Research Article

Comparative evaluation of antioxidant and anti-inflammatory activities of four seaweed species from the east coast of the Gulf of Thailand

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Abstract

Seaweeds are good sources of bioactive secondary metabolites, with utilizations in medicine and the food industry. However, information on the biological activities of seaweed harvested from the east coast of the Gulf of Thailand are limited. We, therefore, conducted a comparatively study on antioxidant and anti-inflammatory activities of four seaweed species (*Dictyota cervicornis*, *Sargassum polycystum*, *Padina australis* and *Caulerpa lentillifera*) collected from Sattahip District, Chonburi Province. *In vitro* antioxidant screening was performed based on DPPH radical scavenging and metal ion chelating activities. Cell-based antioxidant activities were evaluated based on the formation of reactive oxygen species (ROS) by dichlorofluorescein (DCF) assay. Anti-inflammatory activity was determined by assessing suppression of nitric oxide (NO) production in LPS-induced macrophages. Among all seaweed species examined, *P. australis* was the most active as a DPPH scavenger, whereas *P. australis* and *D. cervicornis* showed the highest metal chelating activity. Furthermore, *D. cervicornis* showed the greatest inhibitory activity on ROS and NO production in LPS-stimulated macrophages. Phytochemical screening tests demonstrated the presence of steroids in all four seaweeds. Tannins were found in *P. australis* and *D. cervicornis* and terpenoids in *D. cervicornis*. These results suggest all four seaweeds, especially *D. cervicornis*, are natural sources of antioxidants and anti-inflammatory agents with potential applications in the food and medical industries.

Keywords: Macrophage; Nitric oxide; Phytochemicals; ROS; Seaweed

Introduction

Oxidative stress is an imbalance of antioxidants and reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These play essential roles in the enhancement of cellular signaling pathways of the immune system and regulation of physiological functions. [1] A principal source of ROS production is the mitochondrial electron transport chain. At normal levels, RNS such as nitric oxide (NO) participate in the regulation of physiological processes. [2] Human chronic diseases have one cause from an excess of ROS or RNS intracellular levels that damage to macromolecules. [3] Therefore, inhibiting production of reactive species or increasing use of antioxidant compounds are promising approaches to prevent or treat inflammatory diseases. Currently, there is a trend toward natural food supplements for improved human health. Seaweed is one such source of bioactive secondary metabolites with antioxidant, [4] anti-cancer, [5] antidiabetic, [6] antimicrobial, [7] and anti-inflammatory properties and is of interest to food and pharmaceutical industries. [8, 9, 10] However, there is a limited literature available reporting the biological activity of seaweeds found in Thailand. Thai seaweed species such as *Sargassum binderi*, *Turbinaria conoides*, *Padina minor*, *Caulerpa lentillifera*, *S. oligocystum*, *S. polycystum* and *C. racemose* exhibit antioxidant activities. [11-13] *P. minor* shows anti-inflammatory activities. [12] *S. oligocystum* and *P. australis* exert antibacterial activities and cytotoxic effects against cancer cell lines. [14, 15] Thus, the aim of this research was to comparatively study on antioxidant and anti- inflammatory activities of ethanol extracts from three species of brown seaweed (*Dictyota cervicornis*, *S. polycystum* and *P. australis*) and one green seaweed (*C. lentillifera*) harvested from the eastern coast of the Gulf of Thailand as potential therapeutic agents or food supplements for the preventing or treating oxidative stress - and inflammation-related diseases.

Materials and Methods

Materials

The chemicals 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 3 - (4, 5 - dimethylthiazol -2 - yl) - 2, 5 - diphenyltetrazolium bromide (MTT), 2', 7' dichlorofluorescin diacetate (H_2DCFDA), ferrozine, lipopolysaccharide (LPS; *Escherichia coli* serotype O111: B4) and aminoguanidine bicarbonate were bought from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid was obtained from Merck (Kenilworth, NJ, USA).

