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ORIGINAL PAPER

Larvicidal activity of catechin isolated from *Leucas aspera* against *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* (Diptera: Culicidae)

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Abstract Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Insecticides of plant origin my serve as an alternative biocontrol technique in the future. The aim of the present study was to evaluate the larvicidal activity of fractions and compounds from the whole-plant methanol extracts of *Leucas aspera* on the fourth-instar larvae of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus*. The larvae were exposed to fractions with concentrations ranging from 1.25, 2.25, 5, 10, and 20 ppm and isolated compounds. After 24 h exposure, larval mortality was assessed. Among the eight fractions, four from hexane extractions showed potent larvicidal activity against tested mosquito species at 20 ppm concentration. The isolated compound catechin showed pronounced larvicidal activity at very low concentrations. The LC_{50} and LC_{90} values of catechin were 3.05 and 8.25 ppm against *Ae. aegypti*, 3.44 and 8.89 ppm against *An. stephensi*, and 3.76 and 9.79 ppm against *C. quinquefasciatus*, respectively. The isolated compound was subjected to spectral analyses (GC-MS, FTIR, ^{1}H NMR, and ^{13}C NMR) to elucidate the structure and to compare with spectral data literature.

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Introduction

Mosquito-borne diseases are endemic in more than 100 countries, causing mortality of nearly two million people every year, and at least one million children die of such diseases each year, leaving as many as 2100 million people at risk around the world (Rajkumar and Rahuman 2011). In India, 17 states and six Union territories have been identified to be endemic, with about 553 million people exposed to the risk of infection.

Vectors of the genera *Anopheles*, *Culex*, and *Aedes* are principally responsible for spread of diseases like malaria, filariasis, dengue, and yellow fever (Morens and Fauci 2013). *Anopheles stephensi* is a major vector of malaria in tropical countries. *Aedes aegypti*, a vector of dengue and chikungunya, carries the arbovirus responsible for these diseases in the tropical and subtropical zones. The only way to prevent dengue virus transmission is to combat the disease-carrying mosquitoes (Dean 2001). *Culex quinquefasciatus*, a vector of lymphatic filariasis, is widely distributed in tropical and subtropical countries, with around 120 million people infected worldwide and 44 million people having common chronic manifestation (Bernhard et al. 2003).

Synthetic insecticides have been used as larvicides in several countries for the last 30 years (Chavsses and Yap 1997). However, the non-selective nature of insecticides and their harmful effects on other organisms are the major hindrances with the use of these synthetic insecticides (De Omena et al. 2007). In most parts of the world, synthetic larvicides are continuously applied for controlling mosquitoes but many of these are toxic to human, animal, and plant life (Suman et al. 2012). Repeated use of synthetic insecticides for mosquito control has

disrupted natural biological control systems and has led to resurgence in mosquito populations (Kamaraj et al. 2008). Much effort has been focused on plant extracts or phytochemicals as potential sources of commercial mosquito control agents or bioactive compounds (Dwivedi and Karwasara 2003).

In view of the problems associated with conventional mosquito control methods, great efforts are required to develop new or complementary control techniques for major mosquito species (McGraw and ONeill 2013). This has prompted researchers to look for environment-friendly, cost-effective, biodegradable, and target-specific insecticides against mosquito species (Sharma et al. 2005).

Plants, being a natural source of various compounds, are known to contain mosquito larvicidal agents, which may act in combination or independently. Some phytochemicals act as general toxicants both against the adult as well as larval stages of mosquitoes, while others interfere with the growth and development or with reproduction or produce olfactory stimuli and act as a repellent or as an attractant (ICMR 2003; Ghayal et al. 2010). Mosquito control at the larval stage of development with phytochemicals that occur in the leaves and roots of plants is one technique which is affordable and environment friendly (Mandal 2011). Therefore, the natural products of plant origin with insecticidal properties have been tested in recent years for control of a variety of insect pests and vectors. Hence, they may be considered as an alternative source of mosquito control agents which are preferred due to their innate biodegradability.

Leucas aspera (Willd) belonging to Lamiaceae family is known for its medicinal properties, and the leaves are used in traditional medicine for treating dyspepsia, cough, cold, painful swelling, fevers, ulcers, and chronic skin eruptions (Chopra et al. 2002). The leaves are used as insecticide and mosquito repellent in rural areas (Maheswaran et al. 2008). and as a natural pesticide against *An. stephensi* (Karunamoorthi and Bekele 2009).

Preliminary studies of the weed *L. aspera* revealed very strong and potent mosquito larvicidal activity. Whole plant methanol extracts were found to possess potent larvicidal activity at very low concentrations, when compared to other solvent extracts.

The main objectives of the study were to characterize, quantify, purify, and isolate the active principle from the methanol extracts of *L. aspera.* The compound isolated from *L. aspera* was assessed for its larvicidal potential against the fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus*. Larval susceptibility to the isolated compound was also compared at the histopathological level to elucidate the effects of compounds isolated from *L. aspera*.

Materials and methods

Selection and procurement of plants

Healthy and disease-free *L. aspera* plants were collected from the natural population in and around the suburbs of Chennai, Tamil Nadu, India. The plants were identified and authenticated by Prof. P. Jayaraman and deposited at the Plant Anatomy Research Center (PARC /2013/2110) West Tambaram, Chennai-45, Tamil Nadu, India.

Extraction and fractionation of bioactive compounds

One kilogram of powdered sample of an *L. aspera* plant was added to 4 L methanol (1:4 *w*/*v*), sealed tightly and kept in a shaker at 120 rpm for 4 days at room temperature. Then the extract was filtered through double-layered cheese cloth and reduced to 200 mL using a rotary evaporator at 45 °C. The concentrated crude extract was tranferred to a separating funnel (2 L) and mixed with 2 volumes of glass distilled water, to which was added 1200 mL of hexane; the solution was mixed vigorously and allowed to stand overnight for complete separation at room temperature.

