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MOSQUITO LARVICIDAL AND ANTIMICROBIAL PROPERTIES OF ETHYL ACETATE CRUDE EXTRACTS OF CLADOSPORIUM SPHAEROSPERMUM AND CLADOSPORIUM MACROCARPUM

K PRANAY KUMAR, SHRUTI S DESHPANDE AND SUNIL MISRA*

Biology Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad-500 607, India

ABSTRACT

The main objective of this study is to investigate the ethyl acetate crude extracts of *Cladosporium macrocarpum* (MTCC 2137) and *Cladosporium sphaerospermum* (MTCC 2805) for their larvicidal activity against third and fourth instar larvae of *A. aegypti* and antimicrobial activity on few selected bacterial and fungal strains. *C. macrocarpum* extracts showed more susceptible to the third instar larvae than that of fourth instar larvae after 24hr of exposure (LC_{50} =116.36 ppm and 196.72 ppm; LC_{90} =577.84 ppm and 1604.98ppm) respectively. In the case of *C. sphaerospermum* extract also showed good larvicidal activity with (LC_{50} = 182.90 ppm and 248.96 ppm; LC_{90} = 1243.54 ppm and 1435.3ppm) against third and fourth instar mosquito larvae respectively. In the antimicrobial activity of the extract of *C. sphaerospermum* exhibited good antibacterial activity against *S. aureus*, *P. aeroginosa*, *C. albicans* and *C. rugosa* those are known to cause skin lesions and wound infections. From this study it is inferred that, mosquito larvicidal properties of both the extracts showed potent larvicidal activity against *A.aegypti* which can be exploited further for eco-friendly control of mosquito larvae as well as the effectiveness of the both crude extracts against possible usage in skin lesions and wound infections as therapeutic agents.

KEYWORDS: Cladosp orium sphaerospermum, Cladosporium macrocarpum, Aedes aegypti, larvicidal and antimcrobial activity



SUNIL MISRA

Biology Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad-500 607, India

INTRODUCTION

Dengue is a mosquito-borne disease which has become a great health concerned in recent times in India 1-3. The causative organism is a Flavivirus that transmits by the Aedes aegypti mosquito species 4. Till date, there is no effective drug or licensed vaccine is ready for the treatment of dengue ⁵⁻⁷. As a result of limited therapeutics strategies, vector control is the ideal way for the control of population Entomopathogenic mosquito (mycoinsecticIdes) are gaining increased attention as environmentally friendly insect control agents for the last few decades. Among fungi classes, Ascomycetes are known to be active producers of antimicrobial compounds, which have high therapeutic values. The extracellular secondary metabolites generated by entomopathogenic fungi have become a focus of interest for insect pathologists. However, the effects of extracellular metabolites appear to be very limited in comparison to using of spores and mycelia of the fungi to control mosquito larvae ⁹. Fungi and fungus-derived products have attracted many insect pathologists as they are highly toxic to mosquitoes, yet have low toxicity to non-target organisms and are also biodegradable. Fungi belonging to class Ascomycetes are known to produce many bioactive compounds with pharmaceutical and agriculturally importance. In the present study, Cladosporium species belonging to the fungal division Ascomycetes was investigated for control of Aedes mosquito larvae. Cladosporium is a mitosporic, ascomycetes fungus and cosmopolitan distribution. Many endophytic, fungicolous, human pathogenic, phytopathogenic, saprobic Cladosporium species are known for their medical, commercial industries and agricultural importance 10-11 Many Cladosporium species are capable of producing useful metabolites with interesting biological activity like antibacterial, antifungal, larvicidal activity ¹²⁻¹⁴. Gibberellins production ¹⁵, phototoxic ¹⁶⁻¹⁷, antitumor, antiviral, antimitotic, cytostatic activity ¹⁸, biodegradation of polycyclic aromatic compounds ¹⁹⁻²⁰, and also act as weedicide ²¹. Cladosporium species are also used in the biological control of insects like aphids (plant pest), whiteflies (Crop pest), for insects developed resistance to chemical insecticides ²². From the reported studies on metabolites of Cladosporium species, it is evident that this genus is a rich source of bioactive compounds with exceptional biological activities. However, till now no report available on this fungus used for mosquito control. Based on this, we characterize the larvicidal efficacy of crude ethyl acetate extracts from fungi C. sphaerospermum (MTCC 2805) and C. macrocarpum (MTCC 2137) against third and fourth instar larvae of Aedes aegypti. These extracts were also used to investigate the antimicrobial properties against selected fungal and bacterial strains.

