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Scientific Inquiry

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Walford Anglican School for Girls

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The effect of bacteriophage in combination with curcumin against planktonic and biofilm formations of Methicillin-resistant *Staphylococcus aureus*: an *in vitro* evaluation

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AIM

The aim of this experiment was to assess the destructive (antibacterial) effect of bacteriophage, curcumin and their combination on planktonic Methicillin-resistant *Staphylococcus aureus* (MRSA) and the biofilm formation of MRSA.

WHY THIS EXPERIMENT?

In 2018, I conducted an experiment that tested the effects of household disinfectants on the gram-negative bacteria *Pseudomonas aeruginosa* for the Oliphant Science Awards. Subsequently, in 2019, I investigated whether there were any harmful bacteria on healthcare workers' mobile phones. This was part of my 2019 Oliphant Science Awards. The experiment was conducted with Clinpath Laboratories and I was introduced to the field of multi-drug resistant bacteria. Upon further research, I learnt that there has been an ongoing problem in the medical field in managing patients with multi-drug resistant bacterial infections.

I came across a media release (BioScience Managers, 2017) of a team that had conducted the first human trial on bacteriophages against *Staphylococcus aureus* infections arising from sinus infections of the nose. This team is based in Adelaide attached to the Basil Hetzel Institute (BHI). Additionally, I found out that curcumin, a naturally occurring compound in turmeric, has been tested against multi-drug resistant bacterial infections. This prompted me to experiment whether a combination of the two treatments would offer any antibacterial effect on multi-drug resistant bacterial infections.

INTRODUCTION

When a bacterium is multi-drug resistant, it is resistant to more than one antibiotic. When antibiotics are used, the bacteria mutate in response, with the strongest capable of surviving and becoming immune to them. The emergence of multi-drug resistant (MDR) bacteria has become a major issue for the treatment of pathogenic bacterial infections across the globe. The arbitrary use of antibiotics has resulted in drug-resistance from self-medication, over usage, prolonged administration and random choice of inappropriate antibiotics (Pallavali et al; 2017). Consequently, every year, at least 700, 000 people die from MDR bacteria globally (Dadgostar, 2019). The Organisation for Economic Co-operation Development (OECD) report in 2018 estimated that an average of 290 deaths occur each year in Australia from MDR bacteria and 10, 430 people will die between 2015 and 2050 (AURA 2019, Third Australian Report, 2019). Although it is causing many deaths already, MDR bacteria are said to overtake cancer as the leading cause of death in 2050 if not treated appropriately, causing close to 10 million deaths per year (Dadgostar, 2019). This predicament is amounting to over nine billion euros in Europe alone and $20 billion in the United States (Dadgostar, 2019). The combined expenditure from 2015 to 2050 in the United States, Canada and Australia is approximately $74 billion US dollars (AURA Antimicrobial Use and Resistance in Australia, 2019). Experts have raised caution and warned that the global community is underprepared to deal the crisis of MDR bacteria. Based on these facts, a new report from the WHO is calling for urgent action to prevent the antimicrobial resistance crisis (WHO Joint News Release, 2019).

Over the past few years, there have been several strategies undertaken to combat MDR bacteria (Pacios et al; 2020, Pallavali et al; 2017). These include:

Antimicrobial peptides (alone or in combination with antibiotics)

Anti-virulence compounds

Phage therapy (alone or in combination with antibiotics)

Synthetic retinoids

Non-antibiotic compounds termed as "drug repurposing" - e.g. anti-inflammatory & glucocorticoids

Anti-psychotics, anti-helminthic, anti-cancerous drugs

Statin compounds

Iron chelation therapy

Photodynamic therapy

Nitric oxide base therapy

BACTERIOPHAGE

Bacteriophages (BPs) are viruses that depend on bacterial hosts for survival. They inject their genetic material to the bacteria's cell by binding to receptors on the cell surface and produce BPs inside of the cell body. When enough of them are produced, they secrete an enzyme called 'endolysin' that punch holes in the bacteria with pressure. When the pressure is immensely high, the bacteria bursts and dies. The issue with antibiotics is that they will attack non-pathogenic or the "good" bacteria that reside in our bodies as well as the pathogenic bacteria. There is no worry of BPs attacking non-pathogenic bacteria because they are specialised to attack only the pathogen that they recognise (Romero-Calle et al; 2019).

CURCUMIN

Curcumin is a yellow chemical produced by the ginger root, turmeric. It is natively grown in Southeast Asia and often used as a spice in Indian cuisines. It is also used as treatment agent with anti-inflammatory, antioxidant, antimicrobial and antitumor properties. Independently, it has been shown to be effective against many gram-positive and gram-negative bacteria (Praditya et al; 2019 Moghadamtousi et al; 2014) in various *in vitro* experiments (experiments performed outside the body). Also, a synergistic action with antibiotics has been identified for biofilms of both gram-positive and gram-negative organisms through *in vitro* experiments.

BIOFILM

Biofilms are accumulations of bacteria that grow on surfaces such as biomaterials, medical devices and household pipes. Due to their close proximity, they can exchange substrates and remove toxic end products and even protect the bacteria from antimicrobial attack and the immune system, causing bacteria to become MDR. Biofilms are formed in five steps.

1) **Initial reversible attachment**

The moving planktonic bacteria comes into contact with the human body.

2-3) Irreversible attachment

The bacteria forms a monolayer and produces an extracellular matrix for its protection.

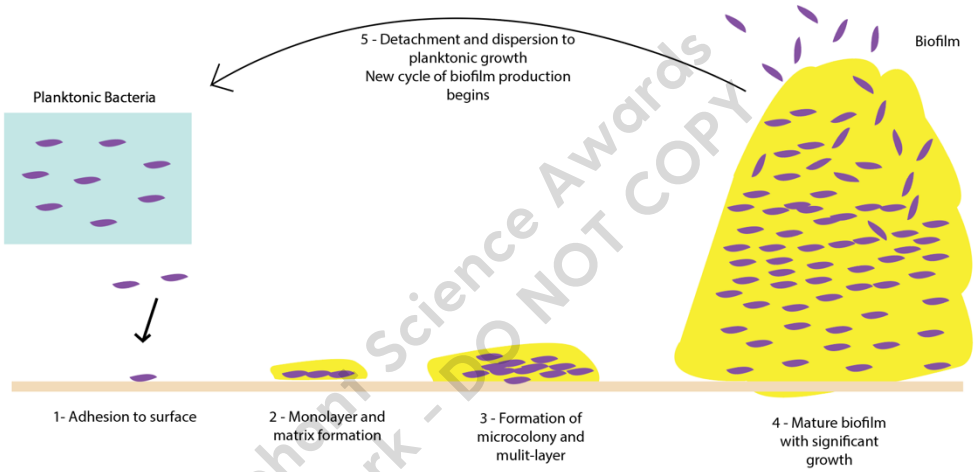
The matrix consists of extracellular polymeric substances (EPS), which are composed of nucleic acids, structural proteins and cell debris.

4) Maturation

Microcolonies which exhibit significant growth and cell-cell communication such as quorum sensing are formed.

5) Dispersion

This mature biofilm detaches and disperses into the environment, creating more planktonic cells for more biofilm formations.



About 65% of all bacterial infections are correlated with bacterial biofilms (Jamal et al; 2018). S. *aureus* biofilms are a leading cause of infections such as osteomyelitis and infections linked to artificial implants and catheters (Kumaran et al; 2018). *In vitro* work has shown that antibiotic induced elimination of S. *aureus* biofilms could be augmented by establishing prior phage infection. Such a scenario would result in higher BP density inside the biofilms and cause disruption of the biofilm composition. Subsequent antibiotics would result in better penetration and greater reduction of bacterial load (Kumaran et al; 2018).

BACTERIOPHAGE EFFECTIVENESS

Bacteriophages (phages) have been extensively experimented in the treatment of resistant bacterial infections (both gram-positive and gram-negative), as well as biofilm-producing organisms (Romero-Calle, 2019). They have been shown to have a synergistic mechanism when combined with antibiotics. In a case study on a patient with cystic fibrosis, a BP targeting *S. aureus*, was administered. It reduced the bacterial load (Kutateladze & Adamia, 2008). *In vitro* experimentation has shown that bacteriophages are effective against MDR bacterial isolates from infected wounds including *S. aureus*.

In vitro lytic activity of recombinant BP has been shown to be effective against biofilms of *S. aureus* (Saas & Bierbaum, 2007). Various laboratory works have indicated that bacteriophages can infect the bacterial cells within biofilms. Antibacterial resistance in biofilm-forming bacteria is presumably due to the presence of an extracellular polymeric substance (EPS) - which is composed of proteins, polysaccharides, nucleic acids and metal ions (Harper et al; 2017 Sutherland et al; 2004). It blocks the entry of drugs (Hayat et al; 2017). The BP secrete an enzyme called depolymerase, that causes EPS degradation. It facilitates the entry of BP into biofilms.

