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Snailicidal, antimicrobial, antioxidant and anticancer activities of *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces lilacinus* fungal extracts

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ABSTRACT

Fungi are capable of producing a wide variety of secondary metabolites. Entomopathogenic fungi along with species which parasitize nematodes represent excellent candidates for bioactive compounds production. This study aimed at exploring the snailicidal, antimicrobial and antioxidant activities of ethyl acetate and acetone extracts of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces lilacinus*. Ethyl acetate and acetone extracts were prepared and tested against *Biomphalaria alexandrina* snails, their antioxidant and antimicrobial activities were also investigated. The anticancer activity of *P. lilacinus* acetone extract was tested on hepatocellular carcinoma (HepG2) cells. Acetone extract of *P. lilacinus* (LC_{50} =120 ppm) was more toxic to *B. alexandrina* snails than that of *B. bassiana* (LC_{50}= 231 ppm). Sublethal concentrations of both extracts adversely affected survival rate, histological pattern of digestive and hermaphrodite glands of the tested snails, besides their genotoxicity to DNA of snails. *P. lilacinus* acetone extract showed the highest antioxidant activity. Also, its antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* was high. Moreover, it was cytotoxic to HepG2 cells (IC_{50} =2.81 µg/ml). GC/MS analysis indicated the presence of 37 compounds in *B. bassiana* acetone extract with 2-methylhept-2,6-dien-4-ol and 1,3-dimethoxy-2-(hydroxymethyl)-9h-xanthene as major compounds. While 48 compounds were identified in *P. lilacinus* acetone extract where hexadecanoic acid, methyl ester and 10, 13 octadecadienoic acid, methyl ester were the major compounds. In conclusion, *B. bassiana* and *P. lilacinus* are excellent sources of bioactive compounds which have multiple biological effects.

INTRODUCTION

Fungi are recognized as prolific producers of secondary metabolites which have high therapeutic value as antibiotics, cytotoxic substances, insecticides,

compounds that promote or inhibit growth, attractors and repellents, besides their biotechnological applications (Demain, 1999; Kishore *et al*., 2007; Mabrouk *et al*., 2008; Abdel-Aziz *et al*., 2018). Entomopathogenic fungi are classified as fungi that infect, invade and eventually kill their insects (Singkaravanit *et al*., 2010). Some entomopathogenic isolates such as *Metarhizium anisopliae* and *Beauveria bassiana* have been investigated for use against a broad range of insect pests (Sheng, 2007; Ezz *et al*., 2008), they are environmentally friendly and not harmful to the public health, hence they have been developed and commercialized for pest control (Francardi *et al*., 2015).

Paecilomyces lilacinus is a soil inhabiting fungus that is capable of parasitizing nematode eggs, juveniles and females thus reducing soil population of plant parasitic nematodes. Strains of this fungus have been formulated to be applied for controlling nematodes in many countries (EPA, 2005; Kiewnick and Sikora, 2003; 2006). Also, a strain of *P. lilacinus* showed snailicidal effect against the invasive apple snail *Pomacea canaliculata* (Maketon *et al*., 2009).

Generally, diverse toxic metabolites have been described in several fungal biological control agents including *Beauveria*, *Metarhizium* and *Pacilomyces* (Vey *et al*., 2001), some of these metabolites have been found to possess antibiotic, fungicidal or insecticidal properties (Kershaw *et al*.,1999; Vey *et al*., 2001; Ross, 2005). Production of oosporein, beauvencin, bassianolide, and cyclosporine A was commonly observed in cultures of some entomopathogenic fungi (Boucias and Pendland, 1998; Strasser *et al*., 2000). Moreover, Parine *et al*. (2010) found that secondary metabolites of *B. bassiana* showed antifungal and antibacterial effects against some pathogens. Thus, the current study aimed at exploring the bioactive compounds produced by *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces lilacinus* by studying the snailicidal, antimicrobial, antioxidant and anticancer activities of their extracts.

MATERIALS AND METHODS

Maintenance and rearing of snails

Biomphalaria alexandrina snails were obtained from irrigation canals in Kafr Hakeem area, Giza Governorate. They were maintained in glass aquaria (40 X20 X25cm) containing dechlorinated tap water, which was changed twice weekly. The snails were kept under laboratory conditions (22 ± 2°C), and fed fresh lettuce leaves. These snails were allowed to acclimatize in the laboratory conditions three weeks before being used in experimental tests.

Cultivation and extraction of fungi

The fungi used in this work were *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces lilacinus*. These fungal strains were obtained from the commercial products; Biover©, Bioranza© and Bio-Nematon©, respectively. They were kindly provided as powders by Central Agricultural Pesticides Laboratory, Agricultural Research Center, Egypt. To get the fungal strains, one g of each powder was dissolved in 9 ml sterilized distilled water and shaken well. Then, one ml of this mixture was transferred and spread, under aseptic condition, onto the surface of Czapek-Dox agar medium of the following composition (g/l): Sucrose (30.00), $NaNO_3$ (3.0), $MgSO_4$ x 7 H_2O (0.50), KCl (0.50), $FeSO_4$ x 7 H_2O (0.01), K_2HPO_4 (1.0), agar (18.0), distilled water (1000.0 ml) and the pH was adjusted to 6.5. The inoculated plates were incubated at 28°C for 7 days. Scale-up fermentation was carried out using three 1 L Erlenmeyer flasks for each fungus, each contains 100 g

rice and 100 ml distilled water, sterilized at 121°C (15 lb) for 20 min. Each flask was inoculated with spore suspension from ten days old cultures. After incubation at 30°C for 15 days, the medium was extracted several times with ethyl acetate till exhaustion (ethyl acetate extract).The mycelial components, in the medium, were extracted by acetone followed by ethyl acetate (acetone extract) (Abdel-Aziz *et al*., 2018).

Toxicity of fungal extracts against *Biomphalaria alexandrina* snails

Six fungal extracts were used in toxicity tests to prepare a series of concentrations using dechlorinated tap water at 22±2°C. Three replicates were used; each of ten snails (8-10 mm in diameter) for each concentration. The exposure period was 24 h followed by another 24 h as a recovery period. Another group of snails was maintained under the same experimental conditions as a control group (WHO, 1965). Dead snails were counted, and LC_{50} values of the most toxic fungal extracts were computed (Litchfield and Wilcoxon, 1949).

