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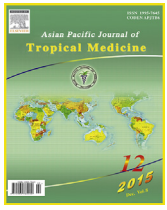

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Larvicidal activity, inhibition effect on development, histopathological alteration and morphological aberration induced by seaweed extracts in *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Objective: To investigate the larvicidal activity, inhibition effect on development, histopathological alteration and morphological aberration induced by the extracts derived from seaweeds *Bryopsis pennata* (*B. pennata*), *Sargassum binderi* (*S. binderi*) and *Padina australis* in *Aedes aegypti* (*Ae. aegypti*) larvae and to characterize the phytochemical components of the three seaweeds.

Methods: Larvicidal activity of the seaweeds towards the larvae of *Ae. aegypti* was determined according to WHO. The inhibition effect of seaweeds was assessed by determining the mortality, adult emergence rate, larval and pupa duration of the treated larvae. Histopathological effect on midgut epithelium of larvae and morphological aberration induced by the methanol extracts were examined. Phytochemical analysis was done to determine the presence of alkaloids, saponins, steroids and terpenoids in the seaweeds.

Results: Chloroform partition of *B. pennata* extract exhibited the strongest larvicidal activity (LC_{50} = 82.55 μg/mL), followed by methanol extract of *B. pennata* (LC_{50} = 160.07 μg/mL) and chloroform partition of *S. binderi* extract (LC_{50} = 192.43 μg/mL). The methanol extract of *S. binderi* exhibited the strongest effect on prolongation of larval period (1.5-fold longer as compared to control) and resulted in strongest inhibition effect in adult emergence (98.67%). The histopathological study showed that larvae treated with seaweed extracts had cytopathological alteration of the midgut epithelium. The morphological observation revealed that the anal papillae and terminal spiracles of larvae were the common sites of aberrations.

Conclusions: The study provided information on various effects of seaweed extracts on *Ae. aegypti*. Further investigation on identifying the active compounds and their mechanisms of action is recommended.

1. Introduction

Aedes aegypti (L.) (Diptera: Culicidae) (*Ae. aegypti*) is a mosquito vector for several important viral diseases of human and animals [1]. Of all the viral diseases carried by *Ae. aegypti*, dengue fever has been reported to increase dramatically around the world. World Health Organization currently estimates over 40% of the world's population are at risk of dengue. Dengue fever cases reported across the Americas, South-east Asia and Western Pacific exceeded 2.3 million in 2010 and continued to increase [2]. However, vaccines and drugs for dengue treatment are not available till date. Disease preventive operation still solely depends on the anti-vector measures. Elimination of mosquito by killing mosquito larvae using larvicide is effective [3]. Larvicides that are commonly used in the mosquito control programme are

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chemical synthetic insecticides, namely organophosphates, organochlorines and carbamates. The extensive and widespread use of synthetic insecticides has caused some concerns on the safety and toxicological impacts towards the environment, human and other organisms. The repetitive application of chemical insecticide results in the development of resistance in mosquitoes globally. Therefore, the search for new insect control agents from natural products which are target specific, biodegradable and of low environmental toxicity is crucial [4].

Seaweeds have been reported to possess primary and secondary metabolites with a wide range of novel biological activities [5]. One of the bioactivity that possessed by seaweed secondary metabolites is the mosquitocidal properties. Many reports have described the pronounced mosquitocidal properties of seaweed. Recently, Yu *et al*. [6] described the mosquito larvicidal activity of 42 extracts and 13 compounds of seaweeds. For instance, halogenated sesquiterpene, elatol isolated from red seaweed *Laurencia dendroidea* has been reported to exhibit active insecticidal activity against *Ae. aegypti* larvae (LC_{50} = 10.7 ppm) [7]. Besides the killing effect, the insecticidal compounds and extracts of seaweed are proven to influence the metabolism of insect in a wide range of diverse ways, such as through toxicity, mortality, growth and development, feeding behavior, utilization of food, oviposition and reproduction system [8,9]. This is evidence in the study of Elbanna and Hegazi [8], when they observed a longer larval duration for mosquito *Culex pipiens* compared to the control larvae, after the treatment of dried ground seaweeds namely *Caulerpa prolifera, Caulerpa serrulata, Jania rubens*, *Nitophyllum punctatum, Cystoseira myrica* and *Padina pavonica* (*P. pavonica*).

