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Scientific Research

Evaluation of chemical properties and antimicrobial effect of *Thymus trautvetteri* essential oil on a number of bacteria causing infection and food poisoning: a laboratory study

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ABSTRACT

Chemical properties and antimicrobial effect of *Thymus trautvetteri* essential oil against some of the bacteria causing infection and food poisoning were evaluated in this study. For this purpose, *T. trautvetteri* essential oil was extracted with the help of hydrodistillation method and its total phenol content was based on Folin-Ciocalteu method, its antioxidant activity was based on DPPH and ABTS free radical inhibition methods and its antibacterial effect against *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* were investigated based on disc diffusion agar, well diffusion agar, minimum inhibitory concentration and minimum bactericidal concentration methods. The results showed that *T. trautvetteri* essential oil contained 6.27 mg of catechin/g total phenol and its inhibitory activity against DPPH and ABTS free radicals was obtained as 12.32 and 10.20 mg/ml, respectively. The results of the antibacterial activity of the essential oil showed that *S. aureus* and *E. coli* were the most sensitive and resistant bacterial strains against *T. trautvetteri* essential oil, respectively. The inhibition zone in the disc diffusion agar and well diffusion agar methods, and the minimum inhibitory and bactericidal concentrations for *S. aureus* were 14.60 mm, 16 mm, 1 mg/ml and 4 mg/ml, respectively. According to the findings of this research, *T. trautvetteri* essential oil can be used as a natural antioxidant and antimicrobial agent to prevent oxidative reactions and microbial spoilage in food.

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1- Introduction

In recent years, there has been significant attention given to the increase of diseases caused by foodborne pathogens. These pathogens pose a threat to the health and safety of human, animal, and plant populations worldwide. Major pathogenic bacteria, including *Campylobacter*, *Salmonella*, *Listeria* monocytogenes, *Escherichia coli* O157:H7, *Staphylococcus*, and *Bacillus cereus*, are responsible for various diseases [1-3]. Contaminated food has been linked to approximately 250 types of foodborne illnesses. In the United States alone, it is estimated that foodborne illnesses affect 76 million people annually, resulting in 5,000 deaths each year [4]. Food degradation is often attributed to oxidation, a complex reaction that leads to detrimental changes in nutritional value, sensory characteristics, overall quality, and the formation of potentially toxic compounds. Foods that undergo extensive oxidation exhibit significant defects and are generally rejected by consumers. Oxidation can manifest as discoloration and the development of off-flavors [5-7]. To combat microbial growth and oxidation during food processing and storage, synthetic additives and antimicrobial/antioxidant agents are commonly employed. These measures aim to prevent contamination and extend the shelf life of food products [8, 9]. However, synthetic chemical additives are associated with certain toxic effects, and the use of preservatives has been linked to respiratory and other health problems. For instance, sodium benzoate has the potential for cumulative toxicity and can induce asthma, while nitrate and nitrite can convert ingested materials into toxins [10]. Furthermore, the emergence of antibiotic resistance among foodborne pathogens has complicated the task of ensuring a safe food supply, as microorganisms have become more resistant to conventional preservatives [12]. Consequently, there is a growing demand for natural inhibitors as alternatives to synthetic antimicrobial agents due to consumer preferences for processed and antibiotic-free foods [3, 9, 13-17]. Essential oils, volatile liquids extracted from various parts of aromatic plants, have garnered attention for their potential antimicrobial and antioxidant properties. These oils are derived from plant sources such as bark, seeds, flowers, fruits, roots, leaves, and whole plants. The International Standard Organization defines essential oil as "a product obtained from natural raw materials of plant origin, by steam distillation, mechanical processes from citrus epicarps, or dry distillation, after separation of water, if any, by physical processes" [18]. Among the plant families known for their medicinal value, the mint family holds significant importance. The Thymus genus consists of approximately 350 species distributed across Eurasia, the northern part of Africa, and southern Greenland. Human activity has also led to their spread worldwide. In Iran, there are 14 species, including Talshi thyme (*T. trautvetteri*), Kermani thyme (*T. carmanicus*), Danai thyme (*T. daenesis*), and

