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Full Paper

Establishment of *Stevia rebaudiana* Cell Cultures and Phytochemical Profiling via GC-MS and HPLC

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Abstract

Stevia rebaudiana, a plant renowned for its high content of non-caloric steviol glycosides, is widely used as a natural sweetener. This study focused on the establishment of Stevia cell suspension cultures from in vitro-derived and the comparative phytochemical analysis of leaves, calli, and cell suspension cultures using Gas Chromatography–Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC). Six-week-old leaf disc-derived calli (~5.0g) were transferred into 150 mL of liquid Murashige and Skoog (MS) medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA, and they were agitated at different speeds (50, 100, 150 rpm) on a rotary shaker at 25±1°C and kept under both light and dark conditions. Phytochemical analysis via GC-MS and HPLC was conducted on three-month-old plant leaves, six-week-old calli, and two-week-old cell cultures. Phytochemical extraction of leaves, callus, and cell pellet obtained by centrifugation was performed using methanol as the solvent. The optimal agitation speed for successful culture establishment was 100 rpm, which ensured effective cell separation. Lower speeds resulted in cell clustering, while higher speeds caused cell damage. HPLC analysis showed that plant leaves accumulated significantly higher levels of steviol glycosides (Rebaudioside A: 8.234±0.04 mg/mL, Stevioside: 10.132±0.03 mg/mL, Rebaudioside C: 1.585±0.02mg/mL, Dulcoside A: 0.477±0.01 mg/mL) compared to callus (Rebaudioside A: 1.793±0.01 mg/mL, Stevioside: 2.228±0.03 mg/mL, Rebaudioside C: 0.365±0.03 mg/mL, Dulcoside A: 0.118±0.01 mg/mL) and cell cultures (Rebaudioside A: 0.272±0.02 mg/mL, Stevioside: 0.384±0.01 mg/mL). This disparity is likely due to the higher levels of photosynthetic pigments in leaf tissues, which positively correlated with steviol glycoside content. The callus exhibited lower glycoside levels, and cell cultures produced minimal amounts, underscoring the essential role of light in glycoside biosynthesis. GC-MS identified distinct phytochemical profiles across the different culture systems (22 in leaves, 16 in callus, and 12 in cell culture), revealing bioactive compounds with pharmacological properties. The results indicate that in vitro systems represent promising platforms for producing bioactive phytochemicals, including steviol glycosides, although further optimization is required.

Keywords: Cell suspension culture, GC-MS, HPLC, *Stevia rebaudiana*, steviol glycosides

Introduction

Stevia rebaudiana (Bert.) *Bertoni*, a perennial herbaceous plant, belongs to the genus Stevia within the family Asteraceae. Commonly referred to as sweet leaf, honey leaf, or candy leaf herb, Stevia is native to the highland regions of northeastern Paraguay, particularly the Rio Monday Valley in the Amambay Mountain

region [1, 2]. The plant naturally grows on marsh edges or grasslands with shallow water tables, flourishing in semi-humid subtropical climates with average temperatures of 23 °C and annual rainfall ranging from 1500 to 1800 mm. From its origin in South America, the cultivation of Stevia expanded globally, with its first commercial use reported in Paraguay in 1964 and its introduction to Japan in 1968. Today, it is grown extensively in countries such as China, Korea, Brazil, and India, driven by its increasing demand as a natural sweetener [3].

Stevia rebaudiana leaves are a rich source of Steviol glycosides, the compounds responsible for their intense sweetness [4]. Additionally, they contain other bioactive compounds such as flavonoid glycosides, coumarins, cinnamic acids, phenylpropanoids, and various essential oils [5]. The leaves also yield triterpenes like amyrin acetate, three esters of lupeol, and sterols such as stigmasterol, sitosterol, and campesterol [2]. Furthermore, *S. rebaudiana* is abundant in phenols, flavonoids, and antioxidants, which enhance its functional and nutritional value.

