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Anti-dengue efficacy of bioactive andrographolide from *Andrographis paniculata* (Lamiales: Acanthaceae) against the primary dengue vector *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

The current study investigated the toxic effect of the leaf extract compound andrographolide from *Andrographis paniculata* (Burm.f) against the dengue vector *Ae. aegypti*. GC–MS analysis revealed that andrographolide was recognized as the major chemical constituent with the prominent peak area compared with other compounds. All isolated toxic compounds were purified and confirmed through RP-HPLC against chemical standards. The larvicidal assays established at 25 ppm of bioactive compound against the treated instars of *Ae. Aegypti* showed prominent mortality compared to other treated concentrations. The percent mortality of larvae was directly proportional to concentration. The lethal concentration (LC_{50}) was observed at 12 ppm treatment concentration. The bioactive andrographolide considerably reduced the detoxifying enzyme regulations of α- and β- carboxylesterases. In contrast, the levels of GST and CYP450 significantly increase in a dose dependent manner. The andrographolide also showed strong oviposition deterrence effects at the sub-lethal dose of 12 ppm. Similarly, the mean number of eggs were also significantly reduced in a dose dependent manner. At the concentration of 12 ppm the effective percentage of repellency was greater than 90% with a protection time of 15–210 min, compared with control. The histopathology study displayed that larvae treated with bioactive andrographolide had cytopathic effects in the midgut epithelium compared with the control. The present study established that bioactive andrographolide served as a potential useful for dengue vector management.

1. Introduction

Mosquitoes are vectors of disease that affect the human health and domestic animals. Malaria, yellow fever, chikungunya and Japanese encephalitis are most of the common mosquito vectors spreading diseases. Among these dengue fever, dengue haemorrhagic fever, yellow fever and chikungunya, endemic in Southeast Asia and Africa are primarily transmitted by *Aedes aegypti* L (Diptera: Culicidae), (Maillard et al., 1993; Lija-Escaline et al., 2015). People living in 128 countries totalling almost 4 billion currently are at risk for infection of dengue viruses (DENV-1 to –4). This is more than any other arthropod-borne virus caused disease associated with human morbidity and mortality worldwide (Brady et al., 2012; Bhatt et al., 2013; Duong et al., 2015). About two-thirds of the world's population live in the presence of mosquito dengue vectors (Thanigaivel et al., 2012). It is estimated that 34% of the dengue infected cases occur in India (Bhatt et al., 2013) where dengue is considered to be endemic (Gupta et al., 2012).

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Currently, heavy synthetic chemical insecticides used to control mosquitoes has produced populations with resistance, which permits mosquito resurgence and can cause undesired effects on beneficial non-target organisms (Devine and Furlong, 2007), comprising natural competitors in the terrestrial ecosystem (Greenwood and Mutabingwa, 2002). Hence, improved chemistries are needed as an alternative product to aid the control of mosquitoes. Plants are known to be chemical factories, defending themselves from insects and other herbivores. Thus, the search for novel natural chemical products isolated from plants or other organisms, have gained renewed importance in recent decades (Senthil-Nathan et al., 2005a,b; Kalaivani et al., 2012).

While it is estimated that more than 2000 plant species have insecticidal properties, few have been thoroughly investigated (Nataya et al., 2010). In the last three decades, plant derived secondary metabolites have been isolated which are more specific in targeting only larval stages in mosquitoes (Senthil-Nathan et al., 2005a,b; Rattan, 2010). Increasingly, plant compounds are being used worldwide in the management of mosquitoes and other pests (Senthil-Nathan, 2007, 2013, 2015). Phytochemicals have demonstrated properties as larvicides, insect growth regulators, repellents, ovipositional attractants or deterrents (Senthil-Nathan, 2015).

Andrographis paniculata Nees (Burm.f) (Acanthaceae) is a shrub that is commonly known as 'King of Bitters' (Nosalovaa et al., 2014). The plant *A. paniculata* is traditionally used as a medicine to treat different diseases in southern Asia, China and India (Govindarajan, 2011). Several studies reports that the plant to contain phytochemicals which have antibacterial, hypoglycemic, hypercholesterolemia, and adaptogenic properties (Shahid, 2011). The main phytochemical compound is a bicyclic diterpenoid lactone (Chao and Lin, 2010) some constituents known are andrographolide, neoandrographolide (NAND), 14-deoxyandrographolide (DAND), and 14-deoxy-11, 12-didehydro andro-grapholide (DDAND) (Rao et al., 2004).

Diterpenoids from plants are considered to be the main source of biological activity (Samy et al., 2008). Previous studies have demonstrated that andrographolide is the primary active diterpenoid component of *A. paniculata* (Zhang et al., 2014), showing anti-inflammatory and antimicrobial activity (Shen et al., 2002; Wang et al., 2014). Andrographolide's have some limitations in direct used as a healing agent due to decreased water solubility (Xiaoa et al., 2013). The crude extract of *A. paniculata* shows larvicidal activity against many insects and specifically mosquitoes such as *Culex quinquefasciatus* (Sheeja et al., 2012) and *Ae. aegypti* (Govindarajan, 2011). Combined effects of *A. paniculata* and *Anasimyia lineata* on insects demonstrated insecticidal activity against *C. quinquefasciatus* and *Ae. aegypti* (Renugadevi et al., 2013), with antifeedant and antioviposition activity against *Plutella xylostella*, the diamondback moth (Lepidoptera) (Hermawan et al., 1994). This study evaluated the biological effects of andrographolide extracted from leaves of *A. paniculata* on the survival, biochemical, and cytopathic effects on the mosquito, *Aedes aegypti*, Linn.

2. Materials and methods

2.1. Mosquito culture

Aedes aegypti culture was maintained at the Biopesticides and Environmental Toxicology Laboratory (BET Lab), SPK Centre for Excellence in Environmental Sciences since at least 2007, without exposure to insecticides. They were maintained at $27 \pm 2\,^{\circ}C$ and 75–85% RH under a 14:10 L/D photoperiod. Larvae were fed a diet of Brewer's yeast, dog biscuits (Choostix Biskies) and algae collected from ponds in a ratio of 3:1:1 respectively. Pupae were transferred from the trays to a cup containing tap water and placed in screened cages of ($50 \times 50 \times 50$ cm dimension) for adult emergence. Adults were provided with 10% sucrose solution. On the fifth day emerged adults were deprived of sugar for twelve hours, then provided with a mouse placed in resting cages overnight for blood feeding by females. Adult mosquitos were maintained at the similar conditions and the larvae from the hatched eggs were used in the experiments.

2.2. Plant collection, extraction and purification

The plant *A. paniculata* (Fig. 1A) was collected from Alwarkurichi village, Tirunelveli district, Tamil Nadu, India, (8.78806′N–77.36088′E) during morning hours and washed twice with tap water and air dried at room temperature. The plant extraction was followed based on the procedure of Thanigaivel et al. (2012) with slight modification. Dried leaves were cut into pieces with electric stainless steel blender. About (200 g) of powdered leaf were laden with 750 ml of ethanol for 48 h using soxhlet equipment. Extractions were concentrated with a rotary evaporator, and yields were stored at 4 °C for further use.