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Iranian thyme (*T. persicus*). Thyme essential oil is known for its antimicrobial and antiseptic properties, attributed to its high phenol content [19]. However, limited information is available regarding the chemical composition, antioxidant effect, and antimicrobial activity of *T. trautvetteri* essential oil in scientific literature. *T. trautvetteri* is characterized as a small shrub or cushion plant with branched stems, a woody base, hairy leaves measuring 5 to 8 mm in length and 2.5 to 5.5 mm in width, petiolate with rarely two or three pairs of veins, and a 3-5.5 mm long narrow calyx. It blooms from mid-spring to mid-summer, with purple flowers. Among the 49 identified compounds, thymol, borneol, parasimene, gammaterpinene, alphapinene, and carvacrol are among the most important components of *T. trautvetteri* essential oil [20]. This study aims to extract the essential oil from *T. trautvetteri*, investigate its phenol content, evaluate its antioxidant effect, and determine its antimicrobial activity against bacteria known to cause infections and food poisoning, such as *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Bacillus cereus*.

2- Materials and methods

2-1- Essential oil extraction

The extraction of *T. trautvetteri* essential oil was performed following the method described by Shahnazi et al. (2006). Firstly, the aerial parts of the plant were dried and then turned into powder using an electric mill. Next, 100 grams of the dried plant powder were placed in the Clevenger, and essential oil extraction was carried out for 4 hours. The resulting essential oil was dehydrated using anhydrous sodium sulfate and then stored in a dark, closed container at a temperature of 4°C [21].

2-2- Total phenol content

The total phenol content of the essential oil was determined using the Folin-Ciocalteu method. Briefly, 300 µL of the essential oil ethanol solution (0.1 mg/mL) was added to a test tube, followed by the addition of 2.5 mL of Folin-Ciocalteu 0.2 normal reagent and 2 mL of sodium carbonate (7.5% w/v). The test tube was then stirred and heated at 50°C for 5 minutes. The absorbance was measured at a wavelength of 760 nm, and the results were expressed as the equivalent amount of catechin per gram of essential oil [22].

2-3- Antioxidant activity

2-3-1- DPPH radical inhibition

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical inhibition ability of the essential oil was investigated following the method described by Fallah et al. (2021). For this purpose, 50 microliters of the ethanolic solution of essential oil with different

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concentrations (5-0.1 mg/ml) were mixed with 1 ml of DPPH ethanolic solution (0.004%). The resulting solution was kept in the dark for 1 hour, and then the absorbance was recorded at a wavelength of 517 nm. The IC_{50} value, which represents the amount of sample required to reduce DPPH uptake by 50%, was calculated by plotting the percent inhibition against the sample concentration. The DPPH radical inhibition percentage was calculated using the following equation:

$$\%inhibition = [(AC-AS)/AC)] \times 100$$

where AC is the absorption of the control sample, and AS is the absorption of the sample [23].

2-3-2- ABTS radical inhibition

The ABTS (2,20-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) analysis was performed according to the method described by Fallah et al. (2021) with minor changes. The ABTS radical was prepared by mixing ABTS and potassium persulfate solutions and keeping them at room temperature in the dark. The ABTS radical stock solution was then diluted in 50 mM potassium phosphate buffer (pH 8.0) until its absorbance reached 0.9 at 734 nm, and the pH of the final mixture was adjusted to 7.4. Next, 100 µl of the ethanolic solution of essential oil with different concentrations (0.1-5 mg/ml) was mixed with 1 ml of ABTS, and the absorbance was measured at 734 nm. The IC_{50} values of the samples, as defined in the DPPH method, were determined using the following equation:

$$\%inhibition = [(AC-AS)/AC)] \times 100$$

where AC is the absorption of the control sample, and AS is the absorption of the sample [24].

