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Original Article

Spirogyra neglecta inhibits the absorption and synthesis of cholesterol *in vitro*

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ABSTRACT

Background: *Spirogyra neglecta* (SN) has many nutritional benefits and it is commonly used to ameliorate different human conditions including inflammation, gastric ulcer, hyperglycemia, and hyperlipidemia. However, the mechanism of the hypocholesterolemic effect of SN still remains unclear. Therefore, the present study was aimed to evaluate the effect of SN extract particularly on cholesterol absorption and synthesis mechanisms.
Methods: For cholesterol absorption, the uptake of cholesterol was measured by using tritium radiolabeling of cholesterol in Caco-2 cells. Bile acid binding, micelles size, and cholesterol solubility were analyzed in *in vitro* assays, while cholesterol synthesis was evaluated by using a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase assay kit.
Results: SN extract was found to decrease cholesterol uptake in Caco-2 cells and decreased the solubility of cholesterol in micelles. The SN extract bound to taurocholate, taurodeoxycholate, and glycodeoxycholate bile acids, and increased micelles size. SN has also demonstrated an inhibitory effect on HMG-CoA reductase (HMGR) enzymatic activity. For further experimentation, the treatment combination of SN and ezetimibe (0.04 mg/mL) showed a greater significant reduction in cholesterol uptake than the extract alone.
Conclusion: These observations suggested that inhibitory cholesterol absorption effects of SN could be mediated through the modulation of size and solubility of cholesterol micelles, resulting in interference of cholesterol uptake. In addition, SN inhibited the rate limiting step of cholesterol synthesis. This study provides supporting evidence for the potential usage of SN as a cholesterol lowering agent.

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1. Introduction

Cholesterol homeostasis is maintained by intestinal cholesterol absorption, cholesterol synthesis, and excretion.[1] Since an excessively high level of plasma cholesterol is one of the major risk factors for atherosclerosis and cardiovascular diseases,[2] the reduction of cholesterol levels is desired for hypercholesterolemia. Besides lifestyle modification, medications could be another recommended alternative for management of hypercholesterolemia. Drugs such as statins and ezetimibe are the common options. A statin, which is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, may exert its effect by suppressing cholesterol biosynthesis pathway.[3] Ezetimibe, a cholesterol absorption inhibitor, plays a role in inhibiting cholesterol uptake into the enterocyte by blocking the Niemann-Pick C1 like 1 (NPC1L1) cholesterol transporter.[3,4] However, several adverse events associated with ezetimibe or statins used, e.g., hepatotoxicity, severe cholestatic hepatitis,[5] rhabdomyolysis, and myopathy,[6] were reported. Therefore, functional foods and nutraceuticals are becoming popular as alternative therapies for lowering plasma cholesterol levels, especially in individuals whose blood cholesterol levels are marginally high, but who are not recommended to undergo drug treatments.[7]

Spirogyra neglecta (SN) is an edible freshwater macroalga and a common ingredient used in Northern Thai cuisine. SN contains many nutritional constituents including fat, proteins, carbohydrate, fibers, multivitamins, minerals, and antioxidants.[8] In addition, it has been reported to exhibit antigastric ulcer, antiinflammatory,[9] antihyperglycemic, and antihyperlipidemic properties.[10] Recently, SN was able to reduce plasma triglyceride in in type 2 diabetic rats.[10] However, evidence to support the hypocholesterolemic effect of SN and its mechanism still remains unclear. Normally, the possible target is intestinal cholesterol absorption, which has a major role in influencing plasma cholesterol level.[11] Cholesterol absorption involves multistep processes including digestion of dietary lipids and micellar solubilization of cholesterol, and protein transporters.[12] Therefore, we determined the impact of SN extract on cholesterol uptake by using Caco-2 cells, *in vitro* bile acid binding, micelles size, and cholesterol solubility. Meanwhile, the cholesterol synthesis study was evaluated by using *in vitro* HMG-CoA reductase activity.

2. Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium/F12, cholesterol, phosphatidylcholine, taurocholic acid (TC) sodium salt hydrate, glycodeoxycholic acid (GC), taurodeoxycholic acid (TD), cholestyramine, and the HMG-CoA reductase assay kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was purchased from Gibco (Carlsbad, CA, USA). [1α,2α(n)-^{3}H]Cholesterol was purchased from PerkinElmer (Wellesley, MA, USA). All other chemical reagents used in this study were of analytical grade.

