Original Article

Larvicidal and histopathological effects of *Piper sarmentosum* leaf extract against *Culex quinquefasciatus*

ผลของสารสกัดจากใบ *Piper sarmentosum* ในการกำจัดลูกน้ำและพยาธิวิทยาต่อ *Culex quinquefasciatus*

ขนิษฐา จิรอังกูรสกุล, วรรณีย์ จิรอังกูรสกุล

Kanitta Jiraungkoorskul, Wannee Jiraungkoorskul

คณะสาธารณสุขศาสตร์ มหาวิทยาลัยกรุงเทพธนบุรี

16/10 ถ.เลียบคลองทวีวัฒนา เขต/แขวงทวีวัฒนา กรุงเทพมหานคร 10170 ประเทศไทย

Faculty of Public Health, Bangkokthonburi University,

16/10 Leabklongtaweewatana Road, Taweewatana District, Bangkok 10170, Thailand.

Abstract The transmitted diseases by mosquitoes are still be classified as one of the serious problems in many countries. The chemical pesticides have been continuously used and affected human health and environments. The objective of this study was to find the alternative to chemical pesticide by evaluate the efficacy of the natural plants. Herb, *Piper sarmentosum* or Cha Phlu in local Thai, was extracted in different interval by using water. The total phenolic content in each interval was assayed and recorded. The results of the total phenolic content after 2, 4, 6, 12 and 24 hours extractions were 8.96, 9.11, 10.8, 12.9 and 17.2 mg/g of dried leaves, respectively. The aqueous extraction of the concentration of total phenolic content was selected for the mosquito larvicidal activity evaluations. Therefore, the 24-hour extract was diluted to the concentrations of 6.25, 12.5, 25 and 50 mg/L and tested individually with *Culex quinquefasciatus* mosquito larvae for 24-h incubation to find the lethal concentrations. The results of the 24-h LC_{50} and LC_{90} values were 32.9 and 61.6 mg/L respectively. The histopathological alterations after incubation with the 50% of the 24-h LC_{50} were observed directly in the midgut tissue. The abnormal lesions were found as swelling enterocytes, separating or lifting from the basement membrane, hyperplasia, blebbing appearance and finally the necrosis of the tissues. The takeaway of this study suggests that using Cha Phlu as mosquito larvicidal herb should be well aspectual consideration.

Keywords Cha Phlu, Histopathology, Larvae, Natural product, Pesticide

Correspondence to: รองศาสตราจารย์ ดร.วรรณีย์ จิรอังกูรสกุล
คณะสาธารณสุขศาสตร์ มหาวิทยาลัยกรุงเทพธนบุรี
E-mail: wannee.jir@bkkthon.ac.th

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บทคัดย่อ โรคติดต่อที่มียุงเป็นพาหะยังคงจัดเป็นปัญหาที่ร้ายแรงในหลายๆประเทศ สารเคมีกำจัดศัตรูพืชถูกนำมาใช้ในการกำจัดยุงอย่างต่อเนื่องซึ่งส่งผลกระทบต่อสุขภาพของมนุษย์และสิ่งแวดล้อม การวิจัยในครั้งนี้จึงมีวัตถุประสงค์ในการศึกษาประสิทธิภาพของพืชสมุนไพรในการกำจัดยุงเพื่อทดแทนการใช้สารเคมี *Piper sarmentosum* หรือ ชะพลู ในภาษาท้องถิ่นไทย นำมาสกัดโดยใช้น้ำในช่วงเวลาต่าง ๆ และวิเคราะห์หาปริมาณฟีนอลิกทั้งหมดในแต่ละช่วงเวลา ผลการทดลองพบว่าปริมาณฟีนอลิกทั้งหมดหลังจากการสกัดเป็นเวลา 2, 4, 6, 12 และ 24 ชั่วโมงมีค่าเท่ากับ 8.96, 9.11, 10.8, 12.9 และ 17.2 มิลลิกรัมต่อกรัมของใบแห้งตามลำดับ การวิเคราะห์ฤทธิ์ในการกำจัดลูกน้ำยุงจะเลือกใช้สารสกัดในช่วงเวลาที่ให้ปริมาณฟีนอลิกทั้งหมดสูงสุด ดังนั้น สารที่สกัดในช่วง 24 ชั่วโมงจึงถูกนำมาทำให้เจือจางจนมีความเข้มข้น 6.25, 12.5, 25 และ 50 มิลลิกรัมต่อลิตร และนำไปทดสอบกับลูกน้ำยุง *Culex quinquefasciatus* เป็นเวลา 24 ชั่วโมง เพื่อหาความเข้มข้นที่ทำให้เสียชีวิต ผลการทดลองค่า LC_{50} และ LC_{90} ที่ 24 ชั่วโมงมีค่าเท่ากับ 32.9 และ 61.6 มิลลิกรัมต่อลิตรตามลำดับ การเปลี่ยนแปลงทางจุลพยาธิวิทยาหลังจากที่ลูกน้ำยุงสัมผัสกับสารสกัดในระดับความเข้มข้นเท่ากับ 50% ของ LC_{50} ที่ 24 ชั่วโมง พบการเปลี่ยนแปลงในในเนื้อเยื่อลำไส้ส่วนกลาง โดยพบรอยโรคที่ผิดปกติ ได้แก่ เซลล์เยื่อบุลำไส้มีลักษณะบวม พบการแยกตัวหรือยกตัวขึ้นจากเยื่อบุฐาน มีการเพิ่มจำนวนของเซลล์เยื่อบุมากเกิดปกติ พบลักษณะตุ่มพองตัว และสุดท้ายคือพบการตายของเนื้อเยื่อ ข้อสรุปจากการศึกษานี้ชี้ให้เห็นว่าการใช้ชะพลูเป็นสมุนไพรฆ่าลูกน้ำยุงควรได้รับการพิจารณาอย่างรอบคอบ

