Antiproliferative and immunomodulatory activity of edible seaweed *Gelidium latifolium*

**Abstract:** Trends in uses of complementary medicines which include dietary supplements to prevent several diseases such as cancer continue to increase. Edible seaweed *Gelidium latifolium* commonly found in Indonesian coastal areas with high bioavailability. However, it remains largely unexplored. This study aims to evaluate *Gelidium latifolium* (GLE) pharmaceutical properties by investigation of its growth inhibitory effects against human cancer cells and immunomodulatory effects in BALB/c mice. Phytochemical profile of GLE revealed presence of various bioactive compounds. Furthermore, GLE demonstrated high antioxidant activity (EC_{50} = 162 µg/mL) analysed by DPPH assay. Cytotoxic assay via Lactate Dehydrogenase (LDH) assay revealed that GLE demonstrated moderate cytotoxicity in HeLa and Caco2 cells with IC_{50} of 175 ± 23 µg/mL and 153 ± 56 µg/mL respectively. Observation by morphological criteria reveals GLE potentially induces apoptosis in HeLa and Caco2 cancer cells. Furthermore, *in vivo* immunomodulatory activity evaluation of *G. latifolium* administered BALB/C mice of shows effective dose of 100 mg/kgbw. *G. latifolium* significantly increases macrophage phagocytic activity and leukocyte content, particularly neutrophils. In addition, toxicity analysis via histological observation suggested that GLE has good safety profile. Thus, GLE provides further utilization as substance foods with medicinal purposes both potent as an antitumor and immune-enhancing agent.

**Keywords:** Antiproliferative; Cytotoxicity; Gelidium latifolium; Edible macroalgae; Immunomodulatory; Medicinal food

1. **INTRODUCTION**

Today, cancer has been regarded mainly as a group of diseases which is rapidly increasing worldwide [1]. Extrinsic factors such as diet and other nutritional aspects together with lifestyle behaviors have been suggested to be associated with cancer [2]–[4]. Thus, some studies suggested cancer occurrence to be reduced by adopting certain lifestyle changes, such as eating a better diet.

In developing countries such as Indonesia, conventional cancer treatments such as chemotherapy and surgery are unaffordable for most people. Therefore, alternative solutions such as development of medicinal foods from natural products would be promising for reduction of cancer risks [5]–[8]. Medicinal foods which are foods that, besides its nutritious effect, have a demonstrated benefit for one or more functions of the human organism, improving the state of health or well-being or reducing risk of diseases [9].
Marine macroalgae, also referred as seaweeds, are well known as a rich source of structurally diverse bioactive compounds with various biological activities and their importance as a source of novel medicinal uses against cancer, cardiovascular-related diseases, diabetes, inflammation, thrombosis, and obesity[13]–[17]. Macrogae in Indonesia are highly abundant yet remains an underexploited plant resource[18], [19]. One example of note is red macroalgaeGelidiumlatifolium. Several species members of Gelidiaceae family have been reported to exhibit therapeutic effects, including preventing cancers, cardiovascular diseases, lowering blood pressure, and blood glucose levels[20]–[24]. Gelidiumamansiihas evidenced growth inhibitory effects against HL-60, Hepa-1 and NIH-3T3 cells [25]. Immunostimulatory effects were also shown by increased phagocytic activity in white shrimp Litopenaeus vannamei fed with G.amansii diet [26]. Other several studies demonstrated Gelidiumelegansbiological effects as antioxidant, anti-inflammatory, and an impact on glucose homeostasis in high fat-induced obese mice [26]. However, there remains no established information regarding Gelidiumlatifolium pharmaceutical properties. This study aims to evaluate the potential of GLE as medicinal food by investigation of its antiproliferative activity against human cancer cells and immunomodulatory effects in BALB/c mice.

