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Molecular detection of *LecA* and *LasA* genes and antibacterial activity of some plant extracts against locally isolated *Pseudomonas aeruginosa*

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بسم الله الرحمن الرحيم

﴿ نَرْفَعُ دَرَجَاتٍ مِّن نَّشَاء وَفَوْقَ كُلِّ ذِي عِلْمٍ عَلِيمٌ ﴾

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Dedication

I am extremely grateful to my family for moral support, without their support, I would not have done this thesis.

To the source of my happiness and strength in this life ,my support…… My Father

To the fountain of unconditional love and sacrifice, warmth, tenderness, and the affection……My Mother

To the one who planted in me all the meanings of sincerity and had support in my scientific and practical life…. My husband

To the two persons who always give me the hope And reason to go on in my life……My lovely angels Brother and Sister

To whom I see optimism in his eyes.. My beloved child

Dhafar

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Dhafar Al-Saady

Summary

Pseudomonas aeruginosa found in most natural environments (soil, water, plants and on the skin). It is distinguished by its color between yellow, green and blue and its smell that resembles the smell of grapes or fruits. It is a gram-negative, aerobic, opportunistic, pathogenic Bacillus that causes diseases in humans and animals. When these bacteria enter wounds, or people who are immunocompromised (burn patients "the most prominent example"), they generally cause severe infections, sepsis and tissue destruction. And if it hits vital body systems such as the lungs, urinary tract or kidneys, it may lead to death.

Ninety samples were collected from clinical samples (61 from burn patients) and 29 environmental samples including soil and water. Identification of *Pseudomonas aeruginosa* isolates were carried out by using biochemical tests. Further these isolates identities were confirmed by PCR technique to amplify *LecA* and *LasA* genes.

An antibiotic sensitivity test was performed using a number of antibiotics using the VITEK device for 24 isolates, 8 of which are environmental and 16 clinical, these isolates were selected according to the diagnostic ratio of the VITEK device.

The isolates for which the sensitivity test was performed showed very high resistance to the antibiotics Ampicillin, Amoxicillin, Cefuroxime, Meropenem it was 100% . The resistance rate for Amikacin and Gentamicin was (72.7%) and (88.8%) respectively, while all isolates were sensitive for Colistin (100%), and then suggested that colistin was the most effective antibiotic.

The results of the electrophoresis of the polymerase chain reaction of *LecA* gene for 24 isolates (5 soil, 3 water, 16 clinical) selected because

of their high antibiotic resistance showed that 19 isolate(environmental isolates and 11 clinical isolates) possess the gene and showed bands in agarose gel electrophoresis with a size of 369 base pairs.

As for the *LasA* gene, the results of the electrophoresis of the polymerase chain reaction of for 24 isolates (5 soil, 3 water, 16 clinical) selected because of their high antibiotic resistance showed that 18 isolate (environmental isolates and 10 clinical isolates) possess the gene and showed bands in agarose gel electrophoresis with a size of 226 base pairs.

Then, ten sample of PCR products were sent to Macrogen company(Korea) for the sequencing, programs such as Mega and Blast were used to compare the obtained isolates with global isolates(NCBI), the results of comparing the obtained isolates with global isolates of the *LecA* gene showed that two isolates were close to previous studies, while the remaining three were new strains. As for the results of the *LasA* gene, it was shown that one isolate is close to previous studies, and four isolates were new strains.

Six plant extracts were extracted by methanol, ethanol and hot water were tested, namely (*Cinnamon, Pimpinella anisum, Nigella sativa, Artemisia, Myrtus communis* and Quercus cortex.), and tested against three isolates from different sources (clinical, soil and water), where the first five of these plant extracts showed no antibacterial activity against *P. aeruginosa*, while Quercus cortex was effective, as the inhibition zone was clear when tested with four concentrations (25, 50, 75 , 100)mg\ml.

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List of Abbreviations

BLASTn	Nucleotide basic local alignment search tool
Mbp	Mega base pair
˚C	Celsius
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
KDa	Kilo Dalton
µg	Microgram
µl	Microliter
ml	Milliliter
%	Percentage
PCR	Polymerase chain reaction
Pmol	Picomole
Sec	Second
TBE	Tis-Borate EDTA
UV	Ultraviolate
LPS	Lipopolysaccharide
β-Lactamases	Beta-Lactamases
P. aeruginosa	*Pseudomonas aeruginosa*
PA-IL	*LecA*

Introduction

Introduction

Pseudomonas aeruginosa is a pathogenic bacterial species that causes infections and diseases in both plants and animals, beside many human diseases, in particular in patients with immune compromises, and several hospital-acquired infections (Azam and Khan,2019). *P. aeruginosa* is a widespread nosocomial pathogen in burn patients and easily acquires antibiotic resistance; hence developing an effective therapeutic approach is the most promising strategy for combating infection (Ranjbar *et al*., 2019).

P. aeruginosa is a Gram-negative bacterium that belongs to the γ-proteobacteria (proteobacteria). Like other members of the *Pseudomonas* genus, it is known for its metabolic flexibility and its ability to colonize a wide variety of ecological niches, human for example is subjected for serious infections (Chevalier *et al*.,2017).

In natural environments, such as soil and water, *P. aeruginosa* is commonly defined as ubiquitous, It was identified by environmental samples screening as being substantially associated with hydrocarbon and pesticide-contaminated environments and feces, as compared to non-contaminated conditions where its prevalence was relatively low (Crone *et al*.,2020).

P. aeruginosa's pathogenicity is regulated by its ability to produce a wide variety of virulence factors, which is improved by its inherent tolerance to environmental stresses and xenobiotics such as antibiotics, disinfectants and heavy metals (Gonzalez *et al*., 2016). Pathogenesis of *P.*

aeruginosa is caused by a combination of endo and exo cellular virulence factors (Zarei *et al*., 2018).

Several virulence factors can cause pathogenicity that, via targeting the extracellular matrix, facilitates adhesion, such as lectins, and/or disrupts host cell signaling pathways. Lipopolysaccharide, flagellum, type IV pili, type III secretion system, exotoxin A, proteases, alginate, quorum sensing, biofilm formation, type VI secretion systems, oxidant generation in airspace are considered among the virulence factors which caused the pathogenicity. These are major virulence factors that function in the immune system in different ways (Rocha *et al*.,2019).

P. aeruginosa lectins are mainly found in the cytoplasm of the cell; some can be found on the surface of the outer membrane (Grishin *et al*.,2015).

LecA is present on the outer membrane of *P. aeruginosa*, which may be involved in adhesion to host cells and play a major role in the intensity of *P. aeruginosa* and the spread of a pathogen, affecting its survival and development of biofilms (Al-wrafy *et al*., 2017).

Among other genes encoding to virulence factor ,The *LasA* gene is the first of *P. aeruginosa* genes involved in proteolysis and elastolysis to be cloned and sequenced. Genetic methods and attempts to purify an active fragment of the protein have been used to investigate its function and significance (Toder *et al*.,1994).

The ability of this bacterium to colonize different anatomical sites as a result of effective adhesion mechanisms, a lack of complex nutritional requirements, and antibiotics resistance which can all contribute to severe infections (Zeng,2004).

The seriousness of *P. aeruginosa* infection and the lack of successful treatment has encouraged researchers over the world to concentrate their efforts on improving diagnostic performance and reducing effort and time. Modern techniques, such as polymerase chain reaction technique, (PCR) has been used bacterial identity in clinical samples as well as food models, especially when epidemics occur, by detecting genes encoding virulence factors and using them as molecular markers in diagnostic studies that cannot be investigated by traditional diagnostic methods (Tsai *et al*., 2006).

The emergence of mutant strains of *P. aeruginosa*, which have resistance factors, are one of the most significant explanations for the emergence of high resistance in *P. aeruginosa*, which has resulted in a decrease in treatment options for infections caused by them (Kipnis *et al*., 2006).

The aim of the study

The high virulence of *P. aeruginosa* and its resistance to most antibiotics, the aim of the study was carried out to investigate the *LecA* and *Las A* genes of bacterial isolated through the following steps:

1. Isolation and diagnosis of *P. aeruginosa* from burn patients and environmental sources.

2. Detection the resistance of isolates to commonly used antibiotics.

3. Screening for *LecA* and *LasA* genes by PCR technique and sequencing.

4. Finding an appropriate anti-bacterial agent.

Chapter One

Literature review

1.Literature review

1.1. History of *P. aeruginosa*

Pseudomonas aeruginosa was isolated first by Sedillot in (1850), who observed the formation of blue-colored pus in the burns of infected soldiers, it was later confirmed by Fords in (1860) (Agnihotri *et al*.,2004). It was isolated by Schroeter in 1872 , who described this microbe as the causative agent of blue pus , and named it *Bacillus Pyocyaneus* . In 1882, Gessard noticed that these bacteria produced two types of pigments, the first of which was pyocyanine , as a green fluorescen pigment and dissolved in water and chloroform. The second was the fluorescein pigment, which is a fluorescent yellowish-green dye and not dissolved in chloroform , but dissolved in water (Blankenfeldt, 2013).

1.2. General characteristics of bacteria:

The ubiquitous Gram-negative bacterium *Pseudomonas aeruginosa* is a member of the Pseudomonadaceae family which is able to live in a wide variety of environments, compared to other sequenced bacteria, such as *Bacillus subtilis* (4.2 Mbp), *Escherichia coli* (4.6 Mbp) and *Mycobacterium tuberculosis* (4.4 Mbp), The genome of *P.aeruginosa* (5.5-7 Mbp) is comparatively large and codes for a large proportion of regulatory enzymes essential for synthesis, transportation, metabolism and efflux of organic compounds (Diggle and Whiteley , 2020). This improved the coding capacity of the genome of *P. aeruginosa* enables great metabolic flexibility and high adaptability to changes in the

environment, the heterotrophic, motile and Gram-negative rod-shaped *P. aeruginosa* is the bacterium with approximately 1-5 µm long and 0.5-1.0 µm wide (Pang *et al*., 2019).

P. aeruginosa grows well at 37 ° C, but it can live at a wide range of temperature 4-42 ° C. It is an important soil bacterium that is capable of breaking down aromatic polycyclic hydrocarbons, but is also often found in animal and human-polluted water bodies, such as sewage and sinks within and outside hospitals (Stover *et al*., 2000).

P. aeruginosa emits a characteristic smell similar to that of grapes in bacterial culture and infected wounds, this smell is emitted because the construction of 2-amino aceto phenone as it is formed early in the growth cycle , so it can be grown after 24 hours of incubation on the blood agar (Todar,2008).

P. aeruginosa has some differential characteristics as it is positive for the oxidase, catalase, urease and gelatinase test. Further, the complete decomposition of the β Hemolytic is considered the blood hemolysis of this bacteria on blood agar form the enzyme hemolysin(Scott-Thomas *et al.,* 2010; Brooks *et al*., 2010).

P. aeruginosa is distinguished by its outer membrane containing two types of lipoprotein oprI and lipoprotein oprL , which are responsible for resistance to antibiotics and antiseptics, The outer membrane can be used for the rapid diagnosis of *P.aeruginosa* bacteria in clinical samples (Nikbin *et al*., 2012).

1.3. Classification of *P.aeruginosa*

Pseudomonas aeruginosa belongs to class Shizomycetes, Order Eubacteriales and family Pseudomonadaceae (Holt *et al*., 1994) , where *Pseudomonas* is subdivided into five groups depending on RNA

homology which was represented by each of rRNA group 1 that included *Pseudomonas* fluorescent bacteria as agroup *P. aeruginosa* , *P. fluorescens* and *P. putida.* In addition to non-fluorescent *Pseudomonas* such as *P. stutzeri* , *P. alcaligenes* and *P. pseudoalcaligenes* (Gillespie and Hawkey, 2006). and group 2 called group (pseudomallei) that was transferred to the genus *Burkholderia* by Yabuuchi and his group in 1992 and which included both species *Burkholderia cepacia* , *B. pseudomallei* and *B. mallei* as well as many pathogens of plants.

The third group included *Comamonas acidovorans* that merged into the genus Delftia , while the fourth and fifth groups were reclassified into *Brevundimonas* and *Stenotrophomonas* respectively, Then this system was developed by (Palleroni, 2003).

The latest classification of *P. aeruginosa* was found by (Kahlon, 2016) as follows:

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: *Pseudomonas*
Species: *aeruginosa*

1.4. Pathogenecity:

P. aeruginosa is known as an opportunistic and diverse bacterium that possesses many virulence factors that enable it to pass the immune defenses of the patient's body, It is often present in all environments such as soil and prefers the surface of moist environments (Aujoulat *et*

al.,2012). Indeed this bacteria has the ability to adapt all conditions and it is resistant to many antibiotics that enable it to survive, being a common cause of urinary tract and lower respiratory tract infections, i.e., ventilator-associated pneumonia (Gellatly and Hancock, 2013) . *P. aeruginosa* maintains chronic infection through the formation of biofilms, which causes a delay in burn wound healing, patient recovery, and it is also a causative agent of graft loss (Karaky *et al*., 2020).

Three stagses can be identified for the pathogenesis of *P. aeruginosa* according to (Qarah, 2004):

1. Bacterial attachment and colonization.

2.Local invasion or infection.

3. Systemic dissemination of disease.

One of the most important genes responsible for bacterial attachment to the host is the *LecA* gene which is produced by *P. aeruginosa* which is a calcium-dependent galactose-specific soluble lectin, also referred to as PA-IL, its production regulated by quorum sensing . *LecA* was initially identified and characterized in the cytoplasm of *P. aeruginosa* but large quantities are present on the outer membrane of the bacterium, suggesting it may play a role in adhesion (Kuhaudomlarp *et al*., 2020).
Lectins are bacterial outer membrane proteins that recognize host glycoconjugates and enable bacteria to bind to host tissues. In addition, Pang *et al*., (2019) mentioned that lectins of *P. aeruginosa* are involved in the formation of biofilms by interacting with host cell glycoconjugates.

The lectin *LecA* (51 kDa) consists of four subunits containing 121 amino acids. Its bonds on oligosaccharides with a mild µM affinity to α-Gal monosaccharide and α-Gal terminals (Kuhaudomlarp *etal*., 2020).

The presence of aromatic group on the sugar anomeric position, either in α or β configurations, yields higher affinity, with KD of 5 μM for β-galactosides by CH-π interaction between His50 and the galactoside aglycone aromatic ring (Kadam *et al*., 2013).

Glycan arrays were showed stronger binding of PA-IL to αGal1–4βGal-terminating structures and weaker binding to αGal1–3βGal structures to determine the human glycoconjugates which may play a role in the bacteria's carbohydrate-mediated adhesion (Blanchard *et al*., 2008).Lectin produced by bacteria also linked to virulence factors and regulated by both quorum sensing and the alternative sigma factor (Grishin *et al*., 2015).

LasA is a metalloproteinase (also known as staphylolysin) with a molecular mass of approximately 22 kDa elastolytic and staphylolytic endopeptidase secreted by *P.aeruginosa*. It plays an important role in bacterial colonization by preventing other bacteria from colonizing the bacteria, For example, when it causes mixed infection with *Staphylococcus aureus* this enzyme lyses the bacteria and then eliminates the competing bacteria for *P. aeruginosa* (Galdino *et al*., 2017). It belonging to the β-lytic family of Zn-metalloendopeptidases LasA possesses a high staphylolytic activity that lyses *Staphylococcus aureus* cells by cleaving the pentaglycine bridges with in the peptidoglycan and may also enhance elastolysis by cleaving Gly-Gly peptide bonds abundant in elastin (Kessler and Ohman, 2004).

This protease has been suggested as a virulence factor secreted by *P. aeruginosa* in animal models of corneal and lung infections. LasA is responsible for the shedding of the host cell surface proteoglycan syndecan-1 (Anderjko , 2013) .

Among such virulence factors are secreted extracellular proteases these enzymes are essential predisposing factors that play a role in bacterial infection establishment and They are known to facilitate bacterial colonization by inducing damage to host tissue and actively subverting immune responses (Potempa and Pike, 2009; Al–Abaadi *et al*., 2015).

1.5. Virulence factors

Many nosocomial infections are caused by Gram-negative pathogens, and interaction of these pathogens with the host is often mediated by the production of virulence factors, Bacteria have evolved mechanisms for the production of virulence factors into the host cell to alter host cell biology and enable bacterial colonization, and these mechanisms typically require that bacteria to be in intimate contact with the host (Bomberger *et al*., 2009).

