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Antimicrobial Activity of *Saussurea costus* Against Bacterial Pathogens Isolated from the Gills of Common Carp (*Cyprinus carpio*) with Gill Disease

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ABSTRACT

Bacterial gill disease (BGD) is a major issue in aquaculture, often causing substantial mortality in farmed fish. This study evaluated the antibacterial efficacy of the Indian costus (*Saussurea costus*) root extract against bacterial isolates from the common carp (*Cyprinus carpio*) affected by BGD. Bacterial isolates, including *Aeromonas sobria*, *Sphingomonas paucimobilis* strain K3, and *Sphingomonas paucimobilis* strain E6-5, were tested against ethanolic *S. costus* root extract at varying concentrations (10%, 7.5%, 5%, and 2.5%) along with the antibiotic tetracycline. The results showed promising dose-dependent antibacterial activity, with *A. sobria* being particularly sensitive. The gas chromatography (GC) mass analysis of the ethanolic extract revealed a high diversity of bioactive compounds, especially lactones, phenols, and terpenes with known antibacterial properties. While tetracycline displayed greater potency, the findings suggest that *S. costus* extracts could potentially serve as a natural alternative for managing BGD and reducing the reliance on conventional antibiotics in aquaculture practices. However, before large-scale application, further research is needed to evaluate the efficacy, safety, and cost-effectiveness of utilizing *S. costus* extracts in the aquaculture field. Nonetheless, this study provides valuable preliminary data highlighting the antibacterial potential of *S. costus* against fish pathogens and the potential of exploring natural products from medicinal plants for sustainable disease management in aquaculture.

INTRODUCTION

The aquaculture sector produced 82.1 million tons of various aquatic animals in 2018, and the common carp (*Cyprinus carpio*) contributed to approximately 4.19 million tons of total production **(FAO, 2020)**. *C. carpio* is the only freshwater fish raised in Iraq and is considered a source of income for the rural community in the country **(Ahmed *et al*., 2020; Ahmed, 2021)**. Aquaculture production has been plagued by many factors including diseases. High stocking density of fish in aquaculture enterprises increases the rate of disease outbreaks **(Irshath *et al*.,**

2023). Besides high density, the deterioration of ecological conditions in the aquatic habitat is also believed to contribute to the occurrence of diseases in fish (**Rajeswari *et al.,* 2019)**.

Bacterial gill disease (BGD) is a common contagious disease that affects the respiratory system of farmed and wild fish populations and can lead to massive mortalities and severe economic losses **(Bakhtiyar *et al.,* 2021)**. BGD is mainly caused by filamentous bacteria within the genus Flavobacterium (most often *F. branchiophilum*) **(Taylor *et al.,* 2023)**. BGD is a fast-moving disease that kills fish at a faster rate than other types of fish infections due to the massive colonization of bacteria on the gill lamellar surfaces **(Bakhtiyar *et al.,* 2021)**.

Antibiotics play a critical role in limiting the spread of infection and disease control in different sectors including human health, livestock, and aquaculture **(Nunes *et al.,* 2015)**. Tetracyclines are the most common antibiotic drugs in the world, and they are known to inhibit protein synthesis in bacteria and combat a variety of bacterial infections **(Ahmad *et al.,* 2022)**. However, using tetracycline to treat BGD can have several negative impacts. In this context, tetracycline can bioaccumulate in the food chain, leading to toxicity in the microbial population, promoting antibiotic resistance and disturbing the gut microbiota in humans **(Amangelsin *et al.,* 2023)**. Tetracycline produces oxidative stress and induces apoptosis in zebrafish embryos, which leads to developmental delay **(Zhang *et al.,* 2015)**. Similarly, oxytetracycline (a member of the tetracycline family) caused oxidative stress in *C. carpio* **(Sharma *et al.,* 2021)**. Another study **(Jawahar *et al.,* 2023)** reported that oral oxytetracycline administration for a long time and high doses produced histopathological lesions in the liver and kidney of the Nile tilapia (*Oreochromis niloticus*). Tissue alterations and histopathological lesions in the gills and liver were observed in the gilthead seabream (*Sparus aurata*) exposed to the antibiotic erythromycin and oxytetracycline **(Rodrigues *et al.,* 2019)**. Sulfamethoxazole (a member of the sulfonamides group of antibiotics), extensively used in aquaculture, caused variations in blood profile and biochemical parameters in *C. carpio* **(Iftikhar *et al.,* 2021)**. Further, ineffective wastewater systems contribute to increased antibiotic concentrations in aquatic environments **(Rhodes *et al.,* 2000; Almeida *et al.,* 2021)**. The global use of antibiotics leads to bacterial resistance, which is a concern in various sectors including aquaculture **(Almeida *et al.,* 2021)**. Living organisms exist in an interconnected environment, where each is influenced by others. Regarding this, the oxytetracycline resistance gene has been isolated from *Aeromonas* bacteria, fish hatchery systems, and untreated hospital wastes **(Rhodes *et al.,* 2000)**. Hence, the excessive use of antibiotics is considered an environmental pollutant and may lead to antibiotic resistance, posing risks to the environment, and animal, and human health **(Kulik *et al.,* 2023)**. This highlights the importance of finding environmentally friendly substances to reduce ecosystem pollution and disease distribution.