Bryopsis pennata (*B. pennata*) is green seaweed with glossy dark green filamentous thallus and feather-like fronds found in tropical to temperate marine waters [10]. *B. pennata* exhibits antibacterial, antifungal [11] and antimicrobial activities [12]. Besides, *B. pennata* has been reported to exhibit cardiac effect by inducing inotropic effect towards ventricular muscle strips of toad and positive chronotropic action towards isolated right atria of rat by Freitas *et al*. [13]. *Padina australis* is brown seaweed with thallus in leaf-like clusters and fan-shaped blades having chalky white alternating with light brown bands [14]. *P. australis* exhibits antibacterial activity against gram-positive and gram-negative bacteria [15], antioxidant activity [16] and *in vitro* cytotoxic effect [17]. *Sargassum binderi* (*S. binderi*) is a bushy brown seaweed that has a differentiated thallus consists of basal holdfast and main axis with blades. *S. binderi* has been studied for its cytotoxic activity on brine shrimp *Artemia salina* [18]. In addition, *Sargassum* spp. have been used in Traditional Chinese Medicine for nearly 2000 years to treat diseases such as goiter, arteriosclerosis, skin diseases, high blood pressure, chronic bronchitis, sore throat, *etc* [19].

In view of the biopotential of seaweed, the present study aimed to determine the larvicidal activity and inhibition effect on development of *B. pennata*, *S. binderi* and *P. australis* against *Ae. aegypti*. Besides, histopathological alteration and morphological aberration induced by the extracts of the three seaweeds in *Ae. aegypti* larvae were evaluated.

2. Materials and methods

2.1. Seaweed material

Fresh seaweeds were collected from Teluk Kemang (Latitude 2° 26.29′ N and Longitude 101° 51.42′ E), Port Dickson, Malaysia. All samples were transported with ice back to the laboratory, washed and air-dried at (26 ± 1)°C. The samples were identified by using the standard taxonomic keys. All voucher specimens were deposited at the Herbarium of Universiti Kebangsaan Malaysia.

2.2. Preparation of extract

Dried samples were ground, sieved and macerated with methanol (60 g/L) for 72 h and stirred with the aid of a magnetic stirrer. The samples were extracted until exhausted. Then, the samples were filtered and concentrated by rotary evaporator at 50 °C to dryness [20]. The crude methanol extracts were liquid–liquid partitioned into hexane, chloroform and aqueous partitions [21]. The partitions were concentrated by rotary evaporator to dryness and kept in vials at 4 °C.

2.3. Larvicidal assay

Laboratory strain of *Ae. aegypti* was obtained from the insectary of the Institute for Medical Research (IMR), Malaysia. The guidelines of Entomology Unit, Infectious Disease Research Centre, IMR for maintain and use of mosquitoes have been followed. All procedures performed in mosquito bioassays were in accordance with the ethical standards of IMR. The larvicidal assay was conducted according to World Health Organization [22]. Batches of 25 of fourth instar larvae were introduced to 200 mL paper cups filled with various concentrations of seaweed extract diluted from stock solution (in methanol and distilled water). Malathion and 0.25% v/v of methanol were used as positive and negative controls, respectively. The experiment was repeated five times with triplicates. The larval mortality was recorded after 24 h.

2.4. Morphological observation

Morphological changes of the treated larvae were studied and recorded and further compared to the control larvae after treatment of the methanol extracts at LC_{50} for 24 h. For scanning electron microscope study, the larvae were washed with distilled water and treated with glutaraldehyde and osmium tetroxide prior to dehydration in graded ethanol and acetone series. Then, the samples were dried by using the critical point dryer, subsequently spurted with 45 nm gold, attached to the stubs and viewed under scanning electron microscope (JSM-7001F, JEOL, Tokyo, Japan) [23].

