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Enzymatic synthesis of 1-*o*-galloylglycerol: Characterization and determination of its antioxidant properties

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ABSTRACT

1-*o*-Galloylglycerol (GG) was synthesized by the enzymatic glycerolysis of propyl gallate (PG) using a food-grade lipase (Lipozyme® 435). The reaction conditions affecting the yield of GG were optimized and a yield of 76.9% ± 1.2% was obtained. GG was characterized by various techniques after being separated from the reaction mixture using liquid-liquid extraction. The water solubility and hydrophilicity of GG were significantly higher than those of gallic acid (GA) and PG. The antioxidant properties, measured by the ferric reducing antioxidant power (FRAP) and hydrogen peroxide (H_2O_2) scavenging assays, showed that GG exhibited the highest scavenging capacity (GG > GA > PG). From the results of the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) assays, GG and GA exhibited greater scavenging capacity than PG (GG = GA > PG). These results suggest that GG may be used as a water-soluble antioxidant alternative to GA for food and cosmetic applications.

1. Introduction

1-*o*-Galloylglycerol (GG) is a water-soluble derivative of gallic acid (GA), and a phenolic secondary metabolite of many plants, such as *Pelargonium reniforme, Phyllanthus emblica,* and *Rheum rhabarbarum* (Amir, 2016; Latté, Kaloga, Schäfer, & Kolodziej, 2008; Nonaka & Nishioka, 1983). The structure of GG consists of one gallate moiety esterified at the sn-1 position of the glycerol backbone with the other two hydroxyl groups remaining unesterified. GG was first proposed as an antioxidant and synthesized *de novo* using arduous chemical methods (Takasago, Horikawa, & Masuyama, 1976). In terms of preventing lipid oxidation, GG has been reported as being more effective than propyl gallate (PG) and tocopherols (Song & Xiao, 1988; Takasago et al., 1976). GG was isolated from a natural source, and also synthesized using direct chemical esterification of gallic acid (GA) and glycerol catalyzed by *p*-toluene-sulfonic acid, to acquire a sufficient quantity to confirm the proposed structure of the natural compound (Nonaka & Nishioka, 1983). GG has been further characterized, revealing its strong ultraviolet (UV) absorbance in the UVB (280–315 nm) and UVC (200–280 nm) regions (Artamonov, Nigmatullina, Aldabergenova, & Dzhiembaev, 1999).

As a GA derivative, GG shares many of its characteristics, such as antioxidant, UV absorbing, and metal chelating properties. Theoretically, the solubility of GG should be greatly enhanced by the hydroxyl groups of the glycerol moiety. GG should not only readily dissolve in aqueous media, but could also be transformed to a lipophilic form through well-established chemical or enzymatic esterification to esterify one or two of the remaining hydroxyl groups with fatty acids. However, the applications of GG as an antioxidant or as a UV filtering agent are limited because of its low level of occurrence from natural sources.

GG is conventionally synthesized by chemical esterifications and transesterifications using strong acids or bases as catalysts at a high temperature (> 100 °C). These reactions often destroy a significant amount of the materials, because the GA moiety is heat sensitive and susceptible to oxidation. As results, the direct chemical esterification and transesterification methods can only achieve yields of 41% and 12%, respectively (Artamonov et al., 1999; Nonaka & Nishioka, 1983). To increase the yield of GG, the GA moiety can be protected using ethyl chloroformate or thionyl chloride to replace the hydrogen atoms of the aromatic hydroxyl groups (Song & Xiao, 1988; Takasago et al., 1976). These protective steps significantly increased the yield of GG, but made the synthesis process more complicated. Another strategy, the Mitsunobu reaction (Mitsunobu & Yamada, 1967), has also been used to synthesize the esters of gallic acid (Appendino, Minassi, Daddario, Bianchi, & Tron, 2002). Even though the reaction conditions were mild, purifying the product was difficult, and the yield was usually low because of the complicated mechanism and delicate nature of the

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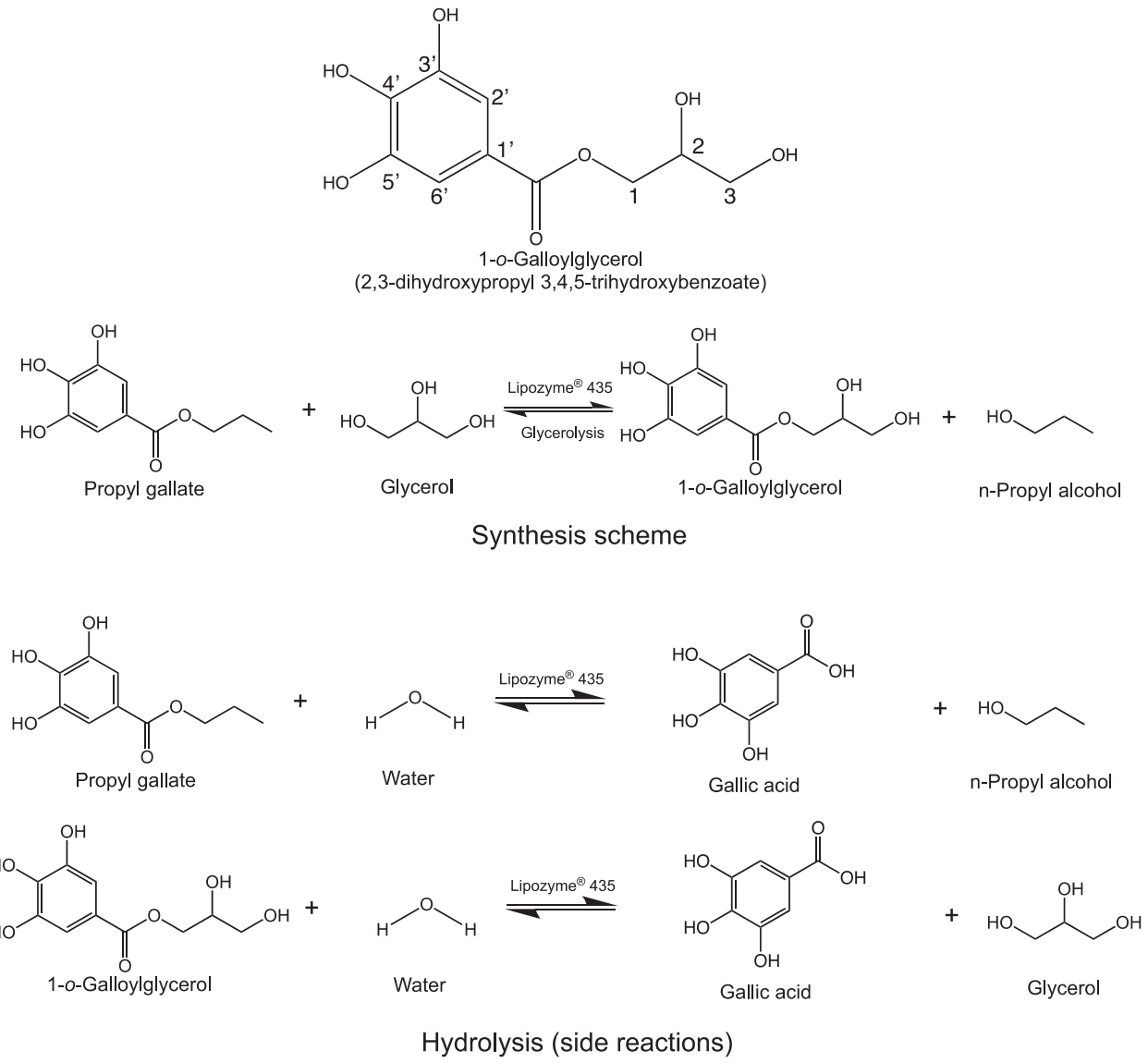

