Sudan University of Science and Technolog
College of Graduate Studies

Antimicrobial activity of *Aloe Barbadensis millar* (Aloe Vera) against *Pseudomonas aeruginosa* isolates in Khartoum state, Sudan

نشاط مستخلص نبات الالفيرا (الصبار الحقيقي) على معزولات الزائفة الزنجبارية في ولاية الخرطوم، السودان

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الاية

بسم الله الرحمن الرحيم

قال تعالى:

{وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُم مِّنَ الْعِلْمِ إِلَّا قَلِيلًا}

[الإسراء : 85]

صدق الله العظيم

Dedication

To my favorite parents...

To my mother (Aisha)

To my father (Musa)

Who supported me

To my favorites...

My sister's and brother's

For all who prayed for me

To my all friends who supported me

For all who helped me to complete this work

MAISONIII

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Abstract

Aloe vera is medicinal plant with antimicrobial properties against bacteria, viruses and fungi.

This study was done in Khartoum state to determine the antimicrobial activity of Aloe vera(Aloe vera) plant extract against *Pseudomonas aeruginosa* ***(P.aeruginosa)***.

The study was carried out on 100 bacterial isolates collected from different Khartoum hospitals in period from May to July 2019 .

The powdered plant material was extracted by using 85% ethanol and different concentrations are (100, 75, 50, 25, 12.5 mg/ml) prepared from plant extract And the antimicrobial activity of Aloe vera was assayed in vitro by agar well diffusion method against *P.aeruginosa* isolates.

P.aeruginosa isolates were collected from urine, wound, sputum, blood, ears, vagina, and ,pleural fluid, from patients with different ages. Conventional Biochemical techniques were used for isolates identification.

Antibiotic discs were used for bacterial *P.aeruginosa* isolates sensitivity on Muller Hinton Agar (MHA). The tested antibiotic discs were Ciprofloxacin, Gentamicin, Ceftazidime, Imipenem, Meropenem and Amikacin.

The concentration 100 mg/ml of the extract inhibited the growth of the all isolates 100(100%), while 75mg/ml inhibited 98(98 %) , 50mg/ml inhibited 75(75%), 25 mg/ml inhibited 18(18%) while the concentration 12.5mg/ml inhibited only 2 (2%) from total isolates.

It is concluded from this study the different concentration of Aloe vera extract can inhibit the growth of *P.aeruginosa*.

الخلاصة

نبات الالوفيرا هو نبات طبي له خواص مضادة للميكروبات كالبكتريا والفيروسات والفطريات. أجريت هذه الدراسة في ولاية الخرطوم لتحديد نشاط المضاد البكتيري في مستخلص **الصبار الحقيقي (الالوفيرا)** كمضاد حيوي لمعزولات بكتريا ***الزائفة الزنجبارية.***

اجريت الدراسة على عدد مائة عينة معزولة من نوع بكتريا *الزائفة الزنجبارية* والتي تم جمعها من مختلف المستشفيات في ولاية الخرطوم.

تم استخلاص مسحوق النبات باستخدام الكحول الايثانولي بتركيز 85% . مختلف التراكيز (100-75 -50 -25- 12.5مليجرام/مل) قد تم تحضيرها من المستخلص. ومن ثم تحديد نشاط مستخلص نبات الصبار عن طريق انتشاره من حفر الوسط الغذائي مولر هنتون علي معزولات بكتريا ***الزائفة الزنجبارية***.

تم جمع معزولات *الزائفة الزنجبارية* من عينات البول – الجروح- البلغم – الدم –الاذن الوسطى – المهبل والغشاء الرئوي من المرضى من أعمار مختلفة. وقد استخدمت الطرق التقليدية لعزل وتحديد نوع المعزولات البكتيرية.

استخدمت اقراص المضادات الحيوية على معزولات *الزائفة الزنجبارية* لتحديد حساسيتها في وسط المولر هنتون .والاقراص المختبرة هي سيبروفلوكساسين – جنتامايسين – سيفيتاذيديم – امبينيم – ميروبينيم و اميكيسين.

اظهرالتركيز 100 مليجرام/مل من المستخلص اظهر تثبيطا لنمو كل المعزولات 100 (100%) بينما اظهر التركيز 75 مليجرام/مل تثبيطا لنمو 98(98%) , 50 مليجرام/مل تثبيطا لنمو 75(75%), 25 مليجرام/مل تثبيطا لنمو18(18%) بينما اعطى التركيز 12.5مليجرام/مل تثبيطا لنمو 2(2%) من عدد المعزولات الكلي.

استنتج من هذه الدراسة ان مختلف التراكيز لمستخلص نبات الصبار الحقيقي يمكنها تثبيط نمو بكتريا ***الزائفة الزنجبارية.***

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List of abbreviations

Abbreviation	Name
Ak	Amikacin
CAZ	Ceftazidime
CIP	Ciprofloxacin
Dw	Distle water
GEN	Gentamicin
g/l	grams per liter
IPM	Imipenem
MDR	Multi Drugs Resistance
MEM	Meropenem
MHA	Muller Hinton Agar
NA	Nutrient Agar
TCAM	Traditional Complementary and Alternative Medicine
UTI	Urinary Tract Infection
WHO	World Health Organization

Chapter One
INTRODUCTION

CHAPTER ONE

INTRODUCTION

1.1. Introduction

Traditional, complementary and alternative medicine (TCAM) refers to a set of healthcare practices (indigenous or imported) that are delivered outside of the mainstream healthcare system (James *et al*, 2018).

The WHO estimates that a considerable number of people in Africa rely on TCAM to meet their primary healthcare needs. Due to increasing uptake of TCAM services across the continent recently has attracted the attention of policy maker, researchers, and healthcare professionals (James *et al*, 2018).

Sudan folklore medicine is characterized by a unique combination of Islamic, Arabic, and African cultures. In poor communities, traditional medicine has remained as the most reasonable source of treatment of several diseases and different infections (Karar and Kuhnert, 2017). Often traditional medicine provides the only available healthcare services to the population in many part of the country specially in rural area (Abdelhafeez, 2017)

Aloe vera is medicinal plant with antimicrobial properties which mentioned in the Bible several times herbalists and medical folklorists recognize the plant for centuries as the medicinal plant (Goudarzi *et al*, 2015).

P.aeruginosa found as a part of normal flora and significant pathogenic effect responsible for a wide range of nosocomial infections including

gastrointestinal infections, urinary tract infections and blood stream Infections (Preeti *et al*, 2019).

