EFFECT OF THE ESSENTIAL OIL OF *ZANTHOXYLUM ARMATUM* DC. (RUTACEAE) AGAINST APHID (*APHIS CITRICIDUS)*

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ABSTRACT

Aphids have been considered as a major pest of cultivated crops and invade a wide range of vegetables, cereals, and fruits. Use of synthetic chemical insecticides are the common aphid management practices in the world. The use of chemical insecticides/pesticides raises many concerns associated with the environment, biodiversity and human health. Therefore, the use of natural products such as essential oils have been tested extensively to assess their insecticidal and repellent activity. In this study different concentrations (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) of essential oil (EO) from seeds of *Zanthoxylum armatum* were tested for their insecticidal and repellent activity for aphids using direct toxicity and repellency method. Results revealed that mortality and repellency were concentrations and exposure time dependent. Mean mortality percentage increased with the increasing concentration and exposure time. 100% mortality was observed in 0.5% concentration after 48 hrs exposure time. However, repellent activity increases with the increasing concentration and decreases with the increasing exposure time. The maximum repellence percentage (96.29%) was achieved in 0.5% oil concentration after 24 hrs of exposure time. Gas Chromatography - Mass Spectrometry (GC-MS) analysis of the essential oil revealed the presence of three major compounds such as Eucalyptol (46.47%), Methyl Cis-Cinnamate (11.13%), and D-Limonene (7.10%) reported as insecticidal activity.

Keywords: aphids, essential oil, GC-MS analysis, repellent, *Zanthoxylum armatum*

INTRODUCTION

Aphids has been considered as one of the significant pest in agriculture and damage almost all crops all over the world. Aphids belonging to the family Aphididae is a common pest infecting a wide range of commercial crops. Theoretically, one female aphids can produce billion of descendants annually without mortality owing in their

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short life cycle (Hughes 1963; Dedryver et al., 2010). Aphids mainly attacked the leaves, stem, flowers and buds by sucking the sap that leads to weakening the host plants (Blackman and Eastop, 2017). The main control and protection of plants against aphids are the application of synthetic chemical insecticides that can cause many negative consequences to the non-target organisms such as pollinators and other beneficials, including humans (Banelli et al., 2017; Costa, 2018). Hence, essential oils extracted from medicinal and aromatic plants can be utilized to reduce the infestation from crop pest including aphids.

Zanthoxylum armatum DC. is a small xerophytic medicinal and aromatic shrub that belongs to family Rutaceae. It is commonly known as Prickly Ash, Winged Prickly Ash. These medicinal plants have been widely utilized in a traditional medicine in the North Eastern India and other neighbouring regions including South-East Asia (Kharshiing, 2012). Essential oils and different extracts of *Z. armatum* such as aqueous ethanol, dichloromethane, acetone, methanol, petroleum ether possesses many biological activities like antifungal, antibacterial, antiviral, larvicidal, pesticidal/insecticidal, keratolytic, anti-protozoan, hepatoprotective, antihelminthic and allelopathic effects (Seidemann, 2005; Singh and Singh, 2011; Tewary et al., 2005).

Essential oils (EO) are aromatic products derived from medicinal and aromatic plants by hydro distillation. EOs have insecticidal, repellent, and growth-reducing effects on various species of insects (Hikal et al., 2017; Farajzadeh et al., 2014). They are widely used as new bio-control alternative agents against insect pests, because of their non-toxicity to human consumption and low environmental impact, specificity of action, biodegradable nature and potential for commercial application (Kerdchoechuen et al., 2010).

MATERIALS AND METHODS

Plant material

Fruits of *Zantoxylum armatum* were collected from Mawsiatknam (Lat-25°39.780’N and Long- 91°.58.542’E), East Khasi Hills, Meghalaya located at an altitude of 1027 m.

Essential oil extraction

Essential oil is extracted by hydro distillation using 5 L Clevenger’s apparatus (Borosil, Code- 3450). 1 kg of freshly collected *Z. armatum* fruits were mixed with water in a round bottom flask and heated in a heating mantle for about 8 hours at 40ºC to evaporate the volatile components which is then collected in a receiver tube (10 ml). The oil were then collected and stored in 4^0 C until use. The yield of essential oil were calculated using the formula,

Yield of essential oil (%)= Volume of essential oil (g)/Volume of sample (g) *100

Oil preparation and analysis

Extracted oil were sent to Guwahati Biotech Park, Science Technology and Climate Change Department (Lat-26º11'39.49" N and Long- 91º40'15.56" E) for GC-MS analysis. Different concentration of essential oil 0.1%, 0.2%, 0.3%, 0.4% and 0.5% (v/v) were prepared. 0.1 ml, 0.2ml, 0.3ml, 0.4ml, and 0.5ml of essential oil were diluted in 99.9, 99.8, 99.7, 99.6 and 99.5 ml of distilled water respectively in a 100ml conical flask. A drop of Tween-20 was added to each concentration as an emulsifier and mixed properly. Water with just Tween-20 was used as a control.

