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ORIGINAL ARTICLE

Plant essential oils induce expression of heat shock proteins and antioxidant enzyme activity in carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae)

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Abstract. Rising concerns about the negative effects of chemical compounds in pest control have made it increasingly necessary to find new environmentally friendly compounds to combat insect pests. In recent years, the use of plant derivatives, such as, essential oils have proved very promising. Despite the many studies on essential oils, there are very few studies on the effect of these oils on induction of Heat shock proteins (HSPs) and antioxidant enzymes. Therefore, it was hypothesized that the exposure of insects to essential oils induce stress, which it is likely induces the production of heat shock proteins and antioxidant enzymes. Thus, *Ectomyelois ceratoniae* Clark (Lepidoptera: Pyralidae) was used as a model insect to study the lethal and sublethal effect of the fumigant toxicity of the essential oils extracted from the plants *Thymus daenensis* Celak and *Satureja khuzistanica* Jamzad. Gas Chromatography/Mass Spectrometry indicated that thymol and carvacrol were the major component of *T. daenensis* and *S. khuzistanica* extracts, respectively. Exposing *E. ceratoniae* to sublethal doses of the essential oils (LC_{30}) and using RT-qPCR indicated that the expression levels of HSP70 and HSP90 were significantly increased. Activities of antioxidant enzymes including SOD, CA, POX, GST and the concentration of MDA increased following exposure to sublethal doses of both essential oils. These findings clearly show that in addition to the reported effects of essential oils, oxidative stress and induction of free radical production could be another mode of the action of essential oils on insects.

INTRODUCTION

Any internal and external factor that mediate a biological response in living organisms is called stress and the factors leading to this response are known as stressors. All living organisms are exposed to a wide array of stressors during their lives, including biotic (pathogens and predators) and abiotic factors (heat, cold, ultrasonic waves, chemicals, and etc.) (Schulte, 2014; Farahani et al., 2020a). Exposing living organisms, including insects, to stress induces the production of reactive oxygen species (ROS), which are able to damage vital molecules in living organisms, including lipids, proteins and nucleic acids (Felton & Summers, 1995; Zhao & Jones, 2012). Because ROS are highly damaging to most biological macromolecules, organisms have robust antioxidant systems to neutralize ROS before they can inflict such damage. Insects have enzymes and non-enzymatic compounds that can detoxify pro-oxidant endobiotics and xenobiotics. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APOX), glutathione peroxidase (GPX) and glucose 6-phosphate dehydrogenase (GPDH) are among the most important antioxidant enzymes (Lukasik et al., 2011; George & Gatehouse, 2013; Jena et al., 2013). Some compounds with low molecular weight such as ascorbic acid (ASA), reduce glutathione (GSH), thiols and α-tocopherol and constitute non-enzymatic components of the antioxidant system (Krishnan et al., 2009). While the antioxidant system can readily handle ROS produced from normal oxidative metabolic activities, insects have the capacity to enhance the antioxidant activity when exposed to oxidizing compounds, including those in their diets. Rahimi et al. (2018) report that feeding *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) larvae on an artificial diet containing agglutinin from *Polygonum persicaria* L. led to an increase in the level of antioxidant enzymes, including CAT and SOD.

Heat shock proteins (HSP) are another large group of multifunctional proteins that help organisms cope with intrinsic and extrinsic stresses (Zhao & Jones, 2012). Essential oils induce HSP synthesis in insects, indicating that these oils cause cellular stress. For example, Chohan et al. (2018) report that topical treatment of adult *Myzus persi-*

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cae (Sulzer) (Hemiptera: Aphididae) with 40% *Melaleuca alternifolia* (Maiden & Betche) oil induced the expression level of HSP60 approximately 10-fold in comparison to the untreated group. The carob moth, *E. ceratoniae*, is a highly destructive polyphagous insect pest of fruit and nut crops, such as, pomegranates, almonds, figs and dates (Gothilf, 1984; Warner et al., 1990; Abid et al., 2021). *E. ceratoniae* can severely damage stored nuts, flour and processed products post-harvest, causing extensive losses in tropical and subtropical regions (Grieshop et al., 2006; Nay, 2006; Izadi et al., 2019).

