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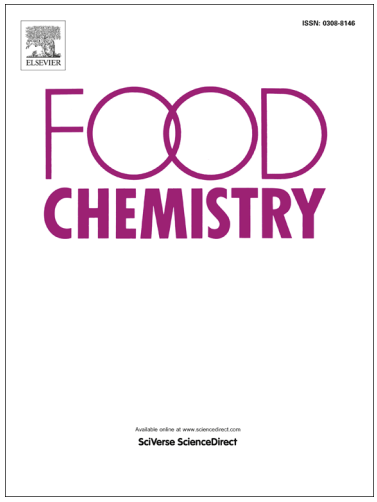

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Epigallocatechin (EGC) esters as potential sources of antioxidants

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Abstract

Epigallocatechin (EGC) was acylated with selected fatty acids, namely propionic acid [C3:0], caprylic acid [C8:0], lauric acid [C12:0], stearic acid [C18:0]) and docosahexaenoic acid (DHA)[C22:6 n-3] in order to increase its lipophilicity. Monoesters were identified as the predominant products (~40 %) followed by diesters (~33 %), triesters (~9 %) and trace amounts of tetra- and pentaesters. ^{1}H NMR, ^{13}C NMR and HPLC-DAD-MS were used to elucidate the acylation sites and structures of new EGC esters. According to the HPLC-MS analysis of the caprylate esters, EGC-4'-*O*- caprylate (27 %), EGC-3'-*O*- caprylate or EGC-5'-*O*- caprylate (12 %) and EGC-3',5'-*O*- dicaprylate (16 %) were the major compounds generated upon the acylation reaction of EGC. The acylation significantly increased the lipophilicity of EGC. In addition, EGC and its esters showed radical scavenging activities against DPPH radical and ABTS radical cation. Therefore, EGC esters could serve as potential sources of antioxidants for application in both hydrophilic and lipophilic media.

Keywords: Epigallocatechin (EGC); Acylation; Fatty acids; Lipophilicity; Antioxidant activities; Green tea catechins

1. **Introduction**

Recently, tea (*Camellia sinensis*) catechins have attracted increased attention of researchers due their myriad of health benefits. Tea is one of the most consumed beverages in the world and a rich source of polyphenols. Tea catechins include (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG). Green tea contains all of the above-mentioned catechins, since it has not been subjected to oxidation by polyphenol oxidase. Meanwhile, oxidized black tea and partially oxidized oolong tea have a much lower content of the aforementioned catechins and primarily contain theaflavins and thearubigins. EGCG is the most abundant flavan-3-ol of green tea and in oolong tea ranging between 22 and 53 mg per gram followed by EGC, ECG and EC, while black tea contains nearly 4 mg/g of EGCG (Zuo, Chen & Deng, 2002).

Several studies have been conducted on green tea catechins for their antioxidant activities (Amarowicz & Shahidi, 1995; Wanasundara & Shahidi, 1996; 1999; Shahidi & Alexander, 1998; Zhong & Shahidi, 2011; 2012; Zhong, Ma & Shahidi, 2012; Shahidi & Zhong, 2015; McKay & Blumberg, 2002; Perera, Ambigaipalan & Shahidi, 2018; Sun, Zhou & Shahidi, 2018) and health benefits such as anti-cancer, anti-inflammatory, anti-obesity, anti-glycation, as well as neuro- and cardioprotective effects (McKay & Blumberg, 2002; Cabrera, Artacho & Giménez, 2006; Rains, Agarwal & Maki, 2011; Wang, Zhang, Zhong, Perera & Shahidi, 2016). Epigallocatechin (EGC) is the second most abundant favan-3-ol of green tea catechins with a three-ring structure and six hydroxyl groups, hence it is quite hydrophilic in nature. Shahidi and Zhong (2015) introduced a novel method to enhance the lipophilicity of EGCG by acylation with various fatty acids such as stearic acid, EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). In addition, several other studies have also used the same method for improvement of lipophilicity of phenolic

compounds such as resveratrol, tyrosol and hydroxytyrosol (Laguerre, Bayrasy, Lecomte, Chabi, Decker, Wrutniak-Cabello, Cabello & Villeneuve, 2013; Oh & Shahidi, 2017; Zhou, Sun & Shahidi, 2017). Myers, Fuller and Yang (2013) demonstrated that the catechin fatty acid ester (catechin 4'-O-palmitoyl EGCG) naturally exists in Chinese green tea and this compound is now approved for use in food products in China. In addition, Okushio, Suzuki, Matsumoto, Nanjo and Hara (1999) showed that only 0.1% of EGCG, 14 % of EGC and 31 % of EC were detected in plasma after oral administration of decaffeinated green tea in rats. This implies that increase in hydrophilicity decreases the bioavailability of tea catechins, hence the necessity of structural modification in order to improve their absorption. The metabolic pathway of tea polyphenols involves methylation by S-adenosyl-methionine that is catalyzed by the enzyme catechol-*O*-methyltransferase (COMT) (Kanwar, Taskeen, Mohammad, Huo, Chan & Dou, 2012). Moreover, tea polyphenols could be converted to glucuronide and sulphate conjugates of catechins by UDP-glucuronoryltransferase (UGT) and sulphotransferase (SULT) enzymes, respectively (Yang, Sang, Lambert & Lee, 2008). In this study, for the first time, EGC was lipophilized with selected fatty acids (propionic acid [C3:0], caprylic acid [C8:0], lauric acid [C12:0], stearic acid [C18:0] and docosahexaenoic acid (DHA)[C22:6 n-3]) and the resultant derivatives were characterized using proton NMR and HPLC-MS. Radical scavenging activities of the novel derivatives were tested against DPPH and ABTS radicals and the efficacy compared with respect to their acyl type, multiplicity, and location. Thus, it is hypothesized that acylation of EGC with fatty acids would enhance the lipophilicity and antioxidant activities *in vitro*.

2. Materials and Methods

2.1 Materials

Epigallocatechin (EGC) was obtained from Chengdu Biopurify Phytochemicals Ltd (Chengdu, Sichuan, China). Acyl chlorides (propionyl [C3:0] chloride, capryl [C8:0] chloride, lauroyl chloride [C12:0] and stearoyl chloride [C18:0]) were purchased from Sigma–Aldrich Canada Ltd (Oakville, ON, Canada). DHA single cell oil (DHASCO) was received from DSM (Columbia, MD, USA). Silica gel and flexible thin layer chromatography (TLC) plates with silica gel 60A (2.5 × 7.5 cm, layer thickness of 250μm) were bought from Selecto Scientific (Suwanee, GA, USA). All chemicals used were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada) or Sigma–Aldrich Canada Ltd. The solvents used were of ACS grade, pesticide grade or HPLC grade and were used without any further purification.

