Research Square

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GC–MS Characterization and In Vitro Antibacterial Activity of a Standardized Multi–Essential Oil Formulation Containing Eucalyptus globulus, Thymus vulgaris, Zataria multiflora, and Mentha piperita Against Food Spoilage Bacteria

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Abstract

Background:

Food spoilage bacteria significantly reduce shelf life and product quality, leading to economic losses in the food industry. Plant-derived essential oils are increasingly considered safe and sustainable alternatives to chemical preservatives due to their broad antimicrobial activity.

Objective:

This study aimed to evaluate the in vitro antibacterial activity of a standardized plant-based spray formulation against food spoilage bacteria and to characterize its chemical composition using GC–MS analysis.

Materials and Methods:

The standardized spray, obtained from Dineh Pharmaceutical Company (Iran), contained Eucalyptus globulus, essential oils of various Eucalyptus species, Thymus vulgaris, Zataria multiflora, Mentha piperita, menthol, ethanol (70%), and purified water. Antibacterial activity was assessed using agar well diffusion and macro-dilution methods against standard strains of Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, and Salmonella typhi, as well as clinical isolates of Klebsiella pneumoniae and Enterobacter aerogenes. All tests were performed in triplicate.

Results:

The formulation exhibited strong antibacterial activity, with the lowest minimum inhibitory concentration (MIC) observed against Staphylococcus aureus (7.81 µg/mL). Other tested microorganisms also showed high sensitivity.

Conclusion:

The standardized plant-based spray demonstrated broad-spectrum antibacterial activity, highlighting its potential application as a natural antimicrobial agent for food safety–related uses, including food packaging and surface sanitation.

Introduction

Food spoilage caused by pathogenic and spoilage-associated bacteria represents a critical challenge for the food industry, leading to substantial economic losses and potential health hazards. Conventional chemical preservatives, although effective, have raised growing concerns regarding consumer safety and environmental impact, motivating the search for natural and safe alternatives. Plant-derived extracts and essential oils have gained attention due to their bioactive compounds with potent antimicrobial properties. Among these, eucalyptus (Eucalyptus globulus) leaf extract, eucalyptus essential oil, garden thyme (Thymus vulgaris) essential oil, Shirazi thyme (Zataria multiflora) essential oil, peppermint (Mentha piperita) essential oil, and menthol have been recognized for their inhibitory effects against a wide range of foodborne bacteria. Incorporating these compounds into a liquid-based spray formulation offers a promising strategy to prevent microbial growth, improve food safety, and align with the increasing consumer demand for natural, clean-label, and sustainable products.

Plant-derived extracts and essential oils are increasingly recognized as natural alternatives to chemical preservatives due to their antimicrobial properties. Compounds such as eucalyptol, thymol, and menthol have demonstrated inhibitory effects against foodborne bacteria. However, the combined antibacterial potential of multiple plant extracts and essential oils in a liquid-based spray formulation remains underexplored. Essential oils are natural volatile compounds derived from aromatic and medicinal plants that have gained increasing attention as potential natural antioxidants and antimicrobial agents for food applications. These bioactive compounds exhibit significant antimicrobial activity against foodborne pathogens, and their incorporation into active food packaging systems—such as emulsions, nanoemulsions, and coating films—can enhance food safety and extend shelf life. A better understanding of the chemical composition and biological properties of essential oils and their constituents is crucial for optimizing their application in food preservation [1].Recent research has demonstrated that combinations of essential oils and plant extracts can exhibit synergistic antimicrobial activity against a wide range of foodborne pathogens, including Escherichia coli, Salmonella enterica, Staphylococcus aureus, Bacillus cereus, and Listeria monocytogenes. While individual essential oils may not be highly effective against all tested species, paired combinations such as peppermint/thyme have shown enhanced inhibitory effects, suggesting their potential as natural alternatives to synthetic preservatives for food safety applications[2].Despite the growing evidence of antimicrobial activity of individual plant extracts and essential oils, the combined effect of multiple essential oils in a liquid-based spray formulation targeting food spoilage bacteria remains largely unexplored. In this study, we evaluated a plant-based spray containing eucalyptus leaf extract, eucalyptus essential oil, garden thyme (Thymus vulgaris) essential oil, Shirazi thyme (Zataria multiflora) essential oil, peppermint (Mentha piperita) essential oil, and menthol, along with ethanol and water, for its antibacterial potential. This work aims to investigate the synergistic antimicrobial effects of these compounds and assess their practical application as a natural preservative strategy to improve food safety, extend shelf life, and support the development of sustainable, clean-label food products. Essential oils are natural volatile compounds from aromatic plants, known for their antioxidant and antimicrobial activities. They are increasingly studied as alternatives to synthetic preservatives in food systems. Incorporating essential oils into emulsions, nanoemulsions, or coating films in active food packaging can help control spoilage and pathogenic bacteria, enhancing food safety and shelf life. Understanding their chemical composition and biological properties is essential to optimize their application in food preservation[3], [4]..Eucalyptus is a genus of evergreen trees whose leaves produce essential oils rich in bioactive compounds, such as 1,8-cineole, with antimicrobial and antioxidant properties, making them useful in food preservation and pharmaceutical applications [10]. Thymus vulgaris (garden thyme) is an aromatic herb whose essential oil contains thymol and carvacrol, compounds with well-documented antimicrobial and antioxidant properties, making it a promising natural preservative for food systems [11].Zataria multiflora (Shirazi thyme), a thyme-like aromatic herb native to Iran, contains carvacrol and thymol, which have strong antimicrobial and antioxidant activities, making it a valuable natural preservative for food systems[12].Mentha piperita (peppermint) essential oil contains menthol and menthone, which exhibit significant antimicrobial and antioxidant activities, making it a promising natural preservative in food systems[13].Eucalyptus globulus essential oil has been shown to exhibit significant antimicrobial activity against a range of food spoilage microorganisms. In vitro studies demonstrated that E. globulus essential oil effectively inhibited the growth of multiple bacterial and yeast strains, indicating its potential use as a natural preservative in food systems[5].