2. MATERIALS AND METHODS

2.1. Sample collection of GLE

Red macroalgae Gelidiumlatifolium was collected from North West Lombok area, (8˚24'11.7396"S, 116˚4'1.9056"E), West Nusa Tenggara Province, Indonesia. The seaweed samples were washed with freshwater to remove adhering debris. The collected samples were dried and powdered.

2.2. Chemicals and Biochemicals

A lactate dehydrogenase (LDH) release assay kit was obtained from Wako Pure Chemical Industries (Osaka, Japan). DMEM, fetal bovine serum (FBS) and penicillin were purchased from Gibco RBL. All organic solvents and other chemicals were of analytical grade or complied with the requirements for cell culture experiments.

2.3. Preparation of GLE macroalgae extracts

Fifty grams of powder samples were mixed with absolute ethanol solvent with 5x volume of sample weight (w/v). Suspensions were then mased by incubation in room temperature for 2x24h. After 24h, suspensions were filtered with Whatman number 1 filter papers. Filtrates were evaporated with rotary evaporators until concentrated ethanol extracts were obtained. These filtrates were then used for seaweed ethanol extracts. The resulting pasty extracts were stored in a refrigerator at 4°C for further use. The extraction was centrifuged at 15,000 × g for 10 min. The supernatant was collected and dried, and stored at 4°C until future use. The dried macroalgae extract was resolved in dimethyl sulfoxide (DMSO) and centrifuged at 15,000 × g for 15 min, then obtained clear macroalgae extract sample [27].

2.4. Phytochemical composition of GLE

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the ethanol extract of Macroalgae extracts was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with an Elite-SMS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 μm ID × 0.25 μm df). Macroalgae extracts were also subjected for phytochemical screening following the methodology of Harborne [28].

2.4.1. Test for alkaloids
One gram powder of macroalgae samples were taken in a conical flask and added ammonia solution (3mL). It was allowed to stand for few minutes to evaluated free alkaloids. Chloroform (10mL) was added to the conical flask shaken by hand and then filtered. The chloroform was evaporated from the crude extract by water bath and added Mayer’s reagent (3mL). a cream colour precipitation was obtained that showed the presence of alkaloids.

2.4.2. Test for flavonoids

The stock solution (1mL) was taken in a test tube and added few drop of dilute NaOH solution. An intense yellow colour was appeared in the test tube. It became colourless when on addition of a few drop of dilute acid that indicated the presence of flavonoids.

2.4.3. Test for saponins

The stock solution (1 mL) was taken in a test tube and diluted with 20 mL of distilled water. It was shaken by hand for 15 min. A foam layer was obtained on the top of the test tube. This foam layer indicated the presence of saponins.

2.4.4. Test for steroids

The crude plant extract (1 mg) was taken in a test tube and dissolved with chloroform (10 mL), then added equal volume of concentrated sulfuric acid to the test tube by sides. The upper layer in the test tube was turns into red and sulfuric acid layer showed yellow with green fluorescence. It showed the presence of steroids.

2.4.5. Test for tannins

The stock solution (3mL) was taken in a test tube and diluted with chloroform and added acetic anhydride (1mL). Finally, sulphuric acid (1mL) was added carefully by the side of test tube to the solution. A green colour was formed which showed the presence of tannins.

2.4.6. Test for terpenoids

The dry crude plant extract (5mg) was dissolved in chloroform (2mL) and then acetic anhydride (1mL) was added to it. Concentrated sulfuric acid (1mL) was added to the solution. Formation of reddish violet colour shows the presence of terpenoids.

2.5. Determination of antioxidant activity

The antioxidant activity of GLEethanol extract was assessed based on the radical scavenging effect of the stable 2,2-diphenyl-2-picrylhydrazyl (DPPH) [29]. The extract was dissolved in EtOH, and solution of 10µL was allowed to react with 200 µL DPPH at 37°C for 30 min in a 96-well plate. After incubation, the decrease in absorbance (optical density, OD) of the extract solution was measured at 490 nm using a microplate reader. Ascorbic acid was used as the positive control. For each sample concentration tested, the percentage of DPPH was calculated using the following formula:

$$\text{Antioxidant activity} \, (\%) = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100\%$$

Where ODsample is the OD of the samples or positive control, and ODcontrol is the negative control OD. The values obtained were plotted against the sample concentration to determine the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC50). The assay was performed in triplicate.

