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ANTIFUNGAL ACTIVITY OF *FICUS RACEMOSA* LEAF EXTRACT AND ISOLATION OF THE ACTIVE COMPOUND

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Abstract: The 50% methylene chloride in hexane flash column fraction of the extract of the leaves of *Ficus racemosa* was found to have antifungal activity. The extract inhibited the growth of several plant pathogens *(Curvularia* sp, *Colletotrichum gloeosporioides*, *Alternaria* sp, *Corynespora cassiicola* and *Fusarium* sp). Psoralen was identified as the active compound and was shown to be biodegradable, having the potential to be developed as a fungicide against pathogens causing diseases on crops of economic importance.

Keywords: *Alternaria* sp., Antifungal activity, *Colletotrichum gloeosporioides*, *Corynespora cassiicola*, *Curvularia* sp., *Ficus racemosa*, *Fusarium* sp.

INTRODUCTION

Tropical forest plant species have served as a source of medicine for the people of the tropics for millennia. Sri Lanka with its great diversity of flora and fauna, has many plants of medicinal value.[1-5] Discovery of metabolites showing biological activity has led to research programmes aimed at the isolation and characterization of biologically active metabolites from Sri Lankan plants. Many extracts of local plants have been screened for antifungal activity.[6-11]

Ficus racemosa which belongs to the family Moraceae, is an evergreen tree 18-20 m high, with glabrous, pubescent or scaberulous shoots. It flowers in November and the flowers are unisexual. The tree is distributed in India, Burma and Sri Lanka. In Sri Lanka it is common in the banks and streams in the moist low country upto an altitude of 610 m. The fruit is reddish and about 3.5 cm long.[4] Many uses of the fruit, root and bark in traditional medicine are reported.[4] The powdered leaves are used for bilious ailments.

There are no previous studies on the antifungal activity of the leaf extracts of *F. racemosa*. We report here the effect of the 1:1 methylene chloride : hexane flash column fraction of the leaf extract of *F. racemosa* on several fungal pathogens causing diseases in crops of economic importance. The active compound was isolated and its biodegradation was also investigated.

METHODS AND MATERIALS

Preparation of the leaf extract: Ficus racemosa leaves (1.3 kg; collected from the University of Colombo) were washed under running water and air dried, cut into small pieces, ground in a laboratory mill and kept immersed in methanol (5l) for 2 weeks. The methanol extract was filtered through cotton wool and solvent removed under reduced pressure at 40-45^{0}C to yield a crude extract (165 g). The crude extract was subject to flash chromatography using G-6 silica gel and solvent systems of increasing polarity(hexane, methylene chloride in hexane, methanol in methylene chloride). These fractions were concentrated under reduced pressure at 40-45^{0}C using a rotary evaporator and tested for antifungal activity by *Cladosporium* thin layer chromatography (tlc) bioassay using *Cladosporium cladosporioides* as the test fungus.[12] All solvents were distilled before use.

Cladosporium tlc-bioassay: Test solutions were spotted on tlc plates (Aldrich, tlc grade silica pre-coated to a thickness of 0.5mm on 20x20 cm glass plates). Six sets of plates were separately developed in a solvent system of 1:1 methylene chloride : hexane in solvent tanks equilibrated with eluant. After air drying at ambient temperature for 24 hours, the plates were sprayed with a conidia suspension of *Cladosporium cladosporioides* and incubated as described by Smith.[12]

The 50% methylene chloride in hexane flash column fraction (containing about 5.3g of compound) showed antifungal activity against *Cladosporium cladosporioides* and was used to study antifungal activity against other plant pathogens.

Organisms: *Cladosporium cladosporioides*, *Curvularia* sp.(isolated from infected leaves of rice plants), *Colletotrichum gloeosporioides* (from rubber leaves affected by Colletotrichum leaf disease), *Fusarium* sp. (from infected tomato plants), *Altenaria* sp. (from infected leaves of potato), *Corynespora cassiicola* (from infected leaves of rubber) were used as test fungi. All fungi were maintained on potato dextrose agar (PDA) at 30^{0}C.

Agar plate assay: Fifteen ml portions of sterile molten PDA were cooled to 45^{0}C and were mixed with volumes of test solution (20 mg/ml of test sample in acetone) so that the final concentration of the test compounds were either 0.01%, 0.02% or 0.05% by weight, and then poured into sterile petri dishes. A 0.5 cm^2 agar square obtained from a periphery of a 7d old fungal culture growing on PDA at room temperature was placed at the centre of the medium. Three plates were used per test fungus. In the control experiments the medium was prepared using only the corresponding volume of acetone. All plates were incubated at room temperature and the diameter of the colony was measured as described in Senaratna *et al.*[13] at 24 h intervals for 7d; growth on day 4 was used for the calculations. The experiments were carried out in triplicate.

The percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Growth area in reference - Growth area in sample}}{\text{Growth area in reference}} \times 100$$

Isolation of the active compound: The 1:1 methylene chloride : hexane flash column fraction was concentrated under vacuum (40-45^{0}C) and column chromatographed on silica gel (Aldrich 70-230 mesh) column, eluting with mixtures of methylene chloride in hexane of increasing polarity. Fractions were collected, pooled together after tlc monitoring and these combined fractions were subjected to *Cladosporium* bioassay. The fractions that showed significant activity and were almost pure with respect to tlc analysis were combined together and the solvent evaporated under vacuum (40-45^{0}C). The crystalline compound obtained was further purified by recrystallisation with methanol in methylene chloride. The structure was elucidated by spectroscopic methods.

Densitometric studies of the crude extract used for agar plate assays: 6.0 mg active compound and 47.0 mg of crude sample were used in this study. The active compound (6.0 mg) was dissolved in 10.0 ml of $CHCl_3$. Crude sample (47.0 mg) was dissolved in 5.0 ml of $CHCl_3$. These samples were applied on to a tlc plate (0.5 mm x 20 cm x 10 cm Merck precoated with silica gel GF254) and the plate was developed with ethyl acetate : hexane 2:1 to give a R_f = 0.57 for the pure compound. 10μl of the crude sample (in triplicate) and 6μl, 8μl, 10μl, 12μl of pure compound (each in triplicate) was spotted on a tlc plate and scanned by a densitometer (λ_{ex} = 365 nm and $\lambda_{emi\ (flou)}$ = 480 nm). A calibration curve of volume (μl) vs peak area was obtained. This was used to calculate the concentration of the active compound in the crude extracts.

Degradation of the active component: Fifteen ml of potato dextrose liquid medium was introduced into 100 ml Erlenmeyer flasks and each flask was inoculated with a 0.5 cm^2 agar square of *Colletotrichum gloeosporioides* obtained from a 7-day old culture on PDA at room temperature. To each liquid culture medium, 20 mg of pure psoralen in 2 ml acetone was added immediately after inoculation. In the control experiments, the pure psoralen was incorporated into liquid medium without inoculating with the fungus. The absorbance of the culture filtrate was measured at 330nm. Biodegradation was estimated by calculating the percentage decrease in absorbance[10] at 330nm assuming that the degradation product has no significant absorbance. The percentage decrease in absorbance at 330nm was calculated as follows:

$$\text{Percentage decrease in absorbance} = \frac{\text{Initial absorbance - Absorbance after harvest}}{\text{Initial absorbance}} \times 100$$

Spectroscopic analysis: NMR was recorded in $CDCl_3$ solution using a Brucker AC 200 MHz spectrometer. Mass spectra was recorded using a Hewlett Packard 5989 A GC-MS spectrometer operating in the EI mode. IR spectrum was recorded in a JASCO 5300 FT-IR spectrometer. UV absorbance was measured in 1 cm cells using a JASCO V560 UV/Visible spectrometer. The Densitometer was a Shimadzu CS 9000 Dual-wavelength scanner.

RESULTS

Antifungal activity of the leaf extract: The preliminary screening of the flash column fractions for antifungal activity was done by tlc plate bioassay method using *Cladosporium cladosporioides*. Two of the flash column fractions (1:1 methylene chloride : hexane and 4:1 hexane : methylene chloride) showed inhibitory zones in the bioassay. The 1: 1 methylene chloride : hexane fraction was chosen for the study of the inhibitory action against plant pathogens because of its higher inhibition and greater weight of residue (5.3 g). The fraction inhibited the growth of all fungi examined. The % inhibition of each fungus by 1: 1 methylene chloride: hexane fraction of *F. racemosa* leaf extract obtained using agar plate bioassay is given in Table 1. The highest inhibition was against *Curvularia* sp. The inhibitory activity of the test samples increased with increase in concentration except in *C. cassiicola*.

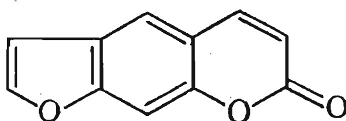
Table 1: Inhibition of fungal growth by 50% methylene chloride in hexane fraction of *Ficus racemosa* leaf extract.

