ACTIVITY EVALUATION OF CURCUMIN COMBINATION WITH PROTAMINE SULFATE AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION

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Dedication

I dedicate my success to my dear **father** and **mother**, without whom I would not have reached what I am now, who always stood by my side with support, supplication and prayer, and thank you with all my heart.

And dedicate my success to my second half and my life partner **Anwar**, for her presence in my life and her continuous support to me with her beautiful words.

And my **sisters**, **brothers**, and **their children** who kept making me happy and making me smile always.

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ACTIVITY EVALUATION OF CURCUMIN COMBINATION WITH PROTAMINE SULFATE AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION

ABSTRACT

A bacterial biofilm is one of the most difficult structures to eradicate and is involved in the enhancement of the virulence and resistance of bacteria. *Pseudomonas aeruginosa (P.aeruginosa)* is one of the most common opportunistic human pathogens that have vigorous biofilm formation, which is responsible for many life-threatening infections like those associated with cystic fibrosis, nosocomial infections, incurable burns and wounds. Several strategies have been used to fight bacterial virulence and biofilm formation through targeting the quorum sensing (QS) mechanism of communication. Curcumin is a natural component extract from turmeric and has antimicrobial and QS inhibitory (QSI) effects on many microbes and their biofilm. This study aimed to evaluate the effect of curcumin-protamine sulfate combination against the biofilm formation by *P. aeruginosa*(ATCC 27853). The MIC and MBC test was detected by 24 well plate method, and the MIC and MBC of Curcumin 625µg/ml, 10mg/ml. and for protamine sulfate 156µg/ml, 312µg/ml. Neither the curcumin nor the protamine sulfate exhibited antibiofilm activity when tested against the *P.aeruginosa* biofilm alone. However, when curcumin was combined with protamine sulfate at different concentrations, a significant reduction in biofilm formation was detected. The highest inhibition percentage was detected

against 48hour biofilm when the biofilm was treated with 62.5µg/ml curcumin with 62.5µg/ml protamine sulfate using the 96-well plate method. When the biofilm was allowed to grow for 72 hours, the highest inhibition percentage, which was 43%, was measured when the biofilm was treated with 62.5µg/ml of curcumin, with 0.125mg/ml of protamine sulfate. The porcine *ex-vivo* model was used to confirm the previous result. The porcine skin explants was used to stimulate the biofilm growth on skin that has wounds and provides an accurate model for assessing the effects of Curcumin and protamine sulfate on mature biofilm. Explants were prepared according to the following groups: curcumin group that was treated with 100 µL of 500 µg/ml curcumin; protamine sulfate group that was treated with 100 µL of 500 µg/ml protamine sulfate; three treatment groups that were treated with 100 µL of 500 µg/ml curcumin combined with different protamine sulfate concentrations (125 µg/ml, 250 µg/ml, and 500 µg/ml); a control group that did not receive any treatment; and a vehicle group that was treated with vehicle without any treatment. The highest inhibition of biofilm was 95% when the porcine skin was treated with 500µg/ml of curcumin with 500µg/ml protamine sulfate. The obtained results from this study highly suggest that the combination of curcumin with protamine sulfate improves the effect against the biofilm, and ensures that the effect is due to the prevention of biofilm formation mainly through interruption of QS rather than killing of the bacteria. Accordingly, the tested combination can be used as an antibiofilm and anti-pathogenic agent against *P.aeruginosa*. Further studies are required to test the effect of this combination over the virulence factors of *P. aeruginosa*.

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LIST OF ABBREVIATIONS

Min	Minutes
%	Percentage
°C	Celsius
Ab	Amyloid-b
AD	Alzheimer's disease
AgNPs	Silver nanoparticles
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
CATH-2	Cathelicidin
CFU	Colony-forming unit
COX-2	Cyclooxygenase-2
Curc-gluc	Curcumin-glucoside
CurNPs	Curcumin nanoparticles
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
HDP	Host defense peptide
IL	Interleukin
IMPDH	Inosine monophosphate dehydrogenase
kDa	Kilo Dalton
LDL	Low density lipoprotein
MBC	Minimum Bactericidal Concentration
MBIC	Minimum biofilm inhibitory concentration
MDR	Multi-drug resistance
Mg	Milligram
MIC	Minimum Inhibitory Concentration
Ml	Milliliter
MRSA	Methicillin-resistant *Staphylococcusaureus*
MW	Molecular weight
NPH	Neutral protamine hagedorn
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffer saline
PD	Parkinson disease
pH	Potency of Hydrogen
QS	Quorum Sensing

S. aureus	*Staphylococcus aureus*
SD	Standard deviation

1. INTRODUCTION

Antibiotic resistance is one of the biggest modern public health challenges; each year, anew strain of bacteria is reported as resistant and this situation becomes more complicated when bacteria grow in biofilm(Li and Webster, 2018).

A biofilm can be defined as a population of microorganisms in which cells adhere to each other and to the living or non-living surfaces, embedded within a self-produced matrix of extracellular polymeric substances, and experience deep changes during their transition from planktonic (free-swimming) cells to cells that are a fraction of a complex community. The matrix, which includes exopolysaccharides, deoxyribonucleic acid (DNA) and proteins, holds the bacterial biofilm together, and protects the bacteria from environmental changes, as well as increases their tolerance and resistance to antibiotics and disinfectant chemicals. It also enables the bacterial cells to resist phagocytosis and any defense system in the body (Donlan, 2002; Berlanga and Guerrero, 2016).The growth in biofilm by any type of bacteria depends on several factors including bacterial cell density and availability of nutrients (Høiby *et al.*, 2010; Gebreyohannes *et al.*, 2019).

1.1 Biofilm formation

The formation of biofilm allows bacteria to live in close proximity to each other, which provides the best chances for Quorum Sensing communication between cells by the production and detection of autoinducers (extracellular signaling molecules) which act as letters of a certain language that are clearly understood by this community. These autoinducers allow bacteria to share information about cell density, availability of nutrients and competitors, by which the

bacteria can adjust their gene expression depending on the environmental cues(Rutherford *et al.*, 2014).

The production and spreading of bacterial biofilm occurs in five stages (Figure 1), starting with bacterial motility; the bacterial flagella propels the cells to the surfaces to which they attach reversibly by physical forces e.g. attractive or repulsive electrostatic interactions, Van der Waals interactions and hydrophobic interactions. The bacteria then irreversibly attach to each other by using cell adhesion structures such as pilli, or they produce exopolymeric material, which is a stronger adhesive compound (Praxis *et al.*, 1997; Tuson and Weibel, 2013). This is followed by the formation of a microcolony, which is the initial step of biofilm maturation. The fourth step involves further maturation of the biofilm, and it becomes a three-dimensional structure that contains colonies of cells with channels between them to deliver the nutrients and water. Cell division does not occur in the mature biofilm due to the fact that most of the cells' energy is utilized to produce the exopolysaccharide. Finally, the bacterial cells in the biofilm are dispersed into the environment to achieve more spreading and utilize more surfaces (Millezi *et al.*, 2012).

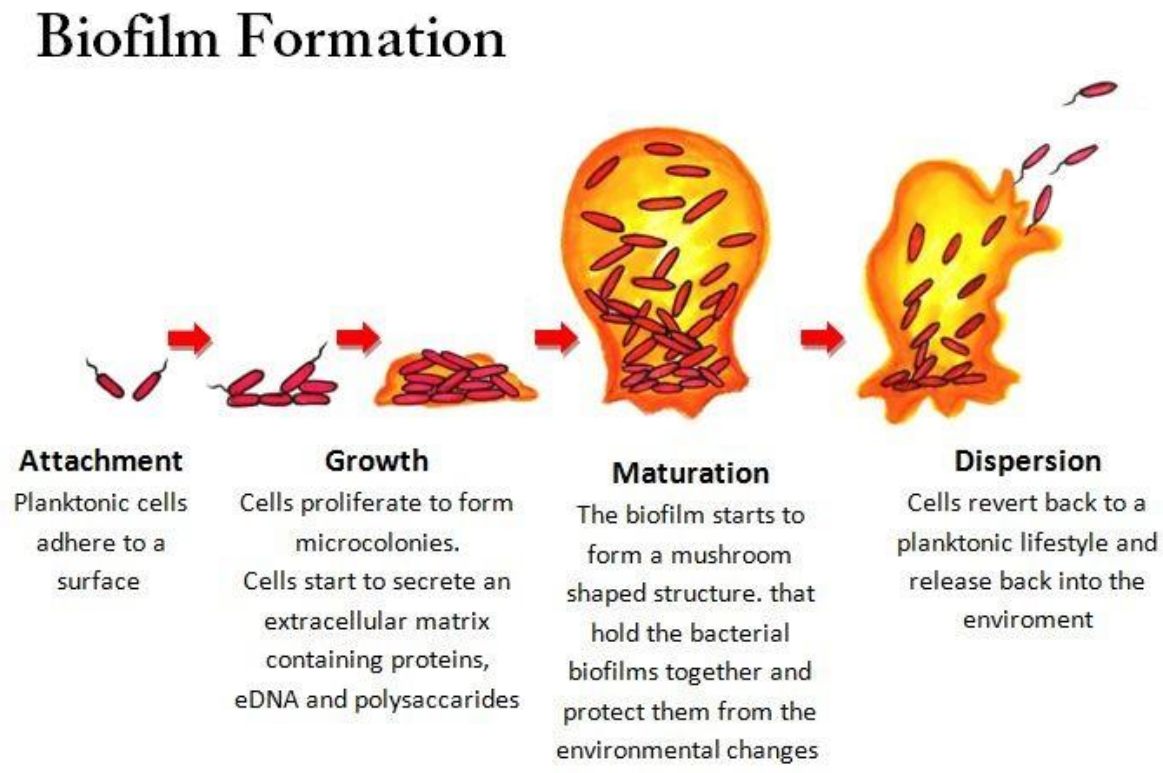

Figure 1. Stages of biofilm formation.

