Isolation of Betulinic acid from the stem bark and root of *Uapaca guineensis*

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Abstract:

**Aims:** To investigate the stem bark and root of *Uapaca guineensis* extracts, with a view of isolating betulinic acid across the various part of the plant based on its reported biological activities.

**Study design:** The design included extraction of *U. guineensis* stem bark and root with ethanol and subsequent partitioning, isolation and characterization of the possible isolated compounds.

**Place and Duration of Study:** Chemistry Department (Organic Unit), Faculty of Science, University of Ibadan, Nigeria and H.E.J. Research Institute of Chemistry, ICCBS, University of Karachi, Pakistan between March 2012 and April 2013.

**Methodology:** The air-dried and pulverized stem bark and root of *U. guineensis* (0.5 kg each) were separately extracted with aqueous ethanol (80%) for 72 hour at room temperature, filtered, concentrated on rotatory evaporator at 37 °C and then stored in a desiccator. The ethanol extracts of the stem bark was partitioned successively with n-hexane, dichloromethane and methanol. The dichloromethane, methanol and ethanol extracts (root) were then chromatographed and the isolated compounds subjected to various spectroscopic analysis.

**Results:** The air-dried and pulverized stem bark and root of *U. guineensis* (0.5 kg each) extracted with ethanol and then partitioned yielded dichloromethane (6.9 g), methanol (17.9) and the ethanol (12.9) extracts. The chromatography of the various extracts results in the formation of whitish powder that are UV inactive and also gave single spot each with cerium sulphate coded as DBG-D13 (25.0 mg), MBG-Q3 (6.1 mg) and ERG-E7 (20.0 mg) for dichloromethane, Methanol and Ethanol extracts respectively. The structures were unequivocally established to be 3α-hydroxy-20(29)-lupen-28-oic acid and 3β-hydroxy-20(29)-lupen-28-oic acid by 1HNMR, 13CNMR, 2D-NMR, IR, UV and MS spectra analysis as well as by comparison of the spectra data with previously reported values.

**Keywords:** *Uapaca guineensis*, Euphorbiaceae, Betulinic acid
1. INTRODUCTION

*Uapaca* belong to a genus of the family Euphorbiaceae [1, 2] and is in the sub-trIBE *Uapacineae*. It comprises approximately 105 species, of which 49 are confined to continental Africa. The species described so far in the genus are trees of 20 to 25 m high with about 60 cm in diameter [3, 4].

Phytochemical studies on extract of the genus revealed the presence of terpenoids, saponins, alkaloids and sterols [5]. The presence of phenolic compounds including tannins, gallotannins and flavonoids from fruits of *Uapaca kirkiana* was also reported [6]. Betulinic acid and antidesmone as constituents of *Uapaca nitida* and *Uapaca robynsii* respectively were also reported [7, 8].

*Uapaca guineensis* Mull. Arg. (Euphorbiaceae) commonly called false mahogany and “Ewe akun” in the western part of Nigeria grows in humid localities, mixed evergreen forest, bush land on steep slopes and sea-level up to 1100 m altitude. They are widely distributed from West Africa to Democratic Republic of Congo. It is a tree of about 30 m high, rarely more, bore to 4 m girth by 13 m long, usually much less, more or less fluted with large stilt-roots up to 3 m high, dense low-branching crown and leaves may reach 30 cm long by 15 cm wide. The wood is hard, durable and moderately heavy, and when quarter-sawn is attractively figured with a silver grain [9, 10].

The isolation of substantial amounts of betulinic acid as almost the sole component from *Uapaca* species as well as the *in vitro* antitrypanosomal activity have been reported [4, 18]. The pharmacological activities of this species in Western and Eastern Africa folk medicine include anti-inflammation, treatment of fever, pain, sexual dysfunction and bacterial diseases [16, 17 and 8].

In Nigeria, the pulp is made into a refreshing drink; leaves are used to wrap kola nuts and the roots to treat male impotence. The wood is used locally in carpentry and to make planks, railway sleepers, beams, furniture, beds and kitchen utensils. It also produces good firewood and charcoal [7]. The stilt-rooted habit of this riverside species has raised the suggestion that it is able to stabilise river-banks, curb banks, by putting them inside desiccators. Successive partitioned was done in the order of n-Hexane, dichloromethane and methanol for the stem bark extract while the root ethanol extract was chromatographed directly.

