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Exploring Stevia Leaf Residue Extracts: Antibacterial and Antioxidant Potential as Natural Food Preservatives

Panata Iawsipo[1,2,*], Jamorn Somana[3], Phattharaphon Patthamawan[1], Thitiporn Kaewmahatthai[1] and Shotabdy Roy[3]

[1]Department of Biochemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand
[2]Unit of Bioactive Natural Compounds for Healthcare Products Development and Centre of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand
[3]Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

([*]Corresponding author's e-mail: panata@go.buu.ac.th)

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Abstract

Chemical preservatives commonly used in food preservation often pose health risks and contribute to bacterial resistance, prompting the search for safer alternatives from natural sources. *Stevia rebaudiana* Bertoni leaves are well-known for their role as natural sweeteners and sugar substitutes in both culinary and medicinal applications. Additionally, their extracts exhibit diverse biological activities, such as anti-diabetic, antibacterial and antioxidant properties, and have previously been confirmed for use in food preservation. Nevertheless, limited research has explored the potential of leaf residues, which are plentiful wastes from steviol glycoside extraction. These residues might still retain valuable bioactive compounds suitable for food preservation, emphasizing the need for additional investigation. Therefore, in this study, stevia leaf residues were subjected to ethanol (RE), a 50 % ethanol/water mixture (REW), and hot water (RW) extraction, and the antibacterial and antioxidant properties of 3 distinct extracts were examined. Evaluation of antibacterial activity against *Staphylococcus aureus* revealed significant inhibition zones for all extracts, with the REW extract exhibiting the highest efficacy, reaching up to 25.58 ± 2.54 mm. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.0125 mg per spot and 0.2 mg per spot, respectively, were recorded for the most effective extract (REW), indicating a bacteriostatic effect. Quantification of total phenolic content (TPC) and total flavonoid content (TFC) revealed a strong correlation between phenolic compounds and the tested biological activities, antibacterial and DPPH radical scavenging. Furthermore, HPLC Orbitrap MS analysis identified 6 compounds in the extract: 1 phenolic acid, 4 flavonoids and 1 fatty amide. These identified compounds may be responsible for the observed biological activities of the stevia leaf residue extract. The results confirm the retained potential of stevia leaf residue extracts as sources of natural preservatives in the food industry, offering both antimicrobial and antioxidant benefits.

Keywords: Stevia leaf residues, Natural preservatives, *Staphylococcus aureus*, Antioxidant activity, Phenolic content

Introduction

S. aureus is a pathogenic bacterium that can cause wound infection and also food poisoning or spoil food [1]. People often use chemical preservatives to prevent bacterial growth, but these might raise many health concerns, such as toxicity and the development of bacterial resistance [2]. Because of these problems, there is a growing need to find safer, natural alternatives to chemical preservatives.

Natural antioxidants, such as phenolic compounds, flavonoids, tocopherols and carotenoids, represent commonly utilized groups of food additives [3]. Numerous plant extracts abundant in these compounds not only demonstrate substantial antioxidant properties but also serve as effective inhibitors against various foodborne pathogens and spoilage bacteria [4-6].

Stevia rebaudiana Bertoni, a plant member in the Asteraceae family, is one of the natural sources of phenolic compounds with antioxidant and antimicrobial properties [7-10]. This plant is well-known for sweet compounds, steviol glycosides, of which the major compounds are stevioside and rebaudioside A. The glycosides are approximately 200 to 350 times sweeter than sugar [11]. It has been used for therapeutic purposes against several diseases such as diabetes mellitus, hypertension, inflammation and cancer [7].

Unfortunately, a significant amount of other bioactive compounds found in stevia leaves are discarded as residues from the steviol glycoside extraction industry. Thus, exploring ways to recover bioactive compounds that could offer effective antioxidant and antimicrobial properties suitable for the food industry would be beneficial. In addition, it might give more profitable resources and reduce waste as a way to promote a bio-circular-green economy. Previous research has validated the antioxidant activity of polyphenol extracts from stevia leaf residues obtained by water extraction, ethanol/water extraction and supercritical CO_2 with ethanol extraction [12]. Moreover, the study has also explored their utility as preservatives in salmon paste. However, there is a lack of information regarding the antibacterial potential of food-related pathogens targeted by stevia leaf residue extract. Hence, this study aims to assess the inhibitory effect of stevia leaf residue extracts from various solvents on *S. aureus*, evaluate their total phenolic and flavonoid contents, and conduct antioxidant testing. Additionally, the analysis was performed to determine if there was any correlation between these secondary compounds and the tested activities. Finally, HPLC-MS/MS analysis was carried out to identify specific compounds present in the extracts.

Materials and methods

Sample extraction

Dried post-hot water extracted stevia residues, sourced from Sugavia Company Limited in Nakhon Ratchasima, Thailand, were utilized for this study. The residues were air-dried in a solar dryer at around 60 °C for 2 days and then pulverized to a coarse powder using a grinder.

For extraction purposes, 300 g of the dried powdered sample were subjected to 3 different methods: Maceration in food-grade ethanol (Union Chemical and Equipment Co. Ltd., Bangkok, Thailand) for 5 days, maceration in a 50 % ethanol/water mixture for 5 days, or boiling in distilled water for 30 min. The residues underwent 2 additional extraction cycles using fresh solvents of the same type.

Subsequently, each aqueous mixture was filtered through Whatman filter paper No. 1 (MilliporeSigma, MA, USA), and the filtered extracts were concentrated using a rotary evaporator at 42 °C for ethanol and 60 °C for water. The concentrated crude extracts were freeze-dried and stored at –20 °C in sterile, foil-wrapped tubes until further use.

