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Echinocystic acid-3-O-β-D-galactopyranosyl (1→5)-O-β-D-Xylofuranoside from Root of *Spondias mangifera* Willd

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A new triterpenoidal saponin has been isolated from the roots of *Spondias mangifera* Willd. Its structure was determined as echinocystic acid 3-O-β-D-galactopyranosyl-(1→5)-O-β-D-xylofuranoside on the basis of chemical and spectral studies.

INTRODUCTION

Spondias mangifera Wild[1] commonly known as Indian Hog Plum in English is widely distributed in India and belongs to natural order Anacardiaceae. The plant is credited to possess reputed medicinal values[2, 3]. Bark refrigerant[2], useful in dysentery, articular and muscular rheumatism; fruits[2, 3] antiscorbutic, astringent, useful in bilious dyspepsia, ulcers, burning sensations, phthisis, blood complaints; leaves[3] astringent, useful in earache. The presence of triterpenoidal compounds[4] has been reported in it by earlier workers in the aerial parts of this plant and this encouraged us to investigate the roots of *S. mangifera* further resulting in the discovery of a new triterpenoidal saponin.

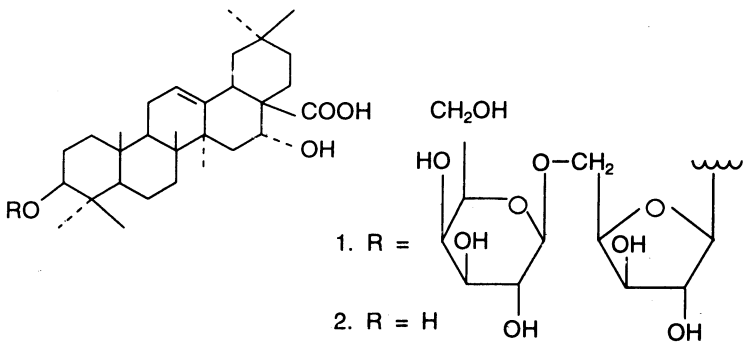
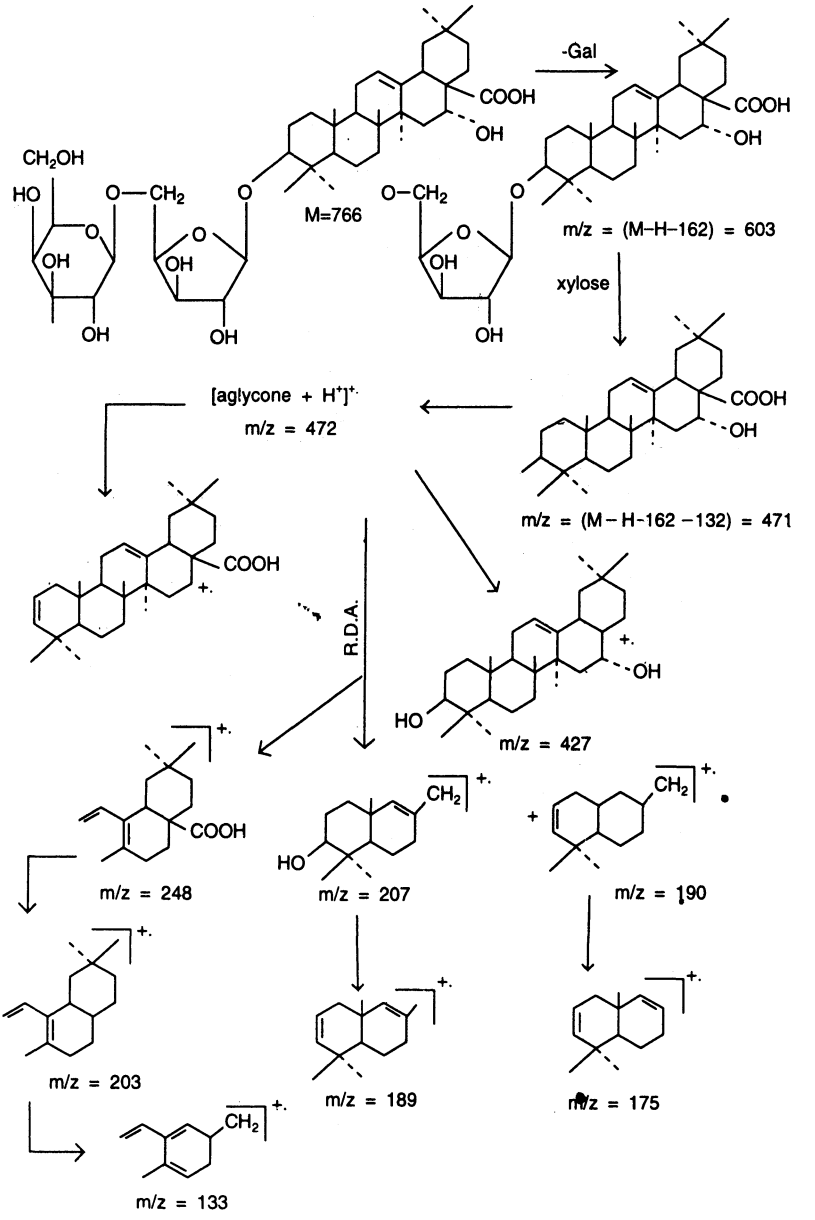


