**In vitro** antifungal activity of ethanol fractions of *Argyreia nervosa* (Burm. f.) Boj. leaves

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*A. nervosa* (Burm. f.) Boj. has been traditionally used in the treatment of skin infections. In the present study, *in vitro* antifungal activity of petroleum ether, benzene, ethyl acetate, chloroform fractions of ethanol extract of leaves of *A. nervosa* was performed. *Candida albicans, Aspergillus niger, A. flavus, Trichoderma spp., Fusarium proliferatum, Microsporum spp.* and *Trichophyton spp.* were the test organisms. The preliminary study was performed using the disc diffusion method. The ethyl acetate fraction showed significant antifungal activity as compared to other fractions. The MIC of the ethyl acetate fraction was found to be in the range of 70-80 µg/ml. The fractions were then analyzed with TLC bio-autographic analysis. The antifungal components were identified by their Rf values and these components showed positive tests for flavonoids.

**Keywords:** *Argyreia nervosa*, HPTLC, Antifungal activity, Bio-autography, Fractionation.

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**Introduction**

The frequency of life threatening infections caused by pathogenic micro organisms has increased worldwide and is becoming an important cause of morbidity and mortality in immune compromised patients in developing countries. Although a large number of antimicrobial agents have been discovered, pathogenic micro organisms are constantly developing resistance to these agents. Since herbal medicines have been used traditionally for therapeutic effects and proven to have lesser side effects, it is the need of the hour to explore and document these facts systematically.

Intact plant parts like leaves, stem and roots produce antimicrobial substances that are probably responsible for the plant’s natural resistance to microbial infection. This medicinal value of plant is due to the secretion of secondary metabolites which might be acting alone or in combination for the therapeutic activities. These secondary metabolites can be extracted, fractionated and further isolated to get the lead molecules for the research and development.

The anti-microbial effect of a drug can be investigated by various bioassay procedures. Bio-autography is a technique that combines, Thin Layer Chromatography (TLC) with bioassay *in situ*. It can be used to the screen active antimicrobial constituents present in a particular natural product extract. Bio-autography combines TLC with bioassay in such a way that it is able to locate the active constituents in a complex matrix such as a plant extract.

Since many years, *Argyreia nervosa* (Burm. f.) Boj. belonging to family Convolvulaceae, has been used in various formulations for its wide range of pharmacological actions. Ethnopharmacologically leaves are reported to be used externally as emollient, vesicant, in the treatment of ringworm, eczema, as local stimulant and rubefacient. Also used internally in the treatment of boils, swelling, as oral contraceptive, antiphlogistic and in UTI for relieve from the burning sensation.

The leaf of *A. nervosa* is reported to contain 1-tricontanol, epifriedelinol and its acetate, β-sitosterol, anthocyanins, quercetin, kaempferol and its glycosides, kaempferol 3-O-L-rhamnopyranoside, 7,8,3',4',5'-pentahydroxyflavone, 5-O-α-D-rhamnopyranoside and 5-O-β-D-glucopyranoside. Literature survey reveals that the chloroform fraction of root shows significant antifungal activity. The alcoholic extract of the leaves of *A. nervosa* has antibacterial activity against *Staphylococcus aureus*. The antifungal activity of *A. nervosa* is however, not

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yet investigated in detail. Thus in the present study an attempt has been made to investigate the antifungal potential of its leaves and to identify the components responsible for the activity.

Materials and Methods

Plant material collection and authentication
Leaves of *A. nervosa* were collected in the month of May from local gardens of Amravati district (Maharashtra). The plant was identified at the Department of Botany, Shri Shivaji Science College, Amravati, and the voucher specimen (AN/14/2010) is placed in the herbarium of Pharmacognosy Laboratory of Government College of Pharmacy, Amravati, Maharashtra, India. The leaves were dried at room temperature and powdered in a multi-mill. The powdered material was stored in an air tight container.

Microorganisms
The standard strains of *Candida albicans* (NCIM No.3471), *Aspergillus niger* (NCIM No. 1196), *Aspergillus flavus* (NCIM No. 535), *Trichoderma spp.* (NCIM No.1059), *Fusarium proliferatum* (NCIM No.1103), *Microsporum spp.* (ATCC 118325), *Trichophyton spp.* (ATCC 2818810) were obtained from the CSIR-National Chemical Laboratory, Pune, India. The diseases caused by the above strains have been enumerated in Table 1.

