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Prevention of oxidative stress in Chang liver cells by gallic acid-*grafted*-chitosans

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ABSTRACT

Gallic acid-*grafted*-chitosans (GA-*g*-chitosans) were prepared according to our previous method, and the *in vitro* protective effect of the various GA-*g*-chitosans on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative stress was investigated in Chang liver cells. Pretreatment of the GA-*g*-chitosans decreased cell damage induced by *t*-BHP in a dose-dependent manner. In addition, *t*-BHP-induced reactive oxygen species (ROS) generation and lipid peroxidation were inhibited by the GA-*g*-chitosans. Also, intracellular glutathione (GSH) was increased in the presence of the GA-*g*-chitosans. And GA-*g*-chitosan (I), which had the highest GA content, showed the highest activity among the tested compounds. Moreover, antioxidant enzyme activities such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were decreased by *t*-BHP-induction., while GA-*g*-chitosan (I) pretreatment increased levels of CAT, SOD, and GPx. Overall, we demonstrated that GA-*g*-chitosans effectively attenuated the oxidative stress induced by *t*-BHP in Chang liver cells by inhibiting ROS generation, lipid peroxidation, and increasing levels of antioxidant enzymes.

1. Introduction

Aerobic cells frequently undergo ill environmental conditions due to the production of excessive reactive oxygen species (ROS), and oxidative stress is the final result (Meydani, Wu, Santos, & Hayek, 1995). This may involve a series of pathological conditions such as ageing, cancer, metabolic disorders, and inflammation (Datta, Sinha, & Chattopadhyay, 2000; Halliwell, 1997). However, oxidative stress is efficiently mediated by antioxidant defence systems minimizing the harmful effects (Dimmeler, Hermann, Galle, & Zeiher, 1999; Hermann, Zeiher, & Dimmeler, 1997). The formation of ROS is enhanced when living organisms are exposed to heavy metals such as Fe^{2+} or to certain organic contaminants such as herbicides, halogenated organic compounds, or organic hydroperoxides.

The organic hydroperoxide, *tert*-butylhydroperoxide (*t*-BHP), is a useful model compound for the study of mechanisms of oxidative cell injury (Altman et al., 1994; Coleman, Gilfor, & Farber, 1989). Organic hydroperoxides form as a result of oxygen additions to alkyl radicals, and/or by hydrogen atom abstraction from peroxyl radicals (Sandstrom, 1991).

Chitosan, a natural-based biopolymer obtained by alkaline deacetylation of chitin, is a nontoxic, biocompatible, and biodegradable material (Muzzarelli, Baldassarre, Conti, Ferrara, & Biagini, 1988). These properties make chitosan a good candidate for the development of novel drugs and an ingredient of functional foods. In recent decades, many researchers have focused on chitosan as a potential source of bioactive material and have used it as an antioxidant and anticancer, antiinflamatory, antibacterial, and antihypertensive agent (Je & Kim, 2006; Lee, Jeong, Kim, Lee, Ahn, & Je, 2009; Lee, Senevirathne, Ahn, Kim, & Je, 2009; Muzzarelli, 2009; Ngo, Qian, Je, Kim, & Kim, 2008). Therefore, much attention has been given to chitosan for its utilization in the biomedical, food, and chemical industries, but the practical use of chitosan has mainly been confined to the unmodified forms (Muzzarelli et al., 1988; Razdan & Pettersson, 1994). However, chemical modifications of chitosan are useful for the association of bioactive molecules to polymers and for enhancing biological properties. In our previous study, we demonstrated improvements in the antioxidant capacity of chitosan by grafting gallic acid onto chitosan (Cho, Kim, Ahn, & Je, 2011). The objective of the present study was to evaluate the protective effects of gallic acid-*grafted*-chitosans (GA-*g*-chitosan) against *t*-BHP induced oxidative stress in Chang liver cells.

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2. Materials and methods

2.1. Materials

Fluorescence probes 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA), diphenyl-1-pyrenylphosphine (DPPP), 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), and monobromobimane (mBBr) were purchased from Sigma Co. (St. Louis, USA). The Chang liver cell line (CCL-13) was obtained from the American Type of Culture Collection (Manassas, VA, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin/streptomycin, and other materials required for culturing the cells were purchased from Gibco BRL (Paisley, UK). An enzyme assay commercial kit was supplied by Biovision (CA, USA). All other chemicals used were of analytical grade.

