

بسم الله الرحمن الرحيم

INTERNATIONAL UNIVERSITY OF AFRICA

FACULTY OF POST GRADUATE STUDIES

FACULTY OF PURE AND APPLIED SCIENCE

**ANTIMICROBIAL ACTIVITY OF SOME SUDANESE MEDICINAL
PLANTS AGAINST DIABETIC WOUNDS INFECTIONS**

BY

EHSAN MOHAMMED ABD EL RAHMAN

M.SC., FACULTY OF SCIENCE, U OF K, 2000

A THESIS SUBMITTED TO INTERNATIONAL UNIVERSITY OF AFRICA IN
FULFILLMENT FOR THE REQUIREMENTS OF THE DEGREE OF Ph.D. IN
APPLIED AND PURE SCIENCE (MICROBIOLOGY)

SUPERVISOR

DR. WALEED SAID KOKO

MEDICINAL AND AROMATIC PLANTS AND TRADITIONAL MEDICINE
RESEARCH INSTITUTE
NATIONAL CENTER FOR RESEARCH
CO SUPERVISOR

PROFESSOR: OSMAN KHALIL

FACULTY OF APPLIED AND PURE SCIENCE
INTERNATIONAL UNIVERSITY OF AFRICA

JANUARY, 2018

DECLARATION

Iam Ehsan Mohammed Abd El Rahman declare that this research work reported in this thesis entitled antimicrobial activity of some Sudanes medicinal plants against diabetic wounds infections, is the product of my own research efforts; undertaken under the supervision of Dr. Waleed Said Koko and has not been prese.

.....
Ehsan Mohammed Abd ElRahman
Student.

.....
Date

DEDICATION

TO

MY GRAND MOTHER , MOTHER , FATHER ,

SMALL FAMILY

UNCLES , UNTS, BROTHERS , AND SISTERS

CLOSE RELATIVES AND Mr. MOHAMMED MUSTAFA AHMAD

WITH LOVE AND RESPECT

ACKNOWLEDGMENT

I am grateful to my supervisor Dr. Waleed Said Koko Medicinal & Aromatic Plants Traditional medicine Research Institute, National Center for Research for his pursuing and valeable advices.

I would like to express my deep and sincere gratitude to my co supervisor Prof Osman Khalil, Department of Microbiology, Faculty of Pure and Applied Sciences, International University of Africa for his patience guidance and co supervision in both practical and thesis preparation and revision.

The ever-lasting thanks to my boss and first instructors, land of patience my father Mr. Mohammed Abd Elrahman and my mother Mrs. Setana Hassan Alhag.

Many thanks to my husband Mohammed Mustafa Ahmad, for his great and continuous pushing, solid support and fruitful suggestions.

I wish to express my tremendous gratitude to my uncle Salah Hssan Alhag for his infinitive and generous support before and during this work, and a strong wave of thanks to my unts, uncles, brothers, sisters, sons and daughters who did not hesitate to offer help.

I am indebted to Mr. Yassin Mirgani for his solid and gentle support and fruitful suggestions for choosing the title of this research .

Furthermore My thanks are extended to Dr. Samah Awad Laboratory Sciences (Microbiology) University of Khartoum, and Dr .Naaim Almubark International University of Africa, for their valuable advices.

I would also like to express my thanks to all workers in Zeenam Center for Diabetes for harnesses assistance for collection specimens.

My grateful to Dr. Mohammad Hassan, Bayero University Kano, Naigeria, for his incorporeally supporting.

I am grateful to Mr. A bubakr, Emad, Ahmad Saeed, Alsadig and Mohammed Ismail Garby, Chiefs Technicians of the Microbiology laboratory in International University of Africa, for their great assistance during processing this research.

Also I don't forget the administrator of Zeenam Center for diabetes Prof. , Mr. Busharha and all workers on the Center whom made this work easier and feasible, helped me in collecting the specimens and for their invaluable help supplying me with searching information from their center.

