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Research article

Toxicity and Anti-Oxidation Capacity of The Extracts from *Caulerpa lentillifera*

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Abstract *Caulerpa lentillifera* (sea grape) has been widely used in pharmaceutical industry and health-care products in Thailand. In this study, we attempted to evaluate the toxicity and antioxidant capacity of sea grape extracts in five fractions (ethanol- CLET, hexane- CLHE, ethyl acetate- CLEA, butanol-CLBU, and aqueous-CLAQ). The extracts were evaluated for cytotoxicity by MTT and LDH assays on four cell lines, fibroblast (L929), macrophages (RAW 264.7), hepatocytes (FL83B), and keratinocytes (HaCaT). Genotoxicity was tested by comet assay and micronucleus assay on human lymphoblast cells (TK6). The antioxidant capacity was measured by DPPH and ABTS scavenging assays. Our results demonstrated low cytotoxicity and genotoxicity of CLET, CLBU and CLAQ. When tested by DPPH and ABTS assays, CLET, CLEA, and CLHE showed high antioxidant activity. In conclusion, CLET, CLBU, and CLAQ demonstrated no toxic effects, and CLET, CLEA, and CLHE exhibited high antioxidant capacity. Therefore, our results indicated that CLET, CLEA, and CLHE could be consumed safely at doses lower than 500 and 200 µg/ml for CLHE and CLEA, respectively.

Keywords: Anti-oxidation, *Caulerpa lentillifera*, Cytotoxicity, Genotoxicity



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INTRODUCTION

Marine algae or seaweeds are important parts of the ecological system. Seaweeds have been used commercially because they grow rapidly with high yield. In 2018, the export value of seaweed products worldwide was about USD 13.3 billion. From 2000 to 2018, the output of seaweeds has more than tripled, up from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018 (FAO, 2020). At present, many products are derived from seaweed, such as food, nutraceuticals, fertilizers, pharmaceuticals, cosmetics, and biochemicals. Seaweeds are also used for waste water treatment. *Caulerpa lentillifera* (sea grape) is a seaweed that can be found in coastal area of Thailand and has been cultured by Phetchaburi Coastal Fisheries Research and Development Center, Department of Fisheries and farmers since 1993. *C. lentillifera* is a popular edible species with high minerals, dietary fibers, vitamin A, vitamin C, and several essential unsaturated fatty acids (Matanjun et al., 2008). Previously, several biological activities of *C. lentillifera* extracts have been reported, including anti-cancer (Maeda et al., 2012), anti-oxidative (Matanjun et al., 2008), anti-diabetes (Sharma and Rhyu, 2014; Sharma et al., 2015; Nguyen et al., 2011), anti-coagulant (Arenajo et al., 2017), immuno-stimulatory (Sun et al., 2018; Maeda et al., 2012), and lipid-lowering activities (AbouZid et al., 2014; Matanjun et al., 2008; Nguyen et al., 2011; Sharma et al. 2015, 2017; Sharma and Rhyu, 2014). These biological activities suggest that *C. lentillifera* has high commercial values which can benefit farmers who grow these seaweeds in Thailand and beyond.

Toxicity or safety tests of raw materials before being processed into food and drug, and for environment safety are necessary. Especially for food and drugs, the selected extracts must not be cytotoxic and genotoxic and cause cell death and cell mutation. Previously, toxicity and genotoxicity were tested in laboratory animals. Because of ethical consideration on the use of animals, the cost and inconvenience wide range of *in vitro* tests have been developed and gain full acceptance to replace *in vivo* tests using animals (Walum et al., 1994; Collins et al., 2008).

Test for antioxidative capacity is also an important requirement for the extracts. Free radicals are atoms or molecules that have one or more unpaired electrons that can react quickly with other micromolecules and macromolecules. Free radicals are the by-products of metabolism and a harmful environment. It caused a lot of problems in an organism, such as DNA damage, mitochondria impairment, and misfolded proteins, induction of tissue injuries and many diseases by its activation of chronic inflammation (Arulselvan et al., 2016). Antioxidants are substances that can take up free radicals which are found in many plants, including *C. lentillifera* (Apostolova and Victor, 2015; Palanisamy et al., 2017). They can prevent and reduce many health problems generated by free radicals and inflammation.

Therefore, in this study, we aim to screen the toxicities and antioxidant capacity of solvent extracts from *C. lentillifera*. No research has been conducted to test each fraction of the extract toxicity and antioxidant. These fractions could be used as safe dietary supplements and raw materials for drug development for human and animal consumptions.

MATERIALS AND METHODS

Plant materials and extraction

C. lentillifera 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). The dried *C. lentillifera* (1.00 kg) was air-dried, milled and macerated successively with 95% EtOH (3 L) at RT for 7 days. Next, EtOH extract was successively partitioned in solvents with increasing polarity from n-hexane, ethyl acetate, n-butanol and distilled water. The yields after evaporation of the solvents under reduced pressure were compared with dried weight of the seaweed, which for CLET (ethanol), CLHE (hexane), CLEA (ethyl acetate), CLBU (butanol), CLAQ (aqueous) were 12, 1, 0.5, 6.4, and 3.2%, 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.

Cell cultures

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). Briefly, L929, FL83B, and HaCaT cells were grown in DMEM culture medium with 1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, penicillin G (10 U/ml), streptomycin (10 µg/ml), and 10% fetal bovine serum. RAW 264.7 and TK6 cells were grown in RPMI 1640 medium with L-glutamine, penicillin G (10 U/ml), streptomycin (10 µg/ml), and 10% fetal bovine serum. All cell lines were cultured at 37°C in a humidified incubator with 5% CO₂ atmosphere. The culture media were changed every 2–3 days. The cells at logarithmic phase were harvested immediately before performing each experiment.