Fractionation and characterization of bioactive compounds

Seventy-five grams of silica G (100–200 mesh, Sisco, Mumbai) was packed with hexane in a column of 25 mm diameter and 900 mm length. The *L. aspera* methanol extract (10 g) was chromatographed over silica column and was eluted with solvents of increasing polarity: hexane, chloroform, ethyl acetate, methanol, and their mixtures. The respective fractions were collected and concentrated by flash evaporation and analyzed by TLC.

The elutes with similar R_f were combined and finally eight fractions were obtained. The fractions were evaluated for mosquito larvicidal activity at concentrations of 1.25, 2.25, 5, 10, and 20 ppm. Fraction 4 eluted with chloroform:ethyl acetate (90:10) showed pronounced larvicidal activity, and was selected for spectral analysis for identification of phytocompounds. The elutes with similar R_f values were pooled and concentrated under reduced pressure in a rotary evaporator at 45 °C. The concentrated elutes obtained from *L. aspera* were tested against the fourth-instar larvae *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus*. The fractions which showed pronounced larvicidal activity were taken for further studies.

Characterization of catechin

The active fraction (fraction 4) was subjected to spectral analysis. The FTIR spectrum was taken on a Perkin-Elmer spectrophotometer in KBr disc. ^{1}H NMR and ^{13}C NMR spectra

were recorded on a NMR spectrometer (model: Bruker) in $CDCl_3$ at 300 and 75 MHz, respectively.

Mosquito larvicidal activity

The larvae at the early fourth-instar stage were used for the assays. The larvicidal activity was evaluated by standard procedures recommended by WHO (2005). The concentrations of 1.25, 2.25, 5, 10, and 20 ppm were prepared using DMSO. Twenty larvae were placed in a glass beaker (250 mL) containing 199 mL of tap water and 1 mL of the respective concentration of the fraction and the isolated compound. Five replicates were maintained for each concentration and the dead larvae were counted after 24 h exposure period. The percent mortality was calculated and corrections were done using the Abbott formula (1925).

Morphology studies

The fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus* were treated with 5 ppm of the isolated compound. The changes in morphological features and behavioral aspects of the treated larvae were studied by light microscopy. The dead larvae were mounted with Hoyer's medium on a microscopic slide and scrutinized by light microscopy (Kamalakannan et al. 2014).

Histopathological studies

The treated and control morbid larvae were fixed in 10 % formalin. The tissues were dehydrated in an ethyl alcohol series, cleared in xylene, embedded in paraplast, and sectioned. Sections were stained using hematoxylin and eosin according to routine staining methods. Untreated larvae were also investigated in the same manner (Kaewnang et al. 2011). The midguts of the treated and control larvae were photographed. The midguts of the treated larvae were examined and compared with those of the control.

Statistical analysis

All the data were analyzed using SPSS version 11.5. The average larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95 % fiducial limits of upper and lower confidence limits, and chi-square values were calculated. Results with $p<0.05$ were considered to be statistically significant.

Results and discussion

Identification and structural elucidation of active principle (catechin)

The fraction obtained from the silica gel column chromatography showed a single band in a thin-layer chromatography separation. The phenolic derivative fraction was subjected to structural elucidation by spectroscopic studies. TLC was performed and a single spot containing the compound was confirmed (Fig. 1).

The elute of λ_{max} 279 nm was subjected to UV, FT-IR, GC-MS, and ^{1}H and ^{13}C NMR spectral analyses (Fig. 2). The elute fraction of compound λ_{max} 279 nm obtained from the column was a pale yellow powder on elution with chloroform:ethyl acetate at (90:10). Two characteristic absorption bands at 219 and 270 nm in the UV spectrum indicated flavan skeleton in the molecule. The mass spectrum of the compound showed the molecular ion peak at *m*/*z* 290 which corresponds to the molecular formula $C_{15}H_{14}O_6$ (Figs. 3 and 4). The other characteristic peak at *m*/*z* 139 indicates loss of $C_7H_7O_3$. The IR spectrum showed a broad absorption band around 3400–2600 cm^{-1} region corresponding to aromatic and aliphatic C–H, phenolic, and alcoholic OH stretching. The band at 1661 cm^{-1} may be due to aromatic C=C stretching.

The 1H NMR spectrum of the compound showed three aromatic proton signals in ring B—one a doublet at δ 6.82 due to H-6', a doublet at δ 6.73 for H-5', and a doublet at δ 6.7 for the proton at H-2'. The other characteristic signals in the ^{1}H NMR spectrum are a doublet for H-2 at δ 4.57, a multiplet at δ 3.99 for the proton at C-3, and two quartets at δ 2.48 Ha and δ 2.86 Hb for the diastereotopic protons at H-4 (Figs. 5 and 6). The C^{13}-NMR spectrum exhibited 15 carbon signals. The chemical shift of a methine at δ 62.07 indicated that hydroxylation had occurred at C-3, while the signal at δ 22.32 was in agreement with the presence of a methylene carbon at C-4 (Figs. 7 and 8). On the basis of the above spectral data and comparative literature values, the compound was identified as catechin ((2*R*, 3*S*)-2-(3,4-dihydroxyphenyl) chroman-3,5,7-triol). ^{1}H NMR data are similar to those reported by Donovan et al. (1999) for the 3′ and 4′-*O*-methylated derivatives of catechin. Protons and carbons were easily assigned according to their ^{1}H and C^{13} NMR chemical shifts by comparison with data published for catechin (Bond et al. 2003). Moreover, the EI-MS shows molecular ion at *m*/*z* 290, which is in agreement with the molecular formula $C_{15}H_{14}O_6$ of catechin (Zhang et al. 1995).

