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Isolation and characterization of β-sitosterol, oleanolic, 19-dehyroursolic and yarumic acids, from *Plectranthus esculentus* leaves and tubers

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Abstract

Plectranthus esculentus N.E.Br. (family Lamiaceae) also known as Livingstone potato (*vat* or *rizga* in Nigeria), is a dicotyledonous perennial shrub growing up to 2 m tall. While it is cultivated mainly for its edible tubers, the plant is potentially valuable as phytomedicine. Three varieties (*vat-long'at, vat-riyom* and *vat-bebot*) are well known among the Berom of Plateau State, Nigeria. The *vat-bebot* variety (which showed good promise in bioactivity studies) was used in this study. The leaves and tubers were extracted successively with hexane, ethyl acetate, methanol and water. Fractionation of the active ethyl acetate extracts was carried out using open column and preparative High Performance Liquid Chromatography (prep HPLC). This led to the isolation of β-sitosterol and oleanolic acid from the leaves; while 19-dehydroursolic acid and yarumic acid, as well as β-sitosterol were isolated from the tubers. Nuclear Magnetic Resonance (NMR) and Electron Impact Mass Spectroscopy (EIMS) were used to characterize isolated compounds. Comparing acquired spectral data of isolated compounds with those from literature helped to confirm the identity of the compounds. The isolation and characterisation of these compounds, from *Plecthranthus esculentus*, have not been hitherto reported in literature.

Keywords: Plectranthus esculenthus; β-Sitosterol; Oleanolic acid; 19-Dehydroursolic acid; Yarumic acid

Introduction

Plectranthus esculentus N.E.Br. is a dicotyledonous perennial shrub that grows up to 2m tall and belongs to the family Lamiaceae [1-3]. Also known as Livingstone potato, it has a strong, pleasant smell with soft hairy leaves, hairy four-angled (square) and succulent stems. The leaves are oval in shape, with toothed margins, 50-70 mm in length and arranged in opposite pairs [4,5]. The flower is small, usually about 1.5 cm long, bilaterally symmetrical, with united petals. It also contains a four-lobed ovary that produces four one-seeded nutlets [5]. The long and cylindrical roots form clusters of short hairy edible tubers at the base of the stem that are 5-10 cm long and up to 2 cm in diameter [1,4,6].

P. esculentus is widespread throughout Africa. In Nigeria, it is common in Plateau State, where it is grown mainly because of its edible tubers [1]. It is locally known as *vat* (in Berom) or *rizga* (in Hausa). Several varieties

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of the plant are known, these differ in the shape and size of the tubers and also in the height of the plant. The different varieties are known by local names of *vat-long'at, vat-riyom* and *vat-bebot* among Berom people. *P. esculentus* is a plant with very good prospects in phytomedicine [1]. Ethnobotanically, it has found use in the treatment of digestive disorders, stomach ache, cancer, and for the relief of pain [7-11]. Also, a bolus of the crushed leaf is used as an anal suppository for oxyuris (human threadworm) [7]. Additionally, the Berom people of Plateau state, Nigeria believe that consumption of *P. esculentus* tubers is responsible for low incidence of diabetes, heart related diseases and goiter among the people. Furthermore, the antioxidant (*in vivo* and *in vitro*) and antidiabetic activities have been reported [1, 10-13]. The antimicrobial activity has also been reported [1]. It is common knowledge that phytochemicals are responsible for the medicinal properties of plants. A search of the literature revealed that no chemical compound has yet been isolated from *P. esculentus.* This work is therefore aimed at kick-starting the research into the chemistry of this plant by isolating phytochemicals from the leaves and tubers of *P. esculentus*.

