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Original Article

ESTIMATION OF SOME SECONDARY METABOLITES FROM THE *IN VITRO* CULTURES OF *CHLOROPHYTUM BORIVILIANUM* SANT. Et. FERN

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ABSTRACT

Objective: To perform phytochemical screening, estimate total saponins and to study the secondary metabolite profiling of *Chlorophytum borivilianum* by ESI-MS (Electron spray ionisation-mass spectroscopy) and GC-MS (Gas chromatography-mass spectroscopy) techniques.

Methods: The powdered samples of leaves and roots (nature grown and freshly harvested and hardened *in vitro* regenerated) and callus of *Chlorophytum borivilianum* (2 g) were extracted exhaustively by soxhlet by refluxing with petroleum ether (60-80 °C) and then with ethanol (85%). Alternatively, the methanol extract was prepared for the samples by the cold maceration method and the filtered extract was used. This extract was further analyzed by gas chromatography-mass spectrometry to identify and characterize the chemical compounds present in the crude extracts. Vanillin sulphuric acid assay was done to estimate the total saponin content of extracts.

Results: Phytochemical analysis of ethanolic extract showed the presence of major classes of phytochemicals. The spectrophotometric analysis as well as the GC-MS results revealed the remarkably high percentage of saponin content in the *in vitro* roots. Besides this, many important secondary metabolites viz. *β-Sitosterol and Taraxerone* were also revealed by GC-MS.

Conclusion: Results of this study showed enhanced production of many important secondary metabolites, especially saponins in tubers of *in vitro* regenerated plantlets vis a vis that of the nature grown plants establishing the fact that micropropagation can pave new vistas for the research in phytopharmaceuticals and bioprospecting of rare and important medicinal plants.

Keywords: *Chlorophytum borivilianum*, Phytochemical, ESIMS, GCMS, *In vitro* cultures

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INTRODUCTION

Since the dawn of mankind, plants with secondary metabolites have been used to treat human infections and diseases. Pharmaceutically significant secondary metabolites or phytopharmaceuticals include alkaloids, glycosides, flavonoids, volatile oils, tannins, resins etc. Currently, most of these secondary metabolites are either isolated from wild or cultivated plants, as their chemical synthesis is either extremely difficult or economically infeasible. Biotechnological production in plant cell cultures is an attractive alternative, but till recently it has had only limited commercial success due to the lack of understanding of how these metabolites are synthesized [1].

Chlorophytum borivilianum Santapau and Fernandes is a small perennial herb belonging to the family Liliaceae. The plant is indigenous to the forests of Rajasthan, western M. P. and north Gujarat. The tubers of this plant are highly reputed for its adaptogenic and aphrodisiac properties [2, 3]. The tubers are particularly rich in steroidal and tri-terpenoidal saponins, alkaloids, vitamins, minerals, proteins, polysaccharides and steroids. It also contains fructans, simple sugars such as glucose and fructose, phenolics, triterpenoids, gallo-tannins and mucilage [4-6]. Because of the great therapeutic importance, *C. borivilianum* tubers are the major constituents of more than a hundred ayurvedic preparations [7]. Attributing to its medicinal properties the demand for *C. borivilianum* tubers has bolstered across the world and India has been a huge market for it [8]. The ever-growing market demand coupled with the over exploitation of this plant has dwindled the population of *C. borivilianum* in its natural habitat [9].

There are a few reports on tissue culture based propagation of *Chlorophytum borivilianum* [10-12]. Albeit there has been a paucity of scientific work regarding the efficient phytochemical investigations from the *in vitro* raised cultures of this plant. Therefore, the estimation of secondary metabolites of *C. borivilianum* in the present investigation proceeded using a combination of various techniques viz. TLC, spectrophotometric (colorimetric) estimation, HPLC, ESI-MS and GC-MS analysis.

MATERIALS AND METHODS

Culture media and growth conditions

The plant material was obtained in the form of dry *C. borivilianum* tubers from Jawaharlal Nehru Agriculture University, Jabalpur (India). The young, shoot bud sprouts on the surface of dried tubers during monsoons were used as explants. After the initial procedure of surface sterilization, the explants were aseptically transferred to Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and fortified with different concentrations of PGRs. The pH of the media was adjusted to 5.6–5.8 before adding agar (8 gm L^{-1}). The media was sterilized at 15 lbs pressure for 15 min by autoclaving. Cultures were incubated at 25±2 °C at photoperiodic cycle of 16 hr light (approx. 1500 lux) and 8 hr dark. All cultures were sub-cultured on the fresh medium at every four-week interval. *In vitro* response from each explant was recorded every week.

Somatic embryo induction and development

The juvenile shoot bud explants were inoculated on MS medium supplemented with varying concentrations (0.1, 0.5, 1 and 5 mg/l) of BAP and KN alone. BAP at 5 mg/l concentration was able to induce a very high frequency of somatic embryogenesis (80.55) with an average of 18.44 embryos per explants. On the other hand, a much lower concentration of BAP (0.5 mg/l) led to the proliferation of somatic embryos. For the multiplication and growth of somatic embryos, the embryos were next sub-cultured on MS medium supplemented with varying concentrations (0.1, 0.5, 1 and 5 mg/l) of BAP and KN alone and in combination, whereas, the hormone free medium i.e. basal medium (BM) served as control. The conversion of somatic embryos with high MRN and MRL was favoured on BM [13].

Preparation of plant samples

Both nature grown and hardened *in vitro* regenerated plants were collected and washed thoroughly under running tap water and allowed to dry for some time. The leaves and roots were separated

and shade dried without any contamination for about 3 to 4 w. The dried plant samples were powdered in a blender and stored in airtight containers.

