

BIOPROSPECTING OF THE ENDOPHYTIC FUNGI FROM STEVIA *REBAUDIANA* FOR THE PRODUCTION OF STEVIOSIDE AND REBAUDIOSIDE A: SCREENING AND CHROMATOGRAPHIC CHARACTERIZATION

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ABSTRACT

Bioactive secondary metabolites produced from the endophytic fungi offering a significant potential in pharmaceutical sectors. Steviol glycosides are the diterpenoids secondary metabolites which are found inside the plant *Stevia rebaudiana* Bertoni. This study represents a systematic and structural framework for the extraction, preliminary screening and chromatographic analysis of the bioactive SMs derived from the endophytic fungal isolates. Total 108 endophytic fungal isolates obtained from plant *S.rebaudiana* in which 48 isolates were morphologically different subjected to the Richard's culture broth fermentation for synthesizing the steviol glycosides followed by thoroughly extraction of secondary metabolites by ethyl acetate in 1:1. The resulting crude extracts were initially screened by anthrone and salkowski test to identify fungal isolates capable of producing carbohydrate and terpenoid associated secondary metabolites. Selected two positive samples were further analyzed on TLC Silica gel 60 F254 plate by using solvent phase ethyl acetate, methanol, formic acid (93:40:1 v/v/v). High performance liquid chromatography showed two different Rt value for both stevioside and rebaudioside A in solvent ACN and water (80:20) by using column 2.50 mm × 4.6 mm. The current findings demonstrate that endophytic fungi resides inside the plant *S.rebaudiana* is a promising microbial resource for identifying alternative routes for the production of stevioside and rebaudioside

KEYWORDS: *Stevia rebaudiana*; Endophytic fungi; Bioprospecting; Stevioside; Rebaudioside; Steviol glycosides; Fungal screening; Secondary metabolites.

INTRODUCTION

Stevia rebaudiana Bertoni is widely used a natural sweetener and important medicinal plant belongs to Asteraceae family, recognized for producing natural and non caloric sweeteners include rebaudioside A and stevioside. These steviol glycosides were listed to be between 300-450 times more sweet than sucrose and fascinate a significant role in various food, pharmaceutical and nutraceuticals industries (Lemus-Mondaca et al., 2012; González et al., 2014). *S. rebaudiana* contains various pharmacological properties like antitumor, antimicrobial, antidiabetic and antihypertensive in addition to sweetening properties. Recent studies further demonstrate its significance as a therapeutic medicinal plant with enhanced metabolite modulation potential under biotic interactions (Chatsudthipong & Muanprasat, 2009; Wahyuni et al., 2026). Endophytes are microorganisms such as bacteria and fungi that reside within plants without affecting them and make beneficial symbiotic associations. In recent years, endophytes garnered attention as essential sources of bioactive compounds usually complementing or imitating the metabolic pathways exhibited by their host plant species (Devi & Kariyanna, 2026).

The interaction between *S. rebaudiana* and its endophytic community enhanced as a crucial research area of study due to its profound biotechnological and pharmaceutical significance. Recent studies have revealed that endophytic fungi derived from stevia are capable of producing a range of bioactive compounds include antimicrobial substances effective against multidrug resistant pathogens (Bansal et al., 2025). Furthermore, particular fungal endophytes such as *colletotrichum siamense*, and *Acremonium sclerotigenum* were known to produce stevioside (Nishant Chandra et al., 2025) and bacterial endophyte *Pantoea vagans* produce rebaudioside A (Simlat M et al., 2020). The trend analytical methods such as HPLC profiling define the production of stevioside and rebaudioside A and other compounds associated with stevia. Endophytes not only contribute to synthesizing the SMs but also the significantly help in promoting the plant growth and provide defence against abiotic and biotic stress conditions. Recent studies also demonstrated that endophyte-mediated signaling can considerably stimulate biosynthesis of steviol glycoside particularly under abiotic stress conditions, thereby enhancing both the production and quality of stevia. Additionally, endophytes have been exhibited potential to enhance plant resilience to environmental stresses such as drought and salinity by regulating secondary metabolic pathways (El-Boukhary et al., 2025; Wahyuni et al., 2026).

In spite of these developments, the depth studies on the endophytic fungal diversity within the stevia plant and their potential to produce steviol glycosides are limited. Therefore, the present study aims to explore the endophytic fungal communities in *S.rebaudiana* plant and their contribution in synthesizing the plant secondary metabolites and analyzing the plant-endophyte interaction. Such type of research might be proved as a development of novel bioinoculants and sustainable strategies for the production of steviol glycosides.

