



Plant Archives

Journal homepage: <http://www.plantarchives.org>

DOI Url : <https://doi.org/10.51470/PLANTARCHIVES.2025.v25.no.2.407>

PHYTOCHEMICAL PROFILING AND ANTIMICROBIAL EFFICACY OF AQUEOUS AND METHANOLIC EXTRACTS OF *STEVIA REBAUDIANA* LEAVES

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(Date of Receiving-19-06-2025; Date of Acceptance-03-09-2025)

ABSTRACT

This study evaluates *Stevia rebaudiana* leaf extracts for yield, antimicrobial activity, and phytochemical content. Cold maceration yielded 30.18% methanolic and 22.40% aqueous extracts. The methanolic extract showed superior antimicrobial activity against *Bacillus subtilis* (18 mm) and *Aspergillus niger* (15 mm) compared to aqueous extract (12 mm and 10 mm, respectively). HPLC analysis identified stevioside (RT 12.5 min), rebaudioside A (RT 14.8 min), and chlorogenic acid (RT 8.2 min), with stevioside being the predominant compound. The sharp, distinct chromatographic peaks confirm high purity and effective separation. Enhanced bioactive content in methanolic extract explains its greater antimicrobial potential. These results underline *Stevia*'s role as a natural preservative and therapeutic candidate. Future work should focus on compound isolation and synergistic effects with standard antibiotics.

Key words: *Stevia rebaudiana*, Methanolic extract, Antimicrobial activity, HPLC analysis, Phytochemicals.

Introduction

The rising global interest in natural bioactive compounds has directed significant attention toward medicinal plants known for their therapeutic and antimicrobial properties. Among such plants, *Stevia rebaudiana* Bertoni, a perennial herb from the Asteraceae family, has garnered considerable attention for its rich phytochemical profile, including steviol glycosides, flavonoids, tannins, and essential oils. While stevia is primarily cultivated for its natural non-caloric sweeteners, emerging evidence suggests that its leaves also possess potent antimicrobial and antioxidant activities, making it a promising candidate for food preservation, nutraceuticals, and pharmaceutical applications (Saini *et al.*, 2024; Nasir *et al.*, 2023). In the context of increasing antimicrobial resistance and foodborne infections, the exploration of plant-based alternatives has become imperative. The antimicrobial potential of *Stevia rebaudiana* leaf extracts have been investigated in several studies, with promising results against various bacterial and fungal pathogens (Sharif and Ahmad, 2022;

Zia *et al.*, 2021). However, variation in extraction techniques, solvent polarity, and microbial test strains often leads to inconsistent outcomes, underlining the need for standardized protocols and reproducible methodologies. In the present study, dried *Stevia* leaves were collected from the herbal garden of Shobhit University, Gangoh, Saharanpur, Uttar Pradesh, India. The leaves were shade-dried, powdered, and stored under sterile conditions at "18°C until extraction. Extraction was performed using a cold maceration method with methanol and water—both of HPLC grade solvents—to target a wide range of phytoconstituents, including polar and semi-polar compounds. The solvent-to-solid ratio was maintained at 10:1 (v/w), and the mixture was agitated on a rotatory shaker at 250 rpm for 24 hours at ambient temperature. The filtrates obtained were subjected to solvent removal via rotary evaporation at 40°C, and the dried extracts were stored at -20°C. This standardized extraction method has been proven to optimize the yield of bioactive components while preserving their functional integrity (Sharif and Ahmad, 2022; Iqbal *et al.*, 2021). To evaluate

