



Phytochemical screening, antioxidant potential, and cytotoxic effects of different extracts of red algae (*Laurencia snyderiae*) on HT29 cells

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Abstract

Background and purpose: Marine algae are potential renewable and sustainable sources of bioactive natural products which can be utilized in nutraceutical and pharmaceutical industries.

Experimental approach: Different extracts (methanol, chloroform, and ethyl acetate) of red algae, *Laurencia snyderiae*, was evaluated for their antioxidant potential, with various antioxidant assessment assays, cytotoxic properties (using MTT colorimetric assay), and phytochemical constituents (total phenolic and flavonoid contents). The GC-MS analyses of the algal methanolic extract and its apoptotic effects on the human colon carcinoma cell line (HT29) were also investigated.

Findings/Results: The total phenolic content in the methanol, chloroform, and ethyl acetate extracts of *L. snyderiae* was 3.6 ± 0.12 , 3.2 ± 0.41 , and 3.3 ± 0.35 $\mu\text{g}/\text{mg}$ of gallic acid, respectively. Among different algae extracts, chloroform extract showed significantly chelating ability ($\text{IC}_{50} = 0.027$ mg/mL) and reducing power activity ($\text{IC}_{50} = 0.082$ mg/mL), while the highest DPPH scavenging activity ($\text{IC}_{50} = 0.058$ mg/mL) exhibited in the methanol extract compared to the other extracts. The methanolic extract was found to have a higher cytotoxicity effect on colon carcinoma cells with IC_{50} 70.2 $\mu\text{g}/\text{mL}$. The viability of the cancer cells was increased with the decrease in the concentration in different extracts. GC-MS analysis of the algal methanolic extract revealed the presence of active antitumor constituents and apoptosis-based cytotoxicity against colon cancer cells through the DNA damage was also confirmed.

Conclusion and implications: Based on these results, the red algae *L. snyderiae* possesses potent bioactive constituents and can use as additional resources as a natural antioxidant and antitumor agent in the pharmaceutical and nutraceutical area.

Keywords: Anticancer; GC-MS; Persian Gulf, Red algae.

INTRODUCTION

It is widely accepted that oxidative stress induced by reactive oxygen species (ROS) can lead to cell-membrane disintegration, protein and DNA oxidative damage which is consequently caused to immune dysregulation and major health problems including cancer, aging-related disorders, and cardiovascular diseases. An imbalance of free radicals and antioxidants in the body due to the chemicals and pollutants exposure could lead to overproduction of free radicals be contributed to reducing the capacity of the biological defense system, causing irreversible oxidative damage (1,2).

Antioxidants with free radical scavenging activity play a crucial role in the prevention or treatment of diseases associated with oxidative stress (3,4). The extensive cellular and molecular studies on cancer cells have created a purposeful approach for the biochemical prevention of cancer, with the aim of cessation or returning cells to precancerous conditions without any toxic doses through nutrients and drug agents (5).

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Several studies have been carried out on using naturally occurring compounds as anticancer agents in relation to appropriate antioxidant activity (6).

Bioactive products, especially those derived from the secondary metabolism of marine organisms, are a potential source of anticancer drugs against malignant disorders. Seaweeds are well known to be one of the richest sources of bioactive compounds. In general, the seaweeds consist of three phyla, namely green, red, and brown algae, and three families, including *Chlorophyceae*, *Rhodophyceae*, and *Phaeophyceae*. The red algae or *Rhodophyceae* are major algae groups in bioactive compounds (6). Moussavou *et al.* investigated the anticancer effects of different algae on human breast and colorectal cancer cells, and can be utilized as potent anticancer agents to control a variety of cancerous diseases (7). Patra and Muthuraman established the anticancer properties and stimulated cell apoptosis by the *Gracilaria arcuata* extract. Their results showed that the inoculation of algae extracts in rats with Ehrlich ascites tumour (EAT) prolonged the survival of rats, and significantly prevented the growth of tumours (8).

Seaweeds in the southern regions of Iran, particularly Chabahar, are highly valuable sources of nutrients and therapeutic potentials. The genus *Laurencia* is classified into 145 species, found in temperate and tropical shore areas in the world (9,10). The phytochemistry of some *Laurencia* species has been proved by the presence of sesquiterpenes and various halogenated secondary metabolites such as diterpenes, and acetogenins (11,12).

Additionally, the biopharmaceutical and nutritional values of these genera related to life expectancy through dietary intake have been considered. So, it could be expected the risk of some chronic diseases clearly be reduced through the consumption of these types of seaweeds (13,14).

The current research is the first to evaluate the phytochemical composition of red algae, *Laurencia snyderiae*, collected from the Persian Gulf and also to investigate the antioxidant and cytotoxic activities of different extracts against human colorectal cancer cells (HT29).

MATERIALS AND METHODS

Sample collection and extract preparation

Red algae samples were collected in May 2016 from Chabahar coasts, at coordinate Oman sea, Iran (25° 18' 53"N; 60° 37' 41"E) in Oman sea, Iran.

Laurencia snyderiae (E. Y. Dawson) was fixed in formalin 5% in seawater and was mounted on herbarium sheet for the following identification. Seaweeds were identified by Dr. Jelveh Sohrabipour (taxonomist) and the voucher specimens (*L. snyderiae*: 55-23R) has been deposited at the Herbarium of Agriculture and Natural Resources Research Center of Hormozgan, Bandar Abbas, Iran. Then the samples were transferred to Lahijan biotechnology laboratory for further experiment.

Marine algae were thoroughly washed to completely remove sand and epiphytic organisms. The samples were immersed in distilled water to remove salts, and the water was renewed every 3 h. Then, air-dried samples were powdered with a grinder before extraction. The extraction was carried out by immersion 2.5 g of algae powder into methanol (1:20 w/v) for 24 h at 60 °C. The extracts were filtered with Whatman grade 42 filter paper, following evaporation at 35 °C under reduced pressure (rotary evaporator BUCHI R-200 model, Germany). Chloroform and ethyl acetate extracts of the remaining samples (algae powder) were prepared in the same way in which the methanolic extracts were prepared. Finally, different obtained extracts (methanolic, ethyl acetate, and chloroform) were frozen at a -20 °C freezer for further study (15).

