An-Najah National University

Faculty of Graduate Studies

THE ANTIBACTERIAL EFFECT OF DIFFERENT PALESTINIAN OAK SPECIES ON MRSA

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Dedication

For those who made this journey worth walking,
I dedicate this thesis to them.

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As I give thanks to God, I would like to express my sincere appreciation to my supervisors, Dr. Walid Basha and Dr. Raed Alkowni, for the guidance and encouragement they provided me during the work on this thesis. All thanks to Abd-Alrazaq Zarour (Research Assistant) in the microbiology research lab, Ahmed Mousa, the supervisor of the central research laboratory in the faculty of medicine, Mohammed Nabeel (Lab technician) in the animal lab, and my dearest friend Hidaya Qash for their valuable assistance.

All the love to those who light up my path and believe in me like no other, my husband and my precious kids, Khaled and Talia.

There are not enough words to express my gratitude to my idols, Mom and Dad, my supporting teammates, my sisters, and brothers.

All thanks and gratitude to my second family, who is always proud of me and supports me in every step I take.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

THE ANTIBACTERIAL EFFECT OF DIFFERENT PALESTINIAN OAK SPECIES ON MRSA

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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List of Contents

List of Tables

List of Figures

List of Appendices

THE ANTIBACTERIAL EFFECT OF DIFFERENT PALESTINIAN OAK SPECIES ON MRSA

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Abstract

Introduction: The most common cause of nosocomial pneumonia is staphylococcus aureus, with substantial mortality and morbidity. The growing bacterial resistance and side effects of antimicrobial medications have led to significant attention being paid to traditional medicinal plants in the past few decades. Compounds, derived from these plants, can be used to treat microbial infections as an alternative treatment.

Objective: The main aim of this study was to determine the potential antimicrobial effects of ethanol and aqueous extracts of three different species of Quercus against MRSA isolates.

Methods: Antibacterial activity of plant extracts was observed, using the agar well diffusion test.After observation, the researcher determined the Minimum Inhibitory Concentration (MIC) by 2-fold dilution of plant extracts yielding a serial dilution of the original extract. To check if the desired compound entered the site of infection in vivo and reached high levels to alter bacterial viability, mice were used as animal models of infection.

Results: Ten extractions (water and ethanol) were prepared from the three oak species. However, only seven showed MICs ranging between 1.56-12.5 mg/ml. In vivo testing showed an indication of bacterial inhibition ability of *Q.coccifera* extract (0.22 mg/ml) when added to the drinking water of a mice group. The bacterial count from a cultured swab from this group of infected mice was 160 CFU, the lowest count of all extracts.

Conclusions: Ethanol extracts of oak have more efficient antibacterial activity than water extracts. Water extracts, when mixed with the drinking water of mice, have a good antibacterial inhibitory ability. Future studies should be conducted to extract oak's active

ingredients using other solvents. Also, combining these extracts with other antibiotics might enhance their effect in vivo.

Keywords: MRSA; Quercus;Palestine; antibiotic resistance; plant extract; MIC;animal model.

Chapter One
Introduction

1.1 Background

Palestine is one of the rich ecosystems as part of the Eastern Mediterranean region (1). Although it is a small country, and due to its varied geography and environment, it has a great variety of wild plants. More than 2600 plant species are covered in the hills and mountains of Palestine, of which more than 700 are noted for their use as medicinal herbs or botanical pesticides (2,3).

Palestinian Quercus is one of the typical evergreen trees belonging to the Fagaceae family, which is considered an ecologically important woody plant family, as Quercus form the backbone of temperate forests in the Middle East (4).

Quercus's various species have been preserved due to its historical and heritage significance in Palestine, where vast sizes of the Quercus trees are found near holy sites and worship places. Also, Quercus has been consumed as food but very limited; the acorns were roasted and eaten in times of great necessity (5).

Like in many developing countries, herbal medicine is an integral part of Palestinian society and plays an essential role in today's public healthcare system (6). Many diseases in Palestine can be treated with herbal medicine, including intestinal diseases, urinary tract infections, infertility, and cutaneous abscesses. In addition, local Palestinian Quercus has been used to treat arthritis pain, ulcer, severe abdomen ache, bloody coughing, diabetes, and prostates issues. Besides, it helps treat urine problems, stomach cancer, and liver atrophy. It also helps lower blood pressure, giving energy to the body, purifying the blood, diarrhea, and breath irregularities (5).

The occurrence of microbial infectious diseases is continuously growing, primarily because microbial drug resistance to antimicrobial agents is currently accessible (7). Furthermore, these microbes have developed resistance in three or more antimicrobial groups to at least one agent (8) therefore named Multidrug-resistant bacteria (MDRB).

The resistance of pathogenic microorganisms to antibiotics has led to extensive research on extracts and biologically active compounds isolated from plant species. As a result, there is a widespread use of medicinal plants for their antiviral, antibacterial, and antifungal properties in many parts of the world (9).

In various parts of the world, Staphylococcus aureus is considered a major human pathogen in both community and health-related settings (10). It has a set of virulence factors and the capacity to develop resistance to most antibiotics. This ability is boosted by the continuous appearance of new clones, resulting in the emergence of methicillin-resistant S. aureus as a "superbug," also, unnecessary clinical use of methicillin has led to the appearance of methicillin-resistant *S. aureus* (MRSA) (11).

Plant-derived antimicrobials are promising sources of safe antimicrobials when contrasted with synthetic compounds because of their natural origin (12), and their phytochemicals may lead to new aspects of drug discovery (7). So There is an increasing interest nowadays in this alternative therapy for microbial infections (12).

Many studies around the world were conducted on traditional medicinal plant extracts to prove their antibacterial activity against MRSA. For instance, a study conducted by Zuo *et al.* in 2008 in china on 19 extracts from Chinese herbs and all the presented plants showed promising anti-MRSA activity with MIC of 1.25–3.07 mg/ml (13).

Another study by Aqil *et al*. in 2006 in India detected four medicinal plants with MICs ranged between (0.32 - 3.25) mg/ml, and phytochemical studies showed flavonoids and phenols to be the key active constituents in these plants (14).

Mickymaray also mentioned in his study, traditional medicinal plants and their bioactive compounds against clinically important pathogens, that the Antimicrobial screening performed on various medicinal plants, including *Quercus infectoria Olivier*, showed MIC equal to 100–200 μg/mL against MRSA. Therefore, he concluded that the effects of conventional antimicrobials could be improved by crude extracts and plant-derived bioactive compounds. This can be cost-effective, have fewer side effects, and increase the quality of therapy (12).

In addition to the above, a research described the tree *Q. infectoria Olivier* as a small tree that grows naturally in Greece, Asia Minor, and Iran. A gall-wasp, Adleria gallae-

tinctoria, attacks the young branches of this tree to form galls. Women use the galls as a drinking remedy after childbirth in combination with other herbs to restore uterine elasticity. In addition, it is widely used as a mouth washing powder for toothache and gingivitis treatment in Indian traditional medicine. *Q. infectoria* galls are also used medicinally as astringents, antidiabetics, antitremorines, local anesthetics, antivirals, antibacterials, antifungals, larvicidal, and anti-inflammatory agents. The primary ingredients of *Q. infectoria* galls are tannin (50-70 %) and a small portion of free gallic acid and ellagic acid (15).

The Quercus wood also has been found to have anti-bacterial properties against many bacteria like nosocomial *Acinetobacter Baumannii* (16), *Escherichia coli* (17), and many others.

Additional study on Quercus fruits showed that the total phenolic content of *Q. brantii* fruit extract was 3.010 mg GAE/g DW. Furthermore, flavonols and flavonoids were 1.813 mg/g and 0.654 mg/g, respectively. *S. aureus* and *E. faecalis* were significantly inhibited by the extract. In addition to its antioxidant properties, *Q. brantii* fruit extract also can act as a dietary supplement (18).

1.2 Palestinian species

Palestine is known for its rich plant variety represented by trees, shrubs, and herbs. For example, oak is considered a common evergreen tree that inhabits heights and plains of different places in Palestine, and sometimes it is found as dense forests that give shelter to a large number of beautiful plants and shade-demanding ferns, providing a habitat for a great diversity of different plants and animals (19).

The genus Quercus (oak), which belongs to the Fagaceae family, consists of over 600 species worldwide of evergreen trees and shrubs that might be monecious or deciduous (20).

There are many natural hybrid species in the genus Quercus, which makes taxonomy confusing. Three groups of oak trees were classified as subgenera: white oaks (*Leucobalanus*), red and black oaks (*Erythrobalanus*), and (*Cyclobalanus*).

In Quercus species, leaves are alternately simple, deciduous, or evergreen, with lobed, toothed, or entire margins. The male flowers appear with or following the leaves as

pendant yellow catkins. On the same tree, female flowers bloom in one to more than two spikes; each flower has a husk of overlapping scales that expands to hold the fruit, called an acorn. There are sometimes glandular margins on the margins of leaves on white oaks, though they are not bristle-tipped. The seeds in their acorns have a sweet flavor and germinate within a few days after they fall. Their acorns mature in one season. During the second growing season, red and black oaks mature their bitter fruits, which are tipped with hairs and have hairy linings on the acorn shells.

From acorns, oaks are easily propagated and thrive in moderately moist rich soil or dry sandy soil. Many trees grow from stump sprouts again. The tree is hardy and long-lived but is not shade-tolerant and susceptible to oak wilt and leaf-eating organisms (21).

Large areas of Palestinian forests are naturally dominated by six known species of Quercus, which are *Q. boissieri*, *Q. cerris*, *Q. look*, *Q. ithaburensis*, *Q. calliprinos*, and *Q. coccifera* (22). Only three species of them were used in this study; *Q. ithaburensis*, *Q. calliprinos*, and *Q. coccifera.*

1.2.1 Quercus boissieri

Quercus boissieri Reut. is the original name of this species, but later it was known as *Quercus boissieri var. latifolia*, but the current accepted name is *Q. infectoria* subsp. veneris. It is also known commonly as gall oak, dyer's oak, and Aleppo oak (23).

A Boissier oak is an evergreen deciduous tree that grows to a height of 10 m or more. A variety of alternate leaf arrangements are present. There are short petioles that carry the leaves. They have triangular points on their margins, rounded at the ends. A glabrous surface covers the upper surface of the leaves. Leaves are covered with hairs on their lower sides as they grow, but become glabrous as they mature. Various species of this species have hairs that fall off when touched by the fingers, which makes them easy to recognize.

March-April are the peak months for the blooming of *Quercus boissieri.* Flowering occurs only once per year, the trees are monoecious, and male flowers are limp catkins. A small pair or single flower is arranged on a short pedicle by the female flowers. It produces elongated, cylindrical acorns that are 3-4 cm long, ovate, and usually found alone or in pairs. Compared to all other oak species in Palestine, the acorn is thinner and

longer. In most cases, the cupule covers less than a quarter of an acorn's length. A cupule is characterized by short, obtuse, tightly attached scales.

There are forests and Maquis in the Mediterranean mountain range that are home to *Quercus boissieri*, which grows at an elevation of at least 800 m. Limey and basalt soils are both suitable for its growth. Many types of insects infect the trees with galls. The galls were used to produce a red dye, which is the origin of its former scientific name. A Swiss botanist who studied Middle East flora in the 19th century is credited with naming this plant in Latin after Pierre Edmond Boissier (24).

1.2.2 Quercus cerris

A large deciduous tree growing to 40 m tall, *Quercus cerris* L., is commonly known as Turkey oak. It has a trunk size between 1.5 m and 2 m, and a well-developed root system. The lifespan of this plant is between 120 and 150 years. Bark fissures are reddish-brown or orange and deeply furrowed in mauve-grey. Aside from shuttering or fuel wood, the wood is inferior to other common oak species. There are 7-9 pairs of triangular lobes on each leaf, which is dark green above and gray-felted below; the leaves are typically 9-12 cm long and 3-5 cm wide. Usually swirled with long twisted whiskers, the egg-shaped buds on the tips of the twigs are hairy and egg-shaped. Flowering occurs between April-May, and the flowers are monoecious and wind-pollinated. The acorns are without a stalk, about 2 cm in diameter and 2 cm long. There are dense bristles covering the acorn cup. There is an abundance of acorns on Turkey oak trees, and the acorns germinate readily and can be propagated easily (25).

1.2.3 Quercus look

Among mountainous deciduous oaks in the Middle East, *Quercus look* represents the southernmost species. It is an evergreen tree, that grows 4–10 m high. Without cutting the trees down, they will only have one trunk, but many trees now have several erect trunks, indicating they have been cut down previously. Usually wavy, with elongated, glossy, rough toothed, 5–11 cm long leaves. In comparison to *Q. libani*, the leaves have triangular teeth that are either regular or irregular, bigger and fewer than in *Q. libani*. Acorns develop during the summer and ripen in September when they bloom in spring. It doesn't take too long for European jays (Garrulus glandarius) and rodents to disperse the acorns by October, or else they are all gone. There are large acorns, with erect cupules

on top, and spreading outward or bending downward on the rest. Nuts are unique, as they are dark brown and flat or even slightly concave at the tip, regardless of whether they are exserted or not (26).

1.2.4 Quercus ithaburensis

Also called the Mount Tabor oak, it grows in a woody park woodland with herbaceous vegetation between the trees, part of a complex of trees and shrubs species adapted to a dry Mediterranean environment. As a thermophilous tree, growing mostly at altitudes below 500 m, and deciduous tree to shed its leaves during the winter months, it is found mainly in the eastern Mediterranean (Turkey, Syria, Lebanon, Palestine, and Jordan).

Q. ithaburensis develops an upright trunk that can reach 5-6 m and forms open formations. A broadly ovoid or globular crown is formed as the branches divaricate upward. Usually, 8-4 cm is the size and shape of the leaves; the buds are ovoid in shape and have upraised scales. The leaves vary in size and shape from one tree to the next but are mainly leathery and toothed. There are usually sexes in different inflorescences on the same main twig, and they have green flowers or inflorescences that are similar in color. Within a perianth of 4-6 lanceolate lobes, it forms staminate catkins of 5 cm in length. Two years later, the acorns mature. They can be found alone or in pairs. A total of five subvarieties were classified by Zohary (1966), each characterized by a different style of cupules and glands: var. calliprinoides, var.subcalva, var. subinclusa, var. dolicholpeis and var.ithaburensis.

Tabor tree was planted as a supplementary food source in the past because of its huge and sweet acorns, and the cupules were largely used for dye industries until the beginning of the 20th century (27–29).

1.2.5 Quercus calliprinos

Also called Palestine oak because it's the most common tree in the wild flora of Palestine. It is known as part of the genus *Cerris*. *Quercus calliprinos* is a medium-sized evergreen tree reaching 5-18 m tall and 1 m trunk diameter. It grows slowly, at less than 25cm each year, and can live for up to 700 years. It is also tolerant to hard environments and regenerates quickly after fires (30).

