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Exploring the Micro-nutritional and Anti-nutritional Aspects of Azolla for its Application as Functional Food Ingredient

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ABSTRACT

Background: Aquatic plants are unexploited sources of bioactive and functional compounds having the potential as food ingredients for developing novel functional food and nutraceutical products. Azolla, an aquatic fern, is an excellent source of nutrients and secondary metabolites and hence finds application possibilities in the human diet. The goal of the current investigation was to expore the micronutrients as well as identify certain anti-nutritional elements in the water fern Azolla.

Methods: In two species of Azolla *viz*., *Azolla pinnata* and *Azolla caroliniana;* the micro and anti-nutritional factors were determined using standard laboratory methods.

Result: The findings demonstrated that both species of Azolla contained hydrocyanide, phytic acid, oxalate, tannin and saponin, all of which were below the critical level. Comparatively, saponin was found to be in the highest concentration of 9.13±0.012% and 7.52±0.031% and hydrocyanide was the lowest with the content of 0.0045±0.0004% and 0.0036±0.0002% for *A. pinnata* and *A. caroliniana*, respectively. *Azolla caroliniana*, however, outperformed *Azolla pinnata* in total caroteniod concentration, demonstrating its superior cardio-protective and anti-cancerous properties. Results indicated *Azolla pinnata* species to be more suitable for fortification as it contained lower anti-nutrients and was high in chlorophyll content when compared to the counterpart.

Key words: Anti-nutritional factors, *Azolla caroliniana*, *Azolla pinnata,* Micronutrients, Water fern.

INTRODUCTION

Azolla is a freshwater vascular plant without seeds or flowers that produce spores (heterosporous). Ponds, ditches, canals and paddy fields are just a few examples of the waterways where it may be found. The nitrogen-fixing blue-green algae Anabaena Azolla has a symbiotic connection with the Azolla. For both itself and its host, the nitrogen-fixing endosymbiont produces adequate nitrogen (Gupta *et al*., 2018). The Azolla fern, on the other hand, protects the alga, along with providing it with a stable carbon source. With its ability to limit light and its symbiotic relationship with a nitrogen-fixing cyanobacterium (Kc *et al*., 2016). Azolla is used as a companion plant in rice cultivation. Azolla has gained attention as a potential food source for humans due to its high nutritional content and rapid growth (DM *et al*., 2014). It is particularly popular in some Asian countries as a traditional food source (Raja *et al*., 2012). Azolla is rich in various nutrients, making it a valuable addition to the human diet. However, like many plants, it also contains some anti-nutritional factors.

Anti-nutritional factors are chemicals that are harmful to humans or, in some other way, restrict the body's access to nutrients (Kołodziej *et al*., 2019). These substances are present in most foods and inhibit the body from using the nutrients optimally, lowering the food's nutritional value (Thakur *et al*., 2017). Depending on the kind of food, various dietary items include anti-nutritional agents in variable degrees. Many plants and vegetables include poisonous chemicals such as cyanide, nitrate, phenols and tannin, as well as anti-nutrients like phytate and oxalate (Hassan *et al*., 2011). A high level of antinutrients in the body may result in symptoms such as nausea, bloating, headaches, rashes, nutritional deficiencies and other issues. On the other hand, these chemical substances may undoubtedly help humans when consumed properly (Samtiya *et al*., 2020).

Foods have been evaluated for several antinutritional components with hazardous potential and proved to be either heat-stable or heat-labile (Emire *et al*., 2013). The biggest quantities of antinutrients may be found in beans, grains, nuts and legumes. Nevertheless, they can also be found in roots, fruits, as well as leaves of other plants (Mattila *et al*., 2018). Plant materials often include saponins, phytate, tannins, protease inhibitors and polyphenolic chemicals, which are all known to be anti-nutrients (Francis *et al*., 2001). By lowering protein digestibility, limiting mineral absorption and producing toxicity and health problems when present in large amounts, these substances reduce the

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nutritional value of food (Popova *et al*., 2019). However, very little is known about the trypsin inhibitor found in Azolla leaf meal. Isolating and identifying the T1 from Azolla leaves has not been attempted very often. Trypsin inhibitor from water fern was isolated and characterized by Maity and Patra (2003).

