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Original Article

Chemical Composition, Antioxidant Properties, and Antimicrobial Activity of *Ferula Assa-foetida L.* Essential Oil against Pathogenic Bacteria

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ABSTRACT

Ferula assa-foetida L. (*Asafoetida*) a medicinal plant with antimicrobial and antioxidant properties, is crucial in the food and pharmaceutical industries. *Ferula* essential oils (EOs), rich in ferulic acid, flavonoids, alkaloids, and glycosides compositions, may offer a potential solution to antibiotic resistance. The aim of this work is to investigate the components of *Ferula* chemical essential oil (EO) and its antioxidant and antimicrobial properties in different habitats of Iran (Tabas, Yazd, Neishabur, and Kerman). Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to determine the composition of the essential oils from the four different sources. The antimicrobial activity, the minimum inhibitory concentration (MIC), and the minimum bactericidal concentration (MBC) of the essential oils against both Gram-positive and Gram-negative pathogenic bacteria were evaluated using the broth microdilution method and the well diffusion test. Likewise, the antioxidant properties of the essential oils were examined using the DPPH radical scavenging method. GC-MS analysis identified the major components of the essential oils, with the Nishabur EO having the highest percentage of components. The Kerman EO had the lowest inhibitory concentration against *Escherichia coli*, at 6.25 mg/ml, while the Yazd EO demonstrated the highest inhibitory concentration against *Staphylococcus aureus*, at a minimum inhibitory concentration of 50 mg/ml. Yazd EO showed the strongest antimicrobial activity, particularly against Gram-positive bacteria, with a halo diameter of 22.5 mm. In contrast, the Kerman EO showed the least amount of antimicrobial activity against *Escherichia coli*, with a halo diameter of 11.5 mm. Kerman EO had the highest antioxidant activity, with 93.9393%. The *Ferula* EO has the ability to inhibit DPPH radicals and demonstrated activity against both Gram-positive and Gram-negative bacteria, making it a strong antioxidant and natural preservative in the pharmaceutical industry.

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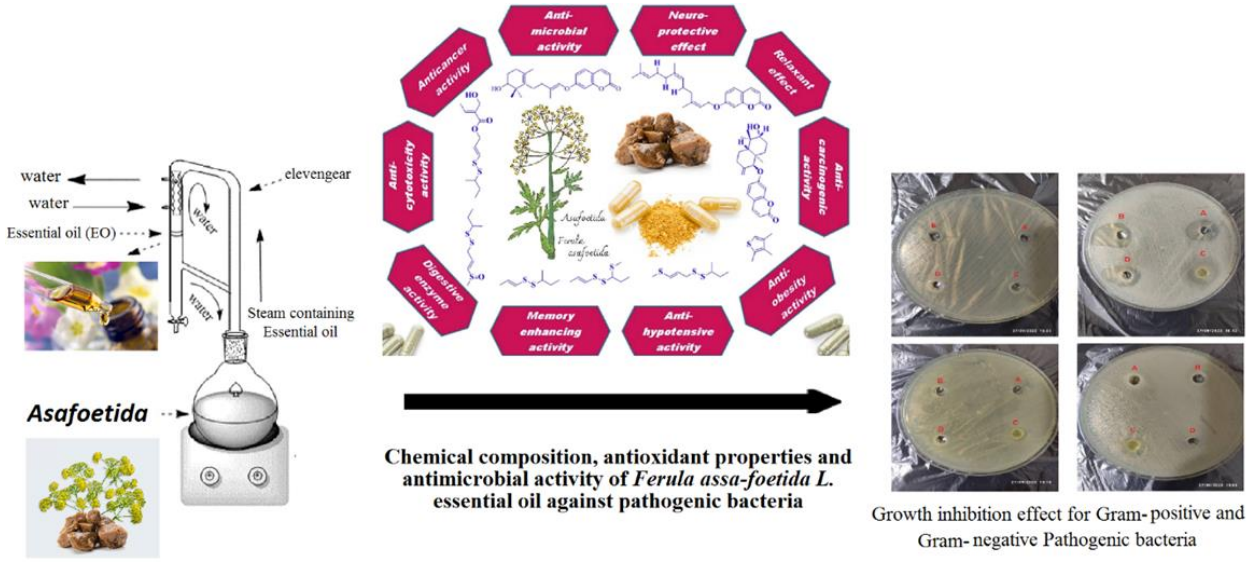
GRAPHICAL ABSTRACT



Introduction

Ferula assa-foetida L., commonly known as "*Ferula*," is recognized as a valuable resource in various industries and provides innovative methods for its cultivation [1]. Many medicinal plants are used in the formulation of various drugs and treatment of some diseases due to their antimicrobial and antioxidant properties, and *Ferula* is included in this category [2-10]. In addition, these medicinal plants are also used in the food industry and food additives due to their properties [11-16].

Ferula emits a pungent, sulfurous odor resembling fermented garlic and possesses a stimulating taste. Depending on the type of plant, *Ferula* can be classified as bitter or sweet. This plant boasts numerous properties, including antiseptic, expectorant, memory enhancement, alleviation of kidney-related pains, digestion improvement, rheumatism treatment, relief from flatulence, bronchitis treatment, and cough relief. Investigating and utilizing the antibacterial and antioxidant properties of *Ferula* in various industries, especially pharmaceuticals and food, is of significant importance. This plant plays a crucial role as a valuable resource in the country's economy and holds potential for expansion and exploitation from various perspectives [1,17-19]. The high-quality type of *Ferula* has various compounds that include 62% resin, 25% gum, 3-7% essential oil, 1.28% free ferulic acid and a very small amount of vanillin. These compounds are crucial for the identification and determination of the superiority of *Ferula*, indicating the quality and properties of this medicinal plant. The resin portion of *Ferula* contains ferulic acid along with various esters, including coumarins, sesquiterpene coumarins, and other terpenoids, arabinose, and rhamnose. These compounds contribute to the various properties of most medicinal plants and act as determining elements in them [19-25].

The gum portion includes compounds such as glucose, galactose, glucuronic acid, polysaccharides, and glycoproteins, which play a significant role in creating various properties and applications in different industries related to *Ferula*. The essential oil part contains sulfuric and monoterpene compounds, which are crucial for determining the scent and taste of *Ferula*, as well as for industrial applications [26-29].

The *Ferula* plant possesses not only a distinctive aroma and pungent taste but also highly valuable antioxidant properties. This plant serves as a natural source of various antioxidants, such as ferulic acid and cadinene. These antioxidants exhibit powerful antioxidant properties, effectively preventing oxidative damage and contributing to the preservation of cellular health. Ferulic acid is an antioxidant compound

present in *Ferula*, assisting in neutralizing free radicals and preventing cellular damage. In addition, cadinene also possesses antioxidant properties, aiding in combating oxidative stress in the body. Furthermore, since these antioxidant compounds can be easily extracted from *Ferula*, they can be utilized in the food and pharmaceutical industries. These compounds can serve as additives in the production of food products or as essential elements in the formulation of drugs and health supplements. Therefore, *Ferula* is recognized as a significant source of natural antioxidants that can contribute to maintaining health and preventing various diseases [30-31].

In a study conducted by Pavela in 2020, the insecticidal effects of essential oils from *Ferula assa-foetida* and F. gummosa were investigated. The oils, primarily composed of sulfides and monoterpenes, were determined through GC-MS analysis. The oils were tested against a panel of insects, including common pests in public health, agriculture, and stored products. Ecotoxicological effects were evaluated on the small crustacean Daphnia magna, the earthworm Eisenia fetida, and human cells. The results indicated that the oils were effective against pests and important insect vectors but exhibited cytotoxicity against fibroblasts and aquatic microcrustaceans, marking them as non-target cytotoxic agents. Further research is required to determine the full spectrum of their environmental toxic effects [32].

