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Larvicidal and Histopathological Effects of *Cassia siamea* Leaf Extract against *Culex quinquefasciatus*

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Abstrak: Suatu ekstrak ubatan tradisional Thai daripada *Cassia siamea* telah dinilai menggunakan sifat-sifat larvisidnya dengan menentukan kepekatan maut median (LC_{50}) pada jam 24, 48, 72 dan 96 terhadap larva instar keempat *Culex quinquefasciatus*, yang merupakan pembawa jangkitan tungau, dengan mengkaji perubahan histopatologikal. Nilai LC_{50} pada jam 24, 48, 72 dan 96 ialah 394.29, 350.24, 319.17 and 272.42 ppm, masing-masing. Lesi histopatologikal selepas pendedahan kepada 25% daripada 24-h LC_{50} telah diperhatikan terutamanya diusus tengah larva. Lesi dengan edema, pembengkakan, dan pencacatan ataupun pemanjangan sel epitilial telah diperhatikan. Selain itu, sel-sel yang protrud ke dalam lumen dan ketiadaan mikrovili juga diperhatikan pada sesetengah kawasan. Kajian ini menunjukkan bahawa ekstrak akueus daun *C. siamea* mempunyai sifat-sifat biopestisid semulajadi.

Kata kunci: Biopestisid, *Cassia siamea*, *Culex quinquefasciatus*, Histopatologi, Daun, Nyamuk

Abstract: A traditional Thai medicinal extract from *Cassia siamea* was evaluated with respect to its larvicidal properties by determining the median lethal concentration (LC_{50}) at 24, 48, 72 and 96 h against the fourth instar larvae of *Culex quinquefasciatus*, which is a carrier of mosquito-borne diseases, by studying the histopathological alterations. The 24, 48, 72 and 96 h LC_{50} values were 394.29, 350.24, 319.17 and 272.42 ppm, respectively. The histopathological lesions after exposure to 25% of the 24-h LC_{50} were observed primarily in the midgut of the larva. Lesions with edema, swelling, and deformation or elongation of the epithelial cells were observed. Moreover, cells protruding into the lumen and absent microvilli were also found in some areas. The present study reveals that aqueous *C. siamea* leaf extracts have natural biopesticide properties.

Keywords: Biopesticide, *Cassia siamea, Culex quinquefasciatus*, Histopathology, Leaf, Mosquito

INTRODUCTION

Culex quinquefasciatus from the family Culicidae is commonly known as the southern house or brown mosquito and is a vector of many microorganisms including the St. Louis encephalitis virus (Hardy *et al.* 1984), the West Nile virus (Zinser *et al.* 2004), the Japanese encephalitis virus (Nitatpattana *et al.* 2005),

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yellow fever (WHO 2006), avian malaria (Reiter & Lapointe 2009), dengue (Calderón-Arguedas *et al.* 2009) and lymphatic filariasis (Aigbodion *et al.* 2011). Adult *C. quinquefasciatus* can be recognised by its body length and brown colour; moreover, the length of six morphological characters, namely, the wing, antenna, proboscis, foreleg, mid leg and hind leg, have been reported by Adeleke *et al.* (2008). Additionally, Acharya *et al.* (2013) reported eight morphological characteristics: the siphon length, width and index; the saddle length, width and index; and the numbers of comb scales and pectin teeth. The life cycle is completed within seven days by passing through the egg, larva, pupa and adult stages.

The most effective prevention of mosquito borne diseases is achieved by reducing the mosquito population in any of the various life cycle stages, such as through the use of ovicidal, larvicidal, pupicidal and adulticidal substances. Currently, problems caused by the usage of chemical insecticides have been reported with respect to the persistence and accumulation of non-biodegradable chemicals in the environment, the biological magnification through the food chain, the development of insecticide resistance and the toxic effect to human health and to non-target organisms (Rawani *et al.* 2009). Many studies into plant or herb extracts targeted at mosquito stages have been conducted in many countries around the world. The larvicidal activity against *C. quinquefasciatus* has been extracted from plants: *Derris indica* from the Fabaceae family in Bangladesh (Mondal *et al.* 2011), *Rosmarinus officinalis* from the Lamiaceae family in China (Yu *et al.* 2013), *Rauvolfia serpentina* from the Apocynaceae family in India (Das & Chandra 2012), *Vernonia adoensis* from the Asteraceae family in Kenya (Swamy *et al.* 2014), *Azadirachta indica* from the Meliaceae family and *Citrus sinensis* from the Rutaceae family in Nigeria (Allison *et al.* 2013), *Melia azedarach* from the Meliaceae family in Pakistan (Ilahi *et al.* 2012), *Moringa oleifera* from Moringaceae family in Tanzania (Nkya *et al.* 2014), *Murraya paniculata* from the Rutaceae family in Thailand (Kjanijou *et al.* 2012), and Euphorbiaceae, Verbenaceae and Meliaceae in West Africa (Azokou *et al.* 2013).

