# Comparative study on antioxidative and macrophage-stimulating activities of polyguluronic acid (PG) and polymannuronic acid (PM) prepared from alginate

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*Abbreviations*: PG, polyguluronate; PM, polymannuronate; CL, chemiluminescence; ESR, electron spin resonance; NO, nitric oxide; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle's minimum essential medium; FBS, fetal bovine serum; HPX, hypoxanthine; XOD, xanthine oxidase; PBS, phosphate buffered saline; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; PHPA, *p*-hydroxyphenyl acetic acid; HRP, horse radish peroxidase; LPS, lipopolysaccharide.

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ABSTRACT

The antioxidant and macrophage-stimulating activities of polyguluronic acid (PG) and polymannuronic acid (PM) prepared from alginate were examined. A chemiluminescence (CL) method using a luminol analog, L-012, showed that both PM and PG scavenge superoxide produced by hypoxanthine–xanthine oxidase system in a concentration-dependent manner. At 100 μg/ml, PG showed slightly stronger superoxide scavenging activity than PM. In an electron spin resonance (ESR)-spin trapping method in which the Fenton reaction was used as hydroxyl radical generation system, we found that both PM and PG showed potent hydroxyl radical scavenging activity to a similar extent. Since PM and PG showed no chelating activity on $Fe^{2+}$, it was confirmed that PM and PG can directly scavenge hydroxyl radical. No significant scavenging activity of PM and PG toward hydrogen peroxide was observed. Interestingly, the macrophage-stimulation activity of PG as measured by nitric oxide (NO)-production from mouse macrophage cell line RAW264.7 cells was evidently stronger than that of PM. Our results suggest that RAW264.7 cells might be able to distinguish the conformational differences between PM and PG, and response differently to them, whereas the effects of such structural differences between PM and PG on the radical scavenging activities may not be so significant.

*Key words*: Alginate oligomer; Polyguluronic acid; Polymannuronic acid; Antioxidative activity; Nitric oxide

## 1. Introduction

Reactive oxygen species (ROS) including superoxide anion ($O_2^-$), hydroxyl radical (・OH), and hydrogen peroxide ($H_2O_2$) are produced in normal cellular metabolic processes such as respiration, as well as by ultraviolet light, ionizing radiation, and various chemical reactions. In living organisms, ROS levels are controlled by certain enzymes such as superoxide dismutase and antioxidant agents. However, overproduction of ROS can often lead to oxidative stress that in turn causes damage to lipids, proteins, and DNA. Thus, ROS are considered to be involved in a number of pathological conditions including cancer and other various severe diseases. [1-4]

The usefulness of polysaccharides and their derivatives in food, agriculture, and medicine has been well documented.[5, 6] Polysaccharides provide various beneficial effects such as lowering blood cholesterol level and blood pressure, and protective effect on infectious and inflammatory diseases.[7] Some polysaccharides are even known as potent immune modulators.[8, 9] In recent years, considerable attention has directed to marine algae as a rich source of polysaccharides with antioxidant activity.[10]

Alginate is an acidic linear polysaccharide that is usually derived from brown seaweeds such as *Macrocystis pyrifera* and *Ascophyllum nodosum*. This polysaccharide is composed of two forms of uronic acids α-L-guluronate (G) and β-D-mannuronate (M) which form three types of polymer blocks: namely homopolymer of guluronate (PG), homopolymer of mannuronate (PM), or heteropolymer (a mixed sequence of these residues); these block structures are expressed as G-blocks, M-blocks, and MG-blocks, respectively.[11] The differences in the molecular weight, the M/G ratio, and the entire sequence seem to be responsible for the diversity of physicochemical properties and bioactivities of alginates.[12-17] Alginates have been utilized for a wide range of commercial applications including thickening agents and dispersion stabilizers. Since alginates have gentle gelling properties in the presence of divalent cations such

as calcium, alginates are also used for live cell encapsulation *in vitro*[18] and *in vivo*[19] and for several tissue engineering applications.[20, 21] Alginate oligosaccharides prepared by the enzymatic degradation of alginate polymers with relatively low molecular weight are also known to have several biological activities including suppression of fibroblast proliferation and collagen synthesis in human skin,[22] stimulation of endothelial cell growth and migration,[23] stimulation of human keratinocyte growth,[24] suppression of Th2 development and IgE secretion through inducing interleukin (IL)-12 secretion.[25] In addition to these mammalian models, it has also been reported that enzymatically depolymerized alginates promote the growth of bifidobacteria, while the original alginate polymer had no such effect.[26] Furthermore, alginate oligomers seem to have some biological effects on plants as well. Alginate oligomers prepared with bacterial alginate lyase increased shoot elongation of komatsuna (*Brassica rapa* var. *pervidis*) seeds[27] and promoted the elongation of barley roots.[28] Our recent studies have demonstrated that alginate polymers induce tumor necrosis factor (TNF)-α secretion from mouse macrophage cell line RAW264.7, and the activity was significantly influenced by the molecular size and M/G ratio.[29] We also found that enzymatically depolymerized alginate oligomers showed even higher activity in terms of TNF-α secretion from RAW264.7 cells as compared to original alginate polymers. Since the alginate oligomers has fairly low viscosity in aqueous solution even at quite high concentration, and has no gel-forming property in the presence of calcium, it is considered that alginate oligomers are more applicable for *in vivo* systems.[29, 30]

In addition to the biological activities of alginate polymers and their oligomers described above, recent studies have demonstrated that alginate oligomers[31] as well as polymers[32] show potent antioxidant activities. However, to our knowledge, the detailed study on the structure-activity relationship of alginate oligomers in terms of the antioxidative and biological activities seems to be deficient. In the present study, we prepared PM (polymannuronate) and PG (polyguluronate) fractions from alginate

polymer, and compared their radical scavenging and macrophage-stimulating activities.

## 2. Results and discussion

### 2.1. ROS scavenging activities of PM and PG

The superoxide-scavenging activities of PM and PG were examined by a luminol analog L-012-dependent chemiluminescence (CL) method. As a source of superoxide, we employed hypoxanthine (HPX)-xanthine oxidase (XOD) system. The L-012-dependent CL method has been used as a highly sensitive CL assay for detecting superoxide.[29] As shown in Fig. 1A, when XOD was added to the reaction mixture, a rapid CL response was observed, and the CL response was significantly reduced by the addition of SOD, indicating that superoxide was generated in the system. In the system, the superoxide scavenging activities of PM and PG were measured (Figs. 1B and C). Both PM and PG showed superoxide scavenging activity in a concentration-dependent manner. In the presence of 1,000 μg/ml of PM or PG, CL response was almost completely inhibited. Regarding the efficiency of radical scavenging activity of alginate oligosaccharides, it has been reported that relatively high concentrations (mg order) of alginate oligosaccharides were required to scavenge superoxide and hydroxyl radical significantly.[31] Thus, the effective concentration of alginate oligosaccharides including PM and PG as a radical scavenger seems to be relatively high. PG at 100 μg/ml showed slightly stronger scavenging activity than PM, while both oligosaccharides at 10 μg/ml had no significant activities. Since PM and PG did not inhibit the production of uric acid from hypoxanthine with the catalytic reaction of xanthine oxidase (data not shown), it is considered that the inhibition of CL response was due to the direct superoxide scavenging activity of PM and PG not the results of the inhibition of xanthine oxidase.

