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Efficiency of some Medicinal Plant Extracts and an Entomopathogenic Fungus, *Metarhizium anisopliae* Separately and in Combination with Proteus® Against the Large Cabbage Butterfly, *Pieris brassicae* L.

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Pieris brassicae (Lepidoptera: Pieridae) causes great qualitative and quantitative damage to cabbage crops. The present research was conducted to assess the synergistic/antagonistic interactions of *Satureja hortensis*, *Trachyspermum ammi*, *Ziziphora tenuior*, *Cuminum cyminum*, and *Foeniculum vulgare* methanolic extracts with *Metarhizium anisopliae* and Proteus® against *P. brassicae* pupae under laboratory conditions. The tested methanolic extracts when combined with *M. anisopliae* and Proteus® possessed synergistic efficacy (except for *M. anisopliae* + ammi). Probit analysis of extracts revealed *S. hortensis* as the most effective extract with LC_{50} value equivalent to 43.49 ppm. Proteus® also exhibited a high efficacy (LC_{50}=48.88). The results support the potential of cumin, fennel, savory and ziziphora methanolic extracts to improve the efficacy of *M. anisopliae*. Results demonstrated that all tested extracts integrated with Proteus® provide more effective control of *P. brassicae* than Proteus® alone.

Keywords: Botanical insecticide, *Metarhizium anisopliae*, *Pieris brassicae*, Proteus®, synergistic effect.

Cabbage (*Brassica oleracea* L.) is a prominent vegetable that is consumed either processed or raw (Rokayya et al., 2013). Brassica crops are attacked by many species of insect pests that cause significant damage to them (Neupane, 1999). One of the most important pests of cabbage is the large cabbage butterfly, *Pieris brassicae* L., which has been recorded as a considerable and cosmopolitan pest of cabbage, cauliflower, and broccoli (Hasan and Ansari, 2010). In addition to a direct damage, the large larvae pollute the host plant with their feces (Pfiffner et al., 2009). Today, applying chemical insecticides are still considered as the most important strategy to manage insect pests. Proteus® is a contact and systemic insecticide with two active ingredients (thiacloprid and deltamethrin) with different modes of action. On the other hand, considering the disadvantages of synthetic pesticides such as adverse effects on environment and human health (acute toxicity, cancer, neurological damage, birth defects, and reproductive and developmental harm) and beneficial organisms suppression (such as parasitoids and predators) (Salunke

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et al., 2005), bio-control agents have attracted scientific interests as safe and low-risk tools (Sharma and Gupta, 2009). There have been several efforts to research and introduce appropriate plant compounds with insecticidal properties (Downum et al., 1993; Rafiee-Dastjerdi et al., 2013). Nowadays, botanical insecticides are receiving attention by researchers because of their low persistence in the environment and little human and animal toxicity (Sampson et al., 2005; Digilio et al., 2008). Therefore, introducing an eco-friendly and low-risk alternative approach is a necessity. Some extracts (Geraniaceae plants) have been shown to control *P. brassicae* (Wawrzyniak, 2009). Alternatives such as entomopathogenic fungi could be good candidates for integrated pest management programs (Sewify et al., 2000). Entomopathogenic fungi are effective microbial control agents as a part of biological control tactics against several insect pests (Kassa et al., 2002; Goettel et al., 2005). The entomopathogenic filamentous fungus, *Metarhizium anisopliae* sensu lato is a well-known pathogen of arthropods employed in biological control strategies against many insect pests in agriculture (Santi et al., 2010). Therefore, the efficacy of *M. anisopliae* also was surveyed against one-day-old pupae of *P. brassicae*. This study was conducted to determine the best methanolic extract and their combinations with Proteus® against one-day-old pupae of the large cabbage butterfly.

Materials and Methods

Insect and insecticide

P. brassicae eggs were collected from research cabbage field of Urmia University, Urmia, Iran, during summer of 2017 and were reared on cabbage leaves in the laboratory. The emerged larvae were transferred to a plastic cage ($150 \times 150 \times 150$ cm) with two open circular sides, covered with a muslin cloth, tied with a rubber band, and reared for two generations at the laboratory of Entomology. One-day-old pupae were treated for experiments. The insecticide applied was Proteus® (110 OD, 100 g/l thiacloprid and 10 g/l deltamethrin, Bayer CropScience, New Zealand), which is a broad-spectrum insecticide.

