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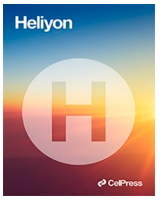

Research article

Extracts of tropical green seaweed *Caulerpa lentillifera* reduce hepatic lipid accumulation by modulating lipid metabolism molecules in HepG2 cells

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ABSTRACT

Seaweed has attracted attention as a bioactive source for preventing different chronic diseases, including liver injury and non-alcoholic fatty liver disease, the leading cause of liver-related mortality. *Caulerpa lentillifera* is characterized as tropical edible seaweed, currently being investigated for health benefits of its extracts and bioactive substances. This study examined the effects of *C. lentillifera* extract in ethyl acetate fraction (CLEA) on controlling lipid accumulation and lipid metabolism in HepG2 cells induced with oleic acid through the *in vitro* hepatic steatosis model. Gas chromatography-mass spectrometry (GC-MS) analysis indicated that CLEA contained diverse organic compounds, including hydrocarbons, amino acids, and carboxylic acids. Docked conformation of dl-2-phenyltryptophane and benzoic acid, two major bioactive CLEA components, showed high affinity binding to SIRT1 and AMPK as target molecules of lipid metabolism. CLEA reduced lipid accumulation and intracellular triglyceride levels in HepG2 cells stimulated with oleic acid. The effect of CLEA on regulating expression of lipid metabolism-related molecules was investigated by qPCR and immunoblotting. CLEA promoted expression of the SIRT1 gene in oleic acid-treated HepG2 cells. CLEA also reduced expression levels of SREBF1, FAS, and ACC genes, which might be related to activation of AMPK signaling in lipid-accumulated HepG2 cells. These findings suggest that CLEA contains bioactive compounds potentially reducing triglyceride accumulation in lipid-accumulated HepG2 hepatocytes by controlling lipid metabolism molecules.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a globally burdensome chronic liver disease, characterized by excessive lipid accumulation in hepatocytes and liver tissues. It is associated with malnutrition. Prevalence of NAFLD worldwide is increasing. A meta-analysis reported in 2022 estimated the overall prevalence of NAFLD worldwide to be 32.4% [1]. NAFLD is closely associated with several chronic diseases, including obesity, diabetes and hyperlipidemia. Increased visceral adiposity and insulin resistance caused by increased free fatty acids (FFA) supplied to the liver play important roles in the pathogenesis of NAFLD [2]. A severe phase of NAFLD, called non-alcoholic steatohepatitis (NASH), results from oxidative stress induction in affected liver tissues. It may result in liver fibrosis, cirrhosis, and hepatocellular carcinoma. Apart from exercise and dietary intervention, statins are the primary therapy for NAFLD patients. However, side effects after long-term administration such as kidney injury and skeletal myotoxicity have been reported.

Marine algae have been mentioned as a great potential bioactive source of beneficial health effects [3]. A systematic review revealed that consumption of seaweed supplements could heal liver injury and maintain metabolic factors such as body mass index, serum triglycerides, and blood sugar level in NAFLD patients [4]. The green seaweed *Caulerpa lentillifera* (CL) is an edible green marine macroalga that is cultivated as food ingredients and for consumable products in tropical regions. Nutritionally, CL has high amounts of essential amino acids equivalent to egg, vitamin B1, vitamin B2, vitamin E, fatty acid, iodine, phosphorus, calcium, magnesium, and copper [5]. Many studies have revealed disease-preventing effects of CL extracts. Previous studies found CL extracts inhibited proliferation and induced apoptosis in A172 human glioblastoma cells [6,7]. Treatment of CL extracts in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages could suppress pro-inflammation mediators and reduce DNA damage [8]. This study aimed to investigate the lipid-lowering effects of CL extracts in the ethyl acetate fraction in lipid-accumulated HepG2 hepatocyte cell line by regulating lipid metabolism-related genes. The results of this study would help expand the application of CL.

2. Materials and methods

2.1. Material collection and extract preparation

Fresh CL was harvested from Phetchaburi coastal fisheries research and development center. Thailand, in January 2019. The CL (1.0 kg) was air-dried, milled, and macerated with 3 L of 95% ethanol at room temperature for 7 days to yield, and the solvents were evaporated under reduced pressure (%yield = 12). After that, ethanol CL extract was partitioned successively with *n*-hexane until colourless, ethyl acetate, butanol and distilled water.