2.2. Preparation of gallic acid-grafted-chitosans

GA-*g*-chitosans were prepared according to our previous report (Cho et al., 2011). Briefly, 0.5 g of chitosan was dissolved in 50 mL of 2% acetic acid. Then, 1 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. After 30 min, gallic acid was added and incubated at room temperature for 24 h. Next, the mixture was dialyzed against water for another 48 h. The molar ratios of chitosan units and gallic acid were 1:1, 1:0.5, 1:0.25, and 1:0.1 for GA-*g*-chitosan (I) to (IV) respectively. ^{1}H NMR spectrometry and TLC analysis were carried out as described by Cho et al. (2011); the gallic acid contents in GA-*g*-chitosans were in the order of GA-*g*-chitosan (I) > GA-*g*-chitosan (II) > GA-*g*-chitosan (III) > GA-*g*-chitosan (IV).

Plain chitosan: ^{1}H NMR (400 MHz, D_2O) δ: 5.30 (1H, H-1), 3.63–4.35 (1H, H-2/6), 2.51 (H-Ac), 4.8 (D_2O). GA-*g*-chitosan: ^{1}H NMR (400 MHz, D_2O) δ: 7.63 (phenyl protons of gallic acid), 5.33 (1H, H-1), 3.65–4.36 (1H, H-2/6), 2.51–2.54 (H-Ac), 4.8 (D_2O).

2.3. Cell culture and treatment

The Chang liver cells purchased from ATCC were cultured in DMEM medium containing heat-inactivated 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL) at 37 °C in an incubator under a humidified atmosphere of 95% air and 5% CO_2. Adherent cells were detached by trypsin-EDTA and plated onto 6- or 96-well plates at 70–80% confluence. The cells were treated in the presence or absence of different concentrations of various GA-*g*-chitosans at final concentrations of 100, 250, and 500 μg/mL.

2.4. Cell viability assay

Cell viability was estimated by MTT assay, which is a test of the normal metabolic status of cells based on the assessment of mitochondrial activity (Hansen, Nielsen, & Berg, 1989).

2.5. Determination of intracellular reactive oxygen species

Intracellular formation of reactive oxygen species (ROS) was assessed as described previously using the oxidation sensitive dye DCFH-DA as a substrate (Takahashi, Shibata, & Niki, 2001). Chang liver cells were seeded in 96-well plates at a concentration of 4×10^5 cells/mL.

2.6. Lipid peroxidation inhibitory assay

Chang liver cells were seeded into 96-well plates at a concentration of 4.0×10^5 cells/mL. Cells growing at confluency were labelled with DPPP (in DMSO) at a final concentration of 25 μM and incubated in a humidified CO_2 incubator at 37 °C for 30 min in the dark (Takahashi et al., 2001). The cells were then treated with GA-*g*-chitosans and incubated another 1 h at 37 °C. Next, the cells were washed three times with HBSS and treated with 100 μL of 150 μM *t*-BHP, except for the normal cell group, and incubated at 37 °C for 30 min. Fluorescence intensity was measured with a spectroflurometer (Spectra Max) at excitation and emission wavelengths of 351 and 380 nm, respectively.

2.7. Determination of intracellular glutathione level

Cellular GSH level was determined using monobromobimane (mBBr) as a thiol-staining reagent via the method described by Poot, Verkerk, Koster, and Jongkind (1986) with slight modifications. Chang liver cells were seeded at a concentration of 4.0×10^5 cells/mL and following confluency were treated with GA-*g*-chitosans for 1 h. The cells were then labelled with 40 μM mBBr for 30 min in a CO_2 incubator at 37 °C in the dark. After staining, mBBr–GSH fluorescence intensity was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm using a spectroflurometer (Spectra Max). The average fluorescence values of cell populations were plotted and compared with a blank group in which cells were grown without treatment of GA-*g*-chitosans.

