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BIOLOGICAL SCREENING OF CRUDE EXTRACT OF *PENICILLIUM* SP. EU0013

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ABSTRACT

Medicinal plants and microbe are a rich source of secondary metabolites. The aim of the current work was to isolate the crude extract from *Penicillium* sp. EU0013 and study its antimicrobial potential at Natural Products Laboratory, The University of Agriculture Peshawar. Fermentation of *Penicillium* EU0013 was carried out using potato dextrose broth. The yield of crude ethyl acetate extract was calculated $1gmL^{-1}$ from static flask fermentation. The fungal extract was screened for bench-top bioassays including antifungal, antibacterial, phytotoxic, insecticidal and nematicidal activities. Results showed that at 100, 200 and 300 $\mu g.mL^{-1}$ the extract exhibit antibacterial activity ranging from 13.4 to 80.7mm against *E. coli*, *Bacillus subtilis*, *Xanthamonas* sp. and *Proteus vulgaris*, and 14.3 to 80.7 mm against *Aspergillus flavus*, *Alternaria alternata*, *A. niger* and *Fusarium oxyspourm*. The phytotoxic, insecticidal and nematicidal activity on 7^{th}day, 24 hour and again 7 days of *Penicillium* extract increased with increased of dose with highest mortality of 16.6, 63.32 and 85.75 % at 200, 200 and 100 $\mu g. mL^{-1}$ respectively.

Key words: *Penicillium sp.*EU0013, natural products, bioassays, phytotoxic, antibacterial, antifungal activities.

INTRODUCTION

Plant health affected by pathogenic fungi is a serious problem all over the world (Carroll and Wicklow, 1992). Mycotoxins produce by pathogenic fungi specifically *Aspergillus* and *Fusarium* species cause the most economic losses and affect human and animal health (Bennett and Klich, 2003).

Presently, the livelihood is mostly dependent on agriculture products to which agrochemicals are applied for high production. However, these agrochemicals may adversely affect crop productivity because environmental concerns affect human health and pathogens are commonly resistant to these applied chemicals (Weger *et al*., 1995). In addition, the growing cost of agrochemicals in developing countries, the incidence of diseases associated with the affected food crops and the consumer demand for pesticide free food products led to the search for alternative substitutes (Gerhardson, 2002). Biological control is thus considered as an alternative way that protect the plants from the invasion of pathogens (Cook and Baker, 1983) reduce the use of chemicals, and enhance crop productivity (Postma *et al*, 2003; Welbaum *et al*, 2004; Adams, 1990).

Fungi known for containing antibacterial, antifungal, phytotoxic properties and promoting plant growth are new sources to provide alternate tool for overcoming these problems (Chitarra *et al*., 2003). Fungal sp *Penicillium* EU0013 isolated from eucalyptus roots (Teshima and Sakamoto, 2006) has been observed to have antibacterial, antifungal, phytotoxic, insecticidal and nematicidal properties against microbes. Thus the aim of this research was to evaluate the potential of secondary metabolites produced by *Penicillium* sp. EU0013and determine their activities against various microbes.

MATERIALS AND METHODS

Collection of *Penicillium* sp. EU0013: The *Penicillium* sp. EU0013 is a newly fungal species isolated from eucalyptus roots. The fungus was obtained from Plant Pathology Department, The University of Agriculture Peshawar. The fungal strain was further sub cultured on potato dextrose agar (PDA) plates to get pure culture of *Penicillium* sp. EU0013 by using procedure of (Siameto *et al*., 2011).

Preparation of PDA and PDB: The PDA media was prepared by taking 200 g of potatoes and were thoroughly washed, peeled and then boiled in distilled water (300 mL) for 30 min. The material was filtered through muslin cloth and then 20 g of dextrose and agar were added to it. Distilled water was added to media till volume reached 1L. The media was sterilized in autoclave for 15 min at 121 °C. Preparation of both PDA and PDB were similar except no agar was added in case of PDB.