Determination of yield

The yield of the extracts was measured with respect to the original mass of red algae powdered according to Mehdinezhad *et al.* (16). The percentage yield was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{Mass of dried extract}}{\text{Mass of seaweed powdered}} \times 100 \quad (1)$$

Determination of total phenolic content

Total flavonoid was determined using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth (17). Briefly, 0.5 mL of algae different extracts (methanol, chloroform,

and ethyl acetate) was mixed with 2 mL of Folin-Ciocalteu reagent. After that, 4 mL of sodium carbonate solution (7.5%, w/v) was added and incubated at room temperature for 30 min. The absorbance of the resulting blue colour was measured at 765 nm using a double beam UV-VIS spectrophotometer (UV Analyst-CT 8200, Taiwan). The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The result was expressed as mg/g gallic acid equivalent (GAE) of dry extract.

The total phenolic concentration of compounds in the extract was specified using the following formula:

$$T = C \times V/M \quad (2)$$

where T is the total phenolic content (mg/g) of seaweed extracts in GAE; C, the concentration of gallic acid from the calibration curve in mg/mL; V, the volume of the extract in mL; and M, the weight of the extract in g. Gallic acid is used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: $y = 0.0061x + 0.0396$, $R^2 = 0.9991$, where y is the absorbance at 760 nm and x is the total phenolic content in the different extracts.

Determination of total flavonoids content

The total flavonoid content of different extracts (methanol, chloroform, and ethyl acetate) was determined using a spectrometer method as described by Chang *et al* (18). Each extract (1 mL) was mixed with aluminum chloride solution (1 mL) and after 10 min, the absorbance of the mixture was recorded at 430 nm. Results were expressed as quercetin equivalent per gram dried weight (mg QE/g DW).

Gas chromatography-mass spectrometry analysis

The analysis of gas chromatography-mass spectrometry (GC-MS) was performed on the methanolic extract using Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a column of 5-DB, 30-mm length, and 0.25-mm internal diameter. The mass spectrometer was equipped with an electron ionization system, was performed with ionization energy of 70 eV in electron impact

mode. The injector temperature was programmed at 250 °C, the ion-source temperature was maintained at 200 °C. The oven temperature was started at 50 °C and increased to 180 °C at a rate of 20 °C/min for 3 min, then raised to 200 °C at 5 °C/min for 5 min and finally held at 280 °C at 10 °C/min for 20 min.

The helium carrier gas (99.999%) at a constant flow rate of 5 mL/min. One μ L of diluted sample in methanol (1/100, v/v) was injected in the split mode with a split ration 120:1. The methanolic extract was injected into the GC-MS device, and the results were then identified based on the Kovats index (KI) of standard references. To define the KI of the components, a commercial aliphatic hydrocarbon mixture (Sigma-Aldrich) was added to the extract under the same conditions (as above) before injecting it into the GC-MS system.

The temperature-programmed KI of the components was calculated by the following quasi-linear equation:

$$KI(x) = 100 \times Z + 100 \times \frac{t(x) - t(z)}{t(z+1) - t(z)} \quad (3)$$

where, KI(x) is the temperature-programmed KI of interest, t(x), t(z), and t(z+1) were the retention times in minutes of interest and the two standards n-alkanes containing z and z+1 carbons and index, respectively: $t(z) < t(x) < t(z+1)$ (14).

Data were calculated in total ion chromatogram mode. An Agilent ChemStation software version 2.53 was applied to handle the mass spectra and chromatograms. The GC-MS spectra and constituents were interpreted using the databases of the National Institute Standard and Technology (NIST) version 2.0, year 2005 library, which has more than 65 000 patterns. Then, were compared and characterized according to available data in the GC-MS library in the literature.

Unknown mass spectra were compared with the known components in the NIST library. The structure, name, and molecular weight of each component of the extract were determined. The chemical components of the methanolic extract were identified based on their structure, molecular formula, retention time, and peak % area.

1-Diphenyl-2-picrylhydrazyl radical scavenging assay

The diphenyl picrylhydrazyl (DPPH) free radical scavenging assay was performed according to the method presented by Lim *et al.* (19). In brief, various concentrations (0.01, 0.025, 0.050, 0.075, and 0.1 mg/mL) of different extracts (chloroform, methanol, and ethyl acetate) were treated by 0.1 M methanolic DPPH solution. The resulting mixture was shaken and incubated at room temperature for 20 min in the dark. The absorbance (A) was read at 517nm wavelength versus the control, and ascorbic acid (vitamin C) was used for comparison. The control sample, containing the same volume without any extract, was prepared, and 95% methanol was used as the blank. The DPPH free radical scavenging activity was measured as a decrease in the absorbance. Ascorbic acid was further used as standard and scavenging activity was calculated by the following equation:

$$\text{DPPH Radical - scavenging (\%)} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100 \quad (4)$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the sample. Ascorbic acid was used as the positive control. IC₅₀ (inhibitory concentration) values, which showed the concentration of the extract that caused 50% neutralization of DPPH radicals, were determined using the plot of inhibition percentages against concentration.

Reducing power assay

The reducing power of the extracts was investigated according to the method described by Oyaizu *et al.* (20). Different concentrations (0.01, 0.025, 0.050, 0.075, and 0.1 mg/mL) of algae extracts (chloroform, methanol, and ethyl acetate) were mixed with sodium phosphate buffer and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, and quickly cooled down to room temperature; 10% trichloroacetic acid was added, and the solution was centrifuged at 3000 rpm for 10 min; 2 mL of the mixture was then combined with the same volume of distilled water and 0.4 mL of 0.1% iron chloride. After 10 min, the absorbance of the solution was read at 700 nm wavelength. Higher absorbance indicates

higher reducing power. The control sample, containing the same volumes of phosphate buffer, was prepared and used as the blank. Butylated hydroxytoluene was used for comparison.

