Preparative-Scale Chromatography of Ecdysteroids: A Class of Biologically Active Steroids

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Abstract

A simple separation procedure is developed for the isolation of the main phytoecdysteroid 20-hydroxyecdysone from the herb *Silene viridiflora*. The purification in four steps uses only a simple preparative-scale separation technique (i.e., liquid–liquid extraction, precipitation, solid-phase extraction on octadecyl silica, and crystallization). This procedure is extended using classical normal-phase liquid column chromatography, rotation planar chromatography, and preparative high-performance liquid chromatography for the isolation of the minor ecdysteroids: integristerone A, 26-hydroxypolypodine B, 2-deoxy-20,26-dihydroxyecdysone, and polypodine B. 2-Deoxy-20,26-dihydroxyecdysone is isolated from this species for the first time. The isolation of these ecdysteroids in adequate amounts makes them readily available for insect physiology experiments and for structure-activity relationship studies. The preparative-scale separation work also results in a minor, as yet unknown ecdysteroid.

Introduction

Ecdysteroids were discovered in insects and recognized as hormones responsible for the onset and regulation of molting and development in almost all classes of arthropods (1). Phytoecdysteroids are structurally similar steroid molecules occurring in plants. The production and occurrence of ecdysteroids in plants is distinguished by higher concentrations and more diverse structural variation (2) than in insects. According to the most accepted hypothesis, phytoecdysteroids have defensive functions against insect predators in plants (3). The relatively high concentrations of ecdysteroids in plants have opened the way to studying their pharmacological activities on mammalian tissues and insects. Based on these experiments, ecdysteroids have shown a wide array of pharmacological effects. Their pharmacological and therapeutic properties are well documented and reviewed (1,3–19).

Ecdysteroids beneficially influence many physiological functions in mammals. Ecdysteroids stimulate the protein synthesis without thymolytic, antigonadotropic, or androgenic side-effects (1,4–9); they influence lipid metabolism (1,5,10,11), carbohydrate metabolism (1,5,12,13), and ion-currents (14,15). Ecdysteroids exert protective, preventive, corrective, and adaptogenic effects (16,17). The use of ecdysteroids is also promising in genetics, where the ecdysteroids are used as inducers for gene-switch systems based on insect ecdysteroid receptors and genes of interest placed under the control of ecdysteroid-response elements (4,20–23). Their acute toxicity to mammals is extremely low (1,4,5).

Because of their pharmacological effects, ecdysteroid-containing preparations are widely available through the Internet (24). These preparations often contain 20-hydroxyecdysone, the main plant ecdysteroid, and are promoted mainly as anabolic and adaptogenic (25,26).

This paper reports a four-step process for the isolation of 20-hydroxyecdysone from the herb *Silene viridiflora* using only simple, unsophisticated separation methods. This procedure was supplemented with some preparative-scale chromatographic methods for the isolation of some minor ecdysteroids from this plant. The structures of the isolated ecdysteroids were elucidated by mass spectrometry (MS) and nuclear magnetic resonance (NMR). The aim of this work was also to study the ecdysteroid composition of *Silene viridiflora* growing in Hungary. The production of the secondary plant metabolite is influenced by the state of development and/or the climatic and soil conditions of the area where the plant is grown (27–29). To determine the differences between the ecdysteroid composition of the Hungarian and the Siberian *Silene viridiflora*, the ecdysteroid spectrums of these plants were compared.

Experimental

Materials

Plant material

The aerial parts of *Silene viridiflora* were collected in June 2002 from Vácrátót, Hungary. A voucher specimen (Silvi 241) was deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

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Chemicals

Solvents and chemicals were purchased from commercial sources in the best available quality. The standard ecdysteroids used for comparison were isolated in our earlier experiments (30).

Solid-phase extraction (SPE) was carried out on an endcapped octadecyl silica (0.06–0.2 mm, Chemie Uetikon, Uetikon, Zurich, Switzerland) packed into a 101 × 65 mm column (Column 1). The entire procedure was carried out at low-pressure (< 1 atm). Normal-phase (NP) column chromatography was achieved on a silica gel 60 (E. Merck, Darmstadt, Germany) 60 × 25 mm column (Column 2), using various mixtures of dichloromethane–ethanol (96 %) for elution.

