QBS
Vol. 35, No. 2, pp. 45-51 (2016)
https://doi.org/10.22283/qbs.2016.35.2.45
pISSN : 2288-1344 **Original Article**

Quantitative Bio-Science
Official Journal of the Institute of Natural Science at Keimyung University
Available Online at http://qbs.kmu.ac.kr

Antioxidative Activity of Gallic Acid Derivatives

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(Received October 25, 2016; Revised November 16, 2016; Accepted November 16, 2016)

ABSTRACT

Antioxidants effectively quench oxidative damage to biomolecules. Therefore, antioxidant supplements have emerged as an alternative to reduce oxidative stress-related diseases. Gallic acid shows a wide range of biological activities, including antioxidant properties, but has inappropriate physicochemical properties for wide spread applications. Herein, the chemical structure of gallic acid was modified, and the antioxidative activities of the derivatives were evaluated. The introduction of a 3,4-dihydroxybenzylamino group onto gallic acid via an amide linkage provided an effective antioxidant that showed potent antioxidative activities comparable to those of propyl gallate.

Key words : Antioxidant, Gallic acid, Propyl gallate, 3,4-Dihydroxybenzylamine

Introduction

Free radical-induced damage in oxidative stress is strongly related to many maladies such as neurodegenerative, cardiovascular and inflammatory diseases, as well as cancer [1]. Oxidative stress is known to weaken various physiological functions, increase disease incidence, and reduce the human life span [2]. It has been understood that the exogenous antioxidants supplied either by diet or injection are complemented by the endogenous antioxidant homeostasis system. Nevertheless, it has been suggested that the exogenous antioxidants would be effective if oxidative stress is above normal levels and cannot be balanced [1].

Phenolic compounds have been recognized as antioxidants that delay or inhibit the initiation step or interrupt the propagation step of oxidation by scavenging free radicals [3,4]. 3,4,5-Trihydroxybenzoic acid, also known as gallic acid, shows strong antioxidant activities and exhibits many cellular activities including anti-proliferative effects on cancer cells [3,5-8]. However, it has high aqueous solubility which is a disadvantage for wide spread applications. Many efforts have been devoted toward the development of a new antioxidant based on gallic acid. For example, the modification of the carboxyl group to an ester provided propyl gallate, which is widely used as an antioxidant in the food industry and has been recognized as an important synthetic antioxidant.

Herein, we report the modification of gallic acid using an amide linkage and the evaluation of the antioxidative activity of the resulting derivatives as an initial step toward identifying potent and highly bioavailable antioxidants.

Materials and Methods

Materials and instruments

^{1}H- and ^{13}C-NMR spectra were obtained on an AVANEC 400 (Bruker, Switzerland) and are reported in parts per million (δ) relative to TMS as an internal standard. TLC was performed on E. Merck silica gel 60 F254 plates (0.25 mm). Silica gel col-

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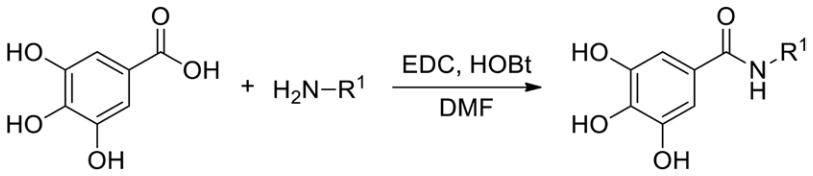


Scheme 1. Reagents and experimental conditions. HOBt, EDC, DMF, RT.

umn chromatography was performed using Merck TA1287685. Unless otherwise noted, all starting materials were obtained from commercially available sources and were used as received without further purification. All reactions were performed under a nitrogen atmosphere. Statistical analyses were performed using IBM SPSS Statistics ver. 23.

