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**Identification of new diterpene esters from green Arabica coffee beans, and their
platelet aggregation accelerating activities**

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Abstract: Eight new *ent*-kaurane diterpene fatty acid esters, namely caffarolides A–H (1–8), were isolated from green beans of *Coffea arabica*. Their chemical structures were confirmed by extensive spectroscopic analysis including 1D, 2D NMR (HSQC, HMBC, ^1H - ^1H COSY, and ROESY), HRMS, IR and CD spectra and by GC-FID analysis. Interestingly, the diterpene moiety of these new compounds first occurred in genus *Coffea*. All the isolates were evaluated for platelet aggregation activity *in vitro*. As the results, caffarolides C, D and F (3, 4 and 6) showed induction effect for platelet aggregation and the possible structure-activity relationships have been discussed briefly.

Keywords: *Coffea arabica*, coffee lipids, diterpene esters, structure elucidation, platelet aggregation

Abbreviations 1D NMR Proton and carbon nuclear magnetic resonance

2D NMR Two-dimensional nuclear magnetic resonance

HSQC Heteronuclear single quantum coherence

HMBC Heteronuclear multiple bond connectivity

^1H - ^1H COSY ^1H - ^1H Homonuclear chemical shift correlated spectroscopy

ROESY Rotating frame nuclear overhauser and exchange spectroscopy

HRMS High resolution mass spectrometry

IR Infrared spectroscopy

CD Circular dichroism

GC-FID Gas chromatography with flame ionization detector

TLC Thin layer chromatography

P-TLC Preparative thin layer chromatography

CC Column chromatography

MeOH Methanol

PRP Platelet-rich plasma

PPP Platelet-poor plasma

ADP

Adenosine diphosphate

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1. Introduction

Coffea arabica (Arabica coffee) is the most economic importance specie around 120 species of the genus *Coffea* (Rubiaceae), occupying 61% of the world's coffee production (ICO, 2017). Coffee is one of the most consumed beverages worldwide which is prepared by the ripe seeds from the coffee plants. Chemical investigations showed that coffee is rich in bioactive compounds, such as caffeine, trigonelline, chlorogenic acids, phenolic compounds, diterpenes and melanoidins (Ludwig & Crozier, 2014). They showed the activities of neuroprotective (Machado-Filho et al., 2014), anti-oxidant (Moreira & Coimbra, 2012; Zhou & Zeng, 2013), hepatoprotective (Baeza & Bravo, 2015) and anti-cancer (Cavin et al., 2002). Therefore, long-time coffee consumption will improve several related chronic diseases, such as cardiovascular diseases (Miranda & Marchioni, 2017; Ranheim & Halvorsen, 2010), , liver diseases (Muriel & Arauz, 2010; Saab & Tong, 2014), cognition disorders (Carman et al., 2014; Nehlig et al., 2015), cancers (Bohn & Paur, 2014; Vitaglione et al., 2012) and diabetes (Chu et al., 2011; Pan & Ho, 2016).

The chemical constituents of coffee brews are directly impacted by the chemical compositions of green coffee beans, because green coffee beans contain all the active components (or their precursors) that existed in coffee brews. However, the studies that characterized the chemical compositions of green coffee beans mainly focused on water-soluble fraction (Chu et al., 2016; Shu et al., 2014), but a few on the lipid fraction, despite that the lipid content of green Arabica coffee beans reached 15 % (Kurzrock & Speer, 2001).

The main compositions of coffee lipid consist of triacylglycerols, diterpene esters, sterols, sterol esters, and free diterpenes (Durán & Maciel, 2010; Kurzrock & Speer, 2001; Speer et al., 2006). Among them, cafestol and kahweol are the well-known diterpenes in coffee with

cholesterol-rising (Urgert et al., 1995) and anti-cancers (Cavin et al., 2002). However, diterpenes in coffee are rarely present in the form of free (0.4% of the coffee lipid), most of them are esterified with different fatty acids (18% of the coffee lipid) (Kurzrock & Speer, 2001). Until now only a few diterpene esters have been reported, including cafestol, kahweol, 16-*O*-methylcafestol and 16-*O*-methylkahweol with fatty acids C₁₆, C₁₈, C_{18:1}, C_{18:2}, C₂₀, and C₂₂ (Speer et al., 2006). Cafestol and kahweol palmitate are the main two diterpene esters that have been reported to enhance glutathione S-transferase (Huber et al., 2010), inhibit angiogenesis (Moeenfarid & Santos, 2016) and COX-2 (Muhammada et al., 2007) activities. The presence of diterpene esters in coffee brew is influenced by roasting degree and methods of preparation. In general, the content of total diterpene esters in coffee beans decreased with an increase degree of roasting (Kurzrock & Speer, 2001; Speer et al., 2006). Meanwhile, the concentrations of total diterpene esters in different coffee brews were in the order boiled coffee > French press coffee > espresso > filtered coffee and instant coffee due to different contact time, grind degree, filter paper and temperature (Moeenfarid & Alves, 2016; Ratnayake et al., 2003).

Recently, several studies found that coffee extracts can inhibit platelet aggregation *in vitro* (Bydlowski et al., 1987; Naito & Sumi, 2011), which is a key step in the development of thrombosis and other cardiovascular diseases. Further studies demonstrated that these water-soluble compounds like phenolic acids (Natella et al., 2008), pyridinium compounds (Kalaska et al., 2014) may be responsible for the antithrombotic effect of coffee extract, but the effect of coffee lipids on platelet aggregation has been poorly studied.

Therefore, as part of our systematic chemical investigations on *Coffea arabica*, the aims of the study presented here were to isolate and structurally characterize new diterpene esters from

coffee lipid fraction and to evaluate their activities of platelet aggregation.

2. Material and Methods

2.1 General

A Jasco P-1020 polarimeter (Jasco, Japan) was used to obtain optical rotations. Ultraviolet spectra were measured by UV-2401 PC spectrophotometers (Shimadzu, Japan). A Bruker Tensor-27 instrument (Bruker, German) was used for recording infrared spectra by using KBr pellets and HRMS data were measured by an API QSTAR Pulsar spectrometer (Waters, UK). The Bruker AM-400, and DRX-600 instruments (Bruker, Zurich, Switzerland) with transcranial magnetic stimulation (TMS) were used to detect ^1H and ^{13}C NMR spectra. Circular dichroism spectra were scanned by a Chirascan spectropolarimeter (Applied Photophysics, UK). Semi-preparative HPLC was performed on an Agilent HP1100 or 1260 series instrument with a UV L-2400 detector (Agilent, USA) and an ZORBAX SB C-18 column ($5\ \mu\text{m}$, $9.4\ \text{mm} \times 250\ \text{mm}$, wavelength detection at 220 and 290 nm). The analysis of fatty acid methyl esters was accomplished by an Agilent 6890N gas chromatograph (Agilent Technologies, Germany) equipped with FID detector. The green coffee beans were ground into 50-300 mesh powder by YB-2500B grinder (Yunbang, Yongkang, China).

