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Phenolics, fatty acids composition and biological activities of various extracts and fractions of Malaysian *Aaptos aaptos*

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ABSTRACT

Objective: To investigate phenolics, fatty acids composition and biological activities of various extracts and fractions of Malaysian *Aaptos aaptos*. **Methods:** Fatty acid methyl ester was analyzed by gas chromatography-flame ionization detector. Antioxidant activity was determined using 2,2-diphenyl-picrylhydrazyl radical scavenging assay and total phenolics content by Folin-Ciocalteu procedure. Vero cells viability was evaluated using methyl thiazole tetrazolium and the inactivation of herpes simplex virus type 1 by neutral red uptake assay. *p*-Hydroxybenzamide isolated by column chromatography was characterized by utilizing nuclear magnetic resonance spectroscopy and electron impact mass spectrometry. **Results:** The chloroform, ethyl acetate and methanol extracts of *Aaptos aaptos* produced higher portions of straight-chain saturated fatty acid, while hexane extract mainly consisted of unsaturated fatty acid. The five majors of fatty acid methyl ester were identified as behenic acid, *cis*-10-heptadecenoic acid and *cis*-10-pentadecenoic acids, palmitic acid and tricosanoic acid. In addition, among all organic extracts, chloroform extract inactivated herpes simplex virus type 1 while exhibited weak cytotoxic activity against normal Vero cells and also exhibited strong cytotoxic activity on HL-60, MCF-7, K562, CEM-SS and WEHI-3B cells. A phenolic compound, *p*-hydroxybenzamide was also isolated from the sponge. **Conclusions:** *Aaptos aaptos* could be a source to derive the potential antiviral and anticancer agents. However, further studies are needed to determine the mechanism involved in the process.

1. Introduction

Sponges are parts of the phylum Poriferae with approximately 15 000 living species available worldwide. Marine sponges have been found to contain the highest number of completely new molecules which are biologically active against human pathogens and other ailments. For examples, these include bioactive marine alkaloids, purines, pyrimidines and their nucleosides, amino acids, peptides,

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guanidine, nitrogenous marine toxins *etc*[1]. In addition, these species are rich of lipid-containing metabolites such as unusual fatty acids (FA) with high percentage of long chain FA (C_{24}-C_{30}), sterols and phospholipid[2–4]. Some novel marine FA has displayed antimycobacterial, antimalarial and antifungal properties[5]. Yakushinamides A and B, prolyl amides of polyoxygenated fatty acids that are isolated from the marine sponge *Theonella swinhoei* are reported as inhibitors of histone deacetylases and sirtuins[6]. Pyrrole alkaloids that are conjugated with various FA obtained from marine sponges from the genera of *Mycale* are reported to have antileshmanial activity against *Leishmania mexicana* promastigotes. The cytotoxicity exhibited by these compounds was affected by the length, number and position of the unsaturations of the fatty acid chains[7]. Besides, hexadecanoic, pentadecanoic, docosanoic, tetracosanoic, octadecanoic, eicosanoic, tetradecanoic and 2-hydroxyhexadecanoic acids isolated are from seaweed *Sargassum granuliferum* and *Dictyota dichotoma* which are shown to have a promising antifouling property[8].

In Malaysia, the studies on marine sponges only have been initiated in the late 1980s, with most of the studies focusing on the bioactivies screening, instead of taxonomic studies[9–11]. Only a few studies emphasized on the isolation of chemical constituents of Malaysian marine sponges which involved *Leucoploeus fenestrata*[12] and *Pseudaxinyssa* sp.[13], *Aaptos* sp.[14–18] and *Xestospongia*[19]. In addition, other previous researchers also have explored the characterization of chemical compounds from marine-sponge derived fungi [20,21], the isolation of marine bacterium associated with *Theonela* sp.[22,23] and *Haliclona amboinensis*[24]. Other than that, studies on the cultivation of sponge *Aaptos* sp. and *Theonella* sp. in open-sea system also has been successfully carried out[25]. Recently, identification of three poly-hydroxyalkanoatesynthase genes (*phaC*) isolated from the marine bacteria metagenome of *Aaptos aaptos* (*A. aaptos*), a marine sponge in the waters of Bidong Island, Terengganu, Malaysia has been reported[26]. In addition to the list, three methanol extracts of Malaysian marine sponges species, namely *Aaptos* sp., *Stryphuous ponderosus* and *Theonella* sp. were reported to exert cytotoxic effects against human breast cancer cell line, MCF-7. Methanol extract of *Stryphuous ponderosus* has revealed apoptotic-induced cytototoxicity against MCF-7 cell line[27].

