

Comparative Evaluation of the Antimicrobial Effects of Xylitol, Aspartame, and Stevia and the Antibiofilm Activity of Stevia against *Escherichia coli*: An *in vitro* Study

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ABSTRACT

Background: Artificial sweeteners are widely used as non-cariogenic alternatives to dietary sugars, and beyond their sweetening properties, certain sweeteners have demonstrated antimicrobial activity, suggesting potential pharmaceutical applications. Thus, they are extensively incorporated into pharmaceutical formulations and oral healthcare products as functional excipients. *Escherichia coli*, a common Gram-negative bacterium capable of forming resilient biofilms, contributes to persistent infections and increased antimicrobial resistance. Although emerging evidence indicates that artificial sweeteners may influence bacterial growth and biofilm formation, their comparative antimicrobial efficacy and antibiofilm potential remain insufficiently characterised. **Objectives:** To evaluate the *in vitro* antimicrobial activity of xylitol, aspartame, and stevia using the agar well diffusion assay and to further investigate the antimicrobial and antibiofilm activities of stevia against *Escherichia coli* using MIC, Crystal Violet (CV), MTT, and CFU assays. **Materials and Methods:** Three sweetener-based formulations, xylitol (5 g/100 mL), stevia (0.2 g/100 mL), and aspartame (0.2 g/100 mL), were prepared in saline, stored at 4°C, and used within seven days. All formulations were initially screened for antimicrobial activity using the agar well diffusion assay. Based on the screening results, the stevia formulation was further evaluated against *Escherichia coli* using Minimum Inhibitory Concentration (MIC), Crystal Violet (CV), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Colony-Forming Unit (CFU) assays. Data were expressed as mean±SD and analysed using one-way ANOVA. **Results:** Stevia demonstrated significant antimicrobial activity, producing the largest Zones of Inhibition (ZOI) against *Escherichia coli* [32 mm] and *Staphylococcus aureus* [28 mm], moderate inhibition of *Klebsiella pneumoniae* [11 mm], and no activity against *Streptococcus mutans*. Xylitol and aspartame showed no detectable antimicrobial effects. Stevia exhibited concentration-dependent inhibition of *E. coli*, with a Minimum Inhibitory Concentration (MIC) of 175 µg/mL. Biofilm formation was reduced to approximately 10% at three times the minimum inhibitory concentration, while metabolic activity decreased by approximately 90%. Colony-Forming Unit (CFU) analysis confirmed complete bactericidal activity at the highest tested concentration. Data were analysed using IBM SPSS Statistics version 21.0. Results were expressed as mean±SD. One-way ANOVA was used to compare groups, with $p < 0.05$ considered statistically significant. **Conclusion:** Among the tested sweeteners, stevia exhibited potent and selective antimicrobial and antibiofilm activity. These findings highlight its potential as a functional sweetener with intrinsic antimicrobial properties and support its possible application as a bioactive ingredient in pharmaceutical and oral healthcare formulations. Further investigations are warranted to validate its therapeutic potential and facilitate the development of natural antimicrobial products.

Keywords: Antimicrobial agents, Artificial sweeteners, Biofilms, *Escherichia coli*, Minimum inhibitory concentration, Stevia.

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INTRODUCTION

Dental caries caused by *Streptococcus mutans* remains one of the most prevalent chronic diseases and is associated with frequent dietary sugar consumption (Shao *et al.*, 2023). Thus, the increasing demand for healthier sugar alternatives has led to the widespread incorporation of artificial sweeteners into pharmaceutical preparations and oral healthcare products (Patil *et al.*, 2023). These sweeteners serve not only as flavour-masking agents and excipients but also contribute additional biological activities that enhance product functionality.



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Unlike fermentable sugars, non-cariogenic sweeteners are not metabolised by oral microorganisms and therefore do not promote acid production, plaque accumulation, or enamel demineralisation (Chattopadhyay *et al.*, 2014). Xylitol, a naturally occurring sugar alcohol, has been extensively investigated for its ability to inhibit *S. mutans*, reduce biofilm formation, stimulate salivary flow, and support caries prevention (Liang *et al.*, 2024). Stevia, a natural sweetener derived from *Stevia rebaudiana*, contains steviol glycosides with reported antioxidant, anti-inflammatory, and antimicrobial properties, making it an attractive candidate for incorporation into pharmaceutical and oral healthcare formulations (Nascimento *et al.*, 2021). Aspartame, a low-calorie synthetic sweetener widely used in pharmaceutical syrups, chewable tablets, and sugar-free products, provides sweetness without promoting bacterial acidogenesis (Fleming *et al.*, 2025).