Fungal pathogen

The entomopathogenic fungus, *Metarhizium anisopliae* sensu lato (isolate AM411) was provided by Mycology collection of Urmia University. Stock cultures of the isolates were grown on potato dextrose agar slants (PDA; Merck, Germany) and stored at 4 °C for further use. Subcultures were prepared by transferring pieces of stock cultures onto new PDA plates and incubating them at 25 ± 1 ºC for 14 days before using in the experiments. Conidia were harvested by flooding the cultures with sterile distilled water containing 0.02% Tween-80 and scraping with a sterile L-shaped glass rod. Concentrations of the resulting stock suspension were determined using a Neubauer hemocytometer (Fuchs–Rosenthal 0.0025 mm 2, depth 0.100 mm, VWR, Sweden). To assess conidial viability, 100 μl of a suspension at a rate of 1×10^4 conidia/ml were placed on PDA plates and incubated at 25 ± 1 °C for 24 h. Germination was checked under a microscope ($\times 400$). A conidium was considered germinated when the germ tube extended beyond the width of the conidium (Inglis et al., 2012). The mean germination rate was 97%.

Plant extract

Five medicinal plants, *Satureja hortensis*, *Trachyspermum ammi*, *Ziziphora tenuior*, *Cuminum cyminum*, and *Foeniculum vulgare,* were purchased from the local market of West Azarbaijan Province. Methanolic extracts obtained from seeds of *T. ammi*, *C. cyminum* and *F. vulgare* and leaves of *S. hortensis* and *Z. tenuior* using a Soxhlet extractor. A total of 40 grams of powdered plants was placed into a Soxhlet apparatus. The apparatus was charged with 300 ml of methanol. The Soxhlet was heated to the boiling point of the solvent and allowed to cycle for 6 h. Excess methanol was evaporated in a rotary evaporator. The collected extracts were diluted and tested for experiments.

Bioassays

Dipping one-day-old pupae in plant extracts/Proteus®

To investigate pupal sensitivity, each one-day-old pupa of *P. brassicae* was immersed individually in 50 ml of methanolic extracts of *S. hortensis*, *T. ammi*, *C. cyminum*, *Z. tenuior*, and *F. vulgare* for 15 sec that was determined by the preliminary dose-setting experiments. The ranges for *S. hortensis*, *T. ammi*, *C. cyminum*, *Z. tenuior* and *F. vulgare* were 12.50–125, 50–350, 25–200, 50–200, and 100–450 ppm, respectively. In controls, the pupae were immersed in methanol alone. In treated and untreated control experiments, when the solvent evaporated and pupae were dried, the pupae were transferred individually into plastic containers with ventilated lids that were kept at 22 ± 2 ºC, 50–70% RH, and a photoperiod of 16 L: 8 D. Each treatment and the untreated control had three replications per experiment. When no adult emergence was observed (the pupae became black and wrinkled), they were considered dead. Mortality counts were taken 7–14 days after exposure. After adult emergence, their survival was recorded and a large number of them died after 2–3 days. There was no mortality in the untreated control of any experiment. It is notable that since the pupa must connect to a base, we transferred 4th-instar larvae of the large cabbage butterfly individually into ventilated plastic containers, followed by placing some wooden branches for pupating. The larvae were fed with fresh cabbage every day. Each branch containing one-day-old pupa was dipped individually in plant extracts. The Proteus® trials were conducted as described in the above section that the range was 12–140 ppm.

Dipping one-day-old pupae in fungal suspension

One-day-old pupae of *P. brassicae* were immersed individually in fungal suspensions of *M. anisopliae* for 15 sec that was determined by the preliminary dose-setting experiments. Finally, the experiments were conducted as described in the previous section.

Methanolic extracts combined with M. anisopliae/*Proteus®*

Sub-lethal concentrations (LC_{25}) of extracts were combined with LC_{25} of *M. anisopliae* (plant extract + fungus)/Proteus® (LC_{25}) (plant extract + Proteus®) separately to examine whether there was a synergistic or antagonistic interaction between plant materials with entomopathogenic fungus/Proteus®. One-day-old pupae were immersed individ-

ually in sub-lethal concentration (LC_{25}) of each plant extract for 15 sec, next they immediately were immersed in LC_{25} of *M. anisopliae* suspension for 15 sec. The combination experiments, LC_{25} of plant + LC_{25} of Proteus® were carried out same as the previous. When the pupae were dried, they were transferred individually into plastic containers with ventilated lids that were kept at 22 ± 2 ºC, 50–70% RH, and a photoperiod of 16 L: 8 D. Each trial had three replications per experiment.