2.5. Histopathological observation

Histopathological changes of the midgut epithelial cells of treated larvae were observed. Larvae that were alive after 24 h of extract treatment at LC_{50} were collected for examination. The larvae were rinsed with distilled water before fixation with bouins solution, followed by dehydration in graded ethanol and toluene series. Then, the larvae were embedded in paraffin, sectioned and stained with Haematoxylin and Eosin before the examination using compound microscope [24].

2.6. Sublethal effect on the growth and development

The surviving larvae from the larvicidal assay treated with methanol extract at concentration of LC_{50} were transferred to distilled water and fed with partially cooked liver. The larvae were monitored daily to determine the mortality, period of pupation and adult emergence rate of treated larvae. Harley mean index was used for comparing the effect of different seaweed extracts towards the growth and survival rates of the treated larvae [25]. The index is calculated as follows. The experiment was repeated five times with triplicates.

$$\text{Harley mean index} = \frac{(\text{Percentage of individuals pupating} + \text{Percentage of individuals reaching adulthood})}{\text{Median day of pupation}}$$

2.7. Phytochemical analysis

Phytochemical screening was performed for the qualitative determination of phytochemical constituents of seaweeds. The presence of alkaloids was determined by formation of precipitation using Mayer's reagent [26]. Froth test was conducted to determine the presence of saponins. Liebermann–Burchard test using acetic anhydride and sulfuric acid was conducted to determine the presence of triterpenes or steroids [27]. Total phenolic content was estimated by using Folin–Ciocalteu method. Phloroglucinol was used as standard for the calibration curve. The results were expressed as mg phloroglucinol equivalent per gram (mg PGE/g) [28].

2.8. Data analysis

The LC_{50} values were calculated using BioStat 2009 (AnalystSoftInc., Alexandria, VA). The effects of different treatments were compared through one way analysis of variance (ANOVA), using Statistically Package for Social Sciences (SPSS version 15, Chicago, IL). $P < 0.05$ is considered statistically significant.

3. Results

3.1. Larvicidal assay

The larvicidal effect of methanol extract and its hexane, chloroform and aqueous partitions of the three seaweeds against *Ae. aegypti* is shown in Table 1. Among all the solutions tested, chloroform partition of *B. pennata* extract exhibited the strongest larvicidal activity (LC_{50} = 82.55 μg/mL), followed by methanol extract of *B. pennata* (LC_{50} = 160.07 μg/mL) and chloroform partition of *S. binderi* extract (LC_{50} = 192.43 μg/mL).

3.2. Morphological observation

Morphological observation of the larvae treated with methanol extract of *B. pennata*, *P. australis* and *S. binderi* revealed a similar manifestation trend of toxicity. Seaweed extract treated mosquito larvae exhibited morphological aberrations, such as damaged anal papillae, distorted body, darken body and pale body. The commonly observed aberrations of the treated larvae were darkening and shrunken cuticle of anal papillae (Figure 1) and destructive structure at the stigmal plate on the siphon apex (Figure 2).

3.3. Histopathological observation

For the histopathological study, the effects of seaweed extracts towards the anterior and posterior midgut epithelial cells

Table 1
Mosquito larvicidal activity of extracts and partition of seaweed towards *Ae. aegypti*.

	Species (voucher no.)	Extract/Partition	LC_{50} value (μg/mL) (95% CL)	Slope (±SE)	X^2
Brown seaweed	*Padina australis* (TKPD/P5)	Methanol	400.46 (378.99–480.52)	8.18 ± 0.70	1.00
		Hexane	1029.47 (889.09–1365.65)	4.47 ± 0.81	0.43
		Chloroform	340.90 (288.52–413.64)	2.68 ± 0.28	3.29
		Aqueous	652.79 (570.01–799.18)	3.46 ± 0.47	0.34
	Sargassum binderi (TKPD/P1)	Methanol	217.04 (176.45–367.85)	3.37 ± 0.33	0.01
		Hexane	718.65 (580.56–1003.70)	2.14 ± 0.30	0.32
		Chloroform	192.43 (184.78–254.13)	3.27 ± 0.28	0.52
		Aqueous	543.23 (443.27–623.13)	2.04 ± 0.63	0.44
Green seaweed	*Bryopsis pennata* (TKPD/C1)	Methanol	160.07 (153.34–190.62)	3.74 ± 0.65	3.57
		Hexane	728.61 (681.04–958.18)	4.23 ± 0.55	2.52
		Chloroform	82.55 (74.10–92.58)	3.39 ± 0.54	2.40
		Aqueous	417.75 (382.19–526.81)	2.82 ± 0.34	1.21

All tests were run in triplicates for five times.

