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Scientific Research

Investigating the chemical properties and antimicrobial activity of *Artemisia kopetdaghensis* essential oil against bacteria that cause infectious diseases *in vitro*

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ABSTRACT

In recent years, in order to improve the hygienic quality and reduce the risks associated with chemical preservatives in the food industry, researchers have focused on the use of natural compounds, especially essential oils. This shift in approach is due to their antioxidant and antimicrobial properties. In this context, the antioxidant activity, total phenolic and flavonoid content, and antimicrobial properties of the essential oil of *Artemisia kopetdaghensis* were investigated. Results showed that *Artemisia kopetdaghensis* essential oil contained high levels of total phenols (60.43 ± 1.41 mg gallic acid equivalent/g) and total flavonoids (24.38 ± 1.32 mg quercetin equivalent/g). Evaluation of antioxidant activity revealed that the essential oil exhibited a strong ability to scavenge DPPH radicals (63.49 ± 1.25%) and ABTS radicals (70.61 ± 1.20%). The antimicrobial activity against pathogenic bacterial strains (Gram-positive: *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Bacillus subtilis*; Gram-negative: *Shigella dysenteriae*, *Klebsiella aerogenes*, and *Escherichia coli*) revealed that Gram-positive bacteria were more susceptible to *Artemisia kopetdaghensis* essential oil compared to Gram-negative bacteria. This research demonstrated that the essential oil of *Artemisia kopetdaghensis* contains bioactive compounds with antimicrobial activity. This finding confirms the potential of this plant-based essential oil as a natural alternative to harmful chemical preservatives in the food industry.

1.Introduction

The growing interest in the beneficial effects of plants, particularly aromatic and medicinal varieties used as herbal remedies for treating or preventing diseases or promoting health, has driven research to determine their properties and phytochemical content. Plant compounds are recognized as a potential source of natural antioxidants [1]. Existing evidence indicates that phenolic compounds extracted from plants act as natural antioxidants. The aim of replacing synthetic antioxidants with these compounds in the food and pharmaceutical industries is to leverage their inherent advantages, such as greater biocompatibility, reduced side effects, and a response to the increasing consumer demand for natural products [2, 3]. Furthermore, these compounds play a role in preventing various oxidative stress-related diseases, including cancers and cardiovascular ailments. Aromatic plants, on the other hand, are rich in essential oils that are widely used in the fragrance, flavor, and aromatherapy industries [4].

Essential oils are volatile compounds typically stored in epidermal tissues. Chemically, they are complex mixtures of low-molecular-weight organic compounds, including monoterpenoids, sesquiterpenoids, phenylpropenes, and isothiocyanates, among others. These oils exhibit a wide range of potent biological activities, such as antifungal, antibacterial, antidiabetic, anticancer, and antiviral effects [5, 6]. Approximately 3,000 different essential oils have been extracted from over 2,000 plant species. In 2018, their annual production ranged between 40,000 and 60,000 tons, generating an income of approximately 7.5 billion dollars, with a projected 9% growth by 2026 [7].

The genus *Artemisia* is one of the largest genera within the Asteraceae family, boasting a widespread distribution and comprising over five hundred distinct species. *Artemisia* species are renowned for producing unique essential oils and exhibiting a broad spectrum of biological activities [8]. These activities encompass antibacterial, antiviral, antiparasitic, antifungal, nematicidal, and insecticidal properties, collectively contributing significantly to the plant's natural defenses against pathogens. Despite these valuable characteristics, certain *Artemisia* species have received less scientific attention due to their scarcity or restricted distribution [9].

One such subspecies is *Artemisia kopetdaghensis*. This subspecies is found in northeastern Iran, Turkmenistan, and Afghanistan, where high valleys and peaks provide unique habitats for this plant, which has limited dispersal capabilities and a narrow tolerance for specific environmental conditions [10, 11].

In this study, the total phenolic and flavonoid content of *Artemisia kopetdaghensis* essential oil, along with its antioxidant properties, were evaluated using the DPPH and ABTS methods. Furthermore, the antimicrobial effect of the essential oil on various Gram-positive and Gram-negative bacterial strains was investigated using disk diffusion, agar well diffusion, and minimum inhibitory and bactericidal concentration assays.