The plant contains approximately ten main Steviol glycosides, including Stevioside, Rebaudioside A, B, C, D, E, F, Dulcoside A, and Steviolbioside [2]. Among these, the four primary sweeteners are Stevioside, Rebaudioside A, Rebaudioside C, and Dulcoside A, with relative sweetness levels of 210, 242, 30, and 30 times that of sucrose, respectively. Stevioside constitutes about 65% of the total glycosides, making it the dominant sweetener compound. However, it can impart a slight licorice-like aftertaste, which some consumers find undesirable [3]. On the other hand, Rebaudioside A is highly favored due to its superior sweetness and lack of bitterness, comprising about 30% of the glycoside content.

In addition to Steviol glycosides, *S. rebaudiana* contains over 100 phytochemicals, including a variety of terpenes and flavonoids. Key compounds identified in *S. rebaudiana* include apigenin, austroinulin, avicularin, beta-sitosterol, caffeic acid, campesterol, caryophyllene, centaureidin, chlorogenic acid, cosmosiin, cynaroside, daucosterol, foeniculin, formic acid, gibberellin, indole-3-acetonitrile, isoquercitrin, jhanol, kaempferol, kaurene, lupeol, luteolin, polystachoside, quercetin, quercitrin, scopoletin, sterebin A-H, stigmasterol, umbelliferone, and xanthophylls [6, 7]. These compounds not only contribute to the plant’s therapeutic and nutritional profile but also make it an attractive candidate for applications in food, medicine, and other industries.

Traditional propagation of *S. rebaudiana* through seeds and vegetative methods faces significant challenges. Seed germination is often poor due to low viability and inconsistent development, making it unsuitable for large-scale cultivation [8-10]. Propagation via stem cuttings, while effective, demands substantial labor and has a low survival rate under field conditions. Additionally, factors such as cutting length, season, and the parent plant's condition heavily influence success rates. Consequently, these traditional methods limit the scalability and quality consistency required for commercial production. To address these limitations, in vitro culture systems offer a promising alternative for the efficient production of Steviol glycosides and other valuable compounds. Tissue culture techniques, such as callus and cell suspension cultures, allow for the controlled synthesis of secondary metabolites under optimal conditions. Callus cultures are initiated from explants such as leaves, nodal segments, and internodes and cultured in media supplemented with

growth regulators. These cultures provide a renewable source of biomass that can be scaled up for metabolite production. Cell suspension cultures, derived from callus, enable even faster proliferation of cell mass and enhance the biosynthetic capacity of plant cells.

This research aims to explore the application of in vitro culture systems, such as callus and cell suspension cultures, for the production of Steviol glycosides and other phytochemicals in *S. rebaudiana*. By addressing the limitations of traditional cultivation, this study seeks to contribute to the development of scalable, efficient, and sustainable production methods for these valuable compounds, supporting the growing demand for natural sweeteners and bioactive phytochemicals in the global market.

Materials and Methods

Maintenance of Mother Stock and in vitro Culture Establishment

S. rebaudiana plants were maintained in a shade house (23-25 °C, 60% shade) at the botanical garden of the Department of Botany, University of Sri Jayewardenepura. Mother plants were used to collect seeds for in vitro culture establishment and phytochemical extraction. The seeds were surface sterilized and cultured on MS medium supplemented with 3.0 mg/L Gibberellic acid (GA_3) for in vitro germination [11]. Two-week-old in vitro germinated seedlings were then used to collect leaves for callus induction.

Establishment of Callus Cultures

Leaf discs were inoculated on MS medium supplemented with 2.0 mg/L 6-Benzylaminopurine (BAP) and 1.0 mg/L Naphthaleneacetic acid (NAA) for callus induction. The nature of calli and the number of days taken to initiate the calli were observed over six weeks.

Establishment of Cell Suspension Cultures

Six-week-old leaf disc-derived calli (about 5.0 g) were transferred into 250 mL conical flasks containing 150.0 mL liquid MS medium with 2.0 mg/L BAP and 1.0 mg/L NAA (pH 5.6 ± 0.5) and agitated at various speeds (50, 100, and 150 rpm) on a rotary shaker under both light and dark conditions at 25±1 °C. Cultures were observed for any contamination in the cell cultures and cell aggregate formation. After one week, a 5.0 mL sample from each culture was microscopically examined for cell dispersion and cluster formation. The remaining suspensions were transferred into 100.0 mL of the same fresh media to make the final volume up to 150.0 mL, and agitated at 100 rpm in both light and dark conditions for another week before being prepared for phytochemical analysis.