Chemistry

3,4,5-Trihydroxybenzoic acid derivatives were synthesized from 3,4,5-trihydroxybenzoic acid in one step (Scheme 1). An amine substituent was introduced into 3,4,5-trihydroxybenzoic acid by amide coupling using *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt). The typical reaction procedure is as follows.

3,4,5-Trihydroxy-*N*-(-4-methoxybenzyl)benzamide (1); Method A

To 100.0 mg (0.532 mmol) of 3,4,5-trihydroxybenzoic acid in 15 mL of N,N-dimethylformamide (DMF) were added EDC 150.0 mg (0.998 mmol) and HOBt 140.0 mg (1.06 mmol), and the reaction mixture was stirred for 20 min. After the addition of 56.0 μL (0.425 mmol) of 4-methoxybenzylamine, the reaction mixture was further stirred for 4 h at room temperature. The residue obtained upon removal of the solvent in vacuo was treated with ethyl acetate (EA). EA solution was washed consecutively with 5% aqueous citric acid solution, saturated aqueous sodium bicarbonate solution, and distilled water. After drying over magnesium sulfate, filtration, and removal of EA, the crude product was purified by flash column chromatography using a dichloromethane/methanol (DCM : MeOH, 90 : 10) mixture to give 76.4 mg of the pure product in 49.6% yield.

3,4,5-Trihydroxy-*N*-(-4-hydroxybenzyl)benzamide (2); Method B

3 mL of boron tribromide was added to 125 mg (0.443 mmol) of compound **1** in 5 mL of DCM at 0°C; the reaction mixture was stirred at RT for 6 h. After quenching with water and removing the solvent, the crude product was dissolved in EA, and the organic layer was washed with water. After drying over magnesium sulfate, filtration, and removal of EA, the crude product was purified by flash column chromatography using a DCM/MeOH/formic acid (90 : 20 : 1) mixture to give 44.1 mg of the pure product in 34.7% yield.

Antioxidation assays

The antioxidant activities of the compounds were evaluated using four different methods. The radical scavenging activity was measured directly using DPPH and ABTS radicals. The effects on lipid peroxidation were measured indirectly using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods.

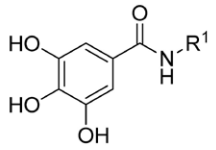
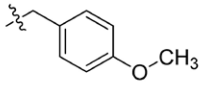
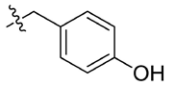
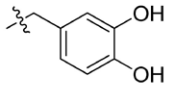
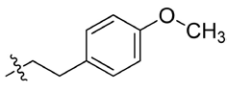
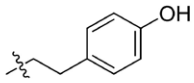
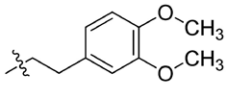
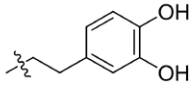
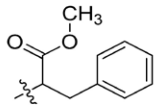
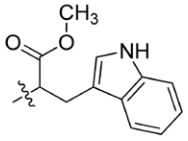
(a) Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH radical)

The free radical scavenging activity of the compound was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH• absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, the absorption decreases. This assay was carried out as described by Erkan [9] with some modifications; 800 μL of various dilutions of the samples in ethanol were mixed with 200 μL of a 0.15 mM ethanolic DPPH• solution. After an incubation period of 30 min at 25°C, the absorbance at 517 nm was recorded. The antioxidant activities of the compounds were expressed as EC_{50}, defined as the concentration of the test material required to cause a 50% decrease in the initial DPPH• concentration.

