EVALUATION OF ACARICIDAL ACTIVITY OF *PURPUREOCILLIUM LILACINUM* ISOLATED FROM EGYPTIAN SOIL AGAINST *TETRANYCHUS URTICAE*

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The study is aimed to isolate and identify a potential entomopathogenic fungus from fields infested with spider mites and evaluate them as a bio-rationale agent against *Tetranychus urticae* Koch., which is an important agricultural pest that infests horticultural crops in both field and greenhouses. Macroscopic and microscopic characteristics showed that the obtained entomogenous isolate was *Purpureocillium lilacinum* (formerly, *Paecilomyces lilacinus*). *P. Lilacinum* demonstrated mortality rates on adult females of *T. urticae* of 71.19 and 77.97% with conidial concentrations of 5×10^7 and 1.6×10^8, respectively, 10 days after application. Median lethal concentration of *P. lilacinum* was 2.85×10^6 conidia/ml. Enzymatic activity of *P. lilacinum* was evaluated. In this context, chitinolytic activity of *P. lilacinum* was relatively weak, since the clear halo obtained was only 4.43 mm in diameter. The activity of chitinase enzyme insignificantly increased over the incubation period of 10 days. In contrast, proteolytic activity of *P. lilacinum* was high and showed a clear zone (25.8 mm) around the colony after 10 days. So, it is concluded that *P. lilacinum* has a potential biological control against *T. urticae*. Finally, evaluation of the ethyl acetate extract of *P. lilacinum* showed acaricidal potency since it exhibited LC_{50} values of 10.49 mg/ml for eggs and 30.75 mg/ml for adults. So, it is concluded that *P. lilacinum* has a potential biological control against *T. urticae.*

Keywords: *Paecilomyces lilacinus*, two spotted spider mite, biological control, secondary metabolites

Family Tetranychidae comprises many species of phytophagous spider mites that usually occupy the lower surface of plant leaves and feed by piercing plant cell to suck out the cell sap. Hence, they cause severe damage to plants. Among them, *Tetranychus urticae* Koch. can invade more than 150 plant species of fruits, vegetables and ornamentals (Fasulo and Denmark, 2000). Feeding of spider mites results in chlorosis, leaves may turn to yellow, leaves shed and eventually, the whole plant collapse especially at high infestation rate (Smith, 1996). Chemical acaricides have been successfully used to control *T. urticae* (Attia et al., 2013). However, pesticides resistance is rapidly developed because of the short lifecycle, rapid reproduction and plentiful offspring of spider mites (Van Leeuwen et al., 2010). The problems of resistance as well as the ecological pollution caused by synthetic pesticides, there is an urgent need for sustainable and eco-friendly management methods.

Different types of biological control agents have been screened and assessed against spider mites. In this context, predator mites such as *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) that specifically feeds on *Tetranychus* sp. as well as *Neoseiulus californicus* (Acari: Phytoseiidae) are effective way to control spider mites (Attia et al., 2013). Studies demonstrated the strong acaricidal potency of plant extracts and essential oils on spider mites. The methanol extract of *Cleome gynandra*, *Capsicum frutescence* and *Urtica dioica* highly reduced *T. urticae* population (Kapsoot et al., 2013). Eucalyptus oils rich in cineole showed potent acaricidal activity against *T. urticae* (Choi et al., 2004). Microorganisms are another group of biocontrol agent that showed high efficacy in spider mites control. For example, thuringiensin produced by *B. thuringiensisis* showed potent acaricidal efficacy on *Tetranychus urticae*, *T. cinnabarinus* (Neal et al., 1987) and *Panonychus ulmi* (Vargas, 1993). *Pseudomonas fluorescens* exhibited a strong efficacy on adults of *Oligonychus coffeae* under laboratory conditions (Roobakkumar et al., 2011). Abamectin (avermectin) produced from *Streptomyces avermitilis* demonstrated the high toxic effect of on *T. cinnabarinus* (Wu and Liu, 1997), *T. urticae*, *Phyllocoptruta oleivora*, *Panonychus citri* and *T. turkestani* (Putter et al., 1981).

