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Evaluation of components from Allamanda leaves extract against *Phomopsis vexans*

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ABSTRACT

Allamanda leaf *(Allamanda cathertica)* extraction were prepared by water at room temperature (25 ± 2°C) as well as by a number of less polar to highly polar solvents at their boiling temperature, that means extract of allamanda at refluxing temperature. The solvents were diethyl ether, methylene chloride, chloroform and ethyl alcohol. These refluxing extracts were applied to determine their inhibition extent against *Phomopsis vexans.* Also TLC of these refluxing extracts was done by using different pure and suitably mixed solvents. Growth inhibition test of P. *vexans* (causal agent of eggplant leaf blight and fruit rot) by each of these refluxing extracts in different solvents revealed different mode of action. Water extracts of allamanda was found satisfactory in inhibiting the growth of *P. vexans* (84.6%). The refluxing extracts of diethyl ether, methylene chloride and ethyl alcohol were statistically similar having inhibition action by 70-74%. Among the extractants, methylene chloride had no considerable effect on *P. vexans.* TLC of these refluxing extracts showed the presence of a number of compounds having polarity very low to high. Based on the above information, refluxing methylene chloride extracts was subjected to column chromatographic separation for compounds in allamanda leaves. Through column chromatography, five single compounds were separated and tested against *P. vexans* individually. Among the separated compounds, one compound was completely inhibitory against *P. vexans* while the other compounds had little inhibitory effect. The other compounds may be active when they exist in mixture with each other in whole extract.

INTRODUCTION

Eggplant *(Solanum melongena),* an economically important and popular vegetable crop of Bangladesh suffers most from *Phomopsis* blight and fruit rot caused by *Phomopsis vexans* (Sacc. and Syd.) Studies have revealed that damping-off and seedling blight phase of the disease can be controlled and fruit rot can be minimized through application of allamanda (*Allamanda cathartica*) leaf extract (Meah et al., 2002). Plants of different species have been explored for the potency of controlling plant diseases. Allamanda is one of the recognized plants, the extract of which has been proved effective in controlling *Phomopsis* blight of eggplant (Khan et al., 2002). However, activity of allamanda against *Phomopsis vexans* has not so far been determined in terms of chemical composition. Rummana (2004) did thin layer chromatography of allamanda leaf extract and detected the presence of at least 14 compounds.

The present study is the continuation of the work of Rummana (2004) aimed at separation of the components of allamanda leaf extracts through column chromatography for identification of the active compound. Chromatographic separation of the active compound of allamanda and exploration of cheap and available substances for formulation of botanical fungicide are essential for inclusion of these components in the management practice. Chemical analyses have indicated that allamanda *(Allamanda cathertica)* leaves are the source of many compounds with medicinal properties with

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varied Pharmacological properties thus supporting many of the recently medicinal uses of allamanda (Anon., 2004).

Khan (1999) assayed allamanda leaf *(Allamanda cathertica)* extract against *Phomopsis vexans* and found it effective in controlling *Phomopsis* fruit rot of eggplant. Meah et al. (2002) tried allamanda extract against *Phomopsis vexans* and found it very effective *in vitro* condition.

Research at IPM Lab, BAU revealed that allamanada leaf extracts were effective against *Phomopsis vexans* both *in vitro* and *in vivo* tests. However, the preparations have been used in crude form, not stable under normal environment and not easy to handle. The products have to be in commercial formulations with properties of stability, durability and economic.

Before going for formulation of a plant product, the nature of action including its active principle should be known. Still now, this information is not available. To know the nature of action and its active principle, chemical analysis of the product is necessary. Therefore, separation of the components of allamanda leaf extracts need to be done by Thin Layer Chromatography (TLC) and column chromatography. The present research was undertaken to identify the component or components in allamanda leaf extract inhibitory to *Phomopsis vexans.*

MATERIALS AND METHODS

Preparation of allamanda leaf extract

Allamanda leaves collected from BAU campus were dried and powdered by mortar and pestle, 100 g powdered leaves were taken and 200 ml ethyl alcohol added to it. The mixture was kept overnight at room temperature (25 ± 2°C). The mixture was then shaken for two hours in a flask shaker and filtered through cheese cloth followed by filtration through filter paper whatman No. 1. Similar procedure was followed for extraction by hexane, benzene, methylene chloride and chloroform.

