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FOENICULUM VULGARE ESSENTIAL OIL AND CONSTITUENTS ACTIVITY AGAINST GRAM-POSITIVE BACTERIA

ACTIVITATEA ANTIMICROBIANĂ ÎMPOTRIVA BACTERIILOR GRAM-POZITIVE A CONSTITUIENȚILOR ȘI ULEIULUI ESENȚIAL DE *FOENICULUM VULGARE*

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ABSTRACT | REZUMAT

The emergence of multi-drug-resistant bacteria is a global problem, so recent studies have focused on finding natural alternatives with increased efficiency without creating resistance. The present study aims to describe the chemical composition and antimicrobial activity against Gram-positive bacteria (*Streptococcus pyogenes* - ATCC 19615, *Staphylococcus aureus* - ATCC 25923, *Listeria monocytogenes* - ATCC 19114, *Bacillus cereus* - ATCC 10876 and *Clostridium perfringens* - ATCC 13124) of *Foeniculum vulgare* essential oil. The minimum inhibitory concentration was determined both for the essential oil itself and for the five standard plant compounds (linalool, cineole, caryophyllene, thujone, and eugenol).

Keywords: *Foeniculum vulgare*, antibacterial, standard

Apariția bacteriilor multi-rezistente la antibiotice este o problemă la nivel mondial, așa că studiile recente își îndreaptă atenția către găsirea de alternative naturale cu eficiență sporită, dar fără a crea rezistență. Prezentul studiu are ca scop descrierea compoziției chimice și activității antimicrobiene împotriva bacteriilor Gram-pozitive (*Streptococcus pyogenes* - ATCC 19615, *Staphylococcus aureus* - ATCC 25923, *Listeria monocytogenes* - ATCC 19114, *Bacillus cereus* -ATCC 10876 și *Clostridium perfringens* -ATCC 13124) a uleiului esențial de *Foeniculum vulgare*. Concentrația minimă inhibitorie a fost determinată atât pentru uleiul esențial în sine, cât și pentru cei cinci compuși standard ai plantei.

Cuvinte cheie: *Foeniculum vulgare*, antibacterial, standard

Due to the emergence and spread of multidrug-resistant, extensive drug-resistant, and pan-drug-resistant infectious agents, finding new natural alternatives to antibiotics is an essential objective for current biomedical research, with *Foeniculum vulgare* being one of the plants of interest due to its antimicrobial activity.

Foeniculum vulgare is a biennial or perennial herb disseminated in the Mediterranean and central European regions, belonging to the *Apiaceae* family. It is considered a herbal medicinal plant not only for its antibacterial and antifungal properties but also for its anti-inflammatory, antithrombotic, antidiabetic, and hepatoprotective activities (2, 11, 18, 19, 20).

It should be emphasised that *F. vulgare* essential oil, one of the most excellent antimicrobial agents against both Gram-positive and Gram-negative strains (10), can be obtained from the different plant parts, so it lies in massive production (19).

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Antimicrobial activity varies depending on the part of the plant from which the essential oil is produced. So, the essential oil obtained from the fruits showed antibacterial activity against *Escherichia coli*, *Bacillus megaterium, Staphylococcus aureus,* and *Listeria monocytogenes* (6, 8, 15), while the one extracted from the seeds is an antimycobacterial and anticandidal agent (1).

However, the antimicrobial activity of essential oil is influenced by the geographical area and the microclimate conditions for plant cultivation, as well as methods of obtaining the essential oil, which determine variations in the chemical composition (10).

In this study, the chemical characterisation, antimicrobial activity, and MIC determination of *Foeniculum vulgare* essential oil (FVEO) and the antibacterial efficacy of five standard plant compounds (linalool, cineole, caryophyllene, thujone, and eugenol) identified in the EO composition were tested against Gram-positive strains: *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19114), *Bacillus cereus* (ATCC 108 76), and *Clostridium perfringens* (ATCC 13124).

