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An investigation into the antioxidant capacity, phenolic and flavonoid compounds, and antibacterial activity of the essential oil of *Artemisia fragrans* against Gram-positive and Gram-negative pathogenic bacteria

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ABSTRACT

In this study, the essential oil of *Artemisia fragrans* was extracted using the hydrodistillation method. Subsequently, the total phenolic content was determined using the Folin-Ciocalteu colorimetric method, total flavonoid content was measured using the aluminum chloride colorimetric method, and antioxidant activity was evaluated using both the DPPH and ABTS radical scavenging assays. Additionally, the antimicrobial activity of the essential oil was evaluated using the agar disk diffusion, agar well diffusion, and standard microdilution methods. The essential oil of *Artemisia dracunculus* contained a total phenolic content of 62.88 ± 1.78 mg GAE/g and a total flavonoid content of 48.14 ± 0.26 mg QE/g. Furthermore, the essential oil exhibited significant antioxidant activity, with DPPH and ABTS radical scavenging activities of 50.1 ± 90.67% and 28.1 ± 95.73%, respectively. In the agar disk diffusion assay, *Streptococcus pyogenes* was found to be the most sensitive strain with a zone of inhibition of 18.80 ± 6.6 mm, while *Salmonella typhimurium* was the most resistant strain with a zone of inhibition of 11.80 ± 2.7 mm. In the agar well diffusion method, *Salmonella typhimurium* exhibited the strongest resistance with a zone of inhibition of 12.70 ± 0.44 mm, while *Streptococcus pyogenes* was the most sensitive strain with a zone of inhibition of 19.50 ± 0.19 mm. The results showed that the MIC for *Bacillus cereus*, *Streptococcus pyogenes*, *Shigella dysentery*, *Klebsiella aerogenes*, and *Salmonella typhimurium* were 16, 4, 4, 64, 32, and 64 mg/ml, respectively, and the MIB for them was, respectively. 256, 32, 64, 256, 128 and 256. Due to its high content of phenolic compounds, the essential oil of tarragon possesses potent antioxidant and antimicrobial properties. These attributes make it a promising natural alternative to synthetic preservatives in the food industry.

1- Introduction

The increasing demand for products with extended shelf life and higher quality has created new challenges within the food industry. Consumers are actively seeking natural alternatives to chemical preservatives, which has consequently fueled a growing interest in using essential oils (EOs) as antibacterial food additives. Numerous studies have investigated the effect of whole EOs on food spoilage microorganisms and foodborne pathogens. Evidence generally indicates that essential oils tend to be more effective against Gram-positive bacteria than Gram-negative bacteria [1]. A significant effect of these oils is the inhibition of growth and reduction in the population of major foodborne pathogens such as *Salmonella spp.*, *Escherichia coli O157:H7*, and *Listeria monocytogenes* [2, 3]. Essential oils, which are complex mixtures comprising a large and diverse group of terpenoid and phenolic compounds derived from aromatic plants, have garnered considerable attention over the past few decades due to their demonstrated broad-spectrum biological properties. Medicinal plants play a crucial role in human life and are utilized for nutritional, health, and therapeutic purposes. The genus Artemisia is one such medicinal plant known for its pungent aroma. This genus belongs to the Asteraceae family, which encompasses approximately 500 species [4]. *Artemisia fragrans* (Fragrant Wormwood) is a perennial herbaceous plant. It is an aromatic herb, both in its leaves and flowers, typically growing to a height of about 45 centimeters. The leaves of this plant initially appear white due to the presence of numerous trichomes (plant hairs), which are shed as the plant matures. *A. fragrans* is widely distributed throughout Iran and is a dominant species in the northern part of Ardabil province, particularly in the pastures of the Moghan region [5]. Essential oils and extracts isolated from various *Artemisia* species serve as a source of active compounds and secondary metabolites demonstrating anti-malarial, anti-tumor, antifungal, antiviral, anti-hepatitis, anti-spasmodic, antioxidant, and anti-inflammatory properties, among others [6]. The essential oil of *A. fragrans*, in addition to aliphatic compounds, contains a variety of terpenic constituents, ranging from hydrocarbon monoterpenes and monoterpenoids to sesquiterpenes. The main compounds identified in this essential oil include 1,8-cineole, α-thujone, α-pinene, β-pinene, camphor, and camphene [5]. Terpenoids represent one of the major families of secondary metabolites that have been shown to have pharmacological applications in human diseases. For instance, artemisinin is prescribed as the sole drug for treating malaria in many regions worldwide where the disease has become resistant to conventional chemical drugs [7]. Previous studies indicate that the essential oil of *A. fragrans* possesses diverse biological effects, including antioxidant, antibacterial, anti-malarial, and even herbicidal activities [8, 9].

