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Larvicidal and Growth Inhibitory Activities of *Ageratum houstonianum* (Mill, 1768) Against the Dengue Vector *Aedes aegypti* (Linnaeus, 1762)

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ABSTRACT

In the present work, *Ageratum houstonianum* leaf acetone extract was tested for the larvicidal and growth inhibitory activity against the third instar larvae of the Dengue vector, *Aedes aegypti.* The extract showed larvicidal activity with LC_{50} and LC_{90} values of 204.79 and 277.57 mg/L, respectively. The larval mortality was increased during subsequent days of treatment. The extract adversely affected larval development, causing a significant reduction in both the formation of fourth instar larvae and pupae. Also, the extract increased the larval duration of *Ae. aegypti* third instar larvae indicating growth inhibitory activities of the extract. The results showed dose-dependent effects of the *A. houstonianum* leaf acetone extract; treatment at higher concentrations inhibited the growth and reduced the viability. This resulted in a decrease in the larval growth index. GC-MS analysis revealed the presence of precocene I and precocene II along with many other components which affect the survival and growth of the insects adversely. This explores the potential of *A. houstonianum* in the management of *Ae. aegypti.*

Keywords: Aedes aegypti, Ageratum houstonianum, larvicidal activity, growth and development.

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INTRODUCTION

Day-biting mosquito, *Aedes aegypti* Linnaeus (Diptera: Culicidae) is a primary vector of dengue, chikungunya, yellow fever, and Zika viruses (Van den Hurk et al, 2012; Dutra et al, 2016; Munusamy, Appadurai, Kuppusamy, Michael, & Savarimuthu, 2016; Thangamani, Huang, Hart, Guzman, & Tesh, 2016; Liu et al, 2020). Transmission of these disease pathogens to human by mosquitoes threaten over 80% of the world's population and is a major global concern (Golding et al, 2015; Leta et al, 2018). In the last few decades, global climate change coupled with environmental conditions such as high relative humidity and rainfall favoured extensive mosquito breeding and outbreak of many mosquito-borne diseases in tropical and subtropical countries (Kumar, Ammani, Jobina, Subhaswaraj, & Siddhardha, 2017). The incidence of dengue has increased dramatically; dengue alone accounted for an estimated 390 million infections annually worldwide of which 96 million people manifested clinical cases (Bhatt et al, 2013). According to the World Health Organization (WHO), the number of dengue cases increased over eight-fold in the last two decades across the globe. Dengue is also considered one of the primary causes of hospital admission (Ahmed & Khan, 2021). In India, the last two decades witnessed repeated outbreaks, significant geographical spread, and almost an eleven-time increase in the number of dengue cases (Prajapati, Singh, Jain, Srivastava, & Prajapati, 2022).

In the absence of an effective vaccine against most mosquito-borne diseases, the main approach to tackle the mosquito-borne diseases is vector control and vector management. Chemical insecticides are widely used to target the larval stages of the vector (Costa, Naspi, Lucia, & Masuh, 2017; Martianasari & Hamid, 2019). However, the constant use of synthetic insecticides affected non-target organisms and the environment by polluting soil, air, and water. Besides, resistance development to the insecticides in insects makes them disadvantageous (Gayathri & Murthy, 2006; Govindarajan, Sivakumar, Rajeswari, & Yogalakshmi, 2012; Saavedra, Romanelli & Duchowicz, 2018). Integrated vector management (IVM) inspires the ideal use of resources for effective, cheap, and ecologically sustainable vector control (Beier et al, 2008).

Botanicals provide an eco-friendly and sustainable approach to managing the mosquito population (Ruiz-Guerrero, Rodríguez-Pérez, & Norzagaray-Campos, 2015; Pavela, Maggi, Iannarelli, & Benelli, 2019). Plant-based chemicals are species-specific, environmentally safe, less toxic to humans and non-target organisms; and have a fewer possibility of resistance development in insects (Isman, 2000; Sharma, Mohan, & Srivastava, 2006; Demirak & Canpolat, 2022). Earlier reports have confirmed the larvicidal activities of several plant extracts. For instance, Macêdo et al (1997) reported the activities of ethanol extracts of 83 plant species against *Aedes fluviatilis* and found that the extracts from *Tagetes minuta* and *Eclipta paniculata* had high larvicidal potential. The methanolic extract of *Chromolaena odorata* leaves was reported to have larvicidal activity against late instar larvae of *Anopheles stephensi, Culex quinquefasciatus* and *Ae. aegypti* (Sukhthankar, Kumar, Godinho, & Kumar, 2014); also, the leaf extract of *Ambrosia arborescens* was found effective against third instar

