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Bioassay-guided Identification of the Nematicidal Secondary Metabolites from *Paecilomyces lilicanus* for the Control of Root-knot Nematode (*Meloidogyne graminicola, Golden and Birchfield*)

Ruben M. Gapasin[1], Erlinda A. Vasquez[2] and Giselle A. Rendon[3]

[1]Department of Pest Management, Visayas State University (VSU), ViSCA, Baybay, Leyte; [2]PhilRootcrops, VSU, ViSCA, Baybay, Leyte; [3]PhilRice, Agusan

ABSTRACT

Meloidogyne graminicola larvae immersed for 48 hours in 100% concentration of *Paecilomyces lilacinus* culture filtrate had 100% larval mortality while only 7.12% and 18.72% mortality was recorded, after 6 and 12 hours immersion, respectively. Toxic metabolites present in the culture filtrate affected the larvae as there were no galls produced in rice seedlings at 100% concentration. The culture filtrate also limited egg hatching as shown by lesser galls produced in rice seedling roots. The mycelial extract was found more potent compared with the culture filtrate extract; however, both fractions affected the larvae and eggs of *M. graminicola*.

The dried ethyl acetate fraction gave 88.51% mortality at concentration of 500 mg/ml, followed by 76.91% at concentration of 400 mg/ml. A lethal concentration (LC) of 50% was attained at 300 mg/ml. Sixteen fractions were recovered from the crude dried extract of the fungus using vacuum liquid chromatography (VLC). Bioassay revealed that fraction 3 (combined fractions 5, 6 and 7) gave 39 percent nematode mortality suggesting that the nematoxic compounds are present in these fractions.

The fragmentation pattern in GC-MS revealed that the active fraction contains more than 20 compounds with 5 major ones. The structure of the main compound was partially identified as derivative of azulene containing two unsaturation points. The limited amount of the fraction subjected to spectroscopic analysis does not warrant its purification to be able to do other analysis such as NMR spectroscopy for structure elucidation and complete compound identification.

Keywords: nematicidal, metabolites, nematophagous, *Paecilomyces lilacinus*, root-knot nematode, *Meloidogyne graminicola*, culture filtrate, azulene

Correspondence: RMGapasin *Address:* Department of Pest Management, Visayas State University, Baybay City, Leyte 6521-A Philippines *Email:* rmgapasin1952@yahoo.com
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INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are worldwide in their distribution and crop losses are estimated to reach about 13% (Sasser, 1979). Infected plants show reduced growth, swollen roots which develop into the typical root-knot galls, are two, or three times larger in diameter as healthy root. Root-knot nematodes are very difficult to control because they are polyphagous; the life cycle is almost completely confined inside the host plant and high reproductive capacity. Although chemical control is still a common method for reducing nematode population, there is a considerable public pressure to limit or even ban the use of nematicides. Many nematicides are highly toxic and sometimes very mobile in the soil because of their solubility in water. Concern over these chemicals has led to an increased interest in biological control in order to achieve more environmentally friendly methods of reducing nematode damage.

The fungal antagonist of nematodes consist of a great variety of organisms which include the nematode-trapping or predaceous fungi, endoparasitic fungi, parasites of nematode eggs, parasites of nematode cyst and fungi which produce toxic metabolites that kill nematodes (Mankau, 1980). *Paecilomyces lilacinus* is one of the many fungi most frequently encountered and is found effective in reducing nematodes in the soil. Their destructive activity includes hyphal colonization and enzymatic disruption of nematode structural elements such as eggshells and larval cuticles, and physiological disturbances brought about by biosynthesis of diffusible toxic metabolites (Morgan-Jones and Rodriguez-Kabana, 1985). In a recent undertaking, *P. lilacinus* culture filtrate incubated for 30 days gave a 100% larval mortality at 50 percent concentration (dela Piza, 2003). However, there was no attempt to identify the nematicidal compound in the culture filtrate.

The ultimate aim of this study is to identify the compounds/ metabolites present in *P. lilacinus*, a fungal biological control agent of plant parasitic nematodes, which could be used to reduce nematode infection in economically important crop plants thereby increasing their yield. Another possible output is the development of agricultural products for pest control. The compounds isolated which have either suppressive

or deterrent properties against pest particularly nematodes could be utilized as pesticide or as templates in the development of more potent activity with reduced toxicity and non-persistent agric-products. This means that we will be developing an environment friendly pesticide.

MATERIALS AND METHODS

Culture of Paecilomyces lilacinus in Liquid Media

Pure culture of *P. lilacinus* acquired from the collections of the Department of Pest Management was sub-cultured in potato dextrose agar (PDA) medium and then incubated at room temperature. An oatmeal liquid medium (oatmeal, 75g; distilled water, 1 liter) was prepared, placed in 500 ml flasks, and sterilized for one hour at 15 psi. Previously grown *P. lilacinus* was inoculated to the liquid media aseptically and incubated under room temperature for 60 days.

Mass-Culture of Meloidogyne graminicola in Rice (UPLRi-5)

Pure culture of the nematode was mass-produced by inoculating 1,000 larvae of the nematode to susceptible UPLRi-5 rice grown in sterilized soil. Inoculated plants were maintained outside the screen house for 60 days.

