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RESEARCH ARTICLE

Anticancer and antioxidant effects of selected Sri Lankan marine algae

H.H. Chaminda Lakmal[1], Kalpa W. Samarakoon[1], WonWoo Lee[1], Ji-Hyeok Lee[1], D.T.U. Abeytunga[2], Hyi-Seung Lee[3*] and You-Jin Jeon[1]

[1] Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea.
[2] Department of Chemistry, Faculty of Science, University of Colombo, Colombo 03.
[3] Marine Natural Products Laboratory, Korea Ocean Research and Development Institute, P.O. Box 29, Ansan 426-744, Republic of Korea.

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Abstract: For the investigation of bioactive components from Sri Lankan seaweeds, three species of red algae (*Chondrophycus ceylanicus*, *Gelidiella acerosa*, *Gracilaria corticata*), two species of green algae (*Chaetomorpha crassa*, *Caulerpa racemosa*) and one species of brown algae (*Sargassum cassifolium*) were evaluated. For the bioactivity evaluations, total phenol content (TPC), free radical scavenging activity using electron spin resonance (ESR) spectroscopy and anticancer activity against different cancer cell lines including a human promyelocytic leukemia (HL-60), a human lung carcinoma (A549) and a mouse melanoma (B16F10) were assessed *in vitro*. Among the extracts, *C. racemosa* showed significantly higher radical scavenging activity against DPPH (34.34 %), alkyl (85.17 %) and hydroxyl radicals (81.16 %), respectively. The MTT assay confirmed that all the extracts were not cytotoxic at 50 and 100 µg/mL concentrations against vero cells *in vitro*. In addition, a significant cancer cell growth inhibitory effect (IC_{50} value 30.17 µg/mL) was observed by *C. racemosa* methanol extract against HL-60 cells and it was the highest anticancer effect compared to the other extracts. Apoptotic body formation in HL-60 cells and the accumulation of DNA in sub-G_1 phase were determined by *C. racemosa* extract in a dose-dependent manner. This study is the first report of Sri Lankan seaweeds with the potential of pharmacological effects including antioxidant and anticancer activity *in vitro.*

Keywords: Anticancer activity, apoptosis, HL-60, *in vitro*, Sri Lankan seaweeds.

INTRODUCTION

At present, there is a great interest on bioactive natural products obtained from marine organisms. The large and structurally diverse array of marine-derived natural resources (Aneiros & Garateix, 2004), can be described as the largest remaining reservoir of secondary metabolites to be evaluated for future therapeutic needs. Marine seaweeds are considered as rich sources of secondary metabolites, which are considered as key ingredients for pharmaceutical applications. Over many years, marine natural products have been used as the lead compounds for drug discovery all over the world (Samarakoon *et al*., 2013).

The Sri Lankan coastal waters are rich in marine flora and the most frequent seaweeds have been identified by Boergesen (1936). In fact, the earliest marine algae collection has been reported in early 19th century by Barton (1903). Baldwin (1991) has reported on 440 taxa of marine algae belonging to 148 genera. More recently, a comprehensive study of marine seaweeds of Sri Lanka has been carried out (Coppejans *et al*., 2009). There has been a tradition of consuming seaweeds from the Puttalam Lagoon area (Jayasuriya, 1987). *Gracilaria* species is a popular food item among fishermen and they believe that it has health promoting effects. Among the Sri Lankan seaweeds, *Saragassum* species (brown algae) are the most abundant type of algae found in the coastal area (Durairatnam, 1961). In addition, *Laminaria, Fucus, Ascophyllum* and *Tubinaria* species are also found in the Sri Lankan waters. Alginic acid has been extracted from seaweeds in 1960/1970 and the cultivation of seaweeds has also been reported (Arumugam *et al*., 1981).

However, bioactivities of the Sri Lankan seaweeds have not been studied extensively and only a few

* Corresponding author (hslee@koist.ac)

reports have been found in the literature. Among them, Premekumara *et al.* (1995) have reported the post-coital contraceptive mechanism of the crude extract of Sri Lankan marine red algae, *Gelidiella acerosa.* As a consequence of the tropical climatic conditions in the seas around Sri Lanka such as extreme salinity levels, temperature variations, low light intensities and nutrient deficient habitats, changes would result in the ecological and physiological properties of the marine seaweeds. Therefore, these algae are a rich source of bioactive secondary metabolites and functional ingredients.

