Original Article

Antifungal response of oral-associated candidal reference strains (American Type Culture Collection) by supercritical fluid extract of nutmeg seeds for geriatric denture wearers: An *in vitro* screening study

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Abstract

Objectives: Since time immemorial, plants have continued to play a predominant role in the maintenance of human health as sources of medicinal compounds. Several effective antifungal agents are available for oral *Candida* infections; the failure is not uncommon because isolates of *Candida albicans* may exhibit resistance to the drug during therapy. The present study aimed to identify an alternative, inexpensive, simple, and effective method of preventing and controlling the candidal infection.

Methodology: All the procured and authenticated nutmeg seeds were dried in shade and cleaned by hand sorting. The crushed seeds were passed through mesh no. 40 individually. About 50 g of powdered nutmeg seeds was loaded in the supercritical fluid extractor unit using supercritical CO_2 as extracting solvent in accordance with the methods of Nguyen *et al*. Supercritical fluid (SFE) extraction was done using CO_2 gas without any cosolvents.

Results: The nutmeg extract displayed antifungal activity with the effective zone of inhibition ranging from 18.0 to 12.0 mm when compared with nystatin as positive control.

Conclusion: This paper described the *in vitro* antibacterial activity, and phytochemical analysis of SFE extract of nutmeg (*Myristica fragrans*) evaluated against *C. albicans* (American Type Culture Collection 10231) through agar well diffusion method. SFE of nutmeg seeds can be used as an adjunct to conventional therapy for oral candidiasis.

Keywords: Alternative medicine, antimicrobial activity, *Candida albicans*, complementary therapies, phytotherapy, plant extracts, supercritical fluid extraction

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INTRODUCTION

Fungi, once dormant and considered as nondisease-causing entity, have become active to be called as chief causative agent of various infections in health and disease. *Candida albicans* is accountable for most fungal infections in humans.[1] Fungi often remain dormant and colonize various surfaces including skin and mucosa.[2-4] Growth on animate and inanimate surfaces is a natural part of the *Candida* lifestyle,[5] and it can inhabit the denture surface.

Oral candidal infections are considered opportunistic having multifactorial etiology such as drug administration (antibiotic/steroid usage), uncontrolled diabetes and/or compromised immunity, denture wearers, patients under chemotherapy, and transplant recipients.[5-8] According to the National Oral Health Survey, denture-related stomatitis is characterized by erythematous inflammatory areas covering the denture surface. This clinical feature is multifactorial with *C. albicans* being the chief etiological agent. Although rational use of synthetic drugs for treatment of candidal infections is justifiable, failure is uncommon due to candidal isolates exhibiting intrinsic (primary) or secondary resistance to the drug during therapy.[9] With the prevalent drawbacks of antifungal drugs, their frequent use has led to drug resistance (altering drug target site, drug modification, or restricted drug penetration). The hazards and negative outcomes of medications in older people have been well documented.[10]

To combat with antimicrobial resistance, antimicrobial alternatives can be thought as an alternative to synthetic drugs.[11-14] Herbal medicines have been into existence since ancient times in treating different ailments. Natural products could be an interesting alternative for the control of fungal diseases due to their lower negative impact, reduced cost, and less adverse reactions as compared to modern synthetic drugs and better patient compliance. Antibacterial, antifungal, and antioxidant activities have been observed in many plants due to the presence of secondary metabolites.

Hence, conventional preventive measures in the form of phytomedicines that have better compliance, availability, and cost need to be promoted. 60%–90% of the population has indulged in phytotherapy as a part of primary health care.[14]

It has been cited that essential oils derived from plants have insecticidal, bactericidal, and fungicidal effects.[15-20] However, the antifungal activity of essential oils more specifically against *Candida* species is at infancy. This prompted us to investigate the antifungal activity of SFE extract of nutmeg seeds against *Candida* species.

Need of the study

1. To identify essential oils from plant products which can prevent and control the growth of *C. albicans*
2. To evaluate the antimicrobial efficacy of SFE of nutmeg seeds on *C. albicans* involved in causing oral candidiasis.

