Indian Journal of Natural Products and Resources
Vol. 12(3), September 2021, pp. 375-383

Larvicidal activity of *Acorus calamus* leaf extracts against the *Aedes aegypti* and *Culex quinquefasciatus*

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Received 03 July 2020; Revised 27 July 2021

Mosquitoes are the important carriers of disease-causing pathogens responsible for serious health effects in humans. The vector control management mainly focuses on synthetic insecticides and their overuse creates resistance in mosquitoes. Henceforth, there is a need to develop insecticides that are effective and help in overcoming resistance development in mosquitoes. An attempt is made here to bring effective management of larval mosquitoes with herbal-based preparations since it is eco-friendly and causes no ill effects on humans. In this study, aqueous and solvent extracts of *Acorus calamus* leaves were tested for their anti-mosquito activity against third instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Among the tested extracts, the crude hexane leaf extract of *A. calamus* showed the highest percentage of mortality with LC_{50} of 151.86 ppm and LC_{90} of 536.36 ppm against *A. aegypti* and LC_{50} of 174.70 ppm and LC_{90} of 696.73 ppm against *C. quinquefasciatus*. The treatment showed significant changes in the larval morphology such as damaged body parts, enlarged mid-gut, and lesions on the thoracic region. Similarly, histopathological analysis of treated larvae after 24 hours of treatment was observed with dislocations of cells, augmented nucleus, and ruptured epithelial cells. GC-MS analysis of the effective crude hexane leaf extract of *A. calamus* showed the presence of asarone to the level of 50.18% and that could have been thought to be insecticidal in nature which is probably responsible for the effective larval anti-mosquito activity.

Keywords: *Acorus calamus*, *Aedes aegypti*, *Culex quinquefasciatus*, Histopathology, Larvicidal activity.
IPC code; Int. cl. (2015.01)-A01N 25/00, A01N 65/00, A01P 7/00, A01P 7/04

Introduction

Mosquitoes are one of the most dreadful groups of arthropods responsible for spreading numerous frightful communicable diseases like malaria, dengue, yellow fever, Japanese encephalitis, filariasis, Zika virus, and elephantiasis[1-3]. Mosquito-borne diseases constitute one of the major health problems both in humans and animals. *Aedes aegypti*, the primary carrier of the dengue virus cause a wide range of diseases like clinically asymptomatic forms such as acute flu-like symptoms, retro-orbital pain, malaise, classic dengue fever, and severe dengue hemorrhagic fever[4]. The study on the prevalence of dengue reported the death of 3.9 million people in over 128 countries. Its prevalence in India i.e., the number of cases and death toll reported in the year 2020 alone is 39419 and 56, respectively. *Culex quinquefasciatus* is found to be the key vector of lymphatic filariasis caused by the parasite *Wuchereria bancrofti*. According to reports, lymphatic filariasis affects 120 million people in 73 countries and in India alone it contributes 40% of the global filarial burden[5]. Hence, mosquito controlling tactics tend to be more important.

For the past several years, the vector control program was based on the application of synthetic insecticides like organochlorides, organophosphates, and pyrethroids. However, repeated application of these chemical insecticides leads to resistance of mosquito species and bioaccumulation of toxic chemicals in the environment which in turn causes serious effects on humans and other organisms[6]. Therefore, the development of plant-based pesticides is a pertinent alternate against synthetic pesticides; yet, the herbal preparations do not show any adverse effects on the non-targeted organisms, which are biodegradable in nature and cost effective[7,8]. Secondary metabolites of plants consist of different types of chemical compounds *viz*., alkaloids, phenolic compounds, terpenes, and steroids which are found to have promising insect control potential[9,10]. Some plant-derived phytochemicals act as insecticides and larvicides. Among them, larvicides play a vital role in controlling mosquitoes compared to any other control strategies[11].