2. MATERIAL AND METHODS

**Plant materials**

The dichloromethane extract (6.9 g) of *U. guineensis* (stem bark) was preadsorbed on silica gel (6.9 g) to form a homogenous mixture which was subjected to column chromatography (weight of silica gel 200 g, length = 70 cm, internal diameter (id) = 4 cm) using gradient of hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) in order of hexane: (100%, 200 mL); hexane: DCM (9:1, 200 mL); (4:1, 100 mL); (3:2, 620 mL); (1:1, 280 mL); (2:3, 460 mL); (3:7, 260 mL); (1:4, 300 mL); (1:9, 220 mL); DCM (100%, 660 mL); DCM: EtOAc: (9:1, 100 mL); (4:1, 320 mL); (7:3, 380 mL); (3:2, 260 mL); (1:1, 180 mL); (2:3, 160 mL); (3:7, 160 mL); (1:4, 180 mL); (1:9, 260 mL); EtOAc (100%, 360 mL); EtOAc: MeOH: (9:1, 620 mL) and (4:1, 200 mL) separately. A total of 328 fractions (20 mL each) were collected and pooled to 16 sub-fractions (D1-16) based on TLC analysis. The sub-fraction D13 eluted with DCM: EtOAc, 4:1 was developed in a TLC tank with solvent ratio of 9.5:0.5 (DCM: MeOH). The isolate is UV inactive and gave a single spot when sprayed with cerium sulphate (Rf = 0.51). The whitish powder was re-crystallised in methanol to give a colourless needles powder coded DBG-D13 (25.0 mg).

**Column Chromatography of Methanol fraction of *U. guineensis* stem bark**

Methanol extract (17.9 g) of *U. guineensis* (stem bark) was mixed with equal weight of silica gel and chromatographed (silica gel weight = 350 g, length = 80 cm, id = 6 cm). Elution was carried out using gradient of hexane, ethyl acetate and methanol in order of hexane: (100%, 500 mL); hexane: ethyl acetate: (1:1, 700 mL); (2:3, 600 mL); (3:7, 500 mL); (1:4, 500 mL); (1:9, 500 mL); ethyl acetate: methanol: (9:1, 300 mL); (4:1, 300 mL) and (7:3, 700 mL). A total of 51 fractions of 100 mL each were collected and pooled to 4 sub-fractions.
(P-S) based on their TLC profile. Fraction Q (2.5 g) eluted between 50-70% EtOAc in Hexane was further subjected to a silica gel column chromatography (weight of silica gel = 70 g, length = 60 cm, id = 2.5 cm) using isocratic elution of hexane: EtOAc (3:7). Fourteen fractions were collected at 30 mL each and pooled to 5 sub-fractions (Q₅) based on their TLC profile. Fraction Q₅ was analyzed on TLC with Hexane: EtOAc system (3:7) to give a single spot on spraying with cerium sulphate (Rₚ = 0.53). It gave a dirty whitish powder which was re-crystallised in dichloromethane to give a clean white powder coded MBG-Q₅ (6.1 mg).

### Column Chromatography of ethanol extract of *U. guineensis* (root)

The ethanol extract (12.9 g) of *U. guineensis* was homogenised with equal weight of silica gel and subjected to column chromatography using silica gel (weight of silica gel = 370 g, length = 80 cm, id = 6 cm). Elution was carried out using gradient of hexane, dichloromethane, ethyl acetate and methanol in order of hexane; (100%, 2800 mL), hexane: dichloromethane; (9:1, 1600 mL); (4:1, 400 mL); (7:3, 600 mL); (3:2, 600 mL); (1:1, 600 mL); (2:3, 400 mL); (3:7, 400 mL); (1:4, 400 mL); (1:9, 400 mL), dichloromethane; (100%, 400 mL), dichloromethane: ethyl acetate; (9:1, 900 mL); (4:1, 900 mL); (7:3, 500 mL); (3:2, 400 mL); (1:1, 300 mL); (2:3, 600 mL); (3:7, 400 mL); (1:4, 600 mL); (1:9, 400 mL), ethyl acetate; (100%, 600 mL), ethyl acetate: methanol; (9:1, 1000 mL); (4:1, 400 mL); (7:3, 2100 mL); separately to give 177 fractions at 100 mL which were pooled to 9 sub-fractions (E₆) using TLC analysis. The sub-fraction E₆ (340.0 mg) eluted with 10% ethyl acetate: dichloromethane was further purified while sub-fraction E₇ eluted between 20-50% ethyl acetate: dichloromethane was developed in TLC tank with solvent ratio 3:7 (EtOAc: Hexane) to give a brown single spot on spraying with cerium sulphate (Rₚ = 0.52). The sample was a dirty white crystalline powder which was re-crystallised in methanol to give clean white powder coded ERG-E₇ (20.0 mg).