Infections caused by this organism are difficult to treat because of the presence of its innate resistance to many antibiotics (B.lactams and penem group of antibiotics including Beta-lactams, aminoglycosides and Fluroquinolons (Preeti *et al*, 2019).

Infections with *P.aeruginosa* have become a real problem in hospital-acquired infections especially in immunocompromised patients. The major problem leading to high mortality lies in the appearance of drugs-resistant strains, therefore , a vast number of approaches to develop novel anti-infective is currently pursued (Bassetti *et al*, 2018).

Treatment is available as eight categories of antibiotics to treat *P.aeruginosa* infections including aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B,(Bassetti *et. al* 2018).

1.2. Rationale

Infections caused by *P.aeruginosa* is worldwide distributed, and it's treatments is available by means of chemical antibiotics include Amikacin ,Cephalosporins, Ciprofloxacin, Imipeneme ,Gentamicin , and Meropenem but the excessive use of an antibiotics without medical instructions lead to many antibiotics resistance .

In recent years, multiple drug resistance in human pathogenic microorganisms is increased and there is need for use of new drugs effective and more cheaper from traditional plants like Aloe vera plant and verification of the antimicrobial activity of these plants to treat infections.

1.3. Objectives

1.3.1. General objectives:

To determine the antimicrobial activity of Aloe vera extract against *P.aeruginosa.*

1.3.2. Specific objectives:

1. To isolate *P.aeruginosa* from patients of different clinical symptoms.
2. To determine antimicrobial activity of Aloe vera alcoholic extract against *P.aeruginosa* isolates by agar diffusion method.
3. To determine the sensitivity of the isolates to commonly used antibiotics by disc diffusion method .
4. To compare between the susceptibility of *P.aeruginosa* isolates to antimicrobials and Aloe vera alcoholic extract.

Chapter Two

LITERATURE REVIEW

CHAPTER TWO

2. LITERATURE REVIEW

2.1.Aloe vera plant

Aloe vera is a cactus-like plant with green dagger shaped leaves filled with a clear viscous couler less gel. Aloe vera is a medicinal plant with anti-inflammatory, antimicrobial, anti diabetic and immune-boosting properties . Aloe vera is also mentioned in the Bible several times herbalists and medical folklorists for centuries as the medicinal plant . Leaf, commonly known as Aloe Vera gel, has been used since early times for a host of curative purposes in humans and animals (Goudarzi *et al*, 2015).

Aloe vera has more than 400 species of Aloe belonging to family liliaceae (Radha and Laxmipriya , 2015)

2.1.1. Plant discription

Aloe vera is succulent plant (Eggli, 2001). The leaf has Three Layers, AAouter layer is called Rind and has a protective function, synthesizes carbohydrates and proteins. The middle layer has a yellow sap which is bitter and contains anthraquinones and glycosides. An inner layer is clear gel and contains 96% water. The rest is made of amino acids, lipids, sterols and vitamins .The succulent property enables the species to survive in areas where there is no enough water supply (Yohannes, 2018) and the plant have ability to store a vast amounts of water in its thick leaves allow to survive long periods of desiccation (Johnson, 2012).

2.1.2. Scientific classification

Botanical name : Aloe barbadensis millar

Kingdom : Plantae

Class : Angiosperms

Order : Asparagales

Family : Asphodelaceae

Subfamily : Asphodeloideae

Genus : Aloe

Species : Aloe Vera

Common names: Aloe Vera Linné; True aloe; Aloe barbadensis (Ravi *et al*, 2011).

2.1.3.Plant distribution :

A.vera is considered to be native only to the south-west Arabian Peninsula. However, it has been widely cultivated around the world, and has become naturalized in North Africa, as well as Sudan and neighboring countries, along with the Canary Islands, Cape Verde, and Madeira Islands. It is also naturalized in wild areas across southern Spain, especially in the region of Murcia, being the only place in Europe where it has been found naturalized(Wikipedia, 2019) .

The species was introduced to China and various parts of southern Europe in the 17th century. It is widely naturalized elsewhere, occurring in temperate and tropical regions of Australia, South America, Mexico, and the Caribbean and southeastern US states. The current distribution may be the result of human cultivation (Wikipedia , 2019)

2.1.4.Therapeutic use of Aloe Vera

Aloe vera is the best source of drugs because this plant can survive in both cold and hot temperature (Santos *et al*, 1995).

Aloe vera is rich source of antioxidant (Hes *et al*, 2019) and Aloe has a history of traditional use by Native Americans for stomach disorders and intestinal disorders including constipation, hemorrhoids, colitis and colon problems. Aloe vera is fungicidal, anti-inflammatory, instrumental in increasing circulation, breaking and digesting dead tissue, and moisturizing tissues and the skin absorbs Aloe vera more than four times faster than water, it helps pores of the skin open and receive nutrients and moisture (Mbajiuka *et.al* , 2014).

Aloe vera have demonstrated as enhancers of immune system functioning within the body. Aloe also has the ability to stimulate macrophages (Mbajiuka *et.al* , 2014).

2.1.5. Chemical Constituents of Aloe Vera

More than 75 ingredients have been identified from the Aloe vera gel each of which may have a range of mechanism of actions, may acting synergistically or individually to explain more than 200 different components which include mucopolysaccharide, monosaccharide, sterols, prostaglandins, minerals, proteins, enzymes and wide variety of vitamins and minerals. It contains several potentially active bioactive compounds including salicylates, magnesium lactate, acemannan, lupeol,campestrol, β-sitosterol, aloin A and anthraquinones .In addition Aloe vera contains at least seven super-oxide dismutases with antioxidant activity (Mbajiuka *et.al* , 2014).

2.1.5.1. Vitamins

Aloe Vera Contains: Vitamin A, C, and D (are antioxidants). Vitamin B and choline , Vitamin B12 (folic acid) is responsible for the production of red blood cells (Yohannes, 2018).

2.1.5.2. Anthraquinones

Anthraquinones are Present in A.vera Like: Aloin, Isobarbaloin, Anthracene, Emodin, Barbaloin, Anthranol, Aloetic acid, Aloe Emodin and Resistannol. They act as natural laxatives, and analgesics and they contain powerful antibacterial, antifungal and antiviral properties (Yohannes, 2018).

2.1.5.3.Minerals

A.vera Contains the Following Minerals: Calcium -Manganese -Sodium-Copper - Magnesium-Potassium-Zinc-Chromium and Iron (Yohannes, 2018).

