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In vitro Antifungal Potentials of Bioactive Compound Methyl Ester of Hexadecanoic Acid Isolated from *Annona muricata* Linn. (Annonaceae) Leaves

M.N. Abubacker[1]* and T. Deepalakshmi[2]

[1]Department of Biotechnology, National College, Tiruchirappalli - 620 001, India.
[2]Department of Botany, National College, Tiruchirappalli - 620 001, India.

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Annona muricata **Linn. (Soursop) of Annonaceae is a medicinal plant. Major bioactive compound methyl ester of hexadecanoic acid was isolated from the leaves. It was tested for antifungal potentials and found to be highly effective at 10.0, 15.0 mg/ml concentrations for *Alternaria solani* (NCBT-118) and *Aspergillus erithrocephalus* (NCBT-124); at 15.0 mg/ml concentration *Aspergillus albicans* (NCBT-120); less effective for *Aspergillus fumigatus* (NCBT-126) and *Penicillium chrysogenum* (NCBT 162). This bioactive compound will find a place in the formulation of herbal based antifungal drugs for the test fungi of this work.**

Key words: Bioactive compound, Methyl ester of hexadecanoic acid, GC-MS analysis.

Annona muricata Linn. commonly known as soursop or graviola, belongs to the family Annonaceae. It is a typical tropical tree with heart shaped edible fruits and widely distributed in most of the tropical countries (De Feo, 1992). The leaves are lanceolate, glossy and dark green in colour had been traditionally used to treat headaches, hypertension, cough, asthma and used as antispasmodic, sedative and nervine for heart condition (Taylor, 2002; Lans, 2006).

Previous reports over the years have demonstrated that the root, stem, bark, leaf fruit and seed extracts of *A. muricata* are anti-bacterial (Misas *et al.*, 1979; Soundarroa *et al.*, 1993; Abubacker and Deepalakshmi, 2012), antifungal (Heinrich *et al.,* 1992) and anti-malarial (Arkcoll, 1990; Antoun *et al*., 1993). Its leaf extract was also found to possess antioxidant (Basker *et al*., 2007; Abubacker and Deepalakshmi, 2012) and molluscicidal properties (Santos and Sant'Ana, 2001; Luna *et al.*, 2006). Recently, it has also been reported to exhibit anti-inflammatory and analgesic effects (De Sousa *et al*., 2010; Roslida *et al*., 2010). Among the chemical constituents found in the leaf of *A. muricata* are alkaloids (Le Bouef *et al*., 1981, 1982), essential oils (Pelissler *et al*., 1994; Kossouh *et al.*, 2007) and acetogenins (Wu *et al*., 1995; Zeng *et al.,* 1996; Kim *et al*., 1998; Chang *et al*., 2003).

All parts of *A. muricata* tree are used in natural medicine in the tropics, but little information is available with respect to antifungal activities of *A. muricata* leaf extract. In view of this, the present study was designed to evaluate the antifungal potentials on fungi.

MATERIALS AND METHODS

Collection and Identification of Plant Material

Fresh leaves of *Annona muricata* Linn. (Annonaceae) were collected from a private

* To whom all correspondence should be addressed.
E-mail: abubacker_nct@yahoo.com

garden in Tiruchirappalli, Tamil Nadu (Fig. a). The taxonomic identities of the plant is confirmed by Flora of the Presidency of Madras (Gamble, 1967). Fresh leaf material was washed under running tap water, air dried in shade and then homogenized to fine powder and stored in sterile air tight bottles for the experimental work.

Fungal Cultures

The fungal cultures tested in this work *Alternaria solani* (NCBT 118), *Aspergillus albicans* (NCBT-120) *A. erithrocephalus* (NCBT-124), *A. fumigatus* (NCBT-126) and *Penicillium chrysogenum* (NCBT 162) were maintained in immobilized condition in polyurethane foam in Microbiology Lab, Department of Biotechnology, National College, Tiruchirappalli.

