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AFLP markers for identification of *Swertia* species (Gentianaceae)

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ABSTRACT. The genus *Swertia* is well known for its medicinal properties, as described in the Indian pharmacopoeia. Different members of this genus, although somewhat similar in morphology, differ widely in their pharmacological and therapeutic properties. The most important species of this genus, with maximal therapeutic properties, is *S. chirayita*, which is often adulterated with other less-potent *Swertia* spp. There is an existing demand in the herbal drug industry for an authentication system for *Swertia* spp, in order to enable their commercial use as genuine phytoceuticals. To this end, we used amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for six *Swertia* species. Nineteen accessions (2 of *S.*

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chirayita, 3 of *S. angustifolia*, 2 of *S. bimaculata*, 5 of *S. ciliata*, 5 of *S. cordata*, and 2 of *S. alata*) were used in the study, which employed 64 AFLP selective primer pairs. Only 46 selective primer pairs were found to be useful for all the accessions. A total of 5312 fragments were produced by these 46 primer pairs. Species-specific markers were identified for all six *Swertia* species (131 for *S. chirayita*, 19 for *S. angustifolia*, 181 for *S. bimaculata*, 47 for *S. ciliata*, 94 for *S. cordata*, and 272 for *S. alata*). These AFLP fingerprints of the *Swertia* species could be used to authenticate drugs made with *Swertia* spp and to resolve adulteration-related problems faced by the commercial users of these herbs.

Key words: Adulteration; DNA fingerprinting; Crude drug

INTRODUCTION

The genus *Swertia* (family Gentianaceae), founded by Linnaeus in 1753 in honor of Emanuele Sweert, an eminent Dutch gardener, comprises about 170 known species that are mainly native to temperate regions of the northern hemisphere (Brahmachari et al., 2004). About 40 species of *Swertia* are found in India, mainly at high altitude (1200-3000 m) in the temperate Himalayan region ranging from Kashmir to Bhutan, and also in the Khasia and Western Ghats hills (Chopra et al., 1956; Anonymous, 1982; Scartezzini and Speroni, 2000). Although plants belonging to various *Swertia* species have been described in different traditional medicine systems for use as crude drugs for the treatment of various ailments, *Swertia chirayita* occupies the most prominent position as regards its medicinal and therapeutic capabilities. It is traditionally used as a bitter tonic in stimulating appetite, as a febrifuge and for treating asthma and liver disorders (Brahmachari et al., 2004). Its medicinal potential is vividly described in various traditional medicine systems such as Ayurveda and Unani and in the American and British pharmacopoeias. The herb is in high demand as evident from its use in various commercially available popular herbal preparations such as Ayush-64, Diabecon, Mensturyl syrup, and Melicon V ointment (Joshi and Dhawan, 2005). However, this high demand of the drug has raised other concerns as well. Most of the herbal industries collect it from the wild, whereby the natural population of the plant in the wild is diminishing continuously, making it an endangered species (IUCN data). This in turn has created another problem, i.e., adulteration of the authentic drug with its less effective and often harmful substitutes. Species of other genera (*Andrographis paniculata*, *Exacum tetragonum*, *E. pedunculatum*, *Slevolgia orientalis*) as well as the genus *Swertia* (*S. alata*, *S. angustifolia*, *S. bimaculata*, *S. ciliata*, *S. densifolia*, *S. elegans*, *S. lawii*, *S. minor*, *S. paniculata*, *S. multiflora*, *S. cordata*) are used as adulterants for *S. chirayita* (Anonymous, 1982). Most of these adulterants/substitutes have inferior biological activity as compared to *S. chirayita*. Although *S. chirayita* has been morphologically and chemotypically differentiated from other species of *Swertia* (Bhatia et al., 2003), it is not possible to use these markers when the herb is present in the crude drug form. This problem could not be resolved by the isozyme markers that have been used to differentiate the *Swertia* species (Verma and Kumar, 2001). It is, therefore, an absolute necessity for the herbal drug industry to have DNA markers for