1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is one of the most common opportunistic human pathogens that might cause life-threatening infections e.g. burn and wound infections, complications in cystic fibrosis patients, septicemia, urinary tract infections and chronic obstructive pulmonary disease. *P. aeruginosa* infection occurs more commonly in patients with some form of immune compromisation. *P. aeruginosa* has become resistant to commonly used antibiotics, and remains one of the main causes of nosocomial infections, due to the production of several virulence

factors including pyocyanin and pyoverdin, and the ability to form intractable biofilms (Adonizio, Kong and Mathee, 2008; Wang *et al*., 2018).

Bacteria within biofilms are considered a significant concern as they are responsible for up to 65% of infections in humans (Haney, 2018). The biofilms cause 100,000 hospital deaths yearly in the United States (Sartelli, Mckimm and Bakar, 2018).Most of the current available antimicrobial agents are active against planktonic bacteria and have poor diffusion across the biofilm matrix because of the exopolysaccharide-coated area and the high communication of cells with each other which make the behavior of the cells resemble that of a multicellular organism. Accordingly, the discovery of a new anti-biofilm or anti-quorum sensing strategy and treatment is highly urgent (Technol and Bueno, 2014).

It should be kept in mind that the emergence of multidrug drug resistant (MDR) pathogens threatens the whole world and this threat increases seriously with time. Different ways to control resistant strains include finding a new antibiotic with no reported resistance, targeting the metabolism of the cells, interrupting the quorum sensing communication, and reconsidering non-antibiotic antimicrobial agents which may serve as adjuvant therapies with antibiotics or may replace antibiotics in some circumstances (Jasovský *et al.*, 2016; Li and Webster, 2018).

P. aeruginosa is a strong biofilm former with high virulence and is associated with nosocomial infections and other serious infections. With their ability to resist several antibiotics, this bacterium should be considered strongly by research and drug development companies in order to find a way to disarm and deal with this pathogen. Since the biofilm type of growth is more protected from antimicrobial agents, a way to enhance antimicrobial uptake by target cells, even if they grow in biofilm, must be found.

Objective

The aim of this study was to evaluate the inhibitory potential of curcumin, which was combined with protamine sulfate in order to enhance its activity, against biofilm formation by *P.aeruginosa*.

- Determination of the minimum biofilm inhibitory concentration of curcumin, protamine sulfate, and curcumin-protamine sulfate combination.
- Evaluation of the antibiofilm activity after 72 hours growth of *P. aeruginosa*.
- Evaluation of the activity of curcumin-protamine sulfate combination against ex-vivo biofilm formation.

2. LITERATURE REVIEW

2.1 Biofilm formation and increasing bacterial virulence

In the presence of harsh environmental circumstances, such as low oxygen, UV damage, low nutrients conditions, pH, metal toxicity, hydrogen peroxide and human immune response, many bacterial species adhere to any surface and switch to the biofilm mode of growth(Witty, College and Myers, 2015).These bacteria show an increased tolerance to antibiotics, disinfectant chemicals, phagocytosis, and other components of the body's defense system. When an antibiotic enters the body, the bacteria contact their neighboring population by releasing chemical signals to promote gene expression for cell aggregation and induce them to switch to biofilm mode of growth in a process called quorum sensing. In addition, the transfer of antibiotic resistance genes between the bacteria cells increases in the biofilm population, hence rendering the antibiotic ineffective (Adolphi *et al.*, 2014).

2.2 The virulence of *P.aeruginosa* biofilm

One bacterium that is particularly notorious for being able to form biofilms is *P. aeruginosa*; it can form it in many environmental conditions, can cause a wide variety of chronic infections like cystic fibrosis and burn infections, and it is responsible for many nosocomial infections. It is estimated that around 3% to 5% of patients leave the hospital having *P. aeruginosa* acquired infections (Mulcahy, Isabella and Lewis, 2013). The pathogenicity of *P.aeruginosa* is increased by its ability to form biofilms and the production of several virulence elements including elastase, pyocyanin and rhamnolipid (Wang *et al.*, 2018). To overcome these infections, antimicrobial drugs are used, the discovery of which is based on activity against planktonic bacteria. However, biofilms can be up to 1,000-times more resistant to common antibiotic

treatment than planktonic bacteria. The mismatch between current antibiotics and biofilm activity has led to a rethinking in the optimal strategy to fight chronic biofilm-related infections and an intensified research for new antimicrobials (Mansour, Fuente-núñez and Hancock, 2014).

2.3 Antibiofilm strategies

Several studies and strategies were aimed to weaken the biofilm and prevent it from forming again. These studies include using chlorogenic acid to repress the biofilm formation by constraining the ability of bacteria to swarm, and inhibiting their virulence factors activity and production (protease, elastase and rhamnolipid) by forming hydrogen bonds with *P. aeruginosa* QS receptors that block them (Wang *et al.*, 2018). Another study of Adolphi, *et al.*(2014), demonstrated the use of iron oxide nanoparticles to deliver antibiotics across *P. aeruginosa* biofilm, and reported that the nanoparticles showed very effective antibacterial activity by repressing the expression of certain genes essential for bacterial biofilm formation (Adolphi *et al.*, 2014).Other strategies include the use of surfactants to remove bacterial biofilm in order to facilitate the entry of the antimicrobial substance into the bacterial biofilm, by solubilizing and disaggregating the proteins responsible for allowing suspended cells to adhere to the surface(Della Porta *et al.*, 2016).

Chitosan has also been used to deactivate *P. aeruginosa*, and inhibit its bacterial growth and biofilm formation by damaging the *P. aeruginosa* membrane (Lwv *et al.*, 2016). Furthermore, using chemicals similar to iron and gallium to inhibit *P. aeruginosa* growth and biofilm formation has been reported(based on the idea that the organisms require iron for growth, and it is critical in pathogenesis and infections). By withholding the iron and transferring the gallium to

the bacteria,iron-dependent processes are inhibited, and, as a result, the growth of *P. aeruginosa* and biofilm formation is inhibited(Kaneko *et al.*, 2007).

An *in vitro* study of the 13 host defense peptides (HDPs) which are derived from different species of *P. aeruginosa* has shown antimicrobial effects on the planktonic bacteria and a capability to prevent their biofilm formation and adherence. Cathelicidin (CATH-2), one of the most potent HDPs, can penetrate the biofilm and kill the bacteria from within. This experiment showed a significant reduction in the number of viable bacteria in the biofilm(Chen *et al.*, 2018).

2.4 Curcumin as an antibacterial agent

2.4.1 Curcumin

Curcumin is a polyphenolic compound naturally derived from the *Curcuma longa* plant, which is a member of the ginger family (Zingiberaceae). It is used as a spice and as a yellow coloring agent in foods. It is relatively insoluble in water, but shows greater solubility in some organic solvents such as acetone, ethyl acetate, acetonitrile, ethanol and Dimethyl sulfoxide (DMSO) (Amalraj *et al.*, 2016). Curcumin has a lot of pharmacological properties, such as: anti-inflammatory properties by cyclooxygenase inhibition; anti-oxidant activity by blocking the reactive oxygen species formation; anti-tumor properties by inhibiting cancer cell proliferation, suppressing the growth of tumors and inducing apoptosis of tumor cells in animal models(Biology, Aggarwal and Harikumar, 2009); laxative effects, anti-helminthic agent, carminative and many benefits in the cosmetic field(Gunes *et al.*, 2016).

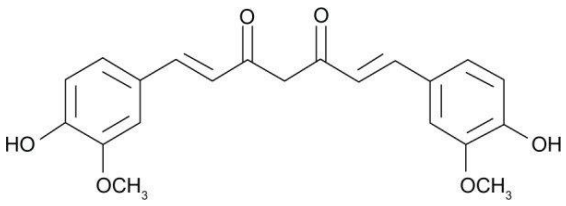

Figure 2. Curcumin structure (Amalraj *et al.*, 2016).

The antibacterial and antimicrobial effects of curcumin have been extensively studied. Curcumin has an antibacterial effect against: *Staphylococcus aureus*, *Enterococcus faecalis*, and *P. aeruginosa* through the interaction of their hydroxyl groups of phenolic compounds with the cell membrane that leads to leakage, alteration of fatty acids and phospholipid profiles in the cells and damage of the energy metabolism and synthesis of genetic materials.