MATERIALS AND METHODS

(i) Fungal strains and cultivation

The fungal cultures *C. macrocarpum* (MTCC 2137) and *C. sphaerospermum* (MTCC 2805) were procured from the

Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The cultures were maintained on Potato Dextrose Agar (PDA) (Himedia laboratories, India). The fresh mycelium of both the cultures were grown on PDA plates and incubated at 27°C for 3-6 days and were then used to inoculate 300ml of Potato Dextrose Broth contained in 1 Lt conical flask. The flasks were incubated at room temperature (27 \pm 2°C) for 15 days.

(ii) Mosquito collection and rearing

The egg rafts of *A. aegypti* were reared in the laboratory condition ($28 \pm 3^{\circ}$ C, 75 to 85% relative humidity and the photoperiod of 14:10h (L:D) ⁸. The larvae were fed with Brewer's yeast–dog biscuit (1:3). Larvae were placed in deionized water at pH 7.0. To counteract evaporation, water was added daily using sterilized glassware.

(iii) Extraction of fungal crude metabolite

The fungal crude metabolite was extracted by solvent extraction procedure using Ethyl acetate as solvent. Equal volumes of the filtrate and Ethyl acetate were taken in a separating funnel and were shaken vigorously for 10 min. The solution was then allowed to stand, and the solvent was collected after the cell biomass was separated. This procedure was repeated thrice. Ethyl acetate was then evaporated using rotary evaporator and the resultant extract was dried and stored in Eppendorf tubes ¹². These extracts were used for their larvicidal assay and antimicrobial activity.

(iv) Larvicidal bioassay

The larval stages (third and fourth instar) were used for the larvicidal bioassay according to the standard procedure recommended by WHO 23 . The extracts (*C. macrocarpum* and *C. sphaerospermum*) were dissolved in acetone. Known volumes of different concentrations (100 - 1500ppm) were added to the glass beakers (250ml) and makeup to 200ml with tap water. The controls are maintained with acetone (blank) and tap water. Mortality was recorded after 24 hours of exposure. The test was performed in triplicates and mean average values were taken. The LC $_{50}$ and LC $_{90}$ were determined by a probit analysis program (6). No mortality was observed in the control (solvent and water).

(v) Antimicrobial activity

Crude extract was screened for its antibacterial activity against an array of 12 microorganisms (obtained from the Microbial Type Culture Collection MTCC), which are as follows: Bacillus subtilis (MTCC 441) and Staphylococcus aureus (MTCC 96) as Gram-positive bacteria; Escherichia coli (MTCC 443), Pseudomonas aeruginosa (MTCC 1688), Chromobacter violeacum (MTCC 2656)) and Klebsiella pneumoniae (MTCC 618) as Gram-negative bacteria. Its antifungal activity was evaluated against yeast Candida albicans (MTCC 227), Candida rugosa and filamentous oryzae(MTCC-262), Aspergillus niger funai Rhizopus (MTCC 1344) and Aspergillus flavus. Bacterial and fungal cultures were maintained on nutrient agar and potato dextrose agar slants (M/S Himedia, Mumbai, India), respectively prior to test performed in Petri dishes. The inhibitory zones (in mm) were determined by agar well method (cup plate method). Antibiotics Streptomycin, Penicillin and Amphotericin B were used as positive controls against bacteria and fungi respectively. The crude extract was assessed for antimicrobial activity at concentrations ranging between 100µg and 2000µg. Each concentration was assessed in triplicates and all values are expressed as means ± standard error.

