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MOLLUSCICIDAL SAPONINS FROM *GUNDELIA TOURNEFORTII*

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Key Word Index—*Gundelia tournefortii*, Asteraceae, triterpenoid saponins, molluscicidal activity

Abstract—From the roots of *Gundelia tournefortii* seven saponins have been isolated mainly by DCCC The main saponins (A and B) were characterized, mainly by ^{13}C and ^{1}H NMR spectroscopy, as oleanolic acid 3-*O*-(2-[α-L-arabinopyranosyl(1 → 3)-β-D-gentiotriosyl(1 → 6)-β-D-glucopyranosyl]β-D-xylopyranoside) (saponin A) and oleanolic acid 3-*O*-(2-[α-L-arabinopyranosyl] (1 → 3)-β-D-gentiobiosyl (1 → 6)-β-D-glucopyranosyl β-D-xylopyranoside) (saponin B) The other saponins are also derived from oleanolic acid and contain more sugar units The saponin mixture and the saponins A and B possess strong molluscicidal activity against the schistosomiasis transmitting snail *Biomphalaria glabrata*

INTRODUCTION

Gundelia tournefortii L † is the only latex-containing representative of the Tubuliflorae [1, 2] This monotypic plant belongs to the tribe Arctotideae and the subtribe Gundeliinae of the Asteraceae family, which occupies a conspicious taxonomic position [3, 4] Chemical investigations have been done with the fruits of the plant, which have been used in Turkey occasionally as a coffee substitute [5] No chemical studies, however, have been carried out on the roots We now report the results of our investigations with a crude saponin mixture, provided by Prof Aynehchi from Tehran, and a drug material of Turkish origin (Hekkari region) provided by Prof Baytop, Istanbul

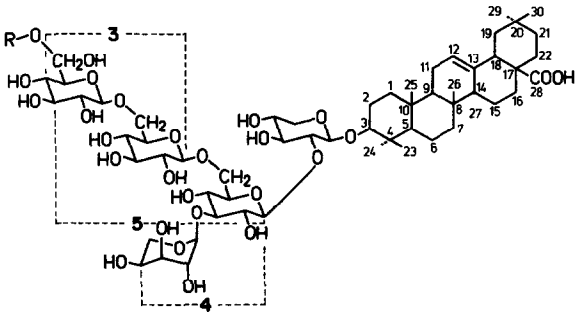
RESULTS

Chromatographic studies showed that only the Iranian specimen had a high saponin content After TLC on silica gel developed with chloroform–*n*-propanol–methanol–water (9 1 12 8, lower phase), Komarowsky reagent as well as citrate blood-suspension showed the presence of at least seven single saponin zones in the R_f range 0 1–0 5 The saponin mixture was successfully resolved by DCCC

Saponin A (1)

On acid hydrolysis, the saponin yielded oleanolic acid and the sugars hexose, xylose and arabinose in a mole ratio of 4 1 1 After $NaBH_4$-treatment no change of the glycoside [6, 7] and no chemical downfield shift higher than 88 2 ppm for the anomeric sugar protons in the ^{13}C NMR [8–10] was observed, therefore the saponin had to be a C-3-*O*-monodesmosidic glycoside A sequence analysis was performed by the permethylation-$NaBD_4$-MS-method [11, 12], and partial acidic and enzymatic hydrolysis followed by ^{13}C NMR-investigations of the degradation products The partially methylated 1-D-alditolacetates identified by MS were assigned to a terminal glucose, a 1,6- and a 1,3,6-linked glucose, whereas it was impossible to differentiate between xylose and arabinose as the terminal and 1,2-linked pentose moieties