It is well known that hydroxyl radical is extremely reactive species that can react with various biological molecules, and damaging action on various biological systems is the strongest among the reactive oxygen species.[33, 34] In the next experiment, the Fenton reaction was used as a hydroxyl radical generation system, in which $Fe^{2+}$ and hydrogen peroxide are reacted to produce hydroxyl radical. Although there are several methods for the detection of hydroxyl radical such as spectrophotometoric or colorimetric methods, ESR method has been used as the most reliable assay for monitoring free radicals because of its high sensitivity and rapidity. Thus, we examined the scavenging activities of PM and PG on the hydroxyl radical by ESR method in this study. When spin-trapping agent DMPO was added to a solution of the Fenton reaction system, the typical 1:2:2:1 ESR signal of the DMPO-OH adduct (an adduct from DMPO and hydroxyl radical) was observed. Figs. 2B and C show the representative ESR spectra of DMPO-OH obtained by the addition of the solvent alone and various concentrations of PM and PG. In the presence of PM or PG, the decreases in the height of the second peak of the spectrum, which represents relative amount of DMPO-OH adduct, were observed. These results clearly indicate that PM and PG have the ability to scavenge hydroxyl radicals. PM and PG showed similar concentration-dependent profiles in terms of scavenging efficiency, but they required higher concentrations to attain the effect similar to mannitol as a known monosaccharide hydroxyl radical scavenger (Fig. 2A). Regarding antioxidant activity of polysaccharides, it was proposed that polysaccharides could inhibit the formation of hydroxyl radicals, probably due to the hydrogen or electron abstraction mechanism.[35] It was also reported that the ease of abstraction of the anomeric hydrogen from the internal monosaccharide units made polysaccharides achieve the scavenging effect.[36] The further studies are obviously required to clarify the molecular basis of the hydroxyl radical scavenging activities of PM and PG.

On the other hand, regarding the hydroxyl radical scavenging mechanism of

certain compounds, it has been pointed out that the scavenging activity was not due to direct scavenging but inhibition of hydroxyl radical formation by chelating iron ions in the reaction system.[37] In fact, it was reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and inhibit the Fenton reaction.[38] Hence, the possible chelation of $Fe^{2+}$ by PM and PG was estimated by the method of Decker and Welch.[39] As shown in Fig. 3, PM and PG had no significant chelating activity on $Fe^{2+}$ even at more than nearly 100 times molar excess to $Fe^{2+}$, which was calculated by uronate bases, while 100 times molar excess of desferal, a Fe-specific chelater, completely inhibited the color development in the reaction system. Similar to our results, it has been reported that alginate oligosaccharides showed no $Fe^{2+}$ chelating activity, while chitosan oligosaccharides and fucoidan oligosaccharides exhibited a potent chelating activity on $Fe^{2+}$.[31] Comparative study on the radical scavenging activities of alginate oligosaccharides, chitosan oligosaccharides, and fucoidan oligosaccharides with similar molecular weight has demonstrated that alginate oligosaccharides showed the highest hydroxyl radical scavenging activity among these marine oligosaccharides, whereas chitosan oligosaccharides had the highest superoxide radical scavenging activity.[31] Although the antioxidant mechanism of the oligosaccharides is not fully understood yet, these findings suggest that chemical structure might be an important factor influencing the antioxidant activity.

Next, we examined the reactivity of PM and PG on hydrogen peroxide. Hydrogen peroxide is not a free radical, but it is cell-membrane permeable and can cause toxic effect on cells. In addition, it reacts with $Fe^{2+}$ to form highly reactive hydroxyl radical by the Fenton reaction as mentioned above. As shown in Fig. 4, no significant scavenging activity of PM and PG toward hydrogen peroxide was observed up to 1,000 μg/ml. Consistent with our results, it has been reported that inhibitory effect of alginate oligosaccharides on hydrogen peroxide-mediated erythrocyte hemolysis was quite low as compared to that of chitosan oligosaccharides.[31]

## 2.2. Macrophage-stimulating activities of PM and PG

It has been known that alginates have an activity to stimulate immune systems. For instance, Otterlei *et al* have reported that alginates induced production of TNF-α, IL-1, and IL-6 from human monocytes in *in vitro* system.[12, 40] It has also been reported that mouse peritoneal monocytes stimulated with alginate i*n vitro* produce nitric oxide (NO) and TNF- α.[41] Thus, in the next experiments, we examined the effects of PM and PG on mouse macrophage cell line RAW264.7 cells in terms of NO production. As shown in Fig. 5, both PM and PG (400 μg/ml) induced NO production from RAW264.7 cells. Interestingly, the activity of PG was higher than that of PM. There is a possibility, however, that PM and PG used contained a low level of endotoxins. It has been reported that alginate isolated from alga is contaminated with polyphenols and endotoxins which have immuno-modulatory effects.[42] Polymyxin B (PMB) is often used as an inhibitor for endotoxins and LPS to confirm the activity of the samples is not due to the contaminated endotoxins.[43, 44] As shown in Fig. 5, PMB at 2 μg/ml slightly reduced the NO production from RAW264.7 cells stimulated with PM and PG, whereas PMB almost completely inhibited the LPS-induced NO production. Thus, it is unlikely that the NO-inducing activities of PM and PG are due to LPS-like endotoxin contamination. In addition, it has been shown that polyphenol inhibits NO and TNF- α production in macrophages rather than stimulation.[45-47] Based on these findings, our results suggest that PM and PG by themselves can directory act on RAW264.7 cells as stimulants of NO production. Regarding cell-surface receptors on immuno-competent cells, it has been proposed that the presence of at least 13 members of Toll-like receptors (TLRs) family. TLRs are evolutionary conserved pattern recognition receptors that can recognize specific pathogen-associated molecular patterns with no apparent structural similarity.[53] TLRs play important roles in signal transduction for the

initiation of mammalian immune responses including cytokine production.[54] In addition to bacterial products, TLRs seem to be responsible for oligosaccharide-mediated stimulation processes.[55] Our previous studies have demonstrated that the activities of alginate oligosaccharides to induce TNF-α production from RAW264.7 cells were inhibited by anti-TLR2 and anti-TLR4 antibodies, suggesting that alginate oligosaccharides may be recognized by these TLRs.[30] Since three-dimensional structures of PM and PG are quite different each other, it is considered that their entire molecular patterns are also different. Probably, cell surface receptors, the most probable candidates for them are TLR2 and TLR4, may be able to distinguish the difference in the molecular patterns between PG and PM. That may be a possible reason why PG showed higher NO-inducing activity than PM.

## 3. Conclusions

In conclusion, our results clearly indicate that PM and PG are capable of scavenging superoxide and hydroxyl radical in a concentration-dependent manner, although they have no effect on hydrogen peroxide. In the macrophage-stimulating activity as measured by NO production, more significant differences between PM and PG were observed. Namely, PG more strongly simulates RAW264.7 cell to induce NO production than that of PM. Probably, certain biological system such as receptors on the cell surface of RAW264.7 cells may more clearly distinguish the conformational differences between PM and PG than the pure chemical antioxidant reactions.

## 4. Experimental section

### 4.1. Preparation of polyguluronate (PG) and polymannuronate (PM)

Sodium alginate (1,000-cps grade) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Polyguluronate (PG) and polymannuronate (PM) (DP = 20-24) were prepared from sodium alginate by the method of Haug *et al*.[48] The homogeneity of the prepared polyuronates was confirmed by the circular dichroic spectral analysis with a Jasco spectropolarimeter J500A coupled with a data processor, based on the method of Morris *et al*.[49]

**4.2. Cell culture**

RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and cultured at 37°C in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere with 5% $CO_2$ and 95% air.

