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RESEARCH ARTICLE

EFFICACY OF FOUR ACETOGENIN CONTAINING MEMBERS OF *ANNONACEAE* AGAINST COLLETOTRICHUM GLOEOSPORIODES (PENZ) PENZ & SACH

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ABSTRACT

The acetogenin containing members of the Annonaceae family *Annona squamosa* L., *Annona reticulata* L., and *Annona muricata* L are known insecticidals. The antifungal potency of these three well studied plants along with a fourth acetogenin containing member, *Uvaria narum* L. were put to test against the fungus *Colletotrichum gloeosporioides* that causes post harvest anthracnose disease in many tropical fruits. Preliminary screening formed the first part of the study where the leaves and seeds of all the four plants were extracted sequentially by soxhelet extraction in four different solvents, viz. (PE) Petroleum ether, (highly non polar), (Chl) Chloroform and (Ac) Acetone, (both medium polar) and (CH_3OH) Methanol (highly polar). The obtained extracts were subjected to antifungal test by paper disc method. None of the plant extracts showed any appreciable activity except the PE and Chl extracts of *Uvaria narum*. Hence this plant was subjected to individual soxhelet extractions in the four above mentioned solvents. Both the above mentioned type of extracts (i.e. sequential and individual) were then subjected to inhibition studies against the same fungus using Poison food technique, in PDA (Potato Dextrose Agar Medium) and the diameter of the hyphal growth measured, to know the inhibition percentage of each extract. It was found that the chloroform and acetone extracts of *Uvaria narum* obtained by individual soxhelet extraction showed a 100% inhibition to the growth of fungus. The effect of the acetone extract on the fungal hyphae and spores was noted by the Scanning Electron Microscope (SEM). It revealed membrane disruptions by pore formations and putative lysis causing cytoplasmic spilling and empty hyphae.

INTRODUCTION

Plant diseases particularly those caused by fungal pathogens are a major cause for huge loss in crop productivity in the whole world. Although synthetic fertilizers are fruitful in controlling most of them, several of them have been found to be carcinogenic in nature. A better alternative will therefore be the use of botanical fungicides that are advocated to be largely non phytotoxic and easily biodegradable in nature. It has been observed that many types of plant diseases are more severe in the tropics causing more severe losses to crops. Among this series, the fungus *Colletotrichum gloeosporioides* (Sach.), and the anthracnose disease caused by it becomes worthy of mention. A few species of Colletotrichum attack almost all tropical and subtropical crops and cause tremendous losses by damaging the fruits of most of them. The symptoms of anthracnose include sunken necrotic lesions on leaves, stems, flowers and fruits as well as crown and stem rots, crown wilts, even blights. It has been shown that even single species of *Colletotrichum* can effect multiple hosts such as *Aloe vera*, Capsicum, turmeric, Bell pepper, Mango, *Zea mays,* Papaya, amomg others.

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Though the fungus comes out as a major villain in the path of agricultural economy, it will unfair not to mention the great role played by this genus in being highly significant for experimental studies of fungal development and infection processes.(Gautum, 2014). Botanicals have been found to be more environment friendly than their chemical counterparts. This is mainly because the former are susceptible to environmental degradation than the latter (Kumar, 2006). Of late, antimicrobial properties of plant extracts are being reported with increasing frequency from around the world (Yazdani, 2011). Annonaceae family is in recent times has been in the limelight due to the discovery of a noble series of a high molecular weight compound called acetogenin. They have been attributed a wide range of activities as antibacterial, antitumorous, insecticidal and antihelminthic qualities and in many reports they have been lauded for being compounds with excellent bioactivities. But there is a dearth of literature highlighting the Annonaceae members or the acetogenins as the ones possessing good antifungal activities. Even though some papers were dug up assigning antifungal virtue to this family, they were too less when compared to the staggering reports for a host of other activities from other journals. Annonaceae family has proved its insecticidal in several papers and it has gained a place among the likes of Solanaceae and Meliaceae for being families with excellent insecticidal activities (Castillo-Sanchez, 2010). *Annona squamosa* L. and

Annona muricata L. have been found to be the best in their family as insecticidal (Isman, 2005). It would therefore be an added advantage if it proved to be an effective antifungal too. As stated before, fungal attacks and insect pests are one of the prime reasons for crop loss every year. The given effort has been undertaken in this direction to research whether the few members among Annonaceae selected are equally effective as antifungals, primarily against the chosen fungus, Colletotrichum. The family Annonaceae is a large family, in fact largest among Magnoliales, comprising about 129 genera and more than 2000 species and is hugely pantropical in distribution (Westra and Maas, 2012). The largest numbers of species lie with Annona which has 120 species mostly tropical in distribution. This family is called as the custard apple family after its representative member, *Annona squamosa* L.

