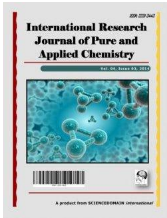

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# Quantification of Amaroswerin and Amarogentin in Different Parts of *Swertia chirayita* by Chromatographic Analysis

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The present study aims to quantify major secoiridoids (amaroswerin and amrogentin) in different parts of *Swertia chirayita* using HPLC to distinguish its parts having a high content of amaroswerin and amarogentin, so that these parts must be included in plant material when the plant is used for estimation or extraction of these phytoconstituents.

**Study Design:** Statistical comparison was performed using OP-STAT software with CRD and was considered statistically significant.

**Place and Duration of Study:** The plant material including all studied parts was procured form field plants were grown at Medicinal and Aromatic Plants Farm, Shilly (latitude-N 30˚54′30" and longitude E 77˚07′30", elevation 1550 m) under Department of Forest Products, UHF, Solan (H.P.) India. The study was undertaken in the Departmental laboratory and in the period between September 2016 and December 2016.

**Methodology:** Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and Empower II software was used for quantification of phytoconstituents under study.

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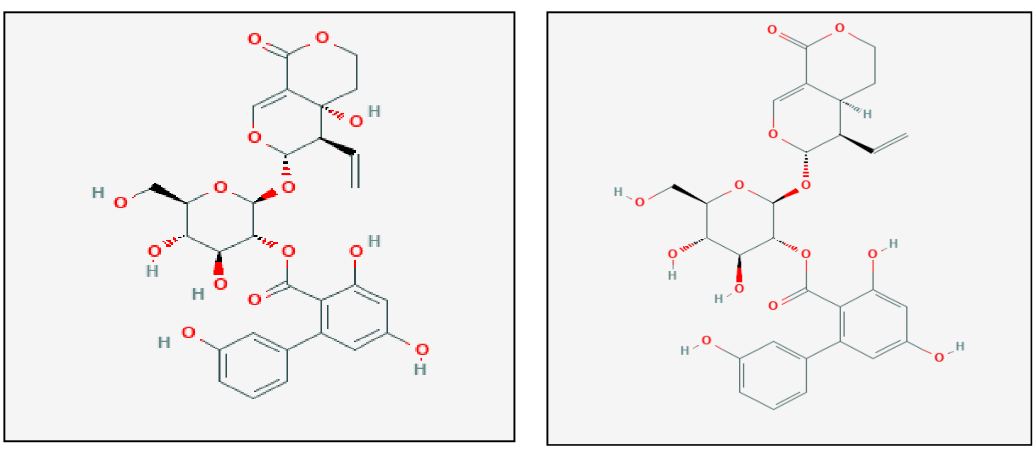
Samples of different plant parts were extracted by using soxhlet method with methanol as solvent.
**Results:** In different parts of *Swertia chirayita,* amaroswerin content (%) was reported highest in flowers (0.741%), followed by leaves (0.386%), roots (0.188%) and lowest in stem part (0.226%) and amarogentin content (%) was reported as highest in flowers (0.617%), followed by leaves (0.447%), stem (0.426%) and lowest in roots (0.369%) of field grown plants.
**Conclusion:** It is conluded that amaroswerin and amarogentin were present in all studied parts of *Swertia chirayita.* Amaroswerin and amarogentin content was ranged from 0.160% to 0.741% and 0.369% to 0.617% respectively in different plant parts under study.

*Keywords: Swertia chirayita; Amaroswerin; Amarogentin; quantification; HPLC.*

## 1. INTRODUCTION

*Swertia chirayita* (Roxb. *ex* Fleming) H. Karst. belonging to the family Gentianaceae, listed in the critically endangered category of IUCN list and is native of Himalayan region [1]. It is known differently in different languages as Chirayata (Hindi), Kairata, Bhunimba (Sanskrit), Chairavata (Urdu) *etc*. Its distribution is from Kashmir to Bhutan at an altitude of 1200-3000 m and in the Khasi hills at 1200-1500 m. It can also be grown in sub-temperate regions between 1500-2100 m altitude [2-5]. It has been used in traditional medicine mainly for skin diseases and chronic fever and also for malaria, anaemia, bronchial asthma, liver disorders, hepatitis, gastritis, constipation, worms, epilepsy, ulcer, scanty urine, hypertension, certain type of mental disorders, secretion of bile, blood purification and diabetes [6-10]. *Chirayita* is high prized medicinal herb in India and is used either alone or as one of the constituents in some polyherbal formulations like Ayush-64, Melicon V *etc*. The plant has also been reported to possess hypoglycaemic activity [11], anti-inflammatory activity [12], hepatoprotective activity [13], wound healing activity [14], anti-carcinogenic activity [10], anti-malarial activity [15] as well as antibacterial activity [16]. Amarogentin is a well-reported compound for antileishmanial, anticancerous, anti-diabetic and gastroprotective activity by different researchers [17-20]. Also, amaroswerin reported having gastroprotective activity [19]. Due to its intense medicinal importance demand for this plant is increasing at the rate of 10 percent per year [21].