Sharp spines surround the edges of the shiny, stiff, dark green leaves, which are 3–5 cm long, and 1.5–3 cm wide (31). Flowers are found as male and female on the same tree. They bloom from March to April and fall soon after. Green male flowers grow as a catkin, a long cylindrical cluster of small flowers without petals, and female flowers grow as a nut called an acorn, borne in a cup-like structure known as a cupule which is 3-4 inches long and 2-3 inches in diameter (32).

1.2.6 Quercus coccifera

Also called kermes oak, it stays green all year long as an evergreen shrub. Although this tree has the potential to grow to a height of just 2 m, depending on where it is grown and how it is cared for, it can reach heights of up to 4 or 5 m and may become a small tree in the process.

Its morphology indicates an impenetrable "wall" with extensive ramifications from its base. There is a pattern of green leaves that alternate between those that fall faster and those that do not fall at the same time. They have a wavy form and are hairless on both sides, with a smooth surface on both sides. To distinguish them, notice how the male blooms are significantly smaller than the female blossoms. Females are born on the same plant as males and might be solitary or in groups of two or three. Flowering occurs in April or later, and fruiting occurs in August of the year following flowering.

Depending on its properties, this shrub can be used for various purposes. Its bark contains a high concentration of tannins and can be used to dye certain wool black. Wood has minimal value, although it can be used as fuel and to make charcoal.

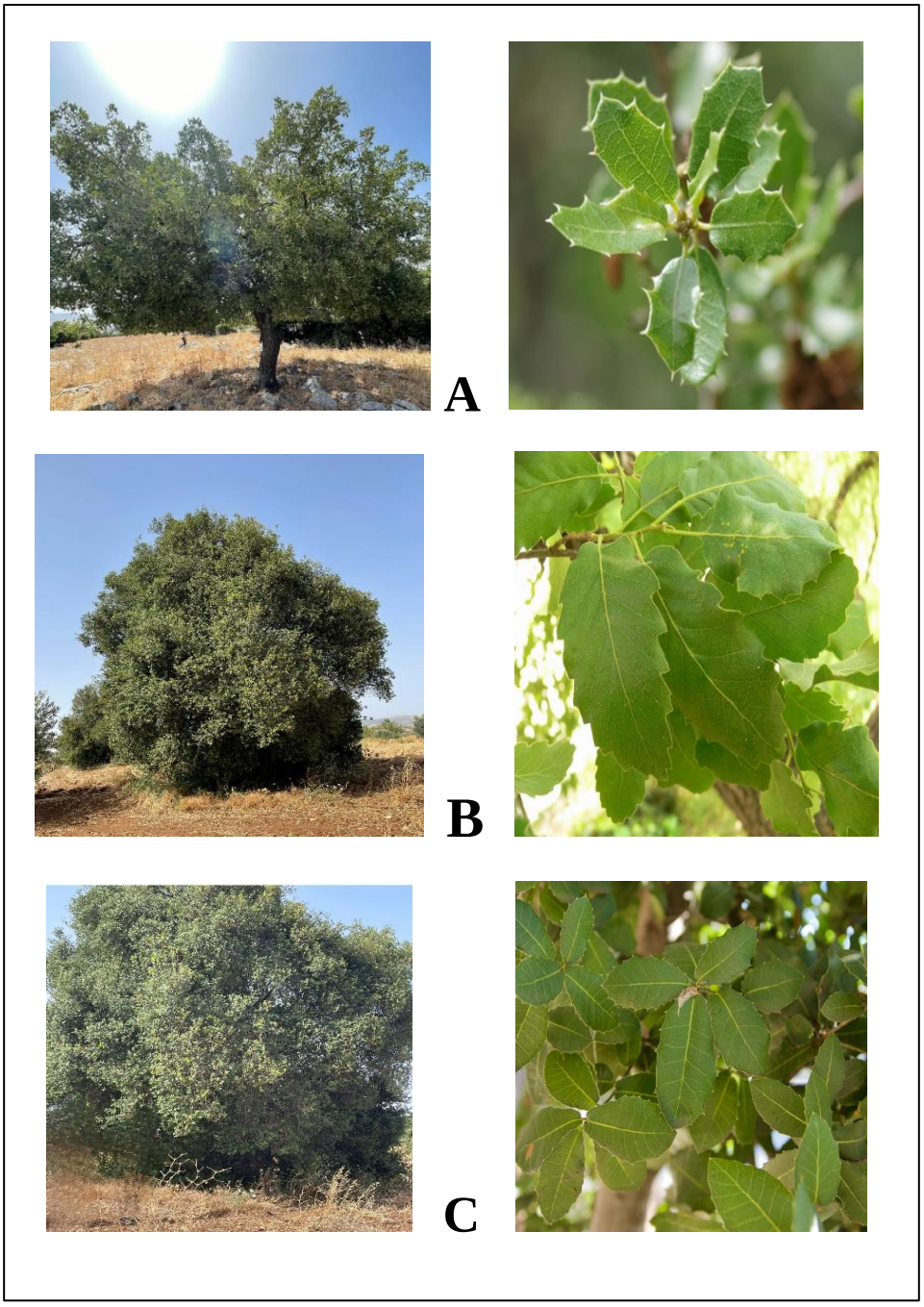
Because of their bitter flavor, they were also used to feed cattle and goats. Finally, it is important to remember the amazing protection that it may provide to impoverished areas, which is why it is critical to avoid causing their degeneration by frequent fires or extensive grazing.

Q. coccifera is also used medicinally. Because of its high tannin content, it can be extracted as a cure for diarrhea and urinary incontinence by decoction of the bark. External use of the decoction can help alleviate hemorrhoids and chilblains. It is also an effective tonic and has anti-inflammatory, antibacterial, and febrifuge qualities (33).

Its common name, kermes, indicates the importance of the species in the past as the food plant of the Kermes insect, whose crushed bodies were used for the production of the original natural crimson dye (34,35).

Figure 1.1

Field photos of Quercus



Quercus coccifera (A), Quercus ithaburensis (B), and Quercus calliprinos (C)

1.3 Phytochemicals and bioactive compounds of Quercus

In addition to primary molecules such as carbohydrates, proteins, and lipids, secondary metabolites play a major role in the pharmacologic action of medicinal plants. Antimicrobial properties in plants are commonly attributed to secondary metabolites that functions naturally as defense mechanisms against pests, pathogens, and predators (36). A significant number of these metabolites have been discovered to be useful for developing safer antibacterial and antifungal chemicals (12).

Quercus species have special compounds that have been isolated by researchers to investigate their biological activities and to validate the usage of genus species as medicinal treatments. Extracts were shown to have a variety of pharmacological actions, including antioxidant, antibacterial, anti-inflammatory, and cytotoxicity activity (37).

Numerous Known polyphenolic compounds were isolated from Quercus, which have been effectively used in several clinical studies. According to Okuda *et al.* (38), polyphenolic compounds have high biological and pharmacological properties. There are several biological effects of these compounds, including antisecretory and antiulcerogenic activities.

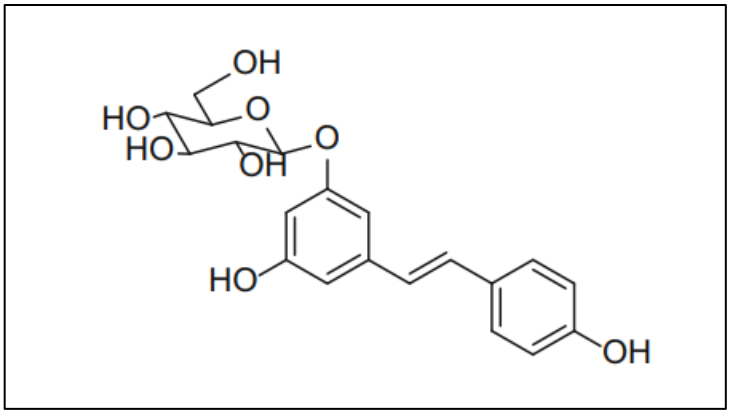
Many Quercus species have phenolic secondary metabolites such as lignans, simple phenols, tannins, flavonoids, and coumarins. Also contains flavonols such as kaempferol, quercetin, and isorhamnetin as aglycon (appendix, figure.1.2), all of which can be found in *Q. cerris*, for example (39).

Quercus species are among the most important tannin-containing plants. Several species of Quercus have high tannin content, for instance *Quercuus rubur* is characterized by approximately 10 % in ethanol (w/w) hexahydroxydiphenoyl esters, most of which are castalagins and vescalagins. Recent studies have identified several polyphenolic compounds in Algerian *Q. coccifera* leaves, including casuarictin, pedunculagin, tellimagrandin I and tellimagrandin II, vescalagin, castalagin, phillyraeoidin E, quercitrin,mongolicain A, acutissimin B, (+)-catechin, quercetin, and kaempferol 3-O-(6″-Ogalloyl)-â-D-glucopyranoside (40).

Epicatechin, gallocatechin, and epigallocatechin, flavan-3-ol monomers, and their derivatives, also trans-resveratrol 3-O-b-glucopyranoside which is a stilbene derivative (figure 1.3) have all been reported in some Quercus species, including *Q. coocifera* (39).

Figure 1.2

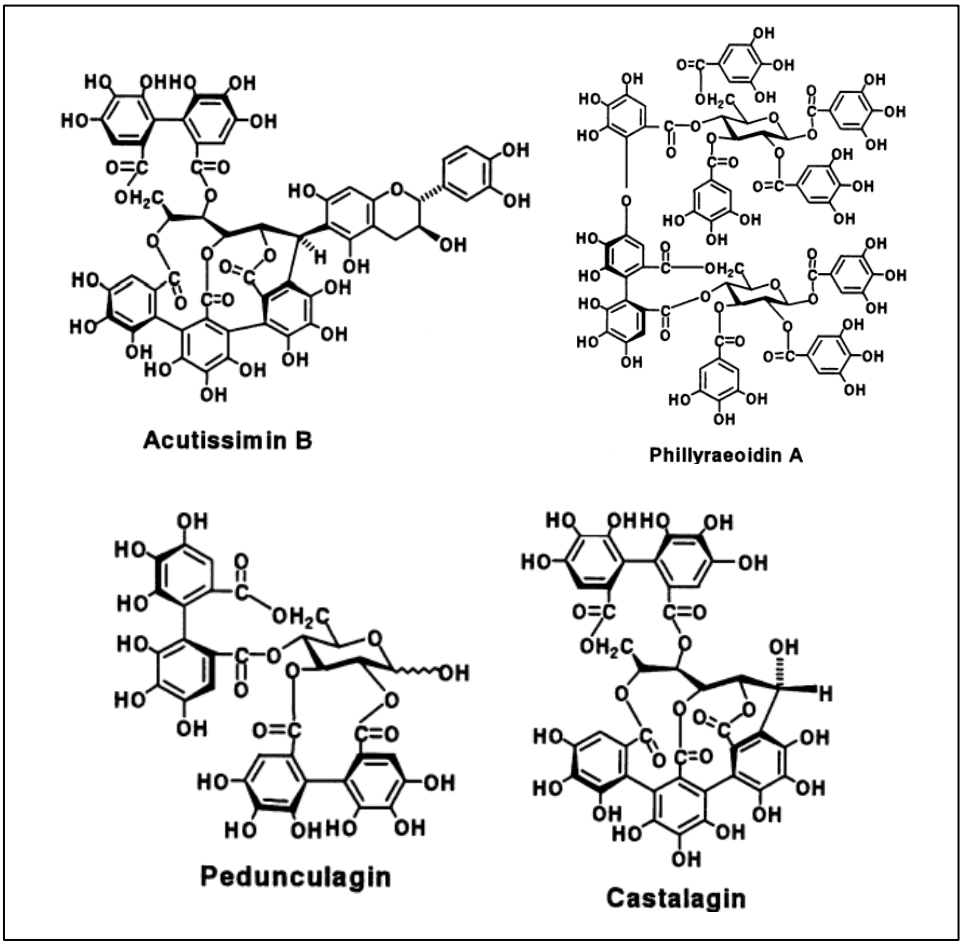
Stilben derivative from Quercus species (39)



The bark and wood of different Quercus species, like *Q. coocifera*, contain some lignans, such as, 5-methoxyisolariciresinol, lyoniresinol, 5,5-dimethylsecoisolariciresinol, matairesinol , olivil and their glycosylated derivatives (39).

Figure 1.3

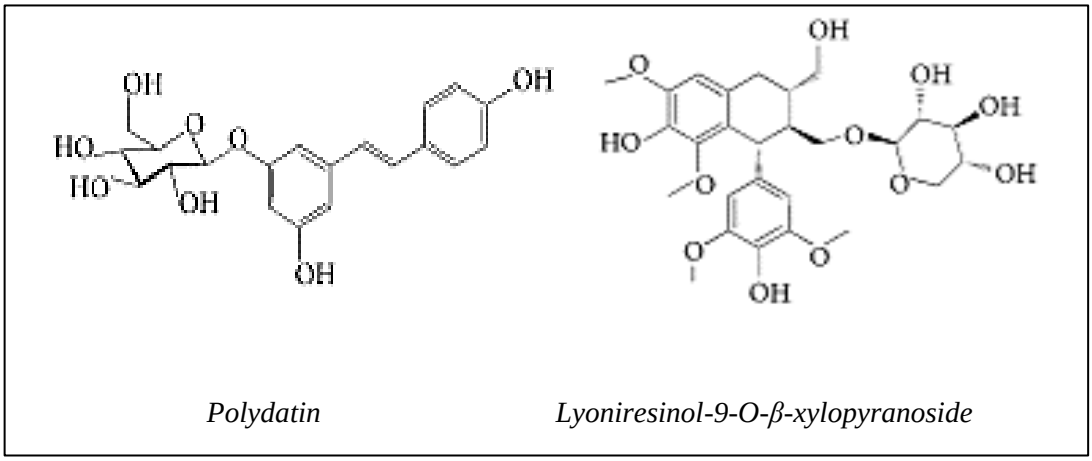
Structures of tannins purified from Q. coccifera (40)



Taib *et al.* reported that flavonoids (particularly flavan-3-ol), phenolic acids (especially gallic and ellagic acids and their derivatives), Only *Q. coccifera* was mentioned to contain two polyphenolic compounds shown in (figure 1.4) (20).

Figure 1.4

Chemical structure of two polyphenolic compounds purified from Q. coccifera (20)



Polydatin *Lyoniresinol-9-O-β-xylopyranoside*

It has been reported that a wide variety of simple phenolic acids and phenols, including ellagitannins and gallo, along with their conjugates epicatechin and catechin, have been isolated in many Quercus species, such as *Q. coocifera* . This type of compound dominates oak woods along with flavan-3-ols (39).