2.6. Cell culture

Human cervical cancer (HeLa) and Colon Adenocarcinoma (Caco2) cell lines were routinely cultivated in Dulbecco’s modified EaGEL medium (DMEM, Wako) supplemented with 10% fetal
bovine serum at 37°C in a 5% CO₂ humidified incubator. For all experiments, cells were plated in DMEM, incubated overnight, and incubated in DMEM supplemented with specific concentrations of GLE. Cell image was obtained from phase-contrast microscopy by BZ-9000 microscope (Keyence, Osaka, Japan).

2.7. Cytotoxicity Assay

Cytotoxicity was estimated by lactate dehydrogenase (LDH) assay[30]. Cells were cultured in 96-well culture plate over-night, then the culture medium discarded and changed new medium containing several concentration of GLE (5-200 µg/mL). After 24 h incubation, a portion of the culture medium transfer the other 96-well plate, which contained 2.5 mM NAD, 50 mM lithium lactate, 100 mMTris (pH 8.0), 0.04 mM 1-Met-PMS, and 1 mM WST-1. Coloring change depended on LDH activity was measured by absorbance at 450 nm. Maximum absorbance (A450 max) was obtained from completely dead cells by addition of 1% Triton X-100 in the cell culture, and minimum absorbance (A450 min) was obtained from the control cell culture. Cytotoxicity was calculated by the following equation; Cytotoxicity (%) = (A450 treatment - A450 min) / (A450 max - A450 min) x 100. Cell proliferation was measured by counting total cell number under microscopic observation. The cell suspension was mixed with equal volume of 0.4% Trypan blue dye. Viability was indicated as the ratio of live cells to total cells. Treated cells were then observed under bright-phase microscope.

2.8. Animals

Healthy male albino mice (20–30 g) of BALB/C strain were obtained from Bioscience and Biotechnology Research Centre University of Mataram. The animals were housed under controlled conditions of temperature 25°C and humidity 55% on a 12h light-dark cycle. Regular food was freely available. Mice had ad libitum access to water. The animal experiments were approved by the Indonesian Association for Laboratory Animal Science (IALAS). Also is in accordance withanimal ethic committee of Medical Faculty of Mataram University (285/UN.21.8/Ethic/2018).

2.9. Immunomodulatory assay

Immunomodulatory activity was assessed by carbon clearance assay [31]. Decrease of carbon intensity in blood shows macrophage phagocytic activity. Carbon ink (5g) evaporated by oven; 105°C for 30 minutes. Then carbon powder (1.6 g) was diluted in 25 mL tween 80 (1%) b/v in NaCl 0.9 % (concentration 64 mg/ml). Crude extract of GLE was suspended by NaCMC 0.5% for per-oral administration (P.O).

BALB/c mice (20–30g) of either sex were housed under standard laboratory conditions prior to experimentation. The animals were divided into three groups those were Group 1: (Control group fed with mice food pellet (MCP) and suspending agent); Group 2: (MCP + 50 mg/kgbw dose of GLE); Group 3: (MCP + 100 mg/kgbw dose of GLE). The treatments were conducted for 7 days in each group. At day 8, blood samples were collected for each animal as the initial value (minute 0), then the mice were induced with a 0.1 ml/10kgbw intravenous suspension in the tail vein. Blood samples were collected (25 µL) at 5, 10, 15, 20, 25 and 30 minutes, then added with 4 ml of 1% acetic acid, followed by measurement of the Optical Density (OD) at 650 nm with UV-Vis spectrophotometry. The phagocytic activity (ĸ) was calculated using the formula:

\[
K = \frac{(\ln \text{OD}_1 - \ln \text{OD}_2)}{(t_2-t_1)}
\]

Where, OD₁ and OD₂ are the optical densities at time t₁ and t₂ respectively.

