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Phytochemical and Thin Layer Chromatographic Evaluation of *Swertia chirayita* Buch.-Hams. Ex Wall at Different Developmental Stages

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A B S T R A C T

Swertia chirayita (family Gentianaceae), imperative medicinal plant in Indian system of medicine known for its bitter principles. However, due to its high demand and scarcity due to its extinction, it is being frequently adulterated with other species of *Swertia* which are more readily available. At present the similar species of *Swertia* are marketed as 'chirayita' which is affecting the potency of the drug. Our studies focused on evaluating *Swertia chirayita* by comparison of its physicochemical characteristics and TLC fingerprinting profile in Chloroform:Methanol:Water as solvent employing amarogentin as a reference marker to distinguish the crude herb *chirayita* from its adulterants. The study revealed methanol soluble fraction of *Swertia* species showed presence of amarogentin (major chemical constituent) in the raw material taken and thus can be further micropropagated on large scale to meet industrial demand.

Introduction

Swertia chirayita a medicinal herb indigenous to temperate Himalaya, belongs to family Gentianaceae, consist of 180 species (Hooker, 1885). The common name of *Swertia chirayita* is chiretta. *Swertia* found from Kashmir to Bhutan at an altitude of 1200-3000 m amsl and in the Khasi hills at 1200-1500 m amsl (Kirtikar and Basu, 1984; Pradhan and Badola, 2010). But, 2000 m altitude is highly preferable range (Bhattarai and Acharya, 1996). It is also found in open ground and recently slash and burnt forests (Edwards, 1993). The genus *Swertia* Linn. consists of annual and perennial herbs. Some authors have described *Swertia chirayita* as an annual (Anonymous, 1982; Kirtikar and Basu, 1984) and others as biennial or pluriannual herb (Edwards, 1993). It has about 2-3 ft long and erect stem, having orange brown or purplish in colour and contain large continuous yellowish pith (Bentley and Trimen, 1880; Joshi and

Dhawan, 2005). The root is tapering, stout, simple, short, almost 7 cm long and usually half an inch thick (Scartezzini and Speroni, 2000).

It is a safe ethnomedicinal herb used for nearly all medical therapies until synthetic drugs were developed (Exarchou *et al.,* 2002). The entire plant is used in traditional medicines for blood pressure, dyspesia, epilepsy, blood purification and liver disorders (Anonymous, 1976; Kirtikar and Basu 1984; de Rus Jucquet *et al.,* 2014; Malla *et al.,* 2015). The herb is very effective against gastrointestinal infection (Mukherji, 1953), used as antipyretic, hypoglycemic (Saxena and Mukherjee, 1992; Bhargava *et al.,* 2009; Verma *et al.,* 2013), antiperiodic, antifungal, (Chakravarty *et al.,* 1994; Rehman *et al.,* 2011), hepatoprotective (Mukherjee *et al.,* 1997), anti-inflammatory (Banerjee *et al.,* 2000), antispasmodic (Saha and Das, 2001), antibacterial (Joshi and Dhawan, 2005), antioxidative (Scartezzini and Speroni, 2000) and used to treat malaria and diabetes (Kumar and Staden, 2016). Recently, *Swertia chirayita* extract has been reported to possess anti-Hepatitis B virus activity (Zhou *et al.,* 2015). Consequently, *Swertia chirayita* has been receiving increasing attention from a wide range of researchers as evident from a number of publications appearing in literature (Bhattacharya *et al.,* 1976; Chakravarty *et al.,* 1994; Chen *et al.,* 2011; Kumar and Chandra, 2013; Padhan *et al.,* 2015).

