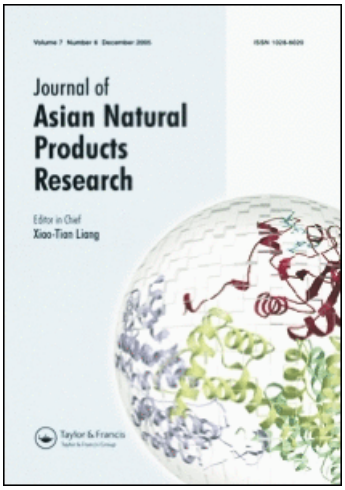

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A new lipid and other constituents from the rhizomes of *Nelumbo nucifera*

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A new lipid and other constituents from the rhizomes of *Nelumbo nucifera*

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A new ursane triterpenoid ester, *urs*-12-*en*-3β-*O*-9*E*,12*E*-octadecadienoate, was identified from the rhizomes of *Nelumbo nucifera* by spectral and chemical analyses along with the isolation of seven known compounds: palmitic acid, linoleic acid, 9*E*,12*E*,15*E*-octadecatrienoic acid, α-amyrin, β-sitosterol, betulinic acid, and β-sitosterol-3-*O*-glucoside.

Keywords: *Nelumbo nucifera*; Nelumbonaceae; triterpenoids; steroids; lipids

1. Introduction

Nelumbo nucifera Gaertn (family Nelumbonaceae), an annual aquatic herb with short rhizomes is grown and consumed over the whole world, especially in Japan, China, and India, both as a dietary supplement and medicinal herb [1]. Almost all parts of the plant are medicinally useful and showed various activities like hypotensive, antispasmodics, anti-ulcer, anti-HIV, pro-apoptosis, antidiabetic, anti-infective, and emulsifier activities [2–8]. The plant was reported to contain polysaccharides, alkaloids, terpenoids, and so on [9]. Betulinic acid is shown to have phytotoxic and antimicrobial activities in our laboratory [10]. There is no systematic chemical screening on the rhizomes of *N. nucifera*, and the present work on its rhizomes culminated in the isolation and identification of a new compound **1** along with the known compounds, palmitic acid, linoleic acid, 9*E*,12*E*,15*E*-octadecatrienoic acid, α-amyrin, β-sitosterol, betulinic acid, and β-sitosterol-3-*O*-β-D-glucoside, by spectral and chemical analyses. The identification of major chemicals is of chemotaxonomic significance with their plausible correlation to its different phenotypic abundance in nature (Figure 1).

2. Results and discussion

The concentrated MeOH extract was partitioned with hexane to afford a viscous mass, which on cooling at 5°C gave a semisolid after centrifugation. The semisolid was purified by flash chromatography to elute a low melting solid from hexane–EtOAc (1:4) fraction and showed positive L–B test for triterpenoid. GLC profile of the solid showed a major peak of compound **1** at retention time 56.75 min with a minor one at 51.00 min, respectively. The major compound **1** was separated by Ag^+-impregnated prep-TLC on Si-gel as an amorphous solid. Compound **1** in its FAB-MS showed $[M]^+$ at *m/z* 688 ($C_{48}H_{80}O_2$) and its IR (KBr) spectrum showed absorption peaks at

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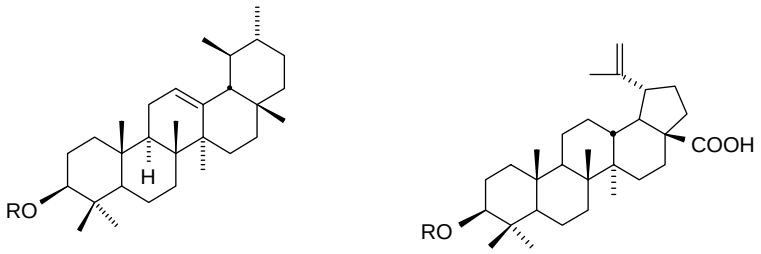
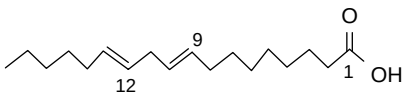


1 *Urs*-12-*en*-3β-*O*-9*E*,12*E*-octadecadienoate
1A *Urs*-12-*en*–3-ol; R = H
2 R = H; Betulinic acid
2A R = Ac; 3-*O*-acetyl betulinic acid



1B 9*E*,12*E*-octadecadienoic acid

Figure 1. Structures of compounds **1**, **2**, **1A**, **2A**, and **1B**.