Various methods have been introduced to date for measuring the antioxidant capacity of foods and biological samples. Antioxidant capacity is a key concept in biosciences, describing the ability of compounds in foods and the body to neutralize harmful free radicals [10]. Rather than focusing on a single antioxidant, antioxidant capacity broadly refers to a system's overall ability to counteract oxidative stress resulting from the complex interplay between reactive

oxygen and nitrogen species and various antioxidant compounds. Consequently, measuring antioxidant capacity can lead to a better understanding of the role of nutrition in the prevention and treatment of diseases [11].

In recent years, a wide array of spectrophotometric methods have been utilized to measure the antioxidant capacity of foods; the most popular among these are the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays [12].

The antimicrobial activity of various plant extracts, essential oils, and pure compounds can be measured using numerous tests, such as agar diffusion methods, as well as the disk diffusion, well diffusion, and microplate dilution methods. Each of these techniques is based on a specific characteristic, such as the inhibition of microbial growth [13, 1].

To investigate the therapeutic, antimicrobial, and antioxidant properties of the essential oil of *Artemisia fragrans*, the oil was prepared from the plant, and its total phenol content, total flavonoid content, and antimicrobial and antioxidant activities were subsequently evaluated.

3. Materials and Methods

2.1. Materials

The materials used in this study included Folin–Ciocalteu reagent, Quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and the culture media Mueller Hinton Agar and Broth. These reagents and media were sourced from Merck (Germany) and Sigma-Aldrich (USA).

2.2. Essential Oil Extraction

To extract the essential oil, 100 g of powdered plant material was subjected to the essential oil extraction process using a Clevenger-type apparatus (hydrodistillation). The extracted essential oil was then dried using anhydrous sodium sulfate and stored at 4 °C in sealed, dark glass containers until subsequent experiments were performed [14].

2.3. Determination of Total Phenol Content

One milliliter (1 mL) of the essential oil was mixed with 1 mL of Folin–Ciocalteu reagent. After 4 min, 1 mL of a 7% sodium carbonate solution was added, and the final volume was adjusted to 10 mL using distilled water. The absorbance of the sample was measured against a blank (containing methanol instead of the extract) using a UV-Vis spectrophotometer at a wavelength of 765 nm. The total phenol content was calculated from a standard calibration curve prepared using gallic acid and expressed in milligrams of Gallic Acid Equivalents (GAE) per gram of sample [15].

2.4. Determination of Total Flavonoid Content

One milliliter (1 mL) of the essential oil was mixed with 4 mL of distilled water and 300µL of a 5% sodium nitrite solution. After 5 minutes, 300µL of a 10% aluminum trichloride solution, 2 mL of a 1 M sodium hydroxide (NaOH) solution, and 10 mL of distilled water were added to the mixture. Finally, the absorbance was measured at a wavelength of 510 nm. Quercetin was used as the standard, and the results were expressed as milligrams of Quercetin Equivalents (QE) per gram of essential oil [16].

