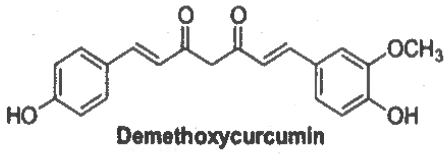
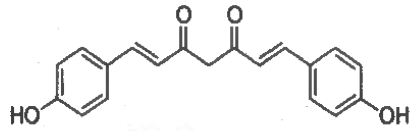
Candidate 2 evidence

The effect of turmeric concentration on inhibition of Micrococcus Luteus by Penicillin

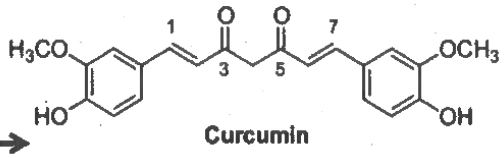
Curcuminoids



Demethoxycurcumin



Bisdemethoxycurcumin



Curcumin

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1 Abstract

This investigation looked into the effect of turmeric concentration on inhibition of M. luteus by penicillin. Turmeric solutions of varying concentrations (0–100%) were added to nutrient broths with M. luteus, and lawn plates were prepared. Penicillin disks were applied, and zones of inhibition were measured after 24 hours. Results showed a clear increase in inhibition of M. luteus by penicillin with higher turmeric concentrations, from 3.0 cm at 0% to 3.6 cm at 100%, with low variation across replicates. Findings suggest turmeric enhances penicillin's inhibition of M. luteus, likely due to curcumin's antimicrobial properties, highlighting its potential as a natural antibiotic enhancer.

2 Introduction

2.1 Aim

To investigate the effect of turmeric concentration on penicillin's inhibition of micrococcus luteus.

2.2 Hypothesis

Increasing turmeric concentration will increase penicillin inhibition against micrococcus luteus.

2.3 Underlying Biology

2.3.1 Bacterial structure: Gram-Positive vs, Gram-Negative

Bacterial cells are single celled organisms, also known as prokaryotes – cells lacking in a nucleus and other membrane-bound organelles. Bacteria can be classified as either gram-positive or gram-negative. The main difference of these different bacterium types is within the cell wall structure. Gram-negative bacteria have a thin peptidoglycan layer surrounded by an outer membrane (also known as the bilayer) consisting of lipopolysaccharides (LPS) and phospholipids whereas gram-positive have a thick peptidoglycan layer with embedded teichoic acids and no outer membrane. This bilayer found in gram negative bacteria acts as a physical barrier to substances (including certain antibiotics) and helps with immune evasion.

2.3.2 Structure and Antibiotic Resistance Mechanisms in M. Luteus

The cell wall of M. Luteus consists of a thick peptidoglycan layer made up of a network of sugars cross-linked by peptide chains (typical of gram-positive bacteria), which gives the cells their shaped shape and helps them to withstand pressure. (1) Covalently linked to the peptidoglycan layer are phosphate rich molecules called teichoic acids (WTAs). WTAs help protect the bacterium from β-lactams as they can shield penicillin-binding proteins (PBPs) by altering the cross-linking of peptidoglycan. In addition, WTAs can increase the activity of bacterial efflux pumps. Efflux pumps are transmembrane transporter proteins that recognize and expel antibiotics, which reduces drug accumulation. (2,3,4)

2.3.3 Quorum sensing and Biofilm Resistance Mechanisms

Microorganisms can live on their own or as part of a consortium i.e., biofilm. Quorum sensing (QS) detects small quantities of hydrophobic signaling molecules called autoinducers (AI) to regulate gene expression for virulence factors (molecules or traits produced by pathogens that enhance its ability to survive and infect a host). QS regulates gene expression for biofilm in

response to fluctuations of cell population density and allows the biofilm to act as a cohesive group by coordinating collective behaviors. During quorum sensing, once the threshold level of an AI is reached, gene expression for virulence factors is turned on. (5,8) Extracellular polymeric substances (EPSs) are then produced. EPS are mostly comprised of polysaccharides, nucleic acids, and proteins, giving the biofilm its gel-like structure and provides physical protection from antibiotics by decreasing antibiotic penetration. This biofilm defense mechanism is further supported by the formation of persister cells. Persister cells are a phenotype of regular cells that are dormant, and neither grow normally nor die in the presence of potent antibiotics. They are also metabolically inert, show slow growth and replication. Persister cells are also able to repopulate the biofilm after antibiotic reduction. (6)