In this study, the main objective was to evaluate the antioxidant and anticancer activities of some selected marine algae from Beruwela, Sri Lanka. In addition, the marine algal species with potent anticancer activity of apoptotic body inducing effect against human promyelocytic leukemia cell line (HL 60) was determined.

METHODS AND MATERIALS

Chemicals and reagents

A monkey kidney cell line (vero), a human promyelocytic leukemia cell line (HL-60), a mouse melanoma cell line (B16F10) and a human lung carcinoma cell line (A549) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Roswell Park Memorial Institute (RPMI-1640) medium, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco/ BRL (Burlington, ON, Canada). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloridetrihydrate (Hoechst 33342) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals and reagents used in these investigations were of analytical grade.

Seaweed collection and preparation of extracts

Fresh marine seaweeds were collected from the sampling sites at Beruwela coral reef in the Southern Province of Sri Lanka. Collected samples were washed well to remove salt, epiphytes and sand. The seaweeds were identified (Figure 1) by one of the co-authors Dr Kalpa W. Samarakoon based on the descriptions on ABC Taxa (Coppejans *et al*., 2009) and the herbarium specimens were kept in the research laboratory, Department of Chemistry, University of Colombo. All the samples were stored at -70 ˚C and air dried at room temperature for 24 h. The frozen and air dried samples were lyophilized and homogenized using a laboratory mechanical blender before extraction. Each homogenized sample was mixed with 80 % methanol and subjected to sonication (ultra-sound assisted extraction) at 25 ˚C for three 90 min periods. The extracts were filtered under vacuum using Whatman No.1 (Whatman Ltd., Maidenstone, England) filter paper and the solvent was evaporated using a rotary evaporator (Fisher Scientific, Loughborough, UK**)**. The collected crude methanolic extracts were used for the *in vitro* bioassays.

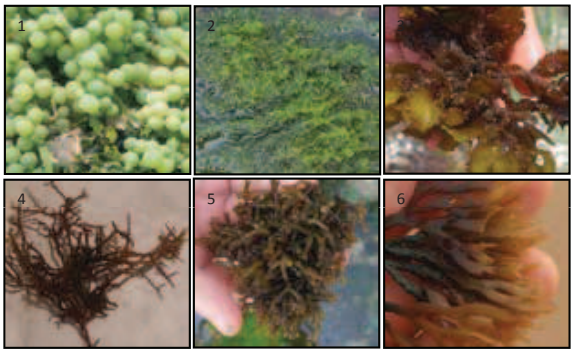

Figure 1: Seaweeds collected from Beruwela coral reef, Sri Lanka (1) *Caulerpa racemosa*; (2) *Chaetomorpha crassai*; (3) *Sargassum crassifolium*; (4) *Gelidiella acerosa*; (5) *Chondrophycus ceylanicus*; (6) *Gracilaria corticata*.

Determination of total phenolic content (TPC)

The total phenolic content of the samples were determined according to Chandler and Dodds (1983). One milliliter of (0.1 mg mL^{-1}) sample was mixed in a test tube containing 1 mL of 95 % ethanol, 5 mL of distilled water and 0.5 mL of 50 % Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min, and then 1 mL of 5 % Na_2CO_3 was added, mixed thoroughly and placed in the dark for 1 h. The absorbance was measured at 725 nm using a UV–vis spectrometer. A gallic acid standard curve was obtained for the calibration of phenolic content.

Cell culture

HL-60 and A549 cells were grown in RPMI-1640 medium. Further more B16F10 and vero cells were cultured in DMEM. Both culture media were supplemented with 1 % antibiotic (100 mL^{-1} penicillin, 100 µg mL^{-1} of streptomycin) and 10 % FBS. The cells were incubated and maintained in an atmosphere of 5 % CO_2 at 37 °C. The cells were sub-cultured every 2 days and exponential phase cells were used throughout the experiments.

Determination of cell viability (MTT assay)

The sample cytotoxicity was evaluated on vero cells and cell growth inhibitory activity of the methanol extracts were assessed by colourimetric MTT assay against cancer cells (HL-60, B16F10, and A549) with different concentrations (50, 100 and 200 µg mL^{-1}). Suspension cells (HL-60 cells) and attached cells (B16F10 and A549) were seeded in a 96-well plate at a concentration of 2×10^4 cells mL^{-1} and incubated for 24 h and treated with samples with different concentrations and further incubated for 24 h before MTT treatment. MTT stock solution (50 µL; 2 mg mL^{-1} in PBS) was added to each well to achieve a total reaction volume of 250 µL. After 3 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO and the amount of purple formazan was assessed by measuring the absorbance at 540 nm.