Phytochemical Analysis of Leaves, Callus, and Cell Suspension Cultures

Leaves of three-month-old *S. rebaudiana* plants and six-week-old calli were washed, sun-dried for five days, oven-dried at 60 °C for one hour, and ground into powder. Two-week-old cell suspension cultures were

also analyzed for phytochemicals, with each extract being analyzed in triplicate (technical replicates).

Analysis of Steviol Glycosides Using High-Performance Liquid Chromatography

Steviol glycosides were analyzed by high-performance liquid chromatography (HPLC) following phytochemical extraction. About 5.0 g of each sample (leaf and calli powder) was extracted using 50.0 mL of methanol (1:10 w/v ratio) for 24 hours in a 150 rpm shaker at 25±1 °C and filtered through the Whatman filter paper. This process was repeated for each sample, and filtrates were collected. Thereafter, methanol was evaporated using a rotary evaporator at 40 °C under reduced pressure. The crude extract was re-dissolved in acetonitrile: deionized water (3:7) ratio solvent mixture and was filtered through micro filters before HPLC analysis.

For cell suspension cultures, initially, suspension cultures (150.0 mL) were centrifuged at 10,000 rpm for five minutes, and the supernatant was decanted. The pellet containing cell mass was dried and redissolved in methanol. The methanol extract was filtered through micro filters and used for HPLC analysis.

Preparation of Standard Solutions

Standards of Stevioside (BCCB 4219), Rebaudioside A (BCCB 1873), Rebaudioside C (BCCB 1872), and Dulcoside A (BCCB 1887) were purchased from Sigma-Aldrich. Standards series were prepared by dissolving four standards in a 3:7 (v/v) mixture of acetonitrile and deionized water separately. For Stevioside, Rebaudioside A, and Rebaudioside C, solutions with 0.1, 0.2, 0.4, 0.5, and 1.0 mg/L concentrations, and for Dulcoside A, solutions with 0.05, 0.1, 0.2, 0.5, and 1.0 mg/L concentrations were prepared. Prepared standard solutions were filtered through micro filters before HPLC analysis.

HPLC Conditions

HPLC under isocratic conditions was performed using an Agilent 100 HPLC system with a G1314A 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.

Screening of Phytochemicals Using Gas Chromatography–Mass Spectrometry (GC-MS)

Crude samples were re-dissolved in absolute methanol and filtered through micro filters before Gas Chromatography–Mass Spectrometry (GC-MS) analysis. An Agilent 7890B GC equipped with MS-5975C inert MSD was used for GC-MS analysis. The GC was equipped with an HP-5MS 5% phenyl methyl siloxane column (length: 30.0 m; width: 250.0 μm; film thickness: 0.25 μm). The oven temperature was initially maintained at 60 °C for 1 min holding time and raised to 280 °C at a rate of 10 °C/min with 4 min holding time. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1.0 mL/min.

Results and Discussion

Establishment of Cell Suspension Cultures

After one week of initiating cell suspension cultures from the callus, the medium turned a brownish-yellow color (Figure 1), likely due to the accumulation of secondary metabolites. Microscopic examination revealed loosely bound, globular cell aggregates around the inoculated calli and dispersed throughout the suspension.

Figure 1. Digital image of cell suspension cultures at 100 rpm agitation speed after one week

Agitation speed significantly influenced cell behavior: at 100 rpm, there were fewer small clusters, and over 70% of cells were separated, indicating an optimal balance for cell separation. At 50 rpm, less than 50% of cells were separated, with more clustering observed, while at 150 rpm, although over 80% of cells were separated, damaged cells were noted (Table 1). Cell growth was absent in growth regulator-free cultures, highlighting the need for exogenous regulators. Light conditions did not affect cell aggregation or separation but influenced secondary metabolite synthesis, as reflected in the color variations between light and dark-incubated cultures.