The phytochemical parameters are most important and reliable criteria for determination of purity of crude drugs which have been reviewed from time to time. The genus is a rich source of flavonoids, xanthonoids, terpenoids and iridoids (Tan *et al.,* 1991; Zhou *et al.,* 1989; Brahmachari *et al.,* 2004; Rajan *et al.,* 2011; Das *et al.,* 2013). The whole plant contains gentianine alkaloid and aerial part contains xanthones (Sharma, 1982). Triterpenoid alkaloid presence was observed in the plant by Chakarvarty *et al.,* (1992). Flavonoids are also active constituent of genus *Swertia* (Negi *et al.,* 2011; Khanal *et al.,* 2015). Early studies also documented presence of flavonoids (Zhou *et al.,* 1989; Tan *et al.,* 1991; Pant *et al.,* 2003). Wide range of biological activities of *Swertia* have been attributed to presence of alkaloids and flavonoids (Wang *et al.,* 1994; Pant *et al.,* 2000; Bhandari *et al.,* 2006; Patil *et al.,* 2013; Lad and Bhatnagar, 2016). Total phenol estimation in the plant was done by a number of researchers (Sultana *et al.,* 2004; Dutta *et al.,* 2012; Patel *et al.,* 2015; Khushwaha *et al.,* 2017). Kumar and Sharma (2015) reported for first time complete biosynthetic pathways for amarogentin in *Swertia chirayita* with detection of intermediate metabolite. Keeping in view, the present investigation has been undertaken with the objective of biochemical estimations of different biochemical contents of micropropagated plants of *Swertia chirayita* Buch.-Hams. ex Wall.

Materials and Methods

Quantitative estimation of macromolecules

Total sugar

One gram of leaf material was homogenized in 5.0 ml distilled water to prepare plant (leaf) extract followed by centrifugation at 5000 rpm for 10 minutes. After that supernatant was collected and the residue was again suspended by adding 5.0 ml distilled water and centrifuged to complete the extraction. The supernatants pooled and the volume was adjusted to 10 ml by dilution with more distilled water.

To 1.0 ml of the leaf extract 1.0 ml of 5% of phenol was added followed by 5.0 ml of sulphuric acid. The sulphuric acid was poured directly in the centre of the test tube to ensure

a proper mixing. The tubes were cooled after 10 minute under running tap water. The absorbance was recorded after another 20 minute at 490 nm against the blank of distilled water replacing the extract. Standard curve prepared by using glucose (10-100 µg/ml) and concentration of total sugars was calculated from this curve and expressed as total sugars mg/g fresh weight. Total sugar content was calculated by using method by Dubois *et al.,* (1956).

Estimation of total protein

Five gram of fresh leaf was homogenized in 5.0 ml of 0.1 N NaOH, centrifuged at 3000 rpm and supernatant was collected. The residue was resuspended in 5.0 ml of 0.1 N NaOH and centrifuged again. The two supernatants were pooled and the final volume was adjusted to 10.0 ml. 2.0 ml of supernatant was treated with 1.0 ml of 15% TCA and kept at 4ºC for 24 hour. Precipitates of protein were formed which were separated by centrifuging at 5000 rpm for 20 minutes. Supernatant was discarded and precipitates were dissolved in 5.0 ml of 0.1N NaOH and used for protein estimation. Estimation of soluble protein content of leaves was done using method given by Lowery *et al.,* (1951). For estimation of protein, 5.0 ml of solution C was added to 1.0 ml of the protein extract taken in a test tube and mixed thoroughly. The solution was left at room temperature for 10 minute and then 0.5 ml of solution D was added to it and mixed. After 30 minutes absorbance was recorded at 660 nm against the blank of distilled water replacing the extract. Protein estimation was made using standard curve prepared by using BSA (10-100 µg/ml).

Total phenol estimation

Two gram fresh leaves homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10,000 rpm for 15 minutes and the supernatant was collected. The residue was extracted twice with 80% ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature. Residues were dissolved in 5.0 ml distilled water. Protocol given by Singleton and Rossi (1965) was followed for estimation of total phenols. 100 µl of this extract was diluted with 3.0 ml water and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 minutes, 2.0 ml of 20% Sodium Carbonate was added and the contents were mixed thoroughly. After 60 minute the absorbance of the solution was taken at 650 nm. The results were expressed as mg/g of fresh weight material. Phenol estimation was made using standard curve prepared by using Catechol (10-100 µg/ml).

Total alkaloid estimation

Protocol given by Harborne (1973) was followed for estimation of total phenols. Plant material was extracted by using successive solvents such as petroleum ether, chloroform and ethanol in increasing polarity for 48 hours respectively. The extracts were concentrated and dried under reduced pressure. 5g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filterd and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Total flavonoid estimation

Ten gram of plant material was extracted with 100 ml of methanol kept on a rotator shaker for 24 hours. Thereafter, the extract was

filtered using Whatmann filter paper No.1 and then concentrated in vaccum at 40-50°C. These extracts were further subjected to the qualitative phytochemical analysis. Protocol given by Koleva *et al.,* (2002) was followed for estimation of total flavonoid.

