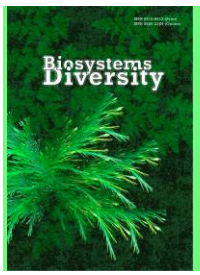

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Antibacterial effect of essential oils of two plants *Eucalyptus camaldulensis* and *Artemisia herba alba* on some bacterial strains

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Essential oils are secondary plant metabolites and have many therapeutic properties. The aim of our study is to determine the antibacterial effect of the essential oils of two plants cultivated in a semi-arid region located in the Northeast of Algeria (Tebessa), *Eucalyptus camaldulensis* (Myrtaceae) and *Artemisia herba alba* (Asteraceae). The yield of essential oils of the two plants were 1.45 ± 0.026 and 1.21 ± 0.061 g/100 g of the dry matter of the aerial part respectively. The test of the antibacterial effect is based on the diffusion method on solid medium (sensitivity), this method allows us to determine the susceptibility or resistance of an organism vis-à-vis the sample studied. Our study reveals that *E. camaldulensis* essential oil had very strong activity on all bacterial strains tested, except on *Pseudomonas aeruginosa* and *Enterococcus faecalis* for which there was no inhibitory effect. However, *A. herba alba* essential oil had very strong activity on all bacterial strains tested except on *Pseudomonas aeruginosa*. The MIC of *Artemisia* essential oil ranged between 0.08 and 1.57 µL/mL, with the lowest activity for *S. aureus* and *P. mirabilis* (1.57 µL/mL) and the highest activity was observed against *E. feacalis*, *E. coli*, and *K. pneumonia* (0.09 µL/mL). The MIC of the second plant EO ranged between 0.08 and 0.36 µL/mL, with the lowest activity for *P. mirabilis* (0.36 µL/mL) and the highest one was observed against *S. saprophyticus* and *E. coli* (0.08 µL/mL). Statistical analysis shows that the two plants have the same efficacy against *S. saprophyticus* while *E. faecalis*, *K. pneumoniae* and *P. mirabilis* species are affected more by the essential oil of *A. herba alba*. While, *E. camaldulensis* has a higher efficiency than that of *A. herba alba* on the species: *S. aureus* and *E. coli*. Therefore, the essential oils of *E. camaldulensis* and *A. herba alba* suggests avenues for further non clinical and clinical studies.

Keywords: yield; antibacterial activity; inhibitory effect; secondary metabolites; sensitivity; aromatogram test.

Introduction

For thousands of years, humankind has used various plants found in its environment, to treat and cure all kinds of diseases (Ghulam et al., 2017). These plants represent an immense reservoir of potential compounds which have the advantage of being of a great diversity of chemical structures and they possess a very wide range of biological activity (Zazharskyi et al., 2019a, 2019b, 2020; Álvarez-Martínez et al., 2020). However, the evaluation of these activities remains a very interesting task which may be of interest to many researchers (Mazari et al., 2010). Medicinal plants are a numerically large group of economically important plants (Pérez-Nicolas et al., 2017). They offer an alternative to drugs and contain active components derived from the secondary metabolites produced from the metabolism of nutrients that are used by human beings in their therapeutic arsenal (Tesche & Metternich, 2008; Al-Jumaili et al., 2018). These active components are distinguished by several categories such as alkaloids, flavonoids, tannins, essential oils, and other compounds (Mehani, 2015). Essential oils are valuable molecules, used in pharmacology because they have a specific effect on other organisms (Tohidi et al., 2019). In cosmetology (Chaudhuri et al., 2020), they are used as a base for the manufacture of perfume and dermatological products (Kumar et al., 2020). In the food industry they are used to enhance the taste, flavour and colour of food and its conservation. Essential oils have a very broad spectrum of activity due mainly to their great affinity due to their nature, for this, the antibacterial activities of these products have been reported in a large number of studies (Nedjai, 2017). The use of essential oils in medicine has never been abandoned despite the discovery of organic synthesis processes and the birth of the pharmaceutical industry (Baptista-Silva et al., 2020). They are considered as a real reservoir of basic molecules that are irreplaceable (Ouraïni et al., 2007). Considerable interest has been generated in essential oils extracted from aromatic plants and endowed with antimicrobial activity against pathogenic microorganisms (Alzoreky & Nakahara, 2003; Traore et al., 2013).

Algeria, by its geographical location, offers a rich and diverse vegetation. A large number of aromatic and medicinal plants grow there spontaneously (Bouvet, 2013).

