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Production of protein hydrolysate from four plant sources and its optimization with α-pinene and tea seed saponin to control olive fruit fly

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ABSTRACT

Types, source of production and optimization with some attractive compounds are the main requirements to produce a commercially efficient protein hydrolysate. The current study was designed to produce protein hydrolysate for the first time from *Camelia sinensis* (Tea) seeds, *Cyamopsis tetragonoloba* (Guar) seeds, *Cucurbita pepo* (Summer squash) seeds, *Glycine max* (Soybean) seeds and *Olea europaea* Kernels (Olive) optimized with α-pinene and *Camelia sinensis* saponin (CSS) aiming to availability of the products from the sources with low economic value and more attractance. Results revealed the highest extracted protein from Guar while the highest inhibition of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and hydroxyl radicals were recorded in the protein hydrolysates extracted from Summer squash, Soybean and Olive. The highest reduction potential of Fe^{3+} ion and radical scavenging activity were found in the protein hydrolysate extracted from Olive and Soybean, respectively. Exposure of protein hydrolysates extracted from plant sources were determined in choice and no choice assays. In choice experiment, the highest amount of ingestion was recorded on Tea, Guar and Summer squash but the adults ingested the most protein hydrolysate extracted from tea optimized with α-pinene. In no choice experiment, the highest ingestion was recorded on Guar while optimization with α-pinene caused the highest ingestion on both Tea and Guar. Bioassay of *C. sinensis* saponin against the adults of *B. oleae* demonstrated the LC_{50} and LT_{50} values of 0.35% and 1.41 days, respectively. Also, CSS decreased activities of digestive enzymes in *B. oleae* except for TAG-lipase and trypsin. The adults of *B. oleae* fed on protein hydrolysate containing CSS showed elevated activity of antioxidant enzymes including catalase, peroxidase, superoxide dismutase and ascorbate peroxidase except for glucose-6-phosphate dehydrogenase. Results of the present study showed that protein hydrolysate can be extracted from plant sources that have little commercial value like tea seeds and olive kernels. In addition, their optimization with compounds that are attractive to the olive fruit fly can provide products with the higher efficiency than the products available in the market.

Abbreviations: CPS, *Cucurbita pepo* seeds; CSS, *Camelia sinensis* saponin; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; CAT, Catalase; POX, Peroxidase; SOD, Superoxide Dismutase; APOX, Ascorbate peroxidase; GPDH, Glucose-6-Phosphate dehydrogenase.

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1. Introduction

Bactrocera oleae (Rossi) (Diptera: Tephritidae) is one of the most devastative pests of olive in the world that was reported for the first time in 2004 from olive orchards in Iran and now it is considered as a key pest in Guilan, Zanjan and Qazvin provinces (Kihanian and Abbassi-Mozhdehi, 2019; Dadras et al., 2021). The olive fruit fly is a single-hosted and multi-generation insect, which causes damages to all olive varieties and spends winter as adult under plant remains, tree trunk and orchard soil (Amini et al., 2018). The feeding of hatched larvae on the flesh of olive fruits causes premature drop and spoilage due to excreta and saprophytic microorganisms (Moazipour et al., 2015). Different control methods have been suggested to control olive fruit fly mainly bait traps containing protein hydrolysate, pheromone traps and sticky yellow traps (Soroush et al., 2010). Chemical control has been directed by incorporating insecticides into protein hydrolysate within traps or a mixture of insecticide and protein hydrolysate is sprayed directly on the foliage of olive trees to impose adult flies (Moazipour et al., 2015).

Olive fruit fly needs sugar and protein sources for survival and sexual development, therefore, one can take advantages of such a feature and mix natural protein compounds with approved insecticides to be used as a toxic bait (Messing, 1999). Protein hydrolysates are an important group of bio-stimulants containing a mixture of poly- and oligopeptides as well as amino acids from protein sources hydrolyzed by chemicals or enzymes (Schaafsma, 2009). Enzymatically produced protein hydrolysates contain free amino acids and soluble peptides that act as molecules effective in signaling and regulating a wide range of physiological processes of *B. oleae* (Colantoni et al., 2017). Supplying some mineral absorbents to protein hydrolysate, yeast and even some plant essential oils can be suitable on the attraction and efficiency of protein hydrolysate solution. The presence of ammonium acetate crystals in the traps containing protein hydrolysate and borax, compared to those containing protein hydrolysate and borax alone, improved the attractance of *Anastrapha oliqua* (Macquart) (Diptera: Tepritidae) and *A. serpentina* (Wiedmann) (Diptera: Tepritidae) in field experiments (Lasa and Williams, 2021). The traps containing three types of synthetic attractants of ammonium acetate, trimethylamine and putrescine, have shown significant performance in attractance of *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) (Hasnain et al., 2022).

Saponins are the bioactive macromolecules in plants that act in the defense systems to deal with pathogens and herbivores (Yucekutlu and Bildaci, 2008; Augustin et al., 2011). Therefore, they have been reported in plant tissues that are sensitive to fungal, bacterial or insect herbivory (Wina et al., 2005). Saponins are the glycosides with high molecular weight, which consist of a sugar part attached to a triterpenoid or steroid aglycone, accordingly they can be divided into three groups: triterpenoid glycosides, steroidal glycosides, and alkaloid-steroidal glycosides (Koczurkiewicz et al., 2015). Saponins are found in different plants, including beans, blackberries, peas, potatoes, sugar beets, and tea (Dini et al., 2001). In order to have features such as reducing the occurrence of resistance and rapid decomposition in the environment, these compounds can partially be the alternatives of synthetic pesticides and play a crucial role in the preservation and sustainable development of agro-environments. Saponins have shown a promising insecticidal activity against a wide range of insect pests by imposing mortality, reducing food consumption, weight loss, delaying growth and reducing reproduction (Roopashree and Dhananjai, 2019; Qasim et al., 2020). Such properties demonstrate an urgent need to improve commercializing methods for saponin extraction and presenting saponin-based insecticide against pests (Qasim et al., 2020).

