بسم الله الرحمن الرحيم

Sudan University of Science and Technology
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Antimicrobial *Activity* of *Nigella sativa* Seeds Extract Against Carbapenem and Colistin Resistant Gram Negative Bacilli in Khartoum State

فعالية مستخلص بذور الحبة السوداء المضاد لماكروبات العصيات السالبة الجرام المقاومة المضاد للكاربابنام والكولستين في ولاية الخرطوم

A dissertation Submitted in Partial Fulfillment for Requirement of
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بسم الله الرحمن الرحيم

الآيـــــــــة

قال تعالي:

(الْحَمْدُ لِله الَّذِي أَنْزَلَ علَى عبْدِهِ الْكتَابَ ولَمْ يَجْعَلْ له عِوَجًا (1) قَيِّمًا لِيُنْذِرَ بَأْسًا شَدِيدًا مِنْ لَدُنْه 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 sister, to my brothers, 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 ward.

Acknowledgment

First and foremost, we must acknowledge our limitless thanks to ALLAH, the Ever-Magnificent the Ever-thankful for His help and bless. Iam totally sure that this work would have never become truth, without His guidance.

All thanks, appreciation and respect to our supervisor Dr. Khawthar Abdelgaleil Mohamedsalih

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I would like to thank all the people who supported me, my family, friends, and classmates. Especially my parent who helped me with everything financially and morally, to my friends those believed that we can finish the study despite of all the struggles, depression and stress I experienced in the making of this research.

Last but not least, deepest thanks go to all people who took part in making this research real.

Abstract

Introduction: One of the most serious issues in medicine is increasing resistance of Gram negative bacilli to antimicrobial agents especially broad spectrum antibiotics such as carbapenems and colistins. This fact is associated with higher mortality and morbidity rates, prolonged hospital stays and increased treatment related costs.Medicinal plants appeared to be the best alternative source for new antimicrobial drugs.**.** ***Nigella sativa*** is an herbaceous plant whose seeds have beenused for centuries for treatment of various ailments, including infectious diseases, and is an important drug of Unani Medicine Narrated Abu Hurairah, "I heard Allah's Apostle saying, 'There is healing in black cumin for all diseases except death'."

Objective: This study aimedto detect antibacterial Activity of *Nigella sativa* extract against carbapenem and colistin resistant gram negative bacilli from Khartoum State

Method: Cross-sectional study and laboratory based study was carried out on 90 isolates from different specimenninety types of pathogenic bacteria were isolated and identified using Gram stain, biochemical reactions and tested for their susceptibility to Carbapenem and colistin antibiotic was performed for all Gram-negative bacilli isolates, then detection of carbapenemase enzyme production for the resistant isolates was performed using modified Hodge test and EDTA combing test. Those were resistant to carbapenem and colistin were tested for their sensitivity to Nigellasativa extracts.

Result: The isolated Gram-negative bacilli comprising of 41(45.6%)*E.coli*, 25 (27.8%) Klebsiella species, 14(15.6%)Proteus species, 4(4.4%) Pseudomonas species, 3(3.3%) Enterobacter species and 3(3.3%) Citrobacter species. . Carbapenem (Imipenem and meropenem) susceptibility testing showed that 31% of Gram-negative rods isolates were Carbapenem resistant. Modified hodge test positive result (63%) EDTA combing test positive result(69%).

Colistin susceptibility testing showed that 16.7% of Gram-negative rods isolates were Colistin resistant. Methanol extractof Nigella sativa showed antibacterial activity all clinical isolates with highest effect in concentration 50.

مستخلص الأطروحة

المقدمة: واحدة من اهم القضا يا في الطب هي زيادة مقاومة العصيات السالبة الجرام للمضادات الحيوية خاصة المضادات واسعة الطيف مثل الكاربابنم والكولستين ونتيجة لذلك ارتفع عدد المرضى وعدد الوفيات مع الاقامة الطويلة في المشفى والزيادة العالية في تكاليف العلاج .النباتات الطبية اظهرت بدائل جيدة للمضادات الحيوية ومصدر جديد لها .الحبة السوداء نبات عشبي يحوي العديدمن العناصر وعلاج لعدد من الامراض في عدد من المناطق وخاصة حبوبه حتى الامراض المعدية وهي من اهم الادوية في الطب النبوي وفي الحديث القدسي الحبة السوداء علاج لجميع الامراض عدا الموت .

الهدف : هذه الدراسة تهدف الى تحديد فعالية الحبة السوداءضد العصيات السالبة الجرام المقاومة لمضاد الكاربابنم والكولستين في ولاية الخرطوم.

الطريقة : اجريت دراسة مقطعية ومعملية الاساس تم فيها عزل 90عزلة من البكتريا من عينات مختلفة وتم التعرف عليها باستخدام التزريع و صبغة الجرام والتفاعلات الكميائية الحيوية و أختبرت حساسيتها لعدد من المضادات الحيوية. تلك التي أظهرت مقاومةلمضاد الكاربابنم والكولستين أختبرت حساسيتها لمستخلصالكمون الأ سود وهي: الاشريكية القولونية (45.6%)41 الكلبسيلا الرئوية (27.8%) 25 ، المتقلبة الاعتيادية 15.6.%) والزائفةالزنجارية (4.4%)4 والليمونية (3.3%) 3.

النتيجة: اختبار الحساسية لمضاد الكاربابنم31%من العصيات سالبة الجرام المعزولة اظهرت قاومتها لمضاد الكاربابنم. كما اجريت اختبارات تاكيدية لمعرفة وجود انزيم الكاربابنميز في البكتريا المعزولة وكانت النسبة 63% لاختبار هودك المطور و69% لاختبارالاديتا المضافة للاميبينيم أوضح اختبار الحساسية لمضاد الكولستين% 17.6من العصيات سالبة الجرام المعزولة مقاومتها لمضاد الكوليستين. مستخلص الكمون الاسود له تأثير مضاد للميكروبات ضد كل العصيات سالبة الجرام مع أعلى تأثير في التركيز 50.

