Aqueous Extracts of the Edible *Gracilaria tenuistipitata* are Protective Against H$_2$O$_2$-Induced DNA Damage, Growth Inhibition, and Cell Cycle Arrest

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**Abstract:** Potential antioxidant properties of an aqueous extract of the edible red seaweed *Gracilaria tenuistipitata* (AEGT) against oxidative DNA damage were evaluated. The AEGT revealed several antioxidant molecules, including phenolics, flavonoids and ascorbic acid. In a cell-free assay, the extract exhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity that significantly reduced H$_2$O$_2$-induced plasmid DNA breaks in a dose-response manner ($P < 0.001$). The AEGT also suppressed H$_2$O$_2$-induced oxidative DNA damage in H1299 cells by reducing the percentage of damaged DNA in a dose-response manner ($P < 0.001$) as measured by a modified alkaline comet-nuclear extract (comet-NE) assay. The MTT assay results showed that AEGT confers significant protection against H$_2$O$_2$-induced cytotoxicity and that AEGT itself is not cytotoxic ($P < 0.001$). Moreover, H$_2$O$_2$-induced cell cycle G2/M arrest was significantly released when cells were co-treated with different concentrations of AEGT ($P < 0.001$). Taken...
together, these findings suggest that edible red algae *Gracilaria* water extract can prevent H$_2$O$_2$-induced oxidative DNA damage and its related cellular responses.

**Keywords:** DNA damage; antioxidant; comet assay; red algae; cell cycle arrest

1. Introduction

The red algal genus *Gracilaria* is distributed worldwide and is the main source of large scale production of food grade agar and phycocolloids [1]. The global use and the importance of *Gracilaria* are well documented [2]. Currently, Taiwan is one of the few countries that produces *Gracilaria*. Because Taiwan can produce more than 30,000 tons of *Gracilaria* annually, this inexpensive algae is now an important aquaculture species [1]. In Taiwan, *Gracilaria* species have been cultivated since 1961 [3]. The major *Gracilaria* species produced by open sea cultivation are *Gracilaria tenuistipitata*, *Gracilaria coforvoides*, *Gracilaria gigas*, *Gracilaria chorda* and *Gracilaria compressa*.

To date, algae represent about 9% of marine biomedical compounds [4]. Moreover, seaweeds are well known sources of bioactive primary and secondary metabolites [5]. Many *Gracilaria* seaweeds, such as *G. gigas* [6], *G. dura* [7] and others [8], contain abundant amino acids, fatty acids, vitamins, minerals, polyphenolic compounds and carbohydrates.

Bioactivities of marine algae of the genus *Gracilaria* have been extensively studied [8]. The many health-promoting properties of genus *Gracilaria* seaweed extracts include the anti-hypercholesterolemic properties of *G. tenuistipitata* [9], the antioxidative properties of *G. tenuistipitata* [10], *G. edulis* [11], *G. salicornia* [12], *G. birdiae* and *G. cornea* [13], the anti-inflammatory properties of *G. verrucosa* [14] and *G. cornea* [15], and the antimicrobial properties of *G. salicornia* [12] and *G. tenuistipitata* [16]. Of these, the antioxidant properties may be helpful for modulating the effects of reactive oxygen species (ROS) generated by cellular metabolism or environmental factors. However, the potential protective effects of *Gracilaria* against ROS are seldom addressed.

The ROS induce formation of 8-oxoguanine, the accumulation of which can induce base pairing mismatch, protein miscoding, DNA mutation and even further genome instability [17]. Excessive free radicals are known to inflict cellular injuries such as gene dysregulation, protein function alteration, lipid oxidation, DNA damage and mutation, and cell growth retardation [18]. Accordingly, reduction of its ROS content and attenuation of its associated effects are important research issues.

This study hypothesized that *Gracilaria* extract has the potential to modulate H$_2$O$_2$-induced DNA damage and its related cellular responses. The hypothesis was tested by using hydrogen peroxide (H$_2$O$_2$) as a DNA damaging agent [19] to examine the antioxidative properties of aqueous extracts of *G. tenuistipitata* (AEGT) and its role in recovery from plasmid and cellular DNA damage, cytotoxicity, and cell cycle arrest.
2. Results

2.1. Polyphenols, Flavonoids, and Ascorbic Acid Contents of AEGT and Its DPPH Radical Scavenging Activity

The amounts of total polyphenol, flavonoid, and ascorbic acid were recorded as 98.94 ± 2.43 μg gallic acid equivalent, 22.59 ± 1.08 μg quercetin equivalent and 1.59 ± 0.18 μg ascorbic acid/mg dry extract, respectively. The DPPH scavenging activity of 4 mg/mL AEGT exceeded 60% (63.37 ± 0.91%), which was significantly higher than those of 10 ppm BHA and 100 ppm ascorbic acid (P < 0.0001) (Figure 1a). Moreover, AEGT significantly (P < 0.05) increased DPPH scavenging activity in a dose-dependent manner (Figure 1b).

