

Evaluation of the Larvicidal Activity of the Fractions of *Hyptis suaveolens* on 4th instar larvae of *Culex quinquefasciatus*

Hausatu Babayi

acadbabayi@futminna.edu.ng

Federal University of Technology Minna <https://orcid.org/0009-0000-2268-9636>

Zainab U. Garba

Federal University of Technology Minna

Israel K. Olayemi

Federal University of Technology Minna

Ibrahim Dawud

Ahmadu Bello University

Aisha Mustapha

Niger State Polytechnic

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Abstract

Mosquitoes are important vectors that transmit many communicable diseases throughout the world. Billions of people face major health concern throughout the world due to vector borne diseases. Synthetic insecticides such as organophosphate and pyrethroids are commonly used for their control. However, insecticidal compounds from natural sources, such as plants are promising tools for managing vectors. The present study was conducted to evaluate the efficacy of *Hyptis suaveolens* on *Culex quinquefasciatus*. Extraction of the plant using hexane and ethyl acetate yielded hexane (coded A) and ethylacetate (coded B) leaf extracts. Extract B which attained 100% mortality at 24 hours of exposing the larvae of *C. quinquefasciatus* (in a previous study) was further fractionated to obtain 5 fractions coded B1-B5. Crude ethylacetate leaf extract of *H. suaveolens* and its fractions were evaluated for their potentials in vector control of mosquito-borne diseases using World Health Organization's recommended protocols for testing the efficacy of insecticides. Crude extract at 0.066g/ml attained 100% mortality and complete inhibition of pupation as well as inhibition of adult emergence of the larvae after 72 hours while that of the fractions B1-B5 was at 0.055g/ml and 0.066g/ml after 24 hours of larval exposure to the extract respectively. Fraction B1 was most potent. The mean LC_{50} and LC_{90} were 0.046 and 0.461, 0.250 and 0.501, 0.040 and 0.478, 0.185 and 0.730, 0.038 and 0.374, 0.080 and 0.401 of crude extract and its fractions respectively. The values of R^2 (coefficient of determination) obtained indicated the rate of mortality linearly increased with increasing dose. The phytochemical constituents detected were alkaloids, anthraquinones, balsams, carbohydrates, glycosides, saponins, terpenes, volatile oils and flavonoids. The present study revealed the potential of fractions of *H. suaveolens* in controlling vector borne diseases (Avian malaria, West Nile virus, St. Louis and Japanese encephalitis).

INTRODUCTION

Mosquitoes are one of the most nuisance creatures of the world and are vectors of many diseases (malaria, dengue, chikungunya, West Nile, Zika, Filariasis, Japanese encephalitis etc.) throughout the world, causing major health concerns and mortality (Prudêncio, 2020). Dengue infections are thought to affect about 400 million people annually world-wide and about 4 billion people in 128 countries are thought to be at risk of contracting dengue virus (WHO, 2021). The approach to combat mosquito-borne diseases largely relied on interruption of disease transmission cycle through chemical, physical, biological methods (Wang et al., 2021).

Chemical methods are the best but due to over dependence on them had led to deleterious effects that include, development of resistance strain (Shroff et al., 2020, Paramanik et al., 2022). Destruction of non-target organisms (Beneli, 2018; Peniche et al., 2022) and bioaccumulation of these synthetic insecticides in some organic tissues (Ngwamah et al., 2018). These insecticides are applied either as aerosol or liquid residual spray against adult mosquitoes in their dwelling places or by spraying the insecticides on larvae breeding sites (e.g. drainage, ponds and grasslands (Ngwamah, 2018)).

The use of plant products as alternative to synthetic insecticides in controlling mosquitoes is attracting attention world wide because they are biodegradable, inexpensive, and readily available (Sowndarya et al., 2017; Ngwamah et al., 2018; Ramkumar et al., 2019; Pittarate et al., 2021).

Several researchers have investigated many plants for their repellent properties against mosquitoes and many have shown high repellence potentials (Ngwamah et al., 2018, Pal et al., 2021, Ramkumar et al., 2021 Paramanik et al., 2022).

Screening locally available plant species for insecticidal activity will provide justification incorporating them into the National Malaria Elimination Programme (NMEP) and contribute to the growing pool of knowledge on Botanicals that can serve as alternatives to synthetic insecticides. Thus, it is important to investigate the locally available plant species for their insecticidal activities in tropical countries like Nigeria with favourable environment for mosquito breeding and abundant natural vegetations that remain largely untapped.

Hyptis suaveolens commonly called *Daddoya-ta-daji* in Hausa, *efiri* in Yoruba, *nchuanwu* in Igbo, and Bush tea in English is of high economic and medicinal value and employed widely for its mosquito-repellent property. *Hyptis suaveolens* is one of the under explored valuable medicinal plant used to treat various ailments in traditional medicine (Mishra et al., 2021). The leaves of the plant are sources of secondary metabolites that have been implicated as antispasmodic, anti-colitis, anti-rheumatic (Mishra et al., 2021). The essential oils in the leaves have potential antimicrobial and antifungal properties (Mishra et al., 2021). The root extract contained antiretroviral compounds-urosoic acid, a triterpenoid that targets retroviral integrases and proteases blocking the replication of retroviruses (e.g., HIV) (Mishra et al., 2021). Most of these bioactives found in *Hyptis suaveolens* are used as therapeutic agent or as precursors of useful drugs (Mishra et al., 2021).

