Madras Agric. J., 105 (1-3): 56-60, March 2018

Antifungal Potential of Myco-molecules of *Coprinopsis cinerea* (Schaeff) S. Gray s.lat. against *Fusarium* spp

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Inky cap mushroom (*Coprinus* sp) possesses vital antifungal properties against the major wilt causing pathogens of banana, tomato and chilli. Wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* in banana, *F. o*. f. sp*. lycopersici* in tomato and *F.brachygibbosum* in chilli were obtained and the antifungal activities of *Coprinus cinerea* was assessed. Methanolic extract from the fruiting bodies of inky cap *C. cinerea* consisted of molecules which could inhibit the growth of *Fusarium* spp at 2000 ppm. Results showed 54.5, 52.2 and 51.1 per cent of mycelia inhibition of *F. oxysporum* f. sp. *cubense*, *F. o*. f. sp*. lycopersici* and *F. brachygibbosum,* respectively when the methanolic extract of *C. cinerea* was used in agar wells in petri dishes. GC-MS analysis of the methanolic extract of the fruiting bodies of inky cap indicated the presence of 9 different compounds *viz*., formic acid, 2-propenyl ester; acetic acid; D-alanine; N-propargyloxycarbonyl-; isohexyl ester; 4H-Pyran-4-one; 2,3-dihydro-3,5-dihydroxy-6-methyl-; n-hexadecanoic acid; octasiloxane; 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-; 2,7-diphenyl-1,6-dioxopyridazino [4,5:2',3'] pyrrolo[4',5'-d]pyridazine; squalene and 2-Nonadecanone 2,4-dinitrophenylhydrazine. Among these compounds, formic acid, 2-propenyl ester, acetic acid; n-hexadecanoic acid; 2,7-diphenyl-1,6-dioxopyridazino [4,5:2',3'] pyrrolo[4',5'-d]pyridazine; squalene; 2-nonadecanone 2,4-dinitrophenylhydrazine are have been reported to possess antifungal activities.

Key words: Inky cap, *Coprinopsis cinerea*, GC-MS, Antifungal biomolecules and *Fusarium* spp

Coprinopsis cinerea (Schaeff) S. Gray s.lat. "Ink cap mushroom" belongs to the family *Psathyrellaceae* (Badalyan *et al.,* 2011), phylum Basidiomycota, is one of that most valued mushroom fungus by pharmaceutical industry. The inky caps are common saprophytic fungi growing on road side. They can also be sighted in places where high levels of ammonia and nitrogen, cellulose and xenobiotic waste materials are dumped. Many times they grow on mushroom beds as weed moulds, especially in oyster, paddy straw and milky mushroom beds when the substrate moisture is excess (Krishnamoorthy and Balan 2016). Rice straw enriched with more of nitrogenous supplements increase *Coprinus* spp (Park and Lee, 2005). A number of bioactive compounds obtained from *Coprinus* spp have been reported to possess several antimicrobial, antinemic, antitumor, anticancer, anti-hypoglycemic, antioxidant and immunomodulatory activities. Yuanzhen *et al*. (2012) reported that the *Coprinus* spp produced several bioactive molecules *viz.,* coprinol, coprinolone, coprinastatin, coprinacins, xanthothone, lagopodins, illudins and armillol. In the present study, the bioactive compounds of fruiting bodies of *C. cinerea* at post capping stage were characterized by GCMS in methanol and the concoction of the biomolecules was tested against the wilt disease causing pathogens *F. brachygibbosum*, *F. o.* f. sp. *cubense* and *F. o.* f. sp. *lycopersici* under *in vitro* condition.

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Material and Methods

Collection and identification of Coprinopsis cinerea

Coprinopsis cinerea fruiting bodies were collected from the contaminated oyster mushroom beds at Mushroom Research Laboratory, Department of plant pathology, TNAU, Coimbatore. The study area fall under the geographical co - ordinates of 11.0 °N**,** 76.9°E and 411.2 MSL**.** For the purpose of tissue isolation, the fruiting bodies of wild isolate was cut into small pieces, surface sterilized with one per cent (w/v) sodium hypochlorite for 60 sec. The tissue bits were washed twice with sterile distilled water for 60 sec and placed in sterile Petri dishes containing 20 mL of PDA. The plates were incubated at 30ºC for 6 d. Following single hyphal tip method (Rangaswamy, 1972), pure cultures were transferred to PDA slants and stored at refrigerated condition.