The production of protein hydrolysate from plant resources with low economic value may be an appropriate source to be used in monitoring or mass trapping of olive fruit fly. This is an important issue because it makes available some natural sources instead of artificial compounds to green management of orchards. On the other hand, mixing the optimized protein hydrolysate with some attractants like α-pinene and botanical compounds such as saponins or even plant essential oils instead of synthetic compounds can be applied in the form of local or widespread bait spraying to population suppression. Therefore, the current study was designed to produce a protein hydrolysate from natural compounds and to optimize with nature-based insecticide against *B. oleae* as an invasive and destructive olive pest in northern Iran. The objectives include (i) production of protein hydrolysate from *Camelia sinensis* (Tea) seeds, *Cyamopsis tetragonoloba* (Guar) seeds, *Cucurbita pepo* (Summer squash) seeds, Glycine max (Soybean) seeds and *Olea europaea* Kernels (Olive) by evaluating its attractiveness to *B. oleae* adults, (ii) determination the effective concentration of α-pinene to increase attraction and feeding performance of *B. oleae* adults and (iii) determination the lethal dose and potential physiological disturbance of tea seed saponin added to the optimized protein hydrolysate against *B. oleae* adults. The obtained results will show whether plant tissues, especially the parts that have little economic value, can be used as a source to produce protein hydrolysate. In addition, optimizing this protein hydrolysate with compounds that increase the attraction of olive fruit fly and adding a plant-based insecticide can be proposed as a commercial package with high efficiency to management of olive fruit fly.

2. Materials and methods

2.1. Preparation of protein hydrolysate

Tea seeds, soybean, guar and olive kernels were provided commercially[1] (Palizkasht company) and dried in the oven for 24 h at a temperature of 40 °C. The dried materials were ground by a grinder and passed through a sieve with a mesh of 30. The resulting powder was mixed with n-hexane (pure 95%) at a ratio of 1–5 (weight/volume) to be defatted by a magnetic stirrer at laboratory temperature for 1 h. The mixture was centrifugated for 15 min at 10,000 rpm and then it was passed through mesh 60 to remove residual solvents (AOAC, 1990). After that, 100 gr of defatted powder was poured into 1 L of distilled water and reached pH to 10 using NaOH (1 N). The sample was incubated for 3 h at room temperature before being centrifugated for 15 min at 4 °C and 12000 rpm. The su-

[1] Ten biological samples were used to extract protein hydrolysates.

pernatant was separated and acidified by HCL to reached pH 3 and kept at room temperature for 30 min. In the next step, centrifugation was performed under the above conditions, the precipitates were washed twice with 20 mL of sterile distilled water and freeze-dried using a freeze dryer (Matsuoka et al., 2012). For enzymatic hydrolysis, initially 350 mL of sterile distilled water was added to 70 mg of frozen sample and reached pH to 8.5 using NaOH. The mixture was kept at 80 °C for 5 min to remove unwanted enzyme activity. After cooling, 3 mg of pepsin was added to be incubated for 4 h at 50 °C. The pH of solution was reached to 8.5 and prolonged the incubation at 80 °C for 10 min to stop the enzymatic reaction. At this stage, pH of solution was decreased to 3.5 before centrifugation for 15 min at 4 °C and 10000 rpm. Finally, the sample was collected and the amount of protein was measured (Matsuoka et al., 2012).

2.2. Measuring antioxidant activity of the produced protein hydrolysates

2.2.1. Inhibition of free radical by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate)

First, 1 mL of the produced protein hydrolysate[2] from all plant sources was separately mixed with 500 μL of DPPH solution (0.02 percent) and 1 mL of ethanol 96%. In control samples, sterilized distilled water was used instead of the protein sample. Both samples were kept at darkness for 15 min before reading the absorbance at 512 nm. The percent inhibition of free radical can be calculated from the following formula (Wang et al., 2013):

$$100 \times \text{absorbance control}/(\text{absorbance sample-absorbance control}) = \text{percent of inhibition}$$

2.2.2. Reduction of Fe^{3+}

First, 100 μL of the produced protein hydrolysate from all plant sources was separately added into 250 μL of phosphate buffer 1X and 250 μL of potassium ferrocyanide 1%. Then, 250 μL of 10% trichlorhydric acid was mixed and centrifuged for 10 min at 5000 rpm at 4 °C. The liquid precipitation was mixed with 50 μL of 0.1% ferric chloride and 250 μL of distilled water. Finally, the absorbance was read at 700 nm after 10 min (Umayaparvathia et al., 2014).

2.2.3. Inhibition of hydroxyl radical (OH)

In this experiment, 100 μL of phenanthroline solution (1.8 M) solution and 200 μL of the produced protein hydrolysate from all plant sources were separately mixed with 100 μL of $FeSO4.7H2O$ (1 mM). Then, 100 μL of hydrogen peroxide (3%) was mixed and incubated for 60 min at 37 °C before reading the absorbance at 534 nm. The percentage inhibition of hydroxyl radical can be calculated from the following formula (Chang-Feng, et al., 2015):

$$\% \text{ of inhibition} = [(\text{As-An})/(\text{Ab-An})] \times 100$$

As = absorption of sample.
An = Absorption of negative control including all reaction compounds except for protein hydrolysate.
Ab = absorbance of blank including all reaction compounds except for protein hydrolysate and hydrogen peroxide.

2.2.4. Total antioxidant capacity

First, 100 μL of the produced protein hydrolysate from all plant sources was separately mixed with 1000 μL of reagent molybdate (sulfuric acid 0.6 mol, sodium phosphate 28 mmol and molybdate ammonium 4 mmol) and kept for 90 min at 95 °C. After cooling, the absorbance was read at 695 nm (Prieto et al., 1999).

