Evaluation of essential oils against red palm weevil, *Rhynchophorus ferrugineus* (Olivier) larvae with reference to protein pattern

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ABSTRACT

Infesting a variety of palm trees throughout Egypt, the Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is regarded as a damaging pest. The objective of the current study was to determine the protein pattern while evaluating six essential oil emulsions as botanical extracts against this pest in a laboratory setting. The method of dipping food was used at concentrations of 2, 3, 4, 5 and 6%. There were notable variations between the various oils and exposure times. The studied oils showed varying fatality rates in response to increasing concentrations. Across all concentrations, the lowest mortality was obtained with citronella oil. After 96 hours, 6% of the clove and orange oils caused 100% death. Totally 90% of insect died when exposed to 4% concentration of chili oil. Even though the tested oils had different fatality rates, longer exposure times resulted in larger percentages. The molecular weights of the bands varied and ranged from 7 to 275 KD in the treated hemolymph and from 6 to 273 KD in the untreated hemolymph, indicating significant variations between the two types of hemolymph. It is stated that essential oils are a good choice for RPW control approaches, as long as the suitable type of oil is used, taking into account exposure time and oil concentration.

Keywords: *Rhynchophorus ferrugineus*, Red Palm Weevil, Protein Pattern and Essential Oils

MS History: 05.04.2023(Received)-20.05.2023(Revised)- 025.05.2023 (Accepted)

Citation: Atwa, A. Atwa, Mohamed Z. A. Rezk, Hala, A. Adel, Heba, S. Abd El-Aty and Ghada M. Ramadan. 2023. Evaluation of essential oils against red palm weevil, *Rhynchophorus ferrugineus* (Olivier) larvae with reference to protein pattern. *Journal of Biopesticides*, 16(1): 68-78.
DOI:10.57182/jbiopestic.16.1.68-78

INTRODUCTION

The most popular and extensively grown plant in the dry parts of the Middle East and North Africa is the date palm, *Phoenix dactylifera* L. For about 7000 years, palm fruits were widely regarded as the primary source of carbohydrates for the indigenous populace (Dawson, 1982). However, according to Nirula (1956), the Arabian Gulf region, the Mediterranean area, and South and Southeast Asia, the red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae), is one of the most destructive pests that infests a variety of palm trees (Atwa and Hegazi, 2014). According to Murphy and Briscoe (1999), the majority of damage is caused by various RPW larval instars feeding on immature fibers and terminal bud tissues. When the larvae are almost fully grown, they enter the palm's center through mines that terminate in cavities, where they pupate inside cocoons composed of dried, brown palm fibers (Gomez and Ferry, 1999). Adult weevils live on the same tree and keep producing eggs there. According to Laćer *et al.* (2009), newly hatched larvae cause additional

severe damage by attacking increasingly delicate apical areas, till the palm eventually dies. In situations of severe and widespread tissue damage, infested offshoots and outer leaves turn dry, pupae congregate around the palm base, the trunk breaks, or the palm crown topples (Vidyasagar and Subaharan, 2000).

There are various approaches that can be used to suppress RPW infestations. The most widely used control method is most likely insecticides. Scientists are searching for safe substitute methods to lessen pesticide dangers due to the overuse of chemical control, particularly undiluted insecticides, against the RPW and the injection of these chemicals into the palm trunk (Muthuraman, 1984; EL-Ezaby, 1997). In certain instances, it was discovered that insecticides harmed biological agents and the environment more than the pest species itself. Recent research on date fruits found pesticide residues beyond allowable limits, most likely as a result of widespread and careless pesticide application for RPW control (El-Saeid and Al-Dosari, 2010).

Botanical insecticides were important tools in the fight against agricultural pests until the invention and commercial success of synthetic insecticides started in the 1940s (Isman, 2004). Many substances that plants produce offer some defense against insect attack (Felton and Gatehouse, 1996). The literature has addressed the potential of certain essential oils as secondary plant substances to regulate specific insects. These substances can act as attractants, repellents, ovicides, insecticides, synergists, juvenile hormone analogues, and antigonadol agents (Golob and Webley, 1980; Schmidt *et al*., 1991). In fact, pyrethrum, rotenone, neem, and essential oils are the four main categories of botanical pesticides that are employed to control insects (Isman, 2006). Essential oils have a brief protection period and are contact insecticides and acaricides. EOs' anti-feedant properties may help stop RPW infestations from starting again. Essential oils derived from the blossoms of *Artemisia nilagirica* (Asteraceae) and snake root, *Eupatorium adenophorum*, were reported by Shukla *et al*. (2012) to exhibit strong anti-feedant effect against adult *R. ferrugineus*. Furthermore, Hussain and AlJabr (2014) demonstrated the great potential of African myrrh's essential oil, *Commiphora myrrha* (Burseraceae), as a bio-pesticide, which may be used in an integrated management plan to combat the red palm weevil. The essential oil of the *Juniperus communis* (Cupressaceae) plant, often known as common juniper, has a harmful effect on RPW at all stages, with particular effects on eggs and larvae due to its fumigant or contact toxicity (Sharaby and EL-Dosary, 2016).

