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# Antioxidant and nitric oxide production inhibitory activities of galacturonyl hydroxamic acid

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## Abstract

The self-prepared pectin hydroxamic acid has been reported to have antioxidant activities [Yang, S. S., Cheng, K. D., Lin, Y. S., Liu, Y. W., & Hou, W. C. (2004). Pectin hydroxamic acids exhibit antioxidant activities in vitro. *Journal of Agricultural and Food Chemistry, 52*, 4270–4273]. In this study, the galacturonic acid (GalA), the monomer unit of the pectin polymer, was esterified with acidic methanol (1 N HCl) at 4 °C with gentle stirring for 5 days to get galacturonic acid methyl ester which was further reacted with alkaline hydroxylamine to get galacturonyl hydroxamic acid (GalA–NHOH). The GalA–NHOH was used to test the antioxidant and antiradical activities in the comparison with GalA. The scavenging activities of GalA–NHOH against DPPH radicals (half-inhibition concentration, $IC_{50}$, was 82 μM), hydroxyl radicals detected by electron spin resonance ($IC_{50}$ was 0.227 nM in the comparison with Trolox of 0.433 μM), superoxide radicals ($IC_{50}$ was 830 μM) were determined. The protection activities of GalA–NHOH against hydroxyl radicals-mediated calf thymus DNA damages, linoleic acid peroxidation and peroxynitrite-mediated dihydrorhodamine 123 oxidations were also investigated. It was found that the GalA–NHOH exhibited dose-dependently antioxidant activity and few or none was found in GalA. The GalA–NHOH was used to evaluate the suppressed activity of nitric oxide (NO) productions of RAW264.7 cells in the presence of lipopolysaccharide (LPS, 100 ng/ml) as inducers. It was found that GalA–NHOH (0.02–0.1 mg/ml) could dose-dependently suppress the NO productions (expressed as nitrite concentrations) in RAW264.7 cells without significant cytotoxicity.
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*Keywords:* Antioxidant activity; Electron spin resonance (ESR); Galacturonylhydroxamic acid (GalA–NHOH); Nitric oxide; RAW264.7 cells

## 1. Introduction

Rective oxygen species and free radical-mediated reactions have involved in degenerative or pathological processes such as aging (Ames, Shigena, & Hegen, 1993; Harman, 1995), cancer, coronary heart disease and Alzheimer's disease (Ames, 1983; Diaz, Frei, Vita, & Keaney, 1997; Gey, 1990; Smith et al., 1996). There were several reports concerning natural compounds in fruit and vegetable for their antioxidant activities, such as anthocyanin (Espin, Soler-Rivas, Wichers, & Viguera-Garcia, 2000), water extracts of roasted Cassia tora (Yen & Chuang, 2000), and the storage proteins of sweet potato root (Hou, Han, Chen, Wen, et al., 2005), yam tuber (Hou et al., 2001), yam mucilages (Hou, Hsu, & Lee, 2002) and potato tuber (Liu, Han, Lee, Hsu, & Hou, 2003).

A variety of hydroxamic acid derivatives have been reported to have biological activities toward cancer, cardiovascular diseases, Alzheimer's disease and tuberculosis etc (Muri, Nieto, Sindelar, & Williamson, 2002). Succinimide hydroxamic acids were proved as potent inhibitors of histone deacetylase and tumor cell proliferation (Curtin et al., 2002). Hydroxamic acid derivatives of salicylic acid were cyclooxygenase (COX)-1 and COX-2 inhibitors (Dooley et al., 2003). Oxal hydroxamic acid derivatives

