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Phytochemical Investigation of the Neem Oil and Its Larvicidal Activity Against the Mosquito Vector *Culex pipiens* (L.)

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ABSTRACT

Mosquitoes play a pivotal role in transmitting various disease agents that pose significant threats to public health. The extensive use of chemical pesticides to control mosquitos has resulted in physiological vector resistance besides negative environmental consequences. Phytochemicals represent bio- safe and eco- friendly alternatives for controlling disease vectors. This study aimed to assess the toxicity of a neem oil formulation (safe oil) against *Culex pipiens* (Diptera: Culicidae) third instar larvae. Mortality was recorded 24 hours post-treatment with four different concentrations of safe oil (0.028, 0.125, 0.25, 0.5ppm). A significant increase in larval mortality was observed with rising concentrations, reaching a median lethal concentration (LC_{50}) of 0.112ppm. In addition, the study investigated the biochemical impact of safe oil on total protein levels and the activities of detoxifying enzymes, acetylcholinesterase, and glutathione S-transferase in *Cx. pipiens* 3rd instar larvae, 24 hours post-treatment with the LC_{50} concentration. Treated larvae exhibited significantly reduced levels and activities across all assessed parameters compared to untreated controls. Histopathological examination of the larval midgut region treated with LC_{50} revealed deformities in epithelial cells, detachment from the basement membrane, and disintegration of nuclei, forming irregular blebs protruding into the lumen. GC-MS analysis of the oil revealed the presence of 38 components with varying concentrations (0.18-8.23%). The neem oil formulation, specifically the chemical constituents present in it, was docked into the active pockets of GST and AChE receptors, providing additional support for the reported activities. These findings imply that safe oil and its components have a promising effect as larvicides for mosquito vector control. Additionally, this study is considered the first study that revealed the molecular docking of one of the neem oil formulations (safe oil).

INTRODUCTION

Mosquito- borne diseases including malaria, filariasis, yellow fever, and other serious human diseases cause significant morbidity and mortality rates annually, leading to substantial economic burdens on regions endemic to these diseases **(An *et al*., 2020;**

Hamama *et al.*, 2022). For many years, synthetic organic chemical insecticides have been effectively used for reducing disease vector populations. However, the widespread use of chemical pesticides for mosquito control has resulted in issues related to physiological vector resistance, in addition to detrimental effects on the environment and human health **(Benelli *et al.*, 2017)**.

Several plant- based products exhibit insecticidal effects and are approved as a safe method for mosquito control, serving as both larvicides and adulticides, as well as mosquito repellents **(Prabhu *et al.*, 2011; Vivekanandhan *et al.*, 2018)**. Azadirachtin derived from the Indian neem tree (*Azadirachta indica* A. Juss, Meliaceae), is known for its anti- mosquito properties and is highly valued in vector control programs across the world due to its effectiveness against a large variety of pests, little influence on the ecosystem, and limited chance of resistance development **(Al-Mehmadi & Al-Khalaf, 2010; Singh *et al.*, 2011; Govindarajan *et al.*, 2016; Ayinde *et al.*, 2020)**. Along with other constituents in neem- based formulations, azadirachtin displays diverse mechanisms of action in relation to insect physiology. These mechanisms encompass antifeedant effects, modulation of growth processes, reduction of fecundity, induction of sterilization, alteration of oviposition behavior, impact on overall biological fitness, and inhibition of the development of vector- borne pathogens (**Mulla & Su, 1999; Dua *et al.*, 2009; Benelli *et al.*, 2015)**. Several fatty acids, including oleic acid (50– 60%), palmitic acid (13– 15%), stearic acid (14– 19%), linoleic acid (8– 16%), and arachidic acid (1–3 %), are the main components of the brownish– yellow oil that makes up the majority of the neem seed kernel. The fat components present in the formulation of neem oil or neem cake contribute to the preservation of the limonoids and, consequently, their bioactivity **(Benelli *et al.*, 2017)**. Neem oil- based insecticides are typically effective against various important insects affecting human and animal health including mosquitoes **(Nicoletti *et al.*, 2016)**. It has been found to have powerful larvicidal activity in the field against several different mosquito species, including *Aedes* sp., *Anopheles* and *Culex* sp. **(Dua *et al.*, 2009; Anjali *et al.*, 2012)**.

Insects' midguts are crucial for digestive enzyme secretion and nutrient absorption (**Christophers, 1960)**. Allelochemicals have been shown to negatively affect the digestive epithelial cells and reduce an insect's ability to survive. It has been demonstrated that mosquito larvae exposed to plant extracts, particularly *Melia azedarach*, *Derris urucu*, and *Capparis cartilaginea*, suffer significant damage **(Gusmo *et al.*, 2002; Al-Mehmadi & Al-Khalaf, 2010; Abutaha & Al-Mekhlafi, 2014)**. According to **Mordue (1993)** and **Nasiruddin and Koul (1996)**, neem oil formulations can directly impair the production of enzymes and the absorption of nutrients by causing necrosis of gut tissues.

Since mosquitoes breed in aquatic environments, it is relatively easy to control them in this habitat. The larval stages of mosquitoes are thus desirable targets for insecticides. Furthermore, the cuticle defence system of the larvae is weakened by the

neem's ability to regulate insect growth, which makes it simpler for pathogenic organisms to enter the insect system. The objective of this study was to assess the potential toxicity of neem oil formulation (safe oil) to *Cx. pipiens* 3rd instar larvae. The metabolic enzymes of *Cx. pipiens* larvae were studied in relation to modifications in the activity of acetylcholinesterase (AChE) and glutathione S- transferase (GST) following exposure to neem oil. Additionally, the histological damage of the midgut of the treated larvae was studied to evaluate its histopathological effects. Analysis and examination of the phytochemical profile using GC-MS were conducted to provide insights into the bioactive components of neem oil that may be responsible for biological activity. Along with the molecular docking of the main ingredients, this study correlated neem oil activity.

MATERIALS AND METHODS

1. Mosquito rearing

The *Cx. pipiens* 3rd larval instar was obtained from laboratory colony established in the insectary of the Entomology Department, Faculty of Science, Ain-Shams University, Cairo, Egypt, following the methods described by **Kauffman *et al.* (2017)**. Larvae were maintained at a temperature of 26.5± 1°C, with a relative humidity of 70-80%, and a photoperiod of 16/ 8 hours light/ dark.

2. Larvicidal bioassay

Neem oil formulation (safe oil) containing azadirachtin at a concentration of 0.03% was provided by the Egyptian Ministry of Agriculture in Dokki. Various concentrations of safe oil (0.028,0.125, 0.25, and 0.5ppm) were prepared with distilled water and tested against 3rd larval instar of the *Cx. pipiens* according to the guidelines of **WHO (2005)**. Twenty- five larvae were transferred to 100ml of each concentration and a control group with only distilled water. The experiment was replicated three times. Mortality was recorded 24 hours post- treatment, and the median lethal concentration (LC_{50}) was calculated using probit analysis **(Finney, 1971)**.