Present investigation was carried out to quantify the major active constituents *i.e.* amaroswerin and amarogentin in different parts (leaves, flowers, stems and roots) of *Swertia chirayita* using HPLC in order to know its parts having high content of both amaroswerin and amarogentin, so that these plant parts must be included in plant material when used for extraction of studied phytoconstituents.



(a) (b)

**Fig. 1. Chemical structure of (a) Amaroswerin and (b) Amarogentin (Fig. 1 a & b source Pubchem)**

## 2. MATERIALS AND METHODS

### 2.1 Sample Source

The plant material including all studied parts was procured form field plants grown at Medicinal and Aromatic Plants Farm, Shilly (30˚54'32.40" N latitude and 77˚09'04.29" E longitude, elevation 1550 m) Department of Forest Products, UHF, Solan (H.P.) India. Identification was done in Herbarium Section of above-said department with reference number 13386.

### 2.2 Extraction, Separation and Purification of Standard compounds *i.e.* Amarogentin and Amaroswerin)

Field grown plants of *Swertia chirayita* were harvested at flowering stage, dried in the open air under shade, coarsely powdered and further dried in the oven for 12 hours at 35-40˚C. The oven dried material (500 gm) was then further powdered and repeatedly extracted (5x3 hours) with petroleum ether (40-60˚C) through reflux method on a boiling water bath. The petroleum ether extracted plant material was dried in the open air (for complete evaporation of solvent) and then repeatedly extracted (5x4 hours) with methanol (3 lts) through reflux method on a boiling water bath. All the filtrates were combined and the total volume was reduced to about 400 ml by partially distilling off the solvent. The contents were then kept overnight in the refrigerator (4˚C) when some brown coloured solid mass separated out which was filtered under vacuum and thoroughly washed with methanol. All the methanol washings were combined with the main filtrate and the solvent was completely distilled off under vacuum. The residue thus obtained was mixed with water (250 ml) and contents were repeatedly extracted with chloroform (5 x 200 ml) in a separating funnel to remove less polar compounds. Each time, after clear separation of solvent layers, lower chloroform layer was withdrawn and upper aqueous layer was further extracted with chloroform. The aqueous layer containing bitter compounds was then extracted with n-butanol (5 x 100 ml) in a separating funnel. Each time after clear separation of the solvent layers, the upper n-butanol layer was taken and aqueous layer re-extracted with n-butanol. All the n-butanol layers were combined and the solvent was distilled off under reduced pressure in a rotary vacuum evaporator.