2.2 Chemical and reagents section

TLC detection was performed on TLC plates (200-250 μm thickness, F254 Si gel 60, Qingdao Marine Chemical, Inc., China). The ordinary column chromatographic materials include Lichroprep RP-18 (40-63 μm , Fuji, Japan), Sephadex LH-20 (20-150 μm , Pharmacia, USA) and Silical gel (200-300 mesh, Qingdao Marine Chemical, Inc., China). The industrial-grade methanol, chloroform, ethyl acetate, acetone, petroleum ether were purchased from Tianjing Chemical

Reagents Co. (Tianjing, China). The analytical-grade acetonitrile, tetrahydrofuran, hydrochloric acid, sodium hydroxide, methyl palmitate (16:0), methyl linoleate (18:2), methyl oleate (18:1), methyl nonadecadienoate (19:2), and methyl eicosadienoate (20:2) were purchased from Aladdin Industrial Corporation (Shanghai, China).

2.3 Plant Material.

The green coffee beans of *Coffea arabica* cultivated in Yunnan province (P. R. China), with a subtropical monsoon climate at the location of 22° 68' north altitude and 100.94' east longitude, were harvested in June 2014. The material was authenticated by Ming-Hua Qiu, Kunming Institute of Botany, Chinese Academy of Sciences. A specimen was deposited in State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.4 Extraction and isolation of the lipid fraction of green Arabica coffee beans.

The powder of dried coffee green beans (50-300 mesh, 10 kg) was soaked in acetone for four days at room temperature, then the acetone extract was evaporated under reduced pressure. The residue (963 g) was dissolved with CHCl_3 , filtered with a Buchner funnel and recrystallized for removing caffeine. Then, the CHCl_3 layer (63 g) was subjected to Silica gel column chromatography (CC, 20.0 × 120 cm) and eluted in a step gradient manner with petroleum ether/acetone (20:1, 5:1, 1:1, 0/100, v/v) to yield four fractions (Fr.): Fr. 1 (16 g), Fr. 2 (9 g), Fr. 3 (5 g), and Fr. 4 (30 g).

Fr. 1 (16 g) was then further separated on a RP C-18 (15.0 × 100 cm) column and eluted in a gradient of MeOH/H₂O (55→80%, v/v) to yield three sub-fractions (Fr. 1-1-1-3). Fr. 1-1 (5 g) was subjected to Silica gel CC (5.0 × 70 cm), eluted with a CHCl_3 /MeOH (80:1→20:1, v/v) gradient

system to afford four minor fractions (Fr. 1-1-1-1-4) on the basis of TLC analysis. Fr. 1-1-2 (205 mg) was separated by preparative thin layer chromatography (P-TLC, eluting with $\text{CHCl}_3/\text{MeOH}$, 30:1, v/v) to gain **7** (14 mg).

Fr. 2 (9 g) was applied to Silica gel CC (5.0×100 cm) and eluted in a gradient of $\text{MeOH}/\text{H}_2\text{O}$ (50 \rightarrow 75%, v/v) to yield four fractions (Fr. 2-1-2-4). Fr. 2-1 (3 g) was separated by Silica gel CC (5.0×70 cm, eluted with $\text{CHCl}_3/\text{MeOH}$, 40:1, v/v), to afford five minor fractions (Fr. 2-1-1-2-1-5). Fr. 2-1-2 (330mg) was subjected to chromatography over RP C-18 (2.0×20 cm, eluted with $\text{MeOH}/\text{H}_2\text{O}$, 40-60%, v/v) to obtain **2** (10 mg) and **5** (8 mg). Fr. 2-2 (2 g) was chromatographed on a Silica gel column (5.0×50 cm), eluted with $\text{CHCl}_3/\text{MeOH}$ (20:1, v/v) to yield four minor fractions (Fr. 2-2-1-2-2-4). Then, Fr. 2-2-3 (400mg) was separated by P-TLC, eluted with $\text{CHCl}_3/\text{MeOH}$ (30:1, v/v) to gain minor fractions, then separated by reverse-phase semi-preparative HPLC ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 40 \rightarrow 70%, 30 min, flow rate = 3.0 mL/min, UV 220, 290 nm) to get **3** (11 mg, t_R = 11.5 min), **6** (9 mg, t_R = 14.7 min), and **8** (8 mg, t_R = 23.4 min). Fr. 2-3 (3 g) was separated by use of Sephadex LH-20 (5.0×200 cm, eluted with MeOH, 100%, 2 L) and divided into three fractions (Fr. 2-3-1-2-3-3), then Fr. 2-3-1 (506 mg) was applied to Silica gel CC (2.0×50 cm), eluted with $\text{CHCl}_3/\text{MeOH}$ (10:1, v/v) to afford four minor fractions (Fr. 2-3-1-1-2-3-1-4). Then, Fr. 2-3-1-2 (88 mg) was treated by reverse-phase semi-preparative HPLC ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$: 45 \rightarrow 80%, 35 min, flow rate = 3.0 mL/min, UV 220, 290 nm) to get **1** (20 mg, t_R = 15.5 min), **4** (9 mg, t_R = 21.3 min).

2.4.1. Caffarolide A (**1**)

White amorphous powder, $[\alpha]_D^{25}$ -246.9 (c = 0.1, CHCl_3); UV (CHCl_3) λ_{max} (log ϵ): 239 (1.59), 201(1.04), 193 (1.05) nm; IR (KBr) ν_{max} : 3511, 3422, 3088, 3922, 1767, 1719, 1654, 1453,

1293, 1161, 943, 875 cm^{-1} ; HREIMS m/z 586.4225 $[\text{M}]^+$ (calcd for $\text{C}_{36}\text{H}_{58}\text{O}_6$, 586.4205); 1D NMR data shown in Tables 1 and 2.

2.4.2. Caffarolide B (2)

White amorphous powder, $[\alpha]_{\text{D}}^{25} -164.8$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 239 (1.63), 218 (1.27), 201 (1.21) nm; IR (KBr) ν_{max} : 3440, 2926, 2854, 1736, 1654, 1451, 1239, 1066, 1105, 928, 753 cm^{-1} ; HREIMS m/z 610.4249 $[\text{M}]^+$ (calcd for $\text{C}_{38}\text{H}_{58}\text{O}_6$, 610.4233); 1D NMR data shown in Tables 1 and 2.