2.2. Gas chromatography-mass spectrometry (GC-MS) analysis

The phytochemical investigation of CLEA extract was performed on GC-MS equipment (Agilent Technologies 7890B). The GC-MS system analysis condition was as follows: HP-5 column 30 m x 0.25 mn i.d with 0.25 μm film thickness. The flow rate of the mobile phase (carrier gas: He) was set at 1.0 mL/min. In the gas chromatography part, the temperature program (oven temperature) was 50 °C raised to 250 °C at 5 °C/min and the injection volume was 1 μL. Samples dissolved in chloroform were run fully at a range of 50–400 *m/z*. The results were compared with references by the Wiley Spectral library search program (NIST MS search 2.0).

2.3. Molecular docking

The ligand structures, dl-2-Phenyltryptophane (D2P: CID 609934) and benzoic acid (BA: CID 243), were downloaded from the PubChem database. Sirtuin 1 (SIRT1) (PDB ID 4IF6) and AMP-activated protein kinase (AMPK) (PDB ID 6bx6), which are the energy metabolism-related molecular 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) (Wolber & Langer, 2005). 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: 8 for exhaustiveness, 9 for the maximum number of binding modes to generate, and 3 for the maximum energy difference between the best binding mode and the worst one. The parameter was validated by redocking the co-crystallized ligands of SIRT1 and AMPK, adenosine-5-diphosphoribose and 2-({5-bromo-2-[(3,4,5-trimethoxyphenyl)amino]pyrimidin-4-yl}oxy)-N-methylbenzene-1-carboximidic acid, into the indicated binding sites, respectively. The redocking RMSD values of those were 0.00 Å, which demonstrated the high reliability of the docking scheme method.

2.4. Determination of cytotoxicity

Cultured HepG2 cells in 96-well plates were incubated with 200 μM sodium oleate (OL) and CLEA diluted in serum-free media at a final concentration of 10–500 μg/mL for 24 h. The cytotoxicity of treated cells was determined by the methyl thiazolyl tetrazolium (MTT) assays. MTT solution (Bio Basic, Canada) was dissolved in treated wells at a final concentration of 30 μg/mL, followed by 3 h of incubation. Dimethyl sulfoxide (DMSO) (Sigma, USA) was added to dissolve MTT formazan. The reaction was measured by a spectrophotometer (Varioskan Flash Microplate Reader, Thermo Fisher Scientific) at the absorbance of 562 and 630 nm.

2.5. Oil red O staining

HepG2 cells were cultured onto glass coverslips and then co-treated with OL and CLEA for 24 h. Treated cells were fixed with 4% paraformaldehyde for 30 min and stained with Oil Red O solution (Sigma, USA) for 15 min at room temperature. Cells were counterstained with Meyer's hematoxylin (Bio Optica, Italy). Cells were observed under a Nikon Ds-Ri1 light microscope (Nikon, Japan). The intracellular lipid droplet area was measured and calculated.

2.6. Triglyceride assay

Treated HepG2 cells with OL and CLEA for 24 h were harvested and homogenized. Cell pellets were heated up to 80 °C then cooled down twice and centrifuged to collect solubilized triglycerides. The concentration of intracellular triglycerides was determined using the Triglyceride Quantification Assay Kit (Abcam, Canada) according to the manufacturer's instruction, and measured using an ELISA plate reader (Molecular Devices LLC, United States) at an absorbance of 570 nm.

2.7. Real time PCR

Total RNA from the cells was extracted using the GENEzol reagent (Geneaid biotech, New Taipei, Taiwan) and isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentrations were quantified using a NanoDrop™ (ND-1000) spectrophotometer. One microgram of RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA), and the first strand of cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Richmond, CA, USA). Real time PCR reaction was performed using the iTaq SYBR green supermix system (Bio-Rad, CA, USA) with the following sets of forward and reverse primers: sirtuin 1 (SIRT1), fatty acid synthase (FAS), sterol regulatory element binding transcription factor 1 (SREBF1), acetyl-CoA carboxylase alpha (ACC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (KiCqStart SYBR Green, Sigma-Aldrich, USA). The reactions were performed on the ABI 7500 real time PCR system (Applied Biosystem, Foster City, CA, USA). The PCR conditions were as follows: pre-incubation at 95 °C for 1 min, 1 cycle; amplification at 95 °C for 30 s, 58 °C for 32 s, and 72 °C for 32 s, 40 cycles; melting at 95 °C for 15 s, at 60 °C for 20 s, at 95 °C for 15 s, 1 cycle; cooling at 60 °C for 15 s, 1 cycle. The fold change of mRNA expression relative to the average of the GAPDH as internal standard and the reference sample was calculated using the $2-\Delta\Delta CT$ method. The relative mRNA expressions of the CLEA-treated group in each concentration were compared with those of the control group treated with OL.