2.3. Insect rearing

The olive fruits infected with *B. oleae* was collected from the orchards of Rudbar Olive Research Station (Zard variety), where no spraying has been done. The fruits were kept in ventilated containers at temperature of 25 ± 2 °C, a relative humidity of 70 ± 5% and a light and dark period of 8:16 h until the larvae became pupae (Akmoutsou et al., 2011; Sánchez-Ramos et al., 2013). The adults were kept in containers of 15 cm long × 12 cm wide × 5 cm high containing a hole in upper side for ventilation. A protein hydrolysate by DACUS BAIT 100® from EVYP company (Greece) was dissolved in distilled water at a ratio of 1:4 to feed adults flies (Akmoutsou et al., 2011). Olive fruits of Zard variety were provided for egg laying of females within the growth containers and kept at the same laboratory condition. Adults (24-h old) from the second generation (Laboratory cohort) was used in the experiments which a 1:1 sex ratio was considered for each replicate.

2.4. Evaluating feeding rate of adults on the produced protein hydrolysates of plant sources

Choice and non-choice experiments was done to study the attraction and ingestion of the protein hydrolysates toward *B. oleae* adults. In choice test, 60 one-day-old males and females (ration 1:1) were released in the five groups of 12 (five replicates) into containers of length 20 cm × width 12 cm × height 15 cm. Also, a container provided by the protein hydrolysate of DACUS BAIT 100® was used as positive control. The protein hydrolysates extracted from seeds of tea, guar, soybean and olive kernel as well as DACUS BAIT 100® were put in each container group (each group contains 60 adults in three replicates, N = 240 adults) in amount of 2 mL. On the other hand, each container includes 60 adults with the two 2 mL volume of desired plant extracted protein hydrolysate and positive control separately. In no choice test, all containers were only provided by plant extracted protein hydrolysate and DACUS BAIT 100® was provided in a separate container including 60 adults. Both experiments were performed at a temperature of

[2] Five different samples were collected to assay all antioxidant assays.

25 ± 2 °C, a relative humidity of 70 ± 5%, and a light and dark period of 8:16 h. The remaining solution was daily evaluated and the same adults were supplied by the new solution. To eliminate the error of evaporation, six containers were provided by each protein hydrolysate separately without insects hence the effect of evaporation is not included as an experimental error (Sánchez-Ramos et al., 2013). The amount of ingestion was calculated based on the following formula:

$$V = (X\text{-}E)\ (2000\ \mu l)/1.5\ cm$$

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.

2.5. The effect of α-pinene on increasing the attraction of olive fruit flies

In this experiment, 60 one-day-old males and females of *B. oleae* in a sex ratio of 1:1 were released in five groups of 12 (five replicates) into containers of 20 cm length × 12 cm width × 15 cm height. For each protein hydrolysate added by α-pinene, three containers were devoted including 20 adults that provided by 1900 μL of plant extracted protein hydrolysate and 100 μL of pure α-pinene. All experiments were performed at a temperature of 25 ± 2 °C, a relative humidity of 70 ± 5%, and a light and dark period of 8:16 h. The remaining solution was daily evaluated and the same adults were supplied by the new solution. To eliminate the error of evaporation, six containers were provided by each protein hydrolysate separately without so that the effect of evaporation is not included as an experimental error (Sánchez-Ramos et al., 2013). The amount of ingestion was calculated based on the following formula:

$$V = (X\text{-}E)\ (2000\ \mu l)/1.5\ cm$$

2.6. Tea saponin extraction

Tea seed saponin was extracted based on the method of Li et al. (2012) using colon-100 variety of *Camellia sinensis* L. Seeds were ground, sieved with 20 meshes apparatus and soaked into water at 80 °C in the ration of 6:1. The mixture was stirred at the given temperature for 6 h before being centrifugated at 5000 rpm at laboratory temperature for 30 min. The supernatant was incubated by polyaluminum chloride (KBR, 1.0547, India) as 1% of the initial extracted solution by weight for 2 h to discard impurities. Then, the mixture was re-centrifugated at 5000 rpm and 25 °C for 30 min. Calcium oxide (10 gr) was added into the supernatant as the precipitation agent and stirred for 4 h before centrifugation at 5000 rpm, for 30 min at room temperature. At this stage, the precipitation was kept and incubated by ammonium bicarbonate (Merck, 1066-33-7, Germany) (30% of the whole mixture) to remove calcium impurities for 2 h on stirrer at 60 °C. The sample was centrifugated at 5000 rpm and room temperature for 30 min then, the supernatant was incubated at 80 °C for 12 h within an oven to obtain CSS crystals. This method led to obtain CSS by 75% of purity (Mirhaghparast et al., 2020).

2.7. The lethal effect of tea seed saponins on B. oleae

Bioassay of CSS (*Camelia sinensis* Saponin) against the adults of *B. oleae* (one-day-old) was done using five concentrations of the saponin against 30 adults in five replicates of each in addition to a control. The required number of adults were randomly selected from the rearing cohort, transferred in the groups of six as replicates to the containers with the dimensions of length 15 cm × width 12 cm × height 5 cm. In each container, the protein hydrolysate was separately mixed with the concentrations of 0.5, 1, 2.5, 5 and 10 percent was exposure to the adults. The protein hydrolysate used in the current experiment had been extracted from olive kernels. In control subjects, distilled water was added to protein hydrolysate (Sánchez-Ramos et al., 2013). For each container that considered as a replicate, 5 drops of the protein hydrolysate mixture containing distilled water as control and CSS as treatment were separately placed on a plastic plate with a diameter of 2 cm in the form of a circle. These plates were not moved or replaced in any way to allow complete feeding (Sánchez-Ramos et al., 2013). The bioassay was done at 25 ± 2 °C, relative humidity of 70 ± 5% and the length of the light and dark period of 16:8 h. Adult mortality was recorded daily till 72 h. The assay was done with five replicates and 180 adults were used for assay.

