



ANTIBIOFILM STUDY ON OSTEOMYELITIC
BACTERIA USING NEW GENTAMICIN-*NIGELLA*
SATIVA FUSION EMULSIONS

BY

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ABSTRACT

The treatments of chronic osteomyelitis are difficult, time-consuming and relatively expensive due to the presence of bacterial biofilm that is highly resistant to antibiotics. This study aimed to assess synergistic antibacterial activities of gentamicin-*Nigella sativa* fusion towards the most common biofilm-bacteria in osteomyelitic infection. Briefly, a total 57 samples (prostheses, bones, tissues and swabs) were taken from 17 cases of osteomyelitic infection at Hospital Tengku Ampuan Afzan, Kuantan. The samples were processed, isolated species were identified, as well as biofilm identification and antibiotic sensitivity assays were performed. Fusion of gentamicin and *N.sativa* were formulated in 4 different types of emulsions (A, B, C, and D) consisting of constant 0.1% (w/v) gentamicin and different *Nigella sativa* oil concentrations from 32.5% to 46.6% (v/v). Antimicrobial activities of the emulsions were evaluated using disc diffusion assay and determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Then, the assessment of antibiofilm activities was carried out as pre- and post-biofilm assays. The pre-biofilm consists of biofilm formation inhibition and minimum biofilm inhibition concentration (MBIC). The post-biofilm assay was done to evaluate the effects of the emulsions on the biofilm, using biofilm penetration test and confocal laser screening microscope (CLSM) analysis. It was found that prosthesis (89%) and bone (66.7%) samples produce the most bacteria growth and *Staphylococcus aureus* (10 out of 16) was the most frequently identified. In the disc diffusion assay, significant synergistic effect of emulsions was seen only in resistant *S.aureus* (clinical isolate) (Tukey's test $p < 0.05$). Additionally, emulsions MIC values were up to 10 times lower than gentamicin alone against all *S.aureus* while MBC values of emulsions were up to 3 times lower towards sensitive *S.aureus* (clinical isolate and control). No bactericidal activity was exhibited by all compounds tested on resistant *S.aureus* (clinical isolate). In pre-biofilm evaluation, there were significant differences in biofilm formation inhibition in comparison between these emulsions with *N.sativa* and gentamicin alone in both clinical isolate *S.aureus* (sensitive and resistant) (Tukey's test $p < 0.05$). MBIC values of emulsions were up to 10 times lower than gentamicin against all *S.aureus*. In contrast, *N.sativa* alone was lesser than emulsions and gentamicin. For post-biofilm assessment, no significant difference in penetration rate was found between emulsions and gentamicin. As opposed to *N.sativa* which showed little penetration. In the CLSM analysis, only emulsion C was used. Results revealed that emulsion C significantly reduced the biofilm thickness compared to gentamicin and *N.sativa* alone (Tukey's test $p < 0.05$). Furthermore, the surface percentage (%) of non-viable bacteria of emulsions is significantly higher than gentamicin and *N.sativa* alone (Tukey's test $p < 0.05$). In conclusion, this new fusion of gentamicin-*N.sativa* have synergistic antimicrobial and antibiofilm properties towards different strains of *S.aureus* including resistant strains, thus, can be developed as a new, and customized, gram-positive-specific treatment for osteomyelitic infection.

