Journal of
PHYTOMEDICINE
AND THERAPEUTICS

Journal of Phytomedicine and Therapeutics 6[1] 2001

Antifungal Activity of *Ancistrophylum secundiflorum* L. (Areceae)

A. A. Adekunle*

Department of Botany and Microbiology
University of Lagos

Abstract
Hexane, ethylacetate, butanol and water extracts of *Ancistrophylum secundiflorum* bark were investigated individually for in vitro antifungal activity by disc diffusion agar technique. The phytochemical properties of th butanol extract was assayed. All the four crude extracts tested showed definite antifungal activity against *Acremonicum strictum*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans*, *Curvularia lunata*, *Fusarium solani* and *Rhizopus oryzae*. The butanol and water extracts were the most active antifungal fractions. Seven pure antifungal compounds were purified from the butanol crude extract using TLC, HPLC and column chromatography. The butanol extract contained flavonoid, phlobatannin, Saponins and tannins. Most of the pure compounds were tannins. The molecular mass of the compounds were between 415 g to 729.5 g as recorded by mass spectrometry. The purer compounds produced from *Ancistrophylum secundiflorum* had more antifungal activity than the check antibiotics, Fulcin, Griseofulvin and Nystatin at 100 µg/ml.

Keywords: Antifungal activity, Tannin, *Ancistrophylum secundiflorum*, Butanol extract.

Introduction

Ancistrophylum secundiflorum L. (Areceae) is a tree of about 20 m high. It is typically a tropical plant, reported to be only in Africa and found in the South-western Nigeria. Apart from its being used as an antifungal agent crudely by local communities in Nigeria, it is also used in stopping or clotting blood in fresh wounds (1).

Fungal related diseases may not be common as other diseases caused by other microbes but when present, they could be difficult to eradicate especially in immunosuppresive situations (2). This has led several workers such as Irobi and Daramola (3), Olukoya *et al.* (4), and Saxena and Mathela (5) in the search for better antifungal substance from *Mitracarpus villosus*, *Emilia coccinea* and *Nepeta leucophylla* respectively. No antifungal activity of *Ancistrophylum secundiflorum* has been reported. Also the phytochemistry of this plant has not been reported. Therefore this study was carried out to investigate the antifungal activity of hexane, ethyl acetate, butanol and water extracts, and isolated pure compounds of some of the extracts of the bark of *Ancistrophylum secundiflorum*. Also the minimum inhibition concentration (MIC) of the extracts against the test organisms will be investigated.

Experimental

Source of micro-organisms: The fungi used in the antifungal assay include *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Acremonium strictum*, *Curvularia lunata*, *Fusarium solani*, *Rhizopus oryzae* and *Candida albicans*. These organisms were obtained from stock cultures of Assoc. Profs. T.K. Tan and T.S. Sim of the Department of Biological Sciences, and

* *Corresponding Author*
 ISSN: 1118-1028

Microbiology, National University of Singapore, Asia. The microorganisms are known to be pathogens of man and other animals. They were stored on potato dextrose agar slants at 4°C prior to use.

Plant materials: The plant bark used were collected from Ota, Ogun State in South-West Nigeria (Lat. 3 4 °N, Long. 13 15 °E). After bringing the plant part to the laboratory, they were shade-dried at room temperature (28-30°C) for 14 days. Samples of the plant was authenticated using the text on vernacular names of Medicinal plants by Gbile (6) and at the Department of Botany and Microbiology, University of Lagos. A voucher specimen of the plant part was deposited at the Department of Botany and Microbiology, University of Lagos.

