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Original research article

Hexane Extract of Seaweed *Caulerpa lentillifera* Inhibits Cell Proliferation and Induces Apoptosis of Human Glioblastoma Cells

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ABSTRACT

Glioblastoma is one the most aggressive types of brain cancers resistant to conventional anti-neoplastic drugs. Marine seaweeds possess valuable bioactive compounds for disease prevention and therapy, including for a number of cancers. *Caulerpa lentillifera* is a tropical marine seaweed containing various minerals and bioactive ingredients. This study demonstrated the activity of *C. lentillifera* hexane extract (CLHE) against A172 human glioblastoma cells. The extract exerted growth- inhibitory effects by inhibiting cell proliferation and inducing apoptosis. CLHE also induced ROS accumulation and altered mitochondrial membrane potential in A172 cells. Taken together, CLHE might be a potential anticancer agent for use against glioblastoma.

Keywords: *Caulerpa lentillifera*; Seaweed; Glioblastoma; Proliferation; Apoptosis

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1. Introduction

Glioblastoma (GBM) is one of the most aggressive types of brain cancers with high lethality. A combination of surgical resection, radiotherapy, and chemotherapy is the standard therapy for GBM treatment [1]. Conventional treatment uses temozolomide, an alkylating agent, to induce DNA damage leading to apoptosis of tumor cells [2]. However, current reports on temozolomide efficacy for GBM therapy show considerable limitations in terms of dosage and effectiveness [3-4].

Marine seaweeds are valuable sources of bioactive compounds including proteins, minerals, vitamins, phytochemicals, polyunsaturated fatty acids and non-starch polysaccharides [5]. Seaweeds are extensively used as functional foods and herbs. Marine seaweeds synthesize a variety of bioactive compounds with potential anticancer activity, including carotenoids, flavonoids, sulfated polysaccharides and terpenoids [6-7]. The partially purified alkaloids from *Caulerpa racemosa* have exhibited cytotoxic activity against breast adenocarcinoma cells in past research [8]. Fucoidan, a major polysaccharide derived from seaweeds, inhibited proliferation and promoted apoptosis in cells of colon carcinoma, melanoma and lung carcinoma [9-10]. Fucoxanthins, carotenoid derivatives contained in the extract of *Undaria pinnatifida* seaweed, showed a cytotoxic effect against lung and colon carcinoma cells [11]. Little evidence on the anticancer effects against GBM, of the bioactive compounds of seaweeds have been presented thus far. Fucoxanthins have showed anti-proliferation and anti-invasion activity against U251 and U-87 GBM cells [12]. Fucoidans extracted from brown seaweed inhibited angiogenesis and promoted nitric oxide production in glioma cells [13-14].

Caulerpa lentillifera (CL), an edible green macroalgae, is cultured in tropical and subtropical regions as well as in Thailand for use in consumable products. It has high nutritional value, containing dietary fibers, vitamin A, vitamin C, calcium, magnesium, iodine and essential unsaturated fatty acids [15-16]. Their extracts possess health promoting effects including antioxidant, anti-diabetic and lipid-lowering properties [17-19]. In our experiment, the extraction was performed by dissolving dried crude *C. lentillifera* in ethanol and then partitioning in various solvents including hexane. Our screening results showed the effective cytotoxicity of the hexane extract. This study presents the anti-proliferative and apoptotic effects of the hexane extract of *C. lentillifera* against human GBM cells.

2. Materials and Methods

2.1 Preparation of seaweed extracts

CL seaweed was cultured and collected from the Phetcha Buri coastal aquaculture research and development center, Phetcha Buri, Thailand. Dried crude CL was soluted and macerated in 95% ethanol for 7 days. The ethanolic extracts were partitioned in hexane and then evaporated to remove residual solvents. The hexane solvent extracts (CLHE) were freeze-dried and collected for further testing. The extraction yield of the sample was 1.27%.

2.2 Cell culture

A172 human GBM cells were purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM containing 10% FBS and a 1% penicillin/streptomycin cocktail in an incubator with an atmosphere of 5% CO_2 at 37°C.