Table 1. Microscopic observations of different cell suspension cultures

Treatment Code	Speed (rpm)	Light or Dark	Microscopic appearance
C1	50	Light	< 50% of the cells were separated. More clusters
C2	100	Light	> 70% of the cells were separated, Few clusters
C3	150	Light	> 80% of the cells were separated, Cells were damaged
C4	50	Dark	< 50% of the cells were separated, More clusters
C5	100	Dark	> 70% of the cells were separated, Few clusters
C6	150	Dark	> 80% of the cells were separated, Cells were damaged

Phytochemical Analysis of Leaves, Callus, and Cell Suspension Cultures

Analysis of steviol glycosides using HPLC

The HPLC method was validated by plotting areas under five peaks against analyte concentrations for four steviol glycosides (rebaudioside A, stevioside, rebaudioside C, dulcoside A). The correlation coefficients for the standard curves exceeded 0.99, confirming linearity across the concentration range and supporting the suitability of the standard curves for quantifying glycoside content. Retention times were found to be 3.099 min for rebaudioside A, 3.288 min for stevioside, 4.207 min for rebaudioside C, and 4.550 min for dulcoside A. However, these retention times differed from those reported in the JECFA method, and rebaudioside A and stevioside peaks were not fully separated, likely due to the use of a C18 column (150 x 4.6 mm).

According to the results of HPLC analysis, the highest rebaudioside A, stevioside, rebaudioside C, and dulcoside A contents were observed in leaves as 8.234±0.04, 10.132±0.03, 1.585±0.02, and 0.477±0.01 mg/mL, respectively, followed by in vitro derived callus as 1.793±0.01, 2.228±0.03, 0.365±0.03, and 0.118±0.01 mg/mL, respectively. The lowest amount of rebaudioside A and stevioside was observed in cell suspension cultures (0.272±0.02 and 0.384±0.01 mg/mL), and rebaudioside C and dulcoside A were not detected (Table 2 and Figure 2).

Table 2. The amount of steviol glycosides present in leaves, callus, and cell suspension cultures

Sample Type	Rebaudioside A (mg/mL)	Stevioside (mg/mL)	Rebaudioside C (mg/mL)	Dulcoside A (mg/mL)
Leaves	8.234 ± 0.04^{c}	10.132 ± 0.03^{c}	1.585 ± 0.02^{b}	0.477 ± 0.01^{b}
Retention time (min)	3.192	3.324	4.237	4.595
In vitro callus	1.793 ± 0.01^{b}	2.228 ± 0.03^{b}	0.365 ± 0.03^{a}	0.118 ± 0.01^{a}
Retention time (min)	3.012	3.198	4.157	4.476
Cell suspension	0.272 ± 0.02^{a}	0.384 ± 0.01^{a}	Not detected	Not detected
Retention time (min)	2.925	3.167	4.169	4.457