3. Biochemical analysis

Untreated and treated third instar larvae, 24- hour post- treatment with LC_{50} of safe oil formulation, were gently washed with distilled water, and then homogenized in phosphate buffer saline (50mg/ 1ml) using a cold glass Teflon tissue homogenizer. The homogenized samples were then centrifuged in a refrigerated centrifuge at 8000rpm for 15 minutes. The resulting supernatants were stored at -20°C for further biochemical analysis. Three replicates were carried out for each biochemical determination.

Similar to insects, mosquitoes can detoxify a variety of invasive pesticides by producing numerous detoxifying enzymes including esterase, oxidase and reductases. In this study, we investigated the activity of two different enzymes, acetylcholinesterase (AChE) and glutathione S- transferase (GST**)**.

3.1. Total protein estimation

The total protein level of untreated and LC_{50}- treated third instar larvae was estimated according to **Bradford (1976)** using a Coomassie Brilliant Blue solution dissolved in 95% ethanol and compared to the untreated control. Briefly, 5mL of Bradford reagent was added to 100µL of sample, and the absorbance was measured at 595nm.

3.2. Acetylcholinesterase (AChE) activity 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 to the method described by **Simpson *et al.* (1964)**. AChE catalyzed the hydrolysis of AChBr, and the resulting enzymatic reaction was detected at a wavelength of 515nm.

3.3. Glutathione S- transferase (GST) activity assay

The activity of glutathione S- transferase of untreated and LC_{50}- treated 3rd instar larvae was measured using the bio- diagnostic glutathione S- transferase assay kit, which quantifies the conjugation of 1- chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione to assess overall GST activity (cytosolic and microsomal) according to the method described by **Habig *et al*. (1974)**. The conjugation is accompanied by an increase in absorbance at 340nm, and the rate of increase is directly proportional to the GST activity in the sample**.**

4. Histopathological studies

In order to assess the effect of neem oil, LC50- treated larvae and control ones were histopathologically investigated.

The larvae were fixed in 0.1M phosphate buffer (pH 7.3) containing 2.5% glutaraldehyde and 4% paraformaldehyde. They were further fixed in a 1% osmium tetroxide solution and dehydrated through a series of acetone solutions according to the method followed by **Disbrey and Rock (1970)**. The specimens were then embedded in epoxy resin (epoan). Semi- thin sections were cut using an EM KMR2 Leica ultra-microtome, stained with toluidine blue, and subsequently inspected using a light microscope.

5. GC– MS analysis of the oil

The neem oil formulation (safe oil) was evaluated for its chemical composition at the Atomic and Molecular Physics Unit, Experimental Nuclear Physics Department, Nuclear Research Center, the Egyptian Atomic Energy Authority, Inshas, Cairo, Egypt, using a TGC- TSQ trace Mass Spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TR- 5MS (30m, 0.25m, and 0.25m film thickness). The temperature of the column oven was first maintained at 60°C, then increased by 5°C/ min to 200°C with a holding period of 2min, and then increased by 10°C/ min to 300°C. The injector's temperature was maintained at 270°C. The flow rate of the carrier gas, helium,

was maintained at 1mL/ min. Auto- sampler AS3000 paired with GC in split mode automatically injected diluted samples of 1L with a 2min. solvent delay. Full- scan EI mass spectra covering the m/ z range of 50– 750 were collected at 70eV ionization voltages. Temperature controls were placed at 200 and 250°C for the ion source and transfer lines, respectively. The elements were recognized by contrasting their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral databases.

6. Molecular docking

The 3D crystalline structures of the proteins AChE (PDB ID: 6XYU) and GST (PDB ID: 1M0U) were downloaded from the protein data bank website (www.rcsb.org). The tested substance was docked in the active pockets of the GST and AChE receptors **(Nachon *et al.*, 2020)**. Discovery Studio 2.5.5 was used to perform docking calculations, and Discovery Studio Visualizer 2016 was used to display the results. Additionaly, the prepare protein methodology was used to prepare the proteins. This was done in order to correct the usual issues with the input protein, such as standardizing atom names, adding missing atoms to residues, introducing loops where they weren't previosly existing, protonating the protein, and using forcefield (CHARMm). Furthermoer, the default settings were chosen. The co- crystallized ligand's active pocket was where docking took place. Ligands were prepared using prepare ligand protocol to add hydrogens, enumerate ionization states, generate tautomers and isomers, remove duplicates, fix bad valencies and generate 3D coordinates. CDOCKER was chosen as a docking algorithm. The best - CDOCKER- scoring poses were chosen. The co- crystallized ligand redocked for docking validation, and the RMSD for AChE and GST, respectively, was 1.3913 and 1.8194.

7. Statistical analysis

The difference between the mean percent mortality of different concentrations of neem oil was statistically calculated using one- way ANOVA in SPSS software (version 26). Lethal concentrations (LC_{50} and LC_{95}) values were determined at 95% confidence limits using probit analysis with the LDP line software. Significance levels between control and treated larvae for the biochemical analyses were determined using Student's t-test with SPSS software (version 26).

RESULTS

1. Larvicidal bioassay

The effects of a neem oil formulation (safe oil) on third larval instar *Cx. pipiens* were assessed after 24 hours of exposure to varying concentrations ranging from 0.028 to 0.5ppm (Table 1 & Fig. 1). The results revealed a notable larvicidal activity of safe oil against *Cx. pipiens* larvae, with mortality rates ranging from 13.33 to 98.66%. A Significant larval mortality was observed by increasing the concentrations ($P< 0.05$). The calculated LC_{50} and LC_{95} values for safe oil were 0.112 and 0.606ppm, respectively.

Table 1. Larvicidal activity of neem oil formulation (safe oil) against 3rd larval instar of *Cx .pipiens* 24- h post-treatment

Conc. ppm	**% Mortality mean±S.E**	**LC50**	**LC95**	***Slope**± S.E	*χ2
0.028	13.33± 3.33[a]	0.112	0.603	2.23±0.24	7.32
0.125	43.33± 3.33[b]				
0.25	76.66± 1.66[c]				
0.5	98.66± 0.66[d]				

Means with different letters are significantly different at $P< 0.05$.
*Slope of the concentration- inhibition regression line ± standard error, *(χ2) Chi- square value. LC values = Lethal concentration value at 95% confidence limits.

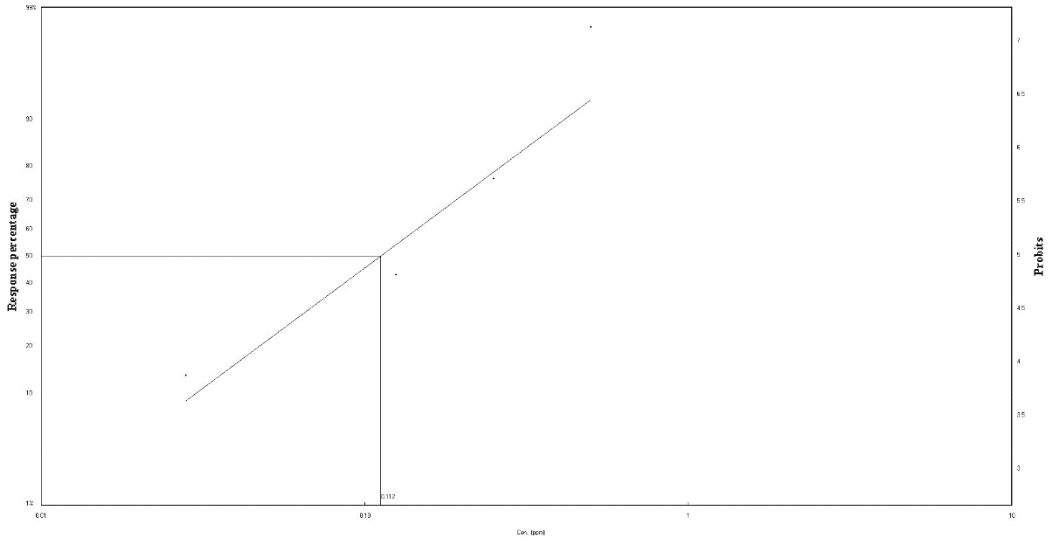

Fig. 1. Probability analysis of mortality of *Cx .pipiens* mosquito larvae by safe oil