2.3 Cytotoxicity

CLHE was dissolved in dimethyl sulfoxide (DMSO). A172 cells in 96-well plates were incubated for 24 h with increasing concentrations of CLHE diluted in serum-free media. The final concentration of DMSO was 0.25%. The cytotoxicity of treated cells was determined by methyl thiazolyl tetrazolium (MTT) assays. MTT

solution (Bio Basic, Canada) was dissolved in treated wells for a final concentration of 30 µg/ml, followed by 3 h incubation. After that, DMSO was added to dissolve MTT formazan. The reaction was measured by a spectrophotometer (Varioskan Flash Microplate Reader, Thermo Fisher Scientific) at the absorbance wavelengths of 562 and 630 nm.

2.4 Cell proliferation assay

Proliferation of A172 cells following CLHE incubation was analyzed by measuring fluorescent intensity of carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described [20]. Briefly, the A172 cells were labelled with CFSE dye (Sigma) in darkness at 37 °C for 10 minutes and then plated on 6-well plates. Cells were treated with CLHE at 150 and 200 µg/ ml for 48 h. After that, cells were retreated with the extract for another 48 h. Next, cells were trypsinized and resuspended in phosphate buffered saline (PBS). Mean fluorescence intensity was measured using a Guava easyCyte flow cytometer (Merck Millipore, USA) at a minimum of 5,000 events/sample.

2.5 Cell cycle analysis

CLHE at 150 and 200 µg/ml was incubated in plated A172 on 6-well plates for 48 h. Treated cells were harvested by 0.1% trypsin-EDTA and fixed in 70% ethanol for 30 min. Cells were incubated with propidium iodide (PI)/RNase (BD Biosciences, USA) as previously described [20]. Fluorescence intensity of PI-stained cells was measured using a Guava easyCyte flow cytometer (Merck Millipore) at a minimum of 10,000 events/sample.

2.6 Apoptotic cell detection

The effects of CLHE on apoptotic cell induction was analyzed by Annexin V staining. A172 cells were treated with CLHE at the final concentration of 250 µg/ml. Treated cells were collected and incubated with Annexin V-FITC (BD Biosciences) for 15 min in darkness at room temperature. The population of apoptotic cells was measured using a Guava easyCyte flow cytometer (Merck Millipore).

2.7 Analysis of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) of A172 cells after CLHE treatment was analyzed using JC-1 MMP assay (Merck Millipore). The assay contains a lipophilic cationic fluorescent dye acting as a mitochondrial activity marker. Treated cells at 24 h were washed with PBS prior to being incubated with JC-1 solution for 10 min. Fluorescence signals were measured using a Guava easyCyte flow cytometer (Merck Millipore) at 2,000 events/sample.

2.8 Analysis of intracellular reactive oxygen species

Induction of ROS production following CLHE treatment was analyzed by 2',7'-dichlorofluorescin diacetate (DCFDA) fluorescence labelling assays. A172 cells were stained with 20 µM DCFDA (Sigma) at 37°C for 30 min. Fluorescence intensity was measured at 0.5, 1, 3 and 6 h by spectrophotometer at the absorbance wavelengths of 490 and 535 nm.

2.9 Statistical analysis

Data are expressed as mean ± SEM. Statistical variations of all experiments were analyzed by GraphPad Prism statistical analysis software (GraphPad Software Inc, USA) using one-way ANOVA test. A *p*-value of less than 0. 05 was considered statistically significant.

3. Results and Discussion

3.1 Cytotoxicity and growth-inhibitory effects of CLHE extract

The prime anticancer effects of many chemotherapeutic agents result from arresting the cancer cell cycle, leading to their anti-proliferative and cytotoxic activity.

The effects of CLHE on toxicity and proliferation of A172 cells were analyzed by MTT and CFSE assays, respectively. After 24 h post-incubation, increasing concentrations of CLHE from 200 to 1,000 µg/ml dramatically decreased the viability of A172 cells when compared with untreated and 0.25% DMSO-treated groups (Fig. 1). The 50% inhibitory concentration (IC_{50}) of CLHE extracts against A172 cells was 224.7±5.205 µg/ml.

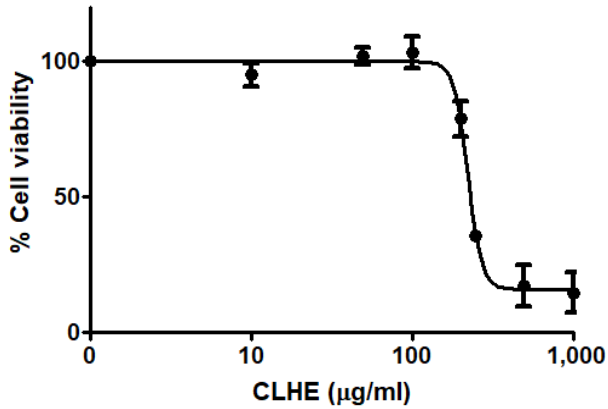

Fig. 1. Effect of CLHE on viability of A172 cells. Cell viability was measured by MTT assays after incubation with various concentrations of CLHE for 24 h.