Evidence suggests that certain sweeteners also possess intrinsic antimicrobial and antibiofilm activities, thereby expanding their potential pharmaceutical applications. Biofilm-associated microorganisms exhibit enhanced resistance to antimicrobial agents, contributing to persistent oral and systemic infections. Consequently, identifying sweeteners with both flavouring and antimicrobial properties may facilitate the development of multifunctional pharmaceutical and oral healthcare products. Despite their widespread use, comparative evidence regarding the direct antimicrobial efficacy of xylitol, aspartame, and stevia remains limited. Furthermore, the antibiofilm activity of stevia against *Escherichia coli*, an important biofilm-forming Gram-negative pathogen, has not been comprehensively investigated.

Therefore, the present study aimed to comparatively evaluate the *in vitro* antimicrobial effects of xylitol, aspartame, and stevia against selected bacterial pathogens using the agar well diffusion assay and to further investigate the antimicrobial and antibiofilm activity of stevia against *E. coli* using Minimum Inhibitory Concentration (MIC), Crystal Violet (CV), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Colony-Forming Unit (CFU) assays.

MATERIALS AND METHODS

Before initiating the study, approval was obtained from the Institutional Scientific Research Board (Approval No.: SRB/SDC/UG-2161/25/PERIO/402). Ethical approval was not required because the study did not involve human participants, human tissues, or animal subjects.

Sample Size

As this was an *in vitro* experimental study, formal clinical sample size calculation was not applicable. Antimicrobial assays were performed in triplicate ($n=3$), whereas MIC, Crystal Violet (CV),

MTT, and Colony-Forming Unit (CFU) assays were conducted according to standard laboratory protocols.

Preparation of Sweetener-Based Test Formulations

Three sweetener-based test formulations containing xylitol, stevia, and aspartame were prepared using analytical-grade materials procured from Sisco Research Laboratories (Mumbai, India), following a previously described protocol (Prashant *et al.*, 2012).

Xylitol-based formulation

Sodium Chloride (NaCl) (0.9 g) and xylitol (5 g) were dissolved in 100 mL of distilled water at room temperature ($25\pm 2^\circ\text{C}$) using a magnetic stirrer for 10-12 min until a clear, homogeneous solution was obtained.

Stevia-based formulation

Sodium Chloride (NaCl) (0.9 g) was dissolved in 100 mL of distilled water at room temperature. Stevia powder (0.2 g) was pre-dissolved in 5 mL of warm distilled water ($40-45^\circ\text{C}$) for 3-5 min to enhance solubility, then added to the saline solution. The mixture was stirred for 8-10 min until a homogeneous formulation was obtained.

Aspartame-based formulation

Sodium Chloride (NaCl) (0.9 g) was dissolved in 100 mL of distilled water at room temperature. Aspartame (0.2 g) was dissolved separately in 5 mL of warm distilled water (approximately 40°C) for 3-4 min, then incorporated into the saline solution and stirred for 8-10 min until homogeneous.

All formulations were stored in airtight amber-coloured glass bottles at 4°C , protected from light, and used within seven days to maintain formulation stability.

In vitro Evaluation of Antimicrobial Activity

Agar Well Diffusion Assay

The antimicrobial activity of the sweetener-based formulations was evaluated using the agar well diffusion assay against *Streptococcus mutans*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli*. Standard antimicrobial agents obtained from HiMedia Laboratories Pvt. Ltd., (Mumbai, India) served as positive controls: amikacin for *S. mutans*, cotrimoxazole for *K. pneumoniae*, levofloxacin for *S. aureus*, and chloramphenicol for *E. coli*. Sterile distilled water served as the negative control.

Fresh bacterial suspensions were uniformly inoculated onto Mueller-Hinton agar plates using sterile swabs. Wells were aseptically prepared, and 100 μL of each test formulation, positive control, or negative control was dispensed into the respective wells. Following incubation at 37°C for 24 hr, the diameters of the inhibition zones were measured in millimetres, and antimicrobial

activity was assessed based on the zone of inhibition (Bharath & Priyanga, 2025; Saravanan & Jeevanandan, 2024).

