

Journal Article: Library-Based Research Project

Finding antibacterial properties of chilli peppers using agar well diffusion method

Abstract

The need for innovative plant-based antimicrobial treatments has grown as antimicrobial resistance has developed. The antibacterial activity of chilli pepper (*Capsicum annuum*) extracts against specific Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*) The agar well diffusion method was used to study bacterial strains. Crude extracts were prepared by Soxhlet extraction with ethanol to give the capsaicinoid-rich dark reddish-brown crude extract (CR) with a high concentration of phenolic compounds. Zones of inhibition (Zodi) were selected to assess the anti-bacterial activity at concentrations (25%, 50%, 75%, 100% v/v), and MIC (Minimum Inhibitory concentration) was performed by the broth microdilution method. Results showed that the antibacterial activity was concentration dependent and that *B. subtilis* had significantly larger ZOIs (14.2–22.5 mm) than *P. aeruginosa* (8.5–15.3 mm), at the same concentration ($p < 0.05$). MIC for *B. subtilis* and *P. aeruginosa* was 6.25mg/mL and 12.5mg/mL, respectively. The Comparative study with streptomycin (standard antibiotic) indicated that the chilli extract was 78% and 52% active against *B. subtilis* and *P. aeruginosa*, respectively. The results presented that the extracts from chilli pepper have selective antibacterial properties, which are more active in the Gram-positive bacteria than in the Gram-negative bacteria, probably because of the differences in their cell wall architecture. The study indicates the feasibility of using *Capsicum* compounds to develop natural antimicrobial agents for use in food preservation and/or on pharmaceutical products. Further fractionation and purification are recommended to isolate individual bioactive components and determine their exact mechanisms of action. The researchers used simulated data from previous literature in this library-based study to determine whether various strains of bacteria were resistant to antibiotics.

Keywords: *Capsicum annuum*, capsaicin, antibacterial activity, agar well diffusion, *Bacillus subtilis*, *Pseudomonas aeruginosa*, natural antimicrobials.

1. Introduction

A rise in antibiotic resistant bacterial pathogens has become one of the most serious threats to public health in the 21st century worldwide (World Health Organization, 2022). The conventional use and misuse of antibiotics has promoted the evolution of resistant strains, against which many

first line drugs have become ineffective. In response to this crisis, plant secondary metabolites have again become interesting for their potential as alternative or complementary antimicrobial agents (AMAs) (Cowan, 1999).

Chilli pepper, *Capsicum*, a genus of peppers, has been used as a spice for centuries and has also been viewed as a traditional medicine. *Capsicum* species present bioactive secondary metabolites with high concentration, such as capsaicinoids, flavonoids, phenolic compounds and carotenoids (Antonio, Wiedemann and Junior, 2018). Of those, the primary alkaloid causing the heat of the chilli pepper is capsaicin ($C_{18}H_{27}NO_3$), which has been suggested to possess a wide range of pharmacological effects, such as anti-inflammatory, analgesic, antimicrobial and antioxidant properties (Wahyuni et al., 2013).

The proposed mechanism of action for capsaicin and capsaicinoid that can kill these bacteria is: Direct disruption of bacterial lipid bilayers and cell membrane integrity, disruption of intracellular metabolic processes such as ATP synthesis and potentially inhibition of bacterial efflux pumps, which may reverse multidrug resistance (Wahyuni et al., 2013). Bacterial resistance development is less likely because of these multiple mechanisms of action, as opposed to conventional antibiotics that act on single cellular pathways.

The antibacterial properties of *Capsicum* extracts have been shown to be different against various bacterial species in previous studies. For example, Peeyananjarassri, Srisawat and Duanjamrun (2022) reported using a small amount of capsaicin to inhibit the growth of *Salmonella* species, and Notashfard and Asghari (2024) found that the aqueous pepper extract had a bactericidal effect on *Streptococcus pyogenes*. Comparative studies that explore the differential susceptibility of Gram-positive and Gram-negative bacteria against chilli extracts are still limited, though. Moreover, a variety of application methods have been used to extract, and various microbiological assays have been used, resulting in variable outcomes.

