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ORIGINAL ARTICLE

ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF *SALVIA OFFICINALIS* GROWING IN MOROCCO

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ABSTRACT

Background. The bacterial infections treatment is complicated by antibiotic resistance. In this fact, the need for new therapeutic approaches to control bacterial infections is crucial. Therefore, discovering new antibiotics from medicinal plants, able to kill drug-resistant bacteria, is essential to saving modern medicine.

Objective. This study was to evaluate the *in vitro* antibacterial activity of *Salvia officinalis* essential oil (SoEO) growing in Morocco.

Material and methods. The essential oil was extracted by hydro distillation, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by agar dilution method. The essential oil was analyzed by Fourier-transform infrared spectroscopy (FTIR) and fractionated/purified using column chromatography followed by thin-layer chromatography (TLC).

Results. The results revealed that SoEO showed higher antimicrobial activity against *Enterococcus faecalis* and *Citrobacter freundii*. Fourier-transform infrared spectroscopy (FTIR) analysis, and purification/fractionation of SoEO, indicates that the most polar fraction F6 is the active fraction of SoEO. This finding can be explained by the existence of polar compounds in this fraction including alcohols, and phenols as thymol, eugenol, globulol, and spathulenol.

Conclusions. It can be conclude that alcohols and phenols from *Salvia officinalis* essential oil (SoEO) have promising antibacterial activity. This action can offer a great possibility of the application of SoEO in the treatment of bacterial diseases.

Key words: *Gram-negative bacteria; bactericidal activity; FTIR analysis; alcohols; phenols*

INTRODUCTION

Gram positive and Gram negative bacteria like *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas* sp, *Staphylococcus aureus*, and *Enterococcus faecalis* are very common human pathogenic microorganisms which can trigger a variety of infectious diseases, including infections of the urinary tract, wounds, sepsis, neonatal meningitis, skin and soft tissue infections, endocarditis, osteomyelitis, lethal pneumonia, chronic bronchial infection, endodontic infection, bloodstream, surgical-site infections, and mixed bacterial infections [1, 2, 3, 4, 5, 6]. The treatment of these bacterial infections is complicated by antibiotic resistance. For instance, this phenomenon kills at least 700 000 people each year. Within 30 years, it is predicted to kill 10 000 000 per year, and estimated to become the greatest challenge in healthcare by 2050 [7]. Indeed, *Pseudomonas aeruginosa*, *E. coli,* and *Staphylococcus aureus* are frequently resistant to several antibiotics, and they were published in the "critical and high" category of the WHO's priority pathogens list for research and development of new antibiotics including *P. aeruginosa* carbapenem-resistant, *E. coli* carbapenem-resistant, and *S. aureus* methicillin-resistant, and vancomycin-resistant [8].

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Moreover, It is worth mentioning that pathogenic *E. faecalis* has acquired extensive antibiotic resistance traits including resistance to "last-resort" antibiotics such as vancomycin, daptomycin, and linezolid [9, 10]. *C. freundii* has also showed strong resistance to β-lactam antibiotics [2, 7]. Thus, the immediate need for new therapeutic approaches to control bacterial infections is crucial.

The use of EOs may constitute an alternative solution to fight against multidrug-resistant bacteria. Many studies have focused on active substances from EOs, which contains natural bioactive substances used as alternative medicines, especially for their strong antibacterial activities [11]. One of them is the EOs obtained from *Salvia officinalis*, which has attracted the attention of microbiologists due to their widespread use against pathogenic bacteria [12].

Salvia officinalis, known in Morocco as "Salmya", has been used since ancient times for medicinal and culinary use. Currently, the essential oil of *S. officinalis*, have shown several chemical and biological activities including anti-proliferative, antibacterial, anti-inflammatory, antioxidant, antiviral, and insecticidal activities [13, 14, 15, 16].

This study aims to investigate the antibacterial activity of *S. officinalis* essential oil (*So*EO) against Gram positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*), and Gram negative bacteria (*Citrobacter freundii*, *Escherichia coli*, *Pseudomonas* sp), it also aims to determine the chemical composition of active fraction of *So*EO.