Herbs and plants are rich sources of a wide variety of compounds that can be used to fight drug-resistant microbes, reduce environmental hazards, and alleviate animal and human illness **(Jubair *et al.,* 2021; Mohamad *et al.,* 2022; Nik *et al.,* 2022)**. Some aquatic plants, such as *Anadendrum microstachyum* and *Selaginella plana*, impeded the growth of pathogenic bacteria (*Edwardsiella ictaluri* and *Streptococcus agalactiae*), which cause severe mortality in farmed

tilapia and catfish worldwide **(Novita *et al*., 2020)**. The methanolic extract derived from duckweeds (*Lemna minor*) has shown effectiveness against *Pseudomonas fluorescens* offering an alternative for treating and controlling septicemia in fish **(González-Renteria *et al*., 2020)**.
S. costus (Falc), a plant widely utilized in traditional medicine, is known for its diverse healing properties **(Nadda *et al*., 2020)**. Extensive pharmacological studies have confirmed that *S. costus* possesses anti-inflammatory, anti-ulcer, anti-cancer and hepatoprotective properties **(Zhen *et al*., 2022)**.

This research aimed to evaluate the effects of the alcoholic extract derived from *S. costus* under controlled laboratory conditions. The study used strains isolated from fish affected by BGD as the target microbes. Additionally, a comparative analysis was conducted to assess the efficacy of the *S. costus* extract compared to tetracycline, an antibiotic used in aquaculture for BGD treatment. The outcomes of this study could offer insights into using *S. costus* as a natural alternative therapy for managing BGD in farmed fish populations.

MATERIALS AND METHODS

Infected fish samples, clinical signs and diagnosis

Infected fish samples were collected from different farms with high mortality rates. The fish were swimming slowly and in scattered groups. They were clearly suffering from suffocation. The fish were examined under a light microscope. Clear histological changes due to inflammation were observed, evidenced by discoloration of the gills and the obvious tissue damage. The gill filaments were enlarged at their ends.

Preparing the alcoholic extract of Indian costus roots

Fig. (1) shows the *S. costus* roots and roots powder. The roots of the plant were purchased from a local market in Basrah province, Iraq. The roots were ground into a fine powder by a milling machine and the powder was stored in a sealed container until further analysis.

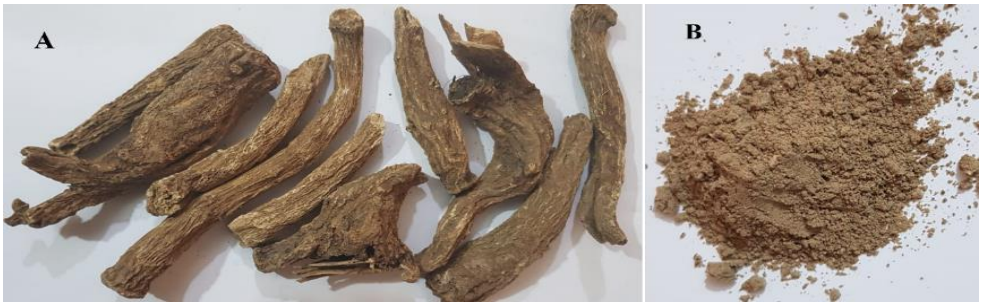

Fig. 1. *S. costus* roots (A) and roots powder (B)

