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GENICULATIN, A TRITERPENOID SAPONIN FROM *EUPHORBIA GENICULATA*

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Key Word Index—*Euphorbia geniculata*; Euphorbiaceae; geniculatin; 3β-[L-rhamnopyranosyl-(1→4)-D-xylopyranosyl-(1→4)-D-β-glucuronopyranosyl-(1→3)]-oxyolean-12-en-28-oic acid.

Abstract—A new triterpenoid saponin, geniculatin, has been isolated from the ethanolic extract of *Euphorbia geniculata* (Euphorbiaceae). The saponin has been identified as 3β-[L-rhamnopyranosyl-(1→4)-D-xylopyranosyl-(1→4)-D-β-glucoronopyranosyl-(1→3)]-oxyolean-12-en-28-oic acid.

INTRODUCTION

E. geniculata is an annual herb, introduced from tropical America. It is often found as a weed in Indian gardens. The narrow floral leaves are green towards the apex and almost white near the base [1]. The literature reports the isolation and identification of new terpenes from species of Euphorbia [2–5] and saponins have also been isolated [6–7]. Some work has been conducted on *E. geniculata* [8–10] and resulted in the isolation of flavonoids and steroids.

This report describes the phytochemical investigation of the aerial parts of *E. geniculata*. A new saponin was isolated and assigned the trivial name geniculatin.

RESULTS AND DISCUSSION

Geniculatin, mp 170–172: $C_{47}H_{74}O_{17}$, gave a copious lather when shaken with water, haemolysed red blood cells and responded to colour reactions characteristic of saponins [11]. It was hydrolysed with 7% H_2SO_4 whereupon the sapogenin was precipitated. The sapogenin was separated from the sugar moiety by filtration and was purified as the potassium salt [12]. It was identified as 3β-hydroxyolean-12-en-28-oic acid (oleanolic acid) by comparison of the IR, ^{1}H NMR and MS data of the sapogenin and its derivatives with those in the literature [13–16]. The structure of the sapogenin was also confirmed by mmp and co-TLC with an authentic sample.

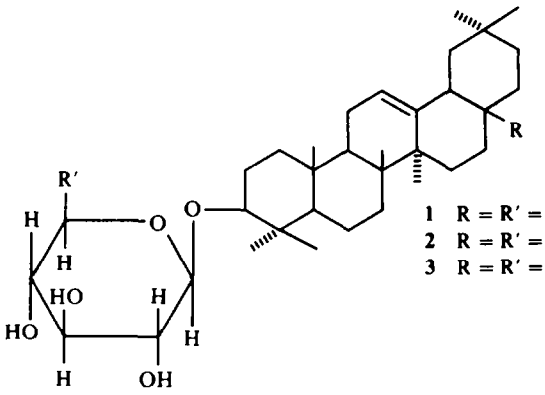
D-(+)-Glucuronic acid, D-(+)-xylose and L-(−)-rhamnose were detected in the aqueous acid hydrolysate of the saponin by PC in BuOH–HOAc–H_2O (4:1:5), sprayed with aniline hydrogen phthalate, at R_f 0.12, 0.27 and 0.37, respectively.

A quantitative estimation [17] of the sugars present in the hydrolysate revealed that the three sugars were present in equimolecular proportions. Hence it was concluded that geniculatin consisted of one molecule each of oleanolic acid, D-(+)-glucuronic acid, D-(+)-xylose and L-(−)-rhamnose.