2-4- Antimicrobial activity

The antimicrobial effect of *T. trautvetteri* essential oil against *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Bacillus cereus* was investigated using the following methods.

2-4-1- disk diffusion agar

In this antimicrobial method, blank disks were immersed in a solution of thyme essential oil for 15 minutes and then placed on the surface of Mueller Hinton agar culture medium containing microbial strains. The Petri dishes were then kept in a greenhouse at 37°C for 24 hours. The diameter of the growth halo around the disks was determined and reported as the antimicrobial effect [25].

2-4-2- well diffusion agar

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In this method, several wells were created on the surface of Mueller Hinton agar culture medium using a sterilized Pasteur pipette. Then, 20 microliters of essential oil were transferred into each well. The plates, which were previously infected with bacterial species, were placed in a greenhouse according to the conditions stated for the agar disk diffusion method. Finally, the diameter of the growth halo around the wells was determined and expressed as the antimicrobial effect of the essential oil [25].

2-4-3- Minimum inhibitory concentration

The minimum inhibitory concentration was determined using the broth microdilution method (96-well plate and triphenyltetrazolium chloride reagent). In this method, the final concentration of *T. trautvetteri* essential oil in the 96-well plates varied from 0.5 to 256 mg/ml. A 96-well plate containing 200 microliters of essential oil and 20 microliters of bacteria was incubated at 37°C for 24 hours. After adding 20 microliters of 5% triphenyltetrazolium chloride to the wells, the plate was placed in the greenhouse again for 30 minutes. Microbial growth causes a dark red color to appear in the wells. The first concentration of essential oil that was able to suppress microbial growth and prevent the formation of the red color was considered as the minimum inhibitory concentration [26-28].

2-4-4- Minimum bactericidal concentration

To determine the minimum bactericidal concentration, the contents (100 µl) of all wells without red color were cultured separately on Mueller Hinton agar medium. The environments containing bacteria were then placed in a greenhouse at 37°C for 24 hours. The minimum concentration of essential oil that prevented the formation of microbial colonies was reported as the minimum bactericidal concentration [26].

2-5- Statistical analysis

All tests were repeated three times. The SPSS statistical software (version 18), one-way analysis of variance, and Duncan's multi-range test were used to compare between groups ($p < 0.05$).

3. Results and discussion

Thyme plant and its essential oil have long been used for the treatment of upper respiratory tract infections, bronchitis symptoms, and parasitic infections. Nowadays, it is commonly employed as an expectorant for coughs associated with colds and as a

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disinfectant in dentistry. Thyme oil exhibits antibacterial effects against both Gram-positive and Gram-negative bacteria, as well as antiviral, antifungal, antioxidant, and anti-inflammatory activities [29].

The total phenolic content of *T. trautvetteri* essential oil was determined to be 6.27 ± 0.19 mg of catechin per gram of essential oil. Upon reviewing scientific sources, it was found that no study has been conducted on the specific number of phenolic compounds in *T. trautvetteri* essence. Shahbazi et al. (2006) reported the composition of *T. trautvetteri* essential oil, which includes 24.43% thymol, 11.36% borneol, 10.09% paracimen, 7.78% gammaterpinene, 5.29% alphapinene, and 5.07% carvacrol. Additionally, oxygenated monoterpenes, hydrocarbon monoterpenes, hydrocarbon sesquiterpenes, and oxygenated sesquiterpenes constitute 53.41%, 36.97%, 6.77%, and 1.9% of the essential oil, respectively [21]. Kandachi and Jamzad (2015) demonstrated that aqueous, methanolic, and chloroform extracts of *T. trautvetteri* contain 141.24 mg/L, 207.64 mg/L, and 146.84 mg/L of total flavonoids, respectively [19].

