Sudan University of Science and Technology

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Antimicrobial Activity of *Vitis vinifera* Leaves Extracts against Multidrug Resistance Bacteria Isolated from Sudanese Patients with Diabetic Foot Infection

اختبار نشاط المضاد الميكروبي لمستخلص أوراق العنب ضد البكتيريا متعددة المقاومة للمضادات الحيوية

المعزولة من المرضى السودانيين المصابين بإلتهاب القدم السكرية

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(قُلْ إِنَّ صَلَاتِي وَنُسُكِي وَمَحْيَايَ وَمَمَاتِي لِلَّهِ رَبِّ الْعَالَمِينَ)

صدق ا لله العظيم

سورة الانعام الآية ١٦٢

DEDICATION

To the candle which burns to light my life

My mother and father

To my beloved sister Mawdda for her support

To my brothers

And

To my teachers and friends who support me

Acknowledgments

First of all, my thanks to ALLAH for giving me health and strength to accomplish this research work.

I would like to express my sincere gratitude to my supervisor Dr. Samar Mohammed Saeed for helping me to accomplish this research, follow up in correcting, as well as her support and encouragement.

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ABSTRACT

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

This study aimed to isolate and identify the bacterial pathogens of diabetic foot wound infection in Khartoum State and test their susceptibility to the most common antibiotics used in treating patients with diabetic foot wound compared to the activity of different concentrations of methanol extract for medicinal plant *vitis vinifera* leaves using Agar disc diffusion method under standard laboratory conditions.

This was a descriptive cross-sectional study conducted in Khartoum State-Sudan, during the period from March 2018 to August 2020.

One hundred wound swabs were collected form diabetic patients of foot infections from both gender (68% males and 32% females).

Out of the hundred specimens 93(93%) showed bacterial growth, from which five types of pathogenic bacteria were isolated and identified.

Fifty-three isolates were Gram negative bacteria (57%) and 40 (43%) were Gram positive bacteria.

The most predominant isolate was *Staphylococcus aureus* 40(43%) followed by *Pseudomonas aeruginosa* 15(16%), *Klebsiella pneumoniae* 13(14%), *Proteus mirabilis* 13 (14%) and *Escherichia coli* 12(13%).

The methanol extract of *Vitis vinifera* leaves carried out on Soxhlet extractor. It was screened for the antibacterial activity against multidrug resistant isolates and the results showed a high antibacterial activity at 100% concentration, moderate activity at 50% and low activity produced by 25 % concentration.

This result authenticates the antibacterial activity of *Vitis vinifera* leaves and support the traditional use of the plant in therapy of bacterial infection.

الخلاصة

الزيادة المقلقة في سلالات البكتيريا المقاومة للمضادات الحيوية الحالية تتطلب جهدا متجددا للحصول علي عناصر فعالة ضد هذه البكتيريا.

هدفت هذه الدراسة الى العزل والتعرف على مسببات الامراض البكتيرية المرتبطة بجروح القدم لدى المرضى المصابين بداء السكري في ولاية الخرطوم واختبار مدى قابليتها للعقاقير الطبية الأكثر استخدامًا في العلاج القرص مقارنة مع نشاط تراكيز مختلفة من مستخلص الميثانول لأوراق العنب الطبية باستخدام طريقة الانتشار علي لوحات الاجار في ظل ظروف المختبر القياسية.

كانت هذه دراسة وصفية مقطعية أجريت في ولاية الخرطوم، خلال الفترة من مارس ٢٠١٨ وحتى أغسطس ٢٠٢٠.

تم جمع مائة مسحه من جروح الاقدام لمرضى السكري من كلا الجنسين (٦٨٪ من الذكور و ٣٢٪ من الاناث).

من بين ١٠٠ عينة ٩٣(٩٣٪) من العينات أظهرت نموًا إيجابيا للبكتيريا، تم عزل خمسه أنواع من البكتيريا المسببة للأمراض والتعرف عليها.

ثلاثة وخمسون (٥٧٪) من العينات البكتيرية سالبه الجرام و٤٠ (٤٣٪) من العينات إيجابية الجرام.

كان الأكثر شيوعا من البكتيريا المعزولة المكورات العنقودية الذهبية ٤٠(٤٣٪) تليها الزائفة الزنجارية ١٥(١٦٪)، الكلبسيلة الرئوية ١٣(%١٤)، المتقلبة الرائعة ١٣(١٤٪) والإشريكية القولونية ١٢(١٣٪).

تم فحص مستخلص الميثانول من أوراق العنب التي أجريت على جهاز السكسوليت. تم فحصها بحثًا عن نشاط مضاد بكتيري ضد البكتريا المعزولة متعددة المقاومة للمضادات الحيوية وأظهر المستخلص نشاط مضاد بكتيري جيد لمعظم السلالات البكتيرية التي تم اختبارها حيث اعطى نشاط جيد عند تركيز ١٠٠٪، نشاط متوسط عند ٥٠٪ ونشاط منخفض عند التركيز ٢٥٪.

هذه النتيجة تؤكد على نشاط مستخلص أوراق العنب كمضاد للميكروبات وتدعم الاستخدام التقليدي للنبات في علاج العدوى البكتيرية.

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List of Abbreviation

MIC	Minimum inhibitory concentration
CoNS	Coagulase-negative staphylococci
DFI	Diabetic foot infection
DM	Diabetes mellitus
DNAse	Deoxyribonuclease
E. coli	*Escherichia coli*
ESBL	Especially of extended-spectrum b-lactamase
HIV	Human immunodeficiency virus
KIA	Kligler Iron Agar
K. pneumoniae	*Klebsiella pneumoniae*
LDL	Low-density lipoprotein
MAPRI	Medicinal and Aromatic Plant Research Institute
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
NCCLS	National Committee for Clinical Laboratory Standards
(OPCs)	Oligomeric proanthocyanidins
S. aureus	*Staphylococcus aureus*
P. mirabilis	*Proteus mirabilis*
PS. aeruginosa	*Pseudomonas aeruginosa*
SPSS	Statistical Package of Social Science
SSIs	Surgical site infections
SSTIs	Skin and soft tissue infections
T1DM	Type 1 diabetes mellitus
USA	United States of America
V.vinifera	*Vitis vinifera*
WHO	The World Health Organization

CHAPTER ONE

INTRODUCTION

CHAPTER ONE
INTRODUCTION

1.1. Introduction

Diabetes mellitus (DM) now is a major health problem all over the world and is increasing globally at an alarming rate, it has been declared an epidemic in developing countries, approximately 347 million people are suffering from (DM) worldwide, which is predicted to double by the year 2025. Diabetic foot infection (DFI) is one of the most serious complications of (DM) and diabetic patients have a 25% increased risk of developing a foot ulcer (Karmaker *et al*., 2016).

Numerous factors related to diabetes can impair wound healing, including wound hypoxia, infection, nutrition deficiencies and the disease itself, also fluctuating blood sugar and hypoxia from poor circulation may impair the ability of white blood cells to destroy pathogenic bacteria and fungi leading to increase infection risk (Alsaimary, 2010).

The control of infectious diseases is badly endangered by rising in the number of microorganisms that are resistant to antimicrobial agent, these infections often fail to respond to conventional treatment, resulting in prolonged illness and greater risk of death (Odonkor and Addo, 2011).

So, searching for newer sources of antibiotics is a global challenge pre-occupying research institutions, pharmaceutical companies, and academia (Aruljothi *et al.,* 2014).

Development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and screening of medicinal plants for antimicrobial activities and photochemical is important for finding potential new compounds for therapeutic use (Abtahi *et al.,* 2011).

Medicinal plants have many healing properties, antibacterial is one of those properties without showing any significant side effect. In most countries of the world, knowledge of plant therapy has become an agenda in scientific researches and use of herbal medicine is constantly growing (Al-Ameri, 2017).

Grapes Fruits (*Vitis vinifera*) have been used for thousands of years because of their nutritional and medicinal benefits. They are rich in sugars, flavonoids, anthocyanins, organic acids, mineral salts (Jassim *et al*., 2010).

