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In Vitro Production and Efficient Quantification of Major Phytopharmaceuticals in an Endangered Medicinal Herb, Swertiachirata

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Abstract

Swertiachirata is an endangered medicinal herb with a wide range of pharmacological activities, particularly as an anti-diabetic in an herbal formulation Diabecon (Himalaya®). The endangered status has resulted in short supply of plant material, thereby, resulting in adulteration practices. It is, therefore, of paramount importance to develop a shoot biomass production system enriched with major chemical constituents, swertiamarin, mangiferin and amarogentin and their rapid quantification. In vitro shoot multiplication occurred within 22-25 days on MS medium supplemented with KN (2mg/L) + GA3 (3mg/L) + IBA (2mg/L) + sucrose 3% (w/v). Swertiamarin content was highest (9.33µg/mg) in nutrient medium containing IBA (2mg/L) and GA3 (3mg/L) at 15±1°C after 30 days of inoculation. Mangiferin and amarogentin contents were highest, 2.99 µg/mg and 1.03 µg/mg, respectively in cultures grown in nutrient media containing IBA(2mg/L) and KN (2mg/L) at 25±1°C in 30 days.

We have optimized a highly sensitive and efficient HPLC method for the quantification of these medicinal compounds simultaneously for quality control and plant material standardization. The retention for peak due to swertiamarin, mangiferin and amarogentin was about 8.5 min, 13.6 min and 21.7 min, respectively. The quantification method is based on rapid ultra-sonic wave assisted extraction with sharp peaks for high limit of detection(LOD) and high limit of quantification(LOQ) simultaneously in 45 minutes of time duration contrary to previous methods.

Keywords: Swertia chirata, In vitro production, Quantification, HPLC.

1. Introduction

Swertiachirata(Gentianaceae) is a medicinal plant indigenous to temperate Himalaya, found at an altitude of 1200–3000 m (4000 to 10,000 ft), from Kashmir to Bhutan, and in the Khasi hills at 1200–1500 m (4000 to 5000 ft). *S. chirata* has an erect 2–3 ft long stem. The plant has ample metabolites which are medicinally very important having anti-inflammatory, hepatoprotective, anti-cholinergic and immunomodulatory properties, which are credited to major secondary metabolites such as swertiamarin, mangiferin and amarogentin(Joshi and Dhawan, 2005). Its bitterness, antihelmintic and hypoglycemic properties are attributed to amarogentin (most bitter compound isolated till date), swerchirin, swertiamarin and other active principles compounds of the herb. Amarogentin is secoiridoid glycoside, and is the most acerbic substance found. Swertiamarin is a secoiridoid glycoside having CNS depression, (Bhattacharya *et. al.*, 1976) antioxidant, antiediematogenic, hepatoprotective, anti-inflammatory and anti-nociceptive (Jaishree and Badami, 2010; Jaishree *et. al.*, 2009). Mangiferin possesses strong hepatoprotective, antidiabetic(Ichiki *et. al.*, 1998; Miura *et. al.*, 2001a; Miura *et. al.*, 2001b), gastroprotective(Carvalho *et. al.*, 2007; Chakravarty *et. al.*, 1994)antiinflammatory activity in arthritic mice and accounted for lowering down TNF-alpha, IL-1beta, IL-6, and IFN-gamma and up regulation of IL-10 in the joint homogenates of mice. It is also found to be a strong chemoprotective agent, antiproliferative(Andreu *et. al.*, 2005b; Saha *et. al.*, 2006), antidiuretic, antiviral, cardiotonic and immunomodulatory activities (Andreu *et. al.*, 2005a; Guha *et. al.*, 1996; Yoshimi *et. al.*, 2001; Zheng and Lu, 1990) . Herbal medicines such as Ayush-64, Diabecon, Mensturyl syrup and Melicon V ointment contain *Swertiachirata* extract in different amounts. The usage of its metabolites in the pharmaceutical industry leads to the reckless collection of *Swertiachirata* and reduces its populations to very low levels resulting in its categorization as an endangered species, thereby, warranting the development of alternative strategies for the production of metabolites of medicinal value.

