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Antioxidant properties of two novel lipophilic gallic acid derivatives

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SUMMARY: The effectiveness of two lipophilic derivatives of the natural phenol, gallic acid (GA), synthesized using methyl gallate as starting material was investigated. The antioxidant activities of these novel phenolics compared to GA, *tert*-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) were evaluated in bulk oil, emulsion and the DPPH systems. The results showed that the new compounds effectively delayed lipid oxidation much better than GA and other antioxidants under Rancimat (100–140 °C) and emulsion tests. In the bulk oil system at 65 °C, they still behaved better than GA, but TBHQ had the highest activity. Thus, replacing the electron-withdrawing carboxylic group on GA by covalently linking sterically hindered phenols to its phenyl ring increased its lipophilicity and also resulted in synergistic effects which improved overall antioxidant activity through stabilization of the phenoxy radical. These new antioxidant variants satisfy industrial demands for bioactive ingredients with strong antioxidant potentials under different food processing conditions.

KEYWORDS: *Antioxidant activity; Free radical scavenging; Gallic acid derivatives; Lipophilicity; Oil-in-water emulsion; Rancimat test*

RESUMEN: ***Propiedades antioxidantes de dos nuevos derivados lipofílicos del ácido gálico.*** Se aporta información sobre de la eficacia de dos derivados lipofílicos de fenoles naturales derivados del ácido gálico (GA) y sintetizados utilizando galato de metilo como material de partida. Las actividades antioxidantes de estos nuevos compuestos fenólicos en comparación con el GA, terc-butilhidroquinona (TBHQ) y butil hidroxitolueno (BHT) se evaluaron en aceites, sistemas emulsionados y mediante DPPH. Los resultados mostraron que los nuevos compuestos retrasaron efectivamente la oxidación de lípidos mucho más fuerte que el GA y otros antioxidantes mediante Rancimat (100–140 °C) y pruebas de emulsión. En el aceite a 65 °C, se comportaron mejor que el GA, pero el TBHQ tuvo la actividad más alta. Por lo tanto, reemplazar el grupo carboxílico en GA al unir covalentemente fenoles impedidos estéricamente a su anillo de fenilo ayudó a aumentar su lipofilia y también dio como resultado efectos sinérgicos que mejoraron la actividad antioxidante general a través de la estabilización del radical fenoxi. Estas nuevas variantes de antioxidantes satisfacen la demanda industrial de ingredientes bioactivos con un fuerte potencial antioxidante en diferentes condiciones de procesamiento de alimentos.

PALABRAS CLAVE: *Actividad antioxidante; Derivados del ácido gálico; Eliminación de radicales libres; Emulsión de aceite en agua; Lipofilia; Rancimat*

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1. INTRODUCTION

Lipid oxidation generally deteriorates vegetable oils and animal fats by producing a wide range of reactive radicals that adversely affect their quality, making them unpalatable and even unfit for human consumption. Several factors affect the rate and/or occurrence of lipid oxidation, such as the composition and degree of unsaturation of fatty acids, the presence of pro-/antioxidants, and processing and storage conditions. Among the different methods employed to prevent lipid oxidation in food, the addition of natural and synthetic antioxidants remains the most effective strategy (Olajide *et al.,* 2018).

Phenolic antioxidants have become an important group of food additives due to their unique ability to conveniently preserve the sensory and nutritional qualities of food. Synthetic antioxidants such as *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate (PG) are widely used in the food industry because of their ready availability and good antioxidant capacity. Nevertheless, due to the sensitivity of consumers to the their presence in foods (Alavi Rafiee *et al.,* 2018) and weak potency during food preparation involving high temperatures (Olajide *et al.,* 2020), continuous efforts are being made to replace them with bioactive, non-toxic, high molecular weight, thermally stable and generally more potent semi-synthetic and/or natural alternatives (Torres de Pinedo *et al.,* 2007).

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a prevalent bioactive phenolic compound found in many edible and medicinal plants (Farhoosh and Nyström, 2018). It is of significant interests to the food and pharmaceutical industries, although hydrophilic groups such as hydroxyl and carboxyl groups generally limit its application under lipophilic conditions (Crauste *et al.,* 2016). Consequently, the need to improve the functionality of GA while retaining its bioactivity has led scientists to synthesize and investigate some gallic acid derivatives including its alkyl esters (Asnaashari *et al.,* 2014; Farhoosh and Nyström, 2018; Mansouri *et al.,* 2020) and bis-gallate analogues with varying length of aliphatic chains (AL Zahrani *et al.,* 2020; Dodo *et al.,* 2008). Interestingly, studies on the *in-vitro* antioxidant investigation of GA derivatives and hybrid molecules in lipid-based foods, especially under high temperature processing conditions are still lacking. To our knowlwdge, there is no report of any derivative or hybrid ever tested in bulk oils at these temperatures (i.e. ≥ 140 °C) except for propyl gallate (PG) which is easily degraded by high heat and thus provides poor carry-through properties (i.e. retardation of rancidity) in foods that are baked or fried.

Lipophenols, as opposed to free polyphenols, contain both polar groups and lipophilic alkyl components in one molecule, and are usually synthesized by joining acids with phenols *via* ester bonds (Zhong and Shahidi, 2012). Similarly, structural modification involving the introduction of *tert*-butyl moiety to the phenyl ring of phenolics has been reported, which is more efficient than long-chain alkanes in producing compounds with better lipid solubility and antioxidant activity at high temperatures (Huang *et al.,* 2014; Silva *et al.,* 2000). Moreover, it has been proven that synergistic effects may result from a medley of phenolic antioxidants linked by a covalent bond in one molecule, which can be greater than individual antioxidants (Kancheva *et al.,* 2010; Teixeira *et al.,* 2013). Additionally, covalent bonds in the form of aliphatic bridge between poly-gallates may increase the Log *P* value (hydrophobicity), resulting in improved bioactivity (Dodo *et al.,* 2008).