various *Swertia* species so as to differentiate and authenticate the herbal material when it is present in the form of a crude drug. Although molecular profiling of the "Chirayat" complex has been recently reported using inter-simple sequence repeat (ISSR) markers, the study generated very few polymorphic bands (Tamhankar et al., 2009). Hence, the present study was undertaken to generate amplified fragment length polymorphism (AFLP)-based DNA markers for 6 *Swertia* species (*S. chirayita*, *S. angustifolia*, *S. bimaculata*, *S. ciliata*, *S. cordata*, and *S. alata*) that are most commonly used in the herbal trade. Although previous attempts have been made to analyze the intra- and interspecific genetic diversity of *Swertia* species at the molecular level using ISSR DNA markers (Joshi and Dhawan, 2007a), this study is the first attempt to generate AFLP-based DNA markers for 6 prominent species of the genus *Swertia* that are commonly used in the herbal drug trade.

MATERIAL AND METHODS

Plant material

The plant material used in this study was collected from the Himalayan region in the Indian States of West Bengal, Sikkim, Uttarakhand, and Himachal Pradesh, and the herbarium was submitted to the National Gene Bank for Medicinal and Aromatic Plants at CIMAP, Lucknow (Table 1). Leaf samples from the selected plants were used for DNA isolation. The samples consisted of two accessions each of *S. chirayita*, *S. bimaculata* and *S. alata*, three accessions of *S. angustifolia* and five accessions each of *S. ciliata* and *S. cordata*.

Table 1. Details of the *Swertia* germplasm collection.

Sample No.	Name	Place of collection
1	*S. chirayita 1*	Sikkim
2	*S. chirayita 2*	Sukhia pokhari, Darjeeling, West Bengal
3	*S. angustifolia 1*	Tehri Garhwal, Uttarakhand
4	*S. angustifolia 2*	Kaddukhal, Chamba, Uttarakhand
5	*S. angustifolia 3*	Sukhia pokhari, Darjeeling, West Bengal
6	*S. bimaculata 1*	Darjeeling, West Bengal
7	*S. bimaculata 2*	Darjeeling, West Bengal
8	*S. ciliata 1*	Kandar, Suki, Uttarkashi, Uttarakhand
s9	*S. ciliata 2*	Raithal, Bhatwari, Uttarkashi, Uttarakhand
10	*S. ciliata 3*	Kandar, Suki, Uttarkashi, Uttarakhand
11	*S. ciliata 4*	Darjeeling, West Bengal
12	*S. ciliata 5*	Manibhanjan, Darjeeling, West Bengal
13	*S. cordata 1*	Raithal, Bhatwari, Uttarkashi, Uttarakhand
14	*S. cordata 2*	Chamba, Uttarakhand
15	*S. cordata 3*	Gangotri, Uttarakhand
16	*S. cordata 4*	Kumaon Himalaya, Uttarakhand
17	*S. cordata 5*	Manali, Himachal Pradesh
18	*S. alata 1*	Chamba, Uttarakhand
19	*S. alata 2*	Darjeeling, West Bengal

DNA isolation

DNA was isolated from the plant leaf samples using the protocol described by Khanuja et al. (1999), and its quality and quantity were analyzed using agarose gel electrophoresis and an ND-1000 spectrophotometer (NanoDropTechnologies, USA).

AFLP

For AFLP analysis, DNA was cleaved using the restriction endonucleases *Eco*RI and Tru9I (an isoschizomer of *Mse*I), and double-stranded adapters were ligated to the ends of DNA fragments, generating a template for subsequent polymerase chain reaction (PCR) amplification (preselective followed by selective). Restriction and ligation reactions were carried out simultaneously in a single reaction (Vos et al., 1995). To carry out the reaction, an enzyme master mix for 10 reactions was prepared containing 1 µL 10X T4 DNA ligase buffer, 1 µL 0.5 M NaCl, 0.5 µL 1 mg/mL BSA, 1 µL Tru9I (10 U/µL), 4.25 µL *Eco*RI (12 U/µL), 0.5 µL T4 DNA ligase (20 U/µL, high concentration) and 1.75 µL water. The restriction ligation reaction mix consisted of 300 ng DNA (5.5 µL), 1 µL 10X T4 DNA ligase buffer, 1 µL 0.5 M NaCl, 0.5 µL 1 mg/mL BSA, 1 µL *Mse*I adapter (Applied Biosystems, USA), 1 µL *Eco*RI adapters (Applied Biosystems) and 1 µL enzyme master, as described above. The reaction was then incubated overnight at room temperature and subsequently diluted 20-fold with $T_{10}E_{0.1}$ buffer. The ligated adaptors served as primer binding sites for low-level selection in the preselective amplification of restriction fragments. The *Mse*I complementary primer had a 3'-C and the *Eco*RI complementary primer had a 3'-A. Only the genomic fragments having an adapter on each end amplified exponentially during PCR. The preselective amplification mix was prepared by adding 4 µL of 20-fold diluted DNA from the restriction ligation reaction, 0.5 µL AFLP preselective primer (*Eco*RI, Applied Biosystems), 0.5 µL AFLP preselective primer (*Mse*I, Applied Biosystems) and 15 µL AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed as follows: 72°C for 2 min; 20 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min; 60°C for 30 min, and 4°C for infinity.

