An-Najah National University

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Synergetic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens

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Dedication

TO MY DEAR PARENTS ,WIFE,DAUTER, BROTHERS , SISTERS AND FRIENDS WITH LOVE AND RESPECT

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I wish to express my deepest gratitude to my thesis supervisor, Dr. Ghaleb Adwan for his supervision, constant encouragement, indispensable guidance throughout this work, constructive comments and for his valuable criticism. My special thanks extended to Dr. Kamel Adwan, Dr. Feras Sawalha,

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إقـــــرار

أنا الموقع/ة أدناه، مقدم/ة الرسالة التي تحمل العنـــوان: **التـــأثيرات التآزريـــة لـــبعض المستخلصات النباتية والمضادات الحيوية على المكورات العنقودية المعزولـــة مـــن عينـــات مرضية**، أقر بأن ما اشتملت عليه الرسالة إنما هي نتاج جهدي الخاص, باستثناء ما تمت الإشارة إليه حيثما ورد , وأن هذه الرسالة ككل، أو أي جزئ منها لم يقدم من قبل لنيل أية درجة أو لقب علمي أو بحثي لدى أية مؤسسة تعليمية أو بحثية.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researchers own work ,and has not been submitted else where for any other degree or qualification.

اسم الطالب: Student's Name:

التوقيع: Signature:

التاريخ: Date:

List of Contents

List of Tables

Abbreviations

MIC: Minimum Inhibitory Concentration

MRSA: Methicillin-Resistant *Staphylococcus aure*us

MSSA: Methicillin-Sensitive *Staphylococcus aure*us

VRSA: Vancomycin-Resistant *S. aureus*

MDR: Multi-Drug Resistant

PABA: Para-aminobenzoic acid

5-MHC-D: 5-methoxyhydnocarpin

NA: Nutrient Agar

NB: Nutrient Broth

MHB: Mueller-Hinton Broth

MSA: Mannitol salt agar

MHA: Meullar Hinton agar

CFU: Colony-forming unit

NCCLS: National Committee for Clinical Laboratory Standard

VRE: Vancomycin-Resistant Enterococci

EMRSA: Epidemic Methicillin-Resistant *S. aureus*

Synergetic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens

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Abstract

This research focuses on antimicrobial activity of different water plant extracts: *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis*, and *Rosa damascena* alone and then synergy testing of these extracts with known antimicrobial agents of different mechanisms (protein synthesis inhibition: oxytetracycline HCl and gentamicin sulfate; cell wall synthesis inhibition: penicillin G and cephalexin; folic acid synthesis inhibition: Sulfadimethoxine as sodium; and nucleic acid synthesis inhibition: enrofloxacin) using both well-diffusion and microdilution method.

The results of the conducted experiments using well-diffusion method demonstrated that these plants extracts contain bioactive compounds some of which has a weak effect. *In vitro* interactions between the above mentioned antibiotics and plant extracts using the previous method were mainly additive against the four strains of *S. aureus*. While *in vitro* study using microdilution method showed synergistic effects with significant reduction in the MICs of the test antibiotics, resulting from the combination of antibiotics with different crude plant extracts against 3 strains of *S. aureus*. The change in MIC was noticed in all plant extracts against test antibiotics including these plants showed weak antibacterial activity by well diffusion method. Also our results showed that synergism effects between antimicrobial agents and plant extracts were occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in resistant strains was higher than the sensitive strains.

Chapter one

Introduction

1. Introduction:

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases, appearance of undesirable side effects of certain antibiotics, as well as the increasing development of resistance to the antibiotics in current clinical use (Cowan, 1999). Therefore, actions must be taken to reduce these problems, for example, to control the use of antibiotic, develop research to a better understanding of the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial agents to the patient.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. That the medicinal actions of plants are unique to particular plant species or groups is consistent with this concept as the combinations of secondary products in a particular plant are often taxonomically distinct. This is in contrast to primary products, such as carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids, which are common to all plants and are involved in the primary metabolic processes of building and maintaining plant cells (Briskin, 2000).

Several diverse lines of evidence indicate that medicinal plants represent the oldest and most widespread form of medication. Higher plants produce hundreds to thousands of diverse chemical compounds with different biological activities. Accordingly, secondary products have both a defensive role against herbivory, pathogen attack, and inter-plant competition and an attractant role toward beneficial organisms such as pollinators or symbionts. Plant secondary products also have protective actions in relation to abiotic stresses such those associated levels, UV exposure, and mineral nutrients. Furthermore, recent work has indicated potential roles of secondary products at the cellular level as plant growth regulators, modulators of gene expression, and in signal transduction (Briskin, 2000).

Although secondary products can have a variety of functions in plants, it is likely that their ecological function may have some bearing on potential medicinal effects for humans. For example, secondary products involved in plant defense through cytotoxicity toward microbial pathogens could prove useful as antimicrobial medicines in humans, if not toxic. Likewise, secondary products involved in defense against herbivores through neurotoxin activity could have beneficial effects in humans as antidepressants, sedatives, muscle relaxants, or anesthetics, through their action on the central nervous system. To promote the ecological survival of plants, structures of secondary products have evolved to interact with

molecular targets affecting the cells, tissues, and physiological functions in competing microorganisms, plants, and animals. In this respect, some plant secondary products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters and thus have beneficial medicinal effects on humans due to similarities in their potential target sites (Briskin, 2000).

Antimicrobial agents produced by plants are active against plant, human and animal pathogens. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. According to WHO, medicinal plants would be the best source to obtain a variety of drugs and herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries (WHO, 2001). Therefore, such plants should be investigated to better understand their properties, safety and efficiency. In recent years, different reports, from different countries were published showing the antimicrobial activities of medicinal plants include studies of medicinal plants from Greece (Proestos *et al*., 2006), Palestine (Abu-Shanab *et al*., 2006), Lebanon (Barbour *et al*., 2004), Qatar (Mahasneh, 2002), Turkey (Uzun *et al*., 2004), Ethiopia (Tadeg *et al*., 2005), Nigeria (Oshodi *et al*., 2004), Malaysia (Wiart *et al*., 2004), Iran (Bonjar 2004), Peruvia (Rojas *et al*., 2003), Brazil (Duarte *et al*., 2005; Machado *et al*.,

2003), India (Ahmad and Beg, 2001; Nair *et al*., 2005). Others prefer to study a wider region that includes different countries such as Asia (Almas, 2001) or Africa (Tshibangu *et al*., 2002), or a wide zone within one country such as Siberia (Kokoska *et al*., 2002).

Drug synergism between known antimicrobial agents and bioactive plant extracts is a novel concept and has been recently reported (Nascimento *et al*., 2000; Aqil *et al*., 2005; Betoni *et al*., 2006). In this study the following plants were used: *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis*, *Rosa damascena*. Some of these plants are growing in Palestine and others collected from Palestinian markets. These plants could be medicinal and/or nutraceutical.

1.1. Objectives of the present study:

The occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and multidrug-resistant (MDR) strains of this organism necessitate the discovery of new classes of anti-staphylococcal drugs. This study aims to:

1. Asses the antimicrobial activity of extracts of these plants against *S. aureus* strains by using well diffusion method

2. Evaluate the possible *in vitro* interaction between extracts of these plants and certain known antimicrobial drugs such as oxytetracycline HCl, gentamicin sulphate, penicillin G, cephalexin, sulfadimethoxine as sodium, and enrofloxacin using well diffusion method and minimum inhibitory concentration (MIC) by microdilution method.

Chapter two

Literature Review

2.1. Major groups of antimicrobial compounds from plants:

Plants have an ability to synthesize thousands of metabolites. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores, give plants their odors, plant flavor or responsible for plant pigment. Some of these plants used by humans to yield useful antimicrobial phytochemicals and can be divided into several categories (Cowan, 1999).

2.1.1. Phenolics and Polyphenols

2.1.1.1. Simple phenols and phenolic acids

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. The common phenolic acid is caffeic acid, which is effective against viruses, bacteria and fungi. Hydroxylated phenols, such as Catechol and pyrogallol shown to be toxic to microorganisms. The site and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins.

Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils such as Eugenol, which is considered bacteriostatic and fungistatic agent (Cowan, 1999).

2.1.1.2. Quinones (diketones)

Quinones are aromatic rings with two ketone substitutions. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. The individual redox potential of the particular quinone-hydroquinone (diphenol) pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in electron transport systems. Vitamin K is a complex naphthoquinone and its antihemorrhagic activity may be related to its ease of oxidation in body tissues. Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase. In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes (Cowan, 1999).

2.1.1.3. Flavones, flavonoids, and flavonols:

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. These compounds have been found to be effective antimicrobial substances in vitro against different microorganisms. Their activity might be due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes. In addition to be effective against bacteria, these compounds exhibit inhibitory effects against viruses, parasites (Cowan, 1999).

2.1.1.4. Tannins:

These are polymeric phenolic compounds capable of tanning leather or precipitating gelatin from solution and they are found in almost every plant part. Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins. One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to

inactivate microbial adhesins, enzymes, cell envelope transport proteins, and also complex with polysaccharide. Tannins can be toxic to filamentous fungi, yeasts, and bacteria, and inhibitory to viral reverse transcriptases (Cowan, 1999).