The mechanism of antibacterial activity of curcumin is determined depending on the strain type. For example, the effect against *Bacillus subtilis* is inhibition of the bacterial proliferation by blocking the assembly dynamics of FtsZ in the Z ring, whereas against *P. aeruginosa*, it functions by interrupting QS mechanism, biofilm initiation and virulence (Negi *et al.*, 1999; Loo *et al.*, 2016). Another mechanism of action of curcumin as an antibacterial agent is thinning of the membrane of *S.aureus* and *Escherichia coli*, as a result disrupting the plasma membrane (Mukhopadhyay *et al.*, 2015).

The studies for synergistic effects of antibiotics in combination with plant derivatives aim to develop antimicrobial activity with a wider spectrum and to reduce adverse side effects of anti-microbial agents. Studies on curcumin have shown it to have a synergistic effect with a lot of antibiotics, thus enhancing bacterial susceptibility towards antibiotics like tetracycline, vancomycin and cefixime against *Staphylococcus aureus*. The synergistic activity of curcumin and ampicillin demonstrated pronounced reduction in the minimum inhibitory concentration (MIC) of ampicillin against a clinical strain of *S. aureus*(ATCC 25923) (Mukhopadhyay *et al.*, 2015).

Pattiyathanee *et al.*(2009) reported that the biofilm formation of *Helicobacter pylori* can be inhibited by curcumin in a dose-dependent manner. However, *H. pylori* could restore biofilm-

forming ability during a prolonged incubation period (Pattiyathanee, Vilaichone and Chaichanawongsaroj, 2009).

2.4.2 The antibiofilm effect of curcumin

In a previous study about the activity of curcumin against biofilm formation, a visible reduction in the numbers of microcolonies, deterioration of the architecture of the biofilm, slight reduction in the size of the bacterial cells, and the cell wall becoming amorphous with unspecific filaments appearing around the cells were all shown to occur. These observational changes serve to prevent biofilm formation (Loo *et al.,* 2016). In addition, the curcumin inhibited the swimming motility and swarming which is associated with biofilm formation by instigating the cell-to-cell surface attachment which plays an important role in the virulence of bacteria (Moshe, Lellouche and Banin, 2012; Packiavathy *et al.,* 2014).

The study of Loo *et al*. (2016) showed that the ionized form of silver has been used as an antibacterial agent but with low efficacy compare to the bactericidal effect of silver nanoparticles (AgNPs); the AgNPs penetrate the dispersed biofilms more efficiently. Curcumin was used as co-therapy due to its anti-bacterial effects against planktonic microbes, and its ability to enhance the bactericidal and anti-biofilm activity of AgNPs by inhibiting bacterial quorum sensing systems. The combination of the AgNPs and curcumin nanoparticles (CurNPs) has been examined as an anti-biofilm agent against the gram negative bacteria *P. aeruginosa* and gram positive bacteria *S. aureus*, and it has shown to be more effective than that of AgNPs or Cur-NPs alone (Loo *et al.*, 2016).

2.4.3 The effect of curcumin in wound healing

Ammayappan and Moses(2009) studied the anti-bacterial and wound healing activity of curcumin in combination with aloevera and chitosan to be a potential suppressor for microbial growth in cotton, wool, and rabbit hair; curcumin in combination with the other two substances showed better antimicrobial activity than aloevera or chitosan alone (Ammayappan and Moses, 2009).

Ba *et al.*(2014), studied the antibacterial, and wound healing activity of curcumin encapsulated into silane hydrogel nanoparticles to overcome poor solubility of the curcumin when applied topically and their ability to load large amount of curcumin. The nanoparticles provide the ability of curcumin to pass through the skin barrier and provide sustained release to prevent reaching to toxicity. The curcumin nanoparticles showed disruption of the cellular architecture of methicillin-resistant *S. aureus* (MRSA), acceleration of wound healing in a murine burn model and enhancement of the granulation tissue formation, collagen deposition and new vessels formation.

2.4.4 Other applications of curcumin

Absence of viable therapeutics for the majority of viral infections, development of anti-viral medication resistance, and significant expense of some antiviral treatments require finding new compelling anti-viral agents. Moreover, the current antiviral treatments are not in every case all around endured or satisfactory. Henceforth, the expanding prerequisite for antiviral substances will be more highlighted. Inosine monophosphate dehydrogenase (IMPDH) is proposed as a therapeutic target for antiviral and anticancer compounds. Curcumin has inhibitory activity against IMPDH effect, and showed inhibition of HIV-1 long terminal repeat-directed gene expression (Airaku *et al.*, 2010).

Curcumin has also been used to relieve osteoarthritis symptoms by inhibiting the inflammatory markers: down-regulating enzymes as phospholipase A2, cyclooxygenase-2 (COX-2), and lipoxygenases, and reducing tumor necrosis factor-alpha and interleukins such as interleukin-1β (IL-1β), IL-6, and IL-8(Su *et al.*, 2015).

Curcumin has shown to reduce heart disease by reducing the "bad" low density lipoprotein (LDL) cholesterol and triglycerides (Qin *et al.*, 2017). It can also regulate body fat and affect diabetes by improving the blood sugar metabolism (Selvi *et al.*, 2014).

For fungal infections, curcumin gives better outcomes in conjunction with fungal medication to treat fungal infections by disrupting fungal cell membranes (Moghadamtousi *et al.*, 2014).

There is a protective effect of extracts of curcumin on the liver and stimulation of bile secretion in animals. Curcumin has been advocated for use in disorders of liver. but the evidence for its effect on liver disease is not yet available (Kim, Kim and Lee, 2015).

Curcumin has been used to suppress prostaglandin synthesis through the inhibition of COX-2 that leads to decreased inflammation and proliferation of cancer cells. Curcumin also has an epidemiological effect that was linked to the high consumption of curcumin India (up to 1.5 g per person daily) which was associated with the overall decrease in incidence of pancreatic, lung, colorectal, prostate, and breast cancers when compared to Western countries where little curcumin is consumed (Biology, Aggarwal and Harikumar, 2009).

The ability of curcumin to decrease the risk of myocardial infarction and other cardiovascular diseases has also been confirmed. The effects of curcumin in cardiovascular diseases are linked to its ability to inhibit the platelet aggregation, their inflammatory response, oxidation of LDL and fibrinogen synthesis(Chen *et al.*, 2006).

The beneficial effect of curcumin has also been documented in various neurological disorders. Parkinson's disease (PD), an age-associated neurodegenerative disease, arises due to selective degeneration of dopaminergic neurons in the substantia nigra of the ventral midbrain thereby depleting the dopamine levels in the striatum. Most of the existing pharmacotherapeutic approaches in PD intend to replenish the striatal dopamine. However, they lack neuroprotective effects. Consequently, novel therapies involving natural antioxidants and plant products/molecules with neuroprotective properties have been exploited for adjunctive therapy. A lot of studies in various experimental models of PD strongly support the clinical application of curcumin in this neurological disorder. In light of this, Gadad *et al*. (2012) synthesized curcumin-glucoside (Curc-gluc), a modified form of curcumin, and demonstrated that addition of Curc-gluc inhibits aggregation of a-synuclein, whose aggregation is centrally implicated in PD (Gadad *et al.*, 2012).

Alzheimer's disease (AD), a progressive neurodegenerative brain disorder, is affecting increasingly older people all around the globe.Amyloid-b (Ab) plaques, widely accepted as the key pathological feature of AD, are mainly constituted by aggregation of the Ab peptide derived from the amyloid precursor protein (APP). In a mutant APP transgenic plaque-forming animal model, curcumin has been shown to reduce amyloid plaques and accumulated Ab(Lim *et al.*, 2001).

Curcumin has also been proven to play a role in diabetes mellitus type II, in which the patients develop a resistance to insulin. Several animal studies have demonstrated that curcumin can overcome insulin resistance (Suryanarayana *et al.*, 2005).

Development of drug resistance against most of the available chemotherapeutic agents represents

a foremost hurdle in cancer treatment. In this setting, Misra and Sahoo (2011) documented the use of curcumin as a chemosensitizer to potentiate the efficacy of doxorubicin in leukemia therapy (Misra and Sahoo, 2011).

2.4.5Curcumin toxicity

A group of studies reported that curcumin does not show any toxic effects in high doses. The curcumin was given to pancreatic cancer patients in an 8g daily dosage, and no toxic effects have been shown in the patients. Also, no toxic effects were seen when rats were administered 2g/kg dose of curcumin. The attractiveness of curcumin as a therapeutic agent is enhanced by its safety, affordability, and history of long-term use (Dudhatra *et al.*, 2012).

2.5 Protamine sulfate

Protamine sulfate is a basic peptide isolated from salmon testicles, used as a natural food preservative which does not influence the smell or taste of the food to which it is added. It is safety approved by sub-long-term toxicity test in rats (Miura *et al*., 2019). Protamine sulfate has been used to reverse the effects of heparin and solve heparin overdose, and to reverse the effects of heparin during delivery and heart surgery. It is given by injection into a vein; because It is broken down by stomach acids when given orally. It is a highly cationic peptide that binds to heparin molecules to form a stable ion pair, which does not have anticoagulant activity. The ionic complex is then removed and broken down by the reticuloendothelial system(Boer *et al.*, 2018). Protamine sulfate has antibacterial effects, especially against gram positive, but it is weak against yeast and fungi. Additionally, it used as co-therapy with ovotransferrin and EDTA, and ciprofloxacin against biofilm formation (Hansen and Gill, 2000).