Iron chelating assay

Iron chelation was determined according to the method of Dinis *et al.* (21). Five different concentrations (0.01, 0.025, 0.050, 0.075, and 0.1 mg/mL) of various algae extracts (chloroform, methanol, and ethyl acetate) were prepared. The solutions were added by 100 µg/mL of 0.4 mM iron chloride. After 3 min, 200 µg/mL of 0.8 mM ferrozine was added to the solution and shaken for 10 min at room temperature. The Absorbance was read at 562nm wavelength. Iron chloride and ferrozine (without extract) were employed as the control groups. Ascorbic acid was also utilized as a standard, and phosphate buffer (pH 6.6) was used as a blank solution. The chelating percentage of iron ion was measured using the following equation:

$$\text{The chelating percentage of iron ion (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (5)$$

where A_{control} is the absorbance of the control reaction mixture without the test compounds, and A_{Sample} is the absorbance of the test compounds. IC₅₀ values, which showed the concentration of the extract that caused 50% of Fe²⁺ ion chelation, were determined from the plot of chelating percentage against concentration.

Cell culture

Colorectal cancer cell lines HT29 and normal fibroblast cell lines L-929 (IBRC C10102) were obtained from the cell bank of the National Center for Biological and Genetic Reserves (Tehran, Iran). The cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS, Sigma-Aldrich, USA), and 1% antibiotic penicillin-streptomycin at 37 °C in a humid atmosphere with 5% CO₂ concentration. Upon reaching at least 70% cell growth, the cells were separated from the flask by trypsin (0.05%) and centrifuged at 1500 rpm for 5 min. The resulting precipitate was prepared in

suspension, and the percentage of cells was specified by Neubauer chamber and Trypan Blue dye through microscopy. After non-contamination was confirmed, cells with a viability of more than 90% were used for the experiment.

Cell viability assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) a colorimetric assay was used to investigate the effect of different extracts on the growth and proliferation of cancer cells (HT29) and normal cells.

Briefly, the cells were cultured in 96-well plates (10000 cells/well) and incubated at 37 °C, 5% CO₂ incubator for 24 h (22). The cells were exposed to various concentrations of different extracts (chloroform, methanol, and ethyl acetate) containing 12.5, 25, 50, 100, and 200 µg/mL for 24 h. The cells with no extract exposure were considered as control, and 20 µL of MTT solution (5 mg/mL) was then added to each well. The plates were incubated in the same condition for 4 h and then centrifuged at 3000 rpm, 4 °C for 3 min. The supernatant was discarded and dimethyl sulfoxide (DMSO) was added to each well to dissolve the resulting formazan precipitation, and the plates were shaken for 20 min. The absorbance of the solutions was obtained at 570-630 nm using ELISA reader (680, Bio-rad, USA).

Apoptosis induction assay

Apoptosis was detected by the dead cell apoptosis kit with annexin V-FITC and propidium iodide (PI) (e-bioscience, San Diego, CA, USA). According to the kit instructions, cells were cultured in 24-well plates at a density of 3×10^5 cells/well. The culture procedure was the same as that in the MTT assay. The cells were trypsinized, harvested, and rinsed twice with calcium buffer. Next, 10 µL of annexin V was mixed with 100 µL of cell suspension and incubated on ice for 20 min in the dark. Finally, the cells were washed, mixed with 10 µL of PI stain, incubated on ice in the dark for 10 min, and analyzed by flow cytometer (Becton Dickinson FACS caliber, USA).

4', 6-Diamidino-2-phenylindole staining

4', 6-diamidino-2-phenylindole (DAPI) is a blue-fluorescent DNA stain binding to adenine and thymine regions of double-strand DNA. The human colorectal cancer cells (1×10^4 cells per well) were seeded into a 96-well plate for 24 h. Then, the cells were treated with the methanolic extract of *Laurencia Synderiae* (at IC₅₀ value) for 24 h. After washing with phosphate-buffered saline (PBS, pH 7.4), the cells were fixed with ice-cold 70% ethanol and resuspended in DAPI. The cells were then incubated for 15 min at 37 °C, washed with PBS, and examined under an Olympus fluorescence microscope (IX 71, Olympus, Japan).

DNA fragmentation

This process was analyzed using the method described by Morgan and Darlin (23). The cells treated with different concentrations of methanolic extract of *L. synderiae* (50, 100, and 70.2 µg/mL) were incubated for 48 h at 37 °C. After that, a cell suspension containing $4-6 \times 10^4$ cells was centrifuged at 2000g for 5 min at 4 °C. The plate containing DNA was mixed with 10 g/mL of RNase, and incubated at 50 °C for an hour. The DNA was extracted using a DNA purification kit (Qiagen, Germany), dissolved in 50 µL of tris-ethylenediaminetetraacetic buffer, and electrophoresed on 8.1% agarose containing ethidium bromide

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software for Windows. The data were presented as mean ± SEM and one-way ANOVA analysis of variance followed by Turkey's posthoc test has been applied to indicate statistically significant differences. The $P < 0.05$ was considered statistically significant.

RESULTS

Yield of extraction, total phenol content, and total flavonoid content

Extraction with methanol, chloroform, and ethyl acetate yielded 6.12 ± 0.16 , 5.78 ± 0.24 , and 5.52 ± 0.36 %, respectively (Table 1).

The total phenol content recorded for methanol extract (3.6 ± 0.12 mg GAE/g) was higher than chloroform and ethyl acetate extracts (3.2 ± 0.41 and 3.3 ± 0.35 mg GAE/g). The total flavonoid content of *L. synderiae* ethyl acetate extract was slightly higher than methanol and ethyl acetate extracts (27.65 ± 2.86 mg quercetin/g DW). There is no significant difference among various extracts in terms of total flavonoid and total phenol contents ($P > 0.05$).