Preparation of extracts

Ethanolic extract

The powdered samples of leaves and roots (nature grown and freshly harvested and hardened *in vitro* regenerated) and callus (2g) were extracted exhaustively by refluxing with petroleum ether (60-80 °C) and then with ethanol (85%) in a soxhlet apparatus. The ethanolic extracts were concentrated and used further.

Methanolic extract

Alternatively, the powdered samples of leaves and roots (nature grown and hardened *in vitro* regenerated) and callus (2g) were extracted in methanol by cold maceration method. The extract was filtered and the filtrate was further used for analysis.

Extraction and isolation of saponins

After evaporation of the ethanolic extract, the gummy residue was suspended in H_2O (100 ml) and extracted with n-butanol (300 ml). The n-butanol fraction was reduced to half and washed twice with 10 ml of 5% NaCl solution to yield crude saponin fraction [14, 15].

Preliminary phytochemical screening of plant extracts

Phytochemical screening was performed for the analysis of different phytochemicals like carbohydrates, saponins, oils, fats, flavonoids, terpenoids, alkaloids *etc*., in methanolic extracts of plant samples. The screening was performed with some modifications from the methods of Harborne [16] and Evans [17].

Estimation of saponins by vanillin–sulfuric acid assay

The method described by Hiai *et al.* [18] was adopted. To 0.5 ml of aqueous solution of the sample, 0.5 ml vanillin solution of 8 % (w/v) and then 5.0 ml of sulfuric acid of 72% (w/v) were added and thoroughly mixed in an ice water bath. The mixture was then warmed in a bath at 60˚C for 10 min and then cooled in ice-cold water. Absorbance at 535 nm was recorded against the blank with the reagents using UV/VIS spectrophotometer (Systronics UV-VIS Spectrophotometer; 108).

Thin layer chromatography (TLC)

Silica gel G, F_{254}, 20 cm x 20 cm plates were used for thin layer chromatography. The extracts of calli as well as the leaves and roots of nature grown and *in vitro* regenerated plants obtained were applied to activated plates and were placed in different solvent systems (table 1) for the detection of secondary metabolites. The developed chromatograms were observed under UV light and their Rfs were calculated.

Table 1: Solvent systems used for different metabolites

S. No.	Secondary metabolites	Solvent systems
1.	Saponins	Chloroform-methanol-water (60:30:10), chloroform-Gl. acetic acid-methanol-water (16:8:3:2) and n-hexane-ethyl acetate (9:1).
2.	Flavonoids	Ethyl acetate-Gl. acetic acid-methanol-water (100:11:11:26)
3.	Alkaloids	Ethyl acetate-methanol-water (10:1.35:1)

High-performance liquid chromatography (HPLC)

The HPLC method was used to estimate the presence saponins in the natural and hardened *in vitro* regenerated leaves and roots, freshly harvested *in vitro* regenerated root and callus. Saponins of *C. borivilianum* were detected with the help of retention time compared with the standard (Saponin from Sigma Aldrich-47036). The fraction analysis was carried out in a Dionex model µ HPLC Focus Ultimate 300 having a pore size of 5 µ. A C_{18} RP Column (150 x 4.6 mm I.D.) was used at a temperature of 30 °C.

A Linear gradient of acetonitrile: water (4:6 to 6:4) over 30 min was used and the mixture was degassed and filtered [19]. The flow rate was maintained at 1 ml per minute. The detection was done in UV at 200-400 nm.

Electron spray ionisation-mass spectroscopy (ESI-MS)

The ESI-MS analysis of the above samples that were subjected to HPLC was performed on Bruker micrOTOF-Q II 10348, mass spectrophotometer equipped with an ESI source in a positive ion mode. High purity nitrogen gas was used as collision, nebulizer and auxillary heated gas. The ESI conditions were set as follows: spray voltage +4500V; nebulizer gas flow, 4.0 l/min; temperature, 180 °C.

Gas chromatography-mass spectroscopy (GC-MS)

The methanolic extracts of nature grown and hardened *in vitro* roots and callus, obtained by cold maceration method were subjected to GC-MS analysis. The analysis was performed on Shimadzu QP-2010 plus with Thermal Desorption System. The MS scan range was 40-650 atomic mass units (AMU). The chromatographic column was fused silica RTX-5MS (95% dimethlpolysiloxane - 5% diphenyl) capillary column (30m X0.25 mm i.d. X0.25 µm). The carrier gas used was helium at a flow rate of 1.21 ml/min. Samples were analyzed with the column held initially at 100 °C for 2 min. and then increased to 280 °C with 15 °C/min heating rate and a hold time of 20 min. The injection was performed in a split mode at 250 °C. The identification of individual compounds was done from Wiley and NIST mass spectral library on the basis of mass fragments and m/z values of each component.

RESULTS

Phytochemical screening of plant extracts

The curative properties of medicinal plants are due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols *etc*. The extracts of hardened *in vitro* and nature grown leaves and roots revealed the presence of carbohydrates, proteins, saponins, flavonoids, cardiac glycosides and gums and mucilaginous substances (table 2). Thus the preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development.

Estimation of Saponin content by vanillin-sulphuric acid assay

Linearity was evident for the regression in the standard saponin (Sigma Aldrich) contents by the vanillin-sulphuric acid assay. The total absorption was observed at 535 nm = 0.2462 x saponin content (mg/ml)+0.009, R^2= 0.9835. On this basis, it was observed that the roots of the freshly harvested *in vitro* regenerated and hardened plants possessed the highest amount of saponin (1. 9 mg/ml) as compared to the nature grown ones (0.77 mg/ml).

This was followed by the callus obtained using the combination of varying concentrations of KN and 2, 4-D in MS medium (1.11 mg/ml) (table 3). Therefore, the callus obtained from MS+KN+2,4-D combination was further used for analysis.