Figure 1. DPPH radical scavenging activity of AEGT. (a) Comparison of DPPH radical scavenging in AEGT (4 mg/mL), positive control BHA (10 ppm) and positive control Vit. C (100 ppm). (b) DPPH radical scavenging activity of AEGT at different concentrations (1–4 mg/mL). Data are means ± S.D. (n = 3). Levels not connected by same big letter significantly differed. With the exception of A vs. D (P < 0.05), all comparisons revealed significant differences (P < 0.0001).

2.2. AEGT Modulates H₂O₂-Induced Plasmid DNA Strand Breaks

This study assessed the protective effect of AEGT against pBR322 plasmid DNA cleavage induced by H₂O₂ treatment. In the absence of H₂O₂, plasmid DNA appeared mainly in supercoiled form (S) (Figure 2a, control). Adding FeSO₄ and H₂O₂ converted the supercoiled form to the relaxed open-circular (OC) and linear forms (L) (Figure 2a, lane 2). However, 1 to 4 mg/mL AEGT retained the supercoiled form of pBR322 in a dose-dependent manner (Figure 2a, lanes 3–5). The relative percentages of supercoiled plasmid DNA decreased to 25.2 ± 4.3 after H₂O₂ treatment and recovered to 54.6 ± 4.1, 63.7 ± 8.3, and 82.9 ± 2.5 in the presence of 1, 2, and 4 mg/mL AEGT, respectively (P < 0.02 to 0.001 between H₂O₂ alone and 1 to 4 mg/mL AEGT treatment) (Figure 2b). These results indicate that AEGT stabilizes DNA molecules by neutralizing oxidative DNA damage by H₂O₂.
**Figure 2.** Modulating effect of AEGT on H$_2$O$_2$-induced plasmid conformational change. (a) Gel view of plasmid DNA cleavage assay. The OC, L and S indicate the open circular, linear, and supercoiled plasmid forms, respectively. (b) The supercoiled content. Plasmid DNA pBR322 was incubated at 37 °C for 30 min with 0.003% H$_2$O$_2$ with and without varying AEGT doses (1, 2, and 4 mg/mL). Control was plasmid alone without treatment with H$_2$O$_2$ or AEGT. Data are means ± S.D. (n = 3). Levels not connected by same big letter significantly differed. Except A vs. D ($P < 0.02$), all comparisons among different letters showed significant differences ($P < 0.005$).

### 2.3. AEGT Modulates H$_2$O$_2$-Induced Cellular DNA Damage

Next, the protective effect of AEGT against oxidative DNA damage was verified by comet-NE assay in H1299 cells. Few “tails” were observed in the untreated controls (Figure 3a). While DNA trailing was evident in the presence of H$_2$O$_2$ (Figure 3b, H$_2$O$_2$ only), it was reduced by the AEGT co-treatments (Figure 3c–e). The average % in tail values (mean ± S.D.) for NE buffer control, H$_2$O$_2$ alone, and AEGT treatments (1, 2, and 4 mg/mL) were 12.4 ± 7.4, 69.6 ± 11.9, 53.6 ± 17.1, 32.5 ± 10.5, and 22.1 ± 1.0 (n = 100; in duplicate), respectively (Figure 3f, $P < 0.0001$ between H$_2$O$_2$ only and 1–4 mg/mL AEGT-treated cells). Again, AEGT showed a dose-dependent protective effect against oxidative DNA damage. These results indicate the DNA modulating effect of AEGT in the cellular DNA context.

### 2.4. AEGT Promotes Cell Survival under H$_2$O$_2$ Treatment

We further tested whether the modulating effect of AEGT also promotes H1299 cell survival under H$_2$O$_2$ treatment. The H1299 cells were treated with or without H$_2$O$_2$ in the presence of 0.5, 1, 2, and 4 mg/mL of AEGT for 24 h. The cell viability was then determined by MTT assay. Treating H1299 with 0.5, 1, and 4 mg/mL AEGT alone showed no significant adverse effects on cell viability ($P > 0.05$ versus control) (Figure 4a). In the presence of H$_2$O$_2$–induced oxidative DNA damage, AEGT increased cell viability in a dose-dependent manner. From a baseline cell viability of 55.7 ± 3.8, treatment with 0.5, 1, 2 and 4 mg/mK AEGT significantly increased the cell viability of H$_2$O$_2$-treated cells to 63.3 ± 8.3, 76.5 ± 4.7, 89.1 ± 3.2 and 100.3 ± 16.0, respectively ($P < 0.05$ to 0.005, Figure 4b). Notably, the viability of H1299 cells treated with both H$_2$O$_2$ and 4 mg/mL AEGT did not significantly differ from that in control cells ($P > 0.05$, Figure 4b).
Figure 3. Modulating effect of AEGT on H$_2$O$_2$-induced cellular DNA strand breaks revealed by comet assay. After 2 h pretreatment with 1, 2, and 4 mg/mL AEGT, H1299 cells were co-incubated with or without 0.003% H$_2$O$_2$ at 37 °C for 2 h. (a–e) Demonstration of PI staining results for H1299 cells incubated with or without 0.003% H$_2$O$_2$ and AEGT. (a) Negative control. (b) Positive control (H$_2$O$_2$ alone). (c–e) H$_2$O$_2$ treatment with 1, 2, and 4 mg/mL AEGT, respectively. (f) Average % of tail DNA for H1299 cells. Data are means ± S.D. (n = 2; 100 cell counts per experiment with two individual replicates). Levels not connected by same big letter significantly differed (P < 0.0001). Electrode polarity is also shown.
Figure 4. Cell viability was determined by MTT assay. The H1299 cells were treated for 24 h with AEGT (0.5, 1, 2, and 4 mg/mL) (a) without H2O2 and (b) with 0.003% H2O2. Data are means ± S.D. (n = 3). Levels not connected by same big letter significantly differed. Except (b) A vs. BC (P < 0.05), comparisons between other different letters in (a) and (b) showed significant differences (P < 0.005).