For preparation of water extract, 100 g allamanda leaves were ground in a mortar and pestle and pulverized mass was mixed with 100 ml water thoroughly. The mixture passed through the cheese cloth. The extracted liquid was used as allamanda extracts (1:1).

Preparation of refluxing extracts of allamanda

Some important compounds in allamanda seemed to be unstable so they decompose readily. Therefore the compounds were extracted by refluxing in low boiling solvents like methylene chloride, ether and ethanol. For preparation of refluxing extract by using sox let's apparatus, 50 g of dried and powdered allamanda leaves were taken in a thimble, added 200 ml methylene chloride in the thimble. This was done by sox let's apparatus in a water bath keeping the temperature not more than 40 ± 2°C. Similar procedure was followed for refluxing extracts of ether and ethanol. These extracts were partially concentrated and used for testing against *Phomopsis vexans.* TLC of these reflux extracts was done.

Thin layer chromatography

Thin layer chromatographic (TLC) technique was employed for the identification of a number of compounds present in these extracts. On the basis of TLC observations, allamanda leaves were extracted by different solvents of suitable polarities. The aim was to divide the components into some fraction. Based on TLC of the residues, five single compounds were tested against *Phomopsis vexans.*

Preparation of culture media

Potato Dextrose Agar (PDA) media was prepared by mixing 200 g Potato (Peeled and Sliced), 20 g Dextrose, 20 g Agar in 1000 ml distilled water. Peeled and sliced potato was boiled to collect the decoction by sieving with a fine piece of cloth. Afterwards, the other ingredients were mixed up on gentle heating. After preparation, the contents were poured into 250 ml Erlenmeyer flasks, plugged with cotton and were sterilized in autoclave at 121°C under 15 psi for 20 minutes. This medium was acidified with 30 drops of 50% lactic acid per 250 ml medium to inhibit the growth of bacteria. Thereafter 20 ml of the

medium was poured in each Petri-plates (9 cm diameter) and allowed to solidify.

Collection and maintenance of culture *of Phomopsis vexans*

Isolate -22 of *Phomopsis vexans* was collected from IPM Lab, Department of Plant Pathology, BAU (Meah, 2002). The fungus was multiplied in PDA and the culture stocked in PDA slant for future use.

Bio-assay of extracts

Refluxing extracts of allamanda prepared in different solvents were tested separately against *Phomopsis vexans in vitro* following growth inhibition technique (Meah et al., 2002). One ml extract was poured on a PDA plate and well spread with a sterile glass rod and kept overnight for diffusion at room temperature (25±2°C). The control plates were maintained where the PDA was not treated with extracts. The plates were inoculated by placing a 5 mm block of 7 days old culture of *Phomopsis vexans* at the centre. The inoculated plates were kept at room temperature and allowed the fungus to grow. Linear growth of the fungus was taken after 5 days when the PDA plates were full in control treatment.

Bio-assay of separated compound

Separated five compounds of allamanda collected through column chromatography were dissolved individually in a polar solvent methylene chloride. Methylene chloride is a volatile solvent and it has no considerable effect on the growth of *Phomopsis vexans.* The compounds were tested separately against *Phomopsis vexans in vitro* following growth inhibition techniques (Meah et al., 2002). One ml of solvent with compound was poured on a PDA plate and well spread with a sterile glass rod and kept overnight for diffusion at room temperature (25 ± 2°C). Therefore, plates were inoculated by placing a 5 mm block of 7 days old *Phomopsis vexans* at the centre. The inoculated plates were kept at room temperature and allowed the fungus to grow. Linear growth of fungus was taken after 5 days when the PDA plate was full in control treatment.

