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Larvicidal and Histopathological Effects of *Andrographis paniculata* Leaf Extract against *Culex quinquefasciatus* Larva

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Abstract

Andrographis paniculata leaf aqueous extract over 0.5, 1, 3, 5, and 24 h were determined to contain the highest amount of total phenolic compound and was used to evaluate mosquito larvicidal properties against *Culex quinquefasciatus* at varies concentrations (3.125, 6.25, 12.5, and 25 ppm) by determining the median and 90 % lethal concentration, LC_{50} and LC_{90}, respectively, within 24 h and by histological analysis. The results revealed that the total phenolic compound measurement in each time extraction were 7.97, 8.68, 7.16, 6.70, and 5.78 mg/g GAE, respectively. The one hour aqueous extract of *A. paniculata* leaf expressed the 24-h LC_{50} and LC_{90} values in *C. quinquefasciatus* which were 15.93 and 28.23 ppm, respectively. Dose dependent lesions were observed. Under histological analysis, midgut lesions, i.e., separation of the epithelial cells from the basement membrane, elongation protruding into the lumen, disruption of the brush border, and the appearance of several vesicles and cytoplasm masses were observed in this study. The present study reveals that the aqueous extract of this leaf has a suitable property for a larvicidal natural product, and may replace harmful chemical pesticides.

Keywords: Leaf, *Andrographis paniculata*, *Culex quinquefasciatus*, mosquito larvae, histology

Introduction

Culex quinquefasciatus (Diptera: Culicidae) is a vector of many human and animal pathogens, including lymphatic filariasis, Japanese encephalitis, avian malaria, and dengue, as well as yellow fever [1]. One method of prevention of mosquito borne diseases is the reduction of the mosquito population in the various stages of development, such as the use of ovicidal, larvicidal, pupicidal, adulticidal, and mosquitocidal substances. The application of chemical insecticides in mosquito control has resulted in the persistence and accumulation of non-biodegradable chemicals in the ecosystem, biological magnification through the food chain, insecticide resistance, and a toxic effect in human health and non-target organisms [2]. Many studies on plant extracts against the mosquito population have been conducted around the world. Larvicidal activity against *C. quinquefasciatus* have been extracted from plants of Asteraceae: *Tagetes erecta* in Bangladesh [3], Fabaceae*: Copaifera reticulate* in Brazil [4], Caricaceae: *Carica papaya* and Phyllanthaceae: *Cleistanthus collinus* [2], and Lamiaceae: *Clerodendrum phlomidis* [5] in India, Myrtaceae: *Syzygium aromaticum* and Pinaceae: *Pinus sylvestris* in Nigeria [6], Acoraceae: *Acorus calamus* and Poaceae: *Cymbopogan citrates* in Pakistan [7], Meliaceae: *Melia azedarach* in Saudi Arabia [8], Asclepiadaceae: *Calotropis procera* in Sudan [9], and Rutaceae: *Murraya paniculata* in Thailand [10].

Andrographis paniculata (Acanthaceae) has been widely used as a "Traditional Medicinal Plant" in Asia and Southeast Asia, including in Thailand. It is traditionally used for antimalarial activity [11], anti-inflammation activity [12], antidiabetic [13] and antiangiogenic activity [14], antimicrobial activity [15], antioxidant activity [16], and associated diseases such as hepatoprotective activity [17], respiratory infection [18], and cardiovascular protection [19]. A number of compounds, including andrographolide,

homoandrographolide, neoandrographolide, dehydroandrographolide, deoxyandrographolide, andrographan, andrographon, andrographosterin, and stigmasterol have been isolated from *A. paniculata* [20]. A plant from the same genus *Andrographis* had been reported as having larvicidal activity against the Japanese encephalitis vector, *Culex tritaeniorhynchus* [21], the dengue vector, *Aedes aegypti* [22,23], and the malarial vector, *Anopheles stephensi* [24]. Furthermore, there has been little research regarding histological analysis after bioinsecticide exposure. The present study was conducted to evaluate the mosquito larvicidal properties of *A. paniculata* leaf aqueous extract against *C. quinquefasciatus* as a target species. The susceptibility of *A. paniculata* was evaluated via histological analysis.

