Phytoecdysteroids Effects on Mammalians, Isolation and Analysis

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Abstract: Ecdysteroids are known insect moulting hormones, regulating the insects' metamorphosis. At the same time, ecdysteroids reveal beneficial effects on humans and animals alike. Medicinal plants have been subjected to an intensive research, addressing the presence of ecdysteroids. The possible utilization of medicinal plant deals with their use as raw materials for health preparations and also for the isolation of new phytoecdysteroids.

Research on the plant ecdysteroids involves two basic lines. Isolation of major compounds and scout their physiological effects; and isolation of minor ecdysteroids to find new compounds.

This review summarizes the recent efforts in the ecdysteroid research including their indication as health improvement preparations, chromatography of ecdysteroids and certain methods for identification of new ecdysteroids.

Key words: Ecdysteroids, 20-Hydroxyecdysone, TLC of ecdysteroids, HPLC of ecdysteroids, Isolation, Health Improvement preparations, Chromatography

1. INTRODUCTION

1.1 Health Improvement Preparations

Medicinal practice has often used preparations of plant origin for hundreds of years. The preparations serve to supply the essential vitamins, trace elements and also the compounds having effects on the body. Vitamin and trace element contents are regulated and strictly controlled, however, the control of all the further active compounds has remained far from the everyday practice. This paper summarizes the possibilities how the ecdysteroid contain of certain health improvement preparations can be analyzed, including the analysis and preparation of 20-hydroxyecdysone, and that of other ecdysteroids.

Health improvement preparations with 20-hydroxyecdysone Fig. (**1**) content have been widely advertised. **Table 1** lists certain commercially available health products with essential ecdysteroid content. As ecdysteroids make the body adaptogenic, they have anabolic actions (without thymolytic, antigonadotropic and androgenic side effects), shields the body from stress. Moreover they are general tonic and wide-spectrum stimulants which also improve the physical, mental and sexual conditions [1-3]. Acute toxicity of ecdysteroids to mammals is extremely low [1].

1.2. Pharmacological and Biological Activity

As the major plant ecdysteroid is 20-hydroxyecdysone (20E), the overwhelming majority of the pharmacological experiments were done with 20E. The literature referred to various pharmacological effects, such as given in the **Table 2.** Effects of ecdysteroid on insects reveal the regulation of insect metamorphosis, with numerous biological consequences.

1.3. Structure-Activity Relationships

As a wide structural diversity of ecdysteroids has been known, certain conclusions on the structure-activity relationship were drawn. The relationships mainly dealt with the activity on insects, for having interactions with the ecdysteroid receptors. There were several conditions for the efficiency, and at least some of them had to be fulfilled:

- cis-fused A/B rings in the steroid skeleton
- 7-en-6-one conjugation in the B ring
- whole sterine side chain on the 17C together with 22R hydroxyl and sometimes with 24α-alkyl
- 3β-hydroxyl
- 14α-hydroxyl
- 2β-hydroxyl

In addition, the C-20 and C-25 positions are often hydroxylated. Further characteristics are that the acetate substitution at C-2 hydroxyl and that of the benzoate at C-25 hydroxyl shows certain activity, while the di- and tri-esthers and the glucosidic derivatives were rather less active or inactive.

One of the basic procedure modeled ecdysteroid(s) using pharmacophore hypothesis, and the procedure was called

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Table 1. Health Improvement Preparations, the Effects Based on their 20-Hydroxyecdysone Content

Name	Indication	Country	Company	20E Content	Dosage Form
Victory Beta Ecdysone	Fitness, Anabolic Body Building	USA	General Nutrition Centers	10 mg/capsule	Capsule
Immunectare®	Adaptogenic for HIV patients	USA	Nature's Plus Product	150 mg Suma®	Beverage
Changing Times™	Adaptogenic	USA	Nature's Plus product		
Retibol	Anabolic	USA	Atletica Sport International		Tablet
Prime One® Prime Plus® Prime Perfect, Prime Quest® Breckhman's Gold®	Shields the Body from Stress to Naturally Heighten Mental, Physical and Sexual Perfomance, Build Lean Muscle Tissue	USA	Prime Firm		Liquid Tablet
Ecdysten®	Anabolic, General Tonic, Prevention from Infectious Diseases	Soviet Union	Thermo Life	5 mg/tablet	Tablet (Pills)
Robofit Drops	Adaptogenic, Against Stress Improvement of Psychical and Physical Conditions	Hungary	Research Institute of Medicinal Plants	Data not given (d.n.g.)	Alcoholic Extract (40%)
Maralan	Stimulant, Improvement of Physical Condition, Resistance against Stress, Stimulation of Functions of CNS	Slovak Republic	J. Kren Firm	0.08% - 0.22%	Green Tea
Leveton	Approches to Sexual Adaptation, Increases Physical Work Capacity of Athletes, Broad-spectrum Adaptogenic	Soviet Union	Bipharm	d.n.g.	Flower Powder
Sumax®	Anabolic			5%	Capsules
Triboxin	Anabolic, Builds muscle	USA	Atletica Sport International	d.n.g.	Capsules
VitiCom N VitiCom P	Adaptogenic	Czech Republic		d.n.g.	d.n.g.