Preparation of P. lilacinus Culture Filtrate

The culture filtrate of *P. lilacinus* from each flask was removed using a pipettor. Culture filtrate was filtered using sterilized filter paper and funnel, placed in sterilized flask. The filtrate was placed in the refrigerator until ready for use in the succeeding bioassay.

Extraction of Culture Filtrate

One-liter culture filtrate was mixed with ethyl acetate in separator funnel and shaken for 3 minutes. The aqueous (water) and ethyl acetate fractions were transferred separately to a beaker and concentrated using rotary evaporator (Rotavap R110) at 30-40°C. The concentrated fractions were placed in vials and refrigerated for use in the succeeding bioassay.

Preparation and Extraction of P. lilacinus Mycelia

The mycelia of the fungus from each flask were collected using sterilized forceps and weighed using the triple beam balance. A known volume (500 ml) of sterile water was added into the mycelial mat, and was osterized for 3 minutes. One liter of methanol (as solvent) was added to the mixture and transferred into the separatory funnel. The aqueous and methanol fractions of the mycelia was transferred separately and filtered using a vacuum pump and the fractions were placed in sterilized beaker. The methanol and aqueous fraction were concentrated using the rotary evaporator (Rotavap R110) at 30-40°C. The concentrated fractions were placed in vials and refrigerated until ready for use in the succeeding bioassay.

In Vitro Bioassay of P. lilacinus Culture Filtrate

Bioassay of the culture filtrate against *M. graminicola* larvae was carried out using the immersion test. This was done by immersing approximately 75 freshly hatched larvae (contained in 0.1 ml nematode suspension) to 2 ml culture filtrate placed in plastic plates at different concentrations of 100%, 50%, 40%, 30%, 20%, 0% (sterile distilled water only). Dead and alive nematodes were counted at 3, 6, 12, 24, and 48 hours under a stereomicroscope with the aid of a multiple tally counter. Erect and non-moving nematodes were counted as dead. The effect of the culture filtrate on *M. graminicola* eggs were assayed following the same procedure as in the bioassay for larvae but eggs were incubated only for 48 hours and later inoculated to previously rooted rice seedlings.

The different treatments were replicated 4 times and arranged in Completely Randomized Design (CRD) in the Laboratory. Differences in the means were compared using the Duncan's Multiple Range Test (DMRT).

In vitro Bioassay of the Different Fractions of Culture Filtrate and Mycelia Extract

The aqueous and ethyl acetate fractions of the culture filtrate and the aqueous and methanol extracts of the mycelia were assayed using approximately 60 nematode larvae following the same procedure as in the culture filtrate bioassay. From the concentrated fractions (stock solution) different concentrations (300 ppm, 500 ppm, 800 ppm and 1,000 ppm) were prepared which were designated as the treatments with 4 replications. A 3% ethyl acetate and 3% methanol were prepared as negative control and the distilled water as the positive control. Dead and alive larvae were counted under the stereomicroscope with the aid of a tally counter after 3, 6, 12 and 24 hours.

Since the stock solution from the concentrated culture filtrate and mycelia were different, the stock solution from the culture filtrate was adjusted by adding sterile water. Different concentrations of 100%, 50%, 25%, 10%, and 0% (distilled water) were then prepared. A solution of 0.2 ml was placed to each plastic plate and approximately 60 larvae and eggs were added. The different treatments with 4 replications were incubated for 24 hours, arranged in CRD in the Laboratory. Differences in the means were compared using DMRT. Dead and alive nematode larvae were counted under the stereomicroscope with the aid of a hand tally counter every 3 hours until the 24th hour. Percent mortality was computed.

Confirmation of Nematode Mortality

The mortality of the nematode was further confirmed by inoculating the nematode-containing filtrate and mycelial extract (treatments corresponding with the *in vitro* bioassay) into two week-old rice seedlings

planted in plastic pots. After 21 days, the rice plants were uprooted and washed of soil debris and examined for the presence of galls and then counted. The presence of galls in roots would indicate that the immersed larvae were still alive and thus, were not affected by the filtrate or extract.

Extraction and Fractionation of the Active Compounds

Culture filtrate of *P. lilacinus* incubated for 60 days in oatmeal liquid culture medium was taken and filtered through a Buchner funnel with gentle suction. The filtrate was extracted using ethyl acetate. The extraction with ethyl acetate was repeated three to four times. The ethyl acetate fraction was then concentrated *in vacou* using a rotary evaporator. The extract was evaluated for nematicidal activity through assay as previously described.

The dried ethyl acetate extract was then subjected to vacuum liquid chromatography (VLC) using the following solvent system; 1:1 ethyl acetate-hexane followed by 100% acetone; 2% methanol in acetone, 4% methanol in acetone, 6% methanol in acetone and 10% methanol in acetone. The extracts were collected in separate containers, and concentrated *in vacou* using a rotary evaporator. The extracts were evaluated against the nematode larvae through a bioassay.