MTT assay

The cell viability was measured by employing MTT assay using L929, RAW 264.7, FL83B and HaCaT cells. The cells were seeded into 96-well plates at 8×10^3 cells per well, and allowed to grow for 24 h at 37°C. Then, the medium was discarded, and cells were treated for 24 h with various concentrations of the extracts at 10, 50, 100, 200, 500, and 1,000 µg/ml in the culture medium, and 1% DMSO diluted in the culture medium as a negative control. MTT salt was dissolved in PBS (stock solution of 5 mg/ml). After treatments, the reaction medium was removed, the MTT was dissolved in culture medium (working solution: 10:100 µl v/v; 0.5 mg/ml), added to each well and incubated for 3 h at 37°C. Finally, the MTT was removed, and DMSO was added to solubilize formazan crystals. The plates were gently shaken for at least 15 min, and the OD were then read with a microplate spectrophotometer (VersaMax microplate reader) at a wavelength of 690 nm (background signal) and 570 nm. The percentage of viable cells was calculated after normalization with the negative control (1% DMSO), which was considered to have 100% cell viability.

LDH release assay

Cytotoxicity was analyzed by LDH assay (Sigma-Aldrich, Roche). L929, RAW 264.7, FL83B and HaCaT cells were seeded into 96-well plates at 8×10^3 cells per well in triplicate wells and allowed to grow for 24 h. Then, the cells were treated with various concentrations of the extracts with 10, 50, 100, 200, 500, and 1,000 µg/ml in the culture medium for 24 h. For controls the cells were incubated in 5 µl/well lysis buffer (positive control), 50 µl culture medium plus 50 µl assay medium (negative control) and incubated the plate for 15 min. A 50 µl of culture medium (sample and control) from each well was collected and transferred to a new 96-well plate. Then, 100 µl of the reaction mixture (freshly prepared) was added to each well on the 96-well plate and incubated for 5–10 min in a dark room. Finally, 50 µl stop solution was added to each well of the 96-well plate. Then, the OD were read by ELISA reader at 492 nm, and percent LDH was calculated for each sample.

Comet assay

Comet assay was carried out to measure the DNA damage. TK6 cells were seeded at 2×10^5 cells/ml in 24-well plate, the plate was maintained in a humidified incubator with 5% CO₂ and 37°C for 24 h and treated with extracts at concentrations 10, 50, 100, 200, 500, and 1,000 µg/ml in the culture medium, hydrogen peroxide- H₂O₂ 10 µM (positive control) and 10% FBS in RPMI medium (negative control). After incubation, the cells were collected, 100 µl of TK6 cell was centrifuged 4,500 rpm for 5 min, and the supernatant was discarded, and cells resuspend with 100 µl PBS pH 7.4. The cell suspension was mixed with 1% low melting point (LMP) agarose at 1:10 ratio at 37°C. And the same volume was deposited on a previously prepared thin layer 1% normal melting point (NMP) agarose, and then covered with a cover glass. The cover glasses were removed, and the slides were immersed in a freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100) for

overnight, at 4°C in the dark. The slides were then transferred to the unwinding solution (300 mM NaOH, 1 mM EDTA, pH > 13) which detected single and double stranded breaks, for 30 min at RT in a dark room. Then, electrophoresis was conducted under standard conditions (20 V; 300 mA; 1 Vcm⁻¹) for 30 min. The slides were neutralized with 0.4 M Tris pH 7.5 for 5 min to remove alkali condition and detergents and stained with SYBR® Green, in the dark. The samples were examined with a fluorescence microscope (Olympus with 40X magnification) immediately, and analyzed with the Comet Score 2.0 software for determining DNA damage. At least 50 randomly non-overlapping cells per culture were examined in double blind observations. These cells were scored based on tail and head (nucleus) sizes. Following parameters were recorded: tail length, % of tail DNA, and tail moment, intensity of the fluorescing DNA. Tail length was the length of the comet tail which indicated the extent of DNA damage. Percent of tail DNA was the intensity of fluorescing caused by damaged DNA in the comet tail, and the tail moment was calculated as (tail length) x (% tail DNA).

Micronucleus test (MN)

TK6 cells were seeded at 2X10⁵ cell per ml in 24-well plate, then the plate was maintained in a humidified incubator with 5% CO₂ at 37°C for 24 h. And then, the cells were treated with the extracts at concentrations 10, 50, 100, 200, 500, and 1,000 µg/ml in the culture medium, with 10 µg/ml mitomycin for positive control and 1% DMSO for negative control. After incubation, 6 µg/ml cytochalasin B was added into each well to obtain dividing binucleated cells for a period of 19 h. The cells were collected and 500 µl of TK6 cells were centrifuged at 4,500 rpm for 5 min, and the supernatant was discarded, cells resuspend in 500 µl PBS pH 7.4, and then centrifuged at 4,500 rpm for 5 min. A 500 µl of hypotonic solution (0.075M KCl) was added into each pellet and incubated at 37°C for 30 min and centrifuge at 4,500 rpm for 5 min. Next, the pelleted cells were fixed in methanol and acetic acid solution with the ratio 3:1. Finally, the slides were prepared by cytospin, stained with 10% Giemsa, and immediately examined in a light microscope (Olympus with 40X magnification). At least 50 cells were counted for each experimental and control sample, and the numbers of binucleated cells with micronucleus were recorded.

