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Comparative thin layer chromatographic evaluation of some Western Himalayan *Swertia* species

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Abstract
Swertia chirayita (family Gentianaceae), source of important Indian Ayurvedic drug "chirayita'', is an imperative medicinal plant in Indian system of medicine known for its bitter principles. The bitterness in *S. chirayita* is due to presence of two secoiridoid glycosides amarogentin and amaroswerin. However, due to its high demand and scarcity, it is being frequently adulterated with other species of *Swertia* which are more readily available. Presently almost all the similar looking species of *Swertia* are marketed as 'chirayita' without any rationale which is affecting the potency of the drug. Present studies focused on evaluating *Swertia chirayita* along with its four common adulterants by developing TLC fingerprinting profile in petroleum ether, chloroform and methanol extracts employing amarogentin as a reference marker to distinguish genuine chirayita from its adulterants. The study revealed methanol soluble fraction of five *Swertia* species *viz. Swertia chirayita, S. alata, S. angustifolia, S. cordata and S. purpurascens* showed presence of amarogentin and amaroswerin (major chemical constituents) only in *S.chirayita* whereas these spots were totally absent in the remaining four species. The results clearly indicated that TLC fingerprinting profiles of five *Swertia* species in methanol soluble fractions using amarogentin as a biomarker can be utilized to distinguish *S. chirayita* from other species due to presence of spot corresponding to amarogentin in *S.chirayita* only. On the other hand, TLC fingerprint profiles of chloroform and petroleum ether extracts were also supportive to mark the similarities and dissimilarities between different *Swertia* species.

Keywords: *Swertia chirayita*, adulteration, TLC fingerprinting profile, Rf values.

1. Introduction
Swertia chirayita (Roxb.) H. Karst, a member of family Gentianaceae commonly known as "chirayita" holds the most prominent position based on its medicinal potential (Chakraborty *et al.,* 2009) [9]. Beside its uses in different traditional systems of medicine including Ayurveda, Unani and Siddha, its medicinal capabilities have also been described in the Indian pharmaceutical codex, as well as the British and American pharmacopoeias (Brahmachari, 2004) [6]. The plant is native of temperate Himalayas found at an altitude of 1200-3000m from Kashmir to Bhutan and in Khasi hills in Meghalaya at 1200-1500m (Blatter, 1984; Clarke, 1885) [5, 10] and can be grown in sub-temperate regions between 1500m and 2100m altitude (Bentley and Trimen, 1880) [3]. Chirayita is one of the most reputed herbal drug extensively used for various health ailments including malaria, gastro-intestinal infection, diabetes, skin diseases, indigestion (Anonymous, 1976; Pant *et al.,* 2010) [1, 25] and also enjoys a special remedy in Western India for bronchial asthma as well as liver disorder (Joshi and Dhawan, 2005) [16]. Reputed Herbal medicines like Ayush-64, Diabecon, Mensturyl syrup, Melicon V ointment & Sudarshanchurna contain *Swertia chirata* extract in varied quantities (Edwin and Chungath, 1988; Valecha *et al.,* 2000; Mitra *et al.,* 1996) [11, 22, 29]. All parts of the plant taste bitter and possess febrifugal, laxative, stomachic, anthelminitic, antipyretic, hypoglycemic, anti-inflammatory, anti-diarrhoeal, anti-cancer, anti-HIV properties (Edwin and Chungath,1988; Valecha *et al.,* 2000; Ichiki *et al.,* 1988; Guha *et al.,* 1996; Lin *et al.,* 1996) [11, 13, 12, 20, 29]. The bitterness, antihelmintic, hypoglycemic and antipyretic properties are attributed to amarogentin (most bitter compound isolated till date) (Joshi and Dhawan, 2005) [16], Swerchirin and swertiamarin (Nadkarni, 1998) [23]. Amarogentin (bioactive compound) is a secoiridoid glycoside and is the most acerbic substance and tastes bitter even at a dilution of 1:58,000,000 (Arino *et al.,* 1997) [2].

The other constitutes of this plant are xanthone, xanthoneglycocides, flovanoids and triterpenoids (Joshi and Dhawan, 2005) [16].

