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Inhibitory potential of ethyl acetate extract from mushrooms against root-knot nematode (*Meloidogyne incognita*)

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Abstract
The present study focused on the nematicidal activities of bio-molecules extracted from mushrooms against *M. incognita*. The experimental results revealed that the highest hatching inhibition and the juvenile mortality were recorded with the ethyl acetate fraction of cell-free culture (CFC) filtrate of *Ganoderma lucidum* followed by *Lentinus edodes*. At 1000 ppm concentration, the bio-active molecules of *G. lucidum* exhibited the maximum inhibition of egg hatching (92.6%) and juvenile mortality (93.2%) of *M. incognita* at 72 hours of incubation. GC –MS analysis of *G. lucidum* revealed the presence of 23 compounds *viz*. ., Octadecane,3-ethyl-5-(2-ethyl butyl), Decane, Undecane, 3,7-dimethyl-, Dihydroartemisinin, Benzaldehyde, 3,4-dimethyl-, Heptadecane, 2,6,10,15-tetramethyl-, 2,4-Di-tert-butyl-phenol, 1-Propanamine, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-,1,2-Benzenedicarboxylic acid, butyl octyl ester, n-Hexadecanoic acid, Dibutyl phthalate, 2,2,4-Trimethyl-3-(3,8,12,16-tetramethylheptadeca-3,7,11,15-tetraenyl)-cyclohexanol, Methyl stearate, Cyclohexane, Octadecanoic acid, Deoxyspergualin, Methyl glycocholate, 3TMS derivative, 1,25-dihydroxyvitamin D3, TMS derivative, Spirost-8-en-11-one, 3-hydroxy-, 1H-Indene, 1-hexadecyl-2,3-dihydro- and Bis(2-Ethylhexyl) phthalate. Among these, 2, 4-Di-tert-butyl-phenol, n-Hexadecanoic acid and Dibutyl phthalate might have been responsible for antinemic activity.

Keywords: *G. lucidum*, biomolecules, cell-free culture filtrate, GC-MS and antinemic activity

Introduction
Root-knot nematodes, *Meloidogyne* spp. constitute one of the major important groups of plant-parasitic nematodes posing a major threat in the cultivation of agricultural and horticultural crops. Root-knot nematodes are sedentary endoparasites and occupy the first position of the top ten plant-parasitic nematodes occurring across the world [11, 2] and their parasitic lifecycle is found to induce feeding sites in the roots of host plants [20].There are more than 100 species of *Meloidogyne* spp. dispersed worldwide and they parasitize both cultivated and uncultivated plants [13]. The four important *Meloidogyne* species *viz., Meloidogyne incognita, M. javanica, M. arenaria, and M. hapla* are economically important and are responsible for 95% of the infestations in cultivated lands [6, 24]. The most favorable condition for survival and multiplication of nematodes are in tropical climates than in temperate climates [4, 12]. The root-knot nematode is soil-borne and the infection starts with the penetration of juveniles (J2) present in soil and modifies the vascular tissues [10, 15, 18]. Infested plants show reduced growth, discoloration of leaves, and wilting due to uptake nutrient partitioning alterations and limited water uptake due to deformations of conducting vessels [12]. The management of plant-parasitic nematodes by biological methods has been very much realized in recent years [1, 7]. Perusal literature showed that mushroom fungi are important as natural sources of medicines and possess several bioactive compounds *viz.*, antibacterial, antifungal, antioxidant, antiviral, anti-nemic, and anti-tumor activity [9, 28, 14, 21, 22]. Owing to the current emphasis on the eco-friendly approaches for plant disease management, mushroom fungi can serve as a promising source of antimicrobials against nematodes as evidenced by the antimicrobial and anti-nemic activity of phenolic compounds and water extracts of *Lentinula edodes, Boletus edulis, Pleurotus ostreatus,* and *Agaricus bisporus* against *M. incognita* [16, 26].

