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Larvicidal Activity of *Citrullus colocynthis* (L.) Schrad (Cucurbitaceae) isolated fractions Against aedes aegypti (L.),Anopheles stephensi Liston and Culex quinquefasciatus Say (Diptera: Culicidae)

KEYWORDS: *Citrullus colocynthis,* larvicidal activity, *Aedes aegypti, Anopheles stephensi, Culex quinquefasciatus*

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ABSTRACT *Vector-borne diseases are one of the greatest contributors to human mortality and morbidity in tropical and subtropical regions. Vector control remains the most effective measure and is often the only way to prevent disease outbreaks since there are no vaccines for many vector-borne diseases. The extensive and repeated use of synthetic organic insecticides have led to disrupted natural biological control systems heading to resurgence and resistance in insects and destruction of non-target beneficial fauna, in turn resulting in environment imbalance and human health concerns. Plants have co-evolved with insects that have equipped them with a plethora of chemical defense, which can, in turn be used against insects. In the present study, the isolated fractions of Citrullus colocynthis dichloromethane whole plant extract were evaluated for larvicidal activity against the vector mosquitoes viz., Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus. Eight fractions viz., A, B, C, D, E, F, G and H were obtained from the residue of dichlorome thane extract by column chromatography. Standard WHO protocols with minor modifications were adopted for the larvicidal bioassay. Larvicidal activity was evaluated against the isolated fractions at concentrations of 25, 50, 75 and 100 ppm. Larval mortality was observed for 24 hours. Amongst the isolated fractions tested, fraction 'C' showed 94.4%, 96.0% and 98.4% mortality against third instar larvae of Aedes aegypti, Anopheles stephensi and Culex quinque fasciatus at 100 ppm and* LC_{50} *values were 18.57, 23.48, 19.26ppm and* LC_{90} *values were 98.65, 92.26, 84.84 ppm , respectively. Further investigations are needed to explore the larvicidal activity of the isolated fraction 'C' of dichlorome thane whole plant extract of this plant and also the active ingredient(s) responsible for larvicidal activity.*

INTRODUCTION

The mosquitoes, commonly called "flying syringes", are sanguivorous vectors, which cause more sufferings to human than any other organism (Thomas *et al.*, 2014). Vector-borne diseases are one of the greatest contributors to human mortality and morbidity in tropical and subtropical regions. Every year, more than one billion people are infected and more than one million people die from vector-borne diseases including malaria, dengue, yellow fever, and lymphatic filariasis (WHO, 2014). Vector control remains the most effective measure and is often the only way to prevent disease outbreaks since there are no vaccines for many vector-borne diseases and drug resistance is an increasing threat. As stated by Amer and Mehlhorn (2006), larval stages of mosquitoes are attractive targets for pesticides to control mosquito populations. The advantage of targeting the larval stages are that mosquitoes are killed before they disperse to human habitations, and larvae, unlike adults, cannot change their behavior to avoid control activities (Killeen *et al.* 2002) and also to reduce overall pesticide use in control of adult mosquitoes by aerial application of adulticidal chemicals (Gleiser and Zygadlo, 2007). The use of conventional larvicides in the aquatic environment poses serious threats *viz.*, undesirable action on human health and other beneficial organisms, environmental sustainability, and higher rate of biological magnification (Brown, 1986). Due to the limitations, such as insecticide resistance, environmental pollution, and high cost of synthetic insecticides, current studies are focused to find out natural substances particularly from plants to control the disease transmitting vectors. Various reports on the use of natural plant products against mosquito vectors have been reported (Sukumar et al., 1991; Arivoli et al., 1999, 2012a, b, 2015; Shaalan et al., 2005; Sakthivadivel and Daniel, 2008; Ghosh et al., 2012; Raveen et al., 2012, 2014; Samuel et al., 2012a, b; Vargas, 2012; Samuel and William, 2014).)

