ORIGINAL ARTICLE

Mosquito Larvicidal Potential of Tropical Seaweeds: Acetylcholinesterase Inhibitory Effects of *Bryopsis pennata, Padina australis* and *Sargassum binderi* on *Aedes aegypti* (L.) and *Aedes albopictus* Skuse

Ke-Xin Yu[1], Rohani Ahmad[2], Ching-Lee Wong[3], Ibrahim Jantan[3]

[1] Department of Diagnostic and Allied Health Sciences, Faculty of Health and Life Sciences, Management and Science University, 40100 Shah Alam, Malaysia
[2] Medical Entomology Unit, Infectious Disease Research Centre, Institute for Medical Research, 50588 Kuala Lumpur, Malaysia
[3] School of Biosciences, Taylor's University, Taylor's Lakeside Campus, 47500 Subang Jaya, Selangor, Malaysia

ABSTRACT

Introduction: Inhibition of the cholinesterase's function leads to paralysis and death. This mechanism is served as a common mode of action of insecticide. The three tropical seaweeds, namely B*ryopsis pennata, Padina australis* and *Sargassum binderi* were reported for its potential mosquito larvicidal effect. In the present study, these seaweeds were evaluated for their potential as a cholinesterase inhibitor in the mechanism of larvicidal action. **Methods:** Acetylcholinsterase (AChE) inhibition assay was carried out based on the colorimetric method using a microplate reader. Phytochemical content of the seaweed extracts was screened by using liquid chromatography-mass spectroscopy (LC-MS). **Results:** Green seaweed *B. pennata* showed the strongest inhibition effect towards *in vitro* AChE by using tissue homogenates of *Aedes aegypti* (IC_{50} value = 0.84 mg mL^{-1}) and *Aedes albopictus* as the enzyme source (IC_{50} value = 0.92 mg mL^{-1}). The pattern of Lineweaver-Burk plots revealed that *B. pennata* was a mixed type inhibitor of AChE, as the readings of *Km, Vmax, Ki* and *Ki'*, indicates that it had a strong inhibition ability with high binding affinity towards both free enzyme and enzyme-substrate complex. **Conclusion:** These findings suggest the compound(s) in *B. pennata* extract serves as a promising source that could be developed into a mosquito larvicidal agent with AChE inhibition effect.

Keywords: Enzyme action, Cholinesterase inhibitor, Mosquitocidal, Green seaweed

Corresponding Author:
Ke-Xin Yu, PhD
Email: kxyu@msu.edu.my, yukxxin@gmail.com
Tel: +603-5521 6478

INTRODUCTION

Cholinesterase is a family of enzymes that catalyzes the hydrolysis of the acetylcholine into choline and acetic acid. Inhibition of cholinesterase leads to overstimulation of cholinergic neuron, hyper-excitableness, paralysis and eventual death. The action of inhibiting nervous system's cholinesterase is a common mode of action of the synthetic insecticides, namely carbamates and organophosphates (1). Introduction of these synthetic chemicals received wide acceptance due to their effectiveness and availability. However, misuse of the insecticides elicited insecticide resistance in mosquito populations and posed detrimental effects on the environment and other organisms (2). Since 2009, increasing number of cases of resistance has been reported and the situation has been identified in 64 countries, mainly due to synthetic insecticides such as pyrethroids, followed by carbamates, dichlorodiphenyltrichloroethane (DDT) and organophosphates. This evolution of insecticide resistance could jeopardize the effectiveness of insecticides in the current and future vector control programmes, as vector control is a crucial facet of mosquito-borne disease control today and is expected to continue to be so, since vaccination and specific drug for most of the viral diseases are not available (1). Discovering novel insecticide with multiple modes of action to encounter the risk of developing insecticide resistance against single class of the insecticide, is of the current interests of research community.

The long history of seaweed-based products in food and pharmaceutical industries supports insecticide research on discovering new active agents in seaweeds. On top of that, many reports have revealed that the seaweeds have profound insecticidal properties on the medical importance mosquitoes, especially on the dengue vector, *Aedes aegypti* (3, 4).

Bryopsis pennata (class Ulvophyceae) is a green seaweed (up to 10.0 cm) with distichous branches with small and erect thallus (5). *B. pennata* exhibits active ovicidal potential, effective lethal effect on mosquito larvae and repellent effect on female oviposition of *Ae. aegypti* and *Ae. albopictus* (6).