2.3.4 Turmeric and Curcumin: Uses and properties

Turmeric is a popular spice derived from the plant Curcuma longa, native to southeast Asia, and has been historically used as a flavoring agent and dyes. It was then adopted into traditional Chinese medicine for its anti-inflammatory properties. Over 100 compounds have been derived from turmeric, including colouring agents coined curcuminoids; curcumin demethoxycurcumin, 5'-methoxycurcumin, and dihydrocurcumin, which are found to be natural antioxidants. (7) The active compound curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a tautomeric compound, meaning that it exists at 2 interconvertible isomers at equilibrium. In the case of curcumin, these compounds are diketone and enol.

2.3.5 Curcumin's Antibacterial and Biofilm disrupting Mechanisms

Curcumin has multiple modes of action against bacteria. It can inhibit cell division in bacteria by binding to microtubules therefore disrupting the mitotic process, and by altering RNA so protein synthesis cannot occur properly, disrupting G2 interphase. Some studies have also shown that curcumin inhibits extracellular proteins, including sortase A, interrupting cell adhesion and biofilm formation. A study by has shown that curcumin can anchor itself deep into the membrane on bacterial cells by forming intermolecular hydrogen bonds with phosphate groups on phospholipids, disrupting the localisation of integral membrane proteins, leading to increased membrane permeability. Curcumin can also combine with peptidoglycan, altering the integrity of the cell wall leading to cell lysis. (9)

In addition, curcumin can also inhibit quorum sensing. Curcumin has been shown to inhibit enzymes involved in AI synthesis, therefore preventing the threshold concentration of AI from being reached, so genes for virulence factors cannot be expressed. This includes EPS

production. Without EPS production this leads to a decrease in biomass in the biofilm and microorganisms in the biofilm have less nutrients to thrive. (8,9) Curcumin also can destabilize preexisting biofilms by weakening the structural integrity of the polysaccharides in the EPS, increasing its permeability and thus susceptibility to antibiotics. (8)

2.3.6 Penicillin mechanism of action

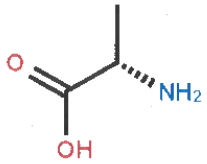


Figure 1: Structure of D-alanine

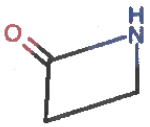


Figure 2: β-lactam ring structure

Penicillin G (benzylpenicillin) belongs to a group of antibiotics called β-lactams which share the common chemical structure β-lactam ring, and work by inhibiting the last stage of peptidoglycan synthesis. Peptidoglycan is made of glycan and peptide cross-links. Glycan chains are long repeating chains of disaccharides consisting of N-acetylglucosamine (GlcNAc) and N-Acetylmuramic acid (MurNAc). The peptide cross-linkers are short chains of amino acids with D-alanine at the end. After the glycan chains are synthesized in the cytoplasm, they move to the bacterial membrane and the peptide cross-linkers attach to MurNAc on the glycan chains. PBPs then catalyze the formation of the cross-link formation between the D-alanine on one peptide to an amino acid on the adjacent peptide. This forms a mesh-like structure which protects bacteria from osmotic pressure and helps act as a physical barrier to antibiotics. Penicillin contains a beta-lactam ring which has a similar structure to D-alanine and therefore can mimic the substrate of PBPs and prevent the bacteria from forming this strong-stable protective wall. As a result, the bacterium can no longer withstand osmotic pressures and causing it to undergo osmotic lysis. (10,11,12)

2.3.7 Biological significance

Potential research for combination therapies of curcumin and penicillin can be done to further study the synergistic effects of them against certain bacteria. This also could lead to more research into natural compounds like turmeric potentially helping to slow the constantly evolving resistance mechanisms of bacteria. Turmeric is also a cheap to produce, readily available substance that could be a cheap addition to antibiotic treatments.