2. Biochemical analysis

Table (2) shows the biochemical effects of neem oil formulation (safe oil) on the total protein level, AChE, and GST activity of the third larval instar *Cx. pipiens* larvae at 24 hours post- treatment with the LC_{50}. A significant decrease in both protein level and enzymatic activity was observed in treated larvae when compared to untreated ones across all the measured parameters ($P< 0.05$).

Table 2. Effect of safe oil on the total protein level and the activity of acetylcholine esterase and glutathione S- transferases of Cx. *pipiens* 3rd larval instar

Group	Total protein body wt.).(mg/ g Mean ± S. E	Acetylcholine esterase (ug AchBr/ min/ g. body weight) Mean ± S. E	Glutathione S-transferases GST (U/g tissue) Mean ± S. E
Control	21.64 ± 0.48[a]	1337.37 ± 6.85[a]	44.65 ± 0.95[a]
Treated	10.43 ± 0.61[b]	282.90 ± 43.22[b]	16.34 ± 0.52[b]

* Means with different letters in the same column are statistically different ($P< 0.05$).

3. Histopathological studies

Fig. (2a) represents the semithin sections of the midgut region in untreated third-instar *Cx. pipiens* larvae, revealing a midgut with an intact single layer of columnar epithelial cells with centric nuclei, resting on an intact basement membrane. The peritrophic membrane was attached to the epithelial cell, and the border membrane of the gut lumen appeared distinct.

Fig. (2b) demonstrates the histopathological effects of safe oil on the midgut of the third larval instar of *Cx. pipiens*. The midgut epithelial cells appeared deformed, losing their normal appearance, detaching from the basement membrane, with disintegration of their nuclei. Epithelial cell destruction was observed in some regions, along with damage to the peritrophic membrane, and irregular bleb formation protruding into the gut lumen.

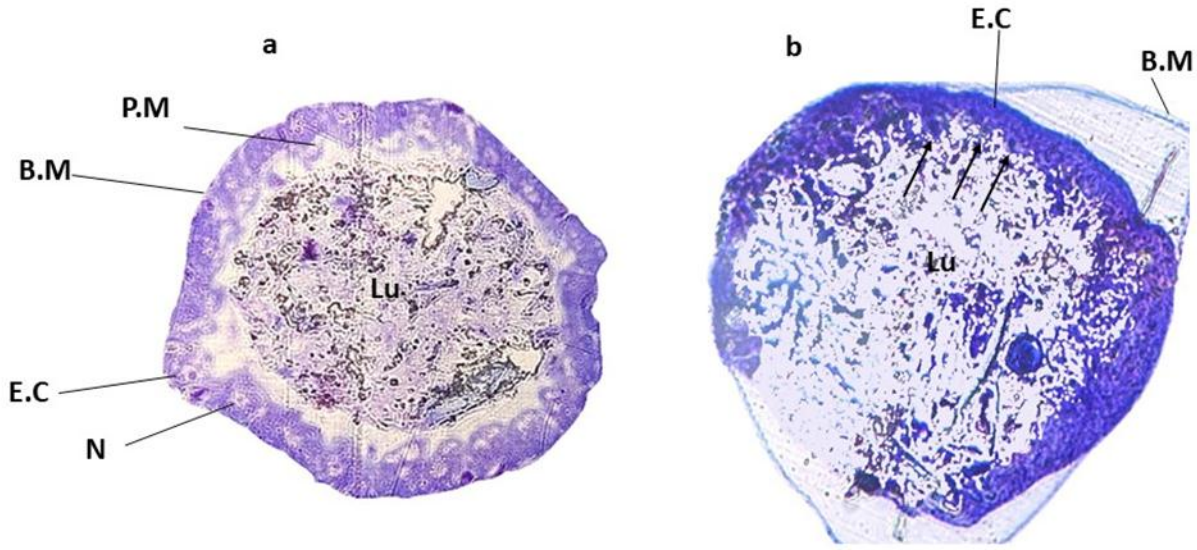

Fig. 2. Semi- thin sections in 3rd larval instar of *Cx .pipiens* midgut showing: **(a)** Untreated control showing normal epithelial cell (E.C), with centric nucleus (N), intact peritrophic membrane (P.M), basement membrane (B.M), gut lumen (Lu), and **(b)** The midgut of treated 3rd instar larva with neem oil formulation (safe oil), the midgut deformed and lost its normal appearance, with disintegrated nuclei and a detached basement membrane. Irregular bleb formation, protruding in the gut lumen (arrows)

4. GC/ MS analysis of oil

The chemical composition of the safe oil, comprising a total of 38 compounds with varying concentrations ranging from 0.18 to 8.23%, is displayed in Table (3) & Figs.(3, 4). Naphthalene, 1, 7- dimethyl, Naphthalene, 2, 3, 6- trimethyl, Naphthalene, 2-methyl , Naphthalene, 1, 8- dimethyl, 2, 2', 5, 5'- tetrachloro- 1, 1biphenyl, Methane, di-p- tolyl , Octadecenoic acid, methyl ester, and vitamin (E) were the predominant chemical components.

Table 3. Chemical constituents and composition (%) of safe oil

Peak	R.t*	Name	Area %	Molecular weight	Molecular formula
1	6.56	o- Cymene	0.18	134	C10H14
2	7.40	Benzene, 1, 2, 4, 5- tetramethyl-	1.29	134	C10H14
3	8.15	Benzene, 1, 2, 3, 4 -tetramethyl-	0.53	134	C10H14
4	9.04	Azulene	1.70	128	C10H8

