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Antibacterial activity of ethanolic and aqueous extract of Ajwain (*Trachyspermum ammi*) and Mountain tea (*Stachys lavandulifolia*) against *Lactococcus garvieae* strains

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Abstract The widespread use of antibiotics in agriculture, aquaculture, and medicine has led to the environmental dispersion of these compounds and the rise of drug-resistant bacterial pathogens. To prevent antibiotic resistance, employing herbal extracts and essential oils against pathogenic bacteria is a promising strategy. This study evaluated the antimicrobial efficacy of plant extracts from *Trachyspermum ammi* (*T. ammi*) and *Stachys lavandulifolia* (*S. lavandulifolia*) against *Lactococcus garvieae* (*L. garvieae*) using the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and disc diffusion test. Results showd that the ethanolic extract of *T. ammi* showed inhibition zones ranging from 22.06 mm to 0 mm, with an MBC of 18.75 mg/mL, while the aqueous extract ranged from 18.2 mm to 0 mm, with an MBC of 75 mg/mL. For comparison, the inhibition zones for streptomycin, erythromycin, and gentamicin were 21.75, 20.7, and 18 mm for the ethanolic extract and 21.32, 19.9, and 18.5 mm for the aqueous extract, respectively. In the case of *S. lavandulifolia*, the ethanolic extract exhibited inhibition zones ranging from 17.2 mm to 0 mm, with an MBC of 75 mg/mL, while the aqueous extract ranged from 16.6 mm to 0 mm, also with an MBC of 75 mg/mL. The inhibition zones for streptomycin, erythromycin, and gentamicin were 22.3, 18.9, and 18.1 mm for the ethanolic extract and 21.8, 20.2, and 17.9 mm for the aqueous extract, respectively. In conclusion, both *T. ammi* and *S. lavandulifolia* possess significant antimicrobial activity against *L. garvieae*, particularly at higher concentrations. Ethanolic extracts generally demonstrated greater efficacy compared to aqueous extracts, suggesting ethanol as a more effective solvent for extracting antimicrobial compounds. These findings support the potential use of these plant extracts as alternative treatments in aquaculture to combat bacterial infections and reduce reliance on traditional antibiotics, thereby mitigating the risk of antibiotic resistance.

Introduction

Lactococcus garvieae (*L. garvieae*) is a Gram-positive ovoid coccus, facultatively anaerobic, and non-motile bacterium. *L. garvieae* is a fish pathogen causing lactococcosis, leading to a decrease in Growth rates, high mortality, and poor body conditions in commercially important aquaculture species such as rainbow trout, yellowtail, tilapia, Japanese eel, olive, gray mullet, damselfly and giant freshwater shrimp; especially when the water temperature is over 15°C. *L. garvieae* is able to adapt to a wide range of environments and can be found in a variety of places including aquaculture, rivers, and wastewater [1].

However, it has detected from subclinical mastitis in cows and water buffalos, and also poultry meat, raw cow's milk, pig blood and the tonsils of cats and dogs [2]. Rare cases of human infection have been reported, with a mortality rate of 25% [3]; it causes endocarditis, urinary tract infections, liver abscesses, septicemia, and peritonitis in humans who consume or use contaminated fish, meat, and milk [4].

Many bacterial diseases in aquaculture are controlled using antibiotics. However, the use of antibiotics leads to drug resistance, resulting in decreased drug efficacy and the risk of selecting antibiotic-resistant bacteria. Antimicrobial resistance (AMR) is a phenomenon where microorganisms acquire the ability to overcome antimicrobial agents. The improper and excessive use of antibiotics exerts selective pressures favoring resistant bacteria, thereby increasing their population. These microorganisms typically employ four basic mechanisms of resistance: (i) limiting drug uptake, (ii) modifying an antibiotic target, (iii) inactivating an antimicrobial agent, and (iv) activating drug efflux pumps [5]. Antibiotic resistance has led to find alternative therapeutic compounds with a broad therapeutic spectrum. One of the strategies to prevent antibiotic resistance is the use of extracts and essential oils. Studies have shown that the antimicrobial activity of plants is generally due to the presence of phenolics, saponins, tannins, and flavonoids in their structure, which affect the plasma and cell membranes of microorganisms or inhibit the structural enzymes of their cell membranes [6].

