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RESEARCH ARTICLE

Biochemical Evaluation of Margosa Oil as an Eco-Friendly Larvicide Against the Mosquito Vector *Culex pipiens*"

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Abstract

Increased climate change and human mobility have led to the ecological expansion of highly harmful insect species. Mosquitoes have a major role in transmitting various disease agents that represent significant threats to human and animal health. Phytochemicals offer bio-safe and eco-friendly alternatives for combating disease vectors. This study investigated the effect of margosa oil extract on the proteins' biochemical components, indicating an increase in total protein values of 0.341 and 0.70 for larvae and pupa, respectively. Both groups of acid and alkaline phosphatase increased to 48.40 and 126.53 after 24 h post-treatment of the third larval instar and increased in the pupa stage with values of 11.82 and 46.66, respectively. In addition, acetylcholinesterase raised to 602.40 in the third larval instar and 297.7 in pupa stages after 48 h. of treatment. Up-and-down regulation of these enzymes in different stages and periods of treatment indicate that they disrupt metabolic homeostasis in target mosquitoes. The results confirm that margosa oil and its components have a promising effect as larvicides for mosquito vector control.

Keywords: biochemical analysis; *Culex pipiens*; *Azadirachta indica*; esterase; phosphatase.

1. Introduction

Mosquitoes transmit mosquito-borne illnesses that cause most life-threatening diseases. Mosquito diseases like yellow fever, Zika, West Nile, malaria, Dengue, Chikungunya, and Lymphatic filariasis affect human health. *Culex pipiens* species infected millions of people worldwide with lymphatic filariasis and yellow fever (Hamama *et al.*, 2022). Beside, using chemical insecticides cause resistance developing species and impact negatively on the environment, human and animal health chemical. It is important to use eco-friendly substances to control mosquitoes. Phytochemicals are promising bio-safe alternatives to control mosquito species, serving as larvicides, adulticides, and mosquito repellents (Vivekanandhan *et al.*, 2018). Azadirachtin derived from the margosa tree (*Azadirachta indica*) has anti-mosquito properties. It is considered in vector control programs worldwide due to its impact on various pests, friendly to the ecosystem. (Govindarajan *et al.*, 2016; Ayinde *et al.*, 2020). Additionally, margosa oil observed many mechanisms of action in insect physiology. These mechanisms included margosa affects immature mosquito larvae primarily due to azadirachtin, which disrupts ecdysteroid hormones responsible for molting. This interference causes abnormal molts, stunted growth, and increased mortality rates (Benelli *et al.*, 2015; Chaudhary *et al.*, 2017). It has significant larvicidal activity against mosquito vectors, including *Culex* sp., *Anopheles*, and *Aedes* sp. (Anjali *et al.*, 2012). Moreover, the chemical composition of margosa plant crude extract exhibits significant insecticidal activity against various insect species from different orders (Alhaithloul *et al.*, 2023; Kaur and Kocher

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2023). On the other side, in insects enzymes play important roles in preserving their normal physiological functions and were established as the defense against foreign compounds (Li and Liu, 2007). There are phosphatases, glutathione S-transferase, and esterase act as detoxifying enzymes that have been reacting against insecticides (Zibaee *et al.,* 2011). Hydrolytic enzymes, including alkaline phosphatases (ALP), and acid phosphatases (ACP), hydrolyze phosphate monoesters within alkaline or acidic conditions, respectively (Janda and Benesova, 1991). Cheung and Low (1975) suggest that in insect hemolymph, acid and alkaline phosphatases may function as hydrolases in the last phases of digestion. According to Dadd (1970), they are also involved in tissue cytolysis during insect development. Toxic compounds' sublethal concentrations may trigger direct sublethal impacts, such as reproduction, growth, development, genetic and morphological changes (Takada *et al.,* 2001 and Willrich and Boethel, 2001), and indirectly via physiological and biochemical changes (Croft, 1990; Sak *et al.,* 2006; Saleem *et al.,* 2013). The transaminase enzymes: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are altered under stress conditions and are involved in protein and carbohydrate metabolism (Etebari *et al.,* 2007). Conversely, acetylcholinesterase (AChE) is responsible for the excitation stage of nerve conduction in the body of an insect; therefore, inhibition of the AChE enzyme in vivo leads to the death of the insect (Ramsey *et al.,* 2010). The present study aimed to evaluate the biochemical changes in *C. pipiens* larvae and pupa after exposure to Margosa oil, investigating the impacts on protein levels and enzyme activities and assessing the potential of Margosa oil as an eco-friendly larvicidal agent.

