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

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Received: 12 Feb 2011, Revised and Accepted: 18 May 2011

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 organisms tested. The MIC80 was found to be the least for Methanol extract 0.237 mg/ml against 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 their disease, of which suffer at least one episode of fungal infection during the course of the system. It is estimated that up to 90% of HIV-infected individuals pathogenic or opportunistic after alteration of the host immune system. 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. MIC80 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 MIC80 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.

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 H2SO4 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 H2SO4 was added. The mixture was then heated for 1 hr 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 Na2CO3 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 (MIC80) 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 180 µl of fresh YPD media was added with 20 µl of the extract. The well contents were kept at 10^4 cells/well. 200 µl of the fresh media were added to the 12th 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.

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 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. Clotrimazone 10µg was taken as positive control.

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 spectrophotometrically at 600nm. The readings were then plotted. Candida culture without any drug added was taken as positive control.

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 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 17 mm in Hexane extracts against Candida albicans and Aspergillus niger respectively and 17 mm and 19mm for Methanolic extract against Candida albicans and Aspergillus niger respectively (Table 2).

<table>
<thead>
<tr>
<th>Spices</th>
<th>Extracts</th>
<th>Saponins</th>
<th>Terpenes</th>
<th>Steroids</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Phenols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristica</td>
<td>Hexane</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>fragrans</td>
<td>Chloroform</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Candida albicans (ZOI)(mm)</th>
<th>Aspergillus niger (ZOI)(mm)</th>
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<tbody>
<tr>
<td>Hexane</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Chloroform</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Methanol</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Ethanol</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Disc diameter: 6mm

MIC experiments performed by standard NCCLS protocol in a 96 well plate, indicated (Table 3) least MIC of 0.237 mg/ml and 0.432 mg/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>
<thead>
<tr>
<th>Extracts</th>
<th>Candida albicans (MIC80)(mg/ml)</th>
<th>Aspergillus niger (MIC80)(mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>0.257</td>
<td>0.547</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.275</td>
<td>2.2</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.237</td>
<td>0.432</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.12</td>
<td>0.950</td>
</tr>
</tbody>
</table>

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.

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. 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 clotrimazole (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.

**CONCLUSION**

The anti fungi 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.

**REFERENCES**

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