The residue containing bitter compounds (obtained after distillation of n-butanol) was dissolved in minimum volume of methanol and then thoroughly mixed with small quantity of column chromatography grade Silica Gel (60-120 mesh) in a beaker. The contents were thoroughly mixed and the solvent was allowed to evaporate in the open and finally the adsorbed mixture was dried in a vacuum desiccator. The adsorbed mixture was then loaded on a clean and dry glass column (5 cm x 100 cm) packed with silica Gel (250 gm, 60-120 mesh) and eluted with chloroform: methanol (98: 2 to 85:15). The eluted fractions of 50 ml each were collected and after distilling off the solvent from each fraction, were monitored on silica Gel G pre-coated TLC plates developed in solvent system chloroform: methanol: water:: 65:20:10. The spots were visualized by iodine and fast red- B salt solution where amarogentin and amaroswerin spots appeared as red spots with the latter reagent and as brown spots with iodine. None of the fractions afforded pure bitter compounds only. All the fractions containing bitter compounds (amarogentin and amaroswerin) were then combined and further chromatographed on a Silica Gel packed narrow glass column (3 cm x 60 cm) using chloroform: methanol (98:2 to 95:5) as the solvent system. This time fractions of 25ml each were collected and monitored through TLC. The fractions containing the bitter compound having higher Rf (0.39) were combined, concentrated, dried under vacuum and dissolved in minimum quantity of methanol. Ethyl acetate was then added dropwise till slight turbidity appeared and the flask was left at room temperature and the bitter compound was crystallized after two days and then dried. Authenticity of the crystallized compound (amarogentin) was ascertained by running TLC and HPLC with a sample of reference amarogentin. The other fraction containing bitter compound having lower Rf (0.34) in the above solvent system could not be crystallized. This fraction was again column chromatographed on small sized column (1 cm x15 cm) using chloroform: methanol (98:2 to 95:5) solvent system for elution. The fractions containing pure amaroswerin (Rf = 0.34) were combined, concentrated and the amaroswerin was crystallized using methanol and ethyl acetate. The authenticity was ascertained by running TLC and HPLC with reference amaroswerin sample. The purity of isolated compounds was established by using HPLC.

### 2.3 Plant Material Preparation

Parts of the plant separated, shade dried and grinded mechanically to form the uniform particle size of the plant material, which was used for quantification of both amaroswerin and amarogentin in the samples under study.

### 2.4 Extraction of Different Plant Parts Samples

Oven-dried and powdered plant material samples (2 gm) of each plant part were repeatedly extracted in three replications with dichloromethane (100 ml) in soxhlet apparatus for the removal of dichloromethane soluble, less polar and non bitter compounds. The samples were then dried in the oven and extracted with methanol (100 ml) for 8 hours extraction duration. After extraction, the solvent from each extracted sample was completely distilled off and the residue was completely dried. Dry residue then dissolved in distilled water (25 ml) and then repeatedly partitioned with n-butanol (20ml). After clear separation of layers, the upper n-butanol layer was collected and aqueous layer was again partitioned with n-butanol. All the n-butanol layers combined and the solvent distilled off in a rotary vacuum evaporator and residue collected. The residue was dried to a constant weight after that the samples for HPLC analysis were prepared with mobile phase (methanol: water:: 45:55, v/v), and filtered through a 0.25µm filter.

### 2.5 Instrumentation

The system used is of Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and program used for data analysis was Empower II software.

### 2.6 Chromatographic Method

HPLC method was used as developed by Sharma [22]. UV detection was done at 235 nm, mobile phase (methanol: water:: 45:55, v/v), isocratic elution at a flow rate of 1ml/min. and injection volume was set to 20 µl. The total run time of standard and sample was 30 min. and 35 min. respectively with a retention time of amaroswerin and amarogentin was 14.449 ± 0.102 min. and 18.610 ± 0.140 min. (Mean ± standard deviation of triplicate analysis) respectively.

### 2.7 Statistical Analysis

Statistical comparison was performed by using OP-STAT software and was considered statistically significant.

## 3. RESULTS AND DISCUSSION

The study results aimed at the determination of the amaroswerin and amarogentin contents in different plant parts of *Swertia chirayita*. The studied contents in different parts of the plant found in present study are given in Table 1. The HPLC chromatograms of standard compounds and sample of different plant parts under study are given in Figs. 1-5. The amaroswerin content was highest in flowers (0.741%), followed by leaves (0.386%), roots (0.188%) and lowest in stem part (0.226%). The amarogentin content (%) in different parts of *Swertia chirayita* was reported as highest in flowers (0.617%), followed by leaves (0.447%), stem (0.426%) and lowest in roots (0.369%) of field grown plants of *Swertia chirayita*. Similar results have also been found for amaroswerin and amarogentin content in *Swertia japonica* [23] and *Swertia chirayita* [24]. Quantification of studied chemical compounds in *Swertia chirayita* fruit/seed is not done in this study, thus further studies can be done in this context.

