Madras Agric. J., 102 (10-12): 353-357, December 2015
https://doi.org/10.29321/MAJ.10.001133

Antifungal Bioactive Compounds from Chinese Caterpillar Fungus (*Ophiocordyceps sinensis* (Berk.) G. H. Sung *et al*.) against Plant Pathogens

C. Sangeetha[1], A.S. Krishnamoorthy[1] and D. Amirtham[2]
[1]Department of Plant Pathology
[2]Department of Biochemistry
Tamil Nadu Agricultural University, Coimbatore - 641 003, India.

Studies were conducted to extract and evaluate the intracellular bioactive molecules of *Ophiocordyceps sinensis* against plant pathogens. Methanolic fraction of mycelium borne bioactive molecules at 200 µL concentration showed the maximum mycelial inhibition of *Fusarium oxysporum* f. sp. *cubense* (48.67 per cent) and *F. o.* f. sp. *lycopersici* (46.47 per cent), which cause wilt disease in banana and tomato, respectively. GC-MS analysis of methanolic fraction of mycelial mat extract indicated the presence of 14 different compounds hydroxylamine; glycerin; oxime-,methoxy-phenyl; adipamide; 1,2,3-Benzenetriol; cyclododecane; imidazole-5-carboxylic acid; 1,3,5-Benzenetriol; 1,3-Cyclohexanedione, 2-propyl; 2-Propenoic acid, tridecyl ester; n-Decanoic acid; 5-Undecene, 9-methyl; L-Ornithine, N5-(aminocarbonyl) and pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro. Among these, the compound hydroxylamine identified at 1.817 RT exhibited the maximum peak area of 35.00 per cent. This compound is also known to possess important pharmacological and antimicrobial properties.

Key words: *O. sinensis*, Antifungal bio-molecules, TLC and GC-MS

Ophiocordyceps sinensis (Berk) G. H. Sung *et al*., is an entomopathogenic fungus belonging to *Ascomycota*, *Sordariomycetidae*, *Hypocreales* and *Ophiocordycepitaceae* (Sung *et al.*, 2007). Sporophores of this fungus are highly valued medicinal mushrooms around the world (Borchers *et al*., 2004 and Zaidman *et al*., 2005). The name *Cordyceps* comes from the two Latin words cord and ceps, meaning club and head. The fungus is known to parasitize the larvae of ghost moth, *Hepialus armoricanus* (Pegler *et al.*, 1994; Wang, 1995 and Yao, 2004). The erstwhile known anamorphic state(s) of the genus *Cordyceps viz*., *Beauveria, Metarhizium* and *Paecilomyces* have been reported to kill nematodes and insect pests by direct parasitism or lysis (St. Leger *et al.,* 1992; Gillespie *et al.,* 1998; De-faria and Wraight, 2007). *Cordyceps* species are parasitic rather specifically on insects, nematodes, sclerotia of *Claviceps*, or hypogeous ascocarps (Jeffries and young, 1994). *Ophiocordyceps* spp are known to produce several novel bioactive compounds like cordycepin, cordymin, adenosine, cordycepic acid, amino acid, ergosterol, sterol, superoxide dismutase, myriocin and multivitamins (Isaka *et al*., 2000; Li *et al*., 2010; Varshney *et al*., 2011; Kumar and Spandana, 2013). In the present study, bioactive compounds from mycelial mat of *O. sinensis* extracted with methanol were characterized by TLC and GC-MS. The combined action of these compounds has also been tested for their efficacy against the root rot, wilt and sheath blight pathogens.

*Corresponding author email : milkmush@rediffmail.com

Materials and Methods

O. sinensis isolate No.1220 was obtained from Forest Research Institute (FRI), Dehradun, India. This fungus was grown on Mushroom Complete Medium (MCM) in 90 mm diameter Petri dishes and incubated at 25°C.

Extraction of bio-active compounds

Four mycelial discs measuring 6 mm diameter each, cut from the margin of a 5 days old colony of *O. sinensis* were inoculated in 250 mL conical flasks containing 100 mL of sterilized MC broth (pH 5.5). The flasks were placed on a rotary shaker maintained at 120 rpm and incubated at 25°C in diffused day light (600 - 800 lux) for 15 days. After incubation, the culture filtrate and the mycelial mat were separated by filtration through Whatman No.40 filter paper. Five gram of mycelial mat was powdered with liquid nitrogen and extracted three times with equal volume of methanol. The extracted sample taken in the Eppendorf tubes was centrifuged at 10,000 rpm for 15 min in a bench centrifuge (at 4°C) and the supernatants were evaporated to dryness, dissolved in methanol and stored at 4°C.