Table 2: Preliminary phytochemical analysis of plant extracts

	Root		Leaves	
	Nature grown	Hardened *in vitro*	Nature grown	Hardened *in vitro*
Carbohydrates (Molisch test)	+	+	+	+
Proteins (Biuret test)	+	+	+	+
Saponin (Foam test)	+	+	-	-
Cardiac glycosides	+	+	-	-
Oils and fats	-	-	+	+
Anthocyanins	-	-	+	+
Terpenoids	+	+	-	-
Alkaloids	-	-	+	+
Flavonoids	+	+	+	+
Sterols	+	+	+	+
Tannins and Phenols	-	-	+	+
Gums and mucilage	+	+	-	-

(+) = present, (-) = absent

Table 3: Saponin content estimation by vanillin-sulphuric acid assay

Samples (Aq. extracts)	Amount of saponin (mg/ml)
Hardened *In vitro* roots	**1.9±0.04**
Nature grown roots	0.77±0.03
Freshly harvested *in vitro* roots	0.55±0.02
MS+2, 4-D callus	0.04±0.01
MS+IAA callus	0.59±0.01
MS+IBA callus	0.52±0.02
MS+NAA callus	0.02±0.01
MS+KN+2, 4-D callus	**1.11±0.11**
MS+BAP+2, 4-D callus	0.61±0.03
MS+KN+NAA callus	0.28±0.02
MS+BAP+NAA callus	0.29±0.02

Results are represented as mean (±standard error) of three independent readings, $P<0.05$.

Thin layer chromatography (TLC)

The TLC analysis of hardened *in vitro* and nature grown roots showed the presence of saponins, flavonoids and alkaloids. The callus showed identical Rfs corresponding to that of the standard and hardened *in vitro* regenerated and nature grown roots for saponins (0.73) and alkaloids (0.98) however, it failed to show up any corresponding Rfs for flavonoids, when compared with the standards (table 4, fig. 1-4).

Table 4: TLC of the callus and nature grown and hardened *in vitro* regenerated roots of *Chlorophytum borivilianum*

Secondary metabolite	Solvent system	Rf values			
		S	C	NR	IVR
Saponins	n Hex: EtAc(9:1)	0.73	0.73, 0.58, 0.56, 0.52, 0.44, 0.22	0.73, 0.58, 0.56	0.73, 0.58, 0.56
	$CHCl_3$:CH_3OH: H_2O (60:30:10)	0.96	-	0.93	0.93
Alkaloids	EtAc: CH_3OH: H_2O (10:1.35:1)	0.98	0.98	0.95	0.98
Flavonoids	EtAc: Formic acid: GAA: H_2O (100:11:11:26)	0.9	-	0.85, 1.0	0.85, 1.0

NR = Nature-grown root, IVR = *In vitro* regenerated root, C = Callus, S = standard

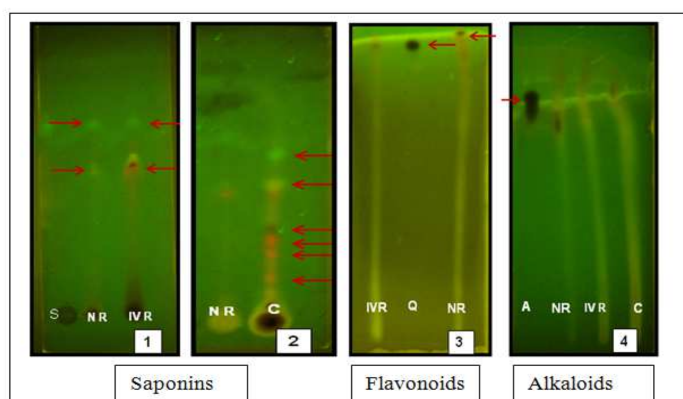

Fig. 1-4: TLC of root samples and callus, S = saponin standard, IVR = *in vitro* root, NR = nature grown root, C = callus, Q = quercitin (flavonoid standard), A = colchicine (alkaloid standard)

High-performance liquid chromatography (HPLC)

The HPLC analysis of the test tube grown roots, callus as well as nature grown and hardened *in vitro* regenerated roots showed an identical Retention time (RT) of 2.10 as compared that of standard saponin (Sigma Aldrich). Other common peaks were also found in the test tube grown roots, callus and nature grown and hardened *in vitro* regenerated roots that were not observed in the standard saponin. The callus and hardened *in vitro* regenerated and nature grown roots showed similar RTs at 2.28, 2.26 and 2.27 respectively. Identical peaks at RT 2.98 and 2.95 for the hardened *in vitro* regenerated and nature grown roots were also observed (fig. 5-7).

