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Article

Assessment of the biological activities of *Azolla pinnata* growing in the North-West of Algeria

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ABSTRACT

Many plants' phytochemical composition and pharmacological activities offer medicinal potential for scientific research. This work aims to assess the bioactivities of *Azolla pinnata* extracts by evaluating their antioxidant, anticancer, antibacterial, and antifungal properties. Ultrasonic pretreatment of the samples was carried out to increase the yield of extracts. Two methods were chosen for extraction (maceration and decoction), using five solvents of different polarities: water, methanol, water/methanol (20:80; v/v), water/acetone (10:90; v/v) and chloroform. The phytochemical contents were determined using chemical assays and HPLC analysis. The antioxidants, anticancer, and antimicrobial capacities of the different extracts were evaluated. The results revealed that the ultrasonic treatment enhanced the extraction yield; the highest rate was noted for the methanolic extract (27.3±1.18%), while the lowest values were reported for those obtained by chloroform (5.8±1.04%). The phytochemical screening has shown that extracts are rich in flavonoids, tannins, and phenolic compounds. The assessment of the bioactivities of extracts reveals that *A. pinnata* possesses a wide range of pharmacological properties, including antioxidant, anticancer, antibacterial, and antifungal. Despite the substantial correlation identified between bioactivity and bioactive contents in the extracts, the specific components accountable for each activity remain unknown.

Keywords: *Azolla pinnata*, bioactive compounds, Ultrasonic treatment, phytochemicals, bioactivities assessment.

INTRODUCTION

In recent years, the search for new processes based mainly on naturally extracted molecules has seen renewed interest in most scientific research. Therefore, it is interesting to study natural resources that aim to exploit new compounds as alternatives to chemicals[1].

The plant kingdom represents a diverse supplier of bioactive compounds utilized in the food industry, cosmetology, and pharmacy[2]. These biologically active compounds have a nutritional value, participating in physiological and cellular function by providing biological activities, such as antioxidants, anti-inflammatories, and anti-carcinogens, to defend against metabolic disorders[3]. They signify a range of molecules not necessary for cell metabolism but play a crucial role in interacting with the environment [4]. Plants produce active substances that have insecticidal, antibacterial, fungicidal, and even growth-regulating properties on plants and insects. Studies have reported that more than 2000 plant species with insecticidal activity have already been identified[5].

We have an aquatic fern called the Azolla among the natural springs available. It is an aquatic fern that floats on the surface of calm, temperate, or tropical waters in watercress fields, rice fields, ponds, and irrigation canals. It has cyanobacteria of the Anabaena genus, which have nitrogen-fixing properties, that is to say, the transformation of atmospheric molecular nitrogen into fixed nitrogen assimilable by plants[6]. It is a common bio-fertilizer in rice cultivation[7].

The development of resistance of microorganisms against antibiotics concerns medical specialists. The development of new therapeutic agents is essential to combat the phenomena of bacterial resistance [8]. Biological control is the most favored method as an alternative in research programs given its economic and agro-environmental interests, which allow the maintenance of a bioecological balance[9]. Natural substances that present a broad spectrum of action in pharmacology, such as bactericides, fungicides, acaricides, etc., can also be used as an alternative to insecticides[10].

The valorization of molecules obtained from Azolla has significant economic potential and is a tool for the emergence and development of the new concept of green chemistry. In this context, this research evaluates the antioxidant, anticancer, antibacterial, and antifungal effects of extracts from Azolla pinnata harvested from the northeastern region of Algeria. Their extraction was optimized using five organic solvents and two different extraction methods.

This initial investigation explores the biochemical interactions between the compounds obtained from Azolla and seven microbial species. This study focuses on a specific bio-effect system in a controlled laboratory setting to ensure precise and accurate observations.

It is important to note that other environmental factors or stressors may also influence the results observed in this study. Furthermore, our findings are based on in vitro assays, and there may be geographical variations in the bioactivities of Azolla that were not accounted for in this investigation.

MATERIAL AND METHODS

Material

The biological material of our Study is *Azolla pinnata*. These are small, floating aquatic ferns native to Southeast Asia and East Africa. It belongs to the Salviniaceae family. The Azolla samples were collected from Honaine–Wilaya of Tlemcen. Samples are collected manually from the culture basins (Figure 1). Samples should be transported in a cooler. Their storage must be done at 4°C and in the dark in the laboratory until preparation for analysis. All the chemicals used in the following analysis were analytical or HPLC grade.

Figure 1. Photograph of the *Azolla pinnata* sampling site (Honaine region, Wilaya of Tlemcen)

Sample preparation

Azolla samples were thoroughly washed with tap water, rinsed with distilled water, and dried in shade for seven days. Fine powder was obtained from the dried material using a kitchen mixer grinder. The plant powder was stored in desiccators to use it to get the extracts.