2.1.5.4.Sugars

Aloe vera provides monosaccharides (Glucose and fructose) and polysaccharides (Glucomannans andpolymannose).These are derived from the mucilage layer of the plant. The most common polysacherides are glucomannans (beta1-4,acetylated mannan). Aglycoprotein with anti-allergic properties, called alprogen has been isolated from Aloe vera gel (Yohannes, 2018).

2.1.5.5. Fatty Acids

A.vera containsFour Plant Steroids: cholesterol, campesterol, beta-sisosterol and lupeol. All these have anti-inflammatory action and lupeol also possesses antiseptic and analgesic properties (Yohannes, 2018).

2.1.5.6. Enzymes

Some Enzymes in the A. Vera Are: peroxidase, alliase, catalase, lipase, cellulose, carboxypeptase, amylase and alkaline phosphatase (Yohannes, 2018).

2.1.5.7. Hormones

Auxins and gibberellins are help in wound healing and have anti-inflammatory actions (Yohannes, 2018).

2.1.5.8. Others

Aloe vera provides 20 of 22 required and 7 essential amino acids. It also contain salicylic acid that possesses anti-inflammatory and anti-bacterial Properties (Yohannes, 2018).

2.1.6. Benefits of Aloe vera plant

Aloe vera gel is very useful for teeth and gum The Dentistry reported that the using of Aloe vera in tooth paste is effective in fighting cavities, constipation treatment, diabetes-induced foot ulcers, antioxidant and possible antimicrobial properties, protection from ultraviolet (UV) irradiation, protection from skin damage after radiation therapy, wounds healing from second-degree burn and irritable bowel syndrome (IBS) (Ruggeri, 2018).

2.2. *Pseudomonas.aeruginosa* (*P.aeruginosa*)

Is a classic opportunistic pathogen widely distributed in soil and water, plants and animals. *P.aeruginosa* colonize humans GIT and skin in small number as normal flora. It is the major human pathogen invasive and toxoginic produce infection in who with abnormal host defense, and is an important nosocomial pathogen, with innate resistance to many antibiotics and disinfectants (Geo *et al*, 2007).

2.2.1. Epidemiology

Pseudomonas species normally inhabit soil, water, and vegetation and can be isolated from the skin, throat, and stool of healthy persons they often colonize hospital food, sinks, taps, mops, and respiratory equipment. Spread is from patient to patient via contact with fomites or by ingestion of contaminated food and water (Iglewski, 1996).

2.2.2.Pathogenesis

P.aeruginosa become pathogenic when introduced into area devoid of normal defenses, which lead to colonization of bacteria and production of systemic disease. These processes promoted by the virulence factor (Geo *et al*, 2007).

Almost all strains of *P.aeruginosa* are hemolysins producers, phospholipase C, leukocidine and Some strains of *P.aeruginosa* produce large amounts of extrac ellular polysaccharide facilitate colonization and persistence.Most strains of *p.aeruginosa* produce aphenazine pigment and fluorescein facilitate colonization by *P.aeruginosa*.Also extracellular protease, toxin which have ability to inhibit protein synthesis in susceptible cells. Exo enzymes is produced by most *pseudomonas* (Iglewski, 1996).

2.2.3.Morphology

Gram-negative non sporing rods, usually motile with single polar flagellum (Cheesbrough, 2004).

2.2.4. Mechanism of drug resistance

One of the most worrisome characteristics of *P.aeruginosa* is its low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (e.g., mexAB, mexXY, etc.). And the low permeability of the bacterial cellular envelopes. In addition to this intrinsic resistance,

P.aeruginosa easily develops.Also acquired resistance either by mutation in chromosomally encoded genes or by the horizontal gene transfer of antibiotic resistance determinants. In addition to production of antibiotic-degrading or antibiotic-inactivating enzymes. And outer membrane proteins to evict the antibiotics and mutations to change antibiotic targets (Collee *et.al*, 1996).

2.2.5. Culture

P.aeruginosa is an obligate aerobe can grow on many type of culture media, sometimes produce a sweet or grape-like or corn taco-like odor. It forms smooth round colonies with fluorescent green color diffusing pigmentation. and it often produce the non fluorescent bluish pigment (pyocyanin). Other pseudomonas species don't produce pyocyanin. Many strains of *P.aeruginosa* also produces the fluorescent pigment (pyoverdin) . Some strains produce the darker pigment (pyorubin) or the black pigment (pyomelanin) (Geo *et al*, 2007).

2.2.6.Growth characteristics

.P.aeruginosa grow well at 37 42°C, the growth at 42°C helps differentiate from other *Pseudomonas* species in the fluorescent group (Geo *et al*, 2007).

2.2.6.1.Blood agar and Chocolate blood agar

Appeared as large colonies with metallic sheen, it may be mucoid, rough or pigmented showing pyocyanin production, and often beta haemolytic (Geo *et al*, 2007).

2.2.6.2.MacConky agar

Appeared as lactose non fermenting with pigmentation or metallic sheen (Geo *et al*, 2007).

2.2.7. Biochemical reactions

P.aeruginosa appear to be inert in typical tests including API20E .But it Characterized by pigment production (Collee *et al*,1996).

Table 1: Biochemical tests of *P.aeruginosa*

Test	**KIA**	**Motility**	**Indole**	**Citrate**	**Urease**	**Oxidase**	**H_2S**
Result	red/red	+	-	+	-	+	-

2.2.8. Control

P.aeruginosa is primarily a nosocomial pathogen, and the method of control similar to that of nosocomial infections (Geo *et al*, 2007).

2.2.9. Treatment

Treatment is available as eight categories of antibiotics to treat *P.aeruginosa* infections including aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), fosfomycin and polymyxins (colistin, polymyxin B) ,(Bassetti *et. al*, 2018).

2.3.Previous Studies

Aloe vera leaf gel is widely used as a traditional folk medicine for the treatment of different infectious diseases. In vitro antimicrobial properties of ethanolic extract of Aloe vera leaf gel were investigated against various common pathogenic bacteria and fungi. The well and disc-diffusion method showed significant zone of inhibition against all the

pathogens studied and the results are comparable to the conventional antibiotics. These results support the ethanomedicinal use of Aloe vera for the treatment of various infection (Subramanian *et.al*, 2006).

