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ORIGINAL ARTICLE

Antibacterial activity of *Cladanthus arabicus* and *Bubonium imbricatum* essential oils alone and in combination with conventional antibiotics against *Enterobacteriaceae* isolates

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Significance and Impact of the Study: The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotics. This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents. Recently, medicinal plants and their derivatives (essential oils, extracts) have become very important in therapeutics because they encounter minimal challenges of the emergence of resistance. In this direction, the antimicrobial activity of the endemic *Bubonium imbricatum* plant and medicinal *Cladanthus arabicus* plant essential oils against multidrug-resistant *Enterobacteriaceae* strains was demonstrated.

Keywords
antibiotics, antimicrobials, bioproducts, enterobacteria, resistance.

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Abstract

Multidrug-resistant bacteria have become common all over the world, necessitating the development of new therapeutic strategies. Synergistic interactions between conventional antibiotics and natural bioactive may have therapeutic benefits in a clinical setting. There are plenty of medicinal plants that have proven efficacy against broad spectrum of micro-organisms. The aim of the work was to assess the antibacterial activity of *Cladanthus arabicus*, a Moroccan medicinal plant, and *Bubonium imbricatum*, a Moroccan endemic plant. The evaluation of the synergistic effect of extracted essential oils (EOs) together with some conventional antibiotics was also investigated. Checkerboard test was used to evaluate the interaction of EOs in combination with amoxicillin and neomycin. The results showed that EOs contain a potent activity against the tested *Enterobacteriaceae* isolates, with inhibition zones values in the range of $8{\cdot}05 \pm 0{\cdot}1$ and $13{\cdot}1 \pm 0{\cdot}11$ mm and MIC values between 200 μg ml^{-1} to 800 μg ml^{-1} for *C. arabicus* and from 400 μg ml^{-1} to 1600 μg ml^{-1} for *B. imbricatum*, respectively. Moreover, the current study allowed concluding that both EOs showed not only satisfactory antibacterial properties but also active effects combined with conventional antibiotics demonstrated by the Fractional Inhibitory Concentration Index (FICI). These findings are very interesting since there are no previous studies on synergistic interactions of these two plants with antibiotics.

Introduction

The frequency of resistance to first-line antimicrobial agents has been rising worldwide during the last decades in both human and veterinary medicine (Paterson 2006; Benameur *et al.* 2016), and now it reaches high proportions in many areas of the world. Infections caused by multidrug-resistant (MDR) *Enterobacteriaceae* are associated with increased morbidity and mortality than those caused by their susceptible counterparts (Rottier *et al.* 2012). The growing of antimicrobial resistance to first-line agents makes the selection of empirical therapy more difficult (Canton *et al.* 2012) and the development of new antimicrobial agents more urgent (Gervasi *et al.* 2014a,b). However, only few new agents have entered full clinical development. In order to find novel antimicrobial agents with new modes of action, medicinal plants have been explored as sources for the identification of new and effective antibacterial. They are also known to produce a variety of compounds as defences against a wide range of micro-organisms (Hayashi *et al.* 2013). It has also been confirmed that medicinal plants especially their essential oils (EOs) are an important source of alternative chemical reagents with potential therapeutic effects (Baytop 1999; Sarikurkcu *et al.* 2013). Recently, the use of EOs in combination with antibiotics against drug-resistant bacteria has gained much interest for scientific research. Several studies have demonstrated that EOs have an appreciable antibiotic spectrum with multiple drug targets (Khan *et al.* 2010; Lu *et al.* 2014). Some authors suggested that association of antibiotics with EOs targeting resistant bacteria may have a different mechanism of action and it may lead to new choices to overcome the problem of bacterial resistance (Yap *et al.* 2014).

Nowadays, EOs combined with antibiotics might be considered as an alternative strategy; the advantage of using a combination is the synergistic effect, in which the antimicrobial activity is greater than the individual contribution of each agent (Freitas *et al.* 2013; Yahiaoui *et al.* 2017).

The antimicrobial activity of *Cladanthus arabicus* and *Bubonium imbricatum* EOs was previously demonstrated (Aghraz *et al.* 2016, 2017). The present study focused on their antibacterial activity against MDR *Enterobacteriaceae* isolates. Furthermore, the possibility of synergistic interaction between essential oils and antibiotics was also explored.

Results and discussion

Bacterial strains identification

The isolates consisted of two *Escherichia coli*, one *Klebsiella pneumoniae*, one *Enterobacter cloacae*, one *Proteus Mirabilis* and one *Salmonella* sp.

Antimicrobial susceptibility testing

The results of antimicrobial susceptibility testing revealed that all the six *Enterobacteriaceae* isolates were MDR (resistant to three or more different antimicrobial agents belonging to different classes of antibiotics). Antimicrobial resistance profiles of the *Enterobacteriaceae* isolates are shown in Table 1. All the isolates were resistant to amoxicillin and neomycin and the majority of them were resistant to nalidixic acid, ciprofloxacin and levofloxacin. In the last few years, various investigations reported high levels of multidrug resistant *Enterobacteriaceae* isolated from poultry in Algeria (Hamoudi and Aggad 2008; Benameur *et al.* 2014, 2016). These data showed a worrying picture but in view of the whole range of antibiotics available in Algeria and their increasing and inappropriate use in poultry farms, the globally high incidence of antibiotic resistance observed in this country is not surprising.

Antibacterial activity of *C. arabicus* and *B. imbricatum* essential oils

The antibacterial activity of *C. arabicus* and *B. imbricatum* EOs extracted from aerial parts of the plants by steam distillation was tested against seven *Enterobacteriaceae* isolates, including six MDR isolates (*E. coli* S33/16, *E. coli* S34/16, *Enterobacter cloacae* S5/16, *Klebsiella pneumoniae* S12/16, *Proteus mirabilis* S32/16 and *Salmonella* sp S12/14) and *E. coli* ATCC 25922. The results of preliminary *in vitro* screening of antibacterial activity of the two EOs by the agar disk diffusion method against seven *Enterobacteriaceae* isolates and their interactions with conventional antibiotics are summarized in Table 2.

Table 1 Antimicrobial resistance patterns of the selected MDR *Enterobacteriaceae* strains

Isolate	Antimicrobial resistance pattern (Inhibition zones in mm)
Escherichia coli (S33/16)	NA (7), CIP (10), AML (7), AMC (12), SXT(7), TE (7), C (10), N (8)
Enterobacter cloacae (S54b/16)	AML(7), AMC (10), TE (8), N (9)
Salmonella sp (S12/14)	NA (8), CIP (18), AML (8), N (10)
Klebsiella pneumoniae (S12b/16)	AML (7), SXT (8), TE (7), C (11), N (9)
Escherichia coli (S34/16)	NA (7), CIP (8), AML (8), N(10)
Proteus mirabilis (S32/16)	NA (8), CIP (8), AML (7), TE (8), SXT (8), N (10)

AML, amoxicillin; NA, nalidixicacid; CIP, ciprofloxacin; AMC, amoxicillin-clavulanic acid; SXT, trimethoprim-sulfamethoxazole, TE, tetracycline; C, chloramphenicol; N, neomycin.

Table 2 Inhibition zones for *C. arabicus* (CA) and *B. imbricatum* (BI) essential oils alone and their synergistic effect in combination with antibiotics

Isolate	Essential oils	Essential oils Inhibition zones (mm)	Synergistic interaction	
			Amoxicillin	Neomycin
Escherichia coli (S33/16)	CA	8·28 ± 0·14	N	N
	BI	8·06 ± 0·2	N	N
Enterobacter cloacae (S5/16)	CA	8·5 ± 0·3	**P**	N
	BI	9·6 ± 0·2	**P**	N
Salmonella sp (S12/14)	CA	10·12 ± 0·2	**P**	N
	BI	13·1 ± 0·11	**P**	N
Klebsiella pneumoniae (S12/16)	CA	10·4 ± 0·2	N	N
	BI	10·45 ± 0·14	N	**P**
Escherichia coli (S34/16)	CA	10·35 ± 0·32	N	N
	BI	9·14 ± 0·15	N	**P**
Proteus mirabilis (S32/16)	CA	8·25 ± 0·12	**P**	N
	BI	8·17 ± 0·3	**P**	**P**
Escherichia coli ATCC 25922	CA	8·23 ± 0·09	**P**	N
	BI	8·05 ± 0·1	**P**	**P**

Values are expressed as mean ± SE (n = 3).
P, Positive synergistic interaction; N, No synergistic interaction.

The results showed that the EOs of *C. arabicus* and *B. imbricatum* have an antibacterial activity against all the tested *Enterobacteriaceae* isolates with different inhibition zones. The diameters of inhibition zones ranged between 8·05 ± 0·1 and 13·1 ± 0·11 mm. Those results confirmed several previous studies, which have shown a potent antibacterial activity of both plants EOs (Aghraz *et al.* 2016, 2017). The agar disk diffusion method allowed not only the determination of EOs inhibition zones diameters against seven bacteria but also the evaluation of synergistic effects between the essential oils and conventional antibiotics. *B. imbricatum* and *C. arabicus* EOs showed a synergistic effect with amoxicillin and neomycin (Table 2). The MIC values obtained for EOs, neomycin and amoxicillin against the seven *Enterobacteriaceae* isolates are shown in the Table 3. Both essential oils exhibited potent antibacterial activity against all the examined strains. However, the sensitivity of these isolates to the EOs was observed to be different depending on the bacterial species. The MIC values of *C. arabicus* EO ranged from 200 μg ml^{-1} to 800 μg ml^{-1} and of *B. imbricatum* EO from 400 μg ml^{-1} to 1600 μg ml^{-1}. Those results are in a good agreement with previous studies conducted with the same essential oil against non-MDR bacterial strains, which revealed that the two essential oils had a potent antimicrobial activity against three Gram-negative bacteria (*E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 2785, *Klebsiella pneumonia*) and three Gram-positive bacteria (*Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* CCMM B3 and *Bacillus cereus* ATCC 14579) (Aghraz *et al.* 2016, 2017).

The antibacterial EOs effect may originate from their major compounds, particularly in *B. imbricatum* EO for the presence of higher proportions of oxygenated monoterpenes (61·4%), particularly, cis-chrysanthenyl acetate (31·4%) and thymolisobutyrate (3·4%) (Aghraz *et al.* 2016) and in *C. arabicus* EO for monoterpenes hydrocarbons (75·8%), such as the main components were sabinene (31·1%), β-pinene (16·7%), myrcene (12·3%) and α-pinene (5·3%) (Aghraz *et al.* 2017). Indeed, it has been reported that EOs with higher amounts of monoterpenes hydrocarbon, such as α-pinene and β-pinene, have good antibacterial properties (Cosentino *et al.* 1999; Wilkinson *et al.* 2003; Elaissi *et al.* 2012; Chikhoune *et al.* 2013), and the mechanism of action determined for the main compounds of *C. arabicus* and *B. imbricatum* EOs, such as p-cymene, γ-terpinene and thymol isobutyrate, is membrane disruption (Di Pasqua *et al.* 2007, 2010; Oyedemi *et al.* 2009). In addition, the majority of EOs harm bacteria by affecting their cell membrane and causing cell death, or leading the permeability of their bacterial membrane, leading the loss of ions and ATP, collapse of proton pumps and finally the release of macromolecules and causing bacterial cell lysis (Bakkali *et al.* 2008; Turgis *et al.* 2012). Furthermore, the antibacterial activity also could result from the synergistic

Table 3 Minimal inhibitory concentration of *Cladanthus arabicus* and *Bubonium imbricatum* essential oils and antibiotics

Isolate	Essential oils (μg ml^{-1})		Antibiotics (μg ml^{-1})	
	C. arabicus	*B. imbricatum*	Amoxicillin	Neomycin
Escherichia coli (S33/16)	200	1600	64	64
Enterobacter cloacae (S5/16)	400	800	64	32
Salmonella sp (S12/14)	800	400	64	64
Klebsiella pneumoniae (S12b/16)	800	800	64	32
Escherichia coli (S34/16)	800	800	64	32
Proteus mirabilis (S32/16)	800	1600	64	64
Escherichia coli ATCC 25922	400	800	64	32

effect between various compounds, as previously reported by many studies (Stefanović *et al.* 2012; Balje *et al.* 2015). Nevertheless, other components or traces could have an effect on this activity.

Synergistic interactions between essential oils and antibiotics

The antibacterial effects of combined EOs with conventional antibiotics against seven *Enterobacteriaceae* isolates, including MDR isolates, were investigated by checkerboard method and the results are reported in Table 4.

The fractional inhibitory concentration (FIC) and the FIC index (FICI) were calculated to determine the interaction of the two EOs in combination with amoxicillin and neomycin. The gains expressed by the MIC of amoxicillin and neomycin in the presence of *B. imbricatum* and *C. arabicus* EOs are summarized in Table 5. The *B. imbricatum* EO showed total synergy in combination with amoxicillin against the majority of tested *Enterobacteriaceae* isolates (FIC < 0·5) and it also revealed a total synergy in combination with neomycin against *Escherichia coli* ATCC 25922 and *Proteus mirabilis*. However, *C. arabicus* EO showed a total synergy with amoxicillin against *Salmonella* sp and *Proteus mirabilis* and a partial synergy against *Enterobacter cloacae*.

Essential oils, as some multicomponent mixtures, can act on different levels unlike many conventional antimicrobials which are pure compounds and they have only a single target site of action (Yap *et al.* 2014). Moreover, several studies showed that the antibacterial activity of EOs or the combinations of compounds are more effective than isolated constituents (Junio *et al.* 2011; Wang *et al.* 2014). Indeed, the combination of tested essential oils and antibiotics showed significant results, the activity of antibiotics increased while the antibiotics MICs decreased. The combination of amoxicillin and *B. imbricatum* essential oil (MIC) allowed to double the activity from four times in case of *Salmonella* sp. to eight times in case of *Proteus mirabilis* (Fig. 1). Neomycin showed also strong interaction with *B. imbricatum* essential oil, and the obtained gain ranged from two fold in case of *E. coli* (S34/16) to 16 fold in case of *Klebsiella pneumoniae* (Fig. 2).

The antibacterial activity of amoxicillin was then examined in combination with *C. arabicus* EO. The highest gain was obtained against *Proteus mirabilis* with 32-fold using EOs at MIC (Fig. 3). However, no effect was noted

Table 4 Synergistic interaction of *Cladanthus arabicus* (CA) and *Bubonium imbricatum* (BI) essential oils in combination with amoxicillin and neomycin against *Enterobacteriaceae* isolates

FICI						
	Enterobacter cloacae (S5/16)	*Salmonella* sp (S12/14)	*Proteus mirabilis* (S32/16)	*Escherichia coli* ATCC 25922	*Klebsiella pneumoniae* (S12b/16)	*Escherichia coli* (S34/16)
AML/CA	0·58*	0·27†	0·03†	1·16‡	NT	NT
AML/BI	0·13†	0·29†	0·13†	0·13†	NT	NT
N/BI	NT	NT	0·13†	0·26†	0·52*	0·52*

NT, Not tested; AML, amoxicillin.
*Partial synergism.
†Total synergism.
‡No effect.

Table 5 Gain of the MICs of amoxicillin and neomycin in synergy with *Cladanthus arabicus* and *Bubonium imbricatum* essential oils

	C. arabicus			*B. imbricatum*			*B. imbricatum*		
Isolate	AML	AML+	Gain	N	N+	Gain	AML	AML+	Gain
Enterobacter cloacae (S5/16)	64	32	2	NT	NT	NT	64	8	8
Salmonella sp (S12/14)	64	16	4	NT	NT	NT	64	16	4
Escherichia coli ATCC 25922	64	64	1	32	8	4	64	8	8
Proteus mirabilis (S32/16)	64	2	32	64	8	8	64	8	8
Escherichia coli (S34/16)	NT	NT	NT	32	16	2	NT	NT	NT
Klebsiella pneumoniae (S12b/16)	NT	NT	NT	32	2	16	NT	NT	NT

NT, Not tested; AML, amoxicillin.

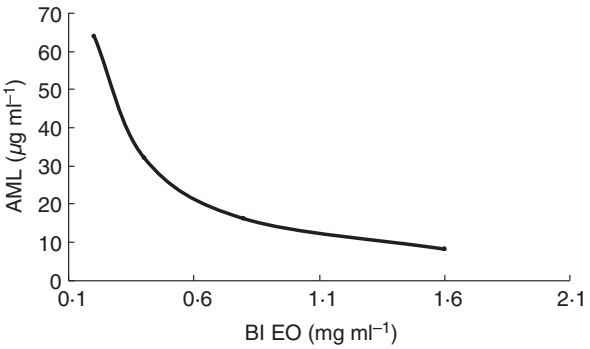

Figure 1 Synergistic interaction of *Bubonium imbricatum* Essential Oil (BI EO) in combination with AML against *Proteusmi rabilis* (MIC, Minimum Inhibitory Concentration; AML, Amoxicillin).

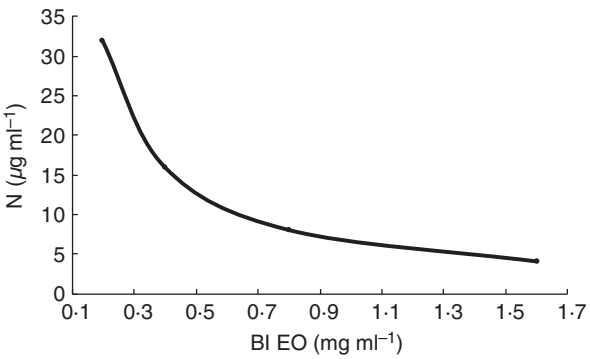

Figure 2 Synergistic interaction of *Bubonium imbricatum* Essential Oil (BI EO) in combination with neomycin against *Klebsiella pneumoniae* (MIC, Minimum Inhibitory Concentration; N, Neomycin).

when amoxicillin was used in the combination with *C. arabicus* essential oil against *E. coli* ATTC 25922.

The study indicates that the interactions between EOs of both plants and the standard antibiotics (amoxicillin and neomycin) have proven to be particularly effective, which may permit to find the treatment of several diseases caused by the micro-organisms. Definitely, the obtained results are in good agreement with previous studies (Ait-Dra *et al.* 2017; Behbahani *et al.* 2018) which investigated the importance of the synergy between antibiotics and EOs as an alternative way to reduce antibiotics consumption and protect the human's health.

No previous study exists on synergistic interaction between these *C. arabicus* and *B. imbricatum* EOs and antibiotics. Hence, the combination of both EOs with neomycin and amoxicillin would probably be a good therapeutic agent against these bacteria, valorizing the effectiveness of antibiotics, and decreasing the rate of antimicrobial resistance. Likewise, the *B. imbricatum* and *C. arabicus* EOs have shown a potent antibacterial activity and strong interaction with amoxicillin. Nevertheless, due

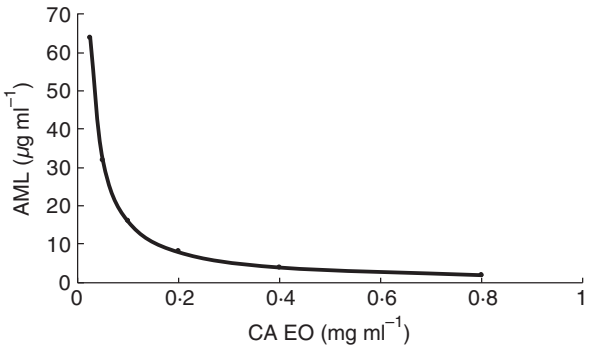

Figure 3 Synergistic interaction of *Cladanthus arabicus* Essential Oil (CA EO) in combination with AML against *Proteus mirabilis* (MIC, Minimum Inhibitory Concentration; AML, Amoxicillin).

to the diversity and the multitude of chemical composition in each plant EOs, the effects of their interaction with antibiotics are equally diverse and may lead to some toxic effects. For this reason, more investigations should be carried out on their probable toxicological effects and also their mode of action in order to optimize their use.

Materials and methods

Plant materials and extraction procedure

The aerial parts of the two plants (*Cladanthus arabicus* L.) Cass and *Bubonium imbricatum* ((Cav.) Litard) were collected in Marrakech and Essaouira regions, respectively, during their flowering period in 2016. The taxonomic identification was performed following the procedure described by Fennane and Ibn-Tattou (2012). Aerial parts were separated and dried at room temperature for 2 weeks in darkness and then stored in sealed paper bags. The EOs were extracted by steam distillation for 3 h (*C. arabicus*) and 4 h (*B. imbricatum*) using a Clevenger-type apparatus and stored in sealed glass vials at 4°C prior to analysis.

Evaluation of antimicrobial activity

Bacterial strains

Seven *Enterobacteriaceae* strains, isolated from poultry in the Regional Veterinary Laboratory of Mostaganem, Algeria, were selected to carry out the study. The strains were identified using Api20E systems (bioMerieux, France). *Escherichia coli* ATCC 25922 (American Type Culture Collection, Rockville, MD) was used as a reference strain.

Antimicrobial susceptibility testing

The *Enterobacteriaceae* isolates were tested for antimicrobial susceptibility using disk diffusion method, on

Muller–Hinton agar (MHA, Oxoid, Milan, Italy), following Clinical and Laboratory Standards Institute guidelines (CLSI 2016). The isolates were tested against a panel of 12 antimicrobials: nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), amoxicillin (AML, 25 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), cefotaxime (CTX, 30 μg), tetracycline (TE, 30 μg), sulphonamides (SSS, 300 μg), trimethoprim (TMP, 5 μg), trimethoprim/sulphamethoxazole (SXT, 1·25/23·75 μg), neomycin (N, 30 μg) and chloramphenicol (C, 30 μg) (Bio-Rad, Marnes la Coquette, France). Results were obtained after incubation for 16–18 h at 37°C and were interpreted according to CLSI previously cited guidelines.

Determination of antibacterial activity of C. arabicus and *B. imbricatum* EOs by disc diffusion assay

The disc diffusion method was conducted to evaluate the antibacterial activity of EOs according to the method previously described (Bauer *et al.* 1966). The suspension is prepared from an overnight culture for each micro-organism then evenly distributed on a Mueller Hinton agar (MHA). Sterile paper discs were impregnated with 10 μl of pure EO. The plates were then placed at 4°C for 2 h and incubated for 24 h at 37°C. After incubation, the diameters of inhibition zones were measured in millimeters.

Disk impregnated with sterile distilled water served as a negative control and disk containing antibiotic (CTX: Cefotaxime) served as positive control. Each test was performed in triplicate. All results have been expressed as mean ± SD.

Determination of Minimal Inhibitory Concentration (MIC) of C. arabicus, *B. imbricatum* EOs and antibiotics

The broth micro-dilution method was used to determine the Minimal Inhibitory Concentration (MIC), according to NCCLS guidelines (2003). The tests were performed in Mueller Hinton broth (MHB, Oxoid). Bacterial cultures of a cool night in log phase were used to prepare the suspension of cells adjusted to 10^6 CFU in MHB, and 100 μl were added to each well of 96 well plates. MHB was used to dissolve the EOs and then diluted to the highest concentration (128 μg ml^{-1}). It was also used to dissolve the antibiotics and the highest concentration for amoxicillin and neomycin was 256 μg ml^{-1} and 128 μg ml^{-1}, respectively.

A positive growth control containing MHB and bacterial culture without compounds, and a negative control containing no bacteria were included in each experiment. The plates were incubated in aerobiosis at 37°C for 24 h, and amount of growth was monitored by measuring the increased turbidity, as indicated by solution optical density (OD) at 630 nm on a plate reader. The MIC is the lowest concentration at which the bacteria failed to grow so that no increase in optical density was detected. Tested EOs and antibiotics were soluble in MHB and each test was performed in triplicate.

Evaluation of synergistic interaction between EOs and antibiotics by checkerboard test

The synergy between conventional antibiotics and EOs has been studied firstly by the evaluation of the inhibition zones between EOs and several antibiotics using the disc diffusion method. The test was performed as described by Jarlier *et al.* (1988) with slight modifications. Briefly, the antibiotics were placed away from the EOs disc depending to their inhibition zone. A clear extension of the edge of the inhibition zone of any of the antibiotics towards the disc EOs was interpreted as positive interaction. The antibiotics showed synergistic effect according to disk diffusion method results were selected for further study by checkerboard test. Checkerboard method was used for the determination of the MIC of antibiotics in the presence of EOs at a low concentration (MIC) as described by Noumedem *et al.* (2013). Fifty microliters of the of six serial twofold dilutions of the two tested EOs (from MIC to MIC/32) were added to each well, containing 50 μl of eight serial twofold dilutions (from MIC to MIC/128) of antibiotic and inoculated after that with 100 μl of cell suspension (10^6 CFU per ml). MIC was defined as the lowest concentration of antibiotic, in combination with EOs at MIC that inhibits the visible growth of tested strains. The combined effects of the antibiotic and essential oils were calculated and expressed in terms of a fractional inhibitor concentration index (FICI) using the following formula:

$$\mathrm{FICI} = \sum \mathrm{FIC} = \mathrm{FIC(A)} + \mathrm{FIC(B)}$$

where FIC (A) = MIC value of antibiotic combined with essential oil/MIC value of antibiotic alone and FIC (B) = MIC value of essential oil combined with antibiotic/MIC value of essential oil alone. Interpretation of FICI values were defined as; total synergism (FICI $\leq$ 0·5), partial synergism (0·5 < FICI $\leq$ 0·75), no effect (0·75 < FICI $\leq$ 2) or antagonism (FICI > 2) (Mulyaningsih *et al.* 2010). Each test was performed in triplicate.

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Conflict of Interest

We declare that we have no conflict of interest.

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