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TRITERPENOIDS FROM THE STEM BARK OF MYRICA ESCULENTA BUCH.–HAM

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ABSTRACT

Phytochemical investigation of stem bark of Myrica esculenta Buch.-Ham. syn. M. nagi Hook.f. (Myricaceae) led to the isolation of four taraxerane-type triterpenoids characterized as 3β, 28, 30-trihydroxytaraxara-23-oic acid (**1**), 3β, 28-dihydroxytaraxerane (**2**), 3β,30-dihydroxy- taraxerane-23-oic acid (**3**) and 3β, 12α, 28, 30-tetrahydroxytaraxeran-23-oic acid (**4**) which were elucidated using spectroscopic and chromatographic analysis.

KEYWORDS: Myrica esculenta, Myricaceae, pentacyclic triterpenoids, phytochemical studies

INTRODUCTION

Complementary and alternative medicine is one of the oldest ways to treat various diseases and disorders. It mainly includes formulations from the plant, animal and mineral origin. Inflammation and rheumatism is the leading ailment of health and affect a large population worldwide. To give authentic information about various herbs useful in inflammation and rheumatism a need was felt to evaluate these herbs scientifically for their potential as anti-inflammatory and antiarthritic agents. As a result a huge data has been generated.

Myrica esculenta Buch.-Ham., syn. M. nagi Hook.f. (Myricaceae), have a common name as kaiphal or box berry, is found at an altitude of 900- 2,100 m. It his useful in various conditions as it is antirheumatic, antiseptic, aromatic, astringent, carminative, ophthalmic and

stimulant. Also, it is having utility in conditions including asthma, bronchitis, ulcers etc.[1,2] A lotion prepared from its bark extract is useful to wash putrid sores. The bark juice relieves toothache and has shown its potential against rheumatism.[3] The major constituents found in the bark include gallic acid, myricanone, myricanol, myricetin 3-rhamnoside, epigallocatechin 3-O-gallate, prodelphinidin dimmers, diarylheptanoid glycosides, n-hexadecanol , eudesmol acetate, palmitic acid, cis-β-caryophyllene, n-pentadecanol and n-octadecanol.[4–7]

The present paper describes the isolation and structure elucidation of taraxerane-type pentacyclic triterpenoids from the stem bark of M. esculenta collected from Mandi, Himachal Pradesh, India.

MATERIAL AND METHODS

General

Melting points were determined on a Perfit melting apparatus (Ambala, Haryana, India) and are uncurrected. UV spectra were measured with a Lambda Bio 20 spectrophotometer (Perkin-Elmer-Rotkreuz, Switzerland) in methanol. Infra red spectra were recorded on Shimadzu FTIR 5000 (FTS 135, Japan) spectrophotometer using KBr pellets; γ_{max} values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were screened on advance DRX 400, Bruker spectrospin 400 and 100 MHz instrument in 5 mm spinning tubes at 27 ºC, respectively (Karlesruthe, Germany) using TMS as an internal standard. Mass spectra were scanned by effecting FAB ionization at 70 eV on a JEOL-JMS-DX 303 spectrometer (Japan) equipped with direct inlet probe system. Column chromatography was performed on silica gel (60-120 mesh; Qualigen, Mumbai, India). TLC was run on silica gel G (Qualigen). Spots were visualised by exposing to iodine vapours, UV radiation, and spraying with ceric sulphate solution.

Collection and authentication of drug material

The stem bark of Myrica esculenta Buch.-Ham., syn. M. nagi Hook.f. (Myricaceae) was collected from the provinces around Sunder Nagar, Dist. Mandi, Himachal Pradesh, India. The herbarium was submitted and the plant material was authenticated at National Bureau of Plant and Genomic Research (NBPGR), Pusa Campus, New Delhi, India (Voucher number: EP 533).

Extraction and isolation

The stem bark (3.5 kg) was coarsely powdered and extracted in a Soxhlet apparatus with methanol for 72 hours. The methanolic extract was concentrated under reduced pressure to get brown colored powder. 60 g extract was subjected to column chromatography and fractions were eluted with varying proportions of hexane, chloroform and methanol. The fractions collected were subjected to thin layer chromatography (TLC) to check homogeneity of various fractions. Chromatographically identical fractions (having same R_f values) were combined together and concentrated. The concentrated fractions were purified by flash chromatography. Then they were crystallized with suitable solvent system.