2. MATERIAL AND METHODS

2.1 Sample collection

Mature and healthy *S.rebaudiana* Bertoni plant samples were collected from six different places in India of different longitudinal regions in different seasons are given in Table 1.1. All the plants was authenticated in the Botany Laboratory according to the natural system of classification at the Department of Bio-Sciences and Technology, M.M (Deemed to be) University Mullana, Ambala. Under the carefully environmental conditions, the plant sample was brought to the laboratory for further processed aimed to isolate the endophytic fungi according to according to Khalil AMA et al., 2021.

Table 1.1 Sample collection of plant *S.rebaudiana* from distinct geographical areas in different weather conditions.

S.No	Month of sample Collection	Sampling site	India region	Geographical coordinates
1.	October	Yamunanagar	Haryana	30° 07'41 N 77° 17' 1 E
2.	November	Cheeka	Haryana	30.05°N 76.33°E
3.	January	Kurukshetra	Haryana	76° 26'41 N 77° 17' 1 E
4.	March	Kharwan	Haryana	30.20°N 77.36°E
5.	April	Jaipur	Rajasthan	69°30' E 23°3' N
6.	June	Dehradun	Uttarakhand	30° 19' N 78° 04'E

2.2. Surface sterilization and isolation of endophytic fungi (EF)

The plant tissues were washed under the tap water for 10 minute and the immersed into 5% hypochlorite solution for 80 second followed by 70% ethanol for 60 seconds and at last 30% for 30 seconds according to Sari M.P, et al., 2025. Finally, the tissues were properly rinsed 3-5 times with distilled water and then washed tissues allowed to dry under the laminar cabinet. The dried plant sample tissues were cut into tiny segments and then placed over the potato dextrose PDA (HIMEDIA) Petri plates. The plates were properly covered with parafilm strip and were incubated at $28 \pm 2^\circ\text{C}$ for 10 – 12 days, checking after 3-4 days at a regular interval. The isolations were sub-cultured on PDA solid media plates and stored at -4°C for further use.

2.3. Production of culture filtrate and extraction of secondary metabolites (SMs)

The endophytic culture broth media were prepared by inoculating a 5mm disc from 7-8 days old culture into the 50 ml Richard's broth media (HI MEDIA) and the culture was incubated for 15 days at $26 \pm 1^\circ\text{C}$ (120 rpm). On the 16th day the mycelial mass was separated from the culture through 0.1 μm Whatmann filter paper (GE Health care Life Sciences, USA). The bioactive SMs were extracted with the help of solvent extraction method by using ethyl acetate. Equal amount of solvent and filtrate (1:1) were added and shaking vigorously for 10 minute until two immiscible layers appeared (Khan A.A, et al., 2017; Celaya L, et al., 2024). The solvent layer was collected and evaporated on water bath at $78 \pm 2^\circ\text{C}$ for 25 minutes to obtain crude residue. The crude extract was dissolved in dimethyl sulphoxide (DMSO) and kept at -4°C for further phytochemical, qualitative and quantitative analysis.

2.3.1. Biochemical test for carbohydrates and terpenoids

Anthrone reagent is prepared by adding 0.1 grams of anthrone to 50 ml of concentrated sulfuric acid. And 1 ml of anthrone reagent was added to 1 ml of ethyl acetate-extracted crude sample and given a 5-minute water bath, and thereafter formation of a blue-green color, which serves as a qualitative indicator for the presence of carbohydrates according to Kumari et al., 2023. To confirm the presence of terpenoids in crude filtrate, the sample, concentrated sulfuric acid (H_2SO_4) and chloroform added in a ratio of 3:1:1. The formation of a reddish brown ring indicates the presence of terpenoids (Kabir D and Lawan I, 2026).

2.3.2. Qualitative analysis by thin layer chromatography (TLC)

TLC mobile phase solvent system was consisting of ethyl acetate, methanol, formic acid (93:40:1 v/v/v) and bands were visualized by putting the precoated silica gel aluminium TLC plate (TLC Silica gel 60 F254, Sigma aldrich) into the iodine chamber, which contained iodine crystals followed by observed under UV spectrophotometer at 254 and 366 nm UV light (Perera W.H, et al., 2021). To ensure accurate identification, high-purity standards of stevioside (Dove Research Analytics, Purity 98.12%) and rebaudioside A (Dove Research Analytics, purity 99.72%) were used as reference.

2.3.3. Quantitative analysis by HPLC

The HPLC analysis of steviol glycosides was carried out by using a column 2.50 mm $\times$ 4.6 mm (5 μ) and column temperature was maintained at ambient (25°C). The analysis was conducted under the isocratic condition with a flow rate of 1 ml/min, detection wavelength was set at 210 nm. The mobile phase was made up of a mixture of acetonitrile and distilled water at a ratio of 80:20 and the pH was adjusted to 3.01 with orthophosphoric acid. The identification was done by comparing the RT, area and height of the peak observed in HPLC chromatogram (Leong S.H, et al., 2026). The quantification of steviol glycosides was calculated by regression equations obtained from the standards.

