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ORIGINAL ARTICLE

New glycosidic constituents from fruits of *Lycium chinense* and their antioxidant activities

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Abstract Potential biologically active new constituents labd-3β, 9β-diol-3α-D-glucopyranosyl-(2a → 1b)-α-D-glucopyranosyl-(2b → 1c)-α-D-glucopyranosyl-(2c → 1d)-α-D-arabinofuranosyl-2d-p-hydroxybenzoate (**1**) and α-D-glucuronopyranosyl (2 → 1′)-α-D-glucuronopyranosyl (2′ → 1″)-α-D-glucopyranosyl-2″-*n*-octadec-9‴-enoate (**2**) along with β-sitosterol-β-D-glucoside were isolated from the fruits of *Lycium chinense*. Their chemical structures were elucidated using detailed spectroscopic studies. The structure assignments are based on two-dimensional (2D)-NMR techniques including COSY, HSQC, HMBC and NOESY experiments. Compounds **1** and **2** were evaluated for antioxidant activities with three assay protocols such as diphenylpicrylhydrazyl (DPPH) radical scavenging activity, reducing power and the phosphomolybdenum activity, compound **2** showed more potential as compared with **1**.

1. Introduction

Lycium chinense Miller fruits (Fructus Lycii) known as 'Gou-Qi-Zi' in Chinese have long history of application as a valuable tonic and health food supplement for improving vision and maintaining good health. It is reputed to have properties like nourishing the blood, enriching the yin, tonifying the kidney and the liver, and moistening the lungs (Peng et al., 2005). Fruits of *L. chinense* (Solanaceae), distributed in northeast Asia, especially China, Japan, Korea, and Taiwan, have been widely used as a tonic in traditional medicine. Potentially hepatoprotective glycolipid constituents and determination of betain in *L. chinense* fruits have been reported (Jung et al., 2005; Shin et al., 1999). Antimicrobial compounds have also been reported from *L. chinense* roots (Lee et al., 2005). The plant is reported to possess antibacterial, anticancer and antioxidant properties (Lee et al., 2005; Zhang et al., 2011; Wang et al., 2010). Antihepatotoxic activity and chemical constituents from *L. chinense* fruits have been reported (Chin et al., 2003). Variation in fruit sugar zcomposition of *Lycium barbarum* and *L. chinense* of different regions and varieties was also reported (Zheng et al., 2010). Evaluation of antioxidant and other activities of compounds from *L. barbarum* and *L. chinense* has also been reported (Li et al., 2007; Ming et al., 2009). Some compounds were reported in recent reports from *L. chinense* fruits (Jung et al., 2012; Chung

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et al., 2013). As part of our ongoing investigations on the biologically active compounds from *L. chinense* fruits, we report here the isolation and identification of two new compounds (**1**–**2**) together with known compound from the methanolic extract of fruits of *L. chinense*. New compounds **1** and **2** were evaluated for antioxidant activities with three assay protocols such as diphenylpicrylhydrazyl (DPPH) radical scavenging activity, reducing power and the phosphomolybdenum activity. The approach developed has proved useful in the study of the active constituents in traditional Chinese medicines like *L. chinense*.

2. Experimental

2.1. General information

All chemicals used were of analytical grade. Hexane, ethyl acetate, chloroform, methanol, ethanol, water, sulphuric acid and vanillin were purchased from Daejung Chemicals and Metals Co. Ltd, Korea. Pre-coated TLC plates (layer thickness 0.25 mm), silica gel for column chromatography (70–230 mesh ASTM) and LiChroprep RP-18 (40–63 μm) were from Merck (Darmstadt, Germany). Previously isolated authentic standard of *β*-sitosterol-*β*-D-glucoside is available. Optical rotation was measured on an AA-10 model polarimeter (Instruments Ltd, Seoul, South Korea). Both ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 600 high resolution spectrometer operating at 600 and 150 MHz, respectively. This NMR machine was available at the Seoul National University (SNU), Seoul, South Korea and all NMR spectra were recorded at SNU. NMR spectra were obtained in deuterated methanol using tetramethylsilane (TMS) as an internal standard, with chemical shifts expressed in ppm (δ) and coupling constants (J) in Hz. FAB MS data were recorded on a JMS-700 (Jeol, Japan) spectrometer instrument which was available at SNU, Seoul, South Korea. IR spectra were recorded on an Infinity Gold FT-IR (Thermo Mattson, USA) spectrophotometer, which was available at Korea Institute of Science and Technology, Seoul, South Korea. The sugars were determined using high performance liquid chromatography (HPLC, Waters Milfords, MA, USA) with a universal evaporative lights scattering detector, column Eurospher 100 NH_2, detector differential refractometer R401, mobile phase acetonitrile:water (4:1), flow rate of 1.0 ml/min, ambient temperature and 2 Mpa pressure standard samples of sugars were obtained from Merck (Germany). The sugar solutions injected into the column calibration lines for each sugars were made, which were later used for assessing the concentrations corresponding to the different peaks in the chromatograms.

2.2. Preparation of the extracts

The fruits of *L. chinense* (3.1 kg) were immersed in methanol (8 L) for three days at room temperature and then the supernatant was concentrated under vacuum to yield 230 g of the extract, which was suspended in water and extracted with hexane, ethyl acetate and *n*-butanol successively to produce 20.0 g, 10.1 g and 40 g of the extracts respectively.

