

Sudan University of Science and Technology

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**Antibacterial Activity of *Nigella sativa* extract against
selected bacterial clinical isolates from Khartoum
State**

**نشاط مستخلص الحبة السوداء علي البكتريا المعزولة من عينات طبية
بولاية الخرطوم**

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الآية

بسم الله الرحمن الرحيم

قال تعالى:

(الْحَمْدُ لِلَّهِ الَّذِي أَنْزَلَ عَلَى عَبْدِهِ الْكِتَابَ وَلَمْ يَجْعَلْ لَهُ عِوَجًا (1)
فَيَّمَّا لِيُنذِرَ بَأْسًا شَدِيدًا مِمَّنْ لَدُنْهُ وَيُبَشِّرَ الْمُؤْمِنِينَ الَّذِينَ يَعْمَلُونَ
الصَّالِحَاتِ أَنَّ لَهُمْ أَجْرًا حَسَنًا (2))

صدق الله العظيم

سورة الكهف

الآية 1,2

Dedication

This research is lovingly dedicated to my mother and my father who have been my constant source of inspiration, without their love and support this project would not have been made possible.

To my sisters Nihad, Suha, Sahwa, Khadiga, Khlood, Jawaher, to my brother Mohammed and my aunts Howida, Ahlam and Nidal, who helped me with their love.

To my teachers and friends who supported me with their confidence.

To those who help me even with a word.

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Last but not least I gratefully appreciate the help of my teachers and colleagues.

ABSTRACT

An alarming increase in bacterial strains resistant to existing antibacterial agents demands a renewed effort to seek agents effective against pathogenic bacteria resistant to antibacterials.

The aim of this study was to study the antibacterial activity of different concentrations of petroleum ether and methanol extracts of medicinal plant *Nigella sativa* (seeds) using cup-plate agar diffusion method on selected clinical isolates of bacteria.

The study was conducted in Khartoum state, Sudan, during the period from June to November 2015.

One hundred forty four samples were collected from urine, wound and stool, 103/144 (72%) showed bacterial growth, from which nine types of pathogenic bacteria were isolated and identified using Gram stain, biochemical reactions and tested for their susceptibility to a number of antibiotics. Those were resistant to three or more antibiotics were tested for their sensitivity to *Nigella sativa* extracts. The tested isolates included, *Klebsiella pneumoniae* 32(31%), *Escherichia coli* 22(21%), *Staphylococcus aureus* 21(20%), *Proteus mirabilis* 11(11%) and *Pseudomonas aeruginosa* 9(9%).

The petroleum ether and methanol extract of *Nigella sativa* were screened for their antibacterial activity against standard and clinical isolates.

Petroleum ether extract of *Nigella sativa* showed pronounced dose dependant antibacterial activity on standard strains and clinical isolates, while methanolic extract showed no activity.

Petroleum ether extract of *Nigella sativa* showed antibacterial activity against standard and clinical isolates of *S.aureus*, *K.pneumoniae* and *E.coli*, while only standard strains of *P. mirabilis* and *Ps. aeruginosa* were sensitive to it.

مستخلص الأطروحة

الزيادة المقلقة في سلالات البكتريا المقاومة للمضادات البكتيرية الحالية تتطلب جهدا متجددا

للحصول علي عناصر فعالة ضد البكتريا الممرضة والمقاومة للمضادات البكتيرية.

هدف هذه الدراسة اختبار فعالية مستخلصي ايثر البترول والميثانول للنبات الطبي الكمون الاسود ، بتراكيز مختلفة باستخدام طريقة الانتشار عن طريق الحفر علي لوحات الاكار، علي البكتريا المعزولة من عينات طبية مختلفة.

اجريت هذه الدراسة في ولاية الخرطوم في السودان في الفترة من يونيو الي نوفمبر 2015.

تم جمع مائة اربعة واربعون عينة من البول والجرح والبراز ، وأظهرت مائة وثلاثة منها نمو بكتيري (72%) 103/144 ، عزلت منها تسعة أنواع من البكتريا الممرضة ، وتم التعرف عليها باستخدام صبغة الجرام والتفاعلات الكيميائية الحيوية و أختبرت حساسيتها لعدد من المضادات الحيوية. تلك التي أظهرت مقاومة لثلاثة أكثر من المضادات الحيوية أختبرت حساسيتها لمستخلصي الكمون السود وهي: الكلبسيلا الرئوية (31%) 32 ، الاشريكية القولونية (21%) 22، العنقودية الذهبية (20%) 21، المتقلبة الاعتيادية (11%) 11 والزائفة الزنجارية (9%) 9.

أختبر النشاط المضاد للبكتريا لمستخلصي ايثر البترول والميثانول للكمون الاسود ضد البكتريا القياسية والمعزولة من العينات طبية .

مستخلص ايثر البترول للكمون الاسود أظهر فعالية ملحوظة حسب الجرعة المستخدمة ضد السلالات القياسية والمعزولة من العينات طبية ، بينما مستخلص الميثانول لم يظهر فعالية.

أظهر مستخلص ايثر البترول للكمون الاسود فعالية ضد البكتريا القياسية والمعزولة : العنقودية الذهبية ، الكلبسيلا الرئوية و الاشريكية القولونية بينما أظهر فعالية ضد السلالات القياسية فقط للمترجمة الاعتيادية والزائفة الزنجارية.

TABLE OF CONTENTS

Topic	Page
الآية	I
Dedication	II
Acknowledgment	III
Abstract	IV
Abstract (Arabic)	V
Table of contents	VI
List of Tables	X
List of Figures	XI
CHAPTER ONE	
1. INTRODUCTION	
1.1. Introduction	1
1.2. Rationale	3
1.3. Objectives	4
1.3.1. General objective	4
1.3.2. Specific objectives	4
CHAPTER TWO	
2. LITERATURE REVIEW	
2.1. Herbal medicine	5
2.1.1 Definition	5
2.1.2. History	5
2.1.3. Antimicrobial Properties of Medicinal Plants	5
2.2. <i>Nigella sativa</i>	6
2.2.1. Distribution	6
2.2.3. Botanical description	6
2.2.4. Chemical constituents	6
2.2.5. Traditional Uses	7
2.2.6 Pharmacological and toxicological properties of <i>Nigella sativa</i>	7

2.2.7. General uses in Herbal medicine	8
2.2.8. Antibacterial effects	8
2.3. General techniques of extraction of medicinal plants	10
2.3.1. Extraction procedures	11
2.3.1.1. Plant tissue homogenization	11
2.3.1.2. Serial exhaustive extraction	11
2.3.1.3. Soxhlet extraction	11
2.3.1.4. Maceration	12
2.3.1.5. Decoction	12
2.3.1.6. Infusion	12
2.3.1.7. Digestion	12
2.3.1.8. Percolation	12
2.3.1.9. Sonication	13
2.4. Using of <i>Nigella sativa</i> in Treatment of Gastrointestinal problems, Wound infection and Urinary tract infection	13
CHAPTER THREE	
3. MATERIALS AND METHODS	
3.1. Study design	15
3.2. Study area	15
3.3. Study population	15
3.4. Inclusion criteria	15
3.5. Exclusion criteria	15
3.6. Sampling	15
3.7. Sample size	15
3.8. Ethical considerations	15
3.9. Specimen processing	16
3.9.1. Collection of the specimens	16

3.9.2. Cultivation of the specimens	16
3.9.3. Identification of the isolates	16
3.9.3.1. Cultural characteristics	16
3.9.3.2. Gram stain	16
3.9.3.3. Biochemical tests	17
3.9.3.3.1. Catalase test	17
3.9.3.3.2. Coagulase test	17
3.9.3.3.3. Deoxyribonuclease (DNase) test	17
3.9.3.3.4. Indole test	17
3.9.3.3.5. Citrate utilization test	17
3.9.3.3.6. Urease test	18
3.9.3.3.7. Motility testing	18
3.9.3.3.8. Kligler Iron Agar (KIA)	18
3.9.3.3.9. Oxidase test	19
3.9.3.3.10. Manitol Salt Agar (MSA)	19
3.9.4. Antibiotics Sensitivity Testing	19
3.9.5. Preservation of Organisms	20
3.10. Extraction of <i>Nigella sativa</i>	20
3.10.1. Collection and Preparation of <i>Nigella sativa</i> specimen	20
3.10.2. Preparation of the extracts	20
3.10.3. Preparation of <i>Nigella Sativa</i> extracts For Testing the Antibacterial Activity	21
3.10.4. Preparation of standard bacterial suspension	21
3.10.5. Testing of extracts for antibacterial activity against standard organisms and clinical isolates	21
3.10.5.1. Cup- plate agar diffusion (inhibition zone) method	21
3.11. Data analysis	22