The preamplified DNA was diluted 20-fold with $T_{10}E_{0.1}$ buffer, and selective amplifications were carried out using different *Mse*I and *Eco*RI primer combinations (Applied Biosystems). Primers chosen for the amplification were from 16 available AFLP selective primers (8 fluorescently tagged *Eco*RI and 8 untagged *Mse*I primers). The *Eco*RI primers contained 3 selective nucleotides with the sequence 5' [Dye-Primer-Axx]-3', while the *Mse*I primers had the 3 selective nucleotides starting with C, i.e., 5'-[Primer-Cxx]-3'. Selective amplification of each sample was done with all 64 (8 x 8)-primer combinations (*Mse*I/*Eco*RI) using multiplex-PCR. For selective amplification, the reactions were set up as follows: 3 µL of 20-fold diluted preselective amplification product, 15 µL AFLP core mix, 1 µL *Mse*I primer 5'-[Primer-Cxx]-3', and 1.5 µL *Eco*RI primers 5'-[Dye-Primer-Axx]-3' (0.5 µL 3 *Eco*RI primers each were pooled here). Selective amplification was carried out in a thermal cycler programmed as follows: 94°C for 2 min; 10 cycles of 94°C for 20 s, 66°C (-1°C/cycle) for 30 s, and 72°C for 2 min; 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min; 60°C for 30 min, and 4°C for infinity. The samples were loaded onto a 5% (29:1) polyacrylamide gel on an ABI Prism 377 DNA Sequencer (Applied Biosystems, USA). For gel electrophoresis, 3 µL of the selective amplification reaction

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product was mixed with 4 μL loading buffer [ROX500 size standard (10%), blue dextran (10%), and deionized formamide (80%)], and 1.5 μL of this mix was finally loaded on the gel. The AFLP amplification modules and the guidelines supplied by Applied Biosystems, USA, were used for setting up the reactions as described above.

Data analysis

Fragment analysis was carried out for bands in the range of 35-400 bp. For diversity analysis, bands were scored as present (1) or absent (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained with Jaccard's similarity coefficient (Jaccard, 1908) using the SPSS v 7.5 software. The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (unweighted pair group method with arithmetic mean) method using NTSys v 2.1.

RESULTS AND DISCUSSION

In the AFLP analysis, of the 64 primer pairs, only 46 responded positively and generated discrete bands with all the plant samples. Of a total of 5312 bands, 8 were monomorphic and 5304 were polymorphic. A polymorphism of 99% was detected among the species. A total of 743 bands were found to be unique for various *Swertia* species. In this analysis, species-specific markers were identified for the 6 *Swertia* species (131 for *S. chirayita*, 19 for *S. angustifolia*, 181 for *S. bimaculata*, 47 for *S. ciliata*, 94 for *S. cordata*, and 272 for *S. alata*) (Table 2). The cluster diagram obtained after analysis indicated 6 major groups, each representing one of the 6 different species of *Swertia* used in the study, as expected (Figure 1). In the first group, two accessions of *S. chirayita* clustered together, showing 46% similarity. The second group consisted of the three accessions of *S. angustifolia* grouping together with 20% similarity within the cluster. Similarly, five accessions of *S. ciliata*, showing 40% similarity, two accessions of *S. alata* showing 58% similarity and two accessions of *S. bimaculata* showing 62% similarity, clustered into the third, fourth and fifth groups, respectively. The last group (sixth) consisted of five accessions of *S. cordata* showing 47% similarity among the accessions. The results obtained in the present study clearly showed that *S. cordata* and *S. angustifolia* did not cluster together as found in a previous ISSR-based study carried out by Tamhankar et al. (2009).