MATERIAL AND METHODS

Plants material

The fresh samples of *Salvia officinalis* were harvested in winter of 2020, at 23:00 nighttime, in El Jadida city in the Kingdom of Morocco, and located at the latitude of 33° 14' 0.0024'' N, and the longitude of 8° 30' 0.0000'' W). Then the leaves were removed and dried in a drying oven at 47°C, and stored in the shade prior to use.

Essential oil extraction

The extraction was carried out by hydrodistillation using a Clevenger-type apparatus, alongside samples of 120 g of Sage leaves dried. The crushed leaves were placed in a 500 ml flask which contains 300 ml of distilled water. The flask was attached to Clevenger apparatus for 4 hours. At the end of hydro-distillation, the pure essential oil was collected and stored in opaque glass bottles at a temperature of 4°C.

Antibacterial assay

Test microorganisms

Five bacteria species obtained from Collection of the Pasteur Institute in Paris (CIP) and American Type Culture Collection (ATCC) were used as the antimicrobial strains: *Citrobacter freundii* ATCC8090, *Escherichia coli* CIP54127, *Pseudomonas* sp ATCC 10145, *Enteroccus faecalis* ATCC19433, *Staphlococcus aureus* CIP 209. The bacterial strains were maintained on the *Mueller-Hinton* agar medium.

Disc diffusion method

Antibacterial activity was examined by the agar disc diffusion method [17]. The bacteria were first grown on *Mueller-Hinton* plates at 37 °C for 18–24 h prior to inoculation onto the gelose of Mueller-Hinton (MH). Then, the inoculums of bacteria were prepared from colonies in exponential growth phase at the concentration of 106 UFC / ml.

Petri dishes containing *Mueller-Hinton* (MHA) agar were inoculated with bacterial inoculums. The disc of *Whatman* paper of 6 mm in diameter was impregnated by essential oil EO) (20 µl /disc). The Petri dishes were placed at 4 °C for 2–3 h and then incubated at 37 °C for 24–48 h. Antibacterial activity was evaluated by measuring the inhibition zone diameter after 48 h of incubation at 37 °C. The positive control was the antibiotic Ampicillin 30 µg. Each experiment was conducted in triplicate and the mean zone diameter value was recorded.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of *So*EO was performed out by micro-dilution method using the resazurin test [18] according to the following steps:

1. 100 µl of *Mueller-Hinton* liquid culture medium are poured into each well;
2. 100 µl of essential oil diluted in Tween 80 is taken to have a concentration of 33 % (v/v);
3. Dilution series of EO were prepared in Bouillon of *Mueller-Hinton*, to obtain a concentration range between 33 and 0.065 % (v/v);
4. Each well of the plate is inoculated with 40 µl of bacterial strain (106 UFC/ml);
5. The plates were incubated at 37 C for 24 hours in the dark;
6. 15 µl of resazurin 0.01 % (w/v) is added to each well.

The MIC is the lowest concentration at which the color changes of the resazurin from blue to fluorescent pink.

- *Minimal Bactericidal Concentration (MBC)*

MBC was determined by inoculating 10 µl aliquots taken from the wells in which the resazurin coloration is not changed. After the incubation of the dishes at

37 °C for 24 to 48 hours, the square without bacterial growth corresponds to MBC value.

• *Antibacterial Effect Interpretation*

MBC/MIC ratio was calculated to assess the bactericidal/bacteriostatic effect on the bacterial growth. If MBC/MIC ≤ 4, then the effect is bactericidal, and if MBC/MIC > 4, the effect is bacteriostatic [19]. The tests were performed in triplicate.

FTIR analysis of S. officinalis essential oil

The chemical composition of *So*EO was analyzed by an FTIR spectrometer of the JASCO 4000 type that is equipped with a detector (TGS), and a ceramic source. Michelson interferometer was also used for IR radiation analysis. The wavelength range used was between 400 - 4000 cm-1 and the resolution was 2 cm-1.

