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Larvicidal activity of *Ipomoea carnea* stem extracts and its active ingredient dibutyl phthalate against *Aedes aegypti* and *Culex quinquefasciatus*

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ABSTRACT

Vector born diseases like Malaria, Filaria and Dengue fever are some major diseases in India. Management of the disease vector for Dengue fever using synthetic chemicals has failed due to vector resurgence and environmental pollution. Considering the recent evidence of these three diseases, there is an urgent need to control vector population of mosquitoes. The development of eco- friendly and target specific agents for the control of mosquito level is of prime importance. Ipomoea carnea, belonging to convolvulaceae family and fistulosa sub-family can be a potential candidate for that. The present work reveals some larvicidal activities of Ipomoea carnea stem extracts, their different fractions and dibutyl phthalate, which is a secondary metabolite isolated from the extract. These were screened against mosquitoe species Aedes aegypti and Culex quinquefasciatus. All exhibited significant chronic mosquito-larval toxicities against the two vectors. Experiments were carried out with 4th instar larvae (0-24 h old) of Aedes aegypti and Culex quinquefasciatus which were cultured and maintained during the experiment at 80± 5% and relative humidity at 27± 2º. LC_{50} and LC_{90} values for test samples were also calculated.

Key words: *Ipomoea carnea, Aedes aegypti, Culex quinquefasciatus,*

INTRODUCTION

Prevalence of Mosquito born diseases is one of the world's most notable health hazards**.** Several mosquito species belonging to genera Anopheles, Culex and Aedes are vectors for the pathogens of various diseases like malaria, filariasis, Japanese encephalitis, dengue, yellow fever and chickungunya [1]. Nearly 300-500 million people are infected worldwide with mosquito-born diseases and 1.5 to 2.0 million die each year [2]. The most efficient approach to control the vector is to target the immature stages of their life cycles. The current mosquito control approach is based on synthetic insecticides of organophosphate compounds and insect growth regulators. Continuous use of synthetic insecticides has disrupted natural enemies and led to outbreak of some insect species, resulted in developing resistance, had undesirable effects on non-targeted organism, environment and human health [3].

There is a continuous and urgent need to discover new environmentally safe, biodegradable indigenous method for vector control. Therefore researchers are increasingly turning their attention to herbal products. Plants may be sources of alternative agents for control of vectors, because they are rich in bioactive chemicals that are

biodegradable. Plant extracts from leaves, flowers and roots were found to have mosquito larvicidal activity [1]. In line with the objectives of the current research work to isolate secondary metabolites that have potential biological activity, dibutyl phthalate – a biologically active molecule was isolated from *Ipomoea carnea* stem and its mosquito larvicidal activity was investigated. There are reports on synergistic effect of insecticides of *Ipomoea carnea* leaves extract against malarial Vector *Anopheles stephensi* [4]. The steam distilled essential oil extracted from the leaves of *Ipomoea cairica* was found highly toxic against *A. aegypti*, *A stephensi* and *C. quinquefasciatus* [5]. Mosquito larvicidal activity of dioctyl phthalate was reported in literature [3]. Mosquito repellent action of Mylol oil, a mixture of dibutyl phthalate and dimethyl phthalate was available in literature [6]. The strong bioactive nature of this secondary metabolite against bacteria as well as fungi was reported [7]. Isolation, characterization and quantification of dibutyl phthalate from *Ipomoea carnea* was reported in authors previous publications [8]. Isolation and characterization of substituted dibutyl phthalate from *Ipomoea carnea* stem was also reported by E. Khatiwora et al [9]. However the mosquito larvicidal activity or mosquito repellent action of pure dibutyl phthalate was not reported. In this work the mosquito larvicidal activity of various extracts of *Ipomoea carnea* stem, their defferent fractions and dibutyl phthalate isolated from the ethyl acetate extract was investigated for the first time against *A. aegypti* and *Culex quinquefasciatus*.

MATERIALS AND METHODS

Collection and identification of plant materials

The plant material was collected from the river sides of Pune, Maharashtra, India. The plant was authenticated at Botanical Survey of India, Pune, India. The authentication number is ELICAI.,BSI/WC/Tech/2009/96.

