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MOLECULAR, PHYTOCHEMICAL, AND BIOLOGICAL STUDIES OF WILD BENE (*Pistacia* spp.) IN DIFFERENT LOCATIONS OF SULAYMANIYAH PROVINCE

A Dissertation

Submitted to the Council of the College of Agricultural Engineering Sciences at the University of Sulaimani in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Horticulture

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By

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﴿قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا

عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ﴾

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Dedication

I would like to dedicate this dissertation to:

My dear father.

My dear mother's soul.

My lovely husband Hoshyar.

My dear brothers and sisters.

My lovely children, Zhyar and Zhella.

My colleagues and friends who always encourage and support me.

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SUMMARY

The present study was carried out at four locations in Sulaymaniyah province, Kurdistan region, Iraq, that entailed three parts. First part: included molecular study of two species of *Pistacia*, namely *Pistacia eurycarpa* (*P. atlantica* subsp. *kurdica*) and *Pistacia khinjuk*. *Pistacia* species were identified morphologically in the locations according to variations in the traits of leaves and stems. In April 2020, leaf samples of *Pistacia* species were collected from forest sites in Qaradagh, Ranya, Sharbazher, and Hawar in Sulaymaniyah province. Using a novel primer set for the biomarker 18S rRNA region, a band of around 600 bp was detected, sequenced, and aligned with 18S rRNA partial sequences of 11 species of *Pistaci*a mined from the National Center for Biotechnology Information (NCBI). Four plants at the four locations were found to be closely related to that recorded for species of *P. atlantica*, while two other plants in Qaradagh and Hawar were seemed to be closely related to that recorded for species *P. khinjuk* on the NCBI. The 18S rRNA sequences of the *Pistacia* species submitted to NCBI GenBank and the accession number were assigned (MW534226, MW534227, MW534228, MW534229, MW534230, MW534231). The neighbor-joining method showed that the phylogenetic relationships of *P. atlantica* and *P. khinjuk* with other *Pistacia* species were closely related.

Second part: estimated the chemical constituents of *P. eurycarpa* parts. For this purpose, leaves, fruits, and rachis in spring and autumn were collected, but bark and gum were collected in spring and summer respectively. From April up to October 2020, samples were taken from the four locations. Concerning the percentage nitrogen, phosphorus, and potassium in leaves, the highest values of nitrogen (6.70%) in spring leaves of Qaradagh. Whereas the highest percentage of phosphorus (2.03%) and potassium (2.25%) occurred in the spring leaves of Hawar. Carbohydrate results showed the highest values of carbohydrate (30.83%) in spring leaves of Qaradagh, while the lowest value (20.31%) in Sharbazher autumn leaves. Essential oil was extracted from gum and autumn rachis of *P. eurycarpa* collected from different locations. Sharbazher gum showed the highest and significant value of essential oil (29.00%), while the lowest value was found in Hawar autumn rachis (2.40%). Regarding GC-MC analysis of essential oil, the highest concentration of alpha-pinene was found in gum taken from Hawar (81.40%). The highest concentrations of the other major constituents in the autumn rachis were beta-pinene (12.03%) in Hawar, camphene (4.42%) in Sharbazher, and beta-myrcene in Qaradagh (3.48%), but beta-phellandrene (2.32%) was found in the gum collected from Sharbazher. Fixed oil was extracted from autumn fruit and bark of *P. eurycarpa* collected from various locations. Qaradagh autumn fruits contained the highest levels of fixed oil (32.08%).

Whereas, the lowest value (3.10%) was in bark collected from Hawar. GC analysis of fixed oil showed the highest values of palmitic acid (11.69%), stearic acid (4.20%), oleic acid (45.39%), linoleic acid (15.36%), and linolenic acid (0.77%) contents appeared in autumn fruit fixed oil collected from Ranya. On the other hand, bark fixed oil collected from Hawar contained lowest amounts of palmitic acid (2.55 %), stearic acid (0.66%), oleic acid (5.89%), linoleic acid (3.69%) and linolenic acid (0.14%). The highest total phenol was 307.057 mg/g recorded at Qaradagh spring leaves, while the lowest level of phenol (1.409 mg/g) occurred at Sharbazher gum. Regarding total flavonoid, the highest level of total flavonoid was 101.483 mg/g obtained by Ranya spring leaves; it was significantly exceeded the lowest level (0.399 mg/g) at Hawar bark. HPLC analysis revealed that the highest concentrations of quercetin, rutin, catechin, ferulic acid, and ellagic acid respectively in Ranya spring leaves were 168.9 µg/g, 149.7 µg/g, 124.5 µg/g, 122.4 µg/g, and 97.4 µg/g. Cinnamic acid, kaempferol, tannic acid, and stilbene in Hawar autumn leaves were 142.6 µg/g, 98.7 µg/g, 97.4 µg/g, and 55.2 µg/g, respectively; while gallic acid was 10.5 µg/g in Hawar gum, and apigenin was 10.2 µg/g in Qaradagh bark.

Third part: included biological activity of *P. eurycarpa* essential oil (PEEO). Biologically active ingredients of essential oils (EOs) provide a promising novel source of antibacterial agents. PEEOs inhibited the growth of Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Acinetobacter baumannii*) and Gram-positive (*Staphylococcus haemolyticus,* and *Enterococcus faecalis*) bacteria, but was not so effective on the growth of *Pseudomonas aeruginosa*. Gram-positive bacteria were more susceptible to PEEOs than Gram-negative bacteria. For further study Hawar gum essential oil will be selected as it has the highest antimicrobial activity against Gram-positive and Gram-negative bacteria compared with other oils. Later, *P. eurycarpa* essential oil of Hawar (PEEO-H) gum were tested against MDR Gram-negative *A. baumannii.* PEEO-H exhibited excellent antibacterial and antibiofilm activities against *A. baumannii*. The minimum inhibition concentration (MIC) of PEEO-H was 2.5 µl/ml. Moreover, the mechanisms of PEEO-H actions against *A. baumannii* were investigated. Protein, phosphate, and potassium ions leakage were increased in the supernatant of the *A. baumannii* after treating the cells with 2.5 µl/ml of PEEO-H. Moreover, transmission electron microscopes (TEM) images showed the distortion of the morphology of the tested bacteria cells, leading to lysis of the cell wall, intracellular ingredient leakage, and consequently, cell death. Finally, PEEO-H exhibited bacterial clearance and anti-adhesion activity using Band-Aids.

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List of Abbreviations

bp	Base Pair
$CaCO_3$	Calcium carbonate
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
Conc	Concentration
CRD	Completely Randomized Design
CTAB	Cetyltrimethylammonium Bromide
DIZ	Diameter Inhibition Zone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Ec	Electrical Conductivity
EOs	Essential Oils
g	Gram
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
GC- MS	Gas Chromatography-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
K	Potassium
L	Litter
LB	Luria–Bertani
MBC	Minimum Bactericidal Concentration assay
MDR	Multidrug Resistance
mg	Milligram (0.001 gram)
µg	Microgram (0.000001 gram)
MIC	Minimum Inhibitory Concentration
ml	Milliliter (0.001 liter)
µl	Microliter (0.000001 liter)
mm	Millimeter (0.1 centimeter)
µm	Micrometer (0.000001 meter)
µmol	Micromole (0.000001 mole)
N	Nitrogen
NA	Nutrient Agar
NB	Nutrient Broth

NCBI	National Center for Biotechnology Information
OD	Optical Density
O.M	Organic Matter
P	Phosphorus
PEEO	*Pistacia eurycarpa* Essential Oil
PCR	Polymerase Chain Reaction
PDR	Pandrug Resistance
pH	Potential of Hydrogen
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rpm	Rotation Per Minute
SMs	Secondary Metabolites
Sp.	Species for Single
Spp.	Species for Plural
TBE	Tris-Borate-EDTA
TEM	Transmission Electron Microscopy
WHO	World Health Organization
XDR	Extensively Drug Resistance

CHAPTER ONE
INTRODUCTION

The genus *Pistacia* is a member of the Anacardiaceae family. *Pistacia* consists of at least 11 species, the majority in Asia and the Mediterranean region, but one species extending into tropical Africa, another species in Central America, some of which have edible nuts and are commercially important (Townsend *et al.*, 1980; Kafkas and Perl-Treves, 2001). Three *Pistacia* species are found in Kurdistan region of Iraq; *P. eurycarpa* (*P. atlantica* subsp. *kurdica*), and *P. khinjuk* are wild species. *P. vera*, on the other hand, is a cultivated species (Shabaz, 2010). *P. eurycarpa* is known as "Daraban" in Kurdistan and is one of the most common *Pistacia* species worldwide (Dyary *et al.*, 2017). In Iran, *P. atlantica* is called "Baneh" and is the most economically important tree species in many rural areas (Saber-Tehrani *et al.*, 2013). *P. eurycarpa* is indigenous to Kurdistan in northern Iraq, southern-east Turkey, northwestern Iran, Afghanistan, Syria, and Armenia (Shabaz, 2010). Environmental factor effect on quantity and quality of primary and secondary metabolites (SMs) in plant. Plant SMs are not only a useful array of natural products but also an important part of plant defense system against pathogenic attacks and environmental stresses. SMs accumulation is strongly dependent on a variety of environmental factors such as light, temperature, soil water, soil fertility and salinity, and for most plants, a change in an individual factor may alter the content of SMs even if other factors remain constant (Yang *et al.*, 2018).

Leaves, fruits, and gum of *Pistacia* are valuable for their medicinal, cosmetic, and nutritional value. Different parts of *Pistacia* species have been found to exhibit antibacterial, antifungal (Hasheminya and Dehghannya, 2020), anti-inflammatory, wound healing (Azeez and Gaphor, 2019), antihyperlipidemic (Hosseini *et al.*, 2020), antidiabetic and antihypertensive (Ahmed *et al.*, 2018) properties. Notably, various types of phytochemical constituents like terpenoids (Najafiasl *et al.*, 2022), phenolic compounds (Hasheminya and Dehghannya, 2020), fatty acids, and sterols (Labdelli *et al.*, 2019) have also been isolated and identified from different parts of *Pistacia* species.

Pistacia species gum produces a considerable yield of essential oil. In addition, gum has been widely used in medicine, dietary supplements, and cosmetics (Pachi *et al.*, 2020). *P. eurycarpa* gum is also used to make natural Kurdish chewing gum. *Pistacia* species fruits are widely consumed as a nutrient by the local population and produce a considerable yield of oil. Also, the oil can be used in traditional medicine, food, and cosmetics (Saber-Tehrani *et al.*, 2013). Its oil contains both saturated and unsaturated fatty acids; the ratio of unsaturated fatty acids is

approximately three times higher than that of saturated fatty acids (Labdelli *et al.,* 2019). The oil is rich in mono-unsaturated fatty acids, particularly oleic acid, which is inversely related to cholesterol levels (Ahmed *et al.,* 2021). *P. atlantica* subsp. *atlantica* leaves are known to be an excellent source of phenolic compounds (Toul *et al.,* 2017). The plant contains various types of SMs like phenolic compounds, tannins, terpenoids, and alkaloids which have strong antimicrobial properties (Barawi *et al.,* 2021).

Finding new antimicrobial drugs against multidrug resistant (MDR) Gram-negative bacterial infections is extremely difficult, mainly due to the complex structure of their cell walls and β-lactamase production (Freitas *et al.,* 2013). Antibiotic resistance in Gram-negative MDR bacteria is most often plasmid-mediated, marking their territory on the World Health Organization's (WHO) critical priority pathogens list. Moreover, WHO reported that *Acinetobacter baumannii* is among the major antibiotic-resistant pathogen priorities, highlighting its serious threat to public health (WHO, 2017). This is due to the ability of the bacterium to quickly adapt to new antibiotics. Also, some strains of the bacterium have been found to be resistant to all commercially available antibiotics (Harding *et al.,* 2018). *A. baumannii* is an aerobic, Gram-negative, non-motile, coccobacilli, ubiquitous organism that exists in water, food, and soil. *A. baumannii* can survive for a long time on abiotic surfaces under desiccated conditions through the presence of cells in a dormant state (Gayoso *et al.,* 2014). Thus, the bacterium can show persistence for the long term in a hospital environment. Strains of *A. baumannii* are problematic hospital pathogens due to their biofilm-forming ability and multiple antibiotic resistance. The biofilm matrix that surrounds bacteria permits germs to withstand extreme circumstances and resist treatments with antibiotics. As a result, medications now available for treating *A. baumannii* biofilm-associated infections are ineffective (Shali *et al.,* 2022).

Despite the natural occurrence of the *Pistacia* tree in Kurdistan region of Iraq, there is no investigation on the molecular identification of *Pistacia* species. Moreover, there is a paucity of literature on the chemical constituents of various parts collected from local locations and the use of *P. eurycarpa* essential oils as potential alternative treatments for infectious diseases caused by MDR pathogens.

The aims of the study were to:

- Identify the wild *Pistacia* species from different locations in Sulaymaniyah province.

- Examine the chemical compounds of plant parts such as leaves, fruits, rachis, bark, and gum at different locations, to determine the best location that contains plant parts with the highest concentrations of active compounds.
- Compare the effect of essential oils, which were extracted from different plant parts collected from different locations in order to find out their effect against a number of both Gram-negative and Gram-positive bacteria.
- Investigate the antibacterial activity of *P. eurycarpa* essential oil, which had the highest antibacterial activity on MDR bacteria, and further appraise the mechanism of action.
- Investigate the antibiofilm and anti-adhesion activity of *P. eurycarpa* essential oil on MDR bacteria.

CHAPTER TWO
LITERATURE REVIEW

2.1 *Pistacia* Species

Pistacia belongs to the family Anacardiaceae with the order of Sapindales under the division Magnoliophyta of the kingdom Planate. The genus consists of at least eleven species (Yaltirik, 1988; Shabaz, 2010). The first classification of the genus *Pistacia* was done by Zohary (1952)**,** who classified the *Pistacia* into four sections including 11 species (Fig. 2.1): Eu-Terebinthus (deciduous and paripinnate/imparipinnate leaves), Eu-Lentiscus (persistent and paripinnate leaves), Lentiscella (sub-persistent leaves) and Butmela (deciduous and imparipinnate leaves). Several others also described and classified *Pistacia* species based on their morphology (Zohary, 1972; Al-Yaf, 1978; Yaltirik, 1988).

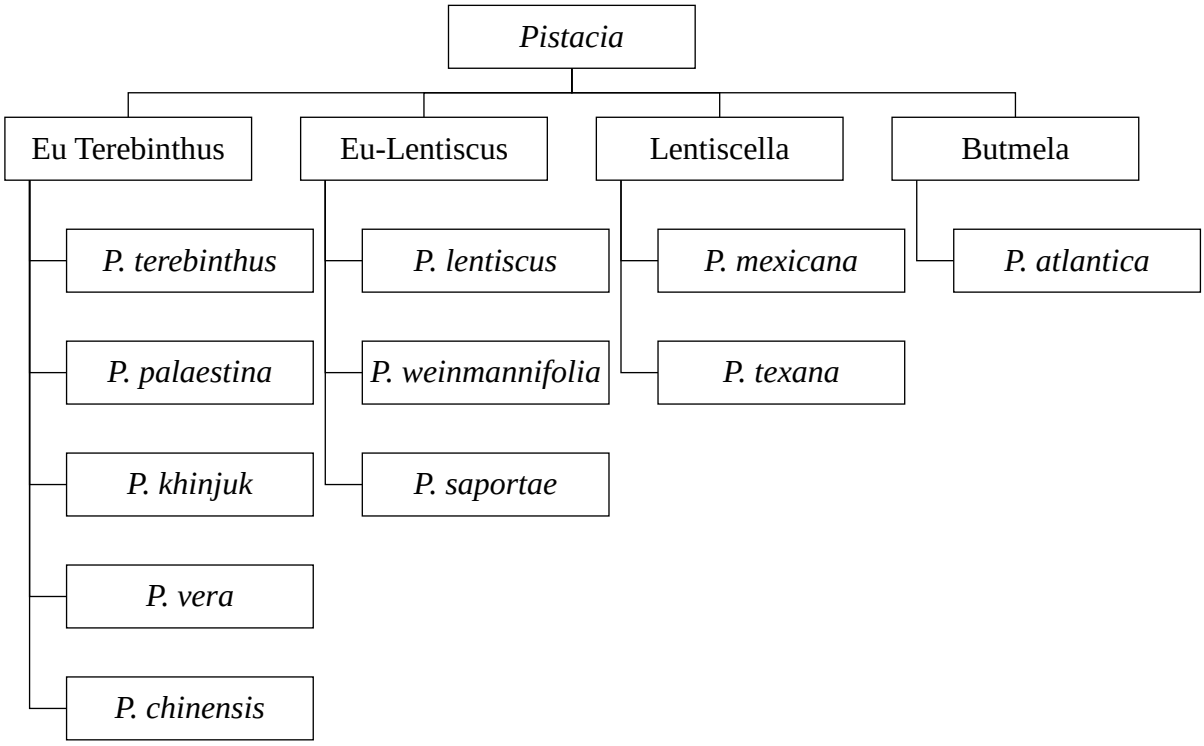


Figure 2.1 Classification of the genus *Pistacia*.

Besides the leaf and seed morphological characters, on the basis of restriction fragment length polymorphism (RFLP) analysis of the *Pistacia* chloroplast DNA, Parfitt and Badenes (1997) suggest to divide the genus into two sections, Terebinthus and Lentiscus (Fig. 2.2). Terebinthus includes deciduous species with imparipinnate leaves and large seeds whereas, Lentiscus consists of the evergreen species with paripinnate leaves and small seeds.

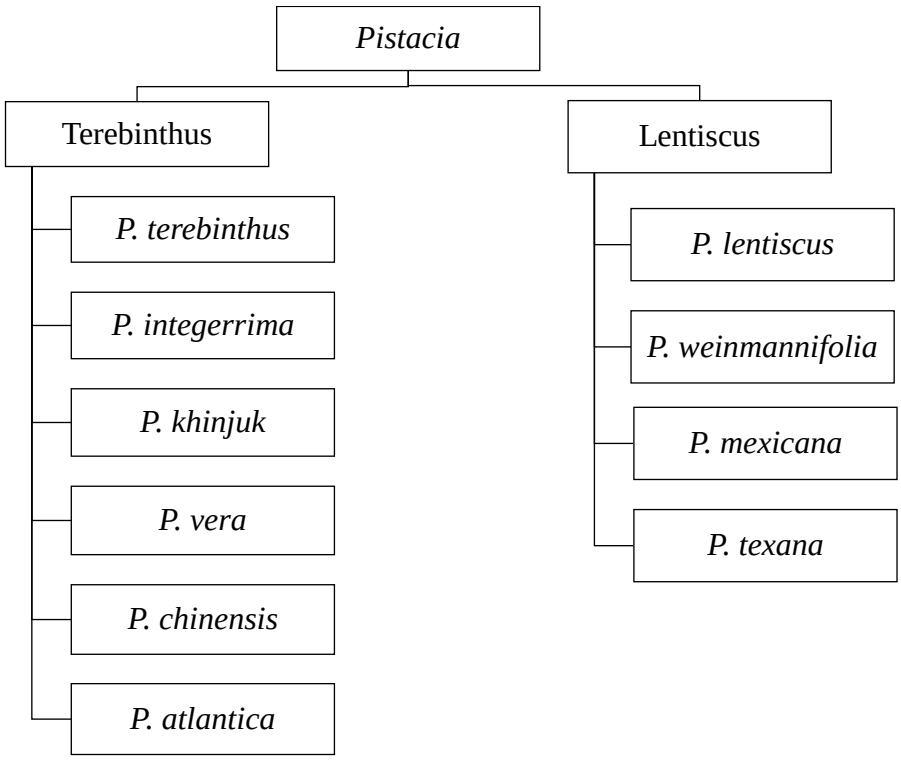


Figure 2.2 Classification of the genus *Pistacia* based on RFLP analysis.

Three *Pistacia* species grown naturally in Iraq, including *P. eurycarpa* (*P. atlantica* subsp. *kurdica*), *P. khinjuk*, and *P. vera.* They are all deciduous tree and dioecious plant. *P. vera* is the only cultivated species in this genus , which produces edible large seeds to be commercially acceptable, but *P. khinjuk* and *P. eurycarpa* species are wild species (Al_Dawoody, 1979). *P. atlantica* subsp. *kurdica* recorded in flora of Iraq as synonym of *P. eurycarpa* (Townsend *et al.*, 1980). Rechinger (1969) in flora of Iranica classifies three subspecies for *P. atlantica*: *P. atlantica* subsp. *mutica*, *P. atlantica* subsp. *kurdica*, and *P. atlantica* subsp. *cabulica*, meanwhile *P. eurycarpa* has been recorded as the synonym of *P. atlantica* subsp. *kurdica.* However, in flora of Turkey, *P. atlantica* was described as a separate species from *P. eurycarpa* (Yaltirik, 1988).

2.2 *Pistacia* Species Uses

2.2.1 Traditional uses of some *Pistacia* species

Different species of *Pistacia* have traditionally been used for a variety of purposes. Among them, *P. lentiscus* is most commonly used in different regions, and it's gum has been used for a variety of gastric ailments in Mediterranean and Middle Eastern countries (Dimas *et al.*, 2009). *P. atlantica* is the main plant medicine widely used in the Middle East and Mediterranean areas since ancient times. Iran is one of the largest producers and exporters of

P. atlantica products (Mahjoub *et al.*, 2018). Various industrial and traditional uses are mentioned for the main parts of wild pistachios (gum and fruit), including food and medicine (Mahjoub *et al.*, 2018). The fruit of the wild pistachio is used by the natives as a flavoring in food after grinding and is used for their oil (Saber-Tehrani *et al.*, 2013). In Kurdistan, the spring fruits are harvested and eaten with sour milk. In addition, gum has been used in traditional Kurdish medicine for treating various disorders such as topical wounds and gastric ulcers. It is also used to make natural Kurdish chewing gum without the addition of any additives, preservatives, or colorings.

2.2.2 Pharmacological uses of *Pistacia* species

Many traditional uses of *Pistacia* species have now been confirmed by pharmacological research. Different parts of *Pistacia* species have been used in the treatment of various diseases in different geographical locations. For example, gum is prescribed for gastric disorders in Morocco, Iraq and Turkey. Similarly, the leaf is used externally as a prescription for antidiabetic drugs in Jordan (Ahmed *et al.*, 2021). Crude extracts and extracted compounds from *Pistacia* species show a broad range of pharmacological properties (Bozorgi *et al.*, 2013). Several researchers have reported wide pharmacological activities of *Pistacia* species in-vivo and in-vitro test models. Different parts of *P. atlantica* have been found to have antibacterial, antifungal, anti-inflammatory, analgesic, wound healing, anticancer, anticholinesterase, antidiabetic, hepatoprotective, erythrocyte protective, urease inhibition, antihypertension, nipple-fissure healing, antileishmanial and antiplasmodial activities (Jaafari-Ashkvandi *et al.*, 2019; Pasban-Aliabadi *et al.*, 2019; Hosseini *et al.*, 2020; Karimi *et al.*, 2020; Ahmed *et al.*, 2021). Fathollahi *et al.* (2019) demonstrated that the *P. atlantica* subsp. *kurdica* essential oil had antibacterial effects against the Gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Shewanella* sp.). In another study, the antimicrobial activity of *P. atlantica* subsp. *kurdica* essential oil gum was found to have positive results on *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Bacillus subtilis*, *Micrococcus luteus*, *E. coli* and, *Klebsiella pneumonia* (Hama Amin *et al.*, 2022).

2.3 Molecular Studies

Morphological and biochemical characteristics are used for distinguishing among populations and for their taxonomical classification. Recently, molecular markers also applicable for the classification of different plant species (Guney *et al.*, 2021). However, morphological and

biochemical characters are strongly influenced by environmental conditions which limit their use in genetic diversity studies, whereas DNA molecular markers are independent of environmental conditions or developmental stages and show a high level of polymorphism (Mirzaei, 2021). Molecular markers have high reliability in determining the diversity in plant populations. Thus, molecular markers have been efficiently used in classification studies of different plant species (Borah *et al.*, 2021).

Among molecular markers, 18S rRNA gene sequencing is the most commonly used marker for taxonomic identification in eukaryotes (Gong and Marchetti, 2019). It contains both conserved and variable regions which can reflect the differences among eukaryotic species. Also, it forms a major component of the small subunit of the eukaryotic ribosome (Bininda-Emonds, 2021). The small subunit 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and it is an important marker for the random target PCR in environmental biodiversity screening. Diversity in *Pistacia* has been evaluated at molecular levels using the internal transcribed spacer 1 marker in three species (*P. atlantica* subsp. *atlantica*, *P. vera* and *P. terebinthus*) compared with other *Pistacia* species (Labdelli *et al.*, 2022).

2.4 Influence of Environmental Factors on Plant Secondary Metabolites

Iraq is now suffering from climatic changes impacts in similar or even worse ways than many other countries of the world (Hassan and Hashim, 2020). Tree species distribution is known to depend mainly on climate variables, particularly temperature, and precipitation (Amissah *et al.*, 2014). In fact, synthesis and accumulation of chemical components in plant depends on environmental conditions. External factors such as light, temperature, soil water, and soil fertility can significantly affect some processes associated with growth and development of the plants, even their ability to synthesize primary and secondary metabolites (SMs), eventually leading to the change of overall phytochemical profiles which play a strategic role in production of bioactive substances (Griesser *et al.*, 2015; Verma and Shukla, 2015). In other words, plant SMs can be gradually generated in response to environmental stress. Hence, plant secondary metabolism be viewed as plant behavior that is in part the ability of adaptation and survival in response to environment stimuli during the lifetime (Metlen *et al.*, 2009). In particular, for medicinal plants, environmental conditions are capable of redirecting the metabolism to consequently regulate the production of active constituents. Herbal medicines have a complex and variable chemical composition, the type and amount of SMs as well as biological effects of medicinal plants were often determined according to the change of environment (Yang *et al.*, 2018). Change in temperature affects plant growth and metabolic pathways involved in

signaling, physiological regulation and defense responses. Temperature as a major weather variable can significantly influence the composition of SMs with disruption in photosynthesis activities to tolerate stressful condition (Pant *et al.*, 2021). High and low temperatures are harmful abiotic stresses that can impact the survival of plants. Therefore, adapted plants alter their metabolisms when faced with such temperatures to increase the level of essential metabolites and thereby tolerate unfavorable conditions (Isah, 2019). High temperatures enhance the biosynthesis of alkaloids (Da Silva *et al.*, 2022); in contrast, low temperatures enhance the synthesis of phenolic compounds (Li *et al.*, 2020).