larvae of *Ae. aegypti* (Morejón et al, 2018). In addition, secondary metabolites present in the plants can provide natural candidates for developing novel larvicides, repellents, oviposition deterrents, and growth inhibitors for the control of insect vectors (Cavalcanti et al, 2004; Ríos, Stashenko, & Duque, 2017; Bekele, 2018; de Souza Wuillda et al, 2019). The larvicidal properties of different classes of compounds of natural origin against malaria mosquitoes were highlighted by Milugo et al (2021). Recent research on plant-extract derived nanoparticles has shown a great promise in the management of vectors. Nanoparticles of aqueous extracts of *Ambrosia arborescens* (Morejón et al, 2018) and leaf extract of *Citrus medica*, *Tagetes lemmonii* and *Tarenna asiatica* (Chandhirasekar et al, 2021) against *Ae. aegypti* showed larvicidal activity. The *Ageratum houstonianum* Mill. (Family: Asteraceae) is an invasive herbaceous weed commonly known as 'Floss flower' or 'blue mink' (BioNet-EAFRINET, 2016). The plant is native to Mexico and Central America (Njateng et al, 2010) and is widely distributed in tropical, subtropical and temperate climatic zones (Bhellum, 2020; Chandraker et al, 2020) including India and Asian countries (Ambasta, 1988; Sharma, 1987). Different species of *Ageratum* are used for relieving sore throats, fever, rheumatism, skin and stomach infections in the aboriginal systems of medicine (Sharma & Sharma, 1995; Andrade-Cetto, 2009). It is also used to cure wounds and burns (Durodola, 1977) and as an anti-inflammatory agent to relieve swelling and pain in the throat (Ming, 1999). Moreover, it is a curative plant reported to possess antifungal (Pandey et al, 1983), antimicrobial (Kurade et al, 2010) and antioxidant activities (Tennyson et al, 2012). Insecticidal properties (Bowers et al, 1976; Ravindran, Samuel, Alex, & William, 2012) and insecticidal compounds (Renuga & Sahayaraj, 2009) including precocenes were reported in this plant (Kumar, 2014). The crude extract of *A. houstonianum* had shown the mosquitocidal properties (Boussaada et al, 2008; Sakthivadivel & Daniel, 2008; Pavela, 2009; Senthilkumar, Varma, & Gurusubramanian, 2009; Elango et al, 2010) including adulticidal (Ravindran et al, 2012), larvicidal (Tennyson, Ravindran, Eapen, & William, 2015a), ovicidal (Tennyson, Ravindran, Eapen, & William, 2015b); the extract also showed repellent (Tennyson, Ravindran, Eapen, & William, 2012a) and oviposition deterrent activities (Tennyson, Ravindran, Eapen, & William, 2012b). The phytochemical composition of *A. houstonianum* showed the presence of chromenes, precocene I and II, which have been described for their anti-JH activity (Haunerland & Bowers, 1985; Binder, Bowers, & Evans, 1991; Lu et al, 2014).

Most of the studies on *A. houstonianum* were focused on cidal activity and lethal effects on mosquitoes (Sharma et al, 2006; Arivoli & Tennyson, 2011; Ravindran et al, 2012; Tennyson et al, 2015a). However, the biological activities of acetone extract of *A. houstonianum* have not been specified against *Ae. aegypti*. The type of solvent used for extraction affects the larvicidal activity (Zhang, Lin, & Ye, 2018). Growth and development are important components of insect life; aberration of these can hamper the insect population. With this aim, larvicidal and growth inhibitory efficacy of the *A. houstonianum* leaf acetone extract against third instar larvae of *Ae. aegypti* were undertaken in the present research work. The phytoconstituents of the *A. houstonianum* leaf acetone crude extract were determined using GC-MS analysis. The larvicidal and

growth inhibitory studies were correlated with the compounds identified in the GC-MS profile of the extract. The present studies are important in the search for effective affordable natural products which can be used in the IVM program of *Ae. aegypti.*

MATERIALS AND METHODS

Rearing and maintenance of *Aedes aegypti* culture

A stock culture of *Ae. aegypti* was procured from 'International Center for Genetic Engineering and Biotechnology (ICGEB)', New Delhi, India, and maintained in an insectary under optimum conditions of temperature 28±1°C, relative humidity 80±5%, and photoperiod 14L:10D, to obtain insects of sustained quality throughout the research work (Shazad et al, 2018). Each larval stage was reared in enamel coated bowl or tray containing dechlorinated water according to the protocol laid by WHO (WHO, 2005). Larval diet was composed of dog biscuits and yeast in a 3:1 ratio. The pupae were separated, transferred into enamel bowls, and kept in the mosquito cages. Adults were fed upon raisins and adult female mosquitoes were blood-fed after 2 days of emergence on albino rats for maturation of eggs.

Plant collection and preparation of acetone extract

The leaves of the *Ageratum houstonianum* were collected during the month of March, 2021 from the fields adjoining the Delhi state, India (geographic coordinates 28°49'14.97"N and 76°46.3776"E). The leaves were washed thoroughly, shade dried at room temperature for a week, and mechanically ground to make a fine powder. The leaves powder was extracted continuously in acetone, using the 'Soxhlet extraction apparatus' at 45°C for 24 h. Subsequently, the extract was filtered through Whatman filter paper No. 1 and finally, concentrated using a 'Rotavapor vacuum evaporator (Buchi)'. The percentage of extraction was calculated by using the Equation No. 1,

$$\text{Percent extraction} = \frac{\text{Weight of the extract}}{\text{Weight of the plant material}} \times 100 \quad \text{.....Eqn. 1}$$

The 10% stock solution of the extract was prepared by mixing 1 part of the extract in 9 parts dimethyl sulfoxide (DMSO) and stored at 4°C for further use.

