# Triterpenoids From Swallow Roots—A Convenient HPLC Method for Separation

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## Abstract

**A convenient semi-preparative high-performance liquid chromatography (HPLC) method for separating a mixture of triterpenoids (α-amyrin, β-amyrin, and lupeol) and their corresponding acetates from the swallow roots (*Decalepis hamiltonii* Wight and Arn), which are known to have potential bioactive properties, is described. The swallow roots are found to be one of the richest natural sources for these compounds. The hexane extract of the dried spent root on column chromatography yields mixtures (i.e., triterpenoids and their acetates) containing at least three compounds in each. These could not be further separated using the routine chromatographic techniques, such as classical column chromatography and preparative thin-layer chromatography using various solvent systems. Therefore, the optimal conditions are determined on reversed-phase HPLC for their separation and are characterized using spectral data, particularly by nuclear magnetic resonance with physical and chemical properties.**

## Introduction

*Decalepis hamiltonii* Wight and Arn, is a monotypic genus belonging to the asclepiadaceae family and is found mostly in the forest areas of south India. Its root has a strong aromatic odor and is used in traditional Indian medicine as an appetite stimulant, blood purifier, and for the treatment of various physiological disorders. The sliced root is pickled as such or along with lime fruit where it acts as an aromatic food preservative (1). In a systematic chemical examination, the spent root [i.e., after the removal of the volatiles (2)] was successively extracted with solvents of different polarities, and the root was subjected to column chromatography for the separation of pure compounds. The hexane extract on column chromatography produced two solids, which were thought to be single constituents, but spectral studies indicated that each of these contain three triterpenoidal components. These compounds could not be further separated through routine chromatographic techniques, such as column or preparative thin-layer chromatography (TLC) with various analytical conditions. Therefore, these were separated by semi-preparative high-performance liquid chromatography (HPLC) and their structures identified by chemical and spectral studies.

HPLC contributes significantly for the separation of a mixture of compounds, which includes steroids and triterpenoids. However, the preparative gas–liquid chromatography method was reported (3) for triterpene methyl ethers (β-amyrin methyl ether and α-amyrin methyl ether using a 1% Apiezon L column). It has limitations in terms of the separation of large quantities.

The HPLC technique had been efficiently used for the separation of the triterpenoids, which had the advantage over the LH-20 columns (lipophilic gravity sephadex columns). The previous report describes that in reverse-phase (RP) HPLC, the sterols had been separated with a baseline resolution (4). Also, the separation of derivatives of lanostanoids had been reported with the gradient system containing methanol, water, and acetic acid (5,6). However, the use of a gradient system and acids is a constraint either for the preparative HPLC or for large-scale separations. Therefore, attempts were made for the separation of these components by HPLC using different solvents and temperatures on an RP column. The analytical conditions are optimized with an isocratic system devoid of acid, which resulted in good resolution, and the separation of compounds was performed on a semi-preparative scale to achieve the samples with purity greater than 95%.

The compounds (viz., lupeol, α-amyrin, β-amyrin, and lupeol acetate) were reported to possess a wide range of inhibiting activity against gram-positive and gram-negative bacteria (7). In addition, the compounds have many bio-active properties, such as the cytotoxic effect of lupeol against a human tumor cell as an indicator of the potential anticancer activity (8,9), and the methanol extract containing lupeol showed significant antibacterial and antifungal activity compared with standard antibiotics (10), and the potential polymerase β-lyase activity of the compounds lupeol, lupeol acetate, and α-amyrin acetate (11), were reported. The administration of pentacyclic triterpene lupeol and its structural analogue, betulin, in hyperoxaluric rats minimized the tubular damage and reduced the markers of crystal deposition in the kidneys.

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Also, lupeol was found to be more effective than betulin (12). Furthermore, the reports indicate that α- and β-amyrins suppress the scratching behavior in a mouse model of pruritus (13). The antibacterial activity against the *Staphylococcus aureus* was reported for the extract containing α-amyrin from leaves of *Chromolaena moritziana* (14). Thus, the significance of the isolated triterpenoids and their acetates is well documented.