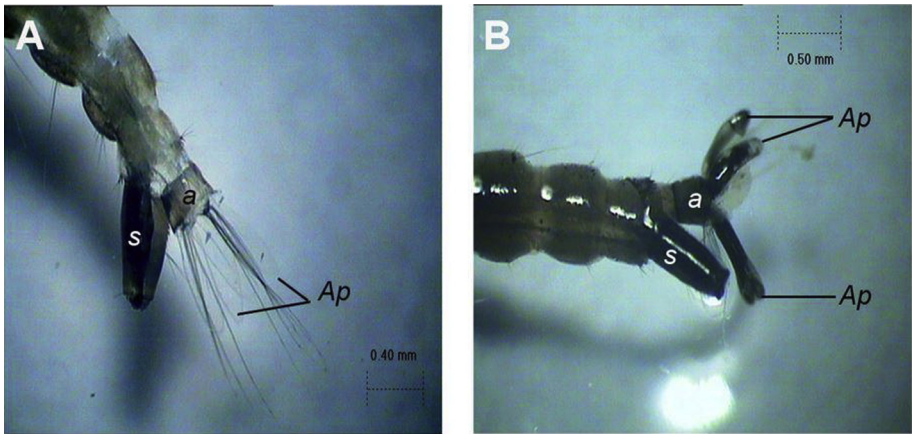

Figure 1. Terminal segment of the *Ae. aegypti* larvae under stereomicroscope (10×).
(A) The control larva with intact anal papillae. (B) The larva treated with methanol extract of *S. binderi* shows darkened and deformed anal papillae. A: anal segment; S: siphon; Ap: anal papillae.

were examined. All treated larvae exhibited similar destruction in the midgut epithelial cells (Figure 3). The anterior midgut epithelium of control larvae exhibited flattened regular cells with pale clear cytoplasm and regular microvilli lining the apical surface, was closely attached to the basal lamina (Figure 3A). In contrast, anterior midgut epithelial cells treated by seaweed extract showed cytopathological alterations, such as the existence of vesicles in various sizes, destruction of microvilli and swollen cells (Figure 3B). On the other hand, the posterior midgut epithelial of control larvae was lined by large, irregular cells with large globular nuclei (Figure 3C). Seaweed extract-treated larvae showed detachment of the posterior midgut cells from the basal lamina, and formation of globular protrusion towards the lumen causes releasing of cellular content (Figure 3D).

3.4. Sublethal effect on the growth and development

The sublethal effects of methanol extracts of *B. pennata*, *P. australis* and *S. binderi* towards *Ae. aegypti* larvae were monitored until the death or emergence of all larvae (Table 2). Larvae treated with methanol extract of *S. binderi* showed the highest inhibition rate of adult emergence [(98.67 ± 0.21)%], longest larval period [(14.50 ± 0.71) d], longest median day of pupation (13 d) and lowest Harley Mean Index (0.58 ± 0.13) among all the treated larvae.