Previous researches on genus *Aaptos* have gained great attention worldwide due to the interesting biological activities of its aaptaminoids compounds. These biological activities include the prevention of neoplasm that acts as an α-adrenoceptor blocker[28], antiamoebic activity[29], the prevention of herpes simplex virus replication[30], an antioxidant activity[31], an activator of p21 promoter stably transfected in MG63 cells[32] and antidepressant-like activity[33], as well as exhibited promising activity against cancer cell lines including A549 (human lung adenocarcinoma), KB16 (human mouth epidermoid carcinoma), P-338 (murine lymphocytic leukemia) and HT-29 (human colon adenocarcinoma)[34]. Recently, a study on *Aaptos* from Pramuka Island, Jakarta has revealed the identification of sponge-associated bacteria based on 16S-rRNA, that was proven to have ability to inhibit *Vibrio* sp. *in vitro* and *in vivo*. Other than that, the study also focused on encoding the genes' bioactive compounds (*NRPS* and *PKS* genes) on *Aaptos* sp. and *Hyrtios*[35].

Aaptos sp. is one of the abundance marine sponges found in the east coast of Peninsular Malaysia, particularly along the Terengganu coast. Previously, we have reported the isolation of cholestanyl myristate, 5α-cholestan-3β-ol, aaptamine, two new derivatives of the aaptamine which are 3-(isopentylamino)demethyl(oxy) aaptamine and 3-(phenethylamino)-demethyl(oxy)aaptamine[14,15]. Aaptaminoids have seemed to be important metabolites for the genera of *Aaptos* since several derivatives which included aaptamine, demethylaaptamine, isoaaptamine, aaptosamine, aaptosine, demethyloxy-aaptamine[28], aaptosine[36], aaptosamine[37], 4-methylaaptamine[30], bisdemethylaaptamine and bisdemethyl-aaptamine-9-*O*-sulfate[38] were isolated from *Aaptos* sp. collected in Indonesia, Philippine and Okinawa. According to summarization of studies from all parts of the world by Larghi *et al*[39], the derivatives that are collected from Indonesia, Philippine and Okinawa, are isolated from *Aaptos* sp. and have gone through the process of derivatization of aaptaminoids. Later, an aromatic alkaloid, *N*-demethylaaptanone has been isolated from Vietnamese marine sponge *A. aaptos*[40]. However, to the best of our knowledge, there were no studies done on the FC constituent and phenolics content of *Aaptos* sp. Thus, this study is conducted to determine the FC constituent, phenolics contents and biological activities of hexane, chloroform, ethyl acetate and methanol extract of *A. aaptos*.

2. Materials and methods

2.1. Extraction

The *A. aaptos* were collected from the coastal waters of Terengganu, on the eastern part of Peninsular Malaysia (Kapas, Perhentian, and Bidong Islands) *via* scuba diving at a depth of 8 to 15 m. Some of the collected specimens were deposited at the Biodiversity Museum, Institute of Oceanography, Universiti Malaysia Terengganu. Five samples from different locations were frozen immediately after the collection. Next, the samples were cleaned and cut into small cubes (1 cm × 1 cm) and they were dried in air-crafted oven at 40 ℃ After the dried samples were extracted with methanol, the extracts were filtered and dried under reduced pressure yielding sample of methanolic extracts (D, G, H, J, K). Furthermore, due to the abundant

supply of extracts, sample from Bidong Island (G) was selected for successive extraction with hexane, chloroform, ethyl acetate and methanol for three times, with each yielding different polarity of extracts. Each extract underwent the process of evaporation under reduced pressure to consequently acquire the hexane fraction extract (ABHE), chloroform fraction extract (ABCE), ethyl acetate fraction extract (ABEE) and methanol fraction extract (ABME).

2.2. Lipid extraction and fatty acid methyl ester (FAME) content analysis

ABHE, ABCE, ABEE and ABME samples were used in the preparation of FAME content analysis. The derivatization of these fatty acids and its FAME analysis were done precisely as the method described by Bazes *et al*[41].

2.3. Total phenolics content

The amount of total phenolics in the extracts was determined according to the Folin-Ciocalteu procedures[42], with some modifications where the concentration of the test samples (ABHE, ABCE, ABEE and ABME) were changed to 1 mg/mL and the absorption at 765 nm was recorded (Bio-rad spectrophotometer). The total phenolic content in methanolic crude extracts in GAE was determined by calculating it using the following formula:

$C = c \times V / m$

Where

C=total phenolic content of methanolic crude extracts (mg/g)

c=concentration of gallic acid established from the calibration curve (mg/mL)

V=volume of extract (mL) and m is the weight of pure plant methanol extract (g).

2.4. Anti-oxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical method was adopted to determine the DPPH free-radical scavenging activity in order to assess the antioxidant activity. The method used was based on Habsah *et al*[15] and Von Gadov *et al*[43], with a modification where the absorbance (Bio-rad spectrophotometer) was read at 517 nm against a blank. Buthylated hydroxyanisole (BHA) and quercetin were used as positive control.

2.5. Culture of cells and cytotoxic activity against cancer cell lines

The samples of the six cell lines used were human acute promyelocytic leukemia (HL-60), humanbreast adenocarcinoma (MCF-7), human chronic myelogenous leukemia (K-562), human cervix adenocarcinoma (HeLa), acute lymphoblastic leukemia (CEM/C2) and murine myelomonocytic leukemia (WEHI-3B). These samples have been supplied by American Type Culture Collection (ATCC). The cell lines were cultured and maintained as described by Ali *et al*[44]. For cytotoxic assay, the microculture cytotoxicity was screened using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a procerdure adopted from Shaari *et al*[14] and Mosmann[45] with some modification where the concentrations of where final concentration of sample ranging from 30 g/mL to 0.46 g/mL. The determination of 50% cytotoxic concentration (CD_{50}) was done in three replicates.