METHODOLOGY

Collection and identification of plant material

Nutmeg seeds were collected from a local market in Mysuru and identified by the botanist Dr. M.N. Naganadini in the Department of Pharmacognosy, JSS College of Pharmacy, Jagadguru Sri Shivarathreeswara University, SS Nagara, Mysuru, India [Table 1].

Preparation of test compound

In the present study, supercritical fluid extract (SCFE) of nutmeg seeds was prepared.

Supercritical fluid plant extract preparation

All the procured and authenticated above seeds were dried in the shade and cleaned by hand sorting. The crushed seeds were passed through mesh no. 40 individually [Figure 1]. About 50 g of powdered nutmeg seeds was loaded in the supercritical fluid extractor unit using supercritical CO_2 as extracting solvent in accordance with the methods of Nguyen *et al*.[21] SFE was done using CO_2 gas without any cosolvents. SCFE involves the use of dense gas as a solvent (e.g., carbon dioxide [CO_2]) for extraction [Figure 2]. The ground plant material was charged into the extractor. Supercritical CO_2 was fed to the extractor through a high-pressure pump (300 bar) at 37°C ± 5°C, which was above its critical temperature (31°C ± 5°C) and pressure (74 bar). The extract laden CO_2 was sent to a separator (60–120 bar) through a pressure reduction valve. The temperature and pressure were reduced so that the extract precipitates into

Table 1: Herbal material used in the present study

Botanical name of the plant	Family	Common name	Part's used
Myristica fragrans	*Myristicaceae*	Nutmeg	Seeds

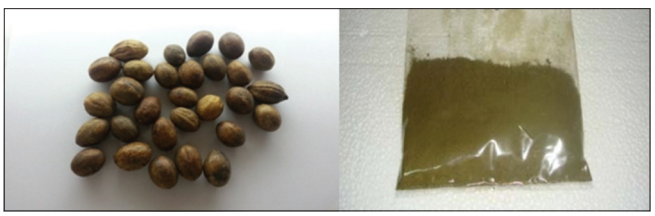


Figure 1: Nutmeg seeds

the separator and gaseous CO_2 is released to the atmosphere. Stock solution of the extract was prepared by dissolving 100 mg of the alcoholic extracts in 10 ml 50% of dimethyl sulfoxide (DMSO). Nystatin was used as a positive control and stock solution was prepared by dissolving 50 mg of the drug in 10 ml of DMSO to get 10 mg/ml.

Chemical reagents

All the chemicals and solvents used were procured from JSS College of Pharmacy.

Evaluation of supercritical fluid extraction of nutmeg seeds

Phytochemical analysis of SFE of nutmeg seeds were evaluated and the findings were in agreement with Trease and Evans[22] and confirmed the presence of tannins, saponins, flavonoids, steroids, and phenols [Table 2].

Procurement of microorganism

Freeze dried form of the microorganism *C. albicans* was obtained from American Type Culture Collection (ATCC 10231), Himalaya Drug Company, Makali, Bengaluru.

Preparation of inoculum

Subculture of ATCC 10231 was performed on yeast peptone dextrose at 25°C ± 2°C for 24 h to form stock culture of the microorganism (inoculum). 30 ml of molten sterile yeast peptone dextrose was poured aseptically in sterile Petri plates and was allowed to solidify at room temperature. Hundred microliters of inoculum was spread with a sterile steel spreader so as to achieve a confluent lawn of fungal growth.