Plant growth and development are largely determined by nutrient availability, nitrogen (N), phosphorus (P), potassium (K), zinc (Zn), sulfur (S), and magnesium (Mg) are some of the vital nutrients required for optimum growth, development, and productivity of plants. The deficiency of any of these nutrients may lead to defects in plant growth and decreased productivity (Kumar *et al.*, 2021). Supplemental plant mineral nutrition may provide a means not only to stimulate plant growth but also influence the content of SMs. Availability of plant nutrients can be an important factor in determining secondary metabolism and antioxidant activity within plants (Yang *et al.*, 2018).

2.5 Chemical Constituents of *Pistacia* Species Parts

Important phytochemicals such as terpenes, essential oils, tannins, flavonoids, steroids, fatty acids, and other compounds are present in the fruit, leaf, stem, bark, and gum of *Pistacia* species. Some of them have shown multiple bioactivities in vivo and in vitro (Bozorgi *et al.*, 2013).

2.5.1 Essential oils in *Pistacia* species gum

Essential oils (EOs) are natural volatile oils with a wide range of bioactive complex compounds characterized by a strong odor and formed by plants as aromatic SMs (Ríos, 2016). EOs are liquid, volatile, limpid, rarely colored, and soluble in lipids and organic solvents that some of them are less dense than water (Antonelli *et al.*, 2020). They can be presented in any plant organ, including buds, leaves, branches, stems, flowers, fruits, seeds, roots, and bark. EOs are generally stored by the plant in secretory cells, canals, cavities, epidermic cells or glandular trichomes (Nazzaro *et al.*, 2013). About 3000 EOs are currently known, 300 of which are commercially important, particularly for the pharmaceutical, agronomic, food, hygiene, cosmetic and perfume industries (Ni *et al.*, 2021). EOs have been used for medicinal purposes and as therapeutics since ancient times (Garzoli *et al.*, 2020). Although food industry uses EOs

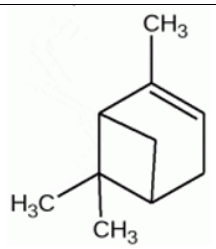
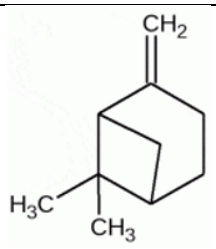
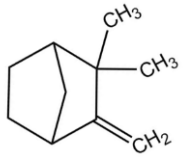
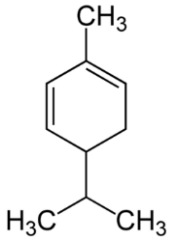
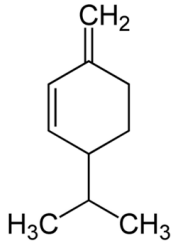
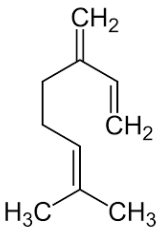
as a flavoring agent, their potential as a natural, food-grade preservative has not been fully explored. EOs represent a valuable tool for food preservation due to their natural antimicrobial properties (Hyldgaard *et al.*, 2012). EOs have a complicated composition, containing from a dozen to several hundred compounds. Maurya *et al.* (2013) reported that essential oils are complex mixtures of volatile substances, typically terpenes, sesquiterpenes, and oxygenated derivatives. EOs are mostly analyzed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). EOs are one of the main components of various parts of *Pistacia* species, including leaves, ripe and unripe fruits, and gum. There are many qualitative and quantitative differences between essential oil content. These variations are related to several parameters such as plant species, plant parts, harvest time, and climatic conditions (Alma *et al.*, 2004).

Pistacia species gum is known to be a rich source of essential oils used in medicine to treat various diseases (Table 2.1). The main ingredients of the essential oils reported by hydrodistillation of the *P. atlantica* subsp. *kurdica* gum based on GC-MS are α-pinene (92.5%) and β-pinene (1.62%) (Ellahi *et al.*, 2019). α–pinene, β–pinene, α-phellandrene, β-phellandrene, camphene, α-terpinene, and limonene were identified as main constituents in essential oils of *P. atlantica* (Labed-Zouad *et al.*, 2017; Didehvar *et al.*, 2020). *P. atlantica* gum essential oil analyzed by GC-MS led to the identification and quantification of 26 compounds representing 95.22% of the total composition. The components characterizing oils were α-pinene (57.06%), β-pinene (9.83%), α -phellandrene (3.81%), trans-verbenol (3.79%), camphene (1.02%) (Kakakhan *et al.*, 2018). Karim (2014) identified eleven ingredients of the essential oils from different plant martials (leaf, seed, outer shell, cluster and gum) of *P. eurycarpa* and *P. khinjuk*, namely α-pinene, β-pinene, phellandrene, limonene, sabinene, aldehyde-citral, 3-carene, β-myrcene, terpinene, carveol, and myrtenol.

In aromatic plants, biosynthesis of essential oils involves mevalonate pathway (MVA) and non-mevalonate (mevalonate independent) or deoxy xylulose phosphate pathway (Fig. 2.3). In the mevalonate pathway, isopentenyl pyrophosphate (IPP) is formed through mevalonic acid that results from the condensation of 3 molecule acetyl coenzyme-A moieties. It takes place in the cytoplasm and leads to the formation of most sesquiterpenes ($C_{15}H_{24}$) (Pulido *et al.*, 2012). In the non-mevalonate pathway, methylerythritol-4-phosphate (MEP) and 1- deoxyxylulose-5-phosphate (DOXP) are involved, resulting from the condensation of glyceraldehyde phosphate and pyruvate. Non-mevalonate pathway occurs in the chloroplasts, producing primarily monoterpenes ($C_{10}H_{16}$) and diterpenes ($C_{20}H_{32}$) (Said-Al Ahl *et al.*, 2017). IPP and dimethylallyl pyrophosphate (DMAPP) lead to geranyl diphosphate (GPP), the immediate

precursor of monoterpenes. The condensation of GPP with IPP leads to farnesyl diphosphate (FPP), the immediate precursor of sesquiterpenes. The condensation of FPP with IPP results in geranyl geranyl diphosphate (GGPP) the immediate precursor of diterpenes (Rehman *et al*., 2016).

Table 2.1 The important monoterpenes in *Pistacia* species essential oils and their medicinal uses.

Name of compound	Chemical formula	Chemical structure	Medicinal uses	Reference
α - Pinene	$C_{10}H_{16}$		They have a wide range of pharmacological activities have been reported anticoagulant, antitumor, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and analgesic effect.	Salehi *et al.*, 2019
β - Pinene	$C_{10}H_{16}$			
Camphene	$C_{10}H_{16}$		Antibacterial, antifungal, antioxidant, anticancer, antidiabetic, and anti-inflammatory properties.	Hachlafi *et al.*, 2021
α -Phellandrene	$C_{10}H_{16}$		Analgesic, anti-inflammatory, anticancer activity, and wound healing activity.	Lin *et al.*, 2016; Siqueira *et al.*, 2016; Scherer *et al.*, 2019
β - Phellandrene	$C_{10}H_{16}$		Antifungal and antibacterial activities.	Mneimne *et al.*, 2016
β - Myrcene	$C_{10}H_{16}$		Analgesic, antioxidant, anti-inflammatory, antibacterial, and anticancer effects.	Surendran *et al.*, 2021

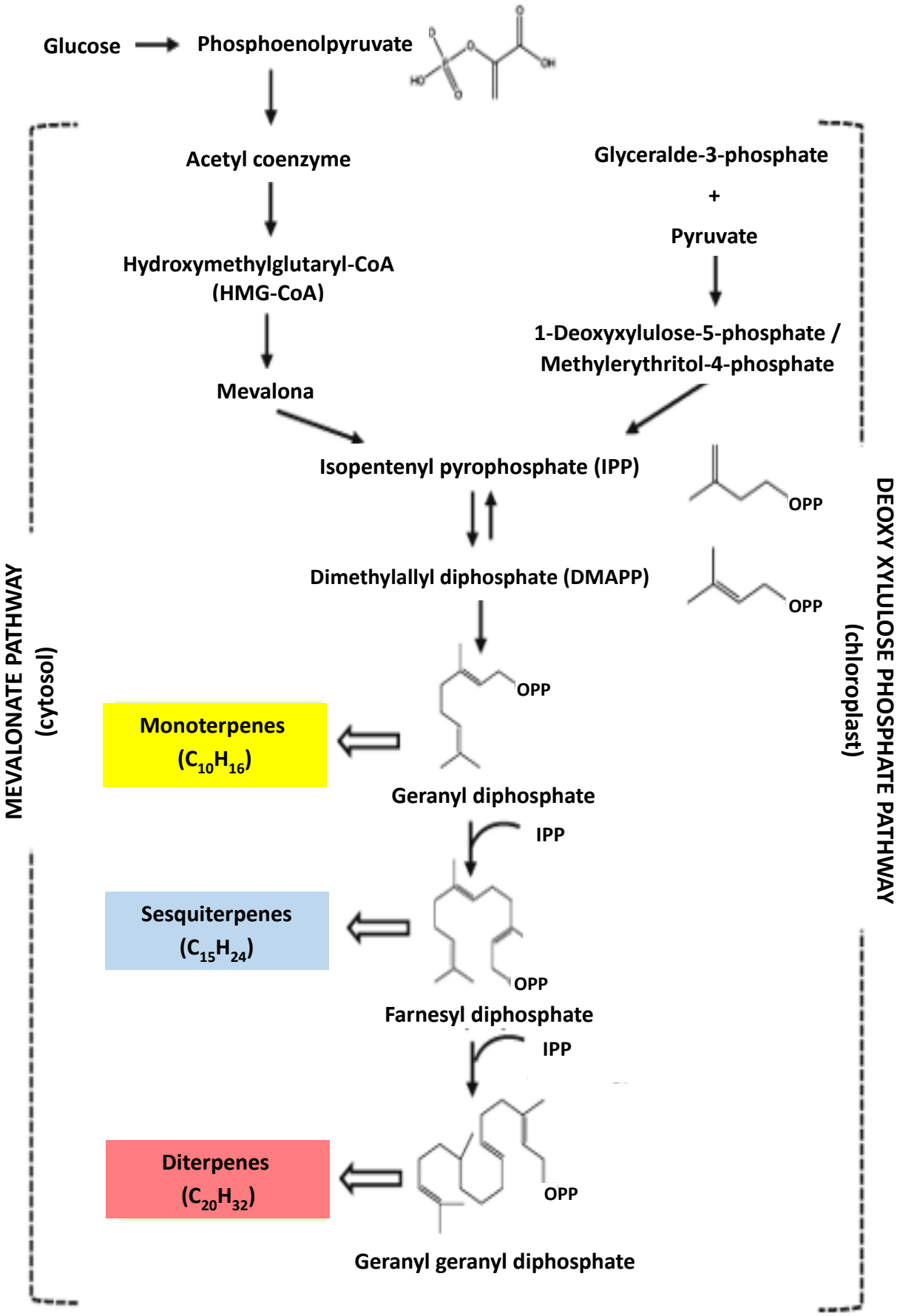


Figure 2.3 Terpenoids biosynthesis in plants. This schematic is adapted from (Said-Al Ahl *et al.*, 2017).

2.5.2 Fixed oil in the fruit of *Pistacia* species

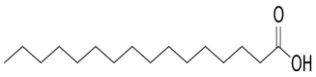
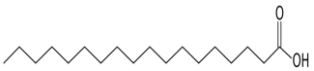
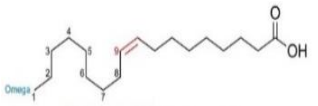
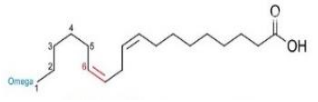
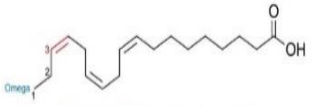
The fruits of *Pistacia* species are edible drupes rich in oil and used in traditional medicine to treat various diseases and widely consumed as a nutrient by the local population (Table 2.2). The seeds contained high levels of oil (32–67%). The most important fatty acids were oleic (39–49%), linoleic (23.6–31%), and palmitic (21.3–26.6%) acids. The ratio of unsaturated fatty acids to saturated fatty acids indicated that the content of unsaturated fatty acids was approximately three times higher than that of saturated fatty acids (Labdelli *et al.*, 2019). Chelghoum *et al.* (2020) analyzed the fatty acid composition in *P. atlantica* fruit oils. The fatty acids were extracted with hexane and then analyzed by gas chromatography. Oleic acid was the main ingredient, followed by linoleic acid and palmitic acid. It has been found that saturated and unsaturated fatty acids show an opposite trend during fruit ripening. Oleic and linoleic acid increased with maturity while palmitic acid decreased. In addition, the fatty acid composition in the oil of *P. atlantica* subsp. *cabulica* and *P. atlantica* subsp. *mutica* seeds was studied by Dorehgirae and Pourabdollah (2015). The oil of seeds was extracted and analyzed by GC mass. The main saturated fatty acid was palmitic acid 13.7% as the predominant followed by stearic acid 2.8%, while oleic acid (55.2%) and linoleic acid (22.6%) were the main unsaturated acids.

On the other hand, Saber-Tehrani *et al.* (2013) characterized 14 fatty acids in oil extracted by the cold press technique from *P. atlantica* seeds and analysis by GC/MS. The main components in the seed oil were oleic acid (50.65%), followed by linoleic acid (29.76%), palmitic acid (13.12%) and stearic acid (2.78%). In addition, the seed oil also contains smaller amounts of various ingredients, such as palmitoleic acid (2.04%), α-linolenic acid (0.59%), gondoic acid (0.32%), behenic acid (0.18%), arachidic acid (0.17%), myristic (0.15%), heptadecenoic acid (0.09%), margaric acid (0.07%), linoleic acid (0.04%) and lignoceric acid (0.04%).

Fatty acids are synthesized in the stroma of plastids, in both leaves and developing seeds (Ohlrogge *et al.* 1979). The steps involved in fatty acid biosynthesis in plants are schematically represented in (Fig. 2.4). Acetyl-CoA is a precursor for the biosynthesis of fatty acids. Acetyl-CoA is obtained by the degradation of photosynthate via glycolysis in the cytosol, followed by the action of pyruvate dehydrogenase complex in mitochondria and plastids (Camp and Randall, 1985). De novo fatty acid biosynthesis involves two enzymes, acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). The end product of this synthesis is usually the saturated fatty acid palmitate (Rajasekharan and Nachiappan, 2010). It can be subject to elongation to produce stearate or desaturation to produce palmitoleate. Longer saturated fatty acids were formed from stearate by elongation. Moreover, oleate was formed from stearate by

desaturation. A variety of unsaturated fatty acids can be formed from oleate by a combination of elongation and desaturation reactions (Dyer *et al.* 2008). Elongases are coded by fatty acid elongase (FAE) genes while the desaturases are coded by fatty acid desaturase (FAD) genes (Harwood, 2020).

Table 2.2 The important fatty acids in *Pistacia* species fruit fixed oils and their medicinal uses.

Name of fatty acids		Chemical formula	Chemical structure	Medicinal uses	Reference
Saturated fatty acids	Palmitic acid	$C_{16}H_{32}O_2$		They are widely used in cosmetics as emollients and emulsifiers because they are chemically or microbially stable.	Kang *et al.*, 2022
	Stearic acid	$C_{18}H_{36}O_2$			
Unsaturated fatty acids	Oleic acid	$C_{18}H_{34}O_2$		anticancer	Malarvizhi *et al.*, 2016
	Linoleic acid	$C_{18}H_{32}O_2$		Reduce blood cholesterol level	Jandacek, 2017
	Linolenic acid	$C_{18}H_{30}O_2$		Reduce the risk of cardiovascular disease	Naghshi *et al.*, 2021

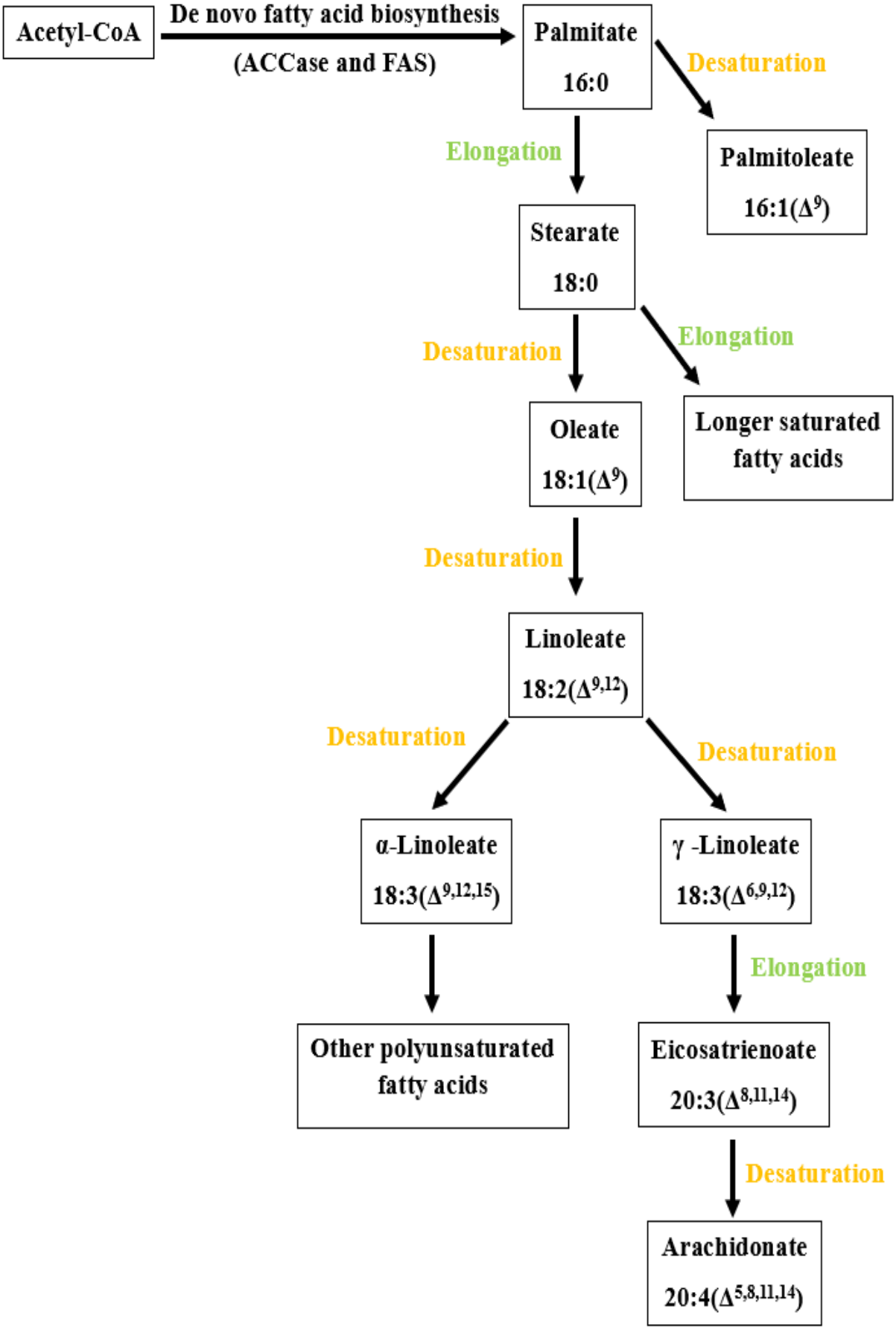


Figure 2.4 Fatty acid biosynthesis in plants.

2.5.3 Phenolic compounds in *Pistacia* species

Phenolic compounds are secondary metabolites, which are generally involved in the adaptation of plants to environmental stress conditions (Naikoo *et al.*, 2019). Phenolic compounds have an aromatic ring with one or more hydroxyl groups and act as antioxidants (Kumar and Goel, 2019). The largest group of naturally occurring polyphenols are the flavonoids (Salehi *et al.*, 2019). In general, two classes of phenolic compounds (hydroxybenzoic acids and hydroxycinnamic acids) are found in plant parts (Saranraj *et al.*, 2019). Much work has been done in recent years on the beneficial effects of phenolic compounds, which act as natural antioxidants and help neutralize free radicals. *Pistacia* species is known to be a rich source of phenolic compounds, which are used for therapeutic purposes (Table 2.3). Toul *et al.* (2017) determined total phenolic content in seven parts of *P. atlantica* Desf. subsp. *atlantica* including; fruits, leaves, buds, stems, roots, and barks. Accordingly, the highest level of phenolic contents has been observed in the leaves' extracts with 255.789 mg/g. In addition, it has been reported that ethanolic extract of *P. atlantica* subsp. *kurdica* leaf originated from the Iran contained phenol (269 mg/g) and flavonoid (40.7 mg/g) (Hashemi *et al.*, 2017). The presence of gallic acid derivatives and glycosylated flavonoids, rutin, quercetin and heterosides in the methanolic extract of *P. atlantica* leaves was confirmed by Amri *et al.* (2018). Karim (2014) identified six phenolic compounds by high-performance liquid chromatography (HPLC) from different plant martials (leaf, seed, outer shell, cluster and gum) of *P. eurycarpa*, namely tannic acid, stilbene, catechin, ellagic acid, gallic acid and cinnamic acid. Moreover, the methanolic extract of *P. atlantica* subsp. *kurdica* fruits showed the presence of free phenolic acids such as gallic acid, ascorbic acid, caffeic acid, rutin, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, ferulic acid, syringic acid and sinapic acid (Hatamnia *et al.*, 2014).

Phenolic compounds are synthesized via the shikimic acid, phenylpropanoid, and flavonoid synthetic pathways. Phenolic compounds, arising from different branch paths are regulated by structural genes multifunctional transcription factor and regulator protein. Final products of the flavonoid pathway such as pelargonidin 3-O-glucoside, are often glycosylated at the position 3 of the C ring of the flavan nucleus (Casañal *et al.*, 2013). There are many key enzymes (PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL,4-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FHT, flavanone 3-hydroxylase; F3'5'H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; F3GT, flavonoid 3-Oglycosyltransferase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase) involved in the biosynthesis pathway of phenolic compounds (Fig. 2.5) (Shi *et al.*, 2021).

Table 2.3 The important phenolic and flavonoid compounds in *Pistacia* species and their medicinal uses.