2.6. Antiviral activity

2.6.1. Cell and virus culture

Vero (ATCC® CCL-81TM) originated from Cercophithecus aethiops African green monkey kidney and herpes simplex type 1 virus (HSV-1) stock was maintained and cultured according to Rashid *et al*[16] and Muench[46].

2.6.2. Neutral red uptake assay

Neutral red uptake assay was done following a procedure as described by Rashid *et al*[16] and McLaren *et al*[47]. Acyclovir was used as positive control.

2.6.3. Cytotoxicity assay by cell viability

Method of Rashid *et al*[16] was adopted with a modification by changing the concentration of samples ranging from 0.5 to 100 μg/mL.

2.6.4. Antiviral assay by cell viability

The same experiment protocol as the cytotoxicity test described by Rashid *et al*[16] and Langlois *et al*[48] was used except that the MEM was replaced by 50 μL HSV-1 virus-infected cell suspensions at multiplicity of infection (MOI) of 0.001 ID_{50}/cells ($2 \times 10^{8.5}$ ID_{50}/mL) HSV-1.

2.7. Statistical analysis

All of the data extracted were determined statistically by using analysis of variance (ANOVA) in SPSS version 11.5 for Windows at 95% confident interval (*CI*) to compare the significant different between doses of sample's treatment, while the independent sample Student *t*-test was used (at 95% *CI*) to compare the significant different between control (untreated) and doses of sample's treatment.

2.8. Purification and characterization of p-hydroxybenzamide

Methanol extract of *A. aaptos* underwent solvent partitioning to

give diethyl ether, butanol and aqueous extract. Approximately 15.2 g of this butanol extract was fractionated using dry vacuum column chromatography on silica gel and gradually eluted with *n*-hexane, *n*-hexane/DCM, DCM, DCM/MeOH, and MeOH. A total of 17 fractions including fractions IK and IL were collected. The combined fraction IK and fraction IL (11.0 g) were chromatographed on silica gel by eluting them with $CHCl_3$ and MeOH with the ratio of 9.5:0.5; 9:1 and 8:2 to give 21 fractions. After that, fractions 8-12 (3.0 g) were further separated on silica gel column eluted with $CHCl_3$/MeOH, 8:2 to yield 12 subfractions including fraction CAMI8-12C. Fraction CAMI8-12C was rechromatographed over LH-20 column chromatography eluted with $CHCl_3$/MeOH, 1:1 to give 5 fractions including fraction CAMI8-12CV. Next, fraction CAMI8-12CV was further purified by washing with MeOH. Separation of soluble and unsoluble compounds in methanol by filtering yielded two compounds. *p*-Hydroxybenzamide which was soluble in MeOH was isolated as white powdered compound. Infrared (IR) spectrum was recorded with Perkin Elmer FTIR (model 1725X) spectrophotometer using KBr discs. Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectra were recorded on Bruker ARX 400 NMR spectrometer with tetramethylsilane as internal standard. Mass spectra were recorded by Direct Induction Probe using a Shimadzu GCMS-QP5050 spectrometer with ionization induced by electron impact at 70 eV.

3. Results

3.1. FAME content analysis

FAME profiles of ABHE, ABCE, ABEE and ABME (Table 1) showed notable differences with predominance of behenic acid (C22:0), *cis*-10-heptadecenoic acid (C17:1), palmitic acid (C16:0), and *cis*-10-pentadecenoic acid (C15:1). Generally, the FAME profiles of all samples were quite similar. Hexane extract was rich in *cis*-10-heptadecenoic acid (C17:1, 19.5%), followed by *cis*-10-pentadecenoic acid (C15:1, 16.4%), palmitic acid (C16:0, 9.2%), palmitoleic acid (C16:1, 7.6%), pentadecanoic acid (C15:0, 7.6%) and behenic acid (C22:0, 6.7%). Meanwhile, the concentration of palmitic acid and behenic acid were much higher in the chloroform extract, which were 18.3% and 10.2% respectively. However, the highest content of behenic acid was detected in ethyl acetate (35.7%) while other FAMEs contained much lower values with palmitic acid, 5.7% and nervonic acid (C24:1), 4.2%. On the other hand, the different major FAMEs were found in methanol extract which included tricosanoic acid (C23:0, 20.0%), heneicosanoic acid (C21:0, 11.1%), behenic acid (19.8%) while higher content of nervonic acid (8.6%) were also detected.

Table 1

Fatty acid compositions of hexane, chloroform, ethyl acetate and methanol extracts of *A. aaptos* quantitatively analysed by GC-FID.