Detailed analysis data of isolated phytoconstituents

Trihydroxytaraxaranoic acid (1)

Elution of the column with toluene: n-butanol (19:1) mixture gave light greenish coloured amorphous powder of **1**, purified by flash chromatography and recrystallized from methanol, 128 mg (0.031%). R_f value: 0.9 (toluene: ethyl acetate, 7:3), melting point 218 – 220 ^{0}C, UV λ_{max} (MeOH) 237 nm (log ε 4.8); IR γ_{max} (KBr) 3510, 3404, 3265, 2929, 2857, 1691, 1636, 1456, 1385, 1261, 1083, 1021, 802 cm $^{-1}$; ^{1}H NMR ($CDCl_3$) δ 1.96 (2H, m, H_2-1), 2.24 (2H, m, H_2-2), 4.03 (1H, dd, J = 4.9, 9.0 Hz, H-3α), 1.69 (1H, m, H-5), 1.30 (2H, m, H_2-6), 1.45 (2H, m, H_2-7), 1.54 (1H, m, H-9), 1.42 (2H, m, H_2-11), 1.69 (2H, m, H_2-12), 5.45 (1H, d, J = 5.7 Hz, H-15), 2.44 (2H, brs, H_2-16), 1.52 (2H, m, H-19), 1.92 (2H, dd, J = 12.6, 4.5 Hz, H_2-21), 1.38 (2H, dd, J = 12.6, 5.8 Hz, H_2-22), 1.18 (3H, brs, Me-24), 0.99 (3H, brs, Me-25), 0.89 (3H, brs, Me-26), 0.82 (3H, brs, Me-27), 3.11 (2H, brs, H_2-28), 0.72 (3H, brs, Me-29), 3.92 (2H, brs, H_2-30); ^{13}C NMR (Table 1); ESI MS (+ve mode) m/z (rel. int.) 489 $[M+H]^+$ ($C_{30}H_{49}O_5$) (10.6).

Dihydroxytaraxerane (2)

Elution of the column with toluene: n-butanol (3:2) mixture furnished colourless powder of **2**, purified by flash chromatography and recrystallized from methanol, 1.8 g (0.48%). R_f value: 0.76 (toluene: ethyl acetate 7:3), melting point: 274 – 275 ^{0}C, UV λ_{max} (MeOH): 203 nm (log ε 3.8), IR γ_{max} (KBr): 3376, 3280, 2943, 2858, 1638, 1466, 1385, 1262, 1027, 811 cm $^{-1,}$, ^{1}H NMR ($CDCl_3$) δ 2.01 (2H, m, H_2-1), 2.50 (2H, m, H_2-2), 4.06 (1H, dd, J = 5.1, 8.8 Hz, H-3α), 1.68 (1H, m, H-5), 1.30 (2H, m, H_2-6), 1.50 (2H, m, H_2-7), 1.60 (1H, m, H-9), 1.44 (2H, m, H_2-11), 1.78 (2H, m, H_2-12), 5.44 (1H, m, H-15), 2.49 (2H, m, H_2-16), 2.06 (1H, m, H-18), 1.50 (2H, m, H_2-19), 1.90 (2H, m, H_2-21), 1.35 (2H, m, H_2-22), 0.99 (3H, brs,

Me-23), 1.18 (3H, brs, Me-24), 0.89 (3H, brs, Me-25), 0.87 (3H, brs, Me-26), 0.82 (3H, brs, Me-27), 3.05 (2H, brs, H_2-28), 0.72 (3H, brs, Me-29), 0.85 (3H, brs, Me-30); ^{13}C NMR (Table 1); ESI MS (+ve mode) m/z (rel. int.): 443 $[M+H]^+$ ($C_{30}H_{51}O_2$) (10.1).

Dihydroxytaraxaranoic acid (3)