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List of Abbreviation:

ACRONYM	STAND FOR
PBPs	Penicillin Pinding Proteins
A.baumannii	*Acinetobacter baumannii*
P.aeruginosa	*Pseudomonas aeruginosa*
EDTA	Ethylene Di amine Tetra Acetic acid
SME	*Serratiamarcescens*Enzyme
S.marcescens	*Serratiamarcescens*
E.cloacae	*Enterobacter cloacae*
IMI	Imipenem-hydrolyzing-β-lactamase
NMC	Not Metalloenzyme Carbapenemase
KPC	*Klebsiella pneumoniae Carbapenemase*
USA	United States of America
K.pneumoniae	*Klebsiella pneumoniae*
GES	Guiana Extended Spectrum
ESBLs	Extended Spectrum Beta Lactamases
E.coli	*Escherichia coli*
OXAs	Oxacillinases
MBL	Metallo-Beta-Lactamase
IMP	Active on Imipenem
VIM	Verona Integron-encoded Metallo-beta-lactamases
SPM	Sao Paulo Metallo-beta-lactamase
GIM	German IMipenemase
SIM	Seoul IMipenemase
NDM	New Delhi Metalo-beta-lactamase
Spp	Species
MDR	Multi Drug Resistant
CDC	Center for Disease Control and prevention
CLSI	Clinical Laboratory Standard Institute

Chapter One

Chapter One

Introduction

1.1. Introduction

Multidrug resistance gram negative bacteria (MDR-GNB) that is, gram negative bacteria resistant to at least three of the following antimicrobials: ampicillin, augmentin, ceftazidime, ciprofloxacin, gentamicin, and/or trimethoprim-sulfamethoxazole (Pop-Vicas*et al*., 2005). Gram negative bacilli are known causative agents of both community and hospital associated infections and responsible for high morbidity and mortality rates across all gender and ages. Clinical significance of gram negative bacteria GNB isolates is further heightened, because of their acquisition and dissemination of resistant genes in both community and hospital-acquired infections and has been known to worsen the antibiotic treatment and management of patients. These organisms are highly responsible for causing urinary tract infections, pneumonia, peritonitis, meningitis, sepsis and medical device associated infections (Longo *et al.,* 2012).They have the propensity to spread easily between humans (hand carriage, contaminated food and water) and to acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons (*Paterson*, 2006).

Carbapenem antibiotics have been used as the last resort salvage treatment for infections caused by multidrug resistance gram negative bacteria (MDR-GNB) (Moquet*et al.*,2011). Thus, resistance to carbapenems becomes a real threat to the survival of

patients with infections caused by MDR-GNB, and the overallmortality in such infections has been reported to be up to 50% (Bratu*et al.*,2005,Brink*et al.*,2012).

Colistinis typically used as last resort treatment when thereare no other therapy option available.

Rationale& Objectives

1.2.Rationale:

At present, one of the most serious issues in medicine is increasing resistance of gram negative bacilli to antimicrobial agent's especially broad spectrum antibiotics such as carbapenems and colistins. This fact is associated with higher mortality and morbidity rates, prolonged hospital stays and increased treatment related costs.

Environmental reservoirs for multi-drug resistant Gram-negative organisms are potentially an important factor in healthcare associated transmission. Patients colonised or infected with CRE widely contaminate their immediate patient's environment.

Antibiotics resistance has become a serious global health concern, with a huge economic burden on the community by increasing the cost of the treatment and raises the rates of hospitalization, particularly in the developing countries which already suffers from economic crises, poor sanitation and misuse of antibiotic drugs. So far, it turns out that the development of new antibiotics, which are costly and time-consuming process, has become useless, as pathogens rabidly develop resistance to these new antibiotics. This has led to an increasing interest in searching for effective alternatives for the current antibiotics with different mode of action on microbes. Hence, medicinal plants appeared to be the best alternative source for new antimicrobial drugs. The antibacterial activities of Nigella sativa against multi-drug resistant bacteria has been reported by researchers so we investigated its activity against carbapenem and colistinresistant isolates to solve this huge problem.

Researchs conduct about Antibacterial Activity of *Nigella sativa* extract against multi drug, carbapenem and colistin resistant gram negative bacilli scantly in Sudan.

1.3. Objectives:

1.3.1. General objective:

Antibacterial Activity of Nigella sativa extract against carbapenem and colistin resistant gram negative bacilli .

1.3.2. Specific objective:

To isolate Gram-negative bacilli from clinical specimens from hospitalized patients in Khartoum state.

To identify the isolated Gram-negative bacilli using conventional biochemical tests.

To determine the antimicrobial susceptibility pattern of the isolates against multi drug, carbapenem and colistin antibiotics.

To detect carbapenemase enzymes production for carbapenem resistant isolates by using modified hodge test and EDTA combination disc.

To detect the effect of *Nigella sativa* plant oncarbapenem and colistinresistant isolates.

Chapter Two

Chapter Two

Literature review

2.1. Carbapenems:

Carbapenems are beta-lactam antibiotics often used as last resort antibiotics for treating infections caused by multidrug resistant Gram-negative bacilli, since they have the broadest spectra among all beta-lactams. (Dahab *etal.*, 2017).

2.1.1. Mode of action of Carbapenems:

Carbapenems enter Gram-negative bacteria through porins and reach periplasmic space, where they permanently acylate the PBPs inhibiting them. This prevents synthesis of peptidoglycan, which results in weakening of cell wall and death of bacteria. (Sridhar *et al.*, 2012)

2.1.2. Mode of resistant to Carbapenems:

Mechanisms of resistance to carbapenems include production of carbapenemase enzymes, efflux pump, mutation in or loss of outer membrane porins and alteration of PBPs.(. Breilh*et al.*,2013)

2.1.3. Treatment options for carbapenem resistant bacteria:

It includes the following:

2.1.3.1Polymyxins:

Colistin (polymyxin E) and polymyxin B are considered to be the most active in vitro agents against carbapenem resistant bacteria; but neurotoxicity was an important concern with the use of polymyxins **(**Morrill *et al.*, 2015**).**

2.1.3.2. Tigecycline:

The Majority of carbapenem resistant bacteria isolates remain active against tigecyline in vitro, however resistance to tigecycline is increasing (Morrill *et al.*, 2015).

2.1.3.3. Aztreonam:

Is stable to MBLs, including IMP, VIM and NDM (Sridhar Rao, 2012).

2.1.3.4. Fosfomycin:

Limited data has demonstrated fosfomycin has activity against KPC-producing *K.pneumoniae* and NDM-1-producing *enterobacteriacea.* (Morrill *et al.*, 2015).

2.1.3.5. Aminoglycosides:

Gentamicin is generally the most active aminoglycoside in vitro againstcarbapenem- resistant K. pneumoniae, however amikacin can be most active against other

carbapenem resistant bacteria (Morrill *et al.*, 2015).