Mosquito larvicidal activity of catechin

The larvicidal activity of catechin at different concentrations (1.25, 2.5, 5.0, 10.0, and 20.0 ppm) was evaluated against the

Fig. 1 TLC profile of isolated E-4 fraction from *Leucas aspera*

early fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus* (Table 1). The compound catechin exhibited 100 % mortality at 20 ppm against *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus*. The LC_{50} and LC_{90} values against *Ae. aegypti* were 3.05 and 8.25 ppm, respectively; against *An. stephensi* 3.44 and 8.89 ppm, respectively; and against *C. quinquefasciatus* 3.76 and 09.79 ppm, respectively. The results were significant at $p<0.05$.

Catechin isolated from the hexane fraction exhibited 100 % mortality against fourth instars of the three mosquito species tested at 20 ppm, which is similar to the activity of methyl-*p*-hydroxybenzoate, a phenolic derivative isolated from the leaves of *Vitex trifolia* that resulted in 100 % mortality against *C. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* at 20 ppm (Kannathasan et al. 2011). Joseph et al. (2004) reported the larvicidal effect of the neotenone, an isoflavonoid isolated from the tubers of *Neorantaenia mitis*, against *Anopheles gambiae* larvae at 20 ppm.

Foliar polyphenols exhibit biocidal effects against mosquito larvae. Ray et al. (1999) reported that the toxicity of polyphenols is exerted on the midgut epithelium of larvae; this observation supports the toxicity of the phenolic derivative, catechin.

The bioactive compound catechin, isolated from methanolic fractions of *L. aspera*, was found to possess potent mosquito larvicidal activity. Catechin, a phenolic derivative, has been reported previously from *Casuarina equisetifolia* and other plants like *Ricinus communis* and *Ulmus davidiana* to possess insecticidal properties (Jang et al. 2002).