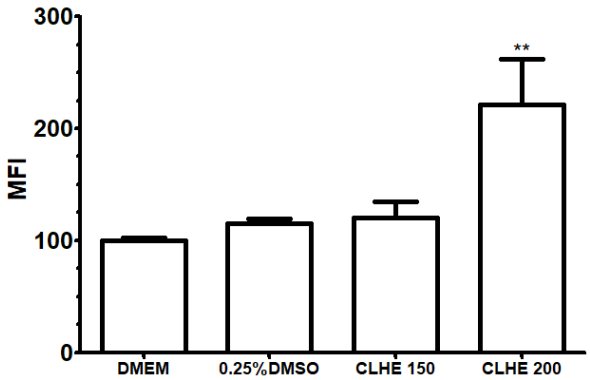

Fig. 2. Growth-inhibitory effect of CLHE on A172 cells. CFSE-labelled cells were treated with CLHE at 150 and 200 µg/ml for 96 h. The change of CFSE signal intensity was measured by flow cytometry and presented as mean fluorescence intensity (MFI). ** $p \leq 0.01$.

The reduction of cell viability could be caused by the inhibition of cell proliferation. CFSE is an intracellular vital green-fluorescent dye that conjugates with cytoplasmic proteins. The dye is partitioned equally between daughter cells upon cell division. Increased levels of CFSE fluorescence intensity indicates the disruption of cell division by the accumulation of dye in daughter cells. The concentration of CLHE at less than IC_{50} was selected for growth-inhibitory effect and determination of the cell cycle to reduce the effect caused by cell death. The mean fluorescence intensity of CLHE-treated cells at levels less than IC_{50} significantly increased at 200 µg/ml after treatment for 96 h compared with DMSO-treated groups (Fig.

2). This indicated that the CLHE extract reduced cell viability and possessed anti-proliferative effects against GBM cells. This effect has been observed in compounds from other seaweeds against brain cancer cells. Fucoxanthins diminished the viability of U251 and U-87 GBM cells [12]. Pheophorbide a isolated from the edible red seaweed *Grateloupia elliptica* inhibited viability of U-87 cells [21]. Hexane and chloroform extracts of the brown Seaweed *Egregia menziesii* promoted toxicity to glioma cell lines [22]. It has been revealed that several seaweed-derived compounds promote cytotoxicity against GBM cells.

Phenolic compounds are enriched in macroalgae, including *Caulerpa* spp. , as secondary metabolites [23]. Natural polyphenols could be used as anticancer agents, acting by suppressing proliferation, inducing apoptosis and promoting oxidative stress [24]. Further, seaweed polyphenols promote cytotoxic activity against HeLa cells [25]. The phlorotannin-rich extract, polyphenol derivatives, from the brown algae *Laminaria japonica* inhibited proliferation of hepatocellular carcinoma and leukemia cells [26]. Determination of the total phenolic content in CLHE showed 23.82 ± 0.32 mg GAE/g (unpublished data). This data indicates that phenolic compounds in CLHE may suppress proliferation of A172 cells.

3.2 Induction of cell cycle arrest by CLHE extract

The cell cycle arrest- induced anti-proliferation activity of CLHE was observed by PI staining in treated A172 cells. CLHE extracts at concentrations less than IC_{50} significantly increased the percentage of A172 cells in G0/G1 phase and decreased the percentage of cells in S and G2/M phases, indicating that CLHE promoted cell cycle arrest at G0/G1 phase at 48 h post- treatment (Fig. 3). This implies that CLHE inhibited GBM cell cycle progression in GBM cells.

Bioactive compounds from other marine seaweeds have also shown the ability to inhibit cell cycle regulation. Methanolic extracts of *C. racemosa* induced cell cycle arrest in G0/G1 phase in promyeloblastic leukemia HL60 cells [27]. The GBM growth-inhibitory activity of Pheophorbide a from *Grateloupia elliptica* was associated with the arrest of cell cycle at G0/G1 phase. Fucoidans isolated from *Fucus vesiculosus* triggered G1 cell cycle arrest of colon cancer cells by downregulating cyclin D1 and cyclin-dependent kinase (CDK) 2 and 4 [28]. Thus, CLHE may arrested GBM cell cycle by suppressing levels of cyclin D1 and Cdk4/Cdk6, cell cycle regulatory molecules, at G1 phase [29].

