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Synergistic effects of *Ruta montana* (Clus.) L. essential oil and antibiotics against some pathogenic bacteria

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ABSTRACT

Antibiotic resistance has been called one of the world's most pressing public health threats. The combination of essential oils with conventional antibiotics is one of the emerging approaches that could help prevent this problem. In light of this, the present study aimed to investigate the impact of the combination of *Ruta montana* essential oil with conventional antibiotics on some pathogenic bacteria. The essential oil isolated by hydrodistillation was first analyzed using GC-MS then tested alone and in combination with five recommended antibiotics against three bacterial strains by the agar disc diffusion and broth micro-dilution methods. Out of forty-nine peaks, thirty-eight components were identified representing 98.17% of the total oil composition. The major components were 2-Undecanone (63.39%), 2-Nonanone (5.65%), 2-Acetoxytetradecane (4.94%), 2-Decanone (4.47%) and 2-Dodecanone (3.35%). While *R. montana* essential oil showed only weak antibacterial activity compared to the antibiotics tested alone, unexpectedly, the combination of RM essential oil with antibiotics remarkably increased the antibacterial activity of the antibiotics through synergistic effects in up to 70% of cases. These results suggest that combining antibiotics with essential oils, even those with low antibacterial activity, may be effective in overcoming problems caused by increasing bacterial resistance.

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INTRODUCTION

The increased prevalence of bacterial resistance is one of the major problems of global health today (Levy & Marshall, 2004). Faced with such a situation, the high antimicrobial potential of essential oils (EOs), their ability to improve the effectiveness of antibiotics (ABs) and the lack of any apparent emergence of bacterial resistance to them or their components make them valuable subjects of research (Bakkali *et al.*, 2008; Sienkiewicz *et al.*, 2017). In fact, essential oils are made up of many molecules, which make bacteria vulnerable to antibiotics (Sienkiewicz *et al.*, 2017). The occurrence of synergism is thought to be the key to the bioactivity of EOs (Boonyanugomol *et al.*, 2017; Chouhan *et al.*, 2017). Therefore, the association between EOs and ABs has emerged as a novel approach in controlling multidrug-resistant (MDR) strains and modulating the activity of ABs (Boonyanugomol *et al.* 2017; Chouhan *et al.*, 2017; Boudjedjou *et al.*, 2018, 2019).

Ruta montana is one of the four species of the genus *Ruta*, from *Rutaceae* family; grow wild in Algeria (Quézel & Santa, 1963). All are perennial herbaceous plants with yellow flowers, characterized by a strong, foul-smelling, nauseating odor, due to an essential oil contained in enormous secretory pockets (Hammiche *et al.*, 2013). In folk medicine, *R. montana* is used for the treatment of persistent cough by fumigation. The infusion or decoction of the aerial parts in milk is used for all problems related to the female genital system such as painful periods and after childbirth. Their decoction in olive oil is used for rheumatism and body aches. Their infusions are used as eye drops for corneal ulcers, as ear drops for otitis and tinnitus, as nasal drops to treat atrophic rhinitis, for fever and vomiting in infants and children (Hammiche *et al.*, 2013).

Previous studies have shown that *R. montana* can be considered as an important source of biologically interesting compounds, namely alkaloids, coumarins, flavonoids, tannins and essential oils (Kambouche *et al.*, 2008; Boutoumi *et al.*, 2009; Belkessame *et al.*, 2011; Bouzidi *et al.*, 2012; Zellagui

et al., 2012; Hammiche *et al.*, 2013; Ferhat *et al.*, 2014; Khadhri *et al.*, 2014; Hammami *et al.*, 2015; Hazzit *et al.*, 2015; Daoudi *et al.*, 2016; Bennaoum *et al.*, 2017; Fekhar *et al.*, 2017; Benali *et al.*, 2020; Drioiche *et al.*, 2020; Mohammedi *et al.*, 2020), with biological activities including antioxidant (Kambouche *et al.*, 2008; Zellagui *et al.*,2012; Benali *et al.*, 2020; Mohammedi *et al.*, 2020), antifungal (Hammami *et al.*, 2015; Hazzit *et al.*, 2015; Fekhar *et al.*, 2017; Benali *et al.*, 2020; Drioiche *et al.*, 2020; Mohammedi *et al.*, 2020), insecticidal and larvicidal (Boutoumi *et al.*, 2009; Fekhar *et al.*, 2017).