One of the most studied organisms with respect of biological control of spider mites is entomopathogenic fungi (EPF). Fifty-six genera comprise about 750 fungal species are known as arthropod pathogens (Hawksworth et al., 1995). Naturally, EPF play an important role in management of spider mite populations. Consequently, they can be used separately as an alternative to synthetic pesticides or in combination with other ways of control in an integrated mite management program (Maniania et al., 2008). Among EPF, several species, e.g. *Hirsutella thompsonii* and *Neozygites floridana* are called acaripathogenic fungi, since they exclusively infect members of Acari while the other entomogenous fungi species kill both insects as well as acari

(Chandler et al., 2000). Some fungi such as *Beauvaria bassiana*, *Metarhizium anisopliae* and *Lecanicellium lecanii* have been screened against spider mites and exhibit acricidal potential; therefore, they could be exploited to develop bio-rationale acaricides (Gatarayiha, 2010).

This study is aimed to isolate potential entomogenous fungal isolates from the rhizosphere of plants infested with spider mites, examine the effectiveness of the obtained isolate/s on adult females of *T. urticae* under laboratory conditions, identification of this isolate/s and finally, evaluation of the virulence factors of this isolate, for instance its ability to produce chitinase and protease enzymes.

MATERIAL AND METHODS

1. Collection of Soil Samples

Seventeen soil samples were collected from the rhizosphere of infested plants from six different fields at El-Khatatba at Alexadria governorate, Egypt. Samples were collected at 10-15 cm under soil surface using sterilized spatula then they were placed individually in sterilized polyethelene bags and then investigated in Department of Plant Protection at Desert Research Centre.

2. Isolation of Entomopathogenic Fungi by Insect Bait Method

Isolation of entomogenous fungi was done by using the insect bait technique developed by Zimmermann (1986) (Fig.1). Soil samples were sieved to remove any debris and placed in sterilized plastic cups with perforated lids. Five larvae of the wax moth, *Galleria mellonella*, (Lepidoptera: Pyralidae) were immersed in water bath at 56ºC to prevent webbing, then placed on the surface of each soil sample. The cups containing larvae were inverted upside down and incubated at 25ºC for two weeks. After the first week, cups were checked for dead larvae daily. The cadavers of larvae were collected, and surface sterilized with 1% sodium hypochlorite for 30 seconds, then rinsed twice with sterilized distilled water prior to incubation in moist chamber (which is sterilized petri dish with moistened filter paper) at 28ºC for 7 days. The cadavers showing fungal growth were transferred to potato dextrose agar (PDA) plates supplemented with chloramphenicol and incubated at 28ºC for 7 days.

3. Morphological Identification of the Entomogenous Fungus

Cultures were grown on Czapek's yeast extract agar and incubated at 28ºC for 7-10 days. Identification of the emtomopathogenic isolate was done on morphological basis using culture characteristics e.g. growth rate, color, pigmentation as well as microscopic features e.g. conidiophores and conidia (Luangsa-ard et al., 2011). The results are confirmed by Mycological Centre, Assiut University, Egypt.

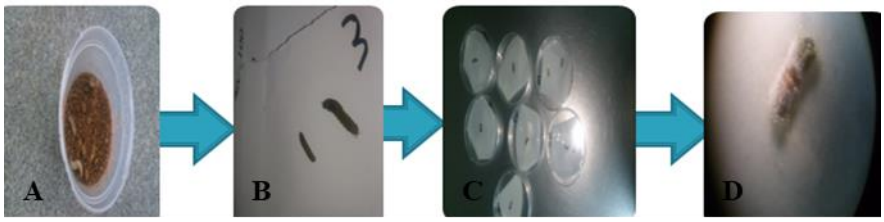

Fig. (1). Steps of baiting technique for isolation of entomopathogenic fungi. **A.** Larvae of *G. mellonella* in soil sample. **B.** Dead larvae. **C.** Dead larvae incubated in moist chamber. **D.** fungal hyphae emerging from dead larvae under dissecting microscope.

4. Effect of *Purpureocillium lilacinum* on Spider Mites

4.1. Preparation of conidial suspension

One entomopathogenic fungus (EPF) was isolated from soil by using baiting technique. The isolate was cultured on PDA plate (11 cm) and incubated at 28±2ºC for 15 days, conidia were harvested by scrapping the surface of the plate and suspend it in 20 ml of sterilized distilled water with 0.05% tween 80, the suspension was vortexed for 3-5 min to produce a homogenous suspension then it was filtered through sterilized muslin cloth in 250 ml conical flask containing 80 ml of sterilized distilled water. Four concentrations of $8\times 10^5, 2.6\times 10^6, 5\times 10^7$ and 1.6×10^8 conidia/ml were prepared using haemocytometer in conidial count.