Recent studies have reported that the grape have the health benefits: reducing the risk of blood clots, reducing low density lipoprotein (bad cholesterol), preventing damage of blood vessels in the heart, and maintaining a healthy blood pressure (NuYi, 2011).

Grape leaves which have astringent and haemostatic properties, are used in the treatment of diarrhea, hemorrhage, varicose veins, hemorrhoids, inflammatory disorder, pain, hepatitis, and free radical related diseases, also externally for to heal wounds, drain furuncles and antiseptic for eye wash (Orhan *et al*., 2009).

1.2. Rationale

Diabetes mellitus (DM) is a major health problem in Sudan and is a leading cause of morbidity and mortality. Diabetic patients are may get diabetic wound infection which is serious problem (Awadalla *et al.,* 2017).

Multidrug-resistant organisms have spread widely, about 70 percent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only are treated with experimental and potentially toxic drugs (Odonkor and Addo, 2011)., so interest in plant-derived drugs is mainly due to the current widespread belief that "green medicine" is safe (Parekh and Chanda, 2006).

Usually in Sudan, plants are one of the main medicinal sources to treat infectious diseases so this study was done to screen the bacterial pathogens present in diabetic foot infection and to determine their antibiotic sensitivity pattern in comparison with plant extract from (*Vitis vinifera*) leaves.

1.3. Objectives

1.3.1. General objectives

To determine the antibacterial activity of *Vitis vinifera* leaves extract against selected multidrug resistant pathogenic bacteria from patients with diabetic foot infection in Khartoum State.

1.3.2. Specific objectives

1- To isolate and identify the most common bacterial from diabetic foot infection patients by conventional methods.

2- To determine the antimicrobial activity of most commonly used antibiotic against the isolated bacteria by Modified Kirby Bauer Disc Diffusion Method.

3- To evaluate the antibacterial activity of *Vitis vinifera* leaves methanol extracts on selected multidrug resistant isolates.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO
LITERATURE REVIEW

2.1. Background

Balandrin *et al.* (1985) defined medicinal plants as a plant in which one or more organs contain substances that can be used for therapeutic purposes or which it precursors for the manufacturing of drugs are useful for disease therapy (Aruljothi *et al.*, 2014). Therapeutic use of plants is as old as 4000 - 5000 B.C. and Chinese used first the natural herbal preparations as medicines (Kayastha, 2014).

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities, local people have a long history of traditional plant usage for medicinal purposes (Maroyi, 2013).

In recent years, the use of traditional medicine information on plant research has received considerable interest, World Health Organization (WHO) has also recognized the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines (Hosseinzadeh *et al.*, 2015).

Plants produce structures such as secondary metabolites (phenolic compounds, alkaloids, flavonoids, cumarins, glycosides, terpens, iso-flavonoid) and their derivatives have antifungal and antimicrobial properties (Sharifi-Rad *et al.*, 2014).

Despite the fact that the antimicrobial properties of plant extracts have undergone extensive evaluations in the field of clinical microbiology, the effects of these agents need to be further investigated in further clinical trials (Mirkarimim *et al.*, 2013).

Total of 82 Indian medical plants traditionally used in medicines were subjected to preliminary antibacterial screening against several pathogenic and opportunistic microorganisms and the results indicated that 56 exhibited antimicrobial activity (Ahmad *et al.*, 1998), the other hand extracts of 111 Sudanese medicinal plants were subjected to preliminary antibacterial screening against four standard organisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *pseudomonas aeruginosa*). Out of the 573 extracts screened, 433 (70%) exhibited inhibitory activity against one or more of the four tested bacteria (Almagboul *et al.*, 1994).

2.2. *Vitis vinifera*

2.2.1 Taxonomic Classification

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta – Vascular plants

Super division: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Monocotyledons

Order: Vitales

Family: Vitaceae

Genus: Vitis

Species: vinifera (Mustafa *et al*., 2008).

Botanical name: *Vitis vinifera*

Common name: Grape (Nuyi, 2011).

2.2.2. Origin and nature

Vitis vinifera is a deciduous woody climber with coiled climbing tendrils and large leaves, it has small, pale, green flowers in the summer followed by bunches of berry fruits that range from green to purple-black (Orhan *et al*., 2009).

They are rich in sugars, flavonoids, anthocyanin and proanthocyanins, organic acids, tannin, mineral salts and vitamins. Grapes skin, especially from the red and black species is rich in resveratrol which is a derivative of stilbene, studies have shown that resveratrol is one of the strongest known natural antioxidants, which found in a large quantity in black grape juice, skin and seed, it is antioxidant potential 50 times higher than vitamin C and E together, also prevents the oxidation of LDL cholesterol, lowers total cholesterol levels, also demonstrates an antibacterial action, reduces the risk of cardiovascular diseases and can prevent cancer development (Jassim *et al*., 2010).

Various studies reported positive health effects of grape products and pharmaceutical preparations from grape leaves are patented and commercialized as drug (Abed *et al*., 2015).

A study in Iran confirmed that *V. vinifera* leave extract has antifungal and antibacterial properties, corroborating the traditional therapeutic and can be used as a preservative in food (Sharifi-Rad *et al.*, 2014).

2.2.3. Distribution

The grapevine (*Vitis vinifera*) was domesticated between the seventh and the fourth millennia BC, in a geographical area between the Black Sea and Iran. It's comprising of around 60 inter-fertile wild Vitis species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental– temperate climatic conditions (Jana *et al*., 2016).

2.2.4. Medical uses

Grape vines and leaves use to treat skin, eye diseases, stop bleeding, anti-inflammation, relief pain, hemorrhoids and sore throats, ripe grape used to treat cancer, cholera, small pox, nausea, kidney and liver diseases, while grape skin use to protect heart, brain damage and inhibit the degenerative nerve diseases. Also grape seed extract is used in treatment of chronic venous insufficiency, blood pools in the legs, pain, swelling, fatigue, visible veins, diabetes and edema. It is used to protect collagen and elastin in skin (anti-ageing), and prevent damage to human liver cells caused by chemotherapy medication (NuYi, 2011).

In addition, it was used in conditions like burning sensations, anemia, leprosy and syphilis (Parekh and Chanda, 2006), also red wine is used to reduce the risk of leukemia, breast and prostate cancers (NuYi, 2011).

2.2.5. Chemical constituent

In recent times, there have been increased waves of interest in the field of research in natural products chemistry, and this can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites (Hosseinzadeh *et al.,* 2015). Previous chemical investigations have shown the presence of nutrients on grapes such as magnesium, vitamins A, B1, B2, B6 and C (Ahmad *et al.,* 2014), organic acids (malic, oxalic, fumaric, ascorbic, citric, tartaric acid and phenolic acids), tannins, anthocyanins, lipid, enzymes, carotenoids, terpenes and reducing or non-reducing sugars in leave of *Vitis vinifera*. Most of the therapeutic properties of the plant may be

attributed to phenolic compounds, which have received considerable attention due to their pharmacological effects including antimicrobial and antioxidant activities (Orhan *et al.,* 2009).

Both leaves and fruit of the grape vine contain a variety of other flavonoid compounds, inclouding (+)-catechin, (-)-epicatechin, (+/-)- gallocatechin, (-)-epicatechin 3-O-gallate, rutin, and luteolin. Grape skins contain the polyphenolic defence compound resveratrol which has attracted attention for its anticancer activity and its serum lipid-lowering and antiplatelet effects. Standardized grape seed extracts are reported to contain 92-95% oligomeric proanthocyanidins (OPCs) (Mustafa *et al*., 2008).

2.2.6 Antimicrobial activity

Several studies dealt with antibacterial activity of grapes extracts obtained from whole fruits, seeds, skins and leaves. Thimothe *et al.* (2007), demonstrated inhibition of glucosyl transferases B and C in *Streptococcus mutans* by all the grape extracts studied. Organic extracts from seeds showed bacteriostatic activity against anaerobic bacteria responsible for different diseases (Mohammed *et al*., 2016).

Among those antimicrobial compounds, phenolic compounds, terpenoids, and alkaloids are very important components in antimicrobial effects (Abtahi *et al*., 2011).