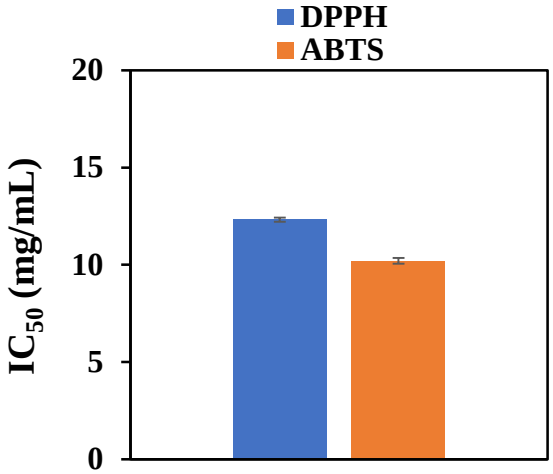

Figure 1. The antioxidant activity of *Thymus trautvetteri* essential oil based on DPPH and ABTS radical scavenging methods.

The results of the antioxidant activity of *T. trautvetteri* essential oil are depicted in Figure 1. Based on the findings, the essential oil exhibited an IC_{50} value of 12.32 mg/ml in inhibiting DPPH free radicals and 10.20 mg/ml in inhibiting ABTS free radicals. The antioxidant activity of *T. trautvetteri* essential oil has not been previously investigated. Motlo-Inguk et al. (2021) reported that *Thymus vulgaris* essential oil had a total phenolic content of 193 mg of gallic acid per liter and showed antioxidant activity against DPPH and ABTS free radicals with IC50 values of 4 mg/ml and 0.08 mg/ml, respectively [30]. Bozin et al. (2006) demonstrated that thyme and oregano essential oils exhibited higher antioxidant activity (with similar IC_{50} values of 0.19 µg/ml and 0.17 µg/ml, respectively) compared to basil essential oil or the synthetic antioxidant BHT [31]. It is worth noting that IC_{50} values obtained using different methods can vary significantly. For instance, Bendaud et al.

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(2010) found that the IC_{50} value determined by the DPPH method for *Schinus molle* L. essential oil was approximately 14 times higher than the IC_{50} value obtained by the ABTS method. According to the study by D'Souza et al. (2019), phenolic compounds such as thymol, eugenol, and carvacrol are potent antioxidant agents in essential oils due to their ability to donate hydrogen atoms to free radicals and convert them into more stable products [32]. Other components, such as specific alcohols, ethers, ketones, aldehydes, and monoterpenes like linalool, 1,8-cineole, geranial/neral, citronelal, isomenthone, and menthone, also contribute to the antioxidant properties of essential oils [33]. The results of the antimicrobial effect of *T. trautvetteri* essential oil against bacterial strains, determined using the agar disk diffusion method, are presented in Figure 2. The antimicrobial activity of the essential oil varied depending on the type of bacteria, with the diameter of the non-growth zone ranging from 10.50 to 14.60 mm. *Staphylococcus aureus* exhibited the highest sensitivity, with a non-growth zone diameter of 14.60 mm, while *Escherichia coli* showed the lowest sensitivity, with a non-growth zone diameter of 10.50 mm. Gram-negative bacteria (*Escherichia coli* and *Shigella dysenteriae*) were found to be more resistant to essential oils compared to Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*).