DPPH assay for antioxidant activity

Four sets of experiments were set up: I) five fractions of *C. lentillifera* extracts were diluted with methanol at 250, 500, 750, 1,000, 1,500, and 2,000 µg/ml. About 0.1 ml of each concentration of each extract was mixed with 0.1 ml of methanol in 96-well plate (designated as Sample+Met); II) A 0.2 ml of methanol was added in 96-well plate (designated as Met); III) 0.1 ml of DPPH working solution was mixed with 0.1 ml of methanol in 96-well plate (designated as DPPH+Met); and IV) each concentration of the extracts as mentioned in I and each concentration of positive controls, vitamin C and quercetin, at concentrations 2, 4, 6, 8, and 10 µg/ml were diluted with 0.1 ml methanol, then they were mixed with 0.1 ml of DPPH working solution (designated as Sample+DPPH). After that, all samples were mixed and let stand at room temperature in the dark for 30 min. Finally, the absorbance of each group 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 assay for antioxidant activity

Similar to DPPH assay, four similar sets of experiments were set up. Instead at the final step each sample was mixed with 0.1 ml of ABTS solution, and samples in the four sets were designated as Sample+Met, Met, ABTS+Met, Sample+ABTS. The samples were mixed and incubated at room temperature in the dark for 20 min, then the absorbance 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

All data were presented as mean ± standard deviation (SD) and analyzed by GraphPad Prism 7 software (version 7.0). Comparisons between the extracts and

controls were conducted using a one-way analysis of variance (ANOVA) followed by Duncan's post hoc comparisons. Differences were considered statistically significant at $P < 0.001$.

RESULTS

Cytotoxicity of the extracts from *C. lentillifera* by MTT and LDH assays.

Cytotoxicity from *C. lentillifera* extracts at 24 h as measured by MTT assays (Figure 1) showed that for all cell types, IC_{50} of CLET, CLBU, and CLAQ were more than 1,000 $\mu\text{g/ml}$, when compared with the negative control. For L929 cells, IC_{50} of CLHE and CLEA were at 575 and 535 $\mu\text{g/ml}$, respectively. For FL83B cells, IC_{50} of CLHE and CLEA were at 514 and 250 $\mu\text{g/ml}$, respectively. For RAW 264.7 cells, IC_{50} of CLHE and CLEA were at 761 and 195 $\mu\text{g/ml}$, respectively. For HaCaT cells, IC_{50} of CLHE and CLEA were at 488 and 246 $\mu\text{g/ml}$, respectively. These results indicated that CLET, CLBU, and CLAQ were not cytotoxicity, while CLHE and CLEA have cytotoxicity at concentrations higher than 200 $\mu\text{g/ml}$.

LDH assay (Figure 2) showed that for all cell types, IC_{50} of CLET, CLBU and, CLAQ were more than 1,000 $\mu\text{g/ml}$ when compared with the negative control. As well, for L929 cells, IC_{50} of CLHE and CLEA were at 566.3 and 435 $\mu\text{g/ml}$, respectively. For FL83B cells, IC_{50} of CLHE and CLEA were at showed as 995.4 and 629.6 $\mu\text{g/ml}$, respectively. For RAW 264.7 cells, IC_{50} of CLHE and CLEA were at 1,025 and 245.6 $\mu\text{g/ml}$, respectively. For HaCaT cells, IC_{50} of CLHE and CLEA were at 544.5 and 390 $\mu\text{g/ml}$, respectively. These results indicated that CLET, CLBU, and CLAQ were not cytotoxic, while CLHE and CLEA have cytotoxicity at concentrations higher than 200 $\mu\text{g/ml}$.