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were potent inhibitors of matrix metalloproteinases (Krumme & Tschesche, 2002). The aspartic acid β-hydroxamate exhibited antitumor activity on L5178Y leukemia (Thomasset et al., 1991), therapeutic effect on friend erythroleukemia (Tournaire et al., 1994a) and antiproliferative activity on friend virus-infected erythropoietic progenitor cells (Tournaire et al., 1994b). We reported that the different degree of esterifications of self-prepared pectin hydroxamic acids exhibited both semicarbazide-sensitive amine oxidase and ACE inhibitory activities (Hou, Lee, Hsu, & Lin, 2003) and antioxidant activity (Yang, Cheng, Lin, Liu, & Hou, 2004). The immobilized pectin hydroxamic acid was used as an immobilized metal affinity resin for trypsin inhibitor purification (Liu, Lu, Cheng, & Hou, 2005). The self-prepared alginic acid hydroxamic acid was reported to have antioxidant activities (Liu, Chuang, & Hou, 2007) and the methanol soluble, β-elimination products of alginic acid hydroxamic acid were reported to have 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities (Liu et al., 2007). Pectin, a polymer of GalA with different methyl esters, exists in middle lamella of cell wall and acts as cells' adhesions (BeMiller, 1986). The ratio of esterified GalA to total GalA is called the degree of esterification (DE) (BeMiller, 1986). DE was correlated with firming effects of vegetables during cooking (Hou & Chang, 1996). Except for the food industry of jams and jellies (Thakur, Singh, & Handa, 1997), there are reports concerning the physiological activities of pectins on the interactions between fibroblast growth factors and receptors (Liu et al., 2001), on the modulation of lung colonization of B16-F1 melanoma cell (Platt & Raz, 1992), and on the inhibition of human cancer cell growth and metastasis in nude mice (Nangia-Makker et al., 2002). Pectin diets could also reduce the incidence of colon cancer in rats (Hardman & Cameron, 1995). In this study, galacturonic acid (GalA), the monomer of pectin polymer, was used as a starting material to synthesize galacturonyl hydroxamic acid (GalA–NHOH) through galacturonic acid methyl ester. Neilands (1967) reported there were several the natural hydroxamic acid derivatives from microbes and plants. Hirsch and Kaplan (1961) reported that the conversion of hydroxamic acids to corresponding amides by mouse liver homogenates. Therefore, the several antioxidant and free radical scavenging models were used to evaluate the GalA–NHOH antioxidant activities in the comparison with GalA. It was found that the GalA–NHOH exhibited dose-dependently antioxidant activity and few or none was found in starting materials of GalA. The NO was overproduced by lipopolysaccharide (LPS) (100 ng/ml) induction in the RAW264.7 cells which the overproduced NO catalyzed by iNOS may reflect the inflammation (Chen, Yang, & Lee, 2000; Chi, Cheon, & Kim, 2001). It was also found that GalA–NHOH could dose-dependently suppress the nitric oxide (NO) productions in RAW264.7 cells in the presence of LPS as inducers and showed none significant cytotoxicity.

## 2. Materials and methods

### 2.1. Materials

Ammonium thiocyanate, butylated hydroxytoluene (BHT), dihydrorhodamine 123 (DHR 123), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), DPPH, ferrous sulfate, galacturonic acid (GalA), hydroxylamine, linoleic acid, LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NADH, xanthine, xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Calf thymus DNA (activated, 25 A260 units/mL) was purchased from Amersham Biosciences (Uppsala, Sweden). Peroxynitrite (200 mM in 4.7% NaOH) was obtained from Calbiochem-Novabiochem Co. (Darmstadt, Germany). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Preparations of GalA–NHOH from GalA

The commercial GalA (5 g) was esterified with 500 ml of ice-cold acidic methanol (1 N HCl) at 4 °C with gentle stirring for 5 days (Sajjaanatakul, van Buren, & Downing, 1989) to get galacturonic acid methyl ester. After being adjusted to neutral pH by KOH in methanol the reacted solution was dried by a rotary evaporator. The reacted substances containing galacturonic acid methyl ester were stirred in 500 ml methanol at room temperature for 20 h with a mixed solution (insoluble salt was removed by filtration) containing 13 g of potassium hydroxide in 50 ml methanol and 12 g of hydroxylamine–HCl in 150 ml methanol (Gee, Reeve, & McCready, 1959; Hou et al., 2003; Yang et al., 2004) to get GalA–NHOH. After being adjusted to neutral pH by HCl in methanol the reacted solution was dried by a rotary evaporator. The dried substances containing GalA–NHOH were stirred in methanol, and the vast amounts of insoluble salt were filtered by a G3 glass filter and then discarded, the filtrates were dried by a rotary evaporator. This procedure was repeated in triplicate. After rinsing with 100% methanol, the GalA–NHOH was dried at 37 °C for biological activity assays.