2.1.1.5. Coumarins:

Coumarins are phenolic substances made of fused benzene and a-pyrone rings. Their fame has come mainly from their antithrombotic, anti-inflammatory, and vasodilatory activities**.** Several coumarins have antimicrobial, antiviral properties, inhibit vaginal candidiasis, contraceptive agent, some coumarins have been found to stimulate macrophages, and others are rodenticides (Cowan, 1999)**.**

2.1.2. Terpenoids and Essential Oils:

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are highly enriched in compounds based on an isoprene structure and are called terpenes. When these compounds contain additional elements, usually oxygen, they are called terpenoids. Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids.

A terpenoid has a wide range of biological activities in humans, affecting the nervous, cardiovascular, and digestive systems as well as finding use as an analgesic. Terpenenes or terpenoids are active against bacteria, fungi, viruses, and protozoa. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Carson *et al*., 2006, Cowan, 1999).

2.1.3. Alkaloids:

Alkaloids are heterocyclic nitrogen compounds commonly found to have antimicrobial properties, may be useful against viral and protozoan infections. The mechanism of action of highly aromatic planar quaternary alkaloids is attributed to their ability to intercalate with DNA (Cowan, 1999).

2.1.4. Lectins and Polypeptides:

Peptides are inhibitory to microorganisms, often positively charged and contain disulfide bonds. Their mechanism of action may be the formation of ion channels in the bacterial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors. Some of these peptides have antifungal, antibacterial effect, and may be antiviral properties.

The lectin molecules which include mannose-specific lectins from several plants are inhibitory to viral proliferation, the mode of action probably by inhibiting viral adhesion with critical host cell components (Cowan, 1999).

2.1.5. Other Compounds:

Other phytochemicals have been found to exert antimicrobial properties. These include polyamines (in particular spermidine), isothiocyanates, thiosulfinates, Polyacetylenes, and glucosides (Cowan, 1999).

2.2. Synergism between plant extract and antimicrobial drugs

Plants are rich in a wide variety of secondary metabolites, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel, 2006; Cowan, 1999). Observations found that phytochemicals are generally have weak antimicrobial properties compared to bacterial, fungal produced antibiotics and semi-synthesized antibiotics; and often show considerable activity against Gram-positive rather than Gram-negative species (Nostro *et al*., 2000; Gibbons, 2004). A number of *in vitro* studies have reported the use of plant extracts in combination with antimicrobial agents, enhance the activity of certain antibiotics against of some resistant and sensitive strains of *S. aureus* and other pathogens (Al-hebshi *et al*., 2006; Betoni *et al*., 2006; Dickson *et al*. 2006; Shibata *et al*., 2005; Braga *et al*., 2005; Gibbons *et al*.,

2003; Darwish *et al*., 2002). The ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics (Lewis and Ausubel, 2006; Zhao *et al*., 2001). Production of efflux pump inhibitors by the plants would be one way to ensure delivery of the antimicrobial compounds. This hypothesis has been supported by the findings of Stermitz *et al*. (2000 a,b), who observed that *Berberis* plants which produce the antimicrobial compound, berberine, also make the multi-drug resistance (MDR) inhibitors 5-methoxyhydnocarpin D (5-MHC-D) and pheophorbide A. These studies have provided the bases for understanding the action of plant antimicrobials, namely that vast majority of such compounds are agents with weak or narrow-spectrum activities that act in synergy with intrinsically produced efflux pump inhibitors. There is reason therefore to believe that, plants could be a source of compounds that can increase the sensitivity of bacterial cells to antibiotics. Such compounds could be useful particularly against antibiotic resistant strains of pathogenic bacteria. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored.

2.2.1. Synergistic interaction evidences using crude plant extracts

Screening the antimicrobial activities in crude extracts is the first step in identifying leads for isolation of such compounds, and some plants have provided good indications of these potentials for use in combination with antimicrobial therapy. Different solvents can be used for active component extraction, these include, water and organic such as ethanol, methanol, chloroform, dichloromethanol, ether, and acetone (Cowan, 1999). Synergistic interactions was observed between methanolic crude extracts of guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), carqueja (*Baccharis trimera*), and mint (*Mentha Pieria*). Some antibiotics which represented inhibitors of protein synthesis, cell wall synthesis, nucleic acid synthesis and folic acid synthesis against *S. aureus* (Betoni *et al*. 2006). Acetone crude extract from *Salvia officinalis* (sage) reduced the MIC of aminoglycosides in vancomycin-resistant enterococci (VRE) (Horiushi *et al*., 2007). A synergistic effect was also observed against different bacterial species, during the association of antibiotics with ethanolic extracts from clove (*Caryophyllus aromaticus*), jambolan (*Syzygyum joabolanum*), pomegranate (*Punica granatum*) and thyme (*Thymus vulgaris*). However, the synergetic effect was observed when plant extracts were combined with the test antibiotics; even those plant

extracts did not show any activity by themselves (Nascimento *et al*., 2000). It was also found that methanolic crude extract of thyme leaves (*Thymus vulgaris*) greatly reduced the MIC of tetracycline against MRSA (Fujita *et al*., 2005). It was also reported that sub-inhibitory levels (200 μg/ml) of methanolic extracts of some Jordanian plants showed synergistic interactions in combination with gentamicin, chloramphenicol, penicillin G and erythromycin against resistant and sensitive *S. aureus* (Darwish *et al*., 2002). Methanolic extract of *Punica granatum* showed synergistic interactions agains both MRSA and MSSA with chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin (Braga *et al*., 2005). Also ethanol extracts of other Chinese plants, *Isatis tinctoria*, *Scutellaria baicalensis* and *Rheum palmatum* can improve the antimicrobial activity of penicillin G, gentamycin, ciprofloxacin, and ceftriaxone used against *S. aureus*. In addition to that, combinations of pencillin with ethanolic extracts of *Paederia scandens* and *Taraxacun monlicum* showed a strong bactericidal activity on two strains of *S. aureus* (Yang *et al*., 2005). *In vitro* synergistic interaction of crude extracts from different India plants *Camellia sinensis*, *Lawsonia inermis*, *Punica granatum*, *Terminalia chebula* and *Terminalia belerica* was detected with tetracycline. Moreover, the extract from *Camellia sinensis* also showed synergism with ampicillin (Aqil *et al*., 2005). Further more, It was also observed that alcoholic crude extracts of other Indian medicinal plants,

Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica and *Plumbago zeylanica* showed synergistic interactions with tetracycline and ciprofloxacin against Extended Spectrum β-lactamase (ESβL), these enteric bacteria with ciprofloxacin showing more synergy with the extracts than tetracycline (Ahmad and Aqil, 2007). Other study revealed that ethanolic rxtracts of *Kola nitida* seeds potentiated the effects of the fluoroquinolones such as ciprofloxacin, pefloxacin and levofloxacin against *E. coli* and the MIC of thes drugs decreased when combined with *Kola nitida* seeds extract (Ibezim *et al*., 2006). Recently, It was showed that the combination of ethanolic extracts of *Rhus coriaria* (leaf), *Psidium guajava* (Leaf), *Lawsonia inermis* (Leaf) and Sacropoterium spinosum (seed) and antimicrobial drugs including oxytetracycline HCl, gentamicin sulphate and sulfadimethoxine against 4 clinical isolates of MRSA was lead to increase these antibiotics to these bacterial strains (Adwan *et al*., 2008). Aqueous extracts of tea (*Camellia sinensis*) have been shown to reverse methicillin resistance in MRSA and also, to some extent, penicillin resistance in β-lactamase-producing *S. aureus* (Stapleton *et al*., 2004; Yam *et al*., 1998). These phenomena are explained by prevention of Penicillin Binding Proteins 2′ (PBP2′) synthesis and inhibition of secretion of β-lactamase, respectively. Forty to one hundred fold dilutions of tea extracts was able to reduce the MICs of high- level resistant MRSA (≥ 256 μg/ml) to less than 0.12 μg/ml

for methicillin and penicillin (Yam *et al*., 1998). Addition of the aqueous crude khat (*Catha edulis*) extracts at a sub- MIC (5 µg/ml) resulted in a 2 to 4-folds potentiation of tetracycline against resistant strains *Streptococcus sanguis* TH-13, *Streptococcus oralis* SH-2, and *Fusobacterium nucleatum* (Al-hebshi *et al*., 2006).