The extraction procedure was followed Extract was washed with benzene and water for recrystallization. Further purification was done in column chromatography. The residue of about (5 g) were chromatographed on a 150 g silica-gel column of pore size 60–120, and are eluted under different gradients. Chloroform and ethanol were used as the mobile phase. The elution's were carried out on different gradients 90:10, 80:20, 70:30, 60:40, 50:50 and 30:60. The fractions collected from the column were one fourth of the column volume. About 10 fractions were collected individually and kept at room temperature for solvent evaporation and tested for their activity and finally then stored at 4 °C. The fraction which was found to be most effective was kept for solvent evaporation. For further purification the fractions were again chromatographed and identification of the sample were analysed by GCMS and HPLC.

2.3. GC–MS analysis of active fractions

Active fractions from *A. paniculata* were used for GC–MS analysis. One ml of sample was dissolved in HPLC grade methanol and then subjected to GC and MS_JEOL_GC mate equipped with secondary electron multiplier. JEOL GCMATE II GC–MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column used (HP5) was fused silica 50 m × 0.25 mm I.D. The run time was 20 min at 100 °C, 3 min at 235 °C for column temperature, 240 °C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1 μl) was evaporated in a splitless injector at 300 °C. The compounds were identified by gas chromatography coupled with mass spectrometry with source at 270 °C at 70 eV. The molecular weight, molecular formula and structure of the compounds of test materials were ascertained by interpretation on mass spectrum of GC–MS using the database of National Institute Standard and Technology (NIST).

2.4. HPLC

The HPLC analyses were carried out in Agilent Technologies LC 8A with C18 column (250 mm × 4.6 mm). Elution was carried out with a mobile phase of methanol: water 65:35 at a flow rate 1 ml/min. The detection was performed with D2 lamp at 224 nm wavelength. Chem32 software was used for integration to find peak area, percentage and purity of the injected sample.

The standard andrographolide $C_{20}H_{30}O_5$ (purity >99%, received from Sigma-Aldrich Chemicals, Chennai) shown in (Fig. 1C), was used as working standard. About 4 mg of standard was dissolved in 1 ml of HPLC grade methanol which gives 3.90 mg/ml concen-

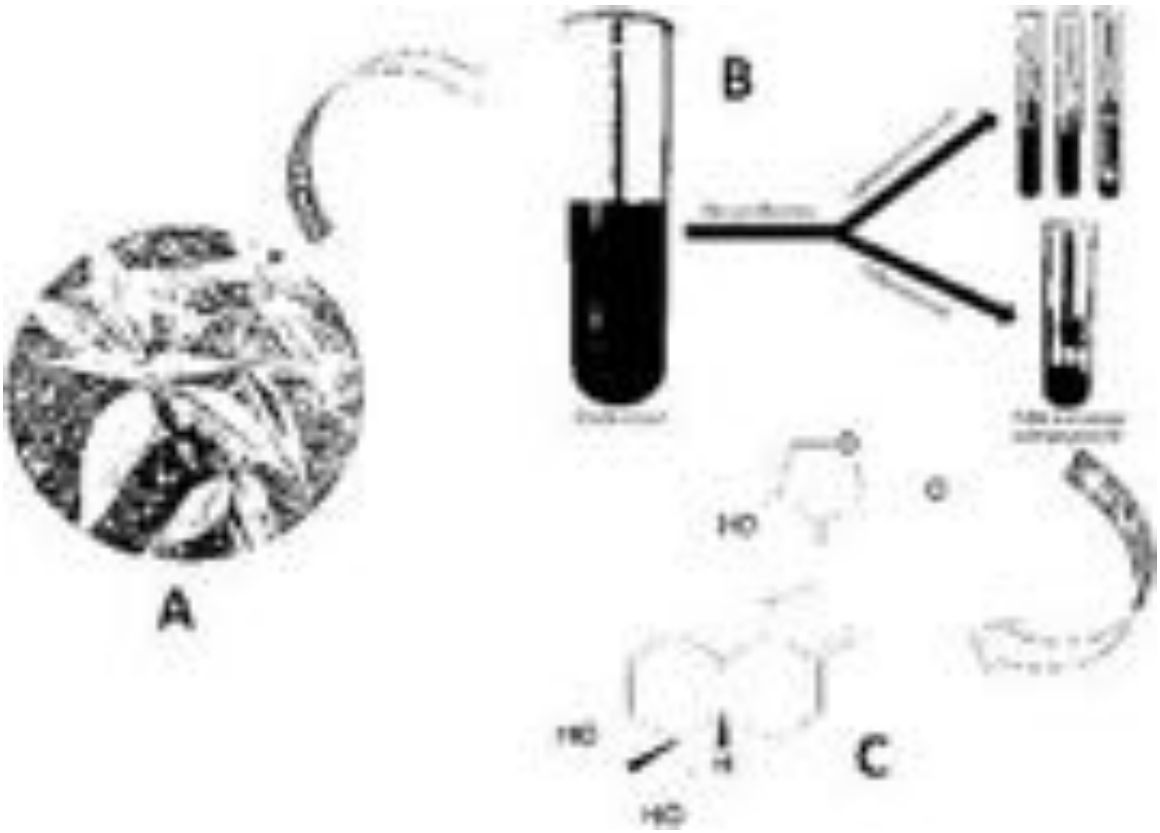

Fig. 1. External morphology of *Andrographis paniculata* (A); Purified plant compound obtained by crystallization process (B); structure of andrographolide (C).

tration. From the above standard solution 20 μl was injected for analysis in 5 replicates to find Relative Standard Deviation (RSD) values.

The active fraction was sonicated for complete dissolution and equilibrated at room temperature. From this the fraction was diluted 1:1 dilution with methanol and injected 20 μl for analysis. The obtained result was compared with standard chromatography.

2.5. Larvicidal assay

Bioassays were performed on first to fourth instars of *Ae. aegypti* at different concentrations of 5, 10, 15 and 25 ppm. Twenty larvae of first, second, third and fourth instar were introduced to a 250-ml glass beaker containing various concentrations of andrographolide for mortality studies. Throughout this experiment, no food was provided to the larvae and the rate of mortality was recorded after 24 h. Probit analysis was used to calculate the lethal concentration (LC_{50} and LC_{90}) (Minitab®17). The treatments were replicated five times, and each replicated set contained one control. Mortality percentage was calculated and corrections for mortality when necessary were done by using Abbott's (1925) formula.

$$\text{Corrected percentage of mortality} = \left(1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}}\right) \times 100 \qquad (1)$$

2.6. Total body homogenates for enzyme assay preparation

Control and Treated larvae of fourth instar were washed with double distilled water, and the adhering water was totally removed from the surface by blotting with tissue paper. The larvae were distinctly homogenized in eppendorf™ tubes using a hand homogenizer in 500 μl of ice-cold sodium phosphate buffer (20 mM, pH 7.0) to enzyme activity. The homogenates were centrifuged (8000 × *g* at 4 °C) for 20 min and supernatants were used for the further analyses. The obtained homogenate were kept on ice for further assays.