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Acorus calamus (sweet flag), a perennial monocot plant present in tropical and subtropical countries was chosen for the present study. The leaves, stem and rhizome of this plant are employed in traditional medicine to treat fever, asthma, and bronchitis. Different parts of this plant contain various biological activities such as antibacterial, anticancer, repellent activity and are also used in larval mosquito control[12]. But, in mosquito control, petroleum ether and ethyl alcohol extracts of *A. calamus* rhizomes were used against the larvae of *A. aegypti*[12]. Phytochemicals derived from the plant vary in their biological potency based on the parts selected. Bio-efficacy of the plant also varies based on their collection site, time of collection, and season. In the present study, the leaves of *A. calamus* were chosen to study mosquito larvicidal activity against *A. aegypti* and *C. quinquefasciatus*.

Materials and Methods

Plant collection and preparation

The leaves of *A. calamus* were collected from Mannavanur hills located (10˚22́ 47 N, 77˚36́ 07 E) in the upper Kodaikanal hill of Western Ghats, Tamil Nadu and it was identified at Botanical Survey of India, Southern Regional Center, Coimbatore, Tamil Nadu, India (voucher specimen no. BSI/SRC/5/23/2019/Tech./244). The freshly collected leaves were then washed in running tap water and shade dried at 28±2 ^{o}C for 10-15 days. The fully dried leaves were powdered using a commercial electrical blender (Philips HL1643/05, India) and sieved to get a fine powder that was used for extraction.

Preparation of extracts

Soxhlet extraction was carried out with 50 g powdered material of plant leaves using 250 mL solvents *viz.*, hexane, diethyl ether and methanol-based on their increasing polarity. After 18 hours of extraction, the extract was filtered using Whatman filter paper No.4 and concentrated in a rotary vacuum evaporator at reduced pressure. For aqueous extraction, 50 g of powdered plant material was boiled with 100 mL of double distilled water at 100 ˚C for 15 minutes. The extract was allowed to cool at room temperature and filtered using Whatman filter paper No.1 and lyophilized (Mini Lyodel Delvac, India).

Maintenance of larvae

The eggs of *A. aegypti* and *C. quinquefasciatus* were procured from the Vector Control Research Centre (VCRC), Pondicherry, India. They were then transferred to enamel trays containing 500 mL of water for hatching. The hatched larvae were fed with pedigree dog biscuits and yeast in a 3:1 ratio and the cultured larvae were maintained according to the standard methodology[13] for experimental studies with plant extracts at the Unit of Entomology, Department of Zoology, University of Madras, Chennai (India) for a period of three months from June to September 2018.

Larvicidal bioassay

Larvicidal bioassay was carried out based on larval susceptibility test as per standard method[14]. Twenty-five third instar larvae of *A. aegypti* and *C. quinquefasciatus* were introduced into the test solution containing different concentrations of plant extracts (100, 200, 300, 400, 500, and 600 ppm). The homogenous test solution was prepared with crude extract, dissolved in 1 mL acetone and mixed with 249 mL of dechlorinated water. The control was set up by mixing 1 mL of acetone with 249 mL of dechlorinated water. Three replicates each of control and treated larvae were maintained. The mortality was recorded after 24 hours of exposure and calculated using Abbott's formula[15]. The average larval mortality data were subjected to probit analysis[16]. The values of LC_{50}, LC_{90}, 95% confidence level and chi-square were calculated using the SPSS statistical software package 21.0 version. Results with P <0.05 were considered to be statistically significant.

Morphological variation

The effect of potential crude hexane leaf extract of *A. calamus* on the larvae post-treatment was subjected to morphological analysis[17]. Briefly, third instar post-treated larvae of *A. aegypti* and *C. quinquefasciatus* were put in plastic cups containing 250 mL of water (control) and 250 mL of water with 500 ppm of hexane crude extract of *A. calamus*. Larvae were considered dead if they stopped moving for a long period, even after gentle probing with a small spatula. The treated larvae were washed with distilled water and observed under a stereomicroscope (Magnus stereo zoom MSZ-TR Trinocular Microscope, India).

Histological studies

The histological variations of the larvae treated with effective crude hexane leaf extract of *A. calamus* were observed following the standard procedure[18]. In brief, the larvae were fixed in 4% formaldehyde for 24 hours, dehydrated using series of different

percentages of ethanol and cleared with xylene solution. The larvae were embedded using paraffin wax and blocks were prepared. The paraffin blocks were sectioned at 5 µm thickness using a rotary microtome, stained with hematoxylin and eosin, and the slides were observed under a bright-field microscope (Nikon Eclipse 80 i, Japan).

