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Evaluation of Toxicity and Anti-Oxidation Activity of the Extracts from *Halymenia durvillei*

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Abstract

Halymenia durvillei (HD), a marine red alga, is believed to have potentials for pharmacological, nutritional and cosmetic applications. However, such potentials are acceptable only when their extracts are devoid of any adverse effects on human health. No previous research has been conducted the toxicity and anti-oxidation capacity of HD. Thus, the aim of this work was to investigate toxicity and anti-oxidation activities of HD extracts. In this study, the toxicity and anti-oxidation capacity of 5 fractions of HD solvent extracts, i.e., ethanol (HDET), hexane (HDHE), ethyl acetate (HDEA), butanol (HDBU), and aqueous (HDAQ) were evaluated. The cytotoxicity was evaluated by MTT and LDH assays on 4 cell types, i.e., fibroblast, macrophage, hepatocyte and keratinocyte. The genotoxicity was evaluated by comet assay and micronucleus test using TK6 lymphoblastoid cell line. The anti-oxidation capacity was investigated by DPPH and ABTS assays. The toxicity studies showed that HDET, HDBU, HDAQ had very low to no toxicity as indicated by cytotoxicity and genotoxicity tests while HDEA, HDHE have some toxicity at high concentrations. HDAQ showed low antioxidant activity while HDET, HDEA, HDHE and HDBU possess relatively high antioxidant activity. Overall, our results indicated that HDET and HDAQ could be consumed as they are not toxic and HDHE, HDEA, and HDBU could be safely consumed at doses lower than 100 µg/mL. Further investigation using in vivo assays are needed to ensure the safety of HD extracts for animal and human consumptions.

Keywords: *Halymenia durvillei*, Cytotoxicity, Genotoxicity, Antioxidant, Safety

Introduction

Macroalgae, including marine algae from the Protista orders, Phaeophyta (brown), Chlorophyta (green), and Rhodophyta (red), have a long history of being used as human diet [1]. Marine algae are a low-calorie food, which contain a high concentration of minerals, vitamins, and proteins and low lipid content [2]. Furthermore, marine algae produce many bioactive secondary metabolites such as polyphenolic compounds, polysaccharides, steroids, fatty acids, carotenoids, mycosporine-like amino acids, halogenated compounds, polyketides, lectins, peptides and their derivatives [3,4] which have been used in biomedical applications. In addition to the above-mentioned compounds, red seaweed of *Halymenia* spp. also contains several other useful compounds, for examples, *H. durvillei* has a high amount of sulfated galactan [5], *Halymenia floresii* (*H. floresii*) has monohydroxy acetylated sterol-halymeniaol which has been reported to exert antimalarial effect [6], Sulfated galactan from *Halymenia dilatate* (*H. dilatate*) showed enhanced the antioxidant properties and prevents *Aeromonas hydrophila* infection [7], and 3-(Hydroxyacetyl)indole and Indole-3-carboxylic acid from ethyl acetate extract of *H. durvillei* showed anti-lung cancer cell and *in vivo* anti-aging activity [8]. Because of the richness of these

natural compounds, *H. durvillei* (HD), the red seaweed that is widely grown in Thailand water, is considered as an excellent starting material which can be processed into pharmaceutical, nutraceutical and cosmeceutical products for humans. However, the extracts from this sea weed must first be tested for their toxicity and oxidative capacity before being developed into safe consumable products. In this study, we tested solvent extracts of HD for their cytotoxicity in causing cell lysis and death by LDH and MTT assays, and possible genotoxicity, especially the breakage of DNA and chromosomes, by Comet and Micronucleus essays. In addition, we also tested the antioxidation capacity of the HD extracts for their possible uses as antioxidants to mop up free radicals that may be damaging to cells. Free radicals are atoms or molecules which contain one or more unpaired electrons. Free radicals consist mainly of reactive oxygen species (ROS), such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), which possess powerful oxidizing capacities that enable them to react with macro- and micro-molecules such as proteins, nucleic acids, or lipids. This event can cause protein misfold, DNA damage, and lipid peroxidation that are highly damaging to cells and cause pathogenesis of several human diseases [9-11]. In the present study we tested the antioxidant capacity of the HD extracts by DPPH and ABTS assays.

Materials and methods

Plant materials and crude extraction

H. durvillei was pond-cultured by Ms. Montakan Tamtin, Phetchaburi Coastal Fisheries Research and Development Center, Thailand. The algae were extensively washed with tap water and dried at room temperature (RT). One kilogram of air-dried *H. durvillei*, was milled and macerated successively with 5 L 95 % EtOH at RT for 7 days. Next, the EtOH extract was successively partitioned in solvents with increasing polarity from n-hexane (HE), ethyl acetate (EA), n-butanol (BU) and distilled water (AQ) according to Manohong *et al.* [8]. The yields after evaporation of the solvents under reduced pressure were compared with dried weight of the seaweed, which for various fractions designated as HDET, HDHE, HDEA, HDBU, HDAQ were 4.47, 5.33, 1.49, 6.34 and 10.89 %, respectively. The extracts were then filtered and evaporated under vacuum. Powders from the extracts were dissolved in 100 % DMSO at 1 mg/mL and kept as stock solutions and stored at −20 °C.

Cells and cell culture

Mouse fibroblast (L929), mouse macrophages (RAW 264.7), mouse hepatocyte (FL83B), keratinocytes (HaCaT), and human lymphoblast cells (TK6) were purchased from American Type Culture Collection (ATCC). RAW264.7 and TK6 cells were cultured in RPMI medium supplemented with 10 % fetal bovine serum (FBS) and 100 U/mL penicillin/100 µg/mL streptomycin. L929, FL83B, and HaCaT cells were cultured in DMEM medium supplemented with 10 % FBS and 100 U/mL penicillin/100 µg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and passaged every 2 - 3 days when they were approximately 90 % confluent. For treatments with HD extracts, cells were collected under exponential growth phase and seeded at adjusted density for different culture flasks or well plates. When they reached the confluence, cells were washed twice with phosphate buffered saline (PBS) and then treated with fresh medium containing HD extracts.