Therefore, taking into account the bioactivity of gallic acid and practical application of novel semi-synthetic GA variants with improved functionalities such as stronger antioxidant efficiency, thermal stability and lipid solubility, two novel gallic acid derivatives, 5-(3,5-di-tert-butyl-4-hydroxybenzyl)benzene-1,2,3-triol (**5a**) and 5-(3(4)-(tert-butyl)-2,5-dihydroxybenzyl)benzene-1,2,3-triol (**5b**), were synthesized and their antioxidant activities were evaluated by Rancimat, DPPH, Schaal oven, and emulsion tests.

2. MATERIALS AND METHODS

2.1. Materials

All solvents and chemicals used in this study were of analytical grade and mainly purchased from Shanghai Macklin Biochemical Co., and Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (USA) and data are presented as chemical shift δ (ppm). Mass spectra were ob-

tained using Thermo Scientific Q Exactive plus LTQ Orbitrap XL (Thermo Fisher Scientific, USA). TLC was carried out on 0.25-mm pre-coated silica gel plates visualized under UV light at 254 nm. Induction periods (IP) were meausred on Rancimat 743 (Metrohm, Herisau, Switzerland) and UV spectra on a UV-2450 spectrophotometer (Shimadzu Corp, Kyoto, Japan).

2.2. General synthesis of compounds

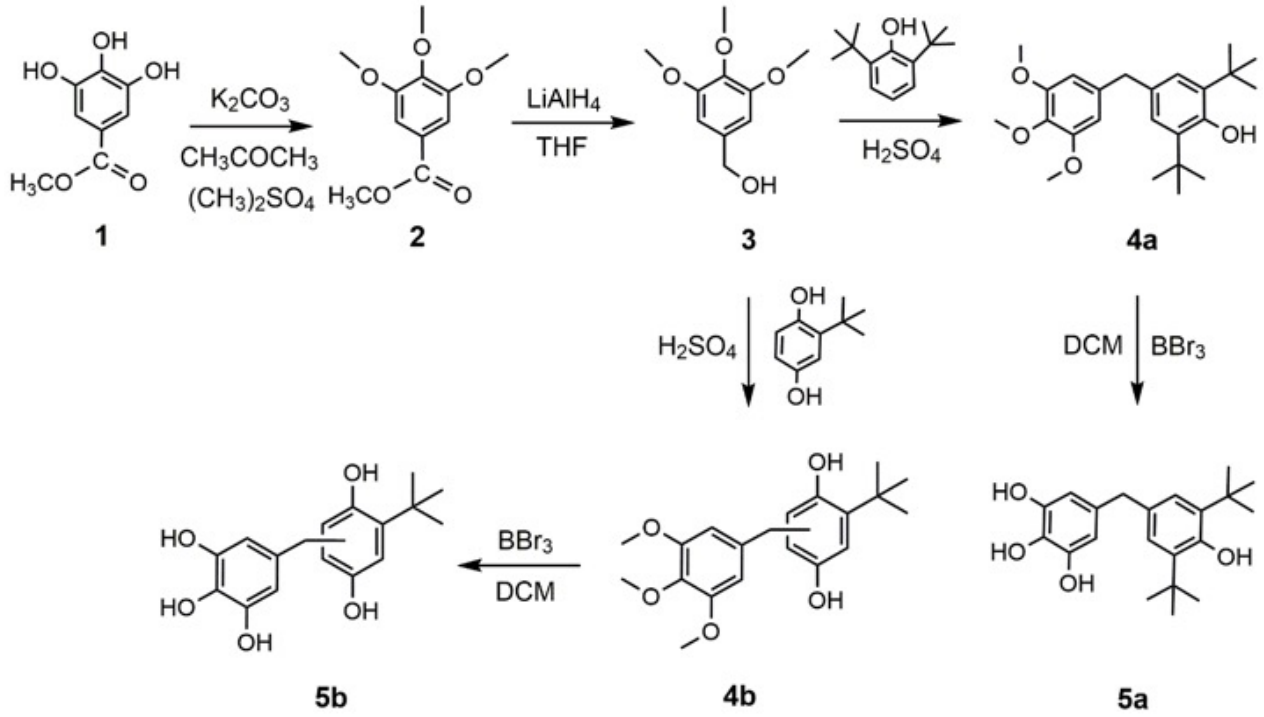
The new compounds were synthesized *via* a four-step reaction starting from methyl gallate (Scheme 1).

Methyl gallate trimethyl ether (**2**): Dimethyl sulfate (7.6 ml, 80 mmol) was slowly and uniformly added to a mixture of MG (7.4 g, 40 mmol) and K_2CO_3 (11.1 g, 80 mmol) in acetone (50 ml), and heated to reflux for 12 h. After completion, the reaction mixture was concentrated under reduced pressure and the desired compound **2** (97% yield) was isolated as a white solid by flash column chromatography using PE/EA (10:1, v/v) as eluent. Analytical data were as reported by Hirose *et al.,* (2014).

3,4,5-trimethoxybenzylalcohol (**3**): **2** (6 g, 30 mmol) was added to a stirred solution of $LiAlH_4$ (2.28 g, 60 mmol) in anhydrous THF (180 ml) at 0 °C under nitrogen conditions. Then, 0.5 M HCl solution (150 ml) was added to the resulting mixture at 0 °C after stirring at room temperature for 7 h. The acidic mixture was washed with EA (50 ml × 3), and concentrated under low pressure and subjected to flash column chromatography using PE/EA (2:1, v/v) as eluent to afford **3** (93% yield) as a colorless solid. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.57 (s, 2H), 4.59 (s, 2H), 3.83 (s, 6H), 3.82 (s, 3H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 153.22, 137.06, 136.88, 103.73, 65.21, 60.82, 56.02.