Nutritional and antinutritional factors like cyanide, tannin and phytin contents of the leaf protein concentrate and other extractives from water fern Azolla and duckweed were investigated by Fasakin *et al*. (1999). There was a drastic reduction in the antinutritional factors of the leaf protein concentrate and extractives compared with the fresh and sun-dried original weeds (Brouwer *et al*., 2019). The results for Azolla reveal that tannin content in leaf protein concentrate and its extractives were more than twice the values obtained for duckweed. Very few investigations have been carried out to look at the micronutrients profile of Azolla, despite the fact that the majority of study inquiries in this area focused on its bio-fertilizing activities. Since there is no published information on Azolla species for its food application and benefits, this research was oriented towards exploring the micronutrients and antinutritional profile of two species of Azolla for its application as functional food ingredient.

MATERIALS AND METHODS

Time and place

The study was conducted from January to July, 2022 in Department of Food Science and Technology at Kerala University of Fisheries and Ocean Studies, Kochi.

Collection and preparation of raw material

Azolla pinnata and *Azolla caroliniana* samples were purchased from Kerala Agriculture University, Mannuthy. Azolla is mainly propagated by vegetative means. Purchased samples were adequately cleaned in cold water to eliminate contaminants and they were grown in cement tanks under-regulated climatic conditions at a temperature of 28-30°C and a light level of 50 k lux. In about 8-10 days, both Azolla species were harvested, dried using a cabinet tray dryer at 40°C for 6 hours and stored under ambient conditions for further studies. For estimation of anti-nutritional factors, 7.5 g of Azolla was extracted using 150 mL of 60% ethanol, filtered, rotary evaporated and stored in chiller (4°C).

Chemicals and reagents

Acetone, ethanol, hydrochloric acid, potassium ferrocyanide, ferric chloride, ammonium thiocyanate, phytin phosphorus, methyl orange indicator, potassium permagnate, alkaline picrate, diethyl ether, n-butanal and sodium chloride reagents of analytical grade procured from Nice Chemicals (Kochi), SRL Chemicals (Chennai) and Sigma-Aldrich, India were used for the study.

Analysis of micronutrients

Estimation of total caroteniods

The standard method of Pandey *et al.* (2003) with slight modification was used. Using 85% acetone, entire carotenoids were extracted from the ferns *Azolla pinnata* and *Azolla caroliniana*. 50 mL falcon tube containing 20 mL of acetone was filled with precisely weighed 5 g of fresh leaves. Two stages of the mixture were separated after being ground in a mortar and pestle and centrifuged for 10 minutes at 3000 rpm. Pigment-containing supernatant was collected and kept at 4°C. Acetone was used to extract the material repeatedly until the supernatant was colorless. The supernatant fractions were collected and combined to get a final known volume. At 450 nm, optical density measurements were made using a blank solution of 85% acetone.

$$\text{Content of carotenoids } (\mu g/g) = \frac{A \times V(ml) \times 10^4}{A^{1\%} \times W(g)}$$

(Leao *et al*., 2017)

Where,

A= Absorbance measured.

V= Total extract volume.

W= Sample weight.

Spectrum was taken in the range of λmax 400-500 nm.

Determination of chlorophyll, carotene and xanthophyll

Methodology by Singh *et al.* (2011) was adopted for this study. Chilled acetone was used for the extraction of carotene and chlorophyll. 40 mL of acetone was added to 5 g of samples *viz*., *Azolla pinnata* and *Azolla caroliniana* in dark condition. The solution was triturated in a mortar and pestle after being kept at -20°C for 18 hours. In a 15 mL falcon tube, the supernatant was filtered and collected. At 470, 645 and 662 nm, xanthophyll, carotene, along with chlorophyll a and b (µg/g) was measured using a spectrophotometer. Calculations was done as per Khuantrairong *et al*. (2012).

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

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

$$\text{Carotene} = \frac{1000A_{450} - 2.270C_a - 81.4C_b}{227}$$

(C_a = Chlorophyll a, C_b = Chlorophyll b)

Xanthophyll = Total carotenoid - Carotene

Estimation of anti-nutritional factors

Determination of tannin content

The standard method by Gupta and Verma (2011) was used for determining tannin content. Mechanically shaken cake residue of two species of *A. pinnata* and *A. caroliniana* amounting to 0.5 g was weighed into a 100 mL container. The following step was adding 50 mL of purified water and shaking the bottle strongly for one hour. The mixture was brought up to specification after filtering it in a 50 mL volumetric flask. A test tube containing 5 mL of the filtrate was filled and 2 mL of 0.1 M $FeCl_3$ in 0.008 M potassium ferrocyanide and 0.1 N HCl were added. The absorbance was measured using spectrophotometer (Spectrum Lab 23A, England) at 725 nm wavelength.