Detection of bioactive compounds

Thin layer chromatography (TLC)

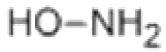
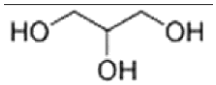
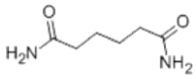
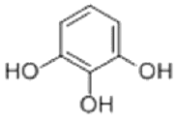
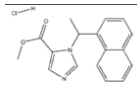
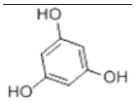
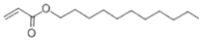
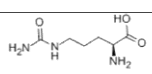
Bioactive compounds of mycelial mat extract were spotted on to silica gel 60 TLC plates (60 F254, 0.12 mm thick, 5×20 cm, Merck, Germany) at the rate of 5 µL /spot. After drying, chromatographs were developed using the solvent system, butanol: water (86:14). The developed TLC plates were air dried overnight to remove the remaining solvents and viewed under UV light at 250 nm. The retention factor (R*f*) values of various compounds resolved on TLC plates were calculated using the formula given by Sadasivam and Manickam (1992).

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

GC - MS analysis

Characterization of biomolecules of methanolic fraction of mycelial mat extract was done by GC – MS analysis. The column Elite-5MS (100 per cent

Table 1. GC-MS analysis of methanolic fraction of mycelial biomass (15 d old) of *O.sinensis*

RT	Compounds	Structure	Molecular formula	MW	Peak area Per cent
1.817	Hydroxylamine		H_3NO	33	35.00
11.09	Glycerin		$C_3H_8O_3$	92	10.01
12.200	Oxime-, methoxy-phenyl	-	-	-	0.60
13.830	Adipamide		$C_6H_{12}N_2O_2$	144	1.40
18.180	1,2,3-Benzenetriol		$C_6H_6O_3$	126	8.51
19.382	Cyclododecane		$C_{12}H_{24}$	168	1.33
21.835	Imidazole-5-carboxylic acid		$C_{17}H_{17}ClN_2O_2$	316.7	0.81
22.945	1,3,5-Benzenetriol		$C_6H_6O_3$	126	3.00
23.407	1,3-Cyclohexanedione, 2-propyl	-	-	-	1.91
23.622	2-Propenoic acid, tridecyl ester		$C_{16}H_{30}O_2$	254	1.09
24.243	n-Decanoic acid		$C_{10}H_{20}O_2$	172.3	3.42
24.545	5-Undecene, 9-methyl	-	-	-	1.14
25.079	L-Ornithine, N5-(aminocarbonyl)		$C_6H_{13}N_3O_3$	175.2	1.24
25.591	Pyrrolo[1,2a]pyrazine-1,4-dione,hexahydro		$C_7H_{10}N_2O_2$	154.17	4.57

RT – Retention time, MW – Molecular weight

Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 μm df equipped with GC clarus 500 Perkin Elmer and turbo mass - gold -Perkin - Elmer detector was used. The carrier gas (helium) flow rate was one mL per min, split 10:1 and injected volumes were 2 μL. The column temperature was maintained initially at 110°C at the rate of 10°C /min followed by increasing upto 280°C at the rate of 5°C /min and hold time 9 min. The injector temperature was 250 °C and this temperature was held constant for 36 min. The electron impact energy was 70 e V, Julet line temperature was set at 2000 °C and the source temperature was set at 200°C. Electron impact (EI) mass scan (m /z) was recorded in the range of 45-450 aMU. Using computer searches on the NIST version 2011 MS data library and comparing the spectrum obtained through GC- MS, the compounds present in the sample were identified.

Bioassay of mycelium borne bioactive compounds

The concoction of mycelium borne bioactive compounds of *O. sinensis* was evaluated against *F. o.* f. sp. *cubense* and *F. o.* f. sp. *lycopersici* causing wilt disease in banana and tomato, respectively; *Macrophomina phaseolina* causing root rot in red gram and *Rhizoctonia solani* causing sheath blight in rice by agar well diffusion method (Stoke and Ridgway, 1980). After solidification of the sterile PDA medium in Petri plates, wells of 10 mm 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 of Petri plates. Secondary metabolites composite in methanolic fraction was poured separately, at the rate of 50, 100, 150 and 200 μL per well using a micro pipette. Actively growing five d old mycelial discs of pathogenic fungi measuring 6 mm in diameter were inoculated separately, at the centre of each of the Petri plate and incubated at 28 ± 2ÚC for seven days. The radial growth of mycelium (mm) and zone of inhibition (mm) were recorded after 7 days of incubation. Based on the observation, per cent inhibition over control was calculated. Each dose was replicated three times. Sterile water served as control.