In a study by Kazemi *et al.* in 2019, the antibacterial activity of *Ferula* (*Ferula assa-foetida*) essential oil obtained from seeds and oleo-gum resin against oral pathogens was investigated. The *Ferula* plants were collected in the summer of 1396 from Tabas, Yazd Province, Iran, and essential oil was extracted by water distillation from seeds and oleo-gum. GC-MS analysis was performed to determine the contents of the essential oils. The GC-MS analysis revealed that both essential oils exhibited similar antibacterial properties to chlorhexidine. The growth inhibition zone was significantly related to the concentration of the essential oil for all bacteria tested [33]. In a study by Hassan Abadi et al. in 2019, oleo-gum resin perforations from *Ferula assa-foetida* in fourteen Iranian and Afghan samples were analyzed. Forty-two compounds were identified in these samples, with six compounds identified as dominant. The samples showed significant differences in the essential oil yield, depending on the sample's height. Generally, an increase in height led to an increase in beta-pinene and a decrease in (Z)-1-propenyl secondary butyl disulfide and thiophene. Various analyses indicated that height was the most influential environmental factor explaining changes in the composition and yield of the essential oil [34]. Zomorodian *et al.* (2018) conducted a study on the essential oil extract of *Ferula assa-foetida*. This oil exhibited activity against both Gram-positive and Gram-negative bacteria at concentrations of 0.5-8 µL/mL and 16-128 µl/m, respectively. In addition, the oil effectively inhibited biofilm formation in Candida albicans, Candida tropicalis, and Candida krusei at a concentration of 4 µl/mL [35].

In the study by Saeidi *et al.* in 2017, Asafetida gum, an oleo-gum resin from the root of *Ferula assa foetida*, was extracted using alcohol and water extraction methods. Various analyses, including colorimetric assays, Fourier-transform infrared spectroscopy, gas chromatography-mass spectrometry, and 1D and 2D nuclear magnetic resonance, identified the components of the gum. Thermal behavior of the gum was evaluated using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), revealing predominant weight loss zones below and above 200 °C [36]. Given that *Ferula* contains active compounds and minerals such as flavonoids, alkaloids, and glucosides, it possesses significant antibacterial and antioxidant properties. These features can be employed in combating bacteria and harmful microorganisms. Moreover, *Ferula* contains aromatic volatile compounds that, in addition to pharmaceutical applications, can serve as natural preservatives in food products. These compounds are also recognized as natural flavor enhancers and can contribute to improving the taste and quality of food items

[37-38]. Therefore, the purpose of this research is to investigate the chemical compounds in the essential oil (EO) of *Ferula assa-foetida L.* (*Asafoetida*) and its antioxidant properties (DPPH radical inhibition) plus to investigate its antimicrobial properties (MIC, MBC and well diffusion test) in different habitats of Iran (Tabas, Yazd, Neishabur, and Kerman).

Experimental

Plant Material and EO Extraction

To prepare and extract the essences of the *Ferula assa-foetida* plant, 100 g of the plant's sap were collected from each of the cities of Tabas, Yazd, Neishabur, and Kerman under the supervision of a pharmacognosy specialist. The goal was to investigate the constituents forming the essences, as well as the antimicrobial (growth inhibition effect) and antioxidant properties. After collection, samples were identified, undesirable samples were eliminated, and the remaining ones were transferred to the laboratory. Subsequently, using the steam distillation method with a Clevenger apparatus, the essences were extracted from the sap of *Ferula*, and then the content and composition of the essences of all four species were identified and determined using Gas Chromatography-Mass Spectrometry (GC-MS).

for the extraction of the essence from the *Ferula* plant, 100 g of *Ferula* plant sap collected from the habitats of Yazd, Tabas, Neishabur, and Kerman were separately placed in 800 ml flasks, and it was dissolved with 400 ml distilled water to create a uniform solution. After dissolving the *Ferula* sap in distilled water, the contents of the solution inside the flask, which was milky in color, were transferred to a 1000 ml separatory funnel. An additional 200 ml of distilled water was added, and it was placed on an electric heater. The distillation process with water vapor for the extraction of *Ferula* essence took place for 6 h. After emptying the essence, which was placed in the graduated cylinder of the Kjeldahl apparatus, it was stored in a brown vial at 4 degrees Celsius for validation using gas chromatography-mass spectrometry (GC-MS). The contents and compounds of the essences from each of the four species were identified and determined using GC-MS.

GC–MS Analyses

In this work, the Hewlett-Packard 5971 GC-MS apparatus (Avondale, PA, USA) available at the Medicinal Plants Research Institute of the University of Tehran was used. The identification of the compounds was also carried out at this center. For the analysis of the essence components, 2 µl of each essence were separately injected into the GC/MS apparatus connected to mass spectrometry. The initial oven temperature was set at 60 °C with a temperature gradient of 4 °C per minute, increasing the temperature to 280-300 °C. The injection temperature was 290 °C, and helium gas was selected as the mobile phase with a flow rate of 0.8 ml per minute. Mass spectrometry was performed with an electron ionization (EI) ionization method at 70 electron volts, and the ionization source temperature was 220 °C. (This process was performed separately for each essence).

Providing Experimental Conditions and Culture Environments

In this study, three types of Gram-negative pathogenic bacteria, *Klebsiella pneumoniae* (13883 PTCC), *Escherichia coli* (25922 PTCC), and *Pseudomonas aeruginosa* (294292 PTCC), cultured in EMB medium, and two types of Gram-positive pathogenic bacteria, *Enterococcus* (29212 ATCC) and *Staphylococcus aureus* (25923 ATCC), cultured in Blood Agar medium, were used for the antibacterial effect of *Ferula* plant essences from Tabas, Nishapur, Kerman, and Yazd in the laboratory. Likewise, Muller-Hinton Agar (MHA) medium was used for the well test. All pathogenic bacteria were standard strains and were obtained from the Genetic Resources Center of Iran. To activate pathogenic bacteria, each of the 5 standard strains of pathogenic bacteria was cultured, and pure bacterial

colonies were obtained using the streak plate method on the appropriate culture medium for each bacterium. Subsequently, the inoculated plates were incubated for 24 h at a temperature of 35.5 °C to facilitate bacterial growth in an incubator. Figure 1 displays the initial Gram-positive pathogenic bacteria obtained for the study and Figure 2 depicts the initial Gram-negative pathogenic bacteria obtained for the study.

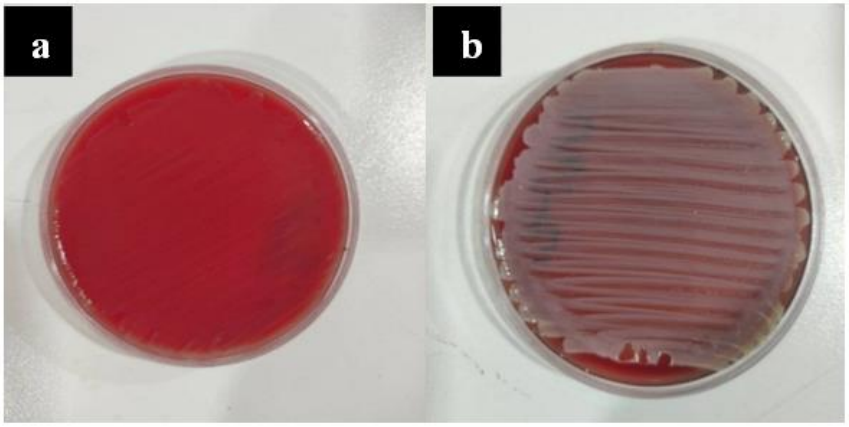


Figure 1: Standard Gram-positive pathogenic bacteria, a) *Enterococcus* bacteria and b) *Staphylococcus aureus* bacteria

