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Evaluation of *Origanum majorana's* Toxicological Effects on Hemolymph and Some Biological Aspects of *Rhynchophorus ferrugineus* Larvae (Coleoptera: Curculionidae)

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ABSTRACT

Red palm weevil (RPW), *Rhynchophorus ferrugineus* is an invasive insect pest of date palm (*Phoenix dactylifera*) and several other palm species. RPW causes heigh damage in most date-producing areas of the world, particularly in the Middle East and North Africa. In this study, we discussed the drastic effects of *Origanum majorana* essential oil on different parameters, including total hemocyte counts (THCs), differential hemocyte counts (DHCs), total protein, lipid, and carbohydrates, as well as some enzyme activity like catalase (CAT), phenol oxidase, super oxide dismutase (SOD),α β-estrase, and acetyl-cholinesterase (AChE) in RPW larvae. Groups of five fourth-stage larvae were submerged for 60 seconds immediately in 9.8% (LC_{50}) of *O. majorana*. Compared to the control group, the THC levels are reduced after treatment with *O. majorana* essential oil. The estimated THCs were 27025 ±1356.54 cells/mm^3 and 23250 ±1035.82 cells/mm^3 for control and treated larvae, respectively. The DHCs showed that granulocytes (Gr) (26.47%) and plasmatocytes (Pl) (31%) were the two hemocyte types that predominated. Compared to the control group, the total protein, lipid, and carbohydrate were significantly lower. In comparison to control larvae, the treated larvae had significantly greater levels of CAT, SOD, and AChE activity. The activity levels of α, β-estrase and phenol oxidase were greatly decreased, but the phenol oxidase level was significantly increased in comparison to the control groups. Through the results obtained can be used *Origanum majorana* in the integrated control program especially in the period before harvest so that there are no residual pesticides in fruits when control palm after conducting some future studies infestation.

Keywords: red palm weevil, essential oil, biochemical studies, hemocytes, enzyme activity.

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INTRODUCTION

The red palm weevil (RPW), *R. ferrugineus* (Olivier), is the most pest that seriously harms date palm trees. (Coleoptera: Curculionidae). RPW is recognized as Egypt's most destructive pest to date and in practically widespread all Egyptian governorates, causing immense damage to palm trees Because of the non-application of an internal quarantine and the non-implementation of an integrated control programme (Merghem, 2011; Merghem & Bibers, 2014).The widespread usage of these compounds has also resulted in issues with the economy and the environment, including threats to mammals, beneficial animals, and arthropods, as well as worldwide pollution (Gloria, Monroy, Gòmez, & Olguìn, 2008; El namaky, El sadawy, Al omari, & Bahareth, 2020). In response to the detrimental effects of the insecticides and the continued economic loss brought on by this borer, other tools that provide effective, safe, and economical control were urged to resolve these issues. It has been demonstrated that plant essential oils and their constituents have the potential to be developed into novel fumigants and that they may have advantages over them in terms of low mammalian toxicity, quick breakdown, and local availability. (Isman, 2008). One of the alternate measurements for the Lamiaceae family, which has 3500 species and 200 genera, is *Origanum* L. The genus contains around 44 species, several of which are oregano variants, sweet marjoram (*O. majorana* L.), and dittany of Crete (*O. dictamnus* L.). (Martins et al, 1999). They have strong antifungal, insecticidal effects antioxidant, and antimicrobial (Belhattab et al, 2005) (Khalfi, Sahraoui, Bentahar, & Boutekedjiret, 2008), (Mechergui et al, 2010:), Rodriguez-garcia et al, 2016).