'Chirayita' was once abundantly growing in wild and was exported from India whereas today it is nearly extinct from Indian hills and is imported from the neighboring countries such as Nepal. Ever increasing demand of *S. chirayita* in national and international markets is a cause of major concern as most of the herbal industries collect crude raw material from wild sources which are becoming critical on account of over harvesting leading to its categorization under 'Critically Endangered' category (Shreshta and Joshi, 1992; Rai *et al.*, 2000; Badola and Pal, 2002) [7, 26, 27]. Natural populations of this species are not sufficient to sustain its commercial demand leading to deliberate and inadvertent adulteration. This is due to the lack of correct information, lack of proper authentification process, intentional or unintentional adulteration and unavailability of the genuine stock. A lot of confusion still prevails in the Indian herbal drug market regarding the true identity of chirayita since "chirayita" is also used for several other species of *Swertia* that also taste bitter. It has been reported that *S. angustifolia, S. corymbosa, S. decussata, S. hookeri, S. macrosperma, S. petiolata, S. lawii, S. paniculata, S. punctata, S. calycina, S. purpurascens, S. bimaculata, S. ciliata, S. densifolia, S. japonica and S. frachetiana*are being used intentionally or unintentionally as its adulterants thereby affecting its potency (Brahmachari, 2004; Joshi, 2008; Negi *et al.*, 2011; Latif *et al.*, 2014) [6, 15, 24, 19]. Besides species belonging to genus *Swertia*, *Swertia chirayita* is also substituted by species like *Andrographis paniculata* (green chirata), *Exacum tetragonum E. bicolor*, *E. pedunculatum* and *Slevolgia orientalis* (Joshi and Dhawan, 2005) [16]. Taking into account the wide spread use and pharmacological importance of *Swertia chirayita* and the issue of adulteration in commercial scale, a strong need was felt to establish TLC fingerprinting profiles to ensure the correct identity of the drug used. Present study was carried to develope TLC fingerprinting profiles of *S. chirayita, S. alata, S. cordata, S. angustifolia* and *S. purpurascens* to define a process for easy identification and differentiation of *Swertia chirayita* from its allied species.

2. Material and Methods

2.1Collection of Plant Material

Swertia chirayita was obtained from the Medicinal and Aromatic farm at Shilly (Distt Solan, HP), *S. alata* as well as *S. angustifolia* were collected from Dharon Ki Dhar (Distt, Solan, HP) while *S. purpurascens* and *S. cordata* were collected from Hattu (Distt. Shimla, HP).

2.2 Thin layer chromatography fingerprinting profile

The chemicals, reagents and solvents used for carrying out thin layer chromatography (TLC) were of analytical grade (AR). Thin layer chromatography of different extracts of *S. chirayita, S. alata, S. cordata, S. angustifolia* and *S. purpurascens* was carried out on silica gel G precoated aluminum plates of Merck Brand. Standard compound amarogentin was isolated through column chromatography using column (3cm x 60cm), silica gel (60-120 mesh) as adsorbent and chloroform: methanol (98:2 to 95:5) as solvent system. Following solvents systems and detection reagents were used for development of spots on plates.

Extracts	Solvent systems	Detection Reagents
Methanol extract	Chloroform: methanol: water (65:25:10) Ethyl acetate: methanol: water (22:2:1) Chloroform: methanol (8:2)	**Iodine:**-The spots on the TLC plates were viewed by keeping the developed plates in TLC jar containing iodine. **Fast red B salt:**- The plates were sprayed with aqueous solution of Fast red B salt followed by spraying with NaOH solution. **Sulphuric Acid:** - The plates were sprayed with aqueous solution of sulphuric acid (5%), heated at 110 ºC for 5 minutes in the oven and then viewed in the visible light.
Petroleum ether extract	Hexane: ethyl acetate (8:2) Chloroform: methanol (9:0.5) Benzene: ethyl acetate(9:1)	Sulphuric acid (5%)
Chloroform Extract	Hexane: ethyl acetate (6:4) Benzene: ethyl acetate (9:1)	Sulphuric acid (5%)

2.2.1 Preparation of extracts for TLC Fingerprint Profile

10g powdered material of whole plant of five *Swertia* species viz., *Swertia chirayita, S. alata, S. cordata, S. purpurascens* and *S angustifolia* was extracted using soxhlet apparatus for 10-12 h sequentially with 100 ml each of methanol, chloroform and petroleum ether to obtain methanol, chloroform and petroleum ether soluble fractions. Each extract was concentrated under reduced and reconstituted with appropriate solvents(s) to develop the TLC fingerprint profile.