2.2.2. Synergistic interaction evidences using pure compounds

Some isolated pure compounds of plant origin have been reported to have resistance modifying activities *in vitro*. This has prompted the search for such compounds from a variety of medicinal plants. Some of these compounds which have been observed to have direct antimicrobial activity, have also been shown to be potentiate against the activity of antibiotics when used at low MIC levels. Diterpene compounds extracted from totara tree have been shown to potentiate methicillin activity against MRSA and reducing the MIC of methicillin against resistant *S. aureus* 256-fold *via* interference with PBP2a expression (Nicolson *et al*., 1999). Epicatechin gallate extracted from leaves of *Camellia sinensis* (green tea) markedly lowered the MIC of oxacillin and other beta-lactams, but not of other antibacterial agents tested, in strains of MRSA (Shiota *et al*., 1999). When catechins were extracted from leaves of *Camellia sinensis* below MIC (25-100µg/ml) and combined with oxacillin (5-12.5 µg/ml) showed antibacterial activity against all MRSA

isolates. Catechin combined with aminobenzylpenicillin, tetracycline, and chloramphenicol showed antibacterial activities against multiple drug resistant MRSA to antibiotics mentioned above (Takahashi *et al*., 1995). The effects of hydrolysable tannins tellimagrandin I and rugosin extracted from petals of *Rosa canina* (rose red) and corilagin extracted from leaves of *Arctostaphylos uva-ursi* showed that these compounds work synergistically with oxacillin, corilagin reduces the MICs of various β-lactams 100- to 2000-fold but not other antimicrobial agents such as vancomycin, the fluoroquinolone ofloxacin or the macrolide erythromycin. Corilagin markedly reduced the MICs of β-lactams in both β-lactamase-positive MRSA and β -lactamase-negative MRSA. The authors suggested that the major action of this natural product is also by the inhibition of PBP2′ activity. In the presence of tellimagrandin I, the MICs of tetracycline against some strains of MRSA were also significantly reduced (Shiota *et al*., 2000, Shimizu *et al*., 2001). The synergy between epigallocatechin gallate in tea catechins which is the main compound responsible for the antimicrobial activity of tea, and oxacillin was attributed to the combined action of epigallocatechin gallate and oxacillin on the biosynthesis of the cell wall thereby bypassing the resistance mechanism resulting from the reduced affinity of PBP to oxacillin. It was found that less than 25 μg epigallocatechin gallate per ml obviously reversed the high level resistance of MRSA to all types of tested β-lactams,

including benzylpenicillin, oxacillin, methicillin, ampicillin, and cephalexin. Epigallocatechin gallate also induced a supersusceptibility to β-lactams in MSSA which does not express *mecA*, encoding PBP2′ (Zhao *et al*. 2001). Combinations of carbapenems, panipenem or meropenem and epigallocatechin gallate showed potent synergy against clinical isolates of methicillin-resistant *S. aureus* (Hu *et al*., 2002). Baicalin extracted from *Scutellaria baicalensis* was reported to be capable of exerting *in vitro* synergistic effect against MRSA when used in combination with β-lactam agents (Liu *et al*., 2000). Also Baicalein extracted from *Thymus vulgaris* remarkably decreased the MIC of tetracycline 66 to 213-fold against MRSA (Fujita *et al*., 2005). Combination of gentamicin and baicalein extracted from *Scutellaria baicalensis* was reported to be capable of exerting *in vitro* synergistic effect against vancomycin-resistant *Enterococcus* (Chang *et al*., 2007). Phenolic diterpene totarol isolated from the immature cones of *Chamaecyparis nootkatensis* had good antimicrobial activity against effluxing strains of *S. aureus*. Subinhibitory concentrations reduced the MICs of Tetracycline, Norfloxacin, and Erythromycina 4 to 8 folds (Smith *et al*., 2007a).). Also active compounds ferruginol and 5-pipisiferol from the cones of *Chamaecyparis Lawsoniana* were screened for resistance modifying activities and observed that these compounds were effective in increasing the efficacy of tetracycline, norfloxacin, erythromycin and Oxacillin against

resistant *S. aureus* (Smith *et al*. 2007b). Ethyl gallate purified from a dried pod of tara (*Caesalpinia spinosa*) synergistically elevated the susceptibility of MRSA and MSSA strains to β-lactam antibiotics. Such a synergistic activity of the alkyl gallates appears to be specific for β-lactam antibiotics, no significant changes were observed in the MICs with other antibiotics examined in this study included norfloxacin, erythromycin, kanamycin, streptomycin, arbekacin, vancomycin, chloramphenicol, fosfomycin, and tetracyclin (Shibata *et al*., 2005). Epicatechin gallate and catechin gallate extracted from Japanese green tea (*Camellia sinensis*) have ability to reduced MIC of oxacillin from 256 and 512 to 1–4 mg/l, respectively in MRSA. Epicatechin gallate, catechin gallate, and epigallocatechin gallate increased the sensitivity of Epidemic Methicillin-Resistant *S. aureus* (EMRSA-15) to oxacillin (Stapleton *et al*., 2004). Incorporation the abietane diterpenes carnosic acid and carnosol isolated from the aerial parts of *Rosmarinus officinalis* by fractionation of the chloroform extract into the growth medium at 10 μg/ml, caused a 32- and 16-fold potentiation of the activity of erythromycin against an erythromycin effluxing *S. aureus* strains, respectively. These strains express the two efflux proteins MsrA and TetK (Oluwatuyi *et al*., 2004). Also Carnosol and Carnosic acid extracted from *Salvia officinalis*, showed a weak antimicrobial activity, and greatly reduced the MICs of various aminoglycosides and some other types of antimicrobial

agents against VRE. Carnosic acid, a related compound, showed the similar activity (Horiuchi *et al*., 2007). The majority of researches on the combinations between plant extracts and antibiotics have been focused on the identification and isolation of potential resistance modifiers from such natural sources which are considered to be positive results. However, it is likely that such combinations could produce antagonistic interactions that most studies have considered irrelevant and therefore ignored (Sibanda and Okoh, 2007).

2.3. Plants used in Study

2.3.1. Psidium guajava (guava)

Psidium guajava is an evergreen shrub or small tree in the family *Myrtaceae*. It grows to 10 m high, occasionally to as much as 20m, roots are shallow; bark smooth, light reddish-brown, bark peels off in large flakes, exposing greenish layer beneath; trunk normally attains a diameter of about 25cm, but can reach 60cm, has a 'bony' appearance; leaves opposite, ovate-elliptic or oblong-elliptic, acute-acuminate, often rather brittle, prominently nerved, lateral nerves 10-20 pairs; blades mostly 7-15 cm long and 3-5 cm wide, rounded at base, dull green, downy on the underside, aromatic when crushed; flowers, hermaphroditic, solitary or 2-4 together in leaf axils, rather large (2.5 cm wide); peduncle about 1-2 cm long, pubescent; calyx 4-5-lobed about 6-8 mm long, petals white, 10-15 mm long, fugacious, usually 4 or 5, obovate,

slightly concave, stamens numerous (200-250), white, about as long as petals; style 10-12 mm long, stigma peltate; fruit begins fruiting a 3-4 years old, fruits globose, ovoid, or pyriform, whitish-yellow or faintly pink, sweet-sour pulpy, many-seeded (100-500/fruit), 2.5-10 cm long; pulp granular-juicy; seeds yellowish, reniform. When immature and unripe, the fruit is hard, green, gummy and astringent. Longevity is 30-40 years (Purseglove, 1968).

Psidium guajava is medicinal plants used in tropical and subtropical countries to treat many disorders. It was reported that *Psidium guajava* leaf extract has a wide spectrum of biological activities such as anticough, antibacterial, haemostasis, antidiarrhoeal and narcotic properties, and antioxidant properties. It also used to treat abdominal pain, convulsions, epilepsy, cholera, insomnia and has hypnotic effect. Some studies reported that the leaf extract and its derivative identified as quercetin has effect on the intracellular calcium levels in gastrointestinal smooth muscle, cardiac muscle, Skeletal muscle, and in neuromuscular junction (Belemtougrir *et al*., 2006).

2.3.2. Rosmarinus officinalis (Rosemary)

Rosmarinus officinalis is a woody, perennial herb with fragrant evergreen needle-like leaves. It is a member of the mint family Lamiaceae, which also

includes many other herbs. Forms range from upright to trailing; the upright forms can reach 1.5 m tall, rarely 2 m. The leaves are 2-4 cm long and 2-5 mm broad, green above, and white below with dense short woolly hairs. The flowers are variable in color, being white, pink, purple, or blue (Fritzweiss and Fintelmann, 2000).

Rosmarinus officinalis, in the dried form, is extremely high in iron, calcium, and Vitamin B6. It was shown that carnosic acid, found in rosemary, shields the brain from free radicals, lowering the risk of strokes and neurodegenerative diseases (Burnham Institute for Medical Research, 2007). It is also used as natural source of food flavouring, acts as a carminative, spasmolytic, thymoleptic, sedative, diuretic and antimicrobial agent. Topically, rubefacient, mild analgestic and parasiticide properties are documented. Traditionally rosemary is indicated for flatulent dyspepsia, headache, and topically for myalgia, antithrombatic, sciatica, and intercostals neuralgia, Kidney stones, sugar in blood, liver-protective, anticancer and ulcer-protective effects . The German commission E approved internal use for dyspeptic complaints and external use as supporting therapy for rheumatic diseases and circulatory problems (Lev, 2006; Yamamoto *et al*., 2005; Vitaglione *et al*., 2004; Barnes *et al*., 2002; Dias *et al*., 2000; Singletary and Nelshoppen, 1991).