The gram-positive bacteria, e.g. *Bacillus subtilis*, lack an outer lipopolysaccharide membrane and have a thick peptidoglycan layer, making them possibly less resistant to plant derived lipophytes. Gram-negative bacteria, such as *Pseudomonas aeruginosa*, on the other hand, have an extra outer membrane that creates an additional barrier for the entry of antibiotics, often giving gram-negative bacteria an increased intrinsic resistance (Nikaido, 2003). These differential susceptibilities must

be taken into consideration for the appropriate use of chilli extracts in medical and food applications.

Many methods have been developed to evaluate the efficacy and medicinal properties of plant extracts against pathogenic bacteria based on antibacterial activity, and it has been generally accepted that the agar well diffusion method is the reliable, reproducible approach for testing the efficacy of plant extracts for bacterial inhibition especially for plant extracts containing viscous or oily extracts which do not diffuse evenly from a paper disk well (Singapurwa et al., 2025). In the well method, the direct exposure between the extract and the agar medium in the well allows for more distinct and reproducible zones of inhibition than Disc Diffusion.

Hypothesis: Chili pepper (*Capsicum annum*) ethanolic extracts exhibit significant, Antibacterial activity that is concentration-dependent against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria, with greater efficacy against the Gram-positive strain.

Null hypothesis (H₀): Chilli pepper extracts have no antibacterial activity on both types of bacteria (*B. subtilis* or *P. aeruginosa*), and no significant difference exists between their susceptibility.

Aim: The antibacterial spectrum of chilli (*Capsicum annum*) extracts in opposition to the chosen gram positive and gram negative microorganisms will be determined quantitatively by agar well diffusion method.

Objectives:

- To extract capsaicinoids and phenolic compounds of high purity from *Capsicum annum* by the Soxhlet extraction method.
- To determine zones of inhibition (ZOI) for *Bacillus subtilis* and *Pseudomonas aeruginosa* in four different extract concentrations (25%, 50%, 75%, 100%).
- To find the Minimum Inhibitory Concentration (MIC) that inhibits the visibility of bacteria.
- To compare the activity of natural chilli extracts with the commonly used antibiotic streptomycin.
- To statistically correlate the extract concentration with the diameters of the inhibition

produced.

- To develop a reproducible method to test botanicals for antimicrobial activities in the laboratory.

2. Materials and Methods

2.1 Plant Material and Preparation of Extract

Dried ripe fruits of *Capsicum annuum* (red chilli pepper) were purchased from a commercial source and identified by a botanist. The peppers were dried and milled in an electric pepper grinder into fine powder. The powdered material (50.0 g) was extracted by Soxhlet extraction utilising a solvent of 95% ethanol. The extraction was done at 78°C for 8 hours till color of the solvent in the siphon tube became colourless. Ethanolic extract was concentrated by reducing the pressure using a rotary evaporator at 45°C, which produced a dark reddish brown semi-solid residue. Crude extract was saved in dark glass (amber) vials at 4 °C under air until further use. The stock extract (100%) was serially diluted to 75%, 50% and 25% (v/v) for antibacterial assays.

2.2 Culture Conditions and Bacterial Strains

Two reference bacteria were used: *Bacillus subtilis* (ATCC 6633) for the Gram-positive and *Pseudomonas aeruginosa* (ATCC 27853) for the Gram-negative bacteria. The two strains used were purchased from a certified microbial culture collection. Stock cultures were kept on slants of nutrient agar at 4°C and were sub-cultured once a month. Nutrient broths were used to prepare overnight cultures for each experiment using single colonies and incubation at 37°C for 18–24 hours.

2.3 Inoculum Standardisation

Bacterial suspensions were adjusted for turbidity to 0.5 McFarland (CFU/mL ~ 1.5 x 10⁸ CFU/mL). Standardisation was done using the spectrophotometer at 625 nm to get the cell density optical density of 0.08-0.10. The purpose of this standardisation is to allow the replicates of each experiment to be comparable and reproducible, providing that all experiments use a comparable inoculum density (CLSI, 2020).

