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ANTIMICROBIAL ACTIVITY OF SOME ESSENTIAL OILS ALONE AND IN COMBINATION WITH AMIKACIN AGAINST *ACINETOBACTER* SP

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ABSTRACT

Acinetobacter sp. as gram negative bacilli is one of the most problematic bacteria in hospital environments. The emergence of multi-drug resistant isolates of *Acinetobacter* sp. encourages the scientists to find the new antimicrobial agent with less side effects. The aim of this study was to evaluate the antibacterial activity of *Cymbopogon olivieri, Heracleum persicum, Juniperus comminus, Azillia eryngioides, Dacus carrota, Ferula gummosa, Acorus calamus, Mentha pulegium, Achillea biebersteinii,* and *Chaerophyllum macropodum* essential oils against clinical trials of *Acinetobacter* sp. by disc diffusion and micro broth dilution assays. The synergistic effect of these essential oils and amikacin (AMI) were determined. The higher inhibition zone diameters were for 2 µl of *C. macropodum* (15.3±0.48 mm). The lower MIC and MBC values were for *C. olivieri* (1.4 and 1.9 µl/ml) and *J. comminus* (1.9 and 2.6 µl/ml), followed by *C. macropodum* (2.01 and 3.2 µl/ml), *D. carrota* (2.1 and 3.8 µl/ml), *A. eryngioides* (2.3 and 3.1 µl/ml) essential oils and *F. gummosa* (2.4 and 4 µl/ml). AMI showed synergistic effect with all of the essential oils. *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*, *J. comminus* and *F. gummosa* essential oils.

Keywords: *Acinetobacter* sp., essential oil, synergistic effect, amikacin

INTRODUCTION

Acinetobacter sp. isolates are problematic pathogens in intensive-care units and other hospital units in recent years. They are the causes of health care associated pneumonia, surgical site infections, bloodstream infections, urinary tract infections **(Tolbat *et al.*, 2006).** *Acinetobacter* sp. isolates with multi drug resistance (MDR) are markedly increasing and treatment of *Acinetobacter* sp. infections have been limited to few broad spectrum antibiotics, including carbapenems, amikacin, doxycycline, minocycline, and ampicillin/sulbactam **(Van Looveren and Guossens, 2004)**. As resistance to antibiotics has emerged, the mortality rates in *Acinetobacter* sp. infected patients have increased. Therefore, the popularity of natural essential oils as alternative treatment has increased **(Sienkiewicz *et al.*, 2011; Mikaili *et al.*, 2011; Candan *et al.*, 2003; Damjanovic- Vratnica *et al.*, 2011)**.
In this research, we isolated 35 clinical isolates of *Acinetobacter* sp. and determined the sensitivity of these isolates to different antibiotics; then we evaluate the anti *Acinetobacter* sp. activity of ten essential oils alone against clinical isolates of *Acinetobacter* sp. The combination of ten different essential oils with amikacin (AMI) was evaluated against one AMI resistant isolates by measuring the FIC and FIC indexes.

MATERIAL AND METHODS

Essential oils and their analysis

10 different essential oils including *Cymbopogon olivieri, Heracleum persicum, Juniperus comminus, Azillia eryngioides, Dacus carrota, Ferula gummosa, Acorus calamus, Mentha pulegium, Achillea biebersteinii* and *Chaerophyllum macropodum* were prepared from Barij Essence Pharmaceutical Company. The essential oils were analyzed using GC-FID and GC-MS. The GC-FID and GC-MS apparatus were conducted on an HP 6890 GC system coupled with 5973 network mass selective detectors with a capillary column of HP-5MS (30 m × 0.25 mm, film thickness 0.25 µm). The oven temperature program was initiated at 60 °C, held for 1 min, then raised up to 245 °C at a rate of 3 °C/min held for 10 min. Helium was used as the carrier gas at a flow rate 1.5 ml/min. The detector and injector temperatures were 250 and 230 °C, respectively. The compounds of the essential oil were identified by comparison of their retention indices (RI), mass spectral fragmentation with those in the stored Wiley 7n.1 mass computer library **(Adams, 2001)**.