Fig. 1

RESULTS AND DISCUSSION

The roots of *S. mangifera* were extracted with 95% ethanol and the extract was treated as described in the experimental part. On chromatographic treatment saponnin 1 was obtained in pure form, crystallised from acetone into colourless crystals. Compound 1, m.p. 238–239°C $[\alpha]_D^{27}$ – 25 (in MeOH), gave characteristic reactions of saponin haemolytic test[5] and foam test[6]. It also gave an apparent molecular ion peak in its mass spectra eims at m/z 766 consistent with the molecular formula $C_{41}H_{66}O_{13}$. Characteristic ions that appeared at m/z

603 $[M-H-162]^-$, 471 $[M-H-162-132]^-$ were generated by subsequent losses from the molecular ion of one hexose and one pentose units suggesting hexose as the terminal sugar and pentose as linked to sapogenin. The various fragments formed and hypothetical scheme are shown in Scheme-I. The IR spectrum indicated peaks at 3405 cm^{-1} (due to hydroxyl group), 1720 cm^{-1} (due to acid group), 3028, 1630 cm^{-1} (due to double bond), 808 cm^{-1} ($HC{=}CH_2$), 1355, 1340 cm^{-1} (triterpenoidal nature). Compound 1 did not display any absorption in the UV spectrum above



Scheme-I

200 nm (in MeOH). The ^{1}H NMR spectrum of methyl ester of acetylated saponin exhibited the presence of seven tertiary methyl groups δ 0.99, 0.79, 0.93, 0.89, 1.25, 0.88, 0.93 each (3H, S); a singlet at δ 2.04 was assigned to 16-OAc; δ 3.1 (1H, dd, J = 8.16, 8 Hz) was assigned to H-3; a double doublet at δ 3.06 (J = 10.3, 9.14 Hz) was assigned to H-16; a double doublet at δ 5.22 (J = 4.9 Hz) was assigned to vinylic proton H-12; two doublets at δ 4.29 J = 7.4 Hz), δ 4.93 (J = 7.1 Hz) were assigned to the anomeric protons of D-xylose and D-galactose respectively.

The spectral data along with ^{13}C NMR·colour with TNM[7] finally suggested the saponin to have β-amyrin-oleanane skeleton[7,8].

To confirm its structure compound 1 was subjected to hydrolysis with 2N-H_2SO_4 when it yielded compound 2 (sapogenin) identified for m.p. 304°C, $[\alpha]_D^{27}$ + 40 (in EtOH), eims m/z 472 M^+, consistent with molecular formula $C_{30}H_{48}O_4$ and sugar moiety. Compound 2 responded to the characteristic colour reactions of triterpenoids, Tschugagew[9], Salkowask[10] and Libermann-Burkard[11] reactions.

A peak at ν_{Max}^{KBr} 1718 cm^{-1} suggested the presence of COOH group which is further confirmed by its monomethyl ester 2a formation, molecular formula $C_{31}H_{50}O_4$, m.p. 218°C, $[\alpha]_D^{27}$ + 30 (in MeOH), M^+ 486. When saponification of 2a was done by ethylene glycol it resulted into sapogenin whereas it was saponified only partially (8–10%) by 10% methanolic KOH suggesting COOH group was hindered[12]. On decarboxylation by heating compound 2 gave decarboxylated sapogenin, m.p. 190°C, molecular formula $C_{29}H_{48}O_2$, ^{1}H NMR spectrum of which showed a multiplet of one proton intensity at δ 2.6 for H-17 while no such signal for H-17 was observed in ^{1}H NMR of methylated diacetyl derivative 2b of 2, thus confirming at C–17 COOH group is present. This is further supported as ^{1}H NMR of compound 2b showed a singlet of 3 proton intensity at δ 3.63 for —COOMe group at position C_{17}.