2.2. Preparation of SN extracts

Fresh whole SN was collected from Na Kuha village, Saun Kuen subdistrict, Muang District, Phrae Province, Thailand. The SN was rinsed and dried in an oven at 50 °C. Dried SN was ground into a fine powder and blended with water. In brief, dried SN (100 g) was added into 2 L of distilled water and boiled at 100 °C for 1 hour. The aqueous solution was then filtered through filter paper. The filtrate was subsequently concentrated by evaporation and lyophilized to dried extract powder by freeze drying. The SN extract was stored at 4 °C prior to subsequent experiments.

2.3. Phytochemical screening of SN extract

The chemical constituents of aqueous extracts of SN were further quantitated by the high-performance with diode array detection and mass spectrometry detector (HPLC-DAD/MSD) method on an Agilent Technologies 1100 series (Waldbronn, Germany), equipped with a Zorbax SB C18 column (4.6 mm × 150 mm × 5 μm) (CA,USA) and diode array detector recording at 270 nm, 330 nm, 350 nm, and 370 nm. The flow rate was 1 mL/min. The mobile phases were a binary solvent system consisting of acetonitrile (Solvent A) and 10 mM ammonium formate (pH 4 with formic acid; Solvent B), with a gradient starting at 0:100 (A:B) for 5 minutes, 20:80 for 15 minutes, and the composition was changed to 40:60 over 40 minutes. Nitrogen was used as the nebulizing gas with a flow rate of 13 L/min at 320 °C. The capillary voltages were set at 4 kV (positive) and 3 kV (negative). A scan time of 0.2 seconds with a range of 100–700 m/z was used. SN extract was analyzed against phenolic standards. Semiquantitative data was analyzed by the peak area under the curve relative to the content of each component in the extract.

2.4. Cholesterol micelles preparation

The micelle solution was prepared and modified from Yamanashi et al.[13] Sodium taurocholate was dissolved in methanol, and cholesterol and phosphatidylcholine were prepared in chloroform. These mixer solutions were dried under nitrogen gas. The hydrated lipid film was dispersed under sonication in phosphate buffered saline (PBS) for 1 hour prior to the experiment.

2.5. Micellar cholesterol solubility assay

The solubility of cholesterol in micelles was adapted from Kirana et al.[14] Briefly, micelle solutions contained 10 mM cholesterol, 1 mM sodium taurocholate, and 0.6 mM phosphatidylcholine. SN extract (0.1 mg/mL, 1 mg/mL, 10 mg/mL) was added to the micelle solution and incubated at 37 °C for 3 hours. Mixed solutions were filtered through a 0.22-μm membrane. The filtrate was taken as micellar cholesterol concentration and the cholesterol content was determined by a cholesterol assay kit, which can be defined as micellar cholesterol solubility. The cholesterol micellar solubility inhibition was calculated by using Eq. (1) as follows:

$$\%\,\text{Cholesterol solubility inhibition} = 100 - \left(\frac{\text{Cholesterol contents (Treated)}}{\text{Cholesterol contents (Control)}} \times 100\right) \quad (1)$$

2.6. Cholesterol micelles size determination

Micelles solutions were prepared as mentioned above, and determination of micelles size in mixed solutions was modified from Kirana, et al.[14] SN extracts (150 μL) at various concentrations (0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL) were added to the micelle solution (1,350 μL) and incubated at 37 °C for 3 hours. Micelles sizes were measured using a particle size analyzer which measures particle size from below 1 nm to several microns using dynamic light scattering. The result was represented as nanometer (nm).

