



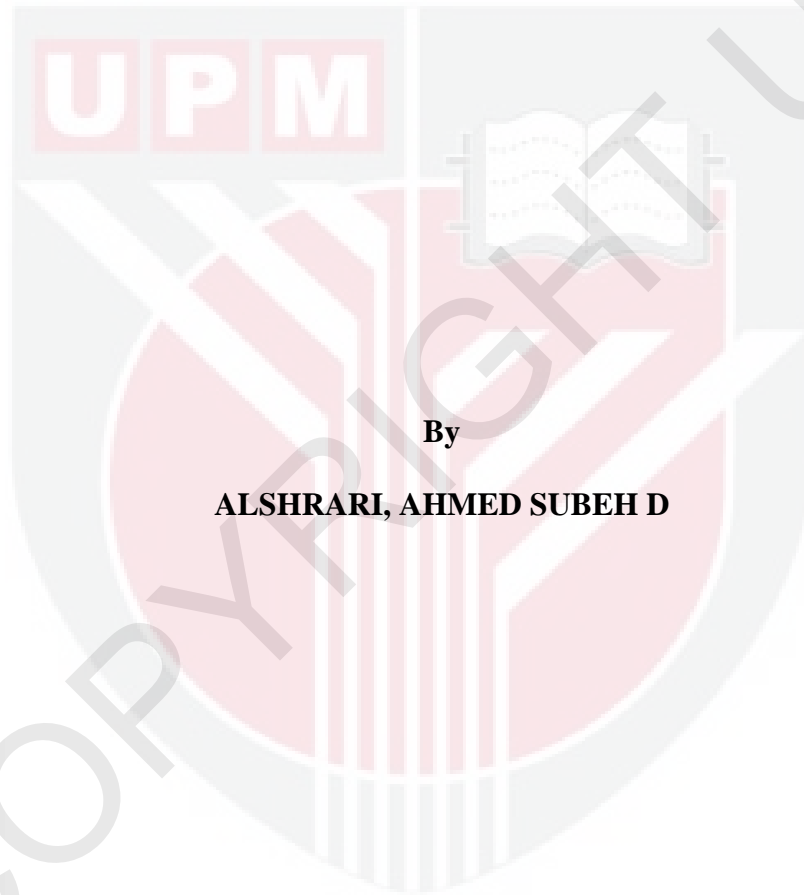
UNIVERSITI PUTRA MALAYSIA

**ANTI-METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND
ANTI-METHICILLIN-SENSITIVE *STAPHYLOCOCCUS AUREUS* EFFECTS
OF *CALLIGONUM COMOSUM* L 'HER. METHANOLIC EXTRACT**

ALSHRARI, AHMED SUBEH D

FPSK(m) 2011 4

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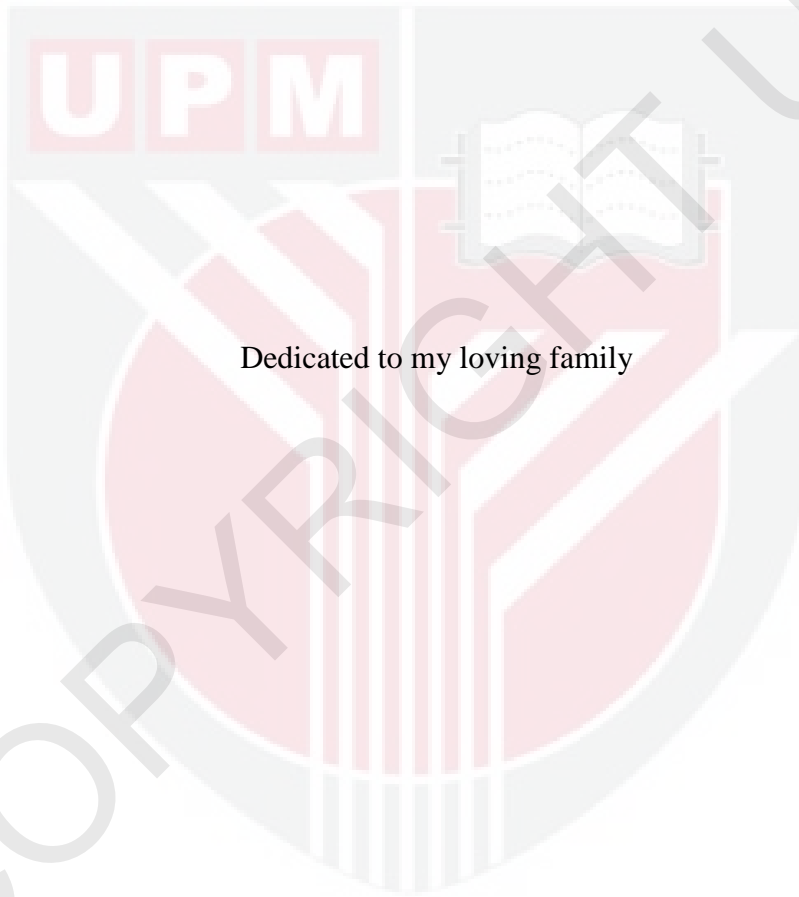
By

ALSHRARI, AHMED SUBEH D

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

MARCH 2011





Dedicated to my loving family

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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the partial requirements of the degree of Master of Science

ANTI-METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND ANTI-METHICILLIN-SENSITIVE *STAPHYLOCOCCUS AUREUS* EFFECTS OF *CALLIGONUM COMOSUM* L 'HER. METHANOLIC EXTRACT

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MARCH 2011

Chairman: Associate Professor Mariana Nor Shamsudin, PhD

Faculty: Medicine and Health Sciences

Methicillin Resistant *Staphylococcus aureus* MRSA is a major life-threatening pathogens causing a variety of serious infections and responsible for the majority of morbidity and mortality in human. Currently, more toxic and more costly antibiotics which are used as last-line agents can only be the choice to cure MRSA infections. Therefore, search for an alternative therapy derived from natural products with effective antimicrobial activity is extensively needed. The aim of this study was to

develop a combination therapy for MRSA infection by using naturally derived extract and antibiotic formulation targeting selected infective proteins.

The antimicrobial activity of *Calligonum comosum* methanolic extract (locally known as Arta), a medicinal plant native to Saudi Arabia, was evaluated using Kirby-Bauer disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), against gram positive and gram negative pathogens. The activity of the extract was determined for various clones of methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA). All MSSA and MRSA strains tested were subtyped based on multilocus sequence typing (MLST) and spa typing to differentiate the clones of these strains. *Escherichia coli* and *Klebsiella pneumoniae* were included as negative controls. The present study also verified the interactions between 17 commercial antibiotics representing 12 classes and the methanolic extract of *C. comosum* against MRSA and MSSA strains. The interaction was assessed using a disc-diffusion test performed on agar plates with or without dilution of *C. comosum* extract at sub-inhibitory concentrations, and the size of the inhibition zones (diameter) were recorded. The bioassay studies revealed that *C. comosum* extract showed inhibitory activity only to gram positive strains whereas gram negative organisms, *Escherichia coli* and *Klebsiella pneumoniae*, showed resistant phenotypic pattern to the methanolic extract. *In vitro*, 10 antibiotics (penicillin, oxacillin, cefoxitin, cloxacillin, ceftriaxone, vancomycin, chloramphenicol, clindamycin, tigecycline and rifampicin) given in combination with the methanolic extract exhibited significant synergistic interaction against most of MRSA and MSSA strains ($P= 0.05$). Namely 4 antibiotics, rifampicin,

vancomycin, ceftioxin and tigecycline ($P= 0.00$), presented the highest synergism rate with the extract in all MRSA and MSSA strains. These antibiotics when exposed in combination with the *C. comosum* extract showed greater inhibition zones when compared to the zones by single application of the antibiotics or extract. However, 6 other antibiotics had indifferent effects with the methanolic extract against most of MRSA and MSSA strains, while the extract showed antagonistic interaction with gentamicin.

In addition to the bioassay experiments, antimicrobial substance activity of *C. comosum* extract was elucidated for effect on the changes in DNA repair gene (*adaB*) sequence in treated *S. aureus* strains through PCR and RT-PCR assay. The effect of *C. comosum* extract inhibitory activity on the selected gene sequence showed several nucleotide changes. The detected changes in nucleotides through substitution, insertion or deletion of the nucleotide base pairs in the *adaB* gene sequence led to several amino acid substitutions at different positions.

Nevertheless, to find out the possibility of using the active compounds in the extract as a new alternative antibacterial agent, cytotoxic activity of *C. comosum* extract was determined using the MTS assay. The toxic effect of the extract was tested on skin fibroblast cells. Various concentrations were selected to evaluate the IC_{50} of the extract at various time intervals of 24h and 48h, respectively. As shown *in vitro*, inhibitory action of the extract used is much lower (250 mg/ml) than that reported for antimicrobials (100 mg/ml) derived naturally. Also, identification of crude extract compounds was carried out using gas chromatography-mass spectrometry (GC-MS).

GC-MS chromatogram of the given crude sample showed 42 different peaks at various retention times ranging from 5 minutes to 28 minutes for different chemical constituents. GC-MS analysis revealed the presence of 1-((allyloxy)methyl)benzene (18.11%), acetic acid (12.57%), 2-sec-butoxybenzene-1,3-diol (10.61%), formic acid (7.76%), 5-(hydroxymethyl)-2-furancarboxaldehyde (6.67%), 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (5.57%), orthomethoxyphenol (3.71%) and 1-tridecanol (1.27%) as the major components.

In conclusion, *C. comosum* methanolic extract is justified in the present study to be a suitable alternative against MSSA and MRSA based on the inhibiting effect and the low toxicity properties. In addition, the extract in combination with several antibiotics showing synergistic activity further enhances the activity of the extract. The extract also revealed to contain potential antimicrobial chemical compounds. The *C. comosum* extract warrants further investigation as a drug for anti MRSA and anti MSSA.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi sebahagian daripada syarat keperluan untuk ijazah Master Sains

**KESAN ANTI-METHACILLIN RENTANG *STAPHYLOCOCCUS AUREUS*
DAN ANTI-METHICILLIN SENSITIF *STAPHYLOCOCCUS AUREUS*
OLEH *CALLIGONUM COMOSUM L'HER* METHANOL EKSTRAK**

Oleh

ALSHRARI, AHMED SUBEH D

Mec 2010

Pengerusi: Profesor Madya Mariana Nor Shamsudin , PhD

Fakulti: Perubatan dan Sains Kesihatan

Methicillin Resistant *Staphylococcus aureus* MRSA merupakan patogen mengancam nyawa utama yang menyebabkan pelbagai jangkitan yang serius dan bertanggungjawab atas sebahagian besar morbiditi dan kematian manusia. Kini, antibiotik-antibiotik lebih beracun dan lebih mahal digunakan sebagai agen baris terakhir yang hanya boleh menjadi pilihan untuk mengubati jangkitan-jangkitan MRSA. Oleh itu, mencari terapi alternatif berasal dari produk semulajadi dengan aktiviti antimikrob yang berkesan diperlukan secara meluas. Tujuan kajian ini adalah

untuk membangunkan satu terapi gabungan untuk jangkitan MRSA dengan menggunakan ekstrak semulajadi dan perumusan antibiotik sasaran pada protein infektif terpilih.

Aktiviti antimikrob ekstrak metanol *C. comosum* (dikenali secara tempatan sebagai Arta), ubat tanaman asli di Arab Saudi, dinilai dengan menggunakan kaedah Kirby-Bauer disc difusi, kepekatan perencatan minimum (MIC) dan kepekatan bakterisida minimum (MBC), terhadap patogen gram positif dan gram negatif. Aktiviti ekstrak ditetapkan untuk pelbagai klon MSSA dan MRSA. Semua strain MSSA dan MRSA diuji subtyped berdasarkan urutan menaip multilocus (MLST) dan Spa untuk membezakan klon strain tersebut. *Escherichia coli* dan *Klebsiella pneumoniae* didigunakan sebagai kawalan negatif. Penyelidikan ini juga mengesahkan interaksi antara 17 antibiotik komersil dan ekstrak metanol *C. comosum* terhadap strain MRSA dan MSSA. Interaksi ditaksir menggunakan ujian disk-difusi yang dilakukan pada plat agar dengan atau tanpa pencairan ekstrak *C. comosum* pada kepekatan sub-perencatan, dan diameter zon perencatan itu telah direkodkan. Kajian bioessai menunjukkan bahawa ekstrak *C. comosum* menunjukkan aktiviti perencatan hanya pada strain gram positif sedangkan organisma gram negatif, *Escherichia coli* dan *Klebsiella pneumoniae*, menunjukkan pola fenotipik pertahanan terhadap ekstrak metanol. 10 antibiotik diberikan dalam kombinasi dengan ekstrak metanol secara *in vitro* mempamerkan interaksi bersinergi menentang kebanyakan daripada strain MRSA dan MSSA. Rifampisin, vankomisin, cefoxitin dan tigecycline, menunjukkan tahap sinergisme tertinggi dengan ekstrak pada semua klon MRSA dan MSSA. Keempat antibiotik ini ketika didedahkan dalam kombinasi dengan ekstrak *C. comosum* menunjukkan zon perencatan yang lebih besar jika dibandingkan dengan

zon aplikasi tunggal dari antibiotik atau ekstrak. Namun, 6 antibiotik yang lain mempunyai kesan tak berkesan dengan ekstrak metanol terhadap sebahagian besar strain MRSA dan MSSA, dan ekstrak menunjukkan interaksi antagonis dengan gentamisin.

Selain eksperimen bioessai, aktiviti antibakterial telah juga dijelaskan mengikut kesan ekstrak *C.comosum* kepada perubahan urutan gen pembaikan DNA (*adaB*) dalam strain *S. aureus* yang ditentukan melalui keadah PCR dan RT-PCR. Kesan aktiviti inhibisi ekstrak *C. comosum* pada urutan gen yang terpilih menunjukkan beberapa perubahan nukleotida.

Namun demikian, untuk mengetahui kemungkinan menggunakan sebatian aktif dalam ekstrak sebagai satu agen antibakteria alternatif yang baru, aktiviti sitotoksik ekstrak *C. comosum* ditentukan dengan menggunakan ujian MTS. Kesan toksik ekstrak diuji pada sel fibroblas kulit. Pelbagai konsentrasi dipilih untuk menilai IC₅₀ ekstrak pada pelbagai selang waktu iaitu 24 jam dan 48 jam masing-masing. Seperti yang ditunjukkan dalam *in vitro*, ekstrak *C. comosum* terbukti sebagai terapi alternatif yang selamat. Selain itu, pengenalpastian sebatian ekstrak kasar dilakukan dengan menggunakan spektrometri jisim gas kromatografi (GC-MS). GC-MS kromatogram dari sampel mentah yang diberikan menunjukkan 42 puncak yang berbeza pada waktu retensi antara 5 minit hingga 28 minit dengan konstituen yang berbeza. GC-MS analisis menunjukkan ada kehadiran 1-((allyloxy)methyl)benzene (18.11%), asid asetik (12,57%), 2-sec-butoxybenzene-1,3-diol (10,61%), asid formik (7,76%), 5-

(hydroxymethyl)-2-furancarboxaldehyde (6,67%), 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (5,57%), orthomethoxyphenol (3,71%) dan 1-tridecanol (1.27%) sebagai komponen-komponen utama.



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I certify that an Examination Committee has met on 15 April 2011 to conduct the final examination of Alshrari, Ahmed Subeh D on his degree of Master science thesis entitled “Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) and anti-methicillin-sensitive *Staphylococcus aureus* (MSSA) effects of *Calligonum comosum* methanolic extract” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U(A) 106] 15 March 1998. The Committee recommends that the student be awarded the relevant degree.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

ALSHRARI, AHMED SUBEH D

Date: 30 March 2011



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