Collection of Insects

Young shoots and leaves of Z. *armatum* infested with *Aphis citricidus* (Aphididae) were collected from net house of Botany Department, North-Eastern Hill University (NEHU). Aphids were then separated from the twig with the help of a soft brush and transferred into a 200 ml glass container for further use. The container was covered with a cloth to prevent the escape of aphids. Only the 3rd and 4th instar nymph were used for the experiment.

Direct toxicity test

Toxicity effects were performed following the method described by Hossain et al. (2021) by dipping *Z. armatum* leaves in prepared oil concentration separately for 5 seconds and then the leaves were air-dried for 30 seconds to remove volatile solvent. Each leaf was placed separately in a Petri dish (90 mm) where 20 nymphs were transferred with the help of a brush. Three replicates containing 20 nymphs were kept for each treatment. They were then kept in a seed germinator (NSW 191-192) where the temperature and Relative Humidity were maintained at 28 ± 2^{0}C and 65 ± 5% respectively under the dark. Mortality was determined at 24, 48 and 72 Hrs after the insect were placed on the dish. Mortality was corrected by Abbott's (1925) formula:

$$P = \frac{P\prime - C}{100 - \text{C}} \text{x}100$$

Where, P = Percentage of corrected mortality; P' = Observed mortality (%); C = Mortality (%) at control

Repellency test

This study was conducted following the method described by Sayed et al. (2022). 9 cm filter papers were cut in half and fitted into a 9 cm petri dishes with the help of a cello tape. 0.5 ml of prepared oil concentration were applied to one half of the filter paper with the help of a 1 ml pipette (Acura 826) and on the other half control was applied. Then, 20 aphids were released in the centre of each dish using a brush. The experiment was carried out in a seed germinator (NSW 191-192) where the temperature and Relative Humidity were maintained at 28 ± 2^{0}C and 65 ± 5% respectively under the dark. Sufficient space was kept to prevent the seepage of the oil to the control and for the aphids to move freely. To avoid the escape of aphids, petri dishes were wrapped with parafilm. Three replicates was kept for each oil concentration. Repellence percentages were calculated according to the following formula: The percent repellence (PR),

$$PR\ \% = \left(\frac{\mathrm{Nc}-\mathrm{Nt}}{\mathrm{Nc}+\mathrm{Nt}}\right) \mathrm{x}\ 100$$ (Tunc and Erler, 2003).

where, Nc is the number of individuals found in the negative control half and Nt is the number found in the treated half. Positive (+) values expressed repellency while negative value represent (-) attractancy. Percent repellencies were subjected to Tukey's test to determine statistically significant differences between concentrations, and between time periods.

Preparation and observation of aphids in Scanning Electron Microscope

Aphids treated in control and 0.5% of oil were subjected to observation of morphological ultra-structures under a Scanning Electron Microscope (JOEL, JSM-6360) done in SAIF, NEHU. Aphids were immersed in 2.5% Gluteraldehyde as primary fixative for about 4 hrs followed by washing in 0.1M Sodium Cacodylate Buffer 3 changes of fifteen minutes each at 4ºC. They were then subjected to sequential dehydration at 4ºC of 30%, 50%, 70%, 80%, 90%, 95%, 100% and finally in dry acetone for two changes and 15 minutes each. For drying the specimens were immersed in Tetra Methyl Silane for 5-10 minutes for two changes at 4ºC. They are then brought to room temperature (25-26ºC) to dry. The specimen was mounted on Brass stubs and coating (FINE COAT- Ion sputter JFC-1100) about 35nm thick was carried out using Gold and then viewed under SEM.

Statistical analysis

The data were analyzed using SPSS v.16 (Chicago, IL, USA) software program. Tukey's test was conducted using one-way analysis of variance (ANOVA) to assess the significant different for direct toxicity and repellent effects. The median lethal values (LC50) were determined by Probit analysis (Finney, 1947) using MS-Excel v.2007 (Microsoft, Washington, DC, USA).

