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Triterpene hexahydroxydiphenoyl esters and a quinic acid purpurogallin carbonyl ester from the leaves of *Castanopsis fissa*

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Triterpene hexahydroxydiphenoyl (HHDP) esters have only been isolated from *Castanopsis* species, and the distribution of these esters in nature is of chemotaxonomical interest. In this study, the chemical constituents of the leaves of *Castanopsis fissa* were examined in detail to identify and isolate potential HHDP esters. Together with 53 known compounds, 3, 4-di-*O*-galloyl-1-*O*-purpurogallin carbonyl quinic acid (**1**) and 3, 24-(*S*)-HHDP-2α, 3β, 23, 24-tetrahydroxytaraxastan-28, 20β-olide (**2**) were isolated and their structures were elucidated by spectroscopic and chemical methods. The polyphenols of the leaves were mainly composed of galloyl quinic acids, triterpenes HHDP esters, ellagitannins and flavonol glycosides. In particular, the isolation yields of 1,3,4-trigalloyl quinic acid and the new compound **2** were 1.53 and 0.27%, respectively, from the fresh leaves. The presence of lipid soluble HHDP esters of oleanane-type triterpenes as one of the major metabolites is an important chemotaxonomical discovery. Lipase inhibition activities and ORAC values of the major constituents were compared. The new triterpene HHDP ester showed moderate lipase inhibition activity and myricitrin gave the largest ORAC value.

Keywords: Castanopsis fissa; Fagaceae; triterpene; ellagitannin; galloyl quinic acid; purpurogallin; benzotropolone

1. Introduction

Ellagitannins are a group of plant polyphenols with various biological activities (Quideau, 2009). The molecules are basically esters of hexahydroxydiphenoyl (HHDP) and related acyl groups with polyalcohols, typically D-glucose (Nonaka, 1989; Okuda et al., 1989). The structural variation in the polyalcohol cores and acyl groups is important from the viewpoint of plant chemotaxonomy (Haddock et al., 1982; Okuda et al., 2000). *Castanopsis* species (Fagaceae) are the only genus containing ellagitannins with triterpene alcohol cores; however, only two species, *C. cuspidata var. sieboldii* in Japan and *C. hystrix* in Taiwan, have been investigated (Ageta et al., 1988; Chen et al., 1993). Therefore, in order to expand the chemotaxonomical study and to develop new procedures that make use of the tree resources of southern China, we have initiated a project to reveal the chemical constituents of Chinese *Castanopsis* species. This paper describes a detailed investigation of the constituents of the leaves of *C. fissa*, which is a fast-growing evergreen tree commonly distributed and planted in the southern regions of China and Vietnam.

2. Results and Discussion

The fresh leaves were extracted with 80% aqueous acetone, and the extract was subjected to a combination of column chromatography using Sephadex LH-20, Diaion HP20SS, Chromatorex ODS and silica gel to give 55 compounds, including two new compounds **1** and **2** (Table 1). The known compounds were identified by comparing their spectral data with data acquired on authentic samples and reported in the literature. The major constituents are polyphenols, including galloyl quinic acids, galloyl and HHDP glucoses, flavonol glycosides, and HHDP esters of triterpenes. In particular, the isolation yields of 1,3,4-trigalloyl quinic acid and the new compound **2** were 1.53 and 0.27%, respectively, from the fresh leaves. The quinic acid derivatives were named according to the IUPAC system (IUPAC, 1976).

The new compound **1** (Fig. 1) was a minor species obtained as a reddish-brown amorphous powder. UV absorptions at 406 and 284 nm indicated the presence of a chromophore with yellow color, and a dark blue coloration with an $FeCl_3$ reagent suggested the presence of 1,2,3-trihydroxybenzene rings. The ^1H NMR spectrum (Table 2) was closely related to the spectral data of 1,3,4-trigalloyl quinic acid (Xu et al., 2008). The location of the acyl groups was apparent from the large low field shifts of the H-3, H-4, and the equatorial protons at C-2 and C-6, which were assigned based on their mutual W-letter type long-range couplings (3 Hz). The low field shifts of these equatorial protons were due to the anisotropic effects of the ester carbonyl group attached to the C-1 hydroxyl group. Two of the three acyl groups were galloyl groups (δ 6.86 and 7.08), and the

remaining one showed three aromatic singlet signals at δ 7.03 (H-f), 7.55 (H-c) and 8.14 (H-e). In the ^{13}C-NMR spectrum (Table 2), twelve signals attributable to the conjugated ester and ketone carbonyls (δ 166.1 and 183.5) and ten aromatic carbons were assigned to the acyl group. The chemical shifts of the signals were in good agreement with those of purpurogallin carboxylic acid (Nonaka et al., 1986). This was consistent with the molecular composition $C_{33}H_{26}O_{20}$ shown by the HR-FAB-MS ion peak $[M+H]^+$ at *m/z* 743.1083 (Calcd for $C_{33}H_{27}O_{20}$ 743.1095). Furthermore, HMBC correlations shown in Fig. 2 confirmed the benzotropolone structure. The location of two galloyl groups at the C-3 and C-4 was apparent from the HMBC correlations of H-3 and H-4 to the galloyl ester carbons; therefore, the purpurogallin carbonyl group was determined to be attached to the C-1 hydroxyl group. Based on these spectroscopic results, the structure of **1** was concluded to be 3, 4-di-*O*-galloyl-1-*O*-purpurogallin carbonyl quinic acid. This compound is presumably formed by oxidative coupling between 1,3,4-trigalloyl quinic acid and gallic acid (Nonaka et al., 1986).

Compound **2** was isolated as one of the major metabolites (1.53% from the fresh leaves) from hydrophobic fractions strongly adsorbed on reversed-phase gels. The fractions also contained castanopsinin E (**4**) and 3,24-(*S*)-hexahydoxydiphenoyl-2α,3β,23,24-tetrahydroxyolean-12-en-28-oic acid (**3**) (Ageta et al., 1988). Compound **3** has previously been reported only as a degradation product of **4** and detailed spectroscopic data were not shown; therefore, the structure was determined independently by chemical and spectroscopic examination, including two-dimensional NMR techniques (see experimental section and Table 3). Compound **2** gave a dark blue coloration when