The present study describes the separation of these bioactive analogous triterpenoids using RP-HPLC, and their identification was made using their $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra, as well as chemical and physical properties.

## Experimental

### Chemicals and materials

The solvents used for extraction were of low-resistivity grade and distilled before use. Silica gel (60–120 mesh size) was obtained from BDH (Mumbai, India). Silica Gel-G (particle size 10–40 µm) and HPLC solvents were procured from Merck (Mumbai, India).

Melting points for the isolated constituents were determined with a PEW (0.75 KW) apparatus (Pathak Electrical Works, Mumbai, Maharashtra, India). Optical rotations were measured in a $CHCl_3$ solution in a 0.5-mL cell with a Perkin Elmer-243 digital polarimeter (Perkin Elmer Co., Rodgua-Jugesheim, India).

$^{1}H$ and $^{13}C$ NMR were recorded at 400 MHz and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (Bruker, Rheinstein, Germany). $^{13}C$ NMR chemical shifts for the isolated constituents were assigned on the basis of a spin-echo Fourier transform (SEFT) spectra.

The spent [i.e., after distillation of the volatile compounds by steam distillation (2)] dry material of *D. hamiltonii* fleshy root was powdered (700 g) and extracted with petroleum ether 60–80 (2.5 L), followed by methanol and 50% aqueous methanol (Figure 1).

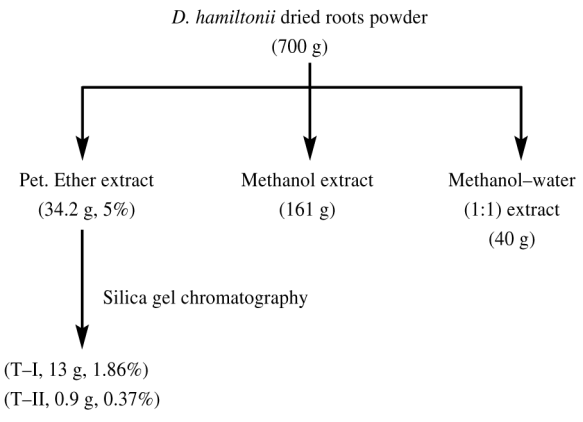


**Figure 1.** Separation of triterpenoids from swallow roots.

The petroleum ether extract (34.2 g) was impregnated with 70 g of silica gel and loaded onto a column of silica gel (300 g) prepared in hexane. The elution started initially with hexane, and it was followed by hexane–ethyl acetate mixtures with a gradual increase in the polarity of the eluting solvent (each fraction 250 mL).

The initial fractions yielded many minor nonvolatile compounds. An elute, using 5% ethyl acetate in hexane, produced a sticky white solid (1.86%, 13 g, designated as T-I) on the solvent removal and was dried in a vacuum desiccator over $P_2O_5$ and paraffin wax. Similarly, the continuation of the elution with the same solvent system produced a white sticky solid (0.37%, 900 mg), which was crystallized in hexane (designated as T-II).

The isolated compounds were a pink color on the Liebermann-Burchardt test (15,16). The solids (T-I and T-II) were found to be mixtures by the study of NMR spectral data, although these were homogenous on TLC.

### Chromatography of the triterpenoid mixtures (T-I and T-II)

Attempts were made to separate these mixtures by column chromatography and preparative TLC using hexane–ethyl acetate, benzene–ethyl acetate, benzene–methanol, and chloroform–methanol mixtures in various proportions, but they could not achieve proper separation.

### HPLC method development for the separation of triterpenoid mixtures (T-I and T-II).

Analytical and semi-preparative HPLC analysis was carried out on a Shimadzu LC (LC 10A, Koyoto, Japan), controlled by a system controller (CBM 10A with PC; CLASS LC10), equipped with a photodiode array detector (PDA) (SPD 10AVP). The C18 column (Shimadzu, CLC, ODS, particle size, 5 µL, 250 × 4.6 mm, i.d.) was interfaced with a guard column packed with Partisphere. Both the columns were operated at 40°C. The detector wavelength was fixed at 205 nm for analysis, and sample peaks were simultaneously monitored by a PDA detector (200–400 nm). The mobile phase was methanol and water (94:6). The flow rate was maintained at 1 mL/min at a pressure range of 50–350 bars. Samples T-I and T-II were analyzed by HPLC (Figures 2–5).