Immunity plays an important role in the development of insects. Many insects belonging to significant orders have exhibited humoral and hemocyte-mediated immune reactions, including phagocytosis, nodulation, and encapsulation (Schmidt, Theopold, & Strand, 2001). Hemocytes that participate in hemocyte-mediated immunity have also been extensively discussed by several authors, along with all other types. The red palm weevil (RPW), *R. ferrugineus* (Olivier, 1790) (Coleoptera: Dryophthorinae), has been suggested as a useful model for studying some aspects of host-pathogen interactions and insect innate immunity (Cappa et al, 2020; Ibrahim et al, 2021). Understanding the immune systems of insects can help improve biological pesticides and increase our understanding of host-pathogen relationships. In this field, the common types of hemocytes discussed in this study include prohemocytes, granular cells (granulocytes), plasmatocytes, spherule cells (spherulocytes) and oenocytoid. These hemocyte forms have been recorded from numerous orders, including Lepidoptera, Diptera, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (Hernandez et al, 1999; De silva, Dunphy, & Rau, 2000). However, changes in the activity of protective enzymes like glutathione-S-transferases (GSTs), - and α-/β-esterases **(**α-/β-ESTs), acetylcholinesterase **(**AChE), acid and alkaline phosphatases (ACP and ALP), and mixed-function oxidases (MFO), which aid organisms in the transformation and/or elimination of endogenous and exogenous compounds (Polson, Brogdon, Rawlins, & Chadeee, 2011; Koodalingam, Mullainadhan, & Arumugam, 2011). On the other hand, superoxide dismutase (SODs) are antioxidant components that can

oxidase reactive oxygen species. In cells, superoxide, a reactive oxygen species, is dissimulated by them into hydrogen peroxide and oxygen. They are regarded as a crucial antioxidant line of defense against superoxide radicals (Bolter & Chefurka, 1990). Also, acetylcholine, a crucial neurotransmitter involved in the transmission of nerve impulses, is converted metabolically by the main enzyme, AChE, which is largely located in insects' synaptic clefts. AChE also serves as the target site for the majority of neurotoxic insecticides. It is sometimes thought that the structural change in AChE is the primary factor causing bugs to become resistant to synthetic chemical insecticides like organophosphates and carbamates (Chaudhari, Singh, Kedia, Das, & Dubey, 2021). This work aimed to investigate the activity of *O. majorana* essential oil against the last instar larvae of RPW. Furthermore, we studied the physiological modifications of enzymes and the amount of total protein, lipids, carbohydrates. Finally, the total and differential haemocyte counts of larvae were evaluated, from an immunological point of view.

MATERIAL AND METHODS

Insect

Red palm weevil larvae obtained from palm trees infestation in Abu Rawash, Egypt's Giza Governorate, RPW last instar larvae were found. The larvae were raised in a lab at a temperature of 25 °C while being fed sugar cane stems.

Essential oil

From a local market in Egypt, *O. majorana* essential oil was bought.

Immersion and contact assays

The study was conducted in the Research Laboratory of the women's college at Ain Shams University's Zoology Department.

Using five groups of fourth-stage larvae were submerged for 60 seconds immediately in 9.8% (LC_{50}) *O. majorana*. This concentration was determined based on previous research that we had conducted (Mady, Ahmed & El namaky, 2021) employing distilled water with 80% Tween at 1 ml/100 ml of emulsion. To increase homogeneity, a homogenizer was used on the generated emulsions. Treatment larvae were placed in plastic boxes (5 larvae per box) with filter paper for 24 hours before being moved to plastic containers with 50g of moist sugar cane sawdust and succulent sugarcane stem pieces under temperature 28°C incubated.

Tween-80 was present in both the treatment and control solutions at 0.02%. Five larvae replicated four times. After 48 hours, the larvae were inspected, and the dead ones were taken out.