2. Materials and Methods

2.1. Materials

All chemicals used were of high purity. Gallic acid, sodium carbonate, Folin-Ciocalteu reagent, aluminum trichloride, quercetin, DPPH radical, ABTS radical, methanol, Mueller Hinton Agar, and Mueller Hinton Broth were purchased from Sigma-Aldrich and Merck.

2.2. Preparation of *Artemisia kopetdaghensis* Essential Oil

The dried plant material (shade-dried) was ground into a powder using a laboratory mill. To preserve its quality and prevent degradation, the resulting powder was stored in sealed containers at 4°C. Essential oil extraction was performed using a Clevenger apparatus via hydrodistillation. The extracted essential oil was then stored in dark glass vials in a refrigerator to protect

it from light exposure and maintain its properties [12].

2.3. Measurement of Total Phenolic Content

The total phenolic content of the Iranian Artemisia essential oil was determined using the Folin-Ciocalteu method at a wavelength of 760 nm. Briefly, 1 mL of the essential oil or a standard gallic acid solution (at concentrations of 20, 40, 60, 80, and 100 mg/L) was added to 3 mL of deionized water. Subsequently, 1 mL of Folin-Ciocalteu reagent was added to the mixture and shaken thoroughly. After 5 minutes, 10 mL of a 7% sodium carbonate solution was added to the mixture. The solution was then incubated at room temperature for 90 minutes. The final results are expressed in milligrams of gallic acid per gram of essential oil (mg GA/g) [13].

2.4. Measurement of Total Flavonoid Content

This method relies on the formation of a flavonoid-aluminum complex, which exhibits maximum absorbance at a wavelength of 510 nm. One milliliter of the extract or a standard quercetin solution at various concentrations (20, 40, 60, 80, and 100 mg/L) was added to 4 mL of deionized water. Then, at specific time intervals, 0.3 mL of a 5% sodium nitrite solution, 0.3 mL of a 10% aluminum trichloride solution, and finally 2 mL of a 1 M sodium hydroxide solution were sequentially added to the mixture. The final solution was thoroughly mixed, and its absorbance was measured. The final results are expressed as milligrams of quercetin equivalents per gram of essential oil (mg QE/g) [14].

2.5. Antioxidant Activity

2.5.1. Measurement of DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the essential oil was determined using a slightly modified method based on Heydari et al. (2020). Briefly, 2 mL of a 0.1 mM DPPH standard solution in pure methanol was added to a test tube containing 2 mL of the sample solution in methanol (200 mg/L). The reaction mixture was stirred for 10 seconds and then incubated in the dark for 30 minutes. The absorbance of the sample was measured at 517 nm using a spectrophotometer. Pure methanol was used for instrument calibration. A decrease in the absorbance of the DPPH solution indicates an increase in DPPH free radical scavenging activity. Antioxidant activity was calculated as the percentage of free radical inhibition using the following formula [15]:

$$TAA\ (\%) = (A_0 - A_s / A_0) \times 100$$

Where TAA represents the total antioxidant activity, A0 is the absorbance of the DPPH solution in methanol, and As is the absorbance of the DPPH solution with the essential oil or gallic acid solution.

2.5.2. Measurement of ABTS Free Radical Scavenging Activity

The ABTS free radical scavenging activity was assessed using a slightly modified method based on Jalilsarghaleh et al. (2023). The ABTS radical cation was pre-generated by oxidizing a 7 mM ABTS solution with an equal volume of a 2.45 mM potassium persulfate solution. This mixture reacted in the dark for 12 hours at 25°C. Then, 1 mL of the resulting solution was diluted in 60 mL of methanol until its absorbance at 734 nm reached 0.7±0.06. One milliliter of this diluted ABTS radical cation solution was added to 3 mL of the essential oil, and the absorbance was measured at 734 nm. The percentage of ABTS free radical inhibition was calculated using an equation similar to the DPPH method [16].