Swertiachirata is a slow growing species with low germination percentage and long gestation period (Abrol *et. al.*, 2012). Reckless collection from wild has considerably decreased its natural populations and it has now been listed in critically endangered category according to International Union for Conservation of Nature (IUCN) criteria. *In vitro* propagation is a viable alternative for production of its bioactive constituents and a ex situ conservation measure (Koul *et. al.*, 2009). Due to their spatial, developmental and ecological requirement, production of bioactive secondary metabolites is low, often 1% of the dry weight. Plant tissue culture is an alternative for sustainable production of uniform quality of these compounds. Elicitation with stressful growth environment has been widely used to enhance the production of secondary metabolites *in vitro*(Vasconsuelo and Boland, 2007).

Domestication of plants using conventional techniques has not yet been successful. A reduced span of seed viability and low germination rate restrict its propagation through seeds. *S. chirata* is difficult to propagate on mass scale via seed sowing due to non-availability of seeds or due to harvesting of plants before the seeds mature. The species is, therefore, deprived of natural regeneration (Pant *et. al.*, 2010). Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technology development and commercial cultivation of the species. The plant material available in the market many a times is adulterated and substituted by close relatives of *S. chirata*. All these aspects account to the need for the *in-vitro* propagation of *S. chirata*and the enhancement of metabolites produced *in vitro* shoot cultures.

2. Materials and Methods

2.1 Sterlization of explant and culturing conditions

Seeds were washed with distilled water to remove dirt and debris and surface sterilization was carried out by trying different concentrations and combinations of Bavistin and Mercuric chloride along with 70% ethanol for different time durations followed by 4-5 washings in distilled sterile water. Surface sterilized seeds were kept in autoclavablepetriplates having filter papers in culture room at 25±1°Cby maintaining optimum moisture. Germinated seeds were transferred in the jars containing 40 ml MS-basal medium supplemented with different growth hormones along with different combinations and concentrations of KN, GA3 and IBA. Basal MS Medium without PGRs was used as the control. This study was carried out at two different temperatures 15±1°C and 25±1°C in the standardized culture room conditions.

2.2 In vitro shoot induction and elicitation of metabolites

Data was collected for shoot growth in different shoot proliferation media (M1-M10) for number of shoots, shoot length,leaf area and shoot biomass. The in vitro grown shoots were exposed to different concentrations of the abiotic elicitors such as chitosan(50 and 100mg/L), methyl jasmonate (25 , 50 , 75 µM), salicylic acid (10mM , 20mM ,30mM), vanadylsulphate (10mM , 20mM ,30mM), biotic elicitors such as Agrobacterium rhizogenes(10, 20µL)and yeast extract (0.2mg/L) and growth hormones to study the metabolite variation in the plantlets. Along with that the plant was exposed to Hoagland solution alone and with Hoagland solution supplemented with yeast extract. For elicitation liquid MS media with 3% sucrose content was prepared in jars with corresponding concentration of the elicitor. The shoots were allowed to grow in the presence of the elicitor for 30 days before harvesting. Observations were recorded and the sampling was done every 10days for a month and the plantlets were stored at -80^{0}C for HPLC. HPLC analysis was done for evaluating the metabolite content.

2.3 HPLC sample preparation and analysis

The accumulation status of metabolites was determined in plants by subjecting samples from different experiments to chemical analysis. The quantification of swertiamarin, mangiferin and amarogentin was carried by reverse phase High Performance Liquid Chromatography (HPLC Waters 515). The plant material was ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was sonicated for 10 min. Following day, the samples were centrifuged at 10,000 rpm for 10-15 min. and the supernatant was filtered through 0.22µ filter. A novel HPLC method was developed and optimized for rapid and simultaneous quantification of major three metabolites (Figure 1, 2). The plant samples were extracted with 80% methanol in a bath sonication for rapid extraction of metabolites. The desired separation and baseline was obtained in a gradient method where mobile phase A was composed of 0.1% TFA in water and mobile phase B was a mixture of acetonitrile: water in the ratio of 70:30. The linear gradient at a flow rate of 1.0ml/min was start with 15% B; 20% B in next 5 min, 70% B in next 25 min, hold for 5 min; 15% B in next 5 and equilibrated for 5 min at 240 nm UV wavelength. The separation was obtained on Waters Spherisorb 5µm ODS2 (250 mm x 4.6 mm). The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex Inc. The quantification was repeated twice for each sample and the data were interpreted in terms of amount of swertiamarin, mangiferin and amarogentin present.