(vi) Statistical Analysis

The zone of inhibition data for each microorganism was analyzed using one-way analysis of variance (ANOVA) and the differences among group means were analyzed using the Dunnett's multiple comparisons test. P value < 0.05 was considered as significant. The software Graph pad Instat Ver 3.10 was employed for the statistical analysis. The average mosquito larval mortality data were subjected to probit analysis 24 for calculating LC $_{50}$, LC $_{90}$, along with 95% fiducial limits of the upper confidence limit (UCL) and lower confidence limit (LCL), and chi-square values using probit analysis software (IICT v1.0). The regression lines were drawn for each of the larval instars using Microsoft Excel.

RESULTS AND DISCUSSION

In past few decades, conventional methods (spraying and fogging of synthetic insecticides) are widely used for control of mosquito population. Negative effects like the release of toxic residues, toxic to other non-target organism and resistance development in mosquitoes due to repeated usage of these synthetic insecticides prompted alternative way of controlling the mosquito population. So at this present circumstance, biological control of mosquitoes proves to be more appropriate in place of indiscriminate use of synthetic pesticides. Use of such products is safe to an environment as well as to nontarget organisms of the ecosystem ²⁷. Much work has been done on plant-based products to control the *A. aegypti* larvae ^{5, 28-29}. Microorganisms are well-known producers of bioactive compounds and they may be an alternative source of mosquito control agents ³⁰. Two insecticidal bacteria (*Bacillus thuringienesis* sp. *israelensis* and Bacillus sphaericus) have been used as larvicides to control mosquito larvae in many countries unfortunately; the development of resistance against these chemicals in various mosquito populations has also been reported ³¹. Vector control still remains the main strategy for control of these diseases, especially in areas where resistance in parasite to drugs is growing 32. The killing of immature stages of A. aegypti mosquitoes and to inhibit the emergence of adults is essential in the process of vector control ³³. These larval stages are suitable targets of most biocontrol agents. Among the biocontrol agents, fungal metabolites have attracted more attention as potential biopesticides because of their target specificity, to overcome pesticide resistance, lack of toxic residue and eco-friendly in nature ³⁴. The secondary metabolites of fungus *Chrysosporium* ³⁵⁻³⁷, *Fusarium* ³⁸ have been