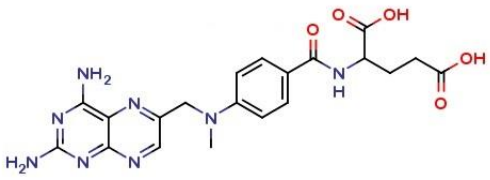

Figure 2. Protamine sulfate structure (Miura *et al*., 2019)

2.5.1 The mechanism of action of protamine sulfate as antibacterial agent

Protamine sulfate has antimicrobial effects against an array of bacterial strains. Its antimicrobial activity depends on the species, and is strongly affected by pH values and salt concentrations. The antimicrobial effect of protamine sulfate is due to the high content of arginine in the protamine. Arginine is a basic amino acid with positively charged guanidine that makes the protamine positively charged to bind with the negatively charged bacterial cell wall by electrostatic attraction. (Uyttendaele and Debevere, 1994).This is followed by disruption of the cytoplasmic membrane by electrostatic interactions between the highly positively charged molecule and the negatively charged cell surface, which leads to leakage of potassium ions, adenosine triphosphate ATP and intracellular enzymes from the cells (Hansen and Gill, 2000).

2.5.2Antimicrobial application of protamine sulfate

In previous studies, ciprofloxacin and protamine sulfate combination was shown to significantly enhance the efficacy of ciprofloxacin against *P. aeruginosa* planktonic cells and biofilm growth, with more potency against planktonic organisms compared with the *P. aeruginosa* biofilms (Davidson *et al.,* 2012).

Protamine sulfate was investigated for its antibacterial activity against periodontal pathogens. The combination between protamine sulfate andchlorhexidine showed inhibitory effects on *E.*

coli and provided a significant synergistic antibiofilm and antimicrobial activity against *E. coli*, *P. aeruginosa* and *Staphylococcus epidermidis* (Yakandawala *et al.*, 2007; Iohara *et al.*, 2010).It is added to dental materials to suppress the growth of many oral pathogens. The MIC and MBC were determined by the microdilution method and showed good inhibition and bactericidal effects (Kim, Kim and Lee, 2015).

Table 1. MIC and MBC against oral pathogens(Kim, Kim and Lee, 2015).

Microorganism	Protamine	
	MIC[a]	MBC[a]
S. anginosus KN427	5	20
S. constellatus KN436	5	10
S. gordonii DL1	0.625	0.625
S. intermedius KN433	1.25	1.25
S. mutans ATCC 25175	0.009	0.019
S. mutans KN405	0.078	0.156
S. oralis KN444	1.25	1.25
S. rattus BHT	5	20
S. salivarius KN440	0.625	1.25
S. sanguinis ATCC 10556	1.25	2.5
S. sanguinis KN420	1.25	2.5
S. sobrinus 6715	0.039	0.625
A. naeslundii CCUG 35333T	1.25	10
A. odontolyticus ATCC 17929	0.625	1.25
L. acidophilus ATCC 4355	2.5	10
E. faecalis KCTC 3206	2.5	40
E. faecalis KCTC 2011	2.5	80
A. actinomycetemcomitans ATCC 33384	20	40
F. nucleatum ATCC 23726	5	5
P. gingivalis ATCC 33277	5	10
C. albicans KCTC 7270	1.25	5

Protamine sulfate showed a good antibacterial effect when combined with chitosan than protamine sulfate alone. It has been reported that decreasing the molecular weight of chitosan may increase its binding affinity to the membrane due to improved mobility, ionic interaction, and attraction; although, an appropriate antibacterial activity can be obtained when the molecular weight (MW) is more than 10 kilo Dalton (kDa). Generally, protamine is made of 20 arginine molecules from a total of 30 amino acids. The MW of the protamine was about 4 kDa when added to chitosan, and showed higher antibacterial activity against *E. coli* than *B. cereus*.(Tamara *et al.*, 2018).

The protamine sulfate has been used for the relief the diabetic foot infection and wounds, one of the foremost complications of diabetic patients that can lead to lower limb amputations. Due to the severe damage of foot tissues with poly-microbial infections, the worst reported problem is *P. aeruginosa* wound invasion, due to its multi-resistance to a lot of antibiotics. Protamine sulfate used against diabetic foot isolated bacteria, and showed suppressed expression of various virulence proteins and cellular activities. Therefore, protamine sulfate can be used as an alternative to antimicrobial agents in wound washing to decrease the microbial load in the infected diabetic foot and wounds (Raza, 2020).

2.5.3 Other application of protamine sulfate

Protamine sulfate is utilized as a carrier of insulin to modify the properties of insulin in order to extend the duration of its blood glucose-lowering effect, decreasing the burden of treatment and minimizing dosing frequency, such as Neutral Protamine Hagedorn (NPH) insulin also known as Humulin N and Novolin N. Andinsulin lispro(Humalog, Admelog)(Owens and Biol, 2011).

The DNA/protamine complex has been reported to enhance bone formation in animal experiments. It is suggested that DNA/protamine sulfate paste will be a useful candidate material for bone regeneration (Yamachika*et al*., 2019).

It is additionally utilized in gene transfer, protein purification and in tissue cultures as a crosslinker for viral transduction. In gene therapy, protamine sulfate has been studied as a means to increase transduction rates by both viral and non-viral-mediated delivery mechanisms (e.g. utilizing cationic liposomes).Protamine sulfate has been shown to be able to condense plasmid

DNA efficiently for delivery into several different types of cells *in vitro* by several different types of cationic liposomes (Sorgi, Bhattacharya and Huang, 1997).

2.6The porcine skin and its similarity with human skin

In the last years, pigs have become more and more important as models to study human diseases due to high anatomic similarities to humans compared to another animals especially porcine skin, which is very structurally similar to human skin in epidermal thickness, blood vessel patterns and hair follicles. Porcine skin contains collagen and elastic content that is very similar to human skin biochemically than other animals (Herron, 2010).

In previous studies, the porcine skin was used as a model to assess the activity of antimicrobial dressing efficacy against mature *P. aeruginosa* biofilm. Many types of antimicrobial agents (silver, ethanol, honey iodine, and polyhexamethylene biguanide) and many types of moisture dressings (cadexomer beads,calcium alginate fiber and cotton gauze) were assessed and the cadexomer iodine and silver gel dressings were the most effective in reducing mature biofilm formation on porcine skin (Phillips *et al.*, 2013).

The porcine skin explants used in this study are incapable of accounting for the contribution of host factors (wound fluid, immune response, etc.) found in skin wounds, and were used as dermal substrate for attachment and the primary source of nutrition (Davis *et al.*, 2008). Also, the porcine skin is low in cost, simple to manipulate, and is compatible with a wide range of techniques and strategies for measuring and visualizing microbial growth and biofilm formation(Alves *et al.*, 2018).

3. MATERIALS AND METHODOLOGY

3.1Materials

The chemicals used in the present study are synthetic curcumin (95% purity) which was purchased from (abcr, Deutschland), protamine sulfate (Sigma Japan), nutrient agar (Biolab, Germany), nutrient broth (Biolab, Germany), crystal violet (ALPHA CHEMIKA, India), Sodium Chloride(Oxford, India), Phosphate buffer saline(OXOID, England), Tween 80(Sigma, Japan), Acetic acid (LABCHEM, India), Dimethyl sulfoxide(GCC,U.K.) and Sodium carbonate anhydrous (JHD,China)

3.2Technical equipment

Table 2.Equipment used in experiment.

Equipment name	Company name
Water bath sonicator	**BANDELIN**
Autoclave	**FALC**
Balance device	**BEL Enginering**
Cell Culture Plate 24 well	**SPL**
Cell Culture Plate 96 well	**SPL**
colony counter	**Wiss-Tech. Werkstatten (WTW)**
Hot plate with stirring	**Accu Plate**
Multi-channel Pipettes	**EPPENDORF**
Petri-dish	**DolphiMD**
Plate micro reader(ELIZA)	**Accu Reader**
Vortex	**N4S**

3.3*Pseudomonas aeruginosa*

P. aeruginosa (ATCC 27853) was used in this study. The *P. aeruginosa* was allowed to grow in nutrient broth and nutrient agar (depending upon the experiment in which it was used) at 37°C for 20 hours before the experiment and then diluted to the final required concentration using nutrient broth.

3.4 Curcumin and protamine sulfate stock solution preparation

Curcumin was prepared as a stock solution with a final concentration of 20 mg/ml using DMSO. The protamine sulfatewas dissolved using phosphate buffer saline (PBS) and a stock solution was prepared with a final concentration of 10 mg/ml. The working concentrations of curcumin were prepared using Tween80 (<1%) and PBS, while the working concentrations of protamine sulfate were prepared using PBS.