Evaluation of extract susceptibility using the agar well diffusion method

S. aureus (ATCC 6538) was subjected to the agar well diffusion method to assess its susceptibility to the extract, following a previously described procedure with some modifications [13]. Initially, the inoculum, containing 1.5×10^8 CFU (OD_{600} 0.08 - 0.1), was evenly spread over the surface of sterile Mueller-Hinton agar (MHA) (HiMedia Laboratories Pvt. Ltd., Maharashtra, India) plates using a sterile cotton swab. Crude extracts at various concentrations were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corp., MO, USA) using a 2-fold dilution method. Wells were subsequently created in the agar plates containing the inoculum using a sterile cork borer (6 mm in diameter), and then 50 µL of each extract was added to the respective wells. The plates were allowed to stand for 15 min to enable the extracts to diffuse into the agar and were then incubated at 37 °C for 18 - 24 h. The antimicrobial activity of the extract was determined by measuring the zone of inhibition that appeared after the incubation period. Ampicillin served as a positive control, while DMSO was used as a negative control. Each extract was tested 6 times across separate plates.

Determination of MIC and MBC

Due to the potential masking of microbial growth by the colouring of the extract in a liquid medium, the agar diffusion method is often favoured over broth dilution for MIC determination [14]. The crude extract from stevia leaf residue demonstrating the highest activity was chosen for MIC determination using a modified method based on Kowalska-Krochmal and Dudek-Wicher [15]. Briefly, the inoculum containing 1×10^4 CFU/spot was pipetted onto the surface of Mueller-Hinton agar (MHA) medium and allowed to dry. Different concentrations of extracts, prepared by 2-fold serial dilution using DMSO, were then spotted on top of the inoculum. After the spots dried, the plates were incubated at 37 °C for 18 - 24 h. The MIC was defined as the lowest concentration of extract at which no visible growth of *S. aureus* was observed.

Subsequently, the MBC of the extract, defined as the lowest concentration that kills 99 % of the inoculum, was determined by subculturing the spots containing extract concentrations of MIC and above onto MHA media. The lowest concentration without bacterial growth was identified as the MBC. All assays were performed 6 times, with DMSO serving as the negative control.

Determination of TPC and TFC

TPC was determined by the Folin-Ciocalteu assay according to Shehata *et al.* [16] with slight modification [16] with slight modification. Briefly, 25 µL of extracts at indicated concentrations were mixed with 100 µL of deionized water and 30 µL of Folin-Ciocalteu reagent (Merck, Darmstadt, Germany). After incubating the mixture in darkness for 6 min, 250 µL of 7 % (w/v) Na_2CO_3 and 195 µL of deionized water were added. The mixtures were then left to incubate for 30 min at room temperature, followed by measurement of absorbance at 765 nm using a microplate reader. Total soluble phenolic compound concentrations were determined using a standard curve of aqueous gallic acid solutions and expressed as mg gallic acid equivalent/g of extract (mg GAE/g).

For TFC determination, the Aluminum chloride method, as described in a previous study [17], was employed with some modifications. Briefly, 100 µL of diluted extracts were mixed with 400 µL of distilled water in individual tubes. Subsequently, 30 µL of 7 % (w/v) $NaNO_2$ and 30 µL of 7 % (w/v) $AlCl_3$ were added sequentially to induce specific reactions, with respective incubation periods of 6 and 5 min. Following this, 200 µL of 1 M NaOH was added to each tube, and the volume was adjusted to 1 mL with distilled water, followed by an additional 5 min incubation in darkness. After the incubation, the contents of each tube were transferred to a 96-well plate. The absorbance at 510 nm was measured and expressed as quercetin equivalent (mg QE/g) using the linear equation based on the standard calibration curve.

Determination of antioxidant activity

The antioxidant capacity of the extracts was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method, following a previously described procedure [18] with some modifications. Initially, 100 µL of freshly prepared DPPH (MedChemExpress, NJ, USA) in methanol (200 µM) was mixed with 50 µL of extract or DMSO (used as a negative control). Methanol was substituted for the DPPH solution in a blank control. After incubating the mixture for 15 min in darkness, absorbance was measured at 517 nm. Ascorbic acid (MilliporeSigma, MA, USA) served as the reference standard. The absorbance of tested extracts and those of DMSO were subtracted from the blank absorbance before further calculations were performed.

The percentage of DPPH radical scavenging activity was calculated by [(Acontrol – Asample)/Acontrol]×100, where Acontrol represents the absorbance of the DMSO and Asample represents the absorbance of the test extract. The results were plotted as the percentage of scavenging activity against the sample concentration, and EC_{50} (the concentration at which 50 % of the DPPH radical was scavenged) was subsequently determined. The experiment was performed in triplicate and repeated 3 times.

Characterization of chemical composition by HPLC-HRESI-MS and MS/MS conditions

The extract was diluted 10 - 100 times with ethanol before HPLC analysis. HPLC quantification was conducted using a Waters e2695 HPLC system with a 2487 dual-wavelength detector (Waters Corporation, Milford, MA), and an Acclaim 120 C18 column (Thermo Fisher Scientific, 4.6×100 mm^2, 3 µm). The column temperature was set at 25 °C. A gradient mobile phase was employed, consisting of 100 % water as solution A and 100 % acetonitrile as solution B. The gradient conditions were adjusted as follows: 0 - 1 min at 95 % A, 1 - 1.5 min at 90 % A, 1.5 - 2 min at 85 % A, 2.5 - 3.5 min at 75 % A, 4 - 5.5 min at 60 % A, 5.5 - 7.5 min at 70 % B, 7.5 - 9.5 min at 90 % B and 10 min at 100 % B, followed by a 5-minute post-run time. An injection volume of 6 µL was introduced at a 1 mL/min flow rate. UV absorbance was monitored between 210 and 280 nm using dual-wavelength detection for compound identification. Each distinct peak was collected individually.