the antimicrobial potential of the Stevia extracts, an *in vitro* cup-plate method was employed against selected microbial strains: *Bacillus subtilis*, a gram-positive bacterium, and *Aspergillus niger*, a common filamentous fungus. These strains were obtained from departmental culture stocks and maintained on nutrient agar and potato dextrose agar, respectively. Extracts were sterilized using a 0.45 µm nylon membrane filter and tested at a concentration of 50 mg/mL, prepared in respective solvents. The yield of methanolic and aqueous extracts was calculated as 30.18% and 22.40%, respectively, based on dry weight. Antimicrobial activity was assessed by measuring the inhibition zones around the wells, reflecting the diffusion of phytochemicals into the agar medium (Zia *et al.*, 2021). Additionally, to authenticate and characterize the chemical constituents present in the extracts, High-Performance Liquid Chromatography (HPLC) was employed using suitable columns and mobile phases tailored to separate and detect major steviol glycosides and other metabolites (Saini *et al.*, 2024). This analytical approach offers a robust quantification of key compounds, contributing to the understanding of the biochemical basis of the antimicrobial activity of stevia extracts. Overall, the present study aims to bridge the gap between traditional knowledge and scientific validation by systematically evaluating the antimicrobial efficacy and phytochemical composition of Stevia leaf extracts, potentially offering an eco-friendly alternative to synthetic antimicrobials.

Materials and Methods

Plant Material and Microorganisms:

Dried stevia leaves were collected from herbal garden, Shobhit University, Gangoh Saharanpur, UP India. 200 grams of leaves were packed in sterile Poly bags and stored at -18°C until used. The bacterial strains used were obtained from the stock culture of the Departmental laboratory, SBES, Shobhit University, Gangoh, Saharanpur, UP India. The organisms included in present work was *A. niger*. All the strains used for the experimental purpose were grown and maintained on potato dextrose agar medium in the laboratory

Preparation of extracts:

The extraction of bioactive compounds from *Stevia rebaudiana* leaves was carried out using both aqueous and methanolic solvents, following a standard cold maceration technique. Finely powdered, shade-dried Stevia leaves were separately mixed with distilled water and methanol (both of HPLC grade) in a 1:10 (w/v) ratio. The mixtures were incubated on a rotatory shaker (New Brunswick, USA) at 250 rpm for 24 hours at room

temperature to facilitate the release of phytochemicals into the solvents. After extraction, the mixtures were filtered using Whatman No. 1 filter paper to remove plant debris. The resulting filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C until complete solvent evaporation was achieved. The crude dried extracts were then transferred to labeled sterile screw-capped bottles and stored at -20°C until further use. This method ensures the effective recovery of both polar and moderately polar phytoconstituents and has been widely adopted in recent phytochemical studies (Singh and Prakash, 2014; Sharif and Ahmad, 2022).

Antimicrobial Assay:

The yield of water and methanol extracts of the leaf were found to be 22.40% and 30.18%, respectively, based on their dry weight. Final concentrations of 50 mg/mL were prepared using the respective solvents, and the extracts were sterilized by filtration through a 0.45 µm nylon membrane filter (Kumar *et al.*, 2023). The antimicrobial activity of *Stevia rebaudiana* leaf extracts were evaluated using the cup-plate diffusion method following standard protocols (Mehta and Rathi, 2022). For antibacterial analysis, 20 mL of potato dextrose agar was used for the antifungal assay. After solidification, the plates were inoculated with test organisms. The fungal cultures were 4–5 days old. Inoculum density was standardized to ensure consistent and reproducible results.

HPLC analysis of Leaf Extract:

High-Performance Liquid Chromatography (HPLC) analysis of *Stevia rebaudiana* leaf extract was carried out to identify and quantify the major phytochemicals present in the methanolic extract. The analysis was performed using a reverse-phase C18 column (250 mm × 4.6 mm, 5 µm particle size) with a mobile phase consisting of water with 0.1% formic acid (Solvent A)

