



Efficacy of the Horse Chestnut, *Aesculus hippocastanum* Seeds Extract on some Biological Activities of *Culex pipiens* and the Detection of its Phytochemical Constituents

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ABSTRACT

In the present study, mosquito larvicidal and repellent activity and phytochemical screening of the horse chestnut, *Aesculus hippocastanum* seeds extract have been evaluated against *Culex pipiens* mosquito. The obtained results indicated that there was a positive correlation between the concentration of *Aesculus hippocastanum* seeds extract and the total larval and pupal mortality percent, the recorded LC₅₀ was 122 and 76 ppm for larvae and pupae, respectively. In addition, a remarkable reduction in both the pupation and adult emergence percent were obtained. Also, the extract showed prolonged activity on both larval and pupal duration. Moreover, various degrees of morphogenic abnormalities were observed in the immature and adult stages. The antifeedant and repellent activity of the present plant extract was investigated, it exhibits the highest protection (100%) from the bites of starved females at the dose of 3mg/cm². the major bioactive chemical constituents in *Aesculus hippocastanum* seeds extract were identified using HPLC and FT-IR analysis. These results suggested the *Aesculus hippocastanum* seeds extract as a promising adult emergence inhibitor and repellent agent against the vector mosquitoes and might be used in small volume aquatic habitats or other breeding sites of mosquitoes.

INTRODUCTION

Mosquitoes constitute a major public health problem as vectors of serious human diseases. They not only cause nuisance by their bites but also transmit deadly diseases like malaria, filariasis, yellow fever, dengue and Japanese encephalitis (Lerdthusnee *et al.*, 1995; Jang *et al.*, 2002 and Nauen, 2007). The mosquito, *Culex pipiens* is the primary vector of several arboviruses, such as the West Nile virus, Japanese encephalitis virus, and Rift Valley fever virus, as well as worms responsible for lymphatic filariasis (Diaz-Badillo *et al.*, 2009).

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and has led to resurgences in the mosquito population. It has also resulted in the development of resistance, ecological imbalance, harmful effect on humans and animals and undesirable effects on non-target organisms (Kamaraj *et al.*, 2008). This has necessitated the need for research and development of an environmentally safe, biodegradable indigenous method for vector control.

Recent studies stimulated the investigation of insecticidal properties of plant-derived extracts and concluded that they are environmentally safe, degradable, and target specific (Senthil *et al.*, 2005). Some plants are known to contain toxic ingredients, which can play a useful role in the control of disease vectors. Sukumar *et al.*, (1991) reviewed the bioactivity observed for 344 plant species against mosquitoes. Some phytochemicals acted as general toxicants to all mosquito stages, whereas others interfere with growth and reproduction.

The present investigation assessed the larvicidal and repellent effect of *Aesculus hippocastanum* seeds extracts against *Culex pipiens* Mosquitoes.

MATERIALS AND METHODS

Mosquito Rearing:

Culex pipiens mosquitoes were used in this study; they were reared and maintained for several generations at the Medical Entomology Research Laboratory, Zoology and Entomology Department, Faculty of Science, Al-Azhar University, Cairo, under controlled conditions using the standard procedures (Kasap and Demirhan, 1992).

Plant Extraction:

The dried seeds of horse chestnut plant, *Aesculus hippocastanum* were pulverized into powder using a grinding machine. Ten grams of powder was weighed and added to 100 ml of prepared solution, consisting of 70% ethanol + 8% ethyl acetate + 22% distilled water in a glass container. The mixture was immersed in a water bath set at 60° C for 5 hours and stirring of the mixture was performed at intervals. The mixture was then removed from the water bath and allowed to cool at room temperature for 72 h; after that, it was filtered. The combined solvent extract was concentrated under vacuum at 40° C until solvents were completely removed. This dried extract of seeds of the plant was used for the biological activity tests.

Extract Analysis:

The major compounds in the plant extract were identified using High-Performance Liquid Chromatography HPLC

(Sykam ROUTINE HPLC System S 500) at EPRI institute.

