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RESEARCH ARTICLE

Toxic effect of nonylphenol on the marine macroalgae *Gracilaria lemaneiformis* (Gracilariales, Rhodophyta): antioxidant system and antitumor activity

Mingqin Zhong[1] · **Pinghe Yin**[1,2] · **Ling Zhao**[3]

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Abstract The objective of the present work was to evaluate the toxic effect of nonylphenol (NP) on the antioxidant response and antitumor activity of *Gracilaria lemaneiformis*. An obvious oxidative damage was observed in this study. The thallus exposed to NP showed 1.2–2.0-fold increase in lipid peroxide and displayed a maximum level of 16.58 μmol g^{-1} Fw on 0.6 mg L^{-1} for 15-day exposure. The activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) enhanced significantly by 1.1–3.2-fold and subsequently diminished at the high concentrations and prolonged exposure. The results of DNA damage in comet assay also supported that NP was obviously toxic on *G. lemaneiformis* with increasing the percentage of tail DNA in a dose-dependent manner. Furthermore, the ethanol extract of *G. lemaneiformis* (EEGL) did exhibit antitumor potential against HepG-2 cells. While decreased in cell inhibition, ROS generation, apoptosis, and caspase-3 in HepG-2 cells treated with the EEGL were observed when *G. lemaneiformis* was exposed to NP for 15 days, and which were related to exposure concentration of NP. These suggested that NP has strongly toxic effect on the antitumor activity of *G. lemaneiformis*. The results revealed in this study imply that macroalgae can be useful biomarkers to evaluate marine pollutions.

Keywords *Gracilaria lemaneiformis* · Nonylphenol · Toxic effect · Antioxidant system · Antitumor activity · DNA damage

Responsible editor: Philippe Garrigues

✉ Pinghe Yin
tyinph@jnu.edu.cn

✉ Ling Zhao
zhaoling@jnu.edu.cn

[1] Department of Chemistry, Jinan University, No. 601, Huangpu Da Dao Xi, Guangzhou 510632, People's Republic of China

[2] Center of Analysis and Test, Jinan University, Guangzhou 510632, China

[3] Key Laboratory of Water/Soil Toxic Pollutants Control and Bioremediation of Guangdong Higher Education Institutes, Department of Environmental Engineering, Jinan University, No. 601, Huangpu Da Dao Xi, Guangzhou 510632, People's Republic of China

Introduction

Marine macroalgae, as a key component of primary producer, have been a focus in recent studies for their pivotal role in coastal and estuarine ecosystems (Wang et al. 2014). Some bioactive substances from macroalgae are regarded economically important. It was documented that macroalgae were potential algicide against the growth of harmful algae blooms (HABs) (Ye et al. 2014). Serial experiments proved that microalgae were efficient in bioremediation of eutrophic waters to improve water quality through absorbing nutrients (Neori et al. 1996; Yang et al. 2015). And some important biologically active compounds with antitumor potential have been extracted from macroalage and are expected to be a novel anticancer drug against cancer (Fan et al. 2012). However, the environment changes, such as temperature, salinity, solar radiation, and hazardous substances, exert an effect on the physiological and biochemical response of seaweeds (Wang et al. 2014).

Nonylphenol (NP), as a kind of typical endocrine disrupting chemicals (EDCs), had been detected in various environmental matrices and was of particular concern for human as well as for aquatic environment because of its persistent toxicity. Nonylphenol is the main biodegradation product of nonylphenol ethoxylate (NPEO) which is massively used in industry and agriculture. In recent years, a higher level of NP had been found at coastal areas. Due to its lipophilicity, NP can accumulate in organisms and pose threat to other organisms through food chains' biomagnification (Lai et al. 2002). Nice et al. (2000) reported that the lethal concentration of NP for oyster larvae was 100 μg L^{-1}. Nonylphenol presented in the environment could be bioconcentrated in microalgae *Isochrysis galbana* and then produced toxic effect on other organisms through the trophic chain (Correa-Reyes et al. 2007). Liu et al. (2010) also reported that NP may inhibit the growth of marine diatom *Navicula incerta* with the EC_{50} value of 0.2 mg L^{-1} (96 h) and will enhance risk to settled larvae. For its persistent toxicity, NP had been included in the list of 27 persistent toxic substances by the United Nations Environment Program (Ibrahim et al. 2002).

Gracilaria lemaneiformis, an edible red alga cultivated in a large scale along southeast coastal region of China for fast growth, are broadly used in food additive, pharmaceutical, and cosmetic industries. It is one of the economically important alga not only for the effective role in the remediation of eutrophication but also for strong growth-inhibiting effect on HABs, for the important raw material of agar extraction and the extractions of other natural products (Wang et al. 2014; Xu and Gao 2012; Ye et al. 2014). Also, the fresh thalli can be used as high-quality feed for abalone or other marine animals and processed into nutrient and healthy sea vegetables for human consumption. Recently, some studies concerning *G. lemaneiformis* were focusing on the severe effects of environmental conditions' alteration and pollutants which were strongly phytotoxic and biodegradation-resistant, such as ultraviolet radiation, metal, and NiO nanoparticles (Han et al. 2012; Simioni et al. 2014; Xia et al. 2004). It has been reported that heavy metals from seawater can accumulate in *G. lemaneiformis* obviously and increase oxidative damage in *G. lemaneiformis* through the accumulation of toxic reactive oxygen species (ROS) and malondiadehyde (MDA) because of lower antioxidant enzyme activities, such as superoxide dismutase (SOD) and glutathione reductase (GR) (Huang et al. 2013; Wang et al. 2014). Other reports also proved that macroalgae is an important species to evaluate the toxicity of contaminants (Babu et al. 2014). But limited studies on the effect of NP on physiological property of *G. lemaneiformis* were conducted. Various studies revealed that water, methanol, ethanol, or ethyl acetate extracts from macroalgae were found to possess cell proliferation-inhibiting effects in cancer (Patra and Muthuraman 2013; Sundaram et al. 2012; Zandi et al. 2013). However, the influence of toxic pollutants on the antitumor activity of macroalgae has not been reported.

