

# Anatomical study of secondary tuberized roots of *Harpagophytum procumbens* DC and quantification of harpagoside by high-performance liquid chromatography method

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## ABSTRACT

**Aim and Background:** A botanical study is conducted to provide a standard diagnostic tool. In order to improve the quality assurance of the secondary tuberized roots of *Harpagophytum procumbens*, derived extract and phytomedicine, a simple, rapid, and accurate high-performance liquid chromatography (HPLC) method was developed to assess the harpagoside. **Material and Methods:** This HPLC assay was performed on a reversedphase C18 column with methanol and water (50/50—V/V) as the mobile phase with a flow rate of 1.5 mL/min and using a monitoring wavelength at 278 nm. **Results and Conclusion:** This method was successfully applied to quantify these bioactive iridoid in an aqueous extract of *H. procumbens* and in its related phytomedicine “harpagophyton.” The result demonstrated that the quantification of harpagoside, indicating that the quality control of the bioactive ingredient in *H. procumbens*, derived extract and phytomedicine, is critical to ensure its clinical benefits.

**Key words:** Anatomical study, harpagophytum, harpagoside, high-performance liquid chromatography quantification

## INTRODUCTION

Indigenous plants, of Kalahari Desert of Africa, *Harpagophytum procumbens* DC (De Candolle—Pedaliaceae), is rare and only found in Southern Africa. It comes only from South Africa, Namibia, Botswana, and South-Eastern Africa, where it was harvested in the wild form.<sup>[1]</sup> The plant named “Devil’s claw” is also cultivated. In Europe, it was introduced for the first time by O. H. Volk in 1953. It was in 1989 that *H. procumbens* was included in the French pharmacopoeia. Traditionally in southern Africa, the *H. procumbens*, also called Devil’s claw, is used among indigenous diseases of the liver, stomach, gallbladder, kidney, and pancreas. In fever, it is used as tonic, in disorders of pregnancy and bleeding. In Europe, *H. procumbens* is also

used in self and without evidence experimental in metabolic disorders, diabetes, and disorders of the elderly.

Secondary tuberized roots are the therapeutic parts of the plant; they contain between 0.5 and 3% of iridoid glycosides assumed to be the active compounds.<sup>[2]</sup>

Harpagoside (8-cinnamoyl harpagide) is the main iridoid. Some other compounds are also present in *Harpagophytum*, gluco-iridoid like Harpagide, procumbide, and its 60-para-coumaroyl ester from the iridoid family, some flavonoids, phenolic acids, quinones, phytosterols, and sugars. They are present in too small amounts or they are not specific for *Harpagophytum*.<sup>[3-5]</sup> Chemical structures of harpagoside [Figure 1] and harpagide have previously been underscored.<sup>[6,7]</sup>

The European Scientific Cooperative on Phytotherapy (ESCOP) monograph summarizes the pharmacological and clinical evidence behind the therapeutic indications for the individual plant materials. ESCOP recommends the

