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Screening for Biological Activities of *Spirogyra neglecta* Water Extract

Atchariya YOSBOONRUANG[1], Acharaporn DUANGJAI[2], Doungporn AMORMLERDPISON[3] and Jarupa VIYOACH[4,*]

[1]*Division of Microbiology and Parasitology, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand*
[2]*Division of Physiology, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand*
[3]*Faculty of Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai 50290, Thailand*
[4]*Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok 65000, Thailand*

([*]Corresponding author's e-mail: jarupaviyoch4@yahoo.com, jarupav@nu.ac.th)

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Abstract

The freshwater green algae, *S. neglecta*, has been commonly used as a Northern Thai local food due to the fact that it is composed of several nutritional components. The aim of the present study was to investigate the phytochemical properties and biological activities of an *S. neglecta* water extract for application as a functional food or pharmaceutical ingredient. The phenolic and flavonoid contents of *S. neglecta* and its biological activities, including anti-oxidant, pancreatic cholesterol esterase, anti-inflammatory, cytotoxic, and antibacterial activities, were investigated *in vitro*. The results showed that the *S. neglecta* extract contained 157.92 mg GAE/g extract of phenolics and 10.10 mg CE/g extract of flavonoids. The radical scavenging activity of the *S. neglecta* extract exhibited an IC_{50} of 7.46±1.17 µg/mL from DPPH assay and 224.20±1.06 µg/mL from ABTS assay. Meanwhile, trolox exhibited an IC_{50} of 7.03±1.08 µg/mL from DPPH assay and 5.24±1.10 µg/mL from ATBS assay. Interestingly, the *S. neglecta* extract at 10 mg/mL inhibited pancreatic cholesterol esterase activity by 60 %. Moreover, the release of TNF-α from macrophages was strongly reduced by incubation with the *S. neglecta* extract in a dose-dependent manner, and all of the study concentrations were non-toxic to primary fibroblast cells. In addition, the *S. neglecta* extract inhibited some gram-positive and -negative bacteria. In summary, *S. neglecta* extract could possibly be used as a potential functional food or pharmaceutical ingredient, for which further studies are required.

Keywords: Spirogyra, algae, anti-inflammation, antioxidant, pancreatic cholesterol esterase

Introduction

Natural compounds from plants are widely used in both direct and indirect applications, such as in the composition of Thai local foods, folk medicines, and cosmetics. One good source of the natural compounds is from algae, which is well known and consumed in Asian countries such as Thailand. There are several reports on the biological activities of various types of algae, including brown algae, blue-green algae, and seaweeds. They contain many bioactive compounds with antioxidant, anti-inflammatory, and antimicrobial activities [1-4]. Both of the biologically active and nutritional compounds of various algae have been illustrated [4]. However, as far as we know, the information on the biological activities of freshwater green algae extract, particularly the water extract, has still not been clarified.

S. neglecta, or Tao (Thai local name), is a filamentous green alga in freshwater found in the North of Thailand. It is a traditional cuisine of the Northern Thai people. *S. neglecta* consists of various nutritional components, including protein, fat, carbohydrate, fiber, sulfate, multivitamins, and minerals

[5]. The pharmacological properties of *S. neglecta* water extract has been reported in rats. *S. neglecta* extract could inhibit the formation of gastric ulcers from physical and chemical stresses in rats [6] and display antihyperlipidemia and antihyperglycemia in type 2 diabetic rats [7]. In addition, *S. neglecta* extract exhibited antioxidant capacity in rat liver [8]. However, other biological activities, including anti-inflammation, antibacterial, and pancreatic cholesterol esterase activities of *S. neglecta* water extract, have never been reported. The aim of the present study was to investigate the phytochemical and biological activities, including antioxidant, anti-inflammatory, antibacterial, cytotoxic, and pancreatic cholesterol esterase activities, of *S. neglecta* water extract. This report could potentially support the biological information claims of *S. neglecta* extract for applications in functional food or other pharmaceutical products.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), lipopolysaccharide (LPS), Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), $AlCl_3$ solution, catechin, *p*-nitrophenyl butyrate (*p*-NPB), taurocholic acid sodium, porcine pancreatic cholesterol esterase, 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and trypsin-EDTA were purchased from Sigma–Aldrich Co. (St. Louis, Missouri, U.S.A.). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco^{TM} (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonicacidhydrate (XTT) was purchased from Roche Diagnostics (Hoffmann-La Roche, Basel, Switzerland). Gentamicin was purchased from Oxoid (Basingstoke, UK), and tryptic soy broth and agar (TSB and TSA) were purchased from Difco Laboratories, Inc. (New Jersey, USA).