خلاصة البحث

العلاجات من التهاب العظم المزمن صعبة، تستغرق وقتا طويلا ومكلفة نسبيا بسبب وجود بكتيريا بيوفيلم التي هي شديدة المقاومة للمضادات الحيوية. هدفت هذه الدراسة إلى تقييم الأنشطة المضادة للبكتيريا المستمرة لانصهار الجنتاميسين-حبة البركة نحو الأكثر شيوعا لبكتيريا بيوفيلم في عدوى التهاب العظم . باختصار، تم اتخاذ مجموع 57 عينة من ال (prostheses والعظام والأنسجة ومسحات)أخذت من 17 حالة من حالات عدوى التهاب العظم في مستشفى تنكو امبوان أفزان، كوانتان. تم تجهيز العينات، تحديد الأنواع المعزولة، وكذلك تم تنفيذ تحديد بيوفيلم وفحوصات الحساسية للمضادات الحيوية. صيغت مزيج من الجنتاميسين وحبة البركة في 4 بأنواع مختلفة من المستحلبات (أ، ب، ج، د) ويتألف من تراكيز ثابتة 0.1% (و / ح) من الجنتاميسين وتركيزات مختلفة من زيت حبة البركة من 32.5% إلى 46.6% (ح / ح) . تم تقييم الأنشطة المضادة للميكروبات من المستحلبات باستخدام فحص القرص نشرها وتحديد الحد الأدنى لتركيز المثبط (MIC) و الحد الأدنى لتركيز مبيد الجراثيم (MBC). ثم جرى تقييم الأنشطة المضادة للبيوفيلم باجراء فحوصات ما قبل وما بعد بيوفيلم. تتكون فحوصات ما قبل بيوفيلم تثبيط تشكيل بيوفيلم و تثبيط الحد الأدنى لتركيز بيوفيلم (MBIC)وقد تم فحص ما بعد بيوفيلم لتقييم الآثار المترتبة على المستحلبات على بيوفيلم، وذلك باستخدام اختبار الاحتراق للبيوفيلم ومتحد البؤر مجهر فحص الليزر (CLSM) وقد تبين أن (89% prosthesis عينة و(66.7% عينة من العظام تنتج معظم النمو للبكتيريا والمكورات العنقودية الذهبية (10 من 16) كان الأكثر تحديدها بشكل متكرر. في قرص نشر الفحص، كان ينظر إلى تأثير متناغم كبير من المستحلبات فقط في مقاومة البكتريا (عزل السريري) (اختبار توكي $p > 0.05$). بالإضافة إلى ذلك، كانت القيم المستحلبات MIC تصل أقل إلى 10 مرات من الجنتاميسين وحده ضد كل البكتريا بينما كانت قيم مستحلبات MBC تصل إلى 3 مرات أقل تجاه البكتريا الحساسة (بالعزل السريري) والمراقبة). وقد عرضت نشاط الجراثيم لجميع مركبات واخضعت للاختبار على مقاومة البكتريا (بالعزل السريري). في تقييم ما قبل بيوفيلم، كانت هناك اختلافات كبيرة في تشكيل تثبيط بيوفيلم بالمقارنة بين هذه المستحلبات مع حبة البركة وجنتاميسين وحدها في كل من عزل البكتريا السريري (الحساسية والمقاومة) (اختبار توكي $p > 0.05$). وكانت قيم MBIC للمستحلبات أقل تصل إلى 10 مرات من جنتاميسين ضد كل البكتريا. في المقابل، كانت حبة البركة وحدها أقل من المستحلبات ومن الجنتاميسين. لتقييم ما بعد بيوفيلم، لم يتم العثور على اختلاف كبير في نسبة الانتشار بين المستحلبات وجنتاميسين. في مقابل حبة البركة التي أظهرت احتراقا قليلا. في تحليل CLSM ، وكان يستخدم فقط مستحلب و. كشفت النتائج أن مستحلب د أدى إلى خفض كبير في سماكة بيوفيلم مقارنة مع الجنتاميسين وحبة البركة وحدها (اختبار توكي $p > 0.05$). وعلاوة على ذلك، فإن النسبة المئوية السطحية (%) من البكتيريا غير قادرة على البقاء من المستحلبات هي أعلى بكثير من الجنتاميسين وحبة البركة وحدها (اختبار توكي $p > 0.05$). في الختام، هذا الانصهار جديد من الجنتاميسين-حبة البركة يكون لهذا التأزر خصائص مضادة للميكروبات ولبيو فيلم نحو سلالات مختلفة من البكتريا بما في ذلك السلالات المقاومة، وبالتالي، يمكن تطويرها باعتبارها العلاج لغرام الإيجابية ومخصصة لعدوى التهاب العظم المزمن.