**4.3. Chemiluminescent (CL) determinations for superoxide generated by hypoxanthine (HPX)-xanthine oxidase (XOD) system**

To study the superoxide scavenging ability of PM and PG, the HPX-XOD reaction was applied for superoxide generation. L-012 is an analogue of luminol, and luminol-CL reaction has been well documented.[50] A reaction mixture containing 10 μl of sample solution in PBS or solvent (PBS) alone and 10 μl of 1.25 mM HPX was dispensed into each well of a 96-well microplate. After preincubation for 2 min, the reaction was initiated by the addition of 80 μl of PBS solution containing 125 μM L-012 and 750 μunits/ml XOD, and the CL intensity of each well was recorded continuously for 8 min using a CL microplate recorder (Mithras LB940, Berthold Technologies GmbH and Co. KG., Bad Wildbad, Germany). To confirm the superoxide

generation in the system, the effect of SOD was examined. In the assay, instead of a sample solution, 10 μl of SOD solution (0.1 ~ 100 units/ml in PBS) was added to the reaction mixture.

### 4.4. Electron spin resonance (ESR)-spin trapping determinations for hydroxyl radical generated by Fenton reaction

The assay used in this study was essentially identical to that described in the previous paper.[51] Twenty μl of 2 mM hydrogen peroxide dissolved in pure water, 20 μl of 8.9 mM DMPO dissolved in pure water, 20 μl of aqueous sample solution or solvent alone, and 20 μl of 0.2 mM $FeSO_4$ dissolved in pure water were placed in a test tube and mixed. Fifty μl of each mixture was transferred to the ESR spectrometry capillary tube, and the spectrum of the DMPO-OH spin adduct in each reaction mixture was measured. Measurement conditions of ESR (The EMX Plus, Bruker, Billerica, Massachusetts, USA) were as follows; field sweep: 3,327 – 3,412 G, field modulation frequency: 100 kHz, modulation amplitude: 3 G, sweep time: 122.880 s, time constant: 163.840 ms, microwave frequency: 9.458 GHz, microwave power: 4 mW. To confirm the hydroxyl radical generation in the system, the effect of D-mannitol was examined. In the assay, instead of a sample solution, 20 μl of D-mannitol solution (40 ~ 4,000 μg/ml in pure water) was added to the reaction mixture.

### 4.5. Chelating activity on $Fe^{2+}$

The chelating activities of PM and PG on $Fe^{2+}$ were measured by the method described previously.[39] Two hundred twenty μl of sample solution in pure water, 814 μl of pure water, 22 μl of 2 mM $FeCl_2$ were mixed and incubated for 10 min at room temperature. The mixture was reacted with 44 μl of 5 mM ferrozine and incubated for

10 min. After incubation at room temperature, the absorbance at 540 nm was measured. Chelating activities of samples on $Fe^{2+}$ was calculated by following equation: Chelating activity (%) = (A-B)/A x 100, where A was the absorbance of the control reaction mixture without sample, and B was absorbance of the test sample. To confirm the specificity of assay system for $Fe^{2+}$, the effect of deferoxaminemesylate (desferal), an iron-specific chelater was examined. In the assay, instead of a sample solution, 220 μl of desferal solution (0.2 ~ 20 mM in pure water) was added to the reaction mixture.

**4.6. Fluorescence determinations for hydrogen peroxide**

To study the hydrogen peroxide ($H_2O_2$) scavenging ability of PM and PG, a known concentration of hydrogen peroxide solution was incubated with PM or PG at room temperature for 1 min. Then the hydrogen peroxide levels in the reaction mixture were determined by *p*-hydroxyphenyl acetic acid (PHPA) method. The reaction mixture containing 360 μl of PBS, 60 μl of sample or solvent (pure water), 60 μl of 0.3 mM $H_2O_2$, 60 μl of 10 units/ml horseradish peroxidase (HRP), and 60 μl of 1 mM PHPA was transferred to cuvette and incubated for 1 min, and the fluorescence intensity of the reaction mixture was measured with fluorescence spectrophotometer (F-2500 fluorescence spectrophotometer, Hitachi High Technologies Inc., Tokyo, Japan). To confirm the specificity of the assay system for $H_2O_2$, the effect of catalase was examined. In the assay, instead of a sample solution, 10 μl of catalase solution (10~10,000 units/ml in pure water) was added to the reaction mixture.

**4.7. Nitrite assay for the estimation of nitric oxide (NO)**

To estimate NO level in RAW264.7 cells, nitrite, a stable reaction product of NO with molecular oxygen, was measured by Griess assay as described previously.[52] In

brief, adherent RAW264.7 cells in 96-well microplates (3 x $10^4$ cells/well) were treated with the indicated concentrations of PM, PG, or Lipopolysaccharide (LPS) in the presence or absence of polymyxin B (2 μg/ml) for 24 h in the growth medium at 37℃, and then the nitrite levels in the culture medium of the treated cells were measured.

### 4.8. Statistical analysis

All the experiments were repeated at least three times. Data were expressed as means ± standard deviation (S.D.), and data were analyzed by paired Student's *t*-test to evaluate significant differences. A level of $p < 0.05$ was considered statistically significant.

## References


1. Hussain, S. P.; Hofseth, L. J.; Harris, C. C.; *Nat. Rev. Cancer* **2003**, *3*, 276-285.
2. Reuter, S.; Gupta, S. C.; Chaturvedi, M. M.; Aggarwal, B. B.; *Free Radic. Biol. Med*. **2010**, *49*, 1603-1616.
3. Barnham, K. J.; Masters, C. L.; Bush, A. I.; *Nat. Rev. Drug Discov*. **2004**, *3*, 205-214.
4. Behl, C.; Davis, J. B.; Lesley, R.; Schubert, D.; *Cell* **1994,** *77*, 817-827.
5. Ajithkumar, A.; Andersson, R.; Siika-aho, M.; Tenkanen, M.; Aman, P.; *Carbohydr. Poly*. **2006**, *64*, 233-238.
6. Surenjav, U.; Zhang, L. N.; Xu, X. J.; Zhang, X. F.; Zeng, F. B.; *Carbohydr. Poly*. **2006**, *63*, 97-104.
7. Hida, S.; Miura, N. N.; Adachi, Y.; Ohno, N.; *J. Autoimmun*. **2005**, *25*, 93-101.
8. Han, E. H.; Choi, J. H.; Hwang, Y. P.; Park, H. J.; Choi, C. Y.; Chung, Y. C.; Seo, J. K.; Jeong, H. G.; *Food Chem. Toxicol*. **2009**, *47*, 62-69.

9. Sun, Y. X.; Liang, H. T.; Cai, G. Z.; Guan, S. W.; Tong, H. B.; Yang, X. D.; Liu, J. C.; *J. Biol. Macromol*. **2009**, *44*, 14-17.
10. Qi, H.; Zhang, Q.; Zhao, T.; Chen, R.; Zhang, H.; Niu, X.; Li, Z.; *Inter. J. Biol. Macromol*. **2005**, *37*, 195-199.
11. Haug, A.; *Methods Carbohydr. Chem*. **1965**, *5*, 69-73.
12. Otterlei, M.; Østgaard, K.; Skjåk-Bræk, G.; Smidsrød, O.; Soon-Shing, P.; Espevik, T.; *J. Immunothe*. **1991**, *10*, 286-291.
13. Kimura, Y.; Watanabe, K.; Okuda, H.; *J. Ethnopharmacol*. **1996**, *54*, 47-54.
14. Logeart, D.; PrigentRichard, S.; Jozefonvicz, J.; Letourneur, D.; *Eur. J. Cell Biol*. **1997**, *74*, 376-384.
15. Logeart, D.; PrigentRichard, S.; BoissonVidal, C.; Chaubert, F.; Durand, P.; Jozefonvicz, J.; Letourneur, D.; *Eur. J. Cell Biol*. **1997**, *74*, 385-390.
16. Asada, M.; Sugie, M.; Inoue, M.; Nakagomi, K.; Hongo, S.; Murata, K.; Irie, S.; Takeuchi, T.; Tomizuka, N.; Oka, S.; *Biosci. Biotechnol. Biochem*. **1997**, *61*, 1030-1032.
17. Jeong, H.-J.; Lee, S.-A.; Moon, P.-D.; Na, H.-J.; Park, R.-K.; Um, J.-Y.; Kim, H.-M.; Hong, S.-H.; *Clinic. Exp. Allergy* **2006**, *36*, 785-794.
18. Fremond, B.; Malandain, C.; Guyomard, C.; Chesne, C.; Guillouzo, A.; Campion, J.-P.; *Cell Transplant*. **1993**, *2*, 453-460.
19. Chang, S. C. N.; Rowley, J. A.; Tobias, G.; Genes, N. G.; Roy, A. K.; Mooney, D. J.; Vacanti, C. A.; Bonassar, L. J.; *J. Biomed. Mater. Res*. **2001**, *55*, 503-511.
20. Hauselmann, H. J.; Masuda, K.; Hunziker, E. B.; Neidhart, M.; Mok, S. S.; Michel, B. A.; Thonar, E. J.; *Am. J. Physiol*. **1996**, *271*, C742-C752.
21. Atala, A.; Kim, W.; Paige, K. T.; Vacanti, C. A.; Retik, A. B.; *J. Urol*. **1994**, *152*, 641-643.
22. Tajima, S.; Inoue, H.; Kawada, A.; Ishibashi, A.; Takahara, H.; Hiura, N.; *Arch. Dermatol. Res*. **1999**, *291*, 432-436.

