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Zizyphus lotus and *Ruta chalepensis* essential oils for combating antimicrobial resistance in pathogenic clinical bacteria and fungi

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Abstract

The antimicrobial activities of essential oils (EOs) isolated from *Zizyphus lotus* (ZL) and *Ruta chalepensis* (RC) harvested in Oran (north-west Algeria) were assessed against pathogenic clinical bacteria and fungi. The EOs were isolated using the steam distillation process, the phenolic and flavonoid contents were determined using colorimetric methods, and the chemical composition was carried out using GC-MS analysis. The antimicrobial activity was evaluated using agar disc diffusion and microdilution methods. The evaluation of the synergistic effect using the combination of *Z. lotus* (ZLEO) and *R. chalepensis* essential oils (RCEO) was done using the checkerboard assay. Effective extraction yields were determined for both plants, with an actual amount in RC than ZL. Concentrations of 8.47 ± 0 mg GAE/g DE and 8.56 ± 0.154 mg CE/g DE of total phenols were determined in ZLEO and RCEO, respectively. Thus, a chemotype of Diisooctyl-phthalate (80.343 %) was determined in ZLEO and the 2-Undecanone (13.236 %) in RC. Both plant EOs exhibited important antimicrobial activity against the selected multidrug-resistant human pathogens. The most potent effect was estimated against *Proteus mirabilis*, *Salmonella enterica* subsp. *arizonae*, and *Hafnia alvei* with growth inhibition zone diameters of 24.06 ± 0.12, 40.1 ± 0.1 and 40.16 ± 0.15 mm using ZLEO, respectively. Also, essential anti-Candida activity was estimated. ZLEO and RCEO did not exhibit either synergistic or additive effects, with fractional inhibition concentration index values greater than 2. Both plants exhibited significant antimicrobial effects alone, while in combination they did not exhibit a synergistic effect but an antagonistic one. Therefore, ZLEO and RCEO can be developed as natural antimicrobial agents in the medical and food industries to combat antimicrobial resistance.

Introduction

The emergence of antimicrobial resistance in most reanimation services, hospitals, and food-borne pathogens is considered a global health concern.

The bacterial and fungal strains developed increasing resistance to many antibiotics, making the therapeutic application less efficient for treating microbial infections. Besides, the significant side effects of these drugs and the dysbiosis phenomena that appeared after the consumption of antibiotics induce essential consequences for human health. So, this allowed us to search for more safe alternative products, especially in the world of medicinal and aromatic plants. An emergent interest in using natural antibacterial and antifungal molecules, such as plant essential oils, as potent alternative antimicrobials for combating the antimicrobial resistance in various pathogenic clinical strains responsible for severe human microbial infections.

The Algerian medicinal plants are known for their contents of bioactive components, and various recent studies have elucidated the antimicrobial effects of Eos and phenolic extracts of a wild variety of medicinal plants (Ayad *et al.* 2022; Bennacer *et al.* 2022). Among the medicinal plants of high interest in traditional medicine in Algeria and used by the local population are *Zizyphus lotus* L. (Rhamnaceae) and *Ruta chalepensis* L. (Rutaceae) species. Both plants contain an immense variety of bioactive substances with various biological properties. Recent studies have demonstrated that *Zizyphus* bioactive components exert various biological activities, such as memory enhancement effects (Zhang *et al.* 2014). Furthermore, the *Ziziphus* species have mainly been used in traditional medicine to treat severe diseases such as respiratory problems, scabies, pimples, and inflammation of the mouth and gums. The flavonoids, saponins, and fatty acids isolated from *Ziziphus* species are responsible for the plant's sedative effects (Xie *et al.* 2012).

Moreover, *Z. lotus* is used in many fields, including nutrition, cosmetics, and healthcare. This plant is widely used in the treatment of urinary tract infections, digestive disorders, and intestinal microbial infections. It is used as a hypoglycemic, hypotensive, antidiarrheal, and anti-ulcer agent in stomach diseases (Bnouham *et al.* 2002; Borgi *et al.* 2007).

Various studies have reported the anticancer and anti-inflammatory properties of this plant. It also exhibited potent antifungal activity, a gastroprotective effect against *Helicobacter pylori* and was used as an analgesic, as well as having a potent antibacterial effect (Wahida *et al.* 2007; Borgi *et al.* 2008; Benammar *et al.* 2010; Bakhtaoui *et al.* 2014; El Hachimi *et al.* 2016; Bencheikh *et al.* 2019). Ghalem *et al.* (2014), Marmouzi *et al.* (2019), and Bencheikh *et al.* (2021) elucidated the antioxidant and nephroprotective effects of *Z. lotus*. Referring to the literature, most of the research is about the biological properties of phenolic extracts of *Z. lotus*, while limited studies demonstrate the chemical composition and the antimicrobial effects of *Z. lotus* essential oils, that is why we are interested in selecting this plant for the isolation of potent antimicrobials that can be used as alternatives to antibiotics.

R. chalepensis is a common species in Algeria and is intensely interested in international traditional medicine. This plant is known for its richness in therapeutic compounds, especially inessential oils (2-Undecanone) (González-Trujano *et al.* 2006; Mejri *et al.* 2010). Recent studies mentioned that *R. chalepensis* leaf extract is known for its essential antioxidant and hypoglycemic activities (Loizzo et al. 2017). Althaher et al. (2020) demonstrated the cytotoxic effect of *R. chalepensis* essential oils on human MCF-7, T47D, and Caco-2 cancer cell lines. The essential oils isolated from the aerial parts of this plant exert an antifungal effect against *Aspergillus* sp., *Fusarium culmorum*, *Fusarium pseudograminearum*, *Fusarium proliferatum*, and *Fusarium graminearum* (Bouajaj et al. 2014). According to Amdouni et al. (2016), the *R. chalepensis* EOs also exert an antibacterial effect against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. González-Trujano et al. (2021) have mentioned the multiuse of *Ruta chalepensis* to treat various disorders such as fever, rheumatism, mental disorders, menstrual problems, anxiety, and epilepsy problems.