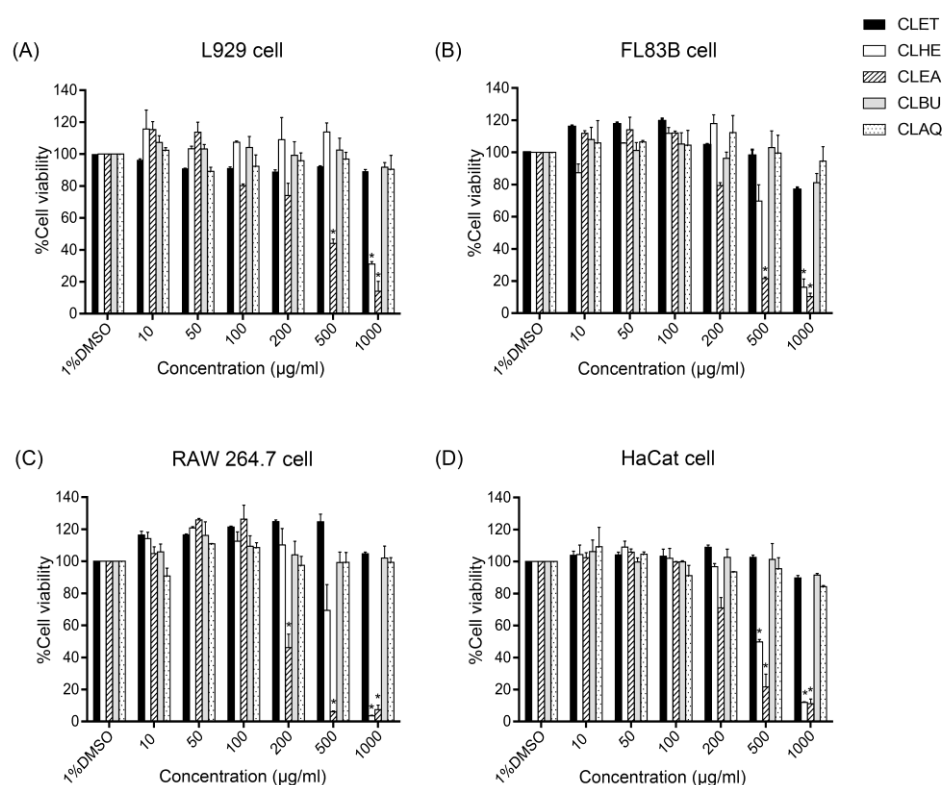


Figure 1. Cell viability of four cell types treated with *C. lentillifera* extract for 24 h, assessed by the MTT assay. (A) L929 cell line; (B) FL83B cell line; (C) RAW 264.7 cell line; (D) HaCaT cell line. Data was expressed as mean $\pm$ standard deviation of the percentage of cell viability in relation to 1%DMSO (100% cell viability), $*P < 0.001$

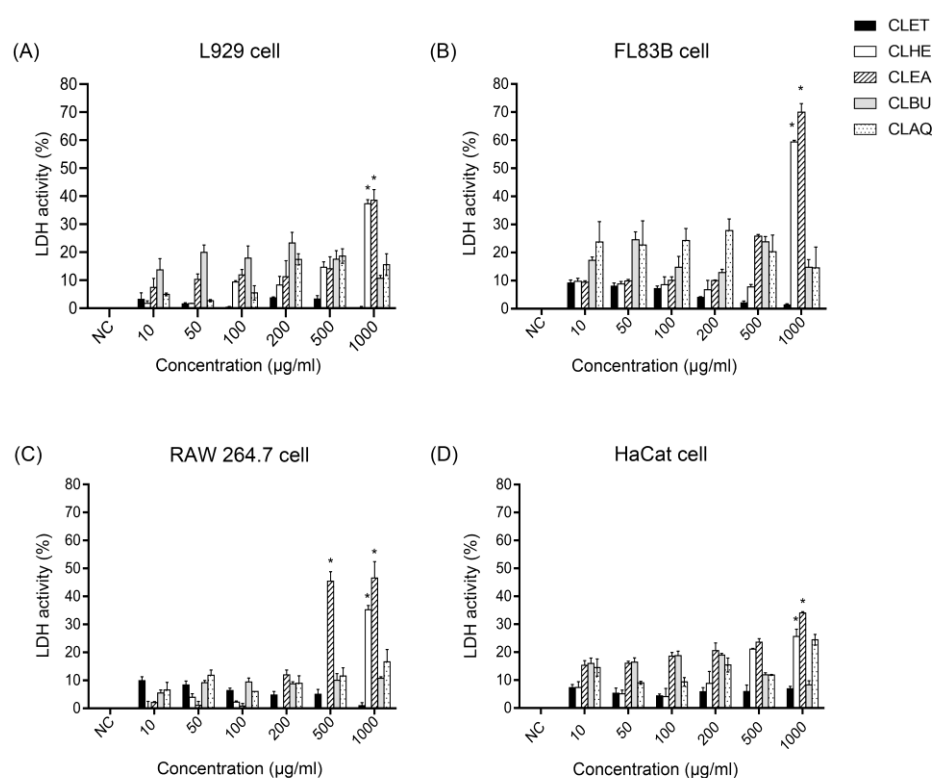


Figure 2. LDH activity of four cell types treated with *C. lentillifera* extracts for 24 h, assessed by the LDH assay. (A) L929 cell line; (B) FL83B cell line; (C) RAW 264.7 cell line; (D) HaCaT cell line. Data were expressed as mean $\pm$ standard deviation of the percentage of LDH activity in relation to the positive control (100% LDH activity), * $P < 0.001$

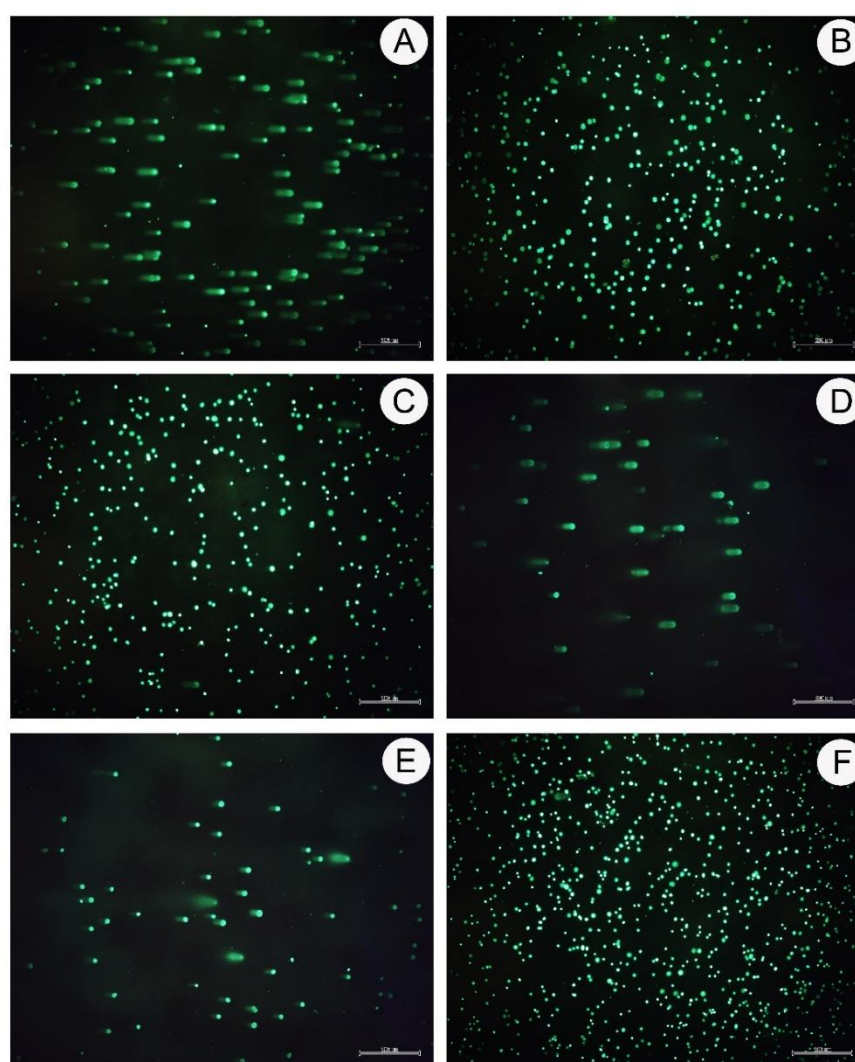
Genotoxicity of extracts from *C. lentillifera* estimated by comet assay

