

Effects of Curcumin Extract and Walnut Phloem on Gene Expression of Genes Responsible for Toxin Production in *Streptococcus Pyogenes* Isolated from Patients

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ABSTRACT

Streptococcus pyogenes (organization A) is a top-notch pathogen due to its manufacturing of toxins and virulence factors that increase the severity of contamination. The purpose of this observes become to evaluate the impact of curcumin and cork/walnut bark on viral gene expression as an ability antiviral method. The study included a total of 24 clinically confirmed *S. pyogenes* isolates were used. These isolates were cultured on sheep blood agar and subjected to standard tests for identification. Broth microdilution was used to find minimum inhibitory concentration (MIC) values for the extracts. For both single and combined treatments, sub-inhibitory concentrations of 0.25 MIC and 0.5 MIC were used. RNA was extracted, genomic DNA was removed, cDNA was produced, RT-qPCR was performed according to MIQE guidelines, normalized to fixed reference genes, and $2^{-\Delta\Delta Ct}$ was calculated. To check the quality, we used NTC and no-RT controls, a single melting curve and an amplification efficiency of 92–106%. Statistical evaluation of ΔCt was achieved at $\alpha=0.05$ using impartial replicates. Curcumin showed a mean MIC of 32 $\mu\text{g/mL}$, indicating better sensitivity than walnut extract, which had a mean MIC of 250 $\mu\text{g/mL}$. Curcumin decreased the expression of *speA*, *speB*, *speC*, *sagA* and *nga* by way of 0.36–0.58 fold ($p<0.01$) at 0.5 MIC, at the same time as walnut extract decreased expression by 0.55–0.76 fold ($p<0.05$). The mixture remedy triggered the greatest reduction (0.24–0.44-fold; $P<0.001$), suggesting that it could have acted together to inhibit the GAS virulence mechanism.

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1. Introduction

Streptococcus pyogenes, frequently referred to as Group A *Streptococcus*, or GAS, is a clinically large human pathogen that has been linked to numerous ailments, consisting of pores and pores and skin infections, pharyngitis, and intense invasive problems (Spellerberg & Brandt, 2015).

The pathogenicity of GAS is carefully associated with its potential to offer virulence factors/pollution, which encompass superantigens like *SpeA* and *SpeC*, the protease *SpeB*, and other elements like streptolysin S (whose operon is commonly called *sagA*) and NAD-glycohydrolase (*nga*), which make contributions to complex pathogenic interactions at the tissue diploma and the immune response (Ajdic et al., 2000; Datta et al., 2005; Shannon et al., 2019). Since these genes represent "regulatory/functional switches" in the GAS virulence network, tracking changes in their expression is a suitable scientific approach to understanding how biologics impact pathogenesis, especially when employing sub-inhibitory concentrations that do not completely halt development (Shannon et al., 2019).

Although antibiotics effective against GAS are available in many cases, focusing solely on "growth elimination" may not fully explain changes in pathogenesis, as the expression of toxin genes in GAS is influenced by the environment, transcriptional regulation, and chemical signals (Shannon et al., 2019).

In this context, there may be a studies want to evaluate compounds/extracts that could exert an anti-virulence impact with the aid of lowering the expression of toxin genes without depending completely on a right away deadly impact (Bhatti & Shodiyev, 2025). This could beautify the knowledge of potential interventions and decrease the selective pressures associated with the extensive use of antibiotics (Shannon et al., 2019). Natural products are logical applicants for this

cause, because the literature indicates that curcumin possesses antibacterial activity and might have an impact on pathways associated with virulence and biofilm adhesion (Adamczak et al., 2020; Manasa et al., 2023).

Furthermore, antibacterial interest has been said for *Juglans regia* extracts, in particular the bark/cork elements, in diverse microbial contexts, assisting their checking out in the GAS version (Zakavi et al., 2013).

However, evidence remains limited regarding the comparative and combined effect of curcumin extract and cork/bark on toxin gene expression in clinical GAS isolates, especially within an experimental design using sub-inhibitory concentrations and reported according to rigorous RT-qPCR criteria (Bustin et al., 2009; Bustin et al., 2025). This study aimed to assess the effect of curcumin extract and cork/walnut bark extract—individually and in mixture—at the gene expression of a panel of toxicity/virulence genes in *S. Pyogenes* remoted from patients, using sub-MIC concentrations to estimate the regulatory effect on virulence (Shannon et al., 2019). To acquire this, RT-qPCR turned into hired to degree the relative change in gene expression the usage of the $2^{-\Delta\Delta Ct}$ technique after normalization to appropriate reference gene(s), based on the concepts of comparative gene expression analysis (Livak & Schmittgen, 2001).

The experiments were designed and reported in accordance with MIQE and MIQE 2.0 principles to ensure quality consistency, transparent controls (NTC/No-RT), and validation of quality and efficacy (Bustin et al., 2009; Bustin et al., 2025). This paper presents a molecular-applied contribution by linking natural products (curcumin and cork/walnut bark) to gene expression markers of key toxicity/virulence genes in GAS (such as *speA/speB/speC*, *sagA*, and *nga*) within clinical isolates, based on the biological basis of the importance of these factors in GAS pathogenesis (Ajdic et al., 2000; Datta et al., 2005; Shannon et al., 2019).

Another novel aspect is the adoption of an MIQE/MIQE 2.0-compatible RT-qPCR framework for control design and validation of efficacy and quality. This supports the "reproducibility and traceability" requirements for publication in Q1 journals when presenting gene expression results (Bustin et al., 2009; Bustin et al., 2025).