EXPERIMENTAL METHODS

Sample collection, authentication and extraction. One of the varieties of *P. esculentus*, locally known as *vat-bebot* was used for this work. This is because, in previous works [1, 11], it showed greater potential in terms of biological activities than the other varieties. Fresh leaves and tubers of this variety of *P. esculentus* was harvested and authenticated by Mrs. Christy D. Gadu of the National Root Crop Research Institute, Vom, Plateau state, Nigeria. Voucher specimens were deposited in the herbarium at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Jos, Nigeria and was assigned the voucher number of UJ/PCG/HSP/17L01. The leaves and tubers were properly washed, sliced and then air-dried. The dried tubers and leaves were powdered. The powdered tubers were individually macerated successively for 48 hours with hexane, ethyl acetate, methanol and water to obtain extracts coded HBT, EBT, MBT and ABT respectively. Similarly, maceration of leaves with hexane, ethyl acetate, methanol and water successively, yielded extracts coded HBL, EBL, MBL and ABL respectively. All extracts were concentrated *in vacuo* and stored in a desiccator. The ethyl acetate extracts showed good bioactivity in previous studies [1,11] and were therefore further processed by fractionation.

Fractionation of extract EBL. Ethyl acetate extract of the leaves, EBL (11 g) was solvent-partitioned into hexane to remove any fatty residue and pigments left in it; after which it was also partitioned into ethyl acetate. The ethyl acetate portion (EBLe) was packed in silica gel column (normal phase silica gel 60, 0.040-0.063 mm) and eluted, stepwise, with hexane, ethyl acetate and methanol. This afforded compounds $EBLe_{18}$ and $EBLe_{33,}$ which gave single and clear spots on TLC plates and also had the same R_f. It was therefore suspected that the two compounds are the same. The Nuclear Magnetic Resonance (NMR) and Mass Spectra (MS) of the two compounds further confirmed them to be same; therefore, the two compounds were joined together and named compound $EBLe_{18}$ (7.7 mg). Impure fraction $EBLe_{180}$ (206 mg) obtained from the fractionation of EBLe was packed in silica gel column and eluted, stepwise, with hexane and ethyl acetate. This yielded compounds $EBLe_{180}$c and d which showed the same TLC, NMR and MS profile, therefore, they were combined and named compound $EBLe_{180}$d (38.1 mg).

Fractionation of Extract EBT. Ethyl acetate extract of the tubers, EBT (5.9 g) was packed on silica gel column and eluted, stepwise, with

hexane, ethyl acetate and methanol. This yielded the impure fraction EBT_{132} (119.3 mg). This was loaded on silica gel column and eluted with hexane, dichloromethane and methanol, yielding fraction EBT_2 which was a mixture of two compounds with very close R_f. Fraction EBT_2 was loaded on silica gel column and eluted with hexane and ethyl acetate. The compounds could not be separated; therefore, the fractions collected were bulked together and loaded on prep HPLC column packed with normal phase silica gel (equipment used was LC-908W-C60, Recycling preparative HPLC, Japan Analytical Industry Co. Ltd). Compounds EBT_2a (4.0 mg) and EBT_2b (7.9 mg) were eluted using hexane and ethyl acetate (1:9) isocratic elution. Impure fraction EBT_{29} (15.5 mg) from extract EBT was loaded on prep HPLC column packed with normal phase silica gel and eluted with hexane: ethyl acetate (8:2) isocratic elution. This yielded compound EBT_5b (12.9 mg).

Spectroscopic analysis. Spectroscopic analysis was carried out using proton NMR (Avance NEO and Avance-III AV equipment from Bruker, Switzerland). Electron Impact Mass Spectrometry (EIMS) was carried out on JMS.600H equipment from JEOL, Japan.

RESULTS

Spectroscopic analysis of isolated compounds. ^{1}H NMR, and EIMS data of the isolated compounds were collected.

Compound EBLe$_{18}$

Appearance: Pale yellow crystals

^{1}H-NMR spectrum ($CDCl_3$+ CD_3OD, 500MHz): (δ_H , ppm) 0.63 (3H, s); 0.77 (3H, d, J = 6.5 Hz); 0.79 (6H, m, 7.5 Hz, 9 Hz); 0.87 (3H,d, J = 6.5 Hz); 0.96 (3H, s); 1.12 (4H, m, J = 6 Hz, 9.5 Hz); 1.17 (2H, t, 7 Hz, 14 Hz); 1.21 (4H, s); 1.24 (3H, d, J = 7 Hz); 1.42 (5H, m, 5.5 Hz, 9.5 Hz); 1.62 (1H, q, J = 6.5 Hz, 12 Hz); 1.78 (3H, m, J = 3.5 Hz, 10 Hz, 13.5 Hz); 2.22 (2H, m, J = 10 Hz, 13.5 Hz, 21 Hz); 3.46 (1H, m, J = 6.5 Hz, 11 Hz, 16.5 Hz); 5.30 (1H, d, J=5Hz)

EIMS: $[M]^+$ m/z value of 414.3.