FAME	Relative amount FAME fractions extract (%)			
	ABHE	ABCE	ABEE	ABME
Undecanoic acid (C11:0)	1.3	nd*	1.4	nd*
Lauric acid (C12:0)	nd*	1.1	0.9	0.1
Tridecanoic acid (C13:0)	0.6	nd*	0.8	nd*
Myristic acid (C14:0)	2.1	1.2	0.9	0.5
Myristoleic acid (C14:1)	3.2	2.6	0.5	0.2
Phenantrene (Control)	13.6	30.4	31.0	22.7
Pentadecanoic acid (C15:0)	7.6	1.7	0.6	1.2
cis-10-Pentadecenoic acid (C15:1)	16.4	2.2	1.4	2.4
Palmitic acid (C16:0)	9.2	18.3	5.7	2.3
Palmitoleic acid (C16:1)	7.6	4.1	2.0	1.3
Heptadecanoic acid (C17:0)	2.4	6.3	5.0	0.6
cis-10-Heptadecenoic acid (C17:1)	19.5	5.9	1.3	1.4
Stearic acid (C18:0)	5.1	4.1	2.6	1.1
Elaidic acid (C18:1n9t)	2.4	1.8	0.7	0.4
Arachidic acid (C20:0)	1.4	nd*	2.2	1.0
Heneicosanoic acid (C21:0)	1.0	3.8	1.5	11.1
Behenic acid (C22:0)	6.7	10.2	35.7	19.8
Tricosanoic acid (C23:0)	nd*	nd*	1.6	20.0
Lignoceric acid (C24:0)	nd*	nd*	nd*	5.6
Nervonic acid (C24:1)	nd*	6.3	4.2	8.6

Note: nd*; not detected, ( ): numbers in parentheses represent positions of unsaturated bonds, FAME: fatty acid metyl ester, ABHE: hexane extract, ABCE: chloroform extract, ABEE: ethyl acetate and ABME: methanol extract.

3.2. Total phenolics content and anti-oxidant activity

The content of phenolic compounds in the samples were determined from regression equation of calibration curve (y=0.007 4x-0.153 7, R^2=0.844 8) and expressed in gallic acid equivalent (GAE; mg/g). The total phenolic contents of methanol crude extracts from five different locations and four solvent fractions varied widely with values ranging from 21.8 to 68.5 mg/g GAE and 6.5 to 14.3 mg/g GAE, respectively (Table 2). In addition, the chloroform (ABCE) and methanol (ABME) fraction extracts showed potential DPPH free radical scavenging activity, with the respective percentage of

inhibition being 67.6% and 78.2% respectively. Their IC_{50} values were 1.71 and 4.62 mg/mL respectively (Table 2).

Table 2

DPPH free-radical scavenging activity and total phenolic content of methanolic extracts of *Aaptos* sp. from different locations.

Code	Specimen	Free radical scavenging activity (%)	IC_{50} (mg/mL)	Total phenolic content (mg/g)
P03/015/04	D	80.5 ± 0.5*	0.13 ± 0.50*	41.60 ± 0.50
B01/010/04	G	78.8 ± 0.5*	0.12 ± 0.50*	29.40 ± 0.50
K01/025/04	H	89.3 ± 0.7*	0.11 ± 0.70*	21.80 ± 0.70
K01/010/05	J	81.6 ± 0.7*	0.26 ± 0.70*	41.20 ± 0.50
K02/011/05	K	81.1 ± 2.3*	0.12 ± 2.30*	68.50 ± 2.30
ABHE		6.7 ± 0.3	NT	13.50 ± 0.30
ABCE		67.6 ± 0.1	4.62 ± 0.10	10.50 ± 0.01
ABEE		44.2 ± 0.1	NT	14.30 ± 0.08
ABME		78.2 ± 0.1	1.71 ± 0.10	6.50 ± 0.04
Buthylated hydroxyanisole	BHA	94.4 ± 0.6	0.04 ± 0.60	-
Quercetin	QCTN	94.2 ± 0.6	0.04 ± 0.60	-

Note: P: Perhentian Island, B: Bidong Island, K: Kapas Island, NT: Not tested, Fraction extracts; ABHE: hexane extract, ABEE: ethyl acetate extract, ABCE: chloroform extract, and ABME: methanol extract, BHA: buthylated hydroxyanisole, QCTN: Quercetin. The results were the means values of three separate experiments (mean ± SD). *Data were cited in Habsah *et al*[10].

3.3. Cytotoxic activity against cancer cell lines

The preliminary cytotoxic screening of methanolic crude extracts (CE) of *A. aaptos* that were collected from various locations of Terengganu islands against HL-60 (human leukemia cell line) and MCF-7 (breast cancer cell line) has revealed that CE from Perhentian Island coast, B and D exhibited the strongest activity against HL-60 cell line (CD_{50}; 7.9 and 10.4 g/mL respectively), moderate activities by J and L (CD_{50}; 12.0 and 13.2 g/mL respectively) from Kapas Island coast, while weak activity was shown by sample A (CD_{50}; 27.6 g/mL) from Perhentian Island coast (Table 3).