2.5. Antioxidant Activity

2.5.1. DPPH Free Radical Scavenging Assay

This method is based on the essential oil's ability to reduce the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical, which results in a color change from purple to yellow. Initially, a 0.2mM DPPH solution was prepared in methanol. Then, 0.1mL of the essential oil was mixed with 3.9 mL of the DPPH solution. The mixture was shaken for 10 seconds and incubated in the dark for 30 min at room temperature. Finally, its absorbance was recorded at a wavelength of 517 nm. The percentage inhibition of the DPPH free radical by the essential oil was calculated using the following equation [17]:

$$\text{DPPH Free Radical Scavenging Activity (\%)} = A_{Control}-A_{essential\ oil}/A_{Control}\times 100$$

Where $A_{Control}$ is the absorbance of the control and $A_{essential\ oil}$ is the absorbance of the essential oil sample.

2.5.2. ABTS Free Radical Scavenging Assay

The ABTS cation radical (ABTS·+) was generated by reacting an ABTS solution (7mM in water) with 2.5mM potassium persulfate (final concentration) for 12 hours at 4 ℃ in the dark (stock solution). Subsequently, the ABTS·+ stock solution was diluted with ethanol until its absorbance at 750 nm was approximately 0.7. The reaction was initiated by adding 10µL of the test sample to 990µL of the diluted ABTS·+ solution. The reduction in absorbance was measured after a 5-minute incubation period at room temperature in the dark. Ethanol and L-ascorbic acid were used as the negative and positive controls, respectively. The radical scavenging activity of the tested sample, expressed as the percentage inhibition of ABTS·+, was calculated using the following formula [18]:

$$\text{ABTS Free Radical Scavenging Activity (\%)} = A_{Control}-A_{essential\ oil}/A_{Control}\times 100$$

Where$A_{Control}$ is the absorbance of the control and $A_{essential\ oil}$ is the absorbance of the essential oil sample.

2.6. Antimicrobial Activity

2.6.1. Agar Disk Diffusion Method

To investigate the antimicrobial effect of the essential oil, the disk diffusion method utilizing 6mm disks was employed. Pathogenic bacteria were cultured on Mueller Hinton Agar (MHA) medium at 37 ℃ overnight. Subsequently, they were adjusted using sterile saline solution to a concentration of 1.5×10^8 colony-forming units per milliliter (CFU/mL). 100µL of the resulting microbial suspension was spread uniformly onto the culture medium. Then, sterile disks saturated with the essential oil were placed on each agar plate (one disk per plate). After 24 hours of incubation at 37∘C, the diameter of the zone of growth inhibition surrounding each disk was measured [19].

2.6.2. Agar Well Diffusion Method

First, Petri plates were prepared by pouring 15 to 20mL of Mueller-Hinton Agar (MHA). Once the medium solidified, its surface was streaked with a cotton swab inoculated with the bacterial suspension. Next, 6mm diameter wells were created in the medium using sterile stainless steel cylinders. The essential oil under investigation was then added into these wells. The plates were incubated at 37 ℃ for 24 hours. Results were recorded by measuring the diameter of the bacterial growth inhibition zones around the wells [20].

2.6. Antimicrobial Activity (Continued)

2.6.3. Determination of Minimum Inhibitory Concentration (MIC)

The microdilution method was used to determine the MIC. The turbidity of the microbial suspension was adjusted using a spectrophotometer at a wavelength of

625nm to match the 0.5McFarland standard (1.5×10^8CFU/mL), reaching an optical density (OD) between 0.8 and 1.0. 50µL of culture medium containing the essential oil, followed by 30µL of the microbial suspension, were sequentially added to each well. The plates were then incubated for 24 hours at 37 ℃. After 24 hours, 20µL of tetrazolium chloride (TTC) (0.5% aqueous solution) was added, and the plates were incubated for an additional hour at 37 ℃. The MIC of the compound was defined as the lowest concentration of that compound that completely prevented visible growth of the cells, which was confirmed by the TTC assay (dead cells do not stain) [21].

2.6.4. Determination of Minimum Bactericidal Concentration (MBC)

To determine the MBC, 100µL from the wells where no visible growth was observed were transferred to Petri dishes containing Mueller-Hinton Agar (MHA) medium. These dishes were then incubated at 37 ℃ for 24 hours. The MBC was defined as a 99.9% reduction (absence of growth) in the number of viable cells [22].