Fractionation and purification of S. officinalis essential oil

• *Column chromatography*

The fractionation of *So*EO was carried out using a Column chromatography, with a diameter of 1.2 cm, and a length of 23 cm. The adsorbent chosen was silica with a fine particle size of 0.063 mm to 0.2 mm. The fixed phase was prepared according to the following steps:

1. Add 10 g of silica to 100 ml of a mixture of hexane/ethyl acetate (95% +5 %).
2. Stirred the mixture was until the gel became homogeneous.
3. Deposit the gel in the column and remove the excess solvent without drying the silica.
   The mobile fixed phase used was the hexane/ethyl acetate (95% + 5%).

• *Thin-layer chromatography (TLC)*

The migration tank was partially filled with the mixture hexane/ethyl acetate (95% + 5%). The samples were deposited by a glass capillary. After that, the plate was brought into contact with the mobile phase. The plates were then revealed by a UV lamp (254 nm), and by sulfuric vanillin.

After the determination of the fractions, the antibacterial activity of each fraction was tested by disc diffusion method to extract the active fraction(s). Moreover, the yield of the active fraction was calculated.

RESULTS

Antibacterial activity

The evaluation of the extraction parameters showed that extraction yield of *So*EO recorded 2%. *So*EO showed antibacterial activity against all the studied bacteria. This activity varies between maximal activity against *E. feacalis* (d= 21.33 ± 5.69 mm) and minimal activity against *E. coli* (d=10.67 ± 0.58 mm) (Figure 1). In the other hand, it is observed that the Gram-positive bacteria are more sensitive to the action of *So*EO than the Gram-negative bacteria. It have recorded a zone of inhibition d=18.16 mm. In contrast, the Gram-negative bacteria have shown more resistant to *So*EO with a weaker zone of inhibition not exceeding d = 12.78 mm (Figure 2).

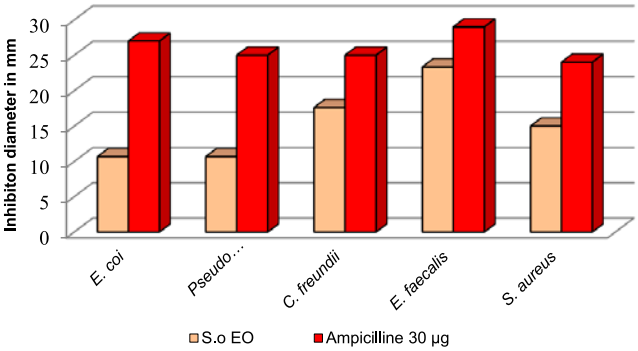

Figure 1. Inhibition diameter of *Salvia officinalis* essential oil (*So*EO)

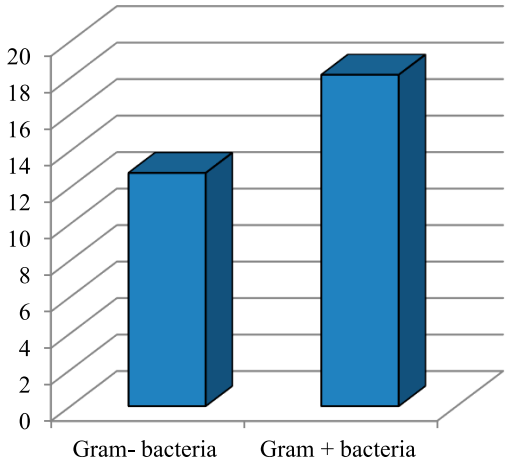

Figure 2. Antibacterial activity of *So*EO against Gram- bacteria, and Gram+ bacteria

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

As indicated in Table 1, *So*EO shows bactericidal activity against *Pseudomonas* sp, and *E. faecalis* (MBC/MIC ≤ 4). However, it shows bacteriostatic activity against *E. coli*, *S. aureus*, and *C. freundii* (MBC/MIC>4). The highest bactericidal activity of *So*EO was observed against *Pseudomonas*. sp which is a Gram-negative bacteria (MBC/MIC = 1.00). The weakest bactericidal effect was found against *E. faecalis* which is a Gram-positive bacteria (MBC/MIC = 2.00). Gram-negative bacteria were more sensitive to *So*EO, with MIC values ranged from 0.13 % v/v to 4.13 % v/v, compared to Gram-positive bacteria which show MIC values between 4.13 % v/v, and 8.33 % v/v.