Table 2. Important Pharmacological Effects of Ecdysteroids

Effect	Species/Cell	Reference	Author
Skin Regeneration	Human	[4]	Meybeck & Bonte
Immune Stimulant	Mice cells	[5]	Chiang *et al.*
Increase of Protein Incorporation	Mice, Japanese Quail, Cow	[6]	Slama *et al.*
Antiarrythmic	Rabbit	[7]	Khusbuktova *et al.*
Hepatoprotective	Rat	[8]	Syrov *et al.*
Decrease of Cholesterol Level	Rat	[9]	Catalan *et al.*
Decrease of Blood Glucose	Rat and Mice	[10]	Yoshida *et al.*
Anti inflammation and Pain Relief	Mice, Rat	[11] [12]	Kurmukov & Syrov Takei *et al.*
Adaptogenic	Rat	[1]	Slama & Lafont
Spasmolytic	Rat	[13]	Babich *et al.*

CoMFA (Comparative Molecular Field Analysis). Dinan [14] sketched the chemical structure of 20-hydroxyecdysone, Fig. (**1**) points out the crucial structural elements (angles and distances), and the required hydrophobic region of the sterine side chain, the hydrophilic and the dipole factors. These elements of various ecdysteroids had to approximate to that of 20-hydoxyecdysone if they bound to the ecdysteroid receptor of *Drosophilia melanogaster*.

