In vitro Cytotoxic Pentacyclic Triterpenoids of Newbouldia laevis

P.A. Onocha, D.A. Okorie, H.C. Krebs and B. Meier
Department of Chemistry, University of Ibadan, Ibadan, Nigeria
Zafar Chemic Analytik and Endokrinologie, Der Tierarzlichen Hochschule, Bischofsloher Damm 15, 30173 Hannover, Germany

Abstract: Bioactivity directed fractionation afforded four cytotoxic constituents from the bioactive methanolic leaf extract (LD₅₀ 30 µg mL⁻¹) of Newbouldia laevis (Bignoniaceae) collected from Ibadan, Nigeria. Four pentacyclic triterpenoids namely 2α, 3β, 19α-trihydroxy-12-ursen-28-oic acid, 3β, 19α-dihydroxy-12-ursen-28-oic acid, 3β-hydroxy-12-ursen-28-oic acid, 3β-hydroxy-12-oleanen-28-oic acid were found to be the cytotoxic principles. These compounds demonstrated significant cytotoxicity in vitro against ST-57 brain tumor transformed fibroblasts with LD₅₀ ranging from 0.4 µg mL⁻¹ (compound 1) to 4 µg mL⁻¹ (compound 4). These ursene and oleanene type triterpenoids are encountered for the first time in this plant. The structures of these compounds were established by spectral data.

Key words: Bignoniaceae, fibroblasts, cytotoxicity, bioactivity, ursenoic acid, oleanenoic acid

INTRODUCTION

In a continuing collaborative search for naturally occurring medicinal agents from plants, the leaves of Newbouldia laevis (Beauv.) Seem. ex Bureau (Bignoniaceae) collected from Ibadan, Nigeria were selected for investigation. Previous phytochemical reports on this species were on the root, root bark, flowers and stem. N. laevis is a common tree of West African origin well known for its ethno medical uses. The leaves, bark, stem and roots are used in the treatment of conjunctivitis, arthritis, dysentery, enlarged spleen, heart burn, wound, ear and stomach ache, sore feet, chest pain, epilepsy and children’s convulsion (Burkill, 1985; 2000; Oliver-Bever, 1986; Iwu, 2000). The anti-inflammatory, antipyretic, analgesic and anti-inflammatory principles of the stem bark and flowers have been studied (Olajide et al., 1997; Usman et al., 2008; Tarko et al., 2008a). Different parts of the plant has also been shown to exhibit antimicrobial, antimalarial, sedative, anti-inflammatory, anticoagulant, anti-diabetic, uterine contractile and antioxidant properties (Gafner et al., 1996; Amos et al., 2002; Eyong et al., 2005; Kuete et al., 2007; Usman and Osuji, 2007; Tarko et al., 2008a; Ogurlana and Ogurlana, 2008; Bafor and Sanni, 2009; Ainoconson et al., 2009; Bafor et al., 2010; Oloyede et al., 2010).

Recent phytochemical analysis of the root, root bark and stem of this plant afforded alkaloids, quinoids, ceramides and phenylpropanoids amongst others (Adesanya et al., 1994; Houghton et al., 1994; Aladesanmi et al., 1998; Gafner et al., 1996, 1997, 1998; Gomann et al., 2003, 2006; Eyong et al., 2005, 2006; Kuete et al., 2007).

There was no extensive report on the isolation of compounds from the leaves (Usman and Osuji, 2007). Bioassay-guided fractionation of the leaf methanolic extract of N. laevis afforded four cytotoxic pentacyclic triterpenoids which are encountered for the first time in the plant.

This study therefore, reports the isolation of four triterpenoids namely 2α, 3β, 19α-trihydroxy-12-ursen-28-oic acid or tormentic acid, 3β, 19α-dihydroxy-12-ursen-28-oic acid or pomolic acid, 3β-hydroxy-12-ursen-28-oic acid or Bungeolic acid, 3β-hydroxy-12-oleanen-28-oic acid or Astrantiagenin-C as cytotoxic constituents of the leaf methanolic extract of N. laevis (Fig. 1).

Fig. 1: Cytotoxic constituents of the leaf methanolic extract of N. laevis

Corresponding Author: P.A. Onocha, Department of Chemistry, University of Ibadan, Ibadan, Nigeria
MATERIALS AND METHODS

General methods: All melting points were determined on a Kofler hot stage apparatus and are uncorrected. IR and UV spectra were recorded on a Perkin Elmer model 983 spectrometer. \(^1\)H and \(^13\)C-NMR including 2D-NMR, DEPT spectra were recorded on a Bruker WP300SY Spectrometer (300.133 MHz for \(^1\)H and 75.468 MHz for \(^13\)C).