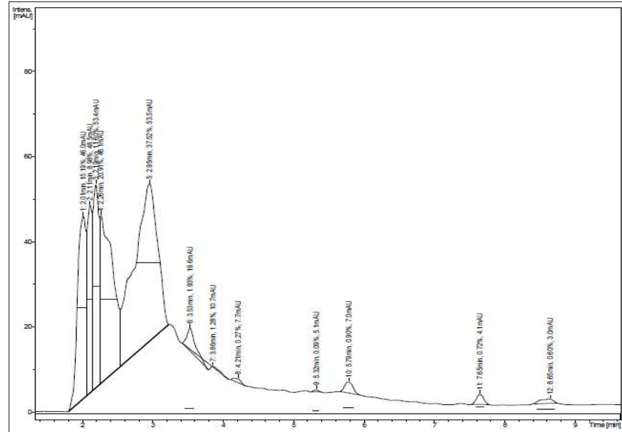

Fig. 5: HPLC of nature grown roots

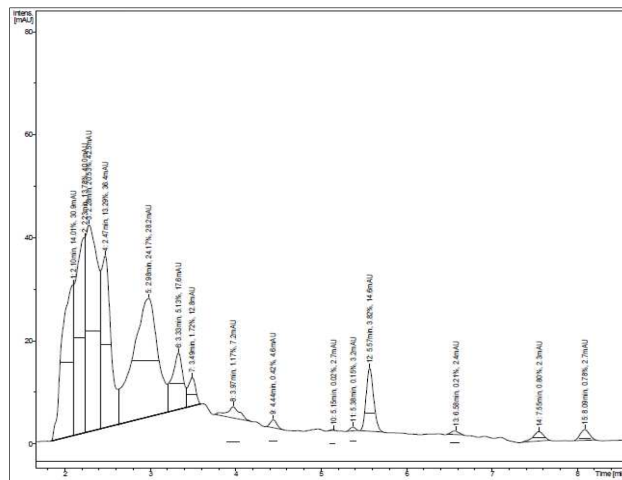

Fig. 6: HPLC of *in vitro* regenerated roots

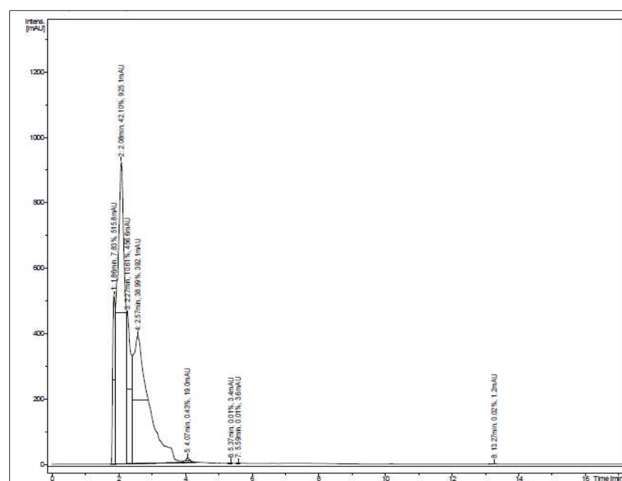

Fig. 7: HPLC of callus

Electron spray ionisation-mass spectroscopy (ESI-MS)

Under the ESI-QTOF-MS the steroidal saponins of *C. borivilianum* were easily ionized and showed the presence of molecular species in the positive ion mode. By comparing the high-resolution mass data based on individual m/z values a good number of discernable peaks were obtained in the roots and leaves of *in vitro* regenerated and nature-grown plants and also in the callus and test-tube grown tubers. Among the vast array of m/z data of the above-mentioned samples, many peaks were found in common. A list of common peaks is summarized in table 5.

Since steroidal saponins are the principle compounds present in the plant under study, the fragmentation pattern of steroidal saponins was taken under review. Based on the literature surveyed the substituents on the steroidal aglycone moiety are observed. If these data are not available then the diagnostic ions at m/z 253 and 271 must be taken under consideration. If the above diagnostic ions are still not detected, this indicates that the saponin possesses a transformed aglycone as dioscorioside A. Furthermore, the aglycone ion can be viewed as the indicator to know whether the steroidal saponins are substituted or not. If ion at m/z 415 was present no substituent should be attached to the aglycone; on the other hand, if ion at m/z 413 was detected, there should be a substituent on the steroid aglycone.

The mass spectra of callus and hardened *in vitro* regenerated and nature-grown roots thus showed identical peaks at m/z 413 which suggested that there was a substitution at the aglycone ion, which upon observation sufficed the probable molecular formula of the aglycone to be $C_{27}H_{47}O_3^+$. The said aglycone lost a water molecule to yield another fragmentation product at m/z 395, which was again observed in the callus and hardened *in vitro* root sample.

Thus based on the intense proton adduct ion $[M+H]^+$of the hardened *in vitro* root saponin at m/z 533 in positive ion mode, the proposed fragmentation would be as follows; m/z 533 $[M+H]^+$, m/z 517 $[M+H-H_2O-2H]^+$, m/z 497 $[M+H-2H_2O]^+$, m/z 435 $[M+H-4H_2O]^+$. As compared to the mass spectra of nature grown root lower m/z values in the hardened *in vitro* regenerated root sample suggested the possibility of loss of glycosides like hexoses and pentoses from it. The proposed fragmentation of the nature grown root sample would be as follows; m/z 901 $[M+H]^+$, m/z 739 $[M+H\text{-hexose}]^+$, m/z 607 $[M+H\text{-hexose-pentose}]^+$, m/z 587 $[M+H\text{-hexose-pentose-}H_2O]^+$, m/z 569 $[M+H\text{-hexose-pentose-}H_2O]^+$, m/z 551 $[M+H\text{-hexose-pentose-}2H_2O]^+$, m/z 533 $[M+H\text{-hexose-pentose-}3H_2O]^+$, m/z 497 $[M+H\text{-hexose-pentose-}5H_2O]^+$.

The fragmentation peak for m/z 533 is also present in the *in vitro* regenerated root sample, suggesting the fact that, this molecule is a fragmentation product of a molecule similar to that of nature grown root (fig. 8-10).