Hyptis is one of the largest genus with more than 300 species of herbs, shrubs, and small trees. It belongs to Lamiaceae (Mishra et al., 2021). It is an annual or perennial herb exhibiting characteristic genotypic polymorphism and plasticity in morphological and physiological attributes (Mishra et al., 2021). It can grow luxuriantly in diverse environmental conditions due to variety of chemical compounds that provide it adaptive and survival advantages. Stems are herbaceous rarely woody prostrate and branched. Stem is quadrangular, velvety, thick and covered with long hairs and small erect glandular dots. Leaves are simple and oppositely arranged, rarely whorled, petiolate or sessile, hairy with characteristic aroma due to the presence of aromatic essential oils. The petioles are 2-4cm long bearing 2-10cm long and 9.6cm wide leaf blades. Inflorescence is considered of panicles or short umbels with 2–5 zygomorphic, blue purplish flowers arising in clusters at axillary positions. The calyx ranges from 4-6mm long with lower lips divided into 3 lobes, 4 didynamous stamens are inserted at the top of corolla tubes and stylar end is filiform bearing small bilobed stigma at the top of carpel (Mishra et al., 2021). This present study investigated the larvicidal activities of *H. suaveolens* crude extract and fractions on 4th instar larvae of *Culex quinquefasciatus*.

MATERIALS AND METHODS

PLANT MATERIAL

Fresh leaves of *Hyptis suaveolens* were collected from a field along Maryam Babangida Girls Secondary School Bosso Road, Minna (long 13° 18' 37.08" E and lat 13° 41' 41" 48.84"N). Niger State, in July, 2014. The plant was identified as *Hyptis suaveolens* by two independent ethnobotanists (Mrs Jemilat Ibrahim and Mallam Muazam Ibrahim) of the Herbarium Department, National Institute for Pharmaceutical Research and Development, NIPRD, Idu, Abuja. The specimen was assigned Voucher number NIPRD/H/7020 at the Institute by Mrs Jemilat Ibrahim.

PRELIMINARY PHYTOCHEMICAL SCREENING

Qualitative phytochemical screening was conducted to detect the presence or absence of various secondary metabolites (steroids, terpenoids, flavonoids, alkaloids, tannins, carbohydrates, etc.) in "B" extract using the standard methods of Harborne (1984), Trease and Evans (1996) and Medicinal Plants Research and Traditional Medicine Practice (MPR-TMP, 2006).

FRACTIONATION OF ETHYLACETATE CRUDE EXTRACT (B)

Crude ethyl acetate leaf extract of *H. suaveolens* (extract B) was selected for fractionation because it displayed marked antimicrobial and larvicidal activities in previous studies (Babayi et al., 2015).

Bioactivity-guided fractionation was performed as described by NIPRID (2014) using flash column chromatography (FCC) and thin layer chromatography (TLC).

Extract B (5.8 g in weight) was fractionated using flash column chromatography. The column was packed by the slurry method using Silica gel mesh size 60–120 (150 g) and n-hexane (100ml) as the stationary and mobile phases respectively. Elution was carried out with varying proportions of hexane: chloroform and chloroform: ethylacetate. Eluents were monitored by TLC, so that identical fractions were pooled and fractions B₁ to B₅ were obtained. The fractions were collected in sterile bottles and were stored at 4°C until required for use.

PHYTOCHEMICAL SCREENING OF FRACTIONS B1-B5

The methodology employed in phytochemical-screening of the crude extract was adopted and the phytochemicals present in B₁–B₅ were determined in the five fractions obtained above.

LARVAE COLLECTION AND IDENTIFICATION

The fourth instar larvae of *Culex quinquefasciatus* were collected at three different locations from Bosso Market Drainage in September 2014. The larvae were identified by an Entomologist, Prof. Israel Olayemi Kayode from the Department of Biological Sciences, Federal University of Technology, Minna.

The biological assay was performed under controlled conditions having kept the larvae at $25 \pm 2^{\circ}\text{C}$, a relative humidity of $75 \pm 5\%$, and a 12h light/dark cycle.

PREPARATION OF STOCK SOLUTION: Stock solution was prepared according to WHO (2005) recommended standard by dissolving the dried extract in the solvent of extraction in ratio 1:9 (2 g of extract in 18 ml of solvent) to obtain a final concentration of 0.11 g/ml. This was done for all the fractions in separate test tubes and all were covered and labeled accordingly. A control plate was prepared containing ethyl acetate only.

PREPARATION OF TEST CONCENTRATION

Test concentration was prepared by adding 1.0ml of the stock solution into 10 ml of sterile distilled water to obtain a final concentration of 0.011 g/ml. The procedure was repeated for all the the fractions separately and labeled accordingly. The test concentration was further increased to 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml and 6.0 ml to obtain the final concentration of 0.022 g/ml, 0.033 g/ml, 0.044 g/ml, 0.055 g/ml and 0.066 g/ml for the crude and all the fractions and were all labeled accordingly (Olayemi, 2013).

LARVICIDAL ACTIVITY OF THE MEDICINAL PLANT AND FRACTIONS

The method of WHO (2005) was employed in the determination of the larvicidal activity of the medicinal plant extract and fractions. The larvae were standardized by measuring one hundred milliliters (100 mL) of sterile distilled water which was transferred to 12 transparent small bowls that were already labeled with different concentrations in two replicates (0.011R1, 0.011R2, 0.022R1, 0.022R2, 0.033R1, 0.033R2, 0.044R1, 0.044R2, 0.055R1, 0.055R2, 0.066R1, and 0.066R2), while 100 ml of sterile distilled water was also measured for the Control in replicates (Control R1 and Control R2). Twenty-five (25) larvae were then counted into each bowl and were ready for the larvicidal bioassay (this was done for the crude extract and its fractions respectively).