5	9.29	Benzene, pentamethyl-	2.20	148	C11H16
6	10.64	1H- Indene, 2, 3- dihydro- 4,7- dimethyl-	0.85	146	C11H14
7	11.86	Naphthalene, 2- methyl-	8.20	142	C11H10
8	14.23	BENZENE, (1- ETHYL- 1- METHYL- 2- PROPEN YL)-	0.18	160	C12H16
9	14.67	Naphthalene, 1, 7- dimethyl-	5.49	156	C12H12
10	14.96	Naphthalene, 1, 8- dimethyl-	8.23	156	C12H12
11	15.25	1, 1'-Biphenyl, 2- methyl-	1.40	168	C13H12
12	15.45	Naphthalene, 1, 3- dimethyl-	1.59	156	C12H12
13	15.78	1,4-DIMETHYLNAPHTHALENE	0.91	156	C12H12
14	17.04	Naphthalene, 1,4,5-trimethyl-	0.93	170	C13H14
15	17.54	1,1'-Biphenyl, 2,4'-dimethyl-	9.55	182	C14H14
16	17.65	Naphthalene, 1,6,7-trimethyl-	1.54	170	C13H14
17	17.85	Benzene, 1-methyl-3- (phenylmethyl)-	0.54	182	C14H14
18	17.97	Naphthalene, 2,3,6-trimethyl-	4.75	170	C13H14
19	19.66	Methane, di-p-tolyl-	7.05	196	C15H16
20	20.79	1,4,5,8-Tetramethylnaphthalene	0.88	184	C14H16
21	21.60	1,1'-Biphenyl, 2,2',5,5'-tetramethyl-	9.20	210	C16H18
22	22.34	2-(p-Tolylmethyl)-p-xylene	0.53	210	C16H18
23	22.81	1,5,6,7-Tetramethyl-3- phenylbicyclo [3.2.0]hepta-2,6-diene	3.80	224	C17H20
24	23.6	Benzene, 1,1'-ethylidenebis[4-ethyl-	2.32	238	C18H22
25	24.02	Tricyclo[8.4.1.1(3,8)]hexadeca- 3,5,7 ,10,12,14-hexaen-2-one, anti-	1.32	222	C16H14O
26	25.06	4H-1-BENZOTHIOPYRAN-4- ONE, 5,8-DIMETHOXY-	1.00	222	C11H10O3S

27	26.22	Palmitic Acid methyl ester	1.80	270	C17H34O2
28	27.8	2-Cyclohexen-1-one, 4,4-diphenyl-	0.88	248	C18H16O
29	28.69	Linoleic acid ethyl ester	0.58	308	C20H36O2
30	29.36	8,11-Octadecadienoic acid, methyl Ester	1.67	294	C19H34O2
31	29.50	9-Octadecenoic acid, methyl ester, (E)-	5.08	296	C19H36O2
32	30.01	Methyl stearate	1.13	298	C19H38O2
33	31.07	Hexadecanoic acid, butyl ester	2.72	312	C20H40O2
34	33.87	n-Propyl 9,12-octadecadienoate	1.22	322	C21H38O2
35	33.99	n-Propyl 9-octadecenoate	1.27	324	C21H40O2
36	34.46	Octadecanoic acid, butyl ester	0.54	340	C22H44O2
37	40.54	Squalene	2.01	410	C30H50
38	43.91	Vitamin E	3.25	430	C29H50O2

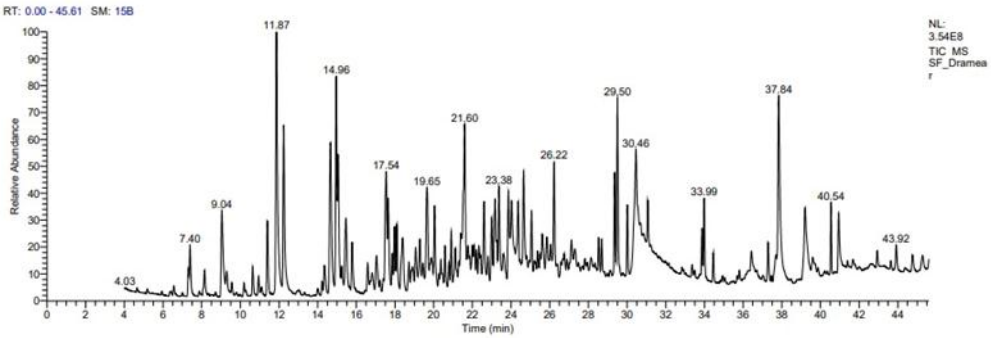

Fig. 3. GC chromatogram of all compounds produced from GC-MS analysis of safe oil

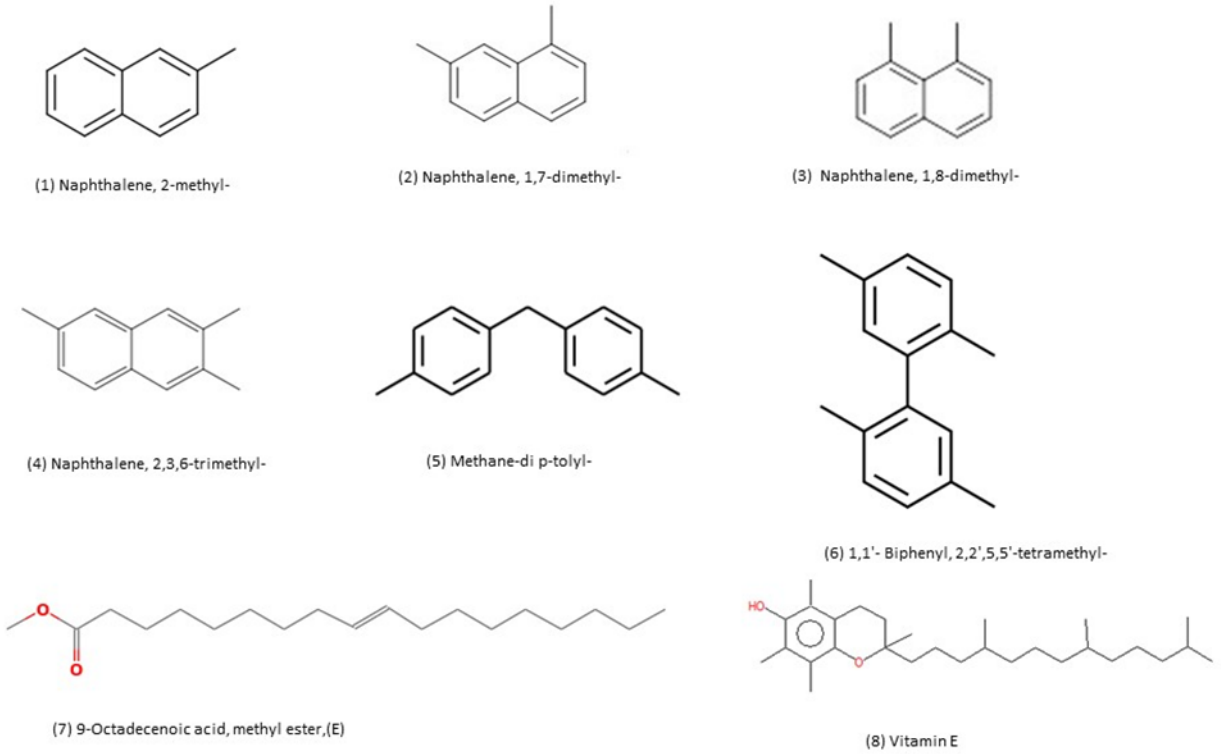

Fig. 4. Structures of major compounds identified in safe oil

5. Binding model analysis

5.1. Binding mode with AChE enzyme

The main compounds in safe oil were docked into the active pocket of AChE (PDB ID: 6XYU). 10 poses were formed for each compound, and the pose with the highest -CDOCKER energy score was selected. This would give us a look over the binding nature of the compounds, and an idea about their mode of action. Data are presented in Table (4) and Figs (5, 6). Methyl 9- octadecenoate showed the highest -CDOCKER energy score (35.4203). This might be attributed to the conventional hydrogen bond between the ester group and GLY150, GLY151 and ALA239 and pi-alkyl interaction with TRP83 and TRP472 and alkyl interaction with LEU479. The di-p-tolylmethane had the second highest score (30.471). One of its phenyl rings formed pi-donor hydrogen bond with TYR370. Moreover, this phenyl ring forms pi- pi interaction