4.2. Bioassay of conidial suspension against *T. urticae*

Conidial suspension was assayed against adult females of *T. urticae* as follows: 250 µl were placed on the surface of mulberry leaf disc 25 mm in diameter and let to dry for 30-45 min then ten adult females of spider mite were transferred to each replicate by using fine hair paint brush. Three replicates were used for each conidial concentration and distilled water with 0.05% tween 80 was used as control. Mortality rates were recorded at the 7^{th} and the 10^{th} day after application and corrected according to Abbott formula (Abbott, 1925).

5. Enzyme Activity of *P. lilacinum*

5.1. Screening for chitinolytic activity of *P. lilacinum*

Chitinolytic activity of *P. lilacinum* was determined by plate assay method. Colloidal chitin was prepared from purified chitin (Qulikems) according to the method developed by Roberts and Selitrennikoff (1988), with few modifications described by Al-Ahmadi et al. (2008), Colloidal chitin is prepared as follows: chitin (5 g) were digested in 90 ml of concentrated HCl and mixed gently on magnetic stirrer for 2 h, 500 ml of ice-cold ethyl alcohol were added then stirred for 30 min, the digested chitin

then incubated for 24 h at 28°C. A dense precipitate is obtained following centrifugation at 2000 rpm, 4°C for 15 min, the colloidal chitin was washed repeatedly with distilled water and pH was adjusted to 7. Chitinase assay medium in which chitin employed as a sole carbon source prepared as follows: Colloidal chitin (1% w/v), 6 g of Na_2HPO_4, 1.0 g of NH_4Cl, 3.0 g of KH_2PO_4, 0.5 g of NaCl, 0.05 g of 15 g of agar, and pH was adjusted to 8 and then autoclaved at 121°C for 15 min (Kuddus and Ahmad, 2013). After cooling the medium was poured into Petri plates and allowed to solidify. The fresh culture plugs of *P. lilacinum* was inoculated onto the medium and incubated at 28 °C for 10 days under observation to detect clear zone formation around fungal colony. Enzymatic activity was measured by the following formula: EA = D – d; (D) diameter of colony plus clear zone; (d) diameter of colony (Hasan et al., 2013).

5.2. Screening for proteolytic activity of *P. lilacinum:*

For proteolytic activity, 1.5% (w/v) of skimmed milk was autoclaved separately at 121°C for 10 min. Nutrient agar medium (Oxoid, Basingstoke, Hampshire, England) 13 g/L is autoclaved at 121°C for 15 min. After cooling, the sterilized skimmed milk was added to the medium and poured into Petri plates and allowed to solidify. The fresh culture plugs of *P. lilacinum* was inoculated onto the medium and incubated at 28 °C for 10 days and observed for the clear zone formation. Enzymatic activity was measured by the following formula: EA = D – d; (D) diameter of colony plus clear zone; (d) diameter of colony.

6. Effect of Crude Extract of *P. lilacinum* on Adult and Eggs of *T. urticae*

Twelve plugs (10 mm) of fresh *P. lilacinum* culture were inoculated into 300 ml of Potato dextrose broth medium (dextrose (20 g/L) and potato extract of 200 g potato) and incubated at 28°C for 10 days at 150 rpm. After incubation time, culture was filtered, centrifuged and subjected to extraction with ethyl acetate (1:1) three times. Ethyl acetate extract was dried under vacuum, weighed and four concentrations were prepared 5, 10, 20, 30 mg/ml using ethanol as solvent. The prepared concentrations were assayed against *T. urticae*. For adulticidal assay, three leaf discs (25 mm) were dipped in each concentration, dried at room temperature and twenty adult females were transferred to each disc by fine hair brush. Mortality rates were recorded three days after application. For ovicidal assay, ten adult females were transferred to each leaf disc (25 mm) and incubated at 25°C. After 24 h, females were removed and leaf discs carrying eggs were immersed crude extract and in ethanol in case of control. Three discs were made for each concentration and control. Number of unhatched eggs was recorded after all eggs on control hatched.

7. Data Analysis

Mortality rates were corrected according to Abbott`s formula (Abbott, 1925).

Corrected mortality = $\frac{T-C}{100-C} \times 100$

Where: T= dead mites in treatment, C= dead mites in control.