In the present study, we investigated (i) the existence of NP-induced oxidative stress by determining lipid peroxidation and antioxidant enzyme activities, (ii) DNA damage in *G. lemaneiformis* induced by NP, and (iii) whether there are any adverse effects on antitumor activity of *G. lemaneiformis*. To the best of our knowledge, the toxic effects of NP on macroalgae and their antitumor potential were reported for the first time.

Material and methods

Algal culture and treatment

G. lemaneiformis were collected from Nanao Island Cultivation Zone (116.6° E, 23.3° N), Shantou, Guangdong, China. After harvesting, the thalli were washed with sterile artificial seawater (ASW) to clean the attached sand, and the rhizoidal portions were removed to eliminate microbial contamination. The cleaned and healthy thalli were acclimatized to laboratory conditions for a week in VSE medium with the pH and salinity of 8.0 and 30‰, respectively. During the acclimatization period, the medium was replenished every other day and the culture conditions were maintained under an illumination of 2000 lx with a 12:12 h light/dark cycle at 20 ± 1 °C. After pre-culture, about 4 g wet weight of healthy thalli was cultured in flask containing 1 L fresh Von Stosch enriched sterile ASW supplemented with different concentrations of NP ranging from 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1} (three replicates per concentration) for 3, 7, and 15 days. And other culture conditions were staying the same with the acclimatization period. Stock solution of NP was prepared in methanol to a concentration of 2 g L^{-1} and stored at −20 °C. The final concentration of methanol in medium was controlled below 0.05% (*v*/*v*) (no observed effect concentration), and there were a set of flasks spiked with 0.05% (*v*/*v*) methanol used as control. Thus, two controls were set in all experiments, namely blank control (CK 1) and methanol control (CK 2), respectively.

Determination of lipid peroxidation, superoxide dismutase, and catalase

Extracts for detecting lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT) were prepared under ice bath cooling in phosphate buffer at a proportion of 1:9 (*w*/*v*). The tissue homogenates were centrifuged at 4000 rpm for 15 min at 4 °C, and the supernatants were used to assay. The lipid peroxidation was determined by thiobarbituric acid reactive substance (TBARS) assay as described by Uchiyama and Mihara (1978). The activities of SOD and CAT were

detected according to the manufacturer's instructions. Total superoxide dismutase (T-SOD) assay kit (No. A001-1) and CAT assay kit (No. A007-1) were purchased from Nanjing Jiancheng Bioengineering Institute, China. And the soluble protein was estimated according to Bradford method (Bradford 1976).

Protoplast isolation and comet assay

Protoplast isolation

Protoplast isolation and purification were performed following previous study with some modifications (Gupta et al. 2011). Briefly, the washed fresh thalli with 15-day exposure to NP were chopped into small pieces (≤1 mm thin). And then, the chopped tissues were rinsed several times in sterile ASW supplemented with 2% NaCl to remove debris prior to plasmolysis. The cleaned tissues were incubated in an enzyme medium (1:10 *w*/*v*) composed of 60% sterile ASW (30‰ sterile ASW diluted with Milli-Q water), 40% deionized water, 2% cellulase, 0.01% agarase, and 0.4 M mannitol and buffered with 50 mM Tris-HCl (pH 7.0) for 6 h on a rotary shaker (50 rpm) in the dark at 25 °C. After enzyme digestion, the protoplasts were filtered through a 300-mesh cell strainer and then centrifuged at 300×*g* for 10 min. The precipitates were rinsed and centrifuged twice and re-suspended in enzyme-lacking medium and kept at 4 °C. The protoplast numbers were estimated using hemocytometer. To achieve plasmolysis effectively, the tissues were treated for 30 min in plasmolysis medium consisting of 60% sterile ASW, 40% deionized water, and 1.0 M mannitol at 25 °C in the dark on a rotary shaker (50 rpm) before enzymatic digestion.

Comet assay

The method of comet assay was performed according to the manufacturer's instructions of comet assay kit (No. KGA240-100) purchased from Keygen Biotech. The protoplast solution (about 1×10^3 protoplasts) was mixed with 0.7% low-melting temperature agarose (LMA) at a proportion of 1:1 (*v*/*v*). Eighty-five microliters of the mixture solution was laid on a slide pre-coated with 100 μL of 0.5% normal-melting temperature agarose and covered with a coverslip and left at 4 °C for 10 min to the solidification of agarose. Subsequently, the coverslip was slipped off gently and horizontally, and the third agarose layer (70 μL, 0.7% LMPA) was layered. And then, the slide covered with a new coverslip was left at 4 °C for another 10 min, removed the coverslip, and the slides were submerged in lysis solution for 2 h at 4 °C. After lysis, the slides were rinsed twice in cold distilled water and set in a horizontal electrophoresis tank with cold electrophoresis buffer for 60 min at room temperature to DNA unwinding. Electrophoresis was accomplished at 25 V and 300 mA in electrophoresis tank under ice bath cooling for 30 min. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5) buffer and dyed with 20 μL of propidium iodide (PI). The samples were analyzed using inverted fluorescence microscope (Olympus IX 53) with an excitation wavelength of 515–560 nm.

Determination of antitumor activity

Preparation of algal extract

Fresh *G. lemaneiformis* treated with different concentrations of NP for 15 days was rinsed with distilled water and shade dried. Ethanol extract of *G. lemaneiformis* (EEGL) was prepared as described by Sundaram et al. (2012). The dried alga was extracted in ethanol solution at a proportion of 1:10 (*w*/*v*). The extract was filtered using Whatman paper No. 1 filter paper and sterilized by Millipore filter with 0.22-μm pore size and stored at −20 °C. The crude extract was diluted with phosphate-buffered saline (PBS) to 1000 μg mL^{-1} prior to use.

Cell culture

HepG-2 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) and incubated at 37 °C in the presence of 5% CO_2 incubator.