Larvicidal bioassay

The larvicidal efficacy of *A. houstonianum* leaf acetone extract was assessed by performing a bioassay against laboratory-bred early third instar larvae of *Ae. aegypti*. The third larval stage was exposed to crude *A. houstonianum* leaf acetone extract of concentrations 50, 100, 150, 200, 250 and 300 mg/L for 24 h using the standard WHO protocol (WHO, 2005). Twenty-five newly emerged third instar larvae of *Ae. aegypti* were placed in enamel-coated bowls containing the 1 ml of test samples diluted in the 249 ml of dechlorinated water. In control, 1ml of DMSO was added to 249 ml of dechlorinated water. The larval mortality was observed after 24 h of the exposure period. The larvae were considered dead when no movement was shown by the larva

on gentle probing with a needle. Also, all moribund larvae were considered as dead. The experiments were repeated four times with each tested concentration and control.

Effects of *A. houstonianum* leaf acetone extract on growth of *Ae. aegypti*

The effect of *A. houstonianum* leaf acetone extract was studied on the survival, growth and development of *Ae. aegypti.* The third instar larvae were treated with 50, 100, 150 and 200 mg/L of *A. houstonianum* leaf acetone extract for 72 h and then transferred to freshwater. The number of larvae found dead, and the number of fourth instar larvae and pupae formed were recorded daily. The data was analyzed to calculate day-wise mortality, time taken by third instar larva to form a pupa, and the percent of third instar larvae moulted into fourth instar larvae and formed pupae. The impact of *A. houstonianum* leaf acetone extract on the development of the third instar larva till pupa formation was measured by considering the larval growth index i.e., ratio of percent pupae formation to the average time taken by the third instar larva to form a pupa. All the experiments were replicated four times; 25 third instar larvae were taken in each replicate.

GC-MS analysis of the *A. houstonianum* leaf acetone extract

The phytochemicals present in the acetone extract of *A. houstonianum* leaves were analyzed through gas chromatography and mass spectroscopy (GC-MS) (Ezhilan & Neelamegam, 2012). The concentrated extract of *A. houstonianum* was dissolved in acetone and injected into the 'Gas chromatography unit (Shimadzu GC-MS QP2010)'. The injector temperature was maintained at 250ºC. The detector used was a flame ionization detector which was maintained at a temperature of 280ºC. The pressure of the carrier gas, nitrogen, was kept at 10 psi. The oven temperature was set from 60ºC to 280ºC with a gradual increment of 10ºC per minute. The injected extracts were eluted in the DB-5 MS column of 30 m long and 0.25 mm inner diameter and the eluted constituents were detected by a flame ionization detector. The GC chromatogram was recorded and the compounds were identified by comparing the data with the existing software libraries like WILEY08, NIST08, and NIST08s (Hübschmann, 2015).

Statistical analysis

The mortality in the experimental tests was corrected by using Abbott's formula presented in Equation No. 2 (Abbott, 1925).

Corrected percent mortality:

$$= \frac{\%\ \text{treated mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100 \quad \text{.....Eqn. 2}$$

The survival data obtained from the bioassay experiment was subjected to regression analysis. The lethal concentration $LC_{90,}$ median lethal concentration $LC_{50,}$ and values of sublethal concentrations (LC_{10} and LC_{30}) with 95% fiducial limits were calculated in bioassay. All quantitative data were analyzed using descriptive statistics in SPSS version 19.0 software (SPSS, Chicago, IL, USA) and MS excel 2016. One-way ANOVA followed by the Tukey's post hoc test was used to determine and identify statistically significant

differences between groups at a *p-value* <0.05. Results with the value of *p*<0.05 were considered to be statistically significant (Finney, 1971; Fisher, 1992).

RESULTS

Extraction of 200 g of the *A. houstonianum* leaves powder was done with 1L of acetone in the Soxhlet apparatus for 24 h, 4-5 cycles per h, at 45°C. This process yielded 17.3 g i.e., 8.65% crude extract.

Distinct effects of *A. houstonianum* leaf acetone extract were reported on the survival of the third instar larva of *Ae. aegypti.* The results indicate that the extract was toxic to the third instar larva and caused larval mortality within 24 h. The regression analysis revealed the toxicity of the extract in a dose-dependent manner (Fig. 1). The LC_{50} and LC_{90} values were 204.79 and 277.57 mg/L, respectively. The values of sublethal concentrations of *A. houstonianum* leaf acetone extract i.e., LC_{10} and LC_{30} were 151.09 and 180.83 mg/L, respectively (Table 1). Treatment of early third instar larvae to the extract of concentrations 50 and 100 mg/L did not show significant mortality after 24 h of exposure.

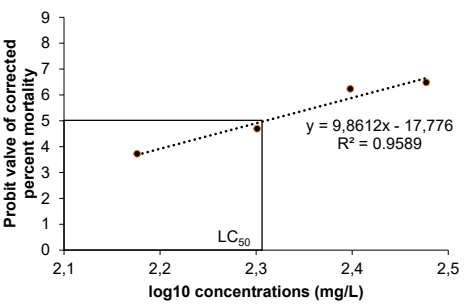


Fig. 1. The response of third instar larvae of *Ae. aegypti* to the acetone extract of *A. houstonianum* leaves. The graph represents regression line of relationship between log concentrations of extract and probit value of corrected per cent mortality.

