Apoptotic and Autophagic Cell Death Effects of the Hexane Extract of Tropical Marine Algae *Halymenia durvillei* against Human Glioblastoma Cells: *In vitro* and *in silico* Studies

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Abstract

Glioblastoma (GBM) considered as aggressive brain cancer with high mortality rate in patients even after surgical resection. Resistant to chemotherapy is the major problem in GBM therapy. Discovery of novel bioactive compounds from algae is being investigated as alternative sources for potential treatment as well as prevention in glioblastoma. This study revealed the effects of marine red algae extract from hexane solvent fraction of *Halymenia durvillei* (HDHE) on proliferation and cell death in A172 human GBM cells. HDHE decreased proliferation and promoted cell cycle arrest at G2/M phase. HDHE induced apoptotic cell death in A172 cells through mitochondrial membrane dysfunction, the decrease of anti-apoptotic Bcl-2 protein expression, and activation of caspase 3/7. Moreover, HDHE increased intracellular reactive oxygen species (ROS) production and accumulation of LC3-II, an autophagic marker. The docked conformation of palmitic acid, a major component of HDHE, showed a high affinity binding to TP53 and Beclin-1 as cell death-related target molecules. This research conclusively demonstrated that HDHE might serve as a potent anticancer agent against glioblastoma by promoting apoptotic and autophagic cell death in A172 human GBM cells.

Keywords: *Halymenia durvillei*, Glioblastoma, Apoptosis, Autophagy, Macroalgae

Introduction

Glioblastoma (GBM) is the aggressive and lethal type of brain tumors, which strongly considered as an incurable disease [1]. GBM tumors classified as cellular heterogeneity and rapidly grow, diffuse and infiltrative tumor cells [1]. Temozolomide (TMZ) is the most common chemotherapeutic drugs used to treat glioma by inhibiting cell growth and inducing apoptosis and autophagy. However, there are reports of resistance to temozolomide and poor prognosis after receiving this drug [2]. Therefore, novel chemotherapeutic substance that effective for GBM therapy with less toxic is urgently explore.

Marine algae are extensively used as functional foods and herbs in several regions including tropical culture. Marine algae contain valuable sources of bioactive compounds that has potential health benefit as well as anticancer activity [3]. Red marine algae are being interested as novel bioactive sources for nutraceuticals and cosmeceuticals [4]. *Halymenia durvillei* (HD) classified as red marine algae is cultivated in the coastline of Indo-Pacific region. It can be used as foods and natural colorants in food and cosmetics.

Red pigments of the algae contain bioactive substances like chlorophyll, carotenes, xanthophyll, zeaxanthins, luteins and phycocyanins [5]. HD extracts might be beneficial to applied as disease-preventing substances as well as anticancer agents due to the enrichment of bioactive ingredients. The hexane solvent extract from HD showed high antioxidant activity tested by DPPH and ABTS assays [6]. The extract, which enriched with n-hexadecanoic acid (palmitic acid) could inhibited proliferation against MDA-MB-231 triple-negative breast cancer cells by induction of apoptotic and autophagic cell death and associated with endoplasmic reticulum stress pathway [7]. Because of chemotherapeutic resistance problem, the investigation of potential anti-GBM compounds derived from marine algae that contained valuable bioactive substances is needed. This study demonstrated potential of HD extracts against human GBM cells on exhibiting anti-proliferation and cell death.

Materials and methods

Preparation of seaweed extracts
Halymenia durvillei (HD) were cultured and collected from Phetcha Buri coastal aquaculture research and development center, Phetcha Buri, Thailand. The authentication of *H. durvillei* (voucher specimens No. SPFR16040) was performed by Department of Fisheries, Coastal Aquatic Feed Research Institute, Coastal Fisheries Research, and Development Bureau, Petchaburi, Thailand. Dried whole crude HD were soluted and macerated in 95 % ethanol for 7 days. The ethanolic extracts were partitioned in hexane and evaporated to remove residual solvents. The hexane solvent extract (HDHE) was freeze-dried and collected for further experiments.

Cell culture
A172 human glioblastoma cells were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin (Gibco, St Louis, MO, USA) in an incubator with an atmosphere 5 % CO_2 at 37 °C.

Determination of cytotoxicity
HDHE was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA). Cultured A172 cells in 96-well plates was incubated with HDHE diluted in serum-free DMEM for 24 h. The final concentration of DMSO was 0.25 %. The toxicity of HDHE in treated cells was determined by incubating with methyl thiazolyl tetrazolium (MTT) solution (Sigma-Aldrich, MO, USA) at the final concentration of 0.4 mg/mL for 2 h, then dissolving formazan product with DMSO. The reaction was measured by a spectrophotometer (Varioskan Flash Microplate Reader, Thermo Fisher Scientific, MA, USA) at the absorbance of 562 and 630 nm.