Minimum Inhibitory Concentration (MIC) Assay

The Minimum Inhibitory Concentration (MIC) of the stevia formulation against *Escherichia coli* was determined using the broth microdilution method. Two-fold serial dilutions (0-200 µg/mL) were prepared in sterile nutrient broth. Each dilution was inoculated with a standardised bacterial suspension (0.5 McFarland standard; approximately 1.5×10^8 CFU/mL) and incubated at 37°C for 24 hr. Wells containing bacterial suspension without stevia served as the untreated control. Bacterial growth was assessed by visual turbidity and optical density measurement at 600 nm. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration producing at least 90% inhibition of bacterial growth (Mahale & Sharma, 2024).

Crystal Violet (CV) Biofilm Assay

The antibiofilm activity of the stevia formulation against *E. coli* was evaluated using the crystal violet assay. Standardised bacterial suspensions (0.5 McFarland) were seeded into 96-well microplates (100 µL/well) and treated with an equal volume of stevia formulation at concentrations corresponding to 0.5×, 1×, 2×, and 3× MIC. Untreated bacterial cultures served as the negative control.

Following incubation at 37°C for 24 hr, non-adherent cells were removed by washing with Phosphate-Buffered Saline (PBS). The remaining biofilm was stained with 0.1% crystal violet for 15 min. Excess stain was removed, and the bound dye was solubilised with 30% acetic acid. Absorbance was measured at 570 nm, and biofilm biomass was expressed relative to the untreated control (Corte *et al.*, 2019).

MTT Assay for Bacterial Viability

The effect of the stevia formulation on the metabolic activity of *E. coli* biofilms was determined using the MTT assay. The experimental procedure was identical to that used for the crystal violet assay, with untreated bacterial cultures serving as the negative control. Following incubation, 10 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated at 37°C for an additional 4 hr. The resulting formazan crystals were dissolved in 100 µL of Dimethyl Sulfoxide (DMSO), and absorbance was recorded at 570 nm. The percentage inhibition of bacterial metabolic activity was calculated relative to the untreated control (Elanthendral, 2024).

Colony-Forming Unit (CFU) Assay

The bactericidal activity of the stevia formulation against *E. coli* was evaluated using the Colony-Forming Unit (CFU) assay. Bacterial cultures were treated with stevia at concentrations corresponding to 0.5×, 1×, 2×, and 3× MIC, while untreated bacterial cultures

served as the negative control. Following incubation at 37°C for 24 hr with shaking, cultures were serially diluted and plated onto nutrient agar. After a further 24-hr incubation at 37°C, visible colonies were counted manually. Results were expressed as CFU/mL and compared with the untreated control.

Statistical Analysis

Data were analysed using IBM SPSS Statistics version 21.0. Results were expressed as mean±Standard Deviation (SD). Differences in the mean zones of inhibition among the experimental groups were analysed using One-Way Analysis of Variance (ANOVA), with $p < 0.05$ considered statistically significant.

RESULTS

Antimicrobial Activity

The antimicrobial activity of the three sweetener-based formulations was evaluated against *Streptococcus mutans*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli* using the agar well diffusion assay (Figure 1). Among the formulations tested, only the stevia-based formulation demonstrated measurable antimicrobial activity, producing the largest zones of inhibition against *E. coli* (32 mm) and *S. aureus* (28 mm), moderate inhibition against *K. pneumoniae* (11 mm), and no detectable activity against *S. mutans*. In contrast, the xylitol- and aspartame-based formulations exhibited no measurable antimicrobial activity under the experimental conditions. The Zone of Inhibition (ZOI) measurements, together with the corresponding mean values, standard deviations, and p -values, are presented in Table 1.

Minimum Inhibitory Concentration (MIC)

The stevia-based formulation exhibited concentration-dependent antibacterial activity against *E. coli*. Lower concentrations (5-25 µg/mL) produced minimal bacterial growth inhibition (7-45%), whereas moderate inhibition was observed at 50 and 100 µg/mL (65-70%). At 150 µg/mL, bacterial growth inhibition increased to approximately 88%, while concentrations of 175 and 200 µg/mL achieved ≥90% inhibition.