Table 1. Lethal and sublethal concentrations of the *A. houstonianum* leaf acetone extract against third instar larvae of *Ae. aegypti* after 24 hours of exposure.

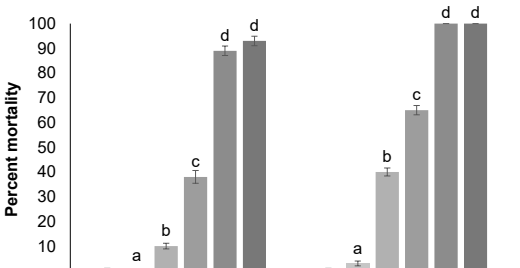
LC levels	Estimated LC values (mg /L)	95% Confidence Limits for Concentrations (mg /L)		Regression Equation	R^2 (Regression coefficient)	χ^2 (Chi-square value)	*p -value*
		Lower Limit	Upper Limit				
LC_{10}	151.09	135.12	168.96	y = 9.8612x -17.776	0.958	0.561	0.021
LC_{30}	180.83	161.71	202.21				
LC_{50}	204.79	183.14	229.00				
LC_{90}	277.57	248.23	310.39				

Quantitative data were analyzed using descriptive statistics in SPSS version 19.0 software (SPSS, Chicago, IL, USA) and MS excel 2016.

Average of four replicates, 25 third instar larvae per replicate.

The null hypothesis is rejected: F value > F critical, and *p* (≤0.001) < α=0.05; LC = Lethal Concentrations; LC10, LC30, LC50, and LC90 = Lethal Concentrations for 10%, 30%, 50% and 90% mortality with 95% confidence limits.

Continued exposure to *Ae. aegypti* third instar larvae to the *A. houstonianum* extract caused an increase in mortality on subsequent days; the larvicidal efficacy of the extract increased as the exposure time increased (Fig. 2). Treatment with the extract of concentrations 250 and 300 mg/L caused a hundred percent larval mortality within 2-3 days of exposure (Table 2). Also, the total mortality of third instar larvae was increased when treated with extract of concentrations 150 and 200 mg/L. The results were significantly different in comparison to the control. The extract of concentrations 50 and 100 mg/L was not effective in causing mortality. No significant increase in mortality was observed at these concentrations during subsequent days of exposure (Fig. 2, Table 2).



	% mortality after 24 hr	Total % mortality
50 mg/L	0	0
100 mg/L	0	3
150mg/L	10	40
200 mg/L	38	65
250 mg/L	89	100
300 mg/L	93	100

Fig. 2. Comparison of percent larval mortality after 24 h of treatment and total mortality of third instar larvae of Ae. aegypti exposed to the A. houstonianum leaf acetone extract for three days. The same letters on the bar graph of one category are not significantly different at $p<0.05$ (ANOVA followed by Tukey's test).

Table 2. Cumulative larvicidal activity of the *A. houstonianum* leaf acetone extracts on third instar larvae of *Ae. aegypti.*

Concentration of the extract (mg/L)	Day-wise cumulative corrected percent mortality* (Mean ± S.E.)			
	Day 1	Day 2	Day 3	Day 4
50	$0^a \pm 0$	$0^a \pm 0$	$0^a \pm 0$	$0^a \pm 0$
100	$0^a \pm 0$	$0^a \pm 0$	$3^a \pm 1$	$3^a \pm 1$
150	$10^b \pm 1.15$	$17^b \pm 2.52$	$35^b \pm 1.91$	$40^b \pm 1.63$
200	$38^c \pm 2.58$	$49^c \pm 1.91$	$60^c \pm 1.63$	$65^c \pm 1.91$
250	$89^d \pm 1.91$	$97^d \pm 1$	$100^d \pm 0$	-
300	$93^d \pm 1.91$	$100^d \pm 0$	-	-
df	5,18		4,15	3,12
F critical value	2.773		3.056	3.490
F value	727.617	1153.182	1192.023	530.545
p value	$p \leq 0.001$			

df-degree of freedom, *Average of four replicates, 25 third instar larvae per replicate. Mortality in the test was corrected by Abbott's formula. Means followed by the same letters in a column are not significantly different at $p<0.05$ (ANOVA followed by Tukey's test). The null hypothesis is rejected: F value > F critical, and p (≤0.001) < α=0.05.

Effect of *A. houstonianum* leaf acetone extract on the growth of third instar larvae of *Ae. aegypti* was evaluated by assessing the day-wise formation of fourth instar larvae and pupae from the treated larvae, time taken by the treated larva to form a pupa, and larval growth index. The results indicate continuous exposure of third instar larvae of *Ae. aegypti* to *A. houstonianum* leaf acetone extract caused a delay in fourth instar larva formation. In control, 86% of the third instar larvae were moulted to fourth-instar larvae within two days and almost all of them molted within 4 days. On the other hand, when treated with the extract of concentrations 50 and 100 mg/L, less than 50% of the third instar larvae moulted to the fourth instar larvae in two days; it was increased during subsequent days. The percent of larvae moulted to the fourth stage remained low throughout the treatments and exceeded for more than six days in the treatments with the extract of concentrations 150 and 200 mg/L (Table 3).