2.8. Effect of exposure time on mortality of B. oleae treated by CSS

The LC_{90} concentration of CSS was selected and added to the protein hydrolysate of *B. oelae* adults. The diet was exposed to 60 adults within the containers with the dimensions of length 15 cm × width 12 cm × height 5 cm. Six containers were provided that included 10 adults. All sex ration was considered as 1:1. Mortality of the adults was recorded from 1 to 6 days that each 60 adults were devoted to each time interval. The experiment was done at 25 ± 2 °C, relative humidity of 70 ± 5% and the length of the light and dark period of 16:8 h. Adult mortality was recorded daily till 72 h.

2.9. Effect of LC_{30} concentration of CSS on digestive enzymes and antioxidant activity of B. oleae

Initially, the adults of *B. oleae* in sex ratio of 1:1 were treated by LC_{30} concentration of CSS in the two groups of 60 for treatment and control separately. After 24 h, they were chilled on ice and their whole body was homogenized in Tris-HCl buffer (20 mM, pH 7.2) in 1.5 ml microtubes using a glass homogenizer. The samples were centrifuged at 20,000 *g* for 20 min at 4 °C. The supernatant was used to assay digestive and antioxidative enzymes.[3]

[3] Five different samples were used to assay enzyme activity.

2.9.1. α-Amylase assay

A mixture containing 50 μL Tris-HCl buffer (20 mM, pH 7), 30 μL of starch (1%) and 20 μL of enzyme sample was prepared and incubated all components for 30 min. Then, 100 μL of dinitrosalisylic acid (DNS) was added and the tubes containing reaction mixture were put in boiling water for 10 min. Finally, the absorbance was recorded at 545 nm. A negative control includes pre-boiled enzyme (for 15 min) and other reaction components was used to validate enzyme activity (Bernfeld, 1955).

2.9.2. α- and β-glucosidases assay

Activity of these enzymes was determined using two substrates of *p*-nitrophenyl- α-D-glucopyranoside (pNαG) and *p*-nitrophenyl-β-D-glucopyranoside (pNβG), respectively. Briefly, 20 μL of enzyme sample was added to 20 μL of each substrate and 50 μL of Tris-HCl buffer (27 mM, pH 7) and incubated for 10 min. Then, the absorbance was read at 405 nm. A negative control includes pre-boiled enzyme (for 15 min) and other reaction components was used to validate enzyme activity (Ferreira and Terra, 1983).

2.9.3. Lipase assay

Lipase assay was done by incubating 20 μL of enzyme sample with 30 μL of *p*-nitrophenyl-butyrate (27 mM) as substrate and Tris-HCl buffer (27 mM, pH 7). The mixture was incubated for 10 min before reading the absorbance at 405 nm. A negative control includes pre-boiled enzyme (for 15 min) and other reaction components was used to validate enzyme activity (Tsujita et al., 1989).

2.9.4. Serine proteases assay

The activities of trypsin, chymotrypsin and elastase were assayed using BApNA (Na-benzoyl-L-arginine-p-nitroanilide, Sigma-Aldrich, 19,362), SAAPPpNA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide, Sigma-Adrich, S7388) and SAAApNA (N-succinyl-alanine-alanine-alanine-p-nitroanilide, Sigma-Aldrich, S4760) (1 mM), respectively. The reaction mixture including 50 μL of Tris-HCl buffer (27 mM, pH 8), 30 μL of each substrate separately and 20 μL of enzyme solution were mixed and incubated for 5 min before reading the absorbance at 405 nm. A negative control includes pre-boiled enzyme (for 15 min) and other reaction components was used to validate enzyme activity (Oppert et al., 2003).

2.9.5. Exopeptidases assay

The two substrates of hippuryl-L-arginine (Sigma-Aldrich, H2508) and hippuryl-*L*-phenylalanine (Sigma-Aldrich, H6875) were used to assay activity of amino- and carboxypeptidases. The reaction mixture including 50 μL of Tris-HCl buffer (27 mM, pH 8), 30 μL of each substrate separately and 20 μL of enzyme solution were mixed and incubated for 5 min before reading the absorbance at 340 nm. A negative control includes pre-boiled enzyme (for 15 min) and other reaction components was used to validate enzyme activity (Oppert et al., 2003).

2.9.6. Catalase (CAT) assay

The enzyme assay was done by mixing 50 μL of sample and 500 μL of hydrogen peroxide (1 %) at 28 °C from 0 to 10 min before reading the absorbance at 240 nm. The reduced activity of enzyme was reported as ΔA (Wang et al., 2001).

2.9.7. Superoxide dismutase (SOD) assay

The enzyme was assayed using 50 μL of sample and 500 μL of reaction including 70 μM of nitro blue tetrazolium (NBT) and 125 μM of xanthine that both have been mixed in phosphate buffer (1X, pH 7.1). The current mixture was incubated with 100 μL of xanthine oxidase solution containing 10 mg of bovine serum albumin. The absorbance was read at 560 nm after 20 min incubation at dark (McCord and Fridovich, 1969a, 1969b).

2.9.8. Peroxidase (POX) assay

The reaction contained 50 μL of sample, 250 μL of buffered pyrogallol [0.05 M pyrogallol in 27 mM Tris-HCl (27 mM, pH 7.0)] and 250 μL of hydrogen peroxide (1%). Absorbance of the mixture was read every 45 s for 3 min at 430 nm (Addy and Goodman, 1972).

2.9.9. Ascorbate peroxidase (APOX) assay

The assay was done by mixing 50 μL of sample, 150 μL of Tris-HCl (27 Mm, pH 7.0), 70 μL ascorbic acid (2.5 mM) and 200 μL of hydrogen peroxide (3%). The whole mixture was incubated for 5 min before reading the absorbance at 290 nm (Asada, 1984).