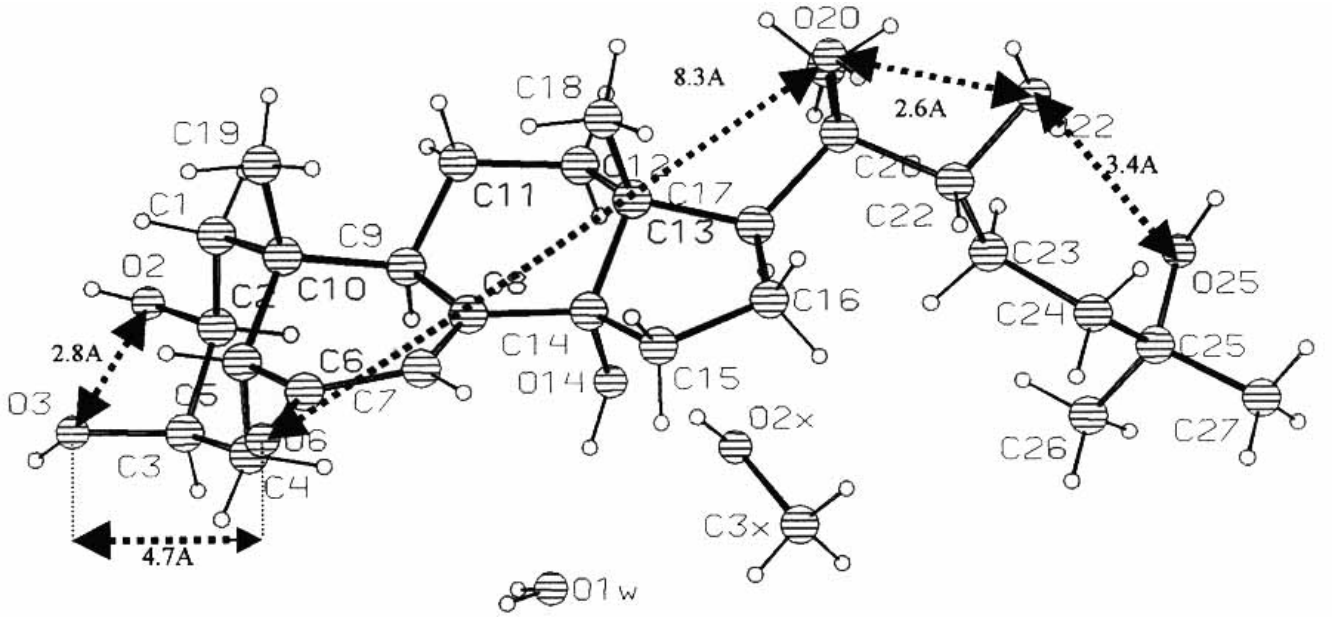

Fig. (1). The chemical structure of 20-hydroxyecdysone with certain important atomic distances. The angle between O-2 to O-3 line and O-3 and O-6 line is 119°, while the angle between O-6 to O-20 line and O-3 and O-6 line is 106°. The angle between O-6 to O-20 line and O-20 and O-22 line is 145°.

Dinan [14] mentioned two other methods, such as

- Homology modeling and docking,
- 4D-QSAR

where 4D-QSAR and CoMFA gave good agreement on their results.

2. ISOLATION OF ECDYSTEROIDS

To perform the biological and pharmacological experiments, a variety of ecdysteroids were consumed, sometimes in large amount. It was the reason that there were extensive researches to find fast and reliable isolation procedure of ecdysteroids that were present in the plants. As the level of phytoecdysteroids covered a wide range (such as from 3.2% down to the trace) a unified method was elaborated to exploit ecdysteroids from the plant1 [15-17].

The method started with sample preparation as given in Fig. (**2**). The isolation was continued with combination of the optimum choice of chromatographic procedures as given in Fig. (**3**)**.** The overground part (herba) of *Silene otites* (L.) Wib. was an adequate source of ecdysteroids, as its 20-hydroxyecdysone content was high enough, and several other ecdysteroids were also present there.

Homogenization, and sample preparation was a crucial phase of both any enrichment and isolation work and they contained three essential steps, such as

1. Crushing, milling and extraction of the plant sample, and filtration of the extract
2. Concentration, fractionated precipitation and/or solvent-solvent partition
3. Solid-phase extraction (SPE).

The 1st step served the physical removal of the "support" matrix of the plant, simply by filtration of the extract, and thereby a ten-fold enrichment was resulted in. It combined both homogenization and sample preparation. Moreover, the sample was made easily accessibly to the further purification. A minimum of ten-fold excess of the solvent (usually, methanol) was used in the 1st extraction step.

The 2nd step was dealing with the sample preparation, and the ecdysteroids were made free from both the rather polar and rather apolar compounds on the basis on the solubility and on the distribution between solvents. In general, fractionated precipitation operated using acetone, and it removed the compounds more polar than ecdysteroids. At the same time, solvents-solvent partition was done using aqueous methanol and hexane (or any other solvents which are plain or mixed apolar hydrocarbons), where hexane removes the majority of components which were more apolar than the ecdysteroids. This 2nd step was generally performed when the aim of ecdysteroid enrichment was either to produce health products or purified ecdysteroids. Both the solvent-solvent partition and the fractionated precipitation left the ecdysteroids and overwhelming majority of the substances with similar chromatographic characteristics (such as flavonoids, iridoids, and certain other biologically active compounds) together.

The 3rd step of prepurification procedure was solid-phase extraction. SPE served also enrichment of ecdysteroids by removing certain compounds from ecdysteroids which had