คำสำคัญ: ชะพลู จุลพยาธิวิทยา ลูกน้ำ ผลิตภัณฑ์ธรรมชาติ สารเคมีกำจัดศัตรูพืช

INTRODUCTION

The mosquito-borne diseases are still been the global health problems including chikungunya, dengue fever, malaria, yellow fever, West Nile virus and Zika virus (Diouf and Nour, 2017; Moreno-Madrinan and Turell, 2018). The use of chemical pesticides has been concerned with health and environmental status such as the resistance, bioaccumulation, and biomagnification (Nicolopoulou-Stamati et al., 2016; Budzinski and Couderchet, 2018). The use of natural pesticides has been popular in worldwide. The efficiency of natural pesticides as an ovicidal, larvicidal, pupicidal and adulticidal substances mean that they have been used to kill or control various stages of several insects (Soonwera and Phasomkusolsil, 2017). The natural pesticides from several plants were studied against *Culex quinquefasciatus* i.e., *Copaifera reticulate* (Leguminosae) from Brazil (Silva et al., 2003); *Cassia occidentalis* (Fabaceae) from India (Kumar et al., 2014); *Cinnamomum verum* (Laurales) from Mexico (Andrade-Ochoa et al., 2018); and *Andrographis paniculata* (Acanthaceae) and *Tinospora crispa* (Menispermaceae) from Thailand (Jiraungkoorskul 2016; 2018). Therefore, the investigation of natural pesticides abilities may be the alternative way for control mosquitoes (George et al., 2014; Damalas and Koutroubas, 2018).

The traditional medicinal plant, *Piper sarmentosuma*, Piperaceae Family used in many countries in Southeast Asia especially in Thailand cuisines. This plant is known as “Cha Phlu” in Thai. The medicinal properties of *P. sarmentosuma* were reported in anti-carcinogenic (Wang et al., 2014), anti-diabetic (Luangpirom et al., 2014), antioxidant (Wang et al., 2016), antimicrobial (Femandez et al., 2012), and antiparasitic activities (Devanthran et al., 2017). It also has the properties for cardiovascular (Thent et al., 2012), hypolipidemia (Kumar et al., 2021), and hypotension treatment (Zainudin et al., 2015). The natural pesticide activities were report against *Aedes aegypti* mosquito vector (Hematpoor et al., 2016), *Brontispa longissima* (Qin et al., 2010), *Rhyzopertha dominica* and *Plodia interpunctella* (Hematpor et al., 2017), and *Sitophilus oryzae* (Vanichpakorn et al., 2017).

The phytochemical compounds in *P. sarmentosuma* are including sesamin, samentine, sarmentosine and samentanide (Tuntiwachwuttikul et al., 2016), alkaloid (Ee et al., 2009), flavonoid (Rahman et al., 2014), phenylpropanoids (Hematpor et al., 2017); and essential oils for the example caryophyllene and myristicin (Chieng

et al., 2008; Qin et al., 2010). No information about the histopathological alteration of insect tissue after the biopesticide treatment. This research was evaluated the efficiency of *Piper sarmentosuma* use as mosquito larvicidal activity by using *Culex quinquefasciatus* as experimental model. The tests had been the lethal concentration bioassay and the histopathological analyses.