MTT cytotoxicity assay

For the MTT assay [12,13], 8×10^3 cells/wells of each cell type were seeded into 96-well plate and incubated for 24 h until cells attached to the plate before adding the extracts. After the incubation period, the cells were treated with HD extracts at concentrations ranging from 10 - 1,000 µg/mL for 24 h. Then culture medium was discarded and 1 mL of MTT reagent (5 mg/mL) (Sigma-Aldrich, U.S.A.) in PBS was added to each well. The plates were incubated at 37 °C for 3 h. At the end of the incubation period, the medium was removed and 100 µL DMSO (analytical grade) was added to each well to dissolve insoluble formazan crystals. The color being formed by formazan crystals was measured by reading the absorbance at 570 nm in a microplate reader (VersaMax microplate reader). The percentage of viable cells was calculated after normalization with the negative control (PBS), which was considered to have 100 % cell viability. The cytotoxicity value was presented as IC50 (the median growth inhibitory concentration) of the extracts. IC50 values were calculated by GraphPad Prism 7 software.

LDH cytotoxicity assay

For LDH assay [12,13], 100 µl of 8×10^3 cells/wells of each cell type in culture medium were seeded into 96-well plate and incubated for 24 h when cell attachment was completed. Then, the cells were treated with seaweed extracts by adding 100 µL/well of various concentrations of the extracts ranging

from 10 to 1000 µg/mL, and incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % of CO_2 for 24 h. At the end of incubation period, 5 µL of lysis solution (Roche, U.S.A.) was added to the well as a positive (high) control while the well without the extracts and lysis solution was used as a negative (low) control. The plate was incubated for an additional 15 min. A 50 µL of culture medium was transferred from each well to a new 96 well plate. Then, 100 µL of the freshly prepared reaction mixture for LDH activity from LDH kit (Roche, U.S.A.) was added to each well on the 96-well plate and incubated for 30 min at room temperature (RT) in the dark. Finally, 50 µL of stop solution (Roche, U.S.A.) was added to each well on the 96-well plate. The reduction of yellow tetrazolium salt, INT, by NADH into a red, water-soluble formazan-class dye was determined by a microplate reader (VersaMax microplate reader) at a wavelength of 490 nm and percent cytotoxicity of each extract was calculated based on the following equation;

$$\text{LDH activity (\%)} = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} x\ 100$$

Comet assay for genotoxicity test

For comet assay [12,13], 100 µL of 2×10^5 cells/well of lymphoblast cells in RPMI medium were seeded into 24-well plate and incubated for 24 h. After the incubation period, the cells were treated with seaweed extracts at concentrations ranging from 10 to 1,000 µg/mL for 24 h. After the incubation, an aliquot of 1×10^5 cells were centrifuged for 5 min at 3,000 rpm, then the incubation medium was removed and the pellet was washed with PBS pH 7.4. The cells in PBS were resuspended in molten low melting point (LMP) agarose at a ratio of 1:10 (v/v). 100 µL of cells in LMP agarose was laid on a glass slide pre-coated with 1 % normal melting point agarose. After gel solidified at RT, the slides were placed in a Coplin jar and immersed in ice-cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base, pH10, supplemented with 1 % Triton X-100) for overnight at 4 °C in the dark, to lyse the cells and separated DNA from histones. The lysis solution was removed and slides were immersed in DNA unwinding solution (0.3M NaOH, 1mM EDTA, pH 13) for 20 min in the dark at room temperature, followed by gel electrophoresis at 20 V for 20 min. Finally, slides were neutralized with neutralization buffer (0.4M Tris, pH 7.5) for 5 min, then stained with an SYBR Green. Images were immediately taken with fluorescence microscope at 400× magnification, and analyzed by the image analysis software CometScore 2.0. The results were recorded by examining at least 50 randomly selected nonoverlapping cells per culture well with a double-blind observation. Following parameters were recorded: tail length, %tail DNA, and tail moment. Tail length was the length of the comet tail indicating the extent of DNA breakage. %Tail DNA was the fluorescence intensity from the UV-illuminated damaged DNA in the comet tail, and the tail moment was calculated from tail length multiply by %tail DNA. The data from the experimental groups were compared with the blank control.

Micronucleus test (MN)

For the micronucleus test [12,13], 2×10^5 cells/wells of lymphoblasts in the RPMI medium were seeded into 24-well plate and incubated for 24 h. At the end of incubation, cells were centrifuged at 3,000 rpm for 5 min. The cells were collected and resuspended in fresh culture medium and transferred to a new 24-well plate. A 6 µg/mL of cytochalasin B was added to each well and incubated for 24 h, after which cells at binucleated stage were collected by centrifugation at 3000 rpm for 5 min, and subjected to a hypotonic solution (5 mL of 0.075M KCl) followed by another centrifugation. The cells were collected and fixed with methanol/glacial acetic acid (3:1), centrifuged, and the pellet was resuspended in a small volume of fresh fixative, and then 20 µL drops were laid on a clean microscope slide. After drying, the cells were stained with 2 % Giemsa-Romanowski solution for 10 min. Micronuclei (MN) formation was scored among 1,000 binucleated cells (BNC) under a light microscope (40×) and the frequency (%) of micronucleated cells were determined.