This is the first time that the antimicrobial effect of the essential oils of *Z. lotus* and *R. chalepensis* collected from the Oran-Tafraoui region in western Algeria has been reported. No studies on the chemical composition of ZLEO in volatile bioactive components have been reported. The present study aimed to determine the chemical

composition and chemotypes of EOs isolated from *Z. lotus* leaves and *R. chalepensis* aerial parts using GC-MS analysis and their antimicrobial effects against pathogenic clinical bacteria and fungi for combating antimicrobial resistance.

Experimental

Plant material

The wild *Z. lotus* leaves and *R. chalepensis* aerial parts were collected in July and April 2017, respectively, during their flowering stages, from the Tafraoui region in Oran (north-west Algeria). Furthermore, both plants were identified by the botanist Pr. Righi K. from the Department of Biology of Mascara University, Algeria.

Clinical microbial strains

The antimicrobial effect was assessed using pathogenic clinical isolates, including Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, and Enterococcus faecalis*), Gram-negative bacteria (*Enteropathogenic Escherichia coli, Salmonella enterica* subsp. *arizonae, Proteus mirabilis, Hafnia alvei*), and pathogenic microscopic fungi (*Candida albicans*). All these microbial strains were isolated from different clinical samples. Stool specimens from gastroenteritis patients, oral cavity samples from the periodontal pocket of patients with periodontal disease, and urine samples from patients with urinary tract infections.

On the other hand, the microbial identification was carried out in the Laboratory of Microbiology of Meslem Taib Hospital in Mascara, Algeria and in the Laboratory of Bioconversion, Microbiological Engineering, and Health Safety Department of Biology, University Mustapha Stambouli of Mascara, Algeria.

Antibiotics susceptibility test

The antibiotic susceptibility testing was carried out using the agar disc diffusion method, following the CLSI (2015). Susceptibility to various antibiotics was tested: Penicillin family: Penicillin G (10 µg/disc), Amoxycillin (25 µg/disc), Oxacillin (5 µg/disc), Aminoglycosides: Neomycin (30 µg/disc), Polymyxin E: Colistin (10 µg/disc), Macrolide: Spiramycin (100 µg/disc), Streptogramin A: Pristinamycin (15 µg/disc), Nitroquinolone: Nitroxolin (20 µg/disc) for bacteria, and Azole antifungal: Fluconazole (25 µg/disc) for *C. albicans* strain.

According to the FMS-AC (2013) and the FMS-AC/EUCAST (2018) guidelines, the growth inhibition zone diameters were interpreted using the critical diameters mentioned in Table 1. The antibiotic resistance profiles allowed us to qualify the isolated clinical microbial strains as pathogenic, multidrug-resistant strains.

Table 1. Antimicrobial resistance profile of clinical isolates.

Clinical isolates	SP	AMX	PT	NI	N	OX	CT	P	P-G	FCA
S. aureus	0^{R}	12^{R}	0^{R}	18^{I}	15^{I}	0^{R}	12^{R}	10^{R}	0^{R}	NT
S. pyogenes	0^{R}	15^{R}	0^{R}	22^{I}	15^{I}	0^{R}	12^{R}	0^{R}	0^{R}	NT
E. faecalis	23^{S}	19^{I}	20^{I}	12^{I}	0^{R}	0^{R}	0^{R}	21^{I}	0^{S}	NT
E. coli	0^{R}	0^{R}	0^{R}	20^{I}	15^{R}	0^{R}	11^{R}	0^{R}	0^{R}	NT
P. mirabilis	0^{R}	15^{R}	0^{R}	14^{I}	16^{R}	0^{R}	0^{R}	10^{R}	0^{R}	NT
S. enterica* subsp. *arizonae	0^{R}	0^{R}	0^{R}	22^{I}	18^{S}	0^{R}	13^{R}	0^{R}	0^{R}	NT
H. alvei	0^{R}	0^{R}	0^{R}	20^{I}	20^{S}	0^{R}	13^{R}	0^{R}	0^{R}	NT
C. albicans	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}	0^{R}

SP – Spiramycin; AMX – Amoxycillin; PT – Pristinamycin; NI – Nitroxolin; N – Neomycin; OX – Oxacillin; CT – Colistin; P – Penicillin-G; FCA – Fluconazole; R – Resistant; S – Sensitive; I – Intermediate sensitivity; NT – Not tested.

Essential oils extraction

The extraction of essential oils (EOs) was carried out using steam distillation. Briefly, each 45 g of the fresh leaves of *Z. lotus* and the aerial parts of *R. chalepensis* were subjected to steam distillation in distilled water for three consecutive hours according to the current European Pharmacopoeia

(2007). The obtained EOs were collected and stored at 4 °C in brown, sealed glass vials until used. This process was done in triplicate, and the extraction yield ($Y_E = w/w$) was expressed as the weight of the EO volume (g) on the weight (g) of the plant used.

Physicochemical index determination

Different physicochemical indices of the EOs were determined: Relative density at 20 °C (NF ISO 279 1999), Refractive index (NF ISO 280 1999), Specific optical rotation (NF ISO 592 1999), Acid index (NF ISO 1242 1999), Ester index (NF ISO 709 2002), and Miscibility with ethanol (NF ISO 875 1999).

Determination of total phenolics content (TPC)

The total phenolic content (TPC) was determined according to Boizot and Charpentier (2006). In brief, 200 µL of each EO sample (ZLEO and RCEO) at a concentration of 1 mg.mL^{-1} was mixed with 1 mL of Folin Ciocalteu reagent and 800 µL of sodium carbonate Na_2CO_3 solution (7.5 %). The mixture was incubated at room temperature in the dark for 10 min, and the absorbance was determined at 735 nm using a spectrophotometer (JENWAY, 6400 spectrophotometer).
Gallic acid (GA) (0.05 – 0.2 mg.mL^{-1}) was used as a standard:$y = 0.751x + 0.0012, R^2 = 9975$.The TPC was expressed as gallic acid equivalents (GAE) per gram of dry extract (mg GAE/g DE). Determinations of TPC were performed in triplicate. Results were expressed as mean ±SD.