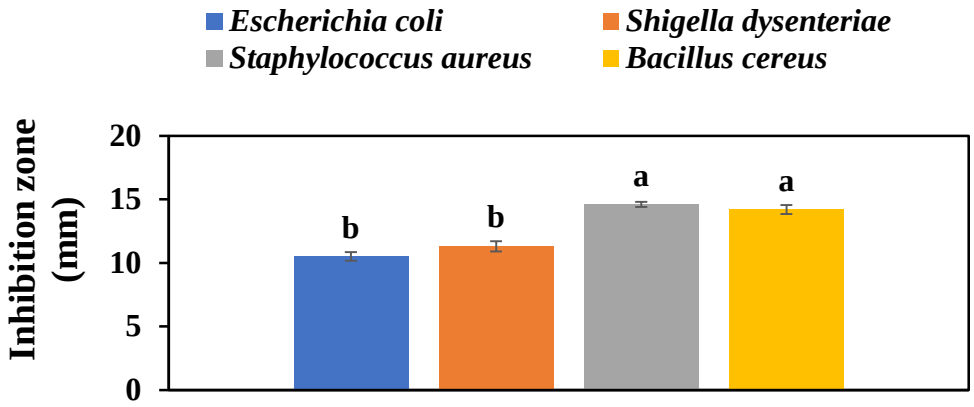

Figure 2. The antibacterial activity of *Thymus trautvetteri* essential oil based on disc diffusion agar method.

According to the antimicrobial results obtained from the agar well method (Figure 3), the diameter of the non-growth halo for bacterial strains ranged from 11.30 to 16 mm. Among the strains, *Staphylococcus aureus* exhibited the largest non-growth halo with a diameter of 16 mm, indicating high sensitivity to *T. trautvetteri* essential oil. On the other hand, *Escherichia coli* showed the smallest non-growth halo with a diameter of 11.30 mm, suggesting resistance to the essential oil. It is worth noting that Gram-positive bacteria showed higher sensitivity to the essential oil

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compared to gram-negative bacteria. The lower sensitivity of Gram-negative bacteria can be attributed to the presence of an outer membrane in their structure, which restricts the diffusion of hydrophobic components of essential oils through the lipopolysaccharide layer [17, 34]. Furthermore, it was observed that the average diameter of the non-growth halo in the agar well method was larger than that in the agar disk diffusion method. This discrepancy can be attributed to the direct contact between microorganisms and the essential oil in the agar well method, whereas in the agar disk diffusion method, the essential oil needs to spread from the surface of the disk into the environment to exhibit its antimicrobial effect [28, 35].

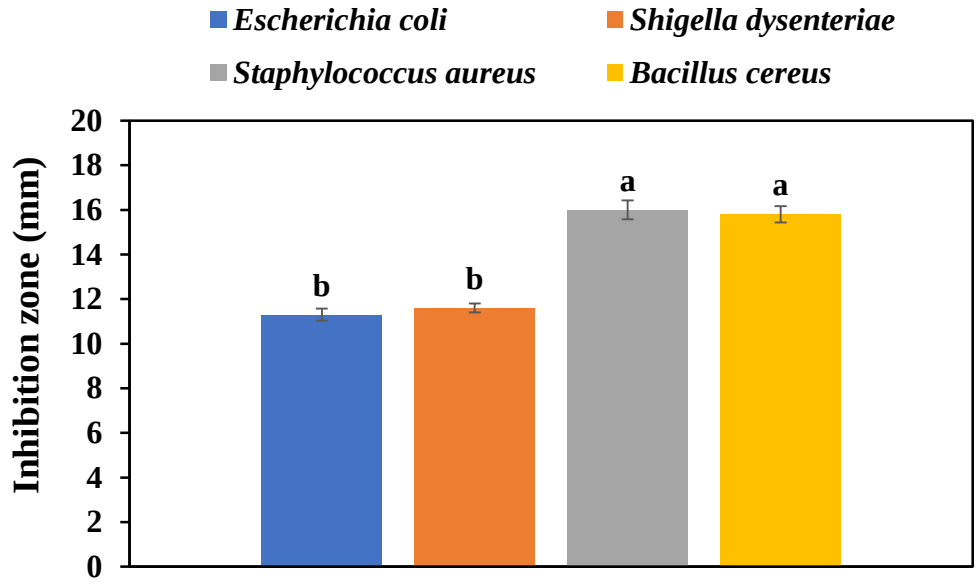

Figure 3. The antibacterial activity of *Thymus trautvetteri* essential oil based on well diffusion agar method.