Instruments
A Camag microlitre sample (Hamilton, Bonaduz, Switzerland) syringe was used for sample application on pre-coated silica gel aluminium plate 60F-254, (5 cm × 10 cm with 0.2 mm thickness, (E.-Merck, Darmstadt, Germany) using a Camag Linomat-V (Switzerland). Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode for all measurements and operated by CATS software (V1.4.3 Camag).

Minimum inhibitory concentration (MIC) was determined by using Digital Spectrophotometer (Model No. EQ820) in the visible range (350-950 nm), Equip-Tronics, (Mumbai). Cultures were incubated in an Incubator (HICON) manufactured by Grover Enterprises, Delhi. Apparatus and nutrient broth were sterilized in Rolex Autoclave Programmable.

Table 1—Diseases caused by the microorganisms used in the study

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Microorganisms</th>
<th>Diseases caused</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Candida albicans</em></td>
<td>Superficial dermatitis, skin abscess, <em>candidiasis</em>, subcutaneous mycoses</td>
</tr>
<tr>
<td>2.</td>
<td><em>Aspergillus niger</em></td>
<td>Onychomycosis (fungal nail infection), otomycosis</td>
</tr>
<tr>
<td>3.</td>
<td><em>Aspergillus flavus</em></td>
<td>Onychomycosis (fungal nail infection), otomycosis</td>
</tr>
<tr>
<td>4.</td>
<td><em>Trichoderma spp.</em></td>
<td>Immunosuppressant</td>
</tr>
<tr>
<td>5.</td>
<td><em>Fusarium proliferatum</em></td>
<td>Fusariosis</td>
</tr>
<tr>
<td>6.</td>
<td><em>Microsporum canis</em></td>
<td>Ringworm of the skin</td>
</tr>
<tr>
<td>7.</td>
<td><em>Trichophyton rubrum</em></td>
<td>Athletes foot, ringworm of the scalp, infection of the armpit, nail infection</td>
</tr>
</tbody>
</table>

Chemicals
Trypan Blue Dye (60%) of Sigma-Aldrich was obtained as a gift sample from Serum Biotech Ltd., Pune. Ethanol, Dimethyl sulphoxide (DMSO), Ethyl acetate, Methanol, and all other chemicals used were of analytical reagent grade obtained from Qualigen, Mumbai (India). Nutrition broth (HIMEDIA M002), Sabouraud Dextrose Agar Medium (HIMEDIA MM063) manufactured by Hi Media Laboratories Pvt. Ltd., Mumbai (India).

Preparation of extracts
The shade dried and powdered leaves of *A. nervosa* were extracted with ethanol by using Soxhlet extractor. The extract was filtered and then solvent was evaporated under reduced pressure to a solvent free concentrated mass, which was then stored in air-tight container in a cool and dry condition.

Preparation of fractions
The ethanol extract (9.5 g) was dispersed in 200 ml of distilled water and sonicated for 30 minutes. This mixture was extracted with petroleum ether (F1), benzene (F2), ethyl acetate (F3) and chloroform (F4) in a separating funnel. The remaining residue was considered as the ethanol fraction (F5). The fractions were concentrated at low temperature and pressure. Even traces of the solvents were removed completely. The fractions so obtained were stored and used for further studies. Preliminary phytochemical screening of all the fractions was performed as per reported methods.

Microbiological Assay

Preparation of subcultures
Fungal cultures were sub-cultured (1% inoculums) in nutrient broth (Himedia M002) at 30°C and
incubated for 24 h. Using sterile saline solution, the cultures were harvested and diluted suitably with the sterile saline solution to bring the count to about $1 \times 10^8$ per mL.

**Sample and standard solution preparation**

Solution (2 mg/mL) in dimethyl sulphoxide (DMSO) of all the fractions was prepared. Nizaral cream, manufactured by Johnson & Johnson Ltd, Mumbai, containing Ketoconazol IP 2% w/w (Batch Number-K120) was used as positive control. Solution (2 mg/mL) of cream in DMSO was also prepared.