(b) Scavenging of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical (ABTS radical)

The assay was carried out according to the procedure described by Re [10]. The ABTS radical cation was produced by mixing 7 mM aqueous ABTS with 2.45 mM (final concentration) potassium persulfate and keeping the mixture in the dark at RT for 16 h. Blue-green $ABTS^{\bullet+}$ was formed at the end of this period. The solution was diluted with ethanol to the con-

Table 1. Structures, synthetic methods, and NMR spectral data of gallate derivatives

	 R[1]	Method (Yield)	NMR Spectral Data ($CDCl_3 + CD_3OD$) ^{1}H NMR (400 MHz) & ^{13}C NMR (100 MHz)
1		A (49.6%)	^{1}H NMR: δ (ppm) 7.24 (s, 1H), 7.21 (s, 1H), 6.85 (s, 3H), 6.83 (s, 1H), 4.48 (s, 2H), 3.78 (s, 3H). ^{13}C NMR: δ (ppm) 168.2, 158.8, 145.1, 136.7, 130.9, 128.5, 124.8, 106.6, 54.7, 42.8
2		B (34.7%)	^{1}H NMR: δ (ppm) 7.8 (d, J = 8.4 Hz, 2H), 6.88 (s, 2H), 6.78 (d, J = 8.4 Hz, 2H), 4.45 (s, 2H).
3		B (20.7%)	^{1}H NMR: δ (ppm) 6.86 (s, 2H), 6.80 (d, J = 2 Hz 1H), 6.78 (dd, J = 2 Hz, 1H), 6.75 (s, J = 8 Hz, 1H), 4.4 (d, J = 4.4, 2H).
4		A (37.8%)	^{1}H NMR: δ (ppm) 7.12 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.8 Hz, 2H), 6.80 (s, 2H), 3.78 (s, 1H), 3.55 (t, J = 6.8 Hz, 2H), 2.8 (t, J = 7.2 Hz, 2H). ^{13}C NMR: δ (ppm) 169.1, 158.2, 145.1, 136.6, 131.2, 129.5, 124.9, 113.7, 106.5, 54.6, 41.65
5		A (20.9%)	^{1}H NMR: δ (ppm) 7.8 (d, J = 8.4 Hz, 2H), 6.82 (s, 2H), 6.75 (d, J = 8.8 Hz 2H), 3.5 (q, J = 6 Hz, 2H), 2.28 (t, J = 7.6 Hz, 2H). ^{13}C NMR: δ (ppm) 160.9, 155.3, 145.1, 136.5, 130.1, 129.6, 124.9, 115.1, 108.9, 34.6, 29.5
6		A (55.3%)	^{1}H NMR: δ (ppm) 6.82 (d, J = 7.2 Hz, 3H), 6.78 (d, J = 1.6 2H), 3.85 (d, J = 3.2 Hz, 2H), 3.55 (t, J = 8 Hz, 2H), 2.83 (t, J = 7.6 Hz, 2H). ^{13}C NMR: δ (ppm) 168.5, 148.8, 147.4, 145.0, 136.5, 131.9, 124.9, 120.8, 112.2, 111.5, 106.5, 55.5, 41.5, 35.0
7		B (58.1%)	^{1}H NMR: δ (ppm) 8.2 (s, 1H), 6.8 (d, J = 8.8 Hz, 4H), 6.9 (s, 2H), 6.85 (s, 4H), 6.3 (d, J = 8 Hz, 1H), 3.80 (s, 6H)
8		A (18.9%)	^{1}H NMR: δ (ppm) 7.28 (s, 2H), 7.23 (t, J = 7.6 Hz, 2H), 7.21 (q, J = 6.8 Hz, 3H), 6.80 (s, 2H), 4.8 (q, J = 2.4 Hz, 1H), 3.7 (s, 3H), 3.32 (q, J = 5.6 Hz, 1H), 3.31 (q, J = 8.8 Hz, 1H)
9		A (41.6%)	^{1}H NMR: δ (ppm) 7.55 (d, J = 8 Hz, 1H), 7.35 (d, J = 8 Hz, 1H), 7.1 (t, J = 4.8 Hz, 2H), 6.88 (t, J = 8 Hz, 1H), 6.8 (s, 2H), 4.88 (m, J = 3.2 Hz, 1H), 3.7 (s, 3H), 3.4 (m, J = 5.6 Hz, 1H)

centration that showed an absorbance of 0.70 ± 0.02 at 734 nm. After adding 35 μL of the ethanol solution to 3.5 mL of the ABTS•$^+$ solution, the absorbance was recorded for 6 min in 1 min intervals.