The phenolic compounds, with an ability to damage microbial cells by exerting an influence on the selective permeability of the plasma membrane, which results in the leakage of vital intracellular substances (Mirkarimi *et al*., 2013).

In Palestine, they found that grape leaves extract was effective against *Ps. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 6538) (Abed *et al*., 2015).

2.3. Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both, the chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Abutaleb, 2016).

According to American Diabetes Association 2014 guidelines there are three schemas to classify the disease: based on the pathophysiology, a specific gene defect itself, or on another common phenotype. No classification scheme is ideal and all have some inconsistences and overlap, until recently, the prevailing conceptual classification was that there were two primary types of diabetes mellitus: autoimmune (type 1) and non-autoimmune (type 2). Advances in classification terminology have included the evolution of autoimmune diabetes from juvenile to insulin dependent to type 1 diabetes mellitus (T1DM) (Thomas and Philipson, 2015).

Foot wounds are now the most common diabetes-related cause of hospitalization and are a frequent precursor to amputation, individuals with diabetes have a 30-fold higher lifetime risk of undergoing a lower-extremity amputation compared with those without diabetes and infected foot wound precedes about two-thirds of lower extremity amputations, and infection is surpassed only by gangrene as an insurance and funding agencies would benefit from knowing the true incidence, the most common types, the clinical and demographic predisposing risk factors, and the outcomes of these infections, this information could help to predict which patients are at highest risk for diabetic foot infections, thereby helping to plan optimally targeted preventative strategies (Lavery *et al.*, 2006).

There are several well accepted predisposing factors that place patients with diabetes at high risk for a lower extremity amputation, the most common components in the causal pathway to limb loose include peripheral neuropathy, ulceration, infection and peripheral vascular disease (Alsaimary, 2010).

2.3.1. Diabetes foot infections

Over the past 25 years, many studies have reported on the bacteriology of diabetic foot infection but the results have varied and have often been contradictory. Foot ulcers and other foot problems are a major cause of morbidity and mortality in people with Diabetes mellitus (Paul *et al*., 2009).

(DFIs) are the leading cause of hospitalization diabetic patients worldwide and in developing countries like India, it accounts about 20% of hospital admissions. DFI is a multifactorial process and three factors predispose to tissue damage, namely neuropathy, peripheral vascular disease and susceptibility to infection whenever there is a direct injury to the foot at risk (Saseedharana *et al*., 2018).

Once an infection has developed in DFI patients, it is difficult to treat because of impaired microvascular circulation to the lower limb, which limits the access of phagocytic cells and antibiotics to infected sites (Karmaker *et al*., 2016).

If the infection progresses, many patients require hospitalization and all too often surgical resections or an amputation (Lipsky *et al*., 2012).

The primary causes of DFI are microbial agents and their early diagnosis is essential for appropriate antimicrobial therapy, these causative agents are usually polymicrobial, caused by aerobic Gram-positive cocci like *S. aureus*, Gram negative bacilli (*Escherichia coli, K. pneumoniae, Pseudomonas aeruginosa*) and anaerobes, proper management of these infections needs appropriate antibiotic selection (Hitam *et al.,* 2019).

Empirical treatment is based on the pathogens and the susceptibility pattern seen in the community where the hospital is located, Beta-lactam antibiotics are the most commonly used antibiotics for bacterial infections, however, the accelerated emergence of antibiotic resistance to these groups of drugs among the prevalent pathogens is the most serious threat to the management of such infections, especially carbapenem resistance. These isolates are usually multidrug resistant, which further complicate the scenario (Saseedharana *et al.,* 2018).

A number of studies have found that *S. aureus* is the main causative but recent investigations reported a predominance of Gram-negative aerobes (Paul *et al*., 2009).

2.3.2. Bacteria isolated from DFIs

2.3.2.1. *Staphylococcus aureus*

Staphylococcus aureus belongs to the family Micrococcaceae and is part of the genus Staphylococcus, *S. aureus* is Gram positive cocci of uniform size, occurring characteristically in groups but also singly and in pairs, non-motile and non-capsulate (Cheesbrough, 2006).

S. aureus both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus*, device related infections and causes a variety of skin and soft tissue infections (SSTIs), ranging from the benign (e.g., impetigo and uncomplicated cellulitis) to the immediately life threatening. It is the most common pathogen isolated from surgical site infections (SSIs), cutaneous abscesses and purulent cellulitis (Tong *et al.*, 2015).

These infections cause over ten million outpatient visits and nearly a half-million hospital admissions per year in the USA. Interestingly, nearly a third of people are colonized with *S. aureus*. Methicillin-resistant *Staphylococcus aureus* (MRSA) first described in 1961. After decades of being a predominantly hospital-associated infection, in the twenty first century, MRSA has spread beyond hospitals to become a significant "community-associated" public health burden, as outlined by Fowler and colleagues in this special issue, that threatens to once again outpace antibiotic development (Myles and Datta, 2012).

2.3.2.2. *Escherichia coli*

E. coli is the best known and most important species of the genus Escherichia, one of the most prevalent members of Enterobacteriaceae.

E. coli is a Gram negative usually motile rod, minorities of strains are capsulated, aerobic and facultative anaerobic. Optimum temperature for growth is 36–37 ºC. It naturally found in the intestinal tract, soil, water and is the commonest pathogen isolated from patients with cystitis (Cheesbrough, 2006).

E. coli is considered as the normal bowel flora of different species of mammals and birds but some strains of *E. coli* possess pathogenic character due to the acquisition of virulent factors. Microbial characteristics associated with virulent *E. coli* include production of enterotoxin, verotoxin, colicins, siderophores, type-1 pili and motility, resistance to the lytic action of the host complement and antibiotics. *E. coli* O157:H7

was first recognized in 1982 as human pathogen and cattle have been identified as a major source of *E. coli* O157:H7 infection of human (Zinnah *et al*., 2007).

2.3.2.3. *Klebsiella pneumoniae*

The medical importance of the genus *Klebsiella* (family Enterobacteriaceae) led to its being subdivided into three species corresponding to the diseases they caused: *K. pneumoniae, K. ozaenae, and K. rhinoscleromatis. Klebsiella* spp are well known to most clinicians as a cause of community-acquired bacterial pneumonia. *K. pneumoniae*, the most medically important species of the genus, its associated with hospitalization primarily attack immunocompromised individuals suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. There has been renewed interest in Klebsiella infections because extensive spread of antibiotic-resistant strains, especially of extended-spectrum b-lactamase (ESBL)-producing strains (Podschun and Ullmann, 1998).

K. pneumoniae is Gram negative, non-motile, usually capsulated rods aerobes and facultative anaerobes and non-motile (Cheesbrough, 2006).

It is ferments glucose and produce urease enzyme, but negative for methyl red (Holt *et al*., 2015).

2.3.2.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a Gram-negative bacterium, which recognized as a serious opportunistic pathogen. While healthy individuals with competent immune systems are usually not at risk of infection by *Ps. aeruginosa*, patients rendered immunocompromised due to burn injuries, surgical procedures, or cancer chemotherapy are highly susceptible to life-threatening infections (Sadikot *et al.,* 2005).

It has been consistently associated with the highest case-fatality rate for any of the bacteraemic, Gram- negative rod infections. An examination of why this organism becomes rapidly bacteraemic has involved identification and study of a number of cellular and extracellular products that have been proposed as *Ps. aeruginosa* virulence factors, including exotoxin A, alkaline proteases, elastase and more recently exoenzyme (exotoxin). It has become apparent that the pathogenesis of infections due to *Ps. aeruginosa* is multifactorial and quite complex, with different virulence

components exhibiting different levels of importance, depending on the type of infection and the animal model studied (Drake and Montie, 1988).

Ps. aeruginosa is responsible for 10–15% of the nosocomial infections worldwide, these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents. *Ps. aeruginosa* represents a phenomenon of antibiotic resistance, and demonstrates practically all known enzymic and mutational mechanisms of bacterial resistance, these mechanisms exist simultaneously, thus conferring combined resistance to many strains (Strateva and Yordanov, 2009).

