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Constituents from *Gymnema sylvestre* Leaves V: Isolation and Preliminary Characterization of the Gymnemic Acids

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Abstract □ The objectives of this investigation were to isolate and characterize the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* leaves, and to make them available for further biological testing. Gymnemic acid was found to be a complex mixture of at least nine closely related acidic glycosides. Solvent extraction and chromatography of gymnemic acid resulted in the isolation of gymnemic acids A–D (the major constituents) and V in crystalline form. Acids A–D are glycosides which yield glucuronic acid on hydrolysis while acids C and D also yield glucose. The gymnemic acids isolated in this study are compared to those described in the literature.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnemic acids—isolation characterization □ Column chromatography—separation □ TLC—separation □ Reverse phase chromatography—separation □ IR spectrophotometry—glycosidic structure □ UV spectrophotometry—glycosidic structure □ NMR spectroscopy—glycosidic structure

While the unique property of the leaves of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) to inhibit temporarily the ability to taste sweet substances has been known in India since antiquity (1, 2), the first such report to be published in the Western literature appears to be that of Falconer (3) in 1847. Chemical investigations were initiated by Hooper (4, 5) who isolated the antisweet principle as an amorphous monobasic acid, $C_{32}H_{55}O_{12}$, which he named gymnemic acid. Further, Hooper described gymnemic acid as a glycoside since it reduced Fehling's solution after treatment with dilute hydrochloric acid. In 1892, Shore (6) reported a modified procedure to isolate gymnemic acid in white crystalline form and suggested the acid to be a derivative of anthracene. Several years later, Power and Tutin (7) isolated racemic glucose as its osazone from the leaves but were unable to detect any sugar after acidic hydrolysis of gymnemic acid. Upon potassium hydroxide fusion of gymnemic acid, Power and Tutin obtained acetic acid and a mixture of protocatechuic and *p*-hydroxybenzoic acids, while alkaline potassium permanganate oxidation afforded formic acid.

In 1958, Khastgir *et al.* (8) reinvestigated Hooper's gymnemic acid but failed to obtain a crystalline product even after silicic-acid chromatography. However, the following year Warren and Pfaffmann (9) isolated the antisweet principle in crystalline form, m.p. 199° (dec.) and, subsequently, Pfaffmann (10) reported the detection of glucose, arabinose, and glucuronolactone upon acidic hydrolysis of gymnemic acid. Recently Yackzan (11, 12), while studying the biological effects of *G. sylvestre* fractions, carried out limited chemical work on gymnemic acid and indicated the presence of hydroxyl and carboxyl groups and one or more glycosidic linkages. Yackzan also assigned a molecular weight of about 600 for gymnemic acid based upon an ultracentrifuge technique.

Thus, at the beginning of the present investigation, knowledge regarding the chemical nature of gymnemic acid was scanty, and its antisweet activity was the only established biological property. However, during the course of this investigation, this situation has undergone considerable change. Cochran and Maassab (13) have observed significant antiviral activity for gymnemic acids made available from these laboratories. Also, Stöcklin (14, 15) has greatly advanced the chemistry of gymnemic acid.

It is the purpose of this paper to describe the isolation and preliminary characterization of the gymnemic acids. This is in support of potential interest in the antiviral field, continuing interest as antisweet compounds, and as a comparison to the acids and methods reported by Stöcklin *et al.* (14).

EXPERIMENTAL[1]

Plant Material—Dry leaves of *G. sylvestre* were purchased from Prachi Gobeson Co., Calcutta, India, and the Himalaya Drug Co., Bombay, India. Identification of the plant material was established through an examination of a flowering top specimen as reported in the previous investigation from these laboratories (16) and through its property of suppressing selectively the ability to taste sugar.

Reagents—All common reagents and solvents utilized in this investigation were of analytical reagent grade. The following reagents were from the sources indicated: silica gel G (Warner-Chilcott Labs.); deuterium oxide, tetramethyl silane, silicic acid (100 mesh), ion-exchange resins (Rohm & Haas), IR 120 (H^+) and IRA 401 (Cl^-) (Mallinckrodt); Woelm neutral alumina, activity grade 1 (Alupharm Chemicals); Teflon-6 (70/80 mesh) (Analabs); Glucostat reagent (Worthington Biochemical Corp.); and D-glucuronolactone, D-glucuronic acid, D-glucose, and D-arabinose (Calbiochem).

Thin-Layer Chromatography—Silica gel G TLC plates were prepared by coating five 20 × 20-cm. glass plates at a thickness of 250 μ with a slurry composed of 18 g. of adsorbent and 45 ml. of water. After air drying for 30 min. at room temperature (27°), plates were activated in an oven at 105° for 1 hr. prior to use.

Activated TLC plates were scored into 1-cm. channels before sample application. Sample size was usually 10–50 mcg. for purified compounds and 100–250 mcg. for crude fractions. Chromatograms were developed to a distance of 13–14 cm. by ascending technique. Developing tanks were lined with filter paper and were saturated with appropriate solvent mixture for at least 30 min. Solvent systems employed for the gymnemic acids are listed in Table I. Spray reagents used for visualizing components on chromatograms were: benzoyl chloride–sulfuric acid reagent (17), ceric sulfate–sulfuric acid reagent (15, 18), and modified Liebermann-Burchard reagent (19).