Analysis and identification of compounds in the active fraction of Paecilomyces lilacinus

The active fraction of the ethyl acetate extract of *P. lilacinus* was analyzed by gas chromatography and gas chromatography-mass spectrometry on a Finnigan Polaris Q GC/MS Ion Mass Spectrometer using a BPX5 capillary column (30 m x 0.32 mm ID, 0.25 mm film). Using helium as carrier gas the column was operated with temperature programmed from 50°C for 5 minutes and raised to 280°C at 10°C/min. with 15 minutes holding time and raised again to 300°C at 10°C/min and holding time of 10 min. Three injections of 0.5 ml from 10 ml concentrated active fraction were done.

Compounds in the fraction were identified by comparison with the retention time and mass spectra of synthetic standard analyzed under the same conditions and comparison with NIST library.

RESULTS AND DISCUSSION

Nematicidal Efficacy of Paecilomyces lilacinus Culture Filtrate

The percent mortality of larvae immersed at different periods (hours) using different concentrations of 60-day-old *P. lilacinus* culture filtrate is shown in Figure 1. No mortality was observed after 3 hours immersion of larvae in the culture filtrate, however after 6 hours 7.12% mortality was recorded at 100% concentration. This caused more than 50% mortality after 24 hours, which was significantly different with the rest of the treatments. Then after 48 hours of immersion in 100% concentration of culture filtrate, there was 100% mortality while at 50% and 40 % concentrations, there was 70% and 50% mortality, respectively, which were significantly different with each other. The results showed that there

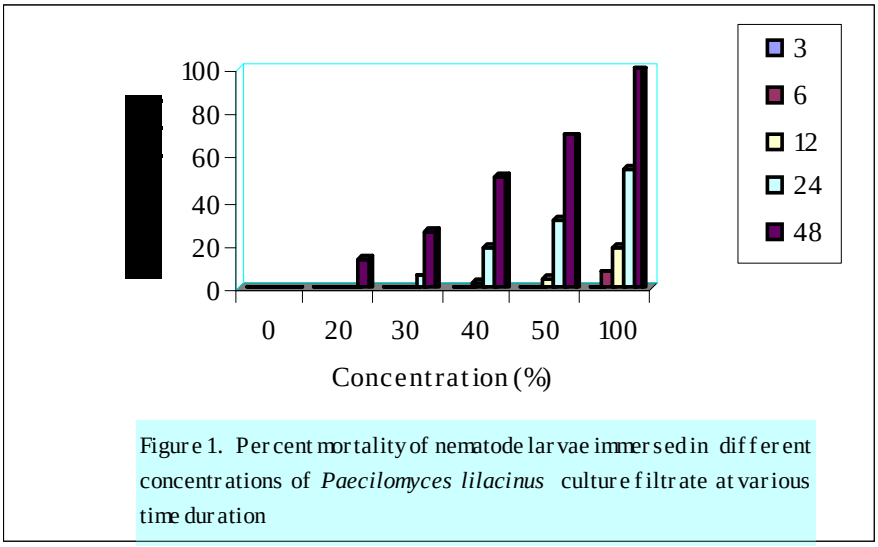


Figure 1. Percent mortality of nematode larvae immersed in different concentrations of *Paecilomyces lilacinus* culture filtrate at various time duration

was an increased mortality with concentrations and immersion time to the culture filtrate. This was in consonance with the results obtained by Dela Piza (2003). However, she observed 100% mortality of *M. incognita* even at 50% concentration of the culture filtrate after 48 hours immersion. This difference in result may be due to the species of nematode used. Apparently, *M. graminicola* larvae are more resistant or tolerant compared with *M. incognita* since at 48 hours immersion at 50% concentration of the culture filtrate only 69.46% mortality was observed.

The paralysis or death of larvae may be attributed to enzyme component of the culture filtrate as reported by Cayrol *et al.,* (1989). In a related report, Park *et al.,* (1988) as cited by Dela Piza (2003) observed protease activity on gelatin medium and chitins activity on colloidal chitin medium of different isolates of *P. lilacinus*. Ode *et al.,* (1997) observed poly (3-hydroxybutyrate) depolymerase from the fungus *P. lilacinus* D218 by column chromatography.

A plant bioassay test was done to verify whether the larvae and eggs immersed in the culture filtrate at different concentrations after 48 hours were either paralyzed or dead. The number of galls produced in rice seedlings 21 days after inoculation with the immersed larvae in culture filtrate is presented in Figure 2. No galls were observed in the roots of plants inoculated with larvae immersed in the 100% concentration of the extract indicating that all larvae died after 48 hours. This was significantly different with the rest of the treatments. The results also showed that as the concentration was decreased, the higher is the number of galls produced. This observation was in consonance with that of Cayrol *et al*., (1989) who found out that toxicity of culture filtrate of *P. lilacinus* decreased rapidly when filtrate was diluted.