Preparation of plant extract and isolation

Air shade dried and powdered stem material of *I. carnea* (500g) was refluxed with hexane (**HSE**), ethyl acetate (**EA**) and acetone (**ACE**) and methanol (**MeOH**) separately for eighteen hours. Solvents were recovered under reduced pressure to obtain the crude extracts. Extractive values were found to be 5%, 7%, 15% and 20% respectively. Hexane extract was found to be inactive .However EA, ACE and MeOH extracts were found to active against the mosquito species. Acetone (**ACE**) extract was broad fractioned on silica gel (60- 120, 10g) using n-hexane (**Fr. 1**), hexane: ethyl acetate (Fr**.2** 1:1), acetone (Fr**. 3**) and residual methanol (**Fr.4**). The solvents were removed under reduced pressure to get their respective extracts. Similarly EA extract was broad fractioned using n-hexane (**Fr.1´**), hexane: ethyl acetate (**Fr.2´** 9.5:0.5), hexane: ethyl acetate (**Fr.3´** 8:2), ethyl acetate (**Fr.4´**) Acetone (**Fr. 5´)** and ethanol fraction (**Fr.6´)**. Compound **1** was isolated from hexane fraction of ethyl acetate extract.

Mosquito Culture

4th instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* were drawn from the laboratory culture of mosquitoes maintained at 27 ± 2 ^{0} C temperature and 80± 5 % relative humidity.

Biological assay

The test samples were dissolved in organic solvent according to their solubility. They were tested to determine the larvicidal activity by making serial dilutions ranging from 1000 to 10 ppm in bioassays against larvae of the mosquito species. All experiments were performed with 4th instar larvae (0- 24 h old) of *Aedes aegypti* and *Culex quinquefasciatus* which were cultured and maintained during the experiment at 80± 5 % relative humidity. The bioassays were performed at room temperature of 27 ± 2 ^{0} C by exposing 10 larvae in each concentration of the extract in the final volume of 50 ml in 100 ml beaker. Larva food, ground dog biscuits/ yeast tablets (1 : 1) was provided every alternate day. Five replicates of each concentration were tested for larval bio-efficasy and each experiment was repeated three times. The larval mortality in each concentration and control was recorded after 24 hours of continuous exposure, where there was no 100 % kill, the larvae was allowed to stay in water for 48 and till 72 hours. The mortality was recorded after 48 hours. Untreated controls were also taken in each test. The corrected mortality was determined using Abbott's formula whenever required [10]. The dose mortality data was analyzed by log Probit – method of Finney [11] and lethal concentration for 50 % and 90 % mortality were calculated (LC_{50} and LC_{90}).

RESULTS AND DISCUSSION

The compound isolated was a colourless transparent liquid. LC-MS of the compound exhibited a molecular ion peak at m/z 279 on positive mode which matches the molecular formula $C_{16}H_{22}O_4$. Fragmentation pattern is in agreement with the structure.

The IR spectrum showed a characteristic absorption frequency at 1726 cm^{-1}(Ester carbonyl); 1600 cm-1 and 1579 cm^{-1} for aromatic stretching and absorption at 1122 cm^{-1} and 1074 cm^{-1} are for C-O stretching.

^{1}H NMR spectrum has displayed an upfield triplet at δ 0.98 (t, *J* = 5 *Hz*, 6H) for (H 4' and H 4") methyl protons. The multiplets at δ 1.47 (m,4H) and δ1.74 (m,4H) are observed for (H3' & H3") and (H2' & H2") protons respectively. A downfield triplet at δ 4.33 (t, *J* = 5 *Hz*, 4 H) is noticed for (H 1' & H 1") protons. The doublet of doublets at δ 7.56 (dd, *J* = 10 & 5 *Hz*, 2 H) and δ 7.73 (dd, *J* = 10 & 5 *Hz*, 2 H) are indicated for (H 3 & H 4) and (H 2 & H 5) aromatic protons respectively.

^{13}C NMR spectrum displays eight signals accounting for sixteen carbon atoms. A quartet at δ 13.70 is assigned to (C 4' and C 4") methyl carbon atoms. The triplets at δ 19.18, δ 30.59 and δ 65.55 are noticed for (C 3' & 3"), (C2' & C2") and (C1' & C1") methylene carbons respectively. The downfield doublets at δ 128.84 and δ130.89 are obversed for (C2 & C5) and (C3 & C4) aromatic carbon atoms. A singlet at δ 132.35 is indicated for (C1 and C6) tetrasubstituted aromatic carbon atoms. The most downfield singlet at δ167.69 is assigned for ester carbonyl carbon atoms.