Preparation of *S. neglecta* extract

Fresh *S. neglecta* was collected from Na Kuha village, Saun Kuen subdistrict, Muang District, Phrae Province, Thailand. It was rinsed and dried at 50 °C in an oven. After grinding the dried *S. neglecta* into fine powder, 50 g of the *S. neglecta* powder was suspended in 1 L of distilled water and boiled at 100 °C for 1 h. The aqueous solution was then filtered through filter paper. The boiling and filtering processes were performed three times, and the pooled solution was concentrated by evaporation. Then, it was lyophilized into dried extract powder by freeze drying, and the obtained yield of the extract was 36.67 %. The *S. neglecta* extract was stored at 4 °C prior to the subsequent experiments.

Total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu method [9]. In brief, *S. neglecta* extract (2 mg/mL) was mixed with 2 µL of Folin-Ciocalteu's reagent. Eighty microliters of Na_2CO_3 solution (15 g/L) was added to the mixture. The mixed solution was incubated at room temperature for 30 min before the absorbance at 750 nm was measured using a spectrophotometer. Gallic acid was used as the standard for the estimation of phenolics. The total phenolic content was performed in triplicate and expressed as mg of gallic acid equivalents (GAE) per g of dry weight.

Total flavonoid content

Total flavonoid content was determined by using the aluminum chloride colorimetric of Chang *et al*. with a slight modification [10]. In brief, *S. neglecta* extract (2 mg/mL) was added into a mixture of 60 µL of 95 % ethanol, 10 µL of 4 % $AlCl_3$ solution, and 10 µL of 0.4 M potassium acetate (CH_3COOK). The solution was incubated for 40 min at room temperature before measuring the absorbance at 415 nm using a spectrophotometer. The standard curve for total flavonoids was made using catechin standard solution. The estimation of the flavonoid compounds was carried out in triplicate. The total flavonoids in the *S. neglecta* extract were expressed in terms of catechin equivalents per g of dry weight.

Antioxidant activity by DPPH assay

The free radical scavenging activity of *S. neglecta* extract was assessed according to the method described earlier with a slight modification [11]. *S. neglecta* extract (10 µL) at various concentrations was mixed with 190 µL of 80 µM DPPH in methanol. All concentrations were prepared in triplicate. The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. Then, the absorbance was measured at 517 nm. The antioxidant capacity of *S. neglecta* extract was expressed as IC_{50}.

Antioxidant activity by ABTS assay

The scavenging activity of ABTS was measured as described previously [12]. Stock solution of ABTS and potassium persulfate was prepared. The mixture solution was kept in the dark at room temperature for 12 h to allow completion of radical generation. ABTS reagent was mixed with the extract or trolox, and the absorbance was taken at 734 nm after 6 min of incubation (n = 3). The percentage of inhibition was calculated as: inhibition % = (1 - absorbance at 734 nm of the extract or positive control/ absorbance at 734 nm of the control) ×100 %. The antioxidant capacity of *S. neglecta* extract was expressed as IC_{50}.

Determination of pancreatic cholesterol esterase activity

Pancreatic cholesterol esterase activity was evaluated using *p*-nitrophenyl butyrate (*p*-NPB) [13,14]. Taurocholic acid and *p*-NPB were prepared in absolute methanol. *S. neglecta* extract was mixed with taurocholic acid (5.16 mM), *p*-NPB (0.2 mM) and 100 mM sodium chloride. Then, porcine pancreatic cholesterol esterase (1 mg/mL) was added to start the reaction. The absorbance at 405 nm was determined after incubation for 5 min at 25 °C. The experiment was performed in triplicate.