Based on these findings, the Minimum Inhibitory Concentration (MIC) of the stevia-based formulation against *E. coli* was determined to be 175 µg/mL, indicating effective antibacterial activity at higher concentrations (Figure 2).

Crystal Violet (CV) Biofilm Assay

The crystal violet assay demonstrated a concentration-dependent reduction in *E. coli* biofilm biomass following treatment with the stevia-based formulation. Biofilm formation remained relatively high at 0.5× MIC (approximately 85%), decreased to approximately 50% at 1× MIC, and was further reduced to approximately 30% at 2× MIC. The greatest antibiofilm activity was observed at 3× MIC, where residual biofilm biomass was

Table 1: Antimicrobial activity of Xylitol (XY), Stevia (ST), and Aspartame (AS) against selected oral pathogens. (mean±SD, n=3). ANOVA and F-values were calculated where variance existed; for *Streptococcus mutans*, all sweetener groups showed zero inhibition, so statistical analysis was not possible.

Microorganism	XY (mm)	ST (mm)	AS (mm)	Antibiotic (mm)	p value	F value
<i>S. mutans</i>	0±0	0±0	0±0	18±1	-	-
<i>K. pneumoniae</i>	0±0	11±0.2	0±0	26±1	0.0010	1800.990
<i>S. aureus</i>	0±0	28±0.5	0±0	26±0.5	0.0080	5848.000
<i>E. coli</i>	0±0	32±0.5	0±0	23±0.2	0.0002	12257.692

Statistical test applied: One-way ANOVA; p-value of <0.05 was considered statistically significant.

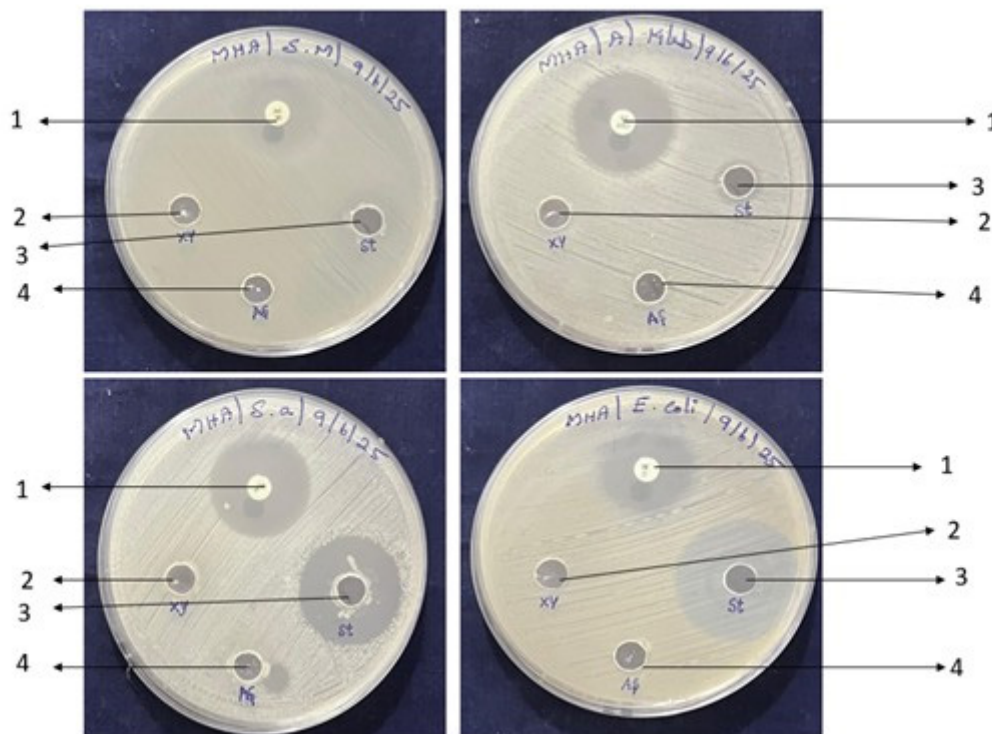


Figure 1: Agar well diffusion assay demonstrating the antimicrobial activity of Xylitol (XY), Stevia (ST), and Aspartame (AS) against four bacterial strains: *Streptococcus mutans* (S.m), *Klebsiella pneumoniae* (K.p), *Staphylococcus aureus* (S.a), and *Escherichia coli* (E. coli). Wells: 1-antibiotic control; 2- xylitol; 3- stevia; 4- aspartame.

reduced to nearly 10% (Figure 3). These findings demonstrate the potent concentration-dependent antibiofilm activity of the stevia-based formulation.

