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Evaluation of the MRSA Sensitivity to Essential Oils Obtained from four Algerian Medicinal Plants

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ABSTRACT

In this work, we evaluated the anti-MRSA activity of essential oils obtained from four Algerian medicinal plants which are: *Ammoides verticillata, Origanum vulgare* subsp. *glandulosum, Lavandula multifida,* and *Thymus munbyanus* subsp. *ciliatus*. The anti-MRSA activity was evaluated by the technique of agar diffusion and determination of MICs. We also studied the kinetics of destruction of these oils against MRSA strain ATCC 43300. The four essential oils showed a good anti-MRSA activity. Oils with the highest activity are; *Origanum vulgare* subsp. *glandulosum, Thymus munbyanus* subsp. *ciliatus, Lavandula multifida* and *Ammoides verticillata* respectively. The kinetics of destruction of all essential oils against MRSA ATCC 43300 strain is dependent on concentration. The Concentration 1% allows a total destruction; while the concentration 0.5% was bactericidal only with *Origanum vulgare*, whereas the concentration 0.25% and 0.125% have had a low destruction respectively. We concluded that the essential oils can be used as an anti-MRSA.

INTRODUCTION

The rapid emergence of antibiotic resistance over the past six decades has been accompanied by a decrease in production of new antimicrobial agents (Talbot et al., 2006). This slowdown is mainly due to the lack of discovery of new molecules of antibiotics (Fischbach and Walsh 2009), but also to the lack of research programs on these molecules, since the pharmaceutical industry has reduced its research in this field because of its low profitability (Nathan 2004, von Nussbaum et al., 2006).

MRSA is now ranked among the top pathogen causing fatal nosocomial infections in hospital and community (Chambers and DeLeo 2009) with several types of skin infections, mucous membranes and endocarditis. It is also responsible for severe lung infections such as fatal necrotizing pneumonia (Tang and Stratton 2010). Initially sensitive to penicillin, *Staphylococcus aureus* was able to adapt quickly to the action of antimicrobial agents by creating of many mechanisms of resistance against most antibiotics (Descloux et al., 2007). The acquisition of the mecA gene that codes for the protein (PBP2') (or PBP 2a) gives *Staphylococcus aureus* resistance to methicillin and most β-lactams (Deurenberg and Stobberingh 2008).

Another mechanism involved in multidrug resistance of methicillin resistance *Staphylococcus aureus* MRSA to many classes of antibiotics other than the β-lactam antibiotics, is the efflux pumps that allow for the pathogen to remove the molecules of antibiotics which penetrate inside the cell to the extracellular medium (Piddock 2006). Glycopeptides are the last lines of defense effective in treating MRSA infection, however strains with reduced susceptibility or resistance to these molecules have been described (Tenover 1998, Tenover 2001)**.**

In addition, there is another problem with these drugs which is their nephrotoxicity (Daurel andLeclercq 2010, Wong-Beringer et al., 2011)**.** That is why the discovery and development of new antimicrobial agents and new classes of antibiotics is a need for emergency (Fischbach and Walsh 2009, Mohtar et al., 2009)**.** In recent years, a growing interest focused on valorization of natural plant extracts which showed anti-bacterial properties, in particular against MRSA (Palaniappan and Holley 2010)**.**

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The natural plant extracts may contain interesting antibacterial compounds that can be considered as a new strategy to fight against nosocomial infections (Palaniappan and Holley 2010, Ramalhete et al., 2011). Many recent studies have evaluated the antimicrobial activity of essential oils on bacteria, including those involved in nosocomial infections (Billerbeck 2008)**,** Nevertheless, a few studies have been conducted on eradication of nosocomial pathogen such as MRSA.

In this work we evaluated the effect of four essential oils obtained from Algerian medicinal plants which are *Amoides verticillata*, *Origanum vulgare* subsp. *glandulosum*, *Lavandula multifida*, and *Thymus munbyanus* subsp. *ciliatus* on 19 clinical MRSA, two references MRSA and three references methicillin sensitive *Staphylococcus aureus* MSSA strains. We also studied the kinetics of destruction of these oils on reference MRSA strain ATCC 43300.