2.1.3.6. Combination Therapy:

Combination therapy for carbapenem resistant bacteria infections may decrease mortality as compared to monotherapy **(**Haley *et al.*, 2015**).**

2.2. Colistin:

Is a polymyxin group of polypeptide antibiotic with a significant activity against Gram-negative bacteria and them target lipopolysaccharide (LPS) in the outer membrane, although the exact mechanism of bacterial killing is still unknown. The paucity of new antibiotics for a decade has allowed clinicians to reconsider colistin as an alternative therapeutic option against infections

caused by Gram-negative bacteria The unconstrained use of colistin has caused the emergence of resistance in the recent times(Manohar ***et*** *al.*,2012) .first reported by Koyama and coworkers was originally thought to be distinct from polymyxins, but was later proven to be identical to polymyxin E .It has been available since 1959 for the treatment of infections caused by Gram-negative bacteria However, when early clinical reports suggested a high incidence of toxicity its use was reduced when the potentially less toxic aminoglycosides and other anti-pseudomonal agents became available. Therefore, from the decline in its use in the early

1970s up until the mid1990s, there have been limited studies on the clinical use of colistin or on its pharmacokinetics and pharmacodynamics.

Two forms of colistin are available commercially: colistinsulphate, chiefly used topically, and sodium colistinmethanesulphonate, used parenterally. Both forms may be given via inhalation. Parenteral administration of sodium colistinmethanesulphonate in humans has been associated with nephrotoxicity, neurotoxicity and hypersensitivity(Li *et al.*, 2005).

2.2.1.Mechanism of action of colistin:

Colistin is a cationic, multicomponent lipopeptide consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid. The two major components of colistin are colistin A (polymyxins E_1) and colistin B (polymyxins E_2). Different pharmaceutical preparations of colistin may contain different amounts of these two components have their antimicrobial activity mainly directed against the bacterial cell membrane. The cationic polypeptides of colistin interact with anionic lipopolysaccharide (LPS) molecules in the outer membrane of gram negative bacteria, leading to displacement of calcium (Ca^{2+}) and magnesium (Mg^{2+}), which stabilize the LPS membrane, thus causing derangement of the cell membrane. This results in an increase in the permeability of the cell membrane, leakage of cell contents, and ultimately cell death. also has potent anti-endotoxin activity. The endotoxin of gram-negative organism is the lipid a portion of LPS molecule and colistin binds and neutralizes this LPS molecule.

2.2.2. The mechanism of resistance to Colistin:

It remains poorly understood. Various studies have suggested that alterations of the outer membrane of bacterial cell-like loss of LPS, reduction of specific outer membrane proteins, reduction in cell envelope Mg^{2+} and Ca^{2+} contents, and lipid alterations are related to the development of resistance. Polymyxin B and E resistance mechanisms appear to be either stable (mutational) or reversible upon removal of selective pressure (adaptive). A few studies have

suggested that hetero resistance to colistin is not as common in MDR *P. aeruginosa* as in MDR *A. baumanii*. (GovilD ,2009,Juri Katchanov,2018).

2.3.Medical plant

The use of medicinal plants as medicine has increased world-wide due to factors such as drug failure. Adverse reactions, cost of medications as well as resistance to antimicrobials by Bacteria. About three-quarters of the world population residing in the developing countries still Use medicinal plant, this is despite the availability of pharmaceutical antibiotics (Vanamala*et al.*, 2012). This use of herbal plants as alternative medicine over centuries remains a popular choice for primary health care (Vanamala *etal.*, 2012). However post antibiotic era, with the emergence and re-emergence of resistant strains of microorganisms, coupled with the side effects of the most conventional drugs, there is a renewed interest in the use of plants and plant products in the management of ailments (Ayogu *etal.*, 2008).

2.4.Plant extraction:

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 andextraction procedure (Amita and Shalini, 2014).

2.4.1. Extraction procedures:

2.4.1.1. 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**).**

2.4.1.2. 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).

2.4.1.3. 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**).**

2.4.1.4. 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).

2.4.1.5. 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).

2.4.1.6. 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).

2.4.1.7. 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. 4.1.8. 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).

2.4.1.9. 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).

2.5. 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 fattyacids gums which are capable of producing definite physiological actionon body. Compounds extracted from different parts of the plants can beused to cure diarrhea, dysentery, cough, cold, cholera, fever andbronchitis (Saranraj and Sivasakthi, 2014).

2 .6. Nigella sativa:

Commonly known as Black Seed or Black Cumin is an herbaceous plant whose seeds have beenused for centuries for treatment of various ailments, including infectious diseases, and is an important drug of Unani Medicine. It has been recommended for use on a regular basis in TibbeNabwi (Prophetic Medicine) as is evident from the following tradition: Narrated Abu Hurairah, “I heard

Allah's Apostle saying, 'There is healing in black cumin for all diseases except death'." (Salman *et al.*,2009).

2.5.1.Taxonomic classification:

Kingdom: Tracheobionta

Superdivision: Spermatophyte

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Magnoliidae

Order: Ranunculales

Family: Ranunculalaceae

Genus: Nigella L.

Species: Nigella sativa L.

2.6.2. 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).

2.6.3. Distribution:

Mediterranean region, North Africa, Middle East and some parts of Asia (Hosseinzadeh *et al*., 2007)

2.6.4.Common names:

Are known as black seed or black cumin (English), black-caraway seeds (US), Al-habba Al-sawda (Arabic), shonaiz (Persian), kalonji (India and Pakistan), kalajira (Bangladesh) and krishnajirika (Southeast Asia) (Abdallah,2017).

2.6.5.Chemical constituents:

These tiny seeds are rich in bioactive compounds. In general, it contains about 32-40% fixed oils, 0.4-0.45% volatile oil 8-9 types of essential amino acids beside some vitamins, and carbohydrates (Forouzanfar*et al*.,2014) In addition, some interesting derivatives of alkaloids, steroids, saponins, terpenes, monoterpenes and phenolic compounds were isolated from the black seed (Abdallah, 2017). Among these pure compounds of pharmacological benefits isolated from the black seed are nigellicine, nigellicimine, nigellicimine N-oxide, carvone, thymoquinone, thymol and many more (Paarakh, 2010)

2.6.6.Medical uses:

Nigella sativa products have been reported to be used in the treatment of diseases such as asthma, bronchitis inflammatory diseases and antifungal. Treatment of respiratory, gastrointestinal, kidney, liver, skin, circulatory and immune system ailments (Abd El-Hack *et al*., 2016). Anti-oxidant and anti-arthritic (Umar *et al*., 2012) Anti-diabetic (Salama,2011) anti-cancer (Salem,2011) .Anti-inflammatory and analgesic (Alemi *et al*., 2012) anti-anxiety (Perveen *et al*., 2009). In treatment of different respiratory diseases