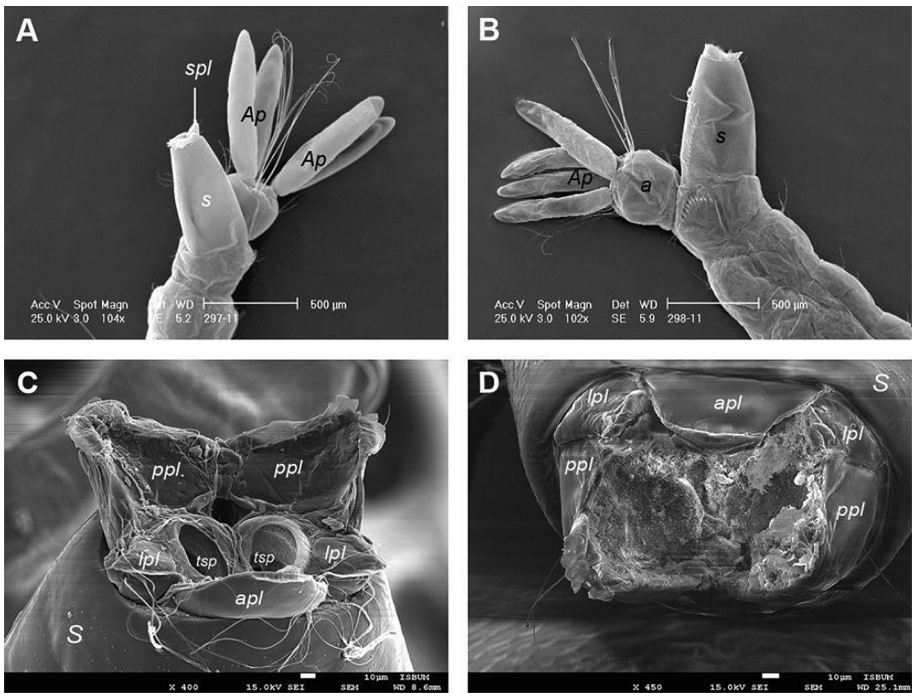

Figure 2. Terminal segment of the *Ae. aegypti* larvae under scanning electron microscope (100× and 400×).
(A) The control larva with smooth cuticle and intact anal papillae. (B) Larva treated with methanol extract of *S. binderi* shows anal papillae with slightly shrunken and billow shaped cuticle. (C) The control larva shows intact opening of terminal spiracles. (D) Larva treated with methanol extract of *S. binderi* shows terminal spiracles with damaged perispiracular lobes. A: anal segment; S: siphon; Ap: anal papillae; spl: perispiracular lobe; ppl: posterior perispiracular lobe; lpl: lateral perispiracular lobe; apl: anterior perispiracular lobe; tsp: terminal spiracle.

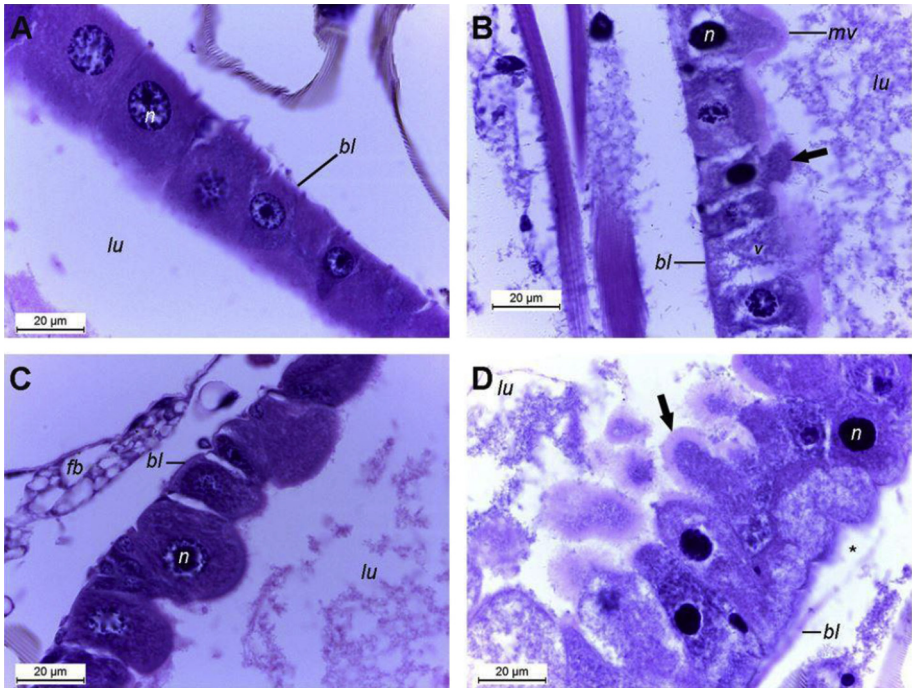

Figure 3. Longitudinal section of midgut of *Ae. aegypti* larvae (40×).
(A) The anterior midgut epithelial cells of the control larva. (B) The larva under treatment of *S. binderi* extract shows anterior midgut epithelium with cell vacuolization and apical protrusion (arrow). (C) The posterior midgut cells of the control larva. (D) The larva treated with *S. binderi* extract has posterior midgut cells that release cellular content through apical protrusion into the lumen (arrow) and cells detached from the basal lamina (*). bl: basal lamina; lu: midgut lumen; n: nucleus; fb: fat body; mv: microvilli; v:vacuole.