2.3.3. Majorana syriaca (Syrian oregano or Za'atar)

Majorana syriaca is a perennial herb or chamaephyte in the family *Lamiaceae*. It grows to between 30-50 cm tall, woolly-canescent and glandular. Stems erect, rigid, paniculately branched, ending in spike-like inflorescences. Leaves short-petiolate to sessile, entire, ovate, obtuse, rather thick, with elevated veins on lower face; floral leaves obovate, as long as calyx. Spikes oblong, 1-2 cm; cymes short-pedunculate. Calyx 2-2.5 mm, slit on the outer side, not dentate. Corolla white, 4 mm; tube exserted (Zohary, 1972a). In Palestine, the *zaatar* herb is used to prepare a spice mixture known by the same name. Leaves of *Majorana syriaca* are traditionally used against abdominal colic, bronchitis, cough (Ghazanfar, 1994)

2.3.4. Laurus nobilis (Bay Laurel)

Laurus nobilis is an aromatic evergreen tree in the family Lauraceae. It is a large shrub reaching 3-6 m or more. The leaves are 5–11 cm long and 2.5–6 cm broad, with a characteristic short petiole, leathery, lanceolate to obovate-oblong, tapering below, acuminate or obtuse, more or less wavy-margined, shining on upper surface. Flowers dioecious, pale yellow-green, pedicellate, in axillary terminal umbels or cymes, staminate inflorescences many-flowered, pistillate for flowered. Bracts involucrate, glabrescent. Perianth segments about 4 mm, obovate obtuse, greenish-white. The fruit is small

about 1.2-1.7 × 0.7-1.2 cm containing single seed 5-6 mm, ellipsoidal to almost globular black, with crustaceous pericarp (Zohary, 1972b).

It is a culinary herb often used to flavor soups, stews, and braises in Mediterranean Cuisine. Extracts from this plant can be used in treatment of indigestion and chronic bronchitis. The oil obtained from the fruits of laurel has been used to kill parasites and decrease rheumatism pains, against various skin diseases and loosing hair, also has been used in veterinary medicine to reduce external pain and protect animals against flies by rubbing them on the animal's skin. In addition to that, it can be used as carminative, analgesic, diuretic, emetic, emmenagogue, narcotic, nervine, immunostimulant, stomachic, antiaggregant, antiviral, antibacterial, fungicide, fungistat, antiseptic, astringent, anticarcinomic, antiestrogenic, anti-inflammatory, hepatoprotective, antiarthritic, lowering blood sugar and sudorific (Lev, 2006; Duke, 2003).

2.3.5. Salvia fruticosa (sage)

*Salvia frutic*osa is an evergreen shrub in the family *Lamiaceae*. It grows to1-1.5 m, appressed-wooly-tomentose galls of this plant, 2-3 cm in diameter, frequently developed on the tips of shoots and known as Habb el mariamiya. Leaves petiolate, 2-5 cm, rugulose, crenulate, ovate-oblong to lanceolate, truncate or nearly cordate at base, grayish-white on lawer face, greenish on

the upper. Leaves beneath the inflorescences with 1-2(-4) small, ovate or elliptic segments at base; floral leaves shorter than verticillasters, ovate, acuminate, sessile, later deciduous. Inflorescences paniculate or racemose, 15-20 cm, viscid; verticillasters 4-6- to many-flowered, mostly remote; bracteoles minute, membranous, deciduous; flowers pedicellate. Calyx campanulate, 7-8 mm, scarcely accrescent, viscid with glandular and partly eglandular hairs and with sessile glands; teeth triangular, acute, nearly equal. Corolla about 3 times as long as calyx, purplish-pink, rarely white (Zohary, 1972a).

The leaves are used as antiseptic, antispasmodic, astringent, carminative, cholagogue, depurative, expectorant, febrifuge, stimulant, tonic and vasodilator, hemorrhages. They are also used internally in the treatment of digestive and respiratory complaints, menstrual problems, infertility, nervous tension and depression (Azaizeh *et al*. 2006; Lev, 2006).

2.3.6. Ocimum basilucum (Basil)

Ocimum basilucum is aromatic plant in the family *Lamiaceae* and has the square stems, branching at the top, two-lipped flowers and abundant fragrance-bearing oil glands typical of many members of the mint family. Basils of the *basilicum* species, which provides most of the culinary varieties, are extremely variable in height, leaf size, color and form. Large-leaved

green basils, known by such names as sweet basil, Italian basil, and lettuce-leaf basil, can grow 2 or 3 feet in height. Small-leaved green forms such as dwarf basil, bush basil, or 'Spicy Globe' will grow 8 to 12 inches in height and as broad. Reddish-purple variations such as 'Dark Opal' or 'Purple Ruffles' tend to be intermediate in size, bearing purple instead of white flowers. These variants of *O. basilicum* have minor nuances of flavor, and are used for the same purposes. Petiolate leaves, ovate, 3–5 cm long and 1–3 cm broad, entire or slightly dentate. The Flowers are quite big, white in color; grow in whorl-like fasciclesin the axils of scales, together forming terminal spikes. Unusual among *Lamiaceae*, the four stamens and the pistil are not pushed under the upper lip of the corolla, but lay over the inferior. The plant tastes somewhat like anise, with a strong, pungent, sweet smell. Most common varieties are treated as annuals. Basil is a highly fragrant plant whose leaves are used as a seasoning herb for many different types of foods; essential oils are used in perfumery. Leaves and flowering parts of *O. basilicum* are traditionally used as antispasmodic, aromatic, carminative, digestive, galactogogue, stomachic, and tonic agents. They have been also used as a folk remedy to treat various ailments such as; feverish illnesses, poor digestion, nausea, abdominal cramps, gastro-enteritis, migraine, insomnia, depression, gonorrhea, dysentery, Cataracts, colds, enteralgia, wounds and chronic diarrhea exhaustion. Externally, they have been applied

for the treatment of acne, loss of smell, insect stings, snake bites, and skin infections (Adiguzel *et al*., 2004; The Herb Society of America, 2003 ;Ghazanfar, 1994; Kresanek, 1989).

2.3.7. Syzygium aromaticum (Cloves)

Syzygium aromaticum is the aromatic dried flower buds of a tree in the family *Myrtaceae*. The clove tree is an evergreen which grows to a height ranging from 10-20 m, having large oval leaves and crimson flowers in numerous groups of terminal clusters. The flower buds are at first of a pale color and gradually become green, after which they develop into a bright red, when they are ready for collecting. Cloves are harvested when 1.5-2 cm long, and consist of a long calyx, terminating in four spreading sepals, and four unopened petals which form a small ball in the centre (Bisset, 2001). Clove is reported as natural source of food flavoring, analgesic, anti-emetic, toothache remedy, anesthetic, antibacterial, antiviral, fungicide, fungistat, antidotal, antioxidant, antiaggregant, antiperspirant, antiseptic, carminative, deodorant, stimulant, stomachic, tonic, vermifuge, antihistaminic, antiinflammatory, antipyretic, antispasmodic, anticough, stomachic, antidermatitic, astringent, have anticarcinogenic property, contraceptive in low doses, useful in cataract and in case of dysmenorrhea (Tajuddin *et al*., 2004; Duke, 2003; Barnes *et al*., 2002; Ghazanfar, 1994).

2.3.8. Rosa damascena (Damask rose)

Rosa damascena belongs to family *Rosaceae*. It is a deciduous shrub growing to 2.2 m tall, the stems densely armed with stout, curved prickles and stiff bristles. The leaves are alternate, pinnate, with sharply toothed oval shaped with five (rarely seven) leaflets. Flowers terminal, solitary, or in corymbs. Hypanthium urceolate, becoming coloured and fleshly in fruit; epicalyx absent; stamens and carpels numerous; styles protruding through the orifice of the disc, sometimes forming a short column; ovules form one fruit, a pseudocarp of numerous achenes in the hypanthium (Tutin *et al*., 1981). They are renowned for their fine fragrance, and their flowers are commercially harvested for rose oil used in perfumery, cleanses facial skin (Lev, 2006). It used also in cardiotonic, febrifuge, nervine, tonic, cough, fever, mouth ulcers, skin disorders, throat pain (Ghazanfar, 1994).

2.4. Staphylococcus aureus:

Taxonomically, the genus *Staphylococcus* is in the bacterial family *Staphylococcaceae*. Staphylococci are Gram-positive spherical bacteria, which usually arranged in grape-like clusters, facultative anaerobes, catalase-positive and oxidase-negative. The genus *Staphylococcus* has at least 30 species. The three main species of clinical importance are *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. *S. aureus* is coagulase-positive which

differentiates it from other species. It has large, round, golden-yellow colonies, often with β-hemolysis, when grown on blood agar.

S. aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. Approximately 20–30% of the human population are *S. aureus* carriers. *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils, cellulitis folliculitis, furuncles, carbuncles, scalded skin syndrome and abscesses, to more serious infections such as mastitis, pneumonia, meningitis, osteomyelitis endocarditis, urinary tract infections, Toxic shock syndrome, and septicemia. Some *S. aureus* strains are able to produce staphylococcal enterotoxins and are the causative agents of staphylococcal food poisonings. This bacterium is a significant cause of nosocomial infections, as well as community-acquired diseases. This organism expresses many potential virulence factors: (1) surface proteins that promote colonization of host tissues; (2) invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase); (3) surface factors that inhibit phagocytic engulfment (capsule, Protein A); (4) biochemical properties that enhance their survival in phagocytes (catalase production); (5) immunological disguises (Protein A, coagulase, clotting factor); and (6) membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin; (7) exotoxins that damage host tissues or otherwise

provoke symptoms of disease (staphylococcal enterotoxins, the exfoliative toxins, and toxic shock syndrome toxin) (8) inherent and acquired resistance (Dinges *et al*., 2000; Brooks *et al*., 2001).

2.5. Antimicrobial agents and antibiotics

2.5.1 General action of antimicrobial drugs and antibiotics:

Antimicrobial drugs either kill microorganisms directly (bactericidal) or prevent their growth (bacteriostatic). The mechanism of action of most antimicrobial drugs is not completely understood. However, these mechanisms of action can placed under the following headings:

a. Inhibit of cell wall synthesis: The cell wall of a bacterium consists of a macromolecular network called peptidoglycan, which is found only in bacterial cell walls. Antibiotics prevent the synthesis of intact peptidoglycan; consequently, the cell wall is greatly weakened and the cell undergoes lysis.

b. Inhibit of protein synthesis: The difference in ribosomal structure between prokaryotes and eukaryotes, accounts for the selective toxicity of antibiotics that affect protein synthesis. Antibiotics reacting with 50S portion of the 70S prokaryotic ribosomes, inhibits the formation of peptide bonds in the growing polypeptide chain. Some antibiotics react with the 30S portion of the prokaryotic ribosome; interfere with the attachment of the tRNA carrying the

amino acids to the ribosome, preventing the addition of amino acids to the growing polypeptide chain. Other antibiotics interfere with the initial steps of protein synthesis by changing the shape of the 30S portion. This interference causes the genetic code to be read incorrectly.

c. Injury to plasma membrane: Certain antibiotics bring about changes in the permeability of the plasma membrane; these result in the lose of important metabolites from the microbial cells. Some antifungal drugs combine with sterols in the fungal plasma membrane to disrupt the membrane.

d. Inhibition of nucleic acid replication and transcription: Some antibiotics inhibit bacterial growth by binding strongly with DNA-dependent RNA polymerase of bacteria. Thus it inhibits RNA synthesis. Other antibiotics inhibit microbial DNA synthesis by blocking DNA gyrase.

e. Inhibit of synthesis of essential metabolites: certain enzymatic activity of microorganisms can be competitively inhibited by a substance that closely resembles the normal substrate for the enzyme. In many microorganisms, para-aminobenzoic acid (PABA) is the substrate for an enzymatic reaction leading to synthesis of folic acid, which functions as a coenzyme for the synthesis of the nitrogenous bases of nucleic acids and many amino acids. In the presence of sulfanilamide, the enzyme that converts PABA to folic acid

combines with the drug instead of with PABA (Tortora *et al*., 1995; Brooks *et al* 2001).

2.5.2. Antimicrobial drugs and antibiotics used in combinations:

In rational drug therapy, the concurrent administration of two or more drugs is often essential and sometimes mandatory in order to achieve the desired therapeutic goal or to treat co-existing diseases. However, the drug interaction may have different effects on the host as well as the infecting microorganism. The potential benefits of using combined antimicrobial therapy can be treatment of mixed infections, therapy of severe infections in which a specific causative organism is known, to achieve bactericidal synergism or to provide bactericidal action, reducing the need time for long-term antimicrobial therapy and prevention of the emergence of resistant (Brooks *et al*., 2001).

Two drugs can interact in several ways. They are usually:

1. Indifference: the combined action is no greater than that of the more effective agent when used alone.

2. Addition: the combined action is equivalent to the sum of the actions of each drug when used alone.

3. Synergism: the combined action is significantly greater than the sum of both effects. This effect can occur in several types of situations:

a. Two drugs may sequentially block a microbial metabolic pathway

b. A drug such as cell wall inhibitor may enhance the entry of an aminoglycoside into bacteria and thus produce synergistic effects.

c. One drug may affect the cell membrane and facilitate the entry of the second drug

d. One drug may prevent the inactivation of a second drug by microbial enzymes

4. Antagonism: the combined action is less than the more effective agent when used alone. Antagonistic effect is rarely occurring in clinical antimicrobial therapy. It occurs when a bacteriostatic drug (inhibits protein synthesis) is given with a bactericidal drug. Antagonism occurs mainly if the bacteriostatic drug reaches the site of infection before the bactericidal drug (Brooks *et al*., 2001; Levinson and Jawetz, 2002).

Chapter Three

Materials and Methods

3.1. Media preparation

3.1.1. Nutrient Agar (NA): A nutrient agar was prepared according to the manufacturer's instructions labeled on the bottle (Acumedia). A 1L bottle containing deionized water (500 mL) and 11.5 g of Nutrient Agar was heated and stirred until the agar dissolved. The solution was allowed to boil for 1 min, then autoclaved at 121 °C for 15 minutes and then was allowed to cool. The agar was poured into sterile Petri dishes (20ml) that were covered and left overnight. The following morning the Petri dishes were turned upside down and refrigerated.

3.1.2. Nutrient Broth (NB): A nutrient broth was prepared according to the manufacturer's instructions labeled on the bottle (Acumedia). A 0.5 L bottle containing deionized water (250 mL) and 2 g of nutrient broth were mixed well and boiled The broth then was divided into tubes each had 5-10 ml and covered by cotton. The tubes were autoclaved at 121 °C for 15 minutes, allowed to cool and then refrigerated.

3.1.3. Mueller-Hinton Broth (MHB): Mueller-Hinton broth was prepared according to the manufacturer's instructions labeled on the bottle (Oxoid). A 0.5 L bottle containing deionized water (250 mL) and 5.3 gm Mueller-Hinton broth were mixed well. The bottle was autoclaved at 121 °C for 15 minutes, allowed to cool and then refrigerated.

3.1.4. Mannitol salt agar (MSA):

Mannitol agar was prepared according to the manufacturer's instructions labeled on the bottle (Acumedia). A 1L bottle containing deionized water (250 mL) and 27.75 g of Manitol salt agar was heated and stirred until the agar dissolved. The solution was allowed to boil for 1 minute, and then was autoclaved at 121 °C for 15 minutes. After that it was allowed to cool, and the agar was poured into sterile Petri dishes (20ml) that were covered and left overnight. The following morning the Petri dishes were turned upside down and refrigerated.

3.1.5. Meullar Hinton agar (MHA):

Meullar agar was prepared according to the manufacturer's instructions labeled on the bottle (Acumedia). A 2L bottle containing 1L of deionized water and 38 g of MHA was heated and stirred until the agar dissolved. The solution was allowed to boil for 1 minute, and then was autoclaved at 121 °C for 15 minutes. After that it was allowed to cool, and the agar was poured into sterile Petri dishes (25-30 ml) that were covered and left overnight. The following morning the Petri dishes were turned upside down and refrigerated.

3.2. Bacterial preparation:

3.2.1. Preparation of starter cultures

A flame sterilized inoculating loop was used to scrape a colony from the nutrient agar plate and then transferred into a tube containing 5-10 ml of nutrient broth. The loop was rotated numerous times to ensure that the tip of the loop came in contact with the bottom of the vial. The inoculated broth was incubated at 37 °C for 4-6 h and gently agitated approximately every half an hour. These cultures were used to inoculate Mueller Hinton Agar to evaluate antibacterial activity by well-diffusion method.

3.2.2. Preparation McFarland turbidity standard No. 0.5

McFarland 0.5 turbidity standard was prepared by adding 50 µl of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl2•2H_2O$) solution to 9.95 ml of 1% (vol/vol) sulfuric acid. McFarland standard tube was then sealed with Parafilm to prevent evaporation and stored in the dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm and water as a blank standard was 0.08 to 0.13. The 0.5 McFarland standard was vigorously agitated the turbidity on a vortex mixer before use. As with the barium

sulfate standards, a 0.5 McFarland Standard is comparable to a bacterial suspension of 1.5 X 10^8 colony-forming units (CFU)/ml (Andrews, 2006).

3.3.3. Preparation of inoculum for MIC determination

Three to four isolated overnight cultured colonies were transferred to a tube of sterile saline. The bacterial suspension was compared to the 0.5 McFarland standard against a sheet of white paper on which sharp black lines were drawn. The bacterial suspension was adjusted to be the proper density as the McFarland 0.5 by adding sterile saline or more bacterial growth. Then bacterial suspension was diluted to obtain 10^4 CFU/ml.