2.4.3. Caffarolide C (3)

White amorphous powder, $[\alpha]_{\text{D}}^{25} -149.8$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 239 (2.43), 207(0.93), 200 (0.96) nm; IR (KBr) ν_{max} : 3440, 3423, 2954, 2925, 2853, 1767, 1720, 1629, 1454, 1378, 1342, 1293, 1242, 1162, 1107, 927, 618 cm^{-1} ; HREIMS m/z 612.4384 $[\text{M}]^+$ (calcd for $\text{C}_{38}\text{H}_{60}\text{O}_6$, 612.4390); 1D NMR data shown in Tables 1 and 2.

2.4.4. Caffarolide D (4)

White amorphous powder, $[\alpha]_{\text{D}}^{25} -67.8$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 275 (1.05), 238 (1.12), 217 (0.97) nm; IR (KBr) ν_{max} : 3441, 3424, 2956, 1735, 1630, 1378, 1127, 620 cm^{-1} ; HREIMS m/z 602.4201 $[\text{M}]^+$ (calcd for $\text{C}_{36}\text{H}_{58}\text{O}_7$, 602.4183); 1D NMR data shown in Tables 1 and 2.

2.4.5. Caffarolide E (5)

White amorphous powder, $[\alpha]_{\text{D}}^{25} -155.4$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 239 (1.26), 217 (0.98) nm; IR (KBr) ν_{max} : 3516, 3451, 2919, 2850, 1748, 1650, 1388, 1239, 1160, 928 cm^{-1} ; HREIMS m/z 616.4170 $[\text{M}]^+$ (calcd for $\text{C}_{37}\text{H}_{60}\text{O}_7$, 616.4186); 1D NMR data shown in Tables 2 and 2.

2.4.6. Caffarolide F (6)

White amorphous powder, $[\alpha]_{\text{D}}^{20} -248.0$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 240.4 (2.28) nm; IR (KBr) ν_{max} : 3485, 3398, 2964, 1721, 1651, 1370, 1240, 1032, 826 cm^{-1} ; HRESIMS m/z 677.9326 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{40}\text{H}_{62}\text{O}_7$, 654.4506); 1D NMR data shown in Tables 1 and 2.

2.4.7. Caffarolide G (7)

White amorphous powder, $[\alpha]_{\text{D}}^{25} -138.5$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 270 (2.34), 232 (2.18) nm; IR (KBr) ν_{max} : 3497, 3108, 2922, 1741, 1612, 1390, 1297, 1177, 1089, 983, 873, 566 cm^{-1} ; HREIMS m/z 584.4068 $[\text{M}]^+$ (calcd for $\text{C}_{36}\text{H}_{56}\text{O}_6$, 584.4077); 1D NMR data shown in Tables 1 and 2.

2.4.8. Caffarolide H (8)

White amorphous powder, $[\alpha]_{\text{D}}^{20} -113.5$ ($c = 0.1$, CHCl_3); UV (CHCl_3) λ_{max} ($\log \epsilon$): 269.8 (2.02), 239.2 (1.97) nm; IR (KBr) ν_{max} : 3485, 3398, 2964, 1721, 1651, 1370, 1240, 1032, 826 cm^{-1} ; HRESIMS m/z 659.9114 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{40}\text{H}_{40}\text{O}_6$, 636.4478); 1D NMR data shown in Tables 1 and 2.

2.5 Mild alkaline hydrolysis of compounds 1-8.

Caffarolides A –H (1–8, each for 3.0 mg, respectively) were dissolved in 1 mL of methanol–tetrahydrofuran (1:1, v/v) and treated with 0.1 M NaOH (0.5 mL) at room temperature for 2 h (Tamaki et al., 2008). After that, 0.01 M HCl (~ 5.0 mL) was added to the reaction mixtures for neutralization. In order to obtain the fatty acid esters, the solutions were extracted with ethyl acetate and then evaporated. The residues were purified by Silica gel column chromatography (1.0 × 10 cm) and eluted in a step gradient manner with $\text{CHCl}_3/\text{MeOH}$ (100:1 → 10:1, v/v). The fatty acid methyl esters were obtained at 80:1 ($\text{CHCl}_3/\text{MeOH}$, v/v) fractions.

2.6 GC comparison analysis for the fatty acid of compounds 1-8.

HP-5 capillary column (30 m × 0.53 mm, 1.0 μm) was used in GC. Column temperature was held initially at 60 °C for 1 min, increased by 10 °C/min to 150 °C and held for 5 min, then by 5 °C/min to 250 °C, then by 20 °C/min to 300 °C and held for 7 min. The carrier gas is helium with the flow rate of 1.1 mL/min and the inlet temperature was set at 200 °C. Then, compared the retention time of the fatty acid methyl esters with those of standards, the GC analysis confirmed that **1, 4, 5, 7** esterified with palmitic acid (16:0, t_R = 23.5 min) and **2, 3, 6, 8** were esterified with linoleic acid (18:2, t_R = 29.8 min), oleic acid (18:1, t_R = 27.1 min), nonadecadienoic acid (19:2, t_R = 30.2 min), eicosadienoic acid (20:2, t_R = 31.7 min), respectively (Zhao et al., 2014).

2.7 Platelet Aggregation Assays.

2.7.1. Preparation of platelets

blood samples from the rabbit ear central artery (Japanese big ear rabbits, Kunming Chu Shang technology co. Ltd., China) were collected into plastic tubes, anticoagulated with 3.8 % sodium citrate acid (9:1, v/v). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifuging the blood at 200 × g (10 min) and 2400 × g (20 min), respectively. Then, cell counter was adjusted to $5 \times 10^8 \text{ mL}^{-1}$ before the measurement (Shen et al., 2003).

2.7.2. Platelet aggregation in vitro

platelet aggregation in PRP was measured as by turbidimetry method (Born, 1964). Firstly, ten disposable cuvettes were prepared. 250 μL PRP and 2.5 μL DMSO were added to each cuvette, similarly, another cuvette with 250 μL PPP and 2.5 μL DMSO was prepared to adjust baseline of aggregometer. Then, all the cuvettes incubated at 37 °C for 5 min. After incubation, eight new compounds (each $3 \times 10^{-4} \text{ g/mL}$) and positive control ADP ($4.3 \times 10^{-6} \text{ g/mL}$, Chrono-log

Corporation, USA) were added to PRP cuvettes, respectively. Finally, the maximal aggregation was monitored by a chrono-log 700 aggregometer (Chrono-log Corporation, USA).

3. Every sample was analyzed five times, and data are reported as the mean \pm standard deviation expressed as platelet maximum aggregation rate (%). *P* values less than 0.05 were considered significant, less than 0.01 highly significant, and less than 0.001 extremely significant.

