Notes

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Hiroshi Mitsuhashi, Marekichi Sasaki, and Yuzuru Shimizu : Studies on the Constituents of Asclepiadaceae Plants. XII.[1] On the Components of *Pergularia extensa* N. E. Br. (*Daemia extensa* R. Br.).

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Our interest in ester glycosides from the Asclepiadaceae[2~4] led to the investigation of a Pakistan member of this family, *Pergularia extensa* N. E. Br. (*Daemia extensa* R. Br.). At about the same time, Reichstein, *et al.*,[5] reported the separation and chemical study of several glycosides from this plant collected in India and Rhodesia. Although they have neary completed their study, we are describing our results briefly since our study started quite independently and our material was collected in a different area.

The whole plant, collected at Peshawar, Pakistan, by Prof. Qazilbash in December 1961, was extracted with chloroform, and the extract was treated as described in the preceding papers for examining ester-glycosides,[1,6,7] but we failed to find the ester-glycosides which were obtained from other Asclepiadaceae plants such as *Cynanchum caudatum*, *Metaplexis japonica* and *Marsdenia tomentosa*. The aglycone fraction gave no remarkable reactions, whereas the sugar fraction showed a positive Keller-Kiliani reaction. On distillation under reduced pressure, D-cymarose, D-sarmentose, and L-oleandrose were identified in the distillate, and the presence of D-allomethylose was assumed. The nondistillable residue was found to contain D-glucose by paper partition chromatography.[8~11]

On the basis of results with the sugars, a further attempt was made to separate cardiac glycosides according to the method of Reichstein.[12,13] One kilogram of powdered material was extracted twice with ethanol after 48 hours fermentation. The extract was dissolved in 50% EtOH, washed with hexane four times, and the ethanolic solution was then treated with freshly prepared lead hydroxide under vigorous agitation. The viscous mixture was filtered, the filtrate was concentrated, and added with 95% ethanol. The resulting precipitate was removed using Celite and the ethanol filtrate was then concentrated under reduced pressure. The ethanolic solution thus obtained was extracted successively with ether (E-1), chloroform (Ch-1), and chloroform-ethanol (2:1) (Ch-E-1). The crude glycoside fractions E-1, Ch-1, and Ch-E-1 were examined by paper partition

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chromatography.[11,14,15] Each fraction thus obtained showed strong, positive Keller-Kiliani, Liebermann-Burchard, Salkowski, and Kedde reactions. Therfore, the presence of a steroidal glycoside containing 2-deoxysugar component was assumed. A crystalline substance (crystal No. 1) was obtained from the E-1 fraction. By comparison with an authentic specimen kindly supplied by Reichstein,[16] crystal No. 1 was identified uzarigenin.

Acetylation with pyridine-acetic anhydride gave 3-O-acetyluzarigenin, m.p. 261~264°. The fraction Ch-1 was chromatographed over an alumina column and its result is shown in Table I.

Table I. Chromatography of the Chloroform Extract (Ch-1)

Fraction No.	Solvent	(ml.)		Eluted product (mg.)	Kedde reaction
1~3	$CHCl_3$-MeOH	(99.5:0.5)	300	10	+
4	〃	(99:1)	400	70	+
5	〃	〃	400	8	+
6	〃	〃	200	23	+
7	〃	〃	200	61	+
8	〃	〃	100	22	+
9	〃	〃	100	10	+
10	〃	〃	200	30	+
11	〃	〃	300	25	+
12	〃	(95:5)	700	10	+
13	MeOH		300	trace	−

Uzarigenin was obtained from fraction No. 2 and 6 mg. of crystalline substance, m.p. 249~255.5° was obtained from No. 10, after repeated chromatography using silica gel. This compound was very similar to *al*-dihydrocalotropagenin.[17,18] Fraction Ch-E-1 was also chromatographed over an alumina column, eluted with methyl ethyl ketone saturated with water, and its results are shown in Table II.

Table II. Chromatography of the Chloroform-Ethanol (2:1) Extract (Ch-E-1)

Fraction No.	Solvent	Eluted product (mg.)	Kedde reaction	Spot[a] (See Fig. 2)
1	MeCOEt saturated with H_2O	291	+	A, (B), (C)
2	〃	203	+	A, B, C
3	〃	76	+	(A), B, C, (D)
4	〃	107	+	B, C, (D)
5	〃	85	+	C, D, (E)
6	〃	41	+	F, G
7	〃	14	+	F, G
8	〃	28	+	F, G
9	〃	22	+	G
10	〃	trace	+	(G)
11~13	〃	0		

Each fraction : 50 ml.
a) Parentheses indicate weak color reaction.

