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Research Article

ACTIVITY OF *MYRISTICA FRAGRANS* AND ITS EFFECT AGAINST *FILAMENTOUS AND NON-FILAMENTOUS* FUNGUS

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ABSTRACT

Mace which is the aril of the fruit of *Myristica fragrans* HOUTT. Asian Indians have traditionally treated stomach pains, dysentery, vomiting, and the symptoms of malaria with mace. It is also chewed to prevent foul breath. The present study was carried out to elucidate the anti fungal effect of 4 extracts; Hexane, Chloroform, methanolic and Ethanolic extract obtained from Mace. The extracts were concentrated and re-suspended in ethanol. Their *in-vitro* susceptibility test was done by disc diffusion method. MIC_{80} was calculated by micro broth dilution method. Results have revealed the extracts to have good activity against both the fungal strain. Methanol and Hexane extracts have been found to be most effective against the organisms tested The MIC_{80} was found to be the least for Methanol extract 0.237 mg/ml against *Candida albicans* and 0.232 mg/ml for *Aspergillus niger.* Phytochemical analysis of the extracts has been conducted to ascertain the active principle associated with the antifungal activity

Keywords: Myristica fragrans, Candida albicans, Aspergillus niger

INTRODUCTION

Antibiotic resistance has become a reason for global concern as clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. Among the infectious diseases fungal pathogens have been associated with animals and humans as commensals, but have lately turned pathogenic or opportunistic after alteration of the host immune system. It is estimated that up to 90% of HIV-infected individuals suffer at least one episode of fungal infection during the course of their disease, of which *Candida albicans* accounts for more than 90% of fungal infections encountered in immuno-compromised patients. The other *Candida* species such as *Candida glabrata, Candida. parapsilosis, Candida. tropicalis*, and *Candida. krusei* are also known to cause symptomatic oral candidiasis in HIV-positive individuals. Other fungal infections seen in HIV-infected individuals include, cryptococcosis due to *Cryptococcus neoformans* and Aspergillosis due to *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger.*

Despite considerable progress in the past few years, the morbidity and mortality of invasive fungal infections are still unacceptably high. It would therefore be novel to evaluate and identify antifungal drugs with new mechanisms of action that not only have a broad spectrum of activity, less toxicity, flexible route of administration but also minimal drug interactions as components of combination therapy regimens for infections that are difficult to treat.

Spices have been used since time immemorable as natural preservative for meat, fish etc. a diverse array of natural phytochemicals that have complementary and overlapping actions, including antioxidant effects, modulation of detoxification enzymes, stimulation of immune system, reduction of inflammation, modulation of steroid metabolism and antibacterial and antiviral effects. *Myristica fragrans* (Mace) belongs to family Myristicaceae is native to the Banda Islands in eastern Indonesia (Moluccas) and is cultivated in the Banda Islands, Grenada, the Caribbean, South India, Sri Lanka, Malaysia, Sumatra, and Brazil. It is spicy and bitter with clove like and piney overtones. Its aroma is due the terpenes present. Depending on its origins, mace has 7% to 14% essential oil and about 30% fixed oil. It contains the same aroma compounds as nutmeg but in different amounts, mainly monoterpenes (87.5%), monoterpene alcohols (5.5%), and other aromatics (7%). Like nutmeg essential oil, the main constituents of mace essential oil are sabinene, α-pinene, myrcene, limonene, 1,8-cineole, terpinen-4-ol, myristicin, γ-terpinene, and safrole.

The current study investigates the anti-microbial potential of *Myristica fragrans* (Mace) against two fungal strains non filamentous *Candida albicans* and filamentous *Aspergillus niger.*[3]

MATERIALS AND METHOD

Spices Collection and Identification

The spices used in this study were procured from local Indian markets and were later authenticated in the botany department of Amity University, NOIDA.

Chemical and reagents

All the chemicals and solvents used were of standard quality and were purchased from commercial vendor.

Preparation of the plant extract

The spice was cleaned under running tap water and dried. In order to obtain the spice extract about 10gms of spice were crushed with mortar and pestle and sieved. The dried powder was then extracted with 40ml n-Hexane, chloroform, methanol and ethanol consecutively for 72hrs/solvent under constant stirring. The extract was then filtered and dried under reduced pressure.

Fungal strains

Candida albicans (SC 2323) and *Aspergillus niger* (ATCC 16404) were obtained from Amity Institute of Microbial Technology. The *Candida* culture was sub cultured on YPD plate and *Aspergillus* culture was sub cultured on PDA plate. The media was prepared by the standard protocol. The plates were grown at 30°C and later maintained as slants stored at 4°C.

Phytochemical analysis

The extracts were subjected to phytochemical screening for the presence of saponins, tannins, terpenes/steroids, phenols and flavonoids. Phytochemical screening of the extracts was carried out according to the methods described by Trease and Evans [4].

Tannins - 1ml of freshly prepared 10% KOH was added to 1ml of the extract. A dirty white precipitate showed the presence of tannins.