MATERIALS AND METHODS

Plant extraction

Fresh *P. sarmentosuma* leaf was dried in 70 degree Celsius for 48 - 72 hours. The 5.0 g of dried leaf was grinding to powder and extracted with 100 mL H_2O. Then, the solution was shaken at 150 rpm room temperature for 2, 4, 6, 12 and 24 hours. After shaking, the solution was centrifuged at 4,000 rpm for 10 min and filtered. The filtered solution was kept as stock solution for further tests. The total phenolic content and the 24-h lethal concentration were performed.

Total phenolic content

The total phenolic contents were used the Folin–Ciocalteu reagent (Mcdonald et al., 2001). The method was done following: the test tube was added with 50 µL Cha Phlu in each extraction time, 250 µL of 10% Folin-Ciocalteus reagent, 200 µL of 0.7M sodium carbonate and 4.5 mL of distilled water. The test tubes were kept at room temperature for 2 hours in dark, and measured OD at wavelength 765 nm. The experiment was repeated three times. The total phenolic contents were reported by using the gallic acid standard curve in the serial concentrations expressed as gallic acid equivalent (GAE) using the following linear equation:

$y = -14.911x^2 + 38.479x - 0.4498$

(y = concentration and x = optical density)

Mosquito larval activity

The filtered solution with the highest total phenolic content was selected for this mosquito larval test. This solution was made to various concentrations: 6.25, 12.5, 25.0 and 50.0 mg/L. The mosquito larval activity was done by following WHO procedure (2005). The mosquito was uninfected laboratory strain and reared by the procedure previously reported (Komalamisra et al., 2005). The late third and early fourth-instar larvae of *C. quinquefasciatus* (n=20, triplicate) were selected for the five groups: control (distilled H_2O) and 6.25, 12.5, 25.0 and 50.0 mg/L of filtered solution. All experiment sample were set in room temperature. The number of larvae death was counted after 24 hours of experiment. Dead larvae showed no movement even after gentle mechanical stimulation. The number of deaths were calculated and reported for the lethal concentration (LC) parameter. The slope of the regression line with its confidence interval ($p<0.05$) was used the probit test from the Statistical Package of Social Sciences software (Finney 1971).

Histopathological alteration

The twenty larvae were exposed with the 50% of 24-h LC_{50} solution through 24 hours. The pathological alteration was analysed (Jiraungkoorskul and Jiraungkoorskul, 2015). The histology protocols were done as following (Presnell and Schreibma, 1997): the experimental specimen was fixed in the neutral buffered formalin for 72 h. Then, the specimen was dipped in the automatic tissue processor step by step: dehydration, clearing, infiltration. The specimen was then embedded in paraffin blocks using an embedding station and was cut by a rotary microtome in 4 µm thick. Finally, the section was stained with hematoxylin and eosin. The stained section was analysed for histopathological alteration using the light microscope. The histopathological report was photographed by a digital camera.

RESULTS AND DISCUSSION

Total phenolic content

The natural antioxidants in herb or medicinal plants are interested in worldwide (Xu et al., 2017). The previous researches have reported the comparative of antioxidant characteristics and total phenolic properties (Dudonne et al., 2009; Faujan et al. 2015). The amount of total phenolic

of *P. sarmentosuma* in 2, 4, 6, 12 and 24 h extractions were 8.96, 9.11, 10.8, 12.9, and 17.2 mg/g GAE, respectively. From this result, the highest total phenolic measurement was the 24-h extraction time. The extraction method and the total phenolic compounds from herb or medicinal plants were previously reported and should be concerned about extracted time and temperature. Moreover, total phenolic amount will be decreased or reduced by extended extraction times. Therefore, the desired extraction time for total phenolic amount should be evaluated (Xu et al., 2017). The present results agreed with earlier studies such as Hafizah et al. (2010) extracted 1:10 of *P. sarmentosuma* leaf dried power by aqueous, methanol and hexane at 80°C for 3 hours and reported that total phenolic content was 90.86 mg/g GAE. Lee et al. (2014) extracted *P. sarmentosuma* leaf with methanol, hexane, chloroform, acetate, butanol and water and reported that total polyphenols were 39, 18, 23, 14, 24 and 18 mg/L, respectively. Rahman et al. (2016) extracted *P. sarmentosuma* leaf with methanol and reported gallic acid was 22.5 µg/g dried weight of plant.