GC-MS Analysis

These compounds were identified with mass spectrometry attached to GC. Different components were present in the entire

methanolic extract of red algae, *L. synderiae*, detected by the GC-MS analysis (Table 2). The mass spectrum revealed the presence of various components with different retention times as shown in Fig. 1. These mass spectra indicated the fingerprint of the compound which was identified and compared with the NIST library data. Among the identified compounds, hexadecanoic acid (9.81%), (E) oleic acid (8.34%), and tetradecanoic acid (6.86%) had the main chemical constituents. Most of these compounds have previously been studied and reported as biologically active molecules in a type of seaweeds (9,11).

Table 1. Percentage of extraction yields TPC and TFC of different extracts of red algae, *L. synderiae*. Data express mean \pm SEM, n = 3. ^aIndicates no significant differences between groups, $P > 0.05$.

Solvent system	Extraction yield (%) ^d	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
Methanol	6.12 ± 0.17	3.6 ± 0.12^a	26.77 ± 0.26^a
Chloroform	5.78 ± 0.24	3.2 ± 0.41^a	23.81 ± 1.20^a
Ethyl acetate	5.52 ± 0.36	3.3 ± 0.35^a	27.65 ± 2.86^a

^d Expressed as 100 g dry extract/g dry algae; GAE, gallic acid equivalent; TPC, total phenol content; TFC, total flavonoid content; QE, quercetin equivalent; DW, dried weight.

Table 2. The chemical composition of the methanolic extract identified by gas chromatography-mass spectrometry and KI.

No.	Chemical constituents	Retention time (min)	Relative amount to total (%)	Molecular weight	Molecular formula	KI obtained	KI literature
1	Diethoxydimethylsilane	3.39	0.75	148	C ₆ H ₁₆ O ₂ Si	665.9	678
2	Benzyl chloride	5.51	1.18	126	C ₇ H ₇ Cl	1096	1015
3	Heptadecane	15.60	3.89	240	C ₁₇ H ₃₆	1087	1072
4	2-Methyl-6-	15.82	3.89	226	C ₁₄ H ₃₄	1455	1453
5	Tetradecanoic acid	17.01	6.87	228	C ₁₄ H ₂₈ O ₂	1755	1762
6	Tetradecanoic acid, ethyl	17.61	1.95	256	C ₁₆ H ₃₂ O ₂	1779	1778
7	2-pentadecanone,6, 10,14 tri methyl	18.67	3.52	268	C ₁₈ H ₃₆ O	1838	1847
8	Pentadecanoic acid	18.98	0.98	242	C ₁₅ H ₃₀ O ₂	1848	1823
9	Dibutyl phthalate	21.04	6.93	278	C ₁₆ H ₂₂ O ₄	1974	1922
10	n-Hexadecanoic acid	21.1	9.81	256	C ₁₆ H ₃₂ O ₂	1967	1967
11	Hexadecanoic acid, ethyl	21.63	8.34	248	C ₁₈ H ₃₆ O ₂	1989	1975
12	Oleic acid	23.41	8.34	282	C ₁₈ H ₃₄ O ₂	2095	2093
13	9-Octadecenoic acid, (E)	24.13	3.35	282	C ₁₈ H ₃₄ O ₂	2100	2084
14	Octadecanoic acid	24.43	3.35	282	C ₁₈ H ₃₆ O ₂	2112	2200
15	9-Octadecenoic acid (Z)-, methyl ester	24.8	2.40	296	C ₁₉ H ₃₆ O	2178	2072
16	Bis(2-ethylhexyl) phthalate	30.21	6.96	390	C ₂₄ H ₃₈ O ₄	2450	2499
17	Phthalic acid, di (2 - propyl-pentyl) ester	30.32	2.40	390	C ₂₄ H ₃₈ O ₄	2513	2527
18	Squalene	30.8	14.5	410	C ₃₀ H ₅₀	2835	2847

KI, Kovats index.

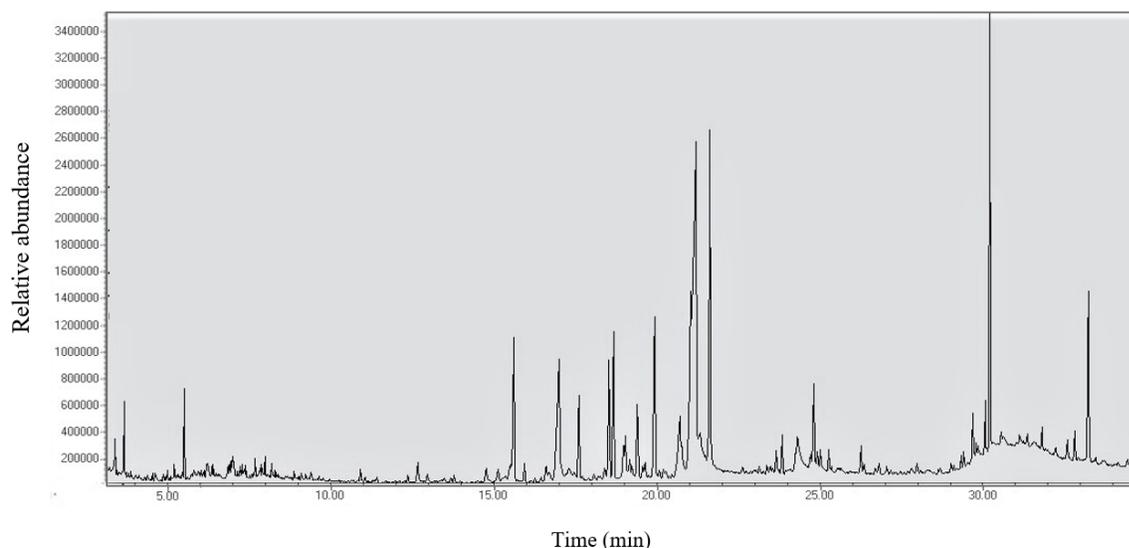


Fig. 1. Gas chromatography-mass spectrometry chromatogram of *Laurencia synderiae* methanolic extract

Antioxidant activities

The free radical DPPH scavenging activity of different red algae extracts and ascorbic acid (0.01-0.1 mg/mL) are presented in Fig. 2A. The highest free radical scavenging activity was observed in the methanolic extract at 0.1 mg/mL with an IC_{50} value of 0.058 mg/mL, while chloroform and ethyl acetate extracts showed less free radical inhibitory effect at this concentration.

