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Research article

Anticandidal activity of essential oils of *Myristica fragrans* and *Syzygium aromaticum*

Thayalini Thileepan[*1], Vasanthi Thevanesam[2], Selvaluxmy Kathirgamanathar. S[3]

[1]*Unit of Siddha Medicine, University of Jaffna, Sri Lanka.*
[2]*Department of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka.*
[3]*Industrial Technology Institute, 363, Bauddhaloka Mawatha Colombo-7, Sri Lanka.*

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***Corresponding Author: Thayalini Thileepan,** Unit of Siddha Medicine, University of Jaffna, Sri Lanka.

Abstract

M. fragrans is commonly known as nutmeg. *S. aromaticum* is called as clove. In Siddha medicine, these are used as one of the ingredients in medicines that are used to treat vaginal diseases. The aim of the study was to evaluate the anticandidal activity of oils of seed and leaf of *M.fragrans* and clove against *C. tropicalis* ATCC 13803, *C. krusei* ATCC 6258, *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019, *C. albicans* 3 clinical isolates. Oils were distilled using the Clevenger apparatus. Anticandidal activity of oils was screened using disc diffusion method. Minimum Inhibitory Concentration (MIC) of the oils was determined in two different method of two fold micro-broth dilution. Minimum Bactericidal Concentration (MBC) was determined wells with no turbidity in the micro-broth MIC method (BHI) were sub cultured onto a blood agar plate. All three oils showed activity against all tested *Candida* sp ZOI from 8.3 ± 0.5 - 30.0 ± 0.0mm. MIC of three oils are similar for all tested *Candida* sp in both methods. The extremely low MIC (0.0045µg/mL) of the oil of *S. aromaticum* for all the tested *Candida* strains is note worthy. However, all the tested oils were active against *Candida* with MICs ranging from 0.0045 –2.5 µg/mL. MBC was the same or differed by only one dilution as the MIC for tested *Candida sp.* suggesting that the oils are fungicidal. Three oils have ability to inhibit *Candida* sp with low MIC.

Introduction

Essential oils are one of the secondary metabolites of aromatic plants and are present in buds, flowers, leaves, seeds, fruits, roots, bark and wood. They are stored in secretary cells, cavities and canals of plants and are soluble in lipids and organic solvents which have a lower density than water. Chemically, essential oils are a combination of benzene derivatives, terpenes, various hydrocarbons and straight chain compounds [1, 4]. Water distillation (hydrodistillation) is one of the techniques used for the distillation of essential oils from aromatic plants. Hydrodistillation is the simplest method for obtaining essential oils. Essential oils are highly volatile at room temperature and have been shown to possess antimicrobial activity [2]. In recent years, many essential oils have been used for the preparation of facial creams, head oils, lotions, herbal soaps and toothpaste. Monoterpenes are present in all essential oils and have been shown to possess antibacterial activity. *M. fragrans* seed**,** leaf and *S. aromaticum* are aromatic plants**.** *M. fragrans* belongs to the family Myristicaeae is commonly known as nutmeg in English, Sadikka in Sinhala and Sathikkai in Tamil [11]. In Siddha medicine (a type of traditional medicine), the seeds and leaves are used as one of the ingredients in medicines that are used to treat skin diseases, respiratory diseases and arthritic conditions [3]. *S. aromaticum* (synonym: *Eugenia carophyllata*) is called as clove in English, karambu-neti in Sinhala and illavangappu or kirambu in Tamil. It belongs to the Family Myrtaceae. In Siddha medicine, along with other ingredients, it is used in the treatment of respiratory, gastro intestinal, urinary and skin diseases. Antimicrobial activity of *S. aromaticum* oil has been previously evaluated against *S. aureus*, *E. coli* and *Bacillus cereus*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Saccharomyces cerevisiae* [5] and *Aspergillus sp* and Dermatophytes [6]. Antimicrobial activity of the essential oils of *M. fragrans* seed has been reported against *Shigella dysenteriae* [8], *E. coli* and *P. aeruginosa* [7] In Siddha medicine, *M. fragrans* leaf, seeds and *S. aromaticum* are used to prepare different medicines for the treatment of vaginal discharge [9]. The aim of the study is to evaluate the anticandidal activity of oils of these plants against selected *Candida*l sp.

Methodology

Plant collection and distillation of oils from plants

M. fragrans seed and leaf and *S. aromaticum* flower buds were collected from Kandy. The specimens of plants were

sent and obtained voucher specimen numbers (*M. fragrans*- S.M/04, *S. aromaticum* – S.M/03) from the National herbarium of Royal Botanical Garden, Peradeniya. These were pounded as coarse powder and transferred to a flask. 100g of *M. fragrans* seed, leaf and flower buds of *S. aromaticum* were distilled separately using Clevenger apparatus for 8 hours and 6mL, 2mL and 14.5mL oils were obtained respectively. Oils were stored at 4 °C until used for the anticandidal assay.