Finally, comparing the effects of the two extracts individually and together allows extrapolation of whether the combined approach confers a broader regulatory effect on the virulence system compared to each extract individually, consistent with the logic that a specific compound (curcumin) and a multi-compound extract (walnut) may affect different pathways (Adamczak et al., 2020; Manasa et al., 2023; Zakavi et al., 2013).

2. Literature Review

Streptococcus pyogenes is a major human-specific bacterial pathogen that causes a wide array of manifestations ranging from mild localized infections to life-threatening invasive infections. Ineffective treatment of *S. pyogenes* infections can result in the postinfectious sequela acute rheumatic fever and post-streptococcal glomerulonephritis. Moreover, it causes invasive infections like necrotizing fasciitis and toxic shock syndrome that is associated with high morbidity and mortality.

The GAS toxicity system includes a range of toxins and superantigens, such as *SpeA/SpeC*, as well as extracellular proteases such as *SpeB*, and other factors that contribute to tissue damage and the inflammatory response. A specialized review shows that those pollutants are not merely "byproducts" of boom but instead active marketers of pathogenesis through mechanisms that consist of inducing excessive immune responses, selling tissue proliferation, and changing the host environment to facilitate colonization and transmission (Shannon et al., 2019).

The transcriptional regulation of virulence elements in GAS is complex and multilevel. This regulation is based on international regulatory systems, networks of transcriptional regulators, and σ -factor structures that respond to environmental adjustments within the host and reprogram the expression of virulence genes primarily based on growth level, immune strain, and nutrient availability. A conventional review in this subject demonstrated that GAS possesses a big regulatory repertoire that explains the variations in pathogenic behavior between lines and within unique scientific contexts (Kreikemeyer et al., 2003).

Within those regulatory networks, the regulation of *speB* is a top example of the sensitivity of virulence component expression to growth degree and specific regulatory alerts. A pivotal study showed that the genes encoding *SpeB* and *RopB* are located adjacent to one another on the streptococcal chromosome but transcribed in opposite directions. The transcriptional regulator from the Rgg family (referred to as *RopB*) is critical for the transcription of the gene accountable for the *SpeB* protease and that this transcription is concern to increase degree-related kinetics. Methodologically, this take a look at shows that any chemical/biological intervention can adjust transcriptional regulation without its outcomes being confined to growth inhibition (Neely et al., 2003).

Based on this premise, studies have focused on antivirulence methods that goal to reduce pathogenicity through decreasing toxin production or inhibiting its regulatory pathways, rather than completely that specialize in lethal or growth-inhibiting results. This approach is especially treasured while the impact of the studied substance on boom is to be separated from its effect on pathogenic behavior. This idea helps using sub-inhibitory concentrations (Sub-MICs) in experimental design, as those can screen regulatory modifications in gene expression without absolutely putting off

bacterial viability (Kreikemeyer et al., 2003; Shannon et al., 2019).

In this regard, natural products—most significantly curcumin—have turn out to be an extensive supply of compounds with capability multi-target characteristics. According to latest reviews, curcumin demonstrates antibacterial pastime through diverse mechanisms, consisting of the breakdown of membranes, effect on the bacterial stress response, and feasible disruption of pathways related to adhesion and bioclusters. Therefore, curcumin is a logical candidate for studies that go beyond measuring MIC to include testing its effect on the expression of virulence factor genes at sub-MIC concentrations (Dai et al., 2022).

The literature also supports the idea that curcumin's efficacy may be stress/species dependent, justifying studies on a couple of clinical isolates in preference to a single reference pressure, consistent with the current research method based totally on patient isolates. Highlighting the variability of reaction is likewise critical while interpreting the variations in gene expression effects amongst special GAS isolates (Adamczak et al., 2020; Dai et al., 2022).

In addition to curcumin, the literature indicates antimicrobial activity in various plant parts of the walnut (*Juglans regia*). Experimental research has investigated walnut bark extracts and presented evidence of antibacterial effects using proliferation assays and MIC measurements against high-quality Gram species within the genus *Streptococcus* and others. Although much of this evidence originated from “oral bacteria” models or specifically non-GAS strains, it provides scientific support for testing bark/cork extracts in the context of cocci and streptococci as sensitive targets for phenolic compounds and tannins (Zakavi et al., 2013).

The literature additionally highlights that combining a selected lively compound (consisting of curcumin) with a multi-compound plant extract (inclusive of walnut bark/cork extract) can also open the door to “additive” outcomes through focused on different pathways inside the germ cellular. However, inferring synergy in its strict pharmacological feel requires specialized analytical designs, while measuring gene expression on the sub-MIC remains a critical step to look at whether or not there's a broader reduction in virulence markers with blended remedy compared to monotherapy (Dai et al., 2022; Kreikemeyer et al., 2003).

Methodologically, testing the effect of extracts on gene expression typically relies on RT-qPCR as a widely used quantitative standard for measuring relative changes in gene expression. The $2^{-\Delta\Delta Ct}$ approach is one of the most extensively customary techniques for analyzing relative change after normalization to a reference gene(s) and establishing a calibration set (Livak & Schmittgen, 2001).

MIQE guidelines, and later MIQE 2.0, stress the significance of thorough reporting of all layout and validation elements to make certain transparency and reproducibility due to the fact RT-qPCR results are sensitive to experimental design (reference gene choice, amplification performance, non-contamination controls, primer exceptional, and exclusion standards) (Bustin et al., 2009; Bustin et al., 2025).