Determination of anti-inflammatory activity

In this study, tumor necrosis factor (TNF)-α was used as a marker of the inflammation produced from macrophages activated by LPS. Briefly, RAW264.7 cells (ATCC®, TIB-71™, VA, USA) were cultured in DMEM containing 10 % FBS and antibiotics; then, they were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 24 h. The medium was replaced with serum-free DMEM containing various concentrations of *S. neglecta* extract. After 30 min of incubation, 1 µg/mL LPS was added to activate pro-inflammatory production from RAW264.7. The cell-free supernatant was collected and TNF-α production was determined using TNF-α enzyme-linked immunosorbent assay kit (eBioscience, CA, USA). Finally, cell viability was determined by XTT-colorimetric assay [15]. The experiment was performed in triplicate.

Determination of cytotoxicity

Cell isolation and culture

Primary skin fibroblasts were used to determine cell viability after incubation with *S. neglecta* extract. Fibroblasts were isolated from the dermal tissue from excess surgery skin. The protocols were approved by the Naresuan University Human Ethics committee. Abdominal skin from a female, aged 55, was aseptically cut into small pieces. Two or three pieces of the skin were placed in a culture flask and subsequently incubated at 37 °C with a 5 % CO_2 humidified atmosphere for 1 h. After that, DMEM supplemented with 10 % FBS and 1 % of a stock penicillin/streptomycin was added, and the flask was subsequently incubated for 3 weeks. After the fibroblasts migrated from the original site, they were detached with a trypsin-EDTA solution and then seeded in new culture flask at 1×10^4 cells/cm^2. Fibroblasts at passage number 4 were used in this study.

Determination of cell viability

Cell viability was performed to evaluate whether *S. neglecta* extract is toxic to human fibroblast cells. Fibroblasts (1×10^4 cell/cm^2) were cultured in DMEM containing 10 % FBS and antibiotics at 37 °C in a humidified atmosphere of 5 % CO_2 for 24 h. Then, the medium was replaced with fresh serum-free DMEM containing various concentrations of *S. neglecta* extract and incubated for 24 h. XTT solution was

added and incubated at 37 °C for 4 h. The cell-free supernatant was collected to measure the enzymatic activity at the absorbance of 490 nm using a spectrophotometer (Model CeresUV900C, Bio-tek Instrument, Winooski, Vermont, USA). Results were expressed as the percentage of cell viability compared to cell viability on the culture flask (control). Cell morphology was also observed under a light microscope. The experiment was performed in triplicate.

Determination of antibacterial activity

The tested microorganisms in this study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. Inoculums were prepared by culturing the microorganisms in TSB at 37 °C for 18 h. Inoculums were swabbed on TSA plates (1×10^7 CFU/mL). After agar punching into the diameter of 6 mm by a sterile cork borer, three replications of the *S. neglecta* extract at a concentration of 200 mg/mL was applied to each well of the inoculated plates. Tetracycline (4 µg/mL) and sterile distilled water were used as a positive and negative control, respectively. All plates were incubated at 37 °C for 18 - 24 h, and the diameter of the inhibition zone was measured in millimeters. A minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) of the extract were determined by broth dilution method.

Statistical analysis

The results were presented as mean ± SD. Analysis of variance (ANOVA) was used for comparing the means using R i386 3.3.2. Significant differences were verified by Tukey's multiple range test at *p*-values < 0.001.

Results and discussion

Phenolic and flavonoid contents

The total phenolic content of *S. neglecta* extract was analyzed using Folin–Ciocalteu's reagent and expressed as gallic acid equivalence (GAE) mg/g extract. The total flavonoid concentrations were detected using aluminum chloride and represented as catechin equivalents (CE) mg/g extract. The results of the total phenolic and flavonoid contents are described in **Table 1**. The phenolic content in the *S. neglecta* extract was 157.92 Gallic acid equivalence (GAE) mg/g extract. The concentrations of flavonoids showed 10.10 catechin equivalence (CE) mg/g extract. These results indicated that the phenolic and flavonoid compounds of the *S. neglecta* extract still remained, although the *S. neglecta* was boiled at 100 °C. Furthermore, a previous study reported that *S. neglecta* is composed of various nutritional components including protein, fat, carbohydrate, fiber, sulfate, multivitamins, and minerals [5].