I would like to thank every body who contributed positively in finding this research. Last but not least my thanks for all the members of the Department of Microbiology, Faculty of Pure and Applied Sciences, International University of Africa.

Ehsam Mohammed Abd El Rahman, January, 2018

ABSTRACT

A total of twelve plant extracts of both chloroform and ethanol from 6 Sudanese medicinal plants *Ambrosia maritima*, *Ammi visnaga*, *Nigella sativa*, *Peganum harmala*, *Punica granatum* and *Trigonella foenum-graecum*, distributed among 6 different families were screened for their antibacterial activity using the disk diffusion method . They were tested against five clinical isolates and standard bacteria, two Gram positive bacteria (*Bacillus subtilis* NCTC 8236 and *Staphylococcus aureus* ATCC 25923) and three Gram negative bacteria (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380 and *Pseudomonas aeruginosa* ATCC 27853).

The extracts exhibited inhibitory activity against one or more of the five tested bacteria. *S.aureus* was the most susceptible organism to the *N.sativa*, *P.harmala* and *P.granatum* chloroformic and ethanolic extracts (21,20; 34,34 and 30,30mm respectivily), while *P. vulgaris* showed the lowest susceptibility to the *A.visnaga* and *T. foenum-graecum* (0,10 and 0 respectivily). The Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of the most active ethanolic extracts of the plants against the standard and clinical isolates bacteria were determined using the agar tube dilution method.

The antibacterial activity of two reference drugs were determined against the tested bacteria, and compared to the antibacterial activity of the tested plant extracts. The extracts were tested against 100 clinical isolates collected randomly from Zeenm Center for Diabetes, Khartoum.

In this study the wound healing effect of *P. harmala* seeds ethanolic extract and *P. granatum* fruit peels ethanolic extract were investigated on open diabetic wound model on rats. Trial was performed using four groups of rats infected with standard *S. aureus*. Treated groups with *P. harmala* and *P. granatum* ointments were compared with non-treated groups and treated groups with tetracycline ointment. Healing was determined by reduction in wound area.

The results of this study confirmed that the 2% *P. harmala* and *P. granatum* ointments were potent healing agent even better than the tested Tetracycline ointment 3%.

ملخص الاطروحة

اشتملت الدراسة على اجراء مسح لفعالية المضادة للبكتيريا ل 12 من مستخلصات الكلوروفورم والايثانول النباتية لستة نباتات طبية سودانية: الدمسisse، الخلة، الكمون، الحرمل، الرمان والحلبة (*Ambrosia maritima, Ammi visnaga, Nigella sativa, Peganum harmala, Punica granatum and Trigonella foenum-graecum*) تتنمي الى ستة عوائل مختلفة باستخدام طريقة الانتشار في الاجار. تم اختبار تأثير جميع المستخلصات ضد خمسة انواع من البكتيريا المعيارية والبكتيريا المعزولة طبيا من جروح مرضى السكري من مركز زينام لمرضى السكري بالخرطوم ، نوعين من البكتيريا الموجبة جرام (العصوية الرقيقة والعنقودية الذهبية) *Bacillus subtilis* NCTC 8236 and *Staphylococcus aureus* وثلاثة انواع من البكتيريا السالبة جرام (الاشريكية القولونية، الزانفة الزنجبارية والمتحلبة الاعتيادية). (*Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6380 and *Pseudomonas aeruginosa* ATCC 27853).

ووجد ان جميع المستخلصات اظهرت فاعلية ضد نوع او اكثر من انواع البكتيريا المختبرة بالإضافة الى ان لها مفعول مثبط لثلاث الانواع البكتيرية ، وكانت العنقودية الذهبية اكثر انواع البكتيريا حساسية لمستخلصات الكلوروفورم والايثانول للكمون، الحرمل والرمان

(34,34 and 30,30mm respectively) ، اما الزانفة الزنجبارية فقد اظهرت اقل حساسية لمستخلصات الكلوروفورم والايثانول للخلة والحلبة بين انواع البكتيريا المختبرة (0,10 and 0 respectively). وكذلك شتملت الدراسة على تحديد اقل تركيز مثبط لنمو البكتيريا وكذلك اقل تركيز قاتل للبكتيريا المعيارية والمعزولة طبيا لاكثر المستخلصات فاعلية ، وهي الاربع مستخلصات الايثانولية لبذور نبات الحرمل وقشور ثمار الرمان بطريقة تخفيف الاجار.