Saponin A was degraded by hydrolysis for 1 hr with 0 5 mol acidic-ethanol to a tetraglycoside, which was identical with saponin B (2) The terminal sugar split off was glucose Hydrolysis afforded mainly a monoglycoside, identified by ^{13}C NMR and hydrolysis as oleanolic acid 3-*O*-β-D-xyloside Consequently, the 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylpentitol obtained by methanolysis was assigned to xylose and the 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol to arabinose The minor degradation products of partial acidic and enzymatic hydrolysis were a trisaccharide (5) and two disaccharides (3, 4), which were isolated in small amounts by DCCC Disaccharide 4 was an arabinosyl-glucopyranose with an α-L-1 → 3 linkage, since the C-3 signal of the glucose in the



1 R = Glucopyranosyl Saponin A
2 R = H Saponin B
3 Gentiobiosyl – Disaccharide
4 Arabinoglucosyl – Disaccharide
5 Gentiotriosyl – Trisaccharide

†Synonymous to *G glabra* Miller syn *G tournefortii* var *glabra* (Miller L), *G tournefortii* var *asperrima* and var *tenuisecta* syn *G tenuisecta* The common name in Iran is Kangar, in Turkey Kengar

Table 1 ^{13}C NMR data for saponin A (DMSO-d_6, TMS as internal standard)

Oleanolic acid C	ppm	Sugars C	ppm
28	178 4	1-Xyl	105 7
13	143 9	1-Glu	104 9
12	122 7	1-Glu	104 5
3	88 2	1-Glu	103 8
5	55 9	1-Glu	103 6
9	47 2	1-Ara	102 6
19	46 1	3-Glu	86 2
18	41 5	2-Xyl	84 5
6	38 8	5-Glu	77 2
1	38 6	5-Glu	77 1
8	38 5	5-Glu	77 0
10	37 1	5-Glu	76 9*
21	33 9	3-Glu	76 8*
29	33 1	3-Glu	76 7
22	32 5	3-Glu	76 6
7	32 4	3-Xyl	76 2†
20	30 7	2-Glu	76 1†
23	28 2	2-Glu	75 5
15	27 8	2-Glu	74 6
2	27 2	2-Ara	71 6‡
27	25 9	4-Glu	71 8‡
30	23 6	4-Glu	71 4
16	23 5	4-Glu	71 4
11	23 2	4-Glu	72 2
6	18 4	4-Xyl	71 1
26	16 9	3-Ara	70 1
24	15 6	6-Glu	70 1
25	15 3	6-Glu	70 1
		4-Ara	69 9
		5-Xyl	67 7
		5-Ara	65 9
		6-Glu	62 4

*,†,‡Assignments are reversible

^{13}C NMR spectrum showed a 9 ppm downfield shift and the C-1 of the arabinose a chemical shift of 102 6 ppm, which corresponded in all respects with data given by Ishii *et al* [13] for α-L-arabinose

The glucose moiety had to be placed on the reducing end, since we found a doubling of the C-1, C-2, C-3 and C-5 signals for the glucose due to mutarotation The ^{13}C NMR spectra of disaccharide **3** and the trisaccharide **5** were superimposable on those published by Usui *et al* [14, 15] for authentic gentiobiose [β-D-glucopyranosyl-(1 → 6) β-D-glucopyranoside] and gentiotriose [β-D-glucopyranosyl (1 → 6) β-D-glucopyranosyl (1 → 6) β-D-glucopyranoside] In both cases again a doubling of the signals for C-1, C-2, C-3 and C-4 of the reducing sugars was observed As far as the sequence of the sugars in the hexasaccharide moiety of saponin A was concerned, the arabinose had to be attached to the glucose next to the xylose moiety, since during enzymatic hydrolysis with β-glucosidase along with gentiobiose and gentiotriose no hydrolysis of the arabinose–glucose bonding was observed On the base of these results, saponin A is represented by the structure **1**

Saponin B (**2**)

This glycoside, after acidic hydrolysis, afforded also oleanolic acid, glucose, arabinose and xylose but the sugar components were in a mole ratio of 3 1 1 This was corroborated by the occurrence of only five signals in the anomeric region of the ^{13}C NMR spectrum (100–108 ppm) compared with the six signals in Saponin A The conversion of saponin A into saponin B by partial acidic hydrolysis confirmed the structure of saponin B