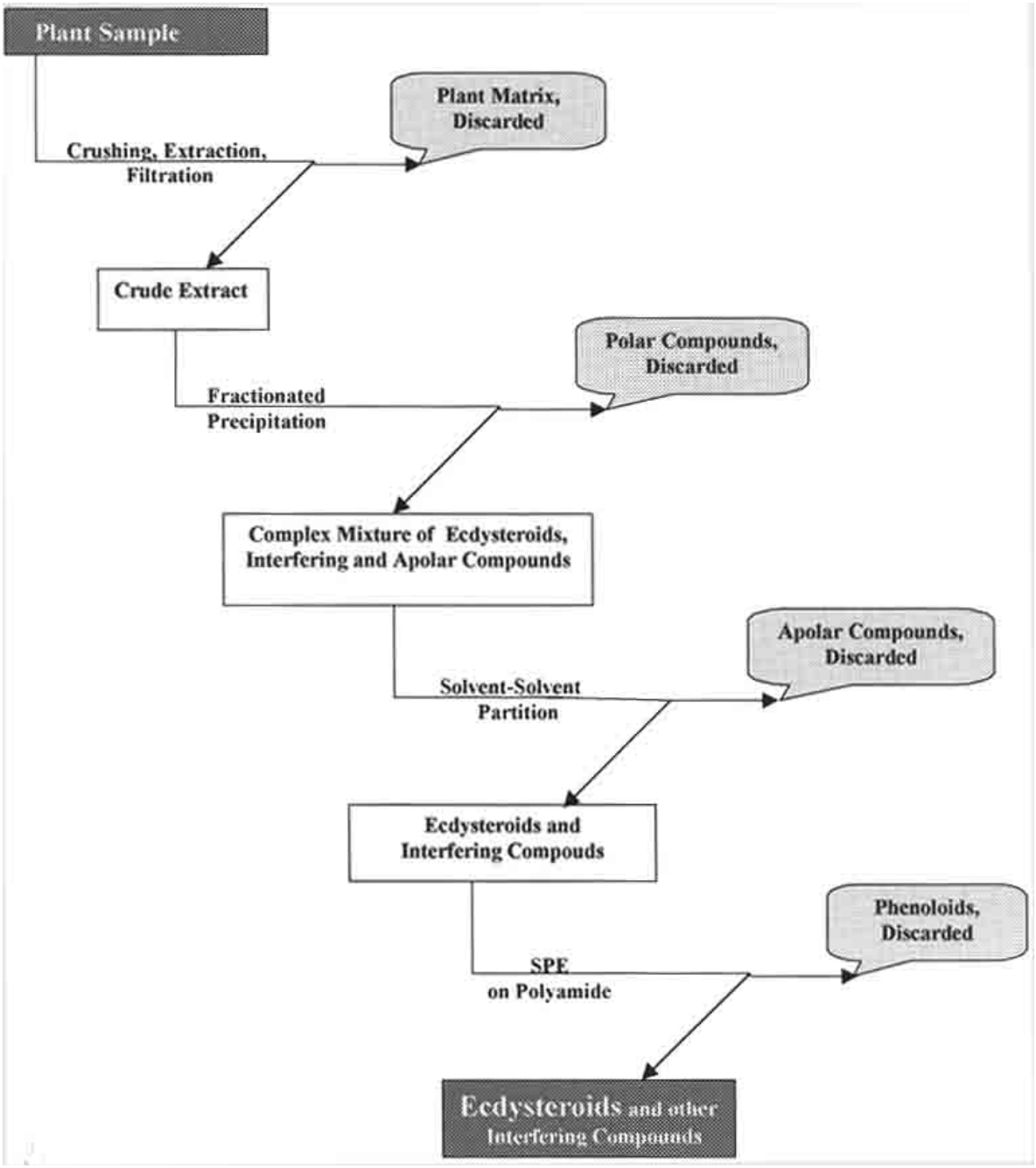

Fig. (2). The process of the ecdysteroid extraction and prepurification from *Silene otites* (L.) Wib.

similar polarity but differed in having their aromatic ring, etc. SPE is mainly used when isolation of pure ecdysteroids was dealt with. The major outcome of the employment of SPE was the effective decrease of the number of contaminants. Polyamide was usually employed for the stationary phase of SPE, and the ratio of sample to polyamide could be about 1:10 (w/w). One of the essential advantages of SPE of ecdysteroids on polyamide was that the ecdysteroids were eluted earlier than the contaminants (phenoloids, etc.), thereby the danger of further contamination was decreased to a minimum.

Fig. (**3**) gives the scheme of separation of the individual ecdysteroids. The basis of the isolation was the proper choice and the adequate order of chromatographic and related methods following the basic rules, such as

- The first steps had to have high sample load and only limited separation power,
- The final purification methods had to show high separation power to separate even the chemically similar compounds,
- The consecutive steps of separation had to be based on the different characteristics of the compounds, such as lipophilicity, adsorption to the stationary