Damage to stored products can occur directly, through larval feeding and indirectly by contamination with the faeces and exuviae of the insects (Nay, 2006). The main method used to control stored product pests is the use of gaseous synthetic chemical pesticides (Mohapatra et al., 2015; Stejskal, 2015; Guru et al., 2022). While these pesticides are effective in controlling stored product pests, growing concerns about their negative effects have prompted researchers to find environmentally friendly and economically feasible alternative methods (Stejskal, 2015; Izadi et al., 2019).

Plants provide a large and diverse reservoir of natural compounds with pesticidal activity known as botanical pesticides, which have received increasing attention in pest management programs because they are less harmful to non-target organisms, safer for humans and quickly biodegraded (Simmonds et al., 2002; Koul et al., 2008; Rana et al., 2016). Currently, several plant-derived pesticides are commercially available for controlling arthropod pests in agriculture and households (Guleria & Tiku, 2009; Jindal et al., 2013). Moura et al. (2019) report that the essential oil of *Vanillosmopsis arborea* Barker (Asteraceae) and its major constituent, α-bisabolol, are insecticidal against *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae) having an LC_{50} of 5.23 and 12.97 $\mu L\,L^{-1}$ of air, respectively. The stronger insecticidal activity of the essential oil, relative to the main constituent, is indicative of other components within the essential oil extract having important insecticidal or synergistic qualities.

Despite extensive studies on the lethal, sublethal, repellent, anti-fertility and anti-nutritional effects of essential oils (Ndomo et al., 2010; Ziaee et al., 2014; Liao et al., 2017; Farahani et al., 2020b; Bulgarini et al., 2021), there are few on how these compounds affect insect heat shock proteins and antioxidant enzymes. Most studies of those done so far indicate that the insecticidal activity of the plant compounds can be due to the disruption of nervous conduction in insects, typically through the inhibition of acetylcholinesterase or antagonism of octopamine receptors (Ryan & Byrne, 1988; Enan, 2001; Jankowska et al., 2017).

This study aimed to assess whether essential oils induce stress in insects and their reactions to stress. For this purpose, carob moth, *E. ceratoniae* (Lepidoptera: Pyralidae), was used as a model insect.

MATERIAL AND METHODS

Insect rearing and maintenance

Pomegranate fruits infested with carob moth larvae were collected from orchards in Tehran, Iran, and transferred to a growth chamber (26 ± 1°C, 70% humidity, and 16L : 8D). When the adult moths emerged, they were collected using an aspirator and transferred to egg-laying containers, which were cylindrical and made of plastic (10 cm diameter × 30 cm height) with internal walls covered with a fine cloth made of polypropylene (32 × 28 cm) for laying eggs. After three days, the cloths along with the moth eggs were collected and transferred to artificial diets. The larval artificial diet was prepared based on Mediouni & Dhouibi (2007) with a slight modification. Briefly, the wheat bran was dry heat sterilized for 2 h at 121°C and the water was autoclaved for 20 min at 121°C. Corn oil was used instead of glycine and 23 g of wheat germ powder was added per 1000 g artificial diet.

Extraction of essential oil

Essential oil extraction was carried out using the method of Papachristos & Stamopoulos (2004). Briefly, the aerial parts of thyme (*Thymus daenensis*) and savoury (*Satureja khuzistanica*) were collected and air-dried for one week in the dark. Then, the essential oil in air-dried material was extracted using hydro distillation in a Clevenger apparatus. The distilled essential oils were stored in a refrigerator at 4°C until required.

Gas chromatography – mass spectrometry profile

A gas chromatograph – mass spectrometer (Model Polaris Q, Thermoelectron Corporation, Germany) equipped with a DB-5 column (30 m × 0.25 mm × 0.25 mm) was used in this analysis. The oven temperature was programmed as isothermal at 40°C for 1 min, then raised to 250°C at 6°C/min and held at this temperature for 4 min. Helium was used as carrier gas at the rate of 1.0 ml/min. The effluent from the GC column, was introduced directly into the MS via a transfer line. Ionization voltage was 70eV and the ion source temperature was 2300°C. The scan range was 41–450 AMUs. The constituents were identified by comparing their retention indices with literature values and their mass spectral data with those in the Wiley mass spectral library (Zribi et al., 2019).