The DEPT pulse sequence demonstrates multiplicities of carbon signals. It is composed of four methine, six methylene and two methyl which, confirms presence of four quaternary carbon atoms. The spectral data (Fig.1) confirms that the isolated compound is dibutyl phthalate [8].

Larvicidal efficacy of extracts

The secondary metabolites have a major role in mosquito control. The active components of the extracts may weaken the cuticle defense system of larvae causing easy penetration of pathogenic molecules into insect bio systems.

Extracts of *I. carenea* stem such as hexane (**HSE**), ethyl acetate (**EA**), acetone (**ACE**) and methanol (**MeOH**) are tested for larvicidal activity against 4th instar larvae of *C. quinquefasciatus* and *A. aegypti*. The results indicate that EA, ACE and MeOH extracts show significant larvicidal activity while HSE is found to be inactive. ACE and EA extracts are fractioned as (Fr **1** to Fr **4**) and (Fr 1´ to Fr 6´) respectively. Compound **1** is isolated from Fr 1´ of ethyl acetate extract. These fractions along with crude extracts and pure compounds **1** were tested against 4th instar larvae of mosquito species . In the first set of experiments six different concentrations (500, 400, 300, 200, 100, 50 ppm) of crude extracts are tested. The results are reported (**Table 1**).

ACE shows 100% mortality at 500 ppm within 24 hours and at 400 ppm after 48 hours against both species. At 300 ppm after 48 hours, 100 % mortality is observed for *Aedes aegypti* and 85% for *Culex quinquefasciatus*. EA shows 100% mortality at 500 ppm and at 400 ppm after 48 hours against both species. At 300 ppm after 48 hours 86 % mortality is observed for *Aedes aegypti* and 84% for *Culex quinquefasciatus*. MeOH shows 100% mortality at 500 ppm and 400 ppm within 48 hours against both species. At 300 ppm after 48 hours 100 % mortality is observed for *Aedes aegypti* and 74% for *Culex quinquefasciatus*.The efficiency of extract decreases at lower concentrations. The order of activity for the total extracts is observed as acetone> methanol >ethyl acetate against both the species. The details are reported (**Table 1**).

Acetone extract and its fractions

In the second set of experiment six different concentrations(500, 400, 300, 200, 100, 50 ppm) of ACE and its fractions, n- hexane (Fr. 1), hexane: ethyl acetate (1:1, Fr**.**2), acetone (Fr**.** 3) and methanol (Fr. 4) are tested for larvicidal activity against 4th instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*.

ACE and Fr 4 show 100% mortility after exposure of 24 hours at 500 ppm whereas Fr 3 shows it at 400 ppm against both mosquito species. ACE and Fr 4 exhibit 100% mortality at 300 ppm against *A.aegypti* and at 400 ppm for

Culex quinquefasciatus after 48 hours exposure whereas Fr **3** shows it at 200 ppm against *A. aegypti* and at 300 ppm against *C. quinquefasciatus*. The efficiency of extract decreases at lower concentrations. Fractions **1** and **2** are found to be inactive. Fraction 3 and 4 show remarkable larval mortility with respect to both species. Results are presented (**Table 1**).

Ethyl acetate extract, its fractions and isolated compound 1.
In the third set of experiment six different concentrations (500, 400, 300, 200, 100, 50 ppm) of EA, its fractions n-hexane (Fr.1´), hexane: ethyl acetate (Fr.2´, 9.5:0.5), hexane: ethyl acetate (Fr.3´, 8:2), ethyl acetate (Fr.4´) acetone (Fr. 5´) and ethanol fraction (Fr.6´) are tested against 4th instar larvae of *A. aegypti* and *C. quinquefasciatus* . 500 ppm to 10 ppm concentrations are used for isolated compound **1**. The results of this study are reported (**Table2**). The results exhibit that extract as well as **compound 1** show significant larvicidal activity. EA shows 100% mortality after exposure of 48 hours at 500 and 400 ppm whereas **Fr1´** shows 100% larvae kill after 24 hours at 500 ppm against both species, *A. aegypti* and *C. quinquefasciatus* larvae**. Fr.6´** exhibits 85% and 75% mortality after exposure of 48 hours at 500 against *A. aegypti* and *C. quinquefasciatus* larvae respectively**.**

