Polyphenol Characterization in *Azolla filiculoides* after Drying and Enzymatic Hydrolysis Processes

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Azolla filiculoides is an aquatic fern with the potential to become a source of raw materials in a biorefinery system, *e.g.*, a source of soluble and insoluble carbohydrates, proteins, carotenoids, and polyphenols. The fiber chemical content was determined as cellulose (19.2% dry basis) and hemicellulose (7.6% dry basis) content. *Azolla* has no lignin as a cell wall structure material. Cellulase treatment showed no effect in ethanolic extraction, but polyphenols were found in the enzyme solution at the end of the reaction. The phenolic acids and flavonoids contents of those with health promoting activity were determined, with gallic, syringic, rosmarinic, and p-coumaric the most abundant acids; kaempferol, apigenin, and quercetin were the most abundant flavonoids. The results show that *A. filiculoides* is a valuable source of bioactive components and cellulosic materials.

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Keywords: Azolla filiculoides; Flavonoids; Phenolic acids; Cellulase; Biorefineries

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INTRODUCTION

Azolla is a genus of tiny floating aquatic ferns. They are 1 cm in diameter on average and have a water content greater than 90%. They develop in static non-saline bodies of water. A characteristic that has made this a successful genus of *Pteridophytes* is the permanent symbiosis between the plant and an atmospheric nitrogen-fixing cyanobacterium of the *Anabaena azollae* species.

Azolla has been used widely as a source of nitrogen fertilization in wetland rice fields throughout Asia, as well as weed control and cover to prevent evaporation (Lumpkin and Plucknett 1980; Kimani *et al.* 2018). Most research has been accomplished in tropical and subtropical systems, but efforts have been made for its mass cultivation in temperate climates (Bocchi and Malgioglio 2010). *Azolla* production must be carried out from vegetative material and can be performed in water bodies such as ditches, wetlands ponds, or canals (Wagner 1997). It can duplicate its population in short times when conditions are optimal, from 4 to 10 days depending on inoculum size, producing up to 26-41 t Ha^{-1} (Singh and Singh 1987). The growth rate of this fern is considerably higher in warmer climate conditions. However, this fern genus is not always welcomed and is considered as part of invasive species in several countries mainly in Europe, Africa, and Asia (Janes 1998;

Cilliers *et al.* 2003; Hashemloian and Azimi 2009; Witt and Luke 2017; Pinero-Rodríguez *et al.* 2021).

Azolla provides a promising source of non-lignified cellulose, which is relevant in pharmaceutical, cosmetic, and food industries as an excipient, rheology modificator or coating material (Shokri and Adibkia 2013; Nechita 2020), as well in tissue engineering as a scaffold material (Hickey and Pelling 2019).

Another important feature is the protein content (with values between 200 and 400 g/kg of biomass on a dry basis), which is not influenced by the growth phase or population density, as well as the protein quality, due to the presence of essential amino acids in adequate concentrations for the animal diet, according to the *Azolla* species (Sanginga and Hove 1989; Brouwer *et al.* 2018). This genus produces other metabolites of interest, *e.g.*, polyphenols, which show a certain chemical homogeneity between different *Azolla* species (Teixeira *et al.* 2001). Such polyphenols can present different activities, *e.g.*, antioxidant activity evaluated *in vitro*, beneficial biological activities as protectants against toxic compounds, antimicrobial activity against bacteria and fungi, and the potential to be applied as bioinsecticides, *etc.* (Pereira *et al.* 2015; Elrasoul *et al.* 2020; Ravi *et al.* 2020; Qian *et al.* 2020; Balasubramaniam *et al.* 2021).

The presence of carotenoid pigments in *Azolla* has allowed its implementation in poultry diets, or as a candidate for industrial extraction (Khatun *et al.* 1999; Lejeune *et al.* 2000; Alalade *et al.* 2007). Species of the genus *Azolla* have been considered and used as a source of nutrients or food supplements in animals intended for human consumption, with favorable results in some species (El-Sayed 1992; Abdelatty *et al.* 2020).

Problems derived from the use of plants for human or animal consumption is the presence of antinutritional factors, which can discourage their use if they are not processed properly (Soetan and Oyewole 2009). These factors in *Azolla* have been studied and have been found to take form in various types, *e.g.*, trypsin inhibitors, tannins, phytic acid, and cyanide, as well as condensed tannins that are associated with protein and cause their use as a supplement a difficult task (Fasakin 1999; Maity and Patra 2003; Brouwer *et al.* 2019).