2.5. AEGT Prevents Cell Cycle Arrest by H2O2

After 24 h treatment with H2O2 in the presence of 0.5, 1, 2, and 4 mg/mL AEGT, the cell cycle distributions of H1299 cells were further analyzed by flow cytometry. The control H1299 displayed a major G1 peak and a minor G2/M peak comprising 62.0% and 18.0% of the cell population, respectively (Figure 5, panel 1). However, the H2O2-treated H1299 showed a significant increase in the G2/M population to 81.7%, which clearly indicated cell cycle arrest caused by DNA damage (Figure 5, panel 2) (P < 0.001). However, this H2O2-induced G2/M arrest was gradually and significantly diminished by co-incubation with AEGT and was virtually eliminated in the presence of 2–4 mg/mL AEGT (Figure 5, panels 3–6) (P < 0.01). This result indicates that AEGT can prevent DNA damage and ensure a normal cell cycle progression.

Figure 5. Cell cycle distribution. The H1299 cells were treated with AEGT (0.5, 1, 2, and 4 mg/mL) without and with 0.003% H2O2 for 24 h. Data are means ± S.D. (n = 3). Levels not connected by same big letter significantly differed (P < 0.01 to 0.0001). * The statistical analyses were performed on the same cell cycle phase % between different treatments (control, H2O2 alone, and AEGT/H2O2) without comparison between different phases.
2.6. Discussion

This study investigated the antioxidant activity of AEGT in terms of its biochemical characteristics, DNA damage protection, cell growth, and cell cycle recovery after treatment with \( \text{H}_2\text{O}_2 \) alone or with \( \text{H}_2\text{O}_2 \) and AEGT. The selected damaging agent in this study was \( \text{H}_2\text{O}_2 \) because it exhibits either direct (oxidation of its target) or indirect (involving peroxiredoxins) signaling [18] and induction of oxidative stress [20–22]. The AEGT effectively suppressed ROS-induced DNA strand break in cell-free assay (Figure 2). Sensitive comet-NE assay of cellular DNA damage (Figure 3) and cell cycle profiling (Figure 5) further showed that AEGT increases H1299 cell survival by modulating oxidative stress (Figure 4). In accordance with many other reports of \( \text{H}_2\text{O}_2 \) treatment in various cell lines [23–25], \( \text{H}_2\text{O}_2 \)-treated H1299 also showed prominent G2/M arrest, and AEGT conferred effective recovery from \( \text{H}_2\text{O}_2 \)-induced G2/M arrest although the sub-G1 population in \( \text{H}_2\text{O}_2/4 \text{ mg/mL} \) AEGT is mildly increased (Figure 5). Earlier reports that ROS correlate with immunity [26], anti-hepatoma activity [27], HCV-related activity [28], and Alzheimer’s disease [29] suggest that AEGT has potential applications in testing recovery from ROS-related cellular responses.

Typical phenolic compounds known to exhibit antioxidant activity include phenolic acids and flavonoids [30,31]. In addition to phenolic compounds, tannins [32], ascorbic acid [33], and pigments [34] are other potential antioxidant compounds contained in seaweeds. In the current study, polyphenols, flavonoids, and ascorbic acid were also detected in AEGT. Algal polyphenols are known to confer the major antioxidant activity of seaweed extracts [35]. Analysis of DPPH radical scavenging is largely performed to assess the free radical scavenging effect of specific compounds or extracts and can quickly indicate antioxidant activity [36,37]. The DPPH radical scavenging and phenolic content reportedly have a strong correlation in plant-based foods such as herbs [38] and seaweeds [39]. Moreover, flavonoids [40] and ascorbic acid [36] also exhibit free radical scavenging activities.