The chemical composition of *Ruta montana* essential oils (RMEOs) from various localities and harvested at different seasons have been reported in several papers. The results showed that RMEOs are characterized by the predominance of 2-ketones, such as 2-undecanone and 2-decanone, whereas terpene components were present in lower amounts, with the exception of caryophyllene oxide which occurs as a major component of RMEOs (Bennaoum *et al.*, 2017). The variation in the content of terpene components can be ascribed to many factors, such as the harvesting period (Bennaoum *et al.*, 2017), plant organ (Khadhri *et al.*, 2014), and geographical origin (Mohammedi *et al.*, 2020).

The antibacterial activity of RMEOs seems a bit controversial. Yet, several studies have reported a moderate to the strong antibacterial activity of RMEOs (Belkessame *et al.*, 2011; Zellagui *et al.*, 2012; Hazzit *et al.*, 2015; Daoudi *et al.*, 2016; Fekhar *et al.*, 2017; Benali *et al.*, 2020; Drioiche *et al.*, 2020; Mohammedi *et al.*, 2020). However, to our knowledge, there are no reports on the combination of RMEOs with antibiotics. Hence, this study aimed to investigate the chemical composition and to compare the antibacterial activity of *R. montana* essential oil alone and in combination with some recommended antibiotics against three pathogenic bacteria.

MATERIALS AND METHODS

Plant Material

Arial parts of *Ruta montana* (Clus.) L. were collected in May 2015, during the period of full flowering, from T'kout, department of Batna in Algeria, at 35.0547 (latitude in decimal degrees) and 6.2235 (longitude in decimal degrees). The identification of plant material has been demonstrated according to the flora of Quezel & Santa (1963). A voucher specimen has been deposited in the Herbarium of our laboratory under the code RUT-001-1-2015. The plant material was cleaned of impurities, dried at room temperature in the dark for two weeks, then cut into small pieces not exceeding 1 cm and kept in paper bags to be used for the extraction of essential oils.

Extraction and Analysis of the Essential Oil

The air-dried plant material (150 g) was subjected to steam hydrodistillation for 3 h using a Clevenger-type apparatus (Clevenger, 1928). The oil obtained was stored in a sealed vial in the dark at 4 °C until analysis. Quantitative and qualitative analyses of the collected oil were determined by gas chromatography coupled with mass spectrometry (GC/MS). Mass spectra of EO were obtained using a GC focus (Thermo) with BR5-MS column (5% phenyl methyl siloxane), 30 m long and 0.32 mm i.d., with 0.25 µm film thickness (Bruker). Coupled to a mass spectrometer (MS) type DSQII (Thermo) with a detector impact of electrons, 70 eV. The carrier gas is helium at a rate of 1.2 mL min^{-1}; the injection volume was 0.1 µL; injector split mode 1:100. The initial temperature of the column was kept at 70 °C for 1 min and programmed to 300 °C at a rate of 10 °C.$min^{-1}$and kept constant at 300 °C for 5 min. The mass spectrum of each compound was recorded between 40 and 500 Da (m/z equivalent unit). Identification of compounds was achieved by comparison of their recorded mass spectra with those of a computer library (NIST2008 v2.0/ Xcalibur data system) provided by the instrument software, and of their retention indices with literature data (Adams, 2001). Retention indices (RI) were calculated by the retention times of a series of n-alkanes.