Fungus	% inhibition of growth* by test sample at 0.01%	0.02%	0.05%
Alternaria sp.	3.37 ± 0.1a	7.49 ± 0.01b	17.42 ± 0.2c
Corynespora cassiicola	10.55 ± 0.06a	16.66 ± 0.02b	16.37 ± 0.07b
Fusarium sp.	7.29 ± 0.07a	10.14 ± 0.06b	25.53 ± 0.01c
Colletotrichum gloeosporioides	2.92 ± 0.06a	17.27 ± 0.18b	32.74 ± 0.12c
Curvularia sp.	27.6 2 ± 0.06a	35.15 ± 0.06b	37.68 ± 0.05c

* Average of three replicates ± standard error of the mean.
Values in a row followed by the same letter are not significantly different at $p = 0.05$ (Duncan's multiple range test)

Isolation of the active compound: The active compound that was separated by chromatography on a silica gel column and purified by recrystallisation with methanol in methylene chloride was identified as psoralen(I) by spectral

analysis. Melting Point 165-167^{0}C from methanol in methylene chloride (lit[6,7] 162-163^{0}C).



I

The following spectral data were obtained for this compound:
U.V (acetone) 330 nm (ε_{max} 8000)
IR($CHCl_3$) 1730, 1635, 1577, 1450 cm^{-1}
^1H NMR($CDCl_3$) δ 7.81(1H, d), 7.70(1H, d), 7.69(1H, s), 7.48(1H, apparent s), 6.84(dd, 1H), 6.38(1H, d).
^1H COSY spectrum showed that the proton at δ 7.48 is coupled to protons at 6.84, 7.69 and 7.81
MS m/e 186, 158, 130, 102

^{13}C NMR ($CDCl_3$): δ 161.05,156.42, 152.2,146.91,144.09,124.87, 119.83, 115.41, 114.66, 106.37, 99.90

This data is in close agreement with that reported for psoralen[14]

Analysis observed: 70.64% C, 2.92%H
Calculated for $C_{11}H_6O_3$: 70.98% C, 3.23%H

Densitometry study: Densitometric study on the crude extract used for the bioassay showed that the 0.01%, 0.02% and 0.05% concentrations contained 93, 186.4 and 466.0 μg respectively of active compound.

Degradation of the active compound: The results in Table 2 show a decrease in absorbance at 330 nm when incubated with the fungus *Colletotrichum gloeosporioides*. The decrease in absorbance at 330 nm indicated the degradation of the active compound psoralen. In the control experiment, without the fungus a decrease in absorbance was not observed.

Table 2: The percentage decrease in absorbance at 330 nm in the culture filtrates of *Colletotrichum gloeosporioides*.

Days after incubation	% Decrease in absorbance
2	44.9 ± 0.7
4	63.3 ± 0.7
7	69.3 ± 1.0
9	100
11	100

Average of three replicates ± standard error of means.
The means are significantly different at p= 0.05 (Duncan's Multiple range test)

DISCUSSION

Isolation and characterization of secondary metabolites of plants which have inhibitory activity against fungal plant pathogens, would enable the development of inexpensive fungicides based on locally available natural products. The 1:1 methylene chloride: hexane flash column fraction of the leaf extract of *F. racemosa* had inhibitory activity against several plant pathogens examined. The active compound was identified as psoralen(I). Coumarins such as psolarens and xanthotoxins are of medicinal interest in the control of leucodermia.[15] Psoralens are powerful phototoxic agents in animals and humans. Psoralen containing pulp of fruits and vegetables can be applied to the skin directly or in the form of cosmetics. It can also be taken orally by ingestion of certain common fruits and vegetables or by the use of certain psoralen containing drug formulations. Psoralen and its derivatives such as 8-methoxy psoralen are increasingly used in photochemotherapy for management of disorders such as vitiligo, psoriasis and mycons fungaids.[16]

Psoralen has been isolated from the leaves, stem bark[7] and unripe fruit shell[6] of *Limonia acidissima* and has been reported to inhibit the growth of *Aspergillus niger, Colletotrichum gloeosporioides, Curvularia* sp., and *Penicillium* sp.[7] Here we have isolated psoralen from the leaves of *F. racemosa* and shown it to be active against several fungal pathogens (*Curvularia* sp, *Colletotrichum gloeosporioides, Alternaria* sp, *Corynespora cassiicola* and *Fusarium* sp.) of plants which cause important diseases in major crops of Sri Lanka. Psoralen underwent biodegradation when incubated with *C.gloeosporioides* in liquid culture. The decrease in absorbance at 330 nm (ϵ_{max} 8000) shows that the degradation occurs in the psoralen nucleus. In conclusion, we show that psoralen which has antifungal activity against several plant pathogens causing diseases in crops of economic importance to Sri Lanka, could be isolated from the leaves of *F. racemosa*. The pathogenic fungus *C. gloeosporioides* was shown to degrade psoralen. Therefore it has the potential to be developed as an effective fungicide.

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