Determination of flavonoids contents (TFC)

The total flavonoid content (TFC) was determined according to Samatha *et al*. (2012). Briefly, 1 mL of 2 % aluminum trichloride ($AlCl_3$) in methanol was mixed with 1 mL of the EO samples. The absorbance values were determined at 430 nm after 40 min against a blank.
Quercetin (Q) (0.05 to 0.25 mg.mL^{-1}) was used as a standard: $y = 4.453x + 0.0115, R^2 = 0.9922$.The TFC of the extracts was expressed as mg of quercetin per gram of dry extract (mg QE/g DE). Determinations of TPC were performed in triplicate. Results were expressed as mean ± SD.

Gas chromatography-mass spectroscopy analysis (GC-MS)

The identification and quantification of volatile bioactive compounds from *Z. lotus* and *R. chalepensis* essential oils was carried out using a Shimadzu gas chromatograph (GC), Agilent GC Model 7890B equipped with the HP 5977A Mass spectrometer. Analytical conditions: Agilent 122-7062 DB-WAXN capillary column (Dimension: 60m, ID: 250 micrometers, 0.25-micrometer film thickness). Helium was used as a carrier gas with a linear velocity in a column of 19 cm.s^{-1} and 37.862 psi of pressure. The carrier gas split flow was about 250 mL.min^{-1}, with a split ratio: of 100 : 1, and septum purge flow of 3 mL.min^{-1}. The oven temperature program was between 70 and 260 °C with an equilibration time of 1 min and was then maintained at 250 °C. The injection in split mode of 2 µL of the substance to be analyzed was carried out using a microsyringe of 10 µL. The components were identified by comparing their relative retention times and mass spectra with the data from the library of EO constituents (Wiley, Mass-Finder, and Adams GC/MS libraries). The percentage composition determination was based on peak area normalization without correction factors.

Antimicrobial activity

The agar disc diffusion method was applied to determine the antimicrobial potency of EOs from *Z. lotus* and *R. chalepensis*. Muller-Hinton agar (MHA) plates were spread by adjusted microbial culture suspensions (0.5 McFarland), and therefore, sterile discs were impregnated in 20 µL of each EO solution. The EOs of 200 mg.mL^{-1} concentration were dissolved in the dimethyl sulfoxide 10 % (DMSO) that was used as a negative control. The discs were aseptically deposited on the inoculated plates. After 2 h at 4°C, plates were incubated at 37 °C for 24 h, and the antimicrobial effect was expressed by measuring the diameters of the microbial growth inhibition zones (ø). Each assay was carried out in triplicate. The EOs effectiveness

assessment was determined as follows:
Ø < 8mm: Resistant, 9 mm < Ø < 14 mm: Sensitive, 15 mm< Ø < 19 mm: Very sensitive, Ø >20 mm: Extremely sensitive (Ponce *et al.* 2003).
The microdilution method was performed to determine the minimum inhibitory (MIC), bactericidal (MBC), and fungicidal (MFC) concentrations. The assays were done in sterile 96-well microplates and examined according to the method described by Chandrasekaran and Venkatesalu (2004). Briefly, 50 µL of Mueller Hinton and Sabouraud broth (for bacteria and yeast tests, respectively) was distributed aseptically in all the sterile microplates' wells. Subsequently, 50 µL of each EO sample of both tested plants, at a concentration of 200 mg.mL^{-1} was added to the first well, and then serial dilutions were obtained to achieve a final concentration of 1.56 mg.mL^{-1}. After that, 50 µL of the adjusted microbial suspensions (0.5 McFarland) were inoculated in each microplate well. The Microplates were incubated at 37 °C, and microbial growth kinetics were measured by reading the optical density at 620 nm for bacteria and 450 nm for fungi at 0-4-18 – 48, and 72 h, using a Microplate Absorbance Reader (Tecan Spectra II Microplate Reader). The microbial tests were prepared in triplicate, and the results were expressed as Log germs.mL^{-1} obtained for each plant extract concentration.

Checkerboard method

During this study, we evaluated the synergistic, additive, or antagonistic effects using the combinations between both plants' EOs: ZLEO/RCEO. The synergistic interaction of both antimicrobial drugs was quantified after the determination of the MIC values for each plant EO (previously determined) by calculating the index of fractional inhibitory concentrations (FICI or ∑FIC), which are the lowest concentrations of the antimicrobial drugs in combination, inhibiting completely the growth of the microbial strains tested.
A total of 50 µL of sterile Mueller Hinton broth was distributed aseptically in all the sterile cupules of the microplates. The first EO solution of *Z. lotus* was serially diluted along the abscissa, while the EO of *R. chalepensis* was diluted along the ordinate. An inoculum equal to 0.5 McFarland turbidity standards was prepared for each culture in sterile saline water. Each suspension cupule was inoculated with 50 µL of the microbial culture, and the microplates were incubated at 37 °C for 18 h.
The value of the association was measured using the FIC in the cupules in which the microbial growth is inhibited and considered an effective MIC for the combination (Orhan *et al.* 2005).
The ∑FICs were calculated as follows Eq. 1:

$$FICI = FIC\,A + FIC\,B \quad (1)$$

where, FIC A = MIC of drug A (PPE or EO of *Z. lotus*) in the combination/MIC of drug A (*Z. lotus* extract) alone, and FIC B = MIC of drug B (PPE or EO of *R. chalepensis*) in the combination/MIC of drug B (*R. chalepensis* extract) alone. The combination is considered synergistic when the FICI is ≤ 0.5, additive when the 0.5 <FICI ≤ 1, indifference: 1 <FICI≤ 4 and antagonism: FICI >4 (Siqueira *et al.* 2021).

Statistical analysis

Replicates were prepared for all experiments. The results were given as means and standard deviations (mean ± SD). The means were compared using one-way and multivariate analysis of variance (ANOVA). The differences between individual means were significant at P <0.05.