An experimental study done in Kenya This study was aimed to determine the anti-microbial activity of aqueous and methanol leaf extracts of Warbugia ugandensis, Moringa oleifera and Aloe vera on standard bacteria and multi drug resistant clinical isolates of *Pseudomonas aeruginosa*, *Staphylococcus.aureus,* and *Escherichia.coli* Tetracycline drug was used as the reference drug.The bacteria were treated with extracts at different concentrations to determine the zones of inhibition Through Agar Diffusion Assay, minimum inhibitory concentration and minimum bactericidal concentration assays. Raw data was analyzed using one-way and two-way analysis of variance followed by Tukey'spost hoc test. Zones of inhibition ranged from 6.5 mm to 9.98 mm on the multi-drug resistant isolates, while those of the standard bacteria ranged from 6.5 mm to 12.0mm (Muhuha *et .al*, 2018).

Methanol extracts of W.ugandensis, M.oleifera and A.vera at the concentration of 400mg/ml had higher zones of inhibition against multi-drug resistant *S.aureus ,P.aeruginosa and E.coli* respectively. The antimicrobial activity of the extracts indicated concentration-dependent response. The minimum bactericidal concentration values obtained were double the minimum inhibitory concentration values. Methanol extracts recorded lower minimum inhibitory and minimum bactericidal concentrations compared to aqueous extracts (Muhuha *et .al*, 2018).

Phytochemicals which were present, included alkaloids, cardenolide glycosides, phenols, flavonoids, coumarins, tannins, saponins and anthracin glycosides. These phytochemicals are associated with antimicrobial activities. This study showed potent antimicrobial activities

of methanol and aqueous extracts of W.ugandensis, M.oleifera and A.vera against the multi-drug resistant and standard bacteria tested.The extracts, therefore, may be used to develop alternative therapeutics in the management of multi drug resistant *Pseudomonas.aeruginosa, Staphylococcus.aureus, and Escherichia.coli* (Muhuha *et .al*, 2018).

A study done in Pakistan to evaluate the antibacterial activity of Aloevera Barbadensis Miller (Aloe vera) by using agar diffusion assay and gel filtration chromatography. The bacterial strains used in this research work were
Escherichia.coli ,*Bacillus.subtilius* ,*Salmonella.typhi* ,*Pseudomonas,Kleb siella.pneumoniae*, *Staphylococcus.epidermidis*.Aloe vera plant leaves and gel were macerated in different organic solvents including ethanol, methanol and distilled water. Then, by using agar diffusion assay and antibacterial activity was estimated. the zones of inhibition were measured by scaling and represented by tables and graphs. The Aloe Vera extract of Methanol showed the maximum antibacterial activity as compared to other solvent extracts. Then, distilled water macerated form Aloevera leaves were used for gel filtration chromatography technique in order to determine the fraction containing the active components. Fraction 8 showed maximum antibacterial activity against all above mentioned bacterial strains. This study reveals the plausibility of the presence of some bioactive components in Aloevera. The further investigation on crude extracts would characterize bioactive components of Aloe Vera which would be done by using High-performance liquid chromatography(HPLC) (Irshad *et al*, 2011).
A study done in Barc university the purpose of this study was to investigate in vitro antimicrobial activity of Aloe Vera leaf and clove extracts against microorganisms isolated from clinical samples. The

antibacterial activities were then compared to commercial antibiotics, Antimicrobial susceptibility testing of a total of 21 clinical isolates (10 *Pseudomonas*, 10 *Klebsiella* and 10 *E.coli*) were carried out using crude ethanol, methanol, acetone and aqueous extracts of Aloe Vera leaf and clove. Kirby-Bauer disk diffusion test of the same organisms was done using the following antibiotics:Imipenem, Meropenem, Gentamicin, Oxacillin, Cloxacillin, Ceftazidime, Ampicillin, Amikacin, Kanamycin and Ciprofloxacin.Extracts of both Aloe Vera leaf and clove were effective against all the isolates, with the highest activity exhibited by acetone extracts. Aqueous plant extracts showed little to no activity against the isolates, except for three *Pseudomonas* samples whose growth were inhibited by aqueous clove extracts. All the bacterial isolates were highly resistant to most of the antibiotics except imipenem, meropenem and ciprofloxacin.It was also observed that 100% of the organisms were resistant to oxacillin, cloxacillin and ampicillin. While some samples were highly susceptible to some antibiotics, others showed little and moderate susceptibility (Afrin, 2017).

Chapter Three

METHODS & MATERIALS

Chapter Three

3.Materials and Methods

3.1. Study Design

Cross-sectional descriptive Study.

3.2. Study Area

Khartoum state hospitals.

3.3. Study duration

From May to July 2019.

3.4. Study Population

One hundred patient males and females with an age varies from one day to 81 years diagnosed according to clinical examination, and the patient had clinical symptoms of urinary tract infection (UTI) or septicemia or otitis media or pneumonia and wound infection.

3.5. Sample size

A total of one hundred (n=100) isolates were detected from 100 patients enrolled in this study.

3.6. Samples collection

The Samples were collected from each of 100 patients having wound infection, or urinary tract infection, or pneumonia, or having septicemia, or otitis media or vaginitis during the study period from May to July 2019.

3.7.Data collection

Data was collected from hospital check lists

3.8. Laboratory procedures (Isolation of *P.aeruginosa*)

The isolates were processed within 2 hours of collection

3.8.1. Cultivation of bacteria

Each isolate was cultivated in Nutrient agar and then aerobically incubated overnight at 37°C.

3.8.2. Colonial morphology

After incubation plates were examined for colonial morphology.

3.8.3. Gram's stain

Method

Smear was prepared, dried and fixed. It was then covered by crystal violet stain for 30-60 seconds, then rapidly washed by tap water then it was flooded with iodine for 1-2min, and then washed by tap water. After that decolorized using alcoholic decolorizer, then washed immediately by water, then the smear was covered with safranine as counter stain for 1-3 min, then washed by tap water, then wiped of the back of slide, cleaned, allowed to air dry or using filter paper, then examined microscopically using oil immersion objective lenses (Cheesbrough, 2004).

3.8.4. Biochemical tests

3.8.4.1. Oxidase test

Done by picking few colonies from tested organism by wooden stick and placed it on oxidase test filter paper (Cheesbrough, 2004).

3.8.4.2.Citrate test

Simmon's citrate agar media was inoculated with tested organism as stabbing in butt and zigzag in slope part by sterile straight wire, then incubated overnight at 37°C (Cheesbrouph, 2004).

3.8.4.3. Urease test

It was done by inoculation of Christensen's urea agar media with the tested organism as zigzag by sterile wire loop, then incubated overnight at 37°C (Cheesbrough, 2004)

3.8.4.4. Kilgler Iron Agar (KIA) :

The KIA agar media was inoculated with tested organism as deep stabbing in butt and zigzag in slope part by sterile straight loop, then incubated overnight at 37°C (Cheesbrough, 2004).