Table 5: A list of common m/z values obtained from the ESI-MS of the callus and root extracts *Chlorophytum borivilianum*

Common m/z values	Saponin standard	Hardened *in vitro* roots	Nature grown roots	Callus
347	+	+	+	
367	+		+	
381	+			+
395	+	+	+	
413*	+	+	+	
437	+			+
497	+	+		
533		+	+	
597	+	+		
613				+
615			+	+
616				+
631				+
633				+
655				+
701				+
893				+
917				+

*Diagnostic m/z of steroidal aglycone [20]

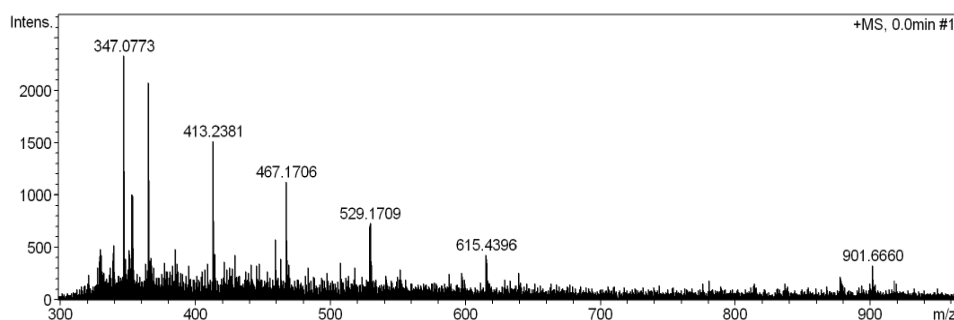

Fig. 8: ESI-MS of nature grown roots of *Chlorophytum borivilianum*

Gas chromatography-mass spectroscopy

All the three samples viz; the roots of nature grown and hardened *in vitro* regenerated plants and callus showed the presence of some of the medicinally important secondary metabolites like *Taraxerone, β-Sitosterol and Lupenol* (Triterpene) (table 6). The *in vitro* roots here showed the highest amount of all the three secondary products, with the remarkably high percentage of *β-sitosterol* (8.16%) and *Taraxerone* (5.47%) (fig. 12). The nature grown roots revealed the presence of sarasapogenin (fig. 11). On the other hand hardened *in vitro* roots showed the presence of squalene; which is an important precursor in the synthesis of steroidal compounds. The callus

revealed the presence of many important secondary metabolites of high medicinal importance like *digitoxin* (0.39%), *β-carotene* (0.37%) and *gamma sitosterol* (1.79) (fig. 13). All the three samples were also found to possess phytosterols like *ergosterol* and *stigmasterol*. The hardened *in vitro* regenerated roots showed a higher amount of ergosterol (2.43%) which was 9.6 fold and *stigmasterol* (8.16%) to be 51 folds higher than the nature grown ones (fig. 14-16). An appreciable amount of carbohydrate-intermediates were observed in the hardened *in vitro* root and callus extracts. The hardened *in vitro* root sample revealed the presence of *α-D-glucopyranoside* (8.27%) and *D-Allose* (2.07%). *D-Allose* was also observed in callus (3.31 %), which was slightly higher as compared to the hardened *in vitro* root sample. *β-D-galactopyranoside* and *β-D-glucopyranose* were observed in both the samples.

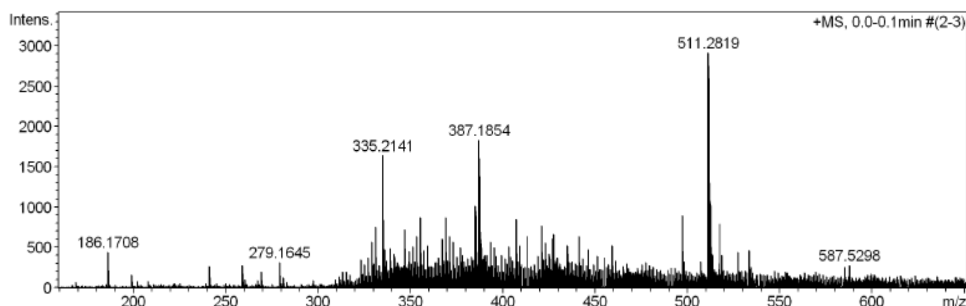

Fig. 9: ESI-MS of *in vitro* regenerated roots of *Chlorophytum borivilianum*

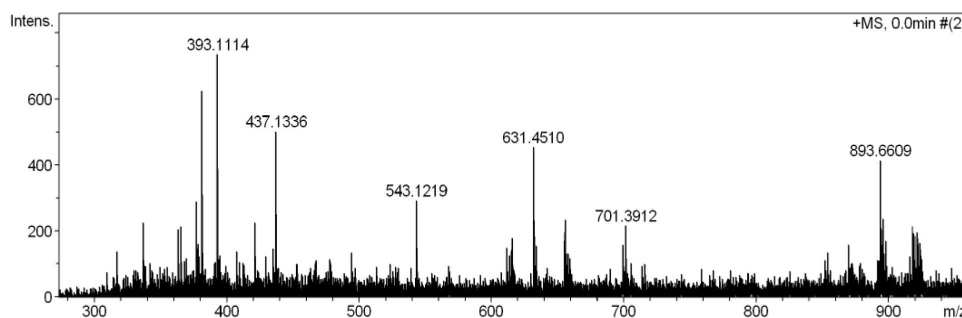

Fig. 10: ESI-MS of callus of *Chlorophytum borivilianum*

Table 6: A comparative analysis by GC-MS of some major secondary products of the nature grown and hardened *in vitro* regenerated roots and callus samples of *Chlorophytum borivilianum*

R time	% area	Compound name
Nature grown roots		
6.1	49.94	*2-furancarboxaldehyde*
29	0.71	*Stigmasterol*
29.4	0.25	*Sarsasapogenin*
29.5	0.16	*Stigmast-5-en-3-ol,*
30.4	0.12	*D: A-Friedoolean-6-ene*
Regenerated roots		
9.8	2.07	*D-allose*
11.8	8.27	*Ethyl. alpha.-d-glucopyranoside*
24.7	1.41	*Squalene*
25.5	5.05	*1-heptacosanol*
27.9	1.24	*Cholest-5-en-3-ol (3. beta.)-*
28.7	2.43	*Ergost-5-en-3-ol*
29	5.47	*D-friedoolean-14-en-3-one*
29.5	8.16	*Stigmast-5-en-3-ol*
Callus		
4.5	8.29	*4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-*
5.8	7.59	*2-Furancarboxaldehyde, 5-(hydroxymethyl*
10.2	3.31	*D-allose*
14.6	14.12	*N-Hexadecanoic acid*
16.3	17.09	*9,12-Octadecadienoic acid*
18.3	0.34	*Lup-20(29)-ene-3,28-diol*
22.2	0.39	*Digitoxin*
24.8	0.37	*Beta. Carotene*
29	1.56	*Stigmasta-5,22-dien-3-ol*
29.4	1.79	*Gamma.-sitosterol*

