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Research Article

Antimicrobial potencies of *Ephedra alata* extracts against marine pathogens

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Abstract: Aquaculture is a fast-growing food sector but is plagued by a plethora of bacterial pathogens that infect water and fish. The current work aims to scrutinize the antibacterial and the antibiofilm potencies of aqueous and methanolic extracts of *Ephedra alata* aerial parts against predominant fish pathogens including *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Aeromonas trota*, *Aeromonas salmonocida* and *Aeromonas hydrophila*. Disk diffusion and microdilution methods were used to test the antibacterial activity. Biofilm inhibition was tested using XTT (2,3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay. The toxicity of extracts was evaluated *in vivo* on Brine shrimp (*Artemia salina*).

Results show that both extracts displayed antibacterial activity against all tested strains. Aqueous extract expressed the great activity against *A. hydrophila* with Minimal Inhibitory Concentration (MIC) value of 3.12 mg/mL and Minimal Bactericidal Concentration (MBC) value of 6.25 mg/mL. While *A. trota* was the most sensitive bacteria towards the methanolic extract with MIC and MBC values of 6.25 mg/mL. The tested extracts were able to inhibit biofilm formation with concentration-dependent manner. Moreover, no cytotoxic effect on Brine shrimp was registered for the extracts. Overall, *Ephedra alata* extracts can be considered as good potentials sources of anti-fish pathogens and safety in an aquatic ecosystem.

Keywords: *Ephedra alata*; Fish pathogens; Antibacterial potency; Antibiofilm power; Cytotoxic activity.

1. Introduction

The last two decades, aquaculture has become an important economic sector in many countries. On a large scale in production facilities, where aquatic animals are exposed to stressful conditions, increasing disease and deteriorating environmental conditions lead to serious economic losses. In recent decades, disease prevention and control have led to a substantial increase in the use of antibiotics (Balcázar & Rojas-Luna, 2007). However, the use of these antibiotics, as a traditional strategy for

disease management, increased the emergence of antibacterial resistance fish pathogens (da S Rocha et al., 2021). The presence of resistant bacteria in aquaculture sites increases the risk of transmission to humans of non pathogenic bacteria containing the resistance gene, and the subsequent transfer of these genes to human pathogens (FAO, 2022). *Vibrio* spp., including *Vibrio anguillarum* and *Vibrio harveyi*, are responsible for vibriosis, a disease that causes hemorrhagic septicemia and internal organ damage in both marine and freshwater fish, particularly farmed salmon, seabass, and shrimp (Novriadi, 2016). Similarly, *Aeromonas hydrophila*, which thrives in stressful conditions such as overcrowding and poor water quality, also causes hemorrhagic septicemia and ulcerative disease, particularly, in tilapia, carp, and catfish (Abdul Kari et al., 2022).

One of the major challenges in managing these pathogens is their ability to form biofilms, which serve as persistent sources of infection. Biofilms allow pathogenic bacteria to recolonize aquaculture systems, where they develop resistance to disinfectants and environmental stressors, making them difficult to eliminate (Bourne et al., 2006; Mizan et al., 2015).

Recent advances have provided innovative solutions to combat biofilm formation. Probiotics such as Lactobacillus have showed potential in outcompeting pathogenic bacteria, inhibiting biofilm formation (Shangguan et al., 2021). Another potential technique is phage treatment, which targets and lyses specific bacteria like *Aeromonas hydrophila* and *Vibrio sp.*, while sparing beneficial microbes (Liu et al., 2022). Enzyme-based treatments, such as DNase I, destroy biofilm matrices, making bacteria more sensible to antibacterial agents (Karatan & Watnick, 2009).

In addition, the use of phytoextracts has gained traction as an environmentally friendly method for preventing bacterial infections and biofilm formation. For example, *Moringa oleifera* leaf extract has has been shown to have antibacterial activity against *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, reducing mortality in farmed fish (Jiang et al., 2023). Similarly, *Allium sativum* (garlic) extracts have been found to reduce *Vibrio* infections in shrimp, improving growth and survival rates (Chirawithayaboon et al., 2020). These findings imply that plant extracts are valuable alternatives to antibiotics, offering a sustainable way to enhance the health and productivity of aquaculture systems while limiting environmental impact (Mariappan et al., 2023).