Saponins - Frothing test: 2ml of the extract was vigorously shaken in the test tube for 2min. Froth stable for a minimum of 3min indicated presence of saponin.

Flavonoids - 1ml of 10% NaOH was added to 3ml of the extract. There was no yellow colouration which is indicative of the absence of flavonoids.

Steroids - 5 drops of concentrated H_2SO_4 was added to 1ml of the extract in a test tube. Red colouration was observed which is indicative for the presence of steroids.

Terpenes - 1ml of the extract was added to 5 drops of acetic anhydride and a drop of concentrated H_2SO_4 was added. The mixture was then steamed for 1hr and neutralized with NaOH followed by addition of chloroform. Absence of blue - green colour indicates the absence of terpenes.

Phenol: Plant extract was mixed with Ciocalteu reagent (0.1 ml, 1N) and allowed to stand for 15 min. Then 5 ml of saturated Na_2CO_3 was added. The mixture was allowed to stand for 30 min at room temperature. Blue colour indicated the presence of phenol.

Minimum Inhibitory Concentration

Determination of the Minimum Inhibitory Concentration (MIC_{80}) was carried out for the extracts that showed inhibitory effect on the test micro-organism. MIC was conducted based on microdilution method with minor modification in a 96 well plate according to NCCLS guidelines. To the first well 180µl of fresh YPD media was added with 20µl of the extract. The well contents were mixed and serially diluted till the 10^{th} well. 100 µl of fresh *Candida* culture were then added to all wells except 11^{th} well which were kept as a media control. The final concentration of the culture was kept at 10^4 cells/well. 200µl of the fresh media were added to the 11^{th} well and 100µl of *Candida* + 100 µl of the media were added in the 12^{th} well which were kept as (+) control. The plate was incubated for 48 hrs at 30°C. The plate was then read both visually and on an ELISA reader at 600 nm. In case of *Aspergillus* the same protocol was followed and the final conidia concentration was adjusted to 10^4/well[5].

Agar Disc Diffusion Method

The disc diffusion method was used for the test. Candida culture was mixed at a final concentration of $5x10^4$ to YPD agar at 40°C. The media was mixed and poured in a glass Petri dish (25ml/plate). The plate was allowed to solidify. Sterile 6mm discs impregnated with 10 µl of the extract were placed on the agar plate. The plates were incubated for 48 hrs at 30°C. The zone of inhibition (ZOI) was measured in mm. For *Aspergillus* ZOI, 10^4 conidial concentration were mixed (final concentration) in PDA. The plates were incubated for 7 days and the ZOI was calculated. Clotrimizole 10µg was taken as positive control[6].

Time Kill assay

The time kill assay of drug potency is based on inhibition of microbial growth as indicated by measurement of the turbidity (transmittance) of a suspension of suitable microorganisms in a fluid medium to which have been added graded amounts of the test compounds and known concentration of reference material.

10ml of *Candida* culture with 10^4 cells was incubated with extract at their MIC concentration. 0 hr. reading was taken and then the readings were taken every 3 hrs till 21 hrs spectroscopically at 600nm. The readings were then plotted. *Candida* culture without any drug added was taken as positive control[7].

RESULTS

In this study, we have tested the hexane, chloroform, ethanol and methanol extracts of *Myristica fragrans* for their antifungal activity against *Candida albicans* and *Aspergillus niger* strains. All the extracts were dried and re-suspended in ethanol. Phytochemical analysis of these extracts showed the presence of terpenes, flavonoids, tannins and phenols, but absence of saponins and steroids (Table 1).

The spice extracts showed antimicrobial activity against both fungal strains. The maximum activity as seen from the Agar disc diffusion assay was observed in Hexane and Methanol extracts with a Zone of Inhibition of 18 mm and 17mm in Hexane extracts against *Candida albicans* and *Aspergillus niger* respectively and 17mm and 19mm for Methanolic extract against *Candida albicans* and *Aspergillus niger* respectively (Table 2).

Table 1: Phytochemical analysis of the extracts of *Myristica fragrans*

Spices	Extracts	Saponins	Terpenes	Steroids	Tannins	Flavonoids	Phenols
Myristica fragrans	Hexane	-	-	-	-	-	+
	Chlorofor m	-	+	-	+	+	-
	Methanol	-	-	-	+	+	+
	Ethanol	-	-	-	-	-	+

(-) indicated no result; (+): indicates high presence

Table 2: Zone of Inhibition (ZOI) of the extracts of *Myristica fragrans* against *Candida albicans* and *Aspergillus niger*

Extracts	*Candida albicans* (ZOI)(mm)	*Aspergillus niger* (ZOI)(mm)
Hexane	18	17
Chloroform	15	12
Methanol	17	19
Ethanol	13	15
Clotrimazole	8	8