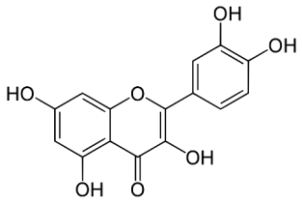
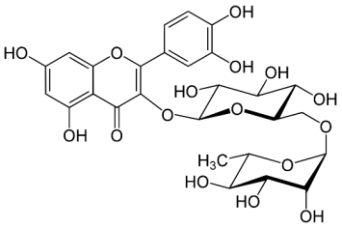
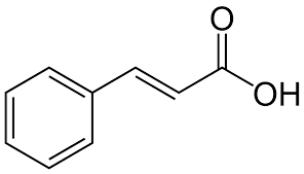
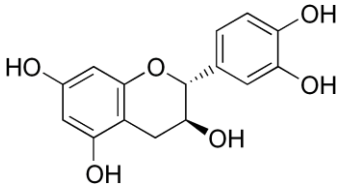
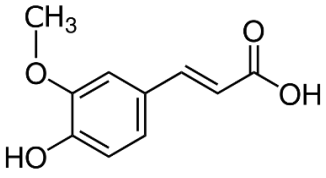
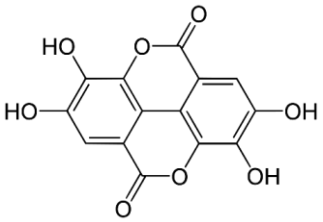
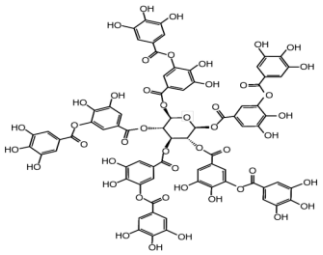
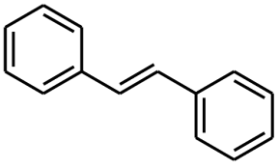
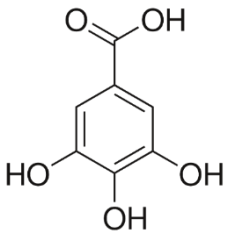
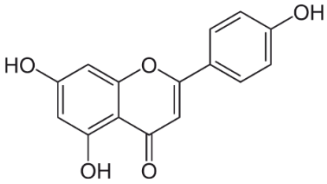
Name of compound	Chemical formula	Chemical structure	Medicinal uses	Reference
Quercetin	$C_{15}H_{10}O_7$		Anticarcinogenic, anti-inflammatory, and antiviral activities.	Li *et al.*, 2016
Rutin	$C_{27}H_{30}O_{16}$		Antioxidant and anti-inflammatory activities, prevent cardiovascular diseases, and skin cancer.	Ganeshpurkar and Saluja, 2017; Negahdari *et al.*, 2021
Cinnamic acid	$C_9H_8O_2$		Anti-cancer, antimicrobial, and anti-inflammatory.	Ruwizhi and Aderibigbe, 2020
Catechin	$C_{15}H_{14}O_6$		Antioxidant and preventing neuro-inflammation.	Cheruku *et al.*, 2018
Ferulic acid	$C_{10}H_{10}O_4$		Antioxidant, antimicrobial, anti-inflammatory, anti-thrombotic, and anti-cancer.	Ou and Kwok, 2004

Table 2.3 Continued

Name of compound	Chemical formula	Chemical structure	Medicinal uses	Reference
Ellagic acid	$C_{14}H_6O_8$		Anti-inflammatory, antiviral, antibacterial, antioxidant, antimutagenic, and anticancer properties.	Ríos *et al.*, 2018
Tannic acid	$C_{76}H_{52}O_{46}$		Antiviral, antibacterial, enhances the cell proliferation, tissue regeneration and wound healing processes.	Kaczmarek, 2020
Stilbene	$C_{14}H_{12}$		Antioxidant and antimicrobial.	Medrano-Padial *et al.*, 2019
Gallic acid	$C_7H_6O_5$		Antioxidant, antimicrobial, anti-inflammatory, and anticancer	Fernandes and Salgado, 2016
Apigenin	$C_{15}H_{10}O_5$		It is an effective therapeutic agent for overcoming diseases such as rheumatoid arthritis, parkinson's disease, and various types of cancer.	Ali *et al.*, 2017

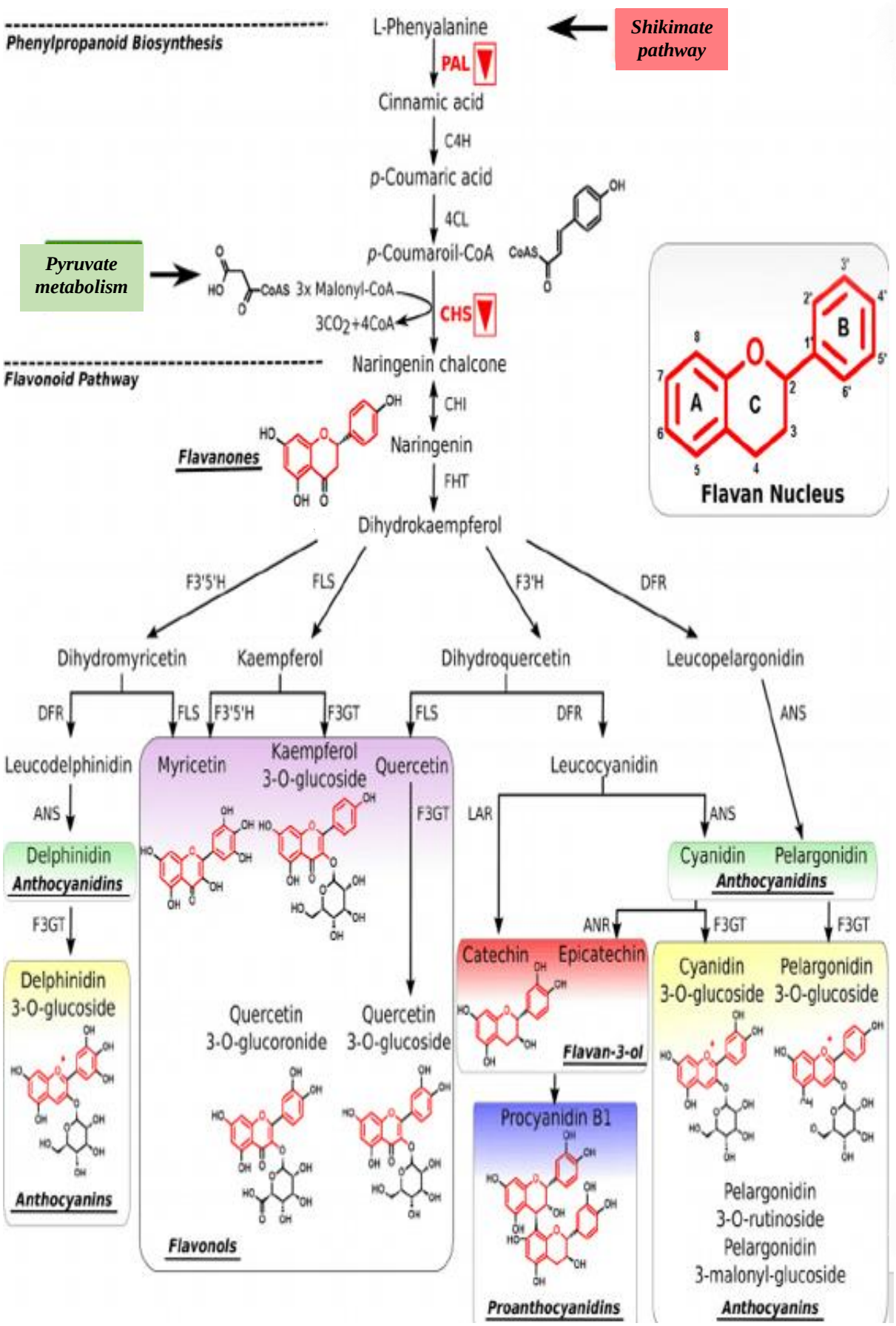


Figure 2.5 The phenolic compound biosynthesis pathway. This schematic is adapted from (Casañal *et al.*, 2013).

2.6 Mode of Actions of Essential Oils as Antibacterial Agents

Various bacterial pathogens are treated with the antibiotics. Today, the bacterial pathogens are becoming resistant to multi-drug antibiotics, which has led to the increase in the severity of infections, and hence scientists are facing challenges of finding alternative ways to treat such infections. In addition, some bacteria have the ability to form biofilm-associated drug tolerance (Tariq *et al.*, 2019). The antimicrobial efficacy of essential oil depends on their chemical composition and the structures of the target bacteria (Swamy *et al.*, 2016) .

The bacterial membrane plays an important role in regulating cellular osmotic pressure and the inflow and outflow of biomolecules. Thus, a compromised membrane will disrupt osmotic pressure, leading to intracellular leakage and eventually destroying the cell (Liao *et al.*, 2022). An important feature of essential oils is their hydrophobicity, which allows them to break down into lipids of the cell membrane of bacteria, disrupting the structure and making it more permeable. This can then lead to the leakage of ions and other cell molecules (Kengne *et al.*, 2019).

One of the most important postulated modes of action of essential oil is its ability to disrupt bacterial membranes (Fig. 2.6). In their work on the antibacterial activity of *Litsea cubeba* essential oil against *Acinetobacter baumannii*, Hao *et al.* (2021) found that the change in the permeability of the cell membrane and the leakage of intracellular biomacromolecules of the cell confirmed that the *L. cubeba* essential oil has an obvious effect on the cell membrane.

Another postulated mode of action of the essential oil is its ability to inhibit the bacterial efflux system. The bacterial efflux system consists of specialized channel proteins that reside on the bacterial membrane and are critical for the removal of harmful compounds such as antibiotics from the intracellular environment. Inhibiting the activity of such pumps is of great importance to counteract antibiotic resistance by restoring the effectiveness of antibiotics (Yang *et al.*, 2021). Chovanová *et al.* (2015), demonstrated the efflux-inhibiting activity of three *Salvia* species essential oils against antibiotic-resistant *S. epidermis*. All three essential oils were found to reduce *S. epidermis* efflux activity when exposed to essential oils.

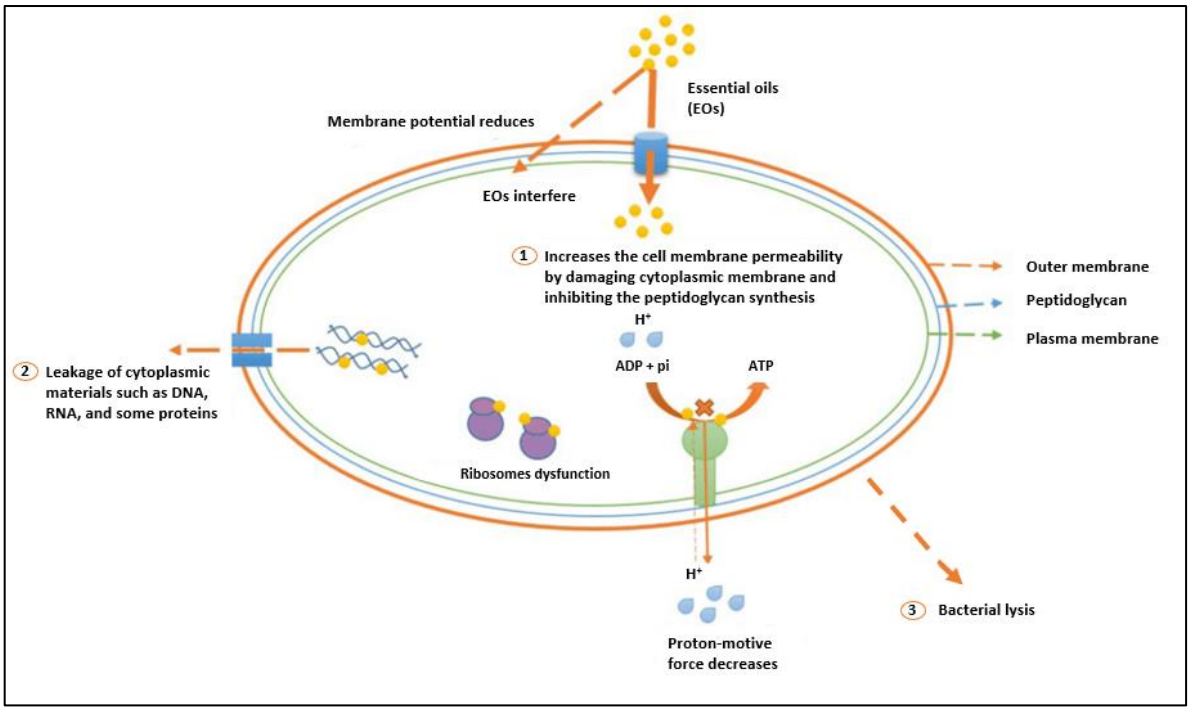


Figure 2.6 Possible antibacterial mechanism of action of essential oils. This figure reproduced from (Maurya *et al.*, 2021)

2.7 Multidrug Resistant Pathogens

E. faecium, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species are ESKAPE pathogens and the most common cause of nosocomial infections worldwide. The majority of them are multidrug resistant forms, which pose the most obstacles to the field of medicine (Mulani *et al.*, 2019). Microorganisms develop resistance to overcome the effects of antibiotics. The term multidrug resistance (MDR) means resistance to at least one agent of three classes of antimicrobial agents. While extensively drug-resistance (XDR) is defined as resistance to at least one agent in all but two or one antimicrobial classes. Also, pandrug-resistance (PDR) is defined as resistance to all agents of all antimicrobial classes (Alotaibi *et al.*, 2021). *A. baumannii* is considered as one of the world's leading drug resistance pathogens (Intorasoot *et al.*, 2017). The bacterium has an extraordinary capacity to quickly adapt to new antibiotics (Sarshar *et al.*, 2021; Talyansky *et al.*, 2021). Therefore, *A. baumannii* is identified as a priority pathogen in the 2017 WHO final report, which urges researchers to take swift action to overcome humanitarian problem (WHO, 2017). All clinically significant isolates must be tested for antibiotic susceptibility testing to select appropriate treatment (Wang *et al.*, 2022). Susceptibility testing is performed by both broth microdilution and disk diffusion, which are acceptable methods listed by the National Committee for Clinical Laboratory Standards for antimicrobial drug susceptibility testing. In 2020, CLSI listed 24 antimicrobials from nine

classes with breakpoints for *A. baumannii* (CLSI, 2020). The mechanisms by which *A. baumannii* is resistant to various antibiotics can be classified into four groups: antimicrobial inactivation by enzymes, control of antibiotic transport across membranes (reducing porin permeability or increasing drug efflux), modification of antibiotic targets, and biofilm formation (Kyriakidis *et al.*, 2021).

The most common mechanism of resistance by *A. baumannii* is the production of numerous *β*-lactamases (extended-spectrum β-lactamase (ESBL), oxacillinase (OXA), and metallo-β-lactamase (MBL) types) and efflux pumps. Beta-lactamases are enzymes that catalyze the beta-lactam hydrolysis ring leading to antimicrobial inactivation and preventing it from being active against the enzymes responsible for bacterial cell wall synthesis (Saranathan *et al.*, 2017). Most *A. baumannii* strains have become progressively resistant to most antibiotics that are currently available due to the existence of mobile genetic elements, such as plasmids (Fallah *et al.*, 2014). Furthermore, the transmission of these determinants between chromosomes and plasmids may participate in the dissemination of resistance among different bacterial strains. Increased rates of antibiotic resistance in *A. baumannii* are of significant importance in health settings all over the world (Shali *et al.*, 2022).

2.8 Classification and Natural Habits of *Acinetobacter baumannii*

The last recent taxonomic studies stated that the genus *Acinetobacter* belongs to the family of Moraxellaceae and order Pseudomonadales that include *A. baumannii* as shown in (Table 2.4) (Kurcik-Trajkovska, 2009).

Table 2.4 Classification of *A. baumannii*.

Classification	Name
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Pseudomonadales
Family	Moraxellaceae
Genus	*Acinetobacter*
Scientific name	*Acinetobacter baumannii*

Acinetobacter species are Gram-negative, nonmotile, coccobacillus, ranging in size from 1 to 1.5 µm (Fig. 2.7). Usually, occurring in pairs or in long chains, their length variable particularly in the stationary phase (Jung and Park, 2015). *Acinetobacter* species are strictly aerobic, they grow well on regular media, such as nutrient agar, Mueller-Hinton agar, Blood agar, Chocolate agar, and MacConckey agar therefore, they are non-fastidious microorganisms. On both Blood and Chocolate agar, they produce colorless, shiny, mucoid colonies (Almasaudi, 2018). On the other hand, colonies of *Acinetobacter* species appear to have bluish to bluish gray color on Eosin Methylene Blue agar. In addition, light lavender color of colonies were observed when these microorganisms grown on MacConkey agar, indicating non-lactose ferments (Peleg *et al.*, 2008). *Acinetobacter* species give negative results for indole and oxidase tests. Besides, many strains are unable to reduce nitrates to nitrites (Kurcik-Trajkovska, 2009). *Acinetobacter* species are typically mesophiles; they grow well at 37 °C (Antunes *et al.*, 2011).

A. baumannii are typically free-living saprophytic organisms that are widespread in diverse environments including soil, water, wastewater, human and animals (Atrouni *et al.*, 2016). *A. baumannii* is able to withstand dry conditions for long periods. Interestingly, *A. baumannii* is frequently isolated from reusable medical devices such as humidifiers, plastic urinals, and respirometers. Moreover, they have been isolated from specimens taken from personal health cares, mattresses, and pillows (Kurcik-Trajkovska, 2009). In addition, they are isolated from different body parts and considered part of the normal flora of the pharynx, mucous membrane, respiratory secretion of human, and skin (Peleg *et al.*, 2008).

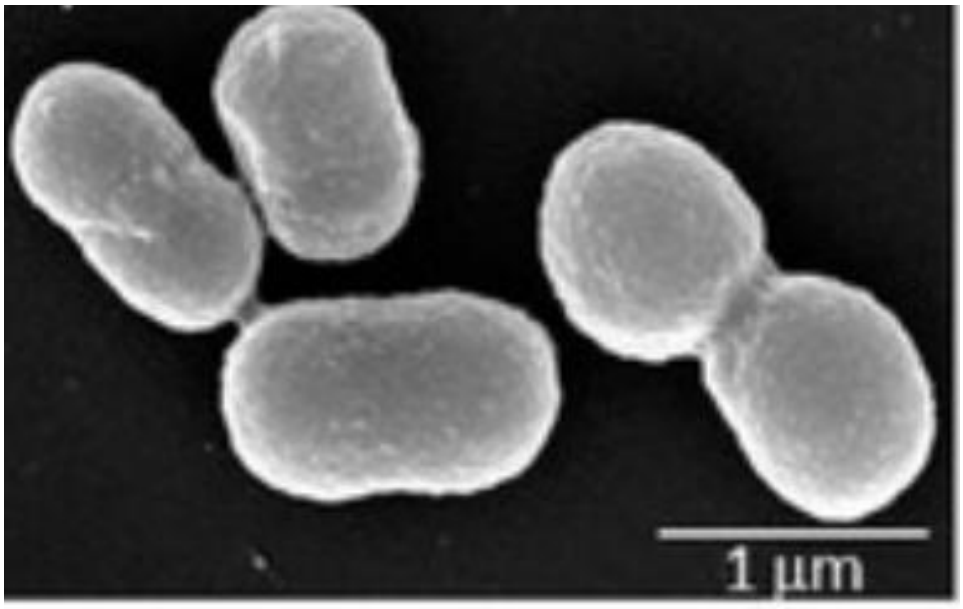


Figure 2.7 Scanning electron microscopy image of *A. baumannii* (Al-Mahmud *et al.*, 2021).

Environmental wound contamination has been investigated in soldiers wounded in Iraq and Vietnam wars, who had a high rate of *A. baumannii* wound and blood infections (Hujer *et al.*, 2006). Clinically relevant strains of *Acinetobacter* are often found colonizing hospital surfaces, hospital stuff and medical instruments. They are able to survive in dry conditions on inanimate objects for long periods, and the main reason responsible for their persistence in the hospital environment is their ability to withstand antimicrobial agents and disinfectants (Ye *et al.,* 2015; Moubareck and Halat, 2020; Ababneh *et al.*, 2022).

2.9 Biofilm Formation by *Acinetobacter baumannii*

Biofilm is an aggregation of microbial cells in a highly self-organized community that resist the action of antibiotics and the human immune system. They strongly attached and adhered to a range of biotic and abiotic surface, enable a stronger cell attachment, that can be formed on wide variety of solid surfaces (Markowska *et al.,* 2013). Microbial communities are enclosed in an extracellular matrix which provides protection and strengthen the structure of the biofilm (Pereira *et al.*, 2016; Dekic *et al.*, 2017). Biofilms are an extracellular matrix consisting of DNA, proteins, and polysaccharides that are constructed by bacterial communities to form complex structures and adhere to living or non-living surfaces (Sonawane *et al.*, 2022).

The process of *A. baumannii* forming biofilm on abiotic surfaces consists of many steps (Fig. 2.8), beginning with attachment to a living or non-living surface, leading to the formation of microcolonies (Bittinger *et al*., 2022). High population density induces microbes to secrete autoinducer molecules, which turns on the N-acyl-homoserine lactone (AHL) type of quorum-sensing system to promote biofilm development (Anbazhagan *et al*., 2012). Microcolonies transition to mature form by multiplication and secretion of polymeric substances, the bacteria biofilm-associated protein (Bap), and extracellular polymeric substances (EPS) (Flemming *et al*., 2007). Biofilm can be dispersed in clumps and individual forms from the attached surface under oxygen- and nutrient-depletion condition.

The polymeric matrix of the biofilm can delay the diffusion of antibiotics, making it more difficult for the antibiotics to penetrate the biofilm to eliminate the pathogens (Yu *et al.,* 2018). The protective architecture of the bacterial biofilm allows the survival of bacteria in hostile environments (Li *et al.,* 2016). Therefore, the application of approaches for controlling biofilm formation for therapeutic approaches is highly desirable (Rasmussen and Givskov, 2006). Biofilm formation is considered a survival strategy against environmental stresses such as pH, UV damage, hydrogen peroxide and metal toxicity, also against the human immune response

to bacterial infections, including phagocytosis (Fuente-Núñez *et al.*, 2016; Chen *et al.*, 2018). Most of the antibiotics currently used are becoming ineffective against the biofilm-associated multidrug-resistant microorganisms (Roy *et al.*, 2018). Therefore, it has become necessary to search for alternative approaches to mitigate problems associated with biofilms (Natan and Banin, 2017).

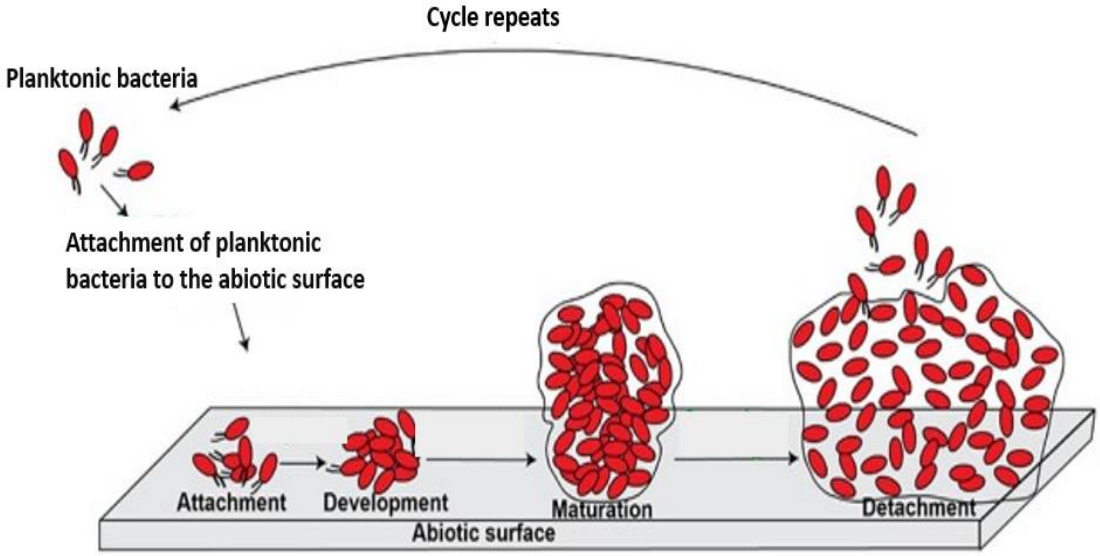


Figure 2.8 Developmental stages involved in *A. baumannii* biofilm formation. This figure is adapted from (Gao *et al.*, 2019).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Instruments and Apparatus

The following instruments and apparatus were used in the present study.

Table 3.1 List of instruments and apparatus.

No.	Instruments and apparatus	Company	Country
1.	Autoclavable tube (15 ml)	ITOTEST	China
2.	Autoclave AE-75-DRY	Raypa	Spain
3.	Auto vortex mixer	Stuart	England
4.	Benzene burner	Jiangsu	China
5.	Clevenger apparatus	Simax	Czechia
6.	Cobas C311	Roche	Germany
7.	Cooling centrifuge	Biofuge stratos	Germany
8.	Disposable petri dishes	CITOTEST	China
9.	Eppendorf (0.5-1.5mL)	Sigma	USA
10.	Filter paper	Whatman	China
11.	Flame photometer	Janeway PFP 7	UK
12.	GC-2010	Shimadzu	Japan
13.	GCMS-QP2010 Ultra	Shimadzu	Japan
14.	Glassware (flask, beaker, cylinder)	CITOGLASS	China
15.	Hood	LABCONCO	USA
16.	Hot plate SP133520-33	Barnstead international	USA
17.	HPLC	SYKNM	Germany
18.	Incubator	Memmert	Germany
19.	Loop	CTTOTEST	China
20.	Micro 200R centrifuge	Hettich	Germany
21.	Micro kjeldahl apparatus	Labconco	Kansas
22.	Micropipette (0.1-3) and (1-3) µl	Human	Germany
23.	Micropipette (10-100) and (100-1000) µl	Human	Germany
24.	Microplate incubator- shaking	BOECO	Germany
25.	Microplate spectrophotometer	Biotech µQuant	USA
26.	Microscope	MOTIC	China
27.	Microwave	Morphy richards	British
28.	Mortar and pestle	CTTOTEST	China
29.	MultiDoc-It™ Imaging System	UVP	USA
30.	Oven	WTC Binder	Germany
31.	Parafilm	Bemis	USA

Table 3.1 Continued

No.	Equipment and instruments	Company	Country
32.	pH meter	Eutech	Singapore
33.	Refrigerator	VESTEL	Turkey
34.	Sensitive balance	Citizen Scale Inc.	USA
35.	Soxhlet apparatus	Simax	Czechia
36.	Spectrophotometer	Thermo Electron	UK
37.	Sterile microplate (96 well)	CITOTEST	China
38.	T100™ Thermal Cycler	Bio-Rad	Singapore
39.	TEM	ZEISS MODEL EM10C-100KV	Germany
40.	Ultra- light gel electrophoresis	Edvotek	USA
41.	Vacuum rotary evaporator	BUCHI	Germany
42.	VITEC 2	BioMerieux	France
43.	Water bath	Memmert	Germany

3.2 Chemical and Standard Compounds

The following chemicals and standard compounds were used in the study.

Table 3.2 List of chemical and standard compounds.

No.	Chemical and standard compounds	Company	Country
1.	Absolute ethanol	AQUA	Turkey
2.	AddPrep Genomic DNA Extraction Kit	Addbio	Korea
3.	Agarose	Addbio	Korea
4.	Aluminum chloride ($AlCl_3$)	CDH	India
5.	Ammonium acetate	Biochem	France
6.	Apigenin	Sigma-Aldrich	Germany
7.	Catechin	Sigma-Aldrich	Germany
8.	Cetyltrimethylammonium bromide (CTAB)	Biochem	France
9.	Chloroform	Scharlau	Spain
10.	Cinnamic acid	Sigma-Aldrich	Germany
11.	Dimethyl sulfoxide (DMSO)	Biochem	France
12.	DNA ladder	Addbio	Korea
13.	EDTA	Biochem	France
14.	Ellagic acid	Sigma-Aldrich	Germany
15.	Ethidium bromide	Addbio	Korea
16.	Ferulic acid	Sigma-Aldrich	Germany
17.	Folin ciocalteu reagent	Thomas Baker	India
18.	Gallic acid	Sigma-Aldrich	Germany
19.	Hydrochloride acid (HCl)	Scharlau	Spain
20.	Hydrogen peroxide (H_2O_2)	Scharlau	Spain

Table 3.2 Continued

No.	Chemical and standard compounds	Company	Country
21	Inorganic phosphorus kit	Agappe	Switzerland
22.	Iso-propanol	Biochem	France
23.	Linolenic acid	Sigma-Aldrich	Germany
24.	Linoleic acid	Sigma-Aldrich	Germany
25.	Loading dye	Addbio	Korea
26.	Master mix	Addbio	Korea
27.	Mercaptoethanol	Biochem	France
28.	Methanol	EMSURE	Germany
29.	n-hexane	Biochem	France
30.	Oleic acid	Sigma-Aldrich	Germany
31.	Palmitic acid	Sigma-Aldrich	Germany
32.	Perchloric acid ($HClO_4$)	Scharlau	Spain
33.	Potassium acetate	Himedia	India
34.	Potassium kit	Agappe	Switzerland
35.	Primer	Macrogene	South Korea
36.	Proteinase K	Addbio	Korea
37.	Protein kit	BIOLABO	France
38.	Quercetin	Sigma-Aldrich	Germany
39.	RNase	Addbio	Korea
40.	Rutin	Sigma-Aldrich	Germany
41.	Sodium carbonate (Na_2CO_3)	Himedia	India
42.	Sodium hydroxide (NaOH)	Scharlau	Spain
43.	Stearic acid	Sigma-Aldrich	Germany
44.	Stilbene	Sigma-Aldrich	Germany
45.	Sulfuric acid (H_2SO_4)	Scharlau	Spain
46.	Tannic acid	Sigma-Aldrich	Germany
47.	Tris-Borate-EDTA Buffer (TBE buffer)	Addbio	Korea
48.	Tris Hcl	Biochem	France

3.3 Culture Media

The following culture media were used in the current study.