3.3 Induction of the mitochondrial-mediated pathway of apoptosis by CLHE extract

Activation of apoptotic cell death is an important target of chemotherapeutic agents. Apoptosis is preceded by arresting the cell cycle in cancer cells [30]. CLHE extracts at 250 µg/ml were selected for toxicity tests as the concentration was higher than IC_{50}. CLHE treatment resulted in a significant increase in the apoptotic cell population, represented as Annexin V-stained cells, when compared with DMSO-treated cells (Fig. 4), indicating that CLHE induced apoptosis in A172 cells.

Next, the pathways underlying apoptosis were investigated. The mitochondrial-mediated pathway of apoptosis depends

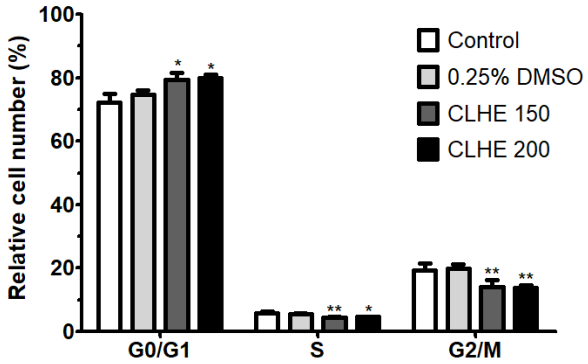

Fig. 3. Cell cycle arrest on CLHE-treated A172 cells. The percentage of cell number in each cell cycle phase at 48 h post-treatment were analyzed by PI staining. * $p \leq 0.05$, ** $p \leq 0.01$.

on the loss of MMP, in turn causing the imbalance of pro- and anti-apoptotic factors. The loss of mitochondria membrane integrity leads to the release of pro-apoptotic molecules from mitochondria and consequently activates executional caspase-3 for activation of apoptotic cell death [31]. Induction of mitochondrial-mediated apoptosis by CLHE was analyzed by JC-1 fluorescence staining assays. At 24 h post-treatment, CLHE extracts at 250 µg/ml promoted the loss of MMP as indicated by the increase in the percentage of green fluorescence compared with DMSO-treated cells (Fig. 5). Our results suggest that CLHE induced the mitochondrial-mediated pathway of apoptosis in GBM cells. Similar to these results, it has been shown that the pepsin digested extract of *Caulerpa microphysa* induced apoptosis in myelomonocytic leukemia cells by decreasing MMP, which in turn led to the upregulation of apoptotic markers [32]. The methanolic crude extract of *Caulerpa taxifolia* inhibited proliferation and was associated with the hyperpolarization of the mitochondrial membrane, indicating apoptosis of breast cancer cells [33]. Induction of apoptosis can be modulated by seaweed polyphenols [34]. Phenolic compounds in the methanolic extract of *Sargassum muticum* inhibited growth and induced apoptosis in breast cancer cells via caspase- 3 activation [35]. The polyphenol derivative phloroglucinol, from the brown seaweed *Ecklonia Cava* Kjellman inhibited proliferation and promoted apoptosis in MCF-7 human breast cancer cells through the activation of caspase-3 and caspase-9, poly-ADP ribose polymerase cleavage, pro-apoptotic p53 and Bax genes [36]. This implies that phenolic compounds in CLHE may induce apoptosis in A172 cells.

3.4 Modulation of intracellular ROS levels by CLHE extract

Many anticancer agents induce intracellular ROS accumulation, which subsequently damages a cancer cell's

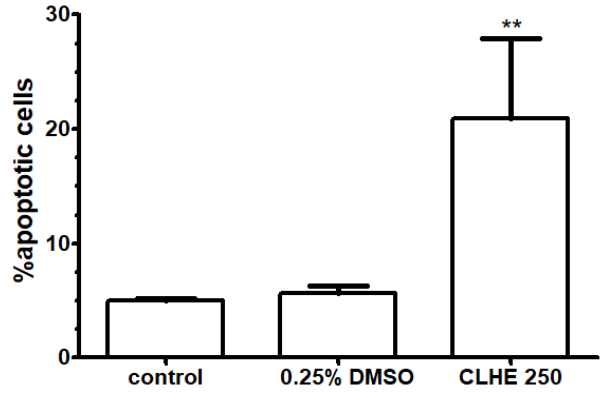

Fig. 4. CLHE induced apoptotic cell death. The percentage of total apoptotic A172 cells after CLHE treatment for 24 h was analyzed by Annexin V-FITC staining. ** $p \leq 0.01$.