Antibiotics

The antibiotic discs that were used in this study including ciprofloxacine (CIPR 5 µg), cefepime (FEP 30 µg), ceftazidime (CAZ 30 µg), levofloxacin (LEVOF 5 µg), amikacin (AMI 30 µg), amoxicillin (AMOXY 30 µg), Imipenem (IMI 10 µg), tobramycin (TOB 10 µg), cefotaxim (CTX 30 µg), norfloxacin (NOR 10 µg), ampicillin+sulbactam (SAM 20 µg (10+10)), meropenem (MRP 10 µg), gentamicin (GEN 10 µg), piperacillin+tazobactum (PI 100+ IZ 10 µg), amoxicillin+clavulonate (AMC 30 µg; (20+10)) were purchased from Rosco (Diagnostica A/S, Taastrupgaardsvej 30 DK-2630 Taastrup).

Acinetobacter isolates and antimicrobial susceptibility testing

A total of 35 clinical isolates cultured from different samples of wounds, trachea, blood, CSF, catheter and other samples of patients at hospitals from Tehran were the subject of this investigation. Antimicrobial susceptibility testing was evaluated using disc diffusion **(NCCLS, 2012)** and micro broth **(CLSI, 2009)** dilution assays. This inoculate of microorganism was adjusted to 0.5 McFarland (1×10^7-1×10^8 CFU/ml) and using a sterile cotton swab, the microbial suspensions were cultured on appropriate media. Subsequently, sterile blank discs (6 mm in diameter) were saturated with 0.5, 1 and 2 µl of essential oil and were put on the cultured media. The plates were incubated at 37 °C for 24 h. The inhibition zones (IZ) diameters were measured in millimeters (mm) and average of IZ was recorded as means ± SD (Standard Deviation).
The minimal inhibitory concentration (MIC) and minimal Bactericidal Concentration (MBC) values of essential oils were determined by micro broth dilution assay. The essential oil was twofold serially diluted (8 - 0.0125 µl/ml of essential oil). Cation adjusted Muller Hinton broth was used as broth media. After shaking, 100 µl of essential oil was added to each well. The above microbial suspensions were diluted to 1×10^6 and then 100 µl were added to each well and incubated at 35±2 °C. MIC was defined as the lowest concentration of essential oil that inhibits bacteria after 24 h. MBC value was the first well that

showed no growth on suitable media. All experiments were done in triplicates.Statistical data analysis was performed by SPSS software (version 17, Chicago, Illinois, USA). Statistical analysis (ANOVA) was applied to determine the differences (P<0.05). Significant differences between the essential oils and microorganisms were determined by Tukey test.

Checkerboard titer test

AMI were purchased from Sigma-Aldrich Co. LLC. and dissolved in water. The dilutions were prepared in water in concentration 64-0.0125 µg/ml and the antimicrobial susceptibility testing was performed as CLSI procedure (CLSI, 2009).

Eight serial twofold dilutions of essential oils and AMI were used. Fifty µl of each dilution of essential oil was added to the wells of 96-well plates in vertical orientation and 10 µl of AMI dilution was added in horizontal orientation. 50 µl of AMI resistant *Acinetobacter sp* (10^6 CFU/ well) was added to each well and incubated for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of essential oil and AMI divided by the MIC of essential oil or AMI alone. The FIC index (FICI) was interpreted as a synergistic effect when it was ≤0.5, as additive or indifferent when it was >0.5-2 and as antagonistic when it was>2.0 **(Rosato *et al*., 2007)**.

Table 1 Antibiotic resistant profile of clinical isolates of *Acinetobacter* sp.

Antibiotics	Resistance (%)
CIPR	(12/35) **65.7**
FEP	(14/35) **60**
CAZ	(9/35) **74.2**
LEVOF	(18/35) **48.6**
AMI	(20/35) **42.9**
AMOXY	(1/35) **97.1**
IMI	(12/35) **65.7**
TOB	(26/35) **25.7**
CTX	(3/35) **91.4**
NORFX	(9/35) **74.3**
SAM	(14/35) **60**
MRP	(12/35) **74.3**
GEN	(10/35) **71.4**
PI+IZ	(13/35) **62.8**
AMC	(9/35) **74.2**

CIPR= ciprofloxacine; FEP= cefepime; CAZ= ceftazidime; LEVOF= levofloxacin; AMI =amikacin; AMOXY= amoxicillin; IMI= Imipenem; TOB= tobramycin; CTX= cefotaxim; NORFX= norfloxacin; SAM= ampicillin+sulbactam; MRP= meropenem; GEN= gentamicin; PI+IZ= piperacillin+Tazobactum; AMC= amoxicillin+clavulonate

RESULTS AND DISCUSSION

Resistance of *Acinetobacter* sp to antibiotics

As the tab 1 is shown, the resistant profile of 35 clinical isolates were included: CIPR (65.7%), CTX (60%), CAZ (74.2%), LEVOF (48.6%), AMI (42.9%), AMOXY (97.1%), IMI (65.7%), TOB (25.7%), CTX (91.4%), NORFX (74.3%), SAM (60%), MRP (74.3%), GEN (71.4%), PI+IZ (62.8%), AMC (74.2%). The higher sensitivity was for TOB and AMI (tab 1).