(Wienkotter *et al*., 2008). Hepato-protective (Yildiz *et al*., 2008) .Nephroprotective (Abul-Nasr *et al*.,2001).Anti-gastric ulcers (Gastro-protective) (Magdy *et al*.,2012) .Immunomodulatory (Majdalawieh *et al*., 2010)

2.7. Previous studies:

A study was conducted by Sahas *et al,* 2018 in India under the title: detection of carbapenem and colistin resistant Gram-negative bacteria the age group 51-60 years (31%). Majority of the isolates like *Klebsiella species* (30.6%) followed by *E.coli* (25.9%) and few isolates reported were *Proteus* species (5.8%). Extended spectrum beta-lactamase production was identified in test isolates at a range of Male predominance (59%) is seen while compared to females (41%). The maximum cases were reported 95.2% by using ceftazidime/clavulanic acid combination antibiotic susceptibility test. The prevalence rate of metallo-beta lactamase isolates was quite high i.e. 66.6% and carbapenemase producers was 77.3%. The major isolates were *Klebsiella spp.* and *E.coli* shows Metallo-beta-lactamase production. The study helps the clinicians in choosing the correct antimicrobial agent which contribute not only to better treatment but their judicious use will also help in preventing the emergence of drug resistant strains which are still sensitive. (Sahas *et al.,* 2018)

In Sudan, 2017 Dahab RA, et al performed a study about Phenotypic andgenotypic detection of carbapenemase enzymes producing gram-negative bacilli isolated from patients in Khartoum State. Hundred and forty nine Gram-negative bacilli were isolated

from 147 different clinical specimens. The most predominant Gram-negative bacilli isolates was E.coli(54.4%), followed by Klebsiella species (29.5%). More than fifty percent of the isolates were carbapenem resistant. Fifty six percent of the resistant isolates were positive by Modified Hodge Test. By using PCR, 17.3% of resistant organisms were harbored bla gene, and 6.7% harbored bla gene. E.coli was the most bacteria that harbored the bla followed by Klebsiella species.bla gene was harbored only by E.coli(Dahabet al. 2017).

Nor' Aishah Hasan*et al* ,Malaysia 2012 did a study about antibacterial activity of Nigella sativa seed extract against some pathogenic bacterial strains, *Streptococcus pyogene*, *Pseudomonas aeruginosa*, *Klebseilla pneumoniae* and *Proteus vulgaris* was evaluated. Methanol extract at the concentration of 100 mg/mL had a remarkable sensitivity towards all tested bacteria in this study. *Klebseilla pneumoniae* and *Proteus vulgaris* showed resistance against aqueous extract at 20 mg/mL. Methanol extract of *Nigella sativa* exhibited significant antibacterial activity at the concentration of 50 mg/mL ($p \leq 0.01$) against *Streptococcus pyogenes* with a greater inhibition zone of 19 mm, while a 15 mm zone of inhibition was observed in *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus vulgaris*. Kruskal Wallis analysis showed that both aqueous and methanol extract of black seed exhibited a greater inhibition on Gram positive bacteria (*Streptococcus pyogenes*) compared with Gram negative bacteria (*Pseudomonas aeruginosa, Klebsiellapneumoniae and Proteus vulgaris*). Our study also showed that species, strains and concentrations of *Nigella sativa* extract are some of the factors that may influence the sensitivity of the tested bacteria (Nor' Aishah *et al.,* 2012).

In July 2016 Amir Riyaz Khan and KirandeepKour in indiaperformed a research under the titleWide spectrum antibacterial activity of *Nigella Sativa L.* seeds**.** Crude extracts of *Nigella sativa* L. (black cumin) seeds were tested in varying dilutions against strains of *Bacillus cereus* 2156, *B. subtilis* 2920, ***E.coli***2065, *Staphylococcus epidermidis* 2493, *Klebsiellapneumoniae*2957, *Pseudomonas aeruginosa* 5029, *Salmonella typhmurium*2501, *Enterobacteraerogens*5139 using agar well diffusion technique in swabbed Muellar Hinton agar plates under standard laboratory conditions. Extract in ethanol and n-hexane showed remarkable dose dependant antibacterial activity against the tested strains as evident from the zones of inhibition. No activity of the extract was observed against *Pseudomonas aeruginosa* 5029 and *Enterobacteraerogens*5139. The most sensitive strain was *S. epidermidis*. No cross resistance was noticed with any of the tested antibiotics.(Khanand kour,2016)

In India, 2005 study titled antimicrobial activity of Nigella sativa oil against multi-drug resistant *Pseudomonas aeruginosa* from clinical specimens was done by Mohd Tariq Salman, et al *Nigella sativa* (black cumin) seed oil and extracts were tested in varying dilutions against strains of *Pseudomonas aeruginosa* resistant to a number of clinically used antibiotics isolated from patients attending JN Medical College Hospital, Aligarh, using disc agar diffusion technique on inoculated Muellar Hinton agar plates under standard laboratory conditions. Both the oil and Methanolic extract showed remarkable dose dependant antibacterial activity against the tested strains upto a dilution of 1:50 as evident from the zones of inhibition. No cross resistance was noticed with any of the tested antibiotics. (Salman *et al*., 2009)

In 2018 Fatima A. Saleh, *et al* performed a study about phytochemical Analysis of *Nigella sativa* L. utilizing GC-MS Exploring its Antimicrobial Effects against Multidrug-Resistant Bacteria among the different preparations used, *N. sativa* oil showed the highest antioxidant and antibacterial activity against highly resistant gram-positive bacteria with the greatest suppression of biofilm formation, which was attributed to its high bioactive contents. Conclusion: This study indicates that *N. sativa* extracts and its oils can be used as natural antibacterial agents to treat infections caused by multidrug resistant bacteria (Saleh F.A *et al*.,2018)