To prepare the ethanolic extracts, 20gm of *S. costus* powder was placed in a special extraction thimble which was placed inside a Soxhlet extractor. The extraction process lasted 24 hours using 250ml of ethanol (95%). After extraction, the solvent extract was filtered and concentrated at 70◦C to half its original volume. The residue was dried at room temperature and was then collected in a sealed container until use **(Souri *et al.*, 2004)**. Different concentrations were prepared for the ethanolic extract, including 10%, 7.5%, 5%, and 2.5%.

Chemical composition of Indian costus by GC mass analysis

The qualitative and quantitative measurement of the ethanol extract of *S. costus* roots were carried out according to **Pongpiachan *et al.*, (2009; 2012)** using gas chromatography/mass spectrometry (GC/MS) (Shimadzu GCMS-QP2010 Ultra). The device is provided with a capillary column Rtx-5MS. The column was 30m × 0.25mm ID with a film thickness of 0.25µm. The extract was diluted in 1 µl of ethanol, ultrasonicated, combined with 0.1 g of anhydrous sulfate, and filtered through a syringe filter (0.22 µm pore size). For chromatographic separation, 1µl of the produced filtrate was put into the capillary column. Helium gas was a carrier at a 1.69ml/ minute flow rate. The injector was set at 200°C, the mass detector at 250°C, and the column oven temperature was set to increase from 50 to 300°C. Initially, the temperature increased by 7°C/ minute until it reached 180°C, and then it was raised to 10°C per minute until it reached the final temperature to achieve optimal peak separation. Electron ionization was conducted at 70 electron Volt. All mass spectra were recorded using scan mode within a 40–500m/ z range over a 28-minute run time. Identification of components in the ethanol extract was done by comparing retention times and mass fragmentation with documented retention indices in the National Institute of Standards and Technology library.

Identification of bacterial strains

The bacteria isolated from the gill of the infected fish were identified biochemically by an automated VITEK 2 compact system and genetically by sequence analysis of the 16S rRNA gene. The 16S rRNA gene sequence of the isolates was compared to reference sequences in the NCBI GenBank database using the BLAST algorithm.

Antibacterial Activity

Three bacterial isolates (*Aeromonas sobria* strain L10, *Sphingomonas paucimobilis* strain K3, and *Sphingomonas paucimobilis* strain E6-5), were used to test the antibacterial activity of the *S. costus* extract. These isolates were obtained from *C. carpio* that were infected with BGD. The infected fish were collected from a local fish farm in Basrah province, southern Iraq and immediately transported to the laboratory at the Marine Science Center, University of Basra, Iraq.

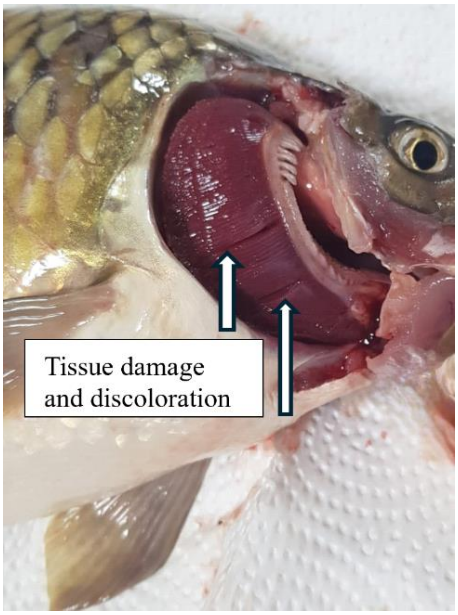

Fig. 2. An infected *C. carpio* gill under a light microscope, showing tissue damage and discoloration

The antibacterial activity of the alcoholic extract was evaluated by adding 50µL of plant extract at different concentrations (2.5%, 5%, 7.5% and 10%) into duplicate wells. The results were compared with those of the antibiotic tetracycline at the same concentrations.