2.10. Total leukocyte count
Total leukocyte count was determined by WBC diluting fluid using haemocytometer. Blood smear was prepared on a clean glass slide. Smear was allowed to dry and was fixed in methanol for 3 minutes. Slide as dried and dipped in Field’s solution B for 5 seconds. It was then rinsed with water and allowed to dry. Then it was stained with Field’s solution A for 15 seconds. After staining slide was rinsed with water and dried again. Stained slide was then observed under microscope.

2.11. Histology study and toxicity evaluation

To evaluate potential toxicity of the treatment, BALB/c mice organs (spleen and stomach) were harvested and fixed with buffered 10% paraformaldehyde for 72h and embedded in paraffin was. 4µm sections were stained with hematoxylin eosin (H&E).

2.12. Statistical analysis

Student’s t-test was used to analyze intergroup differences. Experiments were repeated at least three times, and data are represented as the mean ± SD. A p-value of less than 0.05 was considered to be statistically significant. In vivo data are represented as Mean ± S.E.M. using one way analysis of variance (ANOVA) followed by Tukey-HSD test, P<0.05 was considered as statistical significant, n = 6 in each group.

3. RESULTS

3.1. Phytochemical profile of GLE

Investigation of the chemical constituents in GLE was determined with GC-MS analysis (Table 1). Some main compounds that were detectable in GLE were; Oleic acid (9.27 ± 0.03 %), Myristic acid (4.4 ± 0.02 %), Linolenic acid (5.8 ± 0.2 %), Palmitic acid (40.63 ± 0.01 %). Phytochemical screening in tube reactions shows presence of alkaloids, flavonoids, saponin, terpenoids, and steroids (Table 2).

3.2. Antioxidant activity

Evaluation of GLE antioxidant activity was conducted with DPPH assay. The tested extract ability to scavenge free radicals; concentrations of the extract and control enabling the scavenging of 50% of 300 µM DPPH was detected. As shown in Table 3, the median effective concentration (EC₅₀) of GLE was 167 ± 1.54 µg/mL. However, positive control ascorbic acid demonstrated higher antioxidant activity (95.4 ± 2.1 µg/mL).

3.3. In vitro antiproliferative activity of GLE

Anti-proliferative effects of GLE were investigated via Lactate dehydrogenase (LDH) assay against human cervical cancer (HeLa) and Colon Adenocarcinoma (Caco2) cell line. GLE demonstrate cytotoxicity effect inHeLa cells and Caco2 cells with IC₅₀ of 175 ± 23 µg/mL and 153 ± 23 µg/mL respectively (Figure 1). Morphological observation of the treated cells also revealed that GLE inhibited cell proliferation of HeLa and Caco2 cells. Furthermore, HeLa and Caco2 cells show characteristics of cells undergoing apoptosis, as cells were irregular in size and shape (Figure 2).
Figure 1. Cytotoxic assay in A. HeLa cells and B. Caco2 cells incubated with GLE for 24h analyzed with Lactate Dehydrogenase (LDH) assay.

Figure 2. Morphological observations of HeLa and Caco2 cells incubated in GLE (150 µg/mL) for 24h.

3.4. Immunomodulatory activity of GLE in BALB/c mice blood profiles

Immunomodulatory activity of GLE was determined by macrophage phagocytic activity in BALB/c mice. Carbon ink (64 mg/mL) was separately injected intravenously into mice on the 7th day after GLE crude extract administration. The phagocytic activity of the reticulo-endothelial system is generally measured by the rate of removal of the carbon particles from the blood system. The carbon clearance test is a well-established method to measure this activity [31]. From this result, GLE treated groups exhibited significantly high phagocytic index compared to control (Figure 3). This implies GLE potentially increase immune response. Furthermore, addition of GLE with dose of 100 mg/kgbw in mice diet significantly increases its food intake which results in heavier body weight compared to control and 50 mg/kgbw dose (Figure 4).
Figure 3. Phagocytic index (ĸ) in administered BALB/c mice analysed with carbon clearance assay. The results are expressed as mean ± SD (n=6) using one way analysis of variance (ANOVA) followed by Turkey-HSD test; P<0.05 was considered as statistical significant (*), P<0.01 is considered highly significant (**) compared to control.