One point zero millilitre (1.0 mL) of ethyl acetate was added to controlR1 and controlR2 that had just 100 ml of sterile distilled water and 25 larvae, while 0.11 g/ml, 0.22 g/ml, 0.33 g/ml, 0.44 g/ml, 0.55 g/ml, and 0.66 g/ml of the test concentration was added to the bowls and their replicate bowls according to the labeling. The bowls were then covered with untreated net held tight to the bowls with rubber ring to prevent the emerging adults from escaping. Observation for larval mortality pupal development or mortality and emergence of adults were carried out at different intervals (15 min, 30 min, 1 hour, 2 hours 3 hours, 6 hours, 12 hours, 18 hours, 24 hours, 48 hours and 72 hours). After treatment, dead adult mosquitoes were counted and preserved in a 50% ethanol solution. Control mortality was corrected using Abbott's (1925) formula, when larvae mortality exceeded 5.00 percent.

$$\text{Percentage mortality} = \frac{\% \text{ mortality in treated} - \% \text{ mortality in control}}{100 - \% \text{ mortality in control}} \times 100$$

DATA ANALYSIS

Mortality in the larval bioassay was subjected to the probit analysis to determine LC_{50} , LC_{90} at 95% confidence limits. Mortality in control was corrected using Abbott's formula. Associations between the tested extract concentrations, larvae mortality and time mortality were assessed using multiple regression correlation analysis.

RESULTS

The crude extract and fractions of *Hyptis suaveolens* contained alkaloids, free anthraquinones, saponins, sterols, flavonoids, volatile oils and terpenes. Tannins and glycosides were absent in the fractions (Tables 1 and 3).

The effects of crude ethyl acetate extract and fractions of *Hyptis suaveolens* on Fourth instar larvae of *C. quinquefasciatus* are shown in Fig. 1–6. Exposure of the larvae to lethal concentration of the extract resulted in mortality. All the larvae survived in the negative control group that were exposed to water only, while < 5% mortality was recorded for the positive control exposed to 1.0mL of ethyl acetate. The mortality rate represented by positive values increased with time as well as concentration (0.011g/mL- 0.066g/mL). A significant increase in mortality rate was observed after 24 hours, this increase continued until 72hours (Fig. 1). In fraction B₁, marked increase in mortality rate with increase in concentration at 48 and 72 hours was observed (Fig. 2). An increased mortality rate was observed after 24 hours in fraction B₂ and the increase continued with significant changes between 24 and 48 hours (Fig. 3). Increased mortality rate was observed after 6 hours from fraction B₃ and a significant change was observed between 12 and 72 hours (Fig. 4). In fraction B₄ and B₅, a significant increase in mortality rate with increase in concentration was observed after 24 hours and the highest mortality rate between 48 and 72 hours (Fig. 5–6).

Table 1
Phytochemical constituents of leaf extract of *Hyptis suaveolens*

Phytochemical constituents	Extract of <i>Hyptis suaveolens</i>	
	Hexane (A)	Ethylacetate (B)
Alkaloids	-	+
i. Meyer's reagent	-	+
ii. Wagner's reagent		
Anthraquinones	-	+
Balsam	-	+
Carbohydrates	-	+
Glycosides	-	+
Saponins	-	+
Sterols	+	+
Tannins	-	-
Terpenes	+	+
Flavonoids	-	+
+ = Present and - = Absent		

Table 2
Description of major fractions from B (ethyl acetate fractions of *Hyptis suaveolens*)

Fraction	Solvent system	Code	Physical appearance	Weight (mg)	Number of spots on TLC plates
1-7	Hexane (100%)	B1	Colorless	200	5
8-17	Hex: CHCl ₃ (4:1)	B2	Light yellow oil	500	5
18-30	Hex: CHCl ₃ (1:1)	B3	Greenish black oily mass	340	5
31-35	CHCl ₃ (100%)	B4	Dark green oily mass	600	5
36-71	CHCl ₃ :EtOAc (9:1)	B5	Greenish black gummy mass	700	5

B1 = fraction1, B2 = fraction 2, B3 = fraction 3, B4 = fraction 4, B5 = fraction 5, Hex = Hexane, CHCl₃ = Chloroform, EtOAc = Ethylacetate and mg-milligram.

Table 3
Phytochemical components of fraction B₁-B₅ extract of
Hyptis suaveolens

Phytochemical components	B ₁	B ₂	B ₃	B ₄	B ₅
Alkaloids	-	+	-	-	-
i. Meyer's reagent					
ii. Wagner's reagent	-	+	-	-	-
Anthraquinone	-	+	+	+	+
flavonoids	-	-	-	-	-
Glycosides	-	-	-	-	-
Saponins	-	-	-	-	-
Sterols	+	+	+	-	+
Tannin	-	-	-	-	-
Terpenes	+	+	+	+	+
Volatile oil	+	-	-	-	-
+ = Present and - = Absent					

The LC₅₀ and LC₉₀ of B and B₁-B₅ extracts are represented on Table 4. The extract B had LC₅₀ of 0.366 mg/ml and LC₉₀ of 0.606 mg/ml after 72 hours of exposure. All the fractions had a lower LC₅₀ than the crude extract of *H. suaveolens*. B₁ had LC₅₀ of 0.681 mg/ml and LC₉₀ of 0.320 mg/ml, B₂ had LC₅₀ of 0.262 mg/ml and LC₉₀ of 0.404 mg/ml, B₃ that had LC₅₀ of 0.185 and LC₉₀ of 0.730 mg/ml, B₄ had LC₅₀ of 0.054 mg/ml and LC₉₀ of 0.530 mg/ml and B₅ had LC₅₀ of 0.320 mg/ml and LC₉₀ of 0.360 mg/ml. The LC₅₀ and LC₉₀ for the crude extract and fractions attained all these values after 72 hours of exposure (Table 4).