with TYR71 and TYR374. The other phenyl ring forms pi- pi interaction with TRP83. Its two methyl groups forms alkyl interactions. Vitamin E and 2, 3, 6- trimethylnaphthalene show scores of 19.997 and 18.1083, respectively, without forming any hydrogen bonds. 2, 2', 5, 5'- tetrachloro- 1, 1'- biphenyl and 1, 7- dimethylnaphthalene and 2- methylnaphthalene scores were 16.1662, 12.0517,10.9371, respectively. They form interactions with TYR71, TRP83 and TYR370. 2, 2', 5, 5'- tetrachloro- 1,1'-biphenyl forms additional interaction with TYR374. 1, 8- dimethylnaphthalene showed the least score of 4.42697. It formed no hydrogen bonds. It can be concluded that amino acids TYR71, TRP83, TYR370 and TYR374 are significant in the active pocket. Scores show that these compounds contribute to the activity against AChE enzyme.

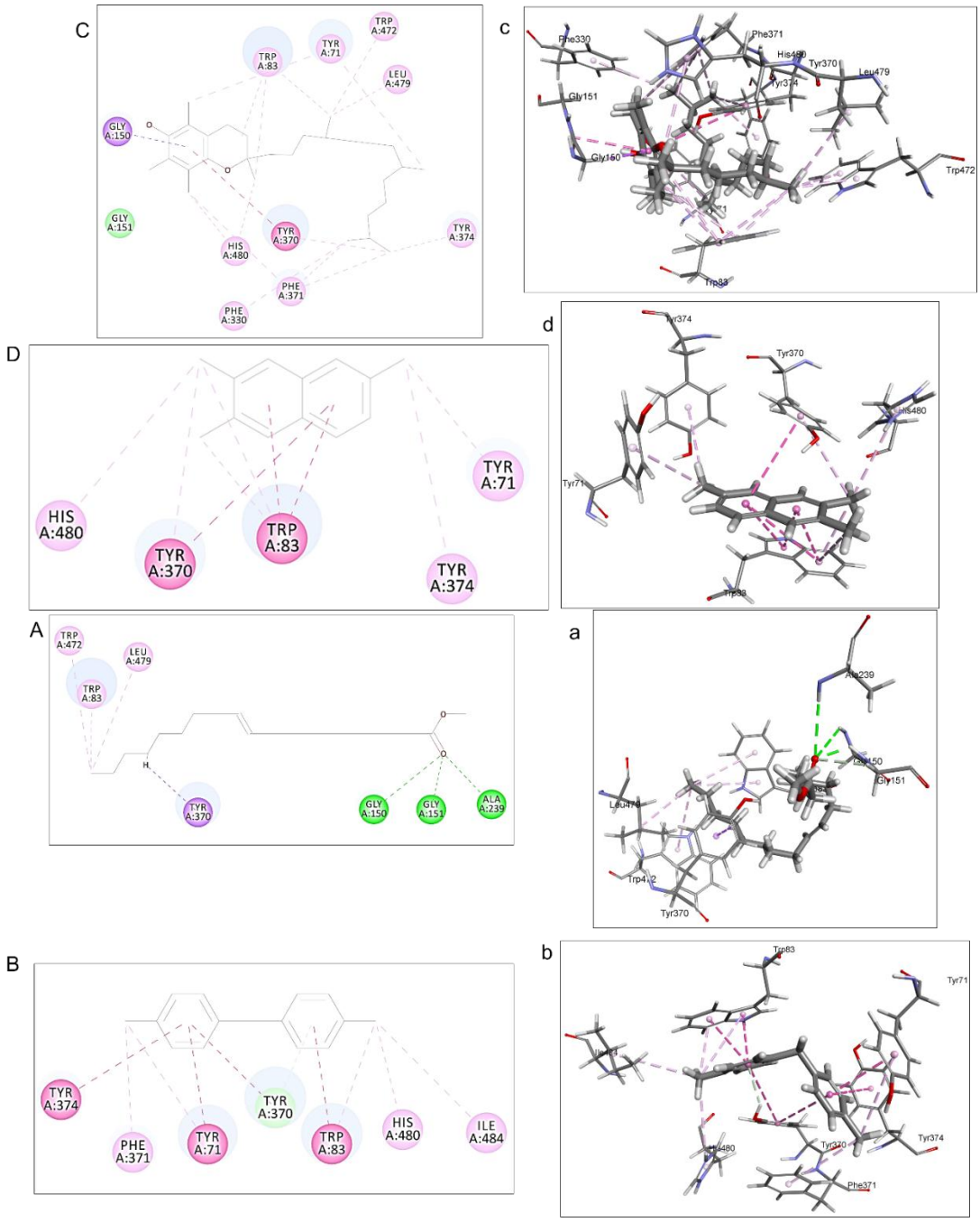

Fig. 5A– **D.** 2D- binding interaction profile for 9-octadecenoic acid, methyl ester, methane, di- p-tolyl, vitamin E, and 2, 3, 6- trimethyl naphthalene, respectively, with the active pocket of the AchE enzyme. **a**– **d** shows in-depth 3D ligand- AchE interaction mode