ABSTRAK

Rawatan untuk kronik osteomyelitis adalah sukar, memakan masa dan mahal kerana bakteria 'biofilm' yang tidak berkesan dengan antibiotik. Kajian ini bertujuan untuk menilai aktiviti sinergi gabungan gentamicin dan *Nigella sativa* terhadap bakteria 'biofilm' di dalam jangkitan osteomyelitis. Sebanyak 57 sampel (implan, tulang, tisu dan 'swab') telah diambil daripada 17 kes osteomyelitis di Hospital Tengku Ampuan Afzan, Kuantan. Sampel diproses dan spesies bakteria bersama dengan biofilm dikenal pasti dan ujian keberkesanan antibiotik telah dijalankan. Formulasi gabungan gentamicin dan *N.sativa* telah dihasilkan di dalam 4 jenis emulsi (A, B, C, dan D) yang terdiri daripada sama kepekatan gentamicin (0.1% w/v) dan pelbagai kepekatan *N.sativa* daripada 32.5 % sehingga 46.4% (v/v). Aktiviti anti-mikrob emulsi dinilai dengan menggunakan 'disc diffusion assay', 'minimum inhibitory concentration' (MIC), dan 'minimum bactericidal concentration' (MBC). Kemudian penialaian aktiviti 'antibiofilm' dijalankan dengan 'pre-biofilm' dan 'post-biofilm'. 'Pre-biofilm' menganalisis kesan agen antimikrobial untuk merencat penghasilan 'biofilm' dengan ujian seperti 'biofilm formation inhibition' dan 'minimum biofilm inhibition concentration' (MBIC). Sementara itu, 'post-biofilm' bertujuan menganalisis kesan agen antimikrobial terhadap 'biofilm' matang menggunakan parameter seperti 'biofilm penetration' dan analisis menggunakan 'confocal laser screening confocal microscope' (CLSM). Keputusan menunjukkan bahawa sampel yang paling banyak menghasilkan bakteria adalah implan (89%) dan tulang (66.7%). Kebanyakan bakteria yang didapati daripada sampel adalah *Staphylococcus aureus*. Ujian 'disc diffusion' telah menunjukkan semua kesan sinergi emulsi hanya pada 'resistant *S.aureus* (clinical isolate)' (Tukey's test $p < 0.05$). Selain itu nilai MIC semua emulsi adalah sehingga 10 kali lebih rendah berbanding gentamicin terhadap semua *S.aureus* dan 3 kali lebih rendah pada kedua-dua 'sensitive *S.aureus* (clinical isolate dan control)'. Tiada 'bactericidal' aktiviti diperhatikan pada semua agen yang ke atas 'resistant *S.aureus* (clinical isolate)'. Untuk 'pre-biofilm', ujian 'biofilm formation inhibition' menunjukkan perbezaan signifikan ke atas semua emulsi diantara gentamicin dan *N.sativa* terhadap kedua-dua 'clinical isolate *S.aureus* (resistant dan sensitive)' (Tukey's test $p < 0.05$). Nilai MBIC semua emulsi adalah sehingga 10 kali lebih rendah berbanding gentamicin terhadap semua *S.aureus*. Untuk penilaian 'post-biofilm', tiada perbezaan signifikan antara emulsi dan gentamicin. Untuk analisis CLSM hanya emulsi C digunakan. Keputusan menunjukkan emulsi C berjaya mengurangkan ketebalan 'biofilm' dan nilai peratus (%) bakteria yang tidak berdaya maju juga adalah paling tinggi serta perbezaan dengan gentamicin dan *N.sativa* adalah signifikan (Tukey's test $p < \text{value } 0.05$). Kesimpulannya, gabungannya gentamicin dan *N.sativa* mempunyai kesan sinergi antimikrobial dan 'antibiofilm' terhadap pelbagai jenis *S.aureus*. Gabungan ini berpotensi untuk dibangunkan sebagai agen baru untuk disesuaikan khusus pada gram- positif bakteria untuk rawatan osteomyelitis.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Health Sciences (Biomedical Sciences).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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For my late father
...My hero and my inspiration...

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TABLE OF CONTENTS

Abstract	iii
Abstract in Arabic	iv
Abstrak	v
Approval Page	vi
Declaration	viii
Acknowledgement	xi
Table of Contents	xii
List of Tables	xv
List of Figures	xvi
List of Formulas	xxi
List of Abbreviations	xxii

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction	1
1.1.1 Research Background and Justification of Study	2
1.2 Objectives and Scope of study	6
1.3 Hypotheses	7
1.4 Literature Review	7
1.4.1 Osteomyelitis	7
1.4.1.1 An Overview	7
1.4.1.2 Stages and Pathophysiology	8
1.4.1.3 Causative Agents	11
1.4.1.4 Treatments	12
1.4.2 Bacterial Biofilm	14
1.4.2.1 History	14
1.4.2.2 Definition and Overview	15
1.4.2.3 Biofilm Development	16
1.4.2.4 Biofilm Resistance	18
1.4.2.5 Others Complications of Biofilm	19
1.4.2.6 Biofilm Related Diseases	20
1.4.3 Gentamicin- <i>N.sativa</i>	23
1.4.3.1 Gentamicin Sulphate	23
1.4.3.2 <i>Nigella sativa</i>	25
1.4.4 Current Challenges	31
1.4.4.1 Biofilm and Drug Resistance	31
1.4.4.2 Microbiological Diagnostic	32
1.4.4.3 Toxicity	33
1.4.4.4 Biofilms on PMMA beads	34
1.4.4.5 High Cost of Treatment	34
1.4.5 Antibacterial Susceptibility Testing	34
1.4.5.1 Antimicrobial Properties	35
1.4.5.2 Antibiofilm Properties	36