Ephedra alata (also known as Alenda in Tunisia) is a medicinal plant belonging to the Ephedraceae family and is widely distributed throughout the steppes and the deserts. Alenda is spread over five continents (Xie et al., 2013). The aerial parts of *E. alata* are edible and are valued in many regions. Alenda is used in traditional medicine to treat a variety of illnesses, including nasal stuffiness, digestive disorders, and respiratory diseases as well as a treatment for bacterial and fungal infections following abortion (Gupta et al., 2008; Nawwar et al., 1984). Indeed, aerial parts of *E. alata* are frequently used in Tunisian traditional medicine as an aqueous hot maceration by cancer patients (Dbeibia et al. 2024). Previous studies have demonstrated that aqueous and methanolic extracts derived from *E. alata* aerial parts have potential antibacterial and antibiofilm activities against numerous humans' bacteria, including *Enterococcus faecalis*, *Salmonella Typhimurium* and *Staphylococcus aureus* methicillin resistant strains isolated from auricular infections (Dbeibia et al., 2022). Dbeibia et al. (2023) have found that the aqueous and methanolic extracts of *E. alata* aerial parts have a significant antifungal activity.

Furthermore, the same group of researchers demonstrated that the essential oil from *E. alata* aerial parts exhibited an antibacterial and antibiofilm activities against a panel of microorganisms (Dbeibia et al., 2023).
The presence of potential phytoconstituents such as alkaloids, phenolic acids, flavonoids, tannins, saponins, reducing sugars, cardiac glycosides, and fatty acids have been reported to be present in the aerial parts of *E. alata* (Dbeibia et al., 2022; Dbeibia et al. 2024).
This study aims to determine the antibacterial and antibiofilm activities of *E. alata* aerial parts extracts against the most pathogenic marine species (*Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Aeromonas trota, Aeromonas salmonocida* and *Aeromonas hydrophila*) as well as their cytotoxic effect on *Artemia salina* larvae.

2. Materials and Methods

2.1. Plant material and extracts preparation

Aerial parts of *Ephedra alata* were collected from the Kef Derbi area (latitude: 34°41', longitude: 9°29') in the Governorate of Gafsa, Tunisia in March 2020. A voucher specimen (Ea-Gaf/20) was deposited at the Laboratory of Analysis, Treatment and Valorization of Environmental Pollutants and Products, Faculty of Pharmacy, University of Monastir, Tunisia.

The air-dried, powdered aerial parts of *E. alata* were extracted with distilled water and methanol (Merck; 97%) at a ratio of (1:10 w/v). The next day, the aqueous maceration was filtered twice with Whatman Grade 1 Qualitative Filter paper and then, lyophilized using a freeze dryer. After 48 h, the resulting methanolic solution was concentrated under vacuum by using a rotating vacuum evaporator. The dried extracts were then weighed and stored at + 4°C in an amber vial for further analyses.

2.2. Antibacterial activity of the plant extracts

The antibacterial activity of *E. alata* extracts was tested against the following marine pathogens strains: *Vibrio alginolyticus* ATCC 17749, *Vibrio parahaemolyticus* ATCC 17802, *Aeromonas trota* ATCC 49657, *Aeromonas salmonocida* ATCC 33658 and *Aeromonas hydrophila* ATCC 7966, using the agar diffusion method according to Dbeibia et al. (2022) with few modifications.
The strains were grown in Mueller-Hinton (MH) broth (Oxoid) supplemented with 10% NaCl at 37°C for 24 h and suspensions were adjusted to 0.5 McFarland standard turbidity. Afterwards,

100 µL of each precultured suspension was spreaded onto plates containing MH agar supplemented with 10% NaCl. Sterile filter paper discs (6 mm in diameter) were impregnated with 20 µL of the different extracts and placed on agar. The treated plates were kept at 4°C for 1 h before being incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zone (clear halo) surrounding the discs was measured. Each sample was tested in triplicate.

