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كليـــة العلوم

ماجستير تحاليل طبية

Antimicrobial, Anti-Biofilm, Anti-Quorum Sensing and Synergistic Effects of Some Medicinal Plants Extracts.

التأثيرات المضادة للميكروبات، للأغشية الحيوية، لإستشعار النصاب والتأثير التآزري لبعض مستخلصات النباتات الطبية.

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إقـــــــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

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Antimicrobial, Anti-Biofilm, Anti-Quorum Sensing and Synergistic Effects of Some Medicinal Plants Extracts

وبعد المناقشة التي تمت اليوم الاثنين 18 ربيع الثاني 1438هـ، الموافق 2017/01/16 الساعة التاسعة صباحاً في قاعة مؤتمرات مبنى اللحيدان، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن يسخر علمها في خدمة دينها ووطنها.

والله ولي التوفيق،،،

نائب الرئيس لشئون البحث العلمي والدراسات العليا

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Abstract

Background: A biofilm is the assembly of microbial cells that are entrapped in an extracellular polymeric substance matrix. Using complex mechanisms produced by specialized genes, biofilm members are able to talk, communicate, exchange experiences and virulence factors with each other according to a phenomenon called quorum sensing (QS). Biofilm formation by bacteria is a major factor in pathogenesis and in survival. Multidrug resistance phenomenon is increasing and no or very few new antibiotics are being discovered so attention is being turned to use other alternatives, including the use of medicinal plants as antimicrobials and/or as anti-biofilm.

Objective: The main objective of this study is to screen locally available medicinal plants for their *In Vitro* antibacterial, anti-biofilm, anti-quorum sensing and their synergistic activity.

Methods: Extracts from the dried aerial parts of plants were prepared using 70% ethanol in soxhlet apparatus. All extracts were screened for their antibacterial and antifungal activity, determination the minimum inhibitory concentration, anti-biofilm activity, anti-quorum sensing using (swarming method, altered pyocyanin production quantitative assay, Las A staphylolytic assay), and synergistic effect in combination with commercially available antimicrobials.

Results: The average diameter of inhibition zone resulted from the various plant extracts against the tested bacteria ranged from 0 to 22 mm, while the range against *Candida albicans* was 0 to 52 mm. The average minimum inhibitory concentration (MIC_{50}) value ranged from 0.3-81.3 mg/ml against *Pseudomonas aeruginosa*, 0.02-20.8 mg/ml against *C. albicans*. *Pelargonium hortorum* exhibited the highest antimicrobial activity. *Agave sisalana* has the highest anti-biofilm activity against the test microorganism, with 87.5% reduction in biofilm formation. Screening for anti-quorum sensing assay using swarming method showed that *P. hortorum* has the best inhibition of motility of the *P. aeruginosa* isolate 1, while *Punica granatum* has the best motility inhibition of *P. aeruginosa* isolate 2. Prominent alteration of pyocyanin production quantitative assay was observed with *Artemisia absinthium* and *Hibiscus sabdariffa* as evident by the reduction of the green colored (pyocyanin) pigment in *P. aeruginosa*. Plant extracts showed variable abilities in reducing the las A protease activity of *P. aeruginosa*. *Momordica charantia* showed the greatest reduction in activity (77.1%). The synergistic effect of plant extracts when combined with different antimicrobials showed variable activity against *Staphylococcus aureus*. *Cinnamomum zeylanicum* had the best synergistic effect against *S. aureus*.

Conclusions: Most of the studied plant extracts showed the ability to inhibit antibacterial and antifungal activities. Anti-biofilm and anti-quorum sensing activity were also demonstrated. In addition, synergistic activity of these extracts with commercial antimicrobials showed promising results. Further studies are required to determine the active components and the LD_{50} of these extracts.

Key words: Anti-biofilm, Antibiotic resistance, Plant extract and biofilm, and Quorum sensing, Gaza- Palestine.

ملخص الدراسة

المقدمة: الأغشية الحيوية (بيوفيلم) هي تجمع الخلايا الميكروبية المحصورة في مادة البولمر النسيجية الغشائية. تنتج الأغشية الحيوية بواسطة آليات معقدة من خلال جينات خاصة، جميع الخلايا البكتيرية المشاركة في تكوين البيوفيلم لها القدرة على التحدث، التواصل، وتبادل الخبرات وعوامل الضراوة فيما بينها وذلك بواسطة ظاهره النصاب الاستشعاري (QS). يعد البيوفيلم المكون بواسطة البكتيريا من أهم عوامل بقائها وقدرتها على التسبب بالأمراض. إن تزايد ظاهرة المقاومة للمضادات الحيوية من قبل البكتيريا وعدم اكتشاف مضادات حيوية جديدة قاد إلى الاهتمام لاستخدام بدائل وتشمل استخدام النباتات الطبية كمضادات ميكروبية أو كمضادات للبيوفيلم.

الهدف: الهدف الرئيسي لهذه الدراسة هو فحص قدرة النباتات الطبية المتاحة محليا للنشاط المضاد للميكروبات، للأغشية الحيوية، لاستشعار النصاب والتأثير التآزري داخل المختبر.

الطرق: جُهزت المستخلصات النباتية من خلال تجفيف أجزاء النبتة في الهواء وتم استخلاصها بواسطة جهاز السكوكسلت واستخدام الايثانول بتركيز 70% كمذيب. وجميع المستخلصات تم فحصها لدراسة النشاط الميكروبي والفطري وتحديد أقل تركيز مثبط، بالإضافة إلى فحص قدراتها المضادة للأغشية الحيوية والنصاب الاستشعاري وذلك بواسطة مجموعة من الفحوصات منها (طريقة فحص الحركة البكتيرية، قدرة البكتيريا على إنتاج صبغة البيوسينين بالطريقة الكمية أيضا من خلال فحص عمل أنزيم (Las A)، كما تم فحص التأثير التآزري عند دمج المضادات الحيوية التجارية المتاحة مع النباتات الطبية.

النتائج: إن متوسط قطر منطقة التثبيط من المستخلصات النباتية المختلفة ضد العزلات البكتيرية المختارة تراوحت ما بين 0-22 ملجرام/ملل وضد الخميرة المسماة بالمبيضات المبيضه (*Candida albicans*) تراوحت بين 0-52 ملجرام/ملل وبلغت قيمه متوسط التركيز المثبط الأدنى (MIC_{50}) ضد الزائفه الزنجاريه (*Pseudomonas aeruginosa*) ما بين 0.3-81.3 ملجرام/ملل. يعد مستخلص نبتة الصقصلي أكثر المستخلصات فعالية ضد العزلات البكتيرية المختارة بينما مستخلص نبتة الصبرة يمتلك أعلى تأثير ضد تكوين الأغشية الحيوية حيث يثبط الأغشية الحيوية بنسبة 87.5%. تم تحديد التأثير ضد النصاب الاستشعاري (QS) بواسطة الفحص ضد الحركة حيث أن مستخلص نبتة الصقصلي كان أفضل مثبط للحركة ضد العزلة رقم (1) الزائفة الزنجارية، أما مستخلص قشر الرمان كان أفضل مثبط للعزلة رقم (2) من الزائفة الزنجارية. وباستخدام القياس الكمي للصبغة الخضراء المسمى بالبيوسيانسن (Procyonine) فان مستخلص نبتة الشيح والكركديه تمتلكان أفضل مثبط ضد عزلات الزائفة الزنجارية المختارة. وجد أن المستخلصات النباتية لها قدرات مختلفة لتثبيط نشاط إنزيم (Las A Protease) الخاص بالزائفة الزنجارية حيث أن مستخلص نبتة الأمبلايا كانت لها أعلى نسبة تثبيط للإنزيم بنسبة 77%. التأثير التآزري للمستخلصات النباتية عند دمجها مع المضادات الحيوية تمتلك نشاط ضد الميكروبات لبكتيريا المكورة العنقودية الذهبية حيث أن مستخلص نبتة القرفة يمتلك أفضل تأثير تآزري ضد العزلات المختارة من المكورة العنقودية الذهبية.

الاستنتاج: وجد أن أغلب المستخلصات النباتية تمتلك نتائج واعدة لما لها من عدة خصائص ضد التأثير التآزري، الفطري، الأغشية الحيوية، والنصاب الاستشعاري. لذلك نوصي بإجراء العديد من الدراسات لتحديد المركبات الفعالة ومتوسط الجرعة القاتلة لهذه المستخلصات النباتية.

الكلمات المفتاحية:التأثير المضاد للأغشية الحيوية، مقاومة المضادات الحيوية، المستخلصات النباتية والأغشية الحيوية والنصاب الاستشعاري،غزه-فلسطين.

DEDICATION

I would like to dedicate this work to my supervisor **Prof. Dr. Abdelraouf Elmanama**, and to pure soul of my father, the martyr **Raed Al Refi**. To the soul that cultivate the real love of knowledge in my heart, the soul who carried the pain and troubles to make me stronger, the soul who taught me love and loyalty, who owns my heart, who left fingerprints of grace on my life, to my honorable **grandmother**, I dedicate my modest work.

For his kindness and devotion, and for his endless support, I would thank my precious, invaluable treasure, my dear brother, **Saleh**. The life could not be pleasant without the presence of my twins. Dear sisters, dear aunt, I am glad to dedicate my work to you. A special dedication is sent to the children of my brother **Raed, Nael** and **Mariam**. To the dear fellow of my path and my thoughts, my friend **Fatma**, and to my dignified **uncles** who I am proud of, I would finally dedicate this work.

Mariam R. Al Refi

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Finally, I can just say thanks for everything and may Allah give all who stood beside me, all the best in return.

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List of Abbreviations

3-oxo-C12-HSL	QSN-(3-oxododecanoyl)-L-homoserine lactone
ACP	Acylated acyl Carrier Protein
acyl HSLs	N-acyl derivatives of homoserine lactone
AHL	Acyl Homoserine Lactone
Bap	biofilm-associated protein
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
C4-HSL	N-butanoyl-homoserine lactone
CF	Cystic fibrosis
ClfB	Clumping factor B
CUP	Chaperone usher pathway
CV	Crystal violet
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
Eap	Extracellular adherence protein
ECM	Extracellular matrix
Edna	Environmental DNA OR Extracellular DNA
EPS	Extracellular Polymeric Substances
FnBP	Fibrinogen-binding proteins
HHL	N-hexanoyl-L-homoserine lactone
HNO_2	Nitrous acid
LD_{50}	Median Lethal Dose
MCE	Microbial consortia engineering
MH	Mueller Hinton Agar
MSHA	Mannose-sensitive hemagglutinin
NB	Nutrient broth
NO	Nitric oxide
OHHL	N-(3-oxohexanoyl) L-homoserine lactone
ONOO-	Peroxynitrite
PGA	Polyglucosamine
$PMIC_{50}$	Planktonic growth inhibiting activity
porin F	Porin F
PQS	*Pseudomonas* Quinolone Signal
QS	Quorum sensing
SAM	S-adenosylmethionine
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	Scanning electron micrograph
SFE	Surface free energy
TTC	2,3,5triphenyltetrazolium chloride

Chapter I
Introduction

Chapter I
Introduction

1.1 Overview

The problem of microbial resistance to antibiotics appeared as soon as the mass-production of penicillin was started in 1945 (Shaughnessy, 2007), and has now become a major concern in medicine throughout the world. Bacterial resistance not only can occur to a single class of antibiotic, but also to multiple classes of antibiotics. The resistance of microorganisms to antibiotics can be categorized in two types: innate resistance, which means that some microorganisms are inherently resistant to many antibiotics; Acquired resistance, which can arise by a number of diverse mechanisms such as mutation and horizontal gene transfer (Tenover, 2006).

Despite the fact that bacteria are unicellular organisms, they often show group behavior. For example, in living biofilms, individual cells at different locations in the biofilm may have different activities. This led to the proposal that biofilm communities may represent an evolutionary step between unicellular non-specialized organisms and multicellular organisms that possess specialized cells (McLean, Whiteley, Stickler, & Fuqua, 1997).

For optimum performance, bacteria have to monitor their own population density. This can be achieved by quorum sensing. This process relies on the production of a low-molecular-mass signal molecule (often called "autoinducer" or quormon) (Fuqua, Winans, & Greenberg, 1996). Biofilm is a community of cells attached to either or to a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS) (Flemming, Wingender, Griegbe, & Mayer, 2000).

Among biofilm producing bacteria, it appears that, some produce biofilms more readily than others do. Biofilms allow microorganisms to trap nutrients and withstand hostile environmental conditions, which are a key feature for survival (Kodali et al., 2013). Surface adhesion of the bacteria is an essential step and is required for the bacteria to arrange themselves favorably in their environment.

Bacterial biofilms have been reported to have useful effects on food chains, sewage treatment plants, eliminate petroleum oil/hydrocarbon spillage from the oceans (Coulon et al., 2012; Kodali, Das, & Sen, 2009). According to a 2011 review, biofilms in drinking water systems can serve as a significant environmental reservoir for pathogenic microorganisms (Wiener-Well et al., 2011).

Biofilms are enclosed within an exopolymer matrix that can restrict the diffusion of substances and bind antimicrobials, this will provide effective resistance for biofilm cells against large molecules such as antimicrobial proteins, lysozyme and complement (Ishida et al., 1998; Shigeta et al., 1997). In fact, it is estimated that approximately 80% of all bacteria in the environment exist in biofilm communities, and more than 65% of human microbial infections involve biofilms (Donlan, 2002). Microbial biofilms cause a variety of problems in different medical, environmental, and industrial settings, ranging from the fouling of ship hulls and the blocking of industrial piping to the colonization of artificial medical implants (Costerton, Stewart, & Greenberg, 1999; Kumar & Anand, 1998), and possibly many other problems.

Plant extracts and other biologically active compounds isolated from plants have gained wide spread interest as they have been known to cure diseases and illnesses since ancient times (LA, 1998). Modern science and technological advances are accelerating the discovery and development of innovative pharmaceuticals with improved therapeutic activity and reduced side-effects from plants. Plant compounds are widely accepted due to the perception that they are safe and they have a long history of use in folk medicine as immune boosters and for the prevention and treatment of several diseases (Jones, 1997).

Over the years, the use of medicinal plants, which forms the backbone of traditional medicine, has grown with an estimated 80% of the populations, mostly in developing countries, relying on traditional medicines for their primary health (Karthick, 2011). Palestine is blessed with diverse flora that need to be investigated for their biological activities.

In this work, 23 plant extracts were investigated for their antimicrobial, anti-biofilm, anti-quorum sensing, and synergistic effects.

1.2 Objectives

The primary objective of this study is to screen different medical plants for their antimicrobial, anti-biofilm, anti-quorum sensing, and synergistic effects

The following specific objectives were achieved:

1. Evaluation of the anti-biofilm of the crude plant extracts on *Pseudomonas aeruginosa*.
2. Determine of the effects of plant extracts on planktonic growth and biofilm formation of *P. aeruginosa In Vitro.*
3. Screening and determination anti-quorum sensing of the plant extracts on *P. aeruginosa.*
4. Determination of anti- *Candida albicans* effects by the various extracts.
5. To determine synergistic activity of plant extracts with commercial antibiotic against many isolates of *Staphylococcus aureus.*

1.3 Significance

Biofilms are not only ubiquitous and costly but poses numerous, problems and risks. The disruption/inhibition of biofilms was shown to be effective in reducing hazards usually associated with biofilm formation. There are no universal or ideal biofilm disruptors and the search for novel ones is on demand. This study has attempted to investigate the effect of locally available plants on inhibiting the ability of well-known pathogens to form biofilms. This would contribute to the accumulating knowledge of biofilms

disruptors/inhibitors and may produce new, effective and low cost agents. Such agents may represent of useful applications both medically and or environmentally.

Quorum sensing is used by microbial communities to orchestrate actions that are essential in pathogenesis e.g., toxin or other virulence factors expression and production. Interfering with quorum sensing may play a role in treating or even preventing infectious diseases. Again, plants should be considered when searching for anti-quorum sensing substance because of the plentiful of natural compounds contained in these plants.

Chapter II
Literature Review

Chapter II

Literature Review

2.1 Biofilm

2.1.1 Biofilm definition

Biofilms are not easily defined as they vary greatly in build and specification from one circumferential niche to another (Percival, Walker, & Hunter, 2000). Biofilm are the microbial communities (Donlan & Costerton, 2002), embedded in a matrix of extracellular polymeric substances (Brooks & Flint, 2008)**,** randomly distributed in a shaped matrix or glycocalyx (Socransky & Haffajee, 2002). Surface free energy (SFE) and surface roughness may contribute to increase rate of microbial adhesion (Weitman & Eames, 1975).

The natural biofilm is like a complex organism with highly differentiated compared to planktonic cells free-swimming, *P. aeruginosa* undergoes irreversible attachment to flatten material but when imparted into approach with the steel, the number adhering fast increased with time in less than one minute (Stanley, 1983). The transition from planktonic growth to biofilm is dependent on the circumstances surrounding the microbe and involves multiple regulatory signal translated into different orders for number of genes (Monds & O'Toole, 2009; G. O'Toole, Kaplan, & Kolter, 2000; Pratt & Kolter, 1998; Prigent-Combaret et al., 2001)**.**

This alters the expression for many genes; for example the expression of *spaP*, *gtfB* and *gbpB* was decreased significantly, when *S. mutans* were grown in biofilm with *L. casei*, but no expression differences of the selected genes was observed when *S. mutans* was double-cultivated with *S. sanguinis* (Wen, Yates, Ahn, & Burne, 2010). The existence of environmental gradients within the "small city" of biofilm, leads to division of labor between subpopulations in response to local nutrient and oxygen availability (Domka, Lee, Bansal, & Wood, 2007; Lewis, 2005)**.**

The ability of biofilm cells to challenge stress is a very important advantage for these. For instance, diversity of biofilm communication, which affects cellular functions (colony morphology, motility and enzyme and pigment secretion) (Boles, Thoendel, & Singh, 2004)**.**

Streptococcus pneumoniae failed to generate capsulae in broth cultures but can make that *In Vitro* biofilm (Waite, Struthers, & Dowson, 2001)**.** Biofilms notoriously resist killing by host defense mechanisms (Singh, Parsek, Greenberg, & Welsh, 2002), and antibiotics because matrix protects biofilm bacteria (Cerca et al., 2007; Jefferson, Goldmann, & Pier, 2005; Leid et al., 2005; Jesaitis et al., 2003; Walters, Roe, Bugnicourt, Franklin, & Stewart, 2003). *S. epidermidis* protects itself against the innate human immune system by using exopolysaccharide intercellular adhesion (PIA). Loss of biofilm matrix that mean lake PIA, which is the first defense of Staphylococci (Vuong et al., 2004).

Some minerals enhanced biofilm formation and growth like calcium, iron and copper. When calcium concentration was increased to 80 ppm, *P. aeruginosa* showed more than 50% rise in its ability to form in biofilm and 15% for *B. subtilis* (Jain, Parida, Mangwani, Dash, & Das, 2013).

2.1.2 Biofilm matrix

Extracellular polymeric substances (EPS) matrix is responsible for making biofilms the most successful forms of life on earth (Flemming & Wingender, 2010), and define the characteristic of biofilms (Blankenship & Mitchell, 2006; Branda, Vik, Friedman, & Kolter, 2005; Sutherland, 2001). Microbial cells make up 10% of a biofilm's total mass, with 90% contributed by the extracellular matrix (Karatan & Watnick, 2009).

A biofilm has become an example for Microbial consortia engineering (MCE) because a common aim of MCE is a mixed population which can perform complex tasks that cannot be done by every individual strain or species alone, and ability to perform function requiring multiple steps, thus increasing productivity (Brenner, You, & Arnold, 2008), for example, when bacteria used dual-strain system, they were able to form strong biofilm, but unable to form biofilm in the same strain. This fact does not apply to all kinds of bacteria. *Acinetobacter calcoaceticus* formed strong biofilms in all single- and dual-strain cultures, by contrast, *P. aeruginosa* prefer the competitive biofilm situations (Andersson, Rajarao, Land, & Dalhammar, 2008).

One study observed strong biofilm formation when *B. cereus* and *B. denitrificans* are mixed together but when *A. calcoaceticus* was added to the two strains, they observed antagonistic interactions (Andersson et al., 2008). EPS matrix (Figure 2.1) consisting of polysaccharides, proteins, DNA, and lipids(Flemming & Wingender, 2010) plasmids (Ikuma, Decho, & Lau, 2013). Studies on the toluene-catabolic (TOL) plasmids (conjugative plasmid) of *Pseudomonas putida* demonstrated that carriage of conjugative plasmids could also increase biofilm formation by increasing the amount of extracellular DNA (eDNA) (Cook & Dunny, 2014). EPS is highly hydrated, and can be both hydrophilic and hydrophobic with varying degrees of solubility (Sutherland, 2001). Biofilm formation by *B. subtilis* culminates in the formation of a structured highly hydrophobic sessile community (Mielich-Süss & Lopez, 2015; Cairns, Hobley, & Stanley-Wall, 2014).

In 2015, a study demonstrated that the main component of *Sulfolobus acidocaldarius* in EPS fraction was carbohydrates followed by proteins, DNA and significant hydrolytic enzyme activity (Jachlewski et al., 2015). In 2010, a study was conducted using two different biofilm model systems; the first is bacteria growing on glass surfaces under continuous flow conditions, the same bacteria grown on agar surfaces, showed different structural and functional aspects for EPS (Tielen et al., 2010).

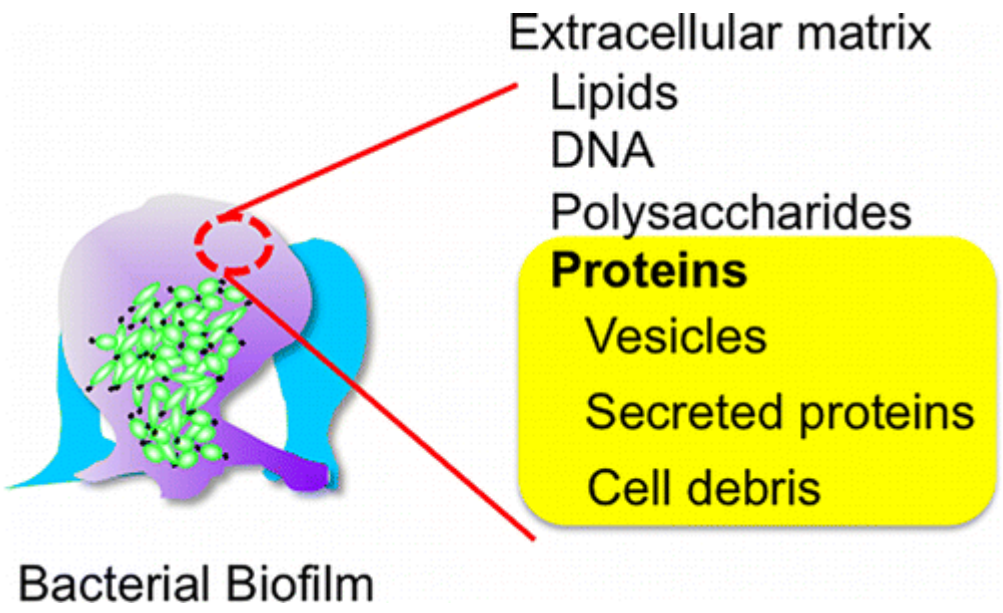

Figure (2.1): Composition of biofilm (Source: Toyofuku, Roschitzki, Riedel, & Eberl, 2012).