*Values followed by different letters are significantly different at $p < 0.05$

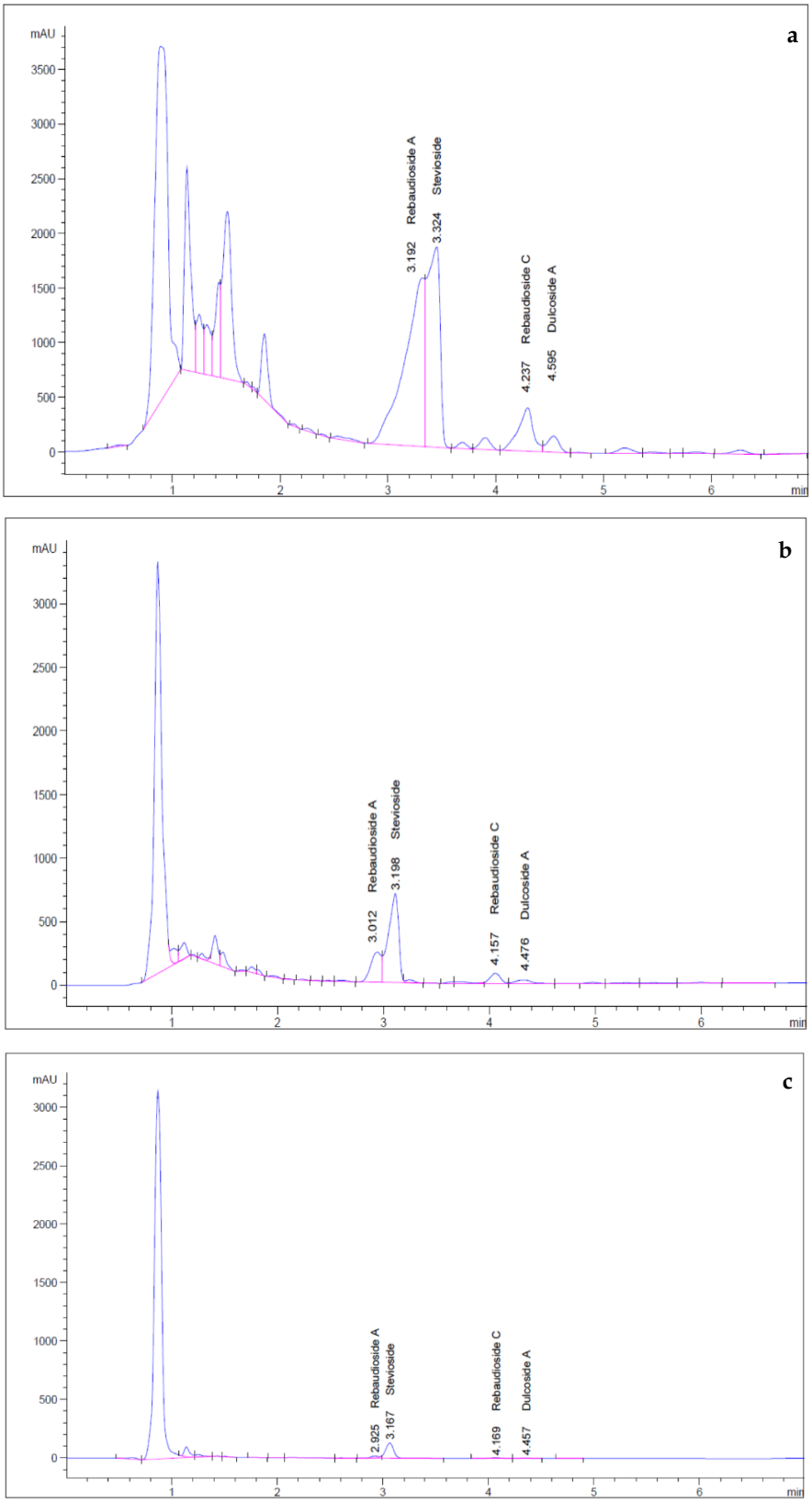
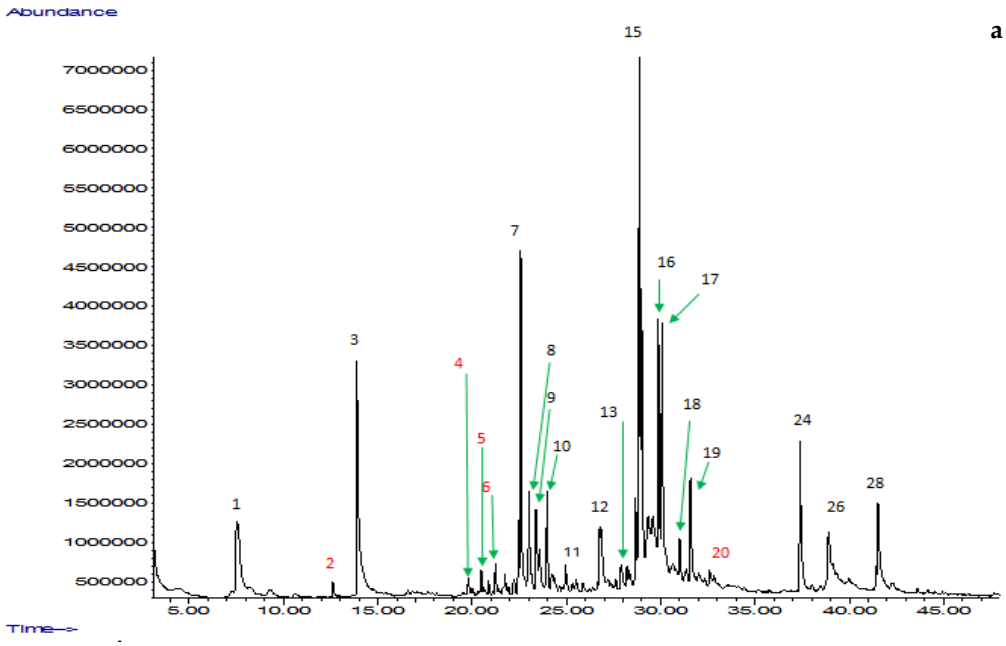