Table 4
LC50 and LC90 of the crude extract and fractions of *Hyptis suaveolens*

Extract	Experimental Duration (h)	LC ₅₀ (mg/mL)	LC ₉₀ (mg/mL)	R ²	Regression Equation
Crude	72	0.366	0.606	0.935	Y = 40.85x – 2.688
Fraction 1	72	-0.681	0.320	0.005	Y = 10.24x + 19.22
Fraction 2	72	-0.262	0.404	0.861	Y = 14.71x + 16.10
Fraction 3	72	-0.185	0.730	0.864	Y = 10.71x + 14.23
Fraction 4	72	-0.054	0.530	0.951	Y = 16.78x + 13.16
Fraction 5	72	-0.320	0.360	0.975	Y = 14.42x + 16.86

Mg/mL: milligram per milliliter, h: hours, LC50: Lethal concentration required to kill 50 per cent of the population exposed, LC90: Lethal concentration required to kill 90 per cent of the population exposed, Significant at P < 0.05 level.

Discussion

Phytochemicals are largely secondary metabolites that help plants endure herbivorous predators, continuous selection pressures and other environmental conditions. They include terpenoids, phenolics e.t.c which have insecticidal properties. *Hyptis suaveolens* extracts studied was found to contain one or more of the following phytochemical constituents; alkaloids, anthraquinones, balsam, carbohydrate, glycosides, saponins, sterols, terpenes, flavonoids and volatile oils (Tables 1 & 3). Extraction of compounds from medicinal plants permits the demonstration of their biological activities. It also facilitates fractionation and monitoring of their biological activities. This may lead to isolation of active compounds and their structural elucidation. These structures are used as lead compounds for the synthesis of a more potent drug with reduced toxicity (MPR-TMP, 2006). Earlier studies observed that phytochemicals have a major role in mosquito control programme. Terpenes are larvae repellent and bind to olfactory glands of female mosquitoes hindering smell perception. Flavonoids inhibit growth and development of insect larvae by interfering with digestion. Anthraquinone interfere with life cycle of insects. Sterols impair reproduction and hormonal regulation. Balsam destroys physiological processes (Adonsou et al., 2016). Thomas et al. (2017) observed the presence of carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins in the plant extract having mosquito larvicidal activity. Ojha and Pattabhiramaiah (2013), reported the use of saponin from *Jatropha Curcas* Seed Oil as a natural larvicidal against *Aedes aegypti* and *Culex pipiens*. Extract A (hexane) contained sterols and terpenes while Extract B (ethylacetate) contained alkaloids, anthraquinones, balsam, carbohydrate, glycosides, saponins, sterols, terpenes and flavonoids, they contaminated different constituents because different solvents were used for the extraction and each solvent has specific affinity for different components.

There was less than 5% mortality of larvae recorded for positive control (group exposed to ethyl acetate only). This is in agreement with WHO standard (2022) for positive control which says that less than 5% mortality indicates that mortality is not due to the solvent but due to the nature of the larvae. 100% survival was observed for the negative control which indicates that the larvae might have been exposed to better feeding, less overcrowding and an enabling environment (Fig. 1).

The results of the present study (Fig. 1) indicate that the crude ethylacetate leaf extract of *H. suaveolens* had a marked larvicidal activity. This may be due to the phytochemical components detected in the crude extract which may be acting singly or synergistically to inhibit the development of larvae into pupae. Agenyi, (2020); Li et al., (2020); Mishra et al., (2021); and Akharaiyi et al., (2023) attributed the activity of *H. suaveolens* to the presence of these phytochemical components. Furthermore, Makuwa and Serepa-Dlamini, (2021). also attributed the activity of crude plant extract to the complex mixture of active compounds.

Hundred percent mortality was observed for crude extract after 72 h of larval exposure (Fig. 1). The mortality of the larvae increased with increased concentration of the crude extract. Shoukat et al. (2020), observed higher larvicidal effect with increased concentration of *Sophora alopecuroides* on *Aedes albopictus*. Plants use their secondary metabolites in defense mechanisms. These secondary metabolites like sterols, triterpenes, phenolics, essential oils, saponins can be used to control mosquito vectors (Balumahendhiran et al., 2019). This study identified the presence of secondary metabolites like terpenoids, sterols, saponins and free glycoside bound anthraquinones in the crude extract of medicinal plant which are responsible for larvae death in *H. suaveolens*.