The antibacterial activity of *S. costus* was determined by the agar diffusion method as recommended by **NCCLS (1993)**. Three to five identical colonies from each culture plate were transferred to a test tube containing 5ml of tryptic soy broth as a culture medium. The turbidity of these cultures was adjusted using a 0.5 McFarland standard. A uniform bacterial lawn was developed by spreading the cultures on the surface of solid nutrient agar plates using sterile cotton swabs. Wells with a diameter of 8mm were made in the agar using a cork borer.

The antibacterial activity of the alcoholic extract was checked by adding 50µL of plant extract with different concentrations (2.5%, 5%, 7.5% and 10%) into duplicate wells. The plates were left at room temperature for 1 hour to let the extract diffuse into the agar. Later, the plates were incubated for 18 hours at 37ºC. Subsequently, the bacterial growth was examined, and the inhibition zone diameter was measured to the nearest millimeter.

RESULTS

The extract analysis by GC mass

Many compounds have been detected in the alcoholic extract of *S. costus* roots by GC mass analysis. These compounds include Phenols, Lactones, Terpenes/Terpenoids, Alkenes, Esters, and other compounds as presented in Table (1) and Fig. (3).

Table 1. Chemical compounds of Indian costus root alcoholic extract detected by GC mass spectrometry analysis

Peak	Library/ID
1	3-Methoxy-2,2-dimethyloxirane
2	Benzene, 1-ethyl-3-methyl-
3	Benzene, 1-ethyl-2-methyl-
4	Mesitylene
5	Benzene, 1-ethyl-2-methyl-
6	Benzene, 1,2-dichloro-
7	2-Methoxy-4-vinylphenol
8	Phenol, 2,6-dimethoxy-
9	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
10	.alpha.-Ionone
11	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
12	Nonanedioic acid, dimethyl ester
13	Cetene
14	Caryophyllene oxide
15	11,11-Dimethyl-spiro[2,9]dodeca-3,7-dien
16	(1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl)cyclohex-2-enol
17	Bergamotol, Z-.alpha.-trans-
18	2-((2R,4aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)prop-2-en-1-ol
19	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol
20	Pentadecanoic acid, methyl ester
21	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-
22	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-
23	Hexadecanoic acid, methyl ester
24	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-
25	Dihydrodehydrocostus lactone
26	3-Oxatricyclo[3.2.1.0(2,4)]octane, (1.alpha.,2.beta.,4.beta.,5.alpha.)-
27	Dehydrocostus lactone
28	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.alpha.,7.beta.,7a.beta.)]-
29	Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-
30	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
31	Dihydrodehydrocostus lactone
32	Methyl 5,7-hexadecadiynoate
33	6-Hydroxy-3,5a,9-trimethyl-3,3a,4,5,6,7,9a,9b-octahydrobenzo[g][1]benzofuran-2-one
34	Santamarine
35	Reynosin
36	9-Octadecenoic acid, 12-hydroxy-, methyl ester, [R-(Z)]-
37	2H-Pyran-2-one, tetrahydro-6-undecyl-
38	2,11-Dodecadiene, 4-chloro-
39	Diazoprogesterone
40	Hexanedioic acid, bis(2-ethylhexyl) ester
41	E-11(13,13-Dimethyl)tetradecen-1-ol acetate
42	Bis(2-ethylhexyl) phthalate
43	15-Tetracosenoic acid, methyl ester, (Z)-
44	Stigmasterol
45	(E)-2-((8R,8aS)-8,8a-Dimethyl-3,4,6,7,8,8a-hexahydronaphthalen-2(1H)-ylidene)propan-1-ol
46	.gamma.-Sitosterol
47	7-Pentadecyne
48	Tris(2,4-di-tert-butylphenyl) phosphate