Major *in vitro* studies proved the antibacterial effect of the plants used in traditional medicine: *Satureja hortesis* (Gulluce et al., 2003), *Albies alba* (Bagci & Digrak, 2003; Yang et al., 2008), *Aloe barbadensis* (Habeeb et al., 2007), *Lavandula angustifolia* (Adam et al., 1998; Rota et al., 2004), *Hypericum perforatum* (Rabanal et al., 2002), *Calendula officinalis* (Dias Barzon et al., 2008), *Mentha piperita* (Silva et al., 1994; Tassou et al., 2000), *Eucalyptus globulus* (Chao et al., 2008), *Cocos nucifera* (Esquenazi et al., 2002), *Eucalyptus* essential oil (Kumar, 1988; Chao et al., 2000; Cermelli et al., 2008) and *Artemisia* (Juteau et al., 2002; Younessi-Hamzekhanlu et al., 2020).

In this study we extracted the essential oil from the leaves of two plants *Eucalyptus camaldulensis* and *Artemisia herba alba* asso and quantified the yield. In a second series of experiments, we investigated under laboratory conditions the efficacy of essential oils of the two plants against the selected Gram-positive and Gram-negative bacteria *Entrococcus faecalis*, *Staphylococcus saprophyticus*, *S. aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, by the aromatogram test (agar diffusion method) and determination of minimal inhibitory concentration (MIC). The organisms like *Escherichia*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* species are known to cause severe infections in humans, as they are found in multiple environmental habitats (Trombetta et al., 2005). The aim was to indicate the potential usefulness of the two plants *E. camaldulensis* and *A. herba alba* as a microbiostatic, antiseptic or as a disinfectant agent. In addition, a statistical analysis was

carried out to determine the significant effect of the essential oils of the two plants on the set of bacteria tested.

Materials and methods

The leaves of *E. camaldulensis* and *A. herba alba* were collected in the month of January 2020 in Tebessa (Northeast Algeria) in the following coordinates: 35°20′46″ N, 8°15′18″ E and 1040 m above sea level for *E. camaldulensis* and 35°29′39″ N, 8°20′12″ E and 1028 m sea level. The harvesting was carried out in the afternoon, and was taken only from the aerial part of the adult tree; chosen at random. The samples were dried well at room temperature and protected from light and moisture for a period of 10 days and were stored in clean bags to remove any impurities.

The essential oil of *E. camaldulensis* and *A. herba alba* was extracted from dried leaves, submitted to hydrodistillation in Clevenger apparatus. After collection, samples of 100 g were subjected to hydrodistillation for 3 h with 1 L distilled water. The essential oil was collected and water droplets were removed using sodium sulfate and stored in amber and refrigerated bottles. The yield of essential oil is the ratio between the weight of the oil extracted and the weight of the dry matter of the plant (Bouabida & Dris, 2020).

Bacterial strains of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis* (Table 1) were provided by the microbilogical laboratory of clinic of Tebessa. The identification of strains was confirmed by the use of biochemical profiles according to the recommendation of the Manual of Clinical Microbiology (Murray, 2003).

Table 1
Characteristics of the bacterial strains tested

Family	Bacterial strains	Bacterial strains
Enterococcaceae	*Enterococcus faecalis*	ATCC 29212
Enterobacteriaceae	*Klebsiella pneumoniae*	ATCC 10031
	Escherichia coli	ATCC 11303
	Proteus mirabilis	ATCC 25933
Staphylococcaceae	*Staphylococcus saprophyticus*	ATCC 15305
	Staphylococcus aureus	ATCC 25923
Pseudomonadaceae	*Pseudomenas aeruginosa*	ATCC 9027

Aromatogram test (agar diffusion method) is an *in vitro* method of measuring the antibacterial power of essential oils. To seed 1 mL of the bacterial inoculum in the petri dishes previously melted with Mueller-Hinton agar a suspension of each of the bacterial isolates, to be used in the tests, was adjusted to a turbidity matching that of a 0.5 McFarland standard, equivalent to 1.5×10^8 CFU/mL (Donay, 2007; Balouiri, 2016). Six disks of blotting paper (0.6 cm diameter) aseptically impregnated with 10, 5, 2.5, 1.25 and 0.62 µL of essential oils were deposited on the agar surface (De Billerbeck, 2002; Pibiri, 2005). All tests were repeated 4 times. After a latency period at 37 °C for 16–18 h, the inhibition zone surrounding the disks was measured (Boland et al., 1991; Euzéby, 1998; Franchomme, 1999; Farah et al., 2001; Cimanga et al., 2002). The sensitivity of bacterial strains depending on the inhibition zones was interpreted according to Table 2.