screened as a potential mosquito larvicide. Fungal phytotoxins cyclopaldic acid and sphaeropsidin A are reported larvicidal activity against *A. aegypti* with LD_{50} = 58.2 and 36.8 ppm respectively ³⁹. Ethyl acetate extract from *C. spherospermum* (MTCC 3719) was reported to exhibit mild larvicidal activity against third and fourth instar mosquito larvae with LD_{50} =464.83 and 565.53µg/ml respectively 40. In contrast to earlier reports, in our study the ethyl acetate of C. sphaerospermum and C. macrocarpum extracts showed significant dose-dependent increase in the larval mortality against third and fourth instar larvae of A. aegypti after 24h post treatment (Fig. 1 and 2). The larvicidal potential of C. macrocarpum was found to be more than the C. sphaerospermum and the third instar larvae were more susceptible with [LC50] =116.36 & 182.90 ppm] and [LC₉₀ = 577.84 & 1243.54 ppm] respectively. Whereas, both the crude extracts showed moderately toxic against the fourth instar larvae with the LC₅₀ values recorded at [196.72 and 248.96 ppm] and LC₉₀ values [1604.98 and 1435.3 ppm] (Table 1). These results suggested that the potential of Cladosporium sp., against A. aegypti larvae and their benefits to developing new types of larvicidal formulation for mosquito control. Mostly, Cladosporium sp., are used to control of insect pest of crops. The death of the insects is mostly due to the spore germination on the insect body. The fungus may also be involved in other mechanisms such as the production of toxic metabolites and/or enzyme that degrade cell wall. Compounds from Cladosporium species were reported as high larvicidal activity that binds to the hydrophobic sites on cell membranes in *B. Amphitrite* ⁴¹. These types of compounds may be present in the extract which are responsible the larvicidal activity against A. aegypti larvae. The present laboratory results strengthen the scope for the utilization of these fungal metabolites as larvicides which can be a better alternative to controlling larval population. Such metabolites can be instrumental in encouraging the use of fungi in mosquito control. The antimicrobial activities of crude extracts produced by C. macrocarpum and C. sphaerospermum are summarized in Table 2 and 3. These secondary metabolites appear to be promising in their activity in vitro against bacteria and fungi. A maximum activity of C. macrocarpum was observed against S. aureus and P. aeroginosa with a zone of inhibition of 25mm and 19mm respectively. Also, a moderate activity was observed against B. sphaericus, S. epidermidis, P. oleovarans and E. coli (zone diameter range from 14 to 16mm) and less active against C. violaceaum and K. pneumoniae. The antifungal activity was best observed for C. rugosa and C. albicans with zone diameter of 21mm and 18 mm respectively. Moderate activity was observed with A. niger, S. cerevisiae, and R. Similarly the extract oryzae. sphaerospermum showed good in vitro potential of antimicrobial activities against all bacterial and fungal species tested except E. coli. The data obtained from the agar diffusion method indicated that the extract displayed the inhibitory effect increased with increase of the concentration from 250 to 2000 µg. Gram-positive S. aureus was the most sensitive strain with the inhibition zones equal to the positive control at 1500-2000µg (1719mm). Gram-negative strains also displayed variable degree of susceptibility. However, a dose dependent increase in the activity observed against all the fungal strain strained. Moderate activity was observed against *K. pneumoniae* (18mm), followed by *P. aeroginosa* (16mm), and *C. violaceaum* (15mm). The extract also exhibited good antifungal activity against yeasts and moderate activity against filamentous fungi. Maximum inhibition zone was observed against *S. cerevisiae* (21-27mm) at 1500 – 2000 µg followed by *C. albicans* (19 mm) and C. rugosa

(18mm) and moderate activity against the filamentous fungi $A.\ niger$ (18mm), $A.\ flavus$ (16mm) and $R.\ oryzae$ (18mm) at 2000 µg. The large zones of inhibition exhibited by both the extracts on $S.\ aureus$ and $P.\ aeruginosa$ justified their use by traditional medical practitioners in the treatment of sores, bore and open wounds ⁴². The antimicrobial activity of the Cladosporium may be due to the production of the potent antibacterial benzene-type and diketopiperazine secondary metabolites, and hexaketides and 12-membered macrolides ¹⁰.

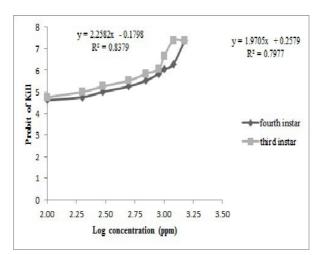
Table 1

Efficacy of selected fungal extracts against third and fourth instars of Aedes aegypti after 24 h of exposure

Fungi	Larval stage	LC ₅₀	LFL-UFL		LC ₉₀	LFL-UFL		y=mx+c		X ²
C.	Fourth	248.96	160.10	-	1435.3	783.96	-	Y=0.0474x	+	4.24
sphaerospermum	instar	240.90	337.82		1433.3	2086.64		34.635		4.24
	Third	182.90	165.51	-	1243.54	750.58	-	Y=0.0437x	+	4.58
	instar		329.26			1736.49		45.049		
C. macrocarpum	Fourth	196.72	104.22	-	1604.00	692.32	-	Y=0.0415x	+	0.56
	instar		290.90		1604.98	2517.65		41.027		9.56
	Third	116.36	58.84	-	EE7 04	381.33	-	Y=0.0334x	+	4.19
	instar		173.88	00.100	557.84	774.37		60.128		4.19

Graph 1

Probit regression line depicting relationship between probit of kill and log concentrations of Crude extract of C. sphaerospermum after 24 h exposure for the third and fourth instars of A. aegpyti.