### 2.3. Scavenging activities of DPPH radicals by spectrophotometry

Every 0.3 ml of GalA (0.01, 0.05, 0.1, 0.5, 1.0, and 1.2 mM) and GalA–NHOH (0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 0.6, and 0.8 mM) were added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 0.6 ml of 100 μM DPPH in methanol to the final concentrations of 60 μM for 20 min under light protection at room temperature (Hou et al., 2002; Lin, Liu, Lu, & Hou, 2005; Liu et al., 2003). The decrease of absorbance at 517 nm was measured and expressed as ΔA517 nm. Deionized water was used as a blank experiment. Means of triplicates were measured. The

scavenging activity of DPPH radicals (%) was calculated with the equation: $(\Delta A517_{blank} - \Delta A517_{sample}) \div \Delta A517_{blank} \times 100\%$. The $IC_{50}$ stands for the concentration of half-inhibition.

### 2.4. Scavenging activity of hydroxyl radicals by ESR spectrometry

The hydroxyl radical was generated by Fenton reaction (Lin et al., 2005; Liu et al., 2007). The total 500 μl mixture included GalA (0.5, 2, and 5 mM) and GalA–NHOH (0.05, 0.11, and 0.5 nM), 5 mM DMPO and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed at the cavity of the ESR spectrometer and hydrogen peroxide was added to a final concentration of 0.25 mM. Deionized water was used instead of sample solution for blank experiments. The Trolox (0.048, 0.2, 0.78, and 1.56 μM) was used as a positive control. After 40 s, the relative intensity of the signal of DMPO-OH spin-adduct was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. The conditions of ESR spectrometry were as follows: center field, $345.4 \pm 5.0$ mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min.

### 2.5. Protecting hydroxyl radicals-induced calf thymus DNA damage

The hydroxyl radical was generated by Fenton reaction (Lin et al., 2005; Liu, Wu, Liang, & Hou, 2007). The 27 μl reaction mixture included 10 μl of GalA or GalA–NHOH (2, 5, and 10 mM), 4 μl of calf thymus DNA, 5 μl of 18 mM $FeSO_4$, and 60 mM hydroxygen peroxide at 37 °C for 20 min. 10 μl of 1 mM EDTA was added to stop the reaction. The only calf thymus DNA was used for blank test, and the control test was without sample additions. After the 1.5% agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light.

### 2.6. Scavenging activity of GalA–NHOH against superoxide radicals

The superoxide radical was generated by the xanthine-xanthine oxidase system (Lin, Chen, Ho, & Lin-Shiau, 2000). Every 355 μl of sample containing different amounts of GalA (0.0025, 0.025, 0.25, and 2.5 mM) and GalA–NHOH (0.0125, 0.625, 1.25, and 1.875 mM) were added in sequence to 200 μl of 400 μM nitroblue tetrazolium,75 μl of 1 mM xanthine, 170 μl of 200 mM phosphate buffer (pH 7.4), and 8 mU xanthine oxidase. Deionized water was used as a blank experiment. The changes of absorbance at 560 nm were recorded during 2 min and expressed as ΔA560 nm/min. The scavenging activity of superoxide radicals was calculated as following: $(\Delta A560\ nm/min_{blank} - \Delta A560\ nm/min_{sample}) \div \Delta A560\ nm/min_{blank} \times 100\%$. $IC_{50}$ stands for the concentration of half-inhibition.

### 2.7. Anti-linoleic acid peroxidation

The 0.1 ml of 0.8 mM GalA or GalA–NHOH against 0.5 ml of 0.02 M linoleic acid emulsion during reaction at 37 °C was measured by thiocyanate method (Pham, Cormier, Farnworth, Tong, & Calsteren, 2000) for anti-lipid peroxidation. At intervals during incubation, a 0.1 ml aliquot of the reaction mixture was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid. Precisely 3 min after the addition of ferrous chloride to the reaction mixture, the absorbance at wavelength 500 nm were determined and expressed asΔA500 nm. The higher value of ΔA500 nm, the lower anti-lipid peroxidation activity was found. Deionized water was used as a control experiment. The BHT was used as a positive control. Increased of absorbance at wavelength 500 nm indicates the decrease of antioxidant activity against linoleic acid peroxidation.

### 2.8. Protecting peroxynitrite-mediated DHR 123 oxidation by GalA–NHOH

The protection against peroxynitrite-mediated DHR 123 oxidation was according to the methods of Kooy, Royall, Ischiropoulos, and Beckman (1994). The total 140 μl reaction mixture included different amounts of GalA or GalA–NHOH (0.0154, 0.154, and 1.54 mM), 10 μl of 0.963 mM dihydrorhodamine (in DMF) and 5 μl peroxynitrite (in 110 μl of 200 mM phosphate buffer (pH 7.4). After 10 min reaction, the fluorescent intensity was measured at the excitation and emission wavelengths of 485 and 530 nm, respectively, and excitation and emission slit widths of 2.5 nm and 3.0 nm, respectively. The result was expressed as the protective effect (%). The control test was without sample additions.