Nuclear staining with Hoechst 33342

The cell permeable DNA dye, Hoechst 33342 stain was used to examine the nuclear morphology of the cells. Hoechst 33342 is excited by uv light and emits blue fluorescence at 460 − 490 nm. This dye binds preferentially to the adenine-thymine (A-T) regions of the DNA. The stained nuclei were considered as viable. In addition, the presence of DNA fragmentations and chromatin condensations were visualized as apoptosis (Lizard *et al*., 1995). HL-60 cells were placed in 24-well plates at a concentration of 2×10^4 cells mL^{-1}. The cells were then treated with different concentrations (50, 100 and 200 µg mL^{-1}) of *Caulerpa racemosa* methanol extract and incubated for an additional 24 h. Next, Hoechst 33342 dye was added to the culture media at a final concentration of 10 µg mL^{-1} and the plates were incubated for an additional 15 min at 37 ˚C. The stained cells were observed under a fluorescent microscope equipped with a CoolSNAP-Pro colour digital camera in order to determine the degree of nuclear condensation.

Cell cycle analysis

The cell cycle analysis was performed to examine the proportion of apoptotic sub-G_1 hypodiploid cells as described by Nicoletti *et al.* (1991). The HL-60 cells were seeded on 6-well plates at a concentration of 2×10^5 cells mL^{-1} and treated with different concentrations (50, 100 and 200 µg mL^{-1}) of *C. racemosa* methanol extract. The cells were harvested after 24 h, fixed in 1 mL of 70 % ethanol for 30 min at 4 ˚C, washed twice with PBS followed by incubation in the dark in 1 mL of PBS containing 100 µg of propidium iodide (PI) and 100 µg RNase A for 30 min at 37 ˚C. The flow cytometric analysis was performed with a FACS caliber flow cytometer (Becton–Dickinson, SanJose, CA, USA). The effect on the cell cycle was examined by changes in the percentage of cell distribution at each cell cycle phase, and assessed by histograms generated by the Quest and Mod-Fit computer programmes (Wang *et al.*, 1999).

Statistical analysis

All data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed *via* a one-way analysis of variance followed by Duncan's multiple range test (DMRT). Probability values of less than 0.05 ($p < 0.05$) were considered as significant.

RESULTS

Total phenolic contents (TPC) and free radical scavenging activity (%)

The total phenolic content (TPC) of methanol extracts of selected Sri Lankan marine algae were determined by Folin–Ciocalteu method and the free radical scavenging activity (%) using electron spin resonance (ESR) spectroscopy (Table 1). The highest TPC (%) was observed as 5.13 (%) from *Gracillaria corticata* and the lowest TPC was recorded from *Gelidiella acerosa* as 3.64 (%). However, the reported TPC % was not significantly different among the species. Free radical scavenging activities were evaluated by ESR spectroscopic technique using three types of radicals including DPPH, hydroxyl and alkyl radicals. Among all the extracts at 1 $mgmL^{-1}$ concentration, *C. racemosa* showed a significant average radical scavenging activity against DPPH (34.34 %), alkyl (85.17 %) and hydroxyl (81.16 %), respectively. In fact, the best alkyl radical scavenging activity was recorded as 91.36 % from *Chondrophycus ceylanicus*. In general, alkyl and hydroxyl radical scavenging activity (%) of the methanol extracts showed significant differences compared to DPPH radical scavenging activity (Table 1).

Sample cytotoxicity assessment against vero cells

Sample cytotoxicity effect against vero cells was determined by the MTT assay. The methanol extract of marine algae samples were incubated at 50, 100 and 200 µg mL^{-1} concentrations for 24 h with vero cells and the cell viability (%) was determined *in vitro* (Figure 2).

