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CHEMOVARIANT OF *MELALEUCA QUINQUENERVIA* (CAV.) S.T.BLAKE AND ITS ANTI-PATHOGEN ACTIVITY

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ABSTRACT

Melaleuca species display chemotypic variations due to biotic and abiotic influence. Characterization of the chemical variant in a volatile oil is of prime importance for authentication of the sample and for evaluating its pharmacological activity. A study aimed at identifying a chemical variant of the essential oil extracted from *Melaleuca quinquenervia*, through Gas Chromatography-Mass spectrometry (GC-MS) analysis was conducted. E-Nerolidol, a sesquiterpene alcohol and this component was identified as the characteristic chemical variant of *Melaleuca quinquenervia* (Niaouli) was identified. Also, the essential oil of *M.quinquenervia* extracted on dry weight basis was screened against Oral and skin pathogens. The volatile oil displayed maximum growth inhibitory activity against dental and skin pathogenic strains. The potent antimicrobial activity exhibited could be due to the enhanced bacterial permeability and susceptibility to Nerolidol, a major component of *M.quinquenervia* essential oil.

KEYWORDS: Niaouli oil, E-Nerolidol, Pathogenicity.

INTRODUCTION

The genus *Melaleuca* of Myrtaceae family comprises of approximately 290 species and a vast majority of them are endemic to Australia.[1,2] Trees and shrubs of this genus are hardy and are adaptable to a wide range of habitats and soils. They are one of the most prominent species established on degraded area for land reclamation, mitigation of salinity, controlling water logging, water and wind erosion, biodiversity improvements, carbon sequestration. Also, potential to increase farm income through essential oils, bark, stem, twigs, dry foliage and bioenergy. Thus they display diversity in form, adaptability and utility. The aromatic trees of this genus are known for their chemical variants and have been identified as distinct chemotypes. Plants synthesize different group of secondary metabolites called chemotypes depending on the environmental conditions, duration and type of stress, genetic plasticity etc. Predominantly, industries are based on extraction of essential oils from leaves of *Melaleuca* species viz., *Melaleuca quinquenervia*, *Melaleuca alternifolia*, *Melaleuca leucadendron*, *Melaleuca ericifolia* and *Melaleuca Cajeputi* sub sp. *Cajeputi*. *Melaleuca quinquenervia* is one of the main commercial species cultivated widely in countries including South America, Central America, Madagascar, Philippines, North America and others. This species was first introduced into Southern florida in the late 1800s. It is also notable that *Melaleuca quinquenervia* comprises of several chemotypes.[1,2,3,4,5,6,7] Four occur in Madagascar, consisting of 1, 8-cineole-37%; Virdiflorol: 20%; terpinolene: 5%; Viridiflorol: 48% and E-nerolidol: 87%. Other two chemotypes are from Australia & New Guinea: one is comprised of E-Nerolidol: 74-95%; Linalool: 14-30%;1,8-Cineole: 10-75%; Viridiflorol: 13-66%, in varying proportions. It is noteworthy to observe that 1,8-cineole and E-Nerolidol chemotype variants represents 70% of the *M.quinquenervia* tree population. The leaves are the richest source of Niaouli oil. This oil is known for its fine woody fragrance and are widely used in floral perfumes. Besides, Niaouli oil has been valued in traditional medicine and French pharmacopoeia against cold, bronchitis, antiseptic against human pathogens, and provides efficient immunization against latent infections. At present, oral/dental, skin and many other new age diseases are increasing at an alarming rate. Response to disease causing microorganisms to chemical synthetic drugs and their side effects are also increasing. These factors motivated to look for alternative medications and their active components. Thus, a study was initiated on identification of the chemical variant of *Melaleuca quinquenervia* essential oil through Gas Chromatography-Mass Spectrometry (GC-MS) and assessment of its inhibitory effects against disease causing microorganisms on Dental pathogens: *Streptococcus mutans* and *Lactobacillus acidophilus*, Gut pathogen: *Escherichia coli* and *Skin pathogen*: *Staphylococcus aureus.*

MATERIALS AND METHODS

Collection of Plant material

Plant material of *Melaleuca quinquenervia* tree was of 8-10m in height, leaves were 1-2cm width, 8cm in length and are silvery grey. The bark is whitish, thick, scaly peels off in large fragments. Fresh leaves were collected from a ten year old tree maintained at the Aromatic garden of Department of Horticulture, UAS(B), GKVK, Bangalore.