Table 3.3 List of culture media.

No.	Culture media	Company	Country
1.	Luria-Bertani (LB) agar	Merck KGaA	Germany
2.	Luria–Bertani (LB) broth	Merck KGaA	Germany
3.	Mueller Hinton agar	Merck KGaA	Germany
4.	Nutrient agar	Lab M (Neogene Europe Ltd)	UK
5.	Nutrient broth	Lab M (Neogene Europe Ltd)	UK

3.4 Locations of the Study

The plant parts were collected from Sulaymaniyah province in Kurdistan region (Northeastern Iraq): Qaradagh, Ranya, Sharbazher, and Hawar (Fig. 3.1). Latitudes, longitudes, and altitudes of the locations are given in (Table 3.4).

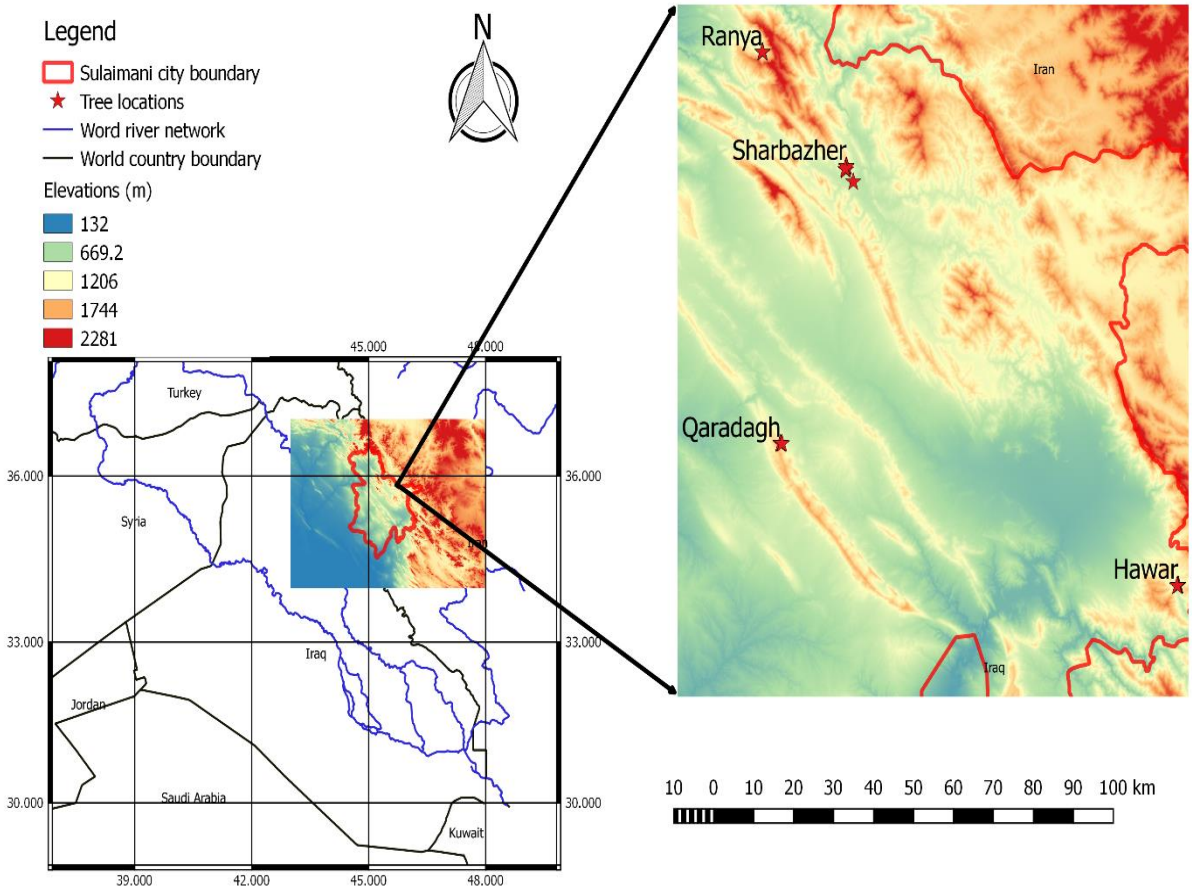


Figure 3.1 Map of the distribution of collection sites of the studied plant parts.

Table 3.4 Species names, locations, latitudes, longitudes, and altitudes.

Species	Locations	Latitudes	Longitudes	Altitudes
Pistacia eurycarpa	Qaradagh	35°38′27″	45°24′07″	1238
Pistacia eurycarpa	Ranya	35°98′78″	45°20′04″	1220
Pistacia eurycarpa	Sharbazher	35°81′09″	45°38′73″	1102
Pistacia eurycarpa	Hawar	35°16′19″	46°12′79″	1040
Pistacia khinjuk	Qaradagh	35°38′24″	45°24′45″	1328
Pistacia khinjuk	Hawar	35°16′16″	46°12′60″	1002

Table (3.5) shows monthly average of the minimum and maximum temperatures, as well as rainfall, in the selected locations.

Table 3.5 Monthly average of minimum and maximum temperatures and rainfall during January to October 2020 for the studied locations.

Month	Qaradagh			Ranya			Sharbazher			Hawar		
	Air temp. °c		Rain fall (mm)	Air temp. °c		Rain fall (mm)	Air temp. °c		Rain fall (mm)	Air temp. °c		Rain fall (mm)
	Min	Max		Min	Max		Min	Max		Min	Max	
January	2.0	9.0	171.0	0.0	7.0	277.4	-2.5	2.4	124.0	3.0	11.4	91.4
February	3.0	12.0	245.5	2.0	10.0	172.8	-5.2	4.1	242.0	4.7	13.8	67.9
March	9.0	19.0	241.3	6.0	15.0	171.8	2.5	11.3	270.0	9.7	20.8	124.5
April	13.0	21.0	101.0	13.0	20.0	71.4	6.3	12.0	94.0	12.9	25.0	83.6
May	19.0	29.0	16.0	16.0	25.0	29.5	11.6	21.0	31.5	18.6	35.3	10.0
June	20.0	35.0	0.0	22.0	33.0	0.0	16.8	30.8	0.0	23.6	42.0	0.0
July	26.0	42.0	0.0	25.0	41.0	0.0	15.9	32.2	0.0	29	45.9	0.0
August	24.0	40.0	0.0	24.0	39.0	0.0	17.1	29.7	0.0	26.4	43.5	0.0
September	22.0	37.0	0.0	20.0	37.0	0.0	19.8	25.9	0.0	24.4	42.0	0.0
October	16.0	30.0	0.0	17.0	27.0	0.0	13.1	20.1	0.0	17.0	34.3	0.0

*The data were obtained from the meteorological station in Sulaimani

Regarding soil characteristics, Table (3.6) depicts some physical and chemical characteristics of the soil taken from the study locations.

Table 3.6 Physical and chemical characteristics of the soil of study locations.

Characteristics	Qaradagh	Ranya	Sharbazher	Hawar
Ec dS m^{-1}	0.20	0.40	0.50	0.20
pH	6.95	7.12	7.08	7.05
Available N mg kg^{-1} soil	45.00	32.00	31.00	39.00
Available P mg kg^{-1} soil	16.65	16.42	15.52	18.23
Available K mg kg^{-1} soil	172.63	160.98	140.53	180.23
O.M g kg^{-1}	58.00	28.30	24.30	48.40
CaCo3 g kg^{-1}	331.20	114.80	103.70	100.00
Sand g kg^{-1}	444.00	439.00	262.00	430.00
Silt g kg^{-1}	367.00	316.00	480.00	380.00
Clay g kg^{-1}	189.00	245.00	258.00	190.00
Texture	Loam	Loam	Loam	Loam

*Soil analysis was carried out in a central laboratory for soil, water and plant analysis in the College of Agricultural Engineering Sciences/University of Baghdad. Samples were sent to Baghdad during covid 19.

3.5 Molecular Study to Identify *Pistacia* Species

3.5.1 Collection of leaves and genomic DNA extraction

Firstly, plant samples were identified morphologically at the locations depending on the variations in the traits of leaves and stems among the plant species. The samples were identified at Iraqi National Herbarium, Directorate of Seed Testing and Certification, Ministry of Agriculture. Forty *Pistacia* specimens were observed from four locations (Qaradagh, Ranya, Sharbazher, and Hawar) in Sulaymaniyah province. Young leaves of *Pistacia* spp. were collected in April 2020 and frozen using liquid nitrogen. The frozen leaves were ground to a fine powder with pestle and mortar. Fig. (3.2) shows the leaves of *Pistacia eurycarpa* and *Pistacia khinjuk.*

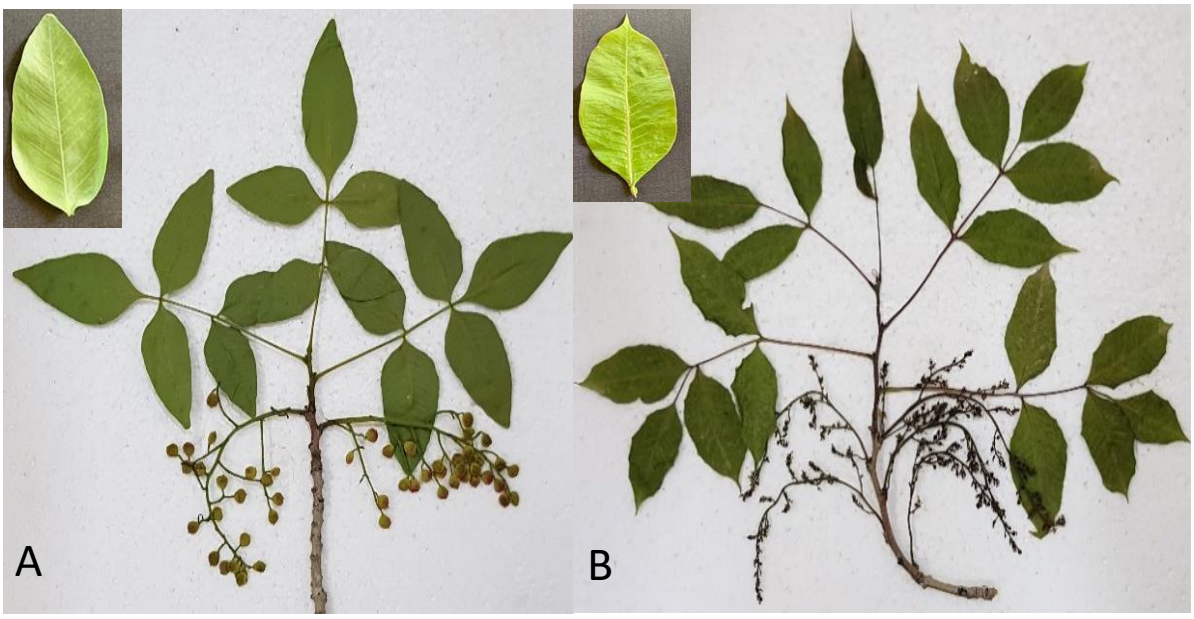


Figure 3.2 *Pistacia* species leaves (A) *P. eurycarpa* (B) *P. khinjuk*.

Young leaf samples were used for DNA extraction and total genomic DNA was isolated using the Cetyl TriMethyl Ammonium Bromide (CTAB) method with minor modifications (Doyle and Doyle, 1987). CTAB buffer was prepared as described in (Appendix 1). Leaf sample (1 g) was ground in liquid nitrogen and mixed with 5 ml of CTAB buffer in the (15 ml) centrifuge tubes and mixed by a vortex. The samples were then incubated at 65 °C for 120 minutes and mixed once after 15 minutes. After that, 5 ml chloroform were added and shaken for 2 minutes to form an emulsion. Samples were then centrifuged at 4000 rpm for 30 minutes at 15 °C. The supernatant was carefully transferred into a new clean labeled centrifuge tube. 0.08 volumes of supernatant of 7.5 M ammonium acetate were added and mixed with 0.54 volumes of ice cold iso-propanol (99%) was also added for the precipitation of DNA and mixed by inverting 20 to 30 times by hand without shake. Tubes were stored in a freezer at -20 °C overnight for a better

yield. Samples were centrifuged at 4000 rpm for 35 minutes at 3 °C, and then supernatant was carefully poured off. One ml of ice-cold 70% ethanol was added to each tube. Samples were centrifuged again at 4000 rpm for 20 minutes at 3 °C, the supernatant was then discarded without dislodging the pellet. For dry samples, tubes with the pellet were left at room temperature for 1 hour. Then, 20 µl of RNase (1.5 mg/ml) were added to each sample, then incubated at 45 °C for 60 minutes, and then directly 20 µl of proteinase K (20 mg/ml) were added and incubated at 45ºC for 40 minutes. Next, the same volume of the sample chloroform (99%) was added and mixed well by inverting and samples were then centrifuged at 4000 rpm for 30 minutes at 15 °C. After that, the aqueous layer was carefully transferred into a clean, labelled tube to avoid contamination by the lower layer**.** The estimated volume of the aqueous layer and 0.08 volumes of 7.5 M ammonium acetate was added and mixed with 0.54 volumes of ice iso-propanol (99%) was also added for the precipitation of DNA and mixed by inverting 20 to 30 times by hand without shake.

Tubes were stored in a freezer at -20 °C overnight for a better yield. Samples were centrifuged at 4000 rpm for 30 minutes at 5 °C, and then isopropanol supernatant was carefully discarded. One ml of ice-cold 70% ethanol was added to each tube. Samples were centrifuged again at 4000 rpm for 20 minutes at 3 °C. The supernatant was then discarded without dislodging the pellet. For dry samples, tubes with the pellet were left at room temperature for 1 hour. The samples were kept at the refrigerator at 4 °C for 1 day and then stored at -20 °C. The quality of DNA was checked by running 3 µl DNA (mixed with 1 µl of loading dye) on a 1 % agarose gel at 80 V.

3.5.2. Polymerase chain reaction

Specific primers for the genus of *Pistacia* were designed based on the multiple sequence alignment of 18S rRNA of 11 *Pistacia* species submitted to NCBI using Clustal Omega. PCR reactions were performed in a total volume of reaction mixtures of 50 µl containing 25 µl master mix, 1μl of forward primer (10 pmol/μl) and 1μl of reverse primer (10 pmol/μl), 1µl DNA template, and 22 μl deionized water. Then, the amplification condition was optimized as shown in (Table 3.7) was used for detection of 18S rRNA. PCR was performed via PCR T100^{TM} Thermo Cycler.

Table 3.7 Primer, amplicon size, and PCR settings used for detection 18S rRNA gene in *Pistacia* species.

Name of primer	Sequence (5' - 3')	PCR product size (~ bp)	PCR condition
18S rRNA	F- ATCATTGTCGAAACCTGCC R- TCGAGGGTCAAAGAGTCC	600	Initial denaturation: 95 °C for 5 min, 30 cycles of denaturation: 95 °C for 30s, annealing: 52 °C for 1 min, extension: 72 °C for 2 min, and final extension: 72 °C for 5 min.

3.5.3 Agarose gel electrophoresis and sequencing

PCR product was visualized by 1% agarose gel electrophoresis in 1x TBE buffer at 80 V for 45 min. The gel was stained with 2 µl of ethidium bromide, and the image was captured via MultiDoc-It™ Imaging System. Finally, purification and sequencing for the PCR products were done by the Sanger sequencing method performed by Macrogen (South Korea). The 18S rRNA sequence of the *Pistacia* species was submitted to NCBI and the accession number was assigned. The taxonomical identification of the species was carried out by aligning the 18S rRNA sequences of the species with closely related species. A phylogenetic tree of the partial sequence of 18S rRNA of *Pistacia* species (~ 600 bp) was proposed using molecular phylogenetic analysis by neighbor-joining tree method using MEGA 11 (Tamura *et al.*, 2021).

3.6 Study the Chemical Constituents of *Pistacia eurycarpa* Parts

3.6.1 *Pistacia eurycarpa* parts collection and preparation

Leaves, fruits, and rachis in spring and autumn were collected, but bark and gum were collected in spring and summer respectively. The samples were taken randomly with five replications. Fig. (3.3) shows the tree, leaf, fruit, and gum of *P. eurycarpa*.

Following collection, samples were prepared for analysis in Research Laboratory/Horticulture Department/College of Agricultural and Engineer Science/University of Sulaimani. All of the leaves, fruits, rachis, and bark were air-dried at room temperature and ground to a fine powder with an electric blender, except the gum. All of the samples were then stored at 4 °C for future research.

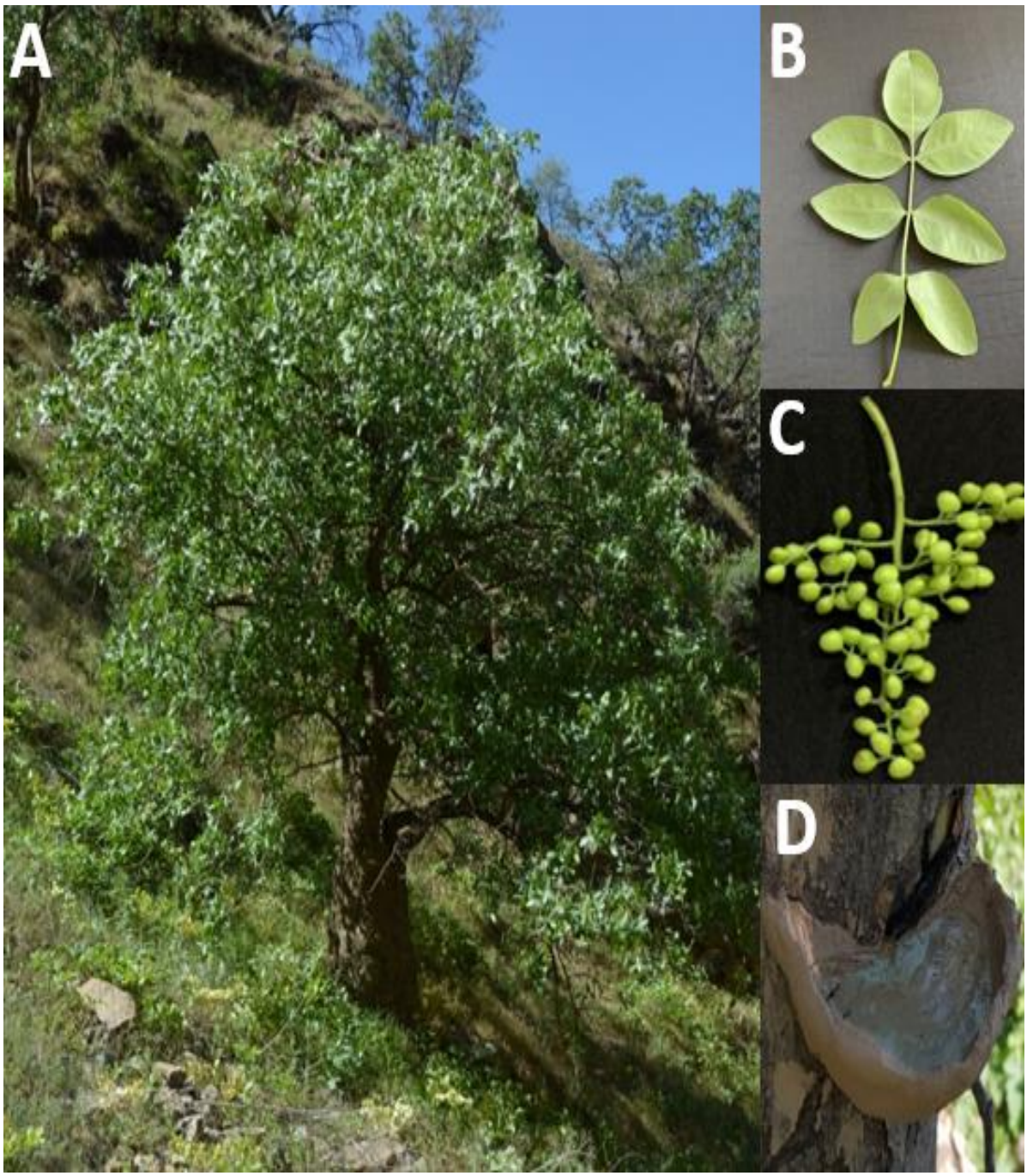


Figure 3.3 *P. eurycarpa* with different parts (A) Tree. (B) Leaf. (C) Fruit. (D) Handmade clay cup with collected gum (resin).

3.6.2 Total nitrogen, phosphorus, potassium, and carbohydrate determination from spring and autumn leaves of *Pistacia eurycarpa*

After digesting 0.5 g of each sample with concentrated sulfuric acid and 30% hydrogen peroxide, total nitrogen in leaves was measured using the Micro kjeldahl apparatus. Phosphorus was determined using a colorimetric method by a spectrophotometer at 410 nm, and potassium was measured using a flame photometer (Estefan *et al.*, 2013). Carbohydrate was measured using a colorimetric method by a spectrophotometer at 490 nm (Joslyn, 1970).

3.6.3 Extraction and analysis the constituents of essential oil from gum and autumn rachis of *Pistacia eurycarpa*

3.6.3.1 Essential oil extraction

The essential oils were extracted from gum and autumn rachis of *P. eurycarpa* by hydrodistillation method (Vidic *et al.*, 2018). Briefly, 50 g of samples were soaked in 500 ml of distilled water in a round flask for 3 hours at 40 °C using a Clevenger apparatus. Finally, the essential oils were collected and preserved in the refrigerator until usage and calculated according to the following equation:

$$\text{Essential oil } \% = \frac{\text{Essential oils weight (g)}}{\text{Sample weight(g)}} \times 100 \quad (3.1)$$

3.6.3.2 Essential oil constituents analysis by gas chromatography-mass spectrometry

To identify and analyze the constituents of the essential oil of *P. eurycarpa* gum and autumn rachis, GC-MS analysis using a Shimadzu GC-QP 2010 Ultra GC-MS system was carried out at the College of Agriculture, University of Basrah, Iraq. The GC oven temperature was initially kept for 2 min at 40 °C, gradually increased to 150 °C at a rate of 10 °C/min, and finally increased to 280 °C at a rate of 50 °C/min. Helium was used as a carrier gas; inlet pressure was 50.4 kPa and linear velocity was 36.3 cm/sec. The column flow rate was 1.01 ml/min, the injector temperature was 250 °C; injection mode was split 1:30 and MS scan conditions were ion source temperature 200 °C, and interface temperature 250 °C. The detector gained 0.70 kV +0.10 kV, and mass range of m/z 50-500. The constituents of the essential oil were identified by matching retention indices with those of standard constituents stowed in the NIST 08 library. Chromatogram of GC-MS of samples showed in (Appendices 2-9).

3.6.4 Extraction and analysis the constituents of fixed oil from autumn fruits and bark of *P. eurycarpa*

3.6.4.1 Fixed oil extraction.

Fixed oil was measured by Soxhlet method (Ferreira-Dias *et al.*, 2003). Briefly, 50 g dried-ground powder fruit or bark were placed in a Soxhlet thimble and extracted with 250 ml n-hexane solvent for 2 hours in Soxhlet extraction system. The remaining solvent was evaporated by a rotary evaporator. After drying, the dried extracts were weighed and the oil content calculated as follows:

$$\text{Fixed oils \%} = \frac{\text{Oil weight (g)}}{\text{Sample weight (g)}} \times 100 \qquad (3.2)$$

3.6.4.2 Fixed oil constituents analysis by gas chromatography

The fixed oil compounds were analyzed using a gas chromatography (GC), where the flame ionization detector (FID) and SE-30 capillary column (30 m length, 0.25 mm inner diameter) were used. Injection area, detector and separator column temperatures were 280 °C, 310 °C and 120–290 °C (10 °C/min), respectively. The gas flow rate was 100 Kpa (Zhang *et al.*, 2015). Chromatogram of different fixed oil compounds of *P. eurycarpa* autumn fruits and bark listed in (Appendices 10-17).

3.6.5 Estimation of total phenolic and flavonoid contents in *Pistacia eurycarpa* parts

3.6.5.1 Preparation of methanolic extract

Samples were prepared using the Michiels *et al.* (2012) method. Briefly, 1 g of samples were placed in 15 ml tubes. Then 10 ml of 80% methanol added, the samples were shaken in water bath for 3 hours at 37 °C before being centrifuged at 5000 rpm for 15 minutes at 4 °C. The upper layer was removed and transferred to a new, clean, labeled 15 ml tube, which was then stored in a refrigerator at 4 °C until use.