Cell proliferation assay
Proliferation of A172 cells following HDHE incubation was analyzed by measuring fluorescent intensity of carboxyfluorescein diacetate succinimidyl ester (CFSE). A172 cells were labelled with CFSE dye (Sigma-Aldrich, MO, USA) in dark at 37 °C for 10 min and then plated on 6-well plates. Cells were treated with HDHE at 150 and 200 µg/mL for 48 h. After that, cells were retreated with the extract for another 48 h. Fluorescence intensity of CFSE of treated cells after trypsinization was measured using a Guava easyCyte flow cytometer (Luminex, TX, USA) at minimum of 5,000 event/sample.

Cell cycle analysis
Cell cycle distribution of A172-treated HDHE was observed by the quantitation of their DNA contents using propidium iodide (PI) staining. HDHE-treated cells for 48 h were harvested and fixed with 70 % ethanol for 30 min. Cells were incubated with propidium iodide (PI)/RNase (BD Biosciences, San Jose, CA, USA) for 30 min at room temperature. Cell cycle distribution was analyzed by a Guava easyCyte flow cytometer Luminex, TX, USA) at minimum of 10,000 event/sample.

Detection of apoptotic cell
Effects of HDHE on induction of apoptotic cell death of A172 cells was observed by fluorescent staining of Annexin V-FITC/7-aminoactinomycin D (7-AAD). HDHE-treated cells for 24 h were harvested and incubated with Annexin V-FITC/7-AAD fluorescence dual staining (BD Biosciences, San Jose, CA, USA) for 15 min in dark at room temperature. Stained cells were measured using a Guava easyCyte flow cytometer Luminex, TX, USA at minimum of 10,000 event/sample and evaluated as apoptotic and necrotic cells.

Analysis of mitochondrial membrane potential

The mitochondrial membrane potential of A172 cells after HDHE treatment was analyzed using JC-1 mitochondrial membrane potential assay (Luminex, TX, USA). Treated cells at 24 h were washed with PBS prior to incubated with JC-1 solution for 10 min. Fluorescence signals were detected using a Guava easyCyte flow cytometer (Luminex, TX, USA) at 2,000 event/sample.

Analysis of intracellular reactive oxygen species

Reactive oxygen species (ROS) production following HDHE treatment was analyzed by 2',7'-dichlorofluorescin diacetate (DCFDA) fluorescence labelling assays. A172 cells treated with HDHE at 0.5, 1, 3 and 6 h on 96-well plates were stained with 20 µM of DCFDA (Sigma-Aldrich, MO, USA) at 37 °C for 30 min. Intracellular ROS production was detected by a spectrophotometer (Varioskan Flash Microplate Reader, Thermo Fisher Scientific, MA, USA, and Synergy™ Neo2 Multi-Mode Microplate Reader, BioTek Instruments, VT, USA) at the absorbance of 490 and 535 nm.

Caspase 3/7 activation assay

HDHE-treated A172 cells for 24 h were collected and stained with caspase 3/7 carboxyfluorescein (FAM) reagents in dark at 37 °C for 1 h according to the manufacturer's protocol (Luminex, TX, USA). Cells were washed and stained with 7-AAD (Luminex, TX, USA). Population of activated caspases and dead cells were determined by a flow cytometer (Luminex, TX, USA) at 5,000 events/sample.

Autophagic cell detection

Treated A172 cells for 24 h were detached and stained with GFP-LC3 using FlowCellect autophagy assay kit (Luminex, TX, USA) according to the manufacturer's instructions. LC3-II fluorescence signals representing autophagosomes were detected using a Guava easyCyte flow cytometer (Luminex, TX, USA).

Immunoblotting

Protein lysates of treated A172 cells were extracted, separated, and transferred onto nitrocellulose membranes as previously described [7]. The primary antibodies, including rabbit anti-Bcl-2, rabbit anti-LC3 and rabbit anti-β-actin (Cell Signaling Technology, MA, USA) were used. The corresponding HRP-conjugated secondary antibodies (Southern Biotech, USA) were used. Expression of targeted proteins were visualized by ECL chemiluminescence system (Thermo Fisher Scientific, Waltham, MA, USA). The relative expression level of targeted proteins was normalized to β-actin expression compared with the control group.