Table 3. Effect of *A. houstonianum* leaf acetone extract on the 4th instar larvae formation from third instar larvae of *Ae. aegypti*.

Concentration of the extract (mg/L)	Day-wise cumulative percent of fourth larva formation* (Mean ± S.E.)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Control	$49^{a} \pm 3.42$	$86^{a} \pm 2.58$	$97^{a} \pm 1$	$100^{a} \pm 0$	-
50	$35^{b} \pm 1.91$	$44^{b} \pm 2.31$	$66^{b} \pm 1.15$	$94^{a} \pm 1.15$	$100^{a} \pm 0$
100	$34^{b} \pm 2.58$	$45^{b} \pm 1$	$61^{bc} \pm 2.52$	$85^{b} \pm 3.42$	$97^{a} \pm 1$
150	$39^{ab} \pm 3.42$	$47^{b} \pm 2.52$	$55^{c} \pm 1$	$57^{c} \pm 1$	$60^{b} \pm 1.63$
200	$17^{c} \pm 3$	$23^{c} \pm 1.91$	$29^{d} \pm 1.91$	$35^{d} \pm 1.91$	-
df	4,15				2,9
F critical value	3.056				4.256
F value	15.727	113.587	223.050	212.745	406.091
p value	$p \leq 0.001$				

df-degree of freedom, *Average of four replicates, 25 third instar larvae per replicate. Means followed by the same letters in a column are not significantly different at $p<0.05$ (ANOVA followed by Tukey's test). The null hypothesis is rejected: F value > F critical, and p (≤0.001) < α=0.05.

Efficacy of the *A. houstonianum* leaf acetone extract was also assessed on pupae formation from treated third instar larvae of *Ae. aegypti*. In control, it was seen that pupae formation was completed within 5 days from the third instar larvae (Table 4). Also, 50% of pupal formation took place within 3 days. On the other hand, in the larvae treated with the extract of concentration 50 mg/L, the pupal formation was delayed; only 11% of pupae formed in three days. The duration for pupa formation was extended up to six days from the day of treatment of the third instar larvae. In the treatment with the extract of concentration 100 mg/L, treated third instar larvae took up to seven days to form pupae. The number of pupae formed on three days was less. The delay in the pupae formation was further increased in the treatments with the extract of concentrations 150 and 200 mg/L (Table 4).

Table 4. Effect *of A. houstonianum* leaf acetone extract on the pupa formation from third instar larvae of *Ae. aegypti*.

Concentration of the extract (mg/L)	Day-wise cumulative percent of pupa formation* (Mean ± S.E.)				
	Day 3	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	50^{a} ± 3.46	76^{a} ± 1.63	100^{a} ± 0	-	-
50	11^{b} ± 1.91	42^{b} ± 2.58	93^{b} ± 1	98^{a} ± 1.15	-
100	9^{b} ± 1.91	23^{c} ± 1.91	58^{c} ± 1.15	76^{b} ± 1.63	90^{b} ± 2
150	2^{b} ± 1.15	8^{d} ± 1.63	26^{d} ± 1.15	41^{c} ± 1.91	57^{c} ± 3
200	4^{b} ± 1.63	10^{d} ± 1.15	14^{e} ± 2	22^{d} ± 2	34^{d} ± 1.15
df	4,15			3,12	2,9
F critical value	3.056			3.490	4.256
F value	83.936	233.588	969.913	400.314	165.837
p value	$p \leq 0.001$				

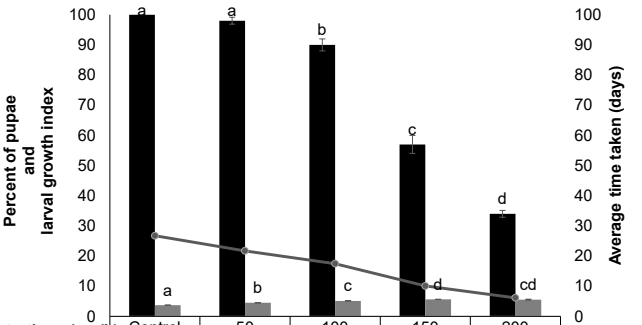
First pupa was formed on day 3. df-degree of freedom, *Average of four replicates, 25 third instar larvae per replicate. Means followed by the same letters in a column on a particular day are not significantly different at $p<0.05$ (ANOVA followed by Tukey's test). The null hypothesis is rejected: F value > F critical, and p (≤0.001) < α=0.05.

It was reported that *A. houstonianum* leaf acetone extract affected the development period of treated third instar larvae of *Ae. aegypti*. Consequently, the larval duration, as measured by the average time taken by the third instar larva to form a pupa, increased in a dose-dependent manner (Fig. 3). In control, the third instar larva took on an average of 3.74 days to complete larval development and form a pupa. However, the larval duration of the treated instars increased to 4.51 and 5.15 days in the treatments with the extract of concentrations 50 and 100 mg/L, respectively. In the treatments with the extract of concentrations 150 and 200 mg/L, the effect was most conspicuous, the larval period of the treated third instar increased to nearly six days which was almost 1.5 times more than the larval period of the third larva in the control (Fig. 3).