Thin-layer chromatography

For NP thin-layer chromatography (TLC), silica gel 60 F_{254} plates (20 × 20 cm) (E. Merck) were used, with the following mobile phases: 1. dichloromethane–ethanol (96%) (8:2, v/v); 2. ethyl acetate–methanol–ammonia (25%) (85:15:5, v/v); 3. toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v); 4. chloroform–methanol–benzene (25:5:3, v/v); and 5. ethyl acetate–ethanol (96%)–water (16:2:1, v/v). Reversed-phase (RP) TLC was carried out on RP-18 WF_{254} on 20 × 20 cm glass plates (E. Merck) with the following mobile phases: 6. methanol–water (4:6, v/v); 7. acetonitrile–water (35:65, v/v); 8. acetonitrile–water–trifluoroacetic acid (35:65:0.1, v/v); and 9. tetrahydrofuran–water (45:55, v/v). Cyano-TLC (CN) was carried out on 10 × 20 cm glass HPTLC plates coated with CN F_{254} (E. Merck) with the following mobile phases: 10. *n*-hexane–acetone (6:4, v/v) and 11. acetonitrile–water (2:8, v/v).

For displacement TLC, silica gel 60 F_{254} plates (20 × 20 cm) (E. Merck) were used with mobile phase 12. dichloromethane–isopropanol–dimethylamino–propylamine (105:30:5, v/v).

Rotation planar chromatography (RPC) was carried out using a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA). The stationary phase was Silica gel 60 GF_{254} (E. Merck), manually coated on the rotor as a 2 mm layer. Stepwise development was carried out with the following mobile phases at 8 mL/min: System 1/A: dichloromethane–methanol–benzene (50:3:2, v/v); System 1/B: dichloromethane–methanol–benzene (50:5:3, v/v); System 1/C: dichloromethane–methanol–benzene (50:7:6, v/v); System 1/D: dichloromethane–methanol–benzene (50:10:6, v/v); or System 2/A: ethyl acetate–ethanol–water (160:10:4, v/v); System 2/B: ethyl acetate–ethanol–water (160: 14:6, v/v); System 2/C: ethyl acetate–ethanol–water (160: 20:10, v/v); System 2/D: ethyl acetate–ethanol–water (160: 40:20, v/v).

The plate was washed with 300 mL of methanol after the development for the desorption of the ecdysteroids remaining absorbed on the stationary phase after the RPC.

HPLC

The HPLC separation was performed on an Agilent 1100 Series Isocratic Pump (Agilent Technologies Inc., Palo Alto, CA) coupled to a Jasco UV-2075 Plus detector (Jasco Corporation, Tokyo, Japan), Zorbax RX Sil (Agilent) 5 µm, 250 × 4.6 mm i.d. column at 1 mL/min flow with mobile phases 1. dichloromethane–isopropanol–water (125:40:3, v/v/v) and 2. cyclohexane–isopropanol–water (100:40:3 v/v/v); and Zorbax ODS C-18 (Agilent) 5 µm, 250 × 9.4 mm i.d. column at 2.5 mL/min flow, using mobile phases 3. water–acetonitrile (77:23, v/v) and 4. water–acetonitrile (78:22, v/v).

Extraction and isolation of the ecdysteroids

The dried herb (1191 g) was milled and percolated with methanol (12.8 L) at room temperature. The methanolic extract was evaporated to dryness (240.3 g) and redissolved in 400 mL methanol–water (50 %). The solution was extracted with *n*-hexane (6 × 600 mL). The aqueous methanol phase was evaporated to dryness. The residue (206.6 g) was dissolved in 350 mL methanol, and acetone (250 mL) was added to the solution. Some precipitate was formed. The precipitation was repeated twice with 300 mL acetone (31). In each case, the resulting precipitate was removed by decantation, and then rinsed three times with 50 mL methanol–acetone (1:1, v/v). The methanol–acetone solution and the supernatant were combined and taken into dryness.

The entire separation was controlled by TLC using mobile phases 3 and 5 and at displacement development using mobile phase 12.

The residue (60.03 g) was dissolved in 50 mL aqueous methanol (30%) and purified by solid-phase extraction (SPE) on an endcapped octadecyl silica column (540 g). The column was washed with 30% aqueous methanol (1000 mL), and the elution was carried out with 40% aqueous methanol (1000 mL). The large amount of 20-hydroxyecdysone (3.4 g) in this fraction was removed by crystallization from ethyl acetate–methanol (2:1, v/v).