2.3. Isolation of the compounds from n-butanol extract

The entire butanol extract was subjected to normal phase column chromatography over silica gel (600 g) to yield 24 fractions (each of 500 mL) with the following eluants: fractions 1–2 with chloroform, fractions 3–4 with chloroform:methanol (9.8:0.2, *V*:*V*), fractions 5–6 with chloroform:methanol (9:6:0.4, *V*:*V*), fractions 7–8 with chloroform:methanol (9.4:0.6, *V*:*V*), fractions 9–10 with chloroform:methanol (9.2:0.8, *V*:*V*), fractions 11–12 with chloroform:methanol (9:1, *V*:*V*), fractions 13–14 with chloroform:methanol (8.8:1.2, *V*:*V*), fractions 15–16 with chloroform:methanol (8.5:1.5, *V*:*V*), fractions 17–18 with chloroform:methanol (8:2, *V*:*V*), fractions 19–20 with chloroform:methanol (8.5:2.5, *V*:*V*) and fractions 21–24 with methanol. All fractions were examined by TLC. Fractions 1–4 were not further separated due to the low amount of the substance. Fractions 7–8 (0.9 g) were crystallized after purification by column chromatography, yielding *β*-sitosterol-*β*-D-glucoside (70 mg) whose identity was confirmed through comparison of TLC and spectroscopic data with those of an authentic sample. Fractions 13–14 (4.4 g) were re-chromatographed over LiChroprep RP-18 (ODS silica gel; 40–63 μm: 200 g; each fraction 100 mL). The elution was sequentially performed with methanol and water to yield 20 fractions with the following eluants: fractions 1–4 with water:methanol (8:2, *V*:*V*), fractions 5–8 with water:methanol (6:4, *V*:*V*), fractions 9–12 with water:methanol (4:6, *V*:*V*), fractions 13–16 with water:methanol (2:8, *V*:*V*), fractions 17–20 with methanol. Fractions 9–12 were rechromatographed over Lichroprep RP18 ODS (80 g, each fraction of 50 mL). The elution was sequentially performed with methanol containing 80%, 60%, 40%, 20%, 10%, and 0% of water to yield two new compounds **1** and **2**.

3. Spectral data

3.1. Labd-3β, 9β-diol-3α-D-glucopyranosyl-(2a → 1b)-α-D-glucopyranosyl-(2b → 1c)-α-D-glucopyranosyl-(2c → 1d)-α-D-arabinofuranosyl-2d-p-hydroxybenzoate (*1*)

Light yellow viscous; $[\alpha]_D^{20}$ + 25.1 (c 0.13, MeOH); ^{1}H NMR (MeOD, 600 MHz) δ: 1.79, 1.84 (m, 2H, H-1), 1.98, 2.03 (m, 2H, H-2), 3.70 (dd, J = 4.9, 9.6 Hz, 1H, H-3), 2.27 (dd, J = 7.2, 7.8 Hz, 1H, H-5), 1.53, 1.58 (m, 2H, H-6), 1.56, 1.60 (m, 2H, H-7), 1.96 (m, 1H, H-8), 1.67 (t, J = 6.0 Hz, 2H, H-11), 1.33, 1.37 (m, 2H, H-12), 1.60 (m, 1H, H-13), 1.41, 1.43 (m, 2H, H-14), 0.90 (t, J = 6.6 Hz, 3H, H-15), 1.25 (br s, 3H, H-16), 1.29 (br s, 3H, H-17), 0.92 (d, J = 7.2 Hz, 3H, H-18), 1.27 (br s, 3H, H-19), 0.95 (d, J = 7.8, 3H, H-20), 5.33 (d, J = 4.8, 1H, H-1a), 3.88 (dd, J = 4.8, 5.2 Hz, 1H, H-2a), 3.87 (m, 1H, H-3a), 3.83 (m, 1H, H-4a), 4.15 (m, 1H, H-5a), 3.30 (br s, 2H, H-6a), 5.31 (d, J = 5.0 Hz, 1H, H-1b), 3.73 (dd, J = 3.6, 5.5 Hz, 1H, H-2b), 3.81 (m, 1H, H-3b), 3.65 (m, 1H, H-4b), 3.82 (m, 1H, H-5b), 3.32 (br s, 2H, H-6b), 4.36 (d, J = 6.0 Hz, 1H, H-1c), 4.01 (dd, J = 4.2, 6.0 Hz, 1H, H-2c), 3.69 (m, 1H, H-3c), 3.56 (m, 1H, H-4c), 3.97 (m, 1H, H-5c), 3.28 (br s, 2H, H-6c), 4.63 (d, J = 5.9 Hz, 1H, H-1d), 4.12 (dd, J = 4.8, 5.9 Hz, 1H, H-2d), 3.62 (m, 1H, H-3d), 4.31 (m, 1H, H-4d), 3.34 (br s, 2H, H-5d), 7.62 (dd, J = 3.0, 7.2 Hz, 1H, H-2′), 7.71 (dd, J = 2.9, 8.5 Hz, 1H, H-3′), 7.70 (dd, J = 2.9, 7.9 Hz, H-5′), 7.60 (dd, J = 3.0, 7.9 Hz, 1H, H-6′); ^{13}C NMR (MeOD, 150 MHz) δ: 30.2 (C-1), 26.2 (C-2), 79.0 (C-3), 56.5 (C-4), 40.3 (C-5), 21.4 (C-6), 30.6 (C-7), 31.7 (C-8), 78.8 (C-9), 35.2 (C-10), 30.9 (C-11), 30.7 (C-12), 33.2 (C-13), 20.3 (C-14),