Studies have shown that Thymus vulgaris essential oil exhibits strong antimicrobial activity against common foodborne pathogens and spoilage bacteria, including Salmonella enterica and Bacillus cereus, and can inhibit biofilm formation, indicating its potential as a natural preservative in food systems [6]. Zataria multiflora essential oil, a thyme-like aromatic herb native to Iran, exhibits significant antimicrobial potential against major foodborne pathogens such as Escherichia coli, Salmonella Typhimurium, Pseudomonas aeruginosa, and Staphylococcus aureus, highlighting its promise as a natural approach to improving food safety and extending shelf life [7]. Peppermint (Mentha piperita) essential oil has shown significant antimicrobial activity against a variety of bacteria, including foodborne and spoilage-associated pathogens. This natural volatile oil, rich in bioactive compounds such as menthol and menthone, has demonstrated antibacterial effects that support its potential as a natural preservative and alternative to synthetic agents in food systems [1]. Menthol, a major component of peppermint essential oil, has been reported to inhibit quorum sensing and biofilm formation in Gram-negative bacteria at sub-MIC concentrations, suggesting its potential as a natural antibacterial agent in food preservation systems [9]. In summary, while essential oils and plant extracts have demonstrated significant antimicrobial and antioxidant activities, the combined effect of multiple plant-derived compounds in a liquid-based spray on food spoilage bacteria remains underexplored. This study aims to evaluate a plant-based spray containing eucalyptus leaf extract, eucalyptus essential oil, garden thyme (Thymus vulgaris) essential oil, Shirazi thyme (Zataria multiflora) essential oil, peppermint (Mentha piperita) essential oil, and menthol, along with ethanol and water, for its antibacterial potential. The findings are expected to provide insights into the development of natural preservative strategies, contributing to food safety, extended shelf life, and the advancement of sustainable, clean-label food products.

Materials and Methods

Plant-based spray

A non-oral plant-based spray containing eucalyptus leaf extract (Eucalyptus globulus), various Eucalyptus species, garden thyme (Thymus vulgaris), Shirazi thyme (Zataria multiflora), peppermint (Mentha piperita), and menthol was used. Additionally, purified water and 70% ethanol are used as solvents and carriers; ethanol helps dissolve the essential oils and provides secondary antimicrobial effects, while water acts as a diluent to ensure a uniform and safe spray formulation. The product was obtained from Dineh Pharmaceutical Company (Tehran, Iran). Each milliliter of the spray contained 100 mg Eucalyptus globulus bark extract (standardized to 200 mg eucalyptol per 100 mL), 6.7 mg essential oil of various Eucalyptus species, 2.5 mg garden thyme (Zataria multiflora), 1 mg Shirazi thyme (Thymus vulgaris), 1 mg peppermint (Mentha × piperita), and 10 mg menthol. Purified water and 70% ethanol were included as carriers and solvents, with ethanol also providing secondary antimicrobial effects. The initial concentration of the extract was 1000 μg/mL and was further diluted to 7.81 μg/mL for the study.