MATERIALS AND METHODS

Samples and chemicals

The *Foeniculum vulgare* essential oil (FVEO) analysed is produced and marketed by Oleya, Romania. The materials, solvents, and standards were of analytical grade (Sigma Aldrich, Austria).

Gas chromatography-mass spectroscopy (GC/MS)

FVEO was analysed by GC-MS using Shimadzu QP 2010 Plus apparatus (Columbia, SC, USA) equipped with an AT WAX 30 m 0.32 mm 1 m capillary column. The discharge rate of the carrier gas, helium, was 1 mL/min, and the temperatures of the injector and ion source were 250°C and 220°C, respectively. For compound separation, a temperature gradient was utilised with an initial oven temperature of 40°C maintained for 1 minute, followed by an increase to 210°C at a rate of 5°C/min and a subsequent 5-minute hold at this temperature. The sample injection volume was 1 µL of a 2% BEO hexane solution, and a split ratio of 1:50 was utilised.

The volatile components of the essential oil evaluated were identified using the NIST 5 Wiley 275 library database. The match of detected compounds to the database was at least 90%. The results were presented as percentages from total compounds. LRI was calculated using normal alkane RI for the same polar column.

The microbiological strains

The reference microbial strains (ATCC) used in this study were obtained from the culture collection of the Microbiology Laboratory of the Interdisciplinary Research Platform within the "King Michael I of Romania" University of Life Sciences in Timisoara.

FVEO samples were tested on the following reference strains: *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19114), *Bacillus cereus* (ATCC 108 76), and *Clostridium perfringens* (ATCC 13124). MIC is defined as the lowest tested concentration that produces no visible, detectable growth of microorganisms. Our previous research described the method as microbial mass loss by measuring OD by spectrophotometry according to ISO 20776-1:2019.

Microbiological method

The bacterial strains were revived overnight in the Brain Heart Infusion (BHI) broth (Oxoid, CM1135) at 37 °C and subsequently switched to BHI Agar (Oxoid, CM1136) for 24 hours at 37 °C. The cultures were then diluted to an optical density (OD) of 0.5 McFarland standard (1.5×10^8CFU×mL) using BHI broth and evaluated with a McFarland densimeter (Grand-Bio, England). The dilutions were spotted at a volume of 100 µL in each well of the 96-well microdilution plate using a Calibra 852 digital multichannel pipette. The tested FVEO was added in the amount of 2.5 µL, 5 µL, 7.5 µL, and 10 µL. The standards tested were the main compounds identified by GC-MS: alpha-Pinene, D-Limonene, and anethole. Plates were covered and left for 24 hours at 37 °C. After 24 hours, the DO was measured at 540 nm using an ELISA reader (BIORAD PR 1100, Hercules, CA, USA). Triplicate tests were performed for all samples. Strain suspensions in BHI were used as a negative control.

Microdilution in broth is one of the most basic methods of testing antimicrobial susceptibility, being used in many studies to highlight the antimicrobial activity of various compounds (4, 13, 17). The technique involves testing double dilutions of the antimicrobial agent analysed in a liquid growth medium distributed in microtitre plates with 96 wells.

MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of the body. CLSI has standardised the broth microdilution method to test aerobically growing bacteria, yeasts, and filamentous fungi (7). The EUCAST broth microdilution method is similar to that of CLSI, with changes that typically refer to some test parameters, such as inoculum preparation, inoculum size, and MIC reading.