MATERIALS AND METHODS

Plant material

Aerial parts of the medicinal plant species (stems, leaves and flowers) used in this work were collected in full inflorescence. *Lavandula multifida* L. and *Thymus munbyanus* subsp. *ciliatus* (Desf.) Greuter & Burdet. were harvested from Bouhanak, located in West of Tlemcen (West of Algeria) at 34 ° 53 North and -1 ° 22 West, 700 m of altitude. While *Origanum vulgare* subsp. *glandulosum* (Desf.) Ietsw. and *Ammoides verticillata* (Desf.) Briq. were harvested from Mefrouche station which is located in the South of Tlemcen at an altitude of 1190 m, between latitudes 34 ° 49 North and longitude 1 ° 19 West. Harvests were made during June 2011. Specimens of all spices in this study were identified by laboratory of ecological management of natural ecosystems, university of Tlemcen. The plants materials were Left to dry for a week by spreading it in open air and away from sunlight.

Obtaining essential oils

Essential oils extraction was performed by hydrodistillation in a Clevenger-type apparatus for three hours. The obtained essential oils was dried with magnesium sulfate (MgSO4) and stored in smoked vials at +4 °C.

GC and GC / MS analysis

GC analyses were performed by a device type Perkins Elmer GC Autosystem equipped with a single injector and two flame ionization detectors (FID).

The device has two columns of fused silica capillary (60 mx 0.22 mm, film thickness 0.25 µm) with different stationary phases: Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethylene glycol). Temperature program: 60-230 ° C at 2 ° C / min and then held isothermal at 230 ° C (30 min). Carrier gas: helium (1 ml / min). Temperatures of injector and detector were held at 280 ° C. Injection was performed with a split ratio of 1:80. Injected volume: 0.1 µl. Analyses GC / MS were made using a detector Perkin Elmer TurboMass, directly coupled to a Perkin Elmer Autosystem XL columns equipped with fused silica capillary (60 m × 0.22 mm, film thickness 0 , 25 um), Rtx-1 (polydimethyl siloxane) and Rtx-Wax (polyethylene glycol).

Analysis GC / MS was performed with the following conditions: temperature of the ion source 150 ° C, ionization energy: 70 eV electron spectra ionization mass were acquired over the mass range of 35 to 350 Da. Scan time: 1 sec. The injection was performed with a division ratio of 1:80.

Bacterial strains

19 Clinical Strains of *Staphylococcus aureus* were isolated from postoperative wound, nasal swabs and central venous catheters incubations of patients hospitalized in several services (surgery, pediatrics, internal medicine, maternity and pulmonology) at the University Hospital of Tlemcen during a period of 14 months ranged from April 2010 to June 2011. These strains were identified by API Staph system (BioMérieux®, France). Reference MRSA strains ATCC 43866 and ATCC 43300, and MSSA strains ATCC 25923 and MSSA ATCC 29213 were also tested.

Selection of strains resistant to methicillin MRSA

Isolation of methicillin resistant *Staphylococcus aureus* strains was performed by two methods as recommended by the Clinical and Laboratory Standards Institute (CLSI 2011), strains of *Staphylococcus aureus* correctly identified have suffered an antibiogram using oxacillin disk and cefoxitin. All strains resistant to oxacillin or cefoxitin were considered MRSA strains (CLSI 2011). The second method is a screening in Muller-Hinton agar (CondaPronadisa™, Spain) supplemented with 6 µg / ml of oxacillin or 4 µg / ml of cefoxitin. The strains were inoculated by spot using a sterile swab, all strains grown on agar were considered MRSA strains (CLSI 2011).

Agar diffusion technique

The strains tested were grown for 18 h in Muller-Hinton broth (CondaPronadisa™, Spain), and then the inoculum was prepared at a concentration of 10^7 CFU / ml. inoculation was done by swabbing on Mueller-Hinton agar according to the recommendations of CLSI (CLSI 2011). A sterile disk of 6 mm in diameter impregnated with 2 µl of oil was placed in the center of the box sown.