The reducing ability of ferric ion was measured at various concentrations (0.01, 0.025, 0.05, 0.075, and 0.1 mg/mL) and compared with the control, ascorbic acid with the same concentrations (Fig. 2B). Chloroform extract showed significantly higher ferric reducing activity at all tested concentrations, and indicated significantly higher reducing power (16.8%) at 0.1 mg/mL compared to methanolic extract (15.3%) and ethyl acetate extract (13.1%). The chelating effects of various extracts were determined and shown in Fig. 2C. Among these extracts, ethyl acetate extract had a significant chelating activity at 0.01 mg/mL compared to the other extracts. But chloroform extract showed significant chelating activity compared to methanolic and ethyl acetate in the other concentration (0.025, 0.05, 0.075, and 0.1 mg/mL). Furthermore, chloroform and methanolic extracts at 0.1 mg/mL exhibited 58.2% (IC_{50} value of 0.082 mg/mL) and 53.4% (IC_{50} value 0.091) chelating ability, respectively.

Cell viability

Figure 3 shows the cell viability of the HT29 cells when exposed to different concentrations of red algae extracts (12.5-200 μ g/mL). Based on the results, the methanol extract had the highest significant effect on the death of cancer cells ($IC_{50} = 70.2 \mu$ g/mL), followed by chloroform extract ($IC_{50} = 98.4 \mu$ g/mL). The methanol extract at 200 mg/mL concentration showed a significant effect compared to the control and lower concentrations of the extract. Other extracts exhibited a significant difference regarding this concentration. The ethyl acetate extract showed a lower effect on cancer cells and growth inhibitory levels compared to the effective concentrations of other extracts. In all extracts, the viability of the cancer cells decreased while the concentration increased.

Apoptosis detection by annexin V-FITC test using flow cytometry

The results of apoptosis with the flow cytometry of colorectal cancer cells exposed to methanolic extract at concentrations of 70.2 μ g/mL (IC_{50} value) were indicated in Fig. 4. In this method, the cells with primary apoptosis were stained only by annexin V stain. The cells with secondary apoptosis and a slightly infiltrated-cell wall were stained with both annexin V and PI stains, and necrotic cells were stained only by PI stain. Each cell group was placed in a separate flow cytometric plot.

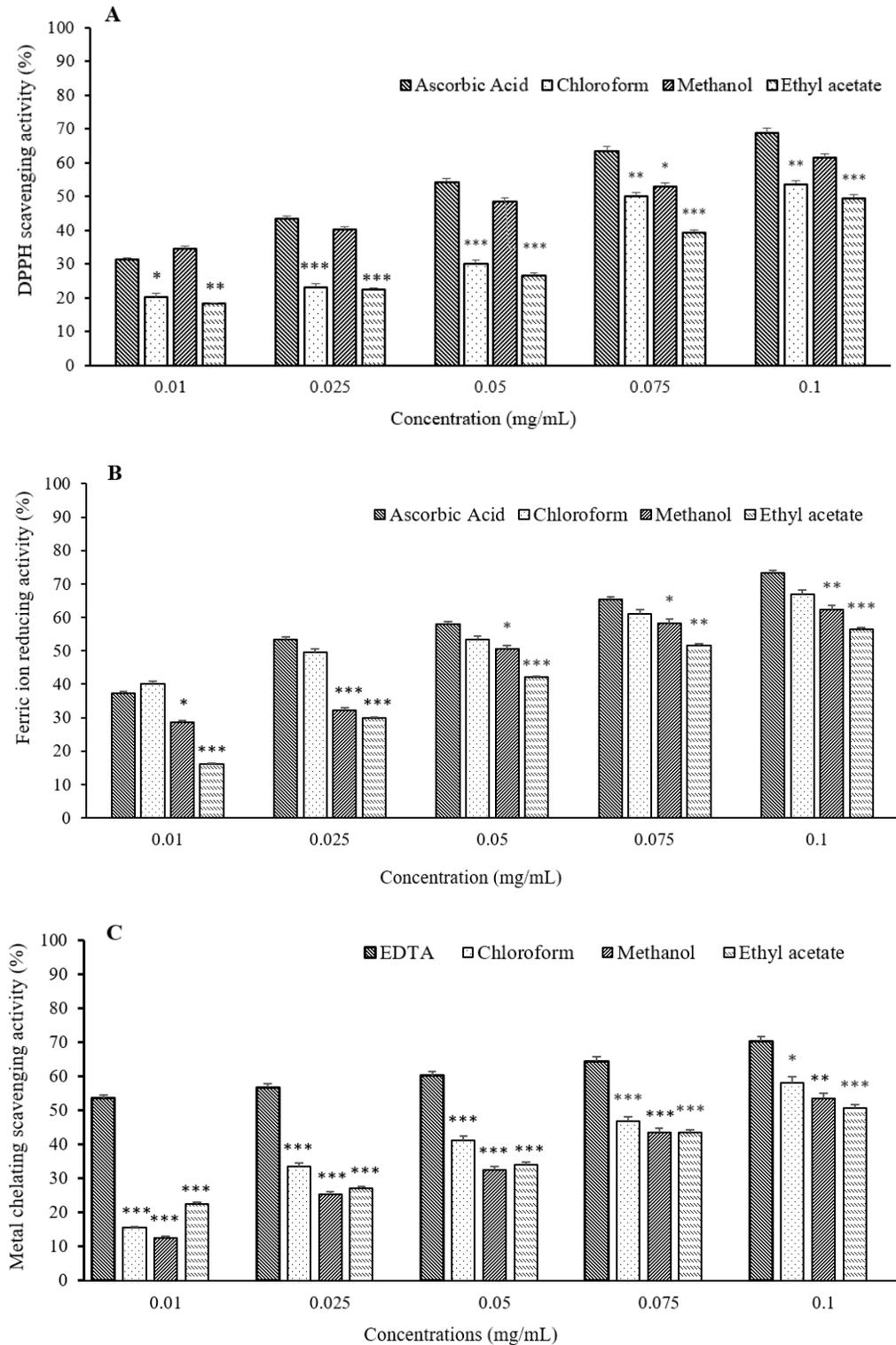


Fig. 2. Antioxidant activity of various extracts of red alga *Laurencia synderiae*. (A) DPPH radical scavenging, (B) ferric reducing antioxidant power assay, and (C) metal-chelating assay. Ascorbic acid and EDTA are used as standard. The data represent as mean \pm SEM, n = 3. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared to the standard. DPPH, Diphenylpicrylhydrazyl; EDTA, ethylenediaminetetraacetic acid.