Extraction and purification: Dried bark of *Ancistrophylum secundiflorum* was ground in a warring commercial blender into powder. The powdered bark (800 g) was suspended in 1.6 liters of 70% aqueous acetone solution and soaked for 24 hrs. Then the solution was filtered with a Whatman No. 1823 filter paper, with the help of a vacuum pump. The residue was resuspended in 80% aqueous methanol in 3 changes, for an hour each. The filtrate of the acetone and methanol were mixed and the organic solvents were evaporated under pressure using a rotatory evaporator. At the end, aqueous extract of the plant was left. This aqueous extract was partitioned with hexane (3 x 100 ml), ethyl acetate (3 x 100 ml) and butanol (3 x 100 ml) to produce hexane, ethyl acetate and butanol extract respectively leaving a water layer or extract at the end of the separation. The organic solvents in the extracts were each evaporated using pressurized rotatory evaporator, Nitrogen, speed vacuum evaporator and/or freeze drier as deem fit for each extract. Each of the dried extracts (hexane, ethyl acetate, butanol and water) were dissolved in its best solvent (50% methanol or water) and tested for antifungal activity on the fungi listed earlier. The best extract with the highest antifungal activity was purified by separating of reverse phase thin layer chromatography (TLC) plates after choosing the right solvent mixture. Then the extract was subjected to column chromatography over sephadex LH-20, five times repeatedly. The fractions were collected manually and then by a Pharmacia-LKB gradifractional collector and a recorder. Each of the fractions was tested for antifungal activity. The fraction with the most antifungal activity was chosen and tested again on TLC plates, which were developed in a chromatographic tank with the suitable solvent mixture. Some of the active fractions were also separated on Bio-card perfusion workstation or Schimadzu CBM-10A, High performance liquid chromatography (HPLC). Fractions with similar Rf value on TLC plates were combined and concentrated. This procedure was followed until the fractions contained pure compounds as shown by the Rf values, peak of the HPLC fractions, or peak of the TLC plates observed under longer short UV light, or developed with 10% H_2SO_4 and heated on an hot plate at 50°C to give visible colours. The pure compounds were then tested for antifungal activity, and their molecular mass were detected and determined with EIMS mass spectrometer.

Antifungal activity assay: The antifungal activity testing was carried out using 3 different methods, the supplemented growth media, disc diffusion agar method and well boring diffusion agar method. Paterson and Bridge (7) modified supplemented growth media method was adopted. The method described by Irobi and Daramola (3) for disc agar diffusion antifungal testing was used while the modified method of Irobi (8) was used for the well boring agar diffusion

method. In addition to the extracts and compounds from *Ancistrophylum secundiflorum*, antibiotics such as Fulcin, Griseofulvin and Nystatin were used as positive controls during the antifungal testing. The experiment was repeated once.

A concentration gradient or minimum inhibitory concentration (MIC) of the antifungal extracts and compounds were determined by varying the concentration of reconstituted extract solution (0.001-1000 µg/ml) and subject to the antifungal testing methods mentioned above.

Preliminary phytochemical study: Preliminary phytochemical studies were carried out using the methods described by Fadeyi *et al*. (9) and Harbone (10). The bark of *Ancistrophylum secundiflorum* were screened for the presence of alkaloid, anthraquinone, flavonoid, phlobatannin, saponins, steroid and tannins.

Result

The 800 g of powdered *Ancistrophylum secundiflorum* bark produced 8 g of Hexane extract, 54 g of Ethylacetate, 240 g of Butanol and 480 g of water extract. The result in Table 1 shows that all the crude extracts showed definite antifungal activity. The water and butanol extracts showed greater antifungal activity than hexane and ethylacetate extracts. The crude extracts have higher inhibitory effect on the fungi tested than the antibiotic, Fulcin, Griseofulvin, Nystatin, used as control.