Cassia siamea from the family Fabaceae has been widely used as a "traditional medicinal plant" in Asia and in southeast Asia including Thailand. *C. siamea* is commonly known by regional names such as: *mezali* (Burmese), *tie dao mu* (Chinese), kassod tree (English), *robles* (Filipino), *bois perdrix* (French), *minjri, manje-konna* (Hindi), *bujuk dulang johar* (Indonesian), *angkanh* (Khmer), *sino-tibetan* (Lao), *sebusok*, *guah jitam*, *juah* (Malay), *manjakonna* (Malayalam), *kassod* (Marathi), *cassia* (Nepali), *amarillo* (Spanish), *manjal konrai* (Tamil), *sima tangedu, kurumbi* (Telugu), *khilek* (Thai), *humbo*, *muoofng xieem* (Vietnamese) (Orwa *et al.* 2009; Singh *et al.* 2013).

The young fruit, leaf and flower are eaten as vegetables and are also used in a popular curry called *Khilek* in Thailand (Teangpook *et al.* 2012). The stem bark is traditionally used for anti-plasmodium treatments (Ajaiyeoba *et al.* 2008), analgesia, anti-inflammation and the treatment of associated diseases, such as fevers and jaundice (Nsonde Ntandou *et al.* 2010). The leaf has anti-diabetic and anti-lipemic effects (Kumar *et al.* 2010), an anti-proliferative effect (Esakkirajan *et al.* 2014) and antibacterial activity (Majji *et al.* 2013). Furthermore, the flower and root have antioxidant activity (Deshpande *et al.* 2013). A

histological analysis after biopesticide exposure has not been performed. The present study was conducted to evaluate the mosquito larvicidal properties of a *C. siamea* leaf aqueous extract against *C. quinquefasciatus* as a target species. The susceptibility of *C. siamea* was evaluated using the median lethal concentration and a histological analysis.

MATERIALS AND METHODS

Plant Collection and Extraction

Fresh, mature, green *C. siamea* leaves were randomly harvested in and around Nakhonpathom Province in the central part of Thailand (13° 45′ 13″ N, 100° 19′ 19″ E). The voucher specimen was numbered and saved in the Faculty of Science, Mahidol University for further reference. The leaves were initially rinsed with distilled water, air dried, and crushed using a mixer-grinder machine. One hundred grams of leaf powder was extracted with 100 ml of distilled water on shaker at 180 rpm for 1 h; the solution was then centrifuged at 4000 rpm for 10 min, the supernatant was filtered using Whatman no. 1 filter paper, and the clear filtrate was used as a stock solution for the bioassay experiments. Required concentrations (0, 60, 120, 240, 480 and 960 ppm) were prepared by mixing the stock extract with various amounts of sterilised distilled water.

Mosquito Larvae Collection

The fourth instar larvae were collected surrounding the Phayathai campus of Mahidol University in Bangkok, which is the capital city of Thailand (13° 45′ 51″ N, 100° 31′ 32″ E), and were transferred into a glass beaker containing distilled water. The larvae were sorted and identified as *C. quinquefasciatus* larvae.

Larval Bioassay Procedure

The larval bioassay was performed using a standard protocol described by the World Health Organization (WHO 1996). The bioassay was repeated three times. Twenty larvae were transferred to beakers containing 100 ml of distilled water and 5 concentrations of leaf extract. The bioassay was maintained at 27±1°C throughout the test. The larval mortality was recorded for a maximum of 96 h of exposure. Larvae were considered dead or moribund if they stopped moving for a prolonged period even after gentle probing with a small spatula. The median lethal concentration (LC_{50}) was analysed using the probit method described by Finney (1971) using the Statistical Package for the Social Sciences (SPSS) 18.0. The lethal concentration and the slope of the regression line with its confidence interval *($p \leq 0.05$)* were determined.