Total flavonoid content was measured by aluminium chloride colorimetric assay. To 1.0 ml of methanolic extract 4 ml of distilled water was added. To above mixture, 0.3 ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added. At 6^{th} min., 2.0 ml of 1.0 M NaOH was added and the total volume was made upto 10 ml with distilled water.

The solution was mixed well and the absorbance was measured against reagent blank at 510 nm. Standard graph was prepared by using different concentration of gallic acid.

Determination of physiochemical characteristics

Total ash content

Accurately weighed air dried and powdered plant material (1g) was taken in each of five previously ignited and weighed silica crucibles. The material was then evenly spread and crucibles were kept in the muffle furnace. The temperature inside was 550°C and the samples were kept until white ash was obtained indicating absence of carbon. After complete burning of organic matter, the muffle furnace was switched off and allowed to cool. Crucibles were then taken out and weighed again. The percentage of total ash was calculated as per the following formula.

$$\text{Total ash \%} = \frac{\text{(Weight of crucible + Ash) - Weight of crucible}}{\text{Weight of sample}} \times 100$$

TLC profile of Swertia plants

Chemicals and reagents used

The chemicals, reagents and solvents used for extraction of bitter compounds from the plant material and carrying out thin layer chromatography (TLC) were of analytical grade (AR) CDH brands. Thin layer chromatography of plant extracts was carried out on Silica gel 60 F254 precoated aluminum plates of Merck Brand.

Solvent system for Chromatography

Lower layer of Chloroform:Methanol:Water (65:25:10) was used for carrying out TLC of different plant extracts.

Detection Reagents for visualization of spots in TLC

1. Iodine: - The spots on the TLC plates were viewed by keeping the developed plates in TLC jar containing iodine.
2. Fast red B salt: - The plates were sprayed with 0.5 % aqueous solution of Fast red B salt.
3. UV light:- The spots on TLC plates were viewed by keeping the developed plates in UV light chamber at wavelength 254 nm.

Results and Discussion

The widespread uses of *Swertia chirayita* as a traditional drug and its commercialization in modern medical systems have led to a rise in scientific exploration of its phytochemistry in order to identify the active phytochemicals. This has resulted in a considerable literature exploring the chemical constituents of *Swertia* plant (Mandal and Chatterjee, 1987; Chakravarty *et al.,* 1994; Mandal *et al.,* 1992; Chatterjee and Pakrashi, 1995; Pant *et al.,* 2000; Patil *et al.,* 2013; Mehjabeen *et al.,* 2017). Samples of various developmental

stages (3, 5, 7 months, 1 year old and flowering stage) of seedling- and micropropagated plants were collected for biochemical estimations. Three replicates were taken for biochemical estimations and their means were calculated.

Under *in situ* conditions total sugar content in the leaves at various growth stages of *Swertia* plants was ranged from 0.046 mg/g to 0.239 mg/g FW showing gradual increase in total sugar content with increase in plant age (Fig. 1a). The lowest sugar content (0.046 mg/g) was recorded at three months old stage. Maximum total sugar content 0.239 mg/g was found at flowering stage of propagation. Similar trend was observed under *in vitro* conditions. It was revealed from Figure 1a that 0.039% of total sugar content was present at 3 months old stage which increased to 0.218% at flowering stage showing gradual increase in total sugar content with advancement of plant age. It may be seen that total sugar content was higher at different stages of propagation under *in situ* as compared to *in vitro* conditions. No significant difference was found within similar stages under *in situ* and *in vitro* conditions when compared for total sugar content. Snehal and Madhukar (2012) also investigated quantitatively total carbohydrates, reducing sugars, protein and amino acids in various leaf extracts of *Stevia rebaudiana.*

The lowest protein content was recorded at three months old stage (0.044 mg/g) followed by five months old stage (0.118 mg/g). And maximum protein content was found in one year old stage (0.307 mg/g). Total protein content for all stages was found significantly different from each other except for seven months old and flowering stage which were found at par with each other with no significant difference as shown in Fig. 1b. On other hand, under *in vitro* conditions in three months old stage 0.033 mg/g of total protein content was observed which was increased to 0.299 mg/g at one year old stage. Thereafter, a decline in total protein content at flowering stage (0.239 mg/g) was occurred. Seven months old and flowering stage were found non-significant while other were significantly different from each other. For similar stages of propagation under *in situ* and *in vitro* conditions there was found no significant difference for total protein content.