3. RESULTS

3.1 Isolation of endophytic fungi

The plant samples collected from six different geographical locations produced a diverse population of endophytic fungi, isolated from surface sterilized leaf and stem part of the plant *S. rebaudiana* after 3-8 days of incubation as shown Fig 1.1. Approximately 108 endophytic fungal isolates were obtained from which around 48 fungi were morphologically different. The number of the isolates obtained from individual locations varied considerably. Dehradun (38) exhibited the highest culturable fungal isolates followed by Yamunanagar (30), whereas the lowest recovered from place Jaipur (5) given in Table 1.1. The mycelial mass of the morphologically distinct fungal isolates was picked and placed on fresh PDA plates to make pure culture.

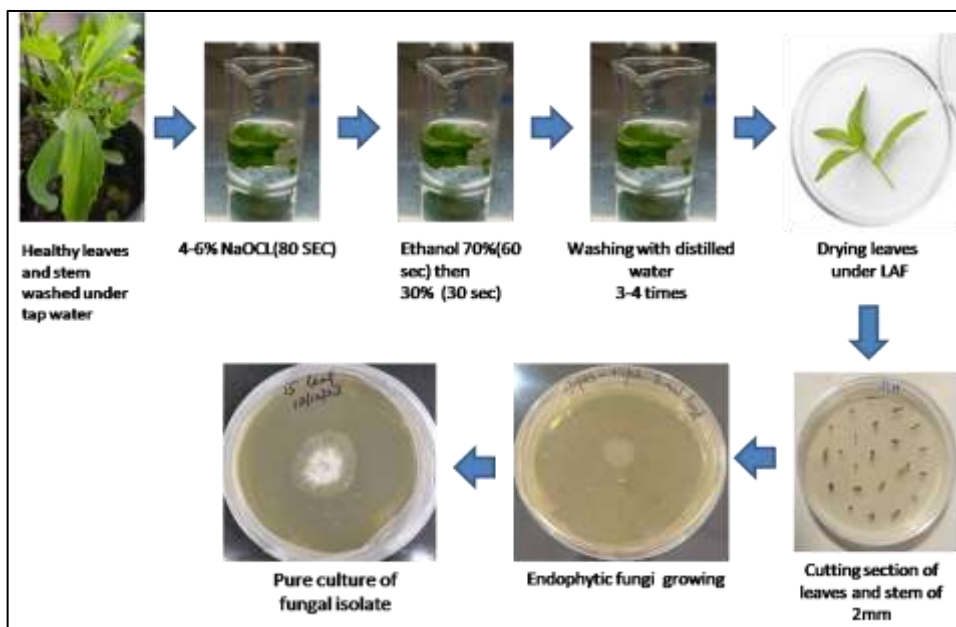


Fig 1.1 Pictorial representation of isolation of endophytic fungi from the surface sterilized tissues of plant *Stevia rebaudiana*

Table 1.2 Distribution of endophytic fungal isolates from *S.rebaudiana* according to sampling location and plant tissue.

S. No	Location of plant sample collection	No. of endophytic fungi isolation		Total
		Leaf	Stem	
1.	Yamunanagar	18	12	30
2.	Cheeka	5	2	7
3.	Kharwan	5	5	10
4.	Jaipur	3	2	5
5.	Kurukshetra	12	6	18
6.	Dehradun	28	10	38

3.2 Characterization of steviol glycosides from culture filtrate

Morphologically distinct purified endophytic isolates were subjected to 15 days of fermentation period for production of secondary metabolites in culture broth under the aseptic conditions. During submerged fermentation, the color of the culture broths varied from pale yellow (0-5 days) to brown (6-10 days) then to reddish brown (11-15 days). On the 16th day, approximately 40 - 45 ml of culture broth was recovered from each flask which gave the distinct yields of crude extract after extraction with ethyl acetate given in Table 1.3. The ethyl acetate crude extracts were subjected to the preliminary qualitative analysis using anthrone and Salkowski tests. Six extracts were showed a positive response in anthrone test, indicating the presence of carbohydrates-containing metabolites, whereas only seven crude extracts developed reddish brown coloration in terpenoid assay, confirms the presence of terpenoid compounds. Based on the color intensity appears after phytochemical tests, two isolates were selected for the further chromatographic analysis.