Graph 2

Probit regression line depicting relationship between probit of kill and log concentration of Crude extract of C. macrocarpum after 24 h exposure for the third and fourth instars of A. aegpyti.

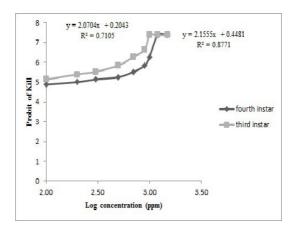


Table 2
Antimicrobial activity of *Cladosporium macrocarpum* crude extract

Test organism's	200µg	400µg	800µg	1200 µg	1600 µg	
Gram Positive Bacteria					Penicillin (G (100 μg)
Bacillus sphaericus	9.66 ± 0.33	11.16 ± 0.76	12.00 ± 0.00	13.83 ± 0.28	15.33 ± 0.33	18.83 ± 0.16
Staphylococus aureus	17.66 ± 0.16	20.83 ± 0.16	22.50 ± 0.28	24.50 ± 0.28	25.16 ± 0.16	21.33 ± 0.33
Staphylococcus epidermidis	9.50 ± 0.28	11.83 ± 0.16	12.66 ± 0.16	15.00 ±0.00	16.50 ± 0.28	22.83 ± 0.16
Gram Negative Bacteria					Streptomy	cin (100 µg)
Pseudomonas aeruginosa	13.00 ± 0.57	14.83 ± 0.16	16.66 ± 0.16	18.00 ± 0.57	19.33 ± 0.16	23.83 ± 0.16
Chromobaterium violaceum	NI	Ni	8.66 ± 0.33	10.50 ± 0.28	12.83 ± 0.16	20.66 ± 0.33
Klebisiella aerogenes	NI	Ni	8.33 ± 0.33	9.83 ± 0.16	11.16 ± 0.16	25.00 ± 0.29
Pseudomonas oleovarans	9.00 ± 0.57	10.83 ± 0.16	12.00 ± 0.00	13.00 ± 0.28	14.50 ± 0.28	24.33 ± 0.16
Escherichia coli	8.75 ± 0.25	10.83 ± 0.16	12.00 ± 0.28	12.83 ± 0.33	15.33 ± 0.33	28.33 ± 0.33
Unicellular Fungi					Amphotericin-E	3 (100µg)
Candida albicans	9.66 ± 0.33	12.00 ± 0.86	13.00 ± 0.00	15.50 ± 0.50	18.00 ± 0.00	23.33±0.44
Candida rugosa	15.00 ± 0.28	16.00 ± 0.57	18.00 ± 0.28	20.00 ± 0.00	21.00 ± 0.50	21.00 ± 0.28
Saccharomyces cerevisiae	7.83 ± 0.16	10.00 ± 0.86	12.00 ± 0.00	12.83 ± 0.28	15.00 ± 0.57	22.00 ± 0.57
Filamentous fungi						
Rhizopusoryzae	8.83 ± 0.33	10.00 ± 0.28	11.83 ± 0.44	13.00 ± 0.00	14.33 ± 0.66	23.83 ± 0.16
Chrysosporium tropicum	NI	8.66 ± 0.16	10.16 ± 0.16	11.50 ± 0.28	12.00 ± 0.00	23.00 ± 0.00
Aspergillus niger	9.83 ± 0.16	11.00 ± 0.28	12.83 ± 0.16	14.00 ± 0.50	15.00 ± 0.00	25.00 ± 0.57
Aspergillus flavus	NI	NI	NI	8.83 ± 0.16	10.00 ± 0.50	23.83 ± 0.16
Aspergillus parasiticus	8.66 ± 0.33	10.00 ± 0.00	11.00 ± 0.50	12.00 ± 0.57	13.33 ± 0.57	22.00 ± 0.50
Asperaillus terrus	NI	NI	8.83 ± 0.16	10.00 ± 0.28	10.66 ± 0.16	` 25.50 ± 0.28