Materials and methods

Plant collection and extraction

The *A. paniculata* leaves were purchased from Nonthaburi province, Thailand. The voucher specimen was numbered and kept in our research laboratory for further reference. The extraction procedure was determined by the method of Sirimongkolvorakul *et al.* [25], with modification. Briefly, they were washed with tap water and rinsed with distilled water, air dried, and crushed with a mixer-grinder machine. Five grams of leaf powder was extracted with 100 ml distilled water with a shaker set at 180 rpm for 0.5, 1, 3, 5, and 24 h at room temperature. After that, they were centrifuged at 750 g for 10 min and filtrated through Whatman No. 1 filter paper. The extraction was kept as stock for total phenolic compound measurement and larval bioassay procedure.

Total phenolic compound measurement

Total phenolic compound was determined using Folin-Ciocalteu reagent, according to methods of Sirimongkolvorakul *et al.* [25] and Mcdonald *et al.* [26], with modification. Briefly, the 50 µl of the extraction in each time (0.5, 1, 3, 5 and 24 h) was mixed with 250 µl of 10 % Folin-Ciocalteu and 200 µl of 0.7 M sodium carbonate, then distilled water added until 5 ml, and incubated at room temperature for 2 h in a dark room; the mixture was measured at 765 nm by using a spectrophotometer. Quantification was based on the standard curve of gallic acid (0, 625, 1250, 2500, 5000 and 10000 mg/l) and expressed as a gallic acid equivalent (GAE) using the following linear equation, based on the calibration curve as shown in equation A: $OD = 0.0005C^2 + 0.0239C + 0.0321$, where OD was the absorbance, and C was concentration as GAE.

Mosquito larvae collection

The fourth instar larvae were collected from the surroundings of the Mahidol University Phayathai campus, Bangkok, Thailand, and transferred into a glass beaker containing distilled water. *Culex* larvae were morphologically identified using the key of Harbach [27] and Rattanarithikul *et al*. [28].

Larval bioassay procedure

The result from the total phenolic compound measurement showed the highest quantity in the 1 h extraction. So, the 1 h extraction was prepared in serial concentrations (3.125, 6.25, 12.5 and 25 ppm) through the mixing up of stock extract with variable amounts of sterilized distilled water**.** The larval bioassay was assessed by following the standard WHO method [1]. The bioassay was repeated three times, using fourth instar larvae of *C. quinquefasciatus*. Twenty larvae were transferred to beakers containing 100 ml of distilled water (control) and 4 concentrations of leaf extract. The bioassay was maintained at 27±1 °C throughout the test. Larval mortality was recorded over 24 h of exposure. A larva was considered dead or moribund if it stopped moving for a prolonged period, even after gentle probing with a small spatula. The LC_{50} and LC_{90} were analyzed by the probit method of Finney [29] using the SPSS 18.0 (Statistical Package of Social Sciences) software [30]. It estimated the lethal concentration and the slope of the regression line with its confidence interval ($p = 0.05$).

Histological analysis

Under the histology technique, only live larvae were examined in each concentration. The procedures were performed following the methods of Kjanijou *et al.* [10] and Pavananundt *et al.* [31]. Briefly, the larvae were fixed in 10 % buffered formaldehyde for 24 h, dehydrated through a graded series of ethanol, and cleared with xylene solutions. They were embedded in a block using melted paraffin at the embedding station. The paraffin blocks were sectioned at 5 μm thickness using a rotary microtome, and stained with Harris's hematoxylin and eosin (H&E). The glass slides were examined for abnormalities using the Olympus CX31 light microscope and photographed by a Canon EOS 1100D digital camera.

Results and discussion

The measurements of the total phenolic compound from leaves of *A. paniculata* in each time extraction, 0.5, 1, 3, 5, and 24 h, were 7.97, 8.68, 7.16, 6.70, and 5.78 mg/g GAE, respectively. No mortality was observed in control group. The result of probit analysis at 95 % confidence level revealed that the 24-h LC_{50} and LC_{90} values were 15.93 and 28.23 ppm, respectively (**Figure 1**). The midgut epithelium consisted of a single layer of digestive cells, exhibiting a well-developed brush border and cytoplasm with acidophilic regions (**Figure 2**). After 24 h exposure to *A. paniculata*, the dose dependent lesions were observed. The partial lysis of the epithelial cells began through local detachment or dilated basal membrane in the lowest concentration, 3.125 ppm (**Figure 3A**). The degeneration of the cell structure, brush border, and elongated cells were found in the 6.25 ppm group (**Figure 3B**). It also contained vesicles of different sizes and broken membranes, with bubbles at the apical side of the epithelial cells in the higher concentration, 12.5 ppm (**Figure 3C**). Finally, cytoplasm masses, which looked like bubbles, were observed in several areas (**Figure 3D**), and hyperplasia of gut epithelial cells was also seen (**Figure 3E**).