2.8. Immunoblotting

Whole proteins of treated HepG2 cells were extracted using RIPA buffer with 1% protease inhibitor cocktail. Lysates at 10 μg concentration were added to an SDS-PAGE gel and subjected to electrophoresis, and transferred onto nitrocellulose membranes (Millipore, USA). The primary antibodies, including rabbit anti-pAMPKα (#2531, Cell Signaling Technology, MA, USA), rabbit anti-AMPKα (#2532, Cell Signaling Technology, MA, USA) and mouse anti-β-actin (ab8226, Abcam, USA) were used. The corresponding HRP-conjugated secondary antibodies (Abcam, USA) were used. Expression of targeted proteins were visualized by TMB 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.

2.9. Statistical analysis

Data were expressed as mean ± SEM. Statistical variations of all experiments were analyzed by GraphPad Prism statistical analysis software version 9.5.1 (GraphPad Software Inc, USA) using a one-way ANOVA test. A p-value less than 0.05 was considered statistically

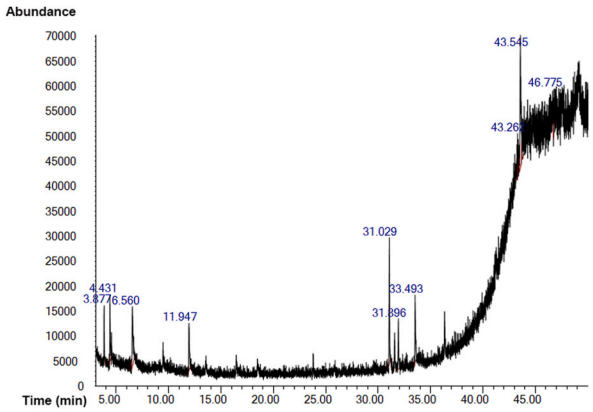

Fig. 1. Chromatogram of CLEA

significant.

3. Results

3.1. Molecular characterization of CLEA

Bioactive compounds in CLEA were characterized by GC-MS. Ten compounds were identified as shown in the chromatogram (Fig. 1). These constituents and their calculated percentage peak area compositions are shown in Table 1. CLEA contains various organic compounds including polycyclic hydrocarbons (compound 4, 5, 6, 7, 10), amino acids (compound 2, 9), and carboxylic acids (compound 1, 3, 8). The two highest amounts of bioactive compounds represented by the percentage of peak area are dl-2-phenyltryptophane [retention time (RT) 43.545 min, 29.28 %] and benzoic acid (RT 6.56 min, 13.79%).

3.2. Docking studies

Molecular docking studies were performed to investigate the molecular interactions between the major bioactive components in CLEA, including dl-2-Phenyltryptophane (D2P) and benzoic acid (BA), and the plausible lipogenesis targets, including SIRT and AMPK. The molecular simulation results regarding the interaction of each ligand with targets are depicted in Fig. 2. The docked conformation of D2P and BA showed high affinity binding to both SIRT1 and AMPK (Table 2). D2P interacted with SIRT1 through hydrogen bonding at the Arg446 residue and hydrophobic interactions with the Phe414 (Fig. 2A). D2P and AMPK formed a hydrogen bond at Asn162 and hydrophobic interactions with the Ala43, Leu22, Tyr95, Val96, Leu146, Lys45, and Val30 (Fig. 2B). BA interacted with SIRT1 by hydrogen bond formation at the Tyr280 residue and hydrophobic interactions with the Phe414 and Val445 (Fig. 2C). BA and AMPK formed hydrophobic interactions with Val96, Ile77, Leu22, Ala43, and Leu146 (Fig. 2D). This implied that major bioactive compounds presented in CLEA could interact with lipid metabolism-related target molecules.

3.3. Viability of lipid accumulated HepG2 cells after CLEA treatment

HepG2 cells were treated with OL in order to stimulate intracellular lipid accumulation. Cell viability after CLEA extracts treatment was determined to evaluate the toxicity of the extracts in lipid-accumulated cells. OL treatment did not affect the viability of HepG2 cells compared with the untreated group. Low and moderate concentrations of CLEA (0–100 μg/ml) did not affect the viability of OL-treated HepG2 cells but high concentration at 200–500 μg/ml reduced OL-treated HepG2 viability compared with the OL-treated control group (Fig. 3). The result implied that a high concentration of CLEA might be toxic to lipid-accumulated HepG2 cells.