The equation below was used to calculate the amount of tannin in the sample:

$$C_T = \frac{A_T \times C_S}{A_S}$$

Where,
C_T = Tannin concentration in mg%.
A_T = Absorbance of the test sample.
C_S = Concentration of tannin in standard.
A_S = Absorbance of the standard.

Determination of phytic acid content

The standard method of Marolt and Kolar (2020) was used for determining phytic acid content. About 4.0 grams of cake residue of two species of *A. pinnata* and *A. caroliniana* was soaked in 100 mL of 2% HCl for three hours before being filtered. Titrated against 0.01 M standard $FeCl_3$, 5 mL of 0.3% NH_4SCN, 25 mL of the filtrate and 53 mL of purified water were combined until a brownish-yellow tint persisted for 5 seconds. The amount of phytin phosphorus (1 mL = 1.19 mg phytin phosphorous) was measured and the amount of phytic acid was estimated by multiplying the result by 3.55.

Phytate content (mg %) =

Tv (mL) × Phytin phosphorous (1.19 g) × 3.55

Where,
Tv= Volume obtained after the color change.

Determination of oxalate content

In accordance with the standard method of Karamad *et al.* (2019), 10 mL of 6 M HCl and 190 mL of purified water were added to a 250 mL volumetric flask together with 2 g of the cake residue of *A. pinnata* and *A. caroliniana.* An hour was spent digesting the combination in a hot water bath before it was cooled, brought to the proper consistency and filtered. A beaker containing 50 mL of the material was filled and 20 mL of 6 M HCl was then added. The solution was filtered after being evaporated to about 1/2 its volume. Following several rounds of washing with warm distilled water, the residue was added to 25 mL of the filtrate together with 3 drops of methyl orange indicator and the mixture was then titrated against a 0.1 M $KMnO_4$ solution until a light pink hue developed and remained for 30 seconds. The following equation was used to get the total oxalate content.

$$\text{Oxalate content (mg \%)} = T_V \times 0.0045$$

Where,
Tv = Volume obtained after the color change.

Determination of hydrocyanic acids (Cyanide) content

The standard method of Fukushima *et al.* (2016) was adopted for the study. The normal technique included weighing 5 g of the cake remnant of *A. pinnata* and *A. caroliniana* in a volumetric flask, adding 50 mL of purified water and corking the flask with cotton wool. After being maintained for 24 hours, the solution was filtered. 1 mL of the filtrate was combined with 4 mL of alkaline picrate and the test tube was sealed. For five minutes, the substance was incubated at 25°C in a water bath. After the solution had cooled, the absorbance at 490 nm was calculated in comparison to a blank for the reagent. The following equation was used to determine the cyanide content.

$$\text{Cyanide content (mg\%)} = \frac{\text{Absorbance of test} \times \text{Concentration of standard}}{\text{Absorbance of standard}}$$

Determination of saponin content

The standard method of Ezeonu and Ejikeme (2016) was used. The normal procedure included weighing 20 grams of the cake remnant of *A. pinnata* and *A. caroliniana* in a conical flask and adding 100 mL of 20 per cent aqueous ethanol. The mixture was continuously stirred and cooked at roughly 55°C over a hot water bath for four hours. The mixture was filtered before being extracted once again using 200 mL of 20% ethanol. Over a water bath set at around 90°C, the combined extract was reduced to 40 mL. 20 mL of diethyl ether was then added to a 250 mL separating funnel along with the concentration and the solution was rapidly mixed. The aqueous layer was collected and the ether layer was removed. The cleansing procedure was performed again. The solution received 60 mL of n-butanol. After being extracted, the mixed n-butanol underwent two items of washing with 10 mL of 5 per cent aqueous NaCl. After evaporating the leftover solution in the water bath, it was dried in an oven until the weight remained consistent. The following equation has been used to determine the saponin content.

$$\% \text{ Saponin} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100$$

Statistical analysis

Values from the experiment were done in triplicates and were expressed as mean ± SD. One-way ANOVA was performed with tukey's significant difference test, based on significant difference of $p \leq 0.05$ to analyse the results.

