ANTIFUNGAL ACTIVITY OF SOME MEDICINAL PLANT MATERIAL EXTRACT AGAINST FUNGUS ASPERGILLUS NIGER

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ABSTRACT

The aim of the study was to evaluate the antifungal activity of extracts of two plant species used in traditional herbal medicine. The plants were selected on the basis of their reported ethnobotanical uses. Aqueous and acetone extracts of two plant species were screened in vitro for their antifungal activity against fungus Aspergillus niger. 50µl concentration MIC of Aloe extract in acetone and 70µl concentration of MIC of Peepal barks extract in acetone also. We concluded from this that these extracts exhibit amazing fungicidal properties that support their traditional uses. The presence of phyto-compound in the extracts including, steroid, triterpenes, alkaloids, tannin, flavnoids, lactones, diterpines, giycosides, sapponins may be responsible for these activities. The acetone extracts of plant are more efficient as compared to the water extract.

KEYWORDS: Aspergillus niger, Ethnobotany, Fungicidal Properties, Traditional Medicine.

INTRODUCTION

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates like monosaccharides (such as glucose) and polysaccharides (such as amylose). Aspergillus species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of Aspergillus demonstrate
oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. In previous reports, *A. niger* (commonly known as black *Aspergillus*), was recorded as a most dominating fungal species to be associated with herbal drugs during storage (Bugno *et al.*, 2006; Gautam and Bhaduria, 2008; Dhale, 2011; 2013). *A. niger* is a saprophytic and filamentous fungus found in soil, forage, organic debris and food product, causing black mould of onion, Shallot; stem rot of Dracaena; root stalk rot of Sansevieria; and boll rot of Cotton; spoilage of cashew kernels, dates, figs, vanilla pods and dried prune (Bobbarala *et al.*, 2009; Panchal and Dhale, 2011).

Medicinal plants represent a rich source of antimicrobial agents (Mahesh and Satish, 2008). Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine. Plants generally produce many secondary metabolites which constitute an important source of micro biocides, pesticides and many pharmaceutical drugs. Plant products still remain the principal source of pharmaceutical agents used in traditional medicine. The effects of plant extracts on bacteria and fungi have been studied by a very large number of researchers in different parts of the world (Reddy *et al.*, 20013; Shinde and Dhale, 2011). Much work has been done on ethno medicinal plants in India. Interest in a large number of traditional natural products has increased. Exploitation of plant metabolites in crop protection and prevention of biodeterioration caused by fungi appear to be promising. In view of these, the author screened some extracts against vegetable pathogenic fungi and the data has been presented in this paper.

**MATERIAL AND METHODS**

**Plant material**

Different plant tissues from plant species used in traditional medicine (Table 1) were collected in 2014 in their natural habitat in around Surat City (Gujarat). The collected plants were identified at the Department of Botany, Veer Narmad South Gujarat University, Surat (Gujarat). The stem bark of Peepal and Aloe leaves were then shade dried and grind in electric mixer grinder. The powder material was kept in air tight glass bottles. This stock powder was used for further extraction.
Table 1: List of plant species

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant Name</th>
<th>Common Name</th>
<th>Family</th>
<th>Part Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Aloe vera</em> (L.) Burm.f. (Syn. <em>A. barbadensis</em> Mill.)</td>
<td>Aloe</td>
<td>Liliaceae</td>
<td>Leaves, gel</td>
</tr>
<tr>
<td>2</td>
<td><em>Ficus religiosa</em> L.</td>
<td>Peepal</td>
<td>Moraceae</td>
<td>Stem Bark</td>
</tr>
</tbody>
</table>

**Preparation of extracts**

After complete dry, fine powder was made by electric grinder. 5g powder sample was added in 50 ml acetone and distilled water then kept it for shaking in orbital shaker for 72h at room temperature. After incubation the extracts were filtered with muslin cloth followed by whatman filter paper. The extracts were added into clean petriplate for evaporation then allowed for evaporation. (The used petriplates were pre-weighted). After evaporation, the plates were weighted. Residual concentrates were dissolved in 5 ml of DMSO. The extracts were collected in screw capped bottles. The extracts were used for antifungal activity, MIC test and phytochemical test. The extracts were stored at 20°C for experimental use. Bioefficacy of the extract was checked *in vitro* by well in agar diffusion method (Onkar *et al.*, 1995).