The calibration curve of the total phenolic compound in this study (OD = $0.0005C^2+0.0239C+0.0321$) was in range with the earlier report of Srivastava *et al.* [32] as per the following: $A = 0.0011C^2+0.0656C+0.001$, where A was the absorbance, and C was concentration as GAE.

This study has shown the potential of *A. paniculata* for use in *C. quinquefasciatus* larvae control. The present 24h-LC_{50} value of *A. paniculata* against *C. quinquefasciatus* larvae was 15.93 ppm. The larvicidal activity of this leaf extract result was comparable with those of activity mentioned in earlier reports [22,33,34]. Govindarajan [22] reported that benzene, hexane, ethyl acetate, methanol, and chloroform leaf extract of *A. paniculata* were found to be effective against *C. quinquefasciatus* in India, and the 24h-LC_{50} values were 112.19, 137.48, 118.67, 102.05, and 91.20 ppm, respectively. Govindarajan and Sivakumar, 2012 [33] reported the adulticidal effect of *A. paniculata* against *C. quinquefasciatus* adults, with the 24-h LC_{50} and LC_{90} values being 149.81 and 172.37 ppm, respectively. Renugadevi *et al.* [34] investigated the 8 h petroleum ether and aqueous extract of *A. paniculata* and *A. lineata* against fourth instar larvae *C. quinquefasciatus* in India, with the 24h-LC_{50} and LC_{90} values of petroleum ether and aqueous extract of *A. paniculata* (LC_{50}=117.73, LC_{90}=239.33; LC_{50}=109.21, LC_{90}=197.53 ppm) and *A. lineata* (LC_{50}=179.03, LC_{90}=253.37; LC_{50}=147.45, LC_{90}=239.16 ppm). From these previous studies, no conclusion was made for an efficient method for the extraction of larvicidal compounds from *A. paniculata*.

Active compounds extracted with methanol from the whole plant, leaf, and stem, including over 20 diterpenoids and over 10 flavonoids, have been reported from *A. paniculata* [35]. Andrographolide, the major diterpenoid, was found to be at about 4 %, in dried whole plant, 1.2 % in stem and 6 % in leaf extracts [36,37]. The fifth instar, sixth instar, larvae, and pupae of the darkling beetle, *Tribolium confusum*, were treated with 1 μg/μl of andrographolide, and exhibited ovarian deformities, variation in the length and size of ovarioles, oocyte degeneration, and inability of the mature oocyte to oviposite [38]. Moreover, several studies have reported the antioxidant activities of *A. paniculata* and its constituents. Verma and Vinayak [39] reported that the aqueous extract of *A. paniculata* significantly increased the activities of antioxidant defense enzymes such as catalase, superoxide dismutase, and glutathione-S-transferase, and reduced glutathione content.

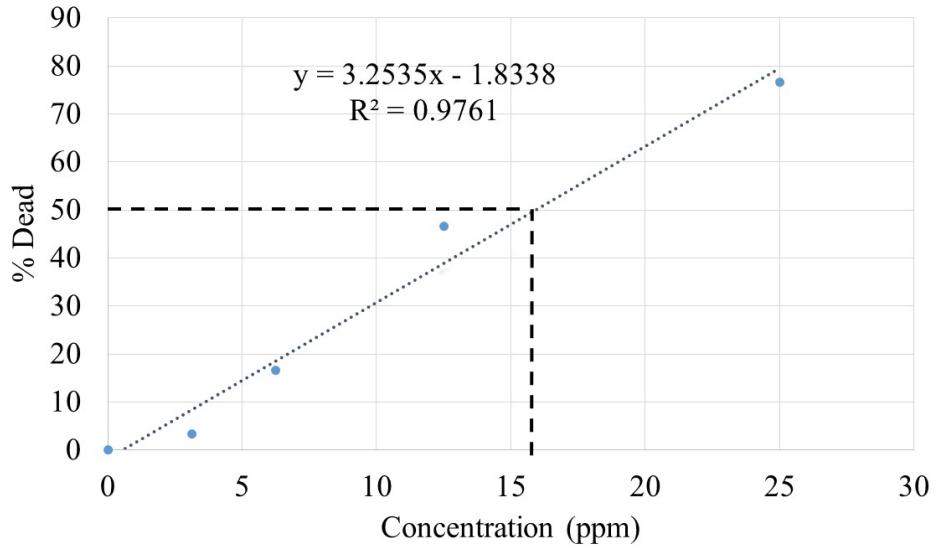

Figure 1 The relationship between the percentage of *Culex quinquefasciatus* larvae dead and *Andrographis paniculata* leaf extract concentration.
Note: The broken line indicates the LC_{50} value.