1.4 The effect of phytochemical compounds of Quercus on different microorganisms

Microorganisms are alleged to be absolutely toxic to phenolic compounds, but increased hydroxylation reactions lead to the destruction of bacterial cells. The presence of highly active flavonoids like quercetin reduces the thickness of lipid bilayers and increases their fluidity levels, thus facilitating the leakage of intracellular proteins and ions by *S. aureus*.

As a cell-cell signaling antagonist, quercetin inhibits enteroaggregative biofilm formation by *E. coli* and *P. aeruginosa* at a concentration-dependent rate. Furthermore, it inhibits 3-hydroxyacyl-ACP dehydrase in *Helicobacter pylori* and eriodictyol, FAS-II, enoyl-ACP-reductase, β-ketoacyl-ACP reductase, and β-hydroxy acyl-ACP dehydratases in Mycobacterium sp., which are responsible for fatty acid synthesis in bacterial cells. Moreover, quercetin inhibits the synthesis of peptidoglycan, another component of the bacterial cell wall. Furthermore, it inhibits ATP synthesis by constraining *E. coli*'s F1FO ATPase system resulting in cell wall damage.

It is believed that catechins inhibit cell wall construction by attracting lipid bilayers of the membrane. These bilayers form hydrogen bonds and attract polar lipid head groups at membrane edges, thereby inactivating or inhibiting enzyme synthesis both intracellularly and extracellularly. In general, antimicrobial agents can target enzymes involved in fatty acid biosynthesis to block the growth of bacteria, and in particular the enzyme fatty acid synthase II (FAS-II) may be of significant importance.

Molecular oxygen can be reduced partially to produce reactive oxygen species (ROS), which are capable of exerting antimicrobial activity, aiding the immune response against various diseases. Catechins' antimicrobial activity is thought to be related to the production of oxidative stress (ROS and RNS), which can alter membrane permeability and damage cell walls. Liposomes also suffer from catechin damage since they contain a high concentration of negatively charged lipids. Catechins were found to support potassium leakage and disturb membrane transport in a methicillin-resistant *S. aureus* isolate.

Antibacterial poisons originate from bacteria like *Vibrio cholerae*, *E. coli*, *S. aureus*, *Vibrio vulnificus*, *Bacillus anthracis*, *Neisseria gonorrhoeae*, and *Clostridium botulinum*, which are neutralized by catchins and quercetins. Due to hyaluronidase-mediated degradation, bacteria produce bacterial hyaluronidases directly targeting host tissues and increasing their permeability of connective tissues. *Streptococcus equisimilis* and *Streptococcus agalactiae* have been shown to respond to quercetin in hyaluronic acid lyase inhibition experiments (12).

1.5 Traditional uses of Quercus

In previous times Quercus acorns were found to be rich in carbohydrates, amino acids, proteins, lipids, and sterols. They have high energy content and are easily digested (41). Also acorns were found to contain fat in low amount, starch in high amount and suggest they are a more nutritious food than cereals, with more than 50% starch content. Furthermore, acorn starch is a natural thickener and stabilizing agent due to its high paste consistency. Thus, acorns might provide food industry with a promising ingredient that has substantial commercial potential. Throughout Italy and Turkey, Quercus is dried and roasted and consumed as coffee. Likewise, livestock in Turkey and Algeria consume the acorns and leaves of *Q. cerris var. cerris*.

Turkey's cities of Adana and Osmaniye recorded the use of *Q. infectoria subsp. boissieri* acorns as a dye. Also Turkish rugs are dyed using the cups of *Q. ithaburensis subsp. Macrolepis* after being boiled in water (39).

In addition to providing skin depigmentation prevention and skin aging treatments, Quercus extracts can also be used as a complementary remedy to acne therapy, as well as in pharmaceutical and cosmetic applications. As a form of hair care, the galls of different species of Quercus are used on the hair alone or combined with henna (42).

Acorns and galls of Quercus have been one of the most important export materials in Anatolia since at least the middle of the first century BC. The galls of Quercus species are most commonly found on the branches, bark, leaves, buds, stems, flowers, even the roots and acorns of the plant species. Galls are comprised of a protective layer that is very hard surrounding a membrane of proteins, fatty substances, and sugar. They were were used for tanning leather, dyeing textiles, inking, and coloring dyes at all times throughout history. Similar to the other parts of Quercus, gall has been used for medical purposes since ancient Greek times (39).

1.5.1 Other uses of Quercus

Quercus tannins nowadays are used in the leather and plastic manufacturing industries, in the glue-producing industry, in the ceramic industry, and in the oil business. Thus, their commercial value is increased as a result of this. Due to their strong antifungal properties and the crucial role that oak barrels play in wine maturation, Quercus polyphenols have been extensively researched (39).

1.6 Biological activity studies of Quercus extracts

1.6.1 Anti-microbial effect

S. aureus and *Enterococcus faecalis* (Gram+ve bacteria), and *E. coli* and *Pseudomonas aeruginosa* (Gram-ve bacteria), as well as three species of yeast *Candida parapsilosis, Candida albicans,* and *Candida krusei*, were all tested for antimicrobial effects of *Q. aucheri* ethylacetate, n-butanol subextract, methanol extract and and water residue.

All the tested microorganisms were most susceptible to the ethylacetate subextract extract. All tested fungi had a high activity against *Candida parapsilosis* with MIC 1.2 mg/ml; *Candida albicans* with MIC 2.4 mg/ml; *Candida krusei* with MIC 4.9 mg/ml and *S. aureus*

(Gram +ve bacteria) with a MIC of 2.4 mg/ml. The subextract which was the most active was analyzed for phytochemical components, which led to the discovery of quercetin-3-O-a-L-arabinopyranoside, quercetin-3-Ob-D-galactopyranoside, and (+)-catechin (39).

Using broth microdilution method and measuring MIC and MBC, 70% ethanolic extract of *Q. brantii* acorn was evaluated for its antibacterial effects. Compared to vancomycin and nitrofurantoin, the extract significantly inhibited *S. aureus* and *E. faecalis*. Extracts had MIC values of (500 and 600) mg/ml, as well as MBC values of (1000 and 800) mg/ml against *S. aureus* and *E faecalis,* respectively. It was found that methanol extracts of *Q. brantii* seed hulls had significant anti-bacterial effects on *E.coli* and *Proteus mirabilis.* Shigella flexneri did not seem to be significantly affected by the extract, which had an effect equivalent to 25 g of cotrimoxazole against *S. typhimurium* (18).

With a 96-well plate containing sterile extracts of *Q. coccifera* stems, the minimum inhibitory concentrations (MICs) of the extracts were determined against *Bacillus subtilis* (ATTC 6633), *E. coli* (ATTC 25922), *Klebsiella pneumonia* (ATCC 27736), *P. aeruginosa* (ATTC 27853*)*, *S. aureus* (ATTC 29213), *P. mirabilis* (ATTC 12453), *Bacillus cereus* (ATTC 11778), *S.epidermidis* (12228) and *Streptococcus agalactiae* (ATCC 12386), using a microdilution method in 96-well plates with gentamicin as a positive control. In tests conducted with tested bacteria strains, aqueous and methanolic extracts were found to have MICs ranging from "0.5 to 256 µg/ml and 0.25 to 256 µg/ml, respectively. *E. coli* was the most resistant bacterium, while *P. mirabilis* and *B. cereus* were most sensitive" (43).

An extract of ethyl acetate from *Q. acuta* leaves was evaluated for antibacterial activities against Gram-positive, Gram-negative, and antibiotic-resistant strains acquired from hospitals, including *P. aeruginosa* with carbapenemase production, methicillin-resistant *S. aureus* (MRSA), *E. coli*, and vancomycin-resistant enterococci with extended spectrum lactamases. MRSA strains showed the best anti-bacterial activity against ethyl acetate extract. Additionally, the same extract also showed antibacterial activity against *E. coli* KCTC 1923, *Micrococcus luteus* ATCC 9341, *S. aureus* KCTC1928, *Salmonella typhimrium* KCTC 1925, and eight MRSA strains exhibiting MIC values of 125 to 500 µg/ml (44).

On *Q. infectoria* gall extract, two fold serial micro dilution broth assays were performed to determine its antimicrobial activity against pathogenic *Leptospira*. An aqueous extract of *Q. infectoria* gall exhibited antimicrobial activity against *L. interrogans* serovars with MIC of 0.125 mg/ml. MBC values ranged from 0.125 mg/ml for *L. interrogans* serovar Javanica, and 0.250 mg/ml for *L. interrogans* serovar Icterohaemorhagiae (45).

Cell division and cell morphology were altered by extracts from *Q. infectoria* galls when they were used to inhibit the growth of gram positive and gram negative bacteria. MRSA and MSSA strains were significantly inhibited by water, 95% ethanol, ethyl acetate, and acetone extracts of the same plant. As for the minimum inhibitory concentrations (MIC), MRSA had MIC of 0.13 and MSSA 1.00 mg/ml. With MIC values of 0.625 mg/ml, 0.625 mg/ml, 1.250 mg/ml, 0.313 mg/ml and 1.250 mg/ml, respectively. An ethanol extract of *Q. infectoria* galls showed antimicrobial activity against, *E. coli, P. aeruginosa, S. typhimurium, S. aureus* and *C. albicans*. Using this extract to disinfect eggshells from pathogenic microorganisms is an effective and natural disinfectant. Using 1% extract solution, eggshell microbial contamination was drastically reduced, including yeasts, molds, and Enterobacteriaceae. As a result of immersion in the extract for 60 minutes, both *E. coli* and *S. aureus* were completely inhibited (46).

In a disc diffusion test, three formulas of a cream contained 10%, 20%, and 30% of *Q. infectoria* gall extract were tested for the antimicrobial activity of them against *C. albicans*. Anti-Candida activity has been observed for all extract cream formulations. Formulated extract creams containing 10%, 20%, and 30% extract showed MIC values of 1.09 mg/ml, 0.55 mg/ml, and 0.07 mg/ml, respectively. Inhibition zone diameters were significantly different between creams containing 10% and 20% extract ($p = 0.0254$). An extract cream with 30% fungucidal concentration (0.068 mg/ml) demonstrated the lowest MFC (47).

Multi-drug resistant bacteria strains were tested using two methods: the disc diffusion method and the microdilution method to evaluate the antibacterial activity of ethanol and water extracts prepared from *Q. infectoria* galls. The extracts significantly inhibited both MRSA coagulase-negative and MSSA coagulase-positive. Acinetobacter species that are multidrug resistant showed a reduced inhibitory zone diameter, while *K. pneumoniae* and *E. coli* isolates with broad-spectrum beta lactamases showed no inhibition effect. According to the results of

the study, extracts are potentially good sources of antimicrobial substances, particularly against multidrug-resistant Gram positive bacteria (48).

Q. infectoria galls extracts in methanol and aqueous medium were analyzed for their anti-Candida activity against *Candida parapsilosis*, *Candida glabrata*, *Candida albicans*, *Candida tropicalis,* and *C. krusei*. The MICs were determined by the serial dilution method at concentrations ranging from 16 to 0.04 mg/ml. In both of their crude extracts, pyrogallol was the major component, and aqueous and methanol extracts both showed significant anti-Candida activity. An aqueous extract displayed good fungistatic activity against *C. parapsilosis*, *C. krusei*, and *C. glabrata* isolates. Almost all Candida species were found to be susceptible to fungicidal activity in methanol extract (49).

Extracts of *Q. infectoria* galls possessed antimicrobial properties in both aqueous and acetone solutions and MIC values between 0.0781 and 1.25 mg/mL. There was a significant difference between the MBC value of the water extract against *S. aureus* and *S. typhimurium* and the MIC value for these bacteria. In addition to having a higher MBC value than its MIC value, acetone extract also had a higher MBC value against *S. aureus*. A 0.25 mg/ml acetone extract had the same MIC and MBC values against *S. typhimurium*. As far as *P. aeruginosa*, *S. epidermidis*, *S. typhimurium*, and *B. subtilis*, , and were concerned, the extracts showed weak inhibitory effects, whereas *E. coli* did not show any inhibition zones (39).

Q. crassifolia bark extract was investigated for its antibacterial properties and potential subacute oral toxicity against probiotics and pathogenic bacteria. In comparison to *Lactobacillus bulgaricus* and *S. thermophylus*, extract selectively inhibited *E. coli.* At 250 and 500 mg/ml, aqueous crude extract and ethyl acetate soluble fraction did not significantly differ in diameters of growth inhibition on *S. thermophilus* and *L. bulgaricus*. In contrast, when ethyl acetate soluble fraction was applied at 500.1 and 750.3 mg/ml, a significantly greater inhibition diameter was observed (p/0.05) in a dose-dependent manner to *E. coli*. Among the subacute adverse effects, no adverse effects were observed at an 11 mg/kg/day dose, while the lowest adverse effects were reported at 33 mg/kg/day for kidney damage (17).

T. gondii, an intracellular parasite that can cause dangerous effects on babies and people with weak immunity, the outer bark extract of *Q. crispula* showed potent anti-toxoplasma

activity. As little as 10 µg/ml of extract resulted in complete inhibition (50). There has been a study investigating the larvicidal effects of the extract of *Q. infectoria* galls on *Anopheles stephensi* larvae which are the main vectors of urban malaria, India's endemic disease. A LC50 value of 116.92 ppm was found for ethanol-acetate extract, the most effective extract among the five extracts tested against larvae of the fourth instar. Gallotannin, nbutanol, acetone, and methanol also showed larvicidal activity against larvae of the fourth instar, respectively (39).

Q. suber leaves and bark methanol extracts were tested on *C. albicans* growth using microbroth dilution parameters, coupled with their MICs.In both cases, the extracts showed antifungal activity. MICs ranged between 12.5 and 50 mg/ml for *Candida albicans* and *Trichophyton rubrum* in the presence of bark methanol extract. Agar well diffusion method was used to test the antibacterial and antifungal properties of catechin derivative isolated from Qincana. Among the bacterial targets, *S. pyogenes* showed the best anti-bacterial activity with 80% inhibition, while *C. glabrata* showed the best anti-fungal activity at 80.5% inhibition (39,51).

1.6.2 Effect on wound healing

In many countries around the world, Quercus species are used to treat wounds. Detailed investigations of their mechanisms of action have been conducted. It was found that ointment prepared with oak bark extract had wound healing, and antibacterial ,anti-inflammatory properties. A clinical study also demonstrated the efficacy of this ointment. Using albino mouse fibroblasts and cell lines of murine macrophage, the effect of aqueous and methanolic extracts of *Q. coccifera stem parts* on different stages of wound healing was investigated. In the presence of 200 µg/ml concentrations of methanol and aqueous extracts, proliferation of fibroblasts was significantly stimulated by 136.7% and 163.4%, respectively. There was a significant increase in wound closure rates between 18.5% and 52% after using an aqueous extract and 23.9% to 75.4% after using a methanol extract (39).

