ORIGINAL ARTICLE

Evaluation of *in vitro* antimicrobial potential and phytochemical analysis of spruce, cajeput and jamrosa essential oil against clinical isolates

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Abstract

Objective: To investigate the phytochemical constituents and thin-layer chromatography (TLC) bioautography of *Picea abies* (spruce), *Melaleuca* spp. (cajeput), and *Cymbopogon khasans* (jamrosa) essential oils. The *in vitro* antimicrobial potential was also determined against fungal isolates and Gram-positive and Gram-negative bacterial strains isolated from human infections. **Materials and Methods:** A preliminary phytochemical analysis was performed. The antimicrobial potential of essential oil from spruce, cajeput and jamrosa was evaluated by agar well diffusion method against clinical isolates. The antibacterial effect was investigated using the TLC-bioautographic method. **Results:** Phytoconstituents analysis demonstrated the presence of few phytochemicals including steroids, reducing sugars and cardiac glycosides in all the tested samples. The essential oils were further investigated for its antimicrobial activity against 11 bacterial clinical isolates and 4 fungi, respectively. The oils showed broad antimicrobial activity against Gram-positive and Gram-negative bacteria and fungal isolates including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter* spp., and *Aspergillus niger*. The highest *in vitro* inhibitory activity was observed for *S. aureus* with a maximum zone of inhibition (27 ± 0.05 mm in diameter) for cajeput essential oil followed by 23 ± 0.07 mm for jamrosa oil against *Acinetobacter* spp. Among fungal isolates tested, the growth of only *A. niger* and *Aspergillus* spp. was inhibited. TLC bioautography assay demonstrated two big spot and one small spot observed at R_f values 0.083 and 0.33 (active spots) and 0.47 (inactive spot) against *S. aureus* 3 and *S. aureus* 1. **Conclusions:** It can be concluded that the essential oils from cajeput and jamrosa possessing antimicrobial potential against clinical isolates can be used in the treatment of various microbial infections.

Key words: Cajeput, jamrosa essential oil, *in vitro* antimicrobial activity, phytochemical analysis, spruce, thin-layer chromatography bioautography

INTRODUCTION

Essential oils are complex mixtures of volatile compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are produced by living organisms from a whole plant or plant part and usually obtained by expression, fermentation or extraction, but the method of steam distillation is most commonly used for commercial production. An estimated 3000 essential oils are known, of which 300 are commercially important in flavors and fragrance market.[1] Essential oils have become an integral part of everyday life and are used as food flavorings, feed additives, flavoring agents and in the compounding of cosmetics and perfumes. Furthermore, they play a crucial role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants.

Antimicrobial resistance is a global problem and is posing serious threat in the treatment of infectious diseases across the world. These infections are currently claiming around 50,000 lives every year across US and Europe, leaving

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around hundreds of thousands dying in other areas of the world. The clinical isolates are becoming multiple drug resistant, *Staphylococcus aureus* (resistant to ciprofloxacin, erythromycin, clindamycin, gentamicin, trimethoprim/ sulphamethoxazole, linezolid, and vancomycin),[2] and *Staphylococcus pyogenes* isolates (resistant to erythromycin and penicillins).[3] When infections become resistant to first-line antimicrobials, treatment has to be switched to second - or third-line drugs, which are always expensive. Faced with such a challenge, there is need to develop alternative approaches in addition to the search for new antimicrobial compounds. Down the ages essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened alternative remedies for the treatment of many infectious diseases.[4] Therefore, researchers are screening natural products, as standardized plant extracts or as pure compounds, for their potential uses as new leads to develop better antimicrobial drugs.

Spruce (*Picea abies*), also known as Norway spruce or Siberian pine, belongs to family Pinaceae, is the most widespread conifer tree in the Romanian forests.[5] They are also found in the northern temperate and boreal (taiga) regions of the earth. Spruces are large trees, about 60-200 feet tall when mature, and can be distinguished by their whorled branches and conical form. The needles of spruce trees are attached singly to the branches in a spiral fashion, each needle on a small peg-like structure known as pulvinus. Spruce oil is mainly made up of chemicals like bornyl acetate with smaller amounts of limonene, borneol, camphor, α- and ß-pinene, camphene, 3-carene, and ß-phellandrene. Spruce essential oil also possess medicinal and healing properties, as it has important phytonutrient, lignin, titled 7-hydroxymatairesinol, having a major role in the health of the human body. Some of the earlier studies had shown the antimicrobial activity of *Picea* spp. against *Proteus mirabilis*, *S. aureus*, and *Staphylococcus epidermidis*.[6]