DHA was prepared from DHASCO (containing 40% DHA) using saponification followed by a urea complexation process (Wanasundara & Shahidi, 1999). Saponification was carried out with DHASCO (30 g), KOH (6.9 g), water (13.2 mL) and 95 % ethyl alcohol (79.2 mL) under nitrogen reflux at 62 ± 2 °C for 1 h. Then distilled water (60 mL) was added to the mixture and the unsaponifiable matters were removed by using a separatory funnel with hexane (100 mL) twice. The aqueous layer containing saponifiable matter was acidified to pH 1 with 3 M HCl followed by the extraction of the released free fatty acid with hexane (50 mL, 4 times). The hexane layer was filtered through a cone of anhydrous sodium sulphate and the solvent was subsequently removed using a rotary evaporator at 40 °C.

For urea complexation, the free fatty acid (FFA) obtained after evaporation (10 g) was stirred with a urea solution (20 %, w/v, in 95 % ethyl alcohol, 150 mL) at 60 °C until a clear

homogenized solution was obtained. The contents were then placed in a cold room at 4-8 °C for 24 h for urea-FFA adduct complex formation, followed by the removal of urea complexed crystals by suction filtration. The filtrate was diluted with an equal volume of distilled water and the pH was subsequently adjusted to 4-5 with 6 M HCl. An equal volume of hexane was then added, stirred for an hour and transferred to a separatory funnel. The hexane layer was passed through anhydrous sodium sulphate, the solvent was removed using evaporation and samples were stored at -60 °C. The identity and relative purity of DHA were confirmed by gas chromatography-mass spectrometry (GC-MS) using DHA methyl ester. The GC-MS (Hewlett-Packard 5890 series II, Agilent, Palo Alto, CA, USA) equipped with a fused capillary column (Supelcowax-10, 30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON, Canada) was used. The temperature of the injector and detector (FID) were both set at 250 °C and the oven temperature increased from 220 to 240 °C at a rate of °C/min. DHA methyl esters were identified by comparing their retention time with standard (Nu-check, Elysian, MN, USA).

2.3 Preparation of DHA chloride

DHA was converted to its corresponding acyl chloride according to the method described by Zhong and Shahidi (2011). Thionyl chloride (4.5 mL) was added dropwise to DHA (9.9 g) in a three-neck round bottom flask under nitrogen reflux at 70 °C in an oil bath for 1 h.

2.4 Preparation of EGC esters

EGC was esterified with each of the five acyl chlorides (propionyl [C3:0] chloride, capryl [C8:0] chloride, lauroyl chloride [C12:0], stearoyl chloride [C18:0]) and DHA chloride [C22:6]) at a mole ratio of 1:1 (Zhong & Shahidi, 2011). EGC dissolved in ethyl acetate (180 mL) in a three-neck round bottom flask was heated in an oil bath at 60 °C under a nitrogen blanket with constant

stirring. When the solution became clear, pyridine (2.44 mL) was added dropwise. Then acyl chloride dissolved in ethyl acetate (20 mL) was added dropwise to the mixture at 50 °C and allowed to react for 3 h. Upon completion of the esterification reaction, the mixture was cooled to room temperature and filtered into a separatory funnel. The filtrate was subsequently washed three times with warm distilled water (60 °C) and the ethyl ester layer was passed through anhydrous sodium sulphate. The solvent was evaporated to dryness and the products containing crude EGC ester mixtures were stored at -60 °C. A thin layer chromatography (TLC) with mobile phase of hexane/ ethyl acetate/ formic acid at the ratio of 3:3:0.12 (v/v/v) was used to identify the reactants and products.

2.5 Purification of EGC esters with column chromatography

EGC derivatives were purified using silica gel column chromatography with gradient elution of hexane/ ethyl acetate/ formic acid (90:10:2; 80:20:2; 70:30:2; 60:40:2 and 50:50:2; v/v/v). The collected fractions were monitored by using thin layer chromatography (TLC) with hexane/ ethyl acetate/ formic acid at a ratio of 3:3:0.12 (v/v/v). Tubes corresponding to a particular compound were collected and evaporated using a rotary evaporator. TLC bands were scrapped off and dissolved in ethyl acetate to extract the pure compounds for NMR analysis.

2.6 Identification of EGC esters with high performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS)

The chemical structures of EGC esters were determined using high-performance liquid chromatography-electrospray ionization-time of flight-mass spectrometry (HPLC-ESI-TOF-MS) on an Agilent 1260 HPLC unit (Agilent Technologies, Palo Alto, CA, USA). Separations were conducted with a SUPERLCOSILTM LC-18 column (4.6 × 250 mm × 5 μm with guard column;

Sigma-Aldrich, Oakville, ON, Canada). The binary mobile phase consisted of methanol/ 5 % acetonitrile in water at the ratio of 80: 20 (v/v) and run for 40-60 min at a flow rate of 0.8 mL/min. The compounds were detected at 280 nm. HPLC-ESI-MS^n analysis was carried out under the same conditions as described above using an Agilent 1100 series capillary liquid chromatography–mass selective detector (LC-MSD) time of flight system in electrospray ionization (ESI) in the positive mode. The data were acquired and analyzed with Agilent LC-MSD software (Agilent Technologies). The area percentage of the HPLC chromatogram was used to calculate the yield ratios and percentages.

2.7 Structure elucidation of EGC esters with nuclear magnetic resonance (NMR) spectroscopy

Compounds purified with TLC were subjected to ^1H NMR and ^{13}C NMR in order to identify their molecular structures and the position of esterification. The ^1H and ^{13}C NMR analyses were recorded on a Bruker Avance 500 MHz NMR spectrometer (Bruker Biospin Co. Billerica, MA, USA) operating at 500.13 and 125.77 MHz, respectively, and the data interpretation was performed with Topspin 3.0 with ICON (Bruker Biospin Co.) and MestRe Nova (Mestrelab Research SL, Santiago De Compostela, Spain). The samples were dissolved in perdeuterated dimethyl sulphoxide (DMSO-d6) containing trimethylsilane (TMS) as internal standard. The position of esterification was confirmed by comparing the chemical shifts of EGC and its derivatives.

2.8 Lipophilicity of EGC derivatives

The lipophilicity of EGC derivatives was computed according to the method established by Tetko and Bruneau (2004) using ALOGPS 2.1 software (http://www.vcclab.org). The structures of EGC

derivatives in simplified molecular input line entry (SMILE) system were drawn by ChemDraw Std 14.0 (CambridgeSoft).