CHAPTER FOUR RESULTS	
4.1. Distribution of clinical specimens according to the hospitals	23
4.2. Distribution of clinical specimens according to the gender	23
4.3. The frequency and percentage of bacterial growth	24
4.4. The frequency and percentage of growth according to specimens	24
4.5. Biochemical identification	25
4.5.1. Biochemical reaction for Gram positive pathogens	25
4.5.2. Biochemical reaction for Gram negative pathogens	25
4.5.3. Frequency and percentage of isolated organisms	26
4.6. Effect of antibiotics used for sensitivity testing on clinical isolates	27
4.7. Result of <i>Nigella sativa</i> extraction	29
4.7.1. The activity of <i>Nigella sativa</i> extracts on standard strains and various clinical isolates	29
4.7.1.1. The activity of petroleum ether extract on standard strains	30
4.7.1.2. The activity of petroleum ether extract on various clinical isolates	31
CHAPTER FIVE 5. DISCUSSION	
5.1. Discussion	35
5.2. Conclusions	39
5.3. Recommendations	39
References	40
Appendixes	49

List of Tables

No.	Title	Page No.
1	Distribution of clinical specimens according to the hospitals	23
2	Distribution of clinical specimens according to the gender	23
3	The frequency and percentage of bacterial growth	24
4	The frequency and percentage of growth according to specimens	24
5	The results of the biochemical reactions for Gram positive pathogens	25
6	The results of the biochemical reactions for Gram negative pathogens	25
7	Frequency and percentage of isolated organisms	26
8	Antibiotics used for sensitivity testing and number of bacterial strains which were resistant to them	28
9	Weight and yield % of <i>Nigella Sativa</i> extracts obtained using petrolum ether and methanol solvents	29
10	Antibacterial activity of <i>Nigella sativa</i> petroleum ether extract on standard strains	30
11	Antibacterial activity of <i>Nigella sativa</i> petroleum ether extract on various clinical isolates	32

List of Figures

No.	Title	Page No.
1	Activity of petroleum ether extract of <i>Nigella sativa</i> against <i>S.aureus</i> isolate	33
2	Activity of petroleum ether extract of <i>Nigella sativa</i> against <i>K.pneumoniae</i> isolate	34
3	Activity of petroleum ether extract of <i>Nigella sativa</i> against <i>E.coli</i> isolate	35

CHAPTER ONE
INTRODUCTION

CHAPTER ONE

1. INTRODUCTION

1.1. Introduction

The use of plants and plant products as medicines could be traced as far back as the beginning of human civilisation (Ahmed *et al.*, 1998).

Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, nutraceuticals, food supplements, modern medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. According to World Health Organization (WHO), up to 80% of the people depend on traditional medicinal plants for their medicines (Arunkumar and Muthuselvam, 2009).

Plant products derived from barks, flowers, roots, leaves, seeds, fruits are the part of phytomedicines (Criagg and David, 2001).

Following the advent of modern medicine, herbal medicine suffered a setback, but during the last two or three decades, advances in phytochemistry and in identification of plant compounds, effective against certain diseases have renewed the interest in herbal medicines (Arora and Kaur, 1999). In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a reevaluation of the therapeutic use of ancient remedies, such as plants and plant-based products (Gupta *et al.*, 2008).

The seeds of *Nigella sativa*, commonly known as black seed or black cumin has been used for medicinal purposes for centuries both as herb and pressed into oil in Asia, Middle East, and Africa (Zohary and Hopf, 2000).

It has been traditionally used for a variety of conditions and treatments related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support and general well-being (Khan *et al.*, 2003).

In Islam, it is regarded as one of the greatest forms of healing medicine available and included in the medicine of the Prophet Mohammed (peace be upon him) (Abn Alqaijem, 2001).

Nigella sativa seeds have been extensively studied in the last 4-5 decades and these studies have reported it to possess a number of medicinal properties (Ali and Blunden, 2003; Randhawa and Al- Ghamdi, 2002).

Their crude extracts (Ali *et al.*, 2001; Mouhajir and Pedersen, 1999) and essential oil (Halwani *et al.*, 1999) have been reported to possess antibacterial activity against several bacteria. These activities have been predominantly attributed to the presence of active, compounds in the fixed oils volatile oils and different extracts studied (Islam *et al.*, 2012).

The present study aimed to screen and evaluate the antibacterial activity of two different extracts of *Nigella sativa* on selected pathogenic bacteria isolated from different clinical specimens.

1.2. Rationale

Antibiotics were introduced in the 1940s and they were thought off as the cure for all emerging diseases. However; resistant strains emerged only two decades after the introduction of penicillins. Even with the introduction of new classes of antibiotics and synthetic drugs the problem of resistance continues as bacteria develop resistance to almost all classes of antibiotics. In recent years pharmaceutical companies has almost stopped producing new antibiotics which has led researchers to look for alternative antimicrobials. Herbs were used for treatment of infectious diseases for many centuries before the introduction of antibiotics and the emergence of resistant strains has renewed the in herbs and medicinal plants to serve as novel antimicrobial agents.

Seeds of *N. sativa* are frequently used in folk medicine for acquiring good health and treating of many ailments and various microbial infections (Al-Jassir, 1992; Al-Ghamadi, 2001). The antimicrobial effects of *N. sativa* seeds against different pathogenic microbes were investigated by many researchers. The diethyl ether extract was found to cause concentration-dependent inhibition of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and a pathogenic yeast *Candida albicans* (Hanafy and Hatem, 1991). The methanol and chloroform extracts also showed high inhibitory effects against *S.aureus*, *P. aeruginosa* and *C. albicans* (Mashhadian and Rakhshandeh, 2005).

The essential oil of the seeds have also shown dose-dependent antibacterial effects on Gram-positive (*S. aureus*) and Gram negative (*E. coli*) bacteria (Hosseinzadeh *et al.*, 2007). The volatile oil and crude extracts of *N. sativa* seeds was also proved to be effective against many strains of bacteria, including those known to be highly resistant to drugs (Salman *et al.*, 2008; Joe *et al.*, 2009; Kamal *et al.*, 2010; Rahman *et al.*, 2011).

1.3. Objectives

1.3.1. General objective

To evaluate the antimicrobial of *Nigella sativa* extracts on selected pathogenic bacteria isolated from different clinical specimens.

1.3.2. Specific objectives

- 1-To isolate and identify the pathogenic bacteria from clinical specimens.
- 2-To study the antibacterial activity of *Nigella sativa* extracts on selected pathogenic bacteria.
- 3-To determine the minimum inhibitory concentration of *Nigella sativa* extracts.

CHAPTER TWO
LITERATURE REVIEW

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Herbal medicine

2.1.1 Definition

Herbal medicine is sometimes referred to as herbalism or traditional medicine. It is the use of herbs for their therapeutic or medicinal value. A herb is a plant or a plant part valued for its medicinal, aromatic or savory qualities. Herb plants produce and contain a variety of chemical substances that act upon the body (Ahmed *et al.*, 1998).

2.1.2. History

Herbs have been used for many centuries by many cultures to enhance the flavor and aroma of food. Early cultures also recognised the value of using herbs in preserving food and for their medicinal value. Scientific experiments since the late 19 century have documented the antimicrobial properties of some herbs and their components (Zakia, 1988).

2.1.3. Antimicrobial Properties of Medicinal Plants

Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins and phenol compounds, flavonoids, steroids, resins fatty acids gums which are capable of producing definite physiological action on body. Compounds extracted from different parts of the plants can be used to cure diarrhea, dysentery, cough, cold, cholera, fever and bronchitis (Saranraj and Sivasakthi, 2014).

2.2. *Nigella sativa*

Nigella sativa is a scientific name, in Arabic it is named AL-habatul al-sauda and Habbatul Baraka, while in English it is called Black seeds, Black cumin and small fennel.

2.2.1. Distribution

Nigella sativa (*N. sativa*) is native to Southern Europe, north Africa and Southwest Asia (Bontnick *et al.*, 2012).

2.2.3. Botanical description

It is small prostrate annual herb belonging to family Ranunculaceae, about 45 cm high 2-3 slender leaves pinnatisect, 2-4 cm long cut into linear segment, segments oblong. Flowers pale, blue on solitary long peduncles, seeds trigonous and black in colour. The plant has a rather stiff, erect, branching stem, bears deeply-cut greyish-green leaves and terminal greyish blue flowers, followed by odd, toothed seed vessels, filled with small somewhat compressed seeds, usually three-cornered, with two sides flat and one convex, black or brown externally white and oleaginous, strong agreeable aromatic odour. The flowers are delicate, and usually coloured pale blue and white, with 5–10 petals. The fruit is a large and inflated capsule composed of 3–7 united follicles, each containing numerous seeds (Dwivedi, 2003).

2.2.4. Chemical constituents

Nigella sativa contains 20.85% protein, 38.20% fat, 4.64% moisture, 4.37% ash, 7.94% crude fibre and 31.94% total carbohydrates. Potassium, phosphorus, sodium and iron were the predominant elements present. Zinc, calcium, magnesium, manganese and copper were found at lower levels.

Unsaturated fatty acids: linoleic and oleic acids. Saturated fatty acids: palmitic acid. Main amino acids: glutamic acid, arginine and aspartic acid. Minor amino acids: cystine and methionine.

Black cumin oil: major fatty acids are linoleic acid (50.2%), oleic acid (19.9%), margaric acid (10.3%), cis-11, 14-eicosadienoic acid (7.7%) and stearic acid (2.5%) (Amin *et al.*, 2010).

