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Assessing of the antibacterial properties of thyme (Thymus vulgaris) essential oil against Streptococcus spp. isolated from clinical cases in Nile tilapia: an in vitro study

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ABSTRACT

In the current study, an agar disc diffusion test was used to determine whether or not the thyme essential oil (TEO) had antibacterial properties against pathogenic fish Streptococcus spp. Using gas chromatography-mass spectrometry (GC-MS), the analyzed essential oil of thyme showed a total of 47 different chemical components. Thymol, P-cymene, terpinene, linalool, and endo-borneol were the most abundant active components that were quantified. Three bacterial strains (*Streptococcus agalactiae*, *Streptococcus iniae*, and *Streptococcus faecalis*) that cause streptococcosis in tilapia were chosen for the purpose of invitro evaluation of the antimicrobial activity of TEO at different concentrations against gentamycin which served as the control positive. The results showed that the zone of inhibition for *Streptococcus agalactiae* was greatest at concentrations of 1:1 and 1:2. However, when compared to gentamycin, the zone of inhibition for concentrations of 1:3 was not significant. Compared to gentamycin, the inhibitory zones produced by TEO against *Streptococcus iniae* and *Streptococcus faecalis* were not significant. In overall, TEO showed efficacy in suppressing the growth of the three strains of Streptococcus, with the most successful outcomes shown in inhibiting the growth of *Streptococcus agalactiae*.

1. INTRODUCTION

Disease outbreaks in the aquaculture sector are thought to have cost more than US$ 6 billion a year worldwide (Cantrell, 2023). The largest problem facing aquaculture is disease management (Naylor et al., 2023). The use of antibiotics is a traditional method to prevent disease outbreaks, especially bacterial infections (Chen et al., 2020). This approach leads to environmental hazards and increases the antibiotic resistance of the bacteria (Sapkota et al., 2008; Assefa and Abunna, 2018). So Particular attention has shifted towards alternative methods using distinctive products, particularly plant-derived natural products, to produce safe, cost-effective, and environmentally friendly substances for the control of fish diseases (Awaad 2017; Brum et al., 2018). There has been a substantial increase in the use of essential oils to manage diseases in fish (Wickramanayake et al., 2022). The essential oils are a blend of organic fat-soluble compounds with strong scents produced by aromatic plants during their metabolism (Carson and Hammer, 2011). The antibacterial properties of essential oil constituents develop from aldehydes and phenolics, which consistently exhibit superior antibacterial activity (Ultee et al., 2002). Additionally, the antibacterial action of essential oils occurs through the disruption of the phospholipid bilayer of the cell membrane, enzyme systems, and genetic material of bacteria (Nazzaro et al., 2013). Thyme essential oil (TEO) is one of the studied alternatives derived from Thymus vulgaris, exhibiting antibacterial, antiviral, and antifungal properties (Badera et al. 2010; Dawood et al., 2021). Thymol constitutes the main component of thyme essential oil, which is accountable for its biological and pharmaceutical characteristics (Dauqan and Abdullah 2017; Halat et al., 2022). Hence Elkomy et al., (2017), proved that TEO was effective in enhancing kidney and liver enzyme functions, increased antioxidant enzyme activity, and protected tissues from oxidative damage in rats subjected to paracetamol toxicity.

In the aquaculture sector, TEO effectively increased the resistance against *Aeromonas hydrophila* infection in Oreochromis niloticus (O. niloticus) (Salam et al., 2021) and common carp (Cyprinus carpio) (Ghafarifarsani et al., 2022). Furthermore, it boosted fish growth performance, antioxidant capacity, and immunity (Abutbul et al. 2004; Valladão et al., 2019; El Euony et al., 2020). Thymol has received approval from the US Food and Drug Administration as a recognized safe molecule for use as a food additive (Johny et al., 2010). This authorization provides researchers with greater potential to incorporate thymol into the treatment regimens for diseases associated with food animals, particularly fish. In the present investigation, the antibacterial activity of TEO at different concentrations was studied against pathogenic streptococcus spp. using agar gel diffusion test.

2. MATERIAL AND METHODS

All procedures used during this study had been approved by the Scientific Research Ethics Committee, Faculty of Veterinary Medicine, Benha University (BUFVTM15-10-22).