3 Procedures

3.1 Pilot study

Different bacteria were plated out for different incubation times to determine which bacteria would give the best growth within the time restrictions in the lab. 24-hour and 48-hour incubation times were tested, and it was found that M. luteus showed the best growth within a 24-hour time period, so this combination was selected for the study.

Another pilot study was carried out to find what concentration of penicillin disks would show the best zones of inhibitions without overlapping to allow the zones to be measured. It was found that 2 micrograms strength of penicillin disks showed maximum inhibition of M. luteus without overlapping zones and so was selected.

Another pilot was carried out to find out what starting concentration of turmeric would give measurable results while not having too much turmeric that it clumps together and invalidates the results. A popular brand of turmeric shots contained 7g turmeric in a 40ml shot, so this dilution was made. Since turmeric is only partially soluble in water the turmeric solution was gently heated on a hot plate and stirred constantly for 20 minutes. Linear dilutions of 75%, 50%, 25% and 0% were made of the original solution and 1ml were added to 9ml nutrient broths along with 1ml m. luteus and left to interact for 2 hours. The solutions were then plated out with P2 penicillin disks and a water disk as a negative control. It was observed in the 100%, 75% and 50% plates showed less inhibition than the 25% plate, as the turmeric was clumping together and not mixing fully with the bacteria. The 25% plate consistently showed larger zones of inhibition than the control. Therefore, for the final study the 25% dilution was used as the maximum concentration.

3.2 Method

Using a 2 decimal point balance, 3.5g of turmeric was added to a small beaker alongside 80ml deionized water and gently heated on a heating plate for 20 minutes while being stirred continuously using a magnetic flea. Different concentrations of turmeric solutions were then made up by mixing various volumes of the original turmeric solution with deionized water using a 10ml graduated pipette as outlined in **Table 1.**

Turmeric Concentration (%)	Water (ml)	Turmeric Solution (ml)
100	0	10
75	2.5	7.5
50	5	5
25	7.5	2.5
0	10	0

Table 1: Turmeric solution dilutions

The lab desks were sprayed down with Virkon. 9ml nutrient broths were appropriately labelled with turmeric concentrations before 1ml of each turmeric solution were added using a 1ml autopipette. The neck of each broth was flamed in a blue flame after opening and before closing, and pipette tips were disposed of in Virkon. The neck of a M. luteus broth was flamed and 1ml was taken into the p1000 autopipette. The neck of the bottle was re flamed and the lid was secured on. One of the nutrient broths containing turmeric was opened and flamed and the 1ml of M. luteus stock broth was pipetted into the broth and the contents of the bottle were pipetted to ensure thorough mixing. The pipette tip was disposed of in Virkon. The neck of the bottle was re flamed and the lid was secured on. This was repeated for the 4 other concentrations of turmeric and the broths were left for 2 hours to interact before plating out. Aseptic techniques were used to clean the desk.

The lab desks were then sprayed down with Virkon again and 0.1ml of each turmeric concentration broth containing M. luteus was plated out onto a simple lawn (nutrient agar) plate using a p100 autopipette, making sure to flame the neck of each broth after opening and before closing and disposing of the autopipette tips in Virkon after each use. The glass lawn spreader was also dipped in ethanol and flamed before and after each use. Sterile metal tweezers were dipped in ethanol and flamed before using to add four 2 microgram penicillin disks to each plate with even separation to ensure no overlapping of zones of inhibition. A penicillin disk was placed on each cross of the template as shown in **Figure 3** to ensure even separation.

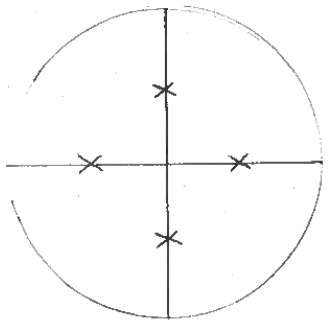


Figure 3: Lawn plate guide

The plates were then left in an incubator set to 30°C for 24 hours and then zones of inhibition around each disk were measured and recorded. The direction of which the zones were measured was kept from north to south for all zones of inhibition. All surfaces were sprayed with Virkon after use.