Plant materials: The leaves of *N. laevis* collected from Jericho forest reserve, Ibadan, Nigeria was authenticated at source and confirmed by Mr. Felix Usang at the Forest Research Institute of Nigeria where a voucher specimen (No. KHI106-427) was deposited in the herbarium.

Extraction and isolation: The air dried leaves of *N. laevis* (800 g) were ground and soxhlet extracted first with hexane for 48 h to afford the hexane extract (34.3 g) and subsequently with methanol to yield the methanolic extract (76.6 g, ST-57, LD\(_{50} 30 \text{ mg mL}^{-1}\)). A portion of the methanol extract (30 g) was subjected to dry flash column (8×10 cm) chromatography on silica gel (230–400 mesh). Gradient elution of the column started with light petroleum (40–60°C) followed by increasing percentages of light petroleum (5, 10 and 15%). Fractions obtained were analysed by silica gel TLC and combined on the basis of similar TLC profiles to give 10 major fractions (NL01–NL10). Compounds were detected by UV light (254 nm), \(I_2\) absorption or Vanillin-H\(_2\)SO\(_4\) spray reagent. Subsequently, further investigations were only on the bioactive fractions (NL 6-8).

Fraction NL08 (250 mg, LD\(_{50}\) 10 \text{ mg mL}^{-1} *in vitro* against ST-57 brain tumor transformed fibroblasts) was obtained from 90% EtoAc in light petroleum and after repeated column and flash chromatographic separation yielded compound 1 which was recrystallized from methanol-acetone giving a white solid (mp 264–266°C). On structural elucidation and comparison with reference data (Doddrell *et al.*, 1974; Nunata *et al.*, 1989; Louksi *et al.*, 1990; Rahman and Ahmad, 1994), compound 1 was resolved as 2x, 3β, 19α-trihydroxy-12-ursen-28-oic acid or tormenic acid.

Fraction NL07 (250 mg, LD\(_{50}\) 15 \text{ mg mL}^{-1} *in vitro* against ST-57 brain tumor transformed fibroblasts) was eluted from 85% EtoAc in hexane yielding compound 2 as pure white crystalline substance on repeated column chromatographic separation. It was recrystallized from methanol-acetone (mp 280–82°C). On structural elucidation and comparison with reference data (Kuang *et al.*, 1989; Guang-Yi *et al.*, 1989; Soares *et al.*, 1998), the structure of compound 2 was deduced as 3β, 19α-dihydroxy-12-ursen-28-oic acid or pomolic acid.

Fraction NL06 (336 mg, LD\(_{50}\) 20 \text{ µg mL}^{-1} *in vitro* against ST-57 brain tumor transformed fibroblasts) was obtained from 85% EtoAc in hexane as a greenish solid which was purified in similar manner to NL08 giving compound 3 as a white crystalline solid (mp 277°C {Decomp}). Compound 3 was identified as 3β-hydroxy-12-ursen-28-oic acid or bungaric acid by spectral data and comparison with reference data (Connolly and Hill, 1991; Rahman and Ahmad, 1994).

Fraction NL06 (336 mg, LD\(_{50}\) 20 \text{ µg mL}^{-1} *in vitro* against ST-57 brain tumor transformed fibroblasts) was obtained as a greenish substance from 85% EtoAc in hexane. Repeated column chromatographic, LH 20 Sephadex (35% chloroform in methanol) separation and recrystallization from acetone-methanol mixture, yielded compound 4 as a white crystalline solid (mp 272°C {Decomp}) compound 4 was identified as 3β-hydroxy-12-oleanen-28-oic acid or Astrantiagenin-C by spectral data and comparison with reference data (Numata *et al.*, 1989; Connolly and Hill, 1991; Rahman and Ahmad, 1994).

