



شبكة المعلومات الجامعية
التوثيق الإلكتروني والميكروفيلم

بسم الله الرحمن الرحيم



HANAA ALY



شبكة المعلومات الجامعية
التوثيق الإلكتروني والميكروفيلم



شبكة المعلومات الجامعية التوثيق الإلكتروني والميكروفيلم



HANAA ALY



شبكة المعلومات الجامعية
التوثيق الإلكتروني والميكروفيلم

جامعة عين شمس

التوثيق الإلكتروني والميكروفيلم

قسم

نقسم بالله العظيم أن المادة التي تم توثيقها وتسجيلها
علي هذه الأقراص المدمجة قد أعدت دون أية تغييرات



يجب أن

تحفظ هذه الأقراص المدمجة بعيدا عن الغبار



HANAA ALY



Faculty of Pharmacy
Microbiology and Immunology

Modulation of Resistance and Virulence of *Acinetobacter baumannii* using Some Selected Natural Products

MSc Thesis presented

By

Mahmoud Magdy Fathy

Bachelor of Pharmaceutical Sciences, 2014

Teaching assistant, Microbiology and Immunology Department

Faculty of Pharmacy, Ahram Canadian University

Submitted in Partial Fulfillment of the Requirements for the Master's degree in
Pharmaceutical Sciences (Microbiology and Immunology)

Under the supervision of

Prof. Dr. Walid Faisal Elkhatib

Professor of Microbiology and Immunology

Faculty of Pharmacy, Ain Shams University

Vice Dean of Faculty of Pharmacy, Galala university

Assist. Prof. Dr. Neveen Ahmed Abdelaziz

Assistant professor of Microbiology and Immunology

Faculty of Pharmacy, Ahram Canadian University

Assist. Prof. Dr. Nooran Sherif Elleboudy

Assistant professor of Microbiology and Immunology

Faculty of Pharmacy, Ain Shams University

2022



Faculty of Pharmacy
Microbiology and Immunology

Modulation of Resistance and Virulence of *Acinetobacter baumannii* using Some Selected Natural Products

A Thesis

Submitted in Partial Fulfillment of the Requirements for the

Master's degree

In Pharmaceutical Sciences
(Microbiology and Immunology)

By

Mahmoud Magdy Fathy

Bachelor of Pharmaceutical Sciences, 2014

Teaching assistant, Microbiology and Immunology Department
Faculty of Pharmacy, Ahram Canadian University

2022

ACKNOWLEDGEMENTS

First and foremost, all praise be to Allah, who helped me, honored me, and led me to where I am, I can never thank him enough for his countless bounties he blessed me with. " And he found you lost and guided you " Quran 93:7. May Allah's blessing goes to His final Prophet Muhammad (peace be up on him), his family and his companions.

I would like to express my thanks to my patient and supportive supervisors without whom I would not have been able to complete this research, and without whom I would not have made it through my master's degree.

I would like to thank **Prof. Dr. Walid Faisal El khatib** for his enthusiasm, scientific supervision, his sincere support, valuable advice, and continuous guidance throughout this work.

From the bottom of my heart, I would like to say big thank you to **Ass. Prof. Neveen Ahmed Abdelaziz** for scientific supervision, throughout the revision of this thesis, offering advice and encouragement with a perfect blend of insight and humor. I'm proud of, and grateful for, my time working with her.

I am greatly grateful to **Ass. Prof. Nooran Sherif Elleboudy** for scientific supervision throughout the revision of this thesis, her constant efforts, encouragement, knowledge and follow up, her motivation have deeply helped me a lot.

I would like to express my gratitude and appreciation for **Dr. Wafaa Khalaf** for her support, guidance and overall insights in this field have made this an inspiring experience for me.

I am also thankful to Faculty of Pharmacy, Ahram Canadian university, and all its member's staff for providing great helping environment and support to work.

Last and not least Great thanks to my **parents, wife and friends** for their sincere love and support.