A peak at ν_{max}^{KBr} 3410 cm^{-1} suggested OH groups(s) confirmed by acetylation of compound 2a to diacetylated 2b product, molecular formula $C_{33}H_{54}O_6$, m.p. 237°C, M^+ 570, thus suggesting two acetylable OH groups in compound 2. The presence of OH group at C-16 and C-3 is also shown by ^{1}H NMR spectrum of compound 2b signals at δ 3.07 (dd, J = 10.3, 9.0 Hz, H–16) and δ 3.5 (dd, J = 11.4, 4.6 Hz, H-3) each for one proton and singlets for three —OAc protons each at δ 2.18 (16-OAc) and 2.07 (3-OAc). α-Configuration was assigned to C-16 OH group because of downfield shift of Me_{27} proton at δ 1.28 (s). β-Configuration was assigned to C-3 OH group due to its large coupling constant (dd, J = 11.4, 4.6 Hz).

Peaks at ν_{Max}^{KBr} 3021, 1620 cm^{-1} indicated the presence of a double bond. Compound 2 gave +ve TNM test and showed high terminal UV absorption (190 nm) characterstic of 12–13 double bond in most of the triterpenes of oleanane series[13]. Further ^{1}H NMR of compound 2b showed upfield shift at δ 5.30 for vinylic proton at H-12.

Peaks at ν_{Max}^{KBr} 2860, 1370 cm^{-1} indicated the presence of methyl group(s). ^{1}H NMR spectra of compound 2b exhibited the presence of 7 tertiary methyl groups indicated by signals at δ 0.98, 0.78, 0.92, 0.88, 1.28, 0.87, 0.94 each (3H, S).

A compilation of available spectral and analytical data concerning echinocystic acid[14–16] finally confirmed the compound 1 as 3β, 16α olean-12-en-28-oic acid.

After acid hydrolysis of saponin the aqueous hydrolysate obtained was neutralised and identified for sugars as D-galactose and D-xylose (co-pc and co-tlc) with authentic samples; solvent used n-butanol : acetic acid : water (4:1:5 v/v); spraying reagent aniline hydrogen phthalate (R_f 0.16 and 0.27 respectively). Quantitative estimation by Mishra and Rao[17] showed that sapogenin, D-galactose, D-xylose were present in equimonomolecular ratio. Periodate oxidation[18] of compound 1 confirmed the presence of disaccharide sugar unit and also suggested one of the sugars was present in pyranose form (D-galactose) and another in furanose form (D-xylose).

Disaccharide sugar unit was attached to either —COOH group at C-28 or OH group at C-16, OH group at C-3. As saponin did not yield sugars on NH_4OH hydrolysis (reagent specific for sugar-ester linkage)[19] and C-16 OH is in strongly hindered position[20] so only left position is C-3 OH for sugar moiety linkage and thus glycosylation was at C-3.

The saponin 1 on partial hydrolysis with Kiliani mixture[21] for 5 days gave two prosapogenins PE_1 (m.p. 255–256°C, m.f. $C_{35}H_{56}O_8$, M^+604) and PE_2 (m.p. 238–239°C m.f. $C_{41}H_{66}O_{13}$ M^+766) which were separated by co-chromatography over si-gel using $CHCl_3$: MeOH in different proportion as eluant.

Sequence of sugars and glycosidic linkages was confirmed by permethylation and hydrolysis PE_1 and PE_2.

On hydrolysis with 7% H_2SO_4, PE_1 yielded a sapogenin and D-xylose (R_f 0.27) while PE_2 yielded sapogenin D-xylose (R_f 0.27) and D-galactose (R_f 0.16) confirmed by co-pc and co-TLC).