DPPH anti-oxidation assay

DPPH assay [14,15] comprised 4 groups: group I (Sample+Met), each of the 5 fractions of *H. durvillei* was diluted with methanol at concentrations 250, 500, 750, 1,000, 1500 and 2,000 µg/mL. A 0.1 mL of each concentration was mixed with 0.1 mL of methanol in 96-well plate; group II (Met), 0.2 mL of methanol was added in each well of the 96-well plate; group III (DPPH+Met), a 0.1 mL of DPPH working solution was mixed with 0.1 mL of methanol in 96-well plate; and group IV (Sample+DPPH), each fraction of *H. durvillei* extracts and positive controls (2, 4, 6, 8, and 10 µg/mL of vitamin C and quercetin) were diluted with 0.1 mL methanol, then they were mixed with 0.1 mL of DPPH working

solution. After that, all samples in each group were thoroughly mixed and left standing at room temperature in the dark for 30 min. Finally, the absorbance of each sample was measured at 517 nm. The EC50 value of each fraction was extrapolated from the linear equation by substituting the percent of scavenging effect (y) at 50 %.

ABTS anti-oxidation assay

ABTS assay [16] also comprised 4-groups: group I (Sample+Met), a 0.1 mL of each concentration of the 5 *H. durvillei* extracts (diluted with methanol at 250, 500, 750, 1,000, 1,500 and 2,000 µg/mL) was mixed with 0.1 mL of methanol in 96-well plates; group II(Met), 0.2 mL of methanol was added to each well of the 96-well plates; group III(ABTS+Met), 0.1 mL of methanol and 0.1 mL of ABTS reagent were mixed in each well on the 96-well plates; and group IV (Sample+ABTS) a 0.1 mL of each concentration of the 5 fractions of H. durvillei and positive controls (2, 4, 6, 8, and 10 µg/mL of vitamin C and quercetin) were diluted with 0.1 mL methanol, then they were mixed with 0.1 mL of DPPH working solution. All samples were thoroughly mixed and incubated at room temperature in the dark for 20 min. After that, the absorbance of each sample was measured at 734 nm. The EC50 value of each fraction was extrapolated from the linear equation by substituting the percent of scavenging effect (y) at 50 %.

Statistical analysis

Results are presented as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Dunnett's test were used to determine significant differences between extracts and controls or vehicles. These analyses were performed using GraphPad Prism 7 for Windows. The significance level was inferred at $p < 0.001$ for all statistical tests.

Results and discussion

Cytotoxicity of extracts from *H. durvillei* by MTT and LDH assays

In MTT assay, after 24 h the IC50 of HDET and HDAQ for all cell types were more than 1,000 µg/mL which indicated that these fractions were non-cytotoxic. IC50 of HDHE, HDEA and HDBU for L929 cells, were 316.7, 514.7, and 531.0 µg/mL, for FL83B cells IC50 were 143.5, 171.3, and 425.6 µg/mL, for RAW 264.7 cells IC50 were 127.6, 229.6, and 456.4 µg/mL, and for HaCaT cells, IC50 were 302.8, 702.1, and 936.7 µg/mL, respectively. These results indicated that HDHE, HDEA and HDBU had some cytotoxic effect ranging from relatively high to low (**Figure 1**). Table of IC50 values of MTT assay were shown in **Table 1**. In LDH assay, IC50 of HDET and HDAQ after 24 h incubation for all cell types were more than 1,000 µg/mL, indicating that these fractions were not toxic. IC50 of HDHE, HDEA and HDBU for L929 cells were 212.4, >1,000, >1,000 µg/mL, for FL83B cells IC50 were 489.4, >1,000, >1,000 µg/mL, for RAW 264.7 cells IC50 were 177.6, >1,000, 475.2 µg/mL, and for HaCaT cells, IC50 were 483.0, >1,000, >1,000 µg/mL. These results indicated that HDHE had some toxicity while HDEA and HDBU were relatively less toxic (**Figure 2**). Table of IC50 values of LDH assay was shown in **Table 2**.

Table 1 IC50 values of MTT assay.

Fractions	IC50 (µg/mL)			
	Cell lines			
	L929	RAW 264.7	FL83B	HaCaT
HD-ET	>1,000.00	>1,000.00	>1,000.00	>1,000.00
HD-HE	316.70	127.60	143.50	302.80
HD-EA	514.70	229.60	171.30	702.10
HD-BU	531.00	456.40	425.60	936.70
HD-AQ	>1,000.00	>1,000.00	>1,000.00	>1,000.00