Identification of separated compound(s)

Infra red (IR) spectra of single *Phomopsis vexans* active compound was recorded as thin liquid film (Chloroform, $CHC1_3$) on a Perkin - Elmer 782 spectrometer at chemistry research Lab, University of Dhaka.

Statistical analysis of data

Data collected on different parameters were analyzed following standard statistical methods in Completely Randomized Design (CRD) using statistical computer package program MSTAT. Means were compared with Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Effect of refluxing allamanda leaf extracts against *Phomopsis vexans*

Effect of refluxing allamanda extract in different solvents in reducing mycelial growth of *Phomopsis vexans* is summarized in Table 1. The inhibitory effect of allamanda extracts in different solvents significantly differed in comparison to control. Among the treatments, water extract showed significantly higher effect in reducing the mycelial growth of *Phomopsis vexans* followed by ethyl alcohol, methylene chloride and diethyl ether. But among themselves, the effect of methylene chloride, ethyl alcohol and diethyl ether extracts were statistically similar.

Effect of used extractants on the growth of *Phomopsis vexans*

In order to know whether the solvents methylene chloride, ethyl alcohol and diethyl ether affected the percent growth reduction of *Phomopsis vexans*, a growth inhibition test was carried out using these three solvents. The results of these tests have been presented in Table 2. It was found that methylene chloride had no considerable inhibition on percent growth reduction of *Phomopsis vexans*, diethyl ether had little inhibition while ethyl alcohol inhibited the growth of the fungus by 50%.

Table 1
Effect of allamanda extract obtained by refluxing solvents against *Phomopsis vexans.*

Solvent of extraction	Mycelial growth (mm)	% Growth reduction
Methylene chloride	23.62	73.50b
Ethyl alcohol	23.12	74.06b
Diethyl ether	26.03	70.79b
Water	13.71	84.61a
Control	89.13	--

Recorded after 5 days of inoculation into Potato Dextrose Agar (PDA) at room temperature (25±2°C). In a column, figures with same letter(s) do not differ at P ≤0.05.

Table 2
Effect of extractants on the mycelial growth of *Phomopsis vexans.*

Solvents of extraction	Mycelial growth (mm)	% Growth reduction
Methylene chloride	76.65	18.48c
Ethyl alcohol	44.00	50.63a
Diethyl ether	69.07	22.50b
Control	89.13	--

*Recorded after 5 days of inoculation into Potato Dextrose Agar (PDA) at room temperature (25±2°C). In a column, figures with same letter(s) do not differ at P≤ 0.05.

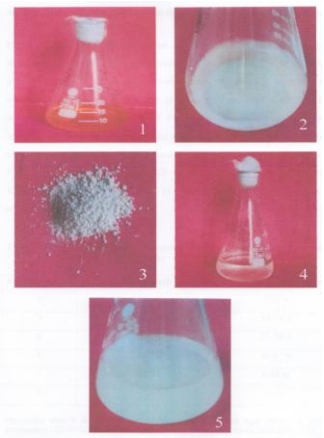

Figure 1
Five different compounds separated from allamanda leaf extract

Effect of compounds separated from allamanda leaf extract against *Phomopsis vexans*

Effect of compounds separated (Figure 1) from allamanda leaf extract on reducing mycelial growth of *Phomopsis vexans* is summarized (Table 3). The inhibitory effect of separated compounds significantly differed in comparison to control. Among the compounds, compound 1 completely inhibited the mycelial growth of *Phomopsis vexans* (Figure 2). The inhibition mode of compounds 3 and 4 was better than compound 2. The effect of compound 3 and compound 4 on percent growth reduction of *Phomopsis vexans* were statistically similar. But among themselves, minimum percent growth reduction of *Phomopsis vexans* occurred in case of compound 5.

Table 3
Effect of separated compounds from allamanda leaf extract against *Phomopsis vexans.*

Compound (s)	Mycelial growth (mm)	% Growth reduction
1	0.000	100 a
2	75.68	13.14 c
3	63.10	27.58 b
4	63.72	26.87 b
5	81.05	6.98 d
6	87.13	—

*Recorded after 7 days of inoculation into Potato Dextrose Agar (PDA) at room temperature (25±2°C). In a column, figures with same letter(s) do not differ at P≤ 0.05.