Effect of fungal extracts on survival rate of adult snails

This experiment was designed to explore the effect of prolonged exposure to sublethal concentrations (LC_5, LC_{15} and LC_{25}) of the most effective fungal extracts on survival rate of *B. alexandrina* snails (8-10 mm in diameter). A group of 20 adult snails was exposed in four replicates to each sublethal concentration. A control group was maintained in clean dechlorinated tap water under the same experimental conditions. These snails were fed on dried lettuce leaves. Dead snails were removed from the containers every day and their number was recorded.

Effect of fungal extracts on digestive and hermaphrodite glands of B. alexandrina snails

Adult *B. alexandrina* snails (five from each treatment, 8-10 mm in diameter) exposed for 4 weeks to LC_{15} of investigated extracts were dissected. The snail shell was gently broken between two slides, shell parts were removed carefully, then the soft part was obtained and slightly washed in a drop of distilled water. The upper portion of the soft part which includes the hermaphrodite and digestive glands was cut, and immediately fixed in Bouin's solution for 24 h. The fixed samples were dehydrated, cleared, and embedded in paraffin. Then they were sectioned serially at 5 µm and stained with hematoxylin and eosin (Mohamed and Saad, 1990).

Determination of genotoxicity of fungal extracts to B. alexandrina snails using COMET assay

Head –foot region of five snails exposed to LC_{15} of the effective extracts and control group were cut and kept at -4°C. For analysis, these samples were crushed, then transferred to one ml ice-cold PBS. This suspension was stirred for 5 min. and filtered. Cell suspension (100 µl) was mixed with 600 µl of low-melting agarose (0.8% in PBS). 100 µl of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2V/cm for 2 min. and 100 mA. They were stained with ethidium bromide (20 µg/ml) at 4°C. The samples were investigated when they were still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (with excitation filter of 420-490 nm [issue 510 nm]). Lengths of comet tails were measured from the middle of the nucleus to the end of the tail, and the size of the comet was determined. For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope. A Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool,

UK) linked to a CCD camera was used to assess the quantitative and qualitative extent of DNA damage in the cells. It was carried out by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculated tail moment. Generally, 50 to 100 randomly selected cells were analyzed per sample.

% DNA in tail = total intensity of tail /total intensity of comet (head and tail) X100, and tail moment = tail length X % DNA in tail. Both % DNA in tail and tail moment are directly proportional to DNA damage (Singh *et al*., 1988).

Antimicrobial activity of fungal extracts

Disc agar plate method was established to evaluate the antimicrobial activities of the six fungal extracts. Four different test microbes, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*, were selected to evaluate the antimicrobial activities as representatives of Gram+ bacteria, Gram− bacteria, yeast, and fungal groups, respectively. The bacterial and yeast test microbes were grown on nutrient agar medium. While, *Aspergillus niger* was cultivated on potato dextrose agar (PDA) medium. The culture of each test microbe was diluted by sterilized distilled water to 10^7 to 10^8 colony forming units (CFUs)/ ml, and then 1 ml of each was used to inoculate 1 L Erlenmeyer flask containing 250 ml of solidified agar media. These media were put onto previously sterilized Petri dishes (25 ml of solidified medium/10 cm diameter Petri dish). Five millimeter filter paper discs loaded with 0.2 mg of each extract were dried at room temperature under sterilized conditions. These paper discs were placed on agar plates seeded with test microbes and incubated for 24 h, at the appropriate temperature of each test organism. Antimicrobial activities were recorded as the diameter of inhibition zones (including the disc itself) that appeared around the discs. Neomycin (100 µg/ disc) and cyclohexamide (100 µg/disc) were used as antibacterial and antifungal standards, respectively (Bauer *et al*., 1966).

Total Antioxidant Capacity (TAC) of fungal extracts

Antioxidant activity of the six extracts was determined according to phosphomolybdenum method using ascorbic acid as a standard. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green colored [phosphate=Mo (V)] complex at acidic pH with a maximal absorption at 695 nm. In this method, 0.5 ml of each compound (100 µg/ ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample, and it was incubated under the same conditions. All experiments were carried out in triplicates. The antioxidant activity of the sample was expressed as the number of ascorbic acid equivalent (AAE) (Prieto *et al*., 1999).

Anticancer activity of acetone extract of *Paecilomyces lilacinus*

Human hepatocellular cancer cell line (HepG2) obtained from the American Type Culture Collection (ATCC, Rockville, MD) were grown on RPMI-1640 medium supplemented with 10 % inactivated fetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week.

The tumor cell lines were suspended in the medium at a concentration of $5x10^4$ cell/well in Corning® 96-well tissue culture plates, then incubated for 24 h. The tested extract was then added into 96-well plates (three replicates) to get twelve

concentrations for each compound. Six vehicle controls with media or 0.5 % DMSO were run for each 96 well plate as a control. After incubation for 24 h, the number of viable cells was determined by measuring the optical density at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA). Then, this equation was applied:

$$\text{The percentage of viability} = (OD_t / OD_c) \times 100$$

Where OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of untreated cells. The relation between surviving cells and drug concentration was plotted to get the survival curve of each tumor cell line after treatment with the specified extract. The concentration required to cause toxic effects in 50% of intact cells (IC_{50}), was estimated from graphic plots of the dose response curve (Mosmann, 1983; Elaasser *et al.*, 2011).

Gas chromatography/mass spectrometry (GC/MS) analysis of fungal extracts

The GC/MS analysis of the most effective extracts was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS,TG-5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1ml/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed to an initial temperature of 50°C (hold 2 min) to 150 °C at an increasing rate of 7°C/min. then to 270 at an increasing rate of 5°C/min (hold 2 min) then to 310°C as a final temperature at an increasing rate of 3.5 °C/min (hold 10 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of the irrelative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

Statistical analysis

Student t-test was used to determine the significance of difference between each two treatments, and between each treatment and control group in COMET assay test. ANOVA was used to determine the significance of difference between treatment groups in antioxidant test. All statistical analyses were carried out using SPSS v. 17.0 for Windows.

RESULTS

Toxicity of fungal extracts against *Biomphalaria alexandrina* snails

Only acetone extracts of *Beauveria bassiana* and *Paecilomyces lilacinus* had molluscicidal effect on *B. alexandrina* snails, as *P. lilacinus* was more toxic than *B. bassiana*, with LC_{50} values of 120 and 231 ppm, respectively (Table 1).

Table 1. The values of LC_{50} and sublethal concentrations of effective fungal extracts on adult *B. alexandrina* snails after 24 h of exposure.