According to War *et al*. (2013), the total amount of protein, serine protease, esterase, and glutathione s-transferase in insect fat bodies are all impacted by essential oils. The toxicity of plant extract is defined by its capacity to lower the total amount of protein in insects. Conversely, Riddiford (1985) verified that the application of essential oil to RPW resulted in a large increase in total protein in the male body and in the gut tissues of both sexes, while other biochemical changes included a decrease in protein synthesis in the haemolymph. Therefore, Sharaby and EL-Dosary (2016) came to the conclusion that the juniper oil's harmful and disrupting effects on the hormonal system and protein synthesis could be the cause of changes in the amount of protein found in the stomach and hemolymph of treated RPW adults. Thus, in the current study, we assessed the effectiveness of six essential oil emulsions as botanical pesticides against red palm weevil larvae in a lab setting. In addition to determining the LC_{50} and LT_{50} for each of the six drugs, the insect protein pattern was also ascertained.

MATERIALS AND METHODS

R. ferrugineus collection

According to measures of the head capsule width, RPW larvae were divided into three categories: young, medium, and full-grown (Jacas *et al.*, 2011). Date palm trees infested with *R. ferrugineus* larvae were harvested at the last instar

or full-grown stage from the orchards. Before being utilized in the lab tests, the samples were properly cleaned with distilled water to get rid of plant fibers. They were then allowed to air dry in plastic containers with sterile filter sheets.

Essential Oils

We purchased oils of peppermint, orange, jasmine, chili (hot pepper), clove and citronella from Egyptian Sugar and Integrated Industries Company in Giza, Egypt. Oils were emulsified individually with 0.25% triton X100. In laboratory experiments, various oil concentrations, 2, 3, 4, 5, and 6% were prepared and utilized. The experiments were conducted at a mean temperature of 25±3ºC in a laboratory setting.

Food Dipping Technique

The efficacy of the six produced oil emulsions against the final larval instar of *R. ferrugineus* was assessed using the dipping technique. Ten 15 cm high by 10 cm diameter cylinders made of plastic, each holding one set of 8–10 cm long sugarcane (*Saccharum officinarum* L.), which serves as the food source. Ten concentrations or replicates, with one larva each were created. In the same manner, control replicates were made using triton x100 (0.25%) in water. After 30 minutes of immersion in oil emulsions, sugarcane pieces were allowed to air dry before being fed to larvae. The plastic boxes were kept in a dark environment at 25±3ºC. There were mortality reports every 48 and 96 hours.

Preparation of Larval Body Homogenates

Four days after treatment, larvae of various concentrations were frozen at -20ºC, weighed and their entire body was homogenized using a glass homogenizer in an ice bath for ten times the volumes (w/v) of 0.02M sodium phosphate buffer, pH 7.0. The homogenate was then centrifuged for fifteen minutes at 12000rpm. After being collected, the supernatant was put into tubes and kept cold until it was needed (Zibaee *et al*., 2008; Fuchs *et al.*, 2010).