2.9 Antioxidant activity of EGC derivatives

2.9.1 DPPH radical scavenging activity of EGC derivatives

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of EGC derivatives was determined according to Zhong and Shahidi (2011). EGC and EGC derivatives, dissolved in ethanol (0.5 mg/mL, 0.1 mL), were added to ethanolic solution of DPPH (0.2 mM, 0.9 mL), vortexed and allowed to stand at room temperature in the dark for 30 min. The mixture (1 mL) was then injected to the sample cavity of a Bruker E-scan electron paramagnetic resonance (EPR) spectrometer (Bruker E-scan, Bruker Biospin Co., Billercia, MA, USA). The operation parameters of Bruker E-scan EPR spectrometer were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.00 G sweep width, 3495.53 G center field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency and 1.86 G modulation amplitude. DPPH radical scavenging capacities of the test compounds were calculated using the following equation:

$$DPPH\ radical\ scavenging\ capacity\ (\%) = \left\{\frac{EPR\ signal\ intensity\ for\ the\ control - EPR\ signal\ intensity\ for\ the\ sample}{EPR\ signal\ intensity\ for\ the\ control}\right\} \times 100$$

Trolox was used as a standard (50-300 μM) and the DPPH radical scavenging activity was expressed as micromoles of trolox equivalents.

2.9.2 ABTS radical cation scavenging activity of EGC derivatives

The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) scavenging activity of EGC and its derivatives was determined according to Liyana-Pathirana and Shahidi

(2006). A solution of $ABTS^{\bullet+}$ was prepared by mixing AAPH (2.5 mM) with ABTS (2.5 mM) stock solution at 1:1 (v/v) ratio, which were prepared in a phosphate buffer (0.1 M) containing sodium chloride (0.15 M, pH 7.4). The mixture was heated for 20 min at 60 °C for colour development, protected from light, stored at room temperature and filtered. Test compounds (0.5 mg/mL, 20 µL) were mixed with $ABTS^{\bullet+}$ solution (980 µL) and the absorbance was read after 6 min at 734 nm. A standard curve was prepared using trolox (0-500 µM). ABTS radical cation scavenging activity of test compounds was expressed as trolox equivalents according to the equation below.

$$ABTS\ cation\ radical\ scavenging\ activity\ (\%) = \left\{\frac{Absorbance\ of\ blank - Absorbance\ of\ sample\ after\ 6\ min}{Absorbance\ of\ blank}\right\} \times\ 100$$

2.10 Statistical Analysis

All experiments were replicated three times and mean values and standard deviations reported. One-way ANOVA was performed, and the mean separations were performed by Tukey's HSD test ($P<0.05$) using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1 Preparation of EGC esters

EGC was acylated with fatty acid chlorides, namely propionyl [C3:0] chloride, capryl [C8:0] chloride, lauroyl chloride [C12:0], stearoyl chloride [C18:0]) and DHA chloride [C22:6]. This reaction produced two monoesters, six diesters and four triesters, at a ratio of 4.3: 3.6: 1. According to the HPLC-DAD-MS data (Figure 2a, 2b) analysis of EGC-caprylic acid ester, monoesters (Figure 1) were the predominant product, with the yield of ~40 % followed by diesters (~33 %), triesters (~9 %) and trace amounts of tetra- and pentaesters. Studies from the literature showed that the yield of these esters varied depending on the type of fatty acid that underwent esterification. For instance, Zhong and Shahidi (2011) reported that tetraesters were

the predominant products when EGCG was esterified with fatty acids (1:1) such as stearic acid, EPA and DHA with yields of 56.9, 42.7 and 30.7 %, respectively. In another recent study, Oh and Shahidi (2017) found that acylation of resveratrol with fatty acids yielded mainly monoesters with a series of fatty acids (C3:0 to C22:6) and the yield varied between 37.7 and 74 % for C3:0 to DHA, respectively. These findings demonstrate that the predominant type of ester (selectivity and specificity of acylation reaction) so produced was primarily determined by the chemical structure of the parent polyphenolic molecule that undergoes esterification with different fatty acids regardless of their chain length. Furthermore, the higher yield obtained for short chain fatty acids compared to long chain fatty acids could be due to the steric hindrance effect imposed by non-linear (bent) unsaturated long chain fatty acids. However, short chain saturated fatty acids could pack closely due their linear configuration and shorter chain length, hence leading to a higher yield during the acylation reaction than long chain fatty acids.

3.2 Identification EGC esters

High performance liquid chromatography–mass selective detector (HPLC-MSD) ion trap system in electrospray ionization (ESI) in the positive mode was used to identify the EGC esters using methanol/water (containing 5% acetonitrile) at ratio of 80:20 (v/v) as a solvent. HPLC chromatogram of crude mixture of EGC-caprylic (C8) ester is shown in Figure 2a. Compound **1** was identified as EGC with the *m/z* value of 307 (Figure 2b-A) that corresponds to the protonated ion of EGC $[M+H]^+$. Similarly, compounds **3** and **4** showed *m/z* of 433 (Figure 2b-B) which is a protonated $[M+H]^+$ species of EGC-C8 monoester with a molecular weight of 432 g/mol. Compounds **4**, **5**, **6**, **7**, **8** and **9** showed *m/z* of 581 (Figure 2b-C), which is identified as a sodium adduct of EGC-C8 diester $[M+23]^+$. A *m/z* of 702 (Figure 2b-D) was observed for compounds **10**, **11**, **12** and **13** in the HPLC-MS (ESI) which reflects the presence of triesters in the EGC-C8 crude

mixture and is the ammonium adduct of EGC-C8 triester $[M+NH_4]^+$ having molecular weight of 684 g/mol. In addition, trace amounts of EGC-C8 tetra- and pentaester were identified with *m/z* values of 833 $[M+Na]^+$ and 959 $[M+Na]^+$, respectively.

3.3 Structure elucidation of EGC esters

The crude mixture of EGC-DHA dissolved in ethyl acetate was run on a silica gel TLC plate using hexane/ ethyl acetate/ formic acid at a ratio of 3:3:0.12 (v/v/v) to identify the reactants and products. TLC plate showed 13 bands that indicated the presence of several esters after acylation reaction (Figure not shown), corresponding to the peaks detected in the HPLC chromatogram (Figure 2a). Compound **1** (band 1) was identified as EGC by comparing the retention factor (R_f) value of pure EGC. EGC was eluted last on the TLC due to its low molecular weight, high hydrophilicity and polarity than EGC-DHA esters. These individual bands were scrapped off, compounds were dissolved in ethyl acetate and their molecular structures identified using ^{1}H NMR and ^{13}C NMR. ^{1}H NMR spectrum of EGC is shown in Figure 3 and the chemical shift (δ ppm) of ^{1}H NMR for EGC and EGC-DHA esters are presented in Table 1. Compound **1** (band 1) was confirmed as EGC by comparing the peaks with those of an EGC standard obtained from Sigma Aldrich. Although HPLC-MS analysis revealed the presence of mono-, di- and triesters in the crude mixture of EGC-DHA, it is difficult to ascertain which hydroxyl groups had participated in the esterification reaction. Hence, ^{1}H NMR analysis and determination of chemical shift (δ ppm) are essential in structure elucidation. The position of esterification is primarily determined by comparison of the esterified compound with the parent molecule as well as the literature data. The chemical shift assignment of alkyl hydrogens and hydroxyl groups for EGC (Figure 3) was obtained from the literature (Davis, Cai, Davies & Lewis, 1996). Hydroxyl proton signals appear at low field i.e. at higher frequency from standard tetramethylsilane (TMS) than alkyl protons.