Chemical composition and antibacterial screening

The antibacterial evaluation of essential oils against clinical isolates of *Acinetobacter* sp. by disc diffusion method showed that the activity was increased dose dependently. Increasing in the amount of essential oils increased the inhibition zone diameter of essential oils (tab 3). The higher inhibition zone diameters were for 2 µl of *C. macropodum* (15.3±0.48 mm), and *H. persicum* (13.3±0.48 mm).

The main components of *H. persicum* was n-octyl acetate (72.3%), 2-methyl-octyl ester butanoic acid (5.5%), 1-octanol (4.2%) while the chemical composition of *C. macropodum* showed the presence of *trans*-ocimene (49.2%), cis-ocimene (23.6%), γ-terpinene (7.7%), β-myrcene (4.4%), p-cymene (5.5%), and fenchyl acetate (2.7%) as the main components (tab 2).

Table 2 Chemical attributes of essential oils

Essential oil	Main components
Cymbopogon olivieri	Piperitone (72.8%), 4-carene (11.8%), β-himachalene (7.6%)
Heracleum persicum	n-octyl acetate (72.3%), 2-methyl-octyl ester butanoic acid (5.5%), 1-octanol (4.2%)
Juniperus comminus	Camphene (37.7%), β-pinene (15.7%), γ-terpinene (12%), murola-4(14),5-diene (trans) (11.8%), α-terpinene (1.89%)
Azillia eryngioides	α-pinene (63.8%), bornyl acetate (18.9%), β-pinene (2.6%), linalool (2.1%), z-citral (1.3%)
Dacus carrota	Carotol (46.1%), 3-octen-5-yne,2,7-dimethyl-(z) (15.7%), α-pinene (10.7%), trans caryophyllene (4.6%), trans-β-farnesene (4.5%), α-bergamotene (2.53%)
Ferula gummosa	β-pinene (62.7%), α-pinene (9.5%), δ-carene (7.5%)
Acorus calamus	Cis-asarone (27.5%), acorenone (17.4%), elemene (8.9%), α-salinene (7.2%), camphor (3.1%), camphene (2.6%)
Mentha pulegium	Piperitone (38.1%), piperitenone (33.1%), α-terpineol (4.8%), 1,8-cineole (4.1%), piperitenone oxide (3.4%), menthone (3.0%)
Achillea biebersteinii	Germacrene-D (46.6%), camphor (6.2%), 1,8-cineole (5.2%), bicyclogemacrene (4.8%), spathulenol (3.8%)
Chaerophyllum macropodum	*trans*-ocimene (49.2%), cis-ocimene (23.6 %), γ-terpinene (7.7 %), β-myrcene (4.4 %), p-cymene (5.8 %), and fenchyl acetate (2.7 %)

As we mentioned before, the higher sensitivity of antibiotics against clinical isolates of *Acinetobacter* sp. was for tobramycin, AMI and levofloxacin. The inhibition zone diameter of these antibiotics was 14.1, 10.5 and 12.5 mm respectively and was lower than *C. macropodum* essential oil. The MIC and MBC evaluation of these essential oils showed the different results with disc diffusion method.

The lower MIC and MBC values were for *C. olivieri* essential oil (1.4 and 1.9 µl/ml) and *J. comminus* (1.9 and 2.6 µl/ml) followed by *C. macropodum* (2.01 and 3.2 µl/ml), *D. carrota* (2.1 and 3.8 µl/ml), *A. eryngioides* (2.3 and 3.1 µl/ml) and *F. gummosa* (2.4 and 4 µl/ml) essential oils. Piperitone (72.8%), 4-carene (11.8%), β-himachalene (7.6%) were found in *C. olivieri* essential oil. Camphene (37.7%), β-pinene (15.7%), γ-terpinene (12%), murola-4(14), 5-diene (trans) (11.8%), α-terpinene (1.89%) were the main components of *J. comminus* essential oil. The MIC values for *H. persicum* (3.5 µl/ml), *A. biebersteinii* (3.6 µl/ml), *A. calamus* (3.9 µl/ml) essential oils were almost the same but the MBC values were 4.8, 5.7 and 6.5 µl/ml, respectively. Therefore, there is no correlation between the inhibition zone diameter and MIC values (P>0.05).