Indeed, the methanolic extract of cell-free culture (CFC) filtrate and mycelium borne bioactive molecules of *Ophiocordyceps sinensis* and *O. neovolkiana* has been known to possess antimicrobial and anti-nemic activity against *M. incognita* [23]. Therefore, the present investigation was conducted to test the efficacy of ethyl acetate extract of cell-free culture (CFC) filtrate bioactive molecules of *Lentinus edodes*, *Ganoderma lucidum*, and *Schizophyllum commune* against root-knot nematode incited by *M. incognita.*

Materials and Methods

Pure culture of root-knot nematode, *M. incognita*

The egg masses were collected from the infected roots of tomato plants by prudently uprooting the plants and the roots with prominently noticeable galls were washed gently in water and the egg masses were then handpicked under the stereo zoom microscope and allowed to hatch by placing the egg masses in 100 ml beaker containing distilled water and incubated at room temperature. Pure culture of root-knot nematode, *M. incognita* was maintained on tomato in earthen pots. The potting mixture was prepared (1:1:2 red earth, sand, and farmyard manure) and sterilized in an autoclave at 121 °C, 15 lbs for 20 min. Then the hatched out second-stage juveniles (J2) of *M. incognita* were inoculated @ 1 J2 / g of soil in the tomato rhizosphere at two weeks after transplanting in the earthen pots and the nematodes were multiplied and maintained in the Nematology glasshouse.

Extraction of Bioactive molecules from mushrooms

Five days old mycelial disc of mushroom fungi *viz., L. edodes, G. lucidum,* and *S. commune* measuring 5mm diameter were cut from the margin of the colony and were inoculated in 250 mL conical flasks containing 100 mL of sterilized mushroom complete (Medium) broth in each flask (adjusted to pH 6.0). The flasks were placed in an incubator cum rotary shaker for incubation at 25°C and agitated at 120 rpm 20 d. Later, the culture filtrate was collected and the mycelial mat was separated by filtration through Whatman No.40 filter paper. Further, the culture filtrate was centrifuged at 10,000 rpm for 15 mins and the cell-free culture filtrate was extracted with ethyl acetate solvent (v/v) sequentially. Liquid-liquid extraction was carried out three to four times for the same solvent. The solvent extract was evaporated separately under reduced pressure using a rotary evaporator to obtain the residues. The condensate of the solvent extract was dried and dissolved in methanol (1mg/mL) and filtered with a membrane filter (0.2 µm), stored at 4°C for further studies.

Bioassay of CFC condensates of mushrooms on egg hatching of *M. incognita*

Two ml of ethyl acetate fraction of CFC condensate of mushroom fungi were prepared from the stock solution (w/v) using distilled water at different concentrations (250, 500, 750, and 1000 ppm) and transferred into 5cm Petri plates. One egg mass of *M. incognita* was placed in each petri dish and incubated at room temperature (28±1°C). One egg mass was placed in distilled water as a control. The number of hatched juveniles was counted after 24, 48, and 72 h intervals. The experiment was conducted in laboratory conditions. Four replications were maintained for each concentration.

Bioassay of CFC condensates of mushrooms on juvenile mortality of *M. incognita*

In a separate experiment, two ml of CFC condensate of mushroom fungi (250, 500, 750, and 1000 ppm) were transferred into a 5cm Petri plate, and 100 freshly hatched juveniles (J_2) of *M. incognita* were placed in each of the Petri dish and incubated at room temperature (28 ± 2ºC). The juveniles placed in the plates containing sterile water served as control and the number of juveniles dead was counted under the microscope after 24, 48, and 72 h of incubation. Four replications were maintained for each treatment.

Characterization of Biomolecules produced by *G. lucidum* through Gas chromatography and Mass Spectrometry (GC-MS)

The effective isolate of *G. lucidum* was analyzed for the detection of active bio-molecules responsible for the suppression of root-knot nematode (*M. incognita*) through GC-MS (GC Clarus 500 Perkin Elmer) using a column Elite-5MS (100% Dimethylpolysiloxane), 30 × 0.25 mm × 0.25 µmdf equipped with GC Clarus 500 Perkin Elmer. The turbo mass gold- Perkin-Elmer detector was used. The carrier gas flow rate was 1 ml per min, split 10:1, and injected volumes were 3 µl. The column temperature was maintained initially at 110ºC at the rate of 10ºC/min-No hold followed by an increase up to 280ºC at the rate of 5ºC/min-9 min (hold). The injector temperature was 250ºC and this temperature was held constant for 36 min. The electron impact energy was 70 eV, Julet line temperature was set at 2000ºC and the source temperature was set at 200ºC. Electron impact (EI) mass scan (m/z) was recorded from the 45-450 aMU range. The GC-MS compounds were identified by comparing the obtained mass spectra with NIST/EPA/NIH Mass Spectral Library.