The residue of the mother liquid (3.1 g) was dissolved in 5 mL methanol and adsorbed onto silica (9 g). A total of 75 g silica was packed into a column, and the silica with the adsorbed sample was added to the top of the sorbent. Stepwise gradient elution was carried out using dichloromethane, dichloromethane–ethanol (96 %) 97:3, 95:5, 93:7, 92:8 (v/v), and methanol (1000, 1000, 900, 4100, and 3200 mL, respectively), and 100 mL of fractions were collected. Fractions 37–54 eluted with dichloromethane–ethanol 93:7 contained polypodine B, and fractions 63–102 eluted with dichloromethane–ethanol 93:7 and 92:8 contained 20-hydroxyecdysone. These compounds finally purified by crystallization from ethyl acetate–methanol (2:1, v/v), and 0.7 g polypodine B and 1.1 g 20-hydroxyecdysone were obtained, respectively.

Fractions 57–60 eluted with dichlorometane–ethanol (96%) (93:7, v/v) also contained a minor unknown ecdysteroid in addition to other ecdysteroids. These fractions were separated by RPC. The residue (0.14 g) of the collected fractions was dissolved in 3 mL methanol and adsorbed onto the rotation plate. The plate was developed stepwise with four mobile phases (Solvent system 1/A–1/D, 100 mL, of each). The effluents were collected manually. In each case, five 20 mL fractions were collected, which were marked 1a through 20a. The sorbent layer was washed with 300 mL of methanol. Fraction 13a contained the pure unknown compound (6 mg).

The methanolic washing solution of the silica column included 20-hydroxyecdysone, 2-deoxy-20,26-dihydroxyecdysone, integristerone A, and 26-hydroxypolypodine B. The

methanol was evaporated and the remaining material was separated by RPC. The residue (0.64 g) was dissolved in 3 mL methanol and adsorbed onto the rotation plate. The plate was developed stepwise with four mobile phases (Solvent 2/A–2/D, 100 mL, of each). In each cases, five 20-mL fractions were collected, which were marked 1b through 20b. The sorbent layer was washed with 300 mL methanol. Fractions 11b–15b (0.14 g), eluted with solvent system 2/C, contained 20-hydroxyecdysone and 2-deoxy-20,26-dihydroxyecdysone; fractions 16b–20b (0.14 g), eluted with solvent system 2/D, contained integristerone A and 26-hydroxypolypodine B. These two compound pairs were separated by HPLC using mobile phase 4 and 1, respectively, yielding 2 mg 20-hydroxyecdysone, 4 mg 2-deoxy-20,26-dihydroxyecdysone, 7 mg integristerone A, and 2 mg 26-hydroxypolypodine B.

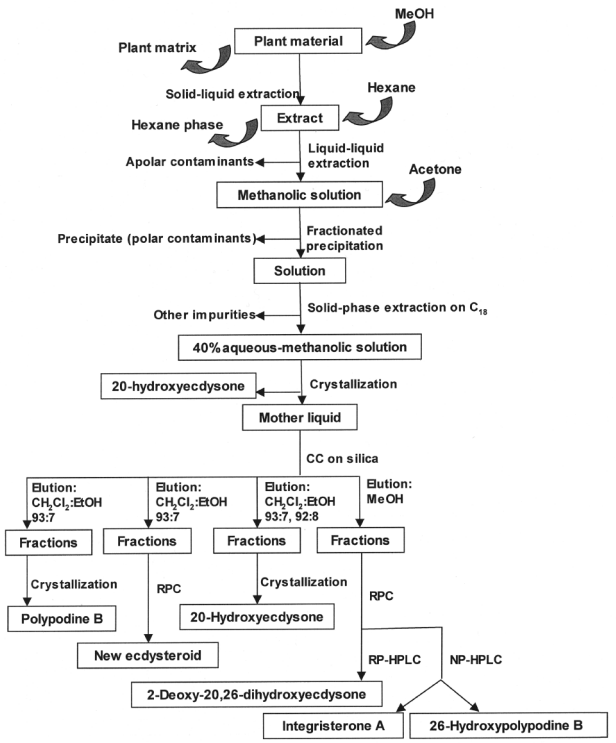

Figure 1. The scheme for ecdysteroid isolation.