The data presented in Figure 3 also indicate that there was a decrease in the larval growth index with the increase in the concentration of extract in the treatments. In control, the larval growth index was 26.75. In treated larvae, the larval growth index continuously decreased with an increase in the extract concentrations; it declined to 6.17 in the treatments with the extract of concentration 200 mg/L. The difference in the results was statistically significant ($p<0.001$). It was also observed that a decrease in the growth index of the larva was due to both decrease in the percentage of total pupae formation and an increase in the average time taken by the third instar larva to form a pupa.

GC-MS analysis of the *A. houstonianum* leaf acetone extract revealed 90 peaks, each representing a specific compound (Fig. 4). Some of the important chemical compounds which affect the life processes of insects are tabulated in Table 5. These include Precocene I, Precocene II, 1h-inden-1-one, Trans ß-caryophyllene, trans-Z-alpha-Bisabolene epoxide, Phytol, n-hexadecanoic acid, α-Linolenic acid, ɣ-Sitosterol, ß-Stigmasterol, Neophytadiene, 2-Pentadecanone, Squalene, Oxymorphone or Morphinan-6-one,

Vitamin E, α-Copaene, ß-copaene, α-farnesene, Cubebol and τ-Cadinol. The biological activities of these compounds in various life processes of insects are listed in Table 5.



Concentrations (mg/L)	Control	50	100	150	200
percent pupae formed	100	98	90	57	34
Average time for pupa formation	3,74	4,51	5,15	5,64	5,53
Larval Growth index	26,75	21,73	17,47	10,09	6,17

Fig. 3. Influence of the acetone extract of *A. houstonianum* leaves on the pupal formation, larval duration, and larval growth index.

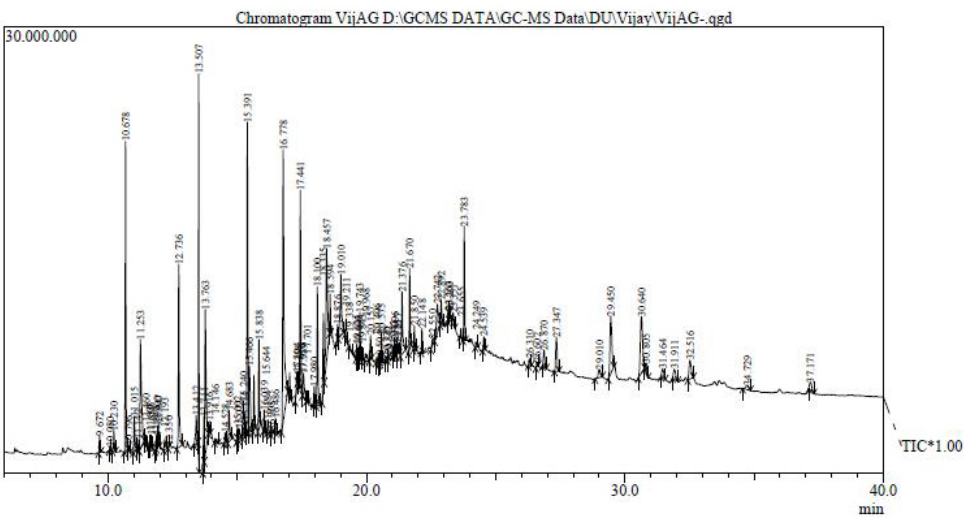


Fig. 4. GC MS chromatogram of the acetone extract of the leaves of *A. houstonianum*, precocene I (arrow) & precocene II (circle) peaks.

Table 5. GC MS analysis of *A houstonianum* leaf acetone extract. Some of the major components present in the extract and their biological activities are represented in the Table.