GC-MS analysis of hexane extract of leaves

Gas chromatography and mass spectrometry (GC-MS) analysis of effective crude leaf extract i.e., hexane extract of *A. calamus* were performed using Perkin Elmer GC-MS (Model Perkin Elmer Clarus 600, USA) at Sophisticated Analytical Instrumentation Facility, Vellore Institute of Technology, India. Experimental conditions of the GC-MS system were as follows: HP 5-MS capillary standard non-polar column, dimension: 30 Mts, ID: 0.25 mm, film thickness: 0.25 µm. The flow rate of the mobile phase (carrier gas: He) was set at 1.0 mL/min. In the gas chromatography, the temperature program (oven temperature) was set at 60 ˚C and raised to 200 ˚C at the rate of 10 ˚C/min. Exactly 10 mg of crude hexane extract was dissolved in 500 µL of hexane and the injection volume of 1 µL run fully at a range of 50-650 m/z and the results were compared using NIST Mass Spectral Library.

Phytochemical screening

Qualitative analyses of the phytochemicals present in crude hexane leaf extract of *A. calamus* were carried out using standard methods[19]. Phytochemical constituents like alkaloids, carbohydrates, proteins, flavonoids, steroids, phenols, tannins, saponins, coumarins, terpenoids, and quinones were analysed.

Results

In the present study, the powdered leaf material of *A. calamus* extracted with water, methanol, diethyl ether and hexane reported with their yield percentage of 2, 8, 1.3, and 4%, respectively. All the extracts were semi-solid in nature except water extract (which was lyophilized). Among the four extracts, methanol extract showed a maximum yield percentage than other extracts (Table 1). Subsequently, the extracts were subjected to larvicidal bioassay to evaluate the effectiveness of the different extracts against the third instar larvae of *A. aegypti* and *C. quinquefasciatus*.

In the larvicidal bioassay, solvent (water, methanol, diethyl ether, and hexane) crude leaf extracts of *A. calamus* exhibited a varied percentage of mortality against third instar larvae of *A. aegypti* and *C. quinquefasciatus* (Fig. 1). The results revealed that crude hexane leaf extract of *A. calamus* treated larvae showed the highest larval mortality of 97 and 90% against the *A. aegypti and C. quinquefasciatus*, respectively, after 24 hours treatment at 600 ppm.

The lethal concentration of hexane, diethyl ether, and methanol crude leaf extracts of *A. calamus* with LC_{50} values determined were 151.86, 295.53, and 445.21 ppm, respectively and LC_{90} values as 536.36, 1774.22, and 3596.75 ppm, respectively against *A. aegypti*. Similarly, the lethal concentration of hexane, diethyl ether, and methanol extracts against the third instar larvae of *C. quinquefasciatus* was observed with LC_{50} values as 174.70, 321.94, and 466.82 ppm, respectively and LC_{90} values as 696.73, 1975, and 3534.62 ppm, respectively. The results indicated that crude hexane leaf extract of *A. calamus* showed the lowest LC_{50} and LC_{90} values against the third instar larvae of *A. aegypti* and *C. quinquefasciatus* after 24 hours treatment (Table 2). Thus, the crude hexane leaf extract of *A. calamus* found to be effective in larvicidal activity was subjected to further experimental analysis.

The higher concentration of *A. calamus* crude hexane leaf extract treated *A. aegypti* larva showed significant morphological alterations with blackened head, thorax, swollen midgut, transparent and

Table 1 — The yield and physical characters/ phase of different solvent extracts of leaves of *A. calamus*

	Plant powder (g)	Solvent	Yield (g)	Phase
A. calamus	50	Hx	4	Semisolid
	50	Et_2O	1.3	Semisolid
	50	MeOH	8	Semisolid
	50	Water	2	Powder

Hx-hexane, Et_2O-diethyl ether, MeOH-methanol, water (lyophilized extract)