2.7. Bile acid binding determination

The bile acid binding assay was slightly modified the procedure according to Yoshie-Stark and Wäsche,[15] and as previously reported.[16] TC, GC, and TD were used as bile acids, while cholestyramine was used as a positive control in this experiment. In brief, 200 μL of SN extract (1 mg/mL) or cholestyramine (1 mg/mL) was incubated with 200 μL of bile acid (2 mM) in 100 mM PBS, pH 7.0, at 37 °C for 2 hours. The mixed solutions were centrifuged at 10,000 rpm for 10 minutes and passed through a 0.22 μm membrane to separate the bound from the free bile acids. The concentration of bile acid was evaluated the following total bile acids methods.[17] The filtrated bile acid solutions (20 μL) were mixed with 170 μL of reaction mixtures [0.133 mol/L tris buffer (pH 9.5), 1 mol/L hydrazine hydrate, and 7.7 mmol/L nicotinamide adenine dinucleotide (NAD)] and 10 μL of 1 unit/mL 3α-hydroxysteroid dehydrogenase. The mixed solutions were incubated at 30 °C for 90 minutes. The rate of formation of thio-nicotinamide adenine dinucleotide (NADH) was determined by measuring specific change of absorbance at 405 nm in a microplate reader.

2.8. Cell culture preparation

Caco-2 cells were obtained from the American Type Culture Collection. Cells were maintained at 37 °C in Dulbecco's modified Eagle's medium/F12 containing 10% fetal bovine serum and 100 units/mL of penicillin, and 100 μg/mL of streptomycin in an incubator with 5% CO_2 and saturated humidity.

2.9. Cell viability assay

The viability of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) assay. Cells were cultured in 96-well plates at 10,000 cells/well and allowed to grow overnight. Cells were treated with the SN extract for 24 hours. Two hours before the end of the treatment, 10 μL of MTT (5 mg/mL) were added. The plate was washed with PBS before the purple formazan crystals were solubilized with 200 μL of Dimethyl sulfoxide : Ethanol (DMSO:ETOH) (1:1; v/v) and measured at 595 nm. Cell viability was expressed as a percentage relative to the untreated.

The % cell viability was calculated by using Eq. (2) as follows:

$$\%\,\text{Cell viability} = \frac{OD_{595(\text{Treated cells})}}{OD_{595(\text{Untreated cells})}} \times 100 \quad (2)$$

where OD is an optical density.

2.10. Cholesterol uptake assay

Cholesterol micelles solutions were prepared as mentioned above. The final concentrations of micelle solutions were 1 μM cholesterol, 2 mM sodium taurocholate, 50 μM phosphatidylcholine, and 1 μCi/mL [1α,2α(n)-^{3}H]cholesterol. Caco-2 cells were plated on a 24-well plate at 50,000 cells/well and cultured for 14–21 days to allow differentiation. Fresh medium was replaced every 2–3 days. The differentiated Caco-2 cells were cultured in serum-free medium overnight before treatment with single [SN extract (0.125 mg/mL, 0.25 mg/mL, 5 mg/mL, 1 mg/mL) or ezetimibe (0.004 mg/mL, 0.04 mg/mL, 0.08 mg/mL)] or combined dose [extract and ezetimibe (0.04 mg/mL)] and [1α,2α(n)-^{3}H]-cholesterol micelles for 4 hours. After washing the plate with ice-cold PBS twice, cells were disrupted with 0.2 N NaOH/0.1% Triton-X 100. The radioactivities of lysates were measured with a Packard β-counter and protein content by a BCA protein assay kit (Pierce, Rockford, IL, USA).

Cholesterol uptake (% of control) was calculated by using Eq. (3): as follows.

$$\%\,\text{Cholesterol uptake} = \frac{\text{Radioactivities of cells/Protein contents (Treated cells)}}{\text{Radioactivities of cells/Protein contents (Untreated cells)}} \times 100 \quad (3)$$

2.11. HMG-CoA reductase inhibitory activity

The HMG-CoA reductase inhibitory activity was determined using an HMG-CoA reductase assay kit from Sigma-Aldrich (St. Louis, MO, USA). Ninety microliters of 1X buffer and 1 μL of SN extract (0.1 mg/mL), 2 μL of nicotinamide adenine dinucleotide phosphate (NADPH), and HMG-CoA concentrations (400 μM, 800 μM, 1,600 μM, and 3,200 μM) were added in 96-well plates. HMG-CoA reductase (1 μL) will then be added to start the reaction. The oxidation of NADPH to NADP+ was measured by using a microplate reader at 340 nm with a kinetic program. The enzyme activity was then expressed as a percentage of inhibition. Pravastatin was used as a positive control in this study.