S. No.	R/T*	Peak Area (%)	Name of the Compound	Biological activities	References
1	11.253	4.29	Precocene I	suppress metamorphosis ovarian activation, aggression and alters sterility signal production	Amsalem, Teal, Grozinger, & Hefetz, 2014; Pener, Orshan, & De Wilde, 1978)
2.	13.507	4.40	Precocene II	suppress metamorphosis, anti-allatal activities, induce precocious metamorphosis	(Bowers & Feldlaufer, 1982; Ohta, Kuhr, & Bowers, 1977; Pener et al, 1978)
3.	13.763	4.76	1h-inden-1-one	antimicrobial, anticancer, anti-inflammatory	(Rovnyak, Millonig, Schwartz, & Shu, 1982; Velaparthi et al, 2008)
4.	10.678	5.63	Trans ß-caryophyllene	weak larvicidal activity	(Dória et al, 2010; Liu & Liu, 2014)
5.	12.736	4.20	trans-Z-alpha-Bisabolene epoxid℮	Unknown	
6.	18.100	2.77	Phytol	antimicrobial, antitumor, anti-teratogenic, antidiabetic, antioxidant, anti-inflammatory, antidepressant, hair fall defense, and antidandruff activities.	(Islam et al, 2015)
7.	16.778	9.35	n-hexadecanoic acid	anti-inflammatory, antibacterial	(Aparna et al, 2012)
8.	18.457	4.73	α-Linolenic acid	antitumor activity	(Xu et al, 2021)
9.	30.640	3.41	γ-Sitosterol	anti-diabetic activity	(Balamurugan, Duraipandiyan & Ignacimuthu, 2011)
10.	29.450	2.84	ß-Stigmasterol	anti-inflammatory effects, anti-diabetic activity	(Morgan et al, 2021; Wang et al, 2017)
11.	15.391	6.57	Neophytadiene	anti-inflammatory potential	(Bhardwaj, Sali, Mani, & Vasanthi, 2020)
12.	15.466	2.59	2-Pentadecanone	repellent, anticancer activity	(Innocent et al, 2008; Swantara et al, 2019)
13.	23.783	2.22	Squalene	anticancer, antioxidant, drug carrier, detoxifier, skin hydrating, and emollient activities	(Kim & Karadeniz, 2012)
14.	18.335	1.83	Oxymorphone or Morphinan-6-one	treating moderate to severe pain, analgesic potency	(Adams, Pieniaszek Jr, Gammaitoni, & Ahdieh, 2005; Prommer, 2006)
15.	27.347	1.29	Vitamin E	antioxidant activity	(Higgins et al, 2020)
16.	9.672	0.47	α-Copaene	antileishmanial activity	(Rodrigues et al, 2018)
17.	10.230	0.54	ß-copaene	anti-inflammatory	(Kadhim, Mohammed, & Hameed, 2016)
18.	10.796	0.10	α-farnesene	oviposition stimulant	(Yan, Bengtsson, Makranczy & Löfqvist, 2003)
19.	11.659	0.21	Cubebol	growth inhibition activities, repellent activities, larvicidal activity	(Chen et al, 2001; Gu et al, 2009; Saijo et al, 2013)
20.	13.412	0.85	τ-Cadinol	antioxidant potential, allelopathic activity	(Abd El-Gawad, El-Amier, & Bonanomi, 2018; Gunes et al, 2021)

*R/T: retention time

DISCUSSION AND CONCLUSION

Extraction of *A. houstonianum* leaves with acetone using Soxhlet apparatus yielded 8.65% crude extract. This yield was high in comparison to the sequential extraction method with hexane, ethyl acetate, and methanol which yielded 0.84%, 2.90%, and 1.21% (w/w) (Ravindran et al, 2012). The high yield of the extract may be due to the choice of extraction methods, agitation, time, and nature of the solvent used in the present study. These factors have been shown to influence extract yield (Mohamad, Ali, Ripin & Ahmad, 2013; Andrade et al, 2015; Zhang et al, 2018).

LC_{50} and LC_{90} values of *A. houstonianum* leaf acetone extract against early third instar larvae of *Ae. aegypti* reported in the present work were 204.79 and 277.57 mg/L, respectively. Also, complete larval mortality was recorded at these concentrations during subsequent days of exposure. Larvicidal activities of hexane, ethyl acetate, and methanol crude leaf extracts of *A. houstonianum* against three vector species *viz., An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* were described by Tennyson et al (2015a). Our studies showed that the *A. houstonianum* leaf acetone extract was more effective in comparison to these studies. The larvicidal activity differs depending on the species of *Ageratum.* Hussaini et al (2018) reported a moderate toxic effect of methanol and n-hexane extract of *A. conyzoides* leaf on the third-fourth instar larvae of *An. gambiae*; the LC_{50} values for methanol and n-hexane extracts were 423.52 and 627.90 ppm, respectively. The LC_{50} values of the crude methanol, petroleum ether and carbon tetrachloride leaf extracts of *A. conyzoides* against *Cx. quinquefasciatus* were 5105.0, 425.6 and 3139.3 ppm, respectively (Sharma et al, 2006). Pintong et al (2020) reported that none of the six types of crude ethanol extracts obtained from *A. conyzoides* had considerable effects at a concentration of 10 mg/L against early fourth instar larvae of *Ae. aegypti*. Sakthivadivel & Daniel (2008) reported that petroleum ether leaf extract of *A. conyzoides* was effective against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus.*

The studies indicated that the *A. houstonianum* leaf acetone extract had a growth inhibitory effect on fourth instar larvae and pupae development. Therefore, there was an increase in the larval duration of the treated third instar larvae of *Ae. aegypti*. Earlier reports have shown similar trends in growth patterns in response to many plant extracts. For instance, Shazad et al (2018) reported that the ethanol extract of *Ocimum sanctum* prolonged the larval duration of *Ae. aegypti* fourth instar larva. Ferdinand (2014) reported that the larval period of *Spodoptera litura* was prolonged by chloroform and ethanol extracts of both *A. conyzoides* and *Artemesia vulgaris*. Muthukrishnan, Pushpalatha, & Kasthuribhai (1997) reported that the active fractions of *Solanum suratense* ethyl acetate extract extended the larval duration of *Cx. quinquefasciatus*. Zhong et al (2001) have also highlighted that ethyl acetate extract of *Rhododendron molle* flower increased the development duration of *Pieris rapae*. The duration of the larval period of *Tribolium castaneum* was extended by methanol extracts of *Raphanu sraphanistrum* and *Peganum harmala* (Jbilou et al, 2008).