Results and Discussion

Extraction yield and quantitative determination of polyphenols

Results of the extraction yields and the quantitative determination of polyphenol and flavonoid content in the essential oils of *Z. lotus* and *R. chalepensis* are shown in Table 2. Statistical analysis showed significant differences between the yields of both plants EOs, while the highest yield was obtained for RCEO: 8.65 ± 0.025 % compared to *Z. lotus*: 4.57 ± 0.015 % (Table 2).
Yields of ZLEO and RCEO were higher than reported in other studies. The EO yield from *R. chalepensis* (8.65 ± 0.025%) originated from the Tafraoui region in Oran (north-west Algeria) was higher than that reported in other studies on the

same plant species originated from different regions of Algeria e.g., from Remchi region (1.17 %), Naama region (0.19 %) (Benammara *et al.* 2006), while from Sidi Bel Abbes region was 7.23 % (Boumediene 2014). According to Attou (2011), the EO yield of *R. chalepensis* harvested in Ain Temouchent in Algeria was 0.42 %, while in Tlemcen it was around 2.21 % (Benammra *et al.* 2006), and in the range 0.28 – 0.78 % (Merghache *et al.* 2009).

Table 2. Extraction yields and quantitative determination of polyphenols in the essential oils of *Z. lotus* and *R. chalepensis*. P <0.05 (significant).

Plant Extracts	Yield [%]	TPC	TFC
ZLEO	4.57 ±0.015	8.47 ±0	3.33 ±0.26
RCEO	8.65 ±0.025	8.56 ±0.154	7.07 ±0

TPC – Total phenol content; TFC – Total flavonoid content.

Compared to our results, a recent study by Bekkar *et al*. (2021) reported that the EO yields were 4.16 ± 0.036 % in *Z. lotus* leaves and 4.99 ± 0.86 % in *R. chalepensis* aerial parts from Mascara in western Algeria. In addition, the harvest region has an immense influence on medicinal plant essential oil yield. Each area is characterized by climatic conditions (precipitation and temperature), which influence the physical qualities of medicinal plants, as well as soil type, moisture retention, and pH. Other ecological factors can intervene in the development of the plant species: altitude, the harvest period, the plant part, the technique, as well as the extraction of the bioactive substances period, which influences not only the yield but also the plant extract chemical composition (Lucchesi 2005; Pinto *et al.* 2006; Zouari *et al.* 2012).

Comparison of our results with other studies on the same plants harvested from other regions outside Algeria. According to Daoudi *et al*. (2016) and Ali *et al*. (2013), the EO yield of *R. chalepensis* from the Meknes region was estimated at 1 % and 0.84 %, respectively.

On the other hand, the essential oil yield of *R. chalepensis* aerial parts (add region) was 0.27 % (Dob *et al.* 2008). While in Tunisia it was 5.51 % (Mejri *et al.* 2010). Moreover, the EO yield of *R. chalepensis* in the current study was higher than that of *Z. lotus*. Also, *Z. lotus* and *R. chalepensis* from the Tafraoui region in Oran contain significant concentrations (P <0.05) of phenol (8.47 mg GAE/g DE) and (8.56 ± 0.154 mg GAE/g DE), respectively. At the same time, RCEO was richer in flavonoids (7.07 ± 0 mg QE/g DE) than ZLEO (3.33 ± 0.26 mg QE/g DE). Althaher *et al*. (2020) determined a TPC of 5 ± 0.005 mg GAE/g of oil extract and a TFC of 34.09 ± 0.140 mg QE/g oil. In addition, it has been reported that the polyphenol content was much higher in both plant phenolic extracts than the essential oils (Bekkar *et al.* 2021), which indicates that the polyphenolic extracts are considered plant drugs, representing a rich and potent reservoir of phenols than EOs.

Physicochemical index of essential oils

The physicochemical results of both plants' EOs carried out according to the French Association for Standardization (1996) method are mentioned in Table 3. The FSM-AC (2013) recommends an EO density between 0.895 – 0.920 for very high-quality EOs, so, referring to our results, *Z. lotus* and *R. chalepensis* EO samples are of good quality. Another parameter that allows us to determine the purity of our EO samples is the refractive index. The FAS standard recommends a refractive index of 1.495 for high-quality EOs and 1.513 for lesser-quality oils.

Indeed, in the present study, we determined an RI for ZLEO 1.441, and for RCEO (4.426) (Table 3). So, according to the results of this study, it appears that our EO samples of *Z. lotus* and *R. chalepensis* are of very good quality. However, a high acid index for RCEO was determined by comparing it with the ZLEO sample, for which a value of 0.933 was obtained. These results indicate that the EO of *Z. lotus* contains fewer free acids compared to *R. chalepensis*. A high ester index also determines the quality of our EO samples.

Table 3. Physicochemical characteristics of *Z. lotus* and *R. chalepensis* essential oils.

	Physicochemical index					
Plant Essential Oils	**$RD_{20°C}$**	**R_I**	**OR_S**	**A_I**	**E_I**	**E_M**
ZLEO	0.6822	1.441	+7.5°	0.933	19.64	1v/1v
RCEO	0.7945	1.426	+10°	4.45	16.85	1v/2v

$R_D^{20°C}$ – Relative density at 20 °C; R_I – Refractive index; OR_S – specific optical rotation; A_I – Acid index; E_I – Ester index, E_M – Miscibility with ethanol.

According to Dummortier (2006), higher E_I values indicate the essential quality of EO samples. For comparison, the study conducted by Alloun (2013) on *R. chalepensis* EO reports physicochemical characteristics with a density of 0.804 and a refractive index of 1.430. Thus, Merghache *et al.* (2009) proved that the physicochemical properties of *R. chalepensis* EO vary according to the harvest period and region. In the literature, no study has been described on *Z. lotus* EO. Therefore, no comparison has been made with previous studies.

Gas chromatography-mass spectroscopy analysis (GC-MS)

The GC-MS analysis was used to determine the chemical composition of volatile bioactive compounds in the EO samples prepared from *Z. lotus* leaves and *R. chalepensis* aerial parts harvested from the Tafraoui region, Oran, in western Algeria. Results are shown in Table 4 and Table 5.