CHAPTER 2: SAMPLE COLLECTION, SPECIES IDENTIFICATION AND BIOFILM IDENTIFICATION..... 38

2.1.Introduction	38
2.1 Materials and reagents	40
2.2 Methodology.....	40
2.3.1 Informed Consent.....	40
2.3.2 Sample Collection	40
2.3.3 Sample Processing	41
2.3.3.1 Swab Sample	41
2.3.3.2 Tissue Sample.....	41
2.3.3.3 Bone, Prosthesis and Bead.....	41
2.3.4 Bacteria Identification	42
2.3.5 Biofilm Identification.....	45
2.3.6 Gentamicin Susceptibility Testing	46
2.3.7 Bacterial Storage	47
2.4 Results and discussion	49
2.5 Conclusions	56

CHAPTER 3: FORMULATION OF GENTAMICIN-*N.SATIVA* FUSIONS AND ANTIMICROBIAL GROWTH SUSCEPTIBILITY STUDY..... 57

3.1 Introduction	57
3.2 Materials and Reagents.....	60
3.2.1 Bacteria Strains.....	60
3.2.2 Formulation of Gentamicin- <i>N.sativa</i> Fusion	60
3.2.3 Antimicrobial Susceptibility Assay	60
3.3 Methodology.....	60
3.3.1 Formulation of Gentamicin- <i>N.sativa</i> Fusion	60
3.3.1.1 Emulsification Process	60
3.3.1.2 Stability Testing	61
3.3.1.3 Centrifugation Test.....	61
3.3.1.4 Organoleptic Characterisation.....	61
3.3.2 Antimicrobial Susceptibility Testing.....	63
3.3.2.1 Preparation of Mueller Hinton Agar (MHA)	63
3.3.2.2 Preparation of Tryptic Soy Agar (TSA).....	63
3.3.2.3 Preparation of Tryptic Soy Broth (TSB)	63
3.3.2.4 Preparation of Bacterial Suspension	63
3.3.2.5 Preparation of Antimicrobial Disc	64
3.3.2.6 Kirby-Bauer Disc Diffusion Assay	64
3.3.2.7 Minimum Inhibition Concentration	67
3.3.2.8 Minimum Bactericidal Concentration (MBC)	67
3.3.2.9 Statistical Analysis	68
3.4 Results and Discussions	70
3.4.1 Gentamicin- <i>N.sativa</i> Formulation	70
3.4.2 Antimicrobial Susceptibility Assay	75
3.4.2.1 Disc Diffusion Assay.....	75
3.4.2.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).	79
3.5 Conclusion.....	87

CHAPTER 4: BIOFILM STUDY	88
4.1 Introduction	88
4.2 Materials and Reagents.....	88
4.3 Methodology.....	90
4.3.1 Preparation of Tryptic Soy Agar (TSA)	90
4.3.2 Preparation of Tryptic Soy Broth (TSB)	90
4.3.3 Preparation of Tryptic Soy Broth 1% Glucose Supplemented (TSBglu)	91
4.3.4 Preparation Bacterial Suspension for Biofilm Formation	91
4.3.5 Biofilm Formation Inhibition	91
4.3.6 Minimum Biofilm Inhibition Concentration (MBIC)	93
4.3.7 Antibiofilm Penetration Study.....	94
4.3.7.1 Biofilm Preparation	94
4.3.7.2 Antibiofilm penetration	94
4.3.8 Confocal Laser Screening Microscope (CLSM) analysis	97
4.3.8.1 Biofilm Preparation on Cover Slip and Treatments	97
4.3.8.2 LIVE/DEAD Baclight Staining.....	98
4.3.8.3 Microscope Viewing	99
4.3.8.4 Image Analysis	100
4.3.9 Statistical Analysis	102
4.4 Result and Discussion.....	103
4.4.1 Pre-Biofilm Assessments.....	103
4.4.1.1 Biofilm Formation Inhibition	103
4.4.1.2 Minimum Biofilm Inhibition Concentration (MBIC)	106
4.4.2 Post-Biofilm Assessments	109
4.4.2.1 Biofilm Penetration	109
4.4.2.2 Biofilm Thickness	114
4.4.2.3 Surface Percentage (%) Viability	116
4.5 Conclusion.....	128
CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION.....	129
5.1 General Discussion	129
5.1.1 Limitations.....	134
5.2 Conclusion.....	135
5.2.1 Further Direction.....	136
BIBLIOGRAPHY	137
PUBLICATIONS AND PRESENTATIONS.....	150
APPENDIX A: ETHICS APPROVAL.....	151
APPENDIX B: CONSENT FORM	152
APPENDIX C: SAMPLE COLLECTION SOP (FLOW CHART)	154

LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
2.1	Colony characteristic and biochemical test for each species.	43
2.2		
2.3	Classification of biofilm formation (Stepanović et al. 2007).	46
2.4	CLSI standard for gentamicin sensitivity (CLSI 2012).	46
2.5	Number of samples with bacterial growth.	49
2.6	Identified bacteria species.	51
2.7	Sample collected and identified species from each patient.	52
3.1	Selected bacteria species for antimicrobial and antibiofilm study.	59
3.2	Concentration of <i>N.sativa</i> and gentamicin in each disc that have been prepared by loading 20 µl of compounds tested.	65
3.3	Composition of different gentamicin- <i>N.sativa</i> emulsions formulated.	70
3.4	Centrifugation effects of the emulsions at different storage temperatures.	71
3.5	Organoleptic characterisation observation (colour changes) of emulsions (A, B, C, and D) from day 0 to day 30 at 8°C, 25°C and 50°C.	72
3.6	Organoleptic characterisation observation (phase separation) of emulsion (A, B, C and D) from day 0 to day 30 at 8°C, 25°C and 50°C.	73
4.1	Numbers of different bacteria strains within biofilm on polycarbonate supporting membrane.	95
4.2	Numbers of different bacteria strains within biofilm on Thermanox cover slip.	98

LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
1.1	Superficial drainage from sinus tract at the infection site (Taken from www.antimicrobe.org).	10
1.2	Surgical finding after plate removal, sequestrum (necrotic bone) and involucrum (Taken from www.stanford.edu).	11
1.3	Implantation of prosthetic material together with antibiotic beads (white beads) (Taken from www.antimicrobe.org).	13
1.4	Implantation of antibiotic beads after debridement (Taken from www.stanford.edu).	14
1.5	Stages of biofilm formation; (1) Initial attachment of planktonic form (blue) bacteria, early production of EPS and irreversible adherent, (2) maturation of biofilm and bacteria multiplication of bacteria within the matrix and (3) dispersal of fraction of biofilm and single bacteria cells to planktonic form (Taken from www.id.cdeworld.com).	22
1.6	<i>Nigella sativa</i> black seed (taken from www.disehat.com).	29
1.7	Binding site at ribosomal unit; gentamicin bind irreversibly to site A at 30s ribosomal subunit, <i>N.sativa</i> binds to site P at 50s ribosomal subunit (Taken from www.webmedcentral.com).	30
1.8	FDA Biopharmaceutical Classification System (BCS) with BCS class of gentamicin and expected BCS class after combination.	31
2.1	Algorithm for bacterial identification.	44
2.2	Timeline of sample collection until storage of the bacteria.	48
2.3	Samples collected from osteomyelitis patients; (A) prosthesis samples (antibiotic beads), (B) tissue sample, (C) swab sample, (D) bone sample and (E) prosthetic implant (screw head).	50
2.4	Isolated pathogens from samples; (A) <i>S.aureus</i> –medium yellowish colonies, (B) <i>P.aeruginosa</i> - swarming green colonies and (C) <i>S.epidermidis</i> small whitish colonies.	53

<u>Figure No.</u>	<u>Page No.</u>
3.1	62
Timeline of centrifugation test and organoleptic characterisation of gentamicin- <i>N.sativa</i> emulsions from day 0 until day 30.	
3.2	66
The position of antimicrobial discs on agar plate (maximum 5 discs per well to minimize error and overlapping inhibition zone).	
3.3	66
The zone of inhibition measurement.	
3.4	69
MIC panel and serial dilutions (2 folds) emulsions (A, B, C, and D), <i>N.sativa</i> with corresponding concentrations and gentamicin from the highest (1) to the lowest concentrations (11).	
3.5	74
Four types of formulated gentamicin- <i>N.sativa</i> emulsions (A, B, C, and D) at day 30.	
3.6	77
Zone of inhibition of different <i>S.aureus</i> strains (n=3). All emulsions showed higher zone of inhibition than gentamicin and <i>N.sativa</i> . Significant difference have been seen in comparison with <i>N.sativa</i> against all <i>S.aureus</i> and in comparison with gentamicin, significant different only seen in gentamicin sensitive <i>S.aureus</i> . (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin, (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i> .	
3.7	78
Images of zone inhibition of emulsion (A, B, C and D), gentamicin and <i>N.sativa</i> alone against gentamicin resistant <i>S.aureus</i> (clinical isolate). Emulsions have larger zone of inhibition than gentamicin and <i>N.sativa</i> .	
3.8	81
MIC (blue) and MBC (red) value of emulsions (A, B, C and D) gentamicin and <i>N.sativa</i> alone against gentamicin sensitive <i>S.aureus</i> (clinical isolate) (n=2). All emulsions have lowered MIC and MBC values than gentamicin alone.	
3.9	82
MIC (blue) and MBC (red) value of emulsions (A, B, C and D), gentamicin and <i>N.sativa</i> alone against gentamicin resistant <i>S.aureus</i> (clinical isolate) (n=2). All emulsions have lowered MIC values than gentamicin alone.	
3.10	83
MIC (blue) and MBC (red) value of emulsions (A, B, C and D), gentamicin and <i>N.sativa</i> alone against <i>S.aureus</i> ATCC 29213 (control) (n=2). All emulsions have lowered MIC and MBC values than gentamicin alone.	

<u>Figure No.</u>	<u>Page No.</u>
4.1 Schematic diagram of membrane assemblies. (A) Test assemblies with biofilm, (B) Control assemblies without biofilm.	96
4.2 Diagram of four microscope view region (yellow circle) on the coverslip with biofilm adherence (grey).	99
4.3 Step by step procedure (1-3) for determination of viable and non-viable bacteria on the coverslip surface (%) by colour threshold in ImageJ software. (1) Normal image before thresholding, (2) Thresholding of non-viable bacteria with hue 0-60 (threshold area was highlighted in white) (3) Thresholding of viable bacteria (with hue 61-100 (threshold area was highlighted in white).	101
4.4 Orthogonal view of biofilm thickness reconstructed from Z-stack images for measurement of biofilm thickness.	102
4.5 Montage view of Z stacks images. Images for surface percentage were selected from (A) top surface of biofilm, (B) middle of biofilm and (C) bottom of biofilm.	102
4.6 Biofilm formation of different <i>S.aureus</i> strains (n=3). Low biofilm formation produced by all <i>S.aureus</i> after treated with emulsion and significant different were seen in comparison with gentamicin against both clinical isolate <i>S.aureus</i> (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin, (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i> .	105
4.7 Minimum biofilm inhibition concentration (MBIC) of different <i>S.aureus</i> strains (n=2). Emulsion A, B, C and D have MBIC values lower than gentamicin against all <i>S.aureus</i> except emulsion C and D that have similar MBIC values with gentamicin against gentamicin sensitive <i>S.aureus</i> .	107
4.8 Minimum biofilm inhibition concentration (MBIC) of gentamicin sensitive <i>S.aureus</i> (clinical isolate). Emulsion A and B showed lower MBIC value than gentamicin while emulsion C and D have similar MBIC values with gentamicin. (◇) denotes the MBIC value – well without biofilm (crystal violet color indicates biofilm formation).	108
4.9 Zone of inhibition that indicates emulsion C and gentamicin penetration through biofilm-producing gentamicin sensitive <i>S.aureus</i> (clinical isolate) by; (a) emulsion A, and B, (b) emulsion C and D and (c) gentamicin.	110

<u>Figure No.</u>	<u>Page No.</u>
4.10 Zone of inhibition that indicates emulsion C and gentamicin penetration through biofilm-producing gentamicin resistant <i>S.aureus</i> (clinical isolate) by; (a) emulsion A, and B, (b) emulsion C and D and (c) gentamicin.	111
4.11 Zone of inhibition that indicates emulsion C and gentamicin penetration through biofilm-producing gentamicin sensitive <i>S.aureus</i> (clinical isolate) by; (a) emulsion A, and B, (b) emulsion C and D and (c) gentamicin.	112
4.12 The biofilm penetration percentage (%) of emulsion C and gentamicin against different <i>S.aureus</i> strains (n=3). Emulsion A, B, C and D have similar penetration rate with gentamicin against both clinical isolate <i>S.aureus</i> while significant different has been seen in comparison with gentamicin against <i>S.aureus</i> ATCC 29213. (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#). As limit of detection is 13 mm, no penetration by <i>N.sativa</i> alone was seen (not included).	113
4.13 The thickness of biofilm after treatment with emulsion C and gentamicin against different <i>S.aureus</i> strains (n=4). Emulsion C has significantly lower biofilm thickness than gentamicin and <i>N.sativa</i> alone against all <i>S.aureus</i> . (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin, (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i> , (◇) denotes (Tukey's test $p < 0.05$) compared to untreated.	115
4.14 Surface percentage (%) of non-viable gentamicin sensitive <i>S.aureus</i> (clinical isolate) (n=4), at the bottom, middle and top surface of biofilm after being exposed to (blue) emulsion C, <i>N.sativa</i> and gentamicin. Emulsion C showed significant higher surface percentage (%) of non-viable <i>S.aureus</i> , (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i> .	117
4.15 Surface percentage (%) of non-viable gentamicin resistant <i>S.aureus</i> (clinical isolate) (n=4), at the bottom, middle and top surface of biofilm after being exposed to emulsion C, gentamicin and <i>N.sativa</i> . Emulsion C showed significant higher surface percentage (%) of non-viable <i>S.aureus</i> . (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i> .	118

<u>Figure No.</u>	<u>Page No.</u>
4.16	119
<p>Surface percentage (%) of non-viable <i>S.aureus</i> ATCC 29213 (control) (n=4) at the bottom, middle and top surface of biofilm after being exposed to emulsion C, <i>N.sativa</i> and gentamicin. Emulsion C showed significant higher surface percentage (%) of non-viable <i>S.aureus</i>. (*) denotes (Tukey's test $p < 0.05$) compared to gentamicin; (#) denotes (Tukey's test $p < 0.05$) compared to <i>N.sativa</i>.</p>	
4.17	120
<p>CLSM images of gentamicin sensitive <i>S.aureus</i> (clinical isolate) after treatment with emulsion C. (A) 3D reconstructed image, (B) montage view, and (C) orthogonal view. Entire images were covered with red stain, yellow and orange those indicate of death bacteria.</p>	
4.18	121
<p>CLSM images of gentamicin resistant <i>S.aureus</i> (clinical isolate) after treatment with emulsion C. (A) 3D reconstructed image, (B) montage view, and (C) orthogonal view. Only few red stains were seen at the tops surface of biofilm (B, topmost).</p>	
4.19	122
<p>CLSM images of <i>S.aureus</i> ATCC 29213 (control) after treatment with emulsion C. (A) 3D reconstructed image, (B) montage view, and (C) orthogonal view. Entire images were covered with red stains, yellow and orange those indicate of death bacteria.</p>	
5.1	131
<p>Evolutionary incline in resistance pattern of <i>S.aureus</i> (Taken from Bhagchandani et al., 2010).</p>	
5.2	132
<p>Three main factors that affected antimicrobial agent development (Wright 2014).</p>	
5.3	132
<p>Four main strategies for development of antimicrobial agents to overcome bacteria resistant (The scientist 2014).</p>	

LIST OF FORMULAS

<u>Formula No.</u>		<u>Page No.</u>
2.1	Cut off (ODc) value calculation (Stepanović et al., 2007)	45
4.1	Calculation of percentage (%) of biofilm formation	92
4.2	Calculation of percentage (%) of biofilm penetration (Singh et al. 2010)	95

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BA	Blood Agar
BCS	Biopharmaceutical Classification System
CA	Cetrimide Agar
CLSM	Confocal Laser Screening Microscope
EPS	Extracellular Polymeric Substances
FDA	Food and Drug Administration
HLB	Hydrophilic Lypophilic Balance
IREC	IIUM Research and Ethics Committee
MBC	Minimum Bactericidal Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MHA	Muller Hinton Agar
MIC	Minimum Inhibition Concentration
MREC	Medical Research and Ethics Committee
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MSA	Mannitol Salt Agar
MSSA	Methicillin Sensitive <i>Staphylococcus aureus</i>
NB	Nutrient Broth
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PJI	Prosthetic Joint Infection
PMMA	Polymethyl methacrylate
SOP	Standard Operating Procedure
SVC	Small Colony Variant
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSBglu	Tryptic Soy Broth with Glucose
VBNC	Viable but Non-Culturable

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Biofilm formation is one of the important characteristics and mechanisms for survival in certain bacteria. Its formation is essential for their survival (Donlan & Costerton, 2002) as it also act as virulence factor that make it highly pathogenic and resistant to antimicrobial agents (Mah & O'Toole, 2001). Although biofilm-producing bacteria may present in many types of chronic diseases, it is highly prevalent in osteomyelitis especially in prosthetic related infections. The increase of orthopaedic implantation contributes to high cases of failed and prolonged treatment (Brady et al. 2008). There is currently no antibiotic that is effective in the treatment against bacterial biofilm. To eradicate biofilm, high concentrations of antibiotic are needed. However, at this high, toxicity become a problem (Mah & O'Toole 2001).

One of the approaches to overcome this issue is by combining antibiotic with natural product to achieve synergistic antibacterial effects (Chanda & Rakholiya 2011). Many natural products are known to contain active metabolite that is effective against bacteria. Even though the natural product alone is not great as antibiotic, the combination is helpful in term of expanding antimicrobial spectrum, prevent emergences of resistant, increase antibiotic effectiveness and minimize the toxicity (Chanda & Rakholiya 2011). This new strategy have focused on the different mechanism of actions, enhanced antibiotic, reverse resistance mechanism and inhibit antibiotic-induce toxicity (Chanda & Rakholiya 2011; Chait et al. 2011).

1.1.1 Research Background and Justification of Study

Osteomyelitis is an inflammation of bone caused by infection. . It is the common problem that is encountered in the orthopaedic settings. Even though bone is not very likely to be susceptible to an infection, high exposure to large number of bacteria inoculation will cause osteomyelitis (Lew & Waldvogel 2004). The presence of foreign bodies such as prosthetic implants also often lead to the high risk of bacteria colonization at the bone (Brady et al. 2008). This infection can occur in acute and chronic stages. Acute stages most commonly happened in children occurring from hematogenous infection while chronic stage is the severe form of osteomyelitis that often take place in adult occurred from contiguous focus or secondary infection (Carek, Dickerson, & Sack, 2011; Cierny & Mader, 1984). Also, high risk of infection in orthopedic setting could occur notably by direct inoculation during open fracture surgery (Brady et al. 2008).

The most common pathogencausing osteomyelitis is *Staphylococcus aureus*, followed by *Enterobacteriaceae* spp. and *Pseudomonas* spp. Osteomyelitis associated with implant is 90% caused by *Staphylocccoccus epidermidis*. Patients who are immunocompromised, generally infected by polymicrobial organisms, mixed between gram-positive and gram-negative bacteria (Ciampolini & Harding 2000). The infection usually characterized by necrotic dead bone tissue that acts as a non-living surface for bacterial attachment and biofilm formation (Mader et al. 1996; Brady et al. 2008). The existence of prosthetic implants also increase high chances of bacteria adherent (Brady et al., 2008).

Microbial film is a community or an aggregation of interactive bacteria attached to a solid surface or to each other and encased in an exopolysaccharide matrix (Toole et al. 2000). These matrixes acts as protective barrier for bacteria and

prevents the access of antibiotics and make it highly resistant. To access the bacteria encased within biofilm, high concentration of antibiotic is required and that often lead to toxicity (Mah & O'Toole 2001).

The treatments of osteomyelitis are so difficult because of biofilm resistant to antibiotic and systemic antibiotic treatment is completely ineffective. Additionally, poor blood flow in the bone and slow healing process increase complication of this disease and Due to these, local implantation of antibiotic is commonly applied during the surgery for extended period of time to optimize healing rate (Barth et al. 2011). However, current available antibiotic bead for implant are very expensive and requires another minor surgery to remove the non-biodegradable beads. Indeed, there is also a possibility of biofilm growth on the surface of the antibiotic beads (Ciampolini & Harding 2000). Due to complicated treatment of osteomyelitis, it decreases patient quality of life.

The use of antibiotic alone is insufficient and not suitable for biofilm-producing bacteria. Combination with natural products might produce better outcome. Plants-derived antibacterial are source for new therapeutic approach. In the nature, infrequency of infective diseases in wild plants is an indication of the successful defence mechanism that is possess by the plant (Hemaiswarya et al. 2008). Plants are also containing many of active compounds that are useful for therapeutic application. The antibiotic combination is effective as the treatment because it has different mechanism of actions, enhance antibiotic effects, neutralise resistance, suppress antibiotic-induce toxicity and low degradation (Chait et al. 2011). Thus, it is beneficial as therapy in terms of expand antimicrobial spectrum, effective at low dosage with low toxicity, and also reduce incidence of bacteria resistant (Chanda & Rakholiya 2011).