Polysaccharide as a major matrix component may be a common theme in biofilms of many bacteria. Polysaccharide is important to initiate biofilm structure by providing cell-cell, cell-surface interactions, and intercellular adhesion for *P. aeruginosa* (Yang et al., 2011; Ma et al., 2009; Ma, Lu, Sprinkle, Parsek, & Wozniak, 2007; Ma, Jackson, Landry, Parsek, & Wozniak, 2006). But Some bacteria does not secret exopolysaccharides, so matrix formation has been shown to rely on outer membrane blebs that intercalate the interbacterial space of the biofilm as observed in *N. gonorrhoeae* (Steichen, Shao, Ketterer, & Apicella, 2008; Edwards & Greiner, 2005).

In *P. aeruginosa*, eDNA is located primarily in the stalks of mushroom-shaped multicellular structures (Montanaro et al., 2011), only a few studies have defined how eDNA interacts with PS in the matrix. It has been suggested that in *Myxococcus xanthus* biofilms, eDNA directly interacts with PS, enhancing the physical strength and resistance to biological stress (Hu et al., 2012).

The matrix is considered as an external digestion system, tarping extracellular enzymes inside the biofilm structure making the biofilm capable of metabolizing dissolved, colloidal and solid biopolymers (Hall-Stoodley, Costerton, & Stoodley, 2004).

The importance of biofilm matrix could be manifested in various forms; the EPS itself is self-produced, cell-cell interaction, cell-surface adhesion, protects the cells from stresses such as desiccation, oxidizing agents (G. A. O'toole, 2003; Ophir & Gutnick, 1994; DeVault, Kimbara, & Chakrabarty, 1990). Polysaccharides from Mixed Species Biofilms (MSP) enhanced stress resistance relative to biofilms formed by the individual species alone (Lee et al., 2014).

The same result was observed in a recent study, this might be due to overproduction of alginate and mucA. *Psl* mutant was less competitive during mixed species growth (Periasamy et al., 2015). In 1998, it was marine isolated Environmental DNA (eDNA) from *Rhodovulum* sp, that was demonstrated the ability to promotes floc stability (Watanabe et al., 1998). eDNA also important for structural stability of young *P. aeruginosa* biofilm, enhance bacterial attachment (Das, Sharma, Busscher, van der Mei, & Krom, 2010; Heijstra, Pichler, Liang, Blaza, & Turner, 2009; Qin et al., 2007), and have antimicrobial properties by chelating cations (Mulcahy, Charron-Mazenod, & Lewenza, 2008).

2.1.3 Biofilm development

Biofilm formation is a dynamic and complex process influenced by several bacterial and/or environmental factors (Vogeleer, Tremblay, Mafu, Jacques, & Harel, 2015)**.** The lifecycle of a biofilm has four stages (Wilson, Gray, Karakiozis, & Thomas, 2012); initial attachment, irreversible attachment, expression & maturation, and dispersion (Vogeleer et al., 2015). Planktonic bacteria reversibly attach to surfaces (Donlan, 2002)**.** *Pseudomonads* required flagellar-mediated motility to swim toward a surface and transient attachment (O'Toole & Kolter, 1998). Brownian motion influences bacterial first step of biofilm formation (Kostakioti, Hadjifrangiskou, & Hultgren, 2013).

Hinsa et al., (2003) examined biofilm formation at three days. Bacterial cells formed a dense monolayer at day one, and from second to third day, cells began developing gradually from microcolonies to macrocolonies, the final shape is visible to the naked eye with more than one species of cells. During the early stage of biofilm formation, bacterial cells are sheltered by hydraulic shear forces, and do not contain channel. Flagella, motility and/or chemotaxis is required for attachment to abiotic surfaces, flagella facilitate the initiation of biofilm formation, and necessary to enable a bacteria to reach the surface (Pratt & Kolter, 1998).

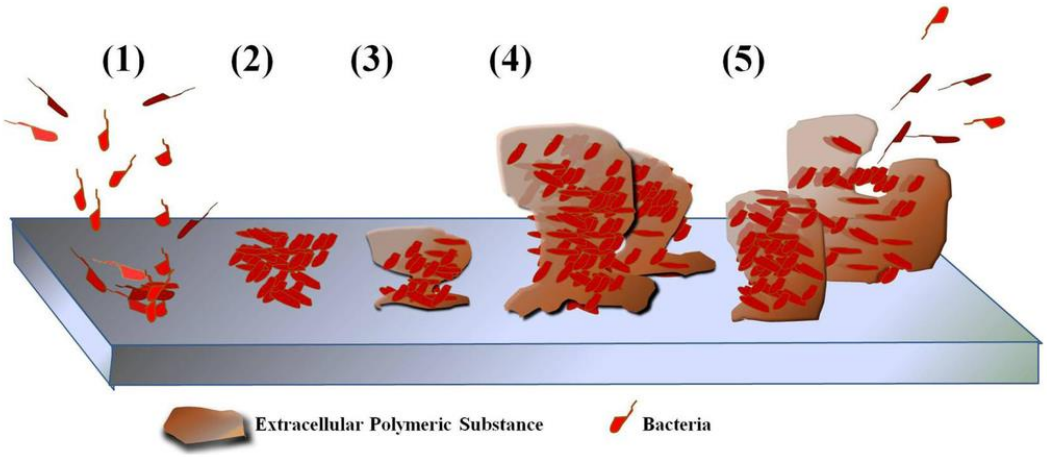

Figure (2.2): Stages of biofilm development: 1. Initial attachment of planktonic cells to the surface, 2. Irreversible (permanent) attachment, 3. Cells proliferation and formation of EPS (maturation I), Formation of biofilm structure (maturation II), and 4. Biofilm spreading (dispersion) (Source: Park, Park, & Hahm, 2011).

In 1999, a study showed that *Vibrio cholerae* does not use the virulence-associated toxin co-regulated pilus to form biofilms but uses the mannose-sensitive hemagglutinin (MSHA) pilus on both nutritive and non-nutritive abiotic surfaces (Watnick, Fullner, & Kolter, 1999). In a separate study, flagella and type IV pili were not necessary for *P. aeruginosa* initial attachment or biofilm formation (Klausen et al., 2003).

In *E. coli*, structural components include the EPS molecules, colonic acid, cellulose, and PGA (polyglucosamine) enhance intercellular interactions, eDNA used as connecting agent well as a nutritional source. Extracellular organelles such as flagella and CUP (chaperone usher pathway) pili enable bacterial aggregation consolidation the biofilm lattice (Kostakioti et al., 2013).

Nutrition level, oxygen and environmental condition (pH, temperature, medium properties, and type of surface) influence biofilm formation. For example, Local oxygen limitation and the presence of nitrate may contribute to the reduced susceptibilities of *P. aeruginosa* biofilms causing infections *In Vivo* (Borriello et al., 2004). Therefore, when the biofilm is of high density, there will be a limited substrate, oxygen uptake rate is high, but removal of acetate rate was reduced (Casey, Glennon, & Hamer, 2000). Some bacteria used repulsive forces for competition. *P. aeruginosa* exploited motility characteristic for dominated co-culture biofilms, in contrast *Agrobacterium tumefaciens* impaired competition in co-culture biofilms (An, Danhorn, Fuqua, & Parsek, 2006).

Second step in biofilm formation is surface-associated cells to form microcolonies by clonal growth and/or aggregation, transition from reversible to irreversible attachment and is thus required for monolayer formation (Hinsa, Espinosa-Urgel, Ramos, & O'Toole, 2003). *P. fluorescens* secreted large amount of proteins (LapA) necessary for irreversible attachment (Hinsa et al., 2003). In *P. aeruginosa*, *fLeR*, *rpoN*, and *flgK* genes responsible for first reversible attachment, *sadB* is important for transition from reversible to irreversible attachment, *sadB* produces a protein for cytoplasmic localization, this explains why it does not act as a cell surface adhesion (Galperin, Nikolskaya, & Koonin, 2001).

P. aeruginosa mature form of biofilm structure is tulip-shaped cell clusters (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995)**.** At this stage, the biofilm has three-dimensional image stacks (Heydorn et al., 2000), and the number of channels are directly proportional to the thickness of the biofilm layer. For example, for a biofilm thickness of about 20 to 300 mm, a large number of vertical and horizontal channels permeated the film was noted. Channels are important in exchange gasses, nutrients and transport of cooperative substrates (Massol-Deyá, Whallon, Hickey, & Tiedje, 1995).

The last stage in the development of biofilms is the detachment of small or large clumps of cells, or by a type of "seeding dispersal" from the biofilm that releases individual cells**.** These cells move on to further colonize sites, and repeat the development cycle. Dispersal of cells from the biofilm colony is an essential stage of the biofilm lifecycle because it enables biofilms to spread and colonize new surfaces (Richards et al., 2009; Hall-Stoodley et al., 2004).

Table (2.1): Phenotypic characteristics of biofilm development

Biofilm stage	Characteristics
Free swarming cells	Motility
Initial attachment	Approach surface, fixed to substratum, gene expression (quorum sensing independent)
Irreversible attachment	Cell organization development, non-motile.
Maturation and expression	Cluster thickness >10 μm in initial time then, most cell cluster development, cell organization thickness up to 100 μm, majority of cells disconnect from substratum, upregulation of protein compered for first stage.
Dispersion	Alteration in cell organization structure observed motile and non motile cells, consistence of pores and conduits, dispersion, down regulation 35% for protein expression in planktonic cells

(Source: Sauer, Camper, Ehrlich, Costerton, & Davies, 2002).

2.2 Quorum sensing

2.2.1 Quorum sensing definition

Quorum sensing (QS) makes the best metabolic and behavioral activities of bacteria for life in close quarters to achieve cell communication by diffusible chemical signals that impact gene regulation response to biochemical molecules called auto inducers, density dependent behavior (Sifri, 2008). QS reflects the needed enough cells to activate certain processes. The increased synthesis of the signal molecule, lead to regulate genes encoding basic metabolic processes and or genes associated with virulence for Gram positive, Gram negative and *Candida albicans* (Sifri, 2008).

The first step in quorum sensing regulation, is the production of the quorum sensing molecules by utilizing the intracellular machinery. Secreted molecule remain bound to the bacterial surface or spread to the surrounding environment. In the second step, QS is cell density-dependent signaling, for example, biofilm population in dental plaque is usually more than 700 cells accumulating the molecules outside (Li & Tian, 2012). This increase is either due to the increase in the number of bacteria, lowering of available area. Impermeable structure near environment with a low level production of the signaling molecules may also increase the effect of signaling molecules (Li & Tian, 2012).

In the third step, when signal molecules exceed a threshold concentration, signal molecules are converted to a specific cellular response (gene expression thought population) (Figure 2.3). Autoinducer/receptor complex bind to DNA by signal-activated intracellular receptor, finally activated quorum sensing process takes actions (Lerat & Moran, 2004; Podbielski & Kreikemeyer, 2004). Example of bacterial quorum sensing systems (Table 2.2) that include the ability to incorporate foreign DNA, improve the efficiency of virulence, formation biofilms (Cvitkovitch, Li, & Ellen, 2003).

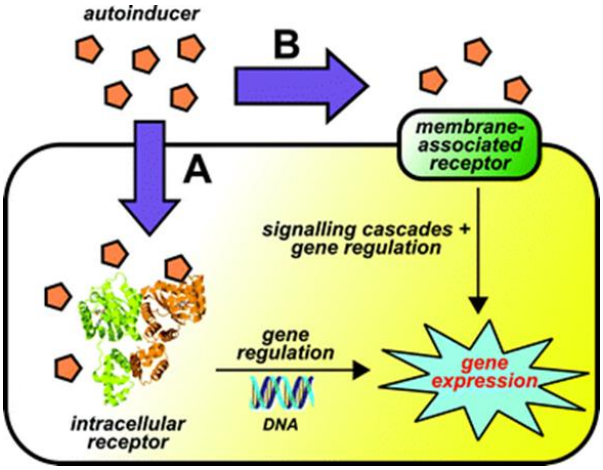

Figure (2.3): Quorum sensing the process of cell-cell communication in bacteria

(Source:Praneenararat, Palmer, & Blackwell, 2012).

V. fischeri benefited from symbiotic relationship with many marine animal hosts (certain fish and squid species). *V. fischeri* produces blue-green light, the host uses this light in avoiding predators, or/and attracting prey (Federle & Bassler, 2003; Miller & Bassler, 2001). This is done through controlling the intensity of the light that it projects downward, thus eliminating the prominent shadow created by moonlight (Graf & Ruby, 1998).

In exchange *V. fischeri* obtain nutrient from the host. Biochemical emission of light occurred only at high cell density (Schauder & Bassler, 2001), and is regulated by the LuxR-LuxI system (Miyashiro & Ruby, 2012). At low cell density, N-(3-oxohexanoyl) L-homoserine lactone (OHHL) as autoinducer produces weak light emission. At high cell density, a critical concentration of OHHL is reached, rapid amplification of the OHHL signal and thus, stronger emission of light (Whitehead, Barnard, Slater, Simpson, & Salmond, 2001). Bacterial species engaging in biofilms or quorum sensing exhibit well-documented social behaviors (Irie & Parsek, 2008).

Table (2.2): Examples of bacterial quorum sensing systems

Microorganism	Benefits
Bacillus subtlis	Competence, sporulation, biofilm formation, antibiotic production (Claverys, Prudhomme, & Martin, 2006; Dunny & Leonard, 1997), access of symbionts to nutrient-rich environments in hosts (Ruby, 1996).
Pseudomonas aeruginosa	Structured biofilm formation, virulence factors (Fuqua & Greenberg, 2002; Taga & Bassler, 2003; Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999).
Staphylococcus aureus	Biofilm formation, virulence factors (Novick, 2003; Waters & Bassler, 2005).

2.2.2 Quorum sensing in Gram-negative and Gram positive bacteria

The most common signaling molecules found in Gram-negative bacteria is N-acyl derivatives of homoserine lactone (acyl HSLs) (Whitehead et al., 2001). It plays a role in swarming (Eberl et al., 1996), stationary-phase survival (Thorne & Williams, 1999), cessation of cell growth (Gray, Pearson, Downie, Boboye, & Greenberg, 1996; Schripsema et al., 1996), capsular polysaccharide synthesis, production of complex molecules (Wood & Pierson, 1996; Jones et al., 1993), and production of exoenzyme (Jones et al., 1993).

Quorum sensing system in Gram-negative bacteria is controlled by LuxI protein family (Moré, Finger, & Stryker, 1996; Swift, Throup, Williams, Salmond, & Stewart, 1996), and LuxR protein family (Salmond, Bycroft, Stewart, & Williams, 1995). The LuxI-type protein catalyzes the synthesis of an AHL autoinducer, by using S-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) as substrate (Schaefer, Val, Hanzelka, Cronan, & Greenberg, 1996).

When number of acyl HSLs increased above a threshold concentration, LuxR-type protein binds to autoinducer then simulation the expression of specific genes (Taga & Bassler, 2003). Two different AHL systems coexist in *P. aeruginosa* the Las and Rhl systems. A lasR of *P. aeruginosa* is important for many virulence factor and is responsible for pathogenicity, for example the las signal molecule required for biofilm differentiation (T. R. De Kievit & Iglewski, 2000). When *P. aeruginosa* lasR-deficient is used to infect neonatal mouse model to cause pneumonia, the virulence was dramatically decreased (Tang et al., 1996).

Acyl homoserine lactone in *Chromobacterium violaceum* as N-hexanoyl-L-homoserine lactone (HHL), is responsible for regulation of exoprotease, chitinase and cyanide production (Throup et al., 1995). A unique specificity between R protein and autoinducers is found. Therefore, antagonizing autoinducers lead to inhibition or weak activation of R proteins (Surette, Miller, & Bassler, 1999; Swift et al., 1997; Schaefer, Hanzelka, Eberhard, & Greenberg, 1996).

Intercellular communication in Gram-positive bacteria are often based on autoinducing peptides. Because the peptide are impermeable to biological membranes (Solomon, Lazazzera, & Grossman, 1996; Håvarstein, Coomaraswamy, & Morrison, 1995; Ji, Beavis, & Novick, 1995), it binds to specific histidine kinase membrane receptor that are part of regulatory systems (Sturme et al., 2002). Table 2.3 list the major differences in QS system for Gram-positive and Gram-negative bacteria.

Table (2.3): Summary of the major differences between Gram-positive and Gram-negative systems.

	Gram positive bacteria	Gram negative bacteria
Autoinducer	Oligopeptides[1]	Homoserine lactone out inducer [2]
Pheromones dependent on	Oligopeptides based quorum sensing system[1]	LuxIR based quorum sensing system[1]
Location of receptor	Membrane bound receptor[1]	Cytoplasmic receptor[6,7]
QS Control	1. Histidine Kinase receptor[1] 2. Cognate cytoplasmic response regulator[1]	1. LuxI protein[3] 2. LuxR protein[3]
Stimulation of target gene expression	Peptide-membrane bound receptor stimulation the intrinsic out phosphorylation activity[8,9]	LuxR-AHL complex[4]
Gene expression dependent on	Cell-density[8,9]	Cell-density[5]

Sources: 1: (Hoch & Silhavy, 1995; Inouye & Dutta, 2002); 2: (Fuqua, Parsek, & Greenberg, 2001); 3: (Engebrecht & Silverman, 1984; Schaefer, Val, et al., 1996) ; 4: (Stevens, Dolan, & Greenberg, 1994); 5: (Kaplan & Greenberg, 1985); 6: (Engebrecht, Nealson, & Silverman, 1983); 7: (Engebrecht & Silverman, 1984) ; 8 : (Peterson, Cline, Tettelin, Sharov, & Morrison, 2000); 9: (Ji et al., 1995).

2.3 Mechanism of antibiotic resistance in bacterial biofilm

Bacterial resistance causes decreased antimicrobial susceptibility through adding penetration barriers, efflux pumps or degrading enzymes. This phenomenon occur for most bacteria, however, decreased antimicrobial susceptibility resulting only from the tolerance characteristic of bacterial biofilms is an added mechanism. It seems that this tolerance arise once a threshold number or density of bacteria has aggregated, whereas the common resistance will develop over time due to intrinsic and external factors such as mutations (Do, 2014).

The mechanisms of biofilm resistance to antimicrobial compounds includes physical or chemical diffusion barriers to antimicrobial penetration into the biofilm . For example, ciprofloxacin binds to certain components of *P. aeruginosa* biofilm, thus, the biofilm acts as a barrier to antimicrobial agents. Piperacillin was not able to penetrate *P. aeruginosa* biofilm. Slow growth of the biofilm owing to nutrient limitation (oxygen availability alone is known to modulate the action of the aminoglycoside) (Figure 2.4).

Because bacteria in biofilms involved in switching metabolism pathways e.g. overproduces the extracellular polymer alginate for *P. aeruginosa*) (Hentzer et al., 2001; Tack & Sabath, 1985), activation of the general stress response and the emergence of a biofilm-specific phenotype (Mah & O'Toole, 2001). In conclusion, antimicrobials resistance of bacterial biofilm could be attributed to the physical, chemical and physiological status of the biofilm.

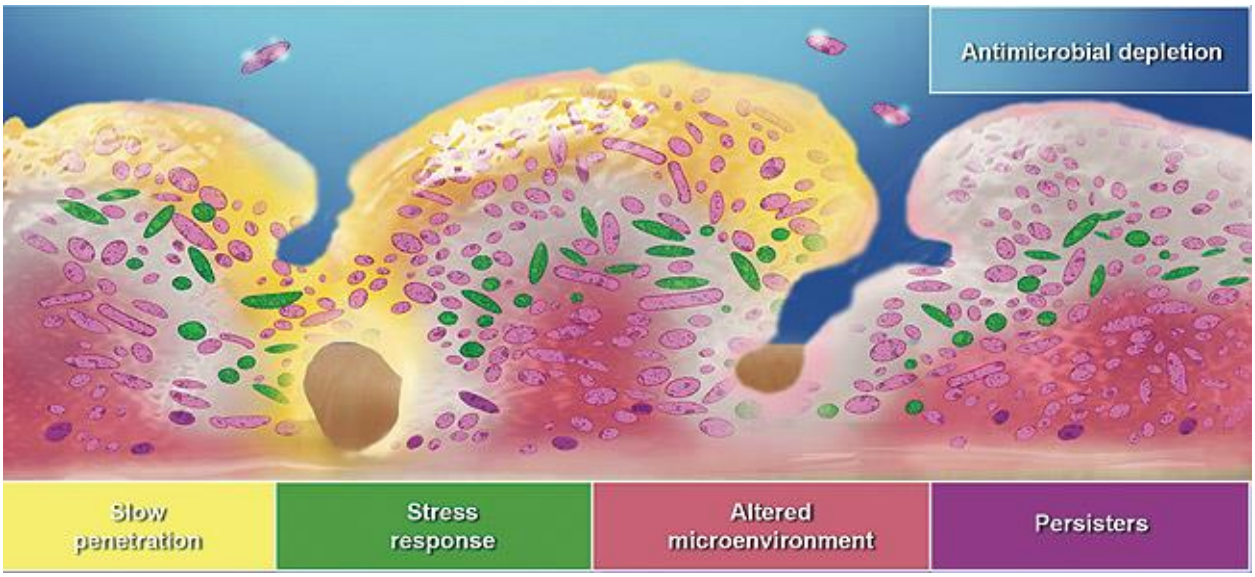

Figure (2.4): Five proposed mechanisms of increased biofilm tolerance to antimicrobials. (Source: Keller & Costerton, 2009)

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The close locative within bacterial cells biofilm lead to the accelerate plasmid transfer, therefore, the biofilm modes of growth facilitate the emergence of resistant mutants or speed the process of gene transfer (Hausner & Wuertz, 1999). Biofilm bacteria not only resist antibiotic, they also resist chemical disinfectants, such as chlorine bleach and glutaraldehyde (Lewis, 2001).