The results are presented as bacterial growth rate (BGR%) and bacterial inhibition rate (BIR%), calculated rates using the formulas (1, 2):

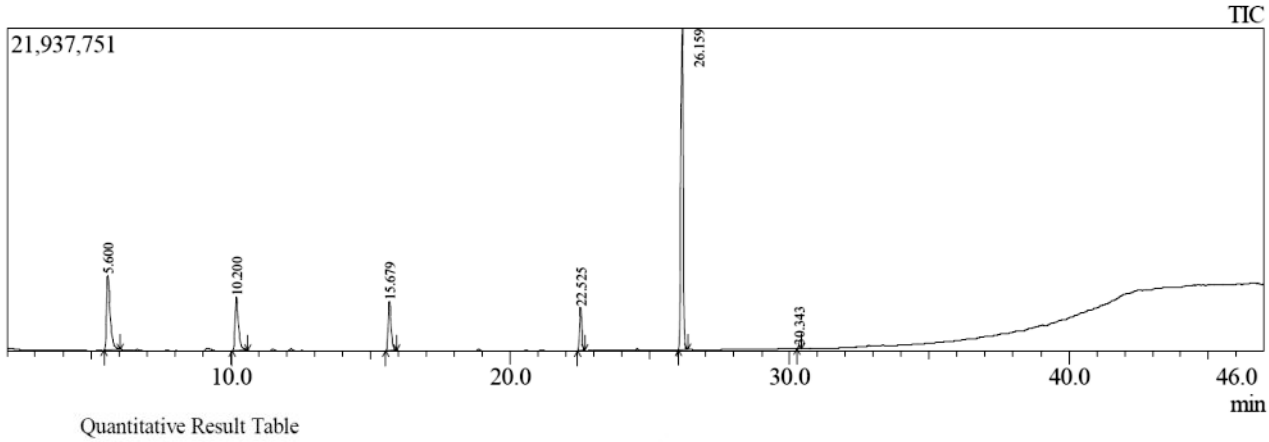
$$\text{BGR\%} = \frac{OD_{SAMPLE}}{OD_{CONTROL}} \times 100\ (\%)$$

$$\text{BIR\%} = 100 - \text{BGR}\ (\%)$$

RESULTS AND DISCUSSIONS

The GC-MS analysis (Fig. 1) identified six chemical constituents, of which the main constituents were anethole (52.74%), alpha-pinene (18.34%), and D-Limonene (12.76%). The other identified compounds were fenchone, isoanethone, and anisaldehyde.

The compound of FVEO highlighted by other researchers is different from the one obtained in the present study (14, 22, 23). The difference can be explained by climate, harvest season, and part of the plant, so various chemotypes of essential oil are obtained. Stefanini et al. (22) reported that the main compounds of essential oils from plant parts were: trans-anethole in dry seeds in summer (78.25%); limonene in steams or leaves in spring (42.30%); fenchone in green seeds in autumn (16.98%) and summer (15.08 %); and methyl-chavicol in green seeds in autumn (3.57%) (22). Milenković et al. (2022) demonstrated that the most common components from fennel stems were E-anethole and methyl chavicol,



Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	Conc.
1	.alpha.-Pinene	5.600	TIC	46005199	5098655	18.349
2	D-Limonene	10.200	TIC	32008147	3652049	12.767
3	Fenchone	15.679	TIC	24542961	3352242	9.789
4	Isoanethole	22.525	TIC	15100495	2932999	6.023
5	Anethole	26.159	TIC	132251693	21850882	52.749
6	Anisaldehide	30.343	TIC	808763	189967	0.323

Fig. 1. Chemical composition of the tested *Foeniculum vulgare* essential oil (FVEO) detected by GC-MS

and from leaves are represented fenchone, α-phellandrene, and methyl o-anisate (14).

Without specifying the part of the plant, Hamada et al. (12) found eleven compounds in the fennel essential oil, among which alpha-phellandrene, dill ether, D-Limonene, and carvone (12). Barrahi et al. (2020) showed that the major compound was the trans-anethole (3). Instead, Di Napoli et al. (2022) obtained similar results to the present study, with α-pinene being the predominant component. However, even in the present study, D-Limonene is the second most abundant compound, Di Napoli et al. (2022) found that estragole has a low concentration of D-Limonene (10). The antimicrobial efficacy testing revealed the OD values in Table 1.