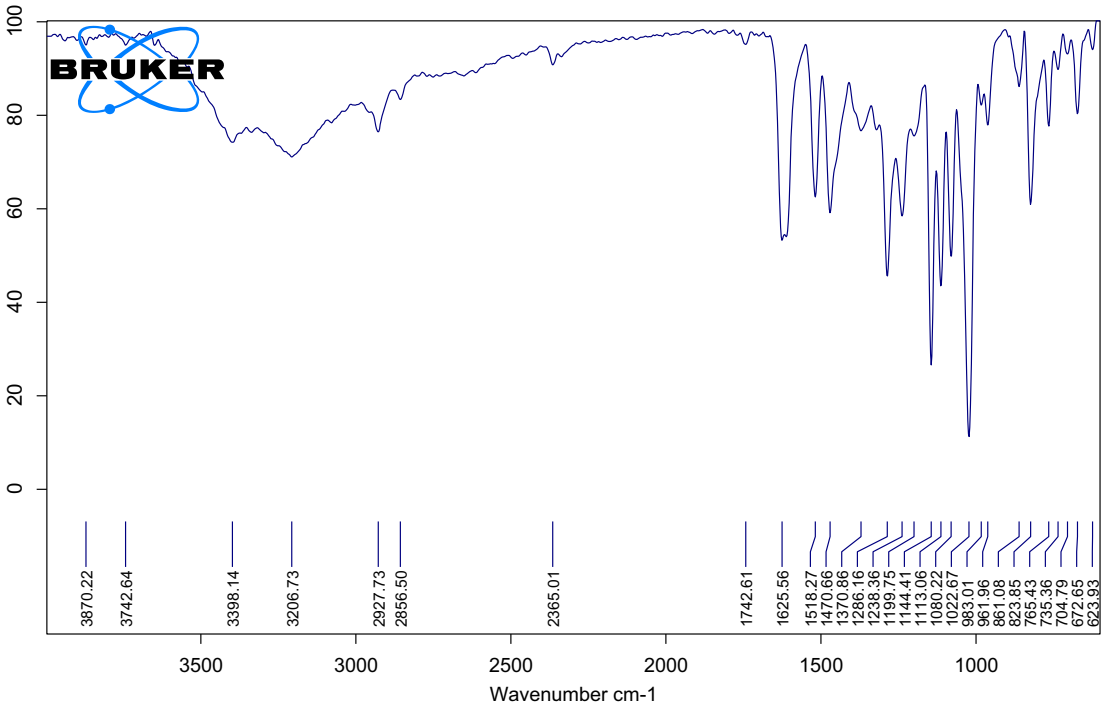


Fig. 2 FTIR spectrum of isolated compound E-4 from *L. aspera*

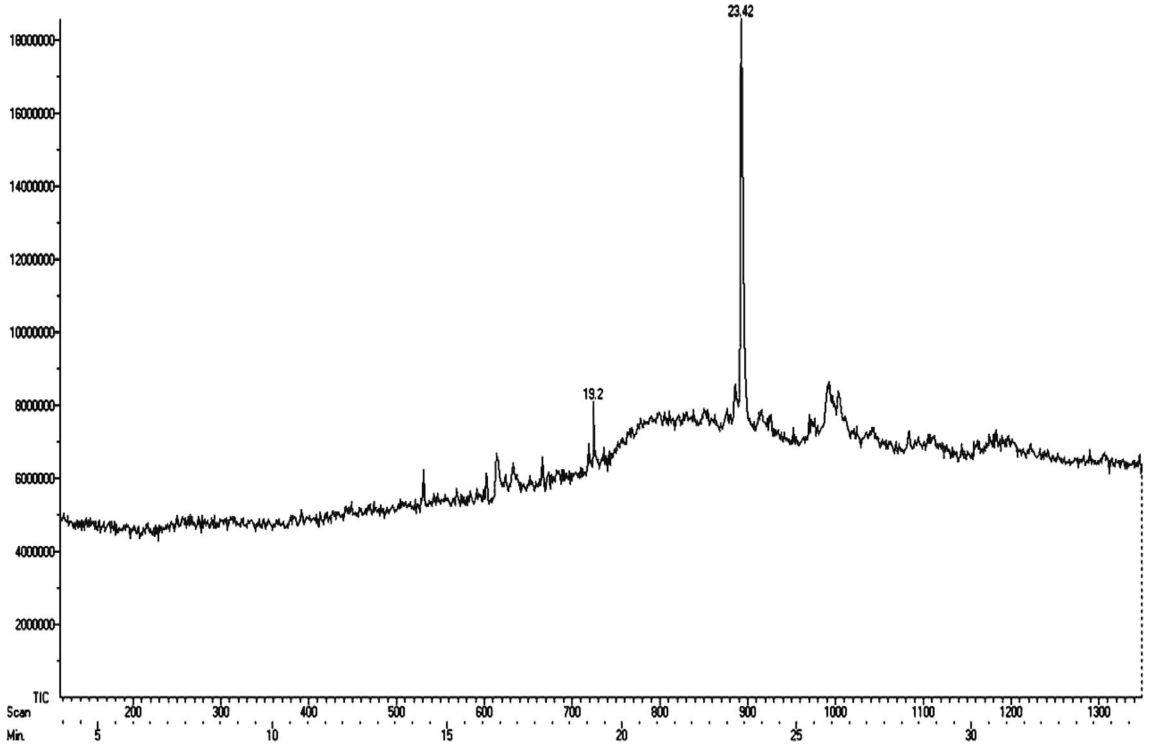


Fig. 3 Gas chromatogram of isolated compound E-4 from *L. aspera*

Yan et al. (2004) has reported the isolation and identification of flavonoids such as catechin and gallocatechin from the roots of *Rosea larvigata*. Catechin derivatives have been isolated from *Prunus grayana* (Shimomura et al. 1989) and