3.6.5.2 Determination of total phenolic contents

Total phenolic content was determined in the methanolic extract with a standard Folin-Ciocalteu reagent described by Rodrigues *et al.* (2019) with some modifications. 100 µl of each methanolic extract is mixed with 4 ml of 10% Folin-Ciocalteu reagent, and allowed to react for 5 minutes at room temperature. After that, 2 ml of 20% saturated Na_2CO_3 solution were added then left for 60 minutes in the dark at 38 °C. Regarding blanks, same previous steps were repeated except 100 µl of water were used instead of the samples, preparation of the solutions showed in (Appendix 18). The measurement was done at 765 nm using a spectrophotometer to calculate the phenolic content using the calibration curve made with gallic acid (Fig. 3.4). The standard curve was prepared by dissolving 100 mg of gallic acid in 50 ml distilled water to prepare stock solution (2 mg/ml), then different volumes (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, and 2.5 ml) were taken from stock solution. Distilled water was added to complete the volume to 5 ml. Measurement of total phenolic content was performed using the following equation:

$$\text{Total phenolic content (mg/g)} = \frac{\text{Concentration (mg/ml)} \times \text{Volume of extract (ml)}}{\text{Sample weight (g)}} \quad (3.3)$$

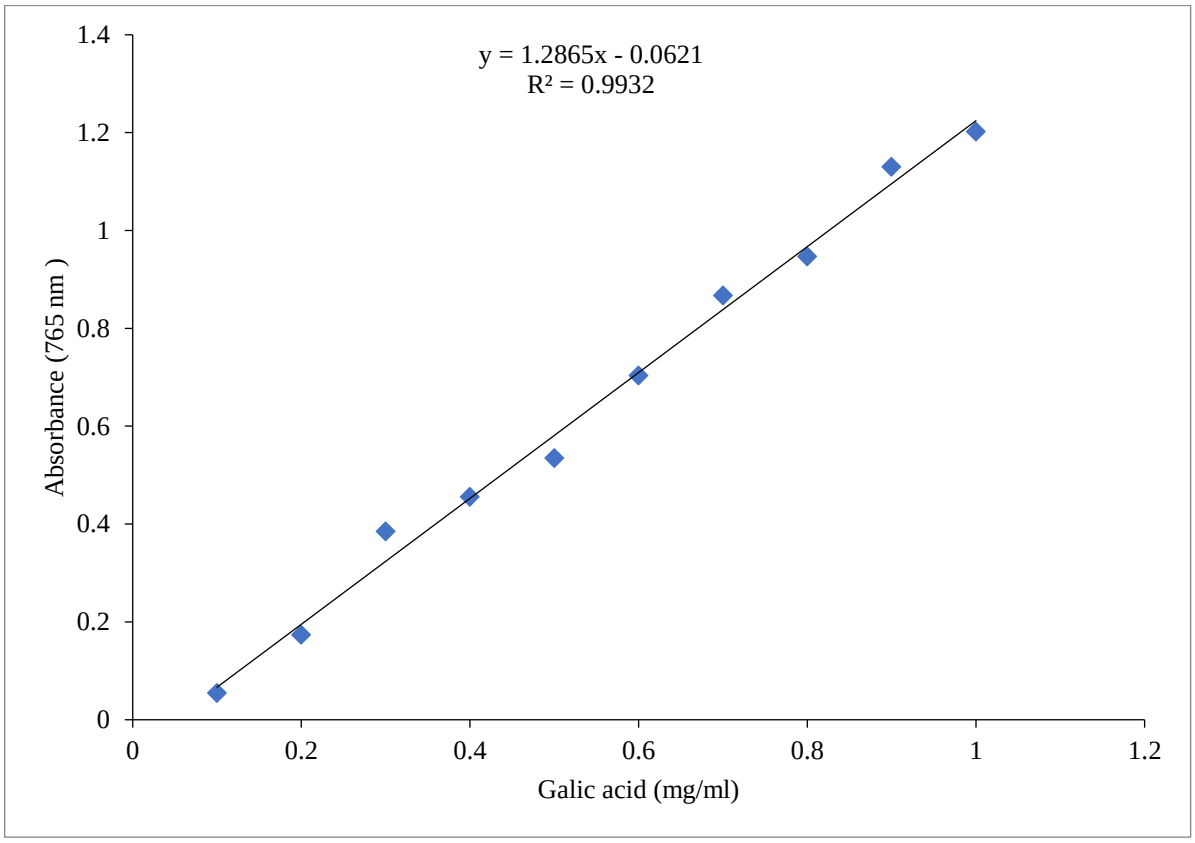


Figure 3.4 Standard curve of gallic acid for determination of total phenols.

3.6.5.3 Determination of total flavonoid content

Total flavonoid content was determined by the aluminum chloride ($AlCl_3$) colorimetric method (Hassan *et al.*, 2020) with minor modifications. In brief, 0.5 ml of each sample extract was mixed with 1.5 ml with 80% methanol, 0.1 ml of 10% (w/v) $AlCl_3$ solution, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water, preparation of the solutions showed in (Appendix 19). Then the mixture was incubated at room temperature for 45 minutes. After that, 0.5 ml of water was mixed with the same amount of chemicals in the previous step as a blank. The absorbance of the reaction mixture was determined at 415 nm using a spectrophotometer. The results of the total flavonoid content were calculated based on a standard curve (Fig. 3.5) prepared using quercetin at different concentrations (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 mg/ ml) and expressed as milligrams of quercetin per gram dry weight of plant parts. Measurement of total flavonoid content was performed using the following equation:

$$\text{Total flavnoid content (mg/g)} = \frac{\text{Concentration (mg/ml)} \times \text{Volume of extract (ml)}}{\text{Sample weight (g)}} \quad (3.4)$$

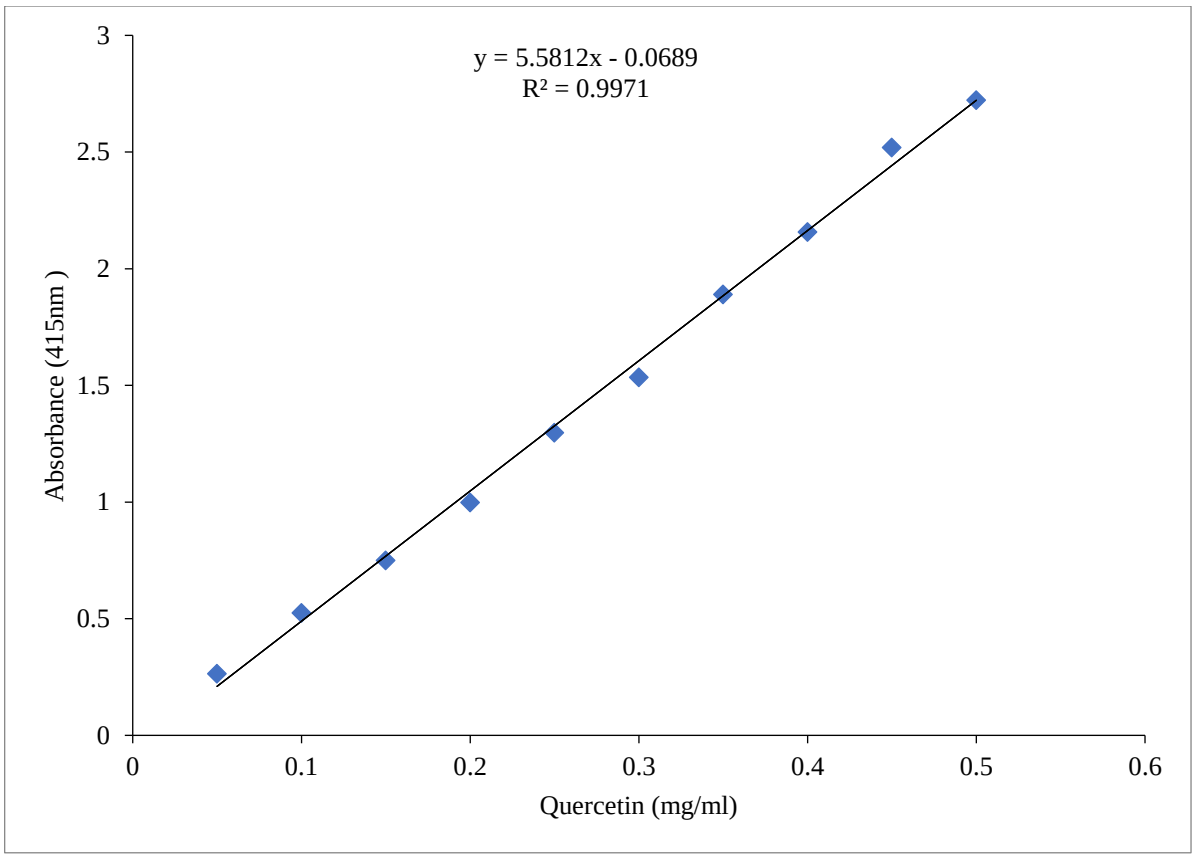


Figure 3.5 Standard curve of quercetin for determination of total flavonoid.

3.6.5.4 Extraction and analysis of phenolic compounds by HPLC

Phenolic compounds were extracted from the homogenized plant sample (3 g) using ethanol/water (70/30) ratio. Extraction process was accomplished using an Ultrasonic Bath at room temperature for 60 sec. After filtration, the solvent was removed by the rotary evaporator under vacuum, and dried at 38 °C to the constant mass. Dry extracts were stored in glass bottles at 4 °C to prevent oxidative damage until analysis. Reversed phase HPLC analysis was used to perform quantification of individual phenolic compounds, using a HPLC chromatographic system equipped with a UV detector, chemstation software, binary pump, online vacuum degasser, autosampler and Zorbax Eclipse Plus-C18-ODS column (4.6 x 250 mm). The gradient elution method, with eluent A (methanol) and eluent B (1% formic acid in water) was performed, as follows: 40 % B (0-4 min); 50 % B (4-10 min). The column temperature was 30 ºC and flow-rate of 0.7 ml/ min. The injected volume of samples and standards was 100 µl and was done automatically using an autosampler. The spectra were acquired in the 280 nm (Radovanović *et al.*, 2015). Quercetin, rutin, cinnamic acid, catechin, ferulic acid, ellagic acid, tannic acid, kaempferol, stilbene, gallic acid, and apigenin were identified by HPLC in the

leaves, fruits, rachis, gum, and bark that collected from different locations. Chromatogram of different phenolic compounds of *P. eurycarpa* leaves listed in (Appendices 20-23).

3.7 Biological Activity of Essential Oil

3.7.1 Source of microorganisms

For the purpose of antimicrobial study, Gram-positive bacteria (*S. haemolyticus* and *Enterococcus faecalis*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Escherichia coli*, and *Acinetobacter baumannii*) were used (Table 3.8). The isolates were identified by VITEK2 (Appendices 24-30). All isolates except *A. baumannii* were stored at the microbiology research laboratory in the Biology Department, College of Science, University of Sulaimani. *A. baumannii* was obtained from the molecular microbiology research laboratory in the Biology Department, College of Science, University of Sulaimani, which was identified molecularly by (Shali, 2022).

Table 3.8 Pathogenic bacteria location and specimens.

Bacterial isolates	Location	Specimen
A. baumannii	Burn and Plastic Surgery Hospital/Emergency	Patients' cabinet surface
P. aeruginosa		Burn
E. coli	Sulaimani Teaching Hospital	Urine
P. mirabilis		Urine
K. pneumonia		Urine
E. faecalis		Urine
S. haemolyticus		Wound

3.7.2 Culture media preparation

All the culture media, which were listed in the table (3.3) prepared according to the manufacturer's instructions. The pH of the media was adjusted if it is required. Culture media were autoclaved at 121°C for 20 minutes based on the instruction of producing companies, and then pouring to Petri dishes (solid media) and tubes (liquid media).

3.7.3 Molecular identification of *Acinetobacter baumannii*

Total genomic DNA of *A. baumannii* was extracted using AddPrep Genomic DNA Extraction Kit following the manufacture's guidelines that recommended for gram negative bacteria. The overnight cultured cell (1 ml) was harvested by centrifuge at 14,000 rpm for 30 sec. and the supernatant was discarded. Amount of 200 µl of lysis buffer were added. The cell pellet re-suspends by pipetting. 20 µl of proteinase K solution (20 mg/ml) was added to the sample tube, mixed by vortexing, and incubated at 56 ℃ for 30 min. in a shaking water bath.

Then spin down the tube briefly to remove any drops form inside of sample tube lid. Later, a volume 200 µl of binding buffer were added to the sample tube, and mixed well by pulse-vortexing for 15 sec. Then, it was incubated at 56 ℃ for 10 min. After that, 200 µl of absolute ethanol were added and mixed well by pulse-vortexing for 15 sec. After this step, the lysate was transferred into the upper reservoir of the spin column with 2 ml collection tube without wetting the rim and was centrifuged at 13,000 rpm for 4 min, then the flow-through was poured off and assembled the spin column with the 2.0 ml collection tube.

A volume of 500 µl of washing buffer 1 was added to the spin column with collection tube and centrifuged at 13,000 rpm for 4 min. The flow-through was discarded and the spin column was placed in the 2.0 ml collection tube. An amount of 500 µl of washing buffer 2 was added to the spin column with collection tube to be centrifuged at 13,000 rpm for 4 min. The flow-through was discarded and the spin column was placed in a clean 1.5 ml micro-centrifuge tube. 50 µl of elution buffer were added to the spin column with micro-centrifuge tube, and was allowed to stand for 5 min. Elute the genomic DNA by centrifugation at 13,000 rpm for 1 min. Lately, the DNA samples were stored in the deep freezer for further application.

PCR was performed via T100^{TM} Thermo Cycler. PCR reaction mixture set-up contained 10 µl master mix, 0.5 µl of forward and reverse primers each, 1 µl of DNA, and 8 µl of distilled water giving a total of 20 µl. Then, the amplification conditions were optimized as shown in (Table 3.9) and used for detection of 16S rRNA, *bla*$_{Oxa-23}$, *bla*$_{Oxa-51}$, and *bla*$_{TEM}$ genes in *A. baumannii.* The PCR products were visualized by 1% agarose gel electrophoresis at 80 V for about 45 min. The gel was stained with 2 µl of ethidium bromide, and the image was captured via MultiDoc-It™ Imaging System.

Table 3.9 Primers, amplicon size, and PCR settings used for detection 16S rRNA, *bla*$_{Oxa-23}$, *bla*$_{Oxa-51}$, and *bla*$_{TEM}$ genes in *A. baumannii*.

Name of primer	Sequence (5' - 3')	PCR product size (~ bp)	PCR condition	Reference
16S rRNA	F- TGGCTCAGATTGAACGCTGGCGGC R- TACCTTGTTACGACTTCACCCCA	1500	Initial denaturation: 94 °C for 2 min, 30 cycles of denaturation: 94 °C for 30s, annealing: 65 °C for 30s, extension: 68 °C for 30s, and final extension: 68 °C for 7 min.	Lee *et al.*, 2008
bla$_{Oxa-23}$	F- GATCGGATTGGAGAACCAGA R- ATTTCTGACCGCATTTCCAT	501	Initial denaturation: 94 °C for 5 min, 33 cycles of denaturation: 94 °C for 25s, annealing: 56.5 °C for 40s, extension: 72 °C for 50s, and final extension: 72 °C for 6 min.	Woodford *et al.*, 2006
bla$_{Oxa-51}$	F- TAATGCTTTGATCGGCCTTG R- TGGATTGCACTTCATCTTGG	353	Initial denaturation: 94 °C for 5 min, 33 cycles of denaturation: 94 °C for 25s, annealing: 56.5 °C for 40s, extension: 72 °C for 50s, and final extension: 72 °C for 6 min.	Shali, 2022
bla$_{TEM}$	F- CGCCGCATACACTATTCTCAGAATGA R- ACGCTCACCGGCTCCAGATTTAT	445	Initial denaturation: 94 °C for 4 min, 30 cycles of denaturation: 94 °C for 60s, annealing: 62.8 °C for 40s, extension: 72 °C for 70s, and final extension: 72 °C for 10 min.	Rajivgandhi *et al.*, 2018

* F: Forward ** R: Reverse

3.7.4 Evaluating the antibacterial effects

3.7.4.1 Agar well diffusion assay

Agar well diffusion assay was conducted to analyze the antimicrobial activity of *P. eurycarpa* essential oils (PEEOs) according to a procedure obtained from a previous work (Ahmed *et al*., 2018). *S. haemolyticus*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, *K. pneumonia*, and *E. coli* were grown in nutrient broth and *A. baumannii* was grown in Luria-Bertani broth (LB) broth and all bacteria were grown over night at 37 °C. The number of cells per milliliter was adjusted to be 1.5 x 10^8 CFU/ml, equivalent to 0.5 McFarland. Then, 100 µl of culture was spread evenly on the surface of Mueller-Hinton agar plates. Gel puncture was used to make wells and 100 µl of the essential oils were loaded into certain wells. Also, 100 µl of the broth were used in a specified well. The plates were incubated at 37 °C for 24 h and the zone of inhibition was measured (mm). The assay was done in triplets. For further study *A. baumannii* was selected due to the multidrug resistant (MDR) pattern.

3.7.4.2 Minimum inhibitory concentration and minimum bactericidal concentration assay

The stock solution of the *P. eurycarpa* essential oil of Hawar (PEEO-H) was prepared according to Clinical and Laboratory Standards Institute protocol (CLSI, 2020). Briefly, the essential oil was dissolved in dimethyl sulfoxide 1% (v/v) (DMSO), and then serial doubling dilutions of the oil were prepared in LB broth with concentrations ranging from 0.08 to 40 µl/ml (Alves-Silva *et al.*, 2016).

To measure the minimum inhibitory concentration **(**MIC) of PEEO-H, the fresh overnight culture of *A. baumannii* was adjusted to be 1.5 x 10^8 CFU/ml as mentioned above. A bacterial culture of 100 µl was dispensed in 96-wells of sterile polystyrene microtiter plates and then 100 µl of an appropriate concentration of essential oil (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10, 20 and 40 µl/ml) was added. In addition, 100 µl LB broth mixed with 100 µl of different concentrations of essential oil were used as blank. Also, 200 µl LB broth was used as a negative control and 100 µl of bacterial culture mixed with 100 µl LB broth was applied as a positive control. The microdilution plates were incubated overnight at 37 °C with a gentle shaking in the microplate incubator-shaker PST-60 HL Plus. The absorbance of each well was measured at 600 nm using a microplate spectrophotometer. Finally, growth percentage was calculated based on the average of triplicate results according to the following formula (Fattah *et al.*, 2022).

$$\text{Growth inhibition (\%)} = \frac{\text{OD}_{(600\text{ nm})}\text{ of treatment} - \text{OD}_{(600\text{ nm})}\text{ of blank}}{\text{OD}_{(600\text{ nm})}\text{ of positive control} - \text{OD}_{(600\text{ nm})}\text{ of negative control}} \times 100 \qquad (3.5)$$

In addition, minimum bactericidal concentration (MBC) was measured by subculturing 5 µl from each well onto LB agar plates. Then, the plates were incubated at 37 °C for 24 h.

3.7.4.3 Biofilm inhibition assay

The biofilm degrading activity of PEEO-H was determined by colorimetric method against *A. baumannii* according to Fattah *et al.* (2022) with a few modifications. A culture of *A. baumannii* was grown in LB broth for 24 h at 37 °C. 100 µl of bacteria culture (1.5 x 10^8 CFU/ml) were placed in a 96-well microtiter plate, with 100 µl of different concentrations of essential oil (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10, 20 and 40 µl/ml) was added. In addition, 200 µl of LB broth was used as a negative control and 100 µl of bacterial culture mixed with 100 µl LB broth was used as a positive control. The plate was then incubated for 24 h at 37 °C with gentle shaking in microplate incubator-shaker PST60 HL Plus. Biofilm was measured by discarding the medium and rinsing the wells three times with 200 µl phosphate buffer saline (PBS, pH 7.2).

After drying, the attached cells were stained with crystal violet (0.1%) for 30 min. After staining, the liquid was discarded and the wells were washed three times with distilled water. The tray was then allowed to dry at room temperature, after that 200 µl ethanol (99%) was added to the wells to solubilize the stain. Then, the wells were read at 595 nm via microplate spectrophotometer. Optical density (OD) readings were used as an indicator of bacterial biofilm formation. The biofilm inhibition percentage was conducted in triplets with averages calculated based on the following equation:

$$\text{Bioflim inhibition (\%)} = \frac{\text{OD of treated cells wtih essential oil}_{(595\ \text{nm})}}{\text{OD of untreated cells (control)}_{(595\ \text{nm})}} \times 100 \quad (3.6)$$

3.7.5 Mode of action of essential oil against *Acinetobacter baumannii*

3.7.5.1 Protein leakage assay

The effect of essential oil on membrane damage was studied by quantifying the leaked cytoplasmic proteins. Protein leakage from bacterial cells was determined using the Biuret method. Briefly, the fresh overnight bacterial cell suspension treated with different concentrations of PEEO-H (0.08, 0.16, 0.32, 0.63, 1.25, 2.5 µl/ml) for about 18 h. Samples were centrifuged at 14,000 rpm for 10 min using Micro 200R centrifuge, and supernatants were subjected for protein quantification (Hamzah *et al*., 2018).

3.7.5.2 Leakage of phosphate and potassium ions

The concentrations of free phosphate and potassium ions were determined according to Zhang *et al.* (2017) with some modifications. The concentrations of free phosphate and potassium ions in bacterial suspensions were measured after the exposure of bacterial cells to PEEO-H at different concentrations (0.08, 0.16, 0.32, 0.63, 1.25, 2.5 µl/ml) for 2 h. Samples were centrifuged at 14,000 rpm for 10 min. The extracellular phosphate and potassium ion concentrations were measured by an inorganic phosphorus kit and potassium kit, respectively. Results were expressed as the amount of extracellular free phosphate and potassium ions (µmol/l).

3.7.5.3 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to assess the morphological changes in *A. baumannii* cells after treatment with PEEO-H according to the method of Peng *et al.* (2017) with some modification. The bacteria were grown for 24 h in LB broth with essential oil at

concentration 1/4 MIC and 1/2 MIC, the pellet obtained after centrifugation was fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer and 1% tannic acid at 4 °C for 1 h and were washed twice with deionized water. A drop containing the bacteria was deposited onto a carbon-coated grid with 2% uranyl acetate, and the grids were examined using a transmission electron microscope. The same procedure was followed without essential oil and served as a control.

3.7.6 Anti-adhesion assay using Band-Aids

PEEO-H was evaluated for its efficiency using applications with the surfaces of Band-Aids. Sterile Band-Aids were purchased from a market and cut into uniformly sized 6 mm sections. 10 µl and 15 µl of essential oil were added to the pieces. Next, the pieces were soaked in 200 µl of *A. baumannii* cultures (1.5 x 10^8 CFU/ml) in a 96-well microtiter plate and incubated at 37 °C overnight. Untreated Band-Aid pieces immersed in bacterial cultures served as controls. The pieces of Band-Aids from the microtiter plate were taken out and washed with 1X PBS (pH 7.2) to remove free floating bacteria from the surfaces. The pieces were then left in PBS for 30 min. Serial dilutions were made from this suspension and 50 µl were then evenly spread on the entire surface of a nutrient agar plate and incubated at 37 °C overnight. Plates were then counted to observe the number of colonies before and after treatment with PEEO (Barawi *et al.*, 2021).

3.8 Statistical Analysis

To perform statistical analysis, the XLSTAT (Version 2016.02.28451) statistical software package was used. For chemical constituent study, Factorial Completely Randomized Design (CRD) was conducted. Two factors with five replicates. The means were compared to determine critical values using the Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. For biological study, Completely Randomized Design (CRD) was used to analyze variation among the experimental groups. The un-paired t-test for the independent variables were applied to make a pairwise comparison between groups. The values were presented as average, with $p \leq 0.01$ considered statistically significant.

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 Molecular Identification of the Plant

Plant samples were identified morphologically according to variation in the traits of leaves and stems. *Pistacia eurycarpa* (*Pistacia atlantica* subsp. *kurdica*) is a tree and has obtuse leaflet's apex, while *Pistacia khinjuk* is a shrub and has acuminate leaflet's apex. Then, samples were identified at the Iraqi National Herbarium, Directorate of Seed Testing and Certification, Ministry of Agriculture (Appendix 31). Four plants in Qaradagh, Ranya, Sharbazher, and Hawar were identified as *P. eurycarpa*, while two other plants in Qaradagh and Hawar were identified as *P. khinjuk.* After preliminary morphological identification, the samples were identified molecularly. To our knowledge, *Pistacia* species have never been classified via 18S rRNA sequence analysis in Kurdistan and Iraq. The 18S rRNA PCR products of the sample with designed primers showed approximately 600 bp band size (Figs. 4.1 and 4.2).

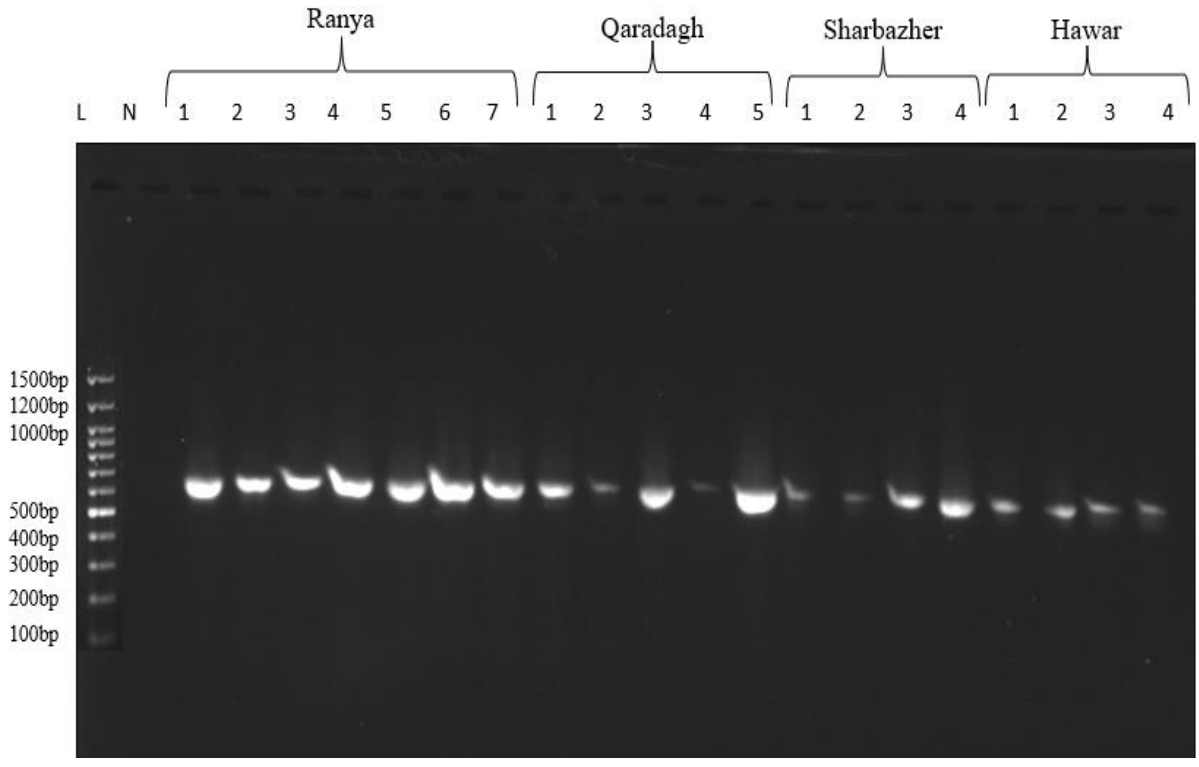


Figure 4.1 Gel electrophoresis of PCR products of 18S rRNA (partial) of *P. atlantica* (L) Ladder. (N) No DNA. (1, 2, 3, 4, 5, 6, and 7) number of samples.