2.6. Antibacterial Activity

The antibacterial activity of *Artemisia kopetdaghensis* essential oil was evaluated against a wide range of both Gram-positive bacteria (*Bacillus cereus*, *Streptococcus pyogenes*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysenteriae*, *Klebsiella aerogenes*, and *Escherichia coli*). This investigation was carried out using four standard methods: disk diffusion, well diffusion agar,

minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC).

2.6.1. Disk Diffusion

For this assay, a bacterial suspension (0.1 mL of a suspension with a concentration of 1.5×108 CFU/mL) was first spread onto the surface of the agar culture medium. Next, filter paper disks (6 mm diameter) were impregnated with 20 µL of the essential oil and placed onto the inoculated agar plates. After a 15-minute pre-incubation at 4°C, the plates were incubated for 24 hours at 37°C. The diameter of the inhibition zones around the essential oil-containing disks was measured as an indicator of antimicrobial activity. The solvent used for essential oil preparation served as a negative control, while disks containing tetracycline (20 µg per disk) were used as a positive control [17].

2.6.2. Well Agar

To investigate the antibacterial effect, the well diffusion agar method, based on Tabatabaei Yazdi et al. (2013), was employed. Wells with a diameter of 6 mm were created in the MHA agar medium, and 40 µL of the essential oil was pipetted into each well. The plates were then incubated for 24 hours at 35°C. Bacterial growth inhibition was determined by measuring the diameter of the resulting inhibition zones in millimeters. A solution of sterile distilled water and 0.5% Tween 80 was used as a negative control [18].

2.6.3. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the essential oil was determined using a micro-dilution assay in 96-well plates, based on a modified method by Alizadeh Behbahani et al. (2014). A stock solution of the essential oil at a concentration of 256 mg/mL was prepared in Mueller-Hinton broth using Tween 80 as an emulsifier. From this stock solution, two-fold serial dilutions ranging from 0.5 to 512 mg/mL were prepared. Subsequently, 20 µL of the final bacterial suspension was added to each well containing 180 µL of the different essential oil concentrations. Thus, the final volume in each well was 200 µL, and the bacterial count was approximately 1.5×10^8 CFU/mL. Finally, the plates were incubated for 24 hours at 37°C. The MIC was defined as the lowest concentration of the essential oil that prevented visible bacterial growth [19].

2.6.4. Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration (MBC), 100 µL samples were taken from the micropellet wells that showed no visible bacterial growth and spread onto MHA agar plates. The plates were then incubated for 24 hours at 37°C. The MBC was defined as the lowest concentration of the essential oil that resulted in the complete death of the bacteria, as indicated by the absence of bacterial colonies on the agar plates [20].

2.7. Statistical Analysis

The comparison of mean data at a 5% probability level was conducted using One-Way ANOVA in SPSS version 17 software. Graphs were plotted using Excel 2016. All experiments were performed in triplicate.

3. Results and Discussion

Phenolic compounds, a group of plant secondary metabolites, are widely recognized as natural antioxidants due to their molecular structure, which is rich in phenolic hydroxyl groups. These compounds not only play a vital role in plant defense against environmental stressors and pathogens but also garner significant research interest due to their broad spectrum of biological activities, including potent antioxidant, anti-inflammatory, anticancer, and cardiovascular properties [21]. The total phenolic content of *Artemisia kopetdaghensis* essential oil was determined to be 60.43±1.41 mg gallic acid per gram, and the total flavonoid content was 24.38±1.32 mg quercetin per gram (Figure 1).