Table 1: Free radical scavenging activity (%) and total phenolic content of Sri Lankan seaweed extracts [a]

Scientific name	Total phenolic content (%)	Free radical scavenging activity (%) [b]		
		DPPH radical	Alkyl radical	Hydroxyl radical
Caulerpa racemosa	3.78 ± 0.10	34.34 ± 0.03*	85.17 ± 0.55*	81.16 ± 0.75*
Chaetomorpha crassa	4.69 ± 0.14	20.12 ± 0.08	36.40 ± 4.00	77.08 ± 0.92
Sargassum crassifolium	3.80 ± 0.12	11.16 ± 1.67	60.56 ± 2.86	76.59 ± 1.56
Gelidiella acerosa	3.64 ± 0.16	6.19 ± 1.53	54.25 ± 0.44	74.95 ± 4.854
Chondrophycus ceylanicus	4.05 ± 0.13	6.39 ± 0.68	91.36 ± 2.26*	80.09 ± 1.36*
Gracilaria corticata	5.13 ± 0.13	18.17 ± 0.90	78.67 ± 0.67*	76.28 ± 2.22

[a] The values were determined by triplicate individual experiments. Values are mean ± SD of three determinations.

[b] Concentration used at 1 mgmL^{-1}

* $p < 0.05$

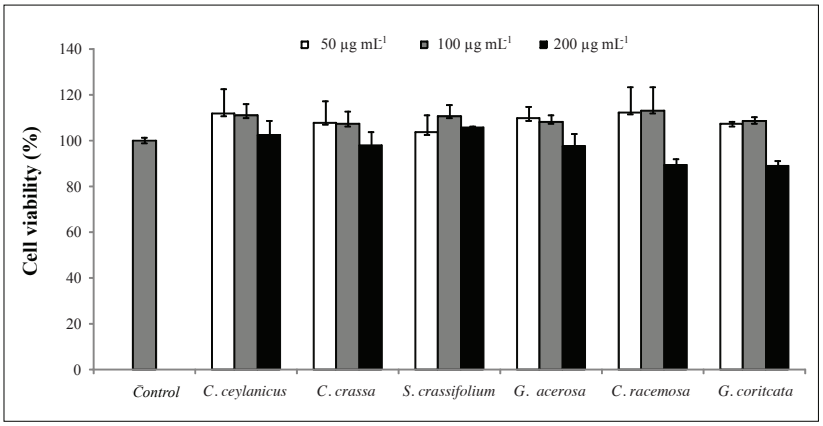

Figure 2: The effect on cell viability (%) of the methanol extracts of selected Sri Lankan seaweeds against vero cells after incubation at 24 hours and evaluated by MTT assay. Values are expressed as means ± SD in triplicate experiments.

Table 2: Cancer cell (HL-60) growth inhibition by methanol extracts from selected Sri Lankan seaweeds

Seaweed samples	*C. racemosa*	*C. crassa*	*S. crassifolium*	*G. acerosa*	*C. ceylanicus*	*G. corticata*
IC_{50} values (µg mL^{-1}) [a]	30.17 ± 0.5*	179.88 ± 1.6	> 200	104.43 ± 0.8	102.17 ± 1.2	> 200

[a] The concentration of samples to 50 % growth inhibition of cancer cell. The values of IC_{50} were determined by triplicate individual experiments.

* $p < 0.05$

All samples at 50 and 100 µg mL^{-1} concentrations showed a high cell viability (%) compared to the controls. Among the samples, *C. ceylanicus, Chaetomorpha crassa, Sargassum cassifolium* and *Gelidiella acerosa* indicated the least cytotoxicity on vero cells at 200 µg mL^{-1} concentration. The methanol extracts of *C. racemosa* and *Gracillaria cortcata* showed significant cytotoxic effects at 200 µg mL^{-1} concentration (Figure 2).