***Beauveria bassiana* acetone extract (ppm)**				***Paecilomyces lilacinus* acetone extract (ppm)**			
LC_5	LC_{15}	LC_{25}	LC_{50}	LC_5	LC_{15}	LC_{25}	LC_{50}
23	69	115	231	12	36	60	120

Effect of sublethal concentrations of acetone extracts of Beauveria bassiana and Paecilomyces lilacinus on survival rate of Biomphalaria alexandrina snails

Generally, survival rate of snails exposed to acetone extract of *B. bassiana* was lower than that of snails exposed to acetone extract of *P. lilacinus*. The survival rate

of snails exposed to LC_5 (23 ppm) and LC_{25} (115 ppm) of *B. bassiana* extract reached 20% at the 4th week of exposure compared to 80% for control snails (Figs. 1 a & b).

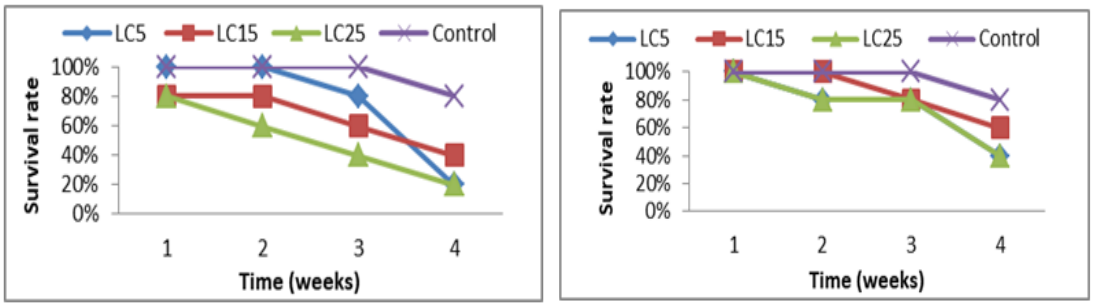

Fig. 1. Effect of sublethal concentrations of (a) *B. bassiana* acetone extract and (b) *P. lilacinus* acetone extract on survival rate of *B. alexandrina* snails

Effect of fungal extracts on digestive and hermaphrodite glands of Biomphalaria alexandrina snails

Fig. (2a) shows that normal digestive gland consists of a number of tubules; each tubule contains two types of cells; digestive cells and secretory cells. Exposure of snails to LC_{15} (69 ppm) of *B. bassiana* acetone extract resulted in extensive vacuolation of digestive cells, and degeneration of secretory ones (Fig. 2b). Also, the effect of LC_{15} (36 ppm) of *P. lilacinus* acetone extract was vacuolation of digestive cells, but some vacuoles contained certain secretions. Moreover, rupture of tubular membrane and degeneration of secretory cells and connective tissue were observed (Fig. 2c).

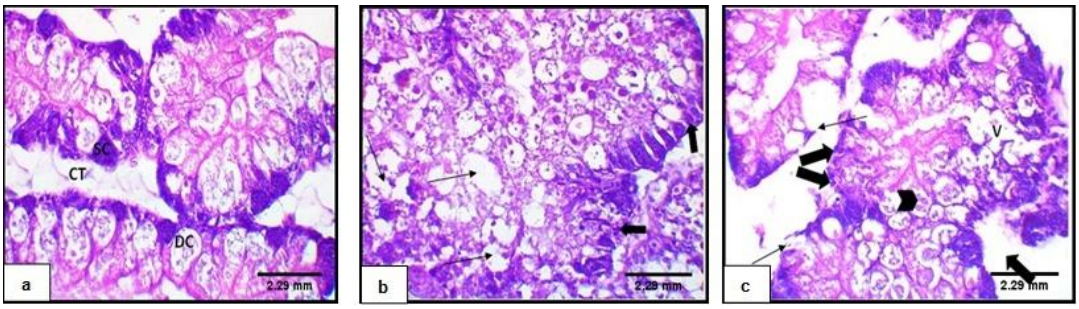

Fig. 2: Photomicrographs of T.S. in the digestive gland of *Biomphalaria alexandrina* snails. a, normal *B. alexandrina* snails showing tubules containing digestive cells (DC) and secretory cells (SC), connective tissue (CT) between tubules; b, snails exposed to LC_{15} of *B. bassiana* acetone extract showing vigorous vacuolation of digestive cells (thin arrows), irregular shape of tubules and degeneration of secretory cells (thick arrows); c, snails exposed to LC_{15} of *P. lilacinus* acetone extract showing rupture of tubular membrane (thin arrows), vacuolation of digestive cells (V), some vacuoles contain certain secretions (head arrow), secretory cells and connective tissue between tubules are degenerated (thick arrows)

Regarding hermaphrodite gland, the normal one consists of a number of acini; each acinus contains the stages of oogenesis (primary oocytes, secondary oocytes and mature ova) and spermatogenesis (primary spermatocytes, secondary spermatocytes and sperms) (Fig. 3a). Exposure of snails to LC_{15} of *B. bassiana* acetone extract resulted in a number of deleterious effects on the gland, as few secondary spermatocyes were scattered in the acinus, they were either degenerated or

densely stained without differentiation between their cytoplasmic inclusions and nuclei (Fig. 3b). Exposure to LC_{15} of *P. lilacinus* acetone extract resulted in vacancy of the acinus, as only secondary spermatocytes were found (Fig. 3c).

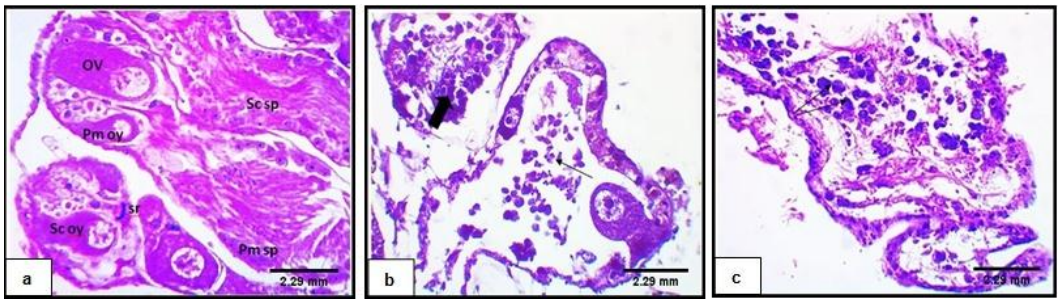

Fig. 3: Photomicrographs of T.S. in the hermaphrodite gland of *Biomphalaria alexandrina* snails. a, normal *B. alexandrina* snails showing primary oocyte (Pm oy), secondary oocyte (Sc oy) and mature ovum (OV), primary spermatocytes (Pm sp), secondary spermatocytes (Sc sp) and sperms (sr); b, snails exposed to LC_{15} of *B. bassiana* acetone extract showing degenerated secondary spermatocytes (thin arrow) and densely stained ones without differentiation between their cytoplasmic inclusions and nuclei (thick arrow); c, snails exposed to LC_{15} of *P. lilacinus* acetone extract showing the presence of only secondary spermatocytes (arrows).