Mass spectral (MS/MS) data for each fraction were obtained using a high-resolution mass spectrometer equipped with electrospray ionization in positive mode (ESI+) from Thermo Fisher Scientific's Orbitrap-based MS systems. The Orbitrap functions as both an analyzer and a detector, featuring an ion trap mass analyzer with 2 external electrodes and a core electrode. The Q-Exactive Plus Orbitrap mass spectrometer offers high-resolution and accurate-mass (HRAM) data analysis capabilities. Properties of Full MS/dd-MS2 Settings: Runtime 0 - 20 min, polarity = positive, default charge = 1, exclusion = on. Full MS/dd-MS2 (full scan with data-dependent acquisition): Scan range = 80 - 1200 m/z, Resolution = 60,000; AGC target = 3 eV, Maximum IT = 500 ms. dd-MS2/dd-SIM (targeted data-dependent acquisition): Resolution = 15,000; AGC target = 5 eV, Maximum IT = 120 ms, loop count = 5, TopN = 5, Isolation window = 2.0 m/z, fixed first mass = 50.0 m/z, stepped nce: 35. dd settings: Minimum AGC target: 1.00 eV, Intensity thresh = 8.3 eV, Peptide match = preferred, Exclude isotope: On, dynamic exclusion: 10.0 s.

Molecular masses and mass fragmentation patterns were searched against MassBank databases (https://massbank.eu/MassBank/Search), PubChem databases (https://pubchem.ncbi.nlm.nih.gov) and ChemSpider (http://www.chemspider.com/). Compound identities were determined by comparing mass spectra with the highest similarity to the same compound across multiple databases.

Statistics

The results were presented as the mean of at least 6 values, with standard deviation (Mean ± SD). All graphs were created using Microsoft Excel 2021. To determine significant differences between groups, a 1-way analysis of variance (ANOVA) was conducted, followed by comparisons using Tukey's test with Minitab 18 software (Minitab Inc., PA, USA). Statistical significance was considered at $p < 0.05$. For Pearson correlation analysis, the correlation coefficient (r) and p-values of 2 variables (quantity of compounds and bioactivity) were determined using Microsoft Excel 2021 software.

Results and discussion

Evaluation of antibacterial activity of stevia leaf residue extracts against *S. aureus*

The antibacterial efficacy of 3 distinct stevia leaf residue extracts obtained via ethanol (RE), a 50 % ethanol/water mixture (REW) and hot water (RW) extraction was investigated against *S. aureus* using the agar well diffusion method. Like disk diffusion, the agar well diffusion method is widely used to evaluate plant extracts' antimicrobial activity based on the antimicrobial agent's diffusion in the agar medium. This method relies on the principle that the antimicrobial compounds present in the extracts diffuse outward from the wells into the agar, resulting in zones of inhibition where bacterial growth is inhibited. The size of the inhibition zones provides a qualitative measure of the effectiveness of the extracts against the tested microbial strain.

In our study, all 3 stevia leaf residue extracts, RE, REW and RW, demonstrated significant antibacterial activity against *S. aureus* in a dose-dependent manner, as evidenced by the presence of clear inhibition zones around the wells (**Figure 1** and **Table 1**). The observed inhibition zones indicate that the extracts contain active compounds capable of inhibiting the growth of *S. aureus*, confirming that the bioactive compounds are still retained in the leaf residues after sweetener extraction.

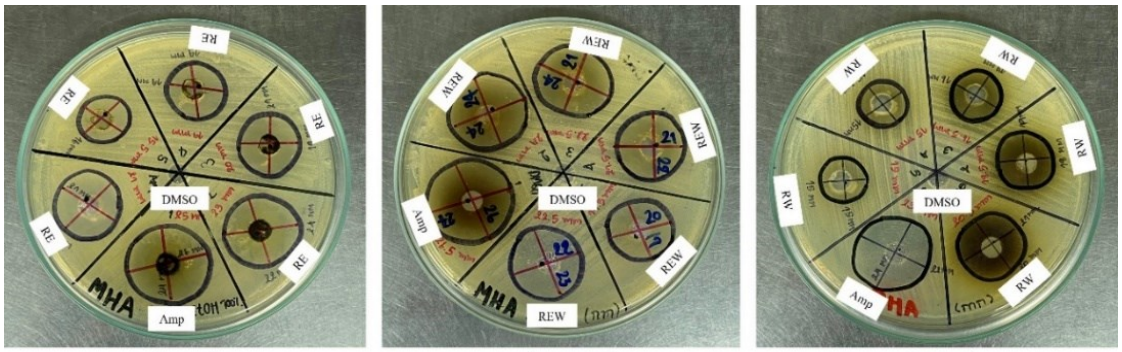

Figure 1 Representative plates from the agar well diffusion assay displaying the inhibition zones against *S. aureus* of 3 extracts obtained via ethanol (RE), a 50 % ethanol/water mixture (REW) and hot water (RW) extraction. Ampicillin serves as the positive control, with DMSO loaded into the well at the center of the plate serving as the negative control.

Table 1 Diameter of inhibition zone (in mm) of various concentrations of stevia leaf residue extracts against *S. aureus*.