Compound EBLe$_{180}$d

Appearance: Colourless powder

^{1}H-NMR (CD_3OD) (δ_H , ppm): 0.75 (1H, d, J = 11.4Hz); 0.78 (3H, s); 0.85 (3H, s); 0.88 (3H, d, J = 6.6 Hz); 0.94 (1H, d, J = 6.6); 0.96 (3H, s); 0.97 (3H, s); 0.98 (3H,s); 1.08 (1H, m, J = 3Hz, 7.2Hz); 1.12 (3H, s); 1.16 (s); 1.31 (1H, m, J = 3.6Hz, 12 Hz, 16.8Hz); 1.39 (3H, m, J = 6.6Hz, 11.4Hz, 22.8Hz); 1.51 (1H, m, J = 4.2Hz, 7.2Hz, 10.2Hz); 1.54 (2H, t, J = 3.6Hz, 7.8Hz); 1.62 (2H, m, J = 3 Hz, 7.8Hz, 10.8Hz); 1.93 (2H, m, J = 6.6Hz, 10.2Hz); 2.19 (1H, d, J = 11.4 Hz); 3.14 (1H, q, J = 7.2 Hz, 11.4 Hz); 5.21 (1H, s)

EIMS gave the molecular mass (m/z) of the compound as 456.3.

Compound EBT$_2$a

Appearance: Light yellow powder

^{1}H-NMR spectrum (CD_3OD, 500 MHz) (δ_H , ppm): 0.78 (3H, s); 0.86 (3H, s); 0.92 (3H, d, J = 6.5 Hz); 0.98 (3H, s); 1.18 (3H, s); 1.25 (2H, t, J = 7.5 Hz, 13.5 Hz); 1.28 (1H, s); 1.30 (1H, s); 1.34 (3H, s); 1.59 (3H, s); 1.85 (1H, q, J = 7 Hz, 10 Hz); 1.98 (1H, d, J = 3 Hz); 2.00 (1H, t, J = 4 Hz, 10.5 Hz); 2.49 (1H, s); 2.57 (1H, ddd, J = 4 Hz, 13 Hz, 17 Hz); 3.92 (1H, t, J = 3 Hz, 11 Hz); 4.56 (s); 5.28 (1H, s)

EIMS: $[M]^+$ m/z value of 488.2.

Compound EBT$_2$b

Appearance: Pale white powder

^{1}H-NMR spectrum (CD_3OD, 500 MHz) (δ_H , ppm): 0.80 (3H, s); 0.86 (3H, s); 0.90 (3H, s); 0.93 (3H, s); 1.00 (3H, s); 1.07 (1H, d, J = 14 Hz); 1.11 (1H, d, J = 4.5 Hz); 1.19 (1H, t, J = 11 Hz, 17 Hz); 1.32 (2H, m, J = 3.5 Hz, 8.5 Hz, 14.5 Hz); 1.41 (2H, m, J = 3.5 Hz, 14.5 Hz, 28 Hz); 1.54 (3H, s); 1.68 (1H, t, J = 14 Hz, 29 Hz); 1.76 (2H, q, J = 13.5 Hz, 27.5 Hz); 1.94 (3H, s); 2.00 (1H, ddd, J = 3.5 Hz, 13.5 Hz, 17 Hz); 2.85 (1H, dd, J = 3.5 Hz, 14 Hz); 2.89 (1H, d, J = 9.5 Hz); 3.61 (1H, ddd, J = 4.5 Hz, 11 Hz, 14 Hz); 5.24 (1H, br s), δ4.56 (s)

