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Original article

Larvicidal activity and chemical compositions of *Aloe ferox* mill, and *Commipora abyssinica* (O.Berg) combination against the mosquito vectors *Culex pipiens* L

Nael Abutaha *, Fahd A. Al-mekhlafi, Mohamed A. Wadaan, Mohammed S. Al-Khalifa

Department of Zoology, College of Science, King Saud University, P.O. Box 2455 Riyadh, 11451 Riyadh, Saudi Arabia

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ABSTRACT

The aqueous extract was prepared from the *Aloe ferox*, and *Commipora abyssinica* combined to develop a botanical mosquito larvicide. The aqueous extract and the solvent fractions obtained using liquid–liquid extraction were tested against *Culex pipiens* larvae for larvicidal potential. The maximum larvicidal activity was recorded for the ethyl acetate (EtOAc) extract with LC_{50} values of 28.24 μg/mL followed by hexane (104.42 μg/mL), water (140.24 μg/mL), and chloroform (211.41 μg/mL) extract against the *Cx. pipiens* third instar 24 h post-treatment. In midguts of EtOAc extract-treated larvae, Longitudinal sections showed edema between the degenerated epithelial cells and degraded microvilli. The extract caused a dose-dependent decrease in the percentage of cell viability of HUVEC cells suing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The IC_{50} value of the EtOAc extract was 143.6 μg/mL and displayed chromosomal condensation. The total phenolic and flavonoid contents calculated were 15.9 GAE/g (gallic acid equivalent per gram) and 3.69 QurE/g (Qurcetin equivalent per gram), respectively. GC–MS analysis showed that the major chemical components of the EtOAc extract were methyl ester of hexadecanoic acid (28%), 3-benzylbutanoate (9.4%), methyl ester of octadecanoic acid (8.6%), and alpha-muurolene (5.3%). The current investigations revealed the possible use of this botanical combination as a larvicide against *Cx. pipiens* larvae.

1. Introduction

Different emerging diseases are transmitted by the mosquitos' -bites such as Dengue Virus, Chikungunya Virus, Eastern Equine Encephalitis Virus, Japanese Encephalitis Virus, La Crosse Encephalitis, Malaria, St. Louis Encephalitis, Yellow Fever, West Nile Virus, and Zika Virus (CDC, 2020).

Around 3500 mosquito species are identified worldwide (Harbach and Howard, 2007); only a small number play a role in arboviruses transmission. *Cx. pipiens* play a critical role in the transmission of viruses, mainly Sindbis Virus (SINV) infection that causes rash and polyarthritis (Lundström, 1999), West Nile Virus that causes a febrile illness that generally resolves without complications (Campbell et al., 2002) and Usutu virus, that causes mortality to Old World blackbirds (Weissenböck et al., 2013) and encephalitis (Pecorari et al., 2009). Vector control is a crucial strategy for managing diseases transmitted by mosquitoes. Larvicides that kill the mosquitoes bound to their habitats (juvenile stages) are much easier before adulthood.

The repeated use of synthetic larvicidal agents resulted in environmental pollution and resistant mosquitoes (Liu, 2015). Insecticide resistance reduces the possibility of eradicating diseases transmitted by vectors (WHO, 1976). Extensive use of mosquitocides to eliminate dengue fever in 2004 (Ayyub et al., 2006), and Rift Valley fever in 2000 (Jupp et al., 2002) increased the level of resistance in mosquitoes. Research conducted by Al-Sarar, 2010 to detect resistance in *Cx. pipiens* populations in Riyadh City found two populations from Wadi Namar were resistant to bifenthrin, deltamethrin, beta-cyfluthrin, and lambda-cyhalothrin. No resistance to fenitrothion was observed (Al-Sarar, 2010). Thus, there is a necessity to search for new Larvicides.

Plant secondary metabolites are a promising source of larvicidal agents for mosquito management/control. Secondary metabolites

* Corresponding author.
E-mail address: nabutaha@ksu.edu.sa (N. Abutaha).

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such as steroids, terpenes, alkaloids, and phenolic compounds are reported for larvicidal without causing deleterious effects to human and animal health and the environment (Hari and Mathew, 2018; Mathew, 2017).