Results

Figure 1 presents the scheme of the ecdysteroid purification. The majority of the main ecdysteroid, 20-hydroxyecdysone, was isolated with a four-step clean-up procedure using simple, preparative scale separation techniques. The principal techniques were liquid–liquid extraction, precipitation, SPE on RP (C-18), and crystallization. The majority of the apolar and polar contaminants were removed by liquid–liquid extraction and precipitation with acetone from the crude methanolic extract of the herb *Silene viridiflora*, as described earlier (31). SPE on a homemade endcapped octadecyl silica column was used to separate the remaining apolar and polar impurities from the ecdysteroids of interest. The ratio of sample to sorbent was relatively large, up to 1:9. The apolar impurities have a greater affinity for the stationary phase; therefore, these impurities were retained on the sorbent. The polar impurities were washed from the column with 30% aqueous methanol, and the ecdysteroids of our interest were eluted with 40% aqueous methanol. The effluent was enriched in 20-hydroxyecdysone but it contained some other ecdysteroids, too. The high excess of 20-hydroxyecdysone was obtained by crystallization from the liquid. The overall simple, four-step procedure resulted in an 18-fold purification.

Adsorption column-liquid chromatography on silica was used for the separation of the other ecdysteroids present in the mother-liquor. Silica column chromatography gave fractions containing only 20-hydroxyecdysone or polypodine B. Additional amounts of 20-hydroxyecdysone or polypodine B were obtained from this fraction by crystallization.

Certain fractions of the silica column contained a minor unknown ecdysteroid (Figure 2A) which was purified by RPC. In this separation, 45 g stationary phase was loaded with 0.14 g of dry sample, giving an adsorbent–sample ratio of 300:1. Fractionation was carried out with stepwise gradient elution in four steps. The separation was controlled by TLC and HPLC on RP (Figure 2A). The HPLC analysis has shown that RPC in a single run resulted in this unknown ecdysteroid in pure form (Figure 2B), which will be identified in the near future.

The last elution step of the silica column was carried out using methanol. The methanolic effluent contained a mixture of four known ecdysteroids which were further purified by the combina-

Table I. TLC Characteristics ($R_F \times 100$) of the Isolated Ecdysteroids

	NP-TLC					RP-TLC				CN-TLC	
	Mobile phase										
Ecdysteroid	1	2	3	4	5	6	7	8	9	10	11
2-Deoxy-20, 26-dihydroxyecdysone	n.d.*	31	n.d.	21	41	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20-Hydroxyecdisone (20E)	24	21	27	30	37	47	56	46	66	26	48
26-Hydroxypolypodine B	12	10	11	15	16	59	69	61	78	8	65
Integriszteron A	18	15	16	24	29	55	63	56	70	11	54
Polypodine B	32	20	25	35	38	48	55	49	66	33	50
New ecdysteroid	n.d.	n.d.	n.d.	41	60	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* n.d. = not determined.

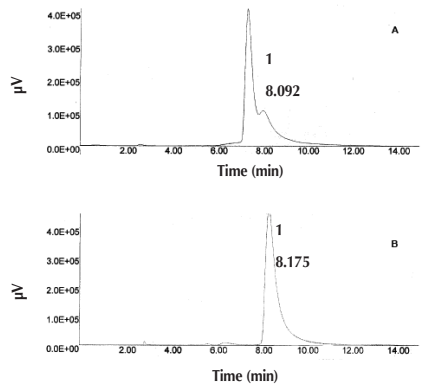

Figure 2. RP-HPLC chromatogram of fraction (A) containing a new ecdysteroid (peak 1), obtained by silica column chromatography, and that of the pure new ecdysteroid (B) obtained by RPC from these fractions. Stationary phase: Zorbax-ODS C18 (5 µm, 250 × 9.2 cm i.d.); mobile phase: water–acetonitrile (77:23, v/v) at a flow rate of 2.5 mL/min.

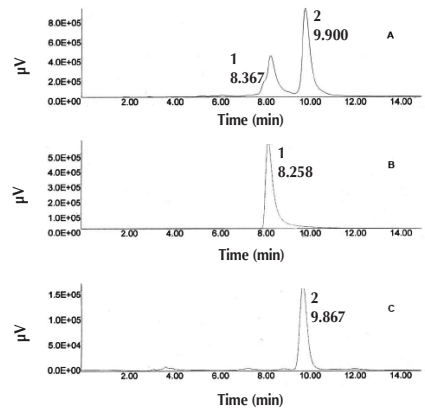

Figure 3. RP-HPLC chromatogram of fraction (A) obtained by RPC on silica column, and those of the pure 20-hydroxyecdysone (peak 1) (B) and 2-deoxy-20,26-dihydroxyecdysone (peak 2) (C) obtained by preparative RP-HPLC from these fractions. Stationary phase: Zorbax-ODS C18 (5 µm, 250 × 9.2 cm i.d.); mobile phase: water–acetonitrile (78:22, v/v) at a flow rate of 2.5 mL/min.