3.4. Identification of S.aureus

Four *S. aureus* isolates used in this study were recovered from urine sample, Thabet Thabet's Hospital, Toulkarm (strain no. 1); semen sample, Al-Zaka Hospital, Toulkarm (strain no. 2); diabetic foot wound, Al-Makased Hospital, Jerusalem (strain no. 3); and chest wound, Al-Makased Hospital, Jerusalem (strain no. 4). Identification of these isolates was confirmed using the following assays:

3.4.1. Gram staininig: Gram staining of bacteria was performed from nutrient broth as described by Cappiccino and Sherman (1996).

3.4.2. Catalase test: Catalase test was carried out by addition 1-2 drops of 3% hydrogen peroxide on bacterial colony cultured on nutrient agar (Cappiccino and Sherman 1996).

3.4.3. Mannitol fermentation:

Aseptically a single line of inoculation of test organism has been carried out on mannitol salt agar. The plate culture has been incubated for 24 hours at 37ºC (Cappiccino and Sherman 1996).

3.4.4. Tube coagulase test: This test was done by inoculating 1ml of diluted (1:4) fresh citrated human plasma with a colony from pure culture of Gram-positive cocci in grape-like clusters that is both catalase- and mannitol-positive. The tube then incubated for 18-24 hours at 37ºC (Cappiccino and Sherman 1996).

3.5. Antimicrobial agents and antibiotics

Six drugs were evaluated for synergism assays. These included oxytetracycline HCl (10%), enrofloxacin (10%), getamicin Sulphate (50%), sulfadimethoxine as sodium (40%), cephalexin (0.15%) and penicillin G (penicillin G procaine 900000 and penicillin G sodium 300,000 I.U). All these Antimicrobial agents and antibiotics were produced by Jerusalem Pharmaceutical CO. Balsam branch except penicillin G was produced by

Birzeit-Palestine Pharamaceutical CO, and were diluted to a final concentration 100 µg/ml except penicillin G to 100 I.U/ml for well diffusion method and 200 µg/ml, and 200 I.U/ml for MIC test.

3.6. Plant collection and Extract preparation:

The plant materials used in this study consisted of *Psidium guajava* (leaf), *Rosmarinus officinalis* (leaf), *Salvia fruticosa* (leaf), *Majorana syriaca* (leaf), *Ocimum basilucum* (leaf), *Rosa damascena* (flower) which are growing in Palestine, *Syzygium aromaticum* (dried flowerbud) and *Laurus nobilis* (leaf) which were collected from Palestinian markets. These plants were identified by Dr. Firas Sawalha, Department of Plant Production, Faculty of Agriculture, An-Najah National University, Nablus, Palestine. The fresh plant materials were dried in an open air protected from direct exposure to sunlight. Water extract was prepared as describe previously (Adwan *et al*., 2006). Approximately of 60 g of dried plant materials were separately powdered, extracted by adding 150-200 ml boiled distilled water, and then kept for 1 h. The extracts were filtered through Whatman No. 2 filter paper under vacuum. Extracts were concentrated to dryness at 37°C. Then, 100 mg of the dry residue was dissolved in 1 ml of sterile distilled water.

3.7. Antimicrobial activity tests

3.7.1. Determination of the combined activity using Well-diffusion method

Antibacterial activity was measured using a well diffusion method according to the National Committee for Clinical Laboratory Standard (NCCLS, 1993). Briefly, Petri plates containing approximately 25-30 ml of Mueller Hinton agar medium were swabed using cotton applicator with a 4-6 h starter culture of the bacterial strains. Wells (6mm diameter) were punched in the agar and filled with 30 µl of plant extracts or antibiotics and in case of synergism effect 30 µl of each has been added into well. Replicate of each plate has been done. The plates were incubated at 37 °C for 18-24 h. The antibacterial activity was assessed by measuring the inhibition zone diameter (mm) around the well. The average of three replicates for each extract, antibiotic, and combination has been calculated. Synergism effect was considered when combinations exhibited with enlargement of combined inhibition zone size by ⩾5 mm (Ahmad and Aqil, 2007).

3.7.2. Determination of MIC by microdilution method

MIC of antibiotic was determined by the microdilution method as described by NCCLS, 2000. The antibiotic was serial diluted in Mueller Hinton broth.

Plant extracts solution were separately added into wells in a final concentration 3mg/ml, then bacterial inoculum size of 10^4 CFU/ml was added to each well. Rows of wells with bacteria and serial diluted antibiotic only were included. Control wells were also included in these experiments, and contain either Mueller Hinton broth only or plant extracts and Mueller Hinton broth without bacteria. Each plant extract was run in duplicate. The test plates were incubated at 37°C for 18 h. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism.

Chapter Four

Results

4.1. Identification of S. aureus isolates

Four strains of bacteria were identified as *S. aureus* using general morphological and biochemical assays. These strains were Gram-positive cocci, in clusters, catalase positive, grow on mannitol salt agar, ferment mannitol and coagulase positive

4.2. Detection combination effect using well-diffusion method

The results of the experiments using well-diffusion method demonstrate that the test plants contain bioactive compounds. The action of combination between aqueous plant extracts (*Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis*, and *Rosa damascena*) and different antimicrobial agents (oxytetracycline HCl, gentamicin sulfate, penicillin G, cephalexin, Sulfadimethoxine as sodium, and enrofloxacin) using well-diffusion method. Against 4 test strains of *S. aureus* did not show an enhancement in the action of antimicrobial agents. The combined effect of both did not exceed significantly the sum of their individual effects. Results of these experiments using well-diffusion method showed that the interactions between the mentioned antibiotics and plant extracts were mainly additive against the four strains of *S. aureus*, which showed that the

inhibitory action of the combined agents were equivalent to the sums of the actions of the single agents (Table 1).

Table 1. The inhibition zone diameter (mm) of antimicrobial agents, plant extracts, and combination against 4 clinical isolates of *S. aureus* using well diffusion method**.**

Antibiotic[a]/plant extrat	Average of Inhibition zone diameter (mm) against *S. aureus* isolates			
	Strain 1	Strain 2	Strain 3	Strain 4
CN	**17.6**	**22**	**14.3**	**16**
H_2O (control)	**0.0**	**0.0**	**0.0**	**0.0**
P. guajava	17.3	15.7	15.3	17.7
P. guajava + CN	16.7	20	13.6	15.0
R. officinalis	12.7	10.7	11.3	12.3
R. officinalis + CN	15	19.3	13.2	13.3
S. fruticosa	15.3	13.7	13.3	15.7
S. fruticosa + CN	13	21.0	13.0	14.5
M. syriaca	11.0	10.7	15.0	12.0
M. syriaca + CN	15.7	18.5	13.3	13.5

O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + CN	15.3	19.0	15.6	13.5
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + CN	15.0	20.0	18.7	15.7
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + CN	14.7	20.7	14.0	15.0
R. damascene	19.3	16.0	17.7	18.0
R. damascena + CN	19.0	19.7	18.0	18.0
ENR	**27**	**28**	**30**	**27.7**
H_2O (control)	**0.0**	**0.0**	**0.0**	**0.0**
P. guajava	17.3	15.7	15.3	17.7
P. guajava + ENR	26.7	28.7	26.7	25
R. officinalis	12.7	10.7	11.3	12.3
R. officinalis + ENR	28	30.7	26.7	26
S. fruticosa	15.3	13.7	13.3	15.9
S. fruticosa + ENR	29.3	29.3	28.3	27.0
M. syriaca	11.0	10.7	15.0	12.0

M. syriaca + ENR	29.0	31.3	29.3	28.7
O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + ENR	28.7	30.7	29.7	29.7
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + ENR	26.3	31.7	29.0	29.3
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + ENR	30.0	32.3	29.0	29.3
R. damascene	19.3	16.0	17.7	18.0
R. damascena + ENR	26	29.3	28.7	29.0
OT	**28.3**	**16.6**	**17.6**	**25.7**
H_2O (control)	**0.0**	**0.0**	**0.0**	**0.0**
P. guajava	17.3	15.7	15.3	17.7
P. guajava + OT	29	17.0	17.0	27.0
R. officinalis	12.7	10.7	11.3	13
R. officinalis + OT	29.3	15.7	15.0	28.3
S. fruticosa	15.3	13.7	13.3	15.9
S. fruticosa + OT	28.0	18.7	15.7	28.7

M. syriaca	11.0	10.7	15.0	12.0
M. syriaca + OT	27.3	16.3	14.7	26.3
O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + OT	26.7	17.0	14.7	25.7
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + OT	27.0	19.0	18.3	27.3
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + OT	27.3	19.3	15.7	26.3
R. damascene	19.3	16.0	17.7	18.0
R. damascena + OT	29.7	19.7	18.7	27.3
CL	**29.7**	**31.0**	**35.0**	**31.3**
H_2O (control)	**0.0**	**0.0**	**0.0**	**0.0**
P. guajava	17.3	15.7	15.3	17.7
P. guajava + CL	33.3	32.0	36.3	29
R. officinalis	12.7	10.7	11.3	12.3
R. officinalis + CL	31.3	30.0	34.0	29.0
S. fruticosa	15.3	13.7	13.3	15.9

