Comparative Biochemistry and Physiology, Part C 271 (2023) 109681

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Research article

Toxicity and physiological interruptions of a proteinaceous toxin from *Metarhizium anisopliae* against the olive fruit pest, *Bacterocera oleae* (Diptera: Tephritidae)

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ARTICLE INFO

Edited by Martin Grosell

Keywords:
Entomotoxicity
Olive fruit fly
Metarhizium anisopliae
Digestion
Antioxidative enzyme

ABSTRACT

The use of natural bioactive compounds mainly proteinaceous secondary metabolites of fungi is one of the promising pest control methods because of their lethal effects on insects in low concentration, limited persistence in environment and easily decomposition into environmentally safe compounds. The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is a destructive pest of olive fruits around the world. In the current study, the proteinaceous compounds were extracted from the two isolates (MASA and MAAI) of *Metarhizium anisopliae* and their effects were evaluated on toxicity, feeding performance and antioxidant system of the adult's olive flies. Both extracts from MASA and MAAI showed entomotoxicity against the adults by 2.47 and 2.38 mg/mL as LC_{50} concentrations. Also, LT_{50} values were recorded 1.15 and 1.31 days for MASA and MAAI, respectively. No statistical differences were recorded in the consumption rate of the adults on control and secondary metabolite contained protein hydrolysate. In contrast, the adults fed on LC_{30} and LC_{50} concentrations of MASA and MAAI demonstrated significant reduction in the activities of digestive alpha-amylase, glucosidases, lipase, trypsin, chymotrypsin, elastase, amino- and carboxypeptidases. Activity of antioxidant enzymes changed in the adults of *B. oleae* fed on the fungal secondary metabolites. Catalase, Peroxidase and Superoxide dismutase elevated in the treated adults with the highest amounts of MAAI. Similar results were found in activity of ascorbate peroxidase and glucose-6-phosphate dehydrogenase except for malondialdehyde amount in which no statistical differences were recorded between treatments and control. Relative gene expression of caspase enzymes revealed the higher expression in the treated *B. oleae* compared to control with the highest level of caspase 8 for MASA and caspases 1 and 8 for MAAI. Results of our study showed that the secondary metabolites extracted from the two isolates of *M. anisopliae* caused mortality, interrupted digestion and induced oxidative stress in the adults of *B. oleae*.

1. Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the most important pest of olive fruits in the Mediterranean, Africa, Canary Islands, Middle East, California and Central America because of exclusively feeding on fruits and imposing annual economic damages (Nardi et al., 2005; Daane and Johnson, 2010; von Gleich and Schröder, 2020). Females lay their eggs under the skin of ripe fruits and the larvae feed on the flesh around the core and finally turn into pupae near the skin to ease of emergence (Nardi et al., 2005). Because of enormous feeding of the larvae, extensive quantitative and qualitative damages are caused, of which the fruit drop, lose weight and reduce the quality of oil (Pereira et al., 2004; Iannota et al., 2012; Malheiro et al., 2015; Pinheiro et al., 2020; Preu et al., 2020). Although *B. oleae* cause 5 % damage of whole olive production worldwide, but it impose 42–75 % damages to olive plantations in Iran (Abbasi-Mojdehi et al., 2019). Integrated pest management programs are highly recommended to alleviate crop loss inflicted by *B. oleae* in which several control techniques are applied worldwide including choosing less susceptible cultivars, chemical treatment to be made only upon exceeding the intervention threshold, preventive treatment with poisoned protein baits, preventive treatments and repellents with copper-based products, precise monitoring of climatic conditions and subsequent pest population and etc. (Abbasi-Mojdehi et al., 2019). In Iran, farmers combat to Olive fruit fly by using

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https://doi.org/10.1016/j.cbpc.2023.109681
Received 10 April 2023; Received in revised form 27 May 2023; Accepted 11 June 2023
Available online 14 June 2023

protein hydrolysate traps and mainly wide spraying by organophosphate insecticides (Abbasi-Mojdehi et al., 2019). The use of chemical insecticides is inevitable in different parts of the world, although their continuous use leads to an increase in pest resistance to insecticides, the creation of chemical residues in the food chain, and the several malfunctions of ecosystem and human health (Keswani et al., 2020; Sharma and Sharma, 2021).

Entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Isaria fumosorosea*, *Nomuraea rileyi*, *Lecanicillium lecanii*, *Purpureocillium* sp. and *Cladosporium* sp., are among the efficient and important microbial control agents of insects which cause mortality by direct consume of body nutrients or indirectly by producing enzymes, toxic proteins and bioactive secondary metabolites (Isaka et al., 2005). Secreted secondary metabolites are a rich source of bioactive chemicals such as polyketides, non-ribosomal peptides, polyketide-peptide hybrid metabolites, and terpenes (Fox and Howlett, 2008). Different studies have shown that many of fungal secondary metabolites have antinutritional and insecticidal properties and affect longevity, fertility, digestion respiration and immunity of insects (Bandani et al., 2000; Molnar et al., 2010). One of the most important metabolites secreted by entomopathogenic fungi are dextroxins produced by *M. anisopliae*, which are cyclic peptides and have shown a wide range of biological properties such as entomotoxicity, cytotoxicity, and antibiotic activity (Pedras et al., 2002). In addition, immunological studies have shown that dextroxins also suppress the immune system of insects (Huxham et al., 1989; Cerenius et al., 1990). Proteins extracted from *M. anisopliae* showed a significant insecticidal activity on *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) by destruction of the midgut after feeding on food containing these proteins (Ortiz-Urquiza et al., 2009). Amiri et al. (1999) investigated the antinutritional property of dextroxin on the larvae of *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) and *Phaedon cochleriae Fbricius* (Stork 1980) (coleoptera) at low concentrations. Cabbage leaves treated with dextroxin significantly reduced larval feeding and imposed death of the insects was due to both starvation of dextroxin and the toxicity of this substance on other physiological processes. The insecticidal activity of dextroxin is also related to the oxidative stress, that affects many antioxidant enzymes, including peroxidase, ascorbate peroxidase, lipoxygenase, superoxide dismutase, total catalase (Sree and Padmaja, 2008).

Because of the severe damage of the Mediterranean and the olive fly to the olive fruits, widespread spraying of infected trees is inevitable in some areas. Bait traps, local and widespread spraying along with agricultural methods have been used to reduce the damage of this insect, but due to environmental reasons and food hygiene, the use of chemical insecticides can harm human health and endanger non-target organisms. The use of new bioactive compounds produced by plants and fungi is one of the most favorable options in pest control that even can prevent the process of resistance to pesticides (Ortiz-Urquiza et al., 2010). Although the use of these compounds has started years ago, they are considered to be the pillars of green pest management because they decompose faster than synthetic chemical compounds and have little negative effects on the environment. The use of toxic proteins produced by entomopathogenic fungi can be a favorable alternative to synthetic pesticides because of their lethal effect in low concentration, limited durability in the environment and their decomposition into environmentally safe compounds. Considering that no research has been done on the effect of *M. anisopliae* protein toxins against olive fruit fly and no physiological details of its effect, so the present research is based on extracting toxic protein from *M. anisopliae* and determining its toxicity and physiological effects against olive fruit fly.

2. Materials and methods

2.1. Insect rearing

Olive fruits infected with olive fly larvae were collected from the garden of Rudbar "Olive Research Station", where no spraying had been done. The collected olives were selected from one variety to avoid bias on the results. These fruits were kept in well-ventilated containers in growth chamber with a temperature of 25 ± 2 °C and relative humidity of 70 ± 5 % and a light and dark period of 16:8 h (Akmoutsou et al., 2011; Sánchez-Ramos et al., 2013). Emerged adults were transferred to separate rectangular cube containers 15 cm long x 12 cm wide x 5 cm high, where a hole of 6 cm long and 4 cm wide is created in the upper part of these containers for ventilation covered with a net to avoid adults exit. Adults were fed on a protein hydrolysate (DACUS BAIT 100®) from EVYP company dissolved in water at a ratio of 1:4 (Akmoutsou et al., 2011).