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration was determined by the method of microplate (96 wells) round bottom (Wiegand et al., 2008). First, we performed successive dilution ½ in Müller-Hinton broth. We have prepared two solutions, the first is the concentrated solution; it contains 40% essential oil, 59% Müller-Hinton broth and 1% Tween 80. The second solution contains only the Müller-Hinton broth and Tween 80 at a concentration of 1%, this solution was used to make the dilutions so that concentration of Tween 80 will remain the same at decreasing. Inocula of

10^8UFC/ml were diluted 1/1000 to the concentration of 10^5UFC/ml. in Microplate, 180 µl of bacterial suspension at 10^5UFC/ml were deposited inside the wells. Then 20 µl of the solution of the oil was added. The final concentration of Tween 80 is 0.1% (v/v) in each well and the final concentrations of the essential oil is ranged from 4% to 0.0078%. Susceptibility testing of essential oils (agar diffusion method and e MICs were repeated three times).

Destruction kinetics of MRSA strain 43300 with essential oils

The kinetics of destruction of MRSA exposed to essential oils tested in this work was evaluated according to the experimental protocol Christoph, et al.,., 2001 (Christoph et al., 2001); The inoculum preparation was done by cultivating the strain tested in 10 ml of trypticase soy TSB (Fluka BioChemika®, France) during 18 h of incubation. Then the culture was centrifuged for 10 min at 250 rpm at 25 ° C. After that, the pellet is recovered and suspended in 0.9 ml of sterile saline for a concentrated suspension of bacteria $3x10^9$ CFU / ml. MRSA ATCC 43300 strain (100 µl of the final inoculum) were inoculated into 50 ml containing the concentrations 1, 0.5, 0.25, and 0.125 % oil and Tween 80 (0.5%) in sterile distilled water and stirred continuously. After incubation for 5, 10, 15, 30, 60,120, and 240 min at 37C °, aliquots of 1 ml were sampled and transferred to a broth inactivation (9 ml of nutrient broth) and vortexed. After 2 min, serial dilutions were prepared in saline, and 100 µl of each dilution and broth inactivation were spread on Mannitol salt agar medium (Merck®, France) for enumerating colonies. For this work we used the reference strain ATCC 43300 MRSA.

RESULTS AND DISCUSSION

GC and GC / MS analysis

The chemical composition of the essential oils is presented in Table 1. Based on this composition, we find that the essential oil of *A. verticillata* is rich in thymol (51.1%), p. cymene (16.4%) and limonene (14.1%). As well as the essential oil of *O. v. subsp. glandulosum* is rich also in thymol (43.3%), p-cymene (15.3%), and γ terpinene (27.0%). In addition, the oil of *A. verticillata* contains more oxygenated monoterpenes than oregano oil while the reverse is true for hydrocarbon monoterpenes. Analysis of *T. m. subsp. Ciliatus* showed a chemotype carvacrol as major compound with a percentage of 80.1% and an abundance of Monoterpenes hydrocarbons, while the chemical composition of *L. multifida* showed that it is rich in carvacrol with a percentage of 57, 1%, but also with the β-bisabolene, with 25.2%.

The results of the antimicrobial activity of essential oils of the four medicinal plants on methicillin resistant *S. aureus* MRSA strains have shown that the four oils showed strong activity against MRSA strains, the level of this activity was variable. The essential oils showed the strongest activities are: *T. m. subsp. Ciliatus, O. v. subsp. glandulosum L. multifida and A. verticillata*, respectively. Results of inhibition diameters and minimum inhibitory concentrations are presented in Tables 2 and 3. The results of the anti-MRSA activity of the four essential oils showed that MRSA strains tested were either sensitive or highly sensitive towards the four essential oils such as the lowest inhibition diameters were 14 mm (Ponce et al., 2003). *O. v. subsp. glandulosum*, showed the highest activity with diameters that reach 55 mm and MIC ≤ 0.06%, which explains the use of this herb by the ancient population and confirms the broad spectrum action of its essential oil (Bendahou et al., 2008). Comparing this with the anti-MRSA effect of other species of the same genus, Chao et al.,, 2008 showed that the species *Origanum majorana* has a remarkable effect on MRSA with diameters of 28 mm, as these authors tested the essential oil of *Thymus vulgaris* species, the latter showed a very strong effect with a diameter of 57 mm (Chao et al., 2008). Indeed Nostro et al.,, 2004 showed the effect of purified carvacrol and thymol essential oil of *Origanum vulgare* on the strain of MRSA ATCC 43300, the results showed a strong inhibition of these compounds against this MRSA strain (Nostro et al., 2004). We obtained the same results with Algerian *O. v. subsp. glandulosum* which has as the major compound the Thymol, while the species *A. verticillata* showed an average anti-MRSA activity although the composition of its essential oil is rich in thymol. In addition, the species *T. m. subsp. Ciliatus* showed a significant anti-MRSA effect, the antibacterial activity of this essential oil is mainly due to its high content of carvacrol (Bousmaha-Marroki et al., 2007).