Proteins Pattern

Using the technique outlined by Hames (1990), a 10% SDS-Polyacrylamide gel was created and utilized to examine the protein pattern of RPW larvae that had been exposed to essential oils. The loading protein sample was made by combining 10 µl of sample buffer (4% SDS, 0.125 M Tris pH 6.8, 5% β-mercaptoethanol, 20% glycerol and 0.005% bromophenol blue) with 40µl of the previously prepared larval homogenate supernatant. After five minutes of incubation at 100ºC, 30µl of the loading sample was added to each individual slap gel slot. SDS (Sigma DS -7-M.P.) was utilized and a broad range of standard proteins, from low molecular weight (M. wt.). The 10% separating gel and the 2.5% stacking gel both contain tris-glycine. Phosphorus 8.3 (15g Tris, 72g Glycine and 5g). SDS employed as the electrode buffer in a slap plate electrophoretic unit (Biometra mingel G 41) and diluted to five liters using distilled H20 buffer system. Gels were operated at 200 volts per hour of circulating current. The staining solution, which included 2.75g of Coomassie blue R 250, 500 methanol, 500ml of acetic acid, and 500 ml of distilled water, was left on the gels for the entire night. The background color was eliminated by the use of a retaining technique. The gels were submerged in distilled water, methanol, and 96% acetic acid for an entire night (2: 12: 28, V: V).

Statistical Analyses

Abbott's formula (1925) was used to adjust all mortality statistics for natural mortality. Software for one-way ANOVA (SPSS, 12) was used to analyze the data in order to identify the primary impacts of essential oils. The SNK method (Steel and Torrie, 1980) was used to separate the means, and $p < 0.05$ was used to determine statistical significance.

RESULTS

Food Dipping Technique

The findings demonstrated that, of all the applied concentrations, citronella oil had the least impact. Even with increased oil concentrations and larval exposure times of up to four days, no greater effects were seen, as RPW mortality remained below 30%. Jasmine oil, on the other hand, had the opposite effect, with fatality rates rising with increasing oil concentrations. After 96 hrs of

exposure, a 3% jasmine concentration caused 70% death. The mortality rates were the same 96hrs after treatment when the concentration of jasmine oil was increased from 5% to 6%; however, two days of exposure resulted in 40 and 60% of deaths, respectively. On the other hand, when larvae were exposed to 2% concentration for 48 hours, clove oil was the sole treatment that resulted in 20% death. After 96 hours, all larvae that were treated with a 6% concentration perished. Chili oil was also distinct from the other treatments in that it resulted in 90% death at a concentration of 4%; however, the mortality rate did not increase when the concentration was increased to 5% or 6%. Surprisingly, lower death rates were associated with increasing peppermint oil concentrations; at 5% concentration, mortality was 90%, whereas at 6% concentration, it was 70%. The only treatments that resulted in 100% mortality were clove and orange oils. Significant differences across oils and exposure times were found using one-way ANOVA ($P \leq 0.001$).

Lethal Concentrations and RPW Mortality Percentages

After 96 hours of exposure, the LC values of the various tested oils in relation to *R. ferrugineus* larval mortality were measured (Table 1). At 2% concentrations, it was noticed that orange, citronella, jasmine, and chili oils could kill 25% of the RPW population. In contrast, the concentrations of peppermint and clove that resulted in 25% mortality were 1.5% and 1.4, respectively. Conversely, when 2.8% concentrations of the jasmine, peppermint, chile, and orange oils were applied, half of the larval population perished. Citronella oil required the maximum dosage to have the same effect, even though clove oil required less concentration to kill 50% of the larvae (Table 1). Among the studied oils, higher concentrations were associated with varying fatality rates. While peppermint required a dosage of 11.7% to kill the same number of larvae, jasmine oil, at the lowest concentration of all the treatments, killed 95% of RPW larvae. For 95% of the larvae to be killed, the amounts of orange and chili oils (7.44) were required.

Table 1. Lethal concentrations (LCs) of essential oils required to kill different percentages of *R. ferrugineus* larvae

Oil treatment	LC_{25}	LC_{50}	LC_{75}	LC_{90}	LC_{95}	LC_{99}
Jasmine	1.947	2.823	4.094	5.719	6.981	10.161
Citronella	2.077	3.048	4.473	6.318	7.768	11.446
Clove	1.408	2.439	4.227	6.929	9.316	16.229
Peppermint	1.556	2.801	5.042	8.560	11.749	21.279
Chili (hot Pepper)	1.928	2.819	4.120	5.799	7.115	10.441
Orange	1.928	2.819	4.120	5.800	7.115	10.441

Exposure Time and RPW Mortality

In general, it has been observed that longer essential oil exposure durations resulted in increased RPW mortality, but with varying values. Of all the studied oils, chili oil required the least amount of exposure time—nearly a day—to kill 25% of the examined larvae. As seen in Table 2, the other five tested oils took nearly equal amounts of time to become effective, never exceeding 1.3 days. The best mortality was still being produced by chili oil, which killed half of the RPW larval population in just 1.58 days as opposed to 1.74 days for orange and jasmine oils and 2.23 days for peppermint oil. To kill 90% of *R. ferrugineus* larvae, jasmine and orange oils required the least amount of exposure time—3.7 days—followed by chili oil and citronella. Table 2 shows that the longest exposure time required to kill 90% of the larvae was found in case of peppermint oil. While peppermint required the highest exposure time 16.3 days—to kill 99% of the studied larvae, jasmine and orange oils required the shortest—6.9 days.