EIMS: $[M]^+$ m/z value of 454.2

Compound EBT$_5$b

Appearance: Pale yellow crystals

^{1}H NMR spectrum ($CDCl_3$, 500MHz) (δ_H , ppm): 0.67 (3H, d, J = 9.5); 0.81 (9H, m); 0.90 (3H, d, J = 6.5 Hz); 0.99 (3H, s); 1.00 (2H, s); 1.06 (3H, m); 1.13 (3H, t, J = 9.5 Hz, 19.5 Hz); 1.25 (2H, q, J = 8 Hz, 14.5 Hz, 21 Hz); 1.43 (3H, m, J = 2.5 Hz, 11.5 Hz, 15.5 Hz); 1.49

(4H, m, J = 6.5 Hz, 13 Hz, 17.5 Hz); 1.53 (s); 1.83 (3H, q, J = 3.5 Hz, 7 Hz, 13.5 Hz); 1.97 (2H, m, J = 3.5 Hz, 13 Hz, 22 Hz); 2.24 (2H, m, J = 3.5Hz, 4.9Hz, 13Hz); 3.49 (1H, m, J = 6 Hz, 11.5 Hz, 15.5 Hz); 5.33 (1H, d, J = 4 Hz)

EIMS: $[M]^+$ m/z value of 414.3

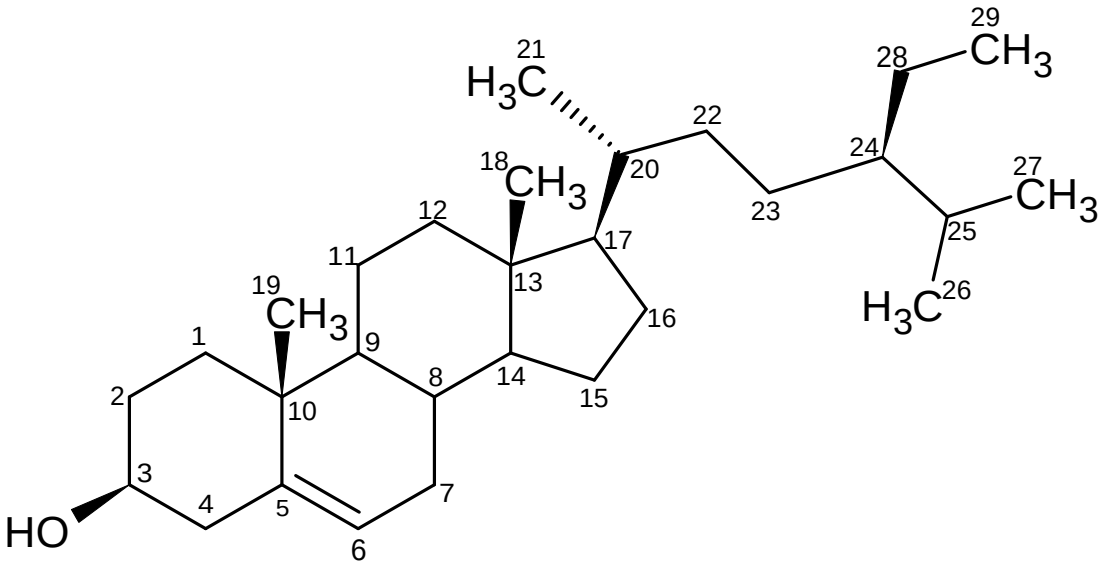

Fig.1: Sitosterol (EBLe18, EBT5a)