3.3 Control of confounding variables

Zones of inhibition were also used to gather data instead of colony counting to try and limit the effect of this natural variation. The same source of turmeric was used for each repeat to limit natural variation. All plates that were used were of the same area and depth of agar. Each penicillin disk used within each repeat was from the same packet and of the same area and thickness. Temperature is a factor that affects m. luteus growth, so the lawn plates were left in an incubator at a set temperature. The quantity of m. luteus also had to remain consistent throughout the experiment, and since it's impossible to control the number of cells of M. luteus in each broth a standardised volume of m. luteus broth was used (1ml in this case, with 0.1ml being plated out each time) and all broths were thoroughly mixed to ensure consistency in bacterial concentration, as well as all turmeric solutions were mixed thoroughly before inoculation. Contamination could also alter the validity of the experiments, so strict aseptic techniques were used including working near a blue flame to reduce airborne contamination, flaming necks of bottles and lawn spreaders, using sterilised equipment and disinfecting surfaces with Virkon. To keep pH constant, agar plates of the same pH were used throughout the experiment.

3.4 Negative Control

A solution containing 9ml nutrient broth, 1ml M. luteus, and 1ml water with no turmeric was plated out with m. luteus and 2 microgram penicillin disks. This was to prove that turmeric was causing an increase in the zones of inhibition.

3.5 Independent Replicate

An independent replicate was also carried out on a different day with fresh nutrient broths, fresh M. luteus stock culture, new pack of antibiotic disks, new bottle of turmeric and fresh agar plates. This was to ensure validity of results in order to compare and eliminate potential reasons for differences.

3.6 Sample size

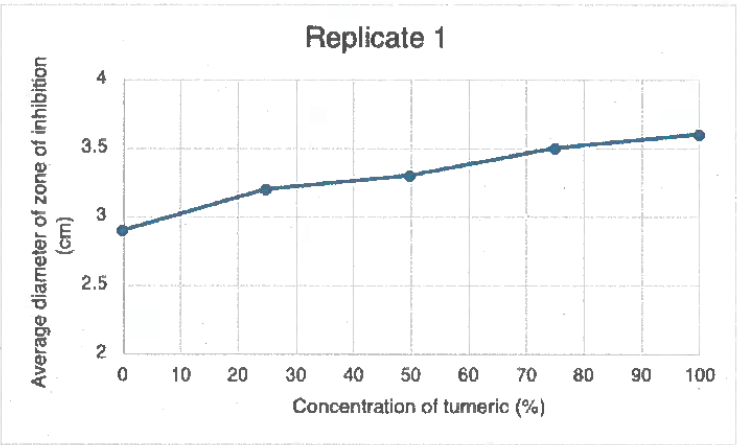
Due to the nature of different numbers of bacterial cells in the assigned volume of m. luteus it was deemed that 4 penicillin disks per plate would be appropriate number of repeats given the variation within a bacterial population. The pilot study also confirmed this to be true.