Hematology assays

Cutting one of the prolegs of fourth-instar *R. ferrugineus* larvae using a pair of tiny scissors allowed for the examination of a drop of hemolymph. We counted the

hemocytes in each individual larva. By using diluted solutions, total hemocyte counts per mm3 (THCs) were performed. The number of cells per µl of hemolymph was used to express THCs. By counting 100 cells and calculating the percentage differential of each type of hemocyte (DHC) found in the hemolymph, the DHCs were calculated. The total cells were counted, and DHCs were expressed as the mean of each hemocyte type. The total and differential hemocyte counts (THCs and DHCs of treated and untreated fourth instar *R. ferrugineus* were calculated. A one-way analysis of variance (ANOVA) was used to analyses the data from the THC and DHC ($P < 0.05$).

Biochemical studies

The Plant Protection Research Institute's Physiology Department conducted the entire biochemical analysis.

Protein assay

Using Coomassie Brilliant Blue G-250, a dye that binds to proteins, and bovine serum albumin as the standard, Bradford's method from 1976 was used to quantify the total amount of protein in the hemolymph.

Lipid assay

A technique adapted from van Handel (1985) and Waburg & Yuval (1996) was utilized for the total lipid analyses.

Total carbohydrates assay

Total carbohydrates were extracted, prepared for assay, and estimated in samples by the phenol-sulphuric acid reaction according to the procedure of Dubios, Gilles, Hamilton, Rebers, & Smith (1956) and Crompton & Birt (1967). Total carbohydrate is expressed as mg glucose/gm larval fresh weight.

Enzyme activity assays

Catalase activity was determined using a catalase assay kit purchased from Biodiagnostic, Co., according to the method described by Aebi (1984).

The activity of superoxide dismutase (SOD) was estimated by using a SOD assay kit purchased from Biodiagnostic, Co., according to the method described by Nishikimi, Roa, & Yogi (1972).

Phenoloxidase activity was determined according to a modification of the method described by Ishaaya (1971).

Acetylcholinesterase (AchE) activity was measured according to the method described by Simpson, Bulland, & Linquist (1964) using acetylcholine bromide (AchBr) as substrate and expressed as ug AchBr/min/g.b.wt.

Alpha esterases (α-EST) and beta esterases (β-EST) were determined according to Van Asperen (1962) using α-naphthyl acetate or β-naphthyl acetate as substrates, respectively. The activity of the EST was presented as ug α-nphthol/min/g.b.wt

RESULTS

Effect of *O. majorana* essential oil on the total and differential haemocyte counts (THCs and DHCs) of last instar larvae of RPW

When compared to the control group, the *O. majorana* essential oil after 48 hours had an impact on cell count. For the control and treatment groups, the estimated circulating hemocyte counts of the RPW, 4th instar larvae, were 27025±1356.54 cells/mm^3 and 23250±1035.82 cells/mm^3, respectively. The overall hemocyte counts are lower after using *O. majorana* essential oil compared to the control group.

After 48 hours, the effect of *O. majorana* essential oil on the various hemocyte counts in RPW, 4th instar larvae was assessed. Differential counts showed that granulocytes (Gr) (26.47%) and plasmatocytes (Pl) (31%) were the two hemocyte types that predominated, while oenocytoides (Oe) (5.88%) had the lowest count. Spherulocytes (Sp), coagulocytes (Co), and prohemocytes (Pr) made up 14.71, 12.61, and 9.24% of the total, respectively (Table 1).

Table 1. Differential hemocyte counts of *Rhynchophorus ferrugineus* 4th larval instar (Mean ± S.E).

Haemocytes	Prohomocyte	Plasmocyte	Graneolocyte	Coagulocytes	Spherulocytes	Oenocyte
Control	7.50 ± 0.29 12.61%	18.50±0.29 31.09%	15.75±0.26 26.47%	8.75±0.48 14.71%	5.50±0.29 9.24%	3.50±0.29 5.88%
Larvae exposed to *Origanum majorana*	9.50 ±0.29 17.43%	11.50±0.33 21.10%	10.25±0.85 18.81%	12.00±0.65 22.02%	5.75±0.60 10.55%	5.50±0.20 10.09%
F	76.0	268.0	44.4	25.8	2.9	56.3

Values represent Mean ±SE of R. ferrugineus. P-values: P<0.05.
Total df = 11

Biochemical studies

The amount of total protein, lipid, and carbohydrates were determined in 4th instar larvae of RPW 48h post treatment. In our study, the total protein, lipid, and carbohydrate contents of *R. ferrugineus* larvae treated with LC_{50} of *O. majorana* essential oil were significantly (P< 0.05) lower than those of the control group. (Fig. 1).