### 3. RESULTS AND DISCUSSION

Compound 1 was obtained as colourless needles powdery powder with yield (25.0 mg) and melting point 279-280 °C. The IR spectrum of DBG-D₁₃ showed diagnostic absorption bands for O-H, C=H₉, C-O, C=C and C-H (bond) at 3447, 2942/2870, 1688, 1648 and 1457 cm⁻¹ respectively. The UV spectrum showed a single band at 207 nm. The ¹H NMR spectrum showed pattern characteristic of lupane type skeleton. The methylene protons on C-16 and C-22 resonated as triplet of triplet at δ 2.23/1.44 (2H, tt, J = 3.0/12.5 Hz, H-16) and 1.53/1.17 (2H, tt, J = 3.0/13.0 Hz, H-22) respectively. The spectrum also showed a one proton broad singlet at δ 3.30 and 10.5 which are characteristic of a hydroxyl and a carboxylic acid signals respectively. The methine protons on C-19 and C-13 also showed signals for two doublet of doublet of doublet (triplet of doublet) at δ 3.04 (1H, ddd, J = 5.0/11.0 Hz, H-19) and 2.33 (1H, ddd, J = 2.5/11.5 Hz, H-13). The triplet at δ 3.10 (1H, t, J = 5.0 Hz, H-3) was assigned to the proton at C-3. There is exomethylene group signal at δ 4.71 and 4.58 (1H, each dd, J = 2.5/2.0 Hz, H-29) which together with an allylic methyl at δ 1.68 (3H, s, H-30) indicated an isopropenyl moiety. The other five methyl groups resonated at δ 1.01, 0.95, 0.94, 0.84 and 0.74. The ¹³C NMR spectrum showed thirty carbon resonances which were sorted using Distortionless Enhancement by Polarisation Transfer (DEPT) experiment as six methyl carbons [δc 28.5 (C-23), 16.1 (C-24), 16.5 (C-25), 16.6 (C-26), 15.0 (C-27) and 19.5 (C-30)], eleven methylene carbons [δc 109.9 (C-29), 39.5 (C-1), 30.4 (C-21), 35.2 (C-7), 32.8 (C-16), 31.3 (C-15), 37.5 (C-22), 28.3 (C-2), 26.4 (C-12), 21.7 (C-11) and 19.1 (C-6)], six methine carbons (78.5 (C-3), 56.3 (C-5), 51.4 (C-9), 49.9 (C-18), 47.9 (C-19) and 39.0 (C-13)], six quaternary carbons [151.6 (C-20), 56.8 (C-17), 43.0 (C-14), 41.5 (C-8), 39.6 (C-4) and 37.9 (C-10)] and one carbonyl carbon [177.5 (C-28)]. The assignment of chemical shifts of the protonated carbons was achieved by a Heteronuclear Quantum Correlation (HSQC) experiment. The ¹H-¹H Correlation Spectroscopy experiment (COSY) showed cross peaks for coupled protons at position 2 and 3, 7a and 7b, 12 and 13, 18, 19, 21, 22, 23, 24 and 25 for DBG-D₁₃. The Nuclear Overhauser Effect Spectroscopy (NOESY) experiment showed non coupling of protons at position 23 and 24 with its neighbouring protons at 3 suggesting β orientation of the hydroxyl group at C-3. The entire long ranged [²J (CH) and ³J (CH)] correlations between the protons and carbons were observed in the Heteronuclear Multiple Bond Correlation (HMBC) spectrum. The EI-MS showed m/z 456.3 calculated for a molecular formula C₃₀H₄₄O₂ with HRMS of 456.3598. Comparison of DBG-D₁₃ spectroscopic data with that of betulinic acid reported in literature [11] showed similarity. The structure of DBG-D₁₃ was elucidated to be 3β-hydroxy-20(29)-lupen-28-oic acid.