2.7. Statistical Analysis

All experiments were performed in triplicate. Excel software was utilized for generating graphs, and SPSS statistical software was used for data analysis. Statistical analysis involved One-Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test.

3- Results and Discussion

The total phenol content of the *Artemisia fragrans* essential oil was determined to be 78.88 ± 1.62 mg Gallic Acid Equivalents (GAE) per gram (g), and the total flavonoid content was 26.40 ± 1.48 mg Quercetin Equivalents (QE) per gram (g) (Figure 1). The values obtained for total phenol and flavonoid content in our study are comparable to those reported for other *Artemisia* species, such as *Artemisia vulgaris* [23]. Polyphenols are secondary metabolites in plants. Plants contain various types of phenolic derivatives, including benzoic acids, cinnamic acid derivatives, flavonoids, isoflavonoids, lignans, and tannins [24]. Bandali et al. (2017) investigated the phenolic and flavonoid content of different parts of the *Artemisia fragrans* plant. Their results indicated that the root, leaf, and flower of the plant contained total phenol contents of 33.51, 117.32, and 110.02 mg GAE per gram, respectively, and total flavonoid contents of 0.8, 3.5, and 5.2 mg QE per gram, respectively. Safaei-Ghomi et al. (2012) reported the total phenol content of the methanolic extract to be 34.50µg/mL [24]. Notably, the total phenol and flavonoid content measured in the essential oil of *Artemisia fragrans* in our current study was higher than the values reported in the aforementioned investigations.

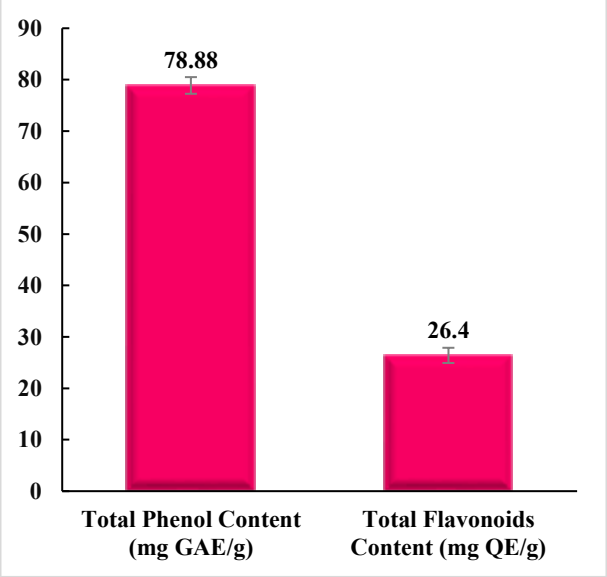

Fig 1. Total phenolics and flavonoids content of *A. fragrans* essential oil.

The results of the antioxidant activity assays demonstrated that the essential oil of *Artemisia fragrans* possesses significant antioxidant potential (Figure 2). This essential oil was able to effectively scavenge the DPPH free radical by 67.90 ± 1.50% and the ABTS free radical by 73.95 ± 1.28%. These two free radical species are known to play a key role in the induction of oxidative stress and cellular damage. These compelling results justify the high antioxidant activity observed in the essential oil derived from this species. Previous studies [23] have confirmed the presence of flavonoid derivatives in *A. fragrans*. Flavonoids are recognized as potent antioxidant compounds due to their unique chemical structure. These compounds are capable of neutralizing free radicals and protecting cells against oxidative damage [25, 26, 27]. Consequently, the presence of these compounds in the *A. fragrans* essential oil can be the reason for its high antioxidant activity. Erdoğan Orhan et al. (2010) investigated the antioxidant properties of ethanolic and acetonic extracts from two *Artemisia* species (*A. herba-alba* and *A. fragrans*). Their findings indicated that the ethanolic extract of *A. fragrans* exhibited the strongest performance in scavenging DPPH radicals and the Ferric Reducing Antioxidant Power (FRAP) assay compared to the other species [28]. Younesi et al. (2020) [1398 AP] compared the antioxidant power of *A. fragrans* essential oil collected in different seasons and showed that the highest antioxidant capacity (28.89%) was obtained from the essential oil harvested in September [29]. Furthermore, the *A. fragrans* essential oil has been consistently reported to possess significantly high antioxidant power [6]. According to a study by Alma et al. (2003), phenolic compounds such as thymol and carvacrol, as well as essential oils rich in phenolic compounds, demonstrate potent antioxidant activity and strong inhibition of DPPH radicals [30]. Therefore, the robust antioxidant activities observed for plant essential oils are often primarily attributed to their major phenolic components.