In the present study, all the five column chromatographic fractions (B₁, B₂, B₃, B₄ and B₅) showed enhanced larvicidal activity at very low concentrations when compared to the crude extract (Fig. 2–6). This suggests that the fractions of the medicinal plant were more potent than the crude. Enhanced larvicidal activity of column chromatographic fractions were also reported by Eukubay et al. (2023) on an ethanol leaf extract from *Ricinus communis* against the late third and fourth instars larvae of *Anopheles arabiensis*. Charria-Girón et al. (2021) observed that the amount of active components in the crude extracts or portions from medicinal plants may be small or too diluted, so that when fractionated, these components become concentrated and therefore exhibit better activity. Fractionation of crude plant extract has reportedly improved the antimicrobial potency of some medicinal plants (Balavijayalakshmi and Ramalakshmi, 2017; Shoukat et al., 2020; Charria-Girón et al., 2021; Chassagne et al., 2021; Narayanan et al., 2023).

The fractions exhibited higher potency than the crude because fractions may contain a compound with higher affinity for target site causing higher inhibition. Again, some fractions may be more efficiently absorbed and thus spreading throughout the insect surface and getting to the target site faster. Fractions may contain compounds that act synergistically to bring about inhibitory effects. Fractionation involves separation and purifying active components from crude extract removing inactive compounds.

Thus, after fractionation, the resulting fractions contain active ingredients that are more concentrated and therefore bring about enhanced action than the crude extract.

Fractionation enhance the removal of inactive substances that may interfere with activity. Thus, fractions contain pure, potent substances with higher activity than the crude. Fractionation may also lead to modification of active compounds that may be more stable and more active thus demonstrating enhanced activity than the crude (Roell et al., 2017; Abubakar and Haque, 2020).

The lethal concentration (LC₅₀ and LC₉₀) is the concentration of the extract that kills 50% or 90% of the larvae population. The crude extract of the *H. suaveolens* exhibited LC₅₀ and LC₉₀ of 0.366 and 0.606, values higher than that observed by Ramkumar et al. (2022) on *Cipadessa baccifera* leaf extract synthesized nano-particles against *C. quinquefasciatus* (0.05 and 0.03). Paramanik et al. (2022) reported LC₅₀ and LC₉₀ values of (0.56 and 1.06), values that are higher than that obtained in the current study. Low LC₅₀ and LC₉₀ values imply high effectiveness of the larvicides (Rahuman *et al.*, 2008; Babayi et al., 2015).

The potency of the crude extract and fractions in terms of LC₅₀ and LC₉₀ are in the order; B₄ > B₂ > B₅ > B₃ > B₁. The low LC₅₀ exhibited by the crude extract and fractions of *H. suaveolens* in the present study indicated their remarkable potentials in reducing the population of mosquito vector species

Conclusion

The present study revealed the larvicidal effects of *Hyptis suaveolens* and its fractions against 4th instar larvae of *Culex quinquefasciatus*, the vector of avian malaria, West Nile virus, St. Louis encephalitis, filariasis and Japanese encephalitis. The crude plant extract and its fractions exhibited remarkable larvicidal activity at low concentrations. The fractions showed better larvicidal activity than the crude suggesting that the larvicidal activity of the crude extract is not due to synergistic effects of its fractions.

Declarations

Conflict of Interest

There is no conflict of interest among the authors

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Figures

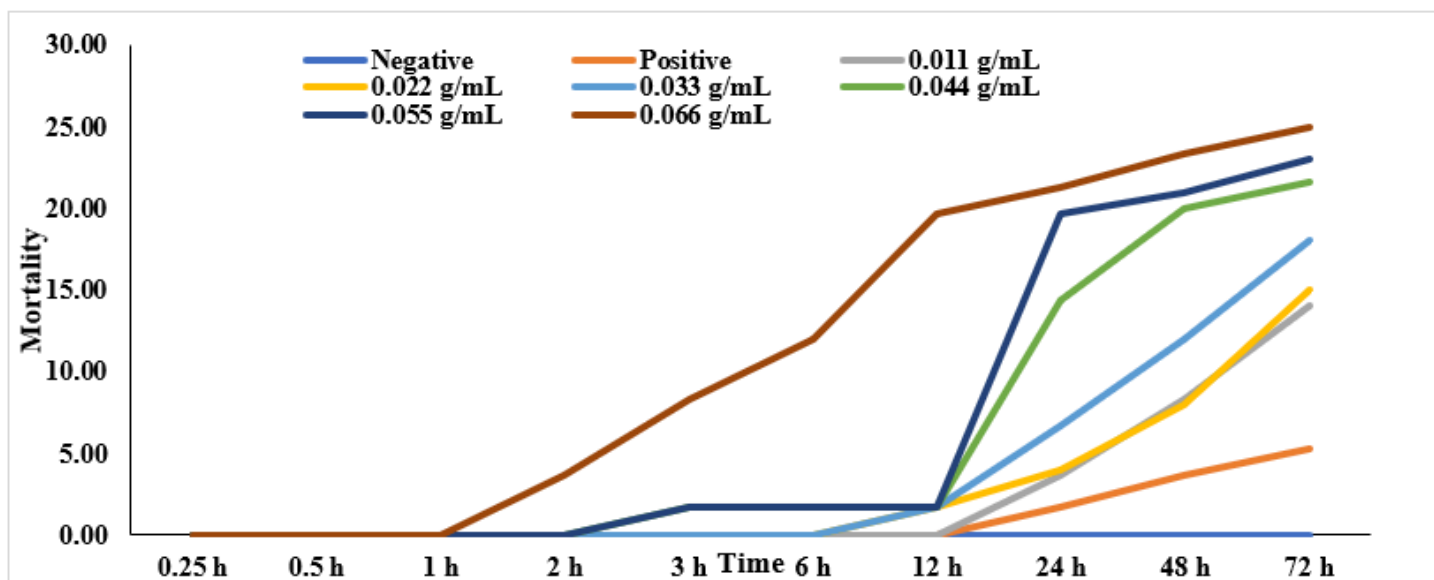


Figure 1

Effect of crude ethyl acetate leaf extract of *Hyptis suaveolens* on the mortality of larvae of *Culex quinquefasciatus*