Earlier research indicated the potential of synergistic plant extract combinations (Benelli et al., 2017). No investigation has been conducted to evaluate the effects of the *Aloe ferox* and *Commipora abyssinica* combination against mosquito larvae. Therefore, this encouraged us to investigate the combined effect of these two plants against the *Cx. pipiens* larvae and chemical constituents analysis. Hence, the outcome of this report could provide a perspective for the development of eco-friendly plant-based alternatives for mosquito control.

2. Materials and methods

2.1. Plant collection and extraction

The leaf *Aloe ferox* Mill. (Asphodelaceae) and resin of *Commipora abyssinica* (O.Berg) Engl. (Burseraceae) were collected from a herbal shop in Riyadh, Kingdom of Saudi Arabia. Voucher specimens were deposited in the department of botany for *A. ferox* (KSU-Bio-225) and *C. abyssinica* (KSU-Bio-226).

2.2. Aqueous extract

Ten grams of each plant powder were mixed and soaked in 500 mL of boiled distilled water and kept on the shaker (250 rpm) (GFL, Germany) for 24 h. Later, the extract was centrifuged (Centurion, UK) at 5000 rpm for 10 min. Three hundred milliliters of the extract were later extracted using the liquid–liquid extraction method in hexane, chloroform, and ethyl acetate (EtOAc). The aqueous extract and the fractions obtained were concentrated using a rotary evaporator (Heidolph, Germany) at 40 °C. All the extracts were reconstituted in DMSO at 50 mg/mL concentration and stored at 4 °C until analysis.

2.3. Collection and maintenance of Cx. Pipiens

The *Cx. pipiens* were collected from Bio-product Research Chair Insectary, King Saud University, Riyadh. The larvae were reared in the standard condition (25 ± 2 °C and 85 ± 5% humidity), maintained in a bowl filled with tap water, and fed on a mixture of fish feed.

2.3.1. Dose-response bioassays on Cx. pipiens

The larvicidal bio-efficacy of the extract was evaluated against the third instar of *Cx. pipiens*. The extract was introduced into 6 well plates containing water (8 mL), and 20 Larvae were released into each well. A well containing water and another well containing DMSO served as control (0.5%). Each test was carried out in triplicate. The number of dead larvae was counted 24, 48, and 72 h post-treatment, and the mortality percentage was calculated. The LC_{50} and LC_{90} values of extracts were evaluated, and the results were analyzed using the SPSS Software.

2.3.2. Histopathology alterations

The treated and control larvae were kept in buffered (pH 7.2) formalin. Ethanol dehydrated larvae were embedded in paraffin wax, sectioned using a microtome (Leica, Germany), stained with hematoxylin and eosin, and mounted. The midgut region was assessed under a microscope (Al-Mekhlafi et al., 2020).

2.4. Cytotoxicity

Noncancerous HUVEC cell lines were purchased from the American Type Cell Culture Collection (ATCC, USA). About 50,000 cells/well was seeded for 24 h in Dulbecco's modified Eagle's medium with 10% FBS in a 24-well plate at 37 °C with a 5% CO_2 atmosphere. Later, different test concentrations (100,200,300,400, and 500 μg/mL) were added to the cell lines separately and incubated as before. MTT reagent (Thermo, USA) was then added to each well and incubated for 3 h. The formazan crystals formed were solubilized in 1 mL dimethyl sulfoxide, and the absorbance (n = 3) was read using a microplate reader at 570 nm.

2.5. Hoechst 33258 staining

Cells were seeded as prepared in the previous section and treated with IC_{50} of the extract. Later the cells were washed twice with phosphate-buffered saline (PBS) before and after fixation and then stained for 20 min at 25 °C with 1 μg/ml Hoechst dye (Invitrogen, USA) and observed under a fluorescent microscope (EVOS, USA).

2.6. Total phenol content

The estimation of total phenol was assessed by the Folins-phenols reagent (FC) method (Abutaha et al., 2021). Extract (12.5 μL) was mixed with 125 μL of 25% FC reagent and 12.5 μL of sodium carbonate and incubated for 1.5 h for color development. The absorbance was read at 725 nm (Thermo Fisher Scientific, USA). The calibration curve was constructed using Gallic acid (5–100 μg/ml). The total phenol of extract content was expressed as gallic acid equivalent per gram (GAE). The results were analyzed in triplicate.