Likewise, the eggs of *M. graminicola* were affected by the culture filtrate when immersed for 48 hours at different concentrations of the filtrate. At 100% concentration, the lowest number of galls of 15.00 was observed which was significantly different with the control and the 20% and 30% concentrations but was not significantly different with the 40% and 50% concentrations. These results showed that the toxic metabolites from the culture filtrate inhibited egg hatching. This affirmed the observations of Park *et al*., (1998) who reported that egg production of

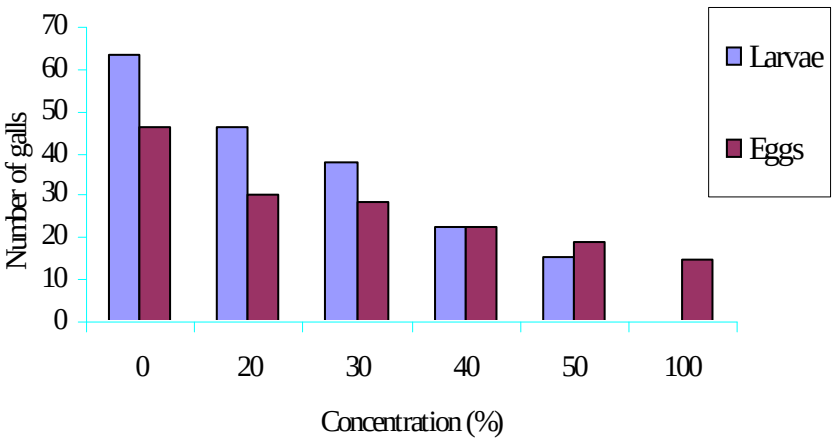


Figure 2. Galls produced in rice after larvae and eggs were immersed in different concentrations of *Paecilomyces lilacinus* culture filtrate.

Caenorhabditis elegans was inhibited when exposed to six isolates of *P. lilacinus* for 7 days. Similarly, Dela Piza (2003) also found suppression of egg hatching of *M. incognita* when exposed to *P. lilacinus* culture filtrate. The eggs' vitaline membrane may also have been disintegrated due to enzyme activity. According to Bonants *et al*., (1995) serine protease from *P. lilacinus* may play a role in the penetration of the fungus through the eggshell of *M. hapla*.

Efficacy of Aqueous Fractions of P. lilacinus Culture Filtrate and Mycelia Extract

The concentrated aqueous fraction (414 mg/12 ml) of the culture filtrate extracted with ethyl acetate showed very high and fast activity against the nematode larvae with more than 50% mortality after 3 hours immersion (Figure 3). After 6 hours, all the larvae were immobile or dead until the 24 hours observation. This was significantly different with the rest of the treatments. There was very low activity of the fraction at

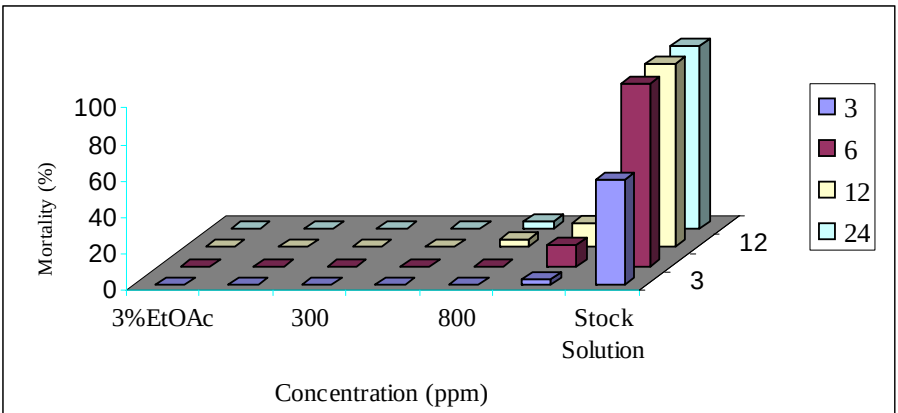


Figure 3. Mortality of nematode larvae immersed in different concentrations of the aqueous fractions of *Paecilomyces lilacinus* culture filtrate at various time duration

1000 ppm even after 24 hours. As there was no mortality observed in the 3% ethyl acetate (negative control) that would indicate its toxicity, death of the larvae could be attributed solely to the toxic compound present in the culture filtrate.

The aqueous fraction of the mycelia (68 mg/10 ml) when extracted with methanol was also toxic with more than 50% mortality after 6 hours and attaining 100% mortality after 24 hours (Figure 4). This was significantly different with the rest of the treatments. At 1000 ppm, 7.59% mortality was recorded which also significantly different with the other treatments (300 ppm, 500 ppm, and 800 ppm) which had zero mortality. This could be due to the very low concentration level or the preparations were too diluted that the nematode could tolerate their nematicidal activity. No mortality was recorded in the 3% methanol, again indicating that larval mortality was due to the toxicity of the mycelia extract and not the methanol.