Table I—Thin-Layer Chromatography of the Gymnemic Acids

Gymnemic Acid[a]	R_f Value in Solvent System[b]						
	I	II	III	IV	V	VI	VII
V	0.28	0.62	0.68	0.59	0.75	0.72	0.38
W	0.25	0.49	0.63	0.28	0.55	0.36	0.32
X	0.22	0.43	0.62	0.24	0.54	0.31	0.29
A	0.20	0.35	0.60	0.18	0.51	0.28	0.27
B	0.17	0.31	0.49	0.15	0.47	0.24	0.21
Y	0.16	0.29	0.38	0.13	0.44	0.21	0.19
Z	0.13	0.17	0.36	0.12	0.43	0.19	0.18
C	0.12	0.14	0.30	0.10	0.38	0.17	0.16
D	0.09	0.10	0.25	0.09	0.35	0.14	0.12

[a] Listed in the decreasing order of R_f values. [b] Solvent Systems: I, chloroform–formic acid–methanol (4:1:1) (aged for 3 hr. at 27°); II, chloroform–acetic acid–methanol (5:1:1); III, chloroform–formic acid–methanol–*t*-butanol (4:1:1:1) (aged for 3 hr. at 27°); IV, isopropanol–ammonium hydroxide–chloroform–*t*-butanol (5:2:1:1); V, isopropanol–ammonium hydroxide–isoamyl alcohol (3:2:1); VI, isopropanol–ammonium hydroxide–diethyl carbonate–isoamyl alcohol–*t*-butanol (3:2:2:1:1); and VII (14), butyl formate–methyl ethyl ketone–formic acid–water (5:3:1:1).

Isolation of Crude Gymnemic Acid—Dry, powdered leaves of *G. sylvestre* (260–280 g.) were wrapped in cheese cloth in bundles of about 40 g. each and were loosely packed in the side chamber of a continuous extractor. Initial defatting of leaves was carried out with 4 l. of petroleum ether (b.p. 30–60°) for 18 hr. This was followed by extraction with either water or 95% ethanol, 4 l., for a period of 18 hr. The extracts were filtered and adjusted to pH 2 with 10% HCl to precipitate Hooper's gymnemic acid (4) in a yield of 3–4% of the dry leaves. These fractions were found to consist of at least nine components upon TLC in Solvent Systems I–VII.

Warren and Pfaffmann's gymnemic acid (9), recrystallized three times from diethyl carbonate, was obtained as white microcrystals, m.p. 199–201° (dec.) [lit. (9) m.p. 199° (dec.)]. This gymnemic acid sample gave two spots during TLC in Solvent System VII.

Preliminary Separation of the Gymnemic Acids—*Acetone Extraction: Silicic-Acid Chromatography*—With the aid of a mechanical shaker, 51 g. of crude gymnemic acid mixture was extracted with 175 ml. of acetone in a glass-stoppered 250-ml. conical flask for 48 hr. The acetone extract was filtered and evaporated to dryness to yield 22.17 g. of acetone-soluble acids. No further investigation of the acetone-insoluble fraction was undertaken.

The acetone-soluble fraction was chromatographed over 450 g. of silicic acid in a glass column (34 × 1200 mm.) prepared with the aid of chloroform. The acetone-soluble fraction, plated on 50 g. of silicic acid with the help of 200 ml. of methanol, was applied to the silicic acid column by the use of chloroform for transfers. Elution was commenced with chloroform at a flow rate of 16–17 ml./hr., while collecting one fraction of 400 ml./day. Polarity of the eluting solvent was gradually increased (as shown in Table II) and all of the fractions collected were analyzed by TLC in Solvent System III.

Ethyl Acetate: Continuous Extraction Separation—In a glass mortar, 20 g. of crude gymnemic acid mixture was finely ground, dissolved in 200 ml. of methanol, and then plated on 100 g. of silicic acid. Two 50-g. portions of silicic acid plated material were first extracted with chloroform (1 l.) and then with ethyl acetate (1 l.), each for 18 hr. in two separate continuous extractors.

The combined chloroform extract, upon evaporation to dryness, yielded 4.15 g. of a green residue which was found to contain only trace amounts of gymnemic acids upon TLC in Solvent System III. However, removal of solvent from the pooled ethyl acetate extract gave 4.86 g. of a light-yellow residue which was observed to be rich in gymnemic acids A–D by TLC. This fraction was named "ethyl acetate acids."

A 4.2-g. quantity of ethyl acetate acids was chromatographed on 100 g. of silicic acid by means of a glass column (23 × 500 mm.).

[1] Melting points were taken on a Kofler hot stage and are uncorrected. Spectra were recorded on Perkin-Elmer models 137B and 337 infrared, Beckman DK-2A UV, and Varian A-60A NMR spectrometers. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Extraction of the plant material was carried out in a Pyrex glass (Corning 3885) side-chamber continuous extraction apparatus. Adjustments of pH were followed with a Beckman Zeromatic pH meter. Centrifugation was carried out with an International Centrifuge model UV at about 2500 r.p.m. All concentrations and evaporations in this investigation were performed under reduced pressure at temperatures not exceeding 50° in either a Buchler Rotary Evapo-Mix (test tube model) or a Rinco flash evaporator.