Table 1. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the MIC/MBC ratio of *Salvia officinalis* essential oil (SoEO)

Microorganisms		MIC (% v/v)	MBC (% v/v)	MBC/MIC		Bacteriostatic or Bactericidal
Gram -bacteria	*E. coli*	2.08	16.67	8.01	>4	Bacteriostatic
	Pseudomonas sp.	0.13	0.13	1.00	≤4	Bactericidal
	C. freundii	4.13	33.33	8.07	>4	Bacteriostatic
Gram+bacteria	*E. faecalis*	8.33	16.67	2.00	≤4	Bactericidal
	S. aureus	4.13	33.33	8.07	>4	Bacteriostatic

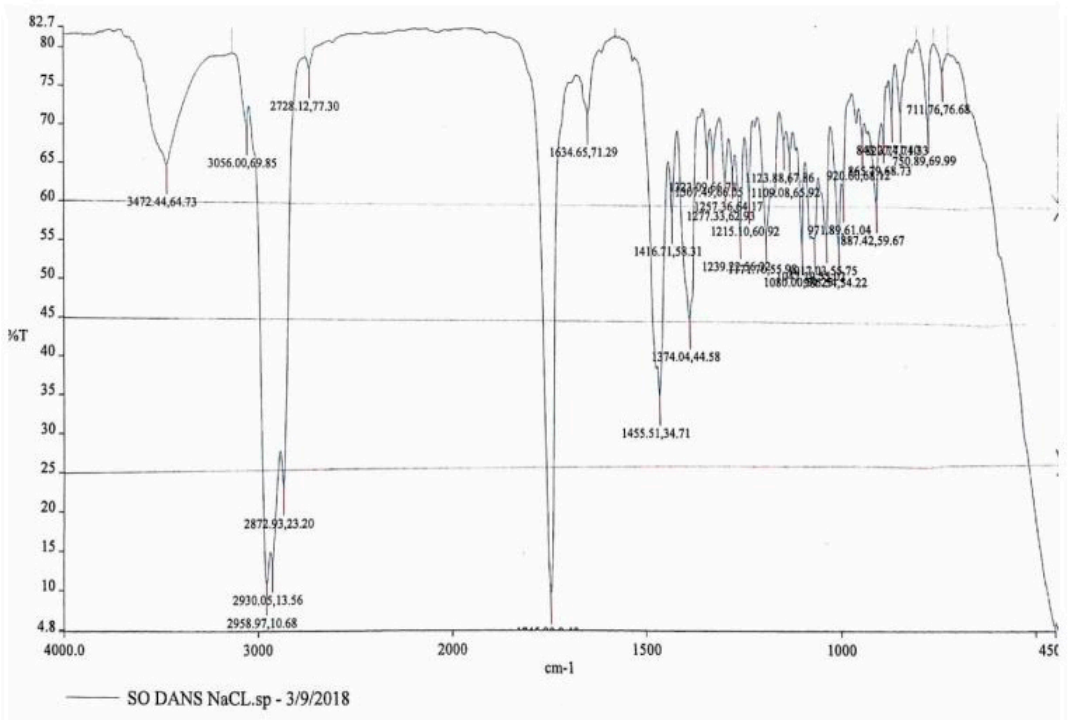

Figure 3. The FTIR spectrum of *S. officinalis* essential oil

Table 2. FTIR absorption band for *Salvia officinalis* essential oil

σ (cm^{-1})	Standards	Intensity	Chemical bond	Functional groups	Interpretation
1634	1675-1645	Medium	C=C	Alkenes	Tricyclene, Myrcene, α-Phellandrene, α-Terpinene, Limonene, α-Copaene, γ and δ-Cadinene [20]
1715	1725-1670	Strong	C=O	Ketones	Thujone, camphor, trans-Pinocamphone, 3-octanone [21, 22]
2872	2900-2800	Strong	C-H	Aldehydes	Benzeneacetaldehyde, myrtenal [22]
1080	1150-1020	Medium to strong	C-O	Ethers	1,8-cineol [23]
1215	1300-1050	Medium	C-O	Esters	Bornyl acetate, trans-sabinyl acetate, myrtenyl acetate [21]
1455	1450-1650	Strong	C=C	Aromatic hydrocarbons	ρ-cymene, ρ-cymenene [20,21]
3056	> 3000	Medium	=C-H		
3472	3300-3600	Medium	O-H	Alcohols and phenols	Phenols: Thymol, carvacrol, eugenol [20, 21]
1239	1000-1300	Medium	C-O		Alcohols: Terpinene-4-ol, linalool, myrtenol, viridiflorol, trans-sabinol, borneol, globulol, terpineol, spathulenol [21]
1171	1200-1050	Medium	C-O		

The most sensitive Gram-negative bacterium was found to be *Pseudomonas*. sp with MIC=0.13 % v/v, and the most sensitive Gram-positive bacterium was *S. aureus* with MIC value of 4.13 % v/v.

• *FTIR Spectroscopy of S. officinalis essential oil*

The Fourier Transform Infrared (FTIR) spectra for the investigated *So*EO are depicted in Figure 3, and the wavelengths values (in the 400–4000 cm−1 range) for all recorded peaks are presented in Table 2. The different chemical compositions of the investigated EOs samples led to obtaining major differences in the intensity of the peaks located at the following wavelengths: 1634 cm−1, 1715 cm−1, 2872 cm−1, 1080 cm−1, 1215 cm−1, 1455 cm−1, 3056 cm−1, and 3472/1239/1171 cm−1