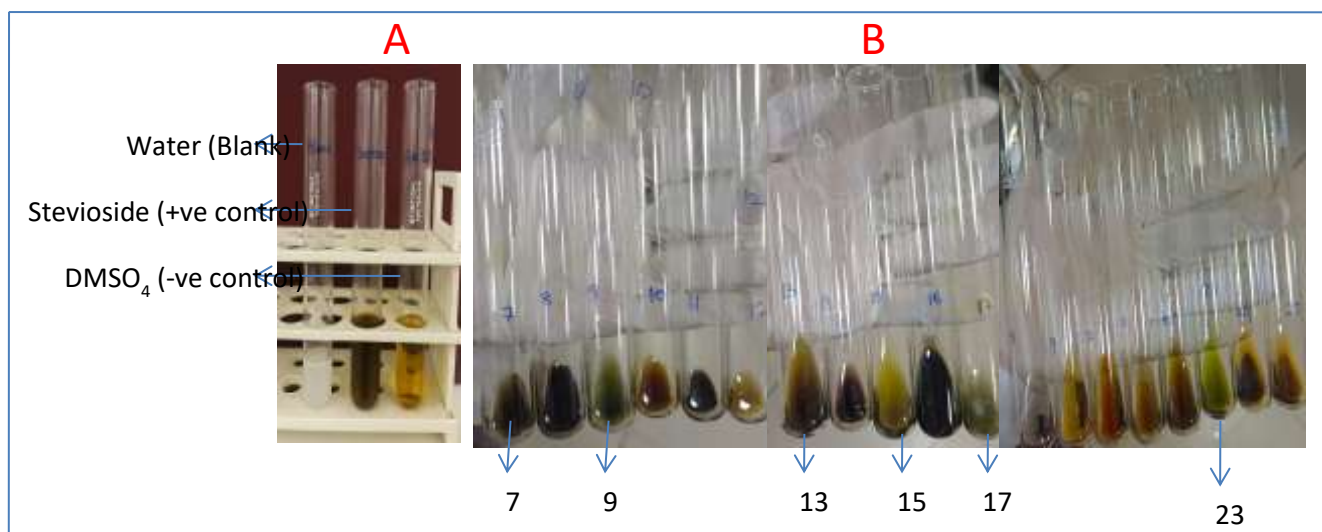


Fig 1.2 Anthrone test analysis (A) Positive & Negative control (B) Sample no. #SR7LC, #SR9LK, #SR13LY, #SR15LC, #SR17LD & #SR23LD showing positive results

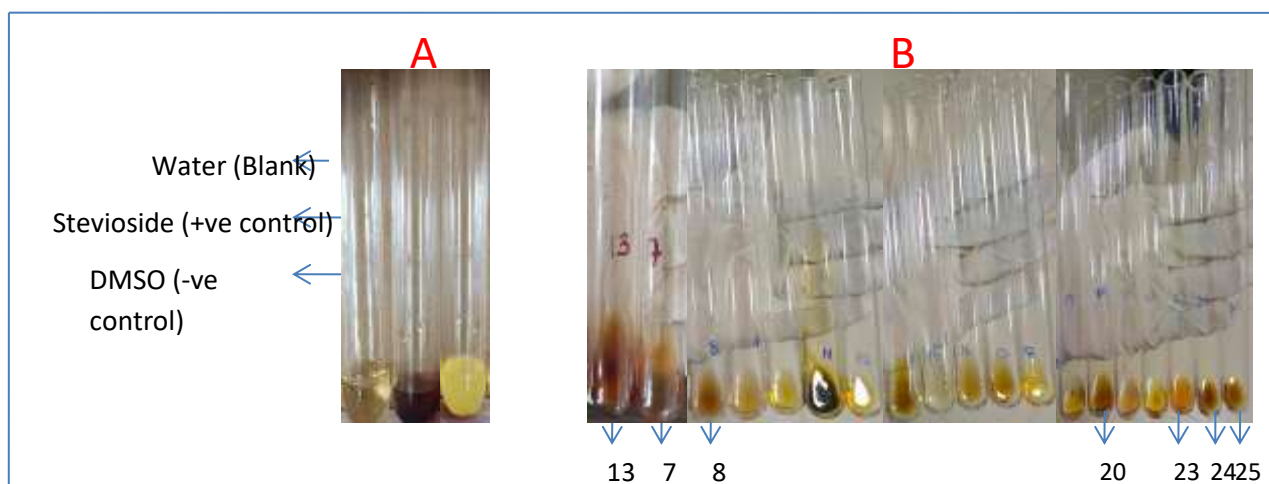


Fig 1.3 Salkowski test analysis (A) +ve and -ve control (B) Sample no. #SR7LC, #SR8SY, #SR13LY, #SR20SJ, #SR23LD, #SR24SD & #SR25SD showing positive response

Table 1.3 Yield of crude extract obtaining after partial purification and their preliminary qualitative analysis for carbohydrates and terpenoids metabolites.

