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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.