11.5 (C-15), 23.8 (C-16), 24.1 (C-17), 14.5 (C-18), 25.1 (C-19), 14.5 (C-20), 105.3 (C-1a), 84.8 (C-2a), 71.6 (C-3a), 64.5 (C-4a), 74.0 (C-5a), 60.6 (C-6a), 101.3 (C-1b), 83.6 (C-2b), 66.9 (C-3b), 64.6 (C-4b), 78.0 (C-5b), 61.6 (C-6b), 101.1 (C-1c), 82.7 (C-2c), 66.8 (C-3c), 64.8 (C-4c), 77.1 (C-5c), 62.9 (C-6c), 109.3 (C-1d), 87.5 (C-2d), 65.4 (C-3d), 89.4 (C-4d), 64.4 (C-5d), 133.7 (C-1′), 132.5 (C-2′), 124.9 (C-3′), 150.0 (C-4′), 123.4 (C-5′), 130.1 (C-6′), 169.4 (C-7′); IR (KBr) ν_{max}: 3510, 3420, 3395, 2922, 2852, 1738, 1625, 1556, 1430, 1380, 1246, 1072, 1028 cm^{-1}; FAB-MS (positive ion mode) *m/z* 1049 $[M + H]^+$ ($C_{50}H_{81}O_{23}$) (1.5), 366 (10.5), 269 (15.7), 366 (10.5), 269 (15.7), 253 (11.3), 137 (23.1), 121 (82.6); HR-FABMS (positive mode) *m/z* 1049.5159 [M + H] (calculated for $C_{50}H_{81}O_{23}$, 1049.5169); ESI Mass (positive mode) *m/z* 413 (M + H -3 glucose + 1furanose).

*3.2. α-D-glucuronopyranosyl (2 → 1)-α-D-glucuronopyranosyl (2 → 1)-α-D-glucopyranosyl -2″-n-octadec-9‴-enoate (**2**)*

Dark yellow semi-solid; $[\alpha]_D^{20}$ + 33.1 (c 0.23, MeOH); ^1H NMR (MeOD, 600 MHz) *δ*: 4.49 (d, *J* = 6.6 Hz , 1H, H-1), 4.09 (dd, *J* = 6.1, 6.6 Hz, 1H, H-2), 3.71 (m, 1H, H-3), 3.64 (m, 1H, H-4), 3.96 (d, *J* = 7.2 Hz, 1H, H-5), 4.83 (d, *J* = 6.8 Hz, H-1′), 4.01 (dd, *J* = 5.8, 6.6 Hz, H-2′), 3.69 (m, 1H, H-3′), 3.57 (m, 1H, H-4′), 3.75 (d, *J* = 6.6 Hz, 1H, H-5′), 5.01 (d, *J* = 6.9 Hz, 1H, H-1″), 4.35 (dd, *J* = 6.6, 6.9 Hz, 1H, H-2″), 3.66 (m, 1H, H-3″), 3.49 (m, 1H, H-4″), 3.78 (m, 1H, H-5″), 3.27 (br s, 2H, H-6″), 2.80 (d, *J* = 4.2 Hz, 2.77, *J* = 4.1 Hz, 2H, H-2‴), 2.42 (m, 2H, H-3‴), 2.20 (m, 2H, H-4‴), 1.98 (m, 2H, H-5‴), 1.96 (m, 4H, H-6‴ & 7‴), 2.62 (m, 2H, H-8‴), 5.02 (m, 2H, H-9‴ & 10‴), 2.58 (m, 2H, H-11‴), 2.30 (m, 2H, H-12‴), 2.01 (m, 2H, H-13‴), 1.96 (m, 2H, H-14‴), 1.49 (m, 2H, H-15‴), 1.33 (m, 2H, H-16‴), 1.25 (br s, 2H, H-17‴), 0.89 (t, *J* = 7.2 Hz, H-18‴); ^{13}C NMR (MeOD, 150 MHz) *δ*: 97.8 (C-1), 74.9 (C-2), 73.2 (C-3), 72.3 (C-4), 78.1 (C-5), 179.4 (C-6), 98.3 (C-1′), 74.3 (C-2′), 73.0 (C-3′), 71.9 (C-4′), 77.7 (C-5′), 176.3 (C-6′), 99.4 (C-1″), 83.3 (C-2″), 71.2 (C-3″), 69.6 (C-4″), 76.4 (C-5″), 62.8 (C-6″), 169.0 (C-1‴), 54.0 (C-2‴), 39.1 (C-3‴), 32.4 (C-4‴), 32.0 (C-5‴), 27.5 (C-6‴ & C-7‴), 41.5 (C-8‴), 120.9 (C-9‴), 116.9 (C-10‴), 40.7 (C-11‴), 27.5 (C-12‴), 26.7 (C-13‴ & C-14‴), 27.0 (C-15‴ & C-16‴), 22.6 (C-17‴), 15.0 (C-18‴); IR ν_{max}: (KBr): 3410, 3375, 3265, 2930, 2843, 1737, 1709, 1628, 1477, 1395, 1334, 1081, 980, 895 cm^{-1}; FAB-MS (positive mode) *m/z* (rel. int.) 797 $[M + H]^+$ ($C_{36}H_{61}O_{19}$ (2.1), 427 (11.3), 370 (6.8), 281 915.8), 265 (9.7), 193 (9.8); HRFAB MS (positive mode) *m/z* 797.3798 $[M + H]^+$ (calculated for $C_{36}H_{61}O_{19}$, 797.3807); ESI Mass (positive mode) *m/z* 426 [M-2 glucoronic acid]$^+$ and 829 $[M\text{-}H + H_2O_2]^+$.

*3.3. Acid hydrolysis of compound **1***

Compound **1** (10 mg) was refluxed with 2 mL of 1 mol/L hydrochloric acid:dioxane (1:1, *V*:*V*) in a water bath for 4 h. The reaction mixture was evaporated to dryness and partitioned with chloroform and water four times, and each extract was concentrated. The chloroform extract contained the aglycone portion, while the water extract possessed glycone portion (co-chromatographed on TLC ($CHCl_3$:CH_3OH:H_2O:AcOH at 16:9:2:2) with authentic sample determined by HPLC.

*3.4. Acid hydrolysis of compound **2***

A solution of compound **2** (10 mg in tetrahydrofuran) was added to 0.5 mL 1 N HCl and stirred at 80 °C for 4 h. After cooling, the reaction mixture was diluted with H_2O and extracted with EtOAc (3 ml × 3), yielded oleic acid and the aqueous layer was subjected to TLC ($CHCl_3$:CH_3OH:H_2O:AcOH at 16:9:2:2) together with an authentic sample of D-glucose and to HPLC.