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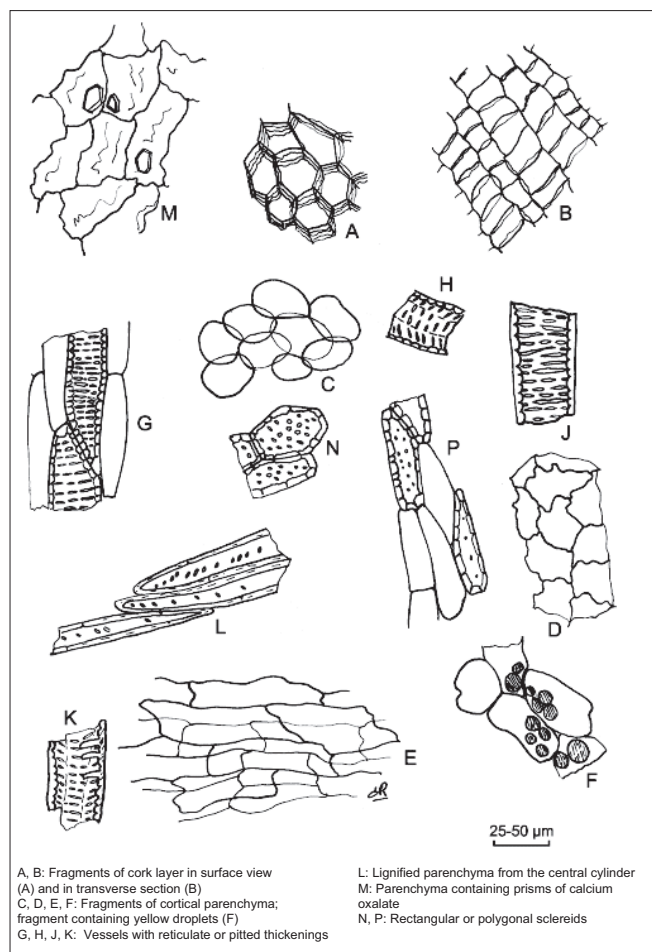
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**Figure 1:** Illustration of powdered herbal drug of devil's claw root (see identification B)

use of Devil's claw for symptomatic treatment of painful osteoarthritis, relief of low back pain, loss of appetite, and dyspepsia.<sup>[5]</sup> The effectiveness of *Harpagophytum* in the treatment of exacerbation of low back pain has been extensively studied.<sup>[8-11]</sup> As a result of a number of recent studies, *Harpagophytum* is proposed as a complementary treatment for chronic rheumatism, tendonitis, osteoarthritis, and arthritis.<sup>[12-16]</sup>

The aim of our work is to propose a quantification method of the harpagoside by high-performance liquid chromatography (HPLC) in Devil's claw and its related phytomedicine "harpagophyton" speciality developed by RandD Pharma laboratory. Therefore, a reliable quality control method<sup>[17,18]</sup> is needed for the qualitative and quantitative determination<sup>[19,20]</sup> of this gluco-iridoid in the secondary tuberized roots of *H. procumbens*.<sup>[21]</sup> A simple HPLC-UV assay using an external standard method has been developed, for the first time. The developed method has been subsequently applied to analyze various extract.

## MATERIALS AND METHODS

### Plant material

Secondary tuberized roots of *H. procumbens* samples were collected in November 2000 from Namibie. Voucher specimens of roots were identified by Professor Isabelle Fouraste and deposited at the herbarium of the Pharmacognosy Laboratory, Faculty of Pharmacy in Toulouse.

### Preparing slides

Observations were based on microscopic study of sectioned and stained tissue materials. Transverse sections were prepared with a sliding microtome (MSE), stained in alun carmine-green combination or Mirande reagent<sup>[22]</sup> for 2–3 minutes, and then washed with water. Following staining, the transverse sections were mounted on glass slides using glycerine gel. Some observations were made using *Chloral hydrate solution R* (European Pharmacopoeia in force). Observations were made with a LEICA Microsystems DMLB microscope (Toulouse, France), and pictures were taken with Digital Camera Power Shot S40 CANON photo-micrographic system (Toulouse, France). For the description purpose, we used some help books.<sup>[23,24]</sup>

### Chemicals and standards

HPLC grade methanol (VWR International, France) was used for the HPLC analysis. All other organic solvents used in this study were of analytical grade from VWR.

Harpagoside reference was furnished by Extrasynthese (®Réf. 02295, Lot 01120515).

The dried roots were crushed into pieces before extraction. The secondary tuberized roots of *H. procumbens* sample collected from Namibie were used for the assessment of the precision and recovery and limits of detection.

### Apparatus

HPLC was performed on Kontron and Merck-Hitachi liquid chromatography systems, equipped with a quaternary solvent delivery system, an auto-sampler, and UV detector. The column configuration consisted of an LICHROSORB, 100 RP-18 reserved-phase column (5 μm, 150 mm × 4.6 mm, i.d.) (Chromoptic Ref: LB613B64—Lot 206 L 849633) with its pre-column Lichrosorb 100 Å RP 18 (5 μm; Ref: LB181505). The colorimetric method was performed on HPLC 430 and 335 Detector spectrometers (UVK-LAB, France).