Result and discussion

3.1 Phytochemical investigation

Caffarolide A (**1**) was isolated as white amorphous powder, whose molecular formula was determined to be $C_{36}H_{58}O_6$ from the $[M]^+$ ion peaks at m/z 586.4225 (calcd for $C_{36}H_{58}O_6$, 586.4205) in the HREIMS. The IR spectrum indicated that **1** possessed hydroxyl (3422 cm^{-1}), and α,β -unsaturated lactone (1767 cm^{-1}) groups. The $^1\text{H-NMR}$ (Table 1) spectrum showed the presence of one singlet methyl (δ_{H} 0.75, s, $\text{H}_3\text{-20}$), one triplet methyl (δ_{H} 0.82, t, $J = 6.8\text{ Hz}$, $\text{H}_3\text{-16'}$), one oxymethylene [δ_{H} 4.52 (d, $J = 11.2\text{ Hz}$, H-17a), 4.68 (d, $J = 11.3\text{ Hz}$, H-17b)], one olefinic methine (δ_{H} 5.80, s, H-18), and huge methylene proton signals at δ_{H} 1.20-1.25 (m, H-2'-15'). The $^{13}\text{C-DEPT}$ spectra (Table 2) displayed a total of 36 carbon resonances. Except for the characteristic huge methylenes, one methyl and one ester carbonyl signals for fatty acid, the remaining resonances were assigned to be one methyl, eight methylenes (one oxygenated), four methines (one olefinic), and seven quaternary carbons (including one olefinic, one carbonyl and two oxygenated). The aforementioned information indicated that compound **1** could be a diterpene ester and the diterpene moiety was exactly similar with tricalysiolide B (Nishimura et al., 2006), which was confirmed by the HMBC correlations of δ_{H} 5.80 (H-18) with δ_{C} 171.4 (C-19), δ_{C} 173.6 (C-4), δ_{C} 105.5 (C-3) and δ_{C} 47.3 (C-5), of δ_{H} 4.68 ($\text{H}_2\text{-17}$) with δ_{C} 79.3 (C-16), δ_{C} 46.3 (C-13)

and δ_C 53.9 (C-15) (Figure 2). Moreover, on the basis of the molecular weight, the fatty acid was determined to be palmitic acid (16:0), which was further confirmed by the alkaline hydrolysis, then, comparing to the retention time of standard methyl palmitate in GC analysis (t_R = 23.5 min) (Novaes, & Neto, 2015). Additionally, the esterification position was located at C-17, which was proved by the key HMBC correlation of δ_H 4.68 and 4.52 (H₂-17) with δ_C 173.6 (C-1') and δ_C 79.3 (C-16) (Figure 2).

The relative configuration of **1** was established by ROESY spectrum as shown in Figure 2. With the solvent of DMSO instead of C₅N₅D (Supporting Information), the ROESY correlations of H-13/H₂-17 and OH-3/H-5 assigned OH-16 and OH-3 as α -orientation and β -orientation, respectively. The absolute configuration at C-3 was established by the CD spectrum (a π - π^* transition in the α,β -unsaturated- γ -lactone moiety). The observed negative Cotton effect at 225 nm revealed the 3R configuration (Supporting Information), which was supporting by literatures (Shen et al., 2015; Yin, Luo & Kong, 2013). Finally, the structure of **1** was established as caffarolide A.

Caffarolide B (**2**) was obtained as white amorphous powder. The molecular formula C₃₈H₅₈O₆ was deduced from the molecular ion peak at m/z [M]⁺ 610.4249 (calcd for C₃₈H₅₈O₆, 610.4233) in HREIMS. The NMR spectra of **2** were similar to those of **1** implying that **2** has the same structure as **1** containing a diterpenoid moiety and a fatty acid fraction. Comparison of the ¹³C-DEPT spectra between **2** and **1** showed that the diterpenoid moiety of **2** was also tricalysiolide B (Nishimura et al., 2006). Similarly, the molecular weight of **2** suggested the presence of 18-carbon fatty acid in **2**. Furthermore, four additional sp^2 methine carbons (δ_C 128.0-130.5) in **2** combined with the HMBC correlations of these aromatic signals (δ_H 5.35, m) with huge sp^3

methylenes (δ_C 22.0-30.0) of fatty acid (Figure 2), together with the 1H - 1H COSY correlations of H-10'/H-11'/H-12', indicated that the fatty acid of **2** could be linoleic acid (18:2). Subsequently, the hydrolysis experiment and GC comparison unambiguously determined the fatty acid to be linoleic acid (18:2, t_R = 29.8 min). In the HMBC experiment, δ_H 4.22 and 4.27 (H₂-17) showed a correlation with δ_C 174.3 (C-1') demonstrated the esterification position of **2** was at C-17. The relative configuration of **2** was the same as that of **1** by the ROESY and CD spectra. Then, the structure of **2** was established and named as caffarolide B.

The HREIMS data for caffarolide C (**3**) indicated a molecular formula of C₃₈H₆₀O₆, with two unites more than **2**. Detailed analysis of the 1D NMR data of **3** and **2** revealed that **3** has the same basic diterpene structure as **2**, expect that the fatty acid in **3** possessed only one double bond. We thus deduced that the fatty acid of **3** could be oleic acid (18:1). The futher evidence was established by the HMBC correlation of the H₂- sp^2 methine signals (δ_H 5.34, m) with huge sp^3 methylenes (δ_C 22.5-31.9) of fatty acid, along with GC comparison analysis (t_R = 27.1 min). Moreover, the esterification location of **3** was at C-17, according to the key HMBC correlation of with C-1'. Its relative configuration was determined to be the same as that of **1** from the ROESY and CD spectra. Therefore, the structure of **3** was determined.

On the basis of HREIMS spectrum, caffarolide D (**4**) was assigned a molecular formula of C₃₆H₅₈O₇. The 1D NMR data of **4** were similar to those of **1** with a palmitoyl group, except that the methylene at C-1 in **1** was replaced by an oxymethine in **4**. The observed HMBC correlations of the singlet methyl δ_H 0.88 (H₃-20), the methylene proton δ_H 2.37 (H-2a) and methine proton δ_H 2.06 (H-9) with the oxygenated methine δ_C 71.1, of oxymethine δ_H 3.84 (H-1) with C-2 (δ_C 40.9) and C-3 (δ_C 107.6), together with the 1H - 1H COSY correlation of H-1/H-2a indicated that a

hydroxyl was located at C-1 (Figure 3). Herein, the planar structure of the cafestol-type lactone moiety in **4** was established. Meanwhile, combined the results of the molecular weight, hydrolysis experiment and GC comparison analysis, the fatty acid of **4** was determined to be palmitic acid (16:0). Furthermore, the esterification position of **4** was located at C-17, which was proved by the key HMBC correlations of δ_{H} 4.22 and 4.28 (H_{2-17}) with δ_{C} 176.0 (C-1'). Its relative configuration was determined to be the same as that of **1**, and the OH-1 group was assigned as β -oriented as indicated by the ROESY correlation of H-1/H-20 (Figure 3). Finally, the structure of **4** was elucidated as shown.