Extracts	Diameter of inhibition zone (mm)			
Concentrations (mg/mL)	**20**	**40**	**80**	**160**
RE	17.75 ± 1.70^{B}	19.08 ± 0.58^{B}	20.33 ± 0.61^{B}	22.25 ± 0.99^{B}
REW	21.08 ± 1.80^{A}	21.67 ± 1.03^{A}	23.08 ± 0.66^{A}	25.58 ± 2.54^{A}
RW	14.58 ± 1.80^{C}	15.92 ± 1.91^{C}	17.66 ± 2.21^{C}	19.33 ± 1.63^{C}

*The results were expressed as Mean ± SD. Each extract was replicated 6 times using separate plates (n = 6). Significant differences among extracts at the same concentration were denoted by different letters (A, B and C) following the values ($p < 0.05$).

The positive control, ampicillin, displayed significant inhibitory activity with an inhibition zone of 22.16 mm at 6.25 µg/mL against the tested bacteria, confirming the sensitivity of the assay. In contrast, the negative control, DMSO alone, exhibited no antibacterial activity, validating the specificity of the assay.

The variation in the size of the inhibition zones among the extracts suggests differences in their antibacterial potency, with the REW extract exhibiting the highest efficacy (21.83 - 25.33 mm in diameter), consistent with findings from a previous study on leaf samples [19]. Differences in activity among extracts may be attributed to variations in the composition and concentration of bioactive compounds extracted using different solvents. The highest antibacterial activity of the REW extract suggests the potential of a 50 % ethanol/water mixture as an effective solvent for extracting bioactive compounds from stevia leaf residues. Interestingly, our results indicated that the RW extract had lower activity compared to the RE extract, which contrasts with previous findings on the activity of stevia leaf extracts against *S. aureus* [19,20]. This difference could be due to some active components, including Steviol glycoside, flavonoids and polyphenols, being extracted during the sweetener extraction process from industry, leaving the remainder for subsequent extraction with ethanol/water mixture, which proved to be the most effective solvent in our study. However, further experiments with different ethanol/water mixtures may be conducted to optimize the extraction process.

MIC and MBC of the effective extract

In this study, the agar dilution method was preferred over broth dilution because the color of the extract might interfere with observation, making it difficult to identify concentrations with no bacterial growth in liquid media. Considering the highest efficacy of the REW extract in inhibiting the growth of *S. aureus*, it was selected for determining the MIC and MBC. The result revealed an MIC value of 2.5 mg/mL (equivalent to 0.0125 mg per spot) and an MBC value of 40 mg/mL (equivalent to 0.2 mg per spot) for the REW extract. The observed MIC and MBC values for the REW extract suggest a moderate antibacterial effect against *S. aureus*. However, the relatively higher concentrations required for inhibition compared to the positive control, ampicillin, which exhibited MIC and MBC values of 1.56 µg/mL, may be due to the REW being a crude extract composed of various compounds, both functional and nonfunctional, in different concentrations, some of which may have antagonistic effects, unlike ampicillin, which is a pure compound. Therefore, fractionation of the REW may enhance its activity. Since the MIC to MBC ratio for the REW extract was higher than 4, its antibacterial activity was considered bacteriostatic [21].

Quantification of phenolic compounds and flavonoids in stevia leaf residue extracts

The Folin-Ciocalteu method is widely used to quantitatively assess phenolic compounds in plant extracts, employing spectrophotometry for measurement due to its simplicity and cost-effectiveness. The intensity of the reduced blue product, with a peak absorbance at 765 nm, correlates with the phenolic groups present in the extract [22]. Various phenolic compounds have been identified in stevia leaves, such as Chlorogenic acid, Caffeic acid, Rutin and trans-Ferulic acid. These phenolics exhibit bioactivities including antimicrobial and antioxidant activities [23,24]. It is plausible that these active phenolics remain in the leaf residue, potentially contributing to the observed biological activities of the stevia leaf residue extracts.

Moreover, TFC was determined utilizing the Aluminum chloride method, commonly employed for quantifying flavonoids in plant extracts. This method involves the formation of a complex between Aluminum chloride and flavonoids, resulting in a colorimetric change measurable by spectrophotometry.

The mean TPC values equivalent to gallic acid varied between 52.51 and 144.90 mg/g, while the mean TFC values equivalent to quercetin ranged from 321.4 to 929.87 mg/g across different stevia leaf residue extracts (**Figure 2**). Notably, the REW extract exhibited significantly higher TPC and TFC values compared to the other 2 extracts, with its TPC and TFC levels being 2 to 3 times higher than those of the other extracts. This highlights the REW extract as a rich source of both phenolic and flavonoid compounds, which is remarkable, especially considering its origin from residual material.

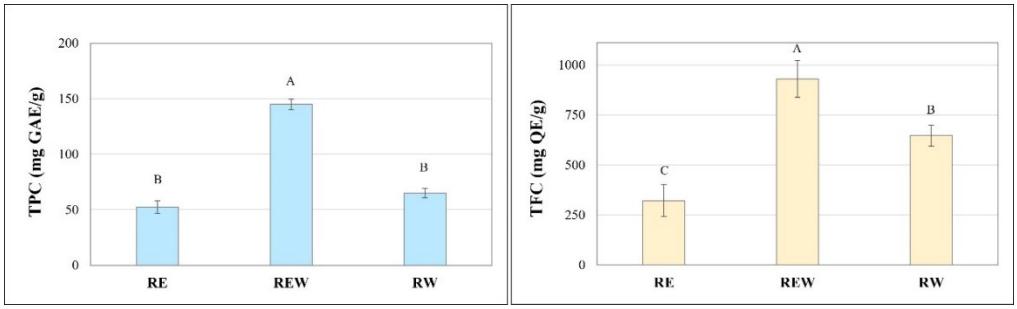

Figure 2 TPC (mg GAE/g) and TFC (mg QE/100 g) of stevia leaf residue extracts were estimated by Folin-Ciocalteau assay and Aluminum chloride method, respectively. Data are expressed as Mean ± SD (n = 6). Bars that do not share a letter (A and B) are significantly different with $p < 0.05$.