2.2.5. Traditional Uses

Traditionally the seeds and its oil are used in several diseases. The seeds are considered as bitter, pungent, aromatic, appetizer, stimulant, diuretic, emmenagogue, galactagogue, anthelmintic, acrid, thermogenic, carminative, anodyne, deodorant, digestive, constipating, sudorific, febrifuge, expectorant, purgative, abortifacient. They are used in ascites, cough, jaundice, hydrophobia, fever, paralysis, conjunctivitis, piles, skin diseases, anorexia, dyspepsia, flatulence, abdominal disorders, diarrhoea, dysentery, intrinsic hemorrhage and amenorrhea. Seed oil is a local anesthetic (Warrier *et al.*, 2004; Sharma *et al.*, 2005).

2.2.6. Pharmacological and toxicological properties of *Nigella sativa*

The seeds contain both fixed and essential oils, proteins, alkaloids and saponin. Much of the biological activity of the seeds has been shown to be due to thymoquinone, the major component of the essential oil, which is also present in the fixed oil. The pharmacological activities of the crude extracts of the seeds (and some of its active constituents, e.g. volatile oil and thymoquinone) that have been reported include protection against nephrotoxicity and hepatotoxicity induced by either disease or chemicals. The seeds oil has anti-inflammatory, analgesic, antipyretic, antimicrobial and antineoplastic activity. The oil decreases blood pressure and increases respiration. Treatment of rats with the seed extract for up to 12 weeks has been reported to induce changes in the haemogram that include an increase in both the packed cell volume (PCV) and haemoglobin (Hb), and decrease in plasma concentrations of cholesterol, triglycerides and glucose. Two cases of contact dermatitis in two individuals have been reported following topical use. Administration of either the seed extract or its oil has been

shown not to induce significant adverse effects on liver or kidney functions .It would appear that the beneficial effects of the use of the seeds and thymoquinone might be related to their cytoprotective and antioxidant action, and to their effect on some mediators of inflammation (Ali and Blunden, 2003).

2.2.7. General uses in Herbal medicine

N. sativa is considered one of the greatest forms of healing medicine available, especially in the Arab countries in which it is medicinal uses are probably related to the incentive from the authentic saying of the prophet Mohammed (peace be upon him): *N.sativa* can heal all disease except death. Ibne-Sina in his famous book *Al-Qanoon fi el-Tibb*, has mentioned many medicinal uses of *N. sativa* including treatment of fever, common cold, headache, asthma, rheumatic diseases, scorpion and spider stings and bites of snake, cat and dog (Bakathir and Abbas, 2011).

2.2.8. Antibacterial effects

The antibacterial activity of *Nigella sativa* essential oil was determined against a panel of strains bacteria, using a broth microdilution method. Results of antibacterial activity confirmed the possibility of using *Nigella sativa* essential oils or some of their components in biology and pharmaceutic preparations (Ainane1 *et al.*, 2014).

Antibacterial activity of two different extracts of *Nigella sativa* L.(Ether and Ethanol) has been evaluated in vitro against five bacterial test species (*S. aureus* , *E. coli*, *E. faecalis*, *Proteus sp* and *M. luteus*) which are known to cause some infections in humans. Among the tested extracts, the most effective one was found to be ether extract. The biggest inhibition zone was observed with ether fraction and it inhibited the growth of two Gram-positive bacteria (Zmen *et al.*, 2007).

The *in-vitro* antimicrobial activity of the volatile oils of *Nigella sativa* Linn Seeds was tested against fourteen pathogenic microbial strains

including three Gram- positive (*S. aureus* ATCC), *S.aureus* (lab. Isolates), *B. subtilis*, eleven Gram negative (*E.coli* ATCC, *E.coli* (lab. Isolates), *S.typhi*, *S.paratyphiA*, *Klebsiella*, *Sh. Flexneri*, *Sh. Dysentriae*, *Pseudomonus*, *Proteus*, *Vibrio cholerae* classical and *Vibrio cholerae* 0139. The volatile oil showed strong sensitivity to all the organisms (Nazma *et al.*, 2005).

The two main components of black seed essential oil, thymoquinone (TQ) and thymohydroquinone (THQ) were investigated for their antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella typhimurium*, *Salmonella enteritidis* and *Staphylococcus aureus*. Both TQ and THQ exerted antibacterial activity against Gram-positive and Gram- negative bacteria regardless to their susceptibility to antibiotics (Halawani, 2009).

In 1991 the antibacterial activity of *Nigella sativa* diethyl ether extract was investigated and found that the extract showed concentration-dependent inhibition of Gram-positive bacteria represented by *Staphylococcus aureus* (*S.aureus*). Gram-negative bacteria represented by *Pseudomonas aeruginosa* (*P.aeruginosa*) and *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) and *Bacillus subtilis* (*B. subtilis*) and a pathogenic yeast *Candida albicans* (*C. albicans*). The extract showed antibacterial synergism with Streptomycin and Gentamycin and showed additive antibacterial action with Spectinomycin, Erythromycin, Tobaramycin, Doxycycline, Chloramphenicol, Nalidixic acid, Ampicillin, and Lincomycin and Sulphamethoxazole-trimethoprim combination (Hanafy and Hatem, 1991).

In a study in 2000, the antimicrobial activity of *Nigella sativa* crude extract was tested against multiple antibiotic-resistant bacteria and found

that the extract showed a promising effect against Gram positive ones (Morsi, 2000).

In author study in 2005, the antimicrobial activity of *Nigella sativa* oil against *S. aureus* obtained from clinical specimens was studied and found to be active against susceptible and multidrug resistant strains of *S. aureus* (Salman *et al.*, 2005). And in author one in 2008, the antibacterial activity of *Nigella sativa* against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) was studied and proved to have strong inhibitory effect (Hannan *et al.*, 2008).

Also in 2008, the antimicrobial activity of *Nigella sativa* Linn seed oil against multidrug resistant-bacteria from clinical isolates was investigated and found that the oil showed pronounced dose dependent antibacterial activity more against Gram positive than in Gram negative bacteria (Salman and Tarig, 2008). The antimicrobial activity of seed oil and extract against multidrug resistant clinical strains of *P. aeruginosa* tested in varying dilutions was studied in 2009, both oil and methanolic extract showed remarkable dose dependent antibacterial activity (Salman *et al.*, 2009).

2.3. General techniques for extraction of medicinal plants

The general techniques of medicinal plants extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter current extraction, microwave-assisted extraction, ultrasound techniques (water distillation, steam extraction (sonication), supercritical fluid extraction, and distillation, phytonic extraction (with hydro fluorocarbon solvents)

The basic parameters influencing the quality of an extract are plant part used as starting material, solvent used for extraction and extraction procedure (Amita and Shalini, 2014).

2.3.1. Extraction procedures

2.3.1.1. Plant tissue homogenization

Plant tissue homogenization in solvent has been widely used by researchers.

Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5-10 min or left for 24 hours after which the extract is filtered. The filtrate then may be dried under reduced pressure and re-dissolved in the solvent to determine the concentration. Some researchers however centrifuged the filtrate for clarification of the extract (Das *et al.*, 2010).

2.3.1.2. Serial exhaustive extraction

It is another common method of extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted.

Some researchers employ soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Das *et al.*, 2010).

2.3.1.3. Soxhlet extraction

Soxhlet extraction is only required where the desired has a compound limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Sutar *et al.*, 2010).

2.3.1.4. Maceration

In maceration (for fluid extract), whole or coarsely powdered plant-drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile drugs (Amita and Shalini, 2014).

2.3.1.5. Decoction

This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume (Bimakr, 2010).

2.3.1.6. Infusion

It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water (Bimakr, 2010).

2.3.1.7. Digestion

This is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstruum is increased thereby (Bimakr, 2010).

2. 3.1.8. Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for the specified menstruum and allowed to stand for approximately 4 hours in a well closed container, after which the mass is packed and the top of the percolator is closed.

Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 hours. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting (Cowan, 1999).

2.3.1.9. Sonication

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules (Cowan, 1999).

2.4. Using *Nigella sativa* in the treatment of gastrointestinal problems, wound and Urinary tract infections

Natural aqueous crude extract of black cumin was screened out against bacterial isolates involved in gastrointestinal problems and found that black cumin seeds could be the alternatives of antibiotics to cure the diseases of gastrointestinal tracts. (Nazir and Latif, 2012)

In a study evaluates the susceptibility of multidrug resistant *Staphylococcus aureus* isolated from diabetic wounds to *Nigella sativa* oil. The oil showed pronounced dose dependent antibacterial activity against the isolates. (Emeka *et al.*, 2015)

Synthesis of silver nanoparticles using seeds of *Nigella sativa* as reducing agent was evaluated in a study. Homogenized extract of *N. sativa* exposed to sunlight gave a better synthesis of nanoparticles when compared to other methods. Antibacterial activity of nanoparticles was studied against urinary tract infection (UTI) causing bacteria by disc diffusion method. The findings suggest that silver nanoparticles from seeds of *N. sativa* may be effectively used against UTI causing bacteria. (Ranjan *et al.*, 2013)

CHAPTER THREE
MATERIALS AND
METHODS

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

This was prospective cross sectional and hospital base study.

3.2. Study area

This study was conducted at Soba University Teaching Hospital, and Omdurman Military Hospital, in Khartoum State, Sudan ,during the period from June to November 2015.