MTT Assay

Treatment with the stevia-based formulation produced a concentration-dependent reduction in the metabolic activity of *E. coli* biofilms (Figure 4). At 0.5× MIC, bacterial viability remained high, with approximately 18% inhibition. Increasing the concentration to 1× MIC resulted in moderate inhibition (approximately 35%), whereas treatment at 2× MIC produced a marked reduction in metabolic activity (approximately 70%). The highest concentration tested (3× MIC) exhibited the greatest antibacterial effect, reducing bacterial viability by approximately 90%.

These findings indicate that the stevia-based formulation effectively suppressed bacterial metabolic activity in a dose-dependent manner and are consistent with the MIC and biofilm assay results.

Colony-Forming Unit (CFU) Assay

The CFU assay demonstrated a clear concentration-dependent bactericidal effect of the stevia-based formulation against *E. coli* (Figure 5). The untreated control and 0.5× MIC groups showed bacterial growth that was too numerous to Count (TNTC), indicating that the lowest concentration did not produce a measurable antibacterial effect.

At 1× MIC, the viable bacterial count decreased to 157 CFU, indicating the onset of antibacterial activity. Further reduction to 47 CFU was observed at 2× MIC, demonstrating enhanced

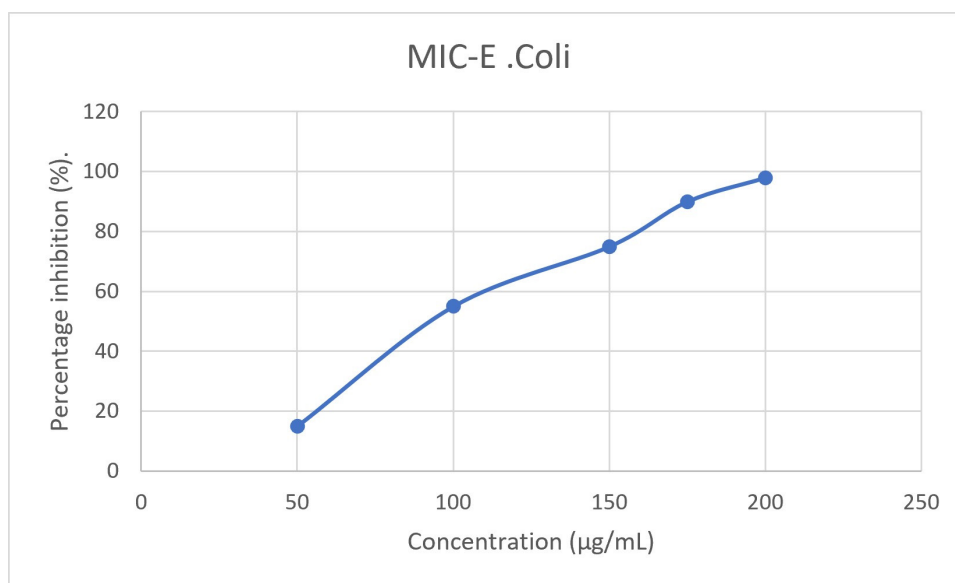


Figure 2: Minimum Inhibitory Concentration evaluation of stevia extract against *Escherichia coli*. The X-axis shows stevia extract concentration (µg/mL), and the Y-axis indicates percentage inhibition of bacterial growth (%). The MIC was recorded at 175 µg/mL.