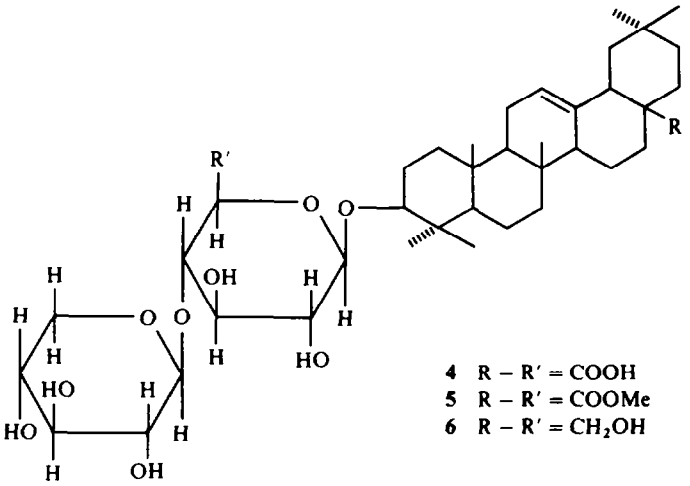
In the sapogenin there are two positions where the sugars can be attached, i.e. at the C-3 hydroxyl group or at the C-17 carboxyl group. The saponin was not hydrolysed with 5N NH_4OH, which is a specific reagent for hydrolysis of sugar esters without attacking any other glycosidic linkage [18]. Hence there could be no sugar unit esterified to the C-17 carboxyl group of the oleanolic acid. On methylation with diazomethane in ethanol solution, the geniculatin furnished a dimethyl ester, $C_{49}H_{78}O_{17}$, which on hydrolysis with 7% H_2SO_4 yielded oleanolic acid methyl ester and not oleanolic acid thus indicating the presence of a free –COOH group in the geniculation.

On the basis of the above experimental observations it was concluded that there was no sugar unit on the C-17 COOH group of oleanolic acid. Hence all the three sugars must be present on C-3 of the genin.

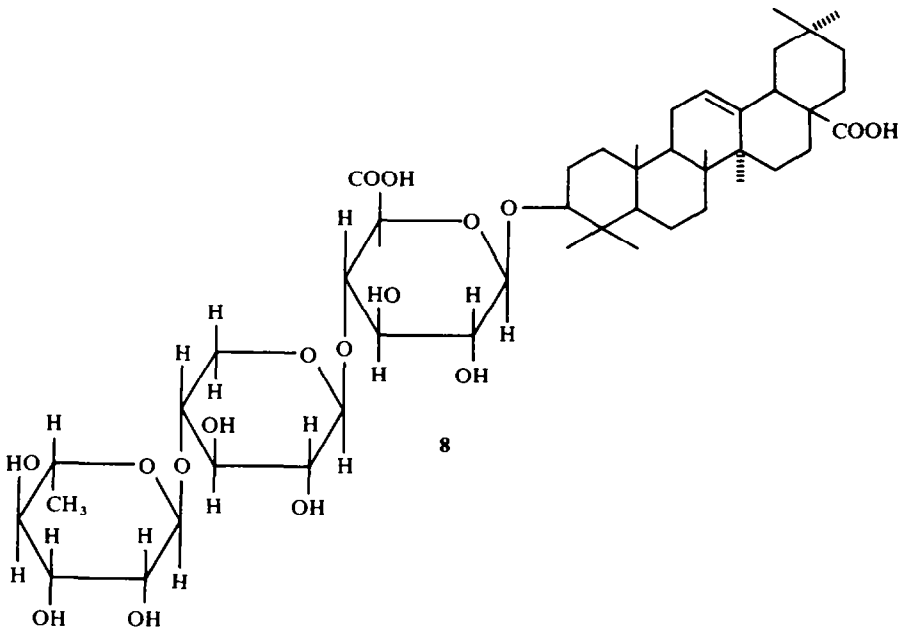
When the dimethyl ester of geniculatin was subjected to partial hydrolysis with 1% H_2SO_4 at 80–90° for 4 hr, a solid product was obtained which, after treatment with diazomethane in methanol, followed by column chromatography over Si gel, yielded oleanolic acid methyl ester and the dimethyl ester of oleanolic acid, β-D-glucurono-pyranoside (**2**), which is the dimethyl ester of PS (**1**) [22–23]. On the basis of the formation of compound **2** of known structure it was inferred that the glucuronic acid in its pyranose form was directly linked to the genin. This was also proved by the reduction of **2** with $LiAlH_4$ to erythrodiol-3β-D-glucoside (**3**) which was hydrolysed by Kiliani's mixture to erythrodiol $C_{30}H_{50}O_2$, mp 242°, $[\alpha]_D$ + 70° ($CHCl_3$). Also, after permethylation of **2** hydrolysis gave 2,3,4,6-tetra-*O*-methyl-D-glucose. The formation of this latter compound conclusively indicated that C-1 of the glucuronic acid was linked to C-3 of the genin.