Methyl thiazolyl tetrazolium assay test

Methyl thiazolyl tetrazolium (MTT) assay test was conducted to evaluate cytotoxicity of EEGL against HepG-2 cells. The cells treated with EEGL (100 μg mL^{-1}) were incubated in a 96-well microplate for 24 and 48 h at 37 °C. Ten microliters of MTT solution (5 mg mL^{-1}) was added to medium, and the cells were incubated at 37 °C for another 4 h. The supernatant was transferred, and 100 μL of DMSO was added. Finally, the microplate was put on a rotary shaker in the dark for 10 min. The absorbance was read by microplate reader (SYNERGY H1) at 490 nm.

Generation of reactive oxygen species

The detection of intracellular ROS was conducted with DCFH-DA method using Reactive Oxygen Species Assay Kit (No. S0033) purchased from Beyotime Biotechnology according to the manufacturer's instruction. DCFH-DA can pass through cell membrane into cells and be hydrolyzed to DCFH, and then, DCFH may be oxidized by intracellular ROS to form highly fluorescent DCF. Briefly, the HepG-2 cells treated or untreated with EEGL (100 μg mL^{-1}) for 6 h in 6-well plate were collected and washed in PBS and then

stained with DCFH-DA. ROS levels were detected by flow cytometry (Gallios, USA).

Analysis of cell apoptosis

HepG-2 cells treated or untreated with EEGL (100 μg mL^{-1}) for 48 h in 6-well plate were collected and washed in PBS, followed by the measurement of apoptosis using FITC Annexin V Apoptosis Detection Kit I (No. 556547) purchased from BD Biosciences as per the manufacturer's instruction. Cells were re-suspended in binding buffer and stained with Annexin V-FITC and PI. Apoptosis was detected by flow cytometry (Gallios, USA).

Determination of caspase-3 activity

Caspase-3 can react with acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA) to produce yellow *p*-nitroanilide (*p*NA), while *p*NA shows strong absorption at 405 nm. The detection of caspase-3 activity was performed according to the manufacturer's instruction of Caspase-3 Activity Assay Kit (No. C115) purchased from Beyotime Biotechnology. HepG-2 cells were incubated with EEGL (100 μg mL^{-1}) for 48 h. Cells collected were washed in PBS and lysed with lysis solution on ice for 15 min. After centrifugation, 50 μL supernatant with 10 μL 2 mM Ac-DEVD-*p*NA (200 μM final concentration) was incubated at 37 °C for 2 h in 96-well plate. The absorbance was measured at 405 nm by microplate reader (SYNERGY H1), and caspase-3 activity was calculated according to *p*NA standard curve.

Statistical analyses

All results are represented as mean ± standard deviation. And significant differences were evaluated using one-way analysis of variance (ANOVA) with the three replicates. The values where $P < 0.01$ or $P < 0.05$; difference were considered significantly.

Results

Effect of NP on antioxidant system in *G. lemaneiformis*

To evaluate the oxidative stress induced by NP, LPO expressed in terms of MDA level, SOD, and CAT activities were determined after 3, 7, and 15-day treatment.

Lipid peroxidation

It can be observed from Fig. 1a that the MDA level in all groups increased significantly by 1.2–2.0-fold when compared to controls, indicating that NP did produce oxidation damage to *G. lemaneiformis*. Its level increased linearly with the increase of concentrations after 3 and 7-day exposure to NP. At the end of 15 days, the MDA increased and showed a maximum level with the increase of 200.24% on 0.6 mg L^{-1} and decreased thereafter gradually, but significant increases could be observed in all the experimental groups ($P < 0.05$). In the last exposure day, there was a tendency of decrease at high concentrations maybe because of the rot in algae body which causes the inactivation of corresponding enzymes.

Superoxide dismutase

The effect of NP on superoxide dismutase (SOD) in *G. lemaneiformis* is presented in Fig. 1b. The SOD activities in the groups exposed to NP were higher than control group, and the trend of variations both in concentration and time was similar to MDA. Under the exposure of NP, the SOD activity was increased continuously in a dose-response and time-response manner, but there was a slight decrease on 1.0 mg L^{-1} of 15 days. It was worth mentioning that the SOD activity attains a peak value with the increase of 3.24-fold on 0.8 mg L^{-1} of 15 days ($P < 0.01$).

Catalase

From Fig. 1c, no obvious variation of CAT activity was observed in the treatment of 3 days, while the CAT level varied evidently under the pressure of NP in the exposure of 7 and 15 days. For 7 days, the CAT activity was gradually induced with increase of exposure dose and reached a maximum at 9.48 U mg^{-1} protein in the concentration of 0.8 mg L^{-1}. However, with the increase of concentrations, the CAT level was increased initially and subsequently decreased invariably for 15 days, and the value in the concentration of 1.0 mg L^{-1} was 0.31 U mg^{-1} protein which was much lower than control group.

Effect of NP on DNA damage in *G. lemaneiformis*

From the comet assay, it was evident that NP may induce DNA damage in their tissues with increasing the percentage of tail DNA (Fig. 2). There was significant difference between the groups with higher concentrations of NP exposure and the control group ($P < 0.05$). And the percentage of tail DNA increased by 1.29–9.59-fold compared to the control. Furthermore, the percentage of tail DNA increased invariably