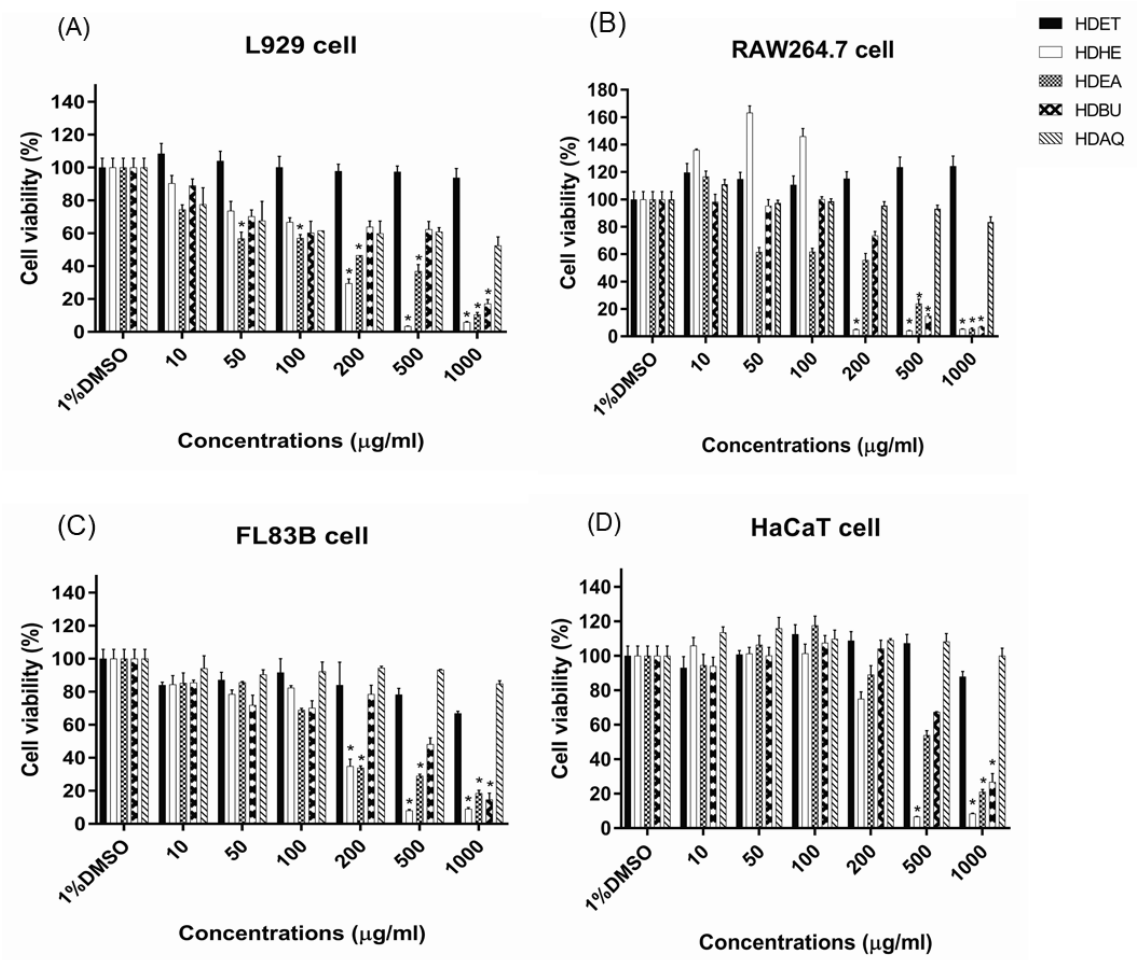

Figure 1 Cell viability of 4 cell types treated with *H. durvillei* extracts for 24 h as determined by the MTT assay. (A) L929 cells; (B) RAW264.7 cells; (C) FL83B cells; (D) HaCaT cells. Data were expressed as mean ± standard deviation of the percentage of cell viability in relation to 1 % DMSO which is used a negative control with 100 % cell viability, *$p < 0.001$.

Table 2 IC50 values of LDH assay.

Fractions	IC50 (µg/ml)			
	Cell lines			
	L929	**RAW 264.7**	**FL83B**	**HaCaT**
HD-ET	>1,000.00	>1,000.00	>1,000.00	>1,000.00
HD-HE	212.40	177.60	489.40	483.00
HD-EA	1,596.00	>1,000.00	>1,000.00	>1,000.00
HD-BU	1,443.00	475.20	>1,000.00	>1,000.00
HD-AQ	>1,000.00	>1,000.00	>1,000.00	>1,000.00