Citrullus colocynthis (L.) Schrad a medicinal plant belonging to the family Cucurbitaceae is commonly called 'bitter apple' or 'wild water melon' (Mahmoud *et al.*, 2009), in Tamil as Paedikari attutummatti', in Hindi as 'Indrayan' and in Sanskrit as 'Indravaruni' (Pravin *et al.*, 2013). The plant is found throughout India and Ceylon, both wild and cultivated. It is also indigenous in the West Asia, tropical Africa and Mediterranean regions (Pravin *et al.*, 2013). In traditional medicine, the plant has been utilized as an abortifacient (Duke, 2006), purgative (Aburjai *et al.*, 2007), in curing tumors, leucoderma, elephantiasis, ulcers and in removing kidney stones (Shah *et al.*, 1989). The powerful medicinal values of the pulp are due to the presence of an amorphous glucosid 'colocynth'. The plant also possesses anti-inflammatory (Belsem *et al.*, 2011), anticandidal and antibacterial (Rasool and Jahanbakhsh, 2011), antioxidant (Saba and Oridupa, 2010), analgesic (Marzouk *et al.*, 2010, 2011), hypoglycemic (Agarwal *et al.*, 2012), hypolipidemic (Rahbar and Nabipour, 2010), antialopecia (Dhanotia *et al.*, 2011), antidiabetic (Huseini *et al.*, 2009) and antifertility (Chaturvedi *et al.*, 2003) properties. The phytochemical compounds present in the plant include stearic acid, myristic acid, palmitic acid, oleic acid, linoleic acid, linolenic acid, flavonoid glycoside quercetin, flavone-3-glucoside *viz.*, iso-vitexin, iso-orentine and isoorentine-3-methyl ether, colocynthoside A and B, cucurbitacin E 2-O-beta-D-glcoside, cucurbitacin E, 2-O-beta-D-glucopyranosyl-cucurbitacin B, 2, 25-di-O-beta-D-glucopyranosyl-cucurbitacin L,

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colocynthin, colocynthein, colocynthetin, pectin gum and albuminoids (Nayab *et al.*, 2005; Yoshikawa *et al.*, 2007; Gurudeepan *et al.*, 2010). Further, *Citrullus colocynthis* gained increasing attention as a natural insecticide and its activity have been evaluated against many important insect species. It possesses deterrent, antifeedant, growth regulating and fertility reducing properties in insects (Prabuseenivasan *et al.*, 2004) and insecticidal effect against the aphid *Aphis craccivora* (Torkey *et al.*, 2009). The whole plant extracts of *Citrullus colocynthis* were assayed for their toxicity against the larvae of *Culex quinquefasciatus* (Rahuman *et al.*, 2008). In addition, Arivoli and Samuel (2011) have reported the larvicidal activity of the crude dichloromethane extracts of *Citrullus colocynthis* whole plants against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* and recently Satti and Edriss (2015) have reported the larvicidal activity of petroleum ether fruit extract of *Citrullus colocynthis* against *Anopheles arabiensis*. Therefore, the present study was carried out to study the larvicidal activity of isolated fractions of *Citrullus colocynthis* dichloromethane whole plant extracts against the vector mosquitoes *viz.*, *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*.

MATERIALS AND METHODS

Plant collection and preparation of crude extract

Citrullus colocynthis whole plants collected in and around Tamil Nadu, India were brought to the laboratory, shade dried under room temperature and powdered using an electric blender. Dried and powdered whole plants (1 kg) was subjected to extraction using 3 L of dichloromethane for a period of 72 hours to obtain the crude extracts using rotary vacuum evaporator. The dichloromethane crude extract thus obtained was refrigerated at 4 ˚C.

Isolation and fractionation of crude extracts by column chromatography

The residue from the crude dichloromethane extract of *Citrullus colocynthis* (32.844g) was mixed with silica gel (60 -120 mesh, 100g) as admixture, subjected to column chromatography (si gel, 100 -200 mesh 300g) to obtain eight fractions *viz.*, A, B, C, D, E, F, G and H by increasing polarity of eluents *viz.*, hexane and ethyl acetate in the ratio of 100:0; 80:20; 60:40; 40:60; 20:80; 0:100 and finally ethyl acetate and acetone in the ratio of 50:50 and 0:100, respectively.