3.5 Minimum inhibitory concentration (MIC) test

3.5.1 Curcumin MIC

The MIC for curcumin was determined using the method described by Wedajo*et al.*(2014) with slight modification. *P. aeruginosa* was cultured in nutrient broth at 37°C overnight and the concentration of the bacteria was adjusted to be equal to 0.5 McFarland (0.08-0.1 OD at 600nm). Twenty-four well plates were used for the serial dilution of curcumin using nutrient broth, where 500µL of nutrient broth were added into all wells first. Afterwards, 500 µL of curcumin from the stock solution previously prepared (20mg/ml final concentration) were added to the first well and mixed by sucking up and down 5 times using the pipette set at 500 µl (carefully without any

splashing). This was followed by serial dilution in the remaining wells except wells 23 and 24, which were used as growth control wells (containing only nutrient broth). The final tested concentrations of curcumin were as follows 20mg/ml, 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 625µg/ml, 312µg/ml, 156µg/ml, 78.1µg/ml, 39µg/ml, 19.5µg/ml, 9.7µg/ml,4.9 µg/ml, 2.4µg/ml, 1.2µg/ml, 0.6µg/ml, 0.3µg/ml, 0.15µg/ml, 0.076µg/ml, 0.038µg/ml, 0.019µg/ml and 0.0095 µg/ml respectively.

One hundred microliters of the bacterial suspension which was adjusted to 0.5 McFarland were added to each well (including wells 23 and 24) and the plates were incubated for 18 hours at 37°C. The MIC of curcumin was determined as the lowest concentration of curcumin where no bacterial growth was detected. This experiment was repeated two times.

3.5.2 Protamine sulfate MIC

MIC of protamine sulfate was tested using the same procedure as that used for curcumin. The tested concentrations of protamine sulfate were as follows: 10 mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 625µg/ml, 312µg/ml, 156µg/ml, 78µg/ml, 39 µg/ml, 19.5µg/ml, 9.75µg/ml, 4.8µg/ml, 2.4µg/ml, 1.2µg/ml, 0.6µg/ml, 0.3µg/ml, 0.15µg/ml, 0.076 µg/ml, 0.038µg/ml, 0.019µg/ml, 0.0095 µg/ml and 0.00475 µg/ml, respectively.

One hundred microliters of the bacterial suspension which was adjusted to 0.5 McFarland were added to each well (including wells 23 and 24) and the plates were incubated for 18 hours at 37°C.

The MIC of protamine sulfate was determined as the lowest concentration of protamine where no bacterial growth was detected. This experiment was repeated two times.

3.6 The minimum bactericidal concentration test (MBC)

3.6.1 Curcumin MBC

The MBC for curcumin was determined using the method described by Kumbar *et al.* (2020) with slight modification. MBC of curcumin was tested by spreading 100µl of the mixture of bacterial cells, curcumin and nutrient broth (taken from all wells that were treated with different concentrations of curcumin, and showed no turbidity in the MIC test) over nutrient agar plates, and incubated at 37°C for 20 hours. After that, the MBC was determined by the lowest concentration of curcumin where no viable bacterial count was detected.

3.6.2 Protamine sulfate MBC

MBC of protamine sulfate was tested using the same procedure as that used for curcumin. MBC of protamine sulfate was tested by spreading 100µl of the mixture of bacterial cells, protamine sulfate and nutrient broth (taken from all wells that were treated with different concentrations of protamine sulfate, and showed no turbidity in the MIC test) over nutrient agar plates, and incubated at 37°C for 20 hours. After that, the MBC was determined by the lowest concentration of protamine sulfate where no viable bacterial count was detected.

3.7 Biofilm inhibition assay

3.7.1 Minimum biofilm inhibitory concentration (MBIC)

The MBIC activity of curcumin, protamine sulfate, and curcumin-protamine sulfate combination was tested according to the method described by Chen *et al.*(2018) with slight modifications.

3.7.1.1 Curcumin MBIC

Using a 96-well plate,50µL of curcumin solution at a concentration of 20 mg/ml were added to the first column of the 96-well plates and diluted using nutrient broth to obtain the following final concentrations of curcumin: 20mg/ml, 10mg/ml, 5mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml,0.125mg/ml, 62.5µg/ml, 31.25 µg/ml, 15.62µg/ml, and 7.8 µg/ml.

The *P. aeruginosa* was adjusted to 0.5 McFarland using nutrient broth and 100µl of this bacterial suspension was added to each well of the 96-well plate and allowed to grow and form biofilm for 48 hours at 37 °C.

At the end of the incubation period, the plates were washed with running tap water three times to remove any planktonic cells into a beaker contain 50% sodium hypochlorite, and then were air dried at room temperature for 15 minutes.

After full dryness of the plates, 200µl of 1% crystal violet solution was added to each well and the plate was incubated at room temperature for another 15 minutes. After that, the crystal violet was washed away, and the wells were allowed to dry. Then, 200µl of absolute ethanol were added to dissolve the stain. The optical density was measured at 600nm and the antibiofilm activity was calculated using the following equation:

$$I\% = ((\text{control } OD_{600} - \text{test } OD_{600}) / \text{control } OD_{600}) \times 100\%$$

Where:

I% - is the biofilm inhibition percentage

OD_{600} - is the absorbance at 600nm

Control is the untreated wells

3.7.1.2 Protamine sulfate MBIC

The MBIC for protamine sulfate was determined using the same procedure as that in section 3.5.1.1 with the following tested concentrations of protamine sulfate (50 µL): 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 62.5µg/ml and 31.25µg/ml.

3.7.1.3 Curcumin-protamine sulfate combination MBIC

Two concentrations of curcumin were prepared (0.125mg/ml and 62.5µg/ml) and three concentrations of protamine sulfate were prepared (0.125mg/ml, 62.5µg/ml and 31.25µg/ml). A volume of 25µl from each preparation (curcumin and protamine sulfate) was aspirated and mixed in the following combinations: 0.125mg/ml curcumin-0.125 mg/ml protamine sulfate, 0.125 mg/ml curcumin-62.5µg/ml protamine sulfate, 0.125 mg/ml curcumin-31.25µg/ml protamine sulfate, 62.5µg/ml curcumin-62.5µg/ml protamine sulfate, 62.5 µg/ml curcumin-31.25µg/ml protamine). The same procedure in section 3.5.1.1 was used to form biofilm and test the activity of the prepared combinations.

3.8 Biofilm formation for 72 hours and the antibiofilm activity

The method used was similar to that described by Crystal (2007) and Chen *et al.*(2018) with slight modifications.

3.8.1 Antibiofilm activity of curcumin and protamine sulfate

The same procedure in section 3.5.1.1 was used with an incubation period of 72 hr. The following concentrations of curcumin (0.125mg/ml, 62.6µg/ml, 31.25µg/ml, 15.625µg/ml, and 7.8µg/ml, and 3.9µg/ml) were used. The same concentrations were prepared for protamine sulfate. The tested volume of added curcumin or protamine sulfate was 50µl. The rest of the wells were considered as untreated control. This test was repeated two times.

3.8.2Antibiofilm activity of curcumin-protamine sulfate combination

Three concentrations of curcumin were prepared (0.125mg/ml, 62.5 µg/ml and 31.25µg/ml) and the same concentrations of protamine sulfate were prepared (0.125mg/ml, 62.5 µg/ml and 31.25µg/ml). A 25µl aliquot from each preparation (curcumin and protamine sulfate) was aspirated and mixed in the following combinations:

0.125mg/ml curcumin-0.125 mg/ml protamine sulfate, 0.125 mg/ml curcumin-62.5µg/ml protamine sulfate, 0.125 mg/ml curcumin-31.25 protamine sulfate, 62.5 µg/ml curcumin-62.5µg/ml protamine sulfate, 62.5 µg/ml curcumin-31.25µg/ml protamine sulfate, 31.25µg/ml curcumin-31.25µg/ml protamine sulfate. The same procedure in section 3.5.1.1 was used to form biofilm and test the activity of the prepared combinations with incubation at 37°C for 72 hours.

3.9 Inhibition of *Pseudomonas aeruginosa* biofilm grown on *ex vivo* skin explants by curcumin-protamine sulfate combination

P. aeruginosa biofilm was grown on an *ex vivo* wound bed according to the method described by Philips *et al.*(2013) with modifications. *P. aeruginosa* (ATCC® 27853™) was cultured in nutrient broth and incubated at 37ºC for 24hours. Porcine skin was cut while frozen by a round

cutter to obtain 12 mm diameter explants that have 3-4 mm thickness. The wound bed was created by a high-speeddrill with a round cutter to be around 3mm in diameter and 1.5 mm in depth (Louxor, China). Explants were prepared according to the following groups: curcumin group that was treated with 100 µL of 500 µg/ml curcumin; protamine sulfate group that was treated with 100 µL of 500 µg/ml protamine sulfate; three treatment groups that were treated with 100 µL of 500 µg/ml curcumin combined with different protamine sulfate concentrations (125 µg/ml, 250 µg/ml, and 500 µg/ml); a control group that did not receive any treatment; and a vehicle group that was treated with vehicle without any treatment. Explants were washed 3 times by normal saline then sterilized by chlorine gas for 45 minutes at room temperature in a sealed plastic box. Chlorine gas was generated by reacting 40 ml of acetic acid with 20ml of sodium hypochlorite commercial grade bleach. Explants were washed 3 times with sterile normal saline before they were placed on nutrient agar (0.5% agar) plates. Explants were treated with 100 µl of the following treatments: 500 µg/ml curcumin, 500 µg/ml protamine sulfate,500 µg/ml curcumin and 125 µg/ml protamine sulfate, 500 µg/ml curcumin and 250 µg/ml protamine sulfate, 500 µg/ml curcumin and 500 µg/ml protamine sulfate. The wound bed was inoculated with 10 µl of 2 McFarland standard solution of *P. Aeruginosa* which was cultured overnight in nutrient broth. Explants were incubated at 37ºC for 48 hours. They were then washed three times with sterile PBS, then aseptically placed into 15mL test tube containing 5ml of cold sterile PBS with 5µl/L Tween 80 before they were sonicated for 30 seconds and vortexed for another 30 seconds. The bacterial suspension was serially diluted then plated on nutrient agar plates and incubated overnight at 37ºC to determine the bacterial load (CFU/ml) (Figure 2).