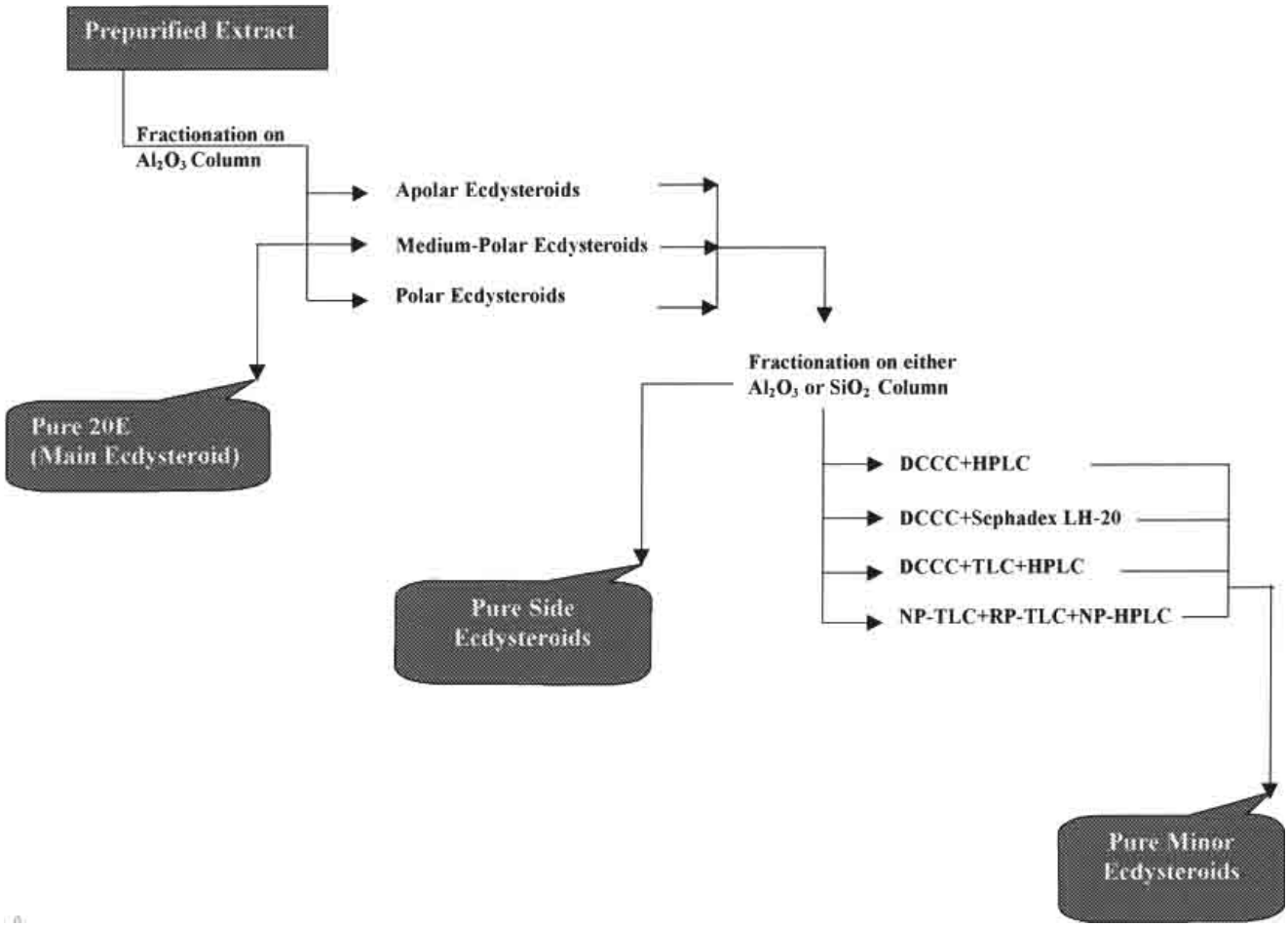

Fig. (3). The scheme of isolation of the individual ecdysteroids.

phase, distribution between two immiscible phases, etc.

3. QUANTIFICATION OF ECDYSTEROIDS

Both separation and detection methods were used to determine the ecdysteroid content [18-19] of plant extracts and health preparations. Chromatographic and related separation methods served to differentiate the ecdysteroids from each other, and also from the other compounds. The separation generated either distinct peaks (at column technique) or distinct spots, however, the detection methods were used to locate of peaks (and spots) belonging to the individual ecdysteroids. When the separation was monitored using UV detection, it offered certain specificity and also the quantitation. The most popular method for ecdysteroid analysis was HPLC combined with UV detection at 254 nm (HPLC/UV_{254}). The sensitivity of HPLC/UV_{254} covered a range from 5 through 100 ng. The detectable signal was the consequence of 7-en-6-one conjugation. As the maximum of ultraviolet absorbance was really at 240 through 245 nm, the specificity could be slightly increased to perform detection somewhere at that wavelength [18-19]. At the same time, HPLC/UV was widely spread, especially by using internal standards for quantitative evaluation [18]. Perfect specificity of a definite ecdysteroid determination was given by HPLC/MS or HPLC/MS/MS combination [20-22]. HPLC/MS improved the sensitivity, made the determination totally reliable, even in the cases of trace amount of ecdysteroids. The detection could also be based on the off-line determination of biological activity of ecdysteroids, thereby RIA (radio immunoassay) was commonly used [23].