Table 2. Time in days required to kill different percentages of *R. ferrugineus* larvae by essential oils

Oil treatments	LT_{25}	LT_{50}	LT_{75}	LT_{90}	LT_{95}	LT_{99}
Jasmine	1.1663	1.7414	2.6002	3.7301	4.6292	6.9405
Citronella	1.249	1.918	2.947	4.337	5.466	8.434
Clove	1.050	1.845	3.240	5.370	7.287	12.872
Peppermint	1.258	2.239	3.983	6.690	9.125	16.331
Chili (hot Pepper)	0.986	1.586	2.550	3.910	5.049	8.158
Orange	1.166	1.741	2.600	3.730	4.629	6.94

Total Protein Pattern

An equivalent portion of the tested larval hemolymph was run on a 10% gel and stained for general protein using Coomassie blue R250, as shown in Figure 1 of the SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. Table 3 displays the electrophoretic patterns of oil hemolymph in treated and untreated larvae 96 hours after treatment. The information showed significant variations between hemolymph that had been treated and that had not; generally speaking, the presence of a particular number of proteins is correlated with the presence of a particular number of bands and their deep staining. The hemolymph of treated and untreated larvae showed 28-bands of the protein pattern. The molecular weights of these bands varied depending on the treated hemolymph; they varied from 7 to 275 KD in treated hemolymph and from 6 to 273 KD in untreated hemolymph.

The two main protein bands in all samples of treated and untreated larval hemolymph were 15 and 23, respectively. The largest band, number 1, had a MW range of 272 to 275 KD. It was only visible in hemolymph treated with orange oil, citronella, and untreated control; it vanished from all other treated larvae hemolymph. Band 7, which has a molecular weight of 95 KD, was present in the hemolymph that was treated but not in the untreated one. In contrast, the hemolymph treated with orange oil only showed the appearance of band 5, which has a molecular weight of 130 KD. Furthermore, the lowest molecular weight band, 28 (6 KD), was entirely eliminated from all treated hemolymph and only showed up in untreated hemolymph. Therefore, the primary cause of the interruption of the effect of essential oils on the neurological system of insects treated is the disappearance of band 28 and the appearance of band 7 in all treated insects.

DISCUSSION

Worldwide, many palm species are thought to be seriously threatened by the red palm weevil (RPW) (Hussain et al. 2013). Severe RPW infections have the potential to destroy palms valued at millions of dollars every year. The current study validates earlier research on the application of aromatic oils to insect management. It was shown that while RPW larvae treated with lesser concentrations of essential oils moved away from the treatment site and stopped feeding, they still died when fed on pieces of sugar cane treated with high concentrations of essential oils.

Our findings showed that using clove and orange oils against RPW larvae could, in certain situations, result in 100% RPW death. At 6% concentration, clove oil killed every larva after four days of exposure. Mona (2020) found similar outcomes with clove seed oil and powder, indicating that 7 mg of clove seed induced 100% death after three days of exposure and that using volatile oils was advised for red palm weevil management. However, as it demonstrated the strongest effect at 9% concentration, Ali *et al.* (2019) demonstrated the superiority of orange and lemon oils against R. ferrugineus larvae. Furthermore, even when concentration and exposure time were increased to 6% and 96 hours, respectively, citronella oil was shown to be the least effective oil among all administered

treatments utilizing the food dipping technique. Its rapid evaporation may be the reason mortality did not rise.