S.No	Culture Code	Culture broth (ml)	Crude extract weight (mg)	Dissolved in DMSO (ml)	Final Conc.(mg/ml)	Anthrone test	Terpenoid test
1	#SR 1LY	50	104.1	5	20.82	-	-
2	#SR 2LY	50	105.1	5	21.02	-	-
3	#SR 3LY	50	205.1	5	41.02	-	-
4	#SR 4SK	50	48.4	5	9.68	-	-
5	#SR 5LY	50	31.5	5	6.3	-	-
6	#SR 6SY	50	30.05	5	6.01	-	-
7	#SR 7LC	50	78.4	5	15.68	+++	+++
8	#SR 8SY	50	69.6	5	13.92	-	+

9	#SR 9LK	50	28	5	5.6	+	-
10	#SR 10LY	50	12.8	5	2.56	-	-
11	#SR 11SK	50	51.4	5	10.28	-	-
12	#SR 12LC	50	25.1	5	5.02	-	-
13	#SR 13LY	50	67.2	5	13.44	+++	+++
14	#SR 14LC	50	22.1	5	4.42	-	-
15	#SR 15LC	50	34.1	5	6.82	++	-
16	#SR16LJ	50	18.2	5	3.64	-	-
17	#SR17LD	50	250.7	5	50.14	+	-
18	#SR18LD	50	265.6	5	53.12	-	-
19	#SR19LJ	50	16	5	3.2	-	-
20	#SR20SJ	50	58.8	5	11.76	-	++
21	#SR21LD	50	321.3	5	64.26	-	-
22	#SR22LD	50	363.5	5	72.7	-	-
23	#SR23LD	50	318.9	5	63.78	+	+
24	#SR24SD	50	59.5	5	11.9	-	++
25	#SR25SD	50	43.5	5	8.7	-	++

3.3 TLC profiling

Crude extract of #SR7LC and #SR13LY which showed positive response during preliminary biochemical tests were subjected to thin layer chromatography (TLC) analysis alongside with authentic stevioside and rebaudioside A standard compounds. The standard stevioside and reb A migrated with retention factor (RT) value 0.36 and 0.19 respectively under the optimized TLC conditions. And, the crude extract of #SR7LC was resolved into two bands, whereas #SR13LY showed four distinct bands with different retention factor as discussed in Table 1.4.

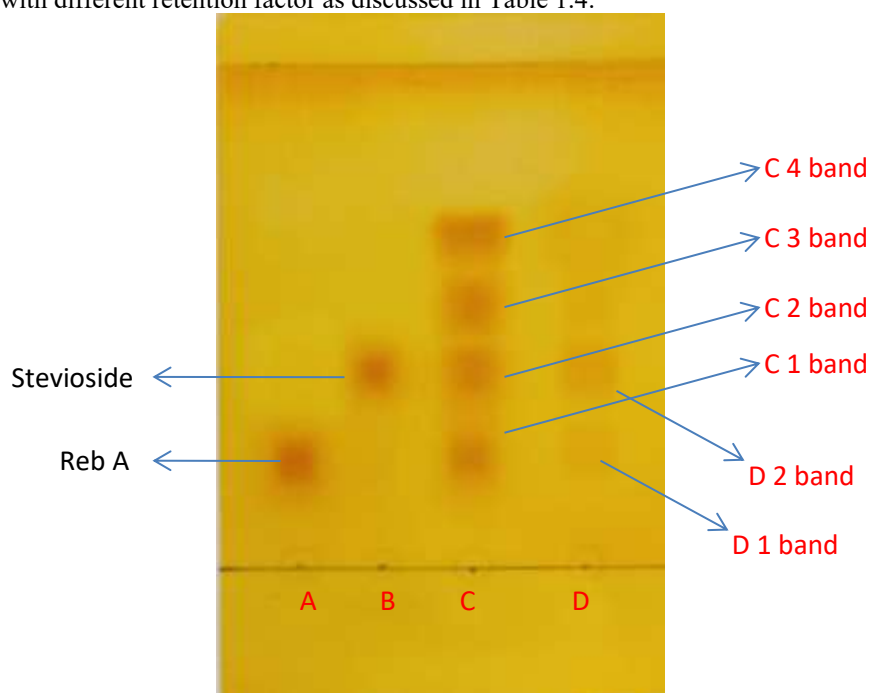


Fig 1.4 Thin layer chromatography (TLC) photography of standards and crude extracts of endophytic fungi isolated from plant *S.rebaudiana*. A - Rebaudioside A, B - Stevioside, C - Fungal crude extract of #SR7LC, D - Fungal crude extract of #SR13LY. The Rf values of different bands are given in Table 1.4.