Mosquito larval activity

The abilities of phenolic compounds act as pesticide, insecticide or larvicidal activities were reported (Vimaladevi et al., 2012; Catelan et al., 2015), that is why, in this study, the extraction for 24 hours was evaluated against *C. quinquefasciatus.* No mortality was observed in control group. The averages of mortality rates were 7%, 25%, 53%, and 67% after treated with 6.25 mg/L, 12.5 mg/L, 25 mg/L and 50 mg/L of leaf extract, respectively. There was positive relation between concentration (x) and percent of mortality (y) in this following equation: $y = 1.3933x + 4.2083$, $R^2 = 0.9034$. The present result of 24-h LC_{50} and LC_{90} values with 95% confidence interval (lower – upper confidence limit) of *P. sarmentosuma* extract against *C. quinquefasciatus* were 33.5 (16.9 - 143.4) and 63.0 (40.6 - 438.7) mg/L, respectively. The mosquito larvae activity of *P. sarmentosuma* was reported in agreement with earlier studies. Intirach et al. (2012) reported the essential oil extracts from *P. sarmentosum* against *Anopheles cracens* with LC_{50} value of 16.0 mg/L. Hematpoor et al. (2016) determined the toxicity of 3 chemical substances of phenylpropanoids: asaricin, isoasarone and transasarone from the hexane extract from roots of *P. sarmentosum* toward *Aedes aegypti, A. albopictus* and *C. quinquefasciatus* mosquito larva. They found that these compounds were highly potent against mosquito larva causing up to complete mortality at 15 µg/mL and also showed strong inhibition on acetylcholinesterase. They suggested this plant may be the neuron toxic compounds toward mosquito. Wang et al. (2014) have reviewed that 32 compounds from *Piper* species such as amide alkaloid, phenylpropanoid, lignan, terpene, flavonoid, alkenylphenol and essential oil were studied about cytotoxic effects. The insect larval toxicities of this plant are due to the presence of phenylpropanoids (Hematpor et al., 2017) and essential oils (Chieng et al., 2008). Many researchers studied the efficiency of medicinal plants in various insects such as termite, *Coptotermes sp.* (Chieng et al., 2008), coconut leaf beetle, *Brontispa longissima* (Qin et al., 2010), rice weevil, *Sitophilus* oryzae (Vanichpakorn et al., 2017), wheat weevil, *Rhyzopertha dominica,* and meal moth, *Plodia interpunctella* (Hematpor et al., 2017).

Histopathological alteration

The enterocytes in the midgut of control group revealed a single layer of columnar epithelial cells with a prominent basophilic nucleus, brush border on apex, and acidophilic cytoplasm (Figure 1A). The histopathological alterations were found in the midgut after exposure the extract of this plant. The enterocytes were shown elongated form and found various vesicles in the cytoplasm (Figure 1B). Moreover, some enterocytes were also shown the cytoplasm masses in balloon form which pushed into the lumen, and no observation of brush borders (Figure 1C). The hyperplasia of enterocytes was also found (Figure 1D). These histopathological alterations were reported in agreement with earlier researches. The mosquito uses the brush border of enterocyte for the

absorption. Chaithong et al. (2006) reported the LC_{50} value against *A. aegypti* of ethanolic extracts from *P. sarmentosum* was 4.06 mg/L. They also found that the other organs, except anal papillae, had shown the normal histology look similar to those of control group. They reported the ultrastructural like external damage with atrophy of the cuticle of the anal papillae (Chaithong et al., 2006). The histopathology in midgut of *C. quinquefasciatus* after exposed with *Melia azedarach* were found pale cytoplasm, loss of brush border, mass protruding into the lumen and cell death (Al-Mehmadi and Al-Khalaf, 2010). The similar histopathological appearance such as pale cytoplasm, tearing of epithelial surface and loss of brush border on the midgut after treated with *Matricharia chamomella* (Al-Mehmadi, 2011). The most important things such as the active compounds, the histopathological alterations will be the main properties for natural mosquito larvicidal substances.