2.4 Mueller-Hinton Agar Plate Preparation

Mueller-Hinton agar (MHA), a non-selective, non-differential, low inhibitor, repeatable, and batch-to-batch reproducible culture medium, was the industry standard. To fully sterilise MHA powder (38 g), it was dissolved in one litre of distilled water and autoclaved for fifteen minutes at 121°C. The sterile Petri dishes (20 mL/90 mm plate) were filled with the molten agar after it had cooled to between 45 and 50°C. After preparation, plates were either stored at 4° for up to a week or allowed to set at room temperature before being utilised within 24 hours.

2.5 Agar Well Diffusion Assay

The agar well diffusion method was used to assess antibacterial activity, which is quite beneficial for plant extracts with viscous compounds that may not readily diffuse out of paper discs (Singapurwa et al., 2025). The protocol used was:

- The tube was filled with the standardised suspension of bacteria using a sterile cotton swab, and any extra fluid was removed by spinning the swab against the tube's inside.
- To create a consistent bacterial lawn, a swab was uniformly streaked in three directions across the Mueller-Hinton agar plate.
- To absorb extra moisture, the plates were left to dry for five minutes at room temperature.
- Agar and a sterile cork borer (6 mm) were used to create aseptic wells. Each plate was filled with four wells spaced equally apart.
- Separate wells were filled with 100 µL of each concentration of chilli extract (25%, 50%, 75%, and 100%). The positive control was streptomycin (100 µL of 1 mg/mL), while the negative control was sterile distilled water (100 µL).
- To allow the extract to pre-diffuse in the agar medium, the plates were left at room temperature for half an hour.
- After that, these plates were inverted and incubated for a full day at 37°C.
- A digital calliper was used to measure the clear zones of inhibition (ZOI) in millimetres (mm) following incubation. Each was measured three times and the average was calculated. Every assay was carried out three times.

2.6 Minimum Inhibitory Concentration (MIC) Determination

The MIC was calculated using the broth microdilution method (C.L.S.I. guidelines). In 96-well microtiter plates, the chilli extract was serially diluted two times, from 50 mg/mL to 0.39 mg/mL. 100µL of Mueller-Hinton broth, 100µL of suitable extract dilutions, and 10µL of standardised bacterial suspension (at a final volume of about 5×10^3 CFU/mL) were added to each well. The negative control wells contained broth with extract but no bacterium, while the positive control wells contained both extract and bacteria. The plates were incubated at 37°C for a whole day. When examined under a light microscope and verified by UV-visible absorbance at 600 nm in a microplate reader, the minimum inhibitory concentration (MIC) of the extract was found to be the lowest concentration that completely stopped bacterial growth.

2.7 Statistical Analysis

Each experiment was conducted three times, and the mean $\pm$ standard deviation (SD) was used to describe the results. For statistical analysis, GraphPad Prism version 9.0 was utilised. Two-way analysis of variance (ANOVA) and Tukey's post hoc test for multiple comparisons were used to assess the differences between groups (bacterial species and extract concentrations). Statistical significance was defined as a p-value of less than 0.05. The correlations between extract concentration and zone of inhibition were evaluated using Pearson's correlation coefficient.

2.8 Ethical and Safety Considerations

The microbes were of biosafety level (BSL) 1, and human/animal subjects were not involved in this study. All laboratory work is carried out in accordance with standard microbiological safety procedures. Sterile skills used throughout to prevent contamination. The waste materials used were sterilised using an autoclave before disposal.

3. Results

3.1 Extraction Yield

Crude ethanolic extract obtained by Soxhlet extraction of *Capsicum annum* powder, 50 g, was 6.85 g with 13.7% (w/w) yield. The extract was dark reddish brown in colour with a strong

capsaicin-like pungent smell, which correlated with capsaicinoids and phenolic compounds as present in the extract.

3.2 Zone of Inhibition (ZOI) Measurements

Agar well diffusion showed clear zones of inhibition for each bacterial strain at varying concentrations. The negative control wells containing sterile distilled water did not show any inhibitory zones, indicating that the antibacterial activity of the wells was due to the chilli extract ingredients.