Extraction of Essential oil

Fresh leaves collected were dried at room temperature for three to four days. 100g of dried leaves in five portions was chopped and then subjected to three-hour distillation in a Clevenger's apparatus. The extracted oil was collected and dried over sodium bisulphate and oil yield (%) was recorded.[2]

Gas Chromatography-Mass spectrometry

The oil was analysed by Agilent equipment, model GC-7890 with FID, MS- 5975 C with inert MSD. Equipped with a DB -17 MS J & W 122-4762, Silica Capillary column of 60 m*0.25 mm* 0.25 um. Helium at a flow rate of 1 mL/min, detector and injector at 280 °C and 250 °C, were maintained respectively; injection volume was 0.2 µL and split ratio was 30:1. Oven temperature was programmed from 70^0C @ 2^0C upto 100^0C, @ 2.6^0C upto 200^0C, @ 5^0C upto 270^0C. The identification of compounds was done with multi databases including Wiley/Nist/ SOM.

Assessment of growth inhibitory activity

Four pathogens affecting dental, gut and skin were taken up for the invitro study viz., dental: *Streptococcus mutan*s and *Lactobacillus acidophilus*, Gut*: Escherichia coli* and Skin*: Staphylococcus aureus*. *Escherichia coli* (MCC 2079), *Staphylococcus aureus* (7443) were procured from National Centre for Cell Science (NCCS), Pune. While, *Streptococcus mutans* (MTCC 497) and *Lactobacillus acidophilus* (MTCC 10307) were procured from MTCC, Institute of Microbial Technology, Chandigarh.

Preparation of inoculum

The bacterial strains of *Escherichia coli* and *Staphylococcus aureus* were inoculated in nutrient broth and incubated overnight at 37ºC was used. While, *S.mutans* was propagated for 24 hours on Brain and Heart Infusion Agar at 37°C under anaerobic condition ; *L.acidophillus* was grown on Lactobacilli MRS agar plates incubates at 37°C for 48 hours. A 24 hour old pure cultures of *E.coli* and *S.aureus* cultured for twenty four hours were used for bacterial suspension as per Mac-Farland Nephelometer Standard.[8] Similarly, 24hrs culture of *S.mutans* was resuspended in peptone water (10%) and *L.acidophillus* in *Lactobacilli* MRS broth. The *M.quinquenervia* essential oil was screened for their antibacterial activity against these microorganisms by Disc Diffusion method (Inhibition Zone Technique).

Disc diffusion assay method

Sterile paper disc of 6mm diameter procured from HiMedia (SD067) was aseptically saturated with 30µl of volatile oil. These discs, were allowed to dry for 1hour in Laminar Air Flow (LAF) chamber for complete absorbance of the sample. The loaded discs were later placed onto respective growth medium. Surface was swabbed with 30µl of respective test organism (ca. 1.5 x 10^8 CFU/ml using 0.5 McFarland's Standard) at equidistance in three locations with a sterilized forceps. The plates were incubated for 24h/48hrs at 37°C. Similarly, standard antibiotic disc of streptomycin, cefoxitin and ampicillin procured from HiMedia, were aseptically placed on the agar plate, swabbed with the respective test organism. The data were recorded as three independent observations by measuring the zone of growth inhibition (mm) around the disc. The recorded inhibition zone (mm) of the sample was compared with the inhibition zone of the standard antibiotics, streptomycin, ampicillin and cefoxitin procured from HiMedia.[8]

