

Pharmacognostic Studies and HPLC Analysis of Roots of *Helicteres isora* (L.)

Pinal A. Harde^{1*} and Mamta B. Shah²

ABSTRACT

Background: The juice of roots of *Helicteres isora* Linn. has been widely used as an anti-diabetic in traditional medicine. **Objective:** The present study deals with pharmacognostical studies and determination of oleanolic acid from the roots of *H. isora* by new HPLC method. **Materials and methods:** Detailed study of morphological, microscopical characteristics, physicochemical parameters and phytochemical screening of roots were carried out. The sapogenins were isolated from the roots of *H. isora*. RP-HPLC method was developed and validated for estimation of oleanolic acid from the sapogenins of roots of *H. isora*. **Results:** Detailed quality control parameters of roots of *H. isora* were reported. Total content of oleanolic acid was 0.075%w/w from roots of *H. isora* determined by HPLC. **Conclusion:** The present study is useful for accurate identification and authentication of roots of *H. isora*. The HPLC method for determination of oleanolic acid from the roots of *H. isora* is efficient, precise, reliable and sensitive and can be adopted for routine analysis.

Key words: HPLC, Oleanolic Acid, Sapogenins, *Helicteres isora*.

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INTRODUCTION

Helicteres isora Linn. (Family: Streuliaceae) is a tall shrub or a small tree commonly known as 'Marophali' in Hindi and the 'East Indian Screw Tree' in English. It is found throughout Central and Western India, Ceylon and China.¹⁻³ The capsule has long been employed in intestinal complaints, colic, flatulence and diarrhea in India. The juice of roots is used in diabetes, emphysema, stomach affections, scabies and snakebite. Roots are also used as expectorant, hepatoprotective, astringent to the bowels and anti galactagogue.¹⁻⁶ Triterpenoid glycosides namely, cucurbitacin B, isocucurbitacin B, oleanolic acid, daucosterol, isorin, 3 β 27diacetyloxylup20(29)en-28-oic methyl ester catechol, gallic acid and betulinic acid have been reported in roots.⁷⁻⁹ The triterpenoid saponins rich butanol extract of roots is shown to possess antihyperglycemic and antilipidemic activities in experimental animals.¹⁰⁻¹³ Oleanolic acid is stated to demonstrate antidiabetic, antilipidemic, cytotoxic, anti-ulcer, anti-inflammatory and antiprotozoal activities in animals.¹⁴⁻¹⁷ Thus, oleanolic acid (Figure 1) would be useful as a biological marker for qualitative assessment of roots of *H. isora*.¹⁸ Literature survey revealed that preliminary pharmacognostical studies and HPTLC method was developed and validated for analysis of oleanolic acid from the roots of *H. isora*.^{19,20} Further there is lack of literature with regard to detailed standardization of roots of *H. isora*. Thus the present study was steered towards pharmacognostic evaluation, and development of rapid and reproducible high-performance liquid chromatographic

(HPLC) method using oleanolic acid as a marker for qualitative assessment of roots of *H. isora*.

MATERIALS AND METHODS

Plant material

The fresh roots of *Helicteres isora* Linn. along with a twig were procured from Dhrampur, Gujarat, India in June-July. Authentication was done by Dr. Minoo Parabha, Ex. Botanist and Head of Bioscience Department, The Veer Narmad South Gujarat University, Surat, India (Voucher specimen no: PAH/23082007/02). The roots were cut and dried properly, powdered to 60-mesh. The powdered sample was stored in an air-tight container at room temperature (28±2°C). Pharmacognostic evaluation including microscopy was carried out by taking free hand sectioning followed by staining with phloroglucinol and hydrochloric acid. Powder characteristics were also studied and photomicrographs were obtained by Magnus MLX-DX photomicroscope provided with Honestech software. Physicochemical constants like ash value, extractive value and phytochemical screening of roots were carried out.¹⁸⁻²¹

HPLC analysis

Chemicals and Standard

Petroleum ether (60-80°C), ethyl acetate, *n*-butanol, methanol, diethyl ether (E. Merck, Mumbai, India) were of analytical grade. The solvents used for HPLC analysis, like methanol, orthophosphoric acid, water

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(Merck, Mumbai, India) were of HPLC grade. Standard oleanolic acid (purity 97% w/w) was purchased from Sigma-Aldrich Chemicals (Germany).

Preparation of standard solution

Standard stock solution of oleanolic acid (0.2 mg/ml) was prepared by dissolving an accurately weighed 10 mg of pure powder of standard oleanolic acid in 50 ml volumetric flask containing methanol and volume was adjusted with methanol up to the mark. Further 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ml of stock solution of oleanolic acid was transferred to 10 ml volumetric flask separately and volume was adjusted with methanol up to the mark to produce working standard solutions of 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 µg/ml.