Fumigant toxicity assays

The toxicity of the essential oil of two species of plants was evaluated using adult carob moths and the fumigation method of Ayvaz et al. (2010). After a preliminary dose setting experiment, filter papers (1 × 1 cm) were treated with different volumes of each essential oil using micropipettes and pasted immediately on the internal surfaces of the lids of the clear plastic cylinder bottles (3 cm diameter × 8 cm height). An untreated paper disc was used as a control. Twenty adult carob moths (< 24-h old) were each transferred into a bottle. The bottles were then sealed with Parafilm to prevent the essential oil from escaping. Treated individuals were maintained in a growth chamber (25 ± 1°C, 60 ± 10% RH, and 16L: 8D h photoperiod) and after 24-h, mortality was recorded. A moth was recorded as dead if it did not move its appendages following stimulation with a fine brush. Mortality data were used for estimating lethal and sublethal concentrations (LCs and subLCs). All bioassays were replicated at least three times and each treatment included 120 adults.

To assess antioxidant enzyme activity and HSP expression levels, the adult carob moths were exposed to sub-lethal concentrations (LC_{30}) of the essential oils for 24 h, as described for the fumigant toxicity assays. The surviving adults were then used in subsequent experiments (i.e. biochemical and molecular assay, as described below).

RNA extraction and cDNA synthesis

Total RNA from 10 larvae was extracted using Trizol reagent (Invitrogen), according to the manufacturer's instructions. The extracted RNAs were treated with RNase-free DNase (TaKaRa, Shinga, Japan). The absence of DNA contamination was confirmed using PCR with the RNA template a 18s ribosomal RNA (Forward: 5'-CCTTTAACGAGGATCTATTGG-3' and Reverse 5'-ATACTTGGCAAATGCTTTCG-3').

cDNA was synthesized by reverse transcription using a cDNA synthesis kit (Takararr037a) according to the manufacturer's instructions. PCR primers were designed using the nucleotide sequences of the HSP70 and HSP90 obtained from the GeneBank database of the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

Analysis of sequences

The sequences of the HSP70 and HSP90 cDNA were confirmed by a homologous search of other HSP70 and HSP90 sequences known within the BLAST program available in the GenBank database of the (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

Quantitative PCR (RT-qPCR)

RT-qPCR was used to determine the expression of HSP70 and HSP90 in treated and non-treated carob moths using RealQ Plus 2× Master Mix Green (ampliqon) on a Rotor-Gene® Q system (QIA-Gene). Gene-specific primers (Table 1) were designed using Primer-Blast. A ribosomal protein gene, RL32, was used as an internal control (Farahani et al., 2020a). The PCR amplifications were carried out using the following cycling conditions: one cycle at 95°C (15 min), followed by 40 cycles of denaturation at 95°C (15 s), annealing at 55°C for 20 s and extension at 72°C for 20 s. Quantitative analysis used a comparative CT (ΔΔCT) method (Livak & Schmittgen, 2001). For each treatment, three replicates of 10 individuals were analysed.

Preparation of samples of antioxidant for assay

The adults from control and treatments were collected and transferred to ice-cold saline solution (NaCl, 10 mM) and homogenized in a glass pestle. The homogenates were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatants were stored at –20°C for subsequent analyses.

Superoxide dismutase (SOD) assay

The procedure described by Beauchamp & Fridovich (1971) was used to assay SOD activity. Briefly, 0.1 ml enzymatic extract was added to 1.5 ml 0.05 M PBS (Phosphate buffer saline, pH 7.8), 0.3 ml 0.1 mM EDTA (Ethylenediaminetetraacetic acid), 0.3 ml 0.13 M methionine, 0.3 ml 0.75 mM NBT (Nitrotetrazolium Blue chloride) and 0.3 ml 0.02 mM riboflavin. Incubation was initiated using fluorescent light (4,000 lux) for 10 min and then the variation in absorbance was recorded at 560 nm.

Catalase assay

This assay was done using the method of Wang et al. (2001) in which 50 µL of sample and 500 µL of hydrogen peroxide (1%) were incubated at 28°C for 10 min before determining optic density as ΔA at 240 nm.

Ascorbate peroxidase assay

The method of Asada (1984) was used to determine ascorbate peroxidase (APOX) activity. 50 µl of sample, 150 µl potassium phosphate buffer (67 mM, pH 7.0), 70 µl ascorbic acid (2.5 mM) and 200 µl H_2O_2 (30 mM) were separately pipetted into each microplate well. The components were mixed and the optic density recorded at 290 nm for 5 min.