Molecular characterization of C. cinerea

Genomic DNA was extracted from suspension cultures of *C. cinerea* by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Martins, 2004; Guimaraes *et al*., 2011). The internal transcribed spacers (ITS) region containing partial portions of the small subunit rRNA (18S), both internal transcribed spacers (ITS1 and 4) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS1 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). PCR reactions were carried out

on a Thermal Cycler (BIO-RAD) and consisted of initial denaturing at 94ºC for 2 min followed by 37 cycles at 94ºC for 30 sec., 58ºC for 30 sec. and 72ºC for one min. The reaction was completed by a 4 min extension at 72ºC. The PCR-amplified products (10 µL) were separated by electrophoresis (Genei Maxi Sub System 03-04, Genei, Bangalore, India) on 1.2 per cent agarose gel using TAE buffer at 80 V constant current for 1h. The gel was stained with ethidium bromide and visualized with gel documentation system (UVITEC, Cambridge, UK). The sizes of the PCR products were determined in comparison with standard 100 bp molecular marker (Genei Pvt. Ltd., Bangalore, India).

Extraction and testing of antifungal biomolecules against Fusarium spp

The bioactive compounds were extracted from the fruiting bodies at post capping stage, utilizing different solvents *viz.,* methanol, ethanol, chloroform, petroleum ether and aqueous phase. Ten grams of freshly picked mushrooms were crushed in a pestle and motor and soaked in 100 mL of specific solvents used and kept in a shaker at 120 rpm for 12 hours. Extract was filtered through a double layered muslin cloth followed by Whatman No. 1 filter paper. The extracts, collected were evaporated under reduced pressure, using a rotary evaporator to obtain concentrated fraction. This was further dried in an already weighed sterile Petri plate in the controlled environment and the per cent recovery of substance was calculated in terms of w/v and dissolved in methanol (mg/mL). The contents were filtered through a membrane filter (0.2µm) and stored at 4°C for further study. Antimicrobial activity of this concoction was evaluated against *F. o.* f. sp. *brachygibbosum*, *F. o.* f. sp. *cubense* and *F. o.* f. sp. *lycopersici* by agar well diffusion method (Stoke and Ridgway, 1980).

After solidification of the sterile PDA medium in Petri dishes, wells of 5 mm in diameter were made on each of the plate using sterile cork borer on all four sides, giving equal distance and also by leaving one cm space from the periphery. Fruiting body fractions were poured separately into the wells at the rate of 1000 ppm per well using a micro pipette. Actively growing ten days old mycelial discs of pathogenic fungi measuring 5 mm in diameter were inoculated separately, at the centre of each of the Petri dish and incubated at 28 ± 2˚C for seven days. Constant observations were made and the per cent growth inhibition of *F. o.* f. sp. *lycopersici*, *F. o.* f. sp. *cubense* and *F. brachygibbosum* were recorded. Based on the result, the effective solvents were used at four different concentrations (500, 1000, 1500 and 2000 ppm) and tested against *Fusarium* spp.

Detection of bioactive molecules of fruiting bodies of C. cinerea by GC - MS analysis

Characterization of biomolecules of fruiting body condensate of *C. cinerea* (Methanolic fraction) was done by GC – MS analysis. In this study, the trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited was engaged for analysis. The instrument was set as follows Injector port temperature set to 250°C, Interface temperature set as 250°C, source kept at 200°C. The oven temperature was programmed as available, 70°C for 2 min, 150°C @ 8°C /min. up to 260°C @ 10°C / min. Split ratio was set as 1:50 and the injector used was in splitless mode. The DB-35 MS non polar column was used, whose dimensions were 0.25 mm OD x0.25 µm ID x 30 meters length procured from Agilent Co., USA. Helium was used as the carrier gas at one mL /min. The MS was set to scan from 50 to 650 Da. The source was maintained at 200°C and < 40 motor vacuum pressure. The ionization energy was -70eV. The MS was also having inbuilt pre-filter, which reduced the neutral particles. The data system had two inbuilt libraries for searching and matching the spectrum. NIST4 and WILEY9 each contain more than five million references. Those compounds with spectral fit values equal to or greater than 700 were considered positive identification. Based on MS data library and comparing the spectrum obtained through GC – MS chromatogram, the compounds present in the sample were identified.