Table 2
Effect of methanol extracts of seaweed (at concentration of LC_{50}) on the growth and development of fourth instar of *Ae. aegypti* mosquito larvae.

Treatment	Mortality (%)				Inhibition of adult emergence (%)	Larval period (day)	Median day of pupation	Mean index
	24 h	48 h	72 h	96 h				
B. pennata	49.33 ± 2.31[a]	22.67 ± 2.31[a]	10.55 ± 2.31[a]	8.00 ± 1.81[a]	90.67 ± 1.23[a]	10.50 ± 0.35[a]	11.5	1.65 ± 0.50[a]
P. australis	53.33 ± 6.11[a]	21.40 ± 4.93[a]	11.67 ± 2.93[a]	6.93 ± 1.93[a]	92.53 ± 1.43[a]	10.00 ± 0.82[a]	11	1.27 ± 0.35[a]
S. binderi	50.67 ± 10.07[a]	28.50 ± 5.66[b]	12.10 ± 4.76[a]	6.00 ± 2.63[a]	98.67 ± 0.21[b]	14.50 ± 0.71[b]	13	0.58 ± 0.13[a]
Control	0.00 ± 0.00[b]	1.00 ± 3.71[c]	1.50 ± 3.27[b]	2.56 ± 0.85[b]	5.14 ± 4.89[c]	9.77 ± 0.87[a]	10	19.77 ± 0.58[b]
Malathion	50.33 ± 5.31[a]	49.65 ± 2.60[d]	–	–	–	–	–	–

Data represent means ± SEM of 5 independent experiments performed in triplicates. Means followed by different letters within the same column are significantly difference at $P < 0.05$.

3.5. Phytochemical analysis

Phytochemical screening revealed the presence of alkaloids, saponins, steroids and terpenoids in all the seaweeds studied. Green seaweed *B. pennata* exhibited a significantly higher total polyphenol content [(20.37 ± 0.20) mg PGE/g extract] ($P < 0.05$) as compared to brown seaweeds, namely *P. australis* [(8.75 ± 0.54) mg PGE/g extract] and *S. binderi* [(10.90 ± 0.33) mg PGE/g extract].

4. Discussion

This is the first report of the mosquito larvicidal activity of *B. pennata*, *S. binderi* and *P. australis*. In our report, the larvicidal activity of chloroform partition of green seaweed *B. pennata* (LC_{50} = 82.55 μg/mL) is considered effective (LC_{50} less than 100 mg/L) according to the classification of Thangam and Kathiresan [29], whilst the other 2 brown seaweeds tested—*S. binderi* and *P. australis* are considered as ineffective larvicide (LC_{50} more than 200 μg/mL). However, other members of the *Sargassum* and *Padina* genus have been reported for their larvicidal activity towards mosquito and the activity reported is more effective compared to the activity in our report. For instance, *Sargassum wightii* has been reported to exhibit larvicidal action against the mosquito larvae of *Ae. aegpyti* and *Culex quinquefasciatus* [30]. Furthermore, ethyl acetate fraction of *Sargassum swartzii* has shown active larvicidal effect against mosquito larvae of *Anopheles stephensi* [31] and *Anopheles sundaicus* [32]. On the other hand, *Padina tetrastromatica* has also exhibited toxic effect against mosquito larvae of *Ae. aegypti* and *C. quinquefasciatus* [30]. Wide difference in bioactivity between the individual species within the same genus can be due to geographical and ecological factors which affect the production of carbon-based bioactive secondary metabolites. Different compositions of chemical constituents in the seaweeds also may result in various degrees of bioactivity.