Figure 2. HPLC chromatograms of a) leaf extract, b) callus extract, and c) cell culture extracts of *S. rebaudiana*

According to the results, it was observed that plants have significantly higher amounts of steviol glycosides, and callus extracts showed the presence of steviol glycosides, yet amounts were significantly lower. Only rebaudioside A and stevioside were found in cell suspension cultures, which were incubated in light. None of the four Steviol glycosides was detected in cell suspension cultures incubated in the dark. Therefore, it can be concluded that light is essential for steviol glycoside biosynthesis as the light initiates cell differentiation. Stevioside accumulation in cell suspension cultures was analyzed by Mathur *et al*., (2013) and the maximum stevioside content (0.381 mg/g) was observed after 7 days of cell suspension culture initiation, and the content remained unchanged until the 14th day [12]. They stated that *S. rebaudiana* cell suspension cultures produce stevioside at different concentrations during their growth cycle.

Screening of phytochemicals using GC-MS

The study analyzed extracts of leaf, callus, and cell cultures using GC-MS, identifying a total of 27 key phytochemicals across all samples (Figure 3 and Table 3). Plant leaves contained 22 bioactive compounds, including 10.alpha.-Eremophilane, known for its anticancer, anti-inflammatory, and antibacterial properties; 4-Quinolinecarboxylic acid, 2-chloro, with antiviral properties; and Pyrido(2,3-d)pyrimidine, 4-phenyl, with anticancer, antimicrobial, anti-inflammatory, antiviral, and antihypertensive properties. Callus included 16 bioactive compounds, including 2-Myristynoyl-glycinamide, which is known for anti-microbial benefits. Cell culture contained 12 phytochemicals such as, 7-Hydroxy-3-(1,1-dimethylprop-2-enyl) coumarin, a compound with anti-quorum sensing antibacterial potential. All three cultures shared 9 common bioactive compounds, such as isopulegol acetate (a flavouring agent), Carbamic acid (antiamoebic), Hexadecanoic acid, methyl ester (antimicrobial, anti-inflammatory, hypercholesteromic and anticancer), and Octadecanoic acid (antimicrobial, anti-inflammatory, hypercholesteromic and anticancer).