23. Kawada, A.; Hiura, N.; Tajima, S.; Takahara, H.; *Arch. Dermatol. Res*. **1999**, *291*, 542-547.

24. Kawada, A.; Hiura, N.; Shiraiwa, M.; Tajima, S.; Hiruma, M.; Hara, K.; Ishibashi, A.; Takahara, H.; *FEBS Lett.* **1997**, *408*, 43-46.

25. Yoshida, T.; Hirano, A.; Wada, H.; Takahashi, K.; Hattori, M.; *Int. Arch. Allergy Immurol*. **2004**, *133*, 239-247.

26. Akiyama, H.; Endo, T.; Nakakita, R.; Murata, K.; Yonemoto, Y.; Okayama, K.; *Biosci. Biotechnol. Biochem*. **1992**, *56*, 355-356.

27. Yonemoto, Y.; Tanaka, H.; Yamashita, T.; Kitabatake, N.; Ishida, Y.; Kimura, A.; Murata, K.; *J. Ferment. Bioeng.* **1993**, *75*, 68-70.

28. Tomoda, Y.; Umemura, K.; Adachi, T.; *Biosci. Biotechnol. Biochem*. **1994**, *58*, 202-203.

29. Kurachi, M.; Nakashima, T.; Miyajima, C.; Iwamoto, Y.; Muramatsu, T.; Yamaguchi, K.; Oda, T.; *J. Infect. Chemother*. **2005**, *11*, 199-203.

30. Iwamoto, M.; Kurachi, M.; Nakashima, T.; Kim, D.; Yamaguchi, K.; Oda, T.; Iwamoto, Y.; Muramatsu, T.; *FEBS Lett*. **2005**, *579*, 4423-4429.

31. Wang, P.; Jiang, X.; Jiang, Y.; Hu, X.; Mou, H.; Li, M.; Guan, H.; *Nat. Product Res*. **2007**, *21*, 646-654.

32. Tomida, H.; Yasufuku, T.; Fujii, T.; Kondo, Y.; Kai, T.; Anraku, M.; *Carbohydr. Res*. **2010**, *345*, 82-86.

33. Aruoma, O. I.; Kaur, H.; Halliwell, B.; *J. R. Soc. Health***, 1991**, *111*, 172–177.

34. Millic, B. L.; Djilas, S. M.; Canadanovic-Brunet, J. M.; *Food Chem*. **1998**, *61*, 443-447

35. Chen, H.; Wang, Z.; Qu, Z.; Fu, L.; Dong, P.; Zhang, X.; *Eur. Food Res. Technol*. **2009**, *229*, 629-635

36. Tsiapali, E.; Whaley, S.; Kalbfleisch, J.; Ensley, H. E.; Browder, I. W.; Williams, D. L.; Free Radic. Bio. Med. **2001**, *30*, 393-402

37. Shon, M. Y.; Kim, T. H.,; Sung, N. J.; *Food Chem*. **2003**, *82*, 593-597.
38. Smith, C.; Halliwell, B.; Aruoma, O. I.; *Food Chem. Toxicol*. **1992**, *30*, 483-489.
39. Decker, E.A.; Welch, B.; *J. Agric. Food Chem*. **1990**, *38*, 674-677.
40. Otterlei, M.; Sudan, A.; Skjåk-Braek, G.; Ryan, L.; Smidsrød, O.; Espevik, T.; *Infect. Immune*. **1993**, *61*, 1917-1925.
41. Pasquali, P.; Zalcman, A.; Murtas, S.; Adone, R.; Brambilla, G.; Marianelli, C.; Cagiola, M.; Ciuchini, F.; *Arch. Pharm. Res*., **2005**, *28*, 936-941.
42. Skjak-Brek G,; Martisen A.; *West Sussex, John Wiley & Sons Ltd*., **1991**, 219-259.
43. Cavaillon, J-M.; Haeffner-Cavaillons N.; *Mol. Immunol*. **1986**, *23*, 965-969.
44. Duff G.W,; Atkins, E.; *J. Immunol. Methods* **1982**, *52*, 333-340.
45. Yang, F.; de. Villiers, W. J.; McClain, C. J.; Varilek, G.W.; *J. Nutr*. **1998**, *128*, 2334-2340.
46. Lin, Y. L.; Lin, J. K.; *Mol. Pharmacol*. **1997**, *52*, 465-472.
47. Chan, M. M.; Ho, C. T.; Huang, H. I. *Cancer Lett*. **1995**, *96*, 23-29.
48. Haung, A.; Larsen, B.; Smidsrøt, O.; *Acta Chem. Scand*. **1966**, *20*, 183-190.
49. Morris, E.; Rees, D. A.; Thom, D.; *Carbohydr. Res*. **1980**, *81*, 305-314.
50. Hodgson, L.; Fridovich, I.; *Photochemist. Photobiol*. **1973**, *18*, 451-455.
51. Niwano, Y.; Sato, E.; Kohno, M.; Matsuyama, Y.; Kim D.; Oda, T.; *Biosci. Biotechnol. Biochem*. **2007**, *71*, 1145-1153.
52. Jiang, Z.; Okimura, T.; Yamaguchi, K.; Oda, T.; *Nitric Oxide*. **2011**, *25*, 407-415.
53. Medzhitov, R.; Janeway, C. Jr.; Innate Immunity. *New. Eng. J. Med*. **2000**, *343*, 338-344.
54. Hirschfeld, M.; Ma, Y.; Weis, J. H.; Vogel, S. N.; Weis, J. J.; *J. Immunol.* **2000**, *165*, 618-622.
55. Shoham, S.; Hung, C.; Chen, J-M.; Golenbock, D. T.; Levitz, S. M.; *J. Immunol*. **2001**, *166*, 4620-4626.

## LEGENDS TO FIGURES

**Figure 1.** Superoxide scavenging activity of PM and PG as measured by L-012-dependent chemiluminescence (CL) method. Effects of various concentrations of SOD (A), PG (B), and PM (C) on CL-responses in the hypoxanthine (HPX)-xanthine oxidase (XOD) system were examined. Insets indicate the CL-response patterns during 10 min in the presence of 0 (○), 0.01 (●), 0.1 (△), 1 (▲), and 10 (□) units/ml of SOD (A), or 0 (○), 10 (●), 100 (△), and 1,000 (▲) μg/ml of PG (B), or 0 (○),10 (●), 100 (△), and 1,000 (▲) μg/ml of PM (C). The columns indicate % inhibition of the integrated CL-response intensity values during 10 min as compared to the control values. Each value represents the means ± standard deviation of triplicate measurements.

