Bioorganic & Medicinal Chemistry Letters 21 (2011) 3070–3073

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Chitosan gallate as potential antioxidant biomaterial

Young-Sook Cho [a], Se-Kwon Kim [b,*], Jae-Young Je [a,*]

[a] School of Food Technology and Nutrition, Chonnam National University, Yeosu 550-749, Republic of Korea
[b] Department of Chemistry and Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea

ARTICLE INFO

Article history:
Received 12 January 2011
Revised 2 March 2011
Accepted 9 March 2011
Available online 16 March 2011

Keywords:
Chitosan
Gallic acid
Antioxidant activity
ROS
DNA damage

ABSTRACT

Chitosan gallate were synthesized using a free radical-induced grafting reaction. Chitosan gallate showed enhanced water-solubility compared to plain chitosan, and exhibited good thermal stability. The IC_{50} value of chitosan gallate against 2,2-diphenyl-1-picrylhydrazyl (DPPH) was 17.86 μg/mL. In addition, chitosan gallate effectively inhibited the generation of intracellular reactive oxygen species (ROS), and also suppressed lipid peroxidation in RAW264.7 macrophage cells. Chitosan gallate also exhibited the protection effect on genomic DNA damage by induced hydroxyl radical, and up-regulated the protein expression of antioxidant enzymes including superoxide dismutase-1 and glutathione reductase under H_2O_2-mediated oxidative stress in RAW264.7 macrophage cells. These results indicate that chitosan gallate might be potential antioxidant biomaterials.

Chitosan, the N-deacetylated derivative from chitin, is a polycationic polymer comprised of mainly glucosamine units. There has been increasing interest in chitosan due to their less toxic nature, biodegradability, and biocompatability as well as its versatile bioactivities such as antibacterial, antitumor, immune-stimulating and antihypertensive characteristics.[1–4] However, chitosan is supposed to be changed to water-soluble property by enzymatic hydrolysis and chemical modification for industrial applications because its insoluble property is major limiting factor for applications. Currently, much attention has been paid to develop for chitosan derivatives with water-soluble property, and further numerous research works had been carried out to develop the antioxidant chitosan derivatives including chitosan sulfates, sulfanilamide derivative of chitosan, *N*-carboxymethyl chitosan oligosaccharides, and quaternized carboxymethyl chitosan.[5–8] It is well known that the antioxidant mechanism of chitosan and their derivatives is attributed to their hydrogen donating ability to free radical and ROS. Therefore, introducing antioxidant compounds with good hydrogen donating ability onto chitosan would be provided a new antioxidant compounds based on chitosan.

Reactive oxygen species (ROS) are produced by abnormal metabolic processes as well as sunlight, ultraviolet light, and chemical reactions, and can change the structure of DNA, membrane lipids, and protein, which may result in disease such as cancer, aging, inflammation, and atherosclerosis.[9] Therefore, supplementation of antioxidants, which can overcome oxidation-mediated problems, may prevent the body from a set of diseases by directly quenched ROS. Therefore, there has been a growing interest to develop natural antioxidant compounds from many sources and to develop new antioxidant compounds from the combination of natural antioxidant compounds. In this study, we prepared chitosan gallate conjugation by green method, and investigate antioxidant properties of chitosan gallate.