4 Results

4.1 Experimental Data

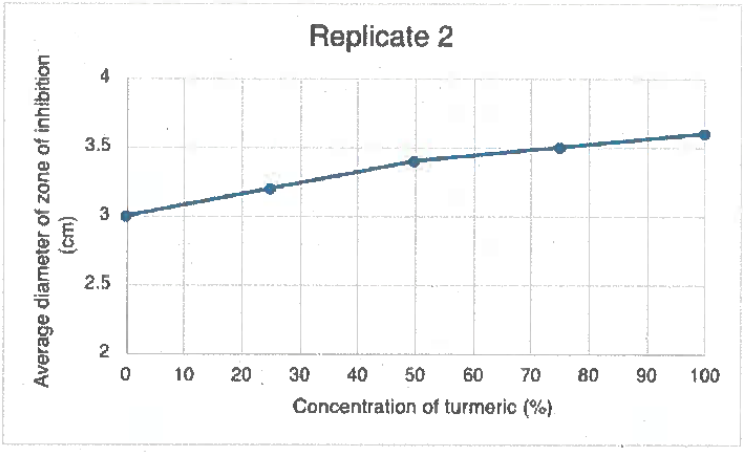
4.1.1 Replicate 1

Turmeric concentration (%)	Diameter of zone of inhibition (cm)				
	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Average
0	2.9	3.0	2.8	2.9	2.9
25	3.1	3.2	3.2	3.2	3.2
50	3.2	3.3	3.2	3.3	3.3
75	3.5	3.4	3.5	3.5	3.5
100	3.6	3.6	3.6	3.6	3.6



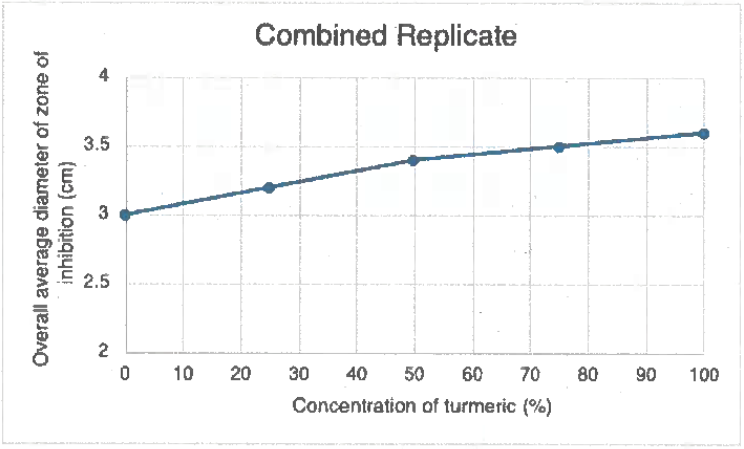
4.1.2 Replicate 2

Turmeric concentration (%)	Diameter of zone of inhibition (cm)				
	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Average
0	3.0	3.0	3.1	3.0	3.0
25	3.2	3.1	3.1	3.2	3.2
50	3.4	3.3	3.4	3.5	3.4
75	3.5	3.5	3.5	3.5	3.5
100	3.7	3.6	3.6	3.6	3.6



4.1.3 Combined Replicate

Turmeric concentration (%)	Diameter of zone of inhibition (cm)		
	Replicate 1average	Replicate2 average	Overall Average
0	2.9	3.0	3.0
25	3.2	3.2	3.2
50	3.3	3.4	3.4
75	3.5	3.5	3.5
100	3.6	3.6	3.6



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Turmeric Concentration (%)	Overall Average (cm)	Standard Deviation (cm)
0	3.0	±0.07
25	3.2	±0.05
50	3.4	±0.08
75	3.5	±0.05
100	3.6	±0.00

4 Discussions

4.1 Conclusion

In conclusion, increasing concentrations of turmeric increases penicillin inhibition against m. luteus. This is supported by the averages across replicates 1 and 2, as the turmeric concentration increases from 0%-100%, the zone of inhibition around the penicillin disk increased from 3.0cm-3.6cm.

4.2 Evaluation of procedures

Controls were effectively used to support the validity of the results. A negative control containing only water, M. luteus, and nutrient broth confirmed that any increase in zone of inhibition was due to the turmeric solution. Consistent use of the same batch of penicillin disks, turmeric source, M. luteus stock and agar plates ensured uniformity in materials, further supporting the validity of the experiment.

All key confounding variables were carefully controlled. Temperature was kept constant by using an incubator set to 30°C, and consistent volumes of M. luteus were added to each broth using the same pipette to maintain a standard bacterial concentration. Thorough mixing of all solutions before inoculation ensured even distribution of turmeric and bacteria. Due to natural variation in M. luteus cultures, it's impossible to guarantee that each turmeric concentration broth had the exact same number of m. luteus cells added to them. This may have caused slight fluctuations in results, but this was maintained as much as possible by adding the same volume of m. luteus stock to each turmeric concentration broth and the same volume of each turmeric concentration broth was plated out.