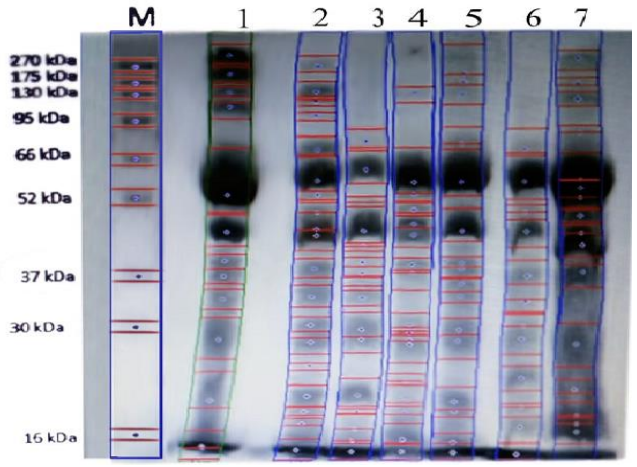

Figure 1. SDS-PAGE Stained for general protein of treated and non-treated hemolymph of red palm weevil larvae using Coomassie blue. Lane M: markers- Lane 1: Non-treaded larva Lanes 2-7 treated larvae with six essential oils (orange,clove, hot-pepper, citronella, jasmine and peppermint, respectively) after 96 hours of treatment.

The mortality rate did not surpass 33% even after a 72-hour exposure, which is consistent with the findings of Pangnakor and Chuenchookli (2018) regarding the toxicity of citronella oil on red flour beetles *Tribolium castaneum* Herbst. It is noteworthy to emphasize that, after 96 hours, only chile oil produced 90% mortality at 4% concentrations. Numerous studies have demonstrated the potency of chili oil as a pesticide; for example, Donald *et al*. (2008) noted that powdered black and red peppers are efficient in eliminating a variety of insect species.

Essential oils have a brief protection period and are contact insecticides and acaricides. They are extremely poisonous to mites, mealybugs, and scales. The ovicidal qualities are insufficient.

Essential oils mostly poison insects, mites, and their eggs because they disrupt an organism's water balance and gas exchange. This is supported by the oil's high activity and high concentration of paraffin and iso-paraffin, which can form stable films on a surface after treatment and are resistant against oxidation. Toxicants are highly pervasive and build persistent shells that block an egg's or an inset's body's ability to metabolize nutrients. Furthermore, oils easily pass through cuticles and wax scale, destroying the integuments of insets and egg shells, disrupting the structure of internal tissues, obstructing the flow of enzyme activities, and causing cytoplasm to coagulate. Thoroughly covering plant surfaces is vital to maintain the high efficiency of essential oils, particularly when fighting pests during their winter hibernation periods (Isman, 2000; Papachristos and Stamopoulos, 2004; Petrakis *et al.,* 2005; Isman *et al.,* 2008).

According to recent findings, jasmine oil at a 20% concentration impacted insect growth, decreased survival, and decreased the pupation percentage. These findings concur with those of Kubo and Klocks (1982), who discovered that the aqueous methanol extract of *Melia azadirachtz* fruits inhibited the exuviate's shedding, hence preventing the completion of larval molting. Certain insect larvae species' development was impeded by azadirachtin, which was isolated from neem and Melia seeds. The amount of energy expended by the larvae to detoxify the extract may be the cause of this growth inhibition and retardation, and as a result, a development delay was seen (Dowd *et al.,* 1983).

The study of protein patterns is thought to be a quickly developing topic that offers special insights into the innate defenses of insect pests. Determining the protein pattern of red palm weevil larvae treated with essential oils was the goal of the current investigation. It was discovered that depending on the oil used, exposure duration, and concentration, some protein bands vanished and others emerged. The presence of harmful chemicals secreted inside larvae through the cuticle or gut may be the reason why certain protein bands develop or disappear in treated insect hemolymph.

The immune system of insects differs greatly from that of mammals and responds to foreign microorganisms or chemical substances through a mix of humoral and cellular reactions. In some

Table 3. Electrophoretic analysis of treated and non-treated hemolymph of red palm weevil larvae with six essential oils (Orange, Clove, Hot-pepper, Citronella, Jasmine and Peppermint, respectively) after 96 hours of treatment