2,6-di-tert-butyl-4-(3,4,5-trimethoxybenzyl)phenol (**4a**): 2.5 mmol of **3** (0.5 g) were added to a mixture of 2,6-DTBP (0.78 g, 3.8 mmol) and H_2SO_4 (0.6 ml) in ethanol under nitrogen conditions. After 24 h at reflux temperature, the mixture was washed with brine (30 ml) followed by EA (30 ml), dried over $MgSO_4$, filtered and evaporated in a vacuum, yielding **4a** (69%) as a white solid after purifying by column chromatography (PE/ EA: 30:1, v/v). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.00 (s, 2H), 6.43 (s, 2H), 3.85 (s, 2H), 3.82 (s, 3H), 3.81 (s, 6H), 1.42 (s, 18H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 153.10, 137.37, 135.88, 125.31, 105.89, 60.89, 56.06, 41.97, 34.33, 30.34.

5-(3,5-di-tert-butyl-4-hydroxybenzyl)benzene-1,2,3-triol (**5a**): BBr_3 (1 mol/L in DCM, 15 ml) was added dropwise to a stirred solution of compound **4a** (0.9 g) in DCM (10 ml) at 0 °C. The reaction mixture after stirring for 16 h at room temperature was



Scheme 1. Synthetic route leading to gallic acid (GA) lipophilic derivatives, 5a and 5b.

poured into ice water and extracted with EA. The organic layers were then concentrated and purified by column chromatography (PE/EA: 4:1, v/v) to afford **5a** as a beige colored oil (92 % yield). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.96 (s, 2H), 6.32 (s, 2H), 3.72 (s, 2H), 1.41 (s, 18H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 152.10, 143.88, 135.83, 134.52, 131.52, 129.64, 125.45, 108.48, 41.32, 34.31, 30.34. HRMS (ESI) m/z calcd for $C_{21}H_{29}O_4^+$ $(M+H)^+$ 345.20604, found 345.20612.

2-(tert-butyl)-5(6)-(3,4,5-trimethoxybenzyl)benzene-1,4-diol (**4b**): 2.5 mmol of **3** (1.0 g) was added to a mixture of TBHQ (1.26 g, 3.8 mmol) and sulfuric acid (1.6 ml) in ethanol under nitrogen conditions. After 12 h at reflux temperature, the mixture was washed twice with brine (50 ml) followed by EA (30 ml), dried over $MgSO_4$, filtered and evaporated in vacuum, yielding **4b** (85%) as a white solid after purifying by column chromatography (PE/ EA: 5:1, v/v). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.73 (s, 1H), 6.46 (s, 5H), 6.41 (s, 1H), 6.37 (s, 1H), 3.97 (s, 4H), 3.83 (s, 3H), 3.82 (s, 6H), 1.38 (s, 18H). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 153.35, 147.97, 135.44, 124.91, 118.28, 114.81, 105.84, 60.87, 56.10, 36.10, 34.32, 29.53.

5-(3(4)-(tert-butyl)-2,5-dihydroxybenzyl)benzene-1,2,3-triol (**5b**): BBr_3 (1 mol/L in DCM, 15 ml) was added dropwise to a stirred solution of compound **4b** (1.0 g) in DCM (15 ml) at 0 °C. The reaction mixture after stirring for 16 h at room temperature was washed with ice water and extracted with EA. The organic layers were then combined, dried over $MgSO_4$, filtered, concentrated at low pressure and the residue was purified by column chromatography (PE/EA: 2:1, v/v) to afford **5b** as a beige colored oil (95% yield). ^{1}H NMR (600 MHz, Methanol-d_4) δ 6.67 (s, 1H), 6.58 (d, J = 3.0 Hz, 1H), 6.35 (s, 1H), 6.27 (d, J = 2.9 Hz, 1H), 6.20 (s, 4H), 3.69 (s, 2H), 3.62 (s, 2H), 1.37 (s, 9H), 1.33 (s, 9H). ^{13}C NMR (150 MHz, Methanol-d_4) δ 148.41, 147.11, 145.51, 133.88, 132.35, 130.70, 125.84, 117.74, 113.56, 107.93, 34.*34*, 33.95, 29.72. HRMS (ESI) m/z calcd for $C_{17}H_{19}O_5^-$ $(M-H)^-$ 303.12380, found 303.12332.

2.3. Soybean oil purification

The soybean oil (250 g) used in this study was bought from the local market and stripped of endogenous antioxidants and pro-oxidants by column chromatography according to an earlier method described by Asnaashari *et al.,* (2014) with minor modification. Briefly, the glass column (50 x 5 cm I.D.) was packed sequentially with aluminum oxide 60 (100 g), silica gel (120 g; 200 mesh) and activated carbon (10 g) at the top. The absorbents were activated by heat prior to purification. The column tube and collection flasks were covered with aluminum foil, and the oil was passed through the column by suction force without solvent.

2.4. Oil and emulsion samples preparation for oven test

Soybean oil samples (50 g) spiked with 200 ppm antioxidants dissolved in acetone were placed in the oven at 65 ± 0.5 °C after agitation for 2 min at room temperature. The peroxide (PV) and *p*-Anisidine (*p*-AV) values of the samples were determined at 3 day intervals. Similarly, a 20% oil-in-water (o/w) stable emulsion was prepared by vigorously mixing purified soybean oil (20 g) containing 200 ppm antioxidant with Tween 80 (10 g) and a phosphate buffer solution (70 g) followed by sonication in an ice bath. The emulsions were oxidized at 65 ± 0.5 °C and PV was measured daily. The IP of samples was dependent on the time taken to reach a PV of 80 meq O_2/kg oil and a *p*-AV of 10 (Maszewska *et al.,* 2018).

2.5. Evaluation of antioxidant activity

2.5.1. Rancimat test

The antioxidant activity compounds were evaluated under Rancimat based on an earlier method reported by Olajide *et al.,* (2020) with some modification. 3 ± 0.05 g lard samples containing 200 ppm antioxidants were subjected to accelerated oxidation between 100 and 140 °C at an air flow rate of 20 L/h. The results were expressed as IP of antioxidant-spiked samples in hours relative to those without antioxidants. The protection factors (Pf) of antioxidants were also calculated with the formula below:

$$Pf = IP_s/IP_{b,}$$

where IP_s is the induction period of oil with added antioxidant, and IP_b is that of oil without antioxidant.