Oxidative DNA damage can result from ROS that are generated by cellular metabolism or environmental stress and it is believed to contribute to aging, carcinogenesis and other diseases [41]. For In vitro assays, the conversion of the supercoiled form of plasmid DNA into either open-circular or linear form has been used as a standard indicator of DNA damage [42]. The literature shows that boiled \( G. \text{tenuistipitata} \) extract exhibits radical scavenging activities with an IC\(_{50}\) of 24.22 mg/mL in DPPH radical scavenging assay [10]. This study shows that AEGT exhibits an even more potent antioxidant activity. A 60% DPPH scavenging activity was observed at only 3 mg/mL. Considering the rapid growth of seaweed and the extensive use of seaweed extracts in the food and cosmetics industries, AEGT represents an attractive multifunctional alternative for such applications. Future studies to identify the bioactive fraction of AEGT would further enhance the economic value of red algal extract. The potential protective role of AEGT to other types of cancer cells warrants further investigation.

Moreover, many algal extracts reportedly exhibit other interesting biological effects, such as the protective effects of enzymatic extracts from microalgae against \( \text{H}_2\text{O}_2 \)-induced DNA damage [43] and the protective effects of \( \text{Phaeodactylum tricornutum} \) lipid-rich algae extract against proteasome activity [44]. Similarly, we found that AEGT protects against \( \text{H}_2\text{O}_2 \)-induced plasmid and cellular DNA damage, cytotoxicity, and cell cycle arrest without affecting cell viability as observed in AEGT alone.
3. Experimental

3.1. Raw Materials

Specimens of *G. tenuistipitata* collected during spring 2009 from a culture farm at Kouhu beach, Yunlin County, Taiwan, were delivered to the laboratory at 0 °C. In the laboratory, the seaweeds were washed with running tap-water to remove epiphytes and encrusting material, immersed twice in distilled water, and then lyophilized. After pulverizing the dried sample and passing it through a 60-mesh sieve, the lyophilized sample was ground to fine powder and stored at −40 °C.

3.2. Extraction and Isolation of Seaweed *G. tenuistipitata*

After the addition of 1,000 mL deionized water, the dried samples (50 g) were agitated in a mechanical shaker at room temperature for 24 h. The extract was then filtered with Whatman No. 1 filter paper. The filtrate solution was evaporated to dryness at 40 ± 2 °C in a rotary evaporator (Buchi Laboratoriums-Technik, Buchs, Switzerland) and then lyophilized. The lyophilized extract was stored in a sealed container at −40 °C until use.

3.3. Determination of Total Phenolics, Flavonoid, and Ascorbic Acid of AEGT

The total phenolic compounds in AEGT extracts were determined with Folin-Ciocalteu reagent as described by Singleton and Rossi [45] using gallic acid as standard. The total phenolic content was expressed as gallic acid equivalent in μg per mg of dry sample. Ascorbic acid was quantitatively determined using the 2,6-dichloroindophenol-Na dye method as described by Jones and Hurghes [46]. Results were presented on a dry matter basis (μg ascorbic acid per mg of dry sample). Flavonoid content was determined by the colorimetric method described by Woisky and Salatino [47]. Total flavonoid content was calculated in quercetin equivalents based on a calibration curve and expressed as μg quercetin equivalents per mg of dry sample.

3.4. Free Radical Scavenging Activity

The ability of AEGT to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined as described earlier [48]. Briefly, a 1 mM methanolic solution of DPPH (1 mL) was mixed with solution of each extract (1, 2, and 4 mg/mL, 3 mL). After vigorous vortexing, the mixture was kept in darkness for 30 min at room temperature. Absorbance was measured at 517 nm, and activity was expressed as percentage of DPPH scavenging compared to control. The percentage of scavenging activity was calculated as [(Ac − As)/Ac] × 100 where As is the absorbance measured with the extract sample in the assay and Ac is the absorbance of control (without extract sample). Butylated hydroxyanisole (BHA) and ascorbic acid (Vit. C) were used as positive controls.

3.5. Cell Cultures

Cells were routinely maintained in complete RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (BenchMark, GEMINI, Bio-Products, West Sacramento, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.03% glutamine (Gibco,
Invitrogen Ltd., Carlsbad, CA, USA). Cells were kept at 37 °C in a humidified atmosphere containing 5% CO₂. The H1299 (human lung adenocarcinoma) cell line was used to test cytotoxicity in the lung cancer cell line.

### 3.6. Plasmid DNA Cleavage Assay

Conversion of the supercoiled (S) form of plasmid DNA to the open-circular (OC) and/or further linear (L) forms was analyzed as an indicator of DNA strand breaks [49]. Reaction mixtures (10 μL) containing 150 ng of pBR322 plasmid DNA, 0.1 mM FeSO₄, 0.05% H₂O₂ and AEGT at concentrations of 0, 1, 2, and 4 mg/mL were incubated at 37 °C for 30 min. After stopping the reaction by adding 2 μL of 6 × gel loading dye (0.05% bromophenol blue, 40 mM EDTA and 50% glycerol (v/v)), electrophoresis was performed on 0.8% agarose gel in 0.5 × TAE buffer at 50 V for 1–2 h. The DNA in the gel was stained with ethidium bromide (final concentration 0.8 μg/mL) and then visualized and photographed under ultraviolet light. The formula used for calculating the percentage of supercoiled DNA was as follows: S% = (band density of S / band density of (S + L + OC), where the band density was determined by the Gel-pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD, USA). Relative S% = S% of test sample/S% of control.