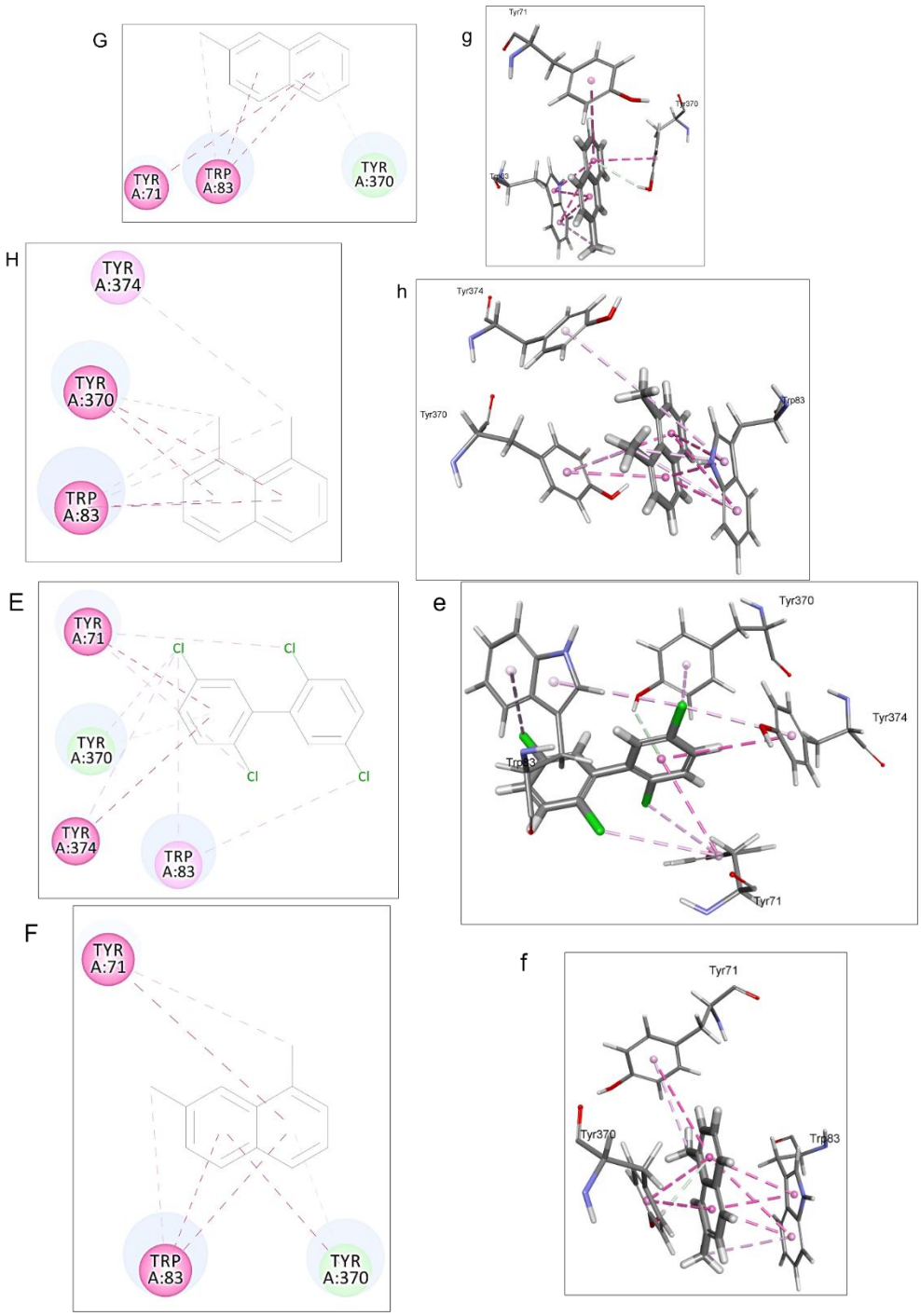

Fig. 6E– H. 2D- binding interaction profile for 2, 2', 5, 5'- tetrachloro- 1, 1biphenyl, 1, 8- dimethyl- naphthalene, 2 methyl naphthalene, 1, 7- dimethyl naphthalene, respectively, with the active pocket of the AchE enzyme. **e– h** shows in- depth 3D ligand- AchE interaction mode

5.2. Binding mode with GST enzyme

Compounds under investigation were docked into GST active pocket (PDB ID: 1M0U). This would contribute to perceiving the mode of action of the tested compounds. Data are presented in Table (4) and Figs (7, 8). Vitamin E shows the best affinity with -

CDOCKER energy of 30.5451. It forms conventional hydrogen between its phenol group and SER110. Additionally, it forms alky interaction between its alkyl chain and LYS89 and ALA149. Moreover, its alkyl chain forms pi- alkyl interaction with TRP85 and TYR153. Methyl 9- octadecenoate showed a good -CDOCKER energy score of 21.1943. It forms a conventional hydrogen bond between its ester carbonyl group and LYS89. Alkyl interaction was observed between its terminal methyl group and PRO98. While, di-p- tolylmethane didn't form any hydrogen bond. It forms pi- alkyl interaction between its two phenyl groups and LEU60. Its -CDOCKER energy is 18.1522. The rest of the compounds showed low scores ranging from 4.35961 to 1.58147. 1, 8-dimethylnaphthalene showed a low affinity with -CDOCKER energy of -6.38238.

These results revealed that Vitamin E, methyl 9- octadecenoate, and di- p-tolylmethane were the major contributors to the activity against the GST enzyme.

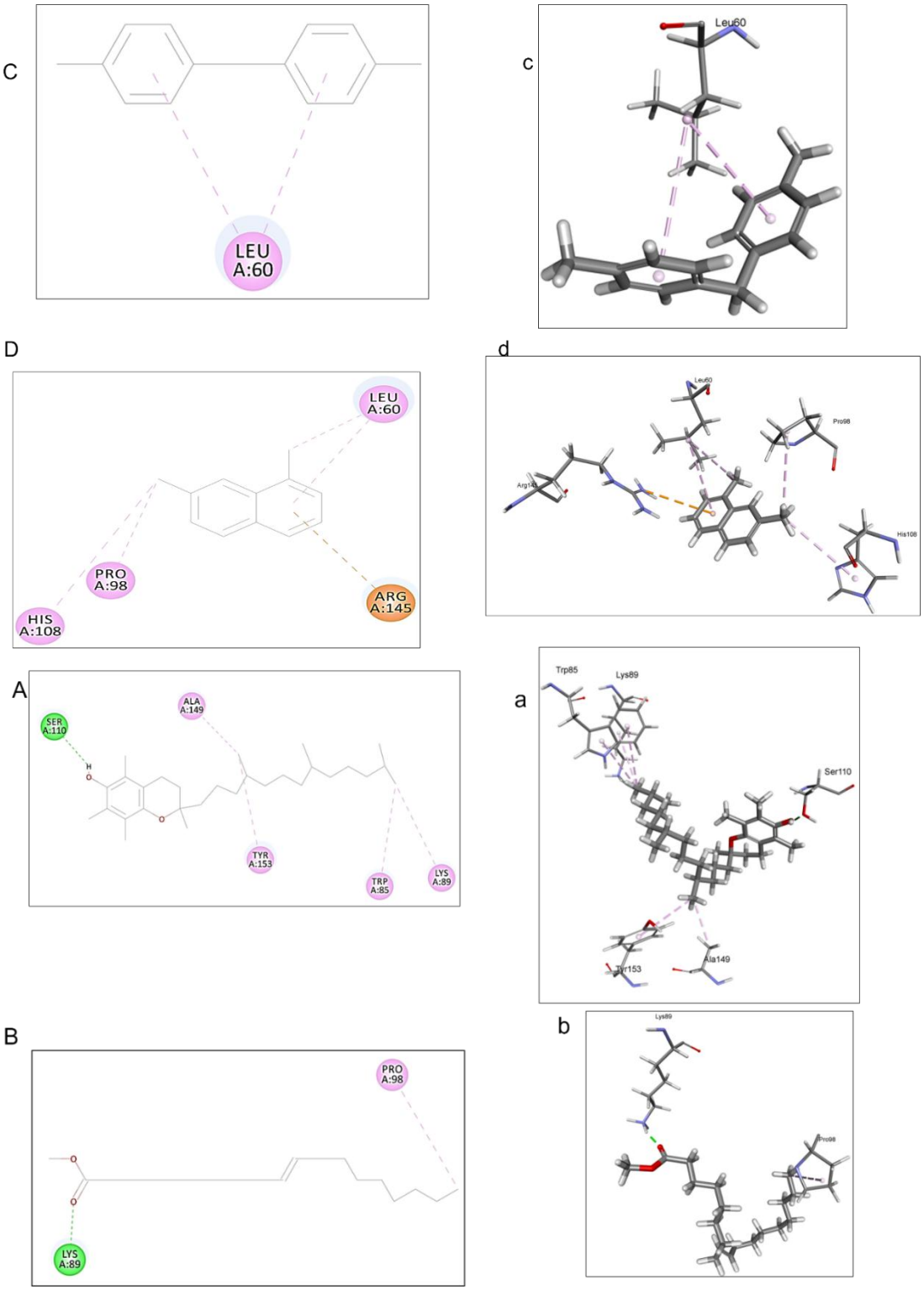

Fig. 7A– **D.** 2D- binding interaction profile for vitamin E, 9- octadecenoic acid, methyl ester, methane, di- p- tolyl- and naphthalene, 1, 7- dimethyl, respectively, with the active pocket of the GST enzyme. **a**– **d** shows in- depth 3D ligand- GST interaction mode