2925, 1742 (ester), and 722 cm^{-1} (long chain moiety), respectively. The ^1H NMR spectrum of compound **1** (300 MHz) showed olefinic protons at δ 5.10–5.36 as a multiplet, an oxymethine proton of the ester group at δ 4.28 as double doublet ($J = 2.0, 7.5$ Hz), oxo-methylene protons at δ 2.28 (—CH_2—CO), and the methylene protons appeared at δ 1.26–1.40 with methyl signals at δ 0.81–1.02.

Compound **1** on alkaline hydrolysis after usual work up gave an alcohol **1A** and acid **1B**. The acid **1B** was identified after methylation from GC–MS analysis as methyl 9*E*,12*E*-octadecadienoate ([M^+] 294) corresponding to molecular formula ($C_{19}H_{34}O_2$). The ion peaks at *m/z* 137 and 223 in GC–MS spectra are characteristic of the double bonds at 9 and 12 positions. Compound **1A**, crystallized as colorless needles, mp 186°C, $[\alpha]_D$ +83.5 ($CHCl_3$), gave positive L–B test for triterpenoid. The mass spectrum showed its $[M]^+$ at *m/z* 426 ($C_{30}H_{50}O$). Its IR (KBr) spectrum showed the presence of OH (3428 cm^{-1}) and double bond (1635 cm^{-1}). The ^1H NMR spectrum of compound **1A** displayed broad doublet at δ 5.34 ($J = 6.1$ Hz) for olefinic proton and a proton at δ 3.23 ($J = 2.0, 7.0$ Hz) as double doublet showed its equatorial disposition at C-3 (δ 79.20), and hence β-configuration. The ^{13}C NMR peaks at δ 124.9 (C-12) and δ 140.0 (C-13) further supported the double bond at $\Delta^{12,13}$ position and the presence of six tertiary methyls at δ 28.3, 15.7, 15.9, 16.9, 23.3, and 28.0, and two secondary methyls at δ 17.3 and 21.5 in its DEPT experiments showed the presence of ursane skeleton [11]. Therefore, compound **1A** was identified as *urs*-12-*en*-3β-ol. The C-3 signal of compound **1** appeared at δ 80.3 lower than C-3 of α-amyrin at δ 79.2. Hence, compound **1** is a fatty ester derivative and characterized as *urs*-12-*en*-3β-*O*-9*E*,12*E*-octadecadienoate. This reports its first occurrence in nature. The lipid 9*E*,12*E*-octadecadienoic acid was earlier reported as its glyceride ester from the seed oil of *Chilopsis linearis* by Chisholm and Hopkins [12]. The separated oil after alkaline hydrolysis and methylation showed the presence of methyl esters of palmitic, linoleic, and 9*E*,12*E*,15*E*-octadecatrienoic acids from their GC–MS analysis by library matching. α-Amyrin, β-sitosterol, betulinic acid, and β-sitosterol-3-*O*-β-D-glucoside were also identified from their spectral study and co-TLC with the authentic samples isolated earlier in our laboratory.

3. Experimental

3.1 General experimental procedures

Melting points were determined with melting point apparatus (Remi, Mumbai, India) and are uncorrected. Elemental analysis was done on Vario EL III. The IR spectra were measured on FT-IR Perkin-Elmer spectrum BX. NMR spectra were obtained on Bruker Avance Spectrometer (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR). Mass spectra were obtained on either Micromass Quattro II triple quadrupole JASCO PU-980 (ESI) or Jeol SX-102 (FAB). GC–MS was carried out on Perkin-Elmer Turbomass with autosystem XLGC fitted with Equity-5 column (5% phenyl, 95% dimethyl polysiloxane, 50 m × 0.22 mm, 0.2 μm). Helium was used as a carrier gas at 10 psi with injection temperature at 70°C and it was gradually increased by 3°C/min up to 250°C and then remained constant. The R_f value of compounds was determined on pre-coated Si-gel 60 F_{254} plates (SRL, Mumbai, India), and iodine vapors or 10% H_2SO_4 solution followed by heating for 10 min at 120°C were used for detection.