2.8. Preparation of cell lysate and determination of protein content

Chang liver cells were seeded in 6-well plates at a concentration of 4.0×10^5 cells/mL. At 70–80% confluence, the cells were treated with GA-*g*-chitosans, except for the blank and control groups, and incubated in a humidified CO_2 incubator at 37 °C for 1 h. Then, 150 μM *t*-BHP was added to the cells, but not to the blank group, as a final concentration, with incubation for another 24 h at 37 °C. The cells were collected and washed three times in HBSS. Then, the HBSS was removed and the cells were lysed in 400 μL of lysis buffer (50 mM Tris–HCl, pH 7.5, 0.5% Triton X-100) for 2 h. The supernatants were obtained by centrifugation at 8000 rpm and 4 °C for 10 min and the protein contents were determined via a BSA assay kit (BioRad, CA, USA), using bovine serum albumin as a standard.

2.9. Determination of antioxidant enzymes

2.9.1. Catalase activity

The CAT (E.C. 1.11.1.6) activity of cell lysates was determined using a commercially available kit (Biovision). One unit of catalase was defined as the amount of enzyme required to decompose 1 μM of H_2O_2 in 1 min. The rate of decomposition of H_2O_2 was measured spectrophotometrically at a wavelength of 570 nm and the enzyme activity was expressed as mU/mg protein.

2.9.2. Superoxide dismutase activity

The SOD (E.C.1.15.1.1) level in cell lysates was estimated using a commercial kit supplied by Biovision (CA, USA). Xanthine and xanthine oxidase were used to generate superoxide anion, which reacts with tetrasolium chloride to form a yellow color formazan dye. SOD activity was measured at a wavelength of 450 nm and the enzyme activity was expressed as mU/mg of protein.

2.9.3. Glutathione peroxidase activity

The enzyme reaction in a tube, which contained NADPH, reduced glutathione, and glutathione reductase, was initiated with the addition of cumene hydroperoxide, and the change in absorbance at 340 nm was monitored by a spectrophotometer. GPx (E.C. 1.11.1.9) activity was determined using a commercial kit supplied by Biovision and the activity was given as mU/mg of protein.

Table 1
Cytotoxicity of *tert*-butyl hydroperoxide in Chang liver cells.

	t-BHP (μM)							
	100	110	120	130	140	150	160	170
Cell viability (%)	86.07 ± 0.73	77.05 ± 1.58	68.80 ± 4.60	62.32 ± 5.75	54.35 ± 1.16	46.69 ± 5.58	45.40 ± 4.17	46.19 ± 3.62

Cells (4×10^5 cells/mL) were cultured in DMEM medium and cell viability was measured by MTT assay after 24 h after each treatment. Results are expressed as means ± SD of three determinations.

Table 2
Cytotoxicity of gallic acid-*grafted*-chitosans in Chang liver cells.

Treatment	Concentration (μg/mL)		
	100	250	500
GA-*g*-chitosan (I)	95.32 ± 4.56	96.69 ± 2.42	97.95 ± 5.17
GA-*g*-chitosan (II)	93.05 ± 5.43	98.68 ± 2.44	98.10 ± 4.61
GA-*g*-chitosan (III)	94.26 ± 3.18	96.89 ± 7.91	99.52 ± 2.41
GA-*g*-chitosan (IV)	95.24 ± 6.24	93.75 ± 1.88	99.44 ± 3.41

Cells (4×10^5 cells/mL) were cultured in DMEM medium and cell viability was measured by MTT assay after 24 h after each treatment. Results are expressed as means ± SD of three determinations.

2.10. Statistical analysis

All values are expressed as means ± SD (n = 3). The statistical significance of differences was analyzed by Student's *t*-test using SPSS (Chicago, IL, USA). *Significant differences at $p < 0.05$ level were compared with *t*-BHP treated group for cell viability, DCF, DPPP and antioxidant enzyme, and compared with blank group for mBBr-GSH.