4. Antioxidant activity

Three assay protocols such as diphenylpicrylhydrazyl (DPPH) radical scavenging activity, reducing power and the phosphomolybdenum activity were used for the evaluation of antioxidant activity of compounds (**1**–**2**) as described below:

4.1. Free radical scavenging activity

The antioxidant activity of compounds **1** and **2** based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl ($DPPH^-$) free radical, was determined by the method described by Katerere and Eloff (2005). The method is based on the reduction of methanolic $DPPH^-$ solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH–H by the reaction (Blois, 1958). Different concentrations (1.0, 2.0, 3.0, 4.0, and 5.0 mg of **1** and 50, 100, 150, 200, and 250 μg of **2**) of the tested samples (0.2 ml; compounds and BHT) were taken in different test tubes with 4 ml of a 0.006% MeOH solution of $DPPH^-$. Water (0.2 ml) in place of the compound was used as control. Absorbance at 517 nm was determined after 30 min of incubation at 37 °C. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula, % Radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the compound/standard.

4.2. Assay of reductive potential

The reductive potential of the compound was determined according to the method of Dorman and Hiltunen (2004). The reaction mixture comprises varying concentrations of the compounds (1.0, 2.0, 3.0, 4.0, and 5.0 mg of **1** and 200, 400, 600, 800 and 1000 μg/ml of **2**) in 1 ml of distilled water, phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. BHT was used as standard. Increased absorbance of the reaction mixture indicated increased reductive potential. All analyses were run in triplicate and averaged.

4.3. Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of compounds **1** and **2** was evaluated by the method (Prieto et al., 1999). An aliquot of 0.1 ml

of sample solution (100 μg/ml) was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The results are expressed as equivalents of α-tocopherol (mg/g of compound).

5. Results and discussion

Compound **1**, was obtained as a light yellow viscous mass from chloroform–methanol (8.8:1.2, *V*:*V*) eluants. Its IR spectrum showed characteristic absorption bands for hydroxyl groups (3510, 3420, 3395 cm^{-1}), ester function (1738 cm^{-1}), and aromatic ring (1625, 1556, 1028 cm^{-1}). The FAB mass and ^{13}C NMR spectral data led to an established molecular formula ion peak at *m*/*z* 1048 consistent with the molecular formula of a diterpenic tetraglycoside esterified with an aromatic acid, $C_{50}H_{80}O_{23}$. The ion peaks arising at *m*/*z* 121 $[HOC_6H_4CO]^+$, 137 $[HOC_6H_4COO]^+$, 253 $[HOC_6H_4CO–C_5H_8O_4]^+$, and 269 $[HOC_6H_4CO–C_5H_8O5]^+$, indicated that the aromatic acid was linked to a hexose sugar unit. The fragmentation pattern of compound **1** is shown in Fig. 2.

The ^1H NMR spectrum of **1** showed four one-proton double doublets at δ 7.62 (*J* = 3.0, 7.2), 7.71 (*J* = 2.9, 8.5 Hz), 7.70 (*J* = 2.9, 7.9) and 7.60 (*J* = 3.0, 7.9 Hz) assigned correspondingly H-2′, H-3′, H-5′ and H-6 suggesting AA′ BB′ system. Four one-proton doublets at δ 5.33 (*J* = 4.8 Hz), 5.31 (*J* = 5.0 Hz), 4.36 (*J* = 6.0 Hz), and 4.63 (*J* = 6.0 Hz) and 4.63 (*J* = 5.9 Hz) were attributed to α-oriented anomeric H-1a, H-1b, H-1c and H-1d protons, respectively. The other sugar protons appeared between δ 4.15 and 3.28. A one proton double doublet at δ 3.70 with coupling interactions of 4.9, 9.6 Hz was accounted to oxygenated methine H-3α proton.

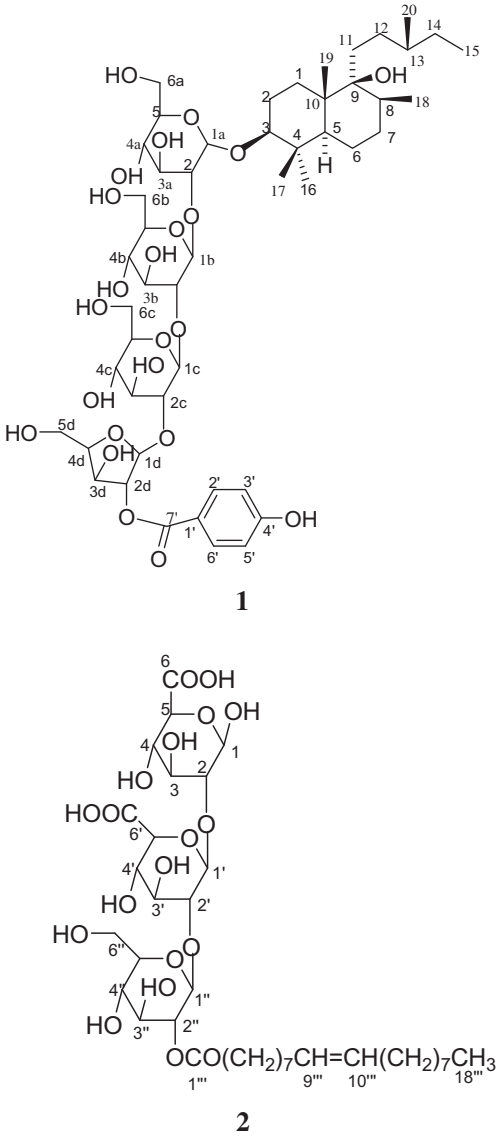

Figure 1 Chemical structures of compounds **1** and **2**.