3.4. Effect of CLEA on lipid droplet accumulation

OL treatment in HepG2 cells led to a significant increase of intracellular lipid droplet accumulation, represented by deposited red dots after staining with oil red O with unchanged cell morphology (Fig. 4A). At 24 h post-treatment with CLEA, intracellular area containing oil red O-stained lipid droplets in OL-treated HepG2 cells were significantly reduced at 100, 250 and 500 μg/ml compared with the OL-stimulated and untreated groups (Fig. 4B). The result indicated that CLEA could reduce lipid accumulation in HepG2 cells.

3.5. Triglyceride accumulation

The intracellular triglyceride accumulation following CLEA treatment in OL-treated HepG2 cells was analyzed after 24 h of incubation by triglyceride quantification assay kit. OL treatment in HepG2 cells mildly increased triglyceride level. Increasing concentrations of CLEA treatment continuously decreased the percentage of triglyceride level (Fig. 5). The result indicated that CLEA effectively decreased intracellular triglyceride accumulation in OL-treated HepG2 cells.

Table 1
Profile of bioactive compound identified in CLEA.

Number of Compounds	RT (min)	Molecular weight	Phytochemical compound	Peak area	% Peak area
1	3.878	130.136	(S)-3-Ethyl-4-methylpentanol	294,186	5.20
2	4.429	206.063	Pyrimidin-2-amine, 4-(2,4-dimethyl-5-thiazolyl)-	314,395	5.56
3	6.562	150.068	Benzoic acid, 2,6-dimethyl-	779,649	13.79
4	11.949	206.025	4-Chloro-6-(2-hydroxyphenyl)pyrimidine	405,552	7.17
5	31.028	206.203	4,7-Methanoazulene, decahydro-1,4,9,9-tetramethyl-	752,012	13.30
6	31.895	206.203	Tetradecahydro-1-methylphenanthrene	388,071	6.86
7	33.491	344.064	2-(4-Methyl-2-nitrophenylcarbamoyl)-terephthalic acid	579,464	10.25
8	43.261	294.256	Butanoic acid, 4′-propyl[1,1′-bicyclohexyl]-4-yl ester	171,253	3.03
9	43.544	280.121	dl-2-Phenyltryptophane	1,655,848	29.28
10	46.774	206.051	1-(4-Methoxyphenyl)imidazoline-2-thione	314,169	5.56

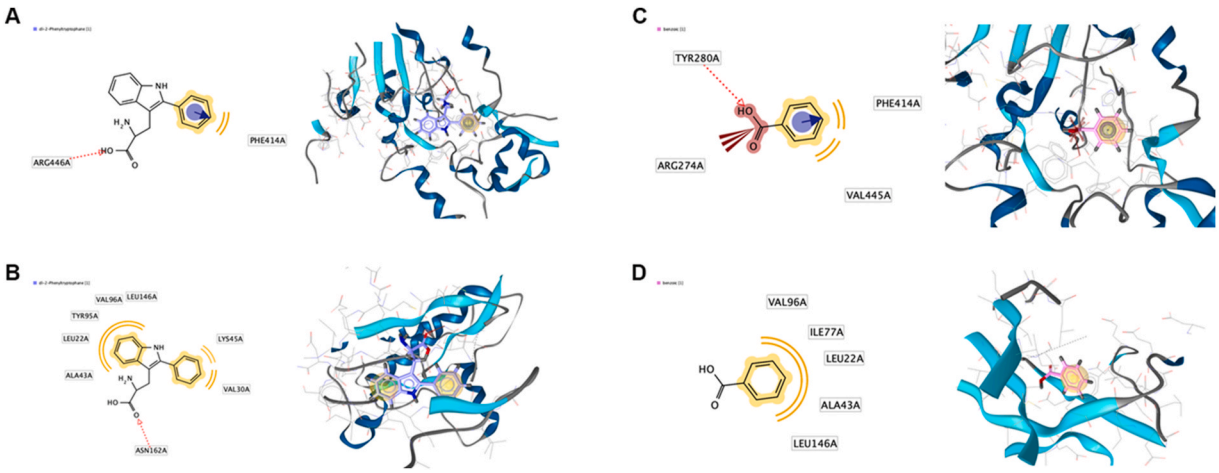

Fig. 2. Interaction of substrate and amino acid residues and docking of (A) D2P and SIRT1, (B) D2P and AMPK, (C) BA and SIRT1, (D) BA and AMPK.

Table 2
Binding affinity values and interacting residues of D2P and BA toward SIRT and AMPK.

Compound-binding sites	Affinity (kcal/mol)	Hydrogen bonds	Hydrophobic interaction	Others
D2P-SIRT1	−11.20	HBA: Arg446	Phe414	AR
D2P-AMPK	−15.10	HBA: Asn162	Ala43, Leu22, Tyr95, Val96, Leu146, Lys45, Val30	–
BA-SIRT1	−12.10	HBA: Tyr280	Phe414, Val445	NI: ARG274, AR
BA-AMPK	−9.20	–	Val96, Ile77, Leu22, Ala43, Leu146	–

HBA: hydrogen bond acceptor, AR: aromatic ring, NI: negative ionizable area.