**Antifungal activity**

Antifungal activity of all fractions was evaluated by disc diffusion method. The inoculate with respective fungi were homogeneously seeded onto the 90 mm petri dishes containing 20 ml cooled molten Sabouraud Dextrose Agar (SDA) medium using sterile swab in such a way as to ensure thorough coverage of the plates and a uniform lawn of growth following incubation. These inoculated plates were left to dry for at least 15 minutes and the three wells were bored in each petri dish at equal distance. Two drops of each fraction and standard solution were added to the respective wells and named accordingly. The plates were left at room temperature for about one hour to allow the extract to diffuse from the disc into the medium and were then incubated at 30°C for 48 h. After incubation the diameter of zone of inhibitions (in mm) were measured. The complete antifungal analysis was carried out under strict aseptic conditions. Each assay was repeated three times.

**Minimum inhibitory concentration (MIC)**

Significant antifungal activity of F3 was observed and thus, the MIC of F3 was determined using the Turbidimetric method. Stock solution of concentration 1000 µg/ml of fraction F3 was prepared in DMSO. Suitable aliquots of this stock solution were taken and solutions in the range of 500 to 1000 µg/ml were prepared. 1 ml of each solution was placed in separate test tubes. To each test tube, 9 ml of nutrient broth medium previously seeded with the appropriate test organism was added. Three control tubes, one containing the uninoculated culture medium (culture control), another identical with it but treated immediately with 0.5 ml of dilute formaldehyde solution (blank) and a third containing un-inoculated culture medium were also prepared. All the test tubes were incubated at the 30°C for 12 h. After incubation 0.5 ml of dilute formaldehyde solution was added to each test tube. The growth of the test organism was measured by determining the absorbance at about 550 nm of each of the solutions in the tubes against the blank. The lowest concentration that had inhibited the growth of the test microorganism was considered as MIC.

**Bio-autography assay**

Direct bio-autography method was used to determine the phytoconstituents responsible for the anti-fungal activity. A 40 mg/ml solution of Fraction F3 in ethanol was prepared and the band of 10 µl was applied on the even pre-coated HPTLC plate of size 2x8 cm. The chromatogram was developed in the ethyl acetate: methanol: water [100: 13.6: 10]. The chromagrams were dried at room temperature under a stream of air overnight, for complete removal of traces of solvents. Fungal cultures were grown on Sabouraud Dextrose Agar for 3 to 5 days. Cultures were transferred in the Nutrition broth from agar with sterile swabs. The developed TLC plates were sprayed with concentrated suspension containing approximately $1.0 \times 10^6$ cells/ml actively growing fungi. The plates were sprayed until they were wet, incubated for 24 h and then sprayed with a 0.2% solution of Trypan Blue Dye. The plates were observed in visual light for the presence of blue coloured bands. The Rf values of the blue bands were then recorded.

**Identification of phytoconstituent**

The phytochemical screening of F3 fraction had revealed the presence of flavonoids and glycosides. Thus, the HPTLC plates were sprayed with 1% AlCl$_3$ solution in ethanol for detection of flavonoids and 1% KOH in ethanol for detection of glycosides. The chromatogram of fraction F3 was developed using the same chromatographic conditions, as used for the bio-autography procedure. The results were observed in visible and at 366 nm and 254 nm in UV chamber.

**Results**

In present study crude ethanol extract was fractionated in various solvents and the percentage yields of the fractions obtained are as presented in Table 2. The preliminary phytochemical screening of fractions revealed the presence of steroids, glycosides, flavonoids and tannins in various fractions as shown in the Table 3. The antifungal activity of all fractions against different strains using the disc diffusion method showed that F3 fraction had significant
Table 2—Yield of various fractions of ethanol extract of *A. nervosa* leaves

<table>
<thead>
<tr>
<th>Solvents</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>39.64</td>
</tr>
<tr>
<td>Benzene</td>
<td>3.61</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>8.43</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.75</td>
</tr>
<tr>
<td>Ethanol</td>
<td>44.95</td>
</tr>
</tbody>
</table>

Table 3—Phytochemical investigation of individual fractions of ethanolic extract of *A. nervosa* leaves

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (Pet. ether fraction)</td>
<td>Steroids, C-glycosides</td>
</tr>
<tr>
<td>F2 (Benzene fraction)</td>
<td>Steroids, C-glycosides</td>
</tr>
<tr>
<td>F3 (Ethyl acetate fraction)</td>
<td>C-glycosides, flavonoids, tannins</td>
</tr>
<tr>
<td>F4 (Chloroform fraction)</td>
<td>C-glycosides</td>
</tr>
<tr>
<td>F5 (Methanol fraction)</td>
<td>C-glycosides, flavonoids</td>
</tr>
</tbody>
</table>

activity against all the fungal strains under study, while F1 fraction also showed moderate activity against Trichophyton, while all other fractions showed very weak or no antifungal activity (Table 4). The results were recorded as shown in the Plate 1.