### 2.9. Suppression of nitric oxide production by GalA–NHOH

RAW 264.7 cells were cultured in Dulbecco's modified eagle medium (DMEM, GibcoBRL, USA) supplemented with 10% fetal calf serum (FCS), 10000 I.U./mL penicillin, 10000 μg/ml streptomycin, 25 μg/ml amphotericin, and 1% L-glutamate. The cell number was adjusted to $2 \times 10^5$ cells/ml. Cell suspension (1 ml) were seeded onto a 96-well microtiter plate and LPS (100 ng/ml) only and LPS (100 ng/ml) plus with various concentrations of GalA–NHOH (0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml, respectively, corresponding to 86, 172, 258, 344, and 430 μM) were added and cultured in 5% $CO_2$ humidified incubator at 37 °C for 24 h. The cell viability was assayed by MTT staining (Allen, Hunter, & Agrawal, 1997; Chuang, Lin, & Hou, 2007) and expressed as relative cell viability (%).

The medium only group was recognized as 100% relative cell viability. The cultured plate was centrifuged at 1500 rpm for 10 min, and supernatants were collected for determinations of NO productions. Each of the 100 μl of cultured supernatant was added onto a 96-well micotiter plate. The 100 μl of Griess reagent was added to each well and stand for 15 min at room temperature. The absorbance at 530 nm was measured and sodium nitrite (0–500 microM) was used to plot the standard curve (Kobuchi, Droy-Lefaix, Christen, & Packer, 1997).

### 2.10. Statistics

Means ± SD of triplicates were measured ($n = 3$). Student's t-test was used for comparison between LPS and LPS plus different concentrations of GalA–NHOH. A difference was considered statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

## 3. Results and discussion

### 3.1. Scavenging activity against DPPH radicals of GalA–NHOH

The hydroxamic acid derivatives exhibited biological activities toward cancer, cardiovascular diseases, Alzheimer's disease and tuberculosis etc. (Muri et al., 2002). The reactive oxygen species and free radical-mediated reactions have been reported to involve in diseases above-mentioned (Ames, 1983; Ames et al., 1993; Diaz et al., 1997; Gey, 1990; Harman, 1995; Smith et al., 1996). In this study, GalA, the monomer of pectin polymer, was used as a starting material to synthesize GalA–NHOH through galacturonic acid methyl ester. The GalA–NHOH was used to test the antioxidant and antiradical activities in the comparison with GalA. It was found that the GalA–NHOH showed dose-dependently scavenging activities against DPPH radicals (Fig. 1), and the half-inhibition concentration, $IC_{50}$, was 82 μM. No or few DPPH scavenging activity of GalA (original material) was found under the same concentrations. The purified five myricetin galloylglycosides from leaves of *Acacia confusa* exhibited $IC_{50}$ value ranged from 591 to 3210 μM (Lee, Liu, Hsu, Wu, & Hou, 2006). The $IC_{50}$ of scavenging activities against DPPH radicals of ascorbic acid and BHT were 13.1 μM and 18.5 μM, respectively. The anti-DPPH radical capacities of GalA–NHOH were about 1/6.3 and 1/4.4 that of ascorbic acid and BHT, respectively. The resonance of hydroxamic acid moiety (Yale, 1943) in GalA–NHOH might contribute its free radical scavenging activities.