To act as antibacterial agents, polyphenols interact effectively with bacterial cell wall components and the bacterial cell membrane. This interaction can prevent and control biofilm formation, inhibit microbial enzymes, interfere with protein regulation and deprive bacterial cell enzymes of substrates and essential metal ions [25]. The presence of phenolic and flavonoid compounds in stevia leaf residue extracts is consistent with the observed inhibition capacity against *S. aureus*, suggesting these compounds may contribute to this antimicrobial activity.

Antioxidant activity of stevia leaf residue extracts

Numerous studies have established correlations between the content of polyphenols in extracts and their antioxidant capacity [26-28]. Antioxidant activity, both *in vitro*, *in vivo* and in food containing stevia extract, has also been extensively documented in the literature [7,29,30].

To confirm the continued existence of antioxidants in stevia leaf residue, the activity of 3 extracts was assessed using a DPPH radical scavenging assay. The DPPH radical scavenging activity is based on 1-electron reduction, representing the free radical reducing activity of antioxidants, which can be observed as

the visually noticeable quenching of the stable purple-colored DPPH radical to the pale yellow-colored DPPH [26]. Ascorbic acid served as a standard reference in this study. The results presented in **Figure 3** and **Table 2** demonstrate the percentage of DPPH radical scavenging activity at 0.0625 - 0.25 mg/mL and the EC_{50} values, respectively. All extracts exhibited over 50 % DPPH radical quenching at 0.25 mg/mL. The REW extract showed the highest DPPH radical scavenging potency with a minimum IC_{50} value of 0.19 mg/mL, followed by the RW and RE extracts. These findings suggest that the antioxidant activity of the extracts is influenced by the level of polyphenols they contain. Therefore, the subsequent analysis focused on investigating the correlation between these phenolic and flavonoid contents and the bioactivities examined in this study.

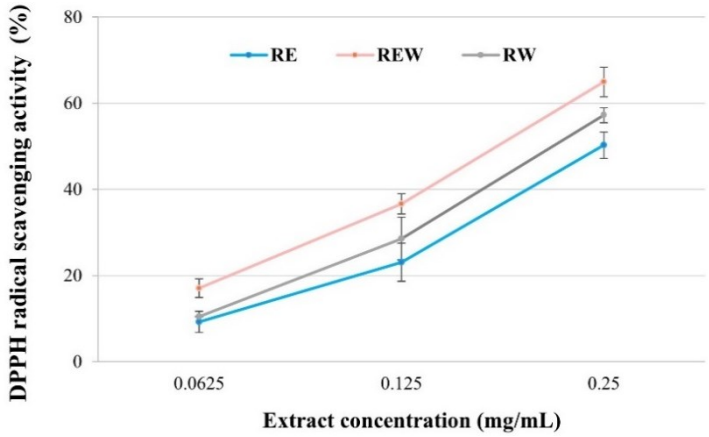

Figure 3 Antioxidant capacity of stevia leaf residue extracts obtained by DPPH radical scavenging assay. Data are expressed as Mean ± SD (n = 9 from 3 independent experiments).

Table 2 The concentration of extracts required to scavenge 50 % of DPPH radical (EC_{50}) of stevia leaf residue extracts.

Extracts	EC_{50} values
RE	0.31 ± 0.04 mg/mL^{A}
REW	0.18 ± 0.02 mg/mL^{C}
RW	0.24 ± 0.03 mg/mL^{B}
Ascorbic acid	14.890.18 µg/mL

*Different letters (A and B) following the values indicate significant differences compared among extracts ($p < 0.05$).

Correlation of bioactivities with phenolic/flavonoid contents

Pearson's correlation coefficient analysis was conducted to investigate the relationship between TPC or TFC and the bioactivities assessed in this study. The results are presented in **Figure 4**.

The results revealed a strong negative correlation between TPC and DPPH radical scavenging activity, as evidenced by a correlation coefficient (r) of –0.894 (p = 0.001) for TPC. Similarly, TFC exhibited a highly negative correlation with DPPH activity, with a correlation coefficient of –0.999 (p = 1.047E-12). In contrast, antibacterial activity displayed a strong positive correlation with TPC, represented by a correlation coefficient of 0.820 (p = 0.045). This finding aligns with the antimicrobial potential of

polyphenol extracts from stevia leaves as previously reported by Myint *et al.* [31]. However, the correlation between antibacterial activity and TFC was weaker, with a correlation coefficient of 0.497 (p = 0.316), indicating a moderate positive correlation.

These findings collectively suggest a significant negative correlation between phenolic and flavonoid contents and DPPH radical scavenging activity, while highlighting a strong positive correlation between antibacterial activity and TPC.

