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Antibacterial Bioactivity of Large Mosquito Fern *Azolla filiculoides* Lam., 1783 (Pteridophyta: Salviniaceae) Against some Pathogenic Bacteria Isolated from common carp *in vitro*

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Abstract

Water extract of large mosquito fern *Azolla filiculoides* used as antibacterial material (as eco-friendly material) against some pathogenic bacteria for common carp *Cyprinus carpio*. The bacteria represent with *Bacillus cereus*, *Escherichia coli* and *Staphyllococcus aureus*. The results shown that the application of 100% of the stock solution gave better inhibition against the bacteria growth *in vitro* (25.7, 26.35 and 28.7mm in *B. cereus*, *E. coli* and *S. aureus*, respectively in comparison with other concentrations of water extract of *A. filiculoides* (25, 50, and 75%). The current encourage results as an inhibition mater against studied bacteria may be applied as alternative eco-friendly material instead of chemical materials to control present and other bacteria pathogenic to fishes.

Keywords: *Azolla filiculoides*, Bacteria, Fish, Inhibition growth.

Introduction

Since ancient times, various medical plants are known for their effect in the treatment of many diseases. The scientific development has led to the knowledge of many of them that have anti-microbial activity. They possess a great inhibitory ability against bacterial species due to they behave like antibiotics in their ability to disrupt or stop some metabolic pathways in the bacterial cells (Al-Rajab, 2007).

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Bacterial diseases caused by some bacterial agents, e.g. *Vibrio cholera*, *Escherichia coli*, *Bacilus cereus* and *Staphylococcus aureus* which resistance toward antibiotics and this resistance is due to excessive doses or short term treatment, as well as incorrect use of them. Therefore, many researchers have developed in medicinal plants that possess the properties of resistance against microbial infection because it is considered safe and effective drug (Bantawa *et al*., 2019), since they contain biologically active compounds that act as anti-infectives and phytochemicals such as flavonoids, tannins and saponins. One example of those plant is the fern *Azolla*, which belong to the family Salviniaceae with six to seven species. A small green flora floats on the surface of the water, tinged with red colour in the winter, growing in slow-running or stagnant water from ponds, benches, waterways, irrigation channels and palm groves (Mahmood *et al*., 2020).

Azolla filiculoides, is locally reported from northern Basrah, Babylon and Sulaymani, Kurdistan, Iraq (Mahmood *et al*., 2020; Alkhafaji *et al*., 2022) and Al-Dalmaj protected area mid-Eastern Iraq (Salim *et al*., 2021). Large mosquito fern *A. filiculoides,* is invasive alien species and present in the Shatt Al-Arab River (Sabbar, 2019). *A. filiculoides* is native to North, Central and South America and spreads in Asia, Australia and Europe (Weber, 2003; Hussner, 2006) and Iran (Hashemloian and Azimi, 2009; Farahpour-Haghani *et al*., 2016).

Azola was initially limited as green manure, but it was used as a drug too, for reclaiming saline soils and as bioremediation. Later it used different levels of *Azolla pinnata* on blood biochemicals, haematology and immunocompetence traits of animals (Mishra *et al*., 2016). It contains methyl alanine; it may treat neurodegenerative disorders (Sjodin, 2012). It has therapeutic, anti-inflammatory, antioxidant and anti-cancer properties (Kumar and Chander, 2017).

Azola is a high biomass and protein aquatic plant that can be used as a direct feed for fish or as a food ingredient as an alternative source of protein. It also contains the highest percentage of crude protein and a rich composition of amino acids (e.g. lysine) compared to other dietary green crops and large aquatic plants; hence Azola has gained importance in aquaculture (Mosha, 2018). Azola has long been used as green manure and a diet for poultry and fishes. It improves fish growth performance, hematopoietic immune system, gastrointestinal enzymes, and disease resistance (Alalade and Iyayi, 2006; Liu *et al*., 2008). Azola has been documented to be a good source of proteins,

minerals, and vitamins. Perfectly adapted to tropical climates and livestock feeding, e.g. *Azolla nolotica* has high amounts of carbohydrates, protein, raw fats, fully digestible nutrients and lower crude fibre content. Due to its high levels of protein, carbohydrates and crude fat, Azola protein concentrate (ACP) can be used as a human food supplement (Mohamed *et al.*, 2018).

The purpose of present study to use the large mosquito fern as substitutive material safely against bacterial diseases of fishes in concentration water extract 25, 50, 75 and 100%.

Materials and Methods

Propagation of Azola

Azola fern was cultured under outdoor weather conditions and exposed to direct sunlight for the period from 1st January to mid-February 2020, six plastic containers (water containers, 23cm in diameter), with a water level of 9 cm were used with organic manure (cow dung). The fern was caught on the water surface and dried away from the sun after washing with clean tap water. The fern are dried in a well-aerated place at room temperature with continuous moving to avoid their decomposition. It is found that the biomass reached twice times after seven days; hence we easily obtained more than 50 gm of live mass (fresh weight) of dry weight of 0.1 gm.

