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Comparison of Phytochemicals in naturally grown plants, tissue cultured plants and calli of Stevia rebaudiana using HPLC and GC-MS

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Research Article

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Abstract

Stevia rebaudiana is a medicinal plant rich in important phytochemicals including steviol glycosides. Its' leaves are the source of non-caloric sweet steviol glycosides and stevioside is the main type. In this study leaves of naturally grown, tissue cultured plants and callus extracts of *S. rebaudiana* were analyzed using HPLC and GC-MS to determine the possibility of obtaining stevioside and other important phytochemicals through in vitro technology. Phytochemical extraction was done using methanol, evaporated using a rotary evaporator and re-dissolved in acetonitrile: deionized water (3:7) ratio solvent mixture. Stevioside contents were analyzed using HPLC system with C18 column. Methanolic extracts of naturally grown plants, tissue cultured plants and calli were screened using GC-MS and compared. According to HPLC analysis, leaves of tissue cultured plants contained highest amount of stevioside content (11.462 ± 0.03 mg/mL) followed by naturally grown plants and calli. Twenty seven important phytochemicals were identified by GC-MS analysis and out of those fourteen compounds were found to be common in all three samples. Results revealed that in vitro cultures can be used as the better alternative to overcome limitations in conventional propagation and can be used to obtain stevioside and other important phytochemicals instead of natural plants. GC-MS analysis of Stevia extracts revealed the presence of terpenes, fatty acids, aldehydes with biological activities such as antimicrobial, anticancer, anti-inflamatory, antioxidant, antimalarial and etc. Bioactivity of those phytochemicals showed the possible use of Stevia extracts in pharmaceutical, food and perfume industries.

INTRODUCTION

Stevia rebaudiana (Bert.) Bertoni is a natural sweetener plant belongs to family of Asteraceae. It is commonly known as sweet leaf herb or candy leaf herb due to the sweetness in its leaves. The chemical compounds produce its sweetness are known as Steviol glycosides and they are non caloric sweeteners [1]. In addition to these Steviol glycosides, many other phytochemicals have been discovered in *S. rebaudiana* including flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids and some essential oils [2]. Triterpenes, sterols like stigmasterol, sitosterol and campesterol are also identified in Stevia leaves [3]. These phytochemicals play an important role in medicinal purpose due to the activity of antimicrobial, anticancer, antimoxident, anti-inflammatory, antiamoebic and antihyperlipidemic etc [4]. Since Stevia extract is rich in other important phytochemicals it can be use in pharmaceutical, food (flavoring agents, food additives) and perfume industries. Present study was designed to determine the possibility of obtaining stevioside and other important phytochemicals through in vitro technology as well as to screen, compare and identify the bioactivity of the important phytochemicals present in *S. rebaudiana.*

MATERIALS AND METHODS

Plant material

Leaves were collected from three months old naturally grown and *in vitro* raised, acclimatized plants. Six weeks old leaf disks derived calli induced on MS medium supplemented with 2.0 BAP and 1.0 NAA were used. All three samples were washed carefully and excess water was removed by blotting. They were initially sun dried for five days in the open air by spreading on papers and then final drying was done in the hot air oven (MEMMERT) at 60 C for one hour. Dried samples were ground into a coarse powder and stored in air tight containers.

Phytochemical extraction

Phytochemical extraction was carried out using simple extraction method. Dried powders were shaken separately with methanol (1:10 w/v ratio) for 24 h in 150 rpm shaker at 25 ± 1°C and filtered through Whatman No.1 paper. This process was repeated again for each sample and filtrates were collected. Thereafter, solvents were evaporated using a rotary evaporator (BUCHI–R124) at 40°C under reduced pressure and crude extracts were collected.

Analysis of stevioside using HPLC

Sample preparation for HPLC analysis

The crude extract was re-dissolved in acetonitrile: deionized water (3:7) ratio solvent mixture. Prepared sample extractions were filtered through micro filters (Agilent Captiva Econofilters, 0.45 µm) to remove any solid residue before HPLC analysis.

Preparation of Standards solution

Stevioside standard was (BCCB 4219) purchased from Sigma-Aldrich and standard solution was prepared by dissolving in a 3:7 (v/v) mixture of acetonitrile and deionized water. Initially, 5.0 mg of standard was dissolved in 5.0 mL of acetonitrile and deionized water mixture to prepare stock solution with 1.0 mg/mL concentration and it was used to prepare the concentration series of 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.5 mg/mL, 1.0 mg/L concentrations. Prepared standard solutions were filtered through micro filters (Agilent Captiva Econofilters, 0.45 µm) to remove any solid residue before HPLC analysis.

HPLC Conditions

Liquid chromatography under isocratic conditions was performed using an Agilent 100 HPLC system with a UV–vis detector set to a wave length of 210 nm. Separation was carried out on a Luna C18 (length, 150 mm; inner diameter, 4.6 mm; particle size, 5 µm) column without temperature control. The mobile phase consisted of a 30:70 mixture of acetonitrile and deionized water used at a constant flow rate of 1 mL/min. The sample injection volume was 20 µL.

Method validation

Prepared standard solutions with different concentrations were injected separately into HPLC machine. To evaluate the linearity, a graph was constructed of the signal produced as a function of analyte concentration and linear regression by the least squares method. Also the correlation coefficient was calculated. Sample solutions were injected into the HPLC machine and stevioside was identified on the basis of their correspondence of retention time using the reference standards. Each solution was injected in triplicate and the mean value was used for quantification.

Screening of phytochemicals using GC-MS

Sample preparation for GC-MS analysis

Crude samples were re-dissolved in methanol. Prepared sample extractions were filtered through micro filters (Agilent Captiva Econofilters, 0.45 µm) to remove any solid residue before GC-MS analysis. About 2.0 µL aliquots were screened for the presence of phytochemicals, using GC-MS. Each chromatogram obtained was compared for identification of important phytochemicals present in each sample.

Column conditions

Agilent 7890A GC (5% Phenyl Methyl Siloxane) capillary column was used for the separation and 5975C inert XL EI/C1 MS detector identified the compounds present (Table 1). An external standard method was used by the GC-MS machine to identify peaks and to find out the relationship between peak areas and analyte concentration in the chromatogram. Each chromatogram obtained for samples was compared for identification of important phytochemicals present in each sample.

Table 1
Column conditions for GC-MS analysis

	Rate (°C/min)	Value (°C)	Hold time (min)	Run time (min)
Initial	-	60	1	4
Ramp	10	280	4	48

Table 1

RESULT AND DISCUSSION

Analysis of Stevioside using HPLC

Method validation

Values of areas under five peaks with different concentrations were obtained and plots were developed by HPLC system using analyte concentration and peak area. The linear regressions were obtained for the standard Stevioside at five concentrations (Table 2). Correlation coefficients of the standard curve were

greater than 0.99, therefore analytical method was linear over the used concentration range and it was suitable for quantifying the stevioside content in different samples (Fig. 1).