To increase sensitivity, another way was the use of fluorescence detection. Preparation of fluorescence derivatives of ecdysteroids allowed the detection of even 10 pg of ecdysteroids. As the preparation of fluorescence derivatives was a time consuming method, but it did not offered specificity (derivatization was based on the hydroxyl

Table 3. Accepted Methods for the Determination of 20-hydroxyecdysone

Determination Method	MDA (Minimum of Detectable Amount	Reference
HPLC	$5x10^{-9}$ g	[18,19]
HPLC/MS	10^{-9} g	[18-22]
RIA	$1x10^{-9}$ g	[23]
HPLC/RIA	10^{-9} g	[23]
Spectrophotometry	10^{-6} g	[24]
TLC	$5x10^{-5}$ g	[25]

groups of ecdysteroids, thereby any hydroxylated compound gave the same reaction), the HPLC/fluorescence detection did not become a popular assay [18-19].

Table 3 depicted several methods for the determination of 20-hydroxyecdysone from either the crude samples or from the purified preparations.

The simplest method of choice for 20-hydroxyecdysone determination was thin-layer chromatography combined with UV densitometry (TLC/UV) [25]. The method used an automated spot application, developments using the adequate mobile phase, and the ultraviolet absorbance scanning densitometry of fluorescent-quenched spots of samples and that of the standards. The quantitative evaluation was performed by reflectance measurements at 254 nm using external standard series for calibration. TLC/UV was especially favorable when a large number of samples had to be analyzed at the same time. Large series analysis was a common requirement for the standardization of health preparations, as well as for determining the seasonal dependence of ecdysteroid content of the medicinal plant. The seasonal dependence was investigated to find the time optimum of harvesting, and thereby the ecdysteroid production could be multiplied.

The selection of an optimal mobile phase was done by a systematic way of trial and error. To ensure the proper selectivity, these following mobile phases could be checked:

A: Dichloromethane-Ethanol 8:2, v/v

B: Ethyl acetate-Methanol- Ammonia (25% solution), 85:10:5, v/v/v/

C: Toluene-Acetone-Ethanol (96%)-Ammonia solution, 100:140:32:9 v/v/v/v

D: Chloroform-Methanol-Benzene 25:5:3, v/v/v

E: Ethyl-acetate-Ethanol (96%)-water, 16:2:1, v/v/v

The proper mobile phase for the determination of 20-hydroxyecdysone in *Silene otites* was the solvent system D which contained benzene, chloroform and methanol, as it gave not only good resolution, but also adequately compact spots for the quantitative evaluation.

Confirming the suitability of this system was possible by measuring and finding the identity of the complete UV spectra of both sample and standard peak; directly on the