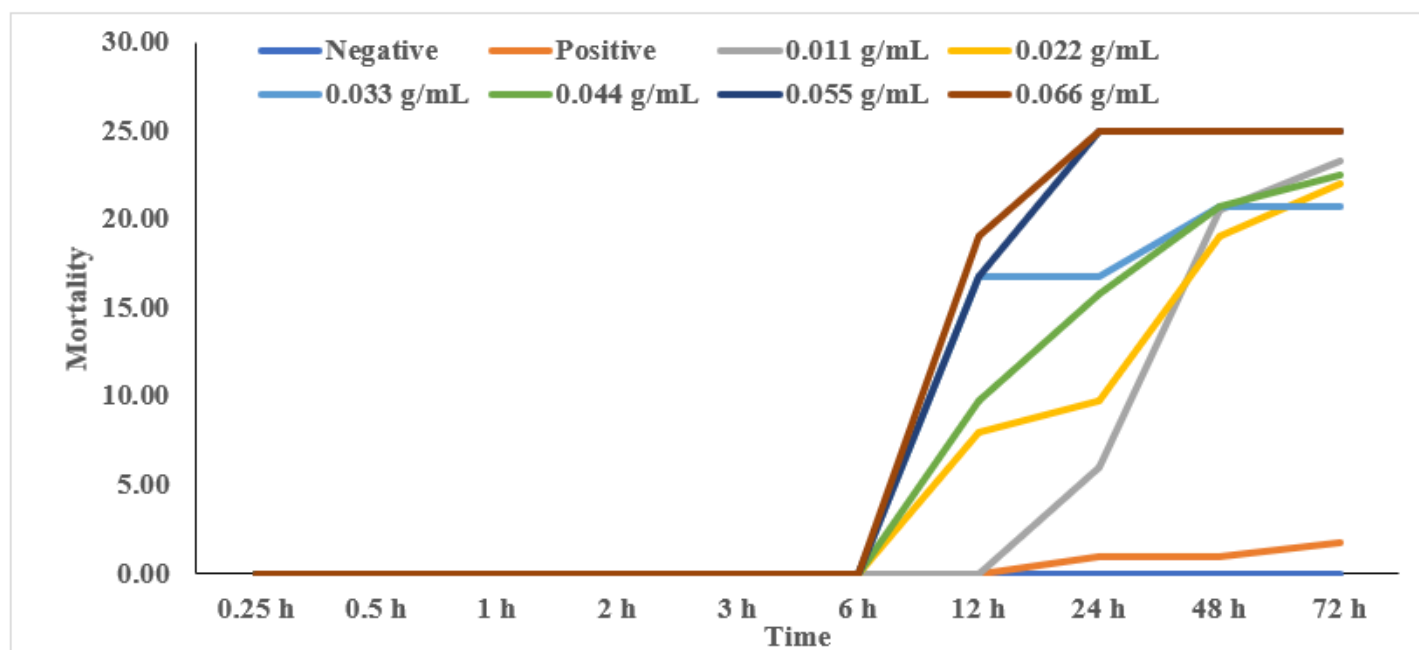


Figure 2

Effect of fraction B₁ on the mortality of larvae of *Culex quinquefasciatus*

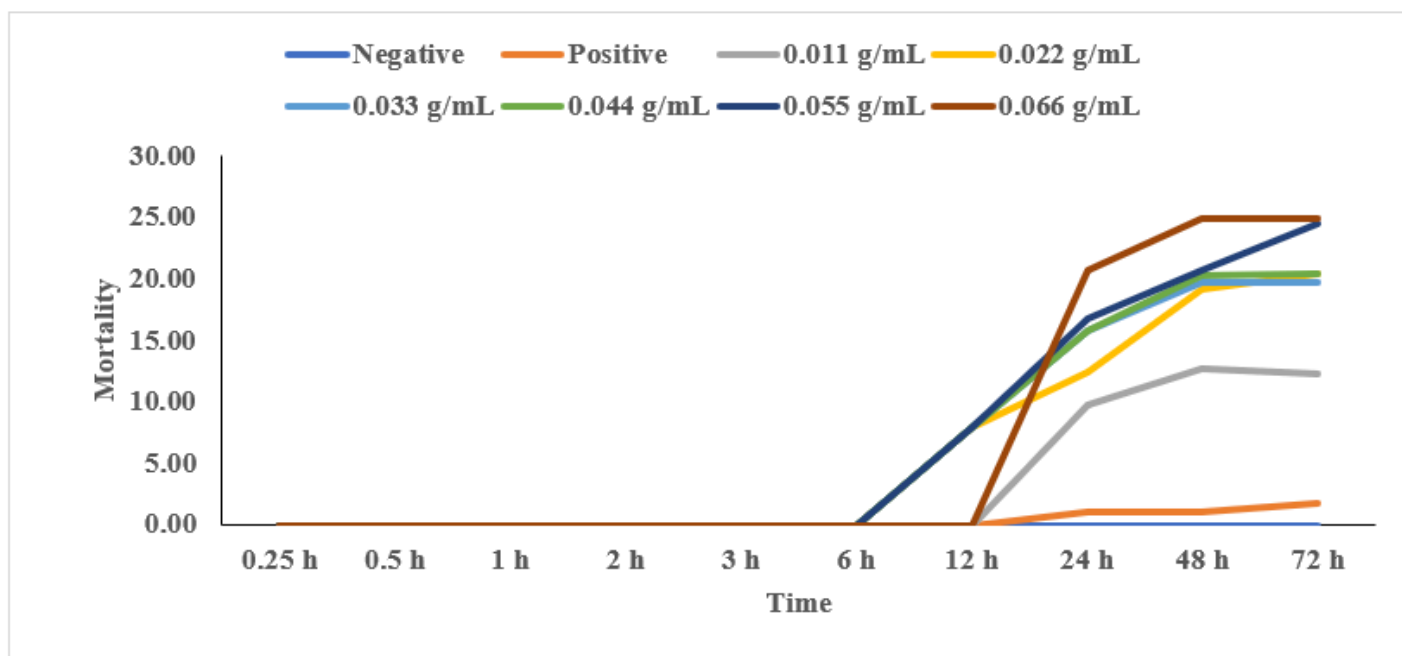


Figure 3

Effect of fraction B₂ on the