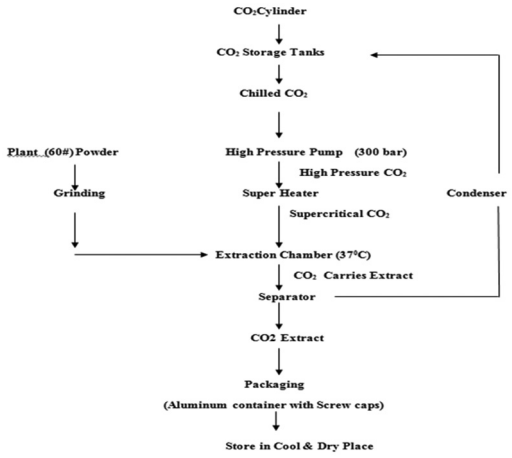


Figure 2: CO_2 extract flowchart

Screening for antimicrobial activity

On the Petri plates with the subculture of the microorganism, wells of 6 mm were made with sterile cork borer. In one of the three plates, three aseptic wells were prepared by sterile cork borer. A 50, 100, and 150 µl volume of nutmeg extract were introduced directly into the wells (in triplicates) of the inoculated specific media agar plates. The plates were allowed to stand for 10 min for diffusion of the extract to take place and incubated at 37°C for 24 h.[23,24] Sterile DMSO (50 µl, 100 µl, and 150 µl) served as the negative control in one of the Petri plates and nystatin (50 µg in 50 µl DMSO and 25 µg in 50 µl DMSO) served as the positive control in another Petri plate. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded[25] and compared with positive and negative controls. Experiments were performed in triplicates, and the mean values of the diameter of inhibition zones with ± standard deviation were calculated.[26] The inhibition zones were measured using Hi-media zone scale available at JSS College of Pharmacy [Figure 3].

Statistical analysis

The collected data were analyzed using statistical tests such as mean value and standard deviation on an SPSS (Version 22, SPSS.inc version 16) software database.

RESULTS

The means of the zones of inhibition of *C. albicans* by SFE of nutmeg seeds at 24 h were measured [Table 3].

Nutmeg oil with 1:1 (50 µl DMSO in 50 µl nutmeg oil) dilution with DMSO showed growth inhibition of *C. albicans*

Table 2: The phytochemical screening of supercritical fluid extract of nutmeg seeds

Reagents and test	Phytochemical constituents	Result-SFE of nutmeg seeds
1 ml of freshly prepared 5% $FeCl_3$ was added to 1 ml of the extract (ferric chloride test)	Tannins	Positive (blue-black coloration)
2 ml of the extract was vigorously shaken in the test tube for 2 min (frothing test)	Saponins	Positive (froth formation)
1% of NH_3 was added to 3 ml of the extract (ammonia test)	Flavonoids	Positive (yellow coloration)
5 drops of concentrated H_2SO_4 was added to 1ml of the extract in a test tube	Steroids	Positive (red coloration)
1 ml of the extract was added to 5 drops of acetic anhydride and a drop of concentrated H_2SO_4 was added.(Salkowski test)	Terpenoids	Negative (no bluish-green precipitate)
Plant extract was mixed with 1% gelatin solution containing 10%NaCl (gelatin test)	Phenol	Positive (bulky white precipitate)

SFE: Supercritical fluid extract

with 18 ± 0.13 mm followed by 1:2 concentration (50 µl DMSO in 100 µl nutmeg oil) with 12 ± 0.15 mm and with 1:3 dilution of DMSO and nutmeg oil (50 µl DMSO in 150 µl nutmeg oil) showed no zone of inhibition at 24 h, respectively; positive control nystatin at a concentration of 50 µg in 50 ml of DMSO showed zone of inhibition of 20 ± 0.14 mm and nystatin at a concentration of 25 µg in 50 µl DMSO showed zone of inhibition of 15 ± 0.12 mm [Graphs 1 and 2]. In general, the plant extract showed antimicrobial activities at the critical difference of 5% ($P \leq 0.05$).

