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In vitro Antifungal Activity of Azaron Isolated from the Rhizome Extract of *Acorus calamus* L.

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Abstract: Azaron or 1, 2, 4-trimethoxy-5-(1-propenyl) Benzene, isolated form the rhizome extract of *Acorus calamus* was screened for antifungal activity against three phytopathogenic fungi viz., *Macrophomina phaseolina* (Maubl) Ash by, *Curvularia lunata* Wakker Boedijn and *Alternaria alternata* (Fr.) Kedissler. This compound completely inhibited the growth (100%) of all the tested fungi at 400 μg mL^{-1}.

Key words: Antifungal activity, azaron, *Acorus calamus*, rhizome

INTRODUCTION

The natural plant product has been interesting and important source of biologically active substance, the major source of which are still left undiscovered. In most of the plant screening programmes described in the literature much emphasis was laid on the detection of antibacterial compounds. The screening of plant material for effective antifungal compounds is rather limited[1-4]. So, we are interested in the antifungal compounds from plants available in Bangladesh. *Acorus calamus* L. is a semi-aquatic perennial herb with creeping and much branched rhizome, grows wild or cultivated in marshes of Chittagong and Chittagong Hill Tracts. The rhizome of this plant, commercially known as bach, has been used traditionally for the treatment of asthma and bronchitis. It is also used as remedy for chronic diarrhoea of children[5]. The rhizome extract of this plant was identified as potential source of antifungal compounds by systematic screening of forty one different Bangladeshi plants using *Alternaria alternata, Curvularia lunata, Fusarim equiseti, Macrophomina phaseolina, Botryodiplodia theobromae* and *Collectotrichum corchori* as test organisms[6]. Moreover Mungkomasawakul *et al.* reported the inhibitory effect of *Acorus calamus* L. extract on some plant pathogenic molds[7]. In the present study tried to isolate and characterize antifungal compounds from rhizome extract of *A. calamus*, which can lead towards the discovery and development of novel antifungal agent.

MATERIALS AND METHODS

Collection and extraction of plant material: Fresh rhizome of *Acorus calamus* was collected from BCSIR, Chittagong Campus. The rhizomes were dried, powdered mechanically and extracted with 80% ethanol. The extracts were filtered and concentrated to near dryness under reduced pressure and low temperature (40-50°C) with the help of rotary vacuum evaporator.

Solvent-solvent partitioning of crude extract: Solvent-solvent partitioning of crude ethanolic extract was done using the protocol designed by Kupchan and modified by Wagenen *et al.*[8]. The extract was dissolved in 90% methanol. It was extracted with petroleum ether (60-80°C), followed by carbon tetrachloride, chloroform and finally with ethyl acetate. All the fractions (organic phases as well as the aqueous phases) were collected separately and concentrated with a rotary evaporator at low temperature (40-50°C) and reduced pressure except aqueous fraction which was freeze dried to a solid mass. These fractions were used for initial antifungal screening and for isolation of compounds.

Vacuum liquid chromatography (VLC): The carbon tetrachloride soluble fraction of crude ehanolic extract was subjected for VLC analysis for fractionation. The column (5×50 cm) was packed with fine TLC grade silica gel (kieselgel 60°H) under vacuum. The column was washed with petroleum ether to facilitate compact packing. The sample to be separated was adsorbed onto silica gel (kieselgel 60, mesh 70-250), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with petroleum ether, petroleum ether-ethyl acetate mixtures of different proportions followed by ethyl acetate and ethyl acetate-methanol mixtures of different proportions of increasing polarity

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and finally with methanol. A total of 30 fractions were collected in an amount of 50 to 100 ml per fraction.

Analysis of VLC fractions by TLC: All the VLC fractions were screened by TLC under UV light at 254 nm and 366 nm wave length to detect the spot/band of any fluorescent compound. For this purpose commercially available precoated silica gel plates were used. Depending on TLC behaviour (Rf value) similar fractions were mixed together and grouped into 14 fractions (A-N). Among them ten fractions- B, C, E, F, G, H, I, J, K, M were found considerable for further investigation.

Fungal test organisms: *Macrophomina phaseolina* (Maubl) Ash by, Curvularia lunata Wakker Boedijn and *Alternaria alternata* (Fr.) Kedissler.

These fungal test organisms were collected from Department of Botany, Chittagong University.