Molecular docking

The ligand structures of palmitic acid (CID 585) was downloaded from the PubChem database. TP53 (PDB ID 7BMG) and Beclin-1 (PDB ID 5VAX) targets were downloaded from the Royal Collaboratory for Structural Bioinformatics Protein Data Bank. The ligand 3D structures were generated and optimized by LigandScout 4.4 Expert (Intel:Ligand GmbH, Vienna, Austria) [8]. Ligand binding, interactions, and binding affinities of the protein-ligand complexes were predicted by the Autodock Vina 1.1 module of LigandScout 4.4 Expert. The parameters of the docking runs were previously described [9].

Statistical analysis

Data were expressed as mean ± SD. Statistical variations of experiments were analyzed by GraphPad Prism statistical analysis software (GraphPad Software Inc, USA) using one-way ANOVA test. Data of mitochondrial membrane potential was analyzed using a Student's *t* test. A *p*-value less than 0.05 was considered statistically significant.

Results and discussion

Cytotoxicity and proliferation effects

Toxicity of HDHE on A172 cells was tested by MTT assays. At 24 h post-treatment, HDHE at increasing concentrations from 50 to 1,000 µg/mL significantly decreased viability of A172 cells compared with untreated and 0.25 % DMSO-treated control groups (**Figure 1A**). The 50 % inhibitory concentration (IC50) of HDHE against A172 cells was 24.57±0.383 µg/mL. The effects of HDHE on proliferation of A172 cells were performed by CFSE assays, an intracellular fluorescent dye that conjugates with cytoplasmic proteins. The dye is partitioned among daughter cells upon normal cell division. Increased mean fluorescence intensity (MFI) represents the inhibition of proliferation in detected cells, resulted from

accumulation of the dye in non-divided cells. After HDHE incubation for 96 h, MFI of HDHE-treated cells at 25 and 50 μg/mL was significantly increased compared with DMSO-treated group (**Figure 1B**). The results implied that HDHE inhibited proliferation of A172 cells at concentrations lower than IC50. HDHE concentration at IC50 level tested in A172 cells has less toxic to normal mammalian cells like mouse macrophage, keratinocyte and hepatocyte as previously reported [6]. Thus, cytotoxic concentration of HDHE may appropriate to inhibit proliferation of A172 cells, while safe to normal cells.

The cytotoxic activity of HDHE may be due to several bioactive components in the extracts, which mediated cell cycle arrest and/or programmed cell death. Several fatty acids were found in marine algae as their main constituents that may mediate anti-proliferative effects. Our previous study revealed several bioactive compounds in HDHE, including fatty acids, mainly n-hexadecanoic acid (Palmitic acid), 6,10,14-Trimethyl-2-pentadecanone, heptadecane, and hexadecenoic acid ethyl ester, could induce apoptotic and autophagic death in human triple-negative breast cancer cells, and associated with the induction of endoplasmic reticulum stress [7]. N-hexadecanoic acid purified from sea pens *Virgularia gustaviana* inhibited growth and promoted apoptosis in MDA-MB-231 breast cancer cells by increasing the expression of caspase-3, caspase-8 and Bax proteins [10]. Previous studies found that fatty acids might be used as potential therapeutic agents against GBM by disrupting lipid metabolism of tumor cells. Treatments with several types of fatty acids, including palmitic acid and eicosapentaenoic acid, enhanced lipotoxicity and reduced proliferation rate of U-87 glioma cells [11]. The acyl-CoA synthetase inhibitor 2-fluoropalmitic acid (2-FPA) suppressed proliferation, stem-like phenotype and invasion of glioma stem cells [12].

In this study, cell cycle distribution of A172-treated HDHE was observed at 48 h post-treatment. HDHE at 25 and 50 μg/mL significantly increased A172 cell number at G2/M phase, accompanied by the decreased cell number at G0/G1 and S phases at 48 h of treatment in concentration-dependent (**Figure 2**). This suggested that HDHE inhibited cell cycle progression in A172 cells by promoting cell arrest at G2/M phase.
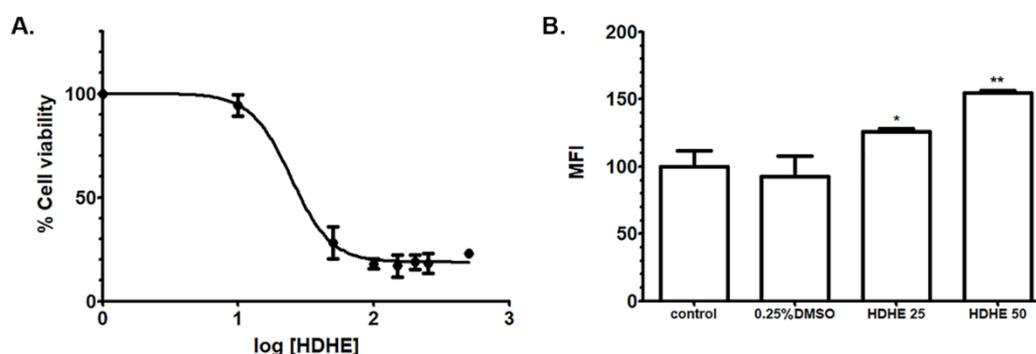