RESULTS AND DISCUSSION

Essential Oil Composition

1Kg of *Z. armatum* fruits produces about 2.6% of essential oil. GC-MS analysis of essential oils showed that 13 compounds were identified which represent 86.33% of total constituents (Table 1). The major constituents were found to be Eucalyptol (46.47%), Methyl Cis-Cinnamate (11.13%), D-Limonene (7.10%), Cyclopenta[C]Pyran-1,3-Dione, 4,4A,5,6-Tetrahydro-4,7-Dimethyl- (6.28%) and Terpinen-4-ol (5.81%). It was observed that the chemical composition of extracted essential oil differ from those that were previously reported by various researcher (Ramidi et al., 1998 and Phuyal et al., 2019) where linalool was the major constituents however in this study eucalyptol was found to be the major components. Similar finding was reported by Kabdal et al. (2023), where linalool was absent in one of their samples and instead, they got *trans*-sabinene hydrate as the major component. Environmental conditions (especially altitude) and collection site could be the reason that indirectly affect the EO yields and chemical compositions (Chrysargyris et al., 2020 and Daferera et al., 2000). Biotic factors such as pathogen attack, herbivory and competition could also affect the chemical components in essential oils (Lämke and Unsicker, 2018).

Table 1. Essential oil composition from seeds of *Zanthoxylum armatum*

Sl No	Compound	RT	Area (%)
1	Alpha.-Pinene	9.68	1.45
2	3-Carene	10.73	2.56
3	D-Limonene	10.92	7.10
4	Cyclopenta[C]Pyran-1,3-Dione, 4,4A,5,6-Tetrahydro-4,7-Dimethyl-	11.92	6.28
5	Eucalyptol	12.41	46.47
6	Terpinolene	13.21	1.31
7	Terpinen-4-ol	15.32	5.81
8	Hexahydro-3-Butylphthalide	15.81	0.40
9	2-Carene	17.39	0.15
10	Cis-Sabinol	17.40	2.37
12	Methyl Cis-Cinnamate	19.60	11.13
13	Ethyl (Z)-Cinnamate	20.81	1.30
		Total Identified	86.33

Direct toxicity effects of essential oils of *Z. armatum*

The direct toxicity of different doses of essential oil and at different hours were shown in table 2. It was observed that mean mortality percentage increased with the increasing concentration and duration of treatment application. The highest mortality (96.29%) was recorded at 24 hrs treatment and the lowest mortality (1.65%) which are statistically significant, similarly at 48 hrs and 72 hrs treatment (Table 2). Complete mortality (100%) of the aphids were observed in 0.5% conc. at duration of 48 hrs after treatment. Average mortality increased with the concentration levels and very little mortality (10%) was recorded in untreated control.

Table 2. Direct toxicity effect of *Z. armatum* oil against aphids at different HAT

Doses (%)	Aphids Mortality (%) at indicated HAT ± SE			Average Mortality (%) ± SE
	24	48	72	
0.1	$1.65^{d} \pm 4.71$	$8.89^{c} \pm 9.49$	$88.89^{a} \pm 11.11$	$33.14^{d} \pm 5.93$
0.2	$12.77^{d} \pm 1.95$	$46.66^{b} \pm 10.18$	$100.00^{a} \pm 0.00$	$53.14^{cd} \pm 5.93$
0.3	$30.81^{c} \pm 3.02$	$71.11^{ab} \pm 4.44$	$100.00^{a} \pm 0.00$	$67.30^{bc} \pm 5.93$
0.4	$65.41^{b} \pm 5.04$	$95.55^{a} \pm 4.44$	$100.00^{a} \pm 0.00$	$86.98^{ab} \pm 5.93$
0.5	$96.29^{a} \pm 1.85$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$98.76^{a} \pm 5.93$
Control	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$10.00^{e} \pm 5.93$
LSD	2.93	2.57	1.81	2.44
CV%	6.29	6.23	6.11	6.21
P	<0.05	<0.05	<0.05	<0.05

HAT= Hours after treatment, SE= Standard Error. LSD= Least Significant Difference, CV= Coefficient of Variation. Within column values followed by different letter(s) are significantly different by Tukey test at 5% level of probability.