Antibacterial Screening

The antibacterial activity of *R. montana* EO was tested against three bacterial strains (*Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, and *Escherichia coli* ATCC25922) using two different methods: the agar disc diffusion and the Broth micro-dilution methods respectively.

Agar disc diffusion assay

The agar disc diffusion assay was performed as recommended by the Food and Drug Administration (FDA) and the Clinical and Laboratory Standards Institute (CLSI) (de Sousa Eduardo *et al.*, 2018). Sterile filter discs (6 mm in diameter) impregnated with 10 µL/disk of the RMEO were placed onto the Petri dishes containing 20 mL of Mueller Hinton Agar (MHA) and inoculated with the tested bacteria (10^8 CFU mL^{-1}). The plates were then incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the discs. Standard discs of five conventional antibiotics: gentamycin 10 µg $disk^{-1}$, amoxicillin 25 µg $disk^{-1}$, cefazolin 30 µg $disk^{-1}$, tetracycline 30 µg $disk^{-1}$, and Amoxicillin/Clavulanic Acid (claventin) 20/10 µg $disk^{-1}$, were used as positive control, whilst discs soaked with 10 µLdimethyl sulfoxide (DMSO) were used as the negative control (no zone inhibition was observed).

Determination of the MIC and MBC

The *in vitro* minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of RMEO were determined using a microdilution assay as described by de Sousa Eduardo *et al.* (2018) with modification. The serial two-fold dilutions of the tested EO were prepared in standard sterile 96-well flat bottom microplates and the layout was designed so that each row covered the final dilution of 500 to 3.9 µL mL^{-1}. One hundred microliters of MH broth media and 10 µL (10^8 CFU mL^{-1}) of the bacterial culture were added to each well containing 100 µL of the serially diluted test

EO, giving a final concentration of the bacteria in the well of approximately 10^5 CFU mL^{-1}. After inoculation overnight at 37 °C, the optical density (OD) was measured. The minimal concentration that has OD less than the OD of the control was defined as the MIC. The MBC was determined as the lowest concentration at which 99.9% of the bacterial population was killed.

Synergistic Interaction

The combinatory effect of RMEOwith conventional ABs was evaluated using an agar-disc diffusion method as previously described (Moussaoui and Alaoui, 2015). The standard discs of antibiotics were impregnated with 10 µL/disc of the RMEO and were put onto the surface of the inoculated MHA. The plates were incubated for 24h at 37°C after which the inhibition zones were measured. If the value of the inhibition zone of the EO/AB combination is significantly higher ($P < 0.05$) than the sum of the individual values, this is considered to be a synergistic effect, but if they are equal ($P \geq 0.05$), then this is considered an additive effect. The antagonistic effect occurs when the values of the inhibition zones of both treatments separately EO/AB are significantly greater than the value of their combination (Boudjedjou *et al.*, 2018, 2019).

Statistical Analysis

All experiments were carried out in triplicate and the data were reported as the mean ± stander deviation of three samples. Statistical analysis was performed using Statistica 8.0 software, StatSoft Inc., USA (Hill and Lewicki, 2007). Differences were tested for significance by using the one-way ANOVA test ($P<0.05$).

RESULTS AND DISCUSSION

Chemical Composition of *R. Montana* EO

The steam-hydrodistillation of *R. montana* aerial parts yielded 2.5% of yellowish essential oil with a strong and penetrating odor. This yield was in the same range as those reported in the literature (0.38 - 6.1%) (Kambouche *et al.*, 2008; Boutoumi *et al.*, 2009; Bouzidi *et al.*, 2012; Zellagui *et al.*, 2012; Ferhat *et al.*, 2014; Khadhri *et al.*, 2014; Hammami *et al.*, 2015; Hazzit *et al.*, 2015; Daoudi *et al.*, 2016; Bennaoum *et al.*, 2017; Fekhar *et al.*, 2017; Mohammedi *et al.*, 2020).