Fractionation and purification of S. officinalis essential oil

Table 3 show that F6 fraction is active of SoEO; It is the most polar fraction (Mf = 0.2). It has a mass of 79.9 mg compared to the mass initial value of the essential oil injected into the column which is 271 mg, which gives a yield of 29.48%.

resistance to antibiotics. Indeed, this resistance can be attributed to the structure of Gram negative bacterial wall. Our results are in agreement with previous studies revealing the potential of EOs to exhibit strong antibacterial activity against other bacterial strains such as *S. mutans, P. fluorescens, A. bohemicus, K. marina, B. cereus* [14, 24].

The results of disk diffusion assay obtained in this study confirm our earlier observations of Gram negative bacteria being more resistant to the antibacterial effect of *So*EO than Gram positive bacteria [25]. This finding could be explained by the fact that, at the membrane level, EOs can disrupt the permeability barrier of bacterial cell membrane structures and lead to the accompanying loss of chemiosmotic control [26]. Moreover, the *So*EO can coagulate the cytoplasm and damage lipids and proteins at the cytoplasmic level, and lead to bactericidal action [27].

The FTIR analysis of *So*EO (Figure 3) shows the existence of:

Two bands of medium intensity between 1050 and 1300 cm−1 (1239 and 1171 cm−1) associated with the C-O group of esters (Table 2), such as bornyl acetate, sabinyl acetate, and myrtenyl acetate [21].

Table 3. Fractionation of *S. officinalis* essential oil by column chromatography, and thin layer chromatography (TLC)

TLC mobile phase	Hexane/ethyl acetate					
Ratio	95%+5%					100%+00%
Fraction	F1	F2	F3	F4	F5	F6
M_f	0.92	0.78	0.64	0.5	0.42	0.2
Inhibition diameter against *Ent. faecalis*	0 mm	0 mm	0 mm	0 mm	0 mm	9.3±1.03 mm
Yield of active fraction	-	-	-	-	-	29.48%