Statistical Analysis

The design of experiments (CRBD) and statistical analysis was followed as suggested by [8]. Statistical software SPSS was used for the analysis of ANOVA and Duncan's Multiple Range Test (DMRT) of the data.

Results

Effect of Bioactive compounds of mushrooms on egg hatching of *M. incognita*

As root-knot nematodes in several cases predispose *Fusarium* infection, this experiment was conducted to test the effect of biomolecules of mushrooms against *M. incognita*. The egg mass exposed to various concentrations (250 ppm, 500 ppm, 750 ppm, and 1000 ppm) of mushroom biomolecules and observed at different time intervals *viz.*,24, 48, and 72 hours. In 24 hours, the ethyl acetate CFC condensate of *G. lucidum* decreased the hatching inhibition by 88.5 percent at 1000 ppm followed by 750 ppm 76.8 percent respectively. In *L. edodes* and *S. commune* the percentage of hatching inhibition was recorded in 1000 ppm concentration at 24 hrs interval which was 80.3 percent and 74.1 percent respectively. The CFC condensate of *G. lucidum* was exhibited more antinemic activity and the highest percentage inhibition was recorded in 72 hrs (92.3 percent) at the concentration of 1000 ppm when compared to other treatments. The least inhibition was observed at 250 ppm when compared with methanol and water control is appended in Table 1 and Figure 1.

Effect of Bioactive compounds of mushrooms on juvenile mortality of *M. incognita*

The CFC condensate of *G. lucidum* showed a significant increase in mortality of J_2 of *M. incognita* at different time intervals. The data showed that a gradual increase in mortality

was observed (24 – 51.2, 48- 73.7, and 72 hours– 93.2 percent, respectively) at 1000 ppm followed by 750 ppm. Therefore, *L. edodes* and *S. commune* also increase the juvenile mortality at the concentration of 1000 ppm at different hour intervals appended in Table 2 and Fig 2. The mortality gradually decreased in the treatment of *S. commune* and *L. edodes* at a concentration of 250 ppm and recorded 3.25 percent and 8.75 percent mortality, respectively at 24 hrs. There was no mortality observed in control at different time intervals.

Detection of compounds present in Cell-free culture filtrate of *G. lucidum*

Among the CFC condensate of different mushrooms tested, *G. lucidum* revealed the highest hatching inhibition and juvenile mortality of *M. incognita*. Further CFC filtrate of *G. lucidum* was subjected to GC-MS analysis for compound identification. The results revealed that the presence of 23 compounds *viz.*,Octadecane,3-ethyl-5-(2-ethyl butyl), Decane, Undecane, 3,7-dimethyl-, Dihydroartemisinin, Benzaldehyde, 3,4-dimethyl-, Heptadecane, 2,6,10,15-tetramethyl-, 2,4-Di-tert-butyl-phenol, 1-Propanamine, Pyrrolo[1,2-a]pyrazine-1,4dione, hexahydro-,1,2-Benzenedicarboxylic acid, butyl octyl ester, n-Hexadecanoic acid, Dibutyl phthalate,2,2,4-Trimethyl-3-(3,8,12,16-tetramethylheptadeca-3,7,11,15-tetraenyl)-cyclohexanol, Methyl stearate, Cyclohexane, Octadecanoic acid, Deoxyspergualin, Methyl glycocholate, 3TMS derivative, 1,25-dihydroxyvitamin D3, TMS derivative, Spirost-8-en-11-one, 3-hydroxy-, 1H-Indene, 1-hexadecyl-2,3-dihydro- and Bis(2-Ethylhexyl) phthalate. Among these, Dibutyl phthalate recorded the highest peak area of 15.57 percent, expressed at 19.92 RT (Retention time).