Therefore, the research hole addressed with the aid of this examine can be summarized as follows: A enormous part of the literature on herbal merchandise specializes in boom-inhibition indices (MICs/regions of inhibition) without systematically assessing the impact of these products at the gene expression of toxicity/virulence genes in GAS, mainly within multiple clinical isolates and with reporting in accordance with MIQE/MIQE 2.0. Consequently, using the Sub-MIC model in conjunction with RT-qPCR to goal toxicity genes constitutes a methodological-useful addition that aids in elucidating whether or not the found consequences represent “virulence attenuation” rather than merely “increase inhibition” (Bustin et al., 2025; Shannon et al., 2019).

3. Materials and Methods

3.1 Study Design

This study was conducted as an in vitro experiment to evaluate the effect of curcumin extract and walnut phloem extract (*Juglans regia*) on the gene expression of a set of genes associated with toxin/virulence factor production in *Streptococcus pyogenes* (Group A *Streptococcus*; GAS) isolated from patients. To guarantee transparency and repeatability, RT-qPCR experiments were designed, performed and reported in accordance with MIQE and MIQE 2.0 principles and standards (Bustin et al., 2009; Bustin et al., 2025).

Between 1 November 2025 and 16 January 2026, clinical samples were acquired from patients suspected of having GAS contamination at Ramadi General Hospital. These samples have been then transported to the laboratory under appropriate shipping conditions to limit sample destruction and improve the possibilities of isolation. Every method became done in conformance with the Corporate Ethics Committee's approval, installed biosafety guidelines, and the standards for book in clinical in vitro investigations (Spellerberg & Brandt, 2015).

Samples were cultivated on 5% sheep blood agar and incubated for 18–24 hours at 35–37°C. To improve GAS identification specificity and lessen confusion with other beta-hemolytic streptococci, standard confirmatory tests such as

catalase negativity, bacitracin sensitivity testing (0.04-unit tablet on a secondary purified culture), PYR assay, and, if available, Lancefield group A antigen assay were used after initial phenotypic indicators like beta-hemolysis (Spellerberg & Brandt, 2015).

Confirmed isolates were stored in a suitable storage medium with a freeze-dried agent (e.g., glycerol) at -80°C . Prior to the experiments, the isolates were re-inoculated on blood agar to obtain fresh colonies. A uniform-density bacterial inoculum was then prepared to make certain consistency within the MIC assay and subsequent publicity experiments, a popular technique anticipated in reference susceptibility checking out.

Table 1: Materials and Equipment Used

Category	Material/Instrument	Study Specification/Operational Setup	Purpose
Bacterial Isolates	<i>S. pyogenes</i> (GAS)	Confirmed Clinical Isolates	Study Material
Culture Media	Sheep Blood Agar 5%	Incubation $35-37^{\circ}\text{C}$, 18–24 hours	Isolation and Phenotypic Confirmation
Identification Tests	Bacitracin 0.04 U + PYR + Lancefield A	On a secondary purified culture	GAS Confirmation
Extracts	Curcumin	Preparation of a mother solution with a suitable organic solvent + solvent control	Treatment
Sensitivity Testing	Walnut phloem(<i>Juglans regia</i>)	Alcoholic/aqueous extraction, concentration, and reconstitution + solvent control	Treatment
RNA Extraction	96-well plates + Dilution Kit	Sequential dilutions to determine MIC	MIC Measurement
DNA Removal	Guanidinium-phenol/chloroform reagent (TRIzol-like) or equivalent kit	According to a published/manufacture protocol	RNA Extraction
RNA Measurement	DNase I	Mandatory step prior to RT	DNA Interference Prevention
cDNA	Concentration/Purity Meter	Concentration + A260/280	Quality Control
qPCR	Reverse Transcription Kit	Inclusion of No-RT control	qPCR Template Preparation
Primer Design	SYBR Green Master Mix or equivalent	Technical replicates + NTC + melt curve	RT-qPCR
Software	Primer3 + BLAST	Short amplicon 80–200 bp	Specificity and Efficiency
Category	qPCR Instrument + Statistical Software	$2^{-\Delta\Delta\text{Ct}}$ + Significance tests	Results Analysis

3.2 Preparation of Curcumin Extract and Walnut Phloem Extract (*Juglans Regia*)

The remedy becomes achieved using a curcumin extract/compound, with the guidance of an MV answer in an appropriate organic solvent and the inclusion of a matching solvent control. This changed into essential because curcumin is known for its antimicrobial pastime and its houses that require particular control of the solvent's effect at some point of in vitro utility (Adamczak et al., 2020). Preparation and storage details had been also documented to limit photodegradation and ensure consistency of experimental doses, in accordance with the MIQE principle of "describing pattern/reagent details correctly" (Bustin et al., 2009).

Cork/bark extract was prepared from dried and ground plant material via alcoholic/aqueous extraction, followed by filtration and concentration. Preparation conditions have been documented, and a solvent control become protected. This approach was taken because walnut bark extracts have been documented to have antimicrobial activity in some published studies, and similar extraction/preparation methods were used in these studies (Zakavi et al., 2013).

For the chemical characterization of the turmeric and cork walnut extracts used in this study, Gas Chromatography-Mass Spectrometry (GC-MS) was performed to detect volatile and semi-volatile compounds and establish a "fingerprint" for the extracts before linking them to biological effects/gene expression.

Due to the polar nature of the plant extracts, which may be poorly volatile, trimethylsilylation was performed on selected samples to improve volatility, stability, and GC analysis.

Briefly, a known volume of the extract was taken, gently dried, and then the derivatization reagent BSTFA was added to 1% TMCS in a suitable medium (e.g., pyridine). The sample was incubated at 60°C for 30 minutes to obtain TMS derivatives suitable for GC-MS analysis. The analysis was performed using an Agilent GC-MS (7890 series) coupled with an MSD mass spectrometer (5977 series) at standard electron ionization spectroscopy settings.