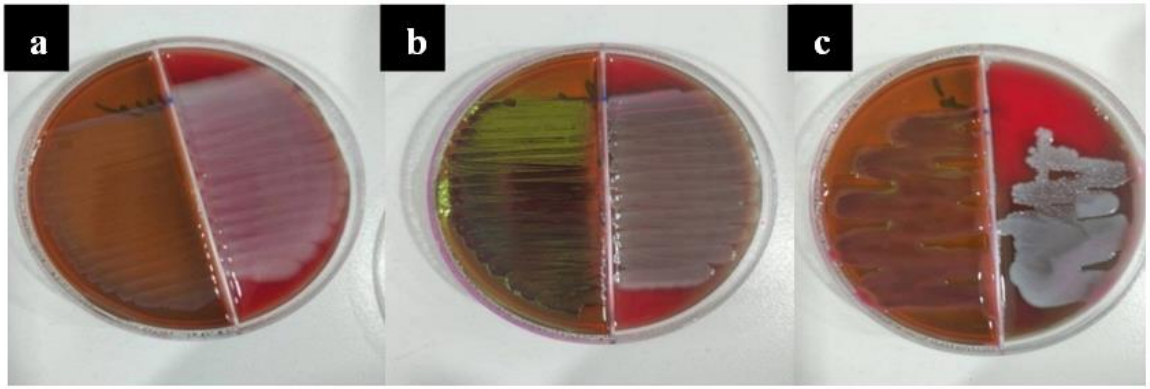


Figure 2: Standard Gram-negative pathogenic bacteria, a) *Pseudomonas aeruginosa* bacteria and b) *Escherichia coli* bacteria, c) *Klebsiella pneumoniae* bacteria

To prepare emulsion liquid of essential oils were mixed with 80 parts of water and 30 parts of glycerin based on the weight of the essential oils. The mixture was emulsified using a high-speed homogenizer (IKA Ultra Digital T25, Turax) at high speed for a specified duration. The homogenization process was conducted in a closed vessel with a high-speed homogenizer set at a constant speed.

For the determination of microbial turbidity, half of the first 175 ml of double-barium chloride powder was dissolved in 100 ml distilled water. Subsequently, 1 ml of concentrated sulfuric acid was added to 100 ml of distilled water, and 10 ml of 1% sulfuric acid was added to the test tube. Then, using a sampler, 50 µl of the acid was removed from the tube, and 50 µl of barium chloride solution was added to the tube. Finally, the absorbance of the solution was measured at 620 nm using a calibrated spectrophotometer with a one-centimeter diameter cuvette. The absorbance of the solution at 620 nm should be in the range of 0.08-0.13.

To prepare microbial suspension, one loop full of each microbial strain under sterile conditions is added to 25 ml of Mulher Hinton Broth (MHB) culture medium to prepare thick microbial suspension will be added. Then, until its OD

optical density was equal to 0.5 McFarland solution (1.10^8 × 5 cfu/mL), it was diluted by Muller-Hinton Broth (MHB) liquid culture medium. For the preparation of a standard microbial suspension, bacterial strains were cultured overnight on a nutrient agar medium one night before the experiment. The cultures were incubated at 37 °C under aerobic conditions. To prepare the microbial suspension, 2-4 h before the experiment, 4-5 separate colonies of the target microorganism were taken from the storage culture and transferred to a tube containing Muller-Hinton Broth (MHB). The bacterial samples were incubated at 37 °C for 2-4 h until their turbidity reached the standard of 0.5 McFarland. The microbial suspension was equivalent to a 0.5 McFarland standard (1.10^8 × 5 cfu/mL). After dilution, 50 µL of the microbial suspension was transferred to the MHA culture medium using a sterile pipette, and wells were created on the agar surface after 30 min, using a sterile pipette, 250 µl of the essence was injected into each well. Then, the plates were incubated at 37 °C, and the zones of no growth were measured after 24 h.

MIC and MBC Test

Using the broth microdilution method, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined. For obtaining the minimum inhibitory concentration (MIC), sterile 96-well plates and the microdilution method were employed, following the Clinical and Laboratory Standards Institute (CLSI) global protocol. Accordingly, each essence from different habitats (Tabas, Neishabur, Kerman, and Yazd) was investigated against five standard pathogenic strains. Initially, 200 µl of each essence with a concentration of 50 mg/ml was added separately to the first well, and then 100 µl of Muller-Hinton Broth (MHB) medium was added to the other wells. Subsequently, 100 µl was taken from the first well and added to the second well containing 100 µl of Muller-Hinton Broth (MHB). This process was repeated until the desired dilution was achieved in the liquid medium, and then 100 µl of the bacterial suspension, equivalent to a 0.5 McFarland standard (1.10^8 × 5 cfu/ml), was taken and added to the wells containing the culture medium and essence.
Finally, all plates were incubated at 37 °C for 24 h. The concentration of the first well with no observed turbidity was considered as the MIC, or the minimum inhibitory concentration, representing the lowest concentration that inhibited the growth of pathogenic bacteria by the mentioned essence. Wells that showed no turbidity in their culture media were streaked on Muller-Hinton Agar (MHA) culture medium (culture medium with pathogenic bacteria as negative control and culture medium with chlorhexidine and pathogenic bacteria as positive control).

Well Diffusion Test

To investigate the antibacterial activity of the essences using the well test method, a standard microbial suspension equivalent to a 0.5 McFarland (1.10^8 × 5 cfu/mL) was prepared for each pathogenic bacterial strain. Pure bacterial colonies were dissolved in physiological serum, and the optical density (OD) was measured at 630 nm using a spectrophotometer (OD should be between 0.18 and 0.13), and then using a sterile swab, the prepared 0.5 McFarland suspension was streaked on Muller-Hinton Agar (MHA) culture medium in four directions on the entire surface. Four wells, each with a diameter of 5 mm and a thickness of 4 mm, were created in each plate using a sterile Pasteur pipette. A volume of 250 µl of each essence from Yazd, Nishapur, Tabas, and Kerman, with concentrations of 50 mg/mL, was added to each well. This procedure was repeated three times based on the coding done on the essences and the experimental design. The plates were then incubated at 37 °C for 24 h, and finally, after 24 h, the results of the antibacterial activity of the essences on pathogenic bacteria were examined and measured. The images of well diffusion test on Mueller–Hinton agar plates is shown in Figure 3.

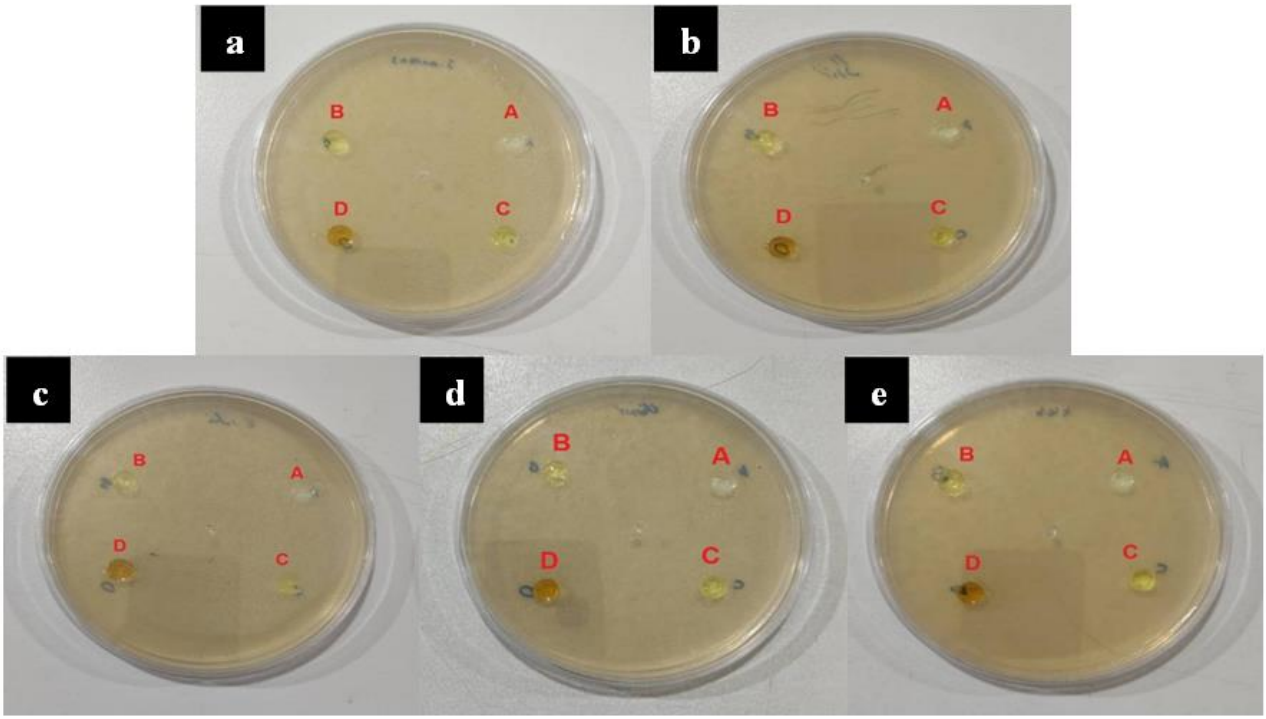


Figure 3: Well diffusion test with the same concentrations for antibacterial testing, a) *Staphylococcus aureus*, b) *Enterococcus*, c) *Escherichia coli*, d) *Pseudomonas aeruginosa*, and e) *Klebsiella pneumoniae* strain