2.7. Total flavonoids

Estimating total flavonoids was carried out by the Aluminium chloride method (Abutaha et al., 2021). An equal volume of the extract and 100 μL of Aluminium Chloride (2%) was mixed and incubated for 10 min at 25 °C for color development, read at 368 nm. The total flavonoid content of the extract was expressed as quercetin equivalent per gram (QURE). The results were carried out in triplicates.

2.8. IR and GC–MS analyses of bioactive principle

As previously reported, a dried sample containing active EtOAc extract was subjected to infrared (IR) spectroscopy and GC–MS analysis (Abutaha et al., 2021).

2.9. Statistical analysis

The results of mortality are means of three replicates. Analysis of variance (ANOVA) was compared using Duncan's multi-range test with SPSS 10.0 software. The significance level was $p < 0.05$.

3. Result

3.1. Bioassays on Cx. pipiens

The larvicidal efficacy of aqueous extract, hexane, chloroform, and ethyl acetate (EtOAc) fractions were tested against the third instar of *Cx. pipiens* (Table 1). All the extracts showed larvicidal potentials against *Cx. pipiens* after 24, 48, and 72 h of treatment

Table 1
Larvicidal activity of aqueous extract of *Aloe ferox* and *Commipora abyssinica* combined and its solvent fractions against *Culex pipiens*.

Extract Type	Time	Mortality% Concentration (µg/mL)					LC_{50} (µg/mL)	LC_{90} (µg/mL)
		15.63	31.25	62.5	125	250		
Hexane	24	-	10.00±5.77d	36.67±3.33c	73.33±3.33b	100.00±0.00a	104.42	206.62
	48	-	40.00±5.77b	76.67±8.82a	90.00±5.77a	100.00±0.00a	34.18	116.22
	72	-	50.00±5.77b	83.33±6.67a	100.00±0.00a	100.00±0.00a	8.33	97.6
Chloroform	24	-	0.00±0.00b	6.67±3.33b	36.67±8.82a	56.67±3.33a	211.41	362.18
	48	-	0.00±0.00b	16.67±3.33b	76.67±6.67a	93.33±26.67a	186.37	217.86
	72	-	20.00±5.77c	76.67±3.33b	86.67±3.33ab	100.00±0.00a	44.5	184.07
Ethyl acetate	24	16.67±3.33d	53.33±3.33c	73.33±3.33b	100.00±0.00a	-	28.24	101.67
	48	23.33±3.33c	70.00±0.00b	93.33±3.33a	100.00±0.00a	-	21.4	90.07
	72	50.00±5.77b	90.00±3.33a	100.00±00a	-	-	5.21	46.88
Aqueous	24	-	10.00±5.77b	10.00±5.77b	63.33±6.67a	83.33±3.33a	140.24	250.83
	48	-	13.33±6.67b	16.67±3.33b	76.67±3.33a	90.00±0.00a	119.42	226.43
	72	-	30.00±5.77b	36.67±3.33b	93.33±3.33a	100.00±0.00a	72.51	191.63

Small horizontal letters indicate significant differences between concentrations. Significant differences were assessed using one-way ANOVA followed by Tukey's test, with $p < 0.05$ considered to indicate significant differences.

(Table 1). No mortality was recorded in the control group. The EtOAc extract showed the highest toxicity when compared to other extracts tested. After 24 h of exposure, the EtOAc extract demonstrated an LC_{50} value of 28.24 µg/mL and an LC_{90} value of 101.67 µg/mL against the *Cx. pipiens*. The larvicidal activity of EtOAc extract was followed by hexane (104.42 µg/mL), water (140.24 µg/mL), and chloroform (211.41 µg/mL) extract (Table 1).

3.2. Histopathology alterations

The larval exposure to LC_{50} of the EtOAc extract showed changes in the midgut architecture of *Cx. pipiens* larvae. The larval exposure to EtOAc extract induced lysis of epithelium layer in the midgut, vacuolization, and destruction of the peritrophic membrane in some regions, loss of epithelial cell, edema between the degenerated epithelial cells, and degraded microvilli (Fig. 1).