**Spectral data**

**Compound 1**

- **IR** v\(_{\text{max}}\) cm\(^{-1}\) (KBr): 3577-2940 br (OH and COOH), 1688 (acid)
- **HRMS** m/z (rel. int. %): 488.077 (0.7, M\(_+\), C\(_{30}\)H\(_{48}\)O\(_{4}\))
- **EIMS** 70 eV: 264(100), 249(36.5, 264-CH\(_3\)), 219(18, 264-COOH), 223 (15.5)
- **\(^1\)H NMR** (CD\(_3\)N, 300.133 MHz): \(\delta\) 5.55 (1H, t, J = 3.5, H-12), 4.09 (1H, dt, J = 6, 9 Hz, H-2β), 3.38 (1H, d, J = 9Hz, H-3), 3.12 (1H, dt, J = 13.5, Hz, H-16α), 3.04 (1H, s, H-18), 2.3 (2H, d, J = 6Hz, H-1), 2.28 (1H, dt, J = 13, 4 Hz, H-15β), 2.25 (1H, dt, J = 13, 5 Hz, H-16β), 1.95 (1H, dt, J = 13, 5 Hz, H-15α), 1.70 (3H, s, 29-Me), 1.42 (3H, s, 27-Me), 1.26 (3H, s, 23-Me), 1.12 (3H, d, J = 6Hz, 30-Me), 1.09 (3H, s, 26-Me), 1.07 (3H, s, 24-Me), 1.01 (3H, s, 25-Me)
- **\(^1\)C NMR** (CD\(_3\)N, 75.468 MHz (Table 1)): \(\delta\) 180.7 (C-28), 140.0 (C-13), 127.9 (C-12), 84.0 (C-3), 72.9 (C-19), 68.6 (C-2), 56.0 (C-5), 54.6 (C-18), 48.3 (C-17), 47.9 (C-1), 47.9 (C-9), 42.4 (C-14), 42.2 (C-20), 40.4 (C-8), 39.8 (C-4), 38.5 (C-22), 38.5 (C-10), 33.5 (C-7), 29.3 (C-23), 26.9 (C-21), 29.1 (C-29), 26.4 (C-16), 29.3 (C-15), 24.7 (C-27), 24.1 (C-11), 19.0 (C-6), 17.7 (C-26), 17.3 (C-30), 16.9 (C-25), 16.8 (C-24)

**Compound 2**

- **IR** v\(_{\text{max}}\) cm\(^{-1}\) (pyridine): 3600-3300 br (OH and COOH), 1697 (acid)
- **HRMS** m/z (rel. int. %): 472.177 (0.87, M\(_+\), C\(_{30}\)H\(_{48}\)O\(_{4}\))
- **EIMS** 70 eV: 390 (M\(^+\)-COOH-H\(_2\)O\(_{2}\)), RDA fragment of ring C, 264 (100), 249 (25.5, 264-CH\(_3\)), 219 (11.264-COOH), 207 (25.5)
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- $^1$H NMR (C$_5$D$_5$N, 300.133 MHz): δ 5.62 (1H, t, J = 3.5 Hz, H-12), 3.41 (1H, t, J = 9 Hz, H-5x), 3.14 (1H, dt, J = 13.5 Hz, H-16a), 3.05 (1H, t, J = 13.5 Hz, H-16b), 2.24 (1H, dt, J = 13.5 Hz, H-15b), 2.08 (1H, m, H-15c), 1.87 (2H, m, H-2), 1.72 (3H, s, Me-29), 1.44 (3H, s, Me-27), 1.21 (3H, s, Me-24), 1.10 (3H, d, J = 6 Hz, Me-30), 1.10 (3H, s, Me-26), 1.02 (3H, s, Me-23), 0.90 (3H, s, Me-25)
- $^1$CNMR (C$_5$D$_5$N, 75.468 MHz): δ 180.6 (C-28), 139.8 (C-13), 128.0 (C-12), 78.1 (C-3), 72.6 (C-19), 55.8 (C-5), 54.5 (C-18), 48.2 (C-17), 47.7 (C-9), 42.0 (C-14), 40.3 (C-8), 39.3 (C-4), 38.9 (C-1), 38.4 (C-22), 37.3 (C-10), 35.5 (C-7), 42.3 (C-20), 29.2 (C-15), 28.7 (C-23), 28.0 (C-20), 27.0 (C-29), 26.8 (C-21), 26.3 (C-16), 24.6 (C-27), 23.9 (C-11), 18.8 (C-6), 17.1 (C-24), 16.7 (C-26), 16.4 (C-25), 15.5 (C-30)

