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In vitro Assessment of Antimicrobial Activity of Honey bees And *Nigella sativa* Against Selected Clinical Isolates from Shendi City.

***A dissertation Submitted in Partial Fulfillment for the Requirement of Degree of M.Sc. in Medical Laboratory Science (Microbiology*)**

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الآيـــــة

* قال تعالى :-

(وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (68) * ثُمَّ كُلِي مِن كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِن بُطُونِهَا شَرَابٌ مُّخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِّلنَّاسِ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ(69).

صدق الله العظيم

(النحل الآية "69","68")

* وقال رسول الله صلى الله عليه وسلم :" عليكم بهذه الحبة السوداء فإن فيها شفاء من كل داء الا السأم" رواه ابو هريرة رضي الله عنه واخرجه البخاري في صحيحه".

Authentic saying of Prophet Mohammed (peace be upon him): use the black seed which is healing for all diseases except death" narrated by Abu Huraira (Allah Taala be pleased from him).

Dedication

This Project is dedicated to

To our teacher prophet Mohammed

Who is sent for all population as a mercy

To My mother

Who have given us endless love

To My father

Who have given us encouragement and strength

To My sisters and My brothers

Who have given us advices

To My colleagues and To My teachers

Who have supported us

And to every one

Has Participated in this project

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Last, but not least I would like to thank all my friends, and my colleagues for their unlimited support and strong advice throughout this study.

Abstract

Background: An alarming increase in bacterial strains resistant to existing antimicrobial agents demands a renewed effort to seek agents effective against pathogenic bacteria resistant to antimicrobials.

Objective: The aim of this study was to study the *in vitro* antimicrobial activity of different concentrations of Honey, petroleum ether extracts of medicinal plant *Nigella sativa* (seeds) and the mixture using cup-plate agar diffusion method.

Methodology: This is a prospective cross sectional study that in Shendi city, Sudan, during the period from March to June 2018, at Microbiology laboratory, Faculty of Medical Laboratory Sciences.

One hundred samples were collected form urine, wound، ear and eye swab, 95/100 (95%) showed bacterial growth, from which six types of pathogenic bacteria were isolated and identified using Gram stain and biochemical reactions.

Results: Out of the total 95 clinical and standard specimens were confirmed as *Staphylococcus aureus* 20(21%), *Escherichia coli* 20(21%), *Pseudomonas aeruginosa* 15(16%), and *Klebsiella pneumoneae* 30 (31%). In addition to *Candida albicans* 10 (11%). Petroleum ether extract of *Nigella sativa* and Honey showed pronounced dose dependant antimicrobial activity on standard strains and clinical isolates, while methanolic extract of *Nigella sativa* showed no activity.

Conclusions: The activity of *Nigella sativa*, honey, and the mixture exhibited a high antimicrobial activity against all types of tested organisms both clinical and standard organisms. Therefore *Nigella sativa*, honey, and the mixture can be regarded as a broad spectrum antimicrobial agent.

Modified diffusion technique was selected to conduct our research; because some trials used disc diffusion technique and proved that discs were source of contamination.

الخلاصة

الملخص: الزيادة المقلقة في سلالات البكتريا المقاومة للمضادات البكتيرية الحالية تتطلب جهدا متجددا للحصول علي عناصر فعالة ضد البكتريا الممرضة والمقاومة للمضادات البكتيرية.

الهدف: تهدف هذه الدراسة على اختبار فعالية العسل ومستخلص الأثير البترولي للنبات الطبي الكمون الاسود (البذور) والخليط بينهما، بتركيز مختلفة خارج جسم الإنسان أنجزت بإستخدام طريقة إنتشال الكأس الطبقي.

المنهجية: هذه الدراسة وصفية احتمالية أجريت في مدينة شندي ، السودان ، خلال الفترة من مارس إلى يونيو 2018 في معمل الأحياء الدقيقة بكلية علوم المختبرات الطبية .

تم جمع مائة عينة من البول والجرح والأذن ومسحة العين ، وأظهرت خمسة وتسعون منها نمو بكتيري (95%) 95/100، عزلت منها ستة أنواع من البكتريا الممرضة ، وتم التعرف عليها باستخدام صبغة الجرام والتفاعلات الكيمائية الحيوية بالطرق الموصوفة في مكى ومكارتيني.

النتيجة: من بين 95 من البكتريا المعزولة والقياسية هي العنقودية الذهبية 20 (21%) والشريكية القولونية 20(21%)، والزائفة الزنجارية 15(16%)، والكلبسيلة الرئوية 30(31%)، وأنواع من الفطريات المبيضة البيضاء 10(11%). مستخلص ايثر البترول للكمون الأسود والعسل أظهر فعالية ملحوظة حسب الجرعة المستخدمة ضد السلالات القياسية والمعزولة من العينات طبية ، بينما مستخلص الميثانول للكمون الاسود لم يظهر فعالية. ومن كل تلك النتائج السابقة يمكن اعتبار أن العسل والكمون الأسود لديه فعالية واسعة على مختلف أنواع البكتريا السريرية سالبة وموجبة الجرام.

الاستنتاجات: زيت الحبة السوداء والعسل والخليط لديهم فاعلية ضد كل أنواع الميكروبات من العينات الإكلينيكية والقياسية .

في هذا البحث تم اختيار تقنيه إنتشال الكأس الطبقي المعدلة ، لأن بعض التجارب استخدمت تقنية انتشار القرص وأثبتت أن الأقراص عرضة للتلوث.

List of contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Explanation
ATCC	American Type Culture Collection
C. albicans	*Candida albicans*
C.L.E.D	Cystine Lysine Electrolyte Deficient
CDC	CDC Centers for Disease Control and Prevention
D.W	Distilled water
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
K .pneumoniae	*Klebsiella pneumoniae*
KIA	Kligler Ion Agar
MBC	Minimum Bactericidal Concentration
MDIZ	Mean diameter of growth inhibition zone MDIZ
MIC	Minimum Inhibitory Concentration
Ml	Milliliter
Mm	Millimeter
N.S	*Nigella sativa*
N.S	Normal saline
NCTC	National Collection of Typing Culture
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
UTI	Urinary Tract Infection

Chapter One

Introduction
Rationale
Objectives

CHAPTER ONE

1.Introduction

1.1. Introduction

Microbial infection is major public health problem in both developed and developing countries. Due to the misuse of antibiotics "used to treat these infections" the incidence of multiple antibiotic resistance among human pathogens is increasing. All this beside the undesirable side effects of antibiotics have forced the scientists to search for new antimicrobial substances from natural sources (WHO, 2016).

Antimicrobial resistance is the ability of microorganisms to resist the effect of drugs leading to resistant infections, which may kill, can spread to other and imposes huge costs to individual and society. Misuse of antibiotics is the most important factor leading to antibiotic resistant around the world (Cassir *et al*., 2014).

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).

Traditional medicine is defined as the health practices, approaches, knowledge and believes incorporating plant, animal and animal based medicines, spiritual therapies, manual technique and exercises, applied singularly or in combination to prevent, diagnose and treat illnesses (WHO,2016).

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).

Since old ancients honey has been used traditionally for the treatment of many diseases including wound infections, respiratory tract infections, urogenital tract infections and many others infections. In this study we want to prove the antibacterial activity of bees honey scientifically using reference laboratory techniques (Cheesbrough, 1991).

The present study aimed to screen and evaluate the antimicrobial activity of Honey bees and *Nigella sativa* against selected clinical isolates from shendi city.

1.2 Rationale

Recently, modern societies face serious problems with using of the synthetic chemotherapeutic agents, in order to their multiple disadvantages such as harmful side effects, high cost and development of multi-resistant due to recurrent usage. So traditional medication started to play an important role as safer and cheaper alternative solution. *Nigella sativa* and honey are have already been cited in elsunna and holly Quran, therefore our study addresses this issue. 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).

In Sudanese culture and as part of the traditional medication, honey is used for treatment of respiratory tract infections and gastrointestinal tract infections so that it is interest to study antibacterial and antifungal activity against common pathogenic organisms in Sudan.

1.3 Objectives

1.3.1 General Objective

To determine the Antimicrobial Activity of Honey bees And *Nigella sativa* Against Selected Clinical Isolates from Shendi City.

1.3.2 Specific Objectives

1- To isolate and identify the pathogenic bacteria from different clinical specimens in patients from Shendi City.
2- To determine the most active extract against the standard strains in order to be used against clinical isolates.
3- To assess synergistic activity of *N. sativa* and Honey bees against tested organisms.

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 (Shelef, 1983).

Olderly: the pharmacological treatment of disease began long time ago with the use of herbs. People did not know why something worked, they just knew what worked. When something worked, it was written down and considered medical knowledge that was passed on from generation to generation (Fahd and Toufic, 1996).

Presently: It has been estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care; where plant based systems still play a vital role in health care. In developed countries, plant drugs are also extremely important, currently at least 119 chemicals derived from plant species can be considered as important drugs in use (Mullholland, 2000).

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

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*:

Black cumin seed (*Nigella sativa*) is annual herbaceous plant and a member of the Rananculacea (buttercup) family. Among various medicinal plants *Nigella sativa* is emerging as miracle herb with rich historical and religious background since many researches revealed its wide spectrum of pharmacological potential. *Nigella sativa* is native to Southern Europe, North Africa and Southwest Asia and it is cultivated in mediterranean region ,South Europe, India , Pakistan , Syria , Turkey, Saudi Arabia.

The largest producers of black cumin seed are Egypt, India, Pakistan, Iran, Iraq, and Turkey. Other species, such as Turkish black cumin (Nigella damascena),Are not used medicinally and one type, Nigella garidella, is even poisonous (Khare, 2004).

2.2.1 Botanical description

N. Sativa is an annual flowering plant which grows to 20-90 cm length, with finely divided leaves, the leaf segments narrowly linear to threadlike. The flowers are delicate, and usually colored white, yellow, pink, pale blue or pale purple, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds(Warrier and Nambiar, 1993).

2.2.2 Components

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).

2.2.3 Scientific classification

Kingdom: Plantae

Division: Magnolipghyta

Class: Magnoliopsida

Order: Ranunculales

Family: Ranunculaceae-Butter cup family

Genera: *Nigella*

Species: *Sativa*

Binomial name: *Nigella sativa*

2.2.4 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 ant-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), 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).

2.2.5 General uses in Herbal medicine

N. sativa is considered one of the greatest forms of healing medicine available, especially in the Arab countries 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 (Randhawa, 2008).

2.2.6 Antibacterial effects

The antibacterial effect of the ground black seeds was studied in modified paper disk diffusion method. Clear inhibition of the growth of *Staphylococcus aureus* was observed in concentration of 300mg/ml with distilled water as control. This inhibition was confirmed by using the positive control azithromycin. The positive inhibition may be attributed to the two active ingredients of the *N. sativa*, thymoquinone and melanin (Bakathir and Abbas, 2011).