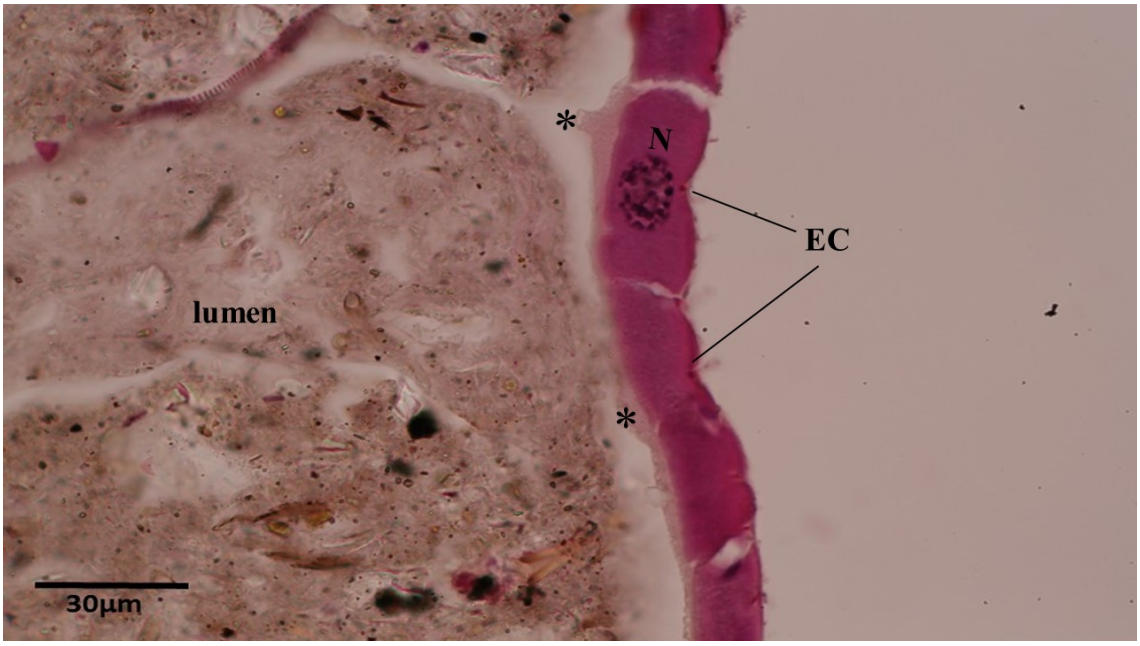

Figure 2 Histology of midgut of *C. quinquefasciatus* larvae in the control group, showing the gut epithelial cells (EC) with nucleus (N) and brush border (*).