Genotoxicity of fungal extracts to Biomphalaria alexandrina snails

Results in Table (2) and Fig. (4) showed the effect of LC_{15} of *B. bassiana* and *P.lilacinus* acetone extracts on DNA of snails. There was a significant difference in the values of tail moment between these two treatments as the highest tail moment was recorded in the DNA of snails exposed to LC_{15} of *B. bassiana* acetone extract. Both extracts resulted in noticeable DNA damage compared to control group, whereas *B. bassiana* acetone extract was more genotoxic than that of *P. lilacinus.*

Table 2: Significance of difference in comet assay results of *B. alexandrina* snails.

Extract	Tailed %	Untailed %	Tail length (µm)	Tail DNA %	Tail Moment (UNIT)
LC_{15} of *Beauveria bassiana* acetone extract	7	93	2.48±0.05*[a]	2.39	5.93±0.03*[a]
LC_{15} of *Paecilomyces lilacinus* acetone extract	9	91	2.55±0.02*[a]	2.29	5.84±0.01*[b]
Control	4	96	1.16±0.01[b]	1.26	1.46±0.02[c]

*The result is significantly different from control at $P< 0.05$

Different letters in the same column indicate significant difference at $P<0.05$, while similar letters indicate no significant difference

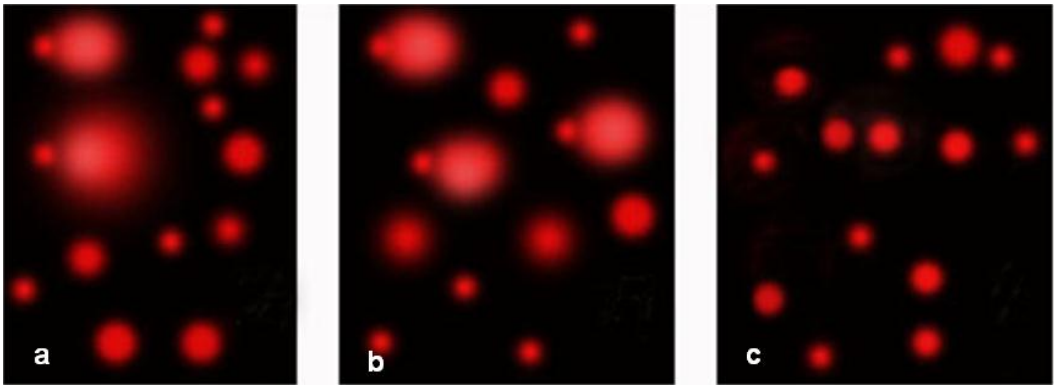

Fig. 4: Comet assay of *B. alexandrina* snails showing (a) snails exposed to LC_{15} of *B. bassiana* acetone extract, (b) snails exposed to LC_{15} of *P. lilacinus* acetone extract and (c) control snails

Antimicrobial activity of fungal extracts

Acetone extracts of *Paecilomyces lilacinus* and *Metarhizium anisopliae* showed the highest activities against the tested bacterial strains and *Candida albicans*. The diameters of inhibition zones for *Staphylpcoccus aureus*, *Escherichia coli* and *C. albicans* treated with *P. lilacinus* acetone extract were 17, 18 and 18 mm, respectively. While *M. anisopliae* acetone extract resulted in 14, 16 and 19 mm inhibition zones against *S. aureus*, *E. coli* and *C. albicans*, respectively (Table 3).

Table 3: Antimicrobial activity of ethyl acetate and acetone extracts of the fungal strains grown on rice medium

Sample	Inhibition zone (mm)			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
EtOAc of *M. anisopliae*	10	7	8	0
EtOAc of *B. bassiana*	9	6	7	0
EtOAc of *P. lilacinus*	9	9	9	0
Acetone of *M. anisopliae*	14	16	19	0
Acetone of *B. bassiana*	8	6	6	0
Acetone of *P. lilacinus*	17	18	18	0
Neomycin	23	22	19	0
Cyclohexamide	0	0	0	28

Total Antioxidant Capacity (TAC) of fungal extracts

Table (4) shows that the highest antioxidant capacity was that of *Paecilomyces lilacinus* acetone extract (154.49), followed by *Beauveria bassiana* acetone extract (82.39).

Table 4: Total antioxidant capacity (TAC) of fungal extracts

Sample	Total antioxidant capacity (mg AAE/g dry extract)[1,2]
EtOAc of *M. anisopliae*	46.81 ± 3.24*
EtOAc of *B. bassiana*	61.79 ± 5.62*
EtOAc of *P. lilacinus*	76.77 ± 7.06*
Acetone extract of *M. anisopliae*	77.71 ± 3.24*
Acetone extract of *B. bassiana*	82.39 ± 5.58*
Acetone extract of *P. lilacinus*	154.49 ± 2.81*

[1]Results are (Mean ± SD) (n = 3)
[2]AAE: Ascorbic acid equivalent
*All the results are significantly different from each other at P< 0.05.

Anticancer activity of *Paecilomyces lilacinus* acetone extract

The results demonstrated that *P. lilacinus* acetone extract exhibited high inhibitory activity against HepG2 cells as IC_{50} was 2.81µg/ml (Fig. 5).

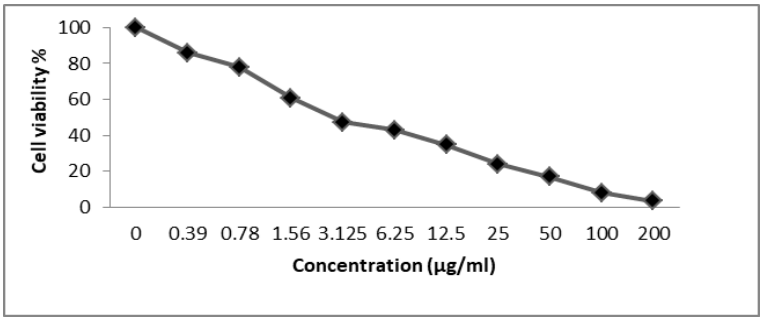

Fig. 5: Effect of *P. lilacinus* acetone extract on HepG2 cell line viability

Gas chromatography/mass spectrometry (GC/MS) analysis of effective fungal extracts

GC/MS analysis indicated that *B. bassiana* acetone extract consists of 37 compounds. The total peak area of the detected compounds is 98.5 %. The probabilities of the structures of the detected compounds are listed in Table (5), where the major peak areas were 41.59% for 2-methylhept-2,6-dien-4-ol ($C_8H_{14}O$) which is an alkene derivative and 33.85% for 1,3-dimethoxy-2- (hydroxymethyl)-9h-xanthene ($C_{16}H_{16}O_4$) (Fig. 6a).