Seed Culture: Before starting the inoculation a Laminar flow cabinet was thoroughly washed with 70 % alcohol. UV (Ultra violet) light was switched on for 15 min for decontamination. After sterilization of PDA medium, streptomycin ($2mg.100\ mL^{-1}$) was added to prevent bacterial growth. Media 25 mL was poured in to each petri dish, and then with the help of a sterilized spatula *Penicillium* sp. (10 mm) was inoculated to each petri dish. The petri dishes were closed with paraffin film and

kept at room temperature for 8 days on a Laboratory bench.

Production Culture: For production culture *Penicillium* was inoculated in broth media. For this purpose 100 mL media was poured in 500 mL flasks and then transferred inoculums in each flask with the help of sterilized spatula. The flasks were covered with aluminum foil to avoid contamination and cultivated at room temperature for 30 °C in the laboratory for 8 days.

Antibacterial screening test: The phytopathogenic bacterial culture was obtained from plant pathology department. Activity of crude was carried out against plant pathogens *E. coli, Bacillus subtilis*, *Xanthamonas* and *Proteus valgaris* by crude diffusion method. The different concentrations used were 100, 200 and 300 ppm. In crude diffusion method the test compounds were expressed by measuring the zone of growth.

Procedure: In crude diffusion method 20 mg of crude extracts was dissolved in DMSO (Dimethyl sulfoxide) and prepared the potato dextrose agar media in petri dishes. Four plates were taken; one for positive control (streptomycin), second for negative control (only media) third for DMSO and in fourth crude was dissolved. In each petri dish 25 mL media was poured and when the temperature of the media lowered to 50 °C, crude was dissolved and then swirled clockwise and anticlockwise ten times. After 72 hours their zone of growth was measured.

Antifungal test: The antifungal test was carried out against pathogenic fungi (*Fusarium oxyspourm*, *Alternaria alternate*, *Aspergillus niger* and *Aspergillus flavus*) which causes wilt disease in various crops (Alam *et al*., 2010). Antifungal activity was carried out by using whole agar diffusion method. Crude extract of 20 mg was dissolved in DMSO (Dimethyl sulfa oxide). Four PDA plates were taken; one for positive control standard fungicide (Diethane M45), second for negative control (only media), third for DMSO and in the fourth petri dish crude was dissolved. In each petri dish 25 mL media was poured and when the temperature of the media lowered to 50 °C then crude was dissolved in each petri dish and swirl clockwise and anticlockwise ten times. After 48 hours their zone of growth was measured.

Brine Shrimp Lethality activity (BSLT): Cytotoxic activity of the crude extract of *Penicillium* sp. EU0013 was determined using the procedure of (Atta-ur-Rahman, 1991).

Phytotoxicity activity: The method of (Einhelling, 1986) was used for phytotoxicity determination.

Insecticidal activity: (Isman *et al*., 2005) method was used for insecticidal effect of natural products on certain insects.

Nematicidal activity: For extraction of nematodes from the soil (Cobb, 1918) method was used.

RESULTS AND DISCUSSION

Growth of *Penicillium* sp. EU0013 was checked in standard laboratory conditions. It was found that a substantial amount of biomass was produced at day 7 (Figure 1). Culture during fermentation changed color with time. At beginning the culture color turned slightly from fluffy white to greenish. On day 7th culture became greenish and then no further change of color was observed. This suggested that the production of natural products starts at day 3 and mostly they are produced until day 7 in the culture. The color change during fermentation corresponds to the production of secondary metabolites (Manjulata, 2011).

Antibacterial effects: Results Table 1 showed that crude *Penicillium* extract exhibited good activity against *E. coli and Proteus vulgaris* at each concentration, at 100 ppm growth of inhibition against *E. coli* recorded was (24.2 %) and *Proteus vulgaris* (10.2 %), at 200 ppm it was (53.2 %), (22.5 %) and at 300 ppm inhibition was (70.5 %) and (37.5 %).

