

Ain Shams University
Faculty of Pharmacy
Microbiology & Immunology department

Characterization of cytotoxic activities of some *Streptomyces* isolates against mammalian cells

A Thesis

Submitted for Partial Fulfillment of the Requirements for the

Master degree

In

Pharmaceutical Sciences
(Microbiology & Immunology)

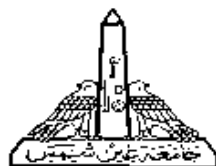
By

Ahmed Said Abu-Zaid Ahmed

Bachelor of Pharmaceutical Sciences,
Faculty of Pharmacy, Ain Shams University, 2005

**Department of Microbiology & Immunology
Faculty of Pharmacy
Ain Shams University**

2012



Ain Shams University
Faculty of Pharmacy
Microbiology & Immunology department

Characterization of cytotoxic activities of some *Streptomyces* isolates against mammalian cells

A Thesis

Submitted for Partial Fulfillment of the Requirements for the
Master degree

In

Pharmaceutical Sciences
(Microbiology & Immunology)

By

Ahmed Said Abu-Zaid Ahmed

Bachelor of Pharmaceutical Sciences,
Faculty of Pharmacy, Ain Shams University, 2005

Under Supervision of

Prof. Dr. Nadia A. El-Haleem Hassouna, PhD

Professor of Microbiology and Immunology,
Faculty of Pharmacy, Ain Shams University

Prof. Dr. Mohammad Mabrouk Aboulwafa, PhD

Professor and head of Microbiology and Immunology Department,
Faculty of Pharmacy, Ain Shams University

Dr. Mohammed Mostafa Hafez, PhD

Lecturer of Microbiology and Immunology
Faculty of Pharmacy, Ain Shams University

2012

Approval Sheet

Characterization of cytotoxic activities of some *Streptomyces* isolates against mammalian cells

By

Ahmed Said Abu-Zaid Ahmed

Bachelor of Pharmaceutical Sciences,
Faculty of Pharmacy, Ain Shams University, 2005

This thesis towards a Master Degree in
Pharmaceutical Science has been approved by:

Prof. Dr. Nadia A. El-Haleem Hassouna

Prof. Dr. Mohammad Mabrouk Aboulwafa

Prof. Dr. Ahmed Ahmed Abdelaziz

Prof. Dr. Ramadan Hassan Ibrahim

(Committee in Charge)

Date: / /

Acknowledgment

Firstly, I thank "**Allah**" for granting me the power to accomplish this work.

I would like to thank **Prof. Dr. Nadia Hassouna**, Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, for her continuous advice and valuable guidance throughout my study.

I would like to express my deepest thanks and sincere appreciation to **Prof. Dr. Mohammad Mabrouk Aboulwafa**, Professor and Head of Microbiology and Immunology Department, Faculty of Pharmacy, Ain Shams University, for suggesting the point, scientific supervision, valuable constructive criticism, continuous guidance throughout the work and thorough revision of the thesis.

I wish to express my thanks to **Dr. Mohammed Mostafa Hafez**, Lecturer of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, for planning the work, guidance, kind support and valuable discussions during this work.

I am greatly indebted to **Dr. Khaled Mohammed Aboshanab**, assistant Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, who taught me different programs that help in identification of selected isolates.

I would like to thank all my **Colleagues** and **Workers** in the Microbiology and Immunology Department, Faculty of Pharmacy, Ain Shams University, for their help and support during this work.

Finally, my deepest everlasting thanks and appreciation are directed to my beloved parents and to my wife for their continuous support, encouragement, and sincere help throughout my life.

وآخر دعوانا أن الحمد لله رب العالمين...

Ahmed Said Abu-Zaid

List of Abbreviations

Abbreviation	Definition
ATCC	American type culture collection
ADM	Adriamycin
CFCS	Cell free culture supernatant
CD ₅₀	Cytotoxic dose 50
DO	Dissolved oxygen
MTT	Di-methyl thiazolyl diphenyl tetrazolium bromid
PBS	Phosphate buffered saline
RTD	Resistance Temperature Detector
SLPM	Standared liter per minute
TC flask	Tissue culture flask
Vvm	Volume per volume per minute