Design of Experiments (DOE)

In this work, the Design of Experiments (DOE) and data analysis were carried out in three repetitions using the Taguchi method and the Minitab software, as presented in Table 1. A comparison of means was conducted using the Taguchi test with 2 factors (type of pathogenic bacteria and type of essence) at 5 levels. In the actual operation of well-creation to evaluate the antibacterial properties of essences on pathogenic bacterial strains, the procedure was repeated three times to determine the average effects based on the halo diameter (inhibition and bactericidal effects) created for each pathogenic strain. For plotting the graphs, Excel software was utilized. The coding of essences and pathogenic bacteria is indicated in Tables 2 and 3, respectively.

Table 1: Design of tests for antibacterial evaluation of essential oils in each run for five levels with two factors

Pathogenic bacteria and the review of essential oils that affect each bacterium					
Type of bacteria / RUNs	***Klebsiella pneumoniae***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Enterococcus***	***Staphylococcus aureus***
R1	Kerman EO	Nishapur EO	Yazd EO	Tabas EO	Control sample
R2	Nishapur EO	Yazd EO	Tabas EO	Control sample	Kerman EO
R3	Yazd EO	Tabas EO	Control sample	Kerman EO	Nishapur EO
R4	Tabas EO	Control sample	Kerman EO	Nishapur EO	Yazd EO
R5	Control sample	Kerman EO	Nishapur EO	Yazd EO	Tabas EO

**Control Sample (Culture medium containing pathogenic bacteria without essence): To examine the culture medium containing pathogenic bacteria concerning external factors such as temperature, nutrient source of the medium, acidity, etc., ensuring that the bacteria remain in a state of growth and activity throughout the duration of the well test.*

Table 2: Coding of essential oils to investigate the effect of growth inhibition based on the diameter of the created halo

Coding of essential oils on the plates to check the halo diameter based on the design of experiments (Taguchi)			
D	**C**	**B**	**A**
Tabas EO	Yazd EO	Nishapur EO	Kerman EO

Table 3: Coding of pathogenic bacteria to investigate the growth inhibition effect based on the diameter of the created halo

Coding of pathogenic bacteria based on design of experiments (Taguchi)				
Gram-positive pathogenic bacteria		**Gram-negative pathogenic bacteria**		
B5	**B4**	**B3**	**B2**	**B1**
Staphylococcus aureus 25923ATCC	Enterococcus 29212ATCC	Pseudomonas aeruginosa 294292PTCC	Escherichia coli 25922PTCC	Klebsiella pneumoniae 13883PTCC
Blood agar Culture		EMB Culture		

Antioxidant Capacity of Samples

The evaluation of antioxidant capacity was analyzed using the DPPH radical assay, a well-known test for measuring the antioxidant power of various compounds, employed in this study. The method of measuring the antioxidant effect of the EOs was performed as follows: 100 µL of each *Ferula* EO from Tabas, Neishabur, Yazd, and Kerman, previously prepared in the extraction phase and confirmed by GC-MS, were poured into the test tube, and then 3.3 ml of DPPH solution was added, and the mixture was kept in darkness for 30 min. The absorption, along with the absorption of the control sample, was measured by Thermo UV-Vis spectrophotometry at a wavelength of 517 nm, showing an absorption of 0.726. The antioxidant activity of the stored EO in dark conditions was assessed as a standard reference. The color of the EO samples will change from purple to yellow. To prepare the DPPH solution for the antioxidant test of the EOs, 4 g of DPPH powder was dissolved in 100 ml of methanol. In addition, for the preparation of the control sample, 100 µl or 0.1 ml of methanol was added to 3.9 g of DPPH. The percentage of DPPH inhibition was calculated using Equation 1.

$$\text{DPPH inhibition (\%) or Antioxidant percentage} = \left(1 - \frac{\text{sample absorption}}{\text{control absorption}}\right) \times 100 \quad (1)$$

Results and Discussion

GC-MS Analysis Results

To analyze the composition of the extracted essences obtained by steam distillation using a Clevenger apparatus, each EO from different plants was individually examined and analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The results are presented in Tables 4, 5, 6, and 7. The results of GC-MS analysis in Table 4 revealed that the predominant compounds present in the essence obtained from *Asafoetida* in Tabas include alpha-di-xylofuranozide, methyl 2/5-di-o-methyl, (*E*)-1-propenyl di-sulfide secondary butyl, (*Z*)-1-propenyl di-sulfide secondary butyl, tri-fluoromethyl tert-butyl di-sulfide, di-sulfide, bis(1-methylpropyl), 10-epi-gamma-eudesmol, 3-mercapto propionitrile, agarospirane, and ethanethioamide.

Table 4: Chemical composition of essential oil obtained from *Ferula* plant extract in Tabas

Row	Chemical compounds that make up essential oils	RI	Percentage of compounds
1	Secondary butyl methyl disulfide	995	0.3
2	Para-cement	1023	0.1
3	limonene	1028	0.2
4	(*Z*)-beta-Eckman	1049	0.1
5	(*E*)-beta-Eciman	1054	0.7
6	Gamma -Terpinen	1056	0.1
7	Secondary butyl propyl disulfide	1152	1.1
8	(*Z*)-1-propenyl secondary butyl disulfide	1173	24.1
9	(*E*)-1-propenyl secondary butyl disulfide	1181	38.2
10	Bis(1-methylpropyl) disulfide	1214	3.1
11	Alpha-Longipinen	1349	1.8
12	Coconut acetate	1358	0.1
13	Alpha –Ilangen	1368	0.3
14	Alpha-copaine	1374	0.1
15	Methyl 1-(methylthio)ethyl disulfide	1392	0.9
16	Sedern	1399	0.3
17	Longifulen	1408	0.2
18	beta -Caryophyllene	1418	0.1
19	Alpha –Gorjonen	1424	2.5
20	Bis[(1-methylthio) propyl] disulfide	1428	1.1
21	Beta-homolene	1444	0.5
22	Alu-aromadren	1451	0.1
23	Alpha-homolene	1454	0.3
24	Methyl 1-(methylthio)propyl disulfide	1469	0.5
25	Germastern D	1474	0.07
26	Valensen	1494	0.3
27	Beta–Himacalen	1499	0.3
28	Methylpentyltetrasulfide	1504	1.2
29	Coparn	1509	0.2
30	Gamma. -Kadinen	1512	0.3
31	Delta –Kadinen	1517	1.1
32	(*E*)-alpha -bisabolene	1539	0.1
33	Germastern B	1559	0.1
34	Spatulenol	1576	0.1
35	Guayol	1598	5.4
36	Carotool	1601	5.1
37	Odsmol 10-epi-gamma	1622	0.8
38	Odsmol 7-epi-alpha	1653	0.3
39	Patchouli alcohol	1693	0.2
Total			**91.57%**

The results of GC-MS analysis in Table 5 demonstrated that the predominant compounds present in the essence obtained from Asafoetida in Neishabur include alpha-pinene, beta-pinene, (*Z*)-1-propenyl secondary butyl disulfide, and (*E*)-1-propenyl secondary butyl disulfide.