2.9.10. Glucose-6-phosphate dehydrogenase (GPDH) assay

The assay was done by incubating 100 μL of Tris-HCl (100 mM, pH 8.2), 50 μL of Nicotinamide adenine dinucleotide phosphate (NADP) (0.2 mM) and 30 μL of $MgCl_2$ (0.1 M). Then, 50 μL of the sample and 100 μL of Glycerol-3-phosphate dehydrogenase (GPDH) (6 mM) were added before reading the absorbance at 340 nm (Balinsky and Bernstein, 1963).

2.10. Protein assay

The assay was done based on the method described by Bradford (1976) using a commercial kit (recommended by Ziest Chem. Co., Tehran- Iran).

2.11. Statistical analyses

The data was compared by one-way analysis of variance (ANOVA), Tukey and *t*-test analyses and all differences were marked by different letters and asterisks at a probability $<5\%$.

3. Results

3.1. Production of protein hydrolysate from plant sources

A multistep process was adopted to extract protein hydrolysates from seeds of Tea, Guar, Summer squash, Soybean and Olive kernels. Results revealed the highest amount of protein hydrolysate in Guar while the least amount was found on Tea although no statistical differences was reported between Soybean and Summer squash (Fig. 1). The highest inhibition of DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) and hydroxyl radicals was recorded in the protein hydrolysates extracted from Summer squash, Soybean and Olive (Fig. 2). Accordingly, the least inhibition was recorded on Guar and Tea in case of DPPH and Tea in case of hydroxyl radical (Fig. 2). The highest reduction potential of Fe^{3+} ion caused by the protein hydrolysate extracted from Olive with the slight statistical differences with Soybean and Tea while the least inhibition was recorded by Guar and Summer squash (Fig. 3). The highest radical scavenging activity was found in the protein hydrolysate extracted from Soybean while Tea showed the least value among the treatments (Fig. 3).

3.2. Effects of the extracted protein hydrolysates on feeding tendency of B. oleae

Choice experiment representing feeding tendency of *B. oleae* demonstrated the highest ingestion of protein hydrolysates extracted from Tea, Guar, Summer squash and Olive compared to commercial protein hydrolysate (Fig. 4). α-Pinene icorporation revealed the highest ingestion of Tea protein hydrolysate compared to other treatments although the least ingestion was recorded in case of commercial protein hydrolysate (Fig. 4). In no choice experiment, the highest ingestion was recorded in the protein hydrolysate extracted from Guar with the slight statistical difference with tea while the least volume was observed on Summer squash (Fig. 4). α-Pinene amendment led to the highest ingestion of the protein hydrolysate extracted from Tea and Guar by *B. oleae* while the adults exposed to Summer squash ingested the least protein solution (Fig. 4).

3.3. Toxicity of saponin extracted from Camelia sinensis (CSS) against B. oleae

Saponin extracted from *C. sinensis* was added into the protein hydrolysate and orally exposured to the adults of *B. oleae* in different concentrations. After 24 h, mortality was recorded and the amounts of 0.061 [Confidence limit (95%) = 0.004–0.158], 0.157 [Confidence limit (95%) = 0.026–0.305] and 0.304% [Confidence limit (95%) = 0.090–0.490] were recorded as LC_{10}, LC_{30} and LC_{50} values (X^2 = 1.647, Df = 4, Slope ± SE = 1.836 ± 0.460). Moreover, the LT_{50} value representing the time requiring to kill 50% of ex-

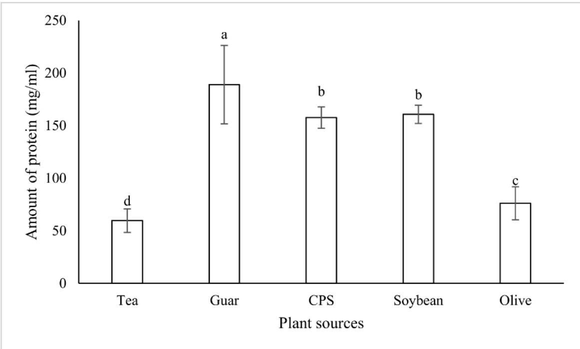

Fig. 1. Amount of protein hydrolysate extraction from the seeds of Tea, Guar, *Cucurbita pepo*, Soybean and Olive. Statistical differences have been marked with different letters (Tukey test, $p \leq 0.05$).

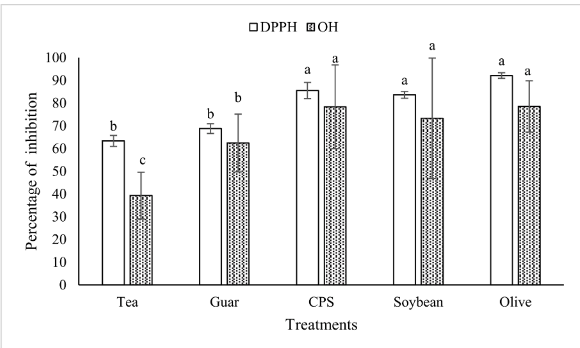

Fig. 2. Percent inhibition of DPPH and OH radicals in the protein hydrolysates extracted from seeds of Tea, Guar, *Cucurbita pepo*, Soybean and Olive. Statistical differences have been marked with different letters (Tukey test, $p \leq 0.05$).