Comparative Efficacy of Culture Filtrate and Mycelial Extract

The aqueous fraction of the mycelia was more toxic compared with the aqueous fraction from culture filtrate (Figure 5). There was higher

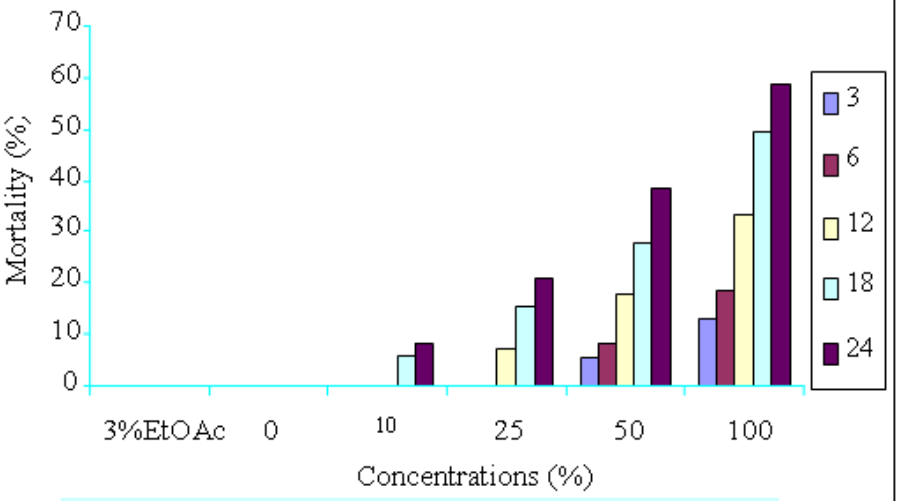


Figure 4. Mortality of nematode larvae immersed in different concentrations of the aqueous fractions of *Paecilomyces lilicanus* culture filtrate at various time duration.

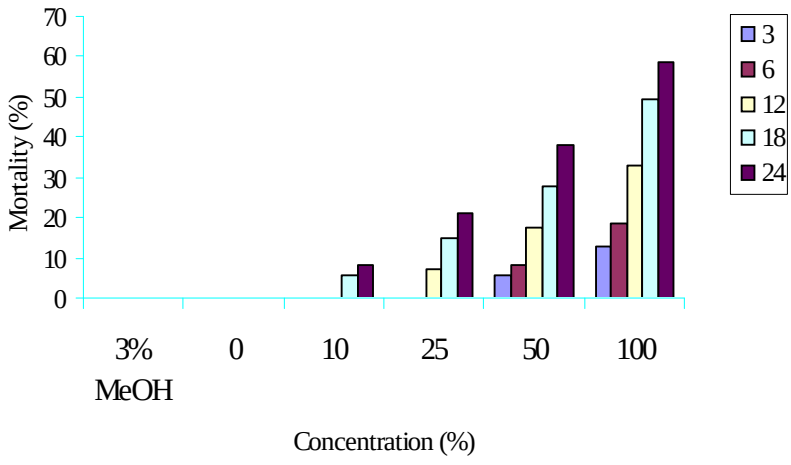


Figure 5. Mortality of nematode larvae immersed in different concentrtions of the aqueous fractions of P*aecilomyces lilacinus* mycelial extract at various time duration

mortality in the mycelia extract regardless of concentration and immersion time. At 6 hours immersion time, more than 50% mortality was recorded at 100%concentration in the mycelial extract while only 18.51% was recorded in the culture filtrate extract. At 24 hours immersion time, 100% mortality was observed in the mycelia extract at 100% concentration while only 58.57% was observed in the culture filtrate.

These results clearly indicate that more toxic compounds were still present in the mycelial extract compared with the culture filtrate extract after 60 days. The medium may have affected maximum toxin production by the fungus. According to Cayrol et al., (1989), the toxic properties of *P. lilacinus* are greatly influenced by the liquid culture medium. In Czapek-Dox modified medium, the toxic metabolites were not produced at any dilution while in the Arconteil modified medium, the toxicity was weak in pure culture filtrate. In the two synthetic media (Czapek-Dox and Mac Coy media) the toxicity was greater. While we can get high toxicity using the synthetic media this may be costly and not readily available compared with the medium used in this experiment.

No galls were produced when inoculated with larvae immersed in 100% concentration of the mycelia extract, indicating that all larvae were dead after 24 hours. However, not all the eggs were affected as 6.25 galls were recorded. This implies that the eggs were resistant due to the vitalline membrane and protein component of the egg shell. Some larvae may have hatched in the soil upon egg inoculation of the immersed egg thereby escaped the effect of toxin thus some galls were observed. Higher number of galls was found in the control and in the 3% ethyl acetate and 3% methanol treatments in both culture filtrate and mycelial extracts indicating a very low toxicity in these treatments. Significant differences were observed among the treatments.

Effect of the Different Fractions on Nematode Larvae

Figure 6 shows the percent mortality of root-knot nematode (RKN) immersed in ethyl acetate (EtOAC) fraction with the highest mortality of 88.51% at concentration of 500 mg/ml, followed by 76.91% at concentration of 400 mg/ml. A lethal concentration (LC) of 50% was attained at 300 mg/ml.