The success of this genus of ferns lies in all the adaptations they have incorporated, which have allowed them to survive throughout their existence on Earth, but which have turned their industrialization into a major challenge. As the need of new raw material increases, vegetal protein sources may provide a suitable option when they are part of a biorefinery process (Dohaei *et al.* 2020). The aim of this work is to contribute to the use of *A. filiculoides* as a source of added-value compounds by determining the chemical proximate analysis, polyphenol content, and the influence of commercial cellulases in the polyphenol extraction.

EXPERIMENTAL

The aquatic fern *A. filiculoides* was previously collected from Ensenada, Baja California, México, and maintained at the Microbiology Department Greenhouse, Soil Science Graduate Program at "Colegio de Postgraduados, Montecillo" (19° 29′ N, 98° 53′ W and 2250 m). Nutrient solution was prepared as described by Yoshida *et al.* (1976) with no nitrogen added. A starting culture consisting in 5 g of plant was placed in a plastic container (42 cm x 32 cm x 10 cm) containing 6 L of nutrient solution at an average day temperature of 22 °C ± 0.20 °C, a humidity of 56% ± 1.1%, and a photosynthetic active radiation (PAR) of 488 $\mu mol \cdot m^{-2} \cdot s^{-1} \pm 3.6$ $\mu mol \cdot m^{-2} \cdot s^{-1}$, for 7 d.

The ferns were harvested and contained in a mesh cloth bag, dried with a kitchen salad spinner, and placed in adsorbent paper to eliminate excess water to determine the fresh weight. Then, the samples were dried at a temperature of 70 °C for 72 h, after which the dry weight was determined. Fresh *Azolla* biomass used in the chemical proximate analysis, fiber, enzyme reactions, phenol extraction, and determinations were dried at room temperature in a dark room for 72 h. The dry *Azolla* was processed in a blade coffee grinder (Fresh Grind, Hamilton Beach, Glen Allen, VA) and the obtained ground material was stored for further analysis.

Chemical proximate analysis

The parameters analyzed were the ash, total fat, and total protein, according to AOAC official analysis methods (AOAC 2005). Results were expressed as a percentage on a dry basis.

Neutral and alkaline fiber analysis

First, 0.5 g of dry ground *Azolla* were placed in filter bags (Ankom Technology, Macedon, NY) and processed according to Soest *et al.* (1991). The parameters obtained were the neutral detergent fiber (NDF) and acid detergent fiber (ADF).

Enzymatic reaction

The *Azolla* enzyme treatment was carried out with commercial cellulase (Celluzyme®, Enmex, Tlalnepantla de Baz, Mexico). The experiment consisted of 3 levels of enzyme solution (0.1% w/v, 0.25% w/v, and 0.5% w/v) as well as the control (0%). First, 3 g of sample were placed in a polyester-fabric bag, sealed with a nylon string tied to the top, and then submerged in a glass jar with 200 mL of enzyme dissolved in citrate buffer (0.05 M, pH 4.8). Jars were incubated at a temperature of 50 °C for 60 min. Sample bags were rinsed with distilled water and dried in a dark room at a temperature of 20 °C for 24 h.

Polyphenol extraction and quantification

Dry sample bags subjected to enzymatic treatment were submerged in 15 mL of 70% v/v absolute ethanol solution in a dark room for 1 hour at a temperature of 20 °C.

Polyphenol quantification

Total polyphenol quantification was carried out using the Folin-Ciocaltieu method with chlorogenic acid as the standard (Kováčik *et al.* 2007).

The total phenolics content of the fronds was evaluated by the Folin-Ciocalteu (0.25 N) reagent assay utilizing chlorogenic acid for the standard curve (Singleton and Rossi 1965; Kováčik *et al.* 2007). The frond extracts were centrifuged for 15 min at 15000 rpm. The reaction mixture procedure consisted of mixing 30 µL of the extract with 90 µL of Na_2CO_3 and 150 µL of Folin–Ciocalteau reagent in a 96-well microplate. After 30 min, the absorbance was measured at 725 nm using a KC-4 spectrophotometer (Biotek Synergy 2® Instruments, Inc., Winooski, VT). The results were expressed as µg of chlorogenic acid equivalents per g of dry weight tissue (µg chlorogenic acid g^{-1} DW).