Elution of the column with n-butanol: ethyl acetate (17:3) mixture afforded greenish brown colored powder of **3**, purified by flash chromatography and recrystallized from methanol, 204 mg (0.052%). R_f value: 0.61 (toluene: ethyl acetate 7:3), melting range: 173 – 174 ^{0}C; UV λ_{max} (MeOH): 207 nm (log ε 5.1), IR γ_{max} (KBr): 3428, 3350, 3245, 2963, 2856, 1704, 1639, 1465, 1385, 1261, 1096, 1021, 862, 802 cm $^{-1}$, ^{1}H NMR: ($CDCl_3$), δ 2.00 (2H, m, H_2-1), 2.10 (2H, m, H_2-2), 3.99 (1H, dd, J = 5.1, 8.8 Hz, H-3α), 1.62 (1H, m, H-5), 1.32 (2H, m, H_2-6), 1.49 (2H, m, H_2-7), 1.53 (1H, m, H-9), 1.41 (2H, m, H_2-11), 1.65 (2H, m, H_2-12), 5.52 (1H, d, J = 5.5 Hz, H-15), 2.34 (2H, m, H_2-16), 2.06 (1H, m, H-18), 1.58 (2H, m, H_2-19), 1.91 (1H, m, H_2-21), 1.37 (2H, m, H_2-22), 1.25 (3H, brs, Me-24), 0.98 (3H, brs, Me-25), 0.89 (3H, brs, Me-26), 0.80 (3H, brs, H-27), 0.62 (3H, brs, Me-28), 0.75 (3H, brs, Me-29), 3.88 (2H, brs, H_2-30); ^{13}C NMR (Table 1); ESI MS (+ve mode) m/z (rel. int.) 472 $[M]^+$ ($C_{30}H_{48}O_4$) (6.3).

Tetrahydroxytaraxenoic acid (4)

Elution of the column with n-butanol: ethyl acetate (9:11) mixture yielded pale yellow coloured powder of **4**, purified by flash chromatography and recrystallized from methanol, 184 mg (0.048%). R_f value: 0.47 (toluene: ethyl acetate, 7:3), melting range: 153 – 155 ^{0}C, UV λ_{max} (MeOH): 230 nm (log ε 3.8), IR γ_{max} (KBr): 3450, 3393, 3250, 2930, 2857, 1707, 1638, 1456, 1376, 1230, 1110, 1030 cm $^{-1}$. ^{1}H NMR ($CDCl_3$): δ 1.88 (2H, m, H_2-1), 2.36 (2H, m, H_2-2), 4.03 (1H, dd, J = 4.8, 9.9 Hz, H-3α), 1.70 (1H, m, H-5), 1.30 (2H, m, H_2-6), 1.46 (2H, m, H_2-7), 1.56 (1H, m, H-9), 1.42 (2H, m, H_2-11), 3.58 (1H, dd, J = 3.6, 5.1 Hz, H-12β), 5.44 (1H, dd, J = 3.0, 4.8 Hz, H-15), 2.70 (2H, m, H_2-16), 2.21 (1H, m, H-18), 1.50 (2H, m, H_2-19), 1.93 (2H, m, H_2-21), 1.40 (2H, m, H_2-22), 10.78 (1H, s, COOH-23), 1.18 (3H, brs, Me-24), 0.89 (3H, brs, Me-25), 0.85 (3H, brs, Me-26), 0.82 (3H, brs, Me-27), 3.08 (2H, brs, $HOCH_2$-28), 0.72 (3H, brs, Me-29), 3.84 (2H, brs, $HOCH_2$-30); ^{13}C NMR (Table 1). ESI MS (+ve mode) m/z (rel. int.) 505 $[M+H]^+$, ($C_{30}H_{49}O_6$) (12.7).

Table 1 ^{13}C NMR spectral data of compounds 1-4

Carbon no.	1	2	3	4	Carbon no.	1	2	3	4
1	37.9	37.87	37.98	38.05	16	33.49	35.75	33.48	33.06
2	27.97	26.22	27.96	27.97	17	40.36	38.43	40.35	32.52
3	79.06	77.43	79.04	79.15	18	49.16	47.58	49.14	49.88
4	38.76	38.7	38.75	39.25	19	41.32	14.2	41.29	41.29
5	55.52	54.05	55.5	55.48	20	28.61	26.64	28.61	27.13
6	18.78	17.17	18.76	18.77	21	32.7	34.3	32.68	32.73
7	35.8	36.28	35.78	35.68	22	27.12	26.22	27.11	27.1
8	39.09	39.26	39.07	39.06	23	183.41	28.97	182.73	180.42
9	44.83	43.5	44.8	45.05	24	21.6	20.02	21.59	21.58
10	37.75	37.09	37.54	37.23	25	15.43	15.64	15.42	15.43
11	17.38	15.64	17.37	17.35	26	29.68	28.28	29.62	29.67
12	30.8	31.23	30.79	68.47	27	26.07	24.33	26.06	26.03
13	37.56	37.59	37.73	37.41	28	61.4	61.45	22.67	61.36
14	159.21	156.63	159.21	159.26	29	29.86	25.54	14.09	28.51
15	115.68	114.56	115.66	115.29	30	65.53	13.73	65.5	65.54