3. Results and discussion

3.1. Effects of gallic acid-grafted-chitosans on cell cytotoxicity and cell viability

The cytotoxicity of *t*-BHP was assessed via MTT assay by exposing Chang liver cells to various concentrations of *t*-BHP (50–200 μM), and the 150 μM concentration was selected as the optimal dose to cause oxidative stress in the Chang liver cells (Table 1). In a similar fashion, different concentrations of GA-*g*-chitosans were tested to determine their optimal dose and cytotoxicity, if any, towards Chang liver cells (Table 2). The GA-*g*-chitosans had no significant ($p < 0.05$) cytotoxicity compared to the control cell group which showed 100% cell viability up to 500 μg/mL, and over 90% viability was observed. Therefore, all experiments were conducted using 100–500 μg/mL of GA-*g*-chitosans.

Cell viability was significantly decreased ($p < 0.05$) in the cells treated with *t*-BHP alone, while pre-treatment of the cells with GA-*g*-chitosans significantly increased ($p < 0.05$) viability in a dose-dependent manner compared to cells treated with *t*-BHP alone (Table 3). This proves that GA-*g*-chitosans are able to attenuate the viability of cells by reacting with the radicals formed by *t*-BHP. Numerous results for GA-*g*-chitosans may be due to the different molar ratios between chitosan units and gallic acid. Moreover, Cho et al. (2011) have confirmed that GA-*g*-chitosans are potential antioxidant compounds with profound activities.

3.2. Effects of gallic acid-grafted-chitosans on reactive oxygen species production, lipid peroxidation, and glutathione level in Chang liver cells

ROS inhibition% of GA-*g*-chitosans is depicted in Table 4. ROS were significantly ($p < 0.05$) increased in Chang liver cells treated only with *t*-BHP. However, ROS were significantly ($p < 0.05$) inhibited in the cells pre-treated with GA-*g*-chitosans. ROS inhibition% was highest in the cells pre-treated with GA-*g*-chitosan (I), which had the highest GA content, and this inhibition indicated the potential of grafting gallic acid against ROS formation. The inhibition of ROS generation was in the order of GA-*g*-chitosan (I) > GA-*g*-chitosan (II) > GA-*g*-chitosan (III) > GA-*g*-chitosan (IV). Moreover, the inhibition% was dose and time dependent (time dependent data was not shown).

DPPP is a fluorescent dye that is used to detect lipid peroxidation products in cell membranes, and it can react with lipid hydroperoxides to give a fluorescent product. The treatment of cells with *t*-BHP alone resulted in an increase in DPPP = O fluorescence indicating increased% of lipid peroxidation. However, the cell groups pre-treated with GA-*g*-chitosans significantly ($p < 0.05$) inhibited lipid peroxidation in a dose-dependent manner as seen in Table 4. GA-*g*-chitosan (I) showed the highest inhibition (45.68%) against lipid peroxidation at a concentration of 500 μg/mL.

A significant ($p < 0.05$) increase in GSH concentration was observed in the cells treated with GA-*g*-chitosans compared to a blank group as measured by increment of mBBr-GSH (Table 4). GA-*g*-chitosan (I) showed the highest increment% compared to those of other GA-*g*-chitosan and also the increment was dose dependent as seen in the table. Furthermore, the GA-*g*-chitosans showed time dependent increases in mBBr-GSH increment (data not shown).

Oxidative stress is defined as a condition in which cellular antioxidant defences are insufficient to keep the ROS levels below a toxic threshold. When a oxidative stress condition is prevailed in

Table 3
Protective effects of gallic acid-*grafted*-chitosans against *t*-BHP-induced oxidative stress in Chang liver cells.

Treatment	Cell viability%			
		Concentration (μg/mL)		
		100	250	500
t-BHP (150 μM)	54.97 ± 4.29			
GA-*g*-chitosan (I)		67.43 ± 0.61*	73.21 ± 2.36*	79.21 ± 2.47*
GA-*g*-chitosan (II)		62.80 ± 1.41*	68.40 ± 1.62*	72.69 ± 2.82*
GA-*g*-chitosan (III)		52.51 ± 2.29	62.49 ± 2.03*	70.95 ± 2.57*
GA-*g*-chitosan (IV)		52.09 ± 5.35	61.25 ± 2.36	68.58 ± 1.44*
Plain chitosan		50.35 ± 4.31	53.87 ± 3.26	59.87 ± 4.22

Cells (4×10^5 cells/mL) were cultured in DMEM medium and treated with GA-*g*-chitosans for 1 h, and then cells were exposed to *t*-BHP further incubated for 24 h. Cell viability was measured by MTT assay, and results are expressed as means ± SD of three determinations.