Fig. 1. Reaction scheme for GG synthesis and side reactions.

Mitsunobu reaction. The Mitsunobu reaction also requires an oxidizing azo reagent (diethyl azodicarboxylate), and a reducing phosphine reagent (triphenylphosphine), which are both toxic, thus preventing the use of this reaction to produce GG for use in the food, cosmetics, and pharmaceutical industries.

Unlike chemical synthesis, enzymatic synthesis does not require harsh reaction conditions, and therefore is more suitable for producing bioactive compounds. In a previous study, GG was successful synthesized at a high yield (67.1% ± 1.9%) by the enzymatic glycerolysis of PG catalyzed by a commercially available food-grade lipase using glycerol as both reactant and solvent (Zhang & Akoh, 2019). Moreover, due to steric hindrance between gallate moiety and the secondary hydroxyl group of glycerol, the ester bond was only formed at the *sn*-1 position of the glycerol backbone, and the formation of *sn*-2 ester was not detected (Zhang & Akoh, 2019). Similar results were also observed in the reaction of ethyl ferulate and glycerol catalyzed by the immobilized *Candida antarctica* lipase B (Novozym 435) under solvent-free condition and in ionic liquids as reaction medium (Sun et al., 2013; Sun, Hu, Song, & Bi, 2015; Sun, Shan, Jin, Liu, & Wang, 2007). This steric hindrance effect was also shown during the reaction of free ferulic acid and glycerol using ferulic acid esterase as catalyst (Matsuo et al., 2008). However, the synthesis was only performed, optimized, and validated at the milligram-scale, and the purification of GG could only be achieved using high-performance liquid chromatography (HPLC) and thin layer chromatography, which are unsuitable for industrial-scale production. Although GG has long been proposed as a novel antioxidant, its antioxidant properties have only been tested using the Schaal oven storage stability test (Song & Xiao, 1988; Takasago et al., 1976). In addition, its water solubility and partition coefficient have not yet been determined, because the original scientific motive for synthesizing GG was to increase the hydrophilicity of GA.

Herein, we report the antioxidant properties, water solubility, n-octanol/water partition coefficient, and molar extinction coefficients of GG produced by an optimized large-scale enzymatic synthesis method. The produced GG was purified from the resultant mixture using liquid-liquid extraction and its purity was tested using HPLC with a diode array detector (DAD). Fourier-transform infrared spectroscopy (FT-IR) was used to determine the functional groups of the synthesized compound. One-dimensional and two-dimensional nuclear magnetic resonance spectroscopy (NMR) were used to determine the exact chemical structure of the compound. The antioxidant properties of GG was evaluated by several *in vitro* assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging

assay, ferric reducing antioxidant power (FRAP) assay, and hydrogen peroxide (H_2O_2) scavenging assay.

2. Materials and methods

2.1. Chemicals and reagents

n-Propyl gallate (99.99% purity) was purchased from HiMedia Laboratories (Nashik, India). Glycerol (99.9% purity) was purchased from Hoefer Inc. (San Francisco, CA, USA). Lipozyme® 435 (recombinant lipase B from *Candida antarctica*, expressed in *Aspergillus niger*, and immobilized on a macroporous hydrophobic resin, with a specific activity of 8000 propyl laurate unit g^{-1}, and a moisture content of 1.0%, *w*/w) was purchased from Novozymes North America, Inc. (Franklinton, NC, USA). DPPH was purchased from Alfa Aesar (Ward Hill, MA, USA). Gallic acid, ABTS diammonium salt, horseradish peroxidase (≥250 units/mg), phosphate buffer solution (PBS, 1.0 M, pH 7.4), and ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, US). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (≥97%, Trolox™), H_2O_2 solution (30% w/w solution), and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were purchased from ACROS Organic (Morris, NJ, USA). Ferric chloride anhydrous ($FeCl_3$) was purchased from Fisher Chemical (Fair Lawn, NJ, USA). All chemicals and reagents were used as received without any further purification.

2.2. Preparation of 1-o-galloylglycerol

GG was synthesized by the enzymatic glycerolysis of PG (Fig. 1). A 100 mL double-layer jacketed glass reactor equipped with a circulating water bath was used to carry out the reaction. A SL 2400 StedFast stirrer (Fischer Scientific Co., Fair Lawn, NJ, USA) fitted with a PTFE anchor paddle stirring rod was used to mix the substrates and the enzyme at 200 rpm. 6.4 g (30 mmol) PG were first dissolved in 69.1 g (750 mmol) glycerol, then 18.0 g (23.8%, *w*/w) Lipozyme® 435 were added into the reactor after the internal temperature of the substrates stabilized. The reaction parameters, such as reaction time (120*h*), reaction temperature (50 °C), substrate ratio (glycerol/PG = 25/1 mol/mol), and enzyme load (23.8% w/w) were chosen based on previous report (Zhang & Akoh, 2019). As heat transfer would likely be less efficient in a large-scale reaction than in a milligram-scale reaction, the reaction temperature and reaction time were further optimized. The reaction mixture was sampled (2 μL) periodically (every 24 h) and the sample was diluted to 1 mL with methanol for quantitative analysis. All experiments were carried out in triplicate.