RESULTS AND DISCUSSION

The methanolic extract of stem bark of M. esculenta was subjected to column chromatographic separations on silica gel (60-120 mesh size) and further purification by flash chromatography, yielding four taraxerane-type triterpenic compounds **1** to **4.**

Compound **1,** named trihydroxy-taraxaranoic acid, was obtained as light greenish coloured amorphous powder from toluene: n-butanol (19:1) eluants. It responded positively to Liebermann –Burchard test for triterpenes and formed effervescence with sodium bicarbonate solution indicating the presence of carboxylic acid group in the molecule. Its IR spectrum showed characteristic absorption bands for hydroxyl groups (3510, 3404 cm^{-1}), carboxyl function (3265, 1691 cm^{-1}) and unsaturation (1636 cm^{-1}). On the basis of mass and ^{13}C NMR spectrum, its molecular weight was established at m/z 489 consistent with the molecular formula of trihydroxytaraxaranoic acid $C_{30}H_{49}O_5$ $[M+H]^+$. It indicated seven double bond equivalents, five of them were adjusted in the pentacyclic carbon skeleton of the triterpene and one each in the vinylic bond and carboxylic group. The ^{1}H NMR spectrum of **1** showed the presence of a one- proton doublet at δ 5.45 (J = 5.7) assigned to vinylic H-15 ptoton. Two one-proton double doublets at δ 4.03 (J = 4.9, 9.0 Hz) and δ 2.08 (J = 7.8, 7.2 Hz) were ascribed to carbinol H-3α and methine H-18β protons, respectively. Nine three-proton broad singlets at δ 1.18, 0.99, 0.89, 0.82, 0.72, and two two-proton broad singlets at δ3,11 and 3.92 were accounted to tertiary C-24, C-25, C-26, C-27, and C-29 tertiary methyl and C28 and C-

30 hydroxymethylene protons respectively, all of them were attached to saturated carbons. The remaining methine and methylene protons resonated between δ 2.44 – 1.30. The ^{13}C NMR spectrum of **1** displayed signals for carboxyl carbon at δ 183.41 (C-23), carbinol carbon at 79.06 (C-3), vinylic carbons at δ 159.21 (C-14), δ 115.68 (C-15), methyl carbons at δ 21.60 (C-24), 15.43 (C-25), 29.68 (C-26), 26.07 (C-27), 29.86 (C-29) and hydroxyl methylene carbons at δ 61.40 (C-28) and 65.53 (C-30). The ^{1}H and ^{13}C NMR spectral values of **1** were compared with the taraxarane type triterpenoids. [8 – 11] On the basis of foregoing account the structure of **1** has been established as 3β, 28, 30-trihydroxytaraxara-23-oic acid.

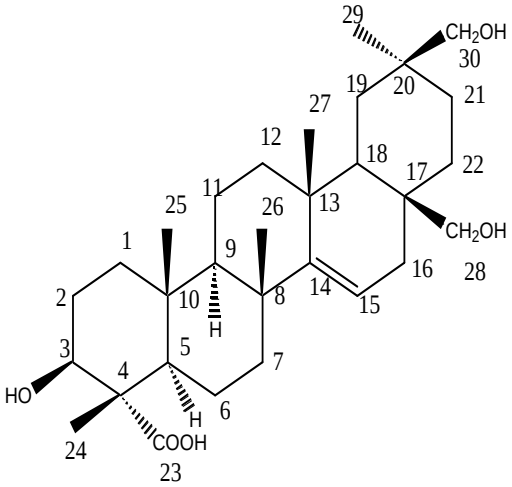
Compound **2,** named dihydroxytaraxerane, was obtained as colourless powder from toluene: n-butanol (3:2) eluants. It responded positively to Liebermann–Burchard test for triterpenes. Its IR spectrum showed characteristic absorption bands for hydroxyl group (3376, 3280 cm^{-1}) and unsaturation (1638 cm^{-1}). On the basis of mass and ^{13}C NMR spectra, its molecular weight was established at m/z 443 $[M+H]^{+}$ consistent with the molecular formula $C_{30}H_{51}O_2$. The ^{1}H NMR spectrum of **2** displayed a one-proton multiplet at δ 5.44 and a one-proton double doublet at δ 4.06 (J = 5.1, 8.8) assigned to vinylic H-15 and carbinol H-3α proton, respectively. Seven three-proton broad singlets at δ 0.99, 1.18, 0.89, 0.87, 0.82, 0.72, 0.85 were accounted to tertiary C-23, C-24, C-25, C-26, C-27, C-29 and C-30 methyl protons, respectively, all of them were attached to saturated carbons. A two-proton broad singlet at δ 3.05 was ascribed to C-28 hydroxyl methylene protons. The remaining methine and methylene protons resonated between δ 1.30 – 2.91. The ^{13}C NMR spectrum of **2** displayed signals for carbinol carbon at δ 77.43 (C-3), vinylic carbons at δ 156.63 (C-14) and δ 114.56 (C-15), hydroxyl methylene carbon at δ 61.45 (C-28) and methyl carbons at δ 28.97 (C-23), 20.02 (C-24), 15.64 (C-25), 28.28 (C-26), 24.33 (C-27), 25.54 (C-29) and 13.73 (C-30). The ^{1}H and ^{13}C NMR spectral data of **2** were compared with the taraxerane-type triterpenoids. [8 – 11] On the basis of the foregoing account the structure of **2** has been established as 3β,28-dihydroxytaraxerane.