Table 5: GC/MS analysis of *Beauveria bassiana* acetone extract

Peak No.	R_t (min)	MW	MF	Area %	Identified compounds
1	10.11	126	$C_8H_{14}O$	41.59	2-Methylhept-2,6-dien-4-ol
2	11.93	142	$C_9H_{18}O$	0.13	7-methyl-6-octen-3-ol
3	20.86	284	$C_{16}H_{13}ClN_2O$	0.21	2H-1,4-Benzodiazepin-2-one,7-chloro-1,3-dihydro-1-methyl-5-phenyl-
4	25.22	414	$C_{30}H_{54}$	0.65	Cyclohexane,1,1',1",1'"-(1,6-hexanediylidene) tetrakis-
5	25.71	542	$C_{40}H_{62}$	0.32	Phytofluene
6	28.12	354	$C_{10}H_{10}Br_2F_2N_2$	0.14	2,6-Dibromo-3,5-difluoro-4-piperidinylpyridine
7	29.15	376	$C_{22}H_{37}BO_4$	1.08	Pregnane-3,11,20,21-tetrol, cyclic20,21-(methylboronate),(3à,5à,11á,20S)
8	29.52	242	$C_{15}H_{30}O_2$	1.11	Tetradecanoic acid, methyl ester
9	30.98	366	$C_{26}H_{54}$	0.57	Octadecane, 3-ethyl-5-(2-ethylbutyl)
10	31.57	228	$C_{14}H_{28}O_2$	0.21	Tetradecanoic acid
11	33.22	490	$C_{35}H_{70}$	0.38	17-Pentatriacontene
12	33.76	272	$C_{16}H_{16}O_4$	33.85	1,3-dimethoxy-2-(hydroxymethyl)-9h-xanthene
13	34.80	300	$C_{17}H_{32}O_4$	1.25	Nonanedioic acid,dibutyl ester
14	35.01	310	$C_{22}H_{46}$	3.48	Docosane
15	35.32	362	$C_{26}H_{50}$	0.17	Cyclohexane,1,1'-dodecylidenebis[4-methyl-
16	35.70	298	$C_{19}H_{38}O_2$	2.07	Octadecanoic acid, methyl ester
17	36.09	378	$C_{27}H_{54}$	0.13	Cyclohexane,1,3,5-trimethyl-2-octadecyl-
18	36.91	294	$C_{19}H_{34}O_2$	0.28	Methyl 10-trans,12-cis-octadecadienoate
19	37.14	296	$C_{19}H_{36}O_2$	0.16	16-Octadecenoic acid, methyl ester
20	38.22	562	$C_{38}H_{74}O_2$	3.50	Oleic acid, eicosyl ester
21	38.68	366	$C_{26}H_{54}$	0.22	Octadecane, 3-ethyl-5-(2-ethylbutyl)
22	40.30	408	$C_{29}H_{60}$	0.14	Nonacosane (CAS)
23	42.00	290	$C_{23}H_{14}$	0.20	8H-Benzo[g]cyclopenta[mno]chrysene
24	42.66	354	$C_{21}H_{38}O_4$	1.07	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester
25	42.73	356	$C_{21}H_{40}O_4$	1.73	2,3-Dihydroxypropylelaidate
26	43.42	304	$C_{21}H_{24}N_2$	0.37	trans-2-Methyl-2-phenyl-1-tert-butyl-1,2-dihydroazeto[2,1-b]quinazoline
27	43.85	490	$C_{35}H_{70}$	0.26	17-Pentatriacontene
28	44.36	390	$C_{24}H_{38}O_4$	0.41	2-Benzenedicarboxylic acid,
29	45.19	460	$C_{25}H_{21}BrN_2O_2$	0.14	2-(3-Bromophenyl)-3,3-diphenyl-4-ethoxycarbonyl-5-amino-3H-pyrrole
30	46.07	610	$C_{27}H_{30}O_{16}$	0.18	Lucenin 2
31	46.60	356	$C_{21}H_{40}O_4$	0.67	9-Octadecenoic acid (Z)-
32	47.71	378	$C_{22}H_{34}O_5$	0.44	Benzenedodecanoic acid
33	48.28	548	$C_{30}H_{44}O_9$	0.14	Carda-4,20(22)-dienolide,3-[(6-deoxy-3-O-methyl-à-D-allopyranosyl)oxy]-1,14-dihydroxy-, (1á,3á)-
34	49.15	354	$C_{23}H_{46}O_2$	0.14	Docosanoic acid, methyl ester
35	50.41	357	$C_{17}H_{12}BrNOS$	0.55	2-[à-(p-bromophenyl)-á-mercaptoethenyl]isoquinolin-1-(2H)-one
36	52.02	442	$C_{26}H_{34}O6$	0.43	Dihydrodeacetylgedunin
37	52.32	414	$C_{29}H_{50}O$	0.13	Stigmast-5-en-3-ol,(3á,24S)-

R_t: Retention time; MW: Molecular weight; MF: Molecular formula

Paecilomyces lilacinus acetone extract consists of 48 compounds (Table 6 and Fig. 6b). The total peak area of the detected compounds is 99.98 %. The probabilities of the structures of the detected compounds are listed in Table (6), where the major peak areas were 54.59% for hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$) and 28.05% for 10,13-octadecadienoic acid, methyl ester ($C_{19}H_{34}O_2$), they are fatty acid methyl esters.