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2.1. Thyme essential oil (TEO)

Thyme essential oil was purchased from the National Center for Agricultural Research, El Dokki, Egypt. The bioactive compounds were analyzed using GC-MS (Agilent Technologies) at the Faculty of Postgraduate Studies for Nanotechnology, Cairo University.

2.2. Determination of thyme oil's active metabolites using GC-MS

A gas chromatography-mass spectrometry (GC-MS) analysis was conducted at the Faculty of Postgraduate Studies for Nanotechnology, Cairo University. Utilizing the Agilent GC-MS-5975C equipped with a Triple-Axis Detector and an autosampler. The gas chromatography (GC) column was integrated with a silica capillary column measuring 30 m in length, 0.25 mm in diameter, and 0.25 µm in film thickness, utilizing helium as the carrier gas at a flow rate of 1 ml/min. The mass spectrometer operated in electron impact (EI) mode at 70 eV, with a scan range of 40-700 m/z. The split ratio was established at 1:10, with an injection volume of 1 µl. The injector temperature was set at 250 °C, and the oven temperature was held at 40 °C for 3 minutes before increasing to 300 °C for 4 minutes. The total duration of the run was 62 minutes.

2.3. Determination of the antibacterial activity

The thyme essential oil was tested against different bacterial pathogens for their antibacterial activity using agar disc diffusion. The tested bacterial strains were *Streptococcus agalactiae*, *Streptococcus iniae*, and *Streptococcus faecalis* which were identified and verified at the Department of Aquatic Animal Medicine, Benha University, Egypt (El-diam et al., 2023), and stored at -80 ºC until use. Bacterial strains were activated using nutrient broth and incubated at 30 ºC for 24 h. The suspensions of organisms were initially adjusted with sterile saline (0.7%) to a density equivalent to the 0.5 McFarland standards.

2.3.1. Agar disc diffusion method

The investigation was carried out in the Laboratory of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Benha University, Egypt. The antimicrobial susceptibility test was conducted using the agar disc diffusion method as described by CLSI (2012) and Balouiri et al. (2016). In this study, Mueller-Hinton agar; MHA (Oxoid, UK) with a pH of 7.4 ± 0.2 was used. Approximately 20 ml of the autoclaved medium was poured into sterile plates and allowed to solidify under aseptic conditions at a laminar flow hood. Approximately 0.2 ml of a 24-h bacterial culture was evenly distributed on the sterile MHA plates with sterile swab. A 100-microliter of the tested TEO was combined with DMSO in ratios of 1:1, 1:2, and 1:3. Subsequently, 100 microliters from each ratio and DMSO (Control negative) were placed onto a sterile Whatman No. 1 filter paper disc with a diameter of 6 millimeters. Following the process of air drying, the discs were positioned onto the MHA plates and exposed to each of the specific bacteria described earlier in triplicate. A disc was saturated with an equivalent amount of standard gentamycin solution (10 µ g) served as the control positive (Paller and Dalmacio, 2022). Following 24 h incubation period at 30 ºC, the inhibition zones were measured to the closest millimeter, and the average of the zones from these replicates was recorded.

The activity index (AI) was applied to compare the antibacterial activity of each concentration of thyme oil against all bacterial pathogens with that obtained from standard antibiotics (Shobier and El Ashry, 2021).

$$AI = \frac{\text{Mean of TEO inhibition zone diameter}}{\text{Mean of the standard antibiotic inhibition zone diameter}}$$

2.4. Statistical analysis

Results are expressed as the mean ± standard error (SE) of three replicates. The statistical tests were conducted to ascertain the level of significance utilizing one-way analysis of variance (ANOVA, Version 22) at a probability limit of $P \leqslant 0.05$. The Duncan post hoc test was employed to evaluate variances among sample means.

3. RESULTS

3.1. Thyme essential oil GC-MS analysis

The principal bioactive compounds identified through GC-MS analysis in the TEO are listed in Table (1) and illustrated in Figure (1), accompanied by their respective retention times (RT), area percentages and molecular weight. The chromatograms obtained from GC-MS analyses have revealed the presence of numerous bioactive compounds in the TEO. The analysis of thyme oil revealed that 83% of the peaks corresponded to the primary chemical constituent, which was identified as an organic compound. The primary metabolites identified included thymol (Rt = 13.91 min) with an area of 0.75%, followed by P-cymene (Rt = 5.54 min) with an area of 5.79%, terpinene (Rt = 6.26 min) with an area of 7.03%, linalool, and endo-borneol. Caryophyllene oxide exhibited a retention time of 19.14 minutes and accounted for 1.56% of the area.