Ultrasonic pretreatment

This part of the study aims to assess the possibility of improving the yield of the extraction of bioactive compounds for possible industrial production. It consists of studying the impact of ultrasound treatment on improving extraction yield by comparing two identical samples of the same Azolla species with or without ultrasound treatment. Ultrasounds are mechanical waves at an intensity between 20 and 100 kHz. They are used to extract aromas and many other molecules from plants. Ultrasonic extraction has shown the capacity to maximize yields and give high-quality extracts with a complete compound structure. The most likely mechanism by which ultrasound operates is the intensification of mass transfer and the facilitation of solvent access to the interior of plant cells[11]. Compared to conventional extraction methods, ultrasonic extraction enhances extraction yield and efficiency, moderates extraction temperature, and increases solvent selection intervals[12]. After drying and grinding the Azolla samples, 30 g were put into glass vials and exposed to ultrasound for 3 to 4 min.

Bioactive compounds extraction

In our study, two methods were chosen to extract the bioactive compounds present in the samples (maceration and decoction), using five solvents of different polarities: water, methanol, methanol/water (80:20; v/v), acetone /water (90:10; v/v) and chloroform.

Extraction by maceration

One (1) g of Azolla powder is subjected to maceration in different mixtures of 60 ml of each solvent (water; methanol; methanol/water 80/20: v/v; acetone /water 90/10: v/v; chloroform), the whole being hermetically closed with a layer of aluminum foil, in the dark with magnetic stirring (25°C for 24 hours). The mixtures were filtered and then evaporated in an oven at 37°C except for distilled water, which was centrifuged. The extracts obtained were stored at 4°C until it is used.

Extraction by decoction

For the decoction, the same amount of sample and the same solvents were used at 60°C for 3 hours. The extracts obtained by filtration are evaporated to remove the solvent using moderate pressure in a rotary evaporator and then stored at 4°C until further used.

Extraction yield determination

The extraction yield is the ratio between the bioactive mass obtained and the Azolla samples' initial mass to be treated. The extraction yield is calculated from the following equation:

$$R\ (\%) = \frac{Me}{Mt} \times 100 \qquad (1)$$

Me: Mass of extracts in g; Mt: Total mass of dry matter in g.

Phytochemical screening

The chemical assays were carried out on the methanolic and distilled water extracts as described in literature 13 for the qualitative and quantitative determination of phytochemical contents.

Determination of total phenolic contents

The total phenolic content in both extracts was determined using the Folin-Ciocalteu reagent method, as mentioned by Singleton and Rossi[14]. Briefly, 200 µL of each Azolla extract was mixed perfectly with 1 mL of Folin-Ciocalteu reagent diluted 10 times. After 5 minutes, 0.8 mL of Na2CO3 solution (7.5%) was added to the mixture. Then, it was allowed to stand for 30 min at 25°C in obscurity. The absorbance was measured at 765 nm. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per g of azolla dry weight.

Determination of total tannins

As per the standard procedure outlined by Zhishen et al.[15], the total flavonoid content was evaluated using quercetin as a standard. Initially, 500 µL of the Azolla extract was combined with 1.5 mL of distilled water, followed by 150 µL of 5% $NaNO_2$ solution, and incubated at 25°C for 5 minutes. Subsequently, 150 µL of 10% AlCl3 was added to the mixture, and after an additional 5-minute incubation, 0.5 mL of 1M NaOH was introduced[16]. The absorbance was then measured at 510 nm, and the flavonoid content was determined from the standard curve, expressed as mg QRE/g dry weight.

HPLC analysis of phenolic compositions

The methanolic extracts were analyzed by injecting a 20µL filtered sample into an HPLC system with a UV-Vis detector set at 280 nm and a reversed-phase column (250 × 4.6 mm, 5 µm) at a 1 ml/min flow rate. The

mobile phase, comprising methanol/H2O acidified to 0.1% (Solvent A) and acetonitrile (Solvent B), was maintained at a temperature of 25°C. The elution followed a specific gradient: 90% solvent A and 10% solvent B for 10 min, then an increase to 30% solvent B after 5 min, followed by increments to 40% for 10 min, 50% for 15 min, and finally 100% solvent B for 10 min[17].

Assessment of bioactivities

Antioxidant activity assessment

DPPH assay

A modified DPPH assay estimated the free radical scavenging activity. Briefly, 250 µL of the sample was mixed with 3.75 mL of a 0.2 mM DPPH methanolic solution and incubated for 30 min in the obscurity. Then, the absorbance was detected at a 517 nm reference with a blank. 300 µg/mL of Trolox was dissolved in ethanol and used as a standard reference. The scavenging activity on the DPPH radical was evaluated as a percentage of inhibition using the following equation[18] :

$$\text{DPPH scavenging-radical (\%)} = [1- ((As - Ab) / Ac)] \times 100 \quad (2)$$

Where:
As is the absorbance of the mixture of the sample and the DPPH solution,
Ab is the absorbance of the blank that contains only the sample,
Ac is the absorbance of the control (DPPH solution).
The IC_{50} value is the extract concentration, which ensures the 50% reduction in DPPH solution, determined graphically by linear regression, for each extract from the curve of the percentage reduction as a function of concentration. The stability and linearity of the DPPH solution should be evaluated before starting the antioxidant test, and the result is presented graphically. Five concentrations of DPPH solutions were prepared (20, 40, 60, 80, 100 µg/mL), then the absorbance of the samples was measured after 30, 60, and 120 min at 517 nm in the dark, with methanol blank by the UV-Visible spectrophotometer[19].