Table 6: GC/MS analysis of *Paecilomyces lilacinus* acetone extract

Peak No.	R_t (min)	MW	MF	Area %	Identified compounds
1	9.97	135	$C_8H_6D_3NO$	0.10	N-Phenylacetamide
2	11.94	143	$C_7H_{13}NO_2$	0.06	7-Methoxy-1-aza-8-oxabicyclo(5.1.3)octane
3	13.77	420	$C_{24}H_{36}O_6$	0.01	8,14-Seco-3,19-epoxyandrostane-8,14-dione,17-acetoxy-3á-methoxy-4,4-dimethyl
4	18.56	442	$C_{30}H_{50}O_2$	0.01	Lanosta-8,24-diene-3,22-diol
5	20.31	308	$C_{20}H_{36}O_2$	0.04	Z,Z-3,15-Octadecadien- 1-ol acetate
6	20.79	145	$C_7H_{15}NO_2$	0.11	Heptane, 1-nitro-
7	22.51	354	$C_{23}H_{46}O_2$	0.03	Docosanoic acid, methyl ester
8	22.96	186	$C_{10}H_{18}O_3$	0.14	Nonanoic acid, 9-oxo-, methyl ester
9	23.72	70	$C_3H_6N_2$	0.01	2-(Methylimino)acetonitrile
10	25.06	294	$C_{19}H_{34}O_2$	0.11	17-Octadecynoic acid, methyl ester
11	25.60	216	$C_{11}H_{20}O_4$	0.14	Nonanedioic acid, dimethyl ester
12	25.83	196	$C_{10}H_{12}O_4$	0.04	Benzoic acid, 2,6-dimethoxy-, methylester
13	26.53	310	$C_{22}H_{46}$	0.04	Docosane
14	26.74	296	$C_{19}H_{36}O_2$	0.04	Cyclopentanetridecanoic acid, methyl ester
15	27.37	224	$C_{13}H_{20}O_3$	0.07	Methyl jasmonate
16	27.93	366	$C_{24}H_{46}O_2$	0.03	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester
17	28.48	251	$C_{18}H_{21}N$	0.03	3,6,9-Triethylcarbazole
18	28.60	310	$C_{20}H_{38}O_2$	0.04	cis-13-Eicosenoic acid
19	29.11	224	$C_{14}H_{24}O_2$	0.44	12-Tridecynoic acid, methyl ester
20	29.55	242	$C_{15}H_{30}O_2$	1.41	Methyl tetradecanoate
21	30.21	400	$C_{29}H_{52}$	0.05	Benzene,2-(1-decylundecyl)-1,4-dimethyl-
22	30.84	266	$C_{19}H_{38}$	0.02	1-Nonadecene
23	31.30	296	$C_{19}H_{36}O_2$	0.01	4-Octadecenoic acid, methyl ester
24	31.63	256	$C_{16}H_{32}O_2$	0.18	Pentadecanoic acid, methyl ester
25	33.36	268	$C_{17}H_{32}O_2$	0.18	11-Hexadecenoic acid, methyl ester
26	34.00	270	$C_{17}H_{34}O_2$	54.59	Hexadecanoic acid, methyl ester
27	34.93	378	$C_{27}H_{54}$	0.01	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-
28	35.01	266	$C_{17}H_{30}O_2$	0.03	Hexadecadienoic acid, methyl ester
29	35.20	282	$C_{18}H_{34}O_2$	0.02	Methyl 9-heptadecenoate
30	35.31	282	$C_{18}H_{34}O_2$	0.16	cis-10-Heptadecenoic acid, methyl ester
31	35.64	284	$C_{18}H_{36}O_2$	0.20	Heptadecanoic acid, methyl ester
32	36.92	294	$C_{19}H_{34}O_2$	0.04	9,12-Octadecadienoic acid, methyl ester, (E,E)-
33	37.44	294	$C_{19}H_{34}O_2$	28.05	10,13-Octadecadienoic acid, methyl ester
34	37.50	296	$C_{19}H_{36}O_2$	12.62	4-Octadecenoic acid, methyl ester
35	37.66	298	$C_{16}H_{26}O_5$	0.52	Dimethyl ester of pentylurofuranoic acid isomer
36	39.03	256	$C_{16}H_{20}N_2O$	0.03	1-Propyl-2-methyl-7-methoxy-5H,6H-pyrido[3,4-b]indole
37	39.34	310	$C_{21}H_{42}O$	0.05	11-Heneicosanone
38	39.79	346	$C_{18}H_{35}BrO$	0.05	Octadecanal, 2-bromo-
39	40.41	400	$C_{21}H_{36}O_7$	0.01	Propanoic acid, 2-[5-(2-hydroxypropyl) tetrahydrofuran-2-yl]-,1-[5-(1-methoxy-1-oxopropan-2-yl)tetrahydrofuran-2-yl]propan-2-yl ester
40	40.74	312	$C_{19}H_{36}O_3$	0.03	Oxiraneoctanoic acid, 3-octyl-, methyl ester,cis
41	41.64	346	$C_{17}H_{18}N_2O_6$	0.01	N-Nitrosohemanthidine
42	42.92	242	$C_{16}H_{34}O$	0.03	2-Hexadecanol
43	43.06	246	$C_{14}H_{14}O_4$	0.01	[1,1'-Biphenyl]-4,4'-diol, 3,3'-dimethoxy-
44	43.44	256	$C_{15}H_{32}OSi$	0.07	1-Ethyl-1-octyloxy-1-silacyclohexane
45	43.55	271	$C_{17}H_{21}NO_2$	0.03	7-[4-(N-Ethyl-N-(2-hydroxyethyl)amino]phenyl]penta-2,4,6-trien-1-al
46	43.78	185	$C_8H_8ClNO_2$	0.02	Benzoic acid, 2-amino-6-chloro-, methyl ester
47	44.33	390	$C_{24}H_{38}O_4$	0.04	1,2-Benzenedicarboxylic acid,bis(2-ethylhexyl) ester
48	54.32	429	$C_{26}H_{23}NO_5$	0.02	12-Phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline

R_t : Retention time; MW : Molecular weight; MF: Molecular formula

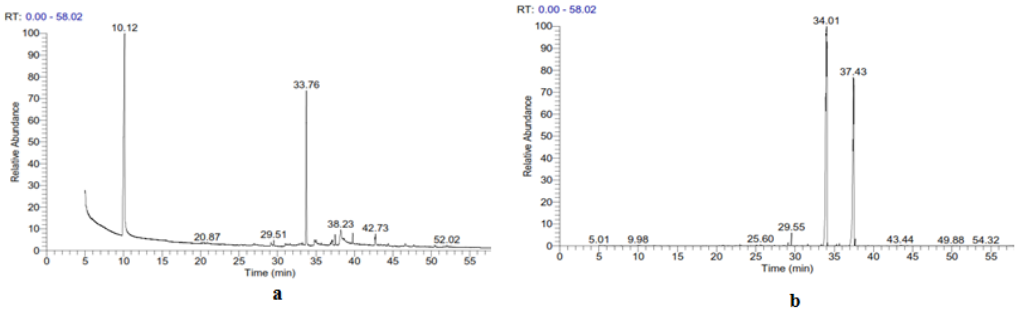

Fig. 6: GC/MS analysis of (a) *B. bassiana* acetone extract, (b) *P. lilacinus* acetone extract.

