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In vitro synergistic efficacy of combination of amphotericin B with *Myrtus communis* essential oil against clinical isolates of *Candida albicans*

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ABSTRACT

In this study, we evaluated the antifungal activity of the essential oil from *Myrtus communis* (myrtle) leaves against *Candida albicans* (eight clinical isolates and one ATCC type strains) and different species of *Aspergillus* sp (*A. niger*, *A. parasiticus*, six isolates of *Aspergillus flavus*) using broth micro dilution assay. In addition, we evaluated the synergistic effect between the essential oil and the antifungal compound amphotericin B by checkboard micro titer assay. The essential oil was obtained from myrtle leaves by hydrodistillation method and the oil was analyzed by GC and GC-MS methods. Chemical analysis of oil revealed the presence of 70 components, representing 99.23% of the total oil. 1,8-cineole (36.1%), α-pinene (22.5%), linalool (8.4%), bornyl acetate (5.2%), α-terpineol (4.4%), linalyl acetate (4.2%) and limonene (3.8%) were found to be the major components of the oil. The antifungal evaluating showed that myrtle oil exhibited good antifungal activity against fungi. Myrtle oil showed significant antifungal activity when combined with amphotericin B.

Introduction

The aim of this study was to find an alternative for amphotericin B currently used in treatment of fungal infections. Fungal infections are one of the major causes of morbidity. Amphotericin B is considered the mainstay of therapy since the 1950s for treatment of fungal infections but it can causes some adverse effects such as hepato and nephrotoxicity (Dupont et al., 1996) and is often combined with azoles. Some *Aspergillus* species are responsible for many cases of food contaminants (Fente et al., 2001) and have been implicated in opportunistic infections of humans (Baker, 2006). *Candida albicans* causes severe opportunistic infections and colonizes mucosal surfaces of the oral, vaginal cavities and in digestive tract. It is able to cause a variety of infections depending on the nature of the host defect (Terlecka et al., 2006).

Myrtus communis (myrtle) is well known in Iran and medicinally believed to have several therapeutic properties such as antioxidant (Yadegarinia et al., 2006; Yoshimura et al., 2008), antimicrobial (Curini et al., 2004; Salih and Nadir, 1994), anti hyperglycemic (Elfellah et al., 1984; Sepici et al., 2004), analgesic (Twaij et al., 1989), anti genotoxic (Hayder et al., 2004).

Yadegarinia et al. (2006) reported the antimicrobial activity for myrtle oil against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. It was revealed that myrtle oil had antibacterial activity against *Salmonella typhimorium* (Gündüz et al., 2009), *Lactobacillus* spp., *Yersinia enterocolitica* (Bouzouita et al., 2003; Sağdiç et al., 2003) and *Helicobacter pylori* (Deriu et al., 2007).The antifungal activity of myrtle oil against *Rhizoctonia solani*, *Fusarium solani* and *Colletotrichum linele muthianum* exhibited weak fungicidal activity (Curini et al., 2004). Myrtle has a long history of use in folk medicine and infusion of its leaves has been employed as anti inflammatory antiseptic for treatment of respiratory and genitourinary disorders (Zargari, 1988).

Several studies has investigated the chemical composition of myrtle oil from leaves (Rasooli et al., 2002; Senatore et al., 2006; Tuberoso et al., 2006; Weyerstahl et al., 2006; Yadegarinia et al., 2006). Myrtle oil can be categorized on the basis of myrtenyl acetate content in two chemotypes and each group can be divided in two subgroups according to the ratio of α-pinene to myrtenyl acetate or α-pinene to 1,8-cineole (Bradesi et al., 1997).

Furthermore, the aim of the present study was to evaluate the antifungal activity of essential oil extracted from myrtle leaves against clinical isolates of *C. albicans* and different species of *Aspergillus* sp as well as to identify the chemical composition responsible for such activity. Also, in attempt to develop a safe and more powerful antifungal agent from medicinal plants, the combined effect of myrtle oil with amphotericin B were determined.

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Material and methods

Plant materials

Fresh leaves of *Myrtus communis* L. were collected from Barij Essence research farm in May 2008. The voucher specimen was prepared and identified by Dr. Mozaffarian, Research Institute of Forest and Rangelands, Tehran, Iran. The herbarium sample was deposited at the Herbarium of Agriculture Department, Research Center of Barij Essence, Kashan, Iran under number 142-1.

Extraction, isolation and identification of the oil

The fresh leaves of myrtle were hydrodistilled for 6 h using a Clevenger type apparatus. The essential oil was dried by anhydrous sulfate and was kept into closed bottle until the analysis.

The oil analysis was carried out using GC and GC/MS. The GC apparatus was Agilent technology (HP) 6890 system, capillary column of HP-5MS (60 m × 0.25 mm, film thickness 0.25 μm). The oven temperature program was initiated at 40 °C, held for 1 min then raised up to 230 °C at a rate of 3 °C/min held for 10 min. Helium was used as the carrier gas at a flow rate 1.0 ml/min. The detector and injector temperatures were 250 and 230 °C, respectively. GC/MS analysis was conducted on a HP 6890 GC system coupled with 5973 network mass selective detector with a capillary column the same as above, carrier gas helium with flow rate 1 ml/min with a split ratio equal to 1/50, injector and oven temperature programmed was identical to GC. The compounds of the oil were identified by comparison of their retention indices (RI), standard materials, mass spectra fragmentation with those on the stored Wiley 7n.1 mass computer library, and NIST (National Institute of Standards and Technology) (Adams, 2001).