Bacterial strains

The antimicrobial activity of the inhalable spray was assessed against standard foodborne pathogenic bacteria, including Bacillus cereus PTCC 1015, Staphylococcus aureus PTCC 1431, Listeria monocytogenes PTCC 1298, Escherichia coli PTCC 1399, Pseudomonas aeruginosa PTCC 1430, and Salmonella typhi PTCC 1596. All reference strains were obtained in lyophilized form from the Iranian Research Organization for Science and Technology (IROST, Karaj, Iran). The strains were reactivated under sterile conditions by adding sterile distilled water, followed by inoculation into tryptic soy broth (TSB; Merck, Germany) and incubation at 37 °C for 24 h. In addition, two clinical isolates (Klebsiella pneumonia and Enterobacter aerogenes) recovered from stool samples of food-poisoned patients were included and identified using standard biochemical methods.

GC–MS analysis of bioactive compounds

The chemical composition of the inhalable spray was characterized using gas chromatography–mass spectrometry (GC–MS) (Agilent Technologies, USA). Separation was performed on an HP-5 capillary column (0.25 mm internal diameter) with helium as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially maintained at 70 °C for 2 min and then increased to 300 °C at a rate of 10 °C/min. The injector temperature was set at 280 °C. Identification of the compounds was carried out using an Agilent mass spectrometer [14].

Agar well diffusion assay

The antimicrobial activity of the bio-based spray was evaluated using the agar well diffusion method. Briefly, 24-h bacterial cultures grown in Mueller–Hinton broth (MHB; Merck, Germany) were adjusted to 0.5 McFarland turbidity. An aliquot of 100 μL of each suspension was spread uniformly onto Mueller–Hinton agar (MHA; Merck, Germany) plates. After 30 min, wells (6 mm in diameter) were aseptically punched into the agar and filled with 90 μL of different spray concentrations. The wells were sealed with 10 μL of molten MHA. Sterile 0.9% saline and ciprofloxacin solution (250 μg/mL; Sigma-Aldrich) were used as negative and positive controls, respectively. Plates were incubated at 37 °C for 24 h, after which inhibition zone diameters were measured. All experiments were performed in triplicate[15].

Broth macrodilution assay

The minimum inhibitory concentration (MIC) of the bio-based spray was determined using the broth macrodilution method in Mueller–Hinton broth (MHB). Bacterial cultures grown for 24 h at 37 °C were adjusted to 0.5 McFarland turbidity. Serial dilutions of the spray (1000 to 7.81 μg/mL) were prepared in MHB, and 1 mL of the bacterial suspension was added to each tube. Tubes were incubated at 37 °C for 24 h. To determine MIC and MBC, the contents of the final tubes were plated onto Mueller–Hinton agar. MIC was defined as the lowest concentration that visibly inhibited bacterial growth, and MBC as the concentration resulting in no growth. Ciprofloxacin and 0.9% saline were used as positive and negative controls, respectively [16].

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using MSTATC software, and mean comparisons were conducted using Duncan's multiple range test at a 5% significance level.

Results – Antibacterial activity of the spray

The in vitro antibacterial effect of the non-oral plant-based spray containing eucalyptus leaf extract, eucalyptus essential oil, garden thyme (Thymus vulgaris) essential oil, Shirazi thyme (Zataria multiflora) essential oil, peppermint (Mentha piperita) essential oil, menthol, with ethanol and water was evaluated in vitro against eight standard and clinical Gram-positive and Gram-negative foodborne pathogens. Qualitative activity was assessed using agar well diffusion, and quantitative effect was determined by broth macrodilution.

Agar well diffusion results

As shown in Table 1 and Figure 1, the spray produced significant inhibition zones compared to the positive control ($p < 0.05$) for all tested bacteria. The largest inhibition zones were observed against Staphylococcus aureus (33 mm) Salmonella typhi and Pseudomonas aeruginosa (21 mm each), Listeria monocytogenes (19 mm).