Fraction No. 1 was chromatographed again over alumina using chloroform-Methanol and a crystalline compound (crystal No. 2) was obtained. The results of elemental analysis and ultraviolet spectrum indicate that this compouod is a cardenolide (carbon 23) containing one molecule of hexose, but the amount of material was too small for further investigation.

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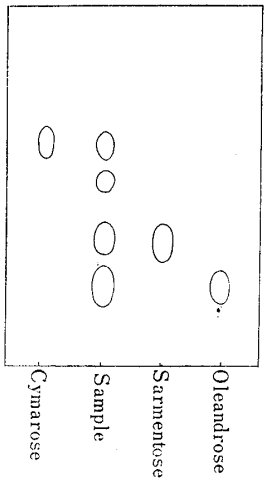

Fig. 1. Chloroform-Formamide Fraction (20% Formamide in acetone)

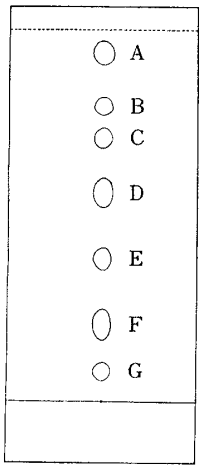

Fig. 2. Chloroform-Ethanol Fraction

Experimental

Extraction I——The whole plant of *Pergularia extensa* was chipped, dried powdered, and 2.2 kg. of the powdered material was extracted with $CHCl_3$ until the extract solution became colorless. The extracts were concentrated and 60 g. of residue was obtained. Yield, 2.7%. This deep green residue was treated with MeOH (200 ml. × 2) and the combined methanolic solution was evaporated below 60° *in vacuo*, giving 41 g. of MeOH-soluble material. This material was treated with hexane and the hexane-soluble part was discarded. The residual substance (5 g.) was dissolved in 75 ml. of MeOH and 25 ml. of 0.2*N* H_2SO_4, and the mixture was refluxed for 25 min. The solvent was removed *in vacuo* at room temperature and the residue extracted with $CHCl_3$. The $CHCl_3$ layer was washed with 5% $NaHCO_3$ solution and H_2O and dried over Na_2SO_4. Removal of the solvent gave 3.9 g. of a viscous substance, which showed a positive Keller-Kiliani reaction and Liebermann-Burchard reaction green → brown. This fraction (3.9 g.) was submitted to chromatography over alumina (120 g.), but gave quite different results in comparing with the cases of *Cynanchum*, and *Metaplexis* plants. The aqueous layer was neutralized with $BaCO_3$ and concentrated to a syrup under reduced pressure. The syrupy sugar fraction thus obtained on hydrolysis of the glycoside, was submitted to paper chromatography for comparison with the authentic samples. The results are shown in Table III. The spot equivalent

Table III. Paper Chromatography of the Sugar Fraction

	BuOH-1% NH_3[9] (25°, 16 hr.)		BuOH-Pyridine-H_2O (3:1:3) (room temp., 16 hr.)	
Sugar from the glycoside	0.34	0.69	0.41	0.65
D-Cymarose		0.68		0.67

to D-cymarose was extracted from the paper with Me_2CO and the solvent removed. The residue was submitted again to paper partition chromatography using $CHCl_3$-formamide system.[11] and this fraction was separated into D-cymarose, D-sarmentose, and L-oleandrose, as shown in Fig. 1.

The syrup (780 mg.) was extracted with Me_2CO and separated into Me_2CO-soluble and insoluble fractions. The Me_2CO-soluble fraction (590 mg.) was distilled at 110~150°/5×10^{-5} mm. Hg (bath temp.) to give 35 mg. distillate. The distillate and the residue were examined by paper chromatography.

Table IV. Paper Chromatography of the Sugar Rf value

	BuOH-AcOH-H_2O (4:1:1)		
Distillate		0.44	0.73
D-Cymarose			0.73
D-Rhamnose	0.34		
	BuOH-Pyridine-H_2O (3:2:1.5)	BuOH-Pyridine-H_2O (3:1:3)	
Residue	0.32	0.23	
D-Glucose	0.33	0.21	

The residue was subjected to the following tests; $SbCl_3$: gray → violet, Legal test : red, Raymond test : (±), and Kedde reaction : violet, these results and the preliminary results of paper chromatographical study suggest that this fraction contained the cardiac glycosides.