The MIC of F3 was found to be 70 µg/ml for C. albicans, A. niger and Trichoderma spp. while 80 µg/ml for A. flavus, F. proliferatum, Microsporum spp. and Trichophyton spp. Due to the observed significant activity of F3 against all the strains, it was further subjected to bio-autography in order to ascertain the possible phytoconstituents responsible for the antifungal property. The fractions were separated on the pre-coated plates and incubated for 48 hours and then sprayed with visualising agent, Trypan Blue Dye, the observations was taken after 20 minutes. Two bands at the Rf values 0.60 and 0.72, showed significant inhibitory effect on the fungal growth (Table 5). Results were recorded as still pictures as presented in the Plate 2.

The phytoconstituents at Rf values 0.60 and 0.72 were then subjected to phytochemical screening using 1% AlCl₃ solution in ethanol. It was observed that yellow fluorescence at approximately the same R values were present. Thus, the two bands showing antifungal activity in F3 were flavonoids. Further screening of F3 by TLC revealed the presence of quercetin.

**Discussion**

Plant derived compounds have been attracting much attention as potent alternatives for infectious diseases. The various secondary metabolites
like flavones, flavonoids, polyphenols, quinones, triterpenoids, alkaloids, lignin, polypeptides and essential oils give potential opportunity for the expansion of modern chemotherapies against wide varieties of microorganisms. A. nervosa has been used in ethno-medicine in the treatment of a number of infections, thus an attempt was made to investigate its antifungal potential and identify the responsible chemical constituent for the effect. It was observed that the ethyl acetate fraction of ethanol extract showed potential antifungal activity with MIC in the range of 70-80 µg/ml. Thus, the same fraction was subjected to bio-autography studies. The plates incubated with fungal strains were treated with trypan blue dye. The principle of visualization was the dye exclusion property of viable cells. Thus only the dead cells allowed trypan blue dye to cross the cell membrane and a dark blue coloured band was visualised. Two antifungal constituents were thus detected using 0.2% solution of Trypan Blue dye. The phytochemical screening of the ethyl acetate fraction showed the presence of flavonoids and glycosides. Thus, the two bands were tested for the presence of both classes of constituents and it was observed that both the antifungal components were flavonoids. Literature reveals that the leaves are rich in quercetin. Reports have revealed that the methanol extract of leaves contain a number of flavonoids, quercetin and kaemperol together with the latter’s glycoside kaemperol-3-O-1rhamnopyranoside. Two new flavone glycosides characterized as 7, 8, 3’, 4’, 5’-penta-hydroxyflavone 5-O-α-l-rhamnopyranoside and 7, 8, 3’, 4’, 5’-pentahydroxyflavone 5-O-α-l-glucopyranoside were also reported from leaves. On performing TLC analysis of ethyl acetate fraction, presence of a quercetin derivative was observed. Quercetin has been reported to have potential anti inflammatory, analgesic, cytotoxic, antioxidant and antimicrobial property. Thus, the significant antifungal activity of the ethyl acetate fraction of ethanol extract of A. nervosa leaves may be due to presence of a quercetin derivative.

Conclusion

A new class of antimicrobial drug is urgently required and the flavonoids represent a novel set of leads. Future optimization of these compounds through structural alteration may allow the development of a pharmacologically acceptable antimicrobial agent or group of agents. The present study gives a good correlation between the reported uses of A. nervosa leaves in skin infections and other infections in traditional Indian system of medicine. Finally, it can be concluded that the flavonoids, mainly a quercetin derivative is responsible for the antifungal property of this plant. Studies need to be performed to further elucidate the structure and properties of this derivative.

Acknowledgement

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References