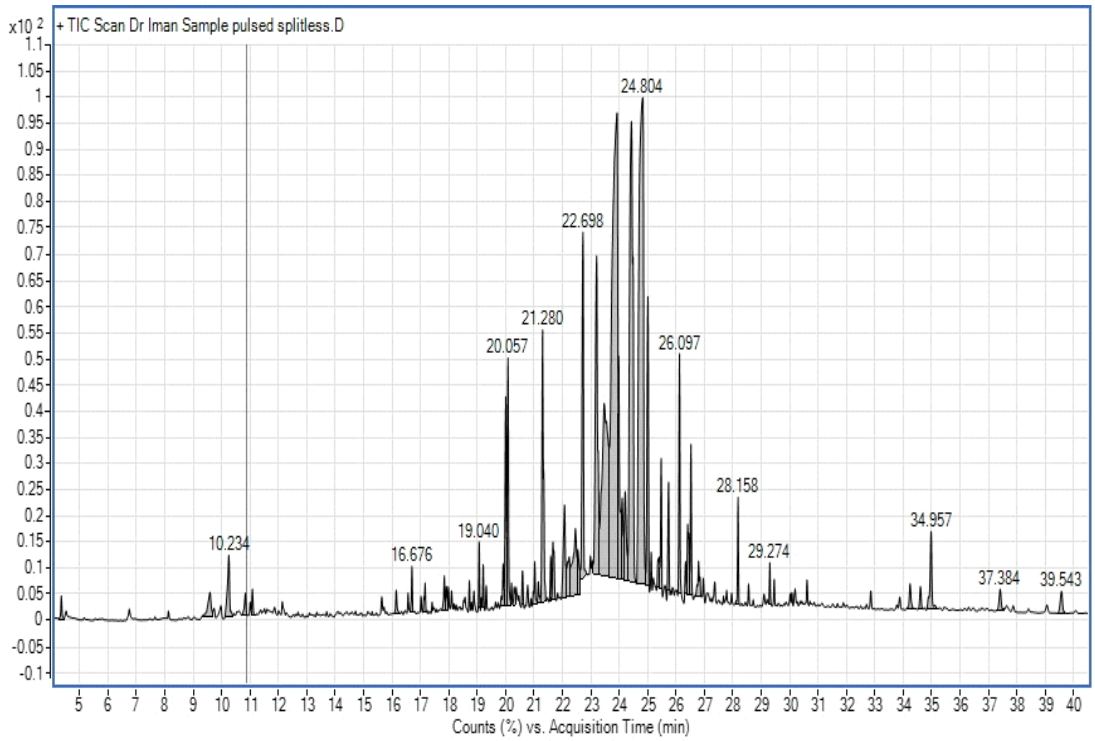

Fig. 3. Chemical composition of Indian costus roots alcoholic extract by GC mass spectrometry analysis

Identification of bacterial strains

The isolates showed a 100% sequence identity to *Aeromonas sobria* strain L10 (GenBank accession MK828155.1). The next closest matches were *Sphingomonas paucimobilis* strain K3 (87% identity, GenBank JN540025.1) and *Sphingomonas paucimobilis* strain E6-5 (81% identity, GenBank KY938114.1).

Antibacterial activity of *S. costus* extract

The findings suggested that the alcoholic extract of *S. costus* roots has antibacterial properties against the bacterial strains tested. The extract was able to inhibit the growth of bacteria and showed dose-dependent responses, with increasing inhibition at higher concentrations (Table 2). *A. sobria* L10 appears to be particularly sensitive to the extract, with inhibition zones approaching those of the antibiotic tetracycline (Fig. 4).

Table 2. Antibacterial activity of *S. costus* ethanolic extract and tetracycline against bacteria isolated from *C. carpio* infected with BGD

Bacterial species	Concentration %							
	2.5		5.0		7.5		10	
	I	T	I	T	I	T	I	T
Aeromonas sobria strain L10	9	17	12	20	25	26	30	30
Sphingmonas paucimobilis strain K3	R	17	5	15	10	26	15	30
Sphingomonas paucimobilis strain E6-5	R	17	R	20	R	26	R	30

*I: Indian costus, T: tetracycline, R: resistant

* Numbers referred to diameters of inhibition zones (mm)