(c) Ferric thiocyanate (FTC) method

The FTC assay was carried out according to the procedure reported by Kikuzaki and Nakatani [11]. A mixture of 1 mL of an ethanolic solution of the synthesized compound (50 μM), 1 mL of 2.52% linoleic acid in ethanol, 2 mL of 0.05 M phosphate buffer (pH 7.0), and 1 mL of water was placed in a vial with a screw cap and then placed in a dark oven at 40°C. 50 μL aliquots were withdrawn from the mixture every 48 h and diluted with 4.85 mL of 75% ethanol, followed by the addition of 50 μL of 30% ammonium thiocyanate. After adding 50 μL of 0.02 M ferrous chloride in 3.5% HCl, the absorbance of the solution was measured at 500 nm. The measurement was repeated until the absorbance reached its maximum. The ratio of the absorbance to the absorbance of a blank without any test material was taken as a measure of the ability of the test material to inhibit lipid peroxidation, which is a measure of antioxidant activity. Propyl gallate and gallic acid were used as positive controls, while a mixture without the compound was used as a negative control.

(d) Thiobarbituric acid (TBA) method

The method described by Ottolenghi was followed [12]. 250 μL aliquots were withdrawn from the mixtures, prepared for

the FTC experiments, every 48 h and were mixed with 500 μL of 20% trichloroacetic acid and 500 μL of 0.8% 2-thiobarbituric acid. The resulting mixture was placed in a boiling water bath for 20 min and then cooled to room temperature. It was centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant at 532 nm was measured. The measurement was repeated until the final day of the FTC experiments. Antioxidant activity was based on the day when the maximum absorbance was observed. Propyl gallate and gallic acid were used as positive controls, while a mixture without the compound was used as a negative control.

Statistical analysis

The experimental data were presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA). Duncan's multiple range test was used for multiple comparisons. Values of $p < 0.05$ were regarded as statistically significant. The Pearson correlation coefficient was computed to identify relationships.

Results and Discussion

Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) is one of the potent natural antioxidants. Because of its low lipophilicity, gallic acid has limited applications as a therapeutic agent or food additive. Efforts to improve the physicochemical properties of gallic acid have been carried out through the conversion of the carboxyl group to ester or amide functionalities. Propyl gallate (PG), a popular antioxidant, is an example of a synthesized gallic acid derivatives with desirable properites.

A novel synthetic modification of gallic acid was performed and the antioxidative activity of the resulting derivatives were examined. Amide coupling with benzylamine and phenethylamine derivatives provided compounds **1** to **9**. The antioxidative activities of the compounds were evaluated by studying the radical scavenging ability and inhibitory activity for lipid peroxidation. The radical scavenging activity was studied using DPPH and ABTS radicals. The FTC method was used to measure the peroxide sequestering activities of the compounds, while the TBA method was used to measure the protective activities of compounds against lipid peroxidation.

Both synthesized and commercially available gallate derivatives showed strong DPPH radical scavenging activities compared to other antioxidants such as BHA, TBHQ, and ascorbic acid. However, there were no statistically meaningful differences in the activities of the gallate derivatives (Fig. 1). Furthermore, the gallate derivatives showed better ABTS radical scavenging activities than BHA, Trolox, and TBAQ except for lauryl gallate (Fig. 2). Slight differences in the activities of gallate derivatives were observed, and gallic acid showed the strongest ABTS radical scavenging activity.