On the other hand, the cytotoxicity against MCF-7 resulted in moderate activity shown by extract J (CD_{50}; 12.7 g/mL) and followed by weak activity of sample D (CD_{50}; 25.5 g/mL). According to the result of the experiment other extracts were not active against HL-60 and MCF-7 cell lines with CD_{50} values of more than 30 μg/mL. Thus, the cytotoxic activity of the extracts became dependent on the locality of the sample. Interestingly, the evaluation of cytotoxic activity of ABCE against panels of cancer cell lines has resulted in positive outcomes (Table 4). ABCE showed strong activity against both HL-60 and MCF-7 cells with CD_{50}; (5.6±0.1) g/mL and (3.3±2.1) g/mL, respectively. Subsequently, ABCE also showed moderate activity against K562, CEM-SS and WEHI-3B with CD_{50} values of 12.5, 11.2 and 12.6 g/mL respectively. However, weak activity was obtained against HeLa cell line with CD_{50} value of 20.5 g/mL. There were some significant differences in cytotoxic percentages between the cell treated with higher doses of ABCE (30, 15 and 7.5 μg/mL) and the untreated in all panels of cell lines at $P<0.05$ (*t*-test) (Figure 1). Meanwhile, a significant difference could be seen between the higher and lower groups of treatment doses in all treated-cell cultures ($P<0.05$).

Table 3

IC_{50} value (g/mL) of 12 crude methanolic extracts of *A. aaptos* (marine sponges) collected off various locations in Terengganu against HL-60 and MCF-7.

Code	Locations	Specimens	CD_{50} value (g/mL)	
			HL60	MCF-7
P01/011/04	Perhentian Island	A	27.6±0.8	>30.0
P02/010/04	Perhentian Island	B	7.9±1.0	>30.0
P02/009/04	Perhentian Island	C	>30.0	>30.0
P03/015/04	Perhentian Island	D	10.4±0.4	25.5±0.5
R01/010/04	Redang Island	E	>30.0	>30.0
R03/007/04	Redang Island	F	>30.0	>30.0
B01/010/04	Bidang Island	G	>30.0	>30.0
K01/025/04	Kapas Island	H	>30.0	>30.0
K01/028/04	Kapas Island	I	>30.0	>30.0
K01/010/05	Kapas Island	J	12.0±0.9	12.7±1.6
K02/011/05	Kapas Island	K	>30.0	>30.0
K03/010/05	Kapas Island	L	13.2±0.4	>30.0

Note: Sample with IC_{50} less than 30 g/mL[46] or compound with CD_{50} less than 20 g/mL[47] could be considered as cytotoxic. In this study, samples with CD_{50} <10 g/mL were classified as having strong cytotoxic activity, 10 to 20 g/mL were considered as moderate activity while CD_{50} ranging from 20 to 30 g/mL were grouped as weak cytotoxic activity.

Table 4

CD_{50} value (g/mL) of hexane, dichloromethane, ethyl acetate and methanol extracts from *A. aaptos* against HL-60, MCF-7, K562, HeLa, CEM-SS and WEHI-3B.

Code	CD_{50} value (g/mL)					
	HL60	MCF-7	WEHI-3B	K562	HeLa	CEM-SS
ABHE	24.0±0.3	23.5±0.5	nd	nd	nd	nd
ABCE	5.6±0.1	3.3±2.1	12.6±1.9	12.5±0.9	20.5±1.4	11.2±3.4
ABEE	>30.0	>30.0	nd	nd	nd	nd
ABME	NT	NT	NT	NT	NT	NT

Note: NT: Not tested, Fraction extracts; ABHE: hexane extract, ABEE: ethyl acetate extract, ABCE: chloroform extract, and ABME: methanol extract. The results were the mean values of three separate experiments (mean ± SD).

3.4. Antiviral against HSV-1

Regarding the determination for anti-HSV-1 activity, the ABHE, ABCE, ABEE and ABME were firstly evaluated for their cytotoxic effect alone against Vero cells (mock-treated cells). The samples were exposed to the cells for 72 h under the same culture condition used in anti-HSV-1 assay. The cytotoxic effect of the samples against Vero cells was microscopically visible with all of the cell monolayers detaching from its culture vessels thus resulting the viability of cells are being compromised. Sample ABHE, ABCE and ABEE (at concentration 100 g/mL) showed low cytotoxic activity with the percentage of reduction (destruction) in dehyrogenase enzyme of

10.2% to 3.3% after 72 h of treatment. However, the cytotoxic effect was not observed in the cells that were exposed to ABME. Thus, CC_{50} of all fractions was not determined due to their percentage of cytotoxic effects being too small and not exceeding 50% (Table 5). After studying the cytotoxicity effect of samples against normal Vero cells, this study was followed by an experiment conducted to assess the antiviral effect of samples against HSV-1. 0.001 ID_{50}/cells of MOI at $2 \times 10^{8.5}$ ID_{50}/mL. Table 5 showed the potential of antiviral activity of ABCE when it was compared to other fractions in which 100% of protection was achieved at 100 g/mL. The EC_{50} value of ABCE was (60.5±1.2) g/mL. No activity was displayed in HSV-1 treated-ABHE, ABEE and ABME with percentage of virus inactivation being lower than 50% (11.6%, 15.7% and 27.8%, respectively).