Disc diameter: 6mm

MIC experiments performed by standard NCCLS protocol in a 96 well plate, indicated (Table 3) least MIC of 0.237mg/ml and 0.432mg/ml in the methanol extracts against *Candida* and *Aspergillus* respectively. This was followed by the MICs of the Hexane extracts 0.257 0.547 mg/ml against *Candida* and *Aspergillus* respectively

Table 3: Minimum Inhibitory Concentration (MIC_{80}) in mg/ml values of various extracts of *Myristica fragrans* against *Candida albicans* and *Aspergillus niger* in crude form

Extracts	*Candida albicans* (MIC_{80}) (mg/ml)	*Aspergillus niger* (MIC_{80})(mg/ml)
Hexane	0.257	0.547
Chloroform	0.275	2.2
Methanol	0.237	0.432
Ethanol	1.12	0.950

Time Kill assay indicated high activity of the extracts in liquid culture as well. The Graph (Fig 1) Indicated reduction of the fungal growth as seen spectrophotometrically to reduce from 18 hrs onwards drastically. *Candida* grown in the presence of Clotrimazole (10 µg/ml) on the other hand shows constant increase in the growth pattern hexane, chloroform and Methanol extracts showed maximum reduction in fungal growth pattern as indicated when compared to the control

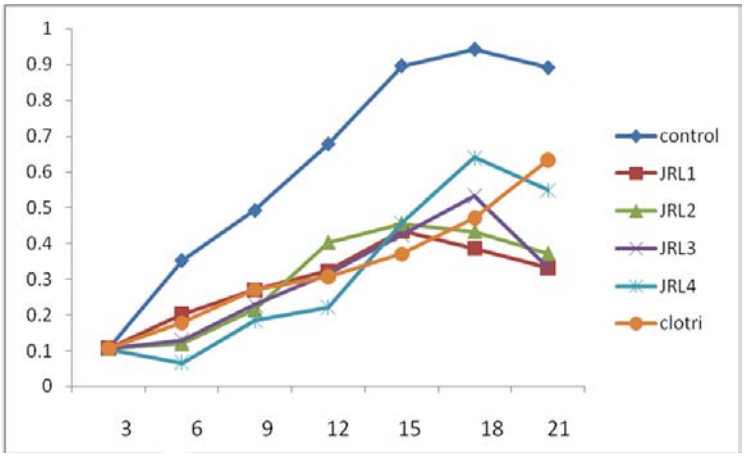

Fig. 1: Growth curve indicating the growth pattern of *Candida albicans* in the presence of extracts of *Myristica fragrans*

X axis indicates time in hrs; Y axis indicates OD at 600nm

- JRL1: hexane, JRL2: chloroform, JRL 03: Methanol , JRL 04: Ethanol extracts of *Myristica fragrans*
- 21 hrs study. Cells grown at MIC concentration of the extracts, control is cells with no drug and positive control is cells with known drug Clotrimazole

DISCUSSION

The medicinal properties of plant species have made an outstanding contribution in the origin and evolution of many traditional herbal therapies. Over the past few years, medicinal plants have regained a wide recognition due to an escalating faith in herbal medicine. In view of its lesser side effects compared to allopathic medicine in addition, the necessity of meeting the requirements of medicine for an increasing human population.

Results of the Phytochemical analysis indicated the presence of phenols in both Hexane and Methanol extract. These results corroborate earlier studies which have reported the antibacterial activity due to phenolic content of spices and herbs[8].

Reason for the high activity of the methanol extracts higher than other extracts may be due to the fact that though ethanol (polarity index: 5.2; viscosity: 1.2) and water (polarity index: 9; viscosity: 0.89) have high polarity but they are highly viscous as compared to methanol (polarity index: 5.1; viscosity: 0.6). Methanol with low viscosity has low density and high diffusibility and can easily diffuse into the pores of the plant material. Methanol was found to be the best extractant, showing high activity in both the fungal species[9]. Furthermore, an interesting observation made during the course of the study was that the antifungal activity of some of the crude extracts tested was more potent than the standard antifungal drug clotrmizole (10µg) against *Candida albicans* and *Aspergillus niger*. Results of the time kill assay has also established the fact that these crude extracts are more potent inhibiting the growth of the microorganism. Earlier studies have proposed that the one of the mechanism of the antimicrobial effects involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally ion leakage from the cells. Due to the emergence of antibiotic resistant pathogens in hospitals and homes, plants are being looked upon as an excellent alternate to combat the further spread of multidrug resistant microorganisms. It is interesting to note that even crude extracts of the spice exhibited good bio activity against the fungal strains[10].

CONCLUSION

The anti fungals that are currently used suffer from several drawbacks such as high toxicity, low absorption, and high cost of treatment. The area of antifungal drug discovery is at a nascent stage. The challenge in antifungal drug discovery is its high toxicity in humans. Therefore the search for alternate treatment modalities is the need of the hour. Our results show high bioactivity of the crude extracts from *Myristica fragrans* against both filamentous and non filamentous fungal pathogens and can be potential candidate for a potent antifungal molecule.

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