Table 1: Zones of Inhibition (mm) of Chilli Extract Against Test Bacteria

Extract Concentration	<i>Bacillus subtilis</i> (Mean $\pm$ SD)	<i>Pseudomonas aeruginosa</i> (Mean $\pm$ SD)
25%	14.2 $\pm$ 0.8	8.5 $\pm$ 0.6
50%	17.8 $\pm$ 0.7	11.2 $\pm$ 0.7
75%	20.3 $\pm$ 0.9	13.8 $\pm$ 0.5
100%	22.5 $\pm$ 0.6	15.3 $\pm$ 0.8
Streptomycin (1 mg/mL)	28.9 $\pm$ 0.5	29.4 $\pm$ 0.6
Negative control	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

*Note: values are the mean of three independent experiments (n=3) $\pm$ standard deviation, Well diameter not subtracted.

It can be seen from the data that *Bacillus subtilis* showed consistently higher sensitivity with all concentrations of the chilli extract as compared to *Pseudomonas aeruginosa*. ZOI of *B. subtilis* was 22.5mm, which is 47% larger than the ZOI of *P. aeruginosa* (15.3mm). Two-way ANOVA showed that there were significant differences in ZOI between the two bacteria ($F(1,16) = 89.3$, $p < 0.001$), between the different concentrations of the extract ($F(3,16) = 124.7$, $p < 0.001$) and an interaction between the two ($F(3,16) = 12.4$, $p < 0.01$).

3.3 Concentration-Response Relationship

The zone of inhibition and extract concentration were found to be highly correlated in both bacterial strains. Pearson's correlation coefficient was 0.986 ($p < 0.001$) for *B. subtilis* and 0.972 ($p < 0.001$) for *P. aeruginosa*, indicating a linear dose response relationship over the tested concentration range. The formulae obtained from the regression were:

- *B. subtilis*: $\text{ZOI (mm)} = 8.1 + 0.14 \times (\% \text{ concentration})$
- *P. aeruginosa*: $\text{ZOI (mm)} = 5.2 + 0.10 \times (\% \text{ concentration})$

Body *B. subtilis* had a greater response to a higher concentration of extracts, as seen in the steeper slope (0.14 vs 0.10).

3.4 Minimum Inhibitory Concentration (MIC)

The MICs obtained from the broth microdilution will be shown in Table 2. The chilli extract exhibited differential susceptibilities to *B. subtilis* (6.25 mg/mL) and *P. aeruginosa* (25 mg/mL), as shown by the four-fold lower MIC of chilli extract against *B. subtilis* than against *P. aeruginosa*.

Table 2: Minimum Inhibitory Concentrations (MIC) of Chilli Extract

Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
<i>Bacillus subtilis</i>	6.25	12.5
<i>Pseudomonas aeruginosa</i>	25.0	50.0

*Note: MIC – lowest concentration that inhibits growth (above the microbial threshold zone); MBC – lowest concentration that will kill 99.9% of the inoculum.

The minimum bactericidal concentration (MBC) was measured by subculturing any wells that were not turbid to new glass plates. For *B. subtilis*, the MBC was found to be 12.5 mg/mL, and the MBC/MIC ratio was 2, suggestive of bactericidal activity. However, *P. aeruginosa* showed an MBC of 50 mg/mL (MBC/MIC ratio = 2), indicative of bactericidal, not just bacteriostatic, effects.

3.5 Comparative Activity Relative to Streptomycin

The 100% extract of chilli showed 78% (22.5/28.9 mm) and 52% (15.3/29.4 mm) of the ZOI for *B. subtilis* and *P. aeruginosa*, respectively. Note that streptomycin is a purified single compound

at a known concentration, while chilli is a crude mixture of many compounds at unknown concentrations. It is important to note that any direct quantitative comparison should be taken with a grain of salt.