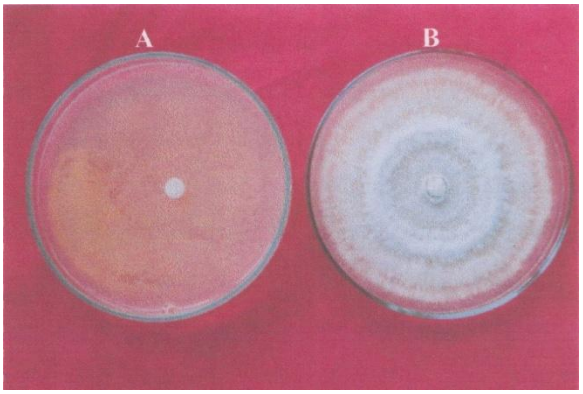

Figure 2
Effect of compounds separated from allamanda leaf extracts against *Phomopsis vexans*. A = Compound 1; B = Control.

Infra red (IR) spectrum of compound 1

According to the aim of this research work, attempts to elucidate the structure of the compound active against *Phomopsis vexans.* As a first step, IR spectrum of the compound 1 was taken in thin liquid film.

Table 4
Peak assignment in IR spectrum of compound 1.

Characteristic (cm^{-1})	Intensity	Probable assignment
860	Small	Benzene ring
1020	Strong	$-\overset{\diagdown}{\underset{\diagup}{C}}-N<$
1100	Strong	$-\overset{\diagdown}{\underset{\diagup}{C}}-N<$
1220	Small	$\gtrless$C-F
1260	Strong	$\gtrless$C-F
1365	Small	- CH_2-
1400	Small	- CH_2-
1440	Medium	- CH_2-
1640	Medium	>c = c<
1720	Medium	>c = o
2850	Strong	C-H
2900	Strong	C-H
3400	Strong	> N-H

According to Chanda (1972). Atomic structure and chemical bond. 2nd ed. Tata McGraw-Hill Publishing company Ltd. Delhi. p. 302.

The IR spectrum of compound 1 revealed a number of functional groups present in the molecule of the compound 1. The band at 860 cm^{-1} indicated the presence of Benzene ring in the compound 1. Two strong bands at 1020 cm^{-1} and 1100 cm^{-1} showed the presence of $-\overset{\diagdown}{\underset{\diagup}{C}}-N<$ group, the band at 1220 cm^{-1} and 1260 cm^{-1} showed the presence of $-\overset{\diagdown}{\underset{\diagup}{C}}-F$ group in the compound 1. The bands at 1365 cm^{-1}, 1400 cm^{-1} and 1440 cm^{-1} indicated the presence of-CH_2- group and band at 1640 cm^{-1} showed the presence of > C = C < group in the compound 1. A medium band at 1720 cm^{-1} indicated the presence of > C = 0 group and two strong bands at 2850 cm^{-1} and 2900 cm^{-1} indicated the presence of C-H linkage in the compound. Finally a strong band at 3400 cm^{-1} showed the presence of >N-H group in the compound 1. However, eight different functional groups were detected in compound 1 (Table 4).

Allamanda extract in water has been found to be very effective against *Phomopsis* fruit rot caused by *Phomopsis vexans*. This was established by Khan (1999) and others. Hawlader (2003) reported that seed treatment with allamanda leaf extracts (1:1) effectively increased germination of eggplant seeds and tremendously decreased nursery diseases. This has conformity with the findings of Panda et al. (1996) who reported leaf extracts of *Allamanda cathertica* as an excellent potential fungicide for control of *Phomopsis* blight of eggplant.