P. aeruginosa has a wide range of virulence factors associated with and excreted from the cell. The integrity of the host's immune system and the absence of other diseases makes the bacteria incapable of causing infection and entering the body's tissues and organs easily unless presented in sufficient numbers to overcome the host's high defenses (He *et al*., 2004).

This bacterium synthesizes several virulence factors including elastase, alkaline protease, exotoxin A, rhamnolipids and pyocyanin, among others, which together allow it to infect humans (Cocotl-Yañez *et al.,* 2020).

The virulence of *P. aeruginosa* depends on both cellular and extracellular factors include Cell-associated pili, flagella, and lipopolysaccharide are important surface components of *P. aeruginosa*

which facilitate attachment of the organism to host cell surfaces and activate immune responses, These cellular bacterial constituents are required for virulence in a number of models of *P. aeruginosa* infection (Ofek and Doyle,2012). Extracellular or secreted virulence factors, such as proteases (elastase and alkaline protease) and toxins (exotoxin A and the exoenzymes S, T, and U), have also been shown to be necessary for virulence in animal models of *P. aeruginosa* infection (Pearson *et al*., 2000).

Exotoxin A (ETA) as a virulence factor is secreted through type II secretion mechanism, which use a pilus-like apparatus to secrete proteins into the extracellular environment, including lipase, phospholipase, alkaline phosphatase and protease. Animal experiments have indicated the significant role of these factors in model infection (Rocha *et al*., 2019).

P. aeruginosa secretes multiple proteases enzyme that has been implicated as virulence factors, the detection of each specific enzyme can be difficult to determine. Unlike the three *Pseudomonas* enzymes that have been well characterized (elastase A, elastase B, and alkaline protease), most strains of *P. aeruginosa* produce various proteolytic enzymes that act on a number of proteins as a base material such as Gelatin , Casein , Elastin , Collagen and Fibrine , which lead to break down the connective tissue and weaken the host's immune factors (Caballero *et al*.,2001; Gillespie and Hawkey , 2006).

Virulence factors of *P. aeruginosa* include hemolysins, which lyse red blood cells and release free iron from heme, allowing the bacteria to grow rapidly and leading to greater colonization of the host tissue. (Fila *et al*.,2017).

The ureases enzyme is considered one of virulence factors for many Gram-positive and Gram- negative bacteria including *Pseudomonas* which is considered as the most dangerous pathogen. This enzyme has an ability to convert urea into CO_2 and NH_3 and increase pH which enhance bacterial growth (Noomi, 2019).

There are two types of pigment produced by *P. aeruginosa* Pyocyanin and Pyocins, Pyocyanin a cytotoxic pigment whose chemical structure is phenazine nucleus and can be soluble in chloroform and water , which is excreted outside the cell. Pyocyanin causes distortion of the respiratory epithelium and has an effect on macrophages (Todar , 2004). Pyocins are substances of a protein nature and have the ability to inhibit the growth of other strains of the same type and can be used in epidemiological studies and in distinguishing the different strains of this type (Line *et al*., 2008).

While the Cell-association virulence factors include Pili, Flagella and Lipopolysaccharide, In relation to virulence factors, the type IV pili of *P. aeruginosa* has a role in adhesion to many cell types and this is likely important in such phenomena as tissue tropism and attachment to particular tissues (Rocha *et al*., 2019). The pili are filiform structures spread on the surface of the bacterial cell, which adhere to the mucous membranes , as *P. aeruginosa* , binds to the epithelial cells and this association occurs in the form of contact with special receptors (Todar, 2004).

P. aeruginosa possesses a single polar flagellum or several flagella which play an important role primarily as a highly complex movement organell in species of flagellated bacteria and they related to biofilm development (Wolfgang *et al*., 2004).

Lipopolysaccharide (LPS) of *P. aeruginosa* is a prominent factor in mediating both bacterial virulence and host responses, The contribution of LPS to pathogenesis and immunity varies depending on the underlying patient basis for increased susceptibility to infection, However, host immunity has been reported to be influenced by the isoform of the LPS, particularly the lipid A component, and structural variation in the O-antigen side chain (Pier, 2007).

1.6.Environmental range

P. aeruginosa is ubiquitous in the environment, being found in soil, water, animals and on plant matter (Tashiro *et al*.,2013). It is a well-recognized phytopathogen and has a predilection for moist environments, and thus is often cultured in very high numbers from drains and similar semi-aquatic environments, It has displayed an ability to use varied substrates as a basis for growth (Bradbury,2009).

P. aeruginosa isolated from environmental samples has been identified as significantly associated with hydrocarbon and pesticide-contaminated environments and feces. It describe as a bacterium largely found in locations associated with human activity, being isolated from different water sources include rivers, streams, lakes and sewage treatment plants (Crone *et al*.,2020; Ramos et al.,2020).

The organism is particularly common in hospital environments having been isolated from soap, disinfectants, respiratory equipment, mattresses, endoscopes, distilled water and suction apparatus (Pier and Ramphal 2005).

1.7. Skin burns

Skin is the organ that gives the body its external shape, covers and protects it from external influences such as injuries, shocks, cold and heat, Among the most important functions for the skin is the production of vitamin D, which is necessary for building bones, regulating temperature, losing water, and its sensory functions such as feeling pain, touch, and defensive functions, that exposure of the skin to damages such as burns, scars, wounds, etc. (Cavanagh, 2004).

Burns are a form of bruising that destroys the skin due to the loss of its defensive function, which results in the possibility of infection, so it is necessary to prompt intervention and necessary medical care to reduce injuries and deaths among burn patients (Ekrami and Kalantar, 2007).

One of the most dangerous skin damages is burns because it leads to necrosis of the skin layers as a result of exposure to heat, then the skin is an appropriate environment for germs to invade due to tissue loss, which facilitates the penetration of microbes into other layers and tissues, thus delaying the healing process and prolonging the treatment period (Muhammad and Muhammad, 2005).

The severe burn injuries interfere with all skin functions and also affect the functions of other organs such as kidneys, lungs and blood circulation. These injuries may lead to the patient losing his life as a result of fluid loss and acute bacterial infections, thus the occurrence of septic shock, especially in cases of untreated (van-Zuijlen, 2002).

Burns are also considered one of the most damaging to physical and mental health conditions due to their severe psychological effects that

require the sick person to undergo health and psychological rehabilitation sessions after burning (Shriner, 2000).

Burns are generally classified as superficial (first-degree burns) , partial-thickness (second-degree burns) or full-thickness (third-degree burns) depending on the degree of tissue damage (Lee *et al*., 2020).

Pathogen colonization and systemic invasion in burn wounds can lead to serious complications and death. The two most common pathogens that cause burn wound infections are *Staphylococcus aureus* and *P. aeruginosa* (Othman *et al*., 2014).

1.8. Resistance of *P. aeruginosa* to antibiotics:

Due to extensive damage to the skin, burn victims probably will be infected with life-threatening infections. Though the skin primarily protects against microbial invasions, a large number of bacteria, fungi, and viruses can be isolated from burn patients, specifically *P. aeruginosa*, a bacterium with both intrinsic and acquired antibiotic resistance (AR) properties (Moosavi *et al*., 2020).

Antibiotics are known to be organic compounds that can kill or inhibit the growth of bacteria; these substances are produced either as a product of the natural metabolic processes of microorganisms or can be produced artificially in the laboratory from some organic compounds (Wright, 2014). On the other hand semi-synthetic antibiotics , are made by adding or removing functional groups of natural products of microorganisms or natural antibiotics, Despite the clinical importance of using these different types of antibiotics to treat a various bacterial infections, the ability of

bacteria to resist these antibiotics has been deemed an obstacle in the treatment of many pathological conditions(Bradford, 2001).

Bacteria have resistance genes carried on both chromosome and bacterial plasmids where the resistance gene carried on the plasmid has the ability to move from one bacterial cell to another during normal gene exchange processes such as conjugation , transduction and transformation. These genes are highly associated with resistance to many antibiotics such as Cephalosporins, Chloramphenicol, Penicillin, Aminoglycosides, Erythromycin, Tetracycline and Sulfonamide (Hemalatha and Dhasarathan, 2010).

All isolates of *P. aeruginosa*, which are resistant to at least anti-microbial agent in three or more anti-pseudomonal anti-microbial categories are called multi-drug resistant (MDR) (Magiorakos *et al.*, 2012). In contraste, extremely drug resistant (XDR) *P. aeruginosa* is known as all those isolates which are resistant to at least one antimicrobial agent in six or more anti-pseudomonal antimicrobial categories. Pan drug resistant (PDR) *P. aeruginosa*, means those isolates which are resistant to all antipseudomonal antimicrobial agents (Gill *et al.*, 2011).

In general, *P. aeruginosa* can be resistant to antibiotics by three type of mechanisms which: intrinsic, acquired, and adaptive resistance. The ability of bacterial species to reduce the effectiveness of a given antibiotic due to inherent structural or functional characteristics is referred to as intrinsic antibiotic resistance (Blair *et al.*, 2015).

P. aeruginosa is shown to possess a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics outside the cell and production of

antibiotic-inactivating enzymes such as β-lactamases. The acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes (Breidenstein *et al*., 2011). The adaptive resistance of *P.aeruginosa* involves formation of biofilm in the lungs of infected patients where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacterial cells (Drenkard, 2003).

One of the mechanisms of the resistance in *P. aeruginosa* is the production of enzymes that degrade some types of antibiotics, where the bacteria secrete many extra-cellular enzymes that destroy the antibiotics, such as Metallo-B-lactamases enzymes that destroy β -lactam antibiotics group and chloramphenicol acetyltransferase which inhibit the chloramphenicol as well as producing aminoglycosidases that modified aminoglycosides (Rodriquez Esparragon *et al*., 2000).

Among other mechanisms that are classified within the acquired resistance is resistance by mutations: mutational changes which are able to cause reduce in antibiotic uptake, modifications of antibiotic targets, and over expression of efflux pumps and antibiotic-inactivating enzymes; all of which allow bacteria to survive in the presence of antimicrobial molecules (Munita and Arias, 2016).

Antibiotic therapy in recent years has faced difficulties due to the increasing rapid emergence of multidrug resistance among bacteria and fungi causing several life-threatening infections and this in turn making the future management of infectious diseases uncertain (Levy and Marshall, 2004). The study of antibacterial activity of some plant extract may identify and portray plant extract as a potential antibacterial medicine and serves as a pointer for pharmaceutical industries in producing the most effective drugs from plant sources (Okla *et al*.,2021).

1.9. Plant extracts

Medicinal plants are important elements of traditional medicine in virtually all cultures, The idea that certain plants had healing potential was known for a long time before humans discovered the existence of pathogens, Medicinal plants which have been used by humans to treat common infectious diseases are important elements of traditional medicine (Sofowora *et al*.,2013). During the last years, traditional medicine plants have not been limited to a specific cultures. They have been used for years in developing countries as well as its using extended to developed countries in daily life to treat disease (Adwan *et al*., 2007).

The characteristics of the plants that inhibit microorganisms and their importance for human health have been investigated in laboratories since 1926. Traditional medical treatments in daily life are now being used with empirical methods (Ates and Turgay, 2003).

Nowadays, environmental problems and high resistance bacteria to antibiotics have reinforced a tendency to replace them with less harmful substances. Among the various agents to replace antibiotics, products of plant origin have recently got un increased attention as alternative disease treatment. Herbal medicines have been the source of disease treatment during the centuries and now with the advancement of sciences and development in the application of synthetic medication, medicinal plants are still used in large-scale (Taheri, 2013).

Recently, there has been a great deal of attention paid in medical treatments to plant extracts and compounds with biological features, because of the resistance and side effects that the pathogens have shown against antibiotics. The anti-bacterial compounds of plants are a significant medical resource. As a result of the spread of infectious

diseases, exploration of more of these compounds will be useful (Jahani *et al*., 2016).

Accordingly, because of the importance of medicinal plants in conventional medicine and the fact that these medicines have few side effects on humans and also due to the changes in the form of the resistance of pathogenic bacteria which requires monitoring of antibacterial effects substances in periodic intervals (Taheri, 2013).

1.9.1 *Pimpinella anisum* (Aniseed)

Anise, aniseed was first cultivated as a spice by the ancient Egyptians and later by the Greeks, Romans and Arabs. Although widely grown commercially, its cultivation has declined in recent years through competition with cheaper anise flavorings such as Illicium verum and synthetic anethole (Duke, 2002). It is a sweet, warming, and stimulant herb that improves digestion, benefits the liver and circulation, and has expectorant and estrogenic effects,the parts of the plant are used including leaves, seeds and oil (Ates and Turgay, 2003).

Seeds of anise are used as analgesic in migraine and also as carminative, aromatic, disinfectant and diuretic in some traditional texts; anise is mentioned for melancholy, nightmare, and also in treatment of epilepsy and seizure (Sun *et al*., 2019).

Some spices have also been stated to have bactericidal or bacteriostatic properties. Spices' inhibitory effects are mostly due to the volatile oils that present in their structure. Origin and composition of the spice, the amount used, type of microorganism, the composition of the food, pH value, temperature of the environment, and proteins, lipids, salts, and phenolic substances present in the food environment all that are

the key factors that contribute in antimicrobial activity (Salim *et al*., 2016).

1.9.2. Cinnamon:

Among the important medicinal plant is Cinnamon which is a common spice used by different cultures around the world for several centuries, it is used internally in western medicine, mainly in preparations for diarrhea, flatulent dyspepsia and colic and colds. in Chinese medicine, it is used for diarrhea, poor appetite, low vitality, kidney weakness, rheumatism and coldness, it is also used for colds, influenza, fevers, arthritic and rheumatic complaints, angina, palpitations and digestive complaints related to cold and chills (Paranagama *etal*.,2001;Ates and Turgay, 2003).

The study of antibacterial activity of Cinnamon may identify and portray Cinnamon as a potential antibacterial medicine and serves as a pointer for pharmaceutical industries in producing the most effective drugs from plant source (Wang *et al*., 2018).

1.9.3. Nigella sativa:

Nigella sativa seeds, also known as black cumin, have been used for medicinal purposes in Asia, Africa, and the Middle East for centuries in the form of herb and oil. Nigella sativa seeds are used to treat gastrointestinal and stomach problems, respiratory problems, immune system help, circulatory problems, liver and kidney problems, and other common health problems. On a dose-dependent basis, the essential oil has antimicrobial properties against a variety of Gram-positive and Gram-negative bacteria (Dhanasekaran, 2019).

1.9.4. Artemisia absinthium (Wormwood):

Artemisia is a diverse Asteraceae family genus with a wide range of medicinally important essential oils and secondary metabolites. Artemisia species essential oils. For several years, essential oils from Artemisia spp. have been commonly used for a number of medicinal purposes. Artemisia nilagirica (Clarke) pamp, also known as Indian wormwood, is widely distributed in India's hilly regions. Insecticidal properties have been identified for A. nilagirica (Ahameethunisa and Hopper , 2010).

The whole plant and leaves of Artemisia absinthium L. (Compositae/Asteraceae), also known as "Wormwood," are commonly used. It's an aromatic, diuretic, bitter herb with anti-inflammatory properties and functions as a tonic for liver, digestive, and nerve. . It stimulates the uterus and expels intestinal worms and used internally for digestion, poor appetite, gall bladder complaints, and roundworms, also externally for bruises and bites. It is only administered to children and pregnant women in small doses for short-term care (Erdogrul ,2002).

Various species of *Artemisia* have been characterized for their biological activities. It is considered to produce the most medicinally important secondary metabolites (Ahameethunisa and Hopper , 2010).

1.9.5. Myrtus communis (Myrtle):

The Mytaseae family's myrtle is a shrub with various stems and branches that is evergreen and aromatic (Taheri, 2013). Medicinal plants, such as Myrtus communis L., have new compounds that can be used in the food industry as well as in medicine, mainly as antimicrobial agents. Polyphenolic compounds, such as phenolic acids, tannins, and flavonoids, found in many vegetal portions of M. communis L., which mediate antioxidant effects and have a remarkable activity against a variety of

harmful microorganisms. According to some findings, phenolic compounds play a significant role in antibacterial activity (Sateriale *et al*., 2020).

1.9.6. Quercus cortex:

The well-known rationale for the therapeutic use of Quercus cortex is its direct antibacterial activity against many pathogenic bacteria for humans and animals, oak bark is usually described as a source of polyphenolic secondary metabolites: hydrolyzable tannins, previously known as pyrogallol tannins, and condensed tannins—proanthocyanidins (Haslam.,2007). The total content of tannins in the dried substance varies from three to 20 percent, depending on the time of harvesting, age of the branches and method of assay used. Their antimicrobial effect is attributed to these tannins (Ebrahimi *et al*,.2012).