The difference between planktonic and biofilm communities is that the frequency of persisters is much higher in the biofilm population. Persisters may form through entering a highly protected perhaps spore-like state.

These cells are unaffected even by prolonged antibiotic treatment, and most of the population is rapidly killed (Brooun, Liu, & Lewis, 2000).

Weakening biofilm resistance may enhance the ability of existing antibiotics to reduce infections involving biofilms that are refractory to current treatments (Stewart, 2002). Four driving forces are depicted by Jefferson (Jefferson, 2004). Initial aggregation, adaptation, available nutritional and environmental conditions.

2.4 Recurrent infection and biofilm

The biofilm-related infections by bacteria had been recognized in human medicine for many bacterial species (Donlan & Costerton, 2002). It is estimated that 80% of human bacterial infections involve biofilm-associated microorganisms. Diseases such as cystic fibrosis (CF) associated infections, pneumonia, infective endocarditis and periodontitis are known for their association with biofilm formation (X. Li, Kolltveit, Tronstad, & Olsen, 2000).

Biofilms cause a significant amount of human microbial infections, especially health care associated infections which are the fourth leading cause of death in the U.S with 2 million infected cases annually (or ~10% of American hospital patients) leading to more than $ 5 billion in added medical cost per annum (Wenzel, 2007). *P. aeruginosa* is one of the most commonly isolated organisms from catheter biofilms. Long term catheters

(30 days) had more biofilm formation on the catheter inner lumen (Donlan, 2001). It was observed that the biofilm formation of *P. aeruginosa* in the sputum of chronically infected CF patients was relevant to CF-associated lung infection compared with single-celled planktonic bacteria (Hoiby et al., 1977).

Biofilm forming bacteria are less susceptible to our immune defense system. Phagocytic cells have difficulty ingesting bacteria within a biofilm. Consequently, a biofilm-associated infection can persist for a long period of time (i.e., progress from an acute to a chronic infection) (Leid, Shirtliff, Costerton, & Stoodley, 2002; Toutain, Caiazza, & O'Toole, 2004).

Because immune cells could not penetrate into the biofilm, the structure of biofilms is such that immune responses may be directed only at those antigens found on the outer surface of the biofilm. Antibodies and other serum proteins often fail to penetrate into the biofilm. Successful treatment of infections involving biofilm forming pathogens depends on long term, high-dose antibiotic therapies and the removal of any foreign-body material (Leid et al., 2002; Toutain et al., 2004).

2.5 Model organisms

2.5.1 *P. aeruginosa* QS and biofilm formation

P. aeruginosa (figure 2.5), is Gram-negative bacteria, aerobic, rod shape, and motile, have pili (fimbriae), and polysaccharide capsules (Elraih, 2015). Found in moist places, hospital environment, and disinfectant solution (Cheesbrough, 1984). It can attack not only human, but a wide variety of hosts (animals, insects, and plants) (Bucher & Stephens, 1957). *P. aeruginosa* causes endogenous infection after 3 weeks of patient stay in hospitals (Collee, Anderw, & Barrie, 1996).

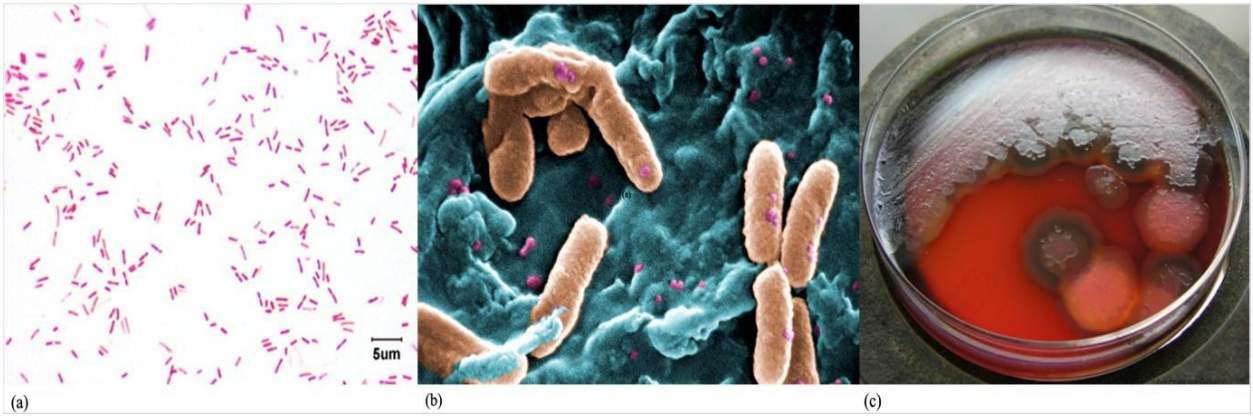

Figure (2.5): Multiple views of *Pseudomonas aeruginosa*. (a) Gram stain of *P. aeruginosa*, (b) scanning electron micrograph (SEM) of *P. aeruginosa*, (c), colonies of *P. aeruginosa* on blood agar plates showing hemolysis (Source: Kaiser, 2009; Carr, 2006).

The best known pigment produced by *P. aeruginosa* is pyocyanine (phenazine pigment) and the pyoverdine fluorescein pigment, which plays an important nutritional role (YABUUCHI & Ohyama, 1972). *P. aeruginosa* initiate an infection by adherence to host cells used porin F (OprF) from the outer membrane (Azghani, Idell, Bains, & Hancock, 2002). It requires many cellular factors to complete bacterial adhesion processes like exoproducts, type IV pili, and flagella (Plotkowski, Bajolet-Laudinat, & Puchelle, 1993).

Swimming motility enable the bacteria to overcome repulsive forces at the surface so the microcolonies may be formed by twitching motility-driven cell aggregation (Heydorn et al., 2002). Despite the evidence that *P. aeruginosa* biofilm development uses motility but the finding that type IV pili are not always necessary for *P. aeruginosa* biofilm or microcolony formation suggests it uses the flagella to mediate transport of the bacteria to the surface. Type IV pili-driven motility along the surface leads to cellular aggregation and microcolony formation, and the subsequent formation of larger sessile mushroom-shaped multicellular structures occurs via a maturation process that requires cell-to-cell signaling (Costerton, Stewart, & Greenberg, 1999; G. O'Toole et al., 2000; Stoodley, Sauer, Davies, & Costerton, 2002).

P. aeruginosa is an opportunistic pathogen that produces a variety of virulence factors. These virulence factors include expression of adhesins, production of biofilms, excretion of hydrolytic enzymes, and production of toxins that may be injected directly into host cells. (Pesci & Iglewski, 1997; Van Delden & Iglewski, 1998).

Biofilm occur naturally in *P. aeruginosa* (Costerton et al., 1999). *P. aeruginosa* can grow anaerobically using molecules as terminal electron acceptors like nitrite (NO_2-) and nitrate (NO_3-), and form biofilm under the same condition, similar to what is believed to occur in the CF airway. Reactive nitrogen intermediates (nitric oxide (NO), peroxynitrite (ONOO-) and nitrous acid (HNO_2)) produced during anaerobic respiration, the possibility that these materials are toxic (Yoon et al., 2002).

Quorum sensing (QS) has been found to play important role in *P. aeruginosa* biofilm formation (De Kievit, 2009). *P. aeruginosa* has two AHL-based QS systems; Las and Rhl signalling system (Gambello & Iglewski, 1991; Passador, Cook, Gambello, Rust, & Iglewski, 1993).

In the las system, LasI catalyzes the synthesis of the 3-oxo-C12-HSL signal molecule, which binds to and activates the transcriptional activator protein *LasR* (Gambello & Iglewski, 1991; Passador et al., 1993). The second QS system, the rhl system, consists of the signal synthase RhlI, which generates N-butanoyl-homoserine lactone (C4-HSL), and the signal receptor RhlR, which induces gene expression when complexed with C4-HSL (Ochsner, Koch, Fiechter, & Reiser, 1994; Parsek, Schaefer, & Greenberg, 1997).

2-Heptyl-3-hydroxy-4(1H)-quinolone, it's the third signalling molecule in *P. aeruginosa* called *Pseudomonas* Quinolone Signal (PQS) (Gallagher, McKnight, Kuznetsova, Pesci, & Manoil, 2002), which encodes for the major virulence factor, LasB elastase, has a role in cell to-cell signaling (Pesci et al., 1999).

P. aeruginosa produced exopolysaccharides (alginate, Psl, and Pel) involved in biofilm formation and development (Ryder, Byrd, & Wozniak, 2007). Alginate is composed of uronic acids (Remminghorst, Hay, & Rehm, 2009), which contribute to decreased

susceptibility of microorganisms inside biofilm to antibiotics and human antibacterial defense mechanisms (Nivens, Ohman, Williams, & Franklin, 2001; Pier, Coleman, Grout, Franklin, & Ohman, 2001).

Pel is a glucose-rich polysaccharide polymer (Friedman & Kolter, 2004), contribute in biofilm biomass accumulation, no role in biofilm attachment (Vasseur, Vallet-Gely, Soscia, Genin, & Filloux, 2005). Pel is critical for maintaining cell-cell interactions in developing *P. aeruginosa* biofilms as well as providing protection against aminoglycoside antibiotics during biofilm growth (Colvin et al., 2011).

Psl is important for biofilm initiation, play a critical role in cell-cell and/or cell-surface interactions (Jackson, Starkey, Kremer, Parsek, & Wozniak, 2004). Study by Colvin et al., (2011), showed loss of Pel production in the laboratory strain PAO1 resulted in no difference in attachment or biofilm development; instead Psl proved to be the primary structural polysaccharide for biofilm maturity.

In conclusion, over the past decade, many studies have been made directed towards understanding *P. aeruginosa* biofilm development, because these bacteria uses different mechanisms for surface colonization in response to the prevailing environmental conditions. Therefore, it failed to reveal a specific mechanism of biofilm development (De Kievit, 2009).

2.5.2 *S. aureus*

S. aureus is Gram-positive cocci, facultative anaerobic bacteria (Devriese et al., 2005; Frank, Kania, Hnilica, Wilkes, & Bemis, 2003; Griffeth, Morris, Abraham, Shofer, & Rankin, 2008), spherical cells, between 0.5 to 1.7 μm in diameter, non-motile and non-spore forming, arranged in grape-like clusters (Figure 2.6). *S. aureus* is the most clinically significant species of staphylococci. *S. aureus* causes various infections (Figure 2.7), skin lesions, pneumonia, mastitis, phlebitis, and meningitis (Taha, 2010). *S. aureus* releasing enterotoxins into food thus causing food poisoning, and can release super-antigens into the blood stream leading to toxic shock syndrome (Todar, 2006).

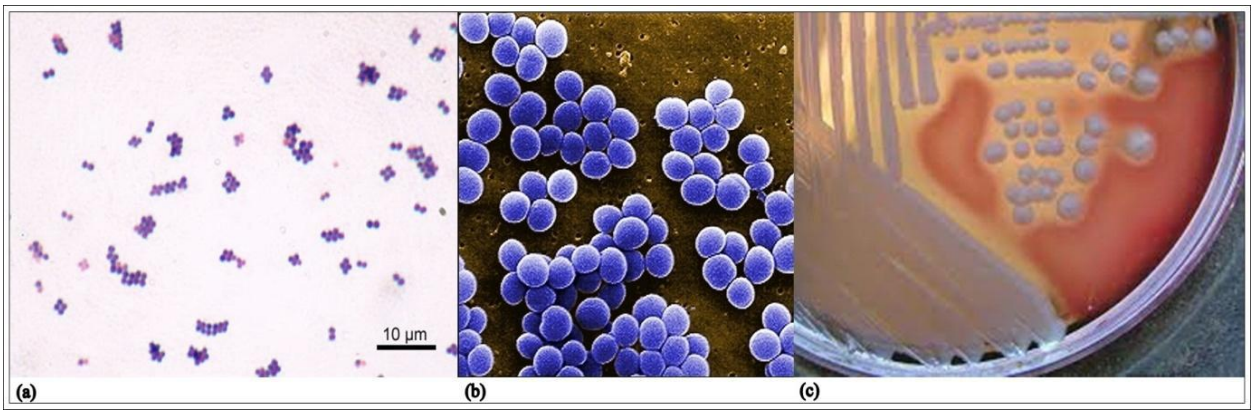

Figure (2.6): Multiple views of *Staphylococcus aureus*. (a) Gram stain of *S. aureus,* (b). scanning electron micrograph (SEM) of *S. aureus*, (c) colonies of *S. aureus* on blood agar plates showing hemolysis (Source: Buxton, 2013).

S. aureus expresses many potential virulence factors, inherent and acquired resistance to antimicrobial agents (Todar, 2011), secreted toxins, exoenzymes, surface, immune-modulating proteins (Tuchscherr et al., 2011; Sendi & Proctor, 2009), and other protein including coagulase. Its biochemical properties that enhance their survival in phagocytes, protein A, alpha-, beta-, gamma-, delta-toxin, and leukocidin greatly contribute to its virulence. These virulence factors serve many function; for evasion of host defense mechanisms, for adherence, cell internalization, and for invasion of host tissue (Harshman et al., 1989).

S. aureus is able to form intricate micro-colonies termed biofilms. Many proteins have been implicated as important components in attachment and biofilm matrix development in *S. aureus*. These include surface-associated proteins such as protein A, fibrinogen-binding proteins (FnBPA and FnBPB), *S. aureus* surface protein (SasG), biofilm-associated protein (Bap), and clumping factor B (ClfB) (Lister & Horswill, 2015).

Many of these factors play a role both in attachment and accumulation. In addition, secreted proteins such as extracellular adherence protein (Eap), and beta toxin (Hlb) play a role in biofilm maturation.

The primary biofilm dispersal strategy utilized by *S. aureus* is the production of various exo-enzymes and surfactants to degrade the extracellular polymeric matrix (Lister & Horswill, 2015).

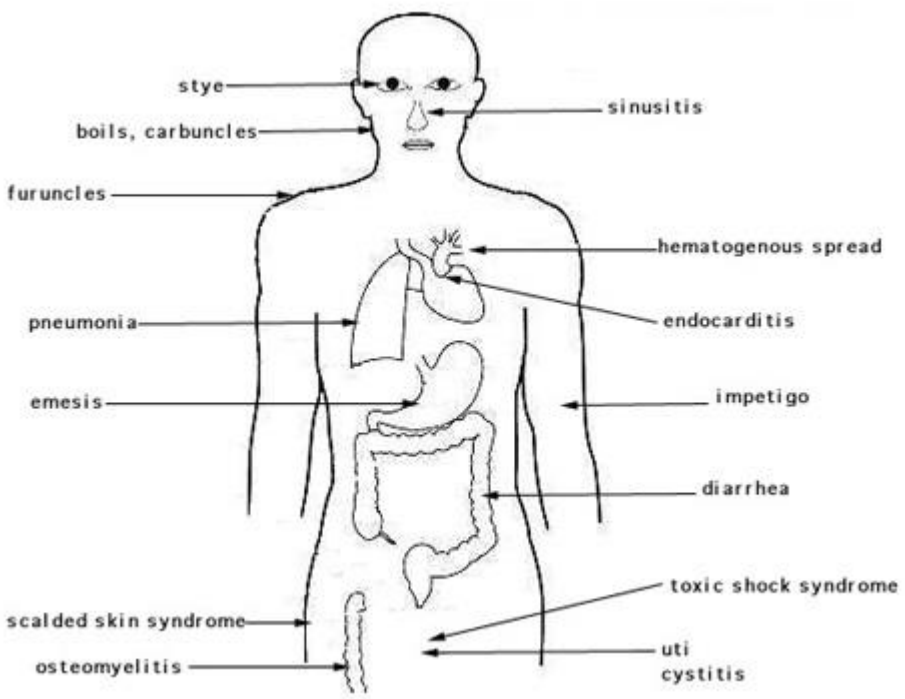

Figure (2.7): Sites of infection and diseases caused by *Staphylococcus aureus* (Source: Todar, 2011).

2.5.3 *C. albicans*

C. albicans belongs to the *Saccharomyceteae* family (Table 2.4). Colonies are cream-colored to yellowish (Figure 2.8) (C. G. Kumar & Anand, 1998).

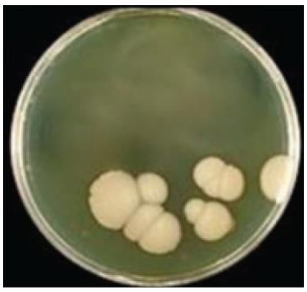

Figure (2.8): *Candida albicans* colonies on sabouraud dextrose agar (Source: Boparai, Amasi, Patil, & Harakuni, 2014).

C. albicans is a commensal of the normal human microflora but can also cause a variety of infections in superficial mucosal infections including vagina. *C. albicans* has to cross physical barriers such as epithelial cell layers by active penetration and/or induced endocytosis (Zakikhany et al., 2007).

Table (2.4): Classification of *Candida albicans* (Pfaller & Diekema, 2007; Source: Dean & Burchard, 1996; Hajjeh et al., 2004).

Kingdom	***Fungi***
Class	*Saccharomycetes*
Family	*Saccharomyceteae*
Order	*Saccharomycetales*
Genus	*Candida*
Species	*C. albicans*

C. albicans is a part of normal microbiota, normally present in vaginal secretions and discharge and colonizer of human mucosal surfaces. However, under unbalanced conditions, candida growth can become excessive. This is referred to as candida or yeast infection (Pappas et al., 2004).

Several virulence factors, such as ability of *C. albicans* morphological transition between yeast and hyphal forms (dimorphic), adhesins and invasins on the cell surface,

secretion of hydrolytic enzymes, and formation of biofilm. *C. albicans* rapid adaptation to fluctuations in environmental pH, powerful nutrient acquisition systems, metabolic flexibility, and robust stress response machineries. All of these factors contribute to *C. albicans* pathogenicity mechanisms (Figure 2.9) (F. L. Mayer, Wilson, & Hube, 2013).

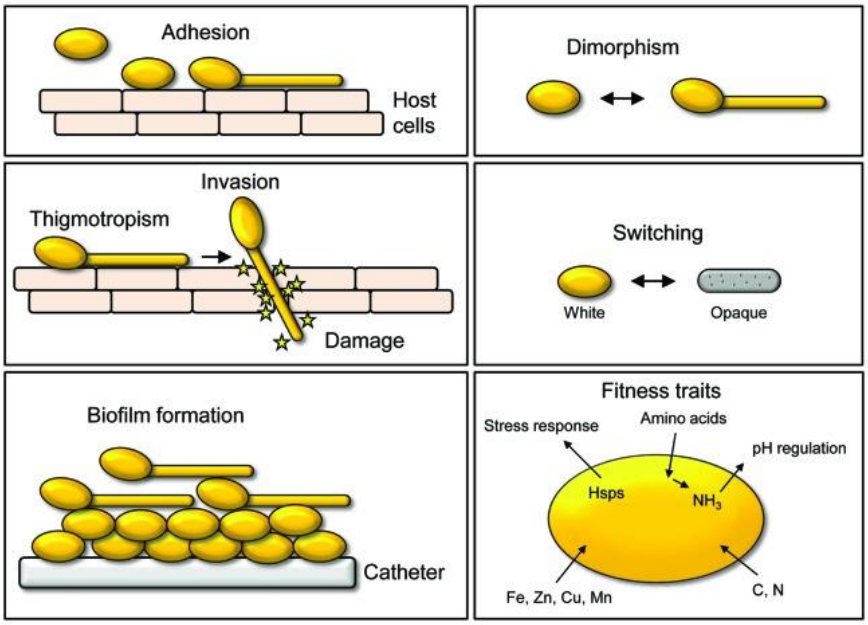

Figure (2.9): An overview of selected *Candida albicans* pathogenicity mechanisms (Source: F. L. Mayer et al., 2013).

Yeast cells attach to a surface via adhesins including, agglutinin. This leads to the formation of microcolonies and the morphology of yeast cells switches to pseudo- and true-hyphae by the regulator Efg1p to form a complex network of hyphal structures with budding yeast cells distributed throughout.

Next, as the biofilm matures, a glucan rich extracellular matrix (ECM), encompasses the structure providing a protective barrier from host defences, antimicrobial agents and environmental stresses.

During the development of a mature biofilm, a hypoxic environment is created which induces the up-regulation of glycolytic genes that control filamentation . Finally,

planktonic yeast cells are able to disperse from the mature biofilm and colonise a new surface to begin the development of a new biofilm (Sherry, 2014).

2.6 Plants used in this study

2.6.1 *Cinnamomum zeylanicum*

C. zeylanicum belongs to the *Lauraceae* family (Table 2.5), small evergreen tree, are 35–50 feet tall. The leaves (7-15 cm) are ovate to oblong in shape. Flowers of this plant are green in color with a distinct odor. The fruit is purple in color containing single seed. Has been known from remote antiquity, with the characteristic odor and a very distinct aromatic taste. The pungent taste and scent come from cinnamic aldehyde or cinnamaldehyde (about 60 % of the essential oil from the bark) (Khusro, 2015).

C. zeylanicum possess anti-inflammatory properties, antimicrobial activity, reducing cardiovascular disease, and reducing risk of colonic cancer (Jayaprakasha & Rao, 2011).

Table (2.5): Classification of *Cinnamomum zeylanicum* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Laurales
Family	*Lauraceae*
Genus	Cinnamomum
Species	*Cinnamomum zeylanicum*

2.6.2 *Marrubium vulgare*

M. vulgare belongs to the *Labiatae* family (Table 2.6), long-lived herbaceous plant usually growing 20-60 cm tall, its small white tubular flowers (6-12 mm long) are borne in dense clusters in the upper leaf forks (Bokaeian et al., 2014).

M. vulgare has been known as a tonic, stomachic tonic, anti-pyretic in external use, and it has been used as an anti-septic and healing agent (Bokaeian et al., 2014). It is helpful for bronchial asthma and nonproductive cough. It also used as vasorelaxant, antihypertensive analgesic. It possesses anti-inflammatory, antioxidant activity, and many other biological activities (El Bardai, Lyoussi, Wibo, & Morel, 2004).