Peak no.	Marker			Untreated hemolymph			Treated hemolymph with six oils																	
							Orange oil			Clove oil			Hot-pepper oil			Citronella oil			Jasmin oil			Pepper mint		
	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf	% band	MW	Rf
1	4.40	270	0.07	5.00	273	0.08	3.51	275	0.07							6.27	272	0.07						
2																2.31	230	0.09				1.54	195	0.10
3	4.56	175	0.12	2.73	175	0.12	4.23	175	0.12				3.90	172	0.12	4.40	175	0.12						
4				4.56	150	0.15	1.57	143	0.14													3.20	140	0.13
5	6.27	130	0.17				2.00	133	0.17															
6							5.39	103	0.25	4.92	98	0.23				3.99	98	0.23	6.95	103	0.25	1.54	94	0.21
7	8.69	95	0.32				13.69	95	0.32	12.33	94	0.30	20.99	97	0.32	19.12	94	0.33	20.34	95	0.33	11.11	89	0.32
8																						8.23	83	0.34
9				23.72	79	0.35				1.85	75	0.38	2.91	71	0.35							4.49	69	0.37
10							3.01	68	0.39				3.12	70	0.39							4.56	7 1	0.41
11	9.32	66	0.43				7.01	64	0.45	16.15	66	0.43	5.07	66	0.43	12.84	66	0.43						
12				10.37	60	0.44	8.13	58	0.45				7.76	60	0.44				11.64	62	0.43	3.47	62	0.43
13										1.38	57	0.49										9.89	59	0.48
14	11.68	52	0.50	4.28	50	0.51				3.00	51	0.51	4.34	50	0.51	3.46	51	0.51						
15				4.05	47	0.55	6.26	47	0.55	2.93	49	0.54	5.64	47	0.55	3.08	48	0.54	8.63	47	0.55	8.62	52	0.50
16										2.60	39	0.58				4.19	42	0.57						
17	9.35	37	0.60	5.69	35	0.61	7.61	37	0.6	5.53	38	0.59				3.43	37	0.60						
18							4.83	34	0.66				5.08	35	0.66									
19										9.49	33	0.68	1.28	33	0.68	5.65	33	0.68						
20				12.49	32	0.71	5.36	31	0.71	6.02	31	0.71	1.95	32	0.71	8.04	30	0.73	10.54	31	0.71			
21	3.85	30	0.70										8.10	30	0.70							16.48	19	0.71
22							1.96	18	0.77				2.31		0.81				10.02	22	0.80			
23				10.51	18	0.86	7.41	17	0.85	11.36	15	0.85	6.26	17	0.86	8.17	18	0.86	1.59	17	0.86	5.15	18	0.81
24	2.90	16	0.85		0.8		2.74	15	0.89	1.79	14	0.89	3.44	14	0.89	2.54	15	0.88				5.90	17	0.88
25							2.41	12	0.92	5.20	12	0.92							10.19	13	0.91	2.90	15	0.90
26				7.29	10	0.98				5.24	10	0.98	9.70	10	0.98	4.08	11	0.99				9.02	12	0.92
27				3.90	8	0.98	10.13	10	0.98	10.20	7	0.98	8.15	9	0.98	8.45	8	0.98	9.20	9	0.99			
28				0.22	6	1.02																		

cases, the immune system of insects can be likened to that of vertebrates. In addition, it's possible that insects' brief lives, tiny size, straightforward neural systems, and, in certain situations, hard cuticles make them a special kind of organism that lacks immunological memory. Insects exposed to stressors or microbial invaders may initiate defense mechanisms. A variety of factors, including injury, bacterial injection, chemical effects, and the invasion of entomopathogenic nematodes, can alter the protein spectrum in the hemolymph and cause other related alterations in the physiology of insects. The hemolymph of these investigated species is the main focus of the majority of experimental techniques (Sheehan *et al.*, 2018).

Aminopeptidase N was discovered by Zulkifli *et al.* (2018) to be the most prevalent enzyme using the NCBI BLAST program. Based on this discovery, techniques for developing pest control could be developed, with the possibility of using anti-nutritional protease inhibitors as bio-control agents. According to Alahmadi *et al.* (2012), when larval hemolymph was analyzed using SDS-PAGE, treated larvae protein pattern bands showed both quantitative and qualitative changes in comparison to the untreated ones. This finding may have implications for the potential role of bio-agents in protein assimilation in the date palm stag beetle, *Lucanus cervusc* (L.). The modifications might result from particular protease inhibitors of the examined bioagents that influence the gut's breakdown of proteins. It is necessary to do more research on oil mixing.

Since essential oils appear to be highly effective alternatives to chemical insecticides with reduced environmental contamination and pest resistance, they can be employed as low-risk pesticides. This study demonstrates that *R. ferrugineus*, can be effectively controlled by selecting the right type, concentration, and exposure duration. To demonstrate the effectiveness of these substances in real-world settings and investigate the impact of varying oil compositions on distinct stages of larvae, more research is required.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the.

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