The characterized function groups and the confirming of the constituents of the extract were analysed by FT-IR spectroscopy (Model 960 Moog, ATI Mattson Infinity Series, USA) at EPRI institute.

Bioassays:

Different concentrations of the plant extract were prepared by dissolving the required amount of the plant extract in distilled water, 1 ppm of PEG 40 Hydrogenated Castor Oil was added as an emulsifier.

Bioassays were conducted in plastic cups containing the needed amount of the plant extract dissolved in 250 ml of distilled water. Twenty-five third instar larvae of the mosquito were then transferred to the test medium; the experiment was performed in three replicates for each concentration. Control larvae received 1 ppm of (PEG40) in 250 ml of distilled water. All larvae were exposed until pupation, and mortality was recorded at 24-h intervals. Dead larvae and pupae were counted and removed immediately.

The morphological aberrations of the dead larvae and pupae were determined and counted; also the larval and pupal duration were calculated.

The growth index was estimated by using the following equation (Saxena and Sumithra, 1985).

$$\text{Growth index} = A / B$$

(where: A = % of adult emergence and B = mean developmental period in days).

Repellent Activity:

Evaluation of the repellent activity of *Aesculus hippocastanum* seeds extract was carried out against the adult females of *Culex pipiens*. Three to four days old blood starved female *Culex pipiens* mosquitoes were kept in a net cage (20 cm x 20 cm x 20 cm). One ml from each concentration was directly applied on 30 cm² of the ventral surface of a pigeon after removal of feathers, and then the pigeon was placed in the cage containing the starved mosquitoes. After two hours the fed

and unfed females were counted. The experiment was conducted three times; fifteen females were used in each experiment. The negative control test was carried out using water and PEG40, and a positive control test was carried out using commercial repellent (DEET) from Jonson Wax Egypt.

The repellency percent was calculated by using the following equation (Abbott, 1925):

Repellent % = $[(\%A - \%B) / (100 - \%B)] \times 100$ (where: A = percent of unfed females in treatment and B = percent of unfed females in control).

Statistical Analysis:

Statistical analysis of the data was carried out according to the method of Lentner *et al.*, (1982). LC₅₀ was calculated using multiple linear regression (Finney, 1971).

RESULTS

Data presented in Table (1) showed that the mortality of *Culex pipiens* larvae exposed to different concentrations of *Aesculus hippocastanum* seeds extract was varied with the exposure time. After 24 hours of contact with the extract, 22.7% of larval mortalities were obtained for the highest concentration (250 ppm) compared to 1.3% dead larvae for the lowest concentration (50 ppm), while after 48 hours of exposure, the concentration of 250 ppm gave 29.3% of larval mortalities. The highest mortalities rates were observed after 72 hours of exposure, where at the concentration of 250 ppm it recorded 45.3%. The data also showed that there was a positive correlation between the concentration of *Aesculus hippocastanum* seeds extract and the total larval mortality percent. The highest concentration (250 ppm) induced complete mortality at the end of larval duration (100%); meanwhile, the lowest value (18.6%) occurred at the lowest concentration (50 ppm) compared to 0.0% for the control group.

The *Aesculus hippocastanum* seeds extract induced some malformation effects on the treated larvae. The malformation percent for larvae was 2.7% at the lowest concentration (50 ppm), while it was 54.7%

at the highest concentration (250 ppm). At the control group, no malformed larvae were observed.

The results indicated that the control third instar larvae reached the pupal stage in 4.6 ± 1.2 days; this duration was significantly prolonged to 7.79 ± 1.53 days at the concentration of 200 ppm.

The pupation rate was found to be considerably decreased as the concentration of *Aesculus hippocastanum* seeds extract increased; it recorded 81.4% at the lowest concentration (50 ppm), while at the highest concentration (250 ppm) it recorded 0.0% compared to 100% for the control group.

The data found in Table (2) revealed that the pupal mortality percent was greatly affected by *Aesculus hippocastanum* seeds extract, it reached to 100% at the concentration of 200 ppm compared to 1.3% for the untreated group.