**Table 1. Quantification of Amaroswerin and Amarogentin in different parts of *Swertia chirayita***

| Sr. no. | Plant part | Amaroswerin content (%) | Amarogentin content (%) |
|---|---|---|---|
| 1 | Flowers | 0.741 (0.860) | 0.617 (0.786) |
| 2 | Leaves | 0.386 (0.621) | 0.447 (0.669) |
| 3 | Stem | 0.160 (0.399) | 0.426 (0.653) |
| 4 | Roots | 0.188 (0.433) | 0.369 (0.607) |
| $CD_{0.05}$ | | 0.017 | 0.021 |
| SE(m) | | 0.006 | 0.007 |

Values in the parentheses are transformed values using square root transformed values

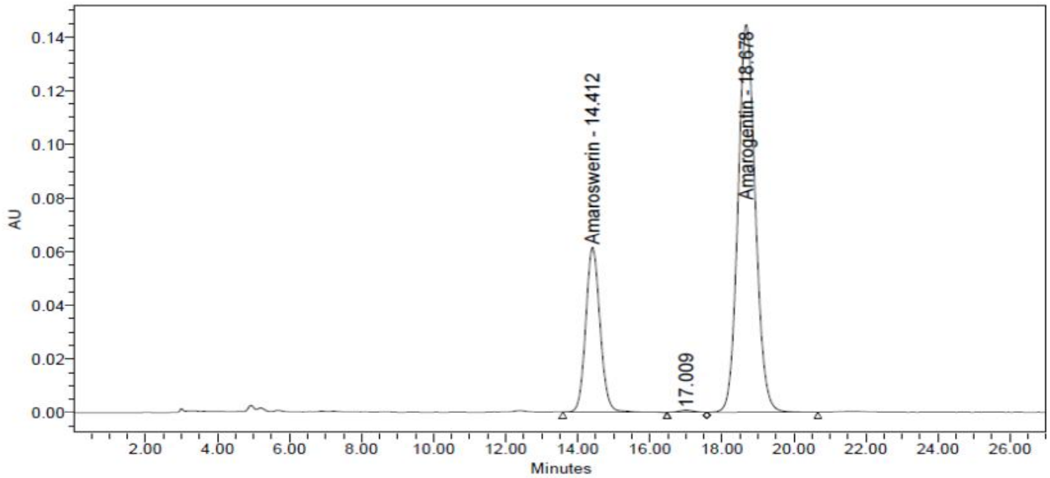



**Fig. 1. HPLC chromatogram of standard compounds amaroswerin (95 µg/ml) and amarogentin (95 µg/ml)**

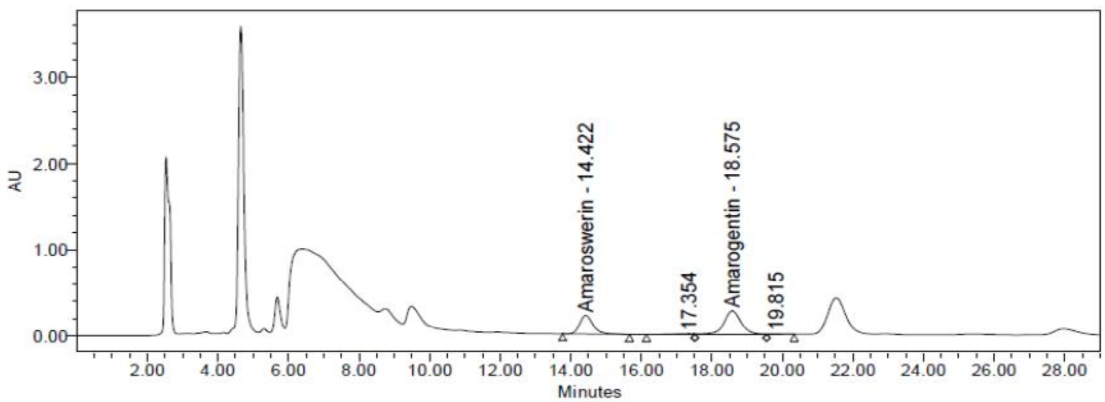



**Fig. 2. HPLC chromatogram of flowers sample of *Swertia chirayita***

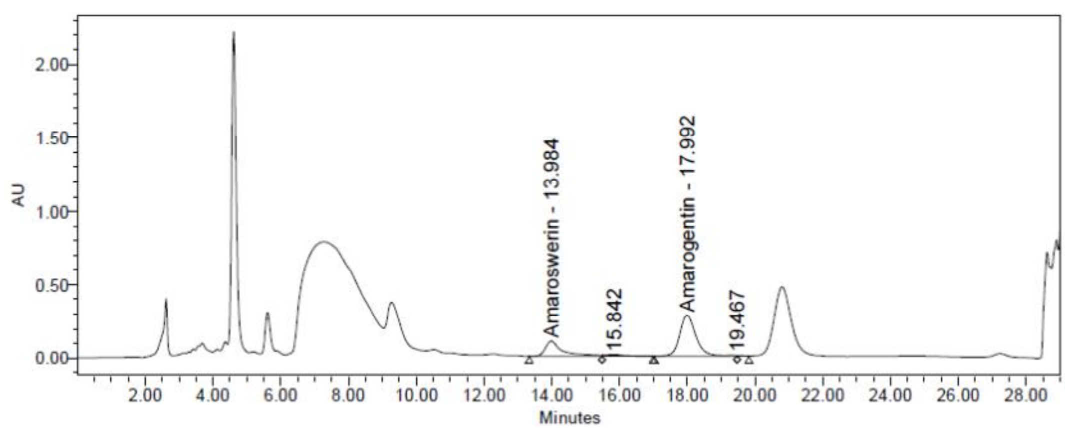