Isolation of saponins and sapogenins and preparation of sample solution

An accurately weighed (10 g) root powder of *H. isora* was extracted successively with petroleum ether (60-80°C) (3X50 ml), ethyl acetate (3X50 ml) and *n*-butanol (3X50 ml) by hot maceration. The *n*-butanol extract was concentrated under vacuum to get semisolid residue that dissolved in 10 ml of methanol and excess diethyl ether was added to yield bulky and sticky precipitates of saponins. The saponins were subjected to acid hydrolysis using 2 M HCl under reflux for 4 h. The hydrosylate was extracted ethyl acetate (3X10 ml) and the pooled fraction on concentrating under reduced pressure yielded sapogenins. The later were dissolved in 8 ml methanol in a 10 ml volumetric flask and the volume was adjusted up to the mark with methanol. From this 1 ml of sample solution was transferred to 10 ml volumetric flask and the volume was adjusted up to the mark with methanol. The afforded solution was filtered through a 0.45 µm syringe filter prior to application to HPLC.

Chromatography

A Shimadzu model of HPLC equipped with quaternary LC-2010 AHT VP pumps with variable wavelength programmable UV/VIS detector, SPD-10AVP column oven and Class-VP software were used. The chromatographic separations were performed using Phenomenex C₁₈ (250 mm x 4.6 mm, 5 µm particle size) column at 40 °C temperature. The optimized mobile phase was found to be methanol-0.1% aqueous orthophosphoric acid 90:10 (%v/v). Auto sampler with injection volume 20 µl was used and sample tray was kept at 15 °C temperature. Analysis was done with flow rate of 0.6 ml/min at 210 nm wavelength using UV detector.

HPLC Method Development and Validation

The proposed RP-HPLC method was validated as per ICH guidelines.²²

Calibration Curve

Graded concentrations of standard oleanolic acid solution 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 µg/ml of 20 µl volume were injected serially by rheodyne injector in triplicates. Immediately, it was scanned at λ_{max} 210 nm by UV/visible detector. Data of peak area was recorded for each injection of standard solution. Standard calibration curve of mean peak area versus concentration of standard oleanolic acid was plotted. The calibration curve was analyzed through linear regression.

Precision (Repeatability)

The precision of the method was checked by repeatedly injecting (*n* = 6) standard solutions of oleanolic acid (6.0 µg/ml). The results were reported in terms of % RSD.

Intermediate precision (Reproducibility)

Intermediate precision was evaluated in terms of intraday and interday precision. The intraday and interday precision of the proposed methods were determined by estimating the corresponding responses three times

on the same day and on three consecutive days for three different concentrations (4.0, 6.0 and 8.0 µg/ml) of working standard solutions of oleanolic acid. The deviations in results were reported in terms of % RSD.

Accuracy (Recovery)

The accuracy of the method was determined by calculating recoveries of oleanolic acid by the standard addition method. Known amounts of standard solutions of oleanolic acid (50, 100 and 150%) were added to pre analyzed sample solution. Here, known amounts of standard solutions of oleanolic acid (1.0, 2.0 and 3.0 µg/ml) were spiked to prequantified sample solutions of roots of *H. isora* and analyzed by proposed method. The amount of oleanolic acid was estimated by applying these values to the regression equation of the calibration curve.

Limit of detection and Limit of quantification

The limit of detection (LOD) was determined by visually evaluating the minimum level at which the oleanolic acid could be reliably detected. The limit of quantification (LOQ) was determined by analyzing the known concentration of oleanolic acid at which it could be quantified with acceptable accuracy and precision.

Robustness

Robustness study of the HPLC method was determined by applying small but deliberate changes in the flow rate, ratio of mobile phase, detection wavelength and column temperature and response of 6.0 µg/ml concentration of oleanolic acid was measured in terms of peak area and retention time. The results were expressed in terms of % RSD.