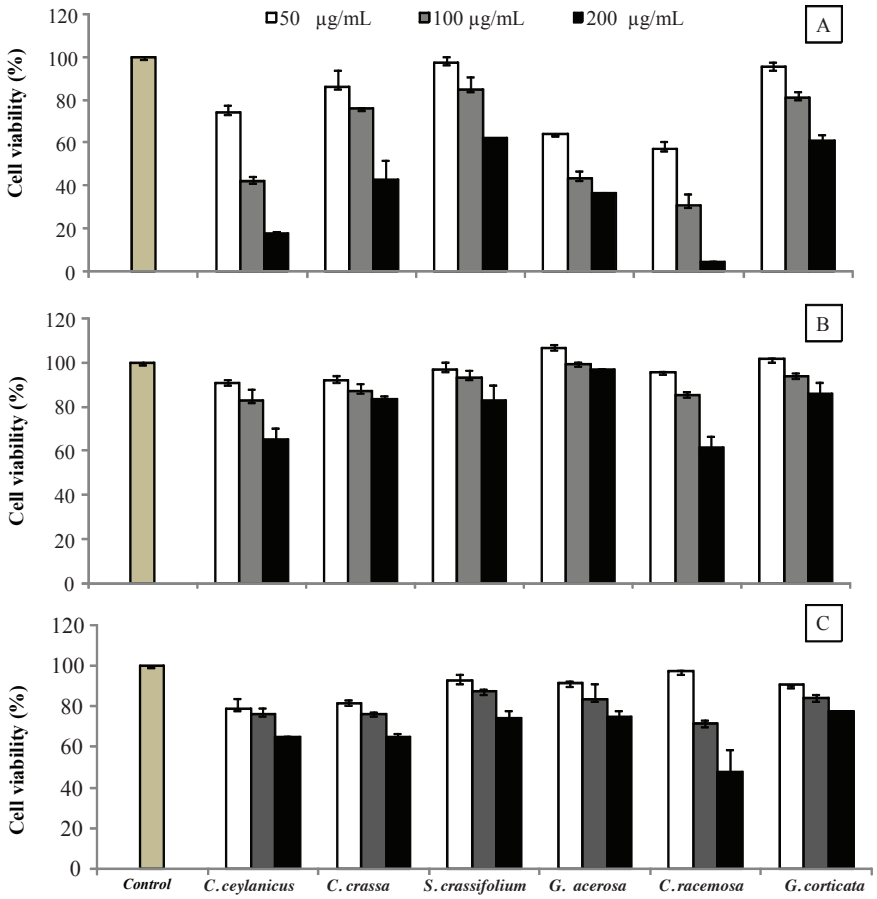

Figure 3: The effect on cell viability (%) of the methanol extracts of collected seaweeds from Sri Lanka against, (A) HL-60; (B) B16F10 and (C) A549 cancer cells for 24 hours. ☐ denotes the control (without sample treatment). Values are expressed as means ± SD in triplicate experiments.

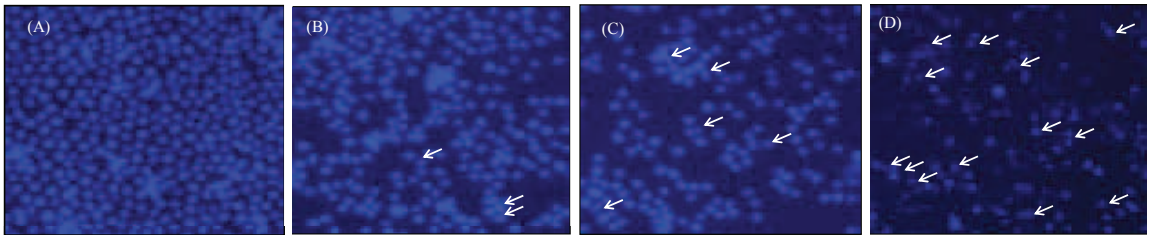

Figure 4: Induction of apoptotic body formation in HL-60 cells was observed under a fluorescent microscope after Hoechest 33342 staining. (A): control (without sample treatment); (B): *Caulerpa racemosa* methanol extract (CRM) treated with 50 µg mL^{-1}; (C): CRM treated with 100 µg mL^{-1}; (D): CRM treated with 200 µg mL^{-1}; Arrows denote a typical apoptotic body formation in HL-60 cells.

Growth inhibitory activity of Sri Lankan seaweed extracts against HL-60, B16F10 and A549 cancer cell lines

The cell viability (%) of the cancer cells HL-60, B16F10 and A549 were determined by MTT assay with incubation of different concentrations (50, 100, 200 µg mL^{-1}) of methanol extract of seaweeds for 24 h. Observed results is shown in Figure 3 (A), (B) and (C), respectively. Among the cancer cells, HL-60 cells were significantly suppressed ($p < 0.05$) with the *C. racemosa* extract and *C. ceylanicus* extract dose-dependently compared to the control. In particular, the highest growth inhibitory activity on HL-60 cells was observed at 200 µg mL^{-1} of *C. racemosa* (Figure 3 A), which was approximately 95 %. The same concentration of *C. racemosa* showed a marked growth inhibitory activity against B16F10 and A459 cancer cells (Figure 3 B and C). The calculated IC_{50} values for the growth inhibitory activity of the cancer cells are listed in Table 2. According to the results, the lowest cancer cell growth inhibitory activity (IC_{50} value 30.17 µg mL^{-1}) was reported in *C. racemosa.* Therefore, the methanol extract of *C. racemosa* was used to determine the mechanism of anticancer activity against HL-60 cancer cells, and the apoptotic body formation and accumulation of DNA content percentage in the sub-G1 phase of the cell cycle of HL-60 were evaluated.