2.7. Cytochrome P450 activity

The control and treated larvae of *Ae. aegypti* were washed with double distilled water, and removed the water adhering on the surface. Larvae were chilled and dissected by placing in 40 mM sodium phosphate buffer (pH 7.2). The heads, last abdominal segment, and digestive system were removed from the larvae. The assay was performed according to Boyer et al. (2005). Effect of andrographolide on cytochrome P450 activity was determined by measuring ethoxycoumarin-*o*-de-ethylase activity in the body walls. Black, round-bottom 96-well microplates were filled with 100 μl of a 0.4 mM 7-ethoxycoumarin solution containing 50 mM sodium phosphate buffer (pH 7.2). Individual larval carcasses were placed in each well and incubated for 4 h at 30 °C. The reaction was stopped with the addition of 50 μl of glycine buffer (1 mM, pH 10.4) and 50 μl of ethanol. Larval carcasses remained at the bottom of the well and were not detached before reading. Six wells containing 100 μl of phosphate buffer, 50 μl of glycine buffer, and 50 μl of ethanol served as the blank. The fluorescence of the reaction medium was measured from the top of the wells using a Synergy HT micro plate reader (Bio-Tek Instruments, India) with 400 nm excitation and 480 nm emission filters. The production of 7-hydroxycoumarin (7-OH) was expressed as μmol 7-OH/mg larvae/min.

2.8. Carboxylesterase assays

The activity of α and β-carboxylesterase in the *Ae. aegypti* was evaluated by the method of Agra-Neto et al. (2014). The larval extracts in 0.1 M phosphate potassium pH 7.2 were used. The

extract (20 μl; 84 μg of protein) was mixed with 500 μl of a solution containing 0.3 mMα- or β-naphthyl acetate in 0.1 M phosphate potassium at pH 7.2 containing 1% acetone. The reaction mixture was incubated for 20 min at 30 °C. Then, 0.1 ml of a mixture containing 0.3% Fast Blue B and 3.3% Sodium Dodecyl Sulphate (SDS) was added. After centrifugation (3000*g*, 28 °C), the supernatant absorbance at 590 nm was recorded. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of α- or β-naphthol per minute.

2.9. Glutathione-S-Transferase activity

GST activity samples were equipped according to the method of Larson et al. (2010). Individual *Ae. aegypti* fourth instar larvae homogenized in 250 μl of 50 mM sodium phosphate buffer (pH 7.2) before being centrifuged at 15,000 × *g* at 4 °C for 20 min. The Sigma-Aldrich (Catalog 0410, Bangalore, IN) GST assay kit was used to evaluate the conjugation of the thiol group of glutathione to the 1-chloro-2, 4-dinotrobenzene (CDNB) substrate. A total of 20 μl of homogenate was added to each well before 200 μl of solution containing Dulbecco's phosphate buffer (Sigma-Aldrich, Bangalore, IN), glutathione reduced (4 mM), and CDNB (2 mM). Rodriguez et al. (2001) determined the saturation concentration of CDNB to be 50 mM and the optimum time for reading to be 3 min. The 96-well flat-bottom UV microplate (Catalog 3635, Corning, Haryana, IN) was immediately loaded onto a Synergy HT microplate reader (BioTek, Mumbai, IN). After a 1-min lag time, the absorbance was read at 340 nm and the samples were read each 60 s for 3 min. Protein concentrations were verified using an albumin standard and bicinchoninic acid protein assay kit (Catalog TP0l00, Sigma Aldrich, Bangalore, IN). The activity of GST was expressed as μmol/mg protein/min substrate conjugated.

2.10. Oviposition deterrence index

Twenty-five gravid females were placed in a cage containing 250 ml glass jars, each jar containing 200 ml of distilled water and placed at diagonally opposite corners of the cage. Different concentrations of andrographolide (5, 10, 15 and 25 ppm) were mixed with distilled water to obtain the desired concentration. A piece of filter paper folded into a cone was placed at the top covering the mouth of the cups, this supports oviposition. During the tests the females were maintained at relative humidity of 14:10 light:dark. The eggs in oviposition paper sheets were manually counted using a stereomicroscope (Optika- Microscope, Italy). The oviposition deterrence index (ODI) was calculated by using the Formula (2). Oviposition assay was performed according to Santos et al. (2012).

$$\mathrm{ODI} = \frac{\mathrm{Nt\text{-}Ns}}{\mathrm{Nt+Ns}} \times 100 \quad (2)$$

Where, Nt = total number of egg rafts in test solution
Ns = total number of egg rafts in control

2.11. Repellent bioassay

2.11.1. Arm-in-cage repellency tests

The bioactive andrographolide were tested for repellency against *Ae. aegypti* mosquitoes at different concentrations 5, 10, 15 and 25 ppm using method cited in World Health Organization (2009). Gravid females 100 mosquitoes (mated, 5–7 days post emergence) were starved for 24 h without a blood meal but previously fed on 10% sucrose solution were kept in net cages (45 cm × 35cm × 45 cm). An area 3 × 10 cm on each forearm of three human volunteers was marked out with a permanent marker and the remaining area covered with paper sleeve with a hole corresponding to the marked area and they had no contact with lotions, perfumed soaps on the day of the assay. As a blank control, ethanol was placed on one forearm of the some volunteer with the same process as the test repellents, whereas the other forearm was untreated. *Ae.* aegypti was test during daytime from 800 h to 1600 h. The control and treated arms were introduced simultaneously into the mosquito cage and gently tapping the sides on the experimental cages, the mosquitoes were activated. Each test concentration was repeated three times. The volunteers conducted their test of each concentration by inserting the treated and control arm into the cages at the same time for one full minute for every 5 min. The mosquitoes that land on the hand were recorded and then shaken off before they imbibes any blood. The percentage of repellency was calculated by the following Formula (2).

$$\%\,\mathrm{Repellency} = [(\mathrm{Ta} - \mathrm{Tb})/\mathrm{Ta}] \times 100 \quad (3)$$

Where **Ta** is the number of mosquitoes in the control group and **Tb** is the number of mosquitoes in the treated group.

2.11.2. Histology and cytopathic effects studies

The andrographolide treated larvae and control larvae were fixed overnight in Bouin's solution, dehydrated and mounted in paraffin wax blocks. The blocks were cooled to −27 °C for 3 h and cut into 1.5 μm ribbons with an ultra-cryo-microtome (Cryocut 1800, Leica, Germany). Ribbon sections were mounted on the glass slide and stained with haematoxylin and eosin for the examination of histopathology under a bright field microscope. The sections of treated and untreated larvae of *Ae. aegypti* were observed and photographed under Optika, Flow series HBO light microscope (model: B-600 TiFl-Italy).

2.11.3. Statistical analysis

Data from mortality experiments were subjected to analysis of variance (ANOVA of arcsine, logarithmic and square root transformed percentages) and data were expressed as a mean of five replicates. Significant differences between treatment groups were analysed by Tukey's multiple range test (significance at $P < 0.05$) using Minitab®17 programme. For enzyme activity, technique of Microcal Software (Sigma plot 11) was used. The lethal concentrations required to kill 50% (LC_{50}) of larvae in 24 h were calculated by Probit analysis with a dependability interval of 95% using the Minitab®17 programme.

3. Results

The product from solid-liquid extraction was washed repeatedly with benzene and water for recrystallization. This had removed the compounds which are soluble in water, benzene and colouring pigments present. The pellet obtained was pale brown from dark brown (Fig. 1B). The pellet was then subjected to column chromatography and the active fraction obtained was analysed by GCMATE II GC–MS (Agilent Technologies 6890N Network GC system for gas chromatography) compounds shown in (Fig. 1C). Compounds identified from fractionation were confirmed by comparing the results obtained from GC–MS against the NIST library. The results identified five compounds, with the active fraction being pentadecanoic acid, 13-methyl, phytol, retinoic acid, andrographolide and ergosterol. Andrographolide was the chief component identified (Fig. 2).