Compound 2 was obtained as a whitish powder with molecular ion peak at m/z 456.0 in EI-MS (positive ion mode) which matched with high-resolution mass spectrometry (HRMS) C₃₀H₄₄O₂ (456.3598). The IR spectrum of MBG-Q₂ showed a broad peak of hydroxyl group at 3454 cm⁻¹, prominent carbonyl absorption at 1687 cm⁻¹, double bonded peaks at 1641 cm⁻¹ and other diagnostic bands like 2942/2871 (C=H₂), 3075 (C=H) and 1452 (C=H₉) cm⁻¹. The UV spectrum showed a single band at 206 nm. The ¹H NMR spectrum of MBG-Q₂ showed pattern characteristic of a lupane type carbon skeleton. The compound indicated six singlet methyl proton signals at δ 0.74 (s, H-25), 0.85 (s, H-24), 0.94 (s, H-26), 0.96 (s, H-27), 0.99 (s, H-23) and 1.69 (s, H-30). It also contains two proton signals corresponding to olefinic methylene proton at δ 4.70 (s, H-29a) and 4.58 (s, H-29b), which correlated with the ¹³CNMR signal at δ 19.6 (C-30).The doublet of doublet at δ 3.11 (1H, dd, J = 4.5/1.0 Hz) could be assigned to H-3 with the vicinal methylene protons suggested β orientation of the hydroxyl group at C-3. The methylene proton at C-16 and C-22 showed triplet of triplet at δ 2.21/1.40 (2H, tt, J = 3.5/13.0 Hz, H-16) and 1.18/1.51 (2H, tt, J = 3.0/13.5 Hz, H-22).
Hz, H-22). The spectrum also showed triplet of doublet (doublet of doublet of doublet) at δ 2.23 (1H, d, d, J = 2.5/11.5 Hz, H-13) and δ 3.02 (1H, d, d, J = 5.0/11.0 Hz, H-19) for the methine proton at C-13 and C-19 respectively. A total of thirty carbons resonances where observed in 13C NMR spectrum which were sorted using DEPT experiment into six methyl carbons, eleven methylene carbons, six methine carbons, six quaternary carbons and one carbonyl carbon. Among the signals are olefinic methylene carbon signal at δ 110.1 (C-29) and carboxylic group carbon signal at δ 180.5 (C-28). Further information about the compound was obtained from the typical EI-MS related to the fragmentation pattern of lupine type triterpenes through the presence of m/z 438 (M-H2O), 411 (M-CO), 248 (C10H18O2), 220 (C10H16O2), 203 (248-COOH), 207 (C10H16O) and 189 (207-H2O) which are characteristics peaks for betulinic acid [13].

Seven degree of unsaturation corresponding to five rings, a carbon signal at δ 180.5 (C=O) in position 28 confirming the presence of a carboxyl group not shown in the structure. The spectrum also showed triplet of OH and δ 10.6 (1H, br. s) for alcoholic OH and δ 3.7 (1H, br. s) for carboxylic OH [19]. The EI-MS showed m/z 456.1 which was confirmed with High Resolution Mass Spectroscopy (HRMS) for a molecular formula C30H48O3 (456.3598). The structure of ERG-E7 was elucidated to be 3α-hydroxy-20(29)-lupen-28-oic acid. Comparison of MBG-Q1, DBG-D13 and ERG-E7 with betulinic acid showed similarity in spectroscopic data [14, 15] as shown in Table 1. They are all betulinic acid except that the hydroxyl group at C-3 is α-oriented for MBG-Q3 and β-oriented for DBG-D13 and ERG-E7. This was also confirmed through NOESY experiment. The structures were elucidated to be 3α-hydroxy-20(29)-lupen-28-oic acid and 3β-hydroxy-20(29)-lupen-28-oic acid as shown in Fig. 1.

![Table 1. 13C NMR Data for DBG-D13, MBG-Q3 and ERG-E7 compared to Betulinic Acid](image)

<table>
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<th>Assignment</th>
<th>DBG-D13</th>
<th>MBG-Q3</th>
<th>ERG-E7</th>
<th>Betulinic acid</th>
<th>DEPT</th>
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26. 16.6 16.7 16.7 16.90q CH₃
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28. 177.5 180.5 180.5 179.32s C=O
29. 109.9 110.1 110.1 110.41t CH₂
30. 19.5 19.6 19.6 19.95q CH₃

Ref. [14, 15]

Compounds 1 and 3
(3-beta-hydroxy-20(29)-lupen-28-oic acid)

Compound 2
(3-alpha-hydroxy-20(29)-lupen-28-oic acid)

**Fig. 1. Structures of Compounds 1, 2 and 3**

4. CONCLUSION

In conclusion, the present work confirms the isolation of large amount of betulinic acid from dichloromethane, methanol, (stem bark) and ethanol extract (root) of *Uapaca guineensis* with α and β orientation. Similarly, the values of ¹H-NMR, IR, UV, EI-MS, HREI-MS, ¹³C-NMR and 2D-NMR obtained were in agreement with the literature values [15].

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**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

**AUTHORS’ CONTRIBUTIONS**

This work was carried out in collaboration between all Authors. ‘Author IO’ designed the study, performed the statistical analysis and wrote the protocol. ‘Author AO’ managed the analyses of the study, wrote the first and final draft of the manuscript. ‘Author PO and AO’ managed the literature searches. ‘Author IC and IO’ monitored the analyses and provided major equipments used. All authors read and approved the final manuscript.”

**CONSENT**

It is not applicable.

**ETHICAL APPROVAL**

It is not applicable.

**REFERENCES**