3.3. Cytotoxicity

Fig. 2 shows the dose-dependent decrease in the percentage of cell viability in the presence of EtOAc extract in HUVEC cells. The LC_{50} value of the extract was 143.6 µg/mL as compared with the control. We also investigated the morphological alternation in HUVEC cells exposed to EtOAc extract using fluorescence microscopy. The nuclei in the control cells were round with normal chromatin and evenly stained. Cells incubated with the EtOAc extract displayed chromosomal condensation. In the control wells, the nuclei of HUVEC cells appeared evenly stained and round (Fig. 2).

3.4. Total phenol and content

The total phenolic and flavonoid contents calculated were 15.9 GAE/g and 3.69 QURE/g, respectively.

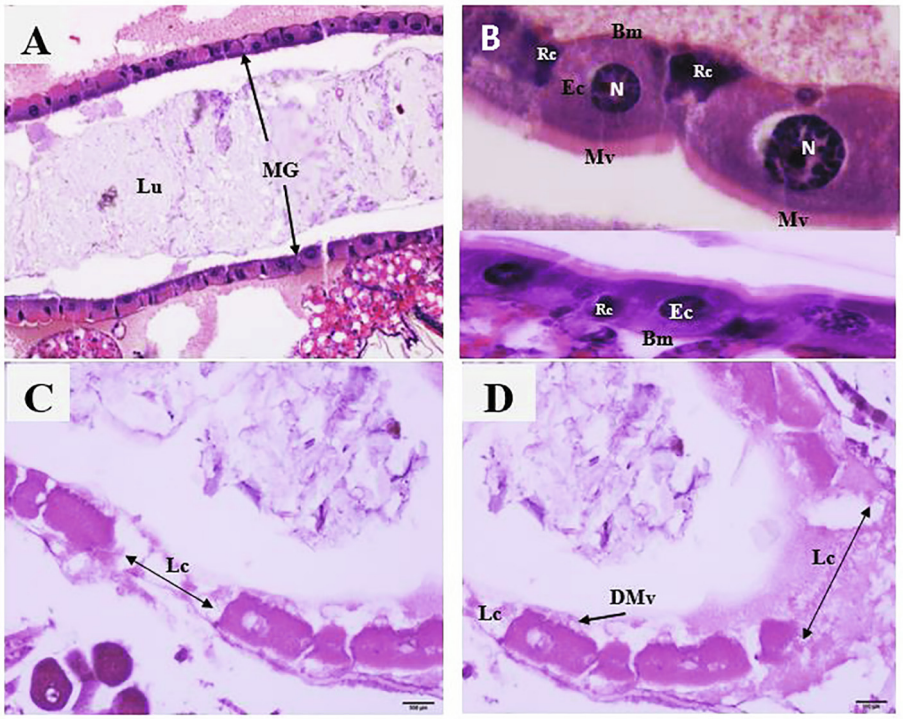

Fig. 1. Photomicrographs of midguts of *Cx. pipiens* larvae treated with EtOAc fraction obtained from aqueous extract of *Aloe ferox* and *Commipora abyssinica* combined. Longitudinal sections in the midguts (MG) of control larvae with normal and healthy epithelial cells (Ec), microvilli (Mv), nuclei (n), and regenerative cells (Rc). Note the absence of the lesions. {C-D}: Longitudinal sections in midguts of EtOAc-treated larvae, with loss of some epithelial cells (Lc) and degraded microvilli (DMv). H&E stain.