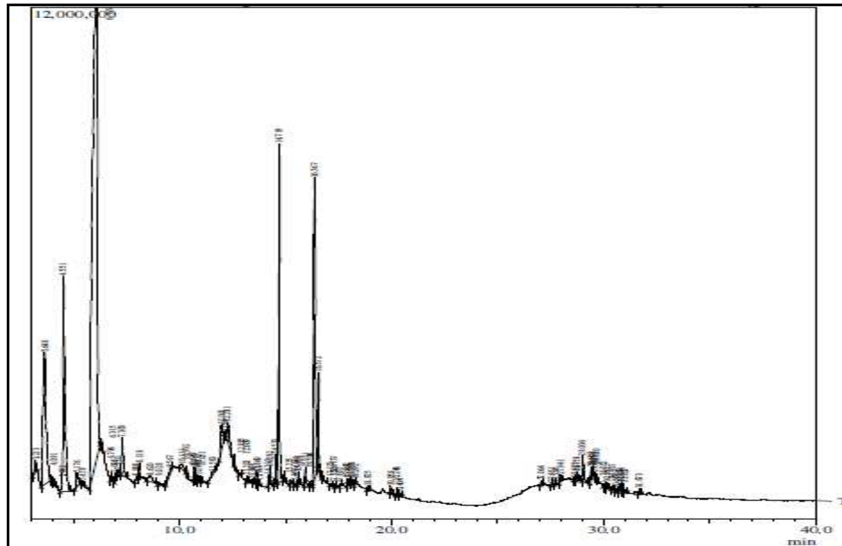

Fig. 11: GC-MS of nature grown roots of *Chlorophytum borivilianum*

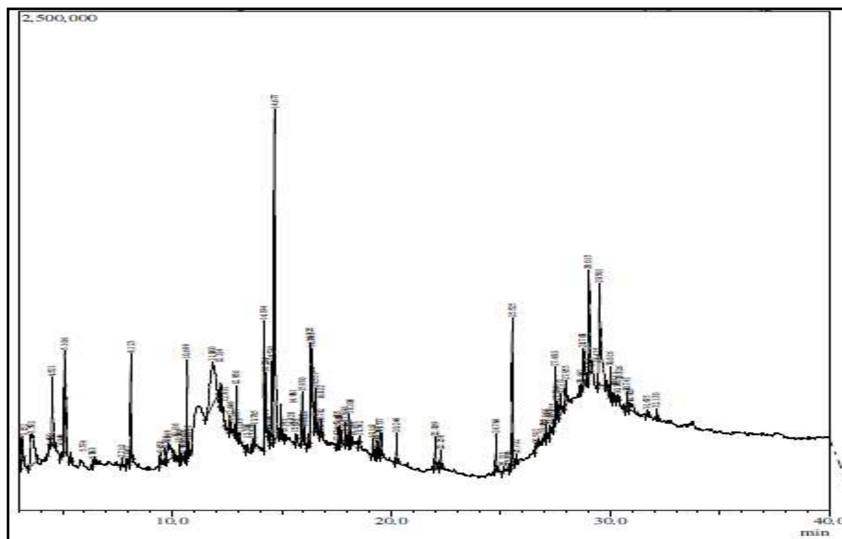

Fig. 12: GC-MS of *in vitro* regenerated roots of *Chlorophytum borivilianum*

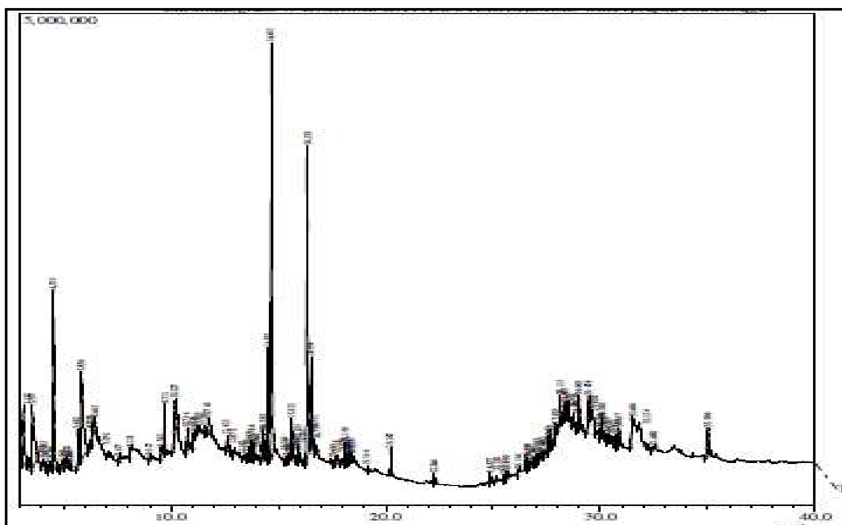

Fig. 13: GC-MS of callus of *Chlorophytum borivilianum*

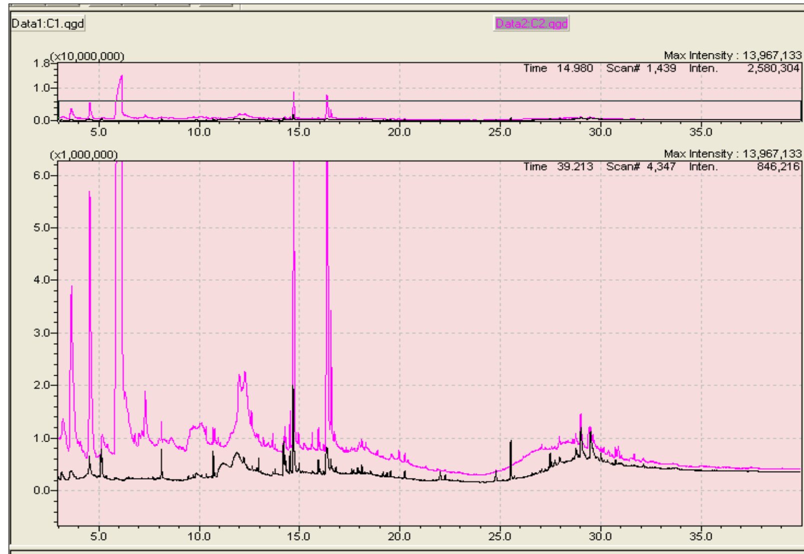

Fig. 14: An overlap chromatogram of GC of *in vitro* regenerated (hardened) and nature grown roots, C1 (Black) = *in vitro* regenerated root, C2 (Pink) = nature grown root

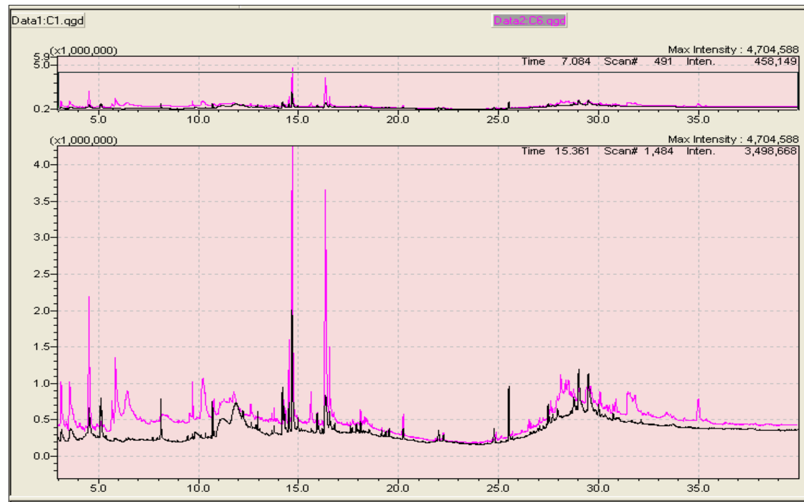

Fig. 15: An overlap chromatogram of GC of *in vitro* (hardened) regenerated roots and callus, C1 (Black) = *In vitro* regenerated root, C6 (Pink) = callus

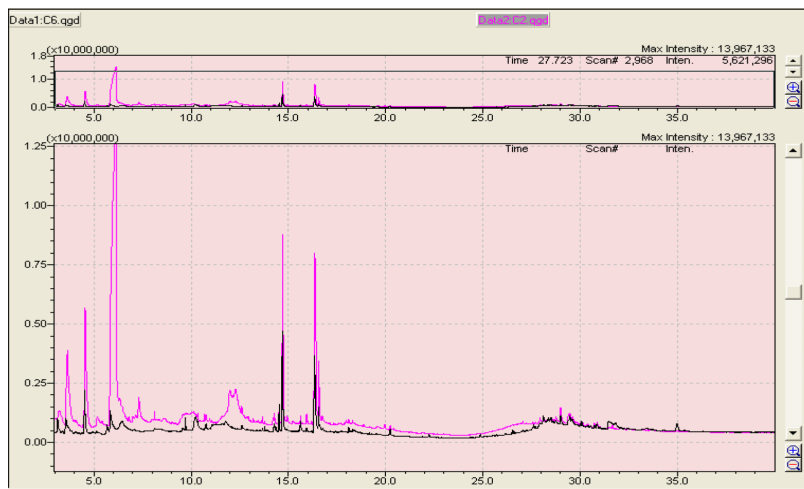

Fig. 16: An overlap chromatogram of GC of callus and nature grown roots, C6(Black) = callus, C2 (Pink) = Nature grown roots

DISCUSSION

The detection of active principles in medicinal plants plays a strategic role in the phytochemical investigation of crude plant extracts and is very important with regards to their potential pharmacological effects [21-23].

Production of high-value natural products by cell and tissue cultures is a matter of great interest among scientists [24]. In cultures, factory type production of natural compounds can be carried out throughout the years, unaffected by season.

A comparative study of saponins in tubers of *in vitro* regenerated plantlets with tubers of nature grown plants showed that there was an enhancement in secondary metabolite content in tubers of micro-propagated plants.

Chromatographic determination of saponins in plant material is still a challenge to the phytochemist. The unique chemical nature of saponins demands tedious and sophisticated techniques for their isolation, structure elucidation and analysis. The task of isolating saponins from plant material is complicated also by the occurrence of many closely related substances in plant tissues, and by the fact that most of the saponins lack a chromophore. There is no single method that can be recommended as a routine procedure for analysis of complex saponin mixtures. None of these methods provides a fingerprint, which allows the quality to be monitored. Also, none of them provides an adequate tool for the separation or preparative isolation of saponin components; a combination of several techniques is required obtain single standard compounds [25, 26]. The estimation of secondary metabolites of *C. borivilianum* in the present investigation, therefore, proceeded using a combination of various techniques viz. TLC, spectrophotometric (colorimetric) estimation, HPLC, ESI-MS and GC-MS analysis.