تم تحديد فاعلية اثنان من مضادات حيوية مرجعية وهي الاريثرومایسين والجنتامايسين ضد الانواع الخمسة البكتيرية المختبرة وقورنت فاعليتها مع المستخلصات النباتية. تلك المستخلصات تم اختبار فاعليتها على البكتيريا الموجودة في عدد 100 عينة معزولة جمعت عشوائيا من مركز زينام لمرضى السكري بالخرطوم.

لقد تم في هذه الدراسة التحقق من ثاثير المستخلصات الايثانولية لنباتي الحرمل والرمان على التئام جروح السكري المفتوحة في 28 من الفئران السويسرية (البيضاء). وتم تحضير المستخلصات الايثانولية للحرمل والرمان ومن ثم تحضير المراهم 2% (وزن ١ وزن) من المستخلصات في البولي ايثلين جليكول ، مع استخدام مرهم التتراسيكلين 3% كحكم .

تم عمل تجربة مكونة من 4 مجموعات من الفئران المصابة بداء السكري والمصابة معمليا بالعنقودية الذهبية المعيارية . قورنت المجموعات المعالجة بالمراهم المعدة من المستخلصات الايثانولية للحرمل والرمان مع مرهم التتراسيكلين والمجموعات المصابة الغير معالجة بالمرهم ، حيث تم تقدير التئام بالنقص في منطقة الجرح . واكتت النتائج ان مرهمي مستخلصي الايثانول بنور الحرمل وقشور ثمرة الرمان 2% هما عاملان التئام فعال ، بل وجد انهما افضل من مرهم التتراسيكلين 3% المختبر.

TABLE OF CONTENTS

	Page
DECLARATION.....	I
Dedication.....	II
Acknowledgements.....	III
Abstract.....	V
Arabic abstract.....	VII
Table of contents.....	IX
List of tables.....	XIII
List of figures.....	XV
 CHAPTER ONE	
 1. INTRODUCTION	
 1.1 Introduction	1
 1.2 Justification	2
 1.3 The objectives of the work	3
 CHAPTER TWO	
 2. LITERATURE REVIEW	
 2 Literature review	4
 2.1 Diabetes	4
 2.1.1 Diabetes in Sudan	4
 2.1.2 Definition of wound	5
 2.1.3 Classification of Wound	5
 2.1.4 Normal wound healing.....	6
 2.1.5 Factors influencing healing.....	7
 2.1.6 Diabetic foot ulcers & diabetic wounds	7
 2.1.7 Diabetic foot ulcer in Sudan	9
 2.1.8 Bacterial pathogens causing wounds infections.....	10
 2.1.9 A wound infection	12
 2.2 Principles of Anti-infective Therapy.....	13
 2.2.1 Antimicrobial agents.....	14
 2.2.2 Antibiotics	15
 2.2.3 Resistant organisms.....	16
 2.3 Definition of medicinal plants	18
 2.3.1 Antimicrobial activity of medicinal plants	20
 2.3.2 Medicinal plants in Sudan	26
 2.3.3 Utilization of plants for wound healing	28
 2.3.4 Extraction of medicinal plants	32
 2.3.5 Definition of extraction	33
 2.3.6 Methods of extraction of medicinal plants	33
 2.4 <i>In vitro</i> antimicrobial activity	34
 2.4.1 Measurement of antimicrobial activity	34