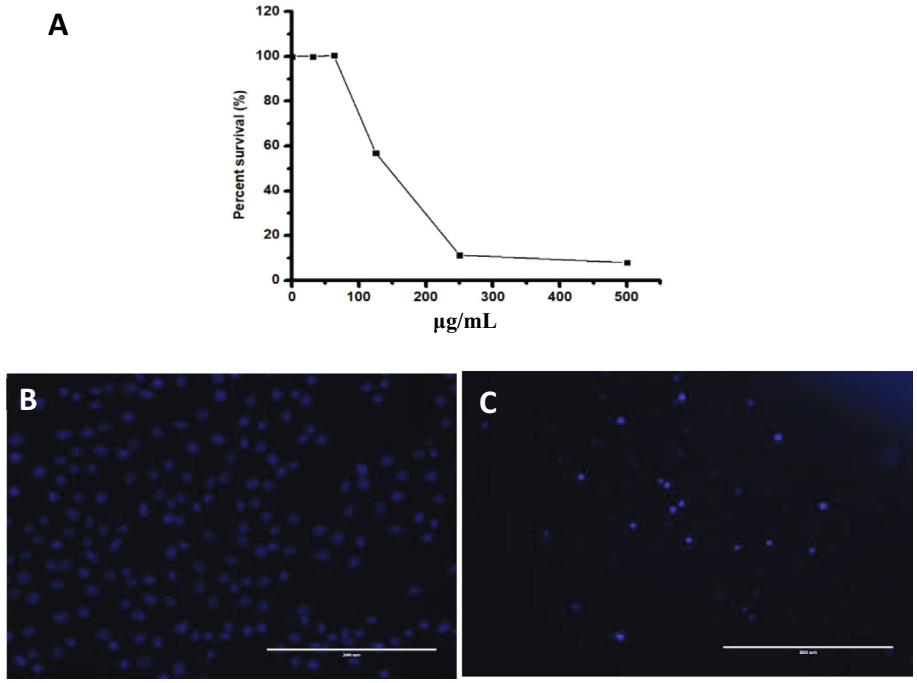

Fig. 2. A; Cytotoxicity of EtOAc fraction obtained from aqueous extract of *Aloe ferox* and *Commipora abyssinica* combined against Noncancerous HUVEC cell lines using MTT assay showing dose-dependent toxicity. Fluorescence microscopy of cells treated with EtOAc extract for 24 h, followed by DAPI staining and then imaged to assess the morphological changes. (B) Untreated control cells showed normal nuclear morphology; (C) treated cells showed fragmented chromatin.

3.5. GC–MS and IR spectra analysis of EtOAc extract

The IR spectrum of EtOAc extract (Fig. 3) showed a hydroxyl peak at ~3430 cm^{-1} and the peaks situated at 2913 and 2996 cm^{-1} belong to the C H stretching vibration of methylene and methyl group. The FTIR spectrum is dominated by monoterpenes' vibrational modes, seen at 952, 1436, and 1654 cm^{-1}. The band in the 1020 cm^{-1} region belongs to the C-O stretching vibration of alcohol. The band at 1654 cm^{-1} could represent amide I, or carboxylic acid. GC–MS analysis showed that the major chemical components of the EtOAc extract were methyl ester of hexadecanoic acid (28%), 3-benzylbutanoate (9.4%), methyl ester of octadecanoic acid (8.6%), and alphamuurolene (5.3%) (Table 2).