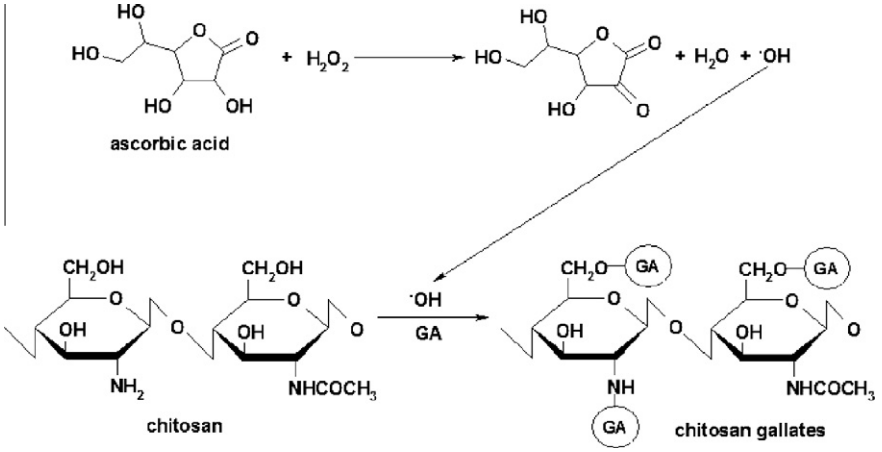
Chitosan was kindly donated from Kitto Life Co. (Seoul, Korea). Average molecular weight and degree of deacetylation of chitosan is 310 kDa and 90%, respectively. Chitosan gallate was prepared according to previous reports with optimum conditions.[10] Briefly chitosan (0.5 g) was dissolved in 50 mL of 2% acetic acid (v/v), and 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 into the mixture with the same molar ratio. Plain chitosan was prepared as the same process without addition of gallic acid. Finally, the mixture was allowed at 25 °C for 24 h under atmospheric air, and then dialyzed with distilled water for 48 h in order to remove unreacted gallic acid (Scheme 1).

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) and chitosan gallate—^{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).

Water-solubility of chitosan gallate was evaluated from the turbidity. Chitosan gallate was dissolved in deionized water, and the transmittance of the solution was recorded on an ELISA reader (SpectraMax® M2/M2^e, CA, USA) using a quartz cell with an optical path length of 1 cm at 600 nm.

* Corresponding authors. Tel.: +82 51 629 7098; fax: +82 51 629 7096 (S.-K.K.); tel.: +82 61 659 7416; fax: +82 61 659 7419 (J.-Y.J.).
E-mail addresses: sknkim@pknu.ac.kr (S.-K. Kim), jjy1915@chonnam.ac.kr (J.-Y. Je).

0960-894X/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.03.033



Scheme 1. Synthesis pathway of chitosan gallate.

DPPH radical scavenging assay was measured according to the method of Blois[11] with slight modifications, and for thermal stability, chitosan gallate was incubated at 100 °C for 30 and 60 min, and then DPPH radical scavenging assay was performed.

For investigation of antioxidant properties in cellular system, RAW264.7 macrophage cells were applied and cytotoxicity of chitosan gallate was assayed using MTT assay.

The lipid peroxidation inhibition activity of chitosan gallate was assessed by the fluorescence probe, DPPP, using a previously described method.[12]

For analysis of intracellular ROS formation, the redox-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was used. The levels of intracellular ROS in the collected cells were determined using FACS Calibur™ flow cytometer (488 nm excitation, 530 nm emission) equipped with CELLQUEST analysis software (Becton Dickinson, Mountain View, CA, USA).

For protection effect of chitosan gallate on radical-mediated DNA damage, genomic DNA was pretreated with various concentrations of chitosan gallate and exposed to hydroxyl radical using Fenton reaction as described by Milne et al.[13]

The effect of chitosan gallate on the expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione reductase (GSR) under H_2O_2-mediated oxidative stress, western blot analysis was performed using standard procedure.

All experiments were performed in triplicate. Where required, statistical analyzes were performed using Student's *t*-test.

Water-solubility of plain chitosan and chitosan gallate was investigated using transmittance by measuring at 600 nm. Each sample was dissolved in water at 2.0 and 1.0 mg/mL. The transmittance of chitosan gallate was recorded 89.5% and 77.2% at 2.0 and 1.0 mg/mL, while plain chitosan was not exceed 30% transmittance through all concentrations. This result suggests that grafting of gallic acid onto chitosan enhanced the water-solublity by disrupting inter- and intra-molecular hydrogen bonding.

DPPH radical scavenging assay is widely accepted as antioxidant assay because the change of color of DPPH solution is easily detected in the presence of antioxidant molecules by hydrogen donating property. As shown in Table 1, IC_{50} values of chitosan gallate toward DPPH radical was improved compared with plain chitosan. Plain chitosan recorded 92.59 μg/mL of IC_{50}, while chitosan gallate showed 17.86 μg/mL of IC_{50}, it was a 5.2-fold increment compared with that of plain chitosan.

