Scientia Pharmaceutica (Sci. Pharm.) 73, 127-137 (2005)

Swertia chirata Buch.-Ham. ex Wall. (Gentianaceae), an endangered Himalayan medicinal plant: comparative study of the secondary compound patterns in market drug, in vitro-cultivated, and micropropagated field grown samples

Christoph Wawrosch [1], Andreas Hugh-Bloch [1], Kurt Hostettmann [2], Brigitte Kopp * [1]

[1] Department of Pharmacognosy, University of Vienna, Althanstr. 14, A-1090, Vienna, Austria

[2] Laboratoire de Pharmacognosie et Phytochimie, École de Pharmacie Genève-Lausanne, Université de Genevè, Quai Ernest Ansermet 30, 1211 Genève 4, Genève, Switzerland

Abstract

Samples of the Himalayan medicinal plant *Swertia chirata* obtained from a local market in Nepal, from a micropropagated field cultivated clone, and from two in vitro-clones were compared by means of HPLC. The substance patterns of methanolic and dichloromethane extracts of the in vivo grown materials showed good conformity while in the samples from tissue culture major compounds were missing. Our findings confirm that the secondary metabolism of in vitro-cultivated plants normally differs from that of plants in their natural environment. Furthermore, the compound pattern of plants produced through micropropagation and subsequently cultivated in the field is comparable to that of plants collected from the wild. As an alternative to the uncontrolled depletion of the natural resources a sustainable use of *Swertia chirata* could hence be achieved by controlled field culture of micropropagated plants.

Keywords

Swertia chirata, Gentianaceae, threatened plants, micropropagation, field culture, compound pattern

Introduction

East indian balmony or chirata (*Swertia chirata* Buch.-Ham. ex Wall., Gentianaceae) is a perennial herb native to the temperate Himalaya region and is found at altitudes between 1,200 to 3,000 m from Kashmir to Bhutan and in the Khasia mountains. The medicinal drug obtained from the whole dried plant has been highly used since centuries by the natives of India and Nepal. Amongst others, the drug possesses bitter tonic, febrifuge and laxative properties and is used against liver and skin diseases as well as illnesses of the respiratory tract [1-4]. Also, *S. chirata* is an important constituent of various Ayurvedic medicines [5] and is included in the British Herbal Pharmacopoeia 1983 [6]. The main compounds include the secoiridoid glucosides swertiamarine, sweroside, gentiopicroside, amarogentin and amaroswerin, and a number of tetrahydroxy-xanthone derivatives [7]. Various studies have shown extracts of the crude drug to possess anticholinergic, antiphlogistic, antidiabetic and CNS activities [7].

Due to indiscriminate and non-systematic collection of very large amounts of *S. chirata* many natural populations are severely threatened [8, 9]: the export trend ranges from 140 – 300 tonnes a year [10]. The production of crude drug through field cultivation, which would allow for the conservation and a sustainable use of chirata, is however depending on an efficient production of seedlings which is difficult by conventional techniques [11]. However, it has been demonstrated that the large scale production of *S. chirata* for field culture can be successfully accomplished through plant tissue culture [12].

The purpose of the present study was to compare the secondary compound pattern of drug material commonly found in the local market in Nepal with that of plants in axenic tissue culture as well as micropropagated plants of one clone which after hardening were cultivated in the field.

Results and Discussion

Methanolic and dichloromethane extracts of four samples of *Swertia chirata* were subject to HPLC analysis, namely: a) locally available market drug from Kathmandu (sample SC-KT) b) material obtained from two in vitro-clones (SC-90 and SC-234) and c) plants of clone SC-90 which were transferred ex vitro and harvested after ca. 17 months of growth in the field. Aerial parts and roots were analysed separately and the compounds used for comparison of the samples are listed in **Table 1**.

The methanolic extracts of the aerial parts of the two "ex vitro" samples correlated well, as did the two samples of the in vitro-clones. Comparing the HPLC-chromatograms of *Swertia chirata* (**Figure 1a**) from the micropropagated plants (SC-MP) and from the Kathmandu market (SC-KT) it can be seen that in the latter the concentration of xanthones (peaks 12 and 13) is increasing to the detriment of the corresponding xanthoneglycosides (peaks 4 and 6). The in vitro-cultures SC-90 and SC-234 did not contain the whole of the compounds that can be seen in the adult plants obtained from tissue culture: the bisxanthones (peaks 10 and 11) and desmethylbellidifolin (peak 12) can't be seen at all. On the contrary swertiamarin (peak 2), mangiferin (peak 3) and bellidifolin-8-O-glucoside (peak 6) already appear in high concentration.