Table 1.4 Comparative analysis of Rf values of positive fungal crude extracts with reference standard compound of stevioside and rebaudioside A on silica gel plate observed under 254 and 366 nm A.

S.No	Compound	Sample name	Solvent run (cm)	Solute run (cm)	Rf factor
1.	Reference standard	Reb A (A)	5.2	1.0	0.19
		Stevioside (B)	5.2	1.9	0.36
2.	Crude extract	#SR7LC (C)	5.2	1.0 (C1 band)	0.19
				1.9 (C2 band)	0.36
		#SR13LY (D)	5.2	1.0 (D1 band)	0.19
				1.9 (D2 band)	0.36
				2.3 (D3 band)	0.44
				2.8 (D4 band)	0.53

3.4 HPLC characterization

Two positive fungal isolates (#SR7LC and #SR13LY) were analyzed through thin layer column chromatography using the column conditions mentioned in section 2.3.3. The presence of stevioside and rebaudioside A in the crude extract of fungal filtrate was confirmed by comparing the RT of the standard compounds and sample. The resulting chromatogram showed well-defined peaks. Both isolates were showing positive peaks corresponding to the retention time (RT) of the stev and reb A standards as shown in Fig. 1.5 and 1.6. In Table 1.5, summarizes the HPLC data obtained during the analysis.