Table 2
Sensitivity of bacterial strains depending on the inhibition zones (Ponce et al., 2003)

Sensitivity	Diameter of inhibition zone, mm
No sensitive or resistant (–)	< 8
Sensitive (+)	9–14
Very sensitive (++)	15–19
Extremely sensitive (+++)	> 20

The micro-dilution broth method was used to determine the Minimal Inhibitory Concentration (MIC) according to Yu et al. (2004). The essential oil diluted in DMSO was mixed with a 100 mL Mueller–Hinton. Serial dilutions of the oil were prepared in a 96-well microtiter plate ranged from 0.08 to 25 µL/mL. Finally, an inoculum containing 1.5×10^8 CFU (10 µL) per well was added to the broth with various oil concentrations. Two columns in each plate were used as controls: one column with a broad-spectrum antibiotic as a positive control (amykacine), and one column containing DMSO as a negative control. The control media containing only DMSO did not inhibit the growth of bacterial strains. The plates were prepared in quadruple, and then they were placed in an incubator at 37 °C for 18–24 hours under aerobic conditions. After incubation time, the growth of cultures were checked visually. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity (Harauma et al., 2007).

The level of significance for the analyses was set at $P < 0.05$. To compare the effectiveness of the two plants on the different bacterial strains, the two way ANOVA followed by multiple comparison of means (Tukey's test) was performed on the lysis diameters of the highest concentration 10 µL. Statistical analyses of the means ± standard deviation were performed using the software program GraphPad Prism 8 (www.graphpad.com).

Results

The yield of essential oil extracted from the dry matter of the aerial part of *E. camaldulensis* and *A. herba alba* of two plants was 1.45 ± 0.026 and 1.21 ± 0.061 g respectively. The following table shows the results of tests of antibacterial activity of essential oils from the plants *E. camaldulensis* and *A. herba alba* on bacterial strains. The antibacterial activity of essential oil is determined by measuring the diameter of the zone of inhibition formed around each disc, the sensitivity of bacteria to essential oil has been classified according to the diameter of the inhibition halos as summarized in Table 3.

Table 3
Zone of inhibition of bacterial strains against essential oil (µL) of *Eucalyptus camaldulensis* (x ± SD, n = 4)

Bacterial strains	Zone of inhibition, mm				
	10 µL	5 µL	2.5 µL	1.25 µL	0.62 µL
E. faecalis	0	0	0	0	0
S. saprophyticus	12.03 ± 0.15	0	0	0	0
S. aureus	17.50 ± 0.50	0	0	0	0
E. coli	15.60 ± 0.53	9.23 ± 0.15	8.32 ± 0.12	0	0
K. pneumonia	13.66 ± 1.52	8.23 ± 0.25	7.31 ± 0.10	6.09 ± 0.10	5.43 ± 0.42
P. aeruginosa	0	0	0	0	0
P. mirabilis	8.20 ± 0.21	0	0	0	0

Table 4
Zone of inhibition of bacterial strains against essential oil (µL) of *Artemisia herba alba* (x ± SD, n = 4)

Bacterial strains	Zone of inhibition, mm				
	10 µL	5 µL	2.5 µL	1.25 µL	0.62 µL
E. faecalis	13.36 ± 1.20	13.01 ± 0.10	12.00 ± 0.17	10.03 ± 0.12	9.33 ± 0.41
S. saprophyticus	12.46 ± 0.58	12.13 ± 0.20	11.20±0.17	10.44±0.10	0
S. aureus	10.40 ± 0.17	9.60 ± 0.10	0	0	0
E. coli	13.03 ± 0,83	9.60 ± 0.10	8.59 ± 0.05	7.77 ± 0.02	6.61 ± 0.20
K. pneumonia	17.56 ± 1.36	13.33 ± 0.98	9.21 ± 0.10	8.83 ± 0.10	8.52 ± 0.10
P. aeruginosa	0	0	0	0	0
P. mirabilis	10.10 ± 0.26	9.26 ± 0.11	8.02 ± 0.10	0	0

E. camaldulensis and *A. herba alba* essential oil had very strong activity on all bacterial strains tested 8 < D < 17 mm (Table 2), except on *P. aeruginosa* and *E. faecalis*, which resisted the effect of *E. camaldulensis* essential oil (Table 3) while only *P. aeruginosa* was resistant to the effect of *Artemisia* essential oil (Table 4). In fact, we noticed a significant decrease in the percentage of inhibition with the following bacterial strains: *S. aureus* and *E. coli*, which were very sensitive. *E. faecalis*, *K. pneumoniae*, *S. saprophyticus* and *P. mirabilis* were sensitive.