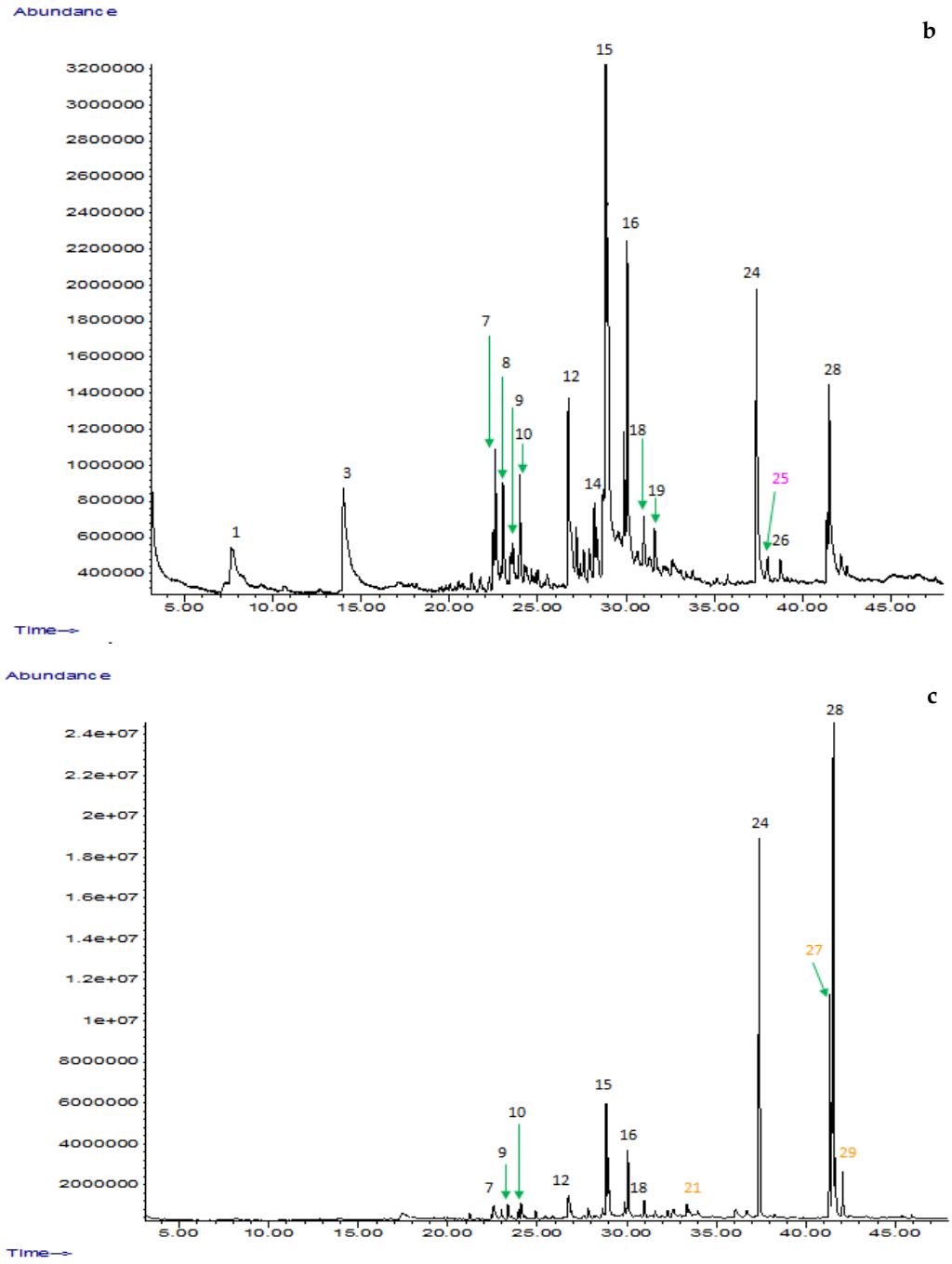


Figure 3. GC-MS chromatograms of a) leaf extract, b) callus extract, and c) cell culture extracts of *S. rebaudiana*

GC-MS analysis of leaf extracts of *S. rebaudiana* revealed the presence of terpenes, fatty acids, aldehydes, ketones, and alcohols [13]. In another study [14] screened the hexane extract of *S. rebaudiana* by GC-MS and identified the saturated fatty acids, hydrocarbons, and esters, which are used as flavoring agents, food additives, and pharmaceutically essential drugs. The present study confirmed their findings.

Table 3. Phytochemicals present in leaves of natural and tissue cultured plants, callus of *S. rebaudiana*, and their potential bio activities