We further investigated thermal stability of chitosan gallate because most water-soluble antioxidant molecules such as vitamin C showed weak thermal stability. Thus, the desired concentration of chitosan gallate was incubated at 100 °C for 30 and 60 min and then DPPH radical scavenging assay was performed. It was observed that the DPPH radical scavenging activity was not altered, and this result indicate that chitosan gallate possess good thermal stability (data not shown).

Prior to investigation of antioxidant properties on cell culture system, cytotoxic levels plain chitosan and chitosan gallate on RAW264.7 macrophage cells were measured using MTT assay. It was observed that chitosan gallate and plain chitosan did not exerted toxic effect on RAW264.7 macrophage cells up to 500 μg/mL.

Unsaturated fatty acids in cell membrane lipids are extremely susceptible for the free radical attack during oxidation. The levels of lipid hydroperoxides were examined in the presence or absence of chitosan gallates using specific fluorescence probe. After 6 h of treatment with AAPH, more than 2-fold increment of DPPP oxide

Table 1
IC_{50} values of chitosan gallate and plain chitosan against DPPH

	IC_{50} (μg/mL)
Plain chitosan	92.59 ± 2.41
Chitosan gallate	17.86 ± 1.62

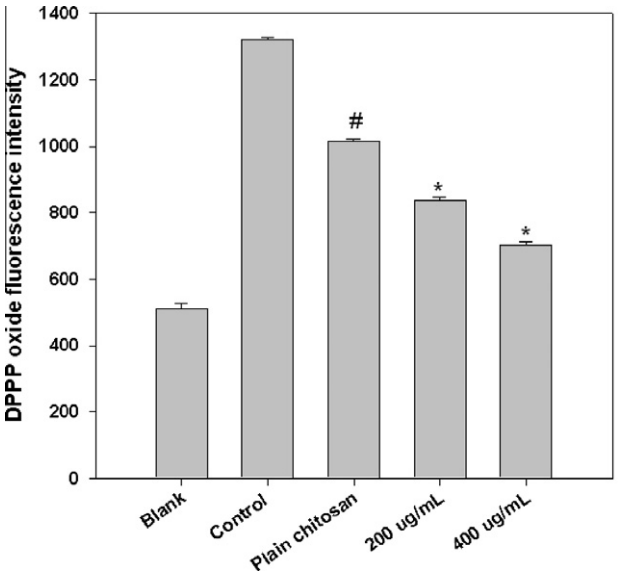


Figure 1. The lipid peroxidation inhibition activity of chitosan gallate on RAW264.7 macrophage cells. All assays were done in triplicate and data are expressed as means ± SE. $^{*}p$ <0.05 versus plain chitosan and $^{\#}p$ <0.05 versus control.

fluorescence was observed in the cells (Fig. 1), and this increment indicated accelerated lipid peroxidation in cell membranes. As shown in Figure 1, plain chitosan exert significant lipid peroxidation inhibition activity compare to control group, however the lipid peroxidation of the treatment groups with chitosan gallate was more inhibited than that of plain chitosan (p <0.05). The decrease in fluorescent intensity was compared to the blank (AAPH non-stimulated), confirming chitosan gallate could exert a significant effect against oxidation of membrane lipids. It is documented that phenolic compounds could inhibit lipid peroxidation by scavenging the lipid-derived radicals including $R^{\cdot}$, $RO^{\cdot}$ or $ROO^{\cdot}$ to stop the chain reactions in a heterogeneous lipid phase.[14] It is also reported that chitosan was a potent scavenger against alkyl radicals that possess $R^{\cdot}$, $RO^{\cdot}$ and $ROO^{\cdot}$ groups.[15] In the present study, plain chitosan also showed the inhibitory effect of lipid peroxidation by quenching lipid-derived radicals, however the inhibition activity was increased by the grafting of gallic acid onto chitosan, and this increase might be induced by the synergistic effect between chitosan and gallic acid.