### 3.7. Comet-NE Assay

The comet-NE is more sensitive than the traditional comet assay [50,51]. The comet-NE assay using nuclear extracts (NEs) prepared from NB4 cells (human acute promyelocytic leukemia) cell line [52–54] was performed using a protocol described previously [52–54]. Aliquots (100 μL) of H1299 cell suspensions (1 × 10⁶ cells/mL in PBS) were mixed with equal volumes of 1.2% low-melting agarose (in PBS, pH 7.4) and immediately pipetted onto a glass slide precoated with 1% regular agarose (in distilled water). The slides were then immersed in freshly prepared ice-cold cell lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% N-laurylsarcosine, 1% Triton X-100 and 10% dimethylsulfoxide or DMSO) incubated at 4 °C for 2 h and then rinsed three times with deionized water. A 20 μL excision mixture containing 0.6 μg NE, 50 mM Hepes-KOH (pH 7.9), 70 mM KCl, 5 mM MgCl₂, 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine and 2.5 mM creatine phosphokinase was then added to each slide. After applying the cover slips, the slides were incubated at 37 °C for 2 h in a humidified space for NE digestion. The slides were denatured in 0.3 N NaOH, 1 mM EDTA for 20 min and then electrophoresed at 20 V, 300 mA for 25 min. After washing with deionized water, the slides were neutralized in 0.4 M Tris-HCl, pH 7.5 and stained with 40 μL propidium iodide (PI, 50 μg/mL). Under a fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan), the migration of DNA from the nucleus of each cell was measured with the CometScore [55] software program. The % tail DNA parameter [56,57] was calculated as the percentage of DNA in the comet tail (sum of intensities of pixels in the tail). The formula used for calculating the % tail DNA was as follows: Tail % DNA = 100 – Head % DNA, where Head % DNA = (Head Optic Intensity / (Head Optic Intensity + Tail Optic Intensity)) × 100.
3.8. Cell Viability Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously [58]. Briefly, fresh medium (100 μL) containing 0.5 mg/mL MTT was added into each well of a 96-well plate containing $5 \times 10^3$ cells/well and incubated for 2 h at 37 °C. After removing MTT-containing medium, 100 μL of DMSO was added into each well to dissolve the purple formazan crystal. The plates were then shaken gently for 20 min in darkness and then read at 595 nm on a microtiter plate reader.

3.9. Cell Cycle Histogram Obtained by Propidium Iodide Staining in Flow Cytometry

The cell cycle histogram was determined as described previously [59]. Briefly, cells with $5 \times 10^5$ cells/100-mm Petri-dish were plated. After recovery, cells were treated for 24 h with 0, 1, 2, and 4 mg/mL AEGT with or without 0.003% H$_2$O$_2$. After treatment, cells were collected, washed twice with PBS, and fixed in 70% ethanol overnight. The cells were then centrifuged at 700 rpm for 5 min at 4 °C and then resuspended in PBS buffer containing 10 μg/mL PI (Sigma, St Louis, MO, USA) and 10 μg/mL RNase A. After 15 min incubation in darkness at room temperature, the cells were analyzed with a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA) in cell counts of 10,000 with gated setting (forward light scatter versus side light scatter such that only single cells were assayed) [60], and the gated data were analyzed by Cell-Quest and Modfit softwares (Becton-Dickinson, Mansfield, MA, USA).

3.10. Statistical Analysis

All data were presented as means ± SEM. Experimental groups were compared by one-way ANOVA with Tukey HSD Post Hoc Test using JMP® 9 software [61]. Levels not connected by the same big letter significantly differed.

4. Conclusions

Taken together, the experimental results in this study confirmed the hypothesis that edible red algae *Gracilaria* extract prevents ROS-induced DNA damage and its related cellular responses.

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Conflicts of Interest

The authors declare no conflict of interest.
References

1. Chiang, Y.M.; Lin, J.L. Nitrate uptake by nitrogen-starved plants of the red alga *Gracilaria tenuistipitata* var. Liui. *Jpn. J. Phycol.* **1989**, *37*, 187–193.

2. Armisen, R. World-wide use and importance of Gracilaria. *J. Appl. Phycol.* **1995**, *7*, 231–243.

3. Ajisaka, T.; Chiang, Y.M. Recent status of Gracilaria cultivation in Taiwan. *Hydrobiologia* **1993**, *260/261*, 335–338.