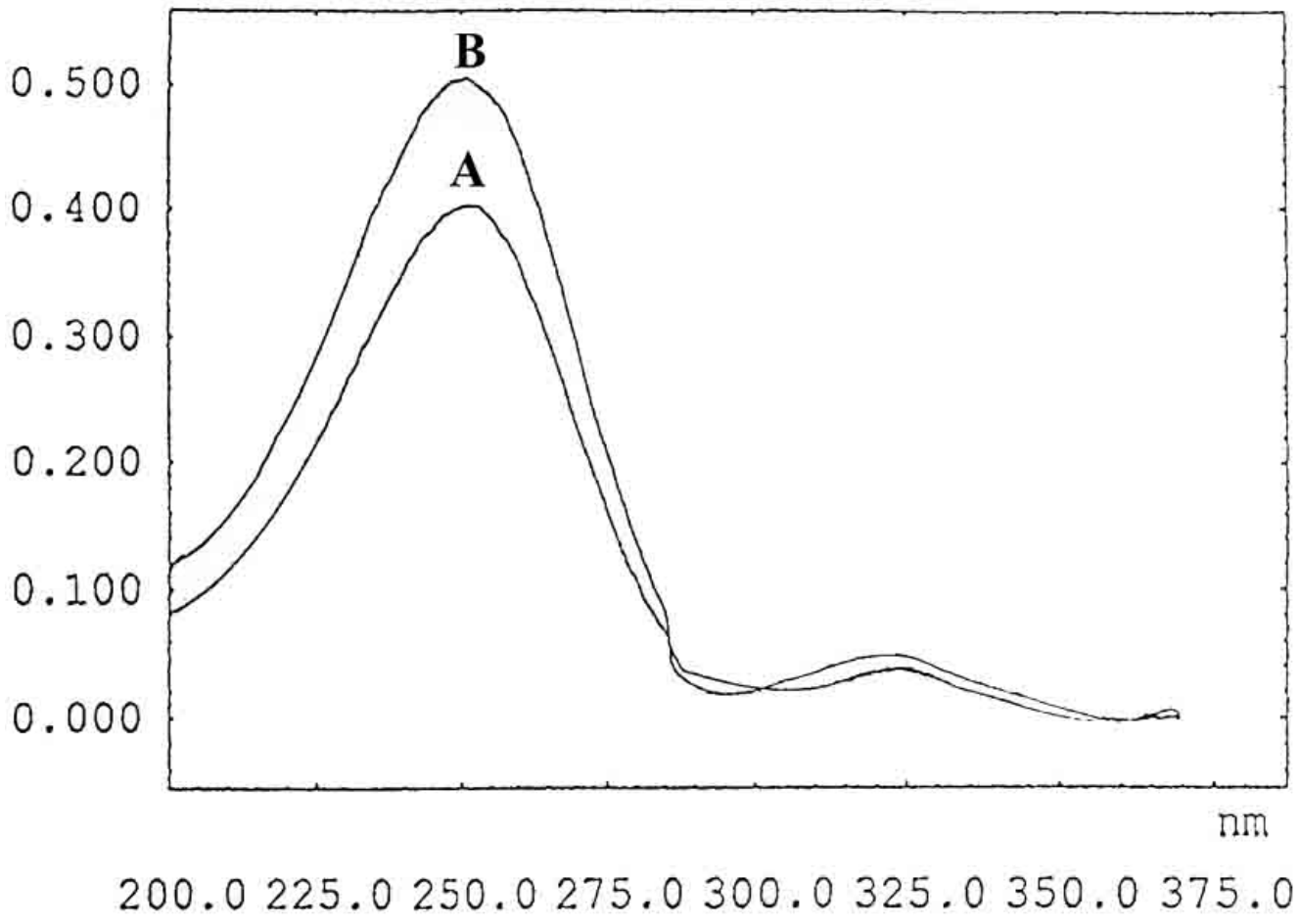

Fig. (4). The UV spectra recorded directly on the TLC plate after development.

- (**A**) 20-hydroxyecdysone from the May's sample of *Silene otites* (L.).
- (**B**) the standard of 20-hydroxyecdysone.

Table 4. Calibration Indicating the Linearity of 20-Hydroxyecdysone Determination

20-Hydrooxyecdysone (μg/spot)	Area under Curve (AUC)	AUC/μg	Mean of AUC/mg	Linearity Between ± 5%
0.90	530.064	588.96		
2.25	1216.449	540.64		
3.15	1741.255	552.78		591.62
4.50	2494.037	554.23	563.45	-
5.85	3338.391	570.67		535.28
6.75	3870.377	573.39		

Table 5. Recovery Determination by the help of Peak Addition Method (n = 6).

Date of Sample	Content of 20-Hydroxyecdysone (mg/Spot)		Recovery (%)
	Expected	Found	
May 31	8.53	8.44	98.94
June 27	4.46	4.23	94.84
July 26	5.11	5.08	99.41
August 12	7.75	7.64	98.58
September 26	5.45	5.22	95.78

TLC plate (Fig. (**4**)). The mobile phase gave an adequate selectivity for 20-hydroxyecdysone to be separated. The working range of the determination was selected on a series of experiments. The upper and the lower level of analyte had to be quantified with suitable precision, accuracy and linearity [26]. The quantitative TLC determinations, the results were highly influenced by the quality of calibration. In order to find a convenient range of calibration graph, the calibration was tested over a wide range at first. A linear range was determined according to this graph. In the range from 0.9 microgram to 6.75 microgram, the calibration could be assumed to be linear. Dividing the area with the sample amount of 20-hydroxyecdysone applied proved the linearity of determination (Table 4). The quantification was controlled by the use of peak addition method. Determining recoveries from pre-analyzed solution, spiked with a known amount of 20-hydroxyecdysone validated the accuracy of the proposed method. Comparison of the results of the samples and spiked samples yielded an accuracy of 94%-99% (Table 5). Stability of the ecdysteroid spots was checked during the developments. Two-dimensional thin-layer chromatography was performed using the same mobile phase (Solvent system D, in both dimensions) in the consecutive developments. As every spot was located on the major diagonal of the chromatogram, no degradation happened through the developments.