Larvicidal bioassay

Bioassay was carried out against laboratory reared vector mosquitoes free of exposure to insecticides. Standard WHO (2005) protocol with minor modifications was adopted for the study. The tests were conducted in glass beakers. Mosquito immature particularly third instar larvae were obtained from laboratory colonized mosquitoes of F_1 generation. Concentrations of 25, 50, 75 and 100 ppm were prepared. Twenty five healthy larvae were released into each 250 ml glass beaker containing 200 ml of water and test concentration. Larval mortality was observed for 24 hours after treatment. Larvae were scored as dead when they showed no signs of movement. A total of five trials with three replicates per trial for each concentration were carried out. Distilled water as control was run simultaneously. The larval per cent mortality was calculated and when control mortality ranged from 5-20% it was corrected using Abbott's formula (Abbott, 1925). SPSS version 11.5 was used for determination of LC_{50} and LC_{90} values (SPSS, 2007). The percentage data obtained was angular transformed. Data from mortality and effect of concentrations were subjected to two way ANOVA followed by Tukey's test ($P<0.05$) to determine the difference in larval mortality between concentrations.

RESULTS

The dichloromethane extract of *Citrullus colocynthis* was subjected to column chromatography with varying proportions of hexane: ethyl acetate and ethyl acetate: acetone. Results revealed that among eight fractions (A, B, C, D, E, F, G and H), fraction 'C' showed 98.4% larval mortality against *Culex quinquefasciatus* followed by 96.0% and 94.4% against *Anopheles stephensi* and *Aedes aegypti*, respectively at 100 ppm. Other fractions showed minimum mortality (Table 1; Figure 1). No mortality was observed in eighth (H) fraction as well as in control. The fraction 'C' exhibited LC_{50} values of 18.57, 19.26, 23.48 ppm against *Aedes aegypti, Culex quinquefasciatus* and *Anopheles stephensi* respectively and LC_{90} values of 84.84, 92.26 and 98.65 ppm, against *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*, respectively (Table 2).

DISCUSSION

In a preliminary investigation, different solvent extracts (hexane, diethyl ether, dichloromethane and ethyl acetate) of *Citrullus colocynthis* whole plants were tested against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* for larvicidal activity where dichloromethane extract was found the to be active (Arivoli and Samuel, 2011) and in the present study the active substances of the dichloromethane extract were found in the 'C' fractionated group, which was indicated by the lowest LC_{50} value reported. The finding of the present study is in line with the high potential of non-polar (dichloromethane, chloroform and hexane) extracts (Chaaib, 2004; Krishnappa *et al.*, 2012) demonstrated against mosquito larvae.

Rahuman *et al.* (2008) reported that the bioassay-guided fractionation of *Citrullus colocynthis* petroleum ether leaf extract led to the separation and identification of fatty acids; oleic acid and linoleic acid which were isolated and identified as mosquito larvicidal compounds. Oleic and linoleic acids were quite potent against fourth instar larvae of *Aedes aegypti* (LC_{50} 8.80 and 18.20 ppm), *Anopheles stephensi* (LC_{50} 9.79 and 11.49 ppm) and *Culex quinquefasciatus* (LC_{50} 7.66 and 27.24 ppm). Similarly, according to Sayed *et al.* (1973), *Citrullus colocynthis* contains oleic and linoleic acids. These substances are known to be more effective against aquatic insects. Alkaloids as well as saponins have also been detected in extracts of this plant and were preferably biologically active against aquatic insects (Hatim *et al.*, 1989) may justify to the action of larvicidal activities. In addition, triterpenoids and saponins in chloroform; saponins in hexane; steroids, saponins, tannins and alkaloids in methanol extracts of *Adansonia digitata* had revealed their toxicity against *Aedes aegypti* and *Culex quinquefasciatus* larvae (Krishnappa *et al.*, 2012).

Basheer (2014) tested the *Ricinus communis* ethyl acetate leaf extract fractions (F1-F7) against the third instar larvae of *Anopheles arabiensis* after 24 hours of exposure and found the fraction F3 to exhibit the highest larvicidal activity whose LC_{50} value was 125.0 ppm. da Silva *et al.* (2007) indicated out of eight fractions each for the hexane (CRH1-CRH8) and methanol (CRM1-CRM8) extracts of *Copaifera reticulata* tested against the third instar larvae of *Aedes aegypti*, two hexane (CRH1, CRH5) and two methanol (CRM1, CRM5) fractions showed the highest toxicity against *Aedes aegypti* larvae and their LC_{50} values were 2.3, 13.9 and 0.8, 10.5 ppm respectively.