Table5: Chemical composition of essential oil obtained from *Ferula* plant extract in Neishabur

Row	Chemical compounds that make up essential oils	RI	Percentage of compounds
1	Dimethyl disulfide	783	0.3
2	Alpha. -pinene	937	2.4
3	Dimethyl trisulfide	970	0.4
4	beta-pinene	977	1.3
5	(*E*)-butyl disulfide	990	0.01
6	Limonene	1026	0.1
7	(*Z*)-beta-Eckman	1035	0.1
8	(*E*)-beta-Eciman	1048	0.3
9	Tetramethylpyrazine	1085	0.1
10	Borneo	1162	0.02
11	(*Z*)-1-propenyl secondary butyl disulfide	1174	34.4
12	(*E*)-1-propenyl secondary butyl disulfide	1179	53.2
13	Alpha-terpineol	1188	0.05
14	Bornyl acetate	1286	0.3
15	beta-element	1391	0.05
16	(*Z*)-caryophyllene	1404	0.1
17	(*E*)-caryophyllene	1415	0.1
18	Alpha-homolene	1453	0.1
19	Gamma -Curcumene	1478	0.05
20	Beta-selenin	1485	0.07
21	Delta -Kadinen	1508	0.1
22	(*Z*)-Azaron	1616	0.09
23	Beta-odsmol	1644	0.3
Total			93.97%

The results of GC-MS analysis for Table 6 revealed that the major compounds present in the essence obtained from *Asafoetida* in Yazd include (*Z*)-1-propenyl secondary butyl disulfide, (*E*)-1-propenyl secondary butyl disulfide, bis(1-methyl propyl) disulfide, alpha-longipinene, alpha-gurjunene, and carotol.

The results of GC-MS analysis for Table 7 revealed that the major compounds present in the essence obtained from *Asafoetida* in Kerman include (*Z*)-1-propenyl sec-butyl disulfide, (*E*)-1-propenyl sec-butyl disulfide, gamma-caryophyllene, germacrene B, (*Z*)-beta-ocimene, bis(1-methylthio) propyl sulfide, and limonene + beta-farnesene. These differences in the major compounds of the essences can be attributed to climatic conditions, the species used, harvest time, and processing methods.

Based on the results obtained from GC-MS analysis of the compositions of the essences in this study, a similar study entitled: ''*Investigating the Antimicrobial Activity of Essences*'', Obtained from Oleo-gum-resin and Seeds of *Ferula assa-foetida* in the Regions of Tabas and Yazd' was conducted. The study revealed a composition close to the current study, with main components identified as alpha-D-Xylofuranoside, methyl 5/2-di-*o*-methyl, (*E*)-1-propenyl sec-butyl disulfide, and (*Z*)-1-propenyl sec-butyl disulfide from seed essence, and (*E*)-1-propenyl sec-butyl disulfide and (*Z*)-1-propenyl sec-butyl disulfide from oleo-gum-resin [33].

Another similar study, ''*Comparison of the Composition of the Essence of Ferula assa-foetida*'', Obtained by supercritical carbon dioxide extraction and steam distillation for *Ferula* in Kerman, reported similar results in terms of percentage composition. The primary components identified in the water-distilled essence were a-pinene, b-pinene, germacrene B, myrcene, *p*-cymene, a-phellandrene, 1,4-cineole, *Z*-b-ocimene, (*E*)-b-ocimene, 1-propyl sec-butyl disulfide, *Z*-1-propenyl sec-butyl disulfide, E-1-propenyl sec-butyl disulfide, bis1-methyl propyl disulfide, bis1-methyl propenyl disulfide, c-gurjunene, a-humulene, viridiflorol, limonene + b-phellandrene, b-caryophyllene, copaene, c-cadinene, d-cadinene, eudesmol (10-epi-c), c-eudesmol, and hinokitiol [17].

Similarly, another study, *''Analysis of the Essence and Antibacterial Activity of Aerial Parts of Ferula assa-foetida L. from the Mountains of Neishabur*'', reported E-1-propenyl sec-butyl disulfide and *Z*-1-propenyl sec-butyl disulfide as the main components. These findings are consistent with the results of our research [39].

Table 6: Chemical composition of the essential oil obtained from the extract of *Ferula* plant in the Yazd

Row	Chemical compounds that make up essential oils	RI	Percentage of compounds
1	Secondary butyl methyl disulfide	995	0.1
2	Para-cement	1023	0.4
3	limonene	1028	0.1
4	(*Z*)-beta-Eckman	1049	0.2
5	(*E*)-beta-Eciman	1054	0.5
6	Gamma. –Terpinen	1056	0.1
7	Secondary butyl propyl disulfide	1152	0.7
8	(*Z*)-1-propenyl secondary butyl disulfide	1173	27.9
9	(*E*)-1-propenyl secondary butyl disulfide	1181	43.3
10	Bis(1-methylpropyl) disulfide	1214	1.5
11	Alpha –Longipinen	1349	1.1
12	Coconut acetate	1358	0.1
13	Alpha –Ilangen	1368	0.1
14	Alpha-copaine	1374	0.1
15	Methyl 1-(methylthio)ethyl disulfide	1392	0.6
16	Sedern	1399	0.1
17	Longifulen	1408	0.2
18	Beta. -Caryophyllene	1418	0.1
19	Alpha. –Gorjonen	1424	1.5
20	Bis[(1-methylthio) propyl] disulfide	1428	1.1
21	Beta-homolene	1444	0.5
22	Alu-aromadren	1451	0.05
23	Alpha-homolene	1454	0.2
24	Methyl 1-(methylthio)propyl disulfide	1469	0.4
25	Germastern D	1474	0.04
26	Valensen	1494	0.3
27	Beta –Himacalen	1499	0.3
28	Methylpentyltetrasulfide	1504	0.9
29	Coparn	1509	0.1
30	Gamma. –Kadinen	1512	0.3
31	Delta –Kadinen	1517	0.8
32	(*E*)-alpha-bisabolene	1539	0.2
33	Germastern B	1559	0.8
34	Spatulenol	1576	0.1
35	Guayol	1598	4.2
36	Carotool	1601	2.7
37	Odsmol 10-epi-gamma	1622	0.1
38	Odsmol 7-epi-alpha	1653	0.4
39	Patchouli alcohol	1693	0.2
Total			92.39%

Table 7: Chemical composition of the essential oil obtained from the extract of *Ferula* plant in Kerman

Row	Chemical compounds that make up essential oils	RI	Percentage of compounds
1	Alpha-pinene	938	1.4
2	Beta-pinene	979	1.1
3	they die	990	1.0
4	Alpha.-Flandren	1004	0.5
5	1,4-Cineol	1016	0.5
6	Para-Cayman	1025	0.2
7	Limonene + beta-flandrene	1030	1.7
8	(*Z*)-beta-Ocimen	1038	3.1
9	(*E*)-beta-Ocimen	1049	0.1
10	1-propyl sec-butyl disulfide	1172	0.7
11	(*Z*)-1-propenyl sec-butyl disulfide	1177	4.8
12	(*E*)-1-propenyl sec-butyl disulfide	1181	53.5
13	Bis(1-methylpropyl) disulfide	1220	1.1
14	Bis(1-methylpropenyl) disulfide	1263	2.1
15	Trans-antol	1281	1.9
16	beta-caryophyllene	1418	0/2
17	Bis(1-methylthio)propyl disulfide	1426	2.9
18	Alpha-homolene	1452	0.7
19	C-Gorjonen	1472	1.1
20	Heat radiation D	1479	0.1
21	Virdifloren	1492	0.3
22	Coparn	1501	0.2
23	C-cadinene	1512	0.4
24	Cys-bisabolene	1514	0.1
25	D-cadinene	1522	0.3
26	Trans-bisabolene	1532	1.1
27	Germastern B	1554	9.6
28	Ovidsmol (10-epi-c)	1619	1.1
29	Ovidsmol	1629	0.1
30	Hinswell	1636	0.3
Total			92.20%

MIC and MBC Results

The antimicrobial activity of essences from Tabas, Neishabur, Kerman, and Yazd was evaluated by examining and measuring the inhibition zone on five different pathogenic bacterial strains. The results indicated that all four essences exhibited distinct antimicrobial activities at various concentrations in both liquid medium diffusion tests (broth microdilution) and well diffusion tests, using a uniform volume of 250 µl and with concentrations of 50 mg/ml. The preliminary results of the MIC (the minimum inhibitory concentration and MBC (minimum bactericidal concentration) for each essence are detailed in Tables 8 and 9.