Glutathione S-Transferase assay

The total reaction volume per well of a 96-well microplate was 165 µL, consisting of 15 µL of enzyme solution, 50 µL CDNB (containing 0.1% (v/v) ethanol)) and 100 µL GSH. Enzyme activity was determined by the change in absorbance measured every 30 s for 5 min at 340 nm using a microplate reader (ELX808 Bio-Tek) (Habig et al., 1974).

PPO Activity

The method of Kumar & Khan (1982) was used to estimate PPO (Polyphenol oxidase) activity in a reaction mixture containing 2 mL of 0.1 M potassium phosphate buffer (pH 6.0), 1 mL of 0.1 M catechol and 0.5 mL of enzyme extract. The purpurogallin formed was read at 495 nm.

Malondialdehyde concentration assay

A method described by Bar-Or et al. (2001) was used to determine the concentration of malondialdehyde (MDA) in control and treated individuals, in which 100 µL of 20% trichloroacetic acid and 50 µL of the sample was mixed and centrifuged at 15,000 g for 10 min at 4°C. Then, the supernatant was mixed with 100 µL of 0.8% TBA reagent and re-incubated at 100°C for 60 min before reading absorbance at 535 nm. The MDA concentration is reported as the amount of MDA produced per mg protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Protein content

Protein content was determined using the Bradford method (1976) and bovine serum albumin as the standard protein.

Statistical analysis

The percentage mortality of essential oil-treated insects was corrected using Abbott's formula as follows: Corrected mortality = $(T - C/100 - C) \times 100$ where T and C are the number of dead insects in treatment and control, respectively (Abbott, 1925). The corrected mortalities were used to estimate lethal concentrations (LC_{30}, LC_{50}) for each essential oil using the Probit analysis in POLO-Plus 1.0 software (LeOra, 1994). Means were compared using the least squared difference (LSD) tests of one-way ANOVAs using SPSS 22 software. Graphs were drawn using MS Excel 2013.

RESULTS

Compound identification

Two essential oils were analysed using GC-MS. The components identified are listed in Table 2 and Table 3. The major components of that of *T. daenensis* were thymol (72.34%), carvacrol (7.07%), ρ-cymene (6.28%), ϒ-terpinene (4.84%) and β-caryophyllene (2.46%), where-

Table 1. Primers used in the RT-qPCR.

Primers	Primer forward	Primer reverse	PCR type
re- HSP70	5'-AACCACTTCGTTCAGGAGT-3'	5'-CTCCTCTGTCGCTCGGTAT-3'	RT-qPCR
re- HSP90	5'-CAGTTCGGTGTGGGTTTCTA-3'	5'-GAGGATGACAAGCCCAAGAT-3'	RT-qPCR
RL32	5'-TGGCACCACACCTTCTAC-3'	5'-CATGATCTGGGTCATCTTCT-3'	RT-qPCR

Table 2. Chemical constituents of the essential oil of *Thymus daenensis*.

No.	Component identified	RI[1]	Concentration (%)	No.	Component identified	RI[1]	Concentration (%)
1	α-thujene	931	2.31	13	1.8-cineol	1026	0.89
2	α-pinene	940	0.74	14	limonene	1033	0.13
3	Camphene	957	0.16	15	E-β-ocymene	1046	0.02
4	1-octene-3-ol	976	0.01	16	γ-terpinene	1062	4.84
5	3-octanol	979	0.02	17	t-sabinene hydrate	1071	0.51
6	β-pinene	979	0.02	18	α-terpinolene	1092	0.03
7	6-methyl-3-heptanon	984	0.03	19	4-terpineol	1184	0.16
8	β-pinene	985	0.09	20	Thymol	1297	72.34
9	β-myrcene	982	0.43	21	Carvacrol	1304	7.07
10	α-phellandrene	1010	0.13	22	β-caryophyllene	1437	2.46
11	α-terpinene	1013	0.73	23	Caryophyllene-oxcide	1601	0.08
12	p-cymene	1017	6.28	24	Spathulenol	1676	0.1
					Total	—	99.58

[1] Retention Index.