3.3. Study population

Patients admitted with symptoms of wound infection, urinary tract infection and gastrointestinal problems.

3.4. Inclusion criteria

Patients admitted with wound infection, urinary tract infection and gastrointestinal problems were recruited and approved to participate in this study.

3.5. Exclusion criteria

Patients under antimicrobial treatment were excluded.

3.6. Sampling

Simple random sample.

3.7. Sample size

A total of one hundred forty four samples (n= 144) were collected.

3.8. Ethical considerations

Permission was issued by Collage of Ethical Committee, Sudan University of Science and Technology. Volunteers were informed and had got all the information about the research study, and all ethical roles were followed during sampling and data collection.

3.9. Specimen processing

3.9.1. Collection of the specimens

Under a septic condition , wound swabs were collected using sterile cotton swabs moistened with sterile normal saline , urine and stool were collected in sterile screw capped universal containers.

3.9.2. Cultivation of the specimens

Urine specimens were cultured on Cystine lactose electrolyte deficient (CLED) agar and MacConky agar using sterile wire loop under a septic condition. Wound specimens were cultured on blood agar and MacConky agar, while stool specimens were transferred from the collected containers to another containers containing Cary- Blair transport medium then cultured on Xylose lysine Deoxy cholate Agar (XLD) using sterile wire loop, inoculated plates were incubated aerobically at 37⁰C for 18-24 hours.

3.9.3. Identification of the isolates

3.9.3.1. Cultural characteristics

After the incubation period the plates were examined for the size, colour, edges, side views, odor and surface of the colonies.

3.9.3.2. Gram stain

Smears from the growth were prepared and stained by Gram stain as follows: fixed by heat, after cooling covered by crystal violet stain for 30-60 seconds, washed off stain by clean water, covered with Iodine for 30-60 seconds, washed with clean water, covered with safranin stain for 2 minutes, then washed and let to air dry and microscopically examined using oil immersion objective (100X) to observe morphological appearance, Gram positive reaction and Gram negative. The results of Gram's stain were reported.

3.9.3.3. Biochemical tests

3.9.3.3.1. Catalase test

A pure of 2-3ml of hydrogen peroxide solution was added in a test tube, by sterile wooden stick several colonies of test organisms was immersed in hydrogen peroxide solution. The positive results indicated by immediate bubbling (Cheesbrough, 2000).

3.9.3.3.2. Coagulase test

A drop of physiological saline was placed on each end of slide, a colony of the test organism was emulsified in each of the drops to make two thick suspensions and a drop of plasma was added to one of the suspensions and mixed gently by rotating. The positive results indicated by producing clump within 10 seconds (Cheesbrough, 2000).

3.9.3.3.3. Deoxyribonuclease (DNase) test

The test organism was cultured on a medium which contains DNA. After overnight incubation, the colonies were tested for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitated unhydrolyzed DNA. DNase producing colonies were therefore surrounded by clear areas due to DNA hydrolysis (Cheesbrough, 2000).

3.9.3.3.4. Indole test

The tested colony was inoculated in sterile peptone water using sterile wire loop and then incubated at 37°C aerobically overnight. Few drops of Kovac's reagent were added to the medium and shaken gently to test for indole. Positive result was indicated by production of red colour in the surface layer within 10 minutes (Cheesbrough, 2000).

3.9.3.3.5. Citrate utilization test

Slopes of Simmon's citrate agar medium were prepared, by using sterile straight wire loop the slope was streaked and the butt was stabbed with a small part of the tested colony. Then the slopes of the medium were

incubated overnight at 35⁰C. Positive reaction was indicated by the change in the colour of the medium into blue colour while negative reaction was indicated by no change in the colour (Cheesbrough, 2000).

3.9.3.3.6. Urease test

The tested colony was inoculated on the surface of the slope of Christensen's urea agar medium by sterile straight loop in zigzagging manner and then incubated overnight at 37⁰C aerobically. Positive reaction was indicated by the colour change in the indicator (phenol red) to pink colour and negative reaction was indicated by no change in the colour (Cheesbrough, 2000).

3.9.3.3.7. Motility testing

The tested colony was taken by a sterile straight loop, and inoculated by stabbing a semisolid media, then incubated aerobically at 37⁰C overnight. The motility was shown by spreading turbidity from the stab-line or turbidity throughout the medium (Cheesbrough, 2000).

3.9.3.3.8. Kligler Iron Agar (KIA)

A small part of the tested colony was picked off using a straight loop and inoculated in KIA medium. First stabbing the butt, then streaking the slope in zigzag pattern, and then incubated at 37⁰C aerobically overnight.

Result

A yellow butt red-pink slope indicated the fermentation of the glucose only.

A yellow slope and yellow butt indicated the fermentation of lactose and glucose.

A red-pink slope and butt indicated no the fermentation of glucose and lactose.

Blackening along the stab line or throughout the medium indicated H₂S production. Cracks and bubbles in the medium indicated gas production from glucose fermentation (Cheesbrough, 2000).

3.9.3.3.9. Oxidase test

A piece of filter paper was placed on a clean glass slide and three to four drops of freshly prepared oxidase reagent (tetra methyl para phenylenediaminedihydrochloride) were added using sterile Pasteur pipette, wooden stick was used to pick a colony of the test organism and placed on the filter paper. The positive reaction was indicated by the production of blue-purple colour within 10 second (Cheesbrough, 2000).

3.9.3.3.10. Manitol Salt Agar (MSA)

This medium was used to differentiate *S.aureus* from other *Staphylococci* species. A portion of colony was inoculated on manitol salt agar containing 75 g/l sodium chloride and incubated aerobically at 37°C for 18-24 hours. *S.aureus* ferments manitol producing yellow colonies (Cheesbrough, 2000).

3.9.4. Antibiotics Sensitivity Testing

By using sterile wire loop 3-5 colonies of similar appearance were selected and emulsified in 3-4 ml of sterile physiological saline and compared with Mc Farland's turbidity standard.

By sterile swab suspension was inoculated in Mueller Hinton agar plate and by sterile forceps an antibiotic discs were placed in the inoculated plate, incubated aerobically at 37°C for overnight, then the inhibition zones measured.

Interpretation of the result was done by aid of interpretative chart. By using the chart, the organism was characterized as being resistant, intermediate, or susceptible to the specific antibiotic. The following antimicrobial agents were tested: Ampicillin (10mcg), Gentamicin (10 mcg), Cotrimoxazole(25mcg), Ciprofloxacin (5mcg), Amoxicillin/clavulanic acid (20/10 mcg) Cefurixime (30mcg), Amikacin (30mcg), Ceftazidime (30mcg), Imipenem (10mcg), Clindamycin (2mcg),

Vancomycin (30mcg), Erythromycin (15 mcg) Tetracyclin (30 mcg), and Oxacilin (5mcg).

3.9.5. Preservation of Organisms

After the identification of the microorganisms (clinical isolates) slopes nutrient agar were prepared, inoculated with organisms and incubated for 24 hours, then preserved in the refrigerator at 4⁰C.

3.10. Extraction of *Nigella sativa*

3.10.1. Collection and Preparation of *Nigella Sativa* specimen

Specimen of *Nigella sativa* was obtained from Omdurman market from "alteman atara". The dried *Nigella sativa* sample was cleaned from dust and grass.

3.10.2. Preparation of the extracts:

Extraction was carried out according to method described by Sukhdev and his colleagues (2008).

Hundred grams of the plant sample was coarsely powdered using mortar and pestle and successively extracted with petroleum ether and methanol using soxhelt extractor apparatus. Extraction carried out for about four hours with petroleum ether and eight hours for methanol till the colour of solvents at the last siphoning time returned colourless. Solvents were evaporated under reduced pressure using rotary evaporator apparatus. Finally the extracts were allowed to dry in Petri dishes till complete dryness.

3.10.3. Preparation of *Nigella sativa* extracts For Testing the Antibacterial Activity

The crude extracts of *Nigella sativa* were diluted into different concentrations as follows: 100%, 50%, 25%, and 12.5% to be used against the selected organisms.

3.10.4. Preparation of standard bacterial suspension

New subcultures of the selected bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) including standard bacteria which brought from microbiology department of National Institute for Research and bacteria isolated from clinical samples were inoculated into 3.0 ml of sterile normal saline. Inoculum density was compared with McFarland standard solution.

3.10.5. Testing of extracts for antibacterial activity against standard organisms and clinical isolates:

3.10.5.1. Cup- plate agar diffusion (inhibition zone) method

Cup plate agar diffusion method was adopted with some minor modification to assess the antibacterial activity of prepared extract. 0.2 ml of bacterial suspension (standard and clinical isolates) were taken with automatic pipettes using sterile tips and added to twenty ml of less than 50°C molten Mueller Hinton media and mixed and poured in sterile plate. The media were allowed to set and solidify for minutes, make wells using sterile Cork borer of 10 mm diameter. Alternated cups were filled with 0.1ml of different concentrations of petroleum ether and methanol extract of *Nigella sativa* (100%, 50%, 25% and 12.5%), using automatic pipettes and allowed to diffuse at room temperature for 30 min then the plates were incubated in an incubator in upright position at 37°C for 18 hours. The bacteria inhibited by the extracts at concentration 12.5% read at 6.25%.