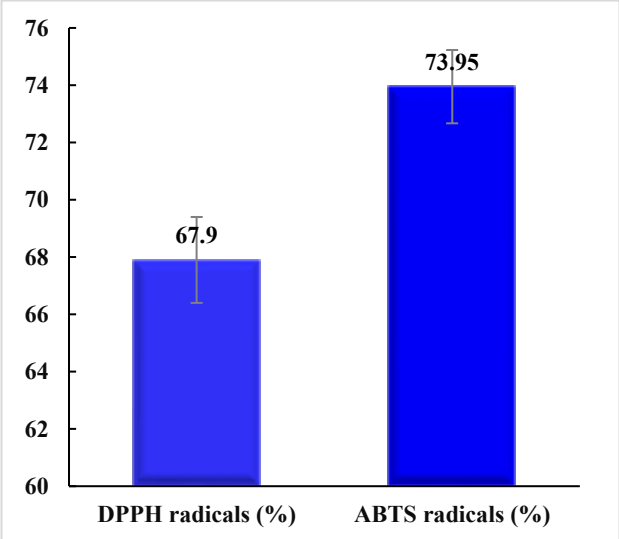

Fig 2. Antioxidant activity of *A. fragrans* essential oil.

The results of the antimicrobial effect of the *Artemisia fragrans* essential oil, based on the agar disk diffusion method, are presented in Figure 3. The diameter of the zone of inhibition (ZOI) observed for the tested bacterial strains ranged from 11.80 to 18.80 mm. *Streptococcus pyogenes*, with a ZOI of 18.80 ± 0.66 mm, was identified as the most sensitive strain to the *A. fragrans* essential oil, while *Salmonella typhimurium*, with a ZOI of 11.80 ± 0.27 mm, was the most resistant strain ($p < 0.05$). The primary reason for the lower sensitivity of Gram-negative bacteria to essential oils is the presence of an outer membrane in their cellular structure. This membrane, a layer of lipopolysaccharide (LPS), acts as a protective shield, effectively preventing the penetration of the essential oil's hydrophobic components into the bacterial cell [31, 32]. Safaei-Ghomi et al. (2012) studied the antimicrobial effect of *A. fragrans* essential oil against a wide range of pathogenic bacteria using the disk diffusion method. Their results clearly showed that the ZOI for Gram-negative bacteria was significantly smaller than that for Gram-positive bacteria [33]. These findings are consistent with the results of our current research.

In contrast, the results reported by Younesi et al. (2020) [1398 AP] indicated that Gram-negative bacteria (*E. coli*, *P. vulgaris*, and *K. pneumoniae*) were more sensitive to the *A. fragrans* essential oil when compared to Gram-positive bacteria (*S. aureus* and *B. subtillus*) [29]. In summary, the findings regarding the efficacy of essential oils against Gram-positive and Gram-negative bacteria vary across different studies and depend on multiple factors, including the type of essential oil, the bacterial species, and the concentration utilized.