The other five saponins were also derivatives of oleanolic acid with glucose, xylose and arabinose as the only sugar moieties, but they contained more sugar units

DISCUSSION AND MOLLUSCICIDAL ACTIVITY

The saponins of *G tournefortii* show structural similarities with those isolated from *Patrinia intermedia* [16], *Clematis mandshurica* [17] or *Gypsophila pacifica* [18], but differ from them in the sugar composition as well as in the type of linkage The *Gundelia*-saponin A seems to be the first example of a saponin which contains a gentiotetrose unit with two additional sugar moieties attached to it The acute oral toxicity of Gundelia saponins in mice is very low, since administration of 2 0 g/kg to mice did not show any sign of toxicity However, the crude saponin mixture of *Gundelia* killed the test snails within 24 hr at a concentration of 40 ppm Saponin A and saponin B were 100% lethal at a much lower concentration (5–6 ppm) but after 12 hr only The oleanolic 3-*O*-xyloside, product of partial hydrolysis of saponin A, possesses the same activity at a concentration of 24 ppm These results correspond with those obtained by Hostettmann *et al* on the saponins of *Cornus florida* [19] and *Hedera helix* [20] The structure–molluscicidal activity relationship of saponins has been discussed in ref [21]

EXPERIMENTAL

Mps corr IR KBr, MS MS-30 (AEI), ^{13}C NMR Bruker WP-80 and WP-250, TLC and HPTLC silica gel $60F_{254}$ For saponins the lower layers of $CHCl_3$–MeOH–H_2O a 12 7 1, b 6 4 5 1 and c $CHCl_3$–MeOH–*n* PrOH–H_2O (9 10 1 8), for sapogenins toluene–Me_2CO (9 1) or toluene–EtOAc (4 1), for monosaccharides $CHCl_3$–MeOH–H_2O (32 25 5) and $CHCl_3$–MeOH–*n*-PrOH–H_2O (12 13 2 8) Spray reagents for saponins, Komarovsky reagent, Godin reagent and citrate blood reagent, for sugars anilinphthalat- and diphenylamine-H_3PO_3 reagent DCCC DCC-A Tokyo Rikakikai Tokyo, 300 tubes (400 × 2 mm), 20 ml/hr, 10 kp/cm^2, 12°

Plant material The roots were collected in April 1979 by Prof Aynehchi 40 km west of Tehran (Karaj) (Voucher Herbarium Department of Pharmacognosy, Tehran) The Turkish material was collected by Prof Baytop (Istanbul) in the Hakkari region in October 1980 (Voucher Nr 221 Herb of Inst of Pharmac Biology, Munich)

Isolation of the saponins The MeOH extract (20 g) corresponding to 500 g root material provided by Prof Aynehchi, was dissolved in 200 ml 50% MeOH and extracted with cyclohexane (3 × 200 ml) and CH_2Cl_2 (3 × 200 ml) to remove lipid material The H_2O–MeOH soln was concd, diluted with H_2O to a vol of 200 ml and extracted (× 3) with *n*-BuOH On evaporation of the solvent, 14 g of a syrupy residue was obtained This was dissolved in a little MeOH and poured into 500 ml Et_2O After centrifugation the residue was dissolved again in a little MeOH and precipitated by adding Et_2O This procedure was repeated three

times The last residue was dissolved in MeOH, the soln treated twice with active charcoal, centrifuged and the supernatant filtered through a deactivated Al_2O_3 column The MeOH soln was evaporated to dryness, dissolved in H_2O and then lypophilized to yield a white powder (saponin mixture, 0 8 g) The further separation (0 5 g) was performed by DCCC in three separation steps using $CHCl_3$–*n*-PrOH–MeOH–EtOH–H_2O (9 1 6 8 8) as solvent in the ascending method (saponins A and B) The saponins C–G were separated with the system $CHCl_3$–*n*-PrOH–MeOH–EtOH–H_2O (9 1 10 2 8) in the descending method In the third separation step mixtures from the first and second procedure (*ca* 0 19 g) were separated Yields saponin A 0 58 g, B 0 026 g, C 0 012 g, D 0 006 g, E 0 014 g, F 0 003 g, G 0 010 g