The Annonaceae family has 52 species spread in 17 genera and Annona has reported 4 tree members, among which, *Annona reticulata* L. is the most widespread one in Kottayam and Alappuzha district (Roy, 2010). Other two members, *Annona squamosa* L. and *Annona muricata* L., both tree members, though not so widespread, do not have their presence in these parts. Uvaria has four recorded members in Kerala of which undoubtedly, the most common is *Uvaria. narum* (Dunal) Wall. that is found in the plains and deciduous forests of Kerala (Roy, 2010). This made its collection easier in comparison to its other members. Recent progress in phytochemical and pharmacological studies on *A.squamosa* seeds has shown that the major bioactive compounds are Annonaceous acetogenins. The total content of acetogenins in the seeds of Annona squamosa has been reported to be higher than in four other species i.e. *Annona reticulata, Annona muricata, Annona glabra and Annona bullata*, (Yang *et al.,* 2009).

***Antifungal activities of Annona squamosa L., Annona reticulata L. and AnnonamuricataL*:**

Only a few reports are present regarding antifungal activities of the three species of Annona under study. The aqueous extract of *Annona reticulata* showed only 41% inhibition against *Botrytis cineria*, only 11% against *Aspergillus niger* and 38% against *Rhizopus stolonifer,* (Mogle, 2013). In *Annona squamosa* antifungal work on the methanolic extract of its root, leaf and seed cotyledons showed activities against *Trichophyton rubrum* and *Aspergillus flavus* (Vidyasagar and Singh 2012). The methanolic crude extract of the seeds of *Annona squamosa* and the acetogenins isolated from this crude extract were found to be active against *Phytophthora infestans* and *Puccinia recondite* (Dang *et al.,* 2011). *Annona muricata* leaves exhibited a significant inhibition against some selected groups of fungi as *Alternaria solani, Alternaria albicans, Aspergillus fumigatus* and *Penicillum chrysogenum* (Abubacker and Deepalakshmi, 2013). The seed extract and its fraction failed to show any significant activity against *Alternaria solani, Curvularia lunata and Fusarium moniliformi* (Basha, 2014). The works in the antifungal properties on *Uvaria narum* (Dunal) Wall. are truely limited. Of the few works unearthed, the root bark of *Uvaria narum* was found to possess antifungal activity against six fungi *Candida albicans, Aspergillus niger, Penicillium notatum*, *Trichophyton floccosum* (of which the last three are dermatophytes). The acetogenins isolated from the crude extracts, uvariamicin I, II and III and panalicin showed the best activity against all the tested fungi (Padmaja *et al.*, 1993). The benzoic acid crystals isolated and essential oils distilled from *Uvaria narum* alcohol extract was tested for antifungal activity against the foot rot disease causal agent of pepper, *Phytophthora capsicii*. (Jose, 2005). Based on the analysis of the review of literature it was decided that we conduct a preliminary screening of the four selected members of Annonaceae, *Annona squamosa L*., *Annona reticulata* L. and *Annona muricata L*. against the selected fungus *Colletotrichum gloeosporioides*.

Objectives of the study

The objectives set were:

- To determine the antifungal activities of the four acetogenin containing members against *Colletotrichum gloeosporioides* (Sach.)
- To conduct a detailed analysis of the most active extract by subjecting it to sequential and individual extraction by soxhelet method and sequential extraction at room temperature.
- If bioactivity is confirmed then to conduct a SEM analysis of the treated colletotrichum hyphae to understand the effect of the active extract on hyphal and spore morphology.