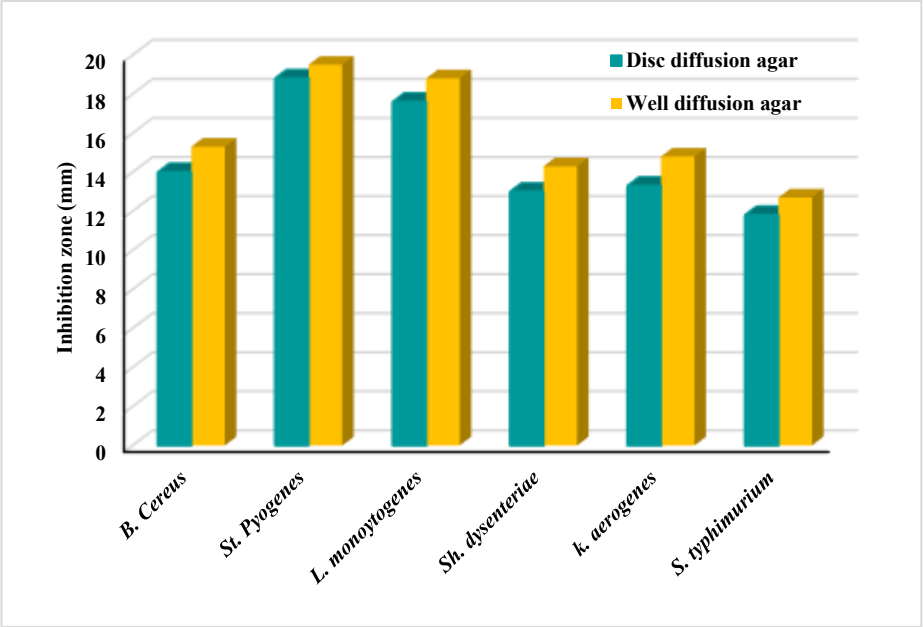

Fig 3. The antibacterial activity of *A. fragrans* essential oil based on disc diffusion agar method and well diffusion agar method.

Figure 3 presents the results of the essential oil's antimicrobial activity based on the agar well diffusion method. The results indicated that the diameter of the zone of inhibition (ZOI) for the tested bacterial strains ranged from 12.70 to 19.50mm. Interestingly, the bacterial strains exhibiting the strongest and most sensitive responses to the *Artemisia fragrans* essential oil were *Salmonella typhimurium* and *Streptococcus pyogenes*, respectively (p <0.05). In one study, the antibacterial activity of the essential oil against two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) was investigated using the agar well diffusion method. The essential oil was found to be effective against both tested Gram-positive bacteria and one Gram-negative bacterium, but it showed no efficacy against *P. aeruginosa* [34]. Other studies have also confirmed the antimicrobial effect of the essential oil against both Gram-negative and Gram-positive bacteria [33, 35].

The results for the MIC (Figure 4) demonstrated that the minimum concentration of the *A. fragrans* essential oil required to inhibit the growth of Gram-positive bacteria was significantly lower than that required for Gram-negative bacteria. This finding highlights the higher sensitivity of Gram-positive bacteria to this essential oil. Furthermore, the minimum concentration of the essential oil required to kill the Gram-positive bacteria (the MBC) was also lower than that needed for the Gram-negative bacteria (Figure 5).