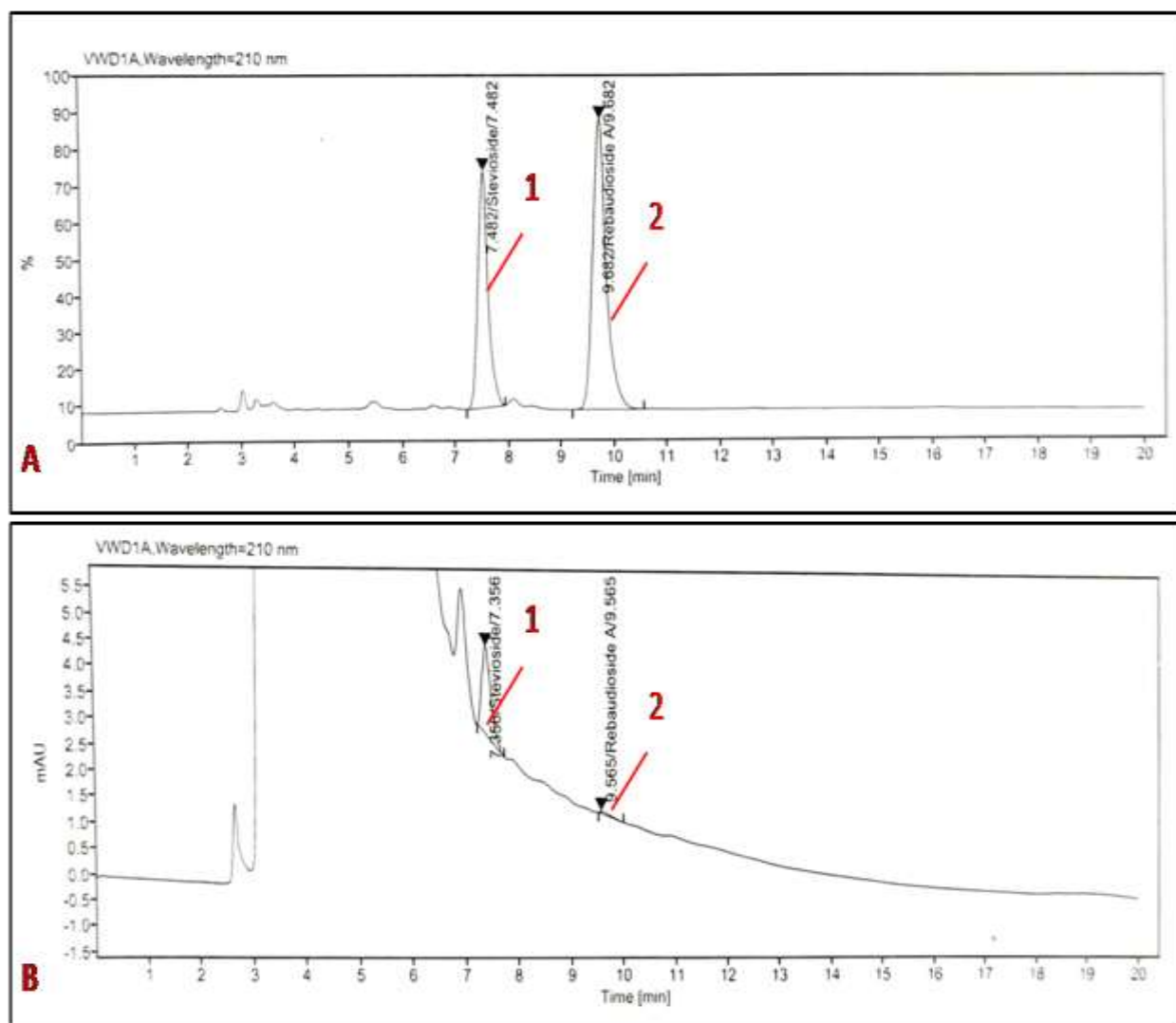


Fig1.5 (a) High-performance liquid chromatography (HPLC) resulting chromatograms observed peaks at 210 nm A. Reference standards (Peak 1, stevioside RT– 7.48 and Peak 2, reb A RT – 9.68), B. Crude extracted sample #SR13LY (Peak 1, stevioside RT– 7.36 and Peak 2, reb A RT– 9.57).

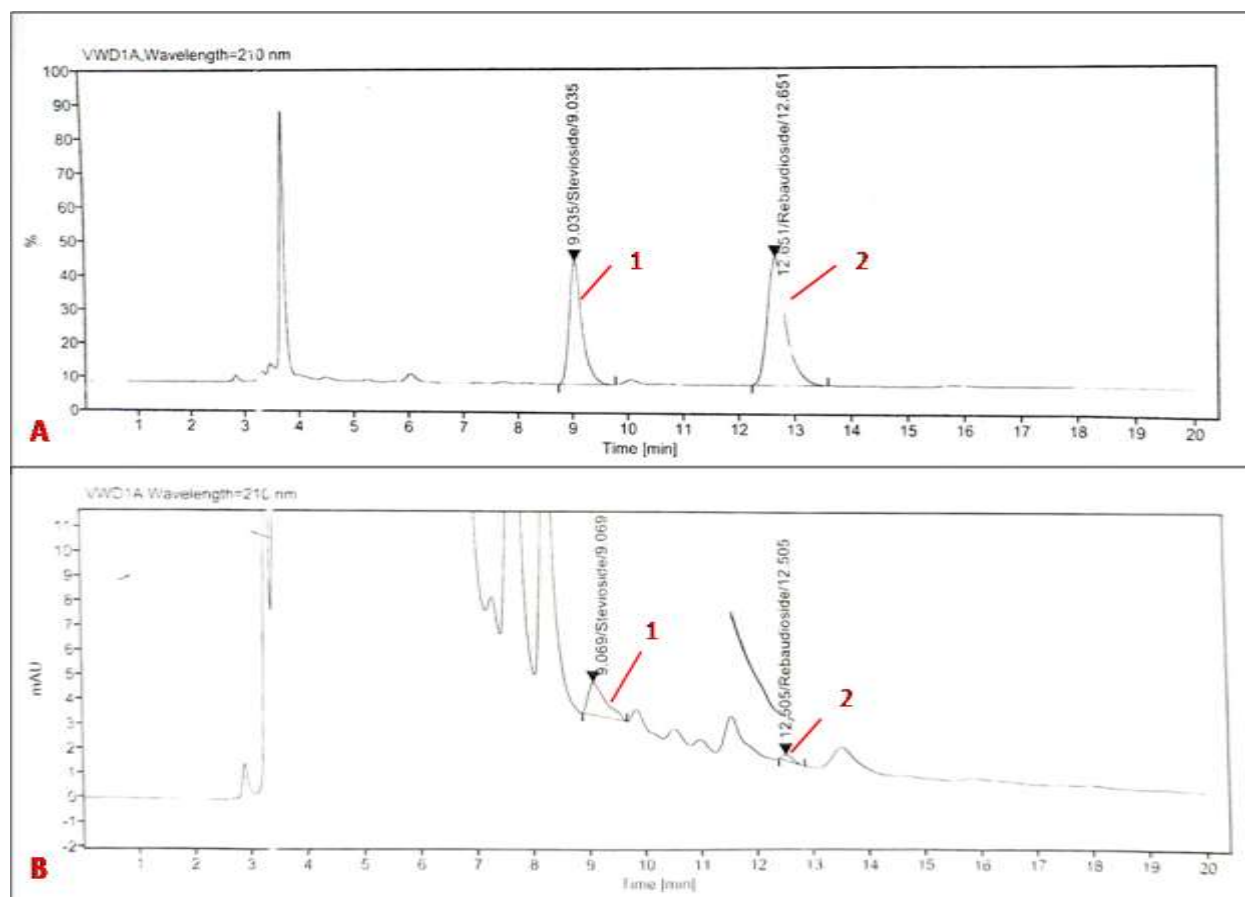


Fig1.5 (b) High-performance liquid chromatography (HPLC) resulting chromatograms observed peaks at 210 nm A. Reference standards (Peak 1, stevioside RT – 9.03 and Peak 2, reb A RT – 12.63), B. Crude extracted sample #SR13LY (Peak 1, stevioside RT – 9.07 and Peak 2, reb A RT – 12