A total of 14 volatile components were identified for *Z. lotus* and 12 compounds for *R. chalepensis*, representing 91.33 % and 58.84 % of the total EOs, respectively. The chromatographic analysis allowed us to determine the majority chemotype of Di-isooctyl phthalate (80.343 %) for ZLEO, while 2-Undecanone was the major component of RCEO (13.236 %), followed by Chalepensine (12.547 %), 2-Nonanol acetate (9.128 %), 2-Nonanone (6.405%) and 2-Undecanol acetate (4.411%) (Table 4 and 5). Other minor compounds were also identified and quantified in ZLEO: Di-Methylene Sulfoxide (7.287 %), Linalol 1.025, Thiourea-Tetramethyle (1.151%), Thymol (0.292 %), Linalyl acetate (0.110 %), 2-Nonanone (0.059 %), P-cymene (0.178 %) and ɤ-Terpinene (0.060 %) (Table 4).

Table 4. Chemical composition of the essential oil of *Zizyphus lotus* leaves harvested in Tafraoui region-Oran.

N°	**R_t**	**Volatil bioactive components**	**[%]**
1	11.190	ɤ-Terpinene	0.060
2	12.075	P-cymene	0.178
3	14.485	Methyl-heptenone	0.077
4	16.295	2-Nonanone	0.059
5	17.580	1,1'-Bicyclohexyl, cas no 92-51-3	0.409
6	20.974	Linalol	1.025
7	21.257	Linalyl Acetate	0.110
8	21.776	Di-Methylene Sulfoxide	7.287
10	22.524	2-Undecanone Methylene nonyl ketone	0.197
11	31.760	Thiourea -Tetramethyle	1.151
12	40.813	Thymol	0.292
13	44.271	Diethyl Phthalate -DEP	0.138
14	56.774	Diisooctyl phthalate	80.343
Components identified			**91.326**

N° – Number; R_t– Retention time.

Table 5. Chemical composition of the essential oil of *Ruta chalepensis* aerial parts harvested in Tafraoui region-Oran.

N°	**R_t**	**Volatil bioactive components**	**[%]**
1	15.443	2-Octanol acetate	0.229
2	16.291	2-Nonanone	6.405
3	16.735	Tetra-decane	0.655
4	18.586	2-Nonanol acetate	9.128
5	19.501	2-Decanone	0.784
6	20.25	2-Nonanol	2.524
7	22.531	2-Undecanone	13.236
8	24.389	2-Undecanol acetate	4.411
9	26.086	2-Undecanol	2.544
10	28.837	2-Tridecanone	0.892
11	31.814	Thiourea -Tetramethyl	5.481
12	61.122	Chalepensine	12.547
Components identified			**58.84**

N°– Number; R_t– Retention time.

To the best of our knowledge, this is the second report on the leaf essential oils of *Z. lotus* collected from Western Algeria, but from another region, for the determination of the harvest region influences

on the chemical profile of bioactive substances in the plant phenolic extracts and essential oils. Moreover, limited studies have been reported on the biological properties of *Z. lotus* essential oils. In contrast, most of the research is directed to this plant's polyphenolic extracts. For this, we were very interested in studying the biological properties of *Z. lotus* essential oils. The study carried out by Ourzeddine *et al*. (2017) on the chemical composition and the antioxidant activity of the fruit's essential oil of *Z. lotus*, harvested from the Ouled Fadhel region of Batna (eastern Algeria) showed that ethyl hexadecanoate (12 %), decanoic acid (11 %), ethyl dodecanoate (9.4 %), ethyl hexadeca-9-enoate (7.9 %), dodecanoic acid (6.5 %), ethyl tetradecanoate (6.1 %), tetradecanoic acid (5 %), ethyl decanoate (4.8 %), octanoic acid (3.1 %), ethyl undecanoate (2.8 %), nonanoic acid (2.4 %) and undecanoic acid (2.1 %) are the predominant components in the EO sample.

Another study by Ghannadi *et al*. (2003) on the volatile constituents of *Z. lotus* EOs from Iran showed different chemical profiles: geranyl acetone (14 %), hexadecanoate (10 %), inethyl-octadecanoate (9.9%), farnesyl acetone C (9.9 %), hexadecanol (9.7 %), and ethyl-octadecanoate (8 %) which were found to be the main constituents.

Thus, Bekkar *et al*. (2021) determined a majority chemotype of Di-isooctyl-phthalate for *Z. lotus* EOs and 2-Undecanone for *R. chalepensis* harvested from the El-Mamounia region in Mascara (western Algeria), which is in agreement with the results of the present study however, a difference was recorded in the EOs diversity in bioactive components, where the plants harvested from Mascara in western Algeria gave a chemical profile very rich in these compounds compared to the plants harvested from Oran. By comparing our results with previous studies of Mejri *et al*. (2010) and Merghache *et al*. (2009) on the essential oil chemical composition of *R. chalepensis* collected from Tunisia and western Algeria (Tlemcen), respectively, showed that the EOs chemical profile is very variable, with 2-Undecanone always being the predominant component (69.23 % and 43.71 %, respectively).

Thus, in their study, Rustaiyan *et al*. (2002) showed that RCEO is dominated by 2-Undecanone (52.5 %). Haddouchi *et al*. (2013) showed the predominance of 2-Nonanone and 2-Undecanone: 32.79 % and 32.58 % respectively, followed by 1-Nonene at 13.95 % and α-Limonene at 5.27 % in the EO of the plant harvested at Ain Temouchent, Algeria. In Jordan, the chemical composition of essential oil from *R. chalepensis* was rich in non-terpenoid aromatic compounds (80.32 %), and the major identified compounds were 2-nonanone (19.45%), methyl hexadecanoate (4.04 %), and 4,5-dimethoxy-6-prop-2-enyl-1,3-benzodioxole (1.87 %) (Althaher *et al*. 2020).