Apoptosis induced anticancer activity

The cell permeable DNA dye Hoechst 33342 staining was used to examine the nuclear morphology of the cells. The methanol extract of *C. racemosa* (CRM) at different concentrations (50 ~ 200 µg mL^{-1}) was incubated with the HL-60 cells for 24 h, and the apoptosis induced nuclear morphology of HL-60 cells stained with Hoechst 33342

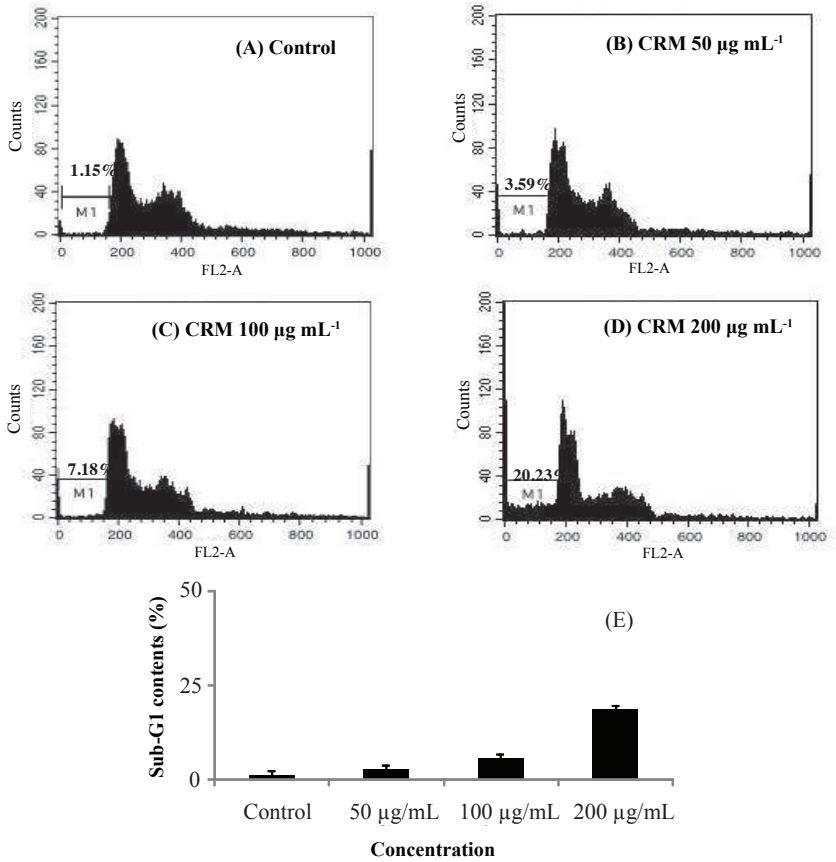

Figure 5: Apoptotic sub-G_1 content was detected by flow cytometry after stained with PI. (A): control (without sample treatment); (B): *Caulerpa racemosa* methanol extract (CRM) treated with 50 µg mL^{-1}; (C): CRM treated with 100 µg mL^{-1}; (D): CRM treated with 200 µg mL^{-1}; (E): Accumulation of DNA content (%) in the sub-G_1 phase of the cell cycle of HL-60 incubated with CRM different concentrations

was visualized by fluorescent microscopy (Figure 4). In this experiment, the control (sample A) showed the intact cell nuclei without DNA damage. However, treatments of CRM at different concentrations showed clear DNA damages and a dramatic increase in the apoptotic body formations [Figures 4 (B), (C) and (D)]. These results were dose-dependently correlated with the HL-60 cell growth inhibition *via* the proportion of apoptotic body formation and the treated CRM concentration.

Induction effect of sub-G_1 contents in HL-60 cells

The inhibitory effects on the proliferation of HL-60 cells were evaluated by determining the sub-G_1 content population (%) with respect to the incubated different concentrations of CRM. According to the results, the cell cycle arrest and the accumulation of cells in the sub-G_1 phase increased in a concentration dependent manner [Figures 5 (B), (C) and (D)]. Moreover, the apoptotic body formations were reflected in the accumulation of sub-G_1 content and further determined as 3.59, 7.18 and 20.13 % of sub-G_1 population against the CRM concentrations 50, 100 and 200 µg mL^{-1}, respectively compared to the control (Figure 4 E). These evidences suggest that CRM induce the cell death due to the apoptotic body formations.