2.2.7 Antifungal activity

Extracts of *Nigella sativa* have the strongest antifungal effect against different strains of *Candida albicans*. Aqueous extract showed no antifungal activity but have antidermatophytes activities against eight species of dermatophytes: four species of *Trichophyto nrubrum* and each of *T. interdigitale*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *microsporum canis* (Randhawa, 2008)..

2.2.8 Antiviral activity

Immune deficiency, (as in AIDS) predisposes to infections including viral infections, and blostering the immune systems may achieve viral control even without antiviral drugs. In view of multiple uses of *N. sativa* it was suspected that it's seed may have some stimulating effect on the immune system of human body and was found to enhance helper T cell (CD4) and cytotoxic T cell (CD8) ratio and increased natural killer (NK) cell activity in healthy volunteers. Besides improvement of immunity, *Nigella sativa* extract had some inhibitory effect on human immune deficiency virus (HIV) protease but the active principle(s) responsible for this activity was not identified. Moreover, *N. sativa* oil when given intra-peritoneally to mice infected with murine cytomegalovirus for 10 days the virus was undetectable in the liver and spleen, while it was still detectable in the control mice. This action was possibly related to increase in number and function of M-phi and $CD4^{+}$Tcells and increased production of INF-gamma (Randhawa, 2008).

2.3 Honey

2.3.1 Bees

Bees are flying insects closely related to wasps and ants, and are known for their role in pollination and for producing honey and beeswax. Bees are amonophyletic lineage within the superfamily Apoidea, presently classified by the unranked taxon name Anthophila. There are nearly 20,000 known species of bees in seven to nine recognized families. Although many are undescribed and the actual number is probably higher (Danforth *et al*., 2006).

2.3.2 Scientific Classification (Michael *et al.*, 2009)

Table 1.Scientific classification of bees

Scientific classification		Families
Kingdom:	*Animalia*	*Andrenidea* *Apidea* *Colletidea* *Dasypodaidea* *Halictidea* *Megachilidea* *Meganomiidea* *Melittidea* *Stenotritidae*
Phylum:	*Arthropoda*	
Class:	*Insecta*	
Order**:**	*Hymenopetra*	
Suborder**:**	*Apocrita*	
Superfamily:	*Apoidea*	
Series:	*Anthophila*	

2.3.3 Honey bees

Honey bees are a subset of bees in the genus *Apis*, primarily distinguished by the production and storage of honey and the construction of perennial, colonial nests out of wax. Honey bees are the only extant numbers of the tribe Apini, all in the genus *Apis*. Currently there are only seven recognized species of honey bees with a total of 44 subspecies; though historically, anywhere from six to eleven species has been recognized. Honey bees represent only a small fraction of the approximately 20000 known species of bees. Some other types of related bees produce and store honey, but only members of the genus *Apis* are true honey bees (Michael *et al.*, 2009).

2.3.3.1 Types of honey bees

Honey bees, like all other living things, vary among themselves in traits such as temperament, disease resistance, and productivity. The environment has a large effect on differences among bees colonies (for example, plants in different areas yield different honey crops), but the genetic makeup of a colony can also impact the characteristics that define a particular group.

2.3.3.2 Origin and distribution

Honey bees are a group that appeared to have their center of origin in South and South East Asia (including the Philippines).The first *Apis* bees appeared in the fossil record at the Eocene-Oligocene boundary, in European deposits. The origin of these historic honey bees does not necessarily indicate that Europe is where the genus is originated, only that it occurred there at that time. There are few known fossil deposits in South Asia, the suspected region of honey bees' origin, and fewer still have been thoroughly studied (Smith *et al.*, 2000).

2.3.3.3 Honey production

Firstly, the foraging bees collect nectar from flowers using tube like structure called proboscis. In the bees stomach the nectar metabolized by certain enzymes such as amylase and glucose oxidase. The metabolized nectar then dropped into the bee wax comb and finally converted into thickened honey after being evaporated by the bees's wing (Subramanian *et al*., 2007).

2.3.3.4 Table 2Chemical composition of honey(Subramanian *et al*., 2007).

Nutrient:	**Amount in 100g of honey**
Water	17.1g
Carbohydrates(total)	82.4g
• Fructose	38.5g
• Glucose	31.0g
• Maltose	7.20g
• Sucrose	1.50g
Proteins, amino acids, vitamins and minerals	0.50g
Energy	304 kcal
Vitamins	**Amount in 100g of honey**
• Thiamin	0.006mg
• Riboflavin	0.06mg
• Niacin	0.36 mg
• Pantothenic	0.11mg

• Pyridoxine (B6)	0.32 mg
• Ascorbic Acid (c)	2.4-2.2 mg
Minerals	**Amount in 100gof honey**
• Calcium	4.4-9.20 mg
• Copper	0.003-0.10 mg
• Iron	0.06-1.5 mg
• Magnesium	1.2-3.50 mg
• Manganese	0.02-0.4 mg
• Phosphorus	1.9-6.30 mg
• Potassium	13.2-16.8 mg
• Sodium	0.0-7.60 mg
• Zinc	0.03-0.4 mg

2.3.3.5 Uses of honey

Honey has many benefits in all body systems and has long medicinal history. The ancient Egyptians not only made offering of honey to their goals, they also used it as embalming fluid and dressing of wound. Antibacterial in laboratory honey has been shown to hamper the growth of food borne pathogens such as *E. coli*, *Salmonella* and to fight certain bacteria, including *S. aureus* and *pseudomonas aeruginosa,* both of which are common in hospital and dressing for wounds. Today many people use honey for its anti-inflammatory properties. Outside the laboratory it is also used as cough suppression. Honey is used in wound healing and as alternative treatment for clinical conditions ranging from GIT problems to ophthalmic conditions(Usama *et al.*, 2012).

2.3.3.6 Certain Precautions of honey

Although there is healing in honey for a variety of medical disorders, certain precautions should be taken for use:

1. Children under the age of one year should not be given honey due to the possibility of infant botulism. This type Of food poisoning can be deadly, however, it only seems to affect infants under one year of age.

2. If you have any known allergies to specific plants, then you should make sure the honey you are using is not produced from that plant.

3. People with allergies to bee stings should be careful when using other bee-related products such as propolis or Royal jelly(Usama *et al.,* 2012).

Many researches to date have addressed honey and *Nigella sativa* antibacterial properties and their effects on many infections. The following Laboratory studies and clinical trials have shown that *honey* and *Nigella sativa* are effective broad-spectrum antibacterial agent.

In a study in 1974, the antimicrobial activity of the volatile oil of *Nigella sativa Linnaeus* seeds was investigated and antimicrobial principle has been isolated and identified as thymohydroquinone, and found to be active against Gram-positive bacteria and yeast (Toama *et al.,* 1974). Another study in 1989 the antifungal activity of *Nigella sativa* oil was studied and found to have significant activity against fungi (Islam *et al.,* 1989). In another study 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, nalidxic acid, ampicillin, and lincomycin and sulphamethoxyzole-trimethoprim combination (Hanafy and Hatem, 1991).

In an *in vitro* study in 1996 on antimicrobial effects of Saudi *Nigella sativa* whole seeds and oil, two hundreds and seventy six organisms, 260 multiple resistant clinical isolates and 12 control strains obtained from the American Type Culture

Collection Control (ATCC) and Center for Disease Control and prevention (CDC).Cultures were tested against *Nigella sativa* crude oils and whole seeds. They found that both crude oils and whole seeds and whole seeds exhibited fairly good activity against most of Gram positive bacteria and some Gram negative ones, the highest activity was found with methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, Beta and Alpha haemolytic, pathogenic *Neisseria*, *Haemophilus influanzae*, *Brucella melitensis,* and *Enterococcus faecalis*.In this study *Nigella sativa* was highly effective against Gram positive cocci compared to Gram negative bacilli (Bilal *et al.,* 1996).

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 the year 2003, the *in vivo* antifungal activity of *Nigella sativa* aqueous extract on candiasis in mice was studied and found that the extract exhibits effect against candidiasis (Khan *et al.,* 2003). In a 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). In a study in 2005 the antimicrobial activity of the volatile oil of *Nigella sativa Linn* seed oil against fifteen pathogenic microbial strains including 3 Gram positive, 11 Gram negative and *C .albicans* was studied and found that the volatile oil showed strong activity against all organisms (Ara *et al.,* 2005). In another study in 2005, the antifungal activity of ether extract of *Nigella sativa* seed and its active principle thymoquinone against eight species of *Dermatophytes* ,four species of *Trichophyton rubrum* and one each of *Trichophyton interdigitale, Trichophyton mentagrophytes, Epidermophyton floccosum* and *Microsporum canis* were studied and found to have strong activity (Aljabre et al., 2005).

In anthor 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).

In another study in 2008, the antimicrobial activity of *Nigella sativa* methanolic extract against *S. aureus, P. aeruginsa, Klebsilla pneumoniae (K. pneumoniae), E. coli* and *Bacillus cereus* (*B. cereus*) was investigated and found that the extract was active against these bacteria including ESBL producing strains of (E. coli, *K. pneumoniae* and *B. cereus*) and multidrug-resistant *S. aureus* and *P. aeruginosa* (Zuridah et al., 2008).

Another study 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).

Another study 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).

In other study in 2008, the *in vitro* antimicrobial activity of *Kalonji, cumin,* and *poppy* seen against Gram positive and Gram negative isolates from oral cavity of healthy individuals was studied and found that the highest antibacterial potential was observed in aqueous extract of *Nigella sativa* (Chaudhry and Tariq, 2008).

In other study in 2008 the antimicrobial activity of *Nigella sativa* seed against multidrug resistant strains of coagulase negative *S. aureus* was studied and the methanolic extract showed remarkable dose dependent antibacterial activity up to dilution of 1:50 (Salman *et al.,* 2008). In another study in 2008, emerging quinolones resistant transfer genes among Gram negative bacteria isolated from faeces of HIV/ AIDS patient was detected (EO and NO, 2006).

In another study in 2009, the antimicrobial activity of seed oil and extract against multidrug resistant clinical strains of *P. aeruginosa* tested in varying dilutions was studied, both oil and methanolic extract showed remarkable dose dependent antibacterial activity **(**Salman *et al*., 2009).

A study in 2005 was done and found that the growth of all coagulase negative *staphylococcus* isolate was inhibited by manuka and pasutuer honey at concentrations of 2.7-5% (v/v) (French *et al*., 2005).

The activity of honey on *Helicobacter pylori* was studied in 2006 proving that all honey tested had inhibitory action against *Helicobacter pylori* (Basil *et al*., 2006).