Caffarolide E (**5**) was assigned a molecular formula of $\text{C}_{37}\text{H}_{60}\text{O}_7$. Compared the 1D NMR data of **5** with those of **4**, showed that a methoxy (δ_{C} 58.9) at C-1 in **5** replaced the hydroxyl in **4**. The deduction was proved by the key HMBC correlations of the methyl δ_{H} 0.86 (H_{3-20}), the methylene proton δ_{H} 2.55 (H_{2a}) and methine proton δ_{H} 2.01 (H_9) with the oxygenated methine δ_{C} 80.4, of oxymethine δ_{H} 3.43 (H_1) with C-2 (δ_{C} 35.3) and C-3 (δ_{C} 102.9). The fatty acid of **5** was assigned as palmitic acid (16:0) by the molecular weight and GC comparison analysis (t_{R} = 23.5 min). The esterification location of **5** was also at C-17, which was confirmed by the HMBC spectrum. In the ROESY spectrum of **5**, the correlations of H-1/H-20 assigned OMe-1 as β -orientation. Therefore, the structure of compound **5** was elucidated as shown.

Caffarolide F (**6**) was assigned a molecular formula as $\text{C}_{40}\text{H}_{62}\text{O}_7$ from its HRESIMS spectrum. Analyzed the 1D NMR data of **6** and **5** indicated that **6** has the same diterpene lactone moiety as **5**, but differing at the fatty acid moiety. Four additional sp^2 methine singals were also observed in the downfield region of **6**. Thus, based on the molecular weight, we deduced that the fatty acid of **6** could be nonadecadienoic acid (19:2). The HMBC correlations of these $\text{H}_4\text{-}sp^2$ methine signals (δ_{H}

5.34) with huge sp^3 methylenes (δ_C 22.7-29.9), together with GC comparison analysis (t_R = 30.2 min) proved the hypothesis. Moreover, H₂-17 showed the HMBC correlation with C-1', demonstrated that the esterification location of **6** was at C-17. The relative configurations of **6** was determined by the ROESY spectrum, which indicated that OMe-1 were β -orientation. Thus, we deduced the structure of compound **6** as caffarolide F.

For support of the absolute configurations assigned for compounds **2–6**, the CD spectra were measured. The CD spectra obtained were in close agreement with that of **1** at 225 nm, thus indicating that the absolute configuration of C-3 in **2–6** is R, the same as that of **1**.

The HREIMS data for caffarolide G (**7**) indicated a molecular formula of C₃₈H₅₆O₆. The 1D NMR data of **7** were generally similar to those of **4**, but an additional double bond was presented at the diterpene lactone moiety in **7**. The HMBC correlation of H-2 (δ 5.72, dd, J = 6.0 and 1.5 Hz) with C-1 (δ_C 69.5), C-3 (δ_C 152.0), C-4 (δ_C 159.1), of H-18 (δ_H 5.76) with C-3 (δ_C 152.0) and C-19 (δ_C 170.0), of H-1 (δ_H 4.20, d, J = 6.0 Hz) with C-20 (δ_C 16.0), C-2 (δ_C 107.1) and C-3 (δ_C 152.0), together with the 1H - 1H COSY correlations H-1/H-2 (Figure 3), demonstrated the double bond was located at C-2 and C-3. Hereto, the planar structure of the diterpene moiety in **7** was determined as tricalysiolide F (Nishimura et al., 2006). Similarly, the fatty acid of **7** was assigned as palmitic acid (16:0) by molecular weight and GC comparison analysis (t_R = 23.5 min). C-17 was confirmed as the esterification location of **7** by the HMBC spectrum. The key ROESY correlations of H-1/H-20 and H-13/H₂-17 were assigned OH-1 and OH-16 as β -orientation and α -orientation, respectively (Figure 3). Therefore, the structure of **7** was determined as caffarolide G.

The molecular formula of caffarolide H (**8**) was assigned to be C₄₀H₄₀O₆. Detailed

comparison of 1D NMR data of **8** and **7** showed that the only difference between them was fatty acid fraction. On the basis of the molecular weight, the fatty acid contains twenty carbons. Meanwhile, four sp^2 methine singals were also observed in the downfield region of **8**. Therefore, we assumed the fatty acid of **8** could be eicosadienoic acid (20:2), which was proved by hydrolysis experiment and GC comparison method ($t_R = 31.7$ min). The esterification location of **8** was also at C-17 which was established by the HMBC spectrum. Based on the ROESY spectrum, OH-1 and OH-16 were assigned as β -orientation and α -orientation, respectively. Thus, we deduced the structure of compound **8** as caffarolide H.

3.2 Platelet Aggregation Assay.

The activity of caffarolides A–H (**1–8**) inducing platelet aggregation *in vitro* were evaluated by turbidimetry method, and the results are showed in Table 3. Compared to control (DMSO, 3.4 ± 1.1 %), compounds **3**, **4** and **6** showed platelet aggregation activity at the concentration of 3×10^{-4} g/mL, with induction rate of 11.4 ± 5.5 , 15.8 ± 5.6 and 7.8 ± 3.3 %, respectively.

From these results, some primary structure-activity relationships can be deduced: the hemiketal at C-3, the substituent groups at C-1 and the types of fatty acids can impact the platelet aggregation effect. If the hemiketal at C-3 in compound **4** was replaced by the enol group in compound **7**, no platelet aggregation effect was shown. When the unsaturated fatty acids were linked to the hydroxyl at C-17, compounds **3** and **6** showed platelet induction activity, whereas, the substitute of saturated fatty acids at C-17, compounds **1** and **5** didn't show any activity. Moreover, the hydroxyl and methoxyl attached to C-1 enhanced the platelet induction activity (**4** vs. **5** vs. **1**).

4. Conclusions

In present study, a systematic chemical research was performed and resulted in the separation of eight new diterpenes esters caffarolides A-H (1–8) from the lipid of green Arabica coffee beans. The structures of these new compounds were identified based on detailed spectroscopic analysis (NMR, HSMS, IR and CD spectra) and GC-FID analysis. Caffarolides C, D and F (3, 4 and 6) showed induction effect for platelet aggregation at 3×10^{-4} g/mL. The results obtained in this study complement the current knowledge of diterpene esters in *Coffea arabica*.