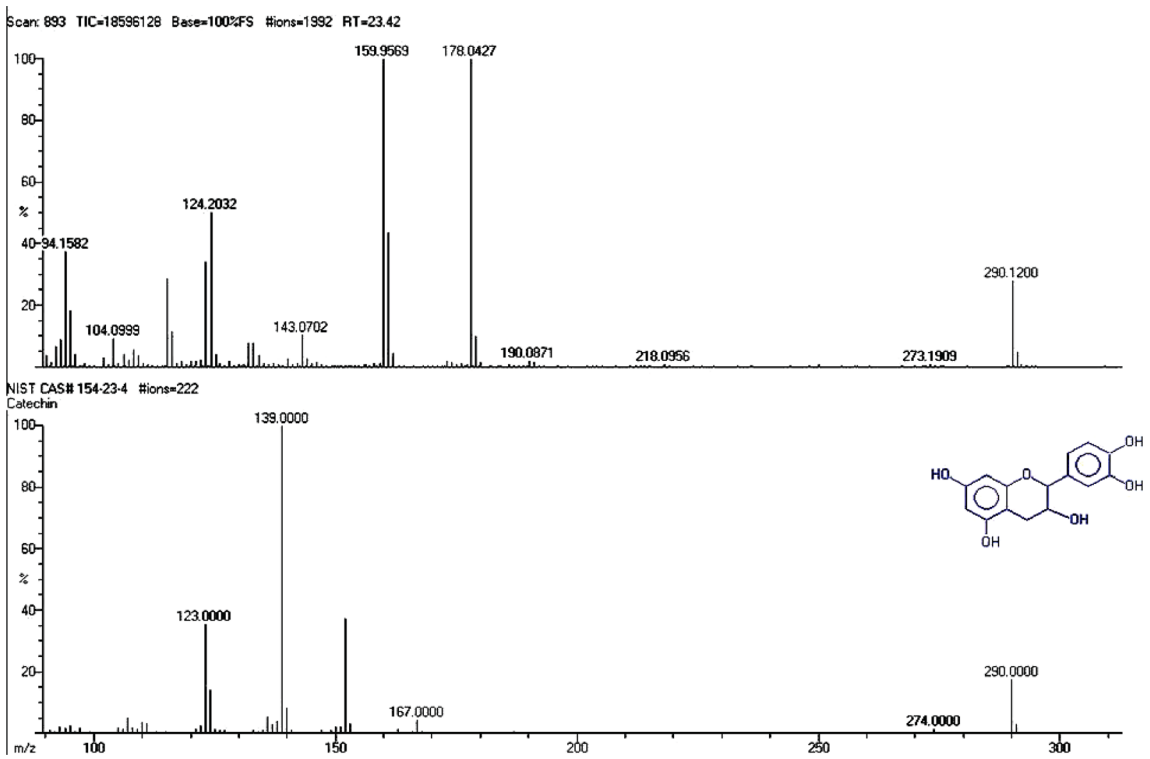


Fig. 4 ESI-MS of isolated compound E-4 from *L. aspera*

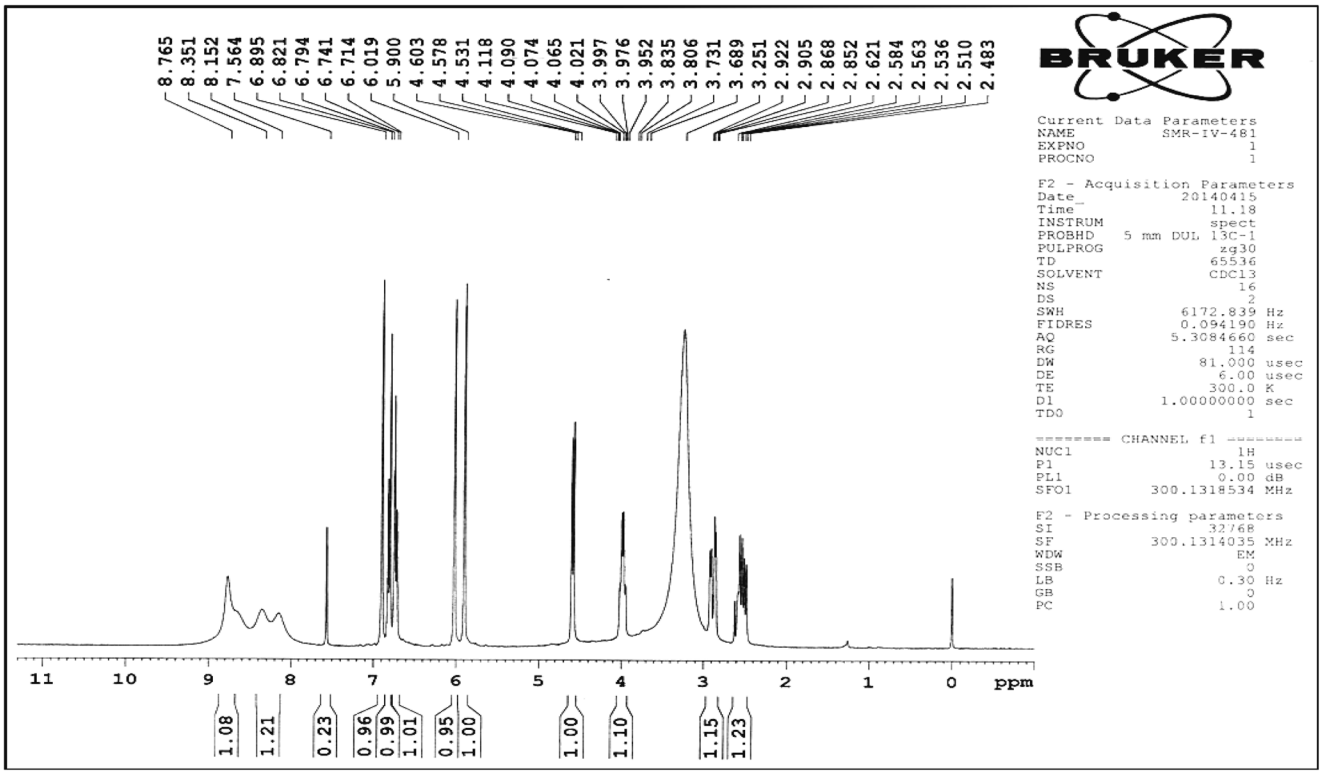


Fig. 5 [1]H NMR spectrum of isolated compound E-4 from *L. aspera*

Quercus ilex (Karioti et al. 2009). Catechin was successfully isolated from the roots and barks of the Chinese herb, *Rhenum tanguticum* (Jin and Tu 2005).

Epicatechin and catechin have been identified as substrates of polyhphenoloxide (Rocha and Morais 2001). Catechin in roots of *Podocarpus nagri* showed growth inhibitory effects