Results and Discussion

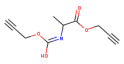
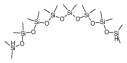
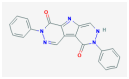
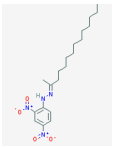
Identification of the fungus - Molecular characterization

The rDNA fragment of locally collected *C. cinerea* was subjected to PCR amplification. ITS-rDNA region of the fungus comprising two non-coding internal transcribed spacers ITS 1 and ITS 4 and 5.8S-rDNA gene was amplified using general primers ITS 1 and ITS 4. The amplified product size measured 600 - 650 bp (Plate1B). Further, the purified PCR product was subjected to partial sequence and nucleotide BLAST (Blastn) analysis was performed in the NCBI. The results revealed that, partial sequence of the DNA region had exhibited 96 per cent homology with *Coprinopsis cinerea* (Nasehi and Javan-Nikkhah) isolates in the GenBank database (MF 161089). Based on the nucleotide BLAST results of ITS-rDNA sequences, the identity of local isolate was confirmed as *Coprinopsis cinerea.* The DNA sequences were registered in NCBI GenBank database with an accession no. MH444367.

Detection of bioactive compounds by GC - MS analysis

Bioactive compounds extracted from the fruiting bodies at post capping stage in methanolic fraction are listed in Table 1. GC – MS analysis of methanolic fraction of fruiting body indicated the presence of 9 different compounds *viz.*, formic acid, 2-propenyl ester (4.06 RT); acetic acid (4.28 RT); D-alanine, N-propargyloxycarbonyl-, isohexyl ester (5.28 RT); 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (6.35 RT); n-hexadecanoic acid (21.18 RT); octasiloxane, 1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (31.39); 2,7-Diphenyl-1,6-d ioxopyridazino[4,5:2',3'] pyrrolo[4',5'-d]pyridazine (33.01 RT); squalene

Table 1. GC-MS analysis of methanolic fraction of fruiting bodies at post capping extract of *C. cinerea*

RT	Compounds	Molecular formula	Molecular weight	Structure	Area %
4.06	Formic acid, 2-propenyl ester	C4H6O2	86.09 g/mol		1.51
4.28	Acitic acid	CH3COOH	60.05 g/mol		1.54
5.28	D-Alanine, N-propargyloxycarbonyl-, isohexyl ester	C13H21NO4	222.37 g/mol		0.82
6.35	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C6H8O4	144.12 g/mol		1.17
21.18	n-Hexadecanoic acid	C16H32O2	257.42 g/mol		2.17
31.39	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15- hexadecamethyl-	C16H48O7Si8	577.23 g/mol		0.82
33.01	2,7-Diphenyl-1,6-dioxopyridazino[4,5:2',3'] pyrrolo[4',5'-d]pyridazine	C20H13N5O2	355.34 g/mol		1.50
34.24	Squalene	C30H50	410.73 g/mol		2.48
35.03	2-Nonadecanone 2,4-dinitrophenylhydrazine	C25H42N4O4	462.63 g/mol		0.75

RT – Retention time

(34.24); 2-nonadecanone 2,4-dinitrophenylhydrazine (35.03). Among the 9 different compounds, formic acid (Şehirli *et al*., 2016), 2-propenyl ester, acetic acid (Sercan *et al*., 2016) and squalene (Godio *et al,*. 2007) are known to possess antifungal activities. In the present experiment