mixed with the $FeCl_3$ reagent and a brown coloration with a $NaNO_2$-AcOH reagent. These observations are similar to those of **3** and **4**, and suggest the presence of a HHDP group in the molecules (Bate-Smith et al., 1972). The ^{1}H and ^{13}C NMR spectra of **2** were also related to those of **3**, and showed signals arising from a HHDP group and a triterpenoid moiety (Table 2). Chemical shifts of the proton and carbon signals attributable to triterpene A/B rings and C-23, 24, and 25 were essentially superimposable with those of **3**, suggesting structural similarities. Large low field shifts of the H-3 (δ 5.86) and H-24 (δ 3.98 and 5.40) indicated the esterification of these positions with the HHDP groups. Clear differences between the NMR spectra were the absence of the signals that were due to the double bond at C-12(13) and the appearance of an oxygenated quaternary carbon signal at δ 83.9 (C-20). The oxygenated carbon showed HMBC correlations with H-19, H-29 and H-30, confirming the assignment of this signal to C-20 (Fig. 3). The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS of **2** (m/z 829, $[M+Na]^{+}$) and elemental analysis revealed that the molecular composition of **2** ($C_{44}H_{54}O_{14}$) is the same as that of **3**. Taking into consideration the same unsaturation index (18) of these compounds strongly suggests that the oxygen atom at the C-20 formed a δ lactone ring with the C-28 carboxyl group. This was also supported by the upfield shift of the C-28 (δ 176.6) moiety when compared to the same carbon in **3** (δ 180.1). The configuration of the substituents on the A- and E-rings was determined from NOESY correlations (Fig. 4): NOEs between the acylated hydroxymethyl protons (H_2-24) and H-2/H-25, and NOEs between the methylene protons with the free hydroxyl group (H_2-23) and H-3/H-5 confirmed the

stereochemistry of the A-ring. As for the E-ring stereochemistry, the α axial orientation of the C-29 methyl group and the β configuration of the C-20 oxygen atom were determined by NOE correlations between H-18 and H-29, H-27 and H-22α, as well as NOEs between H-29 and H-22α. The atropisomerism of the HHDP biphenyl bond was determined by methylation and subsequent methanolysis, which yielded dimethyl (*S*)-hexamethoxydiphenoate (**2a**) ($[\alpha]_D$ −25.5º) (Ikeya et al., 1979) and a tetrahydroxy triterpene lactone **2b**. The triterpene **2b** has not been previously reported. Based on these results, compound **2** was characterized as 3,24-(*S*)-HHDP-2α, 3β, 23, 24-tetrahydroxytaraxastan-28, 20β-olide.

The compounds **2** and **3** were amphiphilic compounds with hydrophobic triterpene and hydrophilic HHDP esters. As such, these compounds were isolated from both the Et_2O and aqueous layers. In contrast, compound **4** was obtained only from the aqueous layer. Partition coefficients between *n*-octanol and water (log $P_{octanol/water}$) at 25 °C of **2**, **3** and **4** were 2.88, 2.64 and 1.78, respectively. Therefore, compounds **2** and **3** are unusual metabolites with both features of water-soluble ellagitannins and lipid soluble triterpenoids (Tanaka et al., 1997). Due to the physicochemical characteristics, we examined whether these compounds showed lipase inhibition activities. Five major compounds, including compound **2** (40.4 ± 15.5% inhibition), 1,3,4-trigalloyl quinic acid (28.5 ± 6.7% inhibition), 1,4-digalloyl quinic acid (29.0 ± 7.1% inhibition), myricitrin (64.7 ± 10.0% inhibition) and pedunculagin (77.6 ± 4.9% inhibition) were compared at 5 μg/ml (Jeevanandam et al., 1989; Han et al., 1999, Kusano et al., in press). Among the compounds tested,

pedunculagin and myricitrin showed inhibition comparable to the positive control (Orlistat, 67.9 ± 1.5% inhibition), and the activity of **2** was moderate. Oxygen radical absorbance capacity (ORAC) of the 10 major compounds were also compared (Table 4) and myricitrin was observed to show the strongest antioxidation activity (Prior et al., 2005). Comparison of the values for **2**–**4** suggested that hydrophilic compounds showed stronger activity among triterpene HHDP esters. Pedunculagin is more hydrophilic compared to its 1-*O*-galloyl ester and gave rise to a larger ORAC value.

3. Conclusions

The presence of triterpene HHDP esters as major components of secondary metabolites (Table 1) is important from a chemotaxonomical viewpoint. Although galloyl esters of triterpenes have been isolated from a few Combretaceous and Fagaceous plants (Ponou et al., 2008; Garcez et al., 2006; Conrad et al., 1998; Romussi et al., 1993), isolation of HHDP esters has only been achieved from two *Castanopsis* species (Ageta et al., 1988, Chen et al., 1993), and the present study represents the third example from the same genus. The compositions of the triterpene HHDP esters of *C. fissa* are much simpler than those of the other two species examined, which contain complex mixtures of isomers, including those with oleanane and ursane type triterpene cores, and positional and configurational isomers of the esters (Ageta et al., 1988). The observed large accumulation of **2** is a characteristic feature of *C. fissa*. The HHDP esters are produced from oxidative coupling of two galloyl groups; however, triterpene galloyl esters were not found. A further difference is that *C. fissa* accumulates 1,3,4-tri-*O*-galloyl quinic acid (25.4% from the extract and 1.53% from fresh leaves), whereas *C. cuspidata* var *sieboldii* contains 3-*O*-galloyl shikimic acid (1.7% from fresh leaves) (Nonaka et al., 1985). Since reports suggest that galloyl quinic acids have inhibitory activities against HIV reverse transcriptase (Nonaka et al., 1990; Nishizawa et al., 1989) and human DNA polymerases (Parker et al., 1989), the leaves of *C. fissa* represent a promising source of galloyl quinic acids. Chemotaxonomical studies of Chinese Castanopsis species are currently underway.

4. Experimental

4.1. General experimental procedures

UV spectra were obtained using a Jasco V-560 UV/Vis spectrophotometer and optical rotations were measured with a Jasco DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra were measured in CD_3OD at 27 ºC using a Varian Unity plus 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Varian, Palo Alto, CA, USA) or a JEOL JNM-AL 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) (JEOL Ltd., Tokyo, Japan). Coupling constants are expressed in Hz and chemical shifts are given on a δ (ppm) scale. MS were recorded on a Voyager DE-PRO (Applied Biosystems, USA) and a JEOL JMS-700N spectrometer (JEOL Ltd.**,** Tokyo, Japan). 2,5-Dihydroxybenzoic acid and glycerol were used as the matrix for MALDI-TOF-MS and FAB-MS measurements, respectively. Column chromatography was performed using Diaion HP20SS (Mitsubishi Chemical, Tokyo, Japan), Sephadex LH-20 (25–100 μm; GE Healthcare Bio-Science AB, Uppsala, Sweden), and Chromatorex ODS (100–200 mesh; Fuji Silysia Chemical, Tokyo, Japan) columns. TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick; Merck, Darmstadt, Germany) with *n*-hexane-EtOAc (9:1, v/v), *n*-hexane-acetone (5:1, v/v) and toluene-ethyl formate-formic acid (1:7:1, v/v) as the solvent, and spots were detected by UV illumination (254 nm) and by spraying with a 2% ethanolic $FeCl_3$ and 10% sulfuric acid reagent, followed by heating. Analytical HPLC was performed on a Cosmosil 5C_{18}-AR II (Nacalai Tesque Inc., Kyoto, Japan) column (4.6 mm i.d. × 250 mm) with gradient elutions from 4–30% (39 min) and

30–75% (15 min) of CH_3CN in 50 mM H_3PO_4 (for polyphenols) or from 70–100% (15 min) and 100% (10 min) CH_3CN in 50 mM H_3PO_4 (for terpenoids). The flow rate was 0.8 ml/min and detection was achieved using a JASCO photodiode array detector MD-910.