mortality of larvae of *Culex quinquefasciatus*

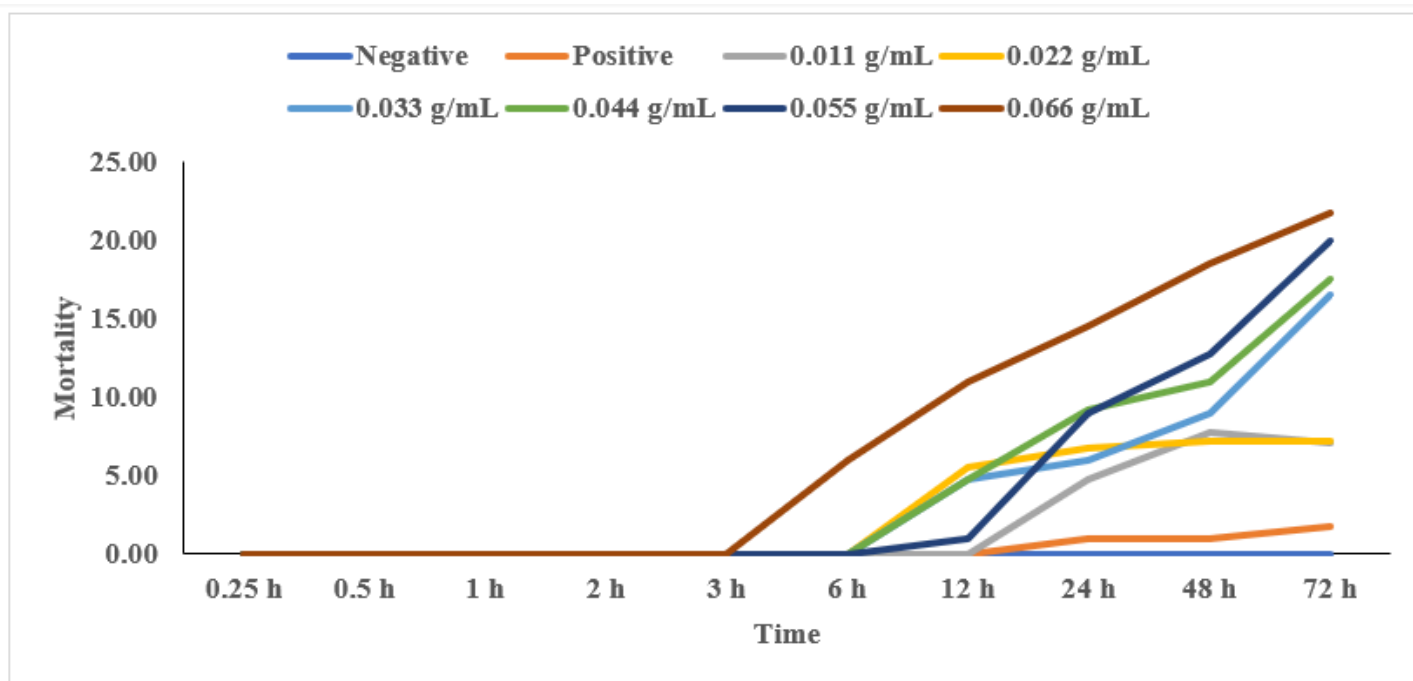


Figure 4

Effect of fraction B₃ on the mortality of larvae of *Culex quinquefasciatus*

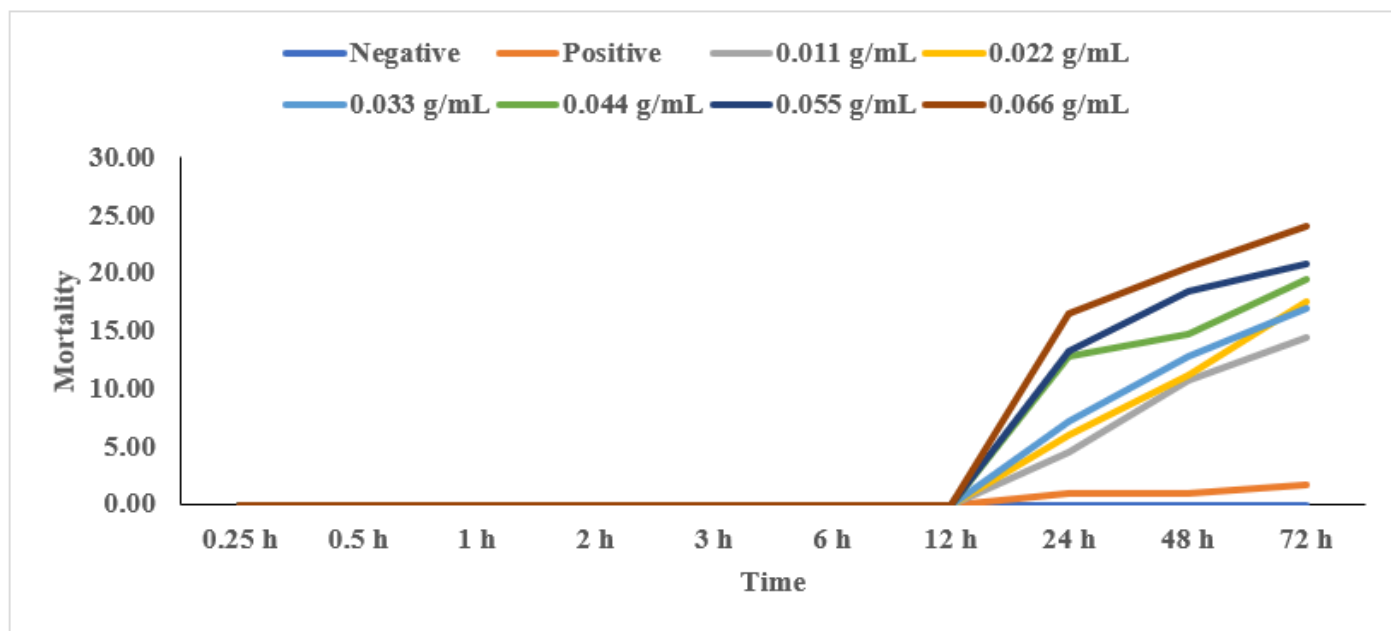