3.8.4.5. Indole test:

The test was carried out by inoculation of sterile peptone water media with tested organism as mixing by sterile wire loop, then incubated overnight at37°C (Cheesbrough, 2004).

3.8.4.6.Motility test

This test was done by inoculation of semisolid agar media with tested organism as stabbing of two third of media by sterile straight wire, then incubated overnight at 37°C (Cheesbrough, 2004).

3.9. Collection of Plant Material

Seven kilograms of the Aloe Vera plant leaves (Aloe barbadensis Miller) were collected from Botanical Garden (Khartoum. Sudan), and they were identified and authenticated by the taxonomist in National Centre for Research, Khartoum.

3.10.Preparation of the Aloe Vera Extract

The Aloe Vera leaves were green when fresh collected. The leaves were thoroughly washed using water, then air dried and swabbed by 70% alcohol. The leaves were grinded and air dried in the shade far away from

direct sun light and dust for 3-7days.The dried leaves were crushed to small pieces using Mortar and Pestle then powdered in an electric grinder.

3.11. Extraction of Plant Material

The powdered plant material was extracted using alcoholic ethanol solution with concentration 85% as the best solution for extraction of Aloe Vera plant (Afrin, 2017).

Seventy grams from dried powder were put in clean conical flask. The powder was soaked in 300 ml of 85% ethanol then mixed by gentle shaking and the flask was covered using aluminum foil to avoid evaporation of ethanol.The flask was incubated at room temperature for at least 24 hours with gentle shaking between hours of incubation. The mixture was filtered in another conical flask using of Whatman No.2 filter paper, then the filtered extract was exposed to air for 6 to 24 hours to evaporate ethanol solution, then the extract was evaporated to dryness and with complete evaporation of solution the concentrated crude extract was taken which become ready for use as antimicrobial substance. The remaining solidified extracts were then collected in clean dry container and preserved in refrigerator and re-suspended in DW when needed for use .

3.12.Determination of Antimicrobial activity of Aloe vera extract

The antimicrobial activity of Aloe vera extract was assayed invitro by agar well diffusion method against 100 *P.aeruginosa* clinical isolates. The isolates of *Pseudomonas* were streaked on freshly prepared Nutrient agar plates and incubated for 24 hours. The Purity of the cultures was maintained by regular sub culturing and after growth was clearly visible the organisms were tested for antimicrobial susceptibility.

3.13. preparation of different concentration from Aloe vera extract

The Aloe vera plant extract was used as antimicrobial agent with the following concentration as 100mg/ml, 75mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml .

3.14. Inoculation an agar well diffusion

3.14.1.Preration of the inoculum

Standardized inoculums of 0.5 McFarland turbidity standard was prepared by taking 1-2 colonies of organisms with loops from 24 hour culture plates and mixed in sterile tube of saline solutions.

3.14.2.Inoculation of the media

The saline tubes were vortexed for homogeneous mixture and the turbidity compared to that of 0.5 McFarland standard solution. Sterile cotton swabs were used, and each of the test bacterial isolates was cultivated on whole surface of labeled Muller Hinton Agar (MHA) plates to achieve even growth. Then the plates were allowed to dry and then a sterile cork borer was used to bore wells in the agar plates. One hundred microliter (100µl/well) from each concentration was used and loaded in to each well using a sterile pasture pipette then the plates were incubated overnight at 37°C.

3.14.3Reading and interpretation of the result

The zone of growth inhibition was measured in millimeters using ruler.

3.15.Inoculation of media and disc diffusion

3.15.1.Preration of the inoculum

Standardized inoculums of 0.5% McFarland standard was prepared by taking 1-2 colonies of organisms with loops from 18-24 hour culture plates and mixing them in sterile saline solutions.

3.15.2.Inoculation of the media

The saline containing tubes were vortexed for homogeneous mixture and the turbidity compared to that of 0.5 McFarland standard solution, then

using sterile cotton swabs, each of the test bacterial isolates was cultured onto labeled MHA plates to achieve even growth. Sterile forcep was carefully used to pick up the antibiotic discs and mount them on to MHA then the plates were incubated overnight at 37°C. .

3.15.3Reading and interpretation of the result

Antimicrobial activity was determined by measuring the diameter of inhibition zone in millimeter produced against the *p.aeruginosa* isolates. The tested antibiotic discs were Ciprofloxacin, Gentamicin, ceftazidime, imipenem, meropenem and amikacin.

3.16. Negative control

One hundred microliter (100µl/well)from diluted ethanol and(100µl)from sterile DW were used and loaded into wells using a sterile pasture pipette then the plates were incubated at 37C for 18-24 hours. Antimicrobial activities of the ethanol and DW were determined by measuring the diameters of inhibition zones in millimeters.

3.17.Data Analysis

All collected data were analyzed using SPSS (Statistical Package of Social science) soft program version 20. Frequency and percentage was used.

Chapter Four

RESULTS

CHAPTE FOUR

4. RESULTS

4.1. prevalance of the clinical infections caused by *P.aeruginosa*

Table 2 showed that the numbers of isolates recovered from different sites of infection. Most of *P.aeruginosa* isolates (42) were recovered from UTI .Only one isolate was recovered from catheter infection and one isolate from vagina

Table 2: Number of *P.aeruginosa* isolates detected from different sites of infection

infection	**isolates No**	**isolates%**
UTI	42	42
Wound infection	17	17
septicemia	16	16
Pneumonia	12	12
otitis media	9	9
plural infection	2	2
vaginitis	1	1
Catheter infection	1	1
Total	100	100

4.2. *P.aeruginosa* infections among different age group of patients

Hundred isolates were detected from different age groups with an age of one day old to 81 years old showed different frequency which presented in table 3.

Table 3: Frequency of sampling according to patient's age group

Age group / Infection type	Up to 1day to 10year	11-20	21-40	41-60	61-81	Total
UTI	5	5	10	15	7	42
Wound infection	3	5	1	6	2	17
Septicemia	9	1	0	2	4	16
Pnemonia	0	0	4	3	5	12
Otitis media	1	3	1	0	4	9
Plural infection	0	0	1	0	1	2
Vaginitis	0	0	1	0	0	1
catheter infection	0	0	0	0	1	1

Total	**18**	**14**	**18**	**26**	**24**	**100**

4.3. Frequency of *P.aeruginosa* Infections according to gender

Out of 100 isolates, 36(36%) of isolates were from females, where as 64(64%) were from males, as shown in table 4.