Mahmoud Magdy

Table of contents

1	List of abbreviations	v
2	List of figures.....	viii
3	List of tables.....	x
4	Abstract.....	11
5	Introduction.....	12
6	Literature review	14
6.1	Antibiotic resistance crisis	14
6.1.1	Types of Antibiotic Resistance	16
6.1.1.1	Intrinsic resistance.....	16
6.1.1.2	Adaptive resistance	17
6.1.1.3	Acquired resistance	18
6.1.2	Mechanisms of antibiotic resistance in bacteria	19
6.1.2.1	Modifications of the antimicrobial molecule	21
6.1.2.2	Limiting drug uptake.....	21
6.1.2.3	Modification of drug targets	22
6.1.2.4	Efflux pumps.....	23
6.2	<i>Acinetobacter baumannii</i> as an urgent threat	26
6.2.1	<i>A. baumannii</i> infections	27
6.2.1.1	Respiratory infections	28
6.2.1.2	Blood stream infections	28
6.2.1.3	Skin and soft tissue infections.....	28
6.2.1.4	Urinary tract infections	29
6.2.1.5	Meningitis	29
6.2.2	<i>A. baumannii</i> virulence factors	30
6.2.2.1	Outer Membrane Proteins (Porins)	30
6.2.2.2	Cell envelope factors (LPS and Capsule).....	31
6.2.2.3	Enzymes	31

6.2.2.4 Motility	31
6.2.2.5 Micronutrient acquisition systems	32
6.2.2.6 Protein secretion systems	32
6.2.2.7 Biofilm production and quorum sensing.....	33
6.2.2.8 Resistance to desiccation and disinfection	33
6.2.3 Antibiotic Resistance mechanisms in <i>A. baumannii</i>.....	34
6.2.3.1 β -lactams	35
6.2.3.2 Fluoroquinolones	37
6.2.3.3 Aminoglycosides.....	38
6.2.3.4 Colistin.....	39
6.3 Strategies to combat antibiotic resistance crisis.....	40
7 Materials and Methods.....	46
7.1 Materials.....	46
7.1.1 Culture Media.....	46
7.1.2 Antibiotics	46
7.1.3 Chemicals	47
7.1.4 Kits and buffers	48
7.1.5 Primers	48
7.1.6 Devices and equipment	50
7.1.7 Buffers and reagents.....	51
7.1.7.1 Phosphate buffer (0.01M, pH7.2)	51
7.1.7.2 Phosphate buffered- saline (PBS, pH 7.4) (Biswas and Mettlach 2019).	51
7.1.7.3 Tris-Acetate EDTA (TAE buffer) (1X)	51
7.1.7.4 Ethidium bromide (10mg /ml)	52
7.1.7.5 Agarose gel (1.5%)	52
7.1.7.6 Resazurin Sodium Salt (0.015%) (Elshikh <i>et al.</i> 2016).	52
7.1.7.7 Crystal violet stain (0.1%)	52
7.1.7.8 Crystal violet stain (0.4%)	52
7.1.7.9 Glutaraldehyde 25 % solution (stock).....	53
7.1.7.10 Glutaraldehyde 2.5 % solution (working solution)	53
7.1.7.11 Buffer solution sodium cacodylate (0.2 M) (stock)	53

7.1.7.12	Buffer solution sodium cacodylate (0.1 M) (working solution).....	53
7.1.7.13	Osmium tetroxide solution (2%) (stock).....	53
7.1.7.14	Osmium tetroxide solution (1%) (working solution).....	53
7.2	Methods	54
7.2.1	Bacterial isolates	54
7.2.1.1	Isolate identification.....	54
7.2.1.2	Genomic DNA extraction	54
7.2.1.3	PCR amplification.....	54
7.2.1.4	Gel electrophoresis.....	54
7.2.1.5	Isolate preservation	54
7.2.2	Antibiogram of <i>A. baumannii</i> isolates	55
7.2.2.1	Inoculum preparation	55
7.2.2.2	Serial dilution of antibiotics	55
7.2.3	Microtiter plate biofilm formation assay	55
7.2.3.1	Inoculum preparation	55
7.2.3.2	Staining technique.....	55
7.2.4	PCR amplification of β -lactamase and biofilm-related genes	56
7.2.5	Antibacterial activities of cinnamic and gallic acids	56
7.2.6	Antibiofilm activities of cinnamic and gallic acids at sub-inhibitory concentrations	57
7.2.7	Growth rate analysis.....	57
7.2.8	Microscopic Analysis of antibiofilm activities of cinnamic and gallic acids	58
7.2.9	Scanning Electron Microscope analysis of antibiofilm activities of cinnamic and gallic acids.....	58
7.2.10	Antibiotic resistance modulating effect of cinnamic and gallic acids ..	58
7.2.11	Quantitative Real-Time PCR	59
7.2.12	Statistical analysis	60
7.2.13	Ethical approval	60
8	Results	61