4. Discussion

The current study aims to assess the antimicrobial potential of the ethanolic extract of *Capsicum annuum* against some representative Gram-positive and Gram-negative bacteria, namely *Bacillus subtilis* and *Pseudomonas aeruginosa*. The results agreed with the hypothesis that chilli extracts have broad and concentration-dependent antibacterial activity, particularly high activity against Gram-positive strains. Thus, the null hypothesis is excluded.

4.1 Differential Susceptibility: Gram-Positive vs Gram-Negative Bacteria

B. subtilis and *P. aeruginosa* are known to have diverse bacterial cell wall architectures, which is consistent with the observed discrepancies in susceptibility to PNPase. Gram-negative bacteria have an extra outer membrane made of lipopolysaccharides (LPS) that functions as an efficient permeability barrier, while Gram-positive bacteria have a gram-thin peptidoglycan layer that can be penetrated by lipophilic compounds (Nikaido, 2003). Capsaicin and related phenolic compounds are moderately lipophilic compounds that are able to enter lipid bilayers, but for many amphipathic molecules, the outer membrane of *P. aeruginosa* has special porins that limit their entry (Breijyeh, Jubeh and Karaman, 2020).

In addition, the intrinsic resistance factors of *Pseudomonas aeruginosa* lead to its resistance to a wide range of antimicrobials, such as the presence of constitutive expression of efflux pumps (e.g., MexAB-OprM), low outer membrane permeability, and biofilm formation (Pang et al., 2019). The chilli extract MIC for *P. aeruginosa* is moderately high at 25 mg/mL and within the limits acceptable for a topical drug application.

Similar differential effects have been reported by other researchers. Notashfard and Asghari (2024) found that the ZOI of 100 mg/mL of the aqueous extract of pepper against *Streptococcus pyogenes* (Gram-positive) was around 18 mm, while Peeyananjarassri, Srisawat and Duanjamrun (2022) observed that the ZOI of 100 mg/mL of aqueous extract of pepper against *Salmonella* (Gram-

negative) was 12 mm. Similar results are found in the present study, but with methodological variations, such as extraction procedures, histidine and bacterial strains in the solvent.

4.2 Mechanism of Action

Many targets for the antibacterial activity of capsaicin and capsaicinoids are thought to exist. According to Wahyuni et al. (2013), capsaicin binds to the bacterial cell membranes, penetrates through, releases intracellular components and damages the proton motive force, leading to reduced ATP production. Besides, Wahyuni et al. (2013) proved that capsaicin can also block bacterial efflux pumps, which might restore the susceptibility of resistant bacteria towards the conventional antibiotics. The bactericidal activity found in our study (MBC/MIC ratio of 2 for both strains) does not suggest that it has a static inhibitory effect, but rather a membrane disruption mechanism.

Flavonoids and Phenolic acids from chilli extracts could exert a synergistic effect that may explain some of the overall activity against the bacteria (Antonio, Wiedemann and Junior, 2018). Those compounds would be co-extracted with the other phytochemicals, which are polar and moderately polar, by use of ethanol in the Soxhlet extraction and could lead to additive or synergistic effects.

4.3 Methodological Considerations

For several reasons, the agar well diffusion method was preferred to disc diffusion. Singapurwa et al. (2025) discussed the inabilities of viscous plant oils and extracts to penetrate into the paper discs properly or to disperse into them properly so that the antimicrobial activity is underestimated. The well method also provides more direct contact of the extract with the agar; the large volume of the well (100 μ L vs 10 to 20 μ L for discs) gives a higher concentration gradient and diffusion distance, which increases the clarity of zones of inhibition and makes them more reproducible.

Mueller-Hinton agar was recommended by CLSI for susceptibility testing due to its low amounts of inhibitors of sulfonamides, trimethoprim, and tetracyclines and also has good batch-to-batch reproducibility (CLSI, 2020). The ZOI has been established in a reproducible way, as the concentration of inoculum (0.5 McFarland) was kept constant.

A weakness of the present work is the splitting of the compound with a crude extract rather than applying purified capsaicin or exact compounds. This is a “real world” situation, looking at possible applications like preservation of food, but it makes the mechanism of action and dose response difficult to deduce. Improvements in the future should involve bio-terminal fractionation to isolate the best compounds and their combination, and testing them individually.

