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Inhibitory and Bactericidal Effect of Aqueous Pepper Extract (*Capsicum annum* L.), Capsaicin, and Capsaicin Combination with Amoxicillin against *Streptococcus pyogenes*

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ABSTRACT

Introduction: *Streptococcus pyogenes* remains a significant human pathogen responsible for a spectrum of infections, including severe invasive diseases such as streptococcal toxic shock syndrome, and serious post-infectious sequelae like acute rheumatic fever and glomerulonephritis. Considering the recognized antimicrobial properties of *Capsicum annuum* L. (*C. annuum* L.) and the persistent clinical challenge presented by *S. pyogenes*, this study investigated the *in vitro* antimicrobial activity of an aqueous extract of *Capsicum annuum* L., Capsaicin ($C_{18}H_{27}NO_3$), amoxicillin, and a combination of Capsaicin with amoxicillin against *S. pyogenes* (ATCC 19615). The primary objective was to evaluate both the inhibitory and bactericidal effects of these agents, specifically exploring the potential for synergistic or additive interactions between a plant-derived compound and a conventional antibiotic to improve therapeutic potential. **Methods**: The antimicrobial activities were evaluated using broth microdilution to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Time-kill kinetics were assessed spectrophotometrically by monitoring bacterial growth. Additionally, disk diffusion assays were performed to evaluate antimicrobial susceptibility. Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine significant differences between treatment groups. **Results**: The aqueous extract of *C. annuum* L. and Capsaicin both demonstrated significant *in vitro* inhibitory and bactericidal activity against *S. pyogenes*. Specifically, MIC and MBC of the aqueous extract were determined to be 12 mg/mL and 14 mg/mL, respectively. For Capsaicin, the MIC was 15 µg/mL, and the MBC was 20 µg/mL. Consistent with these findings, disk diffusion assays revealed distinct zones of inhibition for both agents. Notably, the combination of Capsaicin with amoxicillin exhibited enhanced antimicrobial activity, resulting in a statistically significant reduction in the MIC ($P < 0.05$) and MBC ($P < 0.01$) compared to each agent alone. **Conclusion**: The combination of Capsaicin with amoxicillin demonstrated enhanced bactericidal efficacy in a dose-dependent manner. Notably, Capsaicin exhibited bactericidal activity at microgram concentrations, whereas amoxicillin required milligram concentrations to achieve a comparable effect, highlighting the potent antibacterial properties of Capsaicin. These findings suggest that both the aqueous extract of *C. annuum* L. and Capsaicin hold significant potential as promising candidates for the development of novel antimicrobial therapies against *S. pyogenes*.

INTRODUCTION

The escalating global challenge of antimicrobial resistance has intensified the imperative for discovering novel therapeutic strategies, prompting a renewed focus on natural products as promising sources of antimicrobial agents. Natural sources harbor a diverse

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repertoire of bioactive compounds with antimicrobial potential, potentially offering viable alternatives or adjuncts to conventional antibiotics, particularly by exhibiting distinct mechanisms of action that may circumvent existing resistance mechanisms [1]. These naturally occurring compounds serve as valuable scaffolds for drug development and contribute to elucidating the scientific basis of traditional medicine. Among these, phytochemicals, including alkaloids, flavonoids, tannins, and phenols, are particularly significant due to their well-established antimicrobial properties. These compounds have demonstrated the capacity to combat a wide array of pathogens through diverse mechanisms of action, encompassing antibacterial, antifungal, and anti-inflammatory effects [2]. Their incorporation into modern pharmacology is increasingly driven by their demonstrated efficacy, often coupled with a more favorable safety profile and potential cost-effectiveness compared to synthetic counterparts, thereby playing a crucial role in the innovation of novel pharmaceuticals.

Chili peppers, classified under the genus *Capsicum*, have garnered significant attention within this research domain. Indeed, extracts derived from various *Capsicum* species are currently utilized in both traditional and emerging medicinal applications. Specifically, studies have demonstrated that extracts from cultivars such as Habanero, Serrano, and Morrón effectively inhibit the *in vitro* growth of a spectrum of bacterial pathogens, including *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella enterica* [3]. The bioactive compounds inherent in these peppers, most notably Capsaicin, are responsible not only for their characteristic pungency but also for a diverse range of purported health benefits.

Capsaicin, the principal bioactive compound in chili peppers of the genus *Capsicum*, is well-established for its analgesic properties [4]. Beyond its analgesic effects, Capsaicin has been implicated in a range of other potential health benefits, including anti-inflammatory, antioxidant, and even antineoplastic activities, as well as potential positive effects on cardiovascular and gastrointestinal health, attributed to its diverse physiological and pharmacological mechanisms [5]. The growing interest in Capsaicin stems from its dual functionality as both an analgesic agent and a potential antimicrobial compound, positioning it as a molecule of significant interest in both pharmacological and microbiological research.

Among bacterial pathogens, *S. pyogenes* remains a prominent human pathogen, responsible for a substantial burden of infectious diseases globally [7]. Its capacity to cause a wide spectrum of illnesses, ranging from common pharyngitis to severe invasive infections, underscores the critical need for effective therapeutic interventions.