RESULTS AND DISCUSSION

Hydro-distillation of dried leaves of M.quinquenervia yielded 1.59% of essential oil. The Gas-Chromatography-Mass Spectrometry analysis reported 23 compounds (Table 1; Fig 1), of which the major component found was E-Nerolidol (87.63%) followed by 1,8 Cineole (2.03%). Chemotypes for *Melaleuca quinquenervia* are identified based on the concentrations of 1,8-Cineole, Nerolidol and Viridiflorol. Four chemotypes occur in Madagascar alone consisting of 1, 8-cineole-37%; Virdiflorol: 20%; terpinolene: 5%; Viridiflorol: 48% and E-nerolidol: 87%. Other two chemotypes are from Australia & New Guinea: one is comprised of E-Nerolidol: 74-95%; Linalool: 14-30%;1,8-Cineole: 10-75%; Viridiflorol: 13-66%, in varying proportions. [1,2,3,9] Gas Chromatography-Mass spectrometry analysis confirmed that *Melaleuca* collection at UAS(B), Bangalore corresponds to a Nerolidol Chemotype. Thus, the tree was identified and confirmed as *Melaleuca quinquenervia* (Cav.) S.T.Blake or Niaouli oil, of Madagascar origin. In Madagascar, production of this Niaouli oil is around 7-10metric tons/year and about 1.5-2 tons is being exported to Europe & United States. Besides this, essential oil of E-Nerolidol Chemotype is priced two to four-fold higher than the 1,8-cineole Chemotype.[4]

Nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3ol), a sesquiterpene analogue of linalool, is found naturally in the essential oil of many plants and flowers, e.g. neroli, ginger, jasmine, lavender and tea tree. Its scent is described as pleasant, long-lasting and moderately flowery. Used as a flavoring agent, and as a fragrance additive in cosmetics and detergents. This naturally occurring sesquiterpene is being researched for its high penetration and diffusion capacity for transdermal delivery of therapeutic drugs. Various research articles have reported that Nerolidol increases the diffusion

coefficient of drugs such as 5-Florouracil (Anticancer drug), Diclofenac sodium (Anti-inflammatory drug) etc. This permeation enhancing activity suitable for alignment within lipid lamella of stratum corneum or skin can be attributed to its amphiphilic structure. As natural skin penetration enhancers it provides safe and efficient delivery of therapeutic drugs unlike synthetic chemical enhancers, which are toxic upon long term application.[10,11]

Table 1: Gas Chromatography-Mass spectrometry analysis of *M.quinquenervia* essential oil

S.No.	Components	Area%	S.No.	Components	Area%
1	Alpha Pinene	0.33	12	Eugenol	0.10
2	Benzaldehyde	0.55	13	Caryopyllene	0.60
3	Beta Pinene	0.10	14	Alpha Humulene	0.13
4	Limonene	0.40	**15**	**E-Nerolidol**	**87.63**
5	**1,8 Cineole**	**2.03**	16	Caryophyllene oxide	2.23
6	Methyl Benzoate	0.53	17	Viridiflorol	0.26
7	Camphor	0.78	18	Humulene epoxide (T)	0.28
8	Ethyl Benzoate	0.10	19	Alpha Bisabolol	0.06
9	Styryl Acetate	0.07	20	Beta Bisabolene	0.14
10	Alpha Terpinol	0.67	21	Farnesol	0.33
11	Beta Citronellol	0.07		Others	2.61
				Total	100.00