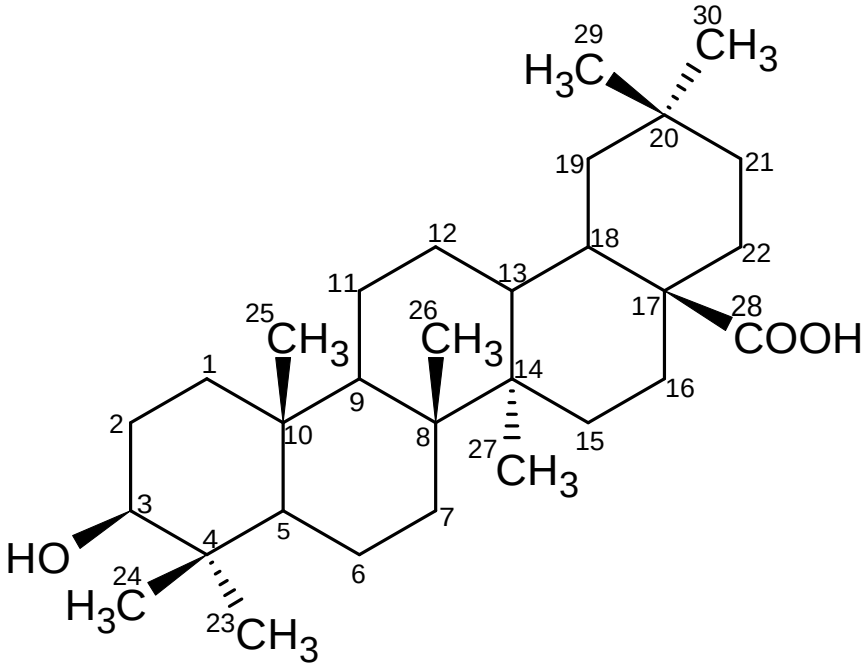

Fig. 2: Oleanolic acid ($EBLe_{180}d$)

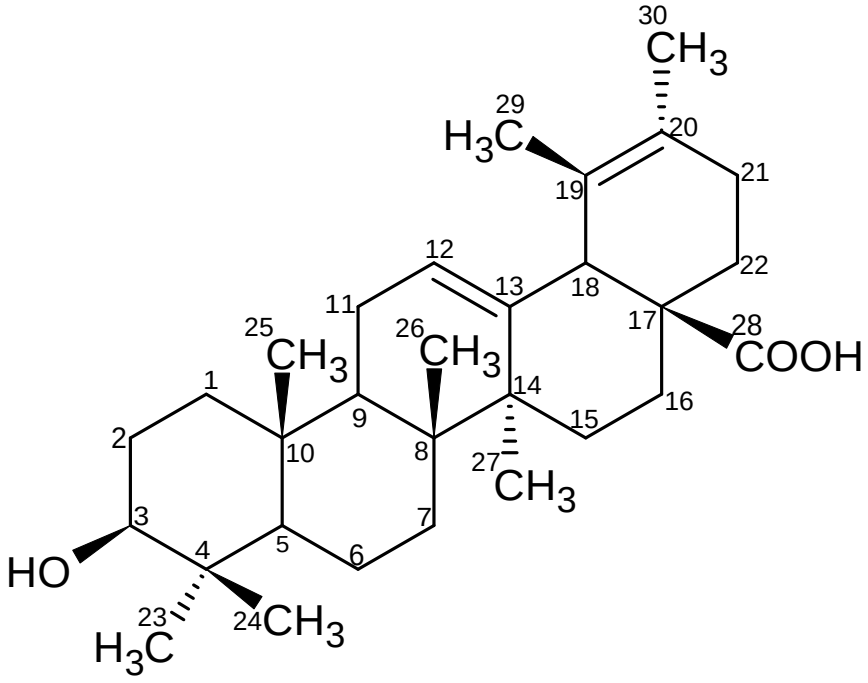

Fig. 3: 19-dehydroursolic acid (EBT_2b)