Antioxidant activity

The antioxidant activity was determined using DPPH assay and expressed as a percentage of inhibition. Trolox was used as a positive control. The *S. neglecta* extract showed DPPH radical scavenging activity with an IC_{50} of 7.46±1.17 µg/mL, while trolox exhibited an IC_{50} of 7.03±1.08 µg/mL. As presented in **Table 1**, the IC_{50} of the *S. neglecta* extract and trolox were 224.20±1.06 µg/mL and 5.24±1.10 µg/mL, respectively. It can be suggested that *S. neglecta* extract was an effective antioxidant by radical scavenging activity. This study demonstrated that *S. neglecta* extract contains phenolics and flavonoids, similar to another study [8]. Accordingly, *S. neglecta* exhibiting high antioxidant capacity might be due to the components of *S. neglecta* extract including Gallic acid, eriodictyol, isoquercetin, kaempferol, quercetin, hydroquinin, rutin, catechin, and tannic acid [16]. In addition, Thumvijit *et al*. reported that the administration of hot water extract of *S. neglecta* presented antioxidant properties in rats by protecting tissue damage due to reactive oxygen species [17].

Table 1 The total phenolic, flavonoid contents and antioxidant activity of *Spirogyra neglecta*

	Phenolic contents (Gallic acid equivalence (GAE) mg/g extract)	Flavonoid contents (Catechin equivalence (CE) mg/g extract)	Antioxidant activity IC_{50} (µg/mL)	
			DPPH	ABTS
Spirogyra neglecta	157.92±0.33	10.10±2.90	7.46±1.17	224.20±1.06
Trolox	-	-	7.03±1.08	5.24±1.10

Pancreatic cholesterol esterase activity

The *S. neglecta* extract played a role as an effective pancreatic cholesterol esterase inhibitor in a dose-dependent manner (**Figure 1**). The *S. neglecta* extract at 10 mg/mL markedly revealed the inhibition of pancreatic cholesterol esterase activity by 60 %. Several studies suggested that the pancreatic cholesterol esterase plays a role in the hydrolysis of cholesterol ester to cholesterol and free fatty acids. The unesterified cholesterol is easily absorbed in the intestine [18]. Inhibition of pancreatic cholesterol esterase enzyme might lead to a reduction of cholesterol absorption. It has been reported that cholesterol absorption was decreased in cholesterol esterase gene-knockout mice [19]. Our study showed the potential of *S. neglecta* extract as an anti-hyperlipidemic agent by the inhibitory effect against pancreatic cholesterol esterase. Ngamukote *et al*. revealed that three major polyphenols (gallic acid, catechin, and epicatechin) present in grape seeds have cholesterol-lowering activity by inhibiting pancreatic cholesterol esterase [13]. The methanol extract of the *Camellia sinensis* leaves containing flavonoids, phenolics, and terpenoids showed the ability to inhibit the pancreatic cholesterol esterase enzyme [20]. Karmar *et al*. suggested that plant-based polyphenols exhibited a potent inhibition of cholesterol esterase [20]. Therefore, it is possible that the inhibitory effect of *S. neglecta* on pancreatic cholesterol esterase is due to the presence of flavonoids and phenolic compounds in the extract.