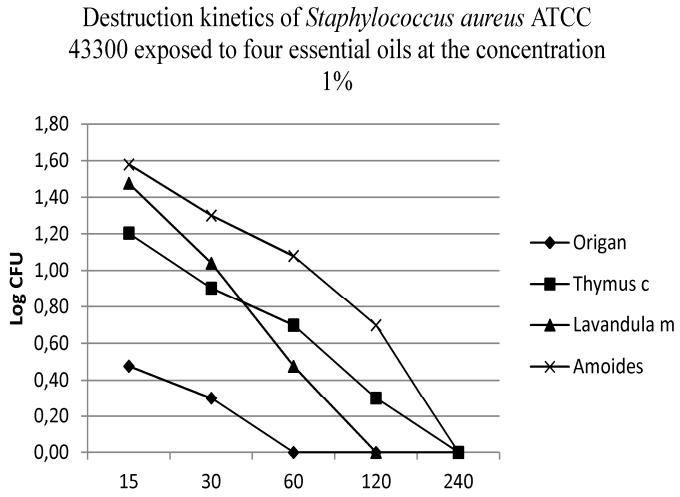
On the active species of lavender we tested, *L. multifida* which presented a remarkable activity. The anti-MRSA effect of this species can be explained by the fact that *L. multifida* is the only species that produces large amounts of carvacrol (Harborne and Williams 2002). Indeed, carvacrol act on the membrane of the bacterium by a reduction in ATP synthesis and membrane electrical potential, and therefore the result is an increase in the permeability of the membrane, which results in the destruction of the bacterial cell (Husnu Can Baser 2008). Moreover, studies have shown that another major component that this plant has; called "β-bisabolene" has an interesting antimicrobial activity on microorganisms and MRSA strains (Nascimento et al., 2007, Simic et al., 2005).

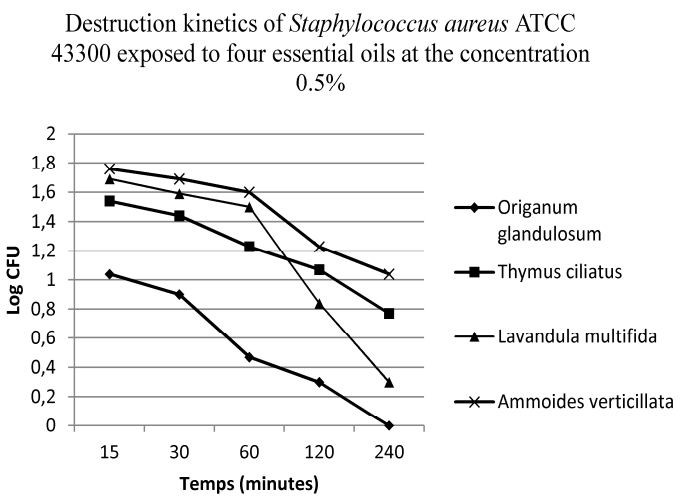
The results for the diameters of inhibition showed that the larger diameters of inhibition were observed with *O. v. subsp. glandulosum* with an average of 29.45 mm, and *T. m. subsp. Ciliatus* of 25.8 mm then less large diameter with *A. verticillata* of 24.33 mm and *L.multifida* with an average of 19.75 mm. Furthermore, we observed some differences in the effect between inhibition zones and MICs. The minimum inhibitory concentrations gave consistent results with the inhibition diameters as smaller MICs were recorded with *O. v. subsp. glandulosum*, however the species *L. multifida* gave more effect at CMI compared with diameters of inhibition, these results can be explained by the fact that certain essential oils have viscosities that affect their broadcasts in the plates and therefore they give MIC greater than the diameters of inhibition, including interest, to compute the CMI assess the effects of these substances (Hood et al., 2003).