Oxidative modification of DNA, proteins, lipids and small cellular molecules by ROS plays an important role in a wide range of common diseases and age-related degenerative conditions.[16] Thus antioxidants that capable of protection to vital molecules against oxidative damage may help prevent the onset and progression of disease.[17] Intracellular ROS were observed by the fluorescence sensitive dye, DCFH-DA using FACS Calibur™ flow cytometer (488 nm excitation, 530 nm emissions). DCFH-DA has been widely used for monitoring various ROS in biological media.[18] During labeling, the non-fluorescent DCFH-DA dye, which freely penetrates into cells, was hydrolyzed by intracellular esterases to DCFH and trapped inside the cells. DCFH further oxidized to DCF by ROS, which emitted fluorescence. As shown in Figure 2, increments in DCF fluorescence intensity due to hydrogen peroxide generation were observed, while pre-treatment with chitosan gallate decreased the DCF fluorescence in a dose-dependent manner, and the level of intracellular ROS was 12.06% compare to control group at 200 μg/mL.

All components of DNA are attracted by ROS and radical species, thus DNA is another biotarget for ROS-mediated oxidative damage.[19] Carcinogenesis initiate by DNA damage, which also affect the pathogenesis of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.[20] To evaluate protective effect of chitosan gallate against DNA oxidative damage induced by Fenton reaction, genomic DNA was prepared from RAW264.7 macrophage cells. As shown in Figure 3A, DNA damage was observed by hydroxyl radical in the control group, which was combined only with Fe(II)–H_2O_2, however, DNA was protected from hydroxyl radical-mediated attack in the presence of chitosan gallate in a dose-dependent manner.

In order to evaluate whether chitosan gallate effect on activity of an antioxidant enzymes such as SOD-1 and glutathione reductase (GSR), chitosan gallate was treated on RAW264.7 macrophage cells for 2 h and then added 500 μM H_2O_2 following incubated further 24 h. The protein expressions of SOD-1 and GSR were evaluated by western blot analysis. As shown in Figure 3B, the control group without chitosan gallate showed lower the protein expressions of SOD-1 and GSR, while chitosan gallate treated group revealed that the protein expression of SOD-1 and GSR recovered as the levels of blank group without H_2O_2 and chitosan gallate.

Cells are possessed of enzymatic system including SOD, glutathione peroxidase, catalase, GSR as a part of the cellular defense system, to preserve stability between oxidant and antioxidant.[21] SODs are antioxidant enzymes that form the first line of defense against ROS. SOD catalyzes superoxide radicals into hydrogen peroxide and molecular oxygen. For this reason, the generation of intracellular H_2O_2 might reduce SOD expression for maintaining the redox system. Glutathione (GSH) is an important factor for defending all cells from oxidative stress and acts on a critical role in regulating intracellular redox system. GSH directly reacts with ROS and functions as a cofactor of glutathione peroxidase. GSH protect all cells by preserving thiol redox potential in cells from oxidative stress. GSR deceased glutathione disulfide (GSSG) to the sulfhydryl form GSH.[22,23] Increased activity of GSR was correlated