In 2007 a study was made by, John J. and randy W. Worobo on the antimicrobial activity of different floral sources of honey against bacterial isolates, the results showed that 92.5% of bacteria was inhibited by honey (Hyungjaer *et al*., 2007).

In 2007 an in vitro study of the effectiveness of honey dressing for healing pressure ulcers showed that after 5 weeks of treatment by honey, patients were completely healed (Yupucu Gunes *et al.*, 2007).

While in 2009 study under the title of the effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms showed that 100% of isolates were effectively inhibited by honey (Alandejani *et al*., 2009).

While in 2010 another study on the antibacterial properties of honey and its effect in wound management was made resulting in that both Gram-negative bacteria isolated were completely inhibited by the honey tested (Nur Azida *et al*., 2010).

In 2013 a study aimed to determine the factors (phenolic compounds, flavonoids, sugar or H_2O_2) that contribute the most to the antimicrobial activity of heather honey samples against four bacteria "*B. cereus*, *S. aureus*, *E. coli* & *P. aeruginosa*, and yeasts *C. krusei*, *C. neoformans*, *C. famat* and *C. albicans*, found that all

microorganisms were inhibited by honey, yeasts were more resistant than bacteria (Feás *et al.*, 2013).

Another study in 2013 found that local Sider & mountain Saudi honey were effective in inhibiting the *in vitro* growth of *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii.* Sider honey was more potent than mountain honey in inhibiting these bacterial growth *in vitro* and both honey samples in the different concentrations were more effective against *E. coli* than other bacteria (Alqurashi *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:

Different hospitals and clinical centers located in Shendi locality, River Nile State, Sudan. Shendi is a town in northern Sudan on the east bank of the River Nile 150 km northeast of Khartoum (16°41'N 33°25'E).

3.3 Study population:

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

3.4 Inclusion criteria:

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

3.5 Exclusion criteria

Patients under antimicrobial treatment were excluded.

3.6 Sampling:

None - Probability Sampling.

3.7 Sample size:

A total of one hundred samples (n=100)were collected.

3.8 Scientific & Ethical considerations

Permission was issued by Collage of Ethical Committee, Shendi University and ethical committee of hospital. Volunteers were informed and had got all the information about the research study**(Appendix I)**.

3.9 Data collection

Data were collected from the patients using structural questionnaire containing all study variables **(Appendix I).**

3.10 Specimen processing

3.10.1 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.

3.10.2 Cultivation of the specimens

Different types of culture media (CLED agar, Blood agar, Macconckey Agar and Chocolate blood Agar) were used for identification and isolation of clinical isolates **(Appendix II).**

3.10.3 Interpretation of culture growth

The plates were examined for any significant bacterial growth. The isolated bacteria were then identified by colonial morphology, Gram stain and biochemical tests.

3.10.4 Identification of the isolates

The isolated organisms were fully identified by Gram stain and the appropriate followed by performing biochemical tests. These include:

3.10.4.1 Gram stain

Dispense three drops of sterile normal saline on a clean dry glass slide using sterile wire loop.

From pure culture of the tested organism touch one colony by sterile wire loop, mix with normal saline and spread it evenly on an area of about 15-20 mm.

Fix the dried smear by heating using the flame.

flood the fixed smear with crystal violet stain for 1 minute.

Rapidly wash off the stain with clean tab water.

flood the smear with Lugol’s iodine for 1 minute, then wash.

Decolorize rapidly (few seconds) with alcohol, then wash.

flood the smear with safranine stain for 2 minutes.

Let the smear air dry, add drop of immersion oil and examine the smear microscopically using X100 (Cheesbrough, 2006).

3.10.4.2 Biochemical tests

3.10.4.2.1 *Catalase test*

-**Required:** Hydrogen peroxide (3% H2O2) and wooden stick.

-**Principle:** Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer.

-**Methods:** Pour 2–3 ml of the hydrogen peroxide solution into a test tube, using a sterile wooden stick or a glass rod, remove a good growth of the test organism and several immerse it in the hydrogen peroxide solution and Look for immediate bubbling (Cheesbrough, 2006).

3.10.4.2.2 *Coagulase test*

-**Required:** undiluted human plasma, slide and physiological saline.

-**Principle:** Coagulase causes plasma to clot by converting fibrinogen to fibrin. Two types of coagulase are produced by most strains of Staphylococcus aureus:

a)Free coagulase which converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma.

b)Bound coagulase (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase-reacting factor. It can be detected by the clumping of bacterial cells in the rapid slide test.

-**Method:** Place a drop of physiological saline on each end of slide, emulsify a colony of the test organism in each of the drops to make two thick suspensions and add a drop of the plasma to one of the suspensions and mix gently by rotating. Look for clumping of the organisms within 10 seconds (Cheesbrough, 2006).

3.10.4.2.3 ***Deoxyribonuclease (DNAse) test***

-**Required:** DNAse agar plate, 1 ml of Hydrochloric acid (1% HCL) was added.

-**Principle:** Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA), the acid precipitate unhydrolyze DNA. DNAse producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

-**Procedure:** 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 precipitates unhydrolyzed DNA. DNAse producing colonies are therefore surrounded by clear areas due to DNA hydrolysis(Cheesbrough, 2006).

3.10.4.2.4 ***Mannitol Salt Agar (MSA)***

This type of medium is both selective and differential. The MSA will select for organisms such as Staphylococcus species which can live in areas of high salt concentration (Cheesbrough, 2006).

3.10.4.2.5 ***Novobiocin Disk***

Staph. aureus is susceptible to novobiocin antibiotics. The test were conducted according to McCartney et al., (2002) by inoculated the target microorganism in Mueller-Hinton agar (Hi-Media, India). Inhibition zones were measured after 24h and as recommended by the National Committee for Clinical Labrotary Standards (NCCL, 2000).

3.10.4.2.6 ***Oxidase test(Cytochrome oxidase test)***

-**Required:** filter paper impregnated with oxidase reagent (tetra methyl para phenylenediaminedihydrochochloride), clean slide and wooden stick.

-**Principle:** It the organism is an oxidase producer, the paraphenylenediamine hydrochloride in the reagent will be oxidized to a deep purple colour.

-**Procedure:** a piece of filter paper in a placed on a clean glass slide and three to four drops of freshly prepared oxidase reagent were added using sterile Pasteur pipette, wooden stick was used to pick a colony of the test organism and placed on the filter paper (Cheesbrough, 2006).

3.10.4.2.7 ***Indole test***

-Required: sterile peptone water in small test tube, Kovac's reagent, wire loop and Pasteur pipette.

-**Principle:** organism having tryptophase enzyme can breakdown amino acid tryptophan to produce Indole as an end product and is detected by adding Kovac's reagent.

-**Procedure**: the tested colony is inoculated in sterile peptone water using sterile wire loop and then incubated at 37oC aerobically overnight. Few drops of kovac's reagent were added to the medium using Pasteur pipette(Cheesbrough, 2006).

3.10.4.2.8 ***Citrate utilization test***

-**Required:** Simmon's citrate agar slope medium and straight loop.

-**Principle**: an organism when culture in medium containing citrate as only source of carbon, it will utilize citrate and give ammonia which change the PH and is detected by change in the colour of indicator in the medium.

-**Procedure:** a small part of the tested colony is picked off using sterile straight loop and inoculated on the surface of the slope of the medium in a zigzag manner, and then incubated at 23Co aerobically overnight(Cheesbrough, 2006).

3.10.4.2.9 ***Urease test***

-Required: Christensen's urea agar medium and straight loop.

Principle: if the organism is urease producer, it will breakdown urea to ammonia and carbon dioxide which is indicated by the change in the colour of indicator.

-Procedure: the tested colony is inoculated on the surface of the slope medium by sterile straight loop in zigzagging manner and then incubated overnight at 37 Co aerobically.

-Results: positive reaction is indicated by the colour change in the indicator (phenol red) to pink colour. Negative reaction is indicated by no change in the colour (Cheesbrough, 2006).

3.10.4.2.10 ***Motility testing:***

-Required: semisolid media and straight loop.

-Principle: organism is motile, it will spread easily within the semisolid.

-Method: the tested colony is taken by a sterile straight loop, and inoculated by stabbing the media, then incubated aerobically at 37 Co overnight (Cheesbrough,2006).

3.10.4.2.11 ***KIA (Kliger Iron Agar):***

-**Required:** KIA slope medium and a straight loop.

-**Principle:** KIA reactions are based on the fermentation of lactose and glucose and the production of hydrogen sulphide (H2S). The fermentation is indicated by colour change of the media to yellow colour due to the phenol red indicator in the media.

H2S is produced when sulphur-containing amino acid are decomposed. It is detected by production of black colour due to ferric citrate indictor in the medium.

-**Method:** 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 the incubate at 37oC aerobically overnight (Cheesbrough,2006).

3.10.5 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 4^0C.

3.11 Extraction of ***Nigella sativa***

3.11.1 Collection and Preparation of ***Nigella Sativa*** **specimen**

Specimen of *Nigella sativa* was obtained from a local supermarket in Shendi city "alteman atara". The dried *Nigella sativa* sample was cleaned from dust and grass**(Appendix II)**.

3.11.2 Preparation of serial dilution of honey and *Nigella Sativa* extracts For Testing the Antimicrobial activity.

The crude extract of *Nigella sativa* and honey was diluted into different concentrations as follows: 100%, 50%, 25%, and 12.5% to be used against the selected organisms **(Appendix II)**.

3.11.3 Preparation of standard bacterial suspension

Clinical isolates were isolated from different samples in sterile slope of nutrient and standard bacteria were brought from microbiology department of National Institute for Research.

Ten ml of normal saline were distributed in test tubes and sterilized in autoclave at 121^{o}C for 15 mins. A loopfull of purified bacterium was inoculated in sterile normal saline. Inoculum density was compared with McFarland standard solution.

3.11.4 Testing of extracts for antimicrobial activity against standard organisms and clinical isolates

3.11.4.1 Cup- plate ager diffusion (inhibition zone) method

Cup plate agar diffusion method was adopted with some minor modification to assess the antimicrobial 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 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 *Nigella sativa* oil, Honey and the mixture (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^{o}C for 18 hours.

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).

3.12 Data analysis

Data were entered, check and analyzed using Microsoft Excel 2007and SPSS (Statistical Package of Social Science) soft program version 11.5.

Proportional data were presented as frequencies and percentages.

Chapter Four

Results

CHAPTER FOUR

4.Results

A total of 100 patients with symptoms of urinary tract infection, wound infection, Ear and Eye problems were enrolled in this study during the period from March to June 2018.

4.1 Distribution of clinical samples according to age group

Out of 100 specimens, 31 samples were taken from patients of with mean age +<20 age group, 42 from 21-40 age group, 15 from 41-60 age group, 9 from 61-80 age group and 3 from >80 age group as shown in table 1.The age grouped in to five grouped majority samples taken from age group 21-40 Year (Table3).