FRAP assay

According to the method of Oyaizu[20], the reducing power of Azolla extracts was measured. One mL of extracts at different dilutions was added to 2.5 mL of phosphate buffer (0.2 M; pH 6.6).) and 2.5 ml of $K3Fe(CN)_6$ (1% w/v); after incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (10% m/v) were added. After centrifugation for 10 min at 2000 rpm, 2.5 mL of supernatant was added to the tubes containing 2.5 mL of distilled water and 0.5 mL of $FeCl_3,6H_2O$ (1% w/v). The absorbance of the obtained solution was read at 700 nm using a water blank. Ascorbic acid (vitamin C) was used as a standard due to its good reducing properties. The heightened absorbance of the reaction mixture signifies an increase in reducing power.

Anticancer activity assessment

To assess the anticancer effect of Azolla extracts, HeLa cell viability assays were used according to the method reported by Niyonizigiye et al.[21]. For the cell viability evaluation, the experiment was conducted by preparing different concentrations of methanolic and aqueous extracts (200, 400, 600, 800, 1000 µg/mL). Then, the IC_{50} was determined.

Evaluation of the antibacterial activity

The determination of the antibacterial activity of the extracts was conducted by using the disc diffusion method[22]. It was estimated in terms of the diameter of the inhibition zone around the discs containing the different concentrations of extracts obtained by the different solvents against Gram (+) bacteria: Staphylococcus aureus, and Gram (-): Escherichia coli, Pseudomonas aeruginosa. Briefly, sterile discs, 6 mm in diameter, impregnated with 20 µL of Azolla extracts obtained by maceration, were placed in Petri dishes on Mueller-Hinton agar, which had been surface spread with 1 mL of logarithmic phase bacteria adjusted to a 108 UFC/mL fixed by the optical density (0.080.1 at 620 nm)[23]. The Petri dishes were then incubated for 18 h at 37°C. The diameter of the inhibition zone was measured. The results of the extracts were compared with those of the negative control (DMSO 5%) and the positive control (antibiotics).

Evaluation of the antifungal activity

Antifungal activity of each Azolla extract was evaluated by the agar well diffusion assay as reported by Quiroga et al.[24]. Briefly, the inocula of filamentous fungi (*A. niger, A. flavus., F. oxysporum* and *F. redolens*) were prepared on potato dextrose agar (PDA) in a 250-ml Erlenmeyer flask at 30°C during 8 days in alternate cycles of 12 h light and dark. Spore suspensions were raised by pouring 5 ml of distilled water into the flask, then vortexing for 1 min, and sieving through 8 layers of cheesecloth. A final inoculum density of 106 spore/ml was calibrated using a hemocytometer.

Aliquots of 50 µl inoculum of each species were mixed with 20 ml melted PDA media cooled at 45°C. The media-spore suspension was smoothly mixed and poured aseptically into Petri dishes. After being left at room temperature for 1 hour, a small 5 mm well was created in the center of each solidified medium using a sterilized cork borer. Using a micropipette, 100 µl of each Azolla extract obtained by maceration was slowly loaded in each well. To allocate a homogeneous diffusion of the extract into the agar, the dishes were then pre-incubated for 2 h at 4°C. The Petri dishes were then incubated under aerobic conditions at 30°C for 72 hours.

Statistical analysis

Analyses of all samples were carried out in triplicate and randomized order, and means were reported. Data were evaluated by Duncan's multiple range test using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) to evaluate differences in mean values.

RESULTS

Extraction yield

The extraction results by both methods and in the different solvents used (water, methanol, water/methanol (20:80; v/v), water/acetone (10:90; v/v), and chloroform) are presented in Table 1. The highest extraction yield was noted for the methanolic extract by decoction (27.3% (g/g); however, the lowest values were reported for the chloroform extracts by maceration of around 6.5% (g/g).

Solvent	Extraction yield (%)			
	Maceration		Decoction	
	Ts	NTs	Ts	NTs
Methanol	24.8 ± 1.02	23.8 ± 0.76	27.3 ± 1.18	26.5 ± 1.04
Methanol (80%)	13.07 ± 0.84	11.4 ± 0.88	14.7 ± 1.06	12.03 ± 0.63
Acetone (90%)	9.5 ± 1.04	8.7 ± 0.91	10.6 ± 0.73	8.4 ± 0.48
Chloroforme	6.5 ± 0.72	5.8 ± 1.04	7.6 ± 1.08	7.02 ± 0.92
Water	14.3 ± 0.58	13.6 ± 0.98	12.5 ± 1.06	12.03 ± 0.37

Ts: Ultrasonic treated sample; NTs: Non-treated sample.Values are presented as mean ± SD (n = 3)

Table 1. Extract yield was obtained using different methods and solvents.

Phytochemical screening

The results of the present study suggest that the *A. pinnata* samples are rich in flavonoids, tannins, and phenolic compounds, as shown in Table 2.