1.6.3 Antioxidant effect

To obtain extracts from *Q.cerris* wood samples, maceration, ultrasound assisted extraction, and accelerated solvent extraction techniques were used to determine anti-oxidant activities. Extraction techniques were found to affect the relative antioxidation capacity index of the extracts obtained from thermo-treated wood in this rate : ultrasound assisted extract > macerate > accelerated solvent extract (52).

Furthermore, a DPPH assay was conducted on extracts from *Q. coccifera* stem parts to determine whether they have an anti-oxidant effect. IC50 values for methanolic extract were 58.7 x 2.42 µg/ml which exhibited more potent antioxidant activity (43).

1.6.4 Neuroprotective effect

In an effort to test whether *Q. coccifera* extracts have neuroprotective effects against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), extracts from cups, and shelled acorns pieces of the plant were tested on the enzyme inhibitory effects of water and ethanol extracts. Between 50% and 74%, there were significant differences in ethanol extracts' cholinesterase inhibition activity on both enzymes (200 µg/ml). An AChE inhibitory test with galantamine at 100 µg/ml showed 93% inhibition and a BChE inhibitory test showed 88% inhibition (53).

1.6.5 Anti-hepatotoxic effect

Recent experiments analyzed the potential antihepatotoxic activity of the acorns of *Q. liaotungensis* on hepatic stellate cells (t-HSC/Cl-6) in humans. It has been found that the acorns are an excellent source of antioxidants and antihepatic fibrosis agents, with galloyl triterpenes being the most significant components of the acorns.

A dose-dependent hepatoprotective effect was observed in an 80% methanol solution extracted from *Q. robur* leaves compared with the group of paracetamol-induced gastric damage. Also in comparison with ethanol-induced gastric damage group, the ethanol-treated group experienced significant gastroprotection (39).

1.6.6 Problems related to the gastrointestinal system

There are several oak species used in folk medicine for problems in gastrointestinal system. In addition to alleviating ulcerative colitis, gastric lesions, Helicobacter pylori infection, and suppressing diarrhea, polar extracts and tannins isolated from them have been demonstrated to be effective. *Q. ilex* aqueous extract was evaluated for its gastrointestinal-physiological activity using phenol-red colorimetry and charcoal/gum arabic in water test meals. Researchers explored the extract's effects on gastrointestinal disorders by inducing colonic constipation and delayed stomach emptying with loperamide. Compared with the control group (70 %), both doses 150 mg/kg and 300 mg/kg of extract reduced gastric emptying GE (66% and 60.8%, respectively) (54).

An experiment involving isolated polyphenols and *Q. ilex* root barks was conducted on rats to investigate the way that the isolated polyphenols affected on gastric lesions resulted from alcohol. Gastric lesions were significantly decreased after oral administration of these extracts and tannic acid (47.7%-76%). According to the study, the anti-lipoperoxidant properties of the compounds are responsible for the effect. Analyses were conducted on rats to determine whether the aqueous extract of *Q. ilex* root bark nor tannic acid were able to protect the gastro-intestinal system from ethanol-induced damage. Haemoglobin from ovines was precipitated in vitro by *Q. ilex* extract and tannic acid. After consuming orally administered tannic acid or extracts, gastric lesions decreased significantly (47.7%-76%). The gastric mucosal barrier can be strengthened by monomeric and polymeric polyphenols, according to the research (39).

1.6.7 Anti-viral effect

Q. infectoria extracts at 100 g/ml showed more than 90% inhibition of HCV protease against methanolic and water extracts. An analysis of linear regression was performed to determine the dose-dependent antiviral effects of *Q. persica* hydroalchoholic extract on the replication of HSV-1 in a hamster kidney cells. Cell death is significantly correlated with extract concentration (p/0.01). As a result of its attachment to baby hamster kidney cells, *Q. persica* L has an IC50 value of 1.02 lg/ml for HSV-1 before and after attachment. Crude extracts of the seeds of *Q. lusitanica* were studied for their potential to inhibit the replication of Dengue virus type 2 (DEN-2). As demonstrated by the absence of cytopathic effects (CPE), methanolic extract of *Q. lusitanica* inhibited 10–1000 TCID50 of virus at its maximum non-toxic concentration of 0.25 mg/ml. When administered at the low dose (15 mg/ml), *Q. lusitanica* inhibited 10 TCID50 of virus totally, but it only inhibited 50% and 25% at 100 TCID50 and 1000 TCID50, respectively. Infected C6/36 cells were also tested for the effect of the extract of *Q. lusitanica* on the expression of NS1. After treatment with the extract, C6/36 cells infected with NS1 protein showed a reduction in expression. (H9N2) virus infection was studied in (MDCK) cells against (Influenza A/turkey/Wisconsin/1/1966) virus reference strain (AMN) as a standard anti-influenza A antiviral drug, comparing the effects with *Q. ilex* wood extract as an antiviral agent. *Q. ilex* extract had a maximum nontoxic concentration of 100 g/ml. As compared to *Q. ilex* extract, Amantadin showed a poor anti-viral effect against H9N2 virus at a dose of 50 TCID50 (39).

1.6.8 Tyrosinase inhibitory effect

Tyrosinase was inhibited by alcohol extract of *Q. coccifera* bark with an IC50 value of 75 µg/ml. Likewise, kojic acid exhibited an IC50 value of 51 µg/ml as a positive control. In contrast, (-)-8-chlorocatechin (IC50 value of 60 g/ml) is not as strong as kojic acid in inhibiting tyrosinase as transresveratrol-3-O-b-glucopyranoside, which exhibited an IC50 value of 4 g/ml. There was a competing response (Ki 50 µg/ml) for (-)-8-chlorocatechin, while there was a noncompeting response (Ki 6.7 µg/ml) for polydatin (55). *Q. infectoria* galls extract 80% methanol did not cause any damage to B16/F10 cells (cells from melanoma-affected mouse skin tissue, this cell line exhibits spindle-shaped and epithelial-like morphology) up to 100 µg/ml. Cell viability at this concentration was reduced by 83% for B16/F10. A 50% decrease in cell viability was observed when kojic acid was used at the same concentration. Melanin content in cells decreased by 66% and 37% when 50 and 100 µg/ml extracts were used as positive controls (39).

Specifically, extract inhibits tyrosinase in a dose-dependent manner, with a maximum inhibition of 59% at 100 g/ml. Similar results were observed with kojic acid. In the dried extract, there were 287 mg gallic acid equivalents per gram of total phenols. A high phenolic content of the extract may be responsible for its melogenesis and tyrosinase inhibitory properties. It inhibited L-DOPA oxidation with *Q. dentate* leaves and stem extract at concentrations of 16 – 666 µg/ml, and it failed to inhibit mushroom tyrosinase activity at similar concentrations. When 50 µg/ml of extract was applied to B16 mouse melanoma cells, melanin synthesis was inhibited (> 50%) by *Q. dentate* (bark) (56).

The decoction of *Q. robur* bark and the tannin fractions from this extract were evaluated as a method for preventing basophilic cells from degranulating (b-hexosaminase activity) in response to allergens. Based on the results of this study, decoctions (0.058- 0.58 g/ml) and semolina fractions (12-100 g/ml) inhibited basophilic degranulation capacity in a dose-dependent way, comparable to azelastine, the positive control. The fraction did not exhibit any cytotoxic effects at the tested concentrations, indicating that the effect was not due to cytotoxicity. Human mast cells released late-phase allergy markers, IL-6, IL-8, and TNF-a, after ingestion of the decoction or fraction D, which had an inhibition rate approximately equal to dexamethasone at high doses (100 mg/ml). Piperonylic acid ester isolated from oak galls also showed antihistamine effects in another study (57).

1.6.9 Anti-hyperlipidemic effect

An extract of *Q. infectoria* gall that was administered at 1.5 g/kg methanol for 45 days markedly reduced total cholesterol, LDL levels and triglycerides, and its ability to lower lipids was comparable to or stronger than that of the atorvastatin drug (positive control). In addition, it reduced aortic valve and thoracic aortic plaque formation more effectively than either atorvastatin or orlistat (39).

1.6.10 Anti-diabetic effect

As compared to the positive control acarbose (IC50: 50 µg/ml), methanol bark extract of *Q. coccifera* significantly inhibited the activity of aglycosidase (IC50: 3 µg/ml). This extract was also tested for its inhibitory potential against a-glucosidase. Based on an IC50 value of 44 µg/ml, (-)-8-chlorocatechin was the most effective a-glucosidase inhibitor of the isolated compounds (Ki of 30 µg/ml). An IC50 value of 50 was observed in the same test using acarbose as a positive control (55). *Q. infectoria* galls methanol extract showed a-glycosidase (amylase, sucrose, maltase, isomaltase) inhibitory activity against 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-bD-glucopyranoside. Acarbose showed lower a-glycosidase inhibitory activity on isomaltase and sucrose, but showed higher inhibitory activity on 3-O-digalloyl-1,2,4,6-tetra-O-galloyl-b-D-glucopyranoside than acarbose. As a result of methanolic extraction of *Q. gilva* leaves, the activity of a-glycosidase was inhibited. An isolated compound named kaempferol-3-O-(600-trans-p-coumaroyl)-bD-glucopyranoside with an IC50 value of 28.1 mM had the highest inhibitory effect on the a-glycosidases, followed by catechin and epicatechin, which have an IC50 of 168.6 mM and 920.6 mM, respectively (39).

Q. dilatata fruit methanol extracts at 200 and 400 mg/kg, respectively, reduced blood glucose levels in alloxan-induced diabetic rats by 114 and 111%. The 200 mg/kg extract also ameliorated the deterioration of liver function markers, including ALT and total bilirubin, and renal function markers, including creatinine and serum urea, as well as changed serum triglycerides, LDL, cholesterol, HDL, and VLDL levels to those of non-diabetic rats (58).

In vitro and in vivo studies demonstrated an antidiabetic effect of different Quercus species. As well as inhibiting the activity of aglycosidase and b-glycosidase enzymes, Quercus extracts also ameliorated diabetic-associated issues through their antioxidant

properties. Furthermore, kaempferol and quercetin derivatives were evaluated for their antioxidative properties. In vitro and in vivo studies also showed an anti-diabetic effect of beverages prepared from some Quercus leaves used in folk medicine (55,59).

1.6.11 Anti-inflammatory effect

The inflammatory cytokines and chemokines were inhibited by extracts and isolated compounds from *Q. mongolica* acorns. Interleukin (IL)-13 release was most inhibited by the ethyl acetate fraction with an IC50 of 5.8 μg/ml. There was a notable decrease in the expression of monocyte chemoattractant protein-1 (MCP-1), IL-10, IL-13, and IL-6 mRNA expression following the administration of pedunculagin (2,3-4,6-bis-(S)-hexahydroxydiphenoylb-D-glucopyranoside) isolated from this extract. Furthermore, it decreased cyclooxygenase-2 (COX-2) and c-Jun N-terminal kinase (p38), ERK, and p38 phosphorylation at concentration-dependent levels (60). NFjB (nuclear factor kappa-light-chain enhancer of activated B cells) is a transcription factor involved in the inflammatory response. *Q. infectoria* extract demonstrated anti-inflammatory activity by downregulation of this pathway. Both high glucose and palmitate medium treated bone marrow-derived macrophages and high fat diet-treated macrophages expressed less Set7, p65, and inflammatory cytokines than vehicle controls. It was demonstrated that the in vivo diabetic environment also lowered inflammatory cytokines, IL-1ß and TNF-a, when macrophages and monocytes from diabetic mice were treated with extract solution (61).

A study was conducted on the effects of infusions of *Q. durifolia*, *Q. eduardii* and *Q. sideroxyla* leaves on inflammation and anticarcinogenesis caused by 1,2-dimethylhydrazine (DMH) in HT29 cells. There was a statistically significant reduction in mean tumor size and multiplicity (1.2 vs. 2.0) in adenocarcinomas compared with DMH groups, and a 2.2-fold increase in bcatenin protein levels (2.2-fold). Adenocarcinoma developed in 68.8% of the animals given only DMH. As compared with adenocarcinomas of DMH-treated groups (4.71 arbitrary units), *Q. sideroxyla* infusion significantly decreased total b-catenin protein expression (1.47 arbitrary units). The expression of b-catenin (3.54 arbitrary units) was reduced by 27% with *Q. durifolia* infusion. The treatment of HT-29 cells with *Q. sideroxyla* effectively decreased COX-2 and IL-8 levels by modulating NF-jB expression (39).

In addition to inhibiting c-Jun N-terminal kinase, NF-jB, and ERK pathways, Polar Quercus extracts and isolated tannins exhibit anti-inflammatory activity in the presence of IL-1b, IL-13, IL-10, and IL-6 and TNF-a inflammatory cytokines. In addition, they inhibited COX-2 activity and suppressed histamine release as well. Inflammatory responses were also reduced by antioxidant mechanisms (60).

1.6.12 Anti-alergic effect

Testing the effect of decoctions and tannin fractions obtained from *Q. robur* bark on the activation of allergen-specific basophilic cells using b-hexosaminidase. Decoctions (0.058 - 0.58 mg/ml) and their tannin fractions (12-100 mg/ml) inhibited basophil degranulation capacity dose-dependently and compared favorably to the positive control azelastine. As tested concentrations of fraction exhibited no cytotoxic effect on cells, it was not related to cytotoxicity. Mast cells of human were inhibited by the decoction and fraction D, with a maximum inhibitory rate (100 mg/ml) comparable to dexamethasone levels at high doses. Another study showed that the anti-histamine properties of the isolated piperonylic acid ester from the galls of oak were based on substances within its structure (57).

1.6.13 Vasorelaxant effect

Inhibitors of nitric oxide synthase (N^{w}-nitro-L-arginine) abolished the vasorelaxing effect of *Q. salicina* leaf extract, which induced endothelium-dependent relaxations. A relaxation degree of 11.2%, 53.5%, and 102.00% was measured at concentrations of 10, 30 and 100 µg/ml, respectively. *Q. salicin* extract had an ED50 of 23.7 µg/mL for vasorelaxing effects. In porcine coronary artery endothelial cells, ethanolic extract activates endothelial nitric oxide synthase (eNOS) in a dose-dependent manner, resulting in increased bioavailability of nitric oxide. It was concluded that the extract could possibly be regarded as a potential herbal cardiovascular protective medicine that may help to prevent cardiovascular diseases and endothelial dysfunction, which can lead to heart attacks (62).

1.6.14 Hemostatic effect

In standardized bone holes prepared in the calvaria of 5 Burgundy rabbits, two groups of cotton pellets were prepared. The first group of pellets were soaked in ferric sulfate solution and the second cotton pellets were soaked in alcohol extract of *Q. persica* fruit.