4.2. Plant material

The leaves of *Castanopsis fissa* were collected at Guangxi Institute of Botany, Guangxi, China, in August 2009, and identified by Prof. Yan Liu. The voucher specimen was deposited in the Herbarium of Guangxi Institute of Botany, China.

4.3. Extraction and separation

The fresh leaves of *C. fissa* (8.15 kg) were cut into small pieces and extracted with acetone-H_2O (8:2, v/v) by maceration at room temperature. After filtration, the plant debris remaining on the filter paper was extracted with the same solvent a further two times. The filtrate was combined and concentrated under reduced pressure to give an aqueous solution with dark green precipitates. The precipitant was mainly composed of chlorophylls and waxes, and removed by filtration. The filtrate was defatted by partitioning with Et_2O (Fr. E 44.7 g). The Et_2O fraction was separated into 80% MeOH insoluble (Fr. E-C, 14.25g) and soluble fractions (Fr. E-ML). The E-C fraction was further separated by silica gel (2 cm i. d. × 15 cm) with $CHCl_3$–MeOH–H_2O (100:0:0, 90:10:1, 80:20:2, 70:30:5, 60:40:10, 50:50:10) to give 2α,3β,23-trihydorxyolean-12-en-28-oic acid

(279 mg) and fraction E-C-2 (487 mg). The E-C-2 fraction was further separated by Chromatorex ODS (3 cm i. d. × 20 cm) with MeOH-H_2O (8:2, 9:1; 10:0) to give 2α,3β,23,24-tetrahydroxyolean-12-en-28-oic acid (132 mg) and β-sitosterol-3-*O*-β-D-glucopyranoside (61 mg). The E-ML fraction was successively separated by Sephadex LH-20 (6 cm i. d. × 40 cm) with MeOH-H_2O (8:2, 9:1, 10:0) and Diaion HP20SS column chromatography (3 cm i. d. × 20 cm) with MeOH-H_2O (6:4, 7:3, 8:2, 9:1, 10:0) to yield **2** (1.79 g), **3** (2.1 mg) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) (17 mg). The aqueous layer was subjected to Sephadex LH-20 column chromatography (10 cm i. d. × 40 cm) with water containing increasing proportions of MeOH (10–100%, 10% stepwise elution) and finally 60% acetone to give nine fractions: frs 1 (156.2 g), 2 (14.1 g), 3 (14.4 g), 4 (19.0 g), 5 (17.1 g), 6 (15.0 g), 7 (140.3 g), 8 (37.9 g) and 9 (1.5 g). Fraction 1 was separated by Diaion HP20SS column chromatography (8 cm i. d. × 40 cm) with MeOH-H_2O (0:100–100:0) to yield fr. 1-1 (140.7 g) and fr. 1-2 (15.5g). Fr. 1-1 was further separated by Sephadex LH-20 column chromatography (10 cm i. d. × 40 cm) with 0–100% MeOH-H_2O containing 0.1% trifluoroacetic acid (TFA) (10% stepwise elution, each 1 L), and the subfractions were purified by Diaion HP20SS column chromatography (3 cm i. d. × 30 cm) with 0–100% MeOH containing 0.1% TFA (10% stepwise elution, each 500 ml) to give 2-(β-D-glucopyranosyloxy)-6-hydroxy benzoic acid (6 mg), gentisic acid 5-*O*-β-D-glucoside (230 mg), 1,3-di-*O*-galloylquinic acid (1.38 g), 3-*O*-galloylquinic acid (928 mg), shikimic acid (276 mg) and cytidine (16 mg). Fr. 1-2 was successively applied to a Diaion HP20SS column (6 cm i. d. × 20

cm) in a manner similar to that described above and the subfractions were separated by Chromatorex ODS column chromatography (4 cm i. d. × 25 cm) with 0–80% MeOH (5% stepwise elution, each 100 ml) or silica gel column chromatography (2 cm i. d. × 20 cm) with $CHCl_3$-MeOH-H_2O (10:0:0, 90:10:1, 80:20:2, 70:30:5, 60:40:10) to give 4-hydroxy-3,5-dimethoxy phenyl β-D-glucoside (27 mg), benzyl-α-L-rhamnopyranosyl (1→6)-β-D-glucoside (360 mg), 3-*O*-coumaroylquinic acid (122 mg), 3'-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (5 mg), 3,3'-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (121 mg), corchoionol C (108 mg), corchoionoside C (46 mg), apocynol A (13 mg) and (1'*S*, 6'*R*)-8'-hydroxyabscisic acid β-D-glucoside (22 mg). Fraction 2 was further fractionated by Diaion HP20SS (4 cm i. d. × 40 cm) chromatography with 10–100% MeOH (10% stepwise elution, each 500 ml) and the subfractions were separated by chromatography using the Sephadex LH-20 column (3 cm i. d. × 40 cm) with EtOH, Chromatorex ODS (2 cm i. d. × 30 cm) with 0–100% MeOH (5% stepwise elution, each 100 ml) and silica gel (2 cm i. d. × 25 cm) with $CHCl_3$-MeOH-H_2O to yield 4,6-(*S*)-HHDP-D-glucose (95 mg), 2,5-dihydroxybenzoic acid (26 mg), 2,6-dimethoxy-*p*-hydroquinone 1-*O*-β-D-glucoside (13 mg), 6-*O*-(*p*-hydroxybenzoyl)-β-D-glucose (36 mg), 4-*O*-coumaroylquinic acid (120 mg), chlorogenic acid (23 mg), ellagic acid (783 mg), 3,4-di-*O*-methylellagic acid (5 mg) and (*Z*)-4-ethylidene-3,5,5-trimethylcyclohex-2-enone (18 mg). Fraction 3 was separated by a Diaion HP20SS column (4 cm i. d. × 40 cm) with 10–100% MeOH (5% stepwise elution) and the subfractions were further separated by column chromatography using Sephadex LH-20 (10–100% MeOH, 10% stepwise elution), Toyopearl Butyl 650C (0–100% MeOH,