2.5.2. DPPH assay

The DPPH radical scavenging capacity was carried out according to the procedure described by Jiang *et al.,* (2014) with slight modification. Briefly, 0.5 ml antioxidant in methanol (1.5, 3, 6, 12, 24, and 48

μM) was added to 2.5 ml DPPH methanolic solution (0.1 mM), vigorously mixed, left to react in a dark chamber for 30 min, and the decreasing absorbance of DPPH was read at 517 nm against a blank on a spectrophotometer. Methanol served as blank and 2.5 ml solution of DPPH plus 0.5 ml methanol were used as the control. EC_{50}, the effective concentration required to obtain 50% antioxidant capacity of compounds was calculated from the linear regression of plots between the scavenging activity (%) of antioxidants and their concentrations. DPPH radical scavenging activity was calculated with the formula below:

$$Scavenging\ activity\ (\%) = [(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$$

2.5.3. Peroxide value (PV), p-*Anisidine value (*p-*AV) and total oxidation value (Totox)*

The peroxide and p-anisidine contents in oil samples were measured according to the AOCS Official Methods Cd 8b-90 (AOCS, 2011) and 18-90 (AOCS, 1995) with some modification.

Finally, Totox was calculated with the following equation:

$$Totox = 2PV + p\text{-}AV$$

2.6. Statistical analysis

All the tests in this study were performed in triplicate and data are expressed as mean ± standard deviation (SD). The level of significance between means was examined by analysis of variance (ANOVA) with Duncan's multiple range test ($P < 0.05$) on OriginPro and IBM SPSS.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of compounds

The new lipophilic GA derivatives were obtained starting from MG following a four-step reaction (Scheme 1). Briefly, the three hydroxyl groups on MG were methylated and the resulting trimethyl ether was reduced using LiAlH4 to trimethoxybenzyl alcohol. Subsequently, the protecting methyl groups were cleaved (Jiang *et al.,* 2014) to yield **5a** and **5b** in good yields, respectively, following alkylation of the trimethoxybenzyl alcohol with 2,6-DTBP and TBHQ.

The identity of the new compounds was confirmed by NMR and HRMS-ESI. Compound **5b** is composed of two structurally similar isomers that were only distinguishable by NMR but chromatographically inseparable on the TLC plate. This means that they displayed the same retention factor (Rf). The retention factors of GA, **5a** and **5b** on TLC were 0.06, 0.62, and 0.44, respectively (PE/ EA: 1:1, v/v) and their theoretical partition coefficient (Log P_{theor}) values on ChemBioDraw Ultra Software were calculated as 0.42, 5.98 and 3.88. Rf correlated with Log P_{theor} values, showing the following order: GA < **5b** < **5a**. This indicates that GA had the highest polarity of all the evaluated antioxidants in this study, with a similar Log P as that reported by Farhoosh *et al.,* (2016). **5a** and **5b** were both less hydrophilic due to the presence of dense carbon skeletons existing in the form of bulky hydrophobic *tert*-butyl substituents and methylene bridges attached to their phenyl rings. Their UV spectra were quite similar, showing absorptions at 273 and 264 nm, respectively.

In the ^{1}H NMR spectrum of **5a**, two singlets around 6.96 and 6.32 ppm were assigned to the aromatic protons, while proton signals at 3.72 and 1.41 ppm were observed for the methylene bridge and the tert-butyl moieties linked to the aromatic ring. The ^{13}C NMR spectrum exhibited 11 carbon signals including eight aromatic carbon signals between 152.10 and 108.48ppm, a methylene group carbon signal at 41.32 ppm, and *tert*-butyl group car-bon signals at 34.31 and 30.34 ppm. The HRMS (ESI) spectrum of **5a** exhibited a protonated specie at m/z 345.20612, which was assigned to $C_{21}H_{29}O_4^+$ $(M+H)^+$. As expected, the ^{1}H NMR spectrum of **5b** revealed it as an isomeric mixture (i.e. 80% 5-(4-(tert-butyl)-2,5-dihydroxybenzyl)benzene-1,2,3-triol (5bi) and 20% 5-(3-(tert-butyl)-2,5-dihydroxybenzyl)benzene-1,2,3-triol (5bii). Two singlets around 6.67 and 6.35 ppm (for 5bi), two doublets around 6.58 and 6.27 ppm (for 5bii) and a sharp singlet at 6.20 (for both) were assigned to the aromatic protons, while proton signals at 3.69 and 3.62 ppm were observed for the methylene groups and *tert*-butyl group proton signals linked to the aromatic ring of 5bii and 5bi, which were observed at 1.37 and 1.33ppm, respectively. The ^{13}C NMR spectrum of **5b** exhibited 13 carbon signals including 10 aromatic carbon signals between 148.41 and 107.93 ppm, a methylene group carbon signal at 34.34 ppm, and tert-butyl group

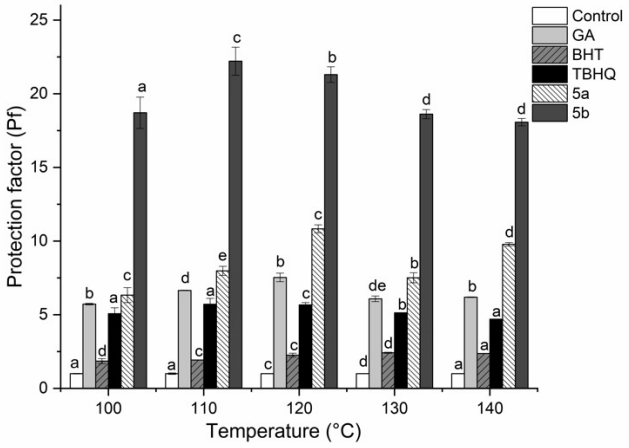


FIGURE 1. Pf values of oil samples containing 0.02% (w/w) antioxidants at different temperatures. Values are expressed as mean ± SD (n=3). Different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

carbon signals at 33.95 and 29.72 ppm. The HRMS (ESI) spectrum of **5b** exhibited a molecular specie at m/z 303.12332, which was assigned to $C_{17}H_{19}O_5^-$ $(M-H)^-$. The spectra data completely characterized **5a** and **5b**, neither of which has been previously described in any literature.