Table 1: Difference in the average diameter of the non-growth zone (in mm) of Eucalyptus, Garden Thyme, Shirazi thyme, peppermint and Menthol around bacteria

According to the tests performed, it is observed that for all bacteria there is a significant difference in the diameter of the no-growth zone in millimeters between the extract and the positive control ($P<0.05$).

	bacteria	Extract	Antibiotic-positive control	physiological Saline-negative control	F	Sig	Significant difference between extract and control+
1	Bacillus Cereus (PTCC 1015)	11.00±3.00	38.00±3.00	00.00±00.00	191.16	0.000	*
2	Staphylococcus aureus (PTCC 1431)	33.00±2.00	22.00±3.00	00.00±00.00	195.46	0.000	*
3	Salmonella Typhi (PTCC 1596)	21.00±1.00	51.00±2.00	00.00±00.00	1182.60	0.000	*
4	Pseudomonas aeruginosa (PTCC 1430)	21.00±2.00	48.66±2.51	00.00±00.00	518.93	0.000	*
5	Escherichia coli (PTCC 1399)	14.33±3.05	34.00±3.60	00.00±00.00	117.41	0.000	*
6	Enterobacter aerogenes (PTCC SPP)	11.00±1.73	49.00±1.00	00.00±00.00	1487.25	0.000	*
7	Listeria monocytogenes (PTCC 1298)	19.00±3.60	38.00±4.00	00.00±00.00	112.03	0.000	*
8	Klebsiella pneumoniae (PTCC SPP)	18.00±0.00	42.00±0.00	00.00±00.00	-	-	*

Broth macrodilution results

The antibacterial activity of the spray, expressed as MIC and MBC, is summarized in Table 2. MIC values ranged from 7.81 to 1000 µg/mL. was the most sensitive bacterium Staphylococcus aureus (MIC 7.81 µg/mL), whereas Bacillus cereus was the most resistant (MIC 1000 µg/mL). Other bacteria, including Pseudomonas aeruginosa, Salmonella typhi, Listeria monocytogenes and Klebsiella pneumonia (15.62 µg/mL each), Escherichia coli and Enterobacter aerogenes (62.5 µg/mL each) were completely inhibited at specific concentrations.

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the phytospray against bacterial pathogens

Number	type of Bacteria	First - concentration of : (MIC) g/ml	Second repetition - concentration: (MBC) /ml
1	*Basillus Cereus (PTCC 1015)*	1000	750
2	*Entetobacter aerogenes (PTCC SPP)*	62.5	31.25
3	*Escherichia coli (PTCC 1399)*	62.5	31.25
4	*Listeria monocytogenes (PTCC 1298)*	15.62	7.81
5	*Salmonella typhi (PTCC 1596)*	15.62	7.81
6	*Klebsiella pneumoniae (PTCC SPP)*	15.62	7.81
7	*Pseudomonas aeruginosa (PTCC 1430)*	15.62	7.81
8	*Staphylococcus areus (PTCC 1431)*	7.81	3.9

GC-MS Analysis of Volatile Compounds in Selected Medicinal Plants (Tables 3 to 6)

Discussion

The antimicrobial potential of natural products, particularly plant-derived formulations, has emerged as a promising avenue for controlling resistant pathogens and mitigating food spoilage. While the bioactive properties of many medicinal plants are well established, evidence regarding the efficacy of non-oral plant-based sprays against foodborne pathogens remains scarce. This study addresses this critical gap by providing the first systematic evaluation of a commercially available non-oral spray produced by Dineh Pharmaceutical Company (Tehran, Iran) against both Gram-positive and Gram-negative foodborne bacteria. The findings highlight not only the broad-spectrum antibacterial activity of this formulation but also its potential application as a natural preservative, offering a feasible alternative to conventional chemical agents. By bridging this knowledge gap, the present research contributes valuable insights into the development of plant-based antimicrobial interventions in food safety and public health.

MIC results demonstrated a clear sensitivity pattern among the tested pathogens. Staphylococcus aureus was the most susceptible Gram-positive bacterium (MIC 7.81 µg/mL), highlighting the spray's efficacy against a major foodborne pathogen. Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, and Listeria monocytogenes were inhibited at 15.62 µg/mL, reflecting activity against bacteria with intrinsic resistance mechanisms. Escherichia coli and Enterobacter aerogenes showed moderate resistance (MIC 62.5 µg/mL), while Bacillus cereus exhibited high resistance (MIC 1000 μg/mL) due to spore formation. Overall, the spray displayed broad-spectrum antibacterial potential against key foodborne pathogens.