10% stepwise elution) and Sephadex LH-20 (EtOH, then 90–60% EtOH, 10% stepwise elution) to yield 2,3-(*S*)-HHDP-D-glucose (534 mg), *trans*-*p*-coumaric acid (18 mg), *cis*-*p*-coumaric acid (19 mg), 1,3,5-tri-*O*-galloylquinic acid (97 mg), 1,4-di-*O*-galloylquinic acid (1.50 g), 3,3'-di-*O*-methylellagic acid (17 mg), 3,3',4-tri-*O*-methylellagic acid (5 mg) and mallophenol B (190 mg). Fractions 4 and 5 were separately purified by Diaion HP-20SS (0 – 60% MeOH, 10% stepwise elution) to yield 1,3,4-tri-*O*-galloylquinic acid (19.0 and 17.0 g, respectively). Similar chromatographic separation of fraction 6 afforded gallic acid **9** (682 mg), 3,5-di-*O*-galloylquinic acid (895 mg), 3,4-di-*O*-galloylquinic acid (650 mg) and 3-*O*-methylellagic acid-3'-*O*-β-D-xylopyranoside (4 mg). Fraction 7 was separated by Diaion HP20SS, Sephadex LH-20, and Chromatorex ODS column chromatography to give 1,3,4-tri-*O*-galloylquinic acid (86.0 g), 1,4-di-*O*-galloylquinic acid (10.7 g), myricitrin (4.3 g), isoquercitrin (2.3 g), **2** (19.5 g), **4** (2.7 g). Separation of fraction 8 using Diaion HP20SS, Sephadex LH-20, and Chromatorex ODS afforded 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (254 mg), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (127 mg), 1,3,4,6-tetra-*O*-galloyl-β-D-glucose (430 mg), pedunculagin (11.69 g), eugeniin (724 mg), 1,3,4,5-tetra-*O*-galloylquinic acid (615 mg), 3,4,5-tri-*O*-galloylquinic acid (1.18 g) and **1** (34 mg). Fraction 9 was purified by Diaion HP20SS chromatography to yield 1(β)-*O*-galloyl pedunculagin (1.45 g).

*4.4. 4, 5-Di-O-galloyl-1-O-purpurogallin carbonyl quinic acid (**1**)*

Reddish-brown amorphous powder; $[\alpha]_D^{21}$ –129.9º (c = 0.11, acetone), MALDI-TOF-MS *m/z*: 765 $[M+Na]^+$, HR-FAB-MS *m/z*: 743.1083 $[M+H]^+$ (Calcd for $C_{33}H_{27}O_{20}$: 743.1095), IR ν_{max} cm^{-1}: 3388, 2360, 1696, 1222, UV λ_{max}^{MeOH} nm (log ε): 219 (5.05), 284 (4.82), 406 (4.19); ^1H and ^{13}C NMR (in actone-d_6), see Table 2.

*4.5. 3,24-(S)-Hexahydroxydiphenoyl-2α,3β,23,24-tetrahydroxytaraxastan-28,20β-olide (**2**).*

Colorless needles (H_2O-MeOH, 5:1, v/v); mp > 300 °C; $[\alpha]_D^{21}$ +0.04° (c 0.1, MeOH); MALDI-TOF-MS m/z: 829 $[M+Na]^+$; IR ν_{max} cm^{-1}: 3390, 2937, 1720, 1448; UV (MeOH) λ_{max} nm (log ε): 221 (4.41), 258 sh. (4.14); ^1H and ^{13}C NMR (in pyridine-d_5), see Table 3 (Found: C, 62.09; H, 6.61. $C_{44}H_{54}O_{14}2.5H_2O$ required: C, 62.03; H, 6.98%.).

*4.5.1. Methylation of **2***

A solution of **2** (201 mg) in MeOH (5 ml) was treated with an ethereal solution of CH_2N_2 at 0 °C for 2 h. After the solvent was evaporated off under reduced pressure, the product was purified by silica gel chromatography with 0–50% acetone in hexane-acetone (5% stepwise elution) to yield a hexamethyl ether of **2** (110 mg) as a colorless amorphous powder; $[\alpha]_D^{23}$ –29.4° (c 0.15, $CHCl_3$); MALDI-TOF-MS *m/z* 913 $[M+Na]^+$; HR-FAB-MS *m/z* 891.4514 $[M+H]^+$ (Calcd for $C_{50}H_{67}O_{14}$: 891.4521); IR ν_{max} cm^{-1}: 3339, 1740, 1465; UV (MeOH) λ_{max} nm (log ε): 213 (5.29), 249 sh. (4.16);

^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.60, 3.64, 3.84, 3.87, 3.91, 3.92 (18H, s, OMe), 6.66 and 6.76 (each 1H, s, HMDP-H).

*4.5.2. Methanolysis of the hexamethyl ether of **2** (**2a** and **2b**)*

The hexamethyl ether of **2** (78 mg) was treated with 3% methanolic MeONa at 50 ºC for 12 h. The reaction mixture was neutralized with ion exchange gel material (Amberlite IR120B, H^+ form) and evaporated off under reduced pressure. The residue was separated by silica gel chromatography with 0–50% acetone in hexane to give dimethyl (*S*)- hexamethoxydiphenoate (**2a**) (21 mg), a colorless syrup, $[\alpha]_D^{22}$ –25.5° (c 1.05, $CHCl_3$), and **2b** (26 mg), $[\alpha]_D^{22}$ +18.0° (c 0.12, $CHCl_3$); MALDI-TOF-MS *m/z* 527 $[M+Na]^+$; HR-FAB-MS *m/z* 505.3535 $[M+H]^+$ (Calcd for $C_{30}H_{49}O_6$: 505.3530); IR ν_{max} cm^{-1}: 3377, 2935, 1739, 1453. ^{1}H NMR (400 MHz, pyridine-d_5) δ: 0.77, 0.89, 1.01, 1.23 (each 3H, s, Me), 0.82 (3H, d, *J* = 7.3 Hz, Me), 2.40 (1H, dd, *J* = 4.4, 12.7 Hz, H-5), 3.97 (1H, d, *J* = 11.2 Hz, H-24a), 4.25 (1H, d, *J* = 11.2 Hz, H-23a), 4.35 (1H, d, *J* = 9.8 Hz, H-3), 4.45 (1H, m, H-2), 4.57 (1H, d, *J* = 11.2 Hz, H-24b) and 4.87 (1H, d, *J* = 11.2 Hz, H-23b); ^{13}C NMR (100 MHz, pyridine-d_5) δ: 14.3 (C-27), 15.8 (C-26), 18.1 (C-29), 18.5 (C-25), 19.1 (C-6), 21.6 (C-11), 24.1 (C-30), 25.3 (C-12), 27.2 (C-21), 27.6 (C-15), 28.0 (C-16), 32.1 (C-22), 34.4 (C-7), 38.2 (C-10), 40.8 (C-8), 41.3 (C-14), 42.1 (C-17), 42.29 (C-19), 42.34 (C-13), 43.2 (C-5), 47.9 (C-1), 48.2 (C-4), 48.3 (C-18), 51.1 (C-9), 62.8 (C-24), 64.2 (C-23), 69.3 (C-2), 79.7 (C-3), 83.8 (C-2) and 176.6 (C-28).