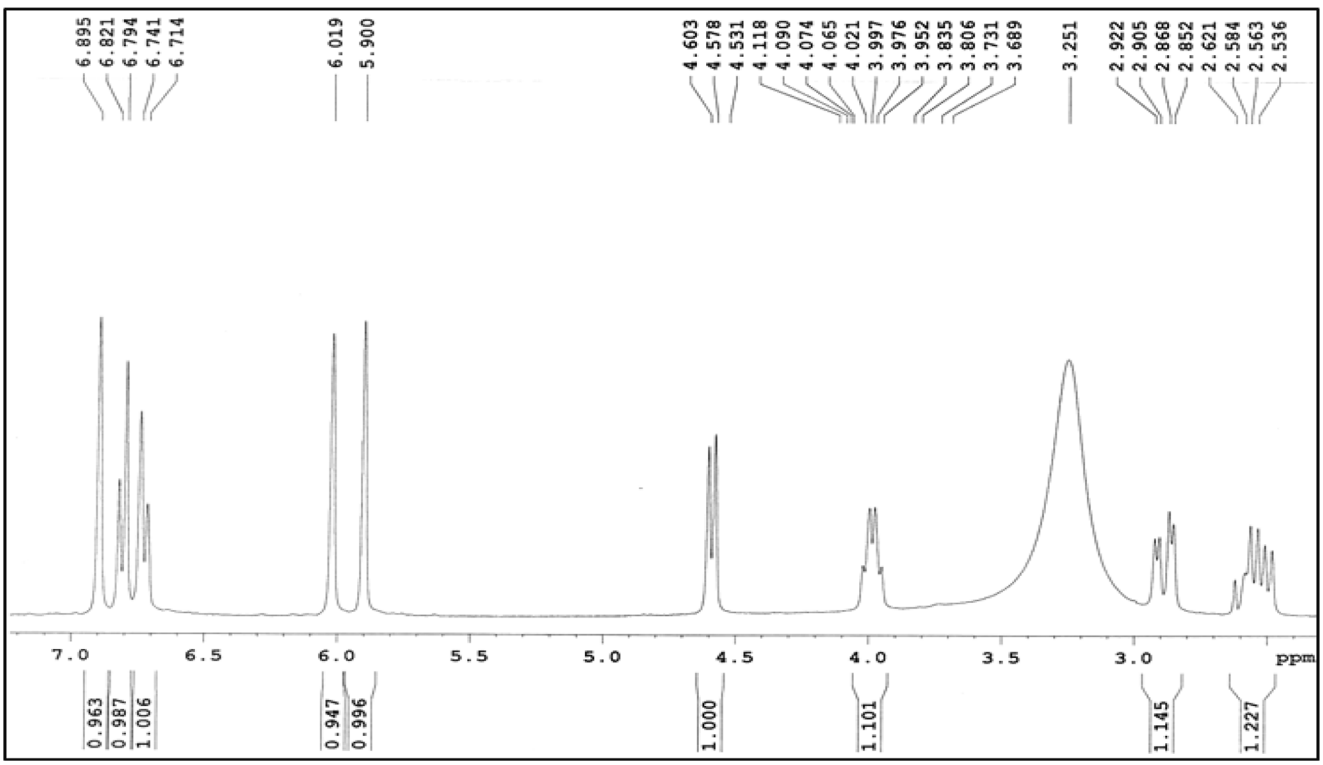


Fig. 6 [1]H NMR spectrum of isolated compound E-4 from *L. aspera*

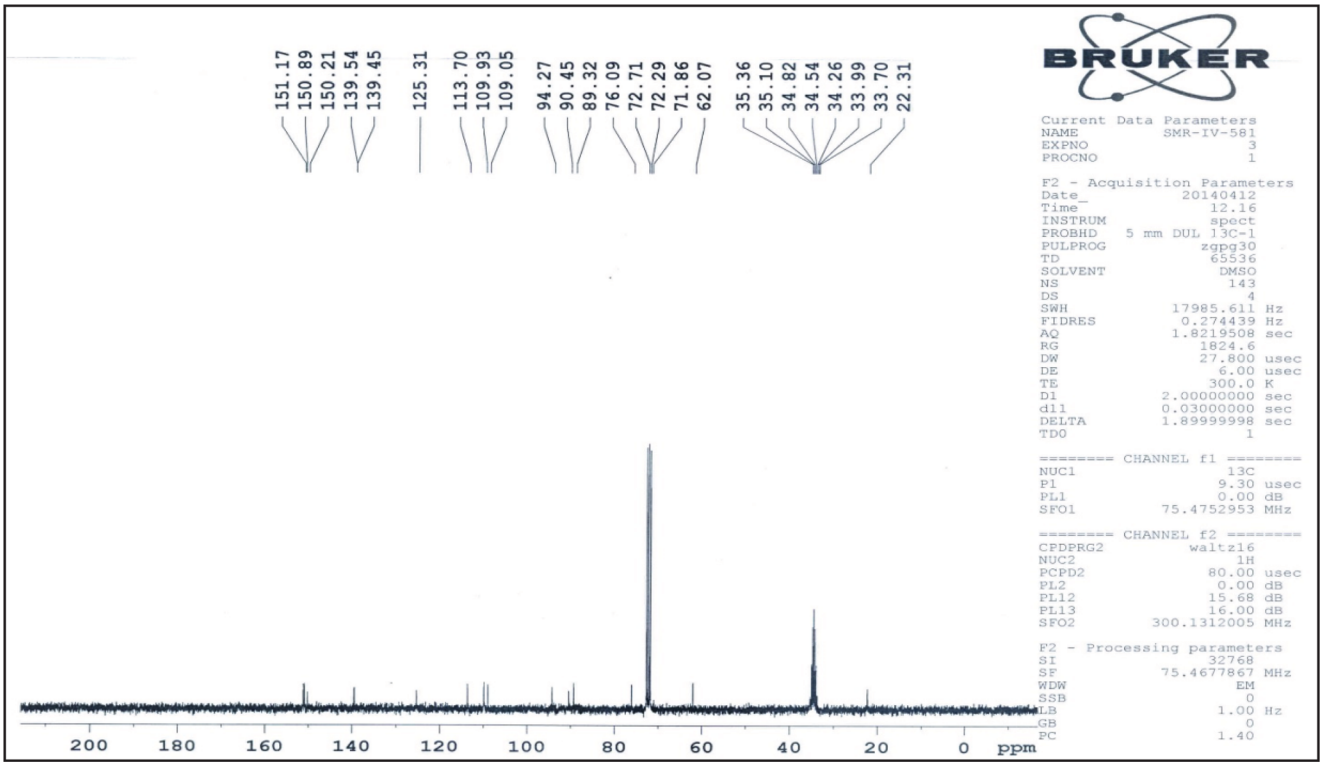


Fig. 7 ^{13}C NMR spectrum of isolated compound E-4 from *L. aspera*

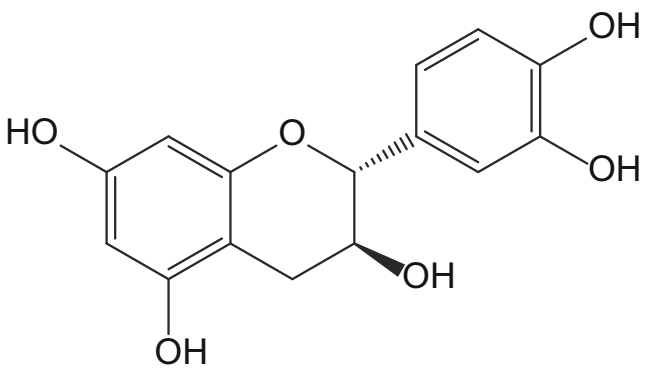
on *Heluothis virescens* larvae (Zhang et al. 1992). Quercetin and catechin members of the flavonoid family possess a variety of beneficial effects, such as anti-fibrotic, anti-inflammatory, and ROS scavenging (Boots et al. 2008; Liu et al. 2010) properties.