3.2 Plant material

The edible rhizomes of *N. nucifera* were collected during August 2005 from the local market in Lucknow, India, which was identified by Dr S. Singh (Botany Division, CIMAP). A voucher specimen (C-1262) has been deposited in the herbarium of the CIMAP.

3.3 Extraction and isolation

The dried rhizomes (2.276 kg) of *N. nucifera* were exhaustively soxhletted with MeOH (6 h ×4). MeOH extract was filtered through G-3 sintered and the filtrate was concentrated *in vacuo* at 45°C to give a MeOH soluble (67 g) and insoluble parts (8 g). The MeOH soluble part was partitioned with hexane (100 ml ×5). Concentrated MeOH part after hexane partitioning was chromatographed over Si-gel (60–120 mesh; SRL), eluting with hexane, EtOAc, and MeOH in their varying proportions in the order of increasing polarities. Fractions collected of 100 ml each were pooled together according to their TLC profiles. Earlier fractions from hexane–EtOAc (2:1) furnished α-amyrin and later fractions from β-sitosterol. EtOAc eluant afforded betulinic acid while EtOAc–MeOH (3:97) afforded β-sitosterol-3-β-*O*-D-glucoside. MeOH insoluble residue (7 g) on acetylation with pyridine and Ac_2O at room temperature gave 3-acetyl betulinic acid after usual work up (2 g). The betulinic acid was found to be the major compound in the rhizomes of *N. nucifera*.

3.3.1 Separation of urs-12-en-3β-O-9E,12E-octadecadienoate (1)

The concentrated hexane part was cooled at 5°C and centrifugation provided a semisolid, which was further separated by flash chromatography on Si-gel (230–400 mesh; SRL) using EtOAc–hexane (1:4) as eluant to yield a white solid. This was further purified on Ag^+ impregnated Si-gel TLC plate, prepared from 32 g Si-gel (GF 254; SRL) and 1.5 g $AgNO_3$ in 60-ml distilled water [13]. TLC plate was partially dried in air and activated at 80°C for 30 min before using. The compound **1** was loaded onto TLC plates and run in $CHCl_3$–hexane (1:2). The major compound was scrapped from TLC plates and dissolved in $CHCl_3$ to yield compound **1** as an amorphous solid, R_f 0.70 (hexane–EtOAc, 2:1); $[\alpha]_D^{20}$ +98.0 ($c = 0.01$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.68 (d, Me), 0.77 (Me), 0.81 (2 × Me), 0.92, 0.97, 1.01, 1.02 (4 × Me), 1.26–1.40 (—CH_2), 2.28 (m, H_2-2′), 2.79 (H_2-11′), 4.28 (dd, $J = 2.0$, 7.5 Hz, H-3), 5.10–5.36 (m, olefinic protons); ^{13}C NMR (75 MHz, $CDCl_3$): δ 38.1 (C-1), 22.9 (C-2), 80.3 (C-3), 37.6 (C-4), 55.1 (C-5), 18.2 (C-6), 32.9 (C-7), 40.0 (C-8), 47.1 (C-9), 36.9 (C-10),

23.3 (C-11), 124.3 (C-12), 139.3 (C-13), 42.0 (C-14), 28.7 (C-15), 26.6 (C-16), 33.7 (C-17), 58.9 (C-18), 39.6 (C-19), 39.6 (C-20), 31.2 (C-21), 41.5 (C-22), 28.3 (C-23), 16.5 (C-24), 15.9 (C-25), 16.9 (C-26), 23.3 (C-27), 28.0 (C-28), 17.3 (C-29), 21.5 (C-30), 173.5 (C-1′), 33.9 (C-2′), 24.7 (C-3′), 28.9 (C-4′), 29.0 (C-5′), 29.0 (C-6′), 29.4 (C-7′), 32.5 (C-8′), 130.7 (C-9′), 128.8 (C-10′), 35.5 (C-11′), 128.4 (C-12′), 131.0 (C-13′), 32.5 (C-14′), 29.2 (C-15′), 31.4 (C-16′), 22.5 (C-17′), 14.0 (C-18′); FAB-MS *m/z*: $[M + Na]^+$ 711, $[M]^+$ 688; elemental analysis: found, C: 83.65%; H, 11.60%; calcd for $C_{48}H_{80}O_2$: C, 83.72%; H, 11.63%.