1 R = R′ = COOH
2 R = R′ = COOMe
3 R = R′ = CH_2OH



4 R – R′ = COOH
5 R – R′ = COOMe
6 R – R′ = CH_2OH



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When geniculatin was subjected to partial hydrolysis with 0.02 N H_2SO_4 at room temperature a prosapogenin, designated PS_2, was obtained. Paper chromatographic examination of the hydrolysate indicated the presence of only L-(−)-rhamnose. Complete hydrolysis of PS_2 with 7% H_2SO_4 yielded oleanolic acid, D-(+)-glucuronic acid and D-(+)-xylose, suggesting that L-(−)-rhamnose was the end sugar of the geniculatin.

On treatment with diazomethane PS_2 formed a dimethyl ester, mp 188–190°, $[\alpha]_D$ − 8.6°. Reduction of the dimethyl ester with $LiAlH_4$ yielded erythrodiol glucosyl xyloside which on hydrolysis with Kiliani's mixture [19] gave erythrodiol, D-(+)-glucose and D-(+)-xylose. The ratio of sugars to erythrodiol was found to be 1:1. This suggested that erythrodiol glycoside was formed from one molecule each of erythrodiol, D-(+)-glucose and D-(+)-xylose. Permethylation of erythrodiol bioside followed by hydrolysis and PC revealed the presence of 2,3,4-tri-*O*-methyl-xylose and 2,3,6-tri-*O*-methyl-D-(+)-glucose. Formation of 2,3,4-tri-*O*-methyl-xylose suggested that it was present in the pyranose form at the terminal end of the bioside unit. The formation of 2,3,6-tri-*O*-methyl-glucose indicated that C-4 of the glucose (which was derived from glucuronic acid) must be linked to C-1 of the xylose. Since the C-1 of the glucuronic acid was linked to C-3 of the genin (see above) the structures **4** and **5** are suggested for PS_2 and its dimethyl ester and **6** for erythrodiol glucosyl xyloside. Thus the sequence of sugars in the saponin must be D-(+)-glucuronic acid, D-(+)-xylose and L-(−)-rhamnose.

When the saponin was hydrolysed by the enzyme diastase, it yielded L-rhamnose which was detected by PC. Thus the L-rhamnose was glycosidically attached to D-(+)-xylose by an α-linkage.

The nature of the other two glycosidic linkages was investigated by hydrolysis of PS_1 and PS_2 with the enzyme emulsin. The nature of the glycosidic linkages was confirmed by comparison of the observed and calculated $[M]_D$ values of the saponin using Klyne's rule [20] as given in Table 1.

The observed and calculated values are in good agreement with the fact that L-(−)-rhamnose was linked by an α-linkage and D-(+)-xylose and D-(+)-glucuronic acid by β-linkages. Hence geniculatin is assigned structure **8**.

EXPERIMENTAL

The air-dried and powdered stem and leaves of *E. geniculata* (4 kg) were extracted with petrol (40–60°) for 48 hr in a Soxhlet. The extract was filtered (3 l.) and concd under red. pres. The defatted plant material was then exhaustively extracted with 95% EtOH. The ethanolic extract (5 l.) was concd under red. pres. and then placed in a refrigerator for 18 hr to yield a ppt. which was separated by filtration. The filtrate was concd and poured into H_2O (2 l.) with constant stirring and the mixture stored overnight. The H_2O soluble and insoluble fractions were separated by filtration. The H_2O soluble fraction was concd under red. pres. to a semi-solid mass which was exhaustively extracted with C_6H_6, $CHCl_3$, Me_2CO and MeOH in a liquid–liquid extractor. All except the methanolic extract were discarded. The MeOH extract was concd and was again dissolved in the minimum amount of MeOH. The ppt. obtained after addition of Et_2O was separated by filtration and further purified by CC on Si gel using *n*-BuOH–EtOAc–H_2O (1:1:1) as solvent. It was crystallized from MeOH to yield a white compound, mp 170–172°.