*4.6. 3,24-(S)-Hexahydroxydiphenoyl-2α,3β,23,24-tetrahydroxyolean-12-en-28-oic acid (**3**)*

Off-white amorphous powder; $[\alpha]_D^{20}$ +19.0° (c 0.15, MeOH); MALDI-TOF-MS m/z: 829 $[M+Na]^+$; IR ν_{max} cm^{-1}: 3395, 2947, 1702, 1450; UV (MeOH) λ_{max} nm (log ε): 227 (4.44), 260 (4.15); ^1H and ^{13}C NMR (pyridine-d_5), see Table 3 (Found: C, 62.92; H, 6.77. $C_{44}H_{54}O_{14}$ $2H_2O$ requires: C, 62.70; H, 6.94%.)

*4.6.1. Methylation of **3***

Methylation of **3** (203 mg) in a manner similar to that described for **2** yielded a hexamethyl ether (102 mg), $[\alpha]_D^{21}$ –35.1° (c 0.11, $CHCl_3$); MALDI-TOF-MS *m/z* 927 $[M+Na]^+$; IR ν_{max} cm^{-1}: 3349, 2942, 1740, 1461; UV (MeOH) λ_{max} nm (log ε): 219 (4.50), 250 (4.11); ^1H NMR (pyridine-d_5) δ: 0.72, 0.88, 0.90, 1.00, 1.12 (each 3H, s, Me), 3.56, 3.59, 3.65, 3.84, 3.87, 3.91, 3.92 (each 3H, s, OMe), 5.28 (1H, br s, 12-H), 6.66 and 6.78 (each 1H, s, HMDP-H)]. (Found: C, 67.37; H, 7.70. $C_{51}H_{68}O_{14}$ $1/4H_2O$ requires: C, 67.68; H, 7.57%.)

*4.6.2. Methanolysis of the methyl ether of **3***

Alkaline methanolysis of the hexamethyl ether of **3** (79 mg) in a manner similar to that described for **2** afforded dimethyl (*S*)-hexamethoxydiphenoate (**2a**) (27 mg), $[\alpha]_D^{22}$ –23.9° (c 1.4, $CHCl_3$), and 2α,3β,23,24-tetrahydroxyolean-12-en-28-oic acid methyl ester (28 mg), $[\alpha]_D^{22}$ +48.1° (c 0.11, $CHCl_3$); MALDI-TOF-MS *m/z* 541 $[M+Na]^+$, HR-FAB-MS m/z 519.3680 $[M+H]^+$ (Calcd for

$C_{31}H_{51}O_6$: 519.3685); IR ν_{max} cm^{-1}: 3387, 2936, 1739, 1453. ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 0.77, 0.79, 0.83, 1.05, 1.06 (15H, s, 5 × Me), 2.25 (1H, dd, J = 4.1, 12.2 Hz, H-5), 2.99 (1H, dd, J = 4.4, 13.7 Hz, H-18), 3.62 (3H, s, -COOMe), 3.94 (1H, d, J = 11.2 Hz, H-24a), 4.21 (1H, d, J = 10.8 Hz, H-23a), 4.26 (1H, d, J = 9.3 Hz, H-3), 4.38 (1H, m, H-2), 4.54 (1H, d, J = 11.2 Hz, H-24b), 4.80 (1H, d, J = 10.8 Hz, H-23b) and 5.28 (1H, br s, H-12). ^{13}C NMR (100 MHz, pyridine-d_5) δ: 17.1 (C-26), 17.2 (C-25), 19.2 (C-6), 23.3 (C-30), 23.6 (C-16), 24.1 (C-11), 26.0 (C-27), 28.0 (C-15), 30.8 (C-20), 32.7 (C-22), 33.06 (C-29), 33.10 (C-7), 33.9 (C-21), 38.1 (C-10), 39.7 (C-8), 41.7 (C-18), 41.9 (C-14), 45.9 (C-17), 46.9 (C-19), 47.8 (C-4), 47.9 (C-1), 48.17 (C-5), 48.23 (C-9), 51.6 (C-OMe), 62.9 (C-24), 64.3 (C-23), 69.0 (C-2), 79.7 (C-3), 122.7 (C-12), 144.1 (C-13) and 178.0 (C-28).

4.7 Measurement of Pancreatic Lipase Inhibitory Activity

Lipase inhibitory activity was measured using previously published methods (Jeevanandam et al., 1989; Han et al., 1999; Kusano et al., in press). Orlistat was used as the positive control. The substrate solution was prepared by sonication (10 min in an ice bath) of a mixture of glyceryl trioleate (80 mg), lecithin (10 mg), and sodium cholate (5 mg) suspended in 9 ml of 0.1 M TES buffer (pH 7.0). Samples were separately dissolved in 0.1 M TES buffer to make 0.2 mg/ml solutions. The substrate (20 µl) and sample solutions (20 µl) in microplate wells were preincubated for 3 min, then 10 µl of a lipase solution (20 µg/ml) was added to each reaction mixture and incubated for 30

min at 37 °C. The amount of released fatty acid was measured by a NEFA C test kit (Wako, Japan) at 550 nm using an Emax microplate reader (Molecular Devices, CA). Inhibitory activity (%) was calculated as follows:

Inhibition (%) = {1 − (Abs 6 − Abs 5)/(Abs 8 − Abs 7)} ×100

Abs 5: absorbance of the incubated solution containing the sample, substrate and lipase

Abs 6: absorbance of the incubated solution containing the sample and substrate

Abs 7: absorbance of the incubated solution containing the substrate and lipase

Abs 8: absorbance of the incubated solution containing only the substrate

Measurements were performed in triplicate and the mean ± SD was shown in the text.

4.8 Measurement of the ORAC values

Measurement of the ORAC values was performed using previously described methods (Prior et al., 2003; Tseye-Oidov et al., 2010). Briefly, test samples were dissolved in acetone–water–acetic acid (70:29.5:0.5, v/v) (1 mg/mL), and then appropriately diluted (10–1.25 μg/mL) with a 75 mM KH_2PO_4–K_2HPO_4 buffer at pH 7.4. Trolox calibration solutions (TCI, Japan) (50, 25, 12.5 and 6.25 μM in KH_2PO_4–K_2HPO_4 buffer (75 mM, pH 7.4)) were made to build a standard curve. Diluted sample solutions, Trolox calibration solutions, or the blank sample (20 μL), 94.4 nM fluorescein sodium salt (TCI, Japan) (200 μL), and 31.7 mM 2,2'-azobis(2-methylpropionamidine) dihydrochloride (Wako, Japan) (75 μL) were incubated in the

KH_2PO_4–K_2HPO_4 buffer (75 mM, pH 7.4) at 37 °C in a 96-well microplate. The fluorescence (Ex: 485 nm; Em: 535 nm) was monitored every 2 min for 90 min using a ARVO *X*2 multilabel reader (PerkinElmer, MA). The net area under the curve (AUC) was calculated by subtracting the AUC of the blank from that of the sample or Trolox. The ORAC value was calculated on the basis of the standard curve for Trolox, and expressed as micromoles of Trolox equivalent to the samples (μmol Trolox equivalent/g). Calculations were performed using WorkOut 2.5 (PerkinElmer, MA). Measurements were performed in duplicate and averages were shown in Table 4.