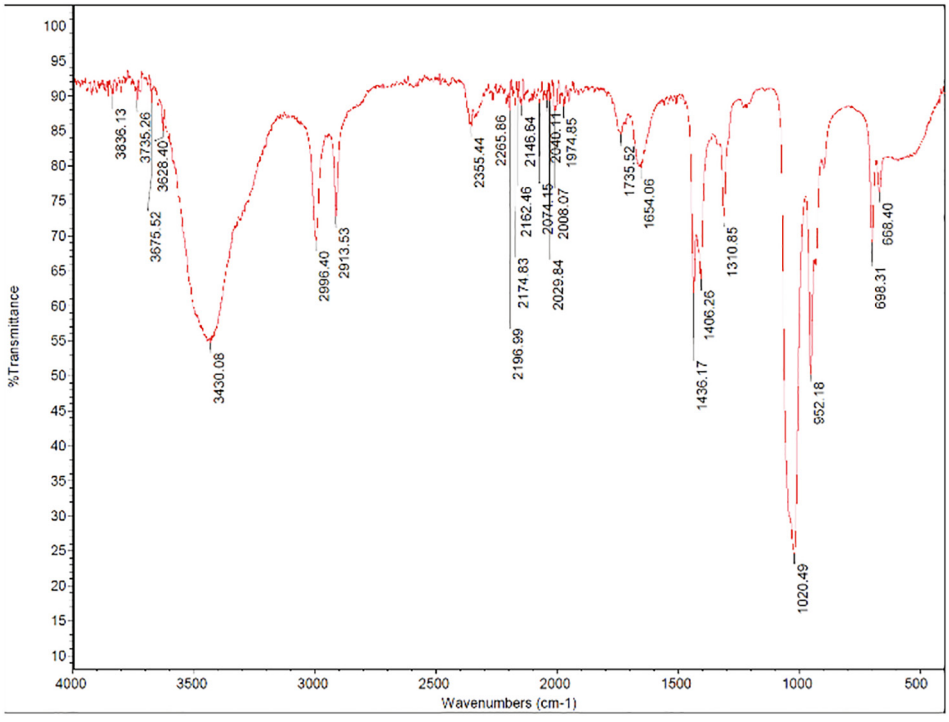

Fig. 3. FTIR spectra of EtOAc fraction obtained from aqueous extract of *Aloe ferox* and *Commipora abyssinica* combined.

Table 2
GC–MS analysis of EtOAc fraction obtained from aqueous extract of *Aloe ferox* and *Commipora abyssinica* combined.

#	Name	Retention time	Area %
1	Octanal	13.31	0.960
2	Methyl ester of octanoic acid	14.03	2.020
3	4-Methyl-benzaldehyde	17.52	0.340
5	alpha.-Muurolene	31.14	5.350
6	Methyl cantharate	31.73	1.190
7	Methyl 2-(1-cyclopentanol)mandelate	32.83	1.980
8	iso-Geraniol	34.00	0.550
9	Methyl ester of hexadecanoic acid	34.27	28.000
10	4,4-Dimethyl-1-phenyl- 2,7-octadien-1-one	34.43	1.520
11	(2-phenylethenylidene)cyclohexene	34.74	0.980
12	Mansonone	34.85	3.640
13	Erythro-2,2-dimethyl-6-nitro-5-Phenylhept-3-ene	35.15	3.110
16	2,5-Dimethylamphetamine	35.95	1.300
17	Methyl 2-phenyl-2,3-dihydroxy-3-Chloromethylbutanoate	36.19	4.230
20	Methyl cantharate	37.29	2.940
21	Methyl ester of 6-octadecenoic acid	37.60	3.220
22	Methyl ester of 11-octadecenoic acid	37.72	2.700
24	Methyl ester of octadecanoic acid	38.09	8.600
25	1,1-Diphenylprop-2-en-1-ol	38.35	4.020
27	Methyl 2-(1-cyclohexanol)mandelate	38.94	2.650
29	Methyl 2-phenyl-2,3-dihydroxy-3-benzylbutanoate	40.10	9.410

4. Discussion

Larviciding is a successful method of managing mosquitoes' spread in their breeding habitant post emerging into adults. Although synthetic larvicides are promising and effective in controlling mosquitos, their frequent use has caused an extensive development of resistance in mosquitoes (Addiss and Lammie, 2013; Kumar et al., 2011). Studies on secondary metabolites of natural origin against mosquito vectors are promising alternative agents to manage the threat of vector-borne diseases that act on mosquitoes' physiological and behavioral. The combination of extracts is more advantageous than individual isolated compounds that prevent mosquito resistance development (Maurya et al., 2007). Though, bioactivity plant extracts vary based on plant species and part used, geographical origin, extraction solvent, chemical nature, mosquito species, and developmental stages (Ghosh et al., 2012; Shaalan et al., 2005).

Results revealed that LC_{50} values decreased with the exposure time, recording the lowest value at 72 h of incubation. Dose-dependent mortality was observed and positively correlated with the concentrations used. In our study, the LC_{50} value of the EtOAc extract was 28.24 μg/mL; this is within the promising range (LC_{50} less than 100 μg/mL) of reported classification (Thangam and Kathiresan, 1996).

Eradicating mosquitoes at the larval stage is considered the best target for mosquitoicides because they breed in water, and therefore, they are easily controlled in this habitat. The use of synthetic mosquitoicides in aquatic sources may pose risks to the environment. Plants' secondary metabolites are very promising sources of compounds that are used in a different fields. Green mosquitoicides may serve as an alternative to synthetic insecticides. The blending of insecticidal agents is practiced and recommended to optimize the effectiveness of the insecticides to solve the issue of insecticidal resistance and preserve the insecticidal efficiency for a longer period. In the current investigation, the binary blending of different plant extracts have been reported in many research articles such as *Callitris glaucophylla* and *Khaya senegalensis* against *Aedes aegypti* (Shaalan et al., 2005), *E. camaldulensis* and *C. rigidus* against *A. gambiae* larvae (Ríos et al., 2017), *Canarium schweinfurthii, Aucoumea klaineana,* and *Dacryodes edulis* against *A. gambiae* (Obame et al., 2016).