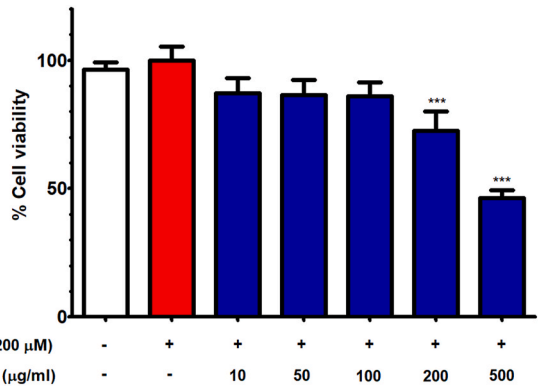

Fig. 3. Viability of HepG2 cells after incubation with 200 μM OL and CLEA at various concentrations. ***$p \leq 0.001$ compared with OL-treated control.

3.6. Expression of hepatic lipid metabolism molecules

To determine the effects of CLEA on the alteration of lipid metabolism in HepG2 cells, mRNA expression of lipogenesis-related genes including SIRT1, FAS, SREBF1, and ACC were determined by qPCR. At 24 h, OL treatment in HepG2 cells decreased SIRT1 while increased FAS, ACC, and SREBF1 compared with the untreated cells. CLEA treatment in OL-treated HepG2 cells promoted SIRT1 significantly at 50 and 100 μg/ml (Fig. 6A) but decreased FAS, ACC and SREBF1 mRNA expression levels in all tested concentrations compared with OL-treated cells (Fig. 6B–D). Protein expression of AMPKα in both phosphorylated and total forms was determined by immunoblotting. The expression ratio of pAMPKα and total AMPKα proteins in OL-treated group is decreased while co-treatment of OL and CLEA at 50 and 100 μg/ml increased pAMPKα/total AMPKα ratio (Fig. 7, supplementary file 1). This result indicated that CLEA promoted lipid lowering activity by regulating lipid metabolism-related molecules.