Table II. HPLC Retention Times (min) of the Isolated Ecdysteroids

	Mobile phase			
Ecdysteroid	1	2	3	4
2-Deoxy-20,26-dihydroxyecdysone	21.8	n.d.*	7.0	9.9
20-Hydroxyecdisone (20E)	16.9	21.5	5.2	8.4
26-Hydroxypolypodine B	30.9	n.d.	4.7	n.d.
Integristeron A	20.0	28.8	4.6	n.d.
Polypodine B	12	21.5	5.8	n.d.
New ecdysteroid	n.d.	n.d.	8.4	n.d.

* n.d. = Not determined.

tion of RPC and HPLC. 20-Hydroxyecdysone and 2-deoxy-20,26-dihydroxyecdysone were eluted together in RPC with solvent system 2/B (Figure 3A). HPLC on RP solved the separation of these two compounds (Figures 3B and 3C). The elution of integristerone A and 26-hydroxypolypodine B by RPC required the use of a more polar solvent such as 2/C. These two ecdysteroids migrated together with other compounds (Figure 4A). The final purification of integristerone A and 26-hydroxypolypodine B was carried out with preparative NP-HPLC (Figure 4B–C).

The chemical structures of the isolated ecdysteroids were elucidated with MS and NMR spectroscopy (1D-, 2D-, heteronuclear multiple-bond correlation, heteronuclear multiple-quantum correlation, correlation spectroscopy, total correlation spectroscopy, nuclear Overhauser enhancement spectroscopy, rotating-frame NOE spectroscopy) (data not shown). Figure 5 shows the chemical structures of ecdysteroids. Tables I and II report the chromatographic characteristics of the isolated ecdysteroids.

Discussion

The steps of the isolation and the type of separation methods used are dependent on the concentration of the ecdysteroids to be isolated. Our isolation process includes three important steps, solid–liquid extraction, clean-up of the crude extract, and chromatographic separations. The whole isolation procedure was controlled by TLC and displacement TLC, a method introduced by Horváth and Kalász (32–34). The isolation of the main ecdysteroid, 20-hydroxyecdysone, which is present in *Silene viridiflora* at relatively high concentration (0.4% relative to the dry weight of the plant), required only the use of an inexpensive, unsophisticated four-step clean-up with simple separation methods. However, the isolation of the other ecdysteroids needed the utilization of a combination of sophisticated preparative-scale chromatographic methods.

For the isolation of pure 20-hydroxyecdysone, an effective clean-up was enough, which involved liquid–liquid extraction, precipitation, SPE on a C-18 column, and crystallization. These prepurification methods made the clean-up procedure rather simple and faster. Capacity is the primary concern for the methods used for clean-up.

The crude extract was made free of apolar contaminants (chlorophyll, terpene) using liquid–liquid extraction between hexane and the aqueous-methanolic extract, with a recovery of ecdysteroids approximating to 100%. The load could be high, because the partition coefficient depends only slightly on concentration. Precipitation with acetone removed the majority of polar contaminants, such as sugars, polysaccharides, and proteins.

SPE on a C-18 column is capable of extracting the ecdysteroids from the aqueous phase. The column was washed with 30% aqueous methanol to remove any remaining polar impurities. Ecdysteroids of our interest

were selectively eluted with 40% aqueous methanol. SPE is an ideal, rapid clean-up method. The final method for the clean-up and also of the isolation of 20-hydroxyecdysone was crystallization. The entire isolation process of 20-hydroxyecdysone is economical and can be easily carried out; it requires only a minimum of technical setup and eliminates the need for complicated apparatus.