Table 2
Chromatogram details of stevioside standard (X: Amount (ng/µL, Y: Area)

Standard	Retention Time (min)	Correlation coefficient	Formula
Stevioside	3.288	0.99903	Y = mx + b m: 2.16375 b: -32.67645

Table 2

Figure 1

According to the chromatogram obtained for stevioside standard, retention time for was 3.288 min (Fig. 2). This is not corresponded with the retention times to that as described in the original JECFA method [5] and peaks were not fully separated. This is due to the use of C18 (150 x 4.6 mm) column other than C18 (250 x 4.6 mm). Matrix load was visible at the beginning of all the chromatograms within 2 min. This might be due to the presence of proteins, organic acids, resins and pigments [6].

Figure 2

According to the results (Fig. 3, Fig. 4, Fig. 5), (Table 3), highest Stevioside content was observed in tissue cultured plants (11.462 ± 0.03 mg/mL) followed by naturally grown plants (10.132 ± 0.03 mg/mL) and in vitro derived callus (2.228 ± 0.03 mg/mL).

Table 3
Stevioside content in naturally grown, tissue cultured plants and callus of *S. rebaudiana*

	Stevioside content ± SD (mg/mL)
Retention Time	3.288
Naturally grown plants	10.132 ± 0.03
Tissue cultured plants	11.462 ± 0.03
Callus	2.228 ± 0.03

Figure 3, Fig. 4, Fig. 5

Table 3

According to the results, it was observed that tissue cultured plants have significantly higher amount of Stevioside than naturally grown plants. Callus extracts also showed the presence of Stevioside yet amount was significantly lower than the naturally grown and tissue cultured plants. This might be due to the low amount of photosynthetic pigments present in calli and incomplete development of photosynthetic mechanism in calli compared to the plants. Furthermore, callus is an undifferentiated cell mass and to obtain maximum productivity cells has to be partially or fully differentiated. According to Bondarev *et al*., (2004) [7] undifferentiated tissues of *S. rebaudiana* were unable to synthesis Stevioside in high amounts like differentiated tissues, therefore calli has lesser amount of Stevioside. Present study confirmed these facts.

According to Gonzalez *et al*., (2015) [8], 8.80 ± 0.14 g/100g Stevioside content was present in dried leaves of three months old *S. rebaudiana*. Jadhao *et al*., (2011) [9] also used aqueous extracts of dried Stevia leaves to determine the Steviol glycoside content and obtained 7.87 ± 0.08% and 7.50 ± 0.04% w/w of Stevioside and Rebaudioside A respectively. In another study it is reported the use of one month old in vitro and ex vitro leaves for the analysis of Steviol glycoside contents [10]. Extractions were analyzed by HPLC using C18 column (250 x 4.6 nm) at 254 nm and 6:4 (v/v) acetonitrile and water as the mobile phase. According to their results Stevioside was only observed in ex vitro leaves (0.20 g/kg). According to Karim *et al*., (2015) [11] methanolic extract of *S. rebaudiana* leaves contained 9.6% of Stevioside and in another study Stevioside content in leaves was found to be vary from 3.17–9.94% [12]. Aman *et al*., (2013) [13] reported a higher stevioside (82.48 µg/g-DW) content in in vitro derived Stevia plants on a medium containing a combination of BA and Kin. Another study reported the highest content of stevioside (8.18%) in plants grown on half MS mediumsupplemented with 0.2 mg/L IBA, and 2 mg/L active carbon [14]. The results of the previous studies suggest that the synthesis of important secondary metabolites in *S. rebaudiana* vary with many external and internal factors.

In a study reported by Javad *et al*., (2016) [15], *S. rebaudiana* callus cultures established on MS media supplemented with different concentrations of plant growth regulators were used for extraction. They were analyzed for the presence of Stevioside using HPLC using an amino C18 column at 210 nm and 80:20 methanol and water as the mobile phase. According to the results it was observed that callus initiated on MS medium supplemented with 1.0 mg/L BAP, 1.0 mg/L NAA and 2.5 mg/L 2,4-D has the highest amount of Stevioside content (33.87 mg/g) and when compared the Stevioside content of green, white and brown calli and the highest amount was observed in green calli. Therefore, they stated that green calli have developed their photosynthetic mechanism which indicates that S. rebaudiana plants require ample amount of sunlight to synthesize Stevioside [15]. Leaf discs and stem derived calli were used to analyzed the Stevioside content using HPLC and Stevioside content was significantly higher in leaf discs derived calli (0.33 mg/g) than in stem derived calli (0.04 mg/g) [16].

Screenings of phytochemicals using GC-MS

Chromatograms and mass spectra obtained by GC analysis for leaves of naturally grown plants, tissue cultured plants and callus illustrated in Fig. 6, Fig. 7 and Fig. 8. Altogether twenty seven (27) important phytochemicals (Table 4) were identified in all three tested samples: twenty two (22) in naturally grown

plants, twenty one (21) in tissue cultured plants and sixteen (16) in callus. Out of those, fourteen (14) compounds were found to be common in all three samples.

Table 4
Phytochemicals present in leaves of natural and tissue cultured plants, callus of *S. rebaudiana* and their potential bio activities

No	Compound name	RT (min)	Natural	Tissue cultured	Callus	Bio activity
1	Oleic acid	7.551	√	√	√	anticancer anti-inflammatory antimicrobial flavouring agent food additive
			5.8%	4.4%	2.6%	
2	3,4-dimethyl-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	12.736	√	-	-	antimicrobial
			0.8%	-	-	
3	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-1-methoxy	13.897	√	√	√	antiallergic antimicrobial anticancer antimalarial antidiabetic
			8.9%	10.0%	7.9%	
4	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	20.494	√	-	-	antimicrobial anti-inflammatory antihyperlipidemic
			0.4%	-	-	
5	Caryophyllene	21.241	√	-	-	anti-inflammatory antidepressive anticancer
			0.7%	-	-	
6	Bicyclo[2.2.1]heptan-2-ol, 3,3-dimethyl-, oxo-	21.751	√	-	-	antimicrobial anti-inflammatory antihyperlipidemic
			0.6%	-	-	
7	2H-Pyrrol-2-one, 4-acetyl-1,5-dihydro-3-hydroxy-1-[2-(1H-imidazol-4-yl)ethyl]-5-(methoxyphenyl)	22.600	√	√	√	antimicrobial anti-inflammatory anticancer
			7.9%	2.4%	3.4%	
8	Tetradecanoic acid	23.019	√	√	√	antioxidant cancer preventive nematicide
			2.9%	1.9%	2.7%	