**Figure 2.** Hydroxyl radical scavenging activity of PM and PG measured by the ESR-spin trapping method. The representative ESR spectra of DMPO-OH obtained by the addition of the indicated concentrations of mannitol (A), PG (B), and PM (C) to the Fenton reaction.

**Figure 3.** Chelating activities of PM and PG on $Fe^{2+}$. The columns indicate $Fe^{2+}$-chelating effects of desferal, PM, or PG as measured by spectrophotometoric method as described in the text. Each value represents the means ± standard deviation of triplicate measurements.

**Figure 4.** Hydrogen peroxide scavenging activities of PM and PG measured by the PHPA-fluorescence method. The columns indicate fluorescence intensity obtained by the addition of indicated concentrations of catalase, PG, or PM to PHPA-hydrogen

peroxide system. Each value represents the means ± standard deviation of triplicate measurements.

**Figure 5.** Macrophage-stimulating activity of PM and PG as measured by NO production by RAW264.7 cells. Adherent RAW264.7 cells (3 x $10^4$ cells/well in 96-well plates) were incubated with the indicated concentrations of PM, PG, or LPS at 37℃ in the absence (□) or presence (■) of polymixin B (2 μg/ml) in the growth medium. After 24 h incubation, the nitrite levels in the culture medium from the treated cells were measured by Griess assay as described in the text. Each value represents the means ± standard deviation of triplicate measurements. Asterisks indicate significant differences between PM and PG in the presence ($^{*}p < 0.05$) or absence ($^{**}p < 0.05$) of polymixin B. [a)]The nitrite level in the medium from the LPS-treated cells in the presence of polymixin B was almost negligible.

**Figure 1 (A)**

**(A)**

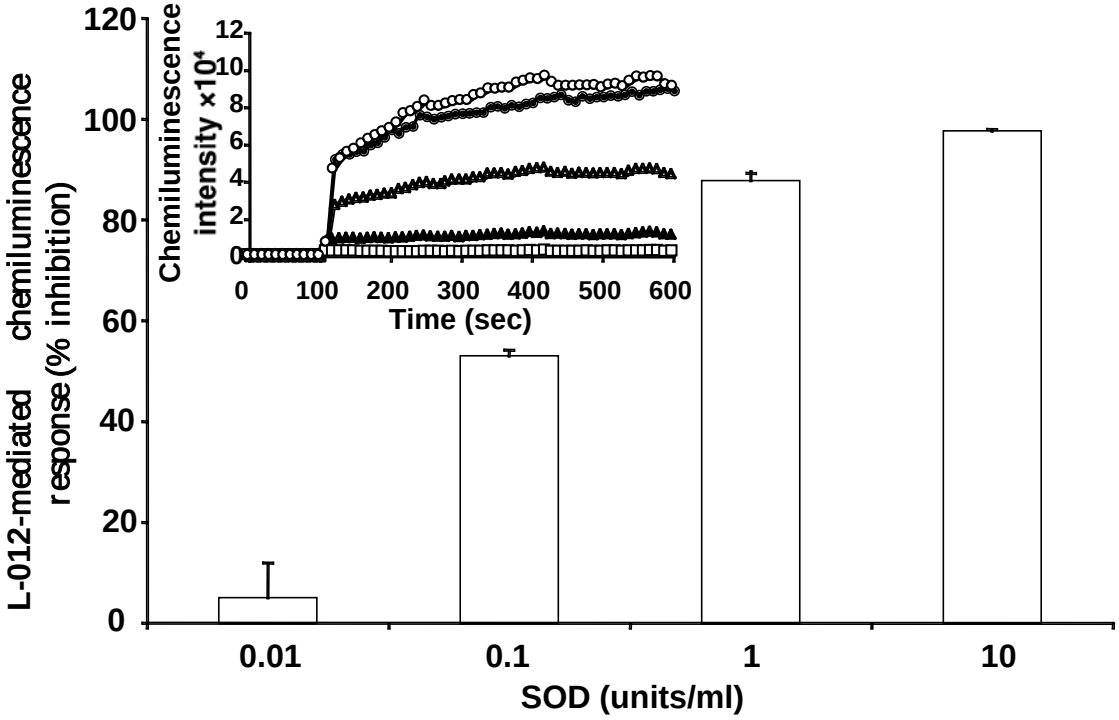

L-012-mediated chemiluminescence response (% inhibition)
120
100
80
60
40
20
0
0.01
0.1
1
10
SOD (units/ml)
Chemiluminescence intensity ×10⁴
12
10
8
6
4
2
0
0
100
200
300
400
500
600
Time (sec)

**Figure 1 (B)**

**(B)**

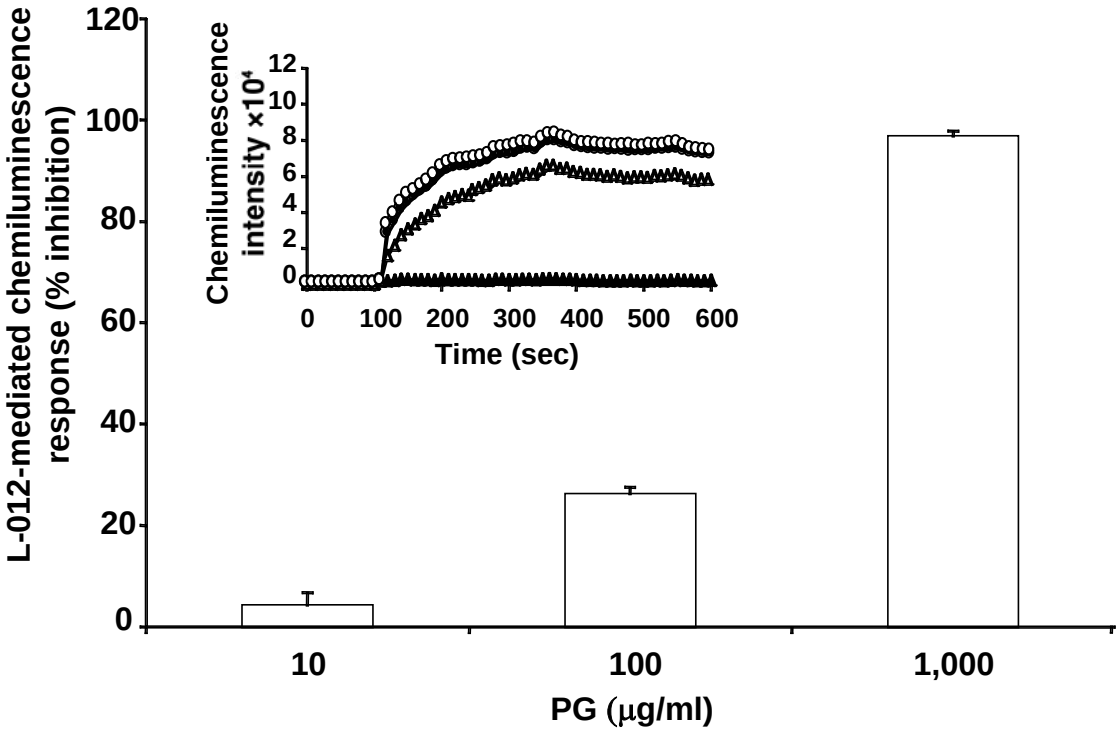

L-012-mediated chemiluminescence response (% inhibition)
120
100
80
60
40
20
0
Chemiluminescence intensity ×10⁴
12
10
8
6
4
2
0
0
100
200
300
400
500
600
Time (sec)
10
100
1,000
PG (μg/ml)