2.12. Statistical analysis

The results were represented as mean ± standard error of the mean (SEM). Each experiments were carried out as duplicate and was repeated three times. The statistical significance of differences was evaluated by analysis of variance and Student t test using statistical software in Microsoft Excel (Microsoft

Table 1 – Phenolic constituents of aqueous *Spirogyra neglecta* (SN) extracts

Phenolic compounds	Aqueous SN extract (mg/kg)
Gallic acid	565.29
Eriodictyol	347.68
Apigenin	ND
Isoquercetin	5,500.44
Kaempferol	621.40
Quercetin	386.73
Hydroquinin	1,118.32
Rutin	700.40
Catechin	3,531.08
Tannic acid	548.91

ND, Not Detected.

Corporation, Redmond, WA, USA). Differences were considered significant at p values < 0.05.

3. Results

3.1. *Phenolic constituents of aqueous SN extracts*

The extraction yield with aqueous extract of SN was 30.5 ± 2.2%. The phytochemical screening of phenolic constituents of aqueous SN extract showed the presence of eriodictyol, isoquercetin, kaempferol, quercetin, hydroquinin, rutin, catechin, and tannic acid (Table 1). Isoquercetin and catechin displayed the main phenolic compounds in the SN extract with 5,500.44 mg/kg and 3,531.08 mg/kg, respectively.

3.2. *Effect of SN extract on micellar cholesterol solubility*

The inhibition of micellar solubility of cholesterol was significantly increased in a dose-dependent manner in the presence of SN extracts (0.1 mg/mL, 1 mg/mL, 10 mg/mL) as shown in Fig. 1. SN extracts inhibited cholesterol solubility in the range of 25–83%.

3.3. *Effect of SN extract on cholesterol micelles size*

SN extracts significantly increased the particle size of cholesterol micelles in a dose-response manner as nanometer ranges (Fig. 2). SN extracts at 0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, and 10 mg/mL increased the size when compared with control by 1.7 times, 2.3 times, 3.5 times, and 11.8 times, respectively.

3.4. *Effect of SN extract on bile acid binding*

The SN extract and bile acids was evaluation total bile acid using the 5th generation Randox total bile acids assay. The rate of formation of Thio-NADH was determined by measuring absorbance at 405 nm in a microplate reader. The percentage of bile acid binding by SN extract (1 mg/mL) is shown in Table 2. TC, GC, and TD were used as bile acids. SN extract bound to TD and GC higher than cholestyramine. The extract bound to TD, GC, and TC by 49.58%, 48.82%, and 24.01%, respectively.

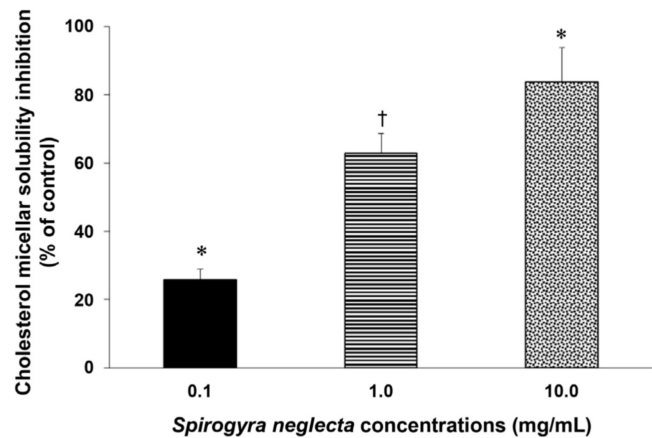

Fig. 1 – Effect of *Spirogyra neglecta* (SN) on the solubility of cholesterol micelles. SN extract (0.1 mg/mL, 1 mg/mL, 10 mg/mL) were incubated with micelle solution at 37 °C for 3 hours. The solutions were filtered and then the cholesterol content determined, which can be defined as micelar cholesterol solubility. Values are represented as mean ± SEM (n = 3).
Significant differences between untreated (control) and treated (SN) percentages as follows:
*** $p < 0.005$.**
† $p < 0.001$.
SEM, standard error of the mean.

Cholestyramine (1 mg/mL), a positive control, bound to TD, GC, and TC by 36.47%, 26.87%, and 83.63%, respectively.