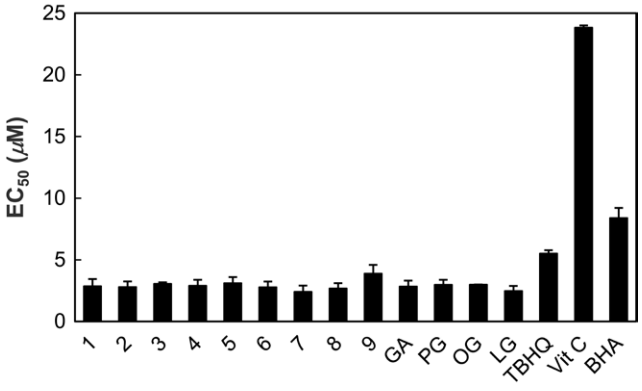


Fig. 1. DPPH radical scavenging activity of synthesized compounds. GA: gallic acid, PG: propyl gallate, OG: octyl gallate, LG: lauryl gallate, TBHQ: tert-butylhydroquinone, Vit C: ascorbic acid, BHA: tert-butylhydroxyanisole.

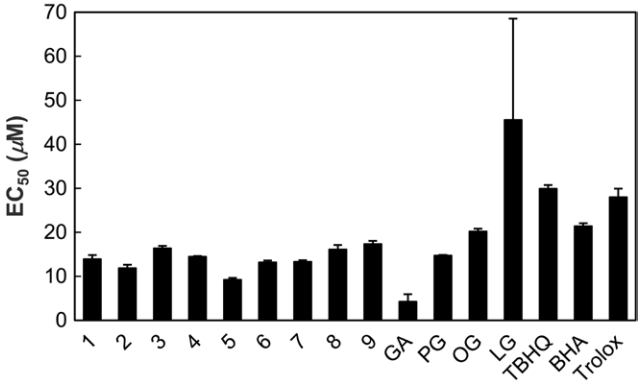


Fig. 2. ABTS radical scavenging activity of synthesized compounds. GA: gallic acid, PG: propyl gallate, OG: octyl gallate, LG: lauryl gallate, TBHQ: tert-butylhydroquinone, BHA: tert-butylhydroxyanisole.

The synthesized gallate derivatives showed improved peroxide quenching activities as compared to gallic acid, according to the results of the FTC assay (Fig. 3). In addition, they retarded the lipid peroxidation according to the results of the TBA assay (Fig. 4). The anti-peroxidation activities varied depending on the structure of the introduced amine derivative. Compounds with a catechol moiety, namely, **3** and **7**, showed better antioxidative activities. In addition, the distance between

the gallate and catechol moieties affected the activity. One methylene unit, **3**, was found to be the optimal condition to afford strong activity comparable to that of PG.

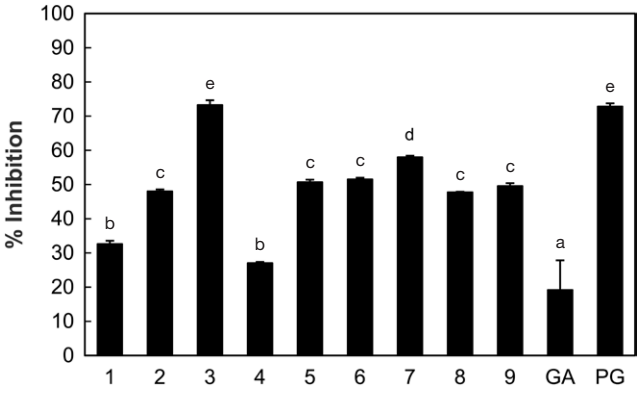


Fig. 3. Inhibition of lipid peroxidation by synthesized compounds (FTC assay). GA: gallic acid, PG: propyl gallate. Values with different letters are significantly ($P<0.05$) different by ANOVA and Duncan's multiple range tests.