The reaction mixture was analyzed as described previously (Zhang & Akoh, 2019). An Agilent1260 Infinity HPLC system (Santa Clara, CA, USA) equipped with a diode-array detector (DAD) scanned at 280 nm were used for quantification purposes. A reverse phase C18 column (Ultrasphere ODS, 5 μm, 250 × 4.6 mm, Hichrom Ltd., Theale, UK) was used at a controlled temperature of 35 °C. The yield of GG, conversion of PG, and hydrolysis during the reaction were calculated as in a previous study (Zhang & Akoh, 2019). The results were expressed as mean values ± standard deviation (SD).

2.3. Separation and purification of reaction product

The reaction product was separated from the reaction mixture using liquid-liquid extraction, according to a previous study with modifications (Holser et al., 2008). Briefly, a volume of sodium chloride solution (200 g/L) twice that of the reaction mixture was added to the reaction mixture to decrease its viscosity. After the enzyme was removed in a Buchner funnel with a filter paper, ethyl acetate was used to extract GG from the mixture using a separation funnel. The ethyl acetate phases were then pooled together and washed with saturated sodium chloride solution (with 1 mM sodium carbonate) to remove glycerol and GA residues. A vacuum-rotary evaporator (40 °C, 50 kPa, 100 rpm) was used to evaporate the solvent. A beige-colored crude GG solid was obtained after removing the solvent. The GG product obtained was then further purified by recrystallization in water to form colorless prisms. After removing the water, a white GG anhydrous powder was obtained (m.p. 179–180 °C). The purity of the product was examined with HPLC-DAD. The recovery of GG was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{\text{Moles of GG obtained}}{\text{Moles of GG in the reaction mixture}} \quad (1)$$

The results were expressed as mean ± SD.

2.4. Structural determination and characterization

The structure of GG was further characterized as described below. A Nicolet Nexus FT-IR 1100 spectrometer (Thermo Fisher Scientific Co. Ltd., Waltham, MA, USA) equipped with a ZnSe attenuated total reflection attachment was used to collect the FT-IR spectra of samples (ν_{max} was reported in cm^{-1}). Before each experiment, the instrument was purged with nitrogen for at least 10 min. Then, 50 mg of the samples were directly placed onto the ZnSe crystal and pressed using the attached accessory. The spectra were collected from 650 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. The data were processed using Omnilab software (Omnilab Group, Bremen, Germany) and the KnowItAll® informatic system (Bio-Rad Laboratories, Hercules, CA, USA).

NMR spectroscopy analysis was done with 10 mg of the sample dissolved in D_2O with 10 mM acetic-2-^{13}C acid sodium salt as the internal standard. ^1H, ^{13}C, $^1H-^1H$ gradient correlation spectroscopy (gCOSY), $^1H-^{13}C$ gradient heteronuclear single quantum coherence (gHSQC), $^1H-^{13}C$ gradient heteronuclear multiple quantum coherence (gHMQC), and $^1H-^{13}C$ gradient heteronuclear multiple bond correlation (gHMBC) spectra were recorded at 25 °C using a Varian Unity Inova 500 MHz NMR Spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 8-mm hydrogen-carbon-nitrogen room temperature probe. The chemical shifts of GG were reported in parts per million (δ/ppm). Acetic-2-^{13}C acid sodium salt ($\delta H/\delta_C$ 1.90/25.63, 164.15 ppm) was used as the internal standard. The chemical shifts were assigned based on the 1D and 2D NMR spectra (supplementary material Figs. S1–S5) as follows: ^1H NMR (500 MHz, D_2O, $^{13}CH_3{}^{13}COONa$) δ: 7.04 (s, 2H, $H_{2',6'}$), 4.37–4.13 (m, 2H, H_1), 3.97 (q, J = 5.6 Hz, 1H, H_2), 3.64 (dd, J = 11.7, 4.6 Hz, 2H, H_3), ^{13}C NMR (125 MHz, D_2O, $^{13}CH_3{}^{13}COONa$) δ: 66.01 (C_1), 69.68 (C_2), 62.82 (C_3), 120.68 (arom. $C_{1'}$), 109.78 (arom. $C_{2',6'}$), 144.58 (arom. $C_{3',5'}$), 139.14 (arom. $C_{4'}$), 168.12 (-COO-).

The UV–Vis spectra of the samples were measured using a UV-1601 UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). The samples were dissolved in methanol at a concentration of 50 μM, then scanned over the range from 190 to 700 nm.

The solubility of GG, PG, and GA were determined as described in previous studies (Daneshfar, Ghaziaskar, & Homayoun, 2008; Tsuchiyama, Sakamoto, Tanimori, Murata, & Kawasaki, 2007). Briefly, an excess of the chemicals was suspended in ultrapure water and incubated in a shaking water bath (C76 Water Bath Shaker, New Brunswick Scientific, Edison, NJ, USA) at 100 rpm and 25 °C for 24 h. The mixture was then centrifuged, and the concentrations of the samples in the supernatant were determined with HPLC-DAD as described previously, using the corresponding standard curves for accurate quantification. The experiments were conducted in triplicate and reported as mean ± SD.

The n-octanol/water partition coefficient was determined using shaking flask method (Sangster, 1989; Short et al., 2010). Equal amount (5 mL) of n-octanol and water were added to a flask, then sealed and stirred in a shaking water bath at 100 rpm and 25 °C for 24 h to reach mutual saturation of the phases. 10 mg of GG, PG, and GA were added to the flasks, respectively. The flasks were sealed again and stirred at

the same condition for 72 h. After the layers were separated using a separating funnel, aliquots of both layers were taken and diluted in methanol. The concentration of compounds in each layer was determined by the method described previously. The distribution coefficient (log D) and partition coefficient (log P) were calculated according to the following equations:

$$\log D_{octanol/water} = \log\left(\frac{\text{Total concentration of the compounds in octanol phase}}{\text{Total concentration of the compounds in water phase}}\right) \quad (2)$$

$$\log P_{octanol/water} = \log\left(\frac{\text{Concentration of the unionized compound in octanol phase}}{\text{Concentration of the unionized compound in water phase}}\right) \quad (3)$$

For unionizable compounds, such as GG and PG, the partition coefficient and distribution coefficient are the same. For ionizable compounds, such as GA, the difference between log P and log D is negligible when the ionization is suppressed by a buffer of suitable pH. This could also be calculated according to the following equation:

$$\log P_{octanol/water} = \log D_{octanol/water} + \log(1 + 10^{(pH - pK_a)}) \quad (4)$$

where pH is the measured pH of the octanol/water system, and pK_a is the dissociation constant of the solute. As shown in the Eq. (4), when the pH is equal or smaller than pK_a, the difference between log P and log D is negligible. The log *P* value for GA was also calculated according to Eq. (3) and measured in the octanol/water system using 0.1% trifluoroacetic acid to suppress the ionization of the GA molecules. The results were expressed as mean ± SD of triplicate determinations.