Hence peaks at chemical shifts of 7.94, 8.69, 8.89 and 9.09 ppm were assigned to 4'-OH, 3' and 5'-OH, 5-OH and 7-OH, respectively. However, 3-OH peak appeared at 4.66 ppm that is due to the deuterated form of the OH group. Since hydroxyl protons rapidly exchange, they do not have enough time to interact with neighbouring protons, hence usually a singlet NMR peak is detected. The singlet at 8.69 ppm is due to the 3' and 5'-OH groups, hence both OH groups have an identical environment and no coupling with other protons exists. Similarly, 2' ad 6' protons showed a doublet peak at 6.37 ppm due to the identical environment and a doublet peak due to the coupling effect from position 2 proton. Two protons in the same carbon at position 4 are not chemically equivalent due to the difference in their environment. Thus, two different doublets were observed at 2.67 and 2.70 ppm and the splitting was due to the geminal coupling i.e. the effect comes from protons from the same carbon. A quartet of doublet peak (6 peaks, qd) at 3.98 ppm was identified as position 3 proton. The quartet coupling effect from two different protons in position 2 and doublet effect from proton in position 4. Two doublet peaks at 5.71 and 5.88 ppm were identified as protons in positions 8 and 6, respectively. Furthermore, all these positions of EGC were reconfirmed with ^{13}C NMR (Table 1).

Table 1 shows the chemical shifts of EGC and various esters (compounds **2**-**9**) obtained from the acylation reactions. Compounds **2** and **3** were identified as monoesters from HPLC-MS and the positions of esterification was further confirmed by alteration in ^{1}HNMR chemical shifts. A hydroxyl peak at 7.94 ppm corresponding to 4'-OH was disappeared in ^{1}HNMR spectra, hence compound **2** was identified as EGC-4'-*O*-caprylate. In addition, a change in chemical shift ($\Delta\delta$) of 0.24 ppm (8.69 to 8.93 ppm, Table 1) was observed for neighbouring OH groups (3' and 5'). The same magnitude of $\Delta\delta$ (0.1 ppm) for hydrogens at H-2' and H-6' reflects that the symmetry of the molecule at ring B was retained after the acylation reaction. Similarly, compound **3** was identified

as EGC-3'-*O*-caprylate or EGC-5'-*O*-caprylate by the missing OH peak at 8.69 ppm as well as split peaks with different chemical shifts for H-2' (6.47 ppm) and H-6' (6.52 ppm). This shows that the molecule is no longer symmetric at ring B following the acylation reaction. Furthermore, integration of the peak at 8.69 ppm was reduced to half compared to its original value, which reflects acylation of OH-3' or OH-5'.

The structures of five diesters (compounds **4**, **6**, **7**, **8** and **9**) were identified and confirmed with ^{1}H-NMR (Table 1). Compounds **4**, **6**, **7**, **8** and **9** were identified as EGC-3,5'-*O*-dicaprylate or EGC-3,3'-*O*-dicaprylate, EGC-4',5'-*O*-dicaprylate or EGC-4',3'-*O*-dicaprylate, EGC-3,4'-*O*-dicaprylate, EGC-3',5'-*O*-dicaprylate and EGC-5,5'-*O*-dicaprylate or EGC-5,3'-*O*-dicaprylate, respectively. Structures of compounds **7** and **8** were further confirmed by the similar chemical shift of H-2' and H-6' that indicates the symmetry of the ring B. According to HPLC-DAD-MS (Figure 2a, 2b) analysis, EGC-4'-*O*-caprylate (compound **2**; 27 %), EGC-3'-*O*-caprylate or EGC-5'-*O*-caprylate (compound **3**; 12 %) and EGC-3',5'-*O*-dicaprylate (compound **8**; 16 %) were the major compounds from the acylation reaction of EGC. These compounds were used for the calculation of lipophilicity of the crude esters.

3.4 Lipophilicity of EGC esters

Previous studies have shown that acylation of phenolic compounds (EGCG, resveratrol) with different chain length fatty acid moieties significantly ($p<0.05$) increases the lipophilicity of the new compounds (Zhong & Shahidi, 2011; Oh & Shahidi, 2017). Various phenolic compounds also exist as their esters in nature such as steryl esters of ferulic and *p*-coumaric acids in soybean and cereals (Evershed, Spooner, Prescott & John Goad, 1988; Wang, Hicks & Moreau, 2002); hexadecyl, octadecyl, and eicosyl esters of *p*-coumaric acid in the vine and root latex of sweet

potato (Snook, Data & Kays, 1994); phenolic fatty acid esters [*E* and *Z* isomers of *p*-coumaryl alcohol with primarily long-chain (C_{16}–C_{26}) saturated fatty acids] in the epicuticular wax of Gala apples (Whitaker, Schmidt, Kirk & Barnes, 2001), etc. (Figueroa-Espinoza & Villeneuve, 2005). Hydrophilic nature of polyphenolic compounds due to the multiplicity of their hydroxyl groups limits their bioavailability and unrestricted food application (stabilizing fats and oils), hence increasing lipophilicity would enhance the bioavailability and antioxidative effect of phenolic compounds in a variety of food systems.

Ascorbyl palmitate is one of the most promising lipophilic alternatives to ascorbic acid that is widely used in the food industries. Pokorski, Marczak, Dymecka and Suchocki (2003) found that lipid-soluble derivative of ascorbic acid, ascorbyl-6-palmitate was able to cross biological barriers and served as a carrier of ascorbate into neural tissues, which are normally hardly accessible to hydrophilic ascorbic acid. In this study, lipophilicity of EGC was increased by acylation with various fatty acid moieties such as C3:0, C8:0, C12:0, C18:0 and DHA. Acylation of EGC with DHA would possess additional benefit such as combined physiological effect of omega 3 that has been shown to exert a myriad of health benefits and is widely used as a dietary supplement (Shahidi & Ambigaipalan, 2018). Table 2 shows the lipophilicity values for monoesters and diesters of EGC. Increase in chain length (C3:0 to C18:0) of fatty acid has increased the lipophilicity of mono- and diesters in comparison to their parent molecule. This is in accordance with the results observed for EGCG esters (Zhong & Shahidi, 2011) and resveratrol esters (Oh & Shahidi, 2017). Oh and Shahidi (2017) reported that the degree of unsaturation of a fatty acid has an inverse relationship with lipophilicity. For example, resveratrol-oleic acid ester showed greater lipophilicity than resveratrol-DHA ester. They suggested that the observed effect could be due to the presence of π- π interaction in unsaturated fatty acids and high density of

electrons, hence more polar than saturated fatty acids. In addition, studies have shown that increased lipophilicity could be confirmed with the increase in retention time of such esters in the HPLC chromatogram (Zhong & Shahidi, 2011; Tan, Le, Moghadasian & Shahidi 2012).