Data analysis

To determine LC_{50} and LC_{25} values, the data were analyzed using the Probit procedures with SPSS for Windows® release 20. The percentage data were transformed into arcsin$\sqrt{x}$ before statistical analysis. To determine synergistic/antagonistic interactions, experiments were performed following Tallarida (2000). The relationship between data was assayed by analysis of variance (ANOVA) and correlation analysis. The means were separated using the Tukey's test. The expected efficacy of a mixture, expressed as percent control ($\%C_{exp}$) can be predicted by the $\%C_{exp} = A + B - (AB/100)$ formula in which A and B are the control levels given by the single insecticides.

Results

Methanolic extract bioassays demonstrated that the applied five botanical materials caused a different amount of mortality of *P. brassicae* pupae (Table 1). The savory was the most effective plant (LC_{50} = 43.49 ppm) as it exhibited the highest potential activity against one-day-old pupae of *P. brassicae*. Fennel extract displayed a low mortality (LC_{50} = 235.63 ppm). Proteus® presented a high efficacy against the pest (LC_{50} = 48.88 ppm) (Table 1). *M. anisopliae* showed high pupal mortality with LC_{50} value equivalent to 1.3×10^3 conidia/ml (Table 2). The inclusion of *M. anisopliae* with all treated methanolic extracts (except for *M. anisopliae* + ammi) led to synergistic interactions against one-day-old pupae of the pest (Table 3). The tested fungus when combined

Table 1

Probit analysis of toxicity of some medicinal plant extracts and Proteus® to pupae of *Pieris brassicae*

Compound	χ^2	Slope ± S. E.	LC_{25} (ppm)	LC_{50} (ppm)	LC_{90} (ppm)
Cumin	3.12	3.27 ± 0.24	27.26 (15.48–37.61)	80.6 (63.34–105.30)	632.42 (354.64–1932.52)
Fennel	1.42	2.14 ± 0.33	106.5 (70.27–134.75)	235.63 (197.62–287.16)	1065.37 (694.01–2455.58)
Ziziphora	2.3	3.74 ± 0.35	48.6 (30.62–62.08)	107.67 (90.20–131.18)	488.15 (313.06–1219.94)
Savory	2.16	3.92 ± 0.21	12.84 (6.66–18.56)	43.49 (33.10–58.48)	441.78 (213.07–1557.04)
Ammi	1.62	2.72 ± 0.25	50.68 (29.29–69.11)	139.92 (11.23–178.22)	963.41 (567.37–2667.18)
Proteus®	2.15	3.58 ± 0.23	15.72 (8.79–21.89)	48.88 (38.07–65.20)	461.86 (227.30–1590.01)

Table 2

Probit analysis of the toxicity of *Metarhizium anisopliae* to pupae of *Pieris brassicae*

Entomopathogenic fungi	LC_{25} (conidia/ml)	LC_{50} (conidia/ml)	LC_{90} (conidia/ml)	Slope ± S. E.	χ^2 (df)
M. anisopliae	1.2×10^2 (3.4×10^1–2.5×10^2)	1.3×10^3 (8×10^2–2.5×10^3)	1.4×10^5 (3.7×10^4–1.8×10^6)	3.93 ± 0.10	1.58 (3)

95% fiducial limit (FL) is shown in parenthesis

Table 3

Synergistic/antagonistic interactions between some medicinal plant extracts with *Metarhizium anisopliae* against one-day-old pupae of *Pieris brassicae*

M. anisopliae + plant compound	%mortality ± S. E.		Interaction
	Expected	Observed	
M. anisopliae + ammi	36.66 ± 2.53	20.33 ± 1.92	antagonism
M. anisopliae + cumin	74 ± 3	90 ± 3.99	synergism
M. anisopliae + fennel	76.33 ± 2.99	90 ± 4.21	synergism
M. anisopliae + savory	72.66 ± 4.56	85 ± 3.45	synergism
M. anisopliae + ziziphora	70.33 ± 2.29	90 ± 4	synergism

Table 4

Combined effects of some medicinal plant extracts with Proteus® against one-day-old pupae of *Pieris brassicae*

Proteus® + plant compound	%mortality ± S. E.		Interaction
	Expected	Observed	
Proteus® + ammi	70.66 ± 2.9	90.33 ± 4.07	synergism
Proteus® + cumin	64 ± 2.01	86.66 ± 3.25	synergism
Proteus® + fennel	76.33 ± 2.8	80 ± 3.91	synergism
Proteus® + savory	72.66 ± 4.34	86 ± 3.22	synergism
Proteus® + ziziphora	66.33 ± 2.09	80.33 ± 3.67	synergism

with ammi methanolic extract possessed antagonistic effect. The tested medicinal plant extracts showed a synergistic effect when combined with Proteus® (Table 4).