CONCLUSIONS

The present study reported the histopathological alteration in the midgut of *C. quinquefasciatus* larvae after treated with *P. sarmentosum*. The aqueous extract of *P. sarmentosum* can be used as a natural larvicidal substance in the mosquito or insect vector control. However, further studies are still need to determine the main active phytochemical substances and mechanism that are responsible for larvicidal activity.

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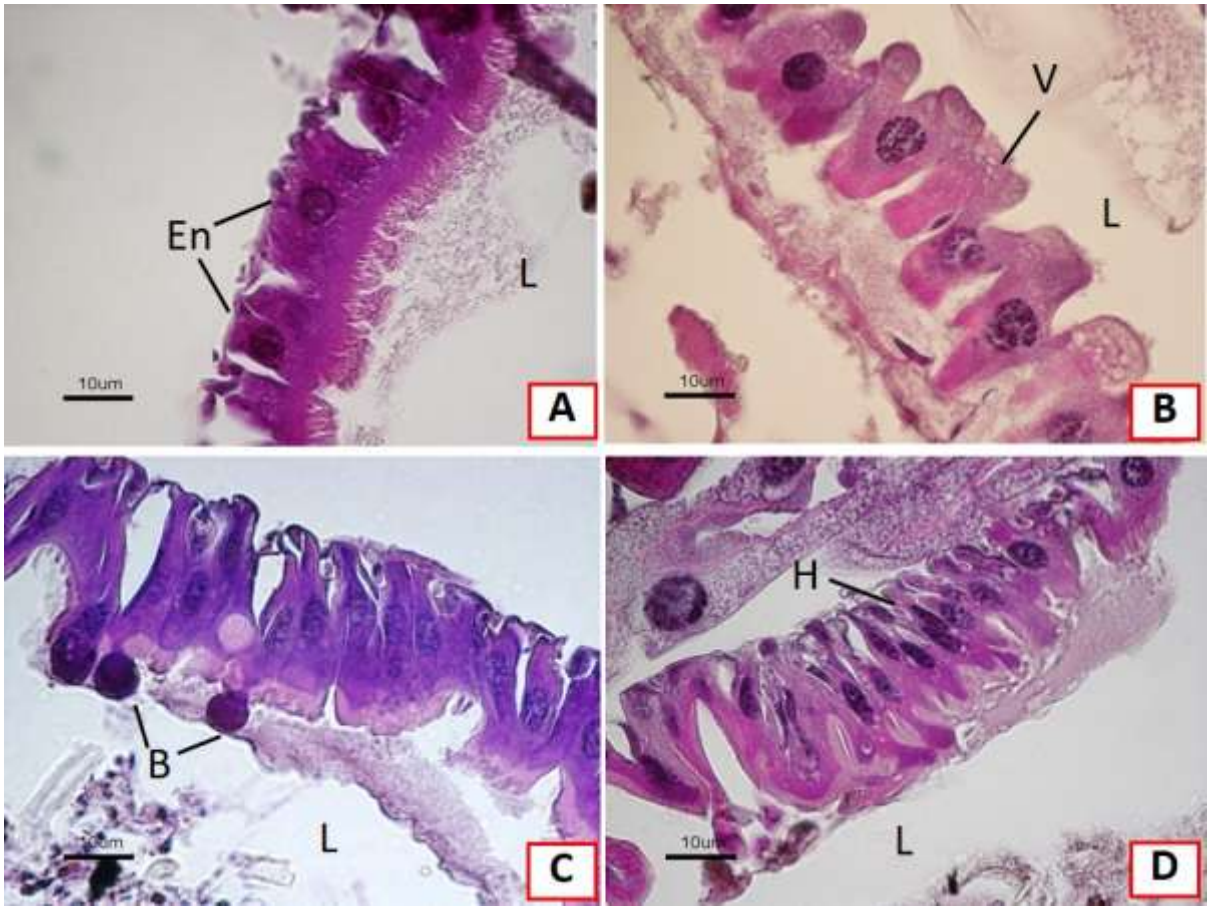


Figure 1. *Culex quinquefasciatus* larvae midgut (A): control larvae midgut with one layer of epithelial cells, the columnar epithelial cell showing the nucleus with basophilic color, brush border on surface, and cytoplasm in acidophilic color: *Piper sarmentosum* groups showing several histopathological alterations such as (B) cytoplasm with numerous pale vesicles, (C) bubble mass push in the lumen, and (D) enterocytes showing hyperplasia appearance. Note: B = bubble; En = enterocyte; H = hyperplasia; L = lumen; V = vesicle.