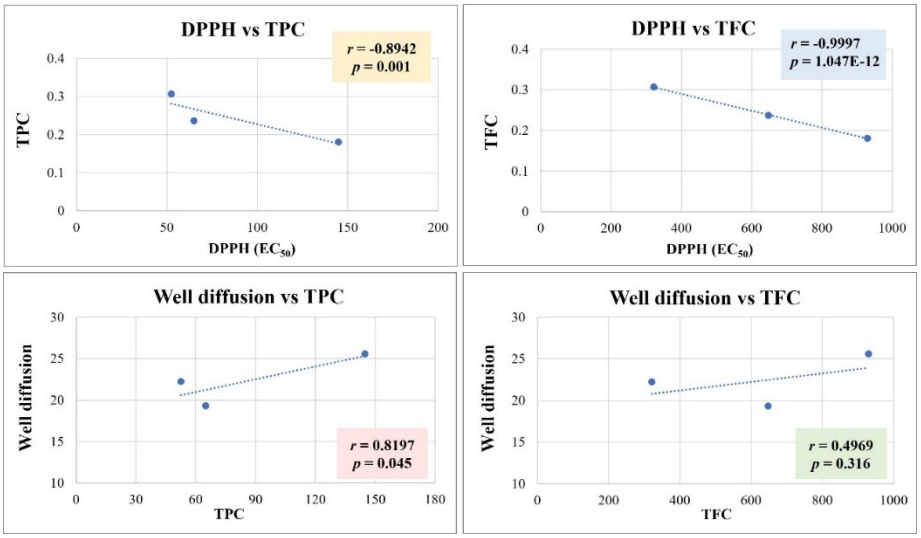

Figure 4 Correlation coefficients between TPC or TFC and the evaluated bioactivities. Pearson's correlation was conducted to analyse the relationship between TPC/TFC and EC_{50} values for antioxidant activity, as well as agar well diffusion results at 160 mg/mL of extracts for antibacterial activity. The correlation coefficients (r) and p-values are provided for each comparison.

Identification of compounds using HPLC-HRESI-MS and MS/MS

From the HPLC chromatogram of the stevia residue extract, only 6 compounds could be identified based on molecular masses and fragmentation patterns matched with databases (**Figure S1** and **Table S1**, supplementary data). Among these compounds, 4 were flavonoids: Apigenin-7-O-glucoside, quercetin-3-O-rhamnoside, pinocembrin-7-O-glucoside and rutin. Additionally, 1 phenolic acid, 3,4-dicaffeoylquinic acid and 1 fatty amide, oleamide, were identified. Consistent with our previous findings, HPLC peak intensities confirmed higher TFC than TPC.

These compounds are known for their potential antibacterial and antioxidant properties. For example, Quercetin-3-O-rhamnoside, a dominant presence in the HPLC chromatogram, is known for its antioxidant activity and could inhibit *S. aureus* adhesion by targeting cysteine transpeptidase [32,33]. Apigenin-7-O-glucoside suppresses biofilm formation by reducing exopolysaccharide production, quorum sensing and cell surface hydrophobicity, resulting in decreased bacterial adhesion and biofilm development [34]. Rutin combats reactive oxygen species through multiple mechanisms, including ROS scavenging, upregulation of antioxidant enzymes and xanthine oxidase inhibition [35]. Furthermore, caffeoyl derivatives, such as 3,4-dicaffeolquinic acid, have demonstrated cytoprotective potential by reducing oxidative stress both *in vitro* and *in vivo*, and also potentially exhibit antibacterial activity [36-38]. This suggests that the compounds identified in stevia residue extracts could be responsible for the observed biological activities.

Conclusions

The current study highlights the significant antibacterial and antioxidant potential of stevia leaf residue extracts, particularly those extracted using a 50 % ethanol/water mixture. At least 5 identified phenolic compounds might contribute to these bioactivities. Our findings suggest that stevia leaf residue extracts are safe and could serve as a promising source of natural alternatives in the food industry, contributing to the transformation of residual materials into valuable resources. Further research is needed to elucidate the mechanisms underlying the observed effects and to explore practical applications in food preservation and functional food development.

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References

[1] A Fetsch and S Johler. *Staphylococcus aureus* as a foodborne pathogen. *Curr. Clin. Microbiol. Rep.* 2018; **5**, 88-96.

[2] AA Mostafa, AA Al-Askar, KS Almaary, TM Dawoud, EN Sholkamy and MM Bakri. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi J. Biol. Sci.* 2018; **25**, 361-6.

[3] A Bensid, NE Abed, A Houicher, JM Regenstein and F Özogul. Antioxidant and antimicrobial preservatives: Properties, mechanism of action and applications in food - A review. *Crit. Rev. Food Sci. Nutr.* 2022; **62**, 2985-3001.

[4] CO Raphaelli, G Dannenberg, GO Dalmazo, ES Pereira, M Radünz, M Vizzotto, AM Fiorentini, EA Gandra and L Nora. Antibacterial and antioxidant properties of phenolic-rich extracts from apple (*Malus domestica* cv. Gala). *Int. Food Res. J.* 2019; **26**, 1133-42.

[5] C Zambrano, EB Kerekes, A Kotogán, T Papp, C Vágvölgyi, J Krisch and M Takó. Antimicrobial activity of grape, apple and pitahaya residue extracts after carbohydrase treatment against food-related bacteria. *LWT Food Sci. Tech.* 2019; **100**, 416-25.

[6] KA Nxumalo, AO Aremu and OA Fawole. Metabolite profiling, antioxidant and antibacterial properties of four medicinal plants from Eswatini and their relevance in food preservation. *S. Afr. J. Bot.* 2023; **162**, 719-29.

[7] J Ahmad, I Khan, R Blundell, J Azzopardi and MF Mahomoodally. *Stevia rebaudiana* Bertoni.: An updated review of its health benefits, industrial applications and safety. *Trends Food Sci. Tech.* 2020; **100**, 177-89.

[8] M Debnath. Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. *J. Med. Plants Res.* 2008; **2**, 45-51.

[9] S Jayaraman, MS Manoharan and S Illanchezian. *In-vitro* antimicrobial and antitumor activities of *Stevia rebaudiana* (Asteraceae) leaf extracts. *Trop. J. Pharmaceut. Res.* 2008; **7**, 1143-9.