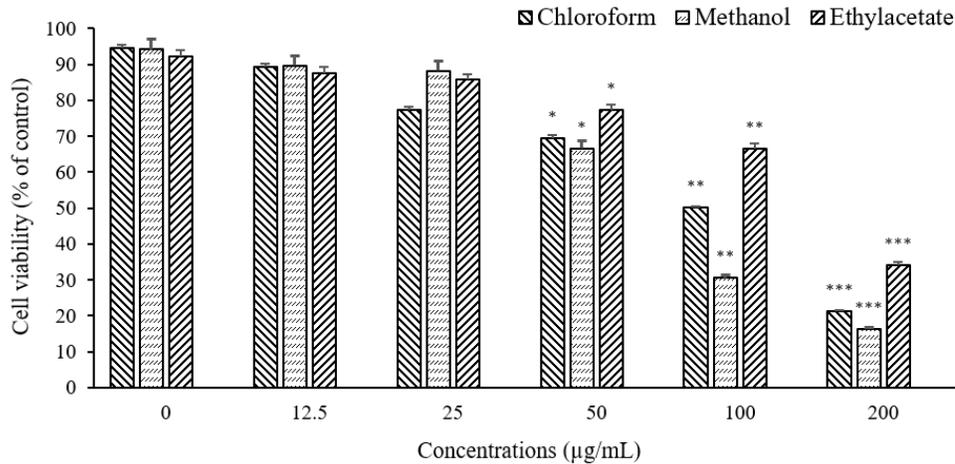


Fig. 3. Cell viability assay of HT29 cells after treatment with *Laurencia synderiae* within 24 h. The data represent as mean \pm SEM, n = 3. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared to the control group (concentration zero).

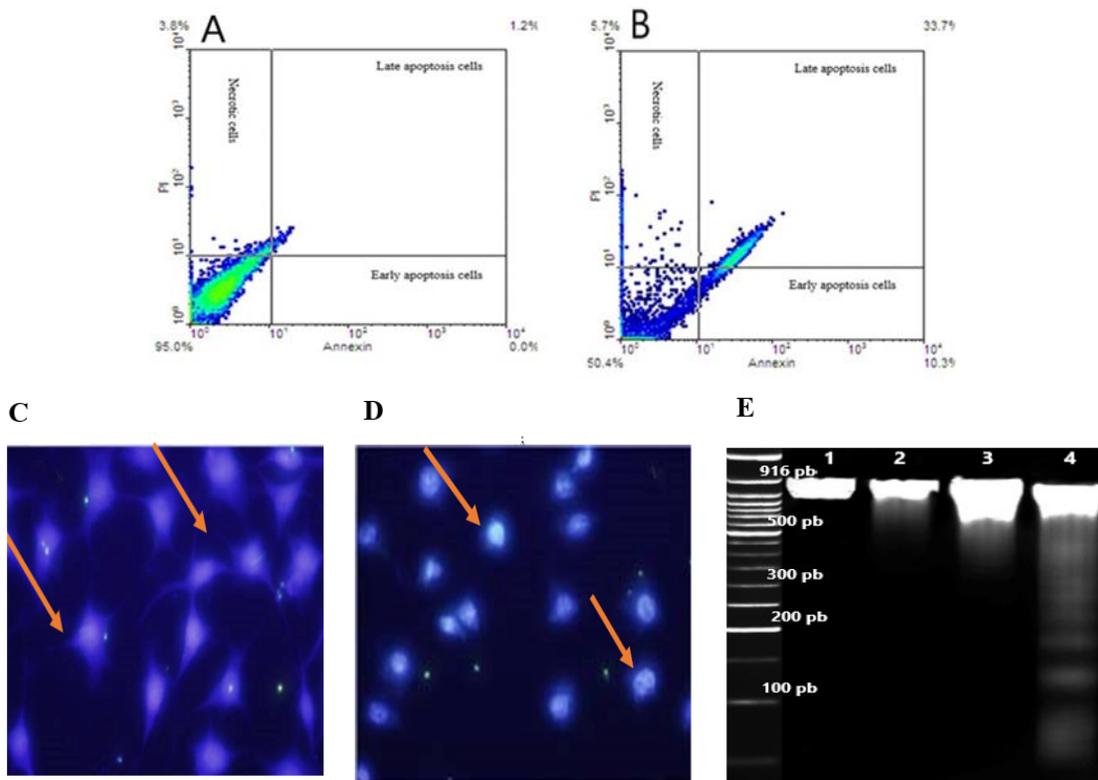


Fig. 4. Flow cytometry charts for evaluating apoptosis induction in HT-29 cells by *Laurencia synderiae* methanolic extract in 24 h. (A) Control cells and (B) IC_{50} concentration of treated cell. Cells were stained with annexin-FITC+ and PI staining index. Morphological changes of (C) untreated cells and (D) treated cells with IC_{50} dose of the methanolic extract of *L. synderiae* using DAPI staining (Magnification 400x). The arrows show nuclear fragmentation and cell shrinkage with are related to the apoptotic mode of cell death. (E) DNA fragmentation of HT-29 cells treated with methanolic extract of *L. synderiae* (50-100 µg/mL) for 24 h observed by agarose gel electrophoresis. 1: Control, 2: HT29 cells treated with 50 µg/mL, 3: HT29 cells treated with 70.2 µg/mL, 4: HT29 cells treated with 100 µg/mL.