2.3.2.5. *Proteus mirabilis*

The genus *Proteus*, which was described for the first time by Hauser in 1885, belongs to the Enterobacteriaceae family. In this family it is placed in the tribe Proteeae, together with the genera Morganella and Providencia. It currently consists of five species: *P. mirabilis, P. vulgaris, P. penneri, P. hauseri* and *P. myxofaciens* (Rozalski *et al*., 2012).

P. mirabilis, a Gram-negative rod-shaped bacterium, is well-known for its urease production and distinctive ability to differentiate into elongated swarm cells and characteristic bull's-eye pattern of motility on agar plates. *P. mirabilis* can be found in a wide variety of environments, including soil, water sources and sewage, but it is predominantly a commensal of the gastrointestinal tracts of humans and animals (Armbruster *et al*., 2018).

2.4. Previous studies

In most of the previous researches, extracts of the various parts of the grapes were used to screen for their antimicrobial activities respective against pathogens bacterial. Ahmad *et al.,* (2014), In Pakistan, who found the leaf extract of *Vitis vinifera* have inhibitory activity against the (*E. coli*, *P. aeruginosa S. aureus* and *E. faecalis*) by use Disk diffusion method, the other hand in Turkey, the water extracts of the grape leaves had shown the anticandidal activity against 3 Candida spp. (*C. albicans, C. glabrata, C. tropicalis)* (Yigit *et al*., 2009), also in Turkey, Orhan and his colleagues (2009), who reported the *Vitis vinifera* have antiviral activity towards Parainfluenza virus than oseltamivir.

Another study in Egypt, screening of antibacterial activity of ethanol, methanol and hexane extracts of grape leaves (*Vitis vinifera*) on *S. aureus*, *E. coli*, *Ps. aeruginosa* and Salmonella spp., were investigated, they were showed antibacterial activities against both Gram-positive and Gram-negative tested bacterial isolates, reported by Abdel-Hamid *et al.,* 2017).

In Iraq study reported, the *Vitis vinifera L.* extract effect on *S. aureus* and the sensitivity of the bacteria was gradually increased with the increasing of extract concentrations (Khashan *et al.,* 2017).

In Sudan, who found that the ethanolic extract *of Vitis vinifera* leaves showed a prominent activity against *Leishmania infantum* promastigotes (Mansour *et al.,* 2013).

CAPTER THREE

Materials and Methods

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Materials and Methods

3.1. Study design

This was a descriptive, cross-sectional hospital-based study.

3.2. Study area

The study was conducted in Khartoum state, Sudan University of Science and Technology (SUST), college of medical laboratory science. Wound swabs were collected from Jabir Abu Al-Izz diabetes Center in Khartoum and Diabetes Center in Khartoum Bahhri.

3.3. Study duration

This study was conducted during March 2018 and August 2020.

3.4. Study population

This study was performed on diabetes mellitus patients of different age groups suffering from wound of foot.

3.5. Inclusion criteria

Patients admitted to hospital with wound of foot infection problems were recruited and approved to participate in this study.

3.6. Exclusion criteria

Patients under antimicrobial treatment were excluded.

3.7. Sample size

One hundred wound swab sample (n =100) were collected from patient's diabetic foot infection (DFI).

3.8. Ethical consideration

Ethical approval to conduct this study was obtained from the college of Graduate Studies, Sudan University of Science and Technology (SUST). Permission was obtained from the hospitals in each of the centers that was included in the study. Verbal consent was taken from the patients to participate in this study.

3.9. Methodology

3.9.1. Collection of wound samples

Under aseptic condition, wound samples were collected using sterile cotton swabs after the wound was washed by sterile normal saline.

3.9.2. Culture of wound samples

The swab specimen was inoculated onto Blood and MacConkey agar (Hi Media laboratories PV+Ltd, India) plates under aseptic technique. The streaked plates were incubated at 37°C for 18-24 hours (Angel *et al.*, 2011).

3.9.3. Identification of the isolates

3.9.3.1 Cultural characteristics

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

3.9.3.2. Gram stain method

Single pure colony was transferred from blood agar plates onto clean dry slide using a sterile loop, the smear was prepared and then fixed by heat. The smear was stained with Gram stain firstly crystal violet stain was applied for 60 seconds, washed with water followed by Lugol"s iodine for 60 secs, washed again then decolorized rapidly by alcohol, washed immediately with water and covered with Safranin for 2 min then washed and dried. Examination was done microscopically by oil immersion lens (X100) to detected Gram reaction and arrangement of bacteria (Carter and Cole, 2012).

3.9.3.3. Biochemical tests

3.9.3.3.1. For Gram positive bacteria

3.9.3.3.1.1. Catalase test

Two ml of 3% hydrogen peroxide solution was poured into a test tube, then by using a sterile wooden stick a colony of the tested organism was removed and immersed in the solution, if immediate active bubbling was observed this indicate for positive result (Cheesbrough, 2006).

3.9.3.3.1.2. Coagulase test (slide method)

A drop of normal saline was placed on a slide, and a colony of the tested organism was emulsifying to make a milky suspension, then a drop of undiluted plasma was added, mixed gently. Clumping of the organism within 10 seconds was observe (Cheesbrough, 2006).

3.9.3.3.1.3. Deoxyribonuclease (DNAse) test

The test was used to differentiate *S. aureus* (positive) from other Staphylococci species (negative). The tested organism was cultured onto a medium which contain DNA, after overnight incubation the colonies were tested by flooding the plate with a weak hydrochloric acid. The acid precipitates un hydrolyzed DNA. DNase produced colonies were surrounded by clear area indicating DNA hydrolysis (Cheesbrough, 2006).

3.9.3.3.1.4. Mannitol salt agar (MSA)

This test was used to differentiate *S. aureus* from other Staphylococci species. A portion of colony was inoculated on mannitol salt agar containing 75 g\l sodium chloride and incubated aerobically at 37ºC for 18-24 hrs. *S. aureus* ferment mannitol producing yellow colonies (Cheesbrough, 2006).

3.9.3.3.2. For Gram negative bacteria

3.9.3.3.2.1. Kligler Iron Agar (KIA)

The tested organism was inoculated in KIA medium, using a straight wire loop, agar butt was stabbed, the opening was closed and then the top slope was streaked (as zigzag). The medium was incubated at 37°Cfor 24 hrs., glucose fermentation, lactose fermentation, H2S production, gas production was looked for (Cheesbrough, 2006).

3.9.3.3.2.2. Indole test

The tested organism was inoculated into peptone water and incubated at 37ºC for 48 hrs. 1 ml of kovac’s reagent was run down along the side of the test tube. Appearance of a pink color in the reagent layer within a minute indicated positive reaction (Ananthanarayan, 2006).

3.9.3.3.2.3. Citrate test

It was done according to Cheesbrough (2006). Simmon’s citrate medium was inoculated with the tested organism and incubated at 37ºC for up to 48 hours; utilization of citrate was recognized by a bluish color.

3.9.3.3.2.4. Urease test

The test organism was inoculated into slope surface of Christensen's urea medium using sterile straight wire, the medium was incubated at 37 °C for 24 hrs. and pink color change indicates a positive result (Ananthanarayan, 2006).

3.10. Susceptibility testing techniques

The test was carried out using Modified Kirby Bauer Disc Diffusion Method. By using sterile wire loop, 3-5 colonies of similar appearance were select and emulsified in 2-4 ml of sterile physiological saline and compared with 0.5 McFarland's turbidity standard, by sterile swab, the suspension was inoculated in Mueller Hinton agar (Hi Media laboratories PV+Ltd, India) plate and by sterile forceps, each antibiotic disc was placed onto the inoculated plate, then incubated aerobically at 37°C for overnight.

The following antibiotic disks (Hi Media laboratories PV+Ltd, India) were used:

3.10.1. Antibiotic disc used for Gram positive bacteria (Table 3.1)

Table 3.1: The antibiotic disc used for Gram positive bacteria:

Antibiotic	Concentrations mc g/disc
Ciprofloxacin	5 mc g
Co-trimoxazole	25 mc g
Erythromycin	15 mc g
Penicillin	10 mc g
Methicillin	5 mc g

3.10.2. Antibiotic disc used for Gram negative bacteria (Table 3.2)

Table 3.2: The antibiotic disc used for Gram negative bacteria:

Antibiotic	Concentrations mc g/disc
Ciprofloxacin	**5 mc g**
Co-trimoxazole	**25 mc g**
Gentamicin	**10mc g**

Tetracycline	**30 mc g**
Imipenem	**10mc g**

Zones of inhibition were measured in mm and the result was interpreted according to standardized chart (Reller *et al.*, 2009).