Padina australis (class Phaeophyceae) is a brown seaweed (9.6-11.0 cm) composed of fan-shaped blades with chalky white and light brown alternating bands and calcified upper surface (7). Members of the Padina genus exhibit lethal effect on various insects (8, 9) and effect on prolongation of larval duration (10).

Sargassum binderi (class Phaeophyceae) is a brown seaweed (8.0-50.0 cm) having main axis, flattened branches, lanceolate blades and air bladders (11). *S. binderi* induces prolongation of larval life cycle and exhibits deleterious effect on mosquito larvae's midgut epithelium and terminal spiracles (12).

The availability of *B. pennata, P. australis* and *S. binderi* in tropical and warm-temperate regions, as well as in dengue endemic areas, together with their reported mosquito larvicidal properties paves the way for the first preliminary study in using the seaweed extracts, as a cholinesterase inhibitor in the potential mosquitocidal agent against dengue vector. On top of that, the characterization of the insecticidal seaweeds by using liquid chromatography-mass spectroscopy (LC-MS) analysis would be informative to further investigate their potential as an effective mosquitocidal agent for future development of product.

MATERIALS AND METHODS

Extract Preparation

Fresh seaweeds were collected from West Malaysia, namely Teluk Kemang in Negeri Sembilan (2° 26.29′ N 101° 51.42′ E) and Cape Rachado (2° 24.95′ N 101° 51.21′ E) in Melaka, from 2010 to 2012. These seaweeds were brought back to the lab in an ice box, cleaned and air-dried. Then, the dried seaweeds were ground, sieved and macerated for 3 days with methanol (60 g L^{-1}) (Merck, Germany). The methanol extract was concentrated by using a rotary evaporator (Buchi, Switzerland) and the concentrated extract was left to air-dry in the fume hood (13). The fresh seaweed samples were authenticated by Dr Ahmad Ismail, Universiti Kebangsaan Malaysia (UKM) and herbarium specimens were deposited in the herbarium, Faculty of Science and Technology, UKM (Voucher number: *B. pennata* – CRM – C1, *P. australis* – TKNS – P5 and *S. binderi* CRM – P1).

Maintaining Mosquito Colony

The mosquito colony was maintained at the insectary of Entomology Unit, Infectious Disease Research Centre, Institute for Medical Research (IMR) following the guidelines under Section 14, Destruction of Disease-Bearing Insects Act 1975. Laboratory strains of *Ae. aegypti* and *Ae. albopictus* were maintained in separate rooms at temperature of 26 ± 2 °C and relative humidity of 80 ± 2 %.

Acetylcholinesterase inhibition assay

Acetylcholinesterase (AChE) assay was carried out (14). Stock solution of seaweed extract (40 mg mL^{-1}) was prepared by dissolving methanol extract to 0.02% methanol solution (v/v) (15). The stock solution was diluted with distilled water to make different concentrations of solution. The tissue homogenate of third instar mosquito larvae from the colony without any treatment was used as source of enzyme. An aliquot of 50 µL of tissue homogenate was incubated with 20 µL of seaweed extract solution (0.25 to 4 mg mL^{-1}) for 5 min, before adding in 50 µL of 0.2 mM acetylcholine iodide solution (as substrate) and 50 µL of 0.3 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, USA) (as indicator solution). Incubation was done at 24 ± 1 °C for 20 min. After that, the absorbance value of reaction was recorded by microplate photometer at 412 nm (Multiskan* FC) (Thermo Scientific, USA). The results were calculated based on Beer-Lambert Law (absorption coefficient of 5-thio-2-nitrobenzoic acid: 1.36 x 10^4 M^{-1} cm^{-1}) and expressed in mole of acetylthiocholine hydrolysed min^{-1} mg^{-1} of protein. Propoxur (AChE inhibitor) (PESTANAL®, USA) was used as standard reference. The experiment was repeated three times with samples tested in triplicates.

Beer-Lambert Law: $A = (\varepsilon\ b)\ C$

A: Absorbance value,
ε: Extinction coefficient,
b: Path length,
C: Concentration (M).