Compound **3,** named dihydroxytaraxeranoic acid, was obtained as greenish brown coloured powder from n-butanol: ethyl acetate (17:3) eluants. It responded positively to Liebermann–Burchard test for triterpenes and formed effervescences with sodium bicarbonate solution indicating the presence of carboxylic acid group in the molecule. Its IR spectrum showed characteristic absorption bands for hydroxyl groups (3428, 3350 cm^{-1}), carboxyl group (3245, 1704 cm-1) and unsaturation (1639 cm^{-1}). On the basis of mass and ^{13}C NMR spectrum, its

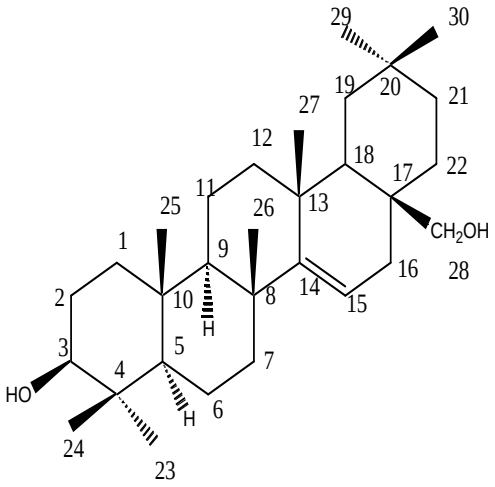
molecular weight was established at m/z 472 consistent with the molecular formula of a triterpenic acid $C_{30}H_{48}O_4$. The ^1H NMR spectrum of **3** displayed a one-proton doublet at δ 5.52 (J = 5.5 Hz) assigned to vinylic H-15. A double doublet at δ 3.99 (J = 5.1, 8.8 Hz) integrated to for one proton was ascribed to carbinol H-3α proton. Six three-proton broad singlets at δ 1.25, 0.98, 0.89, 0.80, 0.62 and 0.75 were accounted to tertiary C-24, C-25, C-26, C-27, C-28 and C-29 methyl protons, respectively, all attached to saturated carbons. A two-proton broad singlet at δ 3.88 was attributed to C-30 hydroxyl methylene protons. The remaining methine and methylene protons resonated between δ 1.31 – 2.34. The ^{13}C NMR spectrum of **3** displayed signals for carboxyl carbon at δ 182.73 (C-23), carbonyl carbon at δ 79.04 (C-3), vinylic carbons at δ 159.21 (C-14) and 115.66 (C-15), hydroxyl methylene carbon at δ 65.50 (C-30) and methyl carbons at δ 21.59 (C-24), 15.42 (C-25), 29.62 (C-26), 26.06 (C-27), 22.67 (C-28) and 14.09 (C-29). The ^1H and ^{13}C NMR spectral data of **3** have been compared with the related taraxerane type triterpenoids. [8 – 11] On the basis of foregoing account the structure of **3** has been established as 3β,30-dihydroxytaraxerane-23-oic acid.