4. Jha, R.K.; Zi-rong, X. Biomedical compounds from marine organisms. *Mar. Drugs* **2004**, *2*, 123–146.

5. Faulkner, D.J. Marine natural products. *Nat. Prod. Rep.* **2002**, *19*, 1–48.

6. Hsu, B.Y.; Tsao, C.Y.; Chiou, T.K.; Hwang, P.A.; Hwang, D.F. HPLC determination for prostaglandins from seaweed *Gracilaria gigas*. *Food Control* **2007**, *18*, 639–645.

7. Marinho-Soriano, E.; Bourret, E. Polysaccharides from the red seaweed *Gracilaria dura* (Gracilariales, Rhodophyta). *Bioresour. Technol.* **2005**, *96*, 379–382.

8. de Almeida, C.L.; Falcao Hde, S.; Lima, G.R.; Montenegro Cde, A.; Lira, N.S.; de Athayde-Filho, P.F.; Rodrigues, L.C.; de Souza Mde, F.; Barbosa-Filho, J.M.; Batista, L.M. Bioactivities from marine algae of the genus gracilaria. *Int. J. Mol. Sci.* **2011**, *12*, 4550–4573.

9. Lin, Y.H.; Tsai, J.S.; Hung, L.B.; Pan, B.S. Hypocholesterolemic effect of compounded freshwater clam protein hydrolysate and Gracilaria. *Food Chem.* **2010**, *123*, 395–399.

10. Yangthong, M.; Hutadilok-Towatana, N.; Phromkunthong, W. Antioxidant activities of four edible seaweeds from the southern coast of Thailand. *Plant Foods Hum. Nutr.* **2009**, *64*, 218–223.

11. Ganesan, P.; Kumar, C.S.; Bhaskar, N. Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresour. Technol.* **2008**, *99*, 2717–2723.

12. Vijayavel, K.; Martinez, J.A. *In vitro* antioxidant and antimicrobial activities of two Hawaiian marine Limu: *Ulva fasciata* (Chlorophyta) and *Gracilaria salicornia* (Rhodophyta). *J. Med. Food* **2010**, *13*, 1494–1499.

13. Souza, B.W.; Cerqueira, M.A.; Martins, J.T.; Quintas, M.A.; Ferreira, A.C.; Teixeira, J.A.; Vicente, A.A. Antioxidant potential of two red seaweeds from the Brazilian coasts. *J. Agric. Food Chem.* **2011**, *59*, 5589–5594.

14. Dang, H.T.; Lee, H.J.; Yoo, E.S.; Shinde, P.B.; Lee, Y.M.; Hong, J.; Kim, D.K.; Jung, J.H. Anti-inflammatory constituents of the red alga *Gracilaria verrucosa* and their synthetic analogues. *J. Nat. Prod.* **2008**, *71*, 232–240.

15. Coura, C.O.; de Araujo, I.W.; Vanderlei, E.S.; Rodrigues, J.A.; Quindere, A.; Fontes, B.P.; de Queiroz, I.N.; de Menezes, D.B.; Bezerra, e Silver, A.A.; et al. Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed gracilaria cornea. *Basic Clin. Pharmacol. Toxicol.* **2011**, *110*, 335–341.

16. Yeh, S.T.; Lin, Y.C.; Huang, C.L.; Chen, J.C. White shrimp *Litopenaeus vannamei* that received the hot-water extract of *Gracilaria tenuistipitata* showed protective innate immunity and up-regulation of gene expressions after low-salinity stress. *Fish Shellfish Immunol.* **2010**, *28*, 887–894.