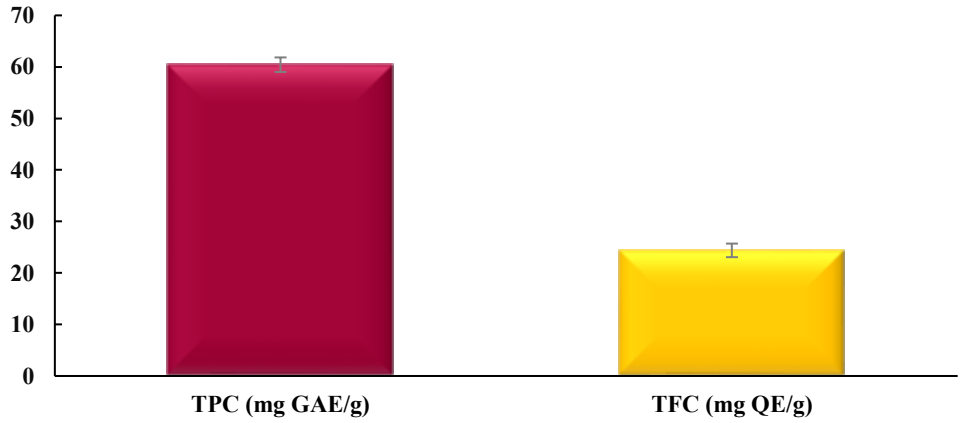

Fig 1. Total phenol content (TPC) and total flavonoid content (TFC) of *Artemisia kopetdaghensis* essential oil

3. Results and Discussion

Plants produce reactive oxygen species (ROS) such as OH•, O2•, and H2O2 when exposed to environmental stresses, which can damage their cells. Antioxidant molecules within plants protect cells by neutralizing these species. Therefore, determining the antioxidant activity of plants is crucial for a better understanding of their defense mechanisms and for discovering new compounds with antioxidant properties [22, 23].In the present study, the antioxidant capacity of *Artemisia kopetdaghensis* essential oil was assessed using two common free radical scavenging assays: DPPH and ABTS. The antioxidant activity, based on the DPPH method, was found to be 63.49±1.25%, while the ABTS method yielded 70.61±1.20%.

Studies have consistently demonstrated a direct correlation between the phenolic content of plant extracts and essential oils and their antioxidant activity. In other words, a higher concentration of phenolic compounds in these essential oils leads to an increased ability to scavenge free radicals. The primary reason for this phenomenon is the presence of multiple hydroxyl groups within the structure of phenolic compounds. These groups can stabilize free radicals by donating hydrogen atoms, thereby preventing cellular damage. Consequently, an increase in the number of hydroxyl groups in extracts is directly linked to an enhanced antioxidant capacity [24]. Research on two *Artemisia species*, *Artemisia sieversiana* (*Siberian wormwood*) and *Artemisia kopetdaghensis*, has indicated that both possess high reducing power. However, *Artemisia kopetdaghensis* demonstrated a stronger antioxidant activity compared to the other species. These researchers attributed this finding to the higher levels of phenolic compounds present in A. kopetdaghensis [25]. Similarly, Salahi et al. (2023) also reported the antioxidant activity of *Artemisia kopetdaghensis.*

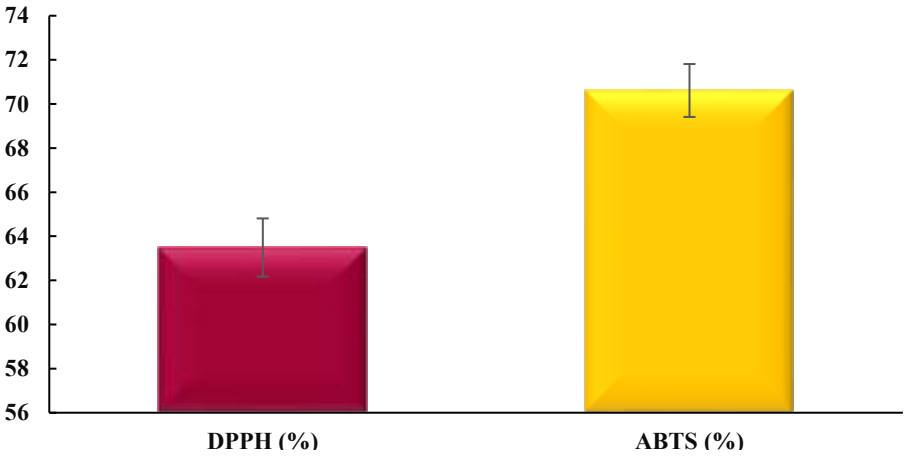

Fig 2. Antioxidant activity of *Artemisia kopetdaghensis* essential oil based on DPPH- and ABTS-radical scavenging methods.

