# Extraction and Monitoring of Phytoecdysteroids Through HPLC

**Dipankar Ghosh*** and **K.S. Laddha**
Medicinal Natural Product Research Laboratory, Pharmaceutical Division, UICT, Mumbai-400 019, India

## Abstract

**The size of the phytoecdysteroids family is rapidly growing. Recent data shows over 250 ecdysteroid analogs have been identified so far in plants. It is theorized that there are over 1000 possible structures, which might occur in nature, but it is a fact that ecdysteroids usually occur in plants as a complex cocktail of structurally different compounds. Among these compounds, the major component is usually the common ecdysteroid-like 20-hydroxyecdysone. Ecdysteroids are polar steroids, almost sugar-like in their solubility properties. Extraction and purification of ecdysteroids (polyhydroxy steroids) is complicated by their polar nature and poor crystallizing properties. These properties make them difficult to separate from other polar plant constituents. Besides, this plant extract is very often processed by multistep procedures to isolate the major and minor ecdysteroids from the new or existing sources. A simplified scheme consisting of a few extraction steps for the purification of ecdysteroid from plants is in great demand. A quantitative approach through high-performance liquid chromatography has been initiated for developing an easy method for the extraction of ecdysteroids from *Ipomoea hederacea (kaladana)* seeds.**

## Introduction

Ecdysteroids include insect moulting hormones, which regulate insect development, and similar chemical structures widely spread throughout plants to affect various biological functions (1). Because they contain ecdysteroids, plant extracts continue to be a significant source for medicinal preparations used to increase the quality of human life. As a means to perform biological and pharmaceutical experiments, a large amount of a variety of ecdysteroids were consumed by people as health improvement preparations (2), but ecdysteroids are not typically consumed as food (3,4). Because of the health benefits, researchers are extensively involved in finding a fast and reliable isolation procedure for the ecdysteroid that are present in the plants. The level of phytoecdysteroids covers a wide range (from 3.4% down to trace levels) (5,6).

Development of a simple and effective method of extraction for the enrichment and subsequent isolation of ecdysteroids from the raw material would be commercially valuable, and the procedure would be used by Western countries, as their plants are abundant in ecdysteroids (7–9). Several researchers proposed various extraction or separation methods (10–14), but none of them are unique or descriptive for the extraction of phytoecdysteroids; they cannot even be followed by the investigators to carry out a proper extraction for any plant containing ecdysteroids. In general, dried, milled samples are usually extracted with MeOH or EtOH. The crude extracts are defatted by a partition between hexane (or light petroleum) and 80% aqueous MeOH. The extracts are partitioned between *n*-BuOH and water, and ecdysteroids are partitioned into the *n*-BuOH phase. Further purification is achieved by column chromatography.

Even though plants contain fairly high levels of 20-hydroxyecdysone (20E) (ecdysterone), the major ecdysteroid (marker compound), its isolation from raw plant sources presents a unique problem. In addition to the necessity of separating ecdysteroids from phenoloids, chlorophyll, lipids, steroids, pigments materials, triterpenes, and amino acids, 20E must also be separated from other ecdysteroids (15). Plant extracts are often complex mixtures of ecdysteroids, with some in very small concentrations.

The method starts with sample preparation and ends with isolation. Homogenization or sample preparation is a crucial phase of both any enrichment and isolation work and contains three essential steps: (*i*) pulverization (size reductions) followed by extraction with a solvent (simple or multiple process), centrifugation, or filtration; (*ii*) concentration, fractionated precipitation, or solvent–solvent partition (or both); and (*iii*) solid-phase extraction. The ultimate goal of any extraction process (viz. enrichment or isolation work) is to separate a part from the whole (16).