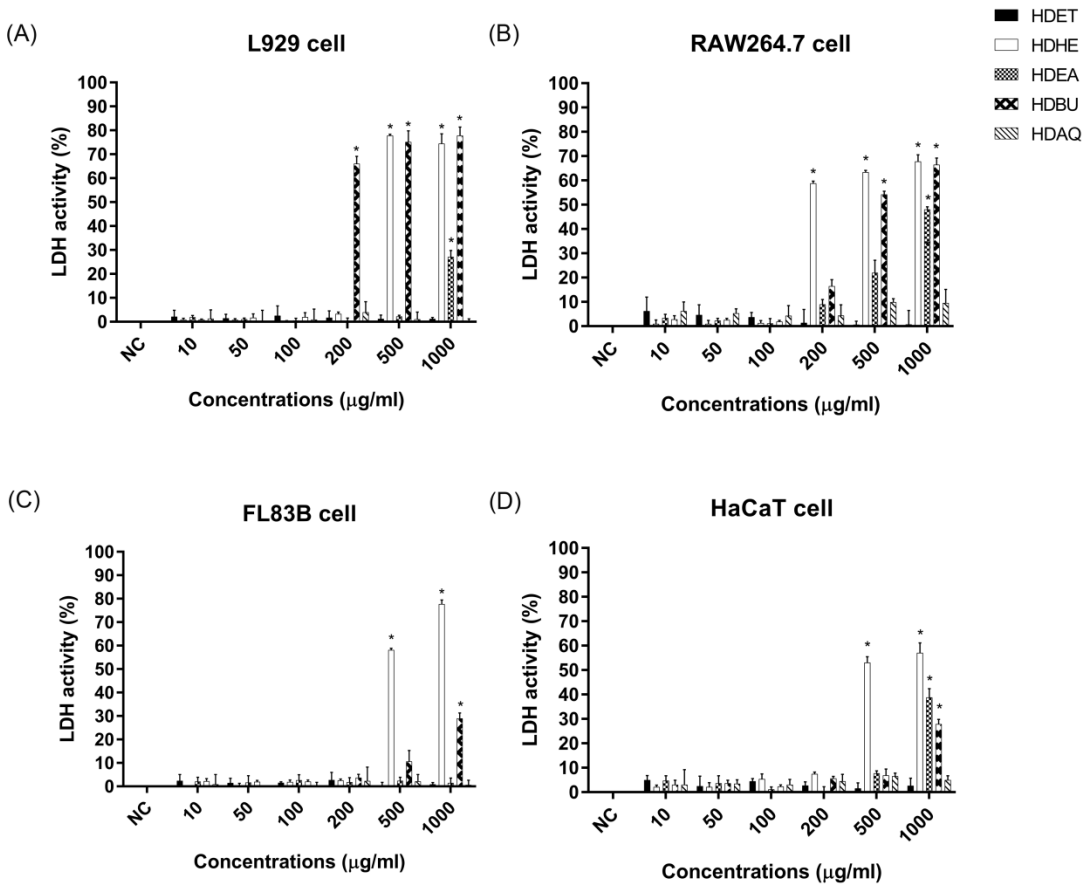

Figure 2 LDH activity of 4 cell types treated with *H. durvillei* extracts for 24 h, as determined by LDH assay: (A) L929 cells; (B) RAW264.7 cells; (C) FL83B cells; (D) HaCaT cells. Data were expressed as mean ± standard deviation of the percentage of LDH activity in relation to the positive control (100 % LDH activity), *$p < 0.001$.

Therefore, from these data it can be concluded that HDET and HDAQ fractions were not toxic with IC50 values more than 1,000 μg/mL, whereas HDHE, HDEA and HDBU showed some degree of cytotoxicity against the 4 cell lines. Our results from the 2 cytotoxic assays showed that the lowest to the highest toxicity of the extracts were HDAQ, HDET, HDBU, HDEA, and HDHE, respectively. Prolonged exposure and higher concentrations of treatments with HDEA and HDHE showed higher toxic effect and lower IC50 value as reported earlier [17]. We found from our initial screening that phytochemicals in extracts from *H. durvillei* contained alkaloids, carotenoids, flavonoids, terpenoids, steroids, glycoside, saponins, and phenolic compounds (unpublished data). These secondary metabolites are exogenous antioxidants that contain in the extract may give either valuable or adverse outcomes in redox balance depending on concentrations being tested or doses being consumed [18,19]. Besides, a few studies showed that exogenous antioxidants gave debatable results, especially at high doses. The type, concentration, and matrix of exogenous antioxidants from a natural compound are the features that affect the balance of the benefits [18]. In general, the cytotoxic activity of a plant against cancer cells is based on their phytochemical properties [19]. These reports are consistent with our study which tend to be at higher concentrations in HDHE, HDEA, and HDBU, can have both useful and harmful effects on cells. Most of these compounds have antioxidant activity which is beneficial to cell survival while some compound like saponin, a membranolytic agent, can exert harmful effect on cells. HDHE, HDEA, and HDBU tended to have higher concentration of saponins than HDET and HDAQ. Saponin has an ability to form complexes with membrane cholesterol leading to pore formation and membrane permeabilization, and it also causes alterations in the negatively charged carbohydrate portions on the cell surface [20-22]. Cytotoxic effects of saponins have been demonstrated in some normal cell lines like fibroblast [23-27], macrophage [28-29], and keratinocyte [30]. These could explain the relatively higher degree of

cytotoxicity of HDHE and HDEA. The above-mentioned substances, including saponin, should also be present in HDET, but the concentrations were too low to cause cytotoxic effect.

Table 3 Genotoxicity was measured by comet assay for tail length, %tail DNA and tail moment in TK6.

Treatment	Tail Length ± SD	%Tail DNA ± SD	Tail moment ± SD
Negative control	0	0	0
Positive control (H_2O_2)	38.97 ± 6.78	44.05 ± 9.04	38.97 ± 6.78
HDET 1,000 µg/mL	1.59 ± 1.55	4.81 ± 2.20	0.14 ± 0.22
HDHE 100 µg/mL	51.93 ± 38.15*	35.13 ± 28.44*	27.56 ± 33.27*
HDHE 200 µg/mL	60.46 ± 21.52*	72.91 ± 10.55*	54.62 ± 29.45*
HDHE 500 µg/mL	72.26 ± 25.26*	79.68 ± 14.55*	75.24 ± 21.06*
HDHE 1,000 µg/mL	N/A	N/A	N/A
HDEA 500 µg/mL	88.93 ± 25.71*	71.70 ± 11.99*	65.83 ± 27.92*
HDEA 1,000 µg/mL	N/A	N/A	N/A
HDBU 500 µg/mL	89.70 ± 11.76*	70.25 ± 9.46*	63.66 ± 14.72*
HDBU 1,000 µg/mL	N/A	N/A	N/A
HDAQ 1,000 µg/mL	1.68 ± 2.21	6.31 ± 3.10	0.07 ± 0.08

S.D = Standard Deviation; H_2O_2 = Hydrogen Peroxide
Significant difference from the control group are calculated and marked with asterisks
(*$p < 0.001$ vs negative control).

Genotoxicity of extracts from *H. durvillei* by Comet assay and Micronucleus test