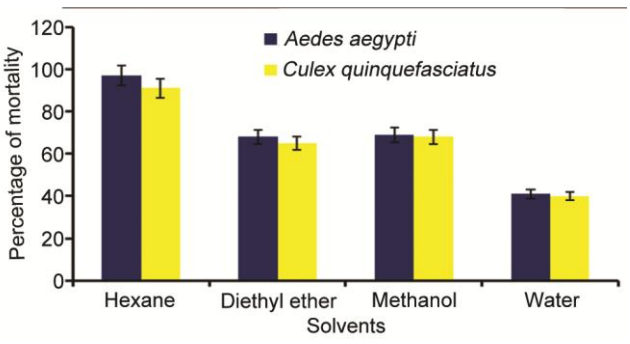

Fig. 1 — Percentage of mortality of different solvent crude leaf extract of *A. calamus* treated *A. aegypti* and *C. quinquefasciatus* third instar larvae after 24 hours treatment.

Table 2 — Larvicidal effect of different solvent leaf extracts of *A. calamus* against the third instar larvae of *A. aegypti* and *C. quinquefasciatus*

	Solvent Used	Dose (ppm)	LC_{50} (ppm) (UCL-LCL)	LC_{90} (ppm) (UCL-LCL)	χ^2
A. aegypti	Hx	100	151.86	536.36	2.6
		200	(98.66 - 195.33)	(404.06 - 908.95)	n.s
		300			
		400			
		500			
		600			
	Et_2O	100	295.53	1774.22	0.15
		200	(208.74-408.30)	(943.64 - 10681.44)	n.s
		300			
		400			
		500			
		600			
	MeOH	100	445.21	3596.75	2.4
		200	(317.44 - 898.19)	(1404.85 - 118054.7)	n.s
		300			
		400			
		500			
		600			
C. quinquefasciatus	Hx	100	174.70	696.73	1.0
		200	(107.66 - 230.06)	(482.98 – 1572.05)	n.s
		300			
		400			
		500			
		600			
	Et_2O	100	321.94	1975.00	0.27
		200	(231.93 - 600.84)	(1012.14 - 13922.80)	n.s
		300			
		400			
		500			
		600			
	MeOH	100	466.82	3534.62	3.2
		200	(335.32 - 950.79)	(1411.00 - 94606.62)	n.s
		300			
		400			
		500			
		600			

*LC-Lethal Concentration, LCL-Lower Confidence Level, UCL-Upper Confidence Level, χ^2- Chi-square value, n.s- non significance (P =0.05), Hx-hexane, Et_2O-diethyl ether, MeOH-methanol

damaged cuticle layer, accumulation of food particles in the gut and lesions in the anal region (Fig. 2). Likewise, the *A. calamus* crude hexane leaf extract treated *C. quinquefasciatus* larva showed darkened gut, presence of lesions in the thorax and anal gills with a thick permeable cuticle layer (Fig. 3). The observed abnormalities confirmed the influence of phytochemicals present in crude hexane leaf extract of *A. calamus* on the tissues of the selected mosquito larvae. The behavioural changes of treated larvae included distressed movement followed by tail biting, lethargy, paralysis and settlement at the bottom of the test containers.

The histopathological analysis of *A. calamus* crude hexane extract-treated larva was observed with disruption of epithelial cells, degraded microvilli, disintegrated peritrophic membrane and augmented nucleus which indicated that the extract had a prominent effect on the midgut epithelium of *A. aegypti*. Also, the foregut of treated *C. quinquefasciatus* larva showed disintegrated columnar cells, fragmented basement membrane, with an augmented nucleus, and vacuolation of epithelial cells after 24 hours treatment (Fig. 4). The phytochemical analysis of crude hexane leaf extract of *A. calamus* revealed the presence of alkaloids,