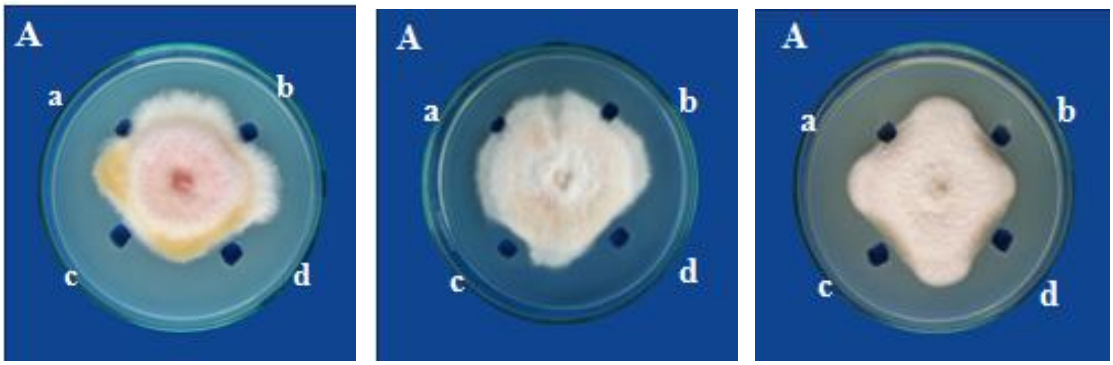
Table 2. Optimization of inhibitory concentration of methanolic fractions of fruiting bodies at post capping stage condensate of *C. cinerea* against *Fusarium* spp

Solvent	Concentration (ppm)	*F. brachygibbosum*		*F. o.* f. sp. *cubense*		*F. o.* f. sp. *lycopersici*	
		Growth (mm)	PI	Growth (mm)	PI	Growth (mm)	PI
Methanolic fraction	500	70.00^{d} (56.20)	22.22	69.00^{d} (56.03)	23.33	72.00^{d} (57.96)	20.00
	1000	62.00^{c} (52.18)	31.11	60.00^{c} (50.68)	33.33	63.00^{c} (52.62)	30.00
	1500	53.00^{b} (46.87)	41.11	50.00^{b} (45.04)	44.44	56.00^{b} (48.86)	37.78
	2000	44.00^{a} (41.52)	51.11	41.00^{a} (39.78)	54.44	43.00^{a} (40.99)	52.22
Control (Water)		90.00^{e} (71.57)	0.00	90.00^{e} (71.57)	0.00	90.00^{e} (71.57)	0.00
CD (p=0.05)	-	0.48	-	1.82	-	1.26	-

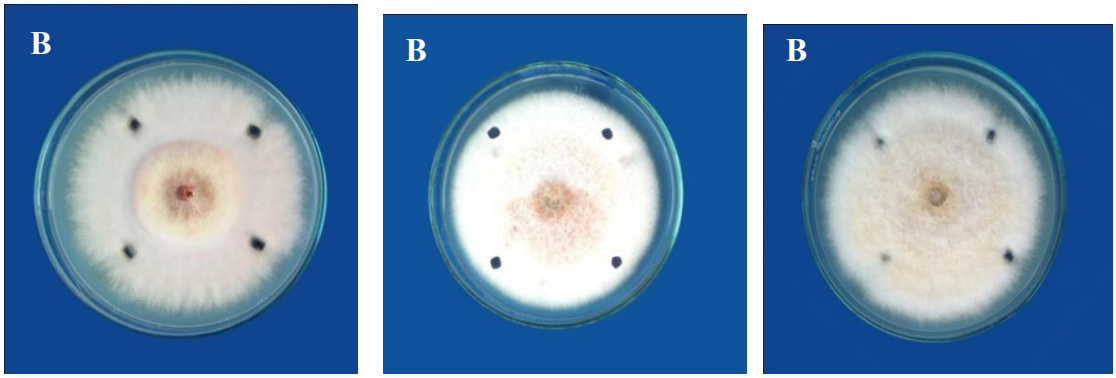
PI - Per cent inhibition

Values are mean of three replications.