Table (3) Distribution of clinical samples according to age group

Age group	Frequency	Percentage
< 20	31	31%
21-40	42	42%
41-60	15	15%
16-80	9	9%
>80	3	3%
Total	100	100%

4.2 Distribution of clinical samples according to the gender

Out of 100 specimens, 53 (53%) were from males and 47 (47%) were from females (Table 5).

Table (4) Distribution of clinical specimens according to the gender

Gender	Frequency	Percentage
Male	57	57%
Female	43	43%
Total	100	100%

4.3 Biochemical identification

4.3.1 Biochemical reaction for Gram positive pathogens

Table (5)The results of the biochemical reactions for Gram postive pathogens

Biochemical Tests / Pathogen	Catalase	DNase	Coaqulase	Mannitol	Lactose fermentation
S.aureus	+	+	+	+	+

4.3.2 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_2S**	**Oxidase**
E.coli	+	–	–	Y/Y	+	–	–
K.pneumoniae	–	–	+	Y/Y	+	–	–
p. mirabilis	–	+	+	R/Y	+	+	–
P. aeruginosa	–	+	+	R/R	–	–	+

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.

4.3.3 Frequency and percentage of isolated organisms

In this study six types of bacteria were isolated (Table 7). The isolated bacteria were *K.pneumoniae* 30/95(31%), *E.coli* 20/95(21%), *S.aureus* 21/95(21%), *Ps.aeruginosa* 15/95(16%), In addition to *Candida albicans*10 (11%). as shown in table 7.

Table (7)Frequency and percentage of isolated organisms

Isolate	**Frequency**	**Percentage**
K.pneumoniae	30	31%
E.coli	20	21%
S.aureus	20	21%
P. aeruginosa	15	16%
Candida albicans	10	11%
Total	95	100%

4.4.1 The activity of *Nigella sativa*, honey and the mixture against *E. coli*.

Out of 20 strains tested. the oil extract of *N. sativa* exhibited showed (90%) activity, Honey which showed low activity when compared with *Nigella sativa* oil (83%), In contrast with *N. sativa* oil and honey the mixture exhibited the highest activity, it showed (100%) against *E. coli* (Table 8 and Figure 1).

Table (8) Antimicrobial activity of *Nigella sativa*, honey and the mixture against twenty *E. coli*.

Concentration	*Nigella Sativa*		Honey		Mixture	
	S	R	S	R	S	R
100%	19	1	20	0	20	0
50%	19	1	20	0	20	0
25%	19	1	19	1	20	0
12.5%	15	5	8	12	20	0
Percentage	90%	10%	83%	16%	100%	

R= resistant.

S=susceptible.

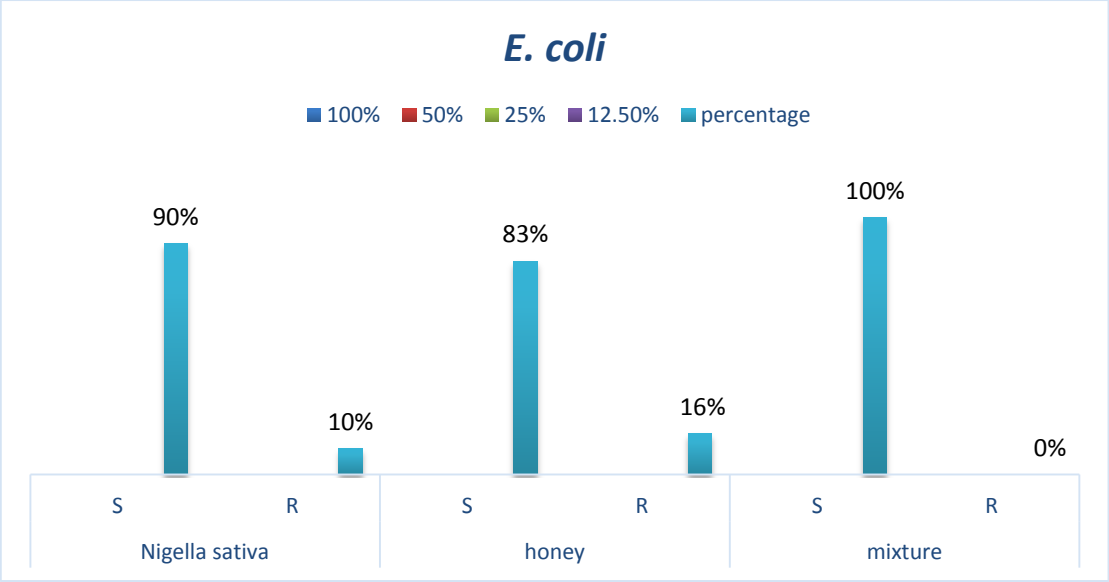

Figure (1) Percentage of extracts activity against *E. coli*.

4.4.2 The activity of *Nigella sativa*, honey and the mixture against *S. aureus*.

Out of 20 strains tested. the oil extract of *N. sativa* exhibited showed activity(100%), Honey which showed low activity when compared with *Nigella sativa* oil (25%), In contrast with *N. sativa* oil and honey the mixture exhibited the highest activity, it showed (100%) against *S. aureus.* (Table 9 and Figure 2).

Table (9) Antimicrobial activity of *Nigella sativa*, honey, and mixture against twenty *S. aureus*.

Concentration	*Nigella Sativa*		Honey		Mixture	
	S	R	S	R	S	R
100%	20	0	20	0	20	0
50%	20	0	0	20	20	0
25%	20	0	0	20	20	0
12.5%	20	0	0	20	20	0
Percentage	100%		25%	75%	100%	

R= resistant.

S=susceptible.

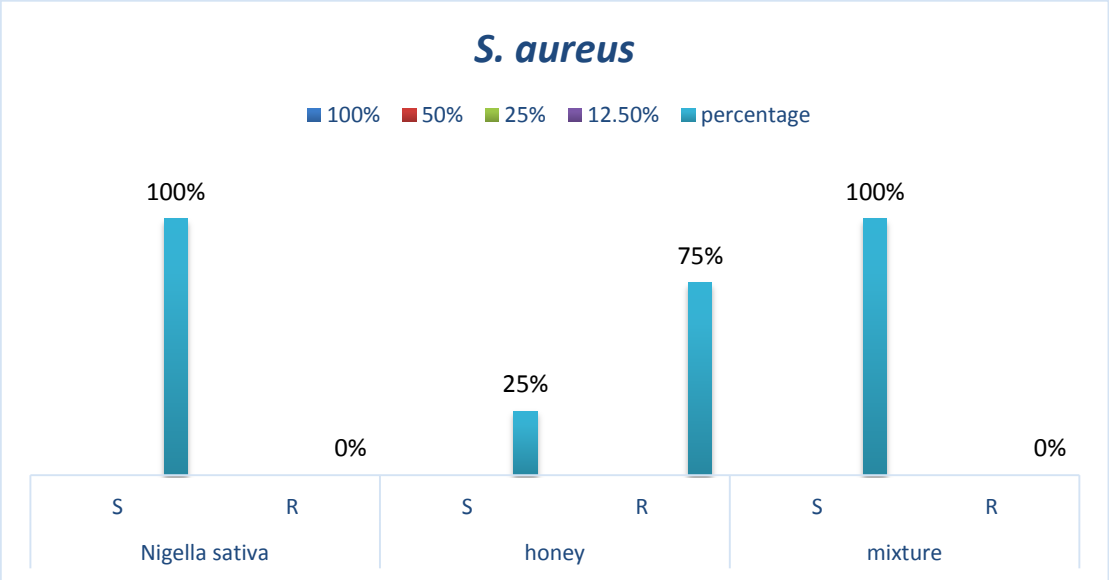

Figure (2) Percentage of extracts activity against *S. aureus*.

4.4.3 The activity of *Nigella sativa*, honey and the mixture against *K. pneumonae.*

Out of 30 strains tested, the oil extract of *N. sativa* exhibited showed activity(66%), Honey which showed low activity when compared with *Nigella sativa* oil (25%), In contrast with *N. sativa* oil and honey the mixture exhibited the highest activity, it showed (72%) against *K. pneumonae.* (Table 10 and Figure 3).

Table (10) Antimicrobial activity of *Nigella sativa*, honey and mixture against thirty *K. pneumonae.*

Concentration	*Nigella Sativa*		Honey		Mixture	
	S	R	S	R	S	R
100%	30	0	30	0	30	0
50%	29	1	0	30	27	3
25%	20	10	0	30	20	10
12.5%	0	30	0	30	10	20
Percentage	66%	34%	25%	75%	72%	28%

R= resistant.
S=susceptible.

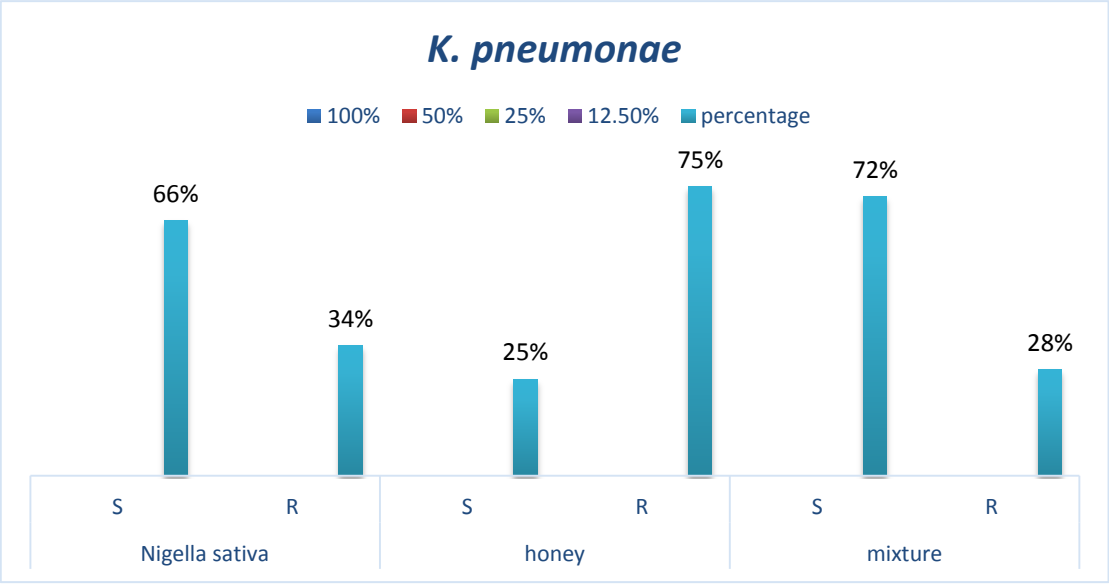

Figure (3) Percentage of extracts activity against *K. pneumonae.*

4.4.4 The activity of *Nigella sativa*, honey and the mixture against *P. aeruginosa*.

Out of 15 strains tested. the oil extract of *N. sativa* exhibited showed (88%) activity, Honey it showed the no activity when compared with *Nigella sativa* oil , In contrast with *N. sativa* oil and honey the mixture exhibited the highest activity, it showed (87%) against *P. aeruginosa*. (Table 11 and Figure 4).