3.5 Antioxidant activity of EGC derivatives

3.5.1 DPPH radical scavenging activity of EGC derivatives

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of EGC derivatives was determined based on their ability to donate a hydrogen atom to the stable DPPH radical, thereby reducing it to the corresponding DPPH hydrazine. EPR spectroscopy was used to evaluate DPPH radical scavenging capacity, where the DPPH signal intensity was inversely correlated to the concentration of antioxidant and reaction time (Sanchez-Moreno, 2002). Since the results from this assay are highly reproducible and comparable to other free radicals, this assay has been widely used by researchers.

DPPH radical scavenging activity of EGC and its esters is shown in Figure 4a. EGC exhibited the highest DPPH radical scavenging activity of 5.87 μmol trolox equivalents/mg of sample followed by EGC-C3:0, EGC-C8:0, EGC-C12:0 and EGC-DHA with values of 1.74, 1.12, 0.82 and 0.43 μmol trolox equivalents/mg of sample, respectively. The decreased radical scavenging capacity of EGC derivatives could be attributed to the loss of hydroxyl groups upon the acylation reaction. A similar phenomenon was also reported in the literature for resveratrol derivatives of fatty acids

(Oh & Shahidi, 2017). Goupy, Dufour, Loonis and Dangles (2003) reported that polyphenols are more effective than simple phenols due to their ability to stabilize phenoxyl radical via hydrogen bonding. In addition, it could be postulated that the poor solubility of EGC-esters in water may be responsible for their poor radical scavenging activity. On the contrary, few other studies have shown that acylated derivatives are better than their parent compounds in terms of radical scavenging capacity. For instance, Zhong and Shahidi (2011) and Perera et al. (2018) reported that EGCG tetraesters of stearic acid, EPA and DHA had greater DPPH radical scavenging activity than their parent EGCG molecule. They postulated that EGCG derivatives have greater affinity towards hydrophobic DPPH radical than hydrophilic EGCG molecule. EGC esterified with stearic acid (EGC-C18:0) did not exhibit any DPPH scavenging activity. The observed variation in radical scavenging activity could be due to the combined effect of electronic and steric hinderance imposed by acylated fatty acids as well as increased lipophilicity. According to the polar paradox theory, hydrophilic antioxidants are more effective in a non-polar medium while lipophilic antioxidants work better in a polar medium (Shahidi & Zhong, 2011). Therefore, in this study, hydrophilic antioxidant EGC was able to scavenge hydrophobic DPPH radical to a greater extent than the lipophilic EGC derivatives. Moreover, EGC-C18:0, which had the highest lipophilicity value (Table 2) showed no scavenging activity against lipophilic DPPH radical. The poor solubility of EGC-C18:0 in ethanol may be responsible for its low DPPH scavenging activity. Furthermore, Zhong and Shahidi (2011) reported that the hydroxyl groups in positions 5 and 7 on the A-ring only had a mild influence on antioxidant activity of EGCG. This seems to be plausible since radical scavenging activity significantly changed after acylation at positions 3', 4' and 5'. Zhu, Wang, Wei, Lin, Yang and Ho (2001) found that the trihydroxy phenyl B-ring of EGC was the predominant active site for DPPH radical scavenging reaction.

3.5.2 ABTS radical cation scavenging activity of EGC derivatives

ABTS radical cation scavenging activity was measured by the decolorization of the bluish-green coloured pre-generated $ABTS^{\bullet+}$ [2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate)] radical cation by the added antioxidative compound using a spectrophotometer and absorbance readings at 734 nm. Both DPPH radical and ABTS radical cation have been shown to react with trolox (water-soluble analog of vitamin E) in a mole ratio of 2: 1 (Sanchez-Moreno, 2002). Valcic, Burr, Timmermann and Liebler (2000) reported that the reaction of EGC with peroxyl radicals would generate products including a seven-membered B-ring anhydride, a symmetrical EGC dimer and an unsymmetrical dimer and confirmed that the trihydroxy phenyl B-ring is the principal site of antioxidant reactions. Figure 4b represents the ABTS radical cation scavenging activity of EGC and its fatty acid esters. Acylation reaction has significantly ($p<0.05$) decreased the ability to scavenge ABTS radical cation by EGC esters than the parent EGC molecule. EGC molecule showed significantly ($p<0.05$) higher ABTS radical cation scavenging activity (15.7 μmol trolox equivalents/mg of sample) than their ester compounds of which values ranged between 8 and 0.6 μmol trolox equivalents/mg of sample. This is similar to the observed effect from DPPH radical scavenging assay, which might be due to the loss of hydroxyl groups and increased steric hinderance by the acylated fatty acids. In this assay, chain length of fatty acids did not show any correlation with the radical scavenging activity. It is noteworthy that EGC-C18:0, which did not show any scavenging activity against DPPH radical exhibited a radical scavenging activity against ABTS radical cation. This implies the importance of using various radicals' assays for the determination antioxidant capacity. This might be due to the highest lipophilicity of EGC-C18:0 among tested compounds. Arnao, Cano and Acosta (2001) reported that ABTS radical cation could be used to detect both lipophilic and hydrophilic antioxidant compounds due to its ability to solubilize in aqueous and acidified ethanol medium. On the

contrary, Tofani, Balducci, Gasperi, Incerpi and Gambacorta (2010) reported that no linear correlation existed between lipophilicity and ABTS radical cation scavenging activity when fatty acid esters of hydroxytyrosol were tested in cell culture assays. Overall, radical scavenging activity of EGC and its derivatives was influenced by the molecular structure, hydrogen donating ability and stabilization of phenoxyl radical. In addition, steric hinderance imposed by alkyl chains of fatty acids also plays a vital role in antioxidant activity.

4. Conclusions

Structural modification of hydrophilic EGC with propionic acid [C3:0], caprylic acid [C8:0], lauric acid [C12:0], stearic acid [C18:0]) and docosahexaenoic acid (DHA)[C22:6 n-3] significantly ($p<0.05$) increased the lipophilicity of the esters so prepared. Mono- and diesters were the predominant products during the acylation reaction. Newly formed esters exhibited both DPPH radical and ABTS radical cation scavenging activities, while the activities were lower than the parent EGC molecule. The efficacy of these derivatives was mainly influenced by the type or chain length of fatty acids. Hence, further studies are required for the assessment of antioxidant capacity as well as synergetic effect of these novel compounds generated from two biomolecules. Furthermore, these compounds could serve as a potential source of antioxidant in both hydrophilic and lipophilic environments.