2.5. DPPH$^{\bullet}$ scavenging assay

The assay was performed according to a previous study (Compton, Laszlo, & Evans, 2012) with some modifications. Samples of GG, PG, and GA were dissolved in methanol at different concentrations (2, 5, 10, and 20 μM). DPPH was dissolved in methanol to make a solution at a concentration of 200 μM. Equal amounts of DPPH$^{\bullet}$ and sample solutions were mixed and then monitored spectrophotometrically at 517 nm at a 1 s interval for 30 min using the UV-1601UV-Vis spectrophotometer mentioned previously. Instead of antioxidant solution, ultrapure water was used in the control groups. The results were expressed as the remaining percentage of DPPH$^{\bullet}$ after being reduced by the samples. All experiments were performed in triplicate. All the samples and reagents were freshly prepared daily.

2.6. ABTS$^{\bullet+}$ scavenging assay

An improved ABTS radical cation decolorization assay (Re et al., 1999) with modifications (Phonsatta et al., 2017) was used. Briefly, ABTS$^{\bullet+}$ was produced by reacting 7 mM ABTS water solution with 2.45 mM potassium persulfate in the dark at room temperature for 16 h. The ABTS$^{\bullet+}$ solution was then diluted with ethanol to obtain an absorbance of 0.70 (± 0.01) at a wavelength of 734 nm. Samples of GG, PG, and GG were dissolved in ethanol to obtain solutions at concentrations of 10, 20, 40, 50, and 100 μM. Trolox™ was used as the standard for measuring the antioxidant activity of the samples. Ethanol solutions of Trolox™ were prepared at concentrations of 10, 20, 50, 100, and 200 μM. Ethanol was used for the control groups. A 100-μL sample was mixed with 900 μL of the ABTS$^{\bullet+}$ solution, then the mixture was incubated in the dark at 30 °C for 6 min. The absorbance of the mixture was measured with the spectrophotometer mentioned previously at 734 nm. The results were expressed as the decrease in absorbance after mixing the samples, compared with the control groups. All experiments were performed in triplicate. All the samples and reagents were freshly prepared daily.

2.7. FRAP assay

The FRAP assay was conducted in accordance with previous studies (Benzie & Strain, 1996; Ozgen, Reese, Tulio, Scheerens, & Miller, 2006) with modifications. The FRAP reagent was prepared by mixing solutions of 10 mM TPTZ (in 40 mM HCl), 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6) at a volumetric ratio of 1:1:10. Aqueous solutions of $FeSO_4{\cdot}7H_2O$ were prepared at concentrations of 100, 200, 500, and 1000 μM, to be used for calibration. Antioxidant samples of GG, PG, and GA were also dissolved in water at various concentrations (20, 40, 100, 200, and 400 μM). Water was used as the blank. Freshly prepared FRAP reagent (900 μL) was mixed with 30 μL of the sample solution, then with 90 μL water. Thus, the final dilution of the sample in the reaction mixture was 34 times. The reaction mixture was then incubated in the dark at 37 °C for 4 min. The absorbance of the reaction mixture was monitored at 593 nm. The increase in absorbance was checked with the calibration curve of $FeSO_4{\bullet}7H_2O$ solution to give the results expressed as Fe^{2+} equivalents.

2.8. H_2O_2 scavenging assay

The hydroxyl radical scavenging ability of the compounds was measured using the H_2O_2 scavenging assay (Pick & Keisari, 1980; Sroka & Cisowski, 2003) with modifications. Solutions of GG, PG, and GA in methanol were prepared at various concentrations (20, 40, 200, and 400 μM). Equal amounts of the sample solution and 0.002% (*w*/w) H_2O_2 solution were mixed with 0.8 mL PBS and incubated in the dark at 37 °C for 10 min. One milliliter assay reagent, containing 0.2 mg/mL phenol red and 0.1 mg/mL horseradish peroxidase in PBS, was added and incubated under the same conditions for 15 min. After incubation, 50 μL of 1 M sodium hydroxide solution were added and the absorbance of the mixture was measured immediately at 610 nm using spectrophotometer. H_2O_2 solutions at different concentrations (0.0002, 0.0005, 0.001, and 0.002%, w/w) were used as the calibration curve. The decrease in absorbance (compared with 0.002% w/w H_2O_2 solution) was expressed against the calibration curve of the H_2O_2 solutions. The results were expressed as the percentages of scavenged H_2O_2.

The results of all four antioxidant assays were calculated using regression analysis and ANOVA by JMP® software (version 13.2.0, SAS Institute, Inc., Cary, NC, USA), and presented as means followed by standard errors.

3. Results and discussion

3.1. Synthesis of 1-o-galloylglycerol and structure confirmation

Initially, the reaction was carried out in a 50-mL double-layer jacketed glass reactor stirred with a magnetic stir bar. In this system, the product yield was only 29.8%, much lower than the previous milligram-scale synthesis (Zhang & Akoh, 2019). Such a low yield could be attributed to the limited mass transfer using the magnetic stir bar in the large reactor. Therefore, the enzymatic glycerolysis of PG was performed in a 100-mL double-layer jacketed glass reactor equipped with a circulating water bath, stirred with a PTFE anchor paddle stirring rod, and using glycerol as both the reactant and solvent. Compared with our previous study (Zhang & Akoh, 2019), the present synthesis was scaled-up by 30 times from our previous report. Thus, the reaction was further optimized by a full factorial design *via* three time-course reactions.

Fig. 2 shows that the yield of GG increased with reaction time, but the rate of increase declined significantly after 120 h. Quadratic (second-order) polynomial models could be used to explain the effect of reaction time on yield at all three temperatures with R^2 values of these models > 0.99, agreeing with our previous study (Zhang & Akoh, 2019). Hydrolysis also increased with reaction time, but the rate of increase did not significantly change with time (low set of lines). Hydrolysis at all three temperatures could be fitted to linear models, with

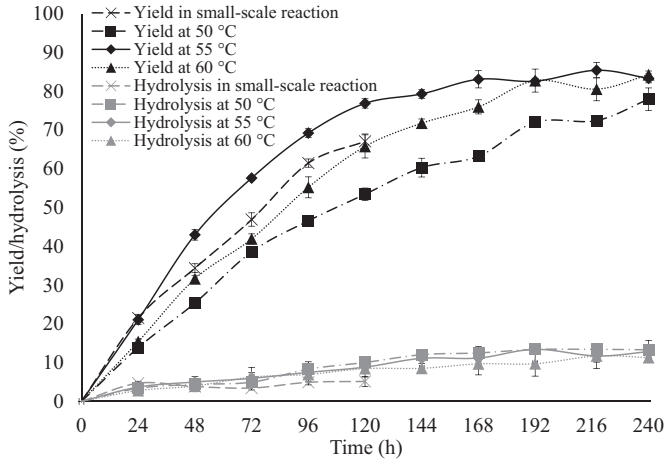

Fig. 2. Effect of the reaction temperature and reaction time on yield and hydrolysis (fixed-reaction conditions: glycerol/PG = 25:1, and enzyme load 23.8%).