Table 1 presents the OD (optical density) values ob-tained, with the red colour indicating the values that reached the MIC. As presented, all the strains presented sensitivity to the FVEO, starting with the lowest concentration tested. Therefore, the 1.25 µL value is set as the MIC and subsequently used as a concentration to analyse the three main compounds identified in the FVEO.

Figure 2 presents a graphical representation of the antibacterial efficacy of FVEO against the *S. pyogenes* ATCC strain expressed as bacterial growth and bacterial inhibition. Regarding the inhibitory potential, the evolution trend is positive, with a positive correlation with the increase in the concentration tested. MIC was achieved at the lowest concentration (1.25 µL), and all the subsequent BIR% values obtained increased alongside the increase in concentration, with values ranging from 7.21% to 29.59%.

S. aureus proved to be highly sensitive to the activity of FVEO, as presented in Fig. 3. The BIR% evolution in correlation to the quantity tested is positive, with values ranging from 39.78% at 1.25 µL to 58.74% at 10 µL.

Concerning the FVEO efficacy against *B. cereus*, we can state that the ATCC strain proved to be more resistant, even if the MIC was also reached at 1.25 µL, the BIR% values were lower (2.34% – 21.93%).

Similar values were obtained in the case of *Cl. perfringens*, with the MIC being present at 1.25 µL but with a small value (0.37) and the highest quantity tested reaching 25.25%.

Tabel 1

The antimicrobial activity of *Foeniculum vulgare* essential oil (FVEO)

	S. pyogenes	*S. aureus*	*B. cereus*	*Cl. perfringens*	*L. monocytogenes*
1.25 µL	0.669	0.489	0.668	0.809	0.612
2.5 µL	0.651	0.443	0.603	0.742	0.588
5µL	0.588	0.398	0.579	0.691	0.511
10 µL	0.508	0.335	0.534	0.607	0.459
Control	0.721	0.812	0.684	0.812	0.632

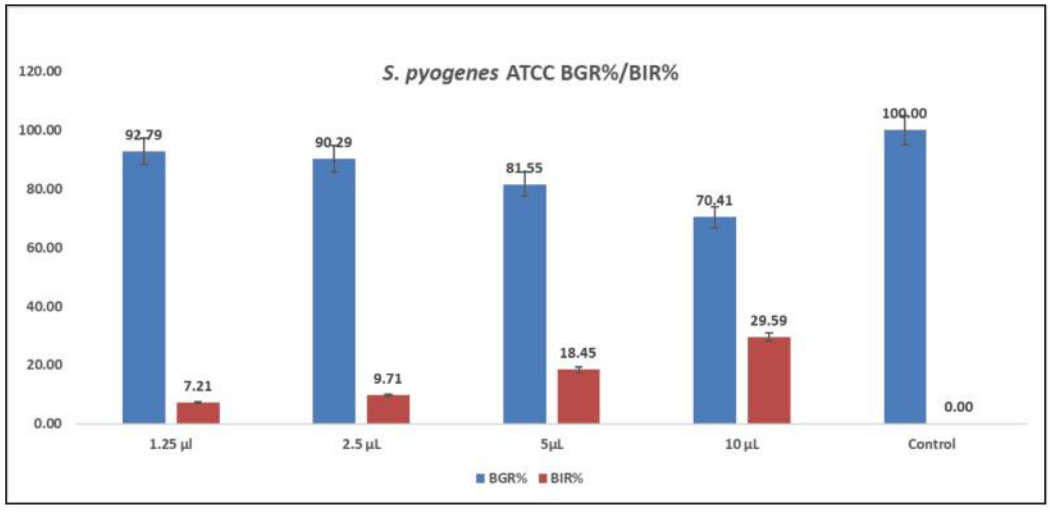


Fig. 2. *Foeniculum vulgare* essential oil (FVE activity against *S. pyogenes* expressed as BGR% and BIR%