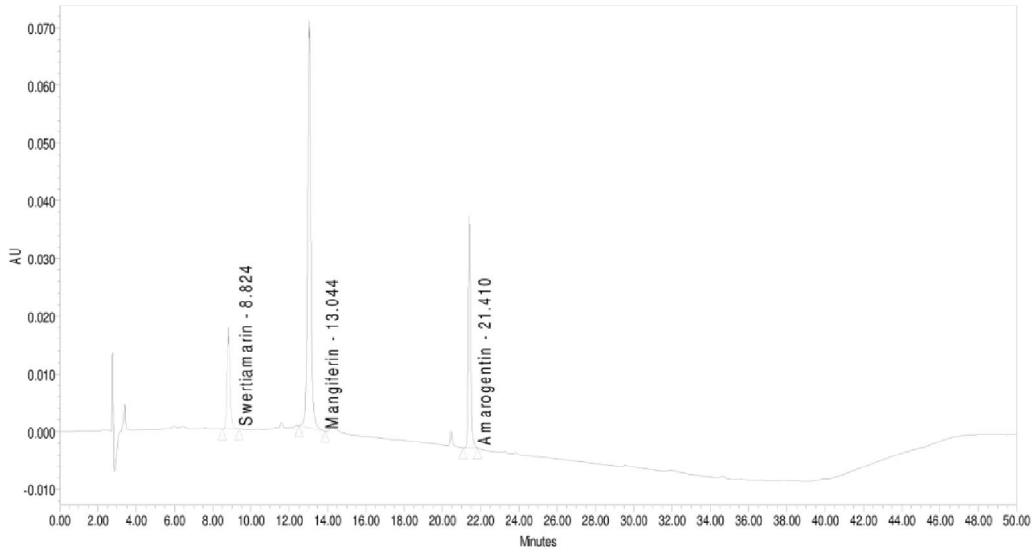

Figure 1: HPLC chromatogram showing standard peaks due to swertiamarin, mangiferin and amarogentin.

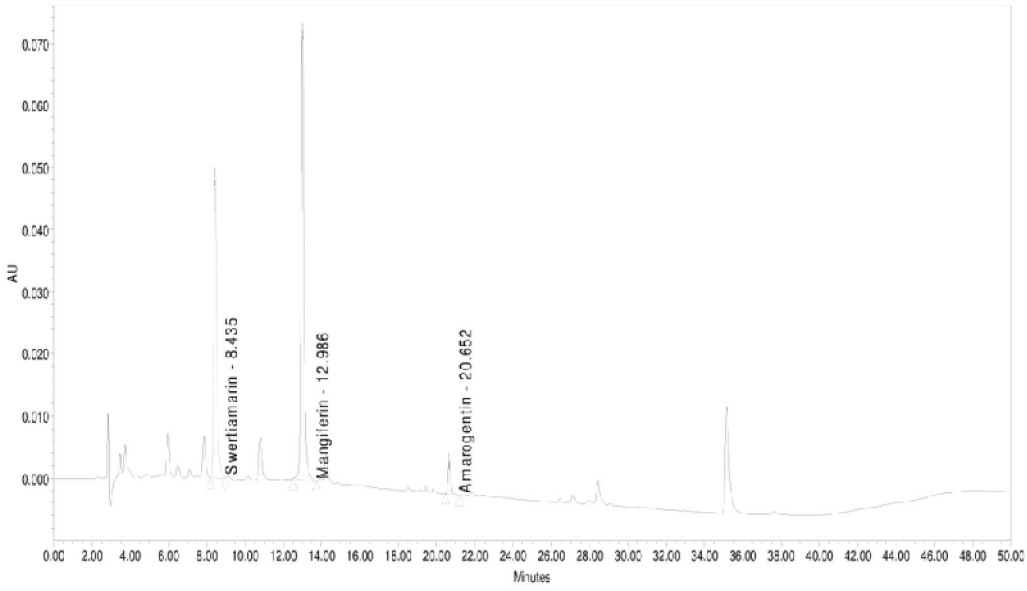

Figure 2: HPLC chromatogram showing sample peaks due to swertiamarin, mangiferin and amarogentin.