No	Compound name	RT (min)	Natural	Tissue cultured	Callus	Bio activity
9	isopulegol acetate	23.581	√	√	√	flavouring agent
			1.4%	0.7%	1.0%	
10	Carbamic acid, tricyclo[2.2.1.0(2,6)]hept-3-yl-, ethyl ester	23.973	√	√	√	antiamoebic
			2.3%	2.0%	2.7%	
11	2,6,10,14-Hexadecatetraen-1-ol,3,7,11,15-tetramethyl-,acetate, (E,E,E)-	24.954	√	√	-	antimicrobial
			7.2%	0.5%	-	anti-inflammatory anticancer
12	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo	26.732	√	√	√	antimicrobial
			2.4%	6.4%	4.7%	
13	1H-pyrazol-5-amine, 3-methyl-1-phenyl-	27.898	√	√	-	antimicrobial
			0.8%	2.1%	-	anti-inflammatory antitumour
14	2(3H)-Furanone, dihydro-4,5-dimethyl-	28.212	-	√	√	antiviral
			-	19.8%	2.1%	
15	Bicyclo[3.1.1]heptanes,2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-	28.987	√	√	√	antimicrobial
			11.8%	10.6%	14.0%	anti-inflammatory antihyperlipidemic antioxidant
16	1,4-Eicosadiene	29.876	√	√	√	antimicrobial
			6.5%	2.2%	7.7%	antioxidant
17	5,9-Undecadien-2-one, 6,10-dimethyl-,)z)-	30.076	√	√	-	flavouring agent
			7.6%	5.1%	-	food additive
18	Hexadecanoic acid, methyl ester	31.008	√	√	√	antimicrobial
			1.0%	1.0%	1.3%	anti-inflammatory hypercholesteromic anticancer
19	7-Choloro-4-methoxy-3-methylquinoline	31.582	√	√	√	antiturbercular
			3.0%	0.9%	1.3%	
20	Kaur-16-ene	32.594	√	-	-	antimicrobial
						anti-inflammatory

No	Compound name	RT (min)	Natural	Tissue cultured	Callus	Bio activity
			0.9%	-	-	
21	10.alpha.-Eremophilane	35.903	-	√	-	anticancer
			-	1.0%	-	anti-inflammatory antibacterial
22	4-Quinolinecarboxylic acid, 2-chloro-	36.094	-	√	-	antiviral
			-	0.7%	-	
23	Octadecanoic acid	37.388	√	√	√	antimicrobial
			4.7%	3.1%	9.5%	anti-inflammatory hypercholesteromic
24	2-Myristynoyl-glycinamide	38.039	-	-	√	antimicrobial
			-	-	0.6%	
25	1H-Indole,5-methyl-2-phenyl	38.896	√	√	√	antibacterial
			1.9%	0.8%	0.6%	anti-inflammatory
26	2-Ethylacridine	41.522	√	√	√	antimicrobial
			3.9%	2.0%	7.5%	antioxidant
27	Pyrido(2,3-d)pyrimidine, 4-phenyl-	44.117	-	√	-	anticancer antimicrobial
			-	0.9%	-	anti-inflammatory antiviral antihypertensive

Figure 6, Fig. 7, Fig. 8

Table 4

GC-MS analysis of leaf extracts of Stevia revealed the presence of terpenes, fatty acids, aldehydes, ketones and alcohols [17]. In another study Maheshwari *et al.*, (2017) [4] screened the hexane extract of Stevia by GC-MS and identified the saturated fatty acids, hydrocarbons and esters which are used as flavoring agents, food additives, pharmaceutically important drugs. Present study confirmed their findings which shows the presence of terpenes: (4,8,8-Trimethyl-2-methylene-4-vinyl bicyclo[5.2.0]nonane, Caryophyllene, Bicyclo[2.2.1]heptan-2-ol, 3,3-dimethyl-, exo-, 2,6,10,14-Hexadecatetraen-1-ol,3,7,11,15-tetramethyl-,acetate, (E,E,E)-, Bicyclo[3.1.1] heptanes,2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-, 10.alpha.-Eremophilane), fatty acids (Oleic acid, Tetradecanoic acid, Octadecanoic acid), esters

(Carbamic acid, tricyclo[2.2.1.0(2,6)]hept-3-yl-, ethyl ester, Hexadecanoic acid, methyl ester). This leads to the conclusion that the possibility of using Stevia extract as a source of natural products other than Steviol glycosides, since the extracts are rich in other important phytochemicals.

According to obtained chromatograms, Oleic acid, 2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-1-methoxy, 2H-Pyrrol-2-one, 4-acetyl-1,5-dihydro-3-hydroxy-1-[2-(1H-imidazol-4-yl)ethyl]-5-(methoxyphenyl), Tetradecanoic acid, isopulegol acetate, Carbamic acid, tricyclo[2.2.1.0(2,6)]hept-3-yl-, ethyl ester, Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo, Bicyclo[3.1.1]heptanes,2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-, 1,4-Eicosadiene, Hexadecanoic acid, methyl ester, 7-Choloro-4-methoxy-3-methylquinoline, Octadecanoic acid, 1H-Indole,5-methyl-2-phenyl and 2-Ethylacridine are present in all three samples at the retention time 7.551, 13.897, 22.460, 23.019, 23.581, 23.973, 26.823, 28.987, 29.876, 31.008, 31.582, 37.388, 39.127 and 41.522 respectively.

Out of those Oleic acid is a fatty acid with several biological properties such as anticancer, anti-inflammatory, antimicrobial and also wound healing ability. It is also use as a flavoring agent and food additive [5]. Jisha *et al*., (2016) [18] stated that 2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-1-methoxy is a ketone compound with several pharmaceutical properties such as antiallergic, antimicrobial, antimoxident, anticancer, antimalarial and antidiabetic etc. 2H-Pyrrol-2-one, 4-acetyl-1,5-dihydro-3-hydroxy-1-[2-(1H-imidazol-4-yl)ethyl]-5-(methoxyphenyl) is an aromatic compound with antimicrobial, anti-inflammatory and anti-tumour activities. Tetradecanoic acid is a common fatty acid found in many plants with antioxidant, cancer preventive and nematicide property [19]. Isopulegol acetate has perppermint flavour thus used as a flavouring agent [5]. Carbamic acid, tricyclo[2.2.1.0(2,6)]hept-3-yl-, ethyl ester is a carbamate derivative and it has antiamoebic activity. Pichardo *et al*., (2005) [20] suggested that these derivatives are a new potential antiamoebic drug. According to the study of Ololade *et al*., (2017) [21] Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo has natural antimicrobial activity against some gram-possitive and gram-negative bacteria. Bicyclo[3.1.1]heptanes,2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]- is a Sesquiterpene known to possess antimicrobial, anti-inflammatory, antihyperlipidemic and antioxidant properties [22]. According to Mehmetoglu *et al*., (2017) [23] and Peng *et al*., (2017) [24] 1,4-Eicosadiene and 2-Ethylacridine have antimicrobial activity against some gram-positive bacteria and also antioxidant activity. Study of Candea *et al*., (2009) [25] revealed the antiturbercular activity of 7-chloro-4-quinolinylhydrazones derivatives; 7-Choloro-4-methoxy-3-methylquinoline. 1H-Indole,5-methyl-2-phenyl is a nitrogen containing heterocyclic molecule widely find in plants, which has antibacterial and anti-inflammatory activity [26]. Hexadecanoic acid, methyl ester and Octadecanoic acid are Decanoic acid derivatives and have antimicrobial, anti-inflamatory, hypercholesteromic, anticancer and lubricant properties. Therefore used in pharmaceutical, cosmetic, perfume industries and also in food industry as a flavouring agent [27].