The results in Tables 8 and 9 showed that the essence from Yazd, with a minimum inhibitory concentration of 50 mg/mL, exhibited the highest inhibitory concentration against *Staphylococcus aureus*, while the essence from Kerman, with a minimum inhibitory concentration of 6.25 mg/mL, showed the lowest inhibitory concentration against *Escherichia coli* among the samples. Furthermore, none of the samples demonstrated antibacterial effects on *Pseudomonas aeruginosa*. The essence assays at concentrations of 50 mg/mL and 25 mg/mL

showed inhibitory and bactericidal effects on Gram-positive pathogenic bacteria, and a better antibacterial capability against Gram-positive bacteria compared to Gram-negative bacteria was observed.

Table 8: The results of the minimum inhibitory concentration of pathogenic bacteria by *Ferula EO* via the microdilution method

	MIC (minimum inhibitory concentration) in mg/ml				
	Type of EO / Type of bacteria	Tabas EO	Neishabur EO	Yazd EO	Kerman EO
Gram-negative	*Klebsiella pneumoniae*	0	0	25	0
	Pseudomonas aeruginosa	0	0	0	0
	Escherichia coli	0	0	25	6.25
Gram-positive	*Enterococcus*	12.5	0	0	25
	Staphylococcus aureus	50	50	50	25

Table 9: The results of the minimum lethal concentration of pathogenic bacteria by *Ferula EO* via the microdilution method

	MBC (minimum bactericidal concentration) in mg/ml				
	Type of EO / Type of bacteria	Tabas EO	Neishabur EO	Yazd EO	Kerman EO
Gram-negative	Klebsiella pneumoniae	0	0	12.5	0
	Pseudomonas aeruginosa	0	0	0	0
	Escherichia coli	0	0	12.5	3.25
Gram-positive	Enterococcus	6.25	0	0	12.5
	Staphylococcus aureus	25	25	25	12.5

The results also highlighted the significant variations in inhibitory and bactericidal effects among the different essences from distinct regions. For instance, the essence from Yazd exhibited superior inhibitory and bactericidal effects against Gram-positive bacteria, whereas the essence from Kerman showed the least inhibitory effects against *Escherichia coli*. Overall, the study suggests that the antimicrobial properties of the essences are influenced by factors such as regional climate, plant species, harvest time, and processing methods. The essence from Yazd demonstrated effective inhibition against Gram-positive bacteria, showcasing its potential as a natural antimicrobial agent. Conversely, the essence from Kerman exhibited the least inhibitory effects.

Well Diffusion Results

The essential oils antibacterial effects were assessed by measuring the diameter of the inhibition zone on Muller-Hinton Agar (MHA) plates is presented in Figure 4. The three repetition of the well diffusion test provided consistent results. The presence of halos around the wells indicated the absence of pathogenic bacterial growth due to the direct inhibitory and bactericidal effects of the essences. Larger halo diameters suggested more potent inhibitory and antibacterial effects of the essences. Figure 4 shows the results of growth inhibition effect and observation of halo diameter created for Gram-positive and Gram-negative bacteria.

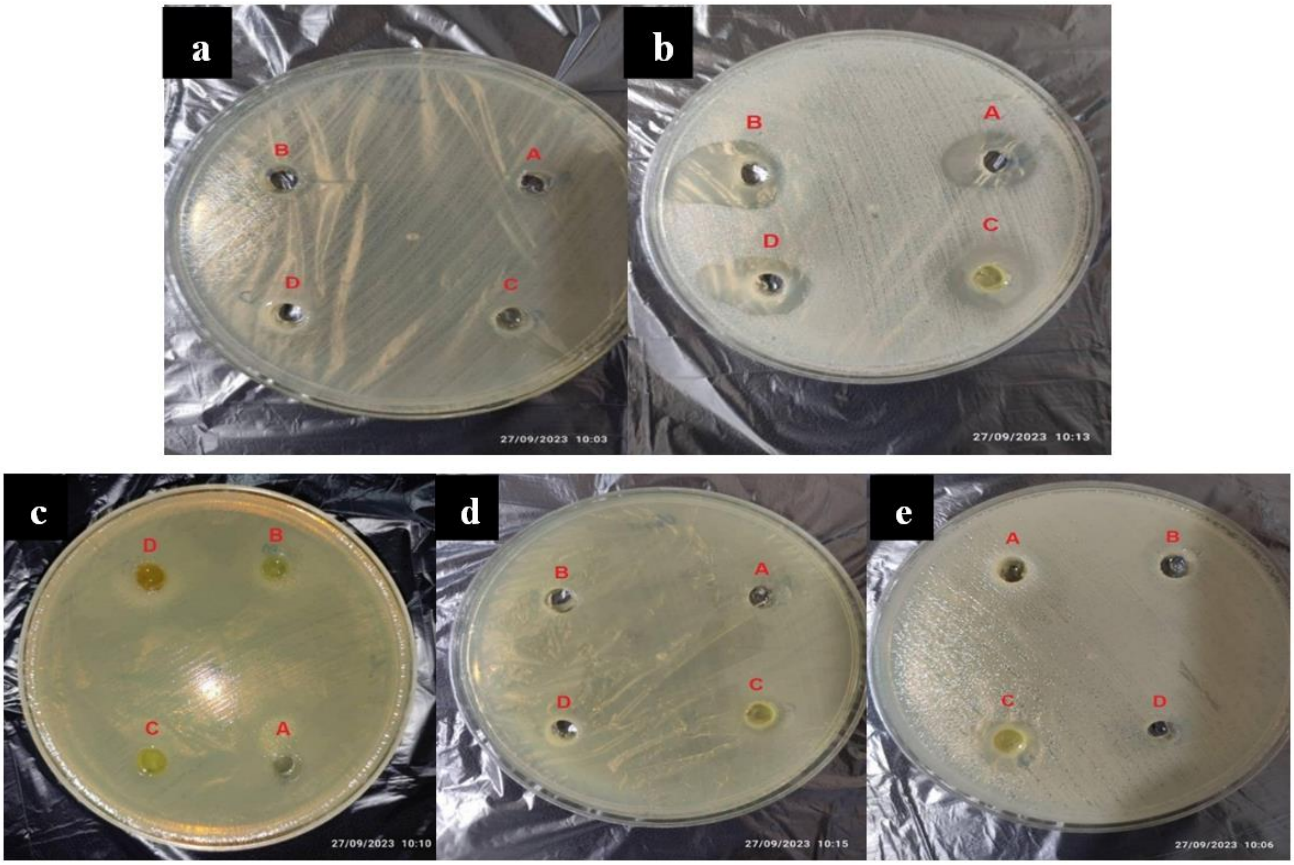


Figure 4: The well diffusion results and growth inhibition effect for Gram-positive pathogen strain, a) *Enterococcus* bacteria, b) *Staphylococcus aureus* bacteria and Gram-negative pathogen strain c) *Pseudomonas aeruginosa* bacteria, d) *Escherichia coli* bacteria, e) *Klebsiella pneumoniae* bacteria

The results of the average effects of essences on pathogenic bacteria for three repetitions are presented in Table 10. Based on the obtained results in the table, the maximum impact and inhibition (antibacterial) by the essence from the Yazd region are evident. The highest level of susceptibility and inhibition is observed against the pathogenic bacteria *Staphylococcus aureus*, with a reported halo diameter of 22.5 mm. In contrast, the least impactful and inhibitory effects (antibacterial) are associated with the essence from the Kerman region, and the lowest susceptibility and inhibition are reported against the pathogenic bacterium Escherichia coli, with a halo diameter of 11.5 mm. A comparative Table of the inhibitory values of the essences based on the halo diameter for three experimental designs is presented in Table 10.