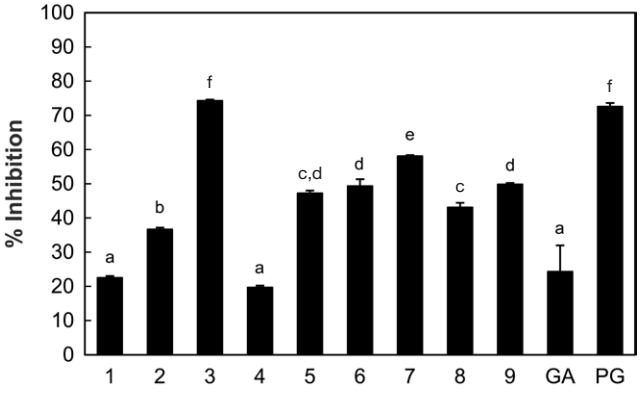


Fig. 4. Inhibition of lipid peroxidation by synthesized compounds (TBA assay). GA: gallic acid, PG: propyl gallate. Values with different letters are significantly ($P<0.05$) different by ANOVA and Duncan's multiple range tests.

Many methods are available for measuring the antioxidant capacity of a molecule, and they can be classified into two types based on the reactions involved: electron transfer (ET) reactions and hydrogen atom transfer (HAT) reactions [13]. However, the correlations between assays and between antioxidative activities and physicochemical properties are not well defined. For the gallate derivatives, there were no significant correlations between the DPPH assay results and the results of the ABTS, FTC, and TBA assays. The ABTS results showed a medium correlation with the FTC and TBA assays ($r=0.56$ and 0.47, respectively). However, the FTC and TBA results were strongly correlated ($r=0.96$), and the correlation was significant at the 0.01 level (2-tailed).

Next, the correlations between the physicochemical properties of the compounds and antioxidative activities were evaluated. It was reported that the hydrophilic or lipophilic character affects the antioxidant activity of gallic acid derivatives [14]. Moreover, the neuroprotective effects of gallic acid derivatives depends more on their molecular polarities than antioxidant activities [7]. Because lipid peroxidation was monitored at the emulsion of water and lipid, the lipophilicity was expected to contribute to the inhibition of lipid peroxidation. In addition, since the introduction of a catechol moiety enhanced both DPPH and ABTS scavenging activities, as mentioned previously with compounds **3** and **7**, the number of phenolic OH moieties was expected to be important. Molecular properties such as partition coefficient (cal'd logP), topological surface area (TPSA), number of oxygen and nitrogen atoms (No. of O and N), and number of phenolic hydroxyl groups (No. of phenolic OH) were evaluated and used for the study of relationships (Table 2). However, significant correlations were not be found.

In general, FTC and TBA assays are carried out simultane-

Table 2. Physicochemical properties of gallate derivatives and days for maximum anti-peroxidation

	MW	*cal'd* LogP*	TPSA**	No. of O and N	No. of phenolic OH	ID_{max} (FTC)***	ID_{max} (TBA)
GA	170.1	0.91	98.0	5	3	6	8
PG	212.2	2.60	87.0	5	3	10	10
1	303.3	2.01	99.0	6	3	4	4
2	289.3	2.08	99.0	6	4	6	6
3	289.3	1.36	110.0	6	5	6	10
4	333.3	1.83	108.3	7	3	6	8
5	370.4	1.95	131.9	8	4	6	8
6	347.3	1.29	136.3	8	3	6	8
7	305.3	0.76	130.2	7	5	8	10
8	275.3	1.43	110.0	6	3	6	8
9	291.3	0.83	130.2	7	3	10	10