Then they were tested and compared for hemostatic effects. There was no significant difference in bleeding control between normal saline and *Q. persica* extract after the following of 4 and 5 minutes when compared with *Q. persica* extract (63).

1.7 Emergence of MRSA

Staphylococcus aureus is a gram-positive coccus (~0.6 μm in diameter), sometimes positive for the reduction of catalase and nitrate, and is an optional anaerobic that can spread without oxygen requirement (64) non-motile, non-spore former, and some strains are capsulated (65). Many skins and/or nares of most humans are colonized with it, and despite the disruption of tissue or impairment of immune function, it can enter other tissues. It has been found that *S. aureus* can cause osteomyelitis, pneumonia, skin and soft tissue infections (SSTIs), endocarditis, and septicemia, just to name a few of the severe clinical manifestations of the infection (66).

With the emergence of hospital-based medicine, *S. aureus* has quickly become a major cause of healthcare-associated infections. Resistance developed just a few years following the introduction of penicillin and was started by the β-lactamase gene (blaZ) by the mid-1940s.

The first semi-synthetic anti-staphylococcal penicillin's were produced in 1960. Unfortunately, MRSA was discovered within a year after their first clinical application. Furthermore, genomic data suggest that methicillin resistance followed the first therapeutic use of anti-staphylococcal penicillins (67).

1.7.1 Evolution and genetic diversity

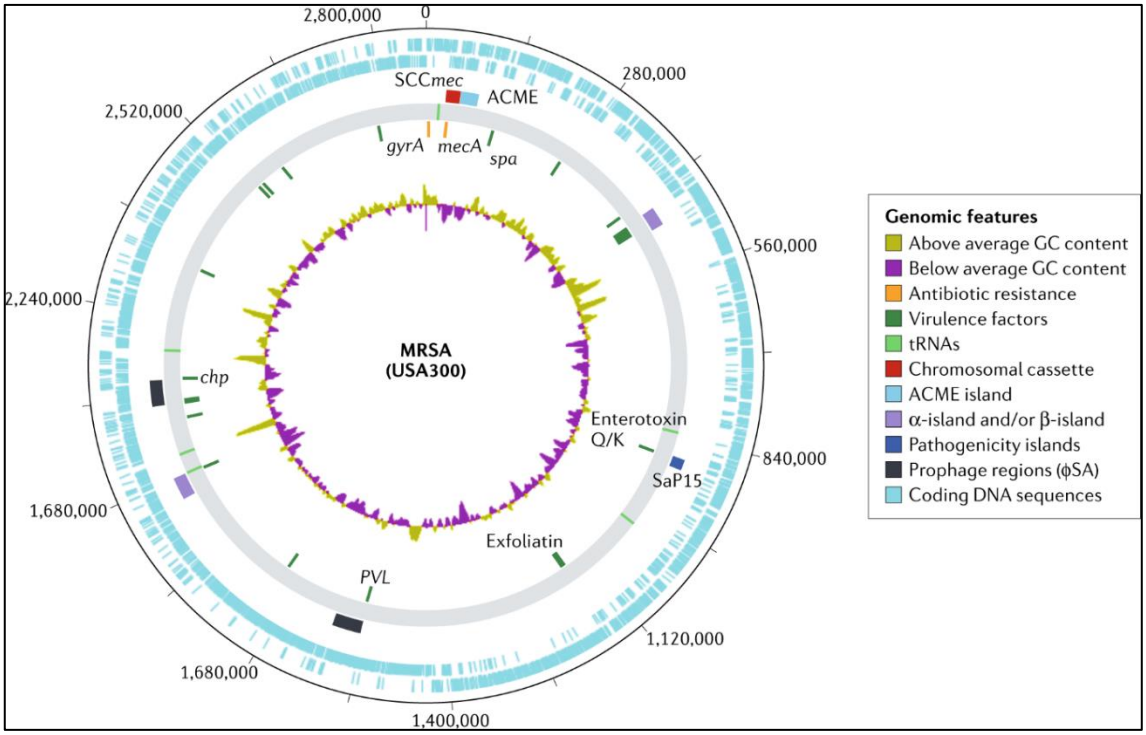
Genomes of bacteria are divided into core components, which hold genes present in all isolates that are related to cellular metabolism and replication, and accessory components, which MRSA and other pathogens have a lot of genetic variation within them like virulence mediators, immunological evasion, and antibiotic resistance (67). Figure (1.5) shows a representative map for the genome of the USA300 strain FPR3757. It displays the genomic content of MRSA as colored circular tracks as shown in the map key.

Methicillin resistance is mediated by the mecA gene and acquired by horizontal transfer of the staphylococcal cassette chromosome mec (SCCmec) by a mobile genetic element (67). SCCmec is a DNA fragment ranging in size from 21 to 67 kb, depending on the type

of SCCmec (68). According to Zong *et al.* (69), SCCmec has two essential components: the mec gene complex and the ccr gene complex. The mec gene complex is made up of mecA, regulatory genes, and associated insertion sequences that have been classified into six classes (A, B, C1, C2, D, and E), as well as cassette chromosome recombinase (ccr) genes (ccrC or the pair of ccrA and ccrB) that encode recombinases that mediate the integration and excision of SCCmec into and from the chro SCCmec, also includes a few additional genes such as insertion sequences, transposons, and plasmids (69).

Figure 1.5

Major genomic elements in methicillin-resistant Staphylococcus aureus (70)



The mecA gene encodes the penicillin-binding protein 2a (PBP2a), an enzyme in the bacterial cell wall that is responsible for crosslinking the peptidoglycans. PBP2a has a low β-lactam affinity, which results in resistance to this whole class of antibiotics (67). Therefore the therapeutic possibilities of MRSA strains are reduced and limited (71).

MRSA infections could be community-acquired (CA-MRSA) or hospital-acquired (HA-MRSA). There are strong variations in phenotypes and genetic history of these strains associated with infection.

1.7.2 Community acquired MRSA (CA-MRSA)

CA-MRSA is a newer and more virulent strain that emerged among healthy and relatively young people that are more likely to contract skin and soft tissue infections. However, current epidemic-molecular investigations have revealed that CA-MRSA may affect a number of patients in health care settings.

Generally, CA-MRSA is defined as any strain of MRSA isolated in an outpatient setting or within 48 hours of admission from a patient who has never been infected with MRSA or colonized, does not have permanent invasive medical devices, and has not experienced the following risk factors in the past year: hemodialysis, surgery, hospitalization, or long-term care residence. It is considered HA-MRSA when the strain does not meet this definition (72).

CA-MRSA differs genetically from HA-MRSA that its strains typically possess a small type of SCCmec (types IV or V), and the majority of them are susceptible to non-β-lactam antimicrobials (73). Additionally, It carries (but not always) LukS-PV and LukF-PV encoding Panton-Valentine Leukocidin (PVL) genes associated with increased virulence (65,74).

S. aureus colonization main site is the front nares. But there are numerous organs where MRSA strains can be colonized, including the inguinal regions (23% of CA- MRSA strains) in children-patients, the rectum in young patients, and the throat in young cases (65).

1.7.3 Hospital acquired MRSA (HA-MRSA)

HA-MRSA strain is characterized by its epidemiological behavior in hospital settings where it is especially successful. In certain health care institutions, *S. aureus* can also cause epidemic levels of infections, usually over the threshold for *S. aureus* infections. The majority of this type of infection is caused by these dominant MRSA clones, with a very small number of clones actually being successful. Practically every geographical region has been affected by epidemics caused by successful clones. The reasons for a pathogen to succeed are many. They can survive in an environment that has a commensal component, they can resist a variety of antimicrobial agents, and they can have a variety of biomarkers for virulence. A broad range of HA-MRSA strains are responsible for the most prevalent infection types in industrialized nations like the United States, including

catheter-associated infections,ventilator-associated pneumonias, nosocomial infections, and surgical wound infections (11).

Genetically HA-MRSA strains carry type I, II, or III of SCCmec and rarely possess genes that encode PVL. HA-MRSA is associated with nosocomial infections such as endocarditis and is often resistant to non-β-lactam antimicrobials, especially aminoglycosides, macrolides, lincosamides, and fluoroquinolones (73).

It is possible to transmit MRSA within hospitals through the air, droplets, direct contact (skin-to-skin), and indirect contact (via fomites). The clothing of healthcare workers could be contaminated with fomite, even if they are not aware of it. All inanimate objects in a health facility are potentially fomite sources (75).

1.8 Resistance to antibiotics on an intrinsic basis

There are three primary aspects to endogenous resistance (appendix figure 1.6):

1.8.1 The efflux system

As Ball and McMurry studied *Escherichia coli* resistance to tetracycline in 1980, they discovered the active efflux system of bacteria (76). The active efflux system was then studied, and its physiological occurrence in sensitive strains was confirmed through many experiments. Genes encoding the efflux system are activated and expressed when substrates in the environment are present for a significant period of time, resulting in a greatly enhanced ability to efflux drugs, resulting in drug resistance. Resistance to multiple drugs is influenced by active drug efflux systems. Staphylococcus aureus cell membranes contain three different types of multidrug-pumping proteins (77).

A proton kinesin is a multidrug pumping protein. Instead of ATP hydrolysis, material exchange takes place via H+ gradients formed across the cell membrane rather than ATP hydrolysis. A reversible process is usually involved, meaning that H+ moves from the extracellular space to the intracellular space, while harmful substances like dyes and antibacterial drugs flow from the intracellular space to the extracellular space (appendix figure 1.6A) (77).

1.8.2 Permeability of the outer membrane

Drug resistance occurs when the bacteria's energy metabolism is affected by lowered cell membrane permeability, resulting in decreased drug absorption. By decreasing membrane permeability, *S. aureus* resists aminoglycosides and eventually consumes less of them, resulting in resistance (appendix figure 1.6B) (77).

1.8.3 Overproduction of β-Lactamase enzyme

It is an enzyme that catalyzes the conversion of various amino acid antibiotics into their cyclic forms, it is encoded by chromosomal genes of bacteria and is transferrable from one species to another. A research showed that β-lactam antibiotics have a deadly effect on bacteria through two mechanisms: first one is by binding to penicillin-binding proteins (PBPs),which inhibits cell wall mucin synthesis, resulting in bacterial expansion and lysis; second, by triggering autolysis and death via the autolytic enzyme activity. Excessive secretion of β-lactamase by MRSA inhibits the effect of antibiotics through two mechanisms that leads to MRSA resistance. The first one is the hydrolysis mechanism, that is, β-lactamase hydrolyzes and inactivates β-lactam antibiotics; the second one is the mechanism of pinching, that is the binding of a large amount of β-lactamase quickly to extracellular antibiotics, preventing the antibiotics from reaching the intracellular space. In turn, MRSA resistance to antibiotics results due to antibiotics being unable to reach the target site (appendix figure 1.6C) (77).

1.9 Acquired antibiotic resistance

1.9.1 Biofilm-mediated resistance

Biofilms are extracellular complex structures consisting of microbial populations attached to the surface of substrates, which are surrounded by a highly hydrated extracellular polymer matrix created by the bacteria themselves, which provides a way for bacteria to adapt to their environment as a protective measure of survival (78,79).

A biofilm is the most prominent bacterial structure in nature. Biofilms have numerous characteristics including strong adhesion and drug resistance, enabling bacteria to evade host immune responses and antibiotics. They can be a thousand times more resistant to antibacterial drugs than plankton. In clinical medicine, antibiotics, as well as chemical synthetic drugs, are used in clinical medicine with some toxic effects, but domestic and foreign anti-biofilm treatments mainly depend on the development of new antibacterial

drugs. Resistance to these conventional drugs is common among biofilm bacteria, and resistant strains have been increasing in recent years (77).

1.9.2 Mutations resistance

Mutations in DNA gyrase can result in drug resistance in *Staphylococcus aureus*, causing the outer membrane proteins to reduce, reducing drug accumulation in the bacteria (80).

A modification of ribosomal RNA methylase is one of the mechanisms which are responsible for the resistance to clindamycin and erythromycin (77).

1.9.3 Acquisition of resistance genes

A plasmid-mediated resistance type called acquired resistance occurs as a result of inherited resistance. Bacteria can develop resistance to antibiotics through gene insertion and transduction through plasmids.

MRSA resistance is mainly attributed to plasmids, or plasmids capable of transmitting drug-resistant genes, which are capable of expanding the genome and transferring resistance genes between bacteria. By acquiring plasmids from Enterococcus, for example, MRSA can expand and enhance its resistance to drugs by obtaining drug-resistant plasmids (80).

1.9.4 The role of persister cells in antibiotic resistance

In microbes, persister cells are a small subset of their genetic homologous counterparts, but they are genetically heterogeneous in their phenotypic traits (81). They grow slowly, remain dormant, and grow in the presence of high antibiotic concentrations. It has been suggested in early studies that bacterial retention is associated with temporary resistance to antibiotic stress, not with any genetic changes. As high-throughput sequencing technology develops, this assumption is being challenged. A small percentage of bacteria will resist external stimuli such as antibiotics by arresting growth and remaining inactive, but most bacteria will be killed immediately by these stimuli. It has been demonstrated that these persister cells can return to normal growth when the external pressure is removed (81). It is difficult to completely eliminate bacterial infections and prevent recurrent infections when persister cells are present. Despite antibiotic treatment, bacteria persister cells demonstrate antibiotic tolerance, slow growth, and the ability to reestablish infection after antibiotic administration. By reducing cell growth, metabolism, and even

becoming dormant, persisters can resist the killing effects of antibiotics. An isogenic subpopulation of bacteria with multidrug tolerance is known as bacterial persistence. It is important to understand that persisters are not mutants, but simply phenotypic variants. According to existing research results, bacterial persistence is a complex process and involves several signaling pathways, including toxins-antitoxin systems, energy metabolism reduction by cells physiologically reduced, protein and nucleic acid synthesis systems, DNA repair and protection systems, protease systems, trans-translation systems, external pumping systems, etc (77,81).

1.10 Virulence Factors of MRSA

1.10.1 Surface associated proteins

1.10.1.1 Staphylococcal protein-A

Staphylococcal protein A is a cell wall component that binds to circulating IgG, preventing opsonization of the complement system and protecting the microbe from phagocytosis.

1.10.1.2 Clumping factors

MRSA cell surface contains clumping factor molecules that are responsible for triggering the S. aureus adherence to the fibrinogen of the extracellular matrix of the host body. MRSA was identified by two unique Clf proteins (Clf A and Clf B). Clf A is present on the surface of MRSA at all stages of growth, and Clf B is mostly detected during the beginning of the exponential phase (65).