Compound **4,** named tetrahydroxytaraxenoic acid, was obtained as pale yellow powder from n-butanol: ethyl acetate (9:11) eluants. It responded positively to Liebermann–Burchard test for triterpenes and formed effervescences with sodium bicarbonate solution indicating the presence of carboxylic acid group in the molecule. Its IR spectrum showed characteristic absorption bands for hydroxyl groups (3450, 3393 cm^{-1}), carboxyl function (3250, 1707 cm^{-1}) and unsaturation (1638 cm^{-1}). On the basis of mass and ^{13}C NMR spectra, its molecular weight was established at m/z 505 consistent with the molecular formula of tetrahydroxytaraxenoic acid $C_{30}H_{49}O_6$. It indicated seven double bond equivalents; five of them were adjusted to the pentacyclic carbon skeleton and one each in the vinylic linkage and carboxylic function. The ^1H NMR spectrum of **4** exhibited a one-proton double doublet in the deshielded region at δ 5.44 (J = 3.0, 4.8 Hz) assigned to vinylic H-15. Two one-proton double doublets at δ 4.03 (J = 4.8, 9.9 Hz) and 3.58 (J = 3.6, 5.1 Hz) were ascribed to α-oriented H-3 and β-oriented H-12 carbinol protons, respectively. Two two-proton broad singlets at δ 3.08 and 3.84 were associated with hydroxyl methylene H_2-28 and H_2-30 protons, respectively. Five three-proton broad singlets between δ 1.18 – 0.72 were accommodated to the tertiary methyl protons, all attached to saturated carbons. The remaining methine and methylene protons resonated between δ 1.30–2.70. The ^{13}C NMR spectrum of **4** displayed signals for carboxyl carbon at δ 180.42 (C-23), carbinol carbons at δ

79.15 (C-3) and 68.47 (C-12), vinylic carbons at δ 159.26 (C-14) and 115.29 (C-15), hydroxyl methylene carbons at δ 61.36 (C-28) and 65.54 (C-30) and methyl carbons at δ 21.58 (C-24), 15.43 (C-25), 29.67 (C-26), 26.03 (C-27) and 28.51 (C-29). The ^{1}H and ^{13}C NMR spectral data of **4** were compared with the reported taraxerane type triterpenoids. [8 – 11] On the basis of the above evidences the structure of **4** has been established as 3β, 12α, 28, 30-tetrahydroxytaraxeran-23-oic acid.



Trihydroxytaraxaranoic acid (1)



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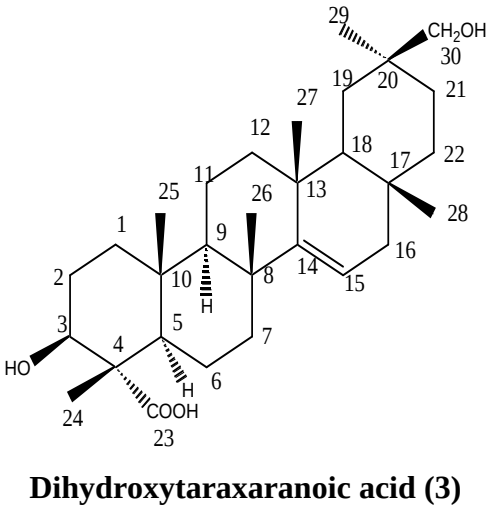
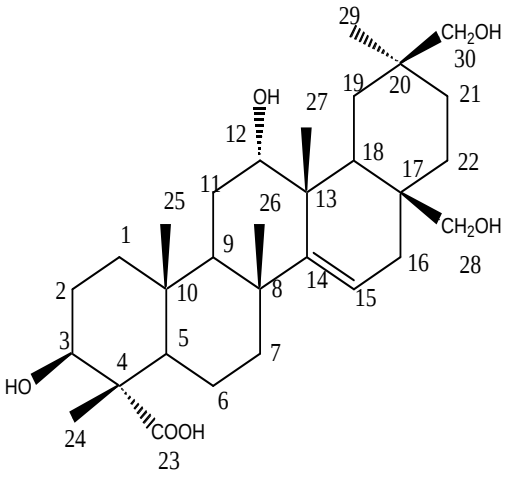


Dihydroxytaraxaranoic acid (3)



Tetrahydroxytaraxenoic acid (4)

Figure: 1 Structures of the phytoconstituents isolated from the stem bark of M. esculenta

CONCLUSION

In the current study taraxerane-type triterpenoids have been isolated from the stem bark of M. esculenta for the first time.

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