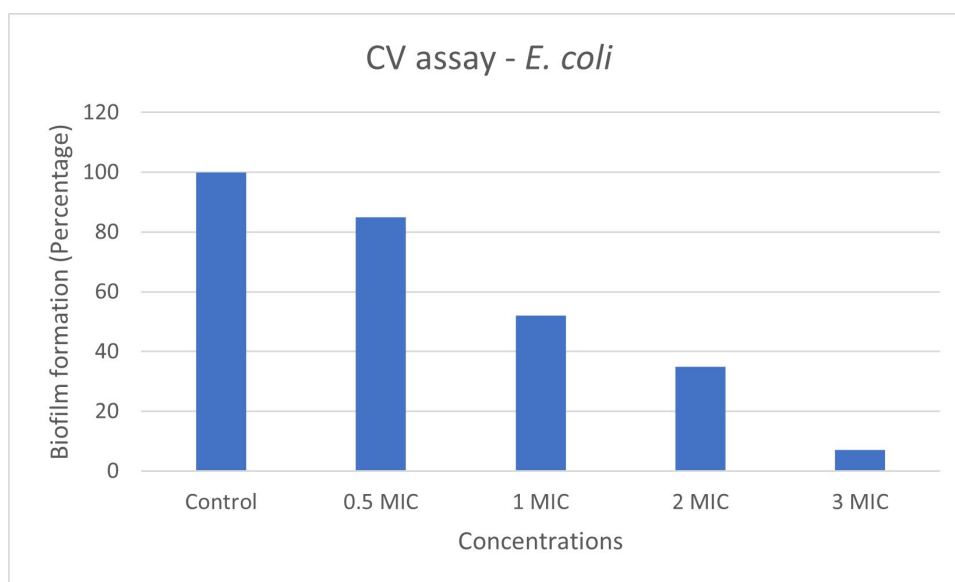


Figure 3: CV assay results of stevia extract against *E. coli*. X-axis: treatment concentrations (MIC); Y-axis: biofilm formation (%).

bactericidal activity with increasing concentration. Complete inhibition of bacterial growth (0 CFU) was achieved at 3× MIC, confirming complete bactericidal activity at the highest concentration evaluated.

Overall, the stevia-based formulation demonstrated potent concentration-dependent antibacterial activity against *E. coli*, with complete bactericidal activity achieved at 3× MIC.

DISCUSSION

Artificial sweeteners are extensively incorporated into foods, pharmaceutical preparations, and oral healthcare products as non-cariogenic alternatives to sucrose (Chattopadhyay *et al.*,

2014). Beyond their primary function as sweetening agents, sweeteners possessing intrinsic biological activities are of particular interest because they enhance the functionality of pharmaceutical and oral healthcare formulations while reducing the risk of microbial colonisation (Mazurel *et al.*, 2025).

Although xylitol, aspartame, and stevia are widely used, their direct antimicrobial activities have not been comprehensively compared under standardised experimental conditions (Myllykangas *et al.*, 2020). Existing reports describing the antibacterial and antibiofilm properties of these sweeteners vary considerably with respect to bacterial species, experimental design, and concentrations employed. Moreover, evidence

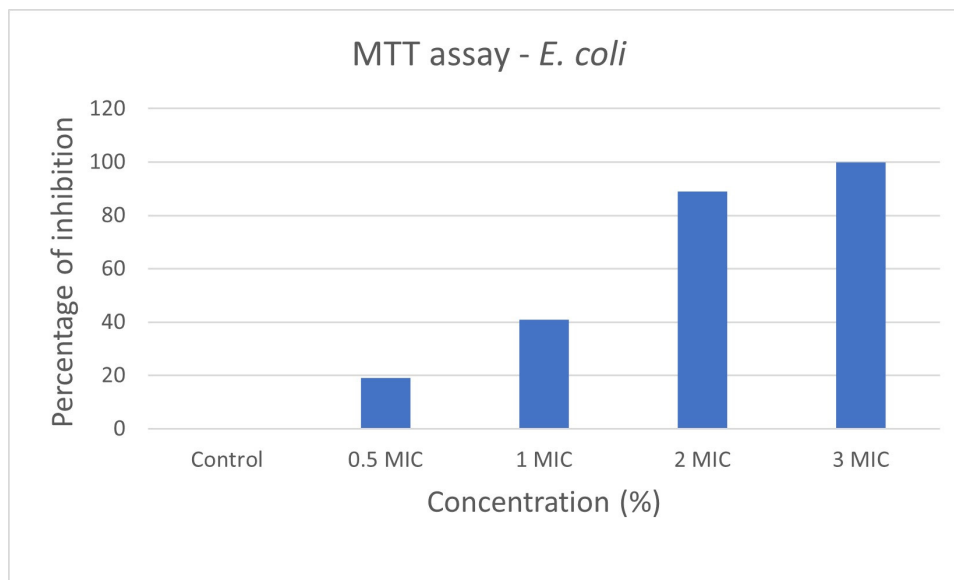


Figure 4: Effect of stevia extract on *Escherichia coli* viability assessed by MTT assay. The X-axis represents increasing concentrations of stevia extract (MIC), while the Y-axis shows the percentage of inhibition relative to the control (%).