Antifungal assay: The *in vitro* antifungal activity of different VLC fractions was done by poisoned food technique[9]. Potato dextrose agar (PDA) medium was used for the culture of fungi. In this method each fraction was redissolved in specific volume of absolute ethanol and mixed with sterilized melted PDA medium to obtain the desired concentration and this was poured in sterilized petriplates. At the center of each petriplate (20 ml/plate) 5 days old fungal mycelial block (5 mm. in dia.) was inoculated and incubated at 25±1°C. A control set was also maintained for each experiment. The diameter of each fungus was measured after 3-5 days of incubation. The percent of mycelial growth inhibition of the test fungus was calculated as follows:

$$I = \frac{(C-T)}{C} \times 100$$

Where:

I = Percent of inhibition.
C = Diameter of fungal colony in the control.
T = Diameter of fungal colony in the treated.

Isolation and purification of compounds from selected VLC fractions: Among the 10 VLC fractions tested for antifungal activity, a highly antifungal fraction (fraction-C) was finally selected for purification through further TLC analysis and characterized by GC/MS. During screening by TLC it was found that VLC fraction-C showed a single spot on the developed TLC plate that was visible under UV light at both 254 and 366 nm.

GC/MS analysis: The GC/MS analysis was carried out in BCSIR Laboratories, Dhaka by electron impact ionization (EI) method on GC-17A gas chromatograph (Shimadzu, Japan) coupled to a GCMS-Qp5050A mass spectrometer (Shimadzu); fused silica capillary column, 30x0.25 mm i.d., coated with DB-1 (J and W), 0.25 μm film thickness; column temperature 80 to 250°C at the rate of 4°C/min, injection port temperature 250°C, constant pressure of carrier gas (hilium) 100 KPa, flow rate (ml/min) 20, acquisition parameters full scan, scan range 60-550 amu, searched library NIST 107, Shimadzu corporation, sample dissolved in n-pentane.

RESULTS AND DISCUSSION

The 80% ethanolic extract of *A. calamus* was separated by petroleum ether (60-80°C), carbon tetrachloride, chloroform, ethyl acetate and aqueous fractions through solvent–solvent partitioning. All the five fractions were concentrated and initially screened for antifungal activity. Carbon tetrachloride soluble fraction exhibited highest antifungal activity compared to other fractions. For this reason further chemical investigation was confined only on carbon tetrachloride fraction. The carbon tetrachloride soluble fraction was subjected for VLC analysis and 30 fractions were collected. After VLC analysis the fractions were subjected to TLC analysis. Through TLC behavior (Rf value) similar fractions were mixed together and grouped into 14 fractions (A-N). Among them the quantity of the fraction number- B, C, E, F, G, H, I, J, K, M were found considerable for further investigation. These ten fractions were tested for antifungal activity using three phytopathogenic fungi, *Alternaria alteranta, Curvularia lunata* and *Macrophomina phaseolina* at two different concentrations, 200 and 400 μg mL^{-1} (Table 1). From the results, it appeared that fraction-C showed the highest inhibition (100%) against all the test pathogens both at 200 and 400 μg mL^{-1} compared to other fractions. So, fraction-C was finally selected for purification through further TLC analysis and characterization by GC/MS. During TLC analysis it was found that VLC fraction-C showed a single spot on the developed TLC plate that was visible under UV lamp at both 254 and 366 nm. The yellow coloured oily material which, was obtained from VLC fraction-C was found pure by further TLC analysis. GC/MS analysis of fraction-C characterizes the constituents (Table 2). The fraction-C consists of three components, Azaron, Asarone and Isoelemicin which covers 99.99% of the fraction. The molecular weight and molecular formula of the three components are identical and structure is slightly changed due to isomerism (Table 2). So, the compounds are isomers. The structure of the major compound is 1, 2, 4-trimethoxy

Table 1: Antifungal activity of VLC fractions of *Acorus calamus* rhizome extract against three phytopathogenic fungi

	Percent growth inhibition					
	Macrophomina phaseolina μg mL^{-1}		Curvularia lunata μg mL^{-1}		*Alternaria alternata* μg mL^{-1}	
VLC fractions	200	400	200	400	200	400
Frac.- B	73.63	nd	68.18	nd	46.36	nd
Frac.- C	90.61	100	82.65	100	81.25	100
Frac.- E	30.61	nd	38.43	nd	24.48	nd
Frac.- F	19.70	43.64	38.85	42.89	26.56	36.15
Frac.- G	16.36	30.91	41.20	46.33	31.40	40.77
Frac.- H	49.09	57.27	30.48	41.52	25.62	40.00
Frac.- I	19.70	65.45	28.32	36.70	19.83	35.38
Frac.- J	17.88	63.64	22.33	31.88	19.55	31.54
Frac.- K	34.55	63.64	31.88	31.50	19.23	28.13
Frac.- M	9.09	nd	7.80	nd	7.69	nd

nd = Not done.