S. fruticosa + CL	30.7	31.3	37.7	31.0
M. syriaca	11.0	10.7	15.0	12.0
M. syriaca + CL	31.0	31.3	33.3	30.0
O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + CL	33	33.3	34.3	30.7
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + CL	34.7	31.0	37.0	32.0
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + CL	32.0	30.7	36.7	33.0
R. damascene	19.3	16.0	17.7	18.0
R. damascena + CL	33.3	31.0	38.0	35.3
P	11.3	12.7	13.3	12.3
H_2O (control)	0.0	0.0	0.0	0.0
P. guajava	17.3	15.7	15.3	17.7
P. guajava + P	20.0	16.3	19.3	18.3
R. officinalis	12.7	10.7	11.3	12.3
R. officinalis + P	16.0	14.0	15.3	15.0

S. fruticosa	15.3	13.7	13.3	15.9
S. fruticosa + P	17.7	15.3	17.3	17.3
M. syriaca	11.0	10.7	15.0	12.0
M. syriaca + P	15.0	14.7	19.0	14.0
O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + P	15.3	15.3	12.6	13.7
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + P	18.7	18.3	23.0	17.0
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + P	15.3	14.3	16	14.3
R. damascene	19.3	16.0	18.7	18.0
R. damascena + P	20.3	19.3	22.7	17.3
SDM	8.3	8.6	9.3	8.7
H_2O (control)	0.0	0.0	0.0	0.0
P. guajava	17.3	15.7	15.3	17.7
P. guajava + SDM	18.3	15.6	16.0	17.3
R. officinalis	12.7	10.7	11.3	12.3

R. officinalis + SDM	11.3	12.6	11.3	13.0
S. fruticosa	15.3	13.7	13.3	15.9
S. fruticosa + SDM	13.7	17.0	13.3	16.3
M. syriaca	11.0	10.7	15.0	12.0
M. syriaca + SDM	9.7	13.3	13.3	11.7
O. basilicum	9.0	7.0	8.0	8.5
O. basilicum + SDM	8.3	8.3	10.7	8.7
S. aromaticum	16.0	14.7	19.7	15.0
S. aromaticum + SDM	19.3	15.3	18.7	15.0
L. nobilis	7.0	7.0	8.0	8.0
L. nobilis + SDM	8.7	9.7	10.7	9.3
R. damascene	19.3	16.0	17.7	18.0
R. damascena + SDM	19.3	17.3	18.3	19.7

[a] P, Penicillin G; CN, Gentamicin sulphate; CL, Cephalexin; SDM, sulfadimethoxine as sodium; ENR, Enrofloxacin; OT, Oxytetracycline HCl.

4.3. Detection combination effect using microdilution method

Our results showed that there is a decrease in the MIC in case of combination between plant extracts (*Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis* and *Rosa damascena*) and different antimicrobial agents (oxytetracycline HCl, gentamicin sulfate, penicillin G, cephalexin, Sulfadimethoxine as sodium, and enrofloxacin) against 3 test strains of *S. aureus* using microdilution method. This implies that these plant extracts increased the antibacterial activity of the antibiotics against the test strains of *S. aureus*, and showed synergistic interaction against test strains. Minimum fold inhibition with drug-plant extract combinations against these strains is presented in Table 2. The change in MIC was noticed in all plant extracts against test antibiotics including these plants showed weak antibacterial activity by well diffusion method. Also our results showed that synergism effect between antimicrobial agent and plant extract was occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in resistant strains was higher than the sensitive strains.

Table 2. Minimum inhibitory concentration of antibiotics alone and in combination with plant extracts against 3 clinical isolates of *S. aureus* using microdilution method.

	MIC (mg/l)			Minimum
Antibiotic [a]/plant extrat	Strain 1	Strain 2	Strain 3	fold inhibition
CN	**3.125-1.563**	**1.563**	**3.125-1.563**	
P. guajava + CN	0.0244	0.0488	0.0244	32
R. officinalis + CN	0.0244	0.0244	0.0244	64
S. fruticosa + CN	0.0122	0.0244	0.0122	64
M. syriaca + CN	0.0244	0.0488-0.0244	0.0244	32
O. basilicum + CN	0.0244	0.0488	0.0244	32
S. aromaticum + CN	0.0244-0.0122	0.0244	0.0122	64
L. nobilis + CN	0.0488	0.0977	0.0488	16
R. damascena + CN	0.0244-0.0122	0.0244	0.0122	64
ENR	**0.195**	**0.39**	**0.39**	
P. guajava + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
R. officinalis + ENR	$<6.1X10^{-3}$	<0.0122	$<6.1X10^{-3}$	>32
S. fruticosa + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
M. syriaca + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32

O. basilicum + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
S. aromaticum + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
L. nobilis + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
R. damascena + ENR	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
OT	**0.78**	**6.25-3.125**	**6.25-3.125**	
P. guajava + OT	0.0244	<0.0244	<0.0244	≥32
R. officinalis + OT	0.0488	<0.0244	<0.0244	≥16
S. fruticosa + OT	<0.0244	<0.0244	<0.0244	>32
M. syriaca + OT	0.0488	0.0488	0.0488	16
O. basilicum + OT	0.0488	0.0488	0.0488	16
S. aromaticum + OT	<0.0244	<0.0244	<0.0244	>32
L. nobilis + OT	0.0488	0.0488	0.0488	16
R. damascena + OT	<0.0244	<0.0244	<0.0244	>32
CL	**0.39**	**0.39**	**0.195**	
P. guajava + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
R. officinalis + CL	$<6.1X10^{-3}$	<0.0122	$<6.1X10^{-3}$	>32

S. fruticosa + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
M. syriaca + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
O. basilicum + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
S. aromaticum + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
L. nobilis + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
R. damascena + CL	$<6.1X10^{-3}$	$<6.1X10^{-3}$	$<6.1X10^{-3}$	>32
P **(**Unit**)**	**>100**	**>100**	**>100**	
P. guajava + P	3.125-1.563	1.563	1.563	>32
R. officinalis + P	1.563	0.78	0.78	>64
S. fruticosa + P	1.563	0.78	0.39	>64
M. syriaca + P	1.563-0.78	0.78	1.563-0.78	>64
O. basilicum + P	3.125	3.125	3.125-1.563	>32
S. aromaticum + P	0.78	0.78	0.78	>128
L. nobilis + P	3.125	3.125	3.125-1.563	>32
R. damascena + P	0.78	0.78	0.78	>128
SDM	**>100**	**>100**	**>100**	

P. guajava + SDM	6.25	6.25	6.25	>16
R. officinalis + SDM	6.25	6.25	6.25	>16
S. fruticosa + SDM	6.25	6.25	6.25-3.125	>16
M. syriaca + SDM	6.25-3.125	6.25-3.125	6.25-3.125	>16
O. basilicum + SDM	6.25-3.125	6.25	6.25	>16
S. aromaticum + SDM	1.563	1.563	1.563-0.78	>64
L. nobilis + SDM	12.5-6.25	12.5-6.25	6.25	>8
R. damascena + SDM	1.563	1.563	1.563	>64

[a] P, Penicillin G; CN, Gentamicin sulphate; CL, Cephalexin; SDM, sulfadimethoxine as sodium; ENR, Enrofloxacin; OT, Oxytetracycline HCl.

Chapter Five

Discussion

Discussion

The need to combat microbial resistance to antibiotics is an increasing global concern. *S. aureus* is one of the gram-positive microorganisms that have been shown to exhibit resistance to a wide range of commonly available antibiotics. Therefore, new chemotherapeutic agents and new approaches are urgently needed to combat such multiple-antibiotic-resistant bacteria. Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacteria infection. 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). Despite the abundant literature about the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics (Gibbons, 2004). This research focuses on antimicrobial activity testing of different plant extracts and then synergy testing of these extracts with known antimicrobial agents of different mechanisms (protein synthesis inhibition: oxytetracycline HCl and gentamicin sulfate; cell wall synthesis inhibition: penicillin G and cephalexin; folic acid synthesis inhibition: Sulfadimethoxine as sodium; and nucleic acid synthesis inhibition: enrofloxacin) using both well-diffusion and

microdilution method. The plants used in this research are widely consumed in Palestine *which are:Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis*, and *Rosa damascena*.

The results of the experiments using well-diffusion method demonstrate that these plants contain bioactive compounds some of them has a weak effect. The interactions between mentioned antibiotics and plant extracts were mainly additive against the four strains of *S. aureus*. This could be attributed to the inability of higher concentrations of plant extracts to diffuse through the nutrient agar medium. This impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial effects of plant extracts using the agar diffusion method (Esimone *et al*., 2006).