The minimum inhibitory concentration (MIC) was determined as recommended by Dbeibia et al. (2022) with few modifications. Briefly, serial dilutions of the extracts (0.05 - 25 mg/mL) were filled in 96 U bottomed-wells polystyrene plates (Nunc, Roskilde, Denmark) with MH broth supplemented with 10% NaCl and each tested pathogenic bacterium. The treated plates were incubated at 37°Cfor 24 hours. The MIC was reported as the lowest concentration of the sample that did not allow the growth of microorganisms and do not show visible turbidity of the broth medium. The minimum bactericidal concentration (MBC) was evaluated by transferring 10 µL from the well showing no bacteria growth after MIC assay, on MH

agar supplemented with 10% NaCl. After incubation at 37°C for 24 hours, the bacterial growth was examined and the MBC was determined as the lowest concentration of the sample having bactericidal activity.

2.3. Evaluation of E. alata Extracts in Inhibiting Biofilm Formation Using XTT Assay

The ability of *E. alata* extracts to inhibit biofilm formation was evaluated using the colorimetric assay XTT (2,3-bis (2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma-Aldrich) as described previously by Dbeibia et al. (2022). The *E. alata* extracts (0.05-25 mg/mL) were co-inoculated with pathogenic bacteria suspension (grown in Tryptic Soy Broth (TSB) supplemented with 10% NaCl for 24 h at 37°C; 10^5 CFU/mL) in 96 U bottomed-wells polystyrene plates (Nunc, Roskilde, Denmark) containing TSB (Oxoid) supplemented with 2% glucose (w/v). Wells containing only TSB with 2% glucose and TSB with 2% glucose inoculated with pathogenic strain were used as the negative and positive controls, respectively. After an overnight incubation at 37°C, the plates were rinsed three times with PBS. Before use, XTT solution (1 mg/mL PBS) and Menadione (Sigma-Aldrich) solution (0.4 mM in acetone) were prepared and filter sterilized. Next, 180 µL of PBS and 20 µL of XTT menadione solution (12.5 v/v) were added to each well. After incubation (3 h, 37°C, in the dark), the optical density at 492 nm was measured using an automated Multiskan reader (GIO, Rome, Italy) to estimatethe reduction of biofilm biomass.

2.4. Cytotoxicity on brine shrimp

The cytotoxic effect of the *E. alata* extracts was evaluated with brine shrimp (*Artemia salina*) lethality bioassay (Taheur et al., 2020). Ten newly hatched nauplii were transferred to each well of 24-well cell culture plate containing 500 µL of seawater. Then, 100 µL of each concentration of diluted plant extracts in seawater (0.1 mg, 0.25 mg, 0.50 mg, 0.75 mg and 1 mg/mL) was added. Ten shrimp nauplii cultivated solely in seawater were used as the control group. Each sample was triple-tested. After 24 hours of incubation at 25°C, the total dead nauplii in each well was counted using an inverted microscope, and the Lethal Concentration LC_{50} was calculated.

2.5. Statistical Analysis

Antibacterial and cytotoxicity tests were performed in triplicate. The averages and standard deviation of the results obtained were calculated using Microsoft Excel.

3. Results

3.1. Antibacterial activities

The antibacterial activity of *E. alata* aerial parts extracts was tested *in vitro* using the disc diffusion and the microdilution assays against 5 references fish pathogenic bacteria. Results are given as diameters of the inhibition zone (DIZ), MICs and MBCs (Table 1). Both aqueous and methanolic extracts showed a dose-dependent antibacterial activity against all tested bacteria strains. The DIZ values of the aqueous extract ranged between 11.5 and 17 mm and the MICs values varied from 3.12 to 12.5 mg/mL. However, values ranged from 13 to 15.8 mm and from 6.25 to 12.5 mg/mL for methanolic extract. *Aeromonas hydrophila* was the most sensitive to the aqueous extract (MIC= 3.12 mg/mL and MBC= 6.25 mg/mL). Furthermore, the methanolic extract was most active against *Aeromonas trota* (MIC= 6.25 mg/mL and MBC= 6.25 mg/mL).