One-way ANOVA ($P< 0.05$) was used. Means were compared by Duncan's test (Duncan, 1955).

RESULTS AND DISCUSSION

1. Isolation of Entomopathogenic Fungi

Isolation by insect bait method using larvae of wax moth *Galleria mellonella* was carried out in the collected soil samples. Dead larvae from seven soil samples out of seventeen showed fungal growth. Three *Penicillium*, five *Aspergillus* and two *Fusarium* isolates were isolated, while only one entomogenous *Purpureocillium lilacinum* isolate were obtained as shown in table (1). Similarly, Barra et al. (2013), isolated thirty-five *Paecilomyces lilacinus* isolates by means of insect baiting method using the flour beetle *Tribolium confusum* (Jacquelin du Val), in addition to other fungal genera like *Penicillium* and *Fusarium* were also recovered along with *Paecilomyces*. Since Fungi in the genera *Mucor*, *Penicillium* sp., *Aspergillus* sp., and some others, were usually present as common opportunistic contaminants (Gouli et al., 2013). Vänninen (1996) recovered a number of entomogenous fungi (*Metarhizium anisopliae*, *Beauveria bassiana*, *Paecilomyces farinosus* and *P. fumosoroseus*) from agricultural soil in Finland by insect bait method.

Table (1). Fungal isolates obtained by insect bait method.

Cultivated crop	Fungal isolates
Eggplant	*Aspergillus sp.* *Fusarium sp.*
Banana	*Aspergillus sp.*
Grape	*Paecilomyces sp.* *Fusarium sp.*
Mango	*Aspergillus sp.*
Eggplant	*Penicillium sp.*
Sweet pepper	*Penicillium sp.* *Aspergillus sp.*
Tomato	*Aspergillus sp.* *Penicillium sp.*

2. Acaricidal Potency of *Purpureocillium lilacinum* on *T. urticae*

Evaluation of the obtained entomopathogenic isolate against adult females of *T. urticae* showed high acricidal potency, in which the fungal hyphae emerged from cadavers of *T. urticae* (Fig. 2). Results in table (2) showed concentration-dependant effectiveness. The highest mortality rates exhibited by concentration of 1.6×10^8 conidia/ml (77.97%) on day 10 however; there is no significant difference with the mortality percent exhibited by 5×10^7 conidia/ml (71.19%) after the same time. Median lethal concentration of *P. lilacinum* is 2.85×10^6 conidia/ml (Fig. 3), which was lower than values obtained by some *Metarhizium anisopliae* and *Beauveria bassiana* isolates that ranged between 0.7×10^7 and 2.5×10^7 conidia/ml (Wekesa et al., 2005). Similar results were demonstrated by Sanjaya et al. (2015), that showed the acricidal potency of *P. lilcainus* with mortality of 70% in concentration of 10^7, while in 10^8 was 74.44% on *T. kanzawai*. Shin et al. (2017) reported that *P. lilacinum* achieved 84% mortality on *T. urticae*. Shin et al. (2011) investigated the effect of *P. lilacinus* on *T. urticae* using SD tower sprayer in application of conidial suspension that killed 73% of tested population after 6 days from application. Whereas, the results illustrated in the current investigation is higher than that caused by four *Beauveria bassiana* isolates studied by Topuz et al. (2016), since the highest mortality exhibited on adults of *T. cinnabarinus* was 50% with concentration of 10^7conidia/ml after 10 days. However, the results obtained in the current study may be less in comparison with mortality rates achieved in some of the previously mentioned reports; this may be because the conidial suspension was applied with spray method while we used the treated food method in the present bioassay. Geroh et al. (2015) supported this conclusion as they found that the direct spray method showed less LC_{50} and LT_{50} than the treated food method.

Fig. (2). Hyphae of *P. lilcinum* emerging from dead cadaver of *T. urticae* adult female as shown under dissecting microscope.

Table (2). Mortality rates of adult females of *T. urticae* after 7 and 10 days after application.

Conc.	Mortality % after 7 days		Mortality % after 10 days	
	Dead females (mean±SE)	Corrected mortality (%)	Dead females (mean±SE)	Corrected mortality (%)
Control	1.00^{c}±0.58	0.0	1.67^{d}±0.67	0.0
8×10^{5}	2.00^{c}± 0.58	10.10	5.00^{c}±0.58	33.90
2.6×10^{6}	4.67^{b}±0.33	37.04	7.33^{b}±0.33	57.63
5×10^{7}	6.67^{a}±0.88	57.24	8.67^{ab}±0.67	71.19
1.6×10^{8}	7.67^{a}±0.33	67.34	9.33^{a}±0.33	77.97

Values are mean no. of dead mite individuals ± standard error of three replicates. Different letters in the same column indicate a significant difference according to Duncan's multiple range test.