DISCUSSION

The research of alternative and effective drugs from medicinal plants against antibiotic resistant bacteria has become a priority for the public health of people around the world. *So*EO displayed highly varying antibacterial activity against tested bacterial strains. It showed a highly variable antibacterial activity, but the highest values were recorded against *E. faecalis* (d = 23.33 mm, MIC= 8.33 % v/v, MBC= 16.67 % v/v), followed by *C. freundii* (d = 17.61 mm, MIC = 4.13 % (v/v), MBC = 33.33 % (v/v) with bactericidal activity against *E. faecalis*, and only bacteriostatic effect against *C. freundii*. Moreover, it should be noted that *So*EO showed a good antibacterial activity against *Pseudomonas* SP (d = 10.67 mm, MIC = 0.13% v/v, MBC = 0.13 % v/v (Figure 1), with considerable bactericidal effect. This is a very interesting result because *Pseudomonas* sp belongs to Gram negative bacteria which are characterized by their strong

The above mentioned compounds are known for their antibacterial power [28, 29].

A band of medium intensity, in the region of 1020-1150 cm−1 (1080 cm−1) (Table XI), indicates a C-O grouping of ethers as 1,8-cineol [23]. Several studies have shown the inhibitory effect of 1,8-Cineol, especially against *E. coli*, *M. catarrhalis*, and *S. aureus* [30, 31].

Two bands exist, one in a strong intensity (1455 cm−1), and the other in a weak intensity (3056 cm−1), which are associated with C=C and =C-H bonds of aromatic hydrocarbons such as ρ-cymene [20,21]. However, based on a recent study, the antibacterial activity of ρ-cymene was negligible on bacterial culturability [32].

Absorption bands are also found in the 1634, 1715 and 2872 cm−1 regions, which are probably associated with the C=C bonds of alkenes, C=O of ketones and C-H of aldehydes, respectively. This observation is confirmed by the composition of *So*EO in terpenes

(tricyclene, myrcene, α-terpinene, limonene), and ketones (thujone, dihydrocarvone, camphor, trans-pinocamphone, 3-octanone).

The *So*EO also contains aldehydes especially acetaldehyde benzene, and myrtenal [20,21]. Indeed, the most commonly reported constituent in the literature which tested positive for antibacterial activity are camphor, thujone, and 1,8-cineole [33, 34]. Other studies revealed that high quantities of 3-octanone exhibited antibacterial activity against *E. coli* and *S. aureus* [35, 36]. The abundance of δ-cadinene, α-copaene, and caryophyllene displayed moderate antibacterial activity against *S. aureus* [37]. Moreover, trans- and cis-pinocamphone were responsible for the antibacterial activity of plant essential oil, demonstrating that they passed through the cell wall and the plasma membrane, disrupting their structure [38]. Benzene acetaldehyde, and myrtenal showed antibacterial activity against *S.aureus* [39,40]. In the other hand, Tricyclene, α-terpinene, myrcene, and limonene are the most terpenes compounds which are responsible for the antibacterial activity of several studied essential oils of the specimens against *B. subtilis*, *S. epidermidis, S. aureus*, and *E. coli* [41-45].

A broad band of medium intensity exists at 3472 cm−1 alongside another medium band at 1239 cm−1, which suggests the existence of the O-H and C-O groups of phenols, including thymol, carvacrol and eugenol [20, 21]. These compounds have demonstrated high antibacterial potential [46, 47].

The existence of another band at an average intensity of 1171 cm−1, was associated with the C-O group of alcohols including terpinene-4-ol, linalool, myrtenol, viridiflorol, trans-Sabinol, borneol, globulol, and spathulenol [21, 23]. Indeed, the antibacterial studies focusing on these compounds have proven against several bacteria as *S. aureus*, *P. aeruginosa* and *E. coli* [48-56].

The combination of the results of the FTIR analysis and those of the purification / fractionation of *So*EO allows us to conclude that the most polar compounds, which are responsible for the antibacterial activity of *So*EO are the alcohols and the phenols. Indeed, the activity of alcohols and phenols from EO against Gram-negative and Gram-positive bacteria has been recently confirmed by several studies focusing on antibacterial activity of EO**.**