R^2 values equal or > 0.92, meaning that over 92% of the variance could be explained by the linear models. Considering the effect of the reaction time on both the yield and hydrolysis, 120 h was selected as the optimal reaction time. A reaction temperature of 55 °C provided a significantly higher yield than that at 50 °C or 60 °C. A reaction temperature of 50 °C produced a temperature at the center of the reaction mixture between 45 and 47 °C after reaching thermal equilibrium. Our previous study showed that the yield of GG was significantly higher when the reaction temperature was 50 °C compared to 45 °C (Zhang & Akoh, 2019). Thus, the lower yield at 50 °C in the present study could be explained by insufficient heat transfer in the larger system. When the reaction temperature was set to 60 °C, the temperature at the center of reaction mixture was 55 to 56 °C. Therefore, this lower yield could be explained, in part, by the partial thermal deactivation of the enzyme at higher temperatures. At 55 °C, the temperature at the center of the reaction mixture was 49 to 52 °C, and these temperatures were closest to the optimum established in our previous report (Zhang & Akoh, 2019). Thus, the optimal reaction temperature was chosen as 55 °C. Fig. 2 shows that the yield from this reaction under optimal conditions (55 °C, 25:1 glycerol:PG substrate molar ratio, 120 h, and 23.8% enzyme load relative to the total weight of the substrates) was higher than the optimal yield (69.1% ± 1.9%) obtained in our previous study. This improvement could be attributed to the more efficient mass transfer using the anchor paddle stirring rod compared with the magnetic stirring bar.

After the extraction, about 92.2% ± 3.6% of the GG was recovered from the reaction mixture. The purified product consisted of > 98% of GG, about 1% GA, and a trace amount of PG. During the extraction, the amount of GA, PG, and GG were monitored using HPLC with the condition mentioned previously. No hydrolysis of GG or PG was observed. The purified GG was structurally characterized by UV–Vis, FT-IR and NMR spectroscopies. The results of the unidimensional NMR and 2D NMR analyses (Supplementary material Figs. S1–S5) indicated that the primary hydroxyl group of glycerol had been esterified with the GA moiety. A detailed interpretation can be found in our previous study (Zhang & Akoh, 2019).

The UV–Vis spectrophotometry results (Table 1 and supplementary material Fig. S6) showed that GG, GA, and PG shared a similar UV–Vis absorption pattern with slightly different wavelengths of maximum absorptions (λ_{max}). For all three compounds, strong UV absorbances were observed around 220 and around 275 nm, with no absorbance in the visible light region. GG has a higher molar attenuation coefficient (ε) at around 275 nm than both PG and GA, which may suggest that GG is a more efficient UV filtering agent than GA and PG. However, as shown in supplementary material Fig. S6, the UV absorption peaks of all three compounds were located in UVC region (200–280 nm), only weak absorbances were observed in UVB region (280–315 nm) while no absorbance was observed in UVA region (315–400 nm). Thus, the potential of using GG as a broad-spectrum sunscreen ingredient maybe limited.

The detailed report of the FT-IR spectra and their interpretation are shown in supplementary material (Fig. S7). The peak at 1686 cm^{-1} indicated the existence of the ester linkage in GG, which shifted from the typical signal at 1740 cm^{-1} owing to the influence of the aromatic ring. The presence of a pair of sharp bands at 1278 and 1193 cm^{-1} indicated the asymmetrical C—O bond of the ester linkage. A sharp signal at 1613 cm^{-1} represented the C=C stretching of the aromatic ring, which shifted from 1609 cm^{-1} in GA, and from 1606 cm^{-1} in PG. The alkyl alcohol of the glycerol moiety (R-CH_2-OH) in the GG molecule was detected in the region from 3400 to 3200 cm^{-1} for the OH hydrogen bond stretching, the medium-weak peak at 1446 cm^{-1} for OH deformation, and the strong peak at 1024 cm^{-1} for C—O stretching. Combining the broad peak in the region from 3400 to 3200 cm^{-1} (OH stretching), the strong peak at 1312 cm^{-1} (OH deformation), and the strong peak at 1024 cm^{-1} (C—O stretching), the secondary alcohol of the glycerol moiety (Ph-CHR-OH) was identified.

3.2. Characterization of GG

The water solubility, log D, and log P of GG, GA and PG are shown in Table 1. The water solubility of GG was > 4 times and 16 times higher than that of GA and PG, respectively. This increase could be attributed to the hydroxyl groups of the glycerol moiety. The solubility of GG is also less likely to be reduced under acidic pH conditions. The most acidic pK_a of GG was 8.11, calculated using Chemicalize (https://chemicalize.com/ developed by ChemAxon http://www.chemaxon.com), indicated that its solubility was stable at pH < 7.0. In alkaline solutions, the solubility of GG would increase further because of the ionization of the hydroxyl groups. GA is sparingly soluble in water, with its solubility being highly dependent on pH. Using the same calculation for GG, the most acidic pK_a of GA was 3.94, while significant ionization of GA molecule starts at pH 2.5 and 99% being ionized at pH 6.0. Thus, the solubility of GA decreased significantly at a pH of < 6.0. Considering its higher solubility and stability in acidic conditions, GG would be a better antioxidant and UV filtering agent in aqueous applications, such as fruit juice and water-based sunscreens.

Log P is the standard logarithmic scale for evaluating the hydrophobicity of compounds (Andersson & Schräder, 1999) and is measured using the concentration of the unionized solute (eq. (3)). For ionizable compounds, log P can be measured either directly by adjusting the pH

Table 1
UV–Vis absorbance, water solubility, distribution coefficient, and partition coefficient of 1-*o*-galloylglycerol (GG), gallic acid (GA), and propyl gallate (PG).