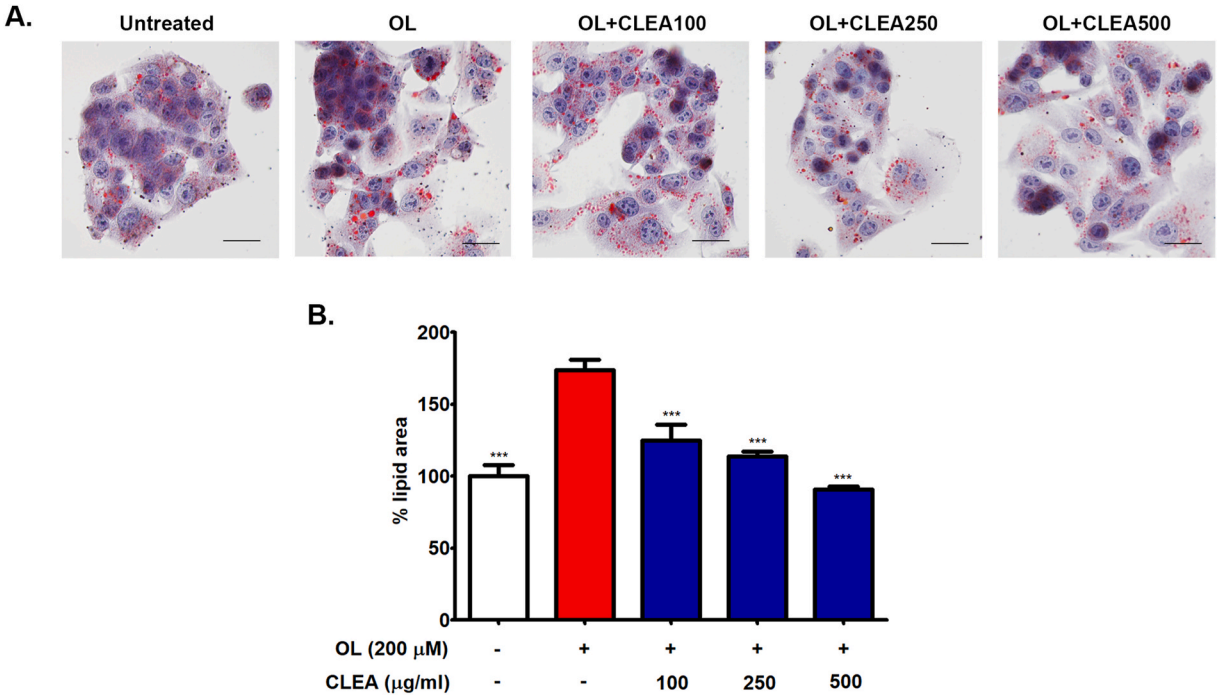

Fig. 4. (A) The photomicrograph of HepG2 cells after treatment with 200 μM OL and CLEA at various concentrations for 24 h, stained with oil red O. (scale bar = 50 μm) (B) The percentage of lipid area in HepG2 cells after treatment with 200 μM OL and CLEA at various concentrations. ***$p \leq 0.001$ compared with OL-treated control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

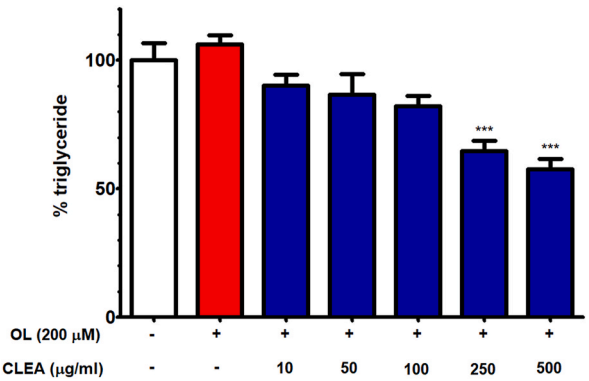

Fig. 5. The percentage of triglyceride in HepG2 cells after treatment with 200 μM OL and CLEA at various concentrations. **$p \leq 0.01$, ***$p \leq 0.001$ compared with OL-treated control.

4. Discussion

NAFLD is classified as a metabolic syndrome that is emerging as the most common liver disease. A large population-based cohort study revealed seaweed consumption is inversely associated with the prevalence of NAFLD [9]. Tropical seaweed C. lentillifera is beneficial as health nutraceuticals and therapeutic pathology supplements because of its high amount of macro and micronutrients [10]. This study revealed for the first time the potential of the extracts from C. lentillifera on hepatic lipid-lowering activity. This effect was demonstrated by OL-induced lipid accumulation in HepG2 cells. CLEA could reduce triglycerides accumulated in HepG2 cells but also mildly reduce its viability at high concentrations (250 μg/mL). These results suggested that CLEA at concentration between 100 and 250 μg/mL may be effective to reduce lipid accumulation in HepG2 cells.

Hepatocytes play a pivotal role in lipid metabolism, which is activated by their corresponding enzymes after their uptake of excessive fatty acids, leading to synthesis of triglycerides. Excessive accumulation of hepatic lipids can cause an imbalance between lipid formation and metabolism and lead to hepatic steatosis, or fatty liver. SIRT1/AMPK is the main signaling pathway involved in