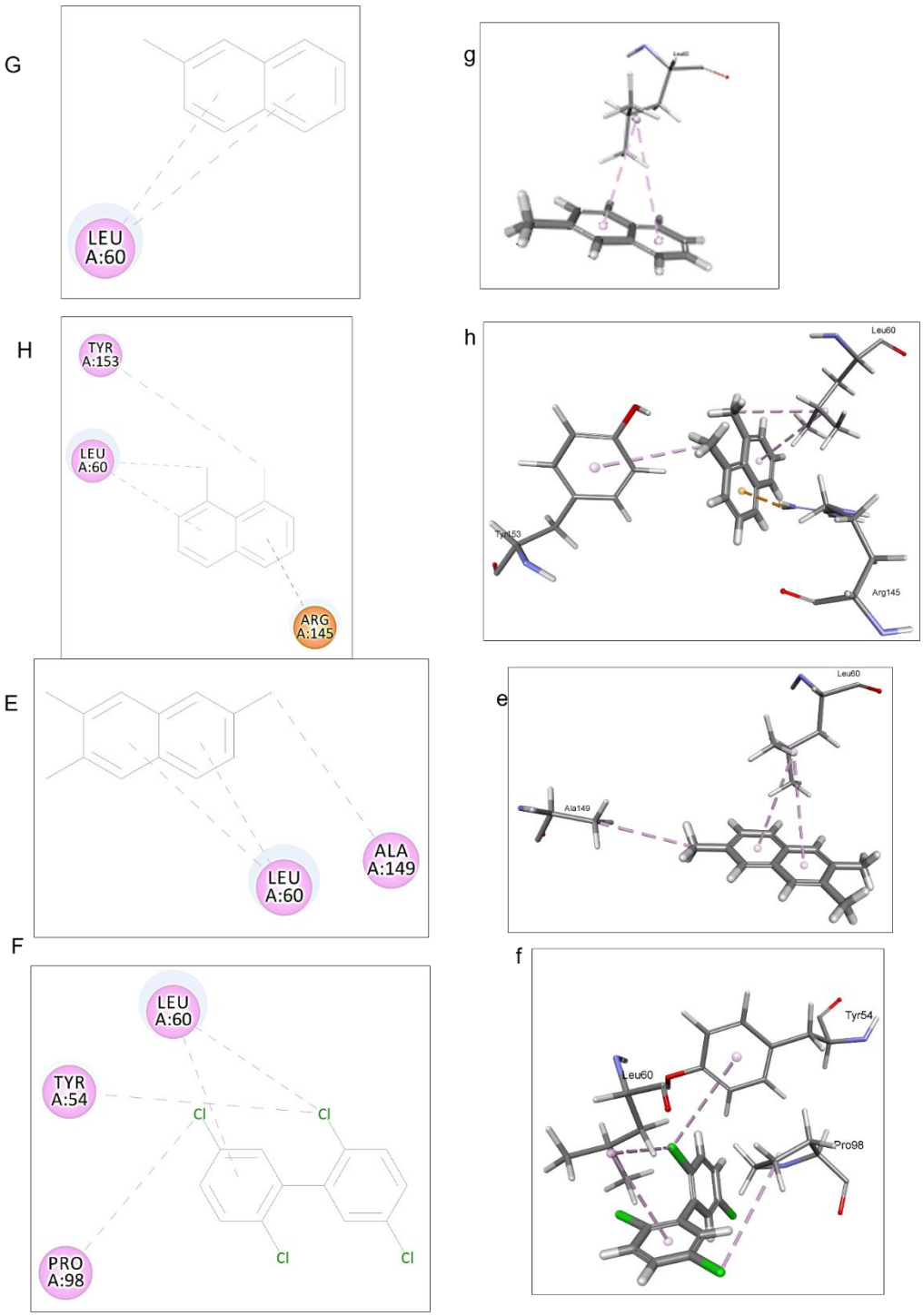

Fig. 8E– H. 2D- binding interaction profile for naphthalene, 2, 3, 6- trimethyl, 2, 2', 5, 5'- tetrachloro- 1, 1biphenyl, naphthalene, 2- methyl and naphthalene, 1, 8- dimethyl, respectively, with the active pocket of the GST enzyme. **e**– **h** shows in-depth 3D ligand- GST interaction mode

Table 4. The binding data for major compounds identified in safe oil with AChE and GST active sites

Code	Code compound name	GST interaction BE	AChE interaction BE
1	Vitamin E	30.5451	19.997
2	9- ctadecenoic acid, methyl ester	21.1943	35.4203
3	Methane, di- p- tolyl	18.1522	30.471
4	Naphthalene, 1, 7- dimethyl	4.35961	12.0517
5	Naphthalene, 2,3,6- trimethyl	4.09486	18.1083
6	2, 2', 5, 5'- tetrachloro- 1, 1biphenyl	3.27494	16.1662
7	Naphthalene, 2- methyl	1.58147	10.9371
8	Naphthalene, 1, 8- dimethyl	-6.38238	4.42697

BE = Binding energy (Kcal/ mol)

DISCUSSION

It is widely known that the extensive use of synthetic pesticides to reduce mosquito populations has led to several adverse consequences, including the development of pesticide resistance and the negative impact of insecticidal residues on both human health and the environment **(Demirak & Canpolat, 2022)**. Several studies support the potential of phytochemicals as a viable alternative to chemical pesticides, especially for mosquito larval control **(Chatterjee *et al.*, 2023; Kaur & Kocher, 2023)**. Besides being biodegradable, plant-based insecticides can effectively reduce mosquito populations without harming the environment or non- target organisms **(Demirak & Canpolat, 2022)**. The chemical composition of *Azadirachta indica's* plant crude extract exhibits significant insecticidal activity against various insect species from different orders (**Lucantoni *et al.*, 2006; Schneider *et al.*, 2017; Alhaithloul *et al.*, 2023**; **Kaur & Kocher 2023)**. Therefore, in the present study, a neem oil formulation (safe oil)

containing 0.03 % azadirachtin was assessed for its efficiency in controlling *Culex pipiens* 3rd larval instar. The results revealed a gradual increase in larval percent mortality as the oil concentration increased. This neem oil formulation exhibited larvicidal effectiveness against *Cx. pipiens* 3rd instar larvae, resulting in mortality rates ranging from 13.33 to 98.66% 24 hours post- treatment. The larvae were exposed to various concentrations (0.028, 0.125, 0.25, 0.5ppm), with a median lethal concentration (LC_{50}) value of 0.112ppm.