The diameters of the resultant growth inhibition zones were measured in mm and the result were recorded. The inhibition zones with diameter less than 12mm were considered as having no antibacterial activity (Srinivasan *et al.*, 2001).

3.11. Data analysis

All collected data were analyzed using Microsoft Office Excel 2007.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4.1. Distribution of clinical specimens according to the hospitals

A total of 144 patients with symptoms of urinary tract infection, wound infection and gastrointestinal problems were enrolled in this study during the period from June to November 2015.

The patients attended Soba University Teaching Hospital (102 samples) and Omdurman Military Hospital (42 samples) (Table 1).

Table 1: Distribution of clinical specimens according to the hospitals

Hospitals	Frequency			Total
	Wound	Urine	Stool	
Soba University Teaching Hospital	56 (55%)	36 (35%)	10 (10%)	102 (100%)
Omdurman Military Hospital	21 (59%)	16 (38%)	5 (12%)	42 (100%)
Total	77 (54%)	52 (36%)	15 (10%)	144 (100%)

4.2. Distribution of clinical specimens according to the gender

Out of 144 specimens, 60 (42%) were from males and 84 (58%) were from females (Table 2).

Table 2: Distribution of clinical specimens according to the gender

Gender	Frequency			Total
	Wound	Urine	Stool	
Male	31(52%)	20(33%)	9(15%)	60(100%)
Female	46(55%)	32(38%)	6(7%)	84(100%)
Total	77(54%)	52(36%)	15(10%)	144(100%)

4.3. The frequency and percentage of bacterial growth

Out of 144 investigated samples, 103 (72%) showed bacterial growth, while 41(28%) showed no bacterial growth (Table 3).

Table 3: The frequency and percentage of bacterial growth

Result of culture	Frequency	Percentage (%)
Growth	103	72
No growth	41	28
Total	144	100

4.4. The frequency and percentage of growth according to specimens

The encountered bacterial growth was distributed in the investigated specimen as follows: 60/77(78%) wound, 36/52(69%) urine, 7/15(47%) stool (Table4).

Table 4: The frequency and percentage of growth according to specimens

Samples	Growth	%	No growth	%
77 Wound	60	78	17	22
52 Urine	36	69	16	31
15 Stool	7	47	8	53

4.5. Biochemical identification

4.5.1. Biochemical reaction for Gram positive pathogens

Table 5: The results of the biochemical reactions for Gram positive pathogens

Pathogen	Biochemical tests				
	Catalase	DNase	Coagulase	Mannitol	Lactose fermentation
<i>S.aureus</i>	+	+	+	+	+

Note: +: Positive.

4.5.2. Biochemical reaction for Gram negative pathogens

Table 6: The results of the biochemical reactions for Gram negative pathogens

Pathogens	Biochemical Tests						
	Ind.	U.	Cit.	KIA Slope/butt	Gas	H ₂ S	Oxidase
<i>Citrobacter</i> species	-	d	+	RorY/Y	+	d	-
<i>E.coli</i>	+	-	-	Y/Y	+	-	-
<i>K.pneumoniae</i>	-	-	+	Y/Y	+	-	-
<i>P. mirabilis</i>	-	+	+	R/Y	+	+	-
<i>Ps.aeruginosa</i>	-	+	+	R/R	-	-	+
<i>Salmonella</i> species	-	-	d	R/Y	d	d	-
<i>Serratia</i> species	-	d	+	RorY/Y	-	d	-
<i>Shigella</i> species	d	-	-	R/Y	-	-	-

Notes: **Ind:** Indole Test, **U:** Urease Test, **Cit:** Citrate Test, **KIA:** Kilger iron agar, **H₂S:** Producing H₂S gas, **Oxidase:** Cytochrome oxidase Test, +: Positive, - Negative, **Y:** Yellow, **R:** Red, **d:** Different strains give different results.

4.5.3. Frequency and percentage of isolated organisms

In this study nine types of bacteria were isolated (Table 7). The isolated bacteria were *K.pneumoniae* 32/103(31%), *E.coli* 22/103(21%), *S.aureus* 21/103(20%), *P.mirabilis* 11/103(11%), *Ps.aeruginosa* 9/103(9%), *Salmonella* species 4/103(4%), *Serratia* species 2/103(2%), *Shigella* species 1/103(1%), and *Citrobacter* species 1/103(1%) as shown in Table 7.

Table 7: Frequency and percentage of isolated organisms

Isolate	Frequency	Percentage (%)
<i>K.pneumoniae</i>	32	31
<i>E.coli</i>	22	21
<i>S.aureus</i>	21	20
<i>p. mirabilis</i>	11	11
<i>P. aeruginosa</i>	9	9
<i>Salmonella</i> species	4	4
<i>Serratia</i> species	2	2
<i>Shigella</i> species	1	1
<i>Citrobacter</i> species	1	1
Total	103	100

4.6. Effect of antibiotics used for sensitivity testing on clinical isolat

Staphylococcus aureus: Twenty one strains were tested which showed variable resistance pattern to the tested antibiotics .Five were resistant to 3 antibiotics and 6 were resistant to 4 antibiotics. Resistance was highest to Clindamycin and Erythromycin followed by Tetracyclin and Cotrimoxazole. Three of these were Methicillin resistant (Table8).

Escherichia coli: Out of 22 strains tested, 7 were resistant to 3 antibiotics, 4 to 4 antibiotics, 2 to 5 antibiotics and 1 to 6 antibiotics. Resistance was highest for Ampicillin and Cefurixime followed by Cotrimoxazole and Amoxicillin/clavulanic acid, Ceftazidime, Ciprofloxacin and Gentamicin (Table8).

Klebsiella pneumoniae: Out of 32 strains tested, 7 were resistant to 3 antibiotics, 5 to 4 antibiotics, 3 to 5 antibiotics and only 1 to 6 antibiotics. Resistance was highest for Ampicillin and Cefurixime followed by Amoxicillin/clavulanic acid, Ceftazidime, Cotrimoxazole, Ciprofloxacin and Gentamicin (Table8).

Proteus mirabilis: Eleven strains were tested, 3 of which showed resistance to 3 antibiotics and 1 to 4 antibiotics. Resistance was highest for Ampicillin, Cefurixime and Amoxicillin/clavulanic acid followed by Cotrimoxazole and Ceftazidime (Table8).

Pseudomonas aeruginosa: Out of 6 strains tested, 3 were resistant to Ciprofloxacin, Ceftazidime and Gentamicin (Table8).

***Salmonella* species**: Out of 4 *Salmonella* species tested, 2 were resistant to Ampicillin and 1 to Cotrimoxazole (Table8).

***Serratia* species**: Two species were tested; both of them were resistant to Ampicillin and Cefurixime (Table8).

***Shigella* and *Citrobacter* species**: They were sensitive to all antibiotics tested.

Table 8: Antibiotics used for sensitivity testing and number of bacterial strains which were resistant to them

No. of isolates showing resistance to antibiotic(no. of resistant strains/ no. of strains tested)															
Code of Name of bacteria	Total	Amikacin	Amoxicillin/clavulanic acid	Ampicillin	Cefazidime	Cefurixime	Cotrimoxazole	Centamicin	Ciprofloxacin	Clindamycin	Erythromycin	Imipenem	Oxacillin	Tetracyclin	Vancomycin
EC	22	0	10	13	8	13	10	3	7	-	-	0	-	-	-
KP	32	0	12	15	11	15	9	3	3	-	-	0	-	-	-
PM	11	0	4	4	2	4	2	0	0	-	-	0	-	-	-
PA	6	0	-	-	3	-	-	3	3	-	-	0	-	-	-
SA	21	-	-	-	0	-	6	0	3	13	13	-	3	7	0
SAL	4	0	0	2	0	0	1	0	0	-	-	0	-	-	-
SE	2	0	0	2	0	2	0	0	0	-	-	0	-	-	-

Notes

EC: *E.coli*, **KP:** *K.pneumoniae*, **PM:** *p.mirabilis*, **PA:** *P. aeruginosa*, **SA:** *S.aureus*, **SAL:** *Salmonella* species, **SE:** *Serratia* species, **No:** Number.

4.7. Result of *Nigella sativa* extraction

Table (9): Weight and yield % of *Nigella sativa* extracts obtained using petroleum ether and methanol solvents

Weight of <i>Nigella</i> <i>Sativa</i> sample	Petroleum ether		Methanol	
	Weight of extract (g)	Yield (%)	Weight of extract (g)	Yield (%)
100 g	13.393	13.393	3.265	3.265 %

4.7.1. The activity of *Nigella sativa* extracts on standard strains and various clinical isolates

Only the isolated bacteria which showed resistant to three antibiotics or more were tested for their sensitivity to *Nigella sativa* extracts, these include *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.

Petroleum ether extract of *Nigella sativa* showed obvious effect on the bacterial growth of standard strains and clinical isolates, while methanolic extract showed no effect.

The efficiency of the antibacterial of petroleum ether extract was found to increase when increasing the extract concentration.