1.10.1.3 Capsular polysaccharides

The capsular polysaccharides are polysaccharide polymers found in MRSA's cell wall. They are produced by 76 – 90 % of clinical MRSA isolates, and 11 serologically different capsular polysaccharide types (CP 1- CP11) have been found, but the two major CP types are CP5 and CP8, which all clinical *S. aureus* strains have the biosynthetic pathways for making either of them (82).

Capsular polysaccharides increase *S. aureus* pathogenicity by interfering with complement and antibody-mediated opsonization and impeding phagocytosis (65).

1.10.2 Extracellular Toxins

1.10.2.1 Staphylococcal hemolysins

MRSA strains have been found to express *alpha*, *beta*, *gamma*, and *delta* toxins, although the levels of synthesis vary amongst strains. Within these toxins, Alpha toxin is produced by the majority of pathogenic MRSA strains and is thought to be a primary virulence factor. MRSA enterotoxins are primarily implicated in both human and animal food poisoning (65).

1.10.2.2 Staphylococcal enterotoxins

Staphylococcal enterotoxins are pyrogenic exotoxins that are superantigens of *Staphylococcus aureus*. Enterotoxin types A, B, C, D, and E are the primary virulence agents implicated in human food poisoning, particularly SEA. Bacterial enterotoxins operate as superantigens (SAgs), stimulating the production of IL-4 and IL-10 genes, resulting in the activation of TH2 cells and the suppression of pathogen clearance. MRSA-produced Panton-Valentine leucocidin can cross the blood-brain barrier, causing severe damage to the cell membrane of human polymorphonuclear cells (65).

1.10.2.3 Panton-Valentine Leukocidin

One of the strongest staphylococcal exotoxins is Panton-Valentine leukocidin, it is a two-component pore-forming toxin that acts mainly on neutrophils, and it is found in high concentrations in *S. aureus* strains obtained from necrotizing infections (83). PVL is regulated by two kinds of secretory proteins, F and S. The genes lukS-PV and lukF-PV, which are found in the staphylococcal chromosome, encode for PVL production. These genes are transferred by PVL-phages, which infect the PVL-negative strains and release toxins.

PVL destroys the plasma membrane of polymorphonuclear cells in humans, increases the release of oxygen metabolites from polymorphonuclear cells, the release of interleukin 8, the synthesis of lysozymes, and the release of histamine from human basophils (65).

1.10.2.4 Staphylococcal exfoliative toxins (ETs)

Exfoliative toxins (also known as "epidermolytic" toxins) are specific serine proteases that recognize and cleave desmosomal cadherins (desmoglein-1 protein) only in the skin's superficial layers resulting in the sloughing of the epidermis, Infected infants and

neonates are mainly affected by staphylococcal scalded skin syndrome (SSSS) due to this bacteria (65,84).

1.10.2.5 Toxic Shock Syndrome Toxin

Toxic shock syndrome toxin (TSST) is one of the most potent *S. aureus* superantigens implicated in toxic shock syndrome (TSS) in humans. The condition developed as a result of the release of TSST, which stimulates the synthesis of TNF-, IL-1, and IL-2. Toxic shock syndrome is a lethal condition with a high morbidity and mortality rate. TSST typically affects women during menstruation, particularly on the second and third days. During this time, *S. aureus* thrives in the vagina and produces toxins. Fever, headache, vomiting, and diarrhea are all symptoms of the condition, which is characterized by both systemic and gastrointestinal issues (65).

1.11 In vivo studies using a mouse model

The purpose of animal experiments with infectious agents is to demonstrate the molecular bases of pathogenesis. Over the past forty years, researchers have used the mouse as a model for human infectious diseases because they are susceptible to various diseases caused by bacteria like MRSA, just like humans. This helped in releasing a multitude of reagents that have allowed rapid advances in the field of infectious disease (85).

Infection models can be constructed using mice because of a number of their characteristics. There are several advantages to them, including their small size, smaller footprint, lower cost, rapid reproduction, and similarities to humans with respect to their immune, nervous, cardiovascular, and endocrine systems (86).

An epidemic like MRSA spread needs to be studied closely in order to be able to control it. So mouse models have been extensively used in studies regarding *S. aureus* infections like infections of soft tissues and skin that can be generated by injecting staphylococci subcutaneously. In addition to understanding the role of virulence factors during infection, mice have also been used to investigate the role of specific host pathways and factors in the response against *Staphylococcus aureus*. In the past few years, several important clinical diseases have been modeled in mice (86,87).

An article published by Tseng *et al.* described and examined skin, subcutaneous, and muscle pathologies from a subcutaneous infection of a mouse model. The article also

indicated that animal model is crucial for understanding how MRSA interacts with immune responses, resulting in a more severe infection (88).

1.12 Statement of the problem and rationale of the study

Due to the potential of methicillin-resistant *Staphylococcus aureus* (MRSA) to spread in hospitals and the population, its growing emergence of it has become a major global burden and a major challenge for infection control. A study published by Kaibni *et al*. in 2009 attempted to examine MRSA colonization in nasal swabs collected at the time of hospital admission from 843 patients without a history of hospitalization and 72 healthcare staff selected in Palestine for comparison. In 2.0 % of patients and 13.9 % of healthcare staff, MRSA was observed. As a result of this study, in healthcare staff, MRSA transport was relatively large compared to that recorded in many other countries (89).

The emergence of MRSA and the increasing existence of a population reservoir for methicillin-resistant strains threaten potential healthcare regulation of antimicrobial resistance; additional antimicrobial resistance could evolve (89) especially due to the lack of effective antimicrobial treatment.

This study will contribute to solving this problem by discovering new, effective, and safe plant-derived antimicrobial that will prevent further spread of MRSA.

1.13 Aim of the study

This study aimed to test the antimicrobial activity of crude extracts from three of the most distributed species of Quercus in Palestine, including *Quercus calliprinos*, *Quercus ithaburensis*, and *Quercus coccifera*, on existing MRSA isolates and determine their MIC and MBC to be then tested in vivo using mice as an animal model.

Chapter Two
Experimental Part

2.1 Sample collection

Fresh leaves of the three Quercus species were collected from Jenin city, North West bank, as shown in table (2.1), in the period between May and December 2021. Fruits were then collected from the same places except for *Q. coccifera*'s tree which didn't have any fruit at that time of the year so only leaves were collected.

The identification of the plant samples was authenticated at An-Najah National University, College of Science, Department of Biology.

Table 2.1

Parts collected from each specie and the place they were collected from

Quercus species	Plant part collected	Collection place
Quercus calliprinos	leaves and fruits	Sir village
Quercus ithaburensis	leaves and fruits	Sir village
Quercus coccifera	Only leaves	Siris village

2.2 Preparation of plants samples for extraction

Quercus leaves were dried at room temperature without exposing them to direct sunlight in a shaded area to bring down the initial large moisture content for two weeks. Table (2.2) shows the amount of water lost from the collected Quercus leaves.

Fruits (acorn + cupule) of *Q. calliprinos* and *Q. ithaburensis* were dried in an oven at 50°C for 48 hours, then they were ground to powder by an electric grinder.

Table 2.2

Amount of water lost in leaves of each Quercus specie

Quercus species	Fresh weight	Dried weight	Water lost
Quercus calliprinos	0.6 kg	0.4 kg	0.2 kg
Quercus ithaburensis	0.56 kg	0.35 kg	kg 0.21
Quercus coccifera	0.55 kg	0.4 kg	0.15 kg

2.3 Extraction method

Two extracts were prepared every time they were needed from each Quercus species: aqueous extracts and alcoholic extracts, at room temperature by infusion method.

Aqueous extracts were prepared by soaking dried leaves (50 g/800 ml) and fruit powder (70 g/400 ml) in distilled water for 48 hours in a covered flask at 4°C. Alcohol extracts were prepared by soaking dried leaves (50 g/800 ml) and fruit powder (70 g/400 ml) in 100% ethanol for 48 hours in a covered flask at 4°C.

After 48 hours, aqueous and alcohol extracts were filtered by a mesh strainer to attain a clear filtrate. Aqueous filtrate was placed in the lyophilizer to be freeze-dried for three days to get a fine powder extract. Alcohol filtrate was placed in a flask in the hood for five weeks to evaporate ethanol and get a fine powder. These concentrated, powdered extracts were labeled and stored in a sealed flask at 4°C for further use (90–93).

2.4 In vitro antibacterial activity testing

2.4.1 Preparation of growth media

In order to prepare Mueller-Hinton agar (MHA), the manufacturer's instructions were followed. With stirring, 38 g of MHA powder was suspended in 1 liter distilled water and allowed to boil until everything was dissolved. After autoclaving the media for 15 minutes at 121°C, it was placed onto sterile Petri dishes and allowed to cool to room temperature before use.

2.4.2 Preparation of bacterial suspension

Available clinical isolate of MRSA was sub-cultured on MHA 24 hours before use. Then a sterile sample was taken gently from the colony surface of the culture and transferred to a sterile tube that contains 5 mL of sterile normal saline. The optical density of the suspension was measured by spectrophotometer at ƛ = 620 nm, where normal saline was used as blank. In order to obtain a bacterial suspension with a 1.5 x 10^8 colony forming units (CFU/mL), the turbidity of the bacterial suspension was adjusted to 0.5 McFarland turbidity standard (94).

2.4.3 Agar well diffusion method

The bacterial inoculum was uniformly spread using a sterile cotton swab on the entire surface of the sterile 12 MHA plates (previously prepared). Then, three wells (holes) with a diameter of 7mm were punched aseptically with a sterile tip in every plate. Two wells for two different volumes of the extract (35 µL, 70 µL) dissolved in its original solvent that it was soaked in at the beginning (distilled water or absolute ethanol), and the third well for the solvent control (distilled water or absolute ethanol).

The test was performed two times only for leaves extracts, every time with a set of six MHA plates with different extract concentrations (0.1 g/ml, 0.2 g/ml). The plates were incubated for 24 hours at 36ºC under aerobic conditions. After incubation, inhibition of the bacterial growth around wells was observed (95,96).

2.4.4 Determination of the minimum inhibitory concentration (MIC)

2.4.4.1 Preparation of extract samples

Every Quercus sample (leaf/fruit) was prepared by dissolving 0.1 g of extract powder in 1ml of their solvent (distilled water/ethanol) in a micro centrifuge tube. Vortex was used for mixing to ensure better dissolving of the extract. Then micro tubes were ultra-centrifuged (15000 rpm) for 7 minutes. Next, the supernatant was transferred to a new tube, and the pellet was discarded.

2.4.4.2 Serial dilution of extracts

Seventy sterile micro tubes were prepared on racks for serial dilution to be done (ten micro tubes for each Quercus specie). 250 µL from the stock solution prepared previously for each Quercus specie was transferred to the first micro tube, and mixed with 250 µL of 2X Mueller Hinton Broth (MHB) solvent to get a total volume of 500 µL. Then serial dilution was done by taking 250 µL from the first tube and transferring it to the second micro tube already containing 250 µL of the solvent and mixing it to obtain (2 fold) serial dilution with a total volume of 500 µL. After that another 250 µL or half of the total volume were taken from the second micro tube and transferred to the third and so on till the 10^{th} micro tube.

A sterile 96 micro-well plate was labelled, and then 100 µL from 2X MHB media were filled in all plate wells using a multichannel pipette. Then, 200 µL from each tube in the serial dilution done earlier was transferred and divided on the first two adjacent rows of the 96 micro-well plate until all dilutions were transferred (100 µL in the test wells and 100 µL in the extract control wells next to the test wells) except columns (11+12). Then, 50 µL from MRSA suspension was added to all wells except the extract control rows next to the test wells and the 12^{th} column.

Wells in column number 11 contained 200 µL of 2X MHB media and 50 µL MRSA suspension without plant extract, which served as positive growth control. Wells in column number 12 contained only the growth media (200 µL of 2X MHB media + 50 µL 1X MHB media) served as the negative growth control. While extract control wells contained only the extract solution and 2X MHB media to ensure that there was no contamination.

The same steps were performed for all Quercus species extracts. The absorbance of each well before incubation was determined using an ELISA reader (BioTek Instruments) at the wavelength of 450 nm. The plates were then incubated for 24 hours at 35 ˚C. Turbidity in the wells as a result of bacterial growth was detected by reading the absorbance of the wells again by the ELISA reader (97). All MIC tests were performed in triplicates.

The absorbance of every extract control well (next to the test well) was subtracted from its diluted extract well (test well) to eliminate the interference of any contaminant (98), which means the remaining absorbance belongs to bacterial cells only, and the result after

subtracting was plotted on Microsoft Excel to draw a chart as shown in figure 3.1. The best MIC was determined as the concentration at which there was a sharp increase in the absorbance curve.

2.4.5 Determination of minimal bactericidal concentration (MBC)

After determining the MIC, the dilutions of the well that has been selected as the best MIC and all the wells preceding it (i.e., those above the MIC) were re-cultured on MHA plates using a cotton swab and incubated at 36˚C for 24 hours.

Plates that did not show any growth of MRSA were considered to be the MBC for that extract.

2.5 In Vivo antimicrobial activity testing using mice

2.5.1 Preparing MRSA for experimental infection

MRSA culture was grown overnight in MHB with rotation at 37°C. On the next day, MRSA culture was diluted 1:100 into a fresh MHB and incubated for 48 hours until absorbance reading at spectrophotometer reached 0.5 at 600 nm.

For 5 minutes, the culture was centrifuged at 5000 rpm to precipitate bacterial cells before being washed twice with 6 ml of phosphate-buffered saline (PBS). Finally, bacteria were diluted with 2 ml PBS to produce a suspension of 10^7 CFU/ml (85,88).

2.5.2 Infecting laboratory mice

The BALB/c mice for this experiment were obtained from the animal house, College of Medicine and Health Sciences at An-Najah National University, and experiments were approved by the research committee and Institutional Review Boards (IRB) at An-Najah National University. Mice were kept in the animal house at a temperature-controlled room under a twelve hour light twelve hour dark cycle and had free access to commercial solid food and water ad libitum.

Twenty-one mice, same in size and of different gender, were distributed randomly into seven groups, including a control group. Every group was caged separately and contained triplets from the same gender, and every cage was labeled. All mice were prepared by shaving a circular area on their backs using an electric razor and sterilizing the area. Then

every mouse was injected subcutaneously with 50 µL of MRSA suspension in the marked area (88).

After in vitro MIC testing, the best two MIC's were determined, which are *Q. calliprinos* leaves water extract and *Q. coccifera* leaves water extract. They were dissolved only in water since the extract was meant to be orally administered to the experimental mice and ethanol cannot be used. 0.1 g of every extract was dissolved in 150 ml tap drinking water and diluted three-fold, as shown in table 2.3. Then every group of mice was provided randomly with a water-extract mixture every day for seven days. Groups were observed for seven days, and changes that might occur in mice were recorded.