The samples of T-I and T-II (300 mg of each) were dissolved

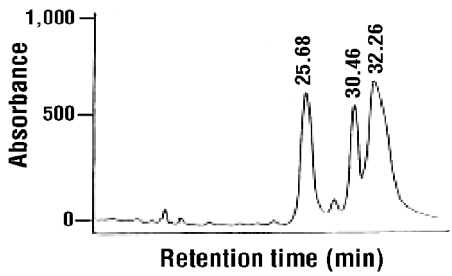


**Figure 2.** HPLC chromatogram of T-I. HPLC analysis with C18 column, 40°C, UV detector at 205 nm. The mobile phase used was methanol–water 94:6, flow rate 1 mL/min.

in 1.5 mL of chloroform. Each time, 20 µL of the solution was loaded onto the column, and the separated constituents were collected in different flasks at respective retention times. The UV spectrum of each eluting peak was fully characterized by continuously monitoring the absorbance between 200–400 nm by a PDA detector. The separations were carried out to produce compounds A, B, and C from T-I and D, E, and F from T-II (at least 40–50 mg of each) (see Tables I and II). The separated compounds A to F were checked for purity by HPLC (Figures 2–5). The retention times, melting points, and percentages of each compound in the mixture are presented in Tables I and II (17).

### Acetylation of T-II

The acetylation of the T-II was carried out according to the reported procedure (18,19). A sample (10 mg) was dissolved in pyridine (0.5 mL), and acetic anhydride (0.5 mL) was added to it. The mixture was stored at room temperature (12 h) for acetylation. It was washed with ice-cold water (5 mL) and extracted with chloroform (5 mL). The chloroform extract was washed with 1N hydrochloric acid and water. The organic layer was passed through anhydrous sodium sulphate, and the solvent was removed to get the acetylated mixture (12 mg). The retention factor ($R_f$) values of the T-II and the acetylated com-

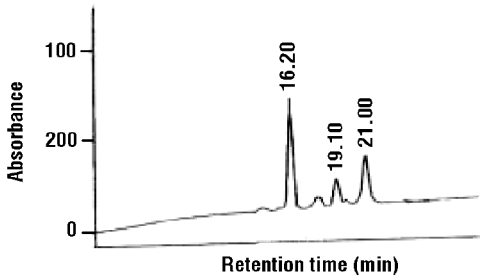


**Figure 4.** HPLC chromatogram of T-II. HPLC analysis with C18 column, 40°C, UV detector at 205 nm. The mobile phase used was methanol–water 94:6, flow rate 1 mL/min.