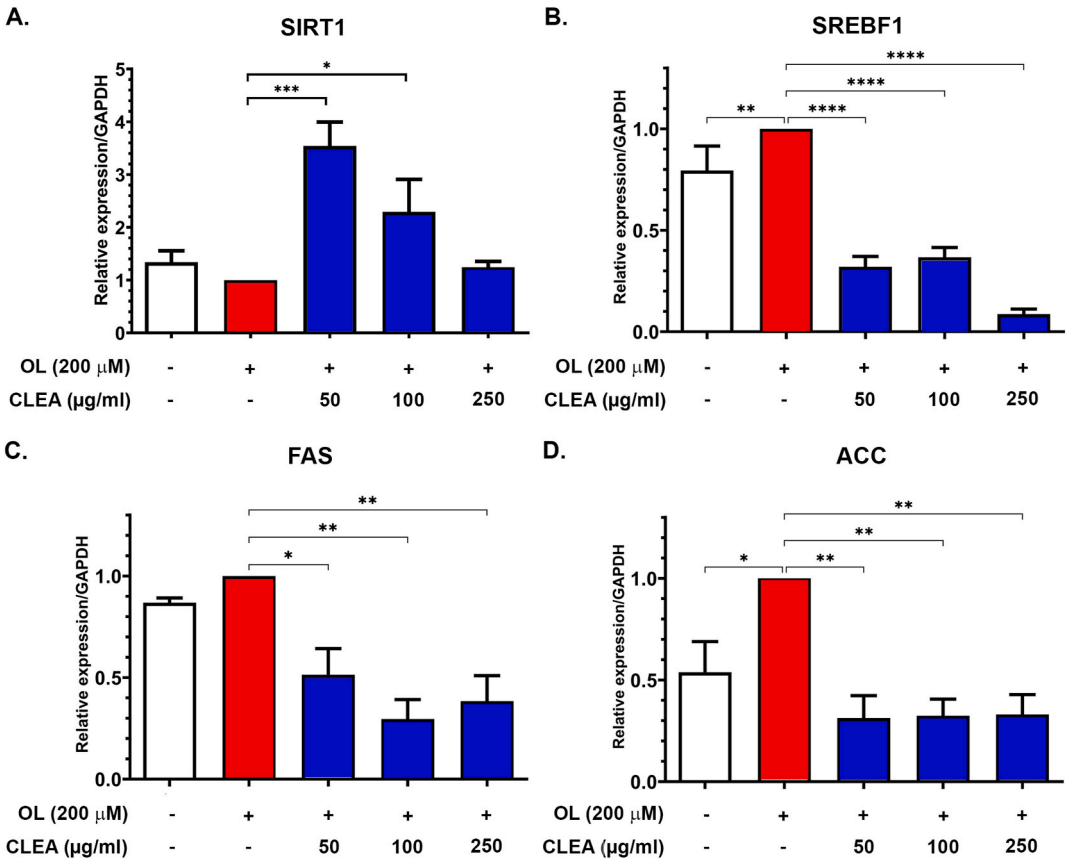

Fig. 6. The relative mRNA expression of (A) SIRT1, (B) SREBF1, (C) FASN, and (D) ACACA in HepG2 cells treated with 200 μM OL and CLEA at 50, 100 and 250 μg/ml. All genes were normalized with GAPDH of each group. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared with OL-treated control.

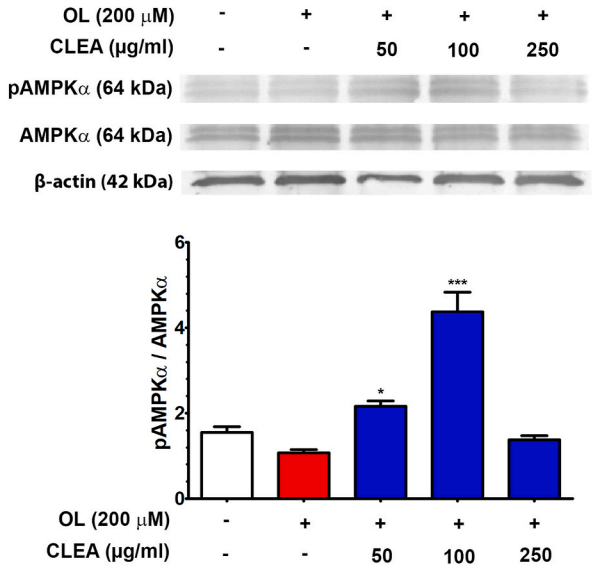

Fig. 7. Representative blots of immunoreactive expression of pAMPKα and total AMPKα in HepG2 cells treated with 200 μM OL and CLEA at 50, 100 and 250 μg/mL. Bar graphs represent the expression ratio of pAMPKα and total AMPKα when normalized with β-actin. *$p \leq 0.05$, ***$p \leq 0.001$ compared with OL-treated control.

regulation of lipid metabolism. Activation of SIRT1/AMPK expression as fuel-sensing molecules could promote fatty acid oxidation and ameliorate hepatic steatosis [11]. The mammalian sirtuins family (SIRT1-7) serve as energy status sensors and protect metabolic stressors. SIRT1 acts as the primary mediator in regulating the expression of lipid metabolism genes. SIRT1 plays a pivotal role in lipid metabolism by inhibiting SREBP during fasting, which results in inhibition of lipid synthesis and fat storage [12]. SIRT1 also acts as metabolic sensor to regulate cellular metabolism and delay age-related degeneration [13]. SIRT1 promotes AMPK phosphorylation as a key pathway to regulate the mitochondrial biogenesis and fatty acid synthesis in hepatocytes [14]. During FFA intake, the upregulation of SREBP1, ACC, and FAS in hepatocytes are associated with fatty acid and triglyceride synthesis, which influenced by AMPK inactivation [15]. SREBP1 is the potent transcription factor activated during *de novo* lipogenesis. ACC and FAS are lipid-metabolizing enzymes that are upregulated as downstream targets following SREBP1 elevation [15]. The result shown that CLEA could promote lipid metabolism by upregulating the expression of SIRT1 significantly at 50 and 100 μg/ml and downregulating SREBF1, ACC, and FAS genes as downstream targets in all tested concentrations. CLEA increased the expression ratio pAMPKα and total AMPKα proteins in OL-treated cells. It might be implied that CLEA could promote AMPK phosphorylation in hepatocytes and lead to inhibit the expression of downstream molecules. Together, the result indicated that CLEA could ameliorate lipid accumulation in OL-treated HepG2 cells by controlling lipid metabolism molecules through activation of SIRT1/AMPK pathway.