Further, the results showed that larval growth index i.e., the ratio of percent pupae formation to the average time taken by the third instar larva to form a pupa, decreased

in a dose-dependent manner. This is due to both the reduction in pupa formation and the increase in the larval development period of the treated third instar larva. The results are in agreement with da Silva et al (2013), who reported that the n-hexane extract of *Hypericum polyanthemum* inhibited the pupa formation and adult emergence of *Ae. aegypti* at sublethal doses LC_{10} and LC_{20}. These studies suggested growth inhibitory activities of the *A. houstonianum* leaf acetone extract on the third instar larvae of *Ae. aegypti*. Jeyabalan et al (2003) described the growth-inhibiting effect of methanol leaf extract of *Pelargonium citrosa* against *An. stephensi*. Consequently, larval and pupal development was completely inhibited by the treatment. It was proposed that the extract contained some compounds which slowed the process of development. The increase in the development period of larva could be related to the disruption of endocrine systems controlling molting, and the synthesis of hormones essential for growth (Lange et al, 1983; Ferdinand, 2014).

Larvicidal and growth inhibitory effects observed in the present study led to the investigation of phytocompounds present in the *A. houstonianum* leaf acetone extract using GC-MS. Our GC-MS chromatogram revealed the presence of 50 components in the crude acetone extract. Chandra et al (1996) found 50 components in the essential oil of *A. houstonianum*. Lu et al (2014) reported a total of 35 components and Hadidy et al (2019) reported 32 components in the essential oil of *A. houstonianum* leaves and flowers, respectively. Further, the GC-MS chromatogram showed the presence of chromene compounds i.e., precocenes; precocene I and precocene II were found most common compounds followed by n-hexadecanoic acid, a pentacyclic triterpene compound and trans-ß-caryophyllene. Precocene I and II were identified as the active components of the essential oil of *A. houstonianum* (Lu et al, 2014). Presence of similar compounds i.e., precocene I, precocene II and β-caryophyllene in the essential oil of *A. houstonianum* was also reported by other researchers (Chandra et al, 1996; Kurade et al, 2010; Lu et al, 2014). Our study also reported the presence of trans-Z-alpha-Bisabolene epoxide, Neophytadiene, 2-Pentadecanone, and Oxymorphone, which were not reported earlier. The difference in components count may be related to the site, habitat, season of plant collection, and organic solvents and techniques used for extractions (Shaalan et al, 2005; Mohamad et al, 2013; Andrade et al, 2015).

Precocenes and cubebol are the most important components of the crude acetone extract considering insect-related activities. The toxicity of the extract to *Ae. aegypti* larvae may be attributed to precocene I and precocene II or synergistic interaction between these components and the other constituents of the extract (da Silva et al, 2013). The results are congruent with studies of Liu & Liu (2014), who reported that precocene I and II exhibited larvicidal activity against the 4th instar larvae of *Ae. albopictus*. Further, cubebol showed growth inhibition activities against *Heterosigma akashiwo* (Saijo et al, 2013) and larvicidal activity against *Ae. aegypti* larvae (Gu et al, 2009). Furthermore, previous studies demonstrated that precocene I and II hinder the juvenile hormone synthesis in several insects. Bowers et al (1976) reported that adults insects treated with precocenes became sterile and juveniles showed precocious metamorphosis and immediate death of premature adults. These compounds may have similar actions on

the dengue vector. Consequently, this can disturb embryonic development, induce premature metamorphosis, decrease the reproductive potential, and affect the insect behavior including the antifeedant and repellent effect (Bowers et al, 1976; Srivastva & Kumar, 1997; Khafagi & Hegazi, 2004; Lu et al, 2014). Moreover, precocene I and II exhibited larvicidal and growth-inhibiting activities against *An. stephensi* (Saxena et al, 1994). Further, the benzopyrans HP1-HP3, the major compounds of *Hypericum polyanthemum*, are structurally similar to precocenes and showed larvicidal and growth-regulating activity against *Ae. aegypti* (da Silva et al, 2013).

The present investigation explored the prospective role of phytochemicals present in the *A. houstonianum* leaf acetone extract as larvicide and growth inhibitor in the management of mosquito, *Ae. aegypti*. Our study suggested that the *A. houstonianum* leaf acetone extract has larvicidal, growth, and developmental disrupting activities against *Ae. aegypti.* The extract showed larvicidal activity against *Ae. aegypti* early third instar larvae; larval mortality was increased with an increase in the concentrations. Moreover, the extract at lower concentrations significantly prolonged the larval duration of the survived third instar larva of *Ae. aegypti*. The GC-MS chromatogram of the *A. houstonianum* leaf acetone extract showed the presence of anti-JH compounds i.e., precocene I and II. Thus, *Ae. aegypti* population could be impeded in the juvenile stages. The results documented showed the potential of *A. houstonianum* leaves as a source of new insecticides for the integrated vector management of *Ae. aegypti*.

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