3.5. *SN extract on cell viability*

To select the concentration of SN extracts for further experiments, the extracts were tested for their cytotoxicity. SN at

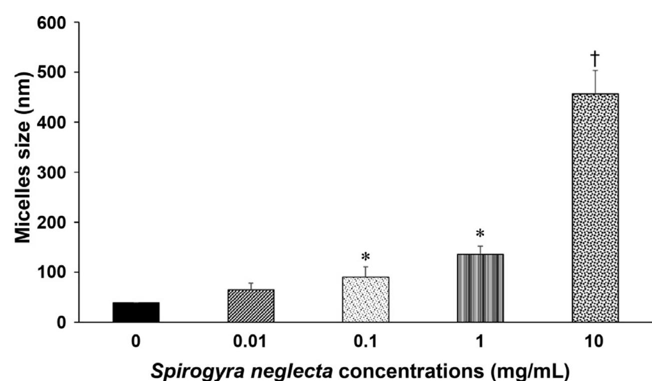

Fig. 2 – Effect of *Spirogyra neglecta* (SN) on micelles sizes. SN extracts (0.01 mg/mL, 0.1 mg/mL, 1 mg/mL, 10 mg/mL) were incubated with micelle solution at 37 °C for 3 hours. The solutions were measured for micelles size. Values are represented as mean ± SEM (n = 3).
Significant differences between untreated (control) and treated (SN) percentages are as follows:
*** $p < 0.001$.**
† $p < 0.05$.
SEM, standard error of the mean.

Table 2 – *In vitro* binding of *Spirogyra neglecta* with bile acids

Samples	Bile acid binding (% of control)		
	Taurocholate	Taurodeoxycholate	Glycodeoxycholate
Spirogyra neglecta	24.01 ± 2.76	49.58 ± 0.29*	48.82 ± 2.88*
Cholestyramine	83.63 ± 0.13*	36.47 ± 2.57*	26.87 ± 2.52*

Values represented as mean ± SEM ($n = 3$). Standard bile acid 2 mM. Sample concentrations 1 mg/mL.
* $p < 0.01$: significant differences between untreated (control) and treated (SN) percentages.
SEM, standard error of the mean.

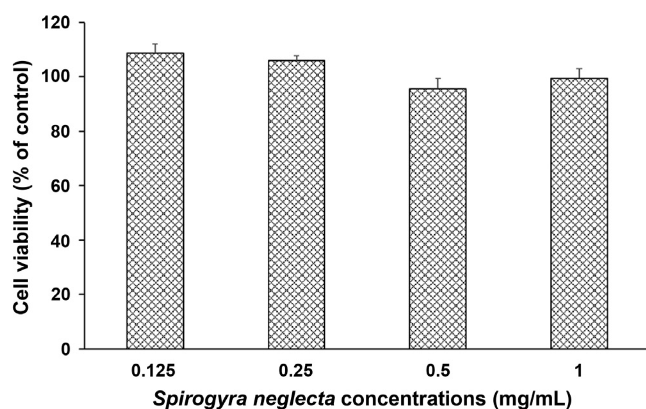

Fig. 3 – Effect of *Spirogyra neglecta* (SN) extract on Caco-2 cells viability. Cells were treated with the SN extract (0.125 mg/mL, 0.25 mg/mL, 5 mg/mL, 1 mg/mL) for 24 hours. Two hours before the end of the treatment, 3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl tetrazolium bromide (MTT) was added. Cell viability was expressed as a percentage relative to the untreated. Values are represented as mean ± SEM ($n = 3$).
SEM, standard error of the mean.

0.125–1 mg/mL was observed to cause no effect on viability of Caco-2 cells (Fig. 3). Therefore, the concentrations of SN at 0.125–1 mg/mL were selected for uptake experiments.

