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Research Letter

Determination of swertiamarin and amarogentin content and evaluation of antibacterial activity in Eastern Himalayan species of *Swertia* L.

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ABSTRACT: **Introduction:** The genus *Swertia* is known to contain many bioactive compounds having pharmacological activities. Biochemical fingerprinting can be useful in screening elite populations within and between *Swertia* species. The present work reports the estimation of two important bioactive compounds: swertiamarin and amarogentin and the evaluation of antibacterial activities in different species of *Swertia* collected from Eastern Himalayan regions of India. **Methods:** Chromatography was performed using a CAMAG HPTLC system for estimation of swertiamarin and amarogentin in *S. chirayita*, *S. bimaculata*, *S. dilatata*, *S. nervosa* and *S. paniculata*, collected from different regions of Eastern Himalayas. Separation was carried out on thin-layer chromatography aluminium plates pre-coated with silica gel 60 F_{254}, eluted with ethyl acetate-methanol-water (77:15:8 v/v/v). Antibacterial activity against selected human clinical pathogens was tested by the disc diffusion method. **Results:** This investigation reports for the first time, the presence of swertiamarin in *S. dilatata* which is conventionally considered as an adulterant species in the chirata trade. The high quantity of swertiamarin detected in *S. bimaculata* leaves (5.80%) of Mungpoo population suggests that this so called inferior species can be a potential and promising source of swertiamarin in herbal and pharmaceutical industries. Antibacterial activity against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* was observed in swertiamarin and amarogentin and in the plant extracts. **Conclusions:** Synthesis of swertiamarin in reproductive shoots of *S. bimaculata*, *S. nervosa*, *S. dilatata* and *S. paniculata* may provide a new source of biomass for future use. Activity against selected bacteria tested revealed promising antibacterial potential of the extracts of *Swertia*.

KEYWORDS: Amarogentin, antibacterial activity, Eastern Himalayas, high performance thin layer chromatography, *Swertia* species, swertiamarin

INTRODUCTION

Swertia L. is a genus of annual, biennial and perennial herbs belonging to the Gentianaceae family. More than 170 species of *Swertia* are found worldwide, that are distributed in the mountains of tropical Asia, Europe, America and Africa. Nearly 40 species are distributed in India of which 13 to 14 are found in the north-eastern region.[1–3]

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Among the different species reported in India, *Swertia chirayita* (Roxb.) H. Karst. (commonly known as chirata) holds the most prominent position based on its medicinal potential.[4] 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 the American pharmacopoeias.[5] *S. chirayita* has medicinal uses in liver disorders, indigestion, malarial fever, bronchial asthma, skin diseases.[6] It is said to possess anthelmintic, febrifugal, stomachic, laxative, anti-inflammatory, anti-diarrhoeal as well as anti-carcinogenic properties.[6,7] Various commercially available popular herbal preparations such as Ayush-64, Diabecon (Himalayan herbal care), Mensturyl syrup and Melicon V ointment (Cadila Parmaceuticals) contain chirata extracts owing to its antipyretic, hypoglycemic, antifungal and antibacterial properties.[8,9]

S. chirayita is considered to be virtually endemic to Himalayas[10] and has been enlisted in the list of natural habitats of prioritized species in the Western and Eastern Himalayan region by the National Medicinal Plants Board, Govt. of India.[11] It has been designated as critically endangered according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria.[3,9,12] The increasing demand for *S. chirayita* in Indian and world markets is a cause of major concern as most of the herbal industries collect it from the wild. Extensive collection and unscientific harvesting is causing the natural population of the plant in the wild to diminish continuously.[7] Hence, various other species of *Swertia* that are known to have some therapeutic potential against fever, dysentery, spasm, pain, malaria, are often used as substitutes or adulterants for *S. chirayita*.[13] *S. alata, S. angustifolia, S. bimaculata, S. ciliata, S. densifolia, S. elegans, S. lawii, S. minor, S. nervosa, S. paniculata, S. multiflora, S. cordata* are the commonly used adulterants and most of them are said to show much inferior biological activity when compared to *S. chirayita*.[8,14] Species of other genera such as *Andrographis paniculata, Exacum tetragonum, Exacum pedunculatum* and *Slevolgia orientalis* are also extensively used as substitutes or adulterants in the chirata trade.[14] Hence, confusion arises over authentic identification of the drug available in the market. This necessitates phytochemical marker studies to differentiate between *S. chirayita* and its adulterants.