The pulverized seed of *Ipomoea hederacea* L. (also called *Ipomoea nil*) is an indigenous source of ecdysteroid (17), as its 20E content is high enough, when compared with other medicinal plants that have been investigated so far in India (18–21), and its 20E content has been quantitatively estimated by HPLC (22). Moreover, the extract of *Ipomoea hederacea* has been reported in sericulture for raising silkworms (23). This experiment concerns the simplification of the extraction process of ecdysteroids from *Ipomoea hederacea* seeds. The seeds are used as a substitute for jalap (*Convolvulus jalap* Linn and *Ipomoea jalap* Linn) (24). The

* Author to whom correspondence should be addressed: email dipankarghosh2001@yahoo.com.

major constituents of the plant contain glycoresin [a macromolecule that is a resinous glycoside of hydroxy fatty acids ($C_{12}$–$C_{16}$) with oligosaccharide; the so-called pharbitinic acid, the latter's hydroxyl group, are estered with (among others) α-methyl butyric acid, tiglic acid, and valeric acid to the fatty acid remnant] (25). The process of extraction and purification varies depending on the plant material chosen, solvents used, method of extraction, temperature and time, and defatting or cleanup process, all of which follow up the conventional methods of extraction (Figure 1). Therefore, by monitoring the extraction procedure through HPLC (in terms of 20E content), the yield was increased to a certain extent (i.e., a simple method of extraction was developed). Also, several ecdysteroids were isolated from the same ecdysteroid-enriched extract (26).

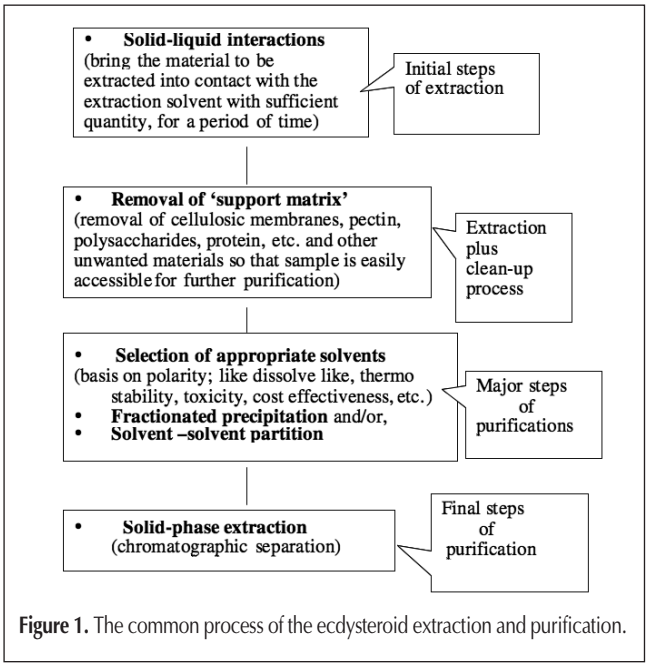


**Figure 1.** The common process of the ecdysteroid extraction and purification.

**Table I. Effect of Time, Temperature, and Method of Extraction**

| Temp. | Solvent (s) | Time (h) | Method of extraction | Total solid content* | % Extractive value* | % ES content*,† |
|---|---|---|---|---|---|---|
| **50–60°C** | Ethyl acetate | 6 h | Soxhlet | 0.589 | 2.95 | 2.46 |
| | | 6 h | Reflux | 0.347 | 1.74 | 1.60 |
| | Methanol | 6 h | Soxhlet | 1.653 | 8.27 | 1.66 |
| | | 6 h | Reflux | 1.577 | 7.89 | 1.33 |
| | Chloroform | 6 h | Soxhlet | 0.715 | 3.58 | 1.18 |
| | | 6 h | Reflux | 0.535 | 2.68 | 0.52 |
| | Acetone | 6 h | Soxhlet | 2.055 | 10.28 | 0.70 |
| | | 6 h | Reflux | 1.895 | 9.48 | 0.92 |
| | Isopropanol | 6 h | Soxhlet | 0.585 | 2.93 | 0.44 |
| | | 6 h | Reflux | 0.237 | 1.19 | 0.12 |
| **50–60°C** | Ethyl acetate | 12 h | Soxhlet | 0.447 | 2.24 | 2.78 |
| | | 12 h | Reflux | 0.385 | 1.93 | 1.57 |
| | Methanol | 12 h | Soxhlet | 1.443 | 7.22 | 2.14 |
| | | 12 h | Reflux | 1.307 | 6.54 | 1.78 |
| | Ethyl acetate | 24 h | Soxhlet | 1.20 | 6.0 | 2.28 |
| | Ethyl acetate | 48 h | Soxhlet | 1.96 | 9.8 | 2.24 |
| **65–70°C** | Ethyl acetate | 18 h | Soxhlet | 0.60 | 3.02 | 3.19 |
| | Methanol | 18 h | Soxhlet | 1.95 | 9.75 | 1.75 |
| | Ethyl acetate–methanol (1:1) | 18 h | Soxhlet | 2.23 | 11.15 | 1.16 |
| **> 85°C** | Ethyl acetate | 3 h | Soxhlet | 0.41 | 2.03 | 2.48 |

* *n* = average of two.
† g/100 g dry weight basis.