Table 1.5 HPLC Analysis of fungal-derived compounds of #SR7LC and # SR13LY with reference standard compounds stevioside and rebaudioside A by compairing their RT, area and height values.

S. no	Sample Code	Name	RT	Area	Height %	Amount (mg/ml)
1.	#SR7LC	Steviosides Std.	7.48	17687019	44.91	498.83
		Crude extract	7.36	338647	98.17	10.06
		Rebaudiosides A Std.	9.68	29192407	55.09	498.80
		Crude extract	9.57	10010	1.83	0.66
2.	#SR13LY	Steviosides Std.	9.03	17954867	49.11	500.67
		Crude extract	9.07	472261	85.47	14.82
		Rebaudiosides A Std.	12.64	26450671	50.89	501.01
		Crude extract	12.51	50757	14.53	2.92

DISCUSSION

Endophytic fungi are important sources of secondary metabolites with a wide range of chemicals. In order to find endophytic fungal isolates that could be able to produce steviol glycosides, the current study used a sequential screening method to examine 48 fungal endophytes linked to *S.rebaudiana* that were obtained from six geographically different areas. The composition of endophytic communities may be influenced by environmental factors, host physiology, climate conditions, soil properties and other ecological factors. The recovery of 48 different morphologically endophytic fungal isolates from different locations may reflect variation in environmental conditions.

The current study used Richard's broth as a fermentation media for the production of secondary metabolites because nutrients induce stress to synthesize the SMs. The ethyl acetate was used extract the diterpenoid secondary metabolites from the culture filtrate due to ability to dissolve a wide range of polar compounds. Same extraction method for the removal of stevioside from the endophytic culture filtrate was used by Sheela Chandra.S, 2025. Preliminary phytochemical screening of steviol glycosides were detected by anthrone and salkowski tests confirms the presence of carbohydrates and terpenoids in fungal crude extract. The positive results for terpenoids are significant because terpenoids are known to produce their antimicrobial, antioxidant and anticancerous properties. Similar studies have been reported in endophytic fungi isolated from medicinal plants. The presence of these compounds reveals the potential of producing as a source of

novel bioactive molecules. Further the TLC was performed to differentiate and detect the number of compounds present in crude extracts. The visualization of different bands with different R_f values on TLC plate indicated the presence of multiple in fungal extracts. The stevioside was detected at the R_f of 0.36 and rebaudioside A was detected at the R_f of 0.19 in solvent phase ethyl acetate, methanol and water (93:40:1), same solvent phase system was used by Perera.H.W and McChesney J.D , 2021, in which stevioside and rebaudioside A were showed the different R_f values i.e, 0.23 and 0.15 respectively. TLC profiling serves as a rapid and cost effective method for characterization. The crude extracts was finally characterized by HPLC technique to assure the presence the stevioside and rebaudioside A. HPLC chromatograms were showed the well defined peaks in solvent system acetonitrile : deionized water (30:70) by using column column C18 indicates the presence of stevioside and rebaudioside A. In recent studies the qualitative and quantitative analysis of stevioside was done by GC-MS, LC-MS and UHPLC-ESI-MS (Nishant and Chadra S et al., 2025) and the current study highlight that endophytic fungi is a big source of producing the SvGLs SMs mainly stevioside and rebaudioside A, whereas some endophytic fungi reported to produce only stevioside (Nishant and Chadra S et al., 2025). The production of diterpenoids by the endophytic fungi supports their role in plante endophyte interactions and their use in pharmaceuticals and other food beaverages.

CONCLUSION

Total 108 endophytic fungal isolates were obtained from the Plant *S.rebaudiana* in which 48 fungi were morphologically distinct subject for the production of culture filtrate. The culture filtrates was screened for the steviol glycosides, with the help of some qualitative analysis tests, stevioside and rebaudioside A were found to be present. TLC and HPLC highlights the biotechnological potential of endophytic fungi as novel source of producing rebaudioside A and stevioside. To the best of our knowledge, this is first endophytic fungus was shows the best production of both glycosides rebaudioside A and stevioside. The present study is providing a platform for synthesizing natural sweeteners (stev and reb A) as an alternative to plant extraction method.

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