Table 2.3

Test groups and extract dilution concentrations

Animal group	No. of mice & gender	Extract provided with water	Extract concentration mg/ml
Group 1	3 females	*Q. calliprinos* leaves	0.22
Group 2	3 females	*Q. coccifera* leaves	0.07
Group 3	3 males	*Q. calliprinos* leaves	0.02
Group 4	3 males	*Q. calliprinos* leaves	0.07
Group 5	3 males	*Q. coccifera* leaves	0.22
Group 6	3 females	*Q. coccifera* leaves	0.02
Group 7 (control)	3 females	NO extract (water only)	_____

On Day 8, post-infection, all mice were sacrificed, and inflammatory tissues were observed.

2.5.3 Bacterial counts in infected mice tissue

To determine the number of viable bacterial cells in the infected tissue (CFU), a swap from the infected area in mice was cultured on MHA plates and incubated for 24 hours at 35°C. Then CFUs were counted manually using Promega colony counter app by Promega Corporation and compared between all groups of mice.

2.6 Statistical analysis

A minimum of three replicates were performed and the data were processed with Microsoft Excel 2013 and expressed as mean ± standard deviation (Standard error of the mean), significant for $p \leq 0.05$.

Chapter Three

Results

3.1 In vitro antibacterial activity testing

In this stage, the agar well diffusion test was only for screening as an initial indicator of the antibacterial activity of the three Palestinian Quercus species extract against MRSA. Therefore, the test was performed twice in different concentrations of the extracts. The first time, test wells were filled with extract concentration of 0.1 g/ml solvent, and zones of inhibition were observed around wells, but they were not clear for some extracts. In the second test, inhibition zones observed were larger due to the higher concentration of extract, which was 0.2 g/ml but also not clear for some extracts. That is why a more sensitive test is needed.

3.2 Determination of MIC

The MICs of the different Quercus species against MRSA were determined using the micro plate spectrophotometric method. When absorbance values showed a sharp increase, they indicated a noticeable increase in bacterial growth at which the concentration is considered the MIC. A chart that illustrates the MIS values of alcohol extracts is shown in figure 3.1, and aqueous extracts in figure 3.2.

Both ethanolic and aqueous extracts of *Q. coccifera* and *Q. calliprinos* leaf showed antibacterial activity against MRSA. However, for *Q. ithaburensis*, only aqueous extract of fruit and ethanol extracts of leaf and fruit showed antibacterial activity against MRSA. MIC values ranged between (12.5-1.56) mg/ml, as shown in Table 3.1 (figure.3.3).

Figure 3.1

Spectrophotometer readings and MIC points of different alcohol Quercus extracts

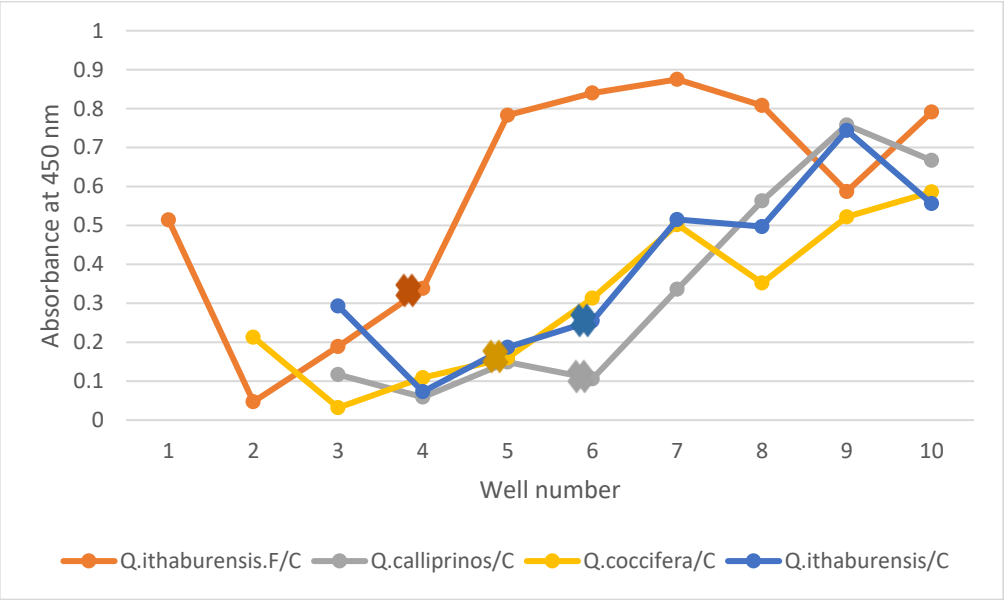


Figure 3.2

Spectrophotometer readings and MIC points of different aqueous Quercus extracts

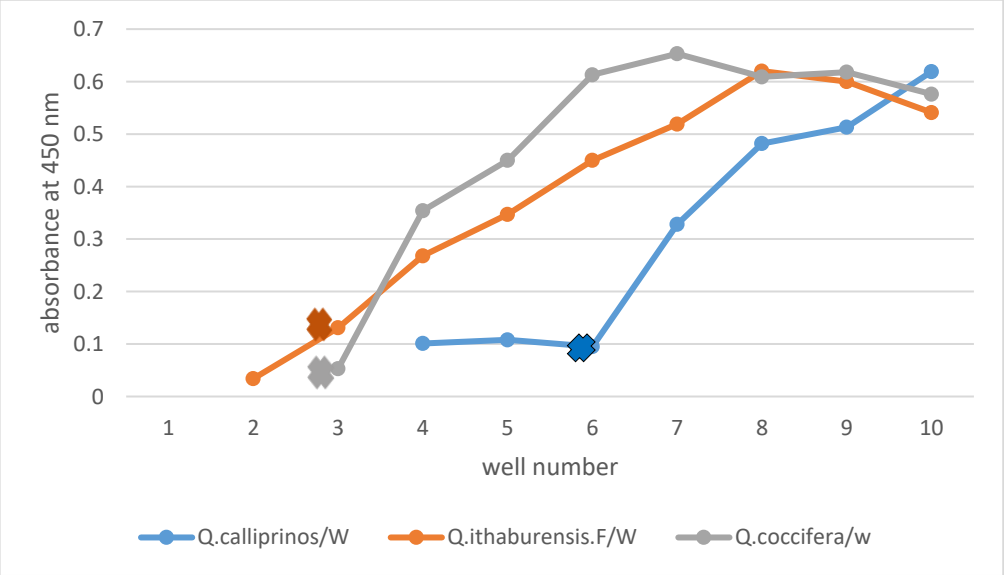


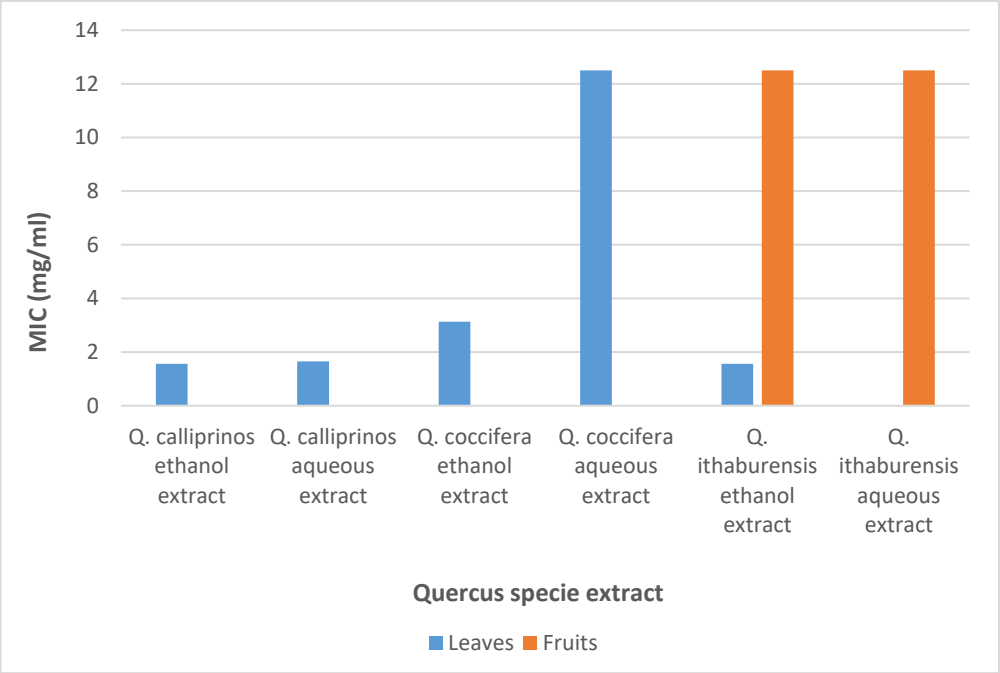
Table 3.1

Mean and standard deviation (M ± SD) MICs of three Quercus species extracts tested against MRSA. Mean of 3 experiments

Quercus Species extract	Plant part used in extract	Type of extract	MIC (mg/ml) ± SEM (Standard error of the mean
Q. calliprinos	Leaves	Ethanol	1.56 ± 0.0907
		Aqueous	1.65 ± 0.0754
	fruit	Ethanol	_____
		Aqueous	_____
Q. coccifera	Leaves	Ethanol	3.13 ± 0.0663
		Aqueous	12.5 ± 0.0866
Q. ithaburensis	Leaves	Ethanol	1.56 ± 0.0811
		Aqueous	_____
	Fruit	Ethanol	12.5 ± 0.0938
		Aqueous	12.5 ± 0.0735

Figure 3.3

Minimum inhibitory concentrations (mg/ml) of three different Quercus extracts against MRSA



3.3 Determination of MBC

After determining the MICs of the extracts, the content of the wells considered as MIC and those above the MIC were cultured. After incubation, ethanolic extracts of the three species' leaves killed MRSA, and no bacterial growth was observed at a concentration of 50 mg/ml, so they were considered as MBCs.

3.4 In vivo antibacterial activity in mice

The in vivo antibacterial activity of Quercus species extracts was examined using a mouse model. Mice were infected with 1 × 10^7 CFU of MRSA subcutaneously. An hour later, the mice were orally administered Quercus extract dissolved in drinking water. *Q. coccifera* and *Q. calliprinos* aqueous extracts were tested due to their inhibitory effect on MRSA, which was obvious in MIC testing previously.

Throw the seven days post-infection; most mice showed lesion formation ranging from very mild to moderate compared with the control group that showed an obvious lesion.

At the end of the experiment, mice were sacrificed, and internal changes in the infected tissues were observed. All mice showed a change in the color of the area of the infected tissue, which appeared white and could be distinguished from the healthy tissues.

After culturing the swabs taken from the infected tissue, bacterial counts appeared on Petri dishes were clear and countable. They were counted manually by eye and recorded in table 3.2.

Table 3.2

Means of bacterial counts from every group of infected mice tissue

Source of culture	**Mean of bacterial counts ± SEM (Standard error of the mean) in CFU**
Control group	TMTC
Q. calliprinos 1st dilution group	120.66 ± 38.4028
Q. calliprinos 2nd dilution group	73.33 ± 33.3783
Q. calliprinos 3rd dilution group	79 ± 5.8594
Q. coccifera 1st dilution group	54 ± 17.0391
Q. coccifera 2nd dilution group	91 ± 45.6544
Q. coccifera 3rd dilution group	73.33 ± 27.8348

Chapter Four
Discussion and Conclusions

Several antibiotics have recently lost their potency due to the emergence of resistant strains of bacteria, which has mostly occurred through the production of resistance genes. Antibiotics cause resistance, Also sometimes associated with adverse consequences such as hypersensitivity, immunological suppression, and allergic responses. As a result, different antimicrobial medicines are required to treat infectious diseases (99). Thus, this experiment was designed to study the antibacterial activity of ethanolic and aqueous extracts of *Q. ithaburensis*, *Q.calliprinos*, and *Q.coccifera* against MRSA, the target pathogen., in addition to testing this activity in vivo using mice as a model.

In this study, ethanol and water were selected as solvents for extracting active compounds from Quercus species based on the results of previous experiments. Previous phytochemical analysis revealed that Quercus (leaf and fruit) contains active substances such as casuarictin, pedunculagin, tellimagrandin I and tellimagrandin II, vescalagin, castalagin, phillyraeoidin E, quercitrin, mongolicain A, acutissimin B, carotechin, quercetin, and quinone. There is evidence that these compounds have antibacterial, antiinflammatory, and antifungal properties. There are several in vitro and in vivo studies mentioned in the literature review that suggest Quercus extracts possess antibacterial properties against a variety of MDR bacteria (40).

4.1 Extraction and in vitro antibacterial activity testing

Polar solvents like ethanol and aqueous are both commonly used as solvents in plant extractions due to their ability to dissolve a wide range of substances, they are highly effective and perfectly safe to use (100). Therefore, in this study, ethanol and aqueous extracts of the three Quercus species were tested against clinical MRSA strain.

Ten extracts were obtained from all Quercus species, five extracts were ethanolic, and five were aqueous. It was noticed that alcoholic extracts, in general, owed a higher antibacterial activity than aqueous extracts (101,102). The ethanolic extract of *Q. calliprinos* and *Q. ithaburensis* leaves owed a MIC equal to 1.56 mg/ml, which is the lowest concentration between MIC values of other extracts that reached 12.5 mg/ml. This result supported the observations of the well diffusion test in the beginning when these extracts showed large inhibition zones. And it contradicted the results of Basri and Fan,

that showed the same concentration of MIC (0.0781 mg/ml) for the aqueous and acetone extracts of *Q. infectoria* galls against *S. aureus* (15).

After MIC testing, the MBC was determined for all extracts (leaves and fruits), and only the first dilutions of the ethanolic extracts of the three species leaves were able to kill MRSA completely. Although the MBC concentration was very high (50 mg/ml), the ethanolic extracts were able to prevent the growth of MRSA in comparison with aqueous extracts. So they were considered as bacteriostatic agents.

Another recent research results have demonstrated that the ethyl acetate extract from *Q. acuta* leaves is capable of exhibiting anti-bacterial properties against hospital-acquired antibiotic resistant strains, such as MRSA. As a result, ethyl acetate extract displayed the highest antibacterial activity. The same extract was also found to be effective against *S. aureus* KCTC1928 and eight MRSA strains, with MICs ranging from 125 to 500 µg/ml, which indicates that it has antibacterial activity (44).

Also extracts of *Q. infectoria* galls were found to inhibit cell growth by impairing cell division and altering cell morphology in both gram-positive and gram-negative bacteria. The galls of *Q. infectoria* were extracted with acetone, ethyl acetate, 95% ethanol, and water and they demonstrated significant antibacterial activity against MRSA and MSSA strains. It was determined that both MRSA and MSSA had minimum inhibitory concentrations (MIC) ranging from 0.13 to 1.01 mg/ml, respectively, and minimum bactericidal concentrations (MBC) that ranged from 0.13 to 1 mg/ml (39), which are very low concentrations compared with our results. Which indicates the high ability of acetone and ethyl acetate to dissolve both polar and nonpolar substances which provides a concentrated final extract full of many plant phytochemicals.