The crude extract also showed promising results against *Bacillus subtilis* and *Xanthamonas* species. Strong inhibition was recorded at 300 ppm against *Xanthamonas* sp (88.8 %) followed by 200 ppm (87.5 %) and lowest was (57.8 %) at 100 ppm while against *Bacillus subtilis* it was (84.5 %), (61.5 %) and (45.6 %) respectively. A 100 % inhibition zone was shown by the bactericide against all the tested bacterial strains. No zone of inhibition was observed in negative control and (DMSO) (which was used as an extract carrier). Ghariaei also conducted antimicrobial disc assay against bacterial strains such as *Candida albicans, Bacillus subtilus, Staphylococcus aureus, Salmonella typhi* and *Escherichia coli* by using the organic extract of four fungal species i.e. *Penicillium verdicatum, Penicillium citrinum, Penicillium aurantiogriseum* and *Penicillium waksmani*. The findings were that these fungal extract exhibited strong activity against bacterial strains by inhibiting their growth. (Ghariaei *et al*., 2009) it was concluded that these fungi produces useful secondary metabolites during the study which suppress the growth of tested bacterial strains.

Antifungal activity: Diethane a fungicide was taken as standard in this assay which showed 100 % inhibition in all concentration. The negative control had no inhibition zone.

After 7th day zones of growth inhibition was observed of all the tested fungi and it was observed that the crude extract of *Penicillium* showed strong inhibition against *Fusarium oxyspourm* i.e. (88.8 %) at 300 ppm and (87.5 %) at 200 ppm. These results were followed by

A. alternata and A. flavus at 300 ppm was (84.5 % and 74.5 %) and at 200 ppm (61.5 % and 52.1 %), respectively. While minimum inhibition was recorded against *A. niger* in all concentration (37.5 %, 300 ppm; 22.5 %, 200 ppm and 10.2 %, 100 ppm).

These results are in favor of those presented (Rosario and Stefano, 2012) who reported that cultural filtrates of several isolates of *Penicillium canescens* and *P. janczewski* produced some fungi toxic extrolites that inhibit the growth of plant pathogenic fungus *Rhizoctonia solani.* Also a new and unreported metabolite curvulinic acid was observed during the study. Sch 642305 another extrolite was detected in five isolates of *Penicillium canescens* and *P. verrucosumstrain.* The purified compound was tested against *Rhizoctonia solani* which completely inhibit its growth and some other plant pathogenic fungi in vitro.

These results also indicated that *Penicillium* sp. EU0013 had the potential to inhibit the growth of *Fusarium oxyspourm* even at low concentrations. This specie is considered as plant growth promoting fungi for the seedlings of cabbage (Teshima and Sakamoto, 2006) and tomato by increasing the supply of nitrogen content (Alams *et al*., 2010)

Phytotoxic activity: The phytotoxic activity of crude extract of *Penicillium* sp. EU0013, Atrazin and control after 7 days is shown in table 3.

It was observed that Atrazin (positive control) exhibit 100 % mortality against *lemna minor*, whereas in highest concentration the percent mortality had reached up to 55.33 %. The least mortality was noticed in control followed by low dose of crude extract of *Penicillium* (10 $\mu g.mL^{-1}$) with mortality of 17.66 %. The LC_{50} calculated was 119.05.

Results showed that with increasing dose of crude extract, percentage mortality of *lemna minor* increased. These results were in a best agreement with the findings of (Iqbal *et al*., 2014 and 2015), who examined the phytotoxic effect of *Rhizopus stolonifer* and *Aspergillus niger* crude extract. Their findings were that at lowest doses the mortality was the lowest, which increased with the increased dose of the crude extract to *Lemma minor* plants.

Insecticidal activity: Table 4 shows the observed effect of control, copex and various crude extract concentrations of *Penicillium* sp. EU0013 on aphid after 24 hours.