2,6,10,14-Hexadecatetraen-1-ol,3,7,11,15-tetramethyl-,acetate, (E,E,E)-, 1H-pyrazol-5-amine, 3-methyl-1-phenyl- and 5,9-Undecadien-2-one, 6,10-dimethyl-,)z)-were identified in both naturally grown and tissue cultured plant extracts while 2(3H)-Furanone, dihydro-4,5-dimethyl- was identified in tissue cultured plant and callus extracts. 2,6,10,14-Hexadecatetraen-1-ol,3,7,11,15-tetramethyl-,acetate, (E,E,E)- is a terpene

alcohol with antimicrobial, anti-inflammatory and anticancer properties. Tantawy *et al.*, (2012) [28] stated the hypolipidemic, antimicrobial, anti-inflammatory and antitumour activities of 1H-pyrazol-5-amine, 3-methyl-1-phenyl- and 5,9-Undecadien-2-one, 6,10-dimethyl-,)z)- is used as a flavouring agent and a food additive [5]. 2(3H)-Furanone, dihydro-4,5-dimethyl- is a ketone compound with antiviral activity [18].

Out of twenty seven compounds four were identified only in extract of naturally grown plants. They were 3,4-dimethyl-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide, Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-, Caryophyllene, Bicyclo[2.2.1]heptan-2-ol, 3,3-dimethyl-, oxo- and Kaur-16-ene. According to Karthikeyan *et al.*, (2016) [19] 3,4-dimethyl-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide is a sulphur compound with antimicrobial activity. Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl and Bicyclo[2.2.1]heptan-2-ol, 3,3-dimethyl-, oxo- are sesquiterpenes with several pharmaceutical properties such as antimicrobial, anti-inflammatory and antihyperlipidemic activity [22]. Francomano *et al.*, (2019) [29] stated that Caryophyllene is a natural bicyclic sesquiterpene with anti-inflammatory action via inhibiting the main inflammatory mediators. According to their study, pre-clinical data highlighted that Caryophyllene is potentially useful in osteoporosis, Streptococcus infections and exerts analgesic, antidepressive effects. Furthermore it has anticancer activity against breast, melanoma, pancreas and glioma cancer. Kaur-16-ene is a precursor of many other structural types of diterpenes such as gibberelic acid, grayonotoxines as well as Steviol glycosides and exhibit biological activities including antimicrobial and anti-inflammatory effects [30].

Pyrido(2,3-d)pyrimidine, 4-phenyl- and 10.alpha.-Eremophilane, 4-Quinolinecarboxylic acid, 2-chloro- were identified only in extracts of tissue cultured plants. A sesquiterpene, 10.alpha.-Eremophilane is a which can be find in both plants and fungi with biological activities including anticancer, anti-inflamatory and antibacterial [31] and 4-Quinolinecarboxylic acid 2-chloro- has antiviral activity [32]. Shamroukh *et al.*, (2016) [33] stated that Pyrido(2,3-d)pyrimidine, 4-phenyl- is a heterocyclic fusion of pyrimidine and pyridine rings with important phytochemical properties such as anticancer, antimicrobial, anti-inflammatory, antiviral and antihypertensive. 2-Myristynoyl-glycinamide was only identified in callus extract of S. rebaudiana and it is is an amino compound with antimicrobial activity [34].

CONCLUSION

According to HPLC analysis tissue cultured plants higher contents of Stevioside than naturally grown plants after three months of growth. Therefore micropropagation, or in vitro culture have high potential to overcome difficulties in conventional mass propagation methods and has the potential to produce genetically uniform, large quantity of better-quality Stevia plantlets to fulfil the current global demand. Identification of individual phytochemical constituents and their biological activities revealed the possibility of using Stevia extract as a source of natural products (in varies pharmaceutical, flavoring agents, food additives and perfume industries) other than Steviol glycosides, since the extracts are rich in other important phytochemicals. Some phytochemicals present in natural plants are also identified in tissue cultured plants and callus. This leads to the conclusion that *in vitro* cultures have potential to extract important phytochemicals used in several industries. It is a green light that possible use of callus

to obtain important phytochemicals instead of plant raw materials as those are more eco friendly and do not rely on environmental changes.

Abbreviations

HPLC
High Performance Liquid Chromatography
GC
MS-Gas Chromatography Mass Spectrometry
UV
Ultraviolet

Declarations

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STATEMENT & DECLARATIONS

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Competing Interests

Authors have no relevant financial or non-financial interests to disclose.

Author Contributuions

Both MDKM Gunasena and WTPSK Senarath contributed to the study conception and design. Material preparation, data collection and analysis were performed by MDKM Gunasena. The firstdraft of the manuscript was written by MDKM Gunasena and both authors commented on previous versions of the manuscript. Both authors read and approved the final manuscript.