This study rigorously evaluated the *in vitro* antibacterial activity of an aqueous extract of *C. annuum* L. and Capsaicin against *S. pyogenes*. The exploration of medicinal plants as a source of novel antimicrobials represents a compelling strategy, owing to their inherent potential for potent antimicrobial activity, coupled with advantages such as relative accessibility and potential cost-effectiveness [8]. Ultimately, understanding the mechanisms by which these natural agents exert their antimicrobial effects is crucial for fully realizing their therapeutic potential.

Therefore, the primary objective of this study was to identify an efficacious agent, ideally exhibiting a reduced incidence of adverse effects, for the treatment of *S. pyogenes* infections. By integrating traditional knowledge with contemporary scientific methodologies, this research endeavors to contribute to the global effort in combating antimicrobial resistance through the exploration of nature-derived therapeutic solutions.

MATERIAL AND METHODS

Evaluation of antibacterial activity. This study investigated the *in vitro* antibacterial effects of an aqueous extract of *C. annuum* L. (pepper) and Capsaicin, both individually and in combination with amoxicillin, against *S. pyogenes* (ATCC 19615). Initially, MIC and MBC of Capsaicin and the aqueous pepper extract were determined for *S. pyogenes* using broth microdilution assays. Subsequently, the susceptibility of *S. pyogenes* to these compounds was assessed using the disk diffusion method by measuring the diameters of the inhibition zones. Furthermore, the impact on bacterial cell density in the presence of Capsaicin, the aqueous pepper extract, and amoxicillin was evaluated using spectrophotometric results to measure optical density at 600 nm for time-kill kinetics analysis.

Preparation of *S. pyogenes* inoculum. The bacterial strain employed in this study was *S. pyogenes* (ATCC 19615), procured from the Iranian Research Organization for Science and Technology (IROST). Initial confirmation of the strain identity involved Gram staining, which revealed Gram-positive cocci upon microscopic examination. Catalase and oxidase tests were subsequently performed, both yielding negative results, indicative of the absence of catalase and cytochrome c oxidase activity. Furthermore, culture on blood agar demonstrated β-hemolysis, corroborating the strain's identity (detailed methodologies are provided in the Supplementary Material) [9-11].

The *S. pyogenes* inoculum was prepared by adjusting the bacterial suspension to a turbidity equivalent to a 0.5 McFarland standard (Refer to Table 1 in Supllementary

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file), as described in detail within the Supplementary Material [12]. This procedure ensured a standardized bacterial concentration for subsequent assays.

Preparation of the aqueous pepper extract. Fresh green chili peppers (*C. annuum* L.) were obtained, and their species was botanically identified at an agricultural research center in Urmia, Iran. The peppers were meticulously washed under running distilled water, air-dried in the dark at ambient temperature, and subsequently pulverized into a fine powder using a sterile electric blender. According to the extraction protocol detailed in the Supplementary Material, 10 g of the powdered pepper material was subjected to extraction with 100 mL of sterile distilled water under constant stirring at room temperature for 24 h. The resulting mixture was then centrifuged at 4000 × *g* for 15 min to separate the solid residue. The supernatant was carefully collected and subjected to sequential filtration using sterile syringe filters with decreasing pore sizes: initially through a 0.65 µm membrane, followed by a 0.45 µm membrane, and culminating in sterilization via filtration through a 0.22 µm membrane filter under aseptic conditions within a laminar flow hood. The resultant sterile aqueous extract was aseptically transferred into sterile Falcon tubes, and stored at 4°C in the dark until further use [13]. Subsequently, serial dilutions of both Capsaicin and the aqueous pepper extract were prepared in appropriate sterile media to achieve the desired concentration ranges for subsequent assays.

Broth microdilution assay for determining antimicrobial susceptibility. The susceptibility of *S. pyogenes* to the various antimicrobial agents was assessed using the broth microdilution method. Briefly, each well of a sterile 96-well microtiter plate was dispensed with 100 µL of sterile Brain Heart Infusion (BHI) broth. The positive control wells contained 100 µL of BHI broth and 20 µL of the standardized bacterial suspension (without any antimicrobial agent). Test wells received 100 µL of BHI broth, 100 µL of Capsaicin solutions prepared in serial dilutions to achieve final concentrations ranging from 1 to 10 µg/mL, and 20 µL of the standardized bacterial suspension. Negative control wells contained 100 µL of BHI broth only (without bacteria or antimicrobial agent). The microplates were then agitated gently on an orbital shaker for 3 min to ensure thorough mixing and incubated at 37°C for 24 h under aerobic conditions. The MIC was visually determined as the lowest concentration of the antimicrobial agent that prevented visible bacterial growth after the 24-hour incubation period [14]. All assays were performed in quadruplicate to ensure reproducibility. For the aqueous pepper extract, an analogous procedure was followed, employing serial dilutions to achieve final concentrations ranging from 0 to 50 mg/mL in one set of experiments and 0 to 20 mg/mL in a separate set of experiments, each tested on individual microplates.

To determine the MBC, aliquots were aseptically withdrawn from the wells exhibiting no visible growth in the MIC assay, as well as from the two subsequent wells containing higher concentrations of Capsaicin. These aliquots were then sub-cultured onto separate sterile BHI agar plates using sterile cotton swabs. The inoculated agar plates were incubated at 37°C for 24 h under aerobic conditions. The MBC was defined as the lowest concentration of the antimicrobial agent that resulted in no visible bacterial growth on the sub-cultured agar plates. This protocol was performed in quadruplicate for both Capsaicin and the aqueous pepper extract to ensure the reliability of the results [15].