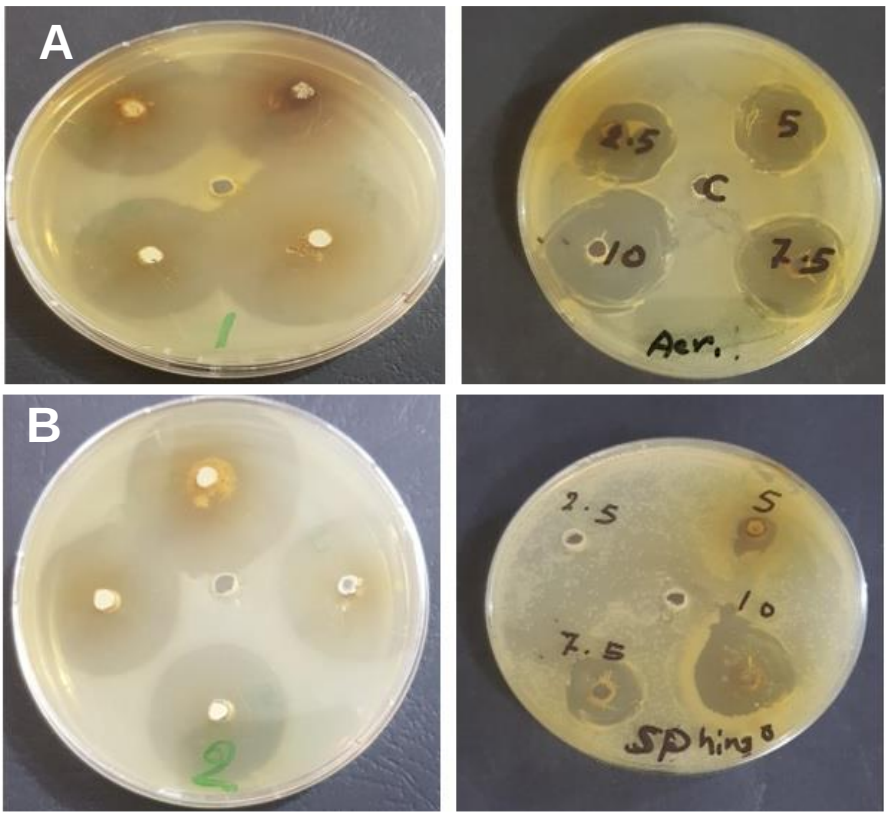

Fig. 4. Comparison of the anti-bacterial efficacy of *S. costus* and tetracycline against bacteria isolated from *C. carpio* infected with BGD. Different concentrations (2.5%, 5%, 7.5% and 10%) were used from *S. costus* and tetracycline

Feasibility study

Based on the prices of Iraqi markets, the cost of using 500 grams of tetracycline at a concentration of 100% is equivalent to 60,000 Iraqi dinars per dunam. In comparison, the cost of producing an extract of 500 grams of *S. costus* at a concentration of 100% is equivalent to 100,000 Iraqi dinars. Therefore, using the same quantity per donum unit equals 30,000 for tetracycline and 50,000 for *S. costus* extract. In other words, using *S. costus* extract is more expensive than using tetracycline by an approximate ratio of 1.67 times for the same quantity and per donum.

DISCUSSION

BGD is a ommon and occasionally devastating disease affecting numerous cultured fish species worldwide **(Starliper, 2012)**. It is well known that *F. branchiophilum* is a major cause of BGD **(Good *et al*., 2015; Pękala-Safińska, 2018)**. However, the bacterial isolates in the current study showed a 100% sequence identity to *A. sobria* (strain L10) followed by *S. paucimobilis strain* K3 (87%) and *S. paucimobilis* strain E6-5 (81%). *Aeromonas* species often cause disease in cultured fish and are responsible for causing significant economic losses in the aquaculture industry **(Pereira *et al*., 2022)**. Similarly, *S. paucimobilis* is common in fish farming and recently isolated from cultured *C. carpio* gills reared in floating cages in the Al-Hilla River, middle of Iraq **(Al-Jubouri *et al*., 2022)**. These species of bacteria contribute to BGD due to the susceptible immune system of cultured fish and poor environmental conditions, especially in industrial-scale fish farms **(Jassim *et al*., 2019)**. To reduce economic losses and increase profitability, different antibiotics are used to treat fish diseases caused by pathogenic bacteria. In this regard, the study of **Al-Jubouri *et al*. (2022)** reported that the six isolates of *S. paucimobilis* from the gills of cultured *C. carpio* were sensitive to Piperacillin/Tazobactam, Imipenem, Meropenem, Ciprofloxacin, and Levofloxacin. However, the excessive antibiotic use can result in potential antibiotic residues in the cultured fish and in the aquatic environment **(Boti *et al*., 2023)**. Therefore, it is essential to find a natural remedy that reduces concerns related to the use of antibiotics in aquaculture.