EXPERIMENTAL

Procedure

100 mg of dry leaf powder mixed with 20 ml of Sabouraud Dextrose Agar (SDA) medium (HI media-M063) constitute 5 mg/ml, 200 mg of dry leaf powder for 10 mg/ml and 300 mg for 15 mg/ml. The Control (a) contained only 20 ml of SDA medium and Control (b) contained 100 mg of bavistin fungicide added to 20 ml of SDA medium constitute 5 mg/ml concentration. The leaf powder is mixed with the SDA medium in Petridish (9 cm) and inoculated with 0.5 ml spore suspension of fungi prepared from 10 days old culture. The experimental petridishes were incubated for 6 days at 28° ± 2°C temperature in dark. Three replicates were prepared and inoculated with fungal spores for each treatment.

Determination of the Minimum Inhibitory Concentration (MIC)

MIC was determined by the liquid dilution method (Irobi *et al*., 1996). Dilution series were prepared with 0.25 to 15.0 mg/ml of Sabouraud Dextrose broth medium (HI media - MU033). To each tube 0.1 ml of standardized suspension of fungal spores (4´10^6 spores/ml) were added and incubated at 28° ± 2° C for 24 hours. The lowest concentration which did not show any growth of the tested fungi after microscopic evaluation was determined as MIC.

Isolation and Identification of Bioactive Compound

Thin Layer Chromatography

Glass plates (4 cm × 12 cm) were used in which 30 gm of silica gel mixed with 60 ml distilled water slurry was prepared and coated on the glass plate to 0.25 cm thickness and dried for an hour at 110°C in an oven (Bothast and Hesseltine, 1975).

Preparation of leaf extract for bioactive compound

The dry powdered leaves (500 mg) of *A. muricata* was mixed with 5.0 ml of chloroform and ground into a paste, dried at room temperature. 1 ml of chloroform was added to the dried samples and spotted on the TLC plates. The TLC plates were kept in several eluent mixture with different polarities to separate the bioactive chemical compounds in chloroform extract has been tried. The eluents used were chloroform : *n*-hexane (8:2), chloroform : ethyl acetate (8:2), chloroform : acetone (8:2), *n*-hexane : acetone (9:1), and chloroform : acetone (9:1). Sample spotting on the TLC plate was done by using a micropipette in which the dot diameter 0.5 mm. The chloroform : acetone (9:1) was the best eluent since it was able to separate the four compounds contained in leaf extract (Komansilan *et al*., 2012).

Gas-chromatography and Mass-spectroscopy (GC-MS) analysis

GC-MS analyses were performed using a GC Clarus 500 Perkin Elmer equipment equipped with a flame ionization detector and injector MS transfer line temperature of 230°C, fused silica capillary column Elite- 5 MS (5% Diphenyl / 95% Dimethyl polysiloxane), 30´0.25 mm df, film thickness, carrier gas Helium at a flow rate of 28 cm/sec was used. 1 ml of extract mixed with methanol (80%) at a split rate of 10:1 was injected. The compound identification was accomplished by comparing the GC relative retention and mass spectra to those of authentic substances analysed under the same conditions, by their Retention Time (RT) and by comparison to reference compounds (Table 2).

RESULTS AND DISCUSSION

The aqueous extract of dried powder of *A. muricata* leaf has shown varied antifungal properties for all the five fungal strains tested in this work (Table 1).