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Notes

The authors declare no competing financial interest.

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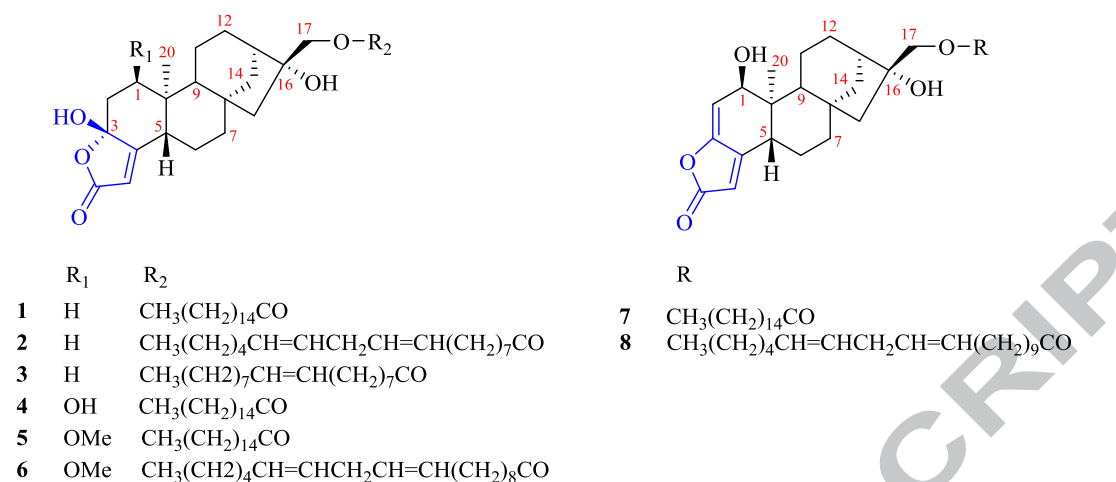


Figure 1. Structures of diterpene esters isolated from green Arabica coffee beans.

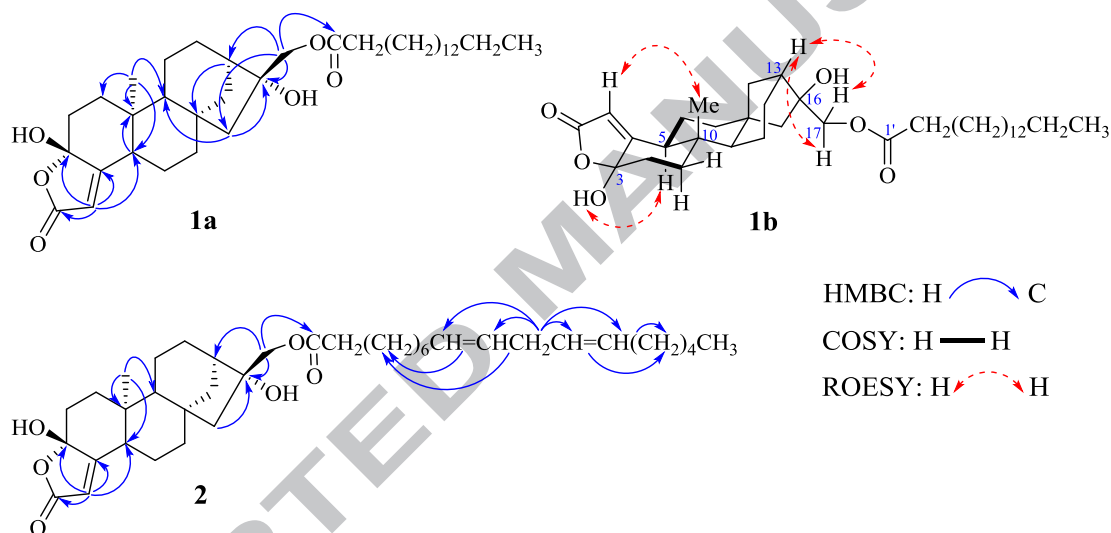


Figure 2. Key HMBC (H \rightarrow C) for **1a** and **2**, ¹H-¹H COSY (—), ROESY (H \leftrightarrow H) correlations for **1b**.

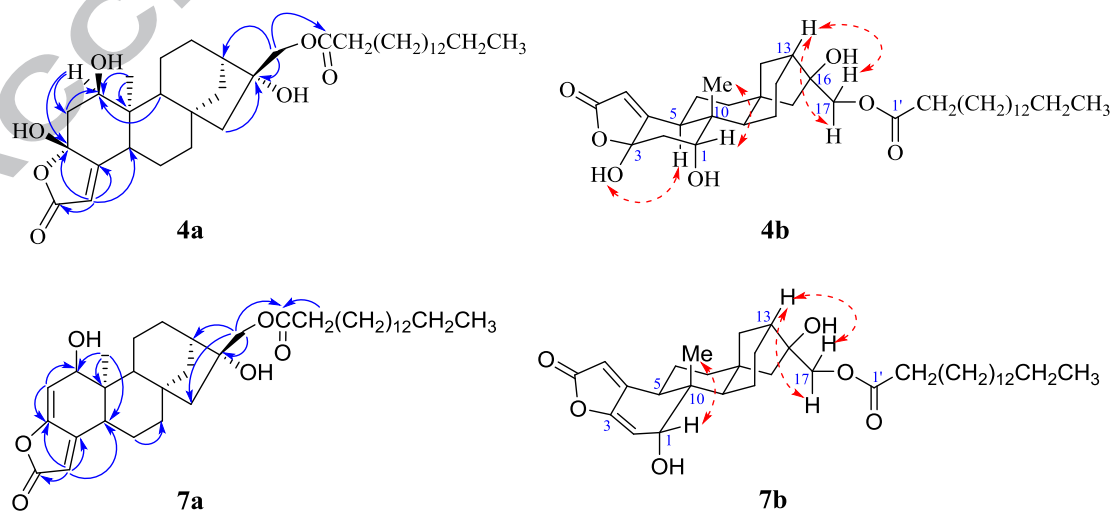


Figure 3. Key HMBC (H $\xrightarrow{\text{blue}}$ C) for **4a** and **7a**, ROESY (H $\xleftrightarrow{\text{red}}$ H) correlations for **4b** and **7b**.