Compound **1** shows potent activity against the mosquito larvae. At 300 ppm concentration 100% kill is noticed after 24 hour exposure for **compound 1** against *A. aegypti*. For *C. quinquefasciatus* 100% kill is noticed after 48 hour exposure at 300 ppm. At lower concentration of 50 ppm also it shows 42.66% mortility after 24 hours against *A. aegypti* and 44.00% mortility after 48 hours against *C. quinquefasciatus*. Remaining fractions are found to be inactive.

Bio efficacy of extracts
All preliminary experiments indicate consequential results. Coming into view lethal concentration for 50 % and 90 % kill (LC_{50} and LC_{90}) were evaluated. The LC_{50} and LC_{90} for ACE, EA and MeOH are (119.61, 122.41, 141.50 ppm) & (603.63, 678.99, 1541.90 ppm) against *A. aegypti* and (166.76, 173.91, 207.94 ppm) & (731.95, 897.52, 1548.27 ppm) against *C. quinquefasciatus*. The details of lethal concentrations, 50 % and 90 % kill using Probit Analysis data are reported (**Table 3**).

Bio efficacy of Fr **3** and Fr **4**
The LC_{50} and LC_{90} for Fr 3 and Fr 4 are (58.41, 98.72 ppm) & (216.12, 440.95 ppm) respectively against *A. aegypti* and (93.11,137.64 ppm) & (314.42 ,559.22 ppm) against *C. quinquefasciatus*.The order of activity for ACE and its fractions is observed as Fr 3 > Fr 4 > ACE against both species.

Bio efficacy of EA extract, its fractions and compound 1.
Bioefficasy of EA extract, its fractions and pure **compound 1** are tested against 4th instar larvae of *C. quinquefasciatus* and *A. aegypti*. The details of lethal concentrations, 50 % and 90 % kill using Probit Analysis data are reported (**Table 3**). The lethal concentrations, LC_{50} and $LC_{90,}$ are (122.41, 169.06, 347.30, 81.43 ppm) and (678.99, 663.16, 1167.16, 269.71 ppm) for EA, Fr1´, Fr.6´ and **compound 1** respectively, when tested against *A. aegypti*. The LC_{50} and LC_{90} values for the same samples when tested against *C. quinquefasciatus* are found as (173.91, 221.43, 444.57, 109.64ppm) and (897.52, 691.15,1611.96, 439.91ppm) respectively. The order of activity for EA and its fractions and pure compound **1** is observed as **1> EA > Fr1´> Fr 6´** against both the species.

DISCUSSION

It is noticed from the results that the percentage mortality is increased with the increase in concentration; hence the effect of the samples is dose dependant. The results are in conformity with the findings of other workers who also reported the dose dependency of plant extract against mosquito larvae [1]. Kaushik R and Saini P reported the laevicidal activity of *Millingtomia hortensis* against three mosquito species [12]. The LC_{50} values of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* were 223, 206 and 138 ppm respectively. Thomus et al reported that the essential oil of *Ipomoea cairica* possessed remarkable larvicidal properties as it could exhibit 100% mortality in the larvae of *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* at concentrations range 100-170 ppm [13]. The results of present experiments are comparable with the above mentioned results.

The outcome of the experiment indicates that the isolated **compound 1** is more active molecule than the fractions and total extracts. The compound exhibits significant activity against both the mosquito species as it shows activity

at lower concentrations also. It is more effective against *A. aegypti* than *C. quinquefasciatus* as LC_{50} and LC_{90} values are are less for A. *aegypti* .