Plant extracts gained significant attention in recent years as a natural antibiotic source owing to their broad antibacterial spectrum, rare side effects, and low probability of producing drug resistance **(Yan *et al*., 2021)**. The antibacterial activity observed in the plant extract might be linked to the biologically active molecules naturally present in the plant. The phytochemical profile of *S. costus* (Table 1) showed that the root extract is rich in lactone and dihydrodehydrocostus lactones. **Negi *et al*. (2014)** reported that samples with high levels of lactones exhibit significant antibacterial potency. Another class of phytochemical molecules, phenolic compounds, was also detected by GC mass analysis in the tested extract (Table 1 & Fig. 3). Phenolic compounds are potential substitutes for

bioactive agents in pharmaceutical and medicinal fields to promote human and animal health by preventing and treating different diseases **(Sun & Shahrajabian, 2023)**. In the current investigation, the alcoholic extract of the studied plant contains different phenolic compounds such as 2-methoxy-4-vinylpheno. It has been reported that 2-methoxy-4-vinylphenol possesses a high ability to interact with DNA and lipoprotein of the bacterial cell wall, which leads to higher antimicrobial efficacy **(Rubab *et al.*, 2020)**. In addition, the antibacterial efficacy of the *S. costus* extract against the tested bacterial strains could be attributed to the presence of terpenoids such as caryophyllene oxide (Table 1). It has been reported that most terpenoids can inhibit oxygen uptake and oxidative phosphorylation, thereby suppressing microbial growth and survival **(Griffin *et al.*, 1999)**.

The tested extract had a notable effect on the growth of *A. sobria* compared to other strains (Fig. 2). This is potentially due to the differences in the components and structure of the bacterial cell wall **(Zhou *et al.*, 2022)**. In addition, the susceptibility of microorganisms may depend on the bioactive constituents extracted by the solvents, because microorganisms respond differently to various phytochemical compounds **(Mohamed *et al.*, 2017)**.

Several studies reported that the phytochemical molecules in medicinal plants can impede the growth of bacteria in different ways. These strategies encompass cellular membrane rupture, signal transmission alteration, interference with intracellular metabolic activities, and gene expression pathways **(Omojate *et al.*, 2014; Mohamed *et al.*, 2017)**.

The difference in the diameters of inhibition zones between the extract and the antibiotic was more significant at lower concentrations (2.5 and 5%) reaching up to 8mm for *Aeromonas sobria* strain L10. This difference decreased to 1mm at 7.5% concentration, and there was no difference between the extract and the antibiotic at 10 % concentration. *Sphingmonas paucimobilis* strain K3 was less susceptible to the extract compared to *Aeromonas sobria* when the same concentrations were used for both bacterial species. The difference in inhibition zones diameters may be attributed to the chemical nature of the extract's ingredients, which had a greater effect on *Aeromonas sobria* strain L10 than on *Sphingmonas paucimobilis* strain K3.

In comparison with tetracycline, the results indicated that an extract of 500 grams of *S. costus* roots at a concentration of 100% is highly effective and is almost identical to the effectiveness of the same concentration of tetracycline, which is widely used in treating bacterial diseases in farmed fish. Considering the economic feasibility, producing *S. costus* extract with the mentioned concentrations is financially expensive compared to the commercial antibiotic. However, improving the extraction method and using advanced

distillation protocols can enhance the extraction efficiency, leading to higher yields and decreased production costs.

In conclusion, antibiotics are extensively used in aquaculture to control bacterial fish infections owing to their high effectiveness against a broad spectrum of pathogenic bacteria and affordable cost. However, the evolution of antibiotics-resistant strains represents a major challenge that impedes the treatment of bacterial diseases. The high levels of bioactive molecules such as lactones, phenols, and terpenes in the *S. costus* extract could be used as an alternative to chemical antibiotics. However, more studies are needed to address the safety and possible side effects of utilizing *S. costus* for treating fish diseases in the aquaculture sector.

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