The genus *Swertia* is known to contain many compounds that are responsible for various pharmacological activities like bitterness, antihelmintic, hypoglycemic and antipyretic properties.[5,15] These therapeutic properties are attributed to the iridoid and secoiridoid bitter principles, including amarogentin, swertiamarin, sweroside, amaroswerin and to other bioactive compounds of the herb such as xanthones, flavonoids, terpenoids.[1,8] Amarogentin and swertiamarin are two common secoiridoid glycosides found in *Swertia* and hence they can be used as reliable marker compounds for the screening of *Swertia* species to reduce intentional and unintentional mixing of adulterants. Amarogentin is considered to be the bitter most substance known[3,8] and acts as a digestive stimulant and gastroprotective agent. Swertiamarin is known to have antidepressant and anticholinergic activities.[16]

High Performance Liquid Chromatography (HPLC) screening of swertiamarin and amarogentin has previously been reported in *Swertia* species.[1,17–19] High Performance Thin Layer Chromatography (HPTLC) detection of swertiamarin and amarogentin has been conducted in *S. chirayita* and *S. cordata* samples of western Himalayas.[20] Detection of swertiamarin from *Enicostemma littorale* and *S. chirayita* has also been studied[16,21] although there is the need for a more extensive study comparing many species with the same technique.

Biochemical fingerprinting can act as a useful tool in screening elite populations within and between *Swertia* species in order to reduce intentional and unintentional mixing with other species in the market. To the best of our knowledge there are no reports on HPTLC determination of swertiamarin and amarogentin in the Indian Eastern Himalayan species of *Swertia*. With respect to the wide range of different pharmacological and medicinal actions of *Swertia*, an analytical assessment of this genus to identify populations with higher content of bioactive compounds must be considered of great importance. Recently we have reported a phytochemical analysis in Eastern Himalayan species of *Swertia* using the xanthone compound mangiferin as a marker compound.[23]

The present paper outlines the estimation of two important bioactive compounds, swertiamarin and amarogentin through HPTLC and the screening to evaluate the antibacterial activity of the crude extracts of different species of *Swertia* collected from the Eastern Himalayan regions of India.

MATERIALS AND METHODS

Sample collection

Whole plants of *Swertia chirayita* (Roxb.) H. Karst., *S. bimaculata* (Sieb. & Zucc.) Hook. f. & Thomson ex C. B. Clarke, *S. nervosa* (Wall. ex G. Don) C. B. Clarke, *S. paniculata* Wall. and *S. dilatata* C. B. Clarke., were collected during the months of September and November, from different natural habitats of the Eastern Himalayas, India. The collection details of the samples are summarized in Table 1. The plants were identified on the basis of morphological characters, herbarium specimens were prepared and authenticated by Prof. S. R. Yadav, Department of Botany, Shivaji University, Kolhapur. Voucher specimens have been deposited in the Herbarium of Shivaji University, Kolhapur, (SUK) Maharashtra, India.

Extraction and quantitative analysis of swertiamarin and amarogentin in plant samples by HPTLC

Root, leaf and reproductive shoots wherever available, were washed, oven dried (45 ± 2 °C) and then powdered in a grinder. A 500 mg amount of dried sample was defatted with 20 ml n-Hexane (AR grade; Spectrochem) for 48 h in 100 ml conical flasks. The residue was subsequently extracted with 20 ml methanol (HPLC grade; Spectrochem)