In India, 2017Tamil Nadu did a study about occurrence of carbapenem- and colistin-resistance among Gram-negative bacteria is increasing worldwide. The aim of this study was to understand the distribution of carbapenem and colistin-resistance. A total of 89 bacterial isolates were studied which included *E.coli* (n=43), *Klebsiella pneumoniae* (n=18), *Pseudomonas aeruginosa* (n=10), *Enterobacter cloacae* (n=6), *Acinetobacter baumannii* (n=5), *Klebsiella oxytoca* (n=4), *Proteus mirabilis* (n=2) and Salmonella paratyphi (n=1). MIC testing showed that 58/89 (65 %) and 29/89 (32 %) isolates were resistant to meropenem and colistin, respectively, whereas 27/89 (30 %) isolates were resistant to both antibiotics. *E.coli*, *K.pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa,* and *Enterobacter cloacae* isolates were blaNDM-1-positive (n=20). Some strains of *E.coli*, K. pneumoniae and K. oxytoca were blaOXA-181-positive (n=4). Class 1, 2 and 3 integrons were found in 24, 20 and 3 isolates, respectively. Nine NDM-1-positive *E.coli* strains could transfer carbapenem resistance via

plasmids to susceptible *E.coli*AB1157. Meropenem and colistin showed synergy in 10/20 (50 %) isolates by 24 htime-kill studies. Our results highlight the distribution of carbapenem- and colistin-resistance in Gram-negative bacteria isolated from the Tamil Nadu region in South India(Manohar *et al.,* 2017)

In Saudi Arabia 2016 Al-Jaafary Maryam***, et al*** performed astudy, different concentrations of Nigella sativa oil were tested for their antibacterial activity against different strains of Gram positive and Gram negative multi-drug resistant bacteria (MRSA, *Acinetobacterbaumannii*, *E.coli*, and *Pseudomonas aeruginosa*) by using well diffusion method. For all the different strains of *citrbaumannii*and *E.coli*that were tested against 100% of N. sativa oil, there was no recorded zone of inhibition. However, for the different strains of MRSA and *Pseudomonas aeruginosa*, different zones of inhibition where obtained for all the different oil dilutions used. Bacterial growth was inhibited at 100%, 80%, 50%, 40%, 30% and 20% N. sativa oil dilutions. **(**Al-Jaafary*etal*.,2016)

Amalia Tri Utami, et al in Indonesia, 2016 did a research which determine the effectiveness of extracts of black cumin seeds (*Nigella sativa* L.) as an antimicrobial against *Salmonella typhi in vitro* andcandetermine minimum inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) from extracts of black cumin seeds (*Nigella sativa* L.) against *Salmonellatyphi*.The MIC (Minimal Inhibition Concentration) is 45% concentration of extract. Step two was plating in NAP (Nutrient Agar Plate) medium. The MBC (Minimal Bactericidal Concentration) is 47.5% concentration of extract. The result of experiment was knew there

are different average of *Salmonella typhi*colony from every group. The conclusion of this study was the addition of extract concentration occurred reduction average of *Salmonella typhi*colony (Amalia Tri Utami, *et al.*, 2016).

Hossein Hosseinzadeh,*et al* in2007 performed study and the purpose of this study was to evaluate the antibacterial activity of total crude extracts and essential oil (EO) of Nigella sativa L. seeds in male mice infected intraperitoneally with Staphylococcus aureus or *E.coli*(0.1mL from 106 colony forming units/ml suspension). After 24 hours, the infected mice were subjected to different doses of TE or EO or received 33 mg/kg of gentamicin (a positive control) or 0.4 mL of normal saline (a negative control). After 24 hours, aspirated specimens from intraperitoneal fluids were cultured on a soybean casein digest agar plate surface. The inhibitory effect of the methanol extract at a dose of 2.14 g/kg in mice infected with S. aureus was 87.5%. The doses of 1.2 and 2.14 g/kg in mice infected with *E.coli*were 100% compared with mice who received saline (the negative control). While the aqueous extract did not show any inhibitory effect on either micro-organism, the effect of the chloroform extract at dose of 2.6 g/kg and 33 mg/kg gentamicin (the positive control) was 100 %. The EO at dose of 0.3 g/kg in mice infected with S. aureus and *E.coli*showed 100% inhibitory effect compared with mice who received saline. N. sativa methanol and chloroform seed extracts as well as its essential oil have dose dependent antibacterial activities on the Gram-positive and Gram-negative organism**.** (Hosseinzadeh*et al.*,2007)

Chapter Three

Chapter Three

Materials and methods

3.1. Study design:

This was cross-sectional study and hospital based study.

3. 2. Study area and study duration

This study was conducted at Omdurman Military Hospital, Mohamed Alamin for children, Alakademy hospital and Bahary hospital in Khartoum state during the period from August to December 2018.

3.3. Study population:

Hospitalized patients admitted with wound infection, urinary tract infection and gastrointestinal problems

3.4. Sample size:

Ninety different clinical samples were collected in the period from August to December 2018.

3.5. Inclusion criteria:

All patients complaining of Gram-negative bacterial infections.

3.6. Exclusion criteria:

All patients not complaining of Gram-negative bacterial infections and non-hospitalized patients.

3.7. Laboratory investigations:

Clinical samples were collected from hospitalized patients. Specimens, such as endotracheal aspiration, pus, and wound swabs were cultured in blood and Macconkey agar while urine were cultured in CLED media. Blood was collected in blood culture bottles containing brain heart infusion broth. Indirect Gram stain

was done and Gram negative rods were selected for further identification and testing, Subcultures for Gram negative bacilli were done on blood agar, MacConkey agar, and CLEDto insure purity and incubated aerobically at 37 C for 24 hours. Then standard biochemical tests were performed including Oxidase tests, KIA, indole test, urease production test, citrate utilization test and motility tests**.**

3.7.1.Gram`s stain:

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

3.7.2Oxidase test:

A piece of filter paper was placed on a clean glass slide and three to fourdrops of freshly prepared oxidase reagent (tetra methyl para Phenylene diamine dihydrochochloride) 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 theproduction of blue-purple colour within 10 second (Cheesbrough, 2005).

3.7.3.kligler iron agar:

A heavy inoculum of the tested colony was streaked over the surface of the slope of KIA media and stabbed into the butt .The tube was incubated aerobically at 37^{o} C overnight, then the result was interpreted as following:

Yellow butt with red slope: glucose, non-lactose fermentation.

Yellow butt with yellow slope: glucose, lactose fermentation.

Red butt with red slope: no fermentation.

Cracks in the medium: gas production.

Blackening of the medium: H_2S production. **(**Mccariney and Mackie, 2006)

3.7.4 Indole test:

The tested colony was inoculated in sterile peptone water using sterilewire loop and then incubated at 37oC aerobically overnight. Few drops of kovac's reagent were added to the medium and shaked gently to test for indole. Positive result was indicated by production of red colour in the surface layer within 10 minutes (Cheesbrough, 2005).