The results of the minimum growth inhibitory concentration of *T. trautvetteri* essential oil are presented in Table 1. The antibacterial activity of the essential oil was observed to be dependent on both its concentration and the specific type of bacteria. Increasing the concentration of the essential oil from 0.5 to 256 mg/ml resulted in a corresponding increase in its antimicrobial effect. At a concentration of 0.5 mg/ml, all bacterial strains were able to grow despite the presence of the essential oil. However, with the exception of the *Staphylococcus aureus* strain,

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the other strains were able to grow even at a concentration of 1 mg/ml. It was only when the concentration of essential oil reached 2 mg/ml and above that the growth of Gram-positive strains was inhibited. Gram-negative bacteria, on the other hand, were able to grow in the presence of a concentration of 2 mg/ml essential oil, but higher concentrations effectively prevented their growth. In summary, the minimum inhibitory concentration for *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus*, and *Bacillus cereus* was determined to be 4, 4, 1, and 2 mg/ml, respectively.

Table 1. Minimum inhibitory concentration of *Thymus trautvetteri* essential oil

Microorganism	**Essential oil concentration (mg/mL)**										**Negative control**	**Positive control**
	0.5	1	2	4	8	16	32	64	128	256		
Escherichia coli	+	+	+	-	-	-	-	-	-	-	-	+
Shigella dysenteriae	+	+	+	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	+	-	-	-	-	-	-	-	-	-	-	+
Bacillus cereus	+	+	-	-	-	-	-	-	-	-	-	+

+ grown; - not grown

Table 2 shows the results of the minimum bactericidal concentration of *T. trautvetteri* essential oil against pathogenic bacteria. In general, Gram-positive bacteria showed more sensitivity to essential oil than gram negative types. So that the minimum bactericidal concentration for the strains of *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* was 16, 16, 4 and 4 mg/ml, respectively.

Table 2. Minimum bactericidal concentration of *Thymus trautvetteri* essential oil.

Microorganism	**Essential oil concentration (mg/mL)**										**Negative control**	**Positive control**
	0.5	1	2	4	8	16	32	64	128	256		
Escherichia coli	+	+	+	+	+	-	-	-	-	-	-	+
Shigella dysenteriae	+	+	+	+	+	-	-	-	-	-	-	+
Staphylococcus aureus	+	+	+	-	-	-	-	-	-	-	-	+
Bacillus cereus	+	+	+	-	-	-	-	-	-	-	-	+

+ grown; - not grown

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According to Shahnazi et al. (2006), the antimicrobial effect of *T. trautvetteri* essential oil is influenced by both its concentration and the specific type of bacteria. Consequently, increasing the concentration of the essential oil results in an enlargement of the non-growth halo diameter for *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Bacillus subtilis*, and *Proteus mirabilis* [21]. In fact, the study found that *Staphylococcus aureus* exhibited the highest sensitivity to the essential oil of thyme, with a minimum inhibitory concentration of 125 µg/ml. Another study has reported that the methanolic extract of *T. trautvetteri* displays stronger antimicrobial activity against both Gram-positive and Gram-negative bacteria compared to various aqueous and chloroform extracts [19]. Notably, *Staphylococcus aureus* was identified as the most sensitive strain to the methanolic extract, exhibiting the highest average diameter of the non-growth halo. The antimicrobial activity of thyme essential oil is influenced by the percentage of its main compounds. Essential oils with a higher proportion of phenolic monoterpene compounds, particularly thymol, possess stronger antibacterial properties due to their structural characteristics. These small and hydrophobic particles can easily penetrate lipid barriers. The essential oil compounds target the cell membrane of pathogens, given its hydrophobic composition. Studies on the mechanism of antibacterial activity of thymol have demonstrated that it has the ability to integrate into the lipid layer of the cell membrane, thereby increasing surface curvature. The hydrophilic part of the molecule interacts with the polar region of the membrane, while the hydrophobic benzene ring and aliphatic side chains penetrate the inner part of the biological membrane. As a result, the membrane structure undergoes various changes, including destabilization of the lipid layer, reduced elasticity, and increased fluidity. These changes lead to increased permeability to potassium and hydrogen ions and affect the activity of inner membrane proteins, such as enzymes and receptors. Thymol also interacts with proteins embedded in the cell membrane through non-specific mechanisms, resulting in alterations to the structure and activity of both internal and membrane proteins. Consequently, the presence of thymol induces tension and destabilization of the cell membrane. Carvacrol acts in a similar manner to thymol, targeting the bacterial membrane [29].