In this study, synergism effect resulting from the combination of antimicrobial agents with aqueous crude extracts was verified for all plants. In vitro studies have reported synergistic effects with significant reduction in the MICs of the antibiotics, resulting from the combination of antibiotics with different crude plant extracts against *S. aureus* strains (Betoni *et al*., 2006; Esimon *et al*., 2006; Darwish *et al*., 2002; Aqil *et al*., 2005; Braga *et al*., 2005; Yam *et al*., 1998; Yang *et al*., 2005) and stand out as veritable sources of potential resistance modifying agents (Sibanda and Okok 2007;

Gibbons *et al*. 2003; Dickson *et al*. 2006). Also Synergistic effects have been documented against other pathoges (Chang *et al*., 2007; Horiuchi *et al*., 2007; Ahmad and Aqil, 2007; Ibezim *et al*., 2006; Nascimento *et al*., 2000).

Synergistic interactions was observed between methanolic extracts of certain Brazilian plants included guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), carqueja (*Baccharis trimera*), and mint (*Mentha Pieria*) and some antibiotics which represented inhibitors of protein synthesis, cell wall synthesis, nucleic acid synthesis and folic acid synthesis against *S. aureus* (Betoni *et al*. 2006). Other study revealed that methanolic extract from *Kola nitida* seeds potentiated the effects of the fluoroquinolones such as ciprofloxacin, pefloxacin and levofloxacin against *E. coli* and the MIC of these drugs decreased when combined with *Kola nitida* seeds extract (Ibezim *et al*., 2006). A synergistic effect was observed against different bacterial species, during the association of antibiotics with ethanolic extracts from clove (*Caryophyllus aromaticus*), jambolan (*Syzygyum joabolanum*), pomegranate (*Punica granatum*) and thyme (*Thymus vulgaris*) (Nascimento *et al*., 2000). It was also reported that sub-inhibitory levels (200 μg/ml) of methanolic extracts of some Jordanian plants showed synergistic interactions in combination with chloramphenicol, gentamicin, erythromycin and penicillin G against *S. aureus* (Darwish *et al*.,

2002). The methanolic extract of *Punica granatum* showed synergistic interactions agains both MRSA and MSSA bacteria with chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin. The bactericidal activity of the combination of *Punica granatum* extract (0.1×MIC) with ampicillin (0.5×MIC) by time-kill assays, reduced cell viability by 99.9 and 72.5% in MSSA and MRSA populations, respectively (Braga *et al*., 2005). Also ethanolic extracts of some Chinese plants, *Isatis tinctoria*, *Scutellaria baicalensis* and *Rheum palmatum* can improve the antimicrobial activity of penicillin G, gentamycin, ciprofloxacin, and ceftriaxone used against *S. aureus* (Yang *et al*., 2005). It was also observed that ethanolic crude extracts of several Indian medicinal plants, *Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica* and *Plumbago zeylanica* showed synergistic interactions with tetracycline and ciprofloxacin against Extended Spectrum β-lactamase (ESβL), producing multidrug-resistant enteric bacteria with ciprofloxacin showing more synergy with the extracts than tetracycline (Ahmad and Aqil, 2006). In addition, aqueous crude khat (*Catha edulis*) extracts at a sub- MIC (5 mg/ml) resulted in a 2 to 4-folds potentiation of tetracycline against resistant strains *Streptococcus sanguis* TH-13, *Streptococcus oralis* SH-2, and *Fusobacterium nucleatum* (Al-hebshi *et al*., 2006). In other study, aqueous extracts of tea (*Camellia sinensis*) have been shown to reverse methicillin resistance in MRSA and also, to some extent,

penicillin resistance in betalactamase-producing *S. aureus*. These phenomena are explained by prevention of PBP2′ synthesis and inhibition of secretion of β-lactamase, respectively. Forty to one hundred fold dilutions of tea extracts was able to reduce the MICs of high- level resistant MRSA (≥ 256 μg/ml) to less than 0.12 μg/ml for methicillin and penicillin (Yam *et al*., 1998). Recently, It was also showed that the combination of ethanolic crude extracts of some Palestinian plants *Rhus coriaria* (leaf), *Psidium guajava* (Leaf), *Lawsonia inermis* (Leaf) and Sacropoterium spinosum (seed) and antimicrobial drugs including oxytetracycline HCl, gentamicin sulphate and sulfadimethoxine against 4 clinical isolates of MRSA was lead to increase the activity of these antibiotics against these bacterial strains (Adwan *et al*., 2008).

In our experiments, the change in MIC was noticed in all plant extracts against test antibiotics including these plants showed weak antibacterial activity by well diffusion method. These results were consistent with a previous report who mentioned a synergetic effect even these extracts did not show any activity by themselves (Nascimento *et al*., 2000). Also our results showed that synergism effect was occurred in both sensitive and resistant strains but the magnitude of minimum fold inhibition in sensitive is less than resistant strains. These results were consistent with that which showed that

synergistic interactions occurred in both resistant and sensitive *S. aureus* (Braga *et al*., 2005; Darwish *et al*., 2002).

All plant extracts showed a decreased in MIC to test antimicrobial agents, and this could be referred to that these crude extracts have many different phytochemicals (Duke *et al*., 2003), which might inhibit bacteria by different mechanisms. This double attack of both agents on different target sites of the bacteria could theoretically lead to either an additive or a synergistic effect (Esimone *et al*., 2006). Screening for such activities in crude extracts is the first step in identifying leads for isolation of such compounds, and some plants have provided good indications of these potentials for use in combination with antimicrobial therapy. Further separation and purification of the crude extracts might show an increased bioactivity than the crude extracts. This may be due to numerous compounds within the crude extracts may have interfered with the actions of one another. Once they were separated by various chromatography methods however, the inhibiting effect of one on the other had reduced significantly (Adwan *et al*., 2008).

Here we recommended the evaluation of the exact drug-plant ratio at which the interaction is maximal between the plant extract and antimicrobial drug. A wider study with increase in the number of drugs in each group, increase number of clinical isolates, and the identification of the effective compounds

in the crude extract are also necessary. Purification of active fractions is needed to isolate and chemically characterize these active compounds in order to establish the mode of action against the *S. aureus* isolates and the mechanism of synergy, which is fundamental to development of pharmacological agents to treat diseases by *S. aureus* using medicinal plants.

In conclusion, the results of this study were encouraging, although clinical controlled studies are needed to define the real efficacy and possible toxic effects *in vivo*. This study probably suggests the possibility of concurrent use of these antimicrobial drugs and extracts in combination in treating infections caused by *S. aureus* strains or at least the concomitant administration of these plants and antimicrobial drugs may not impair the antimicrobial activity of these antibiotics. However, it is hard to predict synergistic effects *in vivo* on the basis of the presented *in vitro* evidence alone because it is difficult to estimate the *in vivo* concentration of active ingredients, especially the bioavailable concentration of free (active) ingredients, after plants have been ingested. Therefore, these results revealed the importance of plant extracts when associated with antibiotics to control bacteria.

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جامعة النجاح الوطنية

عمادة كلية الدراسات العليا

التأثيرات التآزرية لبعض المستخلصات النباتية والمضادات الحيوية على المكورات العنقودية المعزولة من عينات مرضية

إعداد

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قدمت هذه الاطروحة استكمالا لمتطلبات درجة الماجستير في العلوم البيئية بكلية الدراسات العليا في جامعة النجاح الوطنية –نابلس – فلسطين–

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ملخص

تم التركيز في هذا البحث على المضادات البكتيرية لمجموعة من المستخلصات المائية النباتية وهي: الجوافة, اكليل الجبل, المريمية, الزعتر العادي, الريحان, شجر القرنفل, الغار والورد الجوري. أما المضادات الحيوية المستخدمة فهي: اوكسي تتراسيكلين HCL, جينتاميسين سلفات, البنسيلين G, سيفاليكسين, سلفا دايميثوكسين صوديوم و انروفلوكساسين. تم دمج المستخلصات المائية للنباتات المذكورة مع مجموعة من المضادات البكتيرية التي تعمل بآليات مختلفة وذلك لمعرفة تأثير الخليط على سلالات المكورات العنقودية. قد تم استخدام اختباران

خاصان في البحث هما طريقة انتشار الحفرة (well diffusion method) و طريقة التخفيف المتسلسل (Micodilution method). أظهرت نتائج طريقة انتشار الحفرة ان النباتات تحتوي على مواد فعالة بعضها كان تاثيره ضعيف, وأنه عند خلط المستخلصات النباتية مع المضادات الحيوية السابقة الذكر بهذه الطريقة كانت النتائج ذات تاثير اضافي (additive) على السلالات البكتيرية الاربع المستخدمة في التجربة .ولكن عند استخدام طريقة التخفيف المتسلسل كان التاثير تآزري (Synergestic) وذلك لنقصان في قيمة التركيز المانع الادنى (MIC) للمضادات الحيوية المستخدمة. وقد لوحظ تغيير التركيز المانع الادنى للمضادات الحيوية المستخدمة في جميع المستخلصات بما في ذلك التي اظهرت تاثير ضعيف بطريقة انتشار الحفرة. بالاضافة الى ذلك, أظهرت النتائج ان التاثير التآزري بين المضادات والمستخلصات حدث في كلا السلالات الحساسة و المقاومة للمضادات ولكن كان التاثير اكبر للسلالات المقاومة.