Permethylation of PS_1 and PS_2 by Khun *et al.*[22] method gave permethylated prosapogenins which on hydrolysis showed the presence of 2′,3′,5′-tri-O-methyl xylose in the hydrolysate of permethylated PS_1 and 2′,3′,4′,6′-tetra-O-methyl-D-galactose and 2′,3′-di-O-methyl D-xylose in the hydrolysate of permethylated PS_2 (confirmed by co-pc and co-tlc); these results indicated that C_1 of D-xylose was linked to C-1 of D-galactose and further supported D-galactose-pyranose and D-xylose-furanose form.

1 on enzymatic hydrolysis with almond emulsion liberated D-galactose and D-xylose and revealed the linkage between sapogenin and D-xylose as well as between D-xylose and D-galactose as β [23].

Thus structure assigned to saponin was echinocystic acid 3-O-β-D-galactopyranosyl-(1→5)-O-β-D-xylofuranoside.

EXPERIMENTAL

General procedure

IR spectra were recorded on Perkin-Elmer 881 spectrophotometer. ^1H NMR spectra were run on Perkin-Elmer R-32 (90 MHz, in $CDCl_3$, TMS as an internal standard). Mass spectra were determined on Jeol D-300 (F.I.). ^{13}C NMR were measured on Varian CFT-20 (TMS) as internal standard. Electric melting point apparatus was used for determining melting points. Melting points are uncorrected.

Si-gel (60–120 mesh) was used for co-chromatography. All the chemicals used were of A.R. grade.

Plant material

Roots of *Spondias mangifera* Willd was procured from M/s United Chemicals and Allied Products.

Extraction and isolation

The air-dried powdered roots (8 kg) of *S: mangifera* were extracted with 95% ethanol. The extract was concentrated under reduced pressure when the brown-coloured viscous mass after treatment with hexane and petrol was subjected to chromatography on a column with Si-gel. The column was run successively with hexane, petroleum ether, benzene, ethyl acetate, acetone and methanol. The ethyl acetate fraction showed the presence of two spots on TLC [Si-gel, glass plates, chloroform : methanol (1 : 1), visualized by I_2 vapours]. Thus ethyl acetate fraction was subjected to Si-gel column chromatography. The column was run with petroleum ether, C_6H_6, $CHCl_3$, CH_3OH. The benzene : chloroform (4 : 6) layer was evaporated, residue obtained was dissolved in methanol and dimethyl ether was added dropwise. The resulting precipitate contained crude saponin; the process of dissolution and precipitation was repeated and finally saponin was crystallized from acetone and yielded colourless crystals (2.2 g). It showed all the characteristic reactions of saponins[5, 6, 9, 10].

Saponin 1, colourless crystals, m.p. 238–239°C, $[\alpha]_D^{27}$ – 25 in (MeOH) [Found C, 64.10; H, 8.65%; Calc:. for molecular formula $C_{41}H_{66}O_{13}$: C, 64.22; H, 8.61%]; eims $[M]^+$ 766; IR ν_{Max}^{KBr} cm^{-1}: 1720 (acid group), 3028, 1630 (double bond); 808 ($HC{=}CH_2$), 2930 (Me group), 1355, 1340 (triterpenoidal nature), eims m/z 766 $[M]^+$; 603, 471, 440, 427, 248, 207, 203, 190, 189, 175, 133. ^{13}C NMR 39.6 (C-1), 28.5 (C-2), 90.8 (C-3), 40.0 (C-4), 57.4 (C-5), 17.6 (C-6), 33.9 (C-7), 39.8 (C-8), 48.6 (C-9), 37.3 (C-10), 25.1 (C-11), 123.2 (C-12), 144.8 (C-13), 43.2 (C-14), 28.5 (C-15), 66.8 (C-16), 47.8 (C-17), 42.6 (C-18), 47.1 C-19), 30.9 (C-20), 35.1 (C-21), 34.3 (C-22), 28.1 (C-23), 17.4 (C-24), 16.2 (C-25), 18.1 (C-26), 26.8 (C-27), 176.5 (C-28), 33.9 (C-29), 24.2 (C-30), 104.9 (C-1′), 72.6 (C-2′), 74.7 (C-3′), 78.2 (C-4′), 66.4 (C-5′), 103.1 (C-1″), 73.5 (C-2″), 76.1 (C-3″), 75.2 (C-4″), 76.6 (C-5″), 60.9 (C-6″). ^1H NMR (90 MHz in $CDCl_3$) of heptaacetyl derivative of methyl ester of compound 1 δ 0.99, 0.79, 0.93, 0.89, 1.25, 0.88, 0.93 (each δ, 7 × tert Me group), δ 3.1 (1H, dd, J = 8.16, 8 Hz at H-3), δ 3.06 (1H, dd, J = 10.3, 9.14 Hz, H-16), δ 2.04 (3H, S, OAc-16), δ 3.11 (3H, S, $COOCH_3$), δ 5.22 (1H, dd, J = 4.9 Hz, H-12), δ 1.18–2.02 (18H, m, polymethylene and methyl CH_2 and CH) δ 2.03 (dd, J = 4.3 Hz, 2H, CH_2-11), δ 2.47 (d, J = 4.2 Hz, 1H–18B) δ 4.29 (1H, d, J = 7.4 Hz, 1′ anomeric proton) δ 4.99 (1H, d, J = 7.1 Hz, 1″ anomeric proton), δ 3.8–4.29 (11H, m, protons of sugar residue), δ 2.05 (3H, S, 3′OAc), δ 2.12 (3H, S, 3′OAc), δ 2.08 (6H, S, 2″ and 3″ OAc), δ 2.07 (3H, S, 4″ OAc), δ 2.10 (3H, S, 6″ OAc).