Table 1: Chemical composition of plants studied

Plants			*T. m. subsp. Ciliatus*	*O. v. subsp. glandulosum*	*A. verticillata*	*L. multifida*
Yield % (w/w)			6	4	4.5	0.6
Constituents	RIa	RIp				
α-Thujene	928	1023	0.4	1.3	0.2	-
α-Pinene	931	1022	0.5	0.6	0.1	0.6
Camphene	943	1066	0.1	0.1	-	-
1-Octen-3-ol	959	1446	tr	0.1	-	0.2
3-Octanone	963	1253	0.1	0.1	-	-
Sabinen	966	1120	-	-	0.1	-
β-Pinene	970	1110	0.1	0.1	0.1	0.2
Myrcene	979	1159	1.2	2.0	0.7	0.7
α-Phellandrene	997	1164	0.1	0.3	-	-
Δ-3-Carene	1005	1147	0.1	0.1	tr	tr
α-Terpinene	1008	1178	1.3	2.7	0.1	-
p-Cymene	1011	1268	7.1	15.3	16.4	2.2
Limonene	1020	1199	0.3	0.5	14.1	-
Eucalyptol	1020	1209	-	-	-	0.9
α-phellandrene	1021	1212	-	0.2	-	-
(Z)-β-Ocimene	1024	1230	0.1	tr	-	tr
(E)-β-Ocimene	1034	1247	0.1	tr	-	tr
ϒ-Terpinene	1047	1243	4.2	27.0	7.5	-
Cis-hydrate sabinene	1050	1451	-	-	tr	-
trans-Sabinene hydrate	1051	1451	0.2	0.3	-	-
Fenchone	1071	1401	-	-	-	tr
p-cymenene	1073	1469	-	-	-	-
Terpinolene	1078	1280	0.1	0.1	0.1	0.1
Linalool	1081	1544	0.7	0.6	0.2	tr
Borneol	1148	1698	0.1	0.1	-	-
Terpinen-4-ol	1161	1600	0.4	0.2	0.3	-
α-Terpineol	1172	1697	0.2	0.2	0.2	-
Cis-carveol	1208	1863	-	-	tr	-
Thymoquinone	1216	-	-	-	-	-
Carvacrol methyl ether	1231	1603	-	-	-	0.3
Carvone	1235	1739	-	-	-	tr
Thymol	1266	2189	0.2	43.3	51.1	-
Carvacrol	1278	2219	80.1	2.0	7.5	57.1
α-Terpenylacetate	1334	1695	-	-	-	0.1
carvacryl acetate	1345	1876	0.1	-	-	-
Methyleugenol	1367	2009	-	-	-	0.2
β-Caryophyllene	1424	1591	1.2	1.2	-	2.1
β-Farnesene	1448	1661	-	-	-	0.1
α-Humulene	1456	1665	0.1	0.1	-	-
β-Bisabolene	1500	1720	0.1	0.1	-	25.2
β-Sesquiphellandrene	1516	1765	0.1	0.4	-	-
(E)-α-bisabolene	1534	1772	-	tr	-	-
Spathulenol	1560	2119	-	-	-	3.4
Caryophyllene oxide	1576	1980	0.2	0.2	-	3.7
Total (%)			99,5	99,6	98,7	97,1

Percentages, elution order are given on apolar column; Retention index RIa and RIb are given respectively on apolar (Rtx-1) and polar column (Rtx-Waw); tr: trace (<0,05); All compounds are identified by RI, GC/MS from laboratory libraries.