Figure 1 A) Cytotoxic effects of HDHE on A172 cells. Cell viability was measured by MTT assays after incubation with various concentrations of HDHE for 24 h. B) Growth-inhibitory effect of HDHE on A172 cells. Intensity of CFSE-labelled cells after treatment with HDHE at 25 and 50 μg/mL for 96 h was presented as mean fluorescence intensity (MFI) * $p \leq 0.05$, ** $p \leq 0.01$.
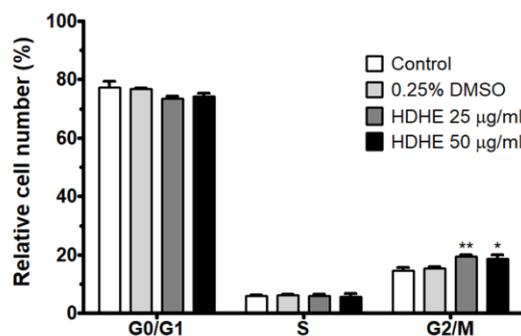

Figure 2 The percentage of cell number in each cell cycle phase (G0/G1, S, G2/M) at 48 h post-treatment were analyzed by PI staining. * $p \leq 0.05$, ** $p \leq 0.01$.

Induction of cellular apoptosis associated with mitochondrial membrane potential change and intracellular ROS level

Effects of HDHE on induction of apoptotic cell death in A172 cells was observed. Apoptotic cell population are identified by cells stained positive to annexin V as presented in the upper and lower right quadrants, while necrotic cells are identified by cells positive to 7-AAD, a DNA binding dye, as presented in the upper left quadrant of dot plots. 7-AAD is not permeable; therefore, the binding of 7-AAD and DNA is indicative of the loss of membrane integrity. At 24 h post-incubation, 50 µg/mL HDHE significantly increased the percentage of apoptotic cells by presenting an increasing number of total apoptotic cells, mostly at late apoptotic stage (the upper right quadrant), compared with controls (**Figure 3A**). Moreover, HDHE induces necrotic cell death as shown in the upper left quadrant of the dot plot. This implied that HDHE could induce apoptosis and necrosis on A172 cells.

The activation of apoptosis is initiated by the change in mitochondrial outer membrane permeabilization [13]. Cellular effects of HDHE on the change of the mitochondrial membrane potential was observed in 50 µg/mL HDHE-treated A172 cells at 48 h. The JC-1 assay contains a lipophilic cationic fluorescent dye acting as a mitochondrial activity marker. HDHE treatment at 50 µg/mL dramatically changed the mitochondrial membrane potentials, revealed by the increase of the percentage of JC-1 green fluorescent intensity (**Figure 3C**). This indicated that HDHE could promote apoptotic cell death in A172 cells by damaging mitochondrial membrane as their target.
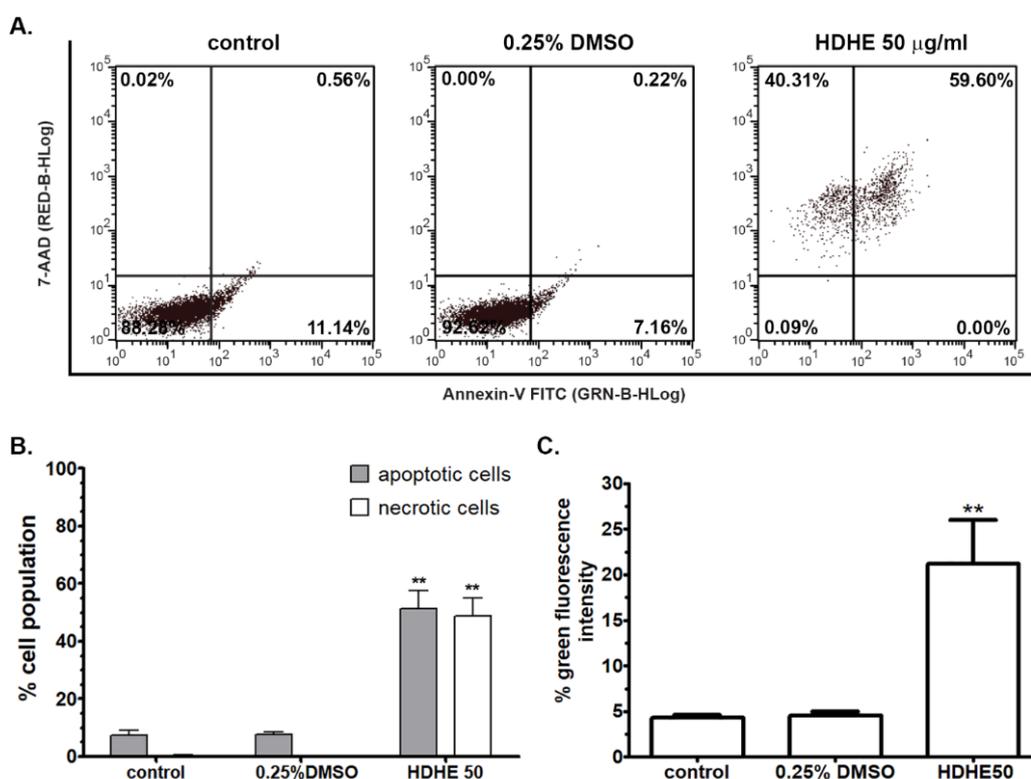