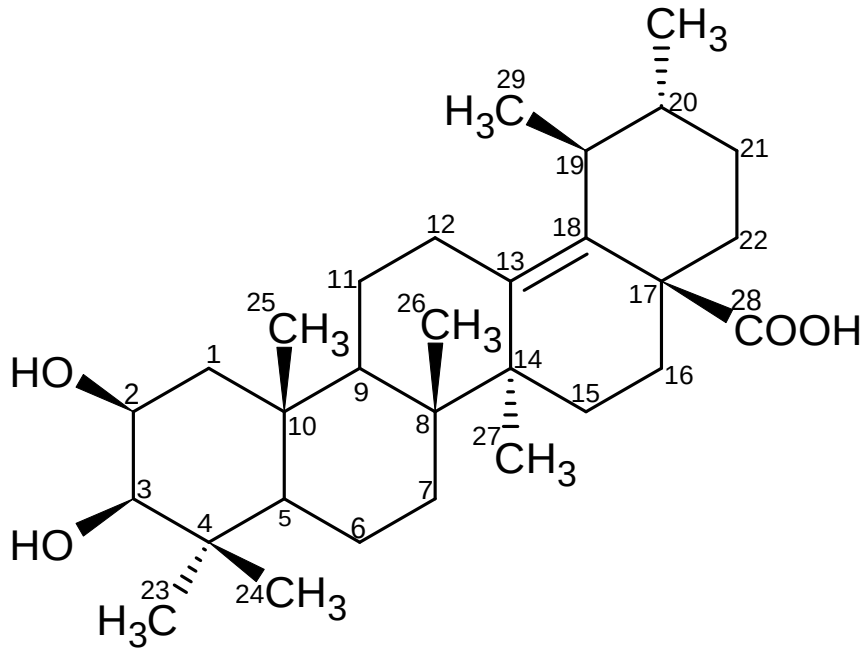

Fig.4: Yarumic acid (EBT_2a)

DISCUSSION

The chemical structures of the compounds were elucidated by comparing the proton NMR spectra with the literature, and also by obtaining suggested compounds from the NIST Mass Spectra library. This was done because the compounds have been previosly isolated and their spectra and chemical structures are already established.

Compound $EBLe_{18}$ is β-sitosterol (Fig. 1)[14,15]. This can be seen from the ^{1}H NMR which shows the two angular methyl protons

(H-18 & H-19) at δ 0.63 and 0.96 respectively, each appearing as a 3H singlet. Terminal methyl group branching off the side chain (H-21) is seen at δ 0.87 while the methyl protons of the isopropyl group (H-26 & H-27) appear at δ 0.79 as overlapping 6H multiplet. The methine proton at H-3, deshielded due to the attached OH, appears at δ 3.46 as a 1H multiplet. The olefinic proton (H-6) appears at δ 5.30 as a 1H doublet and has a small *J* value as expected. Literature reports this signal as 'overlapping triplet' [15] probably because the two neighbouring methylene protons (H-7a,7b) are non-equivalent and therefore split H-6 differently. This was however observed as a poorly resolved doublet at the resolution of the spectrometer employed in this study. Other signals in the upfield region can be seen. All signals compare very well, consistently, with literature values [14,15]. In addition, the mass spectrum (EIMS) gave a molecular ion peak at m/z 414.3 which corresponds to that of β-sitosterol ($C_{29}H_{50}O$) – Fig 1. In the same vein, compound EBT_5b exhibited very similar spectral features (^{1}H NMR and EIMS) as those identified in $EBLe_{18}$. Thus it was also characterised as β-sitosterol [14,15]. β-sitosterol ($EBLe_{18}$; EBT_{5b}) was previously isolated from the root bark of *Synadenium glaucescens* [16], the fresh uncrushed leaves of *Eucalyptus camaldulensis* [17], the root bark of *Terminalia glaucescens* [18], the leaves of *Rumex acetosella* [19], the leaves of *Odontonema strictum* [20] and the root bark of *Malva parviflora* [15]. β-sitosterol has been reported to possess anticancer, antioxidant, antidiabetic, antibacterial, anti-inflammatory, antiarthritic, antipyretic and immunomodulatory activities [21].