Table 1: Collection details of different species of *Swertia*

Sl. no.	Plant species	Population code	Area of collection	Altitude
1	*S. chirayita*	SC1	Lava (Darjeeling)	2200 m
2	*S. chirayita*	SC2	Ghum (Darjeeling)	2438 m
3	*S. chirayita*	SC3	Sillary Gaon (Darjeeling)	1829 m
4	*S. chirayita*	SC4	Lama Camp (Arunachal Pradesh)	2500 m
5	*S. chirayita*	SC5	Ramalingam (Arunachal Pradesh)	1800 m
6	*S. chirayita*	SC6	Bomdila (Arunachal Pradesh)	2530 m
7	*S. bimaculata*	SB1	Lava (Darjeeling)	2200 m
8	*S. bimaculata*	SB2	Mungpoo (Darjeeling)	1146 m
9	*S. bimaculata*	SB3	Lolegaon (Darjeeling)	1675 m
10	*S. nervosa*	SN1	Lava (Darjeeling)	2200 m
11	*S. dilatata*	SD1	Lava (Darjeeling)	2200 m
12	*S. paniculata*	SP1	Tiger Hill (Darjeeling)	2555 m

for 72 h. The extract was concentrated under vacuum, and dissolved in 5 ml HPLC grade methanol.

Chromatography was performed on 20 cm × 10 cm pre-coated silica gel 60 F_{254} TLC plates (Merck) of 0.25 mm layer thickness. A CAMAG HPTLC system (Muttenz, Switzerland) comprising a Linomat-5 automated sample applicator equipped with a 100 µl syringe, CAMAG TLC scanner with winCATS software (version: 1.4.6), a UV cabinet and a twin-trough glass tank was used for the analysis.

Standard solutions of swertiamarin (Day Natural) and amarogentin (Day Natural) were prepared (1 mg/ml) separately. Working solutions of 100 µg/ml (ie, 100 ng/µl) were prepared from both the standard solutions by diluting them with HPLC grade methanol.

For preparation of calibration curves of swertiamarin and amarogentin, different concentrations of working standard solution [1 µl (100 ng), 2 µl (200 ng), 3 µl (300 ng), 4 µl (400 ng), 5 µl (500 ng)] were applied on different tracks using a Linomat-5 applicator to obtain amounts in the range 100–500 ng/band. Peak areas were plotted against the corresponding concentrations and regression analysis was performed to generate the calibration equations. The calibration curves of both swertiamarin and amarogentin were linear in the range of 100–500 ng/spot with good correlation coefficients (Table 2). The Rf value of standard swertiamarin was found to be 0.4 and the spectra had a absorbance maxima at λ = 240 nm. The Rf value of standard amarogentin was found to be 0.6 with absorbance peaks at λ = 228 nm, 272 nm and 307 nm.

Known amounts of samples and standards were applied to the plates as bands of 6 mm width, 8 mm from the bottom of the plate, by the use of the CAMAG Linomat-5 automated TLC applicator, with nitrogen flow. Plates were developed with ethyl acetate–methanol–water (77:15:8, v/v/v),[21] as the mobile phase, in a tank pre-saturated

Table 2: Method validation data for quantification of swertiamarin and amarogentin by HPTLC

Method characteristic	Swertiamarin	Amarogentin
Rf	0.4	0.6
Linearity range (ng/spot)	100–500	100–500
Regression equation (via height)	Y = 3.563 + 0.1707 * X	Y = 13.7 + 0.3569 * X
Linearity (correlation coefficient)	0.99812	0.99894
Average recovery % (n = 3)	98.76	98.68
Standard Deviation %	3.02	1.90
LOD (ng/spot)	53.08	15.97
LOQ (ng/spot)	176.92	53.24
Repeatability (% RSD) (n = 3)	1.16	0.95
Reproducibility (% RSD) (n = 3)	1.40	1.17

n = Number of replica, LOD = Limit of detection, LOQ = Limit of quantification, RSD = Relative standard deviation

with mobile phase vapour for 30 mins. The development distance was 8.5 cm. After development, the plate was removed, dried, and spots were visualized under UV light. Quantitative evaluation of the plates were performed in reflectance/absorbance mode at λ = 228 nm for amarogentin and λ = 240 nm for swertiamarin, with a slit dimension of 5 mm × 0.45 mm, scan speed of 20 mm/s and data resolution at 100 μm/step.