Table II—Silicic-Acid Chromatography of Acetone-Soluble Acids

Solvent	Fractions (400 ml. each)	Weight, g.	Gymnemic Acids Detected[a]	Purification Procedure[b]	Purified[c] Gymnemic Acid Isolated	Weight, g.
Chloroform	1–6	0.54	None			
Ethyl acetate	1–2	0.09	None[d]			
	3–4	0.76	A,V,W,X	(iii)	A	0.15
					V	0.01
					W[e]	0.02
					X[e]	0.03
	5–12	3.05	A,B	(i)	A	2.40
	13–18	0.89	A,B,Y	(i)	A	0.32
					B	0.04
	19–23	0.61	B,Y,Z	(iii)	B	0.04
					Y[e]	0.03
					Z[e]	0.02
	24–36	1.10	B,C,Y,Z			
	37–49	0.47	C,D,Y,Z	(i)	C	0.02
Acetone–ethyl acetate (1:100)	1–3	0.38	C,D,Y,Z	(iii)	C	0.03
					D	0.02
					Y[e]	0.01
					Z[e]	0.01
Acetone–ethyl acetate (5:100)	1–25	1.10	C,D,Y,Z	(ii)	D	0.04

[a] Detection by TLC in Solvent System III. [b] Purification procedures: (i), adsorption chromatography on deactivated silica gel; (ii), reverse phase partition chromatography on Teflon-6; (iii), preparative TLC. [c] Homogeneous by TLC in Solvent Systems I–VII. [d] Stearic acid isolated from these fractions. [e] Isolated as amorphous solid.

The ethyl acetate fraction in 50 ml. of methanol was plated on 10 g. of silicic acid prior to its application to the column. Development of the column was initiated by chloroform followed by solvents with increasing polarity (as shown in Table III). Solvent flow rate was maintained at 10 ml./hr. and fractions (1/hr.) were collected by means of a fraction collector. All fractions collected were individually analyzed by TLC in Solvent System III.

Ethanol Extraction Method—Ethanol (95%) extraction of petroleum ether defatted leaves (105 g.) was performed as described under *Isolation of Crude Gymnemic Acid.* The filtered ethanol extract was concentrated to about 1 l., and petroleum ether was added until no more precipitation occurred (2 l.). The precipitate was collected by centrifugation which, after drying, gave 11.25 g. of a dark-green residue (17.1% of dry leaves).

The residue in 100 ml. of ethanol was plated on 50 g. of silicic acid and the dry plated material was extracted in a continuous extractor, first with chloroform (2 l.) and then with ethyl acetate (2 l.), each for 18 hr. Evaporation of the ethyl acetate extract to dryness afforded 1.93 g. of a light-green residue (1.8% of dry leaves). The presence of gymnemic acids A–D in this ethyl acetate residue was confirmed by TLC in Solvent Systems I–III, while similar TLC analysis of the original ethanol extract of leaves revealed it to contain gymnemic acids A–D and V–Z.

PURIFICATION OF THE GYMNEMIC ACIDS

Adsorption Chromatography on Deactivated Silica Gel—Samples of gymnemic acid mixtures (0.8 g. each) from the preliminary separation procedures rich in acids A, B, or C, as indicated in Tables II and III, were individually chromatographed on 125 g. of deactivated silica gel (125 g. of silicic acid blended with 25 ml. of water) in glass columns[2] (23 × 600 mm.). These columns were prepared with ethyl acetate, and gymnemic acid mixtures were applied after plating them on 1-g. quantities of deactivated silica gel with the help of 25-ml. portions of methanol. Columns were eluted with ethyl acetate at a flow rate of 10 ml./hr., and 250 fractions (5 ml. each) were collected in each case. All fractions were analyzed by TLC in Solvent System III.

Gymnemic Acid A—A total of 4.16 g. of gymnemic acid A was obtained by eight separate deactivated silica gel adsorption chromatographic separations. Acid A was found to be homogeneous by TLC in Solvent Systems I–VII. An analytical sample was prepared by four recrystallizations from ethyl acetate and was obtained as white microcrystals, m.p. 279–283° (dec.) [lit. (14) m.p. 285° (dec.)]; $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 15.92); $\nu_{max.}^{KBr}$ 3450 (intermolecular H-bonded, polymeric O—H), 2990 (C—H), 1710 (C=O), 1640, 855, 810 (trisubstituted C=C), 1384 (methyl of —O—$COCH_3$), 1440 (methylene of —CH_2—O—COR), 1260 (H-bonded O—H), 1070, 1045, (C—O of C—OH), and 920 $cm.^{-1}$ (β-pyranose ring vibration) (20).

Anal.—Found: C, 63.78; H, 8.31.

Gymnemic acid A was found to be identical to gymnemic acid A_1 isolated by Reichstein's group (14) by TLC in Solvent Systems I–VII and by IR spectroscopy.