In the case of the isolation of the minor ecdysteroids, a multistep isolation protocol was achieved. The four-step clean-up was followed by the use of some preparative-scale chromatographic procedures, which included NP-LC on silica, RPC on silica, and preparative HPLC. For the adsorption column LC, a solvent system with increased polarity was utilized, which resulted in the elution of ecdysteroids according to their polarity, thereby contamination was decreased to a minimum. In this way, further pure ecdysteroids (20-hydroxyecdysone and polypodine B) were obtained. The isolation procedure was improved by the use of RPC, which is easy to carry out, and the ecdysteroids contact with the adsorbent layer for a short time. Therefore, the problem associated with adsorbent-assisted decomposition was reduced. RPC is an inexpensive, effective tool for the separation of ecdysteroids, requiring low solvent usage and less time. Our aim was to simplify the earlier isolation procedures with the introduction of RPC and to study the contribution of RPC to this process. The final purification was carried out with preparative-scale NP- and RP-HPLC with good resolution.

The consecutive steps of separation were based on the different characteristics of the ecdysteroids and the accompanying compounds, such as the distribution between two immiscible phases, lipophilicity, and adsorption to the stationary phase.

Silene viridiflora is a suitable source for the isolation of 20-hydroxyecdysone, which is an important ingredient of health improvement preparations. A series of other ecdysteroids was also obtained from this plant in suitable amounts for further biological studies. The ecdysteroid composition of Hungarian *Silene viridiflora* shows some correlation with the ecdysteroid composition of the Siberian one (35,36), but different methods allow us to focus on different minor fractions. The Hungarian and the Siberian *Silene viridiflora* biosynthesize 20-hydroxyecdysone, integristerone A, polypodine B, and 26-hydroxypolypodine B in similar concentrations. However, 2-deoxy-20,26-dihydroxyecdysone was isolated for the first time from the Hungarian species. Isolation of either new or classical ecdysteroids gives a unique advantage. Only the isolated compounds can be used for pharmacological trials. The isolated ecdysteroids can also be used in structure-activity relationship studies.

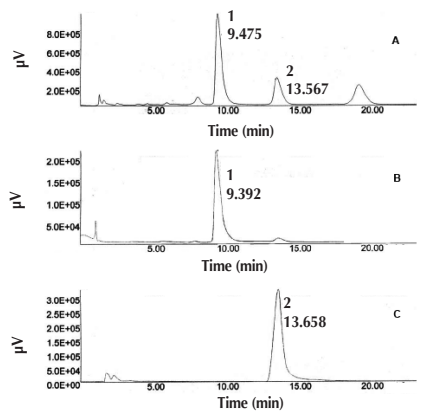

Figure 4. NP-HPLC chromatogram of fraction (A) obtained by RPC on silica, and those of the pure integristerone A (peak 1) (B) and 26-hydroxypolypodine B (peak 2) (C) obtained by preparative NP-HPLC from this fraction. Stationary phase: Zorbax RX Sil (5 µm, 250 × 4.6 cm i.d.); mobile phase: dichloromethane–isopropanol–water (125:40:3, v/v/v) at a flow rate of 1 mL/min.

Conclusions

Silene viridiflora, growing in Hungary, is a rich source of ecdysteroids. The newly established isolation process for the purification of the main ecdysteroid, 20-hydroxyecdysone, used only simple separation methods, and was therefore easy to carry out. This fast, inexpensive procedure is advertised for the purification of 20-hydroxyecdysone from other plants, too.

It is proven that the ecdysteroid composition of plants varies considerably according to the geographical region and ecological environment where the plant is grown. We found some similarities between the ecdysteroid composition of the Hungarian and the Siberian *Silene viridiflora*, but some differences, too. This finding also supports the conclusion mentioned earlier, but further experiments are needed in this area to specify in which proportion these variations are genetically determined.

Acknowledgment

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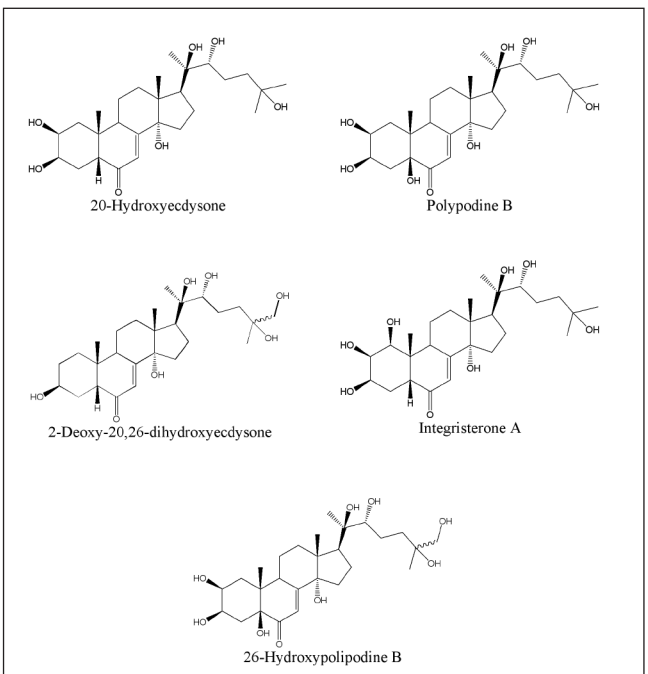

Figure 5. The chemical structures of the isolated ecdysteroids.