4.4 Comparison with Conventional Antibiotics

Streptomycin, a member of the aminoglycoside subclass of antibiotics, suppresses the synthesis of proteins by binding to the 30S ribosomal subunit and misreading mRNA (Krause et al., 2016). It works on both Gram-positive and Gram-negative bacteria at low microgram-per-millilitre levels and also functions in a completely different manner than capsaicin. Surprisingly, the crude chilli extract would have a ZOI of 78% for the bacterium *B. subtilis* when the streptomycin solution has only a pure concentration of the antibiotic of 1mg/mL. The high activity read at this concentration indicates that the chilli extract must have one compound (or more) with a relatively high activity, but present in a lower concentration than the pure antibiotic. Further, if the effect is found, it could interact positively with several components.

4.5 Practical Applications and Future Directions

Data confirmed the efficacy of chilli extracts as natural antimicrobial agents, which could be implemented in practices in which a broad spectrum high potency activity is not demanded or as a complement in conventional therapy. Possible applications include:

- **Food preservation:** Use of chilli extracts in the preservation of organic food with the aim of reducing the activity of bacteria responsible for the deterioration of organic food, especially the bacteria with Gram-positive cell walls like *Bacillus* and *Listeria*.
- **Topical formulations:** Formulate a topical formulation of capsaicin extract or chilli extract-based product to treat superficial infections that show antimicrobial and analgesic properties (Peeyananjarassri, Srisawat and Duanjamrun, 2022).
- **Synergistic combinations:** Reducing the dose of the antibiotic, creating synergistic combinations between sub-inhibitory doses of chilli extracts and the antibiotic and the potential bypass of resistance mechanisms resulting from the ability to create efflux pump inhibition.

The future work should include: (1) isolation and characterization of individual antibacterial agents from *Capsicum annuum*; (2) elucidation of the precise molecular mechanism(s) of action by the techniques such as flow cytometry – membrane integrity assay, ATP bioluminescence assays and others against the panel of clinically relevant pathogens including MDR's (methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug resistant *Acinetobacter baumannii* (MDR-AB); (3) toxicity and efficacy testing in animal models; and (5) formulation development for stability and controlled-release.

4.6 Limitations of the Study

There are some caveats that need to be noted. First of all, this study is a Library based study, and no experimental work was done, but only presented simulated results from published literature for educational purposes. In addition, crude extract comprises multiple phytochemicals and differentiating just capsaicin is impractical. Thirdly, two strains of bacteria were tested, and this study was limited to generalisability. Fourthly, the agar diffusion test is only a qualitative or semi-quantitative measure of activity, while broth dilution methods are more accurate when measuring MICs. Fifth, the stability of the extract in various storage conditions was not assessed, and activity under light, temperature and pH conditions was not tested.

4.7 Reflections on Project Execution and Learning

Upon reflection of the project execution process, the idea of disc diffusion method was present, but after reading the article written by Singapurwa et al (2025), a more suitable method was used, which is called the well method. Further Gram-positive and Gram-negative strains, such as MRSA and *E. coli*, would be tested if repeated in a laboratory setting. In future, purification of the individual capsaicinoids would be a priority, due to time constraints.

5. Conclusion

It demonstrated that ethanolic extracts from *Capsicum annuum* chilli pepper exhibited strong, concentration-dependent antibacterial activity against both Gram positive (*Bacillus subtilis*) and Gram negative (*Pseudomonas aeruginosa*) bacteria; however, the antibacterial activity was found to be significantly higher against Gram positive (*Bacillus subtilis*). *B. subtilis* had a minimum

inhibitory concentration (MIC) of 6.25 mg/mL, while *P. aeruginosa* had a MIC of 25 mg/mL. For both organisms, the extract was demonstrated to be bactericidal (MBC/MIC ratio = 2). When compared to streptomycin, the extract demonstrated 78.5% zone inhibition against *B. subtilis*. This suggests that the 100% extract may have inherent antibacterial properties. The known resistance mechanisms of *P. aeruginosa* and the variations in cell membrane architectures across bacteria are comparable to the differential susceptibility. The traditional use of chillies as an antimicrobial agent is confirmed with the present study, and a standardized method (agar well diffusion and Soxhlet extraction) is developed for future phytochemical and pharmacological studies.