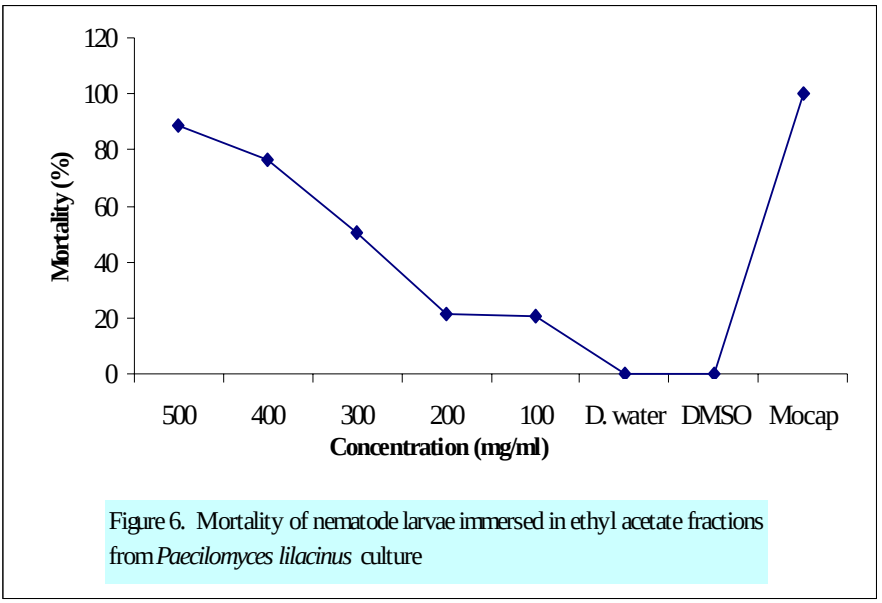


Figure 6. Mortality of nematode larvae immersed in ethyl acetate fractions from *Paecilomyces lilacinus* culture

Using different solvent systems, 16 fractions were produced from the crude dried extract using vacuum liquid chromatography (VLC) (Figure 7). Based on the Rf values as shown in thin layer chromatography (TLC) plates, some fractions were combined resulting in 6 fractions (Figure 8). Bioassay against nematode revealed that fraction 3 (fractions 5, 6, and 7) gave 39 percent mortality. The compound is non polar based on its affinity to the solvent system used.

Identification of the Compound by GC-MS

The active fraction contains more than 20 compounds with 5 major ones [4, 6,7,14, 19]. (Table 1) The structure of the main compound [19] was partially identified as derivative of azulene containing two unsaturation points based on the fragmentation pattern in GC-MS (Figure 9). The main component has a molecular weight of 207 and looks like a derivative of globulol (Figure 10 A and B). However, other functional groups in the molecule would not likely be the same since double bonds are also detected. The limited amount of the fraction subjected to spectroscopic analysis

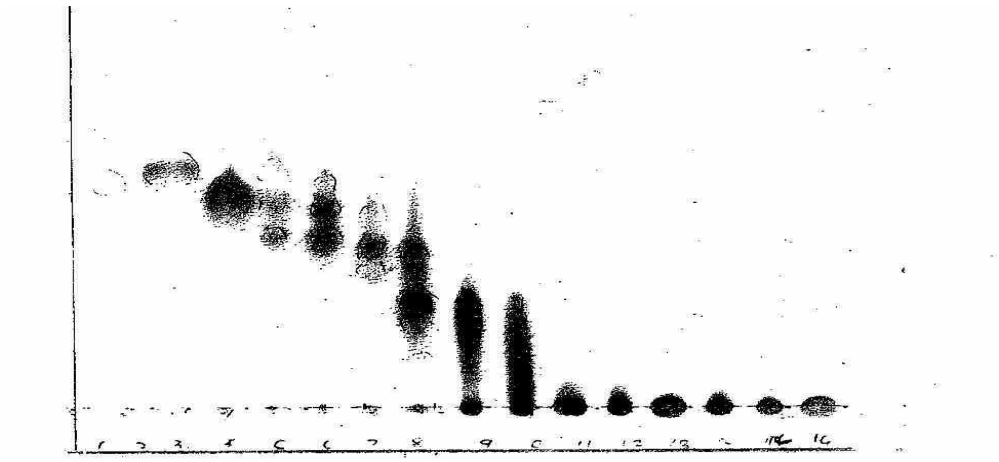


Figure 7. TLC profile of the 16 fractions from dried ethyl acetate extract of *Paecilomyces lilacinus*. Solvent system used for development is 1:1 ethyl acetate, hexane

does not warrant its purification to be able to do other analysis such as NMR spectroscopy for structure elucidation and complete compound identification.