Oak bark due to high tannin is less used in domestics consumption for a long time causes fatigue and pain in the stomach and heart muscle. In addition to stimulating the digestive tract, it also causes other symptoms such as stomach pain and jerky. It is a good antidote to poisoning caused by metals, oak bark tea is recommended for topical baths, lotion and wash in different ways to treat diseases. That tea can be used as local baths for conditions such as cancer ulcers, gland inflammation due to obstruction of the ducts, hematomas melancholy, chronic skin diseases such as eczema and varicose veins (Bahmani, 2015).

Chapter Two
Materials
and
Methods

2.1. Materials

2.1.1. Instruments and Equipment

All the instruments and equipment used in the present study were presented in Table(2-1):

Table (2-1): Equipment and instrument used in the current study

No.	Instruments and tools	Company (origin)
1	Autoclave	Labtech(Korea)
2	Centrifuge	Hettich (Germany)
3	Digital camera	Sony (Japan)
4	Distiller	GFL(Germany)
5	Electrophoresis apparatus	Clever (England)
6	Incubator	Binder (Germany)
7	Laminar flow cabinet	Teio-Tech(Korea)
8	Light microscope	Motic (Germany)
9	Loop	Loop Shandon(England)
10	Magnetic stirrer with hot plate	Labtech(Korea)
11	Micro centrifuge	Sigma(Germany)
12	Micropipettes	Human (Germany)
13	Oven	Memmert(Germany)
14	PCR thermocycler apparatus	Labnet (USA)
15	Refrigerator	LG(Korea)
16	Sensitive balance	Denver-Germany
17	Shaker Incubator	Labtech(Korea)
18	Spectrophotometer	Tudor (Korea)
19	Ultraviolet transilluminator	San.Gabril(USA)
20	VITEK2	Biomeriex (France)
21	Vortex	ROMA (Italy)
22	Water bath	Tafesa (Germany)

2.1.2. Items that are disposed after use

Table (2-2): disposable items used in the present study with their manufacturers :

No.	Name	Origin
1	Cotton	HAD(China)
2	Eppendrof tubes	Sigma (England)
3	Filter paper	Sartoraus (Germany)
4	Latex Gloves	Broche (Malaysa)
5	Para film	BDH(England)
6	Petridishs	Afco-dipo (Gordan)
7	Sterilized cotton swabs	Afco-dipo (Gordan)
8	Sterilized needles	Afco-dipo (Gordan)
9	Tips	SterellinLtd (England)
10	Tubes of 10 ml	Afco-dipo (Gordan)

2.1.3. Biological and Chemical Materials

The chemicals and biological materials used in the present study with their origins are given in Table (2-3):

Table (2-3): Chemicals and biological materials

No.	Chemical materials	Company (origin)
1	Agarose	Promega-USA
2	Barium chloride	BDH (England)
3	Deionize water	EUROPA (India)
4	DNA Loding dye	Promega-USA
5	Ethanol 96%	BDH (England)
6	Ethidium Bromide	Sigma (USA)
7	Glycerol	BDH (England)
8	Gram Stain	VSI(Iraq)
9	H_2SO_4	BDH (England)
10	Hydrogen peroxide (H_2O_2)	VIS (Iraq)
11	Neuclease free water	Promega-USA
12	Normal saline	Pioneer (Iraq)
13	Tetramethyl P-phenylen diamine dihydrochloride	Sigma (USA)

14	Primers	Macrogen (South Korea)
15	Tris Borate-EDTA Buffer solution (TBE) 10X	Bio basic (Korea)
16	Tris –EDTA Buffer solution (TE) 10X	Promega (USA)
17	Gram stain	VIS (Iraq)

2.1.4. Kits

kits used in the present study were illustrated in Table (2-4).

Table (2-4): kits used in the study:

No.	Name of the kits	Purpose of using	The manufacture company
1	VITEK-2 GN Card	Test of Gram negative bacteria	Biomerieux
2	VITEK-2 AST Card	Antibiotic sensitivity test	Biomerieux
3	DNA extraction kit it contains enough solutions for 100 test and it is: G buffer,Binding buffer ,Wash buffer A ,Wash buffer B ,Elution buffer, and contains GD columns and tube collection	Extraction of DNA	INtRoN Biotechnology

2.1.5. Culture Media

All culture media used in the present study were shown in Table (2-5).

Table (2-5): Culture media with manufacture name and purpose of use

No.	Culture media	Manufacturing/Origin	Purpose of use
1	Blood agar base	Oxoid/England	Bacterial activation and Hemolysin production
2	Brain-heart-infusion broth	Himedia/ India	Cultivation of fastidious and non fastidious microorganism
3	Cetrimide agar	Liofilchem/ India	Selective isolation and presumptive identification of *Pseudomonas aeruginosa* from clinical and nonclinical specimens
4	MacConkeys agar	Biolap/Hungary	Designed to isolate and differentiate bacteria based on their ability to ferment lactose
5	Mueller Hinton Agar	Labm/United Kingdom	Antimicrobial sensitivity testing by diffusion method
6	Nutrient agar	Himedia/ India	Used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms

2.1.6. Primers

PCR primers were used in this study were synthesized by BIONER/Korea. The name, and sequence of primers were given in Table (2-6):

Table(2-6): Primers used in the polymerase chain reaction used in present study

Primers	Sequence (5'----3')	Product size (bp)	Reference
LecA forward	**ATGGCTTGGAAAGGTGAG**	369	(Hami *et al*., 2016)
Lec A reverse	**TCAGGACTGATCCTTTCC**		
Las A forward	**CGACAAGAGCGAATACCTGGAG**	226	(Alawad, 2019)
Las A reverse	**CAACTGGTATTCCTCGAAACCGTA**		

2.2. Methods

All main steps of current study are shown in Figure (2-1).

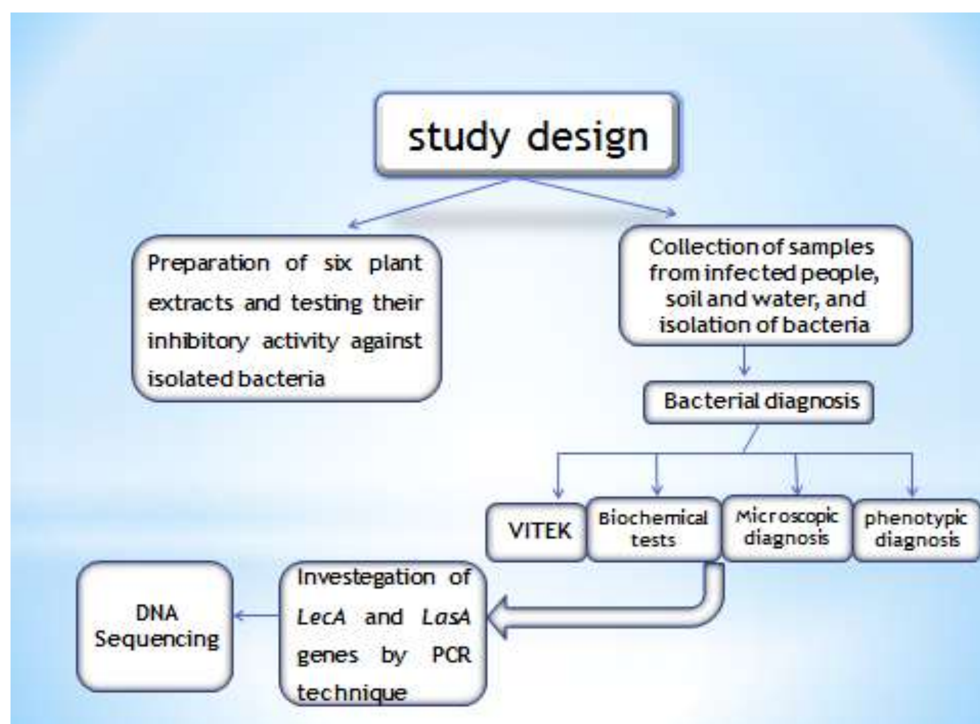


Figure (2-1): Main steps of current study.

2.2.1. Preparation of Culture Media

All media were prepared according to their manufactures instructions, and sterilized by autoclave. Subsequently, the media were poured in sterile Petri dishes.

2.2.1.1. Blood Agar Media

Blood agar media was prepared according to the manufacturer's instructions. 28 grams of the medium were suspended in 1 liter of distilled water and the mixture was shacked to dissolve. Posteriorly, the media was boiled until completely dissolved and homogenous. Media

were sterilized. then left for decreasing its temperature to 45-50 ^{o}C. After that, 5% of human blood was added to the medium and homogenized thoroughly and poured into Petri dishes according to (Coban *et al.*, 2006).

2.2.1.2. MacConkey agar:

This medium was prepared according to the instructions of the manufacturing company, 52 g was dissolved in a liter of distilled water and sterilized by autoclave. This medium was used to isolate Gram-negative bacteria and diagnose them in terms of their ability to ferment lactose sugar.

2.2.1.3. Mueller Hinton Agar

Mueller Hinton agar was prepared by dissolving 38 g of the medium in one liter of distilled water, swirled to mix then heated gently to be completing dissolved. Consequentially, media was sterilized and poured into petri dishes.

2.2.1.4. Brain – Heart infusion broth

This medium was prepared according to the instructions of the manufacturing company, by dissolving 37 g in a liter of distilled water. Media was distributed in tubes and sterilized. This medium was used for the purpose of developing and activation bacteria, as well as for preserving bacterial isolates, by adding 15% glycerol to 85% of the liquid medium after sterilization (Vitko and Richardson, 2013).

2.2.1.5. Cetrimide agar

This medium was prepared according to the instructions of the manufacturing company, 46.7 g was dissolved in 990ml of distilled water and sterilized by autoclave after that 10 ml of glycerol was added.

This medium was used for the purpose of selective isolation and presumptive identification of *Pseudomonas aeruginosa* from clinical and nonclinical specimens (Yilmaz, 2017).

2.2.2.Reagents and solutions used to isolate and diagnose of *P. aeruginosa*:

2.2.2.1.Catalase reagent:

Hydrogen peroxide was used at a concentration of 3% to investigate the ability of bacteria to produce catalase enzyme (Jakubovics *et al*., 2008).

2.2.2.2.Oxidase reagent:

The reagent was prepared by dissolving 0.1 g of Tetramethyl P-phenylen diamine dihydrochloride in 10 ml of distilled water in an opaque vial. This reagent was used to investigate the ability of bacteria to produce oxidase enzyme (Macfaddin, 2000).

2.2.2.3.Gram stain:

This stain was used to study the phenotypic properties of the isolated bacteria, it consists of:

- Crystal Violete
- Iodine Solution
- Decolarizer
- Safranin Pigment

The isolates of bacteria were subjected to a microscopic examination using a gram stain and were examined with the oil lens of a light

microscope to identify the shape of the bacteria, as well as their size and arrangement (Atlas, 2004).

2.2.2.4.Macfarland Solution:

This solution was prepared according to the method described by (Anwer *et al*.,2020) as it consists of two solutions:

A- Barium chloride solution (1.175%): This solution was prepared by dissolving 1.175 g of barium chloride in a quantity of distilled water, and after dissolving the volume was completed to 100 ml with distilled water.

B- Sulfuric acid solution (1%): This solution was prepared by adding 1 ml of concentrated sulfuric acid to 99 ml of distilled water.

McFarland's solution (tube No. 0.5) was prepared by adding 0.5 ml of solution A to 99.5 ml of solution B and the solution was mixed well.

Solution was used to compare the bacterial density used in the sensitivity assay tests where the density of tube No. 0.5 is equivalent to a bacterial density of 1.5×10^8 CFU / ml which is equivalent to an optical absorption of (0.08-0.13) at the wavelength of 625 nm.

2.2.3.Collection of samples

Ninety samples were collected including sixty- one clinical samples from the burn suffering patients who were lying in the Imam Hussain Medical Hospital in Kerbala province, clinical samples were collected by cotton swabs with the media, where a swab was taken from the skin of the burn patient after obtaining the patient’s consen (Mama *et al*.,2014).

Environmental samples included twenty nine samples were collected from soil and water, they were of two types, the first was collected from the soil (agricultural soil) after removing the top layer, and the soil was kept in a sterile plastic bag for the purpose of transporting it to the laboratory (Palese *et al*.,2009). As for the second type of environmental samples, they were collected from river water randomly and were placed in sterilized glass bottles and were also transferred to the laboratory for the purpose of isolation and diagnosis (Momba.,2006).

2.2.4.Sample culturing

At the lab, clinical samples were cultured by streaking method and grown on Petri dishes containing the culture media (blood agar and MacConkey agar) and incubated in air conditions at 37 ° C for 24 h, bacterial isolates were purified on MacConkey agar medium and re-cultivated. Environmental samples were treated in two ways, according to their type, For soil, 17 samples were collected. In the laboratory, 10 g of each sample was placed in a glass flask containing 100 ml of sterile distilled water. The flask was shaken for 5 minutes, then 10 ml of the last suspension was taken and placed in a flask containing 90 ml of sterile distilled water. Three dilutions were made for each sample. Each time 1 ml of the bacterial suspension was added to a test tube containing 9 ml of sterile distilled water and shaken well, then 1 ml of the third dilution was placed on a Petri dish containing cetrimide agar and spread. The process was repeated for all samples. Then the dishes were incubated at 37 ° C for a 24 h (Janssen *et al*.,1997).

As for the water samples, 100 ml of water was taken and passed on a filter membrane. After filtering the water, the filter membrane was transferred by flame-sterilized forceps to the nutrient medium.

After the incubation period, the filter membrane was lifted with sterile forceps to isolate the bacteria on the surface of the membrane, where Pseudomonas aeruginosa is characterized by green color. Due to the secretion of pyocyanin pigment (Adade et al.,2014)

After that a number of phenotypic and biochemical diagnostic tests were performed for the grown bacteria.

2.2.5.Laboratory diagnosis of isolates:

Samples were cultured on selective MacConkey culture media as a primary culture, after which they were cultured on enriched and differential media such as blood agar and selective media Citramide agar (Bonnet *et al*.,2020).

2.2.5.1.Phenotypic diagnosis:

Isolates were cultured on each of MacConkey agar, Citramide agar, blood agar, and nutrient agar, and then dishes were incubated for 24 h at 37 ° C. visible colonies were subjected to study their cultural characteristics of the color, shape, size, smell and edge (Bonnet *et al*.,2020).

2.2.5.2.Microscopic examination

Slides were prepared from pure colonies and subjected to Gram staining to studying the characteristics of the bacterial cells under a combined light microscope by observing the shape, arrangement and color of the cells.

P. aeruginosa isolates were examined under a microscope with a Gram stain to determine the bacteria's form, scale, arrangement, and interaction with the Gram stain (Atlas, 2004) .

2.2.5.3.Biochemical tests:

1.Catalase test

A portion of a pure colony was transferred by wooden sticks to the surface of a clean glass slide. A drop of reagent was placed on the colony, and the appearance of bubbles was evident for the positive test and the ability of bacteria to produce catalase enzyme (Tindall *et al*.,2007).

2.Oxidase test

Pure colony aged 18-24 h was transferred by sterile wooden sticks prepared for this purpose to Whatman (filter paper), A few drops of oxidase reagent were applied to it. The appearance of a dark purple color after 0-5 seconds was considered a positive result of the test and the ability of bacteria to produce oxidase enzyme (Tindall *et al*.,2007).

3. Hemolysis test

Fresh colonies were cultured on a blood agar medium and incubated at 37 °C for 24 h. Then a transparent corona was observed around the bacterial colony which was considered an evidence of the ability of this bacteria to lysis blood (El-Gebaly,2020).

4.Cetrimide test

Dishes containing cetrimide agar (selective medium) were inoculated with isolates to be tested at 24 h of age and incubated at 37 ° C for 24 h. The appearance of bacterial growth indicated to a positive result which means evidence for *P. aeruginosa* growth (Deepalakshmi *et al*.,2010).

5. Bacteria growth test at 42 ° C:

Nutrient agar was inoculated with bacterial colonies, then the dishes were incubated at 42 ° C for 24 h. Growth appearance was indicated for the positivity of the assay (Gull and Hafeez,2012).