Infections and food poisoning caused by both Gram-positive and Gram-negative bacteria represent a significant cause of mortality in developing countries. While chemical compounds effectively control this issue, their repeated use leads to microbial resistance and contamination of the food chain. Consequently, there's a growing interest in natural compounds, such as plant extracts, as safer and more effective alternatives for preserving food safety [26, 27].

The antimicrobial effects of *Artemisia kopetdaghensis* essential oil, based on the agar disk diffusion method, are presented in Figure 3. The findings of this study indicate that the essential oil exerted a stronger inhibitory effect on Gram-positive bacteria than on Gram-negative bacteria. This was evident from the larger zones of inhibition observed around the disks containing the compounds in agar media inoculated with Gram-positive bacteria. Among the Gram-positive bacteria, the largest inhibition zone diameter was observed for *Staphylococcus aureus* (16.00±0.50 mm), while the smallest was for *Bacillus subtilis* (13.10±0.40 mm). Among the Gram-negative bacteria, the largest and smallest inhibition zone diameters were observed for *Escherichia coli* (13.40±0.60 mm) and *Shigella dysenteriae* (11.90±0.30 mm), respectively ($p<0.05$).

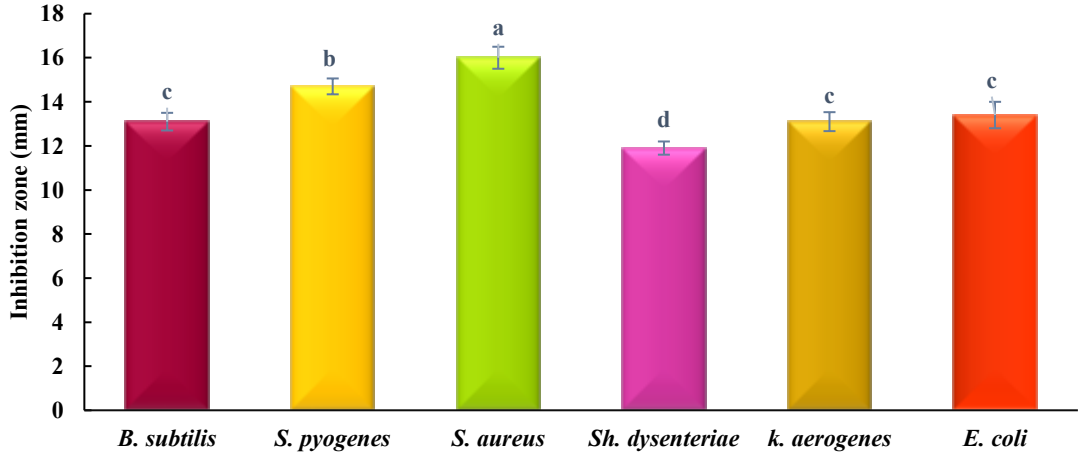

Fig 3. Antimicrobial activity of *Artemisia kopetdaghensis* essential oil based on disc diffusion method.

Figure 4 presents the results of the antimicrobial activity based on the agar well diffusion method. As observed, the diameter of the inhibition zones around the wells in this method was similar to that in the disk diffusion agar method. However, the zone diameters were generally larger in this method, with the largest inhibition zone

for Staphylococcus aureus measuring 17.70±0.49 mm and the smallest for Shigella dysenteriae measuring 13.60±0.27 mm ($p<0.05$).

As previous studies have indicated, this difference is attributed to the varying modes of contact between the extract or essential oil and the microorganisms. In the agar well diffusion method, the extract is directly in contact with the microbes, whereas in the disk diffusion method, the extract gradually diffuses into the culture medium after passing through the paper disk [28]. In another study, the antimicrobial effect of *Artemisia kopetdaghensis* essential oil and its nanoemulsion was investigated against two bacterial strains: *Staphylococcus aureus* and *Escherichia coli*. The findings revealed that *Artemisia kopetdaghensis* essential oil exhibited a stronger inhibitory effect on *Staphylococcus aureus*, with an inhibition zone diameter of 11.1 mm for the essential oil, compared to 8.0 mm for the nanoemulsion. For *Escherichia coli*, the essential oil showed an inhibition zone of 9.0 mm, while no zone of inhibition was observed for the nanoemulsion [21].