Table (2.6): Classification of *Marrubium vulgare* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Labiatae*
Genus	Marrubium
Species	*Marrubium vulgare*

2.6.3 *Tamarix aphylla*

T. aphylla belongs to the *Tamaricaceae* family (Table 2.7). It is fast growing, moderate sized evergreen tree, up to 18 m high. *T. aphylla* leaves are used as an anti-inflammatory agent and in healing the wounds (Emad & Gamal, 2013). Leaves show considerable antioxidant activity (Zain, Awaad, Al-Outhman, & El-Meligy, 2012).

T. aphylla extracts contain biologically active compounds, which work as substitute as the drug remedy used in the treatment of leishmaniasis (Iqbal et al., 2013). *T. aphylla*

leaves and young branches are given externally as lotion (Badshah & Hussain, 2011). *T. aphylla* bark and leaves extract is used to treat Leucorrhea, strengthen the uterus, inflammation of joints and joints pain (Ullah et al., 2014).

Table (2.7): Classification of *Tamarix aphylla* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Violales
Family	*Tamaricaceae*
Genus	Tamarix
Species	*Tamarix aphylla*

2.6.4 *Cuminum cyminum*

C. cyminum belongs to the *Apaiaceae* family (Table 2.8). It is an annual shrub up to 10 to 50cm height, native from the Mediterranean region. Chemical composition of *Cuminum* are monoterpene hydrocarbons, oxygenated monoterpenes, oxygenated sesquiterpenes, saturated and unsaturated fatty acids, aldehydes, ketones and esters (Johri, 2011; Bettaieb et al., 2010; El-Ghorab, Nauman, Anjum, Hussain, & Nadeem, 2010).

Several studies on anti-diabetic (Dhandapani, Subramanian, Rajagopal, & Namasivayam, 2002), antioxidant, anti-bacterial, anti-fungal (Romagnoli, Andreotti, Maietti, Mahendra, & Mares, 2010), effect of *C. cyminum* seed extracts have been reported in literature, prominently considered carminative, eupeptic, antispasmodic, and used in the treatment of mild digestive disorders, diarrhea, dyspepsia, flatulence, morning sickness, and to improve liver function (Sahoo, Sahoo, Sarangi, Sagar, & Kori, 2014).

Table (2.8): Classification of *Cuminum cyminum* (Source: USDA, 2016; Johri, 2011).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	*Apaiaceae*
Genus	Cuminum
Species	*Cuminum cyminum*

2.6.5 *Pelargonium hortorum*

P. hortorum belongs to the *Geraniaceae* family (Table 2.9). Leaves occur in a variety of shapes and sizes, may be covered with fine hairs and may be rough, sticky or have a velvety texture. Leaf edges may appear 'curly' in some species. Flowers may be white, pink, mauve, lavender, pale yellow. The Pelargonium species are divided into 16 recognized sections (Bakker, Culham, Hettiarachi, Touloumenidou, & Gibby, 2004). Used to treatment of diarrhea, dysentery, fever, respiratory tract infections, liver complaints, wounds, gastroenteritis, haemorrhage, kidney and bladder disorders (Bakker et al., 2004).

Table (2.9): Classification of *Pelargonium hortorum* (Source: USDA, 2016; Prickly & Bitter, 2012).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Geraniales
Family	*Geraniaceae*
Genus	Pelargonium
Species	*Pelargonium hortorum*

2.6.6 *Lawsonia inermis*

L. inermis belongs to the *Lythraceae* family (Table 2.10). A much branched or small tree (2 to 6 m in height). Leaves are small, opposite in arrangement along the branches (Chaudhary, Goyal, & Poonia, 2010). *Henna* has a wide spectrum of antimicrobial activity including antibacterial, antiviral, antimycotic and antiparasitic activities (Rayavarapu, Kaladhar, & Kumar, 2011).

Table (2.10): Classification of *Lawsonia inermis* (Source: USDA, 2016; GBIF, 2016)

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	*Lythraceae*
Genus	Lawsonia
Species	*Lawsonia inermis*

2.6.7 *Salvia officinalis*

S.officinalis belongs to the *Lamiaceae* family (Table 2.11). It is aromatic plants with ornamental, culinary, and phytotherapeutic use all over the world (Fournomiti et al., 2015), this plant contain Thujone, 1, 8 cineole, Borneol, Borneol acetate, sesquiterpene, tannins and phenolic acids (Mosafa, Yahyaabadi, & Doudi, 2014).

The herb has anti-tumor and anti-inflammation property and is used vastly in cosmetic and perfumery industries. So, the herb is used for cold, bronchitis, digestive disorders and tuberculosis. *Salvia* extract has antibacterial property due to 1,8-cineol (Ghorbanpour, Hosseini, Khodae Motlagh, & Solgi, 2014).

Table (2.11): Classification of *Salvia officinalis* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	Salvia
Species	*Salvia officinalis*

2.6.8 *Triticum aestivum*

T. aestivum belonging to family *poaceae* (Table 2.12). *T. aestivum* has a long history and is widely used as a health food supplement, have anti-inflammatory, antioxidant, anti-carcinogenic, immune-modulatory, laxative, diuretic, antibacterial, anti-aging properties,(Rana, Kamboj, & Gandhi, 2011; Smith, 2000), and antimicrobial activity against some of the food borne bacterial pathogens (Sundaresan, Selvi, & Manonmani, 2015).

Table (2.12): Classification of *Triticum aestivum* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Liliopsida
Order	Cyperales
Family	*Poaceae*
Genus	Triticum
Species	*Triticum aestivum*

2.6.9 *Artemisia absinthium*

A. absinthium belongs to the *Asteraceae* family (Table 2.13). The plant has a fibrous root system and grows to about 1.2 m in height, contain biologically active compounds ,like camphor, p-cymene, caryophyllene, α-pinene and β-pinene (Moslemi, Hoseinzadeh, Badouei, Kafshdouzan, & Fard, 2012).

A. absinthium widely used mainly for its neuroprotective (Bora & Sharmab, 2010), antifungal antimicrobial, insecticidal, anthelmintic, antimalarial (Irshad, Butt, & Younus, 2011), hepatoprotective , and antidepressant proprieties (Mahmoudi, Ebrahimzadeh, Ansaroudi, Nabavi, & Nabavi, 2009). In addition, the herb is used to make a tea for helping pregnant women during pain of labor and in treating leukaemia and sclerosis (Msaada et al., 2015).

Table (2.13): Classification of *Artemisia absinthium* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Asterales
Family	*Asteraceae*
Genus	Artemisia
Species	*Artemisia absinthium*

2.6.10 *Pelargonium graveolens*

P. graveolens belongs to the *Geraniaceae* family (Table 2.14). It is aromatic and hairy herbaceous shrub, up to 1 m high. Leaves are prickly and carved, flowers are small, usually pink.

Essential oil of *P. graveolens* is used as a fragrant component in perfumery, food and beverages industry, also as antiseptic remedy (Ćavar & Maksimović, 2012; Fayed, 2009; Seo et al., 2009).

Also it regulates the bloodstream, stimulates the adrenal glands and lymphatic system which in combination with diuretic properties makes this essential oil excellent in the fight against cellulite and fluid retention in the body (DzamiÄ et al., 2014), antimicrobial and antimalarial activity (Ćavar & Maksimović, 2012; Fayed, 2009; Seo et al., 2009).

Table (2.14): Classification of *Pelargonium graveolens* (Source: USDA, 2016; Carmen & Hancu, 2014).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Geraniales
Family	*Geraniaceae*
Genus	Pelargonium
Species	*Pelargonium graveolens*

2.6.11 *Foeniculum vulgare*

F. vulgare belongs to the *Apiaceae* family (Table 2.15). It is a medicinal and aromatic plant. It grows to a height of up to 2.5 m, with feathery leaves and golden yellow flowers (Askun, Tumen, Satil, & Ates, 2009; Muckensturm, Foechterlen, Reduron, Danton, & Hildenbrand, 1997; Guillén & Manzanos, 1996).

F. vulgare has significant antimicrobial activity against the bacteria and fungi (Kwon et al., 2002), used as diuretic, analgesic and antipyretic activity, and antioxidant activity (Oktay, Gülçin, & Küfrevioğlu, 2003).

Table (2.15): Classification of *Foeniculum vulgare* (Source: USDA, 2016)

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Apiales
Family	*Apiaceae*
Genus	Foeniculum Mill
Species	*Foeniculum vulgare*

2.6.12 Hibiscus sabdariffa

H. sabdariffa, also known as roselle, belongs to the *Malvaceae* family (Table 2.16), is an ideal crop for developing countries as it is relatively easy to grow, can be grown as part of multi-cropping systems and can be used as food (Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014), can grow up to 2.4 m tall, with red stems.

The main constituents of *H. sabdariffa* are hydroxycitric acid, hibiscus acid, anthocyanins, flavonoids, mucilage, pectin and carbohydrates (polysaccharides), these plant has significant antibacterial, antifungal, antiparasitic activity, antipyretic, anti-inflammatory, antioxidant, hepatoprotective, and nephroprotective activity (Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, & Heinrich, 2014).

Table (2.16): Classification of *Hibiscus sabdariffa* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malvales
Family	*Malvaceae*
Genus	Hibiscus
Species	*Hibiscus sabdariffa*

2.6.13 *Solenostemon scutellarioides*

S. scutellarioides belongs to the *Lamiaceae* family (Table 2.17), is a brightly coloured shrub and leaves heart-shaped. The foliage can be of any combination of yellow, pink, orange, red, or green. *S. scutellarioides* is just one of over 150 species (Khattak & Taher, 2011).

It is famous for ornamental purposes, but it is also used for medicinal purposes in some communities, used for the treatment of various diseased condition such as high blood pressure, cuts and wounds to stop bleeding, treatment of cough, colds and fever (Rout & Panda, 2010). It is also used for the treatment of kidney and bladder stones, bone fractures, headaches, asthma, bronchitis, indigestion, diarrhea, dysentery insect bite, fever and cholera (Kumar, Sucheta, Deepa, Selvamani, & Latha, 2008).

Table (2.17): Classification of *Solenostemon scutellarioides* (Source: USDA, 2016; Khattak & Taher, 2011).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	Solenostemon
Species	*Solenostemon scutellarioides*

2.6.14 *Rosmarinus officinalis*

R. officinalis belongs to the *Lamiaceae* family (Table 2.18). This type has an evergreen bush. which is a local plant of Mediterranean region with pharmacological and decorative value. It is a sustainable plant, so aromatic and has wooden stalks with 50 cm up to 2 m height, main active constituents of *R. officinalis* are flavonoids, phenolic acids, diterpenes, steroids, and triterpene (Frankel, Huang, Aeschbach, & Prior, 1996; Nascimento, Locatelli, Freitas, & Silva, 2000).

Its herb and oil are commonly used as spice and flavoring agents in food processing for its desirable flavor, high antioxidant activity and as antimicrobial agent (Lo, Liang, Lin-Shiau, Ho, & Lin, 2002), rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative bacteria (Moreno, Scheyer, Romano, & Vojnov, 2006).

Table (2.18): Classification of *Rosmarinus officinalis* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	Rosmarinus
Species	*Rosmarinus officinalis*

2.6.15 *Urtica dioica*

U. dioica belongs to the *Urticaceae* family (Table 2.19). Commonly known as stinging nettle, it is widely spread from Europe to Asia. The whole plant is used in folk medicine to treat allergies, kidney stones, burns, anemia, rashes, internal bleeding, diabetes, etc.(Dar, Ganai, Yousuf, Bhat, & Bhat, 2012).

However, only a few of these pharmacological activities have been experimentally proved (Rodriguez-Fragoso, Reyes-Esparza, Burchiel, Herrera-Ruiz, & Torres, 2008). The known phytochemicals of U. dioica include flavonoids, lignans, fatty acids, sterols, polysaccharides, glycoproteins, carotenoids, plastocyanins, and lectins (Sajfrtová, Sovova, Opletal, & Bartlova, 2005).

Table (2.19): Classification of *Urtica dioica* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Urticales
Family	*Urticaceae*
Genus	Urtica
Species	*Urtica dioica*

2.6.16 *Thymus vulgaris*

T. vulgaris belongs to the *Lamiaceae* family (Table 2.20), grow up to 15-30 cm tall by 40 cm wide. Thyme is a tiny perennial shrub, with a semi-evergreen groundcover that seldom grows quite 40 cm tall it's each horizontal and upright habits (Reddy, Kandisa, Varsha, & Satyam, 2014).

Thymol and carvacrol ,flavonoids (e.g. thymonin, cirsilineol and 8-methoxycirsilineol), "Labiatae tannin" (rosmarinic acid), caffeic acid, triterpenoids, long-chain saturated hydrocarbons and aliphatic aldehydesare the most important chemical constituents of *T. vulgaris* (Varga et al., 2015). Thymus species are very efficient disinfectants and can be applied in vaporizers against different human pathogenic Gram-positive and Gram negative bacteria and yeasts (Varga et al., 2015).

Table (2.20): Classification of *Thymus vulgaris* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	Thymus
Species	*Thymus vulgaris*

2.6.17 *Punica granatum*

Punica granatum belongs to the *Lythraceae* family (Table 2.21). It is a large shrub up to 10m in height with smooth dark grey bark. Flowers are scarlet red or sometime yellow, fruits are globose. Rind gives pentose glycosides of malvidin and pentunidin. Fluoride, calcium, magnesium, vitamin C and phosphate are also reported from. Rind of fruit is astringent, fruit is laxative (Yogeeta, Ragavender, & Devaki, 2007). *P. granatum* has antifungal, CNS depressant, diuretic hypothermic, and excellent response to the patients of Giardiasis (Yogeeta et al., 2007).

Table (2.21): Classification of *Punica granatum* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	*Lythraceae*
Genus	Punica
Species	*Punica granatum*

2.4.18 *Agave sisalana*

A. sisalana belongs to the *Agavaceae* family (Table 2.22), also known as sisal, can help in lowering the blood pressure. It is also used as an antiseptic and is taken to stop the growth of bacteria in the stomach and intestine. It is generally cultivated for its fibrous properties yielding a stiff fiber traditionally used in making twine, rope (Debnath, Mukeshwar, Sharma, Thakur, & Lal, 2010).

Table (2.22): Classification of *Agave sisalana* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Liliopsida
Order	Liliales
Family	*Agavaceae*
Genus	Agave
Species	*Agave sisalana*

2.6.19 *Mentha longifolia*

M. longifolia or wild mint is a perennial herb, belongs to the *Lamiaceae* family (Table 2.23), it is aromatic and melliferous herb which has the nice and fresh scent of menthol or lemon (Stanisavljević et al., 2014). The aerial part of the herb in bloom contains the essential oil with the dominant components of piperitone, piperitone oxide, carvone, menthone, limonene, 1,8-cineole (depending on chemo type), flavonoids and tannins.

The preparations show the carminative and stimulating effect towards gastrointestinal tract, alleviate colds, inflammation of respiratory organs, headaches, pains in muscles and joints (Stanisavljević et al., 2014). *M. longifolia* demonstrates a wide range of antibiotic activity against various bacteria, yeasts, insects (Mikaili, Mojaverrostami, Moloudizargari, & Aghajanshakeri, 2013).

Table (2.23): Classification of *Mentha longifolia* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	Mentha
Species	*Mentha spicata*

2.6.20 *Portulaca oleracea*

P. oleracea belongs to the *Portulacaceae* family (Table 2.24). *Purslane* plants are succulent, annual herbaceous, and erect up to 30 cm high (Uddin et al., 2014). *Purslane* is used for cooking or used as a pickle.

Its medicinal value is evident from its use for treatment of burns, headache, and diseases related to the intestine, liver, stomach, cough, shortness of breath, and arthritis. Its use as a purgative, cardiac tonic, muscle relaxant, anti-inflammatory and diuretic treatment (Oliveira et al., 2009).

Table (2.24): Classification of *Portulaca oleracea* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Caryophyllales
Family	*Portulacaceae*
Genus	Portulaca
Species	*Portulaca oleracea*

2.6.21 *Matricaria recutita*

M. recutita belongs to the *Asteraceae* family (Table 2.25). It is flower-head plant contained several groups of compounds having important therapeutic values especially essential oil (Sparkman, 2005). The terpenes, α-bisabolol oxides and chamazulene, are the most important compounds (Kazemi, 2014). *M. recutita* has a diverse range of pharmacological actions including antimicrobial, anti-inflammatory, antioxidant, antispasmodic, antiviral, and antiseptic properties (Webster, Taschereau, Belland, Sand, & Rennie, 2008; Pauli & Schilcher, 2004).

Table (2.25): Classification of *Matricaria recutita* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Asterales
Family	*Asteraceae*
Genus	Matricaria
Species	*Matricaria recutita*

2.4.22 *Erythrina crista-galli*

E. crista-galli trees 5–8 meters tall, called "crybaby tree" because of abundant nectar that sometimes drips from the flowers (Bean, 2008), it is belongs to the *Fabaceae* family (Table 2.26). *E. crista galli* has a compound with antibacterial, antifungal and in vivo antiinflammatory activities (Weber et al., 2005; Weber et al., 2004), used in folk medicine for wound healing, narcotic and analgesic (Redko et al., 2007).

Table (2.26): Classification of *Erythrina crista-galli* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	*Fabaceae*
Genus	Erythrina
Species	*Erythrina crista-galli*

2.6.23 *Momordica charantia*

M. charantia belongs to the *Cucurbitaceae* family (Table 2.27). The Latin name *Momordica* means "to bite," referring to the jagged edges of the leaves, which appear as if they have been bitten. It has shown antibacterial, antileukemic, antiprotozoal, antiviral, antiparasitic, antifungal, anti-obesity, anti-ulcer, hypoglycemi and, immune stimulant activities (Santos et al., 2012; Agrawal & Beohar, 2010; Gupta et al., 2010; Alam, Asad, Asdaq, & Prasad, 2009).

Table (2.27): Classification of *Momordica charantia* (Source: USDA, 2016).

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Violales
Family	*Cucurbitaceae*
Genus	Momordica
Species	*Momordica charantia*

Chapter III
Materials and Methods

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3.1 Materials

3.1.1 Apparatus

The apparatus used in this study are listed in table 3.1

Table (3.1): List of the apparatus used in this work

Apparatus	Manufacture/model	Country
Automated Elisa Analyzer	Multiskan FC	Republic or Korea
Light microscope	Leica	China
Micro-centrifuge	EB series	UK
Centrifuge 50 ml	Hettich	Germany
Heat drying oven	Boxun	China
pH/ Meter	Thermo	Singapore
Soxhlet extractor	Biology department	IUG
Autoclave sterilization	Tuttnauer	Germany
Incubator	Memmert	Germany
IR Concentrator	N- Biotek	Korea
Balance	Ae ADAM	UK

3.1.2 Chemicals, culture media and antibiotics

Eight types of media were used for carrying out this study. Brain Heart Infusion Agar (BHIA), Brain Heart Infusion Broth (BHIB) with 1% glucose, Nutrient broth (NB), Mueller Hinton Agar (MH), Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), and Dulbecco's Modified Eagle Medium (DMEM) without sodium bicarbonate supplemented with L-glutamine. Swarm plates were prepared using 0.5% Bacto agar, 0.5% peptone, 0.2% yeast extract and 1.0% glucose per 100 ml distilled water and used as media for motility test. Ethanol 70% and Dimethyl sulfoxide (DMSO) were used for extraction process and dissolving extracts; 2% crystal violet and 95% methanol for biofilm assay; 0.2 M HCl, chloroform, Na_2PO_4 (pH 4.5) were used. In table (3.2) is a list of antimicrobials used as reference and in synergistic effect testing.

Table (3.2): List of antimicrobials and their potency used as a reference or in synergistic effect.

Antimicrobials	Symbol	Potency	Manufactured by
Cefoxitin	FOX	30 µg	Liofilchem
Ceftaroline	CPT	5 µg	Liofilchem
Chloramphenicol	C	30 µg	Liofilchem
Ciprofoxacin	CIP	5 µg	Liofilchem
Clarithromycin	CLR	15 µg	Liofilchem
Erythromycin	E	15 µg	Liofilchem
Gentamicin	CN	30 µg	Liofilchem
Levofloxacin	LEV	5 µg	Liofilchem
Norfloxacin	NOR	10 µg	Liofilchem
Ofloxacin	OFX	5 µg	Liofilchem
Oxacilin	OX	1 µg	Liofilchem
Penicillin	P	10 IU	Liofilchem
Trimethoprim	TM	5 µg	Liofilchem

3.1.3 Plant materials collection

Four medicinal plants were purchased from Gaza`s local markets, nineteen medicinal plants were collected from different agricultural areas. Plants were identified at the Biology and Biotechnology department of the Islamic University-Gaza. The tested plants are listed in table (3.3).

Table (3.3): List of medicinal plants tested for their anti-microbial, antifungal, anti-biofilm and anti-quorum sensing activity.

No	Binomial Name	Family	Vernacular	Part Tested
1	*Cinnamomum zeylanicum*	*Lauraceae**	قرفه	Cinnamon sticks
2	*Marrubium vulgare*	*Labiatae**	سموه	Leaves
3	*Tamarix aphylla*	*Tamaricaceae**	أثله	Leaves
4	*Cuminum cyminum*	*Apaiaceae**	كمون	Grounded seeds
5	*Pelargonium hortorum*	*Geraniaceae**	صقصلي	leaves, flowers and stalks
6	*Lawsonia inermis*	*Lythraceae**	حناء	Leaves
7	*Salvia officinalis*	*Lamiaceae**	مرميه	Leaves and Flowers
8	*Triticum aestivum*	*Poaceae**	نخاله القمح	Straw
9	*Artemisia absinthium*	*Asteraceae**	الشيح أو الشيبا	Leaves
10	*Pelargonium graveolens*	*Geraniaceae**	العطره	Leaves
11	*Foeniculum vulgare*	*Apiaceae**	شومر	Grounded seeds
12	*Hibiscus sabdariffa*	*Malvaceae**	كركديه	Leaves
13	*Solenostemon scutellarioides*	*Lamiaceae**	سجاده	Leaves
14	*Rosmarinus officinalis*	*Lamiaceae**	اكليل الجبل	Leaves
15	*Urtica dioica*	*Urticaceae**	قريص	Leaves
16	*Thymus vulgaris*	*Lamiaceae**	زعتر	Leaves
17	*Punica granatum*	*Lythraceae**	رمان	Peel
18	*Agave sisalana*	*Agavaceae**	صبره	Leaves
19	*Mentha longifolia*	*Lamiaceae**	نعناع	Leaves
20	*Portulaca oleracea*	*Portulacaceae**	بقله	Leaves
21	*Matricaria recutita*	*Asteraceae**	بابونج	Leaves, flowers
22	*Erythrina crista-galli*	*Fabaceae**	مرجانه	Flowers
23	*Momordica charantia*	*Cucurbitaceae**	القرع المر	Leaves

*Source: (USDA, 2016).