3.6. *SN extract on cholesterol uptake in differentiated Caco-2 cells*

SN extract treatment showed a significant dose-dependent decrease in cholesterol uptake in Caco-2 cells, as illustrated in Fig. 4. The extract at 0.5 mg/mL and 1 mg/mL decreased cholesterol uptake by about 19% and 32%, respectively. Ezetimibe inhibited the uptake of cholesterol approximately 15–54% in a dose range from 0.004 mg/mL to 0.08 mg/mL. Since SN extract and ezetimibe inhibited cholesterol uptake in Caco-2 cells, there is a possibility that a combination of these two compounds might further enhance the uptake inhibition activity. The results showed that the combination of low concentration of SN extract (0.125 mg/mL) and ezetimibe (0.04 mg/mL) caused a significant enhancement in cholesterol uptake inhibition, whereas the combination of a higher dosage of SN extract (0.25 mg/mL and 0.5 mg/mL) and ezetimibe tends to decrease the uptake when compared with SN extract treatment alone (Fig. 4).

3.7. *SN extract on HMG-CoA reductase activity*

The HMG-CoA reductase inhibitory activities of SN extract are shown in Fig. 5. Pravastatin (0.25 μM) was used as a standard treatment agent for HMG-CoA reductase inhibitor activity. From our experiments, SN extract (0.1 mg/mL) inhibited HMGR activity and was believed to cause a direct binding to the catalytic domain of HMGR along an increase of HMG-CoA concentrations (400 μM, 800 μM, 1,600 μM, and 3,200 μM) by 38%, 88%, 75%, and 77% inhibition, respectively, as shown in Fig. 5. However, there is a sign of saturation in HMGR activity as demonstrated in results as a response to increasing HMG-CoA concentration.

4. Discussion

Literature showed SN was well studied for its antigastric ulcer, antiinflammatory,[9] antihyperglycemic, and antihyperlipidemic properties.[10] Although a recent publication reported that SN was found to exert its regulatory effect in animal cholesterol levels, the exact mechanism of action, particularly on the cholesterol absorption inhibition, is still unclear. Cholesterol homeostasis involves a balance in intestinal absorption, endogenous synthesis, bile acid activities, and fecal excretion.[18] Intestinal cholesterol absorption involves several processes which include lipid digestion, micellar solubilization, cholesterol release from micelles into enterocytes, and secretion into lymph.[12] The reduction of cholesterol absorption by reducing micellar cholesterol solubility is suggested for the treatment of hypercholesterolemia.[14] Raederstorff and coworkers[19] suggested that the uptake of cholesterol could interfere with the affinity of micelles into membranes and/or the affinity of cholesterol in micelles. Additionally, the binding of bile acids might act as a possible mechanism in regulating cholesterol levels. In detail, the bile acid sequestrants bind to bile acids in the intestine resulting in the formation of an insoluble complex which can be excreted in the feces. The activation of this process is known to play an important role in regulating the cholesterol levels in hypercholesterolemia condition.[18,20] In order to assess the inhibitory effect of SN extract on cholesterol absorption, Caco-2 cells were used as model of experimentation specifically for the cholesterol absorption study. Our results indicate that SN extract decreased cholesterol uptake in Caco-2 cells. In our attempt to address the possible involvement of the cholesterol absorption mechanism in observed activity, the effect of SN extract on cholesterol micelles size and solubility and its binding property to bile acids was studied. SN also demonstrated a dose-dependent decrease in solubility of cholesterol