3. Results

3.1 Effect of different growth hormones on the growth of plantlets

Shoot growth with maximum number of 26 shoots along with 9.2 cm shoot length and highest leaf area 04 14.2 cm2 with maximum shoot biomass were achieved in M S(M9)supplemented with IBA (2mg/L), GA3 (3mg/L) and KN (2mg/L) whereas MS medium(M1) without growth hormones gave poor growth and development(Table 1). Plantlets regenerated on MS medium supplemented with GA3 (2mg/L) and KN (2mg/L) revealed longer shoots and larger leaf area but a reduced number of shoots per explant. The combination of IBA (3mg/L) and KN (1mg/L), showed a less profused shoot growth, but it led to good root induction and the roots were more elongated as compared to the other media compositions used (Figure 3).

Table 1: Effect of different MS media compositions on in vitro shoot multiplication and growth in Swertiachirata.

Medium	MS+ Growth Hormones (mg/l)			Shoots/ Explant	Shoot Length (cm)	Leaf Area (cm2)	Shoot biomass (gm)
	KN	GA3	IBA				
MI	0	0	0	2± 0.22	3.2± 0.53	2.1± 0.52	0.78± 0.22
M2	0.5	1	1.5	3± 0.53	6.0± 0.34	3.3± 0.65	1.67± 0.65

M3	1.5	0	1	5± 0.54	6.3± 0.33	4.2± 0.74	2.32± 0.52
M4	1	0	3	11± 0.21	6.7± 0.51	4.8± 0.65	4.65± 0.33
M5	3	0	1	19± 0.35	8.2± 0.54	10.4± 0.53	6.89± 0.64
M6	2	0	2	20± 0.55	8.5± 0.23	11.8± 0.34	6.99± 0.56
M7	2	3	0	22± 0.56	8.5± 0.32	11.7± 0.45	7.02± 0.41
M8	2	2	1	22± 0.65	8.0± 0.43	10.2± 0.52	7.67± 0.12
M9	2	3	2	26± 0.45	9.2± 0.12	14.2± 0.24	12.5± 0.21
MI0	0	3	2	22± 0.51	9.2± 0.11	12.3± 0.51	9.34± 0.32