2.2.6.VITEK2 Compact Diagnosis:

The VITEK2 device, equipped by Bio Merieux, was used to conduct biochemical tests for these bacterial isolates. The VITEK2 device includes 48 types of biochemical tests that are used for the diagnosis of bacteria. The diagnostic accuracy of this device reaches approximately 99%. (Pincus, 2006).

2.2.7.Antibiotic sensitivity test with VITEK technology

In order to obtain a more accurate diagnosis and to identify the medication sensitivity of isolates, the diagnostic and medication sensitivity test for 24 bacterial isolates It was chosen according to the highest percentage of diagnosis in the VITEK device, was performed using the developed and modern VITEK device technology, (Mondelli *et al*.,2012).

Table (2-7) shows the antibiotics used in the present study, which included:

No.	Antibiotics	Symbol
1	Amoxicilin	AML
2	Amoxicilin-clavulanic acid	AMC
3	Piperacillin	PRL
4	Cefepime	FEP
5	Ceftazidime	CAZ
6	Imipenem	IPM
7	Meropenem	MEM
8	Amikacin	AK
9	Gentamycin	CN
10	Tobramycin	TOB
11	Ciprofloxacin	CIP
12	Pefloxacin	PEF
13	Colistin	CT
14	Trimethoprim/Sulfamethoxazole	SXT

2.2.8.Preservation of bacterial isolates for a long time in glycerol

Bacteria preservation medium was prepared by dissolving 37 g of the medium in a liter of distilled water and distributed in laboratory tubes, after which it was sterilized adding 15% ml of glycerol to 85% ml of brain-heart infusion broth medium. Finally, these tubes were kept at a temperature of -20 ° C with taking into account its renewal every three months (Vitko and Richardson, 2013).

2.2.9.Molecular diagnosis

Twenty four selected bacterial isolates (according to the range of their antibiotics resistance), Sixteen from clinical source and eight from environmental source, all these isolates were subjected for DNA extraction and polymerase chain reaction (PCR).

2.2.9.1.DNA extraction

DNA was extracted from the isolated bacteria by special DNA kit according to the instructions of the company that manufactured it (iNtRoN Biotechnology, Korea) as the following steps:

1. Broth bacterial culture was centrifuged (1-2 ml) at 13000 rpm for 1 min, and the supernatant was removed.

2. Three hundred µl of buffer G solution was added and mixed well.

3. Incubation was conducted at 65 ° C for 15 minutes, in order to assist in cell lysis, tubes was inverted every 5 min during incubation period.

4.Two hundred fifty µl of binding buffer was added and mixed well by vortex.

5. Cells were degraded loading onto column and centrifuged 13000 rpm for 1 min.

6. (To wash) 500 µl of wash buffer A was added to the column and centrifuged for 1 min. at 13000 rpm.

7.The solution was removed, and then 500 µl of wash buffer B was added to the column and a 1 min centrifugation process was carried out at 13000 rpm.

8. The solution was removed and a 1-min centrifugation process was carried out at 13000rpm.

9. The G-Spin™ column was placed in a 1.5 mL microcentrifuge tube and then 20-200 µl of Elution buffer was added directly onto the membrane.

10. Tube were incubated at room temperature for 1 min, followed by centrifugation for 1 min at 13000 rpm.

11. The DNA was stored at -20 ° C until use.

2.2.9.2. Polymerase chain reaction (PCR)

PCR mixture preparation:

1. After DNA extractions, polymerase chain reaction (PCR) was performed to amplify the target genes. The mixture was prepared for each sample with the same components as Accupower PCR used premix-Bioneer com. This mixture was prepared according to the manufacturer of it according to the following table:

Table(2-8): components of the PCR premix mixture

No.	**PCR Mixture**	**Volum**
1	DNA template	5 µl
2	Forward primer	1.5 pmol/µl
3	Nuclease Free Water	12 µl
4	Reverse primer	1.5 pmol/µl
5	Total volume	20 µl

2. Reaction tubes were placed in a PCR Thermo cycle device to perform the DNA Amplification process under optimal conditions for the thermal cycles (Alawad,2019)

Table (2-9): The number of thermal cycles of PCR

PCR Step	Repeat cycle	Temperature	Time
Initial denaturation	1	95℃	5 min
Denaturation	30	95℃	**30 sec**
Annealing		55℃	**30 sec**
Extension		72 ℃	**45 sec**
Final extension	1	72 ℃	**5 min**

2.2.9.3. Gel Electrophoresis:

PCR product was analyzed by agarose gel electrophoresis (Mladin *et al*., 2009) following the next steps:

1. One percent of the agarose gel was prepared using 1x concentration of TBE buffer by adding 1 g of agarose in 100 ml (TBE buffer) (Tris-borate-EDTA) electrophoresis solution and placed in a water bath to dissolve the agarose.

2. It was thawed in a water bath at 100 ° C for 15 min, after which it was left for decreasing temperature to 50 ° C.

3. Added 0.5 mg / ml of Ethidium bromide stain to the agarose gel solution.

4. The comb was installed in the designated place to locate the locations of the PCR samples. Then the agarose gel was poured into the migration mold and left at the place temperature for 15 min for hardening. Then the comb was removed quietly from the migration template.

5. Six µl of PCR product was added to each well of the comb pits, after which 5 µl of DNA ladder was added to the volumetric guide of the PCR product and placed in the first well.

6. Then the gel mold was installed in the relay basin and filled with 1x (TBE) (Tris-borate- EDTA) , the relay cover was closed, and the device was turned on . Then an electric current of 70 volts and 80 mA was passed for 55 min.

7. After the migration process was completed, the gel containing the PCR product was examined by ultraviolet light(U-V Transillumator).

2.2.10. DNA Sequencing

Sequencing of samples was done by Macrogen company (Korea).Ten isolates sent to Korea for Sequencing, five isolates for sequencing of the *LecA* gene and another five for the *LasA* gene that included clinical and environmental isolates., the results was viewed using NCBI and MEGA7 software.

3.2.11. Bioinformatics Analysis

The bioinformatics programs and websites used in the present study were Mega and Blast.

3.2.11.1. Opine Sample Sequencing Files

Molecular Evolutionary Genetics Analysis (MEGA) was used as described in Tamura *et al*., (2011). Alignment tool in toolbar was selected, then sample sequence file was selected.

3.2.11.2. Alignment Analysis and Phylogenic Tree

Basic Local Alignment Search Tool for nucleotides (Nucleotides BLAST) was used to make alignment to the sequences of the samples (Altschul *et al*., 1990). Also, the phylogenic tree was made by select distance tree of results.

2.2.12.Plant collection and extraction

Six types of plant were used in this study, which were listed in the Table(2-10)

Table (2-10) Types of plant wich used in the present study

No.	**Plant name**	**The extracted part of the plant**
1	Quercus cortex	Stem bark
2	*Artemisia*	All the plant
3	*Nigella sativa*	Seed
4	*Cinnamon*	Bark
5	*Pimpinella anisum*	Seed
6	*Myrtus communis*	Leaves

They were purchased from local markets, saved dry sacs, then grinded by (grinder) to make powder and stored in a dark glass container at room temperature.

Ten gm of each substance were weighed and placed in a volumetric flask with a capacity of one liter, after which 100 ml of ethanol, methanol and hot water were separately added (Adwan *et al*., 2007). The flasks were placed in the shaking incubator for 24 h. After that, the filtration process were took place through several layers of medical gauze and filter papers, and the sterile filtrate was collected in a sterile glass bottle, and then poured into a flat glass dish in the air oven for 24 h at a temperature of 46 °C. The solvent was disposed of in the air oven by evaporation until the sediment became completely sticky powder on glass, they were scraped to obtain a powder-like substance.

The extract was kept in the refrigerator until use. The process was repeated several times to obtain a sufficient amount of the extract (Bokhari, 2009).

Prepare 100 mg/ml stock solution for each plant extract by dissolving 1 gm of plant powder in 9ml of distill water, then it was diluted to obtain four concentrations (25%,50%,75% and 100%) mg/ml of the extract to test the sensitivity of isolates against the extract of these plant (Mitscher *et al.*,1972).

Three bacteria from different sources (clinical, soil and water) to be tested for sensitivity to plant extracts depending on its high resistance and its possession of virulence genes were cultured on brain heart infusion broth for the purpose of activating it. Then, they were incubated at 37 °C for 24 h, after incubation, cell numbers were determined using standard McFarland tubes (Al-Rawi and Altaee, 2019).

The well diffusion method involves spreading 0.1 ml of the bacterial suspension on the medium of Mueller Hinton agar and making four wells of equal distances and with a diameter of 5 mm by means of a cork borer. The extract concentrations were added at the rate of 0.2 mg/ml to each well with different concentrations of the plant extract. Then the dishes were left in the incubator for 24 h at 37 °C for the diffusion of plant extracts in the culture media, after which the diameter of the inhibition zone was measured using a ruler (Saxena *et al.*,1996).

2.2.13. Statistical analysis

Data of antibacterial activity test were presented as mean ± standard deviations (SD). one way analysis of variance (ANOVA) followed by Tukey's HSD multiple range *post hoc* testing to determine the significant differences between concentration of solvents and type of solvents. Statical analysis were carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA). The level for accepted statistical significance was $P < 0.05$

Chapter Three
Results
and
Discussion

3.1.Isolation of *P. aeruginosa*

Ninety samples were collected and they included 61 swabs from burns patients laying in the hospital and 29 samples from soil and water.The results of the current study showed that 32 isolates formed 52.4% were of *P. aeruginosa* which isolated from burn patients, while 29 formed 47.5% isolates were negative culture, represented by no growth appears on the dishes or when bacterial growth appears with characteristics that do not resemble the required bacteria.

The present results are in agreement with the study of Hameed Abboud, (2014), who showed that the highest rate of bacterial isolates from burn patients was *P. aeruginosa*, which accounted for 41 isolates,out of 76 bacterial isolates.

The environmental isolates included 8 out of 29 positive while 21 isolates were negative cultures, this include 5 isolate from soil and 3 isolate from water. The number and percentage of bacteria isolate show in Table(3-1)

Table (3-1):The numbers and percentages of bacteria isolates:

Sample type	Positive culture	Negative culture	Total
Clinical	32 (35.5%)	29 (32.2%)	61 (67.7%)
Environmental	8 (8.9%)	21(23.3%)	29 (32.2%)
Total	40 (44.4%)	50 (55.5%)	90 (100%)

The present study confirmed that *P. aeruginosa* is one of the most common bacteria isolated from the burn patients at Al- Hussein Medical Hospital / Kerbala Governorate due to the spreading of *P. aeruginosa* in the hospital environment at the present time (Todar,2008).

The study of Wolf and Elsasser-Beile, (2009) attributed the high ability of *P. aeruginosa* to cause infection in hospitalized patients to such factors including its virulence factors, its high ability to withstand high extremism in environmental conditions and the dirty and polluted hospitals which are considered as suitable mediums for supporting the growth of these bacteria and increases their opportunity to attack the patients who are dormant in these hospitals. Those patients have weak immune system because they suffer from various diseases and injuries.

3.2.Identification of *P. aeruginosa*

After completing the culture process and incubating for 24 h, bacterial growth appeared on the culture medium, These bacteria are oxidized in metabolic reactions and are not fermenter to common carbohydrates, but may produce acid in the case of oxidation. This characteristic may be used to distinguish these bacteria from the rest of Gram-negative bacteria, which are lactose fermenter family in the intestinal and mobile and grow on the simplest cultivated medium (Alhazmi, 2015).

The grown bacteria on blood agar gave rise to complete lysis (β-Hemolysis) of the blood, which indicates the ability of the bacteria to produce hemolysin and analyze red blood cells (Macfaddin , 2000). Appendix(V) show bacterial colonies growing on Blood agar, after 24 h. at 37Cº.

The cultured isolates of *P. aeruginosa* on nutrient agar showed largely colonies with flat edges and a raised appearance in addition to their ability to produce the pyocyanin pigment.

Then the growing bacteria were transferred to a selective agar media for *P. aeruginosa* (cetrimide agar), due to its containment of cetrimide that inhibit the growth of all bacteria except *P. aeruginosa*.

After the incubation period ended, the colonies appeared clear on the surface of the Cetrimide agar, indicating that the isolates were *P. aeruginosa*. As the Cetrimide medium is a selective medium used to isolate Gram-negative *P.aeruginosa* bacteria. It contains in its composition Cetrimide, which is an inhibitory factor for the growth of other bacterial species as well as increases the bacterial production of external pigments such as fluorescein, which appears in a greenish-yellow color and pyocyanin that appears in a bluish-green color. It is widely used in the examination of medical specimens for the presence of *P. aeruginosa* (Jorgensen *et al*.,2015).

Next, isolates of *P. aeruginosa* were identified further by being cultured on nutrient agar at 42 ^{o}C . The bacterial colonies at the end of incubation time were existing which indicated the ability of these isolates to grow at a degree of 42°C. This temperature do not allow the growth of the rest species in the genus Pseudomonas, therefore, this trait was considered an important diagnostic feature for the species of *P. aeruginosa* (Al-Daraghi and Al-Badrwi, 2020).

Light microscope was applied to investigate the bacterial smear stained with Gram stain, and the bacteria appeared in the form of single bacilli or short chains. Table(3- 2) summarizes the results of biochemical tests applied for the identification of *P. aeruginosa* in the present study.

Table(3- 2): Results of biochemical tests for identification of *P.aeruginosa*

Name of tests	Results
Gram- stain	-
Hemolysis (β- hemolysis)	+
Catalase test	+
Cetrimide agar base	+
Oxidase test	+
Growth at 42°C	+

3.3. Susceptibility test of *P. aeruginosa* to antibiotics

The sensitivity test of bacterial isolates was carried out using VITEK device (VITEK 2 Compact) and using the antibiotic tape prepared by the French manufacturer (Biomerius), where antibiotics of different concentrations were used against the bacterial isolates, Appendix(I) show The results of diagnosis of *P. aeruginosa* bacteria using the VITEK-2 device based on biochemical tests and Appendices(VI) (VII) tables showing the number of clinical isolates and environmental isolates and the rate of diagnosis with the VITEK device.

The VITEK device is one of the best devices to identify microorganisms very accurately in a short period of time .It determines the bacteria automatically and conducts 64 types of biochemical tests without the need for any other tests , and it can distinguish between Gram-positive and Gram-negative bacteria. In addition to sensitivity test for antibiotics , the most important characteristic of this device its ability to identify live cells only by using the identification card , where the level diagnosis of the microorganism is determined by a map of its tests . In comparison to the classification characteristics of the device which help in identifying the organism, its probability ratio and its level of confidence.

Therefore, the VITEK2 device is one of the most recently approved devices in laboratories and this is in agreement with the study of Mondelli *et al*., (2012), who proved that the technique of the VITEK device is an accurate and fast technique that must be used in the diagnosis of microorganisms in all laboratories.

Furthermore, the development of this technique helps in selecting appropriate antibiotics for each pathogen which can be diagnosed by the sensitivity card(Ligozzi *et al*., 2002). The result obtained from the device's diagnosis showed that the bacterial isolates were determined to be *P. aeruginosa* in the present study, Appendix(II) show the results of antibiotic sensitivity test using the VITEK-2 device.

Sensitivity of the bacterial isolates showed that there is a very high resistance revealed by *P. aeruginosa* to the β-lactam antibiotics represented by (penicillin group) such as ampicillin and amoxicillin, where the percentage of resistance to these antibiotics was 100% and this corresponds to what was found by Gad *et al*.,(2008) where the percentage of resistance to these mentioned antibiotics was 100%. The results of antibiotics activity against *P. aeruginosa* are shown in Table (3-3).

Table (3-3):The results of antibiotics activity against *P. aeruginosa*

No.	Name of antibiotic	The resistance ratio%
1	Ampicillin	100
2	Amoxicillin	100
3	Piperacillin	100
4	Cefuroxim	100
5	Cefotaxime	100
6	Meropenem	100
7	Gentamicin	88.8
8	Amikacin	72.7
9	Colistin	0

The resistance ratio of *P. aeruginosa against* piperacillin was 100% which is in agreement with the study of Mohammad, (2013).

Hsueh *et al*., (2005) found that the resistance of bacteria to many antibiotics, including beta-lactams, increase with the increase in the consumption of these antibiotics, and one of the reasons for this resistance is due to the bacteria's production of a large number of beta-lactamase enzymes that break down these antibiotics as a result of continuous and prolonged exposure to these antibiotic.