Gymnemic Acid B—Gymnemic acid B was isolated in a combined yield of 0.09 g. by deactivated silica gel chromatography of two separate fractions indicated in Tables II and III. Acid B was observed to be homogeneous by TLC in Solvent Systems I–VII. When recrystallized three times from ethyl acetate, gymnemic acid B was obtained as white microcrystals, m.p. 220–225° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 15.42); $\nu_{max.}^{KBr}$ 3450 (O—H), 2990 (C—H), 1710 (C=O), 1625 (trisubstituted C=C), 1450 (side-chain methylene), 1375 (side-chain methyl), 1260 (H-bonded O—H), 1080 and 1040 $cm.^{-1}$ (C—O of C—OH).

Anal.—Found: C, 63.94; H, 8.53.

Gymnemic Acid C—Two separate deactivated silica gel chromatographic separations of fractions described in Tables II and III gave a total of 0.05 g. of gymnemic acid C. Acid C was found to be homogeneous by TLC in Solvent Systems I–VII. After three recrystallizations from ethyl acetate containing a few drops of methanol, acid C was obtained as white microcrystals, m.p. 215–220° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 8.18); $\gamma_{max.}^{KBr}$ 3500 (O—H), 2980 (C—H), 1710 (C=O), 1625 (trisubstituted C=C), 1440 (side-chain methylene), 1370 (side-chain methyl), 1250 (H-bonded O—H), 1070 and 1040 $cm.^{-1}$ (C—O of C—OH).

Anal.—Found: C, 57.22; H, 7.70.

Reverse Phase Partition Chromatography on Teflon-6—Teflon-6 (100 g.), which had been left at 0° overnight, was added to 100 ml. of the organic phase of a *n*-butanol–water–methanol (10:10:1) mixture cooled to 0°. The uniform slurry formed by vigorous stirring was immediately transferred to a jacketed glass column[2] maintained at 17 ± 0.5°. The column was allowed to equilibrate by a flow of 100 ml. of the aqueous phase of the above solvent mixture at a rate of 20 ml./hr.

The sample to be chromatographed (0.5–0.8 g.) was dissolved in a minimum amount of methanol and diluted to four times its volume with the aqueous phase of the solvent mixture. This sample

[2] Columns with minimum hold-up volumes below packing and to the tip of the column were required to avoid remixing of separated components and to obtain desired resolution.

Table III—Silicic-Acid Chromatography of Ethyl Acetate Acids

Solvent	Fractions (10 ml. each)	Weight, g.	Gymnemic Acids Detected[a]	Purification Procedure[b]	Purified[c] Gymnemic Acid Isolated	Weight, g.
Ethyl acetate	1–5	0.08	None			
	6–45	1.46	A,B	(i)	A	1.15
	46–58	0.85	A,B,Y	(i)	A	0.29
					B	0.05
	59–76	0.32	B,C,Y,Z			
	77–91	0.79	C,D,Y,Z	(i)	C	0.02
Acetone–ethyl acetate (1:100)	1–10	0.41	C,D,Y,Z	(ii)	D	0.04
Acetone	1–10	0.14	C,D,Y,Z			

[a] Detection by TLC in Solvent System III. [b] Purification procedures: (i), adsorption chromatography on deactivated silica gel; (ii), reverse phase partition chromatography on Teflon-6. [c] Homogeneous by TLC in Solvent Systems I–VII.

solution was then applied to the Teflon-6 column and developed with the aqueous phase of the solvent mixture. A flow rate of 10 ml./hr. was maintained and 5-ml. fractions were collected with the aid of a fraction collector. Fractions were combined on the basis of TLC results in Solvent System III.

Gymnemic Acid D—Column RPC of the fractions rich in acid D from preliminary separation methods (see Tables II and III) yielded a total of 0.08 g. of gymnemic acid D. Acid D was found to be homogeneous by TLC in Solvent Systems I–VII. Three recrystallizations from ethyl acetate containing a few drops of methanol yielded acid D as white microcrystals, m.p. 210–220° (dec.); $\lambda_{max.}^{MeOH}$ 201 mμ (*a* 9.69); $\nu_{max.}^{KBr}$ 3440 (O—H), 2940 (C—H), 1715 (C=O), 1620 (trisubstituted C=C), 1450 (side-chain methylene), 1380 (side-chain methyl), 1265 (H-bonded O—H), 1070 and 1040 cm.$^{-1}$ (C—O of C—OH).

Anal.—Found: C, 59.90; H, 8.00.

Preparative Thin-Layer Chromatography—Preparative TLC plates[3] were activated at 100° for 15 min. prior to use. With the help of a microcapillary, 130 mg. of gymnemic acid mixture in about 0.5 ml. of methanol was applied as a streak at a distance of 2 cm. from the bottom of the plate. Chromatograms were developed in Solvent System II to a distance of 15 cm. Resolved components were located as dark bands against a fluorescent background. Separated bands were individually removed from the chromatograms by means of a razor blade and collected in separate 125-ml. conical flasks. To each flask, 50 ml. of ethyl acetate–methanol mixture (50:1) was added and allowed to stand at room temperature for 12 hr. with occasional shaking. Ethyl acetate–methanol extracts were then filtered through a fine sintered-glass funnel and solvents removed *in vacuo*.