It is cleared from the data that, *Aesculus hippocastanum* seeds extract had a malformation effect against the pupae resulted from the treated larvae at all the used concentrations. The pupal malformation percent was 13.1% at the concentration of 50 ppm, while it was 85.7% at the concentration of 200 ppm. There were no malformed pupae at the control group.

The pupal duration was significantly prolonged to 2.69 ± 0.48 and 2.5 ± 0.55 days at the concentrations of 100 and 150 ppm, respectively. While the pupal duration was 2.3 ± 0.7 days at the concentration 50 ppm compared to 1.56 ± 0.52 days for the untreated pupae.

Moreover, the data in table (2) showed that the total mortality percent of larvae and pupae was 58.7% at the concentration of 50 ppm and 100% at the concentration of 250 ppm compared to 1.3% for the control group.

There was a reduction in the adult emergence percent among the adults produced from the treated third instar larvae at all used concentrations of *Aesculus hippocastanum* seeds extract. The adult emergence percent recorded 0.0% at the two highest concentrations (200 and 250 ppm) compared to 98.7% for the control group. The

results also revealed that there was marked domination in males over females in the emerged adults from larvae treated with different concentrations of *Aesculus hippocastanum* seeds extract. The calculated sex ratio was (5:1) at the concentration of 150 ppm while it was (0.9:1) at the control group.

Moreover, the mean developmental period was significantly prolonged to 9.79 ± 2.18 and 9.57 ± 1.91 days at the concentrations of 100 and 150 ppm, respectively compared to 6.59 ± 2.16 days for the control group. A very retarded effect on the growth of larvae, pupae, and adult *Culex pipiens* was observed. The growth index recorded 6.2, 3.15 and 2.32 at the concentrations of 50, 100 and 150 ppm, respectively, compared to 16 for the untreated group.

The calculated LC_{50} , the Slope (b) and the Correlation coefficient (r^2) for both larval and pupal mortalities were presented in Table (3). The calculated LC_{50} was 122 ppm and 76 for the larval and pupal mortalities, respectively.

Aesculus hippocastanum seeds extract was found to possess a remarkable repellency effect against starved *Culex pipiens* females. As shown in Table (4) *Aesculus hippocastanum* seeds extract induced 100% repellency at the doses of 3 mg/cm² compared to 100% repellence for DEET.

HPLC was used to study the main chemical components of *Aesculus hippocastanum* seeds extract. The retention times, chemical formulas and the compounds

name for the numbered peaks in the chromatogram Figure (1) are listed in Table (5). This extract consists mainly of Quercetin 3,4-diglucoside, Aesculin, escin Ia, escin Ib, isoescin Ia, and isoescin Ib, they are corresponding to Peaks 1, 2, 5, 6, 7 and 8, respectively. Peaks 3, 4, 9, 10 and 11 were identified as Leucocyanidin, Fraxin, Procyanidin A2, Quercetin and Kaempferol. The molecular structures of these compounds are shown in Figure (3).

Figure (2) presented The FT-IR spectrum of *Aesculus hippocastanum* seeds extract. The spectrum is characterized by several absorption bands of which the broad one, centered at about 3400 cm⁻¹ is due to the stretching vibrations of the OH functional groups of alcohols and phenols. The doublet at 2925 and 2850 cm⁻¹ is attributable mainly to the asymmetric and symmetric stretching modes of the CH₂ methylene group, the most abundant structural unit in plant products. The 1730 cm⁻¹ band comes from the stretching vibration of the C=O group indicative of the presence of carbonyl compounds mostly of esteric type (esters of fatty acids). The set of bands between 1650 and 1500 cm⁻¹ is due to vibrational modes of aromatic rings probably in aromatic derivatives and unsaturated conjugated compounds. This set of bands is referred to as the aromatic domain. The absorption bands between 1200 and 950 cm⁻¹ have been assigned to the deformation modes of C-O and O-H groups of alcohol.

Table 1: Effect of *Aesculus hippocastanum* seeds extract on mortality, malformation, duration of larvae and pupation percent of *Culex pipiens*.