Table(11) Antimicrobial activity of *Nigella sativa*, honey and the mixture against fifteen *P. aeruginosa*.

Concentration	*Nigella Sativa*		Honey		Mixture	
	S	R	S	R	S	R
100%	15	0	0	15	15	0
50%	14	1	0	15	14	1
25%	12	3	0	15	12	3
12.5%	12	3	0	15	11	4
Percentage	88%	12%	100%		87%	13%

R= resistant.

S=susceptible.

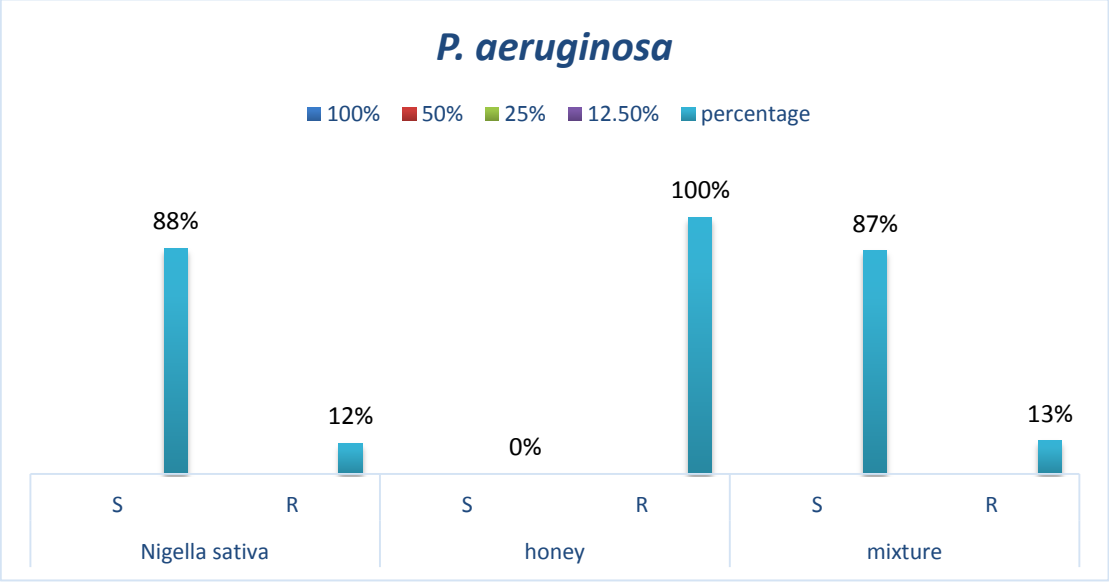

Figure (4) Percentage of extracts activity against *P. aeruginosa*.

4.4.5 The activity of *Nigella sativa*, honey and the mixture against *C. albicans*.

Out of 10 strains tested. the oil extract of *N. sativa* exhibited showed (100%) activity, Honey which showed low activity when compared with *Nigella sativa* oil (27%) , In contrast with *N. sativa* oil and honey the mixture exhibited the highest activity, it showed (100%) against *C. albicans*.(Table 12 and Figure 5).

Table (12) Antimicrobial activity of *Nigella sativa*, honey and the mixture against ten *C. albicans*.

Concentration	*Nigella Sativa*		Honey		Mixture	
	S	R	S	R	S	R
100%	10	0	10	0	10	0
50%	10	0	1	9	10	0
25%	10	0	0	10	10	0
12.5%	10	0	0	10	10	0
Percentage	100%		27%	73%	100%	

R= resistant.

S=susceptible.

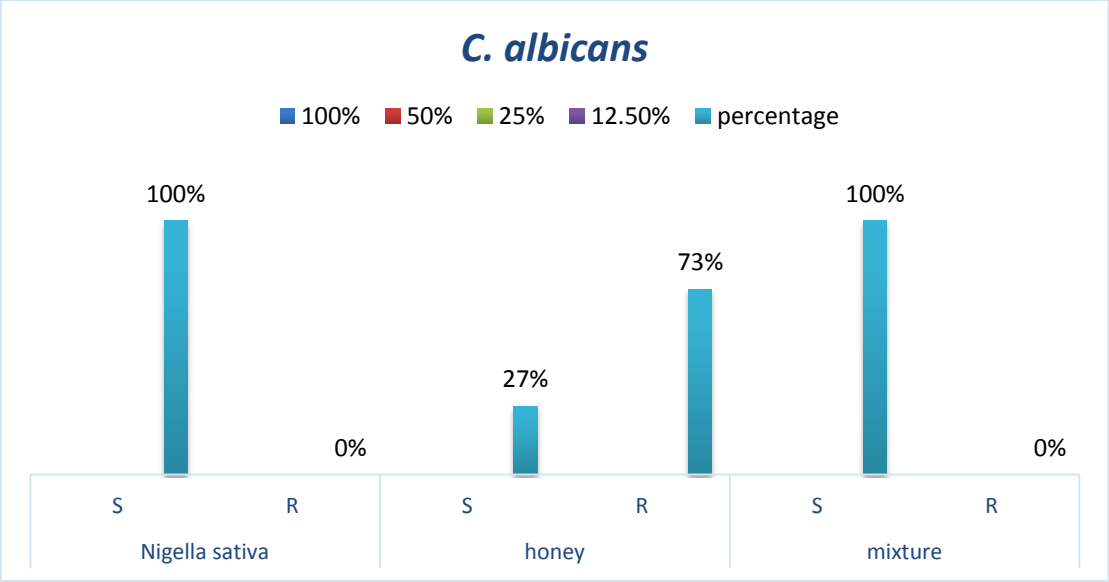

Figure (5) Percentage of extracts activity against *C. albicans*.

4.4.6 The mean diameter of growth inhibition zone (MDIZ) in millimeter (mm)of *Nigella sativa* oil ,honey and mixture against clinical isolates.

The highest mean diameter of the growth inhibition zone in mm (MDIZ) of *N. sativa* oil for *S. aureus* was 47.9mm at conccentration100%, while the lowest MDIZ was 39.8mm at concentration 12.5%. The highest MDIZ was 45.6mm for *C. albicans* at 100%, the lowest MDIZ was 21.5mm at 25%. The highest MDIZ for *P. aeruginosa* was 19.9mm at 100% the lowest MDIZ was 9.4mm at 12.5%. For *E. coli* the highest MDIZ was 17.2mm at 100%, while the lowest was 7mm at 12.5%. The highest MDIZ was 14.2mm at 100% for *K. pneumoniae*, while at concentration 12.5% showed no activity.

The highest MDIZ of honey for *E. coli* was 26.6mm at 100% while the lowest MDIZ was 12.1mm at 12.5%. The highest MDIZ was 19.7mm at 100% for *S. aureus* and the lowest MDIZ showed no activity at 12.5%.For *K. pneumonia* the highest MDIZ was 16.4mm at 100%, the lowest MDIZ showed no activity at 12.5%. The highest MDZI was 12.9mm at 100% for *C. albicans*, it showed no activity at 12.5%, the highest MDIZ was 4mm at 100% for *P. aeruginosa*, and it showed no activity at 12.5%.

The mixture of *N. sativa* and honey showed the highest MDIZ 46.5mm at 100% for *S. aureus*, the lowest MDIZ was 40.2 mm at 25%.For C .*albicans* the highest MDIZ was 41mm at 100%, the lowest MDIZ was 25.3mm at 12.5%. The highest MDIZ for *E. coli* was 28mm at 100%, the lowest MDIZ was 13.7mm at 12.5 % .For *P. aeruginosa* the highest MDIZ was 15.1mm at 100%, and the lowest MDIZ was 4.1mm at 12.5%, while *K. pneumoniae* showed MDIZ 11.7mm at 100% and the lowest MDIZ 3.6mm at 12.5 % (table 13).

Table (13) The mean diameter of growth inhibition zone (MDIZ) in millimeter (mm)of *Nigella sativa* oil ,honey and mixture against clinical isolates.

organisms	*Nigella sativa*				Honey				Mixture			
	100 %	*50 %*	*25 %*	*12.5 %*	*100 %*	*50 %*	*25 %*	*12.5 %*	*100 %*	*50 %*	*25 %*	*12.5 %*
E. coli	17.2	13.4	13.3	7.0	26.6	19.7	16.4	12.1	28	21.9	16.8	13,7
S. aureus	47.9	44.4	42.8	39.8	19.7	0	0	0	46.5	48.2	40.2	44.7
K. pneu[1]	14.2	11.6	7.6	0	16.4	0	0	0	11.7	9.7	4.7	3.6
P. aeru[2]	19.9	15.7	13.5	9.4	0	0	0	0	15.1	12.3	9.1	4.1
C. albic[3]	45.6	43.6	21.5	29	12.9	1.3	0	0	41	41	33.2	25.3

[1] *K. peumoniae.*

[2] *P. aeruginosa.*

[3] *C. albicans*

Interpretation

Mean diameter of growth inhibition zone (MDIZ) in (mm)

MDIZ≥ 12 highly susceptible.

MDIZ< 12 resistance.

4.4.7 The activity of *Nigella sativa*, honey and the mixture on standard strains.

The oil extract of *N. sativa*, honey and the mixture presented variable activity against standard strains *S. aureus* ATCC 25923,followed by *K. pneumoniae* ATCC 53657, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 7596, as shown in Table 14.

Table (14) Antimicrobial activity of *N. sativa*, honey and the mixture against standard organisms.

STD organisms	***Nigella Sativa***				**Honey**				**Mixture**			
	100 %	*50 %*	*25 %*	*12.5 %*	*100 %*	*50 %*	*25 %*	*12.5 %*	*100 %*	*50 %*	*25 %*	*12.5 %*
S. aureus	S	S	S	S	S	S	-	-	S	S	S	S
E. coli	S	S	S	S	S	-	-	-	S	S	S	S
K.peu[1]	S	S	S	R	S	-	-	-	S	S	S	-
P. aeru[2]	S	S	S	S	S	-	-	-	S	S	-	-
C.albi[3]	S	S	S	S	S	-	-	-	S	S	-	-

[1]*K. peumoniae*,

[2]*P. aeruginosa*,

[3]*C. albicans*

(-) No antimicrobial activity.

(S) Susceptible.