Ethical approval

Not applicable

Consent to participate

Not applicable

Consent to publish

Not applicable

Data availability statement

The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

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Figures

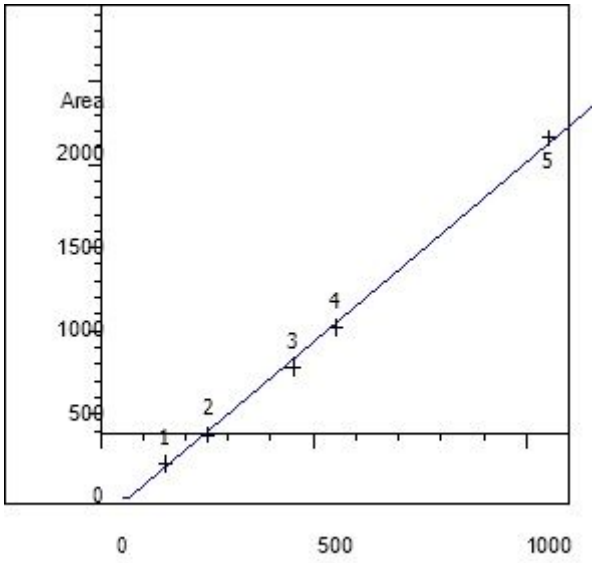