4.7.1.1. The activity of petroleum ether extract on standard strains

The petroleum ether extract showed pronounced dose dependant activity against *S. aureus* ATCC 25923 followed by *K. pneumoniae* ATCC 53657, *E.coli* ATCC 25922, and *P.aeruginosa* ATCC 27853, and *P.mirabilis* ATCC 6380.

S. aureus ATCC 25923 was the most sensitive and *P.mirabilis* was the least sensitive, as shown in Table 10.

Table 10: Activity of *Nigella sativa* petroleum ether extract on stadard strains

Ex.Conc. mg/ml	Mean of inhibition zone including well diameter measured in mm			
	100%	50%	25%	12.5%
Name of standard bacterial strains				
<i>S.aureus</i> (ATCC 25923)	42	25	18	12
<i>E.coli</i> (ATCC 25922)	26	19	12	-
<i>K.pneumoniae</i> (ATCC 53657)	30	23	13	-
<i>P.mirabilis</i> (ATCC 6380)	15	-	-	-
<i>Ps. aeruginosa</i> (ATCC 27853)	16	-	-	-

Note

Ex.Conc.mg/ml: Extract concentration used in mg/ml.

4.7.1.2. The activity of petroleum ether extract on various clinical isolates

Staphylococcus aureus: Seven out of 11 strains tested were inhibited by petroleum ether extract of *Nigella sativa* which showed activity in concentrations of 100%, 50%, 25% and 12.5%. The higher concentration gave the higher effect on the growth of bacterial isolates. Four strains which were resistant to 4 antibiotics including 1 MRSA strains were also found resistant to petroleum ether extract (Table 11 and Figure 1).

Escherichia coli: Petroleum ether extract inhibited 8 out of 14 strains tested, which showed resistant to 3-5 antibiotics. It was active against the 6 strains in concentrations of 100% and 50%, against 2 strains in concentrations of 100% only and no strain inhibited at concentrations 25% and 12.5% (Table 11 and Figure 3).

Klebsiella pneumoniae: Out of 16 strains tested, which showed different resistance pattern to various antibiotics, the petroleum ether extract showed activity against 5 at concentration 100% (Table 11 and Figure 2).

Four strains of *Proteus mirabilis* and 3 strains of *Pseudomonas aeruginosa* which were all resistant to 3-4 antibiotics were not inhibited by petroleum ether extract in any of the concentrations tested.

Table 11: Activity of *Nigella sativa* petroleum ether extract on various clinical isolates

Name of clinical isolates of bacteria	Total	Ex.Conc.mg/ml							
		100%		50%		25%		12.5%	
		N	Zone	N	Zone	N	Zone	N	Zone
<i>S.aureus</i>	11	7	34	6	20	5	14	3	12
<i>E.coli</i>	14	8	21	4	16	-	-	-	-
<i>K.pneumoniae</i>	16	5	24	2	15	-	-	-	-
<i>P.mirabilis</i>	4	0	-	0	-	0	-	0	-
<i>Ps.aeruginosa</i>	3	0	-	0	-	0	-	0	-

Notes:

N: Name of clinical isolates sensitive to different concentrations of petroleum ether extract

Zone: Mean of inhibition zone including well diameter measured in mm

Ex.Conc.mg/ml: Extract concentration used in mg/ml.

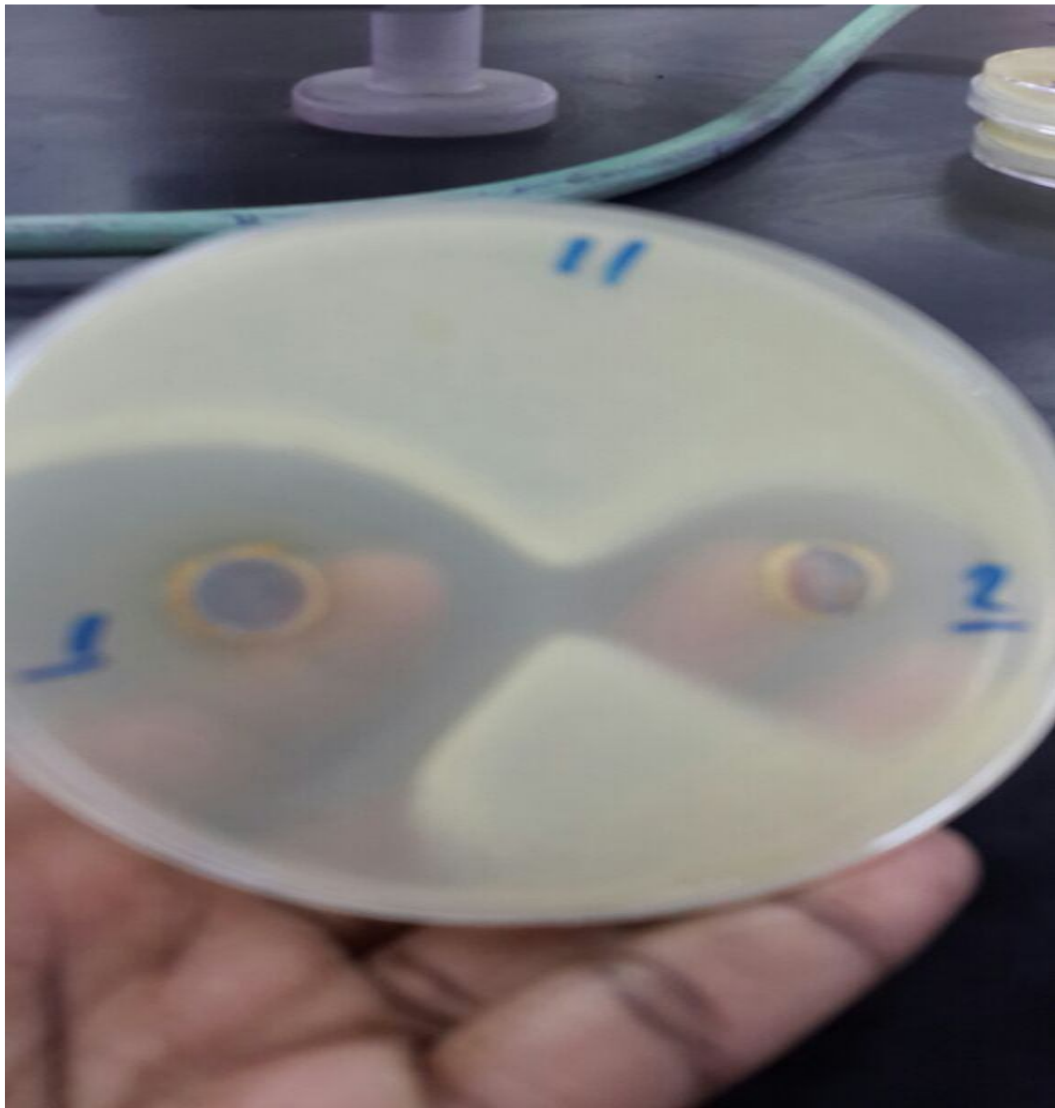


Plate 1: Activity of petroleum ether extract of *Nigella sativa* against *S. aureus* isolate.