Conc. (ppm)	Larval mortality %				Larval mal. %	Larval dur. mean \pm SD.	Pupation %
	24h	48h	72h	total (at the end of l.d.)			
Control	–	–	–	–	–	4.6 \pm 1.2	100
50	1.3	4	6.7	18.6	2.7	5.9 \pm 1.6	81.4
100	4	5.3	12	30.7	12	7.1 \pm 1.7*	69.3
150	13.3	8	30.7	64	24	7.07 \pm 1.36*	36
200	24	17.3	38.7	81.3	37.3	7.79 \pm 1.53*	18.7
250	22.7	29.3	45.3	100	54.7	–	–

Where: Conc = concentration, l.d. = larval duration, mal. = malformation, dur. = duration, SD = Standard Deviation and * = Significant ($P < 0.05$).

Table 2: Effect of *Aesculus hippocastanum* seeds extract on mortality, malformation, duration of pupae, adult emergence percent, sex ratio and growth index of *Culex pipiens*.

Conc. (ppm)	Pupal mort. %	Pupal mal. %	Pupal dur. mean \pm SD.	larval and pupal mort. %	Adult emerg. %	Sex ratio (male: female)	Mean development period (days) \pm SD.	Growth index
Control	1.3	–	1.56 \pm 0.52	1.3	98.7	(0.9 : 1)	6.16 \pm 1.72	16
50	49.2	13.1	2.3 \pm 0.7	58.7	50.8	(2.4 : 1)	8.2 \pm 2.3	6.2
100	69.2	30.8	2.69 \pm 0.48*	78.7	30.8	(3 : 1)	9.79 \pm 2.18*	3.15
150	77.8	55.6	2.5 \pm 0.55*	92	22.2	(5 : 1)	9.57 \pm 1.91*	2.32
200	100	85.7	–	100	–	(1.5 : 1)	–	–
250	–	–	–	100	–	–	–	–

Where: Conc. = concentration, mort. = mortality, mal. = malformation, dur. = duration, emerg. = emergence SD = Standard Deviation and * = Significant ($P < 0.05$).

Table 3: Relative efficiency of *Aesculus hippocastanum* seeds extract against *Culex pipiens* larvae and pupae

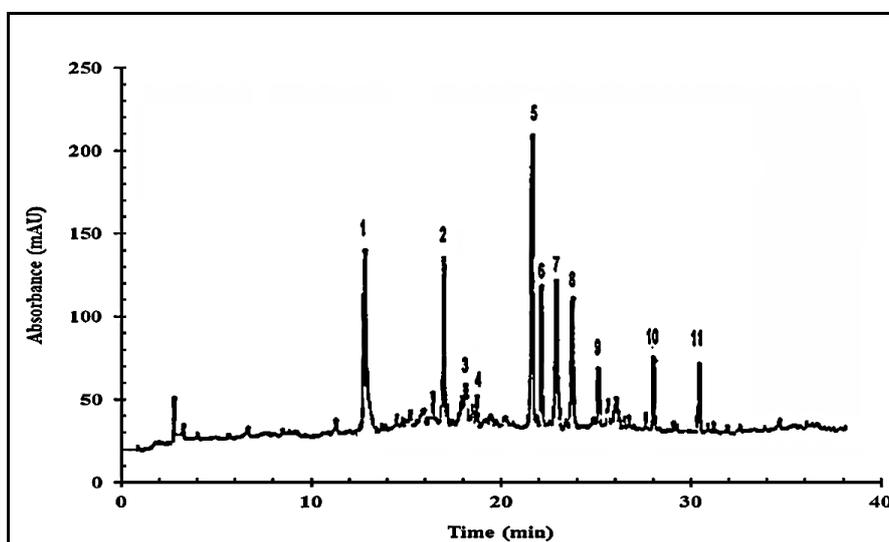
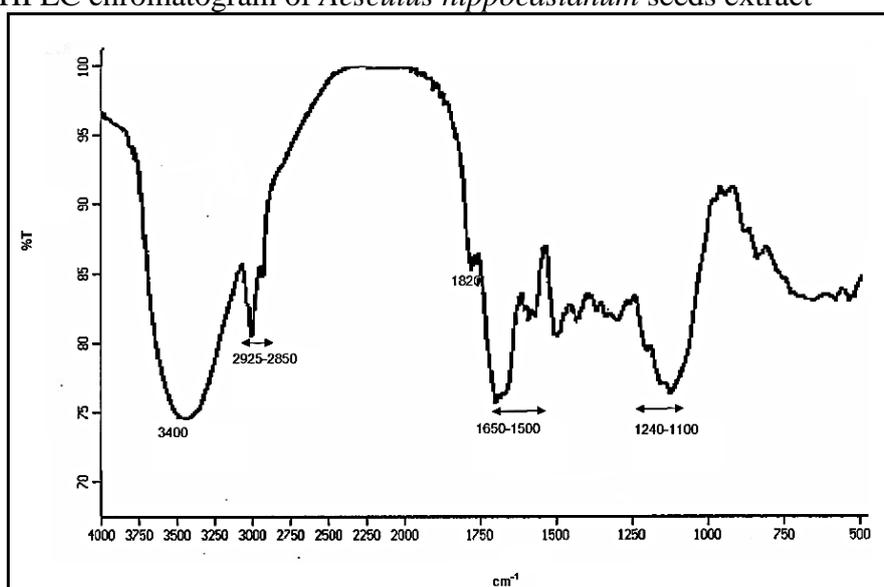
Stage	LC ₅₀ (PPM)	Slope (b)	Correlation coefficient (r ²)
Larvae	122	0.4122	0.9874
Pupae	76	0.452	0.9182