17. Martinet, W.; Knaapen, M.W.; De Meyer, G.R.; Herman, A.G.; Kockx, M.M. Oxidative DNA damage and repair in experimental atherosclerosis are reversed by dietary lipid lowering. *Circ. Res.* **2001**, *88*, 733–739.
18. Rigoulet, M.; Yoboue, E.D.; Devin, A. Mitochondrial ROS generation and its regulation: Mechanisms involved in H(2)O(2) signaling. Antioxid. Redox Signal. 2011, 14, 459–468.
19. Cao, W.; Chen, W.J.; Suo, Z.R.; Yao, Y.P. Protective effects of ethanolic extracts of buckwheat groats on DNA damage caused by hydroxyl radicals. Food Res. Int. 2008, 41, 924–929.
20. Wijeratne, S.S.; Cuppett, S.L.; Schlegel, V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. J. Agric. Food Chem. 2005, 53, 8768–8774.
21. Wood, J.M.; Decker, H.; Hartmann, H.; Chavan, B.; Rokos, H.; Spencer, J.D.; Hasse, S.; Thornton, M.J.; Shalbaf, M.; Paus, R.; et al. Senile hair graying: H2O2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair. FASEB J. 2009, 23, 2065–2075.
22. Spencer, J.D.; Gibbons, N.C.; Rokos, H.; Peters, E.M.; Wood, J.M.; Schallreuter, K.U. Oxidative stress via hydrogen peroxide affects proopiomelanocortin peptides directly in the epidermis of patients with vitiligo. J. Invest. Dermatol. 2007, 127, 411–420.
23. Seomun, Y.; Kim, J.T.; Kim, H.S.; Park, J.Y.; Joo, C.K. Induction of p21Cip1-mediated G2/M arrest in H2O2-treated lens epithelial cells. Mol. Vis. 2005, 11, 764–774.
24. Thorn, T.; Gniadecki, R.; Petersen, A.B.; Vicanova, J.; Wulf, H.C. Differences in activation of G2/M checkpoint in keratinocytes after genotoxic stress induced by hydrogen peroxide and ultraviolet A radiation. Free Radic. Res. 2001, 35, 405–416.
25. Chien, M.; Rinker-Schaeffer, C.; Stadler, W.M. A G2/M growth arrest response to low-dose intermittent H2O2 in normal uroepithelial cells. Int. J. Oncol. 2000, 17, 425–432.
26. Lu, S.; Guo, X.; Zhao, P. Effect of Ginkgo biloba extract 50 on immunity and antioxidant enzyme activities in ischemia reperfusion rats. Molecules 2011, 16, 9194–9206.
27. Li, Q.; Jiang, C.; Zu, Y.; Song, Z.; Zhang, B.; Meng, X.; Qiu, W.; Zhang, L. SFE-CO2 extract from Typhonium giganteum Engl. tubers, induces apoptosis in human hepatoma SMMC-7721 cells involvement of a ROS-mediated mitochondrial pathway. Molecules 2011, 16, 8228–8242.
28. Simula, M.P.; De Re, V. Hepatitis C virus-induced oxidative stress and mitochondrial dysfunction: A focus on recent advances in proteomics. Proteom. Clin. Appl. 2010, 4, 782–793.
29. Dumont, M.; Beal, M.F. Neuroprotective strategies involving ROS in Alzheimer disease. Free Radic. Biol. Med. 2011, 51, 1014–1026.
30. de Oliveira, C.B.; Comunello, L.N.; Lunardelli, A.; Amaral, R.H.; Pires, M.G.; da Silva, G.L.; Manfredini, V.; Vargas, C.R.; Gnoatto, S.C.; de Oliveira, J.R.; et al. Phenolic enriched extract of Baccharis trimera presents anti-inflammatory and antioxidant activities. Molecules 2012, 17, 1113–1123.
31. Pieroni, L.G.; de Rezende, F.M.; Ximenes, V.F.; Dokkedal, A.L. Antioxidant activity and total phenols from the methanolic extract of Miconia albicans (Sw.) Triana leaves. Molecules 2011, 16, 9439–9450.
32. Heo, S.J.; Jeon, Y.J.; Lee, J.; Kim, H.T.; Lee, K.W. Antioxidant effect of enzymatic hydrolyzate from a Kelp, Ecklonia cava. Algae 2003, 18, 341–347.
33. Munda, I.M. Preliminary information on the ascorbic acid content in some Adriatic seaweeds. Hydrobiologia 1987, 151/152, 477–481.
34. Siriwardhana, N.; Lee, K.W.; Kim, S.H.; Ha, J.W.; Park, G.T.; Jeon, Y.J. Lipid peroxidation inhibitory effects of *Hizikia fusiformis* methanolic extract on fish oil and linoleic acid. *Food Sci. Technol. Int.* 2004, 10, 65–72.

35. Nakamura, T.; Nagayama, K.; Uchida, K.; Tanaka, R. Antioxidant activity of phlorotannins from the brown alga *Eisenia bicyclis*. *Fish. Sci.* 1996, 62, 923–926.

36. Scalzo, R.L. Organic acids influence on DPPH scavenging by ascorbic acid. *Food Chem.* 2008, 107, 40–43.

37. Yang, J.I.; Ho, H.Y.; Chu, Y.J.; Chow, C.J. Characteristic and antioxidant activity of retorted gelatin hydrolysate from cobia (*Rachycentron canadum*) skin. *Food Chem.* 2008, 110, 128–136.

38. Zheng, W.; Wang, S.Y. Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* 2001, 49, 5165–5170.

39. Jimenez-Escrig, A.; Rincon, M.; Pulido, R.; Saura-Calixto, F. Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. *J. Agric. Food Chem.* 2001, 49, 5489–5493.

40. Robards, K.; Prenzler, P.D.; Tucker, G.; Swatstitang, P.; Glover, W. Phenolic compounds and their role in oxidative process in fruits. *Food Chem.* 1999, 66, 401–436.

41. Cooke, M.S.; Evans, M.D.; Dizdaroglu, M.; Lunec, J. Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB J.* 2003, 17, 1195–1214.

42. Onuki, J.; Medeiros, M.H.; Bechara, E.J.; Di Mascio, P. 5-Aminolevulinic acid induces single-strand breaks in plasmid pBR322 DNA in the presence of Fe2+ ions. *Biochim. Biophys. Acta* 1994, 1225, 259–263.