Table 1 Toxicity of various extracts and Fr 3 & Fr 4

Test sample	Conc. (ppm)	% Mortality ± S.E after hours			
		A. aegypti		*C. quinquefasciatus*	
		24	48	24	48
ACE	500	100 ± 0	-	100 ± 0	-
	400	80.0 ± 1.95	100 ± 0	70.0 ± 1.38	100 ± 0
	300	70.66 ± 1.81	100 ± 0	61.33 ± 1.65	85.33 ± 1.33
	200	55.33 ± 1.65	74.0 ± 1.31	48.66 ± 1.92	79.33 ± 0.66
	100	45.33 ± 1.92	55.33 ± 1.33	33.33 ± 1.26	68.0 ± 1.11
	50	30.66 ± 1.53	42.66 ± 1.18	22.66 ± 1.18	36.00 ± 1.31
EA	500	92.66 ± 1.18	100 ± 0	90.0 ± 1.69	100 ± 0
	400	82.0 ± 1.74	100 ± 0	74.66 ± 2.15	100 ± 0
	300	69.33 ± 1.81	86.00 ± 1.31	58.0 ± 2.0	84.00 ± 1.31
	200	61.33 ± 1.65	75.33 ± 1.31	45.33 ± 1.65	65.33 ± 1.33
	100	41.33 ± 0.90	64.0 ± 1.31	34.00 ± 1.31	51.33 ± 0.90
	50	30.66 ± 0.66	52.0 ± 1.06	21.33 ± 0.90	35.33 ± 1.33
MeOH	500	84.61 ± 1.34	100 ± 0	81.33 ± 1.65	100 ± 0
	400	72.66 ± 1.81	100 ± 0	65.33 ± 1.33	100 ± 0
	300	58.03 ± 1.11	100 ± 0	53.33 ± 1.26	74.00 ± 1.31
	200	50.00 ± 1.92	80 ± 1.95	40.66 ± 1.53	66.66 ± 1.26
	100	40.66± 1.53	64.0 ± 1.31	33.33± 1.26	53.33 ± 1.26
	50	35.33 ± 1.33	61.33 ± 1.65	22.0 ± 1.06	37.33 ± 1.18
Fr. 3	500	100 ± 0	-	-	100 ± 0
	400	100 ± 0	-	-	100 ± 0
	300	92.0 ± 1.06	100 ± 0	82.0 ± 1.06	100 ± 0
	200	84.61 ± 1.34	100 ± 0	70.0 ± 1.95	91.33 ± 0.90
	100	70.0 ± 1.95	85.33 ± 1.33	52.0 ± 2.0	75.33 ± 1.33
	50	47.33 ± 1.81	63.0 ± 1.06	31.33 ± 1.65	54.0 ± 1.31
Fr. 4	500	100 ± 0	-	-	100 ± 0
	400	90 ± 1.69	100 ± 0	80 ± 1.95	100 ± 0
	300	76.00 ± 1.31	100 ± 0	68.66 ± 2.15	95.33 ± 1.33
	200	63.33 ± 1.26	88.0 ± 0.90	55.33 ± 1.33	86.66 ± 1.26
	100	51.33± 1.65	59.33 ± 0.66	43.33± 1.26	63.33 ± 1.26
	50	33.33 ± 1.26	44.0 ± 1.65	20.0 ± 0.66	25.33 ± 1.33