Table 1. ¹H-NMR Data of Compounds **1-8**^a

Position	1 ^b	1 ^c	2 ^d	3 ^d	4 ^e	5 ^d	6 ^d	7 ^d	8 ^d
1	1.50 (m)	1.20 (m)	1.50 (m)	1.32 (m)	3.84 (t, 3.0)	3.43 s-like	3.44 s-like	4.20 (t, 6.0)	4.20 (t, 6.0)
	1.77 (m)	1.80 (m)	1.58 (m)	1.88 (m)	-	-	-	-	-
2	2.62 (dd, 20.1, 7.6)	2.35 (t, 7.3)	2.36 (ddd, 14.0, 14.0, 3.0)	2.34 (ddd, 14.0, 14.0, 3.0)	2.37 (m)	2.55 (m)	2.55 (m)	5.72 (dd, 6.0, 1.5)	5.72 (dd, 6.0, 1.5)
	1.91 (m)	2.17 (m)	1.88 (m)	1.86 (m)	1.94 (m)	2.02 (m)	1.98 (m)	-	-
5	2.56 (d, 9.0)	2.26 (d, 10.7)	2.34 (m)	2.36 (m)	2.76 (m)	2.67 (m)	2.66 (m)	2.66 (m)	2.69 (m)
6	1.38 (m)	1.56 (m)	1.49 (m)	1.54 (m)	1.75 (m)	1.63 (m)	1.64 (m)	1.57 (m)	1.58 (m)
	1.53 (m)	1.65 (m)	1.64 (m)	1.67 (m)	1.86 (m)	1.76 (m)	1.76 (m)	1.75 (m)	1.74 (m)
7	1.54 (m)	1.30 (m)	1.72 (m)	1.64 (m)	1.62 (m)	1.60 (m)	1.61 (m)	1.64 (m)	1.60 (m)
	1.61 (m)	1.61 (m)	1.83 (m)	1.86 (m)	2.23 (m)	1.75 (m)	1.77 (m)	1.88 (m)	1.87 (m)
9	1.25 (m)	1.30 (m)	1.29 (m)	1.28 (m)	2.06 (m)	2.01 (m)	2.02 (m)	2.08 (m)	2.08 (m)
11	1.45 (m)	1.60 (m)	1.65 (m)	1.69 (m)	1.72 (m)	1.53 (m)	1.54 (m)	1.54 (m)	1.47 (m)
	1.91 (m)	1.74 (m)	1.91 (m)	1.94 (m)	1.94 (m)	1.68 (m)	1.78 (m)	1.90 (m)	1.95 (m)
12	1.50 (m)	1.50 (m)	1.64 (m)	1.53 (m)	1.62 (m)	1.64 (m)	1.54 (m)	1.63 (m)	1.62 (m)
	1.68 (m)	1.59 (m)	1.67 (m)	1.58 (m)	1.66 (m)	1.66 (m)	1.59 (m)	1.66 (m)	1.64 (m)
13	2.52 (s)	2.03 (s)	2.34 (m)	2.08 (s)	2.11 (m)	2.18 (s)	2.18 (s)	1.91 (m)	1.89 (m)
14	1.96 (m)	1.67 (m)	1.74 (m)	1.74 (m)	1.72 (m)	1.68 (m)	1.77 (m)	1.69 (m)	1.68 (m)
	2.05 (m)	1.88 (m)	1.96 (m)	1.96 (m)	2.00 (m)	1.96 (m)	2.00 (m)	1.86 (m)	1.86 (m)
15	1.65 (m)	1.45 (m)	1.50 (m)	1.58 (m)	1.55 (m)	1.62 (m)	1.62 (m)	1.57 (m)	1.57 (m)
	1.77 (m)	1.68 (m)	1.70 (m)	1.74 (m)	1.77 (m)	1.76 (m)	1.72 (m)	1.75 (m)	1.77 (m)

17	4.52 (d, 11.3)	4.13 (d, 11.4)	4.23 (d, 13.0)	4.23 (d, 13.0)	4.22 (d, 13.0)	4.22 (d, 13.0)	4.23 (d, 13.0)	4.16 (d, 13.0)	4.16 (d,13.0)
	4.68 (d, 11.2)	4.22 (d, 11.3)	4.26 (d, 13.0)	4.26 (d, 13.0)	4.28 (d, 13.0)	4.30 (d, 13.0)	4.30 (d, 13.0)	4.18 (d, 13.0)	4.18 (d, 13.0)
18	5.80 (s)	5.78 (s)	5.62 (s)	5.63 (s)	5.72 (s)	5.63 (s)	5.63 (s)	5.76 (s)	5.76 (s)
20	0.75 (s)	0.83 (s)	0.85 (s)	0.86 (s)	0.88 (s)	0.86 (s)	0.86 (s)	0.88 (s)	0.88 (s)
OH-3	-	7.31 (s)	-	-	-	-	-	-	-
OH-16	-	4.43 (s)	-	-	-	-	-	-	-
OMe-1	-	-	-	-	-	3.43 (overlapped)	3.44 (s)	-	-
2'-8'			1.28 (m)	1.28 (m)			1.30 (m)		1.20 (m)
9'			5.35 (m)	5.35 (m)					
10'							5.34 (m)		
11	1.20-1.25	1.21-1.30	2.77 (m)		1.30 (m)	1.28 (m)		1.20 (m)	5.33 (m)
12'	(m)	(m)	5.35 (m)				2.76 (m)		
13'							5.34 (m)		2.77 (m)
14'				1.28 (m)					5.33 (m)
15'									
16'	0.82 (t, 6.8)	0.91 (t, 6.6)	1.28 (m)		0.91 (t, 7.0)	0.86 (t, 7.0)	1.30 (m)	0.78 (t, 7.0)	
17'	-	-			-	-		-	1.20 (m)
18'	-	-	0.89 (t, 7.0)	0.89 (t, 7.0)	-	-		-	
19'	-	-	-	-	-	-	0.90 (t, 7.0)	-	
20'	-	-	-	-	-	-	-	-	0.78 (t, 7.0)

^aδ in parts per million, *J* in Hz, and obtained at 600 Hz. NMR solvent was ^bC₅D₅N, ^cDMSO, ^dCDCl₃, ^eMeOD.