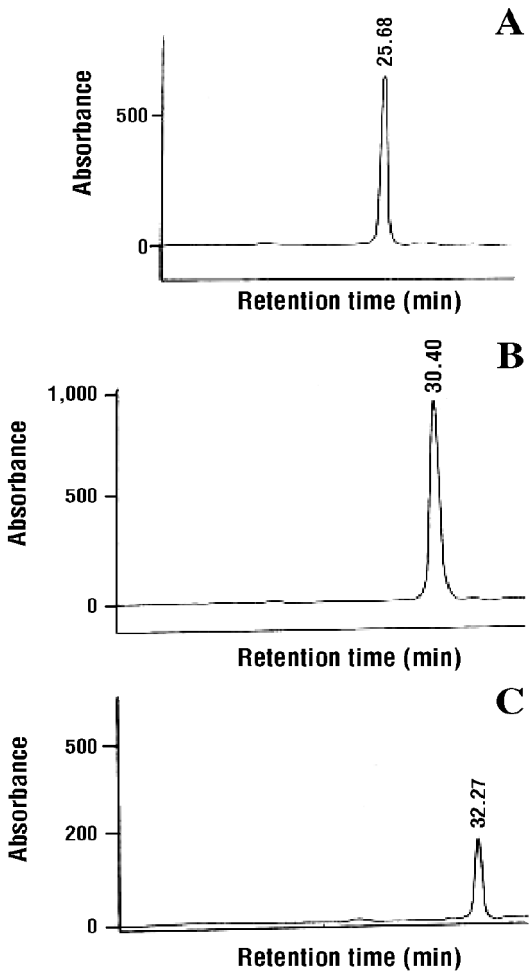


**Figure 3.** HPLC chromatograms A, B, and C from T-I. HPLC analysis with C18 column, 40°C, UV detector at 205 nm. The mobile phase used was methanol–water 94.6, flow rate 1 mL/min. Chromatograms show lupeol acetate (A), β-amyrin acetate (B), and α-amyrin acetate (C).

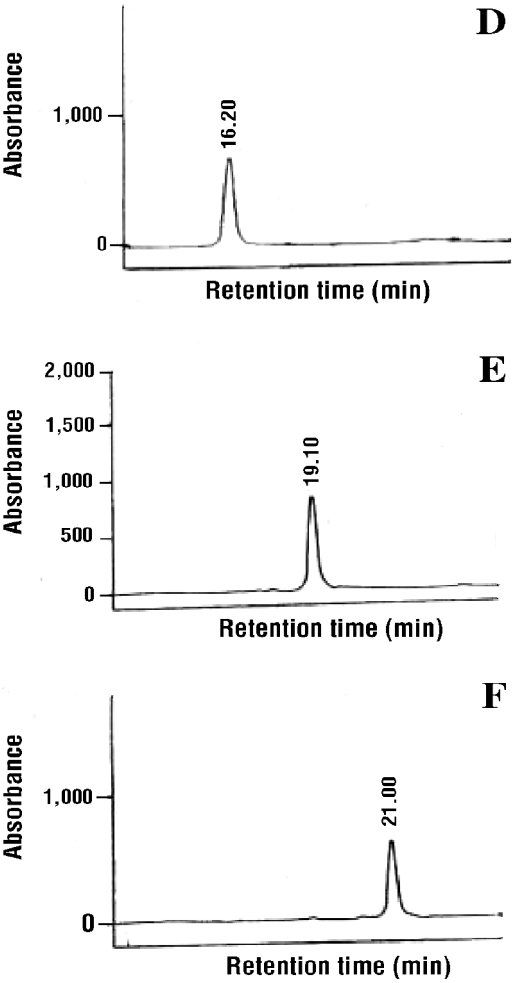


**Figure 5.** HPLC chromatograms D, E, and F from T-II. HPLC analysis with C18 column, 40°C, UV detector at 205 nm. The mobile phase used was methanol–water 94.6, flow rate 1 mL/min. Chromatograms show lupeol (D), β-amyrin (E), and α-amyrin (F).

pound on TLC were 0.23 and 0.79, respectively, with the solvent system hexane–ethyl acetate (95:5). The product was analyzed by HPLC under the previous analytical conditions.

## Results and Discussion

### Method development

The importance of the chromatographic method described is significant because the compounds isolated were reported to possess biological activity, which rendered these compounds potentially beneficial for respective applications, as indicated from the recent reports (7–14). Also, it was significant that these bioactive compounds were isolated in their pure form from the swallow roots, which is an edible material and contains reasonable quantities. The petroleum ether extract was obtained in a 5% yield from the dried roots. The column chromatography of this extract, on elution with 5% ethyl acetate in hexane, afforded the major compound T-I (38% of petroleum ether extract), followed by T-II (2.6% of petroleum ether extract). Both T-I and T-II produced positive results on the Liebermann–Burchardt test, indicating these to be triterpenoids. The $^{1}H$ NMR spectra of T-I and T-II illustrated several resonances, and also the $^{13}C$ NMR spectra showed at least more than 90 carbon resonances in each spectrum, indicating each may contain three constituents. These were subjected to further separation using column chromatography and TLC, and the constituents could not be separated. Other T-I and T-II appeared homogeneous on TLC. This could be because of the close resemblance in their structures and because they were analogous compounds. Therefore, it was decided to use HPLC for the separation.