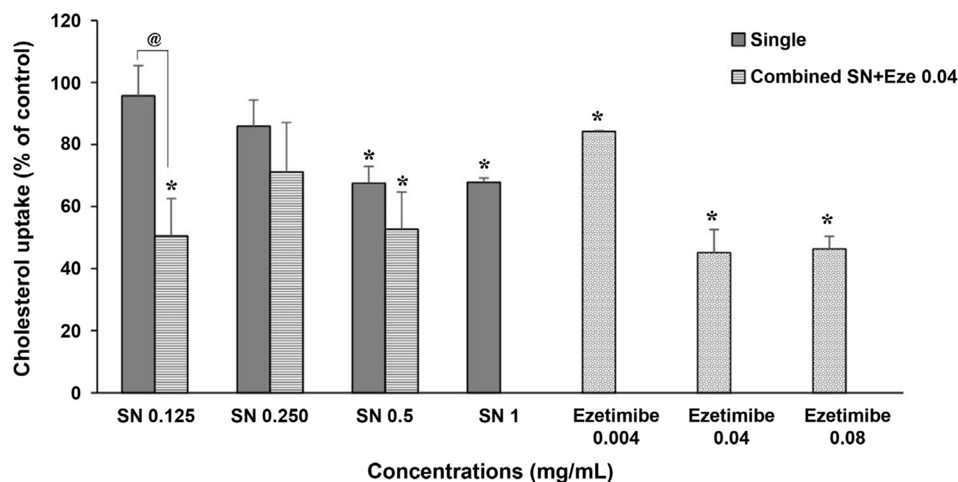

Fig. 4 – Single and combined effects of *Spirogyra neglecta* (SN) extract and ezetimibe on cholesterol uptake in Caco-2 cells. The differentiated Caco-2 cells were treated with single [SN extract (0.125 mg/mL, 0.25 mg/mL, 5 mg/mL, 1 mg/mL) or ezetimibe (0.004 mg/mL, 0.04 mg/mL, 0.08 mg/mL)] or combined dose [extract and ezetimibe (0.04 mg/mL)] and 1 μCi/mL [1a,2a(n)-3H]-cholesterol micelle solution for 4 hours. Cells were disrupted and radioactivities and protein content measured. Significant differences are as follows:

*** $p < 0.05$ [Compared between single dose of SN (0.125 mg/mL, 0.250 mg/mL, 0.5 mg/mL) and combination of SN and Eze 0.04 mg/mL].**

† $p < 0.05$ {Compared between untreated [control] and treated [SN or ezetimibe (Eze)]}.

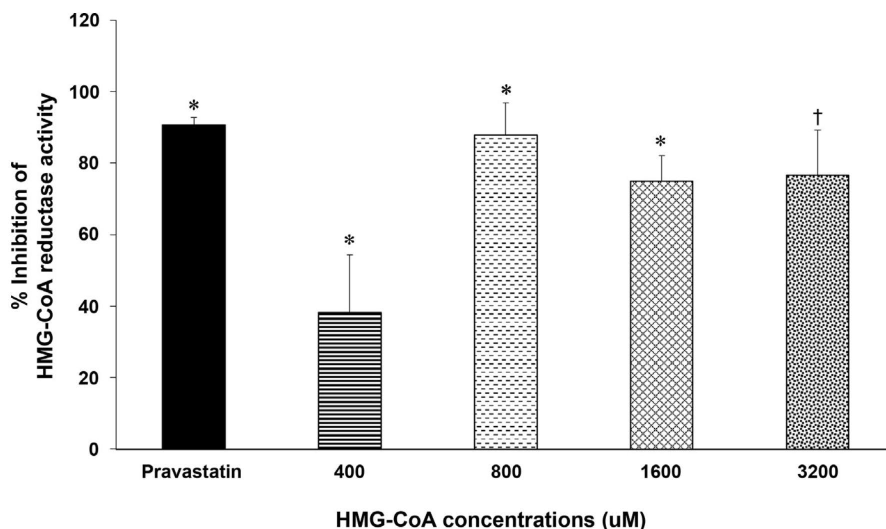

Fig. 5 – Effect of *Spirogyra neglecta* (SN) extract on 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitory activity *in vitro*. The HMG-CoA reductase activity was determined using an HMG-CoA reductase assay kit. SN extracts (0.1 mg/mL), NADPH, HMG-CoA concentrations (400 μM, 800 μM, 1600 μM, and 3200 μM), and HMG-CoA reductase were used in the reaction. Pravastatin was used as a positive control. The oxidation of NADPH was measured at 340 nm with a kinetic program. Enzyme activity is expressed as a percentage of inhibition (mean ± SEM, $n = 3$).

Significant differences between control and SN extract or pravastatin percentages are as follows:

*** $p < 0.001$.**

† $p < 0.005$.

NADPH, nicotinamide adenine dinucleotide phosphate; SEM, standard error of the mean.