The obtained EO was chemically characterized using GC-MS (Table 1, Figure 1). Table 1 depicts the component's identification and their percentages, as well as the RT and RI values, which are listed in order of their elution from the BR5-MS capillary column. Thirty-eight components were identified, representing 98.17% of the total essential oil components. In addition, eleven unidentified compounds were present in the sample, representing 1.7% (from 0.09 to 0.43%) of the total oil. As expected, the aliphatic ketone 2-Undecanone was found to be the major component of RMEO (63.39%), followed by 2-Nonanone (5.65%), 2-Acetoxytetradecane (4.94%), 2-Decanone (4.47%), and 2-Dodecanone (3.35%). Among the main compounds identified, there is also the sesquiterpene caryophyllene oxide (2.34 %). This is in agreement with most of the previous literature on the chemical composition of RMEOs confirming the predominance of 2-Undecanone (Kambouche *et al.*, 2008; Boutoumi *et al.*, 2009; Belkessam *et al.*, 2011; Zellagui *et al.*, 2012; Ferhat *et al.*, 2014; Khadhri *et al.*, 2014; Hazzit *et al.*, 2015; Bennaoum *et al.*, 2017; Benali *et al.*, 2020; Drioiche *et al.*, 2020; Mohammedi *et al.*, 2020). Nevertheless, the difference in the percentage of some minor and major compounds could be attributed to the status of the plant

Table 1: Percentage composition of the essential oil of *Ruta montana* (Clus.) L.

Compounds	RT (mn)	RI	Percentage (%)
β-Terpinene	4.79	975	0.43
O-Cymene	5.70	1027	0.16
Limonene	5.78	1031	0.16
Eucalyptol	5.83	1034	0.24
Acetophenone	6.43	1068	0.18
2-Nonanone	6.88	1094	5.65
Linalool	7.00	1001	0.37
Nonanal	7.06	1104	0.24
Camphor	7.79	1149	0.19
1-Nonanol	8.17	1172	0.09
Terpinen-4-ol	8.32	1182	0.12
2-Decanone	8.53	1194	4.47
2-Undecanone	10.25	1305	63.39
n-Decanoic acid	11.29	1380	0.57
2-Dodecanone	11.46	1392	3.35
α-Methylbenzylpropanoate	11.65	1413	0.23
2-Acetoxytetradecane	11.93	1433	4.94
2-Nonenone-4	12.56	1484	0.18
β-Phenylethylisovalerate	12.68	1494	0.15
2-Tridecanone	12.74	1498	2.18
2-Nonen-4-one	13.18	1533	2.8
1-Cyclododecylethanone	13.33	1545	0.18
Asarone	13.49	1557	0.1
Nerolidol	13.59	1569	0.15
Tridecane-2,4-dione	13.81	1582	1.4
Caryophyllene oxide	13.97	1601	2.34
Cetylglycidyl ether	14.20	1620	0.74
Humulene epoxide II	14.26	1626	0.16
Apiol	14.35	1632	0.34
4,4-Dimethyltetracyclo-(6,3,2,0)(2,5)0(1,8) tridecan-9-ol	14.57	1651	0.9
Isoaromadendrene epoxide	14.93	1685	0.26
1-Cyclopropyl-1-dodecanone	15.86	1770	0.34
2-Bromotetradecane	16.34	1815	0.11
Hexahydrofarnesyl acetone	16.67	1847	0.21
n-Hexadecanoic acid	17.86	1965	0.1
Methoxsalen	18.73	2056	0.34
α-Acetylacetophenone	20.31	2231	0.26
Eicosane	22.51	2499	0.15
Yield (%)			2.5
Monoterpene hydrocarbons (3 compounds)			0.75
Oxygenated monoterpene (6 compounds)			1.25
Oxygenated sesquiterpene (5 compounds)			3.81
Phenylpropenes (2 compounds)			0.44
Ketones (13 compounds)			84.59
Esters (3 compounds)			5.32
Others (6 compounds)			2.01
Total (38 compounds)			98.17

RT: retention time. RI: Kovats index. The percentages illustrated in bold correspond to the major components of *R. montana* essential oil.