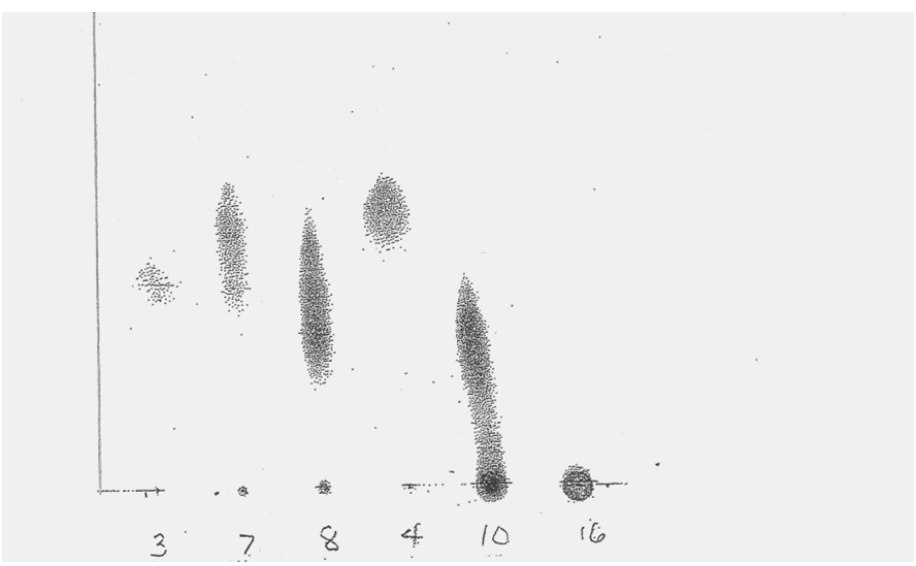
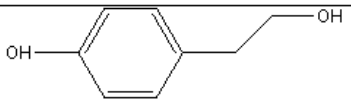
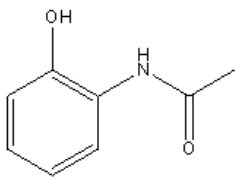
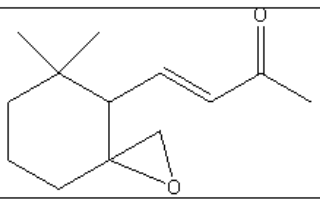
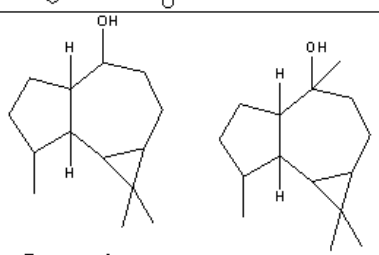
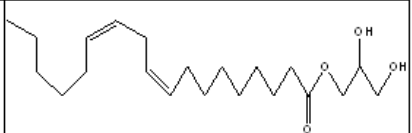


Figure 8. TLC profile of the combined fractions from dried ethyl acetate extract of *Paecilomyces lilacinus*. Solvent system used for development is 1:1 ethyl acetate, hexane

Table 1. Major compounds present in the active fraction of the ethyl acetate extract from *Paecilomyces lilacinus*.

Compound No.	Retention Time	Compound	Mol. Wt.	Structure
4	17.69 -17.79	4-hydroxy benzene ethanol	138	
6	19.52 -19.65	N-(2-hydroxyphenyl)-acetanamide	151	
7	19.74-19.78	4(5,5-dimethyl-1-oxaspirol (2,5)oct-4-yl)3-buten-2-one	208	
14	23.04-23.11	Unidentified, possibly a derivative of azulene which closely resembles Globulol (MW 222)	207	 *Proposed structure* Globulol
20	23.02-23.10	3,5,9-Trimethyl-deca-2,8-trien-1-ol	194	