Protein Assay

Protein content of each tissue homogenate sample used in the enzyme assay was determined (16). This procedure was used as a standard correction factor for the analysis of enzyme activity, in order to account for the variation of size among the individual larva. The assay was done by using protein quantification Kit-rapid (Fluka Analytical, USA). The absorbance value of reaction was measured at 595 nm by using a microplate photometer (Multiskan* FC) (Thermo Scientific, USA). The protein content was determined by using the standard curve of bovine serum albumin (BSA) (concentrations ranging from 1.95 to 250 µg mL^{-1}) and expressed in mg of protein mL^{-1}. Each sample was tested in triplicates and the experiment was repeated three times.

Enzyme Kinetic Studies of Acetylcholinesterases

Enzyme kinetic studies of acetylcholinesterases were carried out with a range of different concentrations of substrate solutions and inhibitors (seaweed extracts and standard drug) (15). The supernatant of tissue

homogenate (50 µL) was incubated with 20 µL of seaweed extract solution (0 to 1.75 mg mL^{-1}) for 5 min, before adding in 50 µL of acetylcholine iodide solution (0.025 to 0.200 mM, as substrate) and 0.3 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, USA) (as indicator solution). The mixture was incubated for 20 min and hydrolyzation of acetylthiocholine was monitored every 10 s by using microplate photometer at 412 nm (Multiskan* FC) (Thermo Scientific, USA). Each sample was tested in triplicates and the experiment was repeated three times.

Enzyme kinetics were analysed by using GraphPad Prism 5 (Graph Software INC., USA): K_m and V_{max} values were determined by using Michaelis-Menten and Lineweaver-Burk plots; K_i (binding constant of free enzyme) and Alpha values were determined by using the nonlinear regression enzyme kinetics (e.g. mixed model, competitive, uncompetitive and noncompetitive inhibitions); K_i' values were obtained from the plots of $V_{max\text{-}1}$ versus concentration of inhibitor (binding constant of enzyme-substrate complex).

Liquid Chromatography-Mass Spectroscopy Profiling

Liquid chromatography-mass spectroscopy (LC-MS) profile of the seaweeds was obtained using Agilent 6530 Accurate-Mass Q-TOF LC-MS system equipped with Zorbax Eclipse XDB-C18 column (2.1 x 50 mm, 1.8 micron) (Agilent Technologies Canada Inc., Canada). The LC-MS sample was prepared in methanol (Thermo Fisher Scientific, USA) with a concentration of 20 µg mL^{-1} and filtered through a 0.45 µm nylon filter. The filtered sample was eluted with acetonitrile (Thermo Scientific, USA) and water (Thermo Scientific, USA) using gradient system at a flow rate of 0.25 mL min^{-1} in the positive ion mode. The flow rate of the drying gas was set at 8 L min^{-1} at the temperature of 350 °C; while the nebulizer pressure was set at 35 PSIG with the capillary and injection volume set at 5 µL (6).

The gradient elution of methanol extract of *B. pennata* consisted of an initial hold at 99.5 % of water and 0.5 % of acetonitrile, followed by a linear gradient to 50 % of water and 50 % of acetonitrile in 10 min, then a further decrement to 100 % of acetonitrile in 25 min, and then a hold at 100 % of acetonitrile for 5 min, and followed by re-equilibration at 99.5 % of water and 0.5 % of acetonitrile for 3 min (for a total run time of 43 min) (6). The LC-MS chromatograph of methanol extract of *P. australis* was obtained by using the gradient elution starting with 99.5 % of water and 0.5 % of acetonitrile for 5 min and decreased to 50 % of water and 50 % of acetonitrile (10 min), followed by 0 % of water and 100 % of acetonitrile (15 min), then held at 0 % of water and 100 % of acetonitrile (10 min), followed by increment to 99.5 % of water and 0.5 % of acetonitrile (3 min), for a total run of 50 min. The LC-MS chromatograph of methanol extract of *S. binderi* was performed in gradient elution, consisting of initial elution of 99.5 % of water and 0.5 % of acetonitrile, and decreased to 90 % of water and 10 % of acetonitrile over 5 min, followed by a linear gradient to 50 % of water and 50 % of acetonitrile in 15 min, then to 100 % of acetonitrile in 15 min, and a hold at 100 % of acetonitrile for 5 min, and finally re-equilibrium at 90 % of water and 10 % of acetonitrile for 5 min, for a total run of 50 min.