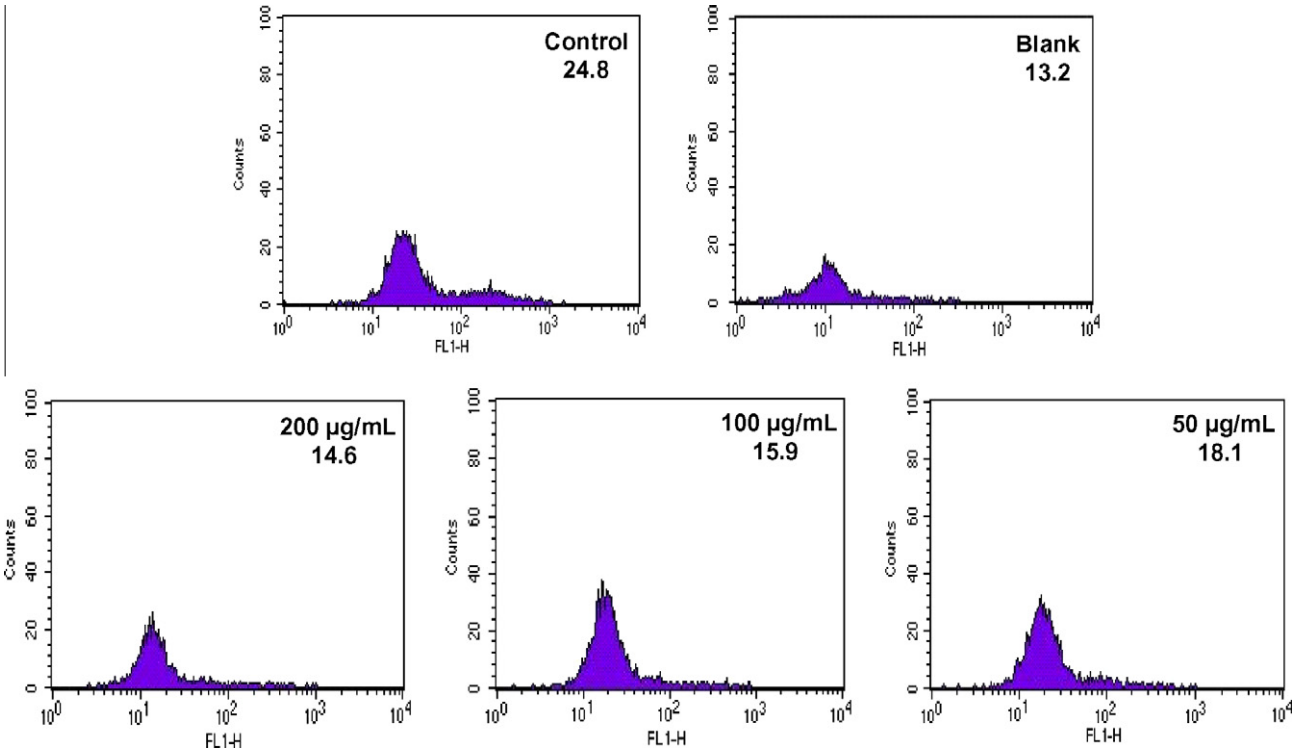


Figure 2. The inhibition effect of chitosan gallate on intracellular ROS generation in RAW264.7 macrophage cells.

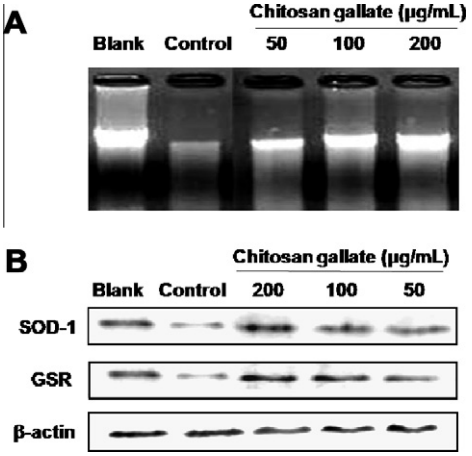


Figure 3. (A) The protection effect of chitosan gallate on hydroxyl radical-induced DNA damage. Genomic DNA was prepared from RAW264.7 macrophage cells. (B) Effect of chitosan gallate on the levels of SOD-1 and glutathione reductase (GSR) under H_2O_2-mediated oxidative stress. The expression levels of protein detected by western blot analysis.

with increased glutathione levels. Our data also demonstrated treatment of chitosan gallate increased protein level of GSR in a dose dependent manner. The expression levels of antioxidant enzymes are related with ROS. When H_2O_2 is generated in the cells, in order to keep the equilibrium of redox system, it might decrease the expression levels of antioxidant enzymes. In this study, we demonstrated that chitosan gallate stimulated the protein expression of antioxidant enzymes such as SOD-1 and GSR.

In conclusion, we prepared chitosan gallate by a free radical-induced grafting reaction and confirmed the structure of chitosan gallates using ^{1}H NMR. Chitosan gallate showed good water-solubility and thermal stability. In addition, the chitosan gallate exhibited good cytocompatibility against RAW264.7 macrophages and strongly inhibited the lipid peroxidation and the formation of intracellular ROS dose-dependent manner. Furthermore chitosan gallate effectively protected DNA damage by induced hydroxyl radical and up-regulated an antioxidant enzymes including SOD-1 and GSR. Our findings showed that chitosan gallate have beneficial effect of protection of radical-mediated cellular damage and could be developed as a candidate for potential use as an antioxidant.