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Figure legends

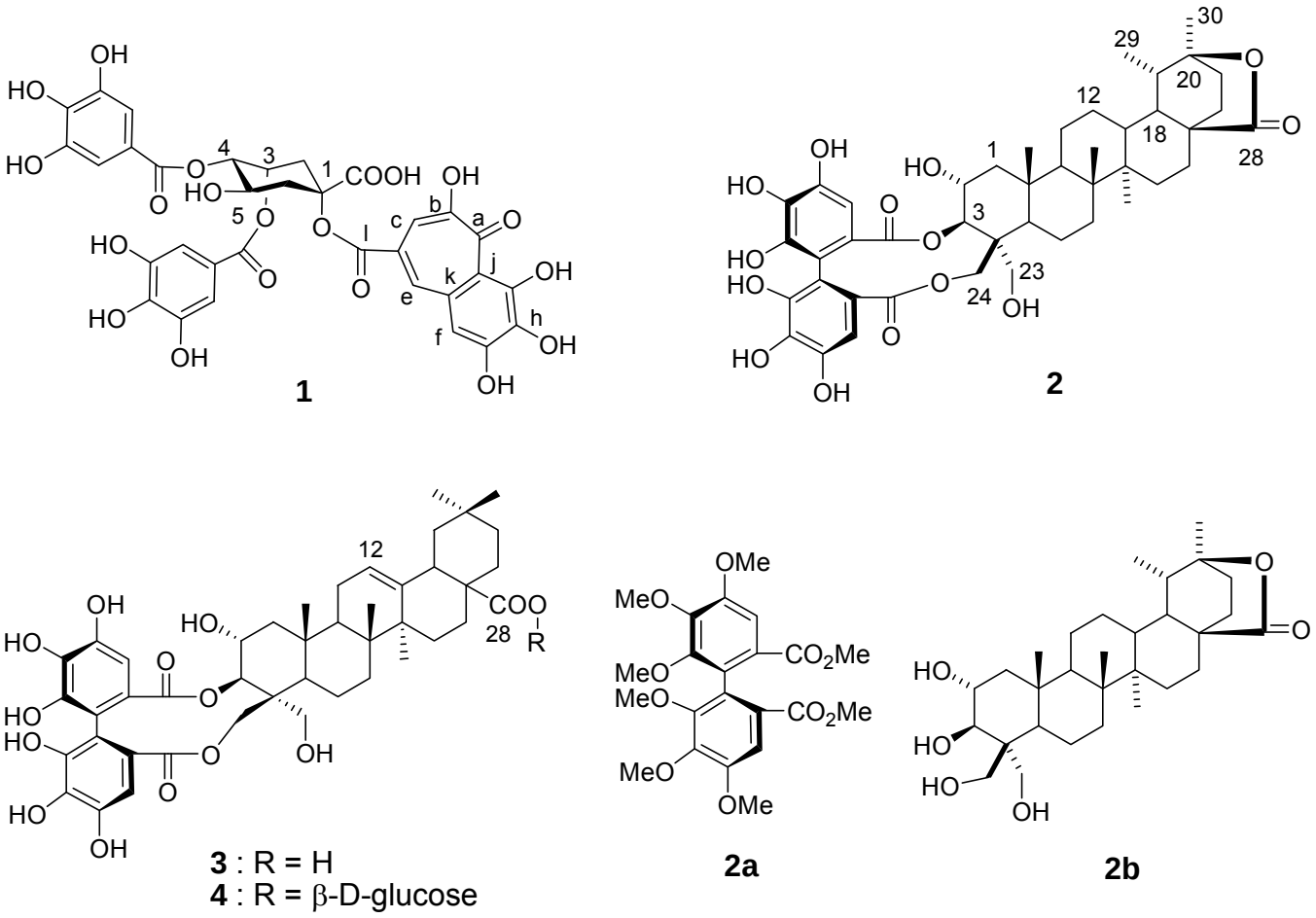

Fig. 1. Structures of **1**–**4**, **2a** and **2b**.

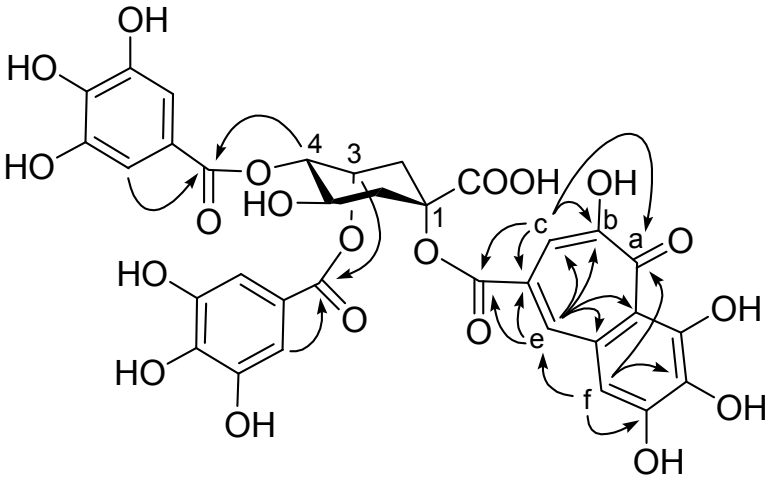

Fig. 2. Selected HMBC correlations (H to C) of **1**.

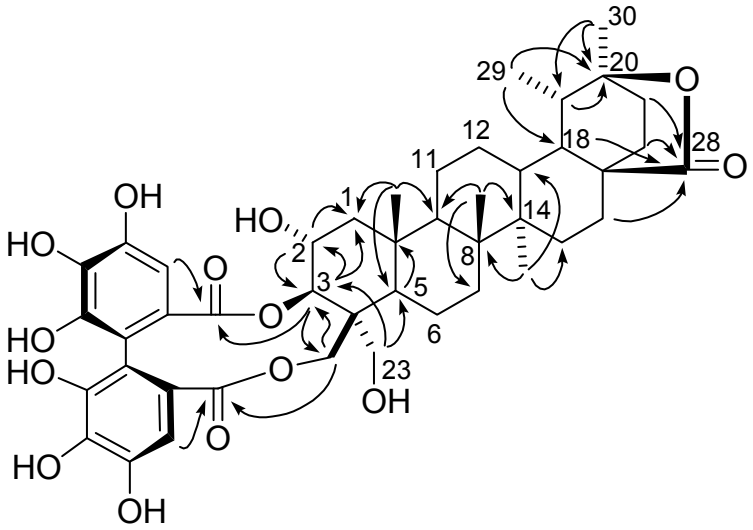

Fig. 3. Selected HMBC correlations of **2**.