2.2. Extraction of the secondary metabolites from M. anisopliae isolates

Fungal secondary metabolites were extracted according to the method of Ortiz-Urquiza et al. (2010). First, two MASA and MAAI isolates [Native to Guilan province, where were collected from the cities of Soemasera and Anzali respectively and registered in the herbarium of mycology of the plant protection department of Gilan University, (Shahriari et al., 2021)] of *M. anisopliae* were cultured separately in the Liquid culture medium containing 40 g of glucose and 20 g of peptone in 1 L of distilled water). The culture medium was inoculated with a concentration of 10^7 conidia/ml and cultured for 4 days at 25 °C on a rotary with a speed of 110 rpm. Then 2 mL of the primary culture was added to 1 L flask containing 250 ml of liquid culture medium. After 7 days, the entire culture medium was passed through filter paper number 3. The cell-free medium was saturated with 90 % ammonium sulfate solution and the resulting sediment was centrifuged at 5000 *g* for 30 min and poured into a 7 kDa dialysis bag (Thermo Fisher Co.). The dialyzed solution was centrifuged at 10000 *g* and the resulting supernatant was filtered with a 0.45 μm syringe filter. Finally, protein concentration was determined using bovine serum albumin (Bradford, 1976).

2.3. Liquid chromatography – Electro Spray Ionization - Mass Spectroscopy/Mass Spectroscopy (LC-ESI-MS/MS)

The crude extracts of *M. anisopliae* isolates were separately dissolved in methanol and purified using preparative HPLC (Waters Alliance 2695). The acetonitrile-soluble residue was flash chromate graphed on a Merck (Darmstadt, Germany) K-60 silica gel column (230–400 mesh, 240,325 mm) employing a 50-ml stepwise methylene dichloride-methanol (95:5, at the beginning) solvent gradient to give desired polarities. Purification was performed on a preparative HPLC column (Merck LiChrosorb, 7 mm, and 250,310 mm). The sample was filtered, diluted with MeOH and analyzed using reverse phase HPLC column and the peaks got separated was detected by UV absorption at 215 nm. The running gradient of 0 min (0 % acetonitrile), 30 min (40 % acetonitrile), 40 min (50 % acetonitrile), 60 min (50 % acetonitrile) was included under the condition to collect the two fractions at 3.2 min and 7.2 min. Fractionated samples were collected and tested for biological activity (Ravindran et al., 2014). The analysis was done by passing the sample through a reverse-phase column (Zorbax 300 SB-C8, 2.1 mm, 6100 mm, 3.5 mm) attached to an electro spray ionization mass spectrometer. Separation of the compounds was eluted in this column via a gradient elution using water and acetonitrile (ACN), each containing 0.1 % formic acid, at a flow rate of 0.2 ml min-1 and the eluting peptides were characterized by conventional MS/MS (LC-ESI-MS; LC-ESI-MS/MS). The mass spectrometer, an HCT Ultra PTM Discovery (Micromass Quattro micro API), an ESI source and housed a classic ion trap (Paul type), using which, tandem MS (LC-ESI-MS/MS) data were acquired. The MS conditions were: capillary temperature 250 °C, source voltage 4.0 kV, source current 80.0 μA, and capillary voltage 7.0 V, in positive mode.

2.4. Bioassay of M. anisopliae secondary metabolite against B. oleae adults

One-day old adult olive flies were used for all bioassay experiments. Ten adults (5 male and 5 female insects) were transported into containers of 15 cm length x 12 cm width x 5 cm height considered as a replicate so that 180 adults were used to determine the effect of each extract on *B. oleae*. Then, different concentrations (0.5, 1, 2, 4, 8 and 10 mg/mL) of the extracted proteins were separately prepared and added into the protein hydrolysate. In control, distilled water was added to protein hydrolysate (Ortiz-Urquiza et al., 2009; Sánchez-Ramos et al., 2013). For each treatment, five drops of this mixture were placed on a plate with a diameter of 2 cm in the form of a circle, which were placed on a plastic piece with a diameter of 4 cm. The adults were allowed to feed on the drops for 24 h at a temperature of 25 ± 2 °C, a relative humidity of 70 ± 5 % and 16 L:8D hours of photoperiod. The number of dead flies were recorded and inserted in POLO-plus software to calculate LC values.

The effect of time on the lethality of protein extracted from *M. anisopliae* were determined using 8 mg/mL concentration of MASA and MAAI isolates as LC_{90} value. The concentration was separately added into the diet of adults based on the above method, and the mortality was recorded in the time intervals of 1 to 3 days. During these intervals, control insects were fed on hydrolyzed protein added by distilled water. For each time period, 50 one-day-old adult flies were used in 5 replicates with a sex ratio of 1:1. Both treated and control protein hydrolysate were replaced daily and the whole experiment in rearing condition explained earlier. The amount of LT_{50} was calculated using the obtained data in POLO-plus software.

2.5. The effect of LC_{50} concentration on feeding tendency of B. oleae

Choice and no-choice tests were done to evaluate the nutritional effects of the secondary metabolites MASA and MAAI isolates against adult of *B. oleae*. In choice test, 60 one-day-old adults male and female (1:1 ratio) were released into cages with dimensions of 20 cm length x 12 cm width × 15 cm height, and two containers with a diameter of 2 cm containing protein hydrolysate and distilled water as control and another containing protein hydrolyzate plus LC_{50} of each extract separately were provided to the adults. In the no-choice test, only one container containing protein hydrolysate and each extracted protein were placed under the mentioned conditions. Both tests were done separately in three groups of 20. The experiment conditions were at temperature of 25 ± 2 °C, a relative humidity of 70 ± 5 %, and 16 L:8D h as photoperiod. In both tests, the volume ingested in the control and treatment was evaluated by measuring the remaining solution. Every three days, the amount of the remaining solution was measured and the new solution was provided to the same group of adults. To eliminate the error of evaporation, six containers containing protein hydrolysate and distilled water (three containers) and protein hydrolysate and extracted protein (three containers) without adults were placed in a separate container so that the effect of evaporation was not considered as an experimental error. Based on the following formula, the amount of ingestion was calculated (Ortiz-Urquiza et al., 2009):

$$V = (X - E)(2000\,\mu l)/1.5\ cm$$

where V is the consumed volume in microliters, X is the height of the remaining solution in the container, E is the evaporated volume in terms of height, 2000 μl is the initial volume and 1.5 cm is the height of the container.

2.6. The effect of LC_{50} concentration on digestive enzymes of B. oleae

The control and treated adults by LC_{50} concentration of each secondary metabolite were randomly selected, chilled on ice and their entire digestive system was isolated under a stereo microscope within saline solution (NaCl, 10 mM). The midgut was separated from the other parts and homogenized in an equal ratio of Tris-HCl buffer (20 mM, pH 7.2) in 1.5 ml microtubes using a glass homogenizer. The homogenized samples were centrifuged at 20,000*g* and 4 °C for 20 min to gain supernatant for enzyme assays. To prepare membrane-bound samples (Glucosidases and Exopeptidases), Triton X-100 was added to the sediment obtained from the first centrifuge at the rate of 10 μg per milligram of protein, incubated overnight and re-centrifuges at 20,000 *g* and 4 °C for 30 min (Ferreira and Terra, 1983). The activity of digestive enzymes will be measured as follows:

2.6.1. α-Amylase assay

α-Amylase activity was assayed by a reaction mixture containing 50 μl Tris-HCl buffer (20 mM, pH 7), 20 μl of starch and 10 μl of enzyme sample. After 30 min, the reaction was terminated by 80 μL of dinitrosalisylic acid (DNS) and incubating in boiling water for 10 min. Finally, the absorbance was recorded at 545 nm. A negative control contained including pre-boiled enzyme (for 15 min) was used to validate enzyme activity (Bernfeld, 1955).