Preparation of ethanol extracts of four seaweed species

Seaweed species were collected between September 2016 and November 2016 from Sattahip District, Chonburi Province, located in eastern Thailand. They were identified by Dr. Jariyavadee Suriyaphan and voucher specimens (S1-59 *Caulerpa lentillifera*, S2-59 *Padina australis*, S4-59 *Dictyota cerviscornis*, S3-59 *Sargussum polycystum*) were maintained at Faculty of Science, Burapha University. Epiphytes were removed from *S. polycystum* and *D. cervicornis* and limestone deposits were removed from *P. australis*. Samples were dried at 50°C and then pulverized. The powdered samples were soaked in 95% ethanol at a ratio of 1:10 (w/v) for five days with shaking twice a day and then filtered through Whatman No.1 filter paper. The seaweed residues were extracted twice. The filtrates for each species were pooled, subjected to evaporation using a rotary evaporator with a vacuum pump until they dried and weighed before storage at -20°C.

DPPH radical scavenging activity

DPPH radical scavenging assay was carried out as described by Srisook *et al*. [16] Briefly, 50 µL of seaweed extracts (0.125-50 mg/mL) were mixed with 100 µL of 0.2 mM DPPH solution and incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm by a microplate reader. Gallic acid was used as the positive control. The scavenging effects (%) were calculated from the absorbance measurements using the following equation:

$$\text{Scavenging effects (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

The effective concentration at which 50% scavenging (EC_{50}) was calculated by equation of linear regression from the calibration curve between extract concentrations and % scavenging effects.

Metal chelating activity

The method used herein to assess a compound's ability to chelate ferrous chloride was described by Srisook *et al*. [16] Two hundred microliters of the seaweed extract solutions (0.25-8 mg/mL) were mixed with 10 µL of 2 mM ferrous chloride solution. Ferrozine solution (20 µL at 5 mM) was then added to the mixture to initiate the reaction. The solution was mixed and incubated for 5 min at room temperature before the absorbance was measured at 562 nm. The chelating effects (%) were calculated from the absorbance measurements using the following equation:

$$\text{Chelating effects (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Cell culture

The murine macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD, USA). Cells were cultured in DMEM containing 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin and were incubated at 37°C in 5% CO_2 atmosphere.

Detection of intracellular ROS by cell-based DCF assay

H_2DCFDA can permeate into the cell membrane and is hydrolyzed intracellularly to 2',7'-dichlorofluorescein (DCFH) carboxylate anion. DCFH was oxidized by ROS forming dichlorofluorescein (DCF). Effect of extracts on ROS production was evaluated as described by Tongyen *et al*. [17] Briefly, cells were pre-treated with seaweed extracts at different four concentrations (6.25-50 µg/mL for *D. cervicornis*, 12.5-100 µg/mL for *S. polycystum*, and 25-200 µg/mL for *C. lentillifera* and *P. australis*) for 1 h before treating with LPS for 12 h. Then, cells were incubated with H_2DCFDA (50 µM) for 30 min and collected by scraping in PBS on ice. The fluorescent intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 521 nm using a fluorescent spectrophotometer (Cary Eclipse, Agilent, USA). Results were expressed as percentages of ROS production calculated via the equation shown below:

$$\text{(\%) ROS production} = (\text{Fluorescent intensity}_{sample} / \text{Fluorescent intensity}_{control}) \times 100$$

Measurement of NO production by Griess reaction

An indirect determination method to determine nitrite, a stable oxidation product of NO, which is determined by the Griess reaction. [18] Macrophages were treated with the seaweed extracts at the same range of concentration as mentioned in cell- based DCF assay before incubation with LPS (1 µg/mL) for 24 h. Thereafter, equal amount of the culture supernatant was mixed with the Griess reagent. The absorbance was measured at 546 nm using a microplate reader. Nitrite concentrations were calculated from a standard curve made from sodium nitrite (0 – 50 µM). The percentage of NO production was calculated using the equation shown below:

$$\text{Production of NO (\%)} = (\text{Nitrite}_{\text{sample}} / \text{Nitritec}_{\text{ontrol}}) \times 100$$