The horizontal axis is annexin V-FITC and the vertical axis is annexin-PI. The results showed that viability in the control group was about 95%, which was reduced by 50% in the cells treated with the methanolic extract at 70.2 µg/mL following 24 h. In the treated cells, about 34% had secondary apoptosis, about 10% had primary apoptosis, and approximately 5% had necrotic death (Fig. 4A and B).

The results of DAPI staining demonstrated changes associated with HT29 cell apoptosis treated with the methanolic extract (Fig. 4C and D). The shape of the nuclei was removed from the normal multi-faceted state and rounded. Moreover, nuclear fragmentation and chromatin condensation were observed in treated HT29 cells, which is related to apoptosis in these cells.

Figure 4B presents the results of the DNA fragmentation of colorectal cancer cells with different concentrations of methanolic extract. The results of apoptosis showed that this extract had more effects on DNA fragmentation in comparison with the control sample (extract-free cancer cell). The various concentrations of algae methanolic extract (50, 70.2, and 100 µg/mL) revealed a dose-dependent effect on DNA fragmentation, and the increase in the extract concentration enhanced DNA fragmentation.

DISCUSSION

Biologically active compounds with a variety of therapeutic applications, such as antibiotic, antiviral, antifungal, and anticancer effects, have been identified and characterized from macro algae (16). The major identified compounds with significant biological effect in red algae, *L. snyderiae*, is considered as future drug development. The hexadecanoic acid (19.81%), (E) is the most predominant followed by oleic acid (8.34%), squalene (6.96%), and tetradecanoic acid (6.86%) alcoholic compounds, glycerin (8.62%), and xylitol (8.46%). Hexadecanoic acid has previously been reported to have anti-inflammatory activity (12).

Similarly, the red algae (*Asparagopsis taxiformis*) has shown to contain major

components such as 4,5-dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester, chlorobenzene, 14-methyl-pentadecanoic acid methyl ester, octadec-9-enoic acid, 2,3-dihydroxy-propyl ester, 9-octadecanoic acid, methyl ester, pentadecanoic acid, and octadecanoic acid which might be involved in synergistic bioactivity (11).

Earlier studies have revealed the presence of the following compounds in the methanol extract of *Laurencia obtuse*: alkaloids, carbohydrates, glycosides, physterols, fixed oils and fats, saponins, phenolic compounds, tannins, proteins, amino acids, and flavonoids (9). Geranyl acetate (13.3%), terpinolene (9.2%), hexadecanoic acid (7.7%), linalool oxide (7.4%), and geraniol (6.4%), as the main compounds, were observed in *Laurencia* species. The foregoing extract compositions obtained in *L. snyderiae* were usually used in therapeutic applications (12).

The stable DPPH free radical levels often provide an appropriate estimation of the radical scavenging activity of the extract (16). In the present investigation, the methanolic extract showed a higher DPPH free radical inhibitory effect (61.42 %) compared to chloroform (53.71%) and ethyl acetate (49.63 %).

Previous studies have shown high DPPH radicals scavenging activities in the methanolic extract of red seaweeds *Gracilaria corticata* (44.32 %), *Gracilaria dura* (33.03 %), *Gracilaria debilis* (53.34 %), and *Gracilaria salicornia* (53.43 %) (24). The phenolic compounds, with OH groups in their structures, are able to donate a proton to free radical DPPH, thereby neutralizing it. In this respect, the present study agrees with the previous findings of Chakraborty *et al.* (25). Besides, there is a significant correlation between DPPH free radical scavenging activity and total phenol content. In fact, the change in the polarity of the solvent changes its ability to dissolve the functional groups of antioxidant compounds and affects the antioxidant activity. The multifunctional compounds extracted from algae could be contributed to their different bioactive metabolites which are recorded in the seaweeds and include brominated phenols, brominated oxygen heterocycles, amino acids

and amines, sterols, sulfated polysaccharides, and prostaglandins (25).

The reducing ability of ferrous ions of various extracts increased with the increase in the concentration. However, chloroform extract of *L. synderiae* exhibited significantly higher reducing ability (16.8 %) compared with methanolic extract (15.3%), ethyl acetate extract (13.1%), and the control. A previous study observed the reducing power of methanol extracts from red seaweeds, *Kappaphycus alvarezii* (0.07-0.74%), which is comparatively much lower than the results of the present study (26). The present study is in good agreement with the results were reported in red algae *G. changii* (17.16 %). They proposed that the reducing power of the compounds related to the degree of hydroxylation and presence of conjugation in phenols in red seaweeds. The antioxidant activity of the reducing agent is based on the free radical scavenging activity and donation of a hydrogen atom. In addition, the reducing agents react with certain precursors of peroxide, thereby preventing peroxide formation (25). The phytochemical composition of algae may act as reducing agents, donate ion electrons, and react with free radicals and convert them into stable compounds which ultimately neutralize the free radical chain.

The metal chelating activities prevent the formation of oxyradicals and consequently hazardous oxidation. The metal chelating capacity plays a major role in the antioxidant mechanism by reducing the concentration of metal conversion (27).

The chelating activity of different extracts demonstrated that among various extracts, the ethyl acetate had higher chelation property (58.2%) at 0.01 mg/mL. The present research is in accordance with the previous studies which showed the methanolic and ethyl acetate extracts of red algae, *Hypnea musciformis*, exhibited 31.02% and 38.29 % (25). It was suggested the phenolic compound and its hydroxyl group's position in seaweeds can be potent as metal chelating ability. Besides, the ethyl acetate extract was revealed better chelation activity among various extracts due to the structure-function configuration (28).