Chapter Five

Discussion
Conclusion
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 essential oils and extracts have been used for thousands of years, in food preservation, pharmaceutical, alternative medicine and natural therapies. Therefore, it is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of health care.

Essential oils are potentials sources of novel antimicrobial compounds especially against bacterial pathogens. *In vitro* studies in this work showed that the essential oils inhibited bacterial growth but in varying degrees of effect. In the present study, *N. sativa* oil, honey and the mixture exhibited activity against some of selected bacterial and fungal strains. The oil extract of *N. sativa* produced wide diameter of inhibition zone because it contains the major active substance **(thymohydroquinone)** required for antimicrobial activity, and the honey contains active component **(methylglyoxal)** which required for antimicrobial activity. The results showed that *N. sativa* oil has the highest activity against *S. aureus*, the lowest activity against *K .pneumoniae* .Honey showed the highest activity againsts *E. coli*, the lowest activity against *P. aeruginosa* .While the mixture showed highest activity against the selected clinical isolates when compared with *N. sativa* and honey, but the highest activity was against *S. aureus*, the lowest activity against *K. pneumoniae.* In this study petroleum ether extract of *Nigella sativa* showed remarkable antimicrobial activity against standard and clinical isolates of *S. aureus, E. coli, Ps. aeruginosa,* and *K.pneumoniae.* While methanolic extact 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. These findings are in agreement with several studies cited below. Toama found that *N. sativa* was active against Gram positive bacteria *(S. aureus)* and yeast cells(Toama *et al.,* 1974).In 1989 Islam Sk, found that *N. sativa* oil has antifungal effect(Islam *et al.,* 1989).In 1996 Bilal and his colleagues found a fairly good activity against most of Gram positive bacteria and some of Gram negative ones (Bilal *et al.,* 1996). In 2005 NazmaAra and his colleagues reported that the volatile oil showed strong activity against Gram negative, Gram positive bacteria and *Candida albicans*(Ara *et al.,* 2005). In 2008 Zuridah. H found that *N. sativa* oil was active against Gram positive cocci *(S. aureus),* Gram negative bacteria(*E. coli, K. pneumoniae* and multidrug resistant clinical strains of *P. aeruginosa*) and Gram positive bacilli (*Bacillus cereus*) (Zuridah et al., 2008). In 2009 Salman MT, Khan R.A. and shuku found that *Nigella sativa* oil has activity against multidrug resistant clinical strains of *P. aeruginosa* (Salman *et al.,* 2008). The best inhibition zone obtained by petroleum ether extract of *Nigella sativa* was 48 mm in diameter against *S.aureus* ATCC 25923 and 63 mm against clinical isolates of *S.aureus* at concentration of 100 mg\ml. Followed by 14 mm against *K. pneumoniae* ATCC 53657 and 18 mm against clinical isolates of *K. pneumoniae* at concentration of 100 mg\ml, then 17 mm against *E. coli* ATCC 25922 and 23 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).

The founding of our study as regards the antimicrobial activity of honey are in agreement with several studies cited below.

Hyungjaerm, reported on the antimicrobial activity of different floral sources of honey against bacterial isolates, the results showed that 92.5% of bacteria was inhibited by honey (Hyungjaer *et al*., 2007).

Alandejani and his colleagues, study under the title of the effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms showed that 100% of isolates were effectively inhibited by honey (Alandejani *et al*., 2009).

NurAzida proved the antibacterial properties of honey and its effect in wound management was made resulting in that both Gram-negative bacteria isolated were completely inhibited by the honey tested (Nur Azida *et al*., 2010). Algurashi found that local Sider and mountain Saudi honey were effective in inhibiting the *in vitro* growth of *E. coli, K. pneumoniae, P. aeruginosa*. Both honey samples at different concentrations were more effective against *E. coli* than other bacteria(Alqurashi *et al*., 2013). Modified diffusion technique was selected to conduct our research; because some trials used disc diffusion technique and proved that discs were source of contamination.

5.2Conclusion

It was concluded that both *Nigella sativa* and honey ; posses antimicrobial activity, but with varying degrees of effectiveness. The mixture was the most potential antimicrobial agent followed by *Nigella sativa* oil and honey.

We believe this investigation together with previous studies provided support to the antimicrobial properties of honey and *Nigella sativa*.

Herbal medicinal practice could provide a source for new drugs and therefore efforts should be directed to evaluate traditional medicinal practice based on scientific methodologies available. Resort new source of antimicrobial agents to treat antibiotic resistant microbes in order to avoid the high cost and side effects of medications. These results Justify the use of some plants as folk medicine.

5.3 Recommendation

1. Further advanced non-cost extraction techniques to determine the active components responsible for the antimicrobial activity. (for example using chromatography that is powerful way to extract the active gradients of any natural herbal material).
2. Confirmatory *in vivo* investigations to evaluate the antimicrobial activity of both *Nigella sativa* and honey in collaboration with health and medical sectors both government and private.
3. Determination of the Minimum Inhibitory Concentration (MIC) using tube dilution method and Minimum Bactericidal Concentration (MBC).
4. Study the toxicity of the active ingredients.

Chapter Six

References
Appendix

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Appendix I

University of Shendi

College of Graduate Studies & Scientific Research

Questionnaire about *In vitro* Assessment of Antimicrobial Activity of Honey bees And *Nigella sativa* Against Selected Clinical Isolates from Shendi City.

Name:…….

Sex……..…….

City:……

Village: ……..

Sample number: ☐

1- **Age:**
   a- <15 – 24 (……) b- 25 – 34 (……). c- 35 – 44 (……...). d- 45 and more (……).
2- **Treatment:**
   a- Treated with antibiotic (……). b- Not treated (……).
3- **If under treatment, the name of treatment used ..**……
4- **Response to treatment:**
   a- Good (…..) b- Few (……) c- No response (……)
5- **Usage of Honey and Black seeds:**
   a- Yes (……) b- No (……)
6- **If answer is Yes :**
   a-Honey only (……) b- Black seeds only (……) c- Combination B/w them (……)
7- **Type of infections:**
   a- Urine (…..) b- Swabs (……) c- Sputum (……) c- Other (……)
8- **Other disease:**
   a-Diabetes (….) b- Hypertension (……) c- Other (……) d- No (……)

إقــرار بالموافقـــة

الاسم.....

العمر..... العنوان.....

أوافق بمحض ارادتى بالمشاركة في البحث العلمي المتعلق بفعالية عسل النحل ومستخلص الحبة السوداء على البكتريا المعزولة من عينات طبية بمدينة شندي.

الطالب.غانم محمد غانم مهجف

بعد أن شرح لي بأنه لا يترتب عليه أي أذي جسدي أو نفسي واعلم أن المشاركة في هذا البحث لن تؤثر بأي حال من الأحوال في الرعاية الطبية التي أتلقاها كما أنه يحق لي بدون أبداء أسباب الإنسحاب من هذا البحث في أي مرحلة من مراحله.

البحث بإشراف:

د. ليلى محمد احمد عبد القادر

التوقيـع ..... التــاريـخ.....

Appendix II

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- Sensitive balance.

11- Straight loops with handle.

12- Water bath.

13- 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-slide.

9- Test tubes.

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-Nutient 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 121ºc for 15 minutes.

2-DNAse agar:

Typical formula g/L:

Conents:

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 121ºc for 15 minutes. Cool to 50ºc 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)

I-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 121ºc for 15 mins. Solidify with the long slant.

II-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 1Lof D.W. Soak for 10 mins. Swirl to mix bring to boil. Distribute into tubes and sterilize by autoclave at 121ºc for 15 mins. Let the medium set as slopes about 3cm deeps.

III-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 121ºc for15 mins. Let it cool to 50-55ºc. Added 50 ml of urea sterile solution 40% (Ref. 06-083) and mix well. Distribute aseptically in tubes and let them solidify slanted.

IV-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 desird quantity and dissolve completely.

2- Dispense in tubes with or without inverted Durhm's tubes and sterilize by autoclaving at 15 Ibs pressure (121ºC) 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 25ºc) 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 121ºc 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 at121 C^0 for 15 minutes. Transfer to a 50 C^0 water bath.

2- When the agar has cooled to 50 C^0, 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 C^0, 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) for15 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 at121 C^0 for 15 minutes.

2 -When the medium has cooled to 50–55 C^0, 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 C^0 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 C^0 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.

9-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 55^0C, mix well, and dispense aseptically in sterile petri dishes.

3- Store the plates at 2–8 ^{0}C, 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

I-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.

II-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.

III-Mc ferland turbidity standard:

Contents:

Concentrared 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 ($Bacl_{2.}2H_2o$) in 200ml of distilled water.
3- Add .5ml of barium chloride solution to 99.5 ml of sulphuric acid solution and mix.

VI- Preparation of the extracts:

Extraction was carried out according to method descried 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. Result of *Nigella sativa* extraction as show (Table 15).

Table (15) Weight and yield % of *Nigella sativa* extracts obtained using petrolum ether and methanol solvents.

Weight of *Nigella Sativa* sample	**Petroleum ether**		**Methanol**	
	Weight of extract	**Yield %**	**Weight of extract**	**Yield %**
150 g	14.3g	14.3%	3.265 g	3.265 %

V- Preparation of serial dilution of honey and *Nigella Sativa* extracts For Testing the Antimicrobial activity.

1- Preparation of serial dilution of honey

The stock honey sample considered as 100 %(stock), then serial dilution was performed as follows:

100% _____10 ml of the stock honey.

50%_____10ml of the stock honey dissolved in 10 ml distilled water (D.W).

25%_____10ml of the 50%diluted honey dissolved in 10 ml D.W.

12.5% _____10ml of 25%diluted honey dissolved in 10 ml D.W.

2- Preparation of serial dilution of *Nigella sativa* oil

The stock of *N. sativa* oil considered as 100%, and then serial dilution was performed as follows:

100% _____10 ml of the stock oil.

50% _____10ml of stock oil dissolved in methanol.

25% _____10ml of 50%diluted oil dissolved in 10ml methanol.

12.5% _____10ml of 25%diuted oil dissolved in 10ml methanol.

3- Preparation of serial dilution of Honey and *Nigella sativa* mixture

100% _____10ml of stock oil + 10ml of stock honey.

50% _____10ml of 50% oil +10 ml of 50% honey (both are diluted with methanol).

25% ____10ml of 25% oil +10 ml of 25% honey (both are diluted with methanol).

12.5% __10ml of 12.5% oil +10 ml of 12.5% honey (both are diluted with methanol).