Measurement of cell viability by MTT assay

MTT assay was performed as described by Srisook *et al*. [18] RAW 264.7 cells were treated with DMEM containing the seaweed extracts at various concentrations in the presence of 1 µg/mL LPS for 24 h. Then, the medium was removed, 0.1 mg/mL MTT solution was added to each well and cells were incubated further for 2 h. Insoluble formazan products in viable cells were dissolved in DMSO. Absorbance of MTT formazan was taken measurement at 550 nm using a microplate reader. The result was expressed as the percentage of cell viability calculated using equation below:

$$\text{Cell viability (\%)} = (A_{\text{sample}} / A_{\text{control}}) \times 100$$

Phytochemical screening of seaweed extracts

Dry extracts were dissolved as 1% solutions in their respective solvents and subjected to phytochemical screening by standard methods. [19-22]

Statistical analysis

All results are shown as means ± SD of at least three independent experiments and analyzed for statistical significance by ANOVA with Tukey test for multiple comparisons. *P* values of <0.05 was considered to be statistically significant.

Results

***In vitro* antioxidant activity of seaweed extracts**

DPPH is a stable radical at room temperature which accepts electron and changes from purple to yellow color of non-radical. It is widely used to evaluate antioxidant activity of active compounds. [23] All seaweed extracts scavenged DPPH radicals in a concentration- dependent manner. *P. australis* showed the most potent antioxidant activity by DPPH radical scavenging, followed by *S. polycystum*, *D. cervicornis* and *C. lentillifera* **(Table 1)**. Gallic acid was used as the positive control, which exhibited an EC_{50} of 8.23 ± 0.15 µg/mL. Since ROS are generated as intermediates of metal-catalyzed oxidation reaction [24], we, therefore, determined metal chelating activity assay. The Fe^{2+} chelating abilities of each seaweed examined herein are presented in **Table 1**. *D. cervicornis* and *P. australis* extracts exhibited the strongest metal chelating activity. EDTA was used as a standard metal chelator.

Table 1 The DPPH radical scavenging and ferrous ion chelating activities of seaweed extracts.

Seaweed extracts	EC50 of DPPH radical scavenging activity (mg/mL)	EC50 of ferrous ion chelating activity (mg/mL)
S. polycystum	5.29 ± 0.29[b]	>8.00
D. cervicornis	6.40 ± 0.16[b]	2.24 ± 0.18[b]
C. lentillifera	22.45 ± 0.90[a]	8.57 ± 0.04[a]
P. australis	0.55 ± 0.02[c]	2.35 ± 0.21[b]
Gallic acid	8.23 ± 0.15 (µg/mL)	
EDTA		41.74 ± 0.81 (µg/mL)

Note: Values marked with different superscript lower-case letters (a, b, c) indicate those in the same column differ significantly ($p < 0.05$) from values for the other seaweeds.

Effect of four seaweed extracts on cell viability

To investigate the cytotoxicity of LPS and all four seaweed extracts, RAW 264.7 cells were incubated with LPS in the presence of seaweed extracts for 24 h and cell viability was measured using MTT assay. As shown in **Figure 1**, the extract at tested concentration did not significantly reduce cell viability of LPS-treated macrophages compared to unstimulated control cells. Thus, the non-toxic concentrations of each seaweed extract were used in the subsequent cell-based assays to exclude the effect of cytotoxicity.

Inhibitory effect of four seaweed extracts on intracellular ROS levels

The H_2DCFDA probe was used to determine the intracellular ROS production due to oxidative stress in LPS-activated macrophages. We determined intracellular ROS scavenging activity of four seaweed extracts at different concentrations to obtain an IC_{50} value of each extract. The accurate IC_{50} value was obtained when there was also at least one response less than the 50% response and one response greater than the 50% response. [25] During incubation with LPS, ROS generation in macrophages dramatically increased **(Figure 2)**. All four seaweed extracts significantly inhibited the production of ROS in a concentration-dependent manner. *D. cervicornis* exerted the most potent inhibitory effect, with concentrations at which 50% inhibition was reached, an IC_{50} value of 36.72 ± 1.53 µg/mL, followed by *S. polycystum*, *P. australis* and *C. lentillifera* with IC_{50} values of 71.75 ± 5.08, 211.38 ± 6.68 and 317.85 ± 6.81 µg/mL, respectively. The positive control used is gallic acid.