Independent replication was carried out on a separate day with fresh supplies, including fresh bacterial cultures, turmeric solution, and antibiotic disks. This independent replicate ensured the reproducibility of the results by confirming that the same effects were observed across multiple experimental runs. This strengthened the validity of the investigation by showing that

the results were reproducible under the same controlled conditions. By looking at replicate 1 and replicate 2 the results were still found to be very consistent.

To account for natural variation in bacterial growth, 4 repeats were used. This helped reduce the effect of any potential outliers and identify the true trends. This ensured that any observed differences in inhibition zones were consistent and repeatable across multiple instances. As can be seen across the 4 repeats in replicate 1 and 2, there was a high degree of consistency across each repeat. This supports that 4 repeats was adequate for clear and interpretable results.

Since turmeric is only partially soluble in water, the initial quantity of turmeric used in the pilot study didn't provide a pipettable solution as the turmeric was clumping together, so the 25% concentration was taken as the new starting point as it dissolved enough to pipette and showed a noticeable difference in zone of inhibition than the control of 0% turmeric. These solutions were also heated to encourage dissolution. This increased the chance of obtaining a uniform dilution during the experiment.

Modifications were made to ensure that the experiment produced consistent and measurable results. By focusing on the 25% concentration, the study was able to avoid the confounding issue of undissolved turmeric, ensuring that the antimicrobial effects of turmeric could be assessed more clearly.

The use of a 10 ml graduated pipette for accurate measurement of the turmeric solution allowed for precise concentrations of the dilutions to be made. The zones of inhibition were measured consistently from north to south, helping to standardize the measurement process across all repeats and replicates. A consistent volume of bacterial broth (1 ml) and nutrient broth (9 ml) was used to ensure equal bacterial concentration in all trials. These all contribute to a high degree of accuracy in measurements. Accurate measurements were achieved using digital balances calibrated to 2 decimal places, 10ml graduated pipettes and p1000/p100 autopipettes, which provided precise control over liquid volumes. All solutions were mixed thoroughly to ensure homogeneity and eliminate variation due to settling or uneven distribution.

4.3 Evaluation of results

In both replicates the variation across each repeat is low, with most variation occurring at 0% in replicate 1, and replicate 2 most variation at 50%, overall, between the 2 replicates, the trend shown is the same and the values for the averages are similar. The standard deviation is very small in the combined replicate, with the lowest being ±0.00 at 100% and the highest being ±0.08 at 50%, showing that there is very little variation in the data between the replicates. The

average zones of inhibition for each turmeric concentration in both Replicate 1 and Replicate 2 are very close, which demonstrates the reliability and reproducibility of the experiment. This consistency indicates that the experimental procedure was well-controlled and that the results are repeatable.

The trend shown in the data of increasing turmeric concentration increasing zones of inhibition supports the hypothesis, that increasing turmeric concentration would increase penicillin inhibition against m. luteus, by increasing permeability of the membrane and inhibiting biofilm formation. The kit used provided enough accuracy of measurements to support this conclusion and the sample size proved the reliability of the results. The trend in the data collected from the two independent replicates showed, as hypothesised, that as the turmeric concentration increased the diameter of zones of inhibition around the penicillin disks increased. The overall average zone increased from 3.0 cm at 0% turmeric to 3.6 cm at 100% turmeric. Each concentration increase (25%, 50%, 75%, 100%) resulted in a gradual increase in inhibition zone size, suggesting that turmeric contributes to enhanced penicillin inhibition of M. luteus.

These findings suggest that turmeric has a synergistic effect when combined with penicillin against M. luteus. This shows that the compound curcumin found within turmeric does in fact alter the integrity of M. luteus membrane, allowing penicillin to penetrate the cell wall easier. This is supported by the findings in an article *British Columbia Medical Journal* written by Zheng, C et al. where they also found that curcumin can disrupt the localisation of integral membrane proteins by embedding itself in the phospholipid bilayer and allowing substances such as penicillin to pass through the membrane, and also inhibit quorum sensing, preventing the bacterium from producing the necessary substances required to form and maintain a biofilm.

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