MATERIALS AND METHODS

The leaves and seeds of the above mentioned four plants were collected from their respective trees and fruits for *Annona squamosa,* (AsL and AsS), *Annona reticulata* (ArL and ArS) and *Annona muricata* (AmL and AmS) and from the woody lianes of *Uvaria narum*, (UnL) from different localities in and around Kottayam and Pathanamthitta, Kerala State during the months of April 2014 and November 2014. The specimen were identified at S.B.College, Changanacherry, Kerala, India. The materials collected were washed and dried in open air, away from intense sunlight. The dried leaves and seeds were then powdered in kitchen blender and the powder collected in air tight bottles and stored in the refrigerator till further use. Soxhelet method of extraction was followed. The extraction was done in four different solvents in terms of increasing polarity from highly non polar Petroleum ether, (PE) to solvents of medium polarity viz. Chloroform (Chl) and acetone (Ac), and to highly polar methanol (CH_3OH). Temperature of extraction was maintained at around 45-50 degree Celsius. The extracts obtained were concentrated in Rotary evaporator and dried off completely to give out residues that were weighed and stored in refrigerator. For an in-depth study it was decided that if the sequential extraction of any plant part (leaf or seed) showed good inhibition then individual extraction using the same solvents would be done by the soxhelet method and the extracts tested against the fungus of interest. The test fungal pathogen was isolated from infected spindle leaf of coconut by single hypha tip method. The colony of colletotrichum was identified by noting the colour of the colony, shape of the spores and was finally confirmed from the Plant pathology departmnt of CPCRI, Kayamkulam. The colletotrichum was subcultured and the stocks wrre maintained on PDA agar slants. The test Organism was obtained as part of training programme conducted at Central Plantations Crop Research Institute, Kayamkulam, Kerala, and was subcultured in Potato

dextrose agar. The slants were maintained and regularly subcultured. All the materials utilized were of Merck (analytical) Grade, purchased from Biotech, Kottayam, Kerala. In vitro susceptibility of the plant extracts were decided to be done by paper disc method as suggested Kirby-Bauer test (Bauer, 1966). The effective extracts were further to be analysed by Poison food technique, (Nene and Thapilyal, 2002) Acetone was a common solvent that could efficiently dissolve all the PE, Chloroform and acetone extracts. It had been reported as the most preferred solvent for dissolving the extractants and had been rated as fairly non toxic (Gurjar, 2012) and hence was decided to be used as the solvent to dissolve the extracts.

Setting of experiment for Preliminary screening

Method of Preliminary screening: Potato infusion was prepared by boiling 200gms of potato chopped into small pieces, for around 20minutes, till soft, then filtering out large pieces to prepare the infusion into which was added 20 gm of Dextrose and finally 15 gms of agar, that was further made upto 1 liter. The agar was dissolved by boiling in the above solution and the whole mixture was kept for autoclaving. The PDA thus prepared was poured into sterile petriplates (9cm diameter) and kept for solidifying. In the centre of the plate, hyphae disc from 7 day old Colletotrichum fungal colony was cut (5mm diameter) and placed in the centre of the plate. Filter paper discs of 5mm were punched out of Whatmann's filter paper and sterilised by autoclaving. Extracts of specified plant parts were dissolved in suitable solvents (acetone/ chloroform/ dmso) filtered with nanofilters (Whatmann's) and loaded onto the disc, maximum upto15µl on either side at concentration of 10mg/ 20mg at 10µl or 20µl depending on the solubility of the extract in the solvent. However in no case did the concentration of solvent exceed 30µl. The plate was covered with sterile thin sheet and left for incubation at the room temperature till the plate got covered or till the hyphae grew over control disc. The extract with activity showed a sort of zone around them where the hyphae grew without overlapping the disc. The extracts that showed a zone of inhibition were further subjected to poison food technique. A specific amount of extract (max.5mg/ml) was added to the agar and after swirling briskly was poured into the petriplates. Once it solidified, fungal discs of 5mm diameter from the periphery of 5 to 7 day old fungal plates were cut and kept on the agar at the centre and plate covered. Control plates were also set with the respective solvents as +ve and distilled water as the negative control. The growth of hyphae was measured by scale in cm. at right angles till the last but one day when the hyphae fully covered the plate and compared to the standard. % growth was taken with the formula:

$$I\ \% = (C-T) / C \times 100$$

Where C= radius of hyphae in control
T= radius of hyphae in extract
I= Inhibition %

If the extract showed an inhibition more than 50%, it was said to be effective in nature. The plant part that showed a promising inhibitory activity in the sequential extract was subjected to exhaustive soxhlet extraction with the same solvents albeit, individually. All the experiments were done in six replicates and the significance was measured statistically at $p<0.05$. The most effective extract was that which inhibited the growth of the fungi the maximum, and the effect of that extract on the fungal hyphae was studied by Scanning Electron Microscope. Small discs of fungal hyphae were immersed in 5% acetone solution of the extract for 48 hours and the effect studied under Scanning Electron Microscope along with the positive control. (ie acetone solvent).