The specificity, sensitivity, accuracy and precision of the HPTLC method were assessed (Table 2). The selectivity of the method was checked by analysis of standard compounds and samples. The bands of swertiamarin and amarogentin from sample solutions were identified by comparing the Rf values and spectra of the bands with that of the respective standards. The sensitivity of the method was determined with respect to the limit of detection (LOD) and limit of quantification (LOQ). The standard solutions were spotted in the range from 100–500 ng. LOD and LOQ were determined[22] from the slope (S) of the calibration curve and standard deviation (SD) of the blank sample using the following equations:

LOD = 3 × (SD/S) and LOQ = 10 × (SD/S).[22] Known quantities of swertiamarin and amarogentin were added to a pre-analyzed sample separately and the sample solutions with added analytes were analysed to determine the accuracy of the method. Three concentrations were tested at three levels (low, middle and high) and the mean recovery (%) was calculated. To determine the variations arising from the method, repeatability and reproducibility were expressed in the form of % RSD of method precision. Identical volumes of the standard solutions were applied three times on TLC plates and analysed by densitometry to determine intraday and intermediate precision of the method.

Disc diffusion test for antibacterial activity

The antibacterial activity of the pure compounds swertiamarin and amarogentin and that of the plant extracts was assessed against four bacterial species, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa*. The methanolic extracts (100 mg/ml) of *S. chirayita, S. bimaculata, S. nervosa, S. dilatata* and *S. paniculata* were air dried and dissolved in 1% DMSO (dimethylsulfoxide) to yield the final concentration of 100 mg/ml. Both methanolic and DMSO extracts of the samples were used for antibacterial assay along with the standards, swertiamarin (1 mg/ml in 1% DMSO) and amarogentin (1 mg/ml in MeOH). The bacterial inoculum was prepared from overnight-grown cultures (24 h) in Lysogeny Broth (LB) (Himedia). A 1 ml aliquot of inocula were added to 100 ml LB agar media and then poured into sterile petri dishes and allowed to solidify for approximately 45–60 min. Circular discs of 5 mm diameter were made from whatman no. 1 filter paper. Sterilized discs were impregnated with standard swertiamarin, amarogentin and plant extracts (1500 μg/disc). The discs were aseptically placed over LB agar, seeded with each of the test pathogens. The plates were incubated at 37 °C for 24 h and the zone of inhibition was measured (in mm diameter). Ampicillin (500 μg/disc) was used as positive control and 1% DMSO, MeOH and distilled water was used as negative control.

RESULTS

Detection and quantitation of swertiamarin and amarogentin in five species of *Swertia*

Root, leaves, and reproductive shoots of five species of *Swertia* were analysed for the presence of swertiamarin and amarogentin. Swertiamarin was detected in all plant parts of all the populations of *S. chirayita* collected. All samples of *S. chirayita* collected from Lava, Ghum and Sillary Gaon and reproductive shoots collected from Lama Camp and Ramalingam showed the presence of amarogentin (Tables 3 and 4).

In *S. bimaculata* all samples collected showed presence of swertiamarin (Table 3). Except root samples of Lava, Mungpoo and Lolegaon. Amarogentin was not detected in *S. bimaculata* samples of Lava, Mungpoo and Lolegaon. Detection of a high quantity of swertiamarin (5.80% in leaves of Mungpoo and 5.13% in leaves of Lava population) suggests that this species which has previously been described as inferior, can be a potential and promising source of swertiamarin. It is evident from the present study that *S. bimaculata* is capable of synthesizing considerable amount of swertiamarin. To our knowledge, this is the first study reporting the detection of swertiamarin in *S. bimaculata*. Thus, this species may be targeted for sustainable use, conservation and future biotechnological improvements.

Chemical analysis for swertiamarin and amarogentin, carried out for the first time in *S. nervosa* collected from Lava, revealed interesting results. Swertiamarin was detected in the reproductive shoots (0.15%), but not in the roots, whilst amarogentin was detected only in roots (0.06%) and not in reproductive shoots of *S. nervosa* collected from the same place (Tables 3 and 4). Our results indicate that the species should not be considered as only adulterant of *S. chirayita* due to its composition, but may be traded separately. The presence of mangiferin has also been reported in a separate study[23] in the same population collected from Lava.