**cal'd* LogP: partition coefficient
**TPSA: topological surface area
***ID_{max}: Day for maximum inhibition

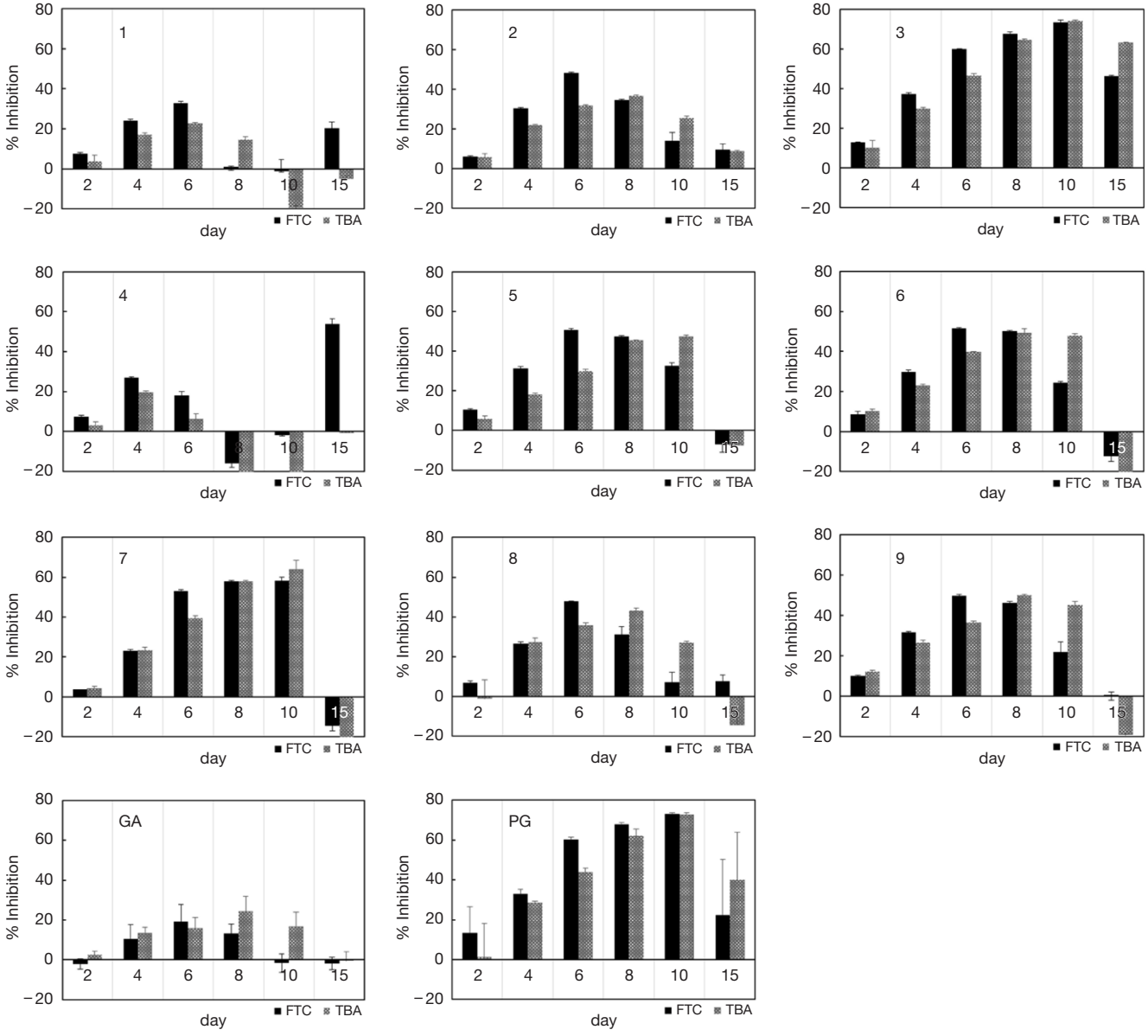


Fig. 5. Time dependent inhibition of lipid peroxidation monitored by FTC and TBA assay. GA: gallic acid, PG: propyl gallate.