Table 4: Frequency of *P.aeruginosa* infections according to gender

Gender	Frequency	Percentage
Male	**65**	**65**
Female	**35**	**35**
Total	**100**	**100**

4.4. Determination of antimicrobial activity of aloe Vera extract

As shown in table 5, all isolates were sensitive to 100mg/ml concentration with 10-22 mm inhibition zone, and only two isolates were sensitive to the lowest concentration 12.5mg/dl with 9-12 mm zone of inhibition.

Table 5: Antimicrobial activity of different concentration of Aloe vera extract on *p.aeruginosa* isolates.

Extract concentration(mg/mL)	100	75	50	25	12.5
sensitive isolates NO	100	98	75	18	2
Inhibition zone of isolates in **(**mm)	22-10	20-9	19-9	12-8	11-9
Inhibition zone of the standard in(mm)	14	13	12		

4.5.The conventional antimicrobial drugs susceptibility

The clinical isolates gave variable results with the common used antibiotics, and a according to table 6, the isolates were 87% sensitive to Meropenem but they were 40% sensitive to Ceftazidime.

Table 6: susceptibility of *P.aeruginosa* to some common used antibiotic

Antibiotics	Sensitivity %	Resistance %	Total %
Ciprofloxacin (5 mcg)	67	33	100
Amikacin (30 mcg)	64	36	100
Ceftazidime (10 mcg)	40	60	100
Meropenem (10 mcg)	87	13	100
Gentamicin (10 mcg)	57	43	100
Imipenem (10 mcg)	85	15	100

Chapter Five

DISSCUSSION, CONCLUSION AND RECOMMENDATIONS

CHAPTER FIVE

5. DISCUSSION

5.1. Discussion

According to the results of this study frequency of *P.aeruginosa* infection was high in males (65%) than females (35%) and this result agree with Yayan , (2015) who found that 67.3% of patients were males and 32.7% were females. The high frequency is due to more contact of males to polluted outside home environment than females.

The study showed that there was higher prevelance of clinical infections caused by *P.aeruginosa* in adult group 21-81 years was 68% than that of younger 1 day -20 years was 32 % and this result was confirmed by Sala *et al* (2019) who found that the prevelance is higher in adult than younger and this due to age associated changes in immune function, exposure to nosocomial pathogens and an increasing number of co-morbidities put the elderly at an increased risk for developing infections.

It was observed that 42(42%) of isolates were from UTI , 16 (16%)Septicemia , 17(17%) wounds infection, 12(12%) pneumonia, 9(9%) otitis media and this result was not in agreement with Lister (2009) who mentioned that *P.aeruginosa* was responsible for 21% of pneumonias, 10% of urinary tract infections, 3% of blood stream infections, and 13% of eye, ear, nose, and throat infection. The increasing in UTI prevelance could be due to increasing of nosocomial

infections, also increasing in catheters use and forgotten to drink plenty of water to flush out the bacteria.

In this study Aloe vera extract 100mg/ml concentration was found effective against the isolates of *P.aeuroginosa* with inhibition zone (10-22mm) and the standered organisim inhibition zone was 14 mm .However 50 mg/ml concentration was also found effective againist some of the isolates and this result is agreed with result of study done by Afrin (2017) who noticed that the activity of different antibiotics were tested against the 21 samples, and it was found that multidrug resistance is a common phenomenon among the organisms.

The negative control showed that the effective action on tested *P.aeruginosa* was from Aloe vera extract not from ethanol.

In this study *p.aeruginosa* isolates showed resistance to different tested antibiotic drugs as 60% for Ceftazidime followed by 43% for Gentamicin and 36% for Amikacin and 33% for Ciprofloxacin and 15% for Imipenem and 13% for Meropenem. The result was in agreement with study of Yayan,(2015).

The higher resistances to Ceftazidime and Gentamicin was due to extensive use of it for treatment of different clinical infections recently.

The Antibiotic activity when compared with Aloe vera extracts, the activity values indicated that the plant extracts were often more effective against the test pathogens while many commercial antibiotics were completely incapable of killing most bacteria, The plant extract showed positive activity against all the bacterial isolates however, in this investigation ,only crude ethanol extract of the plant were used for antimicrobial assay. The concentrations of extracts can be altered, or different extraction methods can be used. Aloe vera also has anthraquinones and acemannan as an active compound, anthraquinones which is similar in structure of tetracycline Afrin (2017).

The anthraquinones act like tetracycline and inhibit bacterial protein synthesis by blocking the ribosomal A site therefore bacteria can not grow in the media contain Aloe vera extracts (Pandey and Mishra, 2010). Acemannan and anthraquinones both work together in vivo, while in vitro only anthraquinones are effective. Anthraquinones are soluble in alcohol, acetone, etc. but poorly soluble or insoluble in water and this explain the effective sensitivity in vivo conditions, but if Aloe vera gel or whole leaf is consumed, then both acemannan and anthraquinones will work together, producing more effective results (Pandey and Mishra, 2010).

The combination of Aloe vera extract with some antibiotics like Ciprofloxacin and Gentamicin gave synergistic activity with clear increasing in the inhibition zone size ranged from 2-4 mm with different concentration of Aloe vera extract.

5.2.Conclusion

P.aeruginosa showed multi drug resistance to most common antimicrobial agent.

The ethanol extracts of Aloe vera exhibited antimicrobial activity on multi-drug resistant *P.aeruginosa* and may be used as an alternative therapeutic agent in the management of clinical multi-drug resistant bacteria.

The Aloe vera crude extract showed synergistic activity with conventional antimicrobial disc which may used in combination for treatment of multidrug resistant *P.aeruginosa* The results of this study support the folkloric use of Aloe vera plants in treating microbial infection and show that Aloe vera could be exploited for new potent antimicrobial agents.

5.3.Recommendation:

1. Aloevera could be used as alternative to chemicals used in medication. This will help to reduce the toxicity of the chemicals used in medications.
2. Use of Aloevera extract with combination of chemical antimicrobial drugs due to synergism activity.
3. More studies should be done on different plant parts separately to determine the antimicrobial activity of any parts like gel and sap.
4. Separation of bioactive component of Aloevera
extract to evaluate the antimicrobial activity of separate substance on different isolates of bacteria.
5. Research is also needed to identify, isolate and purify the extract bioactive components that are responsible for antimicrobial properties of this plant.