Table 3: Swertiamarin and amarogentin content in vegetative parts of five species of *Swertia* collected from Eastern Himalayan region of India

Sl. no.	Plant species	Collected from	Population code	% of Swertiamarin (Mean ± SE, n = 3)		% of Amarogentin (Mean ± SE, n = 3)	
				Root	Leaf	Root	Leaf
1	*S. chirayita*	Lava	SC1	2.60 ± 0.040	1.66 ± 0.011	0.23 ± 0.002	0.24 ± 0.002
2	*S. chirayita*	Ghum	SC2	8.47 ± 0.095	4.67 ± 0.049	0.27 ± 0.022	0.32 ± 0.002
3	*S. chirayita*	Sillary Gaon	SC3	3.65 ± 0.140	2.37 ± 0.049	0.06 ± 0.005	0.23 ± 0.008
4	*S. chirayita*	Lama Camp	SC4	3.55 ± 0.041	2.11 ± 0.029	–	–
5	*S. bimaculata*	Lava	SB1	–	5.13 ± 0.068	–	–
6	*S. bimaculata*	Mungpoo	SB2	–	5.80 ± 0.117	–	–
7	*S. bimaculata*	Lolegaon	SB3	–	0.48 ± 0.006	–	–
8	*S. nervosa*	Lava	SN1	–	–	0.06 ± 0.010	–
9	*S. dilatata*	Lava	SD1	–	2.45 ± 0.010	–	–
10	*S. paniculata*	Tiger Hill	SP1	–	–	–	–

– = Not detected, SE = Standard error, n = number of replica

In *S. paniculata*, collected from Tiger Hill, 0.88% swertiamarin was detected in its reproductive shoots but amarogentin was not detected (Table 4).

All samples of *S. dilatata* showed an absence of amarogentin, whilst swertiamarin was detected only in the leaves (2.45%) and reproductive shoots (0.86%) of the Lava population (Tables 3 and 4).

Among the different Eastern Himalayan populations of *S. chirayita* collected, swertiamarin content varied from 1.65%–8.47%, while amarogentin content varied from 0.06%–0.32%. Variations in swertiamarin content ranged from 0.48%–5.80% among the different populations of *S. bimaculata*.

Potential antibacterial activity of extracts of *Swertia*

The antibacterial activity of the extracts of five species of *Swertia* tested against four bacteria is shown in Table 5. Both the pure compounds swertiamarin and amarogentin were found to inhibit the growth of *E. coli*, *P. aeruginosa* and *B. subtilis*. No zone of inhibition was observed against *S. aureus* in any of the plant extracts or standards. The results show antibacterial activity for *S. chirayita* and *S. dilatata* extracts against *E. coli, P. aeruginosa* and *B. subtilis; S. nervosa* against *P. aeruginosa* and *B. subtilis* and *S. paniculata* against *E. coli* and *P. aeruginosa*. Antibacterial activity of *S. bimaculata* was found only against *E. coli*. The highest zone of inhibition was observed against *P. aeruginosa* by the extracts of *S. chirayita*. Interestingly, these extracts were also found to possess considerably high content of

Table 4: Swertiamarin and amarogentin content in reproductive shoot samples from five species of *Swertia* collected from Eastern Himalayan region of India

Sl. no.	Plant species	Collected from	Population code	% of Swertiamarin (Mean ± SE, n = 3)	% of Amarogentin (Mean ± SE, n = 3)
1	*S. chirayita*	Lava	SC1	2.43 ± 0.072	0.21 ± 0.002
2	*S. chirayita*	Ghum	SC2	2.05 ± 0.135	0.23 ± 0.043
3	*S. chirayita*	Sillary Gaon	SC3	2.12 ± 0.034	0.19 ± 0.011
4	*S. chirayita*	Lama Camp	SC4	1.83 ± 0.014	0.15 ± 0.003
5	*S. chirayita*	Ramalingam	SC5	1.65 ± 0.057	0.07 ± 0.001
6	*S. chirayita*	Bomdila	SC6	1.67 ± 0.054	–
7	*S. bimaculata*	Lava	SB1	3.55 ± 0.069	–
8	*S. bimaculata*	Lolegaon	SB3	2.61 ± 0.008	–
9	*S. nervosa*	Lava	SN1	0.15 ± 0.023	–
10	*S. dilatata*	Lava	SD1	0.86 ± 0.050	–
11	*S. paniculata*	Tiger Hill	SP1	0.88 ± 0.006	–