Table (4): Repellence effect of *Aesculus hippocastanum* seeds extract on *Culex pipiens* females.

Dose mg/cm ²	No. of tested females	Fed females		Unfed females		Repellency %
		No.	%	No.	%	
0.38	45	27	60	18	40	37.2
0.75	45	16	35.6	29	64.4	62.8
1.5	45	11	24.4	34	75.6	74.5
3	45	0	0	45	100	100
DEET	45	0	0	45	100	100
Control	45	43	95.6	2	4.4	–

Table (5): The main chemical components of *Aesculus hippocastanum* seeds extract.

Peak no.	Ret. time (min.)	Chemical formula	Compound name
1	12.5	C ₂₇ H ₃₀ O ₁₇	Quercetin 3,4-diglucoside
2	16.9	C ₁₅ H ₁₆ O ₉	Aesculin
3	18.2	C ₁₅ H ₁₄ O ₇	Leucocyanidin
4	18.7	C ₁₆ H ₁₈ O ₁₀	Fraxin
5	21.4	C ₅₅ H ₈₆ O ₂₄	Escin Ia
6	21.9	C ₅₅ H ₈₆ O ₂₄	Escin Ib
7	22.5	C ₅₅ H ₈₆ O ₂₄	Isoescin Ia
8	23.4	C ₅₅ H ₈₆ O ₂₄	Isoescin Ib
9	24.8	C ₃₀ H ₂₄ O ₁₂	Procyanidin A2
10	27.7	C ₁₅ H ₁₀ O ₇	Quercetin
11	30.8	C ₁₅ H ₁₀ O ₆	Kaempferol

**Fig. 1:** HPLC chromatogram of *Aesculus hippocastanum* seeds extract**Fig. 2:** FT-IR spectrum of *Aesculus hippocastanum* seeds extract