Table 3. Chemical constituents in the essential oil of *Satureja khuzistanica.*

No.	Component identified	RI[1]	Concentration (%)	No.	Component identified	RI[1]	Concentration (%)
1	α-thujene	927	1.53	13	trans-2-caren-4-ol	1145	0.10
2	α-pinene	936	1.04	14	Terpin-4-ol	1163	0.14
3	Camphene	950	0.18	15	α-terpineol	1175	0.26
4	β-pinene	974	0.30	16	Thymol	1266	0.44
5	Myrcene	981	0.96	17	Carvacrol	1282	83.77
6	α-phelandrene	999	0.24	18	carvacryl acetate	1345	2.10
7	p-cymene	1014	2.30	19	β-caryophyllene	1424	0.38
8	1.8-cineole	1023	0.70	20	β-bisabolene	1501	0.70
9	γ-terpinene	1053	1.52	21	Spathulenol	1576	0.10
10	cis-sabinene hydrate	1056	1.32	22	Caryophyllene oxide	1960	0.30
11	Linalool	1085	0.81	23	β-eudesmol	1971	0.37
12	t-sabinene hydrate	1055	0.20	24	trans-2-caren-4-ol	1145	0.10
					Total	—	99.76

[1] Retention Index.

as the percentage concentration of the main components of the *S. khuzistanica* oil were carvacrol (83.77%), ρ-cynene (2.3%), carvacryl acetate (2.1%), α-thujene (1.53%) and α-pinene (1.04%).

Fumigant toxicity

The median lethal concentrations (LC_{50}) of the essential oils of *T. daenensis* and *S. khuzistanica* for adult carob moths was 189.64, and 494.01 µL. L^{-1} air, respectively (Table 4), which indicates essential oil extracted from *T. daenensis* is almost twice as toxic as that of *S. khuzistanica*. However, the slope of the relationship between mortality and concentration of essential oil for *S. khuzistanica* was steeper than that for *T. daenensis* (Table 4). Based on lower and upper confidence intervals, the differences in LC_{50} values of the two extracts were significantly different from one another. The LC_{30} of the essential oils of *T. daenensis* and *S. khuzistanica* for adult carob moths were calculated at 137.09 and 422.96 µL/L air, respectively (Table 4).

Table 4. Fumigant toxicity of the two essential oils recorded for carob moths 24 h after exposure.

Essential oil of	LC30 (µL/L air) (Lower–Upper)	LC50 (µL/L air) (Lower–Upper)	χ^2	Slope
T. daenensis	137.09 (119.64–152.36)	189.64 (171.82–210.12)	6.41	3.71
S. khuzistanica	422.96 (385.1–451.62)	494.01 (464.55–520.4)	4.28	7.78

Quantitative PCR (qRT-PCR)

The expression levels of the two HSP genes were assessed using qRT-PCR. The expression of the two genes increased significantly after exposure to LC_{30} doses of the essential oils ($P < 0.05$), with the *T. daenensis* extract inducing the greatest increase in expression of HSP70 and HSP90. (5.1-fold, 4.7-fold compared to controls, respectively) (Fig. 1).

Effect of treatments on the antioxidant system of carob moth

The activity of SOD increased in moths exposed to LC_{30} doses of each essential oil, with those treated with *T. daenensis* extract showing the greatest increase (1.8-fold) in SOD activity relative to the controls (Fig. 2). Treating adult moths with LC_{30} doses of the essential oils also increased the CAT activity, with extracts of *T. daenensis* inducing the greatest increase (1.6-fold) relative to the control (Fig. 2). Determination of APOX activity showed that activity of this enzyme only increased (1.6-fold) in those adult carob moths treated with a LC_{30} dose of the *T. daenensis* extract, whereas in moths treated with the essential oil of *S. khuzistanica* it did not increase significantly relative to the control (Fig. 2).

Exposure to LC_{30} doses of essential oils increased the GST activity of those insects treated with the essential oils of both *T. daenensis* and *S. khuzistanica* (Fig. 3).

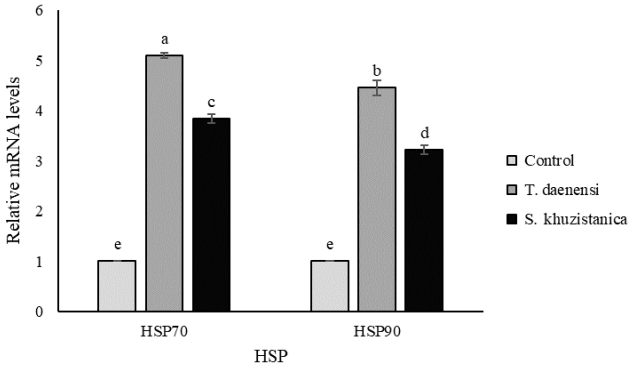

Fig. 1. Level of HSP70 and HSP90 transcripts recorded in adult carob moths following exposure to LC_{30} concentration of essential oils extracted from two plants, relative to their respective levels in untreated moths. The values are means and standard errors for three replicates of 10 individuals. Different letters denote significantly different values (LSD).