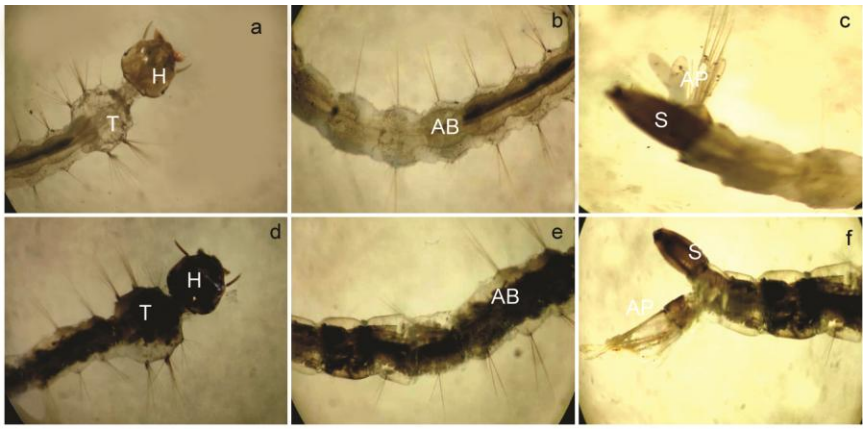

Fig. 2 — Morphological changes of *A. aegypti* larvae treated with crude hexane leaf extract of *A. calamus*, a-c) untreated *A. aegypti* larva, (H) head, (AB) abdomen (gut) and tail region, d) blackened head and (T) thorax, e) swollen gut region with the accumulation of food particles, f) lesions in the anal region after treatment, (AP) anal papillae, (S) siphon.

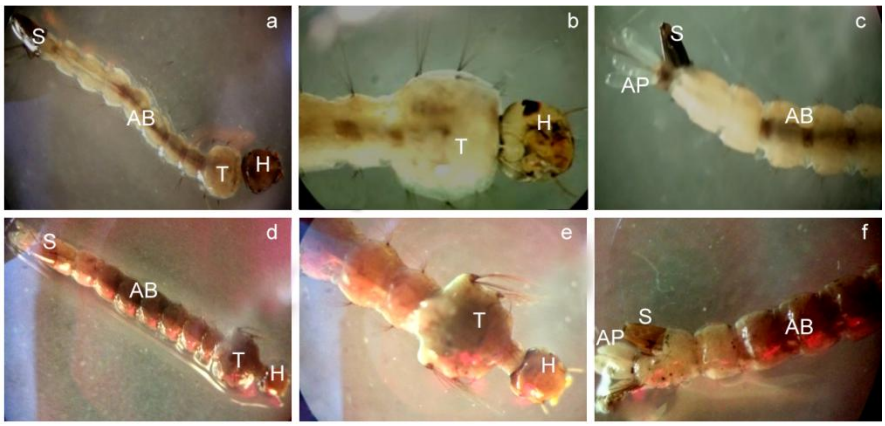

Fig. 3 — Morphological changes of *C. quinquefasciatus* larvae treated with crude hexane leaf extract of *A. calamus*, a-f) untreated *C. quinquefasciatus* larva, (H) head, (AB) abdomen (gut) and tail region, d) darkened gut, e) lesions in the (T) thoracic region, f) anal gills with permeable cuticle layer, (AP) anal papillae, (S) siphon.

flavonoids, steroids, phenols, tannins, coumarins, and terpenoids (Table 3). Likewise, the effective crude hexane extract of *A. calamus* leaf was subjected to GC-MS analysis and the chromatogram showed the presence of 11 different volatile compounds (Fig. 5). The peak area percentage was calculated and is presented in Table 4. The major peak was represented by asarone with 50.18% with other compounds like tetracontane, heptacosane, hentriacontane, heptadecane and 2, 6, 10, 15-tetramethyl.

Discussion

Biological control of mosquitoes in their larval stage is a suitable and effective way of decreasing the mosquito population before entering into a disease-causing adult stage. Nowadays, plant-based larvicides are attracting more concern than synthetic chemical pesticides, due to their biodegradable and harmless nature. Plant-based biopesticides are composed of numerous biologically active phytochemicals which act through different mechanisms on the target organisms. Interestingly, the crude extracts are highly effective than the single compounds because of their synergistic effect on the mosquito larvae which inhibit the development of resistance.