Anti-inflammatory effects of seaweed extracts

LPS treatment increased NO production in macrophages compared with the control unstimulated group. All four seaweed extracts exhibited inhibitory activity on LPS- induced NO production in a concentration- dependent manner **(Figure 3)** . *D. cervicornis* extract displayed the most potent inhibitory effect with an IC_{50} value of 19.97 ± 1.48 µg/mL, while *S. polycystum*, *P. australis* and *C. lentillifera* gave IC_{50} values of 38.23 ± 0.44, 65.74 ± 6.57 and 117.54 ± 7.94 µg/mL, respectively.

Phytochemical screening of seaweed extracts

Phytochemical screening for alkaloids, anthaquinones, flavonoids, coumarin, terpenoids, steroids, tannins and saponins in the extracts was performed based on color and sediment formation **(Table 2)**. Steroids were found in all four extracts, whereas terpenoids were detected only in the *D. cervicornis* extract. Tannins were also found in *P. australis* and *D. cervicornis*.

Table 2. Phytochemical screening of ethanol seaweed extracts

Phytochemicals	Seaweed extracts			
	S. polycystum	***D. cervicornis***	***C. lentillifera***	***P. australis***
Alkaloids	-	-	-	-
Anthraquinone	-	-	-	-
Flavonoid	-	-	-	-
Coumarin	-	-	-	-
Terpenoids	-	+	-	-
Steroid	+	+	+	+
Tannin	-	+	-	+
Saponin	-	-	-	-