Table 10: Mean results for the antibacterial effect of essential oils on pathogenic bacteria for three replicates

	Antibacterial amount based on the diameter of the halo created in the culture medium (mm)					
	Type of EO / Type of bacteria	**Tabas EO (mm)**	**Neishabur EO**	**Yazd EO (mm)**	**Kerman EO (mm)**	**Control sample**
Gram-negative	Klebsiella pneumoniae	-	-	16	-	-
	Escherichia coli	-	-	14.5	11.5	-
	Pseudomonas aeruginosa	-	-	-	-	-
Gram-positive	Enterococcus	13.5	-	-	14.5	-
	Staphylococcus aureus	21	20.5	22.5	16.5	-

The results of growth inhibition and the performance of each essence on pathogenic bacteria, along with the susceptibility of each pathogenic bacterium to the essences in each repetition at different levels, are presented in Figures 5 that illustrates the average performance of the essences on pathogenic bacteria based on the mean diameter of the inhibition zone in each of the three repetitions in the experimental design. In Figure 5, plotted using the Taguchi method, the maximum impact and inhibition (antibacterial) are observed with the essence from growth site C (Yazd region). The highest susceptibility and inhibition are reported against pathogenic bacterium B5 (*Staphylococcus aureus*). While, the least impactful and inhibitory effects (antibacterial) are associated with the essence from growth site B (Neyshabur region), and the lowest susceptibility and inhibition are reported against pathogenic bacterium B3 (*Pseudomonas aeruginosa*).

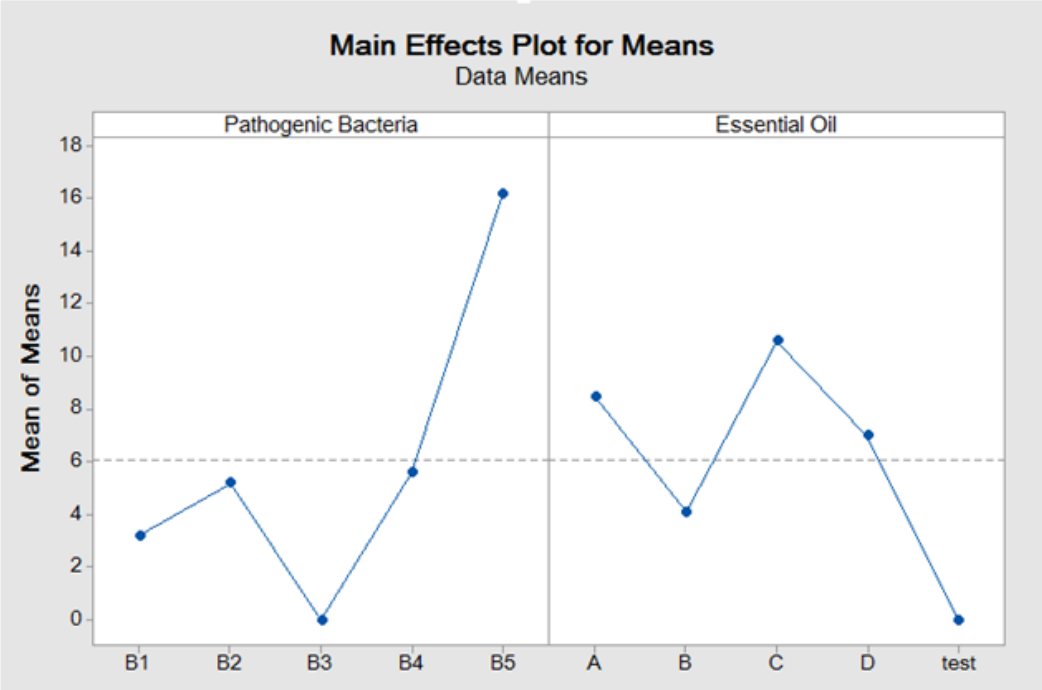


Figure 5: The graph of the averages for the performance of essential oils on pathogenic bacteria based on the average diameter of the halo formed for growth inhibition in all three repetitions of the design of experiments

Antioxidant Results

Antioxidant capacity analysis was conducted using the DPPH radical method. The assessment of antioxidant activity for each sample was determined by measuring the inhibition of free radical using the DPPH method. Equation 1 was employed to calculate the percentage of antioxidant activity for the essences. According to Equation 1, the antioxidant levels of the samples are described in Figures 6 and 7. Figure 6 depicts the graph of absorption percentage of essential oils for antioxidant test of samples, According to Figure 6, the highest absorption was related to the Yazd sample, while the lowest absorption was associated with the Kerman sample.

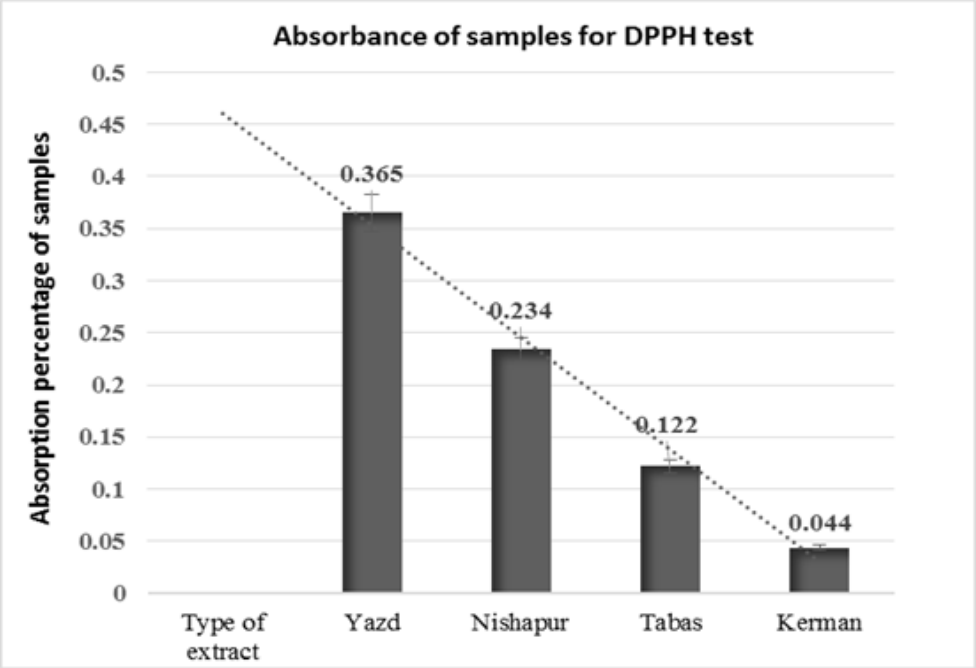


Figure 6: Graph of absorption percentage of essential oils for antioxidant test of samples

Figure 7 shows graph of percentage antioxidant activity of essential oils, In Figure 7, the highest antioxidant activity among the samples was related to the essence from Kerman's region with 93.9393% antioxidant activity. It was followed by the essence from Tabas with 83.1955% antioxidant activity, and then the essence from Neyshabur with 67.768% antioxidant activity. Finally, the least antioxidant activity was reported for the essence from Yazd's region with 49.7245% antioxidant activity.