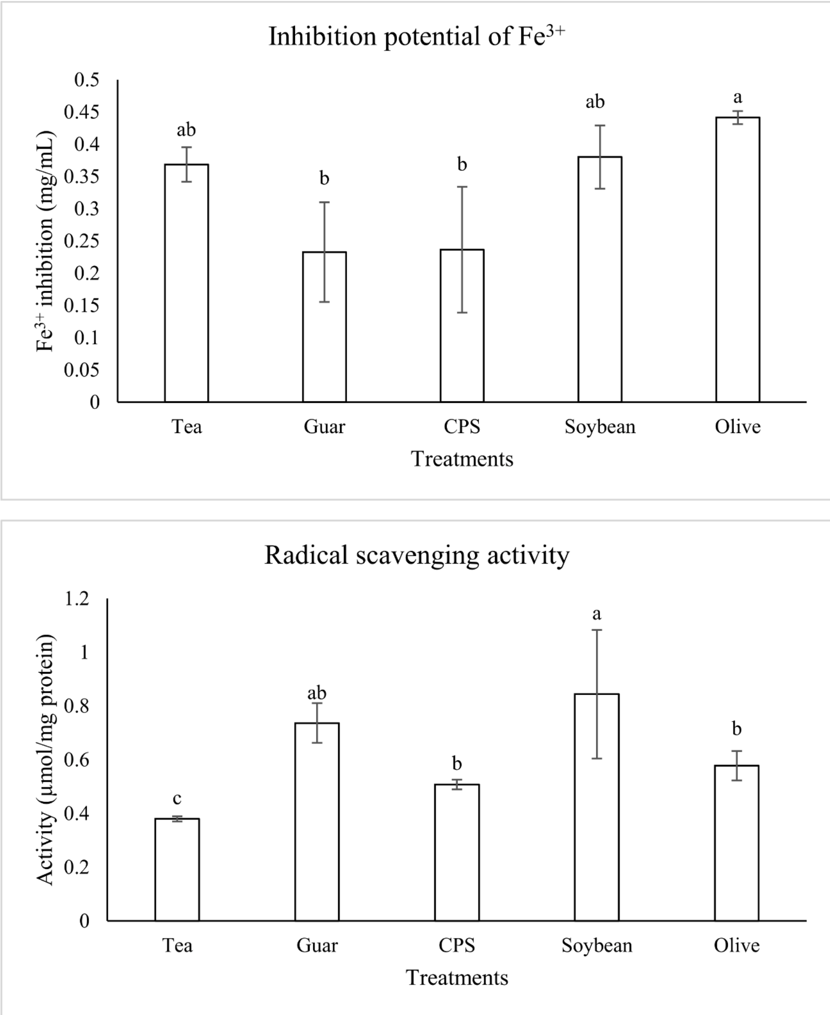

Fig. 3. Inhibition potential of Fe^{3+} and Radical scavenging activity in the protein hydrolysates extracted from seeds of Tea, Guar, *Cucurbita pepo*, Soybean and Olive. Statistical differences have been marked with different letters (Tukey test, $p \leq 0.05$).

posed population was recorded to be 1.41 [Confidence limit (95%) = 0.823–1.875] days (X^2 = 7.50, Df = 4, Slope ± SE = 1.273 ± 0.315).

3.4. Effect of LC_{30} concentration of CSS on digestive enzymes of *B. oleae*

CSS significantly affects several digestive enzymes of *B. oleae* (Table 1). The activities of α-amylase, chymotrypsin, elastase, cathepsin B, Amino- and carboxypeptidase significantly decreased in the CSS treated adults compared to control (Table 1). In contrast, glucosidases showed the higher activity in treatment rather than control (Table 1). TAG-lipase, trypsin and cathepsin L demonstrated no statistical difference between control and treated adults of *B. oleae* by CSS (Table 1).

3.5. Effect of LC_{30} concentration of CSS on antioxidant enzymes of *B. oleae*

Activity of antioxidant enzymes significantly increased in the adults of *B. oleae* treated by CSS including catalase, peroxidase, superoxide dismutase and ascorbate peroxidase but no statistical differences was recorded in case of glucose-6-phosphate dehydrogenase (Table 2).

4. Discussion

The use of protein hydrolysate is one of the main strategies to control fruit flies. The production and optimization of these compounds from suitable sources can lead to production of efficient baits for trapping these insects from one hand and minimize possible environmental pollution by unwanted materials from chemical processes or inflict on non-target organism. In the present study, protein hydrolysates were extracted from four plant sources: Tea, Guar, Summer squash, and Olive to be exposed on *B. oleae* adults. Additionally, some parameters related to the determination of hydrolysate protein quality were evaluated to ensure quality of the prod-