Content (mg/g)	Methanolic extract	Aqueous extract	Ethanolic extract of *A. microphilla*[25].
Total phenols	93.4 ± 1.82	55.8 ± 1.23	90.2 ± 2.85
Total tannins	76.3 ± 4.17	32.2 ± 4.17	82.2 ± 5.25
Total flavonoids	68.41 ± 7.12	38.5 ± 6.37	58.5 ± 1.87

Values are presented as mean ± SD (n = 3)

Table 2. Quantification of bioactive compounds from A. pinnata extracts used

HPLC analysis of phenolic compositions

The concentrations of phenolic compounds identified in methanolic extracts are detailed in Table 3. Syringic acid, Gallic acid, Rosmarinic acid, and chlorogenic acid were identified as phenolic compounds. The results were found to align with those documented by Carballo-Sanchez et al.[26]. Phenolic compounds are known for their antioxidant effect, anticancer[27], anti-inflammatory and anti-diabetic properties[28]. Polyphenols generally function as scavengers of radicals, creating stable molecules when exposed to free radicals[29].

Compounds	µg/g DW
5-Hydroxymethylfurfural (C6H6O3)	18.71 ± 1.28
Chlorogenic acid (C16H18O9)	23.14 ± 0.62
Gallic acid (C7H6O5)	110.44 ± 1.23
Ferulic acid (C10H10O4)	5.8 ± 1.17
9-Octadecenoic acid (C18H34O2)	15.04 ± 0.41
Rosmarinic acid (C18H16O8)	24.69 ± 1.35
Syringic acid (C9H10O5)	124.35 ± 0.23
3,5-dihydroxibenzoic acid (C7H6O4)	18.27 ± 0.43

DW: Dry weight; values are presented as mean ± SD (n = 3)

Table 3. The Phenolic Acids HPLC quantification in the methanolic extract

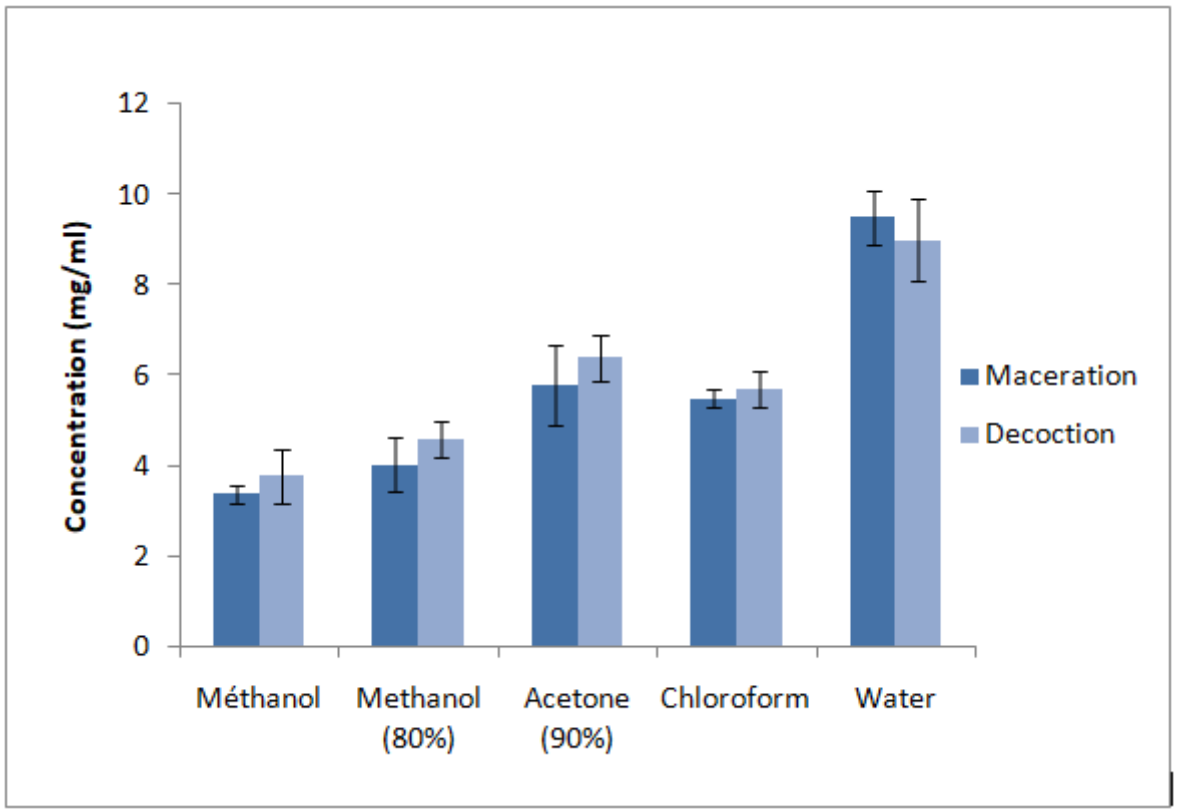
Assessment of bioactivities

Antioxidant activity assessment

In this study, the antioxidant capacity of extracts was assessed using the DPPH and FRAPS tests to accurately gauge the compounds' ability to respond to oxidative stress.