The results of the genotoxic effects of the extracts from HD on TK6 cell line were presented as the changes in tail length (TL), percentage of DNA in tail (%DNA), and tail moment (TM). HDHE, HDEA and HDBU exhibited genotoxic effects on TK6 cells by significant increases of TL, % DNA, and TM at concentrations above 100 µg/mL for HDHE, 500 µg/mL for HDEA and HDBU. On the other hand, TK6 cells treated with HDET and HDAQ showed no significant changes in TL, %DNA and TM even at maximum concentration of 1,000 µg/mL. These results indicated that HDET and HDAQ had no genotoxic effect, while HDHE was safe at concentrations less than 100 µg/mL, and HDEA and HDBU at concentration less than 500 µg/mL (**Figure 3**, **Table 3**). In micronucleus test chromosomal damage in TK6 cells was shown by the formation of micronuclei containing either chromosome fragments or whole chromosomes beside the dividing binucleated nuclei (**Figure 4A**). The result showed that untreated cells (negative control) exhibited a baseline frequency of micronuclei at 0 % and all cells appeared binucleated (**Figure 4C**). In contrast, micronucleus formation was induced by mitomycin c treatment (positive control) at 57.89 % where the abnormal cells appeared binucleated with micronuclei (**Figure 4D**). TK6 cells treated with HDET and HDAQ showed no micronuclei, indicating that these fractions were not genotoxic. On the other hand, TK6 cells treated with HDHE, HDEA, and HDBU exhibited some degree of genotoxicity at concentration between 200 and 500 µg/mL. The percentages of cells bearing micronuclei after treatments with HDHE were 12.44 and 36.94 %, with HDEA were 19.6 and 28.24 %, and with HDBU were 16.18 and 22.41 % (**Table 4**). These results indicated that HDET and HDAQ had no genotoxic effect, while HDHE, HDEA and HDBU were safe at concentrations less than 100 µg/mL at which no micronuclei occurred.

Information on the genotoxicity of *H. durvillei* is scant. Human lymphoblastoid or TK6 cells have been widely used to test genotoxic effect of compounds, with high sensitivity [31]. Comet parameters used in measuring DNA damage in cells included the percentage of DNA in the tail (% tail DNA), tail length (TL), and tail moment (TM) calculated from fluorescence intensity observed under fluorescent microscope. Our result from comet assay showed that HDET, and HDAQ at the highest concentration at 1,000 µg/mL showed no genotoxicity, whereas HDHE, HDEA, and HDBU showed some degree of genotoxicity at concentrations higher than 100, 500 and 500 µg/mL, respectively. In micronucleus assay HDHE, HDEA, and HDBU caused significant increase of micronucleus frequency (%MN) in TK6 cells

at concentrations higher than 500, 1,000 and 1,000 µg/mL, respectively. By contrast, HDET, and HDAQ did not cause chromosome damage even at the highest dose (1,000 µg/mL) being tested. Tonelli *et al.* [32] indicated that the comet and MN assays could detect genotoxic effects by different mechanisms. The comet assay is generally more sensitive than the MN assay [33,34], as it detects DNA raveling and strand breaks [35], while micronucleus originates later during anaphase from acentric chromosomes or chromatid fragments caused by the disrepair of DNA breaks [36]. Thus, this explained the genotoxicity of HDHE, HDEA, and HDBU at lower doses in comet assay.