Morphological and histopathological studies

The fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus* mosquito species when treated with 5 ppm of catechin compound isolated from *L. aspera* showed morphological and behavioral changes. After 15 min of exposure, the larvae were found be restless and exhibited sluggish movements with increased exposure period. Morphological alterations were observed in the anal papillae region of treated larvae and the cuticle layer was found to be damaged (Fig. 9).

The midgut of mosquito larvae is considered as the region of digestion and absorption. The fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus* when treated with catechin isolated from *L. aspera* developed dramatic lesions affecting mainly the epithelial layer of the midgut (Fig. 10). Compared to the control, the larvae treated with catechin suffered various histological changes. The normal

Fig. 8 Chemical structure of catechin



(2R,3S)-2-(3,4-dihydroxyphenyl) chroman-3, 5, 7-triol Catechin

Table 1 Mosquito larvicidal activity of (2*R*-3*S*)-2-(3, 4-dihydroxyphenyl) chroman-3, 5, 7-triol (catechin) against the early fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus*

No.	Mosquito species	Con (ppm)	Mortality±SD	LC_{50}+SE (ppm) (UCL-LCL)	LC_{90}+SE (ppm) (UCL-LCL)	χ^2 (df=4)
1.	*Ae. aegypti*	1.25	16±1.140	3.052±0.163	8.257±0.780	15.5
		2.25	34±1.304	3.372–2.732	9.786–6.728	
		5	63±2.387			
		10	85±1.414			
		20	100±0.000			
2.	*An. stephensi*	1.25	11±0.837	3.447±0.163	8.890±0.846	14.4
		2.25	30±6.548	3.105–3.102	10.54–7.231	
		5	57±1.581			
		10	80±0.0548			
		20	100±0.000			
3.	*C. quinquefasciatus*	1.25	09±0.837	3.765±0.193	9.796±0.950	17.1
		2.25	28±1.140	4.143–3.387	11.65–7.934	
		5	50±0.707			
		10	76±1.342			
		20	100±0.000			

95 % Confidence interval, degrees of freedom (df) significant at $p<0.05$

LC_{50} lethal concentration that kills 50 % of the exposed larvae, LC_{90} lethal concentration that kills 90 % of the exposed larvae, *LCL* lower confidence level, *UCL* upper limit confidence level

midgut wall of fourth-instar larvae of *Aedes*, *Anopheles*, and *Culex* consists of a unicellular epithelial layer resting upon a basement membrane. The epithelial cells are cylindrical, containing a large coarsely granular nucleus that occupies the mid-position within the cells.

The treated larvae developed dramatic lesions affecting mainly the epithelium. The cross section of the midgut showed disarrangement in the appearance of columnar cells, swelling, and extruding masses of cellular material in the mid position of the gut. The columnar cells appeared swollen with distinct protrusion into the gut lumen. Some of the epithelial gut cells were elongated and separated from the basement membrane. The cytoplasm appeared vacuolated with enlarged nuclei of gut cells. Some of the cells were found to be dislodged and detached from each other with separation of cuticle layers. Fused cell mass of undifferentiated epithelial cells and undigested food particles were found in the gut lumen.

These results clearly indicate the toxic nature of the compound which is further supported by histopathological alterations observed in the treated larvae. Similarly, Green et al. (1991) reported distinct features of alteration such as swollen anal papillae of *Ae. aegypti* larvae after treatment with essential oil of *Tagetes minuta*. Structural deformation of anal papillae and cuticle layer may cause dysfunction of the anal papillae, which may be intrinsically associated with the death of mosquito larvae. The anal gills of *Ae. aegypti* larvae serve as the major site for Na^+, Cl^-, and K^+ uptake, complementing the role of the Malphigian tubules and rectum. The natural compound catechin may disturb the ion transport due to damages in the anal papillae and the outer cuticle layer of the treated larvae (Perumalsamy et al. 2013).

The columnar epithelium has striated border microvilli covered by peritrophic membrane. This membrane is surrounded

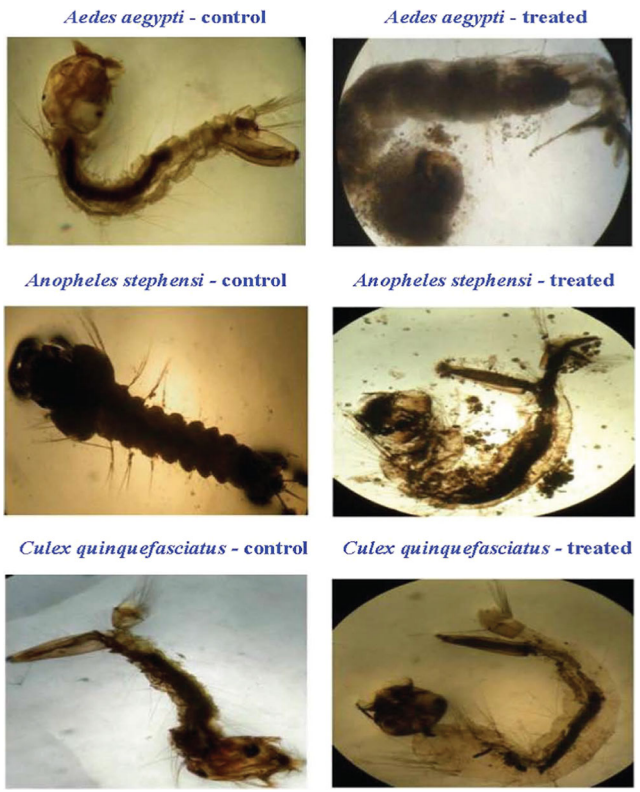


Fig. 9 Light micrographs of control and treated fourth-instar larvae of *An. stephensi*, *Ae. aegypti*, and *C. quinquefasciatus* with catechin (LC_{50}) isolated from *L. aspera* extract after 24 h of exposure (×35 magnification). The treated larvae showed morphological alterations in the anal papillae region, and the cuticle layer was found to be damaged in the treated larvae when compared to control larvae