To evaluate whether the *C. lentillifera* extracts affected genes comet assay was performed with TK6 cell (Table 1). After treatment, three parameters were recorded, the most important of which was % tail DNA. Genotoxicities of CLET, CLBU, and CLAQ was slightly detectable at concentrations of 1,000 µg/ml, which showed comet score of less than 50 percent. For CLHE genotoxicity was detected at concentration of 500 and 1,000 µg/ml. For CLEA genotoxicity was detectable at concentrations of 200, 500, and 1,000 µg/ml. Both fractions showed comet score at more than 50 percent. These results indicated that CLET, CLBU, and CLAQ practically have no genotoxicity, while CLHE and CLEA were safe at concentrations less than 200 µg/ml.

Table 1. Genotoxicity measured by comet assay for tail length, % tail DNA and tail moment in TK6 cells treated with different concentration of *C. lentillifera* extracts for 24 h.

Treatment	Tail Length $\pm$ SD	% Tail DNA $\pm$ SD	Tail moment $\pm$ SD
Negative control	0.70 $\pm$ 1.04	10.66 $\pm$ 2.15	0.08 $\pm$ 0.12
Positive control (H ₂ O ₂)	59.42 $\pm$ 52.39	80.03 $\pm$ 13.83	44.19 $\pm$ 42.56
CLET 500 μ g/ml	0.32 $\pm$ 0.62	10.35 $\pm$ 3.40	0.04 $\pm$ 0.08
CLET1,000 μ g/ml	19.14 $\pm$ 21.08	34.84 $\pm$ 23.75	10.42 $\pm$ 13.18
CLHE 200 μ g/ml	16.20 $\pm$ 26.52	23.31 $\pm$ 16.06	7.50 $\pm$ 14.68
CLHE 500 μ g/ml	25.90 $\pm$ 26.21*	63.86 $\pm$ 12.73*	18.23 $\pm$ 19.15*
CLEA 100 μ g/ml	5.38 $\pm$ 16.16	35.89 $\pm$ 20.12	2.88 $\pm$ 8.95
CLBU 1,000 μ g/ml	1.04 $\pm$ 0.88	13.60 $\pm$ 3.89	0.16 $\pm$ 0.14
CLAQ 1,000 μ g/ml	0.42 $\pm$ 0.57	12.53 $\pm$ 3.90	0.07 $\pm$ 0.11

Note: S.D = Standard Deviation; H₂O₂ = Hydrogen Peroxide
Significant differences from the control group are calculated and marked with asterisks (**P* < 0.001).

**Figure 3.** Genotoxicity measured by comet assay was performed with the TK6 cell. The DNA damage is expressed in a comet pattern due to strand breaking and alkylating agents as analyzed by Comet Score 2.0 software. (A) Positive control; (B) Negative control; (C) CLET at concentration 1,000 μ g/ml; (D) CLHE at concentration 500 μ g/ml; (E) CLEA at concentration 200 μ g/ml; (F) CLAQ at concentration 1,000 μ g/ml. (40x magnification)

Genotoxicity of extracts from *C. lentillifera* by micronucleus test

Analyses of the effect on genetic material by the *C. lentillifera* extracts using the TK6 cell by micronucleus test (Figure 4) showed that in negative control the cell appeared to be binucleated (Figure 4B). In positive control the cell appeared to be binucleated with micronucleus at 42% (Figure 4A). Cells treated with CLET, CLBU, and CLAQ showed no micronucleus at 1,000 $\mu\text{g}/\text{ml}$, indicating that these three fractions were not toxic to the TK6 cells. On the other hand, CLHE and CLEA showed cells with micronucleus. For CLHE micronucleus was observed at concentrations 500 and 1,000 $\mu\text{g}/\text{ml}$ with % of cells with micronucleus at 5.71 and 13.34%, respectively. For CLEA micronucleus was observed at concentrations 200 and 500 $\mu\text{g}/\text{ml}$ with % of cells with micronucleus at 9.72 and 23.80%, respectively. These results confirmed that fractions CLET, CLBU, and CLAQ has no genotoxicity and did not cause any chromosomal damage, while CLHE and CLEA were safe at concentrations lower than 500 and 200 $\mu\text{g}/\text{ml}$, respectively.