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Figure captions

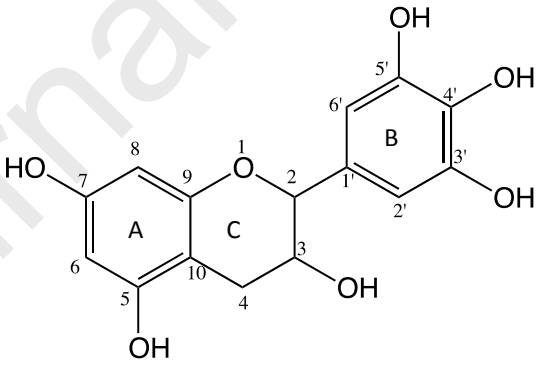
Figure 1. Structure of EGC and its esters

Figure 2. (a) HPLC chromatogram of EGC-caprylic ester; compounds 1-EGC; 2&3-mono esters; 4,5,6,7,8 & 9-di esters and 10, 11, 12 & 13-tri esters; (b)Mass spectra of EGC-caprylic esters; a-EGC, b-mono ester, c-di ester and d-tri ester

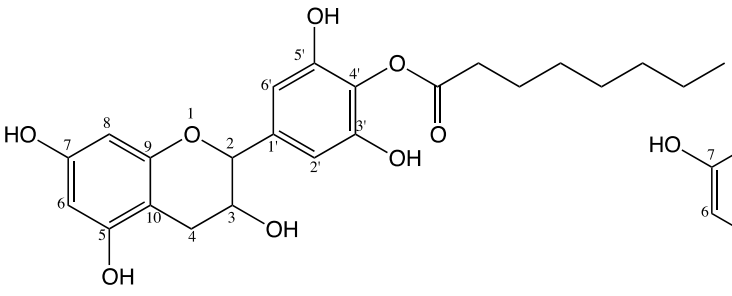
Figure 3. ^{1}H-NMR spectrum of EGC

Figure 4. (a) DPPH radical; (b) ABTS radical cation scavenging activities of EGC and its esters

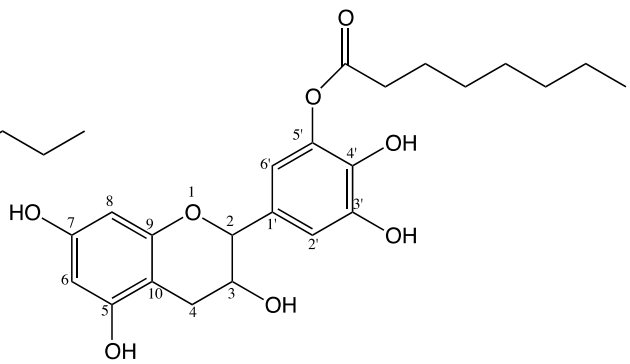
Figures



EGC



EGC-4'-caprylate



EGC-5'-caprylate

Figure 1. Structure of EGC and its esters

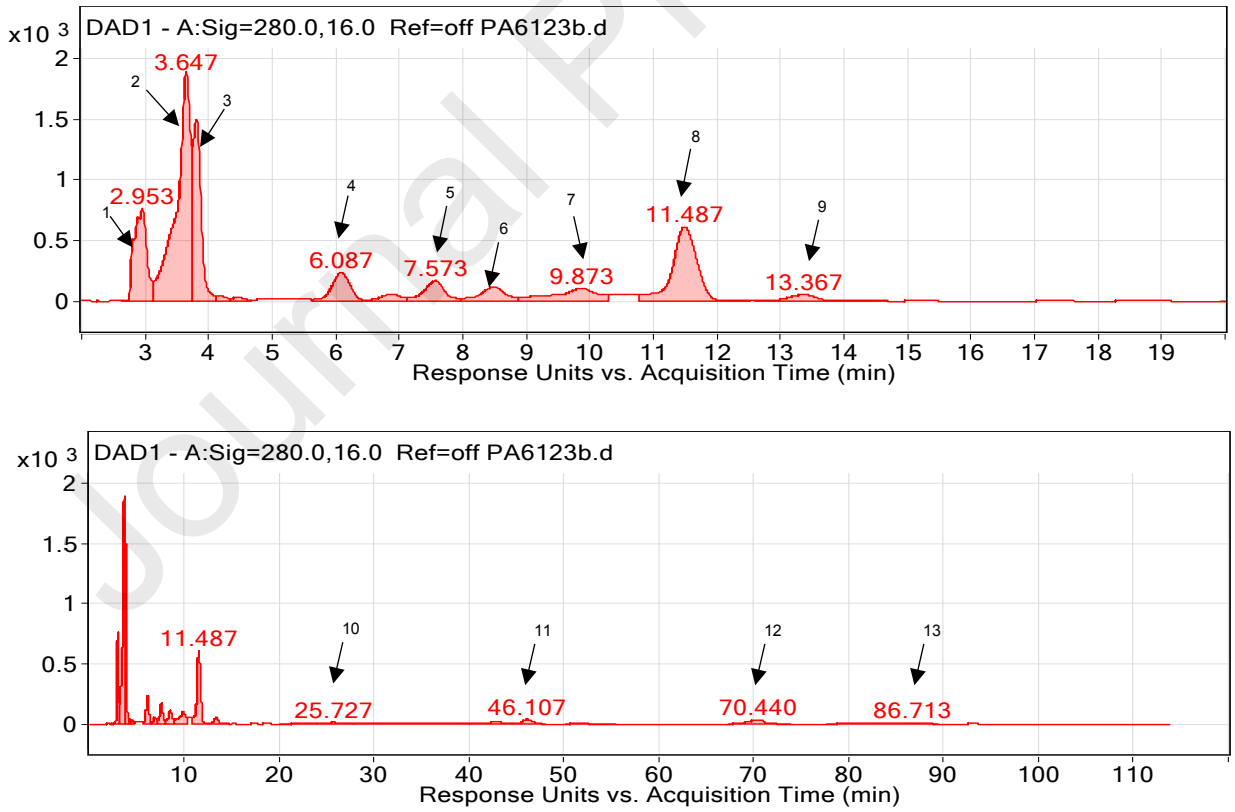
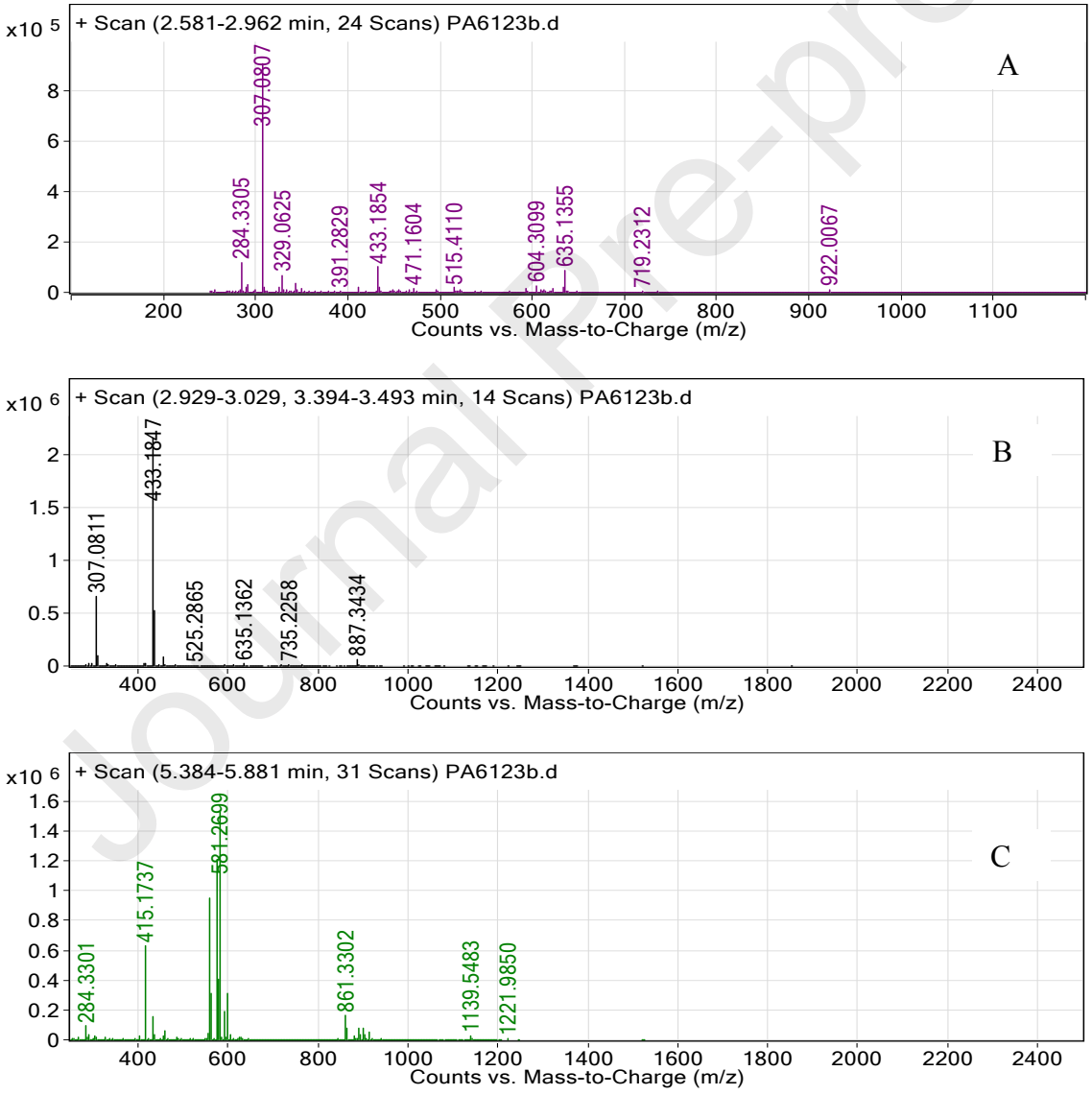