Successful applications of BP therapy have been described against *S. aureus* in burns and diabetic foot ulcers (Morozova et al; 2018). Specific BPs have been shown to kill MDR *S. aureus* from chronic rhinosinusitis patients (Zhang et al; 2018). Used in combination with antibiotics, BPs killed MRSA and have been demonstrated *in vitro* on orthopaedic wires used for the treatment of fractures (Kaur et al; 2016).

CURCUMIN EFFECTIVENESS

The antibiotic, vancomycin, is currently the drug of choice for treating Methicillin resistant *Staph aureus* (MRSA). It acts by preventing cell wall production by disrupting the chemical compound, peptiodoglycan, on the cell wall (McGuinness et al; 2017). *In vitro* studies have shown antibacterial properties of curcumin against Methicillin resistant S. aureus (MRSA). Curcumin has the ability to permeate and damage the cell membrane due to its amphipathic and liphophilic nature, allowing it to easily enter the cell (Tyagi et al; 2015). Membrane leakage and damage has been identified in both gram-positive and gram-negative bacteria (Tyagi et al; 2015). A bactericidal antibiotic is one that can kill bacteria. In this aspect, curcumin acts like a bactericidal antibiotic. When curcumin is combined with an antibiotic, the effect is synergistic. This was observed against an MRSA strain when curcumin was used along with antibiotics such as ampicillin, oxacillin and norfloxacillin. (Mun et al; 2013). If antibiotics undergo lysis and hydroxylation, they lose their efficacy. The curcumin would prevent the bacterial enzymes from causing lysis and hydroxylation of antibiotics. (Praditya et al; 2019).

In vitro studies have shown that curcumin can interfere in various cellular activities in biofilm producing organisms. These include modulating EPS production, adherence and surface hydrophobicity (Hayat et a;. 2018). EPS from Staphylococcus are utilised in surface adhesion and cell-to-cell attachment (Joyce et al; 2003). Curcumin has been shown to reduce the carbohydrate and protein content of EPS in S. *aureus*. It eventually reduces its adherence and biofilm-forming capacity of S. aureus by destroying the EPS linkage (Hayat et al; 2018).

Antibiotic synergy of curcumin is likely from biofilm inhibition, thereby eliminating the biofilm-associated resistance (Kali et al; 2016). While bacterial virulence is reduced by gene modulation and supressing quorum sensing, curcumin also facilitates the influx of antibiotics by causing cell membrane damage (Kali et al; 2016). Curcumin reduces the power of bacteria by gene modulation and interfering quorum sensing. By damaging the cell wall, antibiotics can enter the bacteria and act on them efficiently.

VARIABLES

INDEPENDENT VARIABLE

The independent variable in this experiment was the change in curcumin alone, bacteriophage alone and the combination of the two on the bacteria.

DEPENDENT VARIABLE

The dependent variable in this experiment was the destructive effect that the varying treatments had on the planktonic bacteria and biofilms.

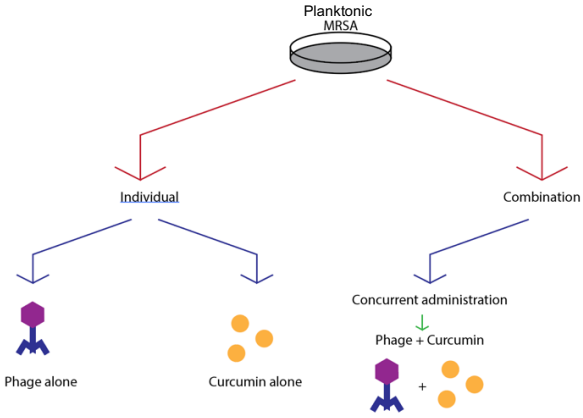
HYPOTHESIS

In this experiment I hypothesised that the combination of BPs and curcumin would result in a synergistic effect against planktonic MRSA and their biofilms. This is because both curcumin and BPs can cause cell wall damage on MRSA and disrupt the EPS on biofilms. Furthermore, curcumin's effect on cell wall damage and altering its permeability may facilitate the BP entry (non-sensitive) as well as lysis.

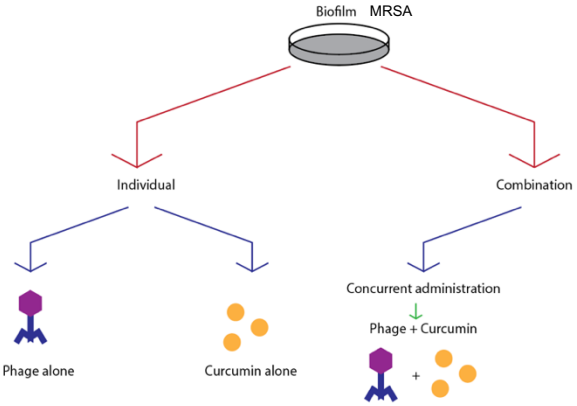
METHOD

a) Testing the antibacterial effects of phage alone on planktonic MRSA and their biofilms
b) Testing the antibacterial effects of curcumin alone on planktonic MRSA and their biofilms
c) Testing the antibacterial effects of the combination of phage and curcumin on planktonic MRSA and their biofilms

EXPERIMENT PROTOCOL



ASSESSING MRSA DESTRUCTION



ASSESSING BIOFILM DESTRUCTION

Creating agar liquids for growing MRSA, MSSA (control) on Petri dishes

An agar plate is a culture medium on a Petri dish that contains agar, a gelatinous substance from red seaweed and supports the growth of various bacteria. Agar plates were used in this experiment to grow the MRSA and MSSA (methicillin sensitive Staph. aureus) bacteria.

The powders TSA (Trypticase soy agar) and TSB (Trypticase soy broth) were separately dissolved in glass containers of 500mL and 1L of water respectively. The containers were placed in an autoclave machine for fifteen minutes at 121°C to sterilise the solutions and ensure that any bacteria that was in them were killed to prevent contamination. When the hot TSA liquid solution was ready, it was poured onto empty Petri dishes. The plates were left to cool for approximately 10 minutes.

Pre-cultured MRSA and MSSA bacteria were each swabbed in the biosafety hood and then streaked onto the agar plates, 6 of which were MRSA and 3 MSSA, as shown below.

Bacteria name	Bacteria
R1	Methicillin resistant Staphylococcus aureus (MRSA) Sample 1
R2	MRSA Sample 2
R3	MRSA Sample 3
R4	MRSA Sample 4
R5	MRSA Sample 5
R6	MRSA Sample 6
S1	Methicillin sensitive Staphylococcus aureus (MSSA) Sample 1
S2	MSSA Sample 2
S3	MSSA Sample 3

Preparation of the curcumin sample

The curcumin was provided by BHI. As it is a light sensitive compound, it was wrapped in foil. Twenty milligrams of curcumin was measured using a calibrator. A calibrator is a special measuring device similar to a weighing scale but specifically designed to measure small quantities.

Forty microlitres of water was added to this sample using a pipette. The sample was foiled and shaken in a centrifuge to mix the solution before placing in the refrigerator. After 3 hours it was evident that curcumin does not dissolve in water and therefore a different liquid agent called DMSO (dimethyl sulfoxide) was used. All further tests were conducted using this created sample of curcumin at a concentration of 10µg/ml.

Spot Assay - Assessment of curcumin and phage interactions

The Spot Assay (Figure 1) was done to assess the nature of the interactions between the curcumin and the phage and see whether one would negatively affect the other when operating with bacteria. Since the synergy of curcumin and phage had never been explored before, this test was important to conduct. Three Spot Assay tests were done: assessing curcumin/phage interactions after 1 hour, 4 hours and 24 hours. For each test, four agar plates were used.

The phage that was used in this experiment was PYO, which has a wide host range and is especially effective against *Staph. aureus* bacteria. Its concentration was 3.33×10^3 PFU/ml (plaque forming units). It was a commercial preparation that was provided by BHI. Each test consisted of five agar plates inoculated with RN4220 *Staph.aureus*. Of these, four agar plates were spot assayed with various concentrations of curcumin (0.2, 0.4, 0.8, 1.0µg/ml) along with orange phage. The fifth agar plate was spot assayed only with orange phage and acted as a control (refer to Figure 2 for concentrations and Figure 3 for the layout of each plate).

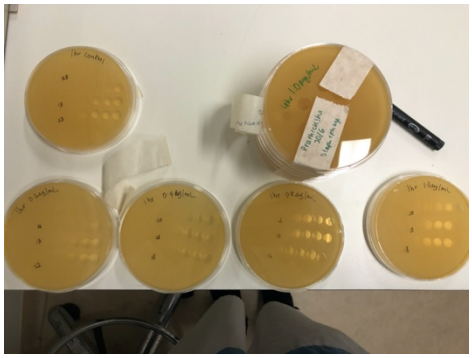


Figure 1: Spot Assays on 1hr agar plates

Three microlitres of diluted phage was spotted in three rows of eight spots on each agar plate. The left-most spots consisted of the largest concentration of phage and through ten-fold dilution across the rows, the eighth spots contained the lowest concentration of phage. This was done because as the concentration decreased, it was easier to count the number of spots of phage (Figure 4) to calculate their phage titers. The curcumin at different concentrations was inserted onto the plates of 20µl each. The same test was used for the 4 hour test and a new batch of plates were used for the 24 hour test. The plates were placed in incubation.