2.2.2 Sample Application

The samples of methanol, chloroform and petroleum ether extracts of each *Swertia* species were spotted with the help of capillary on aluminum TLC plate precoated with Silica gel 60F254 (E. Merck) and developed plates in different mobile phase as mentioned above. The developed plates were dried, visualized in day light and captured with the help of camera. The Rf values of all visualized spots on TLC plates of all three extracts were recorded manually by using following formula.

$$\text{Rf value} = \frac{\text{Distance travelled by the Spot}}{\text{Distance travelled by the Solvent}}$$

3. Results and Discussion

Most of the medicinal plants used in the pharmaceutical industries are procured from their natural habitats excepting few cultivated crops such as Isabgol, *Opium poppy, Andrographis paniculata, Cassia angustifolia, Glycorrhiza glabra, digitalis lanata*, etc. Collection of these plants from naturally growing regions is generally carried out by unskilled persons and this leads to intentional or unintentional adulteration of the genuine material (khatoon *et al.*, 2009) [18]. This ultimately reduces the efficacy of the final products and formulations. Although all the species can be differentiated from each other on the basis of morphological characters when the plants are live with all their parts intact, however, the problem arises when such plants are dried and mixed

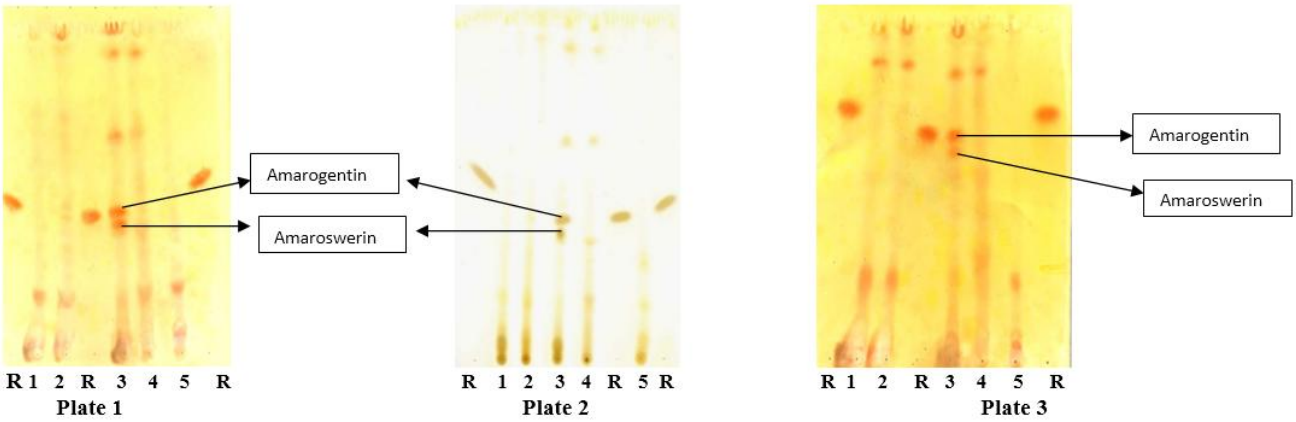
making correct distinction difficult. This is the reason why adulteration in the dry raw form escapes attention and inferior materials get traded as "chirata". Therefore, TLC fingerprinting profile is one of the important tool for detecting the adulteration and judging the quality of drugs in powder form. It is also important in knowing the number and types of compounds present in the plants by using different solvent systems and spraying reagents.