Figure 3 A) Dot plots of apoptotic and necrotic cells of A172 cells after HDHE treatment at 50 µg/mL for 24 h, analyzed by Annexin V-FITC staining. B) The percentage of total apoptotic A172 cells in HDHE-treated group compared with untreated and DMSO-treated groups. ** $p \leq 0.01$. (C) The mitochondrial membrane potential in A172 cells following HDHE treatment at 50 µg/mL for 24 h was indicated by the increase of green fluorescence intensity of JC-1. ** $p \leq 0.01$.

Many anticancer agents induce generation of intracellular ROS in cancerous cells, which triggering cellular organelles as a major modulator of apoptotic cell death [14]. The intracellular ROS after HDHE treatment was detected by DCF assays. The fluorescence intensity of DCF following 50 µg/mL HDHE treatment in A172 cells was measured at 0.5, 1, 3, and 6 h post-exposure. HDHE increased DCF intensity from 1 to 6 h post-incubation compared with untreated and DMSO-treated control group (**Figure 4**). This result indicated that HDHE induced ROS generation in glioblastoma cells.
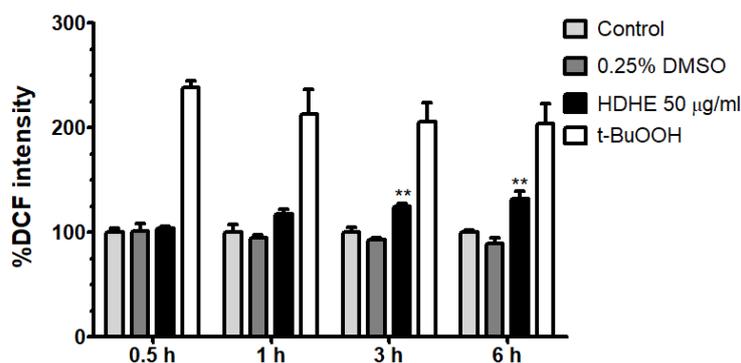
Figure 4 Detection of intracellular ROS generation in A172 cells following HDHE treatment at 50 μg/mL was analyzed by DCFDA assays. The percentage of DCF intensity of HDHE-treated group compared with untreated and DMSO-treated cells as a negative control group, and 1 mM t-BuOOH-treated cells as a positive control group. ** $p \leq 0.01$.