Quantification of oleanolic acid from roots of *H. isora*

The solutions of test sample was injected in triplicates and chromatograms were obtained under the same conditions as for analysis of standard oleanolic acid. The area of the peak corresponding to the retention time (RT) of standard oleanolic acid was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

RESULTS AND DISCUSSION

Macroscopical characters

Exomorphology

A shrub or small tree; young shoots clothed with stellate hairs. Leaves are bifarious, 7.5-12.5 X 5-10 cm oblong, obovate or roundish, cordate, suddenly and shortly acuminate, closely dotted on both surface with stellate hairs, more or less irregularly crenate-serrate; petioles 6-9 mm long; stipules subulate, 6 mm long. Flower is 2.5-3.8 cm long, distinctly bilabiate, in axillary clusters of 2-6 together; pedicels very short, stellately tomentose; bracts small, subulate, hairy. Calyx is tubular, 2 cm long, somewhat 2-lipped stellately pubescent, curved, laterally compressed, mouth wide; teeth triangular, unequal, Corolla is red at first, fading to lead colour, very unequal closely reflexed on the calyx. Separate but with the claws closely hooked together. Androecium is staminal column fused with gynophores, much exerted, suddenly deflexed; anthers 10, in a ring round the ovary. Ovary is conical, 3.8 cm long; style as long as ovary, deflexed. Fruit is follicle 5, beaked, 5-6.3 cm long, linear, twisted together into the form of a screw, stellately tomentose. Seeds are numerous, angular; testa loose wrinkled (Figure 2).

Root is a tap root with wiry rootlets, cylindrical, 5-20 mm in diameter, rough surface that is exfoliated at places, also shows presence of cracks, longitudinal striations and wrinkles. Fracture of root is short in bark and fibrous in wood. Root does not possess any odour and its taste is bitter (Figure 3).

Microscopical characteristics of transverse section of roots of *H. isora*

TS of root shows a cork, phelloderm, secondary phloem and wood region (Figure 4). Cork consists of 10-12 layers of radially arranged tabular

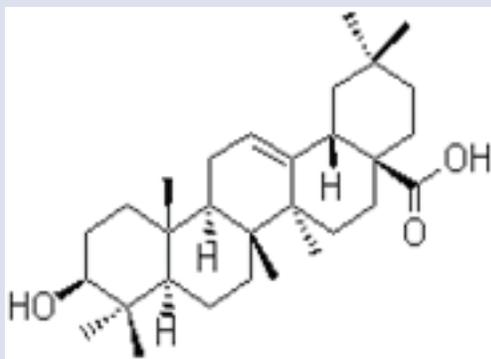


Figure 1: Chemical structure of oleanolic acid

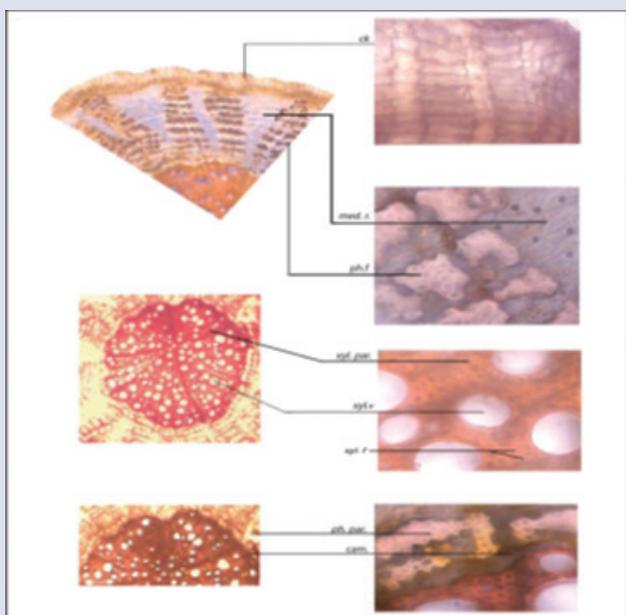


Figure 2: Transverse section of root of *H. isora* cam-cambium, ck-cork, med.r-medullary rays, ph.f- phloem fibre, ph. par.-phloem parenchyma, xyl.par.-xylem parenchyma, xyl.v-xylem vessel

lignified cells. Phellogen is indistinct. Phellogen consists of 3-5 layered parenchymatous cells containing starch grains, crystals of calcium oxalate rosettes and prisms. Phloem is parenchymatous and traversed with groups of concentrically arranged 25-40 phloem fibres (28-116 μ) which are thick walled and lignified. Xylem consists of vessels that are 50-86 μ in diameter, lignified and in radial rows, xylem fibers that are highly thickened and arranged in tangential bands. Medullary rays are bi-to multiseriate, parenchymatous, narrow in the xylem region and wider in the phloem region. Idioblast containing calcium oxalate rosettes and starch grains are found embedded in in the parenchymatous tissue.

Microscopical powder characteristics (Figure 3)

The root powder is fine to semi-coarse, fibrous, cream color, odorless and bitter in taste.