Discussion

Though it is well proven that mushrooms apart from being consumed as food, synthesize numerous molecules that are known to be bioactive. These bioactive compounds found in fruiting bodies, mycelial mat, and cell-free culture broth are polysaccharides, proteins, fats, minerals, glycosides, alkaloids, volatile oils, terpenoids, tocopherols, phenolics, flavonoids, carotenoids, folates, lectins, enzymes, ascorbic, and an organic acid which possess antifungal, antimicrobial, antibacterial, antiviral, anti-inflammatory, antioxidant, insecticidal and nematicidal, anticancer, prebiotic, and immunomodulation properties[25]. Extensive studies have shown that among mushroom molecules tested, the biomolecules extracted from CFC culture filtrate of *G. lucidum* exhibited the highest egg hatching inhibition and juvenile mortality recorded in 72 hours at the concentration of 1000 ppm followed by 750 ppm. Subsequently, the CFC condensate of *L. edodes* and *S. commune* also significantly caused hatching inhibition and juvenile mortality in 72 hrs exposure at the concentration of 1000 ppm .The results are in line with the findings of [23] who reported that the bioactive molecules of *O. sinensis* exhibited maximum inhibition of egg hatching (94%) and juvenile mortality (92%) of *M. incognita* at 72 hours of incubation. In the same way, [5] stated that the culture filtrate of *Amauroder mamacer*, *Laccaria tortilis*, *Peziza* spp., *Omphalotus mucida*, *Pleurotus pulmatus*, and *Tylopilus striatulus* exhibited nematicidal activity against the pinewood nematode *Bursaphelenchus xylophilus*, with over 80 percent mortality within 72 h of exposure. Similarly, bioactive compounds of luminescent mushroom *Neonothopanus nambi* at the concentration 500mg/l caused 99% juvenile mortality [3]. In parallel, basidiomycete fungi *Coprinus comatus* was found to be a nematophagous fungus, which showed nematicidal activity against the free-living nematode, *Paragrellus redivivus*, and the root-knot nematode, *M. arenaria*[17]. The CFC condensate of *G. lucidum* exhibited more nematicidal activity as compared to *L. edodes and S. commune* (Fig 1 and Fig 2). The reason behind the anti-nemic effect of CFC filtrate of *G. lucidum* on eggs and juveniles of *M. incognita* is due to the innate nature and the presence of toxic metabolites such as phenols, esters, and fatty acid which has been confirmed through GC-MS analysis and the presence of 23 compounds expressed in Table 3. Among these compounds, 2, 4-Di-tert-butyl-phenol, n-Hexadecanoic acid and Dibutyl phthalate might have been responsible for anti-nemic activity. Correspondingly, [29] revealed the presence of esters and phenolic derivatives including the compounds L-ascorbyl 2, 6-dipalmitate, dibutyl phthalate, dimethyl phthalate, and 2, 6-di-tert-butyl-p-cresol which suppressed egg hatching and also repelled juvenile mortality in *M. incognita*. The supporting evidence [19] reported that acetic acid, hexadecanoic acid (n-decanoic acid derivatives) and 4H-Pyran-4-one, 2, 3-dihydro-3,5-dihydroxy-6-methyl also exhibited nematicidal activities against root-knot nematode. Consequently, the lipophilic phenol 2, 4-Di-tert butyl-phenol showed nematicidal activity against *Caenorhabditis elegans* during fumigation or soil treatment [27].