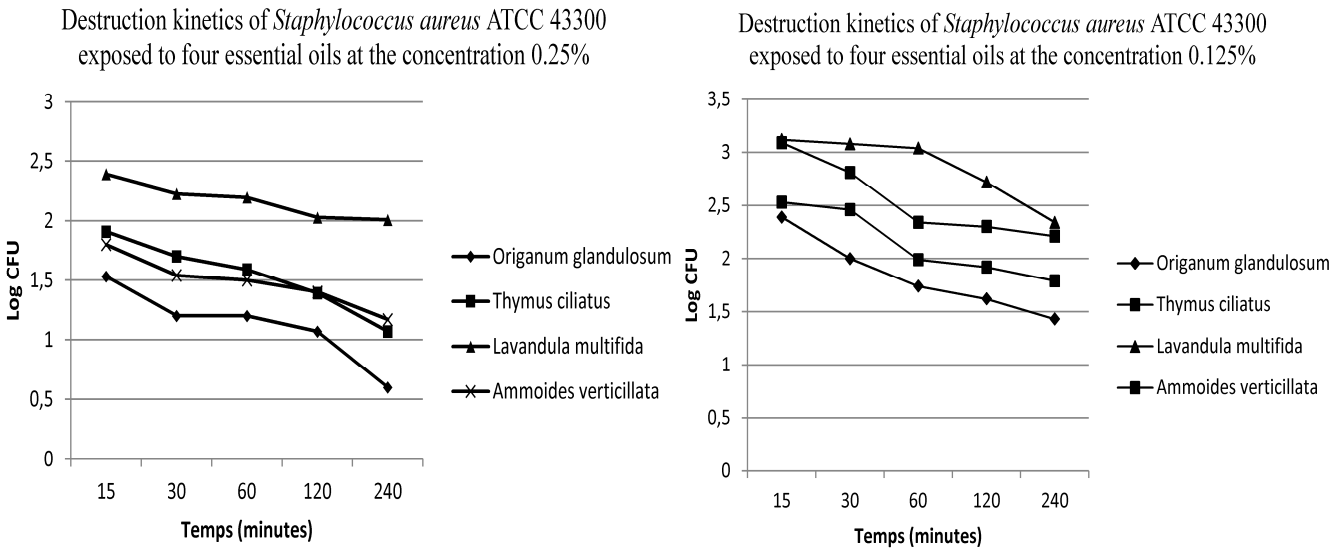


Fig . 1: destruction kinetics of MRSA strain ATCC 43300 exposed to four essential oils (O. v. subsp. Glandulosum, T. m. subsp. Cilliatus, L. multifida and A.verticillata) in time 15; 30; 60; 120; and a four concentration 1%, 0.5%, 0.25% and 0.15%.

Table. 2: Inhibition diameter of essential oils of four medicinal plants collected in western Algeria.

Strains	diameter **(mm)**			
	T. m. subsp. Ciliatus	*O. v. subsp. glandulosum*	*A. verticillata*	*L. multifida*
MRSA1	30	31	25	27
MRSA2	32	34	15	14
MRSA3	34.66	28	28	15
MRSA4	28	28	28	17
MRSA5	16.5	26	24	14
MRSA6	16.5	24	18	23
MRSA7	17.5	23	19	22
MRSA8	16.5	24	15	22
MRSA9	20	25	20	18
MRSA10	20	25	21	20
MRSA11	15	29	22	24
MRSA12	19	28	30	19
MRSA13	36.66	32	27	21
MRSA14	29	25	34	18
MRSA15	36	26	27	21
MRSA16	19	26	25	18
MRSA17	18.5	30	25	21
MRSA18	34	25	16	20
MRSA19	21	25	22	22
MRSA 43300	27	32	30	18
MSSA 25923	40	36	30	19
MSSA 33862	35.5	34	25	22
MSSA 29213	30	36	28	19
MRSA 43866	27	55	30	20

Table. 3: Minimum inhibitory concentration of four medicinal plants collected in western Algeria.

Strains	Minimum inhibitory concentration MIC% (v/v)			
	T. m. subsp. Ciliatus	*O. v. subsp. glandulosum*	*A. verticillata*	*L. multifida*
MRSA1	0.25	0.125	0.25	0.125
MRSA2	0.25	0.125	0.125	0.5
MRSA3	0.125	0.25	0.25	0.25
MRSA4	0.125	0.25	0.25	0.125
MRSA5	0.125	0.25	0.5	0.5
MRSA6	0.5	0.25	0.25	0.125
MRSA7	0.5	0.25	0.125	0.125
MRSA8	0.125	0.25	0.25	0.125
MRSA9	0.125	0.25	0.125	0.0625
MRSA10	0.25	0.125	0.5	0.25
MRSA11	0.5	0.25	0.5	0.5
MRSA12	0.125	0.125	0.25	0.25
MRSA13	0.25	0.25	0.25	0.125
MRSA14	0.125	0.25	0.5	0.5
MRSA15	0.06	0.06	0.5	0.25
MRSA16	0.25	0.25	0.25	0.125