The methanolic extracts of the roots did not coincide as well as the aerial parts. Especially within the area of retention times from 12 to 20 minutes the samples SC-MP and SC-KT revealed striking differences (**Figure 1b**). The former contained a remarkable quantity of various xanthone-O-glycosides which lack in the latter. Only swertiamarin and mangiferin (peaks 2 and 3) appeared in comparable concentrations. Both in vitro-cultures were quite poor in compounds and didn't even contain the main constituents swertiamarin and mangiferin. Similar to sample SC-MP desmethylbellidifolin-8-O-glucoside (peak 4) appeared as major peak.

No.	Retention times	Description
MeOH extracts		
1	5.6	monoterpene with 1 double bond
2	7.16	swertiamarin
3	8.53	mangiferin (1,3,6,7-tetrahydroxy-2-C-glucosyl-xanthone)
4	10.95	desmethylbellidifolin-8-O-glucoside (1,3,5-trihydroxy-8-O-glucosyl-xanthone)
5	15.16	isobellidifolin-8-O-glucoside
6	16.04	bellidifolin-8-O-glucoside (1,5-dihydroxy-3-methoxy-8-O-glucosyl-xanthone)
7	16.24	1,3,7,8-xanthone-O-glycoside with 1 OMe, 3 OH
8	17.98	amarogentin
9	18.16	1,3,7,8-xanthone-O-glycoside with 3 OMe, 1 OH
10	19.81	bisxanthone
11	22.16 + 23.82	swertipunicoside (1,5,8-trihydroxy-3-methoxy-7-(1',3',6',7'-tetrahydroxy-9'-oxo-4'-xanthyl) xanthone-2'-C-β-D-glucoside)
12	26.35	desmethylbellidifolin (1,3,5,8-tetrahydroxy-xanthone)
13	33.58	bellidifolin (1,5,8-trihydroxy-3-methoxy-xanthone)
14	38.09	decussatin (1-hydroxy-3,7,8-trimethoxy-xanthone
15	42.68	swerchirin (1,8-dihydroxy-3,5-dimethoxy-xanthone)
CH_2Cl_2 extracts		
A	7.2	swertiamarin
B	8.40	gentiopicroside
C	8.48	mangiferin
D	26.35	desmethylbellidifolin (1,3,5,8-tetrahydroxy-xanthone)
E	33.62	bellidifolin (1,5,8-trihydroxy-3-methoxy-xanthone)
F	38.68	decussatin (1-hydroxy-3,7,8-trimethoxy-xanthone)
G	42.71	swerchirin (1,8-dihydroxy-3,5-dimethoxy-xanthone)

Tab. 1. Main compounds in the MeOH and CH_2Cl_2 extracts of *S. chirata* used for a comparison of the various samples [13]