Table 5

Inactivation effect of HSV-1 assayed by neutral red uptake method.

Fractions/ Compounds	EC_{50} (μg/mL) *	% Protection	% Destruction
Acyclovir	0.13 ± 0.1	100.0 ± 0.3[a]	24.2 ± 1.8[a]
ABHE	-	11.6 ± 0.1[b]	5.8 ± 0.9[b]
ABCE	60.5 ± 1.2	100.0 ± 11.3[c]	10.2 ± 0.02[c]
ABEE	-	15.7 ± 0.4[b]	3.3 ± 0.3[b]
ABME	-	27.8 ± 0.5[b]	0.0[b]

Note: MOI used was 0.001 ID_{50}/cells ($2 \times 10^{8.5}$ ID_{50}/mL). Vero cells were seeded for 1 h before infected with HSV-1 and incubated with sample for 72 h. EC_{50}: Effective concentration of 50% cells protection, CC_{50}: Cytotoxic concentration of 50% cells destruction; values obtained was too large to be measured (was not inserted in the table). % Protection: Percentage of cells protection at optimal concentration, % Destruction: Percentage of cells destruction at concentration of maximum cells protection. [a]Optimal concentration = 0.5 g/mL, [b] Optimal concentration = 100 g/mL, [c]Optimal concentration = 75 g/mL.

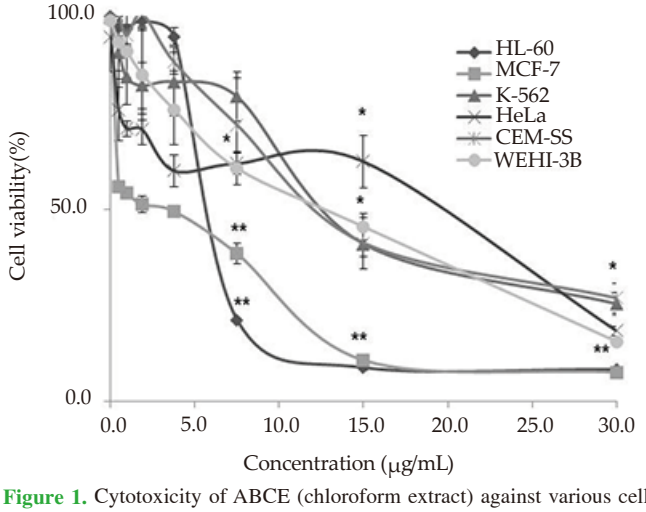

Figure 1. Cytotoxicity of ABCE (chloroform extract) against various cell lines: HL-60, MCF-7, WEHI-3B, K562, HeL and CEM-SS.

Note: The respective CD_{50} obtained were 5.6 g/mL, 3.3 g/mL, 12.6 g/mL, 12.5 g/mL, 20.5 g/mL and 11.2 g/mL. Viability was determined by MTT assay and each standard error bar represents the mean ± SD of triplicate experiments. Asteriks indicate significant difference levels where $^{*}P < 0.05$ and $^{**}P < 0.01$ between the experimental and control values using *t*-test.

3.5. Purification and characterization of p-hydroxybenzamide

The *p*-hydroxybenzamide (Figure 2) was isolated as white powdered compound from butanol fractions obtained after solvent partitioning of methanol extract of *A. aaptos*, followed by a repeated column chromatography on silica gel and Sephadex LH20. Spectroscopic data of *p*-hydroxybenzamide were recorded as below: IR υ_{max} cm^{-1} (KBr disc): 3369 and 3188 (NH_2 group), 3080 (O-H), 1650 (C=O), 1638 (C=C), 1317 (C-O), and 1198 (C-N stretching), EIMS *m/z* (rel. int): 137 $[M]^+$ (31.69), 107 (100.00), 93.05 (17.58), 77.0 (62.98), 63.00 (52.79).

^{1}H-NMR (CD_3OD, 600 MHz) δ ppm: 7.39 (*d*, 7.8 Hz, 2H, H-2 and H-6), 5.61 (*d*, 7.8 Hz, 2H, H-3 and H-5), 1.89 (*s*, 2H, NH_2).

^{13}C-NMR (CD_3OD, 150 MHz) δ ppm: 110.9 (C-1), 142.2 (C-2 and C-6), 100.9 (C-3 and C-5), 152.1 (C-4 (OH)), 166.0 (C=O).

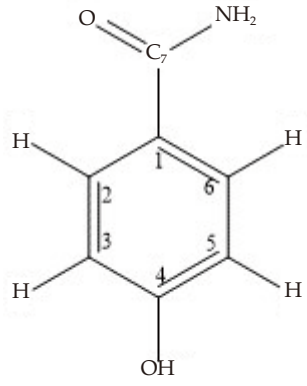

Figure 2. *p*-Hydroxybenzamide.