Title	Page
-------	------

Contents

Abstract	
Introduction	1
Literature Review	3
1. Classical Anticancer Agents and their Mechanisms of Action	3
2. Natural Products as Anticancer Agents	4
3. Bacteria and cancer treatment	5
3.1. Use of bacteria for cancer immunotherapy	5
3.2. Use of bacteria as tumoricidal agents	6
3.3. Use of bacteria as delivery vector for anticancer agents	8
3.4. Bacterial products in the treatment of cancer	8
4. Actinomycetes	9
5. <i>Streptomyces</i>	10
5.1. <i>Streptomyces</i> products	10
5.1.1. Antimicrobials	11
5.1.2. Cytotoxic agents produced by <i>Streptomyces</i>	12
Materials and Methods	36
1. Microorganisms	36
2. Cell lines	36
3. Chemicals	37
4. Culture media	39
4.1. Ready-made culture media	39
4.2. In house formulated culture media	39
4.2.1. Medium used for isolation and maintenance of <i>Streptomyces</i> species	39
4.2.2. Medium used for cultivation of collected isolates to be screened for cytotoxic activities	40
4.2.3. Different culture media used for studying cytotoxic agent(s) production by selected isolates	40
4.2.4. Slant-medium (50:50)	43
4.3 Tissue culture media	43
4.3.1. Eagle's minimum essential medium with Earle's balanced salts (MEM Earle's)	43

Contents

Title	Page
4.3.2. Medium used for maintenance of cell lines	44
4.3.3. Medium used for propagation and monolayer formation of cell lines	45
5. Buffers and solutions	45
5.1. Phosphate buffer (0.2 M)	45
5.2. Phosphate buffered saline (PBS)	46
5.3. Standard Buffers (pH 4 and pH 7)	46
5.4. Trypsin solution	46
5.5. Trypan blue stain solution	46
5.6. MTT solution	46
6. Devices	47
7. Isolation and maintenance of <i>Streptomyces</i>	48
7.1. Collection of soil samples	48
7.2. Enrichment of <i>Streptomyces</i> in soil samples	48
7.3. Cultivation, recovery and maintenance of <i>Streptomyces</i> isolates	48
8. Maintenance of used cell lines	49
9. Preparation of the used cell lines	49
10. Determination of bacterial cytotoxicity to Vero cells using Trypan blue exclusion method	50
10.1. Production of cytotoxic agent(s) by test bacterial isolates	50
10.1.1. Preparation of seed culture	50
10.1.2. Preparation of main culture and cell free culture supernatant	50
10.2. Cytotoxicity assay using trypan blue exclusion method	50
10.2.1. Tissue culture plates preparation	50
10.2.2. Assay procedure	51
11. Screening of bacterial isolates for their cytotoxicity against Vero cells using trypan blue exclusion method	51
12. Determination of cytotoxic activity of selected isolates against Vero and HEP-2 cells using MTT assay	51
12.1. MTT assay procedure	52
12.2. Selection of isolates showing most potent cytotoxicity	53
13. Identification of the selected isolates	53
14. Optimization of cytotoxic agent(s) productivity by <i>Streptomyces</i> isolates S86 and S131 in shake flasks	54
14.1. Effect of different production media	54
14.2. Effect of inoculum size	56

Contents

Title	Page
14.3.Effect of incubation period	56
14.4.Effect of incubation temperature	56
14.5.Effect of rotation speed (RPM)	56
14.6.Effect of initial pH	56
14.7.Effect of different molarities of phosphate buffer	57
14.8.Effect of buffered medium pH	57
14.9.Effect of glucose replacement in soybean meal (M1) with different carbohydrate sources	57
14.10.Effect of different concentrations of some carbohydrate sources	57
14.11.Effect of soybean replacement in soybean meal (M1) with different nitrogen sources	58
14.12.Effect of different concentrations of some nitrogen sources	58
14.13.Effect of addition of metal salts to M1 medium	58
14.14.Effect of addition of amino acids to production medium	59
14.15.Effect of integration of different conditions proved to be optimum for cytotoxic agent(s) production by <i>Streptomyces</i> isolates S86 and S131	59
15. Studying the cytotoxic agent(s) productivity by isolates S86 and S131 in a laboratory fermentor	51
16. Characterization of cytotoxic agent(s) of <i>Streptomyces</i> isolates S86 and S131	51
16.1.Determination of cytotoxic activity spectrum of selected <i>Streptomyces</i> isolates against different mammalian cell lines	63
16.2. Thermal stability of cytotoxic agent(s) in the CFCS	63
16.3. Effect of freezing on cytotoxic agent(s) activity of CFCS	51
17.Extraction of the cytotoxic agent(s) of <i>Streptomyces</i> isolates S86 and S131 at different pH values.	64
17.1. Extraction at pH 7	64
17.2. Extraction at pH 4 and 10	64
Results	65
1.Recovery and microscopical characterization of soil isolates	65
2. Screening of <i>Streptomyces</i> isolates for their cytotoxicity against Vero cells using trypan blue exclusion method	65
3. Determination of cytotoxic activity of selected <i>Streptomyces</i> isolates against Vero and HEP-2 cells using MTT assay	67
3.1. First stage	67