MRSA17	0.5	0.25	0.25	0.25
MRSA18	0.25	0.06	0.125	0.5
MRSA19	0.5	0.06	0.5	0.5
MRSA 43300	0.25	0.5	0.125	0.25
MSSA 25923	0.25	0.25	0.25	0.125
MSSA 33862	0.25	0.25	0.25	0.0625
MSSA 29213	0.25	0.25	0.25	0.125
MRSA 43866	0.5	0.25	0.25	0.25
Average	0.26	0.21	0.28	0.25

The effect of essential oils on different MRSA strains was more or less similar; in general, essential oils with high activities have maintained this activity for the majority of MRSA strains tested. There is not a clear difference between the effect of essential oils on clinical MRSA strains and MSSA references strains, however, the inhibition diameters were the lowest recorded for MRSA strains as it is for MSSA strains case for MRSA11 (15 mm oil *T. m. subsp. Ciliatus*) and MRSA2 (14 mm oil *L. multifida*).

Destruction kinetics of MRSA strain 43300 with essential oils

The study of the kinetics of destruction of the MRSA strain 43300 with the four essential oils showed that after 240 min at the concentration 1%, all four death kinetic curves reached the x-axis with a reduction of 6.8 $\log_{10}$ which means that the four oils exerted a bactericidal effect whereas at the concentration 0.5%, *O. v. subsp. glandulosum* oil exercise a high bactericidal effect with complete destruction of bacterial cells, while *L. multifida* destruction was observed from log 6.8 $\log_{10}$ to 0.25 $\log_{10}$. However *T. m. subsp. Ciliatus* and *A. verticillata* exerted lower reductions of bacterial cells which were from 6.8 $\log_{10}$ to 0.8 $\log_{10}$ and 1.05 $\log_{10}$ respectively. Nevertheless, the concentration 0.25% showed almost complete destruction of *O. v. subsp. glandulosum* and average destruction of *L. multifida*, *A. verticillata* and *T. m. subsp. Ciliatus*. However, the curves of the concentration 0.125% showed a very low activity of the four oils with low decreases of bacterial cells which ranged from 6.8 $\log_{10}$ to 2.34 $\log_{10}$ and 1.43 $\log_{10}$ within 240 min (see fig. 1), it follows that the bactericidal activity of all essential oils tested in this study is dependent on concentration. Indeed, these oils because they have dependent on concentrations, they are bactericidal at concentrations required and destroying cells at the stationary phase, but when concentrations are low they exert more bacteriostatic activity (Christoph et al., 2001).

In fact, the most studies of the antimicrobial activity of essential oils concern inhibition of microbial growth rather than lethal effects (Lattaoui andTantaoui-Elaraki 1994). However there is some studies on time killing kinetics on MRSA strains; Dadalioglu and Evrendilek, 2004, presented the kinetics of destruction of the MRSA strain ATCC 43300 and found a very strong effect of oregano oil followed by the effect of lavender from Turkey (Dadalioglu and Evrendilek 2004). These results agree well with ours as we recorded a faster kinetics of *O. v. subsp. glandulosum* and stronger than that exerted by *L. multifida*. In another study, May et al.,., have studied the kinetics of destruction of MRSA and MSSA strains exerted by tea tree oils, they have found that tea tree oil was more active against methicillin sensitive *Staphylococcus aureus* than methicillin resistant *Staphylococcus aureus* (May et al., 2000).

CONCLUSION

The evaluation of the anti-MRSA activity of four essential oils obtained from Algerian medicinal plants tested in this work showed that oils had a remarkable inhibitory effect on *Staphylococcus auerus*, especially, against MSSA than strains MRSA. In addition, the kinetics of destruction showed that all essential oils tested in this study is dependent on concentration to exercise its bactericides. We also concluded that the essential oils can be used as an anti-MRSA, since our results showed their effectiveness. Further studies are needed to develop formulations for pharmaceutical use based on these essential oils to fight this nosocomial pathogen.

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