When propagation stages both under *in situ* and *in vitro* conditions were compared it was observed that total phenol content significantly increased from 3 months old stage to 7 months old stage and thereafter, there was a significant decline in total phenol content upto flowering stage. Among all the stages highest total phenol content was observed in 7 months old stage (3.916 mg/g) under *in situ* conditions where as lowest phenol content was of three months old stage (0.935 mg/g) under *in vitro* conditions. Total phenol content was found significantly different at similar stages of propagation under both conditions. But one year old stage and flowering stages under *in vitro* conditions were found with no significant difference when both *in situ* and *in vitro* conditions were compared separately (Fig. 1c). Karan *et al.*, (2005) studied about seventeen secoiridoid bitters isolated from different species of *Swertia*. Total phenolic content was found to be 3.57±0.23 mg of GAE/100g (aqueous extract), 2.96±0.25 mg of GAE/100g (hydroalcohol extract), 4.66±0.4199 mg of GAE/100g (ethanol extract).

At different developmental stages under *in situ* conditions there was seen a gradual increase in total alkaloid content from three months old stage (2.134 mg/g DW) to flowering stage (9.621 mg/g DW) as shown in Figure 1d. All stages were found significantly different to each other except for three and

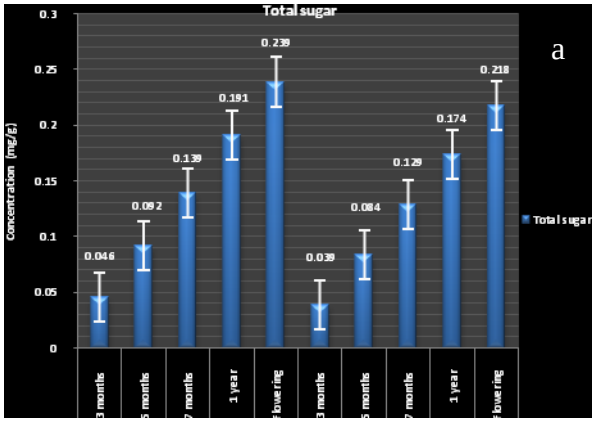
five months old stages which were found to be non-significant. Similarly, for *in vitro* conditions total alkaloid content increase with advancement of plant age. Minimum total alkaloid content was found in three months old stage (1.282 mg/g) whereas maximum total alkaloid content was found in flowering stage (8.664 mg/g). Three months old and five months old stages showed no significant difference while other were found significantly different to each other. When total alkaloid content under *in situ* and *in vitro* conditions was compared at similar growth stages of propagation it was observed that there occurs no significant difference among them for total alkaloid content. On phytochemical analysis revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenols and proteins (Latif and Rehmann, 2014). Shubham *et al.,* (2016) isolated compounds for different pharmacological activities from *Swertia chirayta* plants. Confirmed the presence of tannins, glycosides, alkaloids, flavanoids and reducing sugars in the hydro-alcoholic extract.