2. Materials and methods

2.1. Mosquito rearing and treating

The *C. pipiens* larvae and pupae were reared under laboratory conditions of 27-30 ºC temperature and 80% humidity. The third larval instar and pupa were dipped in Azadictin oil at the mean lethal concentration (LC_{50}) of 10 ppm. All experiments were carried out according to Mostafa and Hashem (2022) who used five concentrations (5, 10, 20, 40, and 80 ppm) of the margosa oil for 120 h to detect the larval mortality and determine LC_{50}.

2.2. Preparation of whole-body homogenates

The live control, third larval instars, and pupae, along with the Neem oil exposed larvae and pupae, were rinsed with double-distilled water at 24- and 48-hours post-treatment. Any remaining water on the body surface was thoroughly removed by blotting with tissue paper. The larvae and pupae (10 individuals each) were homogenized separately in Eppendorf tubes with a Teflon hand homogenizer in 500 µl of 0.9% ice-cold saline, to assess esterase, total protein, and phosphatase activity.

2.3. Quantitative analysis of biochemical constituents

Determine protein concentration. Using 80% ethanol, the precipitation of the proteins in the homogenates of larvae and pupae was done, and proteins in the precipitated were measured according to Lowry *et al.* (1951). The standard used was bovine serum albumin.

2.3.1. Acetylcholinesterase and Carboxylesterase assay

The activity of acetylcholinesterase (AChE) was assessed in untreated and LC_{50}- treated 3rd instar larvae, using acetylcholine bromide (AChBr) as a substrate according to the method described by Ellman *et al.* (1961). AChE catalyzed the hydrolysis of AChBr, and the resulting enzymatic reaction was detected at a wavelength of 515nm. Van Asperen (1962) method was employed to determine the activity of α and β carboxylesterase in the pupae and larvae. Using α-naphthyl acetate and β-naphthyl acetate as substrate, respectively. Naphthol produced as a result of hydrolysis of

substrate can be identified by the addition of diazoblue sodium lauryl sulphate solution to enzyme resource which producing a strong blue purple color in the case of α-naphthol or a strong red purple color in the case of β-naphthol at which colors are measured spectrophorometrically at an absorbency of 600 and 555 nm for α-naphthol and of β-nophthol.

2.3.2. Acid, alkaline phosphatase, GOT and GPT assays

The method of Asakura (1978) was utilized to measure the levels of acid and alkaline phosphatases in the homogenates of larval and pupal homogenates, with minor modifications. In this method, the phenol released by enzymatic hydrolysis of disodium phenylphosphate reacts with 4-aminoantipyrine, and by the addition of potassium ferricyanide, the characteristic brown color is produced. The produced color was measured immediately at 510 nm. The enzyme activity is expressed by unit (U), where 1 unit hydrolyze 1.0 µ mole of p-nitrophenyl phosphate per minute. Additionally, GOT and GPT were determined colorimetrically according to the method of (Reitman and Frankle, 1957).

2.4. Statistical analysis

Using SPSS version 14 software, Student's t-test was employed for the statistical analysis of the experimental data. In all instances, statistical significance was $p \leq 0.05$, and the findings are presented as mean ± standard deviation (SD) of three replicates.