Anti-inflammatory activity

To investigate the effect of *S. neglecta* extract on the production of pro-inflammatory cytokine, TNF-α, Raw 264.7 macrophage cells were treated with/without the *S. neglecta* extract at various concentrations. The result showed that the *S. neglecta* extract significantly inhibited the release of TNF-α by macrophages (**Figure 2**). The percentage of TNF-α release from macrophages treated with the *S. neglecta* extract at various concentrations of 3.125 - 100 mg/mL was reduced to 46.16-7.67±1.8 % (**Figure 2**). In addition, the results of morphology and cell viability indicated that the *S. neglecta* extract at the concentrations of 3.125 - 100 mg/mL was non-toxic to Raw 264.7 macrophage cells (data not shown). These findings exhibited the anti-inflammatory activity of *S. neglecta* extract without toxicity. It has been reported that many cytokines, such as TNF-α, interleukin (IL) -6, and IL-1β, are produced by inflammatory tissues [21]. Heo *et al.* suggested that the major cytokine produced from macrophages is TNF-α. It can stimulate the production of other cytokines, such as IL-6, IL-1β, prostaglandin E_2 (PGE_2), and collagenase, which leads to septic shock, inflammation, and cytotoxicity [21]. Therefore, TNF-α is more important in playing a role in inflammation. The prevention of inflammation, according to the inhibition of cytokine production, particularly TNF-α, is a key mechanism. Our result indicated that the *S. neglecta* extract strongly inhibited TNF-α production in RAW 264.7 macrophages stimulated by LPS, which indicated that *S. neglecta* extract has potential to be an inflammation inhibitor. It has been reported that several natural extract compounds can reduce or inhibit inflammatory cytokine production [1,3,4,22,23]. The effects of the anti-inflammatory activity of these antioxidant compounds are associated with their antioxidant activities. In this study, the active compounds that exhibited anti-inflammatory activity may be associated with phenolic and flavonoid contents in *S. neglecta* extract, which functions as an antioxidant.

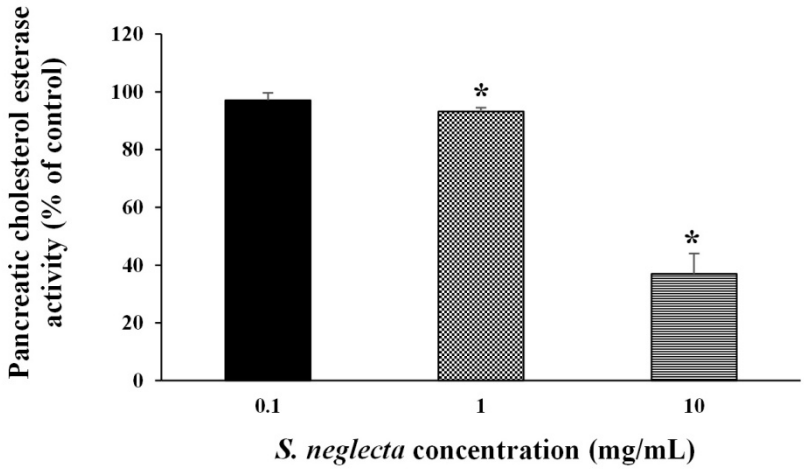

Figure 1 The effect of *spirogyra neglecta* on pancreatic cholesterol esterase activity. Results are expressed as mean ± SEM., n = 3. *p < 0.001