Albaba *et al*. (2015) tested the n-hexane leaf extract fractions (CF1-CF11) of *Chromolaena odorata* against *Aedes vittatus* and found the fractions CF3, CF5 and CF6 to exhibit the highest larvicidal activities and their LC_{50} values were 1.66, 1.81 and 1.30 ppm respectively. Chintem *et al*. (2014) stated that *Datura stramonium* leaf methanol extract fractions (DSEE-F1 to DSEE-F7) when tested against the third instar larvae of *Culex quinquefasciatus*, highest mortality rate was achieved in DSEE-F1 and its LC_{50} value was 4.39 ppm after 24 hours. Nzelibe and Chintem (2013) tested the *Ocimum gratissimum* leaf n-hexane fractions (F1-F6) against *Culex quinquefasciatus* larvae, and found the fraction F1 to be effective with a LC_{50} value of 1.49 ppm. Tomass *et al*. (2011) tested the fractions (F1-F3) of *Jatropha curcas* methanolic leaf extract against *Anopheles arabiensis* and found fraction F1 and F2 to exhibit high larvicidal activity with LC_{50} values of 28.65 and 30.40 ppm respectively. Fraction F3 gave a LC_{50} value of 80.70 ppm.

All fractions (hexane, dichloromethane, acetone, ethyl acetate and methanol) of the methanolic leaf extract of *Spondias mombin* were very effective against *Aedes aegypti*. Hexane, dichloromethane and acetone fractions recorded maximal mortality against *Anopheles gambiae* and it was only hexane fraction that registered maximal mortality against *Culex quinquefasciatus* (Eze *et al*., 2014). The bioassay-guided fractionation of *Achyranthes aspera* led to the separation and identification of a saponin as a potential mosquito larvicidal compound with LC_{50} value of 18.20 and 27.24 ppm against *Aedes aegypti* and *Culex quinquefasciatus* respectively (Bagavan *et al*., 2008). The compounds, ecbolin A and ecbolin B isolated from the ethyl acetate extract of *Ecbolium viride* root showed larvicidal activity against the third instar larvae of *Culex quinquefasciatus* (Cecilia *et al*. 2014).

Botanical pesticides have the advantage of providing novel modes of action against insects that can reduce the risk of cross-resistance as well as offering new leads for design of target specific molecules (Zhou *et al*., 2012). Commonly a connection is extrapolated between plant activity based on traditional experience and insecticidal activity against mosquitoes. In general, extracts of the plants derived from specific solvents can influence the bioactivity, probably because of the active components present in large quantities (Oliveira *et al*., 2010). In addition, insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also due to extraction methodology adopted and the polarity of the solvents used during extraction (Ghosh *et al*., 2012). A considerable number of plants have been extensively screened/ studied for their mosquito larvicidal activity. Though several plant extracts have been reported for mosquitocidal activity, only a few botanicals have been studied for the isolation of active molecule responsible for the activity. In that way, the results of the present study offer a possible way for further investigations to find out the active mosquito larvicidal compound from the dichloromethane extract of *Citrullus colocynthis*. The fractions of dichloromethane extract in the present study contained one or more phytochemical compounds and hence, the larvicidal activity might be due to the presence of those phytoconstituents.