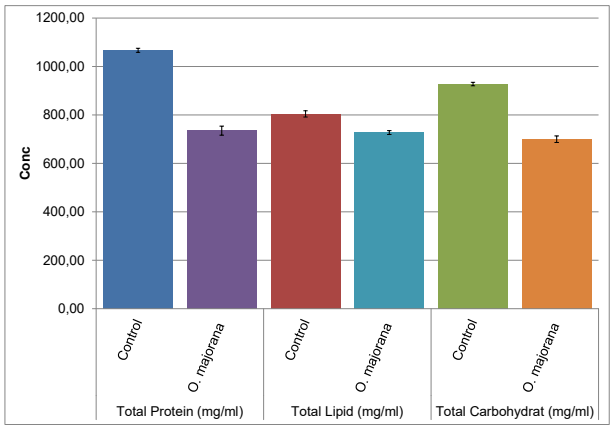

Figure 1. The total protein, lipid, and carbohydrate contents of *Rhynchophorus ferrugineus* 4th larvae treated with LC_{50} of *Origanum majorana* essential oil.

In (Fig.2), it is shown how *O. majorana* essential oil affects the activity of the enzymes involved in detoxification in *R. ferrugineus* larvae. The activity level of the enzymes CAT, SOD, and AChE was significantly higher ($P< 0.05$) in the essential oil-treated 4th instar red palm weevil larvae than in the control larvae.

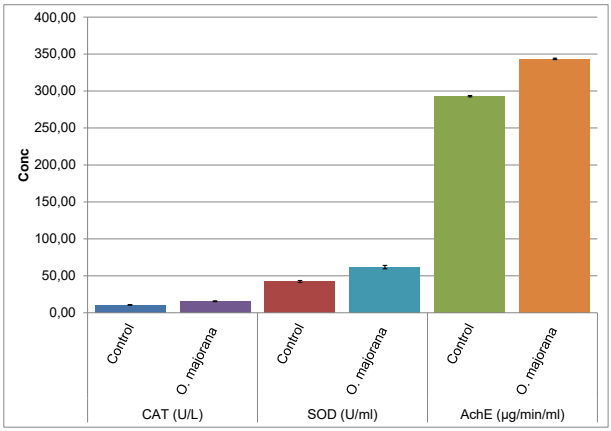

Figure 2. The activity level of the enzymes CAT, SOD, and AChE of *Rhynchophorus ferrugineus* 4th larvae treated with LC50 of *Origanum majorana* essential oil.

It was discovered by comparing the activity levels of α, β-estrase, and phenol oxidase that the level of both α, β-estrase were greatly decreased, but the phenol oxidase level was significantly increased in 4th instar larvae in comparison to the control groups (Fig. 3).

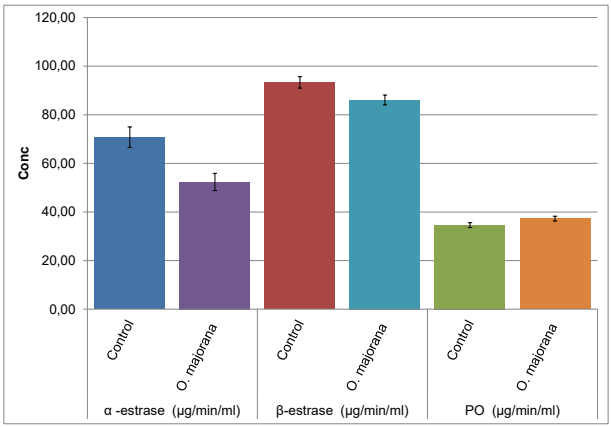

Figure 3. The activity levels of α, β -estrase, and phenol oxidase of *Rhynchophorus ferrugineus* 4th larvae treated with LC50 of *Origanum majorana* essential oil.