This study focuses on the antimicrobial properties of two plant species, including *Trachyspermum ammi* (*T*. *ammi*) and *Stachys lavandulifolia* (*S. lavandulifolia*). *T. ammi*, a member of the *Apiaceae* family, is an aromatic, herbaceous, annual plant used in traditional medicine for various therapeutic effects such as diuretic, antiemetic, and anti-nausea. This plant contains compounds like thymol, p-cymene, γ-terpinene, β-pinene, and carvacrol, which potentially affect both Gram-positive and Gram-negative bacteria [7]. *S. lavandulifolia*, from the mint family (*Lamiaceae*), has demonstrated antimicrobial effects in numerous studies. This plant contains biologically active compounds, including phenylethanoids, terpenoids, and flavonoids.[8]. This study aims to investigate the antimicrobial properties of *T. ammi* and *S. lavandulifolia* using MIC, MBC, and antibiogram methods in the laboratory against *L. garvieae*, one of the significant bacteria in aquaculture.

Materials and Methods

Preparation of aqueous and ethanolic extract

After identifying and collecting the plants, the required parts were separated, dried, and powdered. To prepare the aqueous extract, 50 grams of the plant powder were mixed with 250 mL of distilled water and heated at 90°C with a shaking incubator at 150 rpm for 30 minutes. The mixture was then incubated at 37°C overnight.

For the ethanolic extract, 50 grams of the plant powder were mixed with ethanol (1:9) and incubated in a shaking incubator at 37°C and 150 rpm overnight. The resulting extracts (aqueous and ethanolic) were filtered using Whatman No. 1 filter paper to remove solid residues and subsequently passed through a microbiological filter (Millipore 0.22 µm).

Preparation of bacterial suspension

Lactococcus garvieae bacteria (PTCC No: 1884) were cultured in Mueller-Hinton Broth (MHB; Merck, Germany) and incubated at 35 ± 2°C for 18 to 24 hours. Bacterial suspensions with OD_{600} between 0.08 and 0.13 (equivalent to 0.5 McFarland standard, containing 1×10^8 CFU/mL) were prepared. A suspension of 5×10^6 CFU/mL was then obtained by adding sterile Mueller-Hinton Broth [9].

Determination of MIC

The minimum inhibitory concentration (MIC) was determined using the microdilution broth method in sterile 96-well plates. 100 mL of various concentrations of T. *ammi* and S. *lavandulifolia* were prepared in each well using a 2-fold serial dilution with MHB medium. Then, 10 mL of the bacterial suspension prepared in the previous step were added to each well. The 96-well plate was incubated at 37°C for 24 hours. The lowest concentration without turbidity was considered as the MIC. A well containing the medium and extract without microorganisms, and a well containing the medium and bacteria without the extract, were used as controls.

Determination of MBC

To determine the minimum bactericidal concentration (MBC), 50 mL from each well showing no turbidity were cultured on blood agar plates and incubated for 24 hours at 37°C. The minimum concentration of the extract without colonies on blood agar was considered as the MBC.

Disk diffusion method

The 0.5 McFarland bacterial suspension (*L. garvieae*) was cultured (lawn method) on Mueller-Hinton agar plates. Then, discs containing pure aqueous and ethanolic extracts were placed on the medium. The plates were incubated at 37°C, and the diameter of the inhibition zones was measured every 24 hours for 3 days.

Results

MIC, MBC and inhibition zone of *T. ammi*

The values of MIC, MBC and zone of inhibition of *T. ammi* and *S. lavandulifolia* against *L. garvieae* was shown in Table 1. The inhibition zone diameters (IZDs) associated with the ethanolic extract of *T. ammi* ranged from 22.06 mm to 0 mm, while the aqueous extract ranged from 18.2 to 0 mm. Furthermore, the MBC of the ethanol extract was found to be 18.75 mg/ml, whereas the aqueous extract was 75 mg/ml. In addition, the inhibition zone diameters against streptomycin, erythromycin, and gentamicin were observed to be 21.75, 20.7, and 18 mm for the ethanolic extract and 21.32, 19.9, and 18.5 mm for the aqueous extract, respectively.

MIC, MBC and inhibition zone of *S. lavandulifolia*

IZDs associated with the ethanolic extract of S. lavandulifolia ranged from 17.2 mm to 0 mm, while the aqueous extract ranged from 16.6 to 0 mm. Furthermore, the MBC of the ethanolic and aqueous extract was found to be 75 mg/ml. In addition, the diameter of the inhibition zone diameters against streptomycin, erythromycin, and gentamicin was observed to be 22.3, 18.9, and 18.1 mm for the ethanolic extract and 21.8, 20.2, and 17.9 mm for the aqueous extract, respectively.