Table (1) GC-MS chromatogram thyme essential oil retention time (min) and area (%) of the various compounds:

Retention time	Compound name	Area %	Area	Molecular formula	Molecular weight
13.91	Promecarb; (Carbamult)	0.75	745259314.08	C12H17NO2	207
13.91	Phenol,5-Methyl-2-(1-methyl ethyl) Thymol	0.75	745259314.08	C10H14O	150
5.54	p. cymene	5.79	5744082716.51	C10H14	134
6.26	Terpinene	7.03	6982086504.10	C10H16	136
19.14	Caryophyllene oxide	1.56	1552977557.11	C15H24O	220

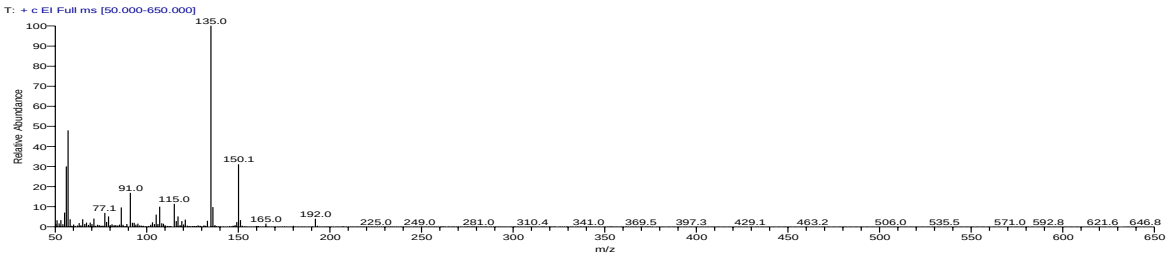

Figure (1). Showing GC-MS curve reading illustrating the active metabolites in thyme oil.

3.2. Antibacterial activity using agar disc diffusion test

The results presented in Figure (2) and Table (2) revealed that bacterial strains responded differently according to the concentration of thyme oil and gentamycin, which served as the positive control. The recorded results revealed that thyme oil at concentrations (1:1 and 1:2) has higher antibacterial activity against S.agalactia with an inhibition zone (1.2±.05 mm and 1.1±.05 mm) respectively, compared to (1:3) (1±.05 mm) and gentamycin (0.9 ±.05 mm). On the other hand, all concentrations give inhibition zone without significance compared to gentamycin against S. iniae. while in S. faecalis plates, the inhibition zones of TEO at concentration 1:2 and 1:3 were significantly decreased than those produced by gentamycin.

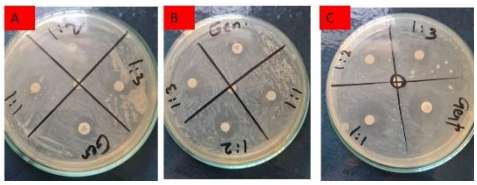

Figure (2). In vitro antibacterial assay of studied thyme oil against A: *S. iniae*, B: *S. faecalis*, C: *S. agalactiae*, where letters Gen= gentamycin (control +ve), central disc was DMSO (Control –ve)

Table (2). Inhibition zones represented as means of thyme oil with different concentration and gentamycin (control +ve) against *S. iniae*, *S. faecalis*, and *S. agalactiae*.

Conc.	*S. faecalis*	*S. iniae*	*S. agalactiae*
1:1	1.7±0.04a	1±.05	1.2±.05a
1:2	1.4±0.04b	1.1±.05	1.1±.05ab
1:3	1.2±.03c	1.2±.05	1±.05bc
Gentamycin (control)	1.8±.05 a	1±.05	0.9±.05c

Means that have different letters in the same column are considered as significantly different at $P < 0.05$.

3.3. Activity Index

As illustrated in Figure (3), the activity index (AI) of the thyme oil revealed that (1:1) and (1:2) concentrations recorded significant differences compared to the positive control antibiotic against S. agalactiae which indicate the sensitivity of S. agalactiae to TEO. while the AI of three TEO concentration against S. faecalis and S. iniae was non-significant.