### Quantification of harpagoside by high-performance liquid chromatography

#### Calibration curves

To prepare the standard solutions, accurately weighed

amounts of the harpagoside standard (0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mg, respectively) were dissolved in methanol (10 mL) for analysis. The standard solutions were injected and run for calibration curves. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with amount of analyte injected.

#### Sample preparation

An aliquot of the dried powder of the secondary tuberized roots of *H. procumbens* (20–40 mesh, 10 g) was extracted, by reflux extraction method, with 8–10 times its weight in purified water for 2 hours at 50°C. The macerate was filtered and then evaporated to dryness under reduced pressure (20 mm Hg) and temperature (under 50°C) to yield an aqueous extract. The dry atomized extract of secondary tuberized roots of *H. procumbens* obtained was then used to prepare (as described in Table 1) coated tablets of the phytomedicine called “harpagophyton.”

The suitable amount of each residue (extracts and phytomedicine) was dissolved in 10 mL of methanol, respectively. The afforded solution was filtered through a 0.45- $\mu$ m syringe filter before HPLC. All separations were

performed at ambient temperature. In addition, assigning peaks were based on their retention times or by spiking the test solution with standard compound.

#### Linearity study

The linearity shows that test results were directly proportional to the amount of harpagoside in *H. procumbens* and “harpagophyton”. The linearity of a HPLC assay was established throughout the measurement interval ranging from 70 to 130% of the concentration test. Each solution is injected once randomly.

#### Precision and recovery studies

The measurements of intra- and interday variability were used to determine repeatability and intermediate precision of the developed assay method. A concentration standard solution containing harpagoside was prepared. Quantity for the analyte was calculated from its corresponding calibration curve.

Each sample was analyzed in six times within the same day to determine the intraday variability. The interday reproducibility was determined by analyzing the sample

**Table 1: Formulated tablets of “harpagophyton” phytomedicine**

Name components	Formula per tablet in mg	Equivalence percentage	Function	Reference to standard
Naked tablet				
Substance active				
Dry aqueous extract nebulized on maltodextrine of secondary tuberized <i>H. procumbens</i> DC <sup>a</sup>	450	66	Active substance	Internal Monograph and French Pharmacopoeia X <sup>th</sup> edition
Excipients				
Microcrystalline cellulose	68	10	Compression agent	European Pharmacopoeia in force
lactose	40	5.88	Thinner	European Pharmacopoeia in force
Croscarmellose sodium	30	4.41	Disintegration agent	European Pharmacopoeia in force
Magnesium stearate	6	0.88	Lubricant	European Pharmacopoeia in force
Silica colloidal anhydrous	6	0.88	Lubricant flow agent	European Pharmacopoeia in force
Total mass of naked tablet	600			
Coating				
First coating				
Coating agent 1 (e)	30		Glazing agent	Technical record
Purified water <sup>b</sup>	220		Solvent	European Pharmacopoeia in force
Second coating				
Methacrylic acid copolymer and ethyl acrylate (1:1) dispersion at 30 percent (f)	114.6		Glazing agent	European Pharmacopoeia in force
Talc	8.75		Lubricant	European Pharmacopoeia in force
Triethyl citrate	6.90		Plasticizer agent	European Pharmacopoeia in force
Purified water <sup>b</sup>	51.3		Solvent	European Pharmacopoeia in force
Total mass of film-coated tablet	680			

<sup>a</sup>Maltodextrin used between 15 and 35% in conformity with the European Pharmacopoeia in force, <sup>b</sup>Evaporated during the stages coating

on two separate days and on two different equipments.

The coefficient of variation was taken as a measure of the repeatability and the test of equal variances (F-test) was taken as a measure of the intermediate precision.