Table 1. Antibacterial activities of the *Ephedra alata* extracts.

Fish pathogenic bacteria	Aqueous extract			Methanolic extract		
	DIZ± SD	MIC	MBC	DIZ± SD	MIC	MBC
V. alginolyticus ATCC 17749	12.2±1	6.25	12.5	13.5±1	12.5	>25
V. parahaemolyticus ATCC 17802	17±0.3	6.25	12.5	15.8± 0.3	12.5	>25
A. trota ATCC 49657	11.5± 0.3	12.5	25	14±0	6.25	6.25
A. salmonocida ATCC 33658	15±0	6.25	6.25	13± 0.3	12.5	12.5
A. hydrophila ATCC 7966	13±0	3.12	6.25	13±0	12.5	>25

DIZ: diameter zone inhibition; SD: Standard deviation; MIC: Minimun inhibitory concentration; MBC: Minimun bactericidal concentration.

3.2. Antibiofilm activity

The effect of *E. alata* extracts on the biofilm formation by *V. alginolyticus, V. parahaemolyticus, A. trota, A. salmonocida* and *A. hydrophila* was summarized in table
2. The extracts were able to inhibit the biofilm formation by all the tested pathogenic bacteria with BIC_{50} values ranging from 1.83 to 15.76 mg/mL. *A. salmonocida* seemed to be the most sensitive towards aqueous extract (BIC_{50}=1.83 mg/mL).

Table 2. Antibiofilm ability of *E. alata* extracts

	BI_{50} (mg/mL)	
Fish pathogenic bacteria	**Aqueous extract**	**Methanolic extract**
V. alginolyticus ATCC 17749	11.63	10.74
V. parahaemolyticus ATCC 17802	9.98	8.54
A. trota ATCC 49657	10.15	3.37
A. salmonocida ATCC 33658	1.83	15.76
A. hydrophila ATCC 7966	7.87	13.25

BI_{50}: Minimum biofilm inhibition concentration of *E. alata* extracts able to inhibit 50% on the biofilm formation.

However, in decreasing order of activity, the methanolic extract was the most active biofilm inhibitor against *A. trota, V. parahaemolyticus, V. alginolyticus, A. hydrophila* and *A. salmonocida*.

3. 3. Cytotoxicity on brine shrimp

After 24 hours of exposure, our results revealed that the mortality rate ranged between 0-10% and 0-20% for aqueous and methanolic extracts, respectively (Figure 1). Obviously, both extracts were always non toxic. However, the 50% concentration death of brine shrimp nauplii was >1000 µg/mL for the tested extracts.

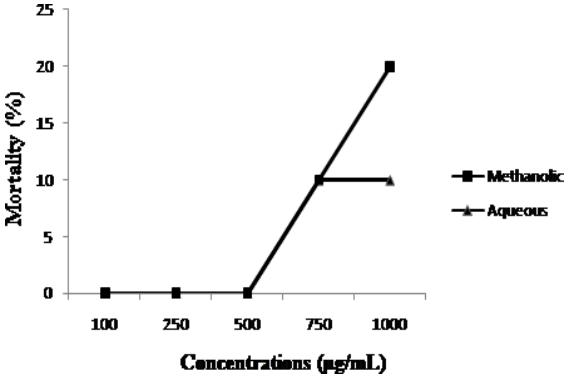

Figure 1. Cytotoxicity of aqueous and methanolic extracts performed on brine shrimp.