The growth of *A. solani* and *A. erithrocephalus* fungi were totally inhibited at

Table 1. Antifungal properties of bioactive compound methyl ester of hexadecanic acid, in leaf extract of *Annona muricata* plant

Fungus	Control-1	Control-2	Concentration of leaf extract (mg/ml)		
			5.0	10.0	15.0
Alternaria solani (NCBT 118)	++++	–	+++	–	–
Aspergillus albicans (NCBT-120)	++++	–	++++	++	–
A. erithrocephalus (NCBT-124)	++++	–	+++	–	–
A. fumigatus (NCBT-126)	++++	–	+++	+++	+
Penicillium chrysogenum (NCBT 162)	++++	+	++	++	++++

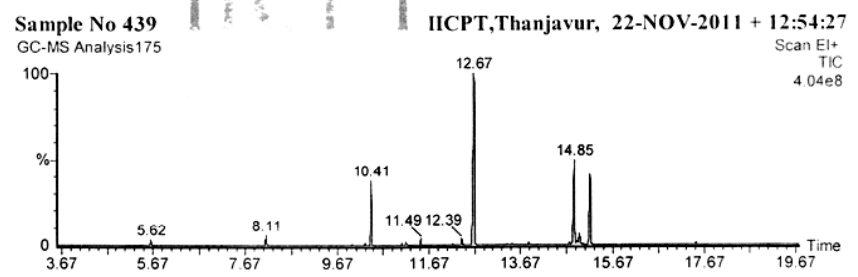
Control-1 : Medium without bioactive compound
Control-2 : Medium with bavistin (5.0 mg/ml)
++++ : Normal growth
+++ : 25% growth inhibition
++ : 50% growth inhibition
+ : 75% growth inhibition
– : Total (100%) growth inhibition

Table 2. Bioactive compounds identified in *Annona muricata* leaf extract

No.	RT	Name of the compound	Molecular formula	MW	Peak Area %
1.	5.62	Hexanal, O-methyloxime	$C_7H_{15}NO$	129	1.07
2.	8.11	Butanal, O-methyloxime	$C_5H_{11}NO$	101	1.68
3.	10.00	2-Propanone, oxime	C_3H_7NO	73	0.12
4.	10.41	5-Methyl-2-hexanone oxime	$C_7H_{15}NO$	129	10.73
5.	11.09	1,2-Ethanediamine, N-(2-aminoethyl)-	$C_4H_{13}N_3$	103	0.24
6.	11.17	Butanamide, 4-cyano-N-methyl-	$C_6H_{10}N_2O$	126	0.46
7.	12.39	1,13-Tridecanediol, diacetate	$C_{17}H_{32}O_4$	300	1.10
8.	12.67	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	44.11
9.	14.85	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	19.60
10.	14.95	10-Undecen-1-yl acetate	$C_{13}H_{24}O_2$	212	2.42
11.	15.19	Acetamide, N-acetyl-N-methyl-	$C_5H_9NO_2$	115	17.98
12.	15.43	3-Cyclohepten-1-one	$C_7H_{10}O$	110	0.09
13.	17.49	5-Hexen-2-one, O-methyloxime	$C_7H_{13}NO$	127	0.37
14.	17.95	Acetic acid, 2-methylpropyl ester	$C_6H_{12}O_2$	116	0.03