Specimen Preparation for Light Microscopic Study

For the histological test, 20 larvae were exposed to 25% of the 24-h LC_{50} for 24 h. Only live larvae were examined. The light microscopy procedures were performed following the methods of Kjanijou *et al.* (2012) and Pavananundt *et al.* (2013). Briefly, the larvae were fixed in 10% buffered formaldehyde for 24 h, dehydrated through a graded series of ethanol and cleared with xylene solutions.

The larvae were embedded in a block using melted paraffin at the embedding station. The paraffin blocks were sectioned at a 5-μm thickness using a rotary microtome and were stained using haematoxylin and eosin. The glass slides were examined for abnormalities using the Olympus CX31 (Bangkok) light microscope and photographed using a Canon EOS 1100D (Bangkok) digital camera.

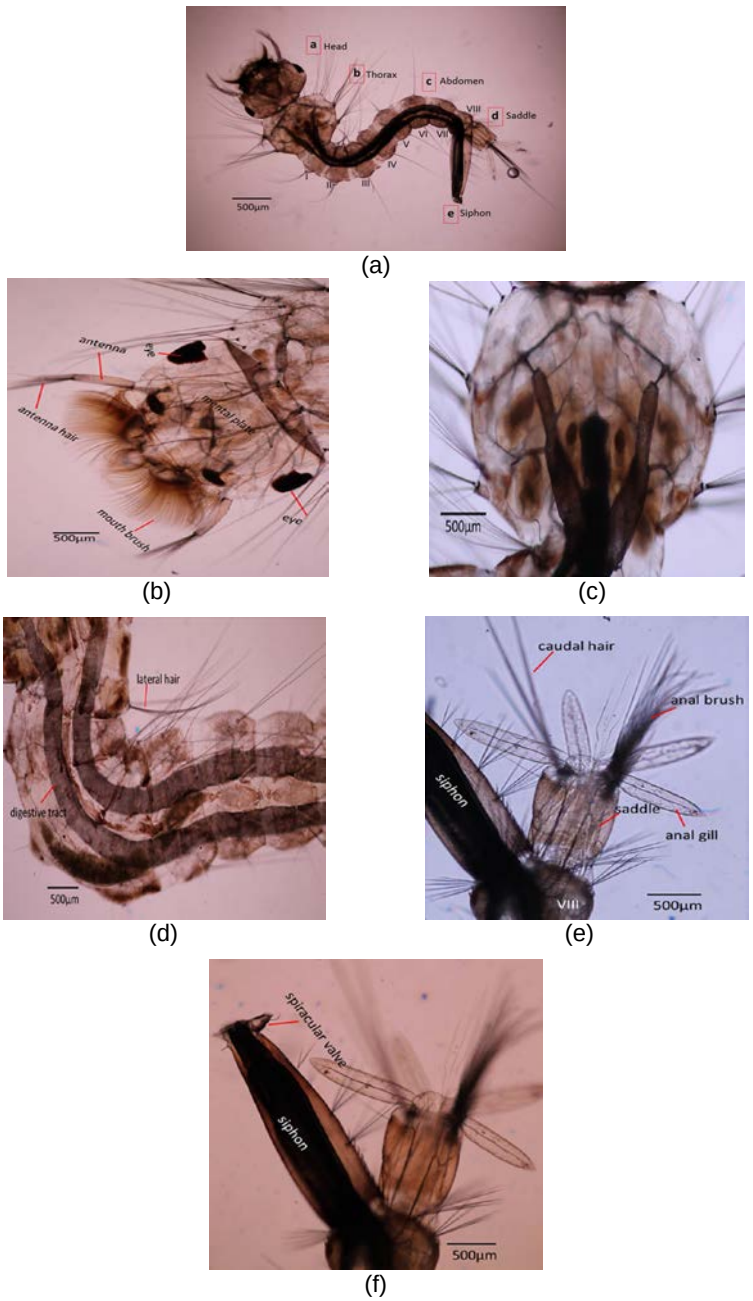
RESULTS

The larvicidal properties of the aqueous leaf extract of *C. siamea* against *C. quinquefasciatus* larvae are presented in Table 1. The result of the probit analysis at a 95% confidence level showed that the LC_{50} and LC_{90} values gradually decreased with the exposure time from 24 to 96 h. A dose dependent mortality was also observed as the rate of mortality (*Y*) was positively correlated with the concentration (*X*) of the leaf extract as demonstrated by the established regression equations. The correlation (R^2) between the concentration and the mortality were observed to range from 0.7667 to 0.8894.