Saponin A (**1**) Mp 218° TLC R_f 0 48 (a) and 0 52 (c), $[\alpha]_D^{24}$ +10 5 (*c*, 0 96, MeOH), ^{13}C NMR see Table 1

Saponin B (**2**) Mp 242° TLC R_f 0 52 (a) and 0 55 (c), $[\alpha]_D^{24}$ +4 3 (*c* 0 84, MeOH)

Acid hydrolysis Acid hydrolysis of saponins A (100 mg) and B (10 mg) with 10% HCl in MeOH for 5 hr under reflux followed by extraction with CH_2Cl_2 gave 25 mg and 4 mg of oleanolic acid respectively The acid was identified by mp (305°) and ^{13}C NMR A sample of the hydrolysate was dissolved in 0 3 M borate buffer, pH 9 1, and subjected to sugar analysis using a ZA 5100 Biotronik apparatus with DA-X4-20, to give the sugar components of saponin A (Glc–Ara–Xyl, 4 1 1) and saponin B (Glc–Ara–Xyl, 3 1 1) Saponin A (5 mg) was refluxed in 1 ml TFA for 1 hr, the soln evaporated to dryness, the residue dissolved in 10 ml H_2O and the soln treated for 3 hr with 10 mg $NaBH_4$ at 24° After separation from Na^+ and H_3BO_3 (Dowex 50 and evaporation) the residue was refluxed with 2 ml HOAc for 15 min and the soln, after removal of toluene, evaporated to dryness A sample was then subjected to GLC (3% 225 Chromosorb-W-AW-DMCS 3, 6 m × 2 mm, column temp 210°, Ar, 30 ml/min) to give the same sugars and mole ratios as above

Partial hydrolysis of saponin A (a) After a test hydrolysis with 5 mg saponin A in 0 5 ml HCl–EtOH (monitored by TLC), 100 mg saponin A was refluxed for 1 hr in 10 ml 0 5 m HCl–EtOH After dilution with MeOH and neutralization with 0 5 M NaOH the soln was chromatographed on a Sephadex LH 20 column using MeOH to obtain in the first fraction the hydrolytic products DCCC separation with $CHCl_3$–*n*-PrOH–MeOH–EtOH–H_2O (9 1 6 8 8) gave 28 mg of a saponin, which showed identity with Saponin B (b) In a second experiment, 100 mg saponin A was hydrolysed with 0 5 ml HCl–EtOH for 3 hr Sephadex chromatography yielded in the first fractions hydrolytic products, which after separation on DCCC in the same system as above yielded 10 mg of a saponin, which was identified as oleanolic acid 3-*O*-β-D-xylopyranoside, mp 283° TLC R_f 0 85 (c), ^{13}C NMR xylosyl part δ105 7 (C-1), 75 5 (C-2), 76 9 (C-3), 71 8 (C-4), 67 7 (C-5), shifts of oleanolic acid see Table 1 The subsequent fractions from Sephadex chromatography, after removal of the saponin split products, yielded a mixture of mono and oligosaccharides On HPTLC *iso*-PrOH–C_5H_5N–H_2O, 3 1 1), the latter gave two major spots (0 12 and 0 35) Oligosaccharide **3** and **5** yielded, after hydrolysis on the HPTLC-plate in a HCl-chamber (30 min at 100°) and subsequent chromatography in the second direction with $CHCl_3$–MeOH–H_2O (32 25 5), glucose only