The diagnostic features of powder include: Lignified, thick walled polygonal cells of cork in surface view; lignified, bordered pitted xylem vessels up

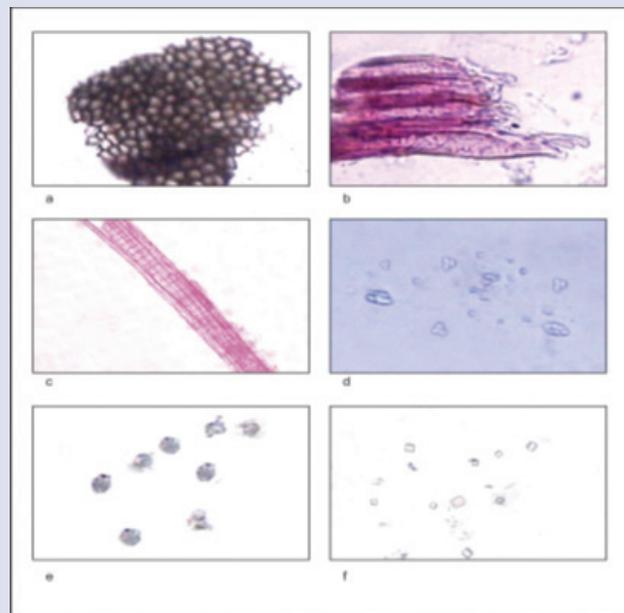


Figure 3: Microscopical powder characteristics of roots of *H. isora*
 a. cork cells in surface view
 b. bordered pitted xylem vessels
 c. phloem fibres
 d. starch grains e. rosettes of calcium oxalate crystals
 e. prisms of calcium oxalate crystals

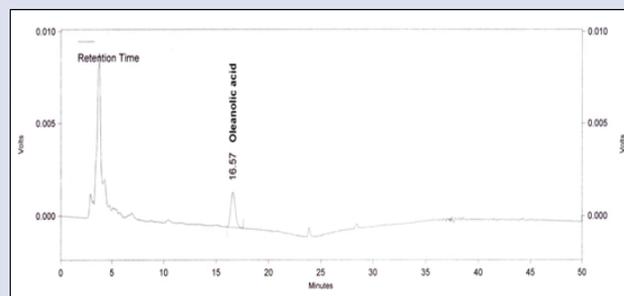


Figure 4: Chromatogram of standard oleanolic acid (2.0 μ g/ml) with corresponding retention time RT=16.57 at 210 nm by RP-HPLC method

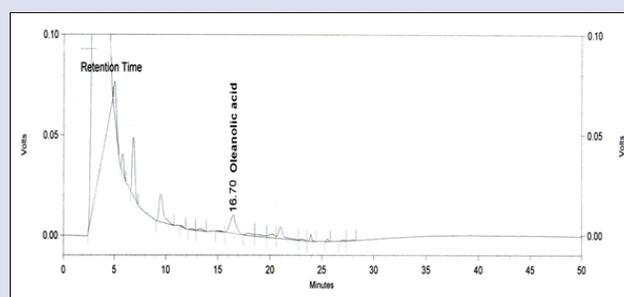


Figure 5: HPLC chromatogram of methanolic sapogenin extract of root of *H. isora* from Gujarat state showing peak of oleanolic acid at RT 16.7 min

to 350 μ in length and 50 μ or more in width; Numerous lignified, thick walled, pitted, phloem fibres that are tapering from both ends and found in groups; abundant, simple and compound (2-8 components) starch grains and numerous rosettes and prisms of calcium oxalate.

Physicochemical parameters like ash value, extractive value and loss on drying are mentioned in Table 1.

Phytochemical screening shows presence of saponins, tannins, steroids, flavonoids, mucilage, proteins and sugars (Table 2).

Method optimization

The HPLC mobile phase was chosen after several trials with acetonitrile, methanol, 0.1% aqueous orthophosphoric acid and water in various proportions. After investigation, it was found that methanol-0.1% aqueous orthophosphoric acid 90:10 (%v/v) revealed better resolution, high sensitivity and good peak shape. A properly separated oleanolic acid was found at RT 16.57 min retention time (Figure 5).

Method Validation

The correlation coefficient of calibration plot of peak area versus concentration of standard oleanolic acid was found to be 0.9991 that was indicative of good linearity over a range of 2.0-12.0 μ g/ml of oleanolic acid (Table 3). The linear regression equation is $y = 28463x + 1420.4$ where y is response and x is amount of oleanolic acid.

Accuracy

The results of recovery of oleanolic acid from root sample of *H. isora* obtained are shown in Table 4. The % recovery at three different levels (50, 100 and 150 %) was found to be 99.16 ± 1.2583 - 100.11 ± 0.6938 . The %RSD was found to be less than 2 %. These results indicate that the proposed method is accurate.