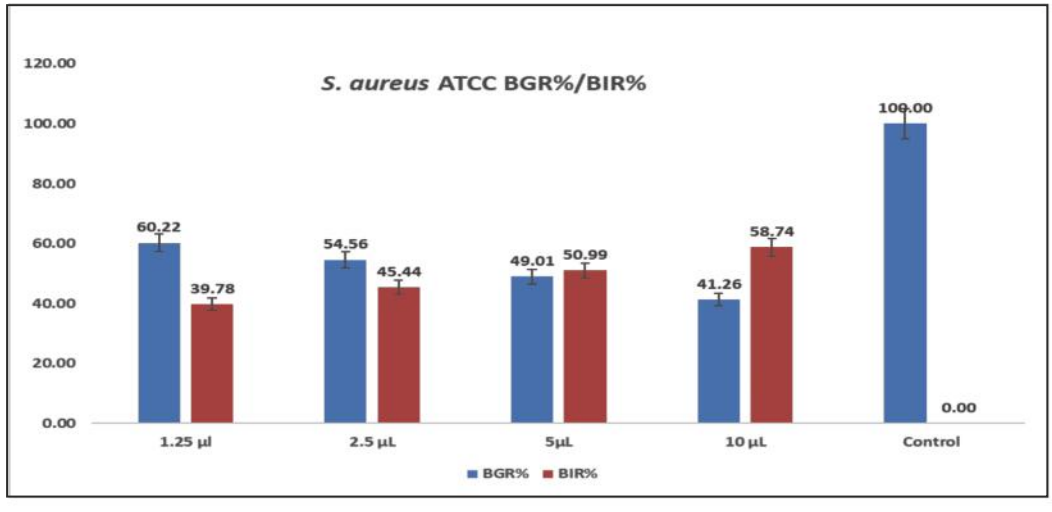


Fig. 3. *Foeniculum vulgare* essential oil (FVEO) activity against *S. aureus* expressed as BGR% and BIR%

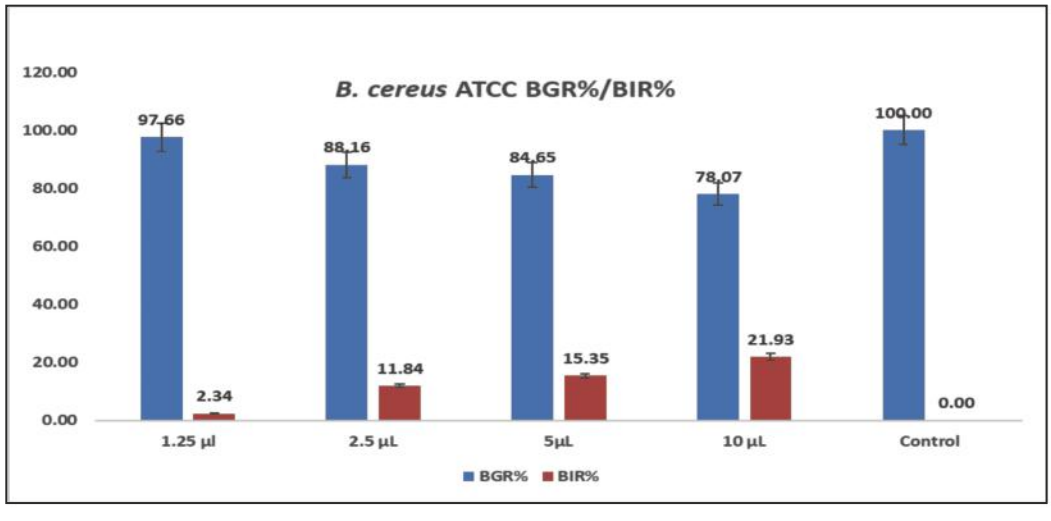


Fig. 4. *Foeniculum vulgare* essential oil (FVEO) activity against *B. cereus* expressed as BGR% and BIR%