Anticandidal assay

Screening for the anticandidal activity

Anticandidal activity of oils was screened using disc diffusion method against selected *Candidal* sp. Three replicates were carried out for the entire procedure [10].

Preparation of paper discs

Whatman'sNo.1 filter paper was used to prepare the discs. Using a borer 6mm diameter discs were cut out from the filter paper and placed in universal bottles and autoclaved.

A broth suspension with turbidity equivalent to 0.5 McFarland standards was prepared from a pure culture of each of the test organisms. A Sabouraud dextrose agar plate was inoculated with 1 mL of the broth suspension and the plate rotated to allow even spreading of the inoculum. After removal of the excess fluid, the plate was allowed to dry at 37°C for 15 min. Sterile blank discs (6mm) placed on the seeded plate were impregnated with 5µL of the oil and left on the bench for 30 min. for absorption of the oil. The plates were incubated at 37°C for 24 h. After incubation, the diameters of the zones of inhibition were measured.

This method was carried out for 8 *Candida* species (Table 1).

Table 1. List of *Candida* strains

Candidal sp.	Strains
C. tropicalis	ATCC 13803
C. krusei	ATCC 6258
C. albicans	ATCC 90028
C. glabrata	ATCC 90030
C. parapsilosis	ATCC 22019
C. albicans	3 clinical isolates

Determination of MIC

Minimum inhibitory concentration of the oils was determined using two micro-broth dilution methods.

i. Using Brain heart infusion (BHI) broth

Under aseptic conditions, U bottomed 96 well microtiter (Corning Life Sciences, Canada) used for this assay. The first column of the microtiter plate was filled with 180 µL BHI, 10 µL of oil and 10 µL of 1/50 diluted Tween 80.Other wells of the microtiter plate were filled with 100 µL of BHI. Two fold serial dilutions were made by transferring 100 µL from the first column to the subsequent well in the next column of the same row so that each well had 100 µL of broth in serially descending concentrations. A broth suspension with turbidity equivalent to 0.5 McFarland standards was prepared from a pure culture of each of the test organisms. One mL of each suspension was transferred to 9 mL of sterile distilled water. Finally, 10 µL of this bacterial suspension was added to each well. The last two columns of each row contained only BHI and Tween 80 respectively which served as controls. The plates were incubated at 37°C for 24 h. After 24 h incubation the turbidity in the well was observed visually. The lowest concentration of the fraction at which no turbidity observed was noted as the MIC value (Figure 1).

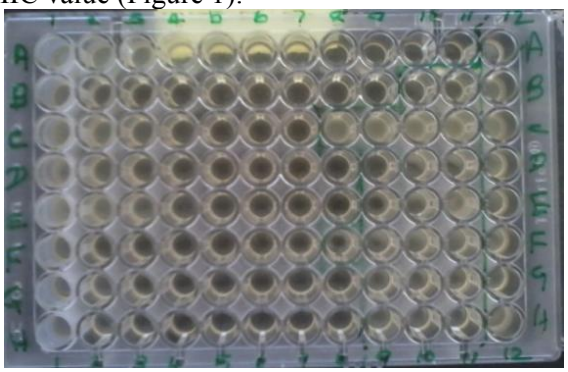

Figure 1. MIC of *S. aromaticum* oil in BHI – turbidity of wells indicate growth of organism.

ii. Using a sugar glucose basal (SGB) medium

The first column of the microtiter plate was filled with 180 µL SGB, 10 µL of oil and 10 µL of 1/50 diluted Tween 80. Other wells of microtitre plates were filled with 100 µL of SGB. Two fold serial dilution was made by starting transferring 100 µL material from first column to the subsequent wells in the next column of the same row and so that each well has 100 µL of material in serially descending concentrations. Finally, 10 µL of bacterial suspensions were added to each well. Last two columns served as the control column for SGB and SGB with Tween80 respectively. The plates were incubated at 37°C for 24 h. After 24 h incubation the colour change to pink was observed in plate. The lowest concentration of oil at which no colour change occurred was recorded as the MIC of the oil for the organisms in the row (Figure 2).

Figure 2. MIC of *S. aromaticum* oil in SGB Pink coloured wells indicate growth of organisms.

Minimum bactericidal concentration (MBC)

Wells with no turbidity in the micro-broth MIC method (BHI) were sub cultured onto a blood agar plate. The MBC was the lowest concentration at which no growth was detected after incubation at 37 °C for 24 hours.

Results and discussion

Table 2 shows that the screening results for anticandidal activity of essential oils using disc diffusion method. All three oils showed activity against all tested *Candida* sp.

Table 3 shows that the MIC of oils (*S. aromaticum, M. fragrans* seed and leaf are similar for all tested *Candida* sp in both methods. Both methods gave results which were identical or varied by one dilution as shown in table 3.