Appendix III

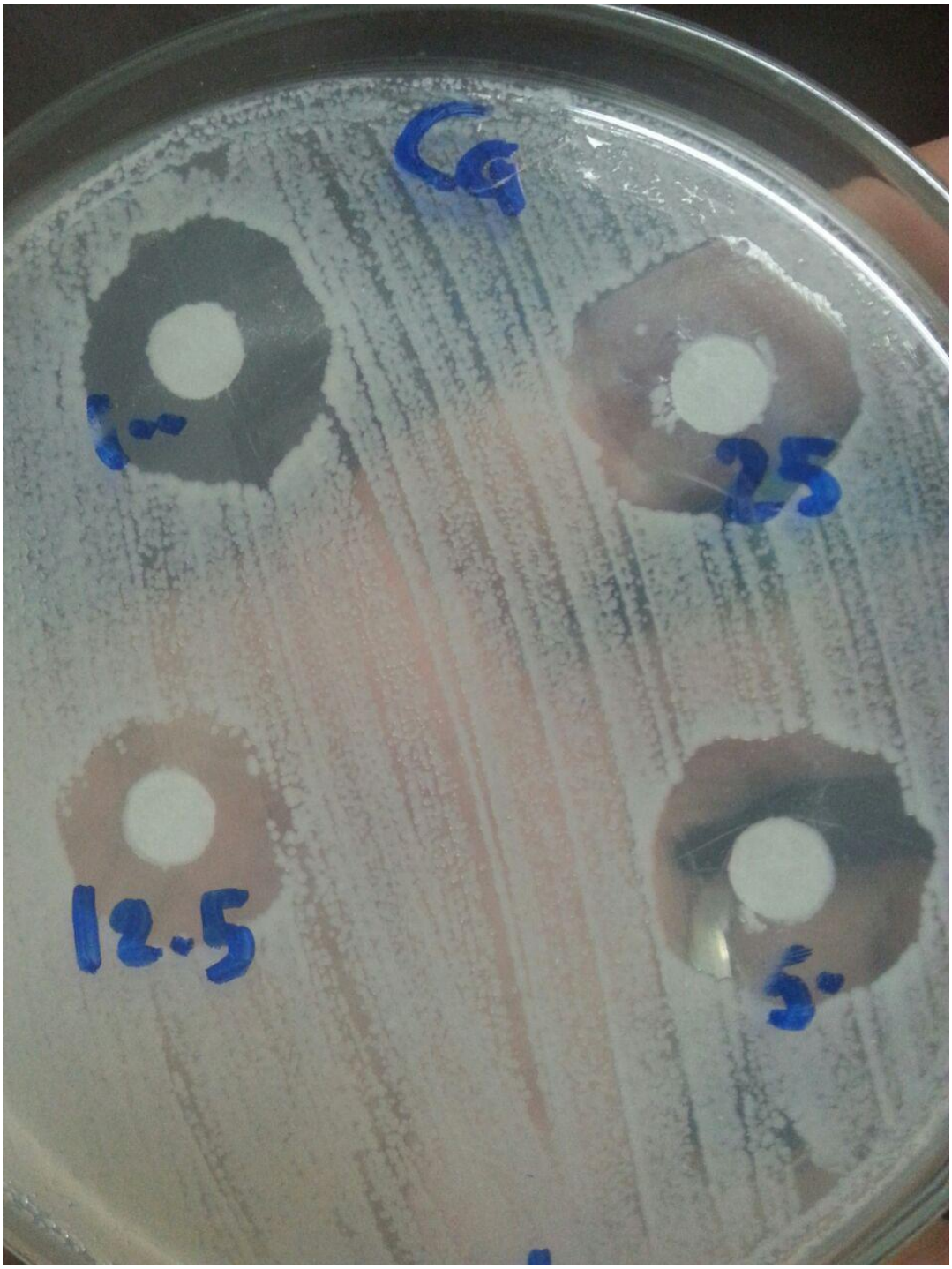


Figure (6) Susceptibility of *C. albicans* ATCC 7596 to *Nigella sativa* at different concentrations.

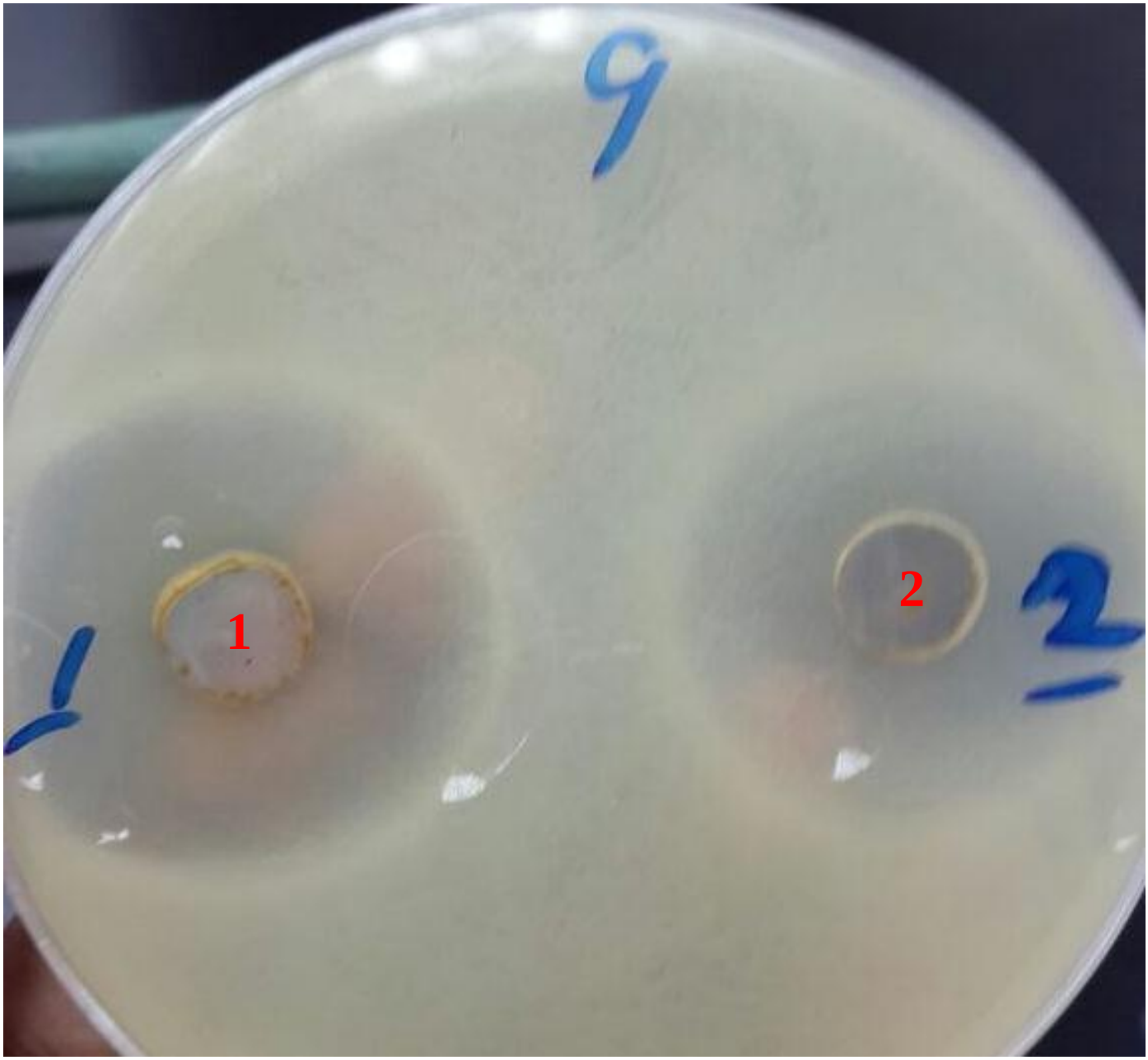


Figure (7) Susceptibility of *E. coli* to *Nigella sativa* at different concentrations

1= Zone of inhibition of *N. sativa at* (100%).

2= Zone of inhibition of *N. Sativa at* (50%).

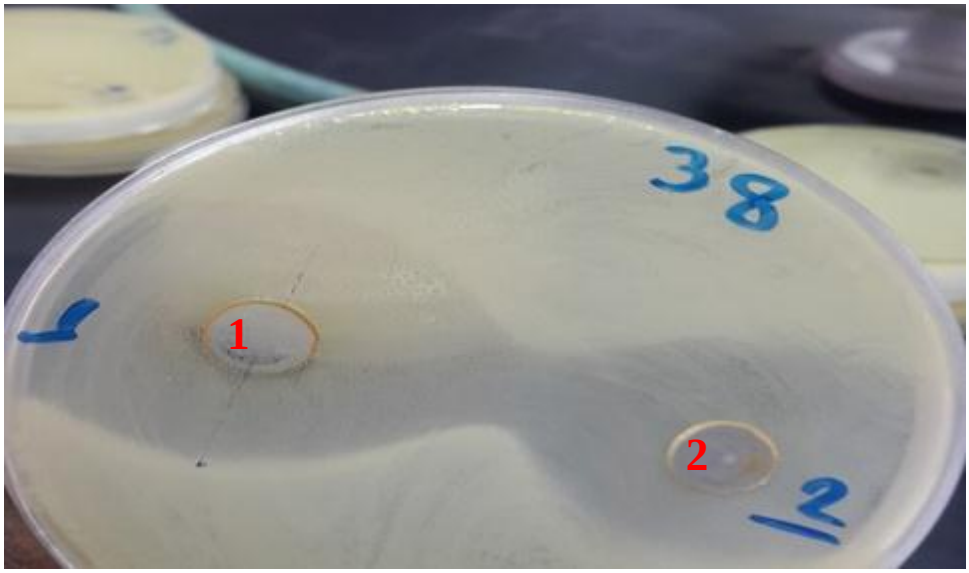
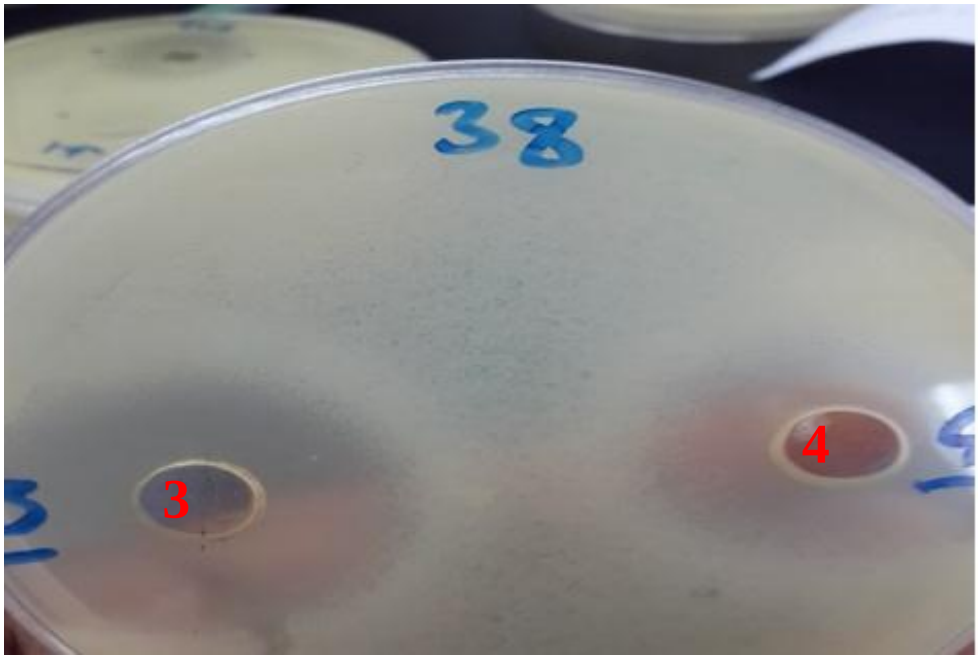


Figure (8) Susceptibility of *E. coli* to Honey at different concentrations

1= Zone of inhibition of Honey at (100%).