Table 2 Toxicity of EA, its fractions and isolated compound 1

Test sample	Conc. (ppm)	% Mortality ± S.E after hours			
		Aedes aegypti		*Culex. quinquefasciatus*	
		24	48	24	48
EA	500	92.66 ± 1.18	100 ± 0	90.0 ± 1.69	100 ± 0
	400	82.0 ± 1.74	100 ± 0	74.66 ± 2.15	100 ± 0
	300	69.33 ± 1.81	86.00 ± 1.31	58.0 ± 2.0	84.00 ± 1.31
	200	61.33 ± 1.65	75.33 ± 1.31	45.33 ± 1.65	65.33 ± 1.33
	100	41.33 ± 0.90	64.0 ± 1.31	34.00 ± 1.31	51.33 ± 0.90
	50	30.66 ± 0.66	52.0 ± 1.06	21.33 ± 0.90	35.33 ± 1.33
Fr1´	500	100 ± 0	-	100 ± 0	-
	400	82.0 ± 1.74	100 ± 0	72.66 ± 1.18	92.66 ± 1.18
	300	55.33 ± 1.65	76.00 ± 1.31	51.33 ± 1.65	81.33 ± 0.90
	200	43.66 ± 1.18	65.33 ± 1.33	32.0 ± 1.06	55.33 ± 1.33
	100	31.33 ± 0.90	52.0 ± 1.06	29.33 ± 0.90	30.66 ± 0.66
	50	20.66 ± 0.66	25.33± 1.65	10.66 ± 10.66	15.33 ± 1.33
Fr.6´	500	61.33 ± 1.65	85.33 ± 1.92	54.0 ± 1.31	75.33 ± 1.33
	400	53.33 ± 1.26	71.33 ± 1.65	47.33 ± 1.81	66.66 ± 1.26
	300	48.66 ± 2.15	62.0 ± 1.74	33.33 ± 1.26	58.00 ± 1.11
	200	33.33 ± 1.26	48.66 ± 1.92	21.33 ± 0.90	34.00 ± 1.31
	100	10± 0	20.0 ± 1.69	10.0± 0	21.33 ± 0.90
	50	0 ± 0	15.33 ± 1.65	0.0 ± 0	0 ± 0
Comp. 1	300	100 ± 0	-	90.0 ± 1.95	100 ± 0
	200	73.33 ± 1.26	89.33 ± 1.81	61.33 ± 1.65	82.66 ± 1.18
	100	50.0 ± 1.38	70.0 ± 1.95	42.0 ± 0.75	65.33 ± 1.33
	50	42.66 ± 1.18	57.33 ± 1.81	30.0 ± 1.06	44.00 ± 1.31
	25	10± 0	30.66± 1.53	10.0± 0	20.66± 0.66
	10	0 ± 0	-	0 ± 0	0 ± 0

Table 3. Lethal concentrations of extracts, Fr 3, Fr 4, Fr 1´, Fr 6´ and Compound 1

Sample	*Aedes aegypti*		*Culex quinquefasciatus*	
	Lethal Conc. (24hrs) (ppm)	**Regression equation**	**Lethal Conc. (24hrs) (ppm)**	**Regression equation**
ACE	LC_{50} =119.61 LC_{90} =603.63	Y=1.212+1.823x	LC_{50} =166.76 LC_{90} =731.95	Y=0.189+2.159x
EA	LC_{50} =122.41 LC_{90} =678.99	Y=1.403+1.722x	LC_{50} =173.91 LC_{90} =897.52	Y=0.971+1.798x
MeOH	LC_{50} =141.50 LC_{90} =1541.90	Y=2.342+1.235x	LC_{50} =207.94 LC_{90} =1548.27	Y=1.592+1.469x
Fr 3	LC_{50} =58.41 LC_{90} =216.12	Y=1.015+2.255x	LC_{50} =93.11 LC_{90} =314.42	Y=0.225+2.424x
Fr 4	LC_{50} =98.72 LC_{90} =440.95	Y=1.067+1.971x	LC_{50} =137.64 LC_{90} =559.22	Y= 0.497+1.971x
Fr 1´	LC_{50} =169.06 LC_{90} =663.16	Y=0.189+2.159x	LC_{50} =221.43 LC_{90} =691.15	Y=-1.080 +2.592x
Fr 6´	LC_{50} =347.30 LC_{90} =1167.16	Y=- 1.182+2.433x	LC_{50} =444.57 LC_{90} =1611.96	Y=- 1.066+2.290x
Comp. 1	LC_{50} =81.43 LC_{90} =269.71	Y=0.291+2.464x	LC_{50} =109.64 LC_{90} =439.91	Y=0.667+2.123x

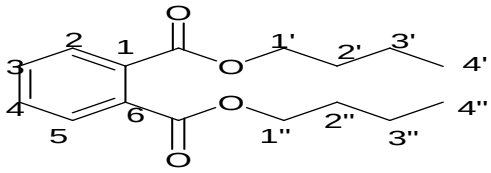

Figure-1: Structure of the compound-1

CONCLUSION

The present study has demonstrated that the crude extracts and pure compound of *I. carnea* stem showed potent larvicidal activity against *C. quinquefasciatus* and *A. aegypti*. The isolated compound as well as extracts were highly effective at lower concentration against the mosquitoes. The plant could be utilized for developing cost effective and environment friendly new type of larvicide for mosquito control. The beneficial effect to the control of vector borne diseases could be employed as a good alternative for synthetic pesticides.

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