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Table 1. Per cent larvicidal activity of *Citrullus colocynthis* isolated fractions of dichloromethane whole plant extract against vector mosquitoes

Concentration (ppm)	Isolated fractions							
	A	B	C	D	E	F	G	H
				Aedes aegypti				
25	42.4 ±2.19 (40.6)[e]	25.6 ± 2.19 (30.4)[d]	56.8 ±4.38 (48.9)[f]	0.0 ±0.0 (0.0)[a]	06.4 ±2.19 (14.7)[b]	14.4 ±2.19 (22.3)[c]	02.4 ±2.19 (8.9)[ab]	0.0 ±0.0 (0.0)[a]
50	48.0 ±2.82 (43.9)[e]	33.6 ±2.19 (35.4)[d]	68.8 ±5.21 (56.0)[f]	04.8 ±1.78 (12.7)[ab]	08.8 ±1.78 (17.3)[b]	20.0 ±2.82 (26.6)[c]	3.2 ±3.34 (10.3)[ab]	0.0 ±0.0 (0.0)[a]
75	59.2 ±3.34 (50.3)[e]	42.4 ±2.19 (40.6)[d]	75.2 ±5.21 (60.1)[f]	08.0 ±2.82 (16.4)[b]	10.4 ±2.19 (18.8)[b]	32.8 ±3.34 (34.9)[c]	5.6 ±2.19 (13.7)[ab]	0.0 ±0.0 (0.0)[a]
100	66.4 ±4.56 (54.6)[f]	52.8 ±3.34 (46.6)[e]	94.4 ±3.57 (76.3)[g]	13.6 ±2.19 (21.6)[bc]	14.4 ±3.57 (22.3)[c]	42.4 ±3.57 (40.6)[d]	7.2 ±3.34 (15.6)[b]	0.0 ±0.0 (0.0)[a]
Control	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]
				Anopheles stephensi				
25	36.0 ±2.82 (36.9)[d]	24.0 ±2.82 (29.3)[c]	55.9 ±5.93 (48.4)[e]	04.8 ±1.78 (12.7)[a]	01.6 ±2.19 (7.39)[a]	16.8 ±3.34 (24.2)[b]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]
50	41.6 ±4.56 (40.2)[c]	35.2 ±3.34 (36.4)[c]	65.6 ±6.69 (54.1)[d]	07.2 ±1.78 (15.6)[a]	08.0 ±2.82 (16.4)[a]	24.0 ±2.82 (29.3)[b]	03.2 ±1.78 (10.3)[a]	02.4 ±2.19 (8.9)[a]
75	54.4 ±5.36 (47.5)[d]	38.2 ±4.56 (38.2)[c]	78.4 ±4.56 (62.3)[e]	12.8 ±3.34 (20.9)[b]	11.2 ±1.78 (19.6)[b]	35.2 ±1.78 (36.4)[c]	06.4 ±2.19 (14.7)[ab]	03.2 ±3.34 (10.3)[a]
100	61.6 ±4.56 (51.7)[e]	49.6 ±6.06 (44.8)[d]	96.0 ±4.00 (78.5)[f]	16.0 ±2.82 (23.6)[b]	12.8 ±3.34 (20.9)[ab]	38.4 ±4.56 (38.3)[c]	08.8 ±1.78 (17.3)[ab]	05.6 ±2.19 (13.69)[a]
Control	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]
				Culex quinquefasciatus				
25	43.2 ±3.34 (41.6)[d]	28.8 ±3.34 (32.5)[c]	59.2 ±5.21 (50.3)[e]	02.4 ±3.57 (8.9)[a]	04.8 ±1.78 (12.7)[a]	14.4 ±2.19 (22.3)[b]	04.8 ±3.34 (12.7)[a]	0.0 ±0.0 (0.0)[a]
50	47.2 ±5.21 (43.3)[e]	36.0 ±4.00 (36.9)[d]	68.8 ± 4.38 (56.0)[f]	05.6 ±2.19 (13.7)[ab]	09.6 ±2.19 (18.1)[b]	20.8 ±3.34 (27.1)[c]	06.4 ±3.57 (14.7)[ab]	0.0 ±0.0 (0.0)[a]
75	59.2 ±1.78 (50.3)[e]	42.4 ±2.19 (40.6)[d]	80.8 ± 5.93 (64.0)[f]	08.8 ±1.78 (17.3)[b]	13.6 ±2.19 (21.6)	29.6 ±3.57 (32.9)[c]	08.8 ±1.78 (17.3)[b]	0.0 ±0.0 (0.0)[a]
100	66.4 ±6.06 (54.6)[f]	52.8 ±4.38 (46.6)[e]	98.4 ± 3.57 (82.7)[g]	14.4 ±2.19 (22.3)[bc]	18.4 ±3.57 (25.4)[c]	35.2 ±3.34 (36.4)[d]	10.4 ±3.57 (18.8)[b]	0.0 ±0.0 (0.0)[a]
Control	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]	0.0 ±0.0 (0.0)[a]

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Values are mean (%) of the five-replicates of three trials ±standard deviation. Figures in parenthesis are angular transformed. Different superscript alphabets indicate statistical significant difference at $P < 0.05$ level by two way ANOVA followed by Tukey's test.