## Experimental

*Ipomoea hederacea* (local name: *kaladana*) seeds are collected from Mumbai local market (India) and authenticated. For future reference, the voucher specimen was deposited in Blatter Herbarium (specimen no E. Blatter 22426, St. Xavier's College, Mumbai, India). For all extractions, distilled solvents were used; for analysis, analytical reagent and HPLC-grade solvents were used.

A Messrs Jasco HPLC system (Tokyo, Japan) with a Rheodyne 7725 loop injector (Rohnert Park, CA), Jasco PU-1580 intelligent pump, Jasco UV-1575 intelligent detector, Jasco MX 2080-31 solvent mixing module, and Borwin software version 1.50 (Jasco) were used. A Lichrospher 100RP-18e (250- × 4.6-mm, 5 µm) column (Merck, Darmstadt, Germany) with a mobile phase of acetonitrile–water (25:75) (0.01% trifluoroacetic acid) and with a flow rate of 1 mL/min was used, followed by isocratic elution at a wavelength of 246 nm. The peak was assigned by spiking with an authentic sample. The concentration was determined from a calibration curve conformed with Lambert–Beer's Law for quantitation.

### Extraction

A specific quantity (20 g) of *Ipomoea hederacea* powder (#36–60) was extracted with different solvents such as methanol, chloroform, acetone, ethyl acetate, isopropanol, etc., in a specific quantity (120 mL) for Soxhlet and reflux extraction at different temperatures and times to determine the total solid content and ecdysteroid (mainly 20E) concentration in the particular extract.

## Results and Discussion

The seeds are coated with a cellulosic layer, mainly polysaccharides. Partial or complete destruction of the polymeric network of the cell wall is necessary to make the extraction process possible. Enzymatic action, as well as mechanical

and thermal treatment, damages the cell wall, favoring the permeability of the solvent to penetrate the cytoplasmic membranes. Therefore, the non-specific, chemical destruction of this matrix during solvent extraction is considered one of the most reliable and efficient methods of extraction.

For a preliminary investigation, solvents were selected based on the principle of "like dissolves like", that is to say, a nonpolar solvent will extract out a nonpolar substance and polar solvent will extract a polar material. These may be truly applicable when extraction is carried out at ambient temperature (room temperature), but the results were found to be different when a similar extraction was carried out in warm temperatures (50–85°C) conditions. Solvent efficacy is distinguishable when extraction is performed in cold conditions along with the solvent, which depends on the affinity between the solvent and substrate molecules. by the solvent through their extractable capability of the chemical analytes from the mass (substances), and evaluated qualitatively or quantitatively.

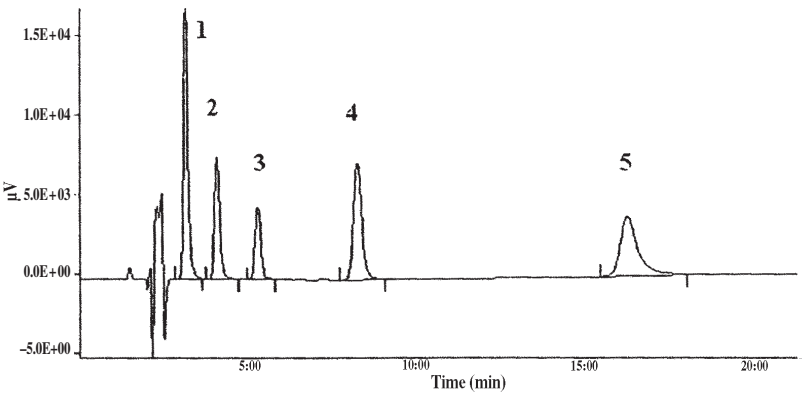


**Figure 2.** Ecdysteroids standards; 20E (1), makisterone-A (2), ecdysone (3), muristerone (4), and kaladasterone (5). Mobile phase: acetonitrile–water (0.01% TFA) (25:75).