Following the guidelines established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), *S. pyogenes* isolates are classified as resistant to amoxicillin when the diameter of the zone of inhibition is less than 18 mm, and susceptible when the zone diameter is 18 mm or greater.

Spectrophotometric analysis of bacterial growth kinetics. Spectrophotometry, based on the Beer-Lambert law [16], was employed to quantitatively assess bacterial growth by measuring the absorbance of the culture medium.

Specifically, bacterial concentration was determined by measuring the optical density at a wavelength of 600 nm, a standard wavelength for monitoring bacterial growth. All experimental conditions were tested in quadruplicate to ensure the reliability of the results. Sterile test tubes were prepared, each containing 2 mL of BHI broth and 100 µL of the standardized bacterial suspension. To evaluate the individual effects of the antimicrobial agents, varying concentrations of Capsaicin (1-10 µg/mL) or amoxicillin (10-50 mg/mL) were added to the respective tubes. Initially, the impact of different concentrations of Capsaicin and the aqueous pepper extract on the growth of *S. pyogenes* was assessed**.** Subsequently, to investigate potential synergistic interactions, combinations of Capsaicin with amoxicillin were tested. Negative control tubes included one containing only BHI broth (serving as a blank) and others containing the individual antimicrobial agents (Capsaicin, aqueous pepper extract, or amoxicillin) without bacterial inoculation to control for any inherent absorbance of the compounds. A positive control tube contained BHI broth and the bacterial suspension, representing uninhibited growth. Each experimental condition was performed in quadruplicate. Following incubation at 37°C for 24 h, the optical density of each

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tube was measured using a spectrophotometer to quantify bacterial growth.

Disk diffusion assay for antimicrobial susceptibility. Mueller Hinton agar plates were prepared with a uniform depth of 4 mm, adhering to the manufacturer's instructions. Sterile paper disks (6 mm in diameter, Padtan Teb Co., Iran) were impregnated with 10 µL of various concentrations of Capsaicin solution and allowed to air dry under sterile conditions for 24 h. Then, these disks were further dried at 37°C for 1 h in a sterile incubator. Subsequently, the disks were aseptically applied onto the surface of Mueller Hinton agar plates that had been uniformly inoculated with a *S. pyogenes* suspension adjusted to a 0.5 McFarland turbidity standard. A sterile cotton swab was used to evenly spread the bacterial inoculum across the entire agar surface, ensuring confluent growth. Each plate included one blank disk impregnated with the solvent used to dissolve Capsaicin (serving as a negative control) and a disk containing a standard concentration of amoxicillin as a positive control. The plates were incubated at 37°C for 24 h under aerobic conditions, after which the diameters of the resulting zones of inhibition were measured in millimeters using a calibrated ruler [17].

According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, an inhibition zone diameter of ≥18 mm indicates susceptibility to amoxicillin for *S. pyogenes*.

The concentrations of Capsaicin, aqueous pepper extract, and amoxicillin utilized in the MIC, MBC, and spectrophotometry assays were determined based on the results of preliminary dose-range finding experiments. These preliminary experiments involved testing a wide range of concentrations to identify the effective ranges for inhibiting the growth of *S. pyogenes*.

Statistical analysis. Statistical analyses were performed using IBM SPSS Statistics, version 26.0**.** To determine if statistically significant differences existed in the antimicrobial efficacy among the various treatment groups (aqueous pepper extract, Capsaicin, amoxicillin, and their combinations), one-way ANOVA was conducted. Where significant differences were detected by ANOVA, Tukey's *post hoc* test was applied for pairwise comparisons to identify which specific treatment groups differed significantly in their antimicrobial effects against *Streptococcus pyogenes*.

Ethical considerations. This study involved *in vitro* experiments with standard strain exclusively and did not involve any research on animals or human subjects. Therefore, formal ethical approval from an institutional review board (IRB) or ethics committee was not required for this specific study. The original thesis from which this manuscript is derived included a statement confirming adherence to ethical guidelines for research not involving human or animal subjects. Furthermore, as a procedural requirement for thesis registration and archiving in Iran, an ethics compliance report pertaining to the original thesis has been submitted to Iran-Doc (in Persian language), as detailed in the supplementary document.

RESULTS

Results for MIC and MBC. The *in vitro* MIC of Capsaicin against *S. pyogenes* was determined to be 15 µg/mL, while the MBC was 20 µg/mL (refer to Supplementary Tables 2 and 3). For the aqueous extract of *C. annuum* L., the *in vitro* MIC was found to be 12 mg/mL, and the MBC was 14 mg/mL (refer to Supplementary Tables 4 and 5).

Disk diffusion assay results for Capsaicin. Based on the determined MBC of Capsaicin for *S. pyogenes* (20 µg/mL), the disk diffusion assay was performed to further evaluate the susceptibility of the bacteria to Capsaicin. Inhibition zone diameters were measured for disks impregnated with Capsaicin at concentrations of 15 µg/mL, 20 µg/mL, and 25 µg/mL. At a Capsaicin concentration of 20 µg/mL, the mean inhibition zone diameter was ≥16 mm, indicating *in vitro* susceptibility. Disks containing 15 µg/mL of Capsaicin resulted in inhibition zone diameters <16 mm. The blank disk, serving as a negative control, did not produce any zone of inhibition (see Table 1).