Fig. 1 shows the peak of one of the pure compounds from the butanol crude extract on an HPLC graphic recorder. In all six other pure compounds were produced from TLC plate and repeated column chromatography of the butanol extract. The compounds were given tags which were: Fr-Ac-6-3134; Fr-Ac-7-3051; Fr-Ac-8-1629; Fr-Ac-10-2258; Fr-Ac-10-6182; Fr-Ac-10-8796 and Fr-Ac-10-96107, relating to the tube fractions they were present in. The molecular mass of these compounds were 498 g for Fr-Ac-6-3134; 504 g for Fr-Ac-7-3051; 729.5 g for Fr-Ac-8-1629; 421 g for Fr-Ac-10-2258; 472.4 g for Fr-Ac-10-6182; 565 g for Fr-Ac-10-8796 and 415 g for Fr-

Table 1: Anti-fungal activity of the crude bark extracts of *Ancistrophylum secundiflorum*

Extracts	* Zone of Inhibition (Mean ± SEM [MM])							
	Acremonium strictum	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus niger*	*Candida albicans*	*Curvularia lunata*	*Fusarium solani*	*Rhizopus oryzae*
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Methanol (50%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.46 ± 0.70	2.44 ± 0.21	0.00 ± 0.00
Hexane	9.79 ± 0.49	10.04 ± 0.32	11.75 ± 0.43	10.33 ± 0.10	10.06 ± 0.68	11.89 ± 0.42	10.30 ± 0.96	10.80 ± 0.17
Ethyl acetate	10.86 ± 0.13	11.78 ± 0.59	14.74 ± 0.71	14.72 ± 1.11	12.00 ± 1.27	13.51 ± 0.91	14.13 ± 1.17	15.92 ± 1.09
Butanol	14.27 ± 0.70	17.39 ± 0.62	18.48 ± 0.88	17.01 ± 0.13	22.24 ± 1.36	25.33 ± 0.83	21.61 ± 0.20	18.99 ± 0.61
Water	16.97 ± 1.04	18.76 ± 0.21	19.38 ± 0.82	18.97 ± 0.59	25.89 ± 0.87	26.30 ± 1.16	24.55 ± 1.29	19.71 ± 1.65
Fulcin	10.78 ± 0.56	12.28 ± 1.31	13.05 ± 0.64	13.83 ± 1.08	13.40 ± 1.26	14.79 ± 0.73	11.15 ± 1.29	11.02 ± 0.90
Griseofulvin	10.10 ± 0.07	10.50 ± 0.50	12.75 ± 1.19	10.38 ± 0.48	10.38 ± 0.48	12.13 ± 0.60	11.19 ± 0.50	14.00 ± 1.12
Nystatin	11.51 ± 0.29	15.58 ± 1.12	16.13 ± 0.60	14.13 ± 0.60	17.75 ± 1.30	17.88 ± 1.50	13.13 ± 1.27	13.13 ± 0.93

* Four replicates and repeated once

Table 2: Anti-fungal activity of compounds from the butanol crude bark extracts of *Ancistrophylum secundiflorum*

Compounds	* Zone of Inhibition (Mean ± SEM [MM])							
	Acremonium strictum	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus niger*	*Candida albicans*	*Curvularia lunata*	*Fusarium solani*	*Rhizopus oryzae*
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Methanol (50%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.46 ± 0.70	2.44 ± 0.21	0.00 ± 0.00
Fr-Ac-6-3134	20.43 ± 0.35	23.00±0.00	21.13 ± 0.60	26.50 ± 0.86	24.88 ± 0.33	29.19 ± 0.50	28.63 ± 0.48	21.38 ± 1.49
Fr-Ac-7-3051	23.65 ± 1.19	24.12±0.93	24.70 ± 0.93	28.87 ± 1.23	25.11 ± 1.04	31.88 ± 0.54	30.56 ± 0.70	23.37 ± 1.15
Fr-Ac-8-1629	19.84 ± 0.83	21.55±0.38	20.34 ± 0.52	22.51 ± 0.81	23.21 ± 0.58	25.75 ± 0.97	24.68 ± 1.14	22.43 ± 0.27
Fr-Ac-10-2258	18.91 ± 0.51	22.98±1.12	21.64 ± 0.68	22.62 ± 1.31	24.25 ± 0.87	26.18 ± 1.16	25.49 ± 0.92	21.41 ± 0.27
Fr-Ac-10-6182	17.65 ± 0.31	0.00 ± 0.00	10.75 ± 0.43	21.00 ± 0.66	21.00 ± 0.71	24.19 ± 0.50	23.00 ± 0.64	21.13 ± 1.03
Fr-Ac-10-8796	18.05 ± 0.79	0.00 ± 0.00	11.88 ± 0.33	22.13 ± 0.33	22.88 ± 0.78	24.88 ± 0.62	25.86 ± 0.96	22.00 ± 1.55
Fr-Ac-10-96107	19.61 ± 0.37	21.63±0.73	0.00 ± 0.00	22.25 ± 0.43	22.34 ± 0.90	25.00 ± 0.00	26.88 ± 0.33	20.13 ± 0.33
Fulcin	10.78 ± 0.56	12.28±1.31	13.05 ± 0.64	13.83 ± 1.08	13.40 ± 1.36	14.79 ± 0.73	11.15 ± 1.29	11.02 ± 0.90
Griseofulvin	10.10 ± 0.07	10.50±0.50	12.75 ± 1.19	10.38 ± 0.48	10.38 ± 0.48	12.13 ± 0.60	11.19 ± 0.50	14.00 ± 1.12
Nystatin	11.51 ± 0.29	15.58±1.12	16.13 ± 1.05	14.13 ± 0.60	17.75 ± 1.30	17.88 ± 1.50	13.13 ± 1.27	13.13 ± 0.93