3.3.2 *Hydrolysis of* urs-*12*-en-*3β*-O-*9*E,*12*E-*octadecadienoate (**1**)*

Compound **1** was hydrolyzed with 10% NaOH for 18 h at 35°C. Partition with dichloromethane gave α-amyrin (**1A**), and further partition with hexane after acidification gave an acid, crystallized from acetone at low temperature to afford 9*E*,12*E*-octadecadienoic acid (**1B**), mp 25°C. This was methylated with diazomethane and identified by GC–MS as methyl 9*E*,12*E*-octadecadienoate. The supernatant oil obtained after removal of semisolid by centrifugation was hydrolyzed by alkali (20% NaOH). The hydrolyzed products after acidic neutralization and methylation were identified as methyl esters of palmitic, linoleic, and 9*E*,12*E*,15*E*-octadecatrienoic acids from GC–MS analysis.

3.3.3 *α-Amyrin (**1A**)*

α-Amyrin was isolated as colorless needles from hexane–EtOAc (2:1); mp 186°C; R_f 0.60 (hexane–EtOAc, 1:1); $[\alpha]_D$ +83.5 ($CHCl_3$); ESI-MS *m/z*: $[M]^+$ 426 (calcd for $C_{30}H_{50}O$).

3.3.4 *Methylation of 9*E,*12*E-*octadecadienoic acid (**1B**)*

Compound **1B** in MeOH was treated with diazomethane and evaporated after 1 h to give its methyl ester. The GC–MS analysis of the methyl ester confirmed its structure as methyl-9*E*,12*E*-octadecadienoate (*m/z* $[M^+]$ 294; $C_{19}H_{34}O_2$) and was confirmed by comparing with the GLC of an authentic methyl linolelaidate (9*E*,12*E*), GC–MS *m/z*: $[M]^+$ 294, 81, 223, 137, 107, 95, 74, 67.

3.3.5 *β-Sitosterol*

β-Sitosterol was isolated from hexane–EtOAc (2:1) eluant and crystallized from $CHCl_3$–hexane as colorless crystals, mp 136°C; R_f 0.40 (hexane–EtOAc, 2:1) $[\alpha]_D$ −37 ($CHCl_3$); ESI-MS *m/z*: $[M]^+$ 414 (calcd for $C_{29}H_{50}O$).

3.3.6 *Betulinic acid (**2**)*

Compound **2** was purified as crude light solid and further purified by column chromatography using eluant EtOAc and crystallized from MeOH–hexane as colorless needles, mp 280°C; R_f 0.40 (EtOAc–MeOH, 97:3) $[\alpha]_D$ +6.89 (MeOH); ESI-MS *m/z*: $[M]^+$ 456.

3.3.7 *Acetylation of betulinic acid (**2**)*

Compound **2** was acetylated with Ac_2O/pyridine at room temperature and after usual work up, 3-*O*-acetyl betulinic acid (**2A**) was crystallized from $CHCl_3$–hexane as colorless crystals, mp 278°C; R_f 0.45 (EtOAc); ^1H NMR (300 MHz, $CDCl_3$): δ 0.80 (Me, s), 0.82 (Me, s), 0.95 (Me, s), 0.97 (3 × Me, s), 1.71 (H_3-30, br s), 2.04 (3H, s, $OCOCH_3$), 4.47 (H-3, dd, $J = 2.0, 7.0$ Hz), 4.74 and 4.61 (2H, br s, H_a 29 and H_b 29); ESI-MS *m/z*: $[M]^+$ 498.

3.3.8 *β-Sitosterol-3*-O-*β-D-glucoside*

β-Sitosterol-3-*O*-glucoside was isolated from 3% MeOH in EtOAc as amorphous

powder, mp 278°C; R_f 0.50 (EtOAc–MeOH, 19:1). Acetylation with Ac_2O/pyridine gave tetra-acetate as colorless flakes from $CHCl_3$–hexane, mp 166°C.

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