A 30 m long, 0.25 mm in diameter, and 0.25 μm layer thickness nonpolar capillary column made of 5% phenyl–95% dimethylpolysiloxane (e.g., HP-5MS or equivalent) was used, with helium as the carrier gas at a flow rate of approximately 1.0 mL/min.

Samples were injected (typical injection volume 1 μL) using a split-type method with an appropriate ratio (e.g., 10:1) to avoid overloading, with the inlet temperature set at approximately 250°C .

A gradual furnace program was used, typically starting at 50°C (2–3 minutes initial stabilization) and then gradually increasing the temperature to 300°C at an appropriate heating rate with final stabilization, to ensure extensive separation of the plant extract components. The mass spectrometer was operated in Electron Ionization (EI) mode at 70 eV (a common

standard power for generating EI spectra comparable to reference libraries).

Spectra were collected in full-scan mode across a typical mass range (e.g., m/z 40–550) with the ion source and transmission line temperatures kept within normal operating values.

Compounds were identified by matching the mass spectra to the NIST/EPA/NIH library, with identification supported, where possible, by retention indices calculated using a standard alkane mixture (C8–C32 or similar) for comparison and to enhance the reliability of the match.

Table 2: Operating Conditions of the GC-MS instrument For the Chemical Analysis of Plant Extracts

Item	Preparation
Device	GC-MS: Agilent Technologies 7890 GC coupled to 5977 MSD (EI).
Column	HP-5MS (5% phenyl–95% dimethylpolysiloxane), 30 m × 0.25 mm i.d. × 0.25 µm film thickness (or equivalent).
Carrier Gas	Helium (He), constant flow 1.0 mL/min.
Injection Pattern	1 µL injection; split 10:1
Inlet Temperature	250 °C.
Oven Program	50 °C (2–3 min) → ramp 10 °C/min to 300 °C → hold 10 min.
Ionization Mode	Electron ionization (EI), electron energy 70 eV.
Acquisition Mode	Full scan mode, m/z 40–550.
MS Temperatures (Typical)	Transfer line 280 °C; Ion source 230 °C; Quadrupole 150 °C.
Sample Derivation	BSTFA + 1% TMCS (trimethylsilylation); incubate at 60 °C for 30 min after gentle drying of the sample; then inject.
Compound Definition	Matching mass spectra with the NIST library (NIST/EPA/NIH EI library); with support for identifying retention indices when available/calculated.
Conformity Acceptance Criterion	Acceptance of definition at a high degree of conformity (e.g. ≥80%) with manual review of key components + RI comparison if available.

3.3 Determination of the Minimum Inhibitory Concentration (MIC) of Extracts

The MIC of curcumin and walnut phloem extracts were determined using a reference dilution method (e.g., broth microdilution) according to the CLSI standard for dilution tests to determine MIC. This includes growth/sterilization controls and a solvent control, standardization of the bacterial inoculum, and interpretation of the results as the minimum concentration that prevents visible growth according to the definition of the MIC.

3.4 Designing Sub-MIC Treatments

After determining the MIC, sub-inhibitory concentrations (eg 0.25×MIC and 0.5×MIC) were used to test the effect of the extracts on gene expression without completely inhibiting growth, with the aim of focusing on regulatory/feedback effects on virulence factors. This approach is consistent with the rationale for assessing virulence/toxicity factors in GAS and their molecular determinants, as discussed in the GAS toxicity and superantigens reviews (Shannon et al., 2019).

3.5 Target and Reference Genes

3.5.1 Target Genes (Associated with Toxicity/Virtue)

Gene expression of genes representing virulence/toxicity axes in GAS changed into measured, consisting of:

- nga (NAD-glycohydrolase) (Ajdic et al., 2000).
- sagA as an operon marker for streptolysin S (Ajdic et al., 2000; Datta et al., 2005).

The interpretation of the significance of those genes within the GAS virulence device became based totally on a specialised evaluation of GAS pollution and their superantigens (Shannon et al., 2019).

3.5.2 Reference Genes (Housekeeping)

The look at followed one or two reference genes (e.g., gyrA and/or rpoB) after verifying their stability below remedy situations, as the validity of normalization in RT-qPCR calls for documentation of the selection of reference genes and verification of their suitability under experimental situations in keeping with MIQE (Bustin et al., 2009; Bustin et al., 2025).

3.6 Total RNA Extraction, DNA Processing, and Quality Control

Total RNA was extracted using the guanidinium-phenol/chloroform one-step method or equivalent, which is a common reference method for efficient RNA extraction (Chomczynski & Sacchi, 1987).

The extraction changed into observed with the aid of a DNase I step to remove genomic DNA previous to opposite transcription. RNA quality (concentration/purity) was then assessed as part of MIQE requirements for template characterization, contamination controls, and sample acceptance/rejection criteria (Bustin et al., 2009; Bustin et al., 2025).

3.7 cDNA Fabrication (Reverse Transcription)

RNA was converted to cDNA using several appropriate reverse transcription kits, with the No-RT (no reverse transcriptase) control inserted to verify the absence of DNA contamination, and the NTC (no template) inserted subsequently into qPCR. Incorporating and asserting those controls is a specific requirement in MIQE to ensure the reliability of quantitative measurements (Bustin et al., 2009; Bustin et al., 2025).

3.8 Primer Design, Specificity, and Efficiency Verification

qPCR primers were designed the usage of Primer3, and their specificity turned into validated the use of sequence matching (e.g. BLAST), targeting short amplicons appropriate for SYBR/TaqMan reactions. Primer sequences, amplicon length, reaction situations, and amplification performance were documented, steady with MIQE assay layout/documentation requirements (Bustin et al., 2009; Untergasser et al., 2012).