The screening of active phytochemicals from plant resources against mosquito species leads to the invention of alternative natural insecticides[20,21]. The difference in toxicity of plant compounds varies primarily based on dose and exposure period. On the other hand, geographical area, time of collection, season etc also contribute to the efficacy of plant compounds. The bioassay in the present investigation carried out with different solvents using leaves of

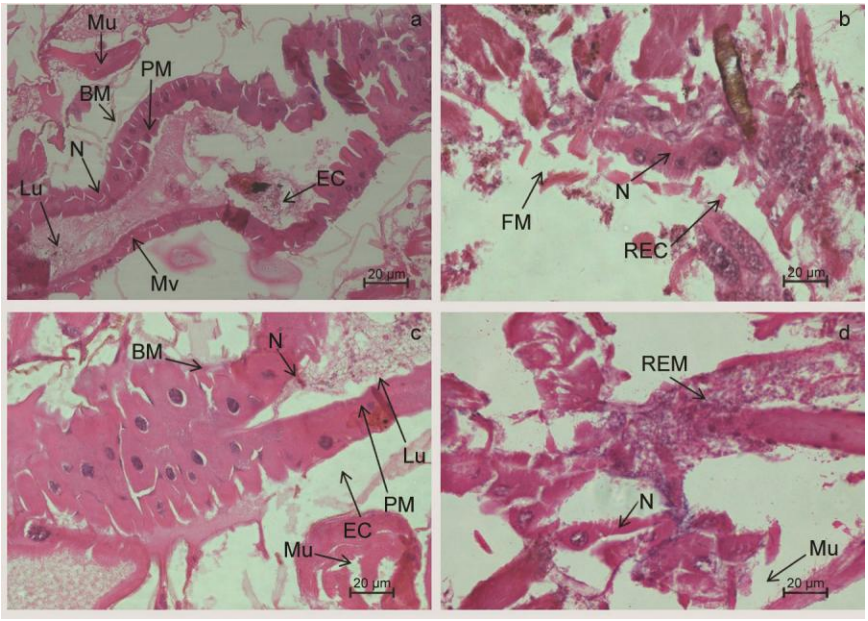

Fig. 4 — Histological observation of third instar larvae of *A. aegypti* and *C. quinquefasciatus* after 24 h treatment with crude hexane leaf extract of *A. calamus*. a) Untreated *A. aegypti* larva with normal cellular organisation like muscle (Mu), basal membrane (BM), peritrophic membrane (PM), luman (LU), nucleus (N), epithelial cells (EC), b) *A. aegypti* showing ruptured epithelial cells (REC), fragmented membrane (FE) and augmented nucleus (N), c) Untreated larval gut of *C. quinquefasciatus* showing a sequential arrangement of columnar epithelial cells and, d) *C. quinquefasciatus* larval gut showing distraction of the columnar epithelial cells (CEC).

Table 3 — Phytochemical analysis of crude hexane leaf extract of *A. calamus*

S. No	Tests	Inference
1	Alkaloids	++
2	Carbohydrates	-
3	Proteins	-
4	Flavonoids	+++
5	Steroids	++
6	Phenols	-
7	Tannins	++
8	Saponins	-
9	Coumarins	++
10	Terpenoids	+++
11	Quinones	-

(-) Absent, (+) Present, (++) Moderate, (+++) High concentration

A. calamus showed promising results with crude hexane extracts when compared to other solvent extracts. The lethal concentration was found to be low in the case of hexane crude leaf extract treated mosquito larvae of *A. aegypti* and *C. quinquefasciatus*. The reason behind the highest efficacy in hexane crude extract is due to the presence of active compounds present in effective quantities in an extract as reported in earlier studies[12,22]. Cheah *et al.*[23] also reported that the crude hexane extract of *A. annua* had larvicidal activity against *A. aegypti*, *A. sinensis*, and *C. quinquefasciatus* and their LC_{50} values are 213.98, 187.10, 304.00 ppm, respectively after 24 hours treatment that is significantly higher than the values observed in this study.