Figure 4. Effects of GLE administration in BALB/c mice for 7 days on its food intake, water intake, and body weight. The results are expressed as mean ± SD (n=6) using one way analysis of variance (ANOVA) followed by Turkey-HSD test; P<0.05 was considered as statistical significant (*), P<0.01 is considered highly significant (**) compared to control.

3.5. Effects of GLE in BALB/c mice blood profiles

Both GLE extracts dose 50 mg/kgbw and 100 mg/kgbw showed increase of leukocytes (Figure 5). In addition, blood differential count revealed increase of neutrophil percentage in higher dose (100 mg/kgbw) of GLE treated mice (Figure 6A). However, neutrophil-to-lymphocyte ratio (NLR) was in normal range (Figure 6B). Which implies the increased neutrophil percentage was not related to the inflammatory status of the tested animal.
Figure 5. Effect of GLE pre-oral adminstration on Leukocyte count in BALB/c mice. Data is represented as Mean ± S.E.M. using one way analysis of variance (ANOVA) followed by Tukey-HSD test, \( P<0.05 \) was considered as statistical significant, \( n = 3 \) in each group. \( P<0.05 \) was considered as statistical significant (*), \( P<0.01 \) is considered highly significant (**) compared to control.

Figure 6. Blood profiles of GLE administered BALB/c mice; A. Leucocyte count, B. Differential leukocyte content.

3.6. Histological study and toxicity evaluation

Hematoxylin eosin (H&E) staining was performed to study the effect of GLE administration on BALB/c mice organs (stomach and spleen). As shown in Figure 7, there was no indication of toxicity effects such as necrotic or inflammatory responses in GLE treated mice organs. This implies GLE crude extract is safe for food consumption.
4. DISCUSSION

The prevalence of chronic non-communicable diseases, such as obesity, osteoporosis, diabetes, and cancer, demonstrates the great possibilities to develop novel medicinal foods[32]–[34]. Natural resources are currently being investigated as potential treatments to reduce risks of these diseases [35]–[38]. Among these, macroalgae has been increasingly viewed as potential sources of bioactive compounds. Macroalgae intake has been associated with lower risks of several cancers with suppressive effects on the development of benign and cancer neoplasms[39]–[42]. In this study, we investigated the therapeutic potentials of GLE as potential medicinal food. For this, the antiproliferative effects of GLE against Human cancer cell lines (HeLa and Caco2) and immunomodulatory activity in mice were evaluated.

Macroalgae or seaweeds are rich in bioactive substances that may be useful in treating a wide spectrum of diseases [43]–[46]. Phytochemical profiling of GLE revealed presence of various secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins which have been documented as natural bioactive products with potent pharmaceutical activities[47]–[49].

Spectrometry analysis with GCMS of GLE showed large concentration of several potential bioactive compounds (Table 1). According to several reports, palmitic acid is predominant in several seaweeds[50]–[52]. Palmitic acid found in marine red algae Amphiroaxonata demonstrated selective cytotoxic activity in human leukemic cells, but no cytotoxicity in normal human dermal fibroblast (HDF) cells[53], [54]. Numerous studies have reported an inhibition in cell proliferation induced by oleic acid in different tumor cell lines [55]. Existence of linolenic acid in certain plants demonstrated cancer chemopreventive ability [56]. In addition, previous study shows that presence of fatty acids such as linoleic acid, myristic acid, and palmitic acid contributes to methanol-water extract of Polygonum Bistorta L. anticancer and cytotoxic activity [57]. Furthermore, phytochemical profiles of GLE show presence of alkaloids, flavonoids, saponins, terpenoids, and steroids (Table 2). Macroalgae are well known to contain terpenes, sterols, alkaloids, flavonoids [58]. GLE demonstrated high antioxidant activity with EC₅₀ of 167 ± 1.54 µg/mL (Table 3). Ascorbic acid had the stronger antioxidant activity (EC₅₀ = 95.4 ± 2.1 µg/mL) compared to GLE because it is a pure antioxidant compound.