3.2. Evaluation of antioxidant activity of compounds

3.2.1. Rancimat test

The structure-antioxidant activity of the new antioxidants, **5a** and **5b** was evaluated in comparison with GA, BHT and TBHQ under temperatures of up to 140 °C at 0.02% (w/w) under air saturation conditions. The results are expressed as the IP of samples compared to the oxidative stability of lard without antioxidants (Table 1), and the Pf of the antioxidants (Figure 1). Pf values greater than 3 indicate strong antioxidant activity and those lower than 1 indicate pro-oxidant activity (Weng and Huang, 2014). In this experiment, the concentration was set at 200 ppm according to the safety limit established for frequently used and emerging commercial antioxidants in oils (Saad *et al.,* 2007).

The IPs of GA, BHT and TBHQ were less than **5a** and **5b** at the different temperatures. i.e., the novel lipophilic GA derivatives exhibited much stronger oxidative stability than GA and the common commercial synthetic antioxidants, TBHQ and BHT. Compound **5b,** however, showed the highest IP, which was 146 h higher than **5a** and 159, 209, and 170 h more than GA, BHT and TBHQ, respectively. For all the antioxidants evaluated at 0.02% (w/w) (Figure 1), a Pf greater than 1 was observed, which affirms a protective capacity against accelerated oxidation in the lipid matrix. Nevertheless, **5b** similarly exhibited the highest Pf (110 °C, Pf = 22.21; 140 °C, Pf = 18.07), followed by **5a** (110 °C, Pf = 7.98; 140 °C, Pf = 9.78), GA (110 °C, Pf = 6.65; 140 °C, Pf = 6.19), TBHQ (110 °C, Pf = 5.71; 140 °C, Pf = 4.70) and finally, BHT (110 °C, Pf = 1.93; 140 °C, Pf = 2.37). Briefly, the antioxidant activity of the evaluated antioxidants under Rancimat conditions decreased as follows: **5b** >> **5a** > GA > TBHQ > BHT > Control.

Previous studies have shown GA and its alkyl esters to exert better antioxidant activities than free polyphenols at moderate temperatures (Farhoosh *et al.,* 2016) and even TBHQ (Jung and Choi, 2016)

TABLE 1. Induction periods (IPs) of oil samples containing 0.02% (w/w) antioxidants at different temperatures under Rancimat accelerated test.

	IP (h)				
Sample	**100 °C**	**110 °C**	**120 °C**	**130 °C**	**140 °C**
Control	6.65 ± 0.04^{a}	3.00 ± 0.11^{a}	1.20 ± 0.01^{c}	0.53 ± 0.00^{d}	0.27 ± 0.00^{a}
GA	38.02 ± 5.26^{b}	19.96 ± 0.04^{d}	9.04 ± 0.57^{b}	3.22 ± 0.38^{de}	1.67 ± 0.03^{b}
BHT	12.31 ± 0.33^{d}	5.78 ± 0.02^{c}	2.72 ± 0.26^{c}	1.28 ± 0.07^{d}	0.62 ± 0.02^{a}
TBHQ	33.74 ± 1.61^{a}	17.13 ± 0.93^{a}	6.80 ± 0.30^{c}	2.72 ± 0.03^{b}	1.27 ± 0.01^{a}
5a	42.11 ± 3.02^{c}	23.94 ± 0.62^{e}	13.0 ± 0.54^{c}	3.98 ± 0.26^{b}	2.64 ± 0.24^{d}
5b	124.45 ± 6.64^{a}	66.64 ± 1.90^{c}	25.56 ± 1.06^{b}	9.87 ± 0.61^{d}	4.88 ± 0.49^{d}

Results are expressed as mean ± SD (n=3). Means in the same row with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$). Control: lard; GA: gallic acid; BHT: butylated hydroxytoluene; TBHQ: *tert*-butylhydroquinone; 5a and 5b: novel gallic acid derivatives.

in lipid matrices. Nonetheless, the much stronger antioxidant effects were exhibited by **5a** and **5b** compared to GA, The TBHQ and BHT in this study may be attributed to their higher molecular weight, which contributed to less partial volatilization/decomposition at high temperatures, especially when air is blown and a large amount of steam is formed (Huang *et al.,* 2014; Olajide *et al.,* 2018; Olajide *et al.,* 2020). In addition, various authors have proven that a synergistic effect may result from the combination of two or more phenolic antioxidants linked by a covalent bond in a single molecule, which is greater than individual antioxidants (Kancheva *et al.,* 2010; Teixeira *et al.,* 2013). Herein, the Log P value (hydrophobicity) of the resulting compounds increased due to the presence of a methylene bridge and bulky *tert*-butyl moiety (an excellent electron-donating group), thus leading to substantial activity of the antioxidants in oil. The electron-withdrawing effect of the carboxylic group on GA generates unstable phenoxy radicals (Torres de Pinedo *et al.,* 2007), however, replacing this with electron-donating molecules (Scheme 1) resulted in a well-stabilized phenoxy radical. Moreover, the excellent antioxidant capacity of the new compounds, especially that of **5b**, can be attributed to the strong steric synergy exhibited separately in the form of hydrogen bonding between hydroxyl groups in the pyrogallol (Farhoosh *et al.,* 2016) and sterically hindered hydroquinone units (Huang *et al.,* 2014) of the molecule, a phenomenal effect which allowed the less stable free radical of **5b** to easily convert to a more stable form intramolecularly. Furthermore, the pyrogallol component may easily synergistically regenerate the sterically-hindered hydroquinone one by donating hydrogen to it radical forms (Guo *et al.,* 2017).