References

1. K. Sláma and R. Lafont. Insect hormones in vertebrates: anabolic effects of 20-hydroxyecdysone in Japanese quails. *Eur. J. Entomol.* **92:** 355–377 (1995).
2. *Ecdybase* (The Ecdysone Handbook, 3rd ed., on-line). R. Lafont, J. Harmatha, L. Dinan, and I.D. Wilson, Eds. Available from http://www.ecdybase.org, 2002.
3. L. Dinan. Phytoecdysteroids: biological aspects. *Phytochem.* **57:** 325–339 (2001).
4. R. Lafont and L. Dinan. Practical uses for ecdysteroids in mammals including humans: an update. *J. Insect Sci.* **3(7):** 1–30 (2003).
5. P. Simon and J. Koolman. In *Ecdysone, from Chemistry to Mode of Action.* J. Koolman, Ed. George Thieme Verlag, Stuttgart, 1989, pp. 254–259.
6. V. N. Syrov. Mechanism of the anabolic action of phytoecdysteroids in mammals. *Biol. Nauki (Moscow)* **11:** 16–20 (1984).
7. V. N. Syrov. Comparative experimental investigations of the anabolic activity of ecdysteroids and steranabols. *Pharmaceutical and Chemical Journal* **34:** 193–197 (2000).
8. V. N. Syrov, Z. Saatov, Sh. Sagdullaev, and A.U. Mamatkhanov. Study of the structure-anabolic activity relationship for the phytoecdysteroids extracted from some plants of Central Asia. *Pharmaceutical Chemistry Journal* **35:** 667–671 (2001).
9. F. Kratky, L. Opletal, J. Hejhalek, and S. Kucharova. Effect of 20-hydroxyecdysone on protein synthesis of pigs. *Zivocisna Vyroba* **42:** 445–451 (1997).
10. R.E. Catalán, A.M. Martinez, M.D. Aragones, B.G. Miguel, A. Robles, and J.E. Godoy. Alteration in rat lipid metabolism following ecdysterone treatment. *Comparative Biochemistry and Physiology* **81B:** 771–775 (1985).
11. V.N. Syrov, Z.A. Khushbaktova, M.Kh. Abzalova, and M.B. Sultanov. On the hypolipidemic and antiatherosclerotic action of phytoecdysteroids. *Doklady Akademii Nauk Uzbeckoy SSR*. **9**: 44–45 (1983).
12. C. Yang, G. Zhang, X. Liu, and C. Wang. Oral antidiabetic composition containing β-ecdysone from *Cyanothis arachnoids*. *Application* CN-2000-10637/20000612. *Chemical Abstracts* **135:** 127188 (2001).
13. V. N. Syrov, Z.A. Khushbaktova, and M.A. Tashmukhamedova. Hypoglycemic action of phytoecdysteroids and some aspects of its mechanism of realization in experimental animals. *Doklady Akademii Nauk Respubliki Uzbekistana* **4:** 46–49 (1997).
14. A.G. Kurmukov and O.A. Yermishina. Effect of ecdysterone on experimental arrythmias, changes in hemodynamics and contractility of the myocardium produced by a coronary artery occlusion. *Farmakologiya i Toksikologiya* **54:** 27–29 (1991).
15. X. Wu. Use of ecdysteroids in preparing medicine for angiocardiopathy. *Application* CN 2000-12119/200000731. *Chemical Abstracts* **135:** 147422 (2001).
16. A. Aikake, T. Matsumoto, and Y. Yamaguchi. Cerebral neuron protective agents containing ecdysteroids. *Application* JP 94-195279/19940819. *Chemical Abstracts* **125:** 1395 (1996).
17. D. K. Najmutdinova and Z. Saatov. Lung local defense in experimental diabetes mellitus and the effect of 11,20-dihydroxyecdysone in combination with manilil. *Archives of Insect Biochemistry and Physiology* **41:** 144–147 (1999).
18. A. Meybeck, F. Bonté, and F. R. Demande. Ecdysteroid-containing liposomes for wound healing and skin regeneration. Demande FR 2,637,182. *Chemical Abstracts* **114:** 30138r (1990).