3.7.5. 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 37oC acerbically. 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, 2005).

3.7.6.Citrate utilization test:

Tested organism were cultured in simmons citrate agar with indicator bromothymol blue. Incubated for 96 h at37C blue colour and streak of growth positive result original green colour and no growth negative result.**(**Mccariney and Mackie, 2006)

3.7.7.Motility:

The organism were cultured in semisolid media aerobically for 24 h by stabing by straight wire loop to3/4 from the media at 37C.motile bacteria typically give diffuse non-motile bacteria generally give growths that were confined to the stab-line,have sharply defined margins and leave the surrounding medium clearly transparent.**(**Mccariney and Mackie, 2006)

3.7.8.Sensitivity testing:

Antimicrobial susceptibility testing to ceftazidime, ciprofloxacin, imipenem, cotrimoxazleamikacin andcolistin antibiotic was performed for all Gram-negative bacilli isolates using the disc diffusion method, according to the Clinical Laboratory Standards Institute Guidelines. Bacterial colonies were suspended in sterile normal saline and compared with McFarland standard and cultured in Muller-Hinton agar media (media were obtained from Pronadisa Laboratories Conda., Spain) using a sterile cottonswab. After overnight incubation at 37°C, zone of inhibition wasmeasured and the reading was compared to the sheet provided bymanufacturerGram-negative isolates showing resistant zones toImipenem were tested for carbapenemase enzyme production using MHT and EDTA Disk synergy test. (Dahab RA, *et al* 2017).

3.7.9.Phenotypic detection of carbapenemases:

3.7.9.1.Modified Hodge Test (MHT):

In Modified Hodge Test the growth was suspended in Normal saline and matched to Mcfarland standard (0.5). It was diluted 1:10 by adding 0.5 ml of the Mcfarland to 4.5 ml of saline. Standard strain of *E. coli* (ATCC 25922) was first inoculated on the Mueller Hinton Agar (MHA) plate as lawn culture. A 10 µgImipenem disk was placed at the centre of the plate

and each clinical isolate was streaked from the disk to the edge of the plate and the later was incubated at 37˚C for 12 hours.

After incubation period, the plates were examined for a clover leaf type of pattern of indentation at the intersection of growth of the test organism and the standard strain *E. coli* ATCC 25922, within the zone of inhibition of the Imipenem disc (12). Interpretation of Modified Hodge Test: A positive test shows a clover leaf like pattern of indentation of *E. coli* ATCC 25922 which grows along the growth of test isolate within the zone of disc diffusion. A negative test shows no growth of *E. coli* ATCC 25922 along the growth of test organism within the zone of disc diffusion[Kaur*et al*.,2016].

3.7.9.2.EDTA Disk synergy test:

EDTA Disk synergy test was used for the detection of metallo - β - lactamases in the imipenem and meropenem resistant clinical isolates. First of all, an EDTA solution with 0.5 M strength was prepared by dissolving 186.1 g of Disodium EDTA in 1000 ml of distilled water. The pH was adjusted to 8.0 and the solution was sterilized by autoclaving. An overnight culture broth of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and was spread on the surface of a Mueller Hinton Agar plate. A 10 μgimipenen disk (HI - MEDIA) was placed on the agar surface. A blank disk (6 mm diameter) was then kept on the inner surface of the lid of the Muller Hinton Agar plate and 10 μl of 0.5 M EDTA was poured onto it with the help of an auto pipette. This EDTA disk was then placed on the surface of agar and was kept about 10 mm edge-to-edge apart from the imipenem disk. After overnight incubation at 37°C, the presence of an expanded growth inhibition zone between the two disks was interpreted as positive for MBL production [Kaur *et al*., 2016].

3. 7. 10. Preservation and storage of isolated organisms:

Isolated organisms were kept in nutrient agar slope at 4 C for further identification and susceptibility tests. Glycerol (20 ml) with peptone water (80 ml) (20% v/v) was used for long preservation of the isolates at -20 C.

3. 7.11. Collection and identification of plant material:

Nigella sativa seeds were procured from sajda minimarket in alarabi market in Khartoum. They were freed of dust and crushed in a domestic grinder and then soaked. The plant extraction was prepared at Medicinal and Aromatic plants and traditional Medicine Research Institute National Center for Khartoum, Sudan.

3.7.12.Preparation of the extracts:

Alcoholic Extraction was carried out according to method descried by Sukhdev *et al.*(2008):97 g of the plant sample was coarsely powdered using mortar and pestle. Coarsely sample was successively extracted with petroleum ether and methanol using soxhelt extractor apparatus. Extraction carried out for about four hours for petroleum ether and six hours for methanol till the color of solvents at the last siphoning time returned colorless. Solvents were evaporated under reduced pressure using rotary evaporator apparatus. Finally extracts allowed to air till complete dryness and the yield percentages were calculated as followed(1)

Weight of extract obtained / weight of plant sample X100

NO	Name of extract	Weight of plant in gm	Weight of extract in gm	Yield%
1	Petroleum ether	97	7	7.2
2	Methanol	97	1	1.03

3.8. Ethical consideration:

Permission taken from ethical committee of faculty of medical laboratory sciences, Sudan University of science and technology.Consent was taken from laboratory manager to collect specimens with insuring all ethical considerations for conducting the research in a way that protects patient's privacy

3.9. Data analysis:

Data collected were analysed using statistical package for the social Sciences (SPSS) version 20, and excel programs.

Chapter Four

Chapter Four

Results

4.1. Results:

Ninetydifferent clinical specimens were collected from patients attended to different hospitals in Khartoum state.Ninety Gram-negative bacilli were isolated. Wound swabs specimens were the most frequent comprising out clinical specimens; 36 Gram negative bacilli were isolated from the 36 wound swabs specimens.

The isolated Gram-negative bacilli comprising of 41 (45.6%) *E.coli*, 25 (27.8%) *Klebsiella* species, 14(15.6%) *Proteus* species, 4(4.4%) *Pseudomona s*species, 3 (3.3%) *Enterobacter* species and3(3.3%) *Citrobacter* species.

Carbapenem (Imipenem) susceptibility testing showed that 83 (92.2%) of Gram-negative rods isolates were Carbapenem resistant, 7 (7.8%) were Carbapenem sensitive.

Colistin susceptibility testing showed that 15 (16.7%) of Gram-negative rods isolates were Colistin resistant, 75 (83.3%) were Colistin sensitive.