Histopathological changes in the midgut of *cx. pipiens* larva post-treatment with EtOAc extract help to understand the mode of action of the extract. The midgut is composed of epidermal cells and muscle. Epidermal cells are responsible for the oxidation process, ionic and osmotic regulation, storage of carbohydrate and lipid, pH control, nutrient absorption, and secretion of the digestive enzyme (Silva et al., 2020; Wang et al., 2019). The third instar of *Cx. pipiens* exposed to EtOAc extract showed morphological deformities. EtOAc extract acted upon epidermal cells and showed damaged epithelial cells, microvilli, muscles, and adipose tissue (Fig. 1C, D). In control, the larval tissues revealed undamaged epithelial cells and adipose tissue (Fig. 1A, B). The results are similar to the previous reports (Abdel-Salam et al., 2018) (FARAG et al., 2018). The toxicity of the plant extract as an insecticide depends on detoxification ability at the developmental stage, target enzymes, and ability to penetrate the insect body (Wang et al., 2019). The third instar of *Cx. pipiens* exposed to EtOAc extract showed histological deformities. The damage in the larval midgut cells treated with EtOAc extract may be attributed to the remarkable feature of compounds; non-polar extract can easily penetrate the larval body and interrupt the intake of feed, cell division, and breathing.

The IC_{50} value of Huvec cells treated with EtOAc extract was 145.5 μg/mL. Plant extract with IC_{50} higher than 30 μg/mL is considered safe based on American National Cancer Institute (Rosidah, 2014). When applying this information to our data, PH2 extract is deemed safe because the concentration needed to be used as larvicides (LC50: 28.2 μg/mL) is 5 times less than the IC_{50} value Huvec cells (145.5 μg/mL). However, the data cannot be generalized, and different models should assess its toxicity.

The biological activity of extracts is mainly attributed to their major compounds (Bakkali et al., 2008; Gbolade and Lockwood, 2008; Vani et al., 2009). Although, in some times, major compounds may not be accountable for the overall activity, the combination of minor and major compounds could have resulted in synergistic, additive, or antagonistic interactions (Bakkali et al., 2008). The present study demonstrated tissue damage in the larvae treated with extract, as it contains several compounds that are common in many plants that have been reported for their insecticidal activity (Mozaffari et al., 2014. Some compounds were individually isolated, such as methyl ester of hexadecenoic acid. This compound was isolated from *Millettia pinnata* and demonstrated potent larvicidal activity against the third instar larvae of *Cx. pipiens* pallens, *A. aegypti* and *A. albopictus* and the acetylcholinesterase was the site of action (Perumalsamy et al., 2015). However, the potent larvicidal activity of EtOAc extract could be attributed to the various compounds present in the extract, such as phenols, flavonoids, terpenoids, aldehyde, and esters.

The phytochemical and GC–MS analysis are the first step towards understanding the nature of larvicidal constituents present in the extract. Nevertheless, further investigations are needed to explore their potentials. Insecticides derived from herbal extract are less active than synthetic ones (Mohan et al., 2010; Shaalan et al., 2005). However, this is justified for being a mixture of inactive or active compounds. The complexity of the extract with a different mechanism of action could increase the bioactivity or prevent the evolution of resistance populations (Tak and Isman, 2015). Phenolics are widely found in the plant kingdom (Pereira et al., 2009), possessing many therapeutic activities (Fukumoto and Mazza, 2000; Germano et al., 2006). The phenolic compounds inactivate enzymes and form a phenol–protein complex that is hard to digest by mosquitoes (Mello and Silva-Filho, 2002).

5. Conclusion

The findings of the present investigation revealed that EtOAc extract has potent larvicidal activity against Cx. pipiens. Further studies are needed to assess its potential in the field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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