Gymnemic acids A–D, isolated by preparative TLC (14 chromatograms) from the various fractions shown in Table II, were found to be identical to those obtained by adsorption and partition chromatographic procedures. Yields of acids A–D secured by the preparative TLC method are shown in Table II.

Gymnemic Acid V—Gymnemic acid V was isolated in a combined yield of 0.01 g. from the fractions indicated in Table II by chromatography on six preparative TLC plates. Acid V was found to be homogeneous by TLC in Solvent Systems I–VII. Recrystallization from ethyl acetate gave acid V as white microcrystals, m.p. 310–312°; $\lambda_{max.}^{EtOH}$ 201 mμ (*a* 3.38); $\nu_{max.}^{KBr}$ 3445 (O—H), 2945 (C—H), 1720 (C=O), 1640 (trisubstituted C=C), 1460 (side-chain methylene), 1385 (side-chain methyl), 1265 (H-bonded O—H), 1075 and 1045 cm.$^{-1}$ (C—O of C—OH).

Anal.—Found: C, 68.94; H, 9.68.

Gymnemic Acids W–Z—Isolation of gymnemic acids W–Z was accomplished by preparative TLC of the various fractions described in Table II; 14 plates were utilized. Acids W–Z were obtained as light-yellow, amorphous solids, and several recrystallizations from ethyl acetate–methanol failed to yield better products. However, acids W–Z were observed to be homogeneous by TLC in Solvent Systems I–VII. Table II summarizes yields of gymnemic acids W–Z. No further investigation of these acids was carried out.

[3] E. Merck AG precoated silica gel GF254 plates (thickness 2 mm.).

Isolation and Characterization of Stearic Acid—Evaporation of solvent from ethyl acetate fractions 1 and 2 obtained during silicic-acid chromatography of acetone-soluble gymnemic acids (Table II) gave a yellow, oily residue (84.6 mg.) which upon four recrystallizations from methanol deposited 14.3 mg. of white crystals, m.p. 69–70°; $\nu_{max.}^{KBr}$ 1700, 1300, 940 (carboxylic acid dimer), eight evenly spaced bands between 1320 and 1190 (16 methylene groups in long-chain *n*-alkyl fatty acid) (21), 728 and 720 cm.$^{-1}$ (methylene chain rocking).

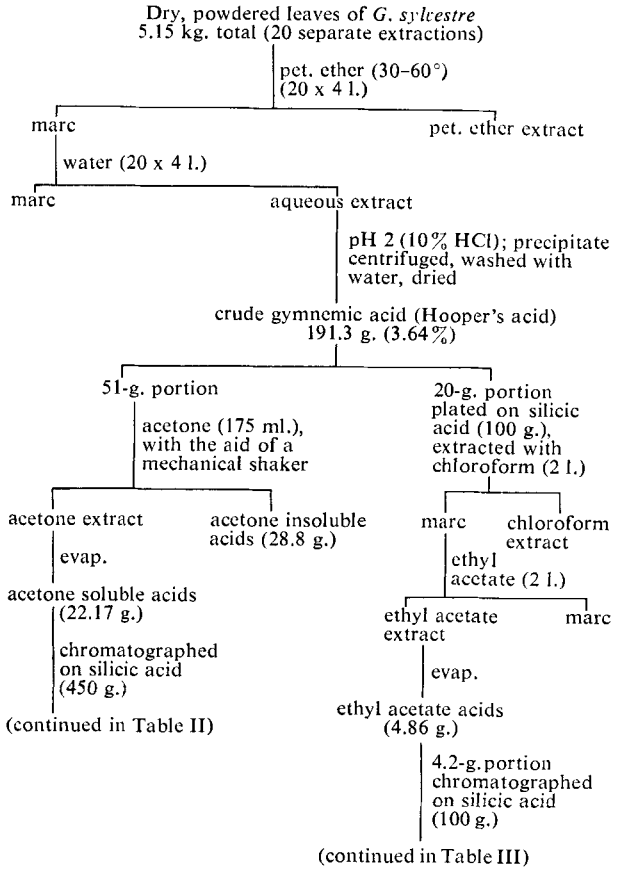
Anal.—Calcd. for $C_{18}H_{36}O_2$: C, 75.99; H, 12.76. Found: C, 75.52; H, 12.96.

An 8-mg. sample of the isolated acid was treated with methanolic boron trifluoride (22), and the reaction product was recrystallized twice from ethanol to give white crystals of methyl ester, m.p. 38–38.5°; $\nu_{max.}^{KBr}$ 1730 cm.$^{-1}$ (ester carbonyl).

The isolated acid and its methyl ester were identified as stearic acid and methyl stearate, respectively, upon comparision with reference compounds, undepressed mixed melting point, and superimposable IR spectra being the criteria for identity.