Thyme essential oil exhibited the strongest antibacterial activity against Staphylococcus aureus, with the lowest minimum inhibitory concentrations (MIC) observed among the tested oils, indicating robust inhibitory effects on this Gram-positive pathogen [17]. Thyme essential oil, along with other plant-derived oils such as peppermint and eucalyptus, has demonstrated significant antibacterial activity against Staphylococcus aureus, with low MIC values reported for thyme oil indicating potent inhibitory effects against this Gram-positive pathogen [18]. Plant essential oils such as thyme and peppermint have demonstrated inhibitory effects against Klebsiella pneumoniae, with reported MIC values in the range of hundreds of micrograms per milliliter, suggesting that bioactive compounds can effectively impede the growth of this Gram-negative pathogen [19]. Essential oils such as tea tree and thyme have demonstrated notable antibacterial activity against Klebsiella pneumoniae, with MIC values ranging from low to moderate micrograms per milliliter, indicating their potential to inhibit growth of this Gram-negative pathogen and support the observed MIC (15.62 μg/mL) in the current study [20]. Plant essential oils, including peppermint and thyme, have shown notable antibacterial effects against Listeria monocytogenes, with studies demonstrating significant inhibition of this pathogen at relatively low MIC values, supporting the antimicrobial potential of mint-rich formulations observed in the current study [21]. Plant essential oils including eucalyptus (Eucalyptus globulus), peppermint (Mentha piperita), and thyme (Thymus vulgaris) have demonstrated antibacterial activity against Pseudomonas aeruginosa, with some studies reporting inhibitory effects even on resistant clinical isolates, highlighting the potential of plant-derived bioactive compounds in limiting growth of this challenging Gram-negative pathogen[22]. Plant essential oils such as cinnamon, thyme and oregano have demonstrated significant antibacterial activity against Salmonella typhi in vitro, with major bioactive components (e.g., carvacrol, thymol, cinnamic aldehyde) contributing to the inhibition of this food-borne pathogen[23]. Essential oils and their key bioactive compounds such as thymol, carvacrol, and eugenol have been demonstrated to possess antibacterial activity against Salmonella typhi by disrupting the bacterial cytoplasmic membrane, suggesting plant-derived oils as promising natural antimicrobial agents [24]. The antibacterial efficacy of essential oils extracted from plants such as Eucalyptus globulus, Mentha pulegium (peppermint), and Thymus capitatus (thyme) against extended-spectrum β-lactamase (ESBL)-producing Escherichia coli isolated from food has been demonstrated, with MIC values of essential oils showing significant inhibitory effects within a bioactive range [25]. Plant-derived essential oils have emerged as promising candidates due to their broad-spectrum antibacterial activity, multi-targeted mechanisms, and capacity to enhance the efficacy of existing antibiotics against drug-resistant Gram-negative bacteria, including members of the Enterobacteriaceae family such as Enterobacter and Escherichia coli [26].

Essential oils and their major components have demonstrated synergistic and broad-spectrum antibacterial activity against Gram-negative bacterial pathogens, including Enterobacter aerogenes. In particular, combinations of compounds such as eugenol, linalool, and menthol have been shown to inhibit growth and reverse resistance mechanisms in Enterobacteriaceae [27]. In contrast to its significant activity against other foodborne pathogens, the plant-based spray showed minimal inhibitory effect against Bacillus cereus (MIC 1000 µg/mL). This high resistance can be attributed to the ability of B. cereus to form endospores and its inherently robust cell wall structure, which often protects it from plant-derived antimicrobial compounds. Similar observations have been reported where Bacillus species exhibited limited susceptibility to essential oils and plant extracts, highlighting the challenge of controlling spore-forming bacteria with botanical formulations [28]. In agreement with our finding that the plant-based spray exhibited minimal inhibitory activity against Bacillus cereus (MIC = 1000 µg/mL), Yang et al. (2023) reported that essential oil vapors required relatively high concentrations to inhibit the growth of B. cereus*, highlighting the intrinsic resistance of this spore-forming pathogen and the challenge in controlling it with plant-derived antimicrobials [29].

This study demonstrates the significant antibacterial potential of a plant-based spray composed of Eucalyptus, Thymus vulgaris, Mentha × piperita, and menthol against a range of foodborne pathogens. While certain bacteria were highly sensitive, others, such as Bacillus cereus, exhibited notable resistance, reflecting their inherent structural and physiological defenses. Importantly, no previous studies have evaluated this specific combination, revealing a clear gap in the literature. These findings highlight the novelty and practical relevance of this formulation as a natural antimicrobial. Overall, the study provides compelling evidence that multi-component plant-based sprays could serve as promising alternatives for controlling foodborne pathogens, supporting future research into formulation optimization, mechanisms of action, and applications in food safety.