– = Not detected, SE = Standard error, n = number of replica

Table 5: Antibacterial activity of extracts of *Swertia*

Samples	Diameter of zone of inhibition of bacteria (mm) (Mean ± SE, n = 3)							
	E. coli		*P. aeruginosa*		*B. subtilis*		*S. aureus*	
	DMSO	MeOH	DMSO	MeOH	DMSO	MeOH	DMSO	MeOH
S. chirayita	7 ± 0.058	7 ± 0.100	8 ± 0.100	8 ± 0.153	6 ± 0.058	5.5 ± 0.120	–	–
S. bimaculata	6.5 ± 0.203	6.5 ± 0.116	–	–	–	–	–	–
S. nervosa	–	–	6 ± 0.288	7 ± 0.145	6 ± 0.033	5.5 ± 0.067	–	–
S. dilatata	7 ± 0.153	–	6 ± 0.067	6 ± 0.100	6 ± 0.033	5.5 ± 0.033	–	–
S. paniculata	7 ± 0.133	6.5 ± 0.088	6.5 ± 0.203	6 ± 0.058	–	–	–	–
Swertiamarin	7 ± 0.100		7 ± 0.145		6.5 ± 0.088		–	
Amarogentin	8 ± 0.153		6 ± 0.067		5.5 ± 0.033		–	
Ampicillin	11 ± 0.100		16 ± 0.033		13.5 ± 0.088		25 ± 0.033	
Water	–		–		–		–	
1% DMSO	–		–		–		–	
Methanol	–		–		–		–	

– = No inhibition observed, SE = Standard error, n = number of replica

swertiamarin (4.67%) and of amarogentin (0.32%). The activities shown by the plant samples are comparable to that of the standards, although they are crude extracts and are expected to contain much lower amount of swertiamarin and amarogentin than that of the standards applied, and to contain bioactive compounds other than swertiamarin and amarogentin. Hence, further efforts are necessary to identify the synergy between different bioactive molecules contributing towards the antibacterial activities.

DISCUSSION

Identification of populations and species of *Swertia* with high contents of active principles is a prerequisite for sustainable utilization and harvesting of these indigenous herbs. Earlier work on *S. chirayita* collected from the Western Himalayas reports 0.76% amarogentin content and 1.22% swertiamarin content.[20] In the present study, a significantly high content of swertiamarin (8.47%) was obtained from the roots of *S. chirayita* collected from Ghum and 0.32% of amarogentin was obtained from the leaves of the same population. In *S. nervosa*, the amount of swertiamarin obtained (0.15%) from the reproductive shoots collected from Lava was found to be higher than that reported previously in the same species.[18]

Species of *Swertia*, other than *S. chirayita* are usually considered to be inferior with respect to the content of bioactive compounds. The present investigation reports the presence of swertiamarin in the reproductive shoots of all species of *Swertia* investigated that are conventionally considered to be adulterant species in chirata trade.

Since, variations in swertiamarin and amarogentin content have been observed between and within species, such studies can prove to be advantageous for identification of elite populations of *Swertia*. These findings can pave way for future exploitation and utilization of these species as potential alternatives of *S. chirayita*.

Exploration of antimicrobials from higher plants can lead to the development of phytomedicines against microbes. Antimicrobials obtained from plant sources have enormous therapeutic potential as they show lesser side effects in comparison to synthetic antimicrobials.[24] The present study of the antibacterial evaluation of some species of *Swertia* forms a primary platform for further phytochemical and pharmacological studies. Further research is necessary to determine the identity of the antibacterial compounds from within these plants and also to determine their full spectrum of efficacy.

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