3.2.2. DPPH assay

This method is frequently used to evaluate the potency of antioxidants as radical scavengers in that it is easily reproducible, rapid and sensitive. The scavenging activities of the antioxidants studied are shown in Figure 2. All the tested compounds showed a steady increase in scavenging activities between 1.5 and 48 µM, except GA, which exhibited a rapid one. At 12 µM, the scavenging abilities of GA, BHT, TBHQ, **5a** and **5b** were 67, 12, 23, 19 and 32%, respectively. Similarly, EC_{50}, the effective concentration needed to reduce the initial DPPH concentration by 50% is provided in Table 2. GA exhibited the highest EC_{50} value (19.09 µM) while BHT expectedly showed the least (54.84 µM). The antioxidant activity of the antioxidants in the DPPH system decreased as follows: GA > **5b** > TBHQ > **5a** > BHT.

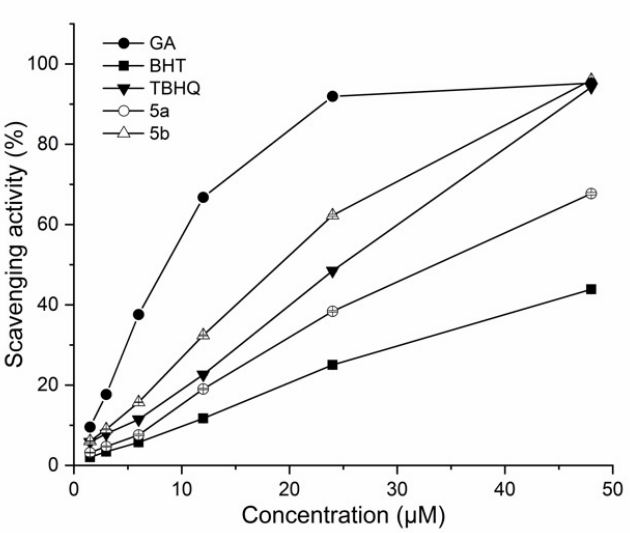


FIGURE 2. Radical scavenging activity of different antioxidants towards DPPH. Values are expressed as mean ± SD (n=3). Statistical significance at $p \leq 0.05$ according to Duncan's multiple range test.

This finding is in agreement with previous studies (Alavi Rafiee *et al.,* 2018; Asnaashari *et al.,* 2014; Farhoosh *et al.,* 2016; Mansouri *et al.,* 2020), where GA was found to be the most potent anti-radical agent compared to a subset of other phenolic antioxidants. The ability of phenolics to scavenge radicals is dependent on the number of electron-donating hydroxyl groups in the phenyl ring, which increase phenoxy

TABLE 2. The EC_{50} values of the lipophilic gallic acid derivatives (5a and 5b), GA, BHT and TBHQ.

Compound	EC_{50} (µM)	Log P_{theor}
GA	19.09 ± 0.04^{b}	0.42
BHT	54.84 ± 0.03^{c}	5.54
TBHQ	25.46 ± 0.05^{a}	2.96
5a	33.66 ± 0.04^{cd}	5.98
5b	23.60 ± 0.07^{b}	3.88

EC_{50}, 50% effective concentration. Values are expressed as mean ± SD (n=3). Means within a column with the same lowercase letters are not significantly different according to Duncan's multiple range test ($P < 0.05$).

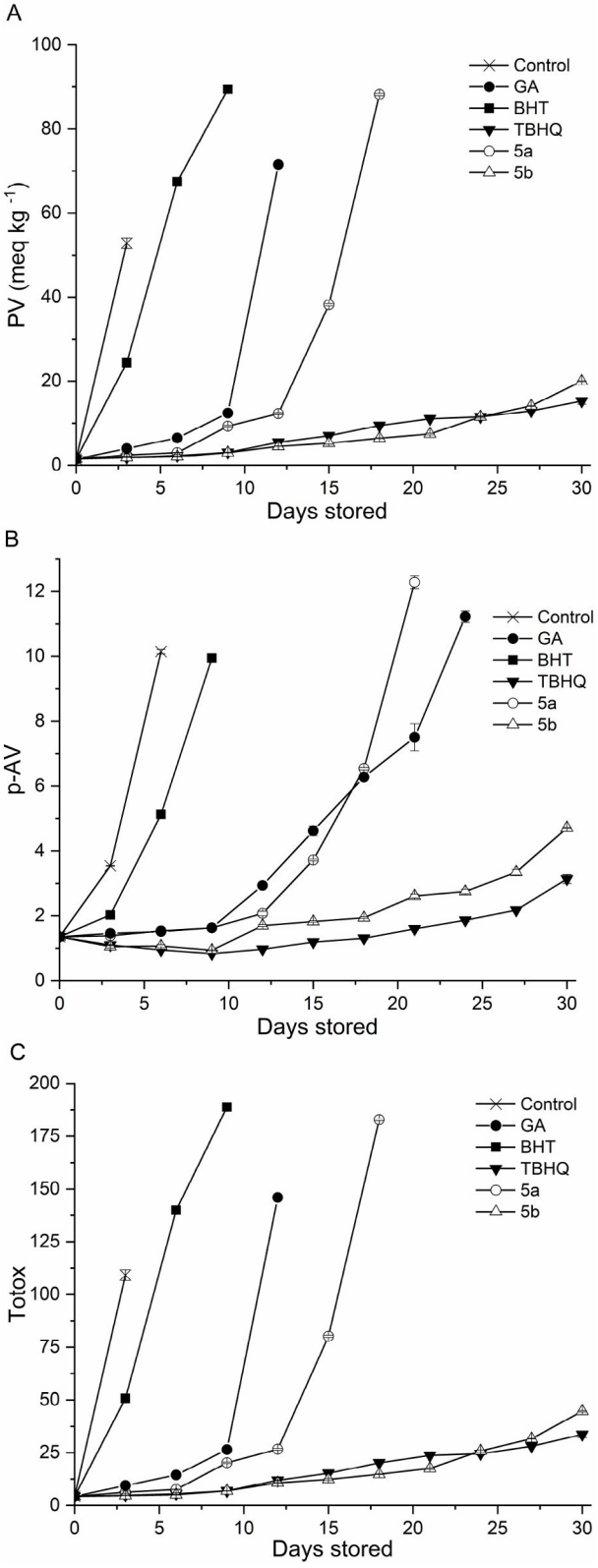


FIGURE 3. Changes in peroxide value (A), p-anisidine value (B) and Totox (C) during oxidation of soybean oil spiked with 0.02% (w/w) antioxidants at 65 °C. Values are expressed as mean ± SD (n=3). Statistical significance at $p \leq 0.05$ according to Duncan's multiple range test.