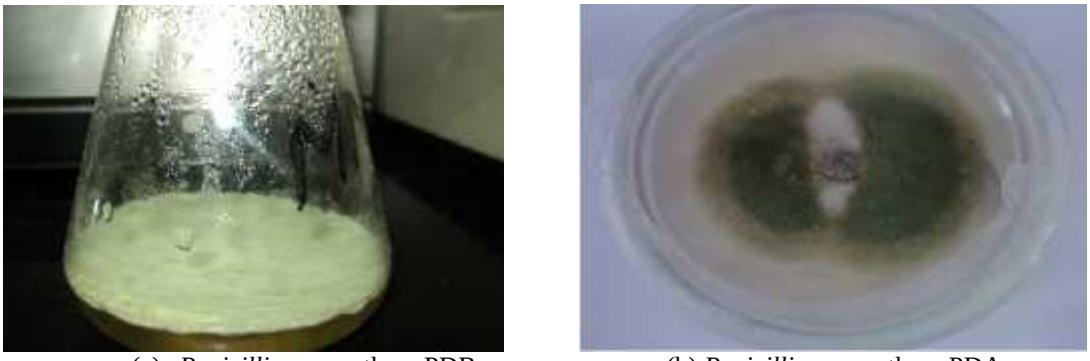
It was observed that copex had the highest percent of mortality (93.32 %). The least mortality was notice in control which was 11.65% after 24 hours. At various doses, highest mortality percent was observed in highest dose of crude extract of *Penicillium* sp. EU0013 200 ppm, similarly the least mortality percent (27.4) was noticed in application of lowest doses (10 $\mu g.mL^{-1}$). The LC_{50} recorded was 8.88.

These results were supported by (Santamarina *et al*., 2002) who examined insecticidal effect of *Penicillium* extract. According to them, *Oncopeltus fasciatus* was cultivated in potato dextrose broth and wickerham broth for three days and their crude extract showed percent mortality ranging from 13.3 to 80, whereas upto 7 days the mortality was reported to reach 100 %.

Nematicidal activity: Table 5 shows the effect of crude extract of *Penicillium* sp. EU0013 against nematodes at various concentrations.

The highest mortality was 85.75 % at100 $\mu g.mL^{-1}$, which is being the high dose. The least percent mortality was 0.00 of that control, followed by lowest dose of crude extract (10$\mu g.mL^{-1}$) and the mortality examined was 19 %. The LC_{50} was observed from logarithmic trendline as it being the clearest line of resultant dots with 13.50.

These results were supported by (Nakahara *et al*., 2004), who determined high nematicidal effect from *Penicillium* extract comparative to the present study. (Nakahara *et al*., 2004) had isolated two compounds from *Penicillium* specie which showed nematicidal effect ranging from 52 to 98 % mortality at300 µg/mL. (Kusano *et al*., 2000) had found alkaloid natural products from the *Penicillium* that had such nematicidal effects.