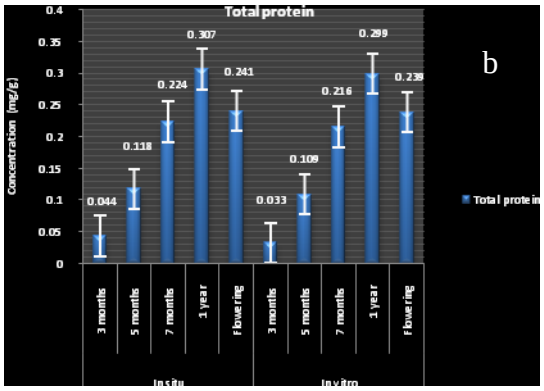
Total flavonoid content at different stages of seedling raised plants was ranged from 3.112 mg/g to 3.432 mg/g DW (Fig. 1e). There was observed a significant difference among all growth stages except five months old and one year old stage. But for micropropagated plants it was ranged from 2.431 mg/g to 2.468 mg/g DW at various developmental stages. All growth stages were significantly different except five months and one year old stage. Similar trend was obtained under *in situ* and *in vitro* conditions for total flavonoids content with advancement of age. It was increased from three months old stage to seven months old stage and then decline upto flowering stage. Total flavonoid content was found to be 96.15±4.26 mg of quercetin equivalent/ 100g (aqueous extract) by Kamtekar *et al.,* (2014). Thus from above results it may be observed that there is not much difference in total content of macromolecules in stages under *in situ* conditions when compared to stages under *in vitro* conditions in all cases. It was also revealed out that total content of macromolecules showed variation with advancement of age. Xanthones, iridoids/secoiridoids and triterpenoids constitutes the major classes of compounds reported from genus *Swertia* (Brahmachari, 2004). Edeoga *et al.,* (2005) reported alkaloids, tannins, saponins, steroid, terpenoid, flavonoids, phlobatannin and cardiac glycoside distribution in ten medicinal plants belonging to different families were assessed and compared. Alkaloid content was 11.53±0.15 (1.15)%. Tewari *et al.,* (2015) established the fingerprint profile of *Swertia chirayita*. Preliminary phytochemical screening of the extracts showed the presence of alkaloids, terpenoids, phenolics, flavonoids, carbohydrates, glycosides, tannins, saponins and lipids in various extracts. Mehjabeen *et al.,* (2017) evaluated the pharmacognostic, phytochemical and some biological studies on *Swertia chirata*. The reactions with chemical reagents showed positive results for the presence of triterpenes, tannin, alkaloids, carbohydrate and sterols.

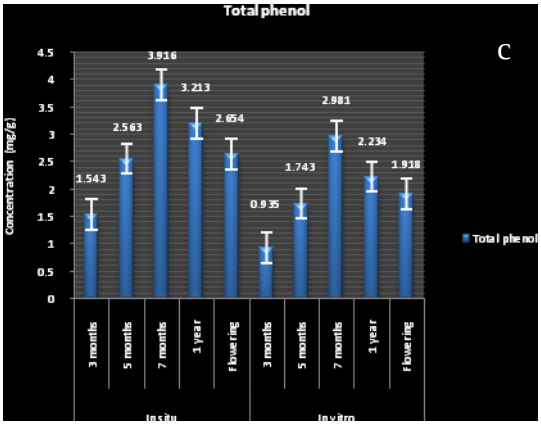
TLC profile of seedling raised and micropropagated plants at different stages

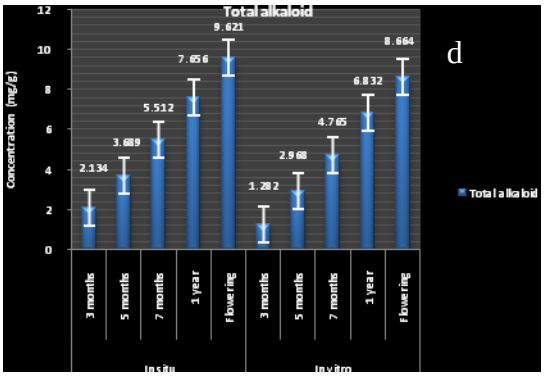
The dried and crushed fine powdered material of leaves of the plants was extracted with methanol and the methanol extract was used for developing TLC profile. The TLC plates were developed in solvent system Chloroform:Methanol:Water (65:25:10). Fast Red B salt and Iodine were used as detection reagents. The developed plates were also viewed under UV light at wavelength 254 nm (Fig. 2a). The plates sprayed with fast Red B salt are presented in Figure 2c. Immediately after spraying with Fast Red B salt, orange coloured spots appeared at Rf 0.59 of the standard compound Ag.

Fig.1 Quantitative estimation of total sugar, total protein, total phenol, total alkaloid and total flavonoid at various stages of seedling-and micropropagated plants of *Swertia chirayita* (Bars represent standard error)









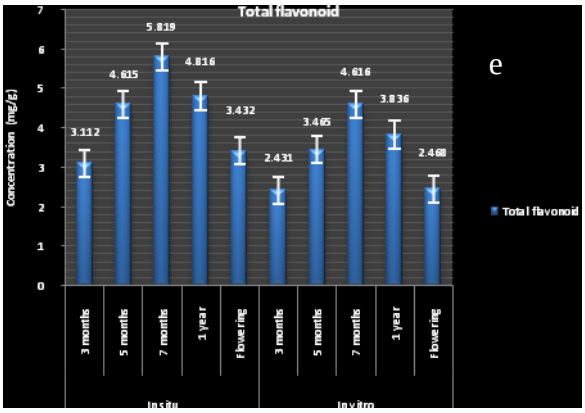


Fig.2 TLC plates viewed under UV light (254 nm), TLC plates developed in iodine, TLC plates sprayed with Fast Red-B Salt (R- Standard Compound, Ag- Amarogentin (1-7- Sample number)