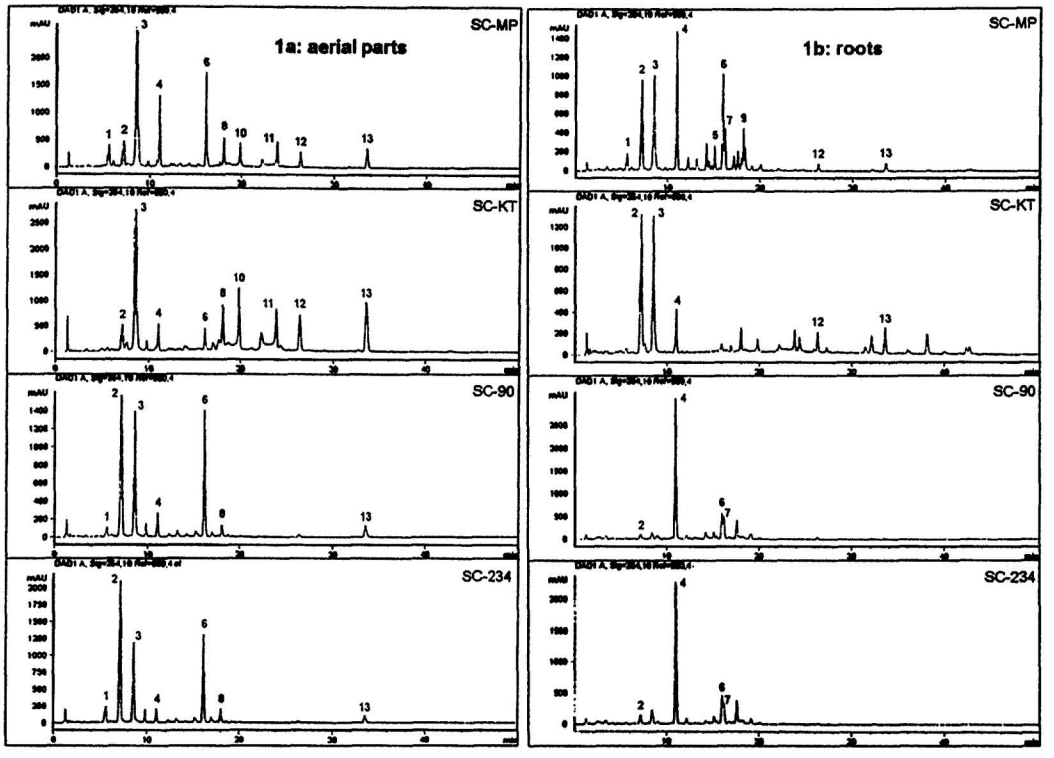

Fig. 1. HPLC chromatograms of methanolic extracts of aerial (**1a**) and root (**1b**) extracts of *S. chirata* (SC-MP: micropropagated field grown clone SC-90; SC-KT: market drug; SC-90 and SC-234: in vitro-clones).

Comparing the aerial parts' dichloromethane extracts of the field cultivated plants from tissue culture(SC-MP) and from Kathmandu (SC-KT) their compound patterns can be considered as nearly identical (**Figure 2a**). Both contained a prevailing quantity of bellidifolin (peak E) and 3 additional peaks, belonging to mangiferin, desmethylbellidifolin and swerchirin (peaks C, D and G). Deviating from that general pattern the extracts of the in vitro-clones possessed a remarkable concentration of swertiamarin (peak A). The root extracts (**Figure 2b**) again showed more obvious differencies and except some reappearing peaks an overlapping substance pattern was difficult to discover. Thus the extracts of the

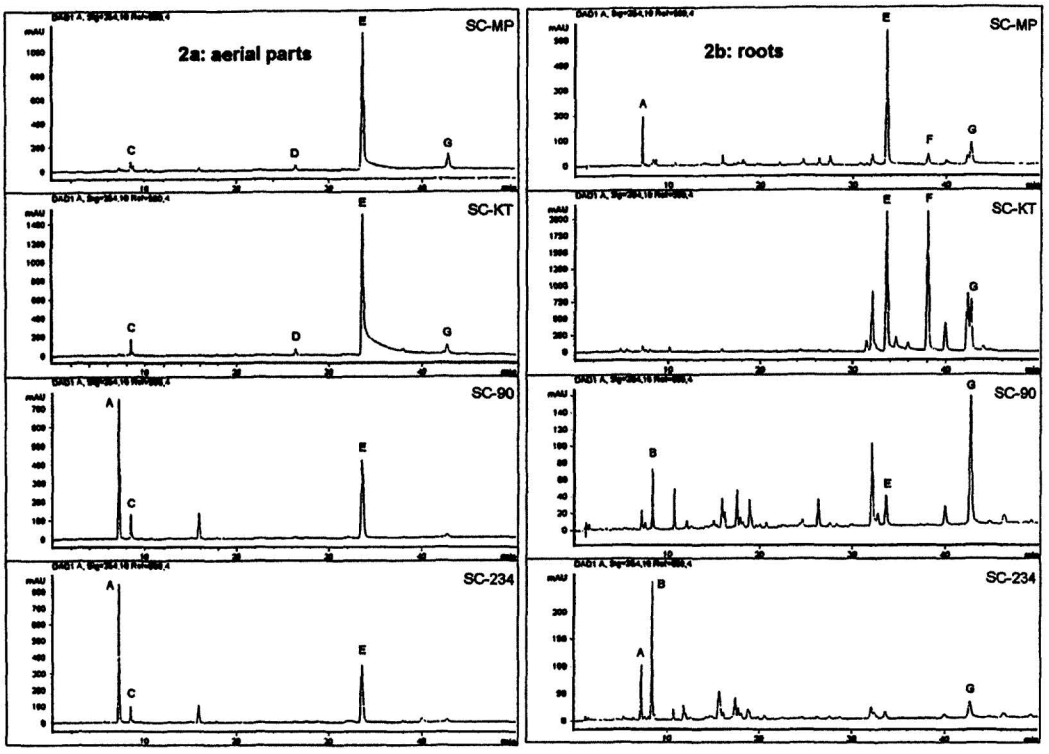

Fig. 2. HPLC chromatograms of dichloromethane extracts of aerial (**2a**) and root (**2b**) extracts of *S. chirata* (SC-MP: micropropagated field grown clone SC-90; SC-KT: market drug; SC-90 and SC-234: in vitro-clones)

ex vitro-grown samples SC-MP and SC-KT only contained 3 common major peaks. These were the xanthones bellidifolin, decussatin and swerchirin (peaks E to G). Apart from these correlations the former sample again exhibited a small peak belonging to swertiamarin (peak A). The in vitro-grown samples SC-90 and SC-234 were clearly distinguishable from the other two by containing only modest substance amounts, among which the secoiridoids swertiamarin (peak A) and gentiopicroside (peak B) as well as the xanthone swerchirin (peak G) should be mentioned. Several other peaks could be observed in low concentrations.

