	13.060	471.73	6.75	33.48	12.76
Azolla caroliniana	50.75	12.7683	354.5723	38.745	43.12	17.09

Amino acid analysis of both the azolla species:

Quantitative amino acid analysis of both the species was carried out using HPTLC. Both essential and non- essential amino acids were found in Azolla microphylla & Azolla caroliniana (Table 7 and 8).

Table7: Essential and Non Essential Amino acids Azolla microphylla:

Essential Amino Acid (µg/g)			Non Essential Amino Acid (µg/g)		
1	Histidin	8.567	1	Serine	23.138
2	Lysine	1.00	2	Tyrosine	166.503
3	Methonine	8.972	3	Glutamic acid	125.372
4	Valine	87.129			

Table 8: Essential and Non Essential Amino acids Azolla caroliniana:

Essential Amino Acid (µg/g)			Non Essential Amino Acid (µg/g)		
1	Tryptophane	20.408	1	Glutamin	30.432
2	Valine	120.836	2	Gluamic acid	45.045

Qualitative amino acid profiling of both the species was carried out using HPTLC. Essential amino acids were found in Azolla microphylla than the Azolla caroliniana.

In A microphylla total six peak where observed with Rf of 0.25, 0.27, 0.44, 0.51, 0.60 and 0.67 resp. in Azolla caroliniana total four peak where observed Rf of 0.41, 0.52, 0.63 and 0.75 on the basis of these HPTLC chromatogram essential amino acid i.e. histidine lysine methionine and valine where as non essential amino acid i.e. serine tyrosine and glutamic acid where found Azolla microphylla. Azolla caroliniana has essential amino acid tryptophan and valine where as non essential glutamine and glutamic acid resp, on the basis of result it was observed that Azolla microphylla is better source of essential and non essential amino acid hence a formulation are a functional food incorporated with Azolla microphylla prepared and check for the nutrient value.

IV. CONCLUSION

The nutritional profiling of Azolla revealed that the fern is rich in non-enzymatic antioxidants and micro-nutrients. Antioxidant properties of Azolla caroliniana eclipsed the properties of Azolla microphylla. However, β-carotene content of Azolla caroliniana overshadowed Azolla microphylla illustrating its better anti-cancerous and cardio protective properties. Broadly, the micro–nutrient profile of Azolla microphylla is higher than Azolla caroliniana. Azolla microphylla is rich in essential amino acids including Histidine, Lysine, Methionine and Valine. Quantitative analysis of B-vitamins showed that Azolla microphylla is rich in Vit B_{12} and Vit $B_{9.}$

Regular intake of B_{12} and B_9 has shown to improve memory in stressed adults. The macro nutrient profile of Azolla microphylla is higher than Azolla caroliniana.

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