3.11. Preservation of Organism

The isolates were Preserved in glycerol at 4°C. The glycerol peptone broth was prepared by adding 80 ml from peptone to 20 ml from glycerol.

3.12. Extraction of *V. vinifera*

3.12.1. Collection and Preparation of *V. vinifera* specimen

Fresh leaves were collected from Northern State in Sudan. The dried *V. vinifera* sample was cleaned from dust and grass. The species of grape (*V. vinifera*) were confirmed by the Medicinal and Aromatic Plant Research Institute (MAPRI).

3.12.2. Preparation of the extracts

Extracts were authenticated by protocol of Medicinal and Aromatic Plant Research Institute (MAPRI).

Extraction was carried out according to method descried by Azwanida, (2015). 50 g of the dried plant sample was coarsely powdered using mortar and pestle and successively extracted with petroleum methanol using Soxhlet extractor apparatus. Extraction carried out for about 2 hours daily to three days at 50°C. for methanol till the color of solvents at the last siphoning time returned colorless. Solvents were evaporated under reduced pressure using rotary evaporator apparatus at 60°C. Finally, the extracts were allowed to dry in Petri dishes till complete dryness.

3.12.3. The Yield percentage of *V. vinifera* leaves extraction

The yield percentage was calculated as followed: Weigh of extract obtained/Weight of plant sample*100. The yield of the extract was presented in (Table 3.3).

Table (3.3): **Weight and yield % of *V. vinifera* extracts obtained using methanol solvents:**

Solvents used	Weight of plant material (g)	Weight of extracts obtained (g)	Percentage Yield (%)
Methanol	50	3.352	6,704

3.12.4. Preparation of *V. vinifera* extracts for Testing the Antibacterial Activity

The crude extracts were diluted into different concentration as follows: 100%, 50% and 25% to be used against the selected organisms.

3.12.5. Preparation of isolation bacterial suspension

Each of the isolate bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) was inoculated into 3.0 ml of sterile normal saline. Inoculum density was compared with 0.5 McFarland stander solution.

3.12.6. Agar disc diffusion assay

The procedure which was accepted by National Committee for Clinical Laboratory Standards (NCCLS) and widely used now days, is a modification of that described by Bauer, Kirby, Sherris and Truck (commonly known as Kirby-Bauer test). The Agar disk diffusion technique has been widely used to assay plant extract for antimicrobial activity (Das *et al.*, 2010).

In this method, One Hundred discs were Prepared (6 mm diameter) of whatman filter paper by used borer. Put each 100 disks in test tub then closed it and sterilized by dry temperature at 140°C to 1h. Dissolved 1g of extracted material in 10 ml of solvent (methanol), then a three-fold serial dilution of extract was prepared in methanol (diluent) to achieve a decreasing range of extract concentrations from 100 mg/ml_ 50 mg/ml_ 25 mg/ml. Under aseptic conditions using sterile forceps to place 3 filter paper discs in agar and pressed down to ensure its contact with the agar then add 15 µl of 3 concentrations of *V. vinifera* leaves extracts were introduced into the 3 discs sequentially. The plates were allowed to stand for 30 min in the refrigerator for diffusion of the extract to take place and incubated at 37°C for 24 hrs. Same researchers done by (Nostro *et al.*, 2000).

3.12.7. Interpretation of results

After 24 hours' incubation antibacterial activity results were expressed in diameters of inhibition zones in millimeter, where 14-19 mm growth inhibition zones are considered to possess relatively high antimicrobial activity, and those resulting 10-13 mm are of intermediate activity, and those resulting in zone below 10 mm are inactive (Galvez, 2016).

3.13. Statically analysis

Analysis of the data was performed by SSPS version 16. to check frequency, mean and standard deviation. Data were presented in form tables and figures.

CHAPTE FOUR

RESULTS

CHAPTE FOUR

RESULTS

4.1. Distribution and frequency of study population according to gender

One hundred swab specimens were collected from patients with DFIs 68(68%) were males while 32(32%) were females as represented on figure (4.1).

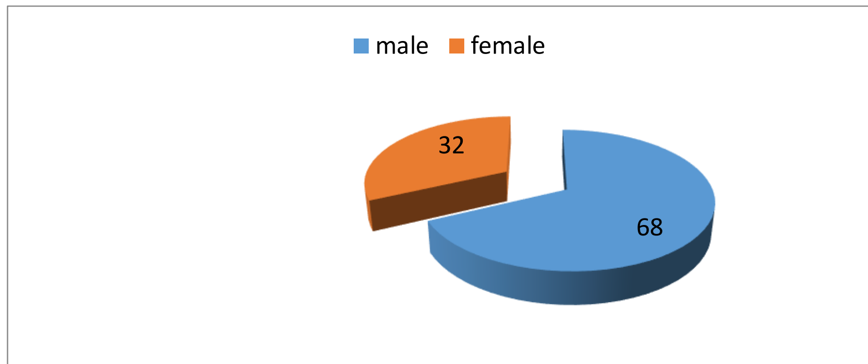

Figure 4.1.: Distribution and frequency of sampling according to gender

Out of 100 patients, 61 specimens were belonging to 20-40 age group, 29 from 41- 60 age group, while10 specimens were belonging to > 60 age group and the mean age was 43.8 ± 1.23 (SD) as shown in (Table 4.1).

Table 4.1: Frequency and percentage of study population according to age groups:

Age group	Frequency	Percentage %
20-40	61	61%
41-60	29	29%
>60	10	10%
Total	100	100%

All swabs were inoculated onto MacConkey agar and Blood agar plates, incubated and examined for growth, 93 (93%) showed significant bacterial growth while the remaining 7(7%) were negative for bacterial growth as displayed in figure (4.2).

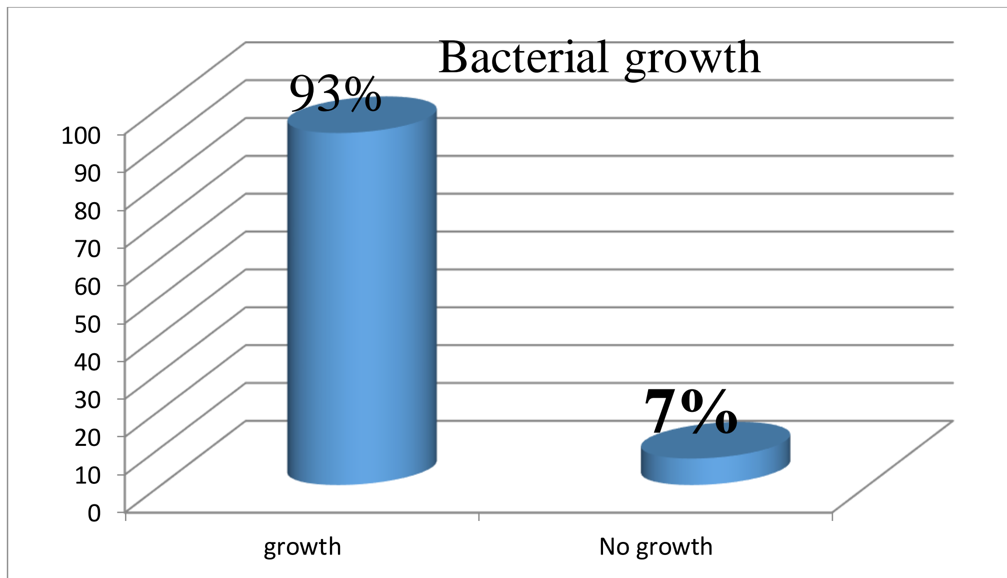

Fig.4.2: **Frequency and Percentage of bacterial growth on Blood and MacConkey agar**

Out of the 93 positive cultures for bacterial growth 40/93 were Gram positive (43%) and 53/93 (57%) are Gram negative shown in (Table 4.2).