A comparison of secondary compound patterns of the four samples reveals that in general the correlations between the extracts of the aerial parts are better than in the case of the root extracts. Obviously changes of the environmental conditions, viz in vitro-culture vs. natural growth ex vitro, are rather reflected in the compound patterns of the underground organs. Moreover it can be seen that the adult plants, although grown in remote regions and under different climatic circumstances (Nepalese Himalayas for SC-KT and Central Europe for SC-MP), are distinguished by similar chromatograms, which sometimes are fully comparable. Certain substances like mangiferin, swertiamarin, bellidifolin and its glucoside reappear frequently and are likely to represent primary compounds, because they already can be found in the young in vitro-cultivated clones. Others like amarogentin, the bisxanthones and desmethylbellidifolin exclusively appear in the fully developed plants.

It is a well known fact that the secondary metabolism is coupled to the degree of differentiation of the plant as well as to the environmental conditions. This not only concerns the total amount of given compounds, but also substance patterns, as has for example been shown for *Tabernaemontana pandacaqui* [14] or *Artemisia dracunculus* [15]. Our results indicate a similar situation for *Swertia chirata*: although being of the same genotype (clone SC-90) in particular the MeOH extracts (**Figure 1**) of the field cultivated plants (SC-MP) differed considerably from those of the in vitro-cultures (SC-90). Certainly the environmental situation in a tissue culture container is very different from a natural environment, and the variety of lacking stress factors like heat, UV irradiation, drought, missing soil nutrients or microorganisms, only to mention a few, might be the reason for the differing compound patterns.

While the patterns of "naturally grown" material from the original habitat in the Himalayas correspond well to those of plants produced in vitro and subsequently grown out of doors for approx. one and a half years, slight differences were still observed. One reason might be that micropropagated plants require a period of time until they physiologically adapt to the natural environment ex vitro. This has for

example been described for peppermint where not until the second year after transfer to the field in vitro-produced plants showed an essential oil pattern typical for vegetatively propagated plants [16]. Furthermore it should be considered that our micropropagated plants were not cultivated in the environment where the natural populations are found. As stated above this might possibly affect the secondary metabolite pattern typical for the species. Finally, sample SC-MP consisted of genetically homogenous plants derived from one clone (which had been established from one seedling [12]) whereas the market sample has to be considered as a mixture of several individual genotypes, as it is usually the case with crude drug collected from the wild.

Nevertheless, our results indicate that crude drug of *Swertia chirata* from tissue culture is comparable to herb collected from wild populations. This might help in the future establishment of domestication programmes as alternatives to the ongoing exploitation of the natural resources.

Experimental

Plant materials

Locally available herb drug of *S. chirata* was purchased at the market in Kathmandu, Nepal (sample SC-KT). Two in vitro-cultivated clones (SC-90 and SC-234) were chosen at random from a stock of genotypes established from single seeds at the Institute of Pharmacognosy, University of Vienna. The protocols for establishment and in vitro-propagation have been described in detail by Wawrosch et al. [12]. For both clones plantlets were taken out of the culture containers and were air dried after thorough removal of adherent nutrient medium. Additional plantlets of clone SC-90 were transferred ex vitro to a plot in the medicinal plant garden at the Institute of Pharmacognosy, Vienna in June (early summer). Fully grown plants were subsequently harvested after 17 months of growth in the field and were air dried (sample SC-MP).

General equipment

HPLC: Hewlett-Packard-1050 instrument with a photodiode array detector. *LC/UV/MS*: Waters 600-MS solvent delivery system ; Waters Nova-Pak C 18 column (4 µm; 155 x 3.9 mm i.d.) preceeded by a Nova-Pak Guard RP-18 precolumn (7 µm); Hewlett-Packard-1050 photodiode array detector; *MS*: Finnigan-MAT-TSQ-700 triple quadrupole instrument equipped with a Thermospray (TSP) 2 (Finnigan-MAT) interface; *UV*: Waters 490-MS programmable multiwavelength detector.