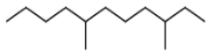
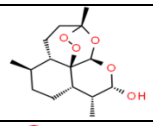
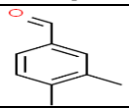
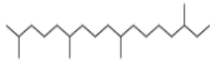
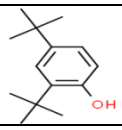
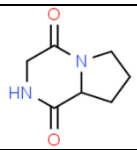
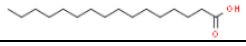
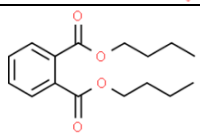
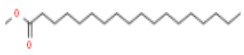
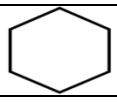
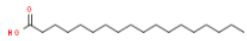
Table 1: Effect of Ethyl acetate extract of CFC filtrate of mushrooms on egg hatching of *M.incognita*

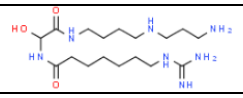
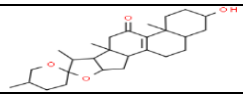
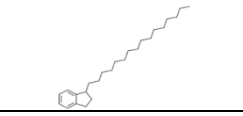
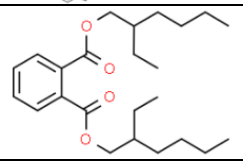
Treatments	Concentration / Incubation period											
	250 ppm			500 ppm			750 ppm			1000 ppm		
	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
CFC Condensate of *L. edodes* (Ethyl acetate fraction)	35.6^{b}	38.6^{d}	42.1^{d}	49.6^{c}	52.1^{d}	60.3^{d}	66.3^{d}	71.6^{d}	78.3^{d}	80.3^{d}	83.6^{d}	85.6^{d}
CFC Condensate of *G. lucidum* (Ethyl acetate fraction)	40.5^{c}	44.1^{e}	48.3^{e}	58.6^{e}	61.2^{e}	65.6^{e}	78.6^{e}	81.3^{e}	84.3^{e}	88.5^{e}	90.1^{e}	92.6^{e}
CFC Condensate of *S. commune* (Ethyl acetate fraction)	29.5^{a}	32.6^{c}	36.1^{c}	44.5^{b}	48.6^{c}	50.1^{c}	59.2^{c}	63.1^{c}	68.3^{c}	70.1^{c}	72.6^{c}	74.1^{c}
Methanol control	53.1^{d}	26.3^{b}	10.3^{b}	53.1^{d}	26.3^{b}	10.3^{b}	53.1^{b}	26.3^{b}	10.3^{b}	53.1^{b}	26.3^{b}	10.3^{b}
Control (Sterile water)	41.8^{c}	12.5^{a}	1.3^{a}	41.8^{a}	12.5^{a}	1.3^{a}	41.8^{a}	12.5^{a}	1.3^{a}	41.8^{a}	12.5^{a}	1.3^{a}
SED	0.56	0.31	0.17	0.36	0.19	0.32	0.59	0.36	0.72	1.14	3.64	1.40
CD (0.05)	1.19	0.66	0.38	0.76	0.42	0.70	1.25	0.76	1.55	2.42	8.11	2.99

Table 2: Effect of Ethyl acetate extract of CFC filtrate of mushrooms on juvenile mortality of *M. incognita*

Treatments	Concentration / Incubation period											
	250 ppm			500 ppm			750 ppm			1000 ppm		
	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
CFC Condensate of *L. edodes* (Ethyl acetate fraction)	8.75^{b}	17.2^{c}	26.5^{c}	17.0^{b}	32.8^{c}	36.2^{c}	27.5^{c}	45.7^{c}	64.5^{d}	35.7^{d}	56.7^{d}	81.2^{d}
CFC Condensate of *G. lucidum* (Ethyl acetate fraction)	20.2^{c}	25.0^{d}	35.7^{d}	33.5^{c}	45.7^{d}	49.0^{d}	40.7^{d}	57.7^{d}	78.7^{e}	51.2^{e}	73.7^{e}	93.2^{e}
CFC Condensate of *S. commune* (Ethyl acetate fraction)	3.25^{b}	12.7^{b}	18.7^{b}	11.5^{b}	23.0^{b}	30.5^{b}	16.5^{b}	34.7^{b}	45.0^{c}	29.7^{c}	44.7^{c}	60.2^{c}
Methanol control	18.2^{c}	27.2^{d}	36.7^{d}	18.2^{b}	27.2^{b}	36.7^{c}	18.2^{b}	27.2^{b}	36.7^{b}	18.2^{b}	27.2^{b}	36.7^{b}
Control (Sterile Water)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
SED	1.57	1.21	1.55	2.70	1.55	1.33	2.53	2.34	1.91	1.94	2.15	1.91
CD (0.05)	3.50	2.59	3.30	5.76	3.30	2.84	5.40	4.98	4.08	4.14	4.58	4.08