Methylation of saponin

Compound 1 (50 mg) was dissolved in ether and treated with etheral CH_2N_2

with constant cooling, reaction mixture allowed to stand for 10–12 h till a yellow colour was produced. The action of excess of CH_2N_2 was destroyed by CH_3COOH addition; then the mixture was washed with H_2O, sodium bicarbonate and dried over anhydrous Na_2SO_4. The residue obtained dissolved in methanol was chromatographed over a column of silica gel, eluted with CH_3OH : CH_3COOH: CH_3COCH_3 (3:2:1) and yielded a homogeneous methyl ester of saponin (35 mg).

Acetylation of methyl ester of saponin

The ester of saponin (30 mg) was treated with Ac_2O (10 mL), pyridine (10 mL) in a round-bottomed flask fitted with reflux condenser and heated on water-bath for 3 h, reaction mixture then allowed to cool and poured into ice cold water to obtain a precipitate which was extracted with ether in a separatory funnel, etheral layer was washed with $NaHCO_3$ and after removal of solvent a colourless acetylated derivative (22 mg) resulted which was recrystallized from acetone.

Acidic hydrolysis of the saponin

The saponin (800 mg) in 15 mL EtOH was treated with 25 mL of 2N-H_2SO_4 and heated on water-bath for 5 h. The solution was then concentrated under reduced pressure, allowed to cool and residue was extracted with ether. The aqueous layer was worked up separately for the identification of sugars. The organic (etheral) layer was washed with water and dried over anhydrous sulphate. Evaporation of solvent ether under reduced pressure yielded the sapogenin 2 which was recrystallised from methanol to give colourless needles, m.p. 304°C $[\alpha]_D^{27}$ + 35, (in EtOH).

The aqueous layer neutralised with $BaCO_3$, $BaSO_4$ filtered off. The filtrate on being concentrated and subjected to paper chromatography showed the presence of D-galactose and D-xylose (nBuOH : AcOH : H_2O 4:1:5, spraying reagent aniline hydrogen phthalate, R_f 0.16 and 0.27 respectively).