Sample preparation

The aerial parts and roots of the plant materials were separately powdered and exhaustively extracted at room temperature under constant agitation. A threefold extraction with dichloromethane, each of them requiring 24 hours, was followed by the same procedure with methanol. The extracts were evaporated to dryness and lyophilized. For the analyses, the concentration was 10 mg/ml in $CHCl_3$ and MeOH (2:8) for the dichloromethane extracts and in MeOH for the methanolic extracts, respectively. The injection volume was 10 µl.

LC/UV/TSP-MS analysis

The extracts were separated using an acetonitrile/H_2O gradient (5:95 to 65:35 in 50 min, 1 ml/min, 0.05 % CF_3COOH). The UV spectra were recorded on-line from 190 to 600 nm. For the TSP-MS, the temperatures of the TSP were: source block 280 °C, vaporizer 100 °C, aerosol 280 – 300 °C (beginnng – end of gradient). The electron multiplier voltage was 1800 V, dynode 15 kV and the filament and discharge were off. Full scan spectra from m/z 150 and 800 in positive ion mode were obtained (scan time 1.2 sec). Post-column addition of buffer (ammonium acetate 0.5 M) was achieved by a Waters 590-MS programmable HPLC pump (0.2 ml/min).

All compounds were isolated and their structure elucidated at the Laboratoire de Pharmacognosie et Phytochimie, École de Pharmacie Genève-Lausanne,

Université de Genevè; the main compounds in the extracts were identified online on the basis of their UV and MS spectra as described earlier [13].

References

[1] Watt G.
A Dictionary of the Economic Products of India, Volume 6.
New Delhi: Periodic Experts, 1972:390-93.

[2] Kannay LD, Rai B.
The Indigenous Drugs of India, 2nd ed.
New Dehli: The Coronica Botanica, 1973:307.

[3] Kirtikar RK, Basu BD, An ICS.
Indian Medicinal Plants, 2nd ed. Volume 3.
Allahabad: Lalit Mohan Basu, 1991:1663-8.

[4] Ghosal S, Sharma PV, Chaudhari RK, Bhattacharya SK.
Chemical constituents of the Gentianaceae. V. Tetraoxygenated xanthones of *Swertia chirata* Buch.-Ham.
J. Pharm. Sci. 1973;62:926-30.

[5] Bajracharya MB.
Ayurvedic Medicinal Plants and General Treatment.
Kathmandu: Piyusavarsi Ausadhalaya, 1979:1-230.

[6] British Herbal Pharmacopoeia 1983.
Bournemouth: British Herbal Medicina Association, 1983:200-1.

[7] Blaschek W, Hänsel R, Keller K, Reichling J, Rimpler H, Schneider G, editors.
Swertia.
In: Hagers Handbuch der Phar, Praxis. 5th ed. Suppl. Volume 3, Drogen L-Z.
Berlin: Springer-Verlag, 1998:584-90.

[8] Rai LK, Prasad P, Sharma E.
Conservation threats to some important medicinal plants of the Sikkim Himalaya.
Biol. Conserv. 2000;93:27-33.

[9] Badola HK, Pal M.
Endangered medicinal plant species in Himachal Pradesh.
Curr. Sci. 2002;83:797-8.

[10] Adhikari AP.
Environmental Conservation and Economic Development in Nepal.
IUCN Nepal Newslett. 1999;3:5-8.

[11] Shrestha JN, Joshi SD.
Tissue culture technique for medicinally important herbs – *Orchis incarnata* and *Swertia chirata*.
Banko Janakari 1992;3:24-6.

[12] Wawrosch C, Maskay N, Kopp B.
Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch.-Ham. ex Wall.
Plant Cell Reports 1999;18:997-1001.

[13] Hugh-Bloch A.
Phytochemical Investigation *of Swertia chirata* (Gentianaceae) and Comparative Study of the Substance Patterns of the Original Plant with Samples Reproduced from Cell Cultures by Means of Hyphenated Techniques HPLC-UV and HPLC-MS.
Thesis, University of Vienna, 1998:1-115.

[14] Sierra MI, van der Heijden R, Schripsema J, Verpoorte R.
Alkaloid Production in Relation to Differentiation in Cell and Tissue Cultures of *Tabernaemontana pandacaqui.*
Planta Med. 1991;57:543-7.

[15] Cotton CM, Evans LV, Gramshaw JW.
The Accumulation of Volatile Oils in Whole Plants and Cell Cultures of Tarragon (*Artemisia dracunculus*).
J. Exp. Bot. 1991;42:365-75.

[16] Holm Y, Hiltunen R, Jokinen K, Törmälä T.
On the Quality of the Volatile Oil in Micropropagated Peppermint.
Flavour Fragr. J. 1989;4:81-4.

Received March 11th, 2005
Accepted July 3rd, 2005