8.1	MIC determination and resistance profiles	61
8.2	Co-existence of antimicrobial resistance	63
8.3	Quantification of biofilm biomass-formation by <i>A. baumannii</i> isolates 64	
8.4	Relationship between antibiotic susceptibility and biofilm-forming ability.....	66
8.5	Antibiotic-resistance correlations of multidrug resistant <i>A. baumannii</i> isolates	68
8.6	Molecular detection of β -lactamase-encoding genes and genotypic- phenotypic correlations	68
8.7	Correlation between biofilm-formation ability and detection of biofilm-related genes.....	70
8.8	Antimicrobial activity of gallic and cinnamic acids	72
8.9	The Anti-biofilm activities of gallic and cinnamic acids.....	73
8.10	Impact of resistance profiles and biofilm-related genes on biofilm susceptibility to gallic and cinnamic acids.....	79
8.11	Microscopic Analysis of antibiofilm activities of cinnamic and gallic acids	79
8.12	SEM Analysis of antibiofilm activities of cinnamic and gallic acids.	80
8.13	Antibiotic resistance modulating effect of cinnamic and gallic acids	80
8.14	Quantitative Real-Time PCR	85
9	Discussion.....	88
10	Summary	95
11	Conclusion	97
12	References	98

1 List of abbreviations

ABC family	ATP-Binding Cassette family
ACB complex	<i>Acinetobacter calcoaceticus</i> – <i>Acinetobacter baumannii</i> complex
AceI	<i>Acinetobacter</i> chlorhexidine efflux protein
AMEs	Aminoglycoside modifying enzymes
AmpC	Ambler class C cephalosporinases
ATCC	American Type Culture Collection
<i>bap</i>	Biofilm-associated proteins
<i>bla</i> _{NDM}	New Delhi metallo-beta-lactamase
<i>bla</i> _{oxa}	Oxacillinase beta lactamase
<i>bla</i> _{PER-1}	<i>Pseudomonas aeruginosa</i> Extended spectrum RND-1 Beta lactamase
<i>bla</i> _{VIM}	Verona integrin associated metallo-beta-lactamase
bp	Base pair
c DNA	Complementary Deoxyribonucleic acid
CDC	Centers for disease control and prevention
CFU	Colony forming unit
CLSI	Clinical and laboratory standard institute
CpaA	glycan-specific adamalysin-like protease
<i>csuE</i>	chaperon/usher pilus system
CT	Cycle threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

List of abbreviations

EPSs	Extracellular polymeric substances
I	Intermediate
Kb	Kilo base
LPS	Lipopolysaccharide
MATE family	Multidrug and Toxic compound Extrusion family
MDR	Multiple drug resistance
MFS	Major Facilitator Superfamily
MIC	Minimum inhibitory concentration
nfs	nitrofurantoin activating genes
ODc	Optical density cut-off value
<i>omp</i>	Outer membrane protein
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDR	Pan drug resistant
PetN	Phosphoryl ethanolamine
PNAG	poly- β -1,6-N-acetylglucosamine
Qnr	Quinolone resistance
qPCR	Quantitative Polymerase chain reaction
QQ	Quorum quenching
QS	Quorum sensing
R	Resistant
RNA	Ribonucleic acid
RND family	The Resistance-Nodulation-Division

List of abbreviations

ROS	Reactive oxygen species
S	Sensitive
SEM	Scanning Electron Microscope
SMR family	Small Multidrug Resistance family
T2SS	type II secretion system
T6SS	type VI secretion system
TAE	Tris-Acetate EDTA
TSB	Trypticase soya broth
VAP	Ventilator associated pneumonia
WFI	Water for injection
WHO	World health organization