The results of the antibacterial broth micro dilution assay are summarised in Table 5. MIC values did not exhibit substantial variations when compared to the trend of inhibition shown with the agar diffusion method. Generally, larger inhibition zone values correlated with lower MIC. *Eucalyptus* and *Artemisia* essential oil showed a high activity against a majority of the selected bacteria. With *Artemisia* essential oil, MIC ranged between 0.08 and 1.57 µL/mL, with the lowest activity for *S. aureus* and *P. mirabilis* (1.57 µL/mL) while the highest activity was observed against *E. feaca-*

lis, *E. coli*, and *K. pneumonia* (0.09 µL/mL).With *Eucalyptus* essential oil, MIC ranged between 0.08 and 0.36 µL/mL, with the lowest activity for *P. mirabilis* (0.36 µL/mL) while the highest activity was observed against *S. saprophyticus*, *E. coli* (0.08 µL/mL) and *S. aureus*, *K. pneumonia* (0.09 µL/mL).

Table 5
Antibacterial activity of *Eucalyptus camaldulensis* and *Artemisia herba alba* essential oil expressed as minimum inhibitory concentration (MIC)

Bacterial strains	Minimum inhibitory concentration (MIC), µL/mL	
	Eucalyptus camaldulensis	*Artemisia herba alba*
E. faecalis	–	0.08
S. saprophyticus	0.08	0.18
S. aureus	0.09	1.57
E. coli	0.08	0.09
K. pneumonia	0.09	0.09
P. aeruginosa	–	–
P. mirabilis	0.36	1.57

Note: "–" – not active.

The multiple comparison of means show that the two plants have the same efficacy against *S. saprophyticus* ($P = 0.985$). In contrast, *E. faecalis*, *K. pneumoniae* and *P. mirabilis* are affected more by the essential oil of *A. herba alba* ($P < 0.0001$; $P < 0.0001$ and $P = 0.039$, respectively). So the essential oil of *E. camalulensis* has a higher efficiency than that of *A. herba alba* on the species *S. aureus* ($P < 0.0001$) and *E. coli* ($P = 0.003$) (Fig. 1).

Discussion

The yield of essential oil extracted from the dry matter of the aerial part of *E. camaldulensis* is 1.45 ± 0.026 g, our results are similar to those of (Mehani, 2015) who found a yield 0.99%. A study by (Ashraf et al., 2010), on *Eucalyptus* leaves in Pakistan and Morocco revealed an essential oil content of between (0.90–0.98%). Some authors including (Chalchat et al., 2000) estimated a return of (0.5%), another study conducted by Bejaia (Makhlouf et al., 2016) found an equivalent yield (3.1%), a considerably high essential oil yield was reported for Taiwan *Eucalyptus* (2.3–3.0%) (Chalchat et al., 2000).

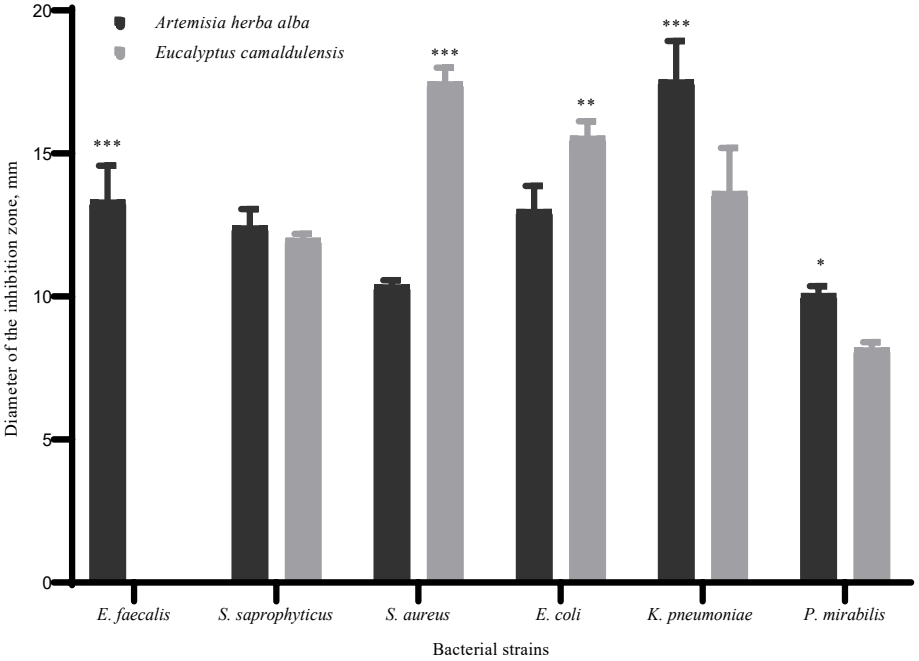

Fig. 1. Comparative efficiency of essential oils extracted from *A. herba alba* and *E. camaldulensis* against bacterial strains (x ± SD, n = 4 repetitions): * – difference compared the diameter of the inhibition zone is statistically reliable at $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$