DPPH assay

The results have proved that all the tested Azolla extracts can trap the DPPH radical and show that the percentage of inhibition significantly increases with higher concentrations of the extract. The concentration necessary to inhibit 50% of free radical from each extract (IC_{50}) was determined, and its value was compared to that of the reference antioxidant (Trolox), which presents potent anti-radical activity, with an IC_{50} of 0.08 mg/ml (Figure 2).



Mean ± standard deviation of three experimental measurements

Figure 2. Inhibition percentage (IC_{50}) in different extracts by maceration and decoction method.

Correlation between bioactive compounds and anti-radical power

To verify the relationship between the anti-radical power of microalgae extracts and their content of polyphenols, flavonoids, and tannins, the correlation factor (r) between the level of these biomolecules and the appropriate anti-radical power values was determined in Table 4.

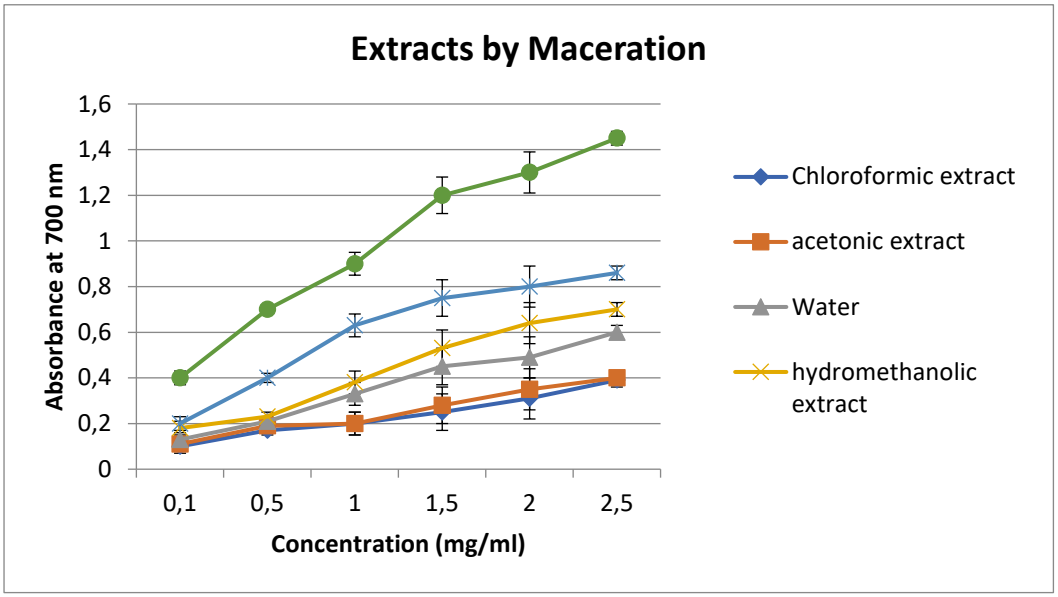
Compound	DPPH	
	Methanolic extracts	Aqueous extracts
Polyphenols	0.741	0.512
Flavonoids	0.341	0.362
Tannins	0.426	0.469

Table 4. Correlation between bioactive compounds and anti-radical power by the DPPH test

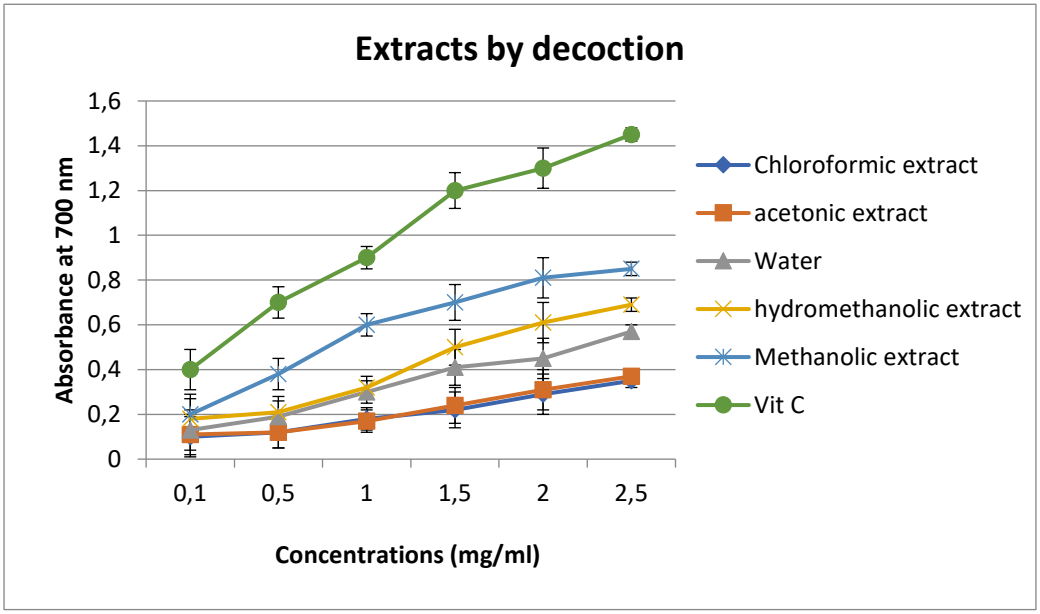
The results obtained show a significant correlation for both of extracts. A notable correlation of 0.741 with the anti-radical activity and in methanolic extracts was found for the polyphenols. An average correlation of 0.426 and 0.469 was observed between the bioactivity and the concentration of tannins in methanolic and aqueous extracts, respectively. Regarding flavonoids, less correlation was found compared with both compounds.