Trace compounds found in the fraction were further purified by column chromatography to improve the purity. These were analysed by HPLC. The analysis revealed that the active fraction showed one major peak and two minor peaks. The results obtained were calculated for purity by comparing with standard chromatogram of andrographolide (Fig. 3). The active fraction showed 88% purity when compared with standard andrographolide (Fig. 4).

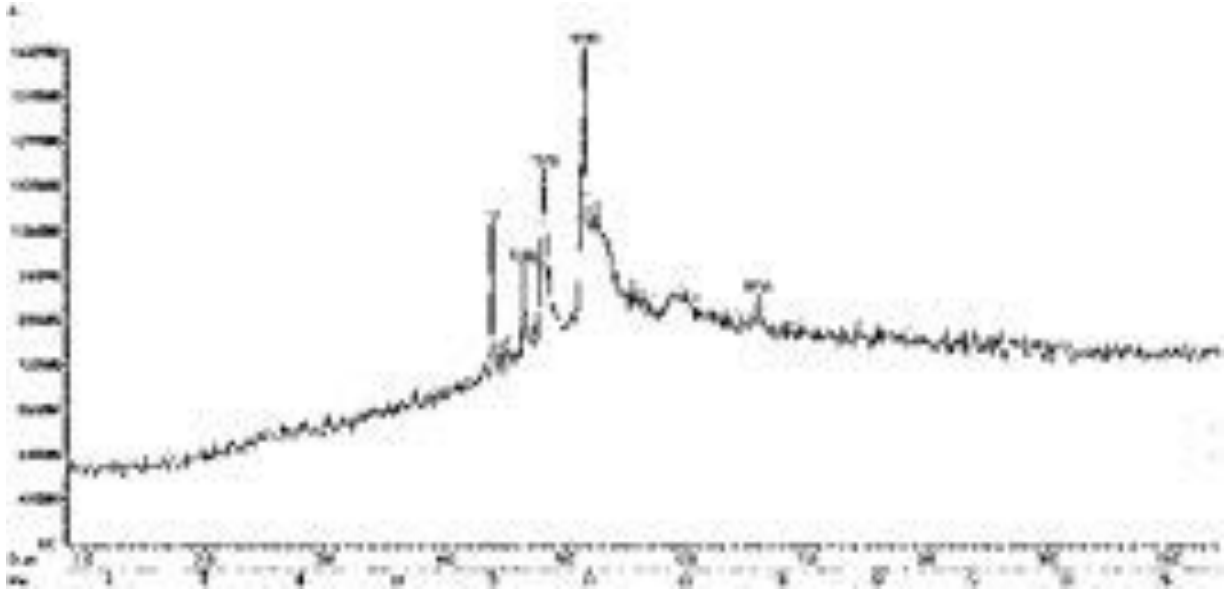

Fig. 2. GC–MS chromatogram performance to identify the compounds present in the active fraction.

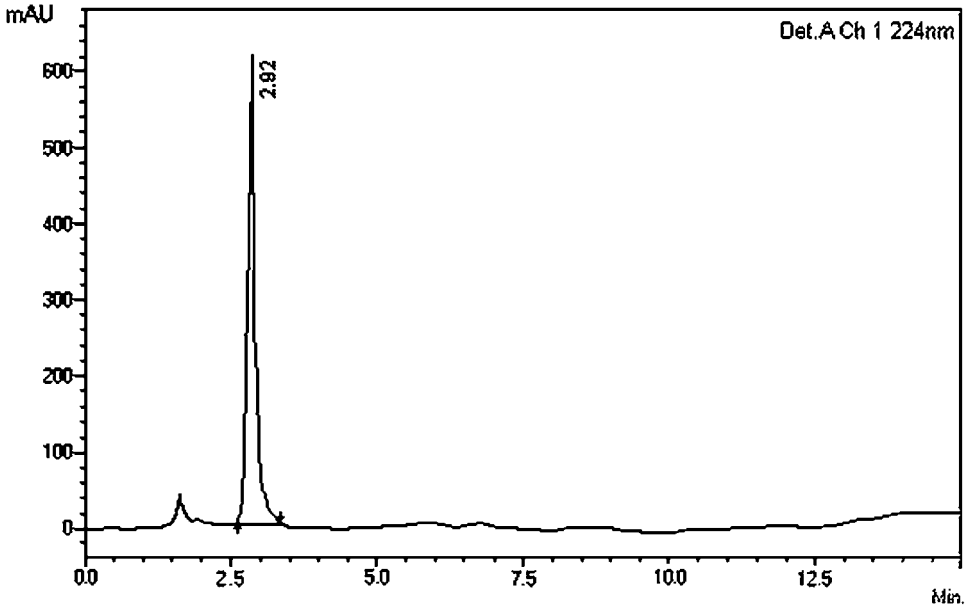

Fig. 3. HPLC chromatogram of standard andrographolide.

At the Rt 3.01 the fraction was eluted and which was spotted at 224 nm as compared with the standard chromatogram. Similarly, the standards of andrographolide was eluted at the Rt of 2.92. This confirmed the compound isolated was andrographolide.

Andrographolide treatments of first- to fourth-instar Ae. aegypti larvae at different concentration (5, 10, 15, and 25 ppm) produce a dose dependent mortality (Fig. 5). Lethal concentration (LC_{50}) of fourth instar larvae of *Ae. aegypti* was observed at 12 ppm (Fig. 6). The first instar larvae shows the greatest mortality rate (91.60%) at the concentration of 25 ppm ($F_{4,20} = 80.99$, $P \leq 0.0001$) and significant difference from test at 15 ppm ($F_{4,20} = 80.99$, $P \leq 0.001$), 10 ppm ($F_{4,20} = 80.99$, $P \leq 0.0001$), 5 ppm ($F_{4,20} = 80.99$, $P \leq 0.0001$) and control larvae ($F_{4,20} = 80.99$, $P \leq 0.0001$) with 66, 42, 21 and 4 percentage mortality respectively.

The effects of andrographolide on enzymatic profile of the fourth instar larvae of *Ae. aegypti* was observed by exposure with different lethal concentrations (3–12 ppm) for 24 h. The α-carboxylesterase level was steadily decreased in the control and maintained the equal level after treatment for 24 h with andrographolide, rate of decline in the enzyme activity at 12 ppm, which was significantly ($F_{4,20} = 11.32$, $P \leq 0.0001$) different than the other treated concentrations. Similarly, the enzyme analysis of β-carboxylesterase also shows the highest declined rate in the protein profile at 12 ppm concentration being significantly different from all other treated concentrations ($F_{4,20} = 9.34$, $P \leq 0.0001$). The different concentrations of bioactive andrographolide significantly decreased the activity of both α- and β-carboxylesterase (Fig. 7). But, the level of cytochrome P450 and GST activities prominently gets increased after *Ae. aegypti* larvae were treated to different concentrations of andrographolide. The GST protein profile significantly increased in concentrations of 12 ppm ($F_{4,20} = 44.37$, $P \leq 0.0001$) and maintained a constant concentration in the 3 and 9 ppm andrographolide treat-