**Figure 1 (C)**

**(C)**

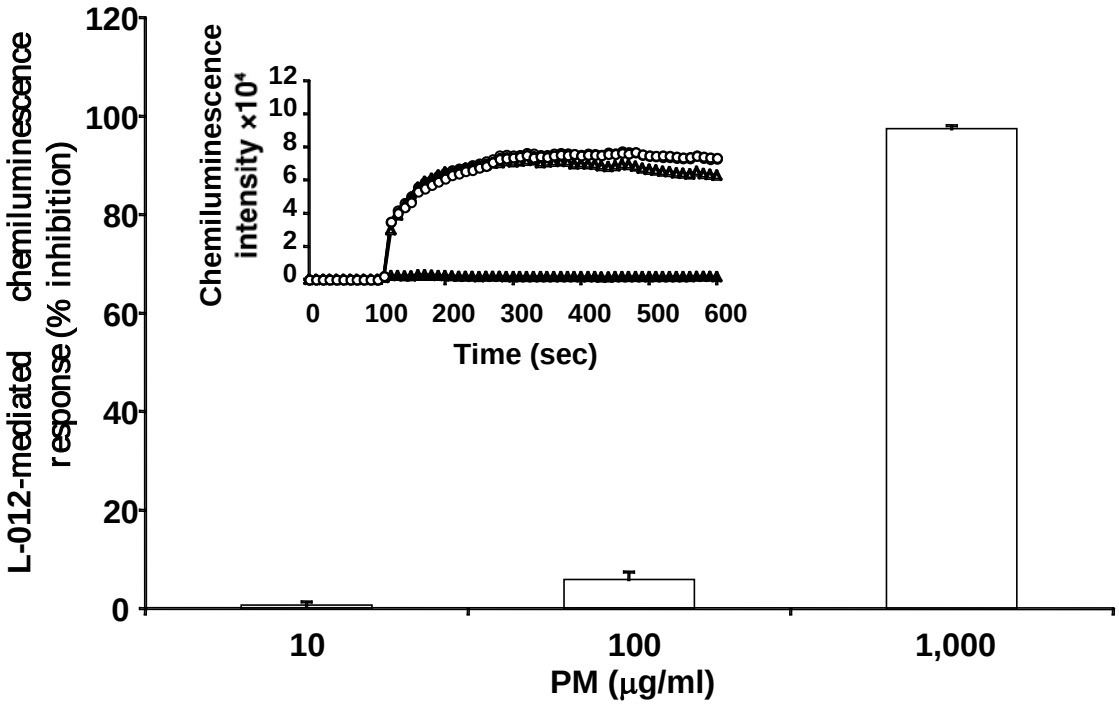

L-012-mediated chemiluminescence response (% inhibition)
120
100
80
60
40
20
0
10
100
1,000
PM (μg/ml)
Chemiluminescence intensity ×10⁴
12
10
8
6
4
2
0
0
100
200
300
400
500
600
Time (sec)