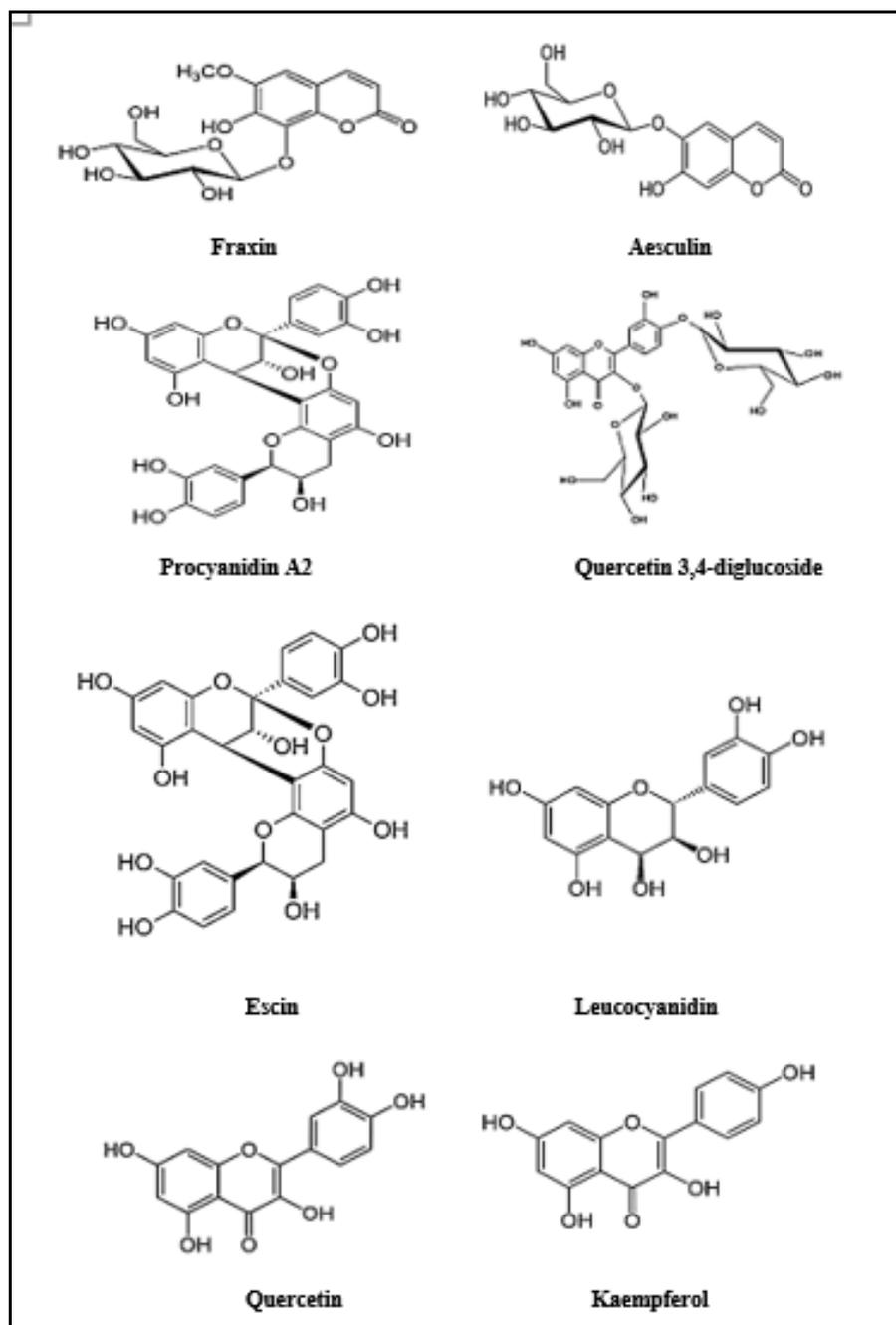


Fig. 3: The molecular structures of *Aesculus hippocastanum* seeds extract components

DISCUSSION

The use of synthetic larvicidal agents to control mosquito larvae presents a potential hazard to other organisms and the environment. Extracts derived from plants often show promising results as larvicidal agents, as they are often inexpensive, biodegradable, and highly effective (Alkofahi *et al.*, 1989; Jang and Ahn, 2002 and Ghosh *et al.*, 2012).