Cajeput (*Melaleuca cajuputi* Powell), belonging to *Melaleuca* species from Myrtacea family. It is native to Peninsular Malaysia. Some *Melaleuca* species are rich inessential oils. The volatile oil is obtained by distillation using the leaves, which is transparent, emerald-green color with a strong, and persistent odor. The essential oil has been reported to be used for medicinal purposes, as insect-repellents and in cosmetics. It also possess antibacterial, anti-inflammatory and insect-repellent properties and are also used in cooking, as fragrance and freshening agent, detergents and perfumes.[7] Carvacrol, thymol, linalool, α- and β-thuyone, camphor, 1,8-cinole,α-phellandrene, limonene, carvone, menthol, and menthone were the major components of cajeput essential oil as reported by Bakkali *et al*.[8]

Jamrosa (*Cymbopogan khasans*), belongs to family Poaceae. It is a medium-sized aromatic grass possessing a rose-grassy scent and is widely grown in central and southern parts of India. The origin of jamrosa grass is in India and it is widely grown in areas such as Chhattisgarh, Maharashtra, and Madhya Pradesh. Jamrosa oil obtained by steam distillation is composed of 75% geraniol and 20% geranyl acetate. The essential oil is extensively used in the perfumery, pharmaceuticals, soap, and cosmetics industries. It also possesses antibacterial and insecticidal activity.[9]

The present study relates to phytochemical analysis, *in vitro* antimicrobial potential and total phenolic content of spruce, cajeput and jamrosa essential oils against fungal isolates and Gram-positive and Gram-negative bacterial strains isolated from human infections.

MATERIALS AND METHODS

Acquisition of Essential Oils

Three essential oils obtained from Ligne International Ltd., India (commercial producer of plant essential oils) were used in this study. The essential oil includes *P. abies* (spruce), *Melaleuca* spp. (cajeput) and *C. khasans* (jamrosa). These oils were selected based on literature survey and their use in traditional medicine. As per manufacturer's information, the essential oils were prepared by steam distillation. The oils were further distilled by rotary evaporator. The essential oils were dissolved in methanol (0.3 ml oil/ 2 ml methanol) and stored at 4°C in sterile vials until further analysis.

Test Organisms and Growth Conditions

The microbial cultures used include clinical isolates of *Escherichia coli*, *Enterobacter* spp., *Salmonella typhi*, *Salmonella paratyphi*, *S. aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures. They are sub-cultured on to nutrient broth for 24 h prior to testing. Three fungal isolates used in the study includes *Aspergillus niger*, *Candida albicans*, and *Rhizopus nigricans*. The cultures were maintained on potato dextrose agar at 4°C. These microbial isolates served as test pathogens for antimicrobial activity assay.

Phytochemical Analysis

All the selected essential oils were dissolved in methanol (0.3 ml oil/ 2 ml methanol) and were subjected to phytochemical analysis to ascertain the presence of various phytoconstituents using wet reactions. The phytoconstituents includes alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatanins, reducing sugars, saponins, steroids, and tannins.[10,11]

Evaluation of Antimicrobial Potential

Qualitative screening of essential oils for antimicrobial properties was done by the agar well diffusion method with minor modifications.[12] Nutrient agar (for bacterial cultures) and potato dextrose agar plates (for fungal cultures) were inoculated with 0.1 ml of each organism (1×10^8 CFU/ml) and spreaded well with sterile swabs. Subsequently, wells of 8 mm diameter were punched on the surface of the agar. Each well was filled with 50 µl of oil in methanol and allowed to diffuse at room temperature for 2 h. The plates were incubated for 24 h at 37°C for bacterial cultures and 28°C for fungal cultures tested. Control was maintained with the same volume of methanol while standard antibiotic discs of vancomycin (30 µg) and imepenem (10 µg) were used as the positive controls. After incubation, the zone of inhibition was measured in millimeters by Vernier scale. All tests were performed in triplicate and the antimicrobial activity was expressed as the mean of inhibition with their standard deviation.

Thin-layer Chromatography (TLC) Bioautography Assay

Cajeput essential oil exhibiting significant antimicrobial potential against *S. aureus* 3 and *S. aureus* 1 as determined by agar well diffusion method was analyzed using TLC bioautography assay.[13]

Statistical Analysis

The antimicrobial activity of oils was indicated by the zone of inhibition. The resultant clear zones around the agar wells were measured in millimeters (mm). All the experiments were conducted in triplicate, and the data are presented as mean values ± standard deviation.