The method used to quantify the phenolic acids and flavonoids *via* high-performance liquid chromatography (HPLC) is described as follows: the HPLC system (Agilent Technologies, Santa Clara, CA) consisted of a quaternary pump model 1100, an automatic injector model 1200, and a diode array detector (model 1100). The extracts were

analyzed *via* HPLC on a HypersilODS (125 mm × 4.0 mm) Agilent column eluted with a gradient of (A) H_2O adjusted to a pH of 2.5 with trifluoroacetic acid and (B) acetonitrile for 0 min to 10 min, in the following mixture ratios: A to B ratio of 85 to 15 for 20 min and A to B ratio of 65 to 35 for 25 min. The following parameters were used: a flow of 1 mL/min at a temperature of 30 °C; detection wavelengths at 254 nm, 280 nm, 330 nm, and 365 nm; an injection volume of 20 μL; and an analysis time of 25 min. Results were expressed as μg of polyphenol compound per μg of polyphenol g^{-1} dry weight (DW)

The standards used for the phenolic acids and flavonoids determination were high purity (Sigma-Aldrich, St. Louis, MO).

RESULTS AND DISCUSSION

The chemical proximate and fiber analysis results are presented in Table 1. As an aquatic fern, azolla presented an elevated moisture content (95.3%), which resulted in a disadvantage for storage and handling, and is the fundamental reason why it was chosen to work with in dry biomass form. Noteworthy, a limiting factor for its use as a bulk forage in livestock applications is the low yield when dry biomass is obtained. However, as a fast-growing aquatic plant, the water surface can be covered in relatively short amounts of time when grown in optimal conditions. In this experiment, 41.2 g of dry *Azolla* can be obtained in a squared meter surface after a week from when the seed culture was established, considering that the aim of this work is not yield increase. One of the most important added values in *Azolla* is the protein content, which was 20.7% according to the analysis carried out in this work and results reported by other authors (Brouwer *et al.* 2019). The ash content in *Azolla* was 21.1%, which is related to its capacity to absorb ions in nutrient solution. One feature of *Azolla* is the toxic ions absorption capacity, which may result an advantage in bioremediation or a detriment if grown for activities related to direct or indirect human consumption. This experiment was established in controlled conditions with analytical-grade reagents in nutrient solutions prepared with distilled water, in order to avoid toxic metal ion uptake in the *Azolla*. The major health concern is related to large scale production in non-technical processes in which translocation of undesired elements is not monitored and controlled, so this aspect must be considered in operating laws and regulations.

The fat content in *Azolla* was 3.2%, which is considered low compared to oilseeds. However, the lipid fraction in *Azolla* contains an important amount of fatty acids and carotenoids, which is relevant in poultry (Abdelatty *et al.* 2020).

Table 1. Chemical Proximate Analysis, Fiber Analysis, and Yield of *A. filiculoides* Dry Biomass

Parameter	Value	Units
Moisture	95.29 ± 0.05	%
Ash	21.13 ± 0.06	% dry basis
Total fat	3.21 ± 0.37	% dry basis
Total protein	20.66 ± 0.77	% dry basis
NDF	26.83 ± 0.86	% dry basis
ADF	19.25 ± 0.18	% dry basis
Hemicellulose	7.58 ± 0.73	% dry basis
Yield	41.17 ± 1.51	g dry weight m^{-2}

In fiber analysis, the cell wall components are quantified, rinsing water soluble polysaccharides and compounds. Neutral detergent fibers are related to cellulose, hemicellulose, and lignins, while ADF values are related to cellulose and lignins. The difference between the NDF and the ADF results in the hemicellulose content. The lignin content in the *Azolla* cells is not abundant; therefore, it can be assumed that the major components in the cell wall are cellulose (19.25% dry basis) and hemicellulose (7.58%) (Nierop *et al.* 2011; Tran *et al.* 2020). With this data, the basis of the enzymatic treatment is cellulases.

Enzyme-assisted polyphenol extraction is a common practice when vegetal material is processed, which presents advantages compared to thermal, ultrasonic, or acid/alkali hydrolysis treatments (Gligor *et al.* 2019). According to the specifications of the manufacturer, the commercial enzymes used in this experiment presented cellulase, as well as hemicellulase and β-glucanase secondary activity. Results from the polyphenol spectrophotometric determination are shown in Table 2, where polyphenols derived from ethanolic extraction presented no significative differences among treatments (p-value equals 0.1882).