The effect of ethanolic extract of *Q. infectoria* on the hydrophobicity of the surface of ten clinically isolated strains of *H. pylori* was evaluated using the salt aggregation test on ten clinically isolated strains. When infected with *Q. infectoria*, all isolates showed significant increases in hydrophobicity, regardless of the anti-biotic resistance pattern. For minimum inhibitory concentrations (MIC), the concentration ranged from 3.12 to 6.25 mg/ml. For minimal bactericidal concentrations (MBCs), the concentration ranged from 3.12 to 12.5 mg/ml (103).

4.2 In vivo antibacterial activity

Although the MIC values of ethanolic extracts of *Q. calliprinos* and *Q. ithaburensis* leaves made them the perfect candidates for in vivo testing, ethanol extracts can't be used in vivo because ethanol can't be used as a solvent and delivered orally to mice. Because oral drug delivery is the most widely used and most readily accepted form of drug administration as it is simple, painless, and self-administered (55). So only aqueous extracts of *Q. calliprinos and Q. coccifera* leaves were used.

50 µL of MRSA suspension containing 1×10^7 CFU was enough to induce infection in mice subcutaneously, and a bleb was noticed directly after injecting to confirm the correct injection depth (85,88). Observations for seven days post-infection were recorded. From day one, lesions on the back skin of mice started to form in all groups of mice. On day seven, lesions became more apparent. In control group the leasions were large and clear due to the subcutaneous injection of MRSA. The groups that took water with diluted *Q. calliprinos* 0.22 mg/ml and *Q. coccifera* 0.02 mg/ml showed very mild lesions compared with other groups. On the other hand, after incubating the cultures from the infected tissue area, bacterial counts from these two groups showed the highest counts of 154 and 73 bacterial count, respectively. The lowest bacterial count was 54 CFU found in the plate of group five, with *Q. coccifera* concentration of 0.22 mg/ml, making it a good plant candidate to inhibit bacterial growth in mice.

4.3 Limitations

This study has two main limitations. The first limitation was in the micro-broth dilution method to test the MIC of extracts; bacterial growth was hard to observe after incubation in the wells of the microplates due to the dark color of the leaf extract. And the optical density had to be repeated many times because of high concentrations of extracts.

The second limitation is that *Quercus coccifera* fruit could not be provided because it was not available in the time period in which the experiment was carried out.

Finally, the availability of experimental animals could not be controlled, so animals were provided randomly with different sexes to be used at the time of the experiment.

4.4 Conclusions

The number of new antibiotics approved by the FDA has dramatically decreased in recent decades. The fact that only a few antibiotics are effective against MRSA as well as the associated resistance to these antibiotics is a cause for concern. It may be possible to overcome resistance through the development of resistance-modifying agents. Based on the current results, it appears that the three species of Quercus were able to inhibit the growth of MRSA in varying proportions and different concentrations. Furthermore, ethanol extracts proved to owe stronger inhibitory abilities than water extracts in the in vitro experiments.

Despite the controlled in vitro experiments results, in vivo studies are important because sometimes they give different results from those predicted due to the uncontrolled environment inside a living animal.

4.5 Recommendations

Additional studies should be conducted to study the synergistic effect of these three Palestinian Quercus species extracts when combined with other antibiotics, as some studies showed a promising additive bactericidal effect on MRSA when combining the methanol and acetone extracts of *Q. infectoria* galls with vancomycin (104). Another similar research also showed the additive activities of acetone extract of Garcinia kola seeds with different antibiotics (105).

Other solvents than water and methanol could also improve the extraction of the active components. Furthermore, more efficient bioautographic procedures are required for the isolation and characterisation of the active phytochemical components of these plant species.

Toxicity tests can also be performed to assess their safety levels. Additionally, in vitro inactive extracts may have qualities comparable to pro-drugs delivered in an inactive form; thus, their metabolites may be active in vivo. As a result, we believe that this advanced research is necessary for extensively verifying the antibacterial activity of Quercus species. (106).

List of Abbreviations

Abbreviation	Meaning
CA-MRSA	Community acquired MRSA
CFU	Colony Forming Unit
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunoassay
HA-MRSA	Hospital acquired MRSA
MBC	Minimmum bactericidal concentration
MDRB	Multi drug resistant bacteria
MHA	Muller Hinton agar
MHB	Muller Hinton broth
MIC	Minimmum inhibitory concentration
PBS	Phosphate buffered saline
PBP2a	Penicillin binding protein 2a
TMTC	Too many to be counted

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Appendices

Appendix A

Figures

Figure 1.5 A

The role of active efflux systems in MRSA resistance (77)

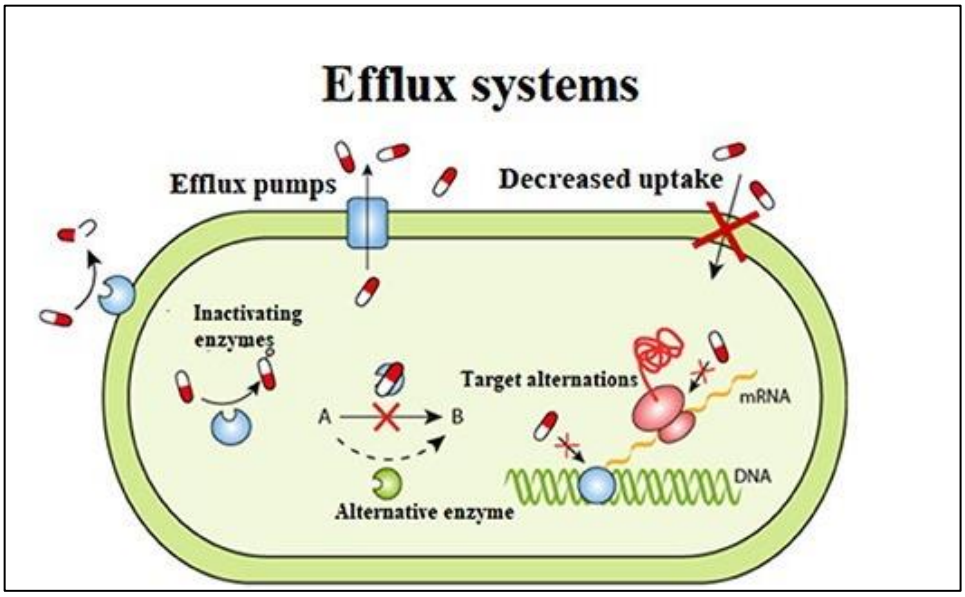


Figure 1.5 B

The scheme of decreased outmembrane permeability caused drug resistance of S. aureus (77)

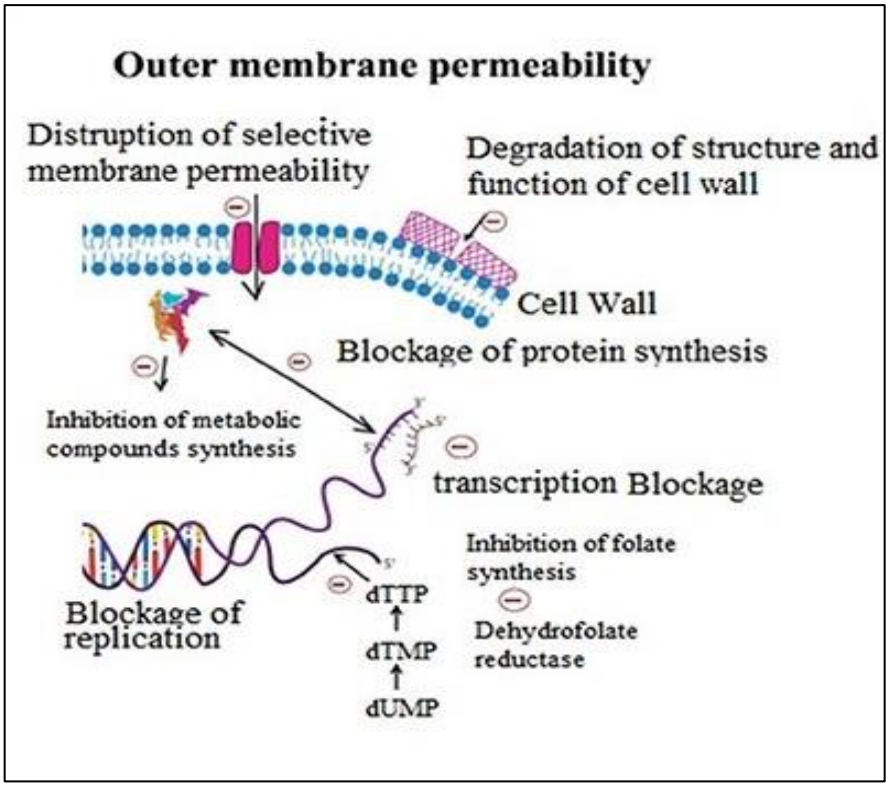
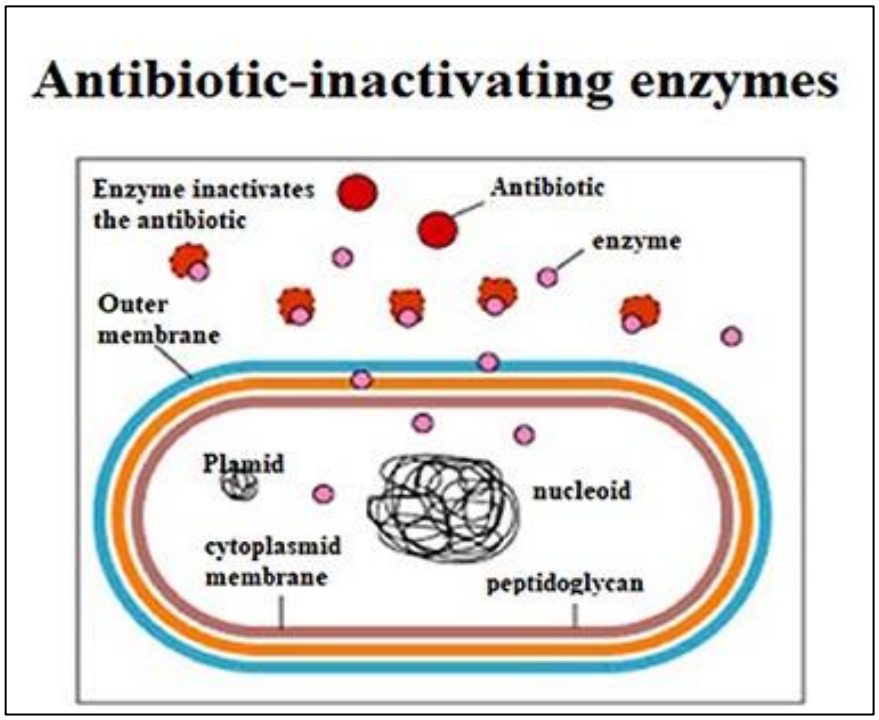


Figure 1.5 C

The role of cellular enzymes in drug resistance of S. aureus (77)



جامعة النجاح الوطنية

كلية الدراسات العليا

التأثير المضاد للبكتيريا لأنواع البلوط الفلسطينية المختلفة على بكتيريا MRSA

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قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الحياتيه، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

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الملخص

المقدمة: اثبتت الدراسات الحديثة ان السبب الأكثر شيوعاً للالتهاب الرئوي في المستشفيات هو المكورات العنقودية الذهبية بمعدل وفيات مرتفع نسبيا. السبب الرئيسي لانتشار هذه البكتيريا يعود الى المقاومة البكتيرية المتزايدة والآثار الجانبية للأدوية المضادة للميكروبات، مما أدى إلى توجه العلماء للبحث عن علاج بديل للأدوية الكيميائية اثاره الجانبية اقل ويمكن استخدامه للتقليل من انتشار هذا المرض. في العقود القليلة الماضية كان هناك اهتمام متزايد بالنباتات الطبية التقليدية. وبالتالي تمكن العلماء من استخدام المركبات الطبيعية المشتقة من النباتات لعلاج الالتهابات الجرثومية كعلاج بديل.

الهدف: الرئيسي من هذا البحث هو دراسة التأثير المضاد للبكتيريا المحتمل لمستخلصات ثلاث أنواع من نبات البلوط المائية والكحولية ضد بكتيريا المكورات العنقودية الذهبية (MRSA).

طرق البحث: تمت دراسة النشاط المضاد للبكتيريا للمستخلصات النباتية باستخدام اختبار الانتشار بالاجار(agar diffusion method)، وتم تحديد الحد الأدنى للتركيز المثبط للبكتيريا (MIC) عن طريق تخفيف تركيز مستخلص البلوط المركز بشكل تسلسلي الى نصف التركيز الأصلي في كل مرة.

بالإضافة الى الدراسة المخبرية تم عمل دراسة باستخدام الفئران الحية كنماذج حيوانية للعدوى للتحقق مما اذا كان هذا المستخلص يمكنه احداث استجابة مناعية في الجسم والوصول الى مستويات تمكنه من القضاء على هذه البكتيريا.

النتائج النهائية: كانت مبشرة جدا فقد تم تحضير عشرة مستخلصات (مائية وكحولية) من أنواع البلوط الثلاثة، وسبعة فقط أظهرت تراكيز مثبطة للبكتيريا (MIC) تراوحت بين (1.56–12.5) مجم / مل. أظهر الاختبار في الفئران مؤشرا على قدرة التثبيط البكتيري لمستخلص البلوط من نوع Q. coccifera (0.22 مجم / مل) عند إضافته إلى مياه الشرب لمجموعة الفئران. كان عدد الخلايا البكتيرية من مسحة المنطقة المصابة من هذه المجموعة من الفئران المصابة بمعدل 54 وحدة عد المستعمرات (CFU)، وهو أقل عدد من جميع المستخلصات.

كنتيجة تم التوصل الى ان المستخلصات الكحولية من البلوط لها نشاط مضاد للجراثيم أكثر كفاءة من المستخلصات المائية. تتمتع المستخلصات المائية عند مزجها بمياه شرب الفئران بقدرة جيدة على تثبيط نمو البكتيريا. يجب إجراء دراسات مستقبلية لاستخراج المكونات النشطة للبلوط باستخدام مذيبات أخرى. أيضاً، قد يؤدي الجمع بين هذه المستخلصات والمضادات الحيوية الأخرى إلى تعزيز تأثيرها في الجسم الحي.

الكلمات المفتاحية: المكورات العنقودية الذهبية، فلسطين، مضاد بكتيري، مستخلص نباتي، نموذج حيواني.