RESULTS

None of the species of Annona genus showed any inhibition zone against *Colletotrichum gloeosporioides* in the paper disc screening method (Table 1). The only plant that showed some appreciable level of inhibition during preliminary screening of sequential extracts was *Uvaria narum*. The colletotrichum hyphae overgrew the paper disc loaded with acetone and methanol extracts; it showed a repulsion towards the petroleum ether and chloroform extract containing paper discs and took a peculiar elliptical shape (Image A.).

Image A. Discs with extracts (sequential) of Uvaria narum PE: Petroleum Ether; Chl: chloroform (Ac):Acetone; CH3OH: methanol

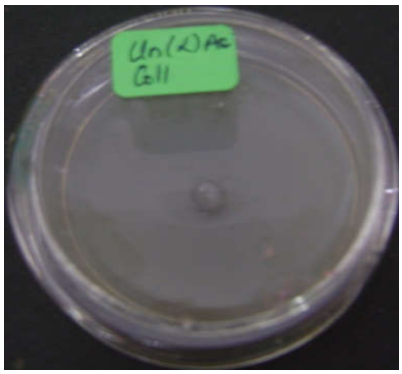


Image B. *Colletotrichum* no growth in acetone extract

Image C. Healthy growth in acetone

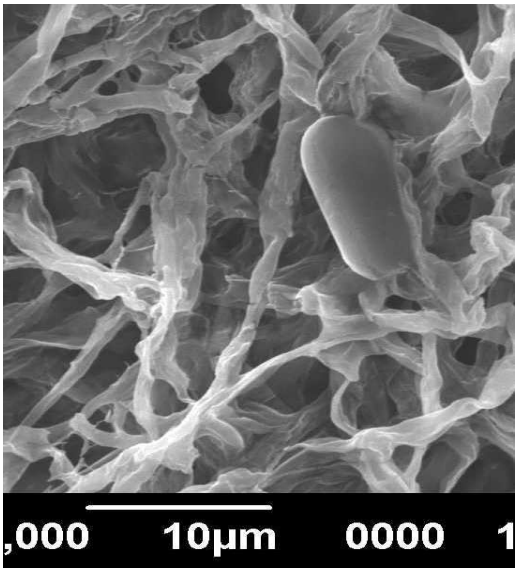


Image D. Hyphae treated with control (acetone)

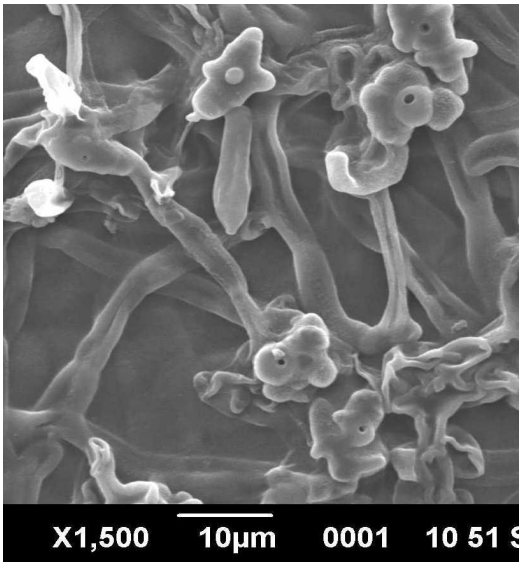


Image E. Hyphae treated with the acetone extract (Individual)

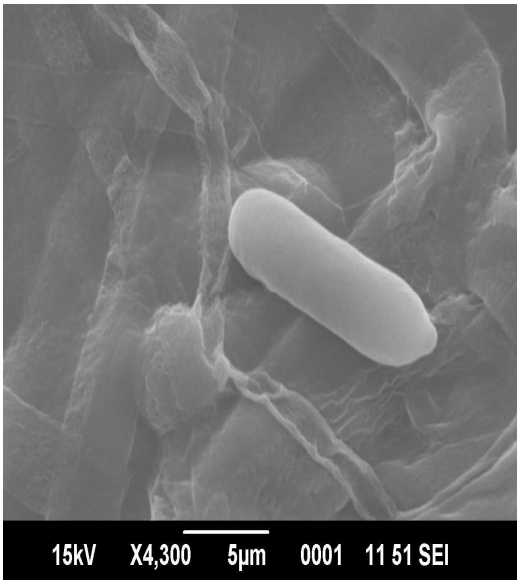


Image F. Spore when treated with control(acetone)