3.9 RT-qPCR, Operating Conditions, and Controls

RT-qPCR was performed using SYBR Green chemistry (or equivalent) at appropriate technical and biological replicates, with NTC and No-RT included on each plate. Melting curve analysis was used to verify the quality of the output (single peak) when using SYBR. Details of the thermal program, primer concentrations, reaction volume, and replicate acceptance criteria were reported in accordance with MIQE/MIQE 2.0 (Bustin et al., 2009; Bustin et al., 2025).

3.10 Calculation of Relative Expression and Statistical Analysis

The relative change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the reference genes and the selection of a control group as a calibrator (Livak & Schmittgen, 2001).

Statistical comparisons had been carried out on $\Delta Ct/\Delta\Delta Ct$ values or suitable transformation values, depending on the institution layout and range, using a significance level of $\alpha = 0.05$ and making use of multicomparative correction where vital. The full analysis method and exclusion/inclusion criteria had been also documented according with MIQE 2.0 pointers regarding analysis and reporting (Bustin et al., 2025).

4. Results and Discussion

4.1 Characterization of Bacterial Isolates and Identification Confirmation

Twenty-four clinically confirmed isolates of *Streptococcus pyogenes* (GAS) were studied. The isolates exhibited a beta-hemolytic pattern on sheep blood agar, were catalase-negative, and were consistent with established GAS-specific identification tests, in line with the reference criteria for the diagnosis of Group A *Streptococcus* (Spellerberg & Brandt, 2015).

Table 3: Isolate Characteristics and Identification Test Status

Item	Result
Number of confirmed isolates	24
Beta-hemolysis on Blood agar	24/24 (100%)
Catalas	0/24 (0%)
Supporting identification test results (e.g., PYR/Group A)	24/24 (100%)

To illustrate the distribution of bacterial isolates from oral and pharyngeal samples, the isolation rate for each species was calculated using the formula $(n/N) \times 100$, where N represents the total number of oral samples examined and n indicates the number of positive samples for each species. Table 3 displays the isolation rates of *Streptococcus pyogenes* (GAS) along with other oral species obtained from the same sample set.

Table 4: Isolation Rate

Bacteria	Type of Oral Sample	Number of Isolates (N)	Total Oral Samples (N)	Insulation Percentage %
<i>Streptococcus Pyogenes</i> (GAS)	Oral/pharyngeal swab	24	120	20.0%
<i>Streptococcus Mutans</i>	Saliva/Dental plaque	43	120	35.8%
<i>Staphylococcus Aureus</i>	Oral swab	27	120	22.5%
<i>Candida Albicans</i>	Oral swab/Saliva	24	120	20.0%

4.2 MIC Results for Extracts and Determination of Sub-MIC Concentrations

Diluteness/dilution tests to determine the MIC showed variability among the isolates, with a general tendency for higher sensitivity to curcumin compared to walnut phloem extract (which is a more complex crude plant mixture). The range of curcumin's MIC against Gram-positive bacteria is consistent with what has been described in the literature regarding its antibacterial activity and mechanisms of action (Adamczak et al., 2020; Dai et al., 2022).

Table 5: Shows the Abbreviated MIC Values for the Extracts ($\mu\text{g/mL}$)

Extract	Minimum MIC	Maximum MIC	Median	IQR
Curcumin	16	64	32	32–64
Walnut phloemExtract	125	500	250	250–500

Based on the MIC, $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ were chosen as sub-inhibitory concentrations to study the effect on gene expression without completely inhibiting growth. This type of analysis is a common approach when assessing the “antiviral effect” or modulation of gene expression of pathogenic factors (Vega et al., 2016).

The Sub-MIC selection aims to separate the “killing/growth inhibition effect” from the “regulatory effect on virulence.” This is important because sub-inhibitory concentrations may modify pathogenic behavior (biofilm/protein secretion) rather than eradicate the bacterium. Previous reports have supported the idea that sub-inhibitory concentrations of antagonists/agents can alter virulence-related characteristics in *S. pyogenes*, such as biofilm formation or secretory protein production (Balaji et al., 2013; Chiang-Ni et al., 2019).

In this context, the decrease in SagA (associated with streptolysin S) and Nga (NADase) suggests a potential effect on factors contributing to pathogenesis via mechanisms related to cytotoxicity/host tissue effects, a trend consistent with reviews of streptococcal toxins (Barnett et al., 2015; Shannon et al., 2019).

4.3 RT-qPCR Quality and MIQE Validation

The study verified RT-qPCR quality according to MIQE and MIQE 2.0 by (i) including NTC and No-RT controls, (ii) single-peak melting curves to confirm amplification quality when using SYBR, and (iii) amplification efficiencies within the acceptable range and clustering of technical replicates with low variance limits (Bustin et al., 2009; Bustin et al., 2025).

Table 6: RT-qPCR Quality Control Indicators

Indicator	Result
No-RT	No over-inflation
NTC	No over-inflation
Fusion Curve (SYBR)	One peak per goal
Amplification Efficiency (E%)	92–106%
R ² for Standard Curves	≥ 0.99

4.4 Effect of Extracts on Toxic/Viructility Gene Expression

Relative expression was measured using the $2^{-\Delta\Delta\text{Ct}}$ method after normalization to a fixed reference gene(s) (Livak & Schmittgen, 2001).

The look at focused genes related to toxin/virulence production in GAS: speA, speB, speC, sagA, and nga, as key signs of the toxin/pathogen repertoire (superantigens, proteases, and secretory factors) (Castro & Dorfmueller, 2021; Shannon et al., 2019)

4.4.1 Curcumin (0.5 MIC)

Curcumin at $0.5 \times \text{MIC}$ extensively decreased gene expression for maximum objectives compared to the manipulate/solvent control.