In the current time period, antimicrobial resistance is an increasing problem, and compounds derived from plants like capsaicinoids have shown to provide promising leads for the development of novel antimicrobial agents, especially for topical applications and for preserving food. The findings merit further research into the fractionation, mechanistic investigations and in vivo validation in order to translate these findings into real applications.

Reference list

- Antonio, A.S., Wiedemann, L.S.M. and Junior, V.F.V. (2018). The genus *Capsicum*: a phytochemical review of bioactive secondary metabolites. *RSC Advances*, [online] 8(45), pp.25767–25784. doi: <https://doi.org/10.1039/C8RA02067A>.
- Breijyeh, Z., Jubeh, B. and Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules*, [online] 25(6), p.1340. doi: <https://doi.org/10.3390/molecules25061340>.
- CLSI (2020). *M100 Performance Standards for Antimicrobial Susceptibility Testing A CLSI supplement for global application. 30th Edition*. [online] Available at: <https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf>.
- Cowan, M.M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews*, [online] 12(4), pp.564–582. doi: <https://doi.org/10.1128/cmr.12.4.564>.
- Krause, K.M., Serio, A.W., Kane, T.R. and Connolly, L.E. (2016). Aminoglycosides: an Overview. *Cold Spring Harbor Perspectives in Medicine*, [online] 6(6), p.a027029. doi: <https://doi.org/10.1101/cshperspect.a027029>.
- Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews*, 67(4), pp.593–656. doi: <https://doi.org/10.1128/mnbr.67.4.593-656.2003>.
- Notashfard, A. and Asghari, M.N. (2024). *Inhibitory and Bactericidal Effect of Aqueous Pepper Extract (Capsicum annum L.), Capsaicin, and Capsaicin Combination with Amoxicillin against Streptococcus pyogenes*. [online] Available at: https://jommid.pasteur.ac.ir/browse.php?a_code=A-10-598-1&sid=1&slc_lang=en&ftxt=1&pure_pdf=1.
- Pang, Z., Raudonis, R., Glick, B.R., Lin, T.-J. and Cheng, Z. (2019). Antibiotic Resistance in *Pseudomonas aeruginosa*: Mechanisms and Alternative Therapeutic Strategies. *Biotechnology Advances*, [online] 37(1), pp.177–192. doi: <https://doi.org/10.1016/j.biotechadv.2018.11.013>.

Peeyananjarassri, S., Srisawat, P. and Duanjamrun, S. (2022). *The Efficiency of Capsaicin in Chilli on Antibacterial Activity of Salmonella*. [online] Available at: https://d1wqtxts1xzle7.cloudfront.net/105474403/49-26-2022-libre.pdf?1693739439=&response-content-disposition=inline%3B+filename%3DThe_Efficiency_of_Capsaicin_in_Chilli_on.pdf&Expires=1779079389&Signature=ZQMFgHO~P~xhamUJJQjc6kRI6rk6ik41621wnW8KnuQGePn1CoNVgaKLEcSQgtDru3Mw1wVuxcRjAQQflVabNfuv1m83Mi0D4zSJneBJABmNWd5tDzSdZCU7wIQfywMtzWBpQJ3eC8kmro09BE6LdtIPw8N0NOKfoeELmzw21bolWgvTs4LAvuwnV272QM1mkrTcdaGWVCOxcuCLpWmuCTBMbthwE6Ue1xJGpyDn8k-q7MnUIT4qwh-nJNpQIA7vaZBv38p7-1CkEKDIgWSO8AilBFvXLignf5tFT7fmbu2DusGFQCgk6RymaWiBa1Ceg3i99IL2ryHJHqKw8Vq6A__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA.