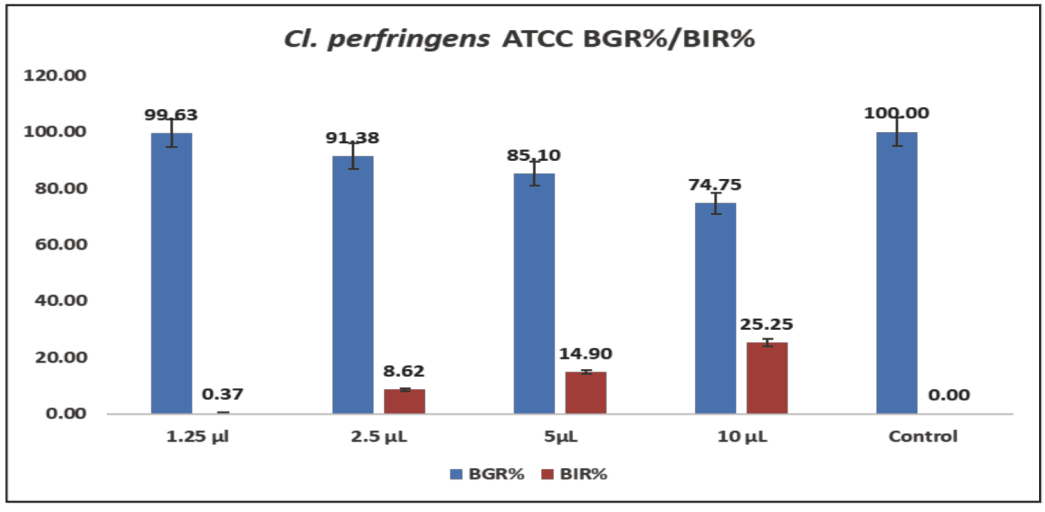


Fig. 5. *Foeniculum vulgare* essential oil (FVEO) activity against *Cl. perfringens* expressed as BGR% and BIR%

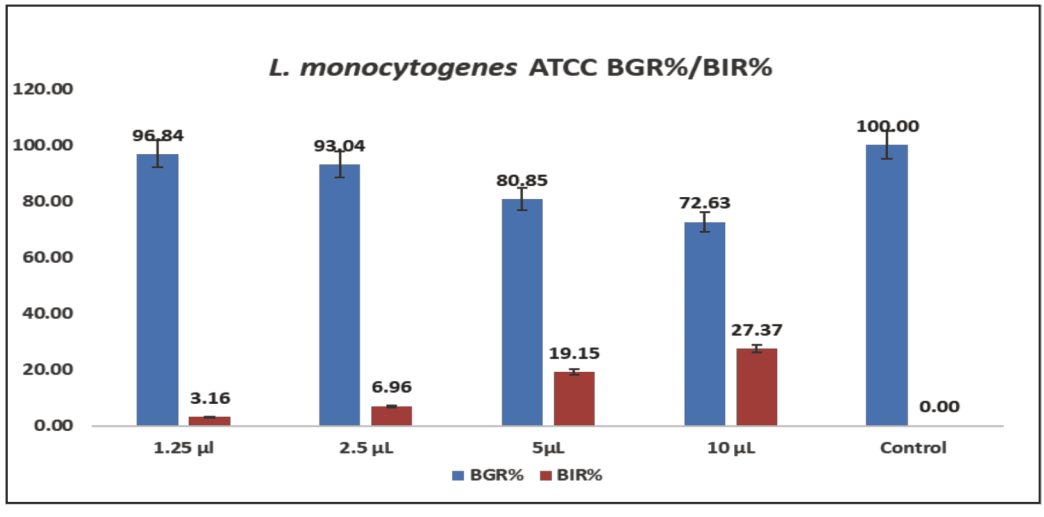


Fig. 6. *Foeniculum vulgare* essential oil (FVEO) activity against *L. monocytogenes* expressed as BGR% and BIR%

Regarding the BIR% values obtained for *L. monocytogenes*, the evolution and values obtained are similar to the ones obtained for *B. cereus* and *Cl. perfringens*. Of all the results obtained, *S. aureus* proved to be the most sensitive to the action of FVEO, followed by *S. pyogenes, L. monocytogenes*, *Cl. Perfringens,* and finally *B. cereus*.