Besides causing death to mosquito larvae, the effect of intoxication also manifests through the aberration of structures. It is evident as *Ae. aegypti* larvae treated with extracts of natural products such as red seaweed *Laurencia dendroidea* and dried fruits of peppercorns have been reported to have darkening and

shrunken cuticle of anal papillae after the treatment [7,33]. Similarly, the mosquito larvae treated with seaweed extract in our report were observed to exhibit the same manner of aberrations on the anal papillae. The deleterious effect on anal papillae interrupts the ion regulation of larvae and further causes the imbalance of homeostasis. Furthermore, the rupture of larval stigmal plate observed in the present report is suggested to cause destruction to the hydrophobe surface of stigma plate, causing water/medium to enter the tracheal trunk which harms the respiration system of the larvae. Similar action has been described in one of the study whereby kerosene entered the tracheal trunks from terminal spiracles and caused the finest capillaries to disappear from the tracheal system [34]. Interruption of the osmo-regulatory system (damaged anal papillae) and the spiracles of respiratory system (disrupted stigma plate) are suggested to contribute to the death of larvae.

The midgut of insect plays an important role in the secretion of digestive enzymes and absorption of nutrients [1]. Allelochemicals are proven to exert detrimental effect on the digestive epithelial cells and further decrease the survivability of the insect. For instance, mosquito larvae treated with plant extracts, namely *Melia azedarach* and *Derris urucu* have been reported to experience extensive damage on the midgut epithelium and peritrophic matrix [24,35]. These observations are in agreement with the findings of present study whereby the destruction and detachment of cells were observed from the midgut epithelium of the seaweed-treated larvae. The severe damage of midgut cells is suggested to disrupt function of midgut, leading to the death of larvae.

Sublethal effects of the seaweed are proven to alter various stages of mosquito life cycle. In fact, delayed larval metamorphosis of the treated larvae is probably caused by the hormonal imbalance of post treatment. For instance, polyphenolic compounds of brown seaweed *P. pavonica* are suggested to prolong the life cycle intervals and pupation duration of mosquito larva *C. pipiens* [8]. Reduction of total body protein and DNA content has also been observed in the red cotton bug *Dysdercus cingulatus* larvae treated with *P. pavonica* extract [9]. This is in line with the observation of the present study where methanol extract of *B. pennata*, *P. australis* and *S. binderi* exhibited their sublethal effects towards mosquito larvae *Ae. aegypti*, resulting in prolongation of larval duration and reduction of adult emergence.

Chemical composition of the seaweed plays important role in bioactivity. Previous studies have shown that brown seaweed *P. australis* contains fucoxanthin, aurantiamide acetate, mannitol D, palmitic acid and fatty acids [36]. Bioactive compounds derived from the *Sargassum* species such as meroterpenoids, phlorotanins, fucoidans, sterols and glycolipids have been reported to have a wide range of pharmacological properties [5]. *Bryopsis* species has been investigated for its pigment, sterol and fatty acid composition [37]. The phytochemical data obtained in the present study is in line with the previously reported data, where alkaloids, saponins, steroids and terpenoids are present in the seaweeds *B. pennata*, *P. australis* and *S. binderi*. This information serves as fundamental data for further investigation of the active constituents that are responsible for toxicity action in the assay. Comparative study of larvicidal extracts and compounds should be carried out to elucidate the possible mode of action in the future studies.

The data obtained in this study provided information on the toxicity of 3 Malaysian seaweeds towards mosquito larvae *Ae. aegypti*. Chloroform fraction of green seaweed *B. pennata* showed the strongest larvicidal activity among all the samples tested, while methanol extract of brown seaweed *S. binderi* showed the strongest inhibition effect towards the development of larvae. Based on the results, it is evident that seaweed which is abundantly found in tropical country possesses potential as a mosquito larvicide. Further investigation aiming at ascertaining the active compounds and/or synergists, which are responsible for the larvicidal action, can be carried out to determine the use of seaweeds extracts and compounds in the implementation of efficient mosquito control strategies.

Conflict of interest statement

We declare that we have no conflict of interest.

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