Antimicrobial activity tests

Results of the antimicrobial activity assessments are shown in Table 6, Fig. 1 and 2. A potent antimicrobial effect was recorded on all bacterial strains, and significant antifungal activity on *C. albicans*, with diameters of the microbial growth inhibition zones exceeding 10 mm. The largest inhibition diameters were recorded using ZLEO: 40.16 ± 0.15mm, 40.1 ± 0.1mm and 24.06 ± 0.12mm against *H. alvei*, *S. enterica* subsp. *arizonae*, and *P. mirabilis*, respectively, with extremely high susceptibility (Table 6). In addition, our results indicated that the essential oils of these plants are more effective in inhibiting the growth of major pathogenic bacteria and yeast isolated from gastroenteritis, urinary tract infections, and periodontitis diseases, which may justify the use of *Z. lotus* and *R. chalepensis* in the treatment of these pathologies.

Regarding the antibiotic susceptibility testing, ZLEO and RCEO were more active against the clinical strains however, the inhibitory potency of *Z. lotus* EOs was more important than *R. chalepensis* EO. *Z. lotus* and *R. chalepensis* EOs were more efficient against all Gram-positive, Gram-negative bacteria, and *C. albicans* with its significant effects (P <0.05), while *S. aureus* and *E. faecalis* among the Gram-positive bacteria were the most susceptible to ZLEO and RCEO respectively. Among the Gram-negative bacteria, *H. alvei*, *S. enterica* subsp. *arizonae*, and *P. mirabilis* were the most sensitive to ZLEO with the qualification as extremely high susceptibility of these germs to ZLEO regarding the important diameters of the growth inhibition zones measured (Table 6).

The most effective effect of RCEO was recorded against *S. enterica* subsp. *arizonae* and *C. albicans*, with diameters of growth inhibition zones of 17.13 ± 0.15mm and 14.06 ± 0.12 respectively (Table 6). So, an important anti-Candida activity was detected. However, both plants' EOs did not exert any antibacterial effect on *S. pyogenes* (Table 6), which explains the high-frequency resistance of this clinical strain to antimicrobials. For Gram-negative bacteria, some strains were distinguished by a very high sensitivity compared to others, as shown by the case of *S. enterica* subsp. *arizonae* and *H. alvei*, whose inhibition diameters were much higher and exceeded 30 mm by applying *Z. lotus* and *R. chalepensis* EOs compared to the antibacterial effect of *R. chalepensis*.

Table 6. Antimicrobial activity of essential oils of *Zizyphus lotus* and *Ruta chalepensis* harvested in Tafraoui region Oran against pathogenic clinical germs. The values are presented as the mean of three replicates ± the standard deviation. P <0.05 (significant).

Clinical strains	**Diameters of growth inhibition zones [ø mm]**			
	ZLEO	**CMI$_{ZLEO}$**	**RCEO**	**CMI$_{RCEO}$**
S. aureus	14.06 ± 0.12^{S}	100	10.06 ± 0.12^{S}	100
S. pyogenes	NE	100	NE	100
E. faecalis	10.03 ± 0.06^{S}	25	13.1 ± 0.1^{S}	100
E. coli (EPEC)	15 ± 0.1^{HS}	100	9.16 ± 0.15^{S}	50
P. mirabilis	24.06 ± 0.12^{EHS}	100	11.06 ± 0.12^{S}	50
S. enterica Subsp. *arizonae*	40.1 ± 0.1^{EHS}	50	17.13 ± 0.15^{HS}	25
H. alvei	40.16 ± 0.15^{EHS}	50	10.03 ± 0.06^{S}	50
C. albicans	15.03 ± 0.06^{HS}	25	14.06 ± 0.12^{S}	25

ø (mm) – Diameters of growth inhibition zone in millimeter; NE – No effect; R – Resistance (Ø < 8mm); S – Sensitivity (9 mm < Ø < 14 mm); HS – High susceptibility (15 mm < Ø <19 mm); EHS – Extremely high susceptibility (Ø >20 mm).

All these results were completed by quantitatively determining an important antimicrobial parameter, the minimum inhibitory concentration (MIC). The results of the MIC values for each clinical strain are mentioned in Table 6. The curves of the microbial growth kinetics in the presence of *Z. lotus* and *R. chalepensis* EOs are mentioned in Fig. 1 and 2.

An important decrease in microbial cell concentration was detected after the 4th hour, expressed by the decrease in the Log germs/mL number (Fig. 1 and 2). The inhibitory properties of the essential oils of *Z. lotus* and *R. chalepensis* against all the microbial strains were determined, with the lowest MIC values of 25 mg.mL^{-1} against *E. faecalis* and *C. albicans* using the ZLEO and against *S. enterica* subsp. *arizonae* and *C. albicans* using the RCEO (Table 6). The bactericidal and fungicidal effects could be explained by the abundant richness of *Z. lotus* leaves in Diisooctyl-phthalate, the major component in ZLEO, and *R. chalepensis* aerial part in 2-Undecanone, the major volatile compound in RCEO. Numerous studies have demonstrated the antimicrobial effect of *R. chalepensis* essential oils with the 2-Undecanone chemotype (Marami *et al.* 2021; Nahar *et al.* 2021). Our results agreed with those of other studies (Belkassam *et al.* 2011; Zellagui *et al.* 2012; Alloun 2013; Arámbula *et al.* 2019). Our results also agree with those of Daoudi *et al.* (2016) on the antibacterial effect exerted by the essential oil of *R. chalepensis* aerial part on *S. aureus* and *P. mirabilis*. However, the results showed that the RCEO has no effect on *E. coli*, which does not agree with our results. During the current study, an important antibacterial effect was recorded on this bacterial strain by applying the EO of this plant. Thus, our results agree with those mentioned in another study that showed that the *R. chalepensis* EO has antibacterial activity against Salmonella, *E. coli*, and *S. aureus* (Amdouni *et al.* 2016). The inhibitory effect of *Z. lotus* and *R. chalepensis* could be explained by the most abundant richness of these plants EOs in secondary metabolites, in particular antimicrobial compounds, which justified specific uses of these medicinal plants in the treatment of several infectious diseases (García-Lafuente *et al.* 2009; Ben-Bnina *et al.* 2010;

Haddouchi *et al.* 2013; Boual *et al.* 2015; Hammi *et al.* 2015).