Table 1

Substance	$[\alpha]_D$	$[M]_D$
Dimethyl ester of oleanolic acid		
3β-D-glucuronopyranoside	+12°	+79°
β-Methyl D-xyloside	−65.9°	−108°
α-Methyl L-rhamnoside	−62°	−111°
Calculated	—	−140°
Observed	−17°	−138.3°

Hydrolysis of saponin. Geniculatin (0.5 g) was hydrolysed by refluxing it with 7% H_2SO_4 (50 ml) for 6 hr on a steam bath. The sapogenin was separated from the hydrolysate and was purified as the K salt [12]. It was crystallized from MeOH–$CHCl_3$ (1:1) into colourless crystals (0.25 g) mp 308–310° $[\alpha]_D$ + 78° ($CHCl_3$). [Found C, 79.09, H, 10.5 MW 456 (MS), required for $C_{30}H_{48}O_3$ C, 78.99, H, 10.52%]. IR ν_{max}^{KBr} cm^{-1}: 3420, 2920, 2840, 1700, 1460, 1390, 1386, 1347, 1325, 1305, 1264, 828, 818, 804; MS *m/e*: 456 (M^+) 441, 411, 410, 395, 300, 248, 207, 203 (base peak), 199, 175, 135. Acetate, $C_{30}H_{47}O_2OCOCH_3$, mp 266–267°, $[\alpha]_D$ + 73° ($CHCl_3$); MS *m/e*: 498 (M^+), 483, 452, 438, 423, 301, 249, 248, 203 (base peak), 189, 133. Methyl ester, $C_{31}H_{50}O_3$, mp 198–199°; MS *m/e*: 470 (M^+), 455, 411, 410, 262, 249, 207, 203 (base peak), 189, 133, ^1H NMR ($CDCl_3$): δ 0.75 (3 H), 0.80 (3 H), 0.97 (6 H), 1.00 (6 H), 1.16 (3 H), 3.60 (3 H), 5.28 (1 H, vinylic).

Estimation of sugars in the saponin hydrolysate. The ratio of the sugars in the saponin was determined with a Klett–Summerson photoelectric colorimeter [16] using a blue filter (420 nm) and with the help of standard curves of authentic sugars. The solns (10, 20, 100 μg in 0.02 ml H_2O) of each of the sugars, D-(+)-glucuronic acid, D-(+)-xylose and L-(−)-rhamnose were applied on Whatman No. 1 filter paper (50 × 55 cm) at 3.5 cm intervals. The chromatograms were developed in BuOH–HOAc–H_2O (4:1:5) for 24 hr, dried in air, sprayed with aniline hydrogen phthalate reagent on both sides and dried at 110° for 10 min. The coloured spots were cut out in equal rectangles, eluted with 50% HOAc (5 ml each) and the colour intensity of each eluate was measured. In order to find out the amount of sugar in the saponin hydrolysate, geniculatin (100 mg) was hydrolysed with 7% H_2SO_4 (25 ml) by reflux on a steam bath for 5 hr. The reaction mixture was extracted with $CHCl_3$ to yield the sapogenin (27 mg). The hydrolysate was neutralized with $BaCO_3$, filtered and concd to a syrup (1 ml). A small amount of the syrup was dissolved in 1 ml H_2O and aliquots applied to Whatman No. 1 filter paper. The chromatograms were developed, sprayed, dried and the coloured spots were cut out in equal rectangles, eluted separately, and assayed as above.

Partial hydrolysis of saponin. Geniculatin (200 mg) was treated with 10 ml 0.02 N H_2SO_4 and the reaction mixture was kept at room temp. for 10 days. It was then extracted with *n*-BuOH and the extract was concd under red. pres. to a syrupy mass which was crystallized from MeOH to an amorphous powder of PS_2 (160 mg). It was recrystallized from MeOH.