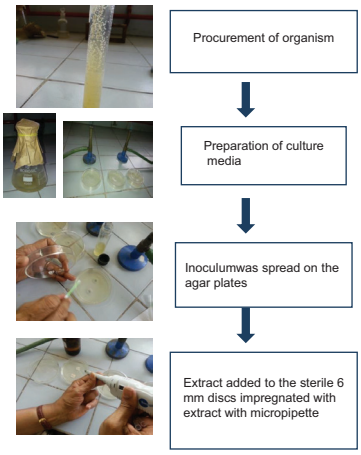
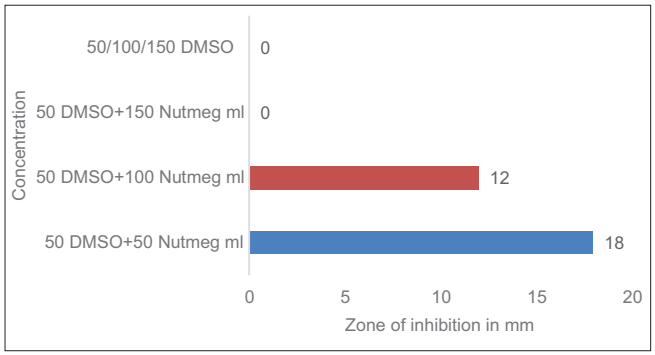
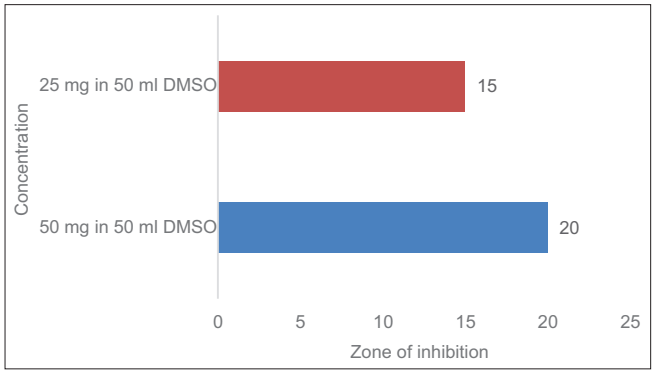


Figure 3: Screening for antimicrobial activity



Graph 1: Zone of inhibition of nutmeg against dimethyl sulfoxide



Graph 2: Zone of inhibition of nystatin

DISCUSSION

C. albicans is one among the 200 species and belongs to the genus *Candida* that accounts for up to 75% of all candidal infections.[27] *C. albicans* is the most common *Candida* species residing in various regions including oral cavity and is in the benign form in health and in disease. Conventional therapeutic options for oral candidiasis range from topical polyene antifungals to azole agents. The increase in the occurrence of resistance of *Candida* spp. to conventional antifungals has been reported in the last few decades. Apart from this, antifungal drugs show relevant limitations such as low spectrum, interaction with other drugs, high cost, and toxic effects; the toxic effects are a result of the similarities between yeast and host cells (both eukaryotic), relevant in the clinical context. In particular, for erythematous candidiasis, the recurrence of the lesion after treatment with conventional antifungals has been reported, especially when associated with poor denture hygiene.[28] Hence, the use of natural products can be opted as an alternative to synthetic medicine.

With various drawbacks of synthetic drugs, there is a paradigm shift from synthetic drugs to plant extracts which exhibit similar antimicrobial activity and are extensively used by the consumers due to low toxicity compared to oral care products containing antimicrobial agents.[27,28] Plant extracts contain many secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids which provide new source of antimicrobial agents that could combat against drug-resistant pathogens.[29-31]

Myristica fragrans Houtt. (family: *Myristicaceae*) locally known as Jaikai in Kannada is commonly in Penang Island, Malaysia, India, and Southeast Asia. Nutmeg is the dried kernel of seed of *Myristica fragrans*.[32] Its pleasant aroma and warm taste make it ideal for usage as spices. It is also used as flavoring agent in beverages, syrups, and perfumes.[32] The main constituents of *M. fragrans* have been found to be alkyl benzene derivatives (myristicin, elemicin, safrole,

Table 3: Zones of inhibition in mm for *Candida albicans* (dimethyl sulfoxide: Nutmeg oil)

Sample name	Solvent	Concentration	Growth SD*±
Nutmeg oil	DMSO	1:1 (DMSO: nutmeg oil) (50 µl DMSO in 50 µl nutmeg oil)	18±0.13 mm
	DMSO	1:2 (DMSO: nutmeg oil) (50 µl DMSO in 100 µl nutmeg oil)	12±0.15 mm
	DMSO	1:3 (DMSO: nutmeg oil) (50 µl DMSO in 150 µl nutmeg oil)	No zone
	DMSO	DMSO 50 µl/100 µl/150 µl	No zone
Nystatin	DMSO	50 µg in 50 µl DMSO	20±0.14 mm
	DMSO	25 µg in 50 µl DMSO	15±0.12 mm