Concentrations of curcumin:

- 0.2µg/ml
- 0.4µg/ml
- 0.8µg/ml
- 1.0µg/ml

Producing these concentrations is discussed further below.

Figure 2: Concentrations of curcumin

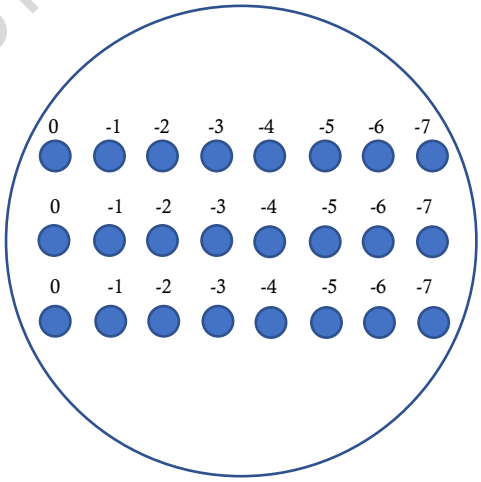


Figure 3: Layout of Spot Assay plates

The phage activity on the bacteria was determined by observing their phage titers as explained below. If this activity produced phage titers of similar values to the controls that were untreated with curcumin, it would have been concluded that the curcumin did not negatively affect the phage's activity on the bacteria.

Plaques are clear areas on fields of bacteria that show that some form of inhibition has occurred to the bacterial cells by some agent, in this case, bacteriophages. It is known that 10 plaques are in the 3µl that were each spotted on the agar and 10 plaques are equivalent to 10 phages. Therefore, there are 3.33 phages in 1µl. Since there are 1000µl in 1 ml, there are 3.33×10^3 phages in 1ml.

Following 1 hour, the plates of the 1 hour test were taken out of incubation. For each plate, the one column that clearly depicted countable spots was used to count them. Most of the time, it was the eight or seventh rows with the lower concentrations of phage that were used to count the plaques. The following formula was used to calculate each plate's phage titer:

$$average\ of\ each\ row's\ number\ of\ spots\ \times 3\ microlitres\ converted\ to\ ml \times concentration\ of\ phage$$

E.g. 4hr - 0.2µg/ml (column 7)

$$\frac{16+14+13}{3} \times \frac{1000}{3} \times 10^4$$

$$5.33 \times 10^7$$

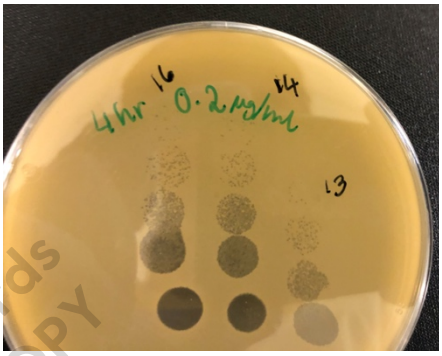


Figure 4: Agar plate 0.2 4hr, number of dots on 1 row

Each time period's control was calculated for its phage titer as well, and those results were compared to the phage titers of those with different concentrations of curcumin. All results for each time period were similar to the control group, implying that curcumin did not play a negative role in the phages' activities to the bacteria.

Adjacent Experiment – Curcumin cytotoxicity

The curcumin cytotoxicity test was conducted to confirm that curcumin does not kill the human nasal epithelial cell line and configure a safe dosage of its concentration. Because this experiment was performed in a different lab, the Tissue Sampling Room, a new lab coat was worn to prevent cross contamination. A patient's cell was collected from The Queen Elizabeth Hospital (human ethics approval was in place) through nasal swabbing. To isolate the cell, this solution was centrifuged with another tube on the opposite side to balance the circular motion. After the cell was isolated in the base of the tube, a vacuum suction pipe extracted the liquid so as to leave just the cell (Figure 5). A cell media was created for the cell to grow in using 50x EX Plus Basal media combined with an EX Plus supplement 2%. Penicillin/Streptomycin, antibiotics and 10% FBS (foetal bovine serum) was also added to the media. This was placed in two containers and incubated over three days.

After incubation, the media in the four plates was aspirated so as to isolate the cell in the bottom of the wells. They were then washed with phosphate-buffered saline (PBS) by inserting 200µl into the wells and aspirating the liquid out, leaving the cells isolated again.

Two microplates were for review after 24 hours and two plates for 48 hours, therefore all plates contained the same layout and quantities of elements in them. Different concentrations of curcumin were added into the wells with the negative control as the cell media and positive control as the DMS using a multi-pipette. Below is a labelled diagram of one of the four plates:

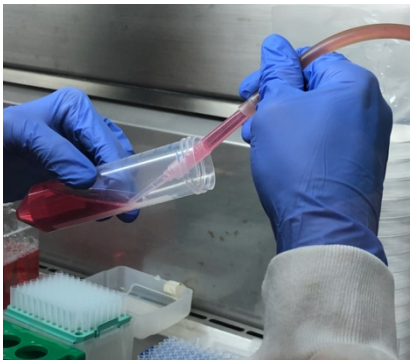


Figure 5: Aspirating the cell media with a vacuum suction to isolate the cell

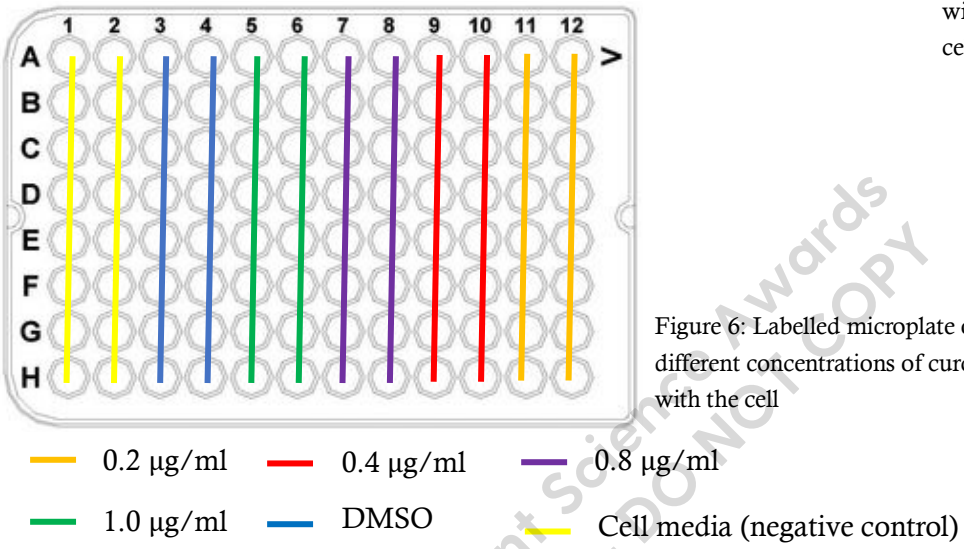


Figure 6: Labelled microplate of different concentrations of curcumin with the cell

Since the total volume of each well was the same (200µl), more volume of the curcumin in comparison to the cell media would increase its concentration, as shown above. The formula below was used to calculate the ratio values of the quantities below:

Concentration of curcumin (µl)	Curcumin (µl)	Media (µl)	Total volume of one well (µl)
1.0	20	180	200
0.8	16	184	200
0.4	8	192	200
0.2	4	196	200

Figure 7: Quantities of each element in each well according to different quantities of curcumin

$$\mathbf{C_1V_1 = C_2V_2}$$

Where C_1 = original concentration of curcumin (10µg/ml)

V_1 = the volume of curcumin that must be added (x)

C_2 = the desired well concentration (e.g 0.2µg/ml)

V_2 = the desired well volume (200µg)

$$10 \times x = 0.2 \times 200$$

Therefore, 4µl of curcumin was required for the wells of 0.2µg/ml curcumin concentration. Hence, the volume of cell media was 196µl to collectively equate to 200µl in one well. These calculations were made for the other concentrations, as shown in the table above.

The concentration of DMSO in the media was to be 1µg/ml, therefore each well of DMSO and media would be in the ratio – 20:180 (DMSO : media), similar to the curcumin concentration of 1µg/ml.

The wells that contained only cell media consisted of 200µl of the solution.

The four plates were incubated overnight. After 24 hours, two plates were taken from the incubator for the LDH (lactate dehydrogenase) assay.

In one of the untreated rows of cell media, 10% of Trident X 100 was poured in three wells for the two plates. Trident X 100 is a solution that has a 100% chance of killing the epithelial cell. Since this is known, the solution was used as a positive control to compare it with the cytotoxicity of curcumin. The negative control was the untreated cell media, where there was no cell affect expected.