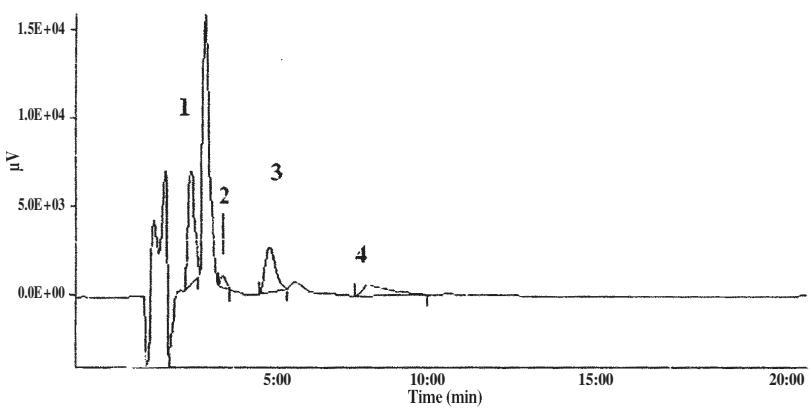


**Figure 3.** Ethyl acetate extract enriched with ecdysteroids after modification.

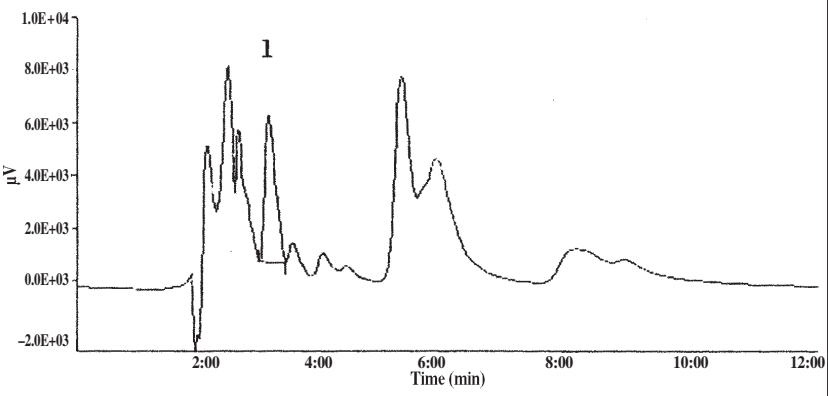


**Figure 4.** Methanolic extract containing major 20E without any modification. Ecdysterone 3.108 (RT) (i.e., 20E) (1).

Manipulation of a method of extraction like Soxhlet or reflux by using different solvent(s), times, and temperatures also intensifies the extraction process, to a certain extent (Table I). The length of extraction (Soxhlation for 18 h) with a solvent (like ethyl acetate) at temperatures between 65°C and 70°C has shown higher values (more quantity of 20E) when compared with methanol and a combination of two solvents [ethyl acetate–methanol (1:1)].

Ethyl acetate has appreciable polarity because both π and lone-pair electrons are present on O. Thus, chemical alteration of the polymeric chain of the cell by any means (heating, enzymatic pretreatment, sonication, etc.) enhances the penetration or leaching effect of the solvent. The resulting effect is the presence of more polar ecdysteroids in ethyl acetate solvent.

Factors like increasing the temperature, length of extraction process, and method of extraction showed a better impact on the extraction of ecdysteroid from the *kaladana* seeds, which rendered the methodology easier for ecdysteroids enrichment (Figures 2–4).

Finally, the extraction process (Figure 5) of the *kaladana* seeds was optimized and found to better yield 20E (ethyl acetate extract contain 26.55% 20E), whereas the plant itself contains only 0.5% 20E on a dry-weight basis (22) (Figure 6). Even the solute-to-solvent ratio (minimum 5-fold) is also an important factor, certainly at the increased temperature of 70–80°C, and a sufficient quantity of solvent (more than 5-fold) released the analytes from the same substrate at a certain time interval. Therefore, the total solid contents (TSC) were varied at different phases of extraction, and the content of ecdsteroids was reciprocal to the TSC (Table II) (i.e., it increased or decreased incrementally to the amount of TSC).