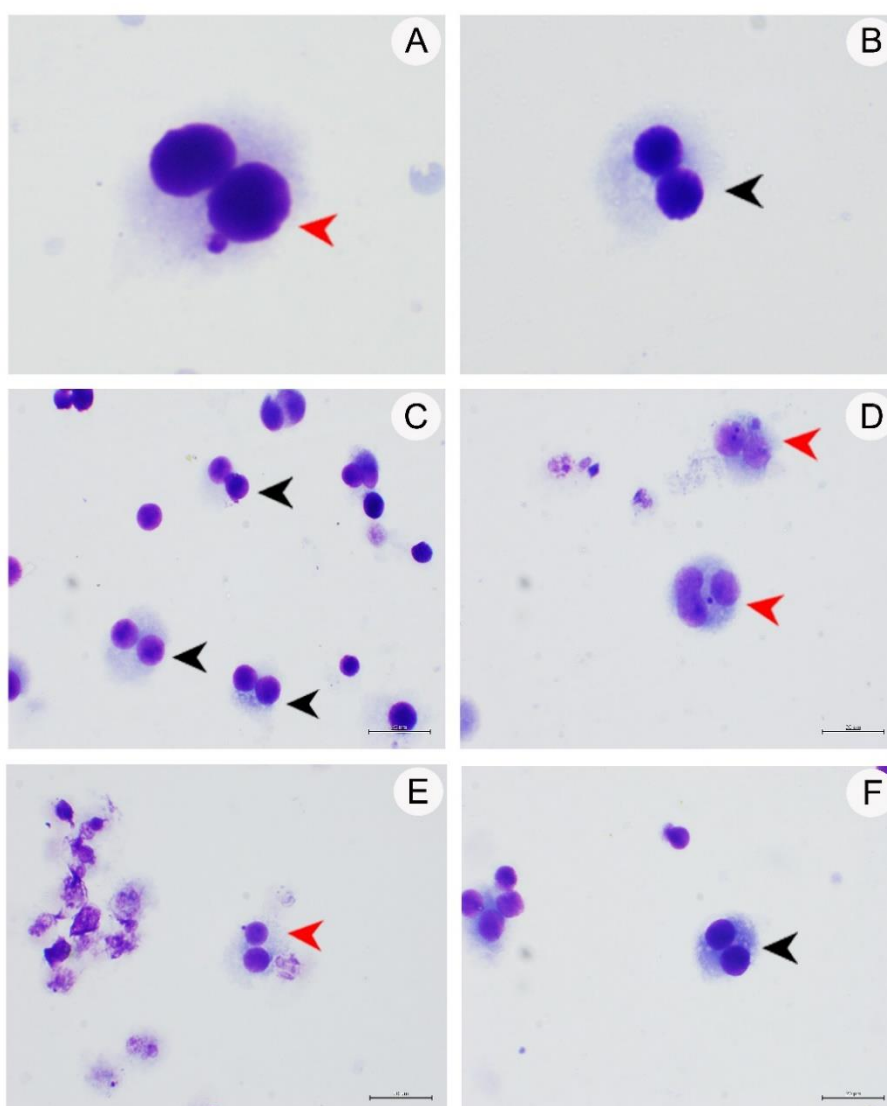


Figure 4. Genotoxicity as measured by micronucleus test indicated chromosome damage. (A) Positive control; (B) Negative control; (C) CLET at concentration 1,000 $\mu\text{g}/\text{ml}$; (D) CLHE at concentration 1,000 $\mu\text{g}/\text{ml}$; (E) CLEA at concentration 500 $\mu\text{g}/\text{ml}$; (F) CLAQ at concentration 1,000 $\mu\text{g}/\text{ml}$. Red and black arrow heads indicate binucleated cells with micronucleus and binucleated cells, respectively. (40x magnification)

Table 2. Genotoxicity measured by micronucleus assay showing % micronucleus in TK6 cells treated with *C. lentillifera* extracts for 24 h. (* $P < 0.001$)

Treatment	Micronucleus (%)
Positive control 0.75 µg/ml of Mitomycin C	42
CLET 1,000 µg/ml	0
CLHE 500 µg/ml	5.71
CLHE 1,000 µg/ml	13.34
CLEA 200 µg/ml	9.72
CLEA 500 µg/ml	23.80
CLEA 1,000 µg/ml	N/A
CLBU 1,000 µg/ml	0
CLAQ 1,000 µg/ml	0

Antioxidant activity of the extracts by DPPH and ABTS assays

In these assays, we have shown free radicals' scavenging or antioxidant activity of *C. lentillifera* extracts by calculating and comparing EC_{50} values. The antioxidant activity of CLET fraction was between 1.21 ± 1.99 to $31.68 \pm 1.65\%$, and CLHE was from 13.97 ± 6.68 to $38.27 \pm 0.59\%$. The CLEA fraction showed highest antioxidant activity between 25.64 ± 1.84 to $91.25 \pm 2.29\%$. The fractions showed the lowest antioxidant activity was CLBU between 4.50 ± 3.17 to $11.00 \pm 5.70\%$. The CLAQ has moderate antioxidant activity at $3.04 \pm 8.56\%$ (Figure 5).