Each sample group (e.g. 0.2 µg/ml) was collected using a pipette and inserted into 2 mini plastic test tubes, 1 for one column of a group and 1 for the other column. Overall, each plate corresponded with 14 mini plastic test tubes.

1 plate:

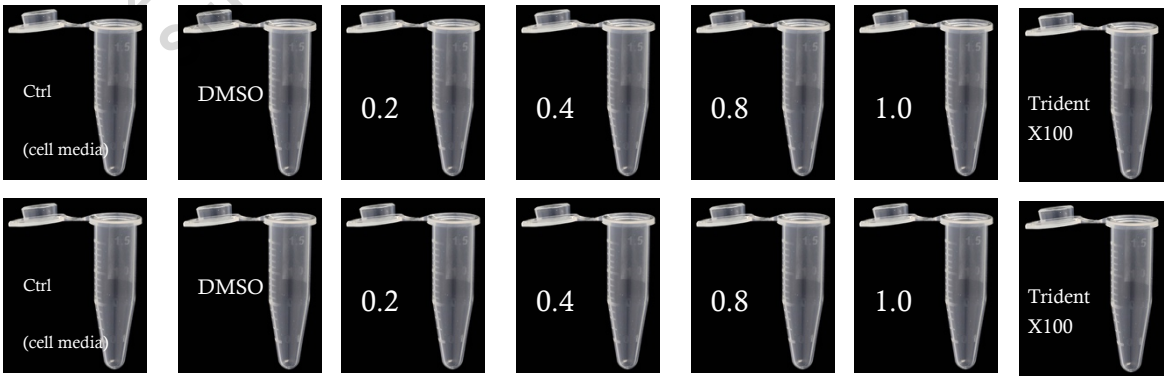


Figure 8: 14 mini plastic tubes of collected solutions from 1 plate of curcumin and the cell

x 2 (for two plates)

All of the tubes were placed in ice (Figure 9) and taken to centrifuge at maximum speed for 1-1 1/2 minutes. Following centrifugation, 50µl of the below elements were placed into the following wells of a fresh plate:

Figure 9: Mini plastic tubes of the cell and curcumin in ice

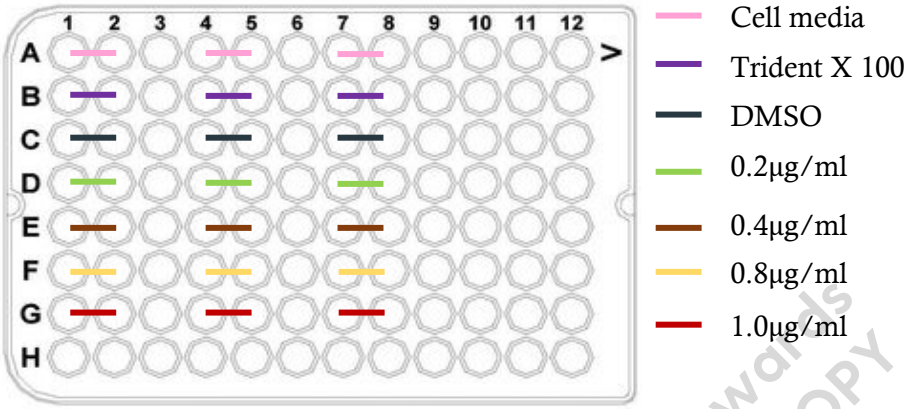


Figure 10: Labelled microplate of the solutions following centrifugation

Each well was then diluted with 50µl of substrate mix, which is an agent that captures released enzymes of dead cells. The plate was incubated for 30 minutes in 37°C. After incubation, 50µl of Stop solution was added to the wells to stop the reaction between the substrate mix and the cell solutions (Figure 11). The plate was then placed in a microplate reader to calculate the wells' OD (optical density) values. If the OD values showed no difference to the negative control, the curcumin did not affect the cell as much as the Trident X 100.

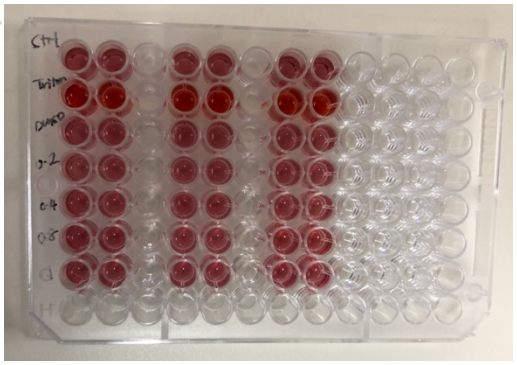


Figure 11: Curcumin and cell solutions after incubation and before OD reading

The same procedure was conducted the next day for the next plate that was observed after 48 hours of incubation.

Microplates of planktonic bacteria

To preserve the curcumin over a period of days, it was refrigerated. However, the refrigeration solidified it. Hence, it was incubated in 37°C for around 15 minutes to warm it to a liquid. Once it was a liquid, 10µl was extracted and diluted in a tube of 10ml sterilised water, making its concentration 10µg/ml. The bacteriophage was also taken out of refrigeration but did not require any dilution in sterilised water. Six plates were used:

Microplate 1: R1-R4 bacteria applied with curcumin alone

Microplate 2: R5-S2 bacteria applied with curcumin alone

Microplate 3: S3 bacteria applied with curcumin alone + R1-R3 bacteria applied with curcumin and phage

Microplate 4: R4-S1 bacteria applied with curcumin and phage

Microplate 5: S2 & S3 bacteria applied with curcumin and phage

Microplate 6: R1-S3 bacteria applied with phage alone

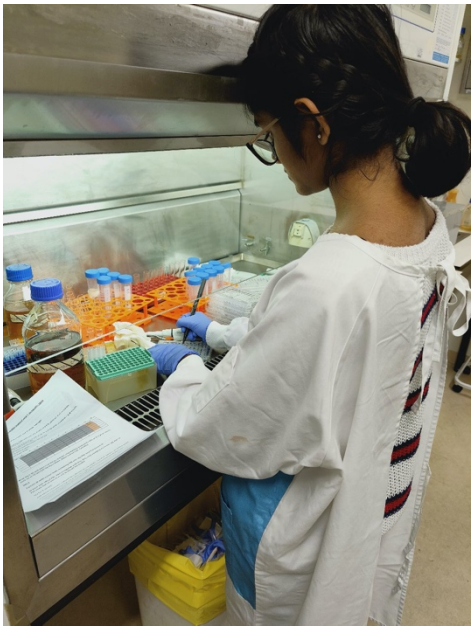


Photo 1: Labelling the microplates of planktonic bacteria

DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
1µg/ml curcumin + R1	1µg/ml curcumin + R1	1µg/ml curcumin + R1	1µg/ml curcumin + R2	1µg/ml curcumin + R2	1µg/ml curcumin + R2	1µg/ml curcumin + R3	1µg/ml curcumin + R3	1µg/ml curcumin + R3	1µg/ml curcumin + R4	1µg/ml curcumin + R4	1µg/ml curcumin + R4
0.8µg/ml curcumin + R1	0.8µg/ml curcumin + R1	0.8µg/ml curcumin + R1	0.8µg/ml curcumin + R2	0.8µg/ml curcumin + R2	0.8µg/ml curcumin + R2	0.8µg/ml curcumin + R3	0.8µg/ml curcumin + R3	0.8µg/ml curcumin + R3	0.8µg/ml curcumin + R4	0.8µg/ml curcumin + R4	0.8µg/ml curcumin + R4
0.4µg/ml curcumin + R1	0.4µg/ml curcumin + R1	0.4µg/ml curcumin + R1	0.4µg/ml curcumin + R2	0.4µg/ml curcumin + R2	0.4µg/ml curcumin + R2	0.4µg/ml curcumin + R3	0.4µg/ml curcumin + R3	0.4µg/ml curcumin + R3	0.4µg/ml curcumin + R4	0.4µg/ml curcumin + R4	0.4µg/ml curcumin + R4
0.2µg/ml curcumin + R1	0.2µg/ml curcumin + R1	0.2µg/ml curcumin + R1	0.2µg/ml curcumin + R2	0.2µg/ml curcumin + R2	0.2µg/ml curcumin + R2	0.2µg/ml curcumin + R3	0.2µg/ml curcumin + R3	0.2µg/ml curcumin + R3	0.2µg/ml curcumin + R4	0.2µg/ml curcumin + R4	0.2µg/ml curcumin + R4
R1 + TSB	R1 + TSB	R1 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB	R2 + TSB
TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB

Figure 12: Labelled diagram of the elements in microplate 1

DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
1µg/ml curcumin + S3	1µg/ml curcumin + S3	1µg/ml curcumin + S3	1µg/ml curcumin + R1	1µg/ml curcumin + R1	1µg/ml curcumin + R1	1µg/ml curcumin + R2	1µg/ml curcumin + R2	1µg/ml curcumin + R2	1µg/ml curcumin + R3	1µg/ml curcumin + R3	1µg/ml curcumin + R3
0.8µg/ml curcumin + S3	0.8µg/ml curcumin + S3	0.8µg/ml curcumin + S3	0.8µg/ml curcumin +phage + R1	0.8µg/ml curcumin +phage + R1	0.8µg/ml curcumin +phage + R1	0.8µg/ml curcumin +phage + R2	0.8µg/ml curcumin +phage + R2	0.8µg/ml curcumin +phage + R2	0.8µg/ml curcumin +phage + R3	0.8µg/ml curcumin +phage + R3	0.8µg/ml curcumin +phage + R3
0.4µg/ml curcumin + S3	0.4µg/ml curcumin + S3	0.4µg/ml curcumin + S3	0.4µg/ml curcumin +phage + R1	0.4µg/ml curcumin +phage + R1	0.4µg/ml curcumin +phage + R1	0.4µg/ml curcumin +phage + R2	0.4µg/ml curcumin +phage + R2	0.4µg/ml curcumin +phage + R2	0.4µg/ml curcumin +phage + R3	0.4µg/ml curcumin +phage + R3	0.4µg/ml curcumin +phage + R3
0.2µg/ml curcumin + S3	0.2µg/ml curcumin + S3	0.2µg/ml curcumin + S3	0.2µg/ml curcumin +phage + R1	0.2µg/ml curcumin +phage + R1	0.2µg/ml curcumin +phage + R1	0.2µg/ml curcumin +phage + R2	0.2µg/ml curcumin +phage + R2	0.2µg/ml curcumin +phage + R2	0.2µg/ml curcumin +phage + R3	0.2µg/ml curcumin +phage + R3	0.2µg/ml curcumin +phage + R3
S3 by itself	S3 by itself	S3 by itself	R1 by itself	R1 by itself	R1 by itself	R2 by itself	R2 by itself	R2 by itself	R3 by itself	R3 by itself	R3 by itself
TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB	TSB

Figure 13: Labelled diagram of the elements in microplate 3

The quantities of curcumin in this test was alike those from the curcumin cytotoxicity on a human cell test. However, the quantities of the TSB in each concentration of curcumin groups was varied to adjust to the control quantities of bacteria (2µl), phage (50µl) and the total volume in each well (200µl).

Concentration of curcumin (µl)	**Volume of element (µl)**			
	Bacteria	Curcumin	Phage	TSB
1.0	2	20	50	128
0.8	2	16	50	132
0.4	2	8	50	140
0.2	2	4	50	144

Figure 14: Table of the quantities of the elements in each microplate of planktonic bacteria

Element	**Volume of element (µl)**			
	Bacteria	Curcumin	Phage	TSB
Bacteria & TSB	2	-	-	198
Bacteria & Phage	2	-	50	148
TSB	-	-	-	200

Figure 15: Table of the quantities of the elements in each microplate of planktonic bacteria without curcumin

The microplates were taped in a stack and incubated overnight on a rotating plate. The next day, the plates were taken to the microplate reader and scanned for OD values. To assess the action of the treatments on the bacteria, observations were made by looking underneath the plates and observing cloudy wells (bacterial wells) and clear wells (bacteria that were killed).

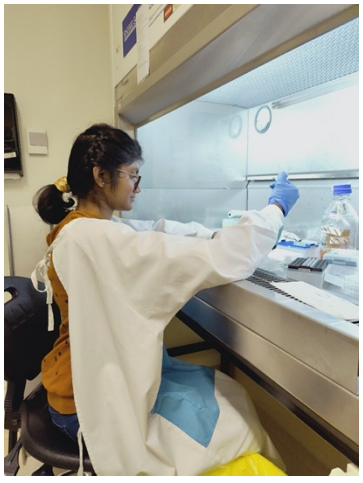


Photo 2: Using a multi-pipette to insert the elements into the microplates

Microplates of biofilm

The biofilms were grown before treating them with curcumin and phage. Biofilm growth takes up to 48 hours, therefore setting up the protocol was essential to ensure that it would grow successfully. Ten microtiter plates of 96 wells were used. Each type of bacteria had its own plate, which, during the treatment, would have just curcumin applied to it and curcumin and phage applied to it (Figure

16). The remaining plate consisted of each bacteria which would just have bacteriophage applied to them (Figure 17). It was important to label the plates with their allocated treatments to provide a clear scaffold for when the treatment was applied in 48 hours.

The outer wells of the plates contained 180µl of PBS. Since the solutions were to be incubated for a long period of time, it was important to prevent them from evaporating. Therefore PBS was applied to the plates.

Each plate contained six wells of only 150µl TSB. This was done to ensure that if there was a contamination in the TSB, we could conclude that the wells' reason for cloudiness was from the TSB's contamination. A cloudy well could denote contamination or bacterial growth.

Figure 16: Example layout of the R6 bacteria's biofilm plate

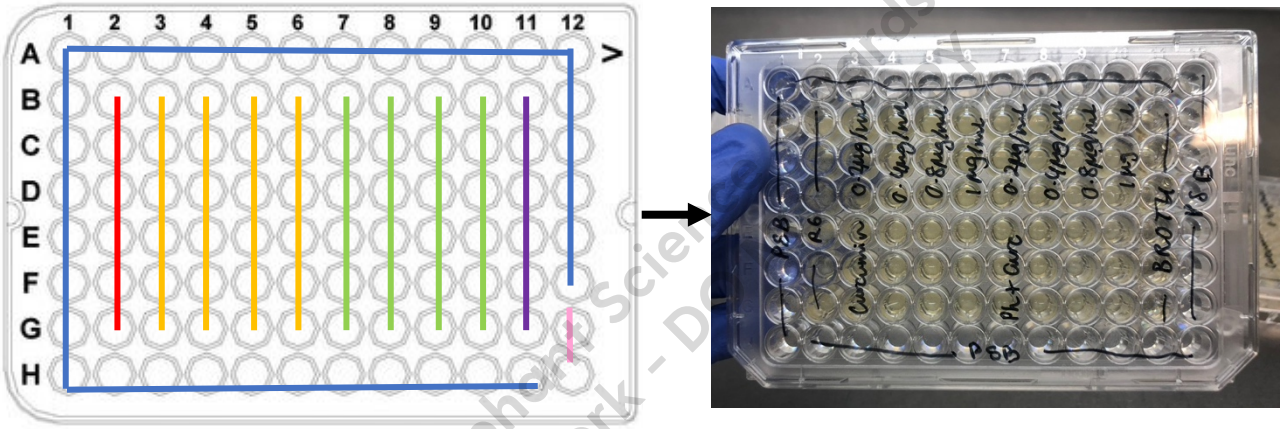
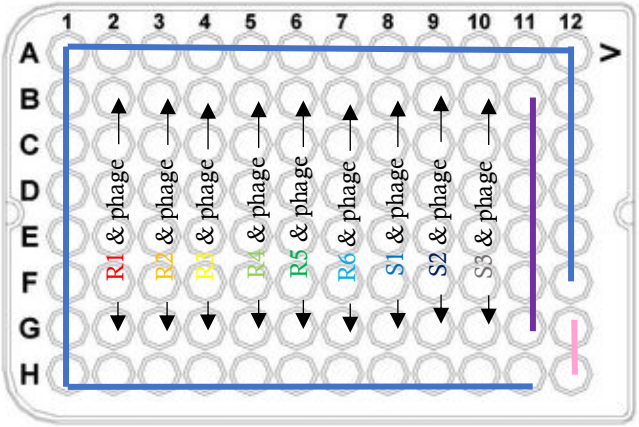


Figure 17: Layout of the phage + bacteria biofilm plate



The ratio of bacteria suspended in the TSB was 1:15, therefore in test tubes, 1.2ml of each bacteria was suspended in 16.8ml of broth before inserting 150µl of bacterial mix into the wells.

Chamber slides of biofilm

The Live/Dead Assay was a staining procedure that was conducted on biofilms to analyse the amount of live bacteria against dead bacteria after treatment. The set up was similar to that of the crystal violet assay. However, the bacteria and treatments were placed on chamber slides (Figure 18) for observations through a confocal telescope. The total quantities in each slide were also different because the slides were larger than wells. Therefore, the total volume in each slide was 350µl rather than 200µl. One chamber was made for one bacteria. Below is a labelled example of S1's chamber slide:

<table>
<tr><td>S1+ 0.2</td><td>S1+ 0.4</td><td>S1+ 0.8</td><td>S1+ 1.0</td><td rowspan="2">S1</td></tr>
<tr><td>S1+ 0.2 + phage</td><td>0.4 + phage</td><td>S1+ Phage alone</td><td>S1 Untreated</td></tr>
</table>

Figure 18: Labelled diagram of the elements on the S1 diagram plate

Figure 19: Chamber slide of S1

The ten plates and nine chambers were wrapped in foil to prevent evaporation and the samples were incubated for 48 hours at 37°C on a rotating plate set a 70rpm (revolutions per minute).