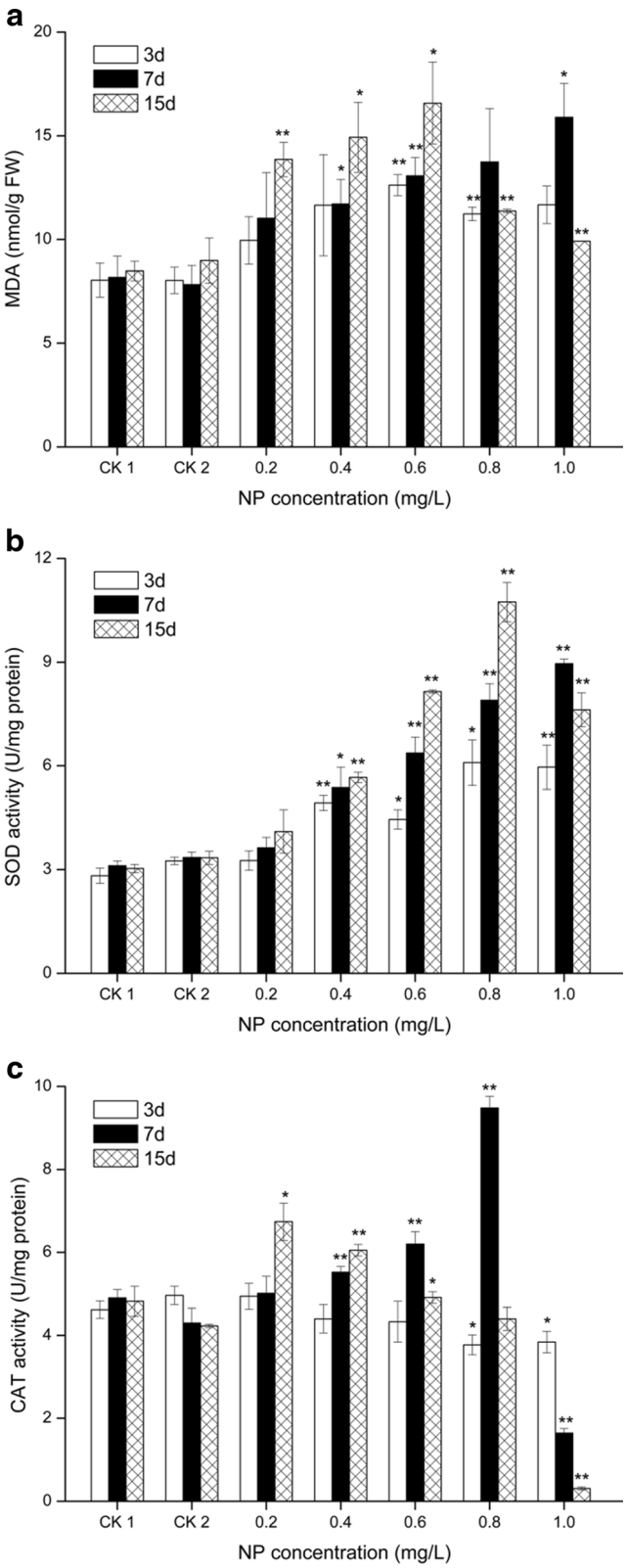

Fig. 1 Effects of NP on **a** MDA, **b** SOD activity, and **c** CAT activity in *G. lemaneiformis*. Data were presented as mean ± SD of three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$

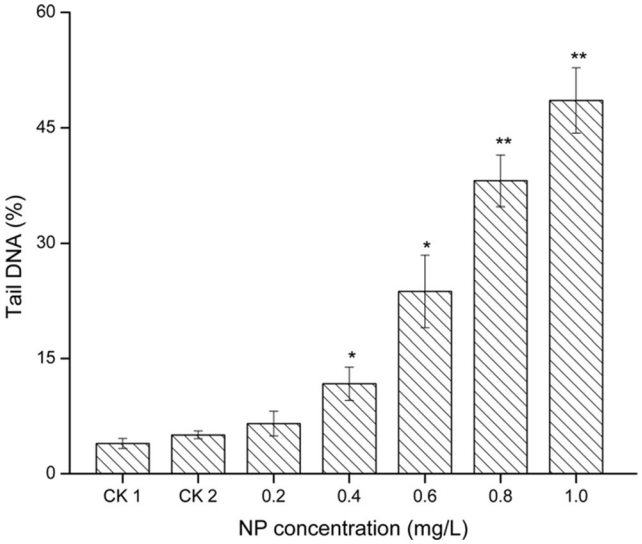

Fig. 2 Tail DNA damage in NP-treated thallus. Data were presented as mean ± SD of three replicates. $^{*}P < 0.05$, $^{**}P < 0.01$

with the increase of NP level, showing that DNA damage was occurred in a dose-dependent manner. These results demonstrated that NP has potential to pose genotoxicity to *G. lemaneiformis*.

Effect of NP on antitumor activity of *G. lemaneiformis*

Cell viability in vitro

Cell viability was monitored by MTT assay test in order to assess the impact of NP on the algal extract against HepG-2 cells after 24 and 48-h treatment with EEGL (Fig. 3). The results indicated that EEGL has cell proliferation-inhibiting effect on HepG-2 cells. And cell inhibition increased slightly with the increase of exposure time at the same exposure dose. However, the extracts of *G. lemaneiformis* which were exposed to NP may lead to significant reduction in cell proliferation inhibition effect and somehow showing apparent dosage effect connection.

The generation of intracellular ROS

ROS is a key role in the modulation of cellular functions, such as the pathways of oxidative signal transduction (Sunassee and Davies-Coleman 2012) and cell damage and apoptosis (Erkekoglu et al. 2010). Figure 4 illustrates the generation of ROS in HepG-2 cells treated with EEGL for 6 h. The results indicated that EEGL plays a significant role in increasing the level of ROS which may result in oxidative stress and then induce cell apoptosis. ROS production increased ~1.7-fold in the group of CK 1 and CK 2 ($P < 0.01$). However,

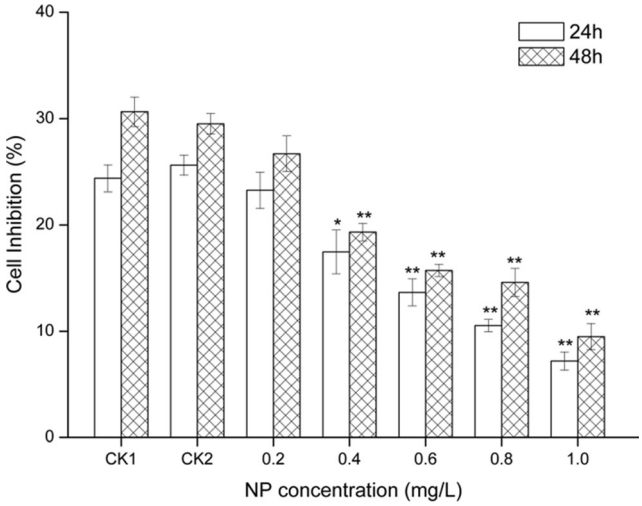

Fig. 3 Cytotoxic of EEGL in HepG-2 cells after 24 or 48-h incubation when *G. lemaneiformis* was exposed to different concentrations of NP. Data were presented as mean ± SD of three replicates. $*P < 0.05$, $**P < 0.01$

G. lemaneiformis treated with high concentrations of NP for 15 days had a lower effect on the generation of ROS when cells treated with the same dose of EEGL.