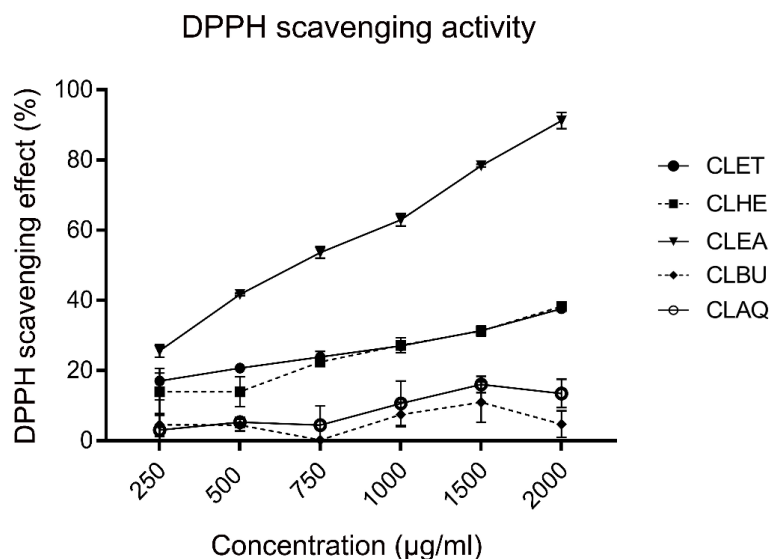


Figure 5. T DPPH scavenging effect of five fractions from *C. lentillifera* extracts. Each value was presented as mean $\pm$ standard error (n=3). The result was used to generate trendlines and for calculating the EC_{50} values.

The high percentage of inhibition of DPPH radicals was CLEA, CLHE, and CLET fractions, while CLAQ and CLBU fractions showed low antioxidant activity. The EC_{50} value of CLEA fraction was highest at 535 µg/ml. The EC_{50} values of CLHE and CLET fractions were 2,947.41 and 3,088.77 µg/ml. In contrast, the EC_{50} values of CLAQ and CLBU fractions were more than 5,000 µg/ml. Positive control (Vitamin C and quercetin) showed EC_{50} as 6.66 µg/ml and 2.37 µg/ml, respectively.

In ABTS assay, the five fractions of *C. lentillifera* extracts inhibit free radicals from ABTS radicals. The antioxidant activity of CLET was between 0.08 ± 2.74 to $46.46 \pm 2.63\%$. The percentage of scavenging activity of CLET was increased when it increased

concentration. The antioxidant activity of CLHE fractions was between 9.70 ± 1.81 to $40.41 \pm 3.02\%$. The CLEA fraction showed the highest antioxidant activity between 28.75 ± 2.55 to $84.37 \pm 0.97\%$. CLBU and CLAQ fractions showed low antioxidant activities at 21.99 ± 8.19 to $22.17 \pm 1.56\%$ and 18.26 ± 8.1 to $29.30 \pm 4.15\%$, respectively (Figure 6).

The EC_{50} values of *C. lentillifera* extracts were calculated the equation of a line from %ABTS scavenging activity at 50% of the radical scavenging effect: CLEA, CLHE and CLET consecutively showed highest to lowest percentage of free radical scavenging activities with EC_{50} values at 712.42, 2,546.60 and 2,022.75 $\mu\text{g/ml}$, respectively. Positive control (Vitamin C and quercetin) showed EC_{50} as 4.82 $\mu\text{g/ml}$ and 8.43 $\mu\text{g/ml}$, respectively.

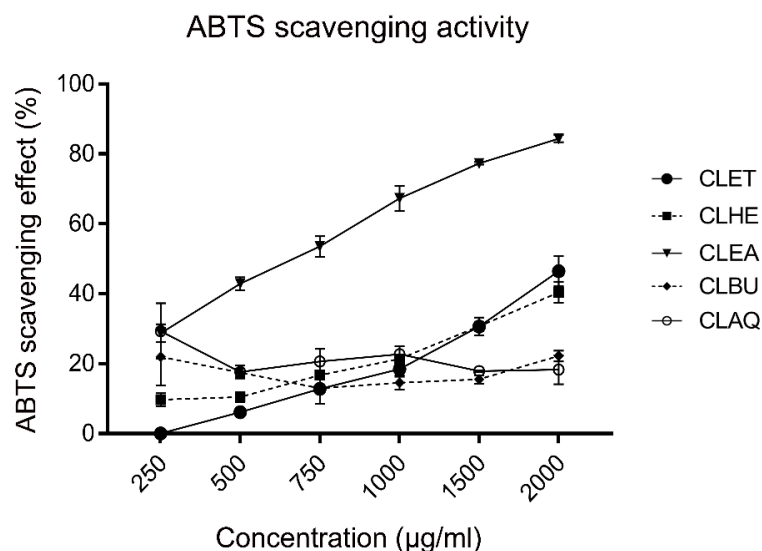


Figure 6. ABTS scavenging activities of five fractions from *C. lentillifera* extracts. Each value represents mean $\pm$ standard error ($n=3$). The data were used to generate trendlines and calculate the EC_{50} value.