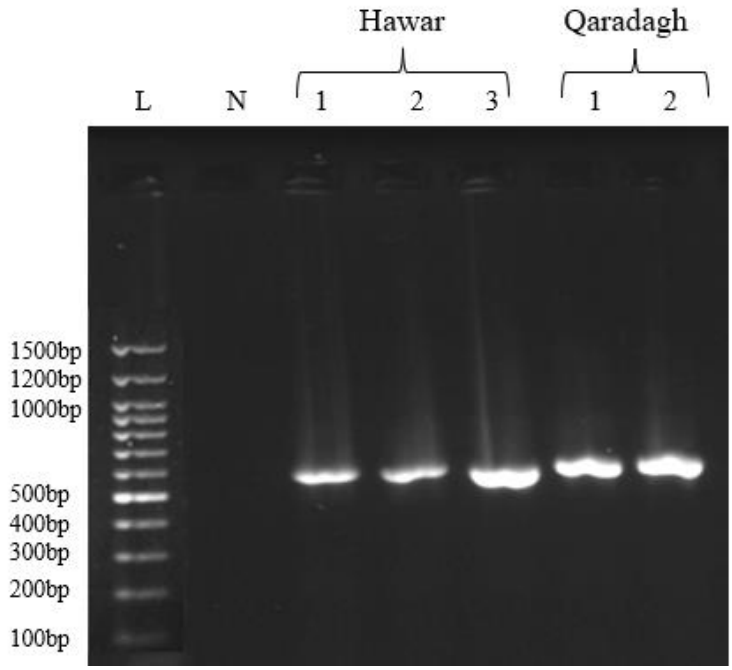


Figure 4.2 Gel electrophoresis of PCR products of 18S rRNA (partial) of *P. khinjuk* (L) Ladder. (N) No DNA. (1, 2, and 3) number of samples.

The homologous sequence searching was performed by using BLASTn tool to find the closest sequence to our 18S rRNA in the NCBI database. Four plants in Qaradagh, Ranya, Sharbazher, and Hawar were found to be closely related to *P. atlantica* as they showed 100%, 100%, 100%, and 99.83%, respectively sequence similarity to others recorded for genus *P. atlantica* around the world, while two plants in Qaradagh and Hawar found to be closely related to *P. khinjuk* as they showed 100% and 99.33%, respectively sequence similarity to others recorded for genus *P. khinjuk*. After confirmation of the genus and species of the plant, the DNA sequence of the 18S rRNA gene was submitted to the BANKIT and GenBank accession numbers were assigned (Table 4.1).

Table 4.1 Accession numbers of the 18S rRNA (novel primers) sequence of *Pistacia* species.

Pistacia species	Locations	Accession numbers	Link in NCBI
P. atlantica	Qaradagh	MW534228	https://www.ncbi.nlm.nih.gov/nuccore/MW534228
P. atlantica	Ranya	MW534226	https://www.ncbi.nlm.nih.gov/nuccore/MW534226
P. atlantica	Sharbazher	MW534229	https://www.ncbi.nlm.nih.gov/nuccore/MW534229
P. atlantica	Hawar	MW534227	https://www.ncbi.nlm.nih.gov/nuccore/MW534227
P. khinjuk	Qaradagh	MW534231	https://www.ncbi.nlm.nih.gov/nuccore/MW534231
P. khinjuk	Hawar	MW534230	https://www.ncbi.nlm.nih.gov/nuccore/MW534230

Furthermore, a phylogenetic tree was created using the DNA sequence of the 18S rRNA gene based on the Neighbor-joining method (Fig. 4.3).

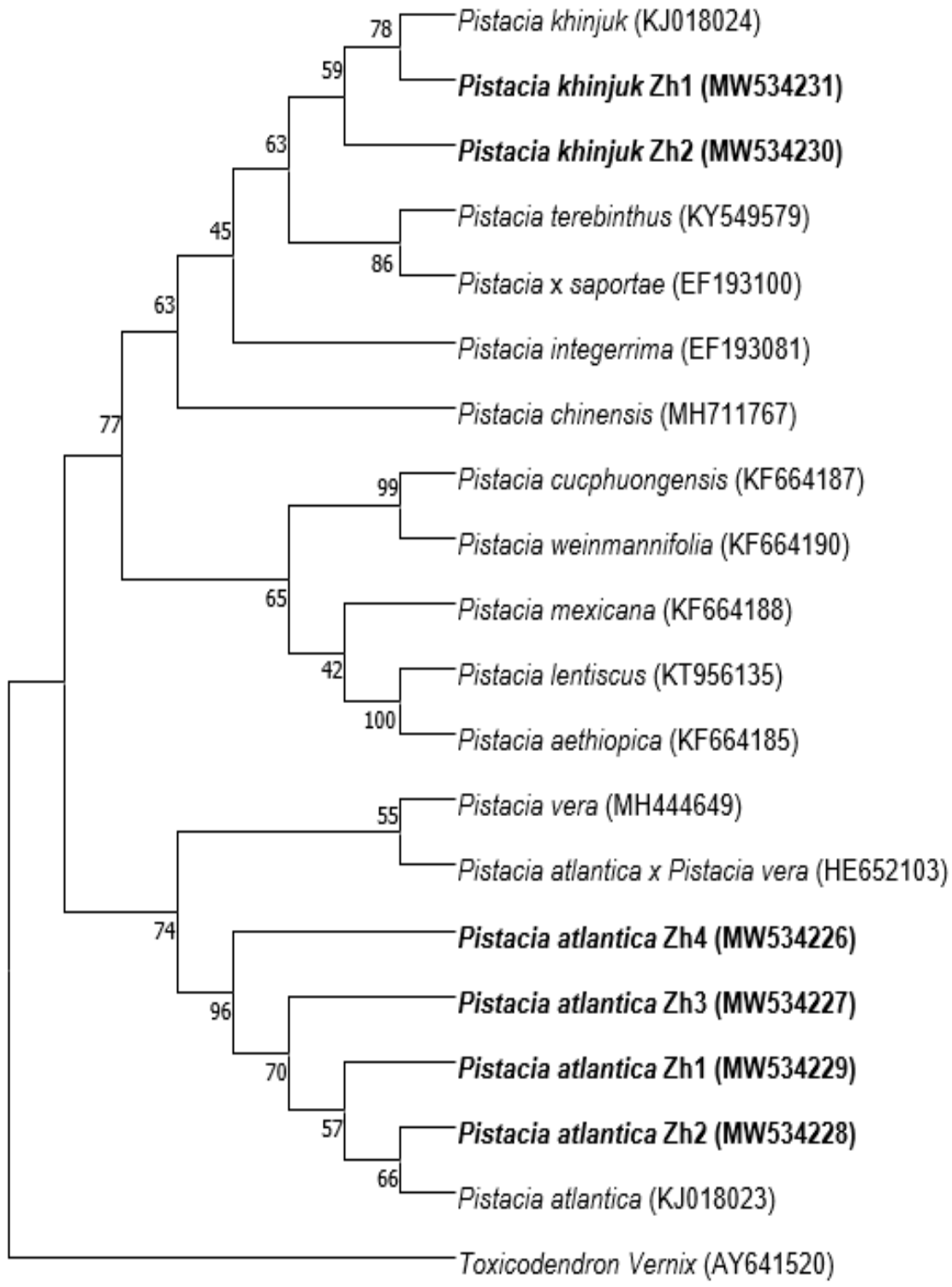


Figure 4.3 Phylogenetic tree of *Pistacia* species based on 18S rRNA gene sequence (partial). The tree was generated by the neighbor-joining method with 1000 bootstrap replicates. *Toxicodendron vernix* served as an outgroup. GenBank accession numbers are provided in parentheses.

For the first time, the molecular identification of *Pistacia* species which collected from different locations by using a novel primer set of the genus *Pistacia* is reported. Nuclear-encoded 18S rRNA sequences have been used widely to infer the phylogenetic relationships among taxa at different hierarchical ranks. Also, due to the presence of numerous DNA sequences that have been deposited in GenBank, the 18S rRNA gene can provide a robust biomarker in the taxonomy of plants. Consequently, a lot of uncertain plants species have been confirmed from the 18S rRNA gene sequences. To obtain more reliable results for the classification and identification of *P. atlantica* and *P. khinjuk*, the 18S rRNA gene sequence has been determined and presented molecular relationships with other *Pistacia* species. *P. atlantica* subsp. *kurdica* recorded in Flora of Iraq as synonym of *P. eurycarpa* (Townsend *et al.*, 1980). Rechinger (1969) in Flora of Iranica classified three subspecies for *P. atlantica*: *P. atlantica* subsp. *mutica*, *P. atlantica* subsp. *kurdica*, and *P. atlantica* subsp. *cabulica*, in addition *P. eurycarpa* has been recorded as the synonym of *P. atlantica* subsp. *kurdica*. It is possible that the species studied in this research could be *P. atlantica* subsp. *kurdica* as suggested by several researchers (Azeez and Gaphor, 2019; Hosseini *et al.*, 2020); although, no sequence of *P. atlantica* subsp. *kurdica* can be found in GenBank for comparison.

4.2 Chemical Profiles of *Pistacia eurycarpa* Parts

4.2.1 Nitrogen, phosphorus, potassium, and carbohydrates content of *Pistacia eurycarpa* leaves

Table (4.2.A) shows the percentages of nitrogen, phosphorus and potassium in the leaves of *P. eurycarpa* that collected from spring and autumn at different locations. The leaves collected from Qaradagh had the highest percentage (4.69%) of nitrogen with significant differences compared to other locations. Concerning the season, spring leaves contain highest amounts of nitrogen (4.40%) which is significantly different from autumn leaves. Regarding the interaction between locations and season, the highest value of nitrogen (6.70%) content appeared in spring leaves collected from Qaradagh followed by Sharbazher (4.90%) and Hawar (3.70%). Whereas, the lowest value was in autumn leaves (1.94%) collected from Ranya, which was not significantly different from spring leaves collected from the same location (2.30%).

Concerning the percentage of phosphorus, the leaves collected from Hawar had the highest percentage of phosphorus (1.36%) with significant difference compared to other locations. Regarding the season, spring leaves contain highest amounts of phosphorus (1.24%) with significant differences compared to autumn leaves (0.65%). The interaction between locations

and season, the highest percentage of phosphorus occurred in the spring leaves collected from Hawar (2.03%) followed by Qaradagh (0.99%), Ranya (0.96%), and Sharbazher (0.96%). The lowest value was in the autumn leaves (0.57%) collected from Ranya, which did not differ significantly with autumn leaves collected from Qaradagh (0.62%).

With regard to the potassium percentage, it was noticed that the leaves collected from Hawar had the highest percentage of potassium (2.20%) with significant difference compared to other locations. Autumn leaves had the highest value of potassium (1.86%) with significant differences compared to spring leaves (1.42%). Spring leaves collected from Hawar gave the highest value (2.25%), which did not significantly differ from autumn leaves collected from the same place (2.14%); the lowest percentage occurred in the spring leaves collected from Ranya (0.48%).

Table (4.2.B) shows the percentages of carbohydrates in the leaves of *P. eurycarpa* collected from spring and autumn at different locations. Carbohydrate results showed the highest value (26.86%) was found in the Ranya leaves, while spring leaves had the highest and significant value (29.78%). Concerning the interaction between locations and season, the highest percentage (30.83%) of carbohydrates was found in the spring leaves collected from Qaradagh with no significant difference with Ranya leaves collected from the same season (30.14%). The lowest percentage was in the autumn leaves (20.31%) that were collected from Sharbazher.

Concerning the spring and autumn leaves, it was discovered that nitrogen, phosphorus, and carbohydrate levels were highest in the spring leaves. The environmental factors such as light, temperature, rainfall and soil fertility have significant effects on the efficiency of photosynthesis and chemical constituents (Muhammad *et al.*, 2021). The leaves were fully expanded with more efficient photosynthesis during spring, so the production of carbohydrate was higher. In addition, the average summer temperature was high, and the plant's respiration rate increased. Autumn leaves showed a gradual decrease in the concentrations of nitrogen, phosphorus, and carbohydrate (Crous *et al.* 2022). The reason for this could be due to senescen, as photosynthetic capacity and efficiency decline with senescen (Bauerle *et al.*, 2020). Qaradagh soil contained the highest percentage of nitrogen (45 mg kg^{-1} soil) (Table 3.6); which may be considered as a significant reason for high nitrogen contents in Qaradagh leaves harvested in the spring. The soil of Hawar location contained the highest percentage of phosphorus and potassium (18.23 and 180.23 mg kg^{-1} soil), respectively (Table 3.6) compared to other locations; this is also considered as a significant reason for high contents of phosphorus and potassium in Hawar spring leaves. The Qaradagh spring leaves contained the highest percentage of carbohydrates.

This finding corroborates those of Hassan (2016), who found that Qaradagh spring leaves contained the highest levels of carbohydrates.

Table 4.2.A Nitrogen, phosphorus and potassium in spring and autumn leaves of *P. eurycarpa* at different locations.

Components	Nitrogen%		Locations mean	Phosphorus%		Locations mean	Potassium%		Locations mean
Season / Locations	Spring	Autumn		Spring	Autumn		Spring	Autumn	
Qaradagh	6.70 a	2.67 de	4.69 a	0.99 b	0.62 de	0.81 bc	2.03 b	1.68 d	1.86 b
Ranya	2.30 ef	1.94 f	2.12 d	0.96 b	0.57 e	0.77 c	0.48 f	1.84 c	1.16 d
Sharbazher	4.90 b	2.56 de	3.73 b	0.96 b	0.73 c	0.85 b	0.92 e	1.76 cd	1.34 c
Hawar	3.70 c	2.74 d	3.22 c	2.03 a	0.68 cd	1.36 a	2.25 a	2.14 ab	2.20 a
Season mean	4.40 a	2.48 b		1.24 a	0.65 b		1.42 b	1.86 a	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

Table 4.2.B Carbohydrates (%) in spring and autumn leaves of *P. eurycarpa* at different locations.

Season / Locations	Spring	Autumn	Locations mean
Qaradagh	30.83 a	22.67 e	26.75 a
Ranya	30.14 ab	23.57 de	26.86 a
Sharbazher	29.26 bc	20.31 f	24.79 b
Hawar	28.89 c	24.21 d	26.55 a
Season mean	29.78 a	22.69 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.2 Essential oil of gum and autumn rachis of *Pistacia eurycarpa*

Essential oil content in gum and autumn rachis of *P. eurycarpa* at different locations varies as demonstrated in (Table. 4.3) and absence of the essential oil in the other parts of the plant. Gum and autumn rachis collected from Sharbazher had the highest value of essential oil (16.50%) with significant differences compared to other locations. On the other hand, gum contains a higher amount of essential oil (25.85%) than autumn rachis (3.25%). Concerning the interaction between locations and plant parts, the gum collected from Sharbazher had the highest value (29.00%) followed by Qaradagh (25.40%) where did not differ significantly from gum collected from Ranya (25.00%). On the other hand, the lowest value was recorded from autumn rachis

collected from Hawar (2.40%), which was not significantly different from autumn rachis collected from Qaradagh (3.00%).

Essential oil (EO) content shows variation in plants at different geographical origin and also in different parts of the tree. The plant part used to abtain EOs should be considered an endogenous factor because is related to the anatomical and physiological characteristics of the plants (Barra, 2009). For example, in the *Pistacia*, the EOs are located in the gum, but in the *Citrus* genus, EOs are located in peels and leaves. The high levels of essential oil was found at various locations in the gum, which was also supported by Naseri *et al.* (2022) and Hashemi *et al.* (2017). In addition, Sharbazher gum provided the highest value for the essential oil. Meteriological data showed that Sharbazher has low temprature and high rainfall. Hence, low temprature and high rainfall increased the rate of the EO (Shams *et al.*, 2016).

Table 4.3 Concentration of essential oil (%) in gum and autumn rachis of *P. eurycarpa* at different locations.

Plant parts / Locations	Gum	Autumn rachis	Locations mean
Qaradagh	25.40 b	3.00 ef	14.20 b
Ranya	25.00 b	3.60 de	14.30 b
Sharbazher	29.00 a	4.00 d	16.50 a
Hawar	24.00 c	2.40 f	13.20 c
Plant parts mean	25.85 a	3.25 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.2.1 Chemical constituents of *Pistacia eurycarpa* gum essential oils

Table (4.4) shows results obtained by GC-MS analysis of *P. eurycarpa* gum essential oils at different locations with their percentage concentrations, in which eighty-one compounds were identified. Alpha-Pinene has been reported as the main compound, the highest concentration of which was found in gum collected from Hawar (81.40%) followed by Ranya and Qaradagh (75.34% and 64.51%, respectively), and the lowest in Sharbazher gum (54.56%). The highest concentrations of other main components were: Beta-Pinene (7.50%), Camphene (4.23%), Beta-Phellandrene (2.32%), and Beta-Myrcene (1.83%) in the gum collected from Sharbazher, whereas the lowest values of Beta-Pinene (3.29%), Camphene (1.50%), Beta-Phellandrene (1.02%), and Beta-Myrcene (0.91%) were in the gum collected from Hawar.

Table 4.4 GC-mass analysis of chemical constituents of *P. eurycarpa* gum essential oils at different locations.

No	Compounds name	Concentration (%)			
		Qaradagh	Ranya	Sharbazher	Hawar
1	Alpha-Pinene	64.51	75.34	54.56	81.40
2	Beta. -Pinene	7.49	4.38	7.50	3.29
3	Camphene	3.30	2.94	4.23	1.50
4	Beta.-Phellandrene	1.90	1.27	2.32	1.02
5	Beta-Myrcene	1.48	0.92	1.83	0.91
6	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-, (.+/-.)-(Alpha-Pinene dimer)	1.80	1.41	2.01	0.27
7	(+)-4-Carene	0.21	0.19	0.28	0.05
8	Benzene, 1-methyl-2-(1-methylethyl)-	0.87	0.55	1.01	0.31
9	Cyclobutane, 1,2-bis(1-methylethenyl)-, trans-	3.44	2.10	4.15	1.13
10	3,5-Methanocyclopentapyrazole, 3,3a,4,5,6,6a-hexahydro-3a,4,4-trimethyl-	0.14	0.12	0.16	0.27
11	3-Oxatricyclo[4.1.1.0(2,4)]octane, 2,7,7-trimethyl-	0.23	0.11	0.23	0.93
12	Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-, [1S-(1.alpha.,2.beta.,5.alpha.)]-	0.31	0.41	0.88	0.07
13	3-Cyclopentene-1-acetaldehyde, 2,2,3-trimethyl-	0.57	0.22	0.60	0.25
14	Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-	1.04	0.14	0.22	0.67
15	Benzenemethanol, .alpha.,.alpha.,4-trimethyl-	0.57	0.40	0.67	0.24
16	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)-	0.85	0.55	1.16	0.67
17	Trans-3(10)-Caren-2-ol	0.06	0.03	0.05	0.07
18	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	0.93	1.15	1.56	0.43
19	2-Oxabicyclo[2.2.2]octan-6-ol, 1,3,3-trimethyl-	0.07	0.06	0.08	0.05
20	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	0.33	0.15	0.33	0.17
21	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	0.21	0.10	0.20	0.04
22	Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	0.68	0.75	0.89	-
23	Alpha-Phellandrene	0.11	0.05	0.12	-
24	Bicyclo[2.2.1]hept-2-ene, 1,7,7-trimethyl-	0.15	0.16	0.16	-
25	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1.alpha.,3.alpha.,5.alpha.)]-	1.62	-	1.58	0.65
26	P-Mentha-1,5-dien-8-ol	0.29	0.14	0.34	-
27	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	0.06	0.06	-	0.05
28	Tricyclo[2.2.1.0(2,6)]heptane, 1,7,7-trimethyl-	0.13	0.06	0.14	-
29	Benzene, 1-methyl-4-(1-methylethenyl)-	0.26	0.19	0.33	-
30	Cyclohexene, 3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl-	0.53	0.31	0.70	-
31	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-, (1S)-	0.10	0.06	-	0.37
32	Cyclohexanol, 2-methylene-5-(1-methylethenyl)-	0.08	0.04	0.09	-
33	Lanosterol	0.26	0.13	4.94	-
34	1,2,3,4,5,8-Hexahydronaphthalene	0.12	0.08	-	-
35	3-Oxabicyclo[3.3.0]octan-2-one, 6-methylene-7-methyl-	0.06	0.05	-	-
36	Cyclohexene, 4-methyl-3-(1-methylethylidene)-	4.33	-	3.56	-
37	1,6-Octadien-3-ol, 3,7-dimethyl-	0.27	0.16	-	-
38	Pseudosolasodine diacetate	-	0.03	0.07	
39	Spiro[2.4]heptane, 1,5-dimethyl-6-methylene-	-	-	0.14	0.06
40	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-	0.14	0.06	-	-
41	3-Isopropylidene-5-methyl-hex-4-en-2-one	0.17	-	0.11	-

Table 4.4 Continued

No	Compounds name	Concentration (%)			
		Qaradagh	Ranya	Sharbazher	Hawar
42	Lanosta-7,9(11)-dien-21-oic acid, 3,16-dihydroxy-24-methylene-, methyl ester, (3.beta.,	0.03	-	-	-
43	1,3-Cyclopentadiene, 5,5-dimethyl-2-ethyl-	0.06			
44	Lupeol	0.13	-	-	-
45	6a,14a-Methanopicene, perhydro-1,2,4a,6b,9,9,12a-heptamethyl-10-hydroxy-	0.11	-	-	-
46	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	-	3.83	-	-
47	Bicyclo[3.2.1]oct-2-ene, 3-methyl-4-methylene-	-	0.02	-	-
48	Pentanal, 5-(methylenecyclopropyl)-	-	0.75	-	-
49	Trans-2-Caren-4-ol	-	0.07		-
50	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (Z)-	-	0.12	-	-
51	Lanostan-11-one, 3-(acetyloxy)-, (13.alpha.,14.beta.,17.alpha.)-	-	0.03	-	-
52	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-	-	0.31	-	-
53	3-(2,5-Bis(benzyloxy)phenyl)-2-formylaminoacrylic acid, benzyl ester	-	-	0.12	-
54	9.alpha.-Hydroxytomatidine, o,N-diacetate	-	-	0.24	-
55	Picolinyl 6,9,12,15,18-heneicosapentaenoate	-	-	0.04	-
56	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)-1,1a,2,3,4,6,7,10,11,11a-decahydro-7,10-dihydr	-	-	0.12	-
57	3'-Butoxy-2-{[3-cyano-6-(2-thienyl)-4-(trifluoromethyl)-2-pyridyl]thio}acetanilide	-	-	0.13	-
58	Urs-12-en-28-al	-	-	0.33	-
59	9,19-Cyclo-9.beta.-lanostane-3.beta.,25-diol	-	-	0.20	-
60	3-Debenzoyloxy-anhydrocarpesteerol	-	-	0.86	-
61	Lanosta-8,24-dien-3-one	-	-	0.33	-
62	Cycloartanol	-	-	0.43	-
63	Eucalyptol	-	-	-	0.39
64	1,3,5-Cycloheptatriene, 3,7,7-trimethyl-	-	-	-	0.04
65	5,8-Dimethylenebicyclo[2.2.2]oct-2-ene	-	-	-	0.11
66	Cis-Ocimene, 8-oxo-	-	-	-	0.02
67	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethyl)-	-	-	-	0.88
68	2(10)-Pinen-3-one, (.+/-.)-	-	-	-	0.21
69	(E)-3(10)-Caren-4-ol	-	-	-	0.04
70	2H-Pyran, 2-(2-heptadecynyloxy)tetrahydro-	-	-	-	0.07
71	Nickel, bis[N,N'-1,2-ethanediylidenebis[cyclohexanamine]-N,N']-, (t-4)-	-	-	-	0.04
72	2(5H)-Furanone, 4-methyl-3-(2-methyl-2-propenyl)-	-	-	-	0.09
73	1-Pentene, 5-(2,2-dimethylcyclopropyl)-2-methyl-4-methylene-	-	-	-	1.41
74	Bicyclo[3.1.0]hexan-3-ol, 4-methylene-1-(1-methylethyl)-, (1.alpha.,3.alpha.,5.alpha.)-	-	-	-	0.43
75	Alpha.-Farnesene	-	-	-	0.77
76	(-)-Myrtenol	-	-	-	0.28
77	Bicyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl-	-	-	-	0.04
78	Bicyclo[3.1.0]hexan-3-ol, 4-methylene-1-(1-methylethyl)-, acetate	-	-	-	0.03
79	4-tert-Butyltoluene	-	-	-	0.13
80	Sulfurous acid, cyclohexylmethyl heptyl ester	-	-	-	0.07
81	Androst-5,15-dien-3ol acetate	-	-	-	0.08
Total		100	100	100	100

4.2.2.2 Chemical constituents of *Pistacia eurycarpa* autumn rachis essential oils

Eighty-one compounds were identified by GC-MS analysis of *P. eurycarpa* autumn rachis essential oils (Table 4.5). Alpha-Pinene was reported as the major compound, the highest value (56.03%) was found in autumn rachis from Qaradagh, followed by Ranya and Hawar (54.69% and 53.38%, respectively), and the lowest in Sharbazher (42.83%). Bicyclo [3.1.1] hept-2-ene, 2,6,6-trimethyl was found only in those of Ranya (17.56%) and Sharbazher (14.53%). The highest concentrations of D-Limonene and Beta-Pinene were found in Hawar (13.59%, 12.03%, respectively). Whereas, the lowest values for these two components in Ranya were (7.87%, 3.55%, respectively)**.** The highest value of Beta-Myrcene was found in Qaradagh (3.48%), followed by Hawar and Sharbazher (3.41% and 2.26%, respectively), and the lowest in Ranya (2.25%).