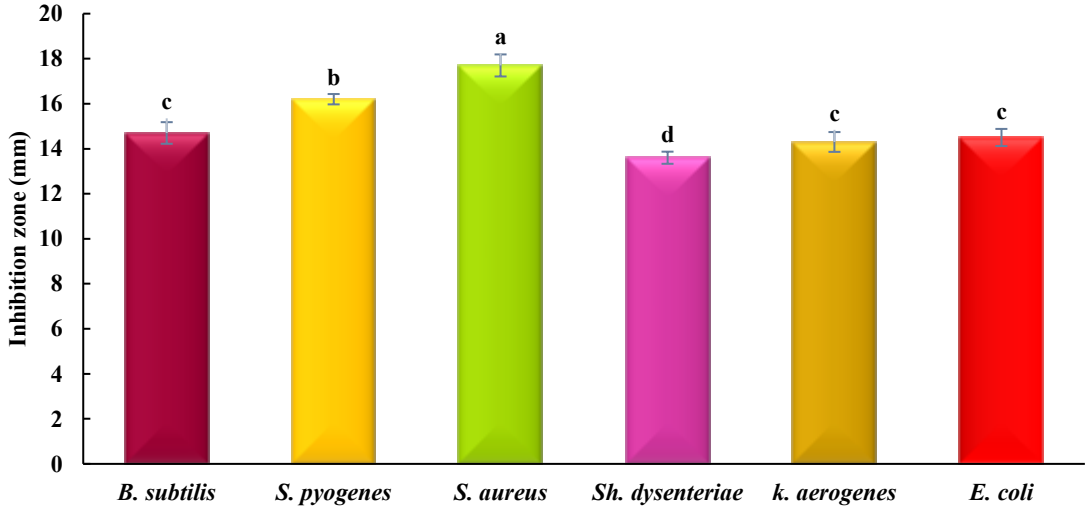

Fig4. Antimicrobial activity of *Artemisia kopetdaghensis* essential oil based on well diffusion method.

Figure 5 presents the results for the minimum inhibitory concentration (MIC) of the *Artemisia* essential oil. Statistical analysis revealed a significant difference in MIC values among the various bacterial strains ($p<0.05$). A gradual increase in essential oil concentration from 2 to 256 mg/mL showed a direct and significant correlation with an enhanced antimicrobial effect. At a concentration of 2 mg/mL, all tested bacterial strains were still able to grow. However, at concentrations of 16 mg/mL and above, the growth of all Gram-positive strains was completely inhibited. Gram-negative bacteria also ceased growth at concentrations of 32 mg/mL and higher. Generally, higher essential oil concentrations effectively prevented the growth of these strains.

Overall, the MIC values for *Artemisia kopetdaghensis* essential oil were determined as follows: *Bacillus subtilis* (8 mg/mL), *Streptococcus pyogenes* (8 mg/mL), *Staphylococcus aureus* (4 mg/mL), *Shigella dysenteriae* (64 mg/mL), *Klebsiella aerogenes* (64 mg/mL), and *Escherichia coli* (32 mg/mL).