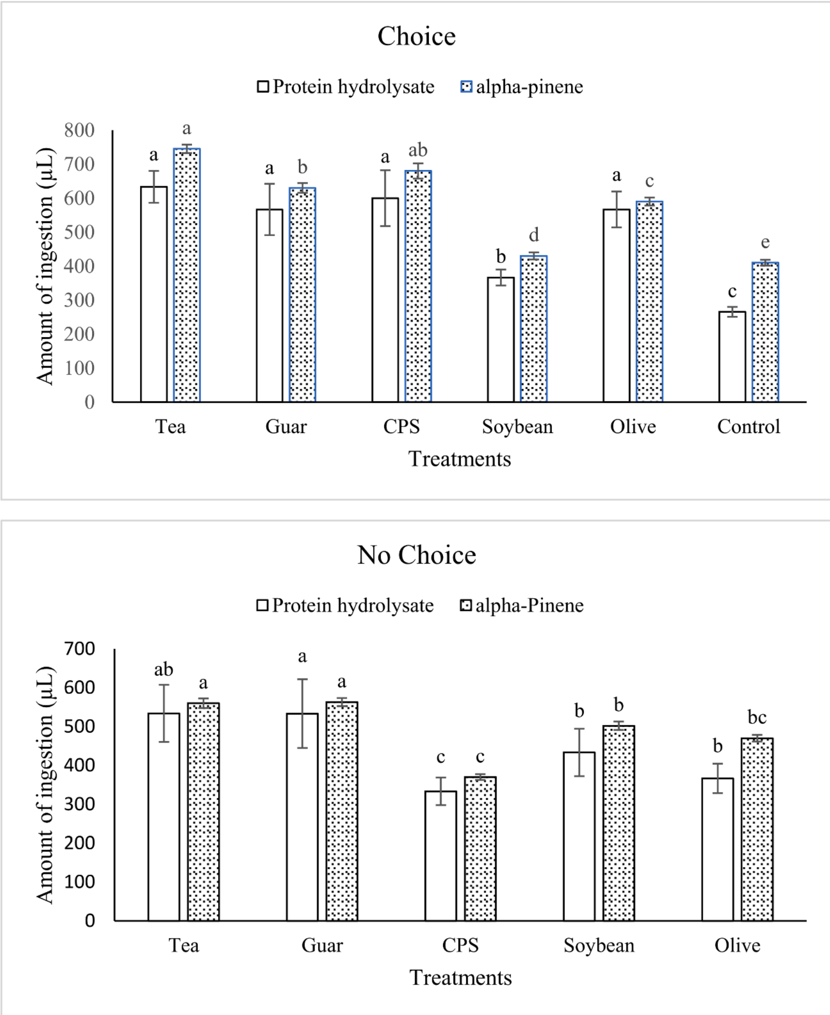

Fig. 4. Choice and no choice feeding performance of *Bacterocera oleae* adults fed on the protein hydrolysates extracted from the seeds of Tea, Guar, *Cucurbita pepo*, Soybean and Olive and those combined with α-pinene. Statistical differences have been marked with different letters (Tukey test, $p \leq 0.05$).

Table 1
Effect of LC_{50} concentration of *Camelia sinensis* saponin (CSS) on digestive enzymes of *Bacterocera oleae*.

Enzyme	Control (μmol/min/mg protein)	Treatment (μmol/min/mg protein)
α-Amylase	0.497 ± 102*	0.338 ± 0.044
α-Glucosidase	0.480 ± 0.034	0.818 ± 0.070*
β-Glucosidase	0.425 ± 0.053	0.972 ± 0.050*
TAG-Lipase	0.333 ± 0.035	0.345 ± 0.032
Trypsin	0.081 ± 0.003	0.082 ± 0.004
Chymotrypsin	0.08 ± 0.004*	0.07 ± 0.004
Elastase	0.051 ± 0.029*	0.042 ± 0.024
Cathepsin B	0.023 ± 0.003*	0.015 ± 0.009
Cathepsin L	0.039 ± 0.002	0.038 ± 0.004
Aminopeptidase	0.149 ± 0.086*	0.078 ± 0.045
Carboxypeptidase	0.081 ± 0.001*	0.062 ± 0.003

*Asterisks show statistical differences between control and treatment (*t*-test, $p \leq 0.05$).

ucts. Our results revealed that the protein hydrolysates extracted from Summer squash, Soybean and Olive had the higher potential of antioxidant properties compared to Guar and tea protein hydrolysates. Accordingly, the results have shown that pepsin enzyme leads to the digestion of peptide bonds by breaking the bond between hydrophobic amino acids such as leucine and aromatic amino acids such as phenylalanine, tryptophan and tyrosine with other amino acids and it is believed that the phenyl group at the end of the residue is able to inhibit free radicals through antioxidant properties (Sun et al., 2011). It is also believed that the destruction of the

Table 2
Effect of LC_{50} concentration of *Camelia sinensis* saponin (CSS) on antioxidant enzymes of *Bacterocera oleae*.

Enzyme	Control (ΔOD/min/mg protein)	Treatment (ΔOD/min/mg protein)
Catalase	0.128 ± 0.046	0.297 ± 0.007*
Peroxidase	0.079 ± 0.003	0.091 ± 0.003*
Superoxide dismutase	0.157 ± 0.016	0.338 ± 0.046*
Ascorbate peroxidase	0.114 ± 0.009	0.205 ± 0.012*
Glucose-6-phosphate dehydrogenase	0.032 ± 0.018	0.038 ± 0.019

*Asterisks show statistical differences between control and treatment (*t*-test, $p \leq 0.05$).

natural structure of proteins due to enzymatic hydrolysis may lead to the opening of the structure and exposure to amino acid active groups that can react with free radicals (Sun et al., 2011). It has been proven that there is a direct relationship between the ability of radical and antioxidant inhibition and the hydrogenation ability of amino acids (Sun et al., 2011).

Plants produce and emit several phytochemicals that contribute as olfactory chemical cues in insects managing many behavioral and physiological responses including signals for food sources, oviposition sites and oviposition behavior (Stensmyr et al., 2001). One of the studied phytochemicals is α-pinene that affect ecological behavior of *B. oleae*. This phytochemical is a monoterpene found in both leaves and fruits of olive also it is one of the essential compounds of sex pheromone in female *B. oleae* (Mazomenos and Haniotakis, 1981). Moreover, α-pinene is involved in attracting males and in stimulating oviposition by boosting strong electrophysiological responses in both males and females of *B. oleae* to increase mating performance (Gerofotis et al., 2013). So, it may be assumed that adding α-pinene may increase efficiency of protein hydrolysates to mass trapping of *B. oleae*. Moreover, Gerofotis et al. (2016) demonstrated that α-pinene increased male longevity and female fecundity by higher egg-laying towards younger ages. They concluded that *B. oleae* adults are required α-pinene to better ecological fitness and population colonization in olive orchards. In our study, α-pinene significantly increased the volume of ingestion that verify earlier findings on necessity of this phytochemical on mating and oviposition of *B. oleae* adults. So, α-pinene in bait spraying may be one of the elicitors to better performance of control measure.