Table 1: Physicochemical parameters of roots of *H. isora*

Physicochemical parameters	Value (%w/w)
Total ash	3.56 \pm 0.15
Water soluble ash	2.51 \pm 0.11
Acid insoluble ash	0.33 \pm 0.05
Water soluble extractive value	5.39 \pm 0.24
Alcohol soluble extractive value (%w/w)	4.51 \pm 0.11
Loss on drying	4.61 \pm 0.03

Table 2: Phytochemical screening of roots of *H. isora*

Phytoconstituents	Value
Tannins	+++
Saponins	++
Steroids	+
Flavonoids	+
Coumarins	-
Alkaloids	-
Mucilage	+
Proteins	++
Sugars	++

–: Not detected, + Present in low concentration; ++Present in moderate concentration; +++ Present in higher concentration

Table 3: Linear regression equation data for oleanolic acid by RP-HPLC method

Concentration of standard oleanolic acid (μ g/ml)	Mean peak area \pm SD (n=3)	% RSD
2.0	58709 \pm 668	1.1391
4.0	115391 \pm 1376	1.1929
6.0	173779 \pm 1671	0.9620
8.0	228334 \pm 1906	0.8349
10.0	280629 \pm 2967	1.0576
12.0	347114 \pm 3459	0.9966

Table 4: Results from determination of recovery of oleanolic acid from root powder of *H. isora*

% Level	Amount of sample taken (mg)	Amount of standard spiked (mg)	Total amount recovered (mg)	%Recovery \pm SD	% RSD (n=3)
50%	2.0	1.0	2.99	99.33 \pm 1.5275	1.5378
100%	2.0	2.0	3.98	99.16 \pm 1.2583	1.2689
150%	2.0	3.0	5.00	100.11 \pm 0.6938	0.6931

Table 5: Results from determination of intra and interday precision for oleanolic acid

Concentration Of standard oleanolic acid (μ g/ml)	Intraday precision (n=3)		Interday precision (n=3)	
	Peak area (Mean \pm SD)	% RSD	Peak area (Mean \pm SD)	% RSD
4.0	115391 \pm 1376	1.1929	115591 \pm 1631	1.4154
6.0	173779 \pm 1671	0.9620	174112 \pm 2182	1.2533
8.0	228334 \pm 1906	0.8341	228067 \pm 2344	1.0279

Table 6: Summary of validation parameters for estimation of oleanolic acid from the roots of *H. isora* by HPLC method

Parameters	Results
Correlation coefficient (R ²)	0.9991
Linearity and range (μ g/ml)	2.0-12.0
Regression equation	$y = 28463x + 1420.4$
Precision (% RSD)	
Repeatability of area	0.4075
Repeatability of retention time	0.2408
Intraday	0.8341-1.1929
Interday	1.0279-1.4154
% Recovery	99.16 \pm 1.2583-100.11 \pm 0.6938
Limit of detection (μ g/ml)	0.03
Limit of quantification (μ g/ml)	0.1
Robustness	Robust

Precision

The % RSD value of instrumental precision was found to be 0.4075. The low value of RSD indicates that the proposed method is precise.

Intermediate precision (Reproducibility)

The results of intraday precision and inter day precision expressed as %RSD are shown in Table 5. The % RSD of intraday and interday precision was in the range of 0.8341-1.1929 and 1.0279-1.4154 respectively. The results show that the method is reproducible.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.03 µg/ml and 0.1 µg/ml respectively. The low value of LOD and LOQ revealed that the given method was found to be sensitive.

Robustness

The results of robustness for each parameter were expressed in terms of % RSD. The %RSD for each parameter was found to be less than 2% indicated that the standard oleanolic acid was not adversely affected by these changes. Hence the given method was found to be robust.

Overall results of method validation parameter were given in Table 6.

Quantification of oleanolic acid in saponinins of roots of *H. isora*

The content of saponins and saponinins found in the roots of *H. isora* 0.425%w/w and 0.13% w/w respectively. The peak of separated oleanolic acid from saponinins was found at retention time 16.70 min in HPLC chromatogram of methanolic saponin extract of root (Figure 5). The amount of oleanolic acid in roots of *H. isora* obtained from Gujarat India is 0.076%w/w.

CONCLUSION

The pharmacognostical study of the roots of *H. isora* will be useful for proper identification of the drug. The proposed HPLC method is accurate, reliable and precise. It can be used as routine quality control method for estimation of oleanolic acid from *H. isora*.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ABBREVIATION USED

HPLC: High Performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantification.

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