Figure 2a. HPLC chromatogram of EGC-caprylic ester; compounds 1-EGC; 2&3-mono esters; 4,5,6,7,8 & 9-di esters and 10, 11, 12 & 13-tri esters.



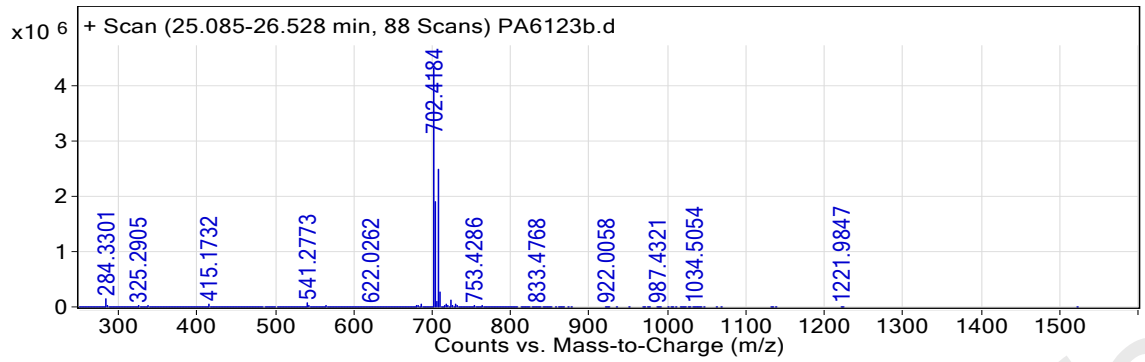


Figure 2b. Mass spectra of EGC-caprylic esters; A-EGC, B-mono ester, C-di ester and D-tri ester

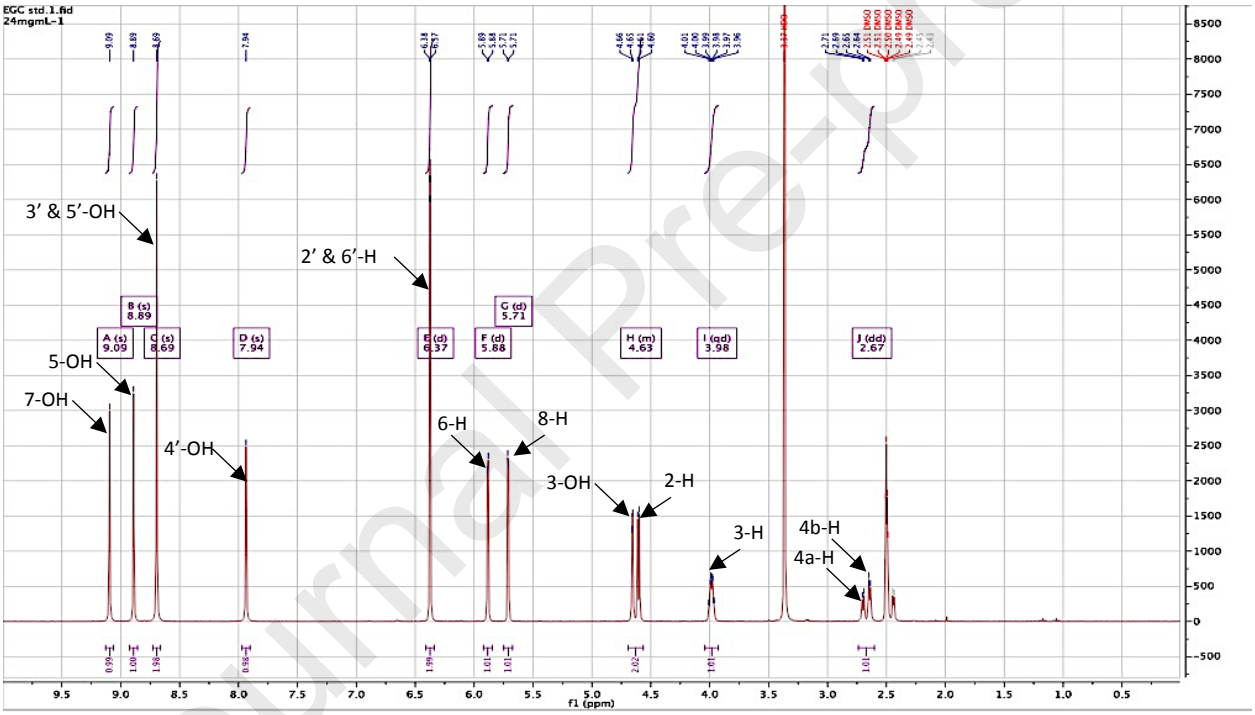
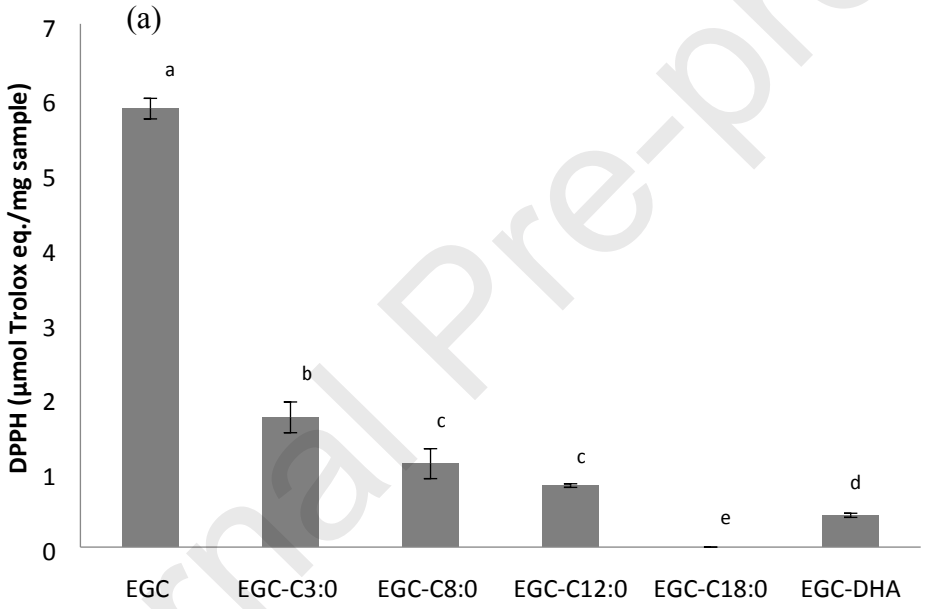


