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Essential oil nanoemulsions of wild patchouli attenuate multi-drug resistant gram-positive, gram-negative and *Candida albicans*

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ABSTRACT

Patchouli essential oil (EO) from *Pogostemon cablin* (PC), is widely used in perfumery, cosmetic and pharmaceutical industries. Being a well-known commodity and used in many products, pharmacological and biological properties of patchouli EO is well studied. However, wild patchouli species, *P. heyneanus* (PH) and *P. plectranthoides* (PP), are least studied in all aspects. The present study is aimed at preparation and characterization of EO nanoemulsions of wild patchouli and to specifically study their antimicrobial and biofilm eradication activities against *Shigella flexneri*, multi-drug resistant (MDR) *Staphylococcus aureus, Streptococcus mutans* and *Candida albicans* in comparison with the commercial patchouli EO. The commercial and wild patchouli EOs showed wide variations in their chemical constituents as patchouli alcohol, acetophenone and atractylone were found to be the principal component of commercial (PC) and wild patchouli (PH and PP) EOs respectively. Among the EO nanoemulsions, PH EO nanoemulsion showed good stability and size on the first day and 45th day of preparation. Similarly, EO nanoemulsion of PH exhibited better antibacterial and anti-candida activities against the studied microbes than the other two EOs. In case of biofilm eradication activity, EOs of all three species eradicated the biofilms of *S. mutans* and *S. aureus* at a range of 30–50%. The study suggests that EO of PH possesses better antibacterial and anti-candida activities compared with the other two species.

1. Introduction

Patchouli [*Pogostemon cablin* (Blanco) Benth.] of the family Lamiaceae, is a medicinal plant used in centuries old Ayurvedic, Greek and Traditional Chinese medical systems for both external and internal applications (Peter, 1997). Being an aromatic plant, this species yields commercially valuable 'Patchouli essential oil' from the leaves. Patchouli essential oil (EO) is one of the important EOs used in perfumery, cosmetic, pharmaceutical and food industries due to its long lasting and strong fixative properties. As it is used in many products, it has great demand globally, and thus, patchouli plant has been commercially cultivated in many parts of the world, particularly in South and Southeast Asian countries.

Apart from many therapeutic uses (Swamy and Sinniah, 2015), patchouli EO is used for treating skin infections and considered as an excellent skin care agent because of its anti-inflammatory, antiseptic, antibacterial, antifungal and antiviral activities (Akhila and Tewari, 1984; Chen et al., 2013; Yang et al., 1996). The extracts, and important constituents (patchouli alcohol and pogostone) of the EO of *P. cablin* (PC) (commercial patchouli plant) have been proved to be effective against many gram positive and gram negative bacteria, fungal species, particularly many strains of *Candida albicans* and viral species (Swamy and Sinniah, 2015). There are two wild *Pogostemon* species namely, *P. heyneanus* Benth. (PH) and *P. plectranthoides* Desf. (PP) in India known as 'wild patchouli' or 'Indian patchouli' (Murugan and Livingstone, 2010; Suganya et al., 2015). Though the chemical constituents of wild patchouli EOs are fairly known (Anjana and Thoppil, 2013; Murugan et al., 2010; Suganya et al., 2015), their antimicrobial activities are least investigated. Except for a recent article on anti-biofilm activity of the EO of PH against *Streptococcus pyogenes* (Nithyanand et al., 2015), there are no antimicrobial studies reported on PH. Similarly, the EO of PP also needs to be evaluated against many microbes in order to assess their suitability to be used as an antimicrobial drug. The present investigation is aimed at the investigation of antibacterial activities against gram positive and gram negative bacteria, *C. albicans* and biofilm eradication effects of the EOs of the wild patchouli (PH and PP) in comparison with the EO of commercial patchouli (PC).

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2. Materials and methods

2.1. Plant materials and extraction of EOs

EO of commercial patchouli was purchased from the Horticulture Division, University of Agricultural Sciences, Bengaluru, India. Leaves of wild patchouli plants, PH and PP were collected from Coorg, Karnataka and Munnar, Kerala respectively. Herbarium specimens of PH (*Murugan* 106) and PP (*Murugan* 73) were prepared and deposited in the Herbarium of SASTRA University, India. The specimens were identified by referring the revision on *Pogostemon* (Bhatti and Ingrouille, 1997) and authenticated by matching the voucher specimens with the authentic herbarium specimens (virtual herbarium) deposited in the Herbaria of the Royal Botanic Gardens (K), Kew, London and Madras Herbarium (MH), Botanical Survey of India, Coimbatore, India.

Leaves of PH and PP were shade dried for two weeks. The shade dried leaf samples were hydro-distilled in a Clevenger apparatus for about 8 h. Leaves to water ratio (w/v) used for distillation was 1:8. After distillation, EOs were collected and a pinch of anhydrous sodium sulphate was added to remove moisture content in the EOs and then stored at 4 °C for further analyses and investigations.