Table 1: Efficiency of *C. siamea* leaf extract on *C. quinquefasciatus* larval mortality (n=20).

Time (h)	*C. siamea* (ppm)						LC_{50}	LC_{90}	Regression equation	*R* value
	960	480	240	120	60	Control				
24	19.33	15.67	8.67	5.33	0.67	0.00	394.29	782.64	*y = 0.0206x + 1.8777*	0.8894
48	19.67	16.67	11.33	5.67	1.67	0.00	350.24	740.49	*y = 0.0205x + 2.8200*	0.8433
72	20.00	17.00	12.33	7.33	2.33	0.00	319.17	719.17	*y = 0.0200x + 3.6166*	0.8172
96	20.00	18.33	13.67	8.00	4.33	0.00	272.42	686.93	*y = 0.0193x + 4.7423*	0.7667

The gross morphology of normal healthy *C. quinquefasciatus* larvae is shown in Figure 1 and is divided into three body regions: head, thorax and abdomen. The head has the antennae, eyes and mouthparts. The antennae are located on each side of the head towards the front. Behind the antennae and near the hind margin of the head are the dark black eyes. The mouthparts are on the underside of the head and consist of a series of numerous brushes, which have long filaments that are used for filtering materials (Fig. 1[a]). The thorax is broader than the head or abdomen and somewhat flattened. The thorax has several groups of hairs (Fig. 1[b]). The abdomen is long and cylindrical and consists of eight segments (Fig. 1[c]), the siphon and the saddle. Each segment has a unique setae pattern. The saddle (Fig. 1[d]) is barrel shaped and is located on the ventral side of the abdomen with four long anal papillae or anal gills protruding from the posterior end. The siphon (Fig. 1[e]) is on the dorsal side of the abdomen, and in *C. quinquefasciatus*, the siphon is four times longer than it is wide, having multiple setae tufts.



(a) (b) (c) (d) (e) (f)

Figure 1: a) Light micrograph of a fresh, whole *C. quinquefasciatus* larva showing the composition of three body regions: (b) head; (c) thorax; (d) abdomen; (e) saddle; (f) siphon.

In the control group, the midgut epithelium consisted of a single layer of digestive cells that exhibited a well-developed brush border or microvilli and a cytoplasm with acidophilic regions (Fig. 2[a] and [b]). The histopathological lesions after exposure to *C. siamea* at 25% of 24-h LC_{50} were observed primarily in the midgut of larva. Lesions with edema, swelling, deformation or elongation of the epithelial cells were observed. Additionally, there were vesicles in the cytoplasm of the epithelial cells (Fig. 2[c]). Moreover, cells protruding into the lumen, blebbing cells and absent microvilli were also observed (Fig. 2[d]). Hyperplasia of the epithelial cells was found in some areas (Fig. 2[e] and [f]).

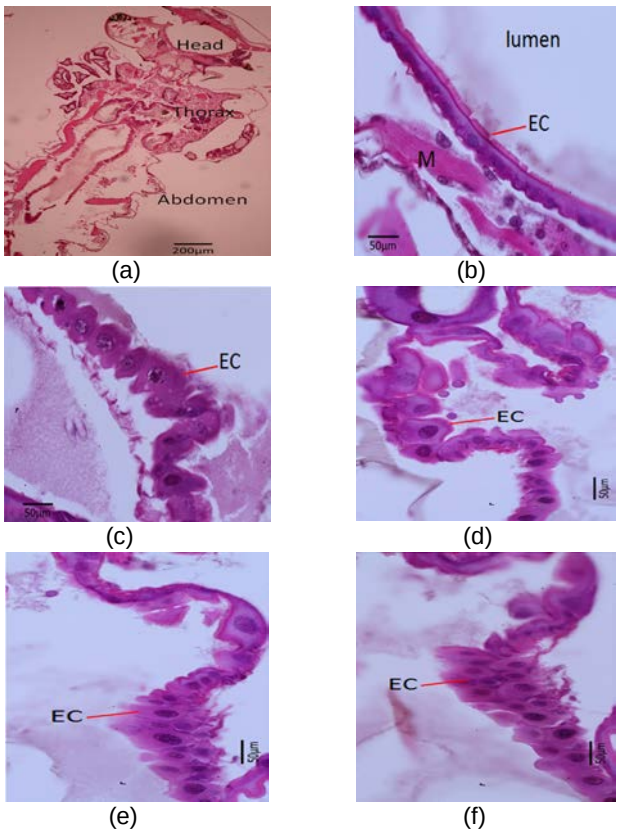


Figure 2: Histology of the *C. quinquefasciatus* larvae in (a) the control group showing the head, the thorax and the abdominal parts and (b) a greater magnification of the midgut. The treated group showing several lesions i.e., (c) vesicles in the cytoplasm of the epithelial cell, (d) blebbing cells, and (e and f) hyperplasia of the epithelial cells.