The essential oil isolated by hydrodistillation of the aerial parts of *A. herba alba* had a yield of 1.21 ± 0.061 g. This yield is lower compared to 1.3% (v/w) from dried tops (Hudaib & Aburjai, 2006) and 1.02% on a dry weight basis for *A. herba alba* growing in the wild in M'sila-Algeria (Dob & Benabdelkader, 2006) and a yield of 0.71% reported in Tafingoult in Morocco (Asdadi et al., 2020). In general, these differences between the different results are due to different factors: plant species, seasons, geographical regions, products and reagents used in the extraction of essential oils, harvest period, the degree conditions time and the drying temperature and the presence of weeds (Ghasemian, 2019).

The essential oils of *E. camaldulensis* and *A. herba alba* have a broad spectrum of antibacterial activity against Gram + and Gram – bacteria. Similar results have been reported by Daroui-Mokaddem (2012) which shows that the essential oil has a very strong activity against *S. aureus* and *E. coli*; also Mehani (2015) showed that *P. aeruginosa* has a resistant power and *Proteus mirabilis* is sensitive to the activity of the essential oil. Indeed, the sensitivity of microorganisms depends on the chemical composition and the concentration of essential oils used and the type of microorganisms tested (Farah et al., 2001). The crude extracts of *E. camaldulensis* and *A. herba alba* acted to perceptibly inhibit the growth of selected pathogenic bacteria. An inhibitory activity of essential oils was found against gram-positive bacteria and gram-negative bacteria. Different investigations have examined the efficacy of essential oils against gram positive and negative bacteria, and shown that gram positive bacteria are more susceptible to oils (Smith-Palmer, 1998; Inouye, 2001). In our research, essential oil of *E. camaldulensis* didn't have any antibacterial activity against *E. feacalis* even at highest concentration. Presence of the polysaccharide capsule in this species of bacteria can act as a barrier to the transmission of active antibacterial compounds. It could also be due to the differences among chemical components of plants that have been cultivated in divergent ecological regions. Tebessa, a town in North East of Algeria, has a very dry and warm climate and the weather condition is very decisive in producing officinal substances (Seyyednejad et al., 2010).

The results also indicated that a higher volume of the essential oil (10 µL) was required to inhibit the growth of all Gram positive and Gram-negative bacteria tested. These results are similar to those found by (Farah et al., 2001; Trivedi & Hotchandani, 2004; Nair et al., 2008). It is worth noting, however, that *P. aeroginosa* isolate was resistant to all the volumes of essential oil used in this study. Since *Pseudomonas* species are known to have the ability to metabolise a wide range of organic compounds and because of this is used extensively in bioremediation, this may explain their high level of resistance. They may simply metabolise the compounds in the oils that are inhibitory to many of the other bacteria (Chao et al., 2000). This and other variations in the sensitivity of different isolates of even the same species are known to be due to different genetic profiles conferring varying resistance patterns. The genetic plasticity of bacteria

allows them to overcome the threat of antibiotics so that only the fittest will survive and adapt to newer environments (Munita, 2016). Although the antibacterial activities of the essential oils from many herb species have been extensively surveyed (Rios, 2005), their antimicrobial mechanisms have not been reported in great detail (Shunying et al., 2005). Any individual essential oil contains complex mixtures of such compounds, however, little is known about the effect of the interaction between the individual constituents on the antimicrobial activity. Interactions between the constituents may lead to additive, synergistic, or antagonistic effects (Delaquis et al., 2002).

Conclusion

The present study confirmed the antibacterial properties of essential oils from *E. camaldulensis* and *A. herba alba*, which showed significant growth inhibition for *S. aureus*, *S. saprophyticus*, *E. coli*, *K. pneumonia* and *P. mirabilis* tested, which present a threat due to the emergence of strains that possess multiple resistance to a range of antibiotics, thereby making them difficult to treat. The encouraging results indicate the *E. camaldulensis* and *A. herba alba* might be exploited as natural antibiotics for the treatment of several infectious diseases caused by these five germs, and could be useful in understanding the relations between traditional cures and current medicines.

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