Cell apoptosis

The active compounds with antitumor potential extracted from macroalgae can induce cell apoptosis (Patra and Muthuraman 2013). To assess the impact of NP on antitumor activity of *G. lemaneiformis*, we examined apoptosis in HepG-2 cells following exposure to EEGL for 48 h by flow cytometry using Annexin V-FITC conjugate and PI labeling. HepG-2 cells were induced to undergo apoptosis significantly by treatment with EEGL compared with the control group (Figs. 5 and 6). Interestingly, the extract from

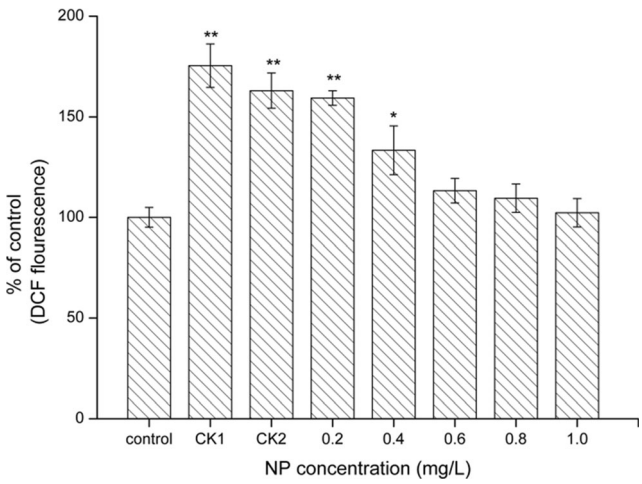

Fig. 4 Effect of EEGL on intracellular ROS generation in HepG-2 cells after 6-h incubation when *G. lemaneiformis* was exposed to different concentrations of NP. Data were presented as mean ± SD of three replicates. $*P < 0.05$, $**P < 0.01$

G. lemaneiformis without exposure to NP induced a higher level of apoptosis in HepG-2 cells (Fig. 5b–c), whereas the apoptosis induced by EEGL decreased significantly with the increase of exposure concentrations of NP to *G. lemaneiformis* (Fig. 5d–h). Those were also supported by the investigations of a higher caspase-3 activity (about 2.7–2.9-fold; Fig. 7) ($P < 0.05$) when *G. lemaneiformis* was not exposed to NP and a decreased caspase-3 activity (about 1.1–2.3-fold) when *G. lemaneiformis* was exposed to NP. The results indicated that NP may exert toxicity effect on antitumor potential of *G. lemaneiformis* via reducing cell apoptosis and the activity of caspase-3.

Discussion

Recently, marine macroalage has received much attention for their positive effects in energy transformation, nutrient cycling, phytoremediation, and medicine (Mendes et al. 2013; Raize et al. 2004; Yeh et al. 2012). The present study indicated that NP may increase the oxidant damage and decrease the antitumor activity of *G. lemaneiformis*. Thereby, the levels of ROS generation, apoptosis, and caspase-3 in tumor cells treated with EEGL were cut down after NP exposure to *G. lemaneiformis* for 15 days.

Antioxidant system plays a vital role in protecting organisms from damage by free radicals. Oxidative stress is one of the important mechanisms involved in the prevention of injury induced by pollutants. Earlier studies have presented enough evidences on the correlation of elevated level of antioxidant enzymes and increased tolerance to toxicity (Kumar et al. 2010; Zhao et al. 2014). SOD is a key enzyme in antioxidant system involved in the process of catalyzing reactive oxygen free radicals to H_2O_2, and subsequently, the produced H_2O_2 could be eliminated to water and molecular oxygen by CAT (Nordberg and Arner 2001). The results showed that the level of SOD increased notably and were much higher than the control group though there was a slight reduction when exposed to 1.0 mg L^{-1} at the end of 15 days. As to CAT, although there was no marked variation in the first day, it was increased sharply and then reduced with the increase of exposure dose and time. Elevated levels of SOD and CAT activities in *G. lemaneiformis* following the NP treatment can be considered as these enzymes could act in combination to adapt the extreme stress against the impact of NP toxicity and oxidative damage (Reddy et al. 2005). Therefore, the reduction of SOD and CAT activities was probably due to the inactivation of enzymes being influenced by the accumulated ROS, which caused damage to antioxidant system through impairing their ability to remove toxic reactive oxygen free radicals and peroxides (Verma and Dubey 2003). The altered antioxidant enzymes were also found in response to the increased oxidative stress on seaweeds with copper and cadmium treatments