[10] SU Singh, VE Garg, DE Yadav, MN Beg and NI Sharma. *In vitro* antioxidative and antibacterial activities of various parts of *Stevia rebaudiana* (Bertoni). *Int. J. Pharm. Pharmaceut. Sci.* 2012; **4**, 468-73.

[11] V Peteliuk, L Rybchuk, M Bayliak, KB Storey and O Lushchak. Natural sweetener *Stevia rebaudiana*: Functionalities, health benefits and potential risks. *EXCLI J.* 2021; **20**, 1412-30.

[12] J Ortiz-Viedma, N Romero, L Puente, K Burgos, M Toro, L Ramirez, A Rodriguez, J Barros-Velazquez and SP Aubourg. Antioxidant and antimicrobial effects of stevia (*Stevia rebaudiana* Bert.) extracts during preservation of refrigerated salmon paste. *Eur. J. Lipid Sci. Tech.* 2017; **119**, 1600467.

[13] S Manandhar, S Luitel and RK Dahal. *In vitro* antimicrobial activity of some medicinal plants against human pathogenic bacteria. *J. Trop. Med.* 2019; **2019**, 1895340.

[14] M Balouiri, M Sadiki and SK Ibnsouda. Methods for *in vitro* evaluating antimicrobial activity: A review. *J. Pharmaceut. Anal.* 2016; **6**, 71-9.

[15] B Kowalska-Krochmal and R Dudek-Wicher. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens* 2021; **10**, 165.

[16] MG Shehata, TS Awad, D Asker, SAE Sohaimy, NMA El-Aziz and MN Youssef. Antioxidant and antimicrobial activities and UPLC-ESI-MS/MS polyphenolic profile of sweet orange peel extracts. *Curr. Res. Food Sci.* 2021; **4**, 326-35.

[17] N Phuyal, PK Jha, PP Raturi and S Rajbhandary. Total phenolic, flavonoid contents, and antioxidant activities of fruit, seed, and bark extracts of *Zanthoxylum armatum* DC. *Sci. World J.* 2020; **2020**, 8780704.

[18] DT Ayele, ML Akele and AT Melese. Analysis of total phenolic contents, flavonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts. *BMC Chem.* 2022; **16**, 30.

[19] A Chakma, F Afrin, MG Rasul, H Maeda, C Yuan and AKMA Shah. Effects of extraction techniques on antioxidant and antibacterial activity of stevia (*Stevia rebaudiana* Bertoni) leaf extracts. *Food Chem. Adv.* 2023; **3**, 100494.

[20] S Ghosh, E Subudhi and S Nayak. Antimicrobial assay of *Stevia rebaudiana* Bertoni leaf extracts against 10 pathogens. *Int. J. Integr. Biol.* 2008; **2**, 27-31.

[21] R Mogana, A Adhikari, MN Tzar, R Ramliza and C Wiart. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates. *BMC Complementary Med. Ther.* 2020; **20**, 55.

[22] M Pérez, I Dominguez-López and RM Lamuela-Raventós. The chemistry behind the Folin-Ciocalteu method for the estimation of (poly)phenol content in food: Total phenolic intake in a mediterranean dietary pattern. *J. Agr. Food Chem.* 2023; **71**, 17543-53.

[23] AK Patra. *An overview of antimicrobial properties of different classes of phytochemicals*. *In*: Dietary phytochemmicals microbes. Springer, Dordrecht, 2012.

[24] R Lemus-Mondaca, A Vega-Galvez, P Rojas, K Stucken, C Delporte, G Valenzuela-Barra, RJ Jagus, MV Agüero and A Pasten. Antioxidant, antimicrobial and anti-inflammatory potential of *Stevia rebaudiana* leaves: Effect of different drying methods. *J. Appl. Res. Med. Aromat. Plants* 2018; **11**, 37-46.

[25] C Papuc, GV Goran, CN Predescu, V Nicorescu and G Stefan. Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: Classification, structures, sources, and action mechanisms. *Compr. Rev. Food Sci. Food Saf.* 2017; **16**, 1243-68.

[26] S Aryal, MK Baniya, K Danekhu, P Kunwar, R Gurung and N Koirala. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants* 2019; **8**, 96.

[27] JS Kim and JH Lee. Correlation between solid content and antioxidant activities in Umbelliferae salad plants. *Prev. Nutr. Food Sci.* 2020; **25**, 84-92.

[28] YM Muflihah, G Gollavelli and YC Ling. Correlation study of antioxidant activity with phenolic and flavonoid compounds in 12 Indonesian indigenous herbs. *Antioxidants* 2021; **10**, 1530.

[29] M Papaefthimiou, PI Kontou, PG Bagos and GG Braliou. Antioxidant activity of leaf extracts from *Stevia rebaudiana* Bertoni exerts attenuating effect on diseased experimental rats: A systematic review and meta-analysis. *Nutrients* 2023; **15**, 3325.

[30] JC Ruiz-Ruiz, YB Moguel-Ordoñez, AJ Matus-Basto and MR Segura-Campos. Antidiabetic and antioxidant activity of *Stevia rebaudiana* extracts (Var. Morita) and their incorporation into a potential functional bread. *J. Food Sci. Tech.* 2015; **52**, 7894-903.

[31] KZ Myint, Z Zhou, Q Shi, J Chen, X Dong and Y Xia. *Stevia* polyphenols, their antimicrobial and anti-inflammatory properties, and inhibitory effect on digestive enzymes. *Molecules* 2023; **28**, 7572.

[32] B Liu, F Chen, C Bi, L Wang, X Zhong, H Cai, X Deng, X Niu and D Wang. Quercitrin, an inhibitor of Sortase A, interferes with the adhesion of Staphylococcal aureus. *Molecules* 2015; **20**, 6533-43.