The LC_{50} values of essential oil tested against aphids at different hour of treatment were presented in Table 3. It was noticed that the LC_{50} values for 24, 48 and 72 hours of treatment were 2.81%, 1.24% and 0.37%, respectively. The comparison between the LC_{50} values in different hrs after treatment showed that they were non-significant of each other. Results from the present study indicates that under laboratory conditions, mortality of the tested essential oil against aphids were concentrations and exposure time dependent. Our results were also similar to the findings of Hossain et al. (2021), where the mortality of *Aphis craccivora* increases with the increase in concentration of tested essential oils of mehogony and karanja. The insecticidal activity of the oils of *Azadirachta indica*, *Eucalyptus camaldulensis* and *Laurus nobilis* was also proportional to dosage in the findings of Ebrahimi et al. (2013). Tewary et al. (2005) also reported that the pesticidal activity of five medicinal plants including *Z. armatum* decreases after 48 Hrs. Similar results were found by Wubie et al. (2014) who worked on *Brevicoryne brassicae* that maximum insecticidal activity of *Mentha piperita* (L.) plant extract was found in the highest concentration and it was increased from 24 hours to 72 hours exposure period. Klingauf et al. (1983) also reported that aphid's mortality were concentration and time dependent. Nia et al. (2015) found that the maximum mortality against *Myzus persicae* was obtained at the highest concentration after 24 hours of exposure period. Prolonged exposure period leads to the greater mortality (Lucca et al., 2015). This may result due to plant essential oils and their major components, monoterpenes that are among the most potential botanicals for alternative use to current commercially available insecticides (Isman and Machial, 2006). Hollingsworth et al. (2005) reported that the presence of D-Limonene as a major constituent, revealed the aphicidal properties against the wooly beech aphid, *Phyllaphis fagi* and the palm aphid, *Cerataphis brasiliensis.* This result also showed eucalyptol as a major chemical constituent along with Terpinen-4-ol which has insecticidal activities against many insects, as shown in previous studies (Koul et al., 2008; Isman, 2000; Carson and Hammer, 2010). Isman (2006) reported that components like monoterpene may affect the digestive and neurological enzymes when they interact with the integument of insects.

Table 3. LC50 values (%) of the tested plant essential oil against aphids using Probit analysis

Tested insect	HAT	LC50 (Confidence Interval Limits)	Intercept ± SE	Slope ± SE	χ2	P
	24	2.81 (1.17-3.09)	-0.23 ± 0.77	2.13 ± 0.34	0.90	0.78
Aphids	48	1.24 (1.28-3.89)	-0.42 ± 1.04	2.59±0.46	0.88	0.71
	72	0.37 (2.56-3.75)	0.07 ± 0.47	3.16 ± 0.21	0.98	0.89

HAT = Hour after treatment. SE= Standard Error. Values were based on five concentrations, three replications of 20 insects each. χ2 = Goodness of fit. P at 5% level of probability.

Observation of aphids in SEM

From the Scanning Electron Micrographs, it has been observed that the aphid treated with oil shows deformed body and shrunken abdomen, which may be due to the loss of bodily fluids (Fig 1). However, aphids treated with control shows no such deformation and all the body parts seemed intact. Similar observations were also reported by Jayaram et al. (2020), where the body and abdomen of *Aphis craccivora* Koch. got shrunk when treated with *Tagetes minuta* oil.