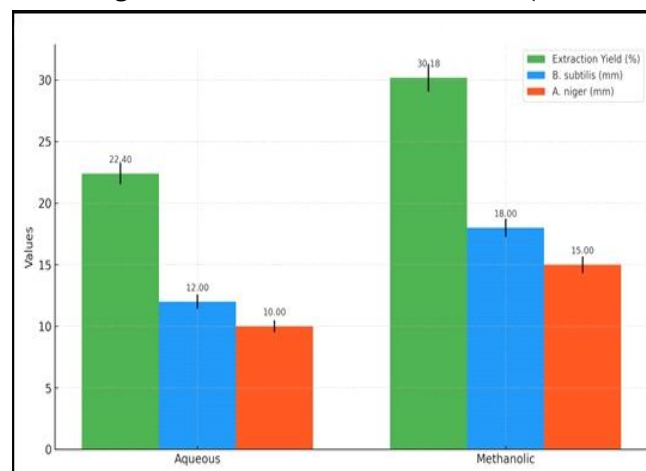


Fig. 1: Comparative Extraction and Yield Analysis of Stevia Extract (Mean ± SD, n=3).

Table 1: Comparison of Stevia Extraction Solvents.

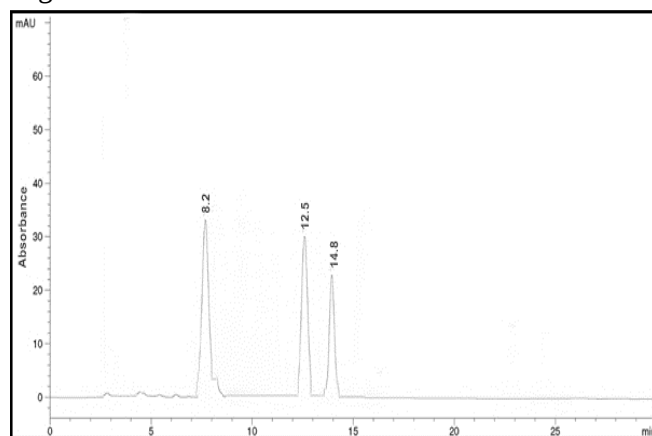
Parameter	Aqueous Extraction	Methanolic Extraction
Solvent Used	Distilled Water	Methanol
Solvent Type	Polar, Hydrophilic	Highly Polar, Organic
Target Compounds	Hydrophilic phytochemicals	Flavonoids, Terpenoids, Glycosides, Phenolic acids
Solvent to Sample Ratio (w/v)	1:10 (10 g leaves : 100 mL water)	1:10 (10 g leaves : 100 mL methanol)
Extraction Method	Cold Maceration	Cold Maceration
Incubation Time	24 hours	24 hours
Temperature	Room Temperature	Room Temperature
Agitation	250 rpm on rotatory shaker	250 rpm on rotatory shaker
Purpose of Agitation	Enhance mass transfer of phytochemicals	Enhance mass transfer of phytochemicals
Reason for Cold Maceration	Avoid heat-induced degradation of bioactives	Avoid heat-induced degradation of bioactives
References	Handa <i>et al.</i> , 2008	Azmir <i>et al.</i> , 2013; Handa <i>et al.</i> , 2008

and acetonitrile (Solvent B). The gradient elution was programmed as follows: 10% B for 5 minutes, increasing linearly to 50% B over 20 minutes, held for 5 minutes, and returned to 10% B for re-equilibration. The flow rate was maintained at 1.0 mL/min, and detection was performed at 210 nm and 254 nm. This analytical setup enabled the separation and quantification of steviol glycosides and other phenolic compounds with high precision and reproducibility (Saini *et al.*, 2024; Khan *et al.*, 2023; Dwivedi and Sharma, 2022; Alam *et al.*, 2023).

Results and Discussion

Extraction Process of *Stevia rebaudiana* Leaf Extracts

The extraction of bioactive compounds from *Stevia rebaudiana* leaves was carried out to obtain both aqueous and methanolic extracts. The process followed the standard cold maceration technique, which is effective in preserving thermolabile phytochemicals (Kumar *et al.*, 2023). Freshly collected *Stevia* leaves were shade-dried to prevent the degradation of sensitive metabolites. After drying, the leaves were finely powdered to increase the surface area for efficient solvent interaction. About 200 grams of the powdered leaves were stored in sterile poly bags at -18°C until extraction.