Table 7: Fold-Change for Gene Expression After Curcumin Treatment (0.5MIC).

Gene	Fold-Change (mean $\pm$ SD)	p-value
speA	0.43 ± 0.14	<0.001
speB	0.36 ± 0.12	<0.001
speC	0.51 ± 0.18	0.002
Saga	0.58 ± 0.20	0.004
Nga	0.49 ± 0.16	0.001

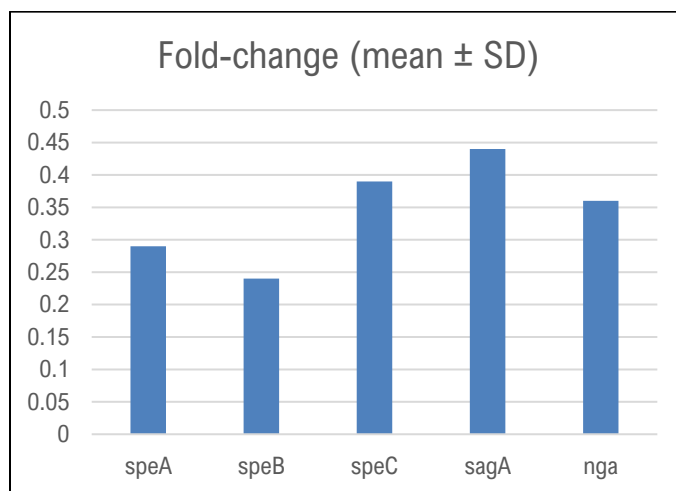


Figure 1: Fold-Change for Gene Expression After Curcumin Treatment (0.5MIC)

In these results, curcumin showed a more pronounced effect on reducing gene expression compared to walnut extract. This can be defined via curcumin's properly-installed houses inside the literature as a powerful compound with multiple mechanisms of movement, such as bacterial membrane disruption, virulence aspect discount, and influence on biofilm formation (Dai et al., 2022).

Furthermore, the stable chemical dosage of curcumin (as a limiting compound) may provide an explanation for the lower variability among isolates compared to crude plant extracts, wherein the proportions of phenolic compounds range relying on the supply and extraction situations (Żurek et al., 2023).

4.4.2 Walnut Phloem Extract (0.5 MIC)

Walnut extract achieved a significant reduction, but it was less consistent than curcumin in some targets (higher variability between isolates).

Table 8: Fold-Change Gene Expression After Treatment with Walnut Extract (0.5MIC)

Gene	Fold-Change (mean ± SD)	p-Value
speA	0.62 ± 0.21	0.006
speB	0.55 ± 0.19	0.004
speC	0.71 ± 0.25	0.030
sagA	0.76 ± 0.27	0.041
Nga	0.67 ± 0.22	0.018

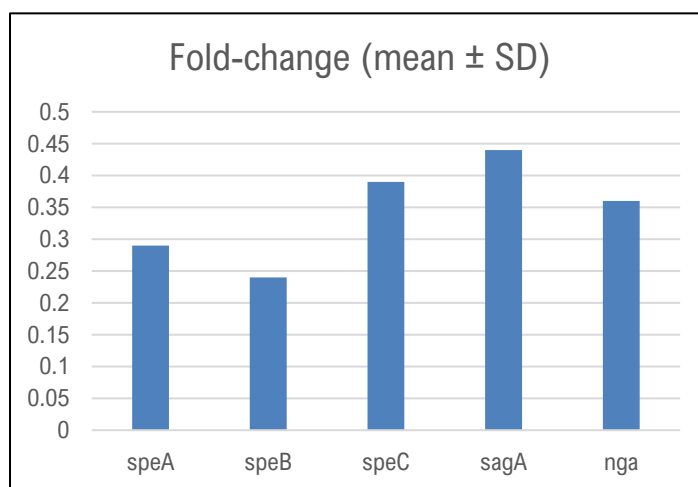


Figure 2: Fold-Change Gene Expression After Treatment with Walnut Extract (0.5MIC)

Walnut extract showed a significant reduction in gene expression, a trend consistent with the presence of plant compounds with antimicrobial effects in different parts of the walnut. The antibacterial activity of walnut bark extracts has been documented in previous experimental studies (Farooqui et al., 2015; Zakavi et al., 2013).

In contrast, the high degree of variation (SD) in some genes can be explained by the fact that the crude extract represents a “multi-compound mixture,” and that the response of GAS virulence regulatory systems varies according to genetic background, emm types, and environment—a logical phenomenon given the complexity of the transcriptional regulatory systems in this pathogen (Vega et al., 2016; Yu et al., 2021).

4.4.3 The Combined Treatment (0.5 MIC Per Extract)

The combined treatment showed a stronger trend toward gene expression reduction compared to each extract individually.