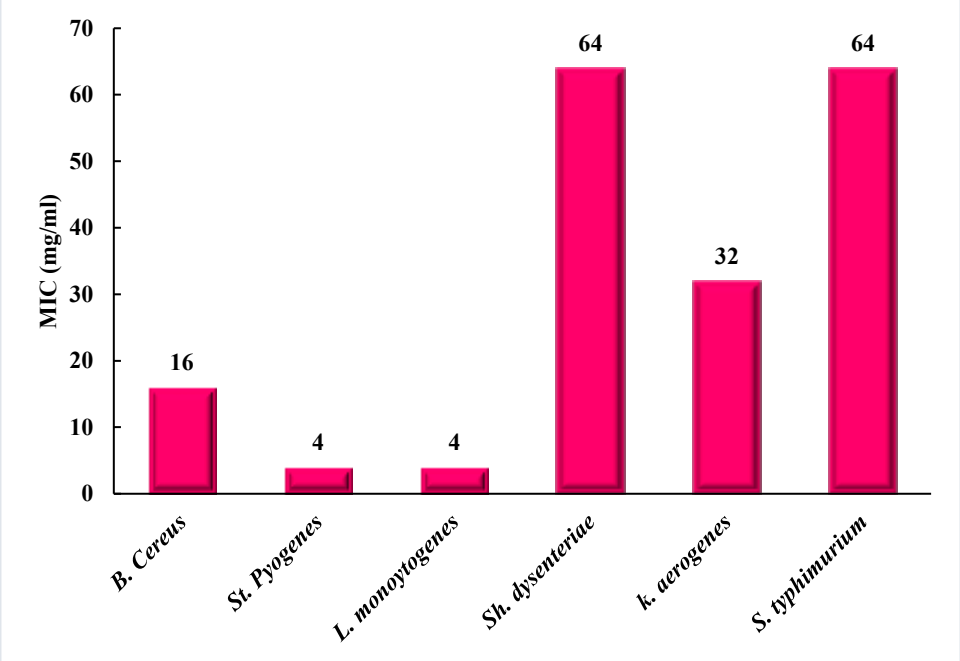

Fig 4. The antibacterial activity of *A. fragrans* essential oil based on minimum inhibitory concentration

The inhibitory effect of the *Artemisia fragrans* essential oil on Gram-positive and Gram-negative bacteria was also investigated by Valizadeh et al. (2012) [35]. In one study [4], the MBC of the *A. fragrans* essential oil was reported for several bacteria: *Salmonella paratyphi* and *typhi* (25mg/mL), *Staphylococcus saprophyticus* (25mg/mL), *E. coli* (25mg/mL), *Shigella flexneri* (12.5mg/mL), *Staphylococcus epidermidis* (6.25mg/mL), *Enterococcus faecalis* (6.25mg/mL), and *S. aureus* (3.12mg/mL). Furthermore, the MIC values for these strains were reported to be 100, 100, 100, 100, 25, 25, and 12.5mg/mL, respectively. In another study [33], the antimicrobial activity of 1,8-cineole, a compound present in *A. fragrans* essential oil, was examined. Their results indicated that this compound exhibited high inhibitory activity against the fungus Candida albicans, with an MBC of 31.25µg/mL. They also stated that the only other sensitive microorganism was Proteus vulgaris, with an MBC of 62.50µg/mL.

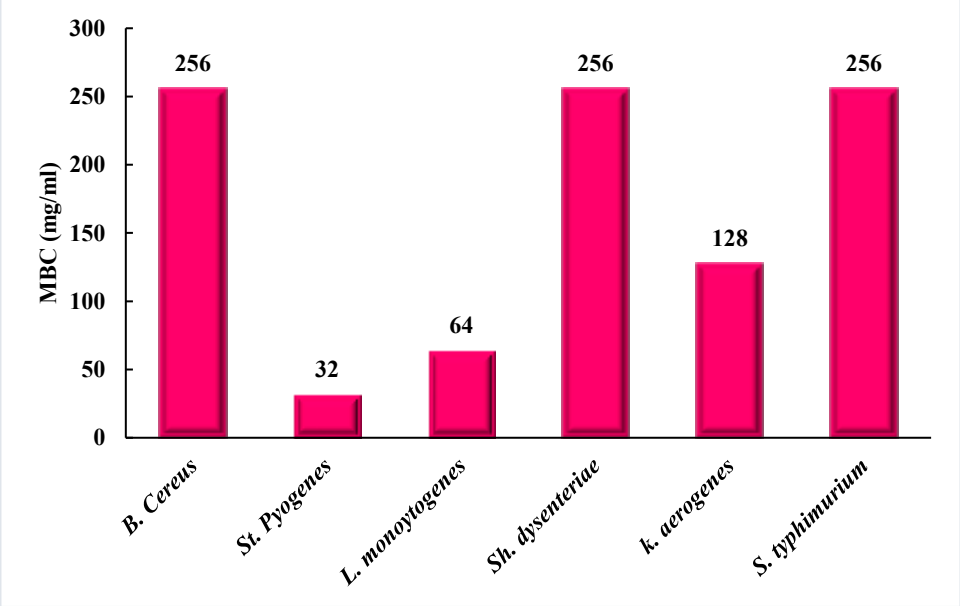

Fig 5. The antibacterial activity of *A. fragrans* essential oil based on minimum antibacterial concentration methods