Figure 5

Effect of fraction B₄ on the mortality of larvae of *Culex quinquefasciatus*

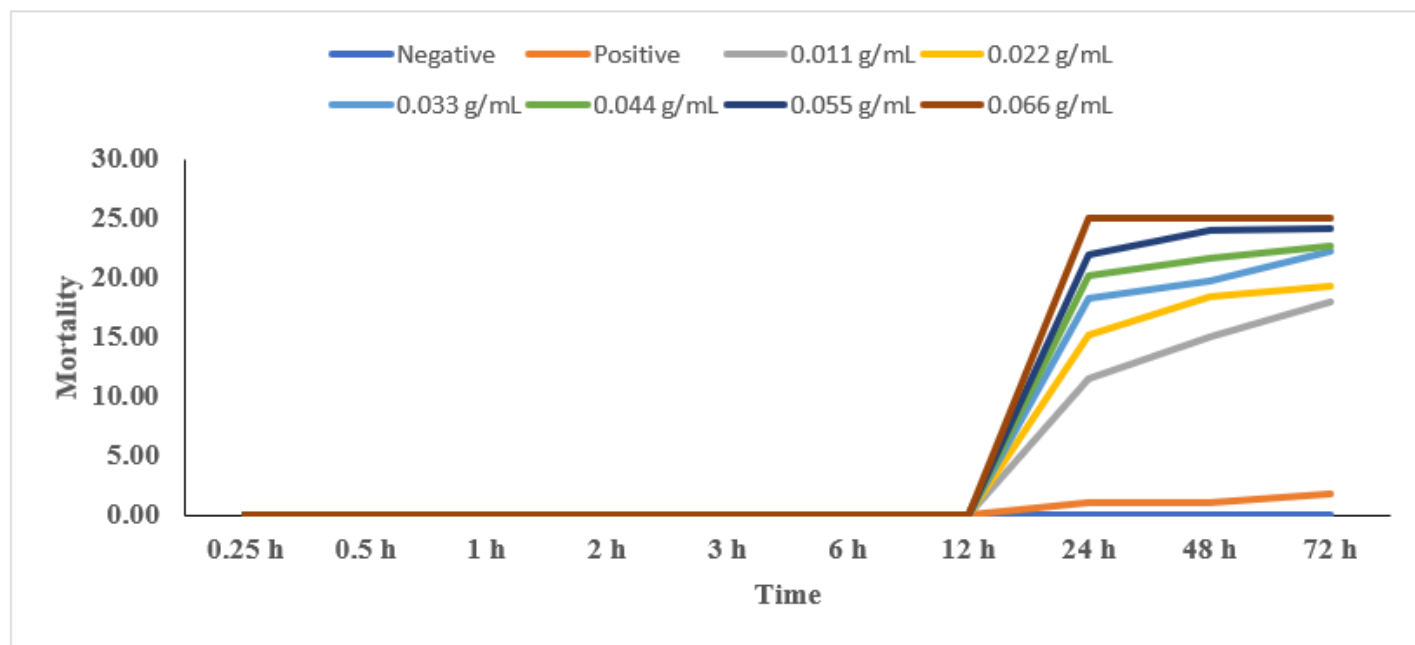


Figure 6

Effect of fraction B₅ on the mortality of larvae of *Culex quinquefasciatus*