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APPENDICES

Appendix 1

1. Equipments and Materials

Light Microscope

lncubator

Hot air oven

Sensitive balance

Refrigrator

Benzene burner

loops Bacteriological

Straight wire

Forceps

Wooden sticks

Cotton

Autoclave

Filter paper

Physiological saline

Distilled water

Sterile cotton swabs

Immersion oil

Racks

Alcohol 70%

Gram stain solutions

Glass wares :

Flasks

Measuring cylinders

Universal containers-20 ml

Test tubes

Slides

petri dishes

pasture pipette

Appendix 2

Media and reagents prepration

KIA agar

Formula

57.5g for 1 liter

Contents	**g/L**
Peptic digest animal tissue	15.0
Yeast extract	3.0
Beef extract	3.0
Peptose peptone	5.0
Dextrose	1.0
Lactose	10.0
Ferrous sulphate	0.20
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.042
Agar	15.0
pH	7.4

Procedure

57.5 g was suspended in 1 Liter of D.w .bring to the boil to dissolve

completely. Mix and sterilize using autoclave at 121°C for 15 minutes. Cooled and poured in glass tube as slope and butt.

Mc Farland turbidity standard 0.5%

Contents

Conc.sulphuric acid	1.0ml
Dehydrated barium chloride	0.5g
DW	99.0ml

Procedure

Prepared as1% v/v sulphuric acid solution by adding 1ml of concentrated Sulphuric acid to 99ml D.W ,mixed then prepared 1% w/v solution of barium chloride by dissolve 0.5 g of dehydrated powder of barium chloride in 50 ml of D.W, then adding of 49 volume from sulphuric acid to 1volume from barium chloride solution and mix well.

Muller Hinton Agar (HI MEDIA)

Formula

38g for 1 liter

Contents	g/L
Casein acid hydrolysate	17.0
Beef heart infusion	2.0
Starch soluble	1.5
Agar	17.0
pH	7.3

Procedure

38g was suspended in 1 Liter of D.w. bringed to the boil to dissolve completely. Mixed and sterilized using autoclave at 121°C for 15 minutes. Cooled and poured into petri dishes.

Nutrient agar (HI MEDIA)

Formula

28 g for 1 liter

Contents:	g/L
Peptone	10.0
Beef extract	10.0
Sodium chloride	5.0
Yeast extract	1.5
Agar	15.0
pH	7.3

Procedure

28g was suspended in 1Liter of D.w. bringed to the boil to dissolve completely. Mixed and sterilized using autoclave at121°Cfor 15 minutes.Cooled and poured into petri dishes.

Oxidase reagent

Prepared fresh before use:

To prepare 10ml

Tetramethyle-p-phenylediaminedihydrochloride	0.1g
DW	10ml

Peptone water

Formula

15 g for 1 liter D.w.

Contents	g/L
Peptic digest animal tissue	10.0
Sodium chloride	5.0
pH	7.2

Procedure

15g was suspended in 1 Liter of D.w. bring to the boil to dissolve completely. Mix and sterilzed using autoclave at 121°Cfor15minutes.Cooled and poured into sterile glass tube.

Simmon's citrate agar

Formula

24.28 g for 1 liter D.w.

Contents	g/L
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.0
Sodium citrate	2.0
Dipotassum phosphate	1.0
Sodium chloride	5.0
Bromothymole blue	0.08
Agar	15.0
pH	6.8

Procedure

24.28 g was suspended in 1 Liter of D.w. bring to the boil to dissolve completely. Mixed and sterilized using autoclave at121°Cfor 15 minutes. Cooled and poured as slope.

Christensen urea agar (oxoid,UK,CodeX456)

Formula

24 g for 950 ml D.w.

Contents	**g/L**
Peptic digest animal tissue	1.0
Sodium phosphate	1.0
Monopotassium phosphate	0.80
Sodium chloride	5.0
Phenol red	0.012
Agar	15.0
pH	6.8

Procedure

24 g was suspended in 950 ml of D.w. bringed to the boil to dissolve completely mixed and sterilized using autoclave at 121°C for 15 minutes. cooled to 50°C and aseptically adding of sterile 40% urea solution and mixed well poured into sterile tube as slope.

Peptone water (Oxoid,UK,Codex4387)

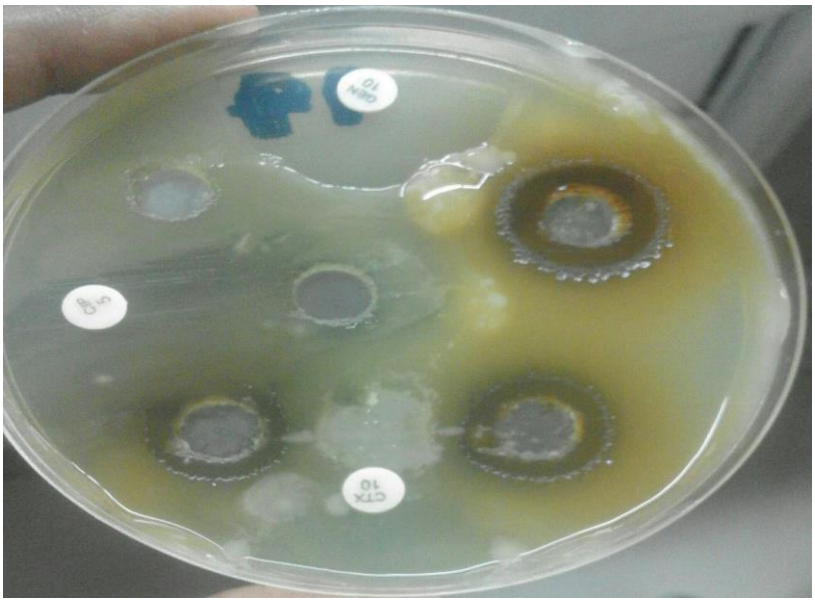
Formula

15 g for 1 Liter D.w.

Contents	**g/L**
Peptone	10.0
Sodium chloride	5.0
pH	7.0

Procedure

Fifteen grams was suspended in 1000 ml of D.w. Mixed and distributed into tubes then sterilized by autoclave at 121°C for 15 min, then cooled to 50°C.



