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Reversal of resistance in bacteria underlies synergistic effect of essential oils with conventional antibiotics

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ABSTRACT

The pervasive of bacterial resistance earnestly threaten the prevention and the treatment of infectious diseases. Therefore, scientific communities take precedence over development of new antimicrobial agents. The aim of the study was to determine antimicrobial potency of three North-African essential oils *Pituranthos chloranthus, Teucruim ramosissimum* and *Pistacia lentiscus* individually, and in combination with antibiotics, to inhibit the growth of highly resistant clinical pathogen. Bacteria clinically isolated from patients, subsequently, challenged to a panel of drugs to determine the antibiotic-resistance profiles. Drugs displaying clinically irrelevant CMI were subjected to further studies in order to rescue antibiotic actions. Singular activity of essential oils and activity when combined with an antibiotic was hence elucidated. The results obtained highlighted the occurrence of strong antibacterial potential of essential oils when administrated alone. In the interactive experiment essential oils were found highly effective in reducing the resistance of Methicillin-resistant *Staphylococcus aureus* to amoxicillin, tetracycline, piperacillin, ofloxacin and oxacillin and resistance of *Acinetobacter baumannii* to amoxicillin and to ofloxacin in interactive manner. Furthermore, the results proved synergism among essential oils and both antibiotics ofloxacin and novobiocin against the Extended-Spectrum Beta-Lactamase producing *E. coli* (ESBL). Time kill kinetics was performed with a combination of sub-inhibitory concentrations to confirm the efficiency and killing rate of the combination over time. Further, the hypothetical toxicity of essential oils against human keratinocytes HaCat and murine spleenocytes were examined. The chemical composition of essential oils was assessed by GC/MS analysis and the major constituents found were sabinene, limonene, terpinen-4-ol, and β-eudesmol.

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1. Introduction

Antimicrobial resistance is a phenomenon occurring worldwide and casting a shadow over the medical miracles [26]. The inappropriate use and sometimes over-prescription of antibiotics in human medicine therapy, are considered to be the major causes for the development of bacterial resistance to antibiotics [7]. This issue has been much more deliberate in the last years with the increase in the prevalence of infections caused by multi-drug-resistant (MDR) strains [28]. Increase in microbial resistance and abundant harmful effects associated with synthetic antimicrobials, motivate researchers toward natural alternative remedies for the treatment of several infectious diseases [16]). Plant-based active compounds are among the alternative agents examined in order to supply

Abbreviation: MDR, multidrug resistant; MTT, bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; MIC, Minimum Inhibitory Concentration; MBC, minimum bactericidal concentration; ESBL, Extended-Spectrum Beta-Lactamase producing *E.coli*; MRSA, Methicillin-Resistant *Staphylococcus aureus*; GC/MS, Gas chromatography–mass spectrometry INT *p*-iodonitrotetrazolium; MHA, Mueller Hinto Agar; CLSI, Clinical and Laboratory Standard; AB, antibiotic; EO, essential oil; RPMI, Roswell Park Memorial Institute medium; DMEM, Dulbcco's Modified eagle medium Modified Eagle Medium; MMC, Minimum Modulatory Concentration; CFU, colony-forming unit.

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traditional antibiotics and synthetic antimicrobials [3]. A promising remedial potency exists for combinations of antibiotics and natural antimicrobial substances, such as plant essential oils. In fact, many studies of essential oils have been carried to develop bio-products and safer drugs of industrial interest [18]. Although, plant derived antimicrobial are less potent, this enhances the need to research a synergism interaction between plant bioactive products and antimicrobial agent. Therefore, the association between essential oils and synthetic drugs has shed light to a novel approach in controlling MDR strains and in modulating the action of antibiotics [2]. Undeniably, the technological application of a specific essential oil must be supported by scientific researches in order to prove its effectiveness as antimicrobial agent and to elucidate health outcomes linked with its future uses.

In our continued strategy to explore new interesting species from Tunisian territory that could be subsequently developed as novel bio-products industrially, we have thus designed the present work to investigate the antimicrobial potential of some commonly medicinal plants. Three plants have been included, among which tow are North-Africa endemic plants (*Pituranthos chloranthus* and *Teucruim ramosissimum*) and one is largely distributed in the mediterranean basin (*Pistacia lentiscus*).

In vitro biological activity of these three species has been reported and reviewed in literature [9,24]. Although, they are still poorly studied with regard to their antimicrobial potential. Furthermore, the interactions between their essential oils and antibiotics have not been definite earlier.

In this investigation, we aimed to determine the chemical composition of three Tunisian essential oils and to assess their antimicrobial activity against MDR isolates. The research of possible existence of a synergism interaction between *Pituranthos chloranthus, Teucruim ramosissimum* and *Pistacia lentiscus* essential oils in combination with some antibiotics was reported and discussed for the first time. Moreover, MTT assay was performed for assessing and anticipating the toxic effects of essential oils upon HaCat and spleen cells.

2. Materials and methods

2.1. Antibiotics

A panel of antibiotics was selected for this study including: amoxicillin, tetracycline, piperacillin, ofloxacin, oxacillin and novobiocin (Sigma Aldrich, St. Louis, MO, USA).

2.2. Bacterial strains

Five multidrug resistant (MDR) stains were clinical isolated from patients in Hospital of Fattouma Bourguiba, Monastir, Tunisia. All strains are isolated from urinary tract from patients, The gram negative bacteria used were Extended-Spectrum Beta-Lactamase producing *E. coli* (ESBL) and ceftazidime-resistant *Acinetobacter baumannii*. The Gram positive bacteria used were methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from three different patients (MRSA 138, MRSA 753 and MRSA 760). Cultures were obtained from Dr Maha Wesleti, Hospital of Fattouma Bourguiba, Monastir, Tunisia.

2.3. Plant material

Aerial parts of *P. chloranthus, T. ramosissimum* and *P. lentiscus* were collected in January 2015, from three different areas of Tunisian territory (ELjem, Gafsa and Zaghouen respectively). The botanical identification of plant material was approved by Professor Fathia Harzallah-Skhiri, a plant taxonomist (Institute of Biotechnology of Monastir, University of Monastir Tunisia). The voucher specimen was deposited in the Herbarium of the Laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir, Tunisia.

2.4. Essential extraction

The aerial parts of plant materials (1 Kg) were powdered and subjected to hydrodistillation for 3 h using a Clevenger apparatus. Essential oils preparations were stored in sterile dark bottle until tested and chemically analyzed by GC/MS.

2.5. Gas chromatography–mass spectrometry (GC–MS)

The analysis of the essential oil was performed with a GC–MS HP model 6980 inert MSD (Agilent Technologies, J&W Scientific Products, Palo Alto, CA, USA), outfitted with an Agilent Technologies capillary HP-5MS column (60 m length; 0.25 mm I.D; 0.25 mm film thickness), and coupled to a mass selective detector (MSD5973, ionization voltage 70 eV; all Agilent, Santa Clara, CA). Helium flow was established at 1 mL/min. The oven temperature program was as follows: 1 min at 100 °C heightened from 100 to 280 °C at 5 °C/min, and 25/min at 280 °C. The chromatograph was equipped with a split/split less injector used in the split less mode. Relative proportion of each compound was expressed as percentage obtained by peak area normalization. Identification of components was assigned by matching their mass spectra with Wiley Registry of Mass Spectral Data 7th edition (Agilent Technologies, Inc.) and National Institute of Standards and Technology 05 MS (NIST) library data. Further identifications were accomplished by comparison of their Kovats retention indexes with reference libraries and from the bibliographies.

2.6. Determination of the antimicrobial agents MICs and MBCs values

Antibacterial activity of nine antimicrobial agents included, three natural compounds (*P. chloranthus*, *T. ramosissimum, P. lentiscus* essential oils) and six antibiotics (amoxicillin, tetracycline, piperacillin, ofloxacin, oxacillin and novobiocin), were assessed by determining their Minimum Inhibitory Concentration (MIC). The micro dilution broth technique was preceded as follows: in a 96 well microtitre plates, 100 μL of the bacterial suspension (at 10^6 colony-forming units/mL) was inoculated with 100 μL of the tested antimicrobial dilution. The concentrations of examined essential oils were two-fold serial dilutions ranging from 1 to 0.015 mg/mL and for antibiotics two-fold serial dilutions ranging from 1024 to 0.25 μg/mL. The microwell plate was placed at the appropriate temperature. After 24 h of incubation, 40 μl of *p*-iodonitrotetrazolium violet (INT) (Sigma) (0.2 mg/mL) were added to each well and plates were incubated for an additional hour to allow the detection of color transformation of the growth indicators [20].

In order to assess the Minimum Bactericidal Concentration (MBC), growth inhibitory test were performed as described by Ref. [12]. 10 μL of broth from the fowling wells corresponded to (MIC value, MIC × 2 and MIC × 4) were inoculated on Muller Hinton Agar (MHA) and incubated for 24 h at 37 °C. The MBC was described as the lowest measured EO concentration of the MIC wells which inhibited the bacterial growth on MHA. All assays of MIC and MBC values were performed in triplicates.

2.7. Broth microdilution checkerboard method

The interaction among two or more antibacterial agents is involved by the checkerboard titration technique [25].

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