The morphological variation in the larvae of the aforementioned mosquito species treated with crude hexane leaf extract of *A. calamus* showed remarkable changes like blackened head and thorax, swollen midgut, transparent and damaged cuticle layer, accumulation of food particles in the gut with lesions. Blackening of the epidermis is caused by damage in the epithelial and peritrophic membranes which are attributable to the elimination of the toxic content from the gut region of the treated larvae[24]. Similar such morphological abnormalities were reported earlier by Thiagaletchumi *et al.*, who observed that the treatment of acetone crude leaf extract of *Ipomoea cairica* against *A. aegypti* larvae with blackened and twisted abdomen[25]. According to Deepak *et al.*, damaged anal papillae and malformed pale body followed by altered spiracular valves in the siphon were observed with *Halymenia palmate* methanolic extract treated *A. aegypti* larvae[26]. Studies indicated that insecticidal activities of the phytochemicals vary based on their mode of actions like interfering with the neuroendocrine system, feeding inhibition and acting on growth regulation[27,28]. The morphological

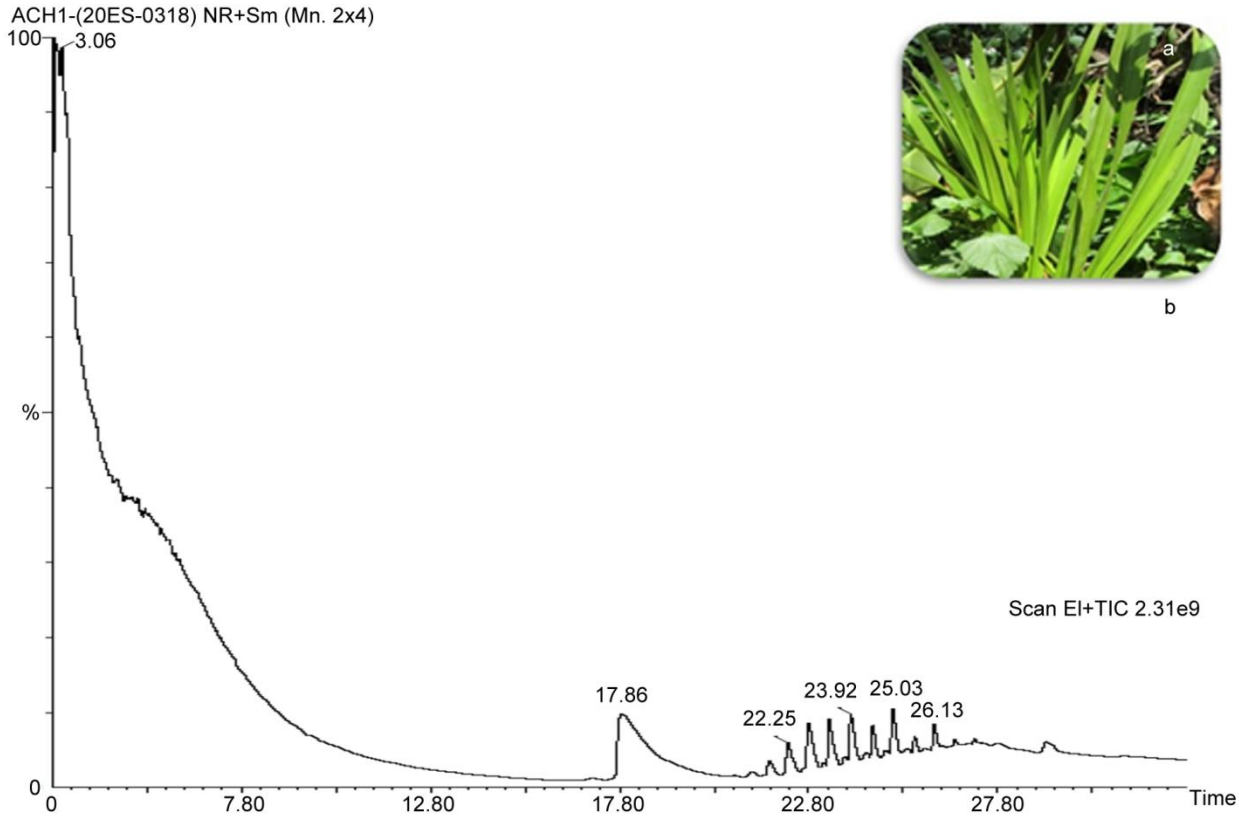

Fig. 5 — GC-MS chromatogram of crude hexane leaf extracts of *A. calamus*. a) *A. calamus* leaf morphology, b) GC-MS chromatogram.