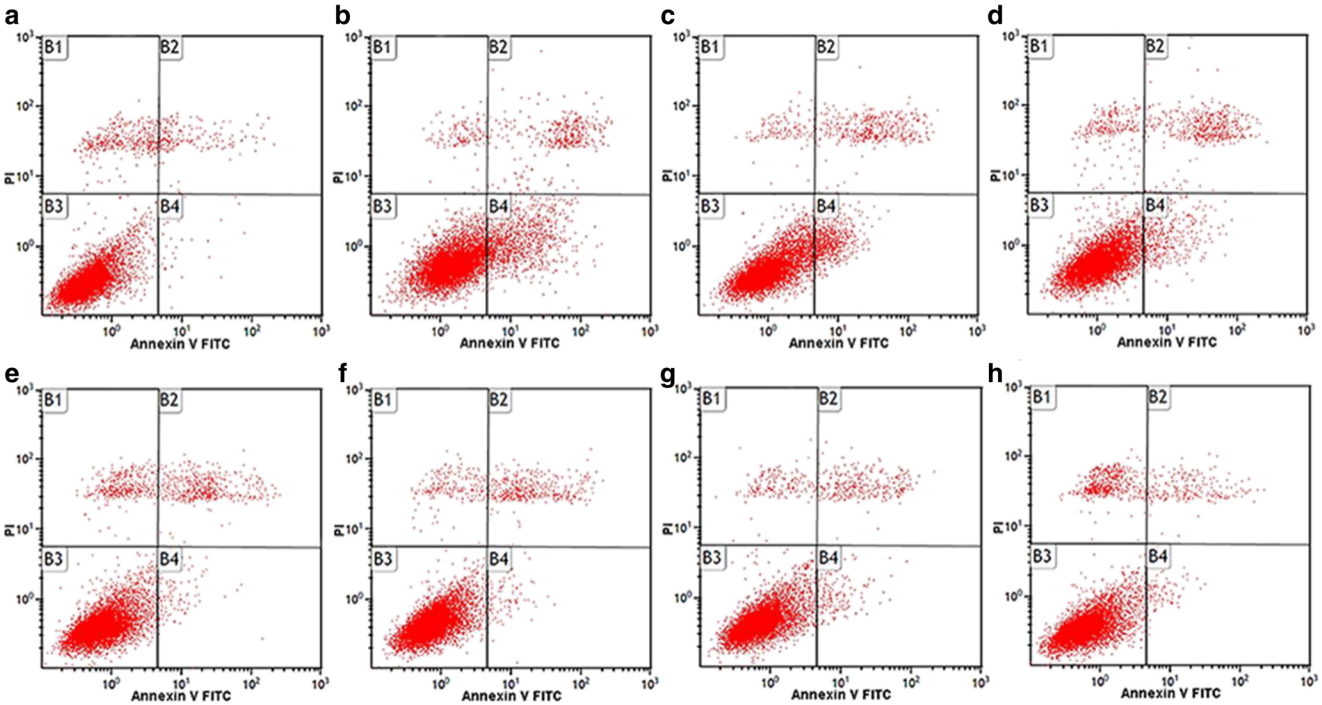

Fig. 5 EEGL-induced apoptosis after 48-h incubation of HepG-2 cells when *G. lemaneiformis* was exposed to different concentrations of NP determined by flow cytometer. **a** Control. **b** CK 1. **c** CK 2. **d** 0.2 mg L^{-1} NP. **e** 0.4 mg L^{-1} NP. **f** 0.6 mg L^{-1} NP. **g** 0.8 mg L^{-1} NP. **h** 1.0 mg L^{-1} NP

(Babu et al. 2014). MDA, one of the important toxic products in lipid peroxidation, may accelerate the membrane damage. And it resulted from free radicals attacking the polyunsaturated fatty (PUFA) in cell membrane by non-enzymatic systems (Papadimitriou and Loumbourdis 2002). Hence, the MDA level can reflect the degree of oxidative damage caused by free radicals indirectly. Results obtained from this study showed that the lipid peroxidation level increased obviously in the majority of experimental groups, indicating that oxidative damage to *G. lemaneiformis* was elicited following the exposure to NP. However, the MDA quantity was decreased under the high concentration exposure of 15 days that was probably due to the rot in algae which causes relevant enzymes inactive.

Alkaline single-cell gel electrophoresis (comet assay) modified by Singh et al. (1988) had been regarded as a useful tool in environmental toxicology to evaluate genotoxicity of pollutants (Papis et al. 2011). In this work, very distinct DNA damages were observed when *G. lemaneiformis* was exposed

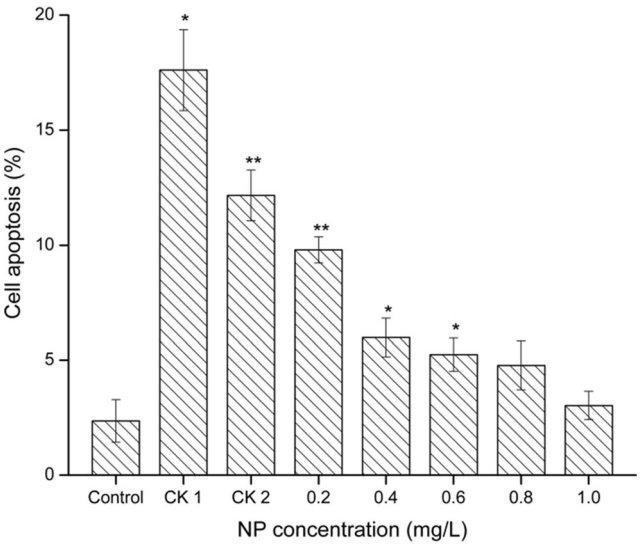

Fig. 6 Effect of EEGL on apoptosis in HepG-2 cells after 48-h incubation when *G. lemaneiformis* was exposed to different concentrations of NP. Data were presented as mean ± SD of three replicates. $*P < 0.05$, $**P < 0.01$

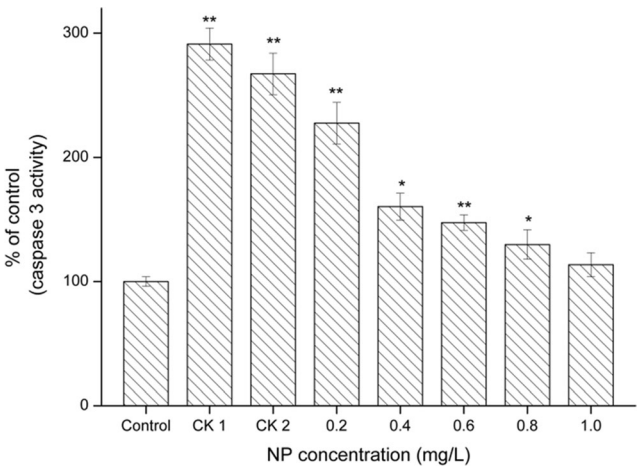

Fig. 7 Effect of EEGL on caspase-3 activity in HepG-2 cells after 48-h incubation when *G. lemaneiformis* was exposed to different concentrations of NP. Data were presented as mean ± SD of three replicates. $*P < 0.05$, $**P < 0.01$

to NP ($P < 0.05$). Babu et al. (2014) also reported that DNA damage was observed in marine macroalgae (*Acanthophora spicifera*, *Chaetomorpha antennina*, and *Ulva reticulata*) with the tail DNA (%) of 12–63% approximately after exposure to cadmium and copper metals. As shown in Fig. 2, the increase of NP concentration increases the percentage of tail DNA in a dose-dependent manner. It turned out that comet assay can be an efficient tool to evaluate the genotoxicity of increasing NP contamination. The results showed that the low concentration of NP induced lower DNA damage in *G. lemaneiformis*. The possible reason was that the enhanced enzymatic or non-enzymatic antioxidants can prevent the generation of ROS and inhibit DAN damage consequently. Therefore, the higher concentrations of NP-induced DNA damage significantly owing to the synergistic effect of antioxidants were not enough to eliminate the generation of ROS and then the DNA stability impaired.