The above biofilm test was done four times for the following assays:

I. Biofilm set up in clear microplates for the Crystal Violet Assay (x2)
II. Biofilm set up in dark microplates for the alamarBlue Assay because the alamarBlue dye is light sensitive (x1)
III. Biofilm set up in chamber slides for the Live/Dead staining (x1)

Treating the biofilms with curcumin and phage

After 48 hours, the bacterial biofilm had grown as a layer on the bottom of each well in the plates. To isolate the biofilm, the planktonic bacteria was aspirated using a vacuum suction with a pipette's tip. It was important to be cautious and avoid removing the thin biofilm layer at the bottom of the well. The biofilms were washed with PBS, adding 180µl in each well before aspirating again, ensuring that the biofilms were not also sucked out. Another round of washing with PBS and aspirating was conducted.

In reference to Figures 16 & 17, the curcumin and phage were applied to the biofilms of the designated wells. This time, the total volume of each well was to be 180µl. Because there was a layer

of biofilm on the bottom of each well, 200µl may have been too high a quantity. Refer to the table below of quantities of each well and chamber slide:

Concentration of curcumin in 180µl (µg/ml)	**Curcumin alone (µl)**		**Curcumin + phage (µl)**		
	Curcumin	**TSB**	**Curcumin**	**Phage**	**TSB**
0.2	3.6	176.4	3.6	50	126.4
0.4	7.2	172.8	7.2	50	122.8
0.8	14.4	165.6	14.4	50	115.6
1.0	18	162	18	50	112

Figure 20: Table of quantities of the elements in the plates of biofilm

E.g. of calculation for curcumin's volume values (0.2 µg/ml calculation):

$$\mathbf{C_1V_1 = C_2V_2}$$

$$10 \times x = 0.2 \times 180$$

$$x = 3.6$$

Therefore, 3.6µl of curcumin was needed for the samples of 0.2 µg/ml concentration.

Phage alone (µl)	
Phage	**TSB**
50	130

Figure 21: table of quantities of the phage and TSB on the bacterial biofilms

Once the treatments of curcumin and phage were applied to the biofilms, the plates and chamber slides were incubated in 37°C on a rotating plate overnight.

Reviewing the biofilm destruction in three methods

The biofilm destruction was assessed in terms of metabolic rate, viability and live vs. dead bacteria through three assays: Crystal violet (biofilm biomass), alamarBlue (metabolic activity) and Live/Dead (ratio between live & dead).

Crystal Violet Assay

Crystal violet is a dye and in this test it was used to stain the remaining surviving bacteria in the biofilms to analyse the biofilms' biomasses following treatment of curcumin, phage and their combination. The Crystal Violet Assay was conducted to stain the biofilm that was attached to the wells and determine the biomass of the bacteria after the curcumin, phage and their combination were introduced.

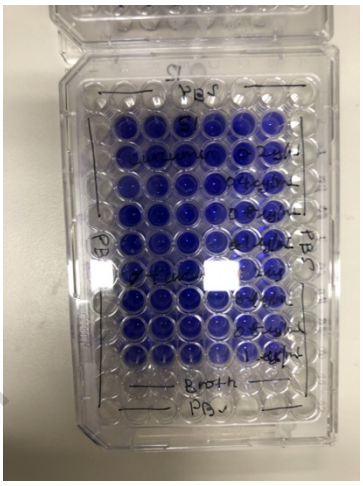


Figure 22: S1's biofilm microplate after treatment and filled with crystal violet

Ten clear plates of biofilm bacteria and treatment were taken out of incubation after 48 hours. The treatments were aspirated using the vacuum suction pipe. The wells were then filled with 180µl of PBS to wash any remaining treatments that might not have been sucked out. The PBS was aspirated and another round of washing and aspiration was conducted. The plates were then left to dry for 10 minutes.

After drying, the wells were filled with 180µl of crystal violet (0.1% in water), except for the PBS wells. The plates were left for 15 minutes whilst the dye stained the bacteria. Subsequently, the crystal violet was aspirated and washed three times with PBS. However, during aspiration, rather than using the suction, they were rotated and flipped quickly onto the sink. After washing with PBS, they were dried overnight. The next day, 180µl was inserted on the wells to absorb the remaining dried crystal violet and shaken on a plate shaker for 1 hour. Then, the plates were scanned through a microplate reader and the results were saved on the computer.

alamarBlue Assay

The alamrBlue assay is used to quantitatively measure the cell viability. This assay involved observing the alamarBlue dye extend through the biofilm's matrix and into the live bacteria over a period of time to observe the bacteria's viability. The procedure of the alamarBlue Assay's aspiration after 48 hours of incubation was conducted similar to the Crystal Violet Assay. After drying the plates for 10 minutes, the alamarBlue solution was made with 1:10 dilution of the dye to the TSB. To sustain for all of the plates, 10.4ml of alamarBlue was diluted in 93.6ml of TSB. This solution of was then added as 200µl to each well, excluding the PBS wells and beginning with the TSB column.

Figure 23: The FLUOstar microplate reader with fluorescent light

The plates were incubated and taken out at 1 hour intervals for 5 hours. These intervals were vital because the biofilm's adhesive matrix prevented the dye from entering and staining the live bacteria immediately. Each time the plates

were removed from incubation, they were inserted in a FLUOstar OPTIMA microplate scanner, specialised for reading plates' fluorescence intensities.

Live/Dead Assay

The Live/Dead assay was conducted to assess the proportions of dead bacteria to live bacteria in the biofilm after treatment. Five percent Glutaraldehyde solution was implemented to 'fix' the biofilm to the bottom of the slides. In other words, the biofilms that were dead remained dead and the biofilms that were alive remained alive. The solution played a fundamental role because it ensured that when looking at the stained bacteria under a microscope, the biofilms were not constantly in motion. This solution was taken out of a -20°C refrigerator and placed in cold water to liquify. Meanwhile, the chamber plates were washed twice like the crystal violet wells with 350µl of saline in each chamber.

Once the Glutaraldehyde solution turned to a liquid, it was diluted in sterile water with a ratio of 1:5. Two millilitres of the solution was diluted and mixed in 8ml of water and 200µl of this solution was added in each chamber before leaving the slides for 45 minutes.

The plates were then washed twice with MilliQ water. The dye that were used to observe alive and dead bacteria were:

Live: SYTO 9 dye

Dead: PI dye

The SYTO 9 dye would stain the alive bacteria in green and PI would stain dead bacteria in red. The two dye were mixed, with 22.5µl of each in 15ml of MilliQ water. This mixture was dispersed as 200µl amongst each chamber and incubated for 15 minutes. The dye was then aspirated and washed once with MilliQ water.

The eight chambers that acted as templates for each group on each plate were removed, leaving just the slides with the biofilms on them. To prevent the biofilm from drying, one drop of mounting oil was meticulously placed on each slide group. Cover slips were then placed over each slide and nail polish acted as a glue to attach the cover slip with its associated slide. The slides were left to dry overnight before observing the stained R3 bacteria through a confocal microscope.

Figure 24: The confocal LSM 700 microscope that was used to view the live/dead bacterial biofilms

RESULTS

1. Assessing the cytotoxicity of curcumin

Based on the LDH assay, curcumin was not found to be toxic to the human cell line. The activity of curcumin at different concentrations (0.2, 0.4, 0.8, 1.0µg/ml) was similar to the negative control (untreated cell media) at both 24 hour and 48 hour periods. As shown in the figures below, the positive control (Trident X 100) displayed a larger OD value, implying that it killed the cell line.

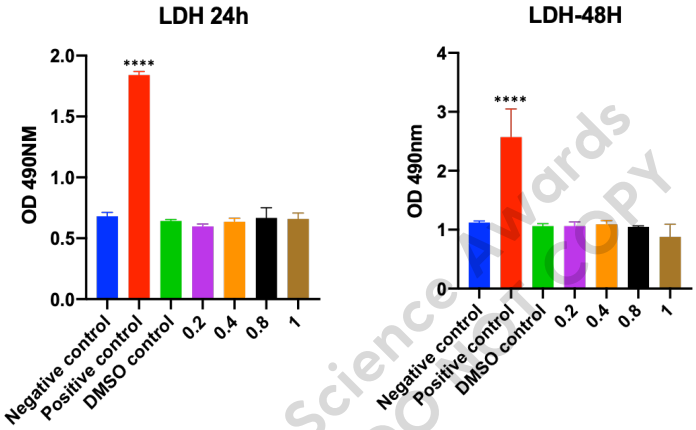


Figure 25: Cytotoxicity of curcumin assessed by LDH assay at 24 and 48 hours

2. The effect of curcumin, phage, curcumin + phage on planktonic bacteria

The effect was assessed by direct observations of the microplates. Curcumin was shown to be effective against MRSA at concentrations less than 0.2, 0.2, 0.8 and 1.0µg/ml. It was also effective against MSSA at less than 0.2, 0.8 and 1.