2= Zone of inhibition of Honey at (50%).

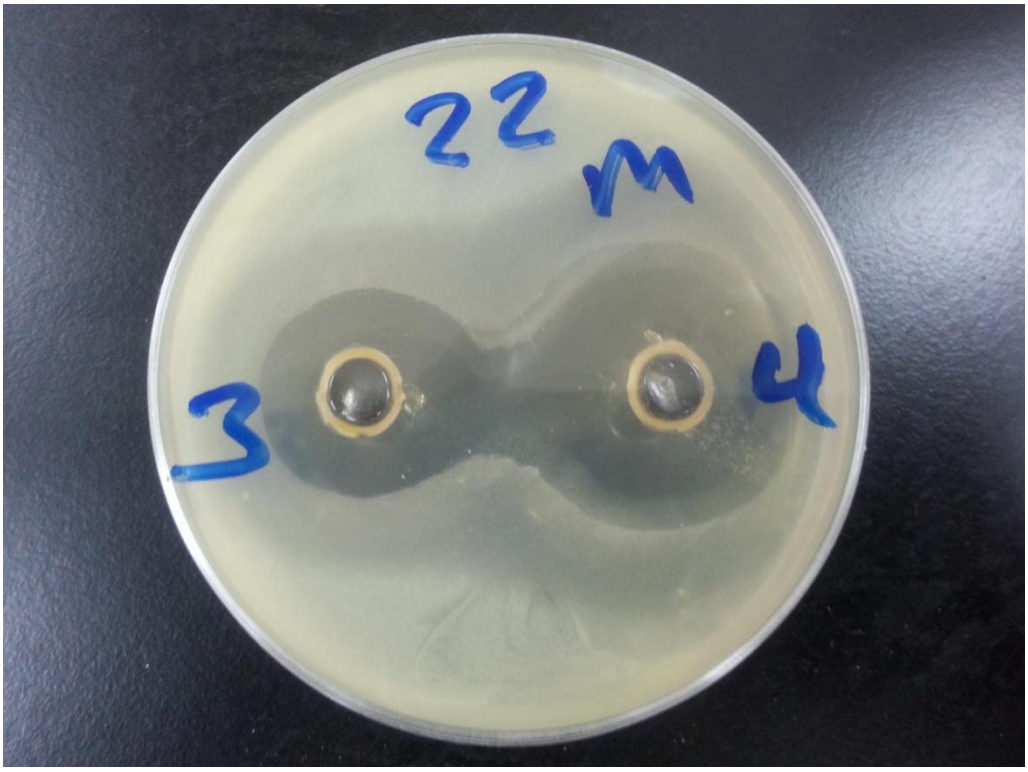


Figure(9) Susceptibility of *E. coli* to Honey at different concentration

3= Zone of inhibition of Honey at (25%).

4= Zone of inhibition of Honey at (12.5%).

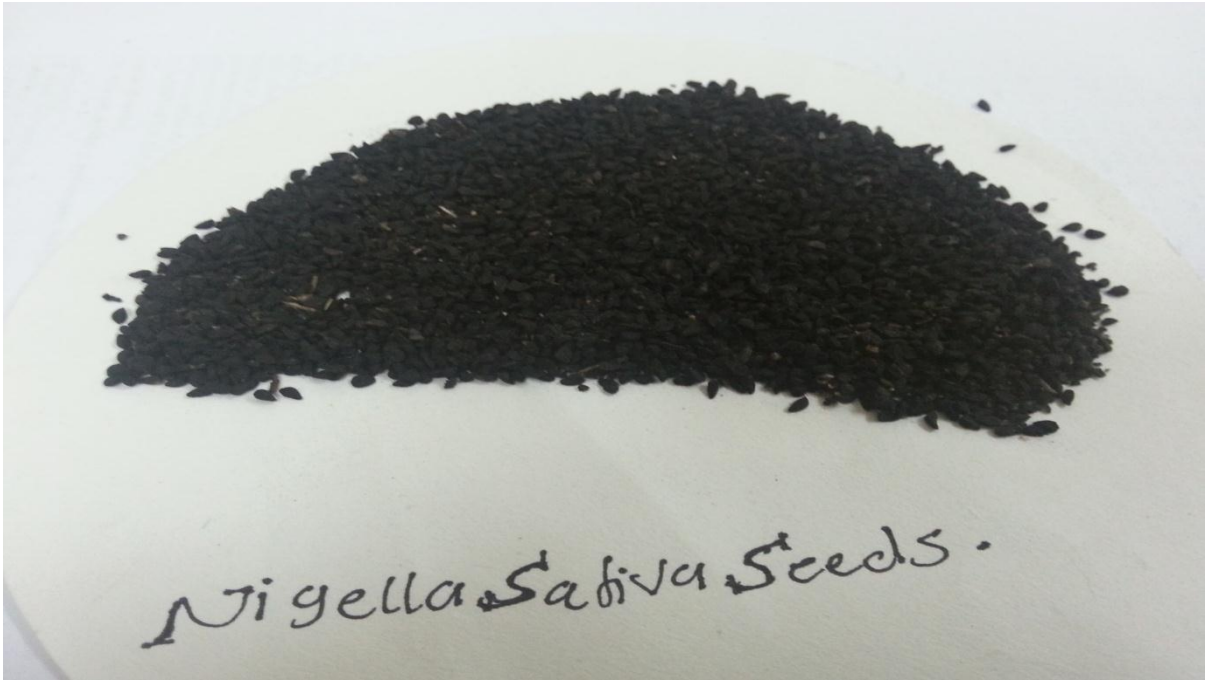
Figure(10) Susceptibility of *E. coli* to Mixture at different concentrations



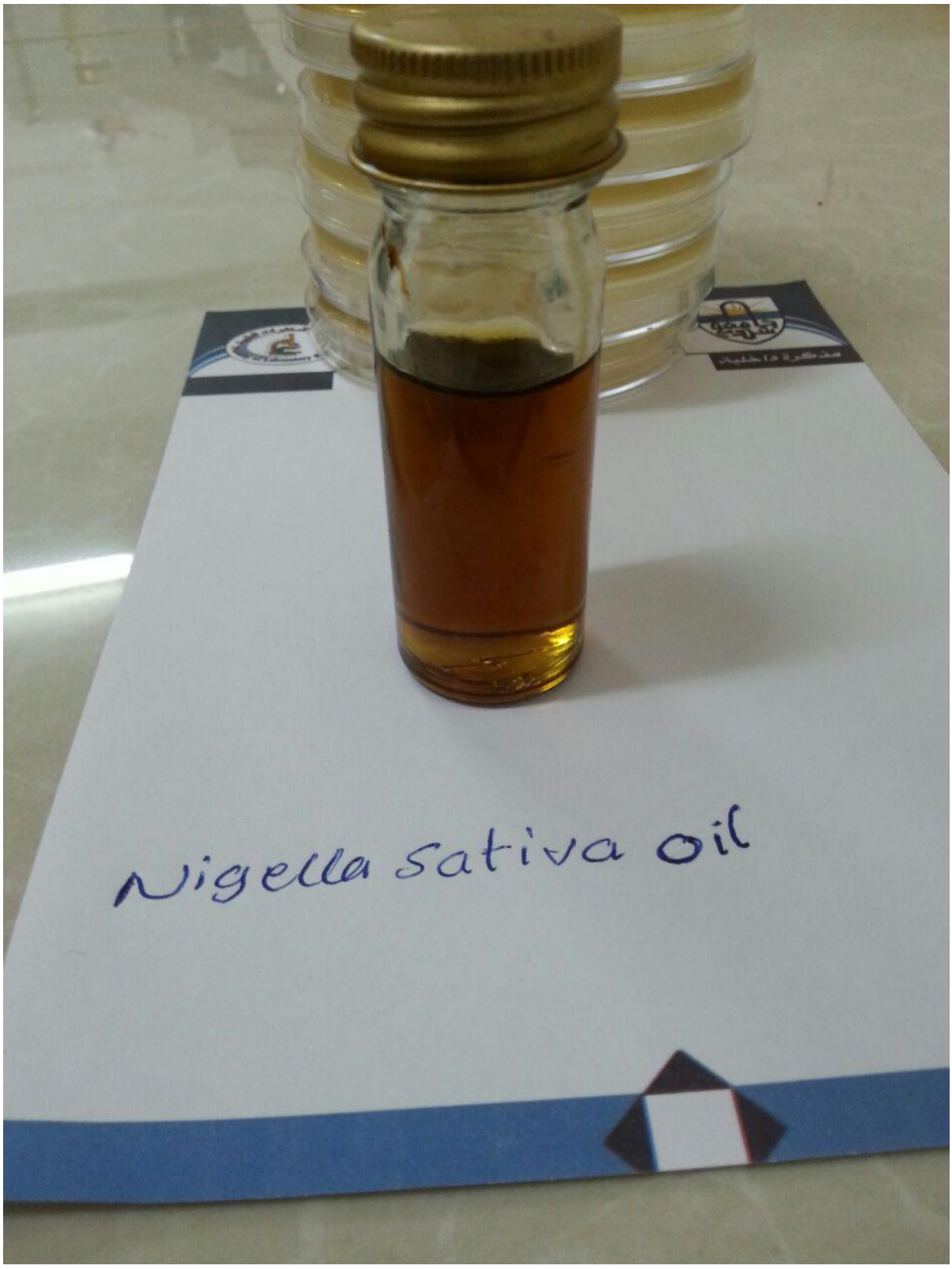
Figure(11) Susceptibility of *C. albicans* to Mixture at different concentrations

Figure(12) Soxhlets Used For Extractions

***Nigella sativa* plant.**



***Nigella sativa* seeds.**



Petroleum ether extract of *Nigella sativa*

Methanol extract of *Nigella sativa*

A bottle of honey obtained from the Western part of Shendi.