**Figure 2 (A)**

**(A)**

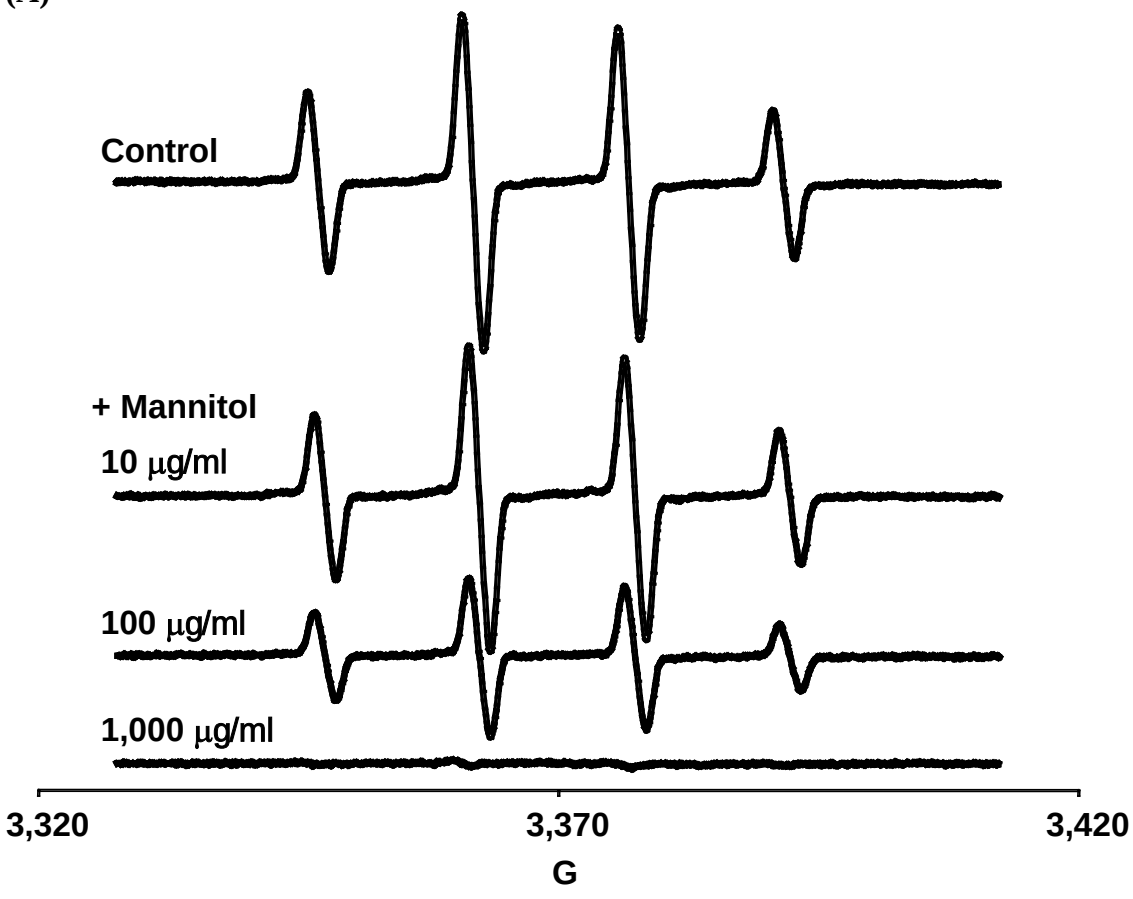


**Figure 2 (B)**

**(B)**

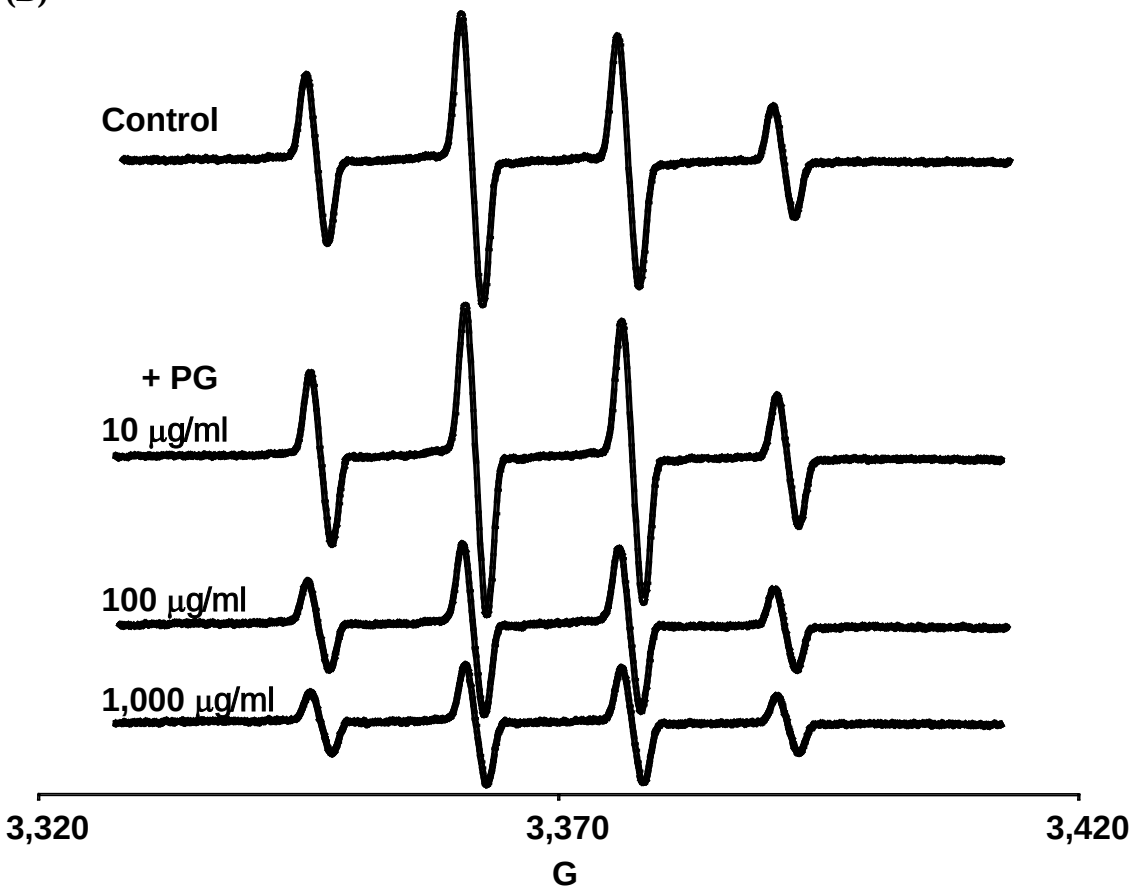


**Figure 2 (C)**

**(C)**

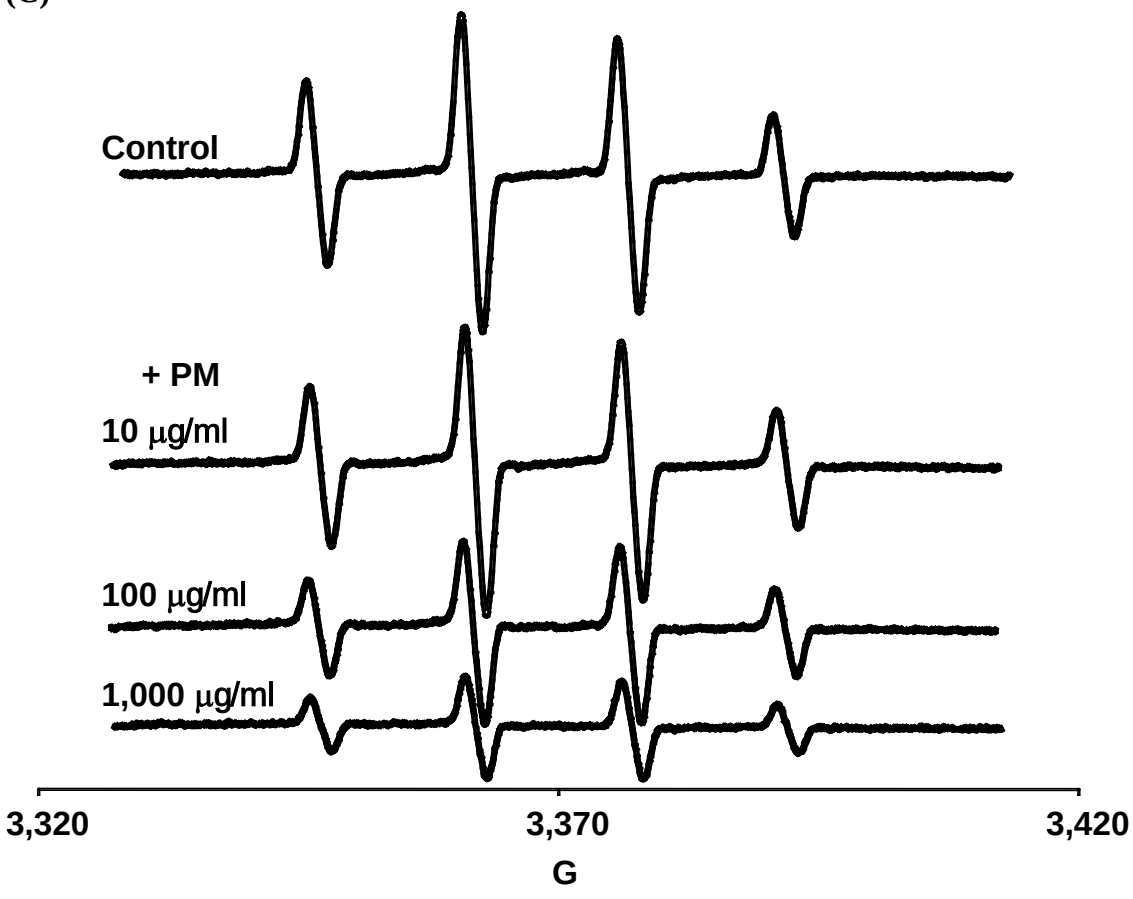


**Figure 3**

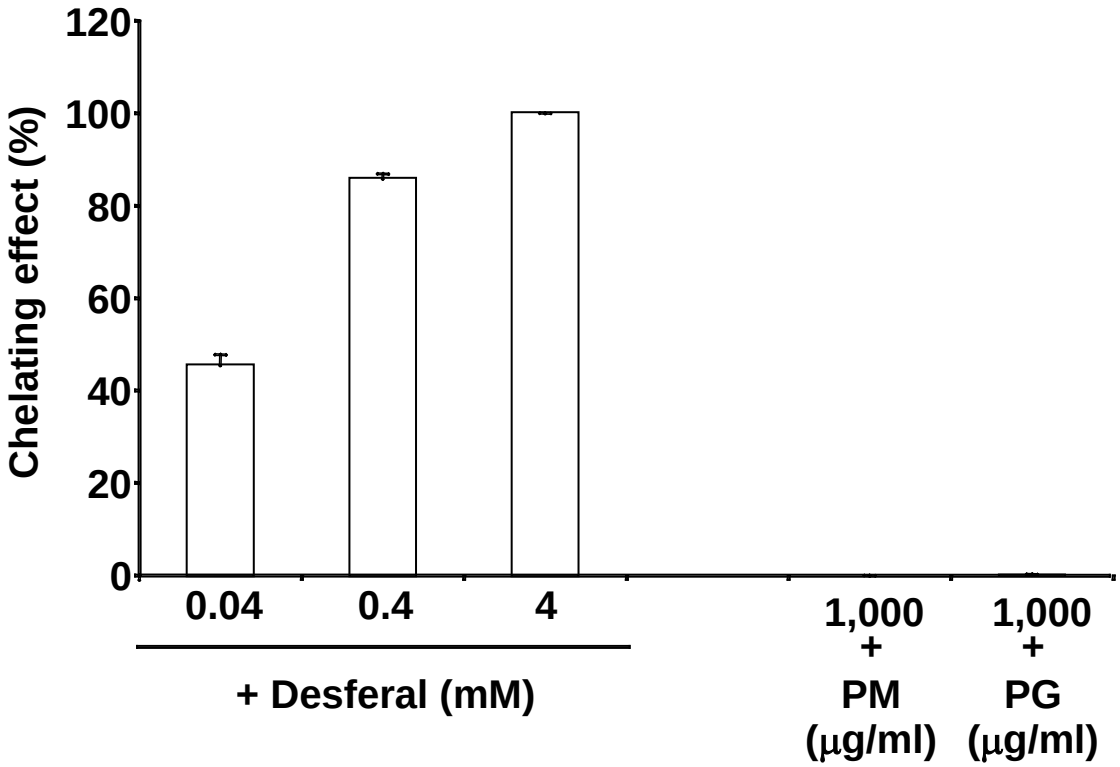

Chelating effect (%)
120
100
80
60
40
20
0
0.04
0.4
4
+ Desferal (mM)
1,000 + PM (μg/ml)
1,000 + PG (μg/ml)