Promotion of cellular autophagy

Autophagic cell death is a multi-step lysosomal degradation that processed normally in long-lived cells to destroy unwanted structures and recycled proteins and organelles. Cell death by autophagy in brain cancers has emerged as tumor-suppressing function for removal of neoplastic cells [15]. Fatty acids trigger autophagy cancer cells that could result to inducing apoptosis [16]. Effects of HDHE on generation of the autophagic flux was determined by detecting the levels of translocated LC3 in the autophagosomes of treated cells. At 24 h post-exposure, 50 μg/mL HDHE increased LC3 fluorescence intensity in A172 cells compared with controls (**Figure 6**). This implied that HDHE promoted autophagic cell death in A172 cells as shown by the increase in the expression of endogenous LC3-II, representing the onset of autophagy. This finding was consistent with a previous report of autophagy inducing effect of HDHE in MDA-MD-231 human breast cancer cells at the same concentration [7]. A previous study found that palmitic acid-enriched chloroform extracts of green algae *Ulva intestinalis* and *Ulva lactuca* could induce autophagic cell death in SiHa cervical cancer cells [17].
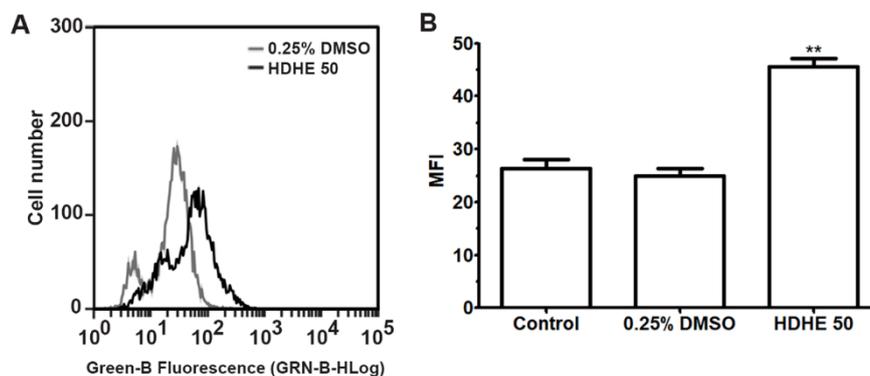

Figure 5 Detection of autophagic flux in A172 cells after HDHE treatment at 50 μg/mL for 24 h. A) Histogram of autophagic flux detection in HDHE-treated group (black line) compared with DMSO-treated control group (grey line). B) Mean fluorescence intensity (MFI) of LC3. ** $p \leq 0.01$.

***In vitro* expression and *in silico* interaction of apoptotic and autophagic molecules**

Our previous study of HDHE effects on MDA-MB-231 cells affirmed that HDHE altered the expression of key molecules contributing to apoptotic cell death including Bax, Bcl-2, and active caspase-3 and caspase-7. In the present study, expression of Bcl-2 and caspase 3/7 were detected. The Bcl-2 family of proteins regulate apoptotic cell death by controlling mitochondrial permeability. Bcl-2 maintains the integrity of the mitochondrial outer membrane in order to prevent cytochrome c release and activate

apoptosis [18]. The executioner caspases (caspase-3, 6 & 7) is a major mediator of apoptosis that cleave several cellular proteins and eventually leads to cell death [11]. Anti-apoptotic protein Bcl-2 expression was significantly decreased in A172-treated cells for 24 h compared with controls (**Figure 6**). Furthermore, the activation of caspase-3 and caspase-7 cascades as the effector molecules mediating apoptotic cell death increased following 50 µg/mL HDHE treatment at 24 h, indicated by the shift of the population of HDHE-treated cells into the right upper quadrant (**Figure 7**). These results revealed the apoptotic-promoting activity of HDHE in A172 cells that modulated via the mitochondrial-mediated pathway. For autophagic-regulatory proteins, LC3 and Beclin-1 are used as markers. LC3-II protein level was significantly increased following HDHE exposure (**Figure 6**). These results showed that HDHE targeted proteins regulating apoptotic and autophagic cell death in A172 cells.
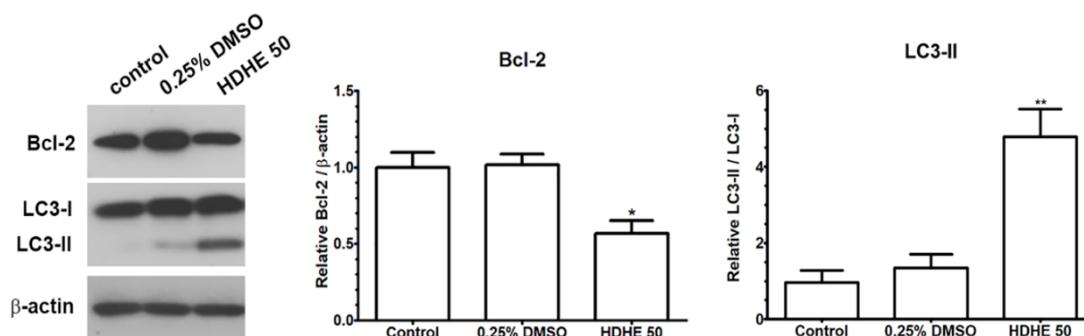