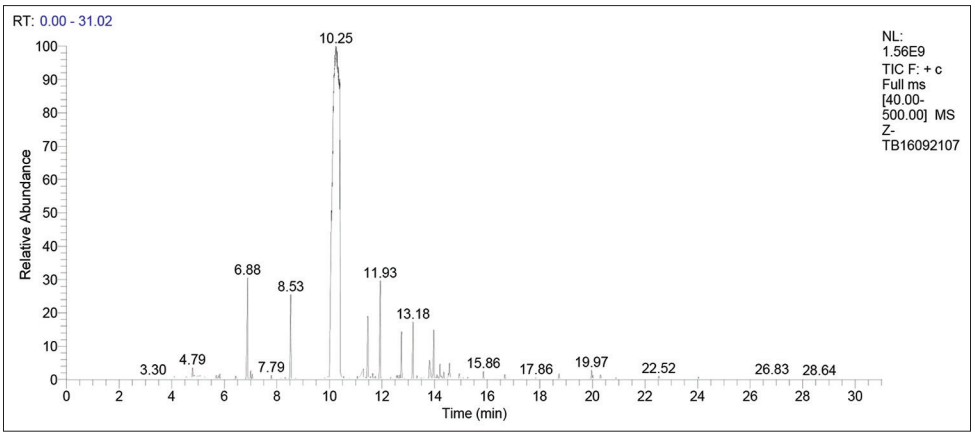

Figure 1: Gas chromatography-mass spectrometry chromatogram of the aerial part essential oil of *R. montana*.

material (dry or fresh), period of harvesting, geographic origin, and the kind of plant material (Kambouche *et al.*, 2008; Boutoumi *et al.*, 2009; Zellagui *et al.*, 2012; Ferhat *et al.*, 2014, Khadhri *et al.*, 2014; Hazzit *et al.*, 2015; Bennaoum *et al.*, 2017; Negri *et al.*, 2020; Mohammedi *et al.*, 2020). Bennaoum *et al.* (2017), from a study conducted on EOs extracted from 11 samples belonging to three species of the genus *Ruta*, it has been suggested that the main factors able to influence the chemical composition of RMEO was the harvested period and the geographical origin (Bennaoum *et al.*, 2017). The EOs extracted from plants harvested in spring and winter, as in our case, were characterized by the predominance of ketones, whereas those harvested on summer and autumn seasons were characterized by the predominance of sesquiterpenes and monoterpenes (Bennaoum *et al.*, 2017).

Antibacterial Activity

The results of the antibacterial activity of RMEO and five standard antibiotics against the three selected pathogenic bacteria are compiled in Table 2. Compared to antibiotics for which all the tested bacteria showed more or less significant sensitivity with the exception of claventin against *P. aeruginosa*, RMEO was found to be ineffective against all the tested strains. In line with this, the evaluation of MIC and MBC of RMEO (Table 3) showed no or very slight antibacterial activity against *E. coli* and *S. aureus*, with MICs of 125µL/mL and 250 µL/mL for *S. aureus* and *E. coli* respectively. These results are consistent with some previous studies, which reported that essential oils of *Ruta* genus displayed no or less antibacterial activity (Merghache *et al.*, 2008; Bnina *et al.*, 2010; Haddouchi *et al.*, 2013). This weak antibacterial activity could be attributed to the high percentage of ketones in the oils (Gibka *et al.*, 2009; Haddouchi *et al.*, 2013). Indeed, the antimicrobial activity of 2-undecanone, the most abundant ketone in this oil, is known to be weak against the bacterial strains (Gibka *et al.*, 2009).