In present study, TLC fingerprinting profiles of five *Swertia* species was analyzed for the presence of bitter secoiridoid (amarogentin) in methanol soluble fraction (Plates 4-6) while TLC fingerprint profiles of petroleum ether (Plates 7-9) and chloroform extracts (Plates 10-11) were developed to mark the similarities and dissimilarities between different *Swertia* species. A large number of solvent systems were tried and which gave good resolution for all the five species were selected. The results revealed methanol soluble fractions of the five *Swertia* species showed distinct red coloured spots of amarogentin and amaroswerin after spraying the plates with fast red B salt solution using chloroform: methanol: water (65:25:10) (plate 1) and ethyl acetate: methanol:water (22:2:1) (Plate 3) as solvent system. Amarogentin and amaroswerin appeared as brown coloured spots on plates after spraying with H_2SO_4 (5%) using chloroform: methanol: water (65:25:10) (Plate 4), ethyl acetate: methanol: water (22:2:1) (Plate 5) and chloroform: methanol (8:2) (Plate 6) as solvent system. Developing the TLC plates in chloroform: methanol: water (65:25:10) and visualization of the spots by keeping the TLC plates in iodine jar gave yellow coloured spots (Plate 2) for the two bitter secoiridoids. Interestingly, it was observed that amarogentin and amaroswerin (principal constituents of *Swertia chirayita*) were detected only in *Swertia chirayita* whereas these were totally absent in other species. Our findings are in conformity with earlier reports (Bhatia *et al*., 2003; Cai *et al*., 2006; Suryawanshi *et al.*, 2006) [4, 8, 28]. The results clearly emphasizes the fact that TLC fingerprinting profiles of five *Swertia* species in methanol soluble fractions using amarogentin as a biomarker can be utilized to distinguish *S. chirayita* from other species due to presence of spot corresponding to amarogentin in *S. chirayita* only. On the other hand, thin layer chromatographic profiles of petroleum ether (Plates 7-9) and chloroform soluble fractions (Plates 10-11) in different solvent system using aqueous sulphuric acid (5%) as spraying reagent were not of much use to distinguish *S. chirayita* from other *Swertia* species. However, Rf values of prominent spots in different solvent systems were useful in recognizing number of compound as well as similarities and dissimilarities among all five *Swertia* species (Tables 1-3). It was observed that methanol extracts of the five *Swertia* species represented maximum number of spots (indicating presence of more components) followed by petroleum ether and chloroform extracts. The TLC fingerprint profile of methanol soluble fraction of the five *Swertia* species using chloroform: methanol: water (65:25:10) as solvent system and spraying the plates with H_2SO_4 (5%) (Plate 4 & Table 1) showed presence of two closely spaced brown coloured spots of amarogentin marked (f) at Rf 0.49 and amaroswerin marked (e) at Rf 0.47 in *S. chirayita* only. The authenticity of amarogentin was ascertained by running a reference sample of amarogentin. Spot marked (d) at Rf 0.44 was visible in *S. chirayita* and *S. purpurascens* while another spots (a) at Rf 0.30 and (g) at Rf 0.56 were visible in *S. alata only*. TLC fingerprint profile of methanol soluble fractions in ethyl acetate: methanol: water (22:2:1) (Plate 5 & Table 1) showed the presence of 8 spots out of which amarogentin marked (g) at Rf 0.69 and amaroswerin marked (f) at Rf 0.63 were present in *S. chirayita* only. The spot (a) at Rf 0.24 was visible in all the *Swertia* species studied except *S. cordata* whereas spot (b) at Rf 0.28 was also present in all *Swertia* species except *S. angustifolia*. Spot (c) at Rf 0.35 and spot (d) at Rf 0.40 were visible in *S. purpurascens* whereas, spots marked (e) at Rf 0.56 and (h) at Rf 0.76 was present in *S.alata* only. Further, TLC fingerprint profile of methanol soluble fractions using chloroform: methanol (8:2) (Plate 5 & Table 1) showed the presence of amarogentin marked (e) at Rf 0.72 and amaroswerin marked (d) at Rf 0.67 in *S. chirayita* only. Another spot (g) at 0.76 was visible in *S. chirayita* and *S. purpurascens* and number of components could be seen only in individual species for example spots marked (a) at Rf 0.22, (c) at Rf 0.56, (f) at Rf 0.73 and (h) at Rf 0.88 were present in *S. alata* only.