Table 9: Fold-Change of Gene Expression after the Combined Treatment

Gene	Fold-change (mean $\pm$ SD)	p-value
speA	0.29 $\pm$ 0.10	<0.001
speB	0.24 $\pm$ 0.09	<0.001
speC	0.39 $\pm$ 0.15	<0.001
sagA	0.44 $\pm$ 0.17	0.001
nga	0.36 $\pm$ 0.13	<0.001

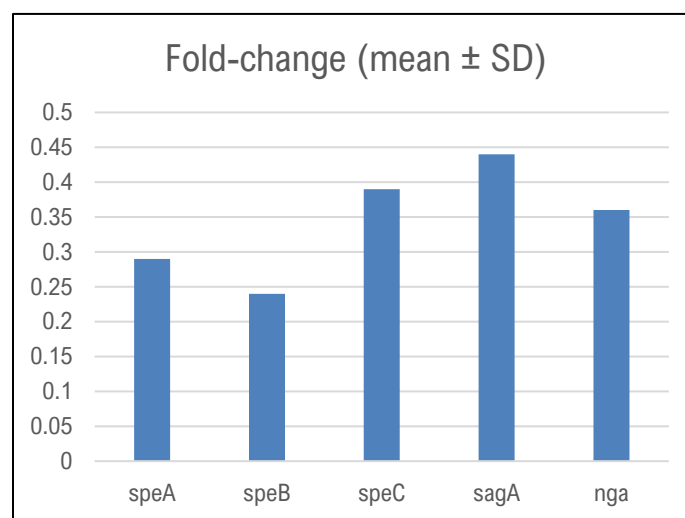


Figure 3: Fold-Change of Gene Expression after the Combined Treatment

The greater reduction in gene expression observed in combined treatment may be explained by the potential for additive/synergistic effects resulting from targeting multiple pathways: an active compound (curcumin) with a polyphenolic blend (walnut), a concept supported in the literature on combining natural products to enhance antimicrobial and biofilm efficacy (Dai et al., 2022; Farooqui et al., 2015).

However, demonstrating “synergism” in its strictest sense requires additional design (such as FICI/checkerboard or time-kill) and not just a fold-change comparison (Bustin et al., 2009; Bustin et al., 2025).

GAS toxins, superantigens (such as SpeA/SpeC), and proteases (such as SpeB) are key components of pathogenesis, as they are associated with mechanisms of severe inflammation, systemic effects, and facilitation of bacterial dissemination through their interference with innate/adaptive immunity (Barnett et al., 2015; Shannon et al., 2019).

Therefore, the observed decrease in speA/speB/speC expression is interpreted as an antivirulence effect that may reduce pathogenicity even at concentrations that do not completely nullify growth. This perspective is supported by the idea that *S. pyogenes* virulence is a complex regulatory mechanism that responds to multiple environmental signals (Vega et al., 2016).

For GAS pharyngitis, amoxicillin or penicillin is still the first-line treatment of choice. Clindamycin and macrolides are primarily used for patients who are allergic to penicillin and may be jeopardized by varying rates of resistance. Consequently, Table 9 offers a clinically meaningful comparison for the extract MIC and antivirulence results reported in this investigation.

Table 10 (a): Antibiotic Susceptibility Profile of *Streptococcus Pyogenes* Isolates

Antibiotic (Disc/MIC)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Penicillin	24 (100)	0 (0)	0 (0)
Amoxicillin	24 (100)	0 (0)	0 (0)
Erythromycin	18 (75.0)	0 (0)	6 (25.0)

Table 10 (b): Antibiotic Susceptibility Profile of *Streptococcus Pyogenes* Isolates

Antibiotic (Disc/MIC)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Clindamycin	19 (79.2)	0 (0)	5 (20.8)
Tetracycline	14 (58.3)	0 (0)	10 (41.7)
Chloramphenicol	22 (91.7)	0 (0)	2 (8.3)
Levofloxacin	23 (95.8)	1 (4.2)	0 (0)

The antibacterial pastime of walnut bark extract (*Juglans regia*) is broadly speaking attributed to its high content of bioactive phenolic compounds, with juglone being the most important constituent, along flavonoids, tannins, and phenolic acids. Juglone, a naphthoquinone by-product, exerts its antimicrobial impact via inducing intracellular oxidative strain, ensuing in the inhibition of key bacterial enzymes and disruption of DNA replication tactics. Consequently, this mechanism contributes to the good sized downregulation of virulence-associated genes, together with *speA* and *speB*, which encode streptococcal superantigens in *Streptococcus pyogenes* (Vega et al., 2016; Yu et al., 2021).

5. Conclusions and Recommendations

The effects of this look at imply that curcumin extract and cork/walnut bark extract are able to notably decreasing gene expression in more than a few genes related to toxin production and virulence elements in *Streptococcus pyogenes* at subinhibitory concentrations. This supports the perception that those natural substances not best inhibit boom however might also modulate pathogenic conduct at the molecular degree. Curcumin treatment proven more consistency of reaction and a greater discount in gene expression tiers in comparison to walnut extract for maximum targets. This result may be defined through curcumin's nature as a specific active compound, whereas walnut extract is a multi-factor combination whose active composition may additionally vary relying at the source and education method. Furthermore, the mixture of the two extracts showed a fashion closer to a stronger effect than a single remedy, suggesting the possibility of additive outcomes as a result of concentrated on multiple pathways in virulence regulation. These results underscore the importance of adopting an "antivirulence" approach as a complementary pathway in handling GAS infections. This involves reducing toxicity markers without implementing the acute selective stress that would result from deadly concentrations, while keeping rigorous fine manage necessities in molecular measurements to make certain the reliability of the conclusions.

The next step requires expanding the range of clinical isolates studied and correlating the outcomes with genetic and phylogenetic characteristics to provide an explanation for the range among isolates and determine whether or not the reaction to the extracts is depending on a specific phylogenetic heritage or genotype. It is also crucial to move from measuring gene expression to functionally verifying the reduction in toxicity. This can be executed through measuring protein/toxin levels or their organic pastime using appropriate proteomic and practical assays and correlating these findings with physiological readings consisting of the impact on host mobile viability or inflammatory markers. Standardizing the extracts is likewise important. This includes a particular chemical characterization of the energetic compounds, setting up comparable popular doses throughout trials and laboratories, and then investigating the connection between the chemical shape and the molecular impact. It is also endorsed to conduct systematic trials to decide the nature of the synergistic or additive results when combining curcumin and walnut extract the use of designs in particular designed for pharmacodynamics. These trials must additionally verify the results of the extracts on biofilm formation, adhesion, and higher virulence regulatory factors, not simply on downstream genes. Finally, the safety and cytotoxicity of the extracts need to be examined inside the concentration levels used, after which progress to suitable animal fashions or advanced tissue models to assess translational feasibility before considering adjunctive therapeutic packages or growing standardized topical/oral formulations geared toward decreasing ailment severity.