**Activation of fungi (*Aspergillus niger*)**

Loopful fungal spores was streaked on potato dextrose agar (Hi-media) plates and incubated at 37°C for 2-3 days. All fungus plates were maintained at 4°C in refrigerator for further use.

**Zone of Inhibition**

For determination of zone of inhibition, basically three methods are used. One of them is well diffusion method which we have used.

**A. Preparation of potato dextrose agar medium (PDA agar medium)**

Preparation of PDA includes the following steps

i. PDA agar medium was prepared from commercially available dehydrated base according to the manufacturer instructions.

ii. Immediately after autoclaving, allowed to cool in 45 to 50°C water bath.

iii. The freshly prepared and cooled medium was poured into the glass or plastic flat bottomed petri dishes till the level, horizontal surface to give uniform depth.

iv. The PDA agar medium should be allowed to cool at room temperature and until the use plates were stored in a refrigerator.
v. Plates should be used within 7 days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of agar.

vi. Representative samples of each batch of plates were examined for sterility by incubating at 30-35°C for 24 hours.

**Preparation of well**
The wells were made using cork borer on N-agar plate. The borer was deeped into the alcohol for sterilization and then was used to make wells. Plates were used for the zone of inhibition test.

**Procedure for performing the well diffusion method**

**Inoculums preparation**
Three to five well-isolated colonies of the fungus were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4-5 ml of PDA broth medium. The broth culture is incubated at 35°C until it achieves turbidity 1-2 x 10^8 CFU/ml. The turbidity of actively growing broth culture was adjusted with sterile saline.

**Inoculum of test plates**
Optimally, within 15 minutes after adjusting the turbidity of the inoculums suspension, loopful of suspension inoculates into flask containing Agar. Mix it well and pour it into plate and rotate the plate for even distribution. On the dry PDA agar plate loopful suspension evenly spreaded by spreader.

**NOTE**
Extremes in inoculums density must be avoided. Never use undiluted over night broth culture or other unstandardized inoculums for streaking plates.

**Inoculum of plant extract into the well**
i. In the plate, wells were made for the inoculation of plant extract. Minimum four wells were made in one plate.

ii. Using micropipette, 30µl of antifungal drug was added and extracts into respective wells.

iii. The plates were first placed at 4°C for 30 min in order to diffusion of extract and antifungal drug.

iv. Then plates were incubated at 37°C for 24 hours at room temperature.
v. The diameter of the inhibition zones were measured in millimeter at the end of the incubation time.

**Determination of Minimal Inhibitory Concentration (MIC)**

Dilution susceptibility testing method was used to determine the minimal concentration of antifungal to inhibit or kill the fungus. This was achieved by dilution of antifungal to inhibit or kill the fungus and was achieved by dilution of antifungal in either agar or broth media (PDA).

**Procedure for performing the minimum inhibitory concentration Test inoculums Preparation**

Inoculums preparation was performed as discuss earlier in well diffusion method.

**Procedure**

i. Different concentration of plant extract in (10µl, 20µl……up to 100µl) to the tube to respective tubes were added.

ii. From the inoculums 10µl of each culture was inoculated separately in each set so that final concentration of fungus in tubes became 10^6 cells/ml. This procedure was performed for all the positive extracts antifungal activity which were obtained by primary screening.

iii. Then all sets of tubes of dilution broth were incubated at 37°C for 24 hours in incubator. All sets of tubes were observed for determination of MIC to the susceptible fungus were tested and note down the results.

**Phytochemical Tests**

I. **Test for steroid**

**Salkowski Test**

Chloroform solution of the extract when shaken with concentrated sulphuric acid and on standing yields red colour.

II. **Test for Triterpene**

**Salkowski test**

Chloroform solution of the extract when shaken with concentrated sulphuric acid, lower layer turns to yellow on standing.
III. Test of Alkaloids
The extracts were mixed with ammonia and then extracted with chloroform solution. To this dilute hydrochloride acid was added. The acid layer was used for chemical tests for alkaloids.

**Hager's Test** (Saturated solution of picric acid): The acid layer with Hager's reagent gives yellow precipitate.

IV. Test for Tannins
**Gelatin test**
Extracts mixed with few drops of 1% solution of gelatin containing 10% sodium chloride gives white precipitate.

V. Test for Flavonoids
**Lead acetate tests**
Alcoholic solution of the extracts mixed with few drops of 10% lead acetate gives yellow precipitate.