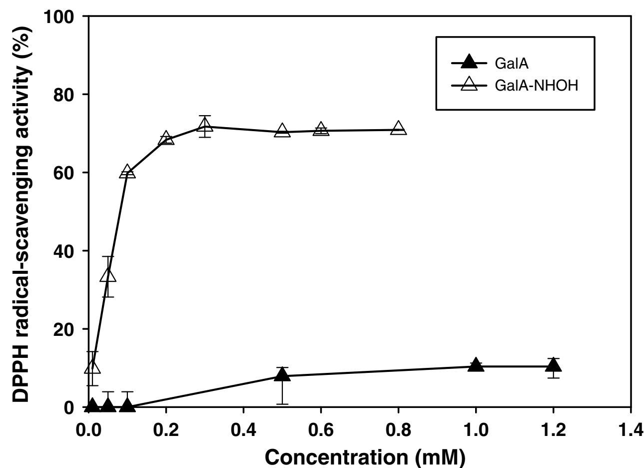


Fig. 1. Effects of different concentrations of GalA (0.01, 0.05, 0.1, 0.5, 1.0, and 1.2 mM) and GalA–NHOH (0.01, 0.05, 0.1, 0.2, 0.3, 0.5, 0.6, and 0.8 mM) on the scavenging activities of DPPH radicals. The scavenging activity of DPPH radicals (%) was calculated with the equation: $(\Delta A517_{blank} - \Delta A517_{sample}) \div \Delta A517_{blank} \times 100\%$.

### 3.2. Scavenging activity of hydroxyl radicals by ESR spectrometry

The changes of the intensity of DMPO-OH spin signal in ESR spectrometry were used to evaluate the scavenging activity of hydroxyl radicals of GalA (Fig. 2A) or GalA–NHOH (Fig. 2B), and the Trolox was used as the positive control (Fig. 2C). The Trolox exhibited dose-dependently scavenging activities against hydroxyl radicals, and the $IC_{50}$ was calculated to be 0.433 μM (Fig. 2C). The GalA showed minor hydroxyl radical scavenging activity. The 200 μM and 500 μM GalA showed 20.83 and 21.03% scavenging activities, respectively (Fig. 2A). However, the GalA–NHOH showed much higher and dose-dependent scavenging activity against hydroxyl radicals, the 0.11 nM and 0.5 nM showed 37.37 and 79.40% scavenging activities, respectively, and the $IC_{50}$ was calculated to be $2.27 \times 10^{-4}$ μM (Fig. 2B) which was about 1900-folds as that of Trolox in hydroxyl radical scavenging activity by ESR assay.

### 3.3. Protecting hydroxyl radicals-induced calf thymus DNA damage of GalA and GalA–NHOH

Free radicals could damage macromolecules in cells, such as DNA, protein and lipids in membranes (Halliwell, 1999). Fig. 3 showed the GalA (2, 5, and 10 mM) and GalA–NHOH (2, 5, and 10 mM) protections against hydroxyl radicals-induced calf thymus DNA damages. The only calf thymus DNA was used for blank test (lane B), and the control test was without sample additions (lane C). Compared to blank test and control test, it was found that the added GalA at 10 mM or GalA–NHOH at 5 mM (Fig. 3) could protect hydroxyl radical-induced calf thymus DNA damages after 20 min reactions.

### 3.4. Scavenging activity of GalA–NHOH against superoxide radicals

The superoxide radical was generated by the xanthine-xanthine oxidase system (Lin et al., 2000). In the preliminary experiment, it was found that both GalA and