Table 3: Biomolecules separated from CFC filtrate condensate (Ethyl acetate fraction) of *G. lucidum* by GCMS analysis**.**

Retention Time	Compounds	Chemical Formula	Molecular Weight (g/mol)	Structure	Area Percentage
3.153	Octadecane, 3-ethyl-5-(2 ethylbutyl)	$C_{18}H_{38}$	254.51		0.300
3.639	Decane	$C_{10}H_{22}$	142.28		1.007
4.409	Undecane, 3,7-dimethyl-	$C_{13}H_{28}$	184.36		0.607
6.400	Dihydroartemisinin	$C_{15}H_{24}$	284.34		1.004
6.830	Benzaldehyde, 3,4-dimethyl-	$C_9H_{10}O$	134.17		0.36
10.53	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	296.57		0.647
10.87	2,4-Di-tert-butylphenol	$C_{14}H_{22}$	206.32		0.631
14.96	1-Propanamine	-	-	-	1.506
16.06	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N$	154.16		2.279
18.05	1,2-Benzenedicarboxylic acid, butyl octyl ester	-	-	-	0.723
19.43	Hexadecanoic acid, methyl ester	$C_{16}H_{32}$	256.42		2.814
19.92	Dibutyl phthalate	$C_{16}H_{22}$	278.34		15.57
23.05	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	-	-	-	1.568
23.36	Methyl stearate	$C_{19}H_{38}$	298.50		6.291
23.36	Cyclohexane	C_6H_{12}	84.16		2.247
23.80	Octadecanoic acid	$C_{18}H_{36}$	284.47		4.763

24.03	Deoxyspergualin	$C_{17}H_{37}$	387.52		0.545
24.66	Methyl glycocholate, 3TMS derivative	-	-	-	0.285
24.88	1,25-Dihydroxyvitamin D3, TMS derivative	-	-	-	0.354
28.36	Spirost-8-en-11-one, 3-hydroxy-	$C_{27}H_{40}$	428.60		0.283
29.33	1H-Indene, 1-hexadecyl-2,3-dihydro-	$C_{25}H_{42}$	342.60		1.116
29.76	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}$	390.55		0.796