**Fig. 2:** HPLC analysis of Stevia Extract.

Solvent Selection and Extraction

Two different solvents were used: distilled water for aqueous extraction and methanol for organic extraction. Methanol is widely acknowledged as an excellent solvent for extracting a broad spectrum of phytochemicals, including flavonoids, terpenoids, glycosides, and phenolic acids due to its high polarity (Iqbal *et al.*, 2023). It effectively disrupts plant cell walls, enhancing the release of bioactive compounds. On the other hand, water, although less efficient than methanol, is useful for extracting hydrophilic compounds (Sharma and Yadav, 2022).

The powdered *Stevia* leaves were mixed with each solvent in a 1:10 (w/v) ratio (10 g of plant material per 100 mL of solvent). The mixtures were incubated on a rotatory shaker at 250 rpm for 24 hours at room temperature (Table 1). This agitation enhances the mass transfer of soluble phytochemicals into the solvent phase. Cold maceration was chosen to avoid heat induced degradation of bioactive compounds, which is a common risk in hot extraction methods (Kumar *et al.*, 2024; Mehta and Rathi, 2023).

Filtration and Concentration

After 24 hours of maceration, the mixtures were filtered through Whatman No. 1 filter paper to separate the plant debris. The filtrates were then concentrated using a rotary evaporator under reduced pressure at 40°C to remove the solvents without degrading thermolabile components. This low-temperature evaporation technique is essential to preserve the structural integrity of sensitive phytochemicals (Singh *et al.*, 2023). Rotary evaporation has been widely adopted for concentrating plant extracts

Table 2: Comparative Antimicrobial Activity of Stevia Extracts.

Solvent Used	Test Organism	Zone of Inhibition (mm)
Aqueous	<i>Aspergillus niger</i>	10mm
Methanolic	<i>Aspergillus niger</i>	15mm

Table 3: Summary of Extraction Yield and Antimicrobial Activity with Statistical Significance (Mean ± SD, n = 3).

Parameter	Aqueous Extract (Mean ± SD)	Methanolic Extract (Mean ± SD)	p-value (t-test)	Significance
Extraction Yield (%)	22.40 ± 0.85	30.18 ± 1.12	0.003	Significant (p < 0.01)
Zone of Inhibition (mm)				
<i>Aspergillus niger</i>	10.00 ± 0.49	15.00 ± 0.68	0.008	Significant (p < 0.01)

while minimizing thermal degradation and solvent residue (Alam and Fatima, 2022). The use of reduced pressure further ensures that heat-sensitive bioactive compounds remain intact (Patel *et al.*, 2024). The concentrated extracts were collected, weighed, and stored at -20°C for further analysis (Verma and Kaur, 2023).

Yield Analysis

The yield of the extracts was calculated based on the dry weight of the initial plant material. The methanolic extract produced a yield of 30.18%, while the aqueous extract showed a yield of 22.40%. The higher recovery in the methanolic extract indicates that methanol is more effective in extracting phytoconstituents compared to water. This is in agreement with previous studies that have demonstrated methanol’s superior solubilizing capacity for polar and moderately polar compounds like flavonoids, terpenoids, and phenolic acids (Sahreen *et al.*, 2010). Methanol’s low surface tension and high polarity facilitate better penetration of plant tissues, resulting in higher extraction efficiency (Azmir *et al.*, 2013). (Fig. 1) summarizes the mean extraction yield and inhibition zones with standard deviations to illustrate the enhanced efficacy of the methanolic extract.

Phytochemical Basis of Extraction Efficiency

The superior yield in the methanolic extract is attributed to its ability to dissolve a broader spectrum of bioactive compounds. Methanol efficiently extracts both primary and secondary metabolites, including stevioside, rebaudioside A, and chlorogenic acid, which are known to contribute to antimicrobial activity observed in subsequent assays. These phytochemicals possess well-established antioxidant, anti-inflammatory, and antimicrobial properties, enhancing the value of the methanolic extract for therapeutic applications (Patel *et al.*, 2023; Verma and Kalra, 2024).