4. Discussion

The present study highlights *Ephedra alata*'s antimicrobial potential and its safety behavior through detailed experimental analysis. Several studies have highlighted *Ephedra alata*'s antimicrobial potential against a wide range of bacterial and fungal pathogens, including *Candida* and *Aspergillus* species (Danciu et al., 2019; Dbeibia et al., 2023). Previous study provides evidence of the potent antibacterial activity of *E. alata* extracts, both aqueous and methanolic, specifically against methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from auricular infections. This bactericidal activity is attributed to the plant's richness in secondary metabolites, including phenols, flavonoids, and tannins, which known with their strong activity against resistant strains.

Comparable findings from Salman et al. (2021) demonstrated *E. alata*'s antibacterial efficacy against wide type of pathogenes including *Klebsiella oxytoca, Staphylococcus aureus, Staphylococcus epidermidis,* and *Escherichia coli,* confirming its broad-spectrum antibacterial capabilities. In this study, active compounds such as ephedrine alkaloids and phenolic compounds were identified as the key contributors to antimicrobial effects, disrupting cell membrane integrity and interfering with microbial viability. This mechanism is consistent with other medicinal plants like *Nigella sativa,* where thymoquinone has been shown to inhibit bacterial and fungal growth (Al-Ameedy & Omran, 2019). However, *E. alata* appears particularly effective against resistant bacterial strains, such as MRSA, compared to other plants like *Moringa oleifera,* which show less consistent activity (Sinaga et al., 2021).

The ability of aqueous and methanolic extracts of *E. alata* to inhibit biofilm formation was also demonstrated. This effect is likely due to bioactive secondary metabolites that interfere with the production of conditioning films essential for microbial adhesion. These findings align with those of Hickl et al. (2018), who reported that phenolic and flavonoid-rich extracts from *Salvia* and *Thymus* species have similar anti-biofilm activity. Such activity is consistent with the idea that secondary metabolites disrupt quorum sensing, the communication mechanism that regulates biofilm formation (Asfour et al., 2018).

Moreover, the inhibitory effects of *Ephedra alata* on biofilms are comparable to those observed in plants like coriander (*Coriander sativum L*), which is also rich in phenolic compounds and has shown efficacy in reducing biofilm formation in both food contaminants and human pathogenic bacteria (Molina et al., 2020). This highlights the broad-spectrum role of plant-based secondary metabolites in preventing biofilm formation and controlling pathogenic infections.

To evaluate the safety of these extracts, we employed the brine shrimp (*A. salina*) lethality assay, a widely used and reliable method for detecting toxicological properties of plant extracts. Previous studies were conducted on the brine shrimp nauplii to evaluate the toxic effects of plant extracts (Hamidi et al., 2014; Jafari et al., 2016; Mayilsamy & Krishnaswamy, 2016; Musa, 2012; Naidu et al., 2014; Nugrahaningsih et al., 2019; Ochanga & Chacha, 2016). Our results showed that both tested extracts were non-toxic, with LC_{50} values exceeding 1000 µg/mL. This categorizes the extracts as non-toxic according to Meyer et al. (1982). Furthermore, these findings are consistent with previous reports (Dbeibia et al., 2022) confirming the safety of *E. alata* extracts against *Vero* cell lines. The absence of toxicity indicates that these extracts are

safe for consumption and highlights their potential application in aquaculture to reduce fish pathogens.

Therefore, these extracts, found to be safe for consumption, can be useful in aquaculture, particularly in the reduction of pathogens of fishes in offshore environments as a perspective.

5. Conclusion

The present study indicates that *Ephedra alata* aqueous and methanolic extracts possess a significant strong and broad-spectrum of antimicrobial activity against the tested bacterial pathogens without any cytotoxic effect on Artemia culture. It is assumed that these extracts can be potentially used in combating fish bacterial diseases and could be a lead for antimicrobial drugs. Therefore, further studies are needed to test synergistic interaction between plant extracts and synthetic antibiotics currently used in aquaculture practice as well as to isolate bioactive constituents of *E. alata* extracts to locate potential antimicrobial agents.

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