DISCUSSION

Reactive oxygen species (ROS) are the major causative agents for oxidative stress in the human body. Some endogenous antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase protect the internal organs and tissues from oxidative damage (Ahn *et al*., 2004). Hence, antioxidants play an important role in the human body by reducing oxidative reactions. However, imbalances between the endogenous antioxidants and ROS cause serious health issues and disorders such as cancer, cardiovascular disease, hypertension, diabetes mellitus, inflammatory diseases, neurodegenerative diseases and ageing (Valko *et al*., 2007). Among them, cancer is the leading threat for the world population; the first-leading cause of death in economically developed countries and the second-leading cause of death in developing countries (Ezzati *et al*., 2002). Therefore, researchers are continually seeking a good source with potent antioxidant ability and cancer suppressing activity as an alternative for dietary supplements.

Among the marine organisms, marine algae are considered as a rich source of natural antioxidants (Ngo *et al*., 2011). In this study, the methanol extracts of some selected Sri Lankan seaweeds were evaluated to determine their medicinal properties. The most sensitive method for the free radical scavenging activity using electron spin resonance spectroscopy showed that the seaweed extracts have the profound free radical quenching effects. In particular, all the extracts showed the best hydroxyl radical scavenging activity (as higher than 75 % of scavenging effect) at the tested concentrations. The total phenolic content of the extracts did not give the supportive facts that correlate with the determined antioxidant activity. It is believed that a different kind of secondary metabolite could be involved for the antioxidant activity.

Finding cancer cure therapeutics from marine food sources would attract great attention from many scientists. Apoptosis is considered as a programmed cell death and characterized cell changes including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Kroemer *et al*., 1995). Apoptosis provides a conceptual framework to understand cancer genetics and cancer therapy (Lowe & Lin, 2000). In fact, the most cytotoxic anticancer agents induce apoptosis that will compel characteristic cell changes and diminishing cancer cells. In this study, *C. racemosa* was identified as the best species with apoptotic inducing potency against HL-60 cancer cells. Apoptosis is also an important biological mechanism that effectively plays a role in maintaining cellular integrity and homeostasis (Yan *et al.*, 2008). This process regulates cell division and intrinsic suicide or programmed cell death (Chang & Yang, 2000). This confirms that the observed typical nuclei morphology characteristics induced with the nuclear condensation or DNA fragmentation and sub-G_1 DNA accumulations are dose-dependently related to CRM.

In addition, Ratnasooriya *et al*. (1991) have investigated the hypotensive activity of a marine red algae, *Gracilaria* sp. extracts on rats. The same group of researchers has identified the gastro-protective effects of the crude extracts of *Jania* sp. on ethanol induced gastric lesions in rats (Ratnasooriya *et al*., 1990). Moreover in another study, the extracts from macro algae collected from different coastal areas of Northern Sri Lanka have been tested for antibacterial activity and some phytochemicals including alkaloids, saponins, flavonoids, tannins and glycosides have been identified (Jayaseelan *et al*., 2012). A sphingosine derivative isolated from the red algae, *G. acerosa* hexane fraction has shown to have non-steroidal, anti progesterone contragestative activity as reported by Premakumara *et al*. (1996). In addition, human sperm mortality stimulation activity has been reported by a sulfono glycolipid isolated from *G. acerosa* (Premakumara *et al*., 2001). This is the first study to determine the antioxidant and anticancer

activity of Sri Lankan marine algae. The results of the study confirms the anticancer and antioxidant effects of the Sri Lankan marine algae. Furthermore, in order to isolate the bioactive natural products, further sequential chromatography and spectroscopy techniques can be employed.

In this study, the potent pharmacological properties of some seaweeds from Sri Lanka were evaluated and confirmed. In conclusion, all the extracts showed potential antioxidant effect with the radical scavenging activity tested by ESR spectroscopy. The determined cell viability was significantly higher and no cytotoxicity was observed against vero cells *in vitro*. Furthermore, apoptotic induced anticancer activity was observed with the *C. racemosa* extract against HL-60 cells.

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