3.1.4 Microorganisms

Two isolates of *P. aeruginosa*, one *S. aureus*, and one *C. albicans* were used. Organism were isolated from clinical specimens submitted to the microbiology laboratories of Al Shifa Hospital, and were maintained on Brain Heart Infusion Agar medium slant at 2-8 ^{o}C. Addition 15 *S. aureus* isolate (MRSA) are obtained from health care workers from hospitals in Gaza strip were kindly provided by Prof. Dr. Mohammad E. Shubair.

3.2 Methodology

3.2.1 Preparation of plant extract

A modification of previously described procedures (Raaman, 2006), was used to prepare the ethanolic extract of the plant as follows. The dried plant materials (30g) were mixed with 300 ml ethanol 70% (v/v) for 8 hours in a soxhlet extractor. The extract was then allowed to evaporate in an oven at 37^{o}C for at least three days (dependent on the type of plant). Some of the extracts were dried using IR Concentrator. The dried extract was stored in a refrigerator until used (extracts were tested within 2 weeks of extraction).

3.2.2 Preparation of stock solutions.

Stock solutions of crude ethanol extracts in DMSO or water were prepared, filter-sterilized (0.45 μm) and stored at 4^{o}C (Zaidan et al., 2005). Table 3.4 shows the concentration of each stock solutions. Concentrations depended on the solubility of the extract in DMSO or DW.

Table (3.4): The concentration of each stock solutions for different plant

NO	Plant	Solvent	Dilution	Conc. mg/ml
1	*Cinnamomum zeylanicum*	DMSO	1:3	250
2	*Marrubium vulgare*	DMSO	1:2	333.3
3	*Tamarix aphylla*	DW	1:6	142.8
4	*Cuminum cyminum*	DW	1:2	333.3
5	*Pelargonium hortorum*	DMSO	1:5	166.6
6	*Lawsonia inermis*	DW	1:3	250
7	*Salvia officinalis*	DMSO	1:5.3	158.7
8	*Triticum aestivum*	DMSO	1:4	200
9	*Artemisia absinthium*	DMSO	1:6	142.8
10	*Pelargonium graveolens*	DMSO	1:3	250
11	*Foeniculum vulgare*	DMSO	1:2	333.3
12	*Hibiscus sabdariffa*	DMSO	1:5	166.6
13	*Solenostemon scutellarioides*	DMSO	1:5	166.6
14	*Rosmarinus officinalis*	DMSO	1:6	142.8
15	*Urtica dioica*	DMSO	1:5	166.6
16	*Thymus vulgaris*	DMSO	1:5	166.6
17	*Punica granatum*	DMSO	1:5	166.6
18	*Agave sisalana*	DMSO	1:5	166.6
19	*Mentha longifolia*	DMSO	1:5	166.6
20	*Portulaca oleracea*	DMSO	1:5	166.6
21	*Matricaria recutita*	DMSO	1:5	166.6
22	*Erythrina crista-galli*	DMSO	1:5	142.8
23	*Momordica charantia*	DMSO	1:5	166.6

3.2.3 Determination of antimicrobial and anti-fungi activity of plant extracts

3.2.3.1 Agar well diffusion method

MHA plates were inoculated with a cotton swab moistened with a McFarland standardized test organism. Holes of 6 to 8 mm diameter were punched and were filled with 20 µL of plant extract. The petri dishes were incubated at 37^{o}C for 24 h. Diameters of inhibition growth zone were measured. Culture of fungi were grown on SD agar. The inoculum was prepared in saline solution. Its turbidity was adjusted in accordance with McFarland scale (0.5) (Lahlah, Meziani, & Maza, 2012; Magaldi et al., 2004).

3.2.3.2 Minimum inhibitory concentration assay

This assay was adapted from (Mazzola, Jozala, Novaes, Moriel, & Penna, 2009), with a modification in the type of media and the additional use of 2,3,5 triphenyltetrazolium chloride (TTC). In a 96 wells micro-titer plate, 100 µL of nutrient broth medium for bacteria and DMAM medium for fungi was distributed to each well, 100 µL of each extract was added to the first well. All wells were shaken and contents mixed well, 100 µL was withdrawn from the first well and transferred to the next well and serial dilution was performed. Then 50µ L of bacterial suspension (adjusted to 0.5 McFarland standard 0.5) were added to each well. Plates were covered and incubated at 37^{o}C for 24h. 20 µl of 0.1% TTC were added to each well, incubated for 15 minutes.

Plates were read and MIC_{50} was determined by recording the concentration of the well that preceded the red color appearance (The development of red color indicate bacterial growth as shown in figure 3.1).

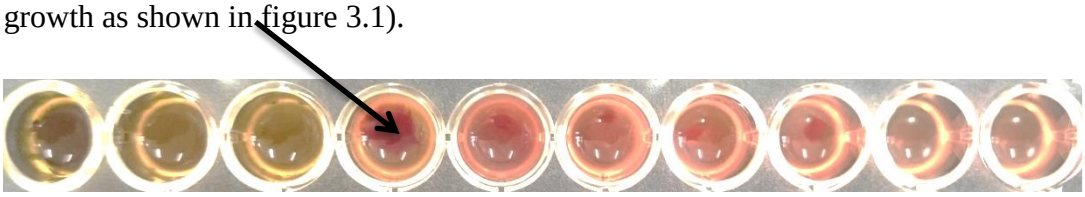

Figure (3.1): Minimum inhibitory concentration determination using microbroth dilution method with TTC as indicator.

3.2.4 Assessment of biofilm formation

3.2.4.1 Tube method

Biofilms can also form in test tubes. For this reason, 0.1 mL of bacterial culture (obtained by adjusting turbidity to 0.5 McFarland standards) was transferred to glass test tubes containing 10 mL NB test tubes which were incubated at 37°C for 72 hours. The medium was then removed and the tubes were washed with distilled water, air dried

and biofilm formation were assayed by crystal violet (Pour et al., 2011). All tests were carried out in triplicates.

3.2.4.2 Tissue culture plate method

Three wells of sterile 96-microtiter polyester U-bottomed plate were filled will 200 µl of bacterial suspension (dilution 1:100 with fresh medium). Negative control contained broth only. After incubation for 24 h at 37°C, wells were washed three times with 250 µl of DW. After 15 min, plates were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was removed and rinsed off by placing the plates under running tap water. The plates were air-dried.

The adherent cells were resolubilized with 160 µl of 95% (V/V) methanol per well (Christensen et al., 1985). The optical density (OD) of each was measured at 570 nm. Results interpreted according to the followings criteria; OD <5.00 (-), OD 0.500-1.500 (+), OD >1.500 (++) (Alcaráz, Satorres, Lucero, & Centorbi, 2003).

3.2.5 The *In Vitro* determination of the effect of plant extract on planktonic growth and biofilm formation of *P. aeruginosa*

3.2.5.1 Biofilm inhibition assay

This method described by (Musleh & Jebur, 2014), with a modification of the used media. *P. aeruginosa* from fresh agar were inoculated in BHI broth with 1% glucose and incubated for overnight at 37^{o}C in stationary condition, dilution 1 in 100 with fresh medium. 96 well U bottom tissue culture plates were filled with 0.2 ml suspension bacteria and 0.2 ml plant extract for serial dilution, plates were incubated overnight at 37^{o}C. After incubation period well was removed by tapping the plate, washed with DW to remove planktonic bacteria.

Adherent organisms in plates were stained with crystal violet (0.1% W/V) for 20 min, excess stain was rinsed off by DW and plates were kept for drying, then resuspended

each well for 200 95%(v/v) methanol, transferred to 96 well-flat bottom plates. Optical density of stained adherent bacteria were determined with a micro-ELISA reader at wavelength of 620 nm.

3.2.6 The *In Vitro* determination of the anti-quorum sensing activities of plant extracts against *P. aeruginosa*

3.2.6.1 Swarming assay

This assay was adapted from (Willis & Kinscherf, 1999). Swarm plates were prepared using 0.5% Bacto agar, 0.5% peptone, 0.2% yeast extract and 1.0% glucose per 100 ml DW. 250 µL of plant extract were added to 5 ml of semisolid agar, gently mixed and poured immediately onto the surface of a 10 ml of pre-warmed agar plate as an overlay. Five µl of a standardized inoculum is placed on the center of the plate. Similar volume was placed on the center of a control plate (overlay agar without extract) and the plates were incubated for overnight at 37^{o}C.

3.2.6.2 Pyocyanin assay

A volume of 7.5 ml of the supernatant, after centrifugion at 10,000 rpm for 10 min from stationary phase culture (~16 hours old) of *P. aeruginosa* in nutrient broth were mixed with 4.5 ml of chloroform. The mixture is vortexed for 10 seconds and then centrifuged at 10,000 rpm for 10 min. Three milliliters of the lower blue layer, containing pyocyanin and chloroform were collected and extracted with 1.5 ml of 0.2 M HCl, vortexed and centrifuged for 2 min at 10,000 rpm. The absorbance of 2 ml from the pink top layer was measured at 520 nm (Essar, Eberly, Hadero, & Crawford, 1990).

3.2.6.3 Las A Staphylolytic assay

A 30-ml volume of an overnight *S. aureus* culture was boiled for 10 min and then centrifuged for 10 min at 10,000 rpm. The resulting pellet was resuspended in 10 mM Na_2PO_4 (pH 4.5) to an OD600 of approximately 0.8.

Various plant extracts were tested for their ability to inhibit Las A staphylolytic ability of *P. aeruginosa*. A 50-µl of sub-lethal concentration of each extract was added to each tube culture containing 1 ml media inoculated with *P. aeruginosa*. A tube with no extract added served as a control for the assay. A 100-µl aliquot of *P. aeruginosa* overnight growth supernatant was added to 900 µl of *S. aureus* suspension.

The OD_{600} was determined after 0, 5, 10, 15, 20, 30, 45, and 60 min. for both the test and control tubes (A. Adonizio, Kong, & Mathee, 2008; A. L. Adonizio, 2008; Kessler, Safrin, Olson, & Ohman, 1993).

3.2.7 Antibacterial and synergistic activity of plant extract with different antibiotic against Gram-positive bacteria.

3.2.7.1 Bacterial strain and media

Multi drug resistant *S. aureus* isolated from health care workers from hospitals in Gaza strip were kindly provided by the team of Prof. Dr. Mohammad E. Shubair. The selected isolates were identified biochemically at the Microbiology Laboratory of the Islamic University-Gaza. Antibiotic susceptibility test was also performed according to CLSI Guidelines. Antibiotic disks were placed on the inoculated MH agar surface. Plates were incubated for 16–24 h at 37°C. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter (Cockerill, 2011).

3.2.7.2 Antibacterial activity test

This was carried out using the previous procedure (Agar well diffusion method). The method measures microbial growth inhibition by plant extracts of *C. zeylanicum*, *L. inermis, and P. hortorum*. Muller Hinton Agar plated were swabbed with a standardized lawn of the test organism. Holes where punched in the agar using sterile pasture pipette. 100 µL of the each of the test extract was added to a specified hole. The plates were

allowed to settle for 30 minutes at the refrigerator to allow for extract diffusion and then incubated at 37^{o}C for 24 hours. The zone of inhibition around each extract was measured and recorded.

3.2.7.3 Evaluation of synergistic activity

Standardized inoculum of the test microorganism was inoculated on the surface of Muller-Hinton agar plate. Commercial antibiotic filter paper discs of 6 mm in diameter were placed on the surface of each inoculated plate, 10 µL of plant extract were dispensed over each disc. Control discs of antibiotic alone and extract alone were also added for comparison. The plates were incubated at 37^{o}C for 24h.

The diameters of clear zones were measured and compared with that of the antibiotic alone and the extract alone (Betoni, Mantovani, Barbosa, Di Stasi, & Fernandes Junior, 2006).

Chapter IV
Results

Chapter IV

Results

This chapter presents the results of the antibacterial, antifungal, anti-biofilm and anti-quorum sensing properties of the tested plant crude extracts against *P. aeruginosa*. In addition, the synergistic effect of three plants with commercial antimicrobials against 15 *S. aureus* isolates are presented in this chapter. Both positive effects and negative effects are illustrated. The results of the synergistic effect were summarized her and the details are listed in annex.

4.1 Screening for antibacterial activity and MIC_{50} of plants crude extracts against two different isolates of *P. aeruginosa.*

The plant crude extracts used in this study showed variable antibacterial activities against *P. aeruginosa* at concentrations ranging from 142.8 to 333.3 mg/ml as crude extracts (stock solution) before dilution. The sizes of the zone of inhibition are listed in Table 4.1.

Bacterial planktonic growth inhibition by crude extracts was also observed from the color change of the 96 microtiter plate wells using the microbroth dilution assay and TTC as growth detection system. A negative control was included in each run and assumed to have 0% activity compared to the extract tested. The $PMIC_{50}$ concentration was calculated in order to determine the concentration that does not affect the microbial growth. These concentrations were used for biofilm and quorum sensing inhibition assays.

Table (4.1): Planktonic growth inhibiting activity ($PMIC_{50}$) in mg/ml effect of different plant crude extract against two isolates of *Pseudomonas aeruginosa.*

ℵ	Plant	Inhibition Zone in mm		Planktonic growth inhibition activity ($PMIC_{50}$) in mg/ml	
		P. aeruginosa **isolate 1**	***P. aeruginosa*** **Isolate 2**	***P. aeruginosa*** **Isolate 1**	***P. aeruginosa*** **Isolate 2**
1	*Cinnamomum zeylanicum*	9	9	15.6	7.8
2	*Marrubium vulgare*	0	0	41.6	41.6
3	*Tamarix aphylla*	0	8	8.9	8.9
4	*Cuminum cyminum*	0	0	83.3	41.6
5	*Pelargonium hortorum*	21	19	0.3	0.3
6	*Lawsonia inermis*	17	17	7.8	7.8
7	*Salvia officinalis*	0	10	19.8	19.8
8	*Triticum aestivum*	0	0	12.5	6.2
9	*Artemisia absinthium*	0	0	17.8	17.8
10	*Pelargonium graveolens*	17	17	15.6	3.9
11	*Foeniculum vulgare*	0	8	83.3	83.3
12	*Hibiscus sabdariffa*	22	22	0.6	0.6
13	*Solenostemon scutellarioides*	0	0	10.4	10.4
14	*Rosmarinus officinalis*	13	8	4.4	17.8
15	*Urtica dioica*	0	0	10.4	20.8
16	*Thymus vulgaris*	0	0	20.8	20.8
17	*Punica granatum*	21	11	1.3	1.3
18	*Agave sisalana*	0	0	20.8	20.8
19	*Mentha longifolia*	0	0	20.8	10.4
20	*Portulaca oleracea*	0	0	20.8	20.8
21	*Matricaria recutita*	0	0	20.8	20.8
22	*Erythrina crista-galli*	0	0	17.8	17.8
23	*Momordica charantia*	0	0	20.8	20.8

The ethanolic crude extract of eleven plant showed various degrees of antibacterial activity against *P. aeruginosa* ranged between 8-22 mm inhibition zone and 0.3 to 83.3 mg/ml Planktonic growth inhibition activity ($PMIC_{50}$).

Antibacterial activities were observed against two isolates of *P. aeruginosa* for C. zeylanicum , *H. sabdariffa, P. hortorum, L. inermis, P. graveolens,* and *R. officinalis, P. granatum* (Figure 4.1). The largest zone of inhibition was observed for *H. sabdariffa* with inhibition zone diameter of 22 mm for both isolates of *P. aeruginosa*. At the

highest concentration tested for *S. officinalis, T. aphylla,* and *F. vulgare* observed that affected the second isolate of *P. aeruginosa* only with no inhibitory zone against the first isolate of *P. aeruginosa.*

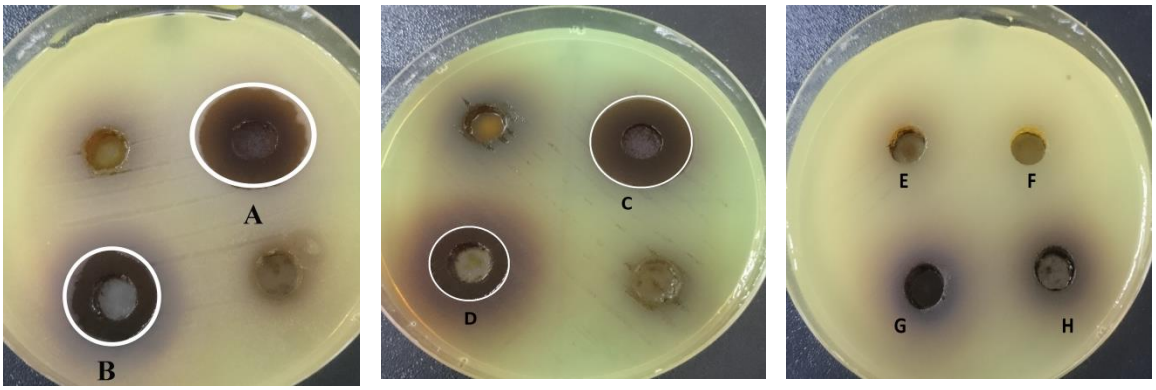

Figure (4.1): Antimicrobial activities against *Pseudomonas aeruginosa* isolate 2, (A) *Hibiscus sabdariffa,* (B) *Punica granatum,* (E) *Mentha longifolia,* (F) *Matricaria recutita,* (H) *Portulaca oleracea* ,and (G) *Solenostemon scutellarioides* extracts. Antimicrobial activities against *Pseudomonas aeruginosa* isolate 1, (C) *Punica granatum,* (D) *Hibiscus sabdariffa* extracts.

The highest planktonic growth inhibition activity ($PMIC_{50}$) in mg/ml against *P. aeruginosa* for *P. hortorum, H. sabdariffa* and *P. granatum* was 0.3 mg/ml, and 0.6 mg/ml, 1.3 mg/ml respectively. The lowest activity was exhibited by *F. vulgare* 83.3 mg/ml. As shown in Table 4.1, the highest $PMIC_{50}$ of *P. hortorum* against of *P. aeruginosa* was 0.3 mg/ml, however, it did not produce the highest inhibitory zone. As also shown in Figure 4.1, *P. aeruginosa* was not affected (no inhibitory zone) by some of plant extract like *P. oleracea, M. recutita, E. crista-galli, M. charantia* but when microdilution technique was used, growth inhibition was observed.

P. hortorum, H. sabdariffa, P. granatum, R. officinalis, L. inermis, T. aphylla, S. scutellarioides, and *U. dioica* (Figure 4.2) presented the highest $PMIC_{50}$ activity respectively at concentrations ranging from 0.3 to 10.4 mg/ml against *P. aeruginosa* isolate 1.

However, different activities against *P. aeruginosa* isolate 2 were observed. The inhibition activity at the same concentration showed different order. *P. hortorum, H. sabdariffa* Figure (4.2), *P. granatum P. graveolens, T. aestivum, C. zeylanicum , L. inermis, M. longifolia*, and *S. scutellarioides* respectively (Figure 4.3).

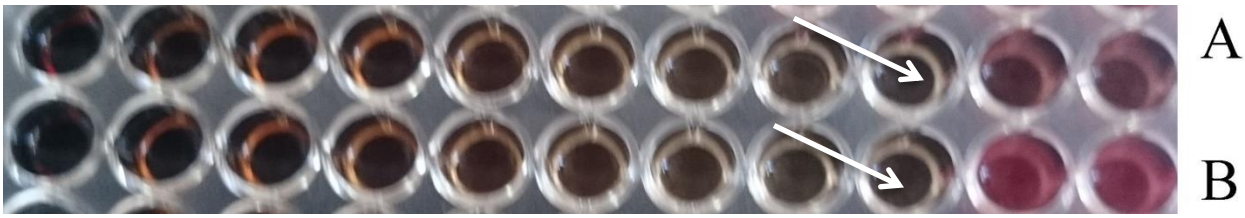

Figure (4.2): Antimicrobial activities against *Pseudomonas aeruginosa* $PMIC_{50}$, (A) *Hibiscus sabdariffa* extract against *P. aeruginosa* isolate 1, (B) *Hibiscus sabdariffa* extract against *P. aeruginosa* isolate 2.

The lowest effect was produced by *M. vulgare* and *C. cyminum* at concentration 41.6 mg/ml against *P. aeruginosa* isolate 2, *C. cyminum* and *F. vulgare* at concentration 83.3 mg/ml against *P. aeruginosa* isolate 1. *E. crista-galli* showed intermediate effect at concentration 17.8 mg/ml for two isolate *P. aeruginosa.*

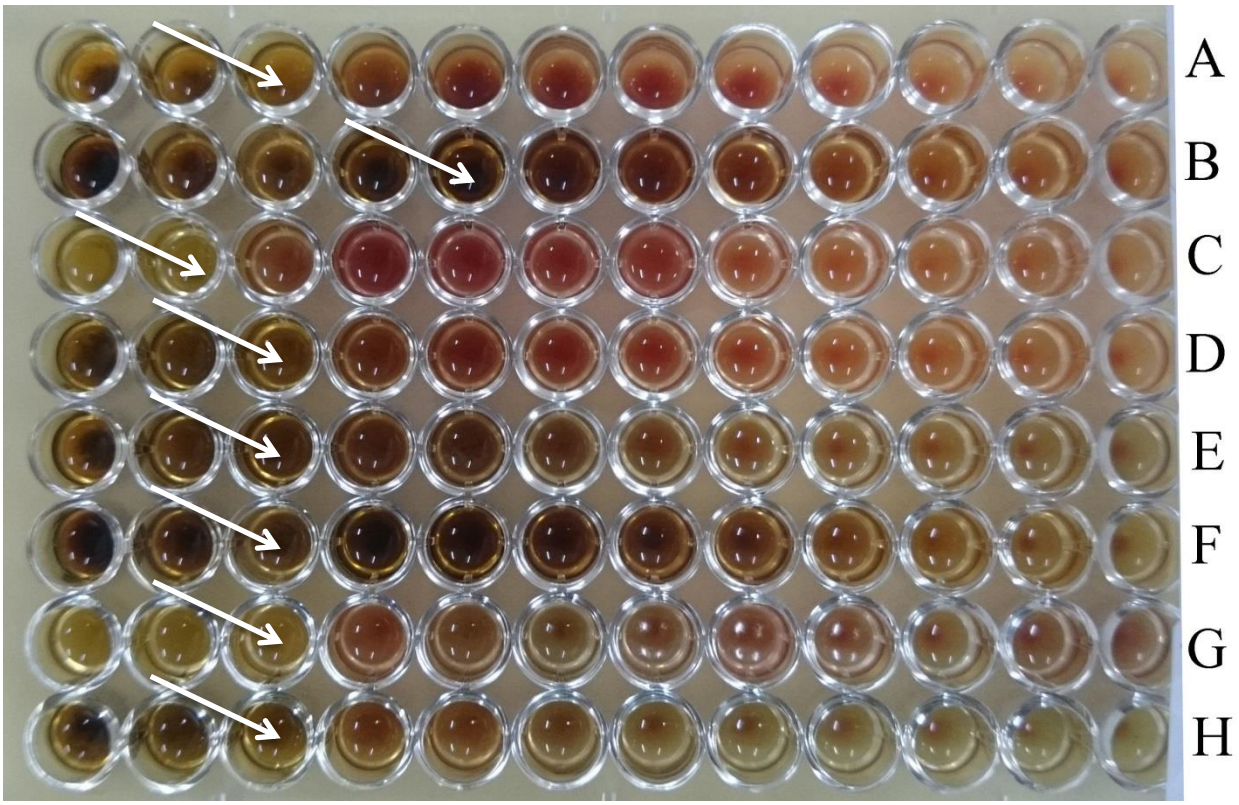

Figure (4.3): Antimicrobial activities against *Pseudomonas aeruginosa* $PMIC_{50}$ activity, from (A to D) for *P. aeruginosa* isolate 2. From (E to H) for *P. aeruginosa* isolate 1 *T. vulgaris, P. granatum, A. sisalana,* and *M. longifolia* extracts respectively.