3. Results and discussion

It is known that azadirachtin has damaged effects on insect physiology; especially by interacting with main growth and metabolic processes (Chatterjee *et al.,* 2023). Changes in the biochemical constituents of *C. pipiens* third instar larvae treated with a margosa oil formulation were investigated using the mean lethal concentration (LC_{50}) (Mostafa and Hashem 2022). The reduction in biochemical components suggests that these extracts can enhance digestion and decrease feeding. Also, they additionally disrupt protein synthesis hormones, leading to a reduction in their levels. Conversely, the rise in specific profiles indicates the physiological stress caused by the extract and the disturbed larvae's metabolic activity. The impact of extracts on the metabolism of treated larvae is influenced by their nature and potentially on the action of various phytochemicals in these extracts. Moreover, proteins are crucial in insects, not only for specific transport functions but also for their enzymatic activities. Increased protein concentration in the tissue suggests heightened metabolic activity and defense reactions (Rajitha and Savithri, 2013). Results represented in Table (1) indicated that the total soluble protein amount in the treated third larvae with margosa oil elevated at 24h after application then, decreased at 48h after application which agree with Ahmed *et al.* (2023) who documented a significant reduction in both protein level and enzymatic activity, noted in treated larvae *C. pipiens* with margosa oil in all related studies. Furthermore, the interaction of azadirachtin with cellular pathways responsible for protein synthesis can prevent the proper assembly of amino acids into proteins, preventing larval growth and development and ultimately larval death (Chatterjee *et al.*, 2023). Also, the result data was showed decrease in total protein of pupa stage. Amylase enzyme is crucial in the first step of maltopolysaccharide digestion. The activity of amylase enzymes decreases at 24h and 48h after application. This outcome is in alignment with Mehrabadi *et al.* (2011), who reported that plant extracts demonstrated inhibitory activity against insect α-amylases, with inhibition levels from approximately 4% to 95%. Jbilou *et al.* (2008) discovered that larvae of *Tribolium castaneum* fed a diet treated with methanol extracts from seven plant species exhibited lower α-amylase activity compared to larvae fed an untreated diet. However, the activity of the amylase enzyme increased in the pupal stage when treated with margosa oil. In the central nervous system,

acetylcholine is hydrolyzed at cholinergic synapses by acetylcholinesterase (AChE). Inhibiting the enzyme in insects can cause rapid mortality; therefore, acetylcholinesterase has been targeted in the development of insecticides (Lushchak, 2018). The acetylcholinesterase activity decreased at 24 hours after application and then increased at 48 hours after application (Table 2). The results indicated a decrease in acetylcholinesterase activity at 24 hours, suggesting that the insects were attempting to counteract the effect, as evidenced by the increase in acetylcholinesterase concentration at 48 hours after application.

Table 1. Influence of margosa oil on the total protein and the activity of amylase of *C. pipiens* 3rd larval instars and pupa.

Biochemical analysis	Larvae (Mean ± S.D)			Pupa (Mean ± S.D)	
	Control	24h	48h	Control	Treatment
Total protein (mg g^{-1})	0.115±0.546	1.796±1.26	0.341±0.182	0.136±0.001	0.70±0.466
Amylase (U g^{-1})	6.255±1.15	3.621±1.42	2.342±0.82	3.09±1.030	8.22±1.39

Table 2. Effect of margosa oil on Acetylcholinestrase, α-Carboxylestrase and β- Carboxylestrase of *C. pipiens* 3rd larval instars and pupa.

Biochemical analysis	3rd Larvae (Mean ± S.D)				Pupa (Mean ± S.D)	
	Control	24h	48h	4th	Control	Treatment
Acetylcholinestrase (U g^{-1})	408.23±6.109	156.07±9.16	602.40±0.154	150.57±2.38	167.7±2.98	297.7±1.09
α-Carboxylestrase (mg phenol per min per g)	0.926±0.82	1.958±1.09	0.750±0.046	1.63±0.60	0.695±0.39	1.94±0.224
β- Carboxylestrase (mg phenol per min per g)	394.08±8.4	323.17±5.05	168.72±4.97	204.81±4.41	218.4±0.36	199.9±1.90

**% of D: percent of increase or decrease of control = (ControlTreatment)/Control * 100.*

In this study, the concentration of acid phosphatase and alkaline phosophatase was increased at 24h and 72h after application, but the concentration of acid phosphatase and alkaline phosophatase decreased at 48h after application (Table 3). These outcomes are in alignment with those of (Hamadah *et al.*, 2016), who observed a rise in the concentration of alkaline and acid phosphatase after infecting larvae of *Spodoptera littoralis* with chitin synthesis inhibitors. Additionally, Mostafa (1993) documented a notable decrease in acid phosphatase activity following the treatment of 4th and 6th instar larvae of the cotton leafworm *Spodoptera littoralis* with the neem formulation Margason-O. Various plant extracts have been identified as inhibitors of acid phosphatase activity in different insects, like *S. littoralis* (Ayyangar and Rao, 1990) and Azadirachtin (Azt.) against the house fly *Musca domestica* (Saeed *et al.*, 1987). In accordance with these findings, Koodalingam *et al.* (2014) and LijaEscaline *et al.* (2015) noted that plant extracts and their derivatives have significantly reduced carboxylesterase (α- β-carboxylesterase) levels in *Aedes aegypti* larvae. Thanigaivel *et al.* (2017a) reported that in addition to its larvicidal activity, *Alangium salvifolium* significantly lowered the levels of superoxide dismutase (SOD) and α, β-carboxylesterase in *A. aegypti*. *Myrrh commiphora molmol* (oil and oleo-resin extract) induced biochemical alterations in *C. pipiens*, leading to decreased in enzyme activity and changes in cellular proteins (Massoud *et al.*, 2001). Conversely, the enzyme phosphatase is utilized as a marker to assess the physiological status of test pests in response to varoius toxicants and plays a role in various physiological functions (Eguchi, 1995; Srinivas *et al.*, 2004). On the other side, Glutamate- Pyruvate Transaminase (GPT) and Glutamate