Table 4 — GC-MS analysis of crude hexane leaf extract of *A. calamus* showing various phytochemical compounds

S. No.	Compound name	R.T. (min)	P. No. %	M.W.	M.F.
1	Ethyl acetate	3.05	5.32	88	$C_4H_80_2$
2	Ethyl acetate	3.21	1.66	88	$C_4H_80_2$
3	Asarone	17.85	50.18	208	$C_{12}H_{16}O_3$
4	Hexa triacontane	21.74	2.48	506	$C_{36}H_{74}$
5	Tetra triacontane	22.25	4.99	618	$C_{44}H_{90}$
6	Tetra tetracontane	22.77	7.58	618	$C_{44}H_{90}$
7	Hepta cosine	23.34	6.30	380	$C_{27}H_{56}$
8	Hepta cosane	23.90	6.55	702	$C_{50}H_{102}$
9	Triaconetane	24.49	4.12	702	$C_{50}H_{102}$
10	Heptadecane	25.01	5.98	506	$C_{36}H_{74}$
11	Heptacosane	25.58	1.86	604	$C_{43}H_{88}$

R.T. – Retention Time; P. No. % - Peak number percentage; M.W. – Molecular weight; M.F. – Molecular formula

abnormalities and mortality of the mosquito larvae may also be due to the variation of growth-regulating hormones[29]. The phytochemicals present in the hexane leaf extract of *A. calamus* showed remarkable histological alterations such as disrupted epithelial cells, augmented nucleus disordered epithelial cells and fragmented cell membrane in the gut region of *A. aegypti* and *C. quinquefasciatus* in the treated larvae. Similar such observations are observed in the mosquito midgut region with compromised and altered integrity of midgut cells due to the toxicity of plant extracts[30].

Similarly, studies on the ethanolic extract of *Catharanthus roseus* treated third instar larvae of *C. quinquefasciatus* showed disorganized cells, shortened microvilli membranes[31]. There is also disorganized vacuolated gut epithelial cells in the third instar larvae of *A. aegypti* when treated with lectin rich seed fraction of *Moringa oleifera*[32]. Some other reports are suggested that larvicidal

activity is linked to destruction of the gut epithelium and imbalanced secretion of the digestive system[33,34]. The histopathological changes in our study also evidently revealed the prominent effect of crude extract of leaves of *A. calamus* on the larval gut of the *A. aegypti* and *C. quinquefasciatus*. Thus, it is confirmed that the plant secondary metabolites could inflict the digestive physiology of mosquito larvae. GC-MS analysis of the effective crude hexane leaf extract was composed of diverse phytocomponents with a high proportion of asarone along with other compounds that could have been responsible for mosquito larvicidal activity. Asarone is a widely known compound due to its biological activities such as anti-fungal, anti-bacterial, anti-helminthic activities with reported insecticidal effects. The rhizome of the plant is mainly used for the extraction of various active compounds. In the present study, the leaf extract of *A. calamus* also possesses many effective phytocompounds as observed in the rhizome. The presence of a higher proportion of asarone in hexane leaf extract of *A. calamus* in the present study is thought to be involved in larvicidal activity against the two mosquito species, *A. aegypti* and *C. quinquefasciatus.*

Conclusion

The crude hexane leaf extract of *A. calamus* exhibited the larvicidal potency against the third instar mosquito larvae of *A. aegypti* and *C. quinquefasciatus*. It induced both morphological and physiological aberrations. Moreover, the presence of a high level of asarone, an insecticidal compound in the extract could have been responsible for the mosquito larvicidal activity. Overall, the crude hexane extract of *A. calamus* had a high potency to be used as a prospective mosquito larvicidal agent. Having characterized the mosquito larvicidal activity, the asarone could be used to augment the synergistic treatments in formulations against the control of mosquito larvae.

Acknowledgement

The authors gratefully acknowledge the funding received through the UGC-UPE Phase II programme under Theme C: Project II. Dr. Shanthi Sathappan thank the University Grants Commission for the award of Dr. D.S. Kothari Postdoctoral Fellowship (No.F.4-2/2006 (BSR)/BL/16-17/0311).

Conflict of interest

The authors declare no conflict of interest.

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