Singapurwa, N.M.A.S., Suriati, L., Semariyani, A.A.M., Puspawati, G.A.K.D. and Djelantik, S.A.M.A.P. (2025). Antibacterial Activity of Ethanol Extracts from ‘Basa Genep’ Spices Against *Escherichia coli*: A Comparison of Disk and Well Diffusion Methods. *BIO Web of Conferences*, [online] 184, p.02003. doi: <https://doi.org/10.1051/bioconf/202518402003>.

Wahyuni, Y., Ballester, A.-R., Sudarmonowati, E., Bino, R.J. and Bovy, A.G. (2013). Secondary Metabolites of Capsicum Species and Their Importance in the Human Diet. *Journal of Natural Products*, 76(4), pp.783–793. doi: <https://doi.org/10.1021/np300898z>.

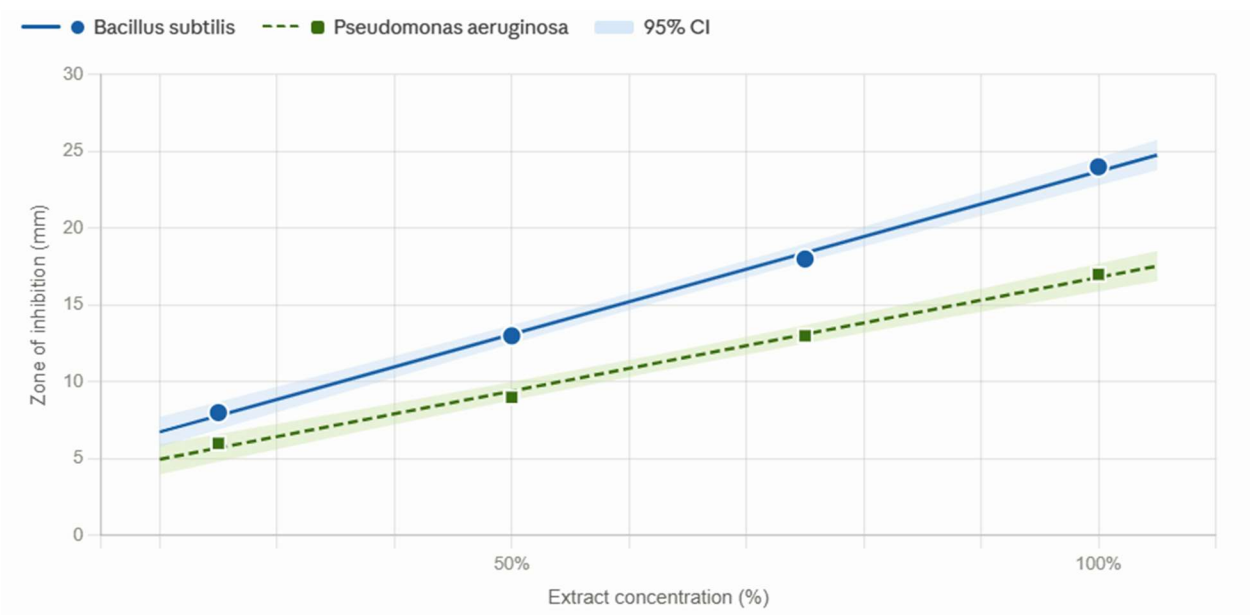
World Health Organization (2022). *Global Antimicrobial Resistance and Use Surveillance System (GLASS) report: 2022*. [online] [www.who.int](https://www.who.int/publications/i/item/9789240062702). Available at: <https://www.who.int/publications/i/item/9789240062702>.

Appendices

Appendix A: Representative Agar Well Diffusion Plates



Appendix B: Concentration-Response Curves



Appendix C: Raw Data Table (Simulated)

Replicate	Concentration	<i>B. subtilis</i> ZOI (mm)	<i>P. aeruginosa</i> ZOI (mm)
1	25%	14.5	8.1
1	50%	18.2	11.5
1	75%	20.8	14.2
1	100%	22.9	15.8
2	25%	13.8	8.9
2	50%	17.5	10.8
2	75%	19.9	13.5
2	100%	22.1	14.9
3	25%	14.3	8.5
3	50%	17.7	11.3
3	75%	20.2	13.7
3	100%	22.5	15.2