The current data also showed that there is a relatively high resistance revealed by *P. aeruginosa* isolates to anti-cephalosporins, where the percentage of bacterial resistance to cefuroxime was 100%, and this percentage was completely consistent with the study of Isichei-Ukah and Enabulele ,(2018).

The percentage of bacterial resistance to cefotaxime was 100% and these results are in agreement with the study of Kahaleq *et al*.,(2015) who found that the percentage of resistance to cefotaxime was 90% .

The present study revealed that *P.* aeruginosa had a high resistance to meropenem (100%) which is in agreement with study of Sid Ahmed *et al*.,(2019). In opposite to data of the present study, the resistance rate against meropenem antibiotics was 41% (Hosu *et al*., 2021).

On the other hand, the bacterial resistance to the aminoglycosides family such as Gentamicin was 88.8%, and this result is similar to what was found by Khosravi *et al.*,(2019). The rate of *P. aeruginosa* resistance against Amikacin was 72.7% and this data is consonant with the study of AL-Salihi *et al*.,(2014) who recorded 75% as the percentage of antibiotic resistance.

Finally, all isolates of the tested bacteria conferred high sensitivity (100%) to colistin which belongs to the family of polypeptides which is in agreement with the study of Goli *et al.,* (2016) who found the resistance rate was 2%.

A number of reasons have been listed by Lambert, (2002) to explain the apparent variation in the sensitivity and resistance of *P. aeruginosa* isolates to different antibiotics including this bacterium which is characterized by having a low permeability cell wall. It also has a genetic ability to express many mechanisms of resistance and to become more resistant through mutations that occur to chromosomal genes. It is also distinguished by its ability to acquire genes responsible for resistance from specific external sources such as plasmids, transposons and bacteriophages.

3.4.Detection of the *LecA* and *LasA* gene

The results of the electrophoresis obtained from PCR products of amplification of the *LecA* gene using the designed primers for this gene on the agarose gel showed bundles within the expected size of the gene (369 base pairs), Figure (3-1) indicates the PCR results for clinical and environmental samples, these results are in agreement with the results of Veeresh *et al*.,(2012), who confirmed the presence of this genes in all *P. aeruginosa* isolates.

The results of the electrophoresis of the polymerase chain reaction of *LecA* gene for 24 isolates (5 soil, 3 water, 16 clinical) selected because of their high antibiotic resistance showed that 19 isolate(environmental isolates and 11 clinical isolates) possess the gene and showed bands in agarose gel electrophoresis with a size of 369 base pairs.

The gene expression of lectin A is complex and it is under the control of the sensitization phenomenon. In addition, its production depends on the sigma factor of the polymerase enzyme (Diggle *et al*., 2002).

The results of the electrophoresis of the polymerase chain reaction of *LasA* gene for 24 isolates (5 soil, 3 water, 16 clinical) selected because of their high antibiotic resistance showed that 18 isolate(environmental isolates and 10 clinical isolates) possess the gene and showed bands in agarose gel electrophoresis with a size of 226 base pairs.

The *LasA* gene is also responsible for an important virulence factor in *P. aeruginosa* where *LasA* expression was correlated with antibiotic resistance in *P. aeruginosa* clinical isolates(Jurado-Martín *et al*.,2021)

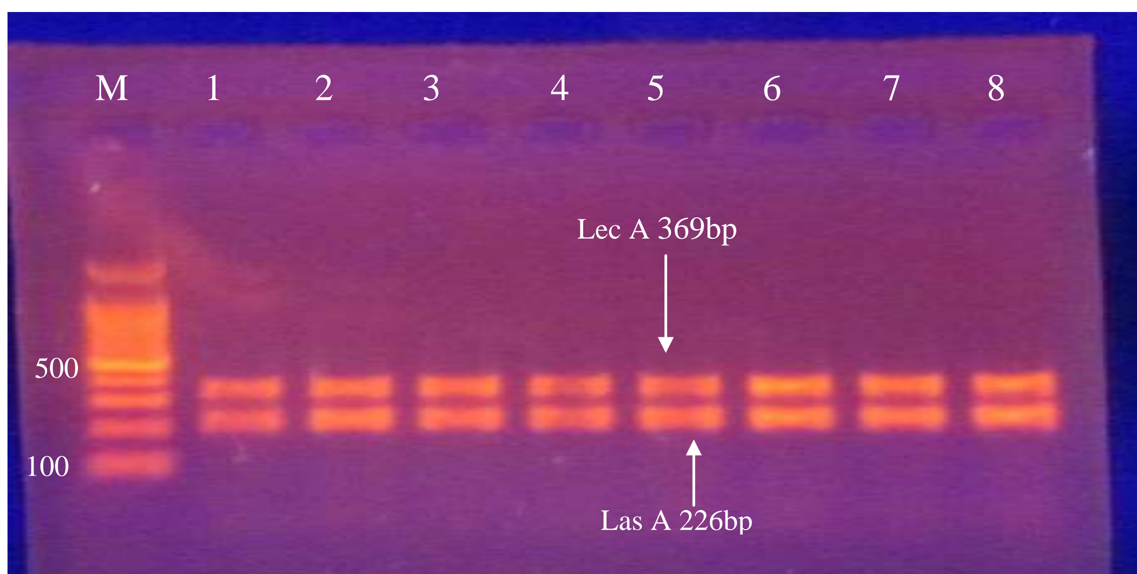


Figure (3-1) : Agarose gel electrophoresis of PCR products on 1% agarose gel at a voltage of 70 V for 55 minutes using specific primers to detect *LecA* and *LasA* genes, M: Marker

3.5.Phylogenetic Analysis of *LecA* and *LasA* Gene

Phylogenetic analysis was conducted for LecA and LasA genes, by comparing local sequences with their corresponding international sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

3.5.1. Sequence result for *LecA* gene

The DNA sequence for *LecA* gene determined the first time from this bacteria as *P.aeruginosa* was aligned with the reference database records (GenBank) using nucleotides BLAST software for nucleotides, that showed a complete match (100%) with the nearest isolated, Appendix(III) show sequence results for one samples of *LecA* gene

Figure (3-2) shows a comparison of one of the isolates, *LecA*5 Iraq, with one of the closely related isolate CP028848 in China and show in the phylogenic tree

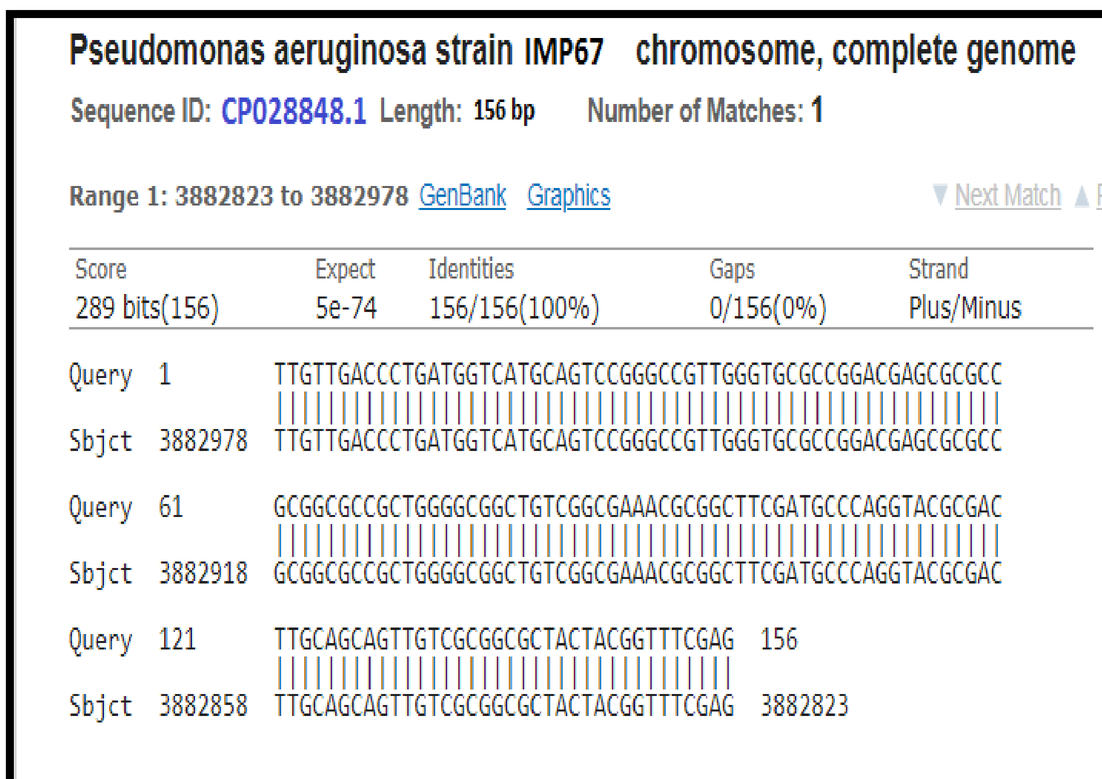
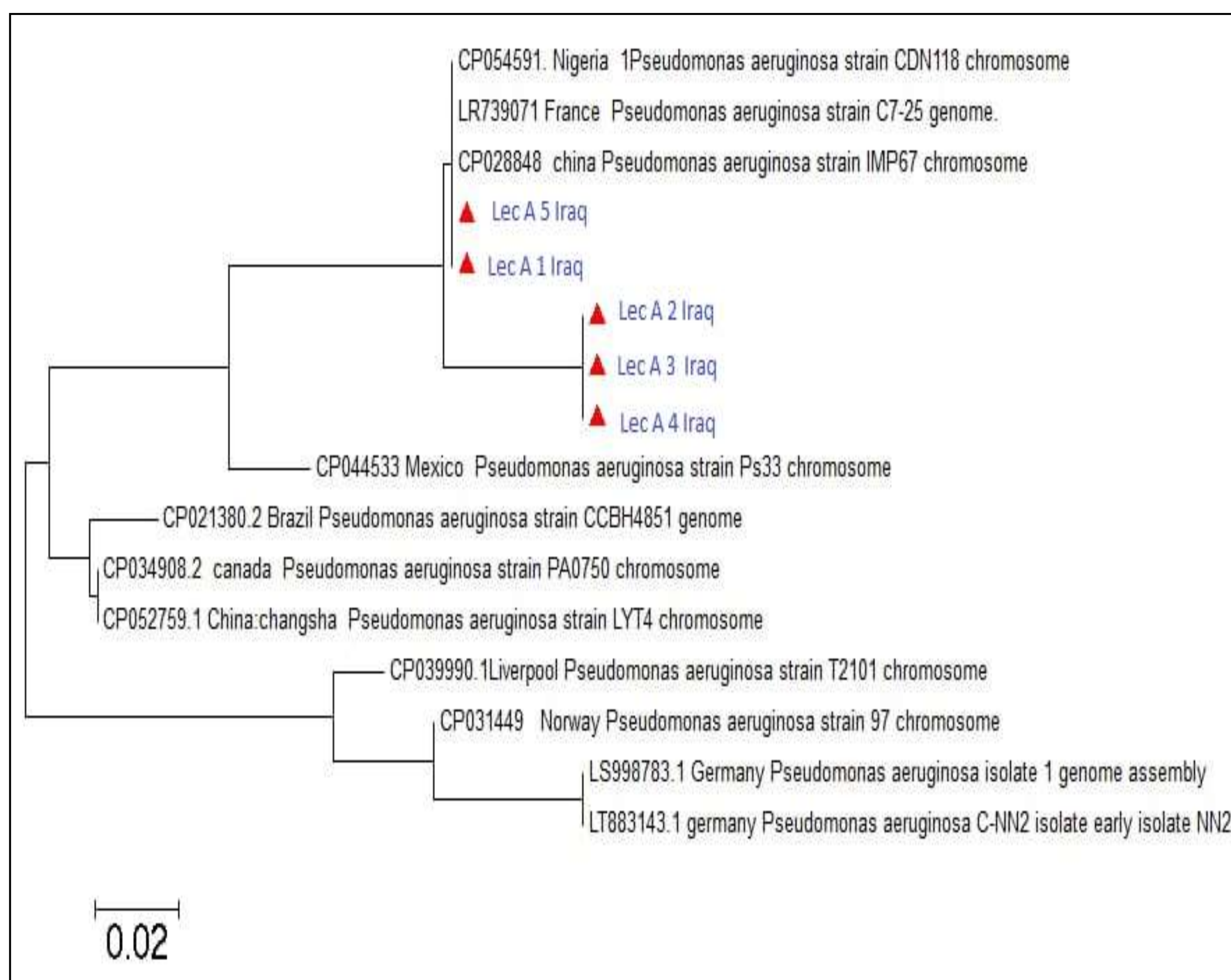


Figure (3-2): Alignment of sample *LecA* gene sequences by nucleotide BLAST which showed complete match (100%) between sequence in this study (Query) LecA5 Iraq comparing with global isolates (Sbjct) CP028848 in China.

When the local and international isolates were directly compared (similarity percentage), the highest similarity was100% which found between LecA5 Iraq and LecA1Iraq and three previous studies (CP054591) was in Nigeria (Shaikh, 2017), (LR739071) in France (Pource1 and Vergnaud, 2019) and the third one was (CP028848) in China by (Xu, 2018) Figure (3-3)

The lowest similarity was (50%) observed between the remaining three isolates, which were LecA2 Iraq, LecA3 Iraq and LecA4 Iraq with the previous studies aforementioned as new strains were considered.



Figure(3-3): Molecular phylogenetic analysis by maximum likelihood method represent the local and international isolates sequences of *LecA* gene.

3.5.2.Sequence result for *LasA* gene

The DNA sequence for the *LasA* gene, which was discovered for the first time in this bacteria as *P.aeruginosa*, was matched with the reference database records (GenBank) using nucleotides BLAST software for nucleotides, which revealed a complete match with the nearest isolated, except for the appearance of a gap between the two nitrogenous bases, guanine thymine, as shown in Figure (3-4)