Table 2. Probit analysis of larvicidal activity of activity of *Citrullus colocynthis* isolated fractions of dichloromethane whole plant extract against vector mosquitoes

Vector mosquitoes	*Aedes aegypti*						*Anopheles stephensi*						*Culex quinquefasciatus*					
Fractions	LC_{50} (ppm)	95% CL LL	95% CL UL	LC_{90} (ppm)	95% CL LL	95% CL UL	LC_{50} (ppm)	95% CL LL	95% CL UL	LC_{90} (ppm)	95% CL LL	95% CL UL	LC_{50} (ppm)	95% CL LL	95% CL UL	LC_{90} (ppm)	95% CL LL	95% CL UL
A	50.35	42.36	56.99	200.87	173.78	244.23	67.02	60.93	73.55	207.14	180.61	248.24	50.12	41.90	56.89	203.67	175.62	248.99
B	93.62	86.13	103.87	226.42	197.57	270.49	102.62	93.14	116.66	250.76	214.57	309.10	94.07	85.41	106.61	249.68	212.52	310.68
C	18.57	3.96	28.17	98.65	88.23	114.76	23.48	11.55	31.70	92.26	83.16	105.79	19.26	4.68	28.66	84.84	75.79	98.71
D	163.81	146.94	190.35	242.14	210.95	291.77	204.78	172.41	264.83	343.75	279.32	464.32	190.91	158.64	258.22	301.19	240.27	429.83
E	281.67	213.11	466.36	497.89	361.46	867.49	200.27	162.31	287.71	323.18	250.53	492.53	190.86	163.35	239.30	323.69	267.41	423.84
F	115.06	106.33	127.09	221.11	196.95	255.82	126.06	113.23	145.84	265.53	227.28	326.77	138.13	123.04	161.92	277.18	236.07	343.83
G	300.10	203.50	864.79	477.23	307.56	1475.68	187.31	162.11	232.21	275.25	230.76	355.27	326.91	220.01	903.78	560.74	358.89	1655.92
H	-	-	-	-	-	-	221.34	166.51	434.44	321.76	229.92	682.50	-	-	-	-	-	-

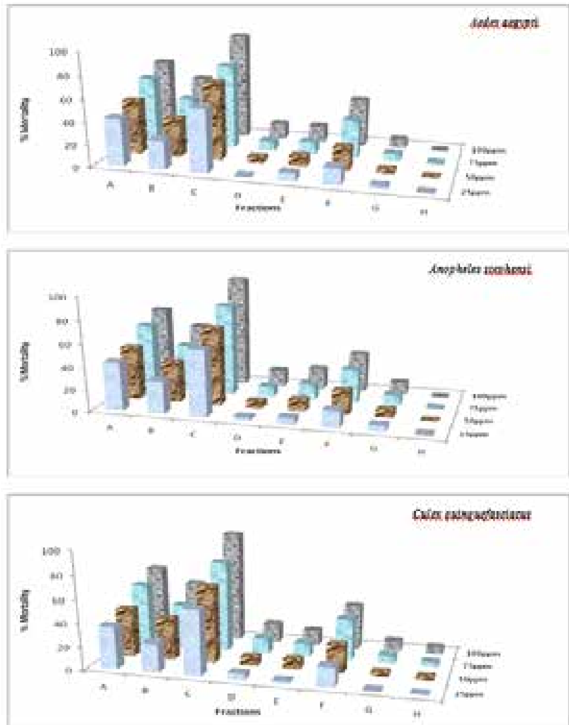

Figure 1. Larvicidal activity of *Citrullus colocynthis* isolated fractions of dichloromethane whole plant extract against vector mosquitoes

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