**Figure 4**

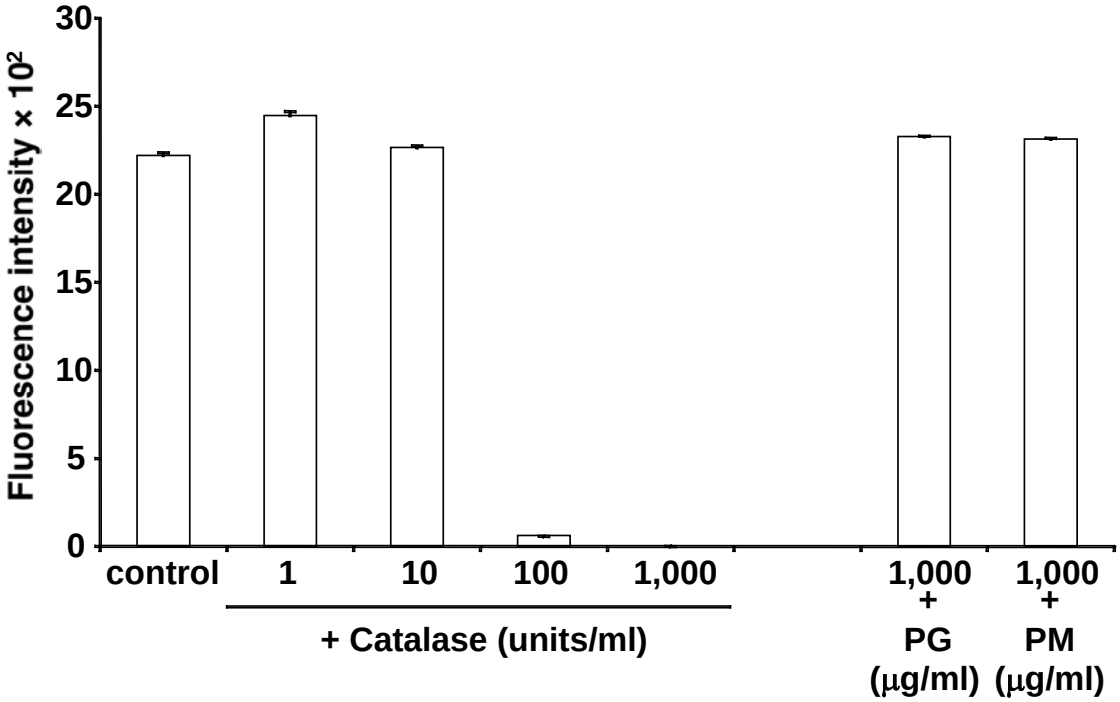
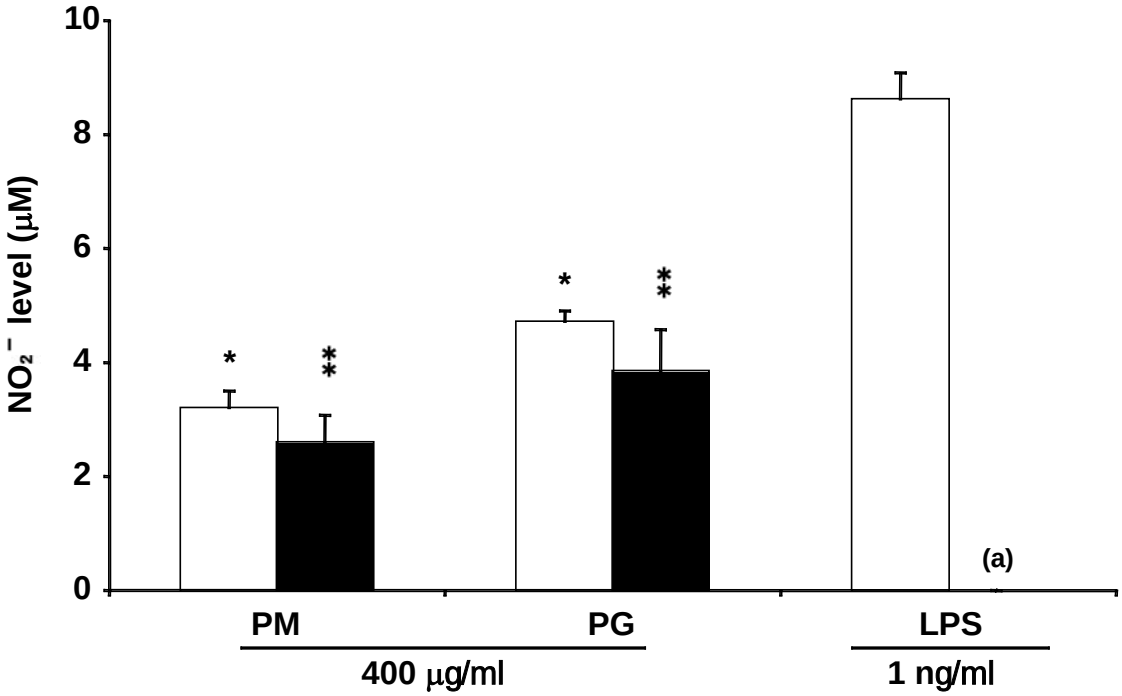
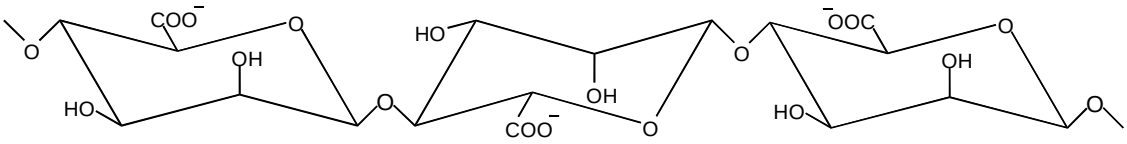

Fluorescence intensity × 10²
30
25
20
15
10
5
0
control
1
10
100
1,000
1,000 + PG (μg/ml)
1,000 + PM (μg/ml)
+ Catalase (units/ml)

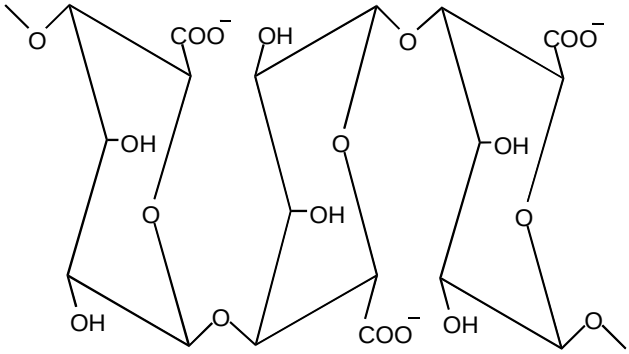
**Figure 5**



**Graphical Abstract**




**Polymannuronate (PM)**




**Polyguluronate (PG)**