The highest percentage of Camphene occurred in Sharbazher (4.42%), followed by each Qaradagh and Hawar (3.07%), and the lowest in Ranya (1.69%). GC-MS analysis of gum and autumn rachis essential oil in various locations showed the main constituent is an Alpha-Pinene. Azeez and Gaphor (2019) corroborates this finding with similar results; 79.76% Alpha-Pinene. Memariani *et al.* (2017) and Ellahi *et al*. (2019) also reported somewhat similar results; 93.17% and 92.08% for Alpha-Pinene, respectively. Spring leaves of Hawar contained the highest percentage of phosphorus; which is also considered to be as a significant reason for high α-Pinene contents in Hawar gum essential oil. Bustamante *et al.* (2020) concluded that increasing phosphorus in the leaf caused the increase in α-Pinene. Phosphorus required as a component of terpenoid precursors (IPP and GDP) and the ATP and NADPH molecules, needed for terpenoid synthesis.

Table 4.5 GC-mass analysis of chemical constituents of *P. eurycarpa* autumn rachis essential oils at different locations.

No	Compounds name	Concentration (%)			
		Qaradagh	Ranya	Sharbazher	Hawar
1	Alpha-Pinene	56.03	54.69	42.83	53.38
2	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-, (.+/-.)- (Alpha-Pinene dimer)	-	17.56	14.53	-
3	D-Limonene	12.03	7.87	10.23	13.59
4	Beta-Pinene	10.03	3.55	9.31	12.03
5	Beta-Myrcene	3.48	2.25	2.26	3.41
6	Camphene	3.07	1.69	4.42	3.07
7	(+)-4-Carene	0.07	0.07	0.04	0.09
8	Alpha-Phellandrene	0.22	0.25	0.52	0.21
9	Benzene, 1-methyl-2-(1-methylethyl)-	0.14	0.08	0.17	0.11
10	1,6-Octadien-3-ol, 3,7-dimethyl-	1.12	0.52	0.69	1.16
11	3-Cyclopentene-1-acetaldehyde, 2,2,3-trimethyl-	0.15	0.23	0.12	0.18
12	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1.alpha.,3.alpha.,5.alpha.)]-	0.29	0.43	0.24	0.30
13	Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-	0.24	0.03	0.20	0.25
14	P-Mentha-1,5-dien-8-ol	0.04	0.08	0.06	0.03
15	Cyclohexene, 3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl-	0.04	0.22	0.18	0.46
16	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)-	0.59	0.58	0.64	0.61
17	Trans-3(10)-Caren-2-ol	0.03	0.04	0.03	0.06
18	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	0.02	1.31	0.06	0.20
19	1,3,6-Octatriene, 3,7-dimethyl-, (E)-	0.04	0.02	0.06	0.30
20	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (Z)-	0.15	0.11	0.06	0.26
21	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	0.09	0.14	0.10	0.10
22	Beta.-Phellandrene	1.23	1.13	1.10	0.15
23	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	0.14	0.07	0.17	0.14
24	2(10)-Pinen-3-one, (.+/-.)-	0.05	0.12	0.09	0.07
25	Alpha.-Caryophyllene	0.04	0.05	0.10	0.10
26	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]-	0.02	0.71	0.43	0.66
27	Gamma.-elemene	0.15	1.16	0.03	0.11
28	Bornyl acetate	3.58	0.13	3.90	2.95
29	3,7-Cyclodecadiene-1-methanol, .alpha.,.alpha.,4,8-tetramethyl-, [s-(Z,Z)]	1.12	0.89	0.90	0.60
30	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alp	0.12	0.39	0.11	0.14
31	2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4	0.56	0.47	0.17	0.31
32	Cyclofenchene	0.67	0.29	1.17	0.48
33	Cyclohexene, 4-methyl-3-(1-methylethylidene)-	0.57	-	2.15	0.52
34	Benzene, 1-methyl-4-(1-methylethenyl)-	0.06	-	0.06	0.06
35	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-, (1S)-	0.07	0.11	0.08	-
36	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	0.31	0.13	0.38	-
37	Isoborneol	0.24	-	0.25	0.28
38	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	0.03	0.04	0.02	-
39	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	0.35	0.33	0.32	-
40	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	0.05	0.10	0.04	-

Table 4.5 Continued

No	Compounds name	Concentration (%)			
		Qaradagh	Ranya	Sharbazher	Hawar
41	Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-	0.05	0.63	0.25	-
42	Caryophyllene oxide	0.06	0.05	0.05	-
43	5-Azulenemethanol, 1,2,3,4,5,6,7,8-octahydro-.alpha.,.alpha.,3,8-tetramethyl-	0.04	0.03	0.04	-
44	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, acetate	0.03	0.06	0.05	-
45	Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-	-	0.03	0.23	0.06
46	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-	0.42	-	0.42	0.42
47	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1R-(1.alpha.,4	-	0.15	0.06	-
48	Caryophyllene	-	-	0.07	0.20
49	Gamma.-Gurjunenepoxide-(2)	-	0.14	0.12	-
50	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-	-	0.04	0.03	-
51	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-	-	0.87	0.31	-
52	3,8,12-Tri-O-acetoxy-7-desoxyingol-7-one	0.05	-	-	-
53	Thymol	0.05	-	-	-
54	1-Cyclopentene-1-carbaldehyde,3,4,5-tri(benzyloxy)	0.02	-	-	-
55	Thujopsene	0.04	-	-	-
56	Cis-.alpha.-Bisabolene	0.03	-	-	-
57	Sulfurous acid, cyclohexylmethyl heptyl ester	0.05	-	-	-
58	Urs-12-en-28-al	0.20	-	-	-
59	Olean-12-en-28-al	0.34	-	-	-
60	9,19-Cyclolanost-23-ene-3,25-diol, (3.beta.,23E)-	0.33	-	-	-
61	3-Debenzoyloxy-anhydrocarpesteerol	0.38	-	-	-
62	1,4-Methanocycloocta[d]pyridazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-, (1.alp	0.21	-	-	-
63	Cholest-7-en-3.beta.,5.beta.-diol-6.beta.-benzoate	0.01	-	-	-
64	(-)-Spathulenol	0.04	-	-	-
65	Cycloartanol	0.42	-	-	-
66	Bicyclo[3.1.0]hexan-3-ol, 4-methylene-1-(1-methylethyl)-, (1.alpha.,3.alpha.,5.alpha.)-	-	0.05	-	-
67	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	-	0.05	-	-
68	5.alpha.-Spirostan, 23-bromo-, (22S,23R,25R)-	-	0.03	-	-
69	Eicosanoic acid, 2-phenyl-1,3-dioxan-5-yl ester	-	0.03	-	-
70	A'-Neogammacer-22(29)-en-3-one	-	-	0.04	-
71	2-Isopropyl-tricyclo[4.3.1.1(2,5)]undec-3-en-10-ol	-	-	0.05	-
72	Phenol, 2-ethyl-4,5-dimethyl-	-	-	0.06	-
73	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-, (S)-	-	-	-	1.81
74	5-Isopropenyl-2-methylcyclopent-1-enecarboxaldehyde	-	-	-	0.09
76	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	-	-	-	0.67
77	Cubenol	-	-	-	0.08
78	Agarospirol	-	-	-	0.10
79	Tau.-cadinol	-	-	-	0.06
80	Cyclohexane, 1,5-diisopropyl-2,3-dimethyl-	-	-	-	0.11
81	1-Heptatriacotanol	-	-	-	0.03
Total		100	100	100	100

4.2.3 Fixed oil of autumn fruits and bark of *Pistacia eurycarpa*

Fixed oil content results only in autumn fruits and bark of *P. eurycarpa* at different locations are illustrated in (Table 4.6), while fixed oil was not noticed in other parts of the plant. Autumn fruits and bark collected from Qaradagh had the highest value of fixed oil (17.85%) compared to other locations with significant differences. Regarding to the plant parts content of fixed oil, autumn fruits contained higher amounts of fixed oil (26.86%) than that of bark (3.38%). Concerning the interaction between locations and plant parts, significant differences were found in the autumn fruit collected from various locations, while slight insignificant differences appeared among the barks collected from various locations. The highest levels of fixed oil found in autumn fruit collected from Qaradagh (32.08%) followed by Ranya (31.16%), Sharbazher (26.16%), and Hawar (18.04%). The lowest level found in the bark collected from Hawar (3.10%). It is noticed that the Qaradagh location gave the highest value of fixed oil in autumn fruit perhaps due to the difference of environmental conditions among the locations. Qaradagh soil contains a high proportion of organic matter which was considered to have a significant impact on the physiochemical properties of the soil. The main consequence was an improvement in the uptake of micro and macroelements (Khoshnaw and Esmail, 2020). Leskovar and Othman (2018) found that increasing the amount of organic matter increases porosity, which decreases soil-specific density and allows microorganisms to penetrate more easily into the soil environment to use organic compounds, providing plants with better access to nutrients.

Table 4.6 Concentration of fixed oil (%) in autumn fruits and bark of *P. eurycarpa* at different locations.

Plant parts / Locations	Autumn fruits	Bark	Locations mean
Qaradagh	32.08 a	3.62 e	17.85 a
Ranya	31.16 b	3.48 e	17.32 b
Sharbazher	26.16 c	3.31 e	14.74 c
Hawar	18.04 d	3.10 e	10.57 d
Plant parts mean	26.86 a	3.38 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.05$.

4.2.3.1 Gas chromatography analysis for fixed oil constituents

GC analysis results of autumn fruit and bark of *P. eurycarpa* at different locations are demonstrated in (Table 4.7 A and B). Autumn fruit and bark collected from Ranya had the highest value of palmitic (7.37%), stearic acids (2.56%), oleic (25.89%), linoleic (10.18%), and

linolenic acids (0.49%) compared to other locations. Concerning the fatty acids content in the plant parts, autumn fruit fixed oil contained more palmitic (11.37%), stearic acids (3.40 %), oleic (44.86 %), linoleic (14.10%), and linolenic acids (0.58%) than bark fixed oil. Regarding the interaction between locations and plant parts, the highest values of palmitic acid (11.69%), stearic acid (4.20%), oleic acid (45.39%), linoleic acid (15.36%), and linolenic acid (0.77%) contents appeared in autumn fruit fixed oil collected from Ranya. Qaradagh autumn fruit fixed oil followed Ranya in the contents of palmitic acid (11.48%), stearic acid (3.60%), oleic acid (45.02%), linoleic acid (14.22%), and linolenic acid (0.61%), while the lowest values of palmitic acid (2.55%), stearic acid (0.66%), oleic acid (5.89%), linoleic acid (3.69%), and linolenic acid (0.14%) contents were found in bark fixed oil collected from Hawar. GC analysis of autumn fruit fixed oil showed the total concentrations of unsaturated fatty acids (oleic, linoleic, and linolenic acids) were approximately four times higher than that of total saturated fatty acids (palmitic and stearic acids) at each four locations. These results were similar to those obtained by (Saber-Tehrani *et al.*, 2013; Dorehgirae and Pourabdollah, 2015).

Table 4.7.A Concentration of saturated fatty acids in *P. eurycarpa* autumn fruits and bark oil at different locations.

Fatty acids	Palmitic acid (%)		Locations mean	Stearic acid (%)		Locations mean
Plant parts / Locations	Autumn fruits	Bark		Autumn fruits	Bark	
Qaradagh	11.48 a	2.97 b	7.23 a	3.60 b	0.85 ef	2.23 b
Ranya	11.69 a	3.05 b	7.37 a	4.20 a	0.91 e	2.56 a
Sharbazher	11.28 a	2.68 b	6.98 a	3.10 c	0.74 ef	1.92 c
Hawar	11.02 a	2.55 b	6.79 a	2.70 d	0.66 f	1.68 d
Plant parts mean	11.37 a	2.81 b		3.40 a	0.79 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.05$.

Table 4.7.B Concentration of unsaturated fatty acids in *P. eurycarpa* autumn fruits and bark oil at different locations.

Fatty acids	Oleic acid (%)		Locations mean	Linoleic acid (%)		Locations mean	Linolenic acid (%)		Locations mean
Plant parts / Locations	Autumn fruits	Bark		Autumn fruits	Bark		Autumn fruits	Bark	
Qaradagh	45.02 ab	6.22 c	25.62 ab	14.22 b	4.88 e	9.55 b	0.61 b	0.29 c	0.45 a
Ranya	45.39 a	6.39 c	25.89 a	15.36 a	5.00 e	10.18 a	0.77 a	0.20 d	0.49 a
Sharbazher	44.79 ab	6.00 c	25.40 ab	13.69 c	3.98 f	8.84 c	0.58 b	0.36 c	0.47 a
Hawar	44.25 b	5.89 c	25.07 b	13.11 d	3.69 f	8.40 d	0.36 c	0.14 d	0.25 b
Plant parts mean	44.86 a	6.13 b		14.10 a	4.39 b		0.58 a	0.25 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.05$.

4.2.4 Total phenolic content of *Pistacia eurycarpa* parts

Table (4.8) shows the values of total phenolic contents in all parts of *P. eurycarpa.* The plant parts collected from Qaradagh gave the highest amount of total phenolic contents (208.098 mg/g) with significant differences compared to other locations. Concerning the total phenolic contents in plant parts, spring (304.953 mg/g) and autumn leaves (300.301 mg/g) are richer in total phenolic content than other parts with significant differences compared to other parts. On the other hand, the gum contained a low amount of total phenol, with no significant differences observed between gum collected from different locations. Regarding the interaction between locations and plant parts, there were significant differences between the maximum concentration in the Qaradagh spring leaves (307.057 mg/g) and the other plant parts at different locations. The minimum concentration (1.409 mg/g) was observed in the gum collected from Sharbazher. Hashemi *et al.* (2017) also found high levels of phenolic compounds at various locations in the leaves. Furthermore, the spring leaves in Qaradagh provided the highest value for the phenolic compound. Accumulation of secondary metabolites is strongly dependent on environmental factors such as light, temperature and soil fertility (Yang *et al.*, 2018). Low temperature promotes the synthesis of phenolic compounds, whereas high temperature breaks the chemical bonds that exist between phenolic molecules because these bonds are temperature sensitive (Jan *et al.*, 2021). When compared to other locations, Qaradagh leaves harvested in the spring contained the highest percentage of nitrogen (Table 4.2.A). The high nitrogen content increases phenylalanine synthesis as a precursor of phenolic compounds, resulting in a high phenol rate (Ibrahim *et al.*, 2011). As a result, a positive relationship exists between the highest percentage of nitrogen and phenolic compounds. Moreover, Leskovar and Othman (2018) concluded that increasing organic matter in the soil increases plant biomass, which contributes to increased phenolic and antioxidant compound production.

Table 4.8 Concentration of total phenolic content (mg/g) GAE in *P. eurycarpa* parts at different locations.

Plant parts / Locations	Spring leaves	Autumn leaves	Spring fruits	Autumn fruits	Spring rachis	Autumn rachis	Bark	Gum	Locations mean
Qaradagh	307.057 a	299.673 e	297.457 f	99.999 u	243.995 n	294.504 h	120.675 t	1.426 x	208.098 a
Ranya	304.546 c	295.981 g	287.267 j	100.295 u	185.658 q	261.865 l	134.706 r	1.415 x	196.467 d
Sharbazher	305.876 b	303.956 c	295.981 g	93.796 w	240.302 o	274.271 k	128.651 s	1.409 x	205.530 b
Hawar	302.331 d	301.593 d	209.436 p	97.784 v	250.493 m	293.322 i	135.444 r	1.442 x	198.981 c
Plant parts effect	304.953 a	300.301 a	272.535 c	97.969 f	230.112 d	280.991 b	129.869 e	1.423 g	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.5 Total flavonoid content of *Pistacia eurycarpa* parts

Table (4.9) shows the concentration of total flavonoid content in all parts of *P. eurycarpa.* The plant parts collected from Qaradagh had the highest amount of total flavonoid (43.190 mg/g) with significant differences compared to other locations. On the other hand, spring leaves contain higher amounts of total flavonoid (90.096 mg/g) than other parts with significant differences compared to other plant parts. Concerning the interaction between locations and plant parts, the highest concentration value (101.483 mg/g) was found in the spring leaves of Ranya followed by Sharbazher (93.953 mg/g), Qaradagh (82.970 mg/g), and Hawar (81.979 mg/g). The lowest concentration value (0.399 mg/g) was recorded in the bark collected from Hawar. Lowering temperatures, according to Wang *et al.* (2015), stimulate the enzymatic activity of some key enzymes involved in flavonoid biosynthesis, such as phenylalanine ammonia-lyase, which presides over the first step of general phenylpropanoid biosynthesis. This could explain why Ranya spring leaves had the highest total flavonoid content. These results could be related to the amount of the organic matter, as previously Leskovar and Othman (2018) revealed that there is a positive relationship between organic matter and biomass which can increase the flavonoid production.

Table 4.9 Concentration of total flavonoids content (mg/g) in *P. eurycarpa* parts at different locations.

Plant parts / Locations	Spring leaves	Autumn leaves	Spring fruits	Autumn fruits	Spring rachis	Autumn rachis	Bark	Gum	Locations mean
Qaradagh	82.970 c	75.624 e	60.528 j	2.715 p	51.713 k	70.739 h	0.687 q	0.548 q	43.190 a
Ranya	101.483 a	63.136 i	49.619 l	2.494 p	40.510 n	45.285 m	0.433 q	0.426 q	37.923 c
Sharbazher	93.953 b	73.053 g	63.063 i	2.118 p	50.978 k	45.138 m	0.441 q	0.478 q	41.153 b
Hawar	81.979 d	74.596 f	26.552 o	2.521 p	51.603 k	60.087 j	0.399 q	0.609 q	37.293 d
Plant parts mean	90.096 a	71.602 b	49.941 d	2.462 f	48.701 e	55.312 c	0.490 g	0.515 g	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.05$.

4.2.6 HPLC analysis for phenolic compounds

4.2.6.1 Quercetin

Fig. (4.4) shows the quercetin concentration of *P. eurycarpa* leaves, fruits and rachis in spring and autumn. The quercetin was not detected in the gum and bark. Spring and autumn leaves from all sites contain high amounts of quercetin. Spring leaves collected from Ranya had the highest level (168.9 µg/g) of quercetin followed by Qaradagh (160.7 µg/g) where did not differ significantly from autumn leaves collected from Hawar (158.9 µg/g). Significant differences

were observed between the maximum value of quercetin in Ranya spring leaves and the other plant parts at different locations. The lowest level (61.4 µg/g) was found in spring rachis collected from Sharbazher.

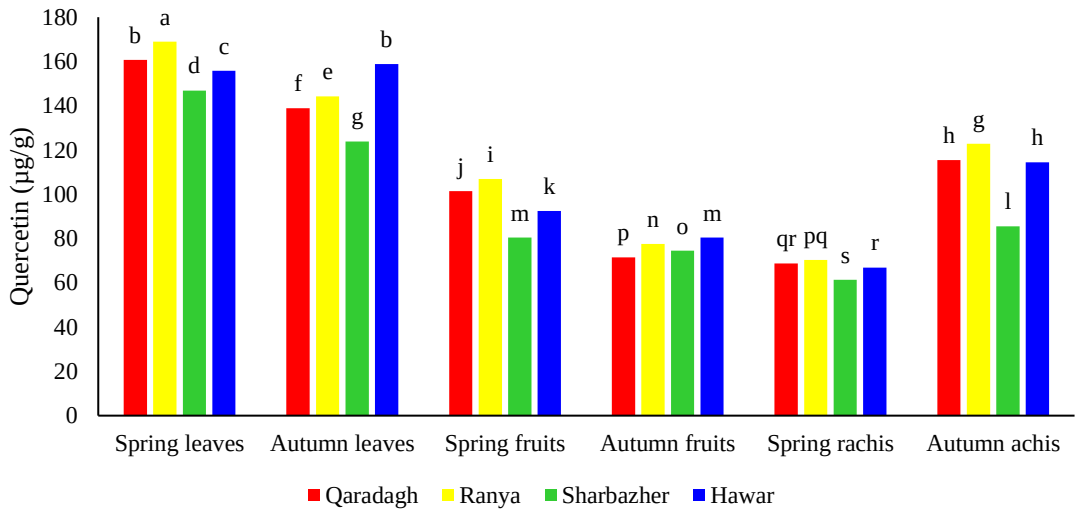


Figure 4.4 Concentration of quercetin (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.2 Rutin

Fig. (4.5) shows significant variations in rutin content among the plant parts of *P. eurycarpa* at different locations. Rutin results showed that the highest value (149.7 µg/g) was found in spring leaves collected from Ranya followed by Qaradagh spring leaves (140.8 µg/g), Hawar autumn leaves (136.9 µg/g), and Hawar spring leaves (133.8 µg/g). The lowest value (11.4 µg/g) was obtained from the bark collected from Ranya.

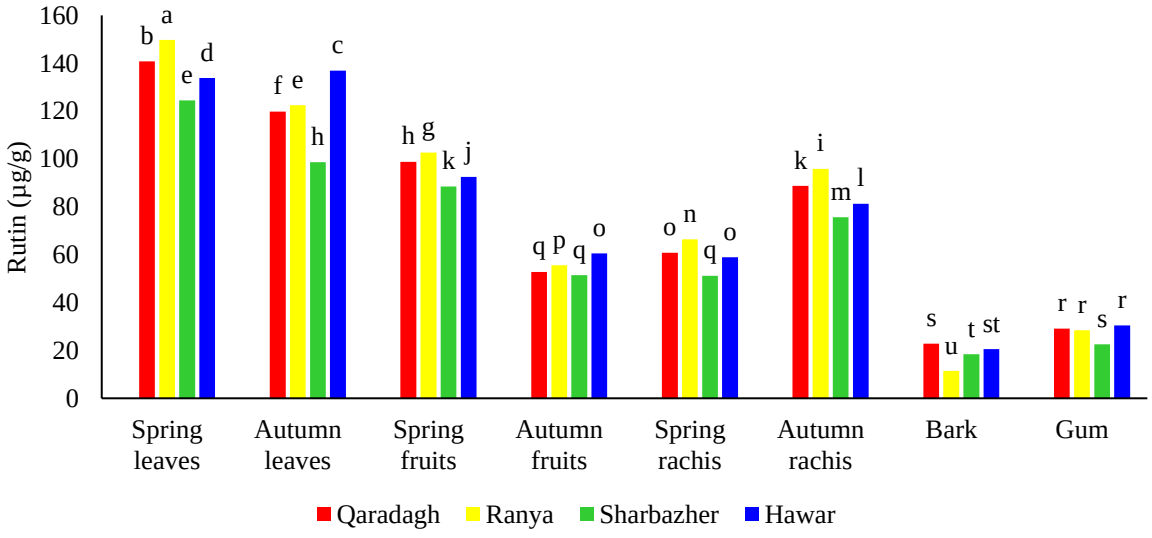


Figure 4.5 Concentration of rutin (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.3 Cinnamic acid

Fig. (4.6) shows cinnamic acid concentration in all parts of *P. eurycarpa* at various locations. The highest value (142.6 µg/g) of cinnamic acid content appeared in autumn leaves collected from Hawar followed by Ranya and Qaradagh (133.5 µg/g and 124.7 µg/g, respectively). Whereas, the lowest concentration was present in bark collected from Ranya and gum collected from Sharbazher (12.8 µg/g).

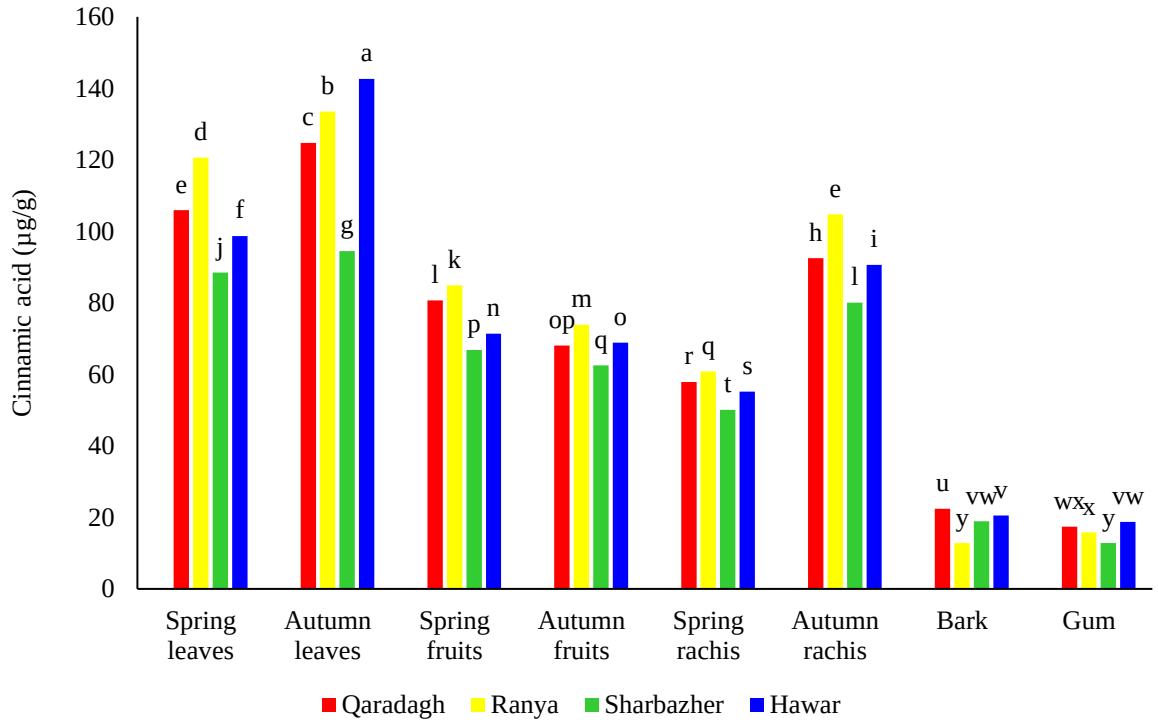


Figure 4.6 Concentration of cinnamic acid (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.4 Catechin

Fig. (4.7) reveals concentrations of catechin in plant parts of *P. eurycarpa* collected in spring from different locations. The catechin was absent in the autumn parts, gum, and bark of *P. eurycarpa* which collected at various locations. Significant differences were observed among spring leaves collected from all locations which have higher amounts of catechin in comparison with spring fruits and rachis. The highest value (124.5 µg/g) was recorded in spring leaves collected from Ranya followed by Qaradagh (120.9 µg/g), Hawar (115.8 µg/g), and Sharbazher (112.5 µg/g). The lowest value (66.2 µg/g) was recorded in spring rachis collected from Sharbazher.