Identification of Gymnemic Acid Sugars—Thirty-milligram quantities of the gymnemic acids were refluxed with 4 ml. of 3 *N* ethanolic hydrochloric acid for 72 hr. The warm hydrolysates were treated with 5 mg. of activated charcoal,[4] filtered, and concentrated to 3-ml. volumes *in vacuo*. The concentrates were then diluted to 30 ml. with water to separate insoluble aglycone substances which were removed by centrifugation after 1 hr. of refrigeration. The supernatants, adjusted to pH 5–5.5 with 5 *N* sodium hydroxide, were evaporated to dryness, the residues extracted with 2.5 ml. of pyridine–water (4:1), and filtered from sodium chloride solids. The filtrates were evaporated to dryness and residues shaken with 10 ml. of water. The yellow aqueous extracts were filtered onto columns containing 5 ml. of an anion-exchange resin.[5] These columns were preconditioned with 10 volumes of 3 *N* hydrochloric acid and washed with water to neutrality prior to use. Column effluents and 25 ml. of water washings were collected and applied to a second column containing 5 ml. of a strongly basic ion-exchange resin.[6] Neutral sugar residues were recovered from the column effluents and 25 ml. of additional water washings by evaporation to dryness. Elution of the basic ion-exchange resin columns with 25 ml. of 1 *N* hydrochloric acid and evaporation of the effluents to dryness gave the acidic sugar fractions. Neutral and acidic sugar fractions were prepared as 2% solutions in 10% isopropanol in water for PC examination in System A (23), ethyl acetate–pyridine–water (12:5:4), and in System B (24), *n*-butanol–benzene–formic acid–water (100:19:10:25) (aged for 3 days at 27°). Detection of sugars was carried out with silver nitrate, aniline diphenylamine, aniline phthalate (23), and modified aniline hydrogen phthalate (24) reagents. Sugars in hydrolysate fractions were identified by direct comparison to reference sugar samples and to reference sugar

[4] Norit, American Norit Co.
[5] Amberlite IR 120(H^+), Rohm & Haas Co.
[6] Amberlite IRA 401(CO_3^-), prepared by converting 5 ml. of Amberlite IRA 401(Cl^-) (Rohm & Haas Co.) to the carbonate form with 50 ml. of 5% sodium carbonate.



Scheme I—Flow sheet for the isolation of gymnemic acids

samples which had been subjected to the ethanolysis procedure.

Five-milligram quantities of the gymnemic acids were also hydrolyzed by the following procedure.

The sample was suspended in 1 ml. of Kiliani mixture (acetic acid–water–hydrochloric acid, 35:55:10) (25, 26) and refluxed for 5 hr. The residue obtained after evaporation of the reaction mixture was dissolved in 1 ml. of water and extracted with chloroform (3 × 2.5 ml.). The aqueous layer was evaporated to dryness to give the sugar residue which was then chromatographed as described previously.

Characterization of Glucuronic Acid—The ethyl acetate acids fraction (3 g.) (Scheme I) was hydrolyzed and an acidic sugar fraction was isolated in a manner similar to that described for the individual gymnemic acids (ethanolysis procedure). Methyl-β-D-glucofuranosidurono-γ-lactone was prepared from both acidic sugar fraction and reference D-glucuronolactone by the following procedure.

To 108 mg. of acidic sugar fraction in 4 ml. of methanol was added 100 mg. of a cation-exchange resin.[7] The suspension was refluxed with stirring for 6 hr., clarified with 10 mg. of activated charcoal,[4] and filtered. The yellow filtrate was evaporated to dryness *in vacuo* and dried over anhydrous calcium sulfate to yield a yellow syrup (95 mg.). This syrup was plated on 0.75 g. of activated silica gel[8] and chromatographed on a column (13 × 230 mm.) of activated silica gel (10 g.). Elution was carried out with 6% methanol in benzene.

Fractions were monitored by silica gel G TLC with development by 20% methanol in benzene and detection with silver nitrate reagent (23).

Fractions containing material of R_f 0.36 were combined to give 18 mg. of a colorless syrup which was dried over anhydrous calcium sulfate under vacuum. The IR spectrum (neat) of this product exhibited an absorption band at 1790 cm.$^{-1}$ (γ-lactone) and the spectrum was superimposable with that of reference methyl D-glucuronolactone. When 10.8 mg. of the product was seeded with a trace of reference crystalline methyl D-glucuronolactone, gradual crystallization occurred. Recrystallization of the product from ethanol yielded large prisms (3 mg.), m.p. 139°. The melting point of the product was not depressed on admixture with reference methyl-β-D-glucofuranosidurono-γ-lactone and the NMR (D_2O) spectra of the two samples were identical.

RESULTS AND DISCUSSION

Isolation of the Gymnemic Acids—Early in this investigation it became apparent that gymnemic acid described in the literature was a mixture of acidic glycosides. TLC of Hooper's (4) gymnemic acid[9] indicated the presence of at least nine components which were named[10] gymnemic acids A–D and V–Z. The R_f values of these nine gymnemic acids in seven TLC systems are listed in Table I. Gymnemic acid as described by Warren and Pfaffmann (9) with its established antisweet property was found by TLC analysis to consist of acids A and B, the former being the major constituent. Indications of antiviral activity were noted for the gymnemic acid fractions rich in acid A (13).

Plant Extraction and Preliminary Separation—Based upon this information, attempts were made to secure gymnemic acid fractions rich in acid A. By following Hooper's (4) procedure, a crude gymnemic acid mixture was obtained from the aqueous extracts of petroleum ether–defatted *G. sylvestre* leaves by precipitation with 10% hydrochloric acid. Preliminary separation of the gymnemic acids was achieved by (*a*) acetone extraction: silicic-acid chromatography and (*b*) ethyl acetate: continuous extraction as summarized in Scheme I. A rapid and milder procedure involving direct ethanol extraction of defatted leaves and precipitation of the acidic glycosides with petroleum ether was also employed to confirm that previously isolated gymnemic acids represented products present in the dried leaves and not artifacts produced during the process of isolation.