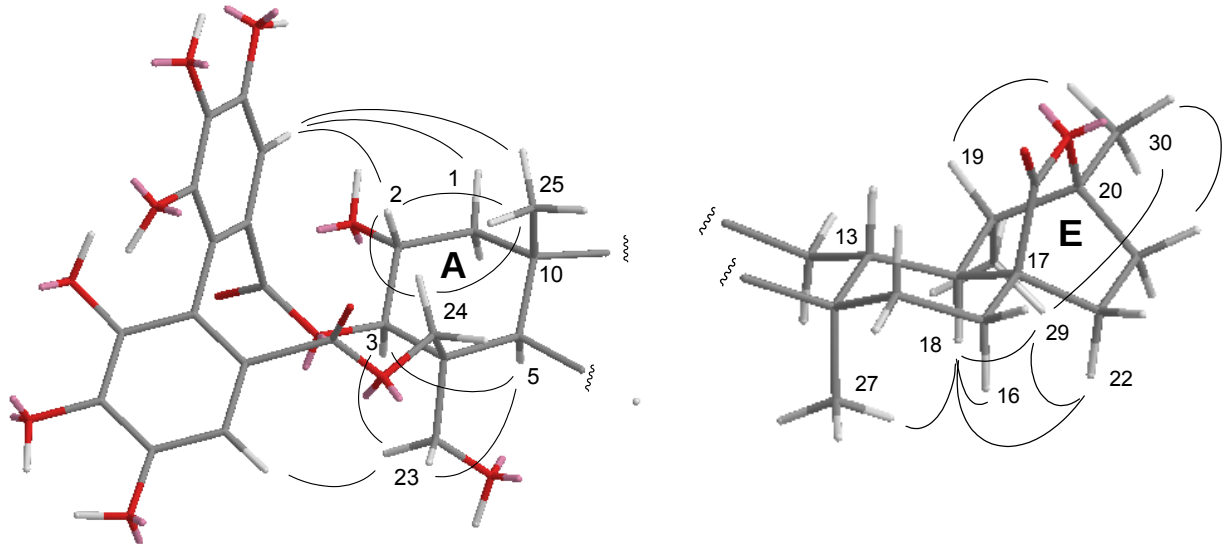

Fig. 4. Selected NOE correlations observed for the A and D/E rings of **2**.

Table 1

Compounds and the yield (% from fresh leaves) isolated from the leaves of *Castanopsis fissa* .

conpound name	yield	references
gallyol and/or hexahydroxydiphenoyl glucoses		
1,2,3,4,6-penta-*O* -galloyl-β-D-glucose	0.0032	Nishizawa et al., 1982
1,2,3,6-tetra-*O* -galloyl-β-D-glucose	0.0016	Lee et al., 1989
1,3,4,6-tetra-*O* -galloyl-β-D-glucose	0.0054	Nishizawa et al., 1983
pedunculagin	0.1461	Lee et al.,1992
2,3-(*S*)-HHDP-D-glucose	0.0067	Lee et al., 1990
4,6-(*S*)-HHDP-D-glucose	0.0012	Ishimatsu et al., 1989
1(β)-*O* -galloyl pedunculagin	0.0181	Tanaka et al., 1993
eugeniin	0.0091	Nonaka et al., 1980
acylated quinic acid		
1,3,4,5-tetra-*O* -galloylquinic acid	0.0077	Xu et al., 2008
1,3,4-tri-*O* -galloylquinic acid	1.5252	Xu et al., 2008
1,3,5-tri-*O* -galloylquinic acid	0.0012	Clifford et al., 2007
3,4,5-tri-*O* -galloylquinic acid	0.0147	Nishimura et al., 1984
3,5-di-*O* -galloylquinic acid	0.0112	Nishimura et al., 1984
1,3-di-*O* -galloylquinic acid	0.0173	Bouchet et al., 1996
1,4-di-*O* -galloylquinic acid	0.1465	Ishimaru et al., 1987
3,4-di-*O* -galloylquinic acid	0.0081	Nishimura et al., 1984
3-*O* -galloylquinic acid	0.0116	Nishimura et al., 1984
3-*O* -coumaroylquinic acid	0.0015	Ossipov et al., 1996
4-*O* -coumaroylquinic acid	0.0015	Whiting et al., 1975
chlorogenic acid (5-*O* -caffeoyl quinic acid)	0.0003	Tamura et al., 2004
3,4-di-*O* -galloyl 1-*O* -purpurogallin carbonyl quinic acid (**1**)	0.0004	
phenolic acids and related compounds		
gallic acid	0.0085	
2,5-dihydroxybenzoic acid	0.0003	
trans -*p* -coumaric acid	0.0002	Yang et al., 2008
cis -*p* -coumaric acid	0.0002	Kort, et al., 1996
2-(β-D-glucopyranosyloxy)-6-hydroxy benzoic acid	0.0001	Gunther, 1959
gentisic acid 5-*O* -β-D-glucoside	0.0029	Sakushima et al., 1995
4-hydroxy-3,5-dimethoxy phenyl β-D-glucoside	0.0003	Zhu et al., 2010
2,6-dimethoxy-*p* -hydroquinone 1-*O* -β-D-glucoside	0.0002	Otsuka et al., 1989
6-*O* -(*p* -hydroxybenzoyl)-β-D-glucose	0.0005	Kaneko et al., 1988
benzyl-α-L-rhamnopyranosyl (1→6)-β-D-glucoside	0.0045	Tommsai et al., 1996
ellagic acid	0.0098	
3,4-di-*O* -methylellagic acid	0.0001	Sato, 1991
3,3'-di-*O* -methylellagic acid	0.0002	Ye et al., 2007
3,3',4-tri-*O* -methylellagic acid	0.0001	Bindra et al., 1988
3'-*O* -methylellagic acid 4-*O* -β-D-xylopyranoside	0.0001	Tanaka et al., 1998
3,3'-di-*O* -methylellagic acid 4-*O* -β-D-xylopyranoside	0.0015	Khac et al., 1990
3-*O* -methylellagic acid-3'-*O* -β-D-xylopyranoside	0.0001	Shi et al., 2009
flavonol glycosides		
myricitrin	0.0534	Fossen et al., 1999
isoquercitrin	0.0281	Lu et al., 1997
triterpenes and related compounds		
2α,3β,23-trihydroxyolean-12-en-28-oic acid	0.0035	Lee et al., 2008
2α,3β,23,24-tetrahydoroxyolean-12-en-28-oic acid	0.0017	Nandy et al., 1989
3,24-(*S*)-hexahydroxydiphenoyl-2α,3β,23,24-tetrahydroxytaraxastan-28,20β-olide (**2**)	0.2655	
3,24-(*S*)-hexahydroxydiphenoyl-2α,3β,23,24-tetrahydroxyolean-12-en-28 oic acid (**3**)	0.0258	Ageta et al., 1988
castanopsinin E (**4**)	0.0341	Ageta et al., 1988
β-sitosterol-3-*O* -β-D-glucopyranoside	0.0008	Sakakibara et al., 1983
megastigmanes and abscisic acid derivative		
(*Z*)-4-ethylidene-3,5,5-trimethylcyclohex-2-enone	0.0002	Ito et al., 1997
corchoionol C	0.0014	Yoshikawa et al., 1997
corchoionoside C	0.0006	Otsuka et al., 1995
mallophenol B	0.0024	Wei et al., 2004
apocynol A	0.0002	Murakami et al., 2001
(1'*S* , 6'*R*)-8'-hydroxyabscisic acid β-D-glucoside	0.0003	Ramos et al., 2004
others		
shikimic acid	0.0035	
3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)	0.0002	Sims et al., 1976
cytidine	0.0002	