DISCUSSION

The results obtained from the present work show that after 48 hours of treatment with LC_{50} of *O. majorana*, the total Hemocyte count (THC) decreased when compared

with untreated 4th instar larvae of *R. ferrugineus*. Many insects have shown reduced THC levels when exposed to various plant extracts or plant-derived materials, including the cotton bollworm *Helicoverpa armigera* when exposed to *Artemisia annua*, *A. conyzoides*, and *Azadirachta indica* oils (Padmaja & Rao, 2000); *S. litura* when exposed to *Acorus calamus* oils (Sharma, Sharma & Saxena, 2008), and the lemon butterfly *Papilio demoleus* when exposed by leaf extracts of *Eucalyptus globules, A. conzoides* and *Allium sativum* (Pandey, Pandey, & Tiwari, 2012).

THC levels were decreased in *E. integriceps* larvae after treatment with *A. annua* extract. (Zibaee & Bandani, 2010). The amount of *Ferula gummosa* oil greatly reduced the THC of the Mediterranean flour moth *Ephestia kuehniella*. (Ghasemi, Yazdib, Tavallaie, & Sendi, 2013). THC levels dropped after *Clerodendron inerme's* aqueous leaf extract was applied to *H. armigera* caterpillars in their sixth instar (Kalyani & Holihosur, 2015). Additionally, after being treated with an acetone extract of the *Calotropis procera* plant, individuals of the desert locust *Schistocerca gregaria* and the migratory locust *Locusta migratoria* showed notable decreases in THC in their hemolymph (*Kaidi, Amroun, Hocine, Doumandji, & Ghezali,* 2017). The amount of THC in *S. littoralis* larvae was substantially reduced after 48 hours of treatment with LC_{25} extracts of *C. procerae* and *Atriplex halimus* (Asiri, 2017).

The reduction in THC could be due to the suppression of larval hematopoietic cell proliferation and function (Zhu et al, 2012). Numerous authors (Sabri & Tariq, 2004; TiwariPandey, & Kumar, 2006; Pandey, Upadhyay,& Tiwari,2007; Sendi & Salehi, 2010; Zhu et al, 2012; Zibaee, Bandani, & Malagoli, 2012) have suggested that the decline in THC levels may be caused by the cytotoxicity of botanicals and the demise of pathologically deteriorated cells.

On the other hand, numerous reports have shown that various insects' THC levels have increased in reaction to botanical treatments. For instance, *azadirachtin* (Azt), *Margosan-O* (neem formulation), and some substances derived from urea waste and rice straw all caused a rise in THC in the hemolymph of the Egyptian cotton leafworm *Spodoptera littoralis* (*Hassan, Bakr, Abd el-bar, Nawar, & Elbanna,* 2013). After being exposed to the acetone solution of *Melia azedarach*, THC levels in the black cutworm *Agrotis ipsilon* larvae rose (El-Shiekh, 2002; Shaurub, & Sabbour, 2017). The non-mulberry silkworm *Antheraea assama* was treated with essential oils of *Ocimum sanctum*, *Ocimum gratissimum*, and *Ageratum conyzoides*, and early treatment results showed a THC rise (Khanikor & Bora, 2012).

The tested *O. majorana* essential oil obviously had an impact on the various hemocyte counts. The evaluated essential oil decreased the number of granulocytes (Gr) and plasmatocytes (Pl). Compared to the control, the percentage of (Gr) dramatically dropped from 26.47 to 18.81%. Additionally, after exposure, the proportion of (Pl) dropped from 31% in the control to 21%. Furthermore, when compared to control levels of 7.6 and 5%, respectively, the percentages of Oe and Sp significantly raised to 19.2 and 13.2%, respectively. These findings corroborate those of Giulianini, Bertolo, Battistella, & Amirante (2003). Granulocytes are the most prevalent phagocytes in the

RPW's hemolymph, just like in other insect species. (Chiang, Gupta, & Han, 1988; Carter & Green, 1987). Oenocytoids identified in the RPW are consistent with those observed in *Pseudomonas aeruginosa* and *Melolontha melolontha* (Devauchelle, 1971), (Giulianini et al, 2003).

The current findings were in agreement with those made by Abou-taleb, Zahran, & Gad (2015), who found that corpora allata (CA) activity and various haemoglobin counts decreased in *Spodoptera littoralis* larvae in their fourth instar after exposure to the insecticides lufenuron and chlorfluazuron. Additionally, Saxena & Tikku (1990) showed that plumbagin therapy damaged haemoglobin and prevented plasmatocytes and granulocytes from elongating their filopodia. (The types that are active in defence mechanisms). While oenocytoids had a high mitotic rate and a quick turnover, Arnold, & Hinks (1983) speculated that this was a method for releasing waste products from their metabolism into the hemolymph. Additionally, Sharma et al (2008) demonstrated that plasmatocytes (Pl) and granular hemoglobinocytes were the main targets of oil treatment. (Gr).

Our findings reveal that, compared to control larvae, the amount of total protein, lipid, and carbohydrates in *R. ferrugineus* 4th instar larvae treated with LC_{50} of *O. majorana* EO after 48 hours significantly decreased (Fig. 1).

Proteins are important biochemical building blocks required for an organism to grow, develop, and carry out its essential functions. Our findings reveal that, the total protein, lipid, and carbohydrates of the hemolymph of treated RPW larvae were significantly decreased after 48 hr. This decrease in protein concentration is likely the result of a decrease in protein synthesis or an increase in protein degradation, as well as a potential EO interaction with hormones that control protein synthesis (Vijayaraghavan, Sivakumar, Zadda kavitha, & Sivasub ramanian, 2010). Nasr, Sendi, Moharramipour, & Zibaee (2017) reported similar outcomes, reporting that the amount of total protein decreased following treatment with the LC_{50} dosage of *O. vulgare* essential oil.

Insect physiology is greatly influenced by carbohydrates, which serve as their primary source of energy. (Chapman, 1998; Kaufmann & Brown, 2008). Also, lipids are a crucial source of energy for ecdysis, cell maintenance, embryonic growth, and reproduction. In fatty bodies, they are conserved. (Chapman, 1998).

Sharma, Mohanm, Dua, & Srivastava (2011) observed a similar trend in the reduction of lipid levels in Anopheline and Culicine larvae following treatment with *A. annua* extract. Because of how plant EOs affect lipid metabolism and how stress-induced lipid reserves are used for energy production, there may be a reduction in lipid levels in plant EO-treated larvae. (Olga, Fevizi, & Ekrem, 2006).

Insect physiology is greatly influenced by carbohydrates, which serve as their primary source of energy. (Chapman, 1998; Kaufmann & Brown, 2008). Similar findings were made by Dris, Tine-djebbar, Bouabida, & Soltani (2017) who found that after treating *Cx. pipiens* larvae and pupae with *O. basilicum* essential oil, glucose levels were decreased. Insects treated for carbohydrate depletion are likely as a result of energy costs during stressful situations.

This outcome was consistent with Tarigan, Dadang, & Sakti harahap (2016) findings that total carbohydrate, protein, and fat contents in *T. castaneum* and *C. maculatus* considerably decreased in all treatments compared to controls.

According to Yazdani, Sendi, & Hajizadeh (2014) all concentrations of *T. vulgaris* and *O. vulgare* essential oils significantly reduced the total protein, fat, and carbohydrate content of larvae. Additionally, Bouguerra & Boukoucha (2021) showed that fourth-instar *Cx. pipiens* larvae fed with *O. glandulosum* (EO) had decreased protein, lipid, and carbohydrate contents.

The findings of our study evaluated the detoxifying activity of the enzymes CAT, SOD, and AChE on RPW larvae in their fourth instar after treatment with oil extract. Our research showed that after being treated with LC_{50}, *R. ferrugineus* fourth-instar larvae had considerably higher CAT, SOD, and AChE activity than the control larvae. In agreement with our findings, Chaudhari et al (2021) found a significant rise in the activity of the enzymatic antioxidant defense systems CAT and SOD which are crucial for preventing the accumulation of free radicals produced in cells in response to oxidative stress and other stresses like exposure to pesticides and detoxifying xenobiotics as well as for maintaining cellular redox homeostasis (Zhao et al, 2017).

Similar to this, treated larvae had significantly higher AChE and SOD activity than control larvae. These findings demonstrate that these enzymes are crucial for the process of detoxifying pollutants. (Gabr, Lemmons, & El-bokl, 2022). Additionally, Agliassa & Maffei (2018) confirmed that the treated larvae had significantly higher AChE and SOD activity than the control larvae. s. These findings show that these enzymes are crucial for the process of detoxifying poisons.

In a recent study, Upadhyay et al (2019) examined the insecticidal effect of Melissa officinalis EO against *T. castaneum* and noted an increase in ROS, SOD, and CAT levels as well as a drop in the GSH/GSSG ratio.

In their examination of the effects of *Carum carvi* EO against *T. castaneum*, Petrovi *et al* (2019) suggested changes to the first line of antioxidant defense (SOD and CAT) are the important parameters whose modification would be the main factor causing the mortality of the pests.

The results of this study demonstrated that RPW 4th instar larvae treated for 48 hours with LC_{50} of *O. majorana* essential oil had significantly lower levels of, α, β -estrase and higher levels of phenol oxidase (PO) than controls. El-Aziz & El-Sayed (2009) showed that the activities of, α, β esterase, and GST of *Tribolium confusum* were significantly reduced in LD_{50} dose of basil, garlic, and sesame essential oils. This finding is consistent with our findings. The extract binds to the enzyme's active site and stops the enzyme from performing its detoxifying function. In most cases, detoxification enzymes in insects have been shown to be the enzymatic defense against foreign substances and to be crucial for the maintenance of their regular physiological processes (Mukanganyama, Figueroa, Hasler, & Niemeyer, 2003).

The concentration of detoxifying enzymes, such as esterase activities on *C. maculatus* and *S. oryzae*, were all considerably decreased in all sub-lethal

concentrations of *A. monophylla* oil, according to similar studies. (Nattudurai et al, 2017). However, Magierowicz, Gòrska-drabik, & Sempruch (2019), observed that carvacrol increased CAT activity in *A. advenella* homogenates while *S. hortensis* essential oil induced polyphenol oxidase (PPO). Deterrent phenols and phenolic acids are detoxified by insect PPO and peroxidase (POX) into less harmful by-products (Urbańska, Tjallingii, Dixon, & Leszczyński, 1998). Accordingly, the findings imply that PPO, but not POX, may take part in the detoxification of carvacrol and/or other *S. hortensis* EO components in the tissues of *A. advenella*. The polyphenol oxidase studies demonstrated that essential oils may be more potent biopesticides than their individual active components (Bakkali, Averbeck, Averbeck, & Idaomar, 2008).

Additionally, it is thought that when an immunological challenge occurs, oenocytoids (OEs) play key roles in the phenoloxidase (PO) cascade. (Strand, 2008). Therefore, the substantial growth in their population may have resulted from the stimulation of the immune system that caused the oil-treated larvae to secrete PO. These findings indicate that the detoxification or metabolism of *O. majorana* essential oil depends on the activities of enzymes and esterases.

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