Extraction of Azola

The fern was sterilized by adding 250 ml of distilled water to 25 g of the dried powder of the ground fern in a 500 ml flask. The mixture was placed in a mixer at room temperature for three hours and then centrifuged for 15 minutes, and the aqueous suspension was completed with distilled water up to 250 ml of four concentrations of each extract 25%, 50%, 75% and 100% of the stock solution (Twaij *et al.*, 1983).

Fish samples

Live fries of common carp *Cyprinus carpio* (10.5-12.4g and 8-11 cm) were randomly collected from farm of University of Basrah.

Bacterial isolation

Bacteria isolation was established by tacking a smear sterilized swab from ulcers of fish's skin (Fig. 1) and placed in sterile Nutrient agar incubated at 37°C for 24 hrs. One colony growth was moved to new media at the same temperature and time as above. By virtue of their

morphology and isolation, bacteria were divided into gram-positive and gram-negative groups. Pure colonies cultured on NA at 37C° for 24 h were then picked for bacterial identification using VITEK II system (Biomerieux- USA) (Benson, 2002).

Test the effectiveness of the fern extract

The Agar well diffusion method is used to test the biological activity of the fern extract against bacterial growth. The areas of inhibition of bacterial growth are measured near 1 mm by using a digital calliper, as the surface of Mueller-Hinton agar (MHA) (Himedia- India) media was inoculated by a cotton swab.

Sterilized in Petri dishes with a diameter of 90 mm, a hole with a diameter of 6 mm was made in the middle of the planting surface by using a sterile cork penetrating and 200 µl of fern extract was placed in the hole, the dishes were incubated at a temperature of 37 °C for 24 hours. The results of the inhibition areas (in mm) were recorded, and the control pit contained only distilled water (Magaldi *et al.*, 2004).

Results and Discussion

The following bacteria are isolated from the skin of the fish and identified: *Bacillus cereus* (9.2%), *Escherichia coli* (13.1%) and *Staphylococcus aureus* (7.2%).

The results in table (1) displayed the inhibition effect of Azola with four concentrations (25, 50, 75 and 100%) on three species of pathogenic bacteria.

Table 1: Inhibition diameter (mm) in three species of pathogenic bacteria growth as a result of using four concentrations of water extract (25, 50, 75 and 100) % of Azola.

Bacteria species	Concentration %	Inhibition diameter Mean (mm)± Sd. Deviation
Bacillus cereus	25	18.57 ±1.289 c
	50	21.40±0.70 b
	75	24.20 ±0.55a
	100	25.70 ±0.55a
Escherichia coli	25	20.50 ±0.61b
	50	22.53±0.93ab
	75	20.97±4.22b
	100	26.53±2.45a
Staphyllococcus aureus	25	23.43±2.84b
	50	23.97±2.26b
	75	25.47±1.95ab
	100	28.70±1.39a

Figure 1: Carp fish infected with skin ulcers.

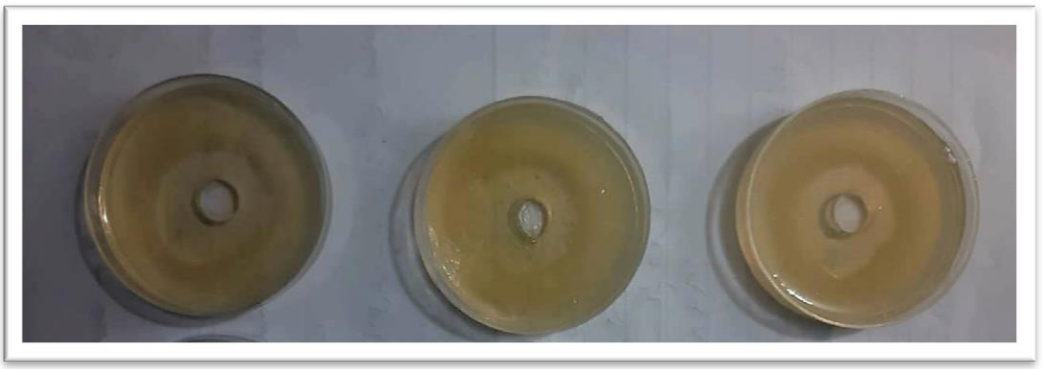

Figure 2: Bacterial inhibition zones

All four concentrations of azola fern extract used in the study showed effectiveness successfully in inhibiting the growth of the three types of pathogenic bacteria (Fig. 2). However, the best inhibition is 100% in all bacteria species (25.7, 26.35 and 28.7mm (Table 1)). The relative increase suggests that the extract of some types of azola possesses antimicrobial activities and phytochemical compounds such as flavonoids, tannins and saponins. Angalao *et al.* (2012) found the ethanolic extract of azola successfully inhibits the fungi *Fusarium oxysporum* and *Tricophyton mentagrophytes*) and actively against the bacteria *E. coli* and *S. aureus*. Gerard (2013) explained the methanolic extract of *Azolla microphylla* displayed inhibitory activity against several strains of *Xanthomonas*. The *Azolla* species contained bioactive agents such as alkaloids, saponins, tannins flavonoids, steroids, and cardiac glycosides, and these agents could be responsible for antimicrobial activity against bacterial species such as *Bacillus*

spp., *Proteus* spp., *Staphylococcus* spp., *Klebsiella* spp., and *E. coli* (Sathammaipriya *et al*., 2018).