The inhibition zone diameters of these antibiotics were 14.1, 10.5 and 12.5 mm, respectively and was lower than *C. macropodum* essential oil. The MIC and MBC evaluation of these essential oils showed the different results with disc diffusion method. The lower MIC and MBC values were for *C. olivieri* essential oil (1.4 and 1.9 µl/ml) and *J. comminus* (1.9 and 2.6 µl/ml) followed by *C. macropodum* (2.01 and 3.2 µl/ml), *D. carrota* (2.1 and 3.8 µl/ml), *A. eryngioides* (2.3 and 3.1 µl/ml) and *F. gummosa* (2.4 and 4 µl/ml) essential oils.

Piperitone (72.8%), 4-carene (11.8%), β-himachalene (7.6%) were found in *C. olivieri* essential oil. Camphene (37.7%), β-pinene (15.7%), γ-terpinene (12%), murola-4(14),5-diene (trans) (11.8%), α-terpinene (1.89%) were the main components of *J. comminus* essential oil. The MIC values for *H. persicum* (3.5 µl/ml), *A. biebersteinii* (3.6 µl/ml), *A. calamus* (3.9 µl/ml) essential oils were almost the same but the MBC values were 4.8, 5.7 and 6.5 µl/ml, respectively. Therefore, there is no correlation between the inhibition zone diameter and MIC values (P>0.05).

Table 3 The antimicrobial activity of essential oils against clinical isolates of *Acinetobacter* sp.

Essential oil	Inhibition Zone (Means±SE mm)				Microbial Concentrate (µl/ml)	
	0.5 µl	1 µl	2 µl	µg	MIC	MBC
C. olivieri	6.2±0.1	8.7±0.23	11.9±0.21	-	1.4±0.07	1.9±0.11
H. persicum	6.2±0.07	8.2±0.22	13.3±0.48	-	3.5±0.07	4.8±0.15
J. comminus	6.3±0.09	8.2±0.19	11.5±0.17	-	1.9±0.1	2.6±0.14
A. eryngioides	6.1±0.03	7.3±0.16	10.2±0.24	-	2.3±0.1	3.1±0.14
D. carrota	6.1±0.03	6.8±0.15	10.6±0.24	-	2.1±0.1	3.8±0.19
F. gummosa	6.1±0.03	6.5±0.74	10.7±0.19	-	2.4±0.09	4±0.25
A. calamus	6.1±0.04	7.7±0.17	11.2±0.19	-	3.9±0.11	6.5±0.25
M. pulegium	6.7±0.12	9.1±0.34	13.6±0.25	-	2.3±0.09	4.1±0.24
A. biebersteinii	6.1±0.04	7.7±0.16	11.4±0.19	-	3.6±0.11	5.7±0.25
C. macropodum	8.4±0.3	10.8±0.36	15.3±0.48	-	2.01±0.15	3.2±0.35
TOB	-	-	-	14.1±1.1		
AMI	-	-	-	10.5±1.2		
LEVOF	-	-	-	12.5±1.2		

LEVOF= levofloxacin; TOB= tobramycin; AMI = amikacin; MIC= Minimal Inhibitory Concentration; MBC= Minimal Bactericidal concentration

Todays, interest in essential oils or extracts as alternative treatment due to their loss or no adverse effects and multifunctional properties such as anti-inflammatory, analgesic, immune enhancing and antimicrobial activities are increasing. There are many investigations that evaluate the antibacterial activities of plant derivatives against *Acinetobacter* sp. as a main human pathological agent. The antibacterial activity of *Foeniculum vulgare* Miller essential oil **(Jazani *et al.*, 2009)**, garlic chloroform extract and allicin **(Jazani *et al.*, 2007)**, green tea aqueous extract **(Hosseini Jazani *et al.*, 2007)**, thyme essential oil **(Lysakowska *et al.*, 2011)**, *Cassia fistula* extract **(Aneja *et al.*, 2011)**, *Satureja hortensis* essential oil **(Mihajilov-Krstev *et al.*, 2009)** were confirmed.

