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2.0 MATERIALS AND METHODS

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2. MATERIALS AND METHODS

The plant *Cassia alata*, Linn., was identified and authenticated at the Regional Herbarium, Southern Circle, Botanical Survey of India, Govt. of India, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in the Botany Department, K.K. College, University of Madras, Tamil Nadu, India.

Leaves of the plant used in the present investigation were collected throughout the year whenever required, from the plants grown in the private garden at Namakkal, Rajaji District, Tamil Nadu, South India, India. For areas outside Namakkal *Cassia alata* leaves available under identical conditions were collected and used in the study.

The study was undertaken among the people of Tamil Nadu, one of the southern states in the coromondel coast of India. The study was extended for a period of 5 years. Patients with less severity and recent origin were treated and cured. The present study represents 327 cases of acute and chronic infection: pityriasis versicolor 210, tinea pedis 63, tinea cruris 38 and tinea corporis 16. Those patients who gave their consent for the study and agreed to the strict compliance and feedback were admitted for this study. They were monitored continuously for relapses even after the complete cure.
2.1 CONFIRMATION OF THE FUNGUS

Direct Microscopic examination method was adopted to confirm the presence of the fungus (Norman *et al.*, 1971; Orlando Canizares, 1975; Robert Berkow, 1982; Margarita Silva-Hunter, 1981; Jan Faergemann, 1989; Bhutani, 1993).

Fine scales scraped from the macules of pityriasis versicolor and at the margins of lesions of dermatophytoses were mounted in 10% KOH solution, stained with methylene blue and observed for the presence of fungal spores (usually in grape like clusters) and hyphal fragments for pityriasis versicolor and highly branched, septate hyphae for dermatophytoses.

2.2 PROCEDURE FOR THE EXTRACTION OF ANTIFUNGAL PRINCIPLES FROM THE LEAVES OF *CASSIA ALATA*

For the extraction of clinically effective antifungal principle(s) from the leaves of *Cassia alata* a simple procedure has been devised.

One hundred grams of fresh, healthy leaflets collected from the leaves of *Cassia alata* were washed first with tap water and then with distilled water. The washed leaflets were mashed by hand in 100ml distilled water (in a stainless steel vessel) and the leaf extract was obtained by squeezing the mash by hands and was filtered using 100 mesh filter cloth. The filtered leaf extract thus obtained was considered as 100% concentrated solution (hereafter referred as *Cassia alata* leaf extract).
2.3 APPLICATION OF THE EXTRACT IN PITYRIASIS VERSICOLOR

For the treatment of Pityriasis versicolor, the different concentrations of the extract were prepared in distilled water and applied to the affected areas of infection as indicated in Table 2.1. The fresh leaf extract was generously applied only once over the infected areas at bed time (4 hours before sleep) and washed off thoroughly with water in the next morning, without using soap. The effect of application of the extract is given in Table 3.1.

2.4 APPLICATION OF THE EXTRACT IN DERMATOPHYTOSES

For the treatment of dermatophytoses, the 100% concentrated solution was applied generously to the affected areas of infection daily at bed time (2 hours before sleep) and washed off in the next morning with water without using soap, until the subsidence of all the clinical manifestations and then once in a week for one month and then once in a month for one year.

The therapeutic effects of application of the extract are given in Table 3.3.

2.5 pH OF THE LEAF EXTRACT

The pH of the leaf extract was measured in Systronics digital pH meter 335 and was found to be 6.1.
Table 2.1  Different concentrations of *Cassia alata* leaf extract used at affected parts in the treatment of Pityriasis versicolor (100 gms of fresh leaves/100 ml of water)

<table>
<thead>
<tr>
<th>Concentration of the leaf extract</th>
<th>Areas applied</th>
</tr>
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<tbody>
<tr>
<td>100%</td>
<td>Trunk only</td>
</tr>
<tr>
<td>90%</td>
<td>Neck and hands</td>
</tr>
<tr>
<td>80%</td>
<td>Face</td>
</tr>
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</table>
2.6 SEPARATION OF ANTIFUNGAL PRINCIPLES

The following procedure was adopted for the separation of antifungal principle(s) present in the leaf extract of *Cassia alata*.