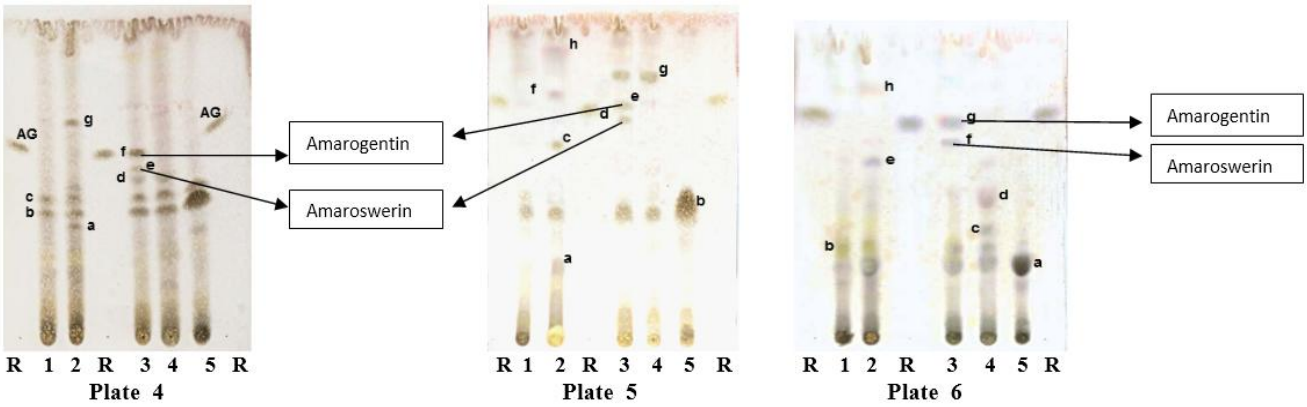
TLC fingerprint profile of petroleum ether soluble fractions using benzene: ethyl acetate (9:1) solvent system and spraying the plate with H_2SO_4 (5%) showed 8 spots (indicating presence of 8 compounds) (Table 2 & Plate 7) and spot marked (b) at Rf 0.29, (e) at Rf 0.69 and (f) at Rf 0.84 were visible in all the S*wertia* species except *S. chirayita* while Spot (c) at Rf (0.42) was present in *S. alata* only. TLC fingerprint profile using hexane: ethyl acetate (8:2) solvent system (Table 2 & Plate 8) showed spot marked (a) at Rf 0.38 and spot (f) at 0.86 and spot (g) at 0.93 were visible in all five *Swertia* species. The spot (b) at Rf 0.45 was visible in *S. cordata* and *S. alata* only. Spots marked (c) at Rf 0.50 and (e) at Rf 0.71 were present in *S. alata* and *S. angustifolia* respectively. TLC profile in solvent system Chloroform: Methanol (9:0.5) (Table 2 & Plate 9) showed spot (a) at Rf 0.45 appeared prominently in bright purple colour in all the five *Swertia* species while, a spot marked (d) at Rf 0.66 was visible in *S. angustifolia* only. On the other hand TLC profile of chloroform extracts of the five *Swertia* species in benzene: ethyl acetate (9:1) (Table 3 & Plate 10) showed total five components out of which a bright prominent purple coloured spot (a) at Rf 0.13 was visible in all the five *Swertia* species. Whereas, the spot (b) at Rf 0.24 was present in *S. cordata* and *S. chirayita* only while spots marked (c) at Rf 0.46, (d) at Rf 0.63 and (e) at Rf 0.92 were visible in *S. alata* only. While, TLC profile of the chloroform soluble fractions in solvent system-hexane:ethyl acetate (6:4) (Table 3 & Plate 11) showed only three spots out of which spot marked (b) at Rf 0.53 was visible in *S. chirayita* only whereas, spot marked (c) at Rf 0.62 could be seen in all the *Swertia* species. Similarly, Karan *et al.* 2012 [17] have also observed similarities and dissimilarities among five different species of *Swertia* viz. *S. angustifolia, S. chirata, S. cordata, S. lurida* and *S. purpurascens* by comparative thin layer chromatographic profile of methanol, hexane and chloroform extracts. TLC fingerprinting profile has also been employed to notice a close chemical relationship between *S. chirata* and *S. lurida* (Jamwal, 2014) [14]. Further, Meena *et al.* 2010 [21] have developed thin layer chromatography for authentification of the fruits of *Terminalia bellerica* and checking the adulterants. TLC fingerprinting profile is simple as well as precise technique and hold potential in not only correctly identifying the potent adulterants of *Swertia chirayita* but also to standardize the raw materials of 'chirayita' for herbal formulations. TLC plates of all the three extracts sprayed with 5% aqueous sulphuric acid are shown in Plates 4, 5, 6 and Rf values of prominent spots are given in Tables 1-3.