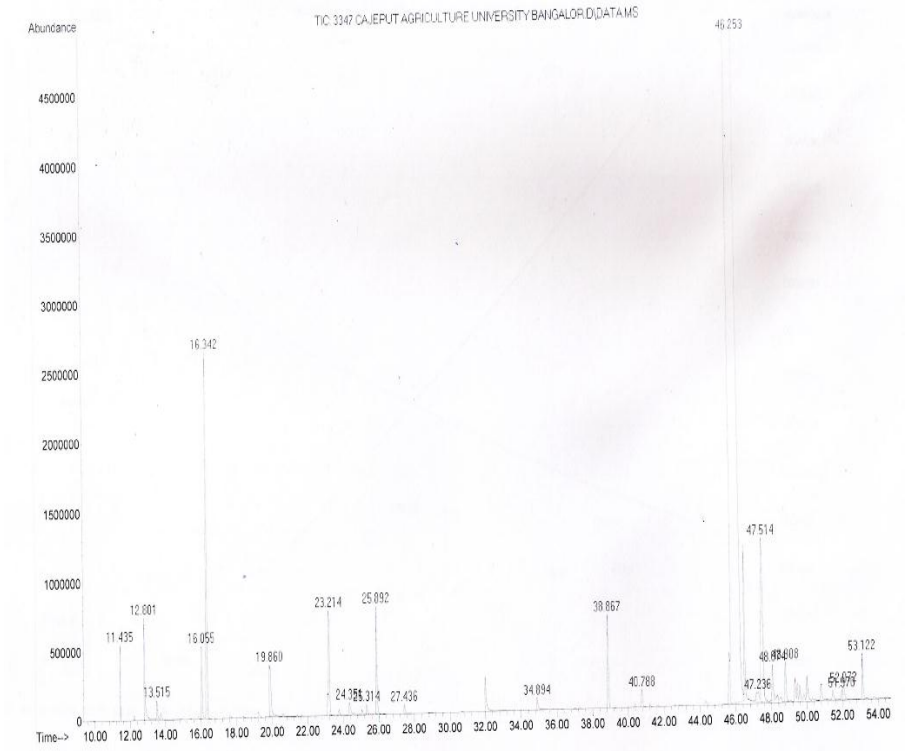

Fig 1: Gas Chromatography-Mass spectrometry of M.quinquenervia essential oil

ANTIMICROBIAL ACTIVITY

The development of bacterial resistance to antibiotics has demanded the search for new antibacterial agents. Although the essential oil composition and antibacterial properties of various *Melaleuca* species from different origins have been studied, it seems that little work has been carried out on pathogens causing dental caries and infectious diseases. The present investigation was carried out to screen the authentic oil sample for antibacterial property against bacterial cultures. 30µl of concentrated Nerolidol chemotype oil was tested by adopting disc diffusion method of screening. Antibacterial activity observed was compared with standard antibiotics.

Efficacy of Niaouli Oil Against Dental Pathogens

Oral pathogens *Streptococci* and *Lactobacillus species* are among the major species which play a crucial role in the formation of dental plaque and development of dental caries respectively. Despite the advent of latest tools for oral hygiene such as tooth pastes and mouth washes, dental science, majority of population suffer from dental caries and periodontal problems amongst which dental caries remains the most important dental health problem

in the developing countries. [12] The present investigation was carried out to screen the Niaouli oil against these dental pathogens. The anti-bacterial efficacy of Niaouli oil in terms of Minimum Inhibitory Concentration (MIC) values, revealed that the dental pathogens were highly susceptible to Niaouli oil, with varying degree of susceptibility. Niaouli essential oil completely inhibited the growth of *S.mutans*, with MIC value of 90±0, and effectively inhibited *L.acidophilus* with MIC value of 8.67±0.41 as compared with the standard antibiotics (Table2, Fig 2). Thus, Niaouli oil seems to be a promising antimicrobial agent against pathogens which are involved in initiation and development of dental caries. The effective antimicrobial activity reported in the present study can be attributed to E-Nerolidol, which constitutes to about 87% of the total composition and to its enhanced bacterial permeability and susceptibility property.[13]

Table 2: Anti-microbial activity of Niaouli oil and Antibiotics against dental pathogens

Microorganism tested	Inhibition zone (mm)		
	Sample	Antibiotics	
	Niaouli oil	Ampicillin	Streptomycin
Streptococcus mutans	90±0	13.67±0.41	28.67±0.82
Lactobacillus acidophilus	8.67±0.41	29.00±1.00	13.33±0.41

[Values are represented as Mean±SEM of three independent replications]

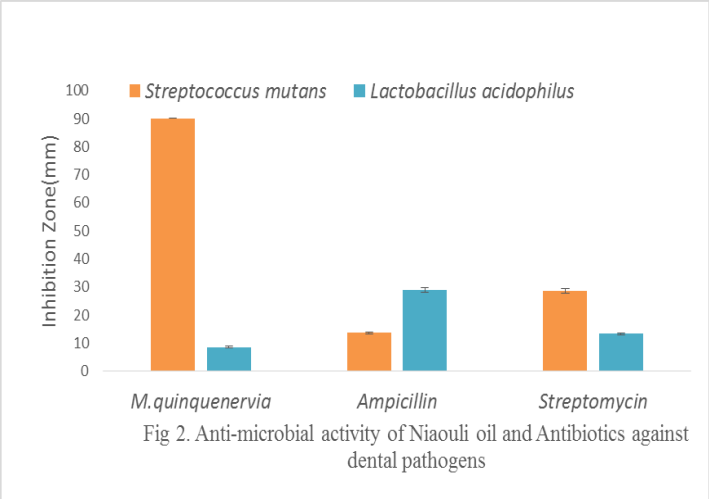

Fig 2. Anti-microbial activity of Niaouli oil and Antibiotics against dental pathogens