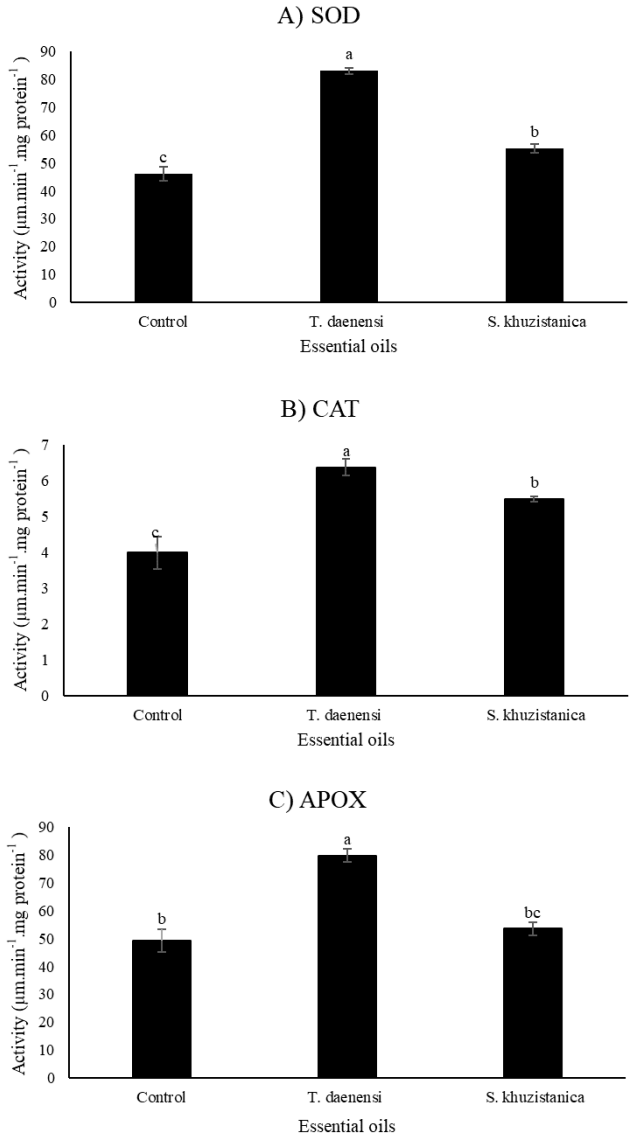

Fig. 2. The effect of the essential oils extracted from the two plants on antioxidant enzymes in the treated moths. A) Superoxide dismutase (SOD) activity (µm. min^{-1}.mg $protein^{-1}$), B) Catalase (CAT) activity (mm.min^{-1}.mg $protein^{-1}$) and C) Ascorbate peroxidase (APOX) activity (µm. min–1.mg $protein^{-1}$). The values are the means and standard errors for three replicates of 10 individuals. Different letters denote significantly different values (LSD).

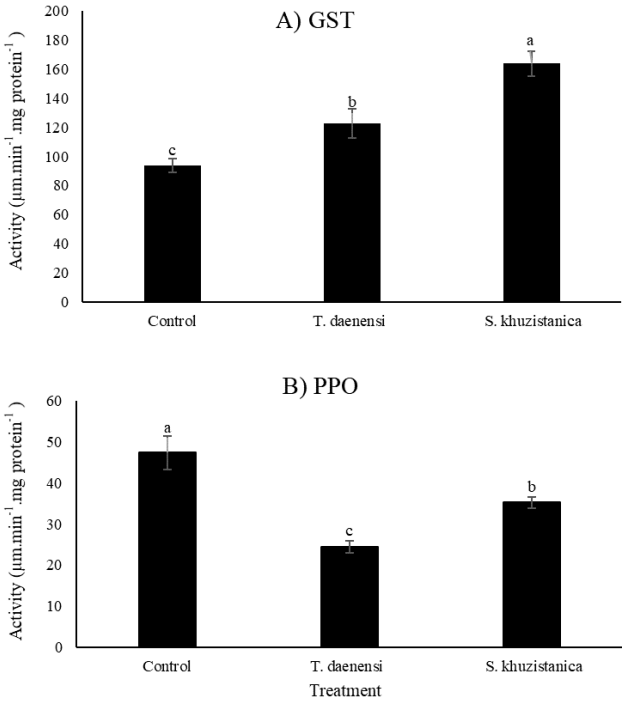

Fig. 3. The effect of the essential oils extracted from the two plants on A) Glutathione S-Transferase (GST) and B) polyphenol oxidase (PPO) activity (µm. min–1.mg $protein^{-1}$) in adult carob moths. The values are the means and standard errors for three replicates of 10 individuals. Different letters denote significantly different values (LSD).