The antimicrobial activity of FVEO is debatable, according to data from the specialised literature.

De-Montijo-Prieto et al. (9) demonstrated that this essential oil had no antimicrobial activity against *S. aureus* and *L. monocytogenes,* while other researchers highlighted its effectiveness against some of the Gram-positive bacteria (9).

Di Napoli et al. (2022) showed that the MIC values for *S. aureus* and *B. cereus* were 250 µg/mL (10), while Mota et al. (2015) claimed that the MIC varies between 250 and 500 µg/mL depending on the chemotypes of the essential oil (16).

In contrast, the present study demonstrated that the tested FVEO had antimicrobial activity against *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19114), *Bacillus cereus* (ATCC 10876), and *Clostridium perfringens* (ATCC 13124) at a concentration of 1.25 µl/mL. Due to the highest BIR% values obtained by the essential oil against *S. aureus*, it can be concluded that FVEO had excellent antimicrobial activity against this strain.

As a follow-up to the first stages of analysis, the main chemical compounds identified through GC-MS were tested at MIC concentrations for each strain to identify the compound responsible for the antibacterial efficacy of FVEO, as presented in Table 2 (BGR% of standards) and Table 3 (BIR% of identified FVEO compounds).

Table 2

BGR% of chemical standards against ATCC strains

	BGR%				
	S. pyogenes	*S. aureus*	*B. cereus*	*Cl. perfringens*	*L. monocytogenes*
alpha.-Pinene	80.70	82.15	98.10	77.58	81.47
D-Limonene	104.64	113.42	131.49	89.04	92.82
Anethole	101.89	101.34	101.58	101.00	100.67
Control	100.00	100.00	100.00	100.00	100.00

Table 3

BIR% of chemical standards against ATCC strains

	BIR%				
	S. pyogenes	*S. aureus*	*B. cereus*	*Cl. perfringens*	*L. monocytogenes*
alpha.-Pinene	19.30	17.85	1.90	22.42	18.53
D-Limonene	-4.64	-13.42	-31.49	10.96	7.18
Anethole	-1.89	-1.34	-1.58	-1.00	-0.67
Control	0.00	0.00	0.00	0.00	0.00

Standards tested showed an antibacterial effect in the following descending order: alpha-Pinene> anethole> D-Limonene. was by far the best antibacterial standard tested, its efficacy proving to be the highest, with all the BIR% values obtained being positive ones.

Those data are similar to those one obtained in the literature, which showed that alpha-Pinene had excellent antimicrobial activity, especially for *S. aureus* (5, 21). Regarding D-Limonene, the only positive value obtained was in the case of *Cl. perfringens* and *L. monocytogenes*, with the results presenting an antibacterial efficacy mostly on food-related pathogens. Anethone demonstrated no inhibitory efficacy against any of the ATCC strains tested.

CONCLUSIONS

Chemical analyses of FVEO by GC-MS, revealed six chemical constituents, three of which were dominant: anethole (52.74%), alpha-pinene (18.34%), and D-Limonene (12.76%).

The antimicrobial activity was correlated with the chemical composition of the oil, a fact demonstrated by the MIC determination of the standards.

Even though the MIC values for all the Gram-positive tested strains were 1.25µL/mL, the best activity was observed against *S. aureus.*

By testing the standards containing in FVEO, the antibacterial efficacy was identified in the following descending order: alpha-Pinene > anethole > D-Limonene.

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