Discussion

The results of this study indicate that both *T. ammi* and *S. lavandulifolia* exhibit significant antimicrobial activity against *L. garvieae*, particularly at higher concentrations. Ethanolic extracts of both plants generally show higher zones of inhibition compared to their aqueous counterparts, suggesting that ethanol is a more effective solvent for extracting antimicrobial compounds. *T. ammi* ethanolic extract, at higher concentrations (150 mg/ml), performs comparably or even better than some common antibiotics like streptomycin and erythromycin. *S. lavandulifolia* also demonstrates considerable antimicrobial activity, although its effectiveness is slightly lower than *T. ammi*. The presence of biologically active compounds such as phenolics, saponins, tannins, and flavonoids in these plants likely contributes to their antimicrobial properties.

These findings support the potential use of *T. ammi* and *S. lavandulifolia* extracts as alternative treatments in aquaculture to combat bacterial infections and reduce reliance on traditional antibiotics, thereby mitigating the risk of antibiotic resistance. Further research and development of these plant extracts could lead to effective, natural antimicrobial agents in aquaculture and other fields. In this study, we aimed to minimize the antimicrobial effects of the solvents by using standard extraction methods.

Table 1. MIC and MBC of extracts against *L. garvieae*

Extract Concentration	Water Extract			Ethanol Extract		
	MIC and MBC (mg / ml)	**Zones of Inhibition (mm)**	**Common Antibiotics**	**MIC and MBC (mg / ml)**	**Zones of Inhibition(mm)**	**Common Antibiotics**
Comparison of antimicrobial effects of ethanolic and aquatic extracts of *Trachyspermum ammi* against *lactococcus garvieae*						
150	-	18.2	21.32 (Streptomycin)	-	22.06	21.75 (Streptomycin)
75	MBC	15.1	19.9 (Erythromycin)	-	18.3	20.7 (Erythromycin)
37.5	MIC	8.8	18.5 (Gentamicin)	-	15.1	18 (Gentamicin)
18.75	+	4.5		MBC	12.9	
9.37	+	0		MIC	6.2	
4.6	+	0		+	0	
2.3	+	0		+	0	
Comparison of antimicrobial effects of ethanolic and aquatic extracts of *Stachys lavandulifolia* against *lactococcus garvieae*						
150	-	16.6	21.8 (Streptomycin)	-	17.2	22.3 (Streptomycin)
75	MBC	14.1	20.2 (Erythromycin)	MBC	15.1	18.9 (Erythromycin)
37.5	MIC	8.7	17.9 (Gentamicin)	MIC	9.3	18.1 (Gentamicin)
18.75	+	0		+	4.2	
9.37	+	0		+	0	
4.6	+	0		+	0	
2.3	+	0		+	0	

+ Indicates the growth of the bacteria in the culture medium
- Indicates the lack of growth of the bacteria in the culture medium

In a study conducted by Khan and Jameel [10], various extracts were examined to evaluate their antibacterial properties. The results indicated that the methanolic and ethanolic extracts derived from *T*. *ammi* seeds exhibited stronger antibacterial effects compared to the aqueous extract. These effects were observed against both food spoilage microorganisms and antibiotic-resistant microorganisms. The primary active ingredients found in *T. ammi* are phenolic compounds, including thymol, β-para-cymene, and alpha-terpinene. Phenolic compounds are known for their diverse properties, including antibacterial activity. They exert their action on bacteria through mechanisms such as lysis of the cell membrane, binding to bacterial cell components and enzyme inactivation, adhesin binding, and formation of cell wall complexes [11]

In a study performed by Javan et al. [12], the MIC of *T. ammi* extract against *S. aureus* was found to be 500 µg/mL. In another investigation by Sharifi Mood et al. [13], the MIC of *T. ammi* extract against *S. aureus* was reported as 1.205 mg/mL. Moreover, in a study reported by Hosseinkhani et al. [14], the MIC of *T. ammi* extract against various foodborne pathogens ranged from 12.5 µg/mL to 5.4 µg/mL. These findings demonstrate that *T. ammi* exhibited higher antimicrobial effects against Gram-positive compared to Gram-negative bacteria. Furthermore, Lee and Park (2011) investigated the antibacterial effect of *T. ammi* extract against several important food pathogens using the microdilution method. They reported that the MIC of *T. ammi* essence ranged from 0.5 mg/mL to 0.03 mg/mL [15].