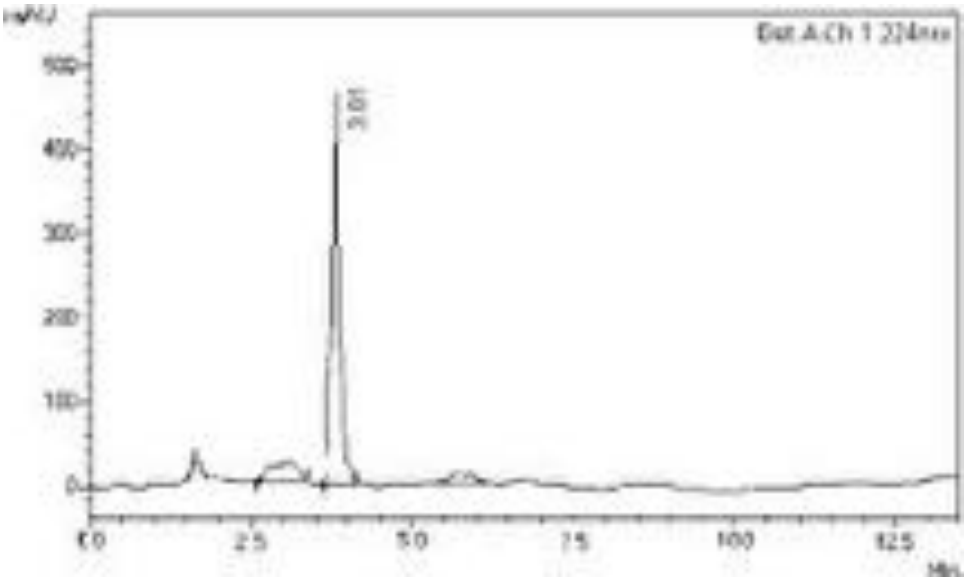

Fig. 4. HPLC Chromatogram of isolated compound andrographolide.

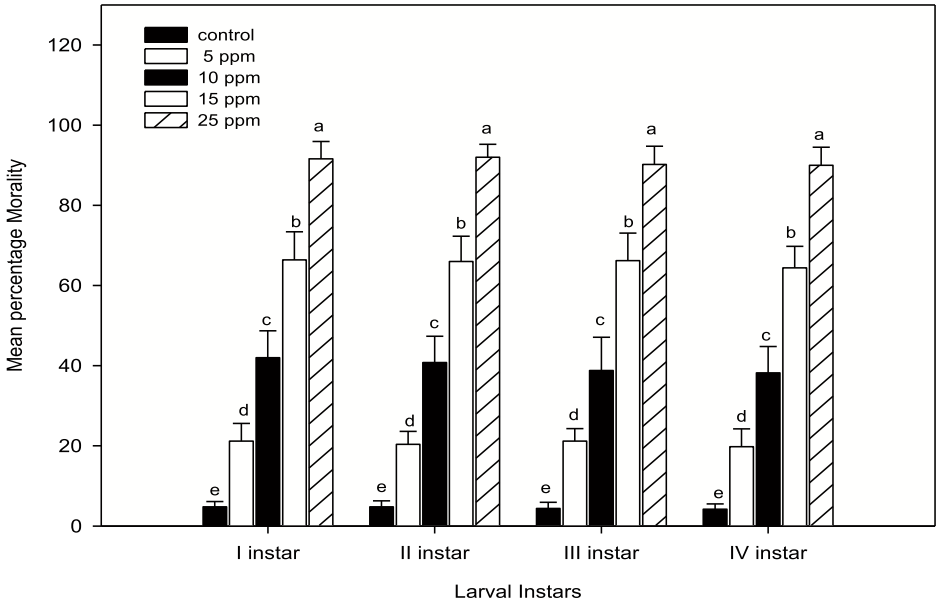

Fig. 5. Percentage mortality of *A. aegypti* after treatment with andrographolide from *A. paniculata*. Means (±standard error (SEM)) followed by the same letters above bars indicate no significant difference ($P < 0.05$) in a Tukey's test.

ments. Similarly, cytochrome P450 level was also up-regulated in the 12 ppm ($F_{4,20} = 30.03$, $P \leq 0.0001$) and it was significantly different from the 3 and 12 ppm of andrographolide (Fig. 8).

The andrographolide affected both the deterrence index (ODI) and egg hatchability of *Ae. aegypti* (Fig. 9). The deterrence index was largest at 12 ppm ($F_{4,20} = 78.58$, $P \leq 0.0001$) being significantly different from 9 ppm, 6 ppm, 3 ppm and control. But there was no significant difference between the effects of the 3 ppm and the control ($F_{4,20} = 78.58$, $P \leq 0.203$). At the concentration of 12 ppm the mean fecundity rate declined significantly comparable to the other treatments ($F_{4,20} = 97.72$, $P \leq 0.0001$). The mean number of eggs laid by females decreased from 127.7 to 31.5 between control and 12 ppm concentration respectively.

Results from the skin repellent activity of andrographolide against blood starved adult females of *Ae. aegypti* showed significant repellent activity with 12 ppm ($F_{4,20} = 3.44$, $P \leq 0.011$) and provided a 98.8% protection rate up to 210 min post treatment, being significantly different from 50 ppm ($F_{4,20} = 19.15$, $P \leq 0.001$), 6 ppm ($F_{4,20} = 9.23$, $P \leq 0.001$) and 10 ppm ($F_{4,20} = 15.01$, $P \leq 0.001$) (Fig. 10). The andrographolide gave protection against mosquito bites without any allergic reaction from the test person. The results clearly show that repellent activity was dose dependent (Fig. 10).

The gut histological evaluation of *Ae. aegypti* shows significant changes between the control and the andrographolide bioactive compound treated larvae. Consistency in cell arrangement was observed in the control larvae (Fig. 11A). But, the cellular components were severely disturbed in the larvae treated with increasing concentrations (Fig. 11B & C). The midgut epithelium of the treated larvae starts to break down, with cells which are vacuolated, but with competent nuclei. In control larvae the gut epithelium remains well formatted and organized. These results revealed that andrographolide treatments severely affects midgut tissues of *Ae. aegypti* larvae.