ously using the same reaction mixture and identical monitoring intervals. In the FTC and TBA assays, the inhibitory activities of the compounds were measured on the day when the inhibition of the positive control reached its maximum. However, the days corresponding to the maximum inhibitory activities of different compounds were not identical. Further, the days corresponding to the maximum inhibitory activities in the FTC and TBA assays were not identical even for the same compound (Fig. 5, Table 2). In addition, there were other abnormal results which cannot be explained at present. Compounds **1**, **4**, **5**, **6**, **7**, **8**, and **9** behaved as oxidants and antioxidants depending on the monitoring day. Compound **7** behaved as an antioxidant until day 10 with up to 60% inhibition in the FTC and TBA assays, but became an oxidant on day 15 with 15 % and 30% enhancements in oxidation in the FTC and TBA assays, respectively. Compounds **1** and **4** showed very different patterns. First, the results of FTC and TBA assays showed different patterns as the incubation time increased. Second, **1** and **4** initially behaved as antioxidants, then became pro-oxidants, and once again returned to antioxidants during the late incubation period. However, compound **3** and PG showed very strong inhibition, and their activities were

persisted up to day 15. According to these results, the effect of an antioxidant on lipid peroxidation should be monitored as a function of incubation time and interpreted based on the time-dependent activity pattern.

Conclusion

A series of gallic acid derivatives were synthesized and their antioxidant activities were evaluated using four assays. The modification of gallate with 2,4-dihydroxybenzyllamine, **3**, provided a potent antioxidant that was comparable to propyl gallate. Some correlations were observed between the results of the ABTS, FTC, and TBA assays for the gallic acid derivatives. However, the DPPH assay results and physicochemical properties of the compounds did not show any correlations with the results of the other assays. The effect of the antioxidants on lipid peroxidation was incubation time-dependent and time course monitoring might be necessary for accurate evaluation of the antioxidative activity of a compound.

References

1. Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: A review. Eur J Med Chem 2015;97:55-74.
2. Maulik N, McFadden D, Otani H, Thirunavukkarasu M, Parinandi NL. Antioxidants in longevity and medicine. Oxid Med Cell Longev 2013;2013:820679.
3. Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. J Funct Foods 2015;18,Part B:820-897.
4. Kim J, Hong VS, Lee J. Antioxidant activity of 3,4,5-trihydroxyphenylacetamide derivatives. Arch Pharmacal Res 2014;37(3):324-331.
5. Locatelli C, Filippin-Monteiro FB, Creczynski-Pasa TB. Alkyl esters of gallic acid as anticancer agents: A review. Eur J Med Chem 2013;60:233-239.
6. Kim JH, Kang NJ, Lee BK, Lee KW, Lee HJ. Gallic acid, a metabolite of the antioxidant propyl gallate, inhibits gap junctional intercellular communication via phosphorylation of connexin 43 and extracellular-signal-regulated kinase1/2 in rat liver epithelial cells. Mutat Res Fund Mol M 2008;638(1-2):175-183.
7. Lu Z, Nie G, Belton PS, Tang H, Zhao B. Structure-activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochem Int 2006;48(4):263-274.
8. Pal C, Bindu S, Dey S, Alam A, Goyal M, Iqbal MS, et al. Tryptamine-Gallic acid hybrid prevents non-steroidal anti-inflammatory drug-induced gastropathy. J Biol Chem 2012;287(5):3495-3509.
9. Erkan N, Ayranci G, Ayranci E. Antioxidant activities of rosemary (*Rosmarinus Officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem 2008;110(1):76-82.
10. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26(9-10):1231-1237.
11. Kikuzaki H, Nakatani N. Antioxidant effects of some ginger constituents. J Food Sci 1993;58(6):1407-1410.
12. Ottolenghi A. Interaction of ascorbic acid and mitochondrial lipides. Arch Biochem Biophys 1959;79:355-363.
13. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53(6):1841-1856.
14. Belin F, Barthélémy P, Ruiz K, Lacombe JM, Pucci B. Synthetic gallic acid derivatives as models for a comprehensive study of antioxidant activity. Helv Chim Acta 2003;86(2):247-265.