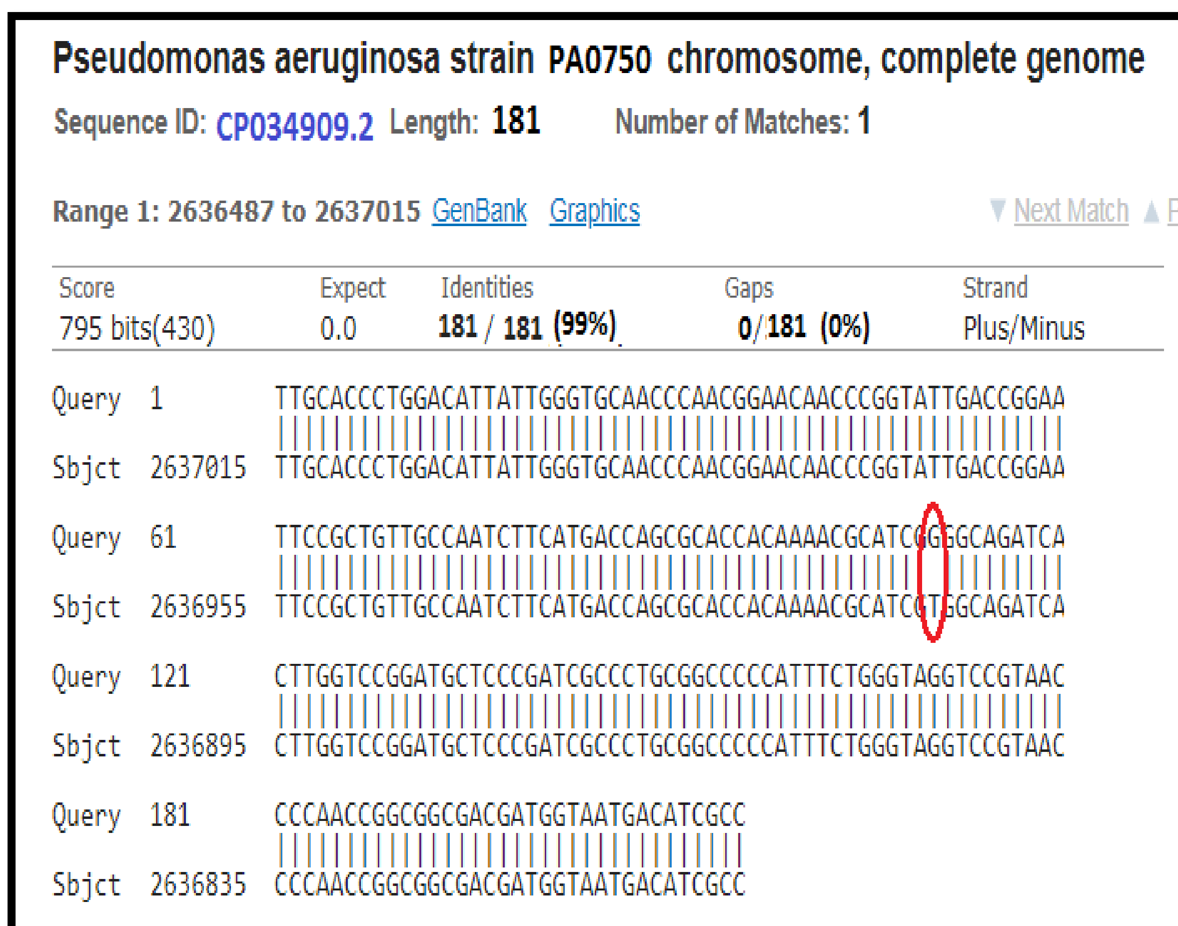
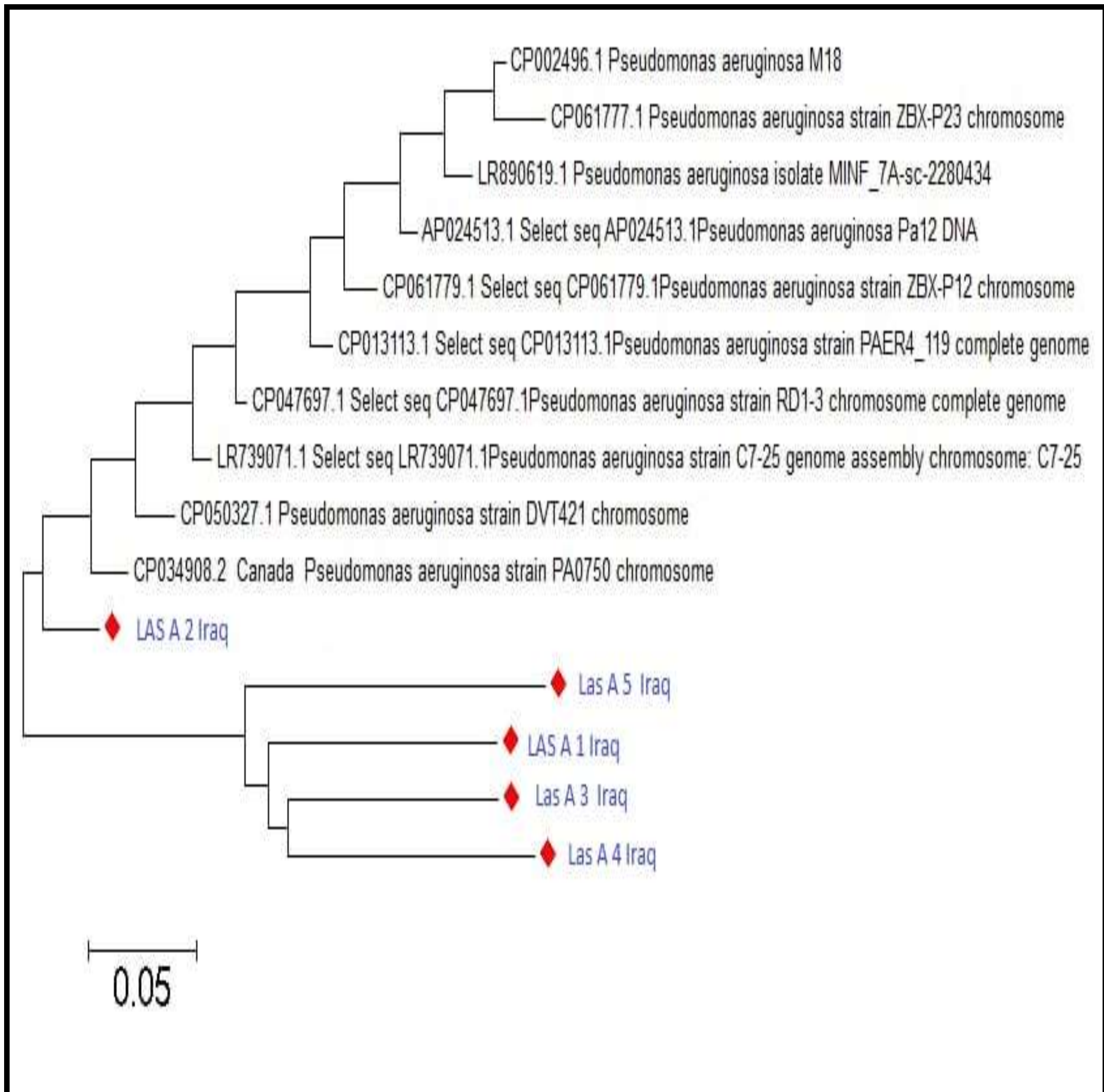


Figure (3-4): Alignment of sample *LasA* gene sequences by nucleotide BLAST which showed complete match between sequence of both isolates LasA2 Iraq and CP034908.2 in Canada except the appearance of a gap between the two nitrogenous bases, guanine thymine.

Sequences results of *LasA* gene showed that only one isolate, LasA2, was close to a previous study (CP034908.2) by Kumar *et al*., (2006) in Canada and the other four isolates, were new strains Figure (3-5) represent the local and international isolates sequences of *LasA* gene. Appendix(IV) show Sequence results for one samples of *LasA* gene.



Figure(3-5): Molecular phylogenetic analysis by maximum likelihood method represent the local and international isolates sequences of *LasA* gene.

Table (3-4):Compairing identities value between local sequences of *LecA and LasA* gene with corresponding homologous international sequences mentioned by their GenBank accessions number.

NO.	Local sequence (Isolates)	BLAST match Accession No.	Source	Identities %	E value
1	Lec A 1	CP054591.1	Nigeria	98.63%	1e-64
2	Lec A 2	CP050335.1	USA	98.45%	2e-55
3	Lec A3	CP053686.1	Kazakhstan	99.41%	4e-79
4	Lec A4	CP050332.1	USA	99.41%	4e-79
5	Lec A5	CP050334.1	USA	97.28%	2e-61
6	Las A1	CP053917.1	South korea	94%	0.0
7	Las A2	CP034908.2	Canada	88.35%	1e-97
8	Las A3	CP053917.1	South korea	92.32%	0.0
9	Las A4	CP050325.1	USA	98.66%	4e-146
10	Las A5	CP045002.1	China	91.21%	0.0

3.6.Susceptibility test of *P. aeruginosa* isolates for some plant extracts

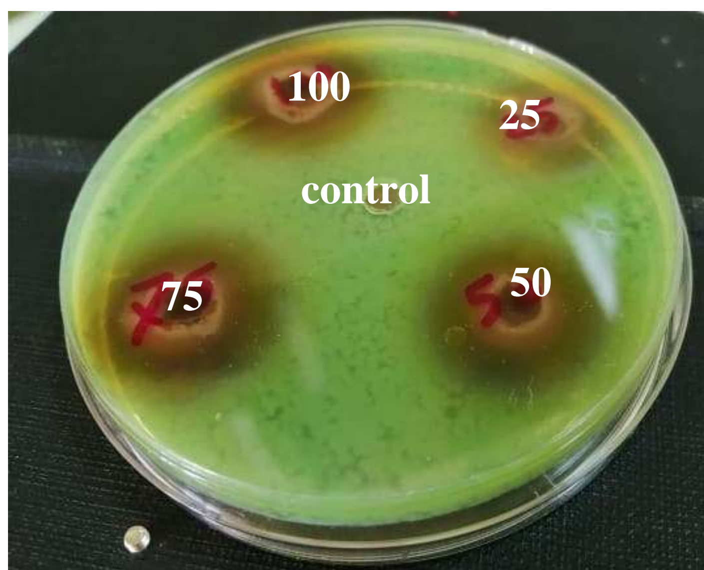
In the current study, six types of plant extracts were tested against three isolates from different sources were selected for their high resistance and possession of the two virulence factors genes *LecA* and *LasA*. including *Cinnamon, Pimpinella anisum, Nigella sativa, Artemisia, Myrtus communis* and Quercus cortex.

The results showed that these bacteria were resistant to five types of extracts and sensitive to only one extract, which is Quercus cortex.

3.7. Antibacterial activity of Quercus cortex against *P.aeruginosa*

The well-known rationale for the therapeutic use of *Quercus* is the direct antibacterial activity against many pathogenic bacteria which causes disease for humans and animals. The antimicrobial effect of *Quercus* bark extracts was previously revealed antibacterial activity against *Pseudomonas, Brucella, Enterobacter, Escherichia, Neisseria* and *Bacillus,* while quite potent antibacterial effects have also be shown against *Escherichia coli* (Aldrich & Cavender, 2011).

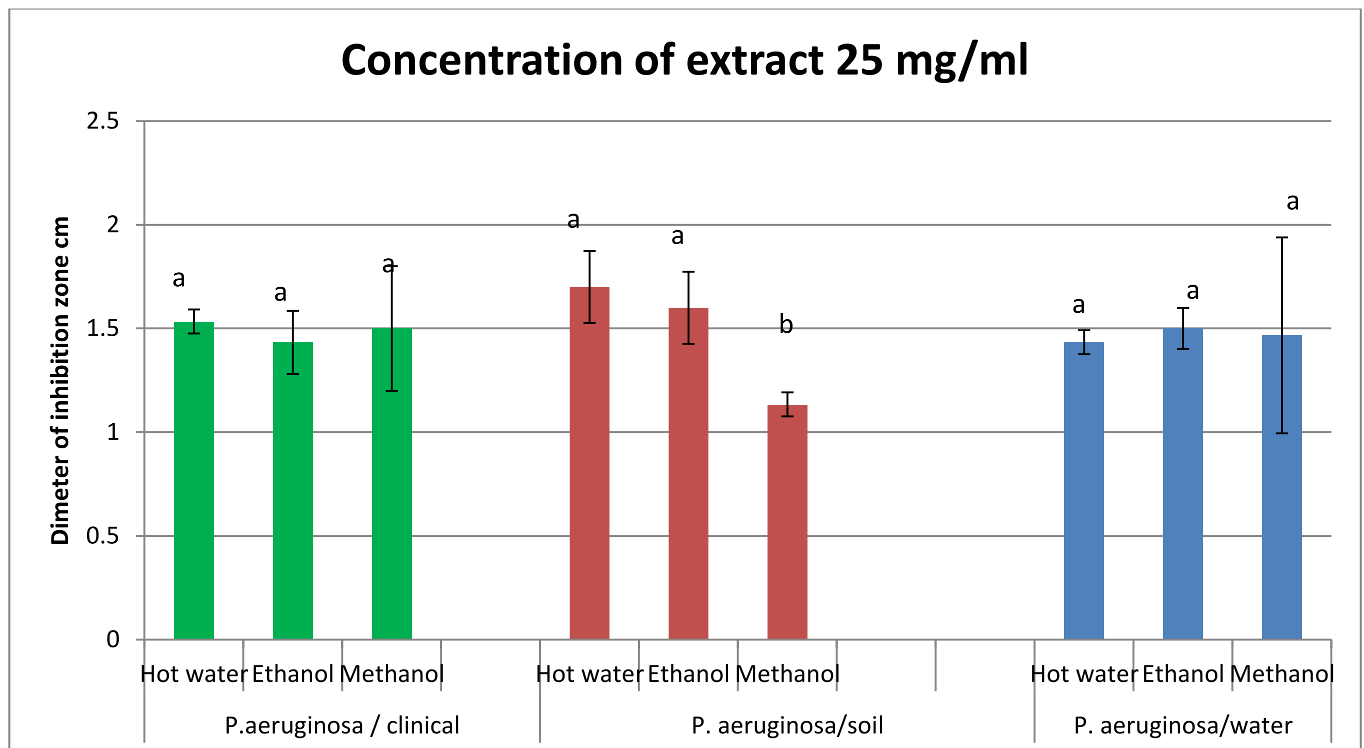
In the present study, Quercus cortex was extracted by ethanol, methanol and hot water and applied to examine bacteria, using 4 concentrations of each extract (25-50-75-100%) mg\ml.



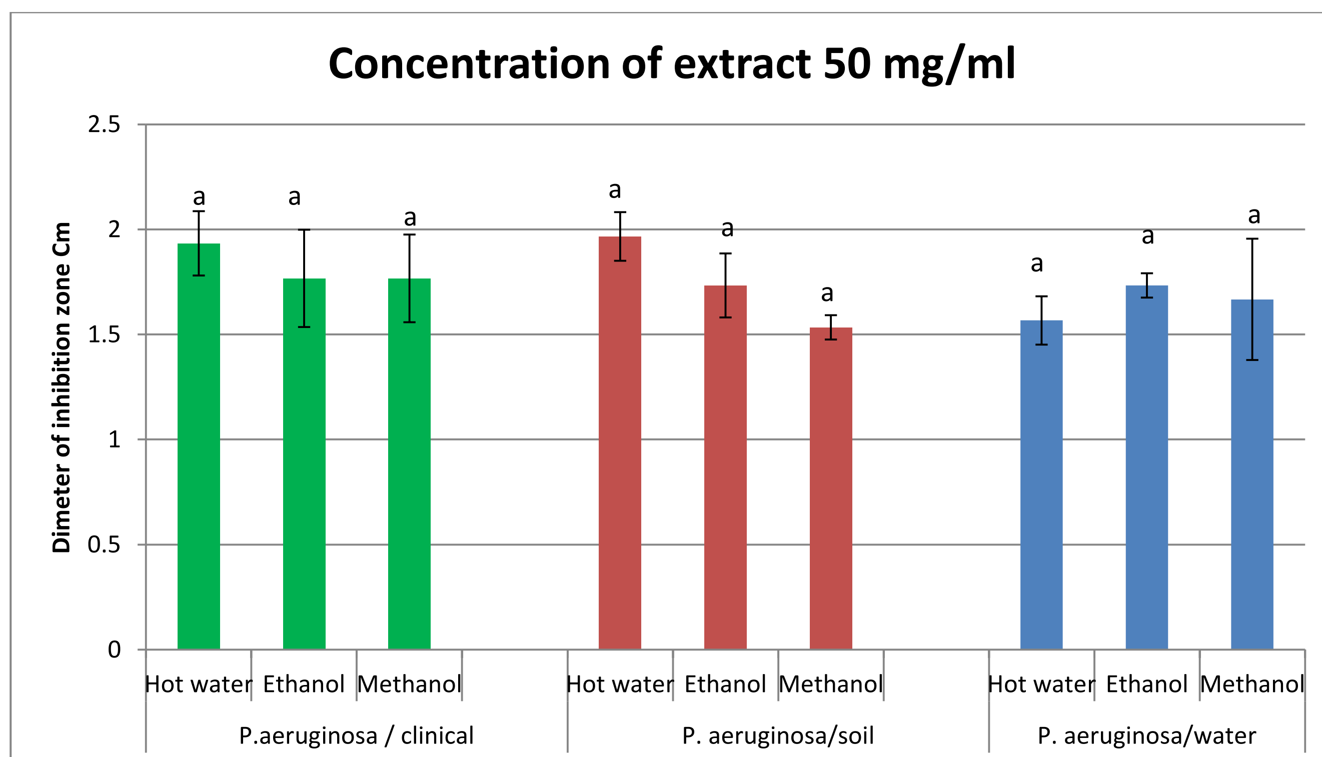
Figure(3-6) The inhibition zone of Quercus cortex extract, The numbers (25,50,75 ,100)mg\ml represent the concentration of the extract in each well. The hole in the center of the petri dish is the control.