*Standard deviation (n=3). DMSO: Dimethyl sulfoxide

etc.), terpenes, alpha-pinene, beta-pinene, myristic acid, trimyristin,[33-35] neolignan (myrislignan), and macelignan.[36]

The phytochemical constituents have been investigated for antimicrobial, antidepressant, aphrodisiac, memory-enhancing, antioxidant, and hepatoprotective properties.[32] The medicinal properties of nutmeg seeds have been extensively investigated and reported for antifungal, anticarcinogenic, anti-spasmodic, and dyspepsia.[32,37-40] Researchers[41] reported that trimyristin, an active compound obtained from the seed of *M. fragrans*, also displayed antibacterial properties against Gram-positive and Gram-negative bacteria. The antimicrobial activities of crude extracts of nutmeg seeds against oral pathogens need attention. Hence, this preliminary screening study was aimed at investigating the antimicrobial activities of SFE of nutmeg seeds against *C. albicans*.

Extraction of bioactive compounds from plant materials forms the main objective where a specific component is targeted which could potentiate the value of herbal medicines. Essential oil is a natural mixture extracted from several aromatic plants. A wide range of technologies could be advocated for extraction of active components from medicinal plants.

Extraction process includes conventional methods such as maceration, infusion, decoction, digestion, percolation, hot Soxhlet extraction, and distillation techniques which are based on the use of certain solvents, heat and shaking for extraction. Nonconventional methods include ultrasound, pulsed electric field, enzyme digestion, extrusion, microwave heating, and SFE. The nonconventional methods have wider advantages over conventional methods with respect to decrease in the extraction time, less usage of chemicals and solvents, and absence of residues which could cause allergic reactions and lack of thermal degradation of plant material with application of heat.[42,43]

Supercritical fluid extraction

SFE is a nonconventional method with the goal of reducing the use organic solvents and increased sample generation. SFE is the process of separating one component (the extractant) from another (the matrix) using supercritical fluids as the extracting solvent. The parameters that govern the extraction process are the temperature and pressure. The word supercritical indicates the temperature and pressure which exceed their critical values (Tc = 31.1°C, Pc = 72.8 atm) at which the substance can exist as a vapor and liquid in equilibrium. In addition, with minor changes in temperature and pressure, the substance could revert to its original form. CO_2 and water are most commonly used supercritical fluids.[42,43]

SFE has gained popularity over conventional methods due to the use of a nontoxic and volatile solvent, such as CO_2 which protects extracts from thermal degradation and solvent contamination.[44,45]

SUMMARY AND CONCLUSION

Antimicrobial resistance, relation to candidal biofilms, is a growing concern for the human population. Through overuse and misuse, antimycotic drugs have become less effective against oral pathogens. In particular, *C. albicans*, in the form of superficial candidal infections, have become less susceptible to standard antifungal agents making treatment much more difficult and less predictable. With the increasing size of the immunocompromised and geriatric populations, the development of potent and safe antifungal agents with as few side effects as possible is needed. It is postulated that nutmeg extract could fill this need. Even though many plants are used as home remedies for oral diseases, their antimicrobial effects on particular pathogenic microorganisms should be evaluated and implied into practice. Thus, the present screening study confirms that plant-based products interfere with the growth and metabolism of *C. albicans* which may prevent oral candidal infections.

Phytochemicals exhibit activities such as invulnerability for microbial growth.[46] Hence, phytotherapy could be chosen as an alternative to allopathic medicine. The need of the hour is to promote phytomedicines and aid in the development of novel herbal preparation which is at nascent stage. Using these plant extracts as home remedies or adding to dentifrices, mouthwashes, and varnishes may create an oral environment which is unfavorable for *C. albicans*.

The research assessing the antimicrobial efficacy of a combination of plant extracts is the need of the hour, and such research will aid the development of a novel, innovative method that can simultaneously inhibit the two most common dental diseases of humanity, apart from lowering the development of drug resistance. Our results show high bioactivity of the SFE of nutmeg seeds against fungal pathogens and can be potential candidate for a potent antifungal molecule. The potential for developing phytomedicine into various health-care products seems to be worthwhile, both from the viewpoint of economy and safety.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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