Extraction II——According to Reichsteins' method,[14] a renewed extraction was attempted. One kg. of powdered leaves and stems were mixed with 3 L. of H_2O of toluene, sealed with CO_2 gas, and kept at 37° for 48 hr. Then 3 L. of EtOH was added and after 24 hr. the mixture was extracted with EtOH (4 L. × 5). The deep green extract was concentrated *in vacuo* below 50°. The residue was dissolved in 2 L. of 50% EtOH, and treated with hexane (2 L. × 4). The hexane layer was washed with 50% EtOH. To the combined residual substance and EtOH solution was added freshly prepared $Pb(OH)_2$[14] under vigorous stirring. The precipitate was removed by filtration and the filtrate was adjusted to pH 6.0 with 5% H_2SO_4. The filtrate was concentrated to 600 ml. under reduced pressure and 1.8 L. of EtOH was added to this solution to remove the insoluble material. The filtrate was concentrated to 500 ml. *in vacuo* below 60° and the solution was extracted successively with 2.0 L. of Et_2O, $CHCl_3$, and $CHCl_3$-EtOH (2:1) mixture. The Et_2O solution was washed with H_2O (total 200 ml.), 5% $NaHCO_3$ (200 ml.). and H_2O (total 200 ml.), and each washing was kept to wash the $CHCl_3$ extract. The $CHCl_3$ solution was washed successively with the above solutions. The $CHCl_3$-EtOH solution was treated in the same manner. Each organic layer was dried over Na_2SO_4 and evaporation of the solvents gave 1.588 g. (Et_2O fraction, E-1), 1.137 g. ($CHCl_3$ fraction, Ch-1), and 1.05 g. ($CHCl_3$-EtOH fraction, Ch-E-1) of pale yellow powder.

Paper Chromatographic Analysis of Fractions E-1, Ch-1 and Ch-E-1——Each fraction was submitted to paper chromatographic analysis. Solvent system : stationary phase 15% formamide in Me_2CO, mobile phase $CHCl_3$ saturated with formamide; Filter paper : Toyo Roshi No. 51; descending. Coloring test : Kedde reagent.[12]

Ch-E-1 fraction was further examined on Toyo Roshi No. 50 using as the stationary phase Me_2CO-H_2O (4:1), MeCOEt saturated with H_2O as the mobile phase by the ascending system. After 6~7 hr. development, the chromatogram was detected by spraying the Kedde reagent. By this analysis, 7 spots were detected (see Fig. 2).

Crystal No. 1 (Uzarigenin)——E-1 fraction was treated with Et_2O and the soluble part was collected (1.072 g.), from which on repeated recrystallization from MeOH crystal No. 1 was obtained (500 mg.), m.p. 239~252. UV : λ_{max} 217 mμ (ε, 14,800). *Anal.* Calcd. for $C_{23}H_{34}O_4$ (uzarigenin) : C, 73.76; H, 9.15. Found : C, 73.55; H, 9.26. The melting point, IR spectrum, and the paper chromatographic analysis suggested that it might be uzarigenin and a mixed melting point determination with an authentic sample of uzarigenin showed no depression.

The acetate was prepared by treatment with Ac_2O in pyridine. Needles from $CHCl_3$-hexane, m.p. 261~264°. *Anal.* Calcd. for $C_{25}H_{36}O_5$ (3-O-acetyluzarigenin) : C, 72.08; H, 8.71. Found : C, 71.97; H, 8.74.

Crystal No. 2——Fraction No. 1 (Table II) was rechromatographed over an alumina column (activated 2 hr., 140°) and eluted with $CHCl_3$-MeOH. Crystal No. 2 was recrystallized from EtOH-Et_2O to a crystalline powder, m.p. 244~248°. Keller-Kiliani reaction, negative. *Anal.* Calcd. for $C_{29}H_{42}O_{10}$: C, 63.25; H, 7.69. Found : C, 63.02; 7.69. UV : λ_{max} 216 mμ (ε, 14,600).

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Summary

The whole plant of *Perugularia extensa* N. E. Br. was proved to contain a glycoside mixture which showed strong Keller-Kiliani reaction, suggesting the presence of a 2-deoxy-sugars in the sugar part. The sugar fraction obtained by acid hydrolysis was found to contain D-cymarose, D-sarmentose, L-oleandrose, and D-glucose by a paper chromatographic comparison with authentic specimens. Uzarigenin and a new glycoside (m.p. 244~248°) were found.

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