PPO activity decreased significantly ($P \leq 0.05$) in carob moths treated with each of the essential oils. Those insects treated with a LC_{30} dose of the essential oil of *T. daenensi* had the greatest (1.9-fold) decrease in PPO activity, whereas those treated with the essential oil of *S. khuzistanica* had the lowest decline in PPO activity, relative to the control (Fig. 3).

Treatment of adult carob moths with the essential oils of *T. daenensis* and *S. khuzistanica* led to increased MDA concentrations of approximately 2.8 and 7.7-fold, respectively, compared to the control (Fig. 4).

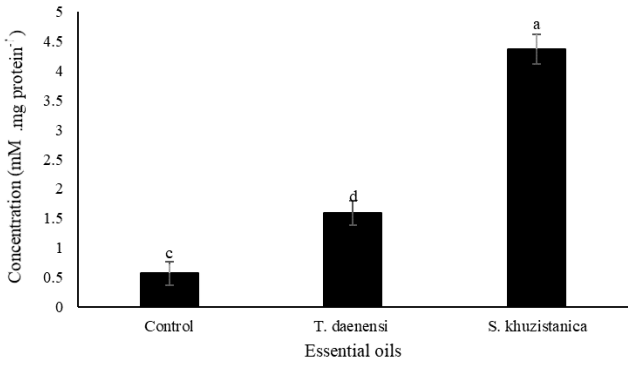

Fig. 4. The effect of essential oils extracted from the two plants on malondialdehyde (MDA) concentration in adult carob moths. The values are the means and standard errors for three replicates of 10 individuals. Different letters denote significantly different values (LSD).

DISCUSSION

Organisms may experience a variety of environmental stresses throughout their lives, which affect their biological parameters and lead to a wide range of changes depending on their severity. Insects, like other organisms, are subject to a wide range of biotic and abiotic stresses including pesticides and plant derivatives such as essential oils. Plant essential oils are potential alternatives to synthetic pesticides due to their shorter persistence in the environment and little effect on mammals and their food (Isman, 2006; Pavela & Benelli, 2016; Boutjagualt et al., 2022). In this study, the toxicity of the essential oils of *T. daenensis* and *S. khuzistanica* was investigated in order to determine the concentrations that induce a biological response in adult carob moths, *E. ceratoniae*. Bioassay results indicate that the mortality of carob moth due to essential oils was dose-dependent, with the rank order of toxicity being, *T. daenensis* > *S. khuzistanica*. Using sub lethal concentrations (LC_{30}), stress-related effects on the insects were recorded. In particular, the expression of two heat shock proteins (HSP70 and HSP90) and activity of antioxidant enzymes increased, indicating that the insects exposed to the essential oils experienced oxidative stress (Lazarević et al., 2020). Interestingly, heat shock protein expression and induction of oxidative enzymes were toxicity-dependent, i.e., essential oils of *T. daenensis* were more effective than those of *S. khuzistanica*.