The extremely low MIC (0.0045µg/mL) of the oil of *S. aromaticum* for all the tested *Candida* strains is noteworthy. However, all the tested oils were active against *Candida* with MICs ranging from 0.0045 – 2.5 µg/mL.

Table 4 shows that the MBC was the same or differed by only one dilution as the MIC for tested Candida sp. suggesting that the oils are fungicidal. MIC and MBC of *S. aromaticum* and *M. fragrans* leaf and seed oils for all *Candida* sp. ranges 0.004–2.5 µg/mL. *S. aromaticum* oil showed very low MIC and MBC for all tested organisms.

In Siddha medicine, *M.fragrans* leaf, seed and *S. aromaticum* are used to prepare different medicines for the treatment of vaginal discharge [9]. Vaginal Candidiasis due to *C. albicans* may lead to vaginal discharge. The current study showed that three oils showed activity against all tested *Candida* sp.

The MIC of oils of *S. aromaticum, M. fragrans* seed and leaf oils for all tested *Candida* sp ranged from 0.004 – 0.31µg/mL. *S. aromaticum* oil was found to have an extremely low MIC of 0.004 µg/mL for all tested *Candida* sp.

Fluconazole is the drug to treat the Candidal infection. Previously, the Subcommittee for Antifungal Testing of the Clinical and Laboratory Standards Institute (CLSI) mentioned MIC interpretive breakpoints for fluconazole on *Candida* sp was 8µg/mL. An organism-drug combination is essential to determine the MIC [12]. The current study shows that the tested oils have lower MIC than Fluconazole.

Table 2. Screening results for anticandidal activity of essential oils with the disc diffusion method (ZOI in mm).

Organisms		M.f.s	M.f.l	S.a
C. tropicalis	ATCC 13803	15.6.±0.5	10.3±0.5	30.0±0.0
C. krusei	ATCC 6258	8.3±0.5	8.3±0.2	20.3±0.5
C. albicans	ATCC 90028	12.3±0.5	12.0±0.0	20.0±0.0
C. glabrata	ATCC 90030	12.0±0.0	12.0±0.0	30.0±0.0
C. parapsilosis	ATCC 22019	12.0±0.0	10.3±0.2	30.0±0.0
C. albicans	Strain 1	12.3±0.5	10.3±0.5	30.0±0.0
C. abicans	Strain 2	12.3±0.5	8.3±0.2	22.0±0.0
C. albicans	Strain 3	12.3±0.5	10.0±0.0	25.3±0.5

M.f.s- *M. fragrans* seed; M.f.l- *M. fragrans* leaf;S.a– *S. aromaticum*.

Table 3. MIC (µg/mL) of plant oils against *Candida* sp. in 2 methods.

	MIC	C.t	C.k	C.a	C.g	C.p	*C. albicans* strains		
							1	2	3
M.f.s	BHI	2.5	0.31	0.31	1.25	1.25	1.25	0.62	1.25
	SGB	2.5	0.62	0.62	0.62	0.62	0.62	1.25	0.62
M.f.l	BHI	0.15	0.037	0.018	0.15	0.018	0.037	0.018	0.018
	SGB	0.15	0.037	0.018	0.15	0.018	0.037	0.018	0.018
S.a	BHI	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045
	SGB	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045

C.t- *C. tropicalis* ATCC 13803; C.k- *C. krusei* ATCC 6258; C.a- *C. albicans* ATCC 90028; C.g- *C. glabrata* ATCC 90030; C.p- *C. parapsilosis* ATCC 22019. M.f.s- *M. fragrans* seed; S.a– *S. aromaticum*; M.f.l- *M. fragrans* leaf.

Table 4. MBC of essential oils against *Candida* sp.

MIC & MBC		C.t	C.k	C.a	C.g	C.p	*C. albicans* - strains		
							1	2	3
M.f.s	MIC	2.50	0.31	0.31	1.25	1.25	1.25	0.62	1.25
	MBC	2.50	0.31	0.31	1.25	1.25	1.25	1.25	1.25
M.f.l	MIC	0.15	0.037	0.018	0.15	0.018	0.037	0.018	0.018
	MBC	0.31	0.075	0.090	0.15	0.037	0.075	0.037	0.037
S.a	MIC	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
	MBC	0.004	0.004	0.004	0.004	0.004	0 .004	0.004	0.004

C.t- *C. tropicalis* ATCC 13803*;* C.k- *C. krusei* ATCC 6258*;* C.a- *C. albicans* ATCC 90028*;* C.g- *C. glabrata* ATCC 90030*;* C.p- *C. parapsilosis* ATCC 22019. M.f.s- *M. fragrans* seed; S.a– *S. aromaticum*; M.f.l- *M. fragrans* leaf.

Conclusion

Three oils have ability to inhibit *Candida* sp with low MIC. Further study on this widely used medicinal plant oils is indicated to explore the possibility of finding novel antifungal compounds.

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