Note: - shows as negative test results, + shows as positive test results

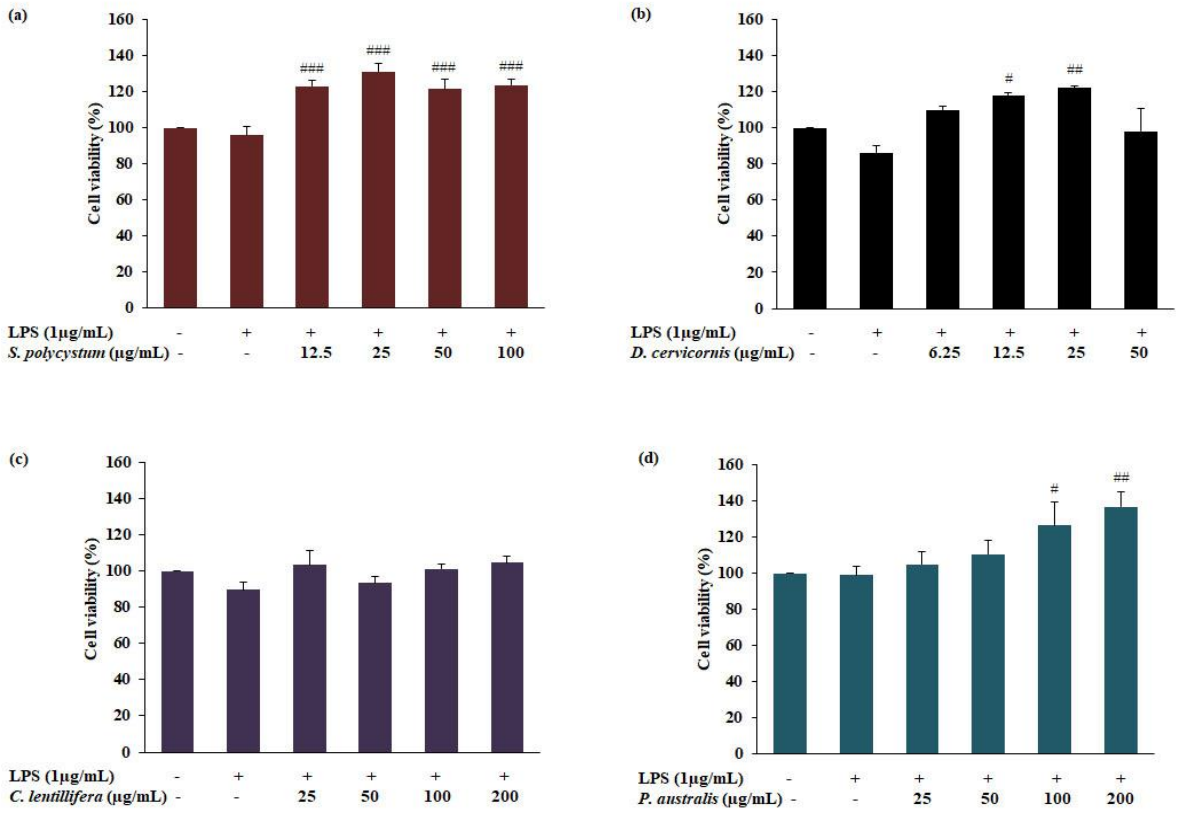

Figure 1 Cell viability of LPS-stimulated cells treated with different seaweed extracts, determined by MTT assay: (a) *S. polycystum*, (b) *D. cervicornis*, (c) *C. lentillifera* and (d) *P. australis*. Cells were pre-treated with indicated concentrations of the extracts for 1 h before LPS stimulation for 24 h. Each column shows the mean ± SD of three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ significant difference from unstimulated cells.

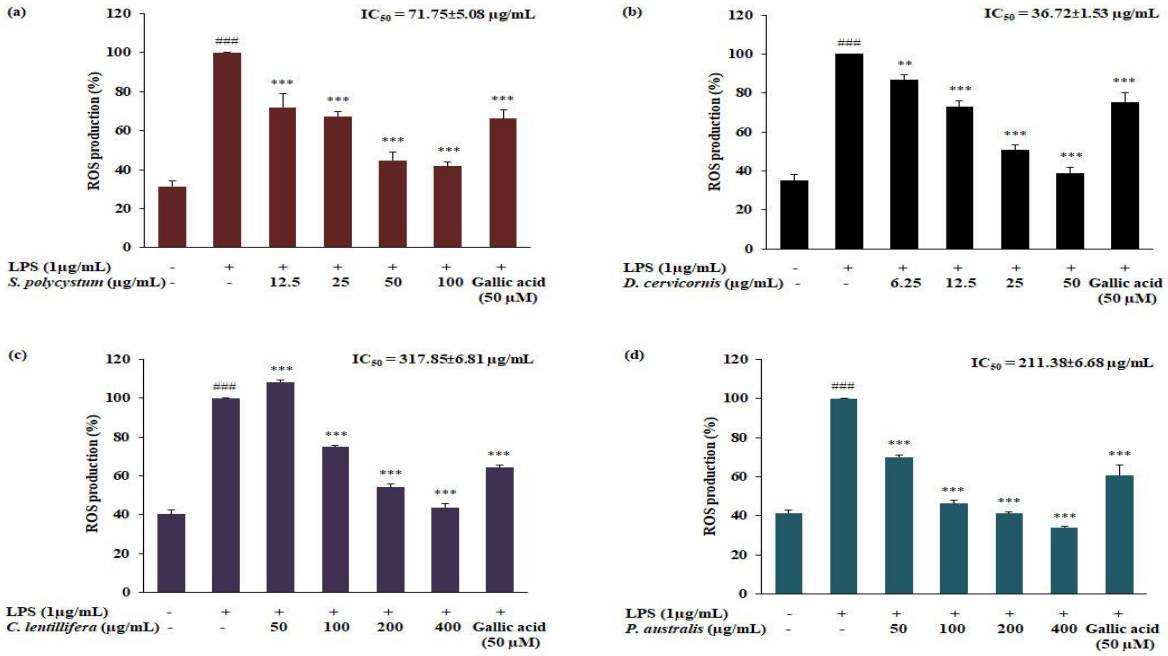

Figure 2 Inhibition of ROS production by seaweed extracts in LPS- stimulated RAW 264. 7 cells: (a) *S. polycystum*, (b) *D. cervicornis*, (c) *C. lentillifera* and (d) *P. australis*. Cells were pre-treated with the different concentrations of the extracts for 1 h before LPS stimulation for 12 h. Each column shows the mean ± SD values of three independent experiments. $^{###}p < 0.001$, significant difference from unstimulated cells. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with LPS alone.