Table 2

NMR spectroscopic data of compound **1** in acetone-d_6 (^{1}H at 500 and ^{13}C at 125 MHz). Chemical shifts are given in δ values; multiplicities and coupling constants (J in Hz) in parentheses.

Position		^{1}H	^{13}C
quinic acid			
C-1			81.9
C-2	ax	2.69 (dd, 3.3, 16.2)	32.3
	eq	3.20 (ddd, 3.0, 3.3, 16.2)	
C-3		5.68 (q, 3.3)	70.1
C-4		5.19 (dd, 3.3, 9.6)	76.6
C-5		4.77 (ddd, 4.1, 9.6, 10.2)	65.2
C-6	ax	2.21 (br dd, 10.2, 12.6)	41.4
	eq	2.83 (ddd, 3.0, 4.1, 12.6)	
COO-			172.2
Benzotropolone moiety			
a			183.5
b			154.1
c		7.55 (s)	113.8
d			125.1
e		8.14 (s)	139.6
f		7.03 (s)	114.9
g			151.4
h			137.2
i			153.1
j			115.9
k			131.9
l			166.1
Galloyl at C-4			
C-1			121.6
C-2, 6		7.08 (s)	110.1
C-3, 5			145.8
C-4			138.8
COO-			166.4
Galloyl at C-5			
C-1			121.1
C-2, 6		6.86 (s)	109.7
C-3, 5			145.5
C-4			138.4
COO-			166.4

Table 3.

^{1}H and ^{13}C NMR spectroscopic data for **2** and **3** in pyridine-d_5.[a]

	2			**3**	
position	δ_H	δ_C	position	δ_H	δ_C
1	1.65 (dd, 5.3, 14.0)	46.8	1	1.60 (m)	46.4
	2.07 (dd, 5.7, 14.0)			2.01-2.03 (m)	
2	4.34 (m)	67.4	2	4.31 (m)	67.6
3	5.86 (d, 5.8)	76.4	3	5.83 (d, 6.6)	77.1
4		46.9	4		47.2
5	2.36 (d, 5.8)	44.1	5	2.40 (d, 5.9)	44.6
6	1.82-1.84 (2H, m)	18.7	6	1.89-1.93 (m)	18.8
7	1.26-1.29 (2H, m)	33.8	7	1.26-1.30 (2H, m)	33.1
8		40.8	8		40.1
9	1.53-1.57 (m)	51.5	9	1.88-1.92 (m)	48.3
10		36.6	10		36.7
11	0.89 (m)	21.9	11	2.01-2.04 (m)	24.1
	1.36 (m)			1.85-1.93 (m)	
12	1.55-1.59 (2H, m)	25.4	12	5.50 (brs)	125.5
13	1.12-1.15 (m)	43.3	13		144.8
14		41.3	14		
15	1.09-1.13 (m)	27.5	15	1.14-1.19 (m)	42.3
	2.19 (m)			2.09 (dd, 3.3, 13.2)	
16	1.21 (m)	27.9	16	1.90-1.95 (2H, m)	23.7
	1.93 (m)				
17		42.2	17		46.7
18	1.00-1.05 (m)	48.2	18	3.27 (dd, 4.3, 13.6)	42.1
19	1.56-1.60 (m)	42.3	19	1.23-1.27 (m)	46.3
				1.72-1.76 (m)	
20		83.9	20		30.9
21	1.42-1.46 (m)	27.2	21	1.11-1.12 (2H, m)	34.2
	1.77 (m)				
22	1.46-1.51 (2H, m)	32.1	22	1.42 (2H, m)	33.1
23	4.13 (d, 10.9)	64.9	23	4.13 (d,10.9)	64.4
	4.41 (d, 10.9)			4.43 (d, 10.9)	
24	3.98 (d, 11.3)	65.1	24	4.00 (d, 11.4)	65.1
	5.40 (d, 11.3)			5.44 (d, 11.4)	
25	1.01 (3H, s)	18.9	25	1.04 (3H, s)	17.8
26	0.84 (3H, s)	15.7	26	0.98 (3H, s)	17.3
27	0.74 (3H, s)	14.2	27	1.14 (3H, s)	26.1
28		176.6	28		180.1
29	0.83-0.84 (3H, m)	18.5	29	0.98 (3H, s)	33.2
30	1.23 (3H, s)	24.1	30	0.90 (3H, s)	23.7
HHDP			HHDP		
1, 1'		115.5, 116.4	1, 1'		115.1, 116.5
2, 2'		126.8, 127.8	2, 2'		126.7, 128.2
3, 3'	7.21 (s), 7.23 (s)	107.9 (2C)	3, 3'	7.24 (s), 7.25 (s)	108.1 (2C)
4, 4'		146.3, 146.4	4, 4'		146.3, 146.4
5, 5'		137.4, 137.9	5, 5'		137.2, 138.1
6, 6'		146.6 (2C)	6, 6'		146.6 (2C)
7, 7'		169.4, 169.6	7, 7'		169.4, 169.7

[a] ^{1}H at 500 and ^{13}C at 125 MHz. Multiplicities and coupling constants (J in Hz) in parentheses.

Table 4.

ORAC values of major compounds obtained from *C. fissa* .

	mmol Trolox equivalent/g
gallic acid	6.4
1,3,4,6-tetra-*O* -galloyl-β-D-glucose	3.1
1,4,5-tri-*O* -galloylquinic acid	6.5
1,4-di-*O* -galloylquinic acid	7.0
pedunculagin	6.4
1(β)-*O* -galloyl pedunculagin	3.5
compound **2**	1.5
compound **3**	2.1
compound **4**	6.0
myricitrin	11.3