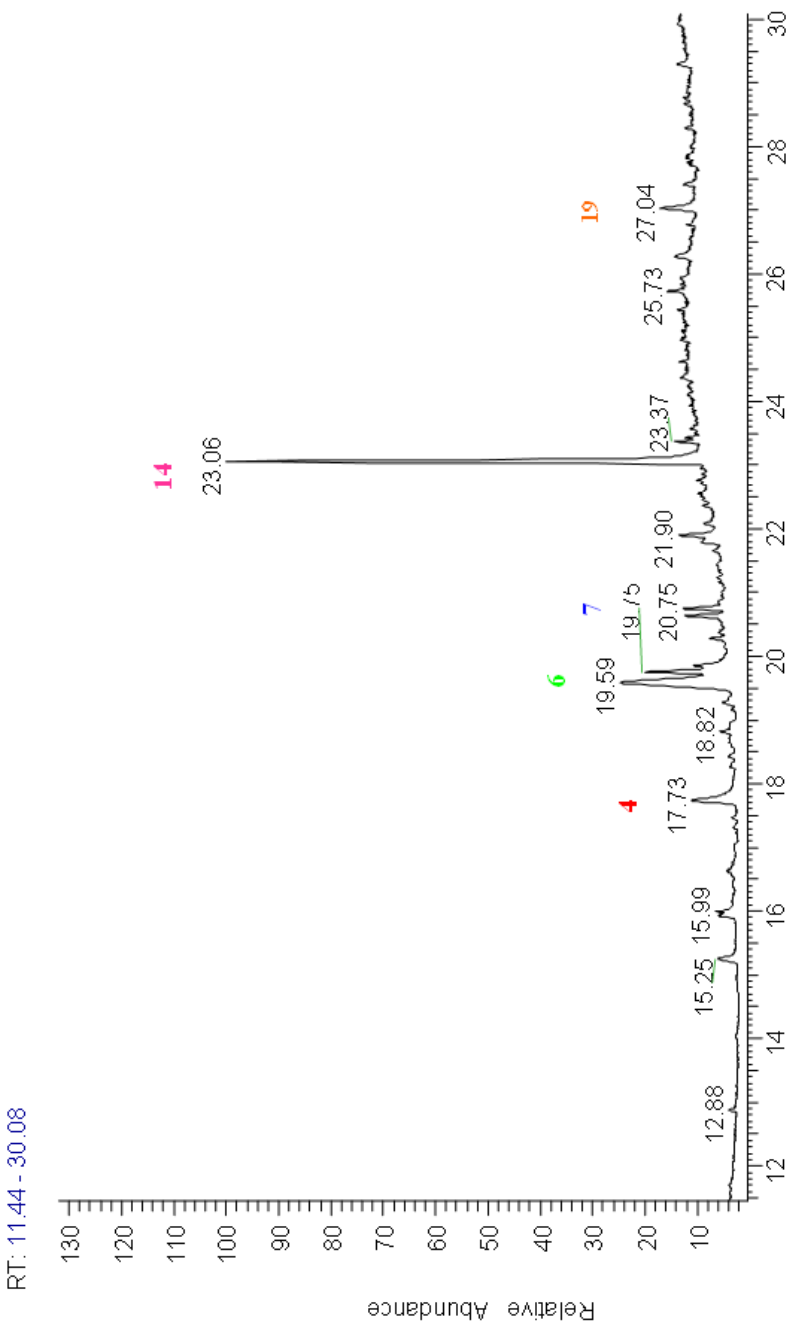


Figure 9. GC spectrum of the active fractions from the ethyl acetate extract of *Paecilomyces lilanus*.

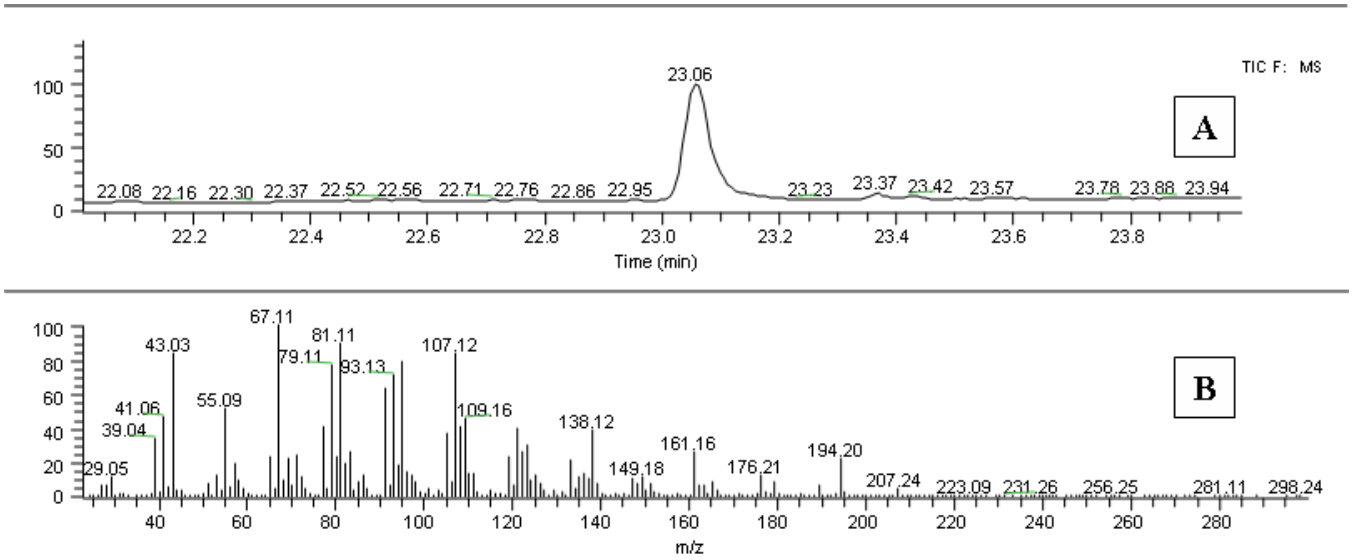


Figure 10. Total ion current (TIC) (A) and MS fragmentation pattern(B) of the main compound of the active fraction in ethyl acetate extract from *Paecilomyces lilacinus*.

CONCLUSION AND RECOMMENDATION

Paecilomyces lilacinus produces metabolites that are nematoxic against the larvae of the rice root-knot nematode, *Meloidogyne graminicola* as exemplified by 100% mortality of larvae even at 50% concentration of the culture filtrate. Extraction of the metabolites with ethyl acetate gave 88.32% mortality at concentration of 500 mg/ml. Fractionation of the dried extract yielded 16 fractions with subfraction 3 (fractions 5, 6, and 7) as the most toxic giving 39% larval mortality.

It can be concluded that based on the available data, metabolites are produced by *P. lilacinus* which are nematoxic against the rice root-knot nematode, *M. graminicola*. The metabolites are non polar. Furthermore, the active fraction contains more than 20 compounds with 5 major ones. The structure of the main compound [19] was partially identified as derivative of azulene containing two unsaturation points. The limited amount of the fraction subjected to spectroscopic analysis allowed us to do partial identification since materials are not enough to proceed purification for other analysis such as NMR spectroscopy for structure elucidation and complete compound identification.

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