Figure 1

Standard curve of Stevioside

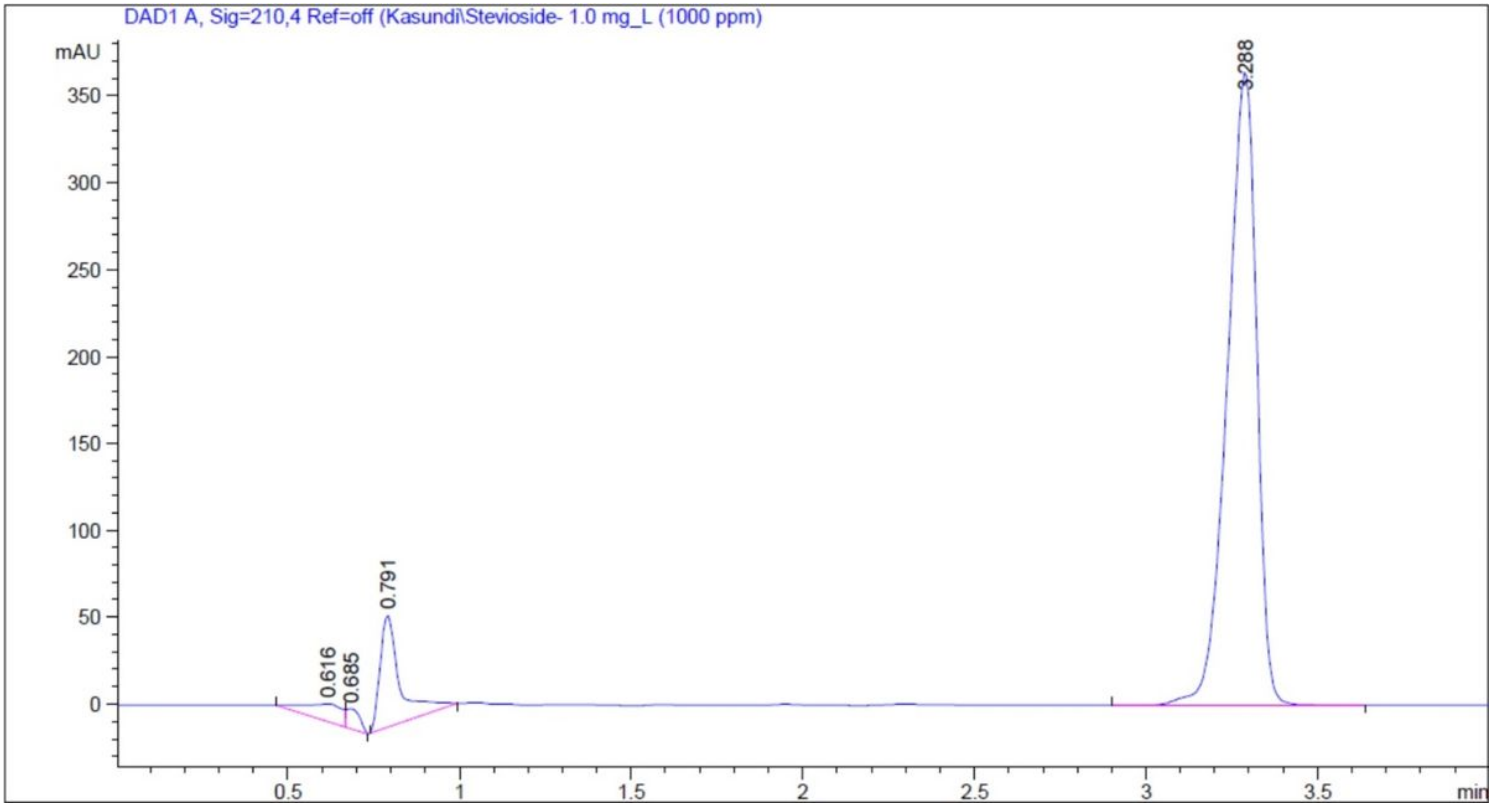

Figure 2

HPLC chromatogram of Stevioside standard

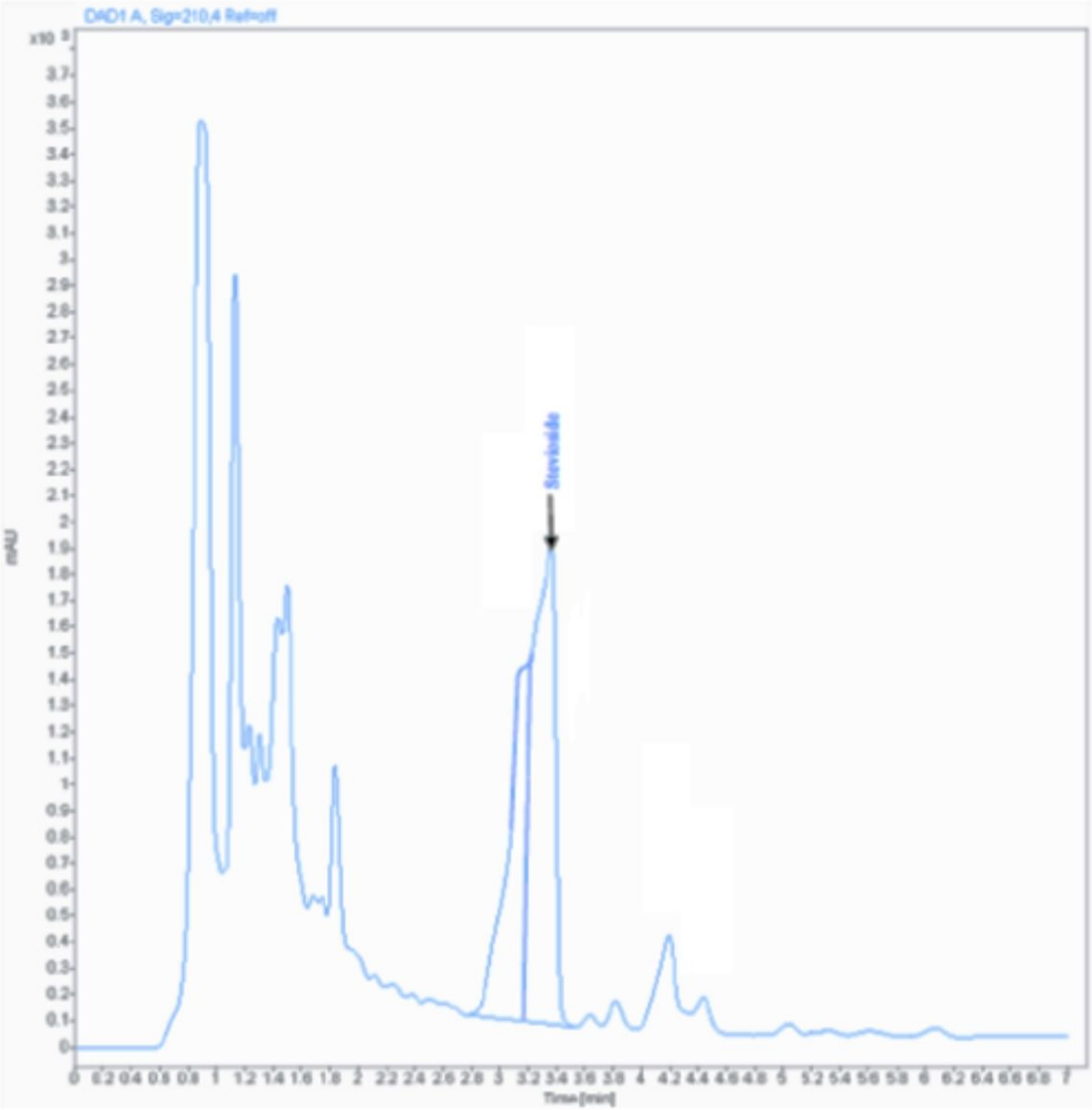

Figure 3

HPLC chromatogram of leaf extracts of naturally grown *S. rebaudiana* plants