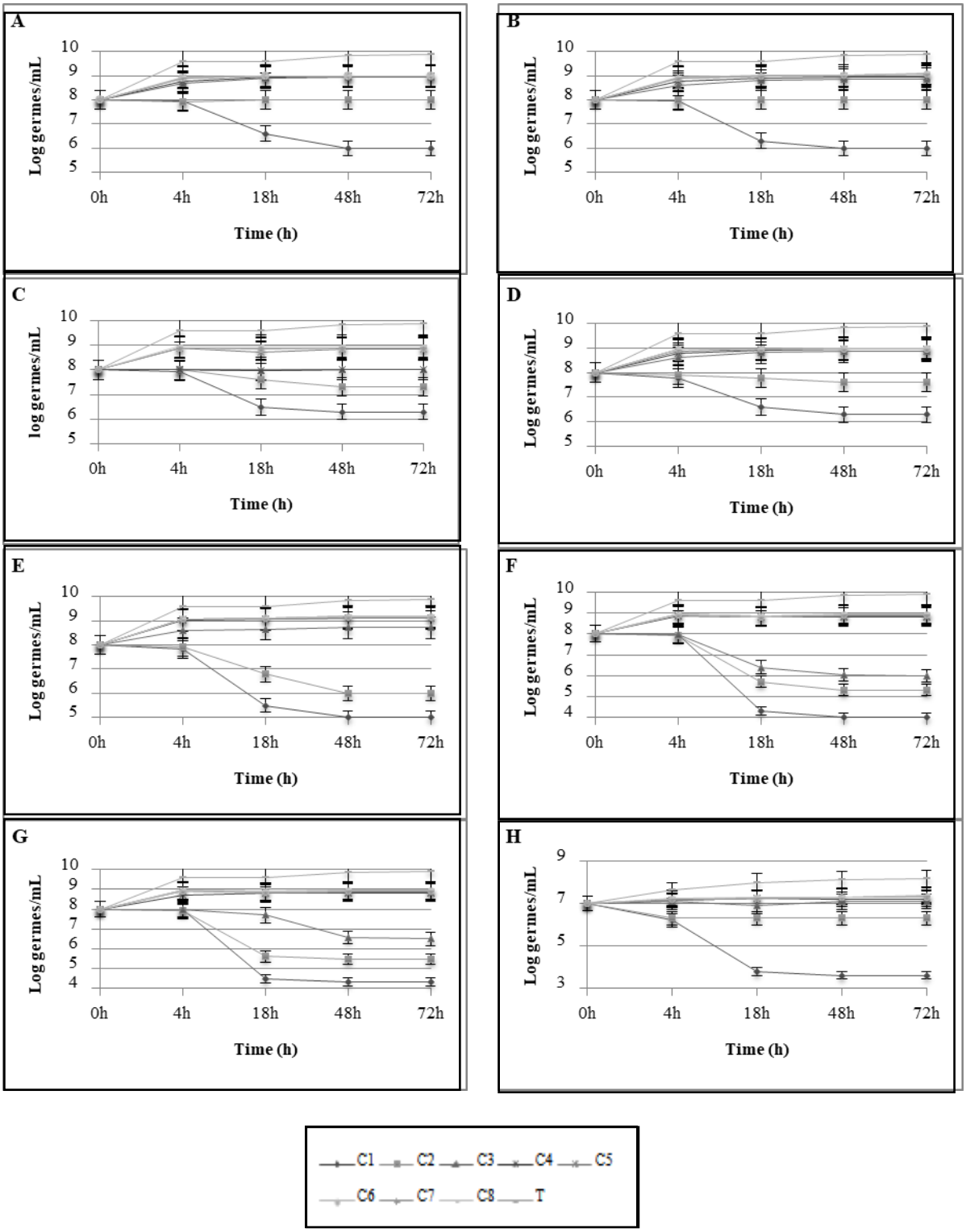

Fig. 1. Antimicrobial effect of *Zizyphus lotus* essential oil on clinical strains isolated from the different biological samples (P <0.05). C1-C8 – Concentrations. C1 – 200 mg.mL^{-1}; C2 – 100 mg.mL^{-1}; C3 – 50 mg.mL^{-1}; C4 – 25 mg.mL^{-1}; C5 – 12.5 mg.mL^{-1}; C6 – 6.25 mg.mL^{-1}; C7 – 3.13 mg.mL^{-1}; C8 – 1.56 mg.mL^{-1}; T – Control test. **A** – *Staphylococcus aureus*; **B** – *Streptococcus pyogenes*; **C** – *Enterococcus faecalis*; **D** – Enteropathogenic *Escherichia coli*; **E** – *Proteus mirabilis*; **F** – *Salmonella enterica* subsp. *arizonae*; **G** – *Hafnia alvei*; **H** – *Candida albicans*.