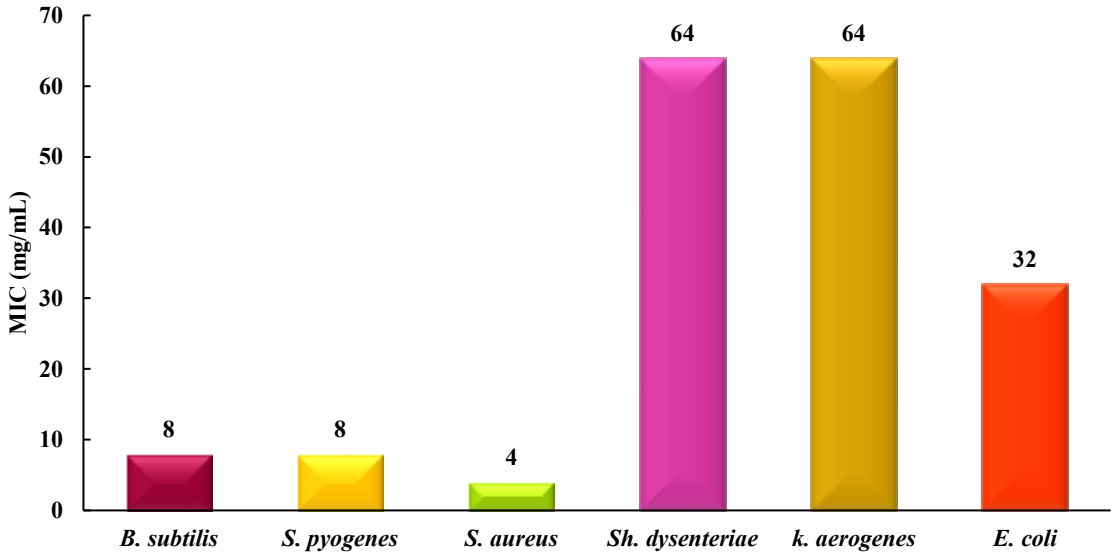

Fig 5. Antimicrobial activity of *Artemisia kopetdaghensis* essential oil based on minimum inhibitory concentration.

The results for the minimum bactericidal concentration (MBC), presented in Figure 6, revealed that the bacterial strains tested exhibited varying sensitivities to the antimicrobial agent. Generally, Gram-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus* showed greater sensitivity with an MBC of 64 mg/mL, compared to Gram-negative bacteria. Among the Gram-positive bacteria, *Bacillus subtilis* demonstrated higher resistance with an MBC of 128 mg/mL. Furthermore, *Shigella dysenteriae* and *Klebsiella aerogenes* were the most resistant strains, both having an MBC of 256 mg/mL. *Escherichia coli* showed intermediate sensitivity with an MBC of 128 mg/mL. The antimicrobial effect of essential oils or plant extracts is primarily due to the presence of bioactive compounds that can interact with the cellular structures of microorganisms. These compounds disrupt vital cellular functions, such as material transport, energy production, and protein synthesis, by penetrating the cell membrane and compromising its integrity, ultimately leading to cell death [29, 30].

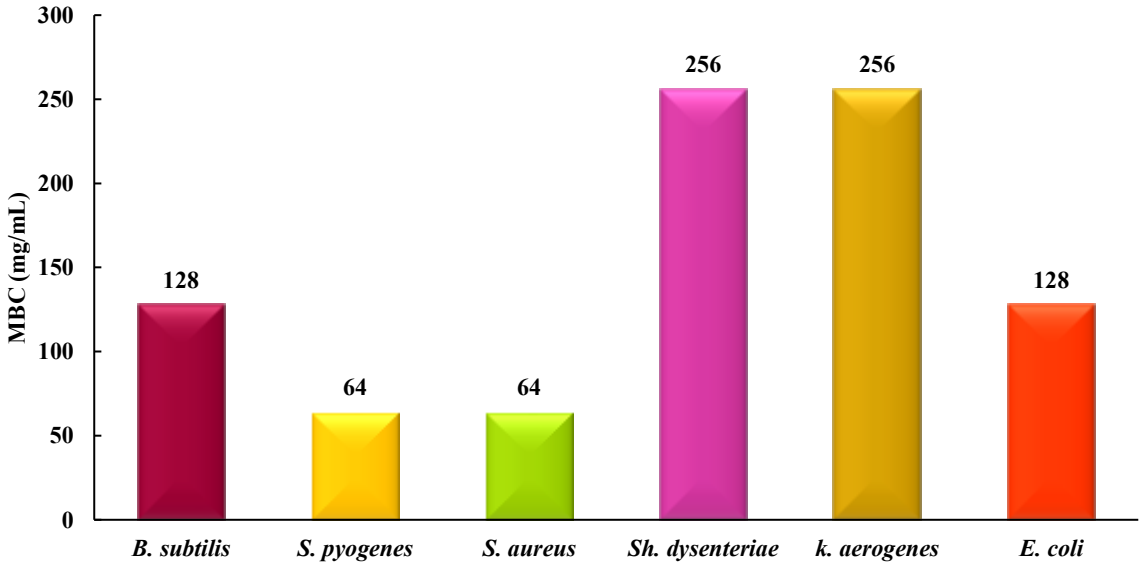

Fig 6. Antimicrobial activity of *Artemisia kopetdaghensis* essential oil based on minimum bactericidal concentration.