F. brachygibbosum *F. o.* f. sp. *cubense* *F. o.* f. sp. *lycopersici*



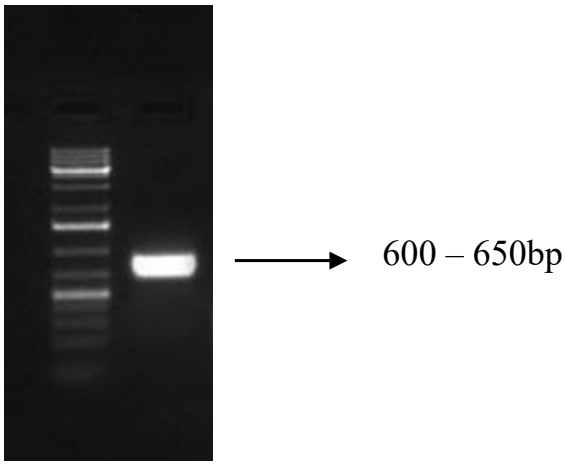
A) Treated plates



B) Untreated plates

C) Note : a, b, c, d – 500, 1000, 1500 and 2000ppm

Plate 1. A. Inhibitory effect of methanolic fractions of fruiting bodies at post capping stage of *C. cinerea* against plant pathogens



1.B. PCR amplification and ITS region of *Coprinus* spp

Means followed by a common letter is not significantly different by one way ANOVA the concoction containing the biomolecules of fruiting bodies of C. *cinerea* at 2000 ppm showed inhibition of F. *brachygibbosum* (51.11 per cent) F. o. f. sp. *cubense* (54.44 per cent) and F. o. f. sp. *lycopersici* (52.22 per cent) (Fig1; Plate1A; and Table 2). Formic acid, 2-propenyl ester and acetic acid are the organic compounds as well as a type of organic acids. These formic and acetic acids were

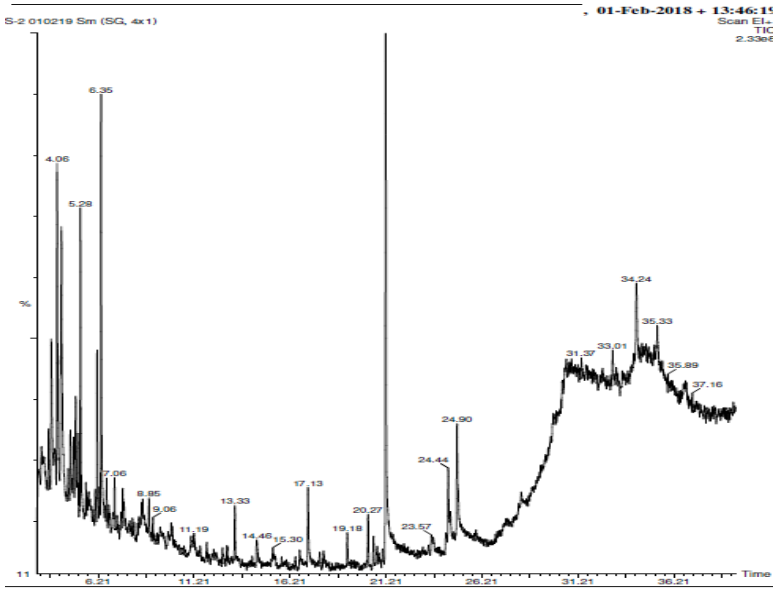


Fig 1. GC-MS analysis of methanolic extract of fruting bodies of *C. cinerea*

reported as an antimicrobial activity and can affect the survival of soil borne pathogens like *Fusarium oxysporum*, *Fusarium solani,* F. o.f. sp. *lycopersici* and bacteria like *Ralstonia solanacearum*. Squalene also identified from the fruiting bodies extract of C. *cinerea* with the peak area per cent of 2.48. Godio *et al*. (2007) working with *Agaricus bisporus* squalene epoxidase indicated that clavaric acid formation during the biosynthesis in the ergosterol biosynthesis of *Apergillus fumigates* which exhibited antifungal activity.

Conclusion

The results of the current study indicated the possibility of extraction of antifungal compounds from the inky cap fungus *C. cinerea* against wilt disease causing *Fusarium* spp. The methanolic extract of the fungus could inhibit the *F. brachygibbosum, F. o.* f. sp. *cubense* and *F. o*. f. sp. *lycopersici* upto 54 per cent.

Acknowledgement

The authors express their thanks and gratitude to UGC-SAP-DRS1 and DST –FIST-GOT Schemes operated in the Department of Plant Pathology, TNAU, Coimbatore for the instrumentation and laboratory support.

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Received : March 01, 2018; Revised : March 15, 2018; Accepted : March 28, 2018