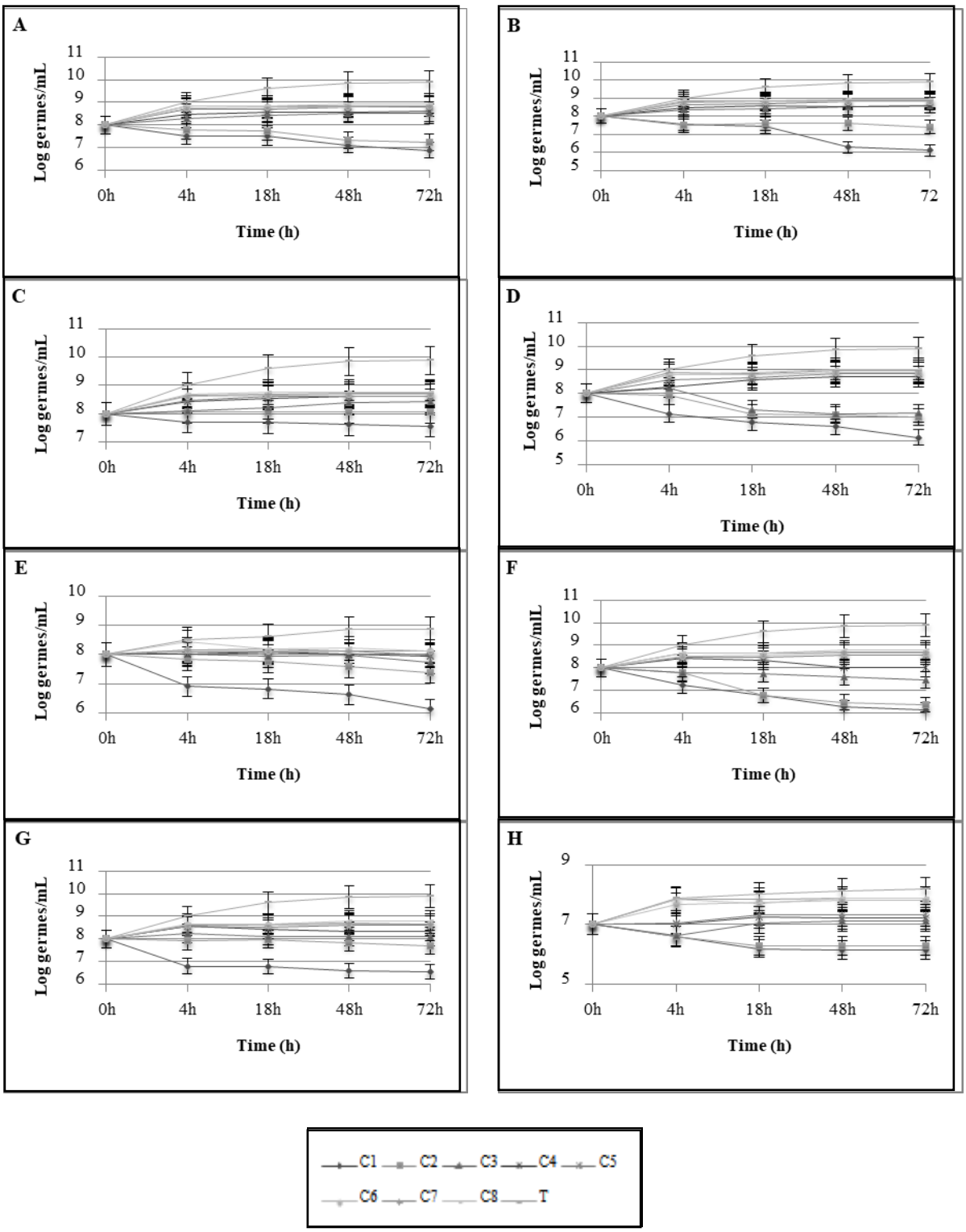

Fig. 2. Antimicrobial effect of *Ruta chalepensis* essential oil on clinical strains isolated from the different biological samples (P <0.05). C1-C8 – Concentrations; C1 – 200 mg.mL^{-1}; C2 – 100 mg.mL^{-1}; C3 – 50 mg.mL^{-1}; C4 – 25 mg.mL^{-1}; C5 – 12.5 mg.mL^{-1}; C6 – 6.25 mg.mL^{-1}; C7 – 3.13 mg.mL^{-1}; C8 – 1.56 mg.mL^{-1}; T – Control test. **A** – *Staphylococcus aureus*; **B** – *Streptococcus pyogenes*; **C** – *Enterococcus faecalis*; **D** – enteropathogenic *Escherichia coli*; **E** – *Proteus mirabilis*; **F** – *Salmonella enterica* subsp. *arizonae*; **G** – *Hafnia alvei*; **H** – *Candida albicans*.

Checkerboard method

The individual and combination minimum inhibitory concentrations, the individual fractional inhibitory concentrations, the index inhibitory fractional concentrations, and the FIC index of *Z. lotus* and *R. chalepensis* EOs combinations against the different microbial strains tested are mentioned in Table 7.

No synergistic effect was determined by applying the combination of *Z. lotus* and *R. chalepensis* EOs against the different microbial strains tested. This combination exerted an antagonistic effect against the microbial strains tested, with FIC_{index} values greater than 4 (FIC_{index}>4). While an indifference interaction was recorded against *S. aureus*, *S. pyogenes*, and *H. alvei* using both plants EOs (Table 7).

These results have enabled us to demonstrate the ineffectiveness of plant essential oils used in combination. In some cases, associations between antimicrobial drugs can be used to broaden their action spectrum on pathogenic germs. Various studies have shown the effectiveness of plant extracts and essential oils combinations in the synergistic effect expression against pathogenic bacteria.

Amirouche and Belkolai (2013) demonstrated that a combination of sage and tea tree essential oils works synergistically against *S. aureus*. However, it was determined during this study that the effect of the EO combinations is lower compared to the effect exerted by the EOs of each plant alone. Therefore, *Z. lotus* and *R. chalepensis* cannot be used in combination due to the antagonism effect exerted on the different microbial strains tested. Thus, the combination of these EOs can limit and reduce the action spectrum of pathogenic bacteria and yeasts.

Table 7. FIC_{index} of the combinations of *Z. lotus* and *R. chalepensis* essential oils on the different microbial strains tested.

Clinical strains	**Essential oils: ZLEO/RCEO**			
	Individual MICs	**MICs in combination**	**Individual FIC**	**FIC_{index}**
S. aureus	100/100	200/200	2/2	4[i]
S. pyogenes	100/100	200/200	2/2	4[i]
E. faecalis	25/100	200/200	8/2	10[a]
E. coli	100/50	200/200	2/4	6[a]
P. mirabilis	100/50	200/200	2/4	6[a]
S. enterica subsp. *arizonae*	50/50	200/200	4/4	8[a]
H. alvei	50/50	100/100	2/2	4[i]
C. albicans	25/25	200/200	8/8	16[a]

[a] – antagonism; [i] – indifference.

Conclusion

Z. lotus and *R. chalepensis* EOs are potent antimicrobials with a comprehensive action spectrum against pathogenic microbial strains. However, both plants' EOs combinations were less effective against all the tested microbial strains, with antagonism effects. So, the impact of each plant EO alone is considered more important in inducing the antimicrobial effect than using of EO combinations in both plants. *Z. lotus* and *R. chalepensis* essential oils could be used in the medical and food industries as potent antimicrobials for combating antimicrobial resistance.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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