Oxaloacetate Transaminases (GOT) are involved in energy production. GPT and GOT act as a strategic link among protein and carbohydrate metabolism and are recognized to be altered in different pathological and physiological circumstances (Ellman *et al.,* 1961). The latter authors reported that changes in transaminase levels are associated with protein anabolism or catabolism. Activities of GOT and GPT may directly influence protein synthesis (Mordue and Goldworthy, 1973). Vidhya *et al.* (2016) reported that the GPT enzyme showed the most significant positive change, which indicated that the change might result from reversible binding among pesticides and enzymatic site of action on the surface of the enzyme. GOT and GPT activities in *S. litura* larvae were significantly increased at 48 hrs with *Metarhizium anisopliae* and *Beauveria bassiana* treated relative to the control larvae.

Table 3. Effect of margosa oil on Acid phosphatase, Alkaline phosphase, GOT and GPT on the 3rd larval instars and pupa of *C. pipiens*.

Biochemical analysis	3rd Larvae (Mean ± S.D)				Pupa (Mean ± S.D)	
	Control	24h	48h	4th	Control	Treatment
Acid phosphatase (U g^{-1})	9.73±2.109	48.40±1.16	15.31 ± 1.25	53.10±0.38	34±0.56	11.82±1.89
Alkaline phosophotase (U g^{-1})	62.62±0.70	126.53±2.09	88.89±1.04	452.49±2.60	11.82±1.43	46.66±2.03
GOT mg pyruvate per min per g	1.37±0.12	1.92±0.52	1.34±0.04	2.22±1.32	0.445±0.35	2.58±0.662
GPT mg pyruvate per min per g	3.45±2.12	7.66±1.02	8.25±0.12	57.10±0.844	5.78±1.23	13.6±1.72

Consistent with these findings, researchers (Al-Dali, 2007; Younes *et al.,* 2011) observed that certain plant oils inhibit ACP and ALP in *Euprepocnemis plorans* nymphs and *Trogoderma ranarium* larvae, respectively. Additionally, Mostafa (1993) reported a substantial decrease in the activity of the ACP following treatment of 4th and 6th instar larvae of the cotton leafworm, *S. littoralis*, with the neem formulation Margason-O. Various plant extracts have been identified as inhibitors of ACP activity in different insects. For example, *Spodoptera littoralis* (Ayyangar and Rao, 1990) and Azadirachtin (Azt.) has been effective against the house fly *Musca domestica* (Saeed *et al.,* 1987). Additionally, Margosan-O and Jojoba oil have shown inhibitory effects against *M. domestica* (Ghoneim *et al.,* 2008). Regarding the effect of time, variations in total lipid and protein levels were observed at different intervals. Similar outcomes were documented by Khosravi and Sendi (2013), who noted comparable effects of neem pesticide (Achook) on lipid and protein levels in the hemolymph of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker. Additionally, Huang *et al.* (2004) discovered that azadirachtin markedly affected protein levels in *Spodoptera litura*. A study by Annandurai and Rembold (1993) indicated that neem oil interfered with protein synthesis in the desert locust.

4. Conclusion

This work explores safe alternative methods, such as margosa oil, for suppressing mosquito vectors. The margosa oil formulation exhibited significant larvicidal and pupicidal activity against *C. pipiens* third larval instars and pupae. It reduced biochemical activities, causing an imbalance in enzyme activities, which confirms the effectiveness of using eco-friendly plant extract oils and their constituent compounds in controlling *C. pipiens* mosquitoes.

Authors contributions

WAM raising the idea and performed the biological tests. FMH supervised the biological tests and wrote the manuscript. WAM and FMH reviewed the

manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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