DISCUSSION

Schistosomiasis is one of the infectious diseases that affect more than 90% of people in the African region (WHO, 2016); the life cycle of the worm necessitates the presence of intermediate hosts which are *Biomphalaria alexandrina* snails. Reducing the numbers of these snails, or debilitating their ability to reproduce is one of the main targets of controlling programs in which chemical molluscicides are currently applied (WHO, 2008). Seeking molluscicidal substances of biological origin is urgently needed to eliminate harmful effects of chemical compounds on both human health and its environment (Oliveria-Filho and Paumgartten, 2000). The present results showed that acetone extract of *Paecilomyces lilacinus* was more toxic to *B. alexandrina* snails than that of *Beauveria bassiana*, with LC_{50} values of 120 and 231 ppm, respectively. This is in agreement with previous studies which showed that some fungal species were toxic to *B. alexandrina* snails such as *Aspergillus fumigatus, A. terreus* and *Penicillium janthinellum* (Osman *et al*., 2013; Saad *et al*., 2014).

The current work showed that survival rate of snails exposed to acetone extract of *B. bassiana* was lower than that of snails exposed to acetone extract of *P. lilacinus*. These results are in coincidence with the findings of Osman *et al*. (2013) who demonstrated that the survival rate of *B. alexandrina* treated with *Aspergillus fumigatus* extract was reversely proportional to the concentration. Moreover, Saad *et al*. (2014) found that after 4 weeks of *B. alexandrina* exposure to LC_{15} and LC_{25} of *Aspergillus terreus* filtrate, their survival rate decreased to 50%, while the survival rate of snails exposed to LC_{25} of *Penicillium janthinellum* filtrate decreased sharply to 45%.

When digestive gland cells which are analogous to hepatic cells are stressed by a xenobiotic, they undergo a series of irreversible histopathological changes, and their study can give indications of the degree of toxicity to the organism (Viarengo and Canesi, 1991). The present results demonstrated that exposure of snails to *B. bassiana* and *P. lilacinus* acetone extracts resulted in vacuolation of digestive cells, and degeneration of secretory ones. These results match the findings of Lajtner *et al*. (1996) on the effect of phenol on digestive gland of *Amphimelania holandrii* snails. The vacoulation of digestive cells was closely related to the digestive and absorptive

processes going on in these cells (Ibrahim *et al*., 2006).These processes increase in snails exposed to molluscicides, and hence, the degree of cytoplasmic vacuolation increases. Hamed *et al*. (2007) studied the ultrastructural changes induced by two carbamate molluscicides on the digestive gland of the snail *Eobania vermiculata*, and found severe cytoplasmic vacuolization. Moreover, exposure of *B. alexandrina* snails to Mirazid resulted in necrosis of connective tissue, digestive tubules lost their normal shape, and noticeable vacuoles were detected in both secretory and digestive cells (Osman *et al*., 2014).

Exposure of snails to *B. bassiana* and *P. lilacinus* acetone extracts in the current study resulted in a number of deleterious effects on the hermaphrodite gland. Our results are in agreement with Saad *et al*. (2014), they found that the more adverse effect on hermaphrodite gland of *B. alexandrina* was that exhibited by LC_{15} of *Penicillium janthinellum* filtrate, where acini lost their architecture and became shrunk. Also, when *B. alexandrina* snails were exposed to Artemether (dihydro-artemisinin methyl ether) for 21 days, a complete destruction of gametogenic cells and severe damage of ovotestis took place (Mossalem *et al*., 2013).

Comet assay is a rapid and sensitive technique that detects DNA strand breaks, measuring the migration of DNA from immobilized individual cell nuclei (Fairbairn *et al*., 1995). It was applied on a wide variety of aquatic species including snails (Sarkar *et al*., 2015; Ibrahim *et al*., 2018). This technique is considered one of the most promising genotoxicity biomarkers as it needs a small number of cells and detects damage at single cell level (Bolognesi and Cirillo, 2014). The present work showed that LC_{15} of both *B. bassiana* and *P. lilacinus* acetone extracts resulted in noticeable DNA damage. Moreover, *B. bassiana* acetone extract was the most genotoxic. To the best of our knowledge, this is the first study to evaluate the genotoxic effect of fungal extracts on *B. alexandrina* snails using comet assay. Previous studies were concerned with the effect of chemical substances; Sarkar *et al*. (2015) evaluated the genotoxicity of cadmium chloride in *Nerita chamaeleon*, and Ibrahim *et al*. (2018) reported high DNA migration in *B. alexandrina* snails exposed to lufenuron insecticide.

In the current work, acetone extracts of *Paecilomyces lilacinus* and *Metarhizium anisopliae* were highly active against the tested bacterial strains and *Candida albicans*. This result is very promising and could be explained according to Hepsibah and Jothi (2017) who reported superior antibacterial activity of acetone extract over ethyl acetate one. The present findings are in coincidence with Lee *et al*. (2005), they declared that a number of *M. anisopliae* strains and *Paecilomyces* spp. were highly effective on *S. aureus*. In addition, dichloromethane extract of *M. anisopliae* showed higher antibacterial activity against *E. coli* than *S. aureus* (Ravindran *et al*., 2014). Ethyl acetate extract of *M. anisopliae* showed moderate antimicrobial effect on *C. albicans* (Fabelico, 2015). Moreover, Mohammadi *et al*. (2016) stated that the extracellular metabolites of two strains of *P. lilacinus* showed antifungal activity against *Saccharomyces cerevisiae*. Similarly, Gulwani *et al*. (2015) demonstrated that *Paecilomyces fumosoroseus* metabolites showed the highest activity against *E. coli*, *Bacillus subtilis* and *Salmonella typhi*.

Oxidative stress is a great health problem leading to several health disorders such as cancer, cardiovascular and liver diseases. This phenomenon is due to an overproduction of free radicals and consequent accumulation of reactive species. Several studies have demonstrated that natural bioactive chemical ingredients derived from medicinal plants and fungal extracts have a great capability to remove hazards of such reactive species and thus are considered promising natural

antioxidant agents (Ghareeb *et al*., 2018 a, 2018 b). The present study demonstrated that the highest antioxidant capacity was that of *Paecilomyces lilacinus* acetone extract, followed by *Beauveria bassiana* acetone extract. These findings are in agreement with Zhang *et al*. (2010) and Kiran and Mohan (2018), they showed that different extracts of *B. bassiana* have high antioxidant capacity. Regarding *P. lilacinus*, to the best of our knowledge, this is the first study to evaluate the antioxidant activity of its extracts.