As regarding to phenols, the thymol (monoterpenoid phenol) exerts relevant antibacterial activity against *S. aureus*. These results may be justified by the hydrophobic nature and low solubility of thymol in the hydrophobic domain of the cytoplasmic membrane of bacterial cells [57]. In addition, the antimicrobial action of carvacrol provides evidence of its rapid antibacterial action. The most frequently reported mechanism of antibacterial action of carvacrol and thymol involves the inhibition of efflux pumps, prevention in the formation and disruption of preformed biofilms, inhibition of bacterial motility, inhibition of membrane ATPases, and the disruption of bacterial membrane that leads to bacterial lysis and leakage of intracellular contents resulting in death. Thus, when carvacrol interacts with the lipid bilayer and aligns itself between fatty acid chains, it leads to the expansion and destabilization of the cytoplasmic membrane [58, 59]. Moreover, Eugenol is a phenolic monoterpenoid belonging to the phenylpropanoids class of natural products (2-Methoxy-4-(prop-2-en-1-yl) phenol). This compound demonstrated a strong antibacterial activity against *S. aureus* strain, by modifying the transmembrane electrochemical potential of the bacteria [60].

The second group of polar compound responsible for the antibacterial activity of *So*EO is alcohols. Several previous studies confirm this finding, the growth of *P. aeruginosa* was inhibited by linalool and the results revealed that this monoterpene alcohol disrupted the normal morphology of the cell by the decrease of membrane potential as well as the release of nucleic acids; and the respiratory chain was also damaged [51]. Other studies show that myrtenol exhibit bactericidal activity against *S.aureus*, the results indicate that PBP2 (penicillin-binding protein 2) is a possible target for myrtenol to act against *S. aureus*. This compound interfered in bacterial cell wall synthesis and inhibited the production of major virulence factors, such as staphyloxanthin, lipase, and hemolysin. It also affected the eDNA production in *S. aureus* [53, 61].

In relation to the sesquiterpenoid viridiflorol, it have been reported that this compound has a strong antibacterial activity [62]. Its mechanism of action can be associated with its lipophilicity, which allows this compound to propagate through cell membranes and cause the death of bacteria by affecting their metabolic paths and organelles or inhibiting syntheses of DNA, RNA, proteins and polysaccharides in bacterial cells [62]. Other research has demonstrated that borneol attracts increasing attention due to its broad-spectrum antibacterial properties via membrane disruption mechanism [63]. In addition, borneol can induce drug accumulation in cells due to its interference with P-glycoprotein (Pgp) that is an efflux protein contributing of multidrug resistance to antibiotics drugs. This finding explains the synergistic effects between borneol and antibiotics [54]. On the other hand, spathulenol, the tricyclic sesquiterpene alcohol, present notable antibacterial activity against *S. aureus, E. coli*, and *P. aeruginosa* [64]. Globulol which is a sesquiterpene alcohol alongside terpinene-4-ol (monoterpene alcohol) were screened to have greater inhibitory effect against *E. coli, P. aeruginosa and S.*

aureus [65, 66]. The specific mechanisms involved in the antimicrobial action of these compounds could be associated with their lipophilic character, monoterpenes are preferentially divided from an aqueous phase into bacterial membrane structures; thus causing structural and functional damage, increasing fluidity and permeability, disturbing of protein function, and inhibiting of ion transport [54].

Accordingly, it is important to note that the antimicrobial activity of active fraction was reduced after fractionation in comparison to the raw *So*EO. This result suggests the possibility of synergism action between molecules presented in *So*EO. Synergistic effects were found between thymol/eugenol, carvacrol/eugenol, and thymol/carvacrol. Moreover, the association between eugenol, and tetracycline indicates a potentiation of antibiotic activity, and a remarkable synergism [60].

Our promising findings provide evidence that phenols and alcohols from essential oil of *Salvia Officinalis* growing in El Jadida city of Morocco exhibit an antibacterial activity against many bacterial strains and it will be clinically valuable.

CONCLUSION

Based on our investigations, it can be concluded that alcohols and phenols from *So*EO have a promising antibacterial activity, especially against *Pseudomonas* sp, *E. faecalis*, *E. faecalis, C. freundii*, and *S. aureus*. In addition, this contribution can offer a distinguished contribution of the application of *So*EO in the field of medicines.

Conflict of interest

The authors declare no conflict of interest.

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