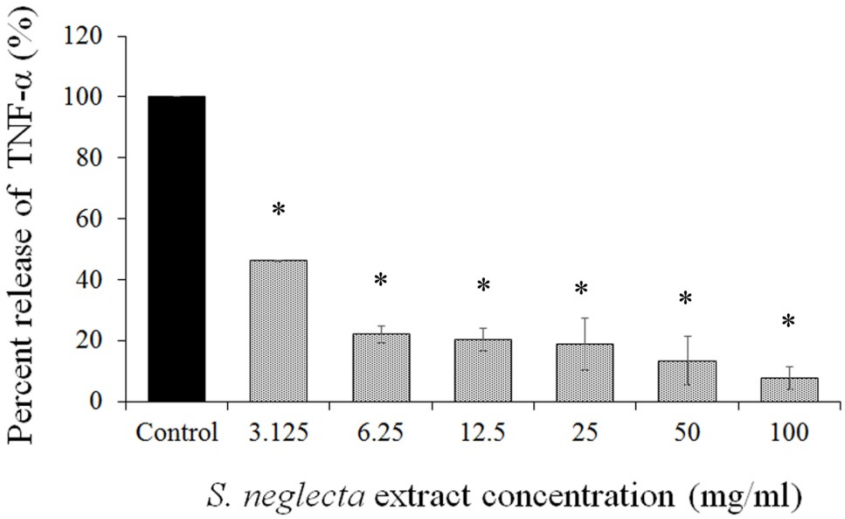

Figure 2 Percent TNF-α production in LPS-induced RAW 264.7 macrophages treated with various concentrations of *S. neglecta* extract. Control is LPS-induced RAW 264.7 macrophage without any treatment. The release of TNF-α was measured in culture media using ELISA technique. Data represented as mean ± SD (n = 3). *p < 0.001

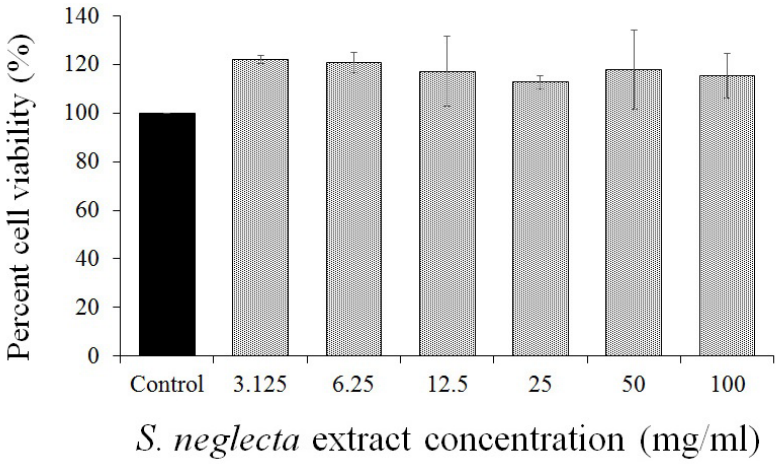

Figure 3 Percentage of fibroblast viability after exposure to various concentration of *S. neglecta* extract. Control is fibroblast unexposed to *S. neglecta* extract. Data represented as mean ± SD (n = 3)

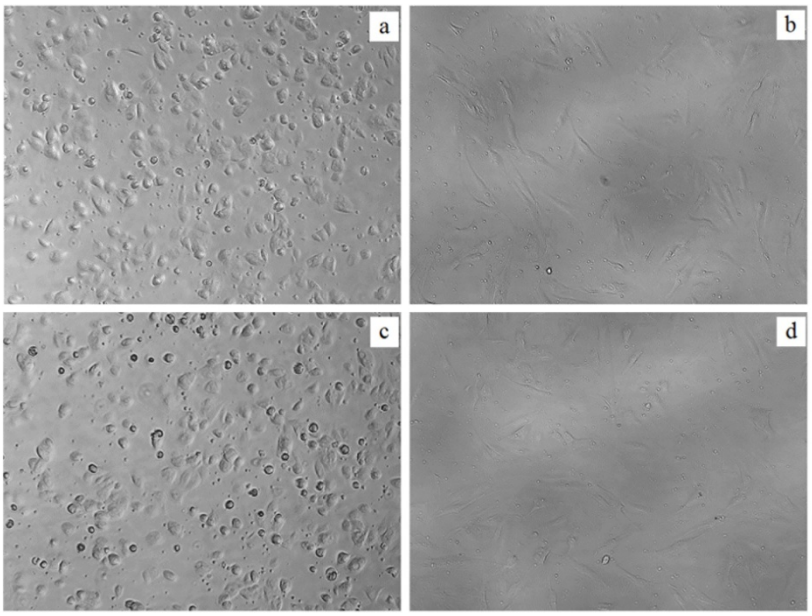

Figure 4 Primary human fibroblast morphology under 100X light microscope after incubation with *S. neglecta* extract for 0 h (a) and 24 h (b), and fibroblast incubation without *S. neglecta* extract at 0 h (c) and 24 h (d)

Cytotoxicity investigation

Cytotoxicity test was performed to determine the toxicity of *S. neglecta* extract at various concentrations on primary human fibroblasts. Cell viability after 24 h exposure to the *S. neglecta* extract was evaluated by measuring the viable cell metabolite using XTT assay. The results showed that, after incubation with the *S. neglecta* extract at concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL, the percentages of cell viability were 115.53, 117.80, 112.72, 117.14, and 120.75 %, respectively (**Figure 3**).