Purification of the Gymnemic Acids—The gymnemic acid fractions, obtained during the preliminary separation step *via* silicic acid chromatography, were repeatedly decolorized with activated charcoal[4] in methanolic solutions until cream-white products were secured. Further purification of the gymnemic acids was carried out by following one of the three procedures: (*a*) adsorption chromatography on deactivated silica gel; (*b*) reverse phase partition chromatography (RPC) on Teflon-6; or (*c*) preparative TLC as summarized in Tables II and III.

In general, preparative TLC procedure was found to be useful for purifying all the gymnemic acids. Gymnemic acids A–D and V were obtained in crystalline form by this procedure. However, gymnemic acids W–Z could be obtained only as amorphous solids despite several recrystallizations. All nine gymnemic acids isolated by preparative TLC were shown to be homogeneous by TLC in seven solvent systems, I–VII. Table II summarizes the results obtained with preparative TLC. Adsorption chromatography on deactivated silica gel proved to be a convenient method for the rapid purification of gymnemic acid A, and to a lesser extent acids B and C, while RPC on Teflon-6 was valuable for obtaining pure acid D. The Teflon-6 RPC was modified from the procedure of Fritz and Hendrick (27) and found to be superior to the silanized diatomaceous earth[11] RPC for retaining the stationary phase with prolonged use.

Final purification of the isolated gymnemic acids was carried out by several recrystallizations from either ethyl acetate alone or ethyl acetate containing a few drops of methanol.

Preliminary Characterization of the Gymnemic Acids—This report is limited to preliminary characterization of the major gymnemic acids A–D. Initial characterization work was performed

[7] Dowex 50(H^+), Dow Chemical Co.

[8] Silicic acid, 100 mesh (Mallinckrodt), was activated at 105° for 24 hr.

[9] Equivalent results were obtained with gymnemic acid commercially available from K & K Laboratories, Inc.

[10] This nomenclature is based upon the order of elution of the components during silicic-acid column chromatography and their abundance in the crude acid mixture. Thus, the four major constituents were named gymnemic acids A–D, acid A being the first major component to elute from the column. Similarly, the minor constituents were named gymnemic acids V–Z, the sequence in which they eluted during chromatographic separation.

[11] Celite 545, Johns-Manville.

Table IV—Sugar Content of the Gymnemic Acids

Sample	Treatment[a]	Glucostat Test	Sugars Detected[b] by Paper Chromatography
Ethyl acetate fraction	Ethanolysis	Positive	Arabinose Glucose Glucuronic acid Glucuronolactone
Gymnemic acid A	Ethanolysis, hydrolysis	Negative	Glucuronic acid Glucuronolactone
Gymnemic acid B	Ethanolysis, hydrolysis	Negative	Glucuronic acid Glucuronolactone
Gymnemic acid C	Ethanolysis, hydrolysis	Positive	Glucose Glucuronic acid Glucuronolactone
Gymnemic acid D	Ethanolysis, hydrolysis	Positive	Glucose Glucuronic acid Glucuronolactone

[a] Ethanolysis was carried out by refluxing with 3 *N* ethanolic HCl for 72 hr. and hydrolysis by refluxing with Kiliani mixture (25) for 5 hr. [b] Colors produced by sugars after treating with modified aniline hydrogen phthalate reagent (24) and heating for 3 min. at 105° were visible (UV): arabinose, red (reddish brown); glucose, green (brown); glucuronic acid and glucuronolactone, brown (brown).

with acid A and with the ethyl acetate acids fraction which represented an enrichment of acids A–D. That the remaining gymnemic acids were closely related to acid A was evident by their various physical properties. Of particular significance was their IR spectra which were remarkably similar except for minor variations in the intensity of absorption peaks.

Glycosidic Properties of the Gymnemic Acids—With the aid of anthrone (28) and chromotropic acid (29) tests, reducing sugars were detected in hydrochloric acid hydrolysates of the gymnemic acids. Neutralized and desalted sugar fractions were then separated into neutral and acidic components. Neutral sugars were collected as effluents from a strongly basic ion-exchange resin in the carbonate form (30). Elution of the anionic resin with hydrochloric acid released sugar acids.

The conditions employed for acidic hydrolysis of the gymnemic acids were dependent upon the subsequent use of the hydrolysates. Thus, while hydrolysis in ethanolic hydrochloric acid was found to be faster and less destructive, it led to formation of ethyl glycosides of the sugars. This limited the detection of free reducing sugars and also gave rise to artifacts during chromatographic analysis. Hydrolyses with the Kiliani mixture (25, 26) were performed as a supplement to ethanolysis studies which assured that the sugars released were in the free reducing state. This procedure had value as a direct route for the identification of sugars. However, the harsh conditions of the Kiliani method led to degradation of both sugar and aglycone moieties, limiting its use in sugar isolations.