In addition, to further evaluate the recovery of the developed assay, amounts of harpagoside calculated ( $m_{\text{calculated}}$ ) were calculated from harpagoside standard calibration curve, and amounts of harpagoside measured ( $m_{\text{measured}}$ ) were measured directly in the tested plant roots. Each sample was analyzed in six times. The analyte concentration was determined from the corresponding calibration curve, and the recovery of the measurement for the analyte was calculated by the following equation:

$$\text{Recovery (\%)} = (m_{\text{measured}} / m_{\text{calculated}}) \times 100$$

where  $m_{\text{measured}}$  is harpagoside amount measured above and  $m_{\text{calculated}}$  is the calculated amount of harpagoside calculated with calibration curve.

#### Limits of detection

The standard solution containing all authentic compounds was diluted with methanol to provide appropriate concentrations. The limit of detection for each analyte was determined when the ratio of the testing peak signal-to-noise was greater than 5.

#### Statistical analysis

All data were expressed as means  $\pm$  standard deviations (SD) of triplicate measurements. The confidence limits were set at  $P < 0.05$ . SD did not exceed 5% for the majority of the values obtained.

## RESULTS AND DISCUSSION

#### Botanical analysis

The drug is made by tuberized, cut and dried *H. procumbens* (N° 206 G146) secondary roots tuberized. The Devil's claw roots are odorless, brown gray to dark brown with bitter taste. These roots consist of thick, fan-shaped or rounded slices or of roughly crushed discs. The outer surface, dark, is crossed by tortuous longitudinal wrinkles. The central cylinder shows fine concentric striations. Seen under a lens, the cut surface presents yellow to brownish-red granules.

Microscopic examination of its paler cut surface shows from the outside to the inside a thin cork consisting of slightly suberized walled cell; a developed phelloderm consisting of cellulosic thin-walled cell, a cortical parenchyma, poorly developed, consisting of polyhedral cells, with thin cellulose walls, separated meatuses. The fibrovascular bundle organized in a continuous ring consists

of secondary structure. Secondary phloem completed by narrow cones is separated by cellulosic pluriseriate rays. A dark cambial zone consists of small rectangular attached cells, aligned in radial files. Secondary xylem includes cellulosic parenchyma, reticulately thickened, or pitted vessels often isolated and/or grouped by two or three and divided into concentric zones. The marrow consists of thin-walled cellulosic cells.

The powder [Figure 1], yellow-brown, is examined under a microscope using chloral hydrate solution R and shows the following:

- Cork fragments consisting of polyhedral cells brown-yellow, thin-walled regular and which are superimposed.
- Fragments consist of cortical parenchyma cells ovoid, thin-walled inclusions sometimes contain granular brown-red (stained orange in the reagent lactic) and yellow droplets isolated.
- Fragments of vessels with reticulate thickenings, of tracheids associated with ligenous parenchyma, from the central cylinder, needles and small crystals of calcium oxalate in the parenchyma. The sclereids punctuated, of rectangular or polygonal, containing dark red-brown. Powder *H. procumbens* DC (206G146 lot) does not contains starch.

#### High-performance liquid chromatography separation optimization

The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time, especially when large amount of samples were analyzed. The solvent system of A–B (A, methanol; B, distillate water) was tested by changing the volume ratio of the components to obtain the optimal composition. After optimizing the separation parameters, the ratio 50:50 (v/v) of the solvent system of A–B at a flow rate of 1.5 mL/min was used so as to ensure that each run of analysis was completed within 25 minutes with better resolution of adjacent peaks and low solvent consumption; 278 nm was chosen as the detection wavelength, as it is close to the maximum absorbency of iridoid.

#### Linearity, precision, and recovery of the high-performance liquid chromatography method

The areas under the curve peak of harpagoside measured linearly dependent quantities of tracer harpagoside, tested in the area between 0.050 and 0.113  $\mu\text{g}/20 \mu\text{L}$  for the aqueous solution of *H. procumbens* DC.

Under the optimal chromatographic conditions used in this study, calibration curve exhibited good linear regression as shown in Table 2, and the limit of detection was in the range of 0.050 and 0.113  $\mu\text{g}/20\mu\text{L}$  for harpagoside.