The multi-component plant-based spray exhibits promising antibacterial activity against key foodborne pathogens, suggesting its potential as a natural antimicrobial to help control food spoilage.

Declarations

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Statements and Declarations

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The authors declare that they have no competing interests.

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Conflict of Interest:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Tables

Tables 3 to 6 are available in the Supplementary Files section.

Figures

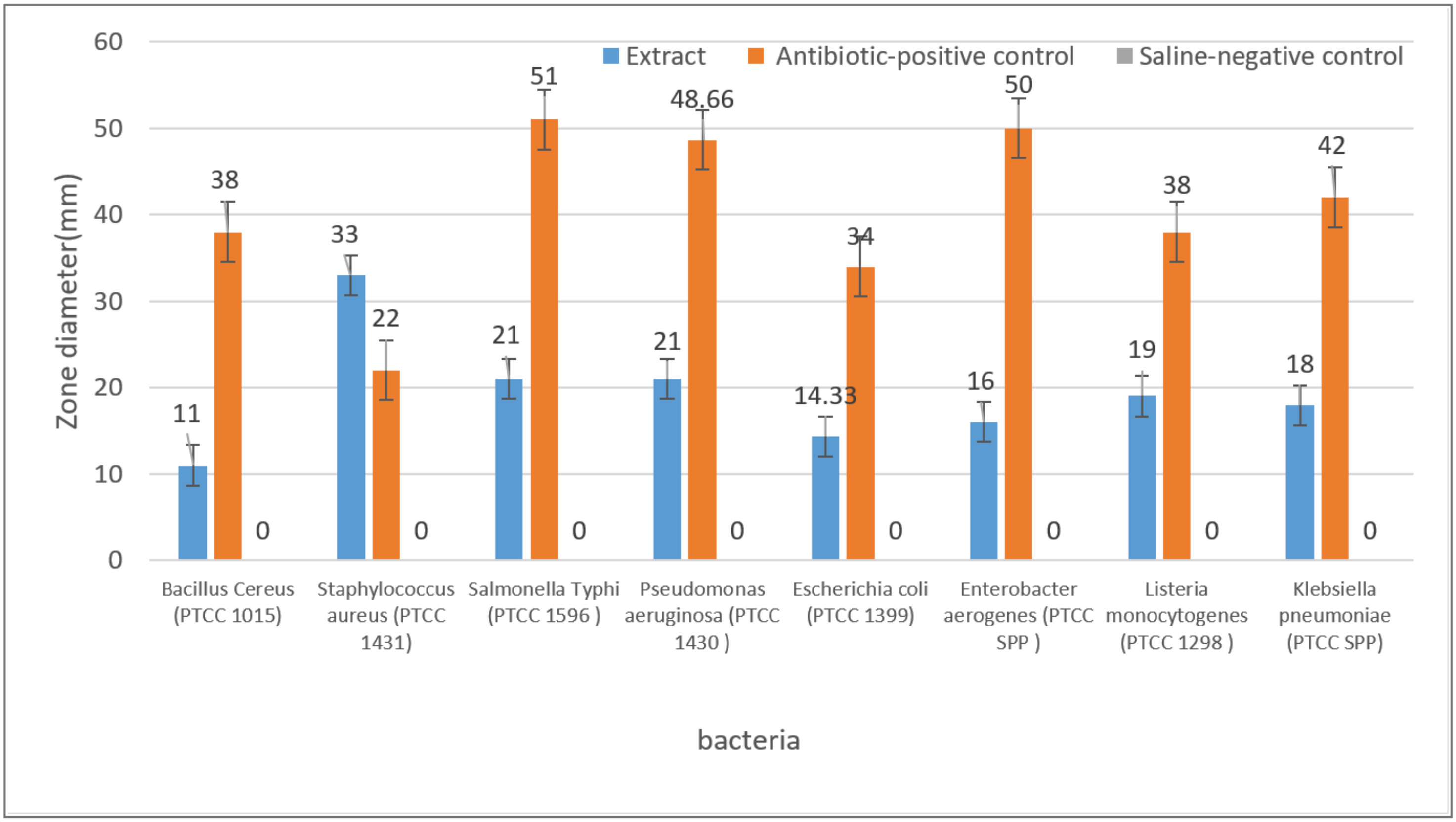

Figure 1

Comparison of the average diameter of the growth inhibition zone in millimeters of Eucalyptus, Garden Thyme, Shirazi thyme, peppermint and on the bacteria studied using the agar well method. Each point on the graph is the average of three replicates

Supplementary Files

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- Table3456.docx