RESULTS

Phytochemical analysis of the three essential oils was tested by colorimetric reactions which revealed the presence of steroids, reducing sugars and cardiac glycosides in all the three tested essential oil samples. It was observed that saponins and coumarins and lactones were positive in both cajeput and jamrosa essential oils while found to be absent in spruce essential oil [Table 1]. None of the essential oil samples tested had shown the presence of anthraquinones, flavonoids, tannins and phlobatannins.

The qualitative screening of the antimicrobial properties of the three essential oils exhibited zones of inhibition of microbial growth. The antimicrobial activity was noticed against a total of 11 different bacterial clinical isolates and 4 fungal species. The results from the agar well diffusion method revealed that all the oils tested showed significant to moderate antimicrobial activity toward all tested strains except *S. typhi*, *S. paratyphi*, *C. albicans*, and *R. nigricans*. Results [Table 2] showed that the most susceptible organism was *S. aureus* which shows the maximum zone of inhibition (27 ± 0.05 mm in diameter) for cajeput essential oil followed by 23 ± 0.07 mm for jamrosa oil against *Acinetobacter* spp. Spruce oil exhibited 21 ± 0.11 mm of the zone of inhibition against *Klebsiella* spp. The growth of *Aspergillus* spp. and *A. niger* was only inhibited by spruce and cajeput essential oil. The control plate did not exhibit inhibition on the tested bacteria. Among the fungi, *A. niger* was the most sensitive while *C. albicans* and *R. nigricans* showed no zones of inhibition [Table 3]. It was observed in most cases, oil was

Table 1: Phytochemical analysis of spruce, cajeput and jamrosa EO

Phytoconstituents	Spruce EO	Cajeput EO	Jamrosa EO
Flavonoids	-	-	-
Steroids	+	+	+
Tannins	-	-	-
Saponins	-	+	-
Reducing sugar	+	+	+
Cardiac glycoside	+	+	+
Anthraquinone	-	-	-
Phlobatanins	-	-	-
Coumarins and lactones	-	+	+

+: Present, -: Not present, EO: Essential oil

Table 2: Antibacterial activity of spruce, cajeput and jamrosa EO determined by agar well diffusion method

Test microorganisms	Zone of inhibition (in mm)		
	Spruce EO	Cajeput EO	Jamrosa EO
Acinetobacter spp.	12±0.085	18±0.12	23±0.07
Escherichia coli 1	12.7±0.05	17±0.085	13±0.11
Klebsiella spp. 1	11±0.12	17±0.07	15±0.05
Klebsiella spp. 2	21±0.11	9.5±0.05	-
Klebsiella spp. 3	19±0.07	-	14±0.12
Salmonella typhi	-	-	-
Salmonella paratyphi	-	-	-
Staphylococcus aureus 1	7±0.13	26±0.12	11±0.13
Staphylococcus aureus 2	9±0.085	23±0.07	10±0.11
Staphylococcus aureus 3	12±0.12	27±0.05	17±0.13
Pseudomonas aeruginosa	-	5±0.085	9.5±0.12

Zone of inhibition is expressed as mean±standard deviation, -: No inhibition, EO: Essential oil

Table 3: Antifungal activity of spruce, cajeput and jamrosa EO

Test microorganisms	Zone of inhibition (in mm)		
	Spruce EO	Cajeput EO	Jamrosa EO
Aspergillus spp.	0.5±0.11	2±0.12	-
Aspergillus niger	2±0.05	19.5±0.12	-
Candida albicans	-	-	-
Rhizopus nigricans	-	-	-

Zone of inhibition is expressed as mean±standard deviation, -: No inhibition, EO: Essential oil

found to be a more effective on bacteria than fungi. No inhibition was observed for two bacterial clinical isolates (*S. typhi*, *S. paratyphi*) and two fungal isolates (*C. albicans* and *R. nigricans*).