In a previous study using ISSR markers, Joshi and Dhawan (2007a) found 98.7% polymorphism among 19 genotypes of *Swertia* species (13 of *S. chirayita* and 2 each of *S. cordata*, *S. paniculata* and *S. purpurascens*). They also found through ISSR analyses that the clonal/genetic fidelity of *S. chirayita* is maintained in micropropagated plantlets generated using an *in vitro* multiplication method (Joshi and Dhawan, 2007b). The genus *Swertia* has been described in the literature as being highly polyphyletic (von Hagen and Kadereit, 2001). Considering the range of different niches occupied by the plant, there is a possibility that many ecotypes and/or chemotypes of even a single species such as *S. chirayita* exist in nature (Joshi and Dhawan, 2005). When making use of the plant for commercial purposes as an herbal drug, it is very important to identify the correct chemotype having the maximal content of the therapeutically significant secondary metabolites. This identification requires the use of molecular markers that are unique to the relevant plant and that are stable under different conditions (plant age, environment, etc.). DNA markers are best suited to serve this purpose.

Table 2. Unique AFLP marker fragments for the 6 *Swertia* species.

Primer combination *Mse*I/*Eco*RI	Unique bands of *Swertia chirayita*	Unique bands of *Swertia angustifolia*	Unique bands of *Swertia bimaculata*	Unique bands of *Swertia ciliata*	Unique bands of *Swertia cordata*	Unique bands of *Swertia alata*
CAA/ACT	69, 102, 108, 151, 332	-	96, 97, 144, 154, 166	68, 190, 303	58, 280, 284, 308, 392	48, 65, 81, 110, 111, 148, 198, 199, 243, 244, 251, 252, 253, 254, 330, 337, 376, 377
CAA/AAC	-	-	55, 60, 193	-	307	46, 300
CAA/ACA	63, 70, 99, 345	-	103, 163, 187, 193, 194	-	-	45, 90, 183, 252, 277, 278, 298, 299, 318
CAA/ACC	114	-	149	331	253	46, 77, 159, 175, 184
CAA/ACG	-	-	-	-	-	128, 168
CAA/AGG	145	-	182, 197, 260	84, 127, 138, 156	55, 132, 183	45, 74, 109, 133, 147, 153, 257, 281
CAC/ACA	-	200	64, 174, 181, 210, 229, 338	111	117, 185, 204, 260	50, 77, 193, 256, 257, 258
CAC/ACT	128, 139, 143	-	-	-	52	251
CAC/AAC	154	-	-	-	-	-
CAC/ACC	75, 163, 250	-	98, 292	238	285	149, 309
CAC/AGG	250, 334	57	79, 102, 103, 122, 153, 236, 253	183	292, 308	127
CAC/AGC	123, 209	41, 61, 321	-	-	-	-
CAC/ACG	98, 168, 334	-	-	-	109	40, 171
CAG/ACT	47, 84	82	54, 60, 88, 92, 103, 127, 134, 175, 193, 205, 208, 214, 224, 234, 249, 294, 304, 332, 343, 345	61, 236, 247	139	110, 117, 173, 230, 259, 289, 349
CAG/ACC	43, 44, 56, 75, 79	-	193, 194, 198	248	163	73, 78, 156, 190, 264, 266, 279
CAG/ACA	77	-	-	69, 247, 256	-	39, 43, 64, 117, 228, 240, 310
CAG/AGG	128, 171, 230	-	95, 224, 229, 309	300	198	93, 148, 156, 201
CAT/ACT	102, 142, 146, 160, 201, 207, 224, 225	-	56, 63, 87, 116, 186, 208, 213, 257, 319	50, 153, 301	57, 58	44, 45, 53, 59, 132, 241, 309, 359
CAT/AAC	224	-	198, 216, 361	52	119, 281, 324	47, 57, 83, 152, 308, 309

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Table 2. Continued.