4.2 Screening and determination of $PMIC_{50}$ plant crude extract for *Candida albicans.*

The screening results for anticandidal activity and determination of $PMIC_{50}$ of plant extract obtained by microdilution method are shown in Table 4.2. Majority of plant extracts tested were found to have a varying degree of anticandidal activity and only three of the extracts (*T. aphylla, M. vulgare,* and *C. cyminum*) failed to inhibit *C. albicans* growth at the highest concentration tested (stock solution).

The anticandidal activity of the crude extract of the tested plants showed zones of inhibition ranging from 0-52 mm. The extract of *C. zeylanicum* exhibited the highest

activity with inhibition zone diameter of 52 mm, followed by *P. granatum* 26 mm, *H. sabdariffa* 24 mm, *P. hortorum* 23 mm, *P. graveolens* 20 mm (figure 4.4).

U. dioica and *M. recutita* have the same activity against *C. albicans* with inhibition zone diameter of 19 mm , *S. scutellarioides, M. longifolia, T. aestivum, M. charantia, T. vulgaris, F. vulgare* which have intermediate activity against *C. albicans* with inhibition zone dimeter of 17, 16, 14, 13 mm respectively. The lowest activity was recorded for *A. absinthium, E. crista-galli* with an inhibition zone diameter of 11 mm, *M. vulgare, T. aphylla*, while *C. cyminum* showed no effect.

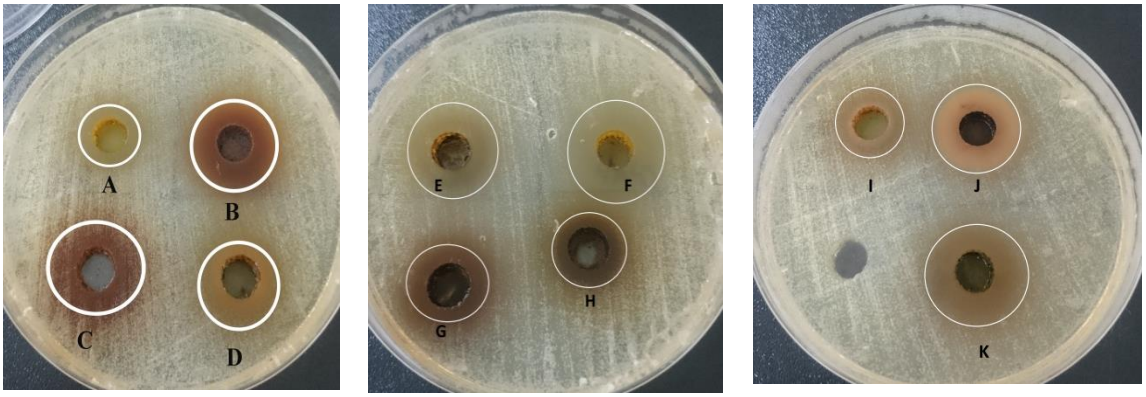

Figure (4.4): Anticandidal activity against *Candida albicans.* (A) *Thymus vulgaris*, (B) *Hibiscus sabdariffa*, (C) *Foeniculum vulgare*, (D) *Lawsonia inermis*, (E) *Rosmarinus officinalis*, (F) *Matricaria recutita*, (G) *Agave sisalana*, (H) *Punica granatum*, (I) *Erythrina* crista-galli, (J) *Pelargonium graveolens*, and (K) *Urtica dioica* extracts.

Table 4.2 showing the highest growth inhibition activity $PMIC_{50}$ against *C. albicans* of the plant extract of *P. graveolens, P. hortorum, C. cyminum, C. zeylanicum, R. officinalis, H. sabdariffa, T. aestivum, T. aphylla*, and *M. charantia* respectively at concentrations ranging from 0.02-2.6 mg/ml.

Table (4.2): Planktonic growth inhibiting activity ($PMIC_{50}$) in mg/ml of different plant crude extracts against *Candida albicans.*

No.	Plant	Stock solution Concentration in mg/ml	Inhibition Zone in mm	$PMIC_{50}$ in mg/ml
1	*Cinnamomum zeylanicum*	250	52	0.2
2	*Marrubium vulgare*	333.3	0	20.8
3	*Tamarix aphylla*	142.8	0	2.2
4	*Cuminum cyminum*	333.3	0	0.12
5	*Pelargonium hortorum*	166.6	23	0.03
6	*Lawsonia inermis*	250	12	7.8
7	*Salvia officinalis*	158.7	12	9.8
8	*Triticum aestivum*	200	14	1.5
9	*Artemisia absinthium*	142.8	11	8.9
10	*Pelargonium graveolens*	250	20	0.02
11	*Foeniculum vulgare*	333.3	13	10.4
12	*Hibiscus sabdariffa*	166.6	24	1.3
13	*Solenostemonscutellarioides*	166.6	17	10.4
14	*Rosmarinus officinalis*	142.8	21	1.1
15	*Urtica dioica*	166.6	19	10.4
16	*Thymus vulgaris*	166.6	13	10.4
17	*Punica granatum*	166.6	26	10.4
18	*Agave sisalana*	166.6	17	20.8
19	*Mentha longifolia*	166.6	16	20.8
20	*Portulaca oleracea*	166.6	12	10.4
21	*Matricaria recutita*	166.6	19	10.4
22	*Erythrina crista-galli*	142.8	11	17.8
23	*Momordica charantia*	166.6	13	2.6

4.3 Assessment of biofilm formation.

4.3.1 Tube method.

Strong biofilm formation by *P. aeruginosa* isolate 2 compared with control as observed by the heavy crystal violate pigment remaining on the test tube.

4.3.2 Screening of the *P. aeruginosa* isolate for biofilm formation by tissue culture plate.

Figure 4.5 is showing the quantitative measurements of adherent biofilm stained by crystal violate dye using the 96 microtiter plates which is read by an ELISA reader. *P. aeruginosa* isolate 2 with absorbance more than 0.5 compared with control OD <0.005.

Figure (4.5): Anti-biofilm activity of plant extracts using the crystal violet assay.

4.4 *In Vitro* effect of crude extract of Plants on Biofilm formation of *Pseudomonas aeruginosa*.

In addition to testing of the plant extracts for inhibition of planktonic growth, their effect on biofilm formation was also investigated. To ensure a concentration that is not affecting the microbial growth, extract concentrations below (sub-$PMIC_{50}$) for anti-biofilm assay were used. Crystal violate staining method is easily and widely used to measure both the formation and inhibition of biofilms. *P. hortorum* ethanol extract concentration of 0.005 mg/mL is the lowest concentration, which showed 85.7% inhibition on *P. aeruginosa* biofilm formation (Table 4.3).

Eleven of the 23 tested extracts inhibited *P. aeruginosa* biofilm formation by ≥ 50%. The ethanolic extract of *A. sisalana* (Figure 4.6) inhibited *P. aeruginosa* biofilm

formation by 87.5%. A 60% inhibition of *P. aeruginosa* biofilm formation was obtained by the extract of *C. zeylanicum* and *T. vulgaris.* As much as 85.7% and 69.2% by *P. hortorum* and *L. inermis* respectively.

The extract of *M. vulgare* and *T. aphylla* inhibited *P. aeruginosa* biofilm formation by 53.3%. Reduction by 84.6%, 81.2%, 66.6%, and 61.5% of *P. aeruginosa* biofilm formation were obtained by the extract of *H. sabdariffa, M. longifolia*, *P. oleracea*, and *P. granatum* respectively (Figure 4.6, table 4.3).

A concentration of 0.005 mg/mL for two extracts obtained from *P. hortorum* and *P. granatum* respectively showed 85.7% and 61.5% of biofilm reduction of *P. aeruginosa* (Table 4.3). *H. sabdariffa* showed the ability to reduce the *P. aeruginosa* biofilm forming ability by 84.6%, at a concentration of 0.01 mg/mL.

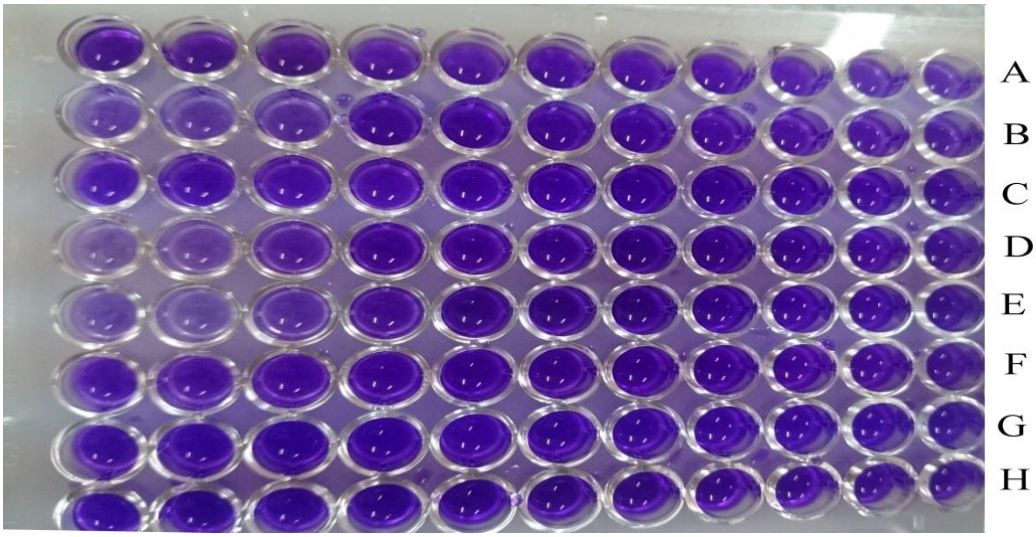

Figure (4.6): Anti-biofilm activities against *Pseudomonas aeruginosa*, crystal violate staining method. (A) *Momordica charantia*, (B) *Thymus vulgaris*, (C) *Punica granatum*, (D) *Agave sisalana*, (E) *Mentha longifolia*, and (H) Negative control.

Four out of the 23 extracts reduced *P. aeruginosa* biofilm formation by <50% (i.e. *C. cyminum, S. officinalis, T. aestivum* and *A. absinthium*) at concentration 0.1, 0.05, 1.5 and 2.2 mg/mL respectively.

At the sub lethal concentrations, *P. graveolens, F. vulgare, S. scutellarioides, R. officinalis, U. dioica, M. recutita*, and *M. charantia* (Figure 4.6, table 4.3) failed to inhibit biofilm formation.

Table (4.3): Effects of ethanolic extracts on biofilm formation and biofilm inhibition of *Pseudomonas aeruginosa* isolate 2.

N	Plant	Sub-PMIC$_{50}$ in mg/Ml	(MBIC$_{50}$) in mg/mL	%Reduction
1	*Cinnamomum zeylanicum*	3.9	0.1	60
2	*Marrubium vulgare*	20.8	0.6	53.3
3	*Tamarix aphylla*	4.4	0.5	53.3
4	*Cuminum cyminum*	20.8	0.1	46.1
5	*Pelargonium hortorum*	0.1	0.005	85.7
6	*Lawsonia inermis*	3.9	0.1	69.2
7	*Salvia officinalis*	9.9	0.05	40
8	*Triticum aestivum*	3.1	1.5	20
9	*Artemisia absinthium*	8.9	2.2	25
10	*Pelargonium graveolens*	1.9	-	-
11	*Foeniculum vulgare*	41.6	-	-
12	*Hibiscus sabdariffa*	0.3	0.01	84.6
13	*Solenostemon scutellarioides*	5.2	-	-
14	*Rosmarinus officinalis*	8.4	-	-
15	*Urtica dioica*	10.4	-	-
16	*Thymus vulgaris*	10.4	0.6	60
17	*Punica granatum*	0.6	0.005	61.5
18	*Agave sisalana*	10.4	0.3	87.5
19	*Mentha longifolia*	5.2	0.3	81.2
20	*Portulaca oleracea*	10.4	1.3	66.6
21	*Matricaria recutita*	10.4	-	-
22	*Erythrina crista-galli*	8.9	-	-
23	*Momordica charantia*	10.4	-	-

* (-); no activity detected within the concentration range tested, MBIC (Minimum Biofilm Inhibition Concentration).

4.5 Screening for anti-quorum sensing assay

Three anti quorum sensing assays were performed to test the crude extracts of 22 plants. The following section presents the results of these assays.

4.5.1 Screening for anti-quorum sensing assay using swarming method

We investigated if the extracts can inhibit quorum sensing through inhibiting the motility in the human opportunistic pathogen *P. aeruginosa*. The results are shown in figures (4.7- 4.9) and Table (4.4).

Extracts of *P. hortorum, P. granatum, T. aphylla, H. sabdariffa, P. graveolens,* and *R. officinalis* exhibited the highest reduction in the swarming motility of *P. aeruginosa* isolate 1 at concentration respectively below $PMIC_{50}$ (sub-$PMIC_{50}$) (Figure 4.7 and 4.8). The activity was divided into four groups; Negative (no activity at the sublethal concentration), low activity (one dilution), moderate activity (2-4 dilutions) and high activity (more than 4 dilutions).

At the sublethal concentrations, the following plant extract (*M. charantia, M. recutita, A. sisalana, T. vulgaris, S. officinalis* and *U. dioica, L. inermis* and *M. longifolia*) failed to reduce the swarming motility of *P. aeruginosa* isolate 1 as shown figure (4.8).

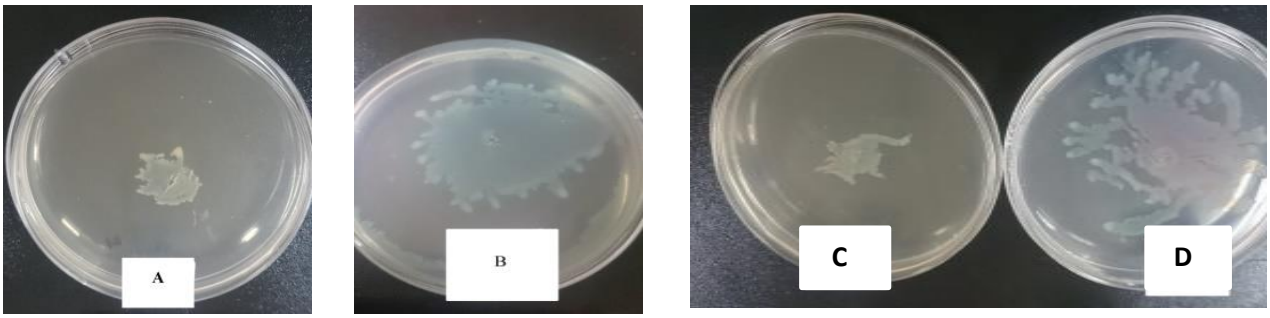

Figure (4.7): Anti quorum sensing activities against *Pseudomonas aeruginosa* isolate 1 swarming motility. (A) *Hibiscus sabdariffa* (0.1 mg/ml), (B) Negative control, (C) *Punica granatum* (0.04 mg/ml), (D) Negative control.

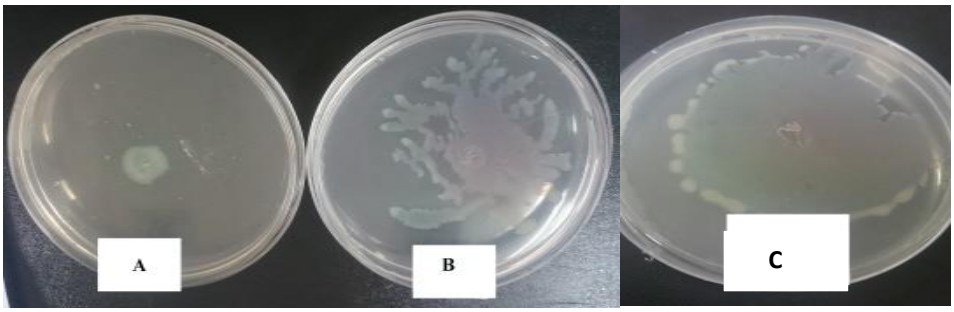

Figure (4.8): Anti-quorum sensing activities against *Pseudomonas aeruginosa* isolate 1 swarming motility. (A) *Pelargonium graveolens* extract (0.9 mg/ml), (B) Negative control, (C) *Momordica charantia* extract.

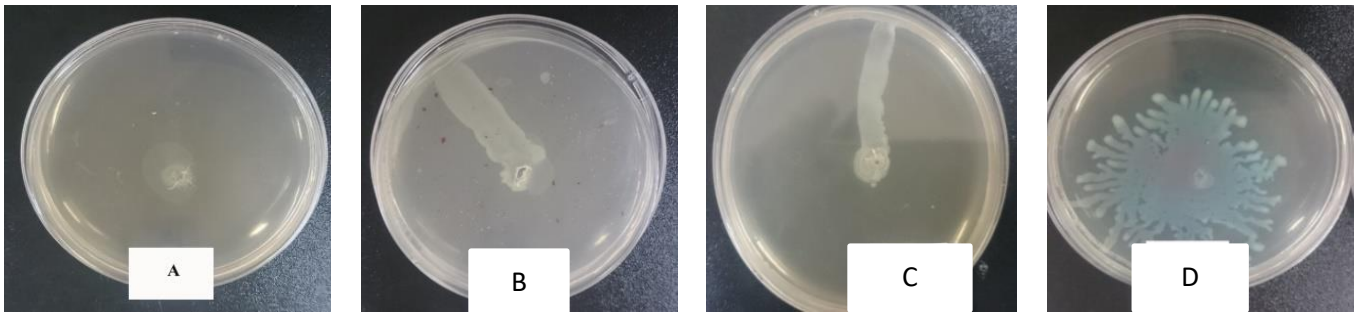

Figure (4.9): Anti-quorum sensing activities against *Pseudomonas aeruginosa* isolate 2 swarming motility. (A) *Pelargonium hortorum* (0.6 mg/ml), (B) *Cinnamomum zeylanicum* (0.9 mg/ml), (C) *Punica granatum* (0.6 mg/ml), (D) Negative control.

C. zeylanicum, T. aestivum, M. vulgare and, *S. scutellarioides* showed low activity against *P. aeruginosa* isolate 1 (only at the first concentration below $PMIC_{50}$) at concentration from 2.6 to 3.9 mg/ml, but *A. absinthium, P. oleracea,* and *M. vulgare* exhibited activities at concentration 4.4, 5.2, 10.4 mg/ml respectively.

The swarming motility of *P. aeruginosa* isolate 1 was also reduced with moderate effect by *F. vulgare* at concentration 5.2 mg/ml after three concentration from the sub-$PMIC_{50}$. As much as 12 out of 22 plant extracts tested failed to inhibit motility of *P. aeruginosa* isolate 2 as shown in Table 4.4.

The highest effect on motility inhibition was for *P. granatum* (con. 0.005 mg/ml), followed by *P. hortorum* (con. 0.02 mg/ml), followed by *P. graveolens* (con. 0.4 mg/ml) against *P. aeruginosa* isolate 2 as shown in figures 4.7 and 4.9. *P. hortorum* showed similar effects on the two *P. aeruginosa* strains test at concentration 0.2 mg/ml.

Table (4.4): Anti-quorum sensing activity of plants crude extracts by plate swarming method.

N	Plant	Concentration(mg/ml) at which motility of *Pseudomonas aeruginosa* was inhibited		Number of dilutions	
		Isolate 1	Isolate 2	Isolate 1	Isolate 2
1	*Cinnamomum zeylanicum*	3.9	0.9	2	3
2	*Marrubium vulgare*	10.4	-	2	-
3	*Tamarix aphylla*	0.06	1.1	7	3
4	*Cuminum cyminum*	10.4	10.4	3	2
5	*Pelargonium hortorum*	0.02	0.02	4	4
6	*Lawsonia inermis*	-	-	-	-
7	*Salvia officinalis*	-	-	-	-
8	*Triticum aestivum*	3.1	1.5	2	2
9	*Artemisia absinthium*	4.4	4.4	2	2
10	*Pelargonium graveolens*	0.2	0.4	6	3
11	*Foeniculum vulgare*	5.2	-	4	-
12	*Hibiscus sabdariffa*	0.08	0.1	3	2
13	*Solenostemon scutellarioides*	2.6	-	2	-
14	*Rosmarinus officinalis*	0.5	1.1	3	4
15	*Urtica dioica*	-	-	-	-
16	*Thymus vulgaris*	-	-	-	-
17	*Punica granatum*	0.04	0.005	5	8
18	*Agave sisalana*	-	-	-	-
19	*Mentha longifolia*	-	-	-	-
20	*Portulaca oleracea*	5.2	-	2	-
21	*Matricaria recutita*	-	-	-	-
22	*Momordica charantia*	-	-	-	-

*(-) represents that first Sub-$PMIC_{50}$ which did not inhibit *P. aeruginosa* motility. *Erythrina crista-galli:* extract was not tested because of limited amount.