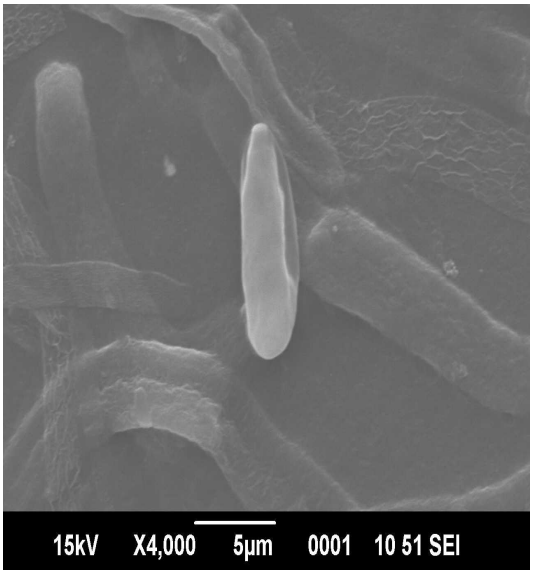


Image G. Spore when treated with acetone extract (Individual) Spores slowly losing their cytoplasm

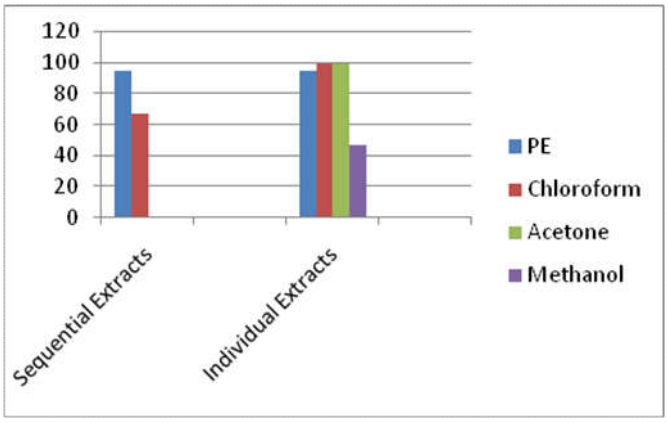
Table 1.

	Annona squamosa		*Annona reticulata*		Annona muricata		*Uvaria narum*
	SEED (AsS)	LEAVES (AsL)	SEED (ArS)	LEAVES (ArL)	SEED (AmS)	LEAVES (AmL)	LEAVES (UnL)
PE	-	-	-	-	-	-	+
Chl	-	-	-	-	-	-	+
Ac	-	-	-	-	-	-	-
Me	-	-	-	-	-	-	-

Determination of inhibition of Colletotrichum by different plant extracts by paper disc method. Here (-) means no inhibition and (+) means inhibition is present.

Table 2.

Uvaria narum	Sequential Extracts	Individual Extracts
UnPE	95±1.3*	95±1.3*
UnChl	67±1.7*	100
UnAc	0	100
UnMe	0	46.62±0.7
DW	0	0
Ac	0	0



Graph showing the inhibition % of the sequential and individual extracts of *Uvaria narum* against *Colletotrichum gloeosporioides*

To study the percentage of inhibition by all the sequential extracts, poison food technique was followed. Sequentially extracted PE extracts showed a mean inhibition of 95% and chloroform extracts showed a mean inhibition of 64% with a standard error of 1.7. (Table 2) while sequentially extracted acetone and methanol extracts showed no inhibition at all. In the testing of activity by extracts obtained by individual extraction, it was found that while UnPE gave a 95% inhibition, both UnChl and UnAc (obtained from individual extraction gave a 100% inhibition) (Image B only acetone inhibition shown.) and UnMe gave only 46% inhibition. Acetone was used as the solvents for dissolving the extracts and it did not have any toxic effects on the growth of the fungus, as is obvious from Image C. The SEM analysis was highly revealing in its images that brought out the activity of the active acetone extract (UnAc) against *Colletotrichum gloeosporioides*. There were putative lysis and development of pores in the walls of the treated hyphae and emptying of the cytoplasmic contents were also observed. The control solvent was acetone. While the hyphae and spores treated in the control showed no changes, (Images D and F), the hyphae in the treated extract showed spilling of the cytoplasmic contents and also the shrinking of the spore and the loss of cytoplasm too (E and G).