Amikacin susceptibility testing showed that 50 (54.5%) of Gram-negative rods isolates were Amikacin resistant, 40 (44.5%) were Amikacin sensitive.

Cotimoxazole susceptibility testing showed that 72 (80%) of Gram-negative rods isolates were Cotimoxazole resistant, 18 (20%) were Cotimoxazole sensitive.

Ceftazidime susceptibility testing showed that 86 (95.6%) of Gram-negative rods isolates were Ceftazidime resistant, 4 (4.4%) were Ceftazidime sensitive.

Ciprofloxacin susceptibility testing showed that 83 (92.2%) of Gram-negative rods isolates were Ciprofloxacin resistant, 7 (7.8%) were Ciprofloxacin sensitive.

Regarding antimicrobial activity of Nigella sativa extract at 100% concentration 51\90 (56.7%) of the isolates were sensitive and 39\90 (43.3%) of the isolates were resistance.

Regarding antimicrobial activity of Nigella sativa extract at 50% 55\90 (61.1%) of the isolates were sensitive and 35\90 (38.9%).of the isolates were resistance. .

Regarding antimicrobial activity of Nigella sativa extract at 25% concentration 51\90 (56.7%) of the isolates were sensitive and 39\90 (43.3%) of the isolates were resistance. .

Table(1): Frequency and percentage of type of specimens.

	Frequency	Percent
Sputum	1	1.1
Blood	2	2.2
Fluid	2	2.2
Sputum	15	16.7
Urine	53	38.9
Wound	35	38.9
Total	90	100.0

Table (2): Frequency and percentage of the isolated Gram-negative bacilli.

Organisms	Frequency	Percent
Pseudomonas aeurginosa	4	4.4
Citrobacterspp	3	3.3
E.coli	41	45.6
Enterobacterspp	3	3.3
Klebsiellapneumonia	25	27.8
Proteus spp	14	15.6
Total	90	100.0

Table(3):**Percentage of Gram-negative bacilli according to susceptibility to antibiotics**

Antibiotic	ensitive	Resistance
Colistin	75\90 (83.3%)	15\90 (16.7%)
Imipenem	83\90 (92.2%)	7\90 (7.8%)
Amikacin	40\90 (44.5%)	50\90 (54.5%)
Cotimoxazole	8\90 (20%)	72\90 (80%)
Ceftazidime	4\90 (4.4%)	86\90 (95.6%)
Ciprofloxacin	7\90 (7.8%)	83\90 (92.2%)

Table (4):Percentage of Gram-negative bacilli according to susceptibility to different *Nigella sativa* concentrations

Concentration	Sensitive	Resistance
100%	51\90 (56.7%)	39\90 (43.3%)
50%	55\90 (61.1%)	35\90 (38.9%)
25%	51\90 (56.7%)	39\90 (43.3%)

Table (5): Crosstabulation between isolated organisms and *Nigella sativa*concentrations

	100%	100%	50%		25%	25%
	Sensitive	Resistance	Sensitive	Resistance	Sensitive	Resistance
Pseudomonasaeurginosa	2\90 (2.2%)	2\90 (2.2%)	3\90 (3.3%)	1\90 (1.1%)	2\90 (2.2%)	2\90 (2.2%)
Citrobacterspp	0\90 (0%)	3\90 (3.3%)	1\90 (1.1%)	2\90 (2.2%)	0\90 (0%)	3\90 (3.3%)
E.coli	19\90 (21%)	22\90 (24.4%)	19\90 (21%)	22\90 (24.4%)	20\90 (22.2%)	21\90 (23.3%)
Enterobacterspp	2\90 (2.2%)	1\90 (1.1%)	2\90 (2.2%)	1\90 (1.1%)	1\90 (1.1%)	2\90 (2.2%)
Klebsiellapneumoniae	20\90 (22.2%)	4\90 (4.4%)	20\90 (22.2%)	4\90 (4.4%)	18\90 (20%)	6\90 (7%)
Proteusspp	8\90 (8.9%)	6\90 (7%)	9\90 (10%)	5\90 (6%)	9\90 (10%)	5\90 (6%)
Total	51\90 (56.7%)	39\90 (43.3%)	55\90 (61.1%)	35\90 (38.9%)	51\90 (56.7%)	39\90 (43.3%)

Figure (1) : Percentage of Gram-negative bacilli according to susceptibility to Colistin antibiotic.

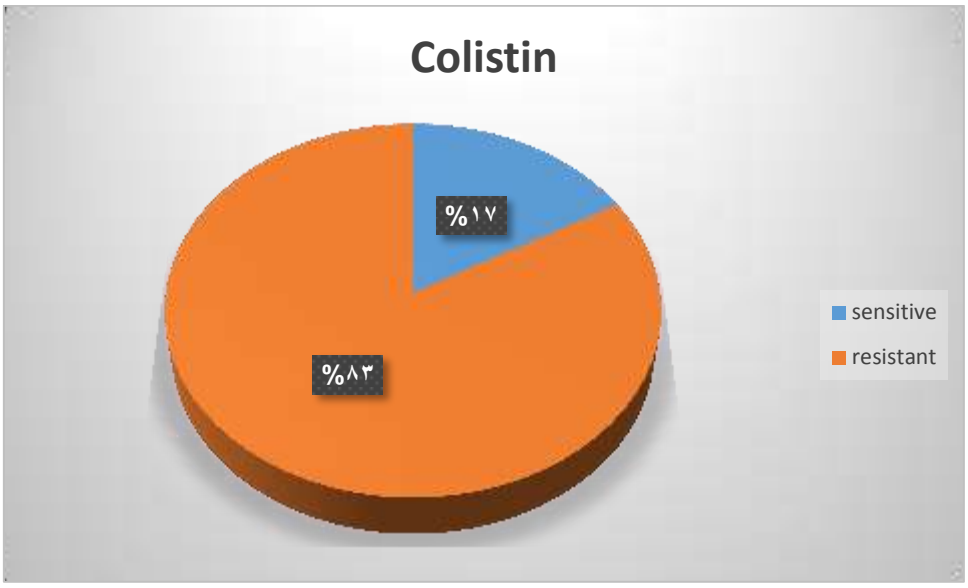

Figure (2): Percentage of Gram-negative bacilli according to susceptibility to Carbapenem antibiotic.