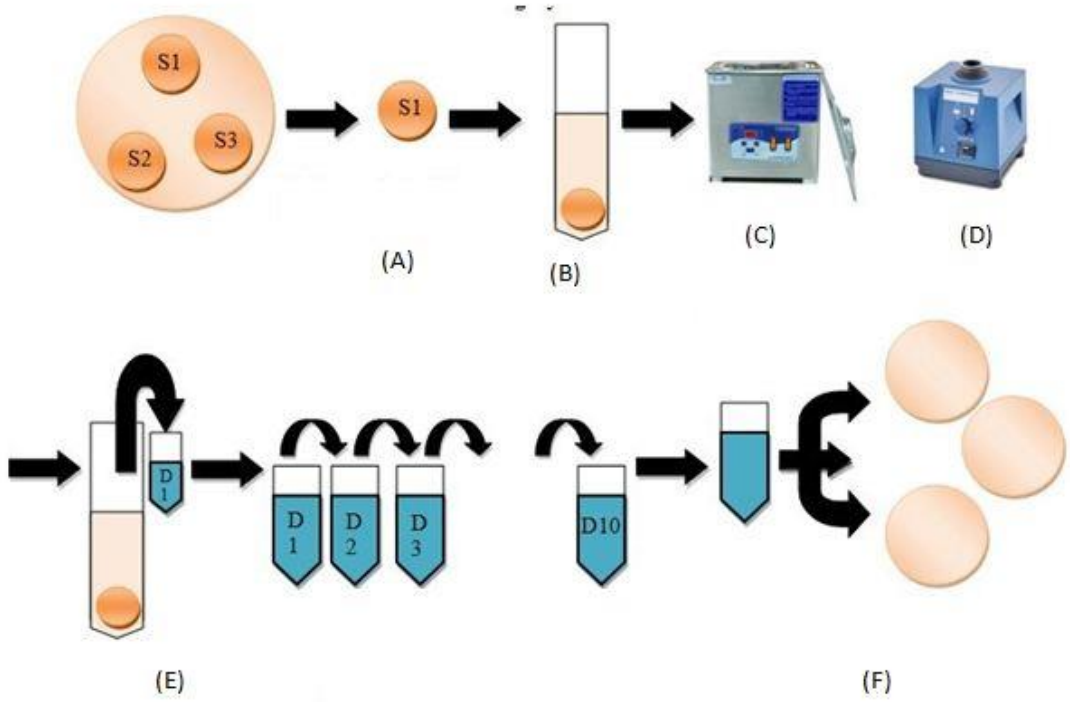

Figure 4. Schematic diagram for bacterial load determination in *ex vivo* skin model. (A) Skin explants were washed three times with sterile PBS. (B) Explants were submerged in 5 ml of cold sterile PBS with 5 µl/L Tween 80. (C) Explants were sonicated for 30 seconds. (D) Explants were vortexed for another 30 seconds. (E) The bacterial suspension was serially diluted. (F) One hundred µL of each dilution was plated on nutrient agar plates and incubated overnight at 37ºC.

3.10 Statistical analyses

The values were presented as means of three readings with standard deviation (SD). Analysis of variance was conducted and differences between variables were tested for significance by one-way ANOVA using the Microsoft Excel. T-test was used for comparisons. Differences at $P< 0.05$ were considered statistically significant.

4. RESULTS

4.1 MIC and MBC results

4.1.1 Curcumin MIC and MBC

To determine the MIC and MBC values of curcumin twenty-four well plates were used in this experiment. The tested concentrations of curcumin were as the following (Table 3).

Table 3. The tested concentrations of curcumin to determine the MIC and MBC value(s).

Curcumin concentrations	Bacterial growth (Turbidity)	Viable count
20mg/ml	No observed growth	No growth
10mg/ml(MBC)	No observed growth	**No growth**
5mg/ml	No observed growth	Viable growth
2.5mg/ml	No observed growth	Viable growth
1.25mg/ml	No observed growth	Viable growth
625µg/ml (MIC)	**No observed growth (MIC)**	Viable growth
312µg/ml	Obvious turbidity	Viable growth
156µg/ml	Obvious turbidity	Viable growth
78.1µg/ml	Obvious turbidity	Viable growth
39µg/ml	Obvious turbidity	Viable growth
19.5µg/ml	Obvious turbidity	Viable growth
9.7µg/ml	Obvious turbidity	Viable growth
4.8µg/ml	Obvious turbidity	Viable growth

2.4µg/ml	Obvious turbidity	Viable growth
1.2µg/ml	Obvious turbidity	Viable growth
0.6µg/ml	Obvious turbidity	Viable growth
0.3µg/ml	Obvious turbidity	Viable growth
0.15µg/ml	Obvious turbidity	Viable growth
0.076µg/ml	Obvious turbidity	Viable growth
0.038µg/ml	Obvious turbidity	Viable growth
0.019µg/ml	Obvious turbidity	Viable growth
0.0095 µg/ml	Obvious turbidity	Viable growth

The MIC of curcumin was 625µg/ml which is the lowest concentration of curcumin with no visible bacterial growth, and the MBC value which is the lowest bactericidal concentration was 10mg/ml.

4.1.2 Protamine sulfate MIC and MBC

To determine the MIC and MBC value of protamine sulfate the following concentrations were tested against *P. aeruginosa* (Table 4).

Table 4. The tested concentrations of protamine sulfate to determine the MIC and MBC value(s).

Protamine sulfate concentrations	Bacterial growth (Turbidity)	Viable count
10mg/ml	No observed growth	No viable count

5mg/ml	No observed growth	No viable count
2.5mg/ml	No observed growth	No viable count
1.25mg/ml	No observed growth	No viable count
625µg/ml	No observed growth	No viable count
312µg/ml (MBC)	No observed growth	**No viable count**
156µg/ml (MIC)	**No obvious growth**	Viable growth
78.1µg/ml	Obvious turbidity	Viable growth
39µg/ml	Obvious turbidity	Viable growth
19.5µg/ml	Obvious turbidity	Viable growth
9.7µg/ml	Obvious turbidity	Viable growth
4.8µg/ml	Obvious turbidity	Viable growth
2.4µg/ml	Obvious turbidity	Viable growth
1.2µg/ml	Obvious turbidity	Viable growth
0.6µg/ml	Obvious turbidity	Viable growth
0.3µg/ml	Obvious turbidity	Viable growth
0.15µg/ml	Obvious turbidity	Viable growth
0.076µg/ml	Obvious turbidity	Viable growth
0.038µg/ml	Obvious turbidity	Viable growth
0.019µg/ml	Obvious turbidity	Viable growth
0.0095 µg/ml	Obvious turbidity	Viable growth
0.00475 µg/ml	Obvious turbidity	Viable growth

The MIC of protamine sulfate was 156µg/ml, which is considered as the lowest tested concentration with no bacterial growth, while the MBC value was 312µg/ml.

4.2Minimum biofilm inhibitory concentration (MBIC)

The MBIC activity of curcumin, protamine sulfate, and curcumin-protamine sulfate combination was tested according to the method of Chen *et al.*(2018)with slight modifications.

4.2.1 Curcumin MBIC

Using 96-well plates, curcumin at different concentrations was tested to determine the minimum biofilm inhibitory concentration (20mg/ml, 10mg/ml, 5mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 62.5µg/ml, 31.25µg/ml, 15.62µg/ml, and 7.8µg/ml).

The concentrations 20mg/ml-0.125 mg/ml were not successfully tested using this essay because of the yellow color of curcumin which affected the reading of the spectrophotometer and so the antibiofilm activity was difficult to measure. The rest of the tested concentrations of curcumin did not show any biofilm inhibitory activity.

4.2.2 Protamine sulfate MBIC

To determine the MBIC of protamine sulfate the following concentrations were tested: 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 62.6µg/ml, 31.25µg/ml). The concentrations 1mg/ml-0.125mg/ml were also difficult to determine because protamine sulfate at these concentrations make a layer that adheres to the wells either in treated or in blank wells. The concentrations 62.6µg/ml-31.25µg/ml did not show any activity.

4.2.3 Inhibition of *Pseudomonas aeruginosa* biofilm (48 hr)

To test the effect of curcumin-protamine sulfate combination against the ability of *P.aeruginosa* to form biofilm, the following concentrations were mixed and the activities of the mixtures were evaluated (Table 5).

Table 5. The biofilm inhibitory percentages of curcumin-protamine sulfate combinations.