Table 2: Percentage of different components with MW and MF present in VLC fraction-C of *Acorus calamus* rhizome extract

Pk No.	R. Time	Total (%)	Name	MW	MF
1	20.45	1.28	Isoelemicin	208	$C_{12}H_{16}O_3$
2	21.89	94.72	Azaron OR 1, 2, 4-trimethoxy-5-(1-propenyl)- Benzene	208	$C_{12}H_{16}O_3$
3	23.57	3.99	Asarone OR 2, 4, 5-Trimethoxypropenyl benzene	208	$C_{12}H_{16}O_3$

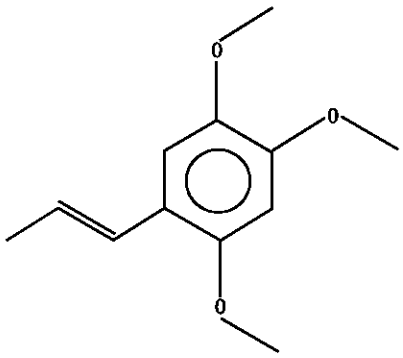

Fig. 1: Structure of Azaron OR 1, 2, 4-trimethoxy -5-(-1-propenyl)-Benzene

-5-(1-propenyl)- Benzene or Azaron is given in Fig. 1 which constitutes 94.72% of the fraction and is responsible for fungal inhibition. Similar type of antifungal compound known as β-asarone was identified previously from dichloromethane extract of *Acorus calamus* using *Cladosporium cladosporioides* as a diagnostic fungus which is consistent with this result[7]. Like *A. calamus*, occurrence of antifungal compound reported from different species of monocot plant (genus *Curcuma*) is a noteworthy feature[3]. Since antifungal compounds from higher plants are advantageous over synthetic fungicides due to their largely non-phytotoxic, systematic and easily biodegradable nature[2,10], Azaron can be exploited as harmless, indigenous and non-polutive source of antifungal agent.

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REFERENCES

1. Khanna, S.G.S., Y.L. Nene, C.K. Banerjee and P.N. Tapliyal, 1967. A note on the isolation and chemical characterization of antifungal agents from extracts of *Anagallis arvensis*. Indian Phytopathol., 20: 64-66.
2. Fawcett, C.H. and D.M. Spencer, 1970. Plant chemotherapy with natural products. Annu. Rev. Phytopathol., 8: 403-418.
3. Banerjee, A.B., S.K. Gupta and D. Roy, 1986. Antifungal Principles of *Curcuma zedoaria* Roscoe. J. Pharmacog. Res. Association of India, 7: 5-8.
4. Rahman, M.S. and M.N. Anwar, 2004. *In vitro* antimicrobial activity of Holarrifine-24ol isolated from the stem bark of *Holarrhena antidysenterica* wall. Pak. J. Biol. Sci., (Submitted).
5. Yusuf, M., J.U. Chowdhury, M.A. Wahab and J. Begum, 1994. Medicinal Plants of Bangladesh. Published by Bangladesh Council of Scientific and Industrial Research, Bangladesh, pp: 8.

6. Begum, J., Md. Yusuf, J.U. Chowdury, S. Khan and M. N. Anwar, 2004. Antifungal activity of forty one higher plants available in Bangladesh. Bangladesh J. Botany (Submitted).
7. Mungkomasawakul, P., D. Supyen, C. Jatisatienr and A. Justisatienr, 2002. Inhibitory effect of *Acorus calamus* L. extract on some plant pathogenic molds. Acta Hort., 576: 341-345.
8. Wagenen, B.C.V., R.J. H. Larsen, H.D. Cardellina, Z.C.I. Randazzo and C. Swithenbank, 1933. Ulo santoin, a potent insecticide from the sponge Ulosa ruetzleri. J. Org. Chem., 58: 335-337.
9. Grover, R.K. and J.D. Moore, 1962. Toximetric studies of fungicides against brown rot organisms *Sclerotina flucticola* and *S. laxa.* Phytopathology, 52: 876-880.
10. Beye, F., 1978. Insecticides from the vegetable Kingdom. Plant Res. Dev., 7: 13-31.