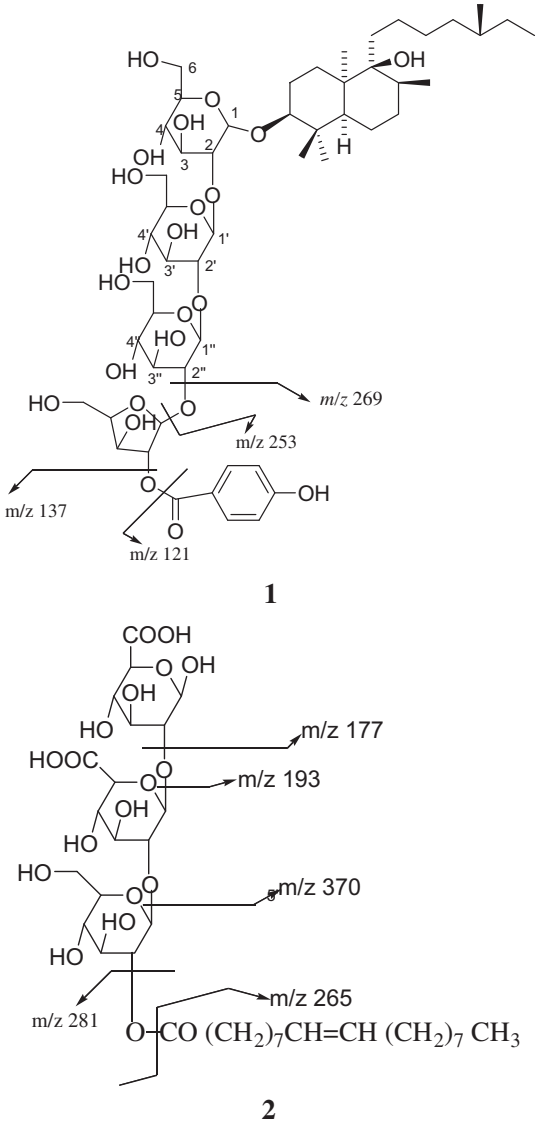

Figure 2 Mass fragmentation pattern of compounds **1** and **2**.

Three broad signals at δ 1.25, 1.29 and 1.27 and two doublets at δ 0.92 (J = 7.2 Hz) and 0.95 (J = 7.8 Hz), all integrated for three protons each were ascribed to tertiary methyl Me-16, Me-17 and Me-19 and secondary methyl Me-18 and Me-20 respectively. A three proton triplet at 0.90 (J = 6.6 Hz), was due to primary methyl Me-15 proton. The remaining methylene and methine resonated from δ 2.27 to 1.33.

The ^{13}C NMR spectrum of **1** and its one-dimensional modifications (ATP) showed that the compound was a glycoside with four sugar residues. The ^{13}C NMR spectrum displayed signals for aromatic carbons from δ 150.03 to 123.40, ester carbon at δ 169.49 (C-7′), anomeric carbon at δ 105.37 (C-1a), 101.35 (C-1b), 101.16 (C-1c), and 109.36 (C-1d), the other sugar carbons between δ 89.42 and 60.68, oxygenated methine carbon at δ 79.08 (C-3), hydroxyl-substituted quaternary carbon at δ 78.84 (C-9), and other labdane carbons from δ 56.55 to 11.55. The presence of two sugar signals in the deshielded region at δ 109.36 (C-1d), and 89.42 (C-4d) suggested α-furanoarabinose moiety in the sugar chain. The presence of C-2a at δ 84.81, C-2b at δ 83.65, C-2c at δ 82.72 and C-2d at δ 87.75 supported $C_{2\rightarrow1}$ linkages of the sugar units. Analysis of the spin–spin coupling constants of the anomeric carbon atoms to anomeric protons established that the sugars adopted the furanose form with an axial anomeric proton.

The ^{1}H–^{1}H COSY spectrum of **1** showed correlations of H-3 with H_2-2, H_3-16 and H-1a; H-2a with H-1a, H-3a and H-1b; H-2c with H-1c, H-3c and H-1d; H-2′ with H-3′ and H-6′; H-5 with Me-16 and H_2-6; and Me-20 with H-13, Me-15 and H_2-14. The HMBC spectrum of **1** exhibited interactions of C-3 with H_2-2, H_3-17 and H-1a; C-9 with H-8, H_3-18 and H_3-19; C-2a with H-3a and H-1b; C-1d with H-2d and H-2c; C-4d with H_2-5d, H-3d and H-2d; and C-7′ with H-2′, H-6′ and H-2d. The HSQC experiment showed key-correlations between the proton H-3 at δ 3.70 and C-3 at δ 79.08; H-1a at δ 5.33 with C-1a at δ 105.37; H-1b at δ 5.31 with C-1b at δ 101.35; H-1c at δ 4.36 with C-1c at δ 101.16; H-1d at δ 4.63 with C-1d at δ 109.36 and aromatic protons with respective carbon signals. The COSY and TCOSY spectra showed close spin systems belonging to sugar protons and enabled a determination of the monosaccharide composition of the carbohydrate part of each glycoside. These spectra exhibited a series of close spin systems for the protons of rings A and B of the aglycone and enabled their partial assignment. The ROESY spectrum showed the bonding sequence of residues in the tetrasaccharide and the site of attachment of the sugars to the aglycone. The ROESY spectrum of **1** contained the usual correlation peaks for a furanose with axial anomeric protons H-1a/H-2a, H-1a/H-3a, H-1a/H-5a, and H-1a/H-6a; in additions to correlation peaks H-1a/H-2b, H-1b/H-1c; H-1c/H-2c, H-1c/H-1d; H-1d/H-2c, H-1d/H-2d, H-1d/H-5d; and H-2d/H-2′ and H-2′ and 6′. This enables the sequence of residues in the tetrasaccharide. The NOESY spectrum of **1** showed correlations of H-3α with Me-16 and H-5α; Me-19 with Me-18, H_2-1 and H_2-11.