Figure 6 Immunoblotting analysis of Bcl-2 and LC3-II proteins level in A172 cells after HDHE treatment at 50 µg/mL for 24 h compared with untreated control and 0.25 % DMSO-treated groups. β-actin was used as an internal loading control. * $p \leq 0.05$, ** $p \leq 0.01$.
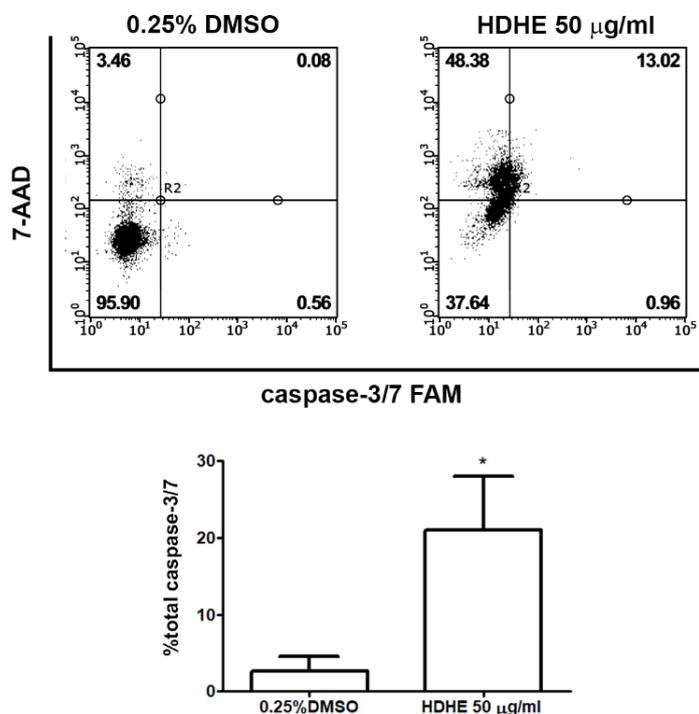

Figure 7 Dot plots of the level of active caspase-3/7 detected in A172 cells after 50 µg/mL HDHE treatment for 24 h. A bar graph represents the percentage of caspase-3/7 FAM in cells treated with 50 µg/mL HDHE compared with 0.25 % DMSO, * $p \leq 0.05$.
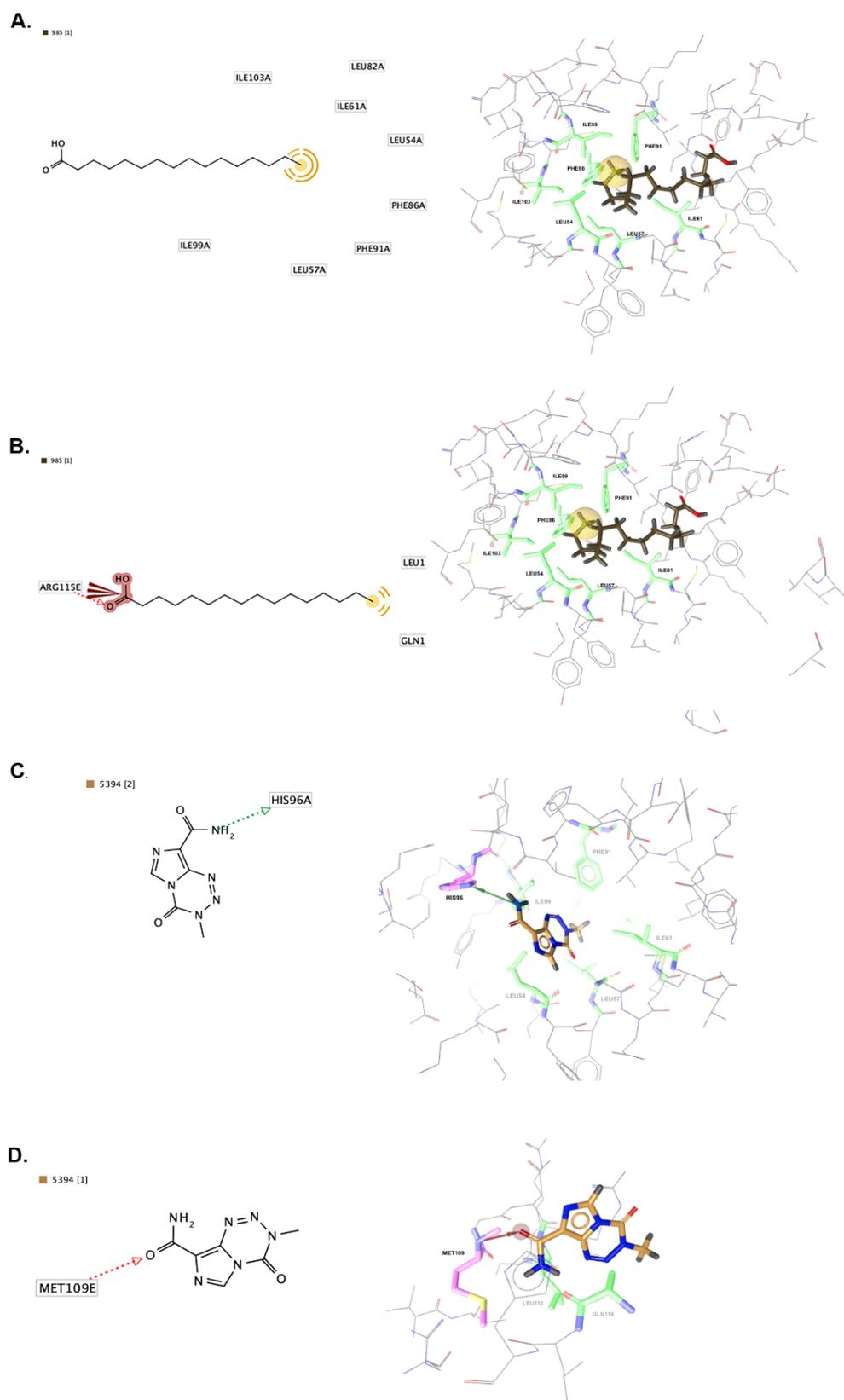
Figure 8 Interaction of substrate and amino acid residues and docking of: A) palmitic acid (PA) and TP53, B) palmitic acid (PA) and Beclin-1, C) temozolomide and TP53, D) temozolomide and Beclin-1.