Compounds	λ_{max}^{MeOH} (nm)	Log ε (log M^{-1} cm^{-1})	Water solubility (g/L)	Log D[a]	Log P[a]
GG	221, 276	4.47, 4.10	55.92 ± 0.30	−0.63 ± 0.01	
GA	220, 274	4.47, 4.05	13.19 ± 0.09	0.28 ± 0.01[b]	0.35 ± 0.03
PG	221, 276	4.39, 4.02	3.39 ± 0.04	1.65 ± 0.03	

[a] Measured in n-octanol/water (5 mL/5 mL) system, log D equals log P when compounds are not ionizable (GG and PG).
[b] The pH of aqueous phase was 3.2 after 72 h.

of the aqueous phase or by calculating it from a known log D value using eq. (4). Unlike log P, log D is the function for all forms of the compound, both ionized and unionized, as shown in Eq. (2). For ionizable compounds, like GA, the concentration of compounds in aqueous phase is contributed by two parts, ionized molecules and unionized molecules. When the pK_a of a compound and the pH of the aqueous phase are significantly different, the concentration of the ionized molecules is not negligible (Sangster, 1989). Table 1 shows that the log *P* value of GA was higher than its log D value. This could be explained by the ionization of GA in the aqueous phase. In particular, the pH of the aqueous phase changed according to the amount of GA used during the shaking flask test. The pH of the aqueous phase, in turn, affected the measured log D values of GA. In many studies (Asnaashari, Farhoosh, & Sharif, 2014; Farhoosh, Johnny, Asnaashari, Molaahmadibahraseman, & Sharif, 2016; Lu, Nie, Belton, Tang, & Zhao, 2006), the difference in the definition between log P and log D was not noticed, so the reported log *P* values of GA were actually their log D values. Because the amount of GA and/or the buffer used as the aqueous phase in these studies were different, the reported log P values were actually their log D values under different pH conditions, thereby causing a significant inconsistency in the reported values. For example, when a smaller amount of GA was used, the aqueous phase would have a higher pH, so that more ionized molecules would have appeared, causing lower log D reading, so that the reading was mistakenly recorded as log P. In the present study, the log P value of GA was measured at the aqueous phase with pH adjusted and also calculated using Eq. (4). While two methods gave the same log P value, it was significantly larger than the log D value measured in n-octanol/water system with no buffer to adjust the pH. Similar to the pattern for water solubility, the log P value of GG was also significantly decreased, compared with those of its acid and propyl-ester forms. A high log P value of PG implies a high penetration of the cellular membrane and hence a high potential for its collapse, which would result in a higher cytotoxicity than GA (Galati, Lin, Sultan, & O'Brien, 2006). With the lowest log P value and a much lower acidity than GA, GG could be a safer alternative to GA for use in food, cosmetic, and pharmaceutical products. Noticeably, no hydrolysis of PG or GG was observed throughout the solubility test or during the measurement of log D and log P.

3.3. Antioxidant activity

The antioxidant properties of GG were determined by four commonly used *in vitro* antioxidant assays. The $DPPH^{\bullet}$, $ABTS^{\bullet+}$, FRAP, and H_2O_2 scavenging assays are homogeneous antioxidant assays which evaluate the hydrogen atom and electron donating ability of antioxidants. For the $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and H_2O_2 scavenging assays, the antioxidant activity of compounds can be expressed as the effective concentration for obtaining a 50% response (EC_{50}) or the inhibitory concentration at 50% response (IC_{50}), depending on how the response is defined. In the present study, IC_{50} (the concentration of a tested compound needed to reduce the free radical to 50% of its initial concentration) was used to quantify the antioxidant activity of the tested compounds, while EC_{50} (the concentration of a tested compound needed to scavenge 50% of the free radicals) was used in the $ABTS^{\bullet+}$ and H_2O_2 scavenging assays. The results of the FRAP assay were expressed as the effective concentration for obtaining 1 mM Fe^{2+} (EC1). The results of all four assays could also be expressed as the standard equivalent antioxidant activity (usually using Trolox equivalent antioxidant activity, TEAC). Trolox is a water-soluble vitamin E analogue. The Trolox equivalent was also used for the $ABTS^{\bullet+}$ and H_2O_2 assays to obtain the relative antioxidant activities of GG, GA, and PG, compared with Trolox.

Table 2 and Fig. 4 show that GG exhibited a higher antioxidant activity than both GA and PG in the FRAP and H_2O_2 assays. From the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, GG and GA exhibited a similar antioxidant activity while PG had a significantly lower antioxidant activity.

The $DPPH^{\bullet}$ assay measures the reducing ability of antioxidants towards $DPPH^{\bullet}$, which is a stable organic nitrogen radical. Figs. 3 and 4a illustrate that GG and GA showed a similar scavenging ability towards $DPPH^{\bullet}$ with PG being slightly lower. The scavenging ability towards $DPPH^{\bullet}$ depends on structural features, such as the dissociation energy of hydrogen atoms from hydroxyl groups, resonance delocalization of the phenol radical ($PheO^{\bullet}$), and steric hindrance arising from bulky groups substituting hydrogen in the aromatic ring (Shahidi & Naczk, 1995). After $H^{\bullet}$ is taken by $DPPH^{\bullet}$, $PheO^{\bullet}$ can react either with $DPPH^{\bullet}$ to form DPPH-PheO or undergo a termination reaction to form PheO-PheO (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998). All three tested compounds had the same number of phenolic hydroxyl groups, so the differential ability to scavenge $DPPH^{\bullet}$ could only be affected by the moiety bonded with the carbonyl group. The lower $DPPH^{\bullet}$ scavenging ability of PG compared with GA has been observed before (Lu et al., 2006). This phenomenon was explained by steric hinderance caused by the n-propyl moiety and the higher hydrophobicity of PG making antioxidants less available for the DPPH radicals in polar systems (Asnaashari et al., 2014; Lu et al., 2006). The secondary $DPPH^{\bullet}$ scavenging reaction of PG could also be terminated *via* the reaction mentioned above, causing lower scavenging ability (Okuda, 1993). The glycerol moiety may have caused steric hindrance, but also increased the hydrophilicity of the compound, so that the $DPPH^{\bullet}$ scavenging ability of GG was not significantly different to that of GA. Fig. 3 shows that GG and GA exhibited a faster reduction towards $DPPH^{\bullet}$ than PG, indicating that the accessibility of the molecule affected its scavenging activity in the $DPPH^{\bullet}$ assay. Compared with α-tocopherol, other phenolic acids and their esters, such as ferulic acid and caffeic acid, GA and its esters usually exhibited a much higher scavenging ability towards $DPPH^{\bullet}$ (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002), suggesting a great potential for GG to be used as an antioxidant.