Table 1. Inhibition zone diameters of Capsaicin against *S. pyogenes* (mm)

Concentration (µg/mL)	Control (blank disk)	Replicate 1	Replicate 2	Replicate 3	Replicate 4
15	0	16.06	16.11	16.09	16.12
20	0	18.03	18.06	18.01	18.09
25	0	19.51	19.53	19.56	19.55

Notes: Data represent the diameters of the inhibition zones measured in millimeters. Experiments were conducted in quadruplicate.

Disk diffusion assay results for aqueous pepper extract. Based on the determined minimum bactericidal concentration (MBC) of the aqueous pepper extract for *S. pyogenes* (14 mg/mL), inhibition zones were evaluated at concentrations of 12 mg/mL, 14 mg/mL, and 15 mg/mL to assess bacterial susceptibility. At a concentration of 15 mg/mL, the aqueous pepper extract produced a mean inhibition zone diameter of ≥14 mm,

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indicating *in vitro* susceptibility. Conversely, concentrations resulting in inhibition zone diameters <14 mm were indicative of resistance. The blank disk, serving as the negative control, did not exhibit any zone of inhibition (refer to Table 2).

Table 2. Inhibition zone diameters of aqueous pepper extract against *S. pyogenes*

Concentration (mg/mL)	Control (blank disk)	Replicate 1	Replicate 2	Replicate 3	Replicate 4
12	0	14.31	14.27	14.30	14.25
14	0	15.35	15.55	15.48	15.52
16	0	17.01	17.17	17.08	16.97

Notes: Data represent the diameters of the inhibition zones measured in millimeters. Experiments were conducted in quadruplicate.

Interpretation of susceptibility based on inhibition zone diameters. Consistent with the EUCAST guidelines, *S. pyogenes* is classified as resistant to amoxicillin if the inhibition zone diameter is less than 18 mm and susceptible if the diameter is 18 mm or greater. Based on these established criteria and the experimental results obtained in this study, *S. pyogenes* demonstrated *in vitro* susceptibility to Capsaicin, exhibiting inhibition zones ≥16 mm, and to the aqueous pepper extract, with inhibition zones ≥14 mm.

Spectrophotometric assessment of the efficacy of the aqueous pepper extract. Optical density measurements were performed to assess the impact of various concentrations of the aqueous pepper extract on the growth of *S. pyogenes*. Six different treatment concentrations were evaluated, with each condition tested in quadruplicate (detailed data are presented in the Supplementary Material). The results demonstrated an inverse relationship between the concentration of the aqueous pepper extract and the optical density, indicating a reduction in bacterial density with increasing extract concentration. The lowest optical density, signifying the greatest inhibition of bacterial growth, was observed at a concentration of 15 mg/mL. It is important to note that the measured optical density includes contributions from the culture medium and the aqueous pepper extract itself (see Figure 1).

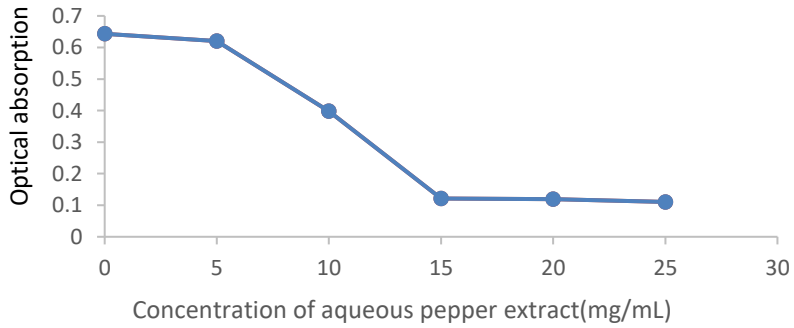


Fig. 1. Optical density measurements of *S. pyogenes* in response to aqueous pepper extract

Tukey's post hoc analysis of aqueous pepper extract efficacy. Pairwise comparisons using Tukey's post hoc test revealed statistically significant differences in bacterial growth inhibition among lower concentrations of the aqueous pepper extract. Specifically, treatments A (0 mg/mL, positive control with bacteria and culture medium), B (5 mg/mL), and C (10 mg/mL) each showed significantly different levels of *S. pyogenes* growth inhibition compared to one another. However, treatments D (15 mg/mL), E (20 mg/mL), and F (25 mg/mL) did not show statistically significant differences in their inhibitory effects on bacterial growth. This suggests that a concentration of 15 mg/mL of the aqueous pepper extract is sufficient to achieve *in vitro* maximal bacterial growth inhibition under the tested conditions, indicating that increasing the concentration beyond this point does not yield a statistically significant improvement in efficacy (see Table 3).

ANOVA results. ANOVA presented in Table 4 revealed a statistically significant difference in mean optical density among the treatment groups ($P < 0.001$; $\alpha = 0.05$). This finding indicates rejection of the null hypothesis and demonstrates a significant effect of the treatments on the inhibition of *S. pyogenes* growth.

Spectrophotometric assessment of Capsaicin's antibacterial activity against *S. pyogenes*. Optical

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density measurements of *S. pyogenes* cultures following inoculation with varying concentrations of Capsaicin were performed on six independent samples, with each concentration tested in quadruplicate (detailed data presented in the supplementary material). As depicted in Figure 2, an inverse relationship between Capsaicin concentration and optical density was observed across the tested range (0 to 25 µg/mL), indicating a concentration-dependent reduction in bacterial density. The lowest mean optical density, suggestive of minimal bacterial presence, was consistently observed at Capsaicin concentrations ranging from 20 to 25 µg/mL. It is important to note that a baseline optical density attributable to the culture medium and the Capsaicin solution itself was accounted for the analysis.