Table 4.2: Bacterial Frequency and Percentage according to Gram stain:

Gram reaction	Frequency	Percentage
Gram positive	40	43%
Gram negative	53	57%
Total	93	100%

The isolates were identified phenotypically as follows: Gram positive species *Staphylococcus aureus* 40/93(43%), 21 of *S. aureus* was found resistant to Methicillin and considered as MRSA, while 4 Gram negative species were identified as follows: *Ps. aeruginosa* 15/93(16%), *K. pneumoniae* 13/93(14%), *P. mirabilis* 13/93(14%) and *E. coli* 12/93(13%) as shown in Table and Figure (4.3).

Table 4.3: Frequency and percentage of isolated bacteria:

Isolate	Frequency	Percentage
S. aureus	40	43%
Ps. aeruginosa	15	16%
K. pneumoniae	13	14%
P. mirabilis	13	14%
E. coli	12	13%
Total	93	100%

4.2. Frequency and percentage of isolated bacteria species according gender

Out of 93 isolated bacteria species 59/93 isolated from males, and 34/ 93 isolated from females, as shown in (Table 4.4).

Table 4.4: Frequency and percentage of isolated bacteria species according gender

Gender	Isolate bacteria					Total
	S. aureus	***Ps. aeruginosa***	***K. pneumoniae***	***P. mirabilis***	***E. coli***	
Male	31 33.3%	9 9.7%	4 4.3%	10 10.7%	5 5.3%	59 63.4%
Female	9 9.7%	6 6.5%	9 9.7%	3 3.2%	7 7.5%	34 36.6%

4.3. Effect of antibiotics used for sensitivity testing on clinical isolate

Antibiotic susceptibility test was performed against different selected antibiotics and the result was:

For *Staphylococcus aureus*: Forty isolates were tested, which showed variable resistance pattern to the tested antibiotics. 25 were resistant to three antibiotic or more. Resistance was higher to Penicillin, Ciprofloxacin and Erythromycin followed by Co-trimoxazole while Twenty-one of these were Methicillin resistant (Table 4.5).

Pseudomonas aeruginosa: Out of 15 isolates tested, 6 were resistant to 3 or more antibiotics. Resistance was high for Tetracycline followed by Co-trimoxazole and Gentamycin (Table 4.6).

Escherichia coli: Out of 12 isolates tested, 7 were resistant to three antibiotic or more. Resistance was high for Tetracycline and Co-trimoxazole followed by Ciprofloxacin (Table 4.6).

Klebsiella pneumoniae: Out of 13 isolates tested, 4 were resistant to 3 antibiotic or more. Resistance was high for Tetracycline followed by Co-trimoxazole and Ciprofloxacin (Table 4.6).

Proteus mirabilis: All thirteen isolates were sensitive to Imipenem. 8/13 antibiotics were resistance to Co-trimoxazole, Tetracycline and Ciprofloxacin (Table 4.6).

Table 4.5: Antibiotics used for sensitivity testing and number of *S. aureus* which were Sensitive and resistant to them:

number of *S. aureus* which were sensitive and resistant to antibiotic					
Antibiotic	**CIP**	**COT**	**ERYTH**	**PEN**	**METH**
Sensitive	16	23	21	7	19
Resistant	24	17	19	33	21

Key: CIP: Ciprofloxacin, COT: Co-trimoxazole, ERYTH: Erythromycin, PEN: Penicillin, METH: Methicillin

Table 4.6: Antibiotics used for sensitivity testing and number of Gram-negative bacteria strains which were Sensitive and resistant to them:

Antibiotic	***E. Coli***		***K . pneumoniae***		***Ps. aeruginosa***		***P. Mirabilis***	
	Sen	**Res**	**Sen**	**Res**	**Sen**	**Res**	**Sen**	**Res**
CIP	6	6	8	5	11	4	7	5
COT	5	7	7	6	7	8	4	8
GEN	11	1	10	3	7	8	11	1
TET	4	8	4	9	4	11	5	7
IMP	12	0	13	0	14	1	13	0
Total	12		13		15		13	

Key: **CIP**: Ciprofloxacin, **COT**: Co-trimoxazole, **TET**: Tetracycline**, IMP**: Imipenem, **GEN**: Gentamycin.

4.4. Results of *V. vinifera* leaves extraction

Thirty multidrug resistant isolates were selected and tested for their sensitivity to *V. vinifera* leaves methanolic extracts, these include 11/30(37%) of *S. aureus, K. pneumoniae* 4/30(13%)*, P. mirabilis* 3/30(10%), 6/30(20%) for each *E. coli* and *Ps. aeruginosa*. Methanolic extract of *V. vinifera* leaves showed obvious effect on the bacterial growth of clinical isolates. The antibacterial activity of methanol extract was found to increase when increasing the extract concentration.

S. aureus: 9/11 tested isolates were affected in concentrations 100% extract of *V. vinifera.* For 50% concentrations, it was active against one isolate and moderate active on 10 isolates. For the 25% concentrations, it has no activity against any of the isolates, moderate activity against 8 isolates and has no activity against 3 isolates. The higher concentration gave the higher effect on the growth of bacterial isolates. Two MRSA strains were found moderate active to methanol extract in concentrations of 100% (Table 4.7).

Table 4.7: **The activity of methanol extract of *V. vinifera* on Gram positive isolates:**

Bacteria Isolated	Total	Ex.Conc.mg/ml								
		100%			50%			25%		
		A	M	I	A	M	I	A	M	I
S. aureus	11	9	2	0	1	10	0	0	8	3

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

A: active, **M**: moderate, **I**: inactive

The antimicrobial test of methanol extract of *V. vinifera* showed the strong activity on 5 isolates tested of *Pseudomonas aeruginosa,* 4 isolates *Klebsiella pneumoniae*, all isolates of *Proteus mirabilis* and *Escherichia coli*, at the highest concentration applied of 100%. It showed activity against 2 isolates of K. *pneumoniae,* 1 of each *Ps. aeruginosa, E. coli* and *P. mirabilis* at the 50% concentration, while showed no activity in 25% concentration for all multidrug resistance isolates as displayed on (Table 4.8).

Table 4.8: **The activity of methanol extract of *V. vinifera* on Gram negative isolates:**

Bacteria Isolated	Total	Ex.Conc.mg/ml								
		100%			50%			25%		
		A	M	I	A	M	I	A	M	I
Ps. aeruginosa	6	5	1	0	1	5	0	0	4	2
E. coli	6	6	0	0	1	5	0	0	2	4
K. pneumoniae	4	4	0	0	2	2	0	0	3	1
P. mirabilis	3	3	0	0	1	2	0	0	2	1

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

A: active, **M**: moderate, **I**: inactive

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

DISCUSSION

5.1. Discussion

Diabetic foot infections cause a major public health problem and impose a heavy burden on health services and own family, it is responsible on patient hospital admission, drain of future earning power, major foot amputations, disability, loss work, family fragmentation and eventually death. Microorganisms can cause the ulcers of DM patients to become very inflamed, sore and delay healing (Al-Hegami *et al*., 2016).

The big problem was that bacteria do not respond well to antibiotic treatment due to high numbers of microorganisms' colonization in foot (Lipsky *et al*., 2005).

In this study, a total of one hundred patients suffering from DFI from both sex most of them were males (68%) followed by females (32%). This high infection is male might be due to outdoor activities of males which make them exposed to infections more than females. These findings are consistent with Kajetan *et al*., (1995), who reported 39 (65%) sample isolate from males and 21(35%) from females. However, the highest frequency of DFI belonging to age group between 20-40(61%) years followed by 41-60 years (29%) and > 60 years (10%).

This study showed that Gram negative aerobic bacteria were the most predominant isolates, similar result was reported by Gadepalli *et al.,* (2006), who found that Gram-negative isolates (51.4%) are greater than Gram-positive pathogens (33.3%) or anaerobes (15.3%). In contrast, this finding disagreed with Sharma *et al.,* (2006), who found that Gram positive bacteria were isolated more often than Gram-negative ones, increase in ratio of Gram-negative to Gram positive in this study may be refer to immunocompromised patient who are highly susceptible to hospital-acquired infections, either after colonization with environmental strains or followed invasive surgical.