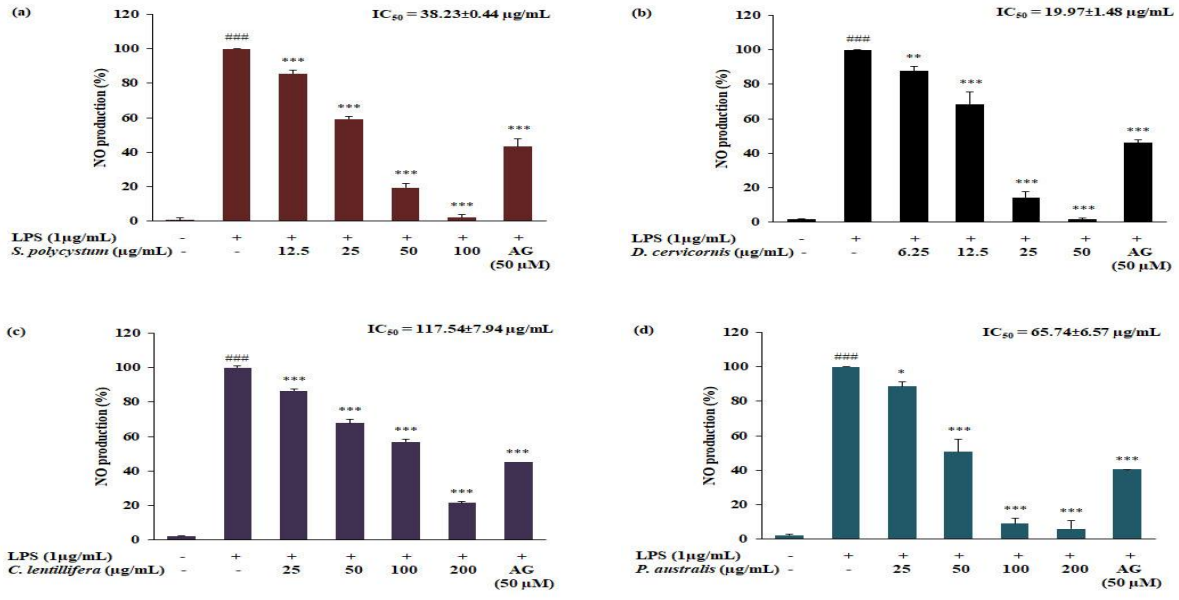

Figure 3 Inhibition of NO production by seaweed extracts in LPS- stimulated cells: (a) *S. polycystum*, (b) *D. cervicornis*, (c) *C. lentillifera* and (d) *P. australis*. Cells were pre-treated with different concentrations of the extracts for 1 h before LPS stimulation for 24 h. Each column shows the mean ± SD values of three independent experiments. $^{###}p < 0.001$, significant difference from unstimulated macrophages. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with LPS alone