The $ABTS^{\bullet+}$ and FRAP assays measure the reducing ability of antioxidants towards two ions (Fe^{3+}-TPTZ and $ABTS^{\bullet+}$, respectively) with a similar redox potential under different pH conditions. The FRAP assay was conducted in acetate buffer (pH 3.6) and the $ABTS^{\bullet+}$ assay in ethanol. In both assays, PG exhibited the lowest antioxidant activity, which agreed with other studies (Phonsatta et al., 2017). GG and GA exhibited similar antioxidant activities in the $ABTS^{\bullet+}$ assay while GG exhibited a higher antioxidant activity than GA in the FRAP assay (Table 2 and Figs. 4b, c). The lower antioxidant activity of GA observed in the FRAP assay could be attributed to the acidic pH of the testing environment as observed previously (Ozgen et al., 2006). As mentioned earlier, the ionization of GA is suppressed in acidic solutions, so the mechanism of the FRAP assay is only electron transfer rather than a combination of electron transfer and hydrogen atom transfer, as in the $DPPH^{+}$ and FRAP assays. These results may imply that it is easier for ionized GA to transfer electrons than for unionized GA. Similar to $DPPH^{\bullet}$ assay, GA showed higher reducing ability than α-tocopherol, ferulic acid, caffeic acid, and ascorbic acid in $ABTS^{\bullet+}$ assay (Miller & Rice-Evans, 1997).

Unlike the $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP assays, the H_2O_2 scavenging assay is a competitive system, where antioxidants have to compete with phenol red to react with $HO^{\bullet}$ radicals generated from H_2O_2. Consequently, the H_2O_2 scavenging assay measure a combination of reducing ability and reactive speed towards the $HO^{\bullet}$ radical. GG exhibited the highest H_2O_2 scavenging ability while GA and PG exhibited similar activities towards $HO^{\bullet}$ radicals (Table 2 and Fig. 4d). The influence of substituents bonded to the benzene ring of GA has been observed before. Pyrogallol (1,2,3-trihydroxybenzene) has been reported to have a lower scavenging ability towards H_2O_2 than GA (Sroka & Cisowski, 2003). The different scavenging abilities of the three compounds tested in the present study may have been caused by the influence of the different moieties bonded with the carbonyl group of the GA (n-propyl alcohol, and glycerol) moiety on the hydroxyl groups of the phenol ring. The glycerol moiety in GG may have increased the electrophilicity of the carbonyl electron, which would increase the

Table 2
Antioxidant activities of 1-*o*-galloylglycerol (GG), gallic acid (GA), and propyl gallate (PG) in DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP, and H_2O_2 assays.

Compounds	DPPH$^{\bullet}$	ABTS$^{\bullet+}$		FRAP	H_2O_2	
	IC_{50} (μM)	EC_{50} (μM)	TEAC*	EC1 (μM)	EC_{50} (μM)	TEAC*
GG	10.51 ± 0.59[a]	28.50 ± 0.76[a]	5.0 ± 0.61[a]	287.91 ± 5.56[a]	197.27 ± 10.75[a]	2.05 ± 0.36[a]
GA	10.50 ± 0.58[a]	28.50 ± 0.65[a]	4.95 ± 0.53[a]	306.64 ± 5.24[b]	237.75 ± 7.89[b]	1.74 ± 0.40[b]
PG	11.90 ± 0.61[b]	32.39 ± 0.59[b]	4.33 ± 0.41[b]	387.55 ± 15.32[c]	235.79 ± 9.63[b]	1.73 ± 0.41[b]

[a,b,c]Different letters indicate significant statistical difference at $p < 0.05$.
All the results are expressed as means ($n = 3$) ± standard errors.
* Trolox equivalent antioxidant activity, molar/molar.

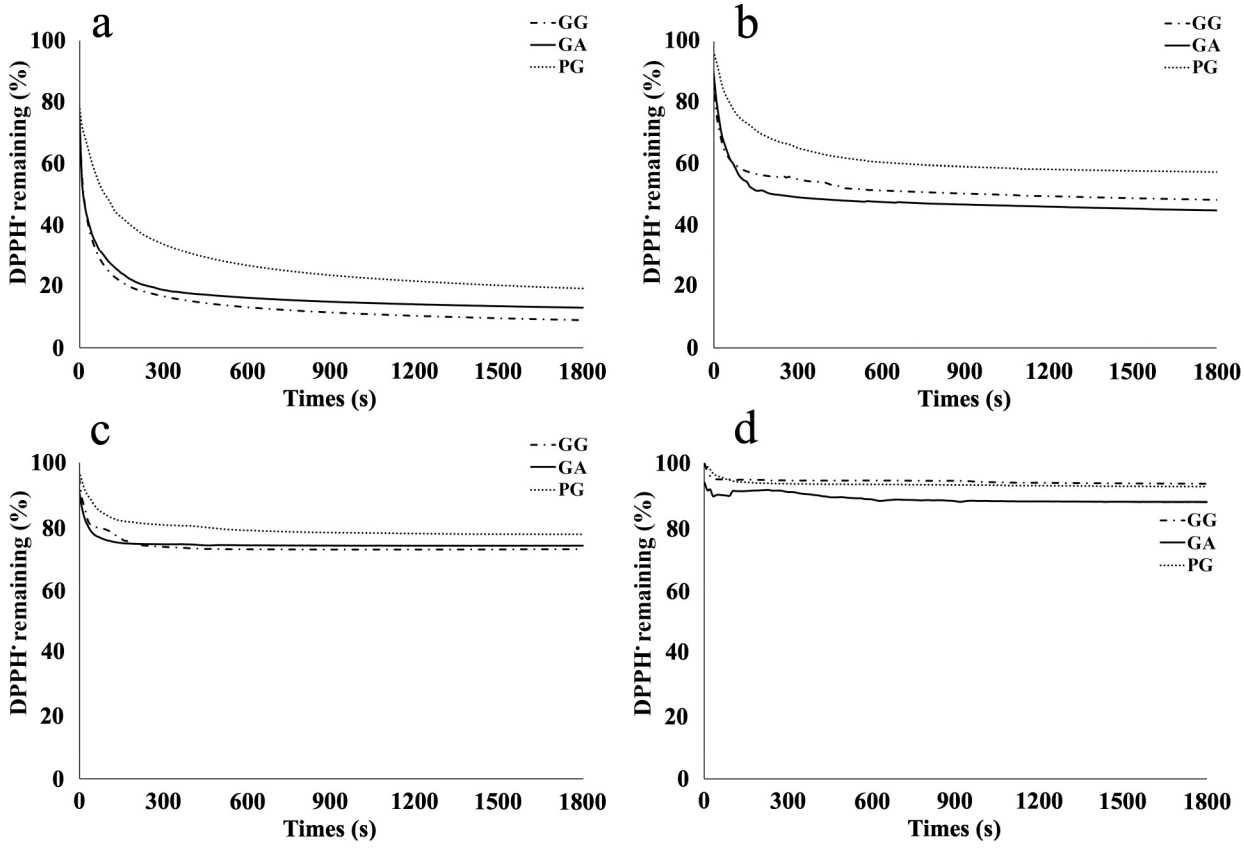

Fig. 3. DPPH$^{\bullet}$ scavenging kinetics in the presence of GG, GA, and PG at 20 (a), 10 (b), 5 (c), and 2 (d) μM in methanol.