The study revealed that the common isolates were *S. aureus* 40(43%)*, Ps. aeruginosa* 15(16%)*, E. coli* 12(13%)*,* while *K. pneumoniae* and *P. mirabilis* 13(14%), this results partially agree with Al-Hegami *et al.,* (2016), in Taiz City, who reported the most commonly isolated microorganisms from the diabetic foot ulcers were *S, aureus*

(18.9%), *Ps. aeruginosa* (37.8%), *E. coli* and *K. pneumoniae* (11.1%), *P. mirabilis* (4.4%) and other isolates were recorded in low frequencies.

The isolates were found to have high level of resistance to Ciprofloxacin, Co-trimoxazole, Gentamicin, Tetracycline and Penicillin. However, they were sensitive to Imipenem which was the most effective antimicrobial against all Gram-negative organisms, these results agreed with that obtained by Paul *et al.,* (2009), who found high levels of resistance to Ciprofloxacin, Co-trimoxazole, Amikacin, Gentamicin and Cephalosporins were found in all isolated organisms. Only Imipenem was the most effective for Gram negative organisms.

The results of antibacterial activity of *V. vinifera* leaves methanolic extract against wound infection bacterial isolates showed broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, using the agar-well diffusion method, these agreed with that report by (Orhan *et al*., 2009) and (Oskay and Sari, 2007), who reported *V. vinifera* leaves methanolic extract had broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria that are resistant to some antibiotics. In Sudan, Sir Elkhatim *et al.,* (2018), who found the grape fruit contain phenolic compound and vitamin C as well as high antioxidant activity rate.

The study showed the antibacterial activity of methanol extract was found to increase when increasing the extract concentration, these results partially agreed with that report by Mohammed *et al.,* (2016), who reported the highest effect of extract was observed at the concentrated extract while the lowest effect was observed at the highest dilution in the both alcoholic and aquatic extracts of *Vitis vinifera*.

This study concluded that this extract may be used in treatment of multidrug resistant aerobic bacterial pathogens after determination of minimum inhibitory concentration (MIC) as well as the toxicity of the active ingredients of the extract.

5.2. Conclusion

-Gram negative bacteria were more frequent than Gram positive bacteria in DFI.

-*S. aureus* is the most predominant isolate from DFI.

-There was high resistance to antimicrobial among the Gram-negative isolates.

-*V. vinifera* leaves methanol extract had antibacterial activity against same bacteria that showed resistant to a number of antibiotics, thus it can be used in the treatment of infectious diseases causes by resistant pathogenic bacteria.

-The efficiency of the antibacterial activity of extract was found to increase by increasing the concentration.

5.3. Recommendations

1- Based on this study and result, it is recommended that to isolate and the active ingredients in the compound extracts responsible for the antibacterial activity using gas chromatography.

2- Determination of the minimum inhibitory concentration (MIC) for active ingredient of each bacteria including in this study.

3- Determination of the toxicity of the active ingredients.

4- More research is required for verify these results and to understand the mode of actions of this plant.

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Appendixes

Appendixes

Appendix (1)

Material

A- Equipment

1-Autoclave

2- Hot air oven.

3-Bunsen burner.

4- Incubator.

5- Freezer dryer.

7- Cork borer.

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- Glassware

1-Petri dishes (plates).

2-Test tubes.

3-Measuring cylinder.

4- Flask with different size.

5- Funnels.

6- Sterile containers (bijou bottles).

7- Slides.

C- Disposable material

1- Filter papers.

2- Sterile cotton swab

3- Disposable syringes.

D- Culture media

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

1- Nutrient ager

Typical formula in g/L

Contents

Peptone…………………………………………..……50

Meat extract…………………………………......…3.0

Agar……………………………………………….…15.0

ṕH7.0± 0.2

Preparation

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

2-Manitol salt agar

Contents

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

1- Prepare the medium as instructed by the manufacturer. Sterilize by autoclaving at 121 ºC for 15 minutes. 2 -When the medium has cooled to 50–55 ºC, mix well, and dispense it aseptically in sterile petridishes. Date the medium and give it a batch number. Store the plates at 2–8 ºC preferably in plastic bags to prevent loss of moisture. pH of medium: 7.3–7.7 at room temperature.

3-DNA agar

Typical formula g/L

Contents

Tryptose………………………………………………20

Deoxyribonucleic acid…………………………………..2

Sodium chloride…………………………………………5

Agar……………………………………………………12

pH 7.2±0.2

Preparation

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

4-MacConkey Agar

Formula in grams per liter Bacteriological peptone.....................................20,00

Lactose... 10,00

Sodium Chloride..5,0

Bile Salts no 2 1,50

Neutral Red 0,05

Crystal Violet 0,001

Bacteriological Agar 13,50

Final pH 7,2 ± 0,2 at 25℃

Preparation

Suspend 50 grams of the medium in one liter of distilled water. Mix well. Heat with frequent agitation and boil until completely dissolved. Dispense into appropriate containers and sterilize at 121° C (15 lbs. sp.) for 15 minutes.

5-Mueller Hinton agar

Typical formula g/L

Contents

Casein acid hydrolysat…………………………17.50

Beef heart infusion……………….……………………...2.00

Starch,soluble……………………………………...1.50

Agar……………………………………………….....17.00

pH (at 25 ºc) 7.3±0.1 Preparation Suspend 38 g of powder in 1000 ml D.W mix well and heat to boiling to dissolve the medium completely. Sterilize by autoclave at 121ºC for 15 min

4-Media for biochemical reactions (Kligler iron agar, simmon's agar, christenensin urea media, media containing tryptophan, semi solid agar medium)

Kligler Iron Agar

Typical formula g/L

Contents

Balanced peptone……………………………………...20.0

Lactose…………………………………………………...10.0

Sodium chloride……………………………………..…1.0

Ferric ammonium……………………………………...…0.5

Sodium thiosulphate…………………………………………0.025

Agar…………………………………………………….....12.0

ṕH 6.9±0.2

Preparation

Dissolve 49g powder in 1IL Soak for 10 min. Swirl to mix bring to boil. Distribute into tubes and sterilize by autoclave at 121℃ for15 minutes. Let's the medium set as slopes about 3cm deeps.

Simmon's citrate medium

Typical formula in g/L

Contents

Magnesium sulfate……………………………………….....0.20

Monoammonium phosphate…………………………….…1.00

Dipostassium phosphate………………………………...1.00

Sodium citrate………………………………………….2.00

Sodium chloride…………………………………………...5.00

Bromothymol blue……………………………...…..0.08

Agar……………………………………………….15.00

ṕH 6.8±0.2

Preparation

Dissolve 24g of powder in IL of D.W. Bring to the boil. Dispense in tubes and sterilize by autoclaving at 121°C minutes. Solidify with the Long slant.

Christensen Urea Medium

Typical formula g/L

Contents

Gelatin peptone……………………………………..….1000

Dextrose…………………………………………………..1.00

Sodium chloride……………………………………..….5.000

Monopotassium phosphate………………………………2.000

Phenol red………………………………………………0.012

Agar……………………………………………….....15.000

ṕH 7.0±0.2

Preparation

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

Peptone water

Typical formula g/L

Contents

Peptic digest of animal……………………………………10.00

Sodium chloride……………………………………………….5.00

Phenol red…………………………………………….…0.02

ṕH 6.8±o.2

Preparation

-Suspend 15.0 g of powder in 100 ml D.W. Add the test carbohydrate in desired quantity and dissolve completely.

Dispense in tube with or without inverted Durham's tubes and sterilize by autoclaving at 121°C for 15Ib pressure (121°C) for 15 minutes.

E-Chemicals and reagents

1- Sodium chloride (normal saline).

2- Methanol.

3-McFarland turbidity standard

McFarland turbidity standard

Contents

Concentrated sulphric acid………………………………1 ml

Dihydrate barium chloride……….………………………0.5 g Distilled water………………..…………………………150 ml

Preparation

1- Prepare 1% (v/v) solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of water and mix well.