Gas chromatography-mass spectrometry (GC-MS) was used to identify the constituents of the essential oil extracts of *T. daenensis* and *S. khuzistanica.* Although there were dozens of compounds identified in each extract, thymol accounted for 72% of that recorded for *T. daenensis* and carvacrol for 84% of that for *S. khuzistanica*. There are reports that the major constituents of essential oils are not the only constituents that have insecticidal activity (Miresmailli et al., 2006). Thymol and carvacrol are monoterpene phenols and exhibit insecticidal activity against various insects (Lee et al., 1997; Mansour et al., 2000). Thymol interacts with GABA receptors in insects and mammals (Priestley et al., 2003; Garcia et al., 2006). In insects, GABA receptors are targets for several pesticides including dieldrin, lindane and fipronil (Bloomquist, 1996) all of which are GABA receptor antagonists. Antagonists such as thymol could also potentially disrupt neural functions in insects (Mohammadi et al., 2001; Priestley et al., 2003). Carvacrol has high binding affinity to octopamine receptors in American cockroaches and can adversely affect the nervous system of these insects (Enan, 2001, 2005a). In houseflies, carvacrol inhibits the binding of nicotine to nicotinic acetylcholine receptors, which indicates that these receptors may also be a target for its insecticidal activity (Tong et al., 2013). In addition to affecting neural tissues, these two compounds may have other effects on insects as demonstrated by Enan (2005b) and De Medeiros et al. (2011). In addition to the two main constituents, thymol and carvacrol, there are minor components such as p-cymene and α-pinene with insecticidal activity in the extracts of *T.daenensis* and *S. khuzistanica* that have an antagonistic effect on octopaminergic receptors and acetylcholinesterase activity, respectively (Dambolena et al., 2016).

In this study, exposing the carob moth to two essential oils induced stereotypical responses to stress, including increased mRNA levels of HSP70 and HSp90. Several studies report a positive correlation between pesticide stress and HSP expression in organisms (Gupta et al., 2010; Yu et al., 2011; Doganlar & Doganlar, 2015; Sahebzadeh & Lau, 2017; Fang et al., 2021). Doganlar & Doganlar (2015) report the effect of pesticides on the expression of several HSP genes in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae). Sarkar et al. (2015) similarly report a significant increase in HSP70 expression in *D. melanogaster* larvae treated with flubendiamide. There are reports that the majority of pesticides strongly induce ROS inside cells (Dutta et al., 2017; Khatun et al., 2017). It is uncertain if EOs can adversely affect beneficial insects, so further studies are needed in this regard. There are some reports that synthetic insecticides such as organophosphate insecticides (Ops), carbamates and phosphine can induce the production of ROS compounds in organisms. Piner & Üner (2012) report that spinosad has oxidative effects in brain tissue by elevating the glutathione content. In addition to HSP synthesis, the essential oils also induced several antioxidant responses. The recorded increases in MDA following exposure to essential oils in the target insect were indicative of lipid peroxidation, which is a clear indicator of oxidative stress (Pinho et al., 2014; Shahriari et al., 2018). Activities of several antioxidant enzymes (SOD, CAT, APOX, and GST), which reduce ROS levels, increased following exposure to the essential oils. Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion (O_2^-, produced during oxidative stress, to H_2O_2 and O_2 (Fridovich, 1995). H_2O_2, despite not being a radical, is still a ROS and needs to be catabolised, or it will accumulate and induce oxidative damage. Catalase (CAT) and ascorbate peroxidase (APOX) are two antioxidant enzymes that convert H_2O_2 into water and O_2. It seems that the elevated activity of CAT and APOX occurred as a consequence of SOD producing more hydrogen peroxide in essential oil-treated individuals (Rahimi et al., 2018). Enhancement of SOD, CAT and APOX activities in response to pesticide stress is reported in various insects. Doganlar & Doganlar (2015) report that exposure to a mixture of pesticides enhanced cellular SOD and catalase in *D. melanogaster*. The higher activity of GST in the treated adults may be attributed to its role in the inactivation of lipid peroxidation products produced during stress. Polyphenol oxidase (PPO) is an oxidoreductase that catalyses the oxidation of a wide range of phenolic compounds using molecular oxygen (Mayer, 1986). PPO activity in insects is typically associated with exoskeleton formation (Halaouli et al., 2006). A decrease in PPO activities was recorded in the adult carob moths treated with both essential oils compared with the control, which indicates that the essential oils inhibited PPO activity in carob moth. This is not unexpected, since all the allelochemicals that were detected in the extracts are comprised of phenolic rings.

Although previous studies demonstrate that essential oils either inhibit acetylcholinesterase or have antagonistic effects on octopamine receptors in the nervous system of insects (Enan, 2001; Kostyukovsky et al., 2002), the findings presented here indicate that there is a linkage between the toxicity of the tested essential oils and induction of ROS and oxidative imbalance. These plant-derived molecules are potential alternatives to some of the current insecticides due in part to their biodegradability and low toxic effect on animals. It is likely that using some plant derived molecules in new insecticide agent formulations may help in suppressing some detoxifying mechanisms and can be effective means of achieving high control efficiency in integrated pest management.

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