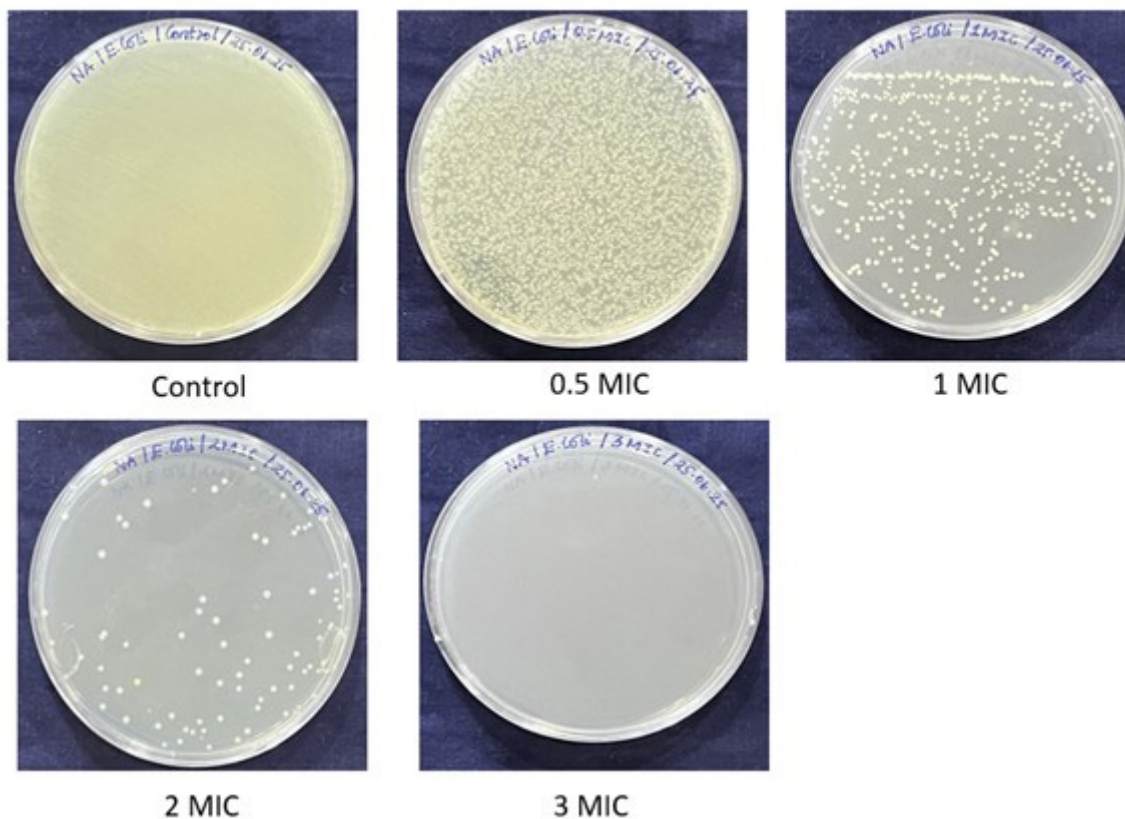


Figure 5: Colony-Forming Unit (CFU) assay of *E. coli* treated with stevia at different MIC concentrations (0.5, 1, 2, 3) and control.

supporting antimicrobial activity for aspartame remains limited. Therefore, comparative evaluation is important to identify sweeteners with potential pharmaceutical applications beyond their conventional role as sweetening agents.

In the present study, only stevia demonstrated measurable antimicrobial activity, exhibiting the greatest inhibition against *Escherichia coli* and *Staphylococcus aureus*, moderate inhibition against *Klebsiella pneumoniae*, and no detectable activity against

Streptococcus mutans. In contrast, xylitol and aspartame showed no direct antibacterial effects under the experimental conditions used. These findings indicate that antimicrobial activity is not a universal characteristic of artificial sweeteners and highlight the distinct pharmacological potential of stevia. Similar findings have been reported previously, where steviol glycosides inhibited bacterial growth, reduced microbial survival, and suppressed biofilm formation (Yang *et al.*, 2023).