2- Prepare 1.175 % (w/v) solution of barium chloride by dissolving 2.35 g of dihydrate barium chloride ($Bacl_2.2H_2o$) in 200 ml of distilled water. 3- Add .5 ml of barium chloride solution to 99.5 ml of sulphuric acid solution and mix.

4- Kovc's reagent.

Kovc's reagent.

Contents

To prepare 20 ml:

dimethylaminobenaldehyde………………………………..1g

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 at 2-8°C.

Appendix (2):

Sudan University of Science and Technology College of Graduate Studies

Antimicrobial Activity of *Vitis vinifera* Leaves Extracts against Multidrug Resistance Bacteria Isolated from Sudanese Patients with Diabetic Foot Infection

Data collection form

Name…………….

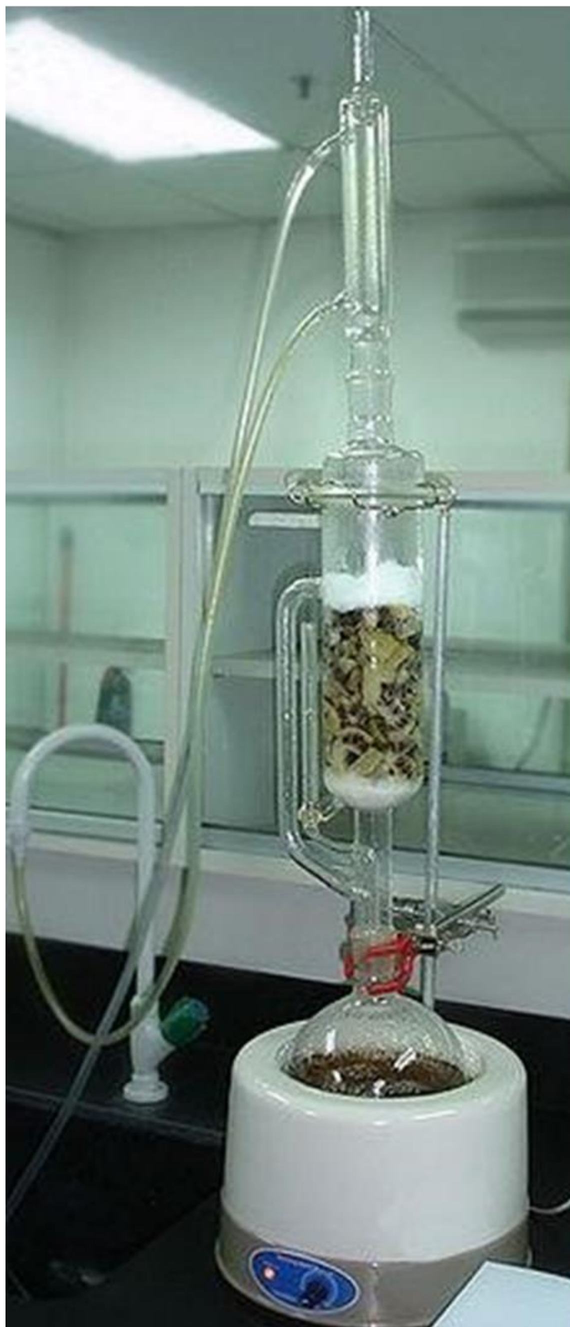
Age……………….

Sex……………….

History of disease……………

Under antibiotics treatment

Yes …...   No…….



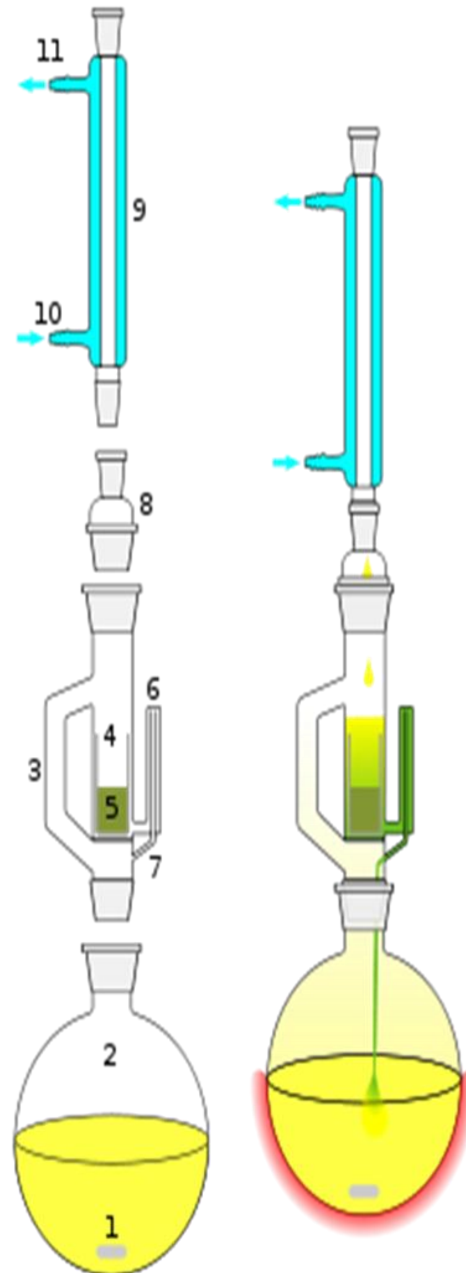


Figure 3: Soxhlet extractor apparatus

Figure 4: Antibiotic sensitivity test

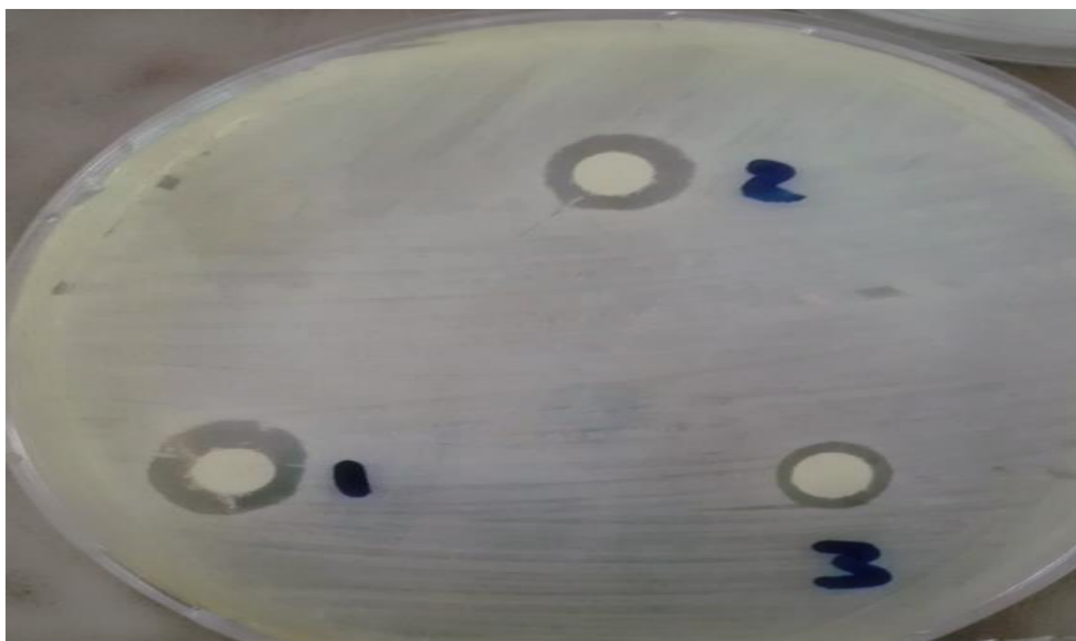


Figure 5: Antimicrobial activity of Methanolic extract of *V.vinifera* leaves against *S. aureus*.

Figure 6: Antimicrobial activity of Methanolic extract of *V.vinifera* leaves against *Pseudomonas aeruginosa*

Appendix (3): Incubator (Trope PicenardiCCRI, Italy)

Appendix (4): Autoclave (Medical Instrumention MFG CO,Mumbia)

Appendix (5): Hot air oven (Leader Engineering Widness Cheshire, UK)