**Fig. 3. HPLC chromatogram of leaves sample of *Swertia chirayita***

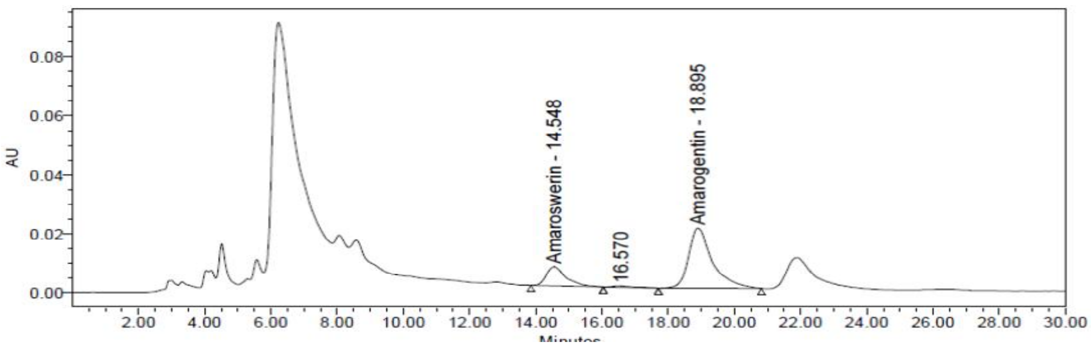



**Fig. 4. HPLC chromatogram of stems sample of *Swertia chirayita***

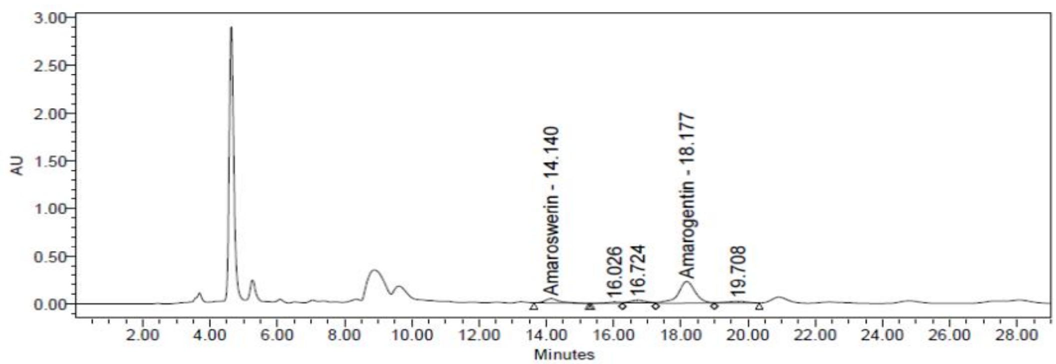



**Fig. 5. HPLC chromatogram of roots sample of *Swertia chirayita***

## 4. CONCLUSION

It is concluded that amaroswerin and amarogentin were present in all studied parts of *Swertia chirayita.* Amaroswerin and amarogentin content was ranged from 0.160% to 0.741% and 0.369% to 0.617% respectively. From the present investigation, it can be reccommended that for extraction purpose, harvesting should be done near to mature stage of the plant, so that good amount of flowers and leaves are present in harvested plant material. It will aid in the conservation of resources as less plant material gives more yield.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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