2 List of figures

Figure 1. Timeline showing the decade new classes of antibiotic reached the clinic, sources of almost known antibiotic classes, and the first reports of resistant isolates.	15
Figure 2. Mode of action of classes of clinically used antibiotics.	15
Figure 3. Pathways of horizontal gene transfer.	19
Figure 4. Mechanisms of antibiotic resistance.	20
Figure 5. General structure of main efflux pump families (Reygaert 2018).	24
Figure 6. The dynamic microbiological nature of <i>Acinetobacter baumannii</i> derives from an interaction between the associated infections, wide range of virulence factors, multidrug-resistance, and animal/environmental spread.	27
Figure 7. An illustration of <i>Acinetobacter baumannii</i> virulence determinants and their functions.	30
Figure 8. Resistance mechanisms of <i>A. baumannii</i> to antimicrobial agents.	35
Figure 9. Sensitivity of the 90 <i>A. baumannii</i> isolates to different antibiotics as analyzed by MIC.	62
Figure 10. Antibiotic-antibiotic correlations. Correlogram representing correlation coefficients between each pair of antibiotics according to the patterns of susceptibility of the <i>A. baumannii</i> 90 isolates..	64
Figure 11. (A) Dendrogram signifying the clustering relatedness of 30 MDR <i>A. baumannii</i> isolates based on their PCR results for biofilm-related genes. (B) Tabular presentation of the PCR screening results of biofilm associated genes and corresponding mean OD ₆₃₀ values.	70
Figure 12. Violin plots showing minimum inhibitory concentrations (mg/mL) of cinnamic and gallic acids against 30 MDR <i>A. baumannii</i> isolates..	72
Figure 13. Boxplots displaying biofilm reduction (%) caused by sub-inhibitory concentrations ($\frac{1}{2}$ MICs and $\frac{1}{4}$ MICs) of cinnamic and gallic acids..	73
Figure 14. Inhibitory effect of cinnamic and gallic acids on biofilm formation of each of the 30 MDR <i>A. baumannii</i> isolates..	74
Figure 15. Antibiofilm activities of sub-MICs of cinnamic and gallic acids. Scatter plot representing biofilm reduction (%) for each sample.	76

List of figures

Figure 16 A. Bacterial growth curve of strong biofilm formers of <i>A. baumannii</i> in the presence of sub-inhibitory concentrations ($\frac{1}{2}$ MICs and $\frac{1}{4}$ MICs) of cinnamic&gallic acids, along with the untreated growth controls.	77
Figure 16 B. Bacterial growth curve of weak biofilm formers of <i>A. baumannii</i> in the presence of sub-inhibitory concentrations ($\frac{1}{2}$ MICs and $\frac{1}{4}$ MICs) of cinnamic&gallic acids, along with the untreated growth controls.	78
Figure 17. Light microscopic visualization of (A) Untreated strong biofilm-former <i>A. baumannii</i> isolate, (B) After a 24-hr treatment with sub-MIC cinnamic acid, and (C) a 24-hr treatment with sub-MIC gallic acid.....	79
Figure 18. Scanning electron microscopy images of (A) Untreated strong biofilm-former <i>A. baumannii</i> isolate, (B) After a 24 h treatment with sub-MIC cinnamic acid, and (C) a 24 h treatment with sub-MIC gallic acid. Magnification x10,000.....	80
Figure 19. Violin plots showing MICs of the selected 30 MDR isolates against colistin in presence/ absence of cinnamic or gallic acids.	81
Figure 20. Violin plots showing MICs of the selected 30 MDR isolates against imipenem in presence/ absence of cinnamic or gallic acids..	82
Figure 21. Violin plots showing MICs of the selected 30 MDR isolates against doxycycline in presence/ absence of cinnamic or gallic acids	83
Figure 22. Violin plots showing MICs of the selected 30 MDR isolates against amikacin in presence/ absence of cinnamic or gallic acids.	84
Figure 23. Violin plots showing MICs of the selected 30 MDR isolates against levofloxacin in presence/ absence of cinnamic or gallic acids.	85
Figure 24. Fold expression values of <i>bap</i> gene in control and treated samples with gallic acid ($\frac{1}{2}$ MIC). Fold expression values proved that gallic acid ($\frac{1}{2}$ MIC) substantially down-regulated <i>bap</i> genes in all five strong biofilm formers ($p=0.001$).	86
Figure 25. Fold expression values of <i>csuE</i> gene in control and treated samples with gallic acid ($\frac{1}{2}$ MIC). Fold expression values proved that gallic acid ($\frac{1}{2}$ MIC) substantially downregulated <i>csuE</i> genes in all five strong biofilm formers ($p=0.003$)......	86
Figure 26. Fold expression values of <i>ompA</i> gene in control and treated samples with gallic acid ($\frac{1}{2}$ MIC). Fold expression values proved that gallic acid ($\frac{1}{2}$ MIC) substantially downregulated <i>ompA</i> genes in all five strong biofilm formers ($p=0.001$)......	87