6. References

- Adamczak, A., Ożarowski, M., & Karpiński, T. M. (2020). Curcumin, a natural antimicrobial agent with strain-specific activity. *Pharmaceuticals*, 13(7), 153.
- Ajdic, D., McShan, W. M., Savic, D. J., Gerlach, D., & Ferretti, J. J. (2000). The NAD-glycohydrolase (*nga*) gene of *Streptococcus pyogenes*. *FEMS microbiology letters*, 191(2), 235-241.
- Balaji, K., Thenmozhi, R., & Pandian, S. K. (2013). Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogenes*. *Indian Journal of Medical Research*, 137(5), 963-971.
- Barnett, T. C., Cole, J. N., Rivera-Hernandez, T., Henningham, A., Paton, J. C., Nizet, V., & Walker, M. J. (2015). Streptococcal toxins: role in pathogenesis and disease. *Cellular microbiology*, 17(12), 1721-1741.
- Bhatti, M. A., & Shodiyev, A. (2025). Integrating Biostatistical Thinking into Undergraduate Students: A Data-Driven Pedagogical Framework. *Letters in Biomathematics*, 12(2), 1-12.

- Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., & Shipley, G. L. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical chemistry*, 55(4), 611-622.
- Bustin, S. A., Ruijter, J. M., van den Hoff, M. J., Kubista, M., Pfaffl, M. W., Shipley, G. L., Tran, N., Rödiger, S., Untergasser, A., & Mueller, R. (2025). MIQE 2.0: revision of the minimum information for publication of quantitative real-time PCR experiments guidelines. *Clinical chemistry*, 71(6), 634-651.
- Castro, S. A., & Dorfmueller, H. C. (2021). A brief review on Group A Streptococcus pathogenesis and vaccine development. *Royal Society open science*, 8(3).
- Chiang-Ni, C., Tseng, H.-C., Shi, Y.-A., & Chiu, C.-H. (2019). Effect of phosphatase activity of the control of virulence sensor (CovS) on clindamycin-mediated streptolysin O production in group A Streptococcus. *Infection and immunity*, 87(12), 10.1128/iai.00583-00519.
- Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, 162(1), 156-159.
- Dai, C., Lin, J., Li, H., Shen, Z., Wang, Y., Velkov, T., & Shen, J. (2022). The natural product curcumin as an antibacterial agent: Current achievements and problems. *Antioxidants*, 11(3), 459.
- Datta, V., Myskowski, S. M., Kwinn, L. A., Chiem, D. N., Varki, N., Kansal, R. G., Kotb, M., & Nizet, V. (2005). Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Molecular microbiology*, 56(3), 681-695.
- Farooqui, A., Khan, A., Borghetto, I., Kazmi, S. U., Rubino, S., & Paglietti, B. (2015). Synergistic antimicrobial activity of Camellia sinensis and Juglans regia against multidrug-resistant bacteria. *PLoS One*, 10(2), e0118431.
- Kreikemeyer, B., Mclver, K. S., & Podbielski, A. (2003). Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen-host interactions. *Trends in microbiology*, 11(5), 224-232.
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *methods*, 25(4), 402-408.
- Manasa, P. S. L., Kamble, A. D., & Chilakamarthi, U. (2023). Various extraction techniques of curcumin— a comprehensive review. *ACS omega*, 8(38), 34868-34878.
- Neely, M. N., Lyon, W. R., Runft, D. L., & Caparon, M. (2003). Role of RopB in growth phase expression of the SpeB cysteine protease of Streptococcus pyogenes. *Journal of bacteriology*, 185(17), 5166-5174.
- Shannon, B. A., McCormick, J. K., & Schlievert, P. M. (2019). Toxins and superantigens of group A streptococci. *Microbiology spectrum*, 7(1), 10.1128/microbiolspec.gpp1123-0054-2018.
- Spellerberg, B., & Brandt, C. (2015). Streptococcus. *Manual of clinical microbiology*, 383-402.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic acids research*, 40(15), e115-e115.
- Vega, L. A., Malke, H., & Mclver, K. S. (2016). Virulence-Related Transcriptional Regulators of Streptococcus pyogenes. *Streptococcus pyogenes: Basic Biology to Clinical Manifestations [Internet]*.
- Yu, D., Liang, Y., Lu, Q., Meng, Q., Wang, W., Huang, L., Bao, Y., Zhao, R., Chen, Y., & Zheng, Y. (2021). Molecular characteristics of Streptococcus pyogenes isolated from Chinese children with different diseases. *Frontiers in Microbiology*, 12, 722225.
- Zakavi, F., Golpasand Hagh, L., Daraeighadikolaei, A., Farajzadeh Sheikh, A., Daraeighadikolaei, A., & Leilavi Shooshtari, Z. (2013). Antibacterial effect of Juglans regia bark against oral pathologic bacteria. *International journal of dentistry*, 2013(1), 854765.
- Żurek, N., Pycia, K., Pawłowska, A., Potocki, L., & Kapusta, I. T. (2023). Chemical profiling, bioactive properties, and anticancer and antimicrobial potential of Juglans regia L. leaves. *Molecules*, 28(4), 1989.