The tannins as an antimicrobial agent by inhibiting extracellular enzymes, deprecating the substrates required for microbial growth or by inhibiting oxidative phosphorylation of microbial metabolism (Vannini *et al* ., 2018). Hili *et al*. (1997) and Al-Jannae'e *et al*. (2017) explained that the active components in the highest concentration (100%) of some aquatic plants extract lead to shrinkage of microorganisms (Bacteria and fungi), while at a lower concentrations (25-75%), the dispersion of the active substances leads to their separation from the cells, which leads to a decrease in the effectiveness of the inhibiting substances. Other studies (e.g. Cowan, 1999; Draughon, 2004; Benkeblia, 2005; Unver *e al*., 2009), added other reasons for the activeness of some aromatic and aquatic plants by inhibiting of microbial enzymes as well as rupture of cell membranes, association with lipophilic compounds. Weiss and Fintelmann (2000) demonstrated that the death of microbial cells might be due to decrease oxygen uptake and inhibit the production of proteins, fats and nucleic acids and consequently affect the production of energy, along with the oxidation of proteins and amino acids in the cellular membrane.

Conclusion

It can conclude this plant has inhibitory activity against fish diseases to be alternative material, eco-friendly instead of chemical material.

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Ethics approval

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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النشاط الحيوي المضاد للسرخس ازولا *Azolla filiculoides* Lam., 1783 ضد بعض انواع البكتيريا المرضية للأسماك المعزولة خارج جسم الكائن الحي

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المستخلص

استخدم المستخلص المائي من سرخس البعوض الكبير *Azolla filiculoides* كمادة مثبطة لنمو البكتيريا (كمادة صديقة للبيئة) ضد بعض البكتيريا المسببة للأمراض في اسماك الكارب الشائع *Cyprinus carpio*. تمثلت بالبكتيريا العصوية *Bacillus cereus* والإشريكية القولونية*Eschereshia coli* والمكورات العنقودية الذهبية *Staphyllococcus aureus*. أظهرت النتائج أن إضافة 100٪ من محلول المستخلص أعطت أفضل تثبيط لنمو البكتريا في المختبر (25.7 و 28.7,26.35 ملم في بكتيريا *B. cereus* و *E. coli* و *S. aureus* على التوالي مقارنة مع التراكيز الأخرى للمستخلص المائي للنوع *A. filiculoides*، (25 و50 و 75%)، اظهرت النتائج الحالية ان المستخلص المائي للرخس كمادة تثبيط فعالة ضد البكتيريا المدروسة ويمكن تطبيقها كمادة بديلة صديقة للبيئة بدلاً من المواد الكيميائية مواد للتحكم في البكتيريا الحالية وغيرها من البكتيريا المسببة للأمراض للأسماك.

الكلمات المفتاحية: *Azolla filiculoides*، بكتيريا، اسماك، تثبيط النمو.

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Microbiology Chart Report

Printed April 5, 2020 5:04:49 PM CDT

Patient Name:
Location:
Lab ID: 2

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	2.40 hours	**Status:**	Final
Selected Organism	**Bionumber:**	**Escherichia coli** 1477410650763031		
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	+	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	+	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	+	61	lMLTa	-	62	ELLM	+	64	lLATa	-			

Location:
Lab ID: 5

Patient ID:
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Bacillus cereus

Source:

Collected:

Comments:	

Identification Information	**Analysis Time:**	2.40 hours	**Status:**	Final
Selected Organism	**Bionumber:**	**Bacillus cereus** 1477410650763031		
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	+	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	+	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	+	61	lMLTa	-	62	ELLM	+	64	lLATa	-			

bioMerieux Customer:
System #:

Laboratory Report

Printed Mar 3, 2020 09:12 CST
Printed by: labadmin

Patient Name:
Isolate Group: 3

Patient ID:

Card Type: GP Testing Instrument: 0000148FEF16 (B300)

Bionumber: 050002021721231
Organism Quantity:

Comments:	

Identification Information	Card:	GP	Lot Number:	242362510	Expires:	Nov 16, 2020 12:00 CST
	Completed:	Mar 2, 2020 16:29 CST	Status:	Final	Analysis Time:	6.00 hours
Selected Organism	94% Probability		Staphylococcus aureus			
	Bionumber: 050002021721231				Confidence:	Very good identification
SRF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Staphylococcus aureus	POLYB(1),					

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	IBGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dCAL	-
38	dRIB	+	39	ILATk	-	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	-	53	dMNE	(-)	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															