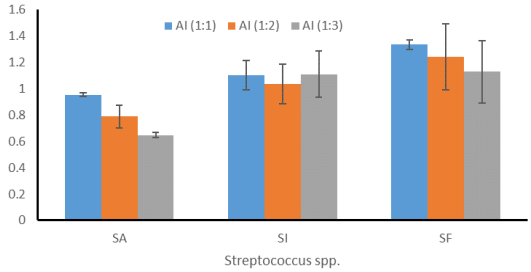

Figure (3). showing Activity Index (AI) of the different thyme oil concentrations (1:1, 1:2 and 1:3) against *S. agalactiae* (SA), *S. iniae* (SI), and *S. faecalis*(SF). Means that have different letters significantly different at $P < 0.05$.

4. DISCUSSION

Studies have mostly focused on the inherent active constituents of botanicals, attributed to their exceptional antibacterial efficacy, ecological sustainability, and safety (Rahman, et al., 2021).

The principal component of thyme essential oil is thymol, which is extracted from Lamiaceae plant families' including Thymus vulgaris. The derivative of this monoterpene finds application across various sectors, including food, medicine, fragrance, and pest control (Rivas et al., 2010; Meeran et al., 2017). Extensive research has demonstrated that thyme exhibits a range of beneficial properties, including anti-inflammatory, anticancer, immunomodulatory, and antioxidant effects, observed in both in vivo and in vitro studies (Alagawany et al. 2021)

Consequently, thymol serves as an effective agent in the treatment of disorders affecting the neurological, respiratory, and cardiovascular systems (Meeran et al., 2017; Salehi et al., 2018). Moreover, thyme exhibits a pronounced inhibitory effect on a range of bacteria, encompassing both gram-positive and gram-negative strains, such as Salmonella spp. (Zhou et al., 2007) *Aeromonas hydrophila* (Liang et al., 2021), *Listeria monocytogen* (Ilhak and Guran, 2014), and *Streptococcus pneumoniae* (Lakis et al., 2012).

The GC-MS analysis conducted in this study revealed that thyme oil contained significant quantities of thymol (51.94%), P-cymene (14.5%), terpinene (10.09%), linalool (3.48%), and endo-borneol (3.95%). Where, thymol with an area of 0.75%, followed by P-cymene with an area of 5.79%, terpinene with an area of 7.03%, and other bioactive compounds. The obtained results were in accordance with those obtained by Moualla, and Naser (2015); Cutillas et al., (2018). Thymol (2-isopropyl-5-methylphenol) constitutes a significant component of essential oils derived from the Lamiaceae family (Abdollahzadeh et al., 2014). The presence of the phenolic hydroxyl group in the thymol may be a contributing factor to its antimicrobial activity (Soleimani et al., 2022).

In the present study thyme oil was effective in inhibiting the growth of three different streptococcus spp. namely; *Streptococcus agalactiae*, *Streptococcus iniae*, and *Streptococcus faecalis* in vitro. Nearly similar findings were reported by Tural et al., (2019) who found that TEO inhibited the growth of Yersinia ruckeri, Pseudomonas fluorescens, Aeromonas sobria, Aeromonas salmonicida, Aeromonas veronii and Lactococcus garvieae with an inhibition zone ranged from 26.50 to 36.0 mm. Similarly, Navarrete et al., (2010) proved that TEO inhibited the growth of *Vibrio ordalii*, *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Flavobacterium psychrophilum* in vitro. In addition to the study of Liang et al., (2021) who confirmed the in vitro antibacterial activity of thymol against *Aeromonas hydrophila*.

The studies concerning the antibacterial activities of TEO against gram-positive fish pathogens are scarce, but in vivo, trials for modulating fish immunity, and antioxidant capacity or for controlling bacterial infection are more abundant (Gulec, 2013; Valladão et al., 2019; El Euony et al., 2020; Salam et al., 2021). In a recent study by Korni et al., (2023), TEO significantly contributed to the prevention of streptococcosis in Nile tilapia.

5. CONCLUSIONS

The study findings indicate that thyme essential oil has substantial antibacterial efficacy against the tested bacterial fish pathogens. As a result, this oil can act as a potential natural alternative for antibiotics in aquaculture. However, more studies are needed to identify the specific active chemical substances that are responsible for the antimicrobial properties and determine their safety and effectiveness in vivo.

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