Statistical analysis

The statistical analyses were carried out by using IBM SPSS 20.0 (IBM, USA). The mean values of each treatment were compared using ANOVA, followed by post hoc Tukey's test. $p < 0.05$ was considered to be statistically significant in the experiment.

RESULTS

Acetylcholinesterase inhibition assay

Maceration extraction of dried seaweeds gave 17.16% (w/w), 4.33% (w/w) and 3.60% (w/w) yield of methanol extract for *B. pennata, P. australis* and *S. binderi*, respectively. In the current study, green seaweed *B. pennata* showed the strongest inhibition effect towards *in vitro* AChE by using tissue homogenates of *Ae. aegypti* (IC_{50} value: 0.84 mg mL^{-1}) and *Ae. albopictus* as the enzyme source (IC_{50} value: 0.92 mg mL^{-1}). On the contrary, the brown seaweeds such as *P. australis* and *S. binderi* had higher IC_{50} values indicating weaker inhibition activity on AChE (IC_{50} values ranging from 1.3 to 1.8 mg mL^{-1}) (Table I).

Table I: Enzyme kinetic parameters of the seaweed extracts on acetylcholinesterase activity using homogenates of Aedes aegypti and Aedes albopictus as enzyme source

Treatment	IC_{50} value (mg mL^{-1})	Type of Inhibition	K_m (µM)	V_{max} [1]	K_i (mg mL^{-1})	Alpha[2]	K_i' (mg mL^{-1})
Aedes aegypti							
Bryopsis pennata	0.84 ± 0.11[a]	Mixed type	**14.28 ± 1.55[a]**	**0.498 ± 0.009[a]**	**0.144** ± 0.025[a]	**2.655**	**0.579 ± 0.058[a]**
Padina australis	1.32 ± 0.12[b]	Mixed type	21.17 ± 3.49[a]	0.578 ± 0.021[a]	0.491 ± 0.143[b]	1.613	0.924 ± 0.067[b]
Sargassum binderi	1.39 ± 0.15[b]	Mixed type	81.31 ± 10.14[b]	0.715 ± 0.032[b]	0.503 ± 0.111[b]	1.785	1.128 ± 0.123[b]
Propoxur	0.10 ± 0.01 x10^{-3c}	Competitive	41.13 ± 3.99[c]	0.580 ± 0.015[a]	0.143 ± 0.011[a]	-	-
Control	-	-	90.32 ± 7.75[b]	0.814 ± 0.031[b]	-	-	-
Aedes albopictus							
Bryopsis pennata	0.92 ± 0.21[a]	Mixed type	**10.19 ± 8.94[a]**	**0.432** ± 0.031[a]	**0.228 ± 0.060[a]**	**3.428**	**0.464 ± 0.035[a]**
Padina australis	1.77 ± 0.15[b]	Mixed type	15.12 ± 3.33[ab]	0.530 ± 0.021[ab]	0.305 ± 0.092[b]	3.335	0.778 ± 0.079[b]
Sargassum binderi	1.30 ± 0.08[ab]	Mixed type	21.16 ± 6.47[b]	0.591 ± 0.039[b]	0.905 ± 0.237[c]	2.025	1.417 ± 0.143[c]
Propoxur	0.14 ± 0.02 x10^{-3c}	Competitive	55.16 ± 6.46[c]	0.608 ± 0.022[b]	0.151 ± 0.014[a]	-	-
Control	-	-	68.34 ± 6.23[d]	0.782 ± 0.026[c]	-	-	-

All the values are mean ± standard deviation (SD) of three independent experiments (n=3)
Means followed by different letters within the same column are significantly different ($p < 0.05$)
[1] V_{max} was expressed as µmole of acetylthiocholine hydrolysed/ min/ mg of protein.
[2] Value indicates the mechanism pattern of mixed-type inhibition. When α = 1, the mixed-model inclines toward the noncompetitive type inhibition; when alpha is very large (α > 1), the mixed-model inclines toward the competitive type inhibition; when Alpha is very small (1> α > 0), the mixed model becomes more similar to uncompetitive model.