Bioactive compounds in CLEA contain various organic compounds, including fatty acids, amino acids, phenolic compounds, and carboxylic acids, which are characterized by GC-MS. The dl-2-phenyltryptophane, aromatic amino acids were the highest proportion of the extract, followed by benzoic acid. Two major ligands in CLEA, dl-2-Phenyltryptophane and benzoic acid, could dock with lipid metabolism target proteins SIRT1 and AMPK as shown by their high affinity binding. A previous study found that CL extracts in hexane solvent fraction had *in vitro* lipase inhibitory activity, presented by a lower half-maximal effective concentration (EC50) than the standard drug orlistat, indicating potent anti-obesity activity [10]. Benzoic acid is widely presented in algae as their metabolites. It is evidenced that phenolic compounds could activate SIRT1 activity, and subsequently promote AMPK activation, inhibiting fatty acid synthesis as a result [16,17]. Therefore, benzoic acid, as major phenolic compound in CLEA, might promote lipolysis in OL-treated HepG2 cells by activating the SIRT1/AMPK pathway. This was supported by the action of benzoic acid derivatives on inhibiting hepatic damage and reducing hepatic lipid accumulation induced by diabetes or a high-fat diet [18–20]. This suggests that the major bioactive compounds in CLEA could interact with molecules regulating lipid metabolism. Tryptophan is one of the essential amino acids found in many species of macroalgae [21]. A supplement of tryptophan in rats promoted fatty acid oxidation and resulted in decreased serum lipid, LDL and VLDL [22]. Dietary tryptophan in humans reduced serum total cholesterol, LDL and apolipoprotein B, and it increased sleep duration, suggesting a protective role against metabolic syndrome [23]. This study has some potential limitations. Protein expression of key signaling molecules controlling lipid metabolism following CLEA treatment in hepatocytes was not investigated in this study. Proteomic analysis of molecules controlling lipid metabolism, energy balance and related pathways will be performed in our further report.

This study conclusively suggested that CLEA extract contains several bioactive compounds, primarily dl-2-phenyltryptophane and benzoic acid, which could reduce triglyceride accumulation in lipid-accumulated HepG2 hepatocytes by controlling lipid metabolism molecules (Fig. 8). The extract might potentially be developed as a lipid-lowering ingredient to prevent NAFLD. This research investigated further evidence of the biological activity of purified substances derived from CL as well as extracts combined with other substances to be more effective against hepatic lipid accumulation.

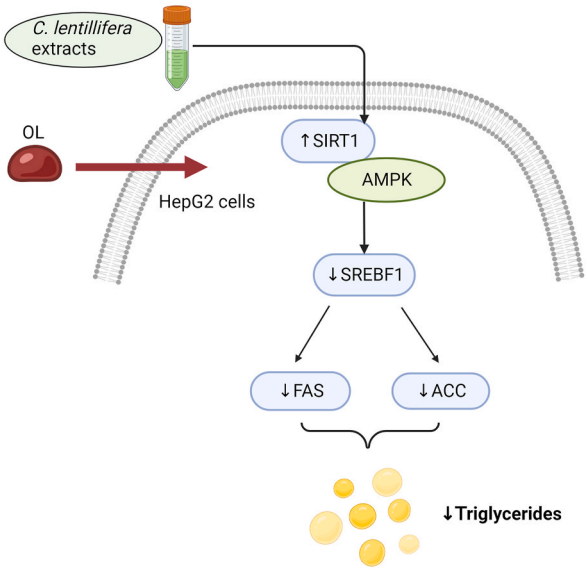

Fig. 8. Schematic summary diagram of the effect of CLEA on amelioration of lipid accumulation in HepG2 cells (Created with BioRender.com).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics declarations

Informed consent was not required for this study.

CRediT authorship contribution statement

Kant Sangpairoj: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kanta Pranweerapaiboon:** Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation. **Chantarawan Saengkhae:** Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Krai Meemon:** Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Nakorn Niamnont:** Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Montakan Tamtin:** Supervision, Resources, Formal analysis, Conceptualization. **Prasert Sobhon:** Supervision, Methodology, Data curation, Conceptualization. **Waranurin Yisarakun:** Supervision, Methodology, Data curation, Conceptualization. **Tanapan Siangcham:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27635.

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