Discussion

Proteus® showed a high efficacy against *P. brassicae* pupae that makes it as a proper component of *P. brassicae* pupae management tactics. Application of botanical sources like medicinal plant extracts could be a low-risk way for controlling pests and they can be an extraordinary replacement for chemical insecticides (Mohan et al., 2011). Botanicals have insecticidal activities because of their physical effect and muscular inhibition (Ali et al., 2017). In the present study, savory and cumin methanolic extracts resulted in the highest pupal mortality of tested botanicals, suggesting that they could

be substantial potential agents in the large cabbage butterfly control. Similarly, several studies have shown that some botanical extracts have insecticidal and repellent properties against lepidopteran insect pests. Sharma et al. (2011) tested repellent efficacies of aqueous and ethanol extracts of some plant species against *P. brassicae*. They reported that both extracts of *Azadirachta indica* A Juss and *Melia azedarach* L. have a higher repellency for 2nd instar larvae of the pest. Their results demonstrated efficacy of plant extracts against the pest that are consistent with our results. Sharma and Gupta (2009) tested antifeedant and toxic effects of ethanol and aqueous extracts of *A. indica*, *M. azedarach* Linn., *Lantana camara* L., *Cannabis sativa* Linn., *Nerium indicum* Mill., *Eucalyptus* sp., *Ricinus communis* Linn. and *Solanum nigrum* Linn. against *P. brassicae*. They reported that aqueous and ethanol extracts of *A. indica* and *M. azedarach* were highly effective against the large cabbage butterfly. They also indicated antifeedant effects of 10% aqueous extracts of *A. indica* and *M. azedarach* on *P. brassicae* 2nd instar larvae (81.7% and 81.8% protection, respectively). Similarly, they reported toxic effects of tested extracts against *P. brassicae*. Khorrami et al. (2017) investigated toxicity of some medicinal plant extracts to one-day-old and 2nd instar larvae of the large cabbage butterfly. They reported that ziziphora methanolic extract was the most effective against *P. brassicae* eggs but neonate larval survival resulted from one-day-old eggs exposed to ammi, cumin and savory methanolic extracts was 0%. They also presented high toxicity of mentioned extracts to 2nd instar larvae of *P. brassicae* (99.01, 98.27 and 97.96% mortality, respectively). Their conclusions are agreed with our results. The present results demonstrated that *M. anisopliae* had a high effect on one-day-old pupae of *P. brassicae*. Similarly, Ansari et al. (2007) reported control of western flower thrips (*Frankliniella occidentalis*) pupae with *M. anisopliae* in peat and peat alternative growing media. Ansari et al. (2004) also demonstrated that *M. anisopliae* CLO 53 is a highly virulent fungal isolate against *Hoplia phylantus* J. that is consistent with our results. In contrast, Garcia et al. (2009) reported less potency of *M. anisopliae* to imported 1st instar larvae of *P. rapae* than *Beauveria bassiana*. They attributed this lower efficacy of *M. anisopliae* to lower persistence of the fungus in field conditions that are probably influenced by environmental agents. For a successful IPM program, it is vital to prevent antagonistic interactions between different control methods. The findings of the present study exhibited that combination of sub-lethal doses of *M. anisopliae* and Proteus® with plant extracts gave synergistic activity, except for ammi + *M. anisopliae*. However, correct time management for their applications can be beneficial to diminish these antagonistic interactions. Generally, Proteus® can provide much better control of *P. brassicae* when integrated with the tested methanolic extracts. The integration of *M. anisopliae* with ziziphora, savory and cumin methanolic extracts can prove a successful alternative to synthetic chemicals and may become efficient component of integrated pest management strategies against lepidopteran pests of cabbage in future. One of the deficiencies of botanical compounds is their quick degradation, which shortens their effectiveness in the field. The entomopathogenic fungi are also susceptible to environmental factors. Perhaps this problem will soon be overcome through recent technological advances such as nanotechnology (Khater, 2012) that will allow future use of these bio-control agents in conventional/commercial crop production systems and field conditions. There are some researches about increased toxicity and effectiveness of some entomopathogenic fungi and plant extracts via nanotechnology approaches (Zahir et al., 2012; Sabbour, 2014) but there is a need for thorough research and study in this area be-

fore recommending nanomaterials to farmers and field conditions. In general, laboratory bioassays have clearly demonstrated the pathogenicity of *M. anisopliae* and tested methanolic extracts for one-day-old pupae of *P. brassicae*. These bio-control agents should be considered for development of a new and environmentally compatible approach to *P. brassicae* management helping at preventing the large cabbage butterfly infestations. It can be concluded that nanomaterials can enhance field performance of lab-tested entomopathogenic fungi and plant extracts but more researches are needed.

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