Results from phytochemical screening of GLE correlates to its cytotoxic activity in HeLa and Caco2 cell lines. Previous study by Chen demonstrated effects of Gelidiumamansii crude methanol extracts in induction of apoptosis in Hepa-1 and NIH-3T3 cells [25]. Based on morphological observation, GLE potentially induces apoptosis as treated HeLa and Caco2 cells show irregular size and circular shape (Figure 2). However, further molecular analysis regarding the mechanisms of apoptotic related genes is needed to confirm this result.
The immune system response is known to be a considerable target for determination of potential medicinal foods. Improved immune system response could provide better management in infections, tumours and other degenerative diseases [59]–[62]. Preliminary approaches include evaluation of immunomodulatory effects of tested substance by determination of phagocytic activity[63], [64]. The role of phagocytosis is the removal of microorganisms and foreign bodies, dead or injured cells. The increase in the phagocytic index in GLE administered mice potentially reflects the enhancement of the phagocytic function of macrophages. GLE at dose of 100 mg/kgbw appeared to enhance the phagocytic function of exhibiting a clearance rate of carbon in mice. Crude extracts of macroalgae are well reported to induce macrophage activity[65]–[67]. Administration of hot-water extract of Gelidium amansii demonstrates immunostimulatory effects in white shrimps Litopenaeus vannamei and resistance against Vibrio alginolyticus[26]. Immunostimulant evaluation is usually based on some parameters such as serum lysozyme, total leucocyte count, monocyte/lymphocyte/granulocyte count, and antibody titers [68]. Hence, increase in lymphocyte count in macroalgae administered mice implies immune-enhancing activity of GLE. Additionally, our study revealed increased food intake and body weight in GLE administered mice. However, mice body weight values were still in the range of BALB/c mice non-obese normal body weight [69]. Overall, GLE consumption induces no toxicity effects to organs in treated mice. Furthermore, supplementation of elderly Japanese men and women with seaweed increases immune responses to seasonal influenza vaccination

Earliest immune response could also be seen and measured by evaluation of the hematological parameters of an animal. Accordingly parameters such as total leukocyte count and differential blood count were measured for control group as well as group which received daily diet of GLE. An immunomodulatory effect of any immune substance would first induce changes in leukocyte contents [70]. GLE administration in mice for 7 days induced leukocyte proliferation. Furthermore, leukocyte contents in 100 mg/kgbw GLE administered mice show high percentage in neutrophil contents. In addition, neutrophil to lymphocyte (NLR) ratio in GLE administered mice was 1.878, which is still in normal range (0.107–3.193) according to Forget et al.[71]. Hence, GLE potentially enhances immune activity without inducing inflammation in mice organs. This result was also supported by the safety profile of histological analyses of stomach and spleen organs of GLE administered mice which show no indication of toxicity (Figure 7).

5. CONCLUSION

In summary, current results show the potential utilizations of Gelidium latifolium constituents towards medicinal applications. The therapeutic effects of Gelidium latifolium is possibly due to existence of various bioactive compounds in its ethanol crude extract. Current results show GLE has an apparent anti-cancer effect by reducing cancer cell proliferation in human cancer cells. Furthermore, in vivo studies evidenced immunomodulatory activity of GLE in mice by increase of phagocytic activity and leukocyte contents. Further studies regarding the mechanisms by which GLE affect immune response would be necessary for development of Gelidium latifolium as potential natural anti-cancer therapeutic agent.

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