4.5.2 Pyocyanin production quantitative assay

The inhibition of quorum sensing activity in the form of altered pyocyanin production by plant crude extracts is shown in figure (4.10) and in Table (4.5). The reducuction of the green colored (pyocyanin) pigment in *P. aeruginosa* is an indication of anti-QS activity by the added plant ethanol extracts.

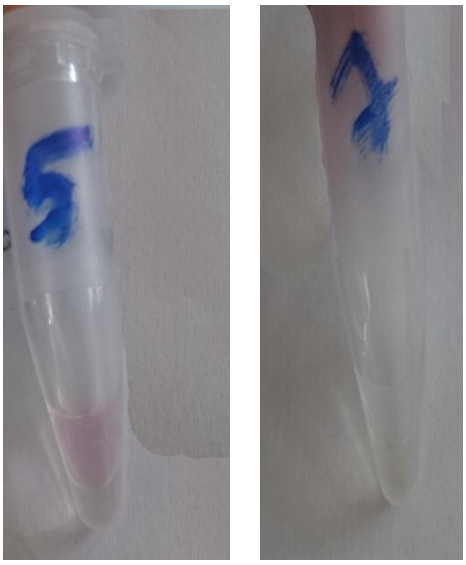

Figure (4.10): Anti-quorum sensing activities against *Pseudomonas aeruginosa*. Pyocyanin production quantitative assay (5), Negative control (7).

Out of 22 crude plant extracts screened for anti-quorum sensing activity, two plant (*P. graveolens* and *S. scutellarioides*) failed to exhibit anti-QS activity, while *P. graveolens* enhanced green pyocynin pigment production compared to the control.

All 20 active extracts showed variable anti-QS activity against *P. aeruginosa* ability to produce pyocyanin. The concentrations used in this assay are not the same since the sublethal concentration of each extract was selected. The results therefore are expressed as percentage reduction regardless of the concentration.

H. sabdariffa and *A. absinthium* were the most effective showing the highest anti-QS activity against *P. aeruginosa* with a 47.8% inhibition followed by *C. zeylanicum, S. officinalis, U. dioica* and *T. aestivum* (44.3%) inhibition. The lowest anti-QS activity for *M. recutita* (6.8%), *R. officinalis*, and *M. charantia* (10.2%).

Table (4.5): Anti-quorum sensing activity of plants ethanol extracts by quantitative pyocyanin production method for *Pseudomonas aeruginosa* isolate 1 only at first concentration of Sub-$PMIC_{50}$ in mg/mL.

N	Plant	Sub-$PMIC_{50}$ in mg/ml	%Reduction
1	*Cinnamomum zeylanicum*	7.8	44.3
2	*Marrubium vulgare*	20.8	42.6
3	*Tamarix aphylla*	4.4	15.3
4	*Cuminum cyminum*	41.6	32.4
5	*Pelargonium hortorum*	0.1	11.9
6	*Lawsonia inermis*	3.9	39.2
7	*Salvia officinalis*	9.9	44.3
8	*Triticum aestivum*	6.2	44.3
9	*Artemisia absinthium*	8.9	47.8
10	*Pelargonium graveolens*	7.8	-119.5
11	*Foeniculum vulgare*	41.6	42.6
12	*Hibiscus sabdariffa*	0.3	47.8
13	*Solenostemon scutellarioides*	5.2	0
14	*Rosmarinus officinalis*	2.2	10.2
15	*Urtica dioica*	5.2	44.3
16	*Thymus vulgaris*	10.4	11.9
17	*Punica granatum*	0.6	13.6
18	*Agave sisalana*	10.4	20.4
19	*Mentha longifolia*	10.4	32.4
20	*Portulaca oleracea*	10.4	23.9
21	*Matricaria recutita*	10.4	6.8
22	*Momordica charantia*	10.4	10.2

4.5.2 Las A protease activity in the presence of plant extract

Plant extracts showed variable abilities in reducing the las A protease activity of *P. aeruginosa* **(**Table 4.6). *M. charantia* showed the greatest reduction in activity (77.1%), followed by *M. longifolia* (66.8%), *A. sisalana* (65.2%), *T. vulgaris* (63.3%), *T. aphylla* (63%), The least reduction in activity was observed with *H. sabdariffa*, *T. aestivum*, and *L. inermis*. In fact, some plant extract has increased the Las A protease activity such as *C. cyminum*, and *S. officinalis*.

Table (4.6): Anti-quorum sensing activity of plants ethanol extracts by quantitative Las A protease activity method for *Pseudomonas aeruginosa* number 1 only at first concentration of Sub-PMIC$_{50}$ in mg/mL.

N	Plant extract	Absorbance at 620 nm		% Reduction of Las A protease activity as compared to control
		P. aeruginosa		
		0 min	45 min	
1	*Cinnamomum zeylanicum*	0.55	0.46	22.9
2	*Marrubium vulgare*	0.55	0.49	48.0
3	*Tamarix aphylla*	0.55	0.51	63.0
4	*Cuminum cyminum*	0.67	0.56	-67.9
5	*Pelargonium hortorum*	0.57	0.52	59.8
6	*Lawsonia inermis*	0.59	0.54	9.4
7	*Salvia officinalis*	0.57	0.43	-18.0
8	*Triticum aestivum*	0.58	0.47	8.8
9	*Artemisia absinthium*	0.54	0.48	52.4
10	*Pelargonium graveoles*	0.56	0.49	33.6
11	*Foeniculum vulgare*	0.54	0.48	44.8
12	*Hibiscus sabdariffa*	0.44	0.39	0.0
13	*Solenostemon scutellarioides*	0.41	0.38	33.7
14	*Rosmarinus officinalis*	0.43	0.40	55.2
15	*Urtica dioica*	0.42	0.39	33.0
16	*Thymus vulgaris*	0.59	0.56	63.3
17	*Punica granatum*	0.62	0.59	58.3
18	*Agave sisalana*	0.62	0.60	65.2
19	*Mentha longifolia*	0.63	0.60	66.8
20	*Portulaca oleracea*	0.46	0.42	20.2
21	*Matricaria recutita*	0.41	0.37	30.6
22	*Momordica charantia*	0.62	0.60	77.1

4.6 Antibacterial and synergistic activity of different plant extracts with different antimicrobials against *S. aureus*.

The crude extract of three plants (*C. zeylanicum, L. inermis*, and *P. hortorum*) showed various degrees of antibacterial activity against *S. aureus* ranged from 8-20 mm in diameter of inhibition zones (Table 4.7). They also exhibited variable synergistic activities when tested with commercially available antibiotics to inhibit the growth of *S. aureus* (Table 4.8).

P. hortorum showed inhibition activity against all *S. aureus* isolates except isolate 7. The highest antibacterial activity for *P. hortorum* was against *S. aureus* isolate 6 and isolate 15 (Figures 4.11) with inhibition zone diameter of 20 mm, followed by isolate 8 with inhibition zone diameter of 19 mm, followed by isolates 2-4 with inhibition zone diameter of 18 mm (Table 4.7). The overall effect of *P. hortorum* as measured by the average zone of inhibition on all tested isolate was the highest.

Table (4.7): Planktonic growth inhibiting activity of different plant crude extract against *Staphylococcus aureus*.

S. aureus Isolate	Zone of inhibition mm		
	Cinnamomum zeylanicum	*Lawsonia inermis*	*Pelargonium hortorum*
1	0	12	17
2	0	0	18
3	0	11	18
4	13	0	18
5	0	11	16
6	0	0	20
7	12	0	0
8	13	0	19
9	0	0	16
10	8	9	17
11	8	8	15
12	10	8	16
13	0	10	16
14	0	7	12
15	9	14	20
Average	5	6	16

Table (4.8) illustrate the synergistic effect of plant extract against *S. aureus*. The three crude extracts, (i.e. *C. zeylanicum, L. inermis,* and *P. hortorum*) were added in a volume of 10 µL/disc with a concentration of 250, 250, 166.6 mg/ml respectively.

The results in Table (4.8) showed that, *C. zeylanicum* had synergistic effect with most of the tested antimicrobials, followed by *L. inermis*, and by *P. hortorum*. *S. aureus* isolate number five showed the highest resistance among all other isolates (E, TM, NOR, OX,

FOX, P, CLR, CN) (annex 1). *P. hortorum* extract, had a synergistic effect with P, OX, E, FOX, NOR, TM against that isolate. The highest synergistic inhibitory effect was observed with CN, CLR when *C. zeylanicum* extract was used, while the highest synergistic activity with FOX was with *P. hortorum* with inhibition zone of 21 mm for isolate 5 (Figures 4.11). The details of the synergistic activity testing is found in Annex 1.

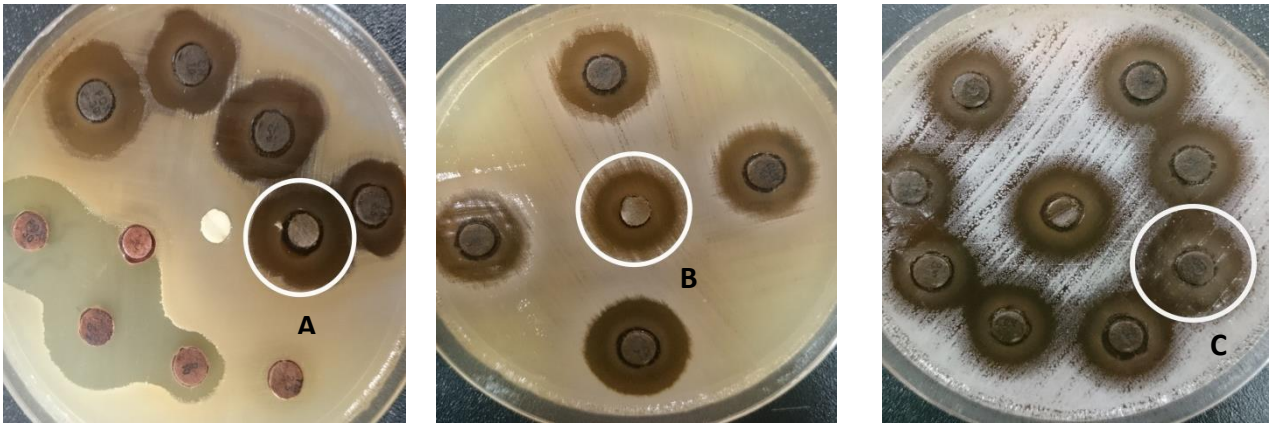

Figure (4.11): Antimicrobial activity against *Staphylococcus aureus.* (A) *S. aureus* isolate 6, (B) *S. aureus* isolate 15, (C) synergistic effect *Pelargonium hortorum* with FOX (Cefoxitin) against that *S. aureus* isolate 5

Table (4.8): Synergistic activity of different plant extracts with different antimicrobials against *Staphylococcus aureus*.

***S. aureus* isolate**	**Antimicrobials tested with**		
	Cinnamomum zeylanicum	***Lawsonia inermis***	***Pelargonium hortorum***
1	-	-	-
2	CN, P	CN	-
3	P, CN	-	P
4	-	E, CLR, P,	-
5	CLR, CN, FOX	CLR, FOX	E, TM, NOR, OX, FOX, P
6	CN, P	TM, CN	P
7	P, FOX, CLR, CN	FOX, CLR, CN	FOX, CLR, CN,
8	C, CN	C, CN	-
9	E, CLR, P	E, CLR	-
10	E, CLR	-	-
11	-	CLR, E, TM	-
12	FOX, P	FOX, P	-
13	CLR, P, CPT	CPT	CPT
14	P, CN	CN	-
15	CLR	CLR	-

(CN: Getamicin, P: Penicillin G, E: Erythromycin, CLR: Clarithromycin, FOX: Cefoxitin, TM: Timethoprim, NOR: Norfloxacin, OX: Oxacillin, CPT: Ceftaroline).

Chapter V
Discussion

Chapter V

Discussion

The increase of microbial resistance to antibiotics created several problems. The severity of multidrug resistance issue is aggravated by the fact that there is no new antibiotics being discovered. The scientific and medical community should turn their efforts to find alternatives for antibiotics. One of the most common approaches is the search for the easiest and the safest to human, which is medicinal plants. Plant extracts do not pose selective pressure issues, and it will unlikely cause resistance problems.

One reason for choosing plants is that they are readily available (Samie, Obi, Bessong, & Namrita, 2005). Of the entire world flora, 250,000 species have been identified and used for curative purposes (Patwardhan, Warude, Pushpangadan, & Bhatt, 2005). This number represents only 15% of those species that have been effectively investigated and found useful (Okeke et al., 2005). Consequently, there is a staggering over 85% of higher plants, which are yet to be investigated.

During this study, twenty three plants were collected from different agricultural areas or purchased from Gaza`s local markets. The extracts of these plants were obtained by soxhlet apparatus in an ethanol solution (70%). The crude ethanol extracts were obtained after filtration and evaporation. Crude extracts of the plants may have mixtures of active compounds which act synergistically and their overall bioactivity is usually greater than individual compounds (Chen, Isman, & Chiu, 1995).

Bioactivity of plants extract significantly varied based on the solvents used for extraction, and depends on geographical source (Cervenka et al., 2006), harvest time (McGimpsey, Douglas, Van Klink, Beauregard, & Perry, 1994), storage conditions, soil conditions, drying method, and rout extract (Bernard et al., 2014). The benefit of plant properties against microorganisms can only be achieved by using a specific solvent and solvent concentration in extracting the plant materials.

In our study, ethanol was selected as the only extraction solvent, because ethanol is relatively safe for human as compared with other organic solvents, such as acetone or methanol, readily available and less expensive. Further, ethanol extraction is widely used to obtain crude extracts of phytochemicals from plant materials in the herbal medicine industry for therapeutic applications (Wendakoon, Calderon, & Gagnon, 2012). The concentration of the solvent could affect the antibacterial activity and other activities. It is necessary to select the appropriate solvent and its concentration.

The methods used to assay plant extracts were selected based on the availability of the test bacteria. For anti-quorum sensing, swarming assay, pyocyanin production quantitative assay, Las A Staphylolytic assay. For anti-biofilm activity, biofilm inhibition assay was used because these methods are suitable for *S. aureus* and *P. aeruginosa.* The concentration of the stock solution of plant extracts ranged from 142.8 - 333 mg/mL were used for testing. This wide range is due to variability of the solubility of various plant extracts in DMSO or DW (Abah & Egwari, 2011).

Although several studies are conducted to investigate the biological activities of plants, most of these determined the antibacterial or antifungal effects. In this study, the focus to determine the anti-QS and anti-biofilm to explore the possibility of finding out new methods for preventing or treatment infectious disease.

5.1 Antibacterial and Antifungal activity of plant crude extract against two different species of *P. aeruginosa* and *C. albicans*.

To determine the antimicrobial and antifungal activity of medicinal plant extract, agar well diffusion method and minimum inhibitory concentration assays were used. Many studies have tested the antifungal effect through agar diffusion or dilution methods (Bhalodia & Shukla, 2011; Dellavalle et al., 2011). In this study, the MIC was selected to test for antifungal activities of plant extracts by micro broth dilution method because it provides quantitative results and is considered by many as the most appropriate and reliable method (Sigei, Muturi, & Bii, 2015).

C. zeylanicum has been studied for its antibacterial activity against *P. aeruginosa*, *C. albicans*, and muli drug resistance *S. aureus*. The activity of *C. zeylanicum* extracts to inhibit the above mentioned microorganisms has been reported previously (Budri et al., 2015).

Our study provided evidence that extract of *C. zeylanicum* possesses significant antibacterial and antifungal activity against *P. aeruginosa*, *C. albicans*, and multi drug resistance *S. aureus*. This activity may be due to the major components for *C. zeylanicum* which include cinnamaldehyde and eugenol (Budri et al., 2015).

Similar results were obtained by Keskin & Toroglu (2011), and they were compatible with the result obtained by Utchariyakiat, Surassmo, Jaturanpinyo, Khuntayaporn, & Chomnawang (2016), where they showed a strong antibacterial activity of *C. zeylanicum* against *P. aeruginosa*. In addition, the results in our study are in agreement with the result obtained by Krishnan & Nair (2016), Pratiwi (2015), which showed that the extract of *C. zeylanicum* had higher inhibitory effect against *P. aeruginosa*. In relation to antifungal activity against *C. albicans*, a study by Castro & Lima (2013), showed MIC_{50} values ranging between 0.3 and 0.6 mg/mL. These results differ from the results of this study, which showed higher inhibitory effect at 0.2 mg/mL.

T. vulgaris extract showed low antibacterial activity against *P. aeruginosa*. Nagy (2010), obtained similar result.

Study by Sulaiman et al., (2014), demonstrated that *H. sabdariffa* extracts tested against *P. aeruginosa* at concentration of 25, 50, 100, 200 mg/mL with diameter of zone of inhibition 9, 11, 12.5, 17 mm respectively. The result obtained by Tolulope (2007), are in agreement with our study, however our study showed slightly higher antibacterial activity of *H. sabdariffa* against *P. aeruginosa.* Similar result obtained by Elmanama, Alyazji, & Abu-Gheneima (2011). Similar effects of *H. sabdariffa* against *C. albicans* were found in a study by Alshami & Alharbi (2014), with MIC_{50}s values ranged from 0.5 to 2.0 mg/mL.

This high potency of *H. sabdariffa* against these bacteria provides the scientific basis for its wide spread use in folk medicine in the treatment of many diseases (Morton, 1987). This may be attributed to the fact that it contains active components (alkaloids, flavonoids, phenolics, and biterpenoids) (Islam et al., 2008; Lee et al., 2007). The antimicrobial activity due to active component flavonoids because of their structure, as they have the ability to form a combined complex with bacterial cell walls, due to number of hydroxyl groups on the phenol group, which increase the toxicity for the microorganism (Cowan, 1999).

A study by Alrumman (2015) showed that *T. aphylla* leaves possessed a significant antimicrobial activity against the human pathogens *S. aureus, Klebsiella pneumoniae, K. oxytoca, Proteus. mirabilis, P. aeruginosa, Micrococcus luteus* and *Shigella sp*., and *Candida sp,*. This is in full agreement with our result against *P. aeruginosa and C. albicans*.

Bioactivity of *T. aphylla* is greatly affected according to the method of extraction. Extractions from fresh materials have a more profound effect in terms of antimicrobial activities compared with dry ones, dried plant materials are characterized by less volatile compounds that have been considered as being good antimicrobial agents (Al-Jaber, Al-Qudah, Barhoumi, Abaza, & Afifi, 2012; Díaz-Maroto, Sánchez Palomo, Castro, Viñas, & Pérez-Coello, 2004).

The result obtained by Zarai et al., (2011), indicated the absence of inhibition zones and no planktonic growth inhibition activity ($PMIC_{50}$) against *P. aeruginosa* was obtained for *M. vulgare* crude extract. This is similar to the case with this study, which also did not show any zone of inhibition. However, it proved effective when tested using MIC (41.6 mg/ml).

5.2 *In Vitro* effect of plant crude extracts on Biofilm formation of *P. aeruginosa.*

The activity of the extracts on the biofilm formation inhibition was tested using the crystal violet (CV) method, which is widely used by microbiologists. It is inexpensive and can be repeated many times in order to ensure accurate results (reproducible). It was noticed that during the optimization of the CV assay, whenever glucose percentage in broth media is increased, the ability of the test bacteria to form biofilm has increased.

In this assay, the use of U-bottom or flat types of 96-well microtiter plates has affected the bacterial adhesion. This was also noted in the literature (Lorite et al., 2011). Adhesion of microbial cells to a surface is an essential step to biofilm formation (Sauer, 2003). The basic disadvantage is that CV, stains binds to both living and dead cells, because it binds to the negatively charged surface molecules and polysaccharides in the extracellular matrix. Therefore it is difficult to evaluate killing of biofilm cells (Burton, Yakandawala, LoVetri, & Madhyastha, 2007).

The crude extracts used in this assay showed antibacterial activity against *P. aeruginosa* at different concentration. To ensure a concentration that is not affecting the microbial growth, extracts concentrations around $PMIC_{50}$ and below (sub-$PMIC_{50}$) were used for anti-biofilm assay. Sub-inhibitory antimicrobial concentrations may produce a variety of effects including altering bacterial morphology and growth, affecting bacterial virulence factors, and altering bacterial susceptibility to host immune defenses (Zhanel, Hoban, & Harding, 1992).

The reason for the selection of *P. aeruginosa* in this assay is the fact that the biofilms-forming ability of this bacterium contribute to its colonization in causing acute infections as well as its presence in burned tissue surrounding blood vessels and adipose cells (Schaber et al., 2007). *P. aeruginosa* reportedly has genes called pel genes that are involved in biofilm formation both at the air-liquid interface in standing cultures (pellicle) and on surfaces (Friedman & Kolter, 2004).

The highest effect on biofilm inhibition was for *A. sisalana, P. hortorum, H. sabdariffa M. longifolia,* and *L. inermis* respectively. Variable results were obtained for the various tested plant extracts. *P. hortorum* showed prominent antimicrobial and anti-biofilm activities. The result obtained by Lotfalian, Ebrahimi, & Mahzoonieh (2016), is in agreement with our study. In this study, the *C. zeylanicum* extracts tested prevented the formation of biofilm at concentration of 0.1 mg/mL, similar to a study by Pratiwi (2015), which prevented the formation of biofilm at concentration of 0.09 mg/mL.

A low concentration of the extract may be required to prevent biofilm first attachment, while higher concentration to disrupt preformed biofilm (Stewart, 2002). Our study indicated that most plant extracts have the antibacterial coupled with anti-biofilm activity, therefore, may prove helpful for developing biofilm inhibitors and increase the effectiveness of infectious diseases treatment.

In this study, *L. inermis* showed remarkable potential as anti-biofilm agent, as it was active upon *P. aeruginosa* in the planktonic state, no articles on anti-biofilm activity of crude extract *L. inermis* was published, however it failed to reduce the QS in most assays.

Leaves of the *L. inermis* are strikingly most effective against the spectrum of bacterial isolates tested as compared with seeds. This may be attributed to the presence of chlorophyll, *L. inermis* leaves contain up to 5% by weight of the compound (2-hydroxy-1,4-naphthoquinone), chemically active constituents such as quinines (Habbal, Al-Jabri, El-Hag, Al-Mahrooqi, & Al-Hashmi, 2005; Maekawa et al., 2007). Therefore, the possibility of using a cream or soaps incorporating *L. inermis* active ingredients may be of great advantage for hygiene purposes for both physicians and patients in hospitals especially in intensive care units or infectious disease units where immuno-compromised patients are treated (Habbal et al., 2011).