Discussion

In this study, we demonstrated antioxidant and anti-inflammatory activities of ethanol extract from four seaweeds, brown seaweed (*D. cervicornis*, *S. polycystum* and *P. australis*) and one green seaweed (*C. lentillifera*) harvested from the eastern coast of the Gulf of Thailand. An *in vitro* antioxidant activity was determined using DPPH radical scavenging and metal ion chelating activities. The DPPH radical scavenging assay is based on the electron transfer between the antioxidant and stable DPPH radicals. [23] Among all seaweeds used in the present study, *P. australis* was the most active as DPPH scavenger. Nursid *et al.* [26] also found that *P. australis* collected from Indonesia showed higher DPPH radical scavenging activity than *S. polycystum*. In addition, the results of our study are in accord with those reported by Farvin *et al.* [4], Sumintilee *et al*., [13] and Cox *et al.* [27] which indicated green seaweeds have a lower DPPH radical scavenging ability than that of brown seaweeds. Initiation and acceleration of oxidation rate in biological systems are induced by excess amounts of transition metals which catalyzed the oxidation of hydrogen peroxide to highly toxic hydroxyl radicals through the Fenton reaction. [24] Therefore, metal chelation is essential because it reduces metal ion bioavailability, decreasing metal - catalyzed macromolecule oxidation. Our results demonstrated *P. australis* and *D. cervicornis* showed the greatest metal chelating activity and brown seaweeds were better than green seaweed in accord with the earlier observations of Farvin *et al.* [4] Further, metal chelation by brown seaweeds from the Arabian Gulf at Kuwait was reported as superior to that by green seaweeds. [4] However, in contrast to our data, Sumintilee *et al.*, [13] and Lima *et al.*, [28] found the amounts of metal chelator in the green seaweeds higher than that in brown seaweeds.

ROS in excess amounts causes cellular damage by oxidizing biomolecules associated with the onset of various chronic diseases in humans. [3] Because activated macrophages produce ROS to eliminate invading pathogens, the effect of seaweed extracts on intracellular ROS production was determined. The ROS inhibitory effect of tested species was as *D. cervicornis* > *S. polycystum* > *P. australis* > *C. lentillifera*. Trends from our ROS inhibition assays conform to data reported by Farvin *et al.* [4] and Yangthong *et al*. [11] in that brown seaweeds had higher antioxidant activities *in vitro* and in cell- based assays than green seaweeds. Collectively, these data suggest that *D. cervicornis* is the most promising seaweed species out of the four, with a good *in vitro* and cell-based antioxidant activity.

NO is a toxic molecule produced by macrophages that enhances their ability to kill ingested microorganisms. However, overproduction of iNOS- catalyzed NO may lead to a variety of inflammatory diseases. [29] Therefore, NO inhibition might be an alternative strategy for disease prevention. Data shown in **Figure 3** indicated that the four seaweed species examined in the present study exhibited anti-inflammatory effect by suppressing NO production. *D. cervicornis* extract was the most potent in inhibitory effect on NO production. Our data are conformed to those reported by Monsur *et al*. [8] and Shanura-Fernando *et al.*, [9] who demonstrated the NO inhibitory effect of *P. australis* and *S. polycystum* in LPS-activated RAW 264.7 macrophages. Moreover, none of the extracts at any tested concentration decreased cell viability of LPS- treated macrophages. Cell viability data indicated reduction

in ROS and NO levels was a result of the extracts and not a cytotoxic effect.

From phytoconstituent analysis, terpenoids, a large group of secondary metabolites, were detected in the *D. cervicornis* specie. As in our study, diterpenes were reported to be abundant in Brazilian *D. cervicornis* [30] Tannins were also found in *P. australis* and *D. cervicornis*. Phlorotannins are a group of tannin compounds that have been found in brown seaweeds [10, 31, 32] and have been reported to exert antioxidant and anti-inflammatory activities. It is likely that the antioxidant and anti- inflammatory effects of *D. cervicornis* are a result of the presence of phlorotannins. However, this was a preliminary analysis of the phytochemicals present in the seaweed extracts. Thus, further investigation should be carried out to isolate the phytochemicals responsible for the antioxidant and anti-inflammatory effects of the selected seaweed extract

In conclusion, this study demonstrated the antioxidant and anti- inflammatory activities of ethanol extracts of brown seaweeds (*S. polycystum*, *D. cervicornis* and *P. australis*) and a green seaweed (*C. lentillifera*) from the eastern coast of the Gulf of Thailand. *D. cervicornis* and *P. australis* extracts demonstrated the potent *in vitro* antioxidant. Furthermore, *D. cervicornis* exerted the most potent inhibitory effects on the production of ROS and NO on LPS- induced macrophage cells. Thus, of the four seaweeds, *D. cervicornis* from Chonburi Province, Thailand, shows the most promise as a natural source of antioxidant and anti-inflammatory agents for therapeutic use or as a dietary supplement.

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