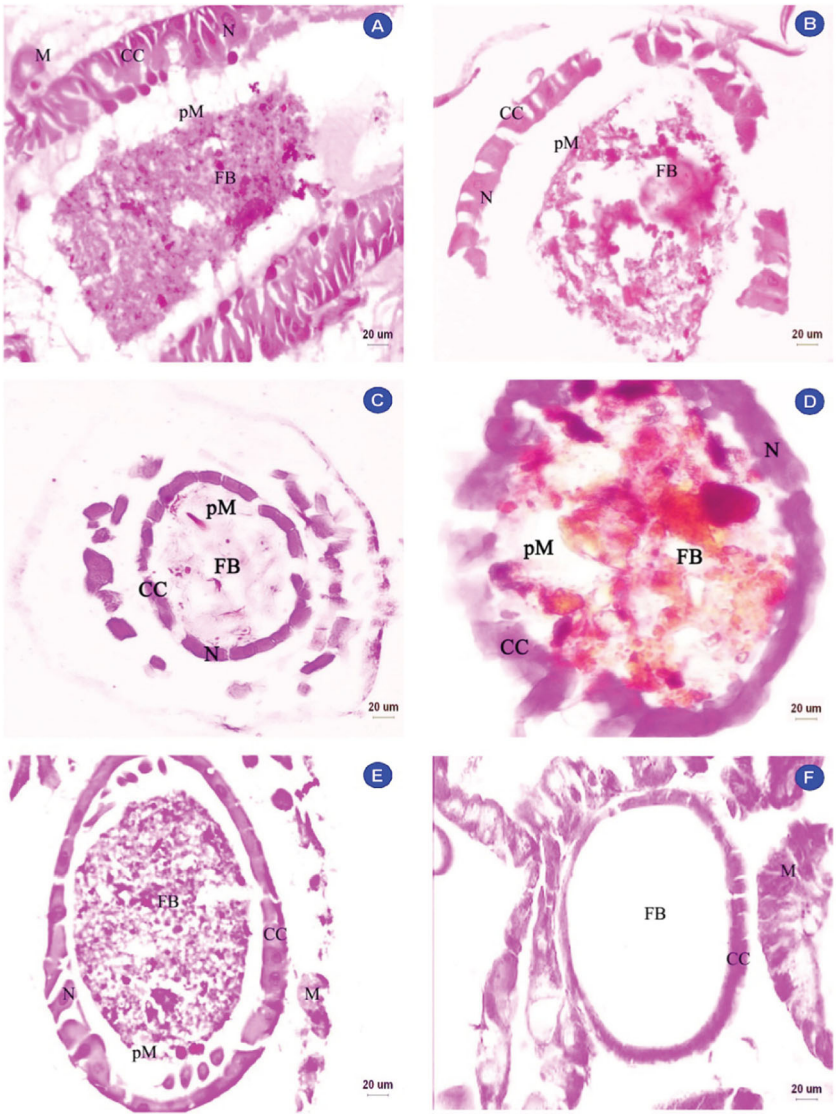


Fig. 10 Cross section through the midgut region of the fourth-instar larvae of *Ae. aegypti*, *An. stephensi*, and *C. quinquefasciatus* treated with LC_{50} of catechin, showing the effects after 24 h of exposure (×400). *N* nucleus, *pM* peritrophic membrane, *FB* food bolus, *CC* cell contents, *M* muscle layers. **a** Control—fourth-instar larvae of *Aedes aegypti*. **c** Control—fourth-instar larvae of *Anopheles stephensi*. **e** Control—fourth-instar larvae of *Culex quinquefasciatus*. **b** Treated—fourth-instar larvae of *Aedes aegypti*. **d** Treated—fourth-instar larvae of *Anopheles stephensi*. **e** Treated—fourth-instar larvae of *Culex quinquefasciatus*

externally by circular and longitudinal muscle layers. These layers cause rhythmic peristaltic movement by which the food moves through the alimentary canal. The epithelium consists of columnar cells with clusters of small regenerate cells with a relatively large nucleus and strongly basophilic cytoplasm. The epithelium is also protected from food particles by the peritrophic membrane surrounding the lumen. Hamouda et al. (1996) stated that the midgut of *C. pipiens* treated with *Artemisa judaica* affected the basement membrane and disrupted the peritrophic membrane. The mixing of the gut contents with the midgut hemolymph could have caused the larval mortality (A1-Mehmadi and A1 Khalaf 2010).

The midgut of the larval mosquito is the animal's interface with the external environment and participates in digestion, absorption, ion transport, and osmoregulation (Bernick et al. 2007). Based on previous histological studies, the midgut region of the treated larvae is the first site where cellular responses are observed (David et al. 2000). Studies by Ray et al. (1999) confirmed that plant extract and isolated compounds primarily affect the midgut epithelium. The observed histopathological effects of the compound catechin on the midgut of the treated larvae are in accordance with earlier studies. The results of the current study indicate that the phenolic derivative, catechin, may be an effective alternative to conventional synthetic insecticides for the control of mosquitoes.

Conclusion

It is evident from the present study that the compound catechin isolated from *L. aspera* could be used as a larvicide in stagnant water bodies which are breeding grounds for mosquitoes. Hence, the large biomass of the weed *L. aspera* available in the wastelands of southern India can be used as a bioresource to commercially produce the active principle catechin which could be used as a potent larvicide for mosquitos.

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