Bacteria	Concentration of the curcumin (µg/ml)		Sensitivity result
	Curcumin	Curcumin + Phage	Phage
R1	0.8	0.2 ↓	(-)
R2	1	0.2 ↓	(-)
R3	1	1	(-)
R4	0.2	0.2	(-)
R5	<0.2	<0.2	(-)
R6	1	0.2 ↓	(-)
S1	<0.2	<0.2	(-)
S2	1	0.4 ↓	(+)
S3	0.8	0.2 ↓	(-)

Figure 26: The appropriate dosages of curcumin based on the effectiveness of curcumin combined with phage

Although a lower dosage than 0.2µg/ml was not experimented with the bacteria, the 0.2 dosage killed all three samples of R5 and S1 and a dosage less than that could have been experimented with those bacteria.

When curcumin was combined with phage, the antibacterial effect was observed at much lower concentrations. Interestingly, not antibacterial effect was observed when phage alone was examined except for S2.

3. Assessing the effect of curcumin, phage and curcumin + phage on biofilms

a) alamarBlue Assay – evaluating the cell viability and proliferation

The viability of the biofilms were not affected by curcumin alone. As shown in the figures below, across the concentrations of curcumin from 0.2 to 1.0µg/ml, the cell viability percentages were similar to the control (TSB, untreated shown in the blue bars). This implies that the biofilm proliferation overtime was not affected. Nonetheless, the combination of curcumin and phage had a remarkable effect on cell viability, reducing the percentages of cell to almost 50% across the curcumin concentrations from 0.2 to 1.0µg/ml.

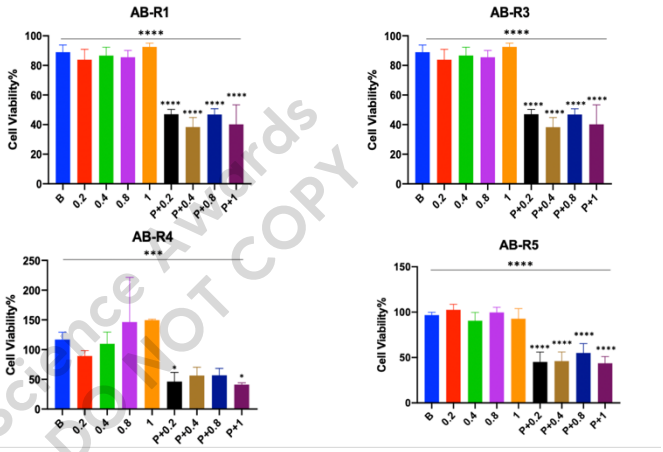


Figure 27: alamarBlue cell viability % of the biofilms across four MRSA with different treatments

b) Crystal Violet Assay

Overall, curcumin alone did not have much of an effect on the biofilm biomass especially on R3, R4 and R5. However, the combination of curcumin and phage reduced the biomass greatly in relation to the control as measured by the OD values. This effect was seen across the concentrations of curcumin from 0.2 to 1.0µg/ml. The larger the OD value, the less light that was passed through the wells, implying that they were cloudy and there was a higher concentration of bacteria.

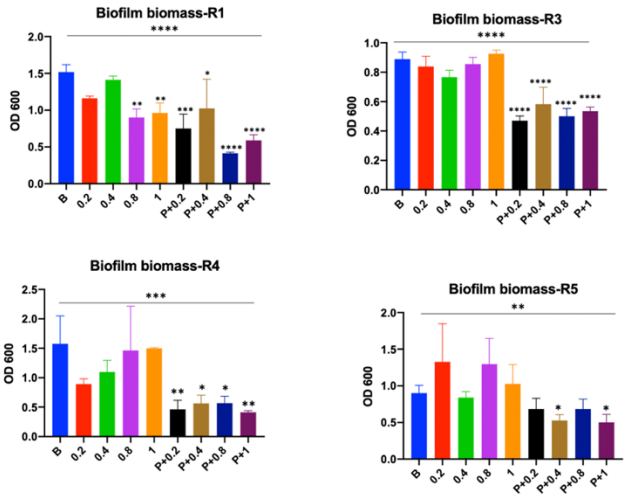


Figure 28: Biofilm biomass OD values across four MRSA biofilms

c) Live/Dead Assay

As compared with the untreated bacterium, phage alone showed minimal killing effect against R3. Curcumin alone at 0.2μg/ml showed a marginally more killing effect against R3. However, when the phage was combined with curcumin of the same concentration, the killing effect was predominantly observed, with a larger mass of dead bacteria dyed red with PI.

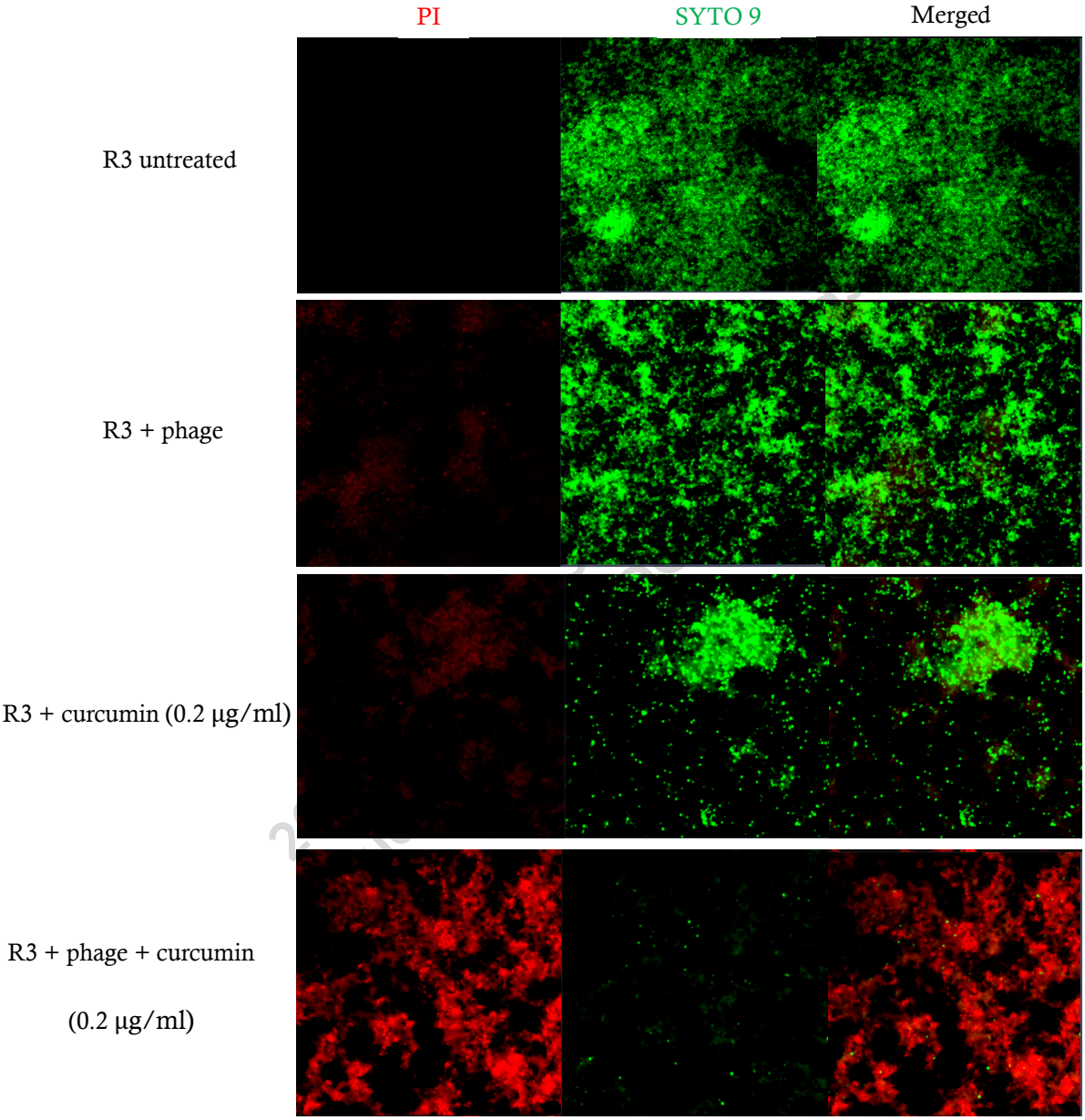


Figure 29: R3's live/dead biofilm comparison images captured by the confocal microscope

DISCUSSION

The experiment showed that the combination of phage and curcumin was effective against the biofilm of MRSA. The effectiveness was observed against cell viability and proliferation, reduction in biomass and a visual representation of direct killing of the bacterial biofilms, supporting my hypothesis. To our knowledge, the combination of curcumin and bacteriophage has never been examined in the context of multi-drug resistant bacteria. The team from BHI conducted the first ever human phase 1 trial of bacteriophage therapy on *Staph. aureus* associated chronic rhinosinusitis in 2017. Results from the trial showed that phage therapy could be an alternative to antibiotics in the treatment of sinus infections (Ooi et al; 2019). Earlier work from this team using Staphylococcus-relevant phages Sa83 and Sa87 have shown phage susceptibility towards antibiotic-sensitive as well as resistant *Staph. aureus* clinical isolates in planktonic and biofilms (Zhang et al; 2018).