The previous reports demonstrated that marine macroalgae are a promising natural product for cancer treatment due to the high antitumor potential against cancer cells (Sundaram et al. 2012). The bioactive components of *Gracilaria* had been researched and were mainly divided into water, ethanol, methanol, or ethyl acetate extracts (Yeh et al. 2012). Extracts exhibited different cytotoxicity in antiproliferative effect for the difference in extracted solvents or nature products. Patra and Muthuraman (2013) described that the ethanol extract of *Gracilaria edulis* showed strongly cytotoxic effect against Ehrlich ascites tumor (EAT) cells with the IC_{50} of 45 μg mL^{-1} (72 h). Yeh et al. (2012) also reported that the IC_{50} value of the methanol extract of *Gracilaria tenuistipitata* for Ca9-22 oral cells was 326 μg mL^{-1} (24 h). And the ethyl acetate extract of *Colpomenia sinuosa*, *Galaxaura oblongata*, and *Halimeda discoidae* for liver cancer (HuH-7) cells were 112.38, 123.54, and 230.53 μg mL^{-1} (72 h), respectively (Huang et al. 2005). Considering the fact that NP exhibited serious toxicity on *G. lemaneiformis*, we attempted to evaluate whether or not there is toxicity on antitumor activity of algae. Accordingly, the EEGL was isolated, and the toxic effects of NP on antitumor activity of EEGL were evaluated in this study.

To validate this hypothesis, the inhibition of cell proliferative was primary considered and it was verified by MTT assay test in HepG-2 cells. A previous study indicated that the extract of *Indigofera linnaei* Ali has a chemopreventive role in liver HepG-2 cell proliferation, which was in agreement with this study (Kumar et al. 2011). By comparing the activities against HepG-2 cells, the result pointed out that EEGL shows less efficience in inhibiting cell proliferative when *G. lemaneiformis* was exposed to different concentrations of NP. Patra and Muthuraman (2013) reported that the increased apoptosis in EAT cells after treatment with the bioactive extract of seaweeds was positively correlated with the elevation of ROS. The results from this study showed that ROS generated in cells may mediate oxidative stress and subsequently lead to cell apoptosis. However, the current study demonstrated that ROS generation and cell apoptosis were depleted significantly following the NP treatment to *G. lemaneiformis* in a dose-response manner. Accordingly, decrease in ROS generation and cell apoptosis in this work clearly indicated that NP did cause irreversible damage to the antitumor activity of seaweed. To further test the negative role in apoptosis, the caspase-3 signaling pathway was also examined. Caspase-3 is a key enzyme in the process of cell apoptosis. A noticeably decreased caspase-3 activity noted in this work was positively correlated with decreased ROS and apoptosis following NP-treated thallus, which may confirm the results of damage to antitumor potential. In summary, the results indicated that the short-time exposure to NP exhibited oxidative toxicity to *G. lemaneiformis* and seriously impaired its potential against HepG-2 cells in vitro. However, there are still some limitations in this study. A further research should be conducted to research these at cellular and molecular level or evaluate the bioaccumulation of NP in *G. lemaneiformis* and its effect on other organisms through food chain.

Conclusion

Results from this study indicated that NP had negative effect on *G. lemaneiformis* which may result in oxidative stress and DNA damage. In addition, the ethanol extract of *G. lemaneiformis* was a potential drug against tumor cells in vitro and did exert role in inhibiting cell proliferation via inducing apoptosis. On the other hand, it was important to mention that the antitumor activity was depleted significantly following the exposure of NP to macroalgae. It suggested that macroalage can be considered as useful biomarkers of NP contamination and efficient bioindicators to evaluate the genotoxicity of increasing contaminations in marine ecosystem.

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References

Babu MY, Palanikumar L, Nagarani N, Devi VJ, Kumar SR, Ramakritinan C, Kumaraguru A (2014) Cadmium and copper toxicity in three marine macroalgae: evaluation of the biochemical responses and DNA damage. Environ Sci Pollut Res 21:9604–9616

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Correa-Reyes G, Viana MT, Marquez-Rocha FJ, Licea AF, Ponce E, Vazquez-Duhalt R (2007) Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere 68:662–670. doi: 10.1016/j.chemosphere.2007.02.030

Erkekoglu P, Rachidi W, Yuzugullu OG, Giray B, Favier A, Ozturk M, Hincal F (2010) Evaluation of cytotoxicity and oxidative DNA damaging effects of di (2-ethylhexyl)-phthalate (DEHP) and mono (2-ethylhexyl)-phthalate (MEHP) on MA-10 Leydig cells and protection by selenium. Toxicol Appl Pharmacol 248:52–62

Fan Y, Wang W, Song W, Chen H, Teng A, Liu A (2012) Partial characterization and anti-tumor activity of an acidic polysaccharide from *Gracilaria lemaneiformis*. Carbohydr Polym 88:1313–1318

Gupta V, Kumar M, Kumari P, Reddy C, Jha B (2011) Optimization of protoplast yields from the red algae Gracilaria dura (C. Agardh) J. Agardh and *G. verrucosa* (Huds.) Papenfuss Journal of applied phycology 23:209–218