Khan (1999) found that the incidence and severity of the disease varied when aqueous leaf of Neem, Allamanda and Bael were applied at flowering and peak fruiting stages in 3 different doses (S, $S/_{10}$ and $S/_{100}$). Allamanda (S) was most effective to reduce percent leaf and fruit infection and lesion size as well followed by Bael (S). However, Khan (1999) did try in vitro lab assay the effectiveness of different plant extracts against *Phomopsis vexans*. Panda et al. (1996) tested efficiency of leaf extracts of Debadaru, Thuza, Allamanda, Bael and Kathgolap and obtained excellent potential from leaf extracts of Allamanda against *Phomopsis vexans.*

These findings prompted to chemical investigation of allamanda extracts. Primary views were to make an extract avoiding unexpected constituents in these extracts. Secondly it was planned to find out the compound or compounds in allamanda which were responsible for such antifungal activity, separation of these compounds and

determination of their chemical formula, structures and configuration. There were many compounds, medium polar to highly polar in allamanda particularly in water extracts. There are some compounds which readily coagulated in water extract and sedimented with active compounds. These turned freshly active water extracts into inactive or less active one.

TLC plates developed in non polar to highly polar pure solvents and by suitable mixed solvents showed that allamanda extracts contained components of high to low R_f values. This indicates the polarity of components in allamanda are of vast range. These TLC plates showed many components were present in allamanda leaves. On the basis of R_f values, refluxing allamanda extract was prepared by less polar to highly polar solvents like diethyl ether, methylene chloride and ethyl alcohol. TLC of the refluxing extract showed each of them contained adequate number of compounds. Growth inhibition test was carried out by all these extracts individually. Results of these extracts showed that refluxing methylene chloride, diethyl ether and ethyl alcohol extracts of allamanda was statistically similar for inhibition of mycelial growth of *Phomopsis vexans.* Their inhibition activity was satisfactory.

Growth inhibition test was also carried out by pure solvents like methylene chloride, diethyl ether and ethyl alcohol. But in case of methylene chloride we found no considerable antifungal activity, diethyl ether have little bit inhibition against *Phomopsis vexans.* So, it indicates that compound or compounds in allamanda leaves are responsible for antifungal activity, not the solvents. Most of the active compound or compounds in refluxing methylene chloride and diethyl ether extracts were inhibitory against *Phomopsis vexans.* Moreover, some unexpected components were not extracted by these solvents. For this reason unlike water and alcoholic extracts, methylene chloride and diethyl ether extracts remained as it was for long time. That is coagulation and sedimentation did not occur in these two extracts.

Based on the above information we selected refluxing methylene chloride extract for column chromatographic separation of active components in allamanda leaves. In column chromatography, we continued elution by non polar to highly polar solvents and mixed solvents and were able to separate five single compounds from allamanda leaves. These were some of the different compounds present in allamanda leaf extract. We tried to crystalize these compounds and got crystals with two residues. The other three single spotted residues were not crystaline. They may or may not be crystaline and effort to crystalize them is continuing. These five single compounds were tested against *Phomopsis vexans.* Among them one of the compounds was most effective that is it was completely inhibitory to *Phomopsis vexans.* But inhibition extent of other single compounds was not satisfactory. TLC plates of these compounds indicated that they are different from each other.

Water extracts of allamanda leaves exhibited higher inhibitory action against *Phomopsis vexans* than any other refluxing solvents. It indicates that some of the components essential for the inhibitory action might have been removed during the preparation of extracts in other solvents. Among the separated compounds, compound 1 showed excellent results against *Phomopsis vexans.*Another compound was also more or less inhibitory against *Phomopsis vexans.* The other compounds may be active when they mixed with each other in water extracts. The compound 1 always showed dark and distinct spot during Thin Layer Chromatography.

The IR spectrum of the compound 1 revealed the functional nature of the compound but for elucidation of complete structure stereochemistry and its mode of action of the compound 1 against *Phomopsis vexans* needs detailed UV, NMR and mass etc. spectroscopic studies.

The future research should separate the active compound in adequate amount through column chromatography or any other easiest direct method and determine the structure of active compound for commercial formulation of botanical fungicide.

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