Note: EC – epithelial cell; M – muscle

DISCUSSION

The larvicidal properties of different plants have been reported in terms of lethal concentrations for 50% mortality. This study has shown the potential of this plant for use in the control of *C. quinquefasciatus* larvae. The present LC_{50} values at 24, 48, 72 and 96 h of *C. siamea* exposure against *C. quinquefasciatus* larvae were 394.29, 350.24, 319.17 and 272.42 ppm, respectively. The larval mortality was greater at the 24th h and continued to increase to the 96th h. The resulting larvicidal activity of this leaf extract was also comparable with earlier reports. Kamaraj *et al.* (2011) reported the LC_{50} and LC_{90} of a *C. siamea* leaf methanol extract against C. *quinquefasciatus* larva were 46.61 and 223.38 ppm, respectively. The methanol solvent was a more effective extraction compared with water as a solvent in the present study. The use of an alternative solvent, such as water, has increased due to environmental, health and safety awareness; moreover, the cost and economics are also a concern (Wang & Weller 2006). Nagappan (2012) reported that the LC_{50} and LC_{90} of a *Cassia didymobotrya* leaf aqueous extract against *C. quinquefasciatus* 4th instar larvae were 82.65 and 213.42 mg/l, respectively. Amerasan *et al.* (2012) reported the LC_{50} of *Cassia tora* leaf hexane, chloroform, benzene, acetone and methanol extracts against adult *C. quinquefasciatus* were 338.81, 315.73, 296.13, 279.23 and 261.03 ppm, respectively. Kumar *et al.* (2014) reported the LC_{50} of *Cassia occidentalis* leaf petroleum ether and butanol extract against C. *quinquefasciatus* 3rd instar larvae were 98.4 and 161.6 µg/ml, respectively.

A number of compounds, such as barakol (Deachapunya *et al.* 2005), flavonoid (Bhadauria & Singh 2001), anthraquinone glycosides and bianthraquinone (Koyama *et al.* 2001), alkaloid, phlobatannin and saponin (Alli Smith 2009), and cassiarin and cassibiphenol (Deguchi *et al.* 2014) have been isolated from *C. siamea.* Another plant from the same genus, *C. didymobotrya*, has been reported to exhibit larvicidal activity against *C. quinquefasciatus* in Ethiopia (Nagappan 2012), *Cassia fistula* against *Culex tritaeniorhynchus* in India (Govindarajan *et al.* 2011), *Cassia nigricans* against mosquito and white flies in West Africa (Georges *et al.* 2008). Therefore, the previous statement may indicate that the demonstrated larvicidal activity of *C. siamea* may also be due to presence of flavonoids, phenols, glycosides and tannins, which is in agreement with Kumar *et al.* (2014).

In this study, histopathological alterations were observed in the midgut including edema, swelling, and the deformation or elongation of epithelial cells. Moreover, cells protruding into the lumen and absent microvilli were also found in some areas. These observations are in agreement with previous reports (Kjanijou *et al.* 2012; Pavananundt *et al.* 2013). Moreover, Hamouda *et al.* (1996) reported the effect of *Artemisia judaica* on the midgut of *Culex pipiens* by demonstrating that vacuolated epithelial cell, swollen cells and debris cells appeared in the lumen, and finally, the epithelium lost its normal appearance. Almehmadi and Alkhalaf (2010) analysed the histological effects of *Melia azedarach* extract on *C. quinquefasciatus*, which included the separation of the epithelial cells from the basement membrane, swelling of the apical portion of the gut, protruding cells, cell detachment and vacuolated cytoplasm. Several reports suggested that the

larvicidal substances led to morphological damage in the epithelial cells of the midgut, which is likely where these substances are absorbed. Regardless of the type of substance used, the similarity of the detrimental changes in the organism indicates that these alterations are a common response to cellular toxicity.

In conclusion, the aqueous extract of *C. siamea* can be recommended in field areas and can be effectively used as a natural larvicidal product in a mosquito control program. However, further studies are needed to determine the active substances and how these substances function and the mechanism of action in the target species.

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