The MTT assay was used to measure the cytotoxic effects of various extracts of

L. synderiae. The yellow MTT turns into an insoluble purple formazan by mitochondrial dehydrogenase enzymes of active cells. The present study demonstrated that the least viability of colorectal cancer cells in algae extract was visible in methanol solvent, where the concentration of 200 µg/mL showed the least cell viability. Wang *et al.* designed an experiment to determine the effect of 12 aqueous extracts of algae species from Hong Kong on breast cancer cell lines of MCF-7 and HL-60. The results showed that algae *Hydroclathrus clathratus* and *Padina arborescens* inhibited the growth of cancer cells with the least effect on healthy cells (29). An *in vitro* study on the cytotoxic activity of *Polysiphonia lanosa* red algae against cancer cells of DLD-1 and HTC-116 (human colorectal cancer) reported better performance of chloroform and methanol extracts (8). In this study, more inhibition of cancer cell lines HT29 observed in methanolic extract, followed by chloroform extract of *L. synderae*. A similar work revealed that the anticancer effect of red alga *Porphyra dentata* was attributed to the presence of sterol structure which has the potential to protect cells against the onset of 4T1 cells tumour (30).

Apoptosis is a process that naturally occurs in specific stages of embryonic growth and development to remove the cells with damaged DNA (31). Uncontrolled apoptosis may cause various diseases in humans. For example, excessive apoptosis causes organ shrinkage, dysfunction, and degenerative neurological conditions. The removal of apoptosis may also lead to an increase in the production of cells and a variety of cancers (32). Molecular studies have shown that cell death is not accidental and is regulated by the expression of specific genes. In addition, the cells in this process undergo certain morphological changes including chromatin condensation, crescent nuclei, cytoskeleton and cell membrane deformation, nucleus fragmentation, cytoplasmic condensation, and conversion of cells into one or more apoptotic bodies surrounded and digested by phagocytes (30).

The previous results of the apoptotic effect of red algae, *G. arcuate* methanol extract showed that the percentage of cell death in

samples treated with different concentrations (710.16, 810.16, and 1010.16 $\mu\text{g/mL}$) was not significantly increased compared to the control sample. In other words, the percentage of apoptosis was 0.76% in the control group (extract-free cell sample), and 7.5% at 1010.16 $\mu\text{g/mL}$ concentration (8).

Patra and Moturaman reported anticancer properties and stimulated cell apoptosis by the red alga, *G. edulis* extract (8). They suggested the properties of the extract were investigated against EAT cells in the *in vitro* and *in vivo* conditions. Finally, *Gracilaria* ethanol extract had a significant toxic effect on EAT cells. In their study, the toxicity index of *Gracilaria* extract was employed against EAT cells. The cancer cells produce ROS, reduce intracellular glutathione levels, and induce oxidative stress. The parameters used to induce cell apoptosis were cellular positive annexin-V, increased levels of DNA fragments, and increased activity of caspases 2, 3, and 9 proteins. The EAT-induced rats with *Gracilaria* extract had increased longevity and significantly prevented tumour growth. In the present study, the results showed that methanol extracts of *L. synderiae* at 70.2 $\mu\text{g/mL}$ had more significant apoptosis compared to the untreated cells (control), which could be attributed to the presence of bioactive compounds such as bromophenols, carotene, steroids, and sulfated compounds such as fucoidan in the algae, which play an important inhibitory role against certain human cancer cells.

Another characteristic of apoptosis is the activation of deoxyribonuclease following the activation of caspase. Deoxyribonuclease is an endonuclease that restricts the DNA strand in histone-noncovered areas. When these fragments are electrophoresed on the agarose gel, they are separated from one another and show patterns similar to the DNA marker, which represents apoptosis (33). In the present study, following the genomic DNA fragmentation of human colorectal cancer cells treated with *L. synderiae* methanol extract after 24 h, DNA fragments were observed *via* electrophoresis on agarose gel (Fig. 4B).

The anticancer effects of red seaweed, *Cladophorosis vaucheriaeformis* was evaluated on mouse leukemia 11210 cells (34). The

results showed the inhibitory effect of algae extract on the growth of several tumours such as B16-BL6 melanoma cells and JVG-B and KPL-1 mammary carcinoma. Intraperitoneal injection of the extracts on B16-BL6 lung metastasis to the vein further corroborated the inhibitory effects of algae and was considered as the first anti-metastatic study of this alga. Akbari et al. reported cytotoxic activity of crude extract and fractions (hexane, trichloroethane, chloroform, and butanol) from red seaweed, *Sargassum boveanum* against cervical cancer cell line (HeLa) (35). They observed the chloroform and hexane extracts were shown the highest selectivity toward HeLa cell line.

Harada and Yamashita found the anti-tumor activity of palmitic acid extracted from red algae as a selective cytotoxic agent in the treatment of human leukemia cells; however, this activity was not observed in human skin fibroblasts (36). Palmitic acid at 12.5-50 $\mu\text{g/mL}$ had cytotoxic effects on leukemia cells, resulting in apoptosis in human leukemia cells (MOLT-4) at 50 $\mu\text{g/mL}$, these effects were also observed on rats in subsequent studies. In the present study, the results of DNA fragmentation in colorectal cancer cells under different concentrations of methanolic extract corresponded to the results of apoptosis and showed that methanolic extract had a greater effect on DNA fragmentation compared with the control (extract-free cancer cell line). Since oxidative stress plays an essential role in inflammatory and cancerous reactions, naturally occurring algae products are potential anti-inflammatory and anticancer drugs. Natural algae-derived products protect cells against the effects of oxidative stress by enhancing the natural ability of cells to adapt to their environment.

CONCLUSION

The present study revealed that various extracts (methanolic, chloroform, and ethyl acetate) of red seaweed, *L. synderiae*, had dose-dependent antioxidant properties. Furthermore, among different extracts, methanolic extract, followed by chloroform extract, showed the highest cytotoxic effect against colorectal cancer cells. Besides, the PI staining and

annexin V-PI showed an increasing amount of apoptotic cells in methanolic extract. The presence of numerous bioactive secondary metabolites such as hexadecanoic acid, (E) oleic acid, squalene and tetradecanoic acid in the red algae was further confirmed by GC-MS analyses. This investigation justifies the antioxidant and anticancer potential of red algae *L. synderiae* which can be considered as a valuable phytochemical source.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

The authors have contributed equally to this work and also, have accepted responsibility for the entire content of this submitted manuscript and approved submission

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