On the other hand, Zellagui *et al.* (2012) and Bouzidi *et al.* (2012) reported that the essential oil of *R. montana* has a strong antibacterial activity against all tested bacterial strains (*E. coli*, *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Mycobacterium kansasii*, and *Mycobacterium vaccae*), with an inhibition

Table 2: Inhibition zone diameters (mm) of ***R. montana*** essential oil and antibiotics.

	E. coli ATCC25922	*S. aureus* ATCC25923	*P. aeruginosa* ATCC27853
RMEO	NI	NI	NI
Gentamycin	18±1.0	21±0.5	21.5±0.5
Amoxicillin	8.7±0.6	15±1.0	10.7±0.6
Cefazolin	22.7±0.6	34.7±0.6	16±1.0
Tetracycline	24±1.0	26.7±0.6	23 ±1.0
Claventin	9.3±0.6	12±1.0	NI

Inhibition zone includes diameter of disk (6 mm). Values of inhibition diameter are given as mean ± standard deviation; RMEO: *R. montana* essential oil; NI: No inhibition; ATCC: American type culture collection.

Table 3: In vitro MICs and MBCs (µL/mL) values of RMEO against tested bacteria

	E. coli ATCC25922	*S. aureus* ATCC25923	*P. aeruginosa* ATCC27853
MIC (µL/mL)	250	125	>500
MBC (µL/mL)	500	500	ND
MBC/MIC ratio	2	4	ND
Effect	Bactericidal	Bactericidal	ND

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ND: not determined; ATCC: American type culture collection.

diameter that increases as the concentration of the oil extract increases. This activity appears to be related to the relative amount of hydrocarbon and alcohol terpenes (Haddouchi *et al.*, 2013). It is widely accepted that essential oils rich in aldehydes or phenols have the highest antibacterial activity, followed by those containing alcoholic terpenes. Essential oils containing high levels of ketones or esters have much weaker activity, while those containing terpene hydrocarbons are often inactive (Bassolé & Juliani, 2012).

Synergistic Interaction

In order to assess whether or not the addition of essential oils improves the effectiveness of antibiotics, the combined effect of RMEO and five antibiotics recommended against three pathogenic strains were investigated. The results indicated a pronounced antibacterial activity of almost all

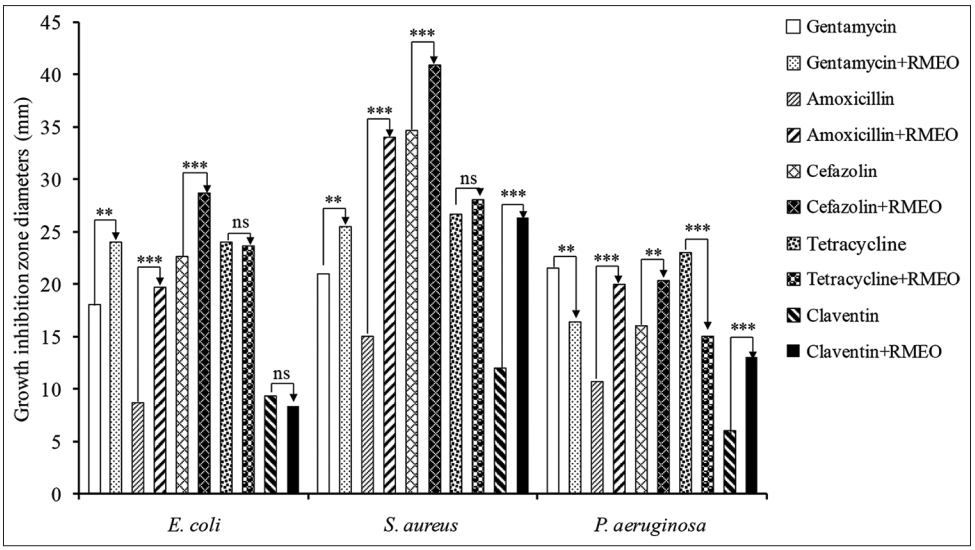

Figure 2: Antibiotic-modulating activity of R. montana essential oil (RMEO) in combination with antibiotics against selected pathogenic strains (E. coli, S. aureus and P. aeruginosa).ns, not significant; *, significant (P<0.05); **, highly significant (P<0.01); ***; very highly significant (P<0.001).