The results of kinetic enzyme assay using different concentrations of substrate solution (0.025-0.2 mM) and seaweed extracts (0–1.75 mg mL^{-1}) shows that all seaweed extracts exhibited the velocity increment of inhibition reaction, in conjunction with the increase of substrate concentration, following the Michealis-Menten kinetics. The mode of inhibition of these seaweed extracts was illustrated by using the Lineweaver-Burk plot (Figure 1). The Lineweaver-Burk plots of all seaweed extracts showed straight double-reciprocal lines which intercepted at the second quadrant (when Km increased, Vmax decreased) (Figure 1a–c), whereas Lineweaver-Burk plots of propoxur displayed lines which intercepted at the Y-axis (when K_m increased, V_{max} remained unaffected) (Figure 1d). The enzyme kinetic parameters of the seaweed extracts on acetylcholinesterase activity are tabulated in Table I. *B. pennata* showed the highest value of Alpha and the lowest values of K_m, V_{max}, K_i and K_i', among the three extracts tested for both assays using homogenates of *Ae. aegypti* and *Ae. albopictus*.

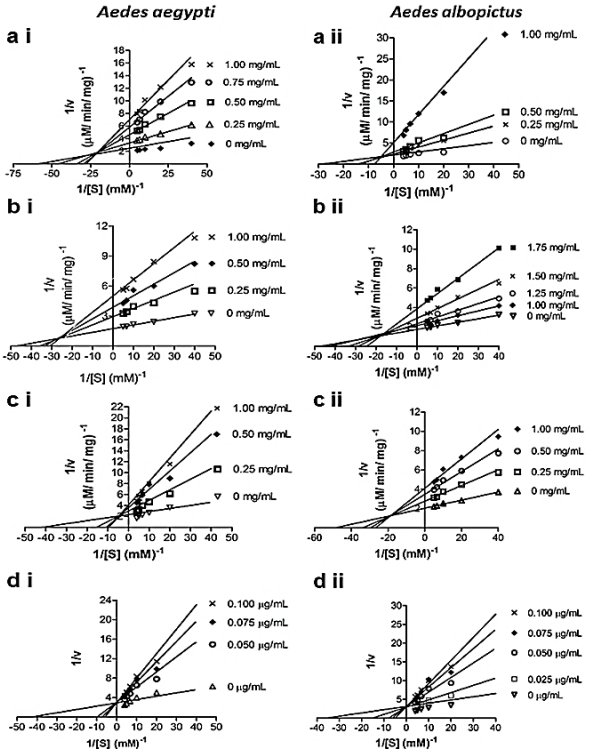

Figure 1: Lineweaver-burk plots showing the AChE inhibition of different treatments by using homogenates of Aedes aegypti (left panel) and Aedes albopictus (right panel) as enzyme source. (a) methanol extracts of *Bryopsis pennata*, (b) *Padina australis*, (c) *Sargassum binderi*, and (d) propoxur (standard inhibitor). V^{-1} was expressed in µmole of acetylthiocholine hydrolysed min^{-1} mg $protein^{-1}$.

LC-MS Analysis of Extracts

The LC-MS chromatograph of *B. pennata* exhibited 22 peaks that were resolved in 36 min (Figure 2A). A total of 15 peaks were resolved in 44 min in the LC-MS chromatograph of *P. australis* (Figure 2B). Figure 2C represents the LC-MS chromatograph of methanol extract of *S. binderi* exhibiting 8 peaks that resolved in 41 min.

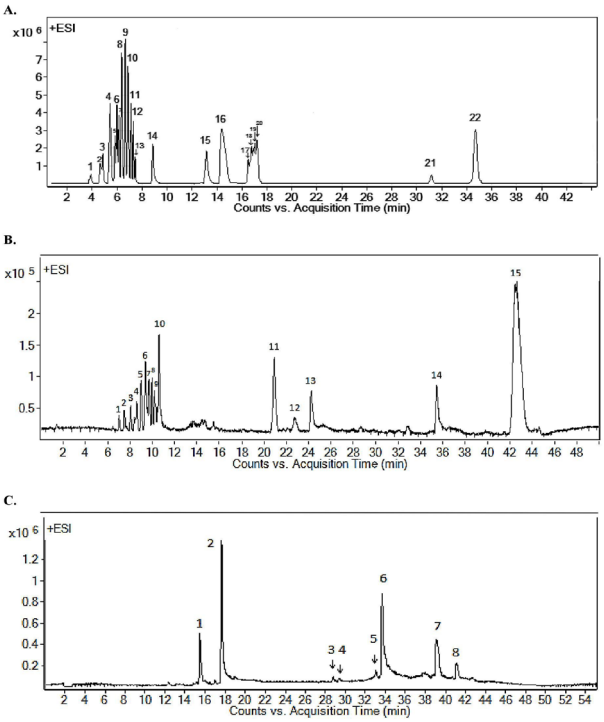

Figure 1: LC-MS chromatographs of methanol extracts of (a) Bryopsis pennata, (b) Padina australis and (c) Sargassum binderi.