No	Compound name	RT (min)	Leaves	Callus	Cell culture	Bio activity	References
1	Oleic acid	7.551	√	√	-	Anticancer, anti-inflammatory, antimicrobial, flavouring agent, food additive	[15]
2	3,4-dimethyl-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	12.736	√	-	-	Antimicrobial	[16]
3	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-1-methoxy	13.897	√	√	-	Antiallergic, antimicrobial, anticancer, antimalarial, antidiabetic	[17]
4	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	20.494	√	-	-	Antimicrobial, anti-inflammatory, antihyperlipidemic	[18]
5	Caryophyllene	21.241	√	-	-	Anti-inflammatory, antidepressive, anticancer	[19]
6	Bicyclo[2.2.1]heptan-2-ol, 3,3-dimethyl-, oxo-	21.751	√	-	-	Antimicrobial, anti-inflammatory, Antihyperlipidemic	[18]
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	[15]
8	Tetradecanoic acid	23.019	√	√	-	Antioxidant, cancer preventive, nematicide	[16]
9	isopulegol acetate	23.581	√	√	√	Flavouring agent	
10	Carbamic acid, tricyclo[2.2.1.0(2,6)]hept-3-yl-, ethyl ester	23.973	√	√	√	Antiamoebic	[20]
11	2,6,10,14-Hexadecatetraen-1-ol,3,7,11,15-tetramethyl-, acetate, (E, E, E)-	24.954	√	-	-	Antimicrobial, anti-inflammatory, anticancer	[21]
12	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo	26.732	√	√	√	Antimicrobial	[22]
13	1H-pyrazol-5-amine, 3-methyl-1-phenyl-	27.898	√	-	-	Antimicrobial, anti-inflammatory, antitumour	[23]

14	2(3H)-Furanone, dihydro-4,5-dimethyl-	28.212	-	√	-	Antiviral	[17]
15	Bicyclo[3.1.1]heptanes,2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-	28.987	√	√	√	Antimicrobial, anti-inflammatory, antihyperlipidemic, antioxidant	[18]
16	1,4-Eicosadiene	29.876	√	√	√	Antimicrobial, antioxidant	[24]
17	5,9-Undecadien-2-one, 6,10-dimethyl-,)z)-	30.076	√	-	-	Flavouring agent, food additive	[15]
18	Hexadecanoic acid, methyl ester	31.008	√	√	√	Antimicrobial, anti-inflammatory, hypercholesteromic, anticancer	[25]
19	7-Choloro-4-methoxy-3-methylquinoline	31.582	√	√	-	Antiturbercular	[26]
20	Kaur-16-ene	32.594	√	-	-	Antimicrobial, anti-inflammatory	[27]
21	7-Hydroxy-3-(1,1-dimethylprop-2-enyl)coumarin	33.599	-	-	√	Antimicrobial, anti-inflammatory	[28]
24	Octadecanoic acid	37.388	√	√	√	Antimicrobial, anti-inflammatory, hypercholesteromic	[25]
25	2-Myristynoyl-glycinamide	38.039	-	√	-	Antimicrobial	[29]
26	1H-Indole,5-methyl-2-phenyl	38.896	√	√	-	Antibacterial, anti-inflammatory	[30]
27	Isoquinoline,1-[3-methoxy-5 hydroxybenzyl]-1,2,3,4,5,8 hexahydro-	41.353	-	-	√	Antimicrobial, antioxidant	[31]
28	2-Ethylacridine	41.522	√	√	√	Antimicrobial, antioxidant	[32]
29	N-Methyl-1-adamantaneacetamide	42.066	-	-	√	Antimicrobial, antioxidant	[33]

Conclusion

For optimal *S. rebaudiana* cell suspension cultures, an agitation speed of 100 rpm is ideal for maintaining effective cell separation. Lower speeds lead to cell clustering, while higher speeds cause cell damage. Light conditions do not affect cell aggregation but are crucial for the biosynthesis of secondary metabolites, including steviol glycosides. The HPLC analysis reveals that leaves accumulate significantly higher amounts of steviol glycosides compared to callus and cell cultures, primarily due to the presence of higher levels of photosynthetic pigments. Callus exhibits lower glycoside content due to incomplete photosynthetic development, while cell cultures produce minimal amounts. Phytochemical analysis indicates variations in compound profiles among leaves, callus, and cell cultures, reflecting differential metabolic activities across these systems. While callus and cell cultures can serve as viable alternatives for specific metabolite production, their overall yield remains low. Therefore, further optimization is necessary to enhance phytochemical production in in vitro cultures.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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