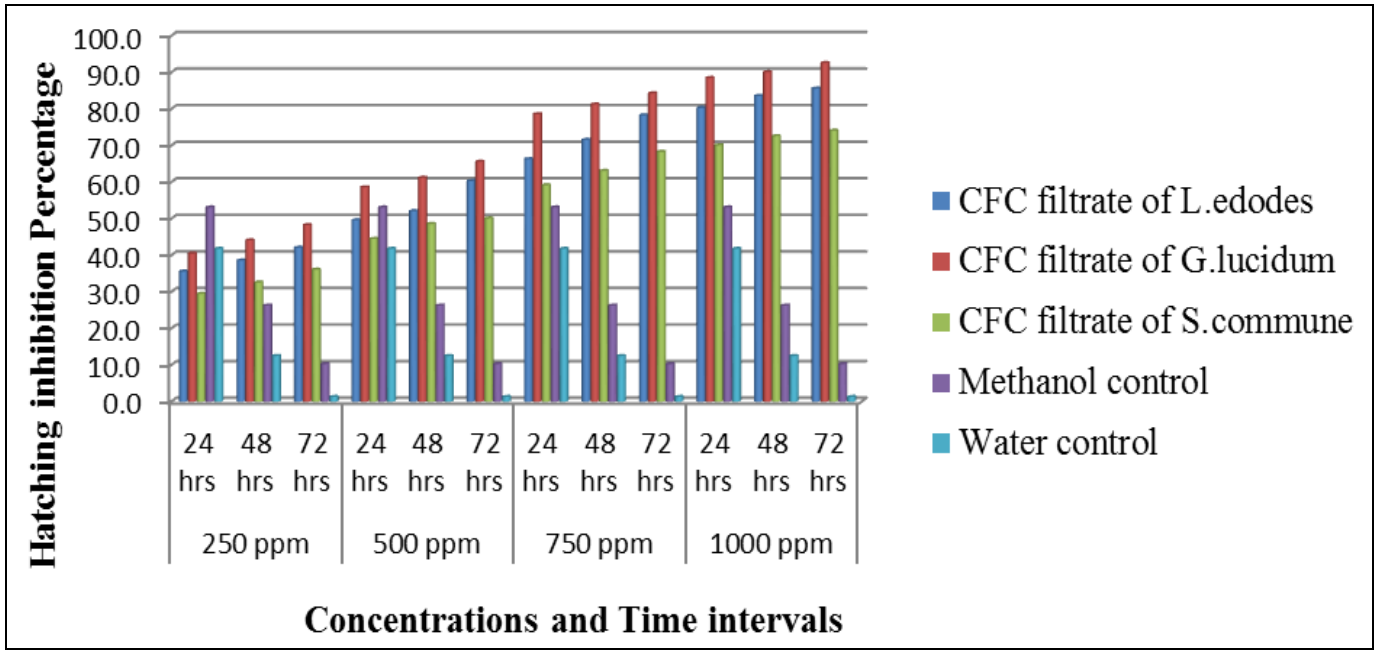


Fig 1: Effect of CFC filtrate of mushrooms on egg hatching of *M. incognita*

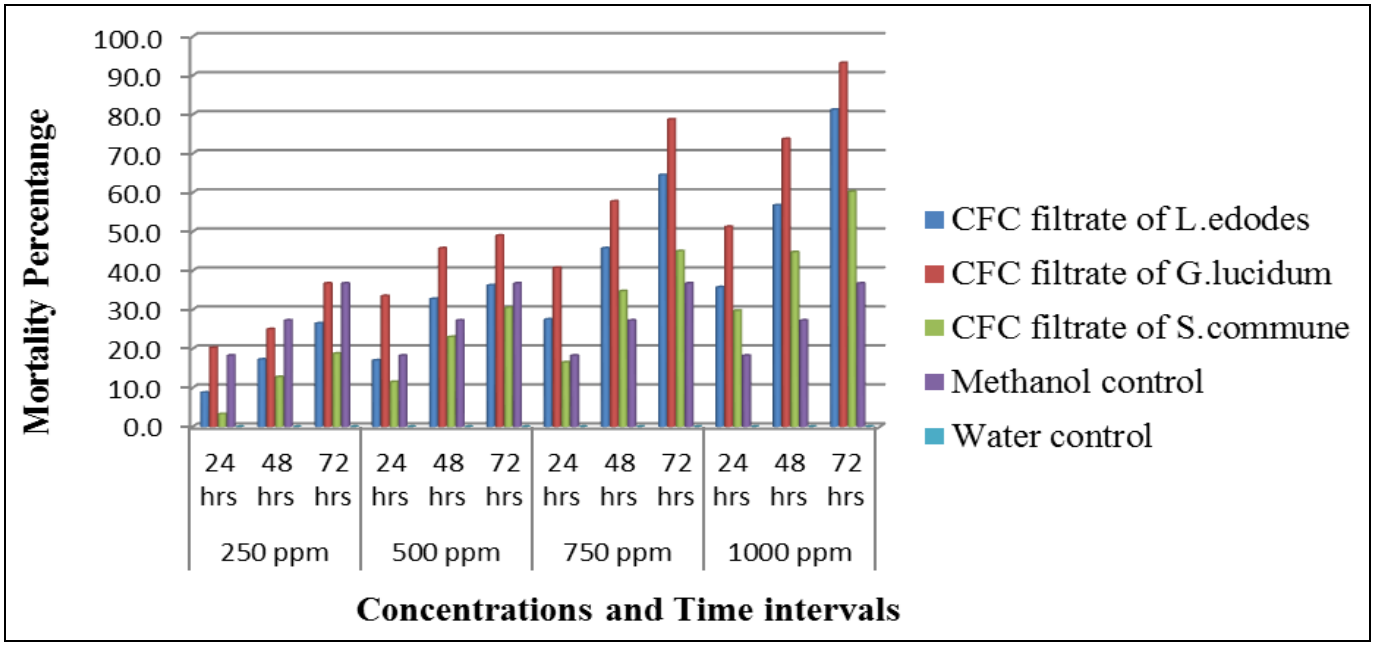


Fig 2: Effect of CFC filtrate of mushrooms on juvenile mortality of *M .incognita*