4. Discussion

The births of hundreds of new metabolites have been discovered every year. Although more than 5 300 different natural products have been purified from marine sponges and their associated microorganisms, sponges still remain as an important marine organism for the discovery of new bioactive natural products. Many substances such as bioactive alkaloids, sterols, terpenes, amino acid derivatives, cyclic peptides, peroxides, unusual nucleosides (most probably halogenated) and FCs have been identified from sponges and their associated microorganisms. Most of these natural products that come from sponges have shown a wide range of pharmacological activities such as antiviral, anticholestrolemic, anthelmintic, anticancer, antifungal, antiprotozoal, anti-inflammatory, immunosuppressive, antifouling activities, antimalarial, antitumour, cardiovascular agent, antihelminthic, muscle relaxant agent and neurosuppressive[49-51].

Subsequently, FCs have been widely distributed in marine sponges

as they play significant functional and structural roles in plasma membrane and biogenesis. Lipids and FA have performed an important role in stress resistance by maintaining proper membrane function so as to to endure the tough environmental changes that bring effects to marine sponges. Sponges with higher composition of storage lipids, phospholipids, sterols, *n*-3 and *n*-6 polyunsaturated fatty acid (PUFA) have displayed the highest level of resistance to ocean warming and ocean acidification[52]. Besides, high levels of polyunsatured long chain fatty acids (C_{24}–C_{30}), high branched and odd-chain fatty acids in marine cold-water sponges of genus *Latrunculia*, namely *Latrunculia bocagei* Ridley and Dendy, 1886, and *Latrunculia biformis*, to some extent have been needed for cell membrane integration so as to survive in low temperatures ocean[53]. In physiological function, long-chain *n*-3 polyunsaturated fatty acids (*n*-3 LC-PUFA), likewise have the potential to decrease inflammation in both *in vitro* and *in vivo* studies. Furthermore, the *n*-3 LC-PUFA eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3) inhibit interleukin-1β and interleukin-6 production in human macrophages. It has been proposed that *n*-3 LC-PUFA may play a prominent role as alimentary therapeutic component for the inhibition and treatment of the inflammatory ailments[54]. In addition, prescribing DHA with UA-lowering medicine may enhance the regulation of blood glucose levels in diabetic patients with high level of UA[55]. EPA and DHA also have improved some cardiovascular risk factors[56].

Many fatty acids have been originated from unusual biosynthetic pathways, thus this displays unusual characteristic of unsaturated patterns, exhibit terminal and/or mid-chain branching. They may happen as mono-, di- and tri- unsaturated and cover an extensive carbon-number range normally C_{24}-C_{30}. Marine sponges also are a rich source of brominated, α-methoxylated, acetylenic, branched fatty acids as well aspolyoxygenated fatty acid amides[3,50,57]. However, based on the result obtained in this study, it has been found that *Aaptos* sp. only contained common saturated and monoenoic fatty acid and it is comparable with *Ircinia spinulosa*[58]. However, its FC content is different compared to marine sponges (*Latrunculia*) and seaweed (*Sargassum granuliferum*, *Ulva armoricana*, and *Solieria chordalis*, *Gracilaria* sp.) which has high PUFA content[8,53,59,60].

Nevertheless, from this study, the five major of FAMEs with each concentration patent in each extract were: behenic acid; ethyl acetate > methanol > chloroform > hexane extract, *cis*-10-heptadecenoic acid; hexane > chloroform > methanol ≥ ethyl acetate, palmitic acid; chloroform > hexane > ethyl acetate > methanol, and *cis*-10-pentadecenoic acids; hexane > methanol ≥ chloroform > ethyl acetate. To summarize, chloroform, ethyl acetate and methanol extracts have shown similarity in higher proportion of straight chain saturated fatty acid, showing 28.5%, 46.4% and 40.9% (total of three highest values) of total FAME content respectively. However, FA composition in hexane extract was more dominated by unsaturated FC with a proportion of 35.9%. The domination of palmitic acid in the chloroform extract of marine sponges has long been proven since 1980s and up to 2000s as reported by Carballeira & Maldonado, who discovered that chloroform/methanol extract of marine sponge *Chondrilla nucula* was rich in palmitic acid which accounted for 26.0% of its total FA[61]. Later, Lee *et al*. reported that the high concentration of saturated fatty acid isolated from Bahamas marine sponge was dominated by palmitic acid and octadecanoic acid[62].

In addition to the determination of FC, the DPPH free radical scavenging activity and total phenolic content of the respective extracts were also carried out. Among the extracts, the highest and lowest content of phenolics that are observed in sample K and H from Kapas Island coast, respectively. There were claims that the antioxidant activity of extracts should be proportional to its total phenolic contents[63]. However, this study has shown that extracts containing high phenolic contents did not always reveal high antioxidant activity. It has been found that ABCE and ABME have displayed high potential of DPPH free radical scavenging (67.6 ± 0.1)% and (8.2 ± 0.1)%, respectively. The total phenolics content of ABCE and ABME were (10.50 ± 0.01) and (6.50 ± 0.04) mg/g GA, respectively. This may be due to the fact that different phenolic compounds have different responses in the experiment conducted by Folin-Ciocalteu[64]. Hence, the molecular antioxidant response of phenolic compounds varied remarkably, depending on their chemical structures[36]. Therefore, the antioxidant activity of an extract cannot be predicted solely on the basis of its total phenolic content. This prediction indicated that there are other factors than the total phenolics which can play a major role in the antioxidant activity of tested materials. Besides that, interference from other chemical components presented in the extract, such as sugar or ascorbic acid also could lead to this possible outcome[65].