Fungal isolates

Tested fungi include eight clinical isolates of *Candida albicans*, ATCC type strains (10231), *Aspergillus niger* ATCC 16404, *Aspergillus parasiticus* ATCC 15517, six isolates of *Aspergillus flavus*.

Determination of minimum inhibitory (MIC) and lethal (MLC) concentrations

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of oil were determined by micro broth dilution assay. The fungi were grown at 30 °C on sabouraud dextrose agar (Merck) plates. Inoculate for antifungal assays were prepared by diluting the colonies in 0.85% NaCl solution, adjusted to 0.5 MacFarland and confirmed by spectrophotometric reading at 530 nm (Transmittance 85%). Cell suspension was finally diluted to 10^5 CFU/ml for use in assay. Minimum inhibitory concentrations were carried out in RPMI 1640 medium using a tissue culture test plates (96 wells). The oil was serially diluted twofold with 10% DMSO which contains 32- 0.0125 μl/ml of myrtle oil. Amphotericin B (Sigma) was used as the reference antimycotic control (64-0.125 μg/ml). After shaking, 100 μl of oil was added to each well. 100 μl of diluted suspensions was added to each well and incubated at 35 °C for 24 h (*C. albicans*) and 48 h (fungi). MIC was defined as the lowest concentration of compound that inhibits fungi after 24 or 48 h. MLC value was the first well that showing no growth on sabouraud dextrose Agar (Marchetti et al., 2000). Each test was performed in duplicate.

Check board titer test for fungi

Eight serial twofold dilutions of myrtle oil and amphotericin B was prepared and used in the MIC tests. Fifty μl of each dilution of essential oil was added to the wells of 96-well plates in vertical orientation and 10 μl of amphotericin B dilution was added in horizontal orientation. 100 μl from suspension of organism (10^5 CFU/ well) was added to each well and incubated for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of oil and amphotericin B divided by the MIC of oil or amphotericin B alone. FIC index (FICI) was calculated total of the FIC of the myrtle oil and FIC of amphotericin B. The FICI was interpreted as a synergistic effect when it was ≤ 0.5, as indifferent when it was > 0.5-2 and as antagonistic when it was > 2.0 (Davidson and Parish, 1989). The synergic effect is shown graphically by applying the Isobole method (Wagner and Ulrich-Merzenich, 2009).

Results and discussion

To identify the comosition of the tested oil, we analyzed the oil derived from hydrodistillation of myrtle oil by GC and GC-MS, even thought myrtle oil has been evaluated extensively. The composition of this oil differs greatly among myrtle oil, possibly due to analytical technique, chemotype, culture climate that may affect on biological activities. The main components of myrtle oil were 1,8-cineole (36.1%), α-pinene (22.5%), linalool (8.4%), bornyl acetate (5.2%), α-terpineol (4.4%), linalyl acetate (4.2%) and limonene (3.8%) (Table 1).

Recent reports have been shown that Turkian myrtle oil contains linalool (36.5%) and linalyl acetate (16.3%); whereas α-pinene, 1,8-cineole are the main components of Italian samples (Senatore et al., 2006; Tuberoso et al., 2006). α-pinene is abundant component of Iranian myrtle oil whereas the second most abundant constituent is different in reports (Weyerstahl et al., 2006; Yadegarinia et al., 2006). Yadegarinia et al. (2006) reported 1,8-cineole as the second major components as like as Italian myrtle oil, while limonene is the second major component of Weyerstahl et al. (2006) study. In our study the amount of linalool and linalyl acetate, limonene is 8.4%, 4.2% and 3.8% respectively and α-pinene is the second most abundant constituent of the myrtle oil in contrast with other studies (Table 1).

The antifungal activity of myrtle oil was determined against filamentous fungi and clinical isolates of *C. albicans*. MIC assay results of the tested oil against fungi (*C. albicans* and *Aspergillus* sp) are shown in Table 2. Amphotericin B was used as the positive control in this experiment. MIC and MLC values of amphotericin B were 1-2 and 2 μg/ml for *C. albicans* and 4-8 and 8 μg/ml for *Aspergillus* sp, respectively. The MICs of myrtle oil did not differ significantly with regard to *C. albicans* and *Aspergillus* sp. The MIC and MLC values of myrtle oil ranged from 8-16, 16-32 μl/ml against tested fungi. The MIC values showed that the oil was quite active against fungi at these concentrations.

To explore the possibility of developing a more powerful combination therapy of myrtle oil with amphotericin B, the checkboard micro titer test was performed (Table 3, Fig. 1). The MIC of amphotericin B alone against *C. albicans* was lowered from two to 0.06 μg/ml when the essential oil was added at concentration 4 μl/ml. The FIC of myrtle oil in combination with amphotericin B against *C. albicans* 10231 were 0.25 μl/ml and 0.03 μg/ml for amphotericin B. The FIC of myrtle oil in combination with amphotericin B against *A. niger* was 0.25 μl/ml and 0.015 μg/ml for amphotericin B. The FIC index showed marked synergism of the oil and amphotericin B combination against *C. albicans* and *A. niger*.