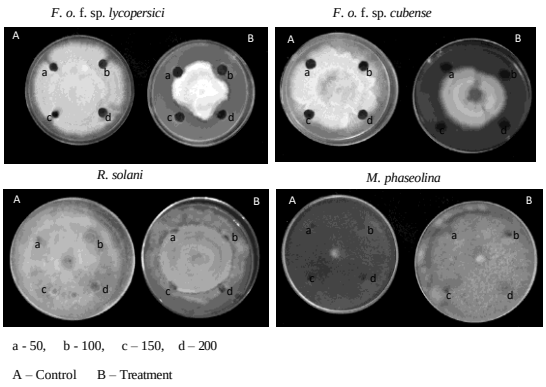
Results and Discussion

The results showed that the methanolic fraction of mycelial mat of *O. sinensis* was highly inhibitory to *F. o*. f. sp*. lycopersici* and *F. o.* f. sp*. cubense* at all concentrations tested, when compared to control (Table 2). At high concentration (200 µL), *F. o.* f. sp*. cubense, F. o*. f. sp*. lycopersici* and *R. solani* exhibited 48.67, 46.47 and 22.24 per cent inhibition, respectively. But, the methanolic fraction is ineffective in reducing the mycelial growth of *Macrophomina phaseolina*. The overall results of bioassay experiment revealed that the wilt pathogens (*Fusarium* spp) are more sensitive when compared to root rot and sheath blight pathogens (Plate 1). Sekaran *et al.* (2011) had evaluated the antifungal activity of methanolic and aqueous extract of *Ganoderma lucidum* and reported inhibition of mycelial growth of five fungal pathogens including *Aspergillus fumigatus* and *Penicillium* sp*.* Ragul (2013) reported that methanolic extract of *Calocybe indica* and *Pleurotus florida* sporophores had effectively inhibited soil borne plant pathogens at a concentration of 150 μL, when tested by agar well diffusion technique.

Table 2. Bioassay of intracellular compounds of *O. sinensis* against plant pathogens

Concentration of bioactive molecules (μL)		*F.o.*f. sp*. lycopersici*		*F.o.*f. sp.*cubense*		*M. phaseolina*		*R. solani*	
		Growth(mm)	PI	Growth(mm)	PI	Growth(mm)	PI	Growth(mm)	PI
Methanol fraction	50	60.27^{c}	33.03	63.05^{c}	29.95	90.00	0.00	87.63^{c}	2.63
	100	54.52^{b}	39.42	51.15^{b}	43.17	90.00	0.00	80.69^{b}	10.34
	150	48.19^{a}	46.46	46.79^{a}	48.01	90.00	0.00	70.98^{a}	21.14
	200	48.17^{a}	46.47	46.19^{a}	48.67	90.00	0.00	69.99^{a}	22.24
Control (water)	50	90.00^{d}	0.00	90.00^{d}	0.00	90.00	0.00	90.00^{d}	0.00
	100	90.00^{d}	0.00	90.00^{d}	0.00	90.00	0.00	90.00^{d}	0.00
	150	90.00^{d}	0.00	90.00^{d}	0.00	90.00	0.00	90.00^{d}	0.00
	200	90.00^{d}	0.00	90.00^{d}	0.00	90.00	0.00	90.00^{d}	0.00
CD (p=0.05)	-	1.04	-	0.75	-	-	-	1.07	-

PI - Per cent Inhibition ; Values are mean of five replications ; Means followed by a common letter is not significantly different by one way ANOVA



a - 50, b - 100, c – 150, d – 200

A – Control B – Treatment

Plate 1. Inhibitory effect of mycelium borne compound of *O. sinensis* against plant pathogens

Detection and characterization of bioactive molecules

Bioactive molecules extracted from mycelial mat of *O. sinensis* were identified by TLC and GC-MS. TLC results exhibited four different compounds (R*f* - 0.12, 0.35, 0.39 and 0.76) as shown in Fig.1. GC-MS analysis indicated the presence of 14 different compounds (Table 1 and Fig. 2) *viz.,* hydroxylamine; glycerin; oxime-,methoxy-phenyl; adipamide; 1,2,3-Benzenetriol; cyclododecane;