FRAP assay

The abilities of the extracts obtained through the two different methods and in the five solvents, as well as the ascorbic acid determined by the FRAP method, are expressed in terms of optical density corresponding to various concentrations (Figure 3). Our results indicate that the enhancement in iron reduction aligns with the concentrations of all extracts.



a)



b)

a) Maceration; b) Decoction (Values are presented as mean ± SD)

Figure 3. Antioxidant effect by FRAP assay of *A. pinnata*

Anticancer activity assessment

In our study, the anticancer activity of two extracts was assessed against HeLa cell lines; the methanolic extracts showed the highest antioxidant activity, and the aqueous extracts with the lowest activity were selected for anticancer assessment. The results are presented in Figure 4. The anticancer activity of the aqueous

extracts was insignificant (IC50 >1000 µg/mL) compared to the methanolic extracts, which exhibited the highest HeLa cell inhibition with an IC_{50} value of 820.47 µg/mL.

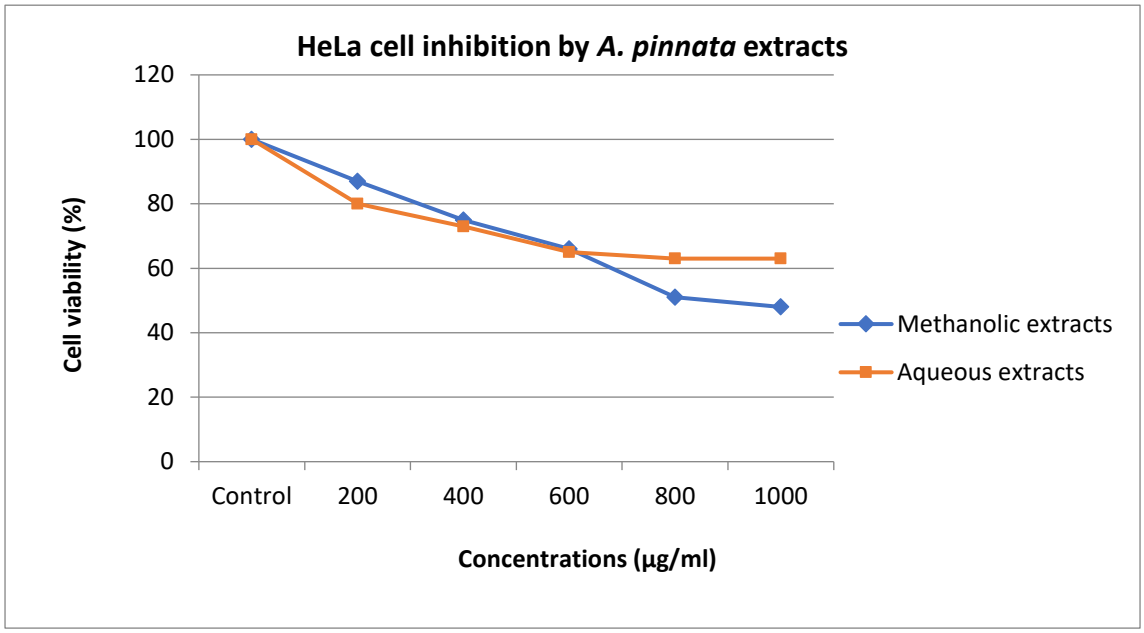


Figure 4. HeLa cell inhibition by methanolic and aqueous *A. pinnata* extracts.

Antibacterial activity assessment

The antimicrobial activity of the solvent extracts obtained through maceration was assessed using the disc diffusion method against Gram (+) and Gram (-) bacteria. The corresponding results are presented in Table 5.

It has been observed that all the samples exhibited antibacterial activity against *S. aureus*, but none of them were effective against *P. aeruginosa* except the methanolic and acetonic extracts. The most exciting effect against *S. aureus* was methanolic extract (12.1 ± 0.8 mm). At the same time, hydromethanolic extract formed the largest average zone of inhibition (3.4 ± 0.7 mm) against *E. coli*.