Note: *Parameters tested are not covered under the scope of NABL accreditation

GC-MS Chromatogram of Annona sp

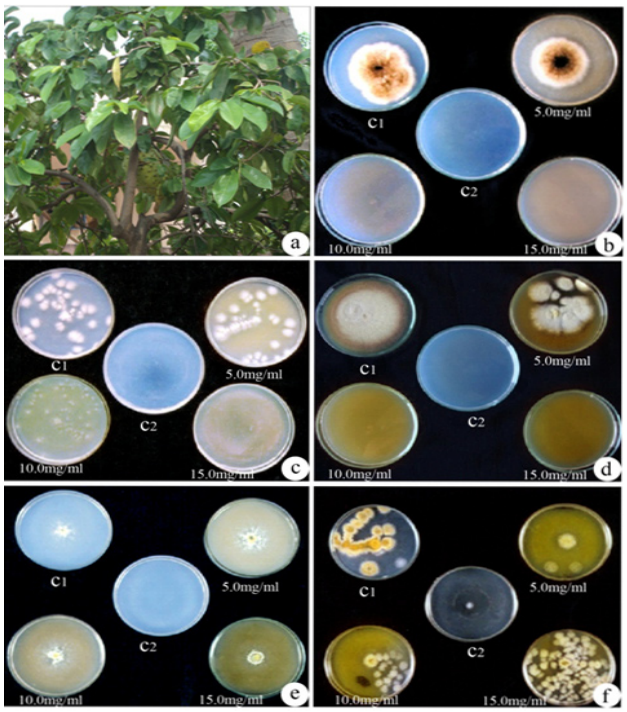


10.0 and 15.0 mg/ml concentration (Fig. b & d). The total inhibition can be comparable to control-b, a standard antifungal agent bavistin at 5 mg/ml, whereas only 25% growth inhibition was noticed at 5.0 mg/ml concentration for these two fungi. For *A. albicans* (Fig. c) total inhibition was seen only at 15.0 mg/ml concentration, but 10.0 mg/ml concentration produced 50% inhibition. *A. fumigatus* (Fig. e) found to show 75% inhibition at 15.0 mg/ml concentration, whereas 5.0 and 10.0 mg/ml showed only 25% inhibition. *P. chrysogenum* (Fig. f) has shown no growth inhibition at 15.0 mg/ml concentration, but 50% growth inhibition was noticed for 5.0 and 10.0 mg/ml concentration.

A. muricata plant extract has been reported to possess antimicrobial activities (Pathak *et al*., 2003). Many fungal pathogens cause soft-rot diseases of the fruit of *A. muricata* such as *Aspergillus* spp., *Colletotrichum* sp. and *Rhizopus* sp. (Nweke and Ibiam, 2012). The present work is one of the first report that *A. muricata* leaf extract with its potential bioactive compound methyl ester of hexadecanoic acid ($C_{17}H_{34}O_2$), and methyl ester of 9-octadecenoic acid (Z) ($C_{19}H_{36}O_2$), an effective plant extracts and act as antifungal agent.

The MIC values of the aqueous extract of leaf varied from 5.50 mg/ml to 9.50 mg/ml for the fungi tested. The MIC value of *A. solani, A. albicans, A. erithrocephalus, A. fumigatus* and *P. chrysogenum* were 5.50, 6.75, 5.50, 8.75 and 9.50 mg/ml respectively. Further investigation was performed to demonstrate the action of the extract on these fungi at different concentrations. The growth of these fungi correspondingly decreased with increasing concentration of the extract and the growth was completely inhibited at their MIC values except *P. chrysogenum*. The reduction

Antifungal properties of bioactive compound methyl ester of hexadecanic acid in leaf extract of *Annona muricata* Linn. plant



a) Habit
b) Alternaraia solani NCBT 118
c) Aspergillus albicans NCBT 120
d) Aspergillus erithrocephalus NCBT 124
e) Aserpgillus fumigatus NCBT 126
f) Penicillium chrysogenum NCBT 162

C1) Medium with out bioactive compound
C2) Medium with bavistin (5.0/ml) 5.0, 10.0 and 15.0 mg/ml concentration of bioactive compund

Plate 1.

of growth was possibly due to the interference by active principles, *i.e.* bioactive compound methyl ester of hexadecanoic acid which is the major compound of *A. muricata* leaf along with methyl ester of hexadecanoic 9-octadecenoic acid (Z) acid. Therefore, the MIC determination is important in giving a guideline of the choice of an appropriate and effective concentration of antifungal therapeutic substance.

CONCLUSION

The aqueous leaf extract of *A. muricata* is a significant inhibitor of growth of certain fungal strains. Therefore, the identification of this potential plant as antifungal agent will help in environmentally safe herbal antifungal formulations to control *Alternaria solani* causing leaf spot disease and *Aspergillus erithrocephalus* causing soft rot disease more effectively.

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