Three samples were randomly selected from different sources include clinical, soil and water for testing the sensitivity of plant extracts to them. The results showed that the bacterial isolates were sensitive to Quercus, where a clear area of inhibition appeared around the well in which the plant extract was placed Figure (3-6)

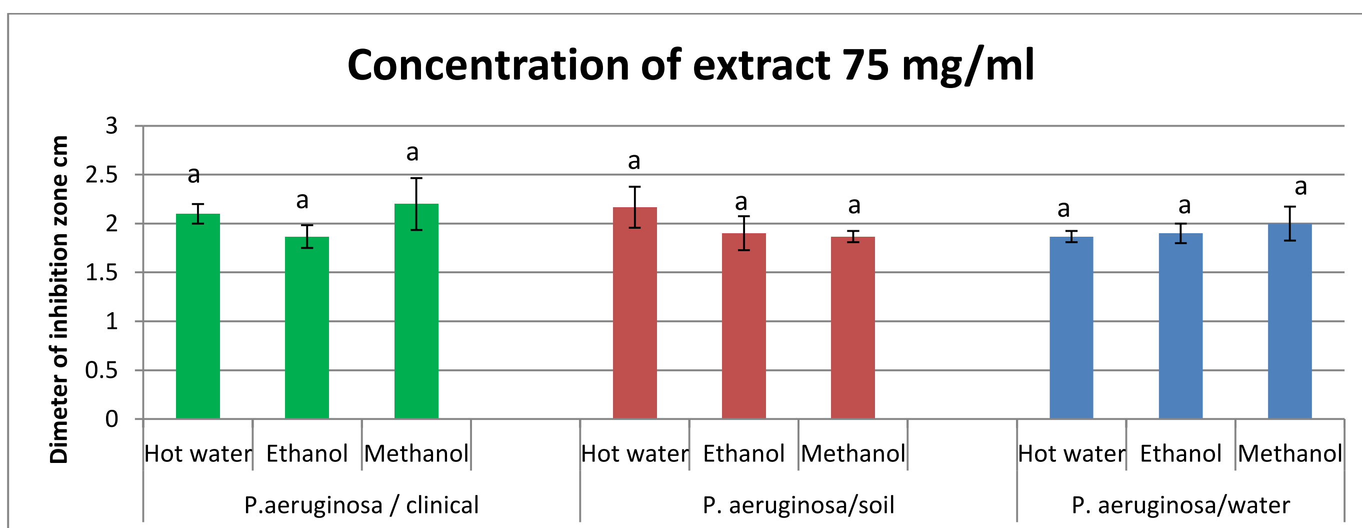
Figure (3-7) showed that no significant differences among solvents in concentration 25 mg/ml aganist isolated bacteria from clinical and water samples. However, the activity of Quercus extracted by methanol in the same concentration was significantly lower against *P. aeruginosa* isolated from soil. On the other hand, no significant differences between the activity of different solvents used in the extraction of this plant in all concentrations used against *P. aeruginosa* isolated from different sources were found in figure (3-8,3-9,3-10). Appendix(VIII),(IX),(X) and (XI) shows effect of each solvent in concentration of 25 mg/ml on *P. aeruginosa* isolated from different sources including clinical, soil and water



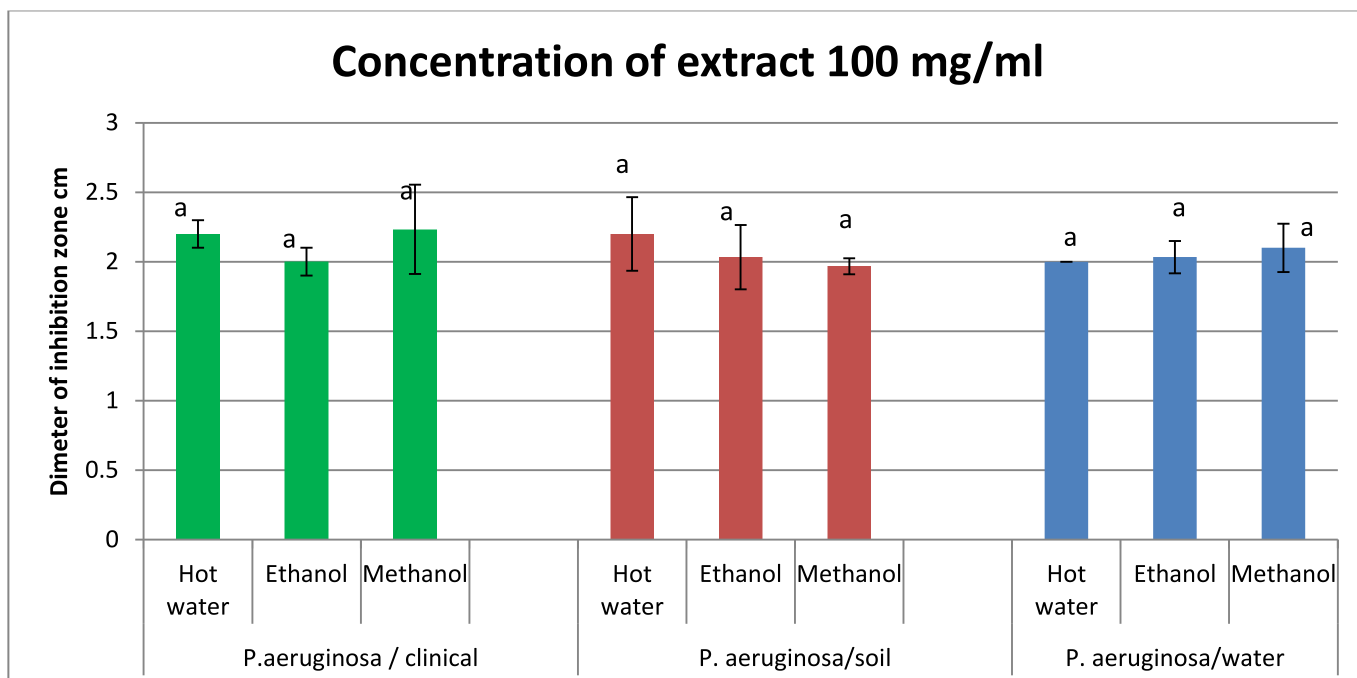
Figure(3-7) Antibacterial activity of different types of Quercus cortex extracts in concentration of 25 mg/ml against *P. aeruginosa* isolated from different source.



Figure(3-8): Antibacterial activity of different types of Quercus cortex extracts in concentration of 50 mg/ml against *P. aeruginosa* isolated from different source.



Figure(3-9): Antibacterial activity of different types of Quercus cortex extracts in concentration of 75 mg/ml against *P. aeruginosa* isolated from different source.



Figure(3-10): Antibacterial activity of different types of Quercus cortex extracts in concentration of 100 mg/ml against *P. aeruginosa* isolated from different source.

Conclusions

And

Recommendations

CONCLUSIONS

1. Emergence of isolates of *P. aeruginosa* isolated from burns resistant to common antibiotics.

2.Colistin has been shown to be effective against *P. aeruginosa.*

3.The results of PCR obtained from the present study showed that the existence of *LecA* and *LasA* genes in some isolates of *P. aeruginosa* could be the reasons behind antibiotic resistance and increased the pathogenicity of these bacteria.

4.Success of Quercus cortex extract in inhibiting the growth of *P. aeruginosa*.

5. The inability of extracts of anise, cinnamon, wormwood, nigella and myrtle to inhibit the growth of bacteria.

RECOMMENDATIONS

1. Adopting the sensitivity test and the PCR reaction to detect the degree of resistance of bacterial isolates as a routine examination for the purpose of selecting appropriate treatments and reducing the random repetition of antibiotics that is responsible for the spread of the phenomenon of multiple resistance.

2. Searching for a new generation of antibacterial that will be more effective in treating infection.

3. Determining the effective groups of cherry bark oak extract that are used as a successful antibacterial.

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Appendices

Appendix(I): The results of diagnosis of *P. aeruginosa* bacteria using the VITEK-2 device based on biochemical tests

bioMérieux Customer:

Microbiology Chart Report

Printed Aug 19, 2020 07:35 CDT

Patient Name: 1, 1
Location:
Lab ID: ab363

Patient ID: 463
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: Burns

Collected:

Comments:	

Identification Information	Analysis Time:	4.88 hours	Status:	Final
Selected Organism	99% Probability	Pseudomonas aeruginosa		
	Bionumber:	0003053001500210		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Appendix(II): The results of antibiotic sensitivity test using the VITEK-2

MEDICA SPECIALIST LAB

Laboratory Report

Printed Oct 27, 2020 09:34 CDT
Autoprint

bioMérieux Customer:
System #:

Patient ID: 1181

Patient Name: 1, b
Isolate: 1181-1 (Approved)

Card Type: AST-N222 Bar Code: 6221345203605311 Testing Instrument: 0000148FFA81 (9271)
Setup Technologist: Laboratory Administrator(Labadmin)

Organism Quantity: **Selected Organism: Pseudomonas aeruginosa**

Susceptibility Information	Card: AST-N222		Lot Number: 6221345203	Expires: Aug 2, 2021 13:00 CDT	
	Completed: Oct 27, 2020 09:34 CDT		Status: Final	Analysis Time: 12.68 hours	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
+Amoxicillin		R	Aztreonam		
+Amoxicillin/Clavulanic Acid		R	Imipenem	8	R
+Ampicillin/Sulbactam		R	Meropenem	>= 16	R
Ticarcillin	>= 128	R	Amikacin	>= 64	R
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	>= 16	R
Piperacillin	>= 128	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
+Cefalexin		R	+Gatifloxacin		R
+Cefalotin		R	+Gemifloxacin		R
+Cefazolin		R	+Levofloxacin		R
+Cefuroxime		R	+Moxifloxacin		R
+Cefixime		R	+Norfloxacin		R
+Cefpodoxime		R	+Ofloxacin		R
+Cefoperazone		R	Pefloxacin		
+Cefotaxime		R	Minocycline		
Ceftazidime	8	*R	Colistin	<= 0.5	S
+Ceftriaxone		R	Rifampicin		
Cefepime	>= 64	R	Trimethoprim/Sulfamethoxazole		

+= Deduced drug *= AES modified **= User modified

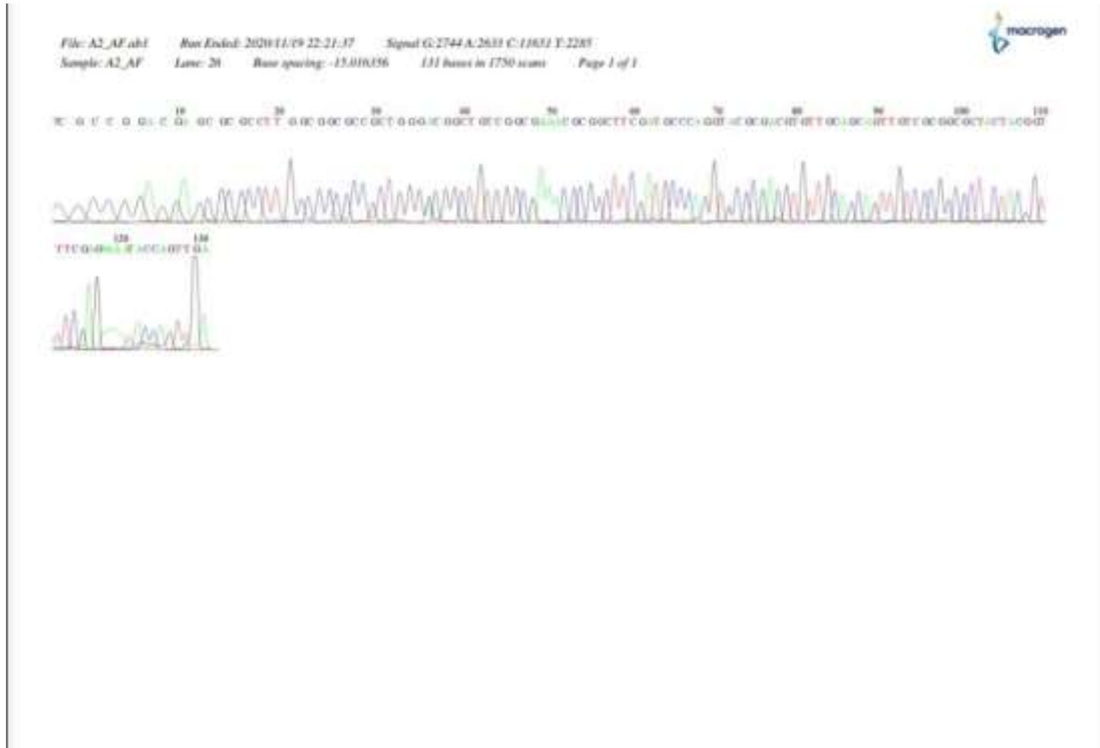
AES Findings:		Last Modified: Oct 12, 2020 13:27 CDT	Parameter Set: Global CLSI-based+Phenotypic 2019
Confidence Level:	Consistent		
Phenotypes flagged for review:	BETA-LACTAMS	CARBAPENEMASE	

Installed VITEK 2 Systems Version: 08.01
MIC Interpretation Guideline: Global CLSI-based 2019
AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019
AES Parameter Last Modified: Oct 12, 2020 13:27 CDT

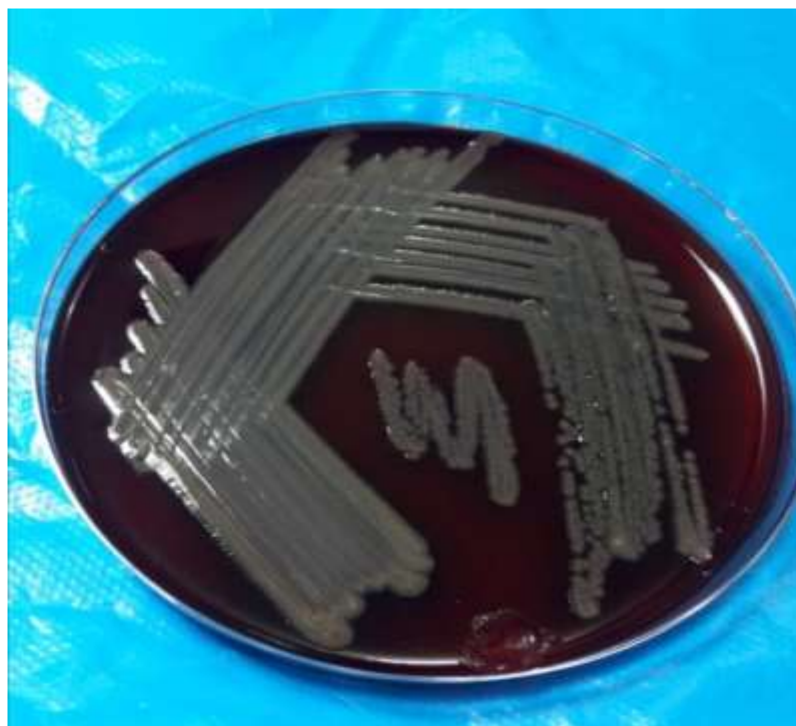
Page 2 of 2

Appendix(III): Sequence results for one samples of *LecA* gene



Appendix(IV): Sequence results for one samples of *LasA* gene

Appendix(V): bacterial colonies growing on Blood agar, after 24 h. at 37Cº



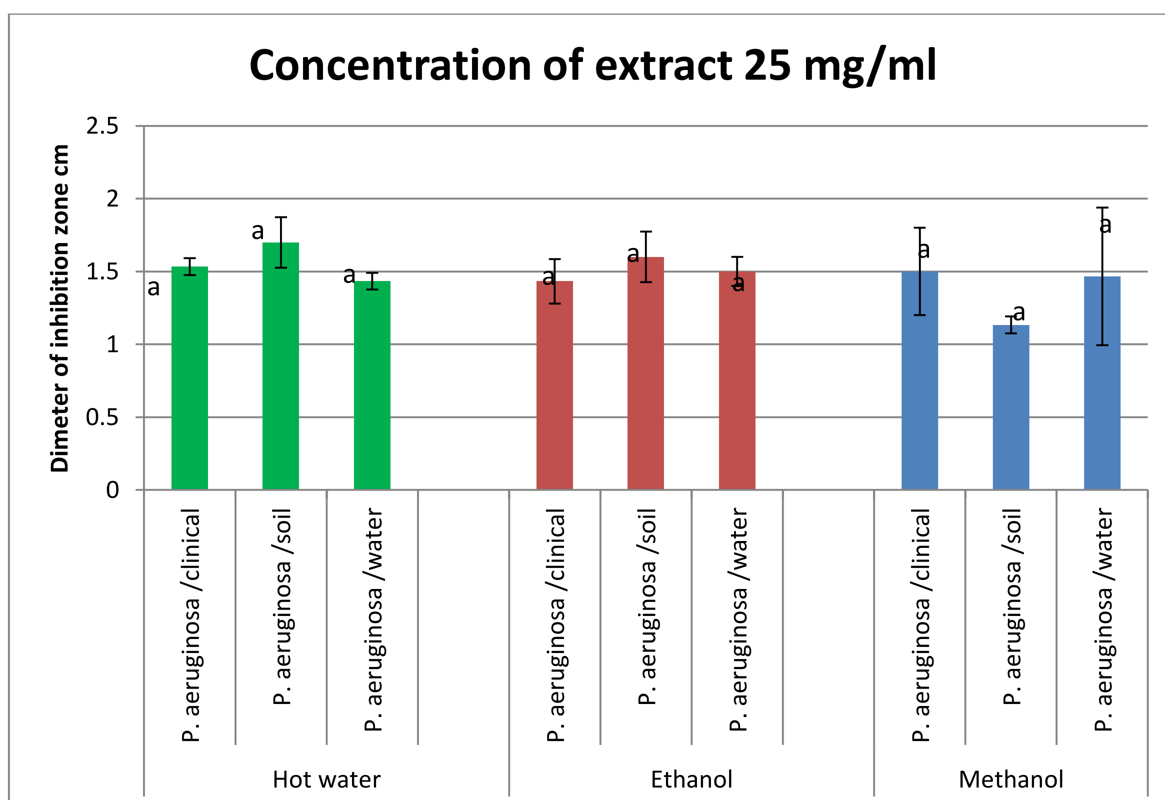
Appendix(VI):Table showing the number of clinical isolates and the rate of diagnosis with the VITEK device

isolation number	VITEK device diagnostic %	isolation number	VITEK device diagnostic %	isolation number	VITEK device diagnostic %	isolation number	VITEK device diagnostic %
1	99	9	97	17	99	25	99
2	97	10	99	18	99	26	97
3	99	11	99	19	97	27	99
4	99	12	97	20	99	28	97
5	99	13	99	21	99	29	99
6	97	14	97	22	99	30	99
7	99	15	99	23	99	31	97
8	99	16	99	24	99	32	99