For centuries, horse chestnut (*Aesculus hippocastanum*) seeds, leaves, bark, and

flowers were used in medical requirements (Sirtori, 2001; Pittler and Ernst, 1998; Konoshima and Lee, 1986 and Niu *et al.*, 2008). The main goal of this work was to evaluate insecticidal and repellent potency of *Aesculus hippocastanum*, as a promising candidate, against *Culex pipiens* mosquitoes. The present investigation indicated a direct relationship between the larval mortality and the exposure time and also between the mortality rate of larvae and the concentration, to which they were exposed. The LC_{50} was

found to be 122 and 76 ppm for larvae and pupae, respectively. In addition, there was a great reduction in pupation and adult emergence percent. These results are in agreement to some extent with Al-Zahrani and Abuldahab (2011), where they used the larvicides formulations of *Bacillus thuringiensis* H-14, *B. sphaericus* 1543-4 and a plant water extract of *Aesculus hippocastanum* they found that the plant extract addition causes a significant increase in larval mortality at the first 24 hr. but with increasing the exposure time no increase in this mortality percentage. Govindarajan *et al.* (2011) tested the effect of *Cardiospermum halicacabum*, which is another member of the family (Sapindaceae), extracts against *Culex quinquefasciatus* and *Aedes aegypti*. He found that the benzene, hexane, ethyl acetate, methanol and chloroform leaf extract of *Cardiospermum halicacabum* was found to be more effective against *C. quinquefasciatus* than *A. aegypti*. The LC₅₀ values were 174.24, 193.31, 183.36, 150.44, 154.95 ppm and 182.51, 200.02, 192.31, 156.80, 164.54 ppm respectively.

Aesculus hippocastanum seeds extract induced some morphological abnormalities in larvae, pupae, and adults (pupal-adult intermediates) and incompletely or half ecdysed adults at all the used concentrations, the malformation percent was increased by increasing the concentration. The malformed larvae, pupae, and adults were not able to develop normally and died. Similar observations were obtained by different plant extracts against different mosquito species in earlier studies. Abahussain (1999) using *Calotropis procera* extracts against *C. pipiens* and *Anopheles multicolour* observed morphological abnormalities. Moreover, El-Bokl (2013) recorded varying degrees of morphogenetic abnormalities in immature and adult stages of *C. pipiens* when larvae were treated with the neem *Azadirachta indica* extract. Shalaby *et al.* (1998) using peel oils of lemon, grapefruit and navel orange against *C. pipiens*, observed adults with paralyzed legs and were not able to survive.

Analysis of data obtained indicated that the sex ratio of *Culex pipiens* was greatly affected by the exposure of the 3rd instar larvae of *Culex pipiens* to all concentrations of *Aesculus hippocastanum* seeds extract, where the sex ratios of emerged adults tended to favour males. In contrast to our results, Kamiabi *et al.* (2015) found that the sex ratio of *Ae. Aegypti* and *Ae. albopictus* was not significantly affected by the exposure of the 3rd instar larvae of each mosquito to the EI₅₀ dose of the crude extract of *C. aromaticus*. El-Husseiny and El Kholy (2015) found that the proportion of emerged adult males was slightly increased with jujube extracted in petroleum ether being 1:0.9 (♂ : ♀). The proportions of emerged adult males and females were equals with jujube oil being 1:1 (♂ : ♀)

Aesculus hippocastanum seed extract had a very retarded effect on the growth of larvae, pupae, and adult *Culex pipiens*, where the growth index was decreased by increasing the concentration of the extract. These results are in agreement with Saxena *et al.* (1993) and Shaalan *et al.* (2005).

The present results revealed that *Aesculus hippocastanum* seeds extract was found to possess a remarkable repellency effect against starved *Culex pipiens* females; it induced 100% repellency at the doses of 3 mg/cm². This result agrees with that of Oyedele *et al.* (2002) who reported that *C. citratus* oil had good repellent protection against mosquitoes bite. Yang *et al.* (2004) recorded the repellent activity of methanol extracts of *Cinnamomum cassia* bark, *Nardostachys chinensis* rhizome, *Paeonia suffruticosa* root-bark and *Cinnamomum camphora* at a dose of 0.1 mg/cm², it was 91, 81, 80 and 94% comparable to Deet (82%) against starved *Ae. aegypti*. Jeyabalan *et al.* (2003) reported that the methanol extracts of *Pelargonium citrosa* leaf exhibited repellency activity 36, 51, 78, 100% against the adult mosquito of *A. stephensi* at the concentrations of 0.5, 1, 2, and 4%.