Table 4 Fractional Inhibitory Concentration (FIC) and FIC indices (FICI)

	FIC	FICI
Cymbopogon olivieri AMI	0.5 0.003	0.503
Heracleum persicum AMI	0.5 0.006	0.506
Juniperus comminus AMI	0.25 0.003	0.253
Azillia eryngioides AMI	0.015 0.05	0.065
Dacus carrota AMI	0.0004 0.05	0.0504
Ferula gummosa AMI	0.25 0.2	0.45
Acorus calamus AMI	0.5 0.003	0.503
Mentha pulegium AMI	0.003 0.5	0.503
Achillea biebersteinii AMI	0.004 0.2	0.204
Chaerophyllum macropodum AMI	0.0004 0.2	0.2004

AMI= amikacin; **FIC of essential oil**=MIC in combination with AMI; **FIC of AMI** =MIC in combination with essential oil, MIC of essential oil alone, MIC of AMI alone, **FICI**= FIC of essential oil+ FIC of AMI

This study evaluates the antibacterial activity of new essential oils against clinical isolates *Acinetobacter* sp. Other studies showed the chemical composition of essential oils play an essential role in their antimicrobial activity. It is shown, different chemotypes of basil essential oil including estragol, linalool-estragol, methyl eugenol-anethol, anethol chemotypes had different antibacterial activity against *Acinetobacter* sp. and exhibited more sensitivity to methyl eugenol chemotype than lonalool or estragol chemotypes **(Koba *et al.*, 2009)**.
Therefore, the different antibacterial activity of essential oils is related to the composition of essential oils. Among the 11 different essential oils, *C. olivieri*, *J. comminus* and *C. macropodum* showed the best antibacterial activity against clinical isolates of *Acinetobacter* sp. Piperitone as the first main component of *C. olivieri* showed antimicrobial activity **(Cardenas-Ortega *et al.*, 2005, Shahverdi *et al.*, 2004)**. Camphene **(Gerige and Ramjaneyulu, 2007)**, β-pinene**(Andrew *et al.*, 1980)**, γ-terpinene **(Cristani *et al.*, 2007)** is responsible for antibacterial activityof *J. comminus* essential oil.

Synergistic evaluation

The synergistic evaluation of essential oils and AMI showed synergistic effect (the FICI was lower than 0.5). *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*,*J. comminus* and *Ferula gummosa* essential oils (tab 4). The results of synergistic evaluation showed that all of the essential oils decreased the MIC value of AMI. The lower FICIs were for *D. carrota* and *A. eryngioides* essential oils. Therefore, it does not mean that the essential oil with higher antibacterial activity has the higher synergistic effect. It is showed that piperitone has increased the antimicrobial activity of Furazolidone and nitrofurantoin **(Shahverdi *et al.*, 2004)**.
Furtheremore, the synergistic effects of AMI with lemon essential oil **(Guerra *et al.*, 2011)**, ciprofloxacin, gentamycin, piperacillin, tetracycline, cefoprazone with *Coriandrum sativum* essential oil **(Duarte *et al.*, 2012)** were reported. Therefore, *C. olivieri*, *J. comminus* and *C. macropodum* essential oils can be used as alternative treatment for controlling of *Acinetobacter* sp. Therefore, *D. carrota* and *A. eryngioides* can be used along with AMI for decreasing the effective dose of this antibiotics. More clinical studies are used for exhibiting the efficacies in clinical trials.

CONCLUSION

This study evaluate the antibacterial activity of ten essential oils against clinical isolates of *Acinetobacter* sp. The results of antibacterial screening showed different essential oils with different chemical composition has different antibacterial activity. Among ten essential oils,*C. macropodum*, *C. olivieri* and *J. comminus* (1.9 and 2.6 µl/ml) has the higher antibacterial activity against *Acinetobacter* sp. AMI showed synergistic effect with all of the essential oils. *D. carrota* and *A. eryngioides* showed the best synergistic effect with AMI, followed by *C. macropodum*, *A. biebersteinii*,*J. comminus* and *F. gummosa* essential oils.Therefore, these essential oils can be as alternative treatment for lowering dose of AMI. More clinical studies are required to providing these essential oils in clinical.

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