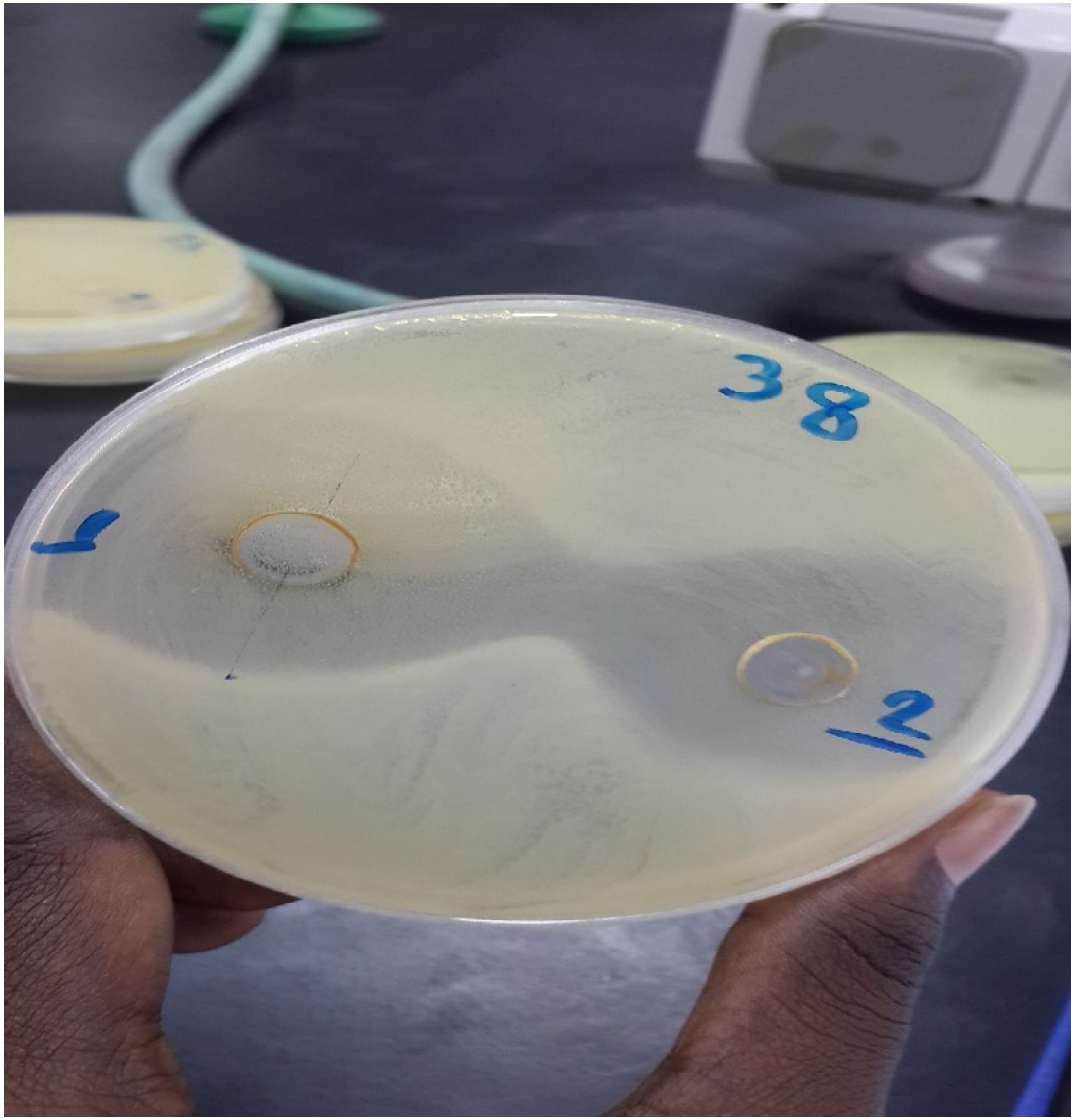


Plate 2: Activity of petroleum ether extract of *Nigella sativa* against *K.pneumoniae* isolate.



Plate 3: Activity of petroleum ether extract of *Nigella sativa* against *E.coli* isolate.

CHAPTER FIVE
DISCUSSION,
CONCLUSIONS AND
RECOMMENDATIONS

CHAPTER FIVE

5. DISCUSSION

5.1. Discussion

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains and this became a major cause of failure of the treatment of infectious disease (Ibrahim *et al.*, 2011). Plants are important sources of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *invitro* antibacterial assays (Mahesh and Satish, 2008).

In this study petroleum ether extract of *Nigella sativa* showed remarkable antibacterial activity against standard strains of *P.mirabilis* and *Ps. aeruginosa*, standard and clinical isolates of *S.aureus*, *E.coli* and *K.pneumoniae*. While methanolic extract showed no activity, these results agreed with that obtained by Kakil (2013). However, negative results do not indicate the absence of bioactive constituents, since active compound (s) may be present in insufficient quantities in the methanolic extract to show activity with the dose levels employed.

Petroleum ether extract was found to be more effective on Gram positive than Gram negative bacteria, which is in conformity with earlier studies (Agarwal *et al.*, 1979; Ali *et al.*, 2001). A number of compounds derived from plants often showed considerable activity against Gram positive bacteria but not against Gram negative species. Gram negative bacteria have an effective permeability barrier, comprised of outer membrane, which restricts the penetration of amphipathic compounds and multidrug resistance pumps that extrude toxins across this barrier. It is possible that the apparent ineffectiveness of plant antibacterials is largely due to the permeability barrier (Tegos *et al.*, 2012).

The means diameter of growth inhibition zone of standard and clinical isolates of bacteria were increased with the increased in extract concentration. This result is in agreement with report of Suleiman (2013). The best inhibition zone obtained by petroleum ether extract of *Nigella sativa* was 42 mm in diameter against *S.aureus* ATCC 25923 and 34 mm against clinical isolates of *S.aureus* at concentration of 100 mg/ml. Followed by 30 mm against *K. pneumoniae* ATCC 53657 and 24 mm against clinical isolates of *K. pneumoniae* at concentration of 100 mg/ml, then 26 mm against *E. coli* ATCC 25922 and 21 mm against clinical isolates of *E.coli* at concentration of 100 mg/ml.

Out of 3 methicillin resistant *Staphylococcus aureus* tested 2 were sensitive to petroleum ether extract of *Nigella sativa*, these results agreed with that report by Hannan *et al.* (2008).

Out of 11 strains of *S. aureus* tested 7 resistant to 3-4 antibiotics were inhibited by petroleum ether extract of *Nigella sativa*.

Minimum zone of inhibition of 12 mm was reported at concentration 12.5 mg/ml.

Eight out of 14 strains tested of *E. coli*, resistant to 3-5 antibiotics were inhibited by the extract and the minimum zone of inhibition was 16 mm at concentration 50 mg/ml.

Five strains of *K. pneumoniae*, which were resistant to a number of antibiotics, were inhibited by the extract with minimum zone of inhibition of 15 mm at concentration 50 mg/ml.

The petroleum ether extract showed antibacterial activity against standard *P.mirabilis* ATCC 6380 and *Ps.aeruginosa* ATCC 27853 at concentration of 100 mg/ml.

The clinical isolates of *P.mirabilis* which were resistant to 3-4 antibiotics were not inhibited by petroleum ether extract in any of the concentration tested, similar results were obtained in a study by Salman *et al.* (2008).

And clinical isolates of *Ps.aeruginosa* (resist to 3 antibiotics) were also not inhibited by extract in any of the concentration tested, this result disagreed with that report by Salman *et al.* (2009).

The antimicrobial activity of petroleum ether extract of *Nigella sativa* may be attributed to the presence of thymoquinone (Kahsai, 2002), thymohydroquinone (El-Fatratry, 1975) and thymol (Randhawa and Al-Ghamdi, 2002) as all of which possess antimicrobial activity (Karapinar and Aktug, 1987).

5.2. Conclusions

- Petroleum ether extract of *Nigella sativa* possessed antibacterial activity against some of tested pathogenic bacteria that showed resistant to a number of antibiotics, thus it can be used in the treatment of infectious diseases causes by resistant pathogenic bacteria.
- The petroleum ether extract was found to be more effective on Gram positive than Gram negative bacteria.
- The efficiency of the antibacterial of petroleum ether extract was found to increase when increasing the extract concentration.

5.3. Recommendations

- To isolate and purify the active ingredients in the extract responsible for antibacterial activity.
- Determination of minimum bactericidal concentrations for the active ingredients on each bacterium including those in this study and others.
- Study the toxicity of the active ingredients.
- More research is required to verify these results.

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REFERENCES

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APPENDIXES

Appendix 1

Materials

A-Equipment

Autoclave.

Bunsen burner.

1- Cork borer (No.10).

2- Deep freezer.

3- Freezer dryer.

4- Hot air oven.

5- Incubator.

6- Light microscope with oil immersion lens.

7- Rack.

8- Refrigerator.

9- Soxhlet apparatus (round bottom, reflex, condenser).

10- Straight loops with handle.

11- Water bath.

12- Wire loops with handle.

B-Glasswares

1- Petri dishes (plates).

2- Flask with different size.

3- Measuring cylinder.

4- Beakers.

5- Funneles.

6- Spoons.

7- Sterile containers (bijou bottles).

8- Test tubes.

9- Slides.

C-Disposable materials

- 1- Disposable syringes.
- 2- Wooden applicator.
- 3- Filter papers.

D-Culture media

Different culture media were used for inoculation, isolation, and identification of organisms. These include:

1-Nutrient agar

Typical formula in g/L

Contents

Peptone.....	5.0
Meat extract.....	3.0
Agar.....	15.0
pH 7.0±0.2	

Preparation

Suspend 23g of powder in 1L of D.W and heat to boiling. Dispense into containers and sterilize in the autoclave at 121°C for 15 minutes.

2-DNAse agar

Typical formula g/L

Contents

Tryptose.....	20
Deoxyribonucleic acid.....	2
Sodium chloride.....	5
Agar.....	12
pH 7.2±0.2	

Preparation

Suspend 3.9g in 1L of D.W. bring to boil to dissolve completely. Sterilize by autoclave at 121°C for 15 minutes. Cool to 50°C and pour into the sterile petridishes. Dry the surface of the medium before inoculation.

3-Media for biochemical reactions (Kligler iron agar, simmon's citrate agar, christensin urea media, media containing treptophan, semi solid agar medium)

Simmons citrate medium

Typical formula in g/L

Contents

Magnesium sulfate.....	0.20
Monoammonium phosphate.....	1.00
Dipotassium phosphate.....	1.00
Sodium citrate.....	2.00
Sodium chloride.....	5.00
Bromothymol blue.....	0.08
Agar.....	15.00

pH 6.8±0.2

Preparation

Dissolve 24g of powder in 1L of D.W. Bring to the boil. Dispense in tubes and sterilize by autoclaving at 121°C for 15 mins. Solidify with the long slant.

Kligler Iron Agar

Typical formula g/L

Contents

Balanced peptone.....	20.0
Lactose.....	10.0
Dextrose.....	1.0
Sodium chloride.....	5.0
Ferric ammonium citrate.....	0.5
Sodium thiosulphate.....	0.3
Phenol red.....	0.025
Agar no2.....	12.0

pH 6.9±0.2

Preparation

Dissolve 49g of powder in 1L of D.W. Soak for 10 mins. Swirl to mix bring to boil. Distribute into tubes and sterilize by autoclave at 121°C for 15 mins. Let the medium set as slopes about 3cm deeps.