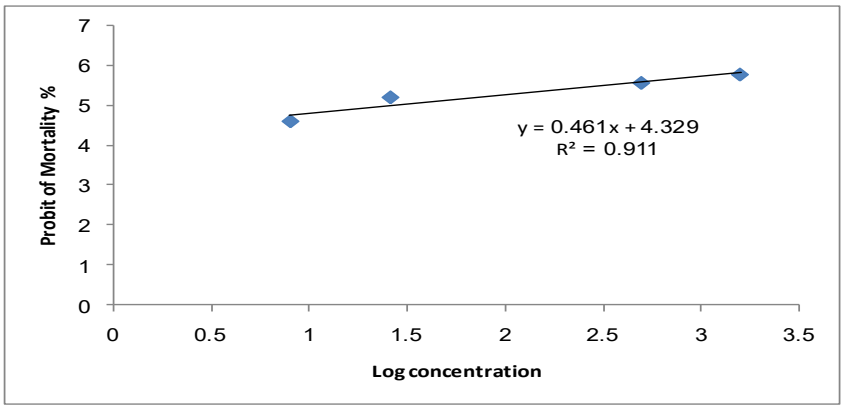

Fig. (3). Lethal concentration of *P. lilacinum* and mortality rate of adult females of *T. urticae*.

3. Identification of the Entomogenous Fungus

Colonies on MEA were growing fast, with a diameter of 25–35 mm after 7 days at 25°C. It consists of a basal felt with or without floccose aerial overgrowth as shown in fig. (4). Colonies were white at first, turns to vinaceous with purpleor reverse. Conidiophores were emerged from submerged hyphae with length of 4-6 µm. Whorls of 2-4 phialides are

carried on verticillate branches. Phialides consist of a swollen basal part tapering into a short distinctive neck (about 1 µm wide). Conidia are in divergent chains, they were ellipsoidal to fusiform in shape. The conidia (2-3×2-4 µm) were smooth-walled to slightly roughened, hyaline, purple en masse (Luangsa-ard et al., 2011).

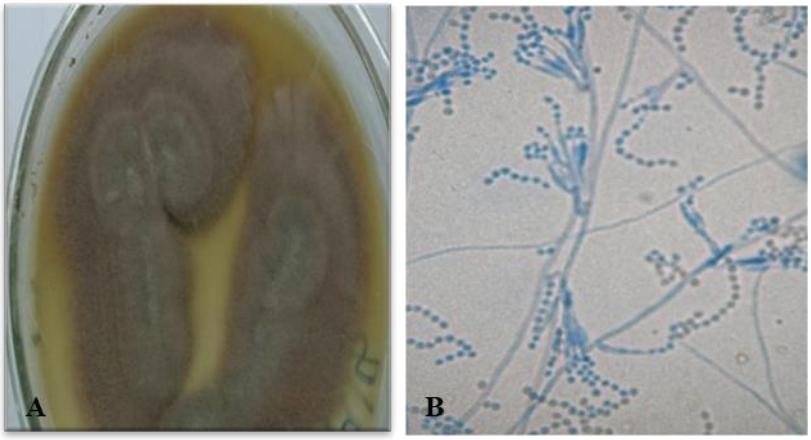

Fig. (4). *P. lilacinum* **A**. on PDA media, **B**. under light microscope (40X) showing it`s phialides and conidia.