The first discovered natural products inhibited QS and biofilm maturation in Gram-negative bacteria are the halogenated furanones from *Delisea pulchra* (Givskov, Eberl,

& Molin, 1997), and other QSI compounds such as cyclic sulfur derivatives obtained from garlic (Persson, Givskov, & Nielsen, 2005), and patulin produced by *Penicillium* sp. (Pei & Lamas-Samanamud, 2014).

M. longifola extract showed strong anti-biofilm activity against *P. aeruginosa* compared to the untreated control. These medicinal plants could be used to manage *Pseudomonas* pathogenesis and hinder its dissemination. To the best of our knowledge, no reports are available regarding the anti-biofilm activity of *P. aeruginosa* by *M. longifola* extract. Menthol is the most important component responsible for most of the pharmacological effects of the *M. longifola*. It is a waxy, crystalline substance, clear or white in color, which is solid at room temperature and melts at slightly high temperatures (Gulluce et al., 2007; Mimica-Dukić, Božin, Soković, Mihajlović, & Matavulj, 2003).

5.3 Screening for anti-quorum sensing (Las A staphylolytic, Pyocyanin, Motility inhibition assays) effect of plant extracts.

One major objective of this study was to determine the anti-QS potential of plant extracts to explore the potential to a possible use in controlling detrimental pathogenic bacteria through the use of three assays against *P. aeruginosa*. This organism has three extracellular signals; QSN-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), N-butyryl-L-homoserine lactone, and 2-heptyl-3-hydroxy-4(1H)-quinoline (Gala & Desai, 2014).

This results in different system/pathway of quorum sensing by *P. aeruginosa*. Therefore, there are different mechanisms of quorum sensing inhibition (Figure 5.1) by active component from plant extract, either by inhibition the production of the virulence factor pyocyanin, inhibition of two QS system effective compounds, and inhibition of QS system receptors (O'Loughlin et al., 2013).

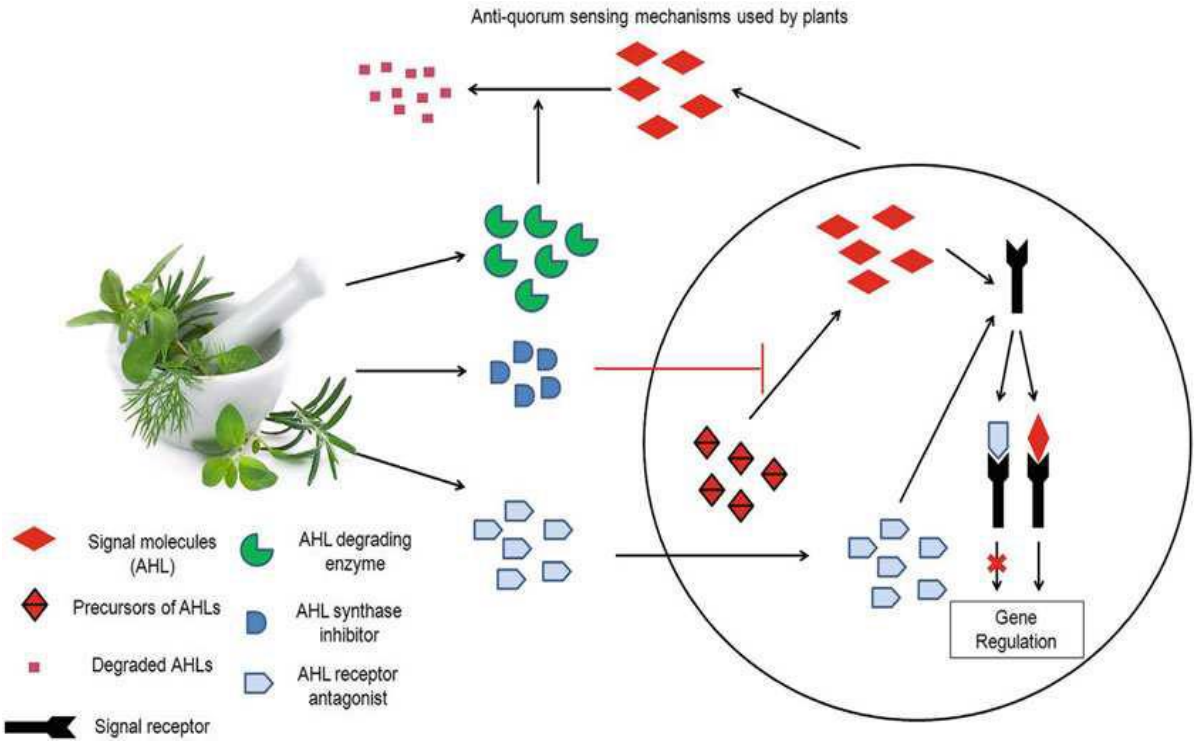

Figure (5.1): Various mechanisms utilized by plants to inhibit quorum sensing (Source: Kalia, 2015).

We investigated if the extracts which inhibited *P. aeruginosa* motility as a mean for determining the inhibition of quorum sensing as an alternative strategy for controlling bacterial virulence as suggested by Vasavi, Arun, & Rekha, (2014). The motility (swarming, and twitching), is considered to be important for cystic fibrosis acute infections, common cause of severe nosocomial infections, involved early stages of biofilm formation. A close link between swarming motility and biofilm formation in *P. aeruginosa* is established (Jimenez et al., 2012).

Three major forms of motility are displayed by *P. aeruginosa* (swarming on semisolid, twitching on solid surfaces, and flagellum-mediated swimming in aqueous environments). There is currently no standardized *In Vitro* assay to visualize and study swarming motility, and the assays used can vary greatly between laboratory groups (Morales-Soto et al., 2015).

The swarming assay depends on factors such as nutrient composition, agar type and composition, sterilization protocol (e.g., autoclaving), and semi-solid media curing among others (Kamatkar & Shrout, 2011; Shrout et al., 2006; Tremblay & Déziel, 2008), thus, it is highly sensitive to environmental factors. At the microscopic level, the edge of the colonies in twitching motility is highly irregular compared with control (Figure 5.2).

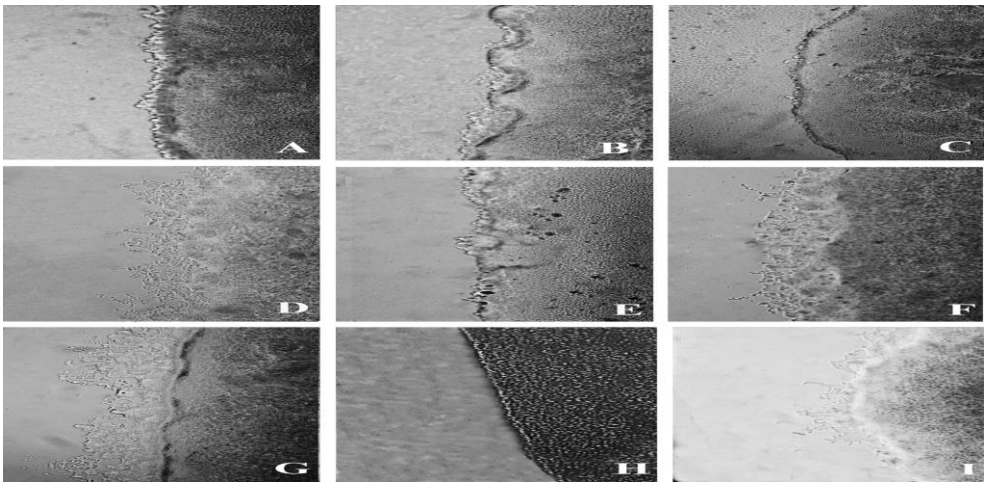

Figure (5.2): Light microscopy of colony edges of *Pseudomonas aeruginosa* in twitching motility. (F) *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony with regular protrusions in the absence of extracts, control, other picture *P. aeruginosa* with different extracts at certain concentration (Soruce: Glamočlija et al., 2015).

The effect of sub-lethal concentration of cinnamon extract on the motility of *P. aeruginosa* was analyzed, the motility test data showed that cinnamon extract at concentrations of 3.9 mg/ml for one *P. aeruginosa* isolate and 0.9 mg/ml for the second isolate, inhibited QS. This result was incompatible with the result obtained by Kalia et al., (2015), where they showed anti QS, inhibition of motility at concentration of 0.2 μg/ml for same bacteria.

The reason for this may be due that they used pure oil instead of crude plant extract, and cinnamon composition may vary depending on the bulk, the species of plant used, and the method of preparation (Herman, Herman, Domagalska, & Młynarczyk, 2013; Ooi et al., 2006).

Our result was compatible with the result obtained by Herman, Herman, Domagalska, & Młynarczyk (2013), where they showed anti QS (inhibition motility) at concentration of 0.3 mg/ml against *P. aeruginosa*, they used Trans-cinnamaldehyde, which is the major component comprising 85% in *C. zeylanicum* (Ooi et al., 2006).

Researchers using pure compounds obtained from plants offer some specific advantages compared to conventional treatments, so phytochemicals identification and development of novel anti-QS compounds beyond crude extract can and will certainly move these treatments from the laboratory bench to market.

In this study, crude extract of *P. granatum* showed a very good inhibition of motility at low concentration against both *P. aeruginosa* isolates. It was stated that *P. granatum* not only used as anti-biofilm and anti QS, but as well used for enhancement of probiotic bacteria in the gut, a hydrolyzable tannin-rich pomegranate by-product (POMx) incubated with faecal bacteria resulted in formation of the dibenzopyranone-type urolithins which enhanced the growth of *Bifidobacterium* spp. and *Lactobacillus spp* (Bialonska et al., 2010). However, the result obtained by Koh & Tham (2011), revealed that, *P. granatum* did not show anti-QS activity against the same bacteria.

The result of this study showed that the crude extract of *T. aphylla* had a high QS inhibitory effect against *P. aeruginosa* motility. Tamaricaceae have many chemical compounds; for example, 62 different chemical compounds were identified in *T. boveana* (Saidana et al., 2008). Composition of polyphenols (phenolic acids and flavonoids) of *T. aphylla* which is a source of bioactive compounds has been studied, and it was found that leaves contain higher amount of polyphenols than the stems (Mahfoudhi, Prencipe, Mighri, & Pellati, 2014).

The result obtained by Anitha & Mahalakshmi (2012), proved that *L. inermis* extract has the ability to inhibit (QS), and this results is incompatible with our result, the reason for this variation may be due different microorganism strain.

P. aeruginosa produce and release blue green pigment (pyocyanin) during biofilm formation (Das & Manefield, 2012). Pyocyanin is considered as a good indicator for investigating the effect of QS inhibition. In this study, a number of plant extracts were shown to be capable of inhibiting pyocyanin production in cultures of *P. aeruginosa*. Studies confirmed the hypothesis that most clinically pathogenic *P. aeruginosa* strains in critically-ill patients have increased expression of two virulence factors, elastase and pyocyanin, which are considered to be regulated by QS (Le Berre et al., 2008).

QS can be measured both qualitatively and quantitatively. Pyocyanin assay is a quantitative method, which measure the pigment reduction. When treated with 0.1, 0.2 and 0.3 mg/ml of trans-cinnamaldehyde, the production of pyocyanin was significantly reduced as the production are 65.54%, 63.03% and 57.94%, respectively (Chang et al., 2014). In our study, the inhibition effect of *Cinnamomum* crude extract significantly reduced the production of pyocyanin to 44.3%, at concentration 7.8 mg/ml.

In a recent study, *M. longifolia* showed moderate anti-quorum sensing effect (Tittikpina et al., 2016). This is in conformity with the results of our study, which reduced the Las A Staphylolytic and pyocyanin production, without affecting *P. aeruginosa* motility. This activity/inactivity against some *P. aeruginosa* phenotypic characteristics could be explained by suggesting different mechanism to control different processes. It was indicated that the effects of the plant extracts studied on *P. aeruginosa* are quite complicated and perhaps extend beyond the domain of the QS control hypothesis (Adonizio, Kong, & Mathee, 2008).

The plant extracts were examined for their ability to interfere with the QS-dependent production of the *P. aeruginosa* virulence factors Las A. The assay of staphylolytic

activity that we used for this purpose is sensitive and specific (Gustin, Kessler, & Ohman, 1996).

The production of Las A is controlled by the las system. *P. aeruginosa* produces at least three well characterized extracellular proteases/peptidases, LasB, Las A, and PrpL (Rawlings, Barrett, & Bateman, 2012), Las A increases significantly the elastolytic activity of other proteases, including that of LasB in the establishment of a *P. aeruginosa* infection (Hoge, Pelzer, Rosenau, & Wilhelm, 2010).

Las A protease activity was measured by determining the ability of *P. aeruginosa* culture supernatants to lyse boiled *S. aureus* cells. Las A has a particular influence on the shedding process in the host tissue which is of particular importance to enhance pathogenesis of *P. aeruginosa* (Naik, Wahidullah, & Meena, 2013).

There was a considerable decrease in Las A activity compared to that of the control when *P. aeruginosa* was grown in the presence of some of the tested plant extract. The most drastic reductions was seen with *M. charantia, M. longifolia, A. sisalana, T. vulgaris,* and *T. aphylla* extracts. No previous studies have considered Las A activity in the presence of anti-QS-compounds against *P. aeruginosa.*

5.4 Antibacterial and synergistic activity of different plant extract with different antimicrobials against *S. aureus.*

The extensive use of antimicrobial agents over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms (Chambers, 2001). *S. aureus* remains a versatile and potent pathogen in humans and animals. It is one of the most common causes of nosocomial and community-acquired infections (Rajbhandari, Manandhar, & Shrestha, 2003).

Drug synergism between known antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome) (Gibbons, 2004). Discovery of new chemical entities

(NCEs) required chemical synthesis or through isolation from natural products (Katiyar, Gupta, Kanjilal, & Katiyar, 2012).

In this study, the synergistic effect resulting from the combination of antimicrobial agents with crude plant extracts was tested and verfied for three plants only. Plant extracts that showed inhibition of the growth of *S. aureus* were selected and this could be explained by the fact that these crude extracts have many different phytochemicals (Duke, JoBogenschutz-Godwin, DuCellier, & Duke, 2003).

The results of this work were found to be consistent with the work done by Ranasinghe et al., (2013) for *C. zeylanicum* extract, the result obtained by Gull, Sohail, Aslam, & Athar, (2013), was compatible with our result against multi drug resistant *S. aureus* for *L. inermis.* The different parts of the *C. zeylanicum* plant possess the same array of hydrocarbons in varying proportions. This chemical diversity is likely to be the reason for the wide-variety of medicinal benefits observed (Gull, Sohail, Aslam, & Athar, 2013).

There is no doubt in the pharmacological properties of *L. inermis,* but its toxicological assessment is also indispensable. *In vivo* acute toxicity of *L. inermis* extracts was checked in mice (Gull et al., 2013). No mortality was observed during their study. All the signs of toxidrome were negative.

Variable results were obtained with *P. hortorum* extract. It showed significant antimicrobial and synergistic activities against some strain of MDR *S. aureus*, these result has not been reported previously in the literature.

In conclusion, *L. inermis*, *P. hortorum* and, *C. zeylanicum* extracts have antibacterial activities and exhibited synergistic effects when used with commercial antimicrobials. Therefore, our data clearly demonstrate the importance of plant extracts in the control of resistant bacteria, which are becoming a threat to human health.

Chapter VI
Conclusions and Recommendations

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Biofilm and QS are major virulence factors of most of the pathogenic microorganism, therefore, particular attention is oriented nowadays from both academic and industrial sectors especially those caused by multidrug resistance strains i.e. *P. aeruginosa* and *S. aureus*. The bioassay of the 23-studied plant extracts revealed promising antimicrobial and antifungal activities. In addition, some of them showed promising anti-biofilm and anti-QS activities. These results may represent a call for examining the possibility of using these plants or their components in ethnomedicine and drug discovery from natural sources. The present study investigated several plant extracts that exhibited various biological activities. Some of these plants have never been studied anywhere against the selected microorganism.

6.1. Conclusions

According to the findings of this study, the following conclusions could be drawn:

- The crude extract of 23 plants showed variable antibacterial and antifungal activity against *P. aeruginosa* and *C. albicans*.

- The highest antimicrobial activity against *P. aeruginosa* was observed for *P. hortorum, H. sabdariffa,* and *P. granatum*.

- The extract of *C. zeylanicum* presented the highest antifungal activity with inhibition zone diameter of 52 mm, but the highest growth inhibition activity $PMIC_{50}$ against *C. albicans* was for the plant extract of *P. graveolens, P. hortorum, C. cyminum,* and *C. zeylanicum* respectively.

- The results obtained in this study showed that eleven of the 23 tested extracts inhibited ≥ 50% of *P. aeruginosa* biofilm formation.

- The extract of *A. sisalana*, *P. hortorum*, and *H. sabdariffa* constitute an interesting source for anti-biofilm agents in the development of new strategies to treat infections caused by *P. aeruginosa* biofilm.

- Plant extracts showed variable abilities in inhibiting quorum sensing through inhibiting the different virulence factors activity of *P. aeruginosa. P. granatum* and *P. hortorum* exhibited the highest reduction in the swarming motility of the two *P. aeruginosa* isolates.

- *H. sabdariffa* and *A. absinthium* were the most effective in inhibiting quorum sensing activity showing the highest reduction in pyocyanin production by *P. aeruginosa.*

- Plant extracts showed variable abilities in reducing the las A protease activity of *P. aeruginosa*. However, *M. charantia* showed the greatest reduction in las A activity.

- The crude extract of three plants (*C. zeylanicum , L. inermis*, and *P. hortorum*) showed various degrees of antibacterial activity against *S. aureus. P. hortorum* showed the highest inhibition activity against *S. aureus* isolates.

- They also exhibited variable synergistic activities when tested with commercially available antibiotics to inhibit the growth of *S. aureus*; *C. zeylanicum* had synergistic effect with most of the tested antimicrobials, followed by *L. inermi*s, and by *P. hortorum*.

6.2 Recommendations

In light of the above conclusions and based on the results of this study, the following recommendations are suggested

1. Further studies to confirm the findings of this study using other methods and experimenting on purified components instead of the crude extract to determine the biologically active component for each plant.
2. Stability studies for plants extracts will be of value before any further testing.
3. Further investigations in animal models to determine plant extract toxicity (LD_{50}) and to human cells.
4. It is recommended that plant extracts with bioactivities against biofilm and QS be further studied to check their efficacies *In Vivo*.
5. Palestine has a unique flora and was not fully explored for their biologically active components, which may represent a cheap resource for future medicine.
6. There are very few reports available on the anti-biofilm and anti QS activities. Hence, we recommend further studies aiming at finding the anti-biofilm and anti QS activities of different plant extracts.

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Annexes

Annex 1: Raw data for the synergistic activity testing for the three extracts with various antimicrobials against 15 *Staphylococcus aureus* clinical isolates.

S. aureus / **Extracts**		**Antimicrobial**												
isolate 1		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		32	14	10	12	0								
X&A		28	12	12	12	12								
Y&A		34	16	12	12	0								
Z&A		18	14	15	13	14								
X	12													
Y	0													
Z	17													
isolate 2		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		16					12							
X&A		16					17							
Y&A		19					22							
Z&A		19					17							
X	0													
Y	0													
Y	18													
isolate 3		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		8					12	0						
X&A		8					18	10						
Y&A		14					23	0						
Z&A		18					16	17						
X	11													

Y	0													
Z	18													
isolate 4		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		9		0	0	0	22	0	23					
X&A		10		0	7	7	17	0	22					
Y&A		15		15	0	0	23	0	26					
Z&A		18		18	18	18	16	16	22					
X	0													
Y	15													
Z	18													
isolate 5		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		0	0		0	0	0	0	0	0	0	20	9	0
X&A		7	7		14	10	7	9	12	7	10	19	7	8
Y&A		0	0		13	0	14	0	13	0	0	16	0	0
Z&A		19	19		16	17	16	17	21	18	14	17	18	16
X	11													
Y	0													
Z	16													
isolate 6		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		10					12	0	21					
X&A		9					17	7	21					
Y&A		13					22	0	21					
Z&A		22					17	17	19					
X	0													
Y	0													
Z	20													

isolate 7		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		9			0	10	12		17					
X&A		7			19	0	18		19					
Y&A		17			20	7	26		24					
Z&A		9			10	7	18		19					
X	0													
Y	12													
Z	0													
isolate 8		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		9		10	0	0	12	0						
X&A		9		12	0	0	18	0						
Y&A		13		15	0	0	23	0						
Z&A		17		17	16	17	17	16						
X	0													
Y	13													
Z	19													
isolate 9		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		26	30		9	0		0	16					
X&A		20	8		12	8		0	14					
Y&A		32	0		17	16		0	18					
Z&A		16	15		16	15		15	16					
X	??													
Y	0													
Z	16													
isolate 10		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A					0	0								

X&A					10	9								
Y&A					20	16								
Z&A					17	17								
X	9													
Y	8													
Z	17													
isolate 11		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A					0	0		0						
X&A					10	11		10						
Y&A					0	0		0						
Z&A					13	14		13						
X	8													
Y	8													
Z	15													
isolate 12		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		0					12	0	21					
X&A		10					13	0	24					
Y&A		15					17	0	26					
Z&A		16					16	16	22					
X	8													
Y	10													
Z	16													
isolate 13		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		0			0	0						17		
X&A		10			12	10						21		
Y&A		14			14	12						20		

Z&A		18			19	17						20		
X	10													
Y	0													
Z	16													
isolate 14		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		0			0	0	11					20		
X&A		0			0	0	16					19		
Y&A		10			0	0	21					21		
Z&A		14			14	12	13					18		
X	7													
Y	0													
Z	12													
isolate 15		**P**	**OX**	**C**	**CLR**	**E**	**CN**	**TM**	**FOX**	**NOR**	**CIP**	**CPT**	**LEV**	**OFX**
A		20		9	0	0								
X&A		21		11	17	12								
Y&A		17		10	11	9								
Z&A		20		14	18	15								
X	14													
Y	9													
Z	20													

A: Antibiotic, X: *Lawsonia inermis*, Y: *Cinnamomum zeylanicum*, Z: *Pelargonium hortorum.*