List of possible molecular formula generated based on data of accurate mass of molecular ions processed using qualitative analysis software of Mass Hunter Acquisition Data (Agilent Technologies Canada Inc., Canada) and hits from Dictionary of Marine Natural Products (17) were shown in supplementary data.

DISCUSSION

AChE is important for the function of neural synapses. Therefore, when the activity of AChE is inhibited, the insect would experience overstimulation of the cholinergic neuron, hyper-excitableness, paralysis or even death (18). From the results, it is evident that the AChE inhibition effect of these seaweed extracts was comparable to other report which reported mostly seaweeds with IC_{50} values ranging from 1-10 mg mL^{-1} (19). For instance, *Sargassum angustifolium* (IC_{50} value: 5.4 mg mL^{-1}), *Sargassum boveanum* (IC_{50} value: 1.0 mg mL^{-1}), *Sargassum oligocystum* (IC_{50} value: 2.5 mg mL^{-1}) (20) and *Sargassum sp.* from India (IC_{50} value: 1.0 mg mL^{-1}) (20). In this study, *P. australis* showed better inhibition effect as compared to *P. australis* from Persian Gulf (IC_{50} value: 6.3 mg mL^{-1}) (21), *Padina gymnospora* (IC_{50} value: 3.5 mg mL^{-1}) (21) and *Padina vickersiae* (IC_{50} value: 7.9 mg mL^{-1}) (22).

The pattern of Lineweaver-Burk plots revealed that *B. pennata, P. australis* and *S. binderi* were mixed type inhibitor of AChE. Thus, these extracts may contain compounds that can bind to free enzyme, as well as enzyme-substrate complex (23). Among the mixed-type

inhibition models shown by the three seaweed extracts, *B. pennata* had the highest value of Alpha, indicating its inhibition mechanism that inclines towards the competitive model (GraphPad Software 2015). Furthermore, the readings of K_m, V_{max}, K_i and K_i' of *B. pennata*, indicates that it had a stronger inhibition ability with high binding affinity towards both free enzyme and enzyme-substrate complex, as compared to the other two seaweed extracts (23).

Similar to the results of present study, the pattern of mixed type AChE inhibiton was reported for other seaweed extracts. *Sargassum sp*. from India was reported to have mixed type inhibition with weaker inhibition ability, by using homogenate of fresh water fish Nile tilapia as source of enzyme (higher K_m, V_{max} and K_i values, where Km was 1.78 mM, V_{max} was 1.01 µmole min^{-1} mg^{-1} of protein and K_i was 0.86 mg mL^{-1}) (21), as compared to *S. binderi* tested in the present study (by using homogenate of *Ae. aegypti*). *P. australis* tested in the current study showed similar inhibition pattern but stronger inhibition ability as compared to Indian *P. gymnospora* (higher K_m, V_{max} and K_i values, where K_m was 1.17 mM, V_{max} was 0.91 µmole min^{-1} mg^{-1} and K_i was 0.90 mg mL^{-1}) (15).

Propoxur, the positive control is well known as an insecticide that acts as AChE inhibitor with competitive inhibition pattern (24) (Figure 1d, Lineweaver-Burk plots of propoxur displayed lines which intercepted at the Y-axis). In the competitive inhibition mechanism, the inhibitor inactivates the free enzyme by binding to the active site of enzyme. The bindings of substrate and inhibitor to the enzyme were mutually exclusive (23). This is in agreement with the mode of action of propoxur, as it is a reversible competitive inhibitor that competes with other substrates for the active site of AChE (25).