Efficacy of Niaouli essential oil against the disease causing micro-organisms

The results revealed that the essential oil effectively inhibited *S.aureus* with the Minimum Inhibitory Concentration value(MIC) of 9.333 ± 0.667 as compared to the standard antibiotics (Table 3, Fig 3). The reason may be attributed to the enhanced bacterial permeability and susceptibility of Nerolidol, a major component of M.quinquenervia essential oil.[13,14, 15] . The above results appear promising for possible use of Niaouli oil as bactericidal agent against *Staphylococcus aureus* an opportunist pathogen, which can colonise in the nasal passages, skin and mucous membranes of humans. They are known to cause infectious diseases like septicaemia, pneumonia, endocarditis, osteomyelitis, gastroenteritis and abscess on its penetrating. On the other hand, *E.coli*, was found to be resistant to the Niaouli essential oil. The cell wall structure of *E.coli*, a gram negative bacteria is complex, the peptidoglycan layer is covered by an outer membrane that contains various proteins and lipopolysaccharides prevents the penetration of antimicrobial agents.[16] This is one of the reason that *E.coli*, gram negative bacteria is resistant to Niaouli oil. The type of interaction occurring between essential oil components also depends on the organism, components examined and component ratios, volume of inoculum, growth phase of microorganisms, culture medium, strain etc, which might have influenced the antimicrobial efficacy. M.quinquenervia essential oil displays promising antimicrobial activity against S.aureus, a skin pathogen.

Table 3: Anti-microbial activity of Niaouli oil and Antibiotics against gut and skin pathogens

Microorganism tested	Inhibition zone (mm)		
	Sample	Antibiotics	
	Niaouli oil	Cefoxitin	Ampicillin
Escherichia coli	No Inhibition	17.333 ± 1.202	15.667 ± 0.333
Staphylococcus aureus	9.333 ± 0.667	21.333 ± 1.202	25.667 ± 2.333

[Values are represented as Mean±SEM of three independent replications]

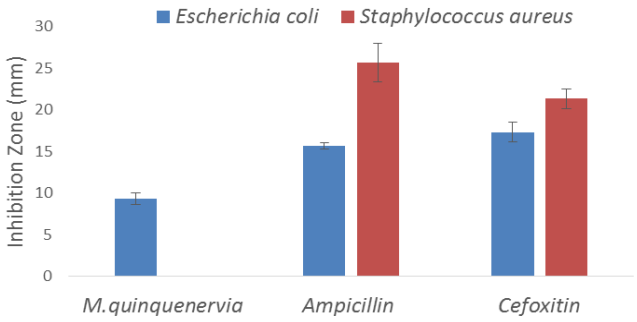

Fig 3: Anti-microbial activity of Niaouli oil and Antibiotics against gut and skin pathogens

CONCLUSION

Understanding the chemical variation of an aromatic tree has a significant impact on its therapeutic value. Besides the pharmaceutical industry, these variants are also of interest to ecologists. E-Nerolidol, the major component (87.63%) of *Melaleuca* sp. is widely used in floral perfumes. As these trees play an important role in carbon sequestration and biodiversity improvements, promoting its cultivation and reclaiming degraded lands is of great value towards conservation. Essential oil of *Melaleuca quinquenervia* displayed promising antimicrobial activity against pathogenic microorganisms which are the main causative agents of dental caries and infectious diseases. The results also indicate the efficacy of Niaouli oil against S.mutans by showing complete inhibition as it is the prime organism to initiate dental plaque. Besides, its efficacy in inhibition Niaouli oil can be a preferred component of dental care product.

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