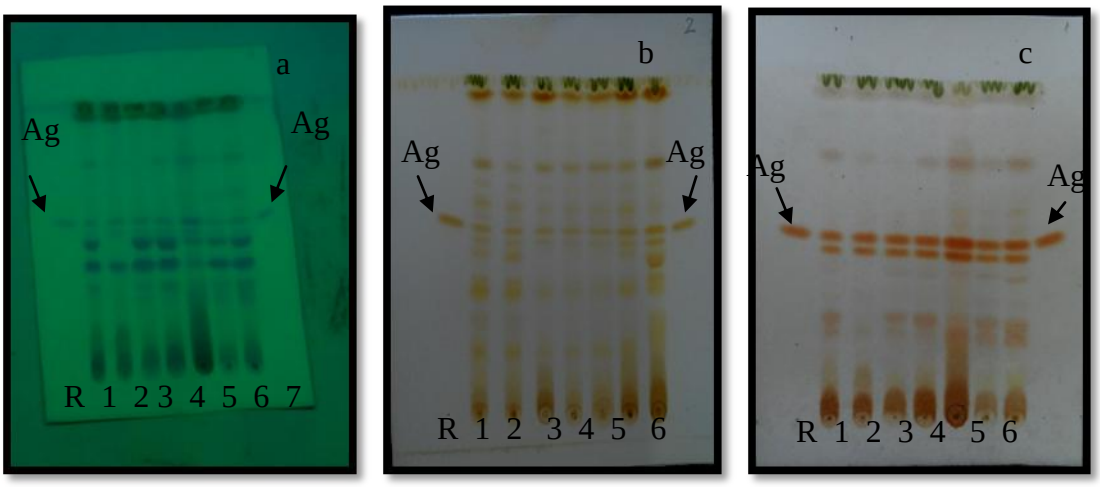


Fig.3 Ash formation from different samples of *Swertia chirayita*

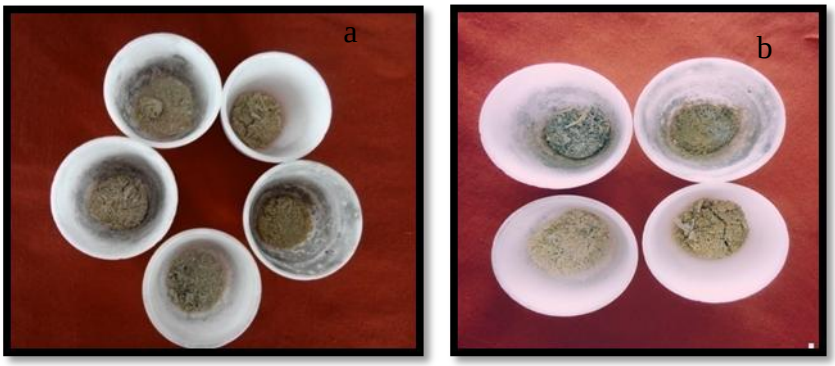
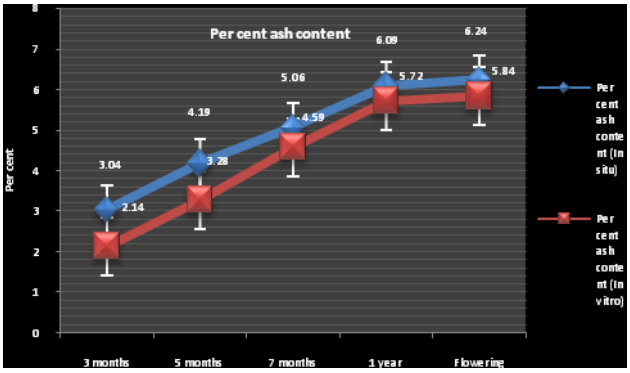


Fig.4 Ash values at various stages of seedling- and micropropagated plants of *Swertia chirayita* (Bars represent standard error)



The prominent dark orange coloured spot at Rf 0.59 was of amarogentin. The presence or absence and intensity of colour was critically observed in the spots of all the treatments in the TLC plate. Critically observing the TLC plates showed that the orange coloured spots belonging to amarogentin. In some treatments very light coloured spots corresponding to the

spots of amarogentin were observed which indicates very low concentration of these compounds in the sample.