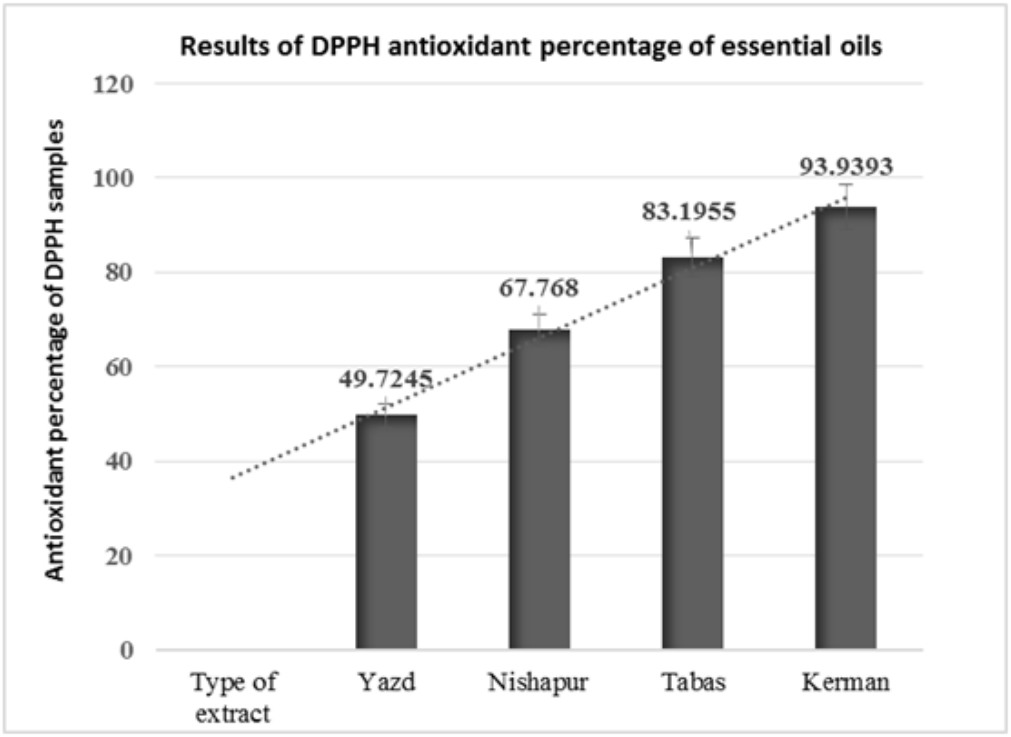


Figure 7: Graph of percentage antioxidant activity of essential oils

In a study conducted to investigate the inhibitory growth and antibacterial efficacy of *Ferula* essence, the minimum inhibitory concentration (MIC) of the essence was examined against both Gram-positive bacteria (*Staphylococcus epidermidis, Staphylococcus aureus,* and *Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*) using the microdilution method. The MIC of the essence ranged from 12 to 24 mg/ml against all tested bacteria. The results also indicated that disulfide compounds could be the primary reason for its antibacterial effect [39].

In another study, the antimicrobial effects of four different concentrations (2.5, 5, 10, and 20 µg/ml) of essences obtained from *Ferula* seeds and oleo-gum resin were examined against four oral pathogenic bacteria. The results demonstrated significant antimicrobial effects at all concentrations. The lowest evaluated concentration (2.5 µg/ml) resulted in an inhibitory zone with a diameter of 8.87 mm for seed essence and 13.5 mm for oleo-gum resin essence against S. mutans [33]. In addition, Kavoosi and Roshan investigated the antioxidant and antibacterial activities of *Ferula* essence, showing its inhibitory effects on the growth of Gram-positive bacteria. The plant exhibited inhibitory effects against Gram-positive bacteria ($P < 0.05$) [40]. In a research study, they reported dose-dependent antimicrobial effects of *Ferula* essence on Gram-positive bacteria. A concentration of 50 µg/ml showed no significant antibacterial effect compared to standard antibiotics, while a concentration of 100 µg/ml was more effective than standard antibiotics [41].

Concerning the antimicrobial properties of essences obtained from medicinal plants, phenolic monoterpenes are the most important effective substances. Due to their ability to increase the permeability and polarity of the cell membrane, they can prevent the growth of bacteria. As a result, proton leakage from cells, imbalance in the electrical potential of membrane cells, reduction in proton motive force, and a decrease in the formation of adenosine triphosphate (ATP) can occur [42]. It is worth noting that the consequence of reduced membrane potential is the loss of ions, ATP, amino acids, and proteins due to cell leakage [43]. One of the signs of membrane damage and cell death is the leakage of ions outside the cell [44]. Extracts from various *Ferula* species exhibit moderate antibacterial activity, possibly due to the presence of phenols, flavonoids, and sesquiterpenes. Higher levels of phenolic and flavonoid compounds are associated with antibacterial activity [45]. The penetration of the membrane and lipid layer is significantly increased by ring-containing compounds through their higher spatial volume, leading to membrane permeability and cell death [46].

In another study evaluating the antioxidant performance of aqueous and hydroalcoholic extracts of *Ferula* gum, the hydroalcoholic extract was found to have higher antioxidant activity due to the higher concentration of phenolic compounds (228 mg GAE/g) compared to the aqueous extract. The IC50 value for the hydroalcoholic extract was 1.714 mg/L, indicating greater antioxidant potential [47]. The increased inhibitory effects at higher concentrations could be attributed to the higher percentage of active compounds and the increased total phenolic content, as phenolic compounds are known to exhibit significant antioxidant activity and play a crucial role in preventing auto-oxidation [48]. Numerous studies have highlighted that the antimicrobial activity depends on the differences in phenolic and flavonoid compositions in essences and extracts, their habitat, and the growth period. Generally, compounds containing phenolic groups are more effective, and phenolic and aromatic compounds alter the function and structure of the cytoplasmic membrane, exerting their antimicrobial effects. Phenolic compounds interfere with cellular metabolism through mechanisms such as membrane disruption, enzyme inactivation, and metal chelation. Their ability to penetrate the cytoplasmic membrane is a key factor in cell resistance or sensitivity, as membrane permeability is a crucial factor in cell death. In addition, non-phenolic compounds in essences also exhibit effectiveness [49].

Hydroxyl groups in phenolic compounds play a vital role in the manifestation of their antimicrobial properties. Chemical compounds with aldehyde groups (-CHO) or hydroxyl groups (-OH) exhibit strong antimicrobial activity. Hydroxyl groups form hydrogen bonds that, when reacting with the enzyme's active site, render it inactive. Moreover, aldehyde groups react with sulfhydryl groups, inhibiting microbial growth. The presence of hydroxyl groups is essential for antimicrobial activity, as they contribute to the formation of hydrogen bonds and reactions with sulfhydryl groups, preventing microbial growth [50].

Conclusion

The study investigates the biological activities of *Ferula assa-foetida* EO, focusing on its antioxidant and antibacterial activities in essential oils from different regions. The GC-MS results show that the essential oils respectively from Neishabur, Yazd, Kerman, and Tabas have the highest concentrations of constituent compounds and sulfur. The Neishabur Nishabur essential oil is the best type of assafoetida in terms of the quantity of constituent compounds. The broth microdilution test for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of four types of essential oils revealed varying levels of antimicrobial activity against both Gram-positive and Gram-negative bacteria. Gram-positive bacteria showed higher inhibitory and bactericidal effects compared to Gram-negative bacteria. The Yazd essential oil of *Ferula assa-foetida* demonstrated a greater inhibitory power against pathogenic bacteria compared to other EOs. The Kerman essential oil of *Ferula assa-foetida* exhibited the lowest inhibitory concentration. The results from the experimental design with three repetitions for the well test showed no significant difference in the inhibitory and bactericidal effects of the essential oils on pathogenic bacteria in each experimental run. The performance chart of the essential oils on pathogenic bacteria and the susceptibility of each pathogenic bacterium to the oils showed that all four types of essential oils had different antimicrobial activities at the same concentrations (50 mg/ml) and volume of 250 µl for the well test. The highest mean impact and inhibition were reported by the Yazd habitat essential oil, with the most significant effect against the pathogenic bacterium *Staphylococcus aureus*. The lowest impact and inhibition were reported by the Kerman habitat essential oil, and the least susceptibility was reported by the pathogenic bacterium *Escherichia* coli. It can be said that the high levels of sulfur compounds and minerals such as flavonoids, alkaloids, and glycosides, especially asafetida and ferulol, in the EO of *Ferula assa-foetida* are likely responsible for its strong antibacterial effects. Furthermore, based on the results obtained from the percentage of antioxidant activity in the essences, the highest antioxidant activity among the samples was associated with the essence from Kerman's region, while the lowest antioxidant activity was attributed to the essence from Yazd's region. The EO has the ability to inhibit DPPH radicals and demonstrated activity against both Gram-positive and Gram-negative bacteria, making it a strong antioxidant and natural preservative in the food industry. In conclusion, the high levels of phenols and flavonoids, especially aromadendrene and cadinene, in the *Ferula assa-foetida* EO are likely responsible for its stronger antioxidant effects.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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