2.4.2	Diffusion methods	35
2.4.3	Factors affecting the <i>In vitro</i> antimicrobial activity	39
2.5	<i>In vivo</i> Antimicrobial activity.....	40
2.5.1	Selection of the appropriate laboratory animals.....	40
2.5.2	Alloxan	41
2.5.3	Chemical Properties	42
2.5.4	Phases of diabetes induction	42
2.5.5	Dosage of alloxan	43
2.5.6	Vehicle of choice, drugs dissolution and volume selection rationale ...	43
2.5.7	Dosage calculation and preparation of stock solutions for salt compound (Alloxan monohydrate)	44
2.5.8	Ointments	46
2.5.9	Polyethylene glycol ointment, (macrogol or carbowaxes)	47
2.5.10	Properties of the Ideal Base	48
2.5.11	Selection of the appropriate base	49
2.5.12	Compounding of Ointments	49
2.5.13	Preparation of the Ointment base by fusion	49
2.5.14	Preparation of medicated ointments and pastes by fusion.....	49
2.5.15	Microbial contents	49
2.6	Botanical, Ethnopharmacological and Phytochemical profiles of the studied medicinal plants.....	50

CHAPTER THREE

3	MATERIALS & METHODS	55
3.1	MATERIALS	55
3.1.1	Chemicals and Reagents	55
3.1.2	Chemotherapeutic agents	56
3.1.3	Culture media	56
3.1.4	Equipments and Instruments	56
3.1.5	Test micro-organisms	57
3.1.5	Bacterial strains	57
3.1.6	Animals	57
3.1.7	Study area	58
3.2	METHODS	58
3.2.1	Plant materials	58
3.2.2	Preparation of the crude extracts	58
3.2.3	Preparation of serial dilution of extracts	59
3.2.4	Phytochemical screening	59
3.2.5	Preparation of the test organisms.....	61
3.2.5.1	Preparation of the bacterial suspensions	61
3.2.5.2	Preparation of inocula.....	61

3.2.5.3	<i>In vitro</i> screening of extracts for antibacterial activity.....	62
3.2.5.4	Quantitative evaluation of antimicrobial susceptibility.....	62
3.2.5.5	Determination of Minimum Inhibitory Concentrations (MICs)	62
3.2.5.6	Determination of Minimum Bactericidal Concentration (MBC).....	64
3.2.6	Clinical isolates	65
3.2.7	Media used for identification of clinical isolates.....	65
3.2.7.1	Microscopical examination of isolated bacteria.....	66
3.2.7.2	Biochemical tests for identification of the isolates.....	66
3.2.8	MTT assay.....	69
3.2.9	<i>In vivo</i> clinical trial for evaluation of antimicrobial and wound healing activities of the extracts	71
3.2.9.1	Experimental animals	71
3.2.9.2	Formulation of the ointment	71
3.2.9.3	Induction of diabetes mellitus	71
3.2.9.4	Glucose monitoring and insulin management	72
3.2.9.5	Wound induction and treatment.....	72
3.2.9.6	Wound healing evaluation	73
2.3	Statistical analysis.....	73

CHAPTER FOUR RESULTS

4	RESULTS.....	74
4.1	Identification of clinical isolates.....	74
4.2	.Identification of organisms.....	74
4.3	The extractive percentage yield of the plants	77
4.4	A preliminary screening for antibacterial activity of six Sudanese medicinal plants	78
4.5	Interpretation of results.....	79
4.6	Detailed investigations of selected Bio-active Sudanese medicinal plants of <i>P. harmala</i> and <i>P.granatum</i>	85
4.7	Comparison of the activity of <i>Punica granatum</i> and <i>Peganum harmala</i> extracts against standard and isolated bacteria with reference antibiotics	85
4.8	Susceptibility of isolated bacteria to different plant extracts.....	90
4.9	Quantitative evaluation of susceptibility to the extracts.....	96
4.10	Qualitative analysis of some chemical constituents.....	97
4.11	MTT assay test.....	98
4.12	Effect of the ointments on excision wounds	90