All sugar fractions were examined by PC and compared to suspected reference sugars which had been subjected to the similar hydrolysis procedures. Chromogens used were: silver nitrate as a nonspecific but sensitive reagent, aniline diphenylamine for its characteristic color variations with sugars, aniline phthalate for its specificity for reducing sugars, and modified aniline hydrogen phthalate for its specificity as well as distinct color production with reducing sugars. The presence of glucose in the neutral sugar fractions was confirmed by the Glucostat test,[12] a specific enzymatic procedure for β-D-glucose.

The procedures applied for the detection of sugars in various gymnemic acid fractions and their results are summarized in Table IV. Glucuronic acid was found in the hydrolysates of gymnemic acids A–D. Identification of glucuronic acid by PC was aided by the presence of an additional highly mobile spot due to glucuronolactone, which arises during hydrolysis as an equilibrium product of glucuronic acid (31). Glucose was observed to be the only neutral sugar constituent in the hydrolysates of gymnemic acids C and D.

Acidic hydrolysis of the ethyl acetate parent fraction gave rise to arabinose in addition to glucose, glucuronic acid, and glucuronolactone. In order to substantiate that arabinose did originate from the ethyl acetate fraction, a mixture of reference glucose and glucuronic acid was subjected to identical acid hydrolysis, and neutral and acidic sugar fractions were isolated as in the case of gymnemic acid sugars. Analysis of the reference neutral sugar fraction by PC failed to detect any trace of arabinose. Since gymnemic acids A–D do not contain arabinose, its origin is assigned to at least one of the minor gymnemic acids. Pfaffmann (10) reported the detection of arabinose along with glucose and glucuronolactone from the hydrolysate of his sample of gymnemic acid which has subsequently been shown to be a mixture (14).

The ethyl acetate parent fraction was also utilized in the present study as a source for the isolation of glucuronic acid to confirm the PC results. Isolation was in the form of methyl-β-D-glucofurano-sidurono-γ-lactone by the method of Osman *et al.* (32). The isolated compound was identical with the methyl glycoside of reference glucuronolactone by undepressed mixed melting point, chromatography, IR, and NMR analyses.

Positive color reactions with Liebermann-Burchard (19) and benzoyl chloride–sulfuric acid (17) reagents suggested gymnemic acids A–D to be glycosides of either steroid or triterpenoid alcohols. Further, the strong hydroxyl absorption around 3500 $cm.^{-1}$ and the characteristic bands in the region 700–1200 $cm.^{-1}$ (33) in their IR spectra, and a high percentage of oxygen content (about 30%) as indicated by elemental analyses, were consistent with the glycosidic character of the gymnemic acids.

Comparison of Gymnemic Acids Isolated by Various Investigators—While the current investigation was in progress, Stöcklin *et al.* (14) reported their isolation of the antisweet principle of *G. sylvestre* leaves, which they named gymnemic acid A_1. This isolation in a yield of about 0.004% was achieved by a complex procedure involving several treatments with both acid and base.

Reichstein's group called their parent fraction, from which gymnemic acid A_1 was secured, gymnemic acid A. Fraction A showed a single spot during TLC in four solvent systems.[13] However, in Solvent System VII, their gymnemic acid A was found to be a mixture composed of acids A_1, A_2, A_3, and A_4. Gymnemic acids A_1 and A_2 were shown to be identical to the two spots produced by Warren and Pfaffmanns' gymnemic acid in the same TLC system. Acid A_1 is the major constituent in both the fractions isolated by the Swiss group and by Warren and Pfaffmann.

Direct TLC comparison of gymnemic acids A–D, isolated in the present investigation, with acids A_1–A_4 obtained by Reichstein's group established that in TLC Systems I–VII acids A and B were identical to acids A_1 and A_2. However, acids C and D did not correspond to acids A_3 and A_4.

Stöcklin *et al.* (14) felt that gymnemic acid A_1 was the native antisweet principle of *G. sylvestre* leaves. Because of the harsh conditions employed in the isolation procedure, there existed the possibility of their acids A_2 and A_3 being artifacts originating from acid A_1. In this study, the ethanol extraction and petroleum ether precipitation method is presented as evidence for the natural occurrence of gym-

[12] Glucostat test was performed according to Method 1-A described in the literature accompanying the reagent kit (Worthington Biochemical Corp.).

[13] Adsorbent, silica gel G; solvent systems: ethanol–water–ammonium hydroxide (18:2:1), isopropanol–water–ammonium hydroxide (9:1:1), chloroform–methanol–ammonium hydroxide (5:1:1), and *n*-butanol–water–acetic acid (10:1:1).

nemic acids A–D. This fact, coupled with the nonidentity of gymnemic acids A_3 and A_4 with acids C and D from these laboratories or any of the minor acids, suggests that acids A_3 and A_4 are degradation products.

Further, gymnemic acid A (A_1–A_4) was shown by the Swiss group to contain glucuronic acid as the sole sugar residue. This is in agreement with the authors' gymnemic acids A and B which also contain only glucuronic acid. In contrast, presence of both glucuronic acid and glucose in gymnemic acids C and D from this study has been established.

Structural elucidation of gymnemic acids A–D will be the subject of forthcoming papers from the authors.

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