[33] A Septembre-Malaterre, A Boumendjel, AS Seteyen, C Boina, P Gasque, P Guiraud and J Sélambarom. Focus on the high therapeutic potentials of quercetin and its derivatives. *Phytomed. Plus* 2022; **2**, 100220.

[34] ZJ Pei, C Li, W Dai, Z Lou, X Sun, H Wang, AA Khan and C Wan. The anti-biofilm activity and mechanism of apigenin-7-o-glucoside against *Staphylococcus aureus* and *Escherichia coli*. *Infect Drug Resist.* 2023; **16**, 2129-40.

[35] AB Enogieru, W Haylett, DC Hiss, S Bardien and OE Ekpo. Rutin as a potent antioxidant: Implications for neurodegenerative disorders. *Oxid. Med. Cell. Longevity* 2018; **27**, 6241017.

[36] YC Fiamegos, PL Kastritis, V Exarchou, H Han, AMJJ Bonvin, J Vervoort, K Lewis, MR Hamblin and GP Tegos. Antimicrobial and efflux pump inhibitory activity of caffeoylquinic acids from Artemisia absinthium against gram-positive pathogenic bacteria. *PLos One* 2011; **6**, e18127.

[37] J Han, QY Lv, SY Jin, TT Zhang, SX Jin, XY Li and HL Yuan. Comparison of anti-bacterial activity of three types of di-O-caffeoylquinic acids in *Lonicera japonica* flowers based on microcalorimetry. *Chin. J. Nat. Med.* 2014; **12**, 108-13.

[38] YJ Hyun, MJ Piao, KA Kang, YS Ryu, AX Zhen, SJ Cho, HK Kang, YS Koh, MJ Ahn, TH Kim and JW Hyun. 3,4-Dicaffeoylquinic acid protects human keratinocytes against environmental oxidative damage. *J. Funct. Foods* 2019; **52**, 430-41.

Supplementary Data

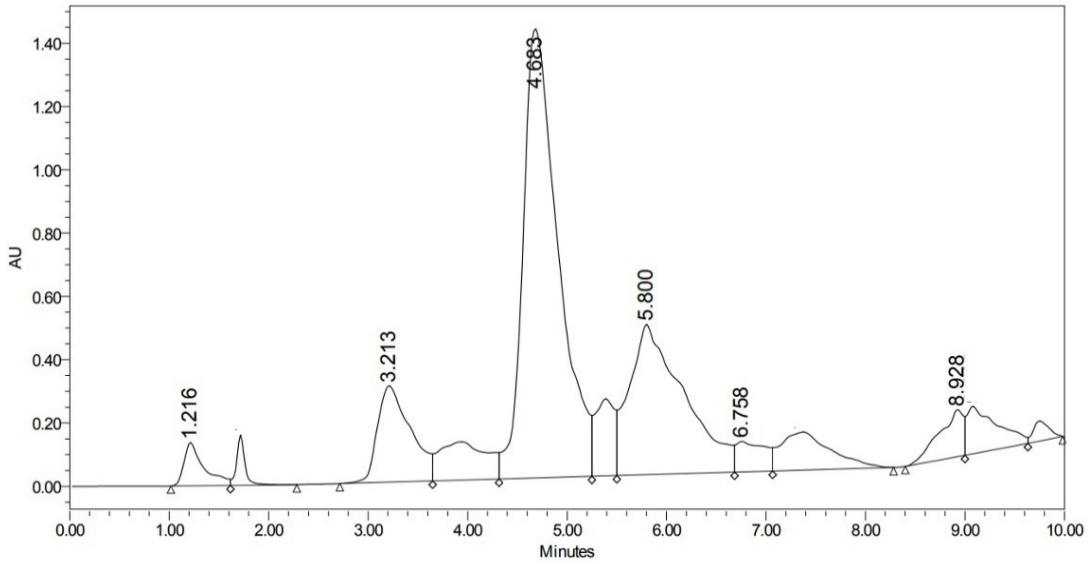

Figure S1 HPLC chromatogram of stevia residue extract. Chromatogram displaying the separation of compounds in stevia residue extract (REW) using High-Performance Liquid Chromatography (HPLC). Peaks represent different compounds identified in the extract based on their retention times (labeled as numbers over the peaks).

Table S1 Tentative Identified Compounds (1-10). Compounds tentatively identified in the stevia residue extract based on their retention time, molecular mass, and mass fragmentation pattern. Identification was conducted using High-Performance Liquid Chromatography-High-Resolution Electrospray Ionization-Mass Spectrometry (HPLC-HRESI-MS) and MS/MS analysis.

Peak No.	RT (min)	[M+H] + Observed	[M+H] + (m/z) Calculated	Molecular Formula	MS/MS	Tentative Identified Compound
1	1.216	281.5	282.2791	$C_{18}H_{35}NO$	265, 149, 247, 109, 93, 69	Oleamide derivatives
2	3.213	432.4	433.3256	$C_{21}H_{20}O_{10}$	271, 149, 85, 71	Apigenin-7-O-glucoside
3	4.683	448.2	449.1063	$C_{21}H_{20}O_{11}$	287, 303, 85	Quercetin-3-O-rhamnoside
4	5.800	516.2	517.134	$C_{25}H_{24}O_{12}$	163, 145	3,4-Dicaffeolquinic acid derivatives
5	6.758	418.2	419.3080	$C_{21}H_{22}O_{9}$	149, 167, 185, 287, 71, 85	Pinocembrin-7-O-glucoside
6	8.928	610.5	611.1530	$C_{27}H_{30}O_{16}$	303, 287, 85, 71	Rutin derivatives