Sapogenin 2, colourless needles, m.p. 304°C $[\alpha]_D^{27}$ + 40 (in EtOH). [Found : C, 75.81; H, 10.11%, Calcd. for molecular formula $C_{30}H_{48}O_4$: C, 76.27; H, 10.16%]; eims M^+ 472, IR ν_{Max}^{KBr} cm^{-1}: 1718 (—COOH group), 3410 (OH group), 3021, 1620 (double bond), 2860, 1370 (Me group); eims m/z M^+ 472, 440, 427, 248, 207, 203, 190, 189, 175, 133. ^{13}C NMR 39.8 (C-1), 28.3 (C-2), 90.7 (C-3), 39.9 (C-4), 57.3 (C-5), 17.5 (C-6), 33.6 (C-7), 40.2 (C-8), 48.0 (C-9), 37.4 (C-10), 24.9 (C-11), 123.5 (C-12), 144.6 (C-13), 43.1 (C-14), 28.4 (C-15), 66.3 (C-16), 47.7 (C-17), 43.0 (C-18), 47.7 (C-19), 31.1 (C-20), 34.7 (C-21), 33.9 (C-22), 27.5 (C-23), 17.2 (C-24), 16.2 (C-25), 17.6 (C-26), 26.2 (C-17), 177.7 (C-28), 33.8 (C-29), 24.2 (C-30). ^1H NMR (90 MHz in $CDCl_3$) of diacetyl derivative of methyl ester of sapogenin: δ 0.89 (S, 3H, Me-23), δ 0.78 (S, 3H, Me-24), δ 0.92 (S, 3H, Me-25), δ 0.88 (S, 3H, Me-26), δ 1.28 (S, 3H, Me-27), δ 0.87 (S, 3H, Me-29), δ 0.94 (S, 3H, Me-30), δ 2.07 (S, 3H, 3-OAc), δ 2.18 (S, 3H, 16-OAc), δ 3.5 (dd, J = 11.4, 4.6 Hz, 1H at C_3), δ 3.07 (dd, J = 10.3; 90 Hz, 1H at C_{16}), δ 5.30 (dd, J = 6.3 Hz, 1H vinylic proton at C_{12}), δ 2.49 (d, J = 4.1 Hz, 1H-18β), δ 2.03 (dd, J = 4.4, 10.8 Hz, 2H, CH_2-11), δ 3.63 (S, 3H, —COOMe), δ 1.29–2.22 (m, 18H, polymethylene and methyl CH_2 and CH).

Methyl ester formation of compound 2

Sapogenin 2 (400 mg) was methylated with CH_2N_2 as described for saponin 1 to yield methyl ester derivative 2a as crystals from methanol (355 mg).

Diacetyl derivative of methyl ester of compound 2

The ester 2a of sapogenin (200 mg) was acetylated with Ac_2O as described for methyl ester of saponin 1a to yield diacetylated methyl ester derivative 2b of sapogenin as colourless crystals from methanol (170 mg).

Partial hydrolysis of the saponin

500 mg of the saponin was treated with 140 mL Kiliani mixture (HCl : AcOH, H_2O; 20:50:70), the reaction mixture was kept for five days at a room temperature and extracted with n-butanol. nBuOH extract revealed the presence of 2 compounds designated as PS_1 and PS_2 (by TLC, co-chromatography on silica gel column, $CHCl_3$: CH_3OH (1 : 1), $CHCl_3$: CH_3OH (3 : 1) as eluants respectively).

Permethylation and hydrolysis of prosapogenin PS_1

25 mg of PS_1 was treated with methyl iodide (10 mL), Ag_2O (20 mg) and dimethyl formamide (5 mL) at room temperature for about 48 h and the contents filtered. The residue was taken up with 15 mL chloroform in a separatory funnel. Chloroform layer was washed with water; on removal of solvents methylated PS_1 precipitated out which was then hydrolysed with acid resulting in methylated sapogenin and the aqueous part was neutralised revealing the presence of methylated sugar identified as 2,3,5-tri-O-xylose (by co-pc and co-TLC).

Permethylation and hydrolysis of prosapogenin PS_2

It was carried out similarly as above for PS_1. It revealed the presence of methylated sapogenin and methylated sugar 2″3″4″6″-tetra-O-methyl-D galactose and 2′3′di-O-methyl-D-xylose (by cc-pc and co-TLC).

Periodate oxidation

30 mg of saponin dissolved in 50 mL MeOH was treated with 15 mL of 0.1 N sodium metaperiodate. The reaction mixture was left for 48 h at room temperature. In a similar manner a blank experiment was carried out. The liberated formic acid and consumed metaperiodate were estimated by titration as described by Jones *et al.*

Saponification of methyl ester of sapogenin

Monomethyl ester (25 mg) was suspended in ethylene glycol (20 mL) and refluxed on sand bath at 150°C for 4 h with 2N KOH (30 mL). The mixture was poured into ice-cold water with constant stirring and then extracted with solvent ether give a gelatinuous mass (20 mg). It was recrystallised from methanol giving colourless needles.

Enzymatic hydrolysis of the saponin.

The (60 mg) saponin in 10 mL methanol was suspended with 25 mg of almond emulsion enzyme aqueous solution at room temperature for 72 h. The hydrolysate on pc when nBuOH : AcOH : H_2O (4:1:5) was used as solvent and aniline hydrogen phthalate as spraying reagent showed two spots corresponding to D-xylose (R_f 0.28) and D-galactose (R_f 0.16) of authentic samples (confirmed by co-pc and co-TLC).

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