4. Conclusion

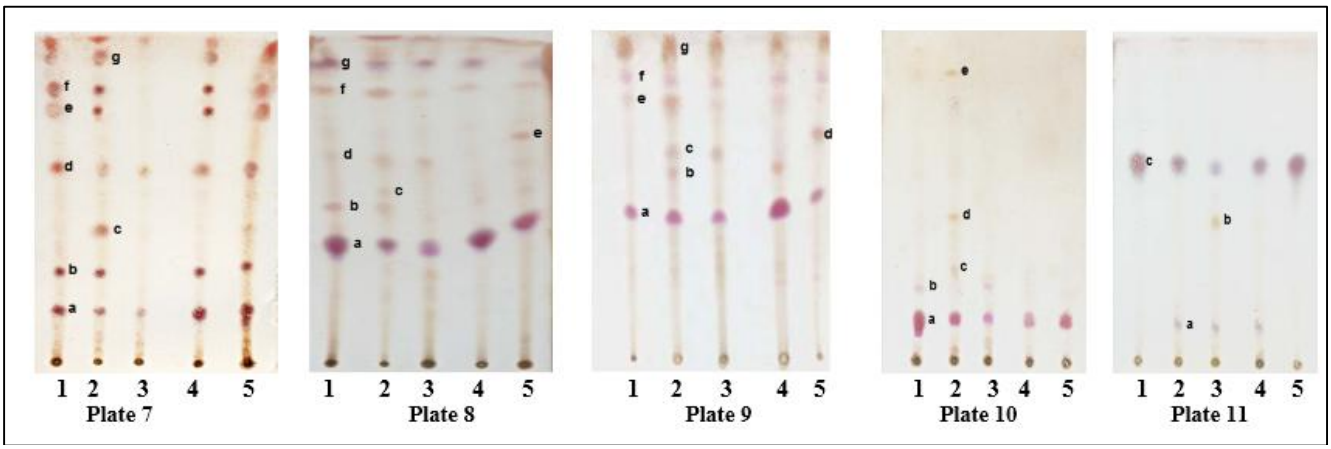
Genuine samples of 'Chirayita' is difficult to obtain in crude drug market of India as different species of *Swertia* are sold under the name chirayita; therefore authentication and standardization is prerequisite to minimize the adulteration in commercial trade. Morphological studies of herbs are not always reliable for checking adulteration due to variation in the chemical content. Thus menace of adulteration of *Swertia chirayita* by other less important *Swertia* species can be checked by studying thin layer chromatographic profile of extracts. Drug 'chirayita' hold its reputation due to presence of major compounds amarogentin and amaroswerin which were found to absent in others investigated *Swertia* species indicating none other species hold the potential to substitute *Swertia chirayita.* The TLC fingerprinting profile five *Swertia* species in petroleum ether and chloroform extracts can be useful to comprehend both similarities and dissimilarities between these species.



TLC fingerprint profile of methanolic extract of different *Swertia* species 1) *S. cordata* 2) *S. alata* 3) *S. chirayita* 4) *S. purpurascens* 5) *S. angustifolia* after spraying with fast red B salt solution (Plate 1&3) and visualization under iodine (Plate 2).
Solvent system- Chloroform: methanol: water (65:25:10) Plate 1&2; Ethyl acetate: methanol: water (22:2:1) Plate 3. **R**-Amarogentin (Reference).