DISCUSSION

In the tests with various extracts of *Uvaria narum*, the peculiar elliptical shape taken by colletotrichum by avoiding the paper discs containing its PE and Chl extracts and growing between them rather than over them is indicative of the inhibitory nature of these extracts. In the poison food technique used to test the percentage inhibition of the extracts obtained by sequential extraction, the bioactive antifungal compounds inhibiting the fungal growth must have been extracted in petroleum ether and the remaining entirely extracted by chloroform solvent. No inhibitory substance was left in the acetone and methanol extract as they supported luxuriant growth of the fungus. At the same time, on individual extraction, this bioactive compound could be completely extracted in chloroform and acetone. This could be the reason why it gave a 100% activity in these solvents. This also suggested that the bioactive compound could be mildly polar in nature. The methanol extract, from sequential extraction gave a luxuriant growth of the fungus, while the individually obtained extract gave a 46% inhibition. All these findings strongly indicate the presence of a bioactive substance that is highly extractable in moderately polar solvents (chloroform and acetone) and fairly extractable in highly non polar petroleum ether, but very poorly extracted in the methanol. The antifungal nature of *Uvaria narum* was proven by its effect on the fungus *Phytophthora capsicii,* that causes foot rot of pepper. Yet, in that experiment it was mainly because of benzoic acid extracted from the leaves of *U.narum* in Petroleum Ether. The acetogenins isolated by Padmaja et al (1993), from the leaves of *Uvaria narum* had also shown a very good antifungal activity towards many dermatophytes that they had subjected to antifungal studies (Padmaja, 1993). While all the four plants in our experiment are excellent sources of acetogenins, yet the antifungal activity exhibited by them against the tested fungus, *Colletotrichum gloeosporioides* was not uniform. It remains thus to be answered whether the antifungal activity thus observed in *Uvaria narum* is due to acetogenins as proven by Padmaja (1993), or due to benzoic acid as proven by Jose, (Jose2003). The SEM analysis gave a good insight into the extent of damage that was done to the colletotrichum hyphae and spore when it was treated with the acetone extract. The putative lysis and development of pores in the walls of the treated hyphae and emptying of cytoplasmic contents all indicate that the bioactive compound/ compounds was/were responsible for the membrane disintegration of the hyphae. This could explain why the fungus failed to show any growth in acetone and chloroform extract thereby giving a 100% inhibition.

Conclusion

Thus it could be deduced from table 1 that none of the Annona species selected for this study exhibited any antifungal activity towards the tested pathogen. This result is at par with the one obtained by Mogle (2013) and Johnny (2011), who also found *Annona reticulata* and *Annona muricata* not much antifungal in nature against different fungi they had selected (Mogle, Jan 2013) (Johnny, 2011). Though Annonaceus acetogenins from seeds of *Annona squamosa* showed their inhibitory effect on Phytophthora in experiment done by Dang (Dang, *et al*., 2011) yet they failed to show any effects in our experiments against Colletotrichum fungus. Thus the antifungal nature of the different extracts of the leaves and seeds of *Annona squamosa* and also of Annonaceous acetogenins from these three species of Annonaceae in different solvents stand challenged in our experiments. It corroborates Kumar's finding that *Annona squamosa* leaves and seeds doesn't show an antifungal nature (Kumar, 2006).

The aim of the given study was to find out whether the members of Annonaceae family, that were insecticidal in nature, were effective as antifungals also, especially against *Colletotrichum gloeosporioides,* the anthracnose fungus. The bioactivity of leaves and seeds of three members of Annona genus namely *Annona squamosa, Annona reticulata* and *Annona muricata* and leaves of *Uvaria narum*, were tested against *Colletotrichum gloeosporioides*. The three selected members of Annona genus, though potent insecticides, (Isman 2014), failed to show effective antifungal activity against our fungus of interest. *Uvaria narum* showed the best antifungal activity of which, the Petroleum ether extract in the sequential with 94% inhibition and Chloroform and acetone extracts in the individual type of extraction showed the best activities with 100% inhibition. The SEM analysis also supported this result

by showing the images of spilled cytoplasm and porous hyphae in acetone treated extracts. Thus we find that the three species of Annona ie, *Annona squamosa* L., *Annona reticulata* L. and *Annona muricata* L. lack the antifungal activity against *Colletotrichum gloeosporioides,* but the chloroform and acetone extracts of *Uvaria narum (extracted individually)* show a very good activity against this anthracnose fungus (100% inhibition). No reports are present regarding the insecticidal potential of *Uvaria narum*. Further studies are going on to isolate and characterize this bioactive compound to find out the nature of the compound involved and whether it is an acetogenin or any other compound that is being responsible for its antifungal activity.

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