Table 3. Tukey's HSD post hoc analysis of mean optical density of *S. pyogenes* exposed to different concentrations of aqueous pepper extract

Treatment group	Number of replicates (n)	Mean optical density	Tukey grouping
1	4	0.64325	A
2	4	0.62050	AB
3	4	0.39800	C
4	4	0.12150	D
5	4	0.11975	E
6	4	0.11050	F

Note: Treatment groups labeled with different letters indicate statistically significant differences at the 95% confidence level, as determined by Tukey's HSD test.

Table 4. ANOVA results for the effect of different treatments on *S. pyogenes* growth

Source	Degrees of freedom (DF)	Adjusted sum of squares (Adj SS)	Adjusted mean square (Adj MS)	*F*- statistic	*P*-value
Treatment	5	1.17599	0.235198	1547.36	< 0.001
Error	18	0.00271	0.000151		
Total	23	1.17870	N/A		

Note: N/A' (Not Applicable) in the 'Total' row indicates that certain statistical measures, such as the *F*-statistic or *P*-value, are not calculated or relevant for the total row in an ANOVA table. The 'Total' row summarizes the overall variation in the data, but it does not require an F-statistic or P-value because it is not used for hypothesis testing.

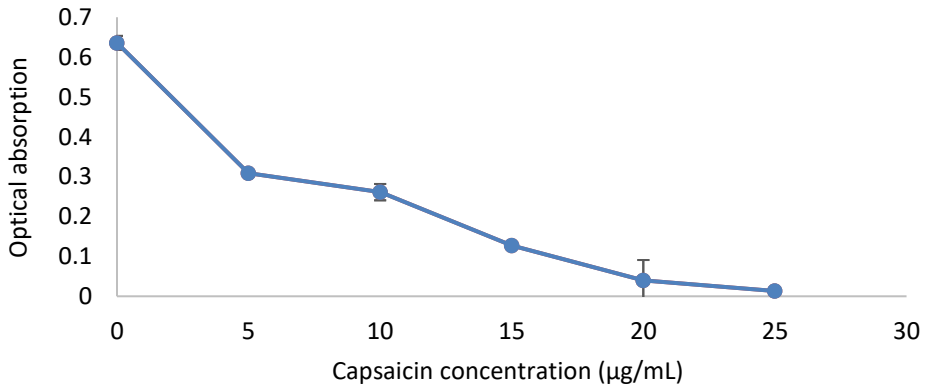


Fig. 2. Optical density measurements illustrating the inhibitory effect of Capsaicin on the growth of *S. pyogenes*

Tukey's HSD test for Capsaicin efficacy. Post-hoc analysis using Tukey's HSD test revealed that Capsaicin concentrations of 20 µg/mL and above resulted in statistically similar levels of *S. pyogenes* growth inhibition. Specifically, treatments with Capsaicin at 0 µg/mL (Group A), 5 µg/mL (Group B), 10 µg/mL (Group C), and 15 µg/mL (Group D) each demonstrated statistically significant differences in their effects on bacterial growth inhibition. However, the inhibitory effects of Capsaicin at 20 µg/mL (Group E) and 25 µg/mL (Group F) were not statistically different from each other, suggesting that 20 µg/mL represents the minimum concentration required to achieve the maximal inhibitory effect under the tested conditions (see Table 5).

ANOVA results for the effect of aqueous pepper extract concentration on *S. pyogenes* growth inhibition. ANOVA presented in Table 6 demonstrated a statistically

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significant effect of aqueous pepper extract concentration on *S. pyogenes* growth inhibition ($P < 0.001$; $\alpha = 0.05$). This finding indicates rejection of the null hypothesis and confirms that the observed variations in bacterial growth are unlikely attributable to random variation.

Table 5. Tukey's HSD post-hoc analysis of Capsaicin concentrations on *S. pyogenes* growth

Capsaicin concentration (µg/mL)	Number of replicates (*n*)	Mean optical density	Tukey grouping
0	4	0.63575	A
5	4	0.30900	B
10	4	0.25450	C
15	4	0.12725	D
20	4	0.01500	E
25	4	0.01300	F

Note: Treatment groups labeled with different letters are significantly different at the 95% confidence level, as determined by Tukey's HSD test.

Table 6. ANOVA results for the effect of different treatments on *S. pyogenes* growth

Source	Degrees of freedom (DF)	Adjusted sum of squares (Adj SS)	Adjusted mean square (Adj MS)	*F*-statistics	*P*-value
Treatment	5	0.396738	0.079348	4428.70	< 0.001
Error	18	0.000323	0.000018		
Total	23	0.397060	N/A		

Note: N/A' (Not Applicable) in the 'Total' row indicates that certain statistical measures, such as the F-statistic or P-value, are not calculated or relevant for the total variation in an ANOVA table. The 'Total' row summarizes the overall variation in the data but is not used for hypothesis testing, so these values are not applicable.

Spectrophotometric assessment of Amoxicillin activity against *S. pyogenes*. The effect of varying concentrations of amoxicillin on *S. pyogenes* growth was evaluated using spectrophotometry. Six independent amoxicillin concentrations were tested, with each concentration assessed in quadruplicate, as illustrated in Figure 3.