Preparation of dimethyl ester of PS_2. PS_2 (50 mg) was treated with an ethereal soln of CH_2N_2. The product was crystallized from $CHCl_3$–MeOH (1:2) into colourless needles (40 mg) mp 188–190°, $[\alpha]_D$ − 8.6°. (Found: C, 65.8, H, 8.9%. Calculated for $C_{43}H_{68}O_{13}$; C, 65.1 and H, 8.5%).

Hydrolysis of PS_2. PS_2 (100 mg) was hydrolysed with 7% H_2SO_4 (5 ml) for 4 hr. The ppt. obtained was extracted with $CHCl_3$. The extract was concd and crystallized from

$CHCl_3$–MeOH (1:1) to yield colourless prismatic crystals, mp 308–310°, identified as oleanolic acid by mmp and co-TLC. The hydrolysate on PC revealed the presence of D-(+)-glucuronic acid and D-(+)-xylose.

Reduction of PS_1 *dimethyl ester* (**2**). Compound **2** (20 mg) was reduced under reflux with LiAH in Et_2O–THF (1:1). The product (**3**) was purified by PLC on Si gel G with $CHCl_3$–MeOH (7:3) and was crystallized from MeOH as a colourless solid (100 mg) mp 208 209°. (Found: C, 71.1, H, 10.6%. Calc. for $C_{36}H_{60}O_7$; C, 71.5 and H, 9.9%).

Dimethyl ester of saponin. Geniculatin (2.4 g) was dissolved in the minimum amount of MeOH and treated with excess of an ethereal soln of CH_2N_2 until a permanent yellow colour was obtained. The reaction mixture was kept in a refrigerator overnight. The product was separated by filtration and was crystallized from $CHCl_3$–MeOH (1:2) as colourless plates, mp 108–110°, $[\alpha]_D$ + 26° (MeOH). (C, 62.61, H, 8.3% $C_{49}H_{78}O_{17}$ required: C 62.6, H, 8.3%).

Partial hydrolysis of saponin dimethyl ester. Geniculatin dimethyl ester (0.6 g) was refluxed with 1% H_2SO_4 in 80% aq. MeOH at 80–90° for 4 hr. MeOH was removed and the reaction mixture was diluted with H_2O. The ppt. so obtained was filtered, dissolved in MeOH and was treated with an ethereal soln of CH_2Cl_2. The product was chromatographed on a column of Si gel and eluted with $CHCl_3$ to yield oleanolic acid methyl ester (90 mg) while elution with $CHCl_3$–MeOH (95:5) furnished the dimethyl ester of oleanolic acid glucuronoside (80 mg), which was crystallized from Et_2O–EtOH (1:2), mp 202–205°, $[\alpha]_D$ + 12° (MeOH). (Found C, 69.23, H, 9.31%. Calc. for $C_{38}H_{60}O_9$; C, 69.09, H, 9.09%).

Permethylation of PS_2. PS_2 (60 mg) was treated with MeI (1 ml) and Ag_2O (1 g) in dimethyl formamide (4 ml) for 48 hr at room temp. The contents were filtered and the residue was washed with a small vol. of dimethyl formamide. The soln was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O and the solvent removed under red. pres. The compound (20 mg) obtained was hydrolysed with 7% H_2SO_4. The hydrolysate showed the presence of 2,3,4-trimethyl-xylose with R_G 0.94 in *n*-BuOH–HOAc–H_2O (4:1:5). The identity of the above compound was also confirmed by co-chromatography with an authentic sample.

Hydrolysis of PS_1 *dimethyl ester* (**2**). The dimethyl ester (50 mg) was hydrolysed with Kiliani's mixture [14] in a sealed tube at 100° for 3 hr. The product was diluted with H_2O and the genin was extracted with $CHCl_3$ and was identified as oleanolic acid methyl ester by mmp and co-TLC. The aq. hydrolysate on PC in BuOH–HoAc–H_2O (4:1:5) showed the presence of D-(+)-glucuronic acid. This identification was further confirmed by PC with an authentic sample of D-(+)-glucuronic acid.

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