To trace the seasonal dependence of 20-hydroxyecdysone in *Silene otites* indicated large difference, such 0.7% in May compared to 0.22% and 0.13% in July and in June, respectively [3]. Both the accuracy and reliability of the method was adequate for this reason.

4. DETECTION OF ECDYSTEROIDS

Several methods have been known for detection and identification of ecdysteroids. The analysis of ecdysteroids was important to

- determine the known ecdysteroid profile of plants which was used as raw materials,
- scout plant sources for new, hitherto unknown ecdysteroids,
- scout new plant sources for ecdysteroid production
- standardize the health improvement preparations.

Ecdysteroid analysis could be started using biological type of determinations, such as RIA and other bioassays [3,23,27,28]. The sensitivity of RIA for 20-hydroxyecdysone was in the range of 10^{-10} through 10^{-12} g. Cross reactions with non-ecdysteroids had to be taken into consideration. The sensitivity of various bioassays covered a range between 10^{-7} through 10^{-9} g. These methods were able to indicate ecdysone-like biological activity, however RIA

and/or bioassay was not capable either specification of the individual ecdysteroid compound, or even verification the occurrence of any ecdysteroid there. It was the reason, that RIA and some other bioassays were widely used to detect the biological activity like to that of 20-hydroxyecdysone, and the positive result was generally followed by the use of either HPLC or TLC or spectroscopic methods [18-19,23].

High-performance liquid chromatography combined with UV detection has been a widely used analytical (separation + detection) method for ecdysteroids [18-19, 29]. The sole clean-up method was generally SPE (solid-phase extraction) using C18 stationary phase. The popularity of HPLC was rooted on its simplicity, as no derivatization was required, the majority of the sample component(s) could be recovered and reentered to another chromatographic system. HPLC operated using either normal-phase (silica) or reversed-phase column. The broad use of plain silica stationary phase was explained by its excellent separation power for the majority of ecdysteroids. At the same time, the consecutive use of normal- and reversed-phase HPLC highly improved the selectivity of the separation system [18].

Another technique of choice was thin-layer chromatography [25,30]. Virtually, TLC was not so efficient as HPLC. However, the choice of mobile phases for TLC was wider than for HPLC, as strong UV absorbing solvents (such as benzene, toluene, acetone, etc.) could also be used. Special methods of planar chromatography might increase the efficiency, such as two-dimensional developments, displacement TLC, programmed multiple developments, etc. [31-34]. The reliability of TLC separation was also further increased by the use of TLC combined with mass spectrometry [30]. The essential advantages of TLC was its combination with the triple detections [15,17]:

(a) Dark spots on silica F_{254} plates; after spraying the plate with vanillin-sulfuric acid, and observing the plates (without any heating) both

(b) On daylight and also the

(c) Fluorescence under 366 nm.

Following the use of vanillin-sulfuric acid, the ecdysteroid spots gave specific color signals, such as 20-hydroxyecdysone gave turquoise spots, and all ecdysteroids also gave characteristically fluorescent spots under 366 nm (e.g. 20-hydroxyecdysone showed violet fluorescence, while the 22-deoxy ecdysteroids showed orange fluorescence). Positive results by triple detection gave a high probability that the spot represented ecdysteroids.

Two-dimensional TLC separated ecdysteroids from interfering substances, and from each other [31]. Silica (straight-phase) plates performed better separation of certain ecdysteroids pairs, such as 20-hydroxyecdysone and polypodine B, than using C18 (reversed-phase) plates. Identification of ecdysteroids required a combination of both straight-phase- and reversed-phase TLC. Ultimate ecdysteroid identification could be performed using two-dimensional TLC separation on silica, with two different mobile phases, and also reversed-phase TLC on C18.

The analysis of an individual ecdysteroids could be done by the use of displacement thin-layer chromatography, and also by forced-flow of the mobile phase [31-34].

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