4. Chitinolytic and Proteolytic Activity of *P. lilacinum*

Conidial adhesion, germination rate and growth on the insect integument influence the virulence of entomopathogenic fungi (Barra et al., 2015). The pathogenicity of entomopathogenic fungi is determined by its proteolytic and chitinolytic activity (Mondal et al., 2016). Thus, the development of effective fungal agents used for insect biological control would require studying the fungal enzyme activities involved in penetration of insect cuticle (Scorsetti et al., 2011). On the other hand, some studies showed no relationship between the fungal pathogenicity and its enzyme activity (Rosato et al., 1981). In the current investigation, the chitinolytic activity of the obtained *P. lilacinum* strain was weak in comparison with its proteolytic activity, as shown in fig. (5). Since chitinolytic activity showed a small halo sized 3.33 mm in diameter (after 5 days), 4.36 mm (after 7 days) and 4.43 mm in diameter (after 10 days). Obviously the chitinolytic activity of *P. lilacinum* increased over time but with insignificant rate. However, the proteolytic activity was strong since, the clear zone obtained was 20.2 mm (after 5 days), 22.6 mm (after 7 days) and 25.8 mm (after 10 days). The difference between the proteolytic activity after 5 days and 7 days was insignificant. The high proteolytic activity of *P. lilacinum* isolate may contribute in its high virulence against *T. urticae*. In view of the fact that protease enzymes are considered as the most important within the infection

process (Mustafa and Kaur, 2009) because proteins are the major component in the exoskeleton of insects (61–70%) (Hepburn, 1985). Once the epicuticle breaks down, the fungus produces large amounts of proteases that enable it to degrade proteins in insect cuticle and use the released amino acids as nutrients (Wang et al., 2002 and de Carolina Sánchez-Pérez et al., 2014). Despite, the weak chitinolytic activity of the obtained *P. lilacinum* isolate, its virulence against *T. urticae* was relatively high which may be due to the soft bodies of *T. urticae*. Since it, unlike insect pests, lacks hard integument. Similarly, Castellanos-Moguel et al. (2008) found that the protease activity of both of the most virulent *P. fumosoroseus* isolates against *T. vaporariorum* reached its peak after 120 h while the chitinase activity was maximum at 264 and 312 h.

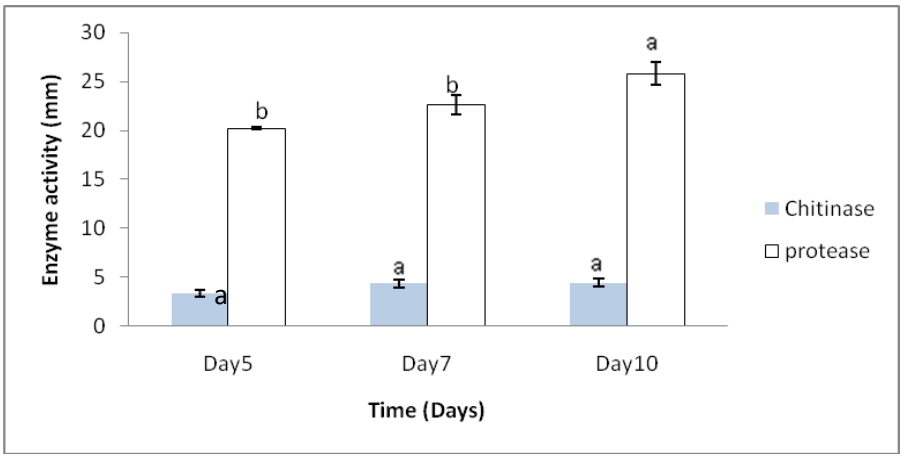

Fig. (5). Activity of chitinase and protease enzymes produced by *P. lilacinum*.

Data are presented as the mean value (diameter of clear zone in mm) ±SE. Error bars are corresponding to standard error. [a,b] Different letters indicate a significant difference according to Duncan's multiple range test.

5. Effect of Crude Extract of *P. lilacinum* on Adult Females and Eggs of *T. urticae*

Secondary metabolites of entomopathogenic fungi show insecticidal or antifeedant effect for pests (Kim et al., 2013). Among them, *P. lilacinum* produce secondary metabolites that show biological control activity against various pests. For instance, extract of *P. lilacinus* shows nematicidal activity against *Meloidogyne incognita* (Liu et al., 2009). Freed et al. (2012) reported the insecticidal activity of *P. lilacinus* protein extract that showed mortality rate of 45.83% against larvae of *Plutella xylostella* (the diamond back moth). In this context, the current investigation revealed the acaricidal potency of *P. lilacinum*. Data from fig. (6) show that the crude extract of *P.*