The LC-MS chromatographs in the study revealed the presence of a wide range of secondary compounds with various types of bioactive properties in the seaweed extracts. β-sitosterol was reported to be found in green seaweed Bryopsis sp. of the Caribbean (26). β-sitosterol is a sterol that has various bioactivities. It was reported to have larvicidal activity towards mosquitoes such as *Ae. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* at LC_{50} values of 11.49, 3.58 and 26.67 ppm, respectively (27). Besides, β-sitosterol was reported to inhibit the growth of human colon cancer cells and alter the membrane lipids (28). Sargachromanol L is a meroterpenoid isolated from Sargassum siliquastrum collected from Jeju Island, Korea. The compound at the concentration of 100 µg mL^{-1}, was reported to have 90.1 % of radical scavenging activity, but weak or negligible inhibition towards butylcholine esterase (29).

In the AChE kinetic study, *B. pennata* was proven to be the strongest AChE inhibitor with mixed type inhibition pattern, as compared to the other two extracts tested. The results of the in vitro assay suggested the potential of the seaweeds to act as enzyme inhibitor in their insecticidal action. The in vitro results of the present study were based on the total activity of enzymes including their isoforms, rather than the specific activity of a single enzyme. Hence, significant qualitative changes between the variants of enzyme following the treatment might have gone undetected. A better characterized enzyme source is recommended in the future study.

CONCLUSION

In the present study, seaweeds collected in Malaysia, namely *B. pennata, P. australis* and *S. binderi* were proven to have inhibition effect of AChE. These findings have brought a step closer in developing the seaweeds as alternative vector control agent against *Aedes aegypti* and *Ae. albopictus*. The chemical constituents of the insecticidal Malaysian seaweeds are warranted to be identified and characterized in the future study. As the seaweeds used in the study are common seaweeds found in tropical regions, availability of the seaweeds eases further investigation and development into potential mosquitocidal agent.

ACKNOWLEDGEMENTS

The authors thankfully acknowledged Universiti Kebangsaan Malaysia (Dana Modal Insan 13-00-09-018), Management and Science University (MSRG SG-035-022018-FHLS), Institute for Medical Research and Taylor's University, for financial and technical supports in carrying out the research.

REFERENCES

1. World Health Organization (2012) Global plan for insecticide resistance management in malaria vectors. Retrieved from http://www.who.int/malaria/publications/atoz/gpirm/en/ on 14 Jan 2015.
2. Paeporn P, Komalamisra N, Deesin V, Rongsriyam Y, Eshita Y, Thongrungkiat S. Temephos resistance in two forms of *Aedes aegypti* and its significance for the resistance mechanism. Southeast Asian Journal of Tropical Medicine and Public Health. 2003;34:786-92.
3. Bianco EM, Pires L, Santos GK, Dutra KA, Reis TN, Vasconcelos ER, Cocentino AL, Navarro DM. Larvicidal activity of seaweeds from northeastern Brazil and of a halogenated sesquiterpene against the dengue mosquito *Aedes aegypti*. Industrial of Crop Production. 2013;43:270-75.
4. Yu K-X, Jantan I, Ahmad R, Wong C-L. The major bioactive components of seaweeds and their mosquitocidal potential. Parasitology Research. 2014;113:3121-41.
5. Coppejans E, Leliaert F, Dargent O, Gunasekara R, De Clerck O. Sri Lankan seaweeds: Methodologies and field guide to the dominant species. 2009;6:60-