Partitioning with *n*-hexane is the correct approach, as opposed to charcolization or adsorption on silica gel. Performing the extraction with different sized particles of the same seed material showed their content variations. Powder materials (coarse or moderate or fine) retained on #60 (fine particle) had shown a maximum yield (0.8% 20E) compared with #36–60 (0.5% 20E) and #10–36 (0.19% 20E). Even after 1 year, the ethyl acetate extract of *kaladana* showed a similar chromato-

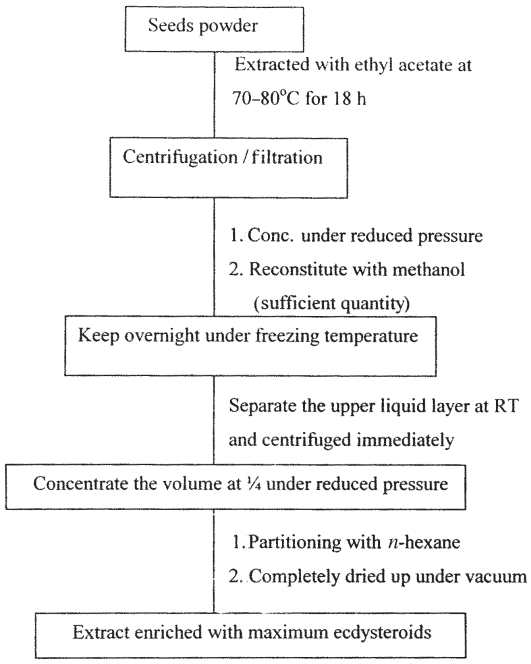


**Figure 5.** Flow chart of ecdysteroids extraction from *kaladana* seeds (modified method).

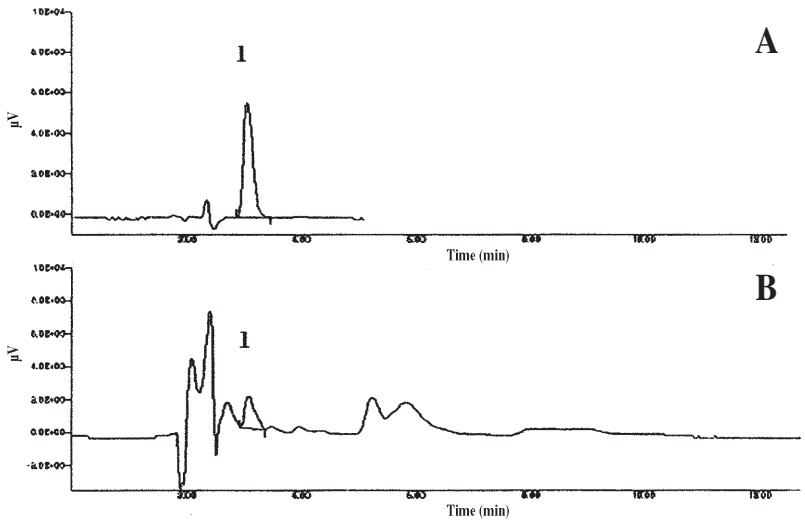


**Figure 6.** Standard compound of 20E (A) and 20E quantitated in *Ipomoea hederacea* extract (B). Ecdysterone 3.117 (RT) (i.e., 20E) (1).

graphic profile, which indicates that the ethyl acetate extract of *kaladana* is quite stable.

## Conclusion

It appears that ethyl acetate is not a very common solvent for the extraction of ecdysteroids, but it has the advantage of avoiding the extraction of unwanted material, specifically in the case of *kaladana*. The yield of 20E in ethyl acetate has been improved because of the length of the extraction at a particular temperature. The concentrated ethyl acetate extract, further fractionated with *n*-hexane, improved the content in the final extract.

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**Table II. Time, Temperature, and Solid-to-Solvent Ratio Effect in the Extraction Process of *kaladana***

| Temp. | Solvent | Time interval (every 6 h) | Total solid content* Before modification (g) | Extractive value (%) | After modification (g) | Extractive value (%) | % ES (HPLC) [after modification]*,† |
|---|---|---|---|---|---|---|---|
| **70–80°C** | Ethyl acetate | After 6 h | 0.761 | 3.81 | 0.238 | 1.19 | 5.87 |
| | | After 12 h | 0.530 | 2.65 | 0.237 | 1.18 | 6.57 |
| | | After 18 h | 0.182 | 0.91 | 0.099 | 0.49 | 7.58 |

* *n* = average of two.
† g/100 g dry weight basis.

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