* Four replicates and repeated once

Fig. 1: HPLC graphic illustrations of one of the pure compounds (peak arrowed) from the butanol extract of *Ancistrophylum secundiflorum*

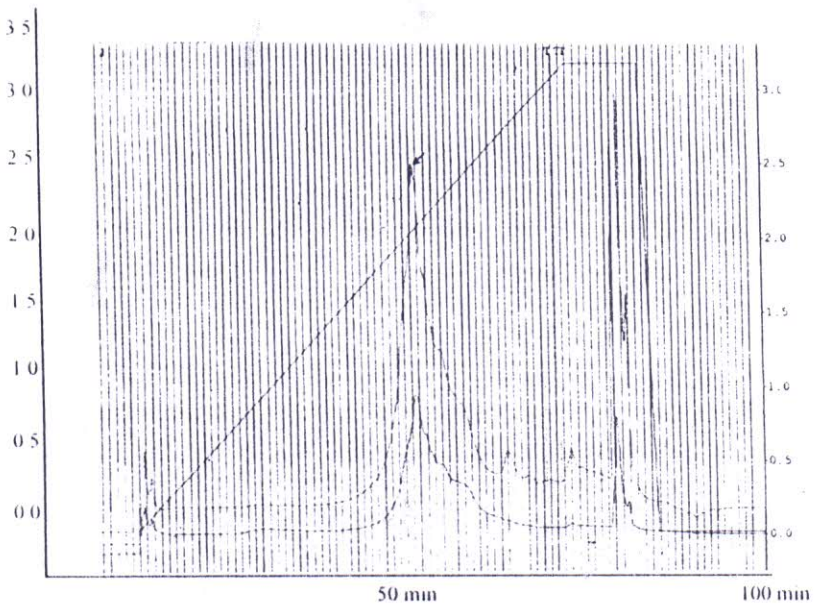


Fig. 2: The mass spectrometer data of a compound, Fr-Ac-10-2258 from *Ancistrophylum secundiflorum*