(a) *Penicillium* growth on PDB (b) *Penicillium* growth on PDA

Figure 1. Growth of *Penicillium* sp. EU0013 on different Medias

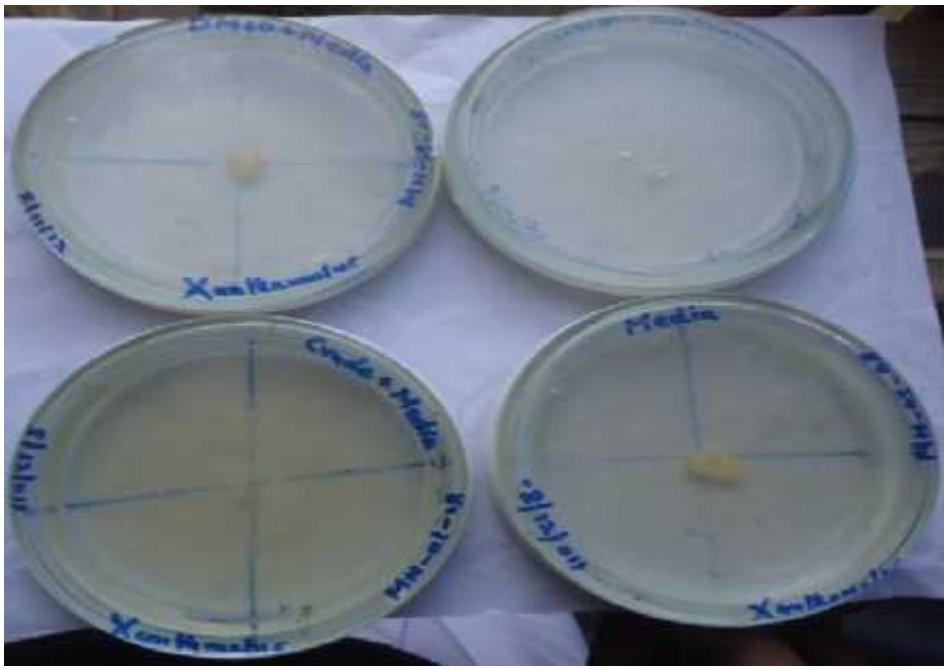


Figure 2. Zone of growth on agar plate against *Xanthamonas* sp.

Table 1. Antibacterial activity of the acetonitrile whole cell extract of *Penicillium* EU0013 against selected bacterial isolates.

	Zone of inhibition ± SEM (mm)				
	Extract concentration (ug.ml $^{-1}$)				
Bacterial isolate	Positive control streptomycin	300	200	100	Negative control
E. coli	0.00 (100%)	22.7 ± 1.4 (70.5)	36.3 ± 1.6 (52.1)	53.0 ± 1.7 (24.2)	68.3 ± 0.5
Bacillus subtilis	0.00 (100%)	15.6 ± 2.8 (84.5)	33.0 ± 3.1 (61.5)	46.0 ± 3.1 (45.6)	80.3 ± 0.2
Xanthamonas sp	0.00 (100%)	13.4 ± 1.5 (88.8)	22.0 ± 2.1 (87.5)	36.3 ± 1.8 (57.8)	79. 3 ± 0.2
Proteus vulgaris	0.00 (100%)	58 .6 ± 3.6 (37.5)	72.3 ± 2.0 (22.5)	80.7 ± 1.7 (10.2)	89.3 ± 0.4

Note: % inhibition data in parenthesis (with reference to negative control. The positive control ciprofloxacin) showed 100 % inhibition even at very low concentration (250 µg ml^{-1})

(a) Crude + media, (b) fungicide + media (c) DMSO + media, (d) media

Figure 3. Zone of growth of fungal species on agar plates using organic extract of *Penicillium* EU0013.

Table 2. Antifungal activity of the Ethyl acetate whole cell extract of *Penicillium* EU0013 against selected fungal isolates.

	Zone of growth ± SEM (mm)				
	Extract concentration (ug.ml $^{-1}$)				
Fungal isolate	Positive control Diethane M 45)	300	200	100	Negative control
A. flavus	0.00 (100%)	23.7 ± 1.3 (70.5)	35.3 ± 1.4 (52.1)	53.0 ± 1.7 (24.2)	68.3 ± 0.5
A. alternata	0.00 (100%)	16.7 ± 2.6 (84.5)	34.0 ± 2.1 (61.5)	46.0 ± 3.1 (45.6)	80.3 ± 0.2
F. oxyspourm	0.00 (100%)	13.3 ± 1.4 (88.8)	21.0 ± 2.1 (87.5)	36.3 ± 1.8 (57.8)	79. 3 ± 0.2
A. niger	0.00 (100%)	57 .6 ± 3.3 (37.5)	70.3 ± 2.0 (22.5)	80.7 ± 1.7 (10.2)	89.3 ± 0.4

(Note % inhibition data in parentheis (with reference to negative control. The positive control Diethane M 45) showed 100 % inhibition even at very low concentration (250 µg ml^{-1}).

Table 3. Phytotoxic activity of crude extract of *Penicillium sp.* EU0013 against *Lemna minor* after 7 days. No. of dead plants show as Means ± SD.