**Compound 3:**
- IR ν$_{max}$ cm$^{-1}$ (KBr): 3430-2920 br (OH and COOH), 1695 (acid)
- HRMS m/z (rel.int. %): 456.696 (3.22, M$^+$, C$_{18}$H$_{24}$O$_{10}$)
- EIMS 70 eV: RDA fragment of ring C, 248 (100), 203 (48.8, 248-COOH), 207 (25.25)
- $^1$CNMR (C$_5$D$_5$N, 75.468 MHz (Table 1)): δ 180.1 (C-28), 144.7 (C-13), 122.4 (C-12), 78.0 (C-3), 55.7 (C-5), 48.0 (C-9), 46.4 (C-17), 42.1 (C-19), 41.9 (C-18), 39.6 (C-8), 39.3 (C-14), 38.8 (C-1), 37.3 (C-4), 37.2 (C-22), 37.2 (C-10), 34.1 (C-21), 33.1 (C-29), 33.1 (C-7), 30.8 (C-20), 28.7 (C-23), 28.2 (C-15), 28.0 (C-26), 26.0 (C-27), 23.6 (C-30), 23.6 (C-16), 23.6 (C-11), 18.7 (C-6), 17.3 (C-26), 16.5 (C-25), 13.6 (C-24)

**Cytotoxicity assay:** The in vitro cytotoxicity assay were carried out to the standard protocols established by the National Cancer Institute (Geran et al., 1972). Different concentration (4 and 0.4 μg/mL$^{-1}$) of the compounds were tested in vitro against ST-57 brain tumor transformed fibroblasts.

Primary culture of fibroblasts was prepared by method of explantate culture (Meier et al., 1990a, b). Fibroblasts were cultured as mono layers on all to a confluency (≤10$^6$ cells cm$^{-2}$) on plastic slips in a 4 well multwell. The cells were inoculated in 2 mL medium containing the compounds (dissolved in methanol and diluted with 0.1 phosphate buffer saline) in sterile atmosphere in multwells. Control cells contained
methanol and phosphate buffer saline. Cytotoxicity was appreciated by evaluation of LD<sub>50</sub> values up to the 3rd day of treatment. LD<sub>50</sub> value ≤4 µg mL<sup>-1</sup> for pure compounds are considered significant. Results are shown in Table 2.

**RESULTS AND DISCUSSION**

In an initial study, the methanol extract of *N. Laevis* was found to be cytotoxic when tested in vitro against ST-57 brain tumor transformed fibroblasts (LD<sub>50</sub> 30 µg mL<sup>-1</sup>). As a result of subjecting this methanol extract to flash column and Sephadex LH 20 chromatography on silica gel and cytotoxic assay, three (NL 06, NL 07, NL 08) of the ten combined fractions were found to demonstrate significant cytotoxic activity (ST 57, LD<sub>50</sub> <20 µg mL<sup>-1</sup>). Fractions NL 08 and NL 07 yielded compounds 1 and 2, respectively while fraction NL 06 yielded compounds 3 and 4.

Compound 1 exhibited a molecular formula of C<sub>29</sub>H<sub>40</sub>O<sub>8</sub> based on its high resolution EIMS data. It gave rise to IR absorption bands for a carboxylic acid and a vinyl bond at 2950-1600 and 900 cm<sup>-1</sup>, respectively. The <sup>13</sup>C-NMR displayed signals for 30 carbon atoms: one carbonyl (δ 180.6), two ethylenic carbon atoms (-C=C-, δ 127.9 and 140.0), two oxygenbearing quaternary carbon, (δ 72.9), seven methyls, eight methylenes, four methines and five quaternary carbon atoms. Six methyls were tertiary and the olefinic proton gave a signal at δ 5.55. The signal for H 18 was a broad singlet indicating its β-diposition relative to the D-ring (Doddrell et al., 1974). The presence of the double bond at C-12 was confirmed by the chemical shifts at δ 127.9 (C-12) and δ 139.9 (C-13), characteristic of Δ<sup>2</sup> uns 12 enes (Doddrell et al., 1974). Six tertiary methyl at δ 1.01, 1.07, 1.09, 1.26, 1.42, 1.70 (each, 3 H, s) and a secondary methyl at δ 1.12 (3H, d, 30-Me) are indicative of a pentacyclic ursene tripterpen (Doddrell et al., 1974). Since, in ursene skeleton, the proton at C-18 appeared as a singlet, the tertiary hydroxy group could only be located at C-19. The two secondary carbinol protons resonated at δ 3.38 (1 H, d, J = 9 Hz) and 4.09 (1 H, dt, J = 6.9 Hz) indicating that both were neighbours coupling with each other (J = 9 Hz) while the doublet of triplet was in addition coupled to two other neighbouring protons (J = 6 Hz). Based on these deductions, the one proton doublet was assigned to C-3 with the neighbouring cortical proton at C-2. This was in accordance with the <sup>13</sup>C-NMR resonances observed at δ 84.0 and 68.6. The value of the coupling constants and carbon resonances confirm that the hydroxy group is attached β to C-3 (J = 9 Hz) and strongly deshielded hence, the high resonance of δ 84.0 observed while the other hydroxyl group is attached α to C-2 and shielded thereby resonating at δ 68.6. These value are consistent with reference data (Connolly and Hill, 1991). From comparison of the <sup>1</sup>H-NMR and <sup>13</sup>C-NMR of compound 1 with reference data (Kuang et al., 1989; Numata et al., 1989), the structure of compound 1 was identified as 2α, 3β, 19α-trihydroxy-12-ursen-28-oic acid or tormentic acid.