Curcumin-protamine sulfate combination (25µl from each concentration)	Biofilm inhibition percentage (%)
0.125mg/mlcurcumin-0.125 mg/ml protamine sulfate	40.6*
0.125 mg/ml curcumin-62.5µg/ml protamine sulfate	29.7*
0.125 mg/ml curcumin-31.25 protamine sulfate	18.8
62.5µg/ml curcumin-62.5µg/ml protamine sulfate	51.2*
62.5 µg/ml curcumin-31.25µg/ml protamine sulfate	36.5*

***** Significant difference $p < 0.05$

4.3 Inhibition of *Pseudomonas aeruginosa* biofilm (72 hr)

4.3.1 Antibiofilm activity of curcumin and protamine sulfate

All the tested concentrations of both curcumin and protamine sulfate did not show any biofilm inhibition activity when the *P.aeruginosa* was allowed to grow and form biofilm for 72 hours. While when curcumin combined with protamine sulfate the following results were obtained (Table 6).

Table 6.The biofilm inhibitory percentages of curcumin-protamine sulfate combination after 72hours biofilm formation.

Curcumin-protamine sulfate combinations (25µl from each concentration)	Biofilm inhibition percentage (%)
0.125mg/mlcurcumin-0.125mg/ml protamine sulfate	38.6*
0.125 mg/ml curcumin-62.5µg/ml protamine sulfate	17.1*
0.125 mg/ml curcumin-31.25 µg/ml protamine sulfate	10
62.5µg/ml curcumin-0.125µg/ml protamine sulfate	43*
62.5µg/ml curcumin-62.5µg/ml protamine sulfate	39.7*
62.5 µg/ml curcumin- 31.25µg/ml protamine sulfate	13.3

***** Significant difference $p < 0.05$

4.4 Inhibition of *Pseudomonas aeruginosa* biofilm grown on *ex vivo* skin explant by curcumin-protamine sulfate combination

P.aeruginosa biofilm susceptibility to curcumin and protamine sulfate combination(s) was tested using *ex vivo* skin model. Neither curcumin alone nor protamine sulfate alone showed significant inhibitory activity against *P.aeruginosa* biofilm. However, the biofilm was susceptible to the combination of curcumin with protamine sulfate (Figure 3). Comparing the results of the combination of curcumin with protamine sulfate, it was apparent that the activity of the combination increased with increasing concentration of protamine sulfate. Combination of curcumin (500 µg/ml) with protamine sulfate (125 µg/ml) showed no significant inhibitory activity against *P.aeruginosa* biofilm. Curcumin (500 µg/ml) in combination with either

protamine sulfate (250 µg/ml) or (500 µg/ml) showed a significant reduction in bioburden (P value < 0.05). Maximum activity was attained by the combination of curcumin (500 µg/ml) and protamine sulfate (500 µg/ml) with approximately 1.3 log cycle reduction (Table 7).

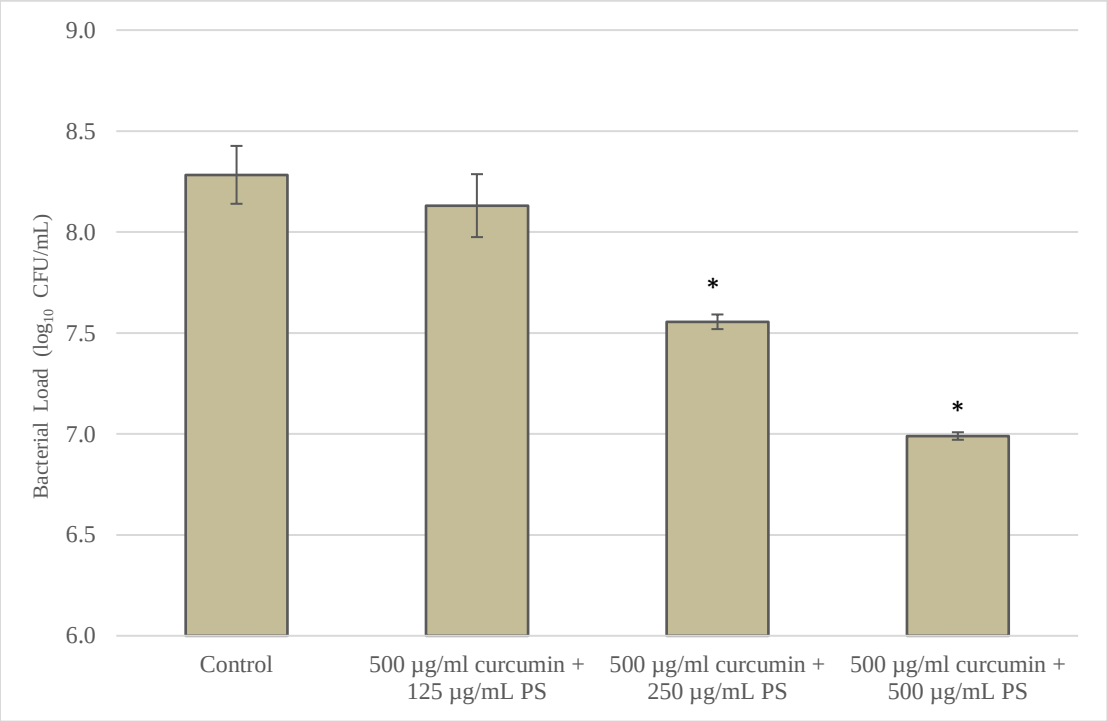

Figure 5. Effect of curcumin treatment (500 µg/ml) in combination with protamine sulfate (125 µg/ml, 250 µg/ml, and 500 µg/ml) on *P. aeruginosa* biofilm formation grown on *ex vivo* skin explants. Results are means of n=3 ± SD (PS: protamine sulfate).

Table 7: Percentage of bacterial load (CFU/ml) reduction of *Pseudomonas aeruginosa* biofilm grown on *ex vivo* skin explants after exposure to curcumin and protamine sulfate combination. Results are means of n=3 (Percentage of bacterial load reduction compared with control as 100%)

	Percentage of CFU/ml reduction (n=2)
500 µg/ml curcumin + 125 µg/ml protamine sulfate	20%
500 µg/ml curcumin + 250 µg/ml protamine sulfate	81%
500 µg/ml curcumin + 500 µg/ml protamine sulfate	95%

5) DISCUSSION

Biofilm formation is one of the most common problems that increase the virulence of bacteria and resistance toward antibiotics. It is estimated that 65% of incurable human infections are related to bacterial biofilm formation (Khatoon *et al.*, 2018). There are many mechanisms by which biofilms increase bacterial resistance to antimicrobial drugs e.g. decreasing the antimicrobial drug diffusion across the polysaccharide matrix, and, most importantly, using quorum sensing (QS) which gives the bacteria the ability to quickly recognize and respond to changes in cell density by using small signaling molecules secreted from the bacteria itself. There are many QS-dependent factors such as production of exopolysaccharide (EPS) and bio-surfactant, and swarming and swimming movement that contribute to the formation of biofilms (Packiavathy*et al.*, 2014).

Curcumin is a natural component of *Curcuma longa* and has many benefits including: anti-inflammation, antioxidant activity, and antibacterial activity against several pathogens as reported in previous studies (Etemadifar and Emtiazi, 2008; Gupta *et al.*, 2013; Hewlings, 2017). Curcumin has amphipathic and lipophilic properties which allows its insertion into the membrane, consequently enhancing membrane permeability. This activity was recorded against both Gram positive and negative bacteria including *P. aeruginosa*. Curcumin's anti-infective activity against *P. aeruginosa* is achieved mainly through affecting virulence, quorum sensing and prevention of biofilm initiation (Tyagi et al., 2015). Curcumin can inhibit the efflux pump of *P. aeruginosa* and so reduce the antimicrobial resistance using this efflux pump as reported by Negi et al., (2014); they found that the effect of combination of curcumin with several

antimicrobial agents against MDR strains of *P. aeruginosa* was through the inhibition of the efflux pump, and that the MIC value was significantly reduced with curcumin combination, while no reduction was detected when curcumin was used alone (Negi et al., 2014).

Another mechanism of action of curcumin was seen against *S. aureus and E.coli*, and was exhibited as thinning of the bacterial membrane which resulted in disruption of the plasma membrane (Mukhopadhyay et al., 2015). Bahari et al. (2017) used curcumin in a combination with azithromycin and gentamicin to improve the activity of these antibiotics and they found that the MIC was significantly reduced using this combination. Additionally, this combination reduced the motility, autoinducer production and biofilm formation (Bahari et al., 2017).

In this study, curcumin has been tested against the *P. aeruginosa* (ATCC 27853). In general, *P. aeruginosa* causes many infections, such as urinary tract infections (UTI) and severe septicemia, pneumonia and incurable burns and wounds especially in patients with immunodeficiency and it is considered a strong biofilm former bacterium. The present study demonstrated that the MIC of curcumin was 625 µg/ml which is considered as the minimum concentration of curcumin that can inhibit the bacteria, while the MBC, which represents the minimum concentration that has a cidal effect against the tested bacteria was 10 mg/ml. These values of MIC and MBC are relatively higher than the results of the previous study by Gunes et al. (2016); they found the MIC of curcumin to be 175 µg/ml (Sigma Chemicals Co., USA). Adahoun et al. (2016), reported the MBC of curcumin (Sigma, USA) to be 1 mg/ml. These variations might be due to the difference in the type and the producer company of curcumin, and the purity of the used curcumin. Protamine sulfate was used to support curcumin activity against the biofilm of the P. aeruginosa, and the MIC value of protamine sulfate in this study was 156 µg/ml (Boussard et al., 1993). The MBC value in this research was 312µg/ml.