Table 4: Inhibition zone diameters (mm) of ***R. montana*** essential oil in combination with conventional antibiotics.

	E. coli ATCC25922	***S. aureus*** ATCC25923	***P. aeruginosa*** ATCC27853
GEN/RMEO	24±1.0	25.5±1.5	16.5±1.0
AMX/RMEO	19.7±0.6	34±1.0	20.3±0.6
CEF/RMEO	28.7±0.6	41±1.0	21.5±0.5
TET/RMEO	23.7±0.6	28±1.0	15±1.0
CLA/RMEO	8.3±0.6	26.3±0.6	13±1.0
P values			
GEN/RMEO	0.0018**	0.0079**	0.0015**
AMX/RMEO	0.00002***	0.00002***	0.00015***
CEF/RMEO	0.0002***	0.00068***	0.0028**
TET/RMEO	0.643 ^{ns}	0.116 ^{ns}	0.0006***
CLA/RMEO	0.1 ^{ns}	0.000028***	0.00026***
Combination effect			
GEN/RMEO	Synergistic	Synergistic	Antagonistic
AMX/RMEO	Synergistic	Synergistic	Synergistic
CEF/RMEO	Synergistic	Synergistic	Synergistic
TET/RMEO	Additive	Additive	Antagonistic
CLA/RMEO	Additive	Synergistic	Synergistic

Values were expressed as mean ± standard deviation. Ns, not significant; *, significant (P<0.05); **, highly significant (P<0.01); ***, very highly significant (P<0.001); RMEO: *R. montana* essential oil; GEN: gentamycin; AMX: amoxicillin; CEF: cefazolin; TET: tetracycline; CLA: claventin; ATCC: American type culture collection.

ABs/RMEO combinations against the three tested bacterial strains, even against *P. aeruginosa* (gram-negative) the most resistant strain among the tested ones (Table 4). RMEO showed significant synergistic effects, making bacterial strains more sensitive when combined with gentamycin, amoxicillin, cefazolin, and claventin, as evidenced by the significant increase in the inhibition zone diameters (Figure 2). It should, however, be noted that the GEN/RMEO and TET/RMEO combinations induced antagonistic effects against *P. aeruginosa*.

It is widely accepted that synergy can occur if the components of a mixture affect different targets (Lewis % Ausubel, 2006; Wagner and Ulrich Merzenich, 2009). Considering the weak RMEO's antibacterial activity, the synergism observed between RMEO and the different ABs is likely due to the fact that EOs facilitates the penetration of ABs into the bacterial cells, since the lipophilic nature of oils can causes expansion of the membrane, increased membrane fluidity and permeability (Sienkiewicz *et al.*, 2017). On the other hand, given the great synergistic effect induced by the interaction between RMEO and the β-lactam antibiotics (amoxicillin, cefazolin, and claventin), which are known to act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls (Elander, 2003), it can be also assumed that these ABs facilitates the diffusion of essential oils into the cell.

CONCLUSION

In the present study, the chemical composition and antimicrobial activity of *R. montana* essential oil alone and in combination with some antibiotics were investigated. The results showed that the combination of *R. montana* EO with conventional antibiotics, particularly with amoxicillin and cefazolin induced significant synergistic effects against all pathogenic strains tested. Therefore, the combination of antibiotics and essential oils have the potential to be used as an alternative therapeutic treatment, not only to reduce possible adverse effects and the cost of antibiotic-based treatments but also to prevent the development of bacterial resistance.

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CONFLICT OF INTERSTS

The authors claim that there is no conflict of interest.

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