* Significant differences at $p < 0.05$ level were compared with *t*-BHP treated group.

Table 4
Effects of gallic acid-*grafted*-chitosans on the formation of intracellular ROS generation, lipid peroxidation, and GSH content.

Sample	Concentration (μg/mL)	Inhibition% DCF intensity	DPPP intensity	Increment% mBBr-GSH intensity
GA-*g*-chitosan (I)	100	14.04 ± 4.70*	18.05 ± 11.63*	6.90 ± 1.72*
	250	21.99 ± 5.26*	43.27 ± 9.28*	8.71 ± 1.98*
	500	34.01 ± 4.89*	45.68 ± 2.98*	10.65 ± 2.26*
GA-*g*-chitosan (II)	100	5.39 ± 5.26	8.11 ± 6.88	−2.43 ± 1.98
	250	12.80 ± 2.32	26.21 ± 7.07*	3.08 ± 1.88*
	500	29.40 ± 0.58*	38.27 ± 4.83*	7.93 ± 1.73*
GA-*g*-chitosan (III)	100	3.52 ± 2.89	8.20 ± 8.05	3.41 ± 1.51
	250	9.04 ± 3.67	24.47 ± 1.35*	3.82 ± 1.75*
	500	15.52 ± 2.98*	36.96 ± 5.25*	4.01 ± 1.84*
GA-*g*-chitosan (IV)	100	ND	7.46 ± 5.42	−0.63 ± 1.73
	250	ND	21.99 ± 3.19*	1.25 ± 1.54
	500	5.20 ± 1.76	34.87 ± 2.17*	1.70 ± 1.94

Intracellular formation of ROS was assessed using the oxidation sensitive dye, DCFH-DA, lipid peroxidation by DPPP and GSH level by mBBr. Results are expressed as means ± SD of three determinations.
ND, not detected.
* Significant differences at $p < 0.05$ level were compared with *t*-BHP treated group for DCF and DPPP and control group for mBBr-GSH.

the cells the most common ROS such as hydroxyl, superoxide, and nitric oxide act as electron acceptors from biomolecules including lipid, proteins and nucleic acids, leading to their oxidation. However, various molecules including chitosan derivatives are able to scavenge the radicals at different levels (Cho et al., 2011). Moreover, the scavenging abilities of grafted chitosan derivatives were related to the number of active hydroxyl groups in the molecules (Li, Jiang, Xue, & Chen, 2002). On the other hand, according to the free radical theory, the amino groups in chitosan can react with free radicals to form stable macromolecules (Sun, Xu, Liu, Xue, & Xie, 2003). Hence, the ROS and lipid peroxidation results obtained in this study are in accordance with the reported information. Since, the GA-*g*-chitosans have gallic acid residues in its molecules they showed profound activities than that of plain chitosan. Moreover, the concentration of GSH in most mammalian cells is relatively high and is continuously synthesized to maintain its level (Orrenius, 1994). The potential of GA-*g*-chitosans to maintain GSH at high levels could be of great importance to combat oxidative stress-induced toxicity in cells. As shown in Table 4, treatment of GA-*g*-chitosans significantly ($p < 0.05$) increased the level of intracellular GSH in Chang liver cells. Therefore, this result indicates that up-regulation of GSH concentration by GA-*g*-chitosans may be due to attenuation of oxidative stress-induced cytotoxicity.