The TLC analysis of the *in vitro* and nature grown roots and leaves of *C. borivilianum* in the present work showed the presence of saponins, flavonoids and alkaloids. The TLC of callus failed to show up any corresponding Rfs for flavonoids. The Rf values obtained in callus sample 0.73, 0.58, 0.56, 0.52, 0.44, 0.22 suggested the presence of a large number of glycosides. Yang *et al.* [27] reported similar RF values for steroidal saponins in the TLCs of *Dioscorea* spp.

Spectrophotometric methods are practical and simple, making them preferable for specification testing. Most frequently used colorants include Ehrlich or vanillin reagents and measurements are made at λ = 515–560 nm. Because of the weak absorbance of saponins, a colorimetric determination is used for their evaluation. To determine the amount of saponin in *C. borivilianum* extract, a spectrophotometric assay using Vanillin and Sulphuric acid was carried out. The result showed that the roots of *in vitro* grown plant possessed the highest saponin (19.7 mg/ml) content followed by that of callus (12.5 mg/ml) relative to the standard saponin used. The amount of saponin in the aqueous and alcoholic extracts of *in vitro* grown calli of *C. borivilianum,* by spectrophotometric method using Vanillin and Sulphuric acid was also studied by [28].

The HPLC analysis of the freshly harvested *in vitro* regenerated roots, callus and nature and hardened (six months) *in vitro* regenerated roots and leaves showed identical peaks at Retention time (RT) 2.10 with that of standard saponin (Sigma). There were some common peaks observed in the test tube grown roots, callus and nature grown and *in vitro* regenerated roots and leaves that were not observed in the standard saponin. The *in vitro* regenerated and nature grown roots and callus also showed similar RTs at 2.28, 2.26 and 2.27 respectively. Besides this, identical peaks were also observed at RT 2.98 and 2.95 for the *in vitro* regenerated and nature grown roots respectively. The leaves of nature grown and *in vitro* regenerated leaves and callus produced similar peaks at RTs 4.07 and 13.29.

Based on the literature by Li *et al.* the ESI-QTOF-MS of the steroidal saponins of *C. borivilianum* revealed the presence of molecular species in the positive ion mode. The diagnostic ions at m/z 413 confirmed that a substituent was attached on the steroid aglycone and its probable molecular formula to be $C_{27}H_{47}O_3^+$which was assumed to be a fission product of glycosides and water molecules. Besides, many peaks in the samples were found to be in common. The fragmentation pattern of the nature grown root revealed the presence of glycosidic moieties, thereby producing peaks at m/z 497 and 533 found in the *in vitro* regenerated root. This observation is in accordance with the m/z values for the compound chlorophytoside, in *Chlorophytum laxum* as reported by Gao *et al.* [29]. Acharya *et al.* [30] reported similar fragmentation of steroidal saponins in the nature grown root samples of *C. borivilianum*.

The GC-MS analysis showed the presence of a large number of phytosterols in the *in vitro* roots and callus extract; along with some of the medicinally important secondary metabolites like *Taraxerone, β-Sitosterol* and *Lupenol* (Triterpene). However, the *in vitro* roots showed the highest amount of all the three secondary products, with the remarkably higher percentage of *β-Sitosterol* (8.16%) and *Taraxerone* (5.47%). The higher amount of *β-Sitosterol* in the *in vitro* regenerated roots suggest the influence of tissue–culture protocols involving the use of different PGRs. *Beta-sitosterol* has been isolated from the cultured tissues of *Terminalia chebula* [31] as well as the callus of several plants viz. *Morus alba* [32], *Vaccinium corymbosum* [33], *Cissus quadrangularis* [34]. Bathoju and Giri [35] (2012) also reported the production of two important sapogenins; *stimasterol* and *hecogenin* from the *in vitro* regenerated root cultures of *C. borivilianum.* While, nature grown roots revealed the presence of sarasapogenin, the *in vitro* roots, on the other hand, showed the presence of squalene, which is an important precursor in the synthesis of steroidal compounds. The callus also revealed the presence of many important secondary metabolites of high medicinal importance like *digitoxin, β-carotene* and *gamma sitosterol* along with other phytosterols like *ergosterol* and *stigmasterol*. Incidentally, the *in vitro* regenerated roots showed a higher amount of *ergosterol* and *stigmasterol* as compared to the nature grown roots and callus.

Phytosterols are responsible for the permeability and fluidity of cell-membranes. Further, they act as a precursor of numerous other metabolites like glycoalkoloides and saponins [36]. The presence of the additional ethyl group branched on the alkyl chain of major phytosterols viz. *sitosterol* and *stigmasterol,* is proposed as reinforcing the membrane cohesion. The fine tuning of sterol structures thus appears to be the evolution response for plant adaptation to large temperature variations [37].

The *in vitro* regenerated roots revealed the presence of higher amount of α-D-glucopyranoside and *D-allose*, albeit, the amount of *D-allose* in callus was slightly higher as compared to the hardened *in vitro* roots which had not been reported in the nature grown plant, which is evident from the overlap chromatograms of the root extracts and callus

Similar examples of high accumulation of secondary products in the *in vitro* cultures have been seen in many plants through the HPLC and GC-MS analysis viz. *Lessertia frutescence* [38], *Rauwolfia serpentine* [39] and *Saussurea involucrate* [40].

CONCLUSION

Presence of a large number of medicinally important secondary metabolites in *Chlorophytum borivilianum* has been widely studied. However, the reports on the enhanced production of these secondary metabolites from the *in vitro* cultures have been scarce. Based on the results established by HPLC, ESI-MS and GCMS it could be concluded that the production of secondary metabolites in *in vitro* cultures could be enhanced in plants of high medicinal importance irrespective of geography and seasonal variation or where natural sources are endangered**,** thus opening new avenues for phytopharmaceuticals**.**

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AUTHORS CONTRIBUTIONS

All experimental work was carried out by Jha A, whereas, Bansal Y. K. supervised them

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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