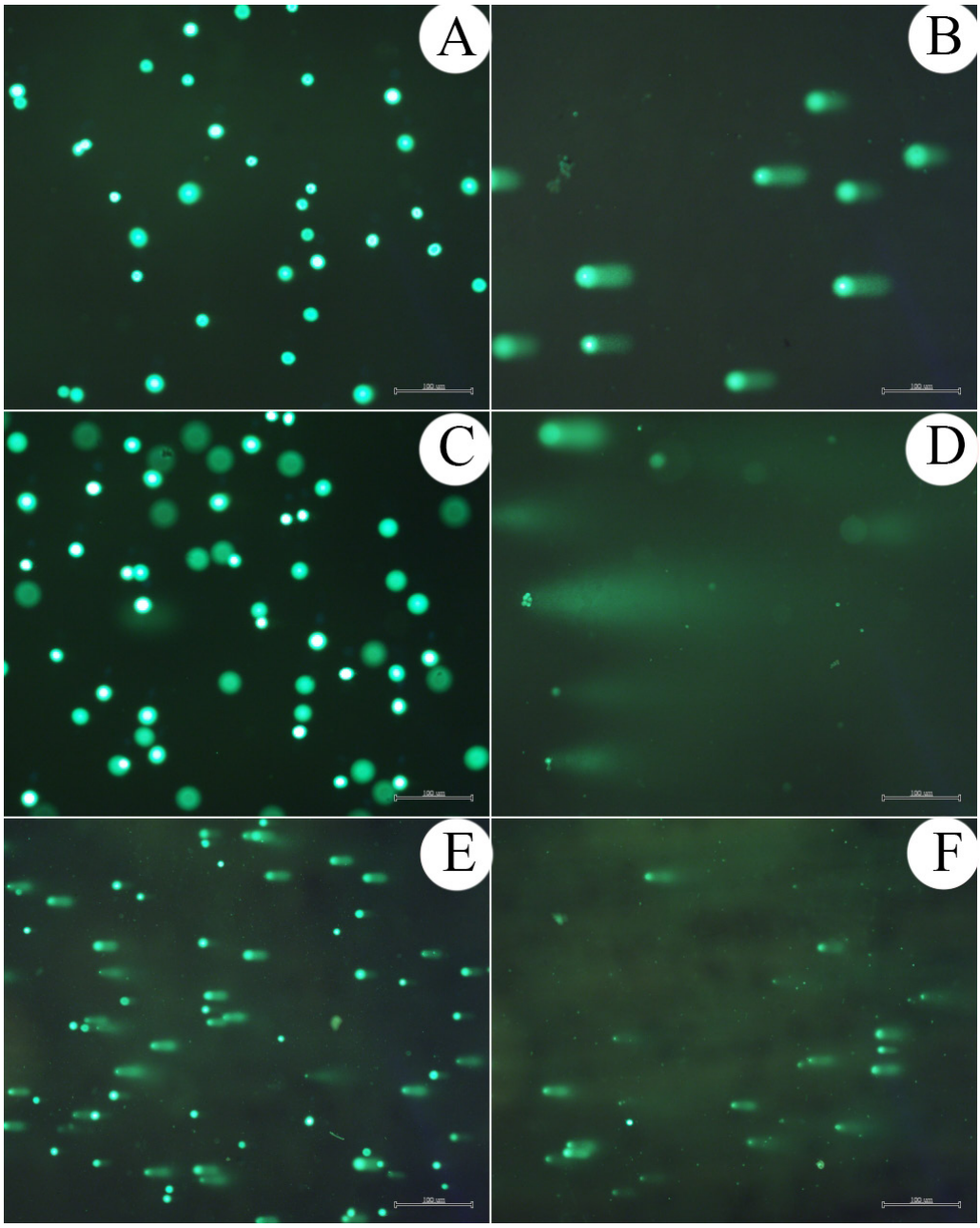

Figure 3 Genotoxicity by comet assay was performed on TK6 cell. The expression of DNA damage as appeared in the comet tail was analyzed by Comet Score 2.0 software. Representative images (at 400× magnification) of TK6 cells after 24 h of exposure to HD extracts and H_2O_2 (50 µM) as a positive control are as follows: A) Negative control, B) Positive control, C) HDET 1000 µg/mL, D) HDHE 500 µg/mL, E) HDEA 500 µg/mL, F) HDBU 500 µg/mL. Long tail of comet-liked appearance is indicative of DNA damage. Scale bar: 100 µm for all images.

Table 4 Genotoxicity as measured by micronucleus assay showing % of TK6 cells with micronuclei after treatment with *H. durvillei* extracts for 24 h. (*$p < 0.001$ vs negative control).

Treatment	The mean values of micronucleus frequencies/ 1000 BNC ± SD	Micronucleus (%)
Mitomycin C 1 µg/mL (Positive control)	578.90 ± 26.45	57.89
HDET 1,000 µg/mL	0	0
HDHE 100 µg/mL	43.05 ± 6.55	4.30
HDHE 200 µg/mL	124.41 ± 19.97	12.44
HDHE 500 µg/mL	369.42 ± 15.93*	36.94*
HDHE 1,000 µg/mL	N/A	N/A
HDEA 100 µg/mL	25.57 ± 6.84	2.55
HDEA 200 µg/mL	32.48 ± 9.27	3.24
HDEA 500 µg/mL	196.03 ± 24.58	19.60
HDEA 1,000 µg/ml	282.24 ± 27.21*	28.22*
HDBU 100 µg/mL	8.13 ± 2.45	0.81
HDBU 200 µg/mL	39.77 ± 10.25	3.97
HDBU 500 µg/mL	161.84 ± 35.29	16.18
HDBU 1,000 µg/mL	224.11 ± 22.43*	22.41*
HDAQ 1,000 µg/mL	0	0

S.D = Standard Deviation
Significant difference from the control group are calculated and marked with asterisks (*$p < 0.001$ vs negative control).

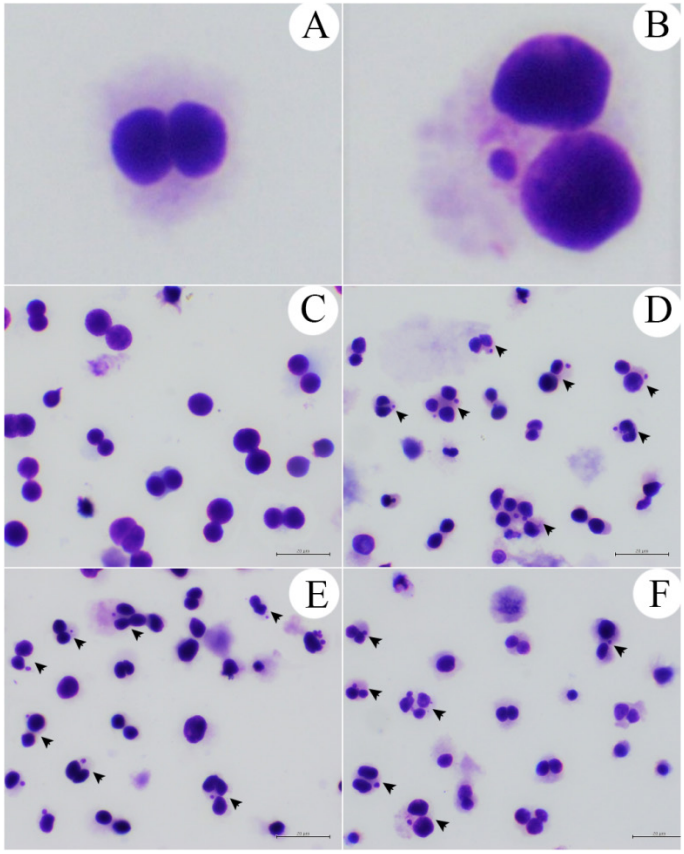

Figure 4 Genotoxicity was measured by micronucleus test. The expression of chromosome damage was displayed by binucleated cells (BNC) with micronucleus. Representative images (400× magnification) of TK6 cells after 24 h of exposure to HD extracts and mitomycin C (1 µg/mL), used as positive control, are as follows: A) BNC (negative control), B) BNC with mitomycin C showing micronucleus (positive control), C) HDET 1,000 µg/mL, D) HDHE 500 µg/mL, E) HDEA 500 µg/mL, F) HDBU 500 µg/mL. Scale bar: 100 µm for all images. Arrow heads indicate binucleated cells with micronucleus.