**Table 2: Calibration curve and accuracy of this iridoid in the secondary tuberized roots of *H. procumbens* and the coated tablet of “harpagophyton” phytomedicine**

Analyte	Retention time (min)	Standard curve	r <sup>2</sup>	Test range (µg/20µL)
Harpagoside in aqueous extract of <i>H. procumbens</i> D.C/	9.9	Y = 18704,6 x - 12,0053333	0.9958	0.050–0.113
Harpagoside in coated tablet of “harpagophyton”	11.1	Y = 14196962,86 x + 5845	0.988	0.045–0.083

Y: Peak area, x: Amount of analyte, r<sup>2</sup>: Correlation coefficient of regression equation, limit of detection: S/N = 5

**Table 3: Intra- and interday precision for the determination of harpagoside**

Analyte	Amount injected (µg/20 µL)	Coefficient of variation (%)	Test F F calculated/F tabbed	
			Intraday (n = 6)	Interday (n = 2) Equipment (n = 2)
Harpagoside in aqueous extract of <i>H. procumbens</i> DC	0.070	8.22	1.22 < 5.05	1.17 < 5.05
Harpagoside in coated tablet of “harpagophyton”				
Lot CPM 5096	0.070	9.21	4.17 < 5.05	0.255 < 5.05
Lot CPM 5097	0.061	3.808	0.83 < 5.05	3.10 < 5.05
Lot CPM 5098	0.064	10.69	1.01 < 5.05	1.58 < 5.05

The results in Table 3 demonstrated that the developed analytical method was reproducible with good accuracy and sensitivity for the analyte examined. The overall intra- and interday variations were less than 10% for the analyte. The recovery assays of harpagoside were carried out by adding the standard to the treated material, and the result is as shown in Table 4, from which it is clear that the recovery for harpagoside determined were in the range of 94–99%.

### Result analysis

Quantification of bioactive component, harpagoside, and its qualitative assessment in the extract of herb and its phytomedicine correspondent

In order to obtain quantitative *extraction*, variables involved in the procedure such as the extraction solvent and extraction time were optimized. The optimal experimental conditions were as follows: the powder of secondary tuberized roots of *H. procumbens* was extracted with 8–10 times its weight in purified water for 2 hours at 50°C. The macerate was filtered and then evaporated to dryness under reduced pressure (20 mm Hg) and temperature (under 50°C) to yield an aqueous extract. A suitable amount of residue was dissolved in 10 mL of methanol. The afforded solution was filtered through a 0.45-µm syringe filter before HPLC. All separations were performed at ambient temperature. This newly developed HPLC assay method was subsequently applied to determination of harpagoside in “harpagophyton”. The yield and purity of harpagoside were determined by HPLC.

Furthermore, to ensure the consistency of therapeutic

**Table 4: Recovery of harpagoside in the secondary tuberized roots of *H. procumbens* and the coated tablet of “harpagophyton” phytomedicine**

Analyte	Amount spiked (µg/20 µL)	Recovery	
		Mean (%)	R.S.D. (%)
Harpagoside in aqueous extract of <i>H. procumbens</i> D.C/	0.0675	97.11	1.94
Harpagoside in coated tablet of “harpagophyton”			
Lot CPM 5096	0.0646	94.39	4.94
Lot CPM 5097	0.0594	96.95	3.11
Lot CPM 5098	0.0635	98.23	3.28

benefits, the determination of the major bioactive component was more important and meaningful than a measurement of the content of bioactive ingredients only before its use as herbal prescription or as the plant source for the manufacture of natural product-based pharmaceutical preparations.

### CONCLUSION

Our botanical study provides a standard diagnostic tool to help for the preliminary identification before the medicinal use of this plant.

Moreover, our work relate on the development of a simple, sensitive, and specific HPLC-UV method to quantify the

bioactive ingredient, harpagoside, in *H. procumbens* DC and in its related phytomedicine. The results demonstrate that the developed method is accurate and reproducible and could be readily used as a suitable quality control method.

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