The TLC plates kept in iodine jar gave yellow coloured spots of main bitter compound that is amarogentin (Fig. 2b) at Rf 0.59. When the developed TLC plates of methanolic extract were viewed under UV light at 254 nm, closely spaced bluish coloured spots of bitter compound were observed at Rf 0.59. The bluish coloured spot at Rf 0.59 corresponding to amarogentin.

Wagner *et al.,* (1984) has developed thin layer chromatography profiles of some medicinal and aromatic plants so that the genuine raw drugs can be distinguished from other species/adulterants. *Swertia chirayita* has been reported to contain amarogentin which is a bitter secoiridoid using thin layer chromatography (Korte, 1955; Cai *et al.,* 2006; Suryawanshi *et al.,* 2006). Gupta *et al.,* (2009) has also used HPTLC along with preliminary phytochemical and UV analysis for the authentification of *Hibiscus rosa sinensis* Linn. Meena *et al.,* (2010) have used thin layer chromatography in authentification of the fruits of *Terminalia bellerica* and knowing the adulterants. Latif and Rehman (2014) carried out TLC analysis using different organic solvent systems in percolated silica gel 60F254 TLC plates. Plates were visualized in day light and UV short and long wavelength. TLC separations were performed at room temperature and detection was carried out by UV light at 354 nm (Yadav, 2017).

Determination of total ash content

The *Swertia* plants of various growth and development stages (3, 5, 7 months, 1 year old and flowering) were analyzed for their physicochemical value (ash content) (Fig. 3) and the results are given in Figure 4 (ash values). Total ash content values for all growth stages of *Swertia* plants were found significant lied between the minimum value 2.14 (%) in 3 months old micropropagation stage and maximum in flowering stage of seedlings (6.24%). Out of the all stages under *in situ* and *in vitro* conditions compared, 7 months old, one year old, flowering stage showed no significant difference for total ash content values (Fig. 4). Whereas, 3 and 5 months old stages of seedling and micropropagation were found significantly different from each other.

Out of the various quality related tests, determination of ash content is one of the most facile means to ascertain authenticity and purity of medicinal plant materials (Trease, 1949). The amount and composition of ash obtained after combustion of plant material varies considerably according to the plant part, age and place of collection (Vermani *et al.,* 2010). In case of *Swertia chirayita* (the actual *Swertia* species used in the pharmaceutical industries), the total ash content has been reported to be less than 6.00 per cent and acid insoluble ash less than 1.00 per cent (Anonymous, 1955; Anonymous, 1998a). Ash contents have also been recommended for quality evaluation of plant based drugs obtained from different plants as *Hibiscus rosa sinensis* Linn. (Gupta *et al.,* 2009), *Terminalia bellerica* (Meena *et al.,* 2010) and *Butea monosperma* (Iqbal *et al.,* 2010). Among the parameters studied by Latif and Rehmann (2014) total ash content was 2.40±0.00 (0.48)%. Similarly, the plants were subjected to determination of various physicochemical parameters including ash values (total ash, water soluble ash) and extractive values (alcohol soluble extractive, water soluble extractive) (Sayyed *et al.,* 2014). A similar study was done by Mehta (2011). Thus, from above results it may be noted that total ash content show variation with age of plant and it shows an increase

with enhancement in age. In conclusion, *Swertia chirayita* (chirata) which is under high demand by the various pharmaceutical industries but its extreme exploitation leads to categorize the herb as an endangered species and is therefore difficult to obtain the crude drug in market in India. Various substitutes of *Swertia chirayita* are being sold out under the trade name of "chirata". These substitutes are needed to be identified on basis of some parameters for which its biochemical parameters are most important and reliable. Besides these, ash content can also be helpful in checking adultration of *Swertia chirayita.* Standardization is an important part for any study and is therefore necessarily required when we are exploring any kind of biological activity of a drug, and to make drug authentic. This study will help in setting down biochemical standards for future reference in determining the purity, quality and authenticity of *Swertia chirayita* Buch.-Hams. ex Wall.

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