Compound 2 exhibited a molecular formula of C<sub>30</sub>H<sub>42</sub>O<sub>8</sub> based on its high resolution EIMS data. It gave rise to IR absorption bands for a carboxylic acid and a vinyl bond at ν<sub>max</sub> 1697 and 1640 cm<sup>-1</sup>, respectively. The <sup>13</sup>C-NMR with DEPT spectrum revealed 30 carbon signals including characteristic signals due to a trisubstituted double bond-δ 128.0 (d) and 140.0 (s), one carbonyl (δ 180.6), one oxymethylene, an oxygen bearing quaternary carbon (δ 72.6), seven methyls, nine methylene, four methines and five quaternary carbon atoms. The difference between compound 1 and 2 was mainly the absence of one oxymethylene which was evident in the low δ value of C-2 (28.0). Based on these and comparison with compound 1 and reference data (Kuang et al., 1989; Gung-Ti et al., 1989), compound 2 was identified as 3β, 19α-dihydroxy-12-ursen-28-oic acid or pomolic acid.

Compound 3 exhibited a molecular formula of C<sub>30</sub>H<sub>44</sub>O<sub>8</sub> based on its high resolution EIMS data. It gave rise to IR absorption band at ν<sub>max</sub> 3430-2920 br (OH and COOH), 1695 (acid) 1695 cm<sup>-1</sup>. The <sup>13</sup>C-NMR gave signals for 30 carbon atoms, one carbonyl (δ 180.6), two ethylenic carbon atoms (-C=C-, δ 125.5 and 138.1), one oxymethylene, seven methyls, nine methylene, four methines and five quaternary carbon atoms. The structure of compound 4 was deduced based on its spectral properties and comparison with reference data (Numata et al., 1989; Rahman and Ahmad, 1994). Comparison of the spectral data (IR, MS, NMR) with those of 3β-hydroxy-12-ursen-28-oic acid or Bungeolic acid confirmed that they were the same compound.

Compound 4 exhibited a molecular formula of C<sub>30</sub>H<sub>46</sub>O<sub>8</sub> based on its high resolution EIMS data. It gave rise to IR absorption bands at ν<sub>max</sub> cm<sup>-1</sup> (KBr): 3500-2941
br (OH and COOH), 1689 (acid). 13C-NMR gave signals for 30 carbon atoms: one carbonyl (δ 180.6), two ethylenic carbon atoms (δ 125.5 and 138.1), one oxymethine, seven methyls, nine methylene, four methines and six quarternary carbon atoms. The structure of compound 4 was deduced based on its spectral properties and comparison with reference data (Rahman and Ahmad, 1994; Spengel, 1996). Comparison of the spectral data (IR, MS, NMR) with those of 3β-hydroxy-12-oleanene-28-oic acid or Astaniageninin-confirmed that they were the same compound.

These compounds were tested for their cytotoxicity and found to possess significant cytotoxicity against ST 35 brain tumor transformed fibroblasts. Compound 1 was the most active (LD50 0.4 μg mL−1) while compound 4 (LD50 4 μg mL−1) was the least active (Table 2). The presence of hydroxy group appears to have some effect on the activity as the activity was observed to increase as the number of hydroxy groups in the molecule increased from compound 4 to compound 1.

CONCLUSION

Although, extensive research had been done on the root, stem and flowers of N. laevis, limited research has been carried out on the leaves of the plant (Usman and Osuji, 2007). This report therefore, establishes the cytotoxicity of the leaf methanol extract. In addition, four of the compound responsible for cytotoxic antitumor activity of the leaf methanol extract has been identified, isolated and characterised. To the best of the knowledge, there is no prior report of the presence of terpenoids in the plant.

ACKNOWLEDGEMENT

P.A.O. is grateful to the Deutscher Akademischer AustauschDienst (DAAD) for a fellowship to Chemisches Institut Der Tierarzlichen Hochschule, Harnover for this study.

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