One hundred ml of the leaf extract, used for the above applications was mixed with 3.5ml of con. hydrochloric acid and heated on a water bath for 15 minutes. After cooling to room temperature it was transferred to a 500ml separating funnel. 100ml of solvent ether was added and shaken well for 15 minutes. The upper yellowish brown ether layer was separated and dried over 20gms of anhydrous sodium sulphate and filtered. The filtrate was evaporated to dryness in a glass dish at room temperature and diffused light. The residue obtained is then dried and weighed. The dried ethereal extract was used for furthur studies (here after referred as antifungal principle(s)).

2.7 IN VITRO ANTIFUNGAL STUDIES OF THE ANTIFUNGAL PRINCIPLE(S)

The anti-fungal principle(s) thus obtained by the above procedure were subjected to *in vitro* antifungal activity against *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum gypseum* by broth dilution method (Arvind Padhye, 1981).

21 days old fungal culture of each organism was separated aseptically from the Sabouraud dextrose agar slants prepared with the following composition (Sigurd Funder, 1968).
Dextrose 40gm
Peptone 10gm
Agar 18gm
Distilled water 1000ml

The fresh culture of the fungi was macerated in sterile saline using a sterile mortar and pestle. Suspensions prepared in this way for each fungus were adjusted spectrophotometrically to an absorbance of 0.600 at 450nm. 10 μl of the fungal inoculum was added to each tube.

One ml of sterile Sabouraud dextrose broth of the following composition was pipetted into 7 sterile test tubes for each organism, out of which one served as control.

Dextrose 40gm
Peptone 10gm
Distilled water 1000cc

The six tubes prepared in the above way for each organism were placed serially in a rack. 1 ml of water containing 400mg of antifungal principle(s) was introduced into the first tube. From this 1ml was taken out and introduced into the second tube and this process was repeated upto the sixth tube. From the sixth tube one ml was withdrawn and discarded.
Both tests as well as control tubes were kept at room temperature (26±1°C) for 20 days. Fungal growth was compared with the controls and the results are presented in Table 3.5.

2.8 IDENTIFICATION OF THE ANTIMICROBIAL PRINCIPLE(S)

To identify the antimicrobial principle(s) which inhibited the above test organisms in vitro, the antimicrobial principle(s) were subjected to Thin Layer Chromatography (TLC) and UV-visible spectroscopic studies.

The T.L.C procedure was adopted as outlined by Egon Stahl (1969) and Wagner et al. (1983).

For the confirmation of the presence of Kaempferol, Aloemodin and Rhein, the principal compounds reported to be present in the leaves of Cassia alata (Hauptmann et al., 1950; Seshagiri Rao et al., 1975) direct comparison of the above authentic samples (Procured from Sigma-Aldrich, U.S.A) with antimicrobial principles isolated from Cassia alata leaves was done by co T.L.C. in precoated plate silica gel 60 F 254 (Marck).

10μl each of Kaempferol, Aloe emodine, Rhein and the antimicrobial principle(s) separated from Cassia alata leaves were applied to the T.L.C plate in 0.1% ethanolic solution. This plate was developed using the solvent mixture n-propanol : ethyl acetate : water (40:40:30). The solvent front was allowed to ascend 10cm from the line of application.
After removal, the solvent from the T.L.C. plate was evaporated under diffused light. The chromatoplate was then observed under visible light and U.V light (365nm). The same chromatoplate was exposed to ammonia vapour (aqueous ammonia sp. gra. 0.91) for 30 min and observed under visible light and U.V. light (365nm.).

The characteristics of the chromatogram are presented in Table 3.6.

The bands corresponding to Kaemphrol, Aloe emodin and Rhein were scrapped carefully from the chromatoplate into the test tubes and the contents were dissolved in 5ml rectified spirit. The contents were centrifuged (Remi C_{24} cooling centrifuge) at 4000 x g for 10 minutes to separate the silica gel.

The blank was prepared by dissolving the silica gel scraped below the line of application.

Absorption maxima was recorded for each band using a double beam spectrophotometer (Jasco 7800. Japan) and presented in Table 3.7.