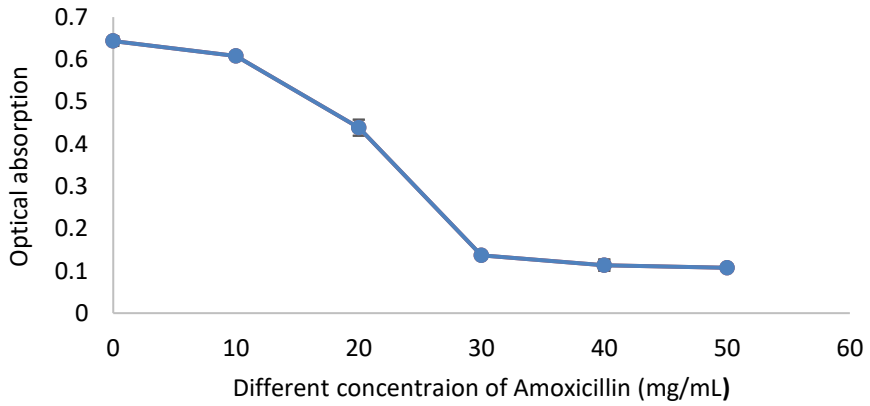


Fig. 3. Amoxicillin-mediated inhibition of *S. pyogenes* growth, assessed via optical density

Tukey's HSD Test for Amoxicillin's efficacy on *S. pyogenes*. Post-hoc analysis using Tukey's Honest Significant Difference (HSD) test revealed that amoxicillin concentrations of 30 mg/mL and above resulted in statistically similar levels of *S. pyogenes* growth inhibition. As shown in Table 7, treatments with amoxicillin at 0 mg/mL (Group A), 10 mg/mL (Group B), and 20 mg/mL (Group C) each demonstrated statistically significant differences in their effects on bacterial growth inhibition. However, the inhibitory effects of amoxicillin at 30 mg/mL (Group D), 40 mg/mL (Group E), and 50 mg/mL (Group F) were not statistically different from each other.

Table 7. Tukey's HSD post-hoc analysis of Amoxicillin concentrations on *S. pyogenes* growth

Amoxicillin concentration (mg/mL)	Number of replicates (*n*)	Mean optical density	Tukey grouping
0	4	0.64325	A
10	4	0.60800	B
20	4	0.43850	C
30	4	0.13400	D
40	4	0.11350	E
50	4	0.10725	F

Note: Treatment groups labeled with different letters indicate statistically significant differences at the 95% confidence level, as determined by Tukey's HSD test.

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ANOVA results for Amoxicillin's inhibitory effect on *S. pyogenes*. Analysis of variance (ANOVA) presented in Table 8 confirmed a statistically significant effect of amoxicillin on *S. pyogenes* growth inhibition ($P < 0.001$; $\alpha = 0.05$). This finding indicates rejection of the null hypothesis and supports the conclusion that the observed reduction in optical density with increasing amoxicillin concentrations is not attributable to random variation.

Table 8. ANOVA for the effect of different treatments on *S. pyogenes* growth

Source	Degrees of freedom (DF)	Adjusted sum of squares (Adj SS)	Adjusted mean square (Adj MS)	*F*- Statistic	*P*-value
Treatment	5	1.28558	0.257115	1507.76	<0.001
Error	18	0.000307	0.000017		
Total	23	1.28865	N/A		

Note: N/A' (Not Applicable) in the 'Total' row indicates that certain statistical measures, such as the *F*-statistic and *P*-value, are not calculated or relevant for the total variation in an ANOVA table. The 'Total' row summarizes the overall variation in the data but is not used for hypothesis testing, so these values are not applicable.