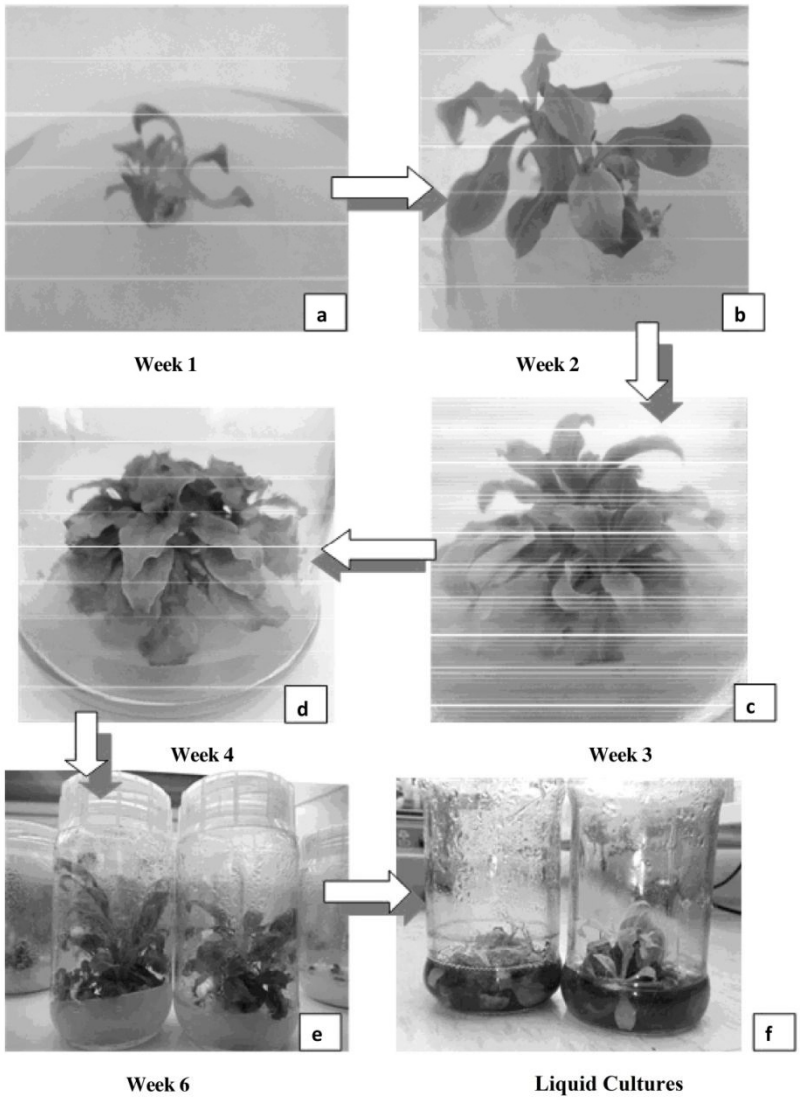

Figure 3: (a) Establishment of cultures of Swertiachirata(b) (c) (d) (e) multiple shoot formation from week2 to week6 (f) Shoots in suspension cultures with elicitors.

3.2 Effect of different growth hormones on the production of secondary metabolites

The quantity of swertiamarin increased significantly (9.33 µg/mg) in the shoots cultured on media supplemented with IBA (2mg/L) and GA3 (3mg/L) in just 20 days(Table 2). In this hormonal variant, the amount of these compounds increased 1.5-fold for swertiamarin and 2-fold for amarogentin over the control. However, the greatest levels of mangiferin (2.99µg/mg) and amarogentin (1.03 µg/mg) were observed in shoots cultured on MSM medium supplemented with a IBA (2mg/L) and KN (2mg/L) combination in 30 days. In this medium, the amount of mangiferin was 1.5-fold higher than the control (MS medium without PGRs) and 1.94-fold higher for amarogentin. The lowest content of swertiamarin was in shoots grownon IBA(2mg/l)+GA3(3mg/l)+KN(2mg/l). In contrast, amarogentin content was extremely low for all hormonal variants.

Table 2: Metabolites biosynthesized and accumulated in Swertiachirata shoots grown at 25±1°C.

Medium	**Media composition**	**Swertiamarin (Mean % ± S.E.)* in days**			**Mangiferin (Mean % ± S.E.)* in days**			**Amarogentin (Mean % ± S.E.)* in days**		
		10	*20*	*30*	*10*	*20*	*30*	*10*	*20*	*30*
M1	MS without growth hormones	0.060±0.002	0.659 ±0.02	0.717 ±0.036	0.072 ±0.002	0.182 ±0.005	0.201 ±0.009	0.033 ±0.005	0.044 ±0.002	0.058 ±0.002
M4	MS +IBA (3 mg/l) +KN (1 mg/l)	0.447±0.026	0.359 ±0.039	0.670 ±0.013	0.088 ±0.002	0.067 ±0.003	0.211 ±0.010	0.051 ±0.001	0.033 ±0.003	0.074 ±0.001
M5	MS+KN (3 mg/l) +IBA (1 mg/l)	0.252±0.051	0.078 ±0.004	0.616 ±0.032	0.108 ±0.011	0.215 ±0.022	0.207 ±0.011	0.049 ±0.003	0.077 ±0.003	0.067 ±0.003
M6	MS+ KN (2 mg/l) +IBAI (2 mg/l)	0.312±0.057	0.308 ±0.020	0.870 ±0.007	0.069 ±0.002	0.059 ±0.005	0.303 ±0.003	0.032 ±0.003	0.051 ±0.004	0.104 ±0.003
M7	MS+ KN (2 mg/l) +GA3 (3 mg/l)	0.349±0.010	0.495 ±0.010	0.694 ±0.009	0.134 ±0.007	0.188 ±0.003	0.086 ±0.002	0.086 ±0.003	0.087 ±0.001	0.104 ±0.001

M9	MS + KN (2 mg/l) + GA3 (3 mg/l) + IBA (2 mg/l)	0.602± 0.029	0.639 ±0.021	0.614 ±0.012	0.102 ±0.004	0.199 ±0.001	0.161 ±0.029	0.060 ±0.001	0.059 ±0.001	0.055 ±0.001
M10	MS + IBA (2 mg/l) + GA3 (3 mg/l)	0.774± 0.019	0.967 ±0.024	0.820 ±0.015	0.172 ±0.011	0.163 ±0.008	0.190 ±0.013	0.098 ±0.004	0.087 ±0.004	0.101 ±0.004
	Media contained MS (Murashige and Skoog,1962)salts + Growth hormones + sucrose (30 gm/L)+Agar-agar (8gm/L)									
	Growth Hormones: KN(Kinetin) ; IBA(Indole-3- butyric acid) ; GA3(Gibberellic acid) *Mean of twenty five replicates repeated three times.									