Primer combination *Mse*I/*Eco*RI	Unique bands of *Swertia chirayita*	Unique bands of *Swertia angustifolia*	Unique bands of *Swertia bimaculata*	Unique bands of *Swertia ciliata*	Unique bands of *Swertia cordata*	Unique bands of *Swertia alata*
CAT/AAG	-	-	179, 274	-	-	49, 273
CAT/AGG	67, 76, 119, 125, 153	146, 147	83, 180, 215, 216, 217, 231, 315, 340	-	-	207, 212, 269, 273
CAT/AGC	62, 82, 144, 157, 168, 205	-	66, 80, 99, 190, 257, 288	-	48, 165, 203, 241, 281, 289, 328	77, 89, 107, 115, 191
CAT/ACG	163, 202	146, 147, 177	78, 125, 144, 183, 274, 318	132	59, 65, 92, 140, 280, 322, 375	43, 73, 113, 116, 138, 173, 194, 207
CTA/ACT	187, 289	-	232, 233	176	189, 190	39, 81, 94, 110, 118, 142, 157, 211, 235, 253, 273, 312
CTA/AAC	81, 114	-	106	-	-	200, 266
CTA/AAG	114, 121, 264, 361	268, 269	214, 216	69, 76	52, 159, 202, 382	144, 166, 242, 254, 276
CTA/ACA	57, 185, 198, 335, 374	-	72, 286, 327	224	192, 207	69, 86, 87, 172, 181
CTA/ACC	-	-	149, 298	127	64	195, 196
CTA/AGG	70, 210, 247, 249, 267	-	65, 107, 139, 284, 286, 287, 296, 397	87	132, 184, 186,	144, 167
CTA/AGC	55, 72, 79, 87, 180, 233	-	49, 185, 192, 193, 208, 209, 214, 296, 297	-	64, 350	50, 61, 62, 94, 201, 244
CTC/AAC	138, 197, 203, 255	-	51, 124, 158	135, 264	60, 106, 163, 252	205
CTC/AAG	-	267	349, 350	251	122, 164	99, 108, 140, 161, 241, 259, 266, 324
CTC/ACA	41, 123	-	75, 86, 91, 165	-	46, 183	60, 61, 64, 65, 113, 122, 132, 156, 161, 184, 217, 274, 294, 335, 338
CTC/ACC	48	-	59, 243	196	116, 190	106, 160, 207, 209, 211, 238
CTC/AGG	-	267	54, 101, 198	251	59, 122, 205	60, 63, 99, 118, 119, 129, 141, 153, 161, 169, 191, 203, 242, 259, 266, 324
CTC/AGC	101, 118, 130, 255, 291, 292, 337	68	79, 198, 223, 304	122, 151, 262	92, 104, 106, 116, 253, 349	75, 76, 163, 174, 182, 183, 204, 219, 239, 286, 344, 374, 391
CTG/AAC	76, 112, 206, 218	-	69, 161, 167, 321	50, 128, 156	-	193

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Table 2. Continued.

Primer combination *Mse*I/*Eco*RI	Unique bands of *Swertia chirayita*	Unique bands of *Swertia angustifolia*	Unique bands of *Swertia bimaculata*	Unique bands of *Swertia ciliata*	Unique bands of *Swertia cordata*	Unique bands of *Swertia alata*
CTG/ACA	191	100	42, 273, 285	162	82, 115	45, 55, 74, 123, 131, 134, 157, 177, 178, 179, 194, 197, 212, 245, 261, 267, 288, 298, 313, 316, 328, 329, 330
CTG/ACC	-	-	85	71	64	46, 47, 142, 143, 159, 217, 311
CTG/AGG	134, 142, 277	112	44, 77, 87, 162, 183, 187, 240, 248, 261, 269, 338	-	63, 67	69, 81, 105, 171, 194, 210, 211, 221, 231, 256, 288
CTG/AGC	78, 116	-	85, 89, 181, 182	75	-	61
CTT/ACT	79, 131, 206, 224, 228, 246, 270, 288, 305, 313, 338, 363, 365	-	137, 179, 204, 205, 297	378	77, 175, 211, 230, 397	59, 111, 123, 245, 278, 287, 291
CTT/AAG	272	-	45, 93	-	78, 242	253
CTT/ACA	53, 74, 95, 114, 183, 316	-	48, 78	-	165, 199	157, 182, 261
CTT/ACC	129, 297, 311	48	55, 123, 146, 184, 186, 232, 330	56	61, 112, 131, 286, 298, 322	41, 107, 111, 189, 208, 235
CTT/AGC	193, 311	-	63, 94, 156, 252	199	-	68, 169, 214, 262, 283
Total	131	19	181	47	94	272