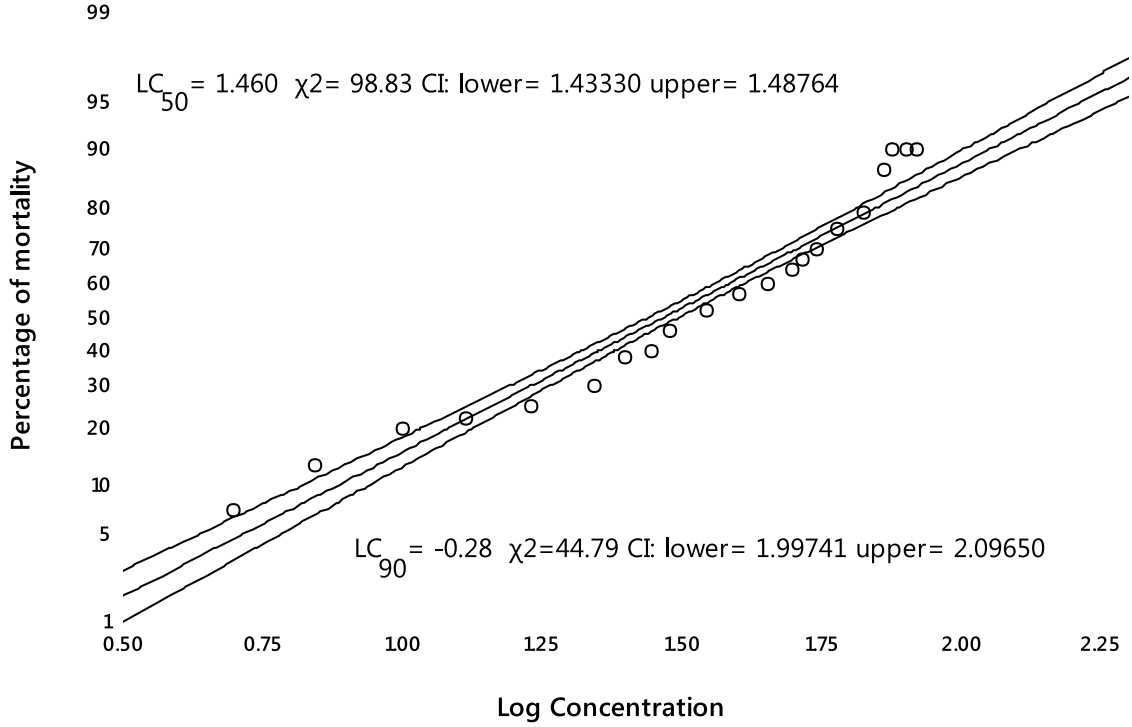

Fig. 6. Lethal concentrations (LC_{50} and LC_{90}) of andrographolide against *A. aegypti.*

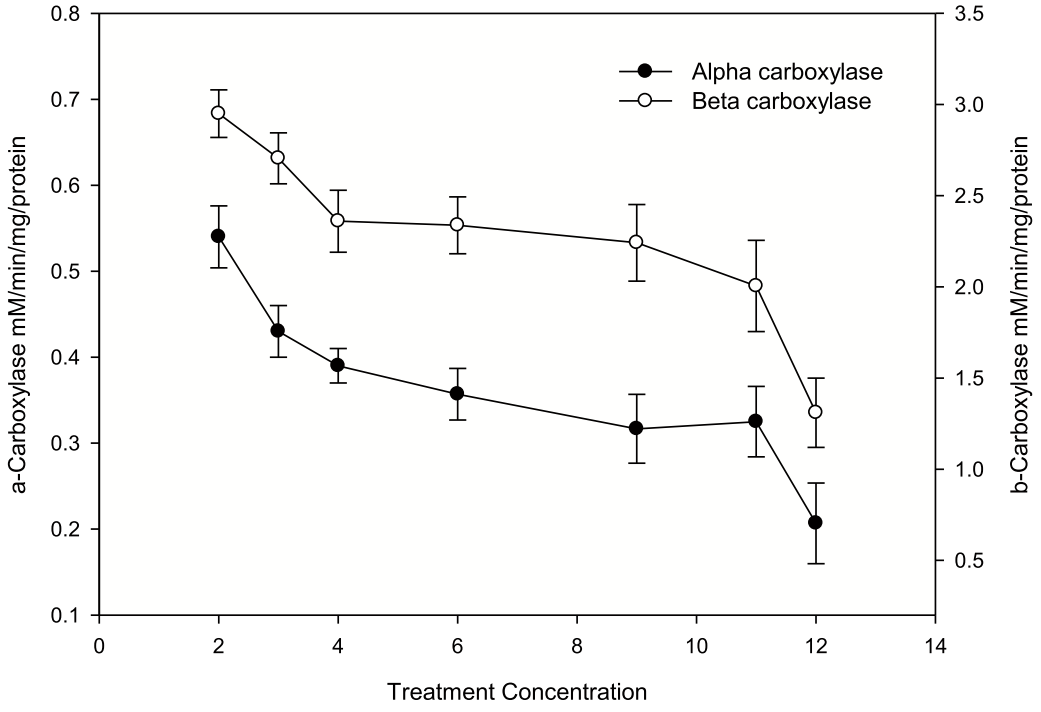

Fig. 7. (A) α, (B) β carboxylestrase enzyme activity of fourth instar larvae of *Ae. aegypti* after treatment with andrographolide.

4. Discussion

Heavy use of synthetic pesticides can cause various environmental pollution disputes (Obermok et al., 2015). Bio pesticides and low toxic chemistries are being further promoted with an international focus on increasing education and incorporation of integrated pest management, IPM, strategies. Better education on the use of biopesticides will hopefully reduce overuse of stockpiled chemicals. Efforts to identify and develop biopesticides from secondary plant metabolites which are non-toxic, or low-toxic chemistries, provides new opportunities for incorporating them into IPM programs. The aim is to develop biopesticides that will also produce less harmful by-products upon degrading, products which biodegrade rapidly, therein providing environmental benefits to humans and beneficial insects (SP-IPM, 2006; Senthil-Nathan et al., 2006a,b; East, 2013). Thus these issues have driven researchers to

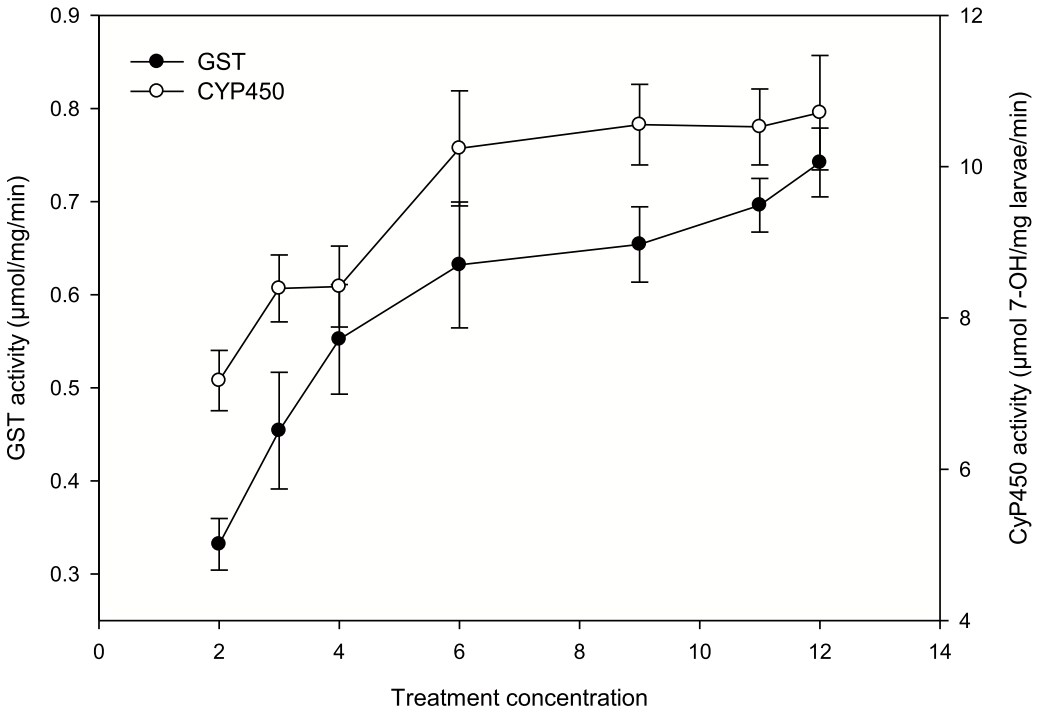

Fig. 8. Impact of andrographolide on Glutathione S Tranferase and cytochrome P450 of fourth instar larvae of vector mosquito *Aedes aegypti*.