The growth of cells unexposed to the *S. neglecta* extract was used as a control (adjusted as 100 %). It was indicated that cell viability after growing with the *S. neglecta* extract was not different from that without the *S. neglecta* extract. In addition, the fibroblast morphology under a light microscope (**Figure 4**) revealed a typical morphology of fibroblast after being treated with 100 mg/mL of the *S. neglecta* extract compared with the fibroblast growth on a plastic plate. They displayed a spindle-shaped phenotype similar to the control. According to a previous report, the extract from brown algae *Sargassum vulgare* was not toxic to erythrocytes and also promoted proliferation of macrophage cells [3]. On the other hand, Kazlowska *et al*. suggested that algae extract was slightly toxic toward RAW264.7 macrophage [1]. In the present study, after the human fibroblasts were treated with various concentrations of the *S. neglecta* extract, their morphology and viability were not different from those of the control (untreated by *S. neglecta* extract). It was indicated that *S. neglecta* extract was not toxic to human fibroblasts.

Investigation of antibacterial activity

The results of antibacterial activity using the agar well diffusion method are presented in **Table 2**. The *S. neglecta* extract was active against the tested microorganisms. The inhibition zones of the *S. neglecta* extract against *S. aureus*, *S. epidermidis*, and *P. aeruginosa* were shown as 28.33±0.58, 26±0, and 8.67±1.15 mm, respectively. Meanwhile, *E. coli* was not inhibited by the *S. neglecta* extract at a concentration of 200 mg/mL. Broth dilution technique could not produce the MIC or MBC due to the interference of an extract turbidity. However, the agar inside the inhibition zone of the positive result was punched and cultured on fresh TSA to determine the bactericidal activity of the extract. The result showed that the *S. neglecta* extract displayed bacteriostatic activity. Previously, the antibacterial activity of marine algae extract toward *Vibrio* strains [24] *E. coli*, *P. aeruginosa*, *S. aureus*, *Enterococcus faecalis*, etc. has been reported [25]. They suggested that the antibacterial effect was observed on the marine algae extract from various solvents, but was not observed on the water extract. In the present study, we found that the *S. neglecta* water extract exhibited activity against *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, which may be related to the phenolic and flavonoid contents of *S. neglecta* extract. Moreover, a previous study on *S. neglecta* water extract [16] exhibited various effective compounds, including isoquercetin, hydroquinin, rutin, catechin, and tannic acid, which may be effective against various bacterial strains. Our results suggested that, after processing *S. neglecta* extract as a food additive or other pharmaceutical product, it can possibly prevent microorganism contamination.

Table 2 Inhibitory effect of *S. neglecta* extract against microorganisms

Microorganisms	Inhibition zone (mm)	
	***S. neglecta* extract (200 mg/mL)**	**Positive control (Tetracycline)**
Staphylococcus aureus	28.33±0.58	40
Staphylococcus epidermidis	26±0	40
Escherichia coli	0	35
Pseudomonas aeruginosa	8.67±1.15	28

Conclusions

The findings suggest that *S. neglecta* contains flavonoids and phenolic compounds, which might cause anti-hyperlipidemic properties by inhibiting pancreatic cholesterol esterase activity. Moreover, at the studied concentrations, *S. neglecta* extract exhibited an anti-inflammatory activity, and also inhibited some gram-positive and gram-negative bacteria. Our results indicate that *S. neglecta* has potential to be developed into an anti-hyperlipidemic agent, functional food, or other pharmaceutical product; however, additional studies, especially preclinical and clinical studies, are required.

Acknowledgements

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