3.3 Effect of elicitors on growth and production of secondary metabolites

The applied elicitors (MeJA and SA) had different effects on S. chirata shoot growth (Table 3). The biomass production of MeJA elicited shoots was lower than the control. Shoot cultures treated with 50 and 75 µM MeJA showed a significant inhibition of biomass production and secondary metabolite production compared to controls and 25 µM MeJA. For comparison, SA showed insignificant effects on biomass production relative to the control, regardless of species and elicitor concentration. Regardless of the elicitor used for S. chirata levels did not exceed those of the control except for MS media containing Agrobacterium rhizogenes(10µL) , which showed a very promising result, the plantlets grown on this media were healthy , their biomass was also high and adding on it , the metabolite content was more than all the elicitors used. It can be stated from the results that Hoagland solution alone and Hoagland solution with Yeast Extract have an inhibitory effect on the metabolites produced by the plant.

Table 3: Effect of elicitors on growth and production of secondary metabolites.

Elicitors	**Time**	**Swertiamarin**	**Mangiferin**	**Amarogentin**
Salicylic acid 25±1°C	10 days	2.43	0.37	0.20
	20 days	2.39	0.16	0.18
	30 days	1.98	0.09	0.00
Vanadylsulphate25±1°C	10 days	2.84	0.92	0.25
	20 days	1.19	0.7	0.10
	30 days	0.79	0.07	0.06
Yeast extract 25±1°C	10 days	1.94	0.34	0.08
	20 days	1.04	0.07	0.00
	30 days	0.35	0.05	0.00
Hoagland solution	10 days	0.41	0.05	0.11
	20 days	0.82	0.15	0.09

	30 days	0.88	0.15	0.05
Hoagland +YE	10 days	0.14	0.00	0.00
	20 days	0.12	0.00	0.00
	30 days	0.00	0.00	0.00
25µM MeJa	10 days	2.75	0.47	0.19
	20 days	2.12	0.22	0.15
	30 days	0.92	0.00	0.10
50 µM MeJa	Week 1	1.22	0.08	0.10
	Week 2	2.45	0.04	0.10
	Week 3	0.21	0.00	0.00
75 µM MeJa	Week 2	1.22	0.15	0.10
	Week 3	0.80	0.10	0.08
	Week 4	1.48	0.23	0.07
AR 10µL	Week 2	1.77	0.09	0.14
	Week 3	3.47	0.32	0.34
	Week 4	4.83	0.62	0.68
AR 20µL	Week 2	0.93	0.05	0.07
	Week 3	0.93	0.04	0.09
	Week 4	1.72	0.32	0.09
Solid control 25±1°C	Week 2	1.35	0.38	0.14
	Week 3	4.3	1.63	0.37
	Week 4	7.55	1.41	0.51
Liquid control 25±1°C	Week 2	1.22	0.22	0.14
	Week 3	1.37	0.35	0.19
	Week 4	3.83	1.46	0.22

3.4 Linearity, Limit of Detection and Limit of Quantification for major metabolites

LOD and LOQ for swertiamarinwere0.10µg and 0.52µg, respectively. The linear regression equation was Y= 8520X+ 32141 and the correlation coefficient (r) was 0.9927.LOD and LOQ for mangiferinwere 0.09µg and 0.44 µg, respectively. The linear regression equation was Y= 35781X-31990 and the correlation coefficient (r) was 0.9942. LOD and LOQ for amarogentinwere0.10µg and 0.52µg, respectively. The linear regression equation was Y= 15665X+ 29207 and the correlation coefficient (r) was 0.9939.

4. Conclusion

Medicinal compounds synthesized in Swertia chiratashoot cultures are of high value but due to unexplored biosynthetic pathway it becomes difficult to elucidate the reasons of biosynthesis and accumulation of these compounds under in vitro conditions. But we have found that utilization of growth hormones would be sufficient

to induce the amount of metabolites in the shoots of Swertia chirata. Moreover developed quantification technique can be used as an efficient and time saving protocol for the estimation of itsmetabolites.

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