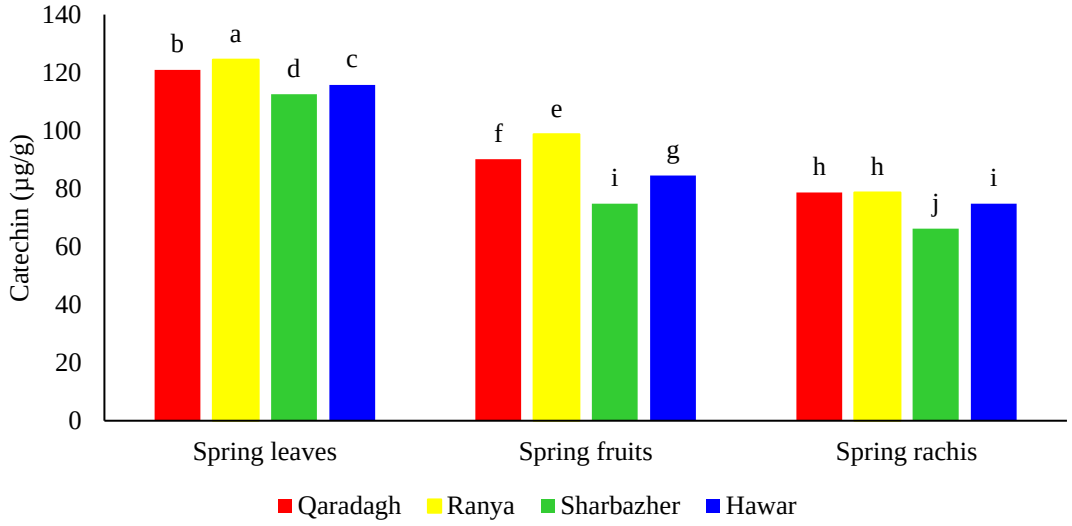


Figure 4.7 Concentration of catechin (µg/g) in *P. eurycarpa* spring parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.5 Ferulic acid

Fig. (4.8) illustrates statistical differences between the plant parts of *P. eurycarpa* at different locations. The ferulic acid was not detected in the autumn plant parts which collected from different locations. Significant differences were observed between the spring leaves that had been collected from different locations which contained higher amounts of ferulic acid in comparison with other plant parts. The highest value (122.4 µg/g) of ferulic acid content was recorded for Ranya spring leaves followed by Qaradagh (120.8 µg/g), Hawar (118.9 µg/g), and Sharbazher (112.8 µg/g). The lowest concentration (6.4 µg/g) was present in bark collected from Ranya.

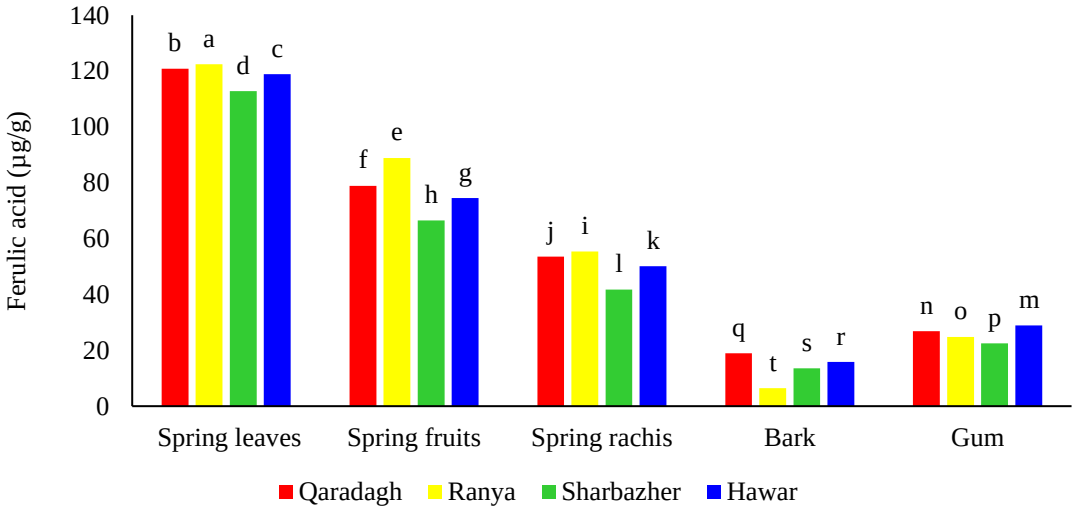


Figure 4.8 Concentration of ferulic acid (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.6 Ellagic acid

Fig. (4.9) shows the concentrations of ellagic acid in different plant parts at various locations except gum which was not detected at all locations. The results exhibit that Ranya spring leaves were significantly superior in ellagic acid content (97.4 µg/g) to other plant parts, followed by Qaradagh and Hawar spring leaves (91.4 µg/g and 88.9 µg/g, respectively), and the lowest value was found in the bark collected from Ranya (18.9 µg/g).

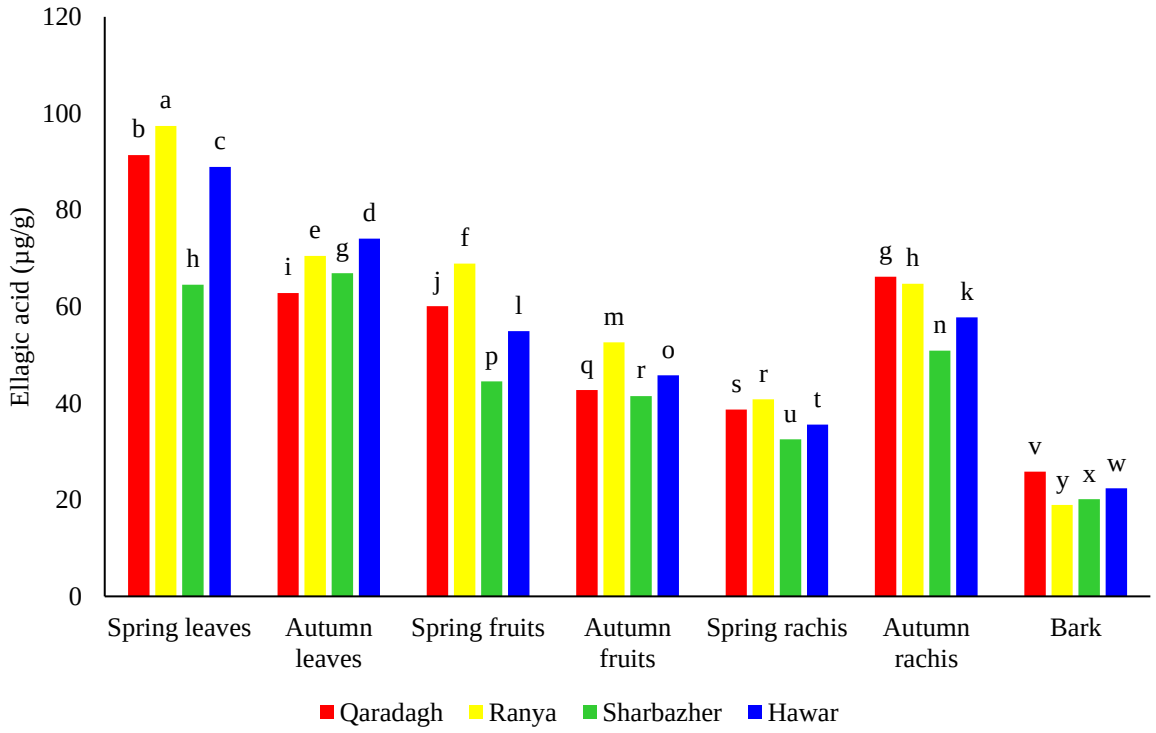


Figure 4.9 Concentration of ellagic acid (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.7 Tannic acid

Fig. (4.10) represents concentrations of tannic acid in autumn parts, bark, and gum. Whereas tannic acid is not detected in the spring parts at all locations. The highest value (97.4 µg/g) of tannic acid appeared in autumn leaves collected from Hawar followed by Ranya autumn leaves (88.5 µg/g) and Ranya autumn rachis (82.4 µg/g) which did not differ significantly from autumn leaves collected from Qaradagh (81.3 µg/g). While, the lowest value was in bark (14.7 µg/g) collected from Ranya.

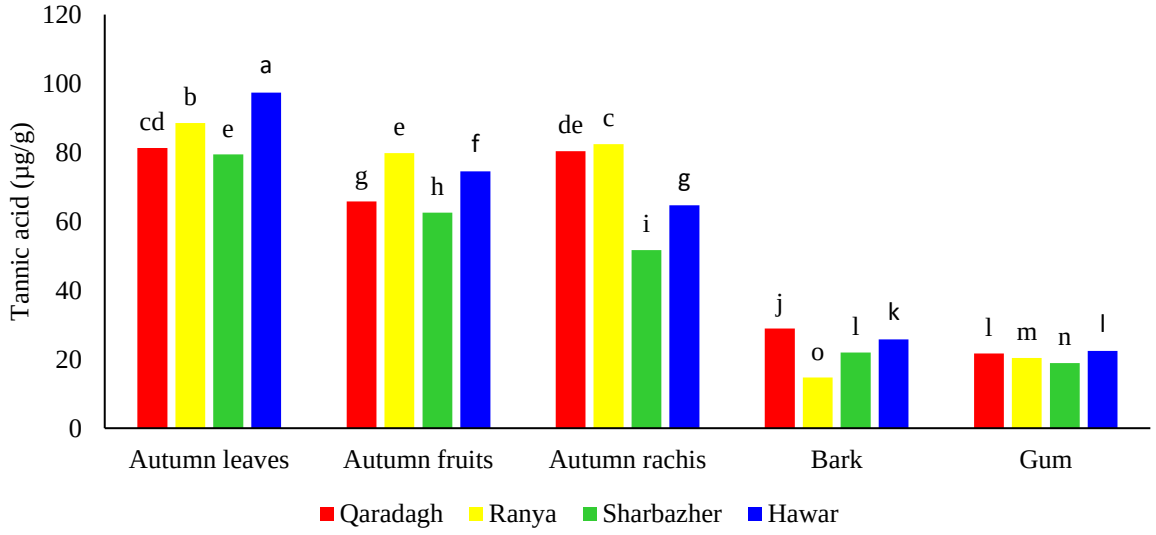


Figure 4.10 Concentration of tannic acid (µg/g) in *P. eurycarpa* parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.8 Kaempferol

Fig. (4.11) illustrates significant differences in the kaempferol concentrations in the autumn parts and gum of *P. eurycarpa* that collected from different locations, while kaempferol was not detected in the spring parts and bark. The highest value (98.7 µg/g) was found in autumn leaves from Hawar, followed by Ranya and Sharbazher (91.4 µg/g and 88.9 µg/g, respectively), and the lowest value was found in Sharbazher gum (25.9 µg/g).

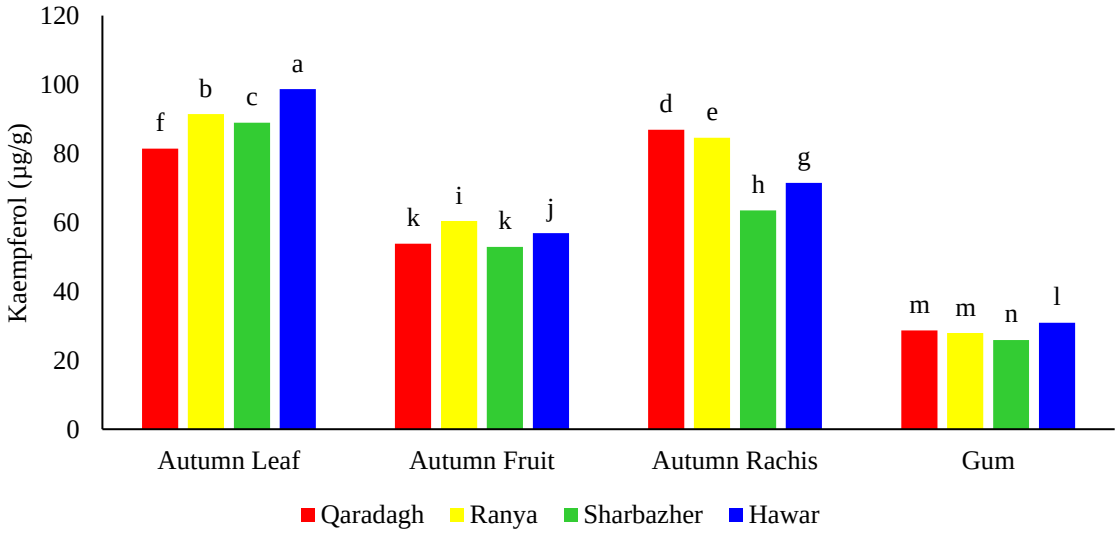


Figure 4.11 Concentration of kaempferol (µg/g) in *P. eurycarpa* autumn parts and gum at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.9 Stilbene

Fig. (4.12) shows the concentrations of stilbene in leaves, fruits and rachis collected from *P. eurycarpa* in the autumn season. The stilbene was absent in the spring parts, gum, and bark of *P. eurycarpa*, which collected at various locations. The maximum level (55.2 µg/g) of stilbene appeared in the autumn leaves collected from Hawar followed by Ranya (50.9 µg/g), Qaradagh (43.9 µg/g), and Sharbazher (42.6 µg/g), which did not differ significantly from autumn rachis collected from Ranya (41.9 µg/g). The lowest concentration was found in autumn fruits at Sharbazher (24.8 µg/g).

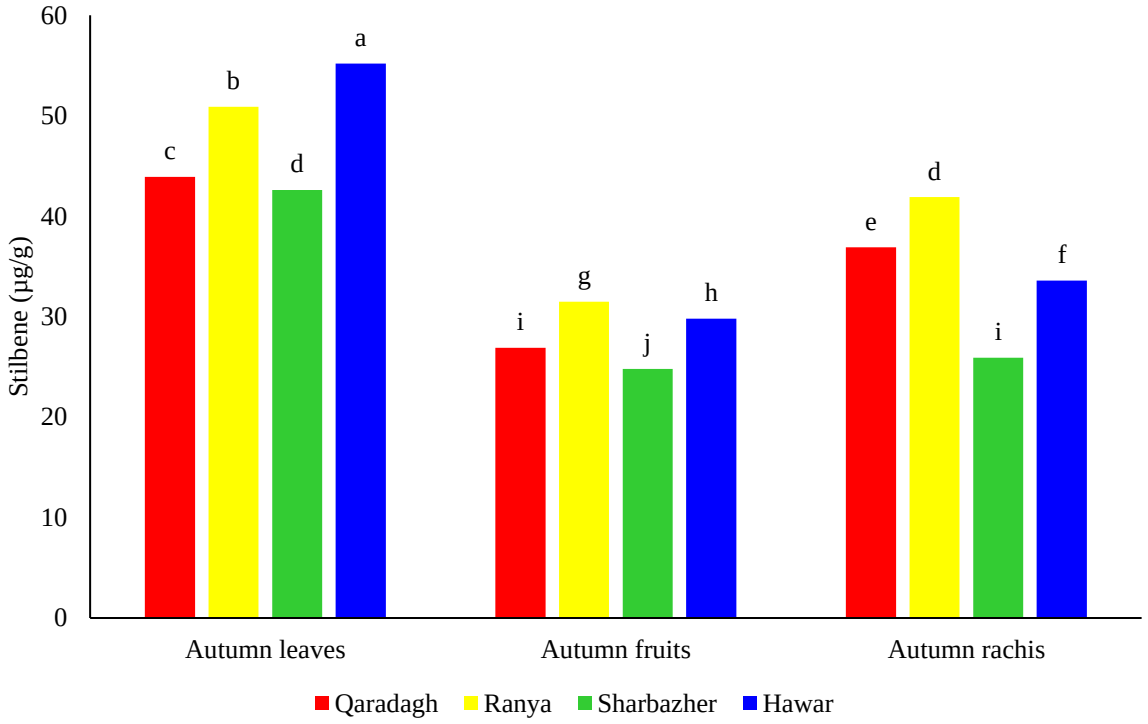


Figure 4.12 Concentration of stilbene (µg/g) in *P. eurycarpa* autumn parts at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.10 Gallic acid

Gallic acid results indicate that there were significant differences in the gallic acid content of the gum collected from different locations (Fig. 4.13). The gallic acid was absent in the other parts of *P. eurycarpa*, which collected at various locations. The highest concentration of gallic acid was found in gum collected from Hawar (10.5 µg/g) followed by Qaradagh (9.7 µg/g), and Ranya (9.2 µg/g). The lowest concentration was found in the gum collected from Sharbazher (8.5 µg/g).

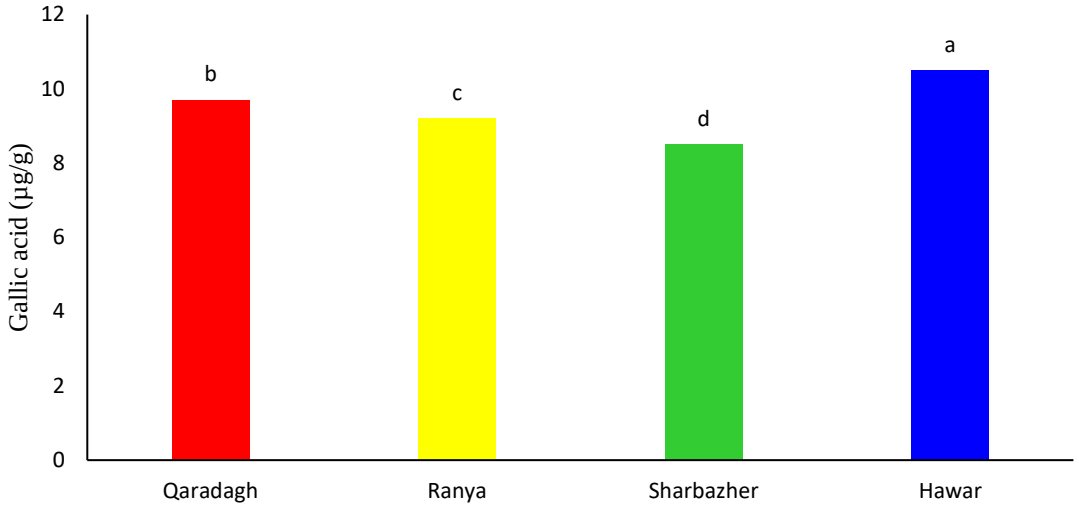


Figure 4.13 Concentration of gallic acid (µg/g) in *P. eurycarpa* gum at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.2.6.11 Apigenin

Apigenin results indicate that there were significant differences in the apigenin content of the bark collected from different locations (Fig. 4.14). The apigenin was not detected in the other parts of *P. eurycarpa* that collected from different locations. The highest value (10.2 µg/g) was found in bark from Qaradagh, followed by Hawar and Sharbazher (8.9 µg/g and 7.8 µg/g, respectively), and the lowest value was found in Ranya bark (6.2 µg/g). HPLC analysis of this study identified eleven phenolic compounds from various plant parts, including quercetin, rutin, cinnamic acid, catechin, ferulic acid, ellagic acid, tannic acid, kaempferol, stilbene, gallic acid, and apigenin. These findings agree with those of Hatamnia *et al.* (2014) and Karim (2014).

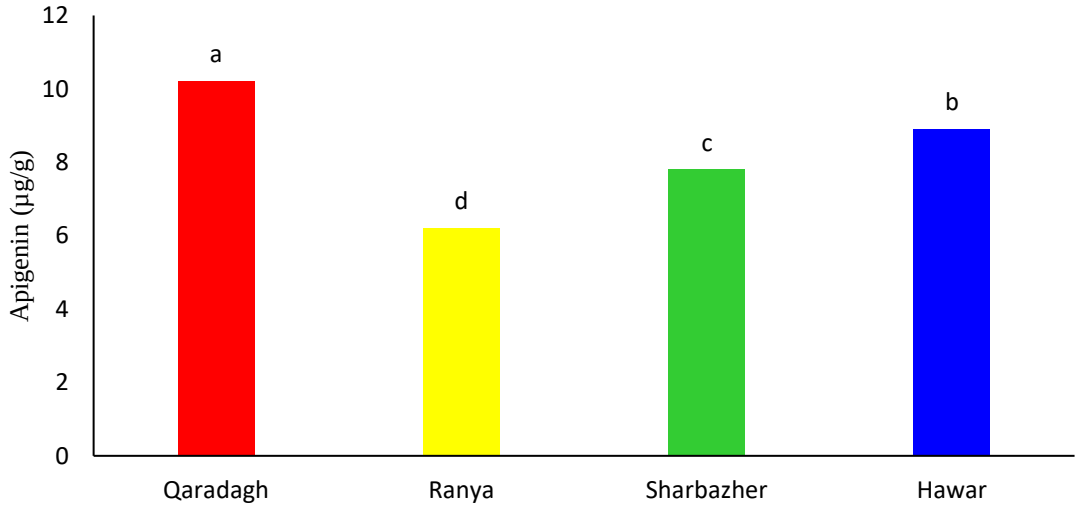


Figure 4.14 Concentration of apigenin (µg/g) in *P. eurycarpa* bark at different locations. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.05.

4.3 Biological Activity of *Pistacia eurycarpa* Essential Oil

4.3.1 Antibacterial activity of essential oil

4.3.1.1 Agar well diffusion assay

The antibacterial activity of essential oil in gum and autumn rachis of *P. eurycarpa* collected from different locations against both *Escherichia coli* (Gram-negative) and *Staphylococcus haemolyticus* (Gram-positive) were determined by the presence of inhibition zones. The antibacterial activity of essential oil in gum at all locations higher than autumn rachis. The highest antibacterial activity observed against *E. coli* and *S. haemolyticus* were 25 mm and 35 mm, respectively, using essential oil gum collected from Hawar (Table 4.10). While, the lowest diameters inhibition zone values were shown against *E. coli* (8 mm) and *S. haemolyticus* (11 mm) when using autumn rachis essential oil collected from Qaradagh.

Table 4.10 Bacterial inhibition zone assay of *E. coli* and *S. haemolyticus* by PEEOs.

Pathogenic bacteria	*E. coli*		Locations mean	*S. haemolyticus*		Locations mean
Plant parts / Locations	Gum Inhibition (mm)	Autumn rachis Inhibition (mm)		Gum Inhibition (mm)	Autumn rachis Inhibition (mm)	
Qaradagh	15 c	8 d	11.5 c	23 c	11 e	17 b
Ranya	21 b	10 d	15.5 b	31 b	15 d	23 a
Sharbazher	14 c	9 d	11.5 c	21 c	13 de	17 b
Hawar	25 a	10 d	17.5 a	35 a	14 d	24.5 a
Plant parts mean	18.75 a	9.25 b		27.5 a	13.25 b	

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.01$.

Gum essential oil from Ranya and Hawar inhibited the growth of other local isolates, which are considered a serious matter of concern. The results show that the Gram-positive bacteria (*Enterococcus faecalis*) seemed to be more susceptible to the essential oil than Gram-negative bacteria (*Klebsiella pneumoniae*, *Proteus mirabilis*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*). The highest inhibition zone observed against *E. faecalis* (35 mm), *K. pneumoniae* (25 mm), *P. mirabilis* (23 mm), and *A. baumannii* (21 mm) using essential oil gum collected from Hawar (Table 4.11). Whereas in the case of *P. aeruginosa* the zone of inhibition for both essential oils were 10 mm. The significant differences were observed between gum essential oil from Ranya and Hawar. Geographical origin, climatic conditions, and harvest time could be possible reasons for the fluctuations in the chemical composition of essential oil percentages. It is important to note that the variation in the chemical composition of essential oils is responsible for their medicinal effects or biological activities, which vary

from one area to another (Dhifi *et al.*, 2016). *P. eurycarpa* essential oils (PEEOs) inhibited the growth of local clinical isolates (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *S. haemolyticus*, and *E. faecalis*), and hospital environmental isolate (*A. baumannii*), which are considered a serious matter of concern. Previous in vitro data showed that the essential oils of gum halted the growth of Gram-negative and positive bacteria (Ellahi *et al.*, 2019). In addition, the oil showed better activity against Gram-positive bacteria than against Gram-negative bacteria. It could be attributed to the structure of the bacterial cell wall that Gram-positive bacteria have a thick layer of peptidoglycan linked to other hydrophobic molecules such as proteins and teichoic acid. This hydrophobic layer surrounding the Gram-positive bacterial cell may facilitate easy entry of hydrophobic molecules (Angane *et al.*, 2022). On the other hand, Gram-negative bacteria have an outer membrane with the presence of lipopolysaccharide molecules that provide a hydrophilic surface (Shakeri *et al.*, 2014). The surface acts as a penetration barrier that prevents macromolecules and hydrophobic compounds from entering the target cell membrane (Kong *et al.*, 2008). Accordingly, Gram-negative bacteria are relatively resistant to hydrophobic molecules in PEEOs. For further study Hawar gum essential oil will be selected as it has the highest antimicrobial activity against Gram-positive and Gram-negative bacteria compared with other oils.

Table 4.11 Bacterial inhibition zone assay of five bacteria by PEEOs.

Bacteria / Locations	*E. faecalis* Inhibition (mm)	*K. pneumoniae* Inhibition (mm)	*P. mirