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Antimicrobial and toxicity profiles evaluation of the Chamomile (*Matricaria recutita* L.) essential oil combination with standard antimicrobial agents

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ABSTRACT

In this present study, commercial Pharmacopeia (PhEur) grade chamomile essential oil (*Matricariae aetheroleum*) was combined with different antimicrobial agents including ampicillin sodium, cefuroxime acetyl, tetracycline hydrochloride, fluconazole and nystatin. All combinations were evaluated *in vitro* against pathogenic standard and clinical resistant Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus)* bacterial isolates as well as against *Candida albicans* for their broad antimicrobial effectiveness. Furthermore, the essential oil was fractioned by column chromatography using *n*-hexane, diethyl ether, dichloromethane and methanol, respectively. Additionally, all fractions of essential oil were tested in combinations for their minimum inhibitory concentrations (MIC) as well as for their fractional inhibitory concentrations (FIC) against the resistant microbial pathogens. Antimicrobial activities were evaluated by microdilution method and antimicrobial interactions were assayed using the checkerboard method. Cytotoxicity of compounds were evaluated using Cytotox-XTT-1 Parameter Kit in WS1 cells and *Aliivibrio fischeri* bioluminescence toxicity assay. The analyses proved that α-bisabolol oxide A (47.7%), *(E)-β*-farnesene (21.5%), *α*-bisabolol oxide B (6.2%), α-bisabolone oxide A (5.8%), chamazulene (4.1%) and α-bisabolol (2.2%), respectively were the major compounds and in compliance with PhEur. The essential oil combination of fluconazole and nystatin showed "synergic and additive inhibitory effects" against the clinical *Candida* strain. According to the IC_{50} values obtained, the inhibitory concentrations of combinations against the clinical *Candida* strain can be considered to be selective when compared with its effect on WS1 cells. Additionally, the essential oil combination of fluconazole and nystatin showed low toxicity against *A. fischeri*.

1. Introduction

Matricaria recutita (Matricaria chamomilla) commonly known as chamomile, German chamomile, is an annual plant of the composite family Asteraceae. *M. recutita* can be found near populated areas all over Europe and Asia, and it has been widely introduced in North America and Australia (Singh et al., 2011). The dried flowers of chamomile contain many terpenoids and flavonoids contributing to its medicinal properties. The principal components of the essential oil extracted from the German chamomile flowers are the terpenoids α-bisabolol and its oxide azulenes including chamazulene and acetylene derivatives (Srivastava et al., 2010).

German chamomile is used in herbal medicine for a sore stomach, irritable bowel syndrome, and as a gentle sleep aid. It is also used as a mild laxative and is anti-inflammatory and bactericidal (McKay and Blumberg, 2006; Ramos-e-Silva et al., 2006; Nayak et al., 2007). *In vitro* chamomile has demonstrated moderate antimicrobial and antioxidant properties and significant antiplatelet activity, as well as preliminary results against cancer (Srivastava and Gupta, 2007).

Several chemical compounds from synthetic or natural sources enhance the activity of specific antibiotics and reverse the natural resistance of specific bacteria to given antibiotics (Santos et al., 2011). Also, there has been an increase in the use of natural substances instead of synthetic chemicals. Essential oils have a broad spectrum antimicrobial activity against pathogenic microorganisms and many studies have been published. But it has not been focused intensively on studying the combinations of these products with antimicrobial agents for overcoming to resistance mechanism. To the best of our knowledge, there are no available reports on the synergistic interactions between antibiotics and antifungals with essential oil of *M. recutita.* Therefore, the aim of the present work was to determine *in vitro* synergistic effects between standard antimicrobials and essential oil of *M. recutita* against

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pathogenic microorganisms. Essential oil was also partially fractionated by column chromatography to determine the active compounds or group in activity. Also, we aimed to evaluate *in vitro* cytotoxic effects of combinations with additive/synergic effects.

2. Materials and methods

2.1. Essential oil

Pharmacopeia (PhEur) grade chamomile essential oil (*M. recutita* L.) purchased from (Phatrade, Cairo in Eygpt).

2.2. Gas chromatography–mass spectrometry (GC–MS) and gas chromatography-flame ionization detector (GC-FID)

2.2.1. GC–MS analysis

The GC–MS analysis was carried out with an Agilent 5975 GC–MSD system. Innowax FSC column (60 m × 0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min that was kept constant at 220 °C for 10 min and followed by elevating the temperature to 240 °C at a rate of 1 °C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. Mass range was *m/z* 35–450.

2.2.2. GC analysis

The GC analysis was carried out using an Agilent 6890N GC system using FID detector temperature of 300 °C. To obtain the same elution order with GC–MS, simultaneous auto-injection was done on a duplicate of the same column at the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.2.3. Identification of components

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty and Stauffer, 1989; Koenig et al., 2004) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils. Additionally, MS literature data (Joulain and Koenig, 1998; ESO 2000, 1999ESO 2000, 1999) was also used for the identification.

2.3. Vacuum column chromatography

Vacuum column chromatography was used to fractionate of *M. recutita* L essential oil. Columns were packed with silica gel using a wet method. The essential oil which previously prepared and then placed on the top of silica gel on the column. Essential oil was fractionated by *n*-hexane, diethly ether, dichloromethane and methanol, respectively. The fractions were collected and evaporated.

2.4. Antimicrobial activity

2.4.1. Microorganisms

Microorganisms used in the assay were; *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Candida albicans* ATCC 90028 standard strains and clinical isolates provided from Akdeniz University, Faculty of Medicine (Antalya, Turkey).

2.4.2. Culture media

Cation adjusted Mueller Hinton Broth-2 (MHB-2, Sigma) and RPMI-1640 medium with L-glutamine (Sigma) buffered pH 7 with 3-[*N*-morpholino]-propansulfonic acid (MOPS) (Sigma) were used for antimicrobial activity and checkerboard microdilution assay.

2.4.3. Antimicrobial drugs

Ampicillin sodium (AMP), cefuroxime acetyl (CEF), tetracycline hydrochloride (TCY) fluconazole (FLU) and nystatin (NS) were used as standard antimicrobial drugs for combination studies were supplied from Deva Pharmaceutical Company.

2.5. Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) was determined using the microdilution broth method (Clinical and Laboratory Standards Institute, 2002, 2006) by automated liquid handling system (Biomek 4000, Beckman & Coulter). The essential oil was diluted–two fold initially, with a final concentration range of (5120–10 μg/mL), for standard antibacterial agents (64–0.125 μg/mL); antifungal agents FLU (64–0.125 μg/mL) and NS (16–0.03 μg/mL).

A fresh overnight culture of the tested microorganism was used to prepare the cell suspensions in twice concentrated Mueller Hinton Broth (MHB) for bacterial strains and RPMI medium for yeasts to obtain 10^6 colony-forming unit (cfu)/ml and 1–2 × 10^3 cells/ml respectively. The tests were carried out in 96-well plates, inoculated microplates were incubated at 37 °C for 24 h for bacteria and at 28 °C for 48 h for yeast, respectively. Microbial growth was observed by adding 20 μL resazurin of 0.01%. MICs were determined as the lowest concentration which showed no fungal growth or no color change from resazurin.

2.6. Checkerboard microdilution assay

The antimicrobial interaction between some conventional antimicrobials (AMP, CEF, TCY, FLU or NS) and *M. recutita* essential oil has been studied by the checkerboard method. Checkerboard method was performed with 96-well plate using an 8-by-8 well configuration. Eight serial dilutions two fold dilutions of *M. recutita* essential oil and antimicrobial agents (AMP, CEF, TCY, FLU or NS) were prepared using the same solvents (medium) as in the MIC test. 25 μL aliquots of *M. recutita* essential oil were added to the wells of a 96-well plate in a vertical orientation and 25 μL aliquots of each antimicrobial agents (AMP, CEF, TCY, FLU or NS) dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. Positive growth controls (to assess the presence of turbidity) were performed in wells not containing antimicrobial. In addition, negative growth control was applied in 96-well plate. Following this, each well was inoculated with 50 μL (5 × 10^3 cfu per well) one of the 6 different microorganisms (both clinical and standard) suspensions and cultivated at 35 °C for 24 or 48 (*Candida*) hours. After incubation 20 μL rezasurin added all wells and again cultivated at 35 °C for 2 h. Growth in the medium is indicated by change in color from blue to pink.

The analysis of the combination was obtained by calculating the fraction inhibitory concentration index (FICI) using the following formula (Van vuuren et al., 2009):

FIC of essential oil = MIC of essential oil in combination with antimicrobial drugs/MIC of essential oil alone,

FIC of antimicrobial drug = MIC of antimicrobial in combination with essential oil/MIC of antimicrobial drug,

FICI = FIC of essential oil + FIC of antimicrobial drug

The types of effects were classified as follows: FICIs ≤ 0.5, synergism; FICIs 0.5 ≤ 1, additive effect; FICIs > 1–4, indifferent effect and FICIs ≥ 4, antagonism.

2.7. In vitro cytotoxicity assay

WS1 (ATCC® CRL-1502™, human normal skin fibroblast cell line) cell line was used for cytotoxicity tests. WS1 cells were incubated in EMEM medium (Wisent Bioproducts, Saint-Jean-Baptiste, Canada) supplemented with fetal bovine serum (Wisent Bioproducts, Saint-Jean-Baptiste, Canada), 100 IU/mL penicillin–100 mg/mL streptomycin (Hyclone, Thermo Scientific, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. WS1 cells were seeded at 1×10^4 cells into each well of 96-well plates. After 24 h of incubating period, the culture mediums were removed and essential oil and oil fractions of essential were added to culture medium at 5–640 μg/mL concentrations, whereas the standard antimicrobial agents were added to culture medium at 3.9–500 μg/mL concentrations. After 24 h of incubation, cytotoxicity test was performed using the In Cytotox-XTT 1 Parameter Cytotoxicity Kit (Xenometrix AG, Gewerbertrasse, Switzerland), which measures mitochondrial activity in WS1 cell line. Firstly, the cells were washed phosphate buffer saline (PBS) and were added 200 μL/well of fresh culture medium. XTTI and XTTII solution were mixed at 1:100 ratios. Then, 50 μL of this mixture was added to all wells. The plate was incubated for 3 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After 3 h, the content of the well was mixed by pipetting up and down. Then, OD of the plate was read at 480 nm with a reference wave length at 690 nm. Percentage inhibition (%) was calculated for each concentration of compound. IC_{50} value was estimated by non-linear regression analysis.

In addition, the cytotoxicity of essential oil/essential oil fractions with standard antimicrobial drug combinations with additive/synergic effects which were obtained by the checkerboard method were investigated against WS1 cell line. For this purpose, 5 series of dilutions were prepared according to the effective concentrations of synergic combinations and were incubated in the culture medium containing WS1 cell line for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The concentrations prepared for each combination were presented in Table 1. After incubation period, cytotoxicity test was performed using In Cytotox-XTT 1 Parameter Cytotoxicity Kit as a described above. Percentage inhibition and IC_{50} values were calculated for each concentration of the combinations by graphical method. The following formula was used to calculate the inhibition percentage for each concentration.

$$\%\ \text{inhibition} = 100 - (\text{mean sample} \times 100/\text{mean solvent})$$

Stock solutions of essential oil/essential oil fractions/antimicrobial drugs were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The final DMSO concentration under 0.1%. All experiments were performed in duplicate.

2.8. Aliivibrio fischeri bioluminescence assay

A. fischeri acute toxicity bioassay measures the decrease in bioluminescence induced in the cell metabolism due to the presence of a toxic substance. The bacterial reagent (*A. fischeri* NRRL-B 11177, a commercially available BioFix®Lumi test from Macherey-Nagel, Germany) is supplied freeze-dried and was reconstituted and stored at 3 °C for an interim period of 5 min before using.

The toxicity assay was carried out in 96-well white polypropylene microplate. The compounds were prepared in 2% NaCl. One hundred microliter of compounds were pipetted into each well, which were supplemented with 100 μL of bacterial suspension. Light was measured during 30 min by using luminometer Synergy HT1 (Bio-Tek Instruments) microplate reader at 20 ± 1 °C. $K_2Cr_2O_7$ was used as toxicity reference compound. After a predefined contact time (5, 15, and 30 min), luminescence inhibition was determined and IC_{50} values were calculated as the effective concentration of inhibitor causing a decrease of 50% in *A. fischeri* bioluminescence.

3. Results and discussion

3.1. Essential oil composition

The results of the chemical composition of *M. recutita* essential oil and fractions are presented in Table 2 according their relative retention indices (RRI) and their relative percentages (%). Overall, 37 compounds representing 95.2% of the oil were identified with the aid of GC–MS and main compounds of *M. recutita* essential oil chromatogram was given in Fig. 1.

The analyses proved that α-bisabolol oxide A (47.7%), *(E)*-β-farnesene (21.5%), α-bisabolol oxide B (6.2%), α-bisabolene oxide A (5.8%), chamazulen (4.1%), and α-bisobolol (2.2%), respectively, were the major compounds and in compliance with PhEur (8.0).

A literature search revealed α-bisabolol oxide A (48.22%), β-farnesene (5.21%), α-bisabolol oxide B (23.31%) and α-bisabolol (12.1%) to be main components of *M. recutita* essential oil (Roby et al., 2013). There is a report showing, *(E)*-β-farnesene (24.19%), chamazulen (10.57%), α-bisabolol oxide A (10.21%), α-farnesene (8.7%) and α-bisabolol (7.27%), respectively in essential oil of *M. recutita* L. (Ayoughi et al., 2010).

Additionally, the chemical compositions were identified for essential oil of fractions. *(E)*-β-farnesene (72.0%) and germacren D (7.0%) for *n-hexane* fraction, α-bisabolol oxide A (57.7%), *(E)*-β-farnesene (13.2%), α-bisabolol oxide B (6.8%), α-bisabolone oxide A (6.7%) for diethyl ether fraction, α-bisabolol oxide A (50.5%), methyl oleate (47.0%) were identified for dichloromethane fraction. There was no chemical components identified for methanol fraction.

Table 1
The cytotoxcity concentrations of *M. recutita* essential oil/fractions of *M. recutita* essential oil + antimicrobial drug combinations with additive/synergic effects.

Combination	IC_{50} (μg/mL)				
EOMR + FLU	5 + 0.06	10 + 0.125	**20 + 0.25**	40 + 0.5	80 + 1
EOMR + NS	5 + 0.125	10 + 0.25	**20 + 0.5**	40 + 1	80 + 2
EOMR + TCY	2.5 + 0.25	5 + 0.5	**10 + 1**	20 + 2	40 + 4
EOMR-H + AMP	10 + 16	20 + 32	**40 + 64**	80 + 128	160 + 256
EOMR-H + FLU	5 + 0.125	10 + 0.25	**20 + 0.5**	40 + 1	80 + 2
EOMR-H + TCY	40 + 0.03	80 + 0.06	**160 + 0.125**	320 + 0.25	640 + 0.5
EOMR-DE + AMP	2.5 + 0.5	5 + 1	**10 + 2**	20 + 4	40 + 8
EOMR-DE + FLU	20 + 0.125	40 + 0.25	**80 + 0.5**	160 + 1	320 + 2
EOMR-DE + TCY	0.625 + 0.06	1.25 + 0.125	**2.5 + 0.25**	5 + 0.5	10 + 1
EOMR-DM + FLU	5 + 0.125	10 + 0.25	**20 + 0.5**	40 + 1	80 + 2
EOMR-DM + TCY	0.625 + 0.06	1.25 + 0.125	**2.5 + 0.25**	5 + 0.5	10 + 1

Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin.

Table 2
Main components of *M. recutita* essential oil and fractions.

No	RRI	Compounds	Relative%			
			EOMR	EOMR-H	EOMR-DE	EOMR-DM
1	1246	*(Z)-β*-Ocimene	–	0.1	–	–
2	1255	γ-Terpinene	0.1	0.3	–	–
3	1266	*(E)-β*-Ocimene	0.3	0.9	–	–
4	1280	*p*-Cymene	–	0.4	–	–
5	1358	Artemisia ketone	0.3	–	–	–
6	1497	*α*-Copaene	–	0.2	–	–
7	1479	*β*-Elemene	0.1	0.4	–	–
8	1510	Artemisia alcohol	0.1	–	–	–
9	1550	*α*-İsocomene	–	0.2	–	–
10	1612	*β*-Caryophyllene	0.1	0.3	–	–
11	1661	Alloaromadendrene	0.1	0.3	–	–
12	1695	*(E)-β*-Farnesene	21.5	72.0	13.2	–
13	1704	γ-Muurolene	0.2	0.7	–	–
14	1708	Leden	0.2	0.5	–	–
15	1726	Germacren D	1.9	7.0	–	–
16	1740	α-Muurolene	0.4	2.2	–	–
17	1755	Bicyclogermacren	1.2	1.8	0.7	–
18	1758	*(E-E)-α*-Farnesene	0.9	1.4	0.6	
19	1773	*δ*-Cadinene	0.3	0.9	–	–
20	1776	*γ*-Cadinene	0.2	0.3	–	–
21	1786	*ar*-Curcumen	–	0.2	–	–
22	2000	Eicosan	–	0.1	–	–
23	2156	α-Bisabolol oxide B	6.2	–	6.8	2.5
24	2200	α-Bisabolon oxide A	5.7	–	6.7	–
25	2226	Methyl hexadecanoate	–	–	3.3	–
26	2232	α-Bisobolol	2.1	–	0.1	–
27	2144	Spathulenol	–	e	0.7	–
28	2200	Docosane	–	0.1	–	–
29	2298	Decanoic acid	0.5	–	–	–
30	2300	Tricosane	–	1.6	–	–
31	2400	Tetracosane	–	0.3	–	–
32	2400	Pentacosane	1.0	3.9	–	–
33	2430	Chamazulen	4.1	–	3.4	–
34	2438	α-Bisabolol oxide A	47.7	–	57.7	50.5
35	2456	Methyl oleate	–	–	5.5	47.0
36	2700	Heptacosane	–	0.1	–	–
37	2900	Nonacosane	–	0.4	–	–
		Total amount	95.2	96.6	98.7	100

(–): Not detected.
Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **RRI**: Relative retention indices calculated against *n*-alkanes; % calculated from FID data.

3.2. Determination of antimicrobial activity of essential oil and its fractions by minimum inhibitory concentrations (MIC)

The antimicrobial activity of essential of *M. recutita* and its fractions were examined against standard with clinical species. As can be seen in Table 3, *Candida albicans* was inhibited at low concentration with MICs, ranging from 160 to 320 µg/mL. In contrast essential oil and its fractions have low activity against Gram positive bacteria *S. aureus* and Gram negative bacteria *E. coli* with MICs, ranging from 640 to 1280 µg/mL.

The essential oil and its fractions tested in this study were found to be generally more effective against standard strains than clinical species in Table 4. *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 were inhibited with MICs, ranging from 320 to 640 µg/mL while *C. albicans* was inhibited with MICs, ranging from 160 to 320 µg/mL. This might be explained by mechanisms of by the resistance in clinical species.

3.3. Checkerboard microdilution assay

Recently, studies on essential oils with antimicrobial combination has been increased especially in the treatment of infectious diseases.

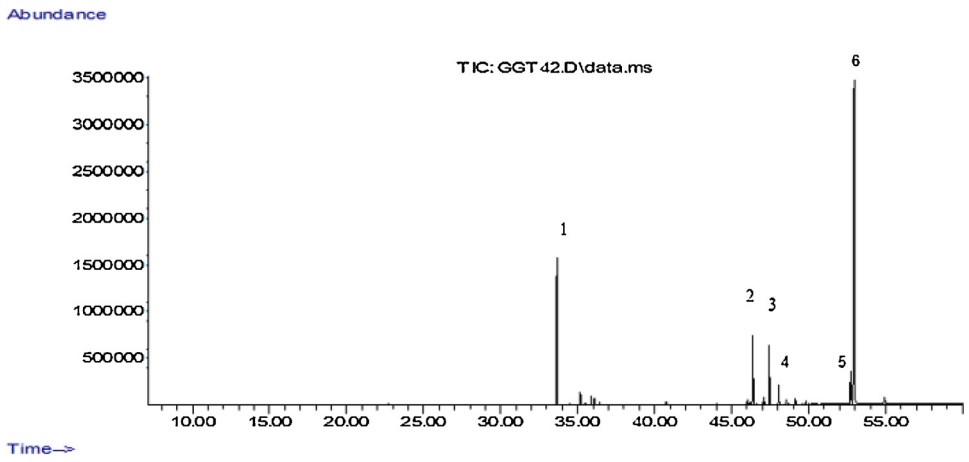
This study was undertaken to the first time to examine a possible effect of between *M. recutita* essential oil and its fractions with ampicillin, cefuroxime, tetracycline, fluconazole and nystatin. Additionally, we aimed to evaluate *in vitro* cytotoxic effects of combinations with additive/synergic effects. The results of antimicrobial combination interactions with antimicrobial drugs are summarized in Table 5. The FICI values were used to evaluate the synergic and additive activity. *M. recutita* essential oil in combination with ampicillin, cefuroxime, tetracycline resulted as “indifferent” against the tested clinical bacterial isolates. In contrast, essential oil combinations with these antimicrobials were found to be effective against ATCC strains.

The results showed that the essential oil provided the best synergic effect with combination of tetracycline and this effect was observed for both Gram positive and Gram-negative bacteria with FIC indexes were 0.26–0.37. MIC of tetracycline decreased from 4 µg/mL alone to 1 µg/mL in the presence of essential oil of *Matricaria against S. aureus and E. coli.* It has been known that possible activity of ampicillin and cefuroxime inhibits on the cell wall and envelope, while tetracycline antibiotics are protein synthesis inhibitors. But essential oils have different chemical compounds, and it might be difficult to identify the molecular pathway of action. Therefore, each of the constituents of the essential oils might be display its own mechanism of action. One of the possibilities for action, particularly destroy to membrane and cytoplasm, and in some cases, they completely change the morphology of the cells (Nazzaro et al., 2013).

There is a report data showed that sesquiterpenoids; nerolidol, farnesol, bisabolol and apritone enhanced the susceptibility of *S. aureus* to the antibiotic tested. Furthermore, it was suggested that the sesquiterpenoid compounds may act by damaging the normal barrier function of the bacterial cell membrane, allowing the permeation into the cell of exogenous solutes such as antibiotics. This effect was found to be more notified for Gram-positive bacteria, probably due to the lack of additional permeability barriers, particularly the outer membrane of Gram-negative bacteria (Brehm-Stecher and Johnson, 2003). We observed that essential oil combination with antibiotics more effective against Gram positive than Gram negative bacteria.

In our study, essential oil combination of fluconazole and nystatin showed synergic and additive inhibitory effects against the clinical isolate of *Candida* with FICI values were calculated as 0.31 and 0.56, respectively. Antifungal drugs, such as; azoles, inhibit ergosterol biosynthesis of the membrane components and polyenes directly interact with the fungal cell membrane (Casalinuovo et al., 2004). Furthermore, mode of action of was reported that *M. recutita* essential oil was primarily affects fungal cell permeability through direct interaction with the plasma membrane in *Aspergillus niger* (Tolouee et al., 2010)

Fluconazole and nystatin are the antifungal drugs for which the use are limited due to side effects and toxicity profiles. However, their combined approaches with essential oils leading to reduce dosages, toxicity and decrease of adverse side effects, may be alternative in order to restore its usage (Cottarel and Wierzbowski, 2007). For this purpose, combinations of nystatin with essential oils of *Origanum vulgare, Pelargonium graveolens* and *Melaleuca alternifolia* were studied against several *Candida* strains. The most synergistic effect was showed in *O. vulgare* essential oil with FIC indexes in range of 0.11–0.17. Combinations between nystatin and *P. graveolens* indicated synergistic effect against some of strains, while combination between nystatin and *M. alternifolia* was only observed additive effect (Rosato et al., 2009). In another report, the essential oil of *Ocimum sanctum* and its combination with two azoles, fluconazole, and ketoconazole, against resistant isolates were studied by Amber et al. (2010). *O. sanctum* combination with fluconazole showed synergistic effect against all the tested isolates with FIC indexes in ranges from 0.24 to 0.53. Many studies reported that combination approaches between essential oils and conventional antimicrobials against different microorganisms. There is only one report showing that antimicrobial interaction with essential oil of *M. recutita.* The authors were studied 26 different essential oils, including of *M.*



1. *(E)-β*-Farnesene
2. α-Bisobolol oxide B
3. α-Bisobolone oxide A
4. *α*-Bisabolol
5. Chamazulen
6. α- Bisabolol oxide A

Fig. 1. Main compounds of *M. recutita* essential oil.

Table 3
MIC values of *M. recutita* essential oil/fractions of *M. recutita* essential oil/the antimicrobial agents against clinical strains (μg/mL).

Samples	*S. aureus**	*E. coli**	*C. albicans***
EOMR	1280-640	1280-640	320-160
EOMR -H	1280-640	1280-640	320-160
EOMR -DE	1280-640	1280-640	320-160
EOMR -DM	1280-640	1280-640	320-160
AMP	> 128-64	> 128-32	–
CEF	128-64	64-32	–
TCY	64-32	128-64	–
FLU	–	–	4-1
NS	–	–	4-1

(–): Not determined.
Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **CEF**: cefuroxime acetyl; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin; *: Ampicilline resistant, **: Fluconazole susceptible.

Table 4
MIC values of *M. recutita* essential oil/fractions of *M. recutita* essential oil/the antimicrobial agents against standard strains (μg/mL).

Samples	*S. aureus* ATCC 6538	*E. coli* ATCC 8739	*C. albicans* ATCC 90028
EOMR	640-320	640-320	320-160
EOMR-H	640-320	640-320	320-160
EOMR-DE	640-320	640-320	320-160
EOMR-DM	640-320	640-320	320-160
AMP	16-4	16-4	–
CEF	16-4	128-64	–
TCY	4-1	4-1	–
FLU	–	–	4-1
NS	–	–	4-1

(–): Not determined.
Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **CEF**: cefuroxime acetyl; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin.

recutita. with combination of Amphotericin B against two isolates of *C. albicans* and one isolate of *C. tropicalis* by the disc diffusion methods. Essential oil of *M. recutita.* showed additive effect against one of the stains of *C. albicans* (Nozaki et al., 2010).

Difference point of in our study, we also fractionated the essential oil with *n*-hexane, diethyl ether, dichloromethane and methanol by column chromatography and demonstrated the antimicrobial interactions at these fractions with antimicrobial drugs to determine the possible active compounds of essential oil. Chemical composition of *n*-hexane fraction was determined (E)-β-farnesene (72.0%) and germacren D (7.0%) as main compounds. This fraction of essential oil combination with ampicillin and tetracycline showed synergic effect. This effect was observed against for only clinical isolate of *S. aureus* with FICI values varying from 0.31 to 0.25. This fraction was more effective against Gram positive than Gram negative bacteria. Synergistic effect might be explained by these chemical components or combination with less abundant chemical components present in *n*-hexane fraction.

Chemical composition of diethyl ether fraction was represented as, α-bisabolol oxide A (57.7%), *(E)-β*-farnesene (13.2%), α-bisabolol oxide B (6.8%), α-bisabolone oxide A (6.7%) main compounds. The strongest synergistic effect was present in diethyl ether fraction with FIC indexes were 0.011 (tetracycline) and 0.022 (ampicillin) only against *S. aureus.* MIC of tetracyline decreased from 32 to 0.25 μg/mL and MIC of ampicillin decreased from 128 to 2 μg/mL. This effect might be explained by difference structures of cell walls of Gram-positive than Gram-negative bacteria. However, no synergistic or additive effect was observed in the experiment with cefuroxime against all tested microorganisms.

In chemical profile of dichloromethane were identified as, α-bisabolol oxide A (50.5%), methyl oleate (47.0%) main compounds. Dichloromethane fraction combination with ampicillin and tetracycline was only effective against clinical isolate of *S. aureus* with FIC indexes were 0.011–0.50. Dichloromethane fraction combination with fluconazole was showed additive effect with FIC index was 0.56. It can be considered that these chemical substances may be responsible for the effect.

From this point of view, *M. recutita* essential oil was found to be

Table 5
M. recutita essential oil/fractions essential oil + antimicrobial drug combinations with additive/synergic effects.

Combinations	Microorganisms	FICI	Results
EOMR + AMP	*S. aureus ATCC 6538*	0.37	**Synergic**
EOMR + CEF	*E.coli ATTC 8739*	0.75	Additive
EOMR + CEF	*S.aureus ATCC 6538*	0.75	Additive
EOMR + TCY	*S.aureus ATCC 6538*	0.26	**Synergic**
EOMR + TCY	*E. coli ATTC 8739*	0.37	**Synergic**
EOMR + NS	Clin. isol. *C. albicans*	0.56	Additive
EOMR + FLU	Clin. isol. *C. albicans*	0.31	**Synergic**
EOMR-H + AMP	Clin. isol. *S.aureus*	0.31	**Synergic**
EOMR-H + TCY	Clin. isol. *S.aureus*	0.25	**Synergic**
EOMR-H + TCY	*S.aureus ATCC 6538*	0.51	Additive
EOMR-H + TCY	*E. coli ATTC 8739*	0.51	Additive
EOMR-H + FLU	Clin. isol. *C. albicans*	0.56	Additive
EOMR-DE + AMP	Clin. isol. *S.aureus*	0.022	**Synergic**
EOMR-DE + TCY	Clin. isol. *S.aureus*	0.011	**Synergic**
EOMR-DE + TCY	*S.aureus ATCC 6538*	0.26	**Synergic**
EOMR-DE + TCY	*E. coli ATTC 8739*	0.51	Additive
EOMR-DE + FLU	Clin. isol. *C. albicans*	0.75	Additive
EOMR-DM + FLU	Clin. isol. *C. albicans*	0.56	Additive
EOMR-DM + AMP	Clin. isol. *S.aureus*	0.50	Additive
EOMR-DM + TCY	Clin. isol. *S.aureus*	0.011	**Synergic**

Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **CEF**: cefuroxime acetyl; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin; Clin. isol.: Clinic isolate.

generally more effective against ATCC strains while essential oil fractions had more synergistic effect against clinical isolates. It is clear that all chemical profile of essential oil did not give a remarkable effect against clinical isolates. Therefore, essential oil fractions may be use to overcome the resistance mechanism instead of *M. recutita* essential oil. It is also important to mention that essential oil and its fractions generally indicated an effective combination with ampicillin and tetracycline. There was no effect with nystatin against *C. albicans*.

3.4. Cytotoxicity results

The cytotoxic activities of the essential oil and essential oil fractions and standard antimicrobial drugs were evaluated on WS1 cell line by XTT assay. The IC_{50} values of the compounds is represented in Table 6. According to these results, the IC_{50} values of standard antimicrobial drugs against WS1 cell line were higher than their MIC values against *Candida* species. So, it may be concluded that compounds are not cytotoxic at MIC values given.

Table 6
IC_{50} values of the antimicrobial drugs, *M. recutita* essential oil/fractions essential oil against WS1 cell line (μg/mL).

Samples	IC_{50}
AMP	> 500
TCY	383.64
NS	83.95
CEF	359.5
FLU	> 500
EOMR	92.54
EOMR-H	115.32
EOMR −DE	129.52
EOMR −DM	> 640

Definition of abbreviations: **AMP**: Ampicillin sodium; **CEF**: cefuroxime acetyl; **TCY**: tetracycline hydrochloride; **FLU**: fluconazole; **NS**: nystatin; **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DE**: dichloromethane fraction of *M. recutita* essential oil.

Table 7
IC_{50} values of *M. recutita* essential oil/fractions of *M. recutita* essential oil + antimicrobial drug combinations with additive/synergic effects against WS1 cell line (μg/mL).

Compounds	IC_{50}
EOMR + FLU	80 + 1 <
EOMR + NS	80 + 2 <
EOMR + TCY	40 + 4 <
EOMR-H + AMP	128 + 204.8
EOMR-H + FLU	80 + 2 <
EOMR-H + TCY	320 + 0.25
EOMR-DE + AMP	160 + 32 <
EOMR-DE + FLU	320 + 2 <
EOMR-DE + TCY	10 + 1 <
EOMR-DM + FLU	80 + 2 <
EOMR-DM + TCY	10 + 1 <

Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DE**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin.

The IC_{50} values of the compounds is represented in Table 7. According to the IC_{50} values obtained, *n*-hexane and diethyl ether fractions of *M. recutita* essential oil against WS1 cell line was lower than their MIC values against *Candida* species. The inhibitory effects of *n*-hexane and diethyl ether fractions of *M. recutita* essential oil against *Candida* species cannot be considered to be selective when compared with their activities on WS1 cell line. Otherwise, the IC_{50} value of dichoromethane fraction of *M. recutita* essential oil on WS1 cell line was higher than its MIC values against *Candida* species. Therefore, it may be concluded that dichoromethane fraction of *M. recutita* essential oil is not cytotoxic at MIC value given.

The cytotoxicity of essential oil/essential oil fractions with standard antimicrobial drug combinations with additive/synergic effects which were obtained by the checkerboard method were investigated against WS1 cell line. The IC_{50} values of all combinations for WS1 cell line were presented in Table 8. The IC_{50} values of *M. recutita* essential oil/*M. recutita* essential oil fractions with standard antimicrobial drug combinations with additive/synergic effects were higher than their concentrations which use in the assay. Therefore, it could be said that combinations with additive/synergic effects are not cytotoxic at concentrations with additive/synergic effects.

3.5. Aliivibrio fischeri Bioluminescence assay

M. recutita essential oil/*M. recutita* essential oil fractions with standard antimicrobial drug combinations with additive/synergic effects which were obtained by the checkerboard method were employed against *A. fischeri*. Main compounds of essential oil farnesene and α-bisabolol were also evaluated. Bioluminescence assay results were represented in Table 8.

A. fischeri bioluminescence inhibition assay, which is often chosen for determination of toxicity because of being rapid, most sensitive, easy to perform, and cost efficient (Westlund et al., 2017). Essential of *M. recutita* and its *n*-hexane fraction generally were obtained low toxicity against *A. fischeri*. Combination of diethyl ether fraction with fluconazole showed high toxicity against *A. fischeri* with 91.42% inhibition in contrast, combination with ampicillin and tetracycline were observed low toxicity, representing 57.84% and 37.20%, respectively. And main compunds of *M. recutita* essential oil, farnesene was indicated

Table 8
Inhibition% levels of *M. recutita* essential oil/fractions of *M. recutita* essential oil + antimicrobial drug combinations with additive/synergic effects against *A. fischeri*.

Combinations	Inhibition%		
	5 min	15 min	30 min
EOMR + FLU (20 + 0.25)	51.03	66.15	72.18
EOMR + NS (20 + 0.5)	45.16	60.55	68.05
EOMR + TCY (10 + 1)	44.12	67.88	74.14
EOMR-H + AMP (40 + 64)	24.79	19.25	15.74
EOMR-H + FLU (20 + 0.5)	38.6	41.44	43.66
EOMR-H + TCY (160 + 0.125)	46.7	53.22	44.54
EOMR-DE + AMP (10 + 2)	32.34	47.44	57.84
EOMR-DE + TCY (2.5 + 0.25)	20.13	29.66	37.20
EOMR-DE + FLU (80 + 0.5)	83.75	90.62	91.42
EOMR-DM + FLU (20 + 0.5)	28.45	31.90	38.88
EOMR-DM + TCY (2.5 + 0.25)	29.12	36.55	32.64
Farnesen (5 mg/mL)	46.63	37.80	30.15
α-bisabolol (5 mg/mL)	11.43	10.99	10.75
$K_2Cr_2O_7$ (4 mg/L)	74.3	77.3	79.4

Definition of abbreviations: **EOMR**: *M. recutita* essential oil; **EOMR-H**: *n*-hexane fraction of *M. recutita* essential oil; **EOMR-DE**: diethyl ether fraction of *M. recutita* essential oil; **EOMR-DM**: dichloromethane fraction of *M. recutita* essential oil; **AMP**: Ampicillin; **TCY**: Tetracycline; **FLU**: Fluconazole; **NS**: Nystatin.

35.15% while α-bisabolol representing, with 10.75%. To the best of our knowledge, essential oil of *M. recutita* and its combination activities against *A. fischeri* are reported for the first time.

4. Conclusion

To the best of our knowledge there is no report for *M. recutita* essential oil and combination with ampicillin, cefuroxime, tetracycline, fluconazole and nystatin against both of strains and clinical isolates. Furthermore, the combinations between essential oil fractions and antimicrobial agents were also demonstrated to show synergistic effect for the majority of clinic isolates of *S. aureus* and *C. albicans*. Additionally, the combinations did not show cytotoxic effects on healthy cell line at antimicrobial concentrations.

These findings are very promising and demonstrate that *M. recutita* essential oil or fraction essential oil might be potential alternatives for resistance mechanisms and would be helpful in the treatment of various infections and might reduce the side effects of synthetic drugs.

Conflict of interest

The authors declare no conflict of interest.

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