RESULTS AND DISCUSSION

Analysis of micronutrients

Micronutrients are essential in plant-based diets for human nutrition because they provide the necessary nutrition that are vital for maintaining good health and preventing various deficiencies and diseases (Bhattacharya *et al*., 2024). They provide a range of health benefits, including antioxidant properties and potential protection against chronic diseases (Elrasoul *et al*., 2020). Total carotenoid, Carotene, Chlorophyll a, Chlorophyll b and Xanthophyll content of *Azolla pinnata* and *Azolla caroliniana* were assessed and depicted in the Fig 1. Compared to *A. pinnata*, *A. caroliniana* had a greater total carotenoid content but a lower carotene concentration. In comparison to *Azolla caroliniana*, *Azolla pinnata* exhibited lower concentrations of xanthophyll and chlorophyll b.

While checking the spectra for total carotenoid, the analysis of the λ_{max} was observed to be 432 nm and

435.65 nm for *Azolla caroliniana* and *Azolla pinnata,* respectively. It was found that total xanthophyll, carotenoids and carotene were 6.75±0.01 µg/g, 38.73±0.05 µg/g and 17.76±0.02 µg/g, respectively, in *Azolla pinnata* while in *Azolla caroliniana* the values were 8.74±0.05 µg/g, 54.57±0.03 µg/g and 12.09±0.02 µg/g respectively. The studies conducted by Lejeune *et al*. (2000) showed carotene in Azolla ranging from 206 to 619 mg/kg and Xanthophyll content of 149 mg/kg was observed in studies conducted by Brouwer *et al*. (2018). Studies indicated *Azolla pinnata* contained 63.27±0.03 µg/g of chlorophyll 'a' and 33.48±0.03 µg/g of chlorophyll 'b' whereas 49.18±0.01 µg/g of chlorophyll 'a' and 43.12±0.05 µg/g of Chlorophyll 'b' was found in *Azolla caroliniana.* Observations by Sánchez-Viveros *et al.* (2011), indicated *Azolla pinnata* and *Azolla caroliniana* species to have higher chlorophyll a and b content when compared to *Azolla fillicullоids*, with 25.55±0.55 µg/g of chlorophyll a and 32±0.21 µg/g of chlorophyll b. In human body, beta-carotene converts into vitamin A (retinol) which gives good vision and eye health, for a strong immune system and for healthy skin and mucous membranes (Ebadi *et al*., 2023).

Estimation of anti-nutritional factors

Aquatic plants are highly valued in both biomedical research and food on account of their wide potential for use as human food, animal feed, bio-fertilizers *etc* (Prabakaran *et al*., 2022). Increased incidence of lifestyle diseases has led to a thoughtful demand for health-oriented, nutraceutical commodities from conventional as well as non-conventional food sources. Azolla extact was studied for its anti-nutritional activities. In general, harmful compounds are present in all foods; what makes a meal edible is how concentrated those poisonous components are (Witczak *et al*., 2017). The amounts that researchers discover when they evaluate a particular item likely rely on the growing circumstances, harvesting practices, processing processes, testing methodologies and even the food's age at the time it is being evaluated since their quantity in raw materials is widely varied (Gupta *et al*., 1988).

The concentrations of tannins, oxalate, phytic acid, hydrocyanide and saponin in *Azolla pinnata* and *Azolla caroliniana* are indicated in Table 1. The qualitative presence of tannin and saponin content was founded by Sathammaipriya *et al.* (2018) in the species *Azolla microphylla* and needed proper quantitative estimation. The saponin was in the highest level with a concentration of 9.13±0.012% and 7.52±0.031% for *A.pinnata* and *A.caroliniana*, respectively followed by tannin with 0.5677±0.033% for *A. pinnata* and 0.3835±0.014% for *A. caroliniana*. Enrichment of food with tannin extracts promotes healthy changes in the human gut microbiota (Molino *et al*., 2021). The anti-nutritional compound in tropical fern *Nephrolepis cordifolia* L. founded by Oloyede *et al.* (2013) included 0.06 mg/100 g oxalate but phytate was not detected and toxic component was 0.16/100 g of hydrogen cyanide. The extremely low levels of both oxalate and hydrogen cyanide in Azolla species ranges from 0.0281% to 0.0349% and 0.0036% to 0.0045% which is alike with this plant are highly remarkable. Thus, the use of this water fern Azolla plant as animal diet and its extract as part of human diet likewise the tropical fern *Nephrolepis cordifolia* L. may not pose any serious health problems to the ruminants eating it.

Table 1: The concentrations of tannin, phytic acid, oxalate, hydrocynide, saponin in two species of Azolla extract.