3.3. Effects of gallic acid-grafted-chitosans on antioxidant enzyme activity in Chang liver cells

Antioxidant enzyme activities were evaluated under oxidative stress induced by *t*-BHP. As shown in Table 5, CAT, GPx, and SOD activities were decreased significantly ($p < 0.05$) by *t*-BHP, while the activities of these enzymes were augmented in Chang liver cells pre-treated with GA-*g*-chitosans. *t*-BHP treatment reduced CAT activity to 20.53 mU/mg of protein; however, pre-treatment of the cells with GA-*g*-chitosans restored CAT activity to above 36 mU/mg of protein, and GA-*g*-chitosan (I) showed the highest enzyme activity (around 50 mU/mg of protein). The exposure of cells to *t*-BHP decreased GPx activity to 8.46 mU/mg of protein but restored activity to 19.95 mU/mg of protein by pre-treatment with GA-*g*-chitosan (I). In regard to SOD activity, GA-*g*-chitosan (I) pre-treatment exhibited 1.8 mU/mg of protein compared to 0.09 mU/mg of protein in *t*-BHP treatment cells. The increment of CAT was much higher compared to SOD and GPx, indicating that CAT plays a more important role in mediating oxidative stress than those of other enzymes. The results showed that the enhancement of enzymatic activities by GA-*g*-chitosans may be related to the inhibition of ROS production. As shown in Table 5, CAT, GPx, and SOD activities were reduced by *t*-BHP treatment alone, while pre-treatment of GA-*g*-chitosans, in particular GA-*g*-chitosan (I), increased CAT, GPx, and SOD activities significantly ($p < 0.05$), suggesting that the scavenging of ROS by GA-*g*-chitosans may in part be related to increased activities of antioxidant enzymes.

Table 5
Effects of gallic acid-*grafted*-chitosans on antioxidant enzyme activities against *t*-BHP-induced oxidative stress in Chang liver cells.

Treatment	CAT	GPx	SOD
	mU/mg protein		
Blank	36.99 ± 1.1	17.79 ± 1.2	1.08 ± 0.02
t-BHP	20.53 ± 1.2	8.46 ± 1.0	0.09 ± 0.03
GA-*g*-chitosan (I)	50.20 ± 1.4*	19.95 ± 0.9*	1.80 ± 0.02*
GA-*g*-chitosan (II)	46.93 ± 1.1*	6.29 ± 0.6	1.20 ± 0.04*
GA-*g*-chitosan (III)	36.81 ± 1.2*	3.69 ± 0.3	0.54 ± 0.05*
GA-*g*-chitosan (IV)	36.26 ± 0.6*	2.82 ± 0.1	0.12 ± 0.01

Results are expressed as means ± SD of three determinations.
* Significant differences at $p < 0.05$ level were compared with *t*-BHP treated group.

The cells possess an intricate network of defence mechanisms including antioxidant compounds such as GSH, and antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidases to neutralize excessive ROS accumulation. A major type of defence system in living tissues against oxidative damage is the use of antioxidant enzymes to convert ROS into non-toxic compounds and the tissue activities of those enzymes have been reported to be changed in response to the oxidative stress (Wispe, Warner, Clark, Dey, & Neuman, 1992; Jaruga, Zastawny, Skokowski, Dizdaroglu, & Olinski, 1994). In this study the antioxidant enzyme activity was significantly ($p < 0.05$) reduced by the stimulation of cells with *t*-BHP. However, due to potential activity of GA-*g*-chitosans to act against oxidative stress, the antioxidant enzyme activity was significantly ($p < 0.05$) increased in Chang liver cells pre-treated with GA-*g*-chitosans and reduced the oxidative stress via inhibition of ROS and lipid peroxidation as well as increment of antioxidant enzymes.

4. Conclusion

In conclusion, under the described experimental conditions, GA-*g*-chitosans afforded protection against *t*-BHP-induced cytotoxicity by inhibiting intracellular ROS generation and lipid peroxidation and by increasing the level of GSH in Chang liver cells. In addition, GA-*g*-chitosans increased antioxidant enzyme activities under *t*-BHP-induced oxidative stress. Thus, scavenging of ROS and effects on antioxidant enzyme defence systems are possible

mechanisms that suppressed oxidative stress in the *t*-BHP-induced Chang liver cells. Moreover, GA-*g*-chitosans exhibited cytocompatibility against Chang liver cells. These results prove that the optimized grafting ratio of GA-*g*-chitosans might provide beneficial effects in the treatment of disorders related to oxidative stress. Therefore, GA-*g*-chitosans may be potential agents in the preparation of pharmaceutical formulations and they may be potential functional foods in the food industry as they prevent or reduce the lipid peroxidation as well.

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