In a study [7], the antimicrobial activity of five compounds (sesquiterpene lactone, sesquiterpene peroxide, phenoxychromone, and two flavonoids) extracted from *Artemisia kopetdaghensis* was investigated against several bacteria. The results showed that sesquiterpene peroxide was the most potent antibacterial compound, exhibiting significant activity against Pseudomonas aeruginosa (MIC: 16 µg/mL), as well as *Salmonella typhi*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Listeria monocytogenes* (MIC: 32 and 64 µg/mL). Salahi et al. (2023) also reported the minimum inhibitory and bactericidal concentrations of the essential oil and nanoemulsion of *Artemisia kopetdaghensis* against *Staphylococcus aureus* and *Escherichia coli*. The antimicrobial activity results of Artemisia essential oil in our report align with other similar studies [21]. Mikaeili et al. (2023) reported the antifungal activity of *Artemisia kopetdaghensis* extract [31].

4. Conclusion

The present study demonstrated that *Artemisia kopetdaghensis* essential oil contains a significant amount of phenolic and flavonoid compounds. Due to this high content, the essential oil exhibits strong antioxidant activity. Furthermore, the tested Artemisia essential oil showed considerable antimicrobial activity, indicating its effectiveness against diseases caused by microbial overgrowth. Therefore, this species warrants further investigation for the development of novel antimicrobial agents. It can also be utilized as a safe and natural additive in food products to increase shelf life and reduce spoilage caused by microbial growth. However, more research is needed to ensure the safety and assess the toxicity of the main compounds present in this essential oil.

5. Acknowledgements

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بررسی ویژگی‌های شیمیایی و فعالیت ضدمیکروبی اسانس درمنه کپه داغی بر باکتری‌های عامل بیماری های عفونی در شرایط برون‌تنی

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چکیده

در سال‌های اخیر، به منظور ارتقاء کیفیت بهداشتی و کاهش خطرات ناشی از نگهدارنده‌های شیمیایی در صنعت غذا، توجه پژوهشگران به استفاده از ترکیبات طبیعی به ویژه اسانس‌های گیاهی معطوف شده است. این تغییر رویکرد به دلیل خواص آنتی‌اکسیدانی و ضدمیکروبی آن‌ها بوده است. در همین راستا، فعالیت آنتی‌اکسیدانی، میزان محتوای فنول و فلاونوئید کل و همچنین خواص ضدمیکروبی اسانس درمنه کپه داغی مورد بررسی قرار گرفت. نتایج نشان داد، اسانس درمنه کپه داغی حاوی فنول کل (٤۳/٤۱±۱/۲۰ ٦۰ میلی‌گرم گالیک اسید در گرم) و فلاونوئید کل (۱/۳۲±۲٤/۳۸ میلی‌گرم کوئرستین در گرم) بالای بود. ارزیابی فعالیت آنتی‌اکسیدانی نشان داد که اسانس توانای بالای در مهار رایکال آزاد DPPH (۱/۲۵±٦۳/٤۹ درصد) و ABTS (۱/۲۰±۷۰/٦۱ درصد) دارد. نتایج اثر ضدمیکروبی بر سویه‌های باکتریایی بیماری‌زا (گرم مثبت؛ *استرپتوکوکوس پیوژنز* ، *استافیلوکوکوس اورئوس* و *باسیلوس سوبتلیس* و گرم منفی؛ *شیگلا دیسانتری*، *کلبسیلا ائروژنز* و *اشرشیا کلی*) نشان داد که باکتری‌های گرم مثبت نسبت به باکتری‌های گرم منفی در برابر اسانس درمنه کپه داغی حساسیت بالاتری داشتند. این پژوهش نشان داد که اسانس درمنه کپه داغی، حاوی ترکیبات زیست‌فعال با فعالیت ضدمیکروبی است. این یافته، پتانسیل این اسانس گیاهی را به عنوان جایگزینی طبیعی برای نگهدارنده‌های شیمیایی مضر در صنایع غذایی تایید می‌کند.