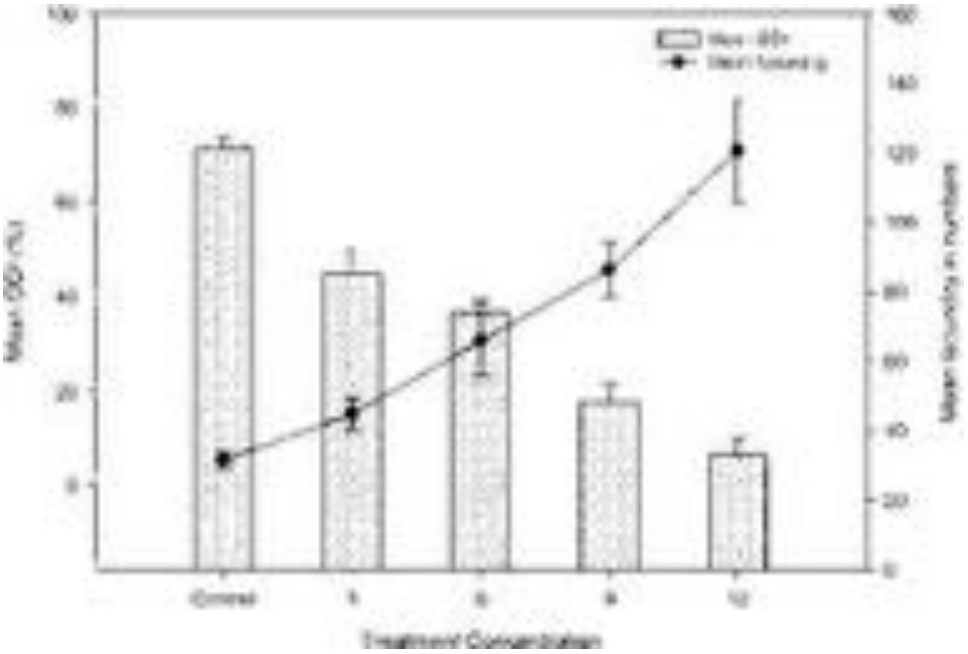

Fig. 9. Oviposition deterrence index and mean fecundity *A. aegypti* after treatment with *andrographolide*.

search for, and develop, more eco-friendly biopesticides. Emerging plant biopesticides are providing some relief from insecticide resistance for some pests (Senthil-Nathan et al., 2007). Historical use of botanical extracts and their secondary metabolites, to manage insect pests provide a proven track record for the potential wealth of products still to be discovered (Senthil-Nathan et al., 2005a, 2006a; Senthil-Nathan, 2013, 2015).

The plant *A. paniculata* while well known for its medicinal value and its therapeutic uses in India, research continues to demonstrate the potential use of crude extracts from the plant as a biopesticide. Results by Govindarajan (2011) and Kurzawa et al. (2015), revealed the crude extract of *A. paniculata* showed larvicidal and ovicidal effects on *Culex quinquefasciatus* (Say.) and *Ae. Aegypti*. Chandrasekaran et al. (2009) found that therapeutically important andrographolide was observed at greater concentrations in aerial parts of *A. paniculata* plants which also provide some protection from herbivorous insects. The phytochemical compounds present in our leaf extract revealed Pentadecanoic acid, 13-methyl, Phytol, Retinoic acid, Andrographolide and Ergosterol. Vasantha et al. (2013) identified five compounds, (Andrographolide, 1,1,3-triethoxy-Propane, n-Hexadecanoic Acid, 9,12-Octadecadienoic acid (Z,Z)-, and Oleic acid) in the ethanolic leaf extract of A. pan-

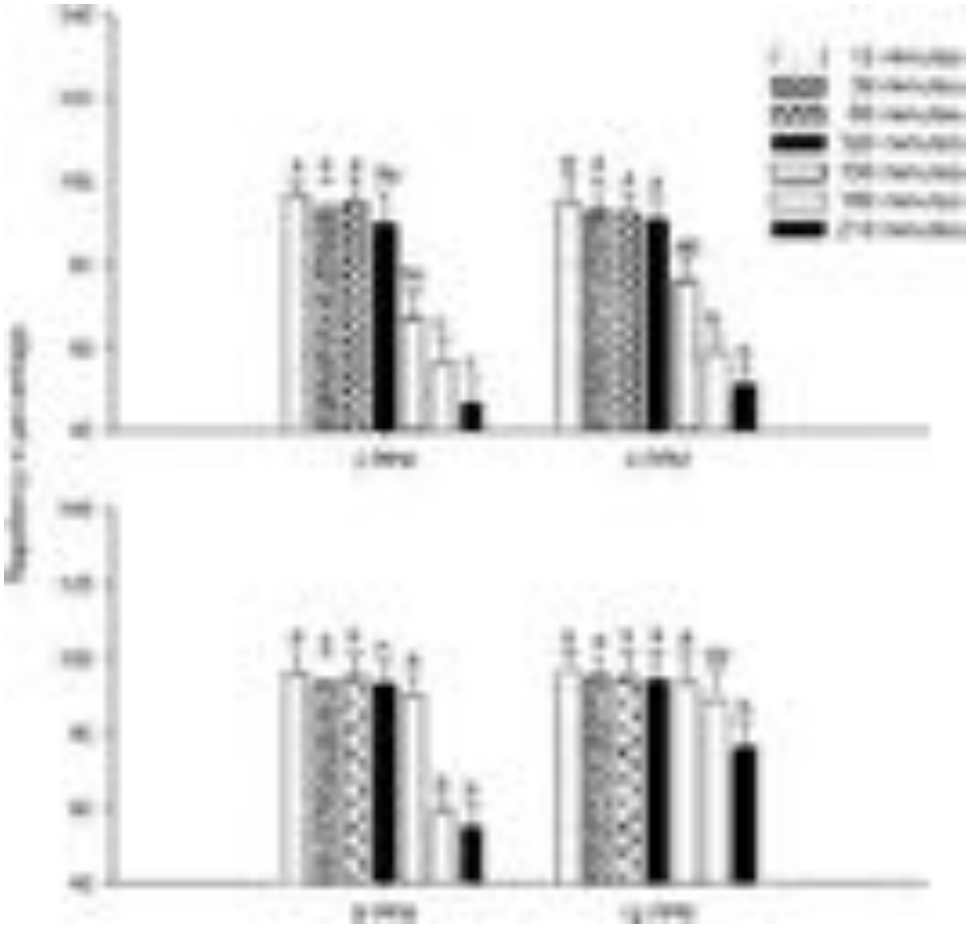

Fig. 10. Repellent activity of andrographolide against *Ae. Aegypti.*

iculata, among which andrographolide was the prominent peak, supporting the results from this study.

Previous studies on mosquito vector control suggest the focus should be on the larval stage due to the limited habitat of the larvae. Sensitivity of mosquitoes to various insecticides are highly variable. Several plant species have demonstrated larvicidal effects on mosquitoes (Waliwitiya et al., 2009). The larvicidal assay of andrographolide produced greater lethal effects on the *Ae. aegypti* fourth instar larvae, with over 90% mortality. Similarly Traboulsi et al. (2002) reported that essential plant oils extracted from leaves and flowers of *Myrtus communis* containing secondary metabolites of Thymol, Carvacrol and (1*R*)-(+)-pinene showed potent larvicidal activity against *Culex pipiens*. Azadirachtin's chief metabolites isolated from *Azadirachta indica* shows good larvicidal activity against all larvae instars of *An. stephensi* (Senthil-Nathan et al., 2005a,b). A natural quinone identified as 2-hydroxy-3-(3-methyl-2-butenyl)-1.4 naphthoquinone (lapachol) from the stem and wood extract of *Cybistax antisyphilitica* resulted in strong larvicidal efficacy against the dengue vector *Ae. aegypti* with LC_{50} value of 26.3 μg/ml (Rodrigues et al., 2005).

The levels of α-and β-carboxylesterase activities show difference throughout the development of *Ae. aegypti* larvae and pupae treated with the leaf extracts of *P. nigrum* (Lija-Escaline et al., 2015). The bio-efficacy of metabolites from plant extracts is determined by the activity levels of esterase enzymes. The esterase activity levels were used as key biomarkers in studies with several insect pests (Breuer et al., 2003; Senthil-Nathan et al., 2008; Koodalingam et al., 2011). The active metabolites of andrographolide inhibit the activity of α- and β-carboxylesterase and the level of detoxifying enzymes are altered significantly in the development of *Ae. aegypti* larvae and pupae. Similar results were found by Agra-Neto et al. (2014) in studies of *Moringa oleifera* Lam. lectins which down-regulated the level of detoxification. The lethality against mosquito larvae is synergistically increased because the secondary metabolites prevent the detoxifying esterases activity (Larson et al., 2010). The level of GST enzymes up-regulated at the greatest concentrations (12 ppm) suggesting their activity involves the major detoxifying process. Jukic et al. (2007) hypothesized that the level of GST expression was the major biomarker suitable to identify resistance and susceptibility of mosquitoes to pesticides. Members of the CYP450 family are also used as key indicators of metabolic resistance and susceptibility to insecticides (David et al., 2013). Our results clearly illustrates that the level of both GST and CYP450 expression increases post treatment with andrographolide.

The oviposition deterrence index results revealed that the andrographolide reduced oviposition in a dose dependent manner. The greatest oviposition deterrent effects were at 12 ppm treatments. The selection of an oviposition site by gravid mosquito females is an important behavioural activity that determines species proliferation and population densities, and dispersion in different geographical areas (Tawatsin et al., 2006). Oviposition deterrence of various essential oils was reported also by Prajapati et al. (2005) for *Ae. aegypti*, *C. quinquefasciatus*, and *An. Stephensi*.

Repellency periods observed against *Ae. aegypti* indicated that andrographolide exhibits a repellent potential (15–210 min) in a dose dependent manner. Similar reports from Gleiser et al. (2011) indicate that essential oils extracted from *Acantholippia salsoloides*, *Aloysia catamarcensis*, *Aloysia polystachya*, *Lippia*

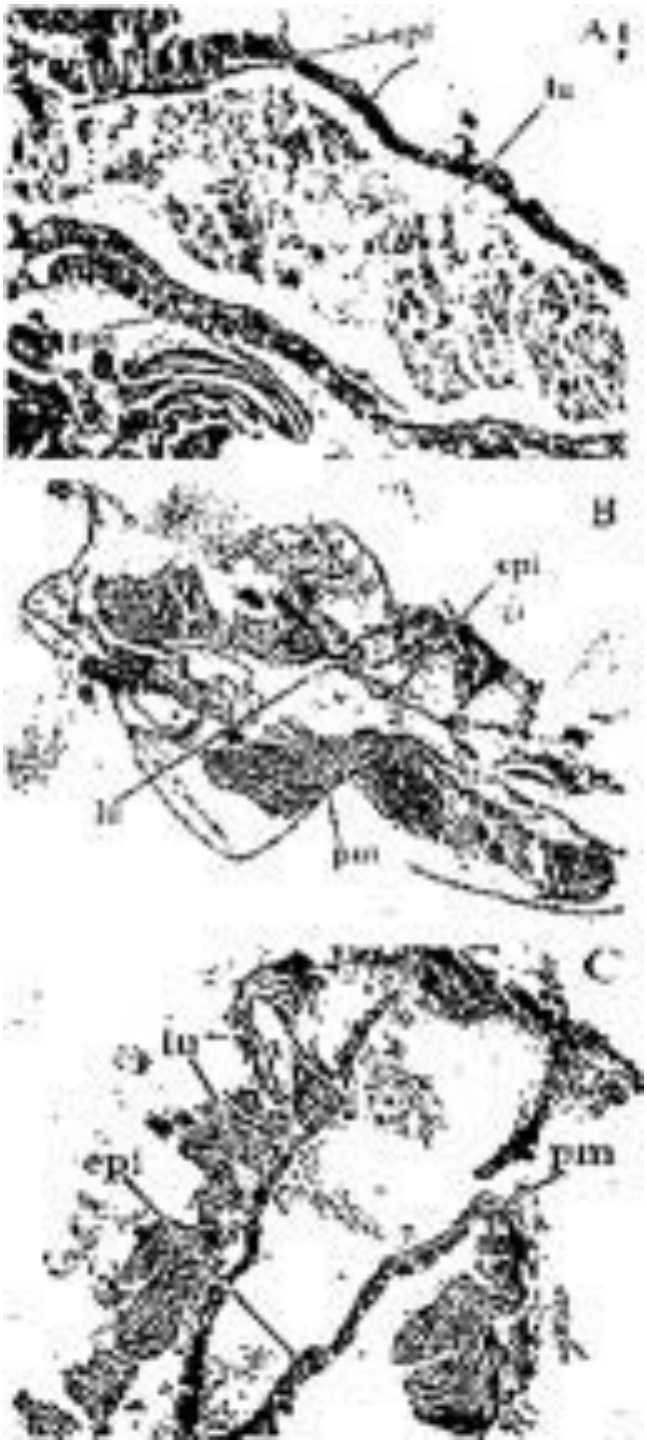

Fig. 11. Histology on mid-gut of 4th instar larvae of *Ae. aegypti* treated with andrographolide (a) Control (b & c) Treated (epi) gut epithelium, (lu) gut lumen, (pm) peritrophic matrix.

integrifolia, *Lippia junelliana* (Verbenaceae), *Baccharis salicifolia*, *Euphatorium buniifolium*, and *Tagetes filifolia* (Asteraceae) produce significant repellent activity against *Ae. aegypti*. The alcohol plant extract of *Lippia javanica* gave 76.7% protection against *An. arabiensis* for a 4-h period (Govere et al., 2001).

Our histological and morphological observations on andrographolide treatments show severe breakdown in the mid-gut peritrophic membrane, and a major shift in the alignment of the epithelial layer and the gut lumen compared to the control. Reports of similar studies by Lija-Escaline et al. (2015) testing crude extracts of *Piper nigrum* L. (Piperaceae) show that the mosquito gut epithelium was severely affected. Damage in the midgut cells directly influences the deregulation in detoxifying enzymes (Senthil-Nathan et al., 2008).

5. Conclusions

In conclusion, the larvae of *Ae. aegypti* are susceptible to andrographolide, the major bioactive derivatives of the *A. paniculata*. Transforming potential bioactive compounds into marketable

products to reduce the spread of dengue by mosquitoes appears to be feasible in light of these results. Overall, this research enhances knowledge to improve novel and harmless natural larvicides against dengue mosquito vector. The selected *A. paniculata* plant generally exists in enormous quantities in Asian countries especially in Indian sub-continent. It is cost effective for preparation comparable to other synthetic pesticides and may minimize the risk of resistance development in vector populations. Thus, andrographolide may be used as an eco-friendly and sustainable insecticides to control mosquito vectors and reduce the chemical burden on the environment.

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