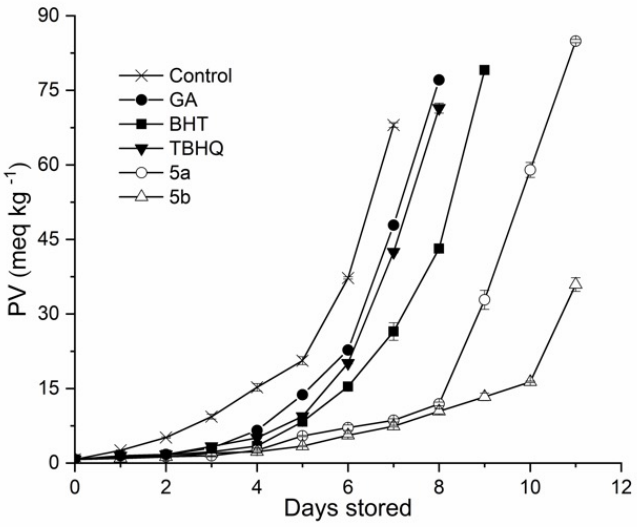


FIGURE 4. Changes in peroxide value during oxidation of soybean oil-in-water emulsion spiked with 0.02% (w/w) antioxidants at 65 °C. Values are expressed as mean ± SD (n=3). Statistical significance at $p \leq 0.05$ according to Duncan's multiple range test.

radical stability. As a result, GA, with a carboxyl and three hydroxyl substituents, was more active than **5b,** TBHQ, **5a** and BHT, as more hydrogen can be donated from the phenolic hydroxyls to stabilize the free radicals (de Pinedo *et al.,* 2007). Moreover, despite the electron-withdrawing effect of the carboxyl group (–COOH), it can readily dissociate in polar media to carboxylate anion (–COO–), an electron-donating moiety which supports the formation of more stable phenoxyl radicals (Mansouri *et al.,* 2020). The pattern of interaction between phenol and the DPPH radical occurs in the form of phenolic hydrogen abstraction and the transfer of a second hydrogen and/or formation of dimers (reactive or non-reactive) from the phenoxyl radical (Guitard *et al.,* 2016). TBHQ, with a hydroquinone structure, can release two hydrogens to DPPH. However, the presence of *tert*-butyl creates a steric hindrance effect on the *ortho*-OH substituent and thus lowers the abstraction of hydrogen by the DPPH (Weng and Huang, 2014).

This effect was apparent in the scavenging effect of **5b**, but nevertheless, the additional presence of a pyrogallol unit clearly increased its overall scavenging ability, thereby surpassing that of TBHQ. Strongly sterically hindered monophenols like BHT can only transfer one hydrogen per molecule and are unable to form reactive dimers (Guitard *et al.,* 2016), leading to low DPPH scavenging activity compared to GA and other antioxidants studied here. Nonetheless, 5a displayed

a higher scavenging activity (EC_{50} = 33.66 μM) compared to BHT (EC_{50} = 54.84 μM), mainly due to the presence of pyrogallol component in its molecule.

Meanwhile, the radical scavenging activity of antioxidants may also be illustrated by the degree of polarity, which determines their solubility and availability to oxidative compounds such as DPPH (Mansouri *et al.,* 2020). Compounds with higher log P values are more hydrophobic. Therefore, based on theoretical partition coefficient (Log P_{theor}) values (Table 2), GA had higher polarity than other compounds studied herein, leading to better interactions between it and DPPH. Briefly, as seen in this study, molecular configuration, ability to donate hydrogen atoms and subsequently stabilize phenoxyl radicals all influenced the antioxidant activity of the different phenolic antioxidants (Silva *et al.,* 2000).

3.2.3. Peroxide (PV), p*-anisidine (*p*-AV) and totox changes in oil*

As shown in Figure 3A, the initial PV of the samples was 1.47 ± 0.06 mEq/Kg oil, depicting that the oil was in good condition (PV < 15 mEq O_2/Kg) (Maszewska *et al.,* 2018). As expected, BHT and control displayed a sharp increase in PV, reaching 80 mEq/kg at 4.6 and 8.5 days of storage, respectively. At the same time, GA and **5a** samples oxidized slowly at the initial stage, and then steeply increased, reaching a maximum on days 12 (71.52 ± 0.85 mEq/kg) and 18 (88.20 ± 0.26 mEq/kg), respectively. However, both TBHQ and **5b** had better oxidative stability by exhibiting a gradual increase in PV throughout the storage period.

A *p*-AV below 10 depicts good quality oil (Maszewska *et al.,* 2018). Results from Figure 3B showed that after 30 days of storage at 65 ± 0.5 °C, most of the samples had a *p*-AV exceeding 10 from an initial value of 1.35 ± 0.03, except for the TBHQ (3.14 ± 0.14) and **5b** (4.71 ± 0.02) groups. The control and BHT samples showed similar result trends as earlier presented in PV with *p*-AV of 10.14 ± 0.07 and 9.94 ± 0.07 on days 6 and 9, respectively. Nevertheless, GA extended the oxidative stability of oil samples in terms of *p*-AV by an additional 12 days compared to what was observed for PV, thereby reaching a maximum value of 11.22 ± 0.18 on the 24th day of storage.

Totox considers both peroxides and aldehydes generated during oxidation and is thus a better indicator of overall oxidative deterioration (i.e. lower Totox value depicts better oil quality and vice versa). The results presented in Figure 3C are similar in trend to those of PV and *p*-AV under the same storage conditions except for GA, which displayed extended oxidative stability by delaying the production of secondary oxidation products for 12 more days. However, this effect showed little significance in terms of total oxidation. Nonetheless, the antioxidant capacity of the compounds evaluated in bulk oil under the Schaal Oven test decreased as follows: TBHQ ≈ **5b** > **5a** > GA > BHT > control.