Han ZX, Zhang M, Lv CX (2012) Bioaccumulation and toxicity of NiO nanoparticles in *Gracilaria lemaneiformis*. In: Advanced Materials Research. Trans Tech Publ, pp 942–945

Huang HL et al (2005) Induction of apoptosis by three marine algae through generation of reactive oxygen species in human leukemic cell lines. J Agric Food Chem 53:1776–1781. doi:10.1021/jf049445n

Huang HZ, Liang JS, Wu XS, Zhang H, Li QQ, Zhang QY (2013) Comparison in copper accumulation and physiological responses of *Gracilaria lemaneiformis* and G-lichenoides (Rhodophyceae). Chin J Oceanol Limnol 31:803–812. doi:10.1007/s00343-013-2261-5

Ibrahim M, Jacinto G, Connell D, Leong L (2002) Regionally based assessment of persistent toxic substances region VIII–Southeast Asia and South Pacific. In: Proceeding of Workshop on Environmental Monitoring of Persistent Organic Pollutants (POPs) in East Asian Countries, pp 146–154

Kumar M, Kumari P, Gupta V, Anisha P, Reddy C, Jha B (2010) Differential responses to cadmium induced oxidative stress in marine macroalga *Ulva lactuca* (Ulvales, Chlorophyta). Biometals 23: 315–325

Kumar RS, Rajkapoor B, Perumal P (2011) Antitumor and cytotoxic activities of methanol extract of Indigofera linnaei. Ali Asian Pac J Cancer Prev 12:613–618

Lai K, Scrimshaw M, Lester J (2002) Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. Sci Total Environ 289:159–168

Liu Y, Guan Y, Gao Q, Tam NFY, Zhu W (2010) Cellular responses, biodegradation and bioaccumulation of endocrine disrupting chemicals in marine diatom *Navicula incerta*. Chemosphere 80: 592–599

Mendes LF, Zambotti-Villela L, Colepicolo P, Marinho-Soriano E, Stevani CV, Yokoya NS (2013) Metal cation toxicity in the alga *Gracilaria domingensis* as evaluated by the daily growth rates in synthetic seawater. J Appl Phycol 25:1939–1947

Neori A et al (1996) Seaweed biofilters as regulators of water quality in integrated fish-seaweed culture units. Aquaculture 141:183–199

Nice H, Thorndyke M, Morritt D, Steele S, Crane M (2000) Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. Mar Pollut Bull 40:491–496

Nordberg J, Arner ES (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 31:1287–1312

Papadimitriou E, Loumbourdis SN (2002) Exposure of the frog *Rana ridibunda* to copper: impact on two biomarkers, lipid peroxidation, and glutathione. Bull Environ Contam Toxicol 69:0885–0891. doi: 10.1007/s00128-002-0142-2

Papis E, Davies SJ, Jha AN (2011) Relative sensitivity of fish and mammalian cells to the antibiotic, trimethoprim: cytotoxic and genotoxic responses as determined by neutral red retention. Comet and micronucleus assays Ecotoxicology 20:208–217

Patra S, Muthuraman MS (2013) *Gracilaria edulis* extract induces apoptosis and inhibits tumor in Ehrlich ascites tumor cells in vivo. BMC Complement Altern Med 13:1

Raize O, Argaman Y, Yannai S (2004) Mechanisms of biosorption of different heavy metals by brown marine macroalgae. Biotechnol Bioeng 87:451–458

Reddy MK, Alexander-Lindo RL, Nair MG (2005) Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation by natural food colors. J Agric Food Chem 53:9268–9273

Simioni C et al (2014) Effects of ultraviolet radiation (UVA+ UVB) on young gametophytes of Gelidium floridanum: growth rate, photosynthetic pigments, carotenoids, photosynthetic performance, and ultrastructure. Photochem Photobiol 90:1050–1060

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Sunassee SN, Davies-Coleman MT (2012) Cytotoxic and antioxidant marine prenylated quinones and hydroquinones. Nat Prod Rep 29: 513–535

Sundaram M, Patra S, Maniarasu G (2012) Antitumor activity of ethanol extract of *Gracilaria edulis* (Gmelin) Silva on Ehrlich ascites carcinoma-bearing mice. Zhong Xi Yi Jie He Xue Bao 10:430–435

Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–278

Verma S, Dubey R (2003) Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci 164:645–655

Wang Z, Wang X, Ke C (2014) Bioaccumulation of trace metals by the live macroalga *Gracilaria lemaneiformis*. J Appl Phycol 26:1889–1897

Xia J, Li Y, Lu J, Chen B (2004) Effects of copper and cadmium on growth, photosynthesis, and pigment content in *Gracilaria lemaneiformis*. Bull Environ Contam Toxicol 73:979–986

Xu Z, Gao K (2012) NH_4^+ enrichment and UV radiation interact to affect the photosynthesis and nitrogen uptake of *Gracilaria lemaneiformis* (Rhodophyta). Mar Pollut Bull 64:99–105

Yang Y, Chai Z, Wang Q, Chen W, He Z, Jiang S (2015) Cultivation of seaweed Gracilaria in Chinese coastal waters and its contribution to environmental improvements. Algal Res 9:236–244

Ye C, Liao H, Yang Y (2014) Allelopathic inhibition of photosynthesis in the red tide-causing marine alga, *Scrippsiella trochoidea* (Pyrrophyta), by the dried macroalga, *Gracilaria lemaneiformis* (Rhodophyta). J Sea Res 90:10–15

Yeh C-C et al (2012) Anti-proliferative effect of methanolic extract of Gracilaria tenuistipitata on oral cancer cells involves apoptosis, DNA damage, and oxidative stress. BMC Complement Altern Med 12:1

Zandi K, Tajbakhsh S, Nabipour I, Rastian Z, Yousefi F, Sharafian S, Sartavi K (2013) In vitro antitumor activity of Gracilaria corticata (a red alga) against Jurkat and molt-4 human cancer cell lines. Afr J Biotechnol 9:6787–6790

Zhao X, Gao Y, Qi M (2014) Toxicity of phthalate esters exposure to carp (*Cyprinus carpio*) and antioxidant response by biomarker. Ecotoxicology 23:626–632