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FULL LENGTH PAPER

Antimicrobial activity of *Mentha piperita* essential oil in combination with silver ions

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Summary Peppermint (*Mentha piperita* L.) is time-honored for its medicinal properties and its antimicrobial characteristics are well established and supported in the literature. In the present study the composition and *in vitro* antimicrobial activity of *Mentha piperita* essential oil (*MpEO*) alone and in combination with silver ions (Ag^+) against the cultures of *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* is highlighted. The nature of the interaction was studied from fractional inhibitory concentration indices (FICIs) for *MpEO* plus Ag^+, calculated from checkerboard microdilution assays. On gas chromatography–mass spectrophotometry (GC–MS) analysis, *MpEO* showed a high content of menthol (34.82%). FICI values depicted a high synergism of *MpEO* with Ag^+ against *C. albicans* ($\sum$FIC = 0.48) and *E. coli* (0.40), while as indifferent effect against *S. aureus* (0.95). No antagonistic activity was seen in the strains tested in the present study. Combinational activity was further confirmed by disk diffusion and time kill curve assays. From these results we suggest that *MpEO* with Ag^+ have great potential as antimicrobial agents required to achieve an effective reduction in opportunistic pathogenic microorganisms.

1. Introduction

There is a steady increase in the incidence of antimicrobial resistance worldwide. Resistance has particularly spread in pathogens causing nosocomial infections, but also in organisms causing community acquired infections. Besides the known pathogens, drug resistance has been observed in opportunistic microorganisms [1]. Resistance to antimicrobial agents has

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resulted in morbidity and mortality from treatment failures and increased health care costs. There is little doubt that emergent antibiotic resistance is a serious global problem highlighting the need for novel antimicrobial agents and therapies. Several studies have demonstrated that natural extracts and essential oils have significant antibiotic properties [2]. *Mentha piperita* essential oil (*MpEO*) has high antimicrobial activity due to its components the major ones being α-terpinene, isomenthone, trans-carveol, pipertitinone oxide and β-caryophyllene [3]. Since ancient times, silver ions have been known to be effective against a broad range of microorganisms. These ions are used to control bacterial growth in a variety of medical applications, including dental work, catheters, and the healing of burn wounds. The antimicrobial property of Ag has been investigated more extensively than any other inorganic antimicrobial compound. Also micromolar concentrations of silver have no harmful effects on humans [4].

In view of the lack of new classes of drugs and molecular targets, drug combination therapy might be considered a viable strategy, considering the multiplicity of microbial targets against which current agents are effective [5]. Drug interactions are described as synergistic, indifferent, additive or antagonistic. An advantage of using a combination is the synergistic effect, in which the antimicrobial activity is greater than the individual contribution of each agent. In the present study, we have evaluated the *in vitro* antimicrobial activity of *MpEO* against three different opportunistic pathogens *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus*. We have also explored the possibility of synergistic interaction between *MpEO* and Ag^+ against these three microbes using checkerboard microdilution assay, disk diffusion assay and time kill curves.

2. Materials and methods

All media constituents were obtained from HiMedia (India). Extra pure grade of *Mentha piperita*essential oil (ISO 9001:2008) was purchased from Mohan Perfumery Co., Tilak Bazar, Delhi, India. DMSO and $AgNO_3$ (99.99%) was purchased from Sigma—Aldrich (USA). All inorganic chemicals were of analytical grade and were procured from E. Merck (India). *MpEO* was dissolved in 1% DMSO and $AgNO_3$ was dissolved in sterile distilled water to make up the desired concentrations.

2.1. Strains and media

Stock cultures of the bacteria and fungi were maintained on nutrient agar and Yeast extract—Peptone—Dextrose (YPD) agar slants and stored at 4 °C. *Staphylococcus aureus* MTCC 902, *Escherichia coli* MTCC 443 and *Candida albicans* ATCC 90028 were grown and sub-cultured in Mueller-Hinton broth, Luria-Bertani broth and YPD media respectively at 37 °C in orbital shaker at 200×rpm (REMI CIS 24 BL).

2.2. Gas chromatography—mass spectrophotometry analysis

Analysis of *MpEO* was carried out by GC—MS as described previously [6]. Briefly, Analysis of *MpEO* was carried out by GC—MS using a Shimadzu 2010 gas chromatograph fitted with an AB-Wax column. Helium was used as the carrier gas. Sample (0.1 mL) was injected in the splitless mode. The chemical components from the *MpEO* were identified by comparing the mass spectra from the total ion chromatogram, and retention indices using NIST and Mass Finder GC—MS libraries.

2.3. Antimicrobial susceptibility test

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the test compound that causes inhibition of visible growth (turbidity). MIC was determined *in vitro* in liquid medium by the macrobroth dilution method as per the guidelines of CLSI reference document M27-A3 [7] for fungi, document NCCLS/CLSI M11-A6 [8] for gram negative bacteria, and document CLSI M100-S15 [9] for gram positive bacteria. Positive control ciprofloxacin for bacteria and fluconazole for yeasts and negative vehicle control (1% DMSO) were also included in every set of experiments. To determine the minimal lethal concentration (MLC) values after reading the corresponding MIC values, 20 μl samples from all optically clear tubes (complete growth inhibition) plus the last tube showing growth were sub-cultured on agar plates. The plates were incubated at 37 °C for a minimum of 3 days, until growth was clearly visible in the control samples, and MLC values were determined as the lowest concentration of the test compounds for which there was no visible growth.

3. Checkerboard microdilution assay

Drug interaction was studied using the checkerboard microdilution assay in 96-well microtitre plates as described previously [10,11]. The initial concentration of cell suspension in the medium was 1×10^3 CFU mL^{-1}, with the following concentration ranges of test compounds: *C. albicans*, 0.00625—0.2 mg/mL *MpEO* and 0.000075—0.0006 mg/mL Ag^+; *S. aureus*, 0.05—0.8 mg/mL *MpEO* and 0.0006—0.0192 mg/mL Ag^+; and *E. coli*, 0.1—6.4 mg/mL *MpEO* and 0.000075—0.0006 mg/mL Ag^+.

To assess the interaction of the combination of *MpEO* with Ag^+ the data obtained spectrophotometrically was further analyzed using fractional inhibitory concentration index (FICI) defined as: FICI = FIC_A + FIC_B = MIC_A in combination/MIC_A tested alone + MIC_B in combination/MIC_B tested alone, where MIC_A and MIC_B are the MICs of compounds A and B respectively. Synergy and antagonism were defined by a FICI of $\leq$0.5 and $>$4, respectively. A FICI result of $>$0.5 but $\leq$4 was considered as a result of indifference.

3.1. Disk diffusion assays

The assay was performed as discussed previously [12]. Briefly, strains were inoculated into liquid media and grown overnight at 37 °C. Cells were then washed three times with distilled water and approximately 1×10^5 cells/ml were inoculated into half-strength molten agar media at 42 °C and poured into 100 mm diameter Petri-plates. After the top layer had solidified; sterile paper discs (4 mm) were impregnated with drugs alone, and in combination, and placed on the agar surface. The concentration range for *MpEO* and Ag^+ when applied alone was 0.16—20.48 mg/mL and 0.00094—0.015 mg/mL respectively while it was 0.0125—0.4 mg/mL and 0.00015—0.0024 mg/mL when applied in