It was reported that compounds, such as triterpenoids, which have a low solubility in methanol and water, have high melting points and produce a broad late peak in RP-HPLC. The chromatographic rules applicable for the sterols may not operate for triterpenoids, and it was also mentioned that the C18 column coupled to a multiple wavelength diode array detector gave better resolution than even the GLC-packed columns coupled to mass spectrometry (4). When attempted at ambient temperature, these mixtures (T-I and T-II) produced a hump-like peak in the chromatogram without any resolution. Hence, the analysis was carried out at various temperatures. A significant and clear resolution with a precise baseline separation was achieved by keeping the column at 40°C under isocratic conditions. These optimized conditions were useful for preparative HPLC, as well as large-scale separations of similar compounds.

### HPLC separation of T-I

The HPLC chromatogram of T-I (Figures 2 and 3) showed three major peaks with retention times ($t_R$) of 25.68, 30.46, and 32.26 min, and each peak corresponded to a single component. These three components were separated on a semi-preparative scale and designated as Figures 3A–3C.

**Table I. HPLC Analysis and Components of T-I**

| T-I | $t_R$* (min) | % in T-I | Melting point (°C) | Identified compound |
|---|---|---|---|---|
| A | 25.68 | 26.19 | 250–51 | Lupeol acetate |
| B | 30.40 | 17.80 | 237–38 | β-Amyrin acetate |
| C | 32.27 | 50.90 | 224–25 | α-Amyrin acetate |

*$t_R$ = Retention time.

**Table II. HPLC Analysis and Components of T-II**

| T-I | $t_R$* (min) | % in T-I | Melting point (°C) | Identified compound |
|---|---|---|---|---|
| D | 16.20 | 47.50 | 213–215 | Lupeol |
| E | 19.10 | 13.22 | 196–197 | β-Amyrin |
| F | 21.00 | 26.38 | 185–186 | α-Amyrin |

*$t_R$ = Retention time.