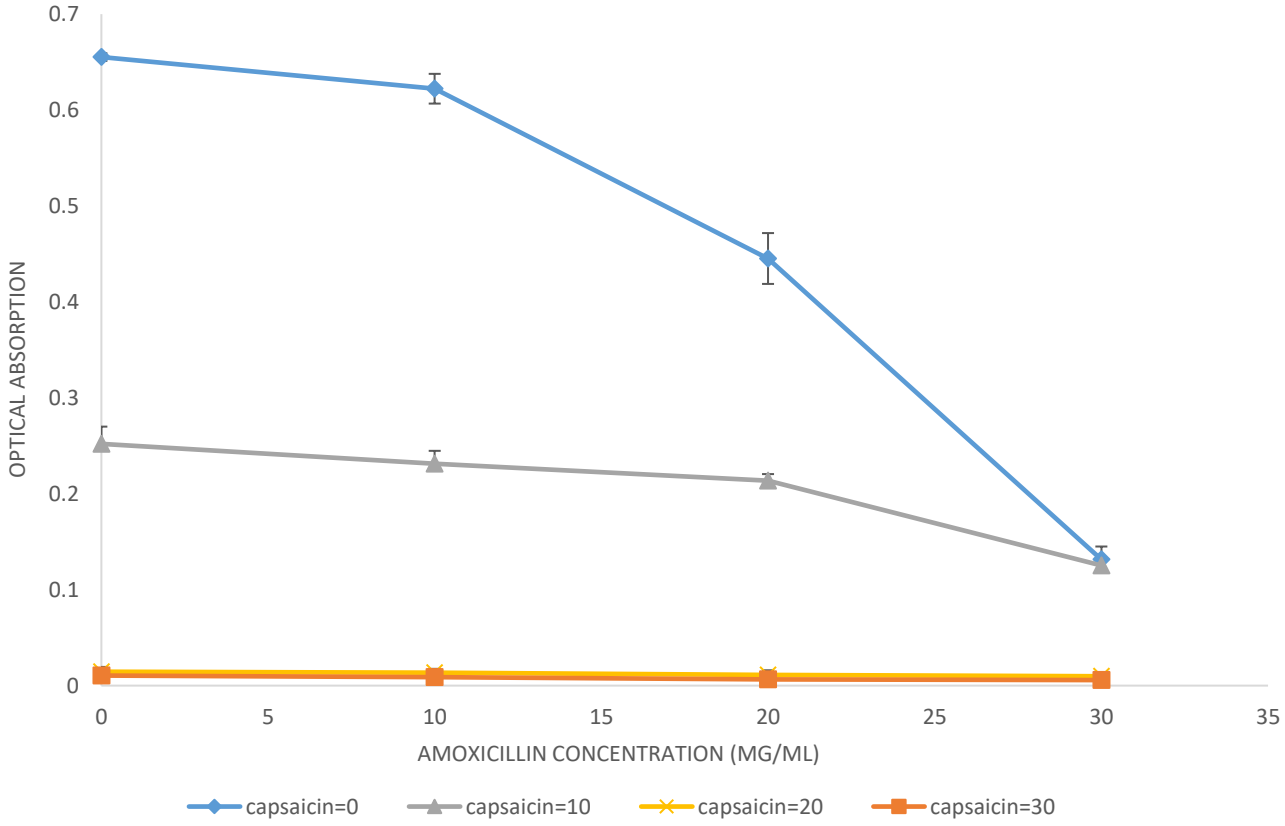


Fig. 4. Optical density of *S. pyogenes* in response to Capsaicin and Amoxicillin

Statistical analysis of treatment interactions. A factorial experimental design was employed to assess the interactive effects of Capsaicin and amoxicillin on *S. pyogenes* growth. ANOVA results revealed a statistically significant effect of Capsaicin treatment ($P < 0.05$; $\alpha = 0.05$). Furthermore, the overall model, as well as the individual and interactive effects of Capsaicin and amoxicillin, were highly significant ($P < 0.001$; $\alpha = 0.01$) (see Table 9).

Synergistic effects of Capsaicin and Amoxicillin on *S. pyogenes* via spectrophotometry. Spectrophotometric analysis revealed the interaction between between Capsaicin and amoxicillin in inhibiting the growth of S. pyogenes , as depicted in Figure 4.

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Table 9. ANOVA for the effects of Capsaicin and Amoxicillin, and their interaction, on *S. pyogenes* growth inhibition

Source	DF	*F*-statistic	*P*-value
Model	19	3215.889	<0.001
R	3	0.560	0.644
Capsaicin (A)	3	9322.101	<0.001
Amoxicillin (B)	3	1123.237	<0.001
Interaction (A×B)	9	641.856	<0.001
Error	45	-	-
Total	64	-	-

Note: The 'Interaction (A×B)' term in the ANOVA table represents the combined effect of Capsaicin (A) and Amoxicillin (B) on S. pyogenes growth inhibition. A statistically significant interaction ($P < 0.001$) indicates that the effect of one factor (e.g., Capsaicin) on bacterial growth inhibition depends on the level of the other factor (e.g., Amoxicillin), and vice versa. This means the two factors do not act independently; their combined effect is greater or different than what would be expected based on their individual effects alone. Dependent variable: bacterial growth inhibition (Y).

DISCUSSION

S. pyogenes represents a major human pathogen implicated in a wide spectrum of human diseases, encompassing both localized superficial skin infections and severe, life-threatening invasive systemic conditions [18].

Globally, statistical analyses indicate that *S. pyogenes* is a significant contributor to bacterial infections, with an estimated 18.1 million individuals experiencing severe manifestations annually and 1.78 million new cases reported each year [19]. Furthermore, *S. pyogenes* is implicated in approximately 616 million cases of pharyngitis worldwide annually [20]. Notably, in developing countries, this pathogen accounts for an estimated 111 million skin infections among children [20]. Within the United States, the prevalence of *S. pyogenes* infections ranges from 15% to 30% in children and 5% to 20% in adults, representing a significant burden of bacterial, rather than fungal, etiology [21].

Currently, intramuscular benzathine penicillin or a ten-day course of oral penicillin remains the first-line treatment for bacterial pharyngitis, owing to its established efficacy and affordability. For patients with penicillin allergies, macrolides and first-generation cephalosporins represent clinically acceptable alternatives [22]; however, the increasing prevalence of macrolide-resistant *S. pyogenes* strains has relegated these agents to second- or third-line options in the management of streptococcal pharyngitis [23]. In the context of severe invasive *S. pyogenes* infections, vancomycin is often employed [24]. Furthermore, surgical debridement of necrotic tissue is a crucial adjunct in managing complicated soft tissue infections caused by this pathogen [24]. The escalating challenge of antibiotic resistance and the prevalence of penicillin allergies underscore the critical need for exploring novel therapeutic strategies, a necessity that forms the foundation of the present investigation.