43. Karawita, R.; Senevirathne, M.; Athukorala, Y.; Affan, A.; Lee, Y.J.; Kim, S.K.; Lee, J.B.; Jeon, Y.J. Protective effect of enzymatic extracts from microalgae against DNA damage induced by H2O2. *Mar. Biotechnol. (NY)* 2007, 9, 479–490.

44. Nizard, C.; Poggioli, S.; Heusele, C.; Bulteau, A.L.; Moreau, M.; Saunois, A.; Schnebert, S.; Mahe, C.; Friguet, B. Algae extract protection effect on oxidized protein level in human stratum corneum. *Ann. NY Acad. Sci.* 2004, 1019, 219–222.

45. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 1965, 16, 144–158.

46. Jones, E.; Hurghes, R.E. Foliar ascorbic acid in some angiosperms. *Phytochemistry* 1983, 22, 2493–2499.

47. Woisky, R.G.; Salatino, A. Analysis of propolis: Some parameters and procedures for chemical quality control. *J. Agric. Food Chem.* 1998, 37, 99–105.

48. Blois, M.S. Antioxidant determinations by the use of a stable free radical. *Nature* 1958, 26, 1199–1200.

49. Chen, B.H.; Chang, H.W.; Huang, H.M.; Chong, I.W.; Chen, J.S.; Chen, C.Y.; Wang, H.M. (−)-Anonaine induces DNA damage and inhibits growth and migration of human lung carcinoma H1299 cells. *J. Agric. Food Chem.* 2011, 59, 2284–2290.

50. Li, P.Y.; Chang, Y.C.; Tzang, B.S.; Chen, C.C.; Liu, Y.C. Antibiotic amoxicillin induces DNA lesions in mammalian cells possibly via the reactive oxygen species. *Mutat. Res.* 2007, 629, 133–139.

51. Wang, A.S.; Ramanathan, B.; Chien, Y.H.; Goparaju, C.M.; Jan, K.Y. Comet assay with nuclear extract incubation. *Anal. Biochem.* 2005, 337, 70–75.
52. Chang, Y.C.; Liao, C.B.; Hsieh, P.Y.; Liou, M.L.; Liu, Y.C. Expression of tumor suppressor p53 facilitates DNA repair but not UV-induced G2/M arrest or apoptosis in Chinese hamster ovary CHO-K1 cells. *J. Cell. Biochem.* **2008**, *103*, 528–537.

53. Chang, Y.C.; Jan, K.Y.; Cheng, C.A.; Liao, C.B.; Liu, Y.C. Direct involvement of the tumor suppressor p53 in nucleotide excision repair. *DNA Repair (Amst)* **2008**, *7*, 751–761.

54. Cemeli, E.; Mirkova, E.; Chiuchiarelli, G.; Alexandrova, E.; Anderson, D. Investigation on the mechanisms of genotoxicity of butadiene, styrene and their combination in human lymphocytes using the Comet assay. *Mutat. Res.* **2009**, *664*, 69–76.

55. CometScore, version 1.5. Available online: http://www.tritekcorp.com (accessed on 11 June 2012).

56. Wong, V.W.C.; Szeto, Y.T.; Collins, A.R.; Benzie, I.F.F. The comet assay: A biomonitoring tool for nutraceutical research. *Curr. Top. Nutraceut. Res.* **2005**, *3*, 1–14.

57. Collins, A.R. The comet assay for DNA damage and repair: Principles, applications, and limitations. *Mol. Biotechnol.* **2004**, *26*, 249–261.

58. Huang, C.Y.; Chen, J.Y.; Wu, J.E.; Pu, Y.S.; Liu, G.Y.; Pan, M.H.; Huang, Y.T.; Huang, A.M.; Hwang, C.C.; Chung, S.J.; *et al.* Ling-Zhi polysaccharides potentiate cytotoxic effects of anticancer drugs against drug-resistant urothelial carcinoma cells. *J. Agric. Food Chem.* **2010**, *58*, 8798–8805.

59. Chiu, C.C.; Li, C.H.; Fuh, T.S.; Chen, W.L.; Huang, C.S.; Chen, L.J.; Ung, W.H.; Fang, K. The suppressed proliferation and premature senescence by ganciclovir in p53-mutated human non-small-lung cancer cells acquiring herpes simplex virus-thymidine kinase cDNA. *Cancer Detect. Prev.* **2005**, *29*, 286–293.

60. Nunez, R. DNA measurement and cell cycle analysis by flow cytometry. *Curr. Issues Mol. Biol.* **2001**, *3*, 67–70.

61. JMP® 9 software. Available online: http://www.jmp.com/ (accessed on 11 June 2012).

Sample Availability: Samples of aqueous extract of *Gracilaria tenuistipitata* (AEGT) are available upon request from Dr. Jing-Iong Yang.

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