Acid hydrolysis of **1** yielded glucose and arabinose as sugars (co-TLC and HPLC comparable). On the basis of the

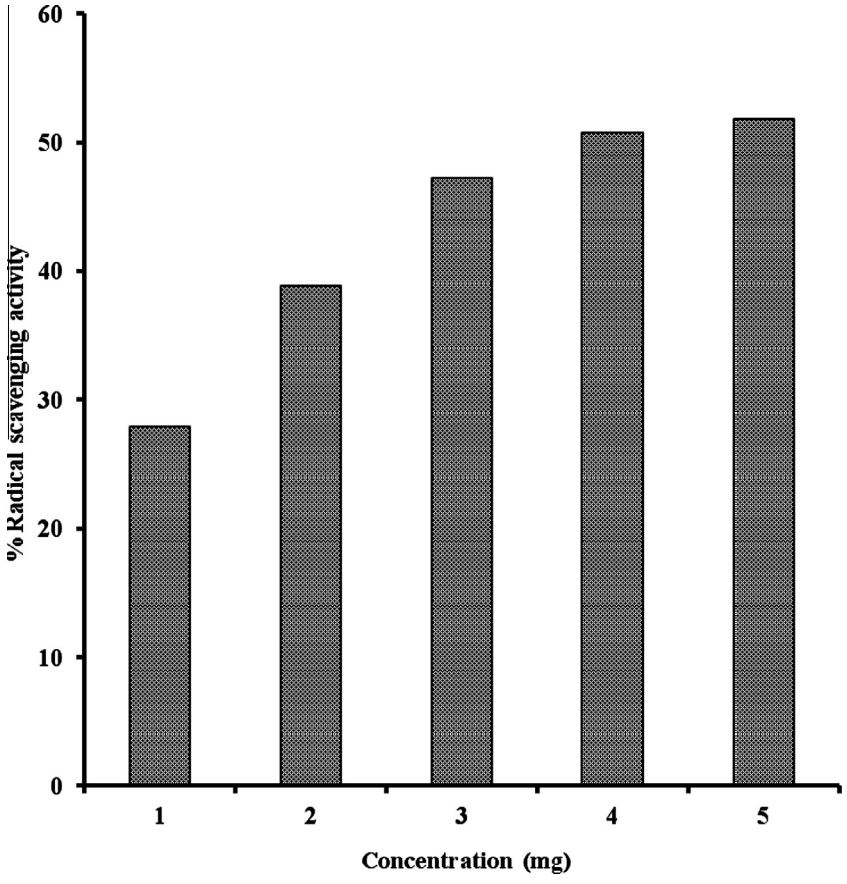

Figure 3 Antioxidant activity of compound **1** at different concentration levels as measured by DPPH radical scavenging activity.

foregoing discussion the structure of **1** has been established as labd-3β, 9β-diol-3α-D-glucopyranosyl-(2 $\rightarrow$ 1)-α-D-glucopyranosyl-(2 $\rightarrow$ 1)-α-D-glucopyranosyl-(2 $\rightarrow$ 1)-α-D-arabinofuranosyl-2d-p-hydroxybenzoate. This is a new diterpene glycoside.

Compound **2** (Fig. 1), was obtained as dark yellow viscous mass from chloroform–methanol (8.8:1.2; *V*:*V*) eluants. It gave positive test for glycosides. Its IR spectrum showed characteristic absorption bands for hydroxyl groups (3410, 3375, 3265 cm^{-1}), ester group (1737 cm^{-1}), carboxylic function (1709 cm^{-1}) and unsaturation (1628 cm^{-1}). On the basis of FAB mass and ^{13}C NMR spectra the molecular weight of **2** was determined at *m*/*z* 797 $[M + H]^+$ corresponding to the molecular formula of a triglycosidic fatty acid ester $C_{36}H_{61}O19$. The ion peaks arising at 265 $[CH_3(CH_2)_7CH{=}CH(CH_2)_7CO]^+$, 281 $[CH_3(CH_2)_7CH{=}CH(CH_2)_7COO]^+$ and 427 $[C_6H_{10}O_6$–oleate$]^+$ suggested that oleic acid moiety was attached to the C_6-sugar unit. The ion peaks arising at 193 $[C_5H_8O_5COOH]^+$, and 370 $[C_5H_8O_5$-$COOH$–$C_5H_7O_4COOH]^+$ supported the presence of two glucoronosidic units in the molecule. The fragmentation pattern of compound **2** is shown in Fig. 2.

The ^1H NMR spectrum of **2** exhibited a two-proton multiplet at δ 5.02 assigned to vinylic H-9‴ and H-10‴ protons respectively. Three one-proton doublets at δ 4.49 (J = 6.6 Hz), 4.83 (J = 6.8 Hz) and δ 5.10 (J = 6.9 Hz) were ascribed to α-oriented H-1, H-1′ and H-1″ anomeric protons respectively. The other sugar protons appeared from δ 4.35 to 3.27. The presence of H-2, H-2′ and H-2″ signals as double doublets in the deshielded region at δ 4.09 (J = 6.6, 6.1 Hz), 4.01 (J = 6.6, 5.8 Hz), 4.35 (J = 6.6, 6.9 Hz) respectively indicated (2 $\rightarrow$ 1) linkage of the sugar units and location of ester function at C-2″. A three-proton triplet at δ 0.89 (J = 7.2 Hz) was accounted to terminal primary C-18‴ methyl protons. The remaining methylene protons resonated between δ 2.80 and 1.25.