Similar larvicidal activity was reported in India against three mosquito larvae species. The LC_{50} for the neem oil formulation tested against *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* was determined 48 hours post- treatment to be valued at 1.6, 1.8, and 1.7ppm, respectively **(Dua *et al.*, 2009)**. The present study is in accordance with several studies conducted to evaluate larvicidal activity with different neem oil formulations against *Culex pipiens* larvae. **Rehimi *et al.* (2011)** reported the larvicidal activity of Azadirachtin (3.2%w/ w) against the early fourth stage of *Cx. pipiens* larva in Algeria, with value of LC_{50} at 0.35mg/ L**.** Furthermore, margosa oil (a neem oil formulation) proved larvicidal activity against *Cx. pipiens* larva with a mortality rate of 30 to 90% after exposure to different concentrations (5 to 80ppm) at 120 hours post- treatment with the LC_{50} value of 11.59ppm **(Mostafa & Hashem, 2022)**. Moreover, the extracted neem oil from *A. indica*, diluted with ethanol, showed larvicidal activity against *Cx. pipiens* with LC_{50} value of 0.44ppm **(Mahmoud *et al.*, 2019)**.

It is important to study the interaction of phytochemicals with the physiological system of the target insect to evaluate their toxicity and develop better insecticides **(Sofi *et al.*, 2022)**. Changes in the biochemical constituents of *Cx. pipiens* 3rd instar larvae treated with a neem oil formulation (safe oil) were investigated using the mean lethal concentration (LC_{50}). In our investigation, a significant decrease in the total protein level of 3rd instar larvae *Cx. pipiens* treated with safe oil was recorded compared to the control group. This decrease in the total protein level can be attributed to the versatile effect of azadirachtin, the main bioactive compound of neem oil. It is worthnoting that, azadirachtin is known for its disruptive effects on insect physiology, particularly by interfering with essential metabolic and growth processes **(Chatterjee *et al.*, 2023)**. In all related studies, the physiological effects of neem oil on the immature stages of mosquito larvae were traced back to the interference of azadirachtin, the main component of neem oil, with the ecdysteroid hormone, the hormone responsible for insect molting, resulting in abnormal molts, growth reduction and subsequently increased mortality rate (**Chaudhary *et al.*, 2017)**. 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)**. The results of the present study are consistent with the previous study of **Mordue (2004)** that showed an effect of azadirachtin on protein synthesis in different insect species, highlighting the importance of azadirachtin effects in

physiological processes. Insects can combat xenobiotics with an enzymatic detoxification system **(Panini *et al.*, 2016)**. Changes in the activity of such detoxifying enzymes are correlated with the lethal effects of phytochemicals due to the disruption of various physicochemical processes in the tested insects **(Parthiban *et al.*, 2020)**. These detoxification enzymes include acetylcholinesterase and glutathione S- transferase. Acetylcholinesterase acts as an esterase that is responsible for breaking down the neurotransmitter acetylcholine and effectively stops nerve impulses. Insects adeptly use this enzyme to reduce pesticide sensitivity at specific target sites **(Lushchak, 2018)**. When acetylcholinesterase activity drops to a critical level, insects become paralyzed and eventually die. Glutathione- S- transferase (GST) is an enzyme involved in phase II detoxification, catalyzing the conjugation of reduced glutathione with various xenobiotics, increasing their solubility, and facilitating their excretion from the body **(Panini *et al.*, 2016)**. In the present investigation, neem oil formulation reduced the activity of detoxifying enzymes, acetylcholinesterase, and GST in 3rd larval instar. This detected decrease is consistent with the finding of **Nathan *et al.* (2008)** who reported a decrease in detoxification enzymes in response to azadirachtin treatment.

Azadirachtin is an anti- nutrient agent and has several gut- disrupting effects on insects with direct adverse effects on enzyme production and nutrient absorption **(Chatterjee *et al.*, 2023)**. In the present study, several histological alterations were observed in the midgut of *Cx. pipiens* larvae. The peritrophic membrane was damaged, midgut epithelial cells were deformed and lost their normal appearance, detached from the basement membrane, and irregular bleb formation protruded into the gut lumen. Similar alterations in the midgut of *Cx. pipiens* treated with plant- based biopesticides were observed by several authors, viz. **Hamouda *et al.* (1996)**, **Hussin and Shoukry (1997)**, **Assar and El- Sobky (2003)** and **Mostafa and Hashem (2022)**. According to **Hamouda *et al.* (1996)**, *Artemisia judaica* impacted the midgut of *Cx. pipiens*, causing the epithelial layer to vacuolate, enlarged cells, masses of cellular material to develop in the lumen, and eventually the epithelium to lose its usual appearance. **Al-Mehmadi and Al- Khalaf (2010)** investigated the effect of *Melia azedarach* (Meliaceae) on the midgut of *Cx. quinquefasciatus*. They found that the most common symptoms were microvilli destruction and midgut vacuolization, with columnar cells occasionally protruding into the lumen. In accordance with our study, treatment of *Cx. pipiens* larvae with combined herbal formulation also induced several histological alterations in the midgut region characterized by the presence of irregular bleb formation **(Abutaha *et al.*, 2022)**.

The chemical structures of the 8 active constituents revealed the presence of four compounds that are polycyclic aromatic compounds with benzene rings. Methyl 9-octadecenoate had the highest -CDOCKER energy score of 35.4. This could be ascribed to the traditional hydrogen connection between the ester group and GLY150, GLY151, and ALA239, as well as the interactions between the pi- alkyl group and TRP83, TRP472, and LEU479. The second- best compound was di- p- tolylmethane with a valye

of 30.471. Its phenyl ring is connected to TYR370 via a pi-donor hydrogen bond. Additionally, this phenyl ring interacts through pi- pi stacking with TYR71 and TYR374, while the other phenyl ring forms a pi- pi connection with TRP83. Its two methyl groups interact with alkyl groups. Without generating any hydrogen bonds, vitamin E and 2, 3, 6- trimethyl naphthalene displayed scores of 19.997 and 18.1083, respectively. Scores indicate that these substances help to increase the activity against the AChE enzyme.

When the 8 compounds were docked with the GST active pocket, it became clear that the main contributors to the activity against the GST enzyme were vitamin E, methyl 9- octadecenoate, and di- p- tolylmethane. **Li *et al.* (2023)** examined the effectiveness of various essential oils and their constituents against *Aedes albopictus*. In this context, **Santos *et al.* (2010)** explored the larvicidal activity of a variety of commercially available aromatic and aliphatic diversely substituted compounds against *Ae. aegypti* and discovered that the presence of lipophilic groups generally increased potency, whereas the presence of hydroxyl groups typically decreased it.

CONCLUSION

In conclusion, this study explores environmentally sustainable alternatives such as neem oil for effective mosquito vector control. The neem oil formulation (safe oil) exhibited remarkable larvicidal activity against *Cx. pipiens* third larval instar, leading to histopathological alterations in the midgut region and reduced biochemical activities. Molecular docking outcomes provided additional evidence of its potential as a potent larvicide, confirming the utility of safe oil and its constituent compounds in controlling *Cx. pipiens* mosquitoes.

ETHICAL APPROVAL

All experiments in this research were approved by the Ethics Committee of the Faculty of Science, Ain Shams University, Cairo, Egypt (Approval code: ASUSCI/ENTO/ 2023/8/4).

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