To the best of our knowledge, this study represents the initial investigation into the *in vitro* antibacterial activity of capsaicin and an aqueous extract of *C. annuum* L. against *S. pyogenes*, with a direct comparison to the effects of amoxicillin. We employed broth microdilution assays to rigorously determine the MIC and MBC of both capsaicin and the *C. annuum* extract against *S. pyogenes*. Each assay was performed in quadruplicate and included a positive control to ensure the validity of the experimental results. Capsaicin was evaluated across a concentration gradient ranging from 5 to 50 µg/mL, while the aqueous pepper extract was tested at concentrations of 0 to 50 mg/mL and 0 to 20 mg/mL in separate experiments**.**

To further refine our concentration assessments and corroborate the MIC and MBC values obtained from the broth microdilution assays, we performed spectrophotometric analysis. Solutions were prepared at the previously tested concentrations, and their optical density was measured using a spectrophotometer. The spectrophotometric data subsequently confirmed our initial findings regarding the MIC and MBC for both capsaicin and the aqueous extract of *C. annuum*. These results were then further validated using the disk diffusion method. This assay revealed that *S. pyogenes* exhibited sensitivity to capsaicin at concentrations ranging from 15 to 25 µg/mL, characterized by inhibition zones of ≥16 mm. Similarly, *S. pyogenes* demonstrated sensitivity to the aqueous pepper extract at concentrations between 12 and 16 mg/mL, producing inhibition zones of ≥14 mm.

Statistical analysis employing Tukey's post hoc test to compare the mean effects of each treatment revealed statistically significant differences among the groups. Specifically, the concentration of the aqueous extract of *C. annuum* L. determined to elicit a significant effect was 15 mg/mL, while for capsaicin, this was established at 20 µg/mL, both warranting further scientific investigation and consideration for potential clinical applications. In contrast, amoxicillin demonstrated efficacy at a concentration of 30 mg/mL. Collectively, the data derived from disk diffusion, broth microdilution, and spectrophotometric assays strongly suggest that

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capsaicin exhibits a potent antibacterial effect against *S. pyogenes* at microgram concentrations, a level of activity notably higher than that observed with amoxicillin or the aqueous pepper extract, which required milligram concentrations. This finding underscores the potential of capsaicin as a promising novel agent in the therapeutic management of *S. pyogenes* infections, offering a compelling perspective on the utility of natural antibacterial compounds.

Previous studies, which largely align with our findings, have predominantly employed the broth microdilution method [25-27]. However, these investigations often reported the efficacy of aqueous pepper extract and capsaicin against various microorganisms without a comprehensive analysis of their concentration-dependent effects. In contrast, our research meticulously demonstrated the concentration-dependent effects of these agents on *S. pyogenes*. The MIC and MBC for the aqueous pepper extract were determined to be 12 mg/mL and 14 mg/mL, respectively. For capsaicin, the MIC was 15 µg/mL, and the MBC was 20 µg/mL. Through disk diffusion assays, we further confirmed the consistent inhibitory and bactericidal activity of both capsaicin and the aqueous pepper extract against *S. pyogenes*. Furthermore, the combination of capsaicin and amoxicillin demonstrated a statistically significant synergistic interaction ($P< 0.05$ and $P< 0.01$), strongly indicating a potentiated antibacterial effect when capsaicin was combined with amoxicillin.

Given the inherent virulence of *S. pyogenes* and the substantial annual morbidity and mortality attributed to streptococcal infections, the significance of our findings is readily apparent. Moreover, the escalating global challenge of antibiotic resistance, coupled with the well-documented and often irreversible adverse effects associated with synthetic antimicrobial agents, underscores the critical importance of this investigation. Our research is strategically focused on exploring the potential of natural botanical extracts as viable alternatives or synergistic complements to conventional antibiotics.

This study is not without limitation, most notably the inherent variability in the cultivation conditions of *C. annuum* L., including factors such as temperature, sunlight exposure, and irrigation practices. These parameters, which are known to differ geographically, could potentially influence the phytochemical profile of the peppers, thereby affecting the reproducibility and generalizability of our findings. Furthermore, a crucial consideration for all antimicrobial agents, including those derived from natural sources, is their safety profile. To comprehensively assess the therapeutic index, future investigations should incorporate larger-scale *in vivo* studies utilizing animal models to establish an efficacious dose with minimal adverse effects. Importantly, sourcing clinical isolates directly from a diverse patient population would be advantageous. This approach would enable the investigation of potential synergistic or antagonistic interactions in the context of polymicrobial infections or when encountering pathogens beyond *S. pyogenes*, facilitating a more comprehensive quantitative and qualitative analysis of the interactions of these botanical agents.

In conclusion**,** this study robustly demonstrates the potential of natural botanical extracts as promising alternatives in addressing the escalating challenge of antimicrobial resistance. Our findings unequivocally show that capsaicin and aqueous *C. annuum* L. extract exhibit significant bactericidal and inhibitory activities against *S. pyogenes*. These results strongly suggest that these natural compounds could serve as fundamental components in the development of novel antimicrobial agents, particularly for combating antibiotic-resistant strains. Furthermore, there is intriguing potential for these extracts to be integrated into food products as natural preservatives to mitigate bacterial contamination and promote public health. However, further rigorous research is imperative to fully elucidate their safety profile, determine optimal dosing regimens, and mitigate any potential adverse effects associated with their consumption.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests associated with this manuscript.

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