The separation of the two oligosaccharides was achieved by DCCC in $CHCl_3$–MeOH–H_2O (7 12 8) to yield *ca* 3 mg of **3** and **5** in one separation step The procedure was repeated 20 times to give 20 and 30 mg of **3** and **5** respectively The identity with gentiobiose and gentiotriose was established by ^{13}C NMR and comparison with synthetic samples ^{13}C NMR disaccharide **3** glucosyl (reducing end) C-1 (δ97 3), C-2 (75 5), C-3 (76 8), C-4 (71 2), C-5 (76 1), C-6 (70 1), glucosyl (middle unit) C-1 (δ103 7), C-2 (74 0), C-3 (77 2), C-4 (71 2), C-5 (76 1), C-6 (62 5) Trisaccharide **5** glucosyl (reducing end) C-1 (δ97 3), C-2 (75 5), C-3 (76 8), C-4 (71 2), C-5 (76 1), C-6 (70 1), glucosyl (middle unit) C-1 (δ103 7), C-2 (74 6), C-3 (77 1), C-4 (71 2), C-5 (76 1), C-6 (70 1), glucosyl (non-reducing end): C-1 (δ103 7), C-2 (74 6), C-3 (77 1), C-4 (71 0), C-5 (77 1), C-6 (62 4)

Enzymatic hydrolysis Saponin A (200 mg) was dissolved in 50 ml 0 1 M HOAc–NaOAc, pH 5 0, and incubed with β-glucosidase (50 mg) (Sigma Chem Co , Nr 6 8625) at 37° for 3 days After adding hot EtOH the suspension was centrifuged and the supernatant evaporated to dryness On TLC, a new oligosaccharide spot appeared at R_f 0 4 Separation on DCCC using $CHCl_3$–MeOH–H_2O (7 12 8, asc method) afforded 4 mg disaccharide **4** Acid hydrolysis yielded glucose and arabinose ^{13}C NMR disaccharide **4** glucosyl (reducing end) C-1 (δ96 7), C-2 (75 0), C-3 (86 1), C-4 (71 0), C-5 (77 1), C-6 (62 4), arabinosyl C-1 (δ102 6), C-2 (71 6), C-3 (70 4), C-4 (69 9), C-5 (65 9)

Permethylation of saponin A and preparation of the partially methylated alditolacetates In a 10 ml injection bottle containing an atmosphere of N_2, 3 mg saponin A was dissolved in DMSO and Na-methanylsulfinylmethanide in DMSO added dropwise After exposure for 30 min at 25° to ultrasound, the soln was kept overnight at room temp and then Me J (1 5 ml) added dropwise into the soln under ultrasound for 1 hr at room temp The excess of Me J was distilled off at 40°, 5 ml H_2O added and the soln extracted (× 4) with CH_2Cl_2 The CH_2Cl_2-phases were washed with H_2O and then evaporated to dryness The residue was hydrolysed with 1 M TFA for 2 hr, the soln evaporated to dryness, the residue dissolved in H_2O, 25 mg $NaBD_4$ added and the soln kept for 2 hr at room temp Then the soln was acidified with Dowex [50 (H^+)] to pH 3 5, filtered, the solvent evaporated, and the residue dissolved in MeOH and distilled (× 3) with MeOH The partially methylated alditols were acetylated with AcOH (1 ml) for 1 hr at 100° The AcOH was removed by co-distillation in the presence of toluene, and the residue dissolved in a little CH_2Cl_2 and evaporated to dryness

The mixture was subjected to GC/MS (Hewlett–Packard 5840 A) using the same system as that used for alditolacetates The following main peaks could be assigned 1-D-1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-hexitol, 1-D-1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-hexitol, 1-D-1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-hexitol, 1-D-1,5-*O*-acetyl-2,3,4-tri-*O*-methyl-pentitol and 1-D-1,2,5-tri-*O*-acetyl-3,4,-di-*O*-methyl-pentitol

Bioassays The molluscicidal activity was measured with snails of the species *Biomphalaria glabrata* (for method see Hostettmann [21]) The Haemolytic Index was determined according to methods described in the Europ Pharmacopoea

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