4- Conclusion

Artemisia fragrans (Fragrant Wormwood) is traditionally employed for medicinal purposes, with most of its pharmacological effects attributed to its essential oil. The present study demonstrated that the *A. fragrans* essential oil possesses potent antioxidant activity, being rich in phenolic and flavonoid compounds and capable of effectively scavenging both DPPH and ABTS free radicals. Furthermore, this essential oil exhibited significant antimicrobial effects against a broad spectrum of pathogenic bacteria, including Streptococcus pyogenes and Salmonella typhimurium. Given these compelling results, the essential oil of *Artemisia fragrans* is proposed as a natural source of bioactive compounds with potential applications in the food industry, serving as a suitable alternative to chemical preservatives.

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بررسی قدرت آنتی‌اکسیدانی، ترکیبات فنولی، فلاونوئیدی و اثر ضدباکتریایی اسانس درمنه معطر روی باکتری‌های بیماری‌زا گرم مثبت و گرم منفی

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چکیده

در این مطالعه، اسانس درمنه معطر با روش تقطیر با آب استخراج گردید. سپس محتوای فنول کل به روش رنگ سنجی فولین سیوکالتو، فلاونوئید کل به روش رنگ سنجی کلرید آلومینیوم و فعالیت آنتی‌اکسیدانی به دو روش مهار رادیکال آزاد DPPH و ABTS اندازه‌گیری شد. علاوه براین، فعالیت ضدمیکروبی اسانس با استفاده از روش‌های دیسک دیفیوژن آگار، چاهک آگار و روش استاندارد میکرودایلوشن صورت گرفت. اسانس درمنه معطر حاوی ۷۸/۸۸±۱/۶۲ mg GAE/g محتوای فنول کل و ۲۶/۱۴±۰/۴۸ mg QE/g محتوای فلاونوئید کل بود. علاوه‌براین، اسانس قادر به مهار رادیکال آزاد DPPH و ABTS به میزان ۱/۵۰ ± ۶۷/۹۰ درصد و ۱/۲۸±۷۳/۹۵ درصد گردید.در روش دیسک دیفیوژن، *استرپتوکوکوس پیوژنز* حساس‌ترین سویه با ناحیه مهار ۱۸/۰±۸۰/۶۶ mm بود، در حالی که *سالمونلا تیفی موریوم* مقاوم‌ترین سویه با ناحیه مهار ۱۱/۸۰±۰/۲۷ mm بود. در روش انتشار چاهک آگار، *سالمونلا تیفی موریوم* قوی ترین مقاومت را با ناحیه مهار ۱۲/۷۰ ±۰/۴۴ mm نشان داد، در حالی که *استرپتوکوکوس پیوژنز* حساس‌ترین سویه با ناحیه مهار ۱۹/۵۰±۰/۲۸mm بود. نتایج نشان داد که MIC برای *باسیلوس سرئوس*، *استرپتوکوکوس پیوژنز*، *شیگلا دیسانتری*، *کلبسیلا ائروژنز* و *سالمونلا تیفی موریوم* به ترتیب ۱۶، ۴، ۴، ۶۴، ۳۲، و ۶۴ mg/ml و MIB به ترتیب برای آن‌ها ۲۵۶ mg/ml، ۳۲، ۶۴، ۲۵۶، ۱۲۸ و ۲۵۶ بود. اسانس درمنه معطر به واسطه دارا بودن مقادیر قابل توجهی از ترکیبات فنلی، از خواص آنتی‌اکسیدانی و ضدمیکروبی قوی برخوردار است. این ویژگی‌ها، این اسانس را به عنوان جایگزینی طبیعی برای نگهدارنده‌های سنتزی در صنایع غذایی معرفی می‌کند.