The ^{13}C NMR spectrum showed important signals for carboxylic carbons at δ 179.40 (C-6) and 176.35 (C-6′), anomeric carbons at δ 97.83 (C-1), δ 98.38 (C-1′), δ 99.45 (C-1″), and other sugar carbons from δ 83.33 to 62.89. The presence of C-2″ in the deshielded region at δ 83.33 supported the existence of the function at this carbon. The signals for fatty acids chain resonated for unsaturated carbons at δ 120.93 (C-9‴) and 116.80 (C-10‴), methylene carbons from δ 54.01 to 22.63 and methyl carbon at δ 15.06 (C-18‴).

The ^1H–^1HCOSY spectrum of **2** showed interactions of H-1′ with H-2′, H-3′ and H-2; H-1″ with H-2″ and H-2′; H-9‴ with H-8‴ and H_2-11‴; and H_2-6″ with H-5″. The HMBC spectrum of **2** exhibited correlations of C-6 with H-5 and H-4; C-1′ with H-2, H-2′, H-3′; C-1″ with H-2′ and H-2″; C-1‴ with H-2″; and

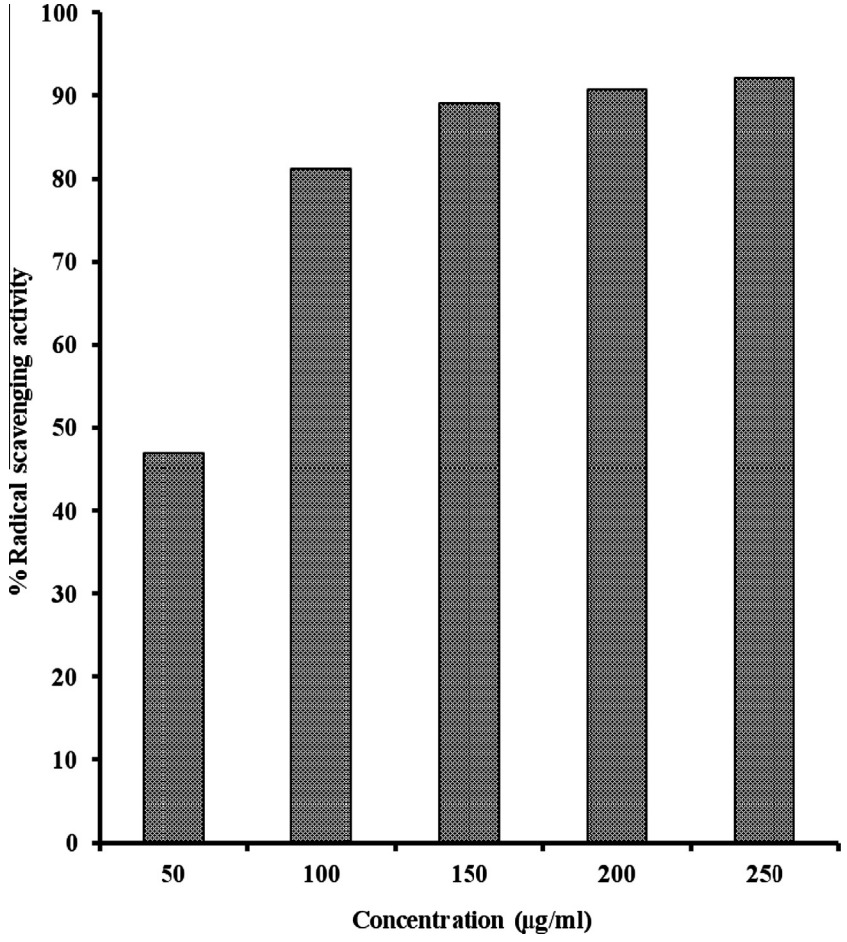

Figure 4 Antioxidant activity of compound **2** at different concentration levels as measured by DPPH radical scavenging activity.

C-9‴ with H-10‴, H_2-8‴ and H-11‴. The HSQC spectrum of **2** showed important correlations of anomeric H-1, H-1′ and H-1″ with C-1, C-1′ and C-1″; H-2 at δ 4.09 with C-2 at δ 74.93; H-2″ at δ 4.35 with C-2″ at δ 83.33; and vinylic protons at δ 5.02 with C-9‴ and C-10‴. The ROESY spectrum of **2** showed correlation signals for axial anomeric protons H-1 with pyranose protons H-2, H-3, H-5 and H-1′; H-2′ with H-1′, H-3′, H-4′ and H-1″; and H-2″ with H-1″, H-3″ and H_2-2‴. This enables the sequence of sugar units and site of attachment of the trisaccharide to the aglycone.

Acid hydrolysis of **2** yielded oleic acid, glucoronic acid and glucose. On the basis of above evidences, the structure of **2** has been elucidated as α-D-glucuronopyranosyl (2 → 1)-α-D-glucuronopyranosyl (2 → 1)-α-D-glucopyranosyl-2″-n-octadec-9‴-enoate. This is a new glycosidic ester.

6. Biological activity

6.1. Free radical scavenging activity

Figs. 3 and 4 show the concentration dependent antioxidant activity of compounds **1** and **2** at different concentration levels as measured by the DPPH-radical scavenging assay. Compounds **1** and **2** were able to reduce the stable radical $DPPH^{-}$ to the yellow-coloured diphenylpicrylhydrazine. The IC_{50} value of compounds **1** and **2** were 3.94 mg/ml and 53.31 μg/ml respectively. The DPPH activity of BHT showed a higher degree of free radical-scavenging activity than that of the compound at very low concentration points. The DPPH activity of BHT exhibited 92.04% at 50 μg/ml concentration with an IC_{50} value of 27.16 μg/ml (data not shown). This is similar to other studies wherein they have reported that only 0.3 mg/ml tocopherol, 0.23 mg/ml BHT and 0.1 mg BHA exhibited a free radical scavenging activity equivalent to 3.9 mg/ml of red bean and 10 mg/ml of sesame coat extract (Chang et al., 2002; Chung et al., 2002).