The stock solution of curcumin was prepared by dissolving curcumin in DMSO while the working concentrations were prepared from the stock solution by adding Tween 80 and PBS. The final concentration of each surfactant does not exceed 1%. Curcumin insolubility in water was considered a limiting factor in this type of research because it reduces the bioavailability of the curcumin and so the activity. In the research of Shariati et al. (2019), curcumin was prepared as nanoparticles to improve its activity and solubility and they found that the nano-preparation of curcumin inhibited MDR *P. aeruginosa* at 128µg/ml, while it was inhibited without nano-preparation at 256 µg/ml.

The main aim of this study was to evaluate the effect of curcumin-protamine sulfate combination against the ability of *P. aeruginosa* to form biofilm. Firstly, the effect of curcumin and protamine sulfate separately was tested to measure the activity of each treatment alone and to compare it later with the effect of combination. The model used for biofilm formation was the 96-well plate method with an incubation period of 48 hours. This method is universally used for biofilm study and is considered a rapid screening assay. The tested concentrations of curcumin were 20 mg/ml down to 7.8µg/ml. It was found that the concentrations from 125 µg/ml to 20 mg/ml were not efficiently tested using this assay because of the yellow color of curcumin which affected the reading using spectrophotometer, even after the plates were washed carefully for three times. Accordingly, any concentration higher than 125µg/ml and its effect against *P.aeruginosa* cannot be examined using this assay. The rest of the tested concentrations exhibited no activity against biofilm formation. This result was consistent with those of Moshe et al. (2012), where they tested the activity of curcumin against several bacterial strains including *P.aeruginosa* and the curcumin did not show any activity against the ability of *P. aeruginosa* to form biofilm.

Neyestani et al. (2019) reported that several studies had different results regarding the effect of curcumin against *P. aeruginosa*; Niamsa and Sittiwet (2009) showed no significant effect of curcumin against *P. aeruginosa*, while others like Rudrappa and Bais (2008) found a significant reduction of the expression of virulence factors of *P.aeruginosa*.

The effect of protamine sulfate against biofilm formation was also tested using the same assay, and again the higher tested concentrations between 1 mg/ml - 0.125µg/ml made a layer inside both the treated and blank wells, which also affected the spectrophotometer reading. The rest of the tested concentrations did not show any biofilm inhibition activity. This result was consistent with that of Haynes et al. (2005).

The most important finding in this study was that when curcumin was combined with protamine sulfate different results were obtained. Separately, neither curcumin nor protamine sulfate showed any activity. However, when they were combined at different concentrations, a significant inhibition of biofilm was recorded. A combination of 1:1 curcumin to protamine sulfate at a concentration of 0.125µg/ml showed 40.6% inhibition of biofilm. A combination of 1:1 curcumin to protamine sulfate at a concentration 62.5 µg/ml showed the inhibition of biofilm formation was 51.2%, which was the highest detected inhibition percentage, even higher than the effect at the higher concentration (0.125 mg/ml) which might be due to the effect of the yellow color of curcumin.

P. aeruginosa was allowed to grow and form biofilm for 72 hours; this time period allowed more growth and stronger biofilm formation with increasing incubation time, and the activity of the combination of curcumin and protamine sulfate at different concentrations was tested. The results of 72 hour biofilm were in sync with those of the 48 hour biofilm, with obvious reduction

in inhibition. It was found that the combination at 0.125 mg/ml (1:1 curcumin to protamine sulfate) showed 38.6% inhibition, while a 1:1 ratio of curcumin at a concentration of 62.5 µg/ml and protamine sulfate at a concentration of 0.125mg/ml resulted in 43% inhibition, and 39.7% inhibition was recorded when 62.5 µg/ml of both curcumin and protamine sulfate at a 1:1 ratio were combined.

Curcumin showed sufficient strength to inhibit the growth of biofilm when protamine sulfate was added to it and demonstrated its potential to be used as a growth inhibitor of biofilm, thereby reducing the resistance of antibiotics by weakening *P. aeruginosa*.

The *ex-vivo* model was used to simulate the biofilm growth on skin that has wounds and provides an accurate model for assessing the effects of curcumin and protamine sulfate combination on mature biofilms. The fresh porcine skin was obtained directly from the slaughterhouse. The tested groups were as follows: untreated control, vehicle group, curcumin 500 µg/ml, protamine sulfate 500 µg/ml and three concentrations of protamine sulfate combined with 500 µl/ml of curcumin. The groups treated with the vehicle, curcumin (alone), and protamine sulfate (alone) each showed no inhibition of biofilm compared with untreated group. However, when was curcumin combined with protamine sulfate, the inhibition significantly improved mainly when a concentration of 500 µg/ml of protamine sulfate was combined with the curcumin; 95% inhibition of viable count was recorded, and the percentage was reduced to 81% when the same concentration of curcumin was combined with 250 µg/ml of protamine sulfate. The lowest inhibition percentage (20%) was detected when curcumin was combined with 125 µg/ml of protamine sulfate.

The enhanced effect of curcumin-protamine sulfate combination may be attributed to the positively charged protamine sulfate (due to high content of arginine amino acid); this allowed binding with the negatively charge bacterial cell wall by electrostatic attraction, followed by disruption of the cytoplasmic membrane. This makes it easier for curcumin to enter the bacterial cells and affect the ability to form biofilm over the surface of either biotic or abiotic substances. These findings are consistent with those of Uyttendaele and Debevere (1994).

Conclusion and Recommendations

The effect of curcumin-protamine sulfate combination mostly affects the QS of *P. aeruginosa*, more specifically by inhibiting the biofilm formation, without affecting the bacterial growth. This result highly supports the effect of curcumin against the QS of *P. aeruginosa* with no cidal effect. The result of this study strongly supports the QSI activity when curcumin is combined with protamine sulfate to enhance its uptake by the bacterial cells through the biofilm. This combination can be used as an anti-pathogenic therapy against *P.aeruginosa*. Further studies are required to test the effect of this combination against the virulence factors of *P.aeruginosa.* The result also leads us to suggest the potential benefit of this combination in wound dressings to protect against *P. aeruginosa* biofilms. The enhanced activity with multiple treatments and different dosage forms with prolonged activity should also be considered.

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الملخص

الغشاء الحيوي البكتيري هو أحد أصعب البنيات التي يمكن إزالتها والتي تشارك في تعزيز ضراوة البكتيريا و مقاومتها. الزائفهالزنجاريه هي واحدة من أكثر مسببات الأمراض الانتهازية شيوعًا القادره على تكوين أغشيه حيويه قويه، وهي مسؤولة عن العديد من أنواع العدوى التي تهدد الحياة مثل تلك المرتبطة بالتليف الكيسي ، والأمراض المنقله من المستشفيات ، والحروق والجروح المستعصية. تم استخدام العديد من الاستراتيجيات لمحاربة الضراوة البكتيرية وتشكيله اللأغشيه الحيويه من خلال أستهداف وسيلة التواصل بين البكتيريا التي تدعى بأستشعار النصاب. الكركمين هو مستخلص طبيعي من الكركم و له تأثيرات مثبطه للميكروبات و لأستشعار النصاب التي بالتالي تِثر على الميكروبات و على تكوينها للأغشيه الحيويه. تهدف هذه الرساله إلى تقييم مركب الكركمين مع كبريتات البروتامين ضد تكوين الزائفه الزنجاريه للأغشيه الحيويه, و مع ذلك عند خلط الكركم مع عدة تراكيز من كبريتات البروتامين, إنخفاض واضح بتكوين الغشاء الحيوي تم ملاحظته. أعلى نسبه تثبيط تم تحديدها ضد 48 ساعه من تكوين الغشاء الحيوي عند استخدام 62.5ميكرو غرام/مل مع 62.5ميكرو غرام/مل من كبريتات البروتامين بأستخدام طريقة ال 96 وعاء. و عندما تم السماح للغشاء الحيوي للنمو ل72 ساعه, أعلى نسبة تثبيط كانت 43% و التي تم قياسها عند معالجة الغشاء الحيوي ب62.5ميكرو غرام/مل مع 0.125ملغرام/مل من كبريتات البروتامين. تم أستخدام عينات مأخوذه من جلد الخنزير لتأكيد النتائج السابقة. أعلى نسبة تثبيط للغشاء الحيوي كانت 95% عندما تمت معالجتها ب 500ميكروغرام/مل من كل من الكركمين و كبريتات البروتامين. تشير النتائج التي تم الحصول عليها من هذه الدراسة بشدة إلى أن الجمع بين الكركمين وكبريتات البروتامين يحسن التأثير ضد الغشاء الحيوي. من خلال توقيف عمليه أستشعار النصاب بدلا من قتل البكتيريا. وفقا لذلك يمكن أستخدام هذا المركب كمضاد للغشاء الحيوي و مضاد للميكروبات مثل البكتيريا الزائفهالزنجاريه. هنالك حاجه إلى المزيد من الدراسات لأختبار تأثير هذا المركب على عوامل ضراوه الزائفه الزنجاريه.