hydrogen atom donating ability of the phenolic hydroxyl groups *via* an inductive effect. Comparing the chemical shift of two hydrogen atoms in the benzene ring, GG has been reported to have a higher chemical shift (7.04 ppm), than GA (6.92 or 6.91 ppm) and PG (6.97 ppm) (Garrido, Garrido, & Borges, 2012; López-Martínez, Santacruz-Ortega, Navarro, Sotelo-Mundo, & González-Aguilar, 2015). A higher chemical shift of H atoms in the benzene ring indicates a decreased electron density for these two atoms (electrons shifting towards carbon atoms), which further indicates an increased electrophilicity of the benzene ring caused by the glycerol moiety. This increased electrophilicity of the benzene ring makes electron transfer and hydrogen atom transfer of Ph-OH easier to occur. The increased H_2O_2 scavenging ability of GG could also be explained by the formation of an intra-molecular hydrogen bond, which could remain stable in an aqueous solution. Comparing the FT-IR spectra of GG and GA (Supplementary material Fig. S7), red shifts were observed in the GG spectrum in the region 3000–4000 cm^{-1}, which indicated the possibility of hydrogen bonds being formed between Ph-OH and the hydroxyl groups of the glycerol moiety. These intra-molecular hydrogen bonds would promote the transfer of hydrogen atoms, thus increasing the H_2O_2 scavenging activity of GG. Notably, the red shifts, observed in the spectrum of GA in the region from 3000 to 4000 cm^{-1} compared with the spectrum of PG, could also have been caused by the inter-molecular hydrogen bond between GA molecules.

4. Conclusions

A readily water-soluble natural plant component, GG, has been synthesized and characterized. Unlike commonly used alkyl gallates, GG is more hydrophilic than GA, and has the potential to be used in aqueous-based foods, cosmetics, and pharmaceutical products. GG was be readily prepared enzymatically and was found to be more effective as an antioxidant in various *in vitro* assays than PG. Its antioxidant activity was similar or higher when compared with GA. As GG is less acidic, less likely to penetrate cellular membranes, and less likely to be cytotoxic, it may be a safer alternative antioxidant than GA and PG.

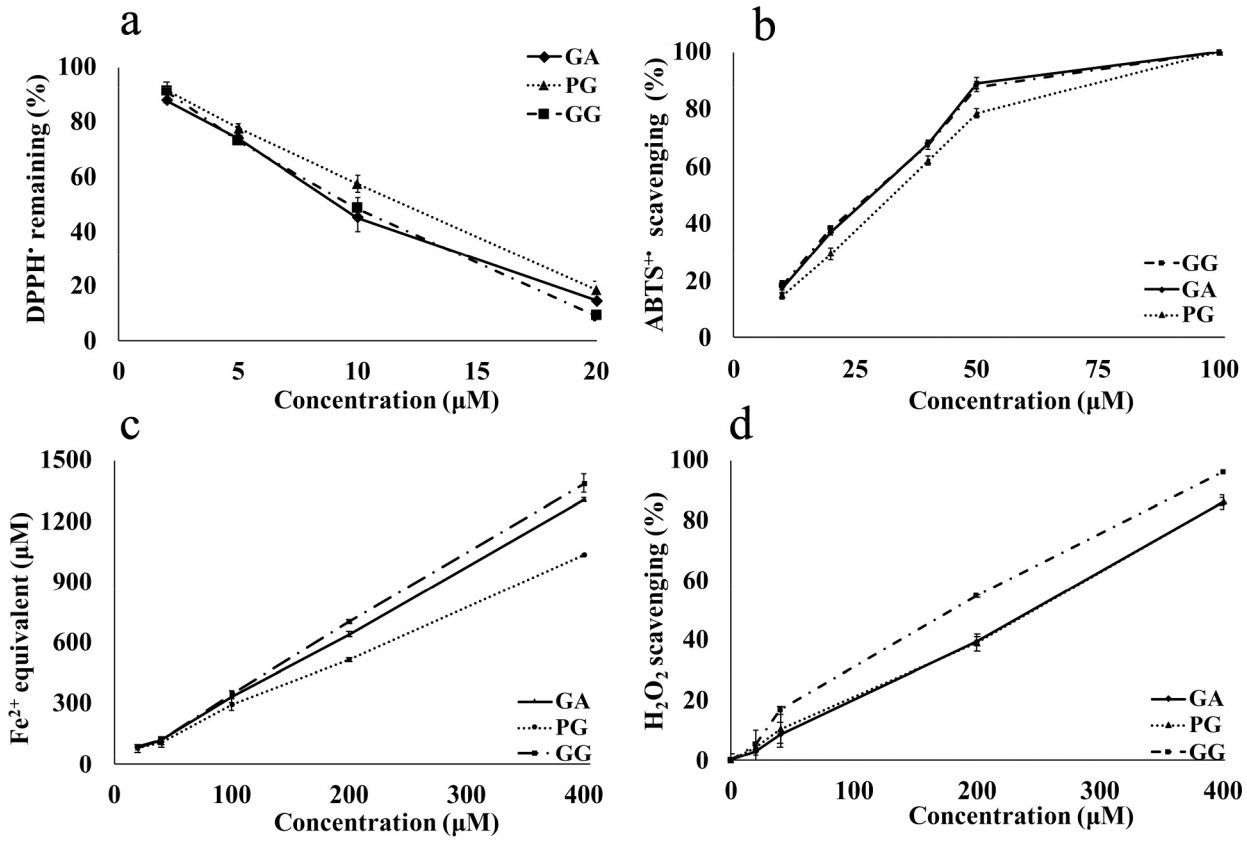

Fig. 4. Antioxidant activity of GG, GA, and PG, evaluated by DPPH• scavenging assay (a), $ABTS^{\bullet+}$ scavenging assay (b), FRAP assay (c), and H_2O_2 scavenging assay (d).

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125479.

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