Anti-oxidation activity of *H. durvillei* extracts by DPPH assay and ABTS assay

The anti-oxidation activity was reflected by the scavenging of DPPH• radicals which appeared in a concentration-dependent manner. The anti-oxidation (scavenging) activity of HDHE ranged from 11.82 to 74.26 %, HDEA from 18.95 to 89.82 %. HDBU from 2.78 to 40.22 %. HDET from 0.00 to 22.22 %, and HDAQ from 6.30 to 5.16 %. The EC50 values were calculated from the linear portions of the dose–response curves of scavenging activity as shown in **Figure 5A**. The EC50 values of HDEA and HDHE fraction were 535 and 1,081 µg/mL, respectively. On the other hand, the EC50 value of HDET, HDBU and HDAQ fractions was more than 3,000 µg/mL. Thus, HDEA and HDHE showed high scavenging activity of DPPH radicals, while HDET, HDBU and HDAQ showed low and very low scavenging activity. By contrast, the EC50 values of vitamin C and quercetin, used as positive controls, were 6.66 and 2.37 µg/mL, respectively, which were 200 - 450 folds higher than HDEA and HDHE fractions since they are pure compounds. The ABTS•+ -scavenging ability reflecting the anti-oxidation capacities of HDHE ranged from 31.28 to 86.18 %, HDEA from 41.28 to 93.27 %, HDBU from 18.61 to 48.22 %, HDET from 0.00 to 25.90 %, and HDAQ, from 15.24 to 20.98 %. The EC50 values were calculated from the linear portions of the dose-response curves as shown in **Figure 5B**. The EC50 values of HDEA and HDHE were 669 and 1,639 µg/mL, respectively. On the other hand, the EC50 value of HDET, HDBU and HDAQ fractions was more than 3,000 µg/mL. Thus, HDHE, HDEA had relatively high while HDET, HDBU and HDAQ fractions showed very low anti-oxidation activity. In comparison EC50 values of vitamin C and quercetin, used as positive controls, were 4.82, 8.43 µg/mL, which indicated that HDEA and HDHE fractions have much lower antioxidation activity when compared to these pure compounds. EC50 values of DPPH and ABTS assay were shown in **Table 5**.

It can be concluded from DPPH and ABTS assays that the highest to lowest antioxidant effect was detected in the HDEA, HDHE, HDET, while HDAQ showed very low antioxidant activity. The high antioxidants were expected to be present in the non-polar extract which might contain non-polar phytochemicals. Antioxidant activity of HDEA being the highest might be because it contained various phytochemicals such as alkaloids, flavonoids, terpenoids, steroids, glycoside, and saponins. The second highest antioxidation activity was observed in HDHE fraction. This fraction also contained phytochemicals that include alkaloids, flavonoids, terpenoids, steroids, glycosides, saponins, and carotenoids with lower concentrations. The third highest antioxidant activity was observed in HDET fraction, which also contained alkaloids, flavonoids, terpenoids, steroids, glycoside, and saponins in lowest concentrations. In a preliminary investigation, we found that HDET, HDHE and HDEA fractions have similar phytochemicals including alkaloids, flavonoids, terpenoids, steroids, glycosides, and saponin but they might be at lowest concentrations in HDET. Thus, HDET has lower antioxidant activity than HDEA and HDHE. Lastly, HDAQ contained low concentrations of flavonoids and glycosides and may be also sulfated polysaccharides, a group of highly polar molecules, which explained its lowest antioxidant capacity.

Table 5 EC50 values of DPPH and ABTS assay.

Fractions	EC50 (µg/mL)	
	DPPH assay	ABTS assay
Vitamin C	6.66	2.37
Quercetin	4.82	8.43
HD-ET	>3,000.00	>3,000.00
HD-HE	1,081.00	1,639.00
HD-EA	535.00	669.00
HD-BU	>3,000.00	>3,000.00
HD-AQ	>3,000.00	>3,000.00

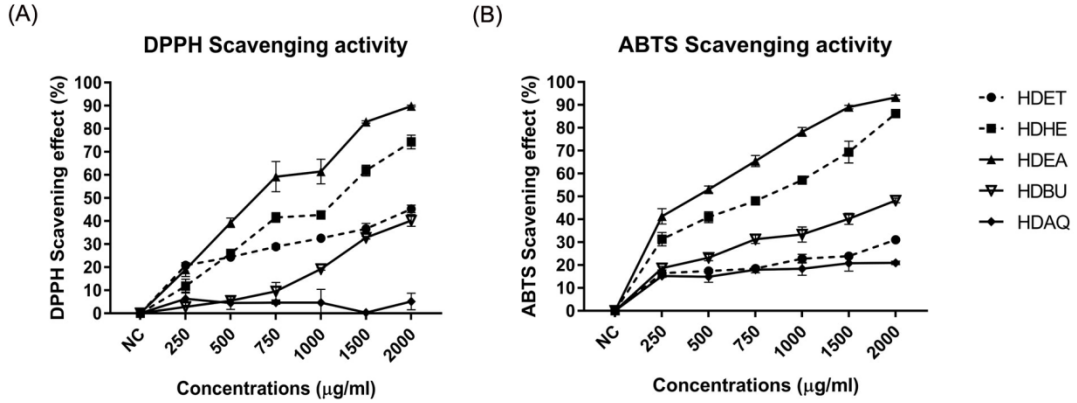

Figure 5 DPPH and ABTS scavenging activity of 5 fractions from *H. durvillei* extracts. Each value is presented as mean ± standard error (n = 3). The results were used to generate trendlines and calculate EC50 values.

Conclusions

In summary, we have investigated cytotoxic, genotoxic, and antioxidant effects of ethanol, hexane, ethyl acetate, butanol and aqueous extract fractions from *H. durvillei* in the *in vitro* models, using different cell types including hepatocyte, fibroblast, macrophage and keratinocyte in order to assess the nontoxic concentrations of the extracts from this sea weed that could be safely utilized in the food, cosmetic and drug developments, and we found that HDET and HDAQ practically had no toxicity and HDHE, HDEA, and HDBU could be safely consumed at doses lower than 100 μg/mL. However, it is crucial to verify whether the effects observed in these *in vitro* assays concur with *in vivo* assays which need to be investigated further.

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