Doses (µg.mL^{-1})	Total	No. of dead plants	% Mortality	LC_{50}µg.mL^{-1}
Control	40	00 ± 00	0	
Atrazine	40	30 ± 00	100	
10	40	5.3 ± 1.1	17.66	119.05
50	40	8.6± 0.5	28.66	
100	40	13± 1.0	43.33	
200	40	16.6± 1.1	55.33	

Figure 4. Phytotoxic activity of *Penicillium sp.* EU0013 crude extract against *Lemna minor* (Duck weed).

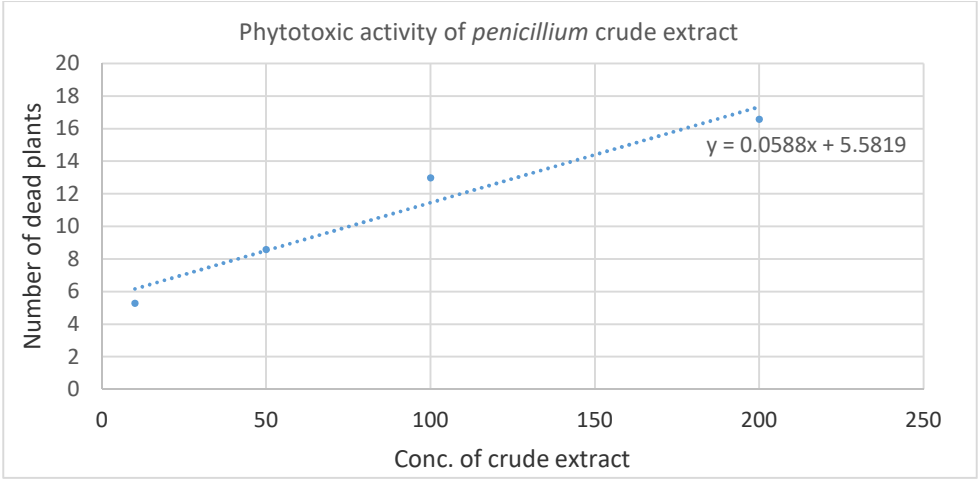


Figure 3. Phytotoxic activity of *Penicillium sp.* EU0013 crude extract against *Lemna minor.*

Table 4. Insecticidal activity of crude extract of *Penicillium sp.* EU0013 after 24 hours against aphids No. of dead aphids show as Means ± SD.

Doses (µg.mL^{-1})	Total (aphids)	No. of dead aphids	% Mortality	LC_{50} (µg.mL^{-1})
Control	40	4.66± 1.15	11.65	
Copex	40	37.33± 1.53	93.32	
10	40	11± 1.00	27.5	
50	40	15± 1.00	37.5	8.88
100	40	17.66± 0.57	44.15	
150	40	21.66± 0.57	54.15	
200	40	25.33± 0.57	63.32	

Figure 4. Insecticidal activity of *Penicillium* EU0013 crude extract against rose aphids.