lilacinum exhibits more toxic effect on eggs than adult females of *T. urticae*. Concentration of 30 mg/ml showed the highest mortality rate of 58.33% on adults and 95.96% on eggs, followed by 20, 10 and 5 mg/ml that caused 26.67, 5 and 3.33% on adult, respectively, and 82.49, 40.40 and 15.15% on eggs, respectively. LC_{50} value exhibited by crude extract of *P. lilacinum* is 10.49 mg/ml for eggs and 30.75 mg/ml for adults. Similarly, the acaricidal effect of secondary metabolites produced by entomopathogenic fungi is reported in some studies. For instance, the culture filtrate of *Hirsutella thompsonii* exhibited mortality rate of 55.90% on *T. urticae* and citrus rust mite, *Phyllocoptruta oleivora* (Aghajanzadeh et al., 2006), the crude extract of *Hypocrella raciborskii* showed residual toxicity of 80% on *T. urticae* (Buttachon and Kijjoa, 2013) and the culture filtrate of *B. bassiana* and *M. anisoplae* highly reduced mite population (Yun et al., 2017).

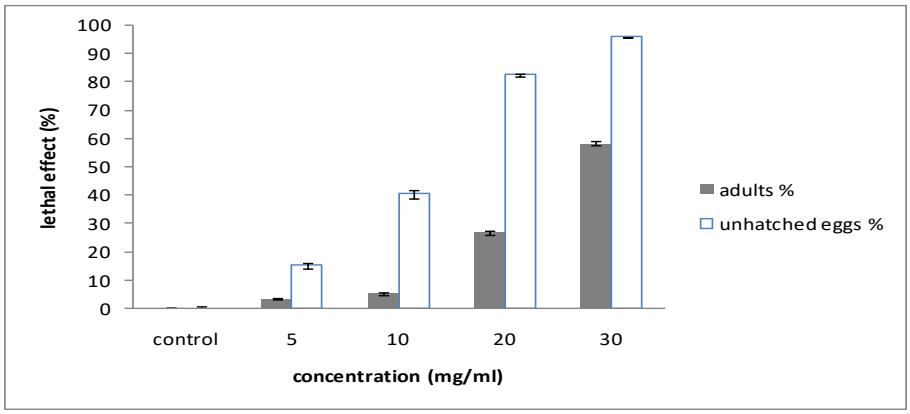

Fig. (6). Effect of crude extract of *P. lilacinum* on adult females and eggs of *T. urticae*.

Error bars are corresponding to SE of the mean value of the dead mite individuals.

CONCLUSION

In conclusion, A *Purpureocillium lilacinum* isolate was successfully recovered by insect baiting technique using larvae of wax moth *Galleria mellonella*. High acaricidal potency has been shown by both the conidia and crude extract of *P. lilacinum* against *T. urticae*. So, *P. lilacinum* could be effectively used as bio-rationale control of spider mites. Nevertheless, further investigations are required to separate active compounds responsible for the acaricidal effect. In addition, compatibility of the obtained isolate with other biocontrol agents should be studied.

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تقييم النشاط الإبادي لفطر *البربريوسيليوم ليلاسينوم* المعزول من التربة المصرية ضد العنكبوت الأحمر ذو البقعتين

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تستهدف هذه الدراسة عزل وتنقية عزلة فطرية ممرضة للحشرات من البيئة المصرية وتقييم فعاليتها لمكافحة العنكبوت الأحمر ذو البقعتين *Tetranychus urticae*. تم عزل عزلة فطرية واحدة ممرضة للحشرات ووضح التعريف المورفولوجي لها أنها *Purpreocillium lilacinum* ثم تم تقييمها ضد العنكبوت الأحمر ذو البقعتين *T. urticae* حيث أظهر محلول الجراثيم بتركيز ١.٦×١٠^{٨}جرثومة/ملل نسبة موت ٧٧.٩٧٪ في الطور البالغ للعنكبوت الأحمر وأيضًا تم تقييم مستخلص هذا الفطر على الطور البالغ والبيض. حيث أظهر نسبة موت ٥٨.٣٣٪ علي الطور البالغ للعنكبوت الأحمر، بينما توقف فقس ٩٥.٩٦٪ من البيض المعامل بهذا المستخلص بتركيز ٣٠ مجم/ملل. وأخيرًا تم تقييم النشاط الأنزيمي لهذه العزلة ووجد أن نشاط الأنزيم المحلل للبروتينات أعلى من نشاط الأنزيم المحلل للكيتين. من هذه النتائج نستنتج أن فطر *P. lilacinum* له فعالية عالية ضد العنكبوت الأحمر ذو البقعتين ويمكن أن يستخدم في المقاومة الحيوية لهذه الآفة.