TLC fingerprint profile of methanolic extract of different *Swertia* species 1). *S. cordata* 2) *S. alata* 3) *S. chirayita* 4) *S. purpurascens* 5) *S. angustifolia* after spraying with H_2SO4 (5%).
Solvent system-Chloroform: methanol: water (65:25:10) Plate 1, Ethyl acetate: methanol: water (22:2:1) Plate 2, Chloroform: methanol (8:2) Plate 3, **R**- Amarogentin (Reference).



TLC fingerprint profile of petroleum ether (Plates 7-9) and chloroform extract (Plates 10-11) of different *Swertia* species 1). *S. cordata* 2) *S. alata* 3) *S. chirayita*, 4) *S. purpurascens* 5) *S. angustifolia* after spraying with H_2SO_4 (5%).
Solvent system: Benzene ethyle acetate (9:1) Plate 7, Hexane: ethyl acetate (8:2) Plate 8, chloroform: methanol (9:0.5) Plate 9 Benzene: ethyl acetate (9:1) Plate 10 and Hexane: ethyl acetate (8:2) Plate 11.

Table 1: TLC details of methanol extract of *Swertia* species visualized under normal light after spray with H_2SO_4 (5%).

Solvent systems	No of spot detected	Rf values	*S. cordata*	*S. alata*	*S. chirayita*	*S. purpurascens*	*S. angustifolia*
Chloroform: methanol: Water (65: 25: 10)	7	0.30	×	√	×	x	x
		0.34	√	√	√	√	√
		0.37	√	√	√	√	√
		0.44	×	x	√	√	×
		0.47	x	x	√	x	x
		0.49	x	x	√	x	x
		0.56	x	√	x	x	x
Ethyl acetate: Methanol: Water (22:2:1)	8	0.22	×	√	x	x	x
		0.38	√	√	√	√	√
		0.56	x	√	×	×	×
		0.67	×	x	√	×	×
		0.72	×	x	√	×	×
		0.73	×	√	x	×	×
		0.76	×	×	√	√	×
		0.88	×	√	×	×	×
Chloroform: methanol (8:2)	8	0.22	×	√	x	x	x
		0.38	√	√	√	√	√
		0.56	x	√	×	×	×
		0.67	×	x	√	×	×
		0.72	×	x	√	×	×
		0.73	×	√	x	×	×
		0.76	×	×	√	√	×
		0.88	×	√	×	×	×

Colour of all the spots brown

Table 2: TLC details of petroleum ether extract of *Swertia* species visualized under normal light after spray with H_2SO_4 (5%).

Solvent systems	No of spot detected	Rf values	*S. cordata*	*S. alata*	*S. chirayita*	*S. purpurascens*	*S. angustifolia*
Benzene: ethyl acetate (9:1)	**7**	0.18	√	√	√	√	√
		0.29	√	√	x	√	x
		0.42	x	√	x	x	x
		0.60	√	√	√	√	√
		0.69	√	√	x	√	√
		0.84	√	√	x	√	√
		0.90	√	√	x	√	x
Hexane: ethyl acetate (8:2)	**7**	0.38	√	√	√	√	√
		0.45	√	√	x	x	X
		0.50	x	√	x	x	x
		0.66	√	√	√	x	x
		0.71	x	x	x	x	√
		0.86	√	√	√	√	√
		0.93	√	√	√	√	√
Chloroform: methanol (9: 0.5)	**7**	0.45	√	√	√	√	√
		0.60	x	√	x	√	x
		0.63	x	√	√	x	x
		0.66	x	x	x	x	√
		0.81	√	√	√	√	√
		0.86	√	√	√	√	√
		0.93	√	√	√	√	√

Colour of all the spots purple

Table 3: TLC details of chloroform extract of *Swertia* species visualized under normal light after spray with H_2SO_4 (5%).

Solvent system	No of spot detected	Rf values	*S. cordata*	*S. alata*	*S. chirayita*	*S. purpurascens*	*S. angustifolia*
Benzene: ethyl acetate (9:1)	5	0.13	√	√	√	√	√
		0.24	√	x	√	x	x
		0.46	x	√	x	x	x
		0.63	x	√	x	x	x
		0.92	x	√	x	x	x
Hexane: ethyl acetate (6:4)	3	0.12	x	√	√	√	x
		0.53	x	x	√	x	x
		0.62	√	√	√	√	√

Colour of all the spots purple

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