DISCUSSION

We analyzed the cytotoxic potential of *C. lentillifera* extracts from low (10 $\mu\text{g/ml}$) to high (1,000 $\mu\text{g/ml}$) concentrations using standard MTT and LDH assays to confirm cytotoxicity in four cell lines. The results showed that RAW 264.7 and FL83B cells exhibited the lowest cell viability as these cells still underwent cell proliferation. Our results from MTT assay showed that fractions with lowest to the highest toxicity were CLAQ, CLET, CLBU, CLHE, and CLEA, respectively. Previous studies also reported that the CLET of the same seaweed did not showed cytotoxicity, including studies conducted in Japan at the highest concentration at 250 $\mu\text{g/ml}$ (Sharma et al., 2015), in China at the highest concentration at 800 $\mu\text{g/ml}$ (Sun et al., 2018) and in the Philippines at the highest concentration at 25 $\mu\text{g/ml}$ (Sharma and Rhyu, 2014), and we have used an even higher concentration at 1,000 $\mu\text{g/ml}$. We found that CLHE and CLEA contains similar substances, including alkaloids, flavonoids, terpenoids, steroids, and glycoside, with tannin being present only in CLEA (our unpublished data). It has been reported that alkaloids, flavonoids, steroids, tannins, saponins, and terpenoids have toxicity around IC_{50} of 9.81 $\mu\text{g/ml}$ (Singh et al., 2019). By contrast, we found the IC_{50} value of CLHE and CLEA to be higher at approximately 200 $\mu\text{g/ml}$ which could be due to the lower concentrations of these substances in the extracts. Noticeably, CLHE and CLEA showed more toxicity than other fractions. Eventhough the above-mentioned substances were also present in CLET this fraction did not showed any cytotoxicity, probably because it contained these various substances in relatively low concentrations. Our results from LDH assay were more sensitive than MTT assay with IC_{50} of CLHE and CLEA being 544-1025 $\mu\text{g/ml}$ and 245-629 $\mu\text{g/ml}$, depending on cell types while CLET and CLAQ were

nontoxic. This is because the MTT assay detected viable cells only (Fotakis and Timbrell, 2006), whereas LDH assay detected cells with damaged membranes which occurred earlier even before cell death. Nevertheless, the results from the two assays followed the same trend.

Comet assay has been used to assess genotoxicity as the result of DNA breakage. TK6 is a well-accepted cell for evaluating DNA damage in comet assay due to its high accuracy (90%) (Bajpayee et al., 2019; Bajpayee et al., 2006). Parameter employed in measuring DNA damage in cells included the percentage of DNA in the tail (% tail DNA), tail length, and tail moment calculated from fluorescence intensity observed under fluorescent microscope. Our data from comet assay showed that CLET, CLBU, and CLAQ at the highest concentration at 1,000 µg/ml showed no toxicity, while but CLHE and CLEA showed some genotoxicity at concentrations higher than 500 µg/ml and 200 µg/ml, respectively.

The micronucleus (MN) test has been used for assessing chromosomal damage in cells by micronucleus formation after cell division after treating TK6 cells for 24 h. The percentage of binucleated cells with micronucleus indicated that fractions or chemicals have cytostatic action on mitogenic activity. This assay was used to confirm comet assay. It was found that CLET, CLBU, and CLAQ did not damage chromosomes but CLHE and CLEA caused significant damage at concentrations higher than 500 µg/ml and 200 µg/ml, respectively.

The antioxidant activity of *C. lentillifera* extracts were measured by DPPH and ABTS assays. The ethanol, butanol, and water are polar solvents whose dielectric constants are 24.3, 18, and 78.5, respectively. They were used to extract hydrophilic antioxidants such as phenolics, flavonoids, and anthocyanins (Xu et al., 2017). On the other hand, hexane is non-polar solvent with dielectric constant at 1.9. It was used for extracting lipo-soluble antioxidants such as carotene, lycopene, and xanthine. The ethyl acetate is a polar aprotic solvent with intermediate dielectric constant and polarity (He et al., 2016; Xu et al., 2017). It is used for extracting both water-soluble as well as lipid-soluble antioxidants. Furthermore, the ethanol is commonly used for extraction because it has medium polarity, that can both dissolve water- and lipid-soluble antioxidants (Xu et al., 2017) and usually employed at the first stage of extraction.

The data from this study showed CLEA fraction showed the highest antioxidant activity followed by CLHE, CLET, CLAQ, and CLBU. CLEA contained alkaloids, tannins, terpenoids, and glycosides. As well, the phytochemicals in CLHE (alkaloids, flavonoids, terpenoids, and glycosides) were similar to CLEA fraction, thus antioxidant activity of CLHE and CLEA fractions were very close. In contrast, The CLET was extracted with ethanol, that contained chlorophyll, alkaloids, flavonoids, phenols, tannins, terpenoids, steroids, glycosides and saponins, at low concentrations in consequence of low antioxidant activity. Finally, the CLBU and CLAQ fractions extracted with high polar, the phytochemicals of these include phenols. The CLBU and CLAQ fractions showed the lowest antioxidant effect because they may contain low concentration of phenol.

In previous studies, *C. lentillifera* was often extracted by using ethanol, chloroform, and water. The high antioxidant activities are expressed by fractions in non-polar solvent. The chloroform extract showed significantly higher antioxidant activity when compared to methanol and water extracts. The EC₅₀ of DPPH scavenging effect with chloroform, methanol, and water extract were about 2.20 ± 0.10 , 9.74 ± 0.59 , and 81.55 ± 4.22 mg/ml (Yap et al., 2019). Compared to our result, The DPPH radical scavenging activity obtained from chloroform extract was lower than CLEA but higher than CLHE and CLET. Thus, the extraction methods affected involve the antioxidant activity. *C. lentillifera* was extracted with ethanol by thermal-drying and freeze-drying methods. The freeze-drying method showed a higher antioxidant effect than thermal-drying methods (Nguyen et al., 2011).

CONCLUSION

In summary, we have investigated cytotoxic, genotoxicity, and antioxidant activity of different extracts from *C. lentillifera* (CLET, CLHE, CLEA, CLBU, CLAQ) in in vitro models of different cell types including hepatocyte, fibroblast, macrophage and keratinocyte in

order to assess the nontoxic concentrations of the extracts from this seaweed that could safely be utilized in the food, cosmetic and drug development industries.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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