Anti-nutritional factors	*Azolla pinnata* (%)	*Azolla caroliniana* (%)	Critical limit (%)
Tannin	0.3675±0.033	0.5837±0.014	<2.5
Phytate	2.5347±0.08	3.3796±0.02	<10
Oxalate	0.0349±0.013	0.0281±0.005	<2.5
Cyanide	0.0045±0.0004	0.0036±0.0002	<0.5
Saponin	9.13±0.12	7.52±0.31	<10

Note: Values are presented as mean±standard deviation of three replicates. Critical values were sourced from Muhammad *et al*. (2011).

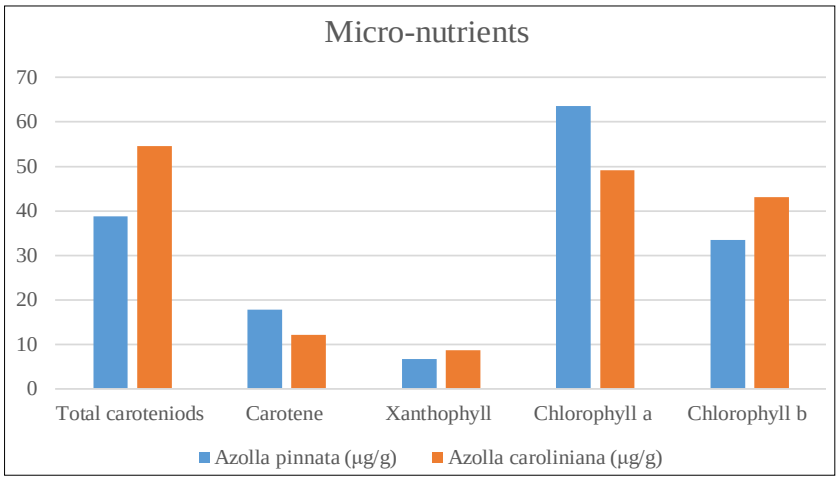

Fig 1: Micro-nutrients in *Azolla pinnata* and *Azolla caroliniana.*

Critical limits of antinutritional factors were sourced from the studies done in *Gardenia aqualla* (Gauden dutse) fruit pulp by Muhammad *et al*. (2011). Both species had anti-nutritional values below the critical limit. The antiinflammatory and antiulceric activities of phytic acid in *Azolla filiculoides* extract was identified by Bhaskaran *et al*. (2015) and quantitive values of phytic acid content in *Azolla pinnata* was 2.5347±0.018% which was lesser than *Azolla caroliniana i.e*., 3.3796±0.02. The extracts of Azolla could be used to fortify food products to enhance overall nutrients due to the efficiency of common processing methods like enzyme treatment, solvent extraction and dry and wet heating in eliminating the harmful consequences of antinutrients from plant-based materials (Natesh *et al*., 2017). Anti-nutritional factors (ANFs) in plants like Azolla, when present below critical limits, can have a limited negative impact on their use in the human diet. ANFs are compounds naturally occurring in many plants that may interfere with nutrient absorption, digestion, or overall utilization of nutrients (Sun *et al*., 2024). However, when the levels of ANFs are low or below critical limits, as observed from the study, their significance is reduced and the plant can still be used in the human diet with minimal adverse effects. These anti-nutrients may be reduced using techniques that have been identified from earlier studies, like solvent extraction, dry and wet heating and enzyme treatment.

CONCLUSION

The present study indicated the suitability of Azolla (water fern) as a nutrient loaded raw material for fortification in human diet. It contained plant pigments like chlorophyll, carotenoids, xanthophylls that can be used for coloration and also associated with potential health-promoting properties. It is also a good source of natural antioxidant and some antinutritional substances like phytic acid that can provide health benefits *viz*., antiulcer and antiinflammatory to the mankind. The presence of low quantity of oxalate in both species of Azolla indicated its suitability to be used in human diet with easy digestibility. The desirable levels of tannin and saponin content indicated beneficial effect as saponin decreases blood lipids, lowers cancer risks and lower blood glucose response. Also a high saponin diet can be used in the inhibition of dental caries and platelet aggregation, in the treatment of hypercalciuria in humans and as an antidote against acute lead poisoning. It contained low level of anti- nutritional substances and toxicant such as oxalate and cyanide and good presence of total carotenoids, xanthophyll and highly useful carotene. Thus, extract from it could be included in food applications to enhance functionality in nutrition, appearance and storage especially in fortification or enrichment in developing countries.

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Conflict of interest

The authors declare that they have no conflict of interest.

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