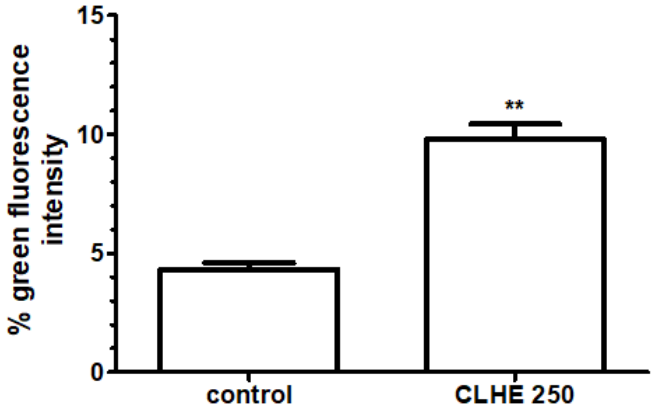

Fig. 5. CLHE promoted the loss of MMP. Mitochondrial membrane damage following CLHE treatment at 250 µg/ml for 24 h was indicated by the increase of green fluorescence intensity of JC-1. ** $p \leq 0.01$.

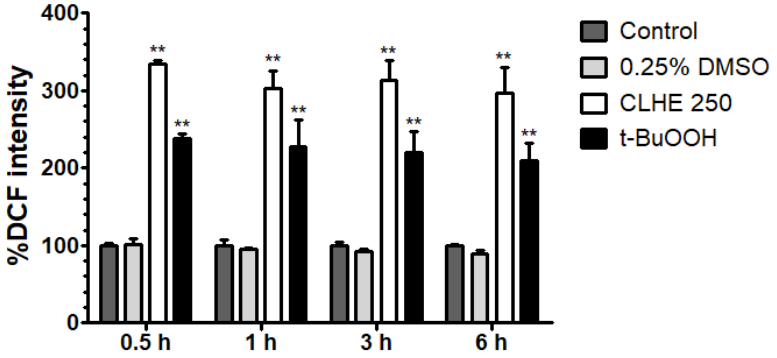

Fig. 6. CLHE induced intracellular ROS accumulation. ROS generation in A172 cells following CLHE incubation at 250 µg/ml was analyzed by DCFDA assays. The percentage of DCF intensity of CLHE-treated group compared with DMSO-treated cells as a negative control group and 1 mM t-BuOOH-treated cells as a positive control group. ** $p \leq 0.01$.

nucleus, mitochondria and membrane proteins, leading to apoptosis [37]. In addition, intracellular ROS accumulation induces cell cycle arrest and thus impedes cancer cell proliferation [38].

Intracellular ROS levels in A172 cells after CLHE treatment for 0.5, 1, 3 and 6 h were analyzed by DCFDA fluorescence labelling. CLHE extracts at 250 µg/ml highly increased ROS levels as was shown by the increase in DCF intensity at all time points, when compared with DMSO-treated cells (Fig. 6). Furthermore, the increase in ROS levels induced by CLHE, was higher than it was in 1 mM tert-buthyl hydroperoxide (t-BuOOH) treatment, used as a positive control. Therefore, CLHE could induce intracellular ROS accumulation in A172 cells. Similarly, ethanolic extracts of the red marine algae *Gracilaria tenuistipitata* induced apoptosis in Ca9–22 oral squamous cancer cells [39]. *C. taxifolia* crude extracts highly increased ROS levels in breast cancer cells, which is correlated with a dose-dependent increase of MMP [40]. The increase in ROS generation may be what gives rise to the growth-inhibitory effects of CLHE, by triggering cell cycle arrest and inducing apoptotic cell death, wtih mitochondria as a pivotal target.

4. Conclusion

CLHE extracts inhibited proliferation and promoted the mitochondrial-mediated pathway of apoptosis, while also inducing intracellular ROS accumulation in A172 human GBM cells. This extract may serve as a source of potential chemo-preventive agents or as a food supplement acting against brain cancers such as glioblastoma. Bioactive compounds in this extract should be further identified and investigated.

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