Christensen Urea Medium

Typical formula g/L

Contents

Gelatin peptone.....	1.000
Dextrose.....	1.00
Sodium chloride.....	5.000
Monopotassium phosphate.....	2.000
Phenol red.....	0.012
Agar.....	15.000

pH 7.0±0.2

Preparation

Suspend 24g in 950ml of D.W and bring to the boil. Sterilize by autoclave at 121°C for 15 mins. Let it cool to 50-55°C. Added 50 ml of urea sterile solution 40% (Ref. 06-083) and mix well. Distribute aseptically in tubes and let them solidify slanted.

Peptone water

Typical formula g/L

Contents

Peptic digest of animal.....	10.00
Sodium chloride.....	5.00
Phenol red.....	0.02

pH 6.8±0.2

Preparation

- 1- Suspend 15.0 grams in 100 ml distilled water. Add the test carbohydrate in desired quantity and dissolve completely.
- 2- Dispense in tubes with or without inverted Durham's tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

4-Mueller Hinton agar

Typical formula g/L

Contents

Casein acid hydrolysate.....	17.50
Beef heart infusion.....	2.00
Starch,soluble.....	1.50
Agar.....	17.00
pH (at 25°C)	7.3±0.1

Preparation

Suspend 38g of powder in 1000ml D.W mix well and heat to boiling to dissolve the medium completely. Sterilize by autoclave at 121°C for 15mins.

5-Blood agar

To make about 35 blood agar plates:

Nutritious agar.....	500 ml
Sterile defibrinated blood.....	25 ml

Preparation

- 1- Prepare the agar medium as instructed by the manufacturer. Sterilize by autoclaving at 121 C⁰ for 15 minutes. Transfer to a 50 C⁰ water bath
- 2- When the agar has cooled to 50 C⁰, add aseptically the sterile blood and mix gently but well. Avoid forming air bubbles.
- 3 -Dispense aseptically in 15 ml amounts in sterile petri dishes as described in subunit 7.4.

4- Date the medium and give it a batch number.

5- Store the plates at 2–8 C⁰, preferably in sealed plastic bags to prevent loss of moisture

pH of medium: 7.2–7.6 at room temperature.

6-Cary-Blair transport medium

Contents

Sodium thioglycollate, *di*-sodium hydrogen phosphate, sodium chloride, agar, calcium chloride.

Preparation

1- Prepare as instructed by the manufacturer. Dispense the medium in 7 ml amounts in screw-cap bottles of 9 ml capacity (large size Bijou bottles).

2- Sterilize by steaming (with caps loosened) for 15 minutes. When cool, tighten the bottle caps. Label the bottles.

3- Date the medium and give it a batch number. Record the expiry date (6 months from preparation) on each bottle.

4- Store in a cool dark place with the bottle tops screwed tightly.

pH of medium: 8.3–8.5 at room temperature.

7-Mannitol salt agar

Contents

Peptone, *Lab-Lemco* powder, mannitol, sodium chloride, phenol red, agar.

Preparation

1- Prepare the medium as instructed by the manufacturer. Sterilize by autoclaving at 121 C⁰ for 15 minutes.

2 -When the medium has cooled to 50–55 C⁰, mix well, and dispense it aseptically in sterile petri dishes. Date the medium and give it a batch number.

3- Store the plates at 2–8 C⁰ preferably in plastic bags to prevent loss of moisture.

pH of medium: 7.3–7.7 at room temperature.

8-nutrient broth

Contents

Peptone, *Lab-Lemco* powder, yeast extract, sodium Chloride.

Preparation

1-Prepare as instructed by the manufacturer. Sterilize by autoclaving at 121 C⁰ for 15 minutes.

2- Dispense aseptically in the required amounts. Date the medium and give it a batch number.

3- Store in a cool dark place. pH of medium: 7.2–7.6 at room temperature.

Xylose lysine deoxycholate (XLD) agar

Contents

Yeast extract, L-lysine HCl, xylose, lactose, sucrose, sodium deoxycholate, sodium chloride, sodium thiosulphate, ferric ammonium citrate, phenol red, agar.

Preparation

1- Prepare as instructed by the manufacturer. Heat the medium with care and do not over heat or autoclave.

2 -As soon as the medium has cooled to about 55⁰C, mix well, and dispense aseptically in sterile petri dishes.

3- Store the plates at 2–8 ⁰C, preferably sealed in plastic bags to prevent loss of moisture.

pH of medium: 7.2–7.6 at room temperature.

E-Chemicals and reagents

1- Petroleum ether.

2- Sodium chloride (normal saline).

- 3- Methanol.
- 4- Oxidase reagent.
- 5- Kovac's reagent.
- 6- Mc ferland turbidity standard

Oxidase reagent

Contents

To prepare 10 ml:

Tetramethyl-p-phenylenediaminedihydrochloride.....	0.1 g
Distilled water.....	10 ml

Preparation

Dissolve the chemical in the D.W. this reagent should be prepared immediately before use because is unstable.

Kovac's reagent

Contents

To prepare 20 ml:

4-dimethylaminobenzaldehyde.....	1 g
Isoamylalcohol (3-methyl-1-butanol).....	15 ml
Concentrated hypochloric acid	5 ml

Preparation

Weight the dimethylaminobenzaldehyde, dissolve in the isoamylalcohol. Added concentrated hydrochloric acid and mix well. Transfer to clean brown bottle and store at 2-8C.

Mc ferland turbidity standard

Contents

Concentrated sulphric acid.....	1 ml
Dihydrate barium chloride.....	0.5 g
Distilled water.....	150 ml

Preparation

- 1- Prepare 1% (v/v) solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99 ml of water and mix well.
- 2- Prepare 1.175 % (w/v) solution of barium chloride by dissolving 2.35g of di-hydrate barium chloride ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) in 200ml of distilled water.
- 3- Add .5ml of barium chloride solution to 99.5 ml of sulphuric acid solution and mix.

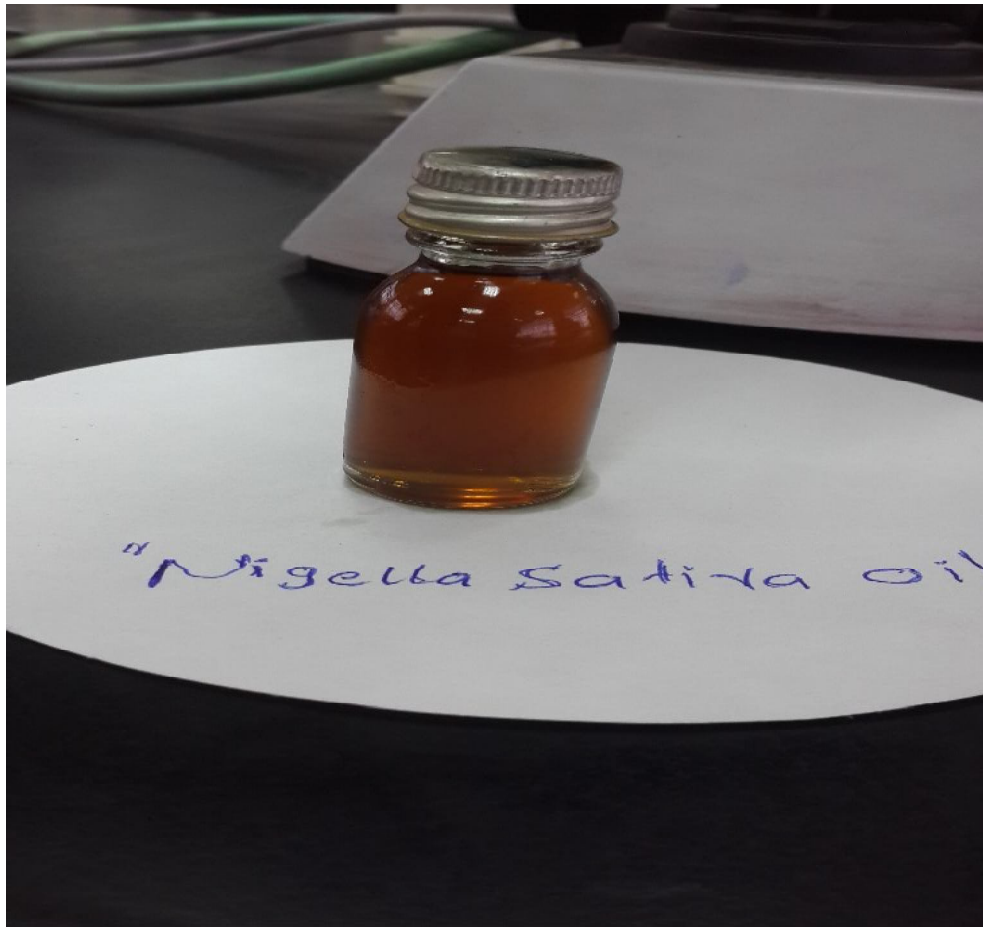
Appendix 2



Nigella sativa plant



Nigella sativa seeds



Petroleum ether extract of *Nigella sativa*



Methanol extract of *Nigella sativa*