The dissimilarity in the antioxidant activities of compounds may depend on their chemical structures, which influence their ability to stabilize their own phenoxy radicals. Compounds **5a** and **5b** both exhibited stronger oxidative stability than TBHQ at high temperatures (> 100 °C) under Rancimat, but at moderate thermal temperatures (65 °C) the protective activity of **5a** was lower and that of **5b** was close to TBHQ. This observation is in accordance with previous reports (Olajide *et al.,* 2020; Zhang *et al.,* 2004). The phenolic hydroxyl groups on TBHQ have strong steric synergy and can transfer hydrogen atoms to active peroxyl radicals in lipid matrices to interrupt the oxidative process. Similar to a previous study (Farhoosh *et al.,* 2016), despite the lower polarity and DPPH scavenging activities of **5a** and **5b** (Figure 2; Table 2), they were more effective antioxidants than GA in bulk oil. This is probably due to their fundamental carry-through properties—i.e., the ability to resist decomposition by heat and/or loss via volatilization. Moreover, in the lipid media, hydrogen atoms from sterically hindered moieties linked to **5a** and **5b** may interact intramolecularly with the meta-hydroxyl substituents of their pyrogallol component, subsequently leading to the stabilization of phenoxy radicals and thus increased antioxidant activities (Asnaashari *et al.,* 2014).

3.2.4. Peroxide value (PV) changes in oil-in-water (O/W) emulsion

The activity of antioxidants evaluated in emulsion following accelerated oxidation in the oven until PV equaled 70 mEq/kg contrasted that observed for bulk oil, and the relative decreasing order was: **5b** > **5a** > BHT > TBHQ > GA > control (Figure 4). Similarly, this finding was different from the trend observed in the DPPH assay which suggests antiox-

idant performance depends greatly on the method of analysis and/or system used (Farhoosh *et al.,* 2016). The effectiveness of antioxidants in the emulsion system were generally reduced compared to those in the soybean bulk oil, indicating lower antioxidant concentration in the hydrophobic center of oil droplets, thus leading to a faster oxidation rate. Gallic acid, with the highest scavenging activity under DPPH assay, was the weakest in the emulsion system. This conforms with previous studies, where the antioxidant activities of GA (Asnaashari *et al.,* 2014) and rosmarinate alkyl esters (Panya *et al.,* 2010; Schwarz *et al.,* 2000) in emulsion increased as their polarity decreased. These lower polar compounds up to their dodecyl derivatives were more effective antioxidants in that they predominantly concentrated themselves at the oil–water interface where oxidation occurs. However, GA and rosmarinic acid with the highest polarity incorporated a larger amount of their molecules to the aqueous phase than the interface.

Comparably, TBHQ, with the strongest antioxidant activity in bulk oil (Figure 3), exhibited weak antioxidant activity compared to the more lipophilic BHT, **5a** and **5b**. Antioxidants partition on the basis of polarity, which is determined by their molecular structural properties (i.e. hydrophobic compounds display higher log P values and vice versa) (Asnaashari *et al.,* 2014). Zhang *et al.,* (2004) also reported weak antioxidant activity for TBHQ, which was due to its low hydrophobicity leading to more migration towards the aqueous phase in the emulsion than the oil-water interface (oxidation site).

According to Panya *et al.,* (2010), butyl rosmarinate, an alkyl ester of rosmarinic acid, with similar molecular structure and Log P_{theor} value to **5b**, predominantly concentrated at the interface and thus exerted stronger antioxidant capacity than the more polar rosmarinic acid and least polar eicosyl rosmarinate (20 carbon atoms). As a result, the combined hydrophobic nature of the methylene bridge and the *tert*-butyl substituent in **5b** must have shifted the molecule to the oil-water interface, leading to its greater oxidative stability compared to other antioxidants studied herein. Meanwhile, BHT, with the highest hydrophobicity among all the phenolics studied, may have partitioned more into the oil phase away from the interface, thus exhibiting reduced effectiveness compared to **5a** and **5b**. This was in agreement with the results obtained by Li *et al.,* (2006) for BHT in soybean oil-in-water emulsions. Hence, contrary to the performance in the DPPH assay, the lipophilic derivatives—**5a** and **5b**, mostly exhibited stronger antioxidant activities than their individual parent molecules in emulsion and bulk oil systems, including the high temperature (100 – 140 °C) Rancimat experiment.

This can be attributed to the fact that alkylation of the pyrogallol unit with sterically hindered hydroquinone or 2,6-DTBP moieties in **5b** and **5a** molecules, respectively, decreased the electron-withdrawing effect of –COOH linked to the phenyl ring of GA, resulting in a phenoxy radical with better stabilization (Farhoosh and Nyström, 2018). Indeed, the effectiveness of phenolic antioxidants in oil indicates that their stabilizing capacity is considerably related to the properties of functional groups present, steric synergy and intramolecular hydrogen bonding. Meanwhile, in the emulsion system, it may depend on variables such as polarity and solubility, emulsifier used, concentration and type of antioxidants, radical-scavenging properties and the complex effects at the oil–water interfaces. Moreover, the polar paradox theory, which denotes that more polar antioxidants exhibit lower capacity in more polar media (Alavi Rafiee *et al.,* 2018), is not applicable in many cases.

4. CONCLUSIONS

The present study showed that the novel lipophilic derivatives of gallic acid, **5a** and **5b**, demonstrated excellent oxidative stability in emulsion and bulk oil systems at high and moderate temperatures. Their efficiency in the alcoholic medium of the polar DPPH system compared to GA slightly decreased, indicating that the polar paradox theory may not always be applicable. Overall, the better antioxidant effectiveness of the new antioxidants compared to other phenolics studied herein justifies the initial premise—i.e., a synergistic effect leading to stronger antioxidant activity may result from a medley of phenolic antioxidants covalently bonded together in one molecule. Thus, these new lipophilic antioxidants may be utilized industrially as functional ingredients with strong antioxidant potential in different food processing conditions following a further study on the proper characterization of their safe consumption.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this work.

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