Table 2. ¹³C-NMR and DEPT Data of Compounds **1-8**^a

Position	1 ^b	1 ^c	2 ^d	3 ^d	4 ^e	5 ^d	6 ^d	7 ^d	8 ^d
1	35.0 (t)	35.1 (t)	35.6 (t)	35.4 (t)	71.1 (d)	80.4 (d)	80.5 (d)	69.6 (d)	69.6 (d)
2	34.5 (t)	33.8 (t)	34.5 (t)	34.3 (t)	40.9 (t)	35.3 (t)	35.3 (t)	107.1 (d)	106.9 (d)
3	105.5 (s)	104.5 (s)	104.3 (s)	104.1 (s)	107.6 (s)	102.9 (s)	102.8 (s)	152.0 (s)	152.1 (s)
4	173.6 (s)	172.5 (s)	172.4 (s)	172.1 (s)	174.5 (s)	172.7 (s)	172.6 (s)	159.1 (s)	159.0 (s)
5	47.3 (d)	46.4 (d)	47.0 (d)	46.9 (d)	41.3 (d)	40.2 (d)	40.3 (d)	38.7 (d)	38.7 (d)
6	22.0 (t)	21.4 (t)	21.8 (t)	21.5 (t)	22.3 (t)	21.8 (t)	21.6 (t)	21.7 (t)	21.8 (t)
7	40.2 (t)	39.5 (overlapped)	39.5 (t)	39.4 (t)	39.7 (t)	39.0 (t)	39.0 (t)	39.0 (t)	39.0 (t)
8	44.7 (s)	43.9 (s)	44.7 (s)	44.5 (s)	45.6 (s)	44.4 (s)	44.5 (s)	44.3 (s)	44.3 (s)
9	53.6 (d)	52.7 (s)	53.3 (d)	53.2 (d)	45.3 (d)	43.6 (d)	43.6 (d)	46.1 (d)	46.1 (d)
10	43.7 (s)	42.9 (s)	43.7 (s)	43.5 (s)	43.7 (s)	47.5 (s)	47.6 (s)	44.0 (s)	44.0 (s)
11	19.3 (t)	18.6 (t)	19.3 (t)	19.2 (t)	19.5 (t)	18.9 (t)	19.0 (t)	17.8 (t)	19.3 (t)
12	26.3 (t)	25.6 (t)	27.4 (t)	27.2 (t)	27.0 (t)	27.3 (t)	27.4 (t)	25.9 (t)	25.9 (t)
13	46.3 (d)	44.8 (d)	46.0 (d)	45.8 (d)	46.8 (d)	45.9 (d)	45.9 (d)	43.9 (d)	43.9(d)
14	37.9 (t)	37.1 (t)	37.6 (t)	37.5 (t)	38.3 (t)	37.5 (t)	37.6 (t)	37.1 (t)	37.1 (t)
15	53.9 (t)	52.8 (t)	52.9 (t)	52.7 (t)	54.3 (t)	53.3 (t)	53.3 (t)	53.0 (t)	53.0 (t)
16	79.3 (s)	78.2 (s)	80.2 (s)	80.0 (s)	80.8 (s)	80.0 (s)	80.0 (s)	80.1 (s)	80.1 (s)
17	69.0 (t)	67.1(t)	68.3 (t)	68.1 (t)	69.3 (t)	68.3 (t)	68.3 (t)	68.3 (t)	68.3 (t)
18	112.5 (d)	111.9 (d)	112.9 (d)	112.8 (d)	113.7 (d)	112.4 (d)	112.4 (d)	112.3 (d)	112.4 (d)
19	171.4 (s)	170.6 (s)	171.1 (s)	170.7 (s)	173.2 (s)	170.8 (s)	170.7 (s)	170.0 (s)	169.8 (s)
20	14.4 (q)	14.0 (q)	14.4 (q)	14.4 (q)	16.0 (q)	15.5 (q)	15.5 (q)	16.0 (q)	16.0 (q)
OMe-1						58.9 (s)	59.0 (s)		
1'	174.0 (s)	173.2 (s)	174.3 (s)	174.1 (s)	176.0 (s)	174.3 (s)	174.2 (s)	174.3 (s)	174.2 (s)

2'	32.1 (t)	31.2 (t)	34.2 (t)	34.0 (t)	31.7 (t)	32.1 (t)	31.7 (t)	34.5 (t)	31.7 (t)
3'-8'			22.8-31.7 (t)	22.5-31.9 (t)			22.7-29.9 (t)		22.8-29.9 (t)
9'			128.0-130.5	129.7-130.					
10'			(d)	1 (d)			128.0-130.4		
11'	22.9-32.1 (t)	22.4-31.2 (t)	27.4	27.3	24.5-31.0 (t)	22.9-29.9 (t)	(d)	22.9-32.1 (t)	128.0-130.4 (d)
12'			128.0-130.5				27.2		
13'			(d)				128.0-130.4		27.4
14'				22.5-31.9 (t)			(d)		128.0-130.4 (d)
15'			22.8-31.7 (t)						
16'	14.3 (q)	13.9 (q)			14.6 (q)	14.2 (q)	22.7-29.9 (t)	14.4 (q)	
17'									22.8-29.9 (t)
18'			14.2 (q)	14.3 (q)					
19'							14.6 (q)		
20'									14.2 (q)

^a δ in parts per million, J in Hz, and obtained at 600 Hz. NMR solvent was ^bC₅D₅N, ^cDMSO, ^dCDCl₃, ^eMeOD.

Table 3. Platelet Aggregation Data of Compounds **1-8**

Compound	Final concentration	Platelet maximum aggregation rate (%)
DMSO (Con.)	1% (v/v)	3.4 ± 1.1
ADP (Pos.)	4.3 × 10 ⁻⁶ g/mL	53.6 ± 5.6 ^{***}
1	3 × 10 ⁻⁴ g/mL	2.0 ± 1.4
2	3 × 10 ⁻⁴ g/mL	6.8 ± 5.3
3	3 × 10 ⁻⁴ g/mL	11.4 ± 5.5 [*]
4	3 × 10 ⁻⁴ g/mL	15.8 ± 5.6 ^{**}
5	3 × 10 ⁻⁴ g/mL	6.0 ± 3.7
6	3 × 10 ⁻⁴ g/mL	7.8 ± 3.3 [*]
7	3 × 10 ⁻⁴ g/mL	2.4 ± 0.9
8	3 × 10 ⁻⁴ g/mL	2.6 ± 0.9

Results are presented as means ± standard deviation of quintuple measurements. Different symbols in rows represent significant differences at ^{*}*P* < 0.05, ^{**}*P* < 0.01, ^{***}*P* < 0.001.

ACCEPTED MANUSCRIPT

The highlights of present study have been refined as follow:

Highlights:

1. Eight new diterpene esters were isolated from the lipid fraction of green Arabica coffee beans.
2. The qualitative analysis of new compounds was based on NMR spectra and GC-FID data.
3. The diterpene moiety of new compounds possessing an additional γ -lactone moiety fusing with ring A was first identified from genus *Coffea*.
4. Caffarolides C, D and F showed platelet aggregation activity *in vitro* with induction rate of 11.4 ± 5.5 , 15.8 ± 5.6 and 7.8 ± 3.3 %, compare to control, respectively.