Saponins are the bioactive macromolecules in plants and act as one of the chemical compounds in the defense system of plants to deal with pathogens and herbivores (Augustin et al., 2011). Therefore, they are found in plant tissues that are vulnerable to fungal, bacterial or insect predation (Wina et al., 2005). These compounds are glycosides with high molecular weight, which consist of a sugar part attached to a triterpenoid or steroid aglycone (genin). Depending on the type of genin, saponins can be divided into three groups: triterpenoid glycosides, steroidal glycosides, and alkaloid-steroidal glycosides (Koczurkiewicz et al., 2015). In order to have properties such as reducing the occurrence of resistance and rapid decomposition in the environment, these compounds can partially replace synthetic pesticides and play an important role in the preservation and sustainable development of the environment (Mirhaghparast et al., 2020). Saponins have shown proper insecticidal activity because they act quickly and they are effective against a wide range of pest insects (Roopashree and Dhananjai, 2019), which has the greatest effect on increasing mortality, reducing food consumption, weight, delaying growth and reproduction (Qasim et al., 2020). By disrupting the enzymatic activities of various pests, saponins play an important role in inhibiting the growth of pest insects, so commercializing pesticides based on saponins is of interest to control insect pests by nature-based materials (Qasim et al., 2020). In the current study, CSS imposed mortality against half of treated population by a concentration of 0.3% and it was able to cause such a mortality in 1.4 days. Also, it decreased activity of digestive enzymes except for TAG-lipase and tryspin. In contrast, it elevated the activity of glucosidases which it may be attributed to the role of these enzymes to naturally hydrolyse plant phytochemicals (Zibaee et al., 2009). Insecticidal activity of saponins against different pests including *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae), *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae), *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) has been reported (Nozzorillo et al., 1997; Adel et al., 2000; Szczepanik et al., 2004; Dolma et al., 2018). Treatment of *S. littoralis* larvae with 100 ppm alfalfa leaf saponin caused 90% mortality in the larval stages (Adel et al., 2000). Saponin extracted from *Ilex opacea* (Aquifoliales: Aquifoliaceae) showed the anti-nutritional activity and prevented food absorption in *Lymantria dispar* (Linnaeus) (Lepidoptera: Erebidae) (Barbosa et al., 1990). Spraying of Alfalfa saponin on tomato leaves reduced the number of *Tetranychus urticae* Koch mites (Acari: Tetranychidae) and showed egg mortality on the treated leaves (Chaieb, 2010). Application of tea seed saponin against *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) decreased growth rate, feeding efficiency, weight loss of pupae, adult emergence and fecundity (Cai et al., 2016; Mirhaghparast et al., 2020) demonstrated that tea seed saponin cause larval mortality against *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). Also, interruption in digestive enzyme activities reduced the absorption of nutrients and the growth of larvae, thus affecting the reproduction and population of *H. armigera* in the next generations.

The adults of *B. oleae* fed on the protein hydrolysate containing CSS showed the elevated activity of antioxidant enzymes including CAT, POX, SOD and APOX except for GPDH. It is believed that increased activity of these enzymes showed induction of reactive oxygenate radicals in the treated *B. oleae* by CSS. The three enzymes of SOD, CAT and POX works together to convert superoxide radical O_2^- into H_2O_2 and subsequently H_2O_2 to H_2O (Lyakhovich et al., 2006). Elevation the activity of these enzymes in the treated *B. oleae* indicates oxidative stress in the midgut gut through increasing the concentrations of superoxide radicals and hydrogen peroxide and subsequently hydrogen peroxides. APOX is another antioxidant enzyme that reduces H_2O_2 and simultaneously oxidized ascorbate (Asada, 1992). Also, GPDH has a crucial role in glycerol-3-phosphate shuttle by transferring the reduced equivalents from cytoplasmic pool of NADPH to the mitochondrial membrane and by re-oxidizing them through transferring electrons across the mitochondrial membrane (Nation, 2008). There is no report on effects of saponins on antioxidant enzymes of insects but elevation the activity of these enzymes severally reported on other phytochemicals. It has been shown the involvement of APOX in oxidative stress of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) treated by orthoquinones (Felton and Duffey, 1992), but it had no significant increase in *Helicoverpa armigera* (Lepidoptera: Noctuidae) treated by *Polygonum persicaria* agglutinin (Rahimi et al., 2018). Hypericin elevated the activities of CAT and SOD in the larvae of *Asota plagiata* Walker (Lepidoptera: Noctuidae) (Aucoin et al., 1991). The grain aphid, *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae) showed the increased activity of CAT after feeding on DIMBOA-containing artificial diets (Figueroa et al., 1999).

5. Conclusions

Regarding the importance of *B. oleae* control using bait traps containing protein hydrolysate and natural compounds instead of synthetic insecticides, the current study was done to produce protein hydrolysate from plant sources. Also, mixing the optimized protein hydrolysate with the botanical insecticides like saponins and essential oils may be of interest to local or wide bait spraying to suppress population outbreaks of fruit flies mainly *B. oleae*. Our results revealed production of protein hydrolysate from Tea, Guar, Summer squash, Soybean and Olive optimized by α-pinene which increased attractance for *B. oleae*. Moreover, saponin extracted from *C. sinensis* showed significant mortality against adults. So, it may be concluded that such protein hydrolysate formulation might be a good candidate to be used in olive orchards but it should be insisted on determination its efficiency in environmental experiments. The results of the present study showed that protein hydrolysate can be extracted from plant sources, especially parts that have little commercial value like tea seeds and olive kernels. In addition, their optimization with compounds that are attractive to the olive fruit fly can produce products with the higher efficiency than the products available in the market to control this important pest.

CRediT authorship contribution statement

Amirmohammad Zadjafar: Resources, Methodology, Investigation. **Arash Zibaee:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Conceptualization. **Mohammad Reza Abbasi Mozhdehi:** Writing – review & editing, Visualization, Validation. **Alireza Mehregan Nikoo:** Writing – review & editing, Validation, Data curation.

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.

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