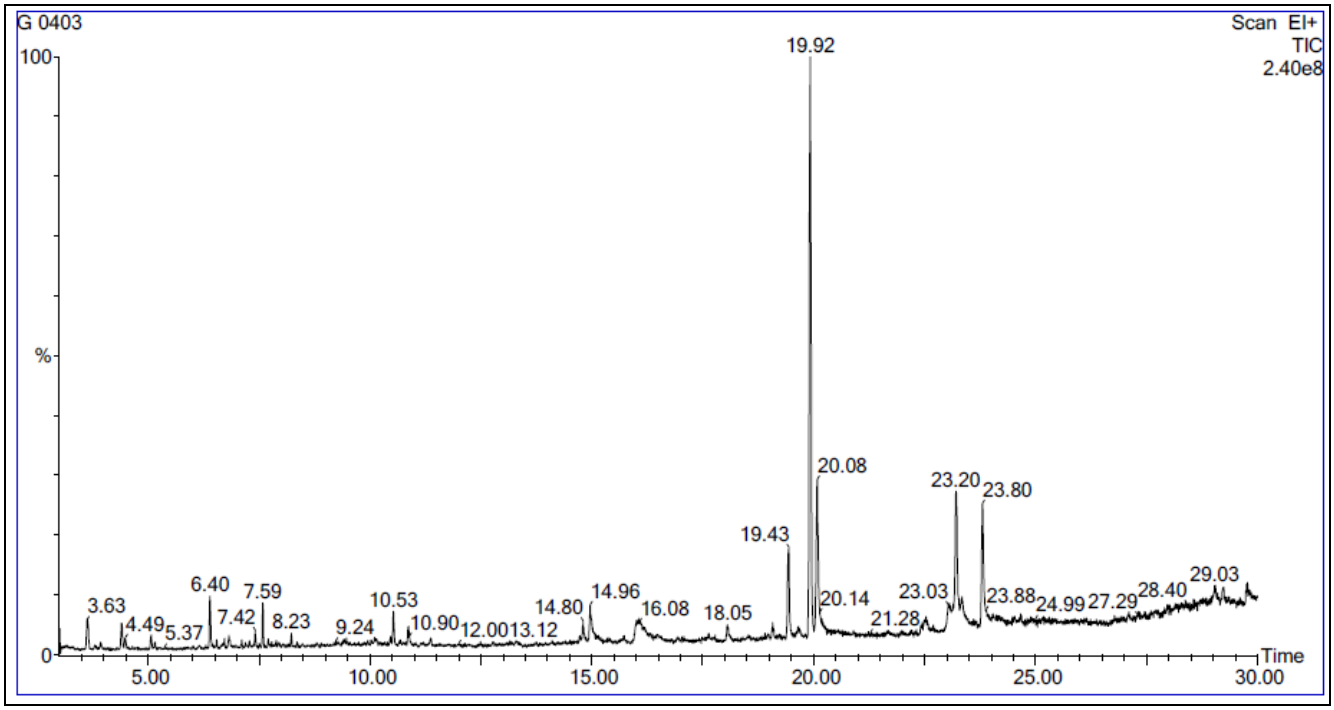


Fig 3: Chromatogram depicting the GC- MS studies of ethyl acetate fraction of *G. lucidum*

Conclusion

To summarize the present findings of ethyl acetate fraction of the cell-free culture filtrate of *G. lucidum* is known to produce plentiful bioactive compounds that could be potentially used for the management of root-knot nematode, *Meloidogyne incognita*.

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