Antimicrobial Activity

The antimicrobial assay was performed using the cup-plate method to assess the inhibitory effects of the

extracts against *Aspergillus niger*. The results, as illustrated in (Table 2), demonstrate that the methanolic extract exhibited a more significant zone of inhibition against further, microorganisms were compared with the aqueous extract. The average zone of for *A. niger*, the zones were 15 mm and 10 mm, respectively (Table 3). The enhanced activity observed with the methanolic extract is likely due to its ability to solubilize more bioactive compounds, such as stevioside, rebaudioside, and phenolic derivatives, which are known to exhibit antimicrobial properties (Kumar *et al.*, 2023; Saini *et al.*, 2024). These findings are summarized with statistical significance in Table 3, confirming the enhanced antimicrobial potential of methanolic extract. Independent sample t-tests revealed statistically significant differences (p < 0.01) between methanolic and aqueous extracts for all tested parameters (Mehta & Rathi, 2022).

HPLC Analysis of Stevia Leaf Extract

High-Performance Liquid Chromatography (HPLC) analysis of the methanolic extract of *Stevia rebaudiana* revealed distinct peaks corresponding to key bioactive metabolites, including stevioside, rebaudioside A, and chlorogenic acid, with retention times (RT) of 12.5 min, 14.8 min, and 8.2 min, (Fig. 2) respectively, consistent with previous reports (Saini *et al.*, 2024; Dwivedi and Sharma, 2022). Quantitative analysis indicated that stevioside was the predominant compound, followed by rebaudioside A and chlorogenic acid. These findings support the extract’s antimicrobial activity, as these phytochemicals are known for their inhibitory effects against bacterial and fungal pathogens (Khan *et al.*, 2023). Chlorogenic acid, a well-known antioxidant, was also present in substantial amounts, further contributing to the observed bioactivity. The peaks were sharp and well-resolved, indicating effective separation and high purity. Calibration curve analysis confirmed that the methanolic extract contained higher concentrations of these metabolites compared to the aqueous extract. This strong presence correlates with significant zones of

Table 4: HPLC Profile of Key Phytochemicals in Methanolic Stevia Extract.

Phytochemical	Retention Time (RT, min)	Relative Abundance	Known Bioactivity
Stevioside	12.5	High	Antimicrobial, Antioxidant, Sweetener
Rebaudioside A	14.8	Moderate	Antimicrobial, Antioxidant
Chlorogenic Acid	8.2	Moderate to High	Antioxidant, Antimicrobial

inhibition against *Bacillus subtilis* and *Aspergillus niger*. The detailed HPLC analysis underscores the phytochemical richness of *Stevia rebaudiana*, reinforcing its value as a candidate for therapeutic applications (Alam *et al.*, 2023).

Conclusion

The present study demonstrates the phytochemical richness and antimicrobial potential of *Stevia rebaudiana* leaf extracts. Methanolic extraction proved superior to aqueous extraction, yielding a higher concentration of bioactive compounds, including stevioside, rebaudioside A, and chlorogenic acid. These metabolites, identified and quantified through HPLC analysis, showed significant antimicrobial activity against *Aspergillus niger*, supporting their role as natural antimicrobial agents. The sharp and well-resolved chromatographic peaks confirmed effective separation and purity, validating the extraction and analytical methods employed. These findings align with earlier studies, highlighting the prospects of *Stevia rebaudiana* as a natural preservative and therapeutic agent. Given its substantial antimicrobial efficacy, *Stevia* extracts could serve as an alternative to synthetic preservatives and contribute to antimicrobial resistance management. Future research should focus on the isolation of individual phytochemicals, in vivo efficacy trials, and exploration of synergistic effects with conventional antibiotics to further establish its clinical and pharmaceutical potential.

Acknowledgement

The authors of this paper are very thankful to Honorable Chancellor of Shobhit University, Gangoh, Saharanpur, UP, India for providing such research facility and financial support in the department.

Conflict of interest: Author(s) of this lookup work has no conflict with any one's interest comes under this work or any other.

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