2.6.2. α- and β-Glucosidases assay

The two substrates of *p*-nitrophenyl- α-Dglucopyranoside (pNαG) and *p*-nitrophenyl-β-D-glucopyranoside (pNβG) were used to determine activities of α- and β-glucosidases, respectively. Samples of membrane-bound fractions were incubated with 10 μL of substrate and 40 μL of Tris-HCl buffer. After 10 min of incubation, the absorbance was reported at 405 nm. A negative control contained including pre-boiled enzyme (for 15 min) was used to validate enzyme activity (Ferreira and Terra, 1983).

2.6.3. Lipase assay

For lipase assay, 15 μL of sample was added to 30 μL of *p*-nitrophenyl-butyrate (27 mM) as substrate already dissolved in Tris-HCl buffer (pH 7). After 10 min of incubation, the absorbance was reported at 405 nm. A negative control contained including pre-boiled enzyme (for 15 min) was used to validate enzyme activity (Tsujita et al., 1989).

2.6.4. Serine proteases assay

The activities of serine proteases including trypsin-, chymotrypsin- and elastase were determined by 1 mM concentration of BApNA (Na-benzoyl-L-arginine-p-nitroanilide, Sigma-Aldrich, 19,362), 1 mM SAAPPpNA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide, Sigma-Adrich, S7388) and 1 mM SAAApNA (N-succinyl-alanine-alanine-alanine-p-nitroanilide, Sigma-Aldrich, S4760), respectively. The reaction mixture contained 30 μL of Tris-HCl buffer (pH 8), 15 μL of each substrate separately and 10 μL of enzyme solution. A negative control contained including pre-boiled enzyme (for 15 min) was used to validate enzyme activity (Oppert et al., 2003).

2.6.5. Exopeptidases assay

Amino- and carboxypeptidases were assayed by hippuryl-L-arginine (Sigma-Aldrich, H2508) and hippuryl-*L*-phenylalanine (Sigma-Aldrich, H6875). The reaction mixture contained 30 μL of Tris-HCl buffer (pH 8), 15 μL of each substrate separately and 10 μL of enzyme solution. A negative control contained including pre-boiled enzyme (for 15 min) was used to validate enzyme activity (Oppert et al., 2003).

2.7. Antioxidant assays

The control and treated adults (whole body preparation) by LC_{50} concentration of each secondary metabolite were randomly selected and homogenized in an equal ratio of Tris-HCl buffer (20 mM, pH 7.2) in 1.5 ml microtubes using a glass homogenizer. The homogenized samples were centrifuged at 20,000 *g* and 4 °C for 20 min to gain supernatant for

enzyme assays.

2.7.1. Catalase assay

The enzyme was assayed by incubating 50 μL of sample and 500 μL of hydrogen peroxide (1 %) at 28 °C for 10 min. Then, the absorbance was reported as ΔA at 240 nm (Wang et al., 2001a, 2001b).

2.7.2. Superoxide dismutase (SOD) assay

A 50 μL of sample and 500 μL of reaction solution containing 70 μM of NBT, 125 μM of xanthine, both dissolved in PBS were mixed with 100 μl of xanthine oxidase solution containing 10 mg of bovine serum albumin was mixed before adding 100 μL of xanthine oxidase (5.87 units/ml) dissolved in 2 ml of PBS. After 20 min incubation at dark, the absorbance was reported as the ΔA at 560 nm (McCord and Fridovich, 1969a, 1969b).

2.7.3. Peroxidase assay

Peroxidase assay was done by 50 μL of sample, 250 μL of buffered pyrogallol [0.05 M pyrogallol in 27 mM Tris-HCl (pH 7.0)] and 250 μL of H_2O_2 (1 %). The absorbance was continually read every 30 s for 2 min at 430 nm. Also, the activity was calculated by an extinction coefficient of oxidized pyrogallol (4.5 l/mol) (Addy and Goodman, 1972).

2.7.4. Ascorbate peroxidase assay

The enzyme was assayed by 50 μL of sample, 150 μL of Tris-HCl (27 Mm, pH 7.0), 70 μL ascorbic acid (2.5 mM) and 200 μL H_2O_2 (30 mM). After 5 min of incubation, the absorbance as recorded at 290 nm for 5 min (Asada, 1984).

2.7.5. Glucose-6-phosphate dehydrogenase assay

The assay was done by 100 μL of Tris-HCl (100 mM, pH 8.2), 50 μL of NADP (0.2 mM) and 30 μL of $MgCl_2$ (0.1 M). After mixing the mentioned components, 50 μL of the sample and 100 μL of GPDH (6 mM) was added to the mixture and absorbance was recorded at 340 nm (Balinsky and Bernstein, 1963).

2.7.6. MDA concentration

A reaction mixture containing 100 μL of 20 % trichloroacetic acid and 50 μL of the sample was prepared and centrifuged at 15000*g* and 4 °C for 10 min. The gained supernatant was added into 100 μL of 0.8 % TBA reagent and re-incubated at 100 °C for 60 min. After that, the absorbance was recorded at 535 nm. The MDA concentration is reported as amount of MDA produced per mg protein using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Bar-Or et al., 2001).

2.7.7. Assay of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate)

Initially, 1 mL of midgut supernatant was added to 500 μL of DPPH solution (0.02) and incubated at darkness for 15 min after adding 1 mL of ethanol (96 %). After that, absorbance was recorded at 512 nm. In control samples, 1 mL of distilled water was used instead of midgut supernatant. Percentage of inhibition was calculated as following (Wang et al., 2013):

$$\% = \frac{(OD\ control - OD\ sample)}{OD\ control} \times 100$$

2.8. Expressions of caspase genes involved in cell death

2.8.1. RNA extraction

Both control and secondary metabolite-treated adults of *B. oleae* were subjected to RNA extraction and cDNA synthesis in which five groups containing 10 adults were separately considered for the process. A commercial kit containing guanidine–phenol solution (RNX-Plus, SinaClon, Cat. No.: RN7713C) was used for RNA extraction. At first, 10 adults (separately for control and treatment for each replicate) were rinsed in 500 μL of ice cold RNXPLUS solution within microtubes, gently homogenized with a glass pestle, vortexed for 5–10 s and incubated at room temperature for 5 min. Then, 200 μL of chloroform was added to the mixture and shaken for 15 s prior to further incubation on ice for 15 min. The first step of centrifugation was done at 18,000*g* for 15 min at 4 °C resulting in an aqueous phase. The phase was transferred to a new RNase-free tube and mixed with an equal volume of isopropanol. The mixture was gently mixed and incubated on ice for 15 min before being centrifuged at 18000 *g* for 15 min at 4 °C. Afterward, 1 mL of 75 % ethanol was added to samples, gently shaken and re-centrifuged at 5000 *g* for 8 min at 4 °C. At this step, supernatant was discarded and the pellets dried at room temperature for 3 min before being immersed in 50 μL of DEPC (diethylpyrocarbonate)-treated water. Estimation of RNA quality was done by UV/VI'S spectrophotometer at multiple wavelengths. The ratio of OD 260/280 was used for quality and the ratio of OD 260/240 was used for purity of RNA. Moreover, an agarose gel electrophoresis (1 %) was done to determine quality of the extracted RNA.

Table 1
Primers used to determine expressions of genes involved in pyridalyl toxicity againt *Bacterocera oleae*.

Gene[a]	Primers	Access code	Product length
Caspase 1	F: TTTCATACCGAACCGACTCC R: TCACAGTTGCTATGGCGTTC	XM-014246332.1	117 bp
Caspase 3	F: TAAAGGCTCCGATCTGTTGC R: GAAGGTTTTGGTGAGCGTTG	XM-014231270.1	139 bp
Caspase 8	F: CGGGACAATTGGTATTACGC R: GTAATAATGCGCCGCGATAG	XM-014236812.1	121 bp
Caspase 9	F: GCCAACACAAAACCGCTAAC R: TAGTGACTTTTGCGGCTGAG	XM-014244989.1	111 bp
18srRNA	F: CACGGGAAATCTCACCAGG R: CAGACAAATCGCTCCACCAACTA	Liu et al. (2013)	114

[a] All primers were designed using an online software, Primer3 plus, (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and their specificity was checked by BLASTX and TBLASTN at NCBI.