Appendix 3:Figures

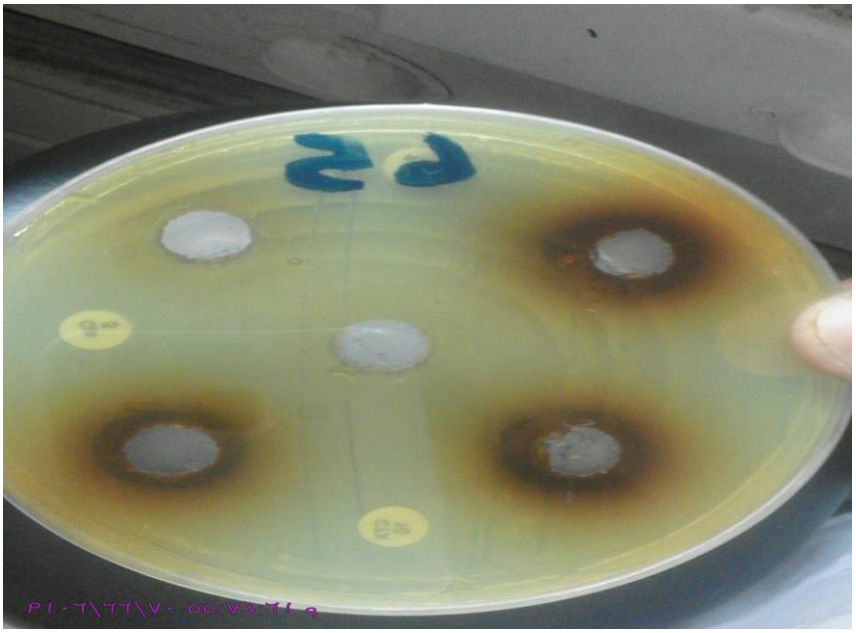


Fig1: Aloe vera antimicrobial activity with Inhibition zone of 100, 75 and 50mg/ml concentrations.

Fig 2: Effective antimicrobial activity of Aloe vera extract and resistance to Ceftazidime, Gentamicin, and Ciprofloxacin

Fig 3: synergisim between Ciprofloxacin and Aloe vera extract.

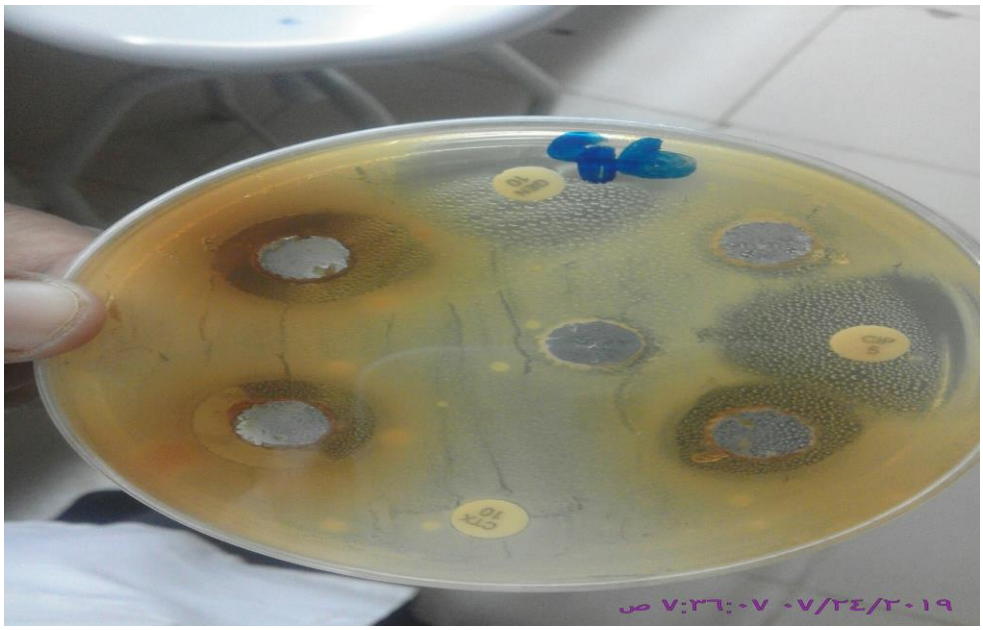
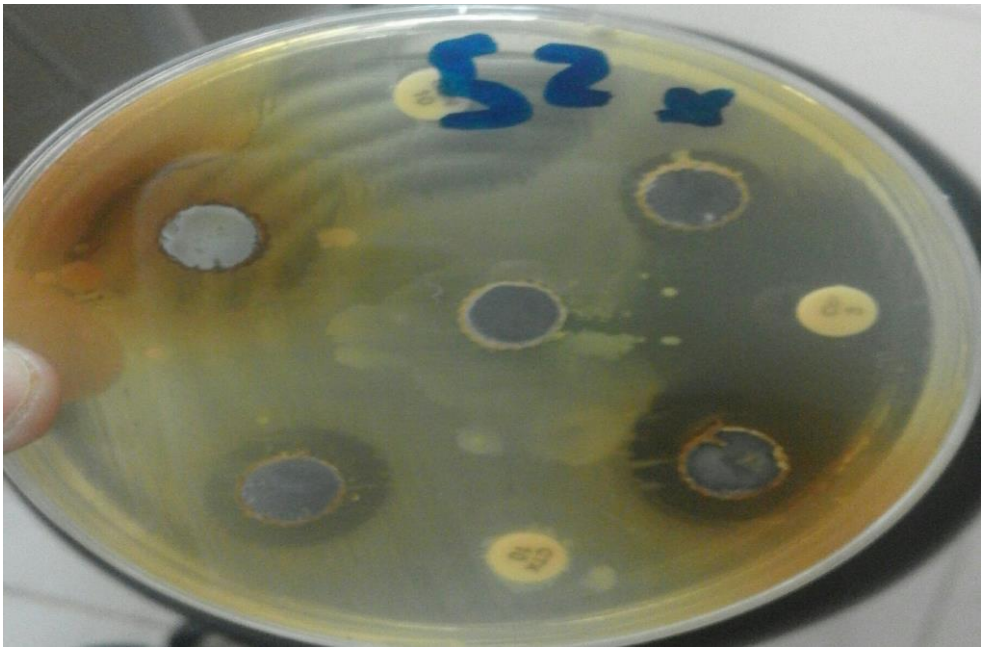


Fig 4: synergisim between Aloevera extract and Gentamicin (upper left) and with Ciprofloxacin (right).



بسم الله الرحمن الرحيم

Sudan University for Science and Technology

Faculty of Medical Laboratory Science

Questionnaire about clinical infections caused by *p.aeruginosa*.

1. **Serial no**:.....

2. **Date**:.....

3. **Patient name**:.....

4. **Age**:.....

4. **Gender**:.....Male( ) Female( )

5. **Complain other chronic disease** Yes ( ) No ( )

6. **Using of antimicrobial drugs** YeS ( ) No ( )