Compound $EBLE_{180}d$ is oleanolic acid (Fig. 2) [22]. This is evident from the ^{1}H NMR which showed seven characteristic angular (tertiary) methyl groups, appearing as 3H singlets, at δ 0.78, 0.85, 0.88, 0.96, 0.97, 0.98 and 1.12 ppm. The characteristic vinyl proton (H-12) is seen at δ 5.21 ppm while another characteristic signal, H-18 methine proton, appears at δ 2.19 ppm. These point to the presence of olean-12-ene skeleton [22]. The methine proton at H-3, deshielded due to attached OH, has its signal moved downfield, as expected, to δ 3.14 ppm. These signals are consistent with those cited in literature for oleanolic acid [22]. In addition, EIMS data gave $[M]^+$ at m/z 456.3 which corresponds to $C_{30}H_{48}O_3$ thus confirming oleanolic acid (Fig. 2). Oleanolic acid ($EBLE_{180}d$) was previously isolated from the aerial parts of *Satureja mutca* [22], the leaves of *Rosa woodsia,* the leaves & twigs of *Prosopis glandulosa*, the whole plant of *Phoradendon juniperinum*, the leaves of *Syzgium claviflorum*, the whole plant of *Hyptis capitata*, the aerial parts of *Ternstromia gymnanthera*, others are *Plantago major* and *Ludwigia octavalvis* [17]. This compound reportedly demonstrated antiviral, anti-HIV, antidiabetic, antibacterial, antifungal, anticarcinogenic, anti-inflammatory, hepatoprotective effects [23]. Others include α-glucosidase inhibitory activity [24] and immunomodulatory activity [25].

Compound EBT_2b was found to be 19-dehydroursolic acid (Fig. 3) [26,27]. This is evident from the ^{1}H NMR which portrays seven angular methyl groups, appearing as 3H singlets at δ 0.80, 0.86, 0.90, 0.93, 1.00, 1.54 and 1.94 ppm. The 3-H methine proton, strongly deshielded by presence of OH is seen at δ 3.61 ppm. The H-18 methine proton, being allylic to both double bonds, is significantly deshielded, seen at δ 2.89 ppm. The olefinic proton (H-12) is seen as a broad singlet at δ 5.24 ppm. These signals compare very well with those cited in literature for 19-dehydroursolic acid [26,27]. In addition, the mass spectrum (EIMS) gave a molecular ion peak at m/z 454.2 which corresponds to that of 19-dehydroursolic acid ($C_{29}H_{50}O$) – Fig 3. 19-dehydro-ursolic acid (EBT_{2b}) has been previously isolated from the root of *Ilex asprella* [26,27] and the leaves of *Empetrum nigrum* [28].

Compound EBT_2a was found to be yarumic acid (Fig. 4) [29]. This was deduced from the ^1H NMR and EIMS when compared with data obtained from literature. Like ursolic acid, yarumic acid also has the α-amyrin skeleton. Yarumic acid however differs from ursolic acid in that it has two extra hydroxyl groups (2-OH and 20-OH). Also the $\Delta^{12/13}$ double bond of ursolic acid switches to $\Delta^{13/18}$ in yarumic acid (Fig 4). The ^1H NMR data of the isolated compound reveals six angular (tertiary) methyl groups, appearing as 3H singlets, at δ 0.78, 0.86, 0.98, 1.18, 1.34, and 1.59 while the methyl at position 29, being secondary, is seen as a 3H doublet (split by H-19) and appears at δ 1.92 ppm. The strongly deshielded H-2 and H-3 methine protons are seen at δ 3.92 & 2.57 respectively. Similarly, H-19 methine proton, deshielded as a result of its close proximity to 20-OH, appears at δ 2.49 ppm. These signals compare very well with those given in literature for yarumic acid [29]. Confirmation was obtained from EIMS data which gave $[M]^+$ at m/z 488.2 corresponding to $C_{30}H_{48}O_5$ for yarumic acid (Fig 4). Yarumic acid (EBT_2a) has been previously isolated from the root of *Cercropia telenitida* [30] and the aerial parts of *Fagopyrum dibotrys* [31]. This compound possesses immunodulatory and anti-inflammatory activity [29].

In conclusion, the present work reports the isolation of four compounds, three triterpenes and a sterol from both the leaves and tubers of *P. esculentus* for the first time. The compounds were identified as oleanolic acid, 19-dehydroursolic acid, yarumic acid and β-sitosterol. Characterization was carried out on the basis of ^1H NMR and mass spectrometric data and by comparing them with those reported in the literature. The biological properties of *P. esculentus* cannot but be connected to the presence of these phytochemicals. These phytochemicals have been known to be responsible for various biological activities in plants, whether as isolated compounds or extracts that possess them.

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