5. Belgian Development Cooperation. Retrieved from http://www.abctaxa.be/volumes/volume-6-sri-lankan-seaweeds-methodologies-and on 19 December 2014.
6. Yu K-X, Wong C-L, Ahmad R, Jantan I. Mosquitocidal and Oviposition Repellent Activities of the Extracts of Seaweed *Bryopsis pennata* on *Aedes aegypti* and *Aedes albopictus*. Molecules. 2015;20:14082-102.
7. Geraldino PJL, Liao LM, Boo SM. Morphological study of the marine algal genus Padina (Dictyotales, Phaeophyceae) from southern Philippines: 3 species new to Philippines. ALGAE-INCHON. 2015;20:99.
8. Guedes, EAC, Carvalho, C. M. D., Junior, R., Lisboa, K. A., Lisboa Ribeiro, T. F., de Barros, LD & Goulart Sant'Ana, AE. Larvicidal activity against *Aedes aegypti* and molluscicidal activity against Biomphalaria glabrata of Brazilian marine algae. Journal of Parasitology Research. 2014;501328.
9. Sahayaraj K, Kalidas S. Evaluation of nymphicidal and ovicidal effect of a seaweed, Padina pavonica (Linn.) (Phaeophyceae) on cotton pest, Dysdercus cingulatus (Fab.). Indian Journal of Marine Science. 2011;40:125.
10. Elbanna SM, Hegazi MM. Screening of some seaweeds species from South Sinai, Red Sea as potential bioinsecticides against mosquito larvae; Culex pipiens. Egyptian Academic Journal of Biological Science. 2011;4:21-30.
11. Sharma OP. Algae. Series on diversity of microbes and cryptogams Tata McGraw Hill, New Dehli. 2011.
12. Yu K-X, Wong C-L, Ahmad R, Jantan I. Larvicidal activity, inhibition effect on development, histopathological alteration and morphological aberration induced by seaweed extracts in *Aedes aegypti* (Diptera: Culicidae). Asian Pacific Journal of Tropical Medicine. 2015; 8:1006-12.
13. Sheikh TZB, Yong CL, Lian MS. In vitro antioxidant activity of the hexane and methanolic extracts of Sargassum baccularia and Cladophora patentiramea. Journal of Applied Science. 2009;9:2490-3.
14. Brogdon WG, Hobbs JH, St Jean Y, Jacques JR, Charles LB. Microplate assay analysis of reduced fenitrothion susceptibility in Haitian Anopheles albimanus. Journal of American Mosquito Control Association. 1988;4:152-8.
15. Suganthy N, Karutha Pandian S, Pandima Devi K. Neuroprotective effect of seaweeds inhabiting South Indian coastal area (Hare Island, Gulf of Mannar Marine Biosphere Reserve): Cholinesterase inhibitory effect of Hypnea valentiae and Ulva reticulata. Neuroscience Letter. 2010;468:216-9.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72:248-54.
17. Blunt J, Munro MH. Dictionary of marine natural products with CD-ROM. Chapman and Hall, London, UK. 2008.
18. Doctor BP, Taylor P, Quinn DM, Rotundo RL, Gentry MK (Eds.). Structure and function of cholinesterases and related proteins. Springer Science & Business Media. 2013.
19. Stirk WA, Reinecke DL, Van Staden J. Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. Journal of Applied Phycology. 2007;19(3):271-6.
20. Ghannadi A, Plubrukarn A, Zandi K, Sartavi K, Yegdaneh A. Screening for antimalarial and acetylcholinesterase inhibitory activities of some Iranian seaweeds. Research in Pharmaceutical Sciences. 2013;8(2):113.
21. Natarajan S, Shanmugiahthevar KP, Kasi PD. Cholinesterase inhibitors from Sargassum and Gracilaria gracilis: Seaweeds inhabiting South Indian coastal areas (Hare Island, Gulf of Mannar). Natural Product Research. 2009;23:355-69.
22. Kartal M, Orhan I, Abu-Asaker M, Senol FS, Atici T, Sener B Antioxidant and anticholinesterase assets and liquid chromatography-mass spectrometry preface of various fresh-water and marine macroalgae. Pharmacog Magazine. 2009;5:291-7.
23. Sauro HM. Enzyme kinetics for systems biology. Ambrosius Publishing and Future Skill Software, USA. 2012.
24. Colovic MB, Krstic DZ, Lazarevic-Pasti TD, Bondzic AM, Vasic VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Current Neuropharmacology. 2013;11:315-35.
25. Côté E. Organophosphate ad carbamate insecticide toxicosis. In: Clinical veterinary advisor: Dogs and cats. 3rd Edn. Elsevier Health Sciences, US. 2014;732.
26. Govindan M, McClean AE, Brown KA. Chemical Constituents of the Caribbean Marine Alga Bryopsis plumosa. Microchemistry Journal. 1993;47:193-6.
27. Rahuman AA, Gopalakrishnan G, Venkatesan P, Geetha K. Isolation and identification of mosquito larvicidal compound from Abutilon indicum (Linn.) Sweet. Parasitolgy Research. 2008;102:981-8.
28. Awad AB, Chen YC, Fink CS, Hennessey T. beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. Anticancer Research. 1995;16:2797-804.
29. Jang KH, Lee BH, Choi BW, Lee H-S, Shin J. Chromenes from the Brown Alga Sargassum siliquastrum. Journal of Natural Product. 2005;68:716-23.