According to the American Cancer Institute (USNCI), the crude extracts are considered potent anticancer agents when their IC_{50} values are less than 20 µg/ml (Suffness and Pezzuto, 1990; Boik, 2001). Cancer is considered the third cause of death after cardiovascular and infectious diseases (Shaikh *et al*., 2014; Doll and Peto, 2003). From the point of controlling the side effects of chemical drugs used in the treatment of cancer, seeking drugs of natural origin becomes mandatory. Hepatocellular carcinoma (HepG2) cells represent one of the most widely used experimental models for *in vitro* studies on liver cancer (Haggag *et al*., 2011). Our results are in accordance with previous results on cytotoxic activity of entomopathogenic fungi; Zhang *et al*. (2004) demonstrated that the cytotoxic activity of ethyl acetate extracts of *Cordyceps sinensis* on human leukemia cell line was high. Also, a noticeable cytotoxic activity of the entomopathogenic fungus *Hirsutella* sp. ethyl acetate extract was recorded against Chinese hamster ovary cells at 100 µg/ml. A significant cytotoxic activity of *Beauveria feline* was reported against leukaemia, melanoma and colon cancer cell lines. Recently, Kiran and Mohan (2018) demonstrated that ethyl acetate extract of *B. bassiana* showed the highest cytotoxic activity against lung carcinoma. Also, Abdel-Hady *et al*. (2016) found that ethyl acetate extracts of both *Aspergillus tamarii* and *Penicillium islandicum* showed high cytotoxic effect on HepG2 cells.

The present GC/MS analysis of the effective extracts showed that on the one hand, *B. bassiana* acetone extract consists of 37 compounds, where the major compounds were 2-methylhept-2,6-dien-4-ol which is an alkene derivative and 1,3-dimethoxy-2-(hydroxymethyl)-9h-xanthene. Chibale *et al*. (2003) declared that xanthene derivatives are essential heterocyclic compounds in medicinal chemistry because of their biological properties as they exhibit anti-inflammatory and antibacterial activities. In addition, Ma *et al*. (2016) reported that four alkenes isolated from the plant *Murraya koenigiithem* showed significant antioxidant activities. On the other hand, *Paecilomyces lilacinus* acetone extract consists of 48 compounds where the major compounds were hexadecanoic acid, methyl ester and 10,13-octadecadienoic acid, methyl ester , they are fatty acid methyl esters. Antibacterial and antifungal activities of fatty acids methyl esters were previously reported (Agoramoorthy *et al*., 2007; Chandrasekaran *et al*., 2008). Sharma *et al*. (2014) recorded the presence of phenolic and alcoholic compounds in the filtrate of *P. lilacinus*, to which the nematicidal activity of this strain was attributed.

CONCLUSION

In conclusion, *Paecilomyces lilacinus* acetone extract is very promising as it showed the highest activities as snailicidal, antimicrobial, antioxidant and anticancer agent followed by *Beauveria bassiana* acetone extract. *Metarhizium anisopliae* acetone extract also exhibited high antimicrobial activity.

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ARABIC SUMMARY

النشاطات الإبادية للقواقع و المضادة للميكروبات و الأكسدة و السرطان للمستخلصات الفطرية لكل من بوفاريا باسيانا و ميتاريزيوم أنيسوبليى و بايسلومايسيس ليلاسيناس

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تتميز الفطريات بقدرتها على إنتاج أنواع كثيرة من مركبات الأيض الثانوية. تمثل الفطريات الممرضة للحشرات و كذلك الأنواع الفطرية المتطفلة على النيماتودا مصادر ممتازة لإنتاج المركبات ذات الفعالية البيولوجية . تهدف الدراسة لإستكشاف التأثير المبيد للقواقع والتأثير المضاد للميكروبات و كذلك التأثير المضاد للأكسدة لكل من الفطريات الآتية : بوفاريا باسيانا وميتاريزيوم أنيسوبليى و بايسلومايسيس ليلاسيناس. تم تحضير نوعين من المستخلصات من كل فطر و هما مستخلص أسيتات الإيثيل و مستخلص الأسيتون. تم إختبار تأثير هذه المستخلصات ضد قواقع بيومفلاريا ألكسندرينا ، كذلك تم إختبار تأثيراتهم المضادة للميكروبات و المضادة للأكسدة. بالإضافة لذلك تم إختبار التأثير المضاد للسرطان لمستخلص الأسيتون من فطر بايسلوميسيس ليلاسيناس على خلايا كبد سرطانية . أوضحت النتائج أن مستخلص الأسيتون لفطر بايسلوميسيس ليلاسيناس كان أكثر سمية لقواقع بيومفلاريا ألكسندرينا من مستخلص الأسيتون لفطر بوفاريا باسيانا حيث كانت قيم التركيز المميت لنصف عدد القواقع هى ١٢٠ و ٢٣١ على التوالى . أظهرت التركيزات تحت المميتة من هذين المستخلصين تأثيرات ضارة على معدل حياة القواقع و الحالة النسيجية لكل من الغدة الهاضمة و الغدة الخنثوية ، هذا بالإضافة لتأثيراتهم السامة جينيا على المادة الوراثية للقواقع. أظهر مستخلص الأسيتون لفطر بايسلومايسيس ليلاسيناس أعلى تأثير مضاد للأكسدة. كذلك كان تأثيره ضد ستافيلو كوككاس أوريوس و إيشيريشيا كولاى و كانديدا ألبيكانس عاليا . فضلا عن ذلك أظهر هذا المستخلص تأثيرا خلويا ساما ضد خلايا الكبد السرطانية حيث كانت القيمة المثبطة للخلايا المعرضة لكى تصل للنصف هى ٢,٨١ ميكروجرام/مل . أوضحت نتائج الفصل الكروماتوجرافى الغازى مع قياس الطيف الكتلى وجود ٣٧مركب فى مستخلص الأسيتون لفطر بوفاريا باسيانا حيث كان المركبان الأعلى تواجدا هما مركب" ٢ ميثيل هيبت٢ و ٦ دايين ٤ أول " و مركب" ١و ٣ دايميثوكسى ٢ هيدروميثوكسى ٩ إتش ذانثين " . بينما تم تعريف ٤٨ مركب فى مستخلص الأسيتون من فطر بايسلومايسيس ليلاسيناس حيث كان مركب حمض هيكساديكانويك ميثيل إستر و مركب ١٠ و ١٣ حمض أوكتاديكادايينويك ميثيل إستر هما الأعلى تركيزا. نخلص من ذلك بأن الأنواع الفطرية : بوفاريا باسيانا و بايسلوميسيس ليلاسيناس يمثلان مصدرين ممتازين للمركبات الناشطة بيولوجيا و التى لها الكثير من التأثيرات البيولوجية .