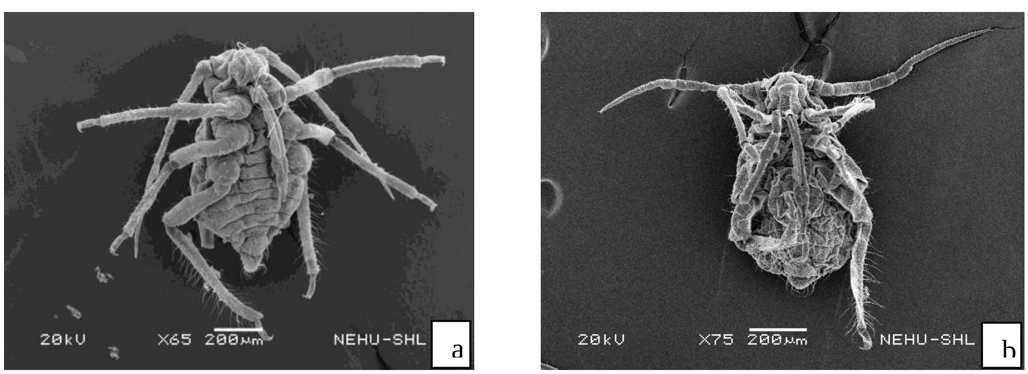

Figure 1. SEM image of Aphids (a) Control (b) Treated

Repellence Test

The repellent activity of essential oil at different concentrations is presented in table 4. The repellence of the tested EO increased with the concentration. However, the repellent activity of the EO decreases with the progress of exposure time. The repellent class of tested oil at different concentrations level varied between classes I to III. The average repellency (58.3%) was obtained in 0.5% oil concentration and the lowest (14.1%) at 0.1% and both were significantly different. These findings are in agreement with the results reported by Hossain et al. (2021), where the repellency rate of mehogony, neem and karanja essential oil decreases with the increasing exposure time. Govere et al. (2000) also reported that the repellency activity of *Cymbopogon excavatus* against *Anopheles arabiensis* decreases when the exposure time was increased. Sharma and Gupta (2011) also reported that repellent effect is dose dependent and reduces with the passage of time. The decrease in effectiveness of EOs may result due to the dissipation of their protective effects hours after their application (Trongtokit et al., 2005).

Table 4. Repellent activity of *Z. armatum* against Aphids at different HAT

Doses (%)	Repellency (%) at indicated HAT ± SE				Average Repellency (%)± SE	Repellent Class
	2	4	6	8		
0.1	$26.6^{d} \pm 2.88$	$16.6^{c} \pm 2.88$	$10.0^{c} \pm 5.00$	$3.3^{c} \pm 2.88$	$14.1^{c} \pm 4.96$	I
0.2	$36.6^{cd} \pm 2.88$	$30.0^{bc} \pm 2.50$	$13.3^{bc} \pm 2.88$	$6.6^{bc} \pm 2.88$	$21.6^{b} \pm 7.00$	II
0.3	$46.6^{bc} \pm 2.88$	$33.3^{b} \pm 2.88$	$23.3^{bc} \pm 2.88$	$10.0^{bc} \pm 5.00$	$28.3^{b} \pm 7.74$	II
0.4	$58.3^{b} \pm 1.44$	$53.3^{a} \pm 2.88$	$33.3^{ab} \pm 2.88$	$23.3^{b} \pm 2.88$	$42.0^{a} \pm 8.26$	III
0.5	$76.6^{a} \pm 2.88$	$63.3^{a} \pm 2.88$	$50.0^{a} \pm 2.88$	$43.3^{a} \pm 2.88$	$58.3^{a} \pm 7.67$	III
LSD	0.26	0.27	0.28	0.27	0.27	
CV%	0.05	0.06	0.11	0.13	0.08	
P	<0.05	<0.05	<0.05	<0.05	<0.05	

HAT= Hours after treatment, SE= Standard Error, LSD= Least Significant Difference, CV= Coefficient of Variation. Within column values followed by different letter(s) are significantly different by Tukey test at 5% level of probability.

Liu et al. (2006) reported that essential oils of *Artemisia princeps* Pamp and *Cinnamomum comphora* L. possess repellency effect on *Sitophilus oryzae* and *Bruchus rugimanus*. Similarly, Hossain et al. (2014) and Manzoor et al. (2015) reported a significant level of success on the suppression of aphids due to repellency of plant oils. Similar results were also obtained by Wubie et al. (2014) that repellent activity of *M. piperita* against *B. brassicae* increased up to 48 hours and it was decreased after 72 hours of exposure period. Wubie also stated that the increased in mortality rate may be due to the arrest of metabolic activity when the odour of the extract enter through the spiracle thereby blocking the respiratory activity. The repellence activity for essential oils is highly dependent on their chemical composition. Some monoterpenes such as alpha-pinene, limonene, terpinolene, 3-Carene which are common constituents of a number of EO described in the literature possess mosquito repellent activity (Jaenson et al., 2006; Park et al., 2005; Yang et al., 2004).

CONCLUSION

The results on direct toxicity of the EO showed that with increasing duration and oil concentration there was an increase in aphids' mortality. Exposure period has a great impact on the mortality of the aphids. Their mortality can be attributed to the presence of effective monoterpenes such as limonene, eucalyptol and terpinen-4-ol and an ester such as methyl cinnamate which has strong insecticidal and aphicidal activity. On the other hand, results showed that *Z. armatum* essential oil has an average repellency rate towards the aphids. Therefore, the present study proves that *Z. armatum* essential oil show insecticidal and repellent activity and further studies can be done on several factors that could enhance the insecticidal and repellency of essential oil. These findings indicates that essential oils can be used as a safer alternatives to the synthetic insecticides and show potential for the development of bio-insecticides in insects and pest management.

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