VI. Test for Lactones
**Balje's tests**
The extracts mixed with solution of sodium picrate give yellow orange colour.

VII. Test for Diterpenes
**Copper acetate test**
The extracts, mixed with solution of copper acetate gives green colour.

VIII. Test for Glycosides
**Kellar Killani's test**
Dissolve the extract in water with Glacial acetic acid and ferric chloride and concentrated sulphuric acid. They give brown ring at the junction.

IX. Test for Saponins
**Foam test**
A small amount of extract is shaken with little quantity of water. The foam produced persists for 10 min. It confirms the presence of saponins.
RESULTS

Screening and evaluation of antifungal activity

The screening and evaluation of antifungal activity was carried out by agar well diffusion method and determination of MIC values, which was carried out by using different concentration. The test fungus was *Aspergillus niger*.

Table 2: concentration of each extract used to check antifungal activity.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Plant Sample</th>
<th>Water (Conc. mg/ml )</th>
<th>Acetone (Conc. mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aloe gel</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Peepal bark</td>
<td>13.1</td>
<td>14.3</td>
</tr>
</tbody>
</table>

Results of diameter of inhibition zone (DIZ value)

The measured DIZ of various extracts of plants with different solvents against *Aspergillus niger* are shown in table - 3.

Table 3: Results of diameter of inhibition zone

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Extract</th>
<th>Acetone extract (DIZ) (mm)</th>
<th>Water extract (DIZ) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aloe</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Peepal bark</td>
<td>23.3</td>
<td>20</td>
</tr>
</tbody>
</table>

Results of determination of MIC value

After evaluating the DIZ values extracts (Aloe and Peepal bark extract prepared with acetone) were taken which shows higher antifungal activity for MIC test by taking different concentration (Table 4 and Fig. 1). The test fungus was inoculated in different concentration of plant extracts i.e.10µl, 20µl, ……100µl.

Table 4: Results of MIC for acetone extracts

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Extracts</th>
<th>Different volume of plant extracts (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>Aloe</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Peepal bark</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig1: Results of MIC for plant extract prepared by using acetone (Aloe and Peepal bark)

The growth of *A. niger* in Aloe extract in acetone was seen below 50µl concentration. So, 50µl can be said MIC of Aloe extract. The growth of *A. niger* in Peepal bark extract in acetone was seen below 70µl concentration. So, 70µl can be said MIC of Peepal barks extract.

**Phytochemical Test**

The results of qualitative screening of phytochemical components in revealed the presence of alkaloids, steroid, glyceroids, triterpenes, diterpines and flavonoids were present in both plants while tannin is extra chemical present in Peepal plant (Table 5).

**Table 5: Result of phytochemical test**

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Phytochemical</th>
<th>Aloe</th>
<th>Peepal bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Steroid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Triterepenes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Tannin</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Lactones</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>7</td>
<td>Diterpines</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Saponin</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

**DISCUSSION**

There are several medicinal plants used by villagers years ago. They have good knowledge of uses of these medicinal plants. In the present *in vitro* study, plant extracts of Aloe (powder) and Peepal bark with medicinal values are screened for evaluating their antifungal activity.
against *A. niger*. Aloe and Peepal bark extract showed higher antifungal activity. So, MIC test was performed for that two plants (acetone extract).

**SUMMARY AND CONCLUSION**

Aloe (powder) and Peepal bark, are plants which are used in medicine for various disease. The above plants involves important phyto chemicals, biological properties, therapeutic application like, Antifungal activity, Antimicrobial activity, Other biological activity and Clinical trials.

These plants were used for antifungal screening against *Aspergillus niger*. The plant materials were extracted with acetone. For the diameter of inhibition zone, minimum inhibitory concentration, were determined by well diffusion method and potato dextrose broth dilutions. The phytochemical analysis was made to determine active inhibitors present in extracts including, steroid, triterpenes, alkaloids, tannin, flavnoids, lactones, diterpines, glycosides, sapponins.

In conclusion, the result obtained in this study clearly demonstrate broad spectrum antifungal activity of Aloe and Peepal bark extract against *Aspergillus niger*. The presence of phyto-compound in the extracts including, (steroid, triterpenes, alkaloids, tannin, flavnoids, lactones, diterpines, glycosides, sapponins) may be responsible for these activities. The acetone extracts of plant are more efficient as compared to the water extract.

**REFERENCES**

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