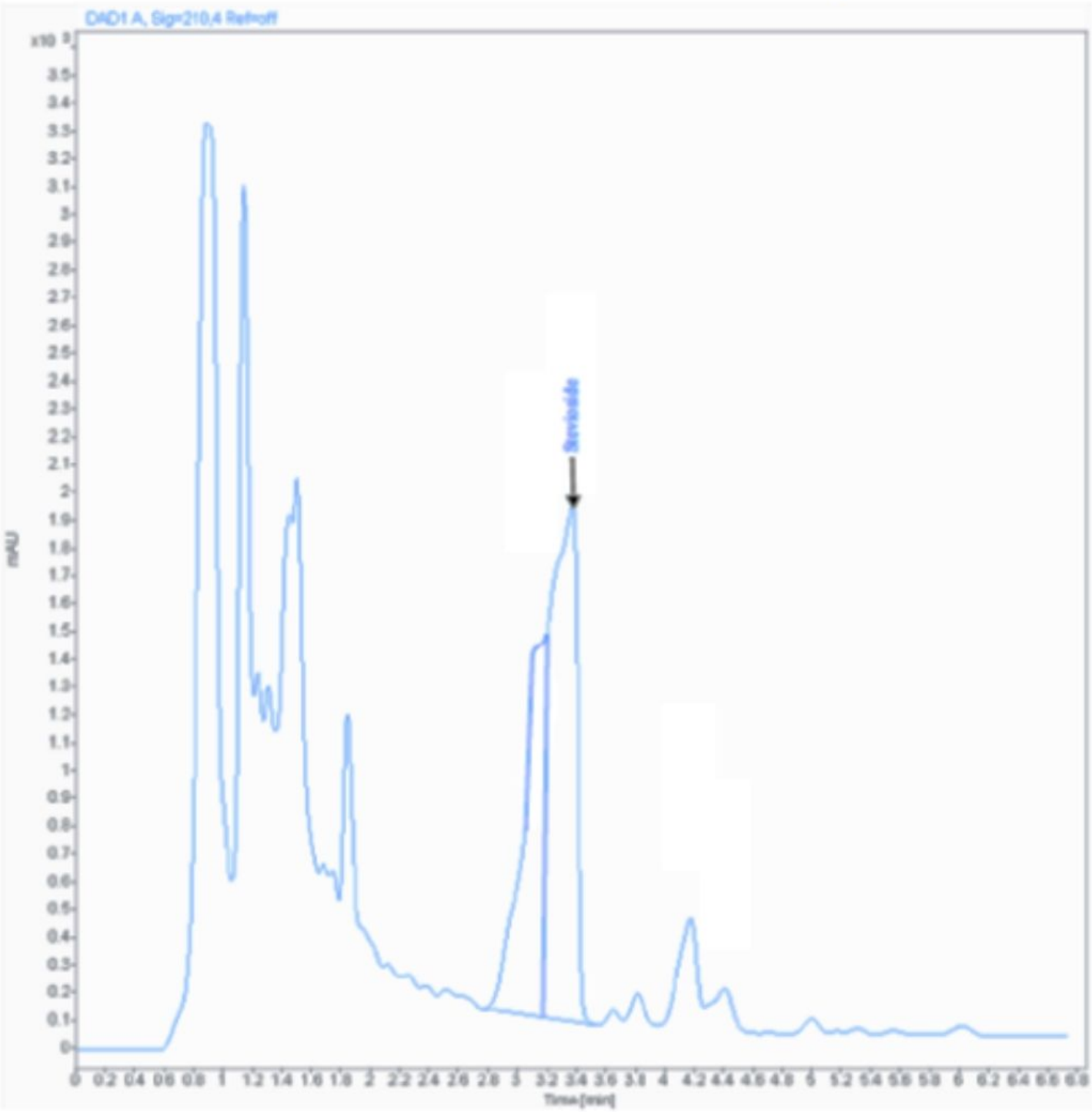

Figure 4

HPLC chromatogram of leaf extracts of tissue cultured *S. rebaudiana* plants

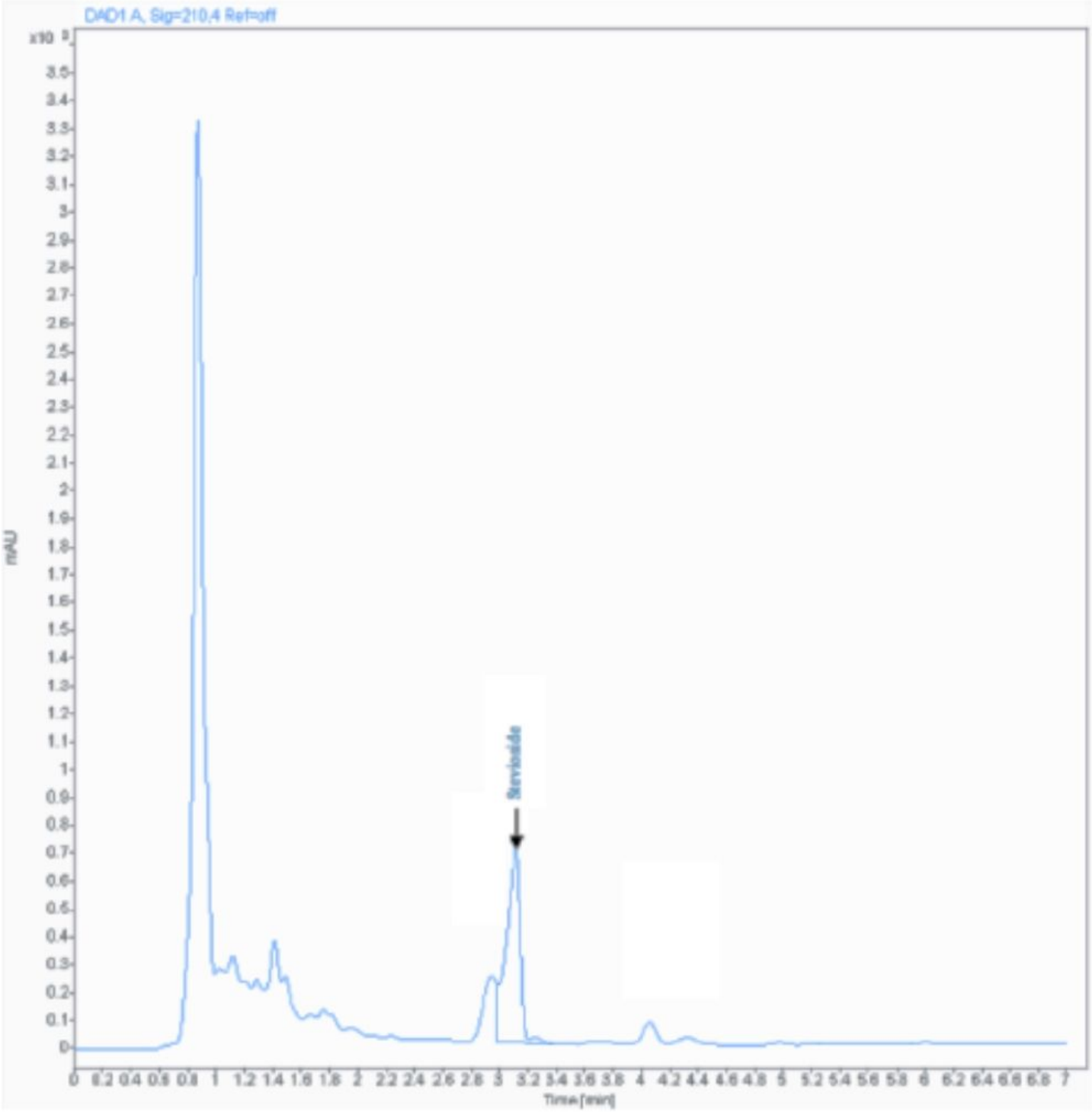

Figure 5

HPLC chromatogram of extracts of in vitro derived *S. rebaudiana* calli

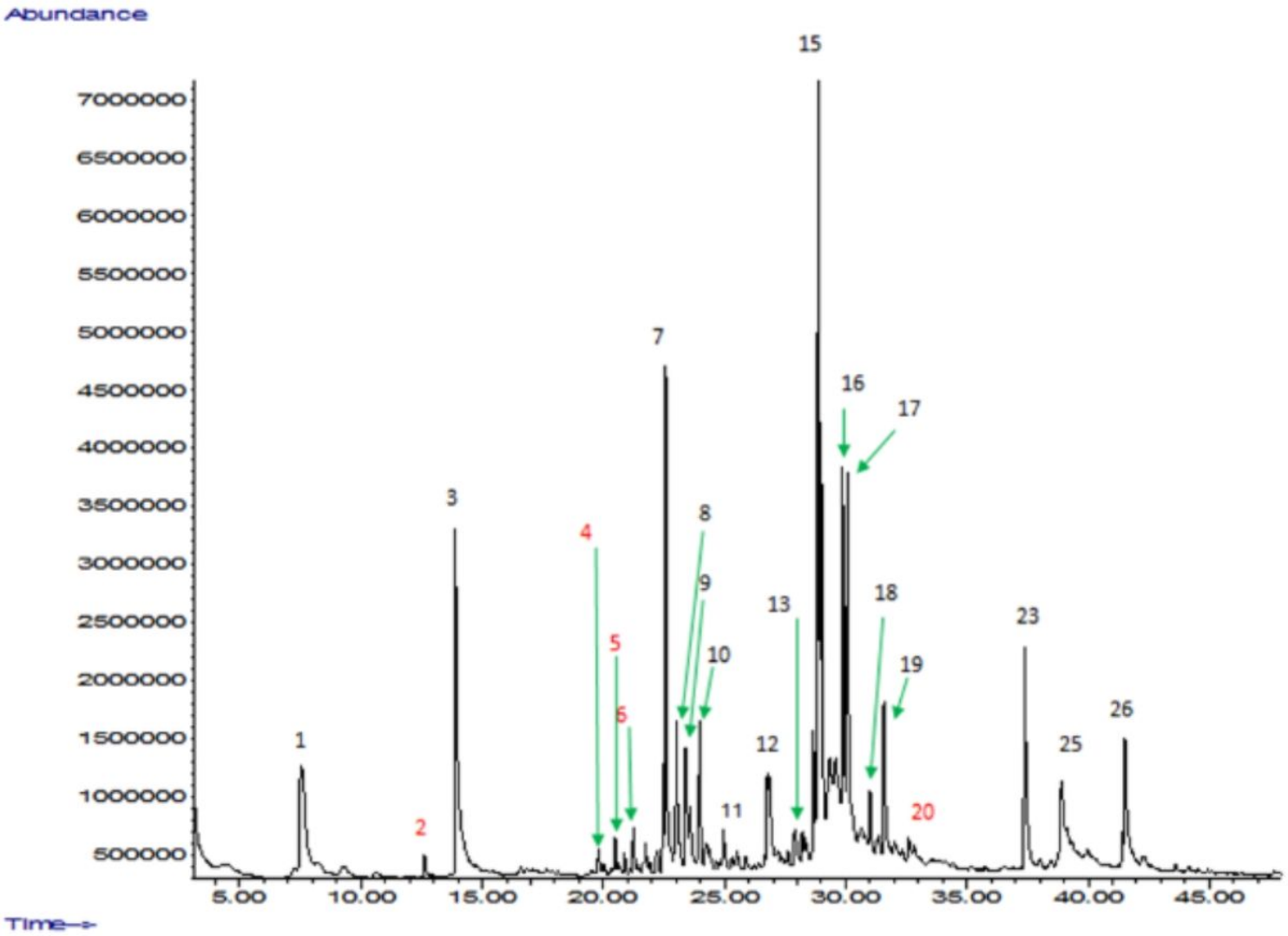

Figure 6