4- Conclusion

The essential oils of thyme species have a high antioxidant and antimicrobial capacity due to their rich composition of non-volatile molecules. *T. trautvetteri* essential oil exhibited a significant phenol content, and its antioxidant capacity against DPPH and ABTS free radicals was noteworthy. Furthermore, *T. trautvetteri* essential oil demonstrated effectiveness against both Gram-negative and Gram-positive bacteria, with *Staphylococcus aureus* being the most susceptible bacterial strain to the essential oil. However, further studies are required to identify the primary compounds responsible for the antioxidant and antimicrobial activity of *T. trautvetteri* essential oil, as well as to understand their mechanism of action. Additionally, future studies should explore the potential utilization of thyme essential oil in various food products and its effectiveness in treating infectious diseases.

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ارزیابی ویژگی‌های شیمیایی و اثر ضدمیکروبی اسانس آویشن تالشی بر تعدادی از باکتری‌های عامل عفونت و مسمومیت غذایی: یک مطالعه آزمایشگاهی

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چکیده

ارزیابی ویژگی‌های شیمیایی و اثر ضدمیکروبی اسانس آویشن تالشی بر تعدادی از باکتری‌های عامل عفونت و مسمومیت غذایی در این مطالعه صورت گرفت. برای این منظور، اسانس آویشن تالشی با کمک روش تقطیر با آب استخراج گردید و مقدار فنول کل آن بر پایه روش فولین سیوکالتو، فعالیت آنتی‌اکسیدانی آن بر اساس روش‌های مهار رادیکال آزاد DPPH و ABTS و اثر ضد باکتریایی آن در برابر باکتری‌های *اشرشیا کلی*، *شیگلا دیسانتری*، *استافیلوکوکوس اورئوس* و *باسیلوس سرئوس* بر پایه روش‌های دیسک دیفیوژن آگار، چاهک آگار، حداقل غلظت مهارکنندگی و حداقل غلظت کشندگی بررسی گردید. نتایج نشان داد که اسانس آویشن تالشی حاوی ۶/۲۷ میلی‌گرم کاتچین در گرم فنول کل بود و فعالیت مهارکنندگی آن در برابر رادیکال‌های آزاد DPPH و ABTS به ترتیب برابر با ۱۲/۳۲ و ۱۰/۲۰ میلی‌گرم در میلی‌لیتر به دست آمد. نتایج فعالیت ضد باکتریایی اسانس نشان داد که باکتری‌های *استافیلوکوکوس اورئوس* و *اشرشیا کلی* به ترتیب حساس‌ترین و مقاوم‌ترین سویه‌های باکتریایی در برابر اسانس آویشن تالشی بودند. میانگین قطر هاله عدم رشد در روش‌های دیسک دیفیوژن آگار و چاهک آگار و حداقل غلظت مهارکنندگی و کشندگی برای *استافیلوکوکوس اورئوس* به ترتیب برابر با ۱۴/۶۰ میلی‌متر، ۱۶ میلی‌متر، ۱ میلی‌گرم در میلی‌لیتر و ۴ میلی‌گرم در میلی‌لیتر مشاهده گردید. مطابق یافته‌های این پژوهش، اسانس آویشن تالشی می‌تواند بعنوان ماده آنتی‌اکسیدان و ضد میکروب طبیعی جهت جلوگیری از واکنش‌های اکسایشی و فساد میکروبی در مواد غذایی مورد استفاده قرار گیرد.

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