in micelles and a significant increase in size of micelles. In addition, SN revealed bile acids binding property (TC, GC, and TD). In fact, Fernandez[21] suggested that soluble-fiber polysaccharides may play an important role in modifying the volume, bulk, and viscosity of the intestinal lumen. These effects were believed to interfere with the enterohepatic circulation and lipoprotein metabolism which may cause a resulting effect on lower cholesterol levels.[21] Oat bran with native beta-glucans, polysaccharides, increased fecal bile acid excretion, as the β-glucans in oat bran entrap or encapsulate whole micelles in the small intestine.[22] Besides, tea polyphenols (epigallocatechin gallate) are known to interact with TCs in

micellar solutions, eventually inhibiting the solubilization of cholesterol.[23] Meanwhile, the polyphenols in grape seed (gallic acid, catechin, and epicatechin) have high binding tendency to bile acids, which will cause a reduction in the solubilization of cholesterol in micelles.[24] A similar activity was also found in black tea polyphenols as they decrease the *in vitro* micellar solubility of cholesterol and have been shown to interrupt intestinal cholesterol absorption in rats.[25]

In the present study, we found that SN contains polyphenolic compounds such as isoquercetin and catechin. Besides polyphenolic compounds, SN was also known to possess polysaccharides.[26] Taken altogether, we postulated that the activities exhibited by SN extract could be possibly linked to the presence of polyphenols and polysaccharides. By contrast, intestinal cholesterol absorption can be specifically inhibited by cholesterol absorption inhibitors (ezetimibe). However, the treatment with ezetimibe showed no effect in NPC1L1 knockout mice, suggesting that NPC1L1plays a role in the uptake of cholesterol.[4] Our results show for the first time that SN extract inhibited cholesterol uptake, and the observed activity possibly acted through an inhibitory effect on cholesterol transporters. Therefore, the experiment evaluated the combinatory effect of SN extract and ezetimibe. The results showed that the combination of SN extract and ezetimibe (0.04 mg/mL) achieved a significantly greater reduction in cholesterol uptake than SN extract alone. However, the combinatory effect was still lower as compared to ezetimibe treatment alone. In the combination, additive effect refers to the sum of their effects, the synergistic effect refers to the effect which is greater than the sum of their effect, whereas an antagonistic effect is the opposite of synergistic effect. Our results suggest that SN and ezetimibe decreased cholesterol uptake and there was no synergetic effect with a combination of SN extracts and ezetimibe. Normally, ezetimibe does not completely inhibit cholesterol absorption, thus our results implied that SN may share the same mechanistic action sites with ezetimibe by inhibiting cholesterol transporter (NPC1L1) and/or effects on the internalization of transporter (NPC1L1) between intracellular compartments and membrane.

In cholesterol synthesis, HMG-CoA reductase is the rate limiting step in cholesterol biosynthesis. Statins or HMG-CoA reductase inhibitors are commonly used for management of hypercholesterolemia. The presence of an HMG-like moiety on these drugs allows them to competitively bind to the catalytic domain of the target enzyme, which results in an observed inhibitory effect as shown in HMG-CoA reductase.[27] In order to assess the effect of SN extract on cholesterol synthesis inhibition we examined the effect of SNF on the activity of HMG-CoA reductase. SN extract and pravastatin showed a significant reduction in HMGR activity. The inhibition effect was observed to be increased in response to increasing HMG-CoA concentration and progressively reached its maximum activity or saturation. It can be noted that SN extract may have caused a direct binding to the catalytic domain of HMGR. Islam and colleges[28] reported that dietary polyphenols can reduce cholesterol biosynthesis by binding to HMGR and blocking the binding of nicotinamide adenine dinucleotide phosphate.[28] Curcumin binds to the HMG-CoA site on the enzyme similar to the statins.[28] Epigallocatechin gallate potently inhibits HMGR activity by competitively binding to the cofactor site of the reductase.[28] Peng and coworkers[29] suggested that polyphenols-rich mulberry water extracts have hypolipidemic properties via inhibiting lipid synthesis (decreased the expression of HMGR) and promoting lipid degradation.[29] It can be inferred that SN extract containing polyphenols may contribute to inhibiting HMGR activity.

The current study demonstrated that SN extract could inhibit cholesterol absorption and synthesis activities by modifying the size and solubility of cholesterol micelles with involvement of the binding effect of bile acids. It was observed to cause an effect by interrupting the cholesterol uptake, at the same time inhibiting HMG-CoA reductase activity. The presence of bioactive ingredients in SN could be responsible for the observed cholesterol lowering effect. Taken altogether, this study provides the evidence for potential SN usage and possible development into a natural supplement for a cholesterol lowering product. However, more preclinical and perhaps clinical studies might be needed for the safe use of this product.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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