GC-MS chromatogram of leaf extract of naturally grown plants

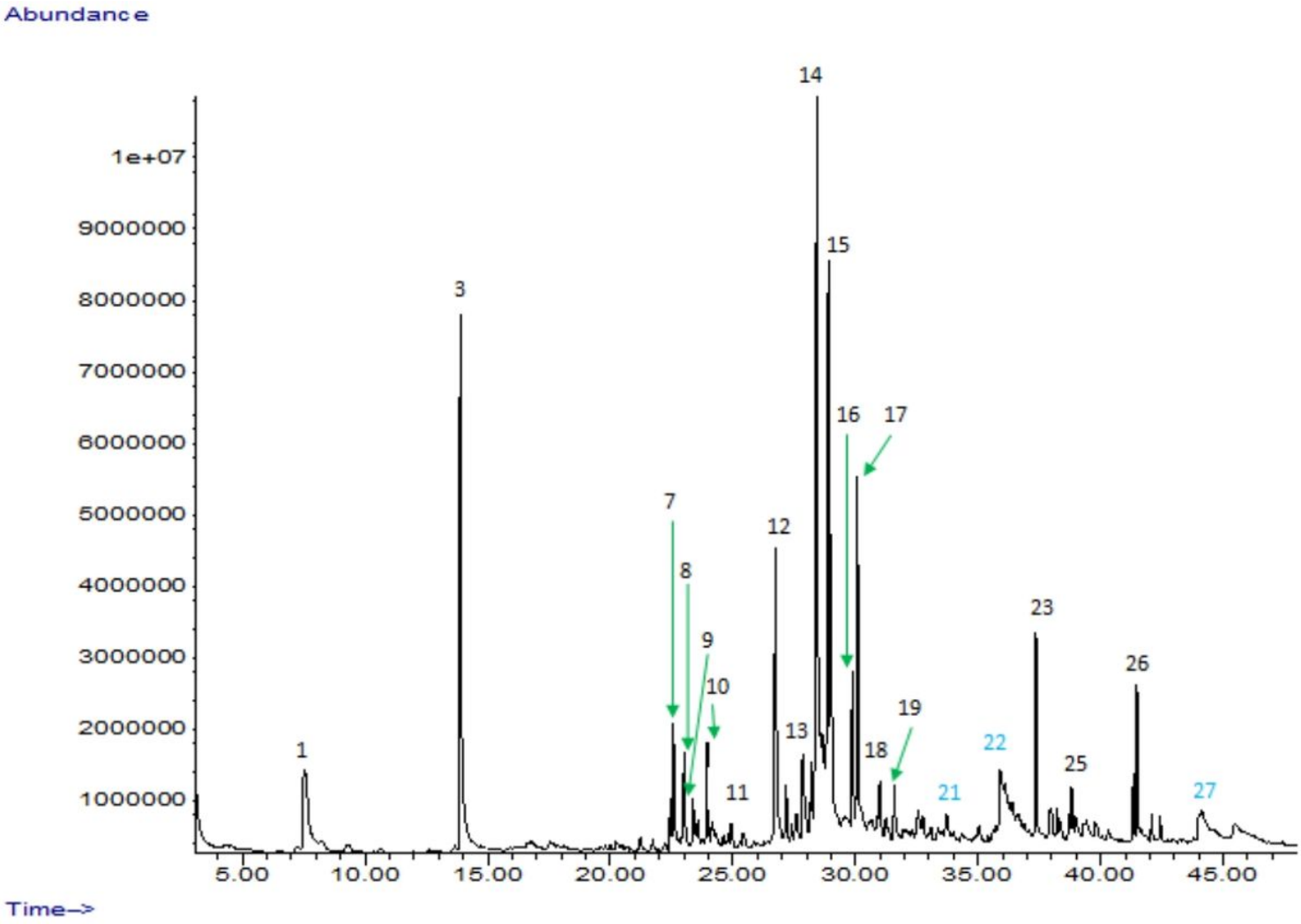

Figure 7

GC-MS chromatogram of leaf extract of tissue cultured plants

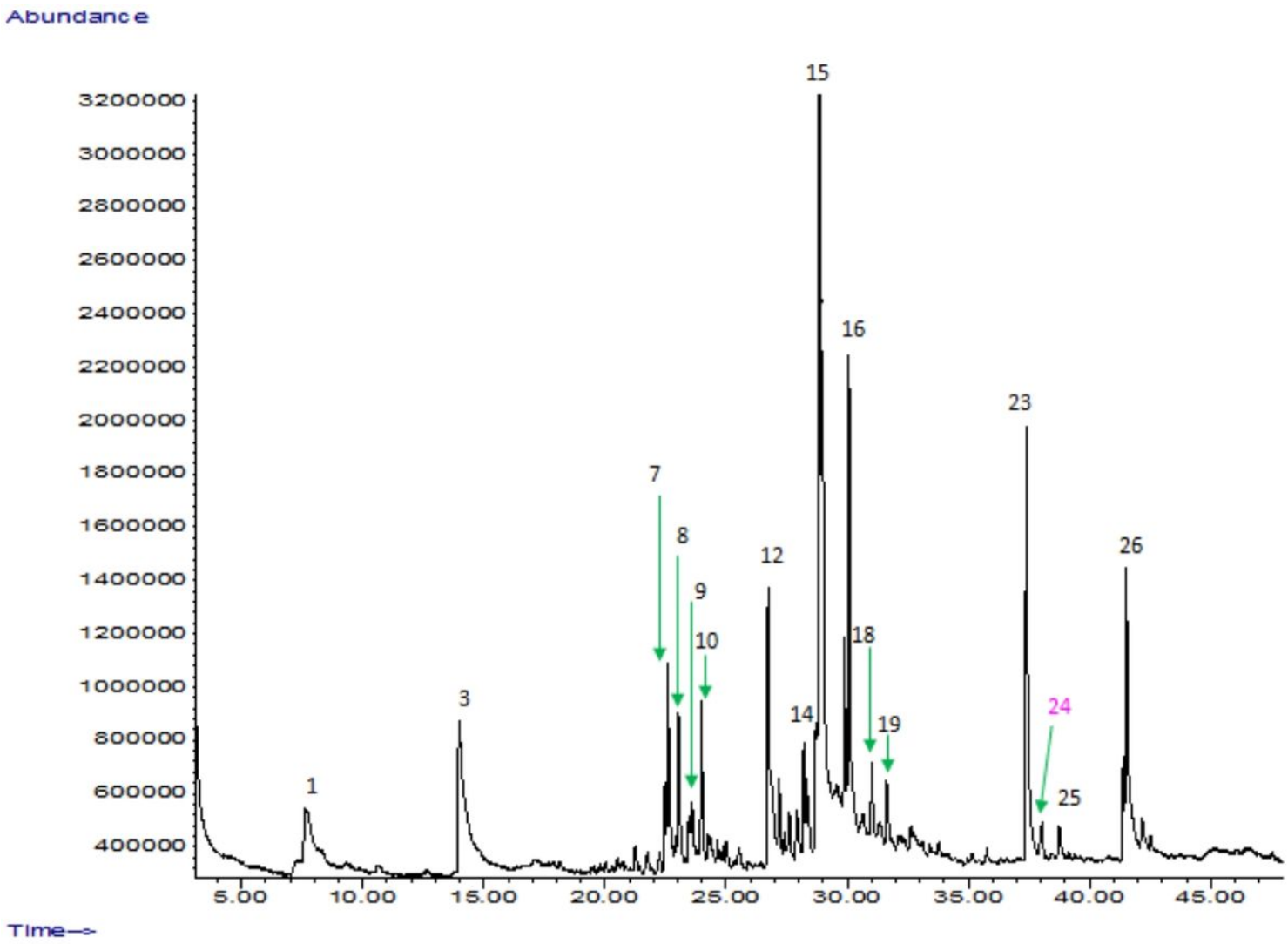

Figure 8

GC-MS chromatogram of extract of in vitro derived *S. rebaudiana* calli