In the present study, the phytochemical constituents of *Aesculus hippocastanum* seeds extract have been

explored using HPLC and FT-IR analysis. The major compound identified was the saponin mixture (escin Ia, Escin Ib, Isoescin Ia, and Isoescin Ib). Saponins are reported as extremely poisonous compounds that cause hemolysis of blood (Kar, 2007). They are Plant secondary metabolites that are known to be effective repellents for a number of harmful insects (Milanovic, 2006; Erturk *et al.*, 2006 and Shields *et al.*, 2006) which is in line with our results. Pelah *et al.* (2002) studied the larvicidal activity of commercial bark saponin extract from *Quillaja saponaria* on 3rd-4th instar larvae of *Aedes aegypti* and *Culex pipiens*. In agreement with our study, their results indicated that commercial bark saponin is toxic, causing 100% larval mortality in *A. aegypti* and *C. pipiens* after 1 and 5 days at a dosage of 800 and 1000 mg/l, respectively. Bagavan *et al.* (2008) reported that saponin is a potential mosquito larvicidal compound, with LC₅₀ value of 18.20 and 27.24 ppm against *A. aegypti* and *C. quinquefasciatus*, respectively.

On the other hand, aesculin and fraxin are from coumarins. Aesculin, also called esculin, which is a coumarin glucoside reported to has antifeeding effect According to Markovic *et al.* (1996). Coumarins also found to exhibit anti-HIV (Bedoya *et al.*, 2005), antifungal (Deng and Nicholson, 2005) and antibacterial (Ouahouo *et al.*, 2004 and Verotta *et al.*, 2004) activities.

Moreover, Quercetin and kaempferol are common flavonoids that present in nearly 70% of plants. Flavonoids are an important group of polyphenols widely distributed among the plants. And they are being used as antioxidants (Kar, 2007). Quercetin and kaempferol were reported to induce cell cycle arrest by inhibiting tyrosine phosphate and/or inducing apoptosis (Aligiannis *et al.*, 2001). They are reported as flavonoids present in *Derris trifoliata* extract which exhibited strong larvicidal activity against the mosquito species, *Aedes aegypti* (Yenesew *et al.*, 2009). Quercetin isolated from *Bobgunnia madagascariensis* has also been reported to have an antifeedant effect on *Tribolium castaneum*, which is a maize and flour weevil

(Freeman and Beattie, 2008 and Adeyemi *et al.*, 2010). Gressel, and Ammann, 2008 stated that quercetin has an insecticidal effect against many insects.

In addition, procyanidin A2 is a type of proanthocyanidin which is a polymeric polyphenolic compound popularly known as condensed tannins. It exhibits antimicrobial effects that inhibit adhesion of bacteria and viruses to host cells thus blocking subsequent propagation (Iglesia *et al.*, 2010 and Daglia, 2012). Also, it showed wound healing, anti-oxidant, anti-inflammatory and anti-enzymatic activities (Wilkinson, and Brown, 1999). Muema *et al.* (2016) assayed the effects of proanthocyanidins isolated from *C. sinensis* leaves on *An. Gambiae* larval development and female mosquito reproductive fitness. They found that it perturbed larval development and impaired mosquito reproductive fitness through repression of juvenile hormone biosynthesis, xenobiotic metabolism, and signal transduction responsive genes. Interestingly, the structure of proanthocyanidins lacks similarity to any insect developmental hormone. Thus, proanthocyanidins could have interfered with hormonal balance via other mechanisms culminating in an extended larval development period and failed adult emergence. It may exert growth reducing effects similar to those exerted by insect growth regulators, potentially disrupting metamorphosis in the mosquito (Dhadialla *et al.*, 1998).

Conclusion

The findings of the present investigation revealed that the *Aesculus hippocastanum* seeds extract possess remarkable larvicidal and adult emergence inhibition activity besides its repellent activity against mosquito *Culex pipiens*. We can conclude from this study that the presence of these phytochemicals in *Aesculus hippocastanum* seeds extract might be the reason for its larvicidal and repellent activity against *Culex pipiens* mosquito.

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