6.2. Reducing power

As shown in Figs. 5 and 6 reducing power of compounds **1** and **2** increased with increasing concentration from 1.0 to 5.0 mg/ml for compound **1** and 200 to 1000 μg/ml for compound **2**. The activity of BHT was higher than the test samples at each concentration points (data not shown). This is in accordance with the observations of several other workers wherein the reducing power of BHT and tocopherol (Chung et al., 2002) and BHA (Oktay et al., 2003) was higher than the extracts. In the present study, compounds **1** and **2** from the butanol fraction of methanol extract of lycium fruits exhibited a good reducing power.

6.3. Antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of compounds **1** and **2** was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of compounds **1** and **2** was found to be 115. 95 and 131.74 mg/g respectively.

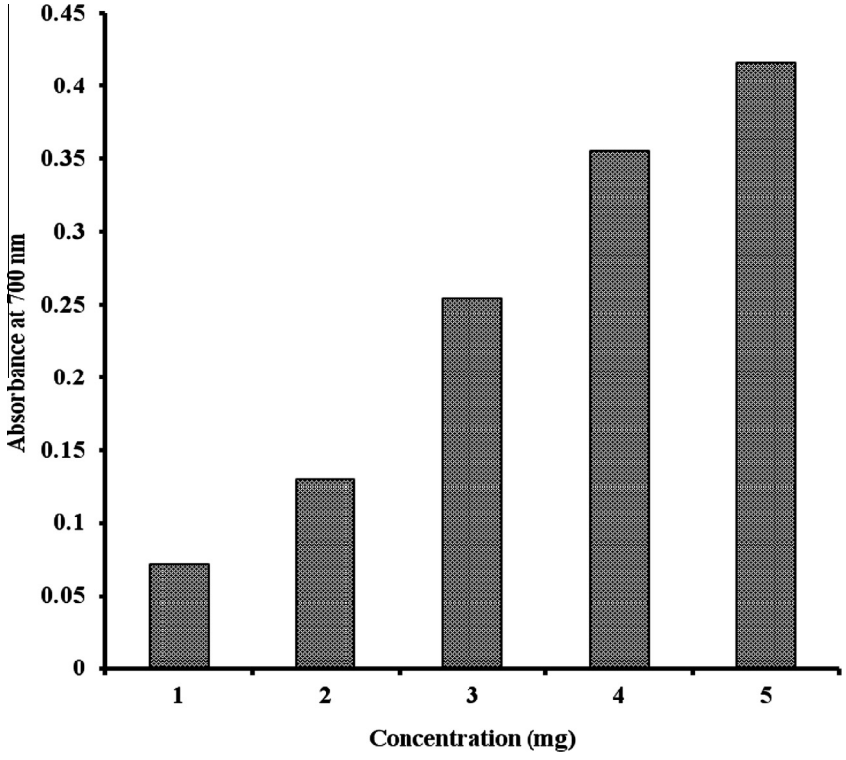

Figure 5 Reducing power of compound **1** at different concentration levels.

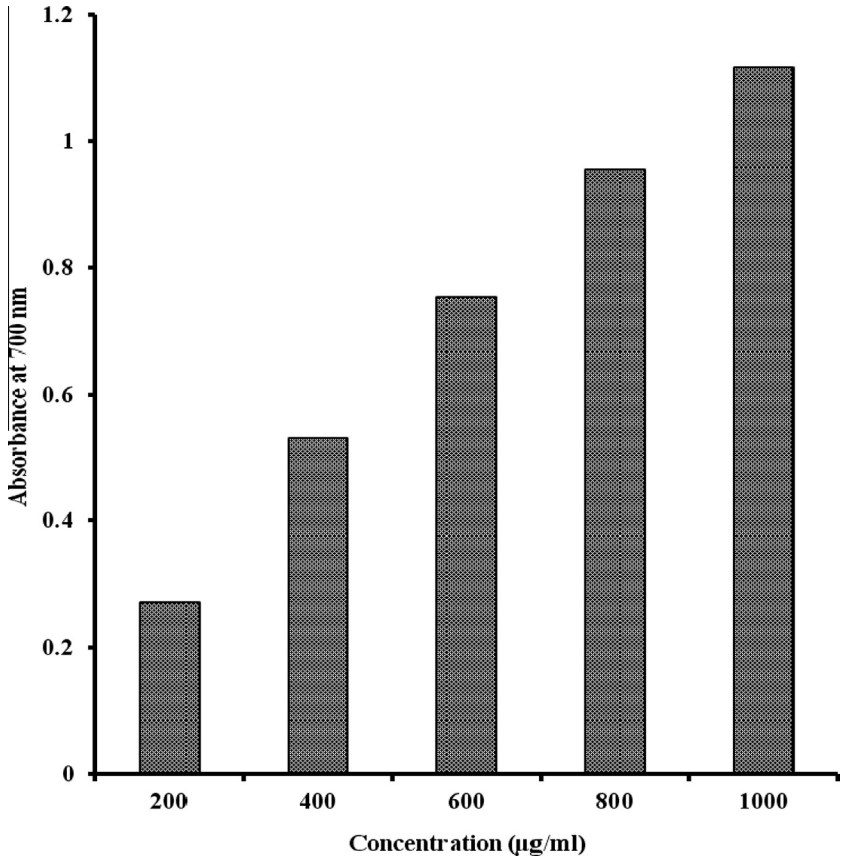

Figure 6 Reducing power of compound **2** at different concentration levels.

7. Conclusion

The new compounds **1** and **2** were isolated from the butanol fraction of methanolic extract of *L. chinense* fruits along with known compound. Compounds **1** and **2** were evaluated for antioxidant activities with three assay protocols as radical scavenging activity, reducing power and phosphomolybdenum activity, compound **2** showed more potential as a natural antioxidant as compared with **1**. The approach developed has proved useful in the study of the active constituents in traditional Chinese medicines.

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