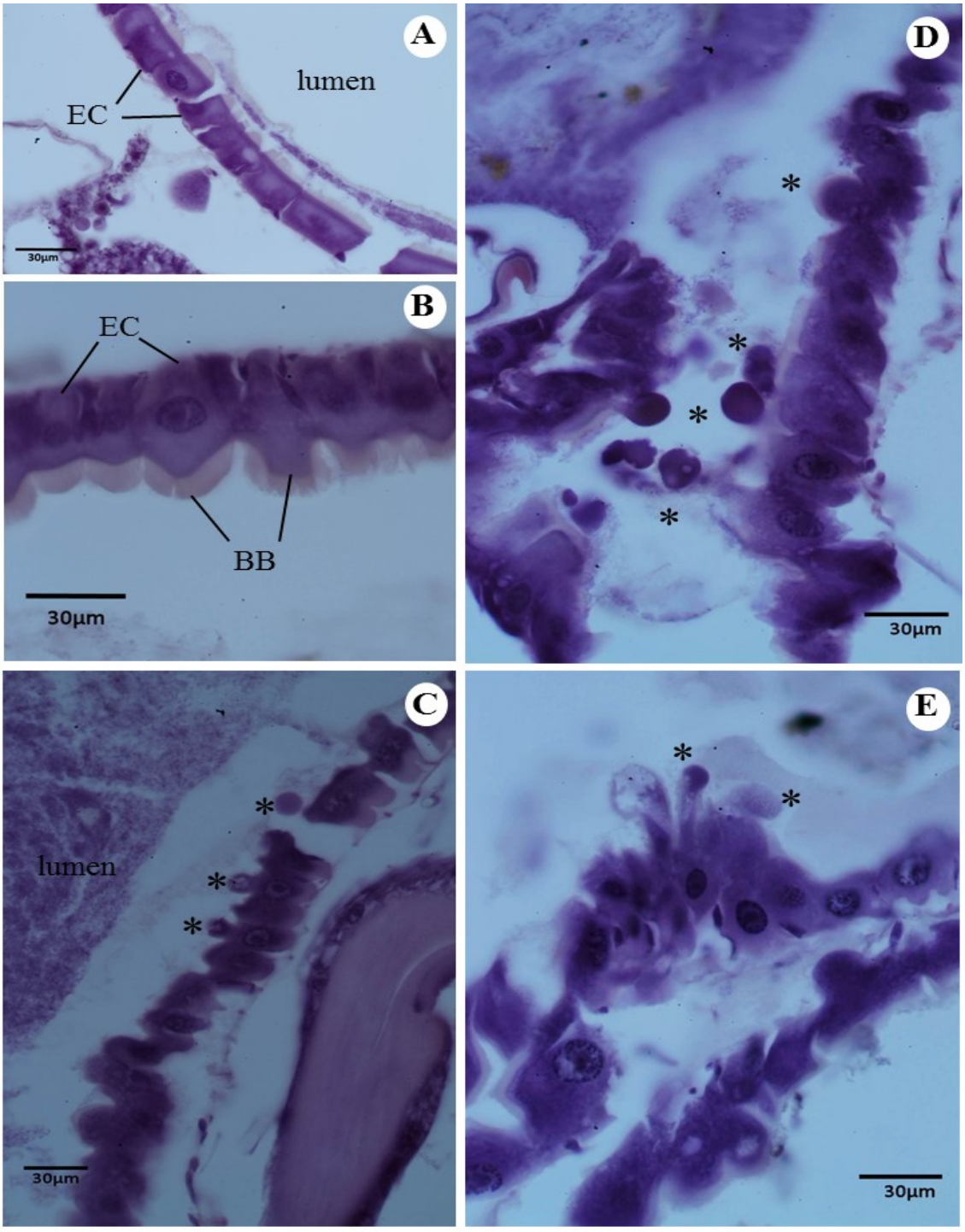

Figure 3 Histology lesions in the midgut of *C. quinquefasciatus* larvae in (A) 3.125 ppm, (B) 6.25 ppm, (C) 12.5 ppm, and (D and E) 25 ppm of *Andrographis paniculata* leaf extract concentrations.
Note: * = bubbles, BB = brush border, EC = epithelial cell.

In this study, histological alterations were seen in the midgut, included separation of the epithelial cells from the basement membrane, sometime distinct elongations protruding into the lumen, and disruption of the brush border. These observations were in agreement with earlier reports. The midgut possesses a well-developed brush border in the cell apex, because it is the main absorption area in the mosquito gut [40]. The midgut, hindgut, and muscles of *C. quinquefasciatus* that were treated with fenugreek (*Trigonella foenumgraceum*) in Saudi Arabia were affected, and the epithelial layer was affected by rupture, disintegration, and cellular vacuolization [41]. The histological studies of *Matricharia chamomella* extract against *C. quinquefasciatus* were analyzed [42]. Lesions were seen in the anterior and posterior regions of the midgut, including cell vacuolization and rupture of the epithelial walls, brush border damage, and the epithelium cell contents passing into the midgut lumen. Regardless of the type of substances used, the similarity of detrimental changes in the organism indicates that these alterations are a common response to cellular intoxication.

Conclusions

In conclusion, the aqueous extract of *A. paniculata* can be recommended in field areas, and can be effectively used as a natural larvicidal product in the mosquito control program. However, further studies are needed to find out what the active substances are, and what the mechanism of delivery into the target species is.

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