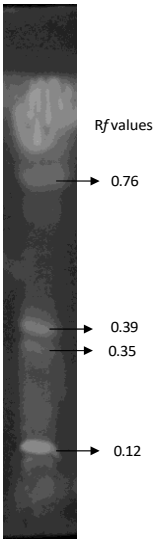


Fig 1. Bioactive molecules of mycelial mat extract of *O. sinensis* detected by TLC

imidazole-5-carboxylic acid; 1,3,5-Benzenetriol; 1,3-Cyclohexanedione, 2-propyl; 2-Propenoic acid, tridecyl ester; n-Decanoic acid; 5-Undecene, 9-methyl; L-Ornithine, N5- (aminocarbonyl) and pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro. The compound hydroxylamine identified at 1.817 RT exhibited the maximum peak area of 35.00 per cent. Several heterocyclic compounds (diazepines, imidazoles and thiadiazole), alpha substituted hydroxlamine derivatives and related compounds are known to possess important pharmacological properties and antimicrobial properties (Baregama *et al*., 2004). Presence of glycerine (10.01 per cent expressed at 11.09 RT) in the CFC filtrate and mycelial preparations of *O. sinensis* would hypothetically indicate the antibacterial nature of the fungus as rightly pointed out by Kern (1971), which could be used as a form of preservative in bio-extract preparations. Another compound namely, 1, 2, 3-benzenetriol and its derivatives 1, 3, 5-benzenetriol pyrogallol compound (8.51 and 3.00 per cent, respectively) detected at 18.18 and 22.94 RT (Table 1) are aromatic alcoholic compounds known for fungicidal, insecticidal, antioxidant and antiseptic activities (Euginamala and Jeyaraj, 2014). In addition, Sangeetha *et al*. (2015a and b) reported that the bioactive molecules present cell free culture (CFC) filtrates and mycelium of *Ophiocordyceps sinensis* could be potentially explored for the management of root knot nematode, *Meloidogyne incognita* and *Fusarium* spp.

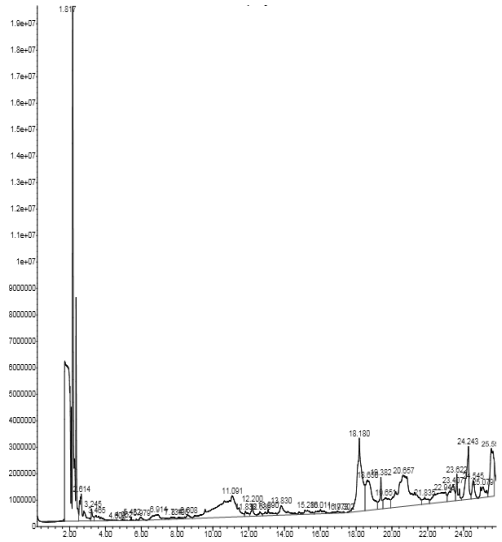


Fig 2. GC-MS analysis of methanolic extract of mycelial mat of *O. sinensis*

Conclusion

The results of the current investigation conclusively indicate that the bioactive compounds of *O. sinensis* could be potentially explored to contain *Fusarium* spp. which cause wilt disease in crop plants. The results also pave way to explore hitherto unutilized biomolecules of *O. sinensis* in a more potential way against plant pathogens. Further systematic insight into individual molecules and developing step up protocols to enhance their level of production and innovative formulation will have a greater stake hold in agrochemical industry.

Acknowledgement

The authors express their gratitude to FRI, Dehradun and Department of Plant Pathology, TNAU, Coimbatore for the valuable support rendered during the course of investigation.

References

Baregama, L., Ahmed, M., Dak, G., Sharma, K. and Talesara, G. L. 2004. Evaluation of the antimicrobial activity of some novel alpha substituted hydroxylamine derivatives. *Indian J Pharmacol.,* **36**(5): 312-320.

Borchers, A. T., Keen, C. L., Gershwin, M. E. 2004. Mushroom, tumor, and immunity: an update. *Exp Biol Med.*, **229**: 393 – 406

De-faria, M. R. and Wraight, S. P. 2007. Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Bio Control,* **43**: 237-256.

Euginamala, V. and Jeyaraj, M. 2014. Determination of antibacterial, antifungal, bioactive constituents of triphala by FT-IR and GC-MS analysis. *Int. J. Pharm. Pharm. Sci.,* **6**: 1- 4.

Gillespie, J. P., Bateman, R. and Charnley, A. K. 1998. Role of cuticle degrading proteases in the virulence of *Metarhizium* spp for the desert locust, *Schistocerca gregaria*. *J. Invertebr. Pathol.,* **71**: 128-137.

Isaka, M., Punya, J., Lertwerawat, Y., Tanticharoen, M. and Theptaranonth. Y. 2000. Antiplasmodial compounds from wood decay fungus *Xylaria* BCC 1067. *Plant Medicine J.,* **66**: 473 - 475.

Jeffries, P. and Young, T. W. K. 1994. *Interfungal Parasitic Relationships*. CAB International, Wallingford, United Kingdom, 296p.

Kern, J. C.1971. Sterility testing and antimicrobial activity of commercial grade glycerine. Final report, University of Massachusetts Amherst, Massachusetts. p. 1-27.

Kumar, H. and Spandana, M. 2013. Simultaneous extraction, determination and analysis of adenosine, cordycepin and other derivatives of *Cordyceps sinensis* of Nepal by new validated HPLC method. *J. Pharmacolognosy and Phytochem.*, **2**(4): 43-45.

Li, H. C., Sun, P. and Feng, C. Q. 2010. The research of cordycepin as an active component in *Cordyceps*," *J. Nat. Sci*., **31**(2): 93–96.

Pegler, D. N., Yao, Y.J. and Li, Y. 1994.The Chinese'Caterpillar Fungus'. *Mycologist*, **8** (1): 3-5.

Ragul, M. 2013. Exploration of antimicrobial potentials of fungal chitosan and secondary metabolites against soil borne plant pathogens. M.Sc (Ag.) Thesis, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, p. 83.

Sadasivam, S. and Manickam, A. 1992. *Biochemical methods for Agricultural Science.* Wiley Eastern Ltd., Tamil Nadu Agricultural University, Coimbatore, India. pp. 139.

Sangeetha, C., Krishnamoorthy, A.S. and Ramakrishnan, S. 2015a. Testing bioactive compounds of Chinese caterpillar fungus, *Ophiocordyceps* spp against root knot nematode (*Meloidogyne incognita*). Research Journal of Agricultural Sciences, **6**(5): 1129-1133.

Sangeetha, C.**,** Krishnamoorthy, A.S.**,** Nakkeeran, S., Ramakrishnan, S. and Amirtham, D. 2015b. Evaluation of bioactive compounds of *Ophiocordyceps sinensis* [berk.] sacc. against *Fusarium* spp. *Biochem. Cell. Arch.,* **15**(1): 431-435.

Sekaran, S., Elumalai, T., Ramalingam, B. and Devendiran, K. 2011. Evaluation of antibacterial and antifungal activity of *Ganoderma lucidum* (curtis) p. Karst fruit bodies extracts. *World J. Sci. Technol.,* **1**(6): 8-11.

St. Leger, R. J., Frank, D. C., Roberts, D. W. and Staples, R. C. 1992. Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur. J. Biochem*., **204**: 991-1001.

Stoke, J. E. and Ridgway, G. L. 1980. Clinical Bacteriology. Edward Arnold Ltd. London.

Sung, G. H., Hywel-Jones, N. L., Sung, J. M., Luangsa-ard, J. J., Shrestha, B. and Spatafora, J. W. 2007. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud. Mycol.,* **57**: 5-59.

Varshney, V. K., Pandey, A., Kumar, A., Rathod., D. and Kannoji, P. 2011. Chemical screening and identification of ordycepin cultured isolate(s) of medicinal chinese caterpillar mushroom, *Ophiocordyceps sinesis* (Berk.). *Int. J. Med. Mushrooms*, **13**(4): 327-333.

Wang, G. D. 1995. *Cordyceps* spp: *Ecology, Cultivation and Application.* Science and Technology Reference Press, Beijing, 307 pp.

Yao, Y. J. 2004. Conservation and rational use of the natural resources of Cordyceps sinensis. *Science News*, **15**: 28 –29.

Zaidman, B. Z., Yassin, M., Mahajna, J., Wasser, S. P. 2005. Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Appl Microbiol Biotechnol*., **67**:453–468.

Received after revision: December 23, 2015; Accepted: December 28, 2015