19. M. Báthori, I. Zupkó, A. Hunyadi, E. Gácsné-Baitz, Z. Dinya, and P. Forgó. Monitoring the antioxidant activity of extracts originated from various *Serratula* species and isolation of flavonoids from *Serratula coronata. Fitoterapia* **75:** 162–167 (2004).
20. D. No, T.P. Yao, and R.M. Evans. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93:** 3346–3351 (1996).
21. S. R. Palli, R. E. Hormann, U. Schlattner, and M. Lezzi. Ecdysteroid receptors and their applications in agriculture and medicine. *Vitamins and Hormones* **73:** 59–100 (2005).
22. L. Dinan, R. E. Hormann, and T. Fujimoto. An extensive ecdysteroid CoMFA. *J. Comput. Aid. Mol. Des.* **13:** 185–207 (1999).
23. M. Ravi, A. J. Hopfinger, R. E. Hormann, and L. Dinan. 4D-QSAR of a set of ecdysteroids and a comparison to CoMFA modelling. *J. Med. Chem.* **41:** 1587–1604 (2001).
24. M. Báthori and Z. Pongrácz. Phytoecdysteroids—From isolation to their effects on humans. *Curr. Med. Chem.* **12:** 153–172 (2005).
25. L. Opletal, M. Sovová, M. Dittrich, P. Solich, J. Dvorák, F. Krátký, J. Cerovský, and J. Hofbauer. Phytotherapeutic aspects of disease of the circulatory system. 6. *Leuzea carthamoides* (Willd.) DC. *Ces. a Slov. Farm.* **46(6):** 247–255 (1997).
26. http://www.diabestea.com/velocity/active.html; http://mdhealthline.com/cgi/sgx/store/web_store.cgi?_id=134.157.163.233&page=elite.html; http://www.marigarden.com/hgw/index.html; http://www.phytolongevity.com/products/performance/performance.htm.
27. L. Zibareva, V. Volodin, Z. Saatov, T. Savchenko, P. Whiting, R. Lafont, and L. Dinan. Distribution of phytoecdysterois in the Caryophyllaceae. *Phytochem.* **64:** 499–517 (2003).
28. L. Zibareva. Distribution and levels of phytoecdysteroids in plants of the genus *Silene* during development. *Arch. Insect Biochem. Physiol.* **43:** 1–8 (2000).
29. M.F. Corio-Costet, L. Chapnis, J.F. Mouillet, and J.P. Delbecque. Sterol and ecdysteroid profiles of *Serratula tinctoria* (L.) plant and cell cultures producing steroids. *Insect Biochem. Molec. Biol.* **23:** 175–180 (1993).
30. J.P. Girault, M. Báthori, E. Varga, K. Szendrei, and R. Lafont. Isolation and identification of new ecdysteroids from the Caryophyllaceae. *J. Nat. Prod.* **53:** 279–293 (1990).
31. H. Kalász, E. Liktor-Busa, G. Janicsák, and M. Báthori. Role of preparative rotation planar chromatography in the isolation of ecdysteroids. *J. Liq. Chrom.* **29:** 2095–2109 (2006).
32. H. Kalász, L. Kerecsen, and J. Nagy. Conditions dominating displacement thin-layer chromatography. *J. Chromatogr.* **316:** 95–104 (1984).
33. H. Kalász. Planar displacement chromatography. *J. Planar Chromatogr.* **17:** 464–467 (2004).
34. H. Kalász. Spot and mobile-phase-front anomalies in planar (thin-layer) chromatography. *Chromatographia* **62:** S57–S62 (2005).
35. N.Z. Mamadalieva, L.N. Zibareva, Z. Saatov, and R. Lafont Phytoecdysteroids of *Silene viridiflora. Chemistry of Natural Compounds* **39(2):** 199–203 (2003).
36. N.Z. Mamadalieva, L.N. Zibareva, N. Edvrard-Todeschi, J.P. Girault, A.M. Nurmurod, Sh. Ramazanov, Z. Saatov, and R. Lafont. New minor ecdysteroids from *Silene viridiflora. Collect. Czech. Chem. Commun.* **69(8):** 1675–1680 (2004).

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