Phenolic compounds are rarely distributed in marine sponges. Most of the phenolic compounds obtained from marine sponges were produced by their microbial symbionts such as cytotoxic phenolic bisabolanesesquiterpenoid dimers disydonol A-C which have been isolated from an endophytic *Aspergillus* sp. (sponge *Xestospongia testudinaria*, Weizho Is., South China Sea)[66] and two antifungal and antioxidative sesquiterpene phenols, (+)-curcupheno1 and (+)-curcudiol, from both deep and shallow water collections of the sponge *Didiscus flavus* van Soest[67,68]. Being said that, this study also wish to report the isolation of a phenolic composition, *p*-hydroxybenzamide from methanol extract of *A. aaptos*. However, the result from the isolation only showed low free radical scavenging activity (22% inhibition) with IC_{50}>10 mg/mL. Hydroxybenzamides are important and well-known phenolic compounds. Owing to the wide range of biological activities of their derivatives, considerable interest has been placed on derivatization of these compounds.

Benzamides derivatives were reported to display cerebroprotective activity, anti-leishmanial activity, antibacterial and antifungal, antipsychotic, and have potential for treating atheroschlerotic and cardiovascular diseases[69-73].

The cytotoxic activity screenings of marine sponge crude extract has attracted the attention of the marine natural product research community. For example, Seleghim *et al*. discussed in his study, that out of 215 Brazilian sponge crude extracts, 11% have displayed cytotoxic activity against MCF-7 breast cancer cell, while 18 % against HCT-8 colon cancer cells, and 8% against B16 murine melanoma cancer cells[74]. Previously, the crude methanolic extract of *A. aaptos* was collected at different dates and locations exhibited cytotoxic activity against HL-60, MCF-7, CEM-SS, HeLa, HT-29 and L929[9].

Compared to other fractions such ABHE, ABEE and ABEE, the sample ABCE exhibited potential antiviral activity. A previous study also reported that 2 g/mL of methanol-methylene chloride extract of *Aaptos* sp. from Abrolhos, Brazil could inhibit 76% of HSV-1 replication in Vero cells[30]. It has been reported that several derivatives of myristic acid and palmitic acid have been effective in inhibiting herpes viruses[75], whereas lauric acids inhibit the arena virus production[76]. Recently, fatty acid esters of antiviral drug entecavir were prepared by going through the process of esterification by reacting *n*-tetradecanoic acid, *n*-hexadecanoic acid, and *n*-octadecanoic acid with entecavir to produce novel lipidic prodrug of entecavir for parenteral sustained delivery[77]. In response to that experiment, octacosanoic acid was found to be cytotoxic against HL-60 cell line[19]. Whereas, Ito *et al*[78] suggested that palmitoleic acid induced the change in the lipid composition of tumour cells hence resulting in the damage of the cells. As stated by Ito, palmitic acid could trigger an increase of dose-dependent in apoptosis of MG 63 cell line[79]. Other FCs, 4-Me-6E, 8E-hexadecadienoic is reported to reduce the viability of MCF-7 breast cancer cells in a dose dependent manner (up to 63%) and the gene expression of two lipogenic enzymes, the acetyl CoA carboxylase and the fatty acid synthase[80]. Thus, this study suggested that the fatty acid constituents as the results shown might have some contribution in the cytotoxic and antiviral properties of the extracts, in addition to the aaptaminoids. Previously, we reported that 3-(phenethylamino)dimethyl(oxy) aaptamine isolated from this *A. aaptos* induced the apoptosis and contributed in anti-HSV-1 activity[16]. Besides, the isoaaptamine from *A. aaptos* was also reported to induce T-47D cells apoptosis and autophagy via oxidative stress making it is a good candidate for breast cancer treatment[81]. In another study, nucleosides (Ara-A and Ara-C, mycalamide A, mycalamide B), sesquiterpene hydroquinones (Avarol), cyclic depsipeptides (papuamide A, B, C, and D, microspinosamide), alkaloid (4-methylaaptamine, dragmacidin F, manzamine A), phenolic macrolides (hamigeran B) were among the antiviral substances that belong to marine sponges[82]. In 2015, a broad spectrum of natural antiviral drugs have been reviewed by Martinez *et al*[83], only mycophenolic acid has a short chain fatty residue. In the chemical structure of mycophenolic acid, 4-methyl-4-hexenoic acid residue was attached to a phthalanylmoeity that was fused to an oxoisobenzofuran-6-yl.

Conflict of interest statement

We declare that we have no conflict of interest.

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