2.8.2. cDNA synthesis

cDNA synthesis was done using a Thermo Scientific RevertAid First Strand Kit (Thermo Scientific, K1621) in which a mixture containing 4 μL of 5× reaction buffer, 1 mL (20 U/μl) of RiboLock RNase Inhibitor, 2 mL (10 Mm) of dNTP Mix, 1 mL (200 U/μl) of RevertAid H Minus Reverse Transcriptase, 1 μL of Oligo-dT primers, 11 μL of DEPC-treated water, and 1 μL of the extracted RNA of control and treated adults separately was used for synthesis of first-strand cDNA. After sample preparations, microtubes were incubated for 60 min at 42 °C and the reaction was stopped at 70 °C for 10 min. The second strand cDNA synthesis was achieved by reverse transcription product prior to being stored at −20 °C.

2.8.3. cDNA amplification

After reverse transcription, we performed ordinary PCR reactions for each of the chosen genes. As a reference gene for successive qRT-PCR experiments, we have chosen 18srRNA accordingly to Liu et al. (2013). The levels of ribosomal 18S rRNA, checked both by semi-quantitative PCR and during qRT-PCR reactions were overlapping for all the tested samples. The target genes included cytochrome P450, insulin receptor, catalase, peroxidase, superoxide dismutase, caspases 1, 3, 8 and 9 using specific primers given in Table 1. PCR was done for 40 cycles at a denaturing temperature of 95 °C for 2 min, 95 °C for 30 s, at an annealing temperature of 53 °C for 30 s, 72 °C for 30 s, and at an extending temperature of 72 °C for 5 min. The PCR constituents contained 25 μL of cDNA (1 μL) templates, 5× reaction buffer (2.5 μL), dNTPs (0.5 μL), $MgCl_2$ (0.75 μL), Taq polymerase (0.25 μL), DEPC-treated water (19 μL), and 0.5 μL of each forward and reverse primers separately for each gene in a thermocycler.

Table 2
Toxicity (Lethal dose) of the secondary metabolites extracted from *Metarhizium anisopliae* secondary metabolites isolates against *Bacterocera oleae* adults.*

Isolates	LC_{30} (mg) (Confidence limit 95 %)	LC_{50} (mg) (Confidence limit 95 %)	LC_{90} (mg) (Confidence limit 95 %)	Slope ± SE	X^2	Df
MASA	1.52 (0.827–2.112)	2.474 (1.690–3.278)	8.123 (5.674–16.38)	2.482 ± 0.381	3.615	3
MAAI	1.411 (0.572–2.189)	2.384 (2.189–1.77)	8.599 (5.056–32.968)	2.300 ± 0.325	7.0752	3

* All data were analyzed POLO-Plus software.

Table 3
LT_{50} value of the secondary metabolites extracted from *Metarhizium anisopliae* secondary metabolites isolates against *Bacterocera oleae* adults.

Isolates	LT_{50} (mg) (Confidence limit 95 %)	Slope ± SE	X^2	Df
MASA	1.15 (0.48–2.63)	0.879 ± 0.035	3.928	2
MAAI	1.31 (0.75–2.24)	0.71 ± 0.114	4.236	2

2.9. Protein assay

The method described by Bradford (1976) was used to find protein contents of each sample (recommended by Ziest Chem. Co., Tehran-Iran).

2.10. Statistical analyses

All data were compared by one-way analysis of variance (ANOVA), Tukey's test. The statistical differences were considered at a probability <5 % and marked by different letters among treatments.

3. Results

3.1. LC-MS analysis

The LC-MS analysis of the extracts from *M. anisopliae* isolates was done to identify the number of chemical components. Three prominent peaks were detected during a period of 40 min after injection that identified as destruxin B (Molecular weight = 533.35), destruxin C

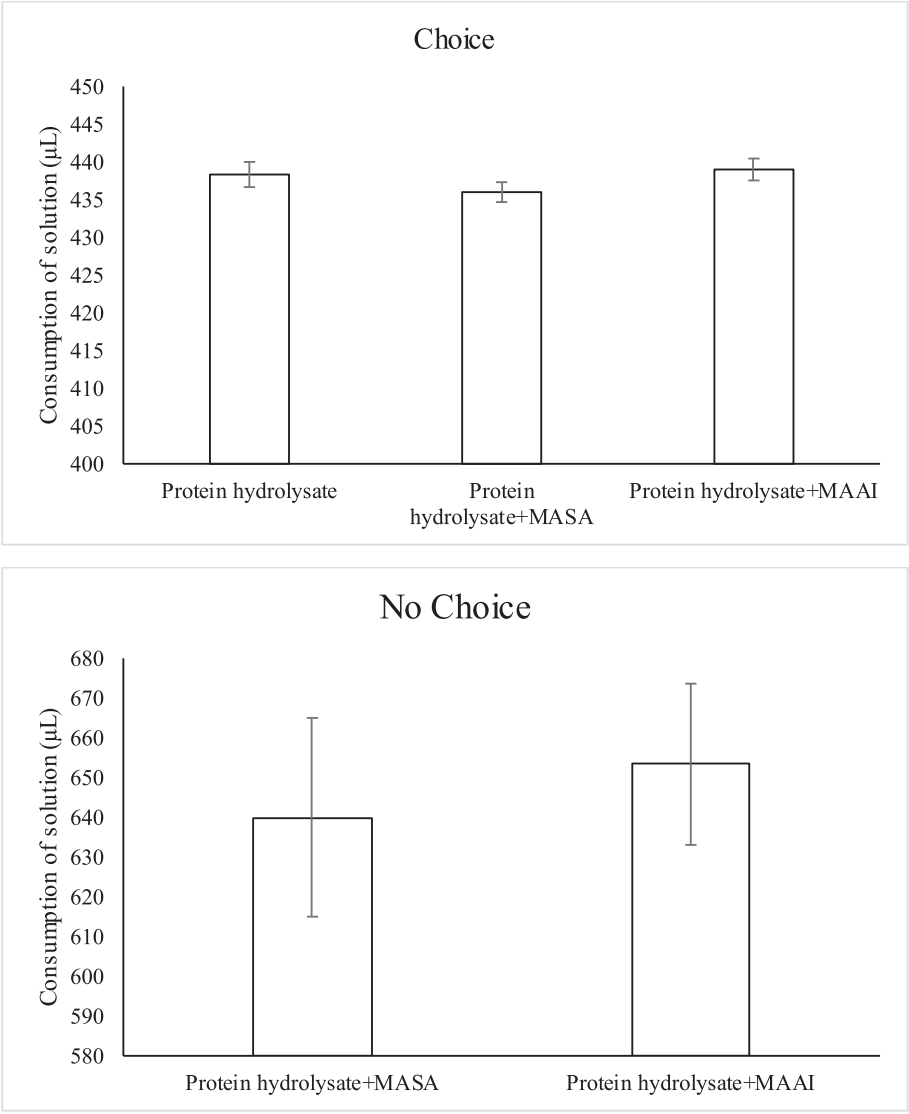

Fig. 1. Effects of the secondary metabolites extracted from *Metarhizium anisopliae* isolates (MASA and MAAI) on feeding performance of *Bacterocera oleae* adults. Statistical differences have been done between control and treatments and marked by different letters.

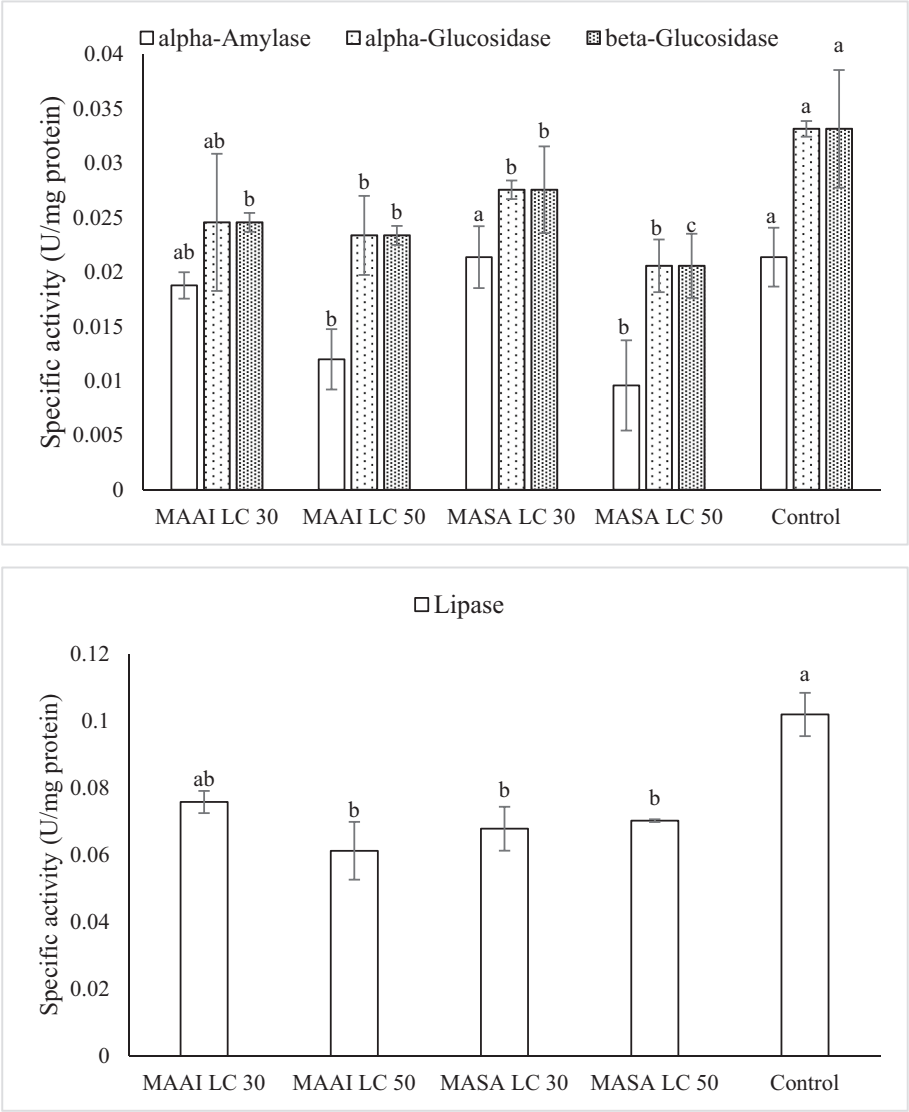

Fig. 2. Activity of digestive carbohydrases and lipase in the control and the treated adults of *Bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

(Molecular weight = 546.28), destruxin E DTX C (Molecular weight = 563.41) (Supplementary 1).

3.2. Effects of M. anisopliae extracts on feeding tendency of B. oleae

The extracted proteins were diluted, added to the protein hydrolysate and exposured to the adults of *B. oleae*. Results revealed the entomotoxicity of these two molecules against the adults by LC_{50} values of 2.474 mg/mL and 2.384 mg/mL for MASA and MAAI respectively (Table 2). Moreover, these molecules afflicted adults of *B. oleae* within 1.15 and 1.31 days as LT50 values, respectively (Table 3). Choice and no-choice experiments to determine feeding deterrence of the proteinaceous extract of MASA and MAAI showed no statistical differences with the protein hydrolysate diluted by distilled water (Fig. 1).

3.3. Effects of M. anisopliae extracts on digestive enzymes of B. oleae

In contrast with choice and no-choice results, the adults fed on control and treated protein hydrolysate by MASA and MAAI proteinaceous extracts showed the statistical differences in the activities of digestive lipase, carbohydrases and proteases. The activity of alpha-amylase significantly decreased in the adults treated by LC_{50} concentrations of the extract from both fungal isolates but the adults treated by LC_{30} concentrations of MASA had no statistical differences with control (Fig. 2, F = 5.93, Pr > f = 0.018, df = 4, 14). The adults were fed on control diet showed the highest alpha-glucosidase activity as it significantly reduced in treated adults by both LC30 and LC50 concentrations of MASA and MAAI (Fig. 2, F = 3.14, Pr > f = 0.011, df = 4, 14). The activity of beta-glucosidase significantly decreased in the treated adults compared to control in which the least activity was found in the adults by LC_{50} concentration of MASA (Fig. 2, F = 4.18, Pr > f = 0.014, df = 4, 14). Finally, lipase activity statistically reduced in the treated adults of *B. oleae* compared to control for all concentrations (Fig. 2, F = 5.01, Pr > f = 0.018, df = 4, 14).

The activity of all digestive proteases decreased in the adults treated by secondary metabolites of MASA and MAAI compared to control (Fig. 3). Trypsin activity follows the mentioned trends as it sharply decreased in the treated adults with the least activity in LC50 concentration of MASA (Fig. 3, F = 5.69, Pr > f = 0.019, df = 4, 14). Moreover, the least activity of chymotrypsin (Fig. 3, F = 6.32, Pr > f = 0.014, df = 4, 14) and elastase (Fig. 3, F = 3.45, Pr > f = 0.016, df = 4, 14) was found in the adults treated by LC_{50} concentration of MASA. The two exopeptidases, amino- and carboxypeptidases, significantly afflicted by adding proteinaceous extract of MASA and MAAI. All treatments led to

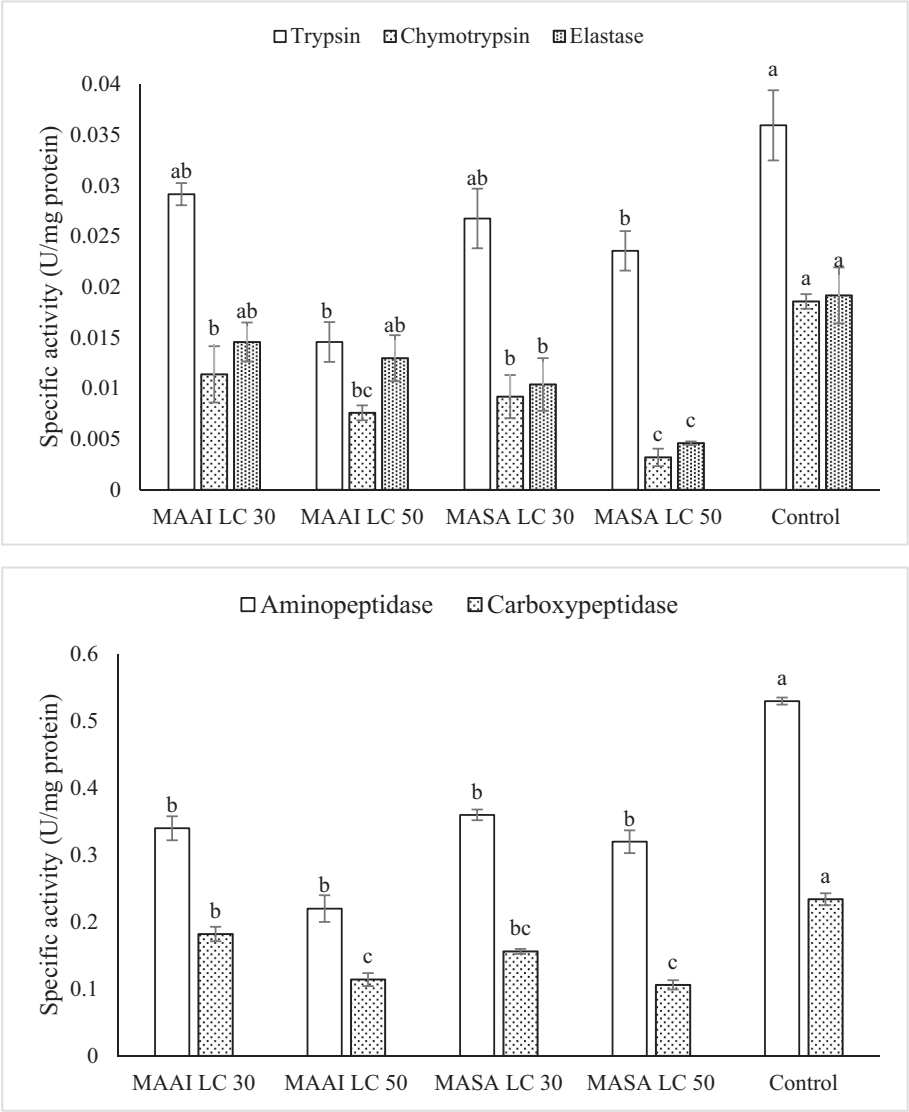

Fig. 3. Activity of digestive serine proteinases and exopeptidases in the control and the treated adults of *Bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

the lower activity of aminopeptidase compared to control (Fig. 3, F = 7.11, Pr > f = 0.019, df = 4, 14) while carboxypeptidase showed the least activity in LC_{50} of both isolates (Fig. 3, F = 11.29, Pr > f = 0.003, df = 4, 14).

3.4. Effects of M. anisopliae extracts on antioxidant activity of B. oleae

Activity of antioxidant enzymes in the treated adults of *B. oleae* significantly enhanced compared to control except for MDA (Figs. 4 and 5). The highest activity of CAT (Fig. 4, F = 56.44, Pr > f = 0.0001, df = 2, 8), POX and SOD (Fig. 4, F = 71.59, Pr > f = 0.0001, df = 2, 8) was recorded in the adults fed on the protein hydrolysate containing MAAI while the least activity was in control adults except for POX that no statistical differences was found between MASA and control (Fig. 4, F = 36.62, Pr > f = 0.0001, df = 2, 8). Activity of APOX significantly increased in the treated adults mainly MAAI (Fig. 5, F = 21.98, Pr > f = 0.0002, df = 2, 8). Similar results were recorded in case of GPDH while no statistical differences were found between MASA and control (Fig. 5, F = 9.58, Pr > f = 0.014, df = 2, 8). In contrast to other antioxidant components, no statistical differences were recorded between control and treated adults by MASA and MAAI in MDA (Fig. 5, F = 4.94, Pr > f = 0.0054, df = 2, 8). Finally, determination of DPPH inhibition revealed significant increase in the treated adults by MASA and MAAI (Fig. 6, F = 14.72, Pr > F = 0.0027, df = 2, 8).

3.5. Effects of M. anisopliae extracts on caspase gene expression of B. oleae

Evaluation of caspase gene expression in the control and secondary metabolite-treated adults of *B. oleae* showed the elevated expression ratio of caspases in the treated adults. The highest expression ratio was recorded in caspase 8 (F = 85.28, Pr > f = 0.0001, df = 2, 8) for MASA and caspase 1 (F = 260.89, Pr > f = 0.0001, df = 2, 8) for MAAI treatments (Fig. 7).

4. Discussion

Rapid increase of pest resistance and environmental toxicity caused by common synthetic pesticides requires precisely and increasingly development of environmentally friendly alternatives including microbial agents and natural products (Keswani et al., 2020). Toxins produced by entomopathogenic fungi have many advantages over chemical

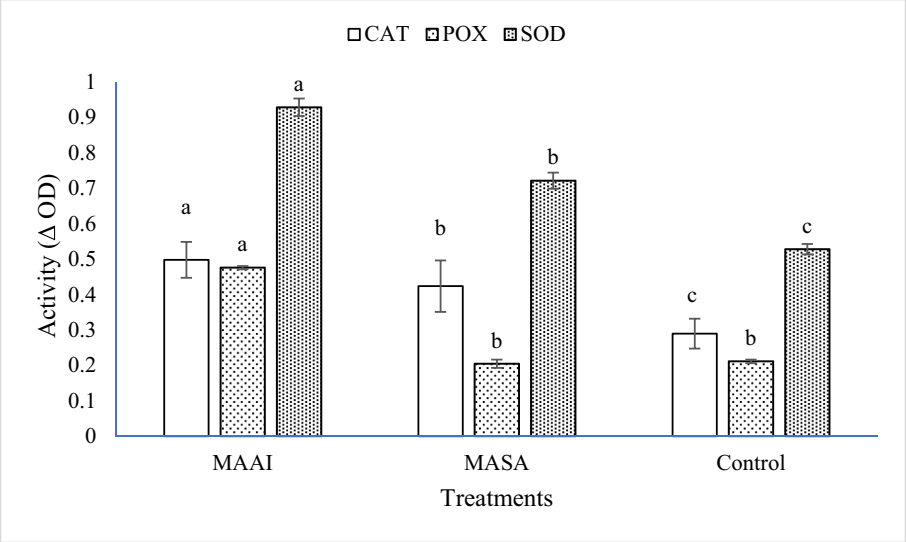

Fig. 4. Activity of Catalase (CAT), Peroxidase (POX) and Superoxide dismutase (SOD) in the control and the treated adults of *Bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

insecticides in that they kill insects at different stages in both laboratory and field conditions, have lower insecticidal activity on non-target organisms, and remain stable for some months in extreme hot and cold conditions. Moreover, their toxicity in low concentrations make them promising to be produced and applied against vast number of agricultural pests (Ortiz-Urquiza et al., 2008; Lozano-Tovar et al., 2015; Keppanan et al., 2019; Vivekanandhan et al., 2022). In the present study, ingestion of the proteinaceous secondary metabolites extracted from MASA and MAAI demonstrated mortality on *B. oleae* adults so that MAAI has more potent insecticidal property than that of MASA. This finding may be attributed to the different origin of these geographical isolates that caused different mortality on our previous study and unpublished data of *B. oleae* (Shahriari et al., 2021). Also, protein gain of the secreted secondary metabolite was 27.2 mg/mL for MAAI and 24.45 mg/mL for MASA. The correlation between content protein and insecticidal activity of crude secondary metabolites from these isolates leads to the conception that content protein can be considered as a virulence factor in addition to possibly different nature of existed proteins in each secondary metabolite. The obtained results were similar to the findings already reported from the protein contents and insecticidal activities of M-19 and M-10 isolates of *M. anisopliae* on *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) larvae (Sowjanya Sree et al., 2008). Insecticidal activity of proteins produced by entomopathogenic fungi has been reported in several previous studies. For example, Ortiz-Urquiza et al. (2008, 2009) showed that the soluble crude protein extracted from *M. anisopliae* (EAMa 01/58-Su) had a strong toxicity against *S. littoralis* and *C. capitata*. Lozano-Tovar et al. (2015) studied virulence of proteins produced by eight entomopathogenic fungi against the adults of *C. capitata*. The protein produced from the isolate of EAMb 09/01- Su (*M. anisopliae*) caused the highest mortality ranging between 95 and 100 % at 48 h post-treatment (Lozano-Tovar et al., 2015). Balachander et al. (2012) reported that the crude toxin proteins from the six isolates of *M. anisopliae* had insecticidal activity against *Hypsipyla robusta* Moore (Lepidoptera: Pyralidae). Also, the concentrations of 1, 2 and 3 % protein extracted from IF8 isolate of *Isaria fumosorosea* caused the mortality >80 % on Asian citrus psyllid at 48–120 h post application (Keppanan et al., 2019). Diao et al. (2022) extracted the crude toxin protein from IF-1106 isolate of *Cordyceps fumosorosea*, and studied its insecticidal activity on *Myzus persicae* by oral exposure, injection, and topical treatment. The crude toxin protein had a high toxicity on *M. persicae* specially by oral exposure and injection than contact sprays (Diao et al., 2022). The crude extracts of the *Metarhizium* sp. contained secondary metabolites, including destruxins m(destruxin-A, destruxin-B and destruxin-E), showed insecticidal and antifeedant properties against some insects of Diptera, Lepidoptera, Homoptera, Coleoptera and Isoptera (Amiri et al., 1999; Hu et al., 2007; Sowjanya Sree et al., 2008; Ortiz-Urquiza et al., 2009; Balachander et al., 2012; Lozano-Tovar et al., 2015; Vivekanandhan et al., 2022).

In the choice and no-choice experiments, there were no significant differences in the ingested volume between treated and controls *B. oleae* adults. These results shows that insects cannot distinguish between toxin soluble and control soluble. This is important because ensure ingestion of toxic materials by flies. Similarly, Ortiz-Urquiza et al. (2009) demonstrated that crude toxin protein *M. anisopliae* had no antifeedant activity in the adults of *C. capitata* during the three first days of the experiment. Although the antifeedant activity of fungal secondary metabolites has previously been reported on numerous insects (Bandani and Butt, 1999; Quesada-Moraga et al., 2006; Hu et al., 2007; Freed et al., 2012). Antifeedant compounds have generally been classified as feeding deterrents or suppressants. Whereas in several insects the ingestion of the crude toxin proteins induced a quick suppression of ingestion, no suppression was observed in *B. oleae*. This disagreement may be a result of a progressive deterioration of the midgut (toxicant), or of a slow accumulation of sensory information (deterrent) in *B. oleae*.

Appropriate nutrition generally contributes in insects' ecological fitness to highly utilization of vital nutrients for reproduction and colonization (Bezzar-Bendjazia et al., 2017). Alimentary canal of insects promotes digestion and absorption of ingested foods so any disturbance may lead to reduction in efficiency of nutrient utilization (Shahriari et al., 2019). Our results here demonstrated a clear disturbance of digestive performance in *B. oleae* adults after treatments by crude toxin proteins of MAAI and MASA isolates compared to control. Feeding of *B. oleae* adults on these metabolites led to lower activities of a-amylase, glucosidases, lipase, and proteases. So far, there are no reports on the effect of fungal toxins on digestive enzymes activities of insects, but the destruction of the epithelium cells of insect midgut observed in histological studies (Quesada-Moraga et al., 2006; Ortiz-Urquiza et al., 2009). Histological analysis indicates a complete destruction of the midgut of *C. capitata* adults and *S. littoralis* after feeding of the crude toxin protein of *M. anisopliae* EAMa 01/58-Su isolate (Quesada-Moraga et al., 2006; Ortiz-Urquiza et al., 2009). Since digestive enzymes are produced in midgut epithelial cells through secretagogue mechanism,

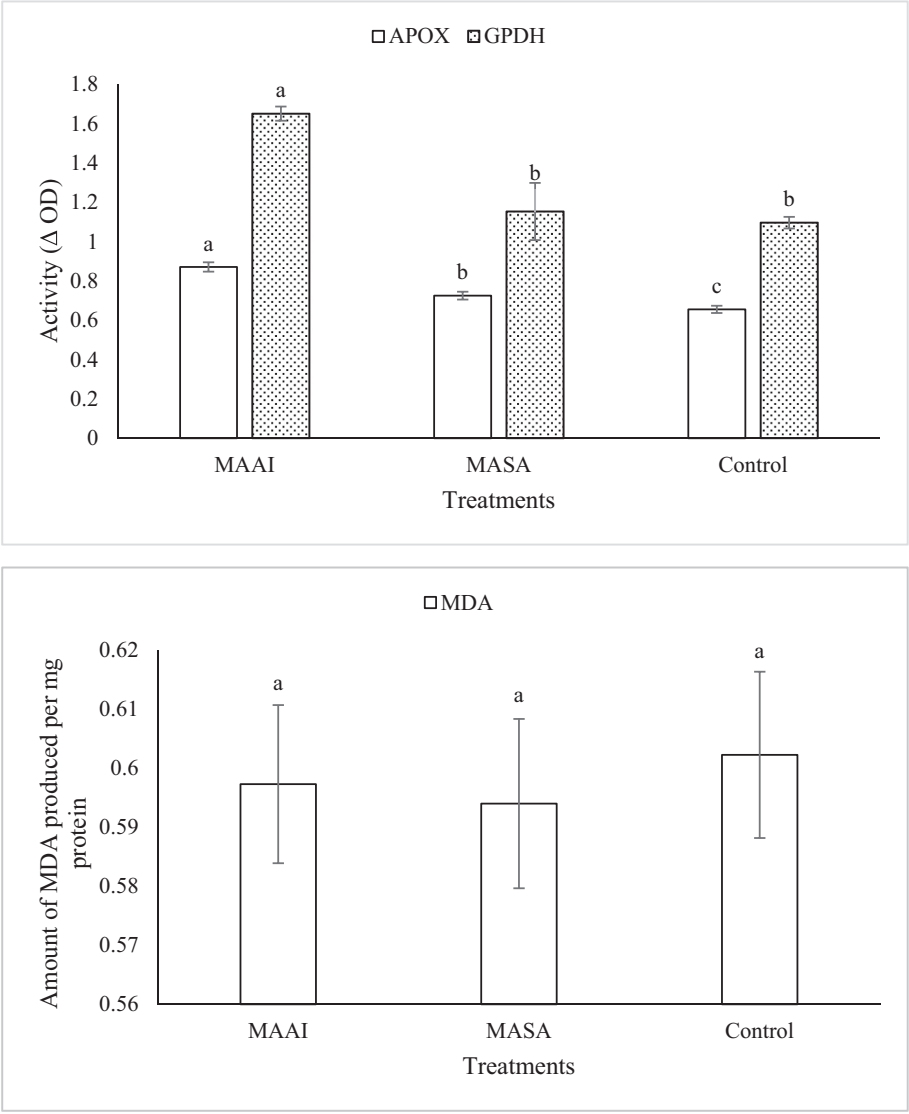

Fig. 5. Activity of Ascorbate peroxidase (APOX), Glucose-6-phosphate dehydrogenase (GPDH) and the produced amount of Malondialdehyde (MDA) in the control and the treated adults of *Bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

any destruction of cells may impose digestive enzyme performance compared to control insects. Also similar results were observed by other microbial toxins such as *Bacillus thuringiensis* (Berliner) or plant-derived active compounds such as diallyl disulfide and azadirachtin (Nathan et al., 2006; Shahriari and Sahebzadeh, 2017). In our study, the lower digestive enzymes activities of *B. oleae* following crude toxin proteins administration could be attributed cytotoxic effects toward epithelial cells of alimentary canal which the present one reduced leakage rate of these enzymes. This conclusion is in accordance with our findings on gene expression of caspases in the treated *B. oleae* with MASA and MAAI secondary metabolites. These enzymes are involved in apoptosis or programmed cell death that may cause during development or damaged cells by physiological or environmental causes (Accorsi et al., 2015). Activation of these genes may indicate cytotoxicity effects of MASA and MAAI on midgut epithelial cells of *B. oleae* adults after exposure.

Entomopathogens and their metabolites have been known as one of the significant exogenous resources that produce free radicals (Glupov et al., 2003; Sowjanya Sree et al., 2008; Shamakhi et al., 2020). Production of reactive oxygen species (ROS) by toxicant compounds, resulted in oxidative stress that finally leads to DNA damage and lipid peroxidation (Sowjanya Sree et al., 2008). Several studies have shown involvements of CAT, SOD, POX, APOX, GPDH and MDA and α-tocopherol in the antioxidant defenses of insects (Shahriari et al., 2019; Shamakhi et al., 2020). Superoxidase dismutase has a key role to catalyze superoxide (O_2^-) radicals into hydrogen peroxide (H_2O_2). Afterward, H_2O_2 converted to H_2O and O_2 by CAT and POD activities (McCord and Fridovich, 1969a, 1969b). Results of the present study confirm findings of previous studies on effect of fungal conidia and crude toxin extracts on antioxidant enzymes of insects (Glupov et al., 2003; Sowjanya Sree et al., 2008; Jia et al., 2016; Karthi et al., 2018; Shamakhi et al., 2020). These investigations clearly demonstrate that microbial agents especially entomopathogenic fungi are a significant factor in activity of antioxidant enzymes in insects. In our study, treatments of *B. oleae* adults by metabolites of the entomopathogenic fungus *M. anisopliae* (MAAI and MASA isolates), increased activities of SOD, CAT and POX. It seems that crude toxin proteins may increase levels of O_2^- and H_2O_2 which subsequently induced antioxidant enzymes activation. Moreover, the increased CAT and POD activities may be attributed to the higher activity of SOD which produces H_2O_2 in the *B. oleae* adults. Similar findings were indicated regarding the effects of dextroxin produced by *M. anisopliae* on *S. litura* larvae (Sowjanya Sree et al., 2008). The response of the antioxidant enzymes, such as SOD, SOD and POX has

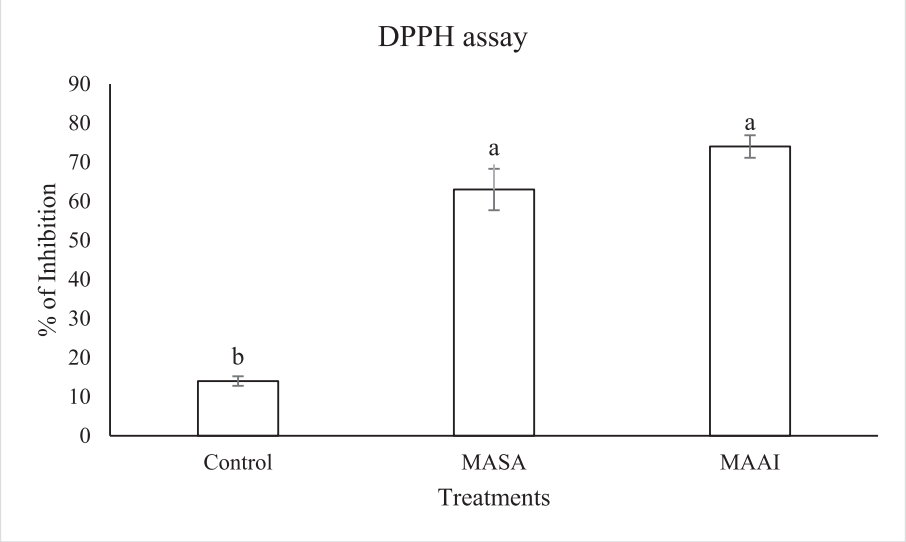

Fig. 6. percentage of DPPH inhibition in the treated adults of *bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

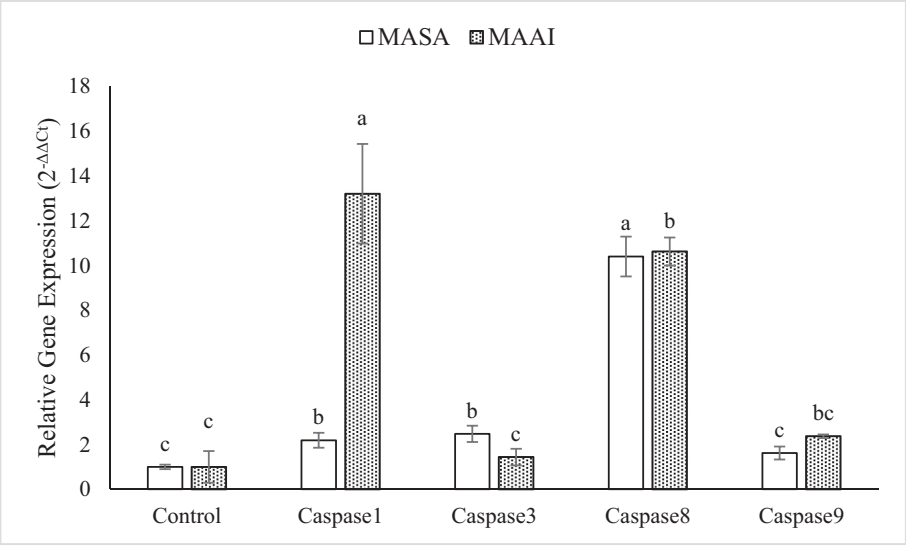

Fig. 7. Relative gene expression (2-ΔΔCt) of caspases in the control and the treated adults of *Bacterocera oleae* by the secondary metabolites of *Metarhizium anisopliae* isolates (MASA and MAAI). Statistical differences have been done between control and treatments and marked by different letters (Tukey test, $p \leq 0.05$).

been demonstrated in the *S. litura* larvae exposed to dextroxin (Sowjanya Sree et al., 2008).

Ascorbate peroxidase and glucose-6-phosphate dehydrogenase are the significant antioxidant enzymes which involved in elimination of toxicant compounds within insects (Asada, 1992). APOX removes H_2O_2 in cytoplasm, chloroplasts and mitochondria, while GPDH engaged in eliminating oxidative compounds in cytosol through change of NADPH to $NADP^+$ (Asada, 1992; Shamakhi et al., 2020). Similar to our results, the larvae of *S. litura* infected by dextroxin demonstrated also the higher APOX activity compared to control (Sowjanya Sree et al., 2008). Shahriari et al. (2023) reported significant higher activities of GPDH and APOX in *C. suppressalis* treated with 6 entomopathogenic fungi isolates. The higher activity of GPDH led to enhancement of NADPH synthesis to delete products of APX activity. Moreover, NADPH decreases the toxicant effects through shifting electrons to free radicals (Dubovskiy et al., 2008).

Malondialdehyde is an indicator of oxidative stress to reveal elevation in radical oxidative stresses. The enhanced MDA level showed occurrence of oxidative stress subsequent lipid peroxidation (Shahriari et al. 2023). On the other hand, free radicals cause break down of polyunsaturated acids, production of MDA and increment of toxicant compounds within cells (Lacan and Baccou, 1998; Wang et al., 2001a, 2001b). Several investigations indicated a direct correlation between microbial infection and lipid peroxidation in various insects (Dubovskiy et al., 2008; Sowjanya Sree et al., 2008; Karthi et al., 2018; Shamakhi et al., 2020; Shahriari et al. 2023).

5. Conclusions

The current study revealed the lethality of proteinaceous toxins of *M. anisopliae* on the key olive trees pest by intervening in digestive and antioxidative systems of the exposed adults. The extracts caused mortality on adults without any changes on toxic bait solution while it significantly decreases activities of digestive enzymes. Moreover, the extracts imposes oxidative stress on midgut of treated adults by elevating the activities of antioxidative enzymes and expression of

caspase genes. The use of toxic proteins produced by entomopathogenic fungi, due to their lethal effect in low concentration, limited durability in the environment, and their decomposition into safe compounds for the environment, can be a favorable alternative to synthetic pesticides. This is worthwhile to highlight that further studies and attention are needed on large-scale production, optimized formulation, storage condition, and introduction among gardeners.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2023.109681.

CRediT authorship contribution statement

Mehraneh Motamedi Juibari: Methodology, Formal analysis. **Arash Zibaee:** Conceptualization, Project administration. **Mohammad Reza Abbasi Mozhdehi:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Arash Zibaee reports was provided by University of Guilan. Arash Zibaee reports a relationship with University of Guilan that includes: employment.

Data availability

Data will be made available on request.

Acknowledgment

This work is based upon research funded the Iran National Science Foundation (INSF) under project No. 4004413.

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