The size is reported in bp.

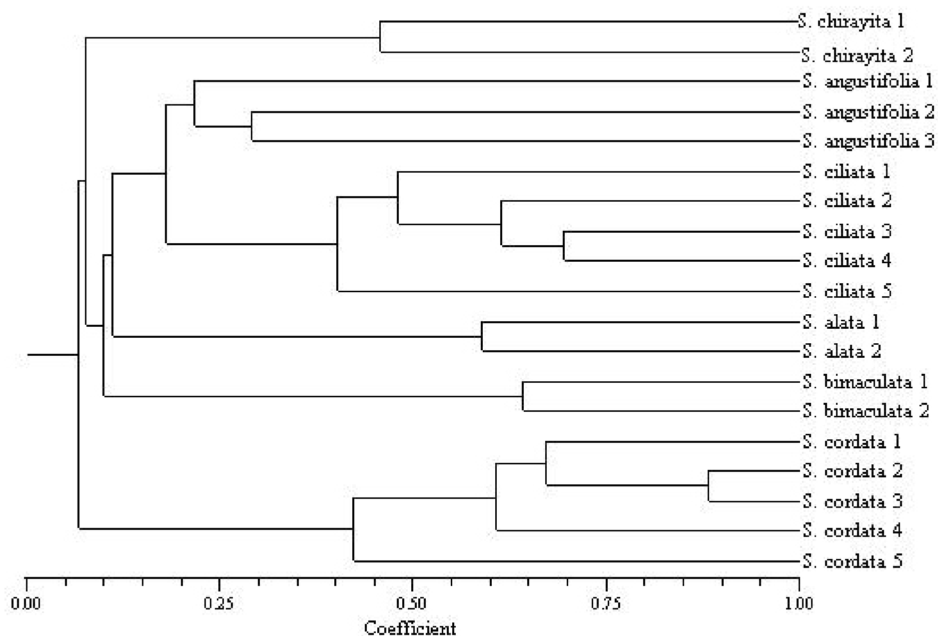

Figure 1. Cluster diagram showing relationship among 6 *Swertia* species.

The DNA molecular markers (unique bands) for *Swertia* species generated in the present study (Table 2) would provide a useful reference tool to identify the herbal material when present in the form of crude drug and would circumvent the problems associated with morphological, chemotypic and isozyme markers. The frequency of the occurrence of these unique bands in the analysis of the DNA isolated from the crude drug preparation could be used in an assay for the presence of a specific species population. In the past, AFLP and other DNA markers have also been used to resolve complex polyherbal mixtures and to identify specific species present in them. In an earlier study, we used the same approach to resolve the "Safed Musli" complex and detect the presence of adulterants in crude drug preparations of the herb, which is commonly known to contain *Chlorophytum* species along with *Asparagus adscendens* (Misra et al., 2007). Recently, species-specific SCAR markers have been used to tag specific *Phyllanthus* species that are prevalent in the herbal drug trade (Jain et al., 2008). AFLP, in particular, has been the method of choice for discriminating closely related species and authentication of herbs, as demonstrated for *Plectranthus* genus in an earlier study (Passinho-Soares et al., 2006). In the present study, too, a well-defined grouping pattern was obtained for all 6 *Swertia* species analyzed. The significance of this study stems from the fact that it provides an authentication tool to detect adulterants in the crude drug preparations of *Swertia* and to maintain the quality standards in the herbal drug industry.

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