Figure 3. ^{1}H-NMR spectrum of EGC



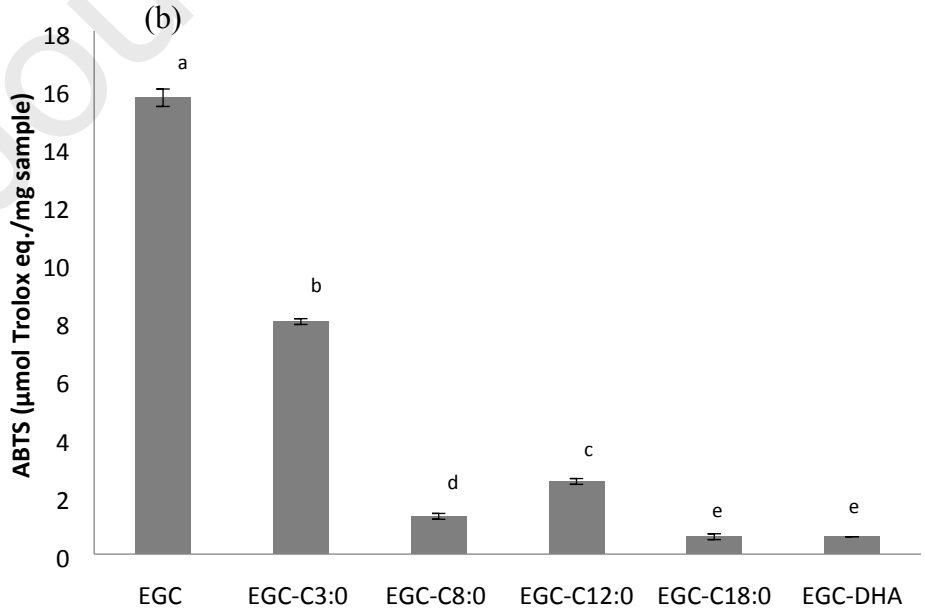


Figure 4. (a) DPPH radical; (b) ABTS radical cation scavenging activities of EGC and its esters

Tables

Table 1. Chemical shifts (δ ppm) of ^{1}H NMR/^{13}C NMR for EGC and EGC-DHA esters*.

		Chemical Shift (δ)							
^{13}C NMR EGC	**C/H position**	**EGC**	**2 (1)**	**3(2)**	**4(3)**	**6(4)**	**7**	**8**	**9**
78.10	**2-H/C**	4.60	4.76	4.76	4.85	4.84	4.95	4.91	4.91
64.96	**3-H/C**	3.98	4.04	4.04	4.04	4.05	4.03	4.00	4.07
28.14	**4a-H/C**	2.64	Overlap	Overlap	Overlap	Overlap	Overlap	Overlap	Overlap
	4b-H	2.70	Overlap	Overlap	Overlap	Overlap	Overlap	Overlap	Overlap
	6-H	5.88	5.90	5.90	5.93	5.91	5.93	5.93	5.93
95.00	**8-H/C**	5.71	5.73	5.73	5.74	5.74	5.77	5.78	5.77
106.02	**2'-H/C**	6.37	6.47	6.47	6.37	6.48	6.45	6.47	6.48
94.04	**6'-H/C**	6.37	6.47	6.52	6.47	6.70	6.45	6.47	6.71
	3-OH	4.66	4.73	4.72		4.86		4.94	4.94
156.49	**5-OH/C**	8.89	9.14	9.12	8.91	9.14	9.02	9.29	
156.49	**7-OH/C**	9.09	9.31	9.29	9.14	9.89	9.37	9.37	9.17
145.34	**3'-OH/C**	8.69	8.93			8.92	8.74		8.94
132.09	**4'-OH/C**	7.94		8.16	8.16			8.14	8.14
145.34	**5'-OH/C**	8.69	8.93	8.91	8.77		8.74		
155.73	**9-C**								
98.54	**10-C**								
129.70	**1'-C**								
106.02	**2'-C**								
106.02	**6'-C**								

*Compounds 2&3-mono esters; 4,6,7, 8 & 9-di esters

Table 2. Lipophilicity of EGC and its mono- and diesters[a]

Compound	Lipophilicity (log *p*)	
EGC	0.71	
	Monoesters	Diesters
EGC-C3:0	1.64	2.62
EGC-C8:0	3.46	6.38
EGC-C12:0	5.25	8.25
EGC-C18:0	7.34	9.91
EGC-DHA	7.34	8.81

[a]The lipophilicity of EGC derivatives was calculated using ALOGPS 2.1. The structures of EGC derivatives in the SMILE system were drawn by ChemDraw Standard 14.0.

Highlights

Acylation of EGC increased its lipophilicity of EGC esters

Mono- and diesters were the predominant acylation products of EGC

EGC and its derivatives effectively scavenged $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals