UNIVERSITI PUTRA MALAYSIA

EFFLUX INHIBITORY ACTIVITY OF SELECTED PHYTO-COMPOUNDS AGAINST CLINICAL ISOLATES OF MULTIDRUG-RESISTANT *STAPHYLOCOCCUS AUREUS*

SAIFUL AZMI BIN JOHARI

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By

SAIFUL AZMI BIN JOHARI

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

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Chairman: Professor Abdul Manaf Ali, PhD

Faculty: Biotechnology and Biomolecular Sciences

Multidrug-resistant *Staphylococcus aureus* (MRSA) has been reported as one of the main cause of nosocomial infection in the world and ranks as one of the most difficult bacteria to treat in hospitalized patients. Apparently, these multidrug resistance (MDR) traits are caused by an array of MDR efflux pumps in *S. aureus*. In this study, a panel of identified clinical *S. aureus* isolates were tested for their multidrug-resistance profile, detection of efflux genes and evaluated against selected phyto-compounds. From the 26 bacterial isolates acquired from two teaching hospitals (HUKM and UMMC) and three ATCC *S. aureus* reference strains, 19 were confirmed as *S. aureus* isolates. Out of the 19 isolates, 14 were confirmed as methicillin-resistant *S. aureus* (MetRSA) via phenotypic and genotypic methods. Fourteen MetRSA isolates exhibit multidrug-resistance against amikacin, erythromycin, gentamicin, norfloxacin, tetracycline and trimethoprim. A methicillin-sensitive *S. aureus* (MetSSA) with multidrug-resistant trait was also detected. Apart from vancomycin, mupirocin seems to be the most effective antibiotic against all *S. aureus* isolates. Two MDR efflux genes (*mdeA* and *norA*) were detected in all isolates tested. Out of the 19 isolates, 18 harboured the *mdeA* gene while 16 isolates contained the *norA* gene. Active efflux activity in *S. aureus* was detected using modified minimum inhibitory concentration (MIC) assay with ethidium bromide and reserpine as the efflux substrate and efflux inhibitor respectively. From this assay, two MRSA clinical isolates and one ATCC 25923 *S. aureus* reference strain were selected as test strains against 37 selected phyto-compounds consisting of alkaloids, flavonoids, coumarins and essential oils. Nine compounds namely quinine, harmaline, piperine, cinnamon oil, dicumarol, eriodictyol-7,4’-dimethyl ether, 2’,4-dihydroxy-4’,5’,6’-trimethoxychalcone and naringenin-4’-methyl ether exhibited good efflux inhibitory activity as compared to reserpine. The first two are alkaloids with a methoxyl group at position C6 of an indole and quinolone skeleton, respectively. The last three are flavonoids from different sub-classes of flavanone (eriodictyol-7,4’-dimethyl and ether naringenin-4’-methyl ether) and chalcone (2’,4-dihydroxy-4’,5’,6’-trimethoxychalcone). The similarity observed amongst member of the latter group is the presence of two hydroxyl group attached to their skeletal structures.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI PERENCATAN EFLUKS DARI KOMPOUN-FITO TERPILIH TERHADAP PENCILAN KLINIKAL *STAPHYLOCOCCUS AUREUS* RINTANG-PELBAGAI-DADAH

Oleh

SAIFUL AZMI BIN JOHARI

Jun 2007

Pengerusi: **Profesor Abdul Manaf Ali, PhD**

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Staphylococcus aureus rintang-pelbagai-dadah (MRSA) telah dilaporkan menjadi punca utama jangkitan nosokomial di dunia dan tersenarai sebagai salah satu bakteria yang paling susah untuk di rawat pada pesakit hospital. Sifat rintang-pelbagai-dadah (MDR) ini adalah disebabkan oleh sekumpulan pam efluks pada *S. aureus*. Di dalam kajian ini, satu panel pencilan klinikal *S. aureus* yang telah dikenalpasti sebagai rintang-pelbagai-dadah dengan gen-gen efluks telah digunakan untuk menilai potensi perencatan efluks pada sebatian-fito terpilih. Daripada 26 pencilan bakteria yang diperoleh dari dua hospital pembelajaran (HUKM dan UMMC), 19 telah dipastikan sebagai pencilan *S. aureus*. Daripada 19 pencilan tersebut, 14 merupakan *S. aureus* rintang-methicilin (MetRSA) melalui ujian-ujian fenotipik dan genotipik. Empat belas pencilan MetRSA menunjukkan ciri rintang-pelbagai-dadah terhadap amikacin, erythromycin, gentamycin, norfloxacin, tetracycline dan trimethoprim. Satu *S. aureus* rintang-methicilin (MetSSA) dengan ciri rintang-pelbagai-dadah juga dikesan. Selain daripada vancomycin, mupirocin didapati yang paling efektif terhadap semua pencilan *S. aureus*. Dua gen efluks MDR (*mdeA* dan *norA*) di kesan daripada pencilan *S. aureus* yang ada. Lapan belas pencilan menunjukkan kehadiran gen *mdeA* manakala 16 pencilan mengandungi gen *norA*. Akiviti efluks yang aktif di kesan menggunakan kaedah kepekatan perencatan minimum (MIC) yang telah diubahsuai dengan ethidium bromide sebagai substrat efluks dan reserpine sebagai perencat efluks. Dari kaedah tersebut, dua pencilan klinikal MRSA dan satu strain rujukan *S. aureus* ATCC 25923 telah di pilih sebagai pencilan ujian terhadap 37 kompoun-fito terdiri dari kumpulan-kumpulan kompoun tumbuhan yang berbeza iaitu alkaloids, flavonoids, coumarins dan minyak pati. Sembilan sebatian iaitu quinine, harmaline, piperine, cinnamon oil, dicumarol, eriodictyol-7,4’-dimethyl ether, 2’,4-dihydroxy-4’,5’,6’-trimethoxychalcone dan naringenin-4’-methyl ether menunjukkan aktiviti perencatan efluks yang baik berbanding reserpine. Dua sebatian pertama adalah sebatian alkaloid dengan kumpulan metoksil pada posisi C6 di struktur asas indole dan quinolone. Tiga sebatian terakhir merupakan sebatian flavonoid dari sub-kelas flavanone (eriodictyol-7,4’-dimethyl dan ether naringenin-4’-methyl ether) dan chalcone (2’,4-dihydroxy-4’,5’,6’-trimethoxychalcone). Persamaan yang dapat diperhatikan adalah kehadiran dua kumpulan hidroksil pada struktur asas sebatian-sebatian tersebut.

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I certify that an Examination Committee has met on _____________ to conduct the final examination of Saiful Azmi Johari on his Master of Science thesis entitled "Efflux inhibitory Profile of Selected Phyto-compounds against Clinical Isolates of Multidrug-resistant *Staphylococcus aureus* (MRSA)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 13 September 2007

DECLARATION

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

SAIFUL AZMI JOHARI

Date: 19 July 2007

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LIST OF ABBREVIATIONS

β	beta
CDC	Centers for Disease Control and Prevention
MetRSA	methicillin-resistant *Staphylococcus aureus*
MRSA	multidrug-resistant *Staphylococcus aureus*
MetSSA	methicillin-susceptible *Staphylococcus aureus*
VRSA	vancomycin-resistant *Staphylococcus aureus*
%	percentage
>	more than
<	less than
≥	more than or equal to
≤	less than or equal to
PBP2a	Penicillin binding protein 2a
ATP	Adenosine triphosphate
MDR	multidrug resistant
UMMC	University Malaya Medical Center
HUKM	Hospital Universiti Kebangsaan Malaysia
sp	species
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
h	hour
min	Minutes
NCCLS	National Committee on Clinical Laboratory Standards

BSAC	British Society for Antimicrobial Chemotherapy
PCR	polymerase chain reaction
TSB	Trypticase soy broth
TSA	Trypticase soy agar
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
VJA	Vogel-Johnson agar
ATCC	American Type Culture Collection
^{o}C	degree celcius
µl	microliter
µg	microgram
X g	times gravity
bp	base pair
V	volt
DNA	deoxyribonucleic acid
NaCl	sodium chloride
MIC	minimum inhibitory concentration
PMF	proton motif force
BLASTN	Nucleotide-nucleotide Basic Local Alignment Search Tool
EtBr	ethidium bromide
µM	micromolar
H+	proton

Table 1: **Results of the Gram differentiation, isolation and detection of *S. aureus* using phenotypic methods. Gram positive bacteria will have no colour change (transparent) and watery suspension in the L-alanine peptidase and 3% KOH tests, respectively. Only *S. aureus* isolates would produce black colonies with yellow ring growth and agglutinates in the Vogel-Johnson agar and the Pastorex Staph-Plus Test respectively.**

Isolates	Gram differentitation		Vogel-Johnson Agar	Pastorex Staph-Plus Test
	L-alanine peptidase test	3% KOH		
N 391	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
N 441	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
N 829	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
N 850	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
N 1406	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
U 949	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 1	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 2	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 3	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 6	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 7	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 9	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 10	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 11	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 13	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
UM 14	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
ATCC 25923 *S. aureus*	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
ATCC 29213 *S. aureus*	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
ATCC 33591 MetRSA	No color change	Watery suspension	Black colonies with yellow ring growth	Agglutination
ATCC 35218 *E. coli*	Yellow coloration	Sticky suspension	N/A	No agglutination
ATCC 700728 *E. coli*	Yellow coloration	Sticky suspension	N/A	No agglutination

Note: N/A = Not Applicable

Table 3: **Antibiogram profile of *S. aureus* clinical isolates and ATCC strains against selected antibiotics. Disc diffusion breakpoints are based on the NCCLS guidelines except for * which are based on the BSAC guidelines.**

ISOLATES	Antibiotics used / Diameter of inhibition zone (mm)															
	AK	AMP	C	DA	E	FD*	CN	MUP*	NOR	OX	RD	S	TEC	TE	W	V
N 391	R	R	S	R	R	R	R	S	R	R	S	R	S	R	R	S
N 441	R	R	S	S	R	S	R	S	I	R	R	R	S	R	R	S
N 829; U 949	R	R	S	S	R	S	R	S	R	R	S	R	S	R	R	S
N 850	R	R	S	S	R	R	R	S	R	R	R	R	S	R	R	S
N 1406	R	R	S	S	R	R	R	S	R	R	S	R	S	R	R	S
UM 1	R	R	R	S	R	I	R	S	R	R	S	R	S	R	R	S
UM 2; UM 9	R	R	R	S	R	S	R	S	R	R	S	R	S	R	R	S
UM 3	R	R	S	S	S	S	R	S	R	R	R	ND	S	R	R	S
UM 6	S	S	S	S	S	R	S	S	S	S	S	ND	S	S	S	S
UM 7	S	S	S	S	S	S	S	S	S	S	S	ND	S	S	S	S
UM 10	R	R	R	R	R	S	R	S	R	R	S	R	S	R	R	S
UM 11	S	R	S	S	S	R	S	S	R	S	S	ND	I	I	S	S
UM 13	R	R	S	S	R	R	R	S	R	R	R	R	I	R	R	S
UM 14	R	R	R	R	R	R	R	S	R	R	S	R	S	R	R	S
ATCC 25923	S	S	S	S	I	R	S	S	S	S	S	ND	S	S	S	S
ATCC 29213	S	S	S	S	I	R	S	I	S	S	S	ND	I	S	S	S
ATCC 33591	S	R	R	R	R	R	S	S	S	R	S	R	S	R	S	S

Degree of susceptibility: R = resistant; I = intermediate; S = susceptible; ND = Not determined

Antibiotics used in this study : AK = amikacin, 30 µg; AMP = ampicillin, 10 µg; C = chloramphenicol, 30 µg;
DA = clindamycin, 2 µg; E = erythromycin, 15 µg; FD = fusidic acid, 10 µg;
CN = gentamicin, 10 µg; MUP = mupirocin, 5 µg; NOR = norfloxacin, 10 µg;
OX = oxacillin, 1 µg; RD = rifampicin, 5 µg; S = Streptomycin, 10 µg;
TEC = teicoplanin, 30 µg; TE = tetracycline, 30 µg; W = trimethoprim, 5 µg;
V = vancomycin, 30 µg.

Table 5: *In silico* PCR results using the primers for *norA* and *mdeA* detection.

Target Genes	*norA*		*mdeA*	
Staphylococci Strains	No. of nucleotides mismatched allowed	Amplification Results	No. of nucleotides mismatched allowed	Amplification Results
S. aureus RF122	0	+	0	+
S. aureus strain Mu50	0	+	0	+
S. aureus subsp. *aureus* COL	0	+	0	+
S. aureus subsp. *aureus* MRSA252	2	-	0	+
S. aureus subsp. *aureus* MSSA476	0	+	0	+
S. aureus subsp. *aureus* MW2	0	+	0	+
S. aureus subsp. *aureus* N315	0	+	0	+
S. aureus subsp. *aureus* NCTC 8325	0	+	0	+
S. aureus subsp. *aureus* USA300	0	+	0	+
S. epidermidis ATCC 12228*	2	-	2	-

* *S. epidermidis* ATCC 12228 was used a negative control isolate in the actual experiment.

Table 6: Summary of the *in silico* nucleotide sequence alignment analysis for MSSA476 (*norA*) and MRSA252 (*mdeA*) against all available *S. aureus* database in NCBI using the BLASTN programme.

No.	Ascension number	*S. aureus* strains/gene/plasmid	Nucleotide sequence alignment analysis (% of homology)	
			MSSA476 (*norA*)	MRSA252 (*mdeA*)
1	gi|47208328|dbj|BA000017.4|	Mu50	100	99
2	gi|49243355|emb|BX571857.1|	MSSA476	100	98
3	gi|47118312|dbj|BA000033.2|	MW2	100	98
4	gi|47118324|dbj|BA000018.3|	N315	100	99
5	gi|216974|dbj|D90119.1|STANORA	N/A	100	ND
6	gi|57284222|gb|CP000046.1|	COL	99	98
7	gi|87201381|gb|CP000253.1|	NCTC8325	99	98
8	gi|87125858|gb|CP000255.1|	USA300	99	98
9	gi|693734|gb|S74031.1|	ISP794	99	ND
10	gi|82655308|emb|AJ938182.1|	RF122	99	99
11	gi|152647|gb|M62960.1|SA2NORA	pSA209	99	ND
12	gi|21328207|dbj|AB086042.1|	N/A	100	ND
13	gi|4115706|dbj|AB019536.1|	norA23	94.5	ND
14	gi|49240382|emb|BX571856.1|	MRSA252	94	100
15	gi|153054|gb|M97169.1|STANORAX	N/A	93	ND
16	gi|295163|gb|M80252.1|STANORA11	norA1199	93	ND
17	gi|19745057|gb|AC090968.14|	NCTC8325 (sabac-126)	ND	98
18	gi|27316888|gb|AE015929.1|*	ATCC12228*	84.7	100 (only 20 nucleotides)

N/A = Not Available ND = Not Detected

* *S. epidermidis* (control strain)

Table 7: Summary of the nucleotide sequence alignment analysis for *norA* using N441 and U949 against all available *S. aureus* database in NCBI using the BLASTN programme.

No.	Ascension number	*S. aureus* strains/gene/plasmid	Nucleotide sequence alignment analysis for *norA* (% of homology)	
			N441	U949
1	gi|57284222|gb|CP000046.1|	COL	100	100
2	gi|87201381|gb|CP000253.1|	NCTC8325	100	100
3	gi|87125858|gb|CP000255.1|	USA300	100	100
4	gi|693734|gb|S74031.1|	ISP794	100	100
5	gi|47208328|dbj|BA000017.4|	Mu50	99	99
6	gi|49243355|emb|BX571857.1|	MSSA476	99	99
7	gi|47118312|dbj|BA000033.2|	MW2	99	99
8	gi|47118324|dbj|BA000018.3|	N315	99	99
9	gi|216974|dbj|D90119.1|STANORA	N/A	99	99
10	gi|82655308|emb|AJ938182.1|	RF122	98	98
11	gi|152647|gb|M62960.1|SA2NORA	pSA209	98	98
12	gi|21328207|dbj|AB086042.1|	N/A	99	99
13	gi|4115706|dbj|AB019536.1|	norA23	93.5	93.5
14	gi|49240382|emb|BX571856.1|	MRSA252	93.5	93.5
15	gi|153054|gb|M97169.1|STANORAX	N/A	92.5	92.5
16	gi|295163|gb|M80252.1|STANORA11	norA1199	92	92.5
17	gi|27316888|gb|AE015929.1|*	ATCC12228*	81	81

N/A = Not Available

* *S. epidermidis* (control strain)

Table 8: **Summary of the nucleotide sequence alignment analysis for *mdeA* using N829 and UM2 against all available *S. aureus* database in NCBI using the BLASTN programme.**

No.	Ascension number	*S. aureus* strains/gene/plasmid	Nucleotide sequence alignment analysis for *mdeA* (% of homology)	
			N829	UM2
1	gi|47208328|dbj|BA000017.4|	Mu50	100	100
2	gi|57284222|gb|CP000046.1|	COL	100	100
3a	gi|87201381|gb|CP000253.1|	NCTC8325	100	100
3b	gi|19745057|gb|AC090968.14|	NCTC8325 (sabac-126)	100	100
4	gi|87125858|gb|CP000255.1|	USA300	100	100
5	gi|49243355|emb|BX571857.1|	MSSA476	100	100
6	gi|47118312|dbj|BA000033.2|	MW2	100	100
7	gi|47118324|dbj|BA000018.3|	N315	100	100
8	gi|82655308|emb|AJ938182.1|	RF122	100	100
9	gi|49240382|emb|BX571856.1|	MRSA252	99	99