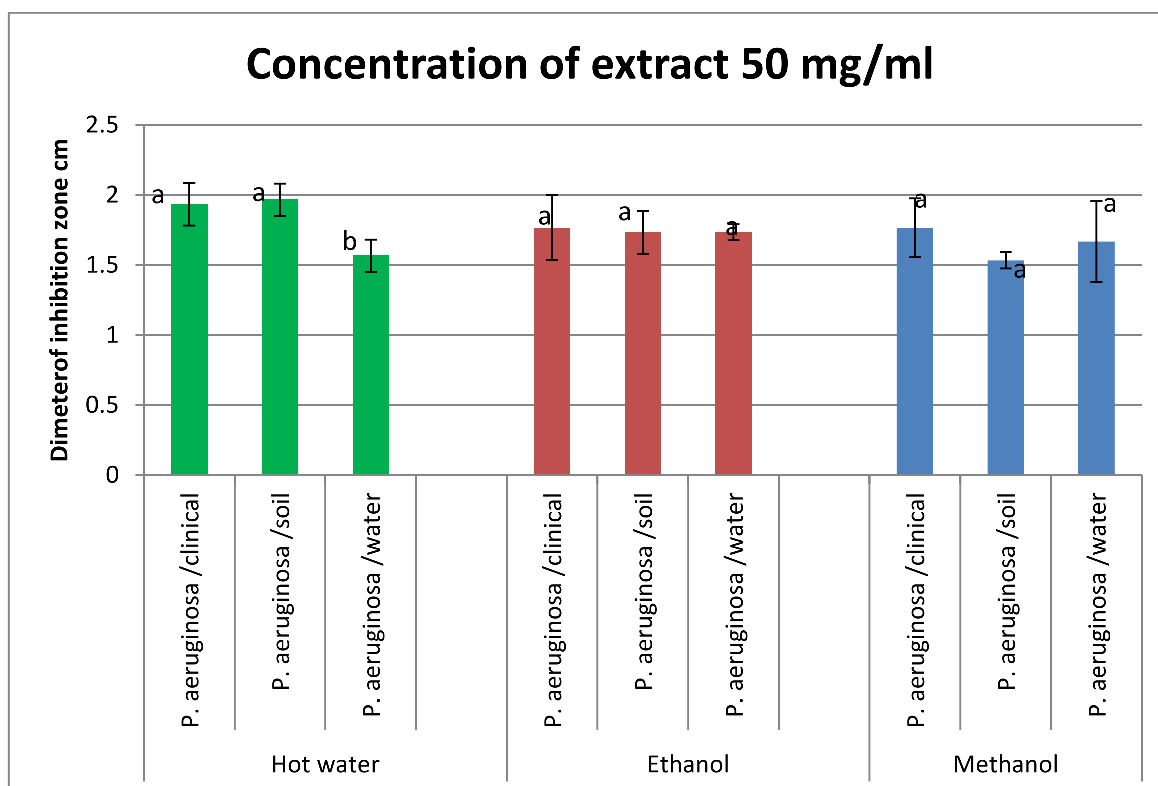
Appendix(VII):Table showing the number of environmental isolates and the rate of diagnosis with the VITEK device

isolation number	source of isolation	VITEK device diagnostic %
1	Soil	95
2	Soil	95
3	Soil	97
4	Soil	97
5	Soil	99
6	water	99
7	water	97
8	water	99

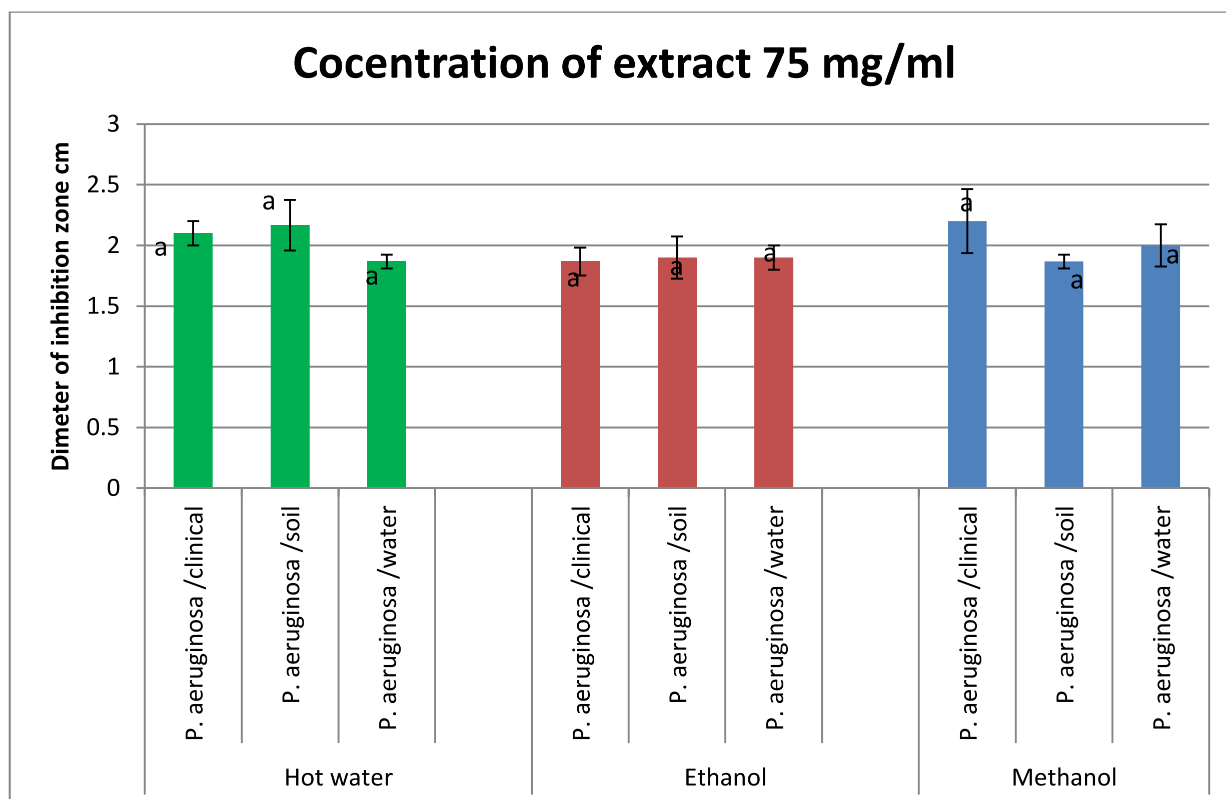
Appendix(VIII): Antibacterial activity of different types of Quercus cortex extracts in concentration of 25 mg/ml against *P. aeruginosa* isolated from different source.



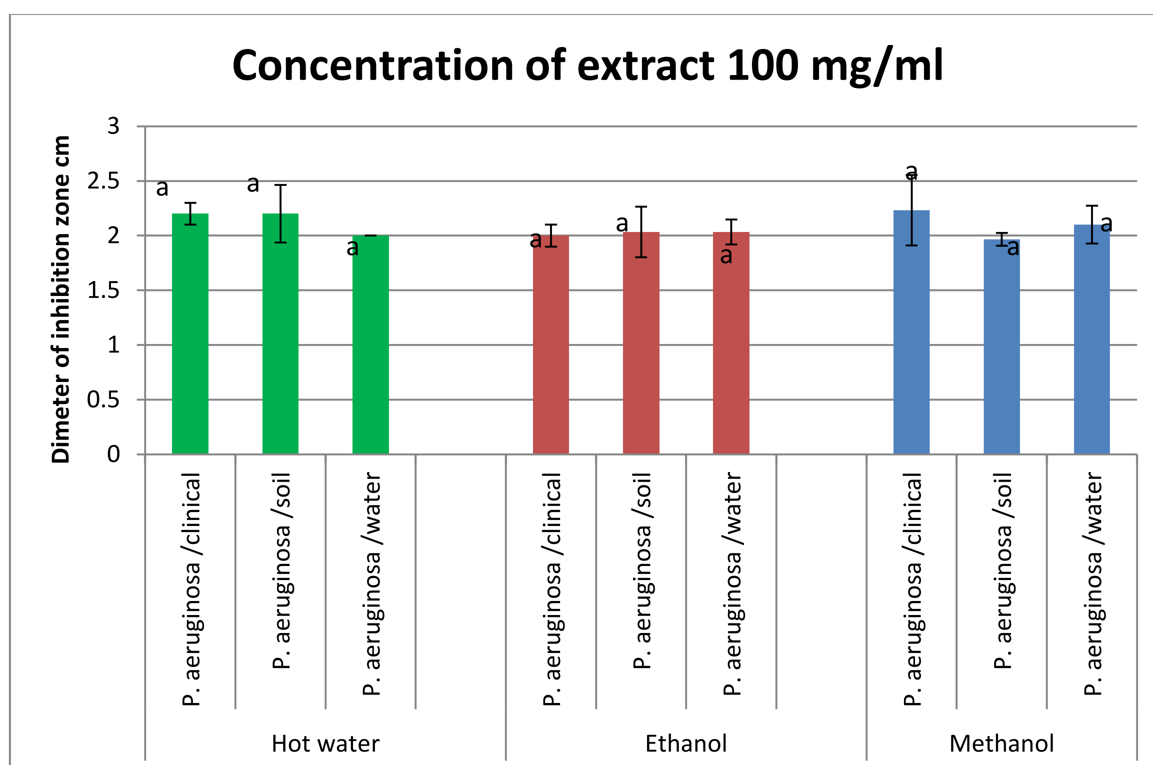
Appendix(IX): Antibacterial activity of different types of Quercus cortex extracts in concentration of 50 mg/ml against *P. aeruginosa* isolated from different source.



Appendix(X): Antibacterial activity of different types of Quercus cortex extracts in concentration of 75 mg/ml against *P. aeruginosa* isolated from different source.



Appendix(XI): Antibacterial activity of different types of Quercus cortex extracts in concentration of 100 mg/ml against *P. aeruginosa* isolated from different source.



الخلاصة

تتواجد بكتريا الزوائف الزنجارية في معظم البيئات الطبيعية (التربة ،المياه ،النباتات وعلى الجلد). تمتاز بلونها ما بين الأصفر والأخضر والأزرق ورائحتها شبيهة برائحة العنب أو الفاكهة. وهي عصية سالبة لصبغة جرام ، هوائية ، انتهازية ، مسببة أمراض للإنسان والحيوان. عندما تدخل هذه البكتيريا الجروح ، أو الأشخاص الذين يعانون من نقص المناعة (مرضى الحروق "المثال الأبرز") ، فإنها تسبب عمومًا التهابات شديدة ، وتعفن الدم ، وتدمير الأنسجة و تؤثر على أجهزة الجسم الحيوية مثل الرئتين أو المسالك البولية أو الكلى ، وقد تؤدي إلى الوفاة.

تم جمع تسعين عينة من العينات السريرية 61 (من مرضى الحروق) و 29 عينة بيئية بما في ذلك التربة والمياه. تم التعرف على عزلات بكتريا الزوائف الزنجارية باستخدام الاختبارات البايوكيميائية وتم التشخيص بجهاز الفايتك.

تم إجراء اختبار حساسية للمضادات الحيوية باستخدام عدد من المضادات الحيوية باستخدام جهاز VITEK ل 24 عزلة 8 منها بيئية و16 سريرية تم اختيار هذه العزلات اعتمادا على نسبة التشخيص بجهاز الفايتك.

أظهرت العزلات التي تم إجراء اختبار الحساسية لها مقاومة عالية جدا للمضادات الحيوية Ampicillin, Amoxicillin, Cefuroxime, Meropenem بنسبة 100٪. كان معدل المقاومة لكل من Amikacin و Gentamicin 72.7٪و 88.8٪ على التوالي ، بينما كانت جميع العزلات حساسة للمضاد Colistin 100٪ ، لذلك كان ال Colistin هو المضاد الحيوي الأكثر فعالية.

أظهرت نتائج الترحيل الكهربائي لتفاعل البلمرة المتسلسل لجين *LecA* لـ 24 عزلة (5 تربة ، 3 ماء ، 16 سريريًا) تم اختيارها بسبب مقاومتها العالية للمضادات الحيوية أن 19 عزلة (8عزلات بيئية و 11 عزلة سريرية) تمتلك الجين وظهرت الحزم بعد الترحيل الكهربائي للهلام بحجم 369 زوج قاعدي.

اما بالنسبة لجين *LasA* فقد أظهرت نتائج الترحيل الكهربائي لتفاعل البلمرة المتسلسل لـ 24 عزلة (5 تربة ، 3 ماء ، 16 سريريًا) تم اختيارها بسبب مقاومتها العالية للمضادات الحيوية أن 18 عزلة (8عزلات بيئية و 10 عزلة سريرية) تمتلك الجين وظهرت الحزم بعد الترحيل الكهربائي للهلام بحجم 226 زوج قاعدي.

تم ارسال عشر عزلات من نتائج تفاعل البلمرة المتسلسل الى شركة Macrogen الكورية لغرض عمل sequence وتم استخدام برامج مثل Mega و Blast لغرض مقارنة النتائج مع نتائج عالمية فاظهرت نتائج المقارنة لجين *LecA* ان عزلتين كانتا قريبتين من دراسات سابقة وان ثلاث عزلات كانت سلالات جديدة اما نتائج المقارنة لجين *LasA* اظهرت ان عزلة واحدة قريبة من دراسات سابقة اما العزلات الاخرى هي سلالات جديدة.

تم استخلاص ستة مستخلصات نباتية بالميثانول والإيثانول والماء الساخن وهي القرفة ، اليانسون ، حبة البركة ، الشيح ، الاس وقلف اشجار البلوط واختبارها اتجاه ثلاث عزلات من مصادر مختلفة (سريرية، تربة و ماء)، حيث ان الخمسة الاولى من هذه المستخلصات لم تظهر اي فعالية تجاه البكتريا بينما كان قلف اشجار البلوط فعالا وظهرت منطقة تثبيط واضحة عند تجربته بأربعة تراكيز , (25 ، 50 ، 75 ، 100) مجم / مل.

جـمـــــــهـوريـــــة العــــــراق
وزارة التعليم العالي والبحث العلمي
جامـعــــــــة كربــــــــــــــلاء
كلــية الــعلوم- قسم علــوم الـحيــاة

الكشف الجزيئي عن جيني *LecA* و *LasA* واختبار فعالية بعض المستخلصات النباتية ضد بكتريا الزوائف الزنجارية المعزولة محلياً

رسالة مقدمة من قبل الطالبة

ظفر نجم خضير

إلى مجلس كلية العلوم-جامعة كربلاء
وهي جزء من متطلبات نيل درجة ماجستير علوم
في علوم الحياة

بإشراف

أ.م.د. علي عطية الحسناوي

1443هـ **2021م**