CHAPTER FIVE

5.1	DISCUSSION.....	105
5.2	CONCLUSION	115
	References.....	116
	Appendixis	139

List of Table

Table 1 Stock solution preparation for alloxan monohydrate and required doses for animals of different body weights.....	45
Table 2 Morphological and biochemical results for identification of the isolated bacteria	76
Table 3 Total number and percentages of the isolated hundred bacteria.....	77
Table 4 Yield percentages of the six studied Sudanese medicinal plants	78
Table 5.1 A preliminary screening of antibacterial activity of <i>A.martima</i> shoot extracts against standard and isolates bacteria using disc diffusion assay*	81
Table 5.2 A Preliminary screening of antibacterial activity of <i>A. visnaga</i> seeds extracts against standard and isolates bacteria using disc diffusion assay*.....	82
Table 5.3 A preliminary screening of antibacterial activity of <i>N. sativa</i> seeds extracts against standard and isolates bacteria using disc diffusion assay*.....	83
Table 5.4 A preliminary screening of antibacterial activity of <i>T. foenum-groecum</i> seeds extracts against standard and isolates bacteria using disc diffusion assay*....	84
Table 5.5 A preliminary screening of antibacterial activity of <i>p.harmala</i> seeds extracts against standard and isolates bacteria using disc diffusion assay*.....	87
Table 5.6 A preliminary screening of antibacterial activity of <i>P.granatum</i> peels extracts against standard and isolates bacteria using disc diffusion assay*... ..	88
Table 5.7 Antibacterial activity of Erythromycin and Gentamicin against standard and isolates bacteria using disc diffusion assay*.....	89
Table 6 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of <i>P.harmala</i> & <i>P.granatum</i> extracts against the isolated.....	97
Table 7 Phytochemical screening of the six tested plants	98
Table 8 MTT assay test result	98

Table 9-1 Wound reduction due to the application of extracts ointments in excision wound model*.....	99
Table 9.2 Multivariate Analysis of Variance *of the <i>in vivo</i> trial	101
Appendix:	
Table 1.1 Susceptibility of <i>S.aureus</i> clinical isolates to the extracts with concentration of 100mg/ml (mm).....	140
Table 1. 2 Susceptibility of <i>P . aeruginosa</i> clinical isolates to the extracts with concentration of 100mg/ml (mm)	141
Table 1.3 Susceptibility of <i>E.coli</i> clinical isolates to the extracts with concentration of 100mg/ml (mm)	142
Table 1.4 Susceptibility of <i>P.valgaris</i> clinical isolates to the extracts with concentration of 100mg/ml (mm)	143
Table 1. 5 Susceptibility of <i>B.subtilis</i> clinical isolates to the extracts with concentration of 100mg/ml (mm)	144
Table 2 Percentages of wound reduction due to the application of extracts ointments in excision wound model.....	145

List of figures

Figure 1: Antibacterial activity of <i>A.martima</i> shoot extracts against isolated bacteria.....	92
Figure 2: Antibacterial activity of <i>A.visnaga</i> seeds extracts against isolated bacteria.....	93
Figure 3: Antibacterial activity of <i>N.sativa</i> seeds extracts against isolated bacteria.....	94
Figure 4: The antibacterial activity of <i>P. harmala</i> against clinical isolates.....	95
F 5: Antibacterial activity of <i>P.granatum</i> fruit peels extracts against clinical isolates	96
F 6: Antibacterial activity of <i>T. foenum-groecum</i> seeds extracts against isolated bacteria.....	97

APPENDICES

Fig 1.1: <i>In vitro</i> activity of <i>P. granatum</i> peels ethanolic extract against <i>B.subtilis</i> and <i>S. aureus</i> (standard bacteria)	140
Fig 1.2 <i>In vitro</i> activity of <i>P. granatum</i> peels ethanolic extract against <i>B. subtilis</i> and <i>S. aureous</i> (clinical isolates)	140