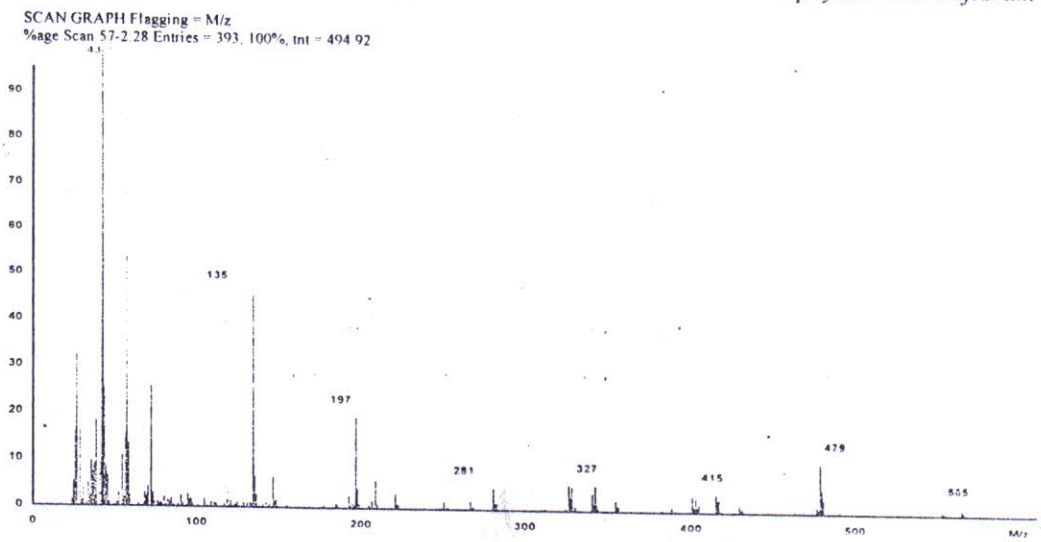
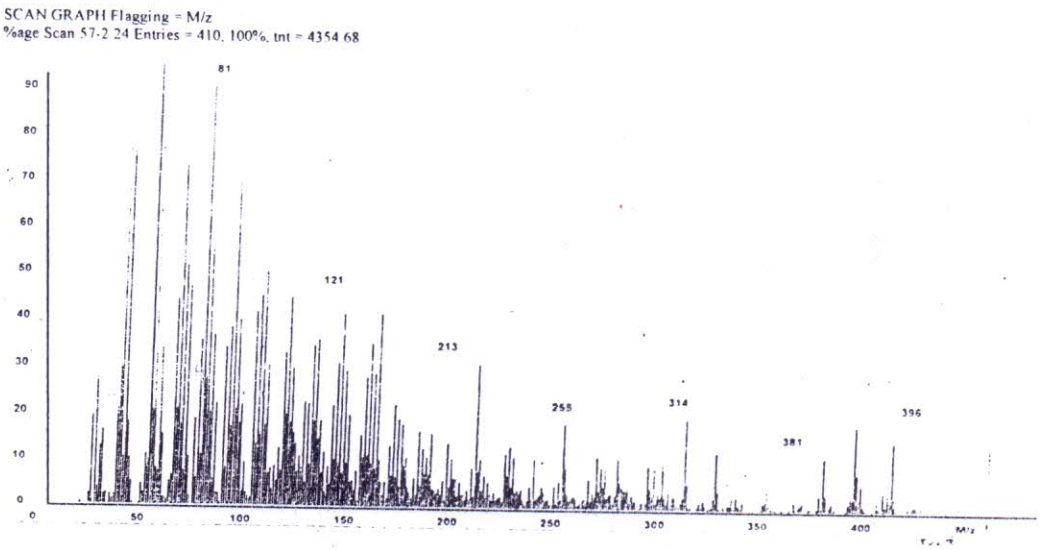


Fig. 3: The mass spectrometer data of a compound, Fr-Ac-6-3134 from *Ancistrophylum secundiflorum*



Ac-10-96107 (Figs. 2 and 3). The antifungal activity result shown on Table 2 reveals that all the pure compounds isolated had inhibitory effect on the seven fungi tested. The compound Fr-Ac-7-3051 was the most active. The compounds were more active than the antibiotic tested.

Some of the phytochemical compounds present in the butanol extract were flavonoid, phlobatannin, saponins and tannins. The compounds Fr-Ac-6-3134; Fr-Ac-7-3051; Fr-Ac-10-2258 and Fr-Ac-10-8796 are tannin compounds.