2.2. Chemical analyses of essential oils and compounds identification

EOs of PC, PH and PP were qualitatively and quantitatively analyzed by GC–MS and GC-FID respectively following the same conditions used in our recent publication (Sriramavaratharajan et al., 2016). The volatile components of the EOs were identified by comparing the calculated retention index (RI) of the GC peaks obtained using homologous series of *n*-alkanes (carbon range from C_8 to C_{20}) with those reported in literature (Adams, 2007). The mass spectra of the peaks were also matched with standard spectra (Adams, 2007) and NIST 2005 MS library. Peak area percentages were calculated from FID response without the use of correction factors.

2.3. Preparation and characterization of nanoemulsions

EO nanoemulsions were prepared using EOs, non-ionic surfactant (Tween 20), co-surfactant (Propylene glycol), emulsifier (Lecithin) and water. Initially, coarse emulsions were prepared by adding EOs with surfactant followed by adding co-surfactant, emulsifier and water under gentle stirrer at room temperature. Then, the coarse emulsions were finely homogenized with a Homogenizer (Remi Instruments, RQT-127A, India) operated at 8000 rpm for 10 min. After, homogenization, the emulsions were stored at room temperature under dark condition.

The EO nanoemulsions were characterized with the help of Dynamic Light Scattering (DLS) experiment using Malvern Zetasizer (Malvern Instruments Ltd.) which determines the particle size distribution and stability (zeta potential). The nanoemulsions were diluted 20 times in deionised water to reduce the multiple scattering effects. Zeta potentials of the nanemulsions were measured using the same instrument. The measurements were carried out in triplicate.

2.4. Microbial strains

For the present study, a gram-negative bacterium *Shigella flexneri* (MTCC 1457), gram-positive *Streptococcus mutans* (MTCC 497), multi-drug resistant *Staphylococcus aureus* (clinical isolate) and a pathogenic fungi *Candida albicans* (MTCC 7253) were used.

2.5. Anti-bacterial activity

Minimum Inhibitory Concentration (MIC) was determined by two fold dilution method described in Courvalin et al. (2006). Briefly, *S. flexneri*, *S. aureus*, *S. mutans* were inoculated in LB broth, Tryptic Soy broth, Brain Heart Infusion broth respectively and incubated overnight at 37 °C. The bacterial suspensions were adjusted to 0.5 Mcfarland standard (approximately 10^7 cfu/ml) in PBS. In a 96 well plate, EO nanoemulsions in a concentration ranging from 0.3 to 25 mg/ml were serially diluted in media (100 μl in each well) and 10 μl of inoculum were added and incubated for 24 h at 37 °C. After 24 h, the plates were analyzed for bacterial growth by measuring OD at 595 nm in microtitre plate reader (iMarkBIORAD).

2.6. Anti-candida activity

Minimum Inhibitory Concentration (MIC) against *C. albicans* was determined by microdilution technique (Ellof, 1998) with slight modifications. The inoculum of *C. albicans* was prepared using saline solution and adjusted to the absorbance of 0.08–0.1 at 625 nm (approx. 5×10^5 CFU/ml). Briefly, 100 μl of prepared EO nanoemulsion was added to 100 μl of Sabouraud Dextrose Broth (SDB) and serially diluted with a concentration ranging from 3.125 to 50 mg/ml. 10 μl of inoculums was added to each well and incubated at room temperature for 48 h. After 48 h, MIC was determined by visual method, the culture from the wells were transferred to Sabouraud Dextrose Agar (SDA) plates and incubated for 48 h to confirm the MIC.

2.7. Biofilm eradication

The Minimum Biofilm Eradication Concentration (MBEC) assay was done as described in Kwieciński et al. (2009) with slight modification. Briefly, in 96-well microtiter plate, the biofilm formation was allowed for 24 h, followed by removal of spent media from the wells and washed gently with phosphate-buffer saline (PBS) to remove the planktonic cells. A 100 μl of EO nanoemulsions serially diluted with media in a concentration ranging from 1.56 to 50 mg/ml were added to each well and incubated for 4 h. The wells without EO nanoemulsions were taken as negative control. After 4 h, the wells were stained using crystal violet to determine the MBEC.

2.8. Crystal violet assay

Crystal violet assay was done as described by Yadav et al. (2015). In 96-well microtiter plates, the biofilms treated with EO nanoemulsions were stained with crystal violet. Briefly, the plates were fixed with methanol followed by addition of 200 μl of 0.2% crystal violet to the wells to stain the biofilm for 15 mins. The excess stain was removed using PBS and the plates were allowed to dry for 30 mins. The absorbed stain was eluted using 33% acetic acid and biofilm formation was quantified at absorbance of 570 nm in microtitre plate reader (iMarkBIORAD).

2.9. Confocal laser scanning microscopy

Confocal Laser Scanning Microscope (CLSM) was done for qualitative assessment of EO nanoemulsions against *S. flexneri, S. aureus, S. mutans and C. albicans*. The biofilm was allowed to form on the glass slides similar to the 96 well plate. The preformed biofilms were treated with EO nanoemulsions for 4 h. The slides were taken out, wiped off one side of the slide and dried. The stock solution of the dye [Baclight LIVE/DEAD stain kit (L7012 molecular probes)] were prepared according to the manufacturer's instructions. Then the slides were rinsed using the stock solution and air dried for