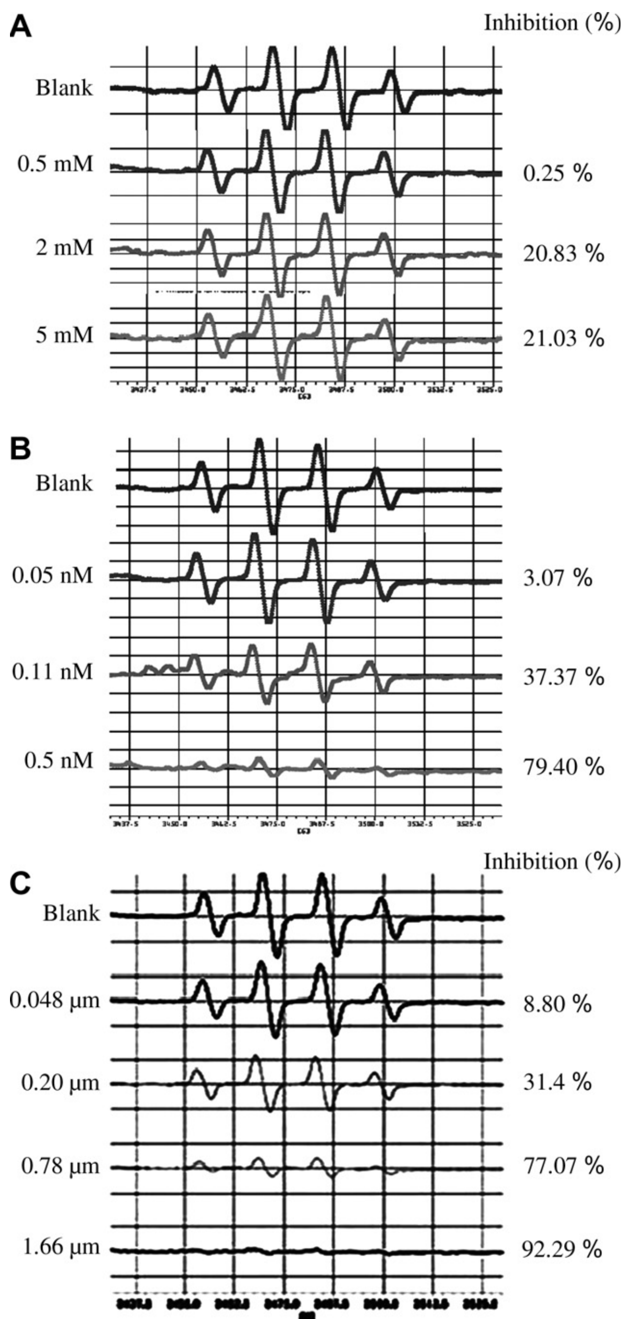


Fig. 2. The scavenging activity of different concentrations of (A) GalA (0.5, 2, and 5 mM), (B) GalA–NHOH (0.05, 0.11, and 0.5 nM), and (C) Trolox (0.048, 0.2, 0.78, and 1.56 μM) against the hydroxyl radicals measured by electron spin resonance spectrometry. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. The conditions of ESR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min.

GalA–NHOH did not inhibit the uric acid formation from xanthine catalyzed by xanthine oxidase, therefore, the use of xanthine-xanthine oxidase system for scavenging activity of GalA and GalA–NHOH against superoxide radicals was performed and showed at Fig. 4. The GalA had no effects on superoxide radical scavenging activity up to 2.5 mM, however, the GalA–NHOH exhibited dose-dependently scavenging activity against superoxide radicals and the $IC_{50}$ was calculated to be 0.823 mM (Fig. 4).

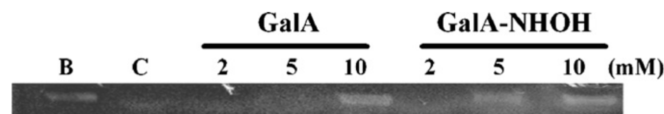


Fig. 3. The effects of GalA and GalA–NHOH (2, 5, and 10 mM) on the protections of hydroxyl radicals-induced calf thymus DNA damages after reaction at 37 °C for 20 min. After the agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light. The only calf thymus DNA was used for the blank test, and the control test (C) was without sample additions.

### 3.5. Anti-linoleic acid peroxidation

The products of the lipid peroxidation (such as malondialdehyde) could cause damage to proteins and DNA (Esterbauer, Schaur, & Zollner, 1991). The linoleic acid emulsion during reaction at 37 °C was measured by thiocyanate method (Pham et al., 2000) as models for lipid peroxidations (Fig. 5) and expressed as ΔA500 nm which the higher value of ΔA500 nm the more lipid peroxidation activity was found. From the results of Fig. 5, it was clear that the BHT and GalA–NHOH exhibited the similar anti-lipid peroxidation activity up to 14 h reaction, while, the lipid peroxidation in the blank and GalA group was increased gradually and kept the maximum after 6 h reaction.

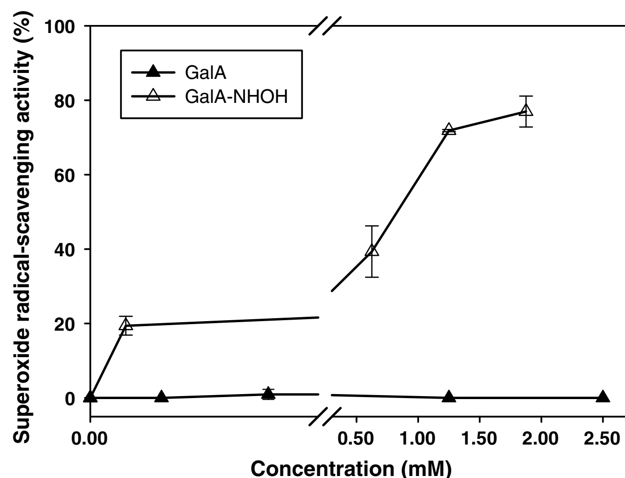


Fig. 4. Effects of different concentrations of GalA (0.0025, 0.025, 0.25, and 2.5 mM) and GalA–NHOH (0.0125, 0.625, 1.25, and 1.875 mM) on the scavenging activities of superoxide radical generating by the xanthine-xanthine oxidase generating system. The scavenging activity of superoxide radicals (%) was calculated as following: $(\Delta A560\ \mathrm{nm/min_{blank}} - \Delta A560\ \mathrm{nm/min_{sample}}) \div \Delta A560\ \mathrm{nm/min_{blank}} \times 100\%$.