However, when the determinations were carried out in enzyme solutions at the end of the reaction, polyphenols were found as having the highest enzyme amount. Such concentration increase can be correlated to enzyme activity, despite having no effect in the ethanolic extraction. A higher enzyme concentration will not result in cost-effectiveness in case process upscale is considered. This effect may be related to interactions between the polyphenols and soluble polysaccharides in *Azolla* mucilages, as well the 70% ethanol solution. The origin of the polysaccharides inherent to mucilage from fern-algal packets may be vegetal (related to pectin), endophyte, or bacterial (Forni *et al.* 1998). Pectinases are commercial enzymes commonly used in food processing, which hydrolyze polygalacturonic acid and rhamnogalacturonan. This may result in a suitable candidate for soluble polysaccharide hydrolysis. However, according to the polysaccharide composition in *Azolla filiculoides* reported by Forni *et al.* (1998), the galactose and rhamnose concentration is lower than 5.2%, which is a minor fraction compared to the major fraction represented by glucose and fucose.

In addition, alcohol utility as a solvent in soluble polysaccharide precipitation may affect interactions between the polyphenols and these biopolymers, whose presence induces solubility and structure changes in polysaccharides (Xu *et al.* 2014).

Table 2. Polyphenol Concentration in Enzyme Solution at the End of Reaction and in Ethanolic Extraction

Treatment	Enzyme Solution	EtOH Extraction
	μg eq. chlorogenic acid mL^{-1}	
0	0	441.44 ± 21.94 a
0.1	0	542.00 ± 69.64 a
0.25	33.67 ± 1.67	478.11 ± 69.46 a
0.5	224.22 ± 25.04	370.33 ± 39.45 a
Note: different letters show significative differences among groups (*p*-value equals 0.05).		

Results related to the chromatographic polyphenol determinations are shown in Table 3, considering the ethanolic extracts from the 0% enzyme treatment because no differences were observed in the polyphenol concentration between treatments during the

HPLC analysis. This analysis was focused on phenolic acids and glycosylated flavonoids (flavonols and flavones). Polyphenols are part of plant secondary metabolism, and they take part in several processes, primarily as internal signaling, signaling between plants and symbiotic microorganisms, or protection against biotic and abiotic stress (Cheynier *et al.* 2013). However, most polyphenols may have biological activity promoting human health. In general, polyphenols act as radical scavengers that form stable molecules when in the presence of free radicals (Cory *et al.* 2018).

Flavonoids, *e.g.*, isorhamnetin, are active ingredients in several plant species, with antibacterial and antiviral effects, effects against cardiovascular and cerebrovascular diseases, as well as immunity regulation effects (Gong *et al.* 2020). Kaempferol is recognized as a chemopreventive agent against cancer, apigenin and quercetin are being studied for preventing chronic diseases in humans, and myricetin may have several activities, *e.g.*, chemoprotective activity against central nervous system diseases (David *et al.* 2016; Dormán *et al.* 2016; Semwal *et al.* 2016; Salehi *et al.* 2019). Phenolic acids, *e.g.*, ferulic, gallic, p-coumaric, 3,5-dihydroxibenzoic, and p-hydroxibenzoic, are common in several plants and fruits, and present antioxidant activity (Jitan *et al.* 2018). Rosmarinic acid is found in ferns and presents antiviral, antibacterial, anti-inflammatory, and antioxidant activities (Petersen and Simmonds 2003).

Table 3. Phenolic Acids and Flavonoid High-performance Liquid Chromatography (HPLC) Quantification in *A. filiculoides* Ethanolic Extraction

Compound	Classification	μg g^{-1} DW
Ferulic acid	Phenolic acid	5.88 ± 0.17
Rosmarinic acid	Phenolic acid	24.69 ± 0.39
3,5-dihydroxibenzoic acid	Phenolic acid	16.37 ± 0.83
Gallic acid	Phenolic acid	110.44 ± 28.48
p-coumaric acid	Phenolic acid	24.52 ± 0.42
Syringic acid	Phenolic acid	124.35 ± 0.35
p-hydroxibenzoic acid	Phenolic acid	1.70 ± 0.24
Myricetin	Flavonol	13.89 ± 0.08
Kaempferol	Flavonol	33.68 ± 1.12
Isorhamnetin	Flavonol	9.62 ± 0.11
Apigenin	Flavone	27.48 ± 1.40
Quercetin	Flavonol	26.15 ± 0.21

CONCLUSIONS

1. The cellulose (19.2% dry basis) and hemicellulose (7.6% dry basis) content were determined. In addition, *Azolla* has no lignin as part of the cell wall structure.
2. The cellulase treatment showed no effect on ethanolic extraction, but polyphenols were found in the enzyme solution at the end of the reaction.
3. The phenolic acids and flavonoids contents of those with health promoting activity were determined, with gallic, syringic, rosmarinic, and p-coumaric the most abundant acids. Kaempferol, apigenin, and quercetin were the most abundant flavonoids.

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