In a study by Mohseni et al. (2019), the ethanolic extract of *Juglans regia* (walnut) demonstrated bactericidal effects against *L. garvieae* at concentrations of 37.5 mg/mL and 18.75 mg/mL [16]. Similarly, Taheri et al. (2014) investigated the antibacterial activity of *S. lavandulifolia* and found that both ethanolic and methanolic extracts exhibited strong bactericidal properties at high dilutions, with the exception of *E. coli*, which showed resistance to the extracts [17]. These findings underscore the potential antimicrobial efficacy of *S. lavandulifolia* and other plant extracts against various bacteria, including *L. garvieae*. The presence of specific secondary metabolites, such as flavonoids, in these extracts may contribute to their antibacterial activity.

In this study, extracts of *T. ammi* and *S. lavandulifolia* were prepared using both aqueous and ethanol solvents. The aqueous extract primarily contains water-soluble substances, whereas the ethanolic extract can encompass compounds with lower polarity. Alcoholic solvents are more effective at extracting higher levels of plant materials. It is noteworthy that most known antimicrobial compounds are herbal, cyclic, or organic compounds typically extracted using ethanol or methanol solvents. The relatively low activity of aqueous extracts can be attributed to the lower concentration of water-soluble phenolic compounds.

In a study conducted by Bashyal and Guha [18], different extracts including methanol, acetone, chloroform, and aqueous extracts of *T. ammi* seeds were analyzed for their antimicrobial activity against *E. coli*. Each extract was tested using 70 μL, and it was found that the methanolic extract exhibited the highest antibacterial activity against *E. coli* compared to the other three extracts. Phytochemical analysis of the methanolic extract revealed the presence of alkaloids, carbohydrates, saponins, flavonoids, proteins, and glycosides. On the other hand, the aqueous extract only contained alkaloids. Based on these findings, the researchers concluded that the diverse phytochemical constituents present in *T. ammi* seeds might contribute to their antibacterial properties [18]. It is crucial to consider the properties of the solvent used for extractin. Ideally, solvents should have low toxicity, evaporate easily at low temperatures, and not form bonds with the active compounds or lead to their degradation. The choice of solvent is influenced by the polarity of the target compounds to be extracted. Ethanol and methanol are commonly employed for extracting aromatic or organic antimicrobial compounds. Extracts obtained using organic solvents generally demonstrate enhanced stability and antimicrobial [10].

Gram-positive bacteria generally exhibit greater sensitivity to antibacterial compounds compared to gram-negative bacteria. The presence of an outer membrane surrounding the cell wall in gram-negative bacteria may contribute to their reduced susceptibility to the effects of these extracts. This outer membrane acts as a barrier, restricting the penetration of the extracts and potentially limiting the release of water through the lipopolysaccharide lining (outer membrane component) [19].

Phytochemical analysis of S. *lavandulifolia* has revealed the presence of several secondary metabolites, including diterpenes, phenyl ethanoid glycosides, flavonoids, and saponins. Among these compounds, flavonoids have been suggested to be responsible for the antimicrobial activity of the plant [20].

These in vitro studies provide valuable insights into the effective concentrations of these extracts against target bacteria and clinical strains, as well as the potential side effects associated with their use. The goal of future research should be to evaluate the precise formulation of these extracts and conduct in vivo studies accordingly. Such research will increase our understanding of the potential applications of these extracts in the treatment of bacterial infections.

Conclusion

In conclusion, the findings of this study demonstrate that both *T. ammi* and *S.*

lavandulifolia exhibit significant antimicrobial activity against *L. garvieae*, especially at higher concentrations. The ethanolic extracts of both plants show greater zones of inhibition compared to their aqueous counterparts, indicating that ethanol is a more effective solvent for extracting antimicrobial compounds. Notably, the ethanolic extract of *T. ammi* at higher concentrations (150 mg/mL) performs comparably or even better than some common antibiotics like streptomycin and erythromycin. Although *S. lavandulifolia* exhibits substantial antimicrobial activity, its effectiveness is slightly lower than that of *T. ammi*. These results support the potential effect of *T. ammi* and *S. lavandulifolia* extracts as alternative treatments in aquaculture to combat bacterial infections and reduce reliance on traditional antibiotics, thereby mitigating the risk of antibiotic resistance. Continued research and development of these plant extracts could lead to the creation of effective, natural antimicrobial agents for use in aquaculture and other fields.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

Ethics approval was not required for this study.

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