In animal-infected models of *P. aeruginosa*, antibiofilm activity of curcumin has been demonstrated. It is attributed to inhibition of quorum sensing genes, reduction of biofilm initiation genes, down regulation of quorum sensing-dependent virulence factors such as alginate production, swarming and motility (Packiavathy et al; 2014, Rudrappa & Bais, 2008). Also, a strong biofilm disaggregation effect ruining the viability of cells and reduction of bacterial motility have also been shown (Marini et al; 2018, Packiavathy et al; 2013).

An *in vitro* synergistic effect of curcumin in combination with antibiotics have been shown against biofilms of various organisms including *Staph. aureus* (Kali et al; 2016, Kumaran et al; 2018). Antibiotic synergy of curcumin is likely from biofilm inhibition, thereby eliminating the biofilm-associated resistance (Kali et al; 2016). While bacterial virulence is reduced by gene modulation and supressing quorum sensing, curcumin also facilitates the influx of antibiotics by causing cell membrane damage (Kali et al; 2016). Curcumin reduces the power of bacteria by gene modulation and interfering quorum sensing. By damaging the cell wall, antibiotics can enter the bacteria and act on them efficiently.

Curcumin is a naturally occurring compound with very minimal toxicity and is widely available. Various *in vitro* and *in vivo* studies have shown its efficacy against different viruses, bacteria and fungi, including multi-drug resistance strains (Praditya et al; 2019). The mechanism of action is yet to be completely understood. Even at high oral doses, it is not toxic. Its broad spectrum anti-infective property makes it a viable approach in dealing with multi-drug resistant bacteria.
The limitations of curcumin therapy are:

- poor solubility
- curcumin is poorly absorbed by the human digestive system
- rapid metabolism

Curcumin formulation as nanoparticles is likely to overcome the above mentioned limitations and might facilitate its clinical use in the future.

As both phage and curcumin are safe to be used, the results of the study might stimulate more interest among researchers to explore the combination.

Strengths

The first ever *in vitro* experiment to investigate the combination of phage and curcumin against multi-drug resistant bacteria.

Both planktonic and biofilms of MRSA were analysed with appropriate controls.

The assays included assessed a range of bacterial properties and effects post-treatment.

Curcumin was analysed across four different concentrations after confirming that it is not cytotoxic.

Adjacent tests were conducted to see the effectiveness of phage and curcumin together and the cytotoxicity of curcumin on a human epithelial cell.

Weaknesses

Due to time constraints, the activity of phage alone could not be examined using the Crystal Violet and alamarBlue assays

Similarly, the Live/Death Assay was read on only R3.

Further Inquiry

The results of this experiment need to be validated by repeating the experiment at least three times and repeated analysis from these repeated experiments.

The concept can be explored against other gram-positive as well as gram-negative bacteria.

A synergistic effect of adding an antibiotic to the combination of curcumin and phage can be explored.

Sequential administration of phage and curcumin can also be explored.

Controlled variables

Variable	How it was controlled	Why it was controlled
The different concentrations of curcumin in each test	Meticulous measurements using reliable technology (multi-pipettes) were used to dilute the right amounts of curcumin in DMSO	Errors in concentrations would not have made the experiment reliable amongst each sample group
The concentration of phage in each test	A pre-prepared concentration of phage was used for each test	Errors in concentrations would not have made the experiment reliable amongst each sample group
How the analysis of biofilm destruction was conducted	All sample groups of biofilm were analysed with the same assays	The interpretation would have been difficult if different tests incorporated different assays

CONCLUSION

The combination of bacteriophage and curcumin was shown to be more effective against the planktonic MRSA strains as well as the biofilm forms of MRSA in comparison to the two treatments alone. The results supported the hypothesis that the combination of bacteriophage and curcumin would have a synergistic antibacterial effect on multi-drug resistant bacteria and biofilms. If the results are further validated by more refined future experiments, the combination may play a role in the clinical management of patients with multi-drug resistant bacterial infections.

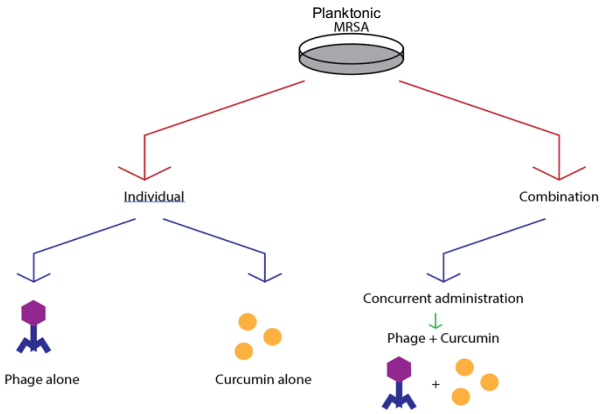
SUMMARY

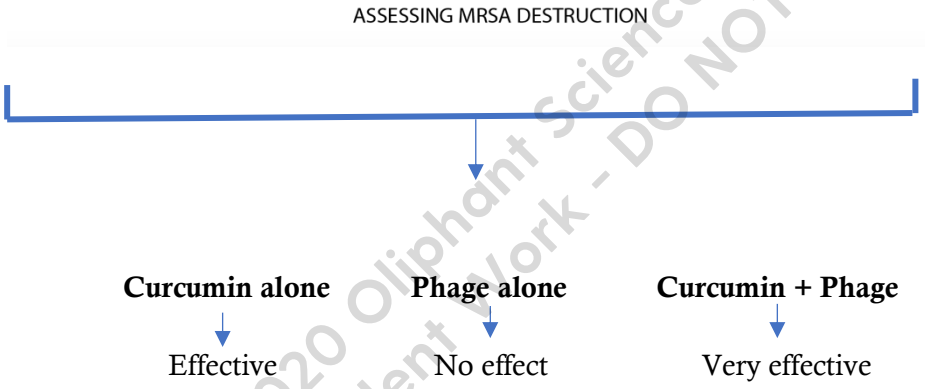
S.aureus bacteriophage

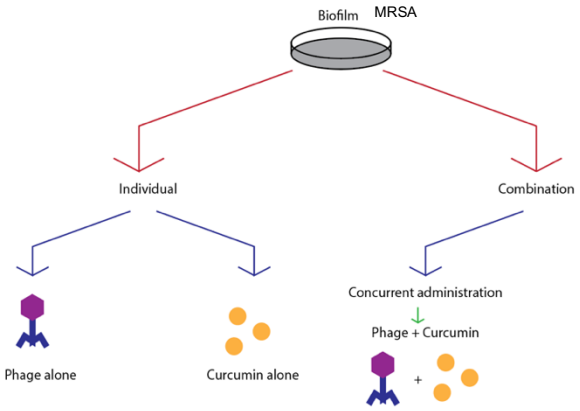
3.33×10^3 PFU/ml

Curcumin concentrations

0.2, 0.4, 0.8, 1.0 µg/ml







***S.aureus* bacteriophage**

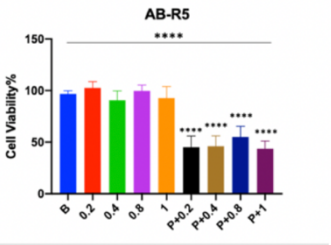
3.33×10^3 PFU/ml

Curcumin concentrations

0.2, 0.4, 0.8, 1.0 µg/ml

ASSESSING BIOFILM DESTRUCTION

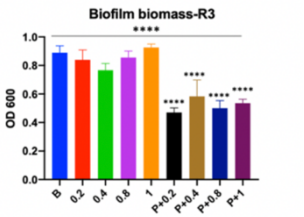
a) alamarBlue – cell viability and proliferation



Curcumin alone – viability was not affected

Curcumin + phage – viability and proliferation was reduced by almost 50%

b) Crystal Violet – assessing the biomass effect



Curcumin alone – did not impact the biomass as much as the combination

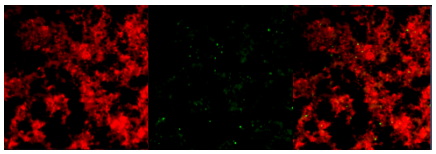
Curcumin + phage – the biomass was reduced to a greater extent

c) Live/Dead – visual assessment of live vs. dead bacterial biofilms

Phage alone – very minimal effect on bacteria

Curcumin alone – minimal dead bacteria

Curcumin + phage – predominant killing effect, larger mass of dead bacteria



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