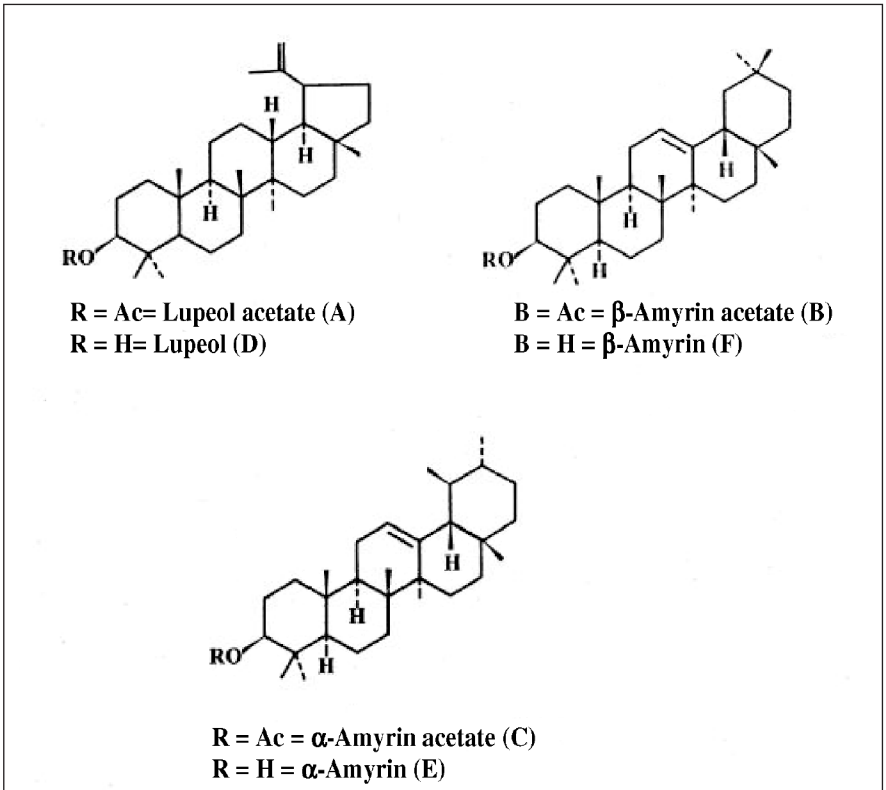


**Figure 6.** Triterpenoids from the roots of *Decalepis hamiltonii*.

**HPLC separation of T-II**

The HPLC chromatogram of T-II (Figures 4 and 5) in analytical mode showed three major peaks, with $t_R$ of 16.2, 19.1, and 21.0 min. These three compounds were separated on the semi-preparative scale, and the purity of the isolated constituents was checked by HPLC, with each showing a single peak labeled as D, E, and F (Figure 5). The retention times and melting points of the isolated compounds A to F are given in Tables I and II. Compounds A to F showed positive results on the Liebermann–Burchardt test, indicating these to be triterpenoids.

**Identification of compounds A to F**

The $^{13}C$ (20) and proton NMR spectrum of compounds A to F indicated that these compounds were triterpenoids, and they were identified as lupeol acetate (A) (21, 22), β-amyrin acetate (B) (23), α-amyrin acetate (C) (25), lupeol (D) (24), β-amyrin (E) (23), and α-amyrin (F) (25). Other $^{13}C$ NMR data were completely identical to the reported values (20), and its SEFT spectrum confirmed the same results. Thus, from the $^{13}C$, $^{1}H$ NMR, and other physicochemical analyses, the compounds were identified (Figure 6).

The elution in the RP-HPLC was found to be in the order of more polar compounds to less polar (i.e., lupeol, β-amyrin, α-amyrin, lupeol acetate, β-amyrin acetate, and α-amyrin acetate) with $t_R$ of 16.20, 19.10, 21.0, 25.68, 30.40, and 32.27, respectively. For example, the lupeol was eluted first, possibly because of the presence of an exocyclic methylene group with the five-membered ring E, and the polarity was stabilized by the cyclic carbonium ion at C18, C19, and C20 (25). Similarly, β-amyrin had the gem-dimethyl (at C20) in the six-membered ring E, whereas in α-amyrin, it was at C19 and C20, respectively. It was also reported that the double bond was more resistant than that in β-amyrin, causing α-amyrin to be unaffected by perbenzoic acid. (26).

The NMR ($^{13}C$ and $^{1}H$) and other spectral data of the isolated compounds (A to F) confirmed these as lupeol, α-amyrin, and β-amyrin (from T-II), as well as their corresponding acetates (from T-I) (17). The acetylation of T-II (7) provided T-I (i.e., the acetates of lupeol, α-amyrin, and β-amyrin). This was confirmed by using TLC with T-I components and also by comparing their $t_R$ using the HPLC analysis.

## Conclusion

The chemical investigation on the spent root of *D. hamiltonii* was carried out by extracting with solvents of different polarities. The hexane extract on column chromatography yielded the two mixtures (i.e., triterpenoids and their acetates). These compounds were obtained as mixtures and could not be separated by either column chromatography or by preparative TLC. These triterpenoids and their acetates, being closely related analogous compounds, were separated using HPLC on a semi-preparative scale. Under optimized conditions, the analysis gave a clear resolution among the triterpenoids lupeol, α-amyrin, β-amyrin, and their corresponding acetates, which have potential bioactive properties. The structures established by the spectral studies, $^{13}C$ NMR spectral data, in particular, were used. SEFT spectra confirmed these assignments. In addition, the conditions employed in the HPLC separation of triterpenoids in the present study (as such or with minor modifications) may be useful in separating other triterpenoids from different sources. Because of the growing importance of the triterpenoids as a result of their biological activities, the separation of individual compounds as described here is of the utmost importance to obtain pure components to carry out structure–activity relationship studies.

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