Extract	Strain	Diameter (mm)
Aqueous	*S. aureus*	3.5 ± 0.6
	E. coli	2.3 ± 0.48
	P. aeruginosa	NA
Methanolic	*S. aureus*	12.1 ± 0.8
	E. coli	2.7 ± 1.02
	P. aeruginosa	1.4 ± 1.1
Hydromethanolic	*S. aureus*	8.8 ± 1.4
	E. coli	3.4 ± 0.7

	P. aeruginosa	NA
Acetonic	*S. aureus*	1.8 ± 0.5
	E. coli	NA
	P. aeruginosa	1.4 ± 0.8
Chloroformic	*S. aureus*	2.3 ± 0.6
	E. coli	NA
	P. aeruginosa	NA
Positive control	*S. aureus*	14.7 ± 0.41
	E. coli	18.00 ± 1.5
	P. aeruginosa	13.24 ± 1.8
Negative control	*All strains*	NA

Positive control: Streptomycin; Negative control: DMSO; NA: not available ; Values are mean ± SD of 3 replications

Table 5. Inhibition zone diameter of A. pinnata extracts obtained by maceration.

Antifungal activity assessment

The antifungal capacity of Azolla extracts was assessed on four filamentous fungi by the agar well diffusion assay. The results regarding the diameter of inhibition zones ensuing by *A. pinnata* extracts effect are summarized in Table 6.

	Inhibition zone diameter (mm)			
Extract	***A. niger***	***A. flavus***	***F. oxysporum***	***F. redolens***
Aqueous	2.1 ± 0.8	3.5 ± 0.6	NA	12.1 ± 0.8
Methanolic	7.5 ± 1.4	6.4 ± 1.34	9.8 ± 1.21	14.7 ± 0.41
Hydromethanolic	8.2 ± 1.7	2.8 ± 1.9	4.2 ± 1.8	10.7 ± 0.41
Acetonic	NA	1.8 ± 0.5	NA	1.7 ± 1.8
Chloroformic	NA	NA	1.6± 1.8	2.3 ± 1.4
Positive control	18.5 ± 0.32	20.1 ± 0.71	18.5 ± 0.32	23.1 ± 0.43
Negative control	NA	NA	NA	NA

Positive control: Econasole; Negative control: DMSO; NA: not available; Values are mean ± SD of 3 replications

Table 6. In vitro antifungal activity of A. pinnata extracts obtained by maceration

Results have shown that all the extracts tested showed an inhibitory activity against the growth of myceliums used for this assessment. Methanolic extracts were the most potent against all fungal strains; the largest average zone of inhibition (14.7 ± 0.41) was against *F. redolens*. However, aqueous extracts were found to have reduced effect against the assayed fungal strains except for *F. redolens,* which was very sensitive with (12.1 ± 0.8 mm). In addition, the hydromethanolic extract was more effective against *A. niger* and *F. redolens* with (8.2 ± 1.7 mm) and (10.7 ± 0.41 mm) respectively. However, the activity of the acetonic extracts was not significant, mainly against *A. niger*; showing a good resistance against the materials diffused from the extracts. The chloroformic extract provided a low activity against the other fungal strains, varied from (1.6± 1.8 mm) to (2.3 ± 1.4 mm).

DISCUSSION

The findings of this study indicated that using ultrasonic treatment increased the extraction yield of bioactive compounds for both methods and the five solvents used. The highest yield was observed for the methanolic extract, while the lowest values were found for those obtained by chloroform. These results agree with those found with microalgal extracts by Kherraf,[30,] and Djamai,[19]. It should be noted that the solvents were chosen based on previous research indicating an increase in extraction yield significantly with the use of aqueous ethanol or aqueous methanol compared to extractions with pure organic solvents[30,31]. Chaouche[32] explained this behavior by the fact that the presence of water destabilizes the cell walls, promoting deep penetration into the cellular matrix, and consequently, the solvent will be in contact with a greater quantity of solute, thus favoring a good extraction yield. Thus, the amount of extracted material is influenced by various factors, including the type of solvent, pH level, temperature, extraction duration, and composition of the sample[33].
Also, ultrasonic treatment has enhanced the extraction efficiency of bioactive compounds from Azolla powder, regardless of the method or solvent employed. Ultrasonic waves create cavitation bubbles in the solvent, forming microjets and shock waves upon their collapse. These intense physical forces disrupt the cell walls of Azolla, facilitating the release of bioactive compounds into the solvent[34].

Depending on their chemical composition, aquatic plants are significant for the environment and the economy. Humans consume some, while others possess medicinal properties[35]. The pharmacological potential of these plants is determined by their unique composition of secondary metabolites, which varies across different species[36]. The results of phytochemical screening have confirmed that *A. pinnata* is rich in flavonoids, tannins, and phenolic compounds. Previous studies[25] reported that the Azolla species are rich in nutrients, such as proteins, sugars, polyphenols, flavonoids, and alkaloids. The results obtained in those studies are in complete agreement with our findings[36].