Contents

Title	Page
۳.۲. Second stage	69
۳.۳. Third stage	71
4. Identification of the two selected <i>Streptomyces</i> isolates (S86 & S131)	71
۵. Optimization of cytotoxic agent(s) productivity by <i>Streptomyces parvus</i> and <i>Streptomyces griseus</i> in shake flasks	74
6. Studying the production of cytotoxic agent(s) by <i>Streptomyces parvus</i> and <i>Streptomyces griseus</i> isolates in a laboratory fermentor.	105
6.1. Fermentation process under uncontrolled pH	105
6.2. Fermentation process at pH 7	107
6.3. Fermentation process at pH 6	109
6.4. Fermentation process at pH 8	111
7. Characterization of cytotoxic agent(s) produced by <i>Streptomyces parvus</i> and <i>Streptomyces griseus</i> test isolates	113
7.1. Activity spectrum against different mammalian cell lines	113
7.2. Thermal stability	115
7.3. Effect of freezing	116
8. Extraction of the cytotoxic agent(s) of <i>Streptomyces parvus</i> and <i>Streptomyces griseus</i> test isolates	116
Discussion	118
Summary	139
References	146

List of Tables

Table No.	Page
Table 1: Different chemicals used throughout this study and their sources.	37
Table 2: Composition of 0.2 M phosphate buffer solution at different pH values.....	45
Table 3: Additional devices used throughout the present study and their manufacturers.....	47
Table 4: Media used for studying the cytotoxic agent(s) productivity by <i>Streptomyces</i> isolates S86 and S131.	55
Table 5: Medium composition and culture conditions proved to be optimum for cytotoxic agent(s) production by S86 and S131 <i>Streptomyces</i> isolates.....	60
Table 6: Cytotoxic activity of <i>Streptomyces</i> isolates that showed complete detachment of <i>Vero</i> cells after 8 hrs of incubation at shorter incubation periods using trypan blue exclusion method.....	66
Table 7: Cytotoxic activities of selected <i>Streptomyces</i> isolates against <i>Vero</i> and HEP-2 cells as determined by MTT assay after 8 hrs incubation.	68
Table 8: Cytotoxic activities of selected <i>Streptomyces</i> isolates against <i>Vero</i> and HEP-2 cells as determined by MTT assay after 2 hrs incubation.	70

List of Figures

Figure No.	Page
Fig.1: Chemical structure of daunorubicin and doxorubicin	13
Fig.2: General structure of actinomycins.	14
Fig.3: Chemical structure of Bleomycin.	16
Fig.4: General structure of mitomycins.	17
Fig.5: production of epothilone B and epothilone D.....	21
Fig.6: chemical structure of UK-1	23
Fig.7: Chemical structure of telomestatin.	24
Fig.8: chemical structure of pladienolide B.....	26
Fig.9: Chemical structure of migrastatin and isomigrastatin.....	27
Fig.10: Migrastatin analogues, core macroketone and core macrolactam.	28
Fig.11: Chemical structure of YM-216391.....	29
Fig.12: General structure of Oligomycins	30
Fig.13: Structures of ansamycin antibiotics, geldanamycin (A) and 17- allylamino-demethoxy geldanamycin (B).....	32
Fig.14: Chemical structure of mayamycin.....	35
Fig.15: 3-hydroxy-1-keto-3-methyl-8-methoxy-1, 2, 3, 4-tetrahydro- benz[α]anthracene.....	35
Fig.16: comparison between cytotoxic activities of CFCS of selected <i>Streptomyces</i> isolates against HEP-2 cells as determined by MTT assay after 2 hrs incubation.	71
Fig.17: Alignment of the nucleotides sequence of 16S rRNA gene for <i>Streptomyces</i> isolate S86 in comparison to the respective nucleotides sequence of 16S rRNA gene for <i>Streptomyces parvus</i> . Query: nucleotides sequence of 16S rRNA gene for <i>Streptomyces</i> isolate S86. Sbjct: nucleotides sequence of 16S rRNA gene for <i>Streptomyces</i> <i>parvus</i>Error! Bookmark	