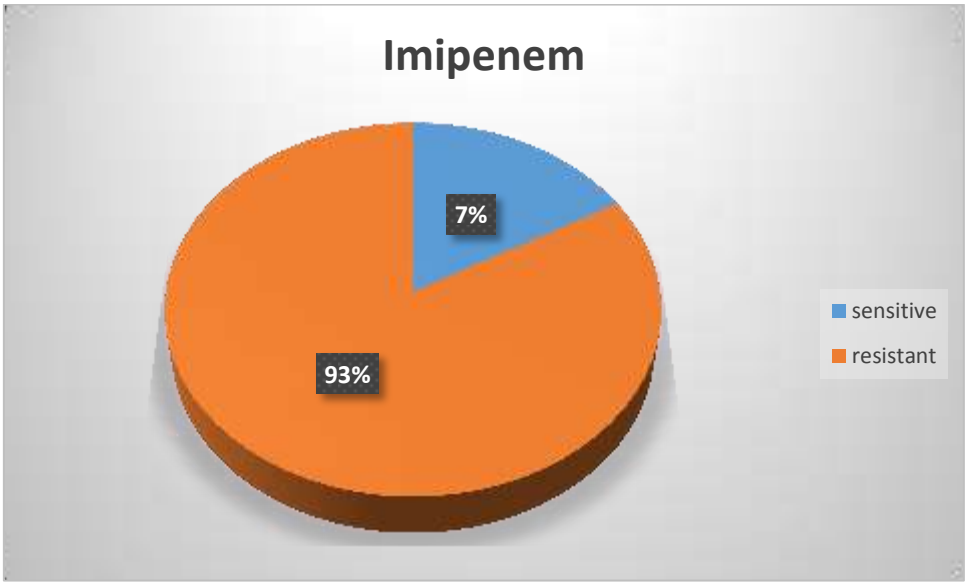

Table (6): **Cross tabulation between isolated organisms multidrug resistant antibiotics**

Organism	caz sensitive	caz resistant	co sensitive	Co Resistant	cip sensitive	cip resistive	Ak sensitive	ak resistant
Pseudomonas aeurginosa	0	4	0	4	0	4	2	2
Citrobactersp p	0	3	0	3	0	3	0	3
E.coli	2	39	11	30	2	39	19	22
Enterobacters pp	0	3	0	3	2	1	2	1
Klebsiellapneumoniae	0	25	5	20	2	23	14	11
Proteus spp	2	12	2	12	1	13	3	11
total	4 (8%)	86 (92%)	18 (20%)	72 (80%)	7 (8%)	83 (92%)	40 (44.5 %)	50 (55.5 %)

caz:ceftazidime

co:cotrimoxazole

cip:ciprofloxacin

ak: amikacin

Chapter Five

Chapter Five

Discussion, Conclusion and Recommendation

5.1. Discussion:

This study aimed to detect multidrug resistant Gram-negative rods from hospitalized patients in Khartoum state and the antimicrobial effect of *Nigella sativa* against the resistant isolates.

In this study *E.coli* was the most predominant organism among the isolated Gram-negative bacteria followed by *Klebsiella species*, that was agreed with study done by Dahab RA, *et al* in Sudan,2017, and Tamil Nadu, *et al* in India, 2017. This was not in agreement with the study conducted bySahas, *et al* in India, 2018 with showed that *Klebsiella species* was the most predominant isolates followed by *E.coli.*

In this study methanolic extract of *Nigella sativa* showed remarkable antibacterial activity isolated gram negative bacilli these results agreed with that obtained by Fatima A. Saleh ,*etal* (2018), the study conducted in India 2005 by Salman, *et al* and the study conducted by Nor' Aishah Hasan *et al* 2012. This is disagreed with the study obtained by Amir Riyaz Khan and Kirandeep Kour (2016) However, negative results do not indicate the absence of bioactive constituents, since active compound (s) may be present in insufficient quantities in the ethanolic extract to show activity with the dose levels employed.

The study conducted in by Salman *et al*., reported that methanolic extract showed remarkable dose dependant antibacterial activity

against isolates up to a dilution of 1:50 which is agreed with our study.

The clinical isolates of *P.aeruginosa*were also inhibited in any of the concentration tested, this result agreed with that report by Al-Jaafary *et al*. (2016).and clinical isolates of E.coli which were inhibited in any of the concentration tested, not similar results were obtained in a study by Al-Jaafary et al. (2016); this may be due to use of oil not methanol extraction.

5.2. Conclusion:

This study concludes that:

-The resistance of Gram negative bacilli to carbapenem and colistin is high.

- E.coli was the most resistant isolate to carbapenems and the *proteus*species most resistant isolate to colistin.

-Modified hodge test is simple, cheap method for detection of carbapenemase enzymes production but it can't determine the type ofcarbapenemase enzymes specifically.

-Nigella sativa has an antimicrobialeffect against carbapenemsand colistin resistant Gram negative bacilli with highest effect in concentration 50.

5.3. Recommendation:

-larger sample size should be tested to cover wider range of isolates.

-specific methods for detection carbapenem and colistin resistance bacteria isolates should be used routinely.

-More specific tests including PCR should be used.

-Using of another extraction methods to know the best extraction methods which gives the best effect.

-Proper control strategy to control the spread of the resistance isolates.

-More researches in herbs antimicrobial effect to increase the use of herbs in treatment of bacterial infection and to find suitable alternatives herbal treatment that can be used as first line before antibiotics use.

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Appendix 1

Materials

A-Equipment

Autoclave.

Bunsen burner.

1- Cork borer

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-Nutient agar

Typical formula in g/L

Contents

Peptone……5.0
Meat extract……3.0
Agar……..15.0
pH7.0±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-Media for biochemical reactions (Kligler iron agar, simmon‘s citrateagar, christensin urea media, media containing treptophan, semi solidagar 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 121ºc 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 1Lof D.W. Soak for 10 mins. Swirl to mix bring to boil. Ditribute into tubes and serilize by autoclave at 121ºc 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 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.

Peptone water

Typical formula g/L

Contents

Peptic digest of animal……...10.00

Sodium choride…….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.

3-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 C0 for 15 minutes. Transfer to a 50 C0water bat

2- When the agar has cooled to 50 C0, add aseptically the sterile bloodand 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 preventloss 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-phenyl enediamine dihydrochloride……0.1 g

Distilled water……10 ml

Preparation

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

Kovac's reagent

Contents

To prepare 20 ml:

4-dimethylaminobenzaldehyde……...1 g

Isoamyl alcohol (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

Concentraredsulphric 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 2.35gof di-hydrate barium chloride (Bacl2.2H2o) in 200ml of distilled water.

3- Add .5ml of barium chloride solution to 99.5 ml of sulphuric acid solution and mix