Regarding the bioactivities assessment, the findings show that the extracts have remarkable antioxidant power, significant anticancer activity, and potential antimicrobial effect.
The importance and applicability of natural antioxidants, which often have multiple functions, primarily rely on the specific test used[37]. Thus, the antioxidant capacity of the different extracts was carried out simultaneously by the DPPH and FRAPS tests to properly evaluate the ability of the compounds to react to oxidative stress. The highest anti-radical power was obtained by the maceration method in the methanolic extract (IC_{50} = 3.4 ± 0.2 mg ES mL-1), while the aqueous extract obtained by the same method of extraction

(maceration) presents the lowest activity (IC_{50}= 9.5 ± 0.6 mg ES mL-1). Potent inhibition was also observed in the hydromethanolic extract of the two extraction methods with an IC_{50} value of 4.05 ±0.9 and 4.6 ±0.4 mg ES mL-1, maceration, and decoction, respectively. As for the other extracts, they seem to have moderate to weak trapping powers, taking into account the IC50s they obtained. Furthermore, a non-significant difference between the two extraction methods was observed, showing that the high temperature for extraction did not have a considerable effect on the extraction of compounds with radical scavenging properties. Djamai,19 with microalgal extracts, reported the same findings.

On the other hand, the findings of FRAP method suggest that the increase in iron reduction corresponds with the concentrations of all extracts. This result is consistent with Safafar et al. [38], who also noted that the rise in iron reduction is directly proportional to the concentrations of *Nannochloropsis salina* extract.
The decrease in reducing power can be primarily ascribed to bioactive compounds linked to antioxidant activity, including total phenolics, flavonoids, and other hydrophilic and hydrophobic antioxidants. These compounds serve as influential electron donors and can halt the free radical reaction chain and convert them into more stable products[39]. This can explain the reducing potential of the azolla extracts tested.
Furthermore, significant correlations were found between the two extracts in the obtained results. A notable correlation was observed between polyphenols and anti-radical activity in methanolic extracts. An average correlation was found between bioactivity and tannin concentration in methanolic and aqueous extracts. However, less correlation was found for flavonoids compared to both compounds.

Recently, several bioactive compounds obtained from marine sources were identified as anticancer agents and advanced to preclinical and clinical trials[40]. As far as we know, no research has shown the anticancer effects of azolla extracts. Willcox et al. [41] have suggested in the previous study that the antioxidant ability of natural resources can be evidence for their anticancer potential. Our results revealed that the aqueous extracts did not show significant anticancer activity compared to the methanolic extracts, which had the highest HeLa cell inhibition.

All samples showed an inhibition effect against S. aureus for the antibacterial activity assessment, but only the methanolic and acetonic extracts were effective against *P. aeruginosa*. Researchers have observed that the organic extracts obtained from some species of Azolla hindered the growth of *B. subtilis*; the work has been done on *A. rubra, A. caroliniana and A. filiculoides* [42]. It is understood that Gram-negative bacteria are significantly more resilient to antimicrobial substances than Gram-positive bacteria[43]. Also, previous studies indicated that the active fractions gained from *A. pinnata* extracts include bioactive compounds with antibacterial properties[44].

About the antifungal assessment, the results indicate that all tested extracts demonstrated an inhibitory action against the growth of the mycelium used in this evaluation. Rahman et al. [45] have reported that all tested fungi species used in their study were found to be resistant to methanol extracts from *A. caroliniana* and *A. filiculoides*, except for *Geotrichum candidum*, which exhibited sensitivity to the extracts, showing a greater susceptibility to the *A. caroliniana* extract, resulting in a larger zone of inhibition compared to the *A. filiculoides* extract.

The growth of several fungi and yeasts has been shown to be inhibited by tannins. Their chemical structure gives them a highly developed capacity to attach to molecules such as alkaloids, gelatin, polysaccharides, and

proteins[19]. Likewise, polyphenols are endowed with significant and diverse antimicrobial activities, probably due to their structural diversities. Cowan[46] has reported that the number of hydroxyl groups on phenolic compounds is believed to be associated with their relative toxicity towards microorganisms. The determination of the bioactive compounds inducing such effects from the extracts is necessary to establish whether the effects were due to a single compound or the interaction of several. Therefore, isolating the available compounds in these ferns' organic and aqueous extract is necessary to precisely identify the responsible molecule or in case of possible synergistic interactions between several bioactive substances in the extract. Determination of the specific non-toxic dose should be carried out to evaluate the antifungal effectiveness of the extracts[47].

While our findings provide valuable insights into the bioactivities of *Azolla pinnata*, it is essential to note that these results may not be directly applicable to other bio-systems or in vivo settings. Further research in diverse environmental contexts and with different species is necessary to understand these effects' broader implications fully.

CONCLUSIONS

The results obtained at the end of this work confirm the interest in studying the different possibilities of valorizing this aquatic fern and using it as a source of products of interest in various economic sectors. Despite the significant correlation estimated between the bioactivity and the bioactive contents in extracts, the components responsible for each activity are currently unknown. Additional studies are necessary to isolate these compounds to test them in vivo on different biological models, aiming to seek possible applications in the fields of health and agri-food. Also, the knowledge of these extracts' manner and inhibition processes could lead to the creation and effective manufacturing of new chemical entities with similar inhibitory effects from large compound libraries, developing novel metabolites with therapeutic potential.

The findings suggest several future directions, such as the performance of in vivo studies using animal models or clinical trials to validate the therapeutic potential of Azolla extracts. This is followed by identifying and isolating the specific active compounds responsible for the observed bioactivities to investigate the underlying mechanisms of their actions.

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