Table 1 Binding affinity values and interacting residues of palmitic acid (PA) and temozolomide toward TP53 and Beclin-1.

Compound-binding sites	Affinity (kcal/mol)	Hydrophobic interaction	Others
PA-TP53	−9.90	Leu54, Leu57, IIe61, Leu82, Phe86, Phe91, IIe99, IIe103	-
PA-Beclin1	−5.60	Leu112, Gln118	NI: Arg115
Temozolomide-TP53	−8.18	-	Hydrogen bond donor: HIS96
Temozolomide-Beclin1	−4.83	-	Hydrogen bond acceptor: Met109

HDHE contains palmitic acid as the major bioactive substance [7]. Palmitic acid provided as exogenous source of fatty acid is rapidly taken up into the brain by crossing the blood-brain barrier after phospholipid incorporation [19]. Several studies reported the association between fatty acid consumption and prevention of brain cancers. Metabolic alteration by ketogenic diets with caloric restriction may provide adjuvant therapy of glioblastoma by inhibited proliferation of GBM both *in vitro* and *in vivo* as well as reduced tumor stem cell expansion [20,21]. The molecular interactions between palmitic acid and the plausible cell death-inducing targets, including TP53 and Beclin-1 were investigated by molecular docking. TP53 transcription factor plays a critical role in tumor suppression mainly by induction of apoptosis and cell cycle arrest, increasing tumor susceptibility to chemotherapy in several cancers including GBM [22]. The interaction of each ligand with targets are depicted (**Figures 8A** and **8B**). The docked conformation of palmitic acid showed a high affinity binding to both TP53 and Beclin-1 (**Table 1**). Palmitic acid interacted with TP53 through hydrophobic interactions at the Leu54, Leu57, IIe61, IIe82, Phe86, Phe91, IIe99, IIe103 residues. Palmitic acid and Beclin-1 formed hydrophobic interactions with Leu112 and Gln118 and also formed a negative ionization (NI) with Arg115. In comparison, docking interactions between temozolomide, a positive chemotherapeutic drug used for GBM treatment, and TP53 and Beclin-1 were also analyzed. The docked conformation of temozolomide also showed a high affinity binding to both TP53 and Beclin-1 but lower than palmitic acid between each molecule. Temozolomide only formed hydrogen bonding with TP53 at HIS96 and with Beclin-1 at Met109 residues (**Figures 8C** and **8D**, **Table 1**). This implied that major bioactive compounds presented in HDHE has a high interaction with cell death-related target molecules, which greater than temozolomide, a positive GBM drug.

Conclusions

The hexane extract of *H. durvillei*, containing a mixture of fatty acids, may play a protective role against glioblastoma by inducing ROS generation, cell cycle arrest, apoptotic and autophagic cell death through modulation of key molecules. This algae extract could be developed as chemopreventive agents or food supplements against glioblastoma and other brain cancers. The limitation of the study is the lack of information on the toxicity of HDHE on normal human astrocytes and other glioblastoma cell lines. Further studies on signaling pathways, identification of novel bioactive ingredients of this extract and validation of anticancer activity and safety in tumor organoid and *in vivo* models should be performed.

Acknowledgements

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