In bioautography assay, the components were separated onto the surface of chromatographic plates which are then overlaid with molten bacterial agar to screen for antibacterial activity. In this method, cajeput essential oil that exhibited large inhibitory zone against *S. aureus* 3 and *S. aureus* 1, was separated by solvent system toluene and ethyl acetate (93:7 v/v) on TLC. Inhibition zones against the growth of *S. aureus* 3 and *S. aureus* 1 was observed on the TLC plates of cajeput oil as clear spots on pink background when sprayed with aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride. The components of the cajeput oil separated into two big spots and one small spot. The two big spots at R_f values 0.083 and 0.33 were found to be active. Both the active spots were found to exhibit antibacterial activity against tested *S. aureus* 3 and 1. Whereas, the small spot at R_f value.0.47 showed no visible inhibitory zone against *S. aureus* 3 and 1, respectively. TLC analysis revealed the presence of saponins when sprayed with Ehrlich reagent which on heating at 100°C for 10 min will give red-brown zones in visible light (data not shown). It is likely that the zone of inhibition observed was due to one or more active compounds which overlap possibly due to the solvent system used for screening.

DISCUSSION

Medicinal plant essential oils have attracted a lot of attention within the scientific community mainly due to the growing concern of multidrug resistance stains. Plant extract and oils are thus can be used in the treatment of infectious diseases caused by the numerous resistant microorganisms present around us. Based on these facts, the present study emphasizes on three plant essential oils and their screening for the presence of phytoconstituents, antimicrobial potential and separation of bioactive fractions by TLC bioautography.

Phytochemical analysis conducted on the essential oils revealed the presence of active medicinal chemical constituents. The three tested oils possess important phytochemicals such as steroids, reducing sugars, cardiac glycosides, saponins and coumarins and lactones. Glycosides possess vast therapeutic efficacy as they are found in almost every medicinal plant. Extracts containing carbohydrates, glycosides and coumarins are known to exert a beneficial action on immune system by increasing body strength and hence are valuable as dietary supplements. Our results were in agreement with Amin[14] who observed that cajeput oil contains saponins but absence in alkaloid and flavonoid. Saponins have the property of precipitating and coagulating red blood cells. Saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties, and bitterness.[15,16] Steroids have been reported to have antibacterial properties[17] and they also help in regulating the immune response.[18] Steroids are found to be helping in Coumarins can be suggested to be beneficial for hyperproliferative skin diseases on the basis of their antimicrobial and anti-inflammatory effects.[19]

In the present study, agar well diffusion method was used to analyze the *in vitro* antimicrobial potential of the essential oils. The three essential oils tested in the present study exhibited varying degree of inhibitory effect against the selected bacterial and fungal clinical isolates. The antimicrobial activity of spruce essential oil tested against *S. aureus*, *Klebsiella* spp. and *A. niger* has also been reported by Valeria *et al*.[5] Better susceptibility towards *C. albicans*, than the other tested mold strain (*A. niger*) for Spruce oil was reported by Valeria *et al*.[5] which is contrary to our results. In this study, cajeput and jamrosa oils exhibited medium to strong antimicrobial activity against *S. aureus*, *Acinetobacter* spp., *Klebsiella* spp. and *A. niger*. Several studies[20,21] have shown that cajeput and jamrosa essential oils had strong and consistent inhibitory effects against various tested isolates. Carson *et al*.[20] reported antifungal activity against *C. albicans* for cajeput oil. However, the present study showed no inhibition in case of fungal isolates *C. albicans* and *R. nigricans*. This difference is may be due to the composition of essential oil. Essential oil composition has been found to be influenced by the climatic, seasonal and geographical conditions, growth regulators, and harvest periods.[22,23]

TLC bioautography method is an easy and cost-efficient technique used to separate and detect the active components present. The important benefits of this technique include low-cost analysis, high-throughput screening of samples, and minimal sample preparation.[24] The bioactive components of cajeput essential oil were separated on TLC followed by TLC bioautography against two clinical isolates, *S. aureus* 3 and *S. aureus* 1. In the present study, TLC analysis showed the presence of steroids as a main component by spraying with spray reagent. These findings corroborated with the observations of Horvath *et al*.[25] who reported the two blue zones detected at R_f = 0.24 and R_f = 0.37 which showed significant antibacterial effects against *Vibrio fischeri*. The

observed inhibition zones were may be due to one or more active compounds which overlap possibly due to the solvent system used for screening. No zone of inhibition was observed for spots with R_f value of 0.47 in cajeput on reference chromatogram. This could be attributed to evaporation of the active components, photo-oxidation or insufficient amount of the active component.[26]

Based on the findings of this investigation, it may be concluded that the essential oils from cajeput and jamrosa possessing antimicrobial potential against various clinical isolates, can be used in the treatment of various microbial infections.

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