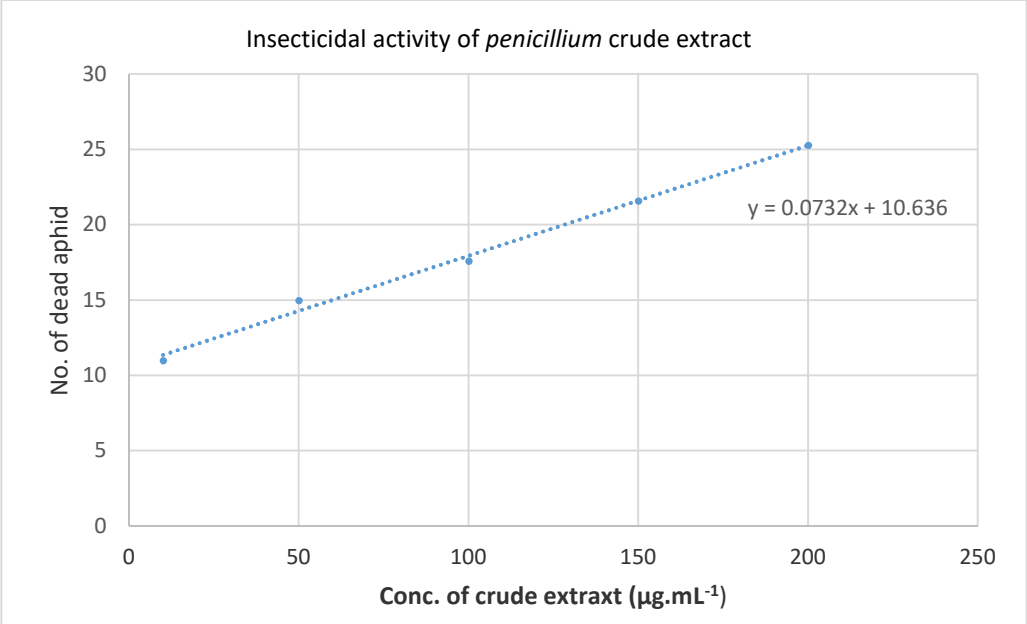


Figure 4. Insecticidal activity of *Penicillium* EU0013 crude extract on aphids

Table 5. Nematicidal activity of crude extract of *Penicillium* sp. EU0013 against eel worm No. of dead nematodes shows as Means ± SD.

Dose (µg.mL^{-1})	Total	No. of dead nematodes	% Mortality	LC_{50} (µg.mL^{-1})
Control	40	0.00± 000	0.00	
5	40	7.6± 1.52	19	
10	40	15± 1.73	37.5	13.50
20	40	21.60± 1.53	54	
50	40	28.3± 1.15	70.75	
100	40	34.3± 1.52	85.75	

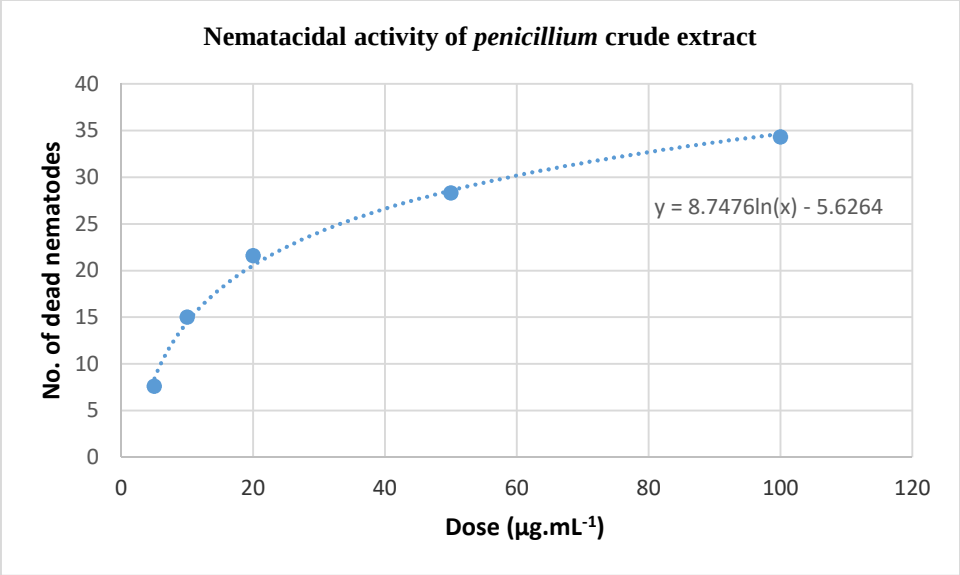


Figure 5. Nematacidal activity of *Penicillium* sp. EU0013 crude extract.

Conclusion: It is concluded from these results that the *Penicillium* crude extract exited strong activities against different bacteria fungi and nematodes and can be used as a biocontrol agent.

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