References and notes

1. Jeon, Y. J.; Park, P. J.; Kim, S. K. *Carbohydr. Polym.* **2001**, *44*, 71–76.
2. Sugano, M.; Yoshida, K.; Hashimoto, H.; Enomoto, K.; Hirano, S. *Advances in Chitin and Chitosan*; Elsevier Applied Science: London and New York, 1992. pp. 472–478.
3. Jeon, Y. J.; Kim, S. K. *J. Chitin Chitosan* **2001**, *6*, 163–167.
4. Park, P. J.; Je, J. Y.; Kim, S. K. *J. Agric. Food Chem.* **2003**, *51*, 4930–4934.
5. Xing, R.; Liu, S.; Yu, H. H.; Zhang, Q. B.; Li, Z.; Li, P. C. *Carbohydr. Res.* **2004**, *339*, 2515–2519.
6. Zhong, Z. M.; Ji, X.; Xing, R.; Liu, S.; Guo, Z. Y.; Chen, X. L.; Li, P. C. *Bioorg. Med. Chem.* **2007**, *15*, 3775–3782.
7. Sun, T.; Yao, Q.; Zhou, D.; Mao, F. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5774–5776.
8. Guo, Z.; Xing, R.; Liu, S.; Zhong, Z.; Li, P. *Carbohydr. Polym.* **2008**, *73*, 173–177.
9. Moskovitz, L.; Yim, M. B.; Chock, P. B. *Arch. Biochem. Biophys.* **2002**, *397*, 354–359.
10. Cho, Y. S.; Kim, S. K.; Ahn, C. B.; Je, J. Y. *Carbohydr. Polym.* **2011**, *83*, 1617–1622.
11. Blois, M. S. *Nature* **1958**, *181*, 1533–1535.
12. Takahashi, M.; Shibata, M.; Niki, E. *Free Rad. Biol. Med.* **2001**, *31*, 164–174.
13. Milne, L.; Nicotera, P.; Orrenius, S.; Burkitt, M. J. *Arch. Biochem. Biophys.* **1993**, *304*, 102–109.
14. Cheng, Z.; Ren, J.; Li, Y.; Chang, W.; Chen, Z. *J. Pharm. Sci.* **2003**, *92*, 475–484.
15. Park, P. J.; Je, J. Y.; Kim, S. K. *Carbohydr. Polym.* **2004**, *55*, 17–22.
16. Lee, S. O.; Kim, S. Y.; Han, S. M.; Kim, H. M.; Ham, S. S.; Kang, I. J. *J. Med. Food* **2006**, *9*, 594–598.
17. Borek, C. *Free Radical Res. Commun.* **1991**, *12*, 745–750.
18. Okimoto, Y.; Watanabe, A.; Niki, E.; Yamashita, T.; Noguchi, N. *FEBS Lett.* **2000**, *474*, 137–140.
19. Martinez, G. R.; Loureiro, A. P.; Marques, S. A.; Miyamoto, S.; Yamaguchi, L. F.; Onuki, J.; Almeida, E. A.; Garcia, C. C.; Barbosa, L. F.; Medeiros, M. H.; Di Mascio, P. *Mutat. Res.* **2003**, *544*, 115–127.
20. You, H. J.; Oh, D. H.; Choi, C. Y.; Lee, D. G.; Hahm, K. S.; Moon, A. R.; Jeong, H. G. *Biochim. Biophys. Acta* **2002**, *1573*, 33–38.
21. Rosen, G. M.; Rauckman, J. *Methods Enzymol.* **1984**, *105*, 198–209.
22. Kong, C. S.; Kim, J. A.; Qian, Z. J.; Kim, Y. A.; Lee, J. I.; Kim, S. K.; Nam, T. J.; Seo, Y. *Food. Chem. Toxiol.* **2009**, *47*, 1914–1920.
23. Gennaro, G.; Zhara, A.; Marina, G.; Annabella, V.; Kavanagh, T. J.; Lucio, G. C. *Toxicol. Appl. Pharmacol.* **2007**, *219*, 181–189.