The MIC assay further demonstrated concentration-dependent antibacterial activity of stevia against *E. coli*, with a minimum inhibitory concentration of 175 µg/mL. Increasing concentrations produced progressively greater bacterial inhibition, consistent with previous studies reporting the antimicrobial activity of stevia-derived compounds against oral and non-oral microorganisms, including *Streptococcus sanguinis* (Al Bayan *et al.*, 2025). These observations suggest that the bioactive phytochemicals present in *Stevia rebaudiana* may contribute to its antimicrobial efficacy.

Evaluation of antibiofilm activity using crystal violet, MTT, and CFU assays consistently demonstrated concentration-dependent inhibition of *E. coli* biofilms. Stevia significantly reduced biofilm biomass, suppressed bacterial metabolic activity, and eliminated viable bacterial colonies at the highest concentration tested. The agreement among these complementary assays strengthens the evidence that stevia exhibits both antibacterial and antibiofilm properties, which are desirable characteristics for bioactive pharmaceutical ingredients and functional excipients intended for oral healthcare applications.

Previous investigations have similarly reported antimicrobial activity of stevia against microorganisms including *Streptococcus mutans*, *Lactobacillus acidophilus*, *Streptococcus gordonii*, *Staphylococcus aureus*, and *Escherichia coli* (Chen *et al.*, 2024; Deviyanti, 2022). In addition, stevia has been shown to reduce bacterial adherence and inhibit biofilm development while maintaining a non-acidogenic environment (AlKanderi *et al.*, 2023; Singh & Sharma, 2024). Although the underlying molecular mechanisms were not investigated in the present study, these findings collectively support the growing pharmacological evidence for stevia as a naturally derived antimicrobial agent with potential applications in pharmaceutical and oral healthcare formulations.

The selective antimicrobial activity observed in the present study indicates that stevia may exhibit organism-specific effects rather than broad-spectrum antibacterial activity (Markus *et al.*, 2020). Consequently, its greatest potential may lie in its incorporation as a multifunctional bioactive ingredient within pharmaceutical formulations, oral care products, and preventive healthcare

preparations, where antimicrobial activity can complement its established sweetening function.

The present study was limited by its *in vitro* design, evaluation of selected bacterial species, and assessment at a single experimental time point. Furthermore, mechanistic investigations were not performed to elucidate the molecular pathways underlying the observed antimicrobial effects. Future studies should include mechanistic analyses, broader microbial evaluation, cytocompatibility assessments, formulation optimisation, stability studies, and *in vivo* validation to facilitate the translation of stevia into pharmaceutical and oral healthcare products.

CONCLUSION

Among the three sweeteners evaluated, stevia exhibited significant concentration-dependent antimicrobial and antibiofilm activity, whereas xylitol and aspartame demonstrated no measurable antibacterial effects under the experimental conditions employed. Stevia effectively inhibited *Escherichia coli* growth, reduced biofilm biomass, suppressed bacterial metabolic activity, and eliminated viable bacterial colonies, with a minimum inhibitory concentration of 175 µg/mL. These findings demonstrate that stevia possesses bioactive properties beyond its conventional sweetening function and highlight its potential as a naturally derived antimicrobial ingredient for pharmaceutical and oral healthcare formulations. Further pharmacological investigations, formulation development, and *in vivo* studies are warranted to establish its therapeutic efficacy, safety, and clinical applicability.

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ABBREVIATIONS

MIC: Minimum Inhibitory Concentration; **CV:** Crystal Violet; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide; **CFU:** Colony-forming unit; **NaCl:** Sodium Chloride; **PBS:** Phosphate-Buffered Saline; **DMSO:** Dimethyl Sulfoxide; **ZOI:** Zone of Inhibition; **TNTC:** Too Numerous to Count.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Dr. Varsha Mugundhan: Methodology, Data curation, Writing - original draft. **Dr. Priyanga Ponnudi Thangavel:** Conceptualisation, Supervision, Validation, Writing - review & editing.

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