Aspergillus terrus NI NI Negative Control: DMSO: No activity NI: No Inhibition

Table 3
Antimicrobial activity of *Cladosporium sphearospermum* crude extract

Test organism	250 µg	500 μg	1000 µg	1500 µg	2000 µg	
Gram Positive Organisms						Penicillin (100 µg)
Bacillus Subtitis	8.16 ± 0.44	8.67 ± 0.17	10.00 ± 0.57	14.00 ± 0.00	15.00 ± 0.50	19.87 ± 0.14
Staphylococcus aureus	11.83 ± 0.16	13.00 ± 0.76	15.00 ± 0.00	17.00 ± 0.00	19.00 ± 0.05	18.00 ± 0.28
Gram Negative Organisms						Streptomycin (100 µg)
Chromobacter violaceaum	8.50 ±0.29	12.00 ± 0.00	12.67 ± 0.16	14.00 ±0.29	15.00 ±0.57	27.80 ±0.15
Escherichia coli	NI	Ni	NI	NI	NI	28.16 ±0.44
Pseudomonas aeroginosa	7.83 ± 0.16	10.00 ± 0.29	12.00 ± 0.57	14.00 ±0.57	15.66 ± 0.66	33.90 ±0.05
Klebsiella pneumoniae	7.83 ± 0.16	10.66 ± 0.29	14.66 ±0.66	16.0 ± 0.00	18.00 ±0.57	29.86 ±0.18
Unicellular Fungi						
Candidia albicans	12.00 ± 0.50	13.00	15.00 ±0.57	17.00 ± 0.57	19.00 ± 0.12	19.83 ±0.08
Candida rugosa	12.00 ± 0.00	13.00 ± 0.00	15.00 ± 0.00	16.00 ±0.50	18.00 ± 0.00	20.60 ±0.23
Saccharomyces cerevisiae	10.00 ± 0.29	12.00 ± 0.29	17.00 ±0.58	21.00 ±1.00	27.00 ± 0.57	21.83 ± 0.16
Filamentous Fungi						
Aspergillus niger	8.50 ± 00	11.00 ± 0.58	15.00 ± 0.29	17.00 ± 0.50	18.00 ± 0.29	22.83 ±0.12
Aspergillus flavus	NI	8.93 ± 0.03	12.00 ± 0.00	14.00 ± 0.50	18.00 ± 0.00	23.70 ± 0.15
Rhizopus oryzae	7.83 ± 0.16	11.00 ± 0.50	15.00 ± 0.58	16.00 ± 0.57	18.00 ± 0.00	22.76 ± 0.14

Negative Control: DMSO: No activity NI: No Inhibition

Zone of Inhibition measured in (mm); ± = SE

CONCLUSION

Ethyl acetate extracts from *C. macrocarpum* and *C. sphaerospermum* appear to be promising larvicidal activity against *A. aegypti* mosquito larvae, and may signify a potential natural product for the control of mosquitoes. This is the first report on the mosquito larvicidal potential of *C. macrocarpum* and *C. sphaerospermum* against *A. aegypti* and thus indicating for further research on the biocontrol properties of this genus. Both the fungal extracts also showed potent antimicrobial activity against *S. aureus*, *P. aeroginosa*, *C. albicans*, *C. rugosa* those are known to cause skin lesions, wound infections and possible usage in therapeutic agents, especially topical medications. The

antimicrobial and mosquitocidal activities may be due to the mechanism of actions of a wide variety of compounds from these species warranting a further purification and characterization of the active compounds from these fungal species.

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