Discussion

The results here reveal that most of the pure antifungal compounds produced from *Ancistrophylum secundiflorum* are tannins. It is of significance that the compounds isolated here were more potent than the check antibiotics, Fulcin, Griseofulvin and Nystatin at 100 µg/ml. The antifungal assay results show that the pure compounds and the crude extracts have a broad antifungal spectrum. According to Irobi and Daramola (3) any extract which can cause an inhibition of more than 10mm is said to be active. Hence all the compounds isolated here are active, although the potency of the compounds vary as shown in their varying zones of inhibition. This finding is similar to the work of Valenciennes *et al.* (11) who worked on the extracts of *Euodia borbonica* var. *borbonica*.

Presence of tannins in the bark extracts of *Ancistrophylum secundiflorum* might have contributed to its high antifungal activity. Burapadaja and Bunchoo (12) suggested that the presence of tannins in the extract of *Terminalia circina* might have been responsible for the inhibition of cell wall formation in fungi leading to the death of the microorganism. Tannins are water soluble thus this might be responsible for the higher antifungal activity of the butanol and water extracts of the plant under study.

The fact that the extracts and compounds of *Ancistrophylum secundiflorum* produced inhibitory activities against some of the fungi implicated in the pathogenesis of skin diseases and eye irritations, provides some scientific basis for the utilization of this plant in the Nigerian traditional medicine for the treatment of skin diseases. The antifungal compounds isolated could be exploited to treat skin diseases and eye irritations.

Acknowledgment

This investigation received financial support from UNESCO fellowship for biotechnology. The author wishes to thank Dr. R.M. Kini, Assoc. Profs. T.K. Tan and T.S. Sim of the National University, Singapore, Asia for their advice and providing laboratory facilities.

References

1. Hutchinson, I. and Dalziel, J.M. (1954). *Flora of Tropical West Afr.* Vol. 2 Crown agents, London.
2. Bryce Kendrick (1992). *The Fifth Kingdom*. Mycologue publications, Ontario. 412 pp.
3. Irobi, O.N. and Daramola, S.O. (1994). Antifungal activities of crude extracts of *Mitracarpus villosus (Rubiaceae)*. *J. Ethnopharmacol;* 40: 604-610.
4. Olukoya, D.K., Idika, N. and Odugbemi, I. (1993). Antibacterial activity of some medicinal plants from Nigeria. *J. Ethnopharmacol;* 39: 69-72.
5. Saxena, J. and Mathela, C.S. (1996). Antifungal activity of New compounds from *Nepeta leucophylla* and *Nepeta clakei*. *Appl. Environmental Microbiol.;* 62(2): 702-704.
6. Gbile, Z. O. (1984). Vernacular names of Nigerian plants in Yoruba. Forest Research Institute of Nigeria, Ibadan, 85 pp.
7. Paterson, R.R.M. and Bridge, P.D. (1994). *Biochemical analysis in Natural Product Chemistry*. CAB International Kew, U.K. 125 pp.
8. Irobi, O.N. (1992). Activities of Chromolaenaodorata (Compositae) leaf extract against *Pseudomonas aeruginosa* and *Streptococcus faecalis*. *J. Ethnopharmacol;* 38: 81-83.
9. Fadeyi, M.O., Adeoye, A.O. and Olowokudejo, J.D. (1987). Epidermal and phytochemical studies in the genus *Boerhavia* (Nyctaginaceae) in Nigeria. *Intl. J. Crude Drug Res.*, 27: 178-184.
10. Harbone, J.B. (1992). Phytochemical Methods: A guide to modern techniques of plant analysis. Chapman and Hill, London. 279 pp.

11. Valenciennes, E., Smadja, J. and Conan, J.Y. (1999). Screening for biological activity and chemical composition of *Euodia borbonica* var. *borbonica* (Rutaceae), a medicinal plant in Reunion Island. *J. Ethnopharmacol;* 64: 283-288.

12. Burapadja, S. and Bunchoo, A. (1995). Antimicrobial activity of tannins of the essential oil of *Origanum syriacum* L. *J. Food Protection;* 58(10): 1147-1149.