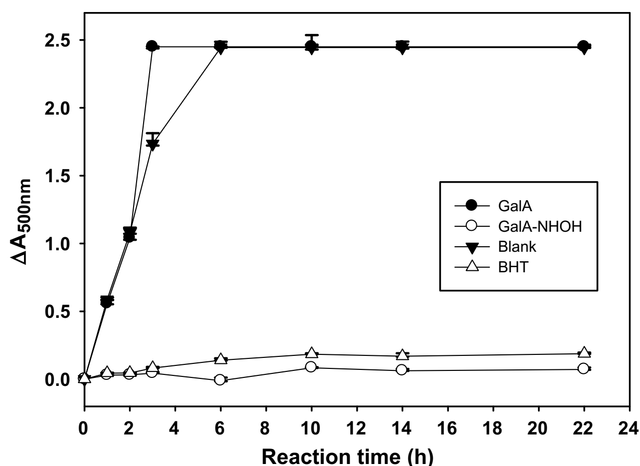


Fig. 5. Antioxidant activity of GalA and GalA–NHOH against linoleic acid peroxidation reactions during the interval of 14 h at 37 °C by thiocyanate method. Deionized water was used as a blank experiment. The BHT was used as a positive control. Means of triplicates of the absorbance at wavelength 500 nm were determined.

### 3.6. Protecting peroxynitrite-mediated DHR 123 oxidation by GalA–NHOH

Peroxynitrite is formed from nearly diffusion-limited reaction between nitric oxide and superoxide and acts as an initiator of potentially harmful oxidation reaction (Brannan, Connolly, & Decker, 2001). From the results of Fig. 6, it was found that the protected effect of peroxynitrite-mediated DHR oxidation of GalA–NHOH was dose-dependent and the protective effect was 39.79, 41.87, and 57.25%, respectively, for 0.0154, 0.154, and 1.54 mM, but not found in GalA which was closed to 20% among 0.0154 ∼ 1.54 mM. It was calculated that the $IC_{50}$ of GalA–NHOH against peroxynitrite-mediated DHR 123 oxidation was 0.887 mM (Fig. 6).

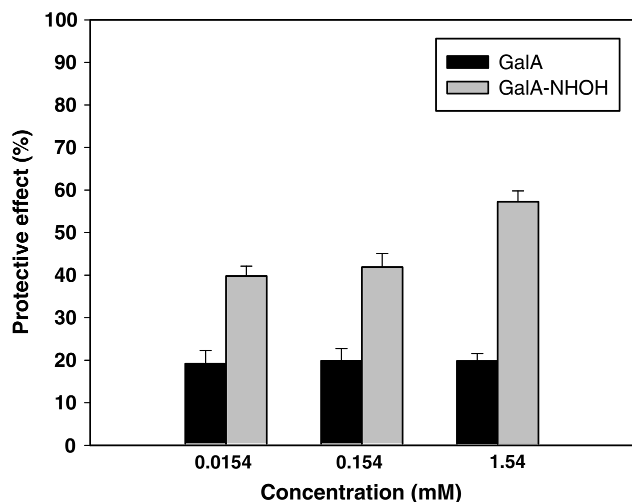


Fig. 6. The GalA and GalA–NHOH (0.0154, 0.154, and 1.54 mM) protected peroxynitrite-mediated dihydrorhodamine 123 oxiadtion. The total 140 μl reaction mixture contained GalA or GalA–NHOH, 10 μl of 0.963 mM dihydrorhodamine (in DMF) and 5 μl peroxynitrite in 110 μl of 200 mM phosphate buffer (pH 7.4). After 10 min reaction, the fluorescent intensity was measured at the excitation and emission wavelengths of 485 and 530 nm, respectively, and excitation and emission slit widths of 2.5 nm and 3.0 nm, respectively.

### 3.7. Suppression of nitric oxide production by GalA–NHOH

The effects of GalA–NHOH on RAW 264.7 cell viability were determined by a MTT assay (Fig. 7A). Compared to the control, the GalA–NHOH (0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml) did not show the significant cytotoxicity toward RAW264.1 cells. Therefore, the effects of GalA–NHOH on LPS-induced NO production in macrophage was examined. The nitrite in the cultured medium was detected by the Griess reagent, an indirect method to measure the release of NO (Fig. 7B). Macrophages cultured with different concentrations of GalA–NHOH (0.02, 0.04, 0.06, 0.08,

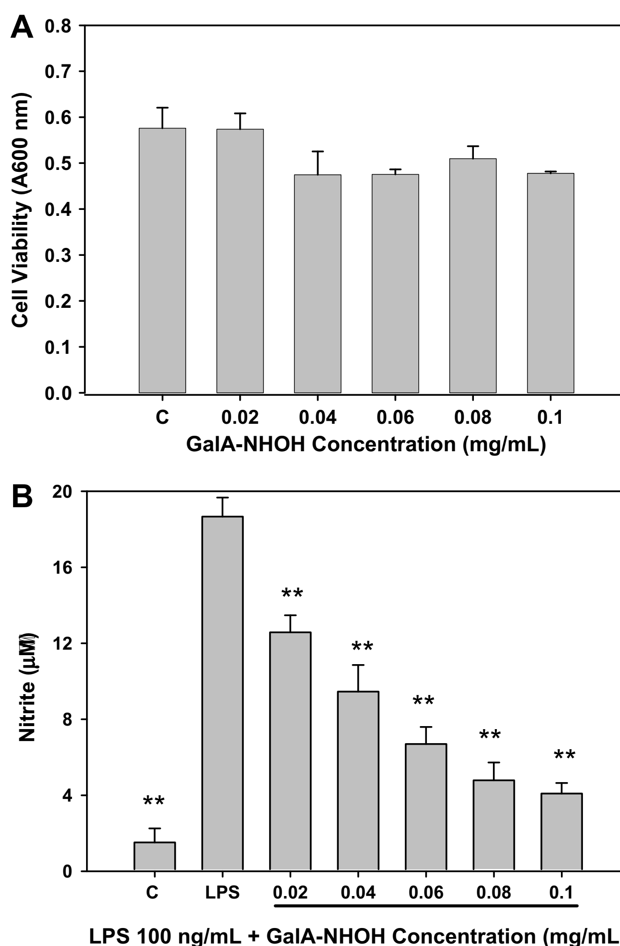


Fig. 7. (A) Effects of GalA–NHOH (0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml) in the cytotoxicity of RAW264.7 cells by MTT staining and (B) the effects of GalA–NHOH (0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml) on the suppressive effects against LPS-induced nitric oxide productions (expressed as nitrite) in RAW264.7 cells. LPS (100 ng/ml) only and LPS (100 ng/ml) plus with various concentrations of GalA–NHOH were added and cultured in 5% CO2 humidified incubator at 37 °C for 24 h. The cultured plate was centrifuged at 1500 rpm for 10 min, and supernatants were collected for determinations of NO productions by Griess reagent.

and 0.1 mg/ml, respectively, corresponding to 86, 172, 258, 344, and 430 μM) in the presence of LPS (100 ng/ml) resulted in dose-dependently suppressed NO productions ($P < 0.01$, each treatment compared with LPS alone). NO is an important molecule which is the biosynthesis via the conversion from L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS, E.C. 1.14.13.39). NO plays a number of physiological effects including blood pressure modulation, neural signal transduction, platelet function, and antimicrobial and antitumor activities. However, over-produced NO by iNOS can be induced quantitatively in macrophages, smooth muscle cells and hepatocytes to trigger several disadvantage cellular responses and caused some diseases including inflammation, sepsis, stroke, and with the development of atherosclerosis (Bruckdorfer, 2005). In the results of Fig. 7, it was clear that the GalA–NHOH exhibited suppressed effects on LPS-induced NO production in macrophage.

## 4. Conclusion

In conclusions, the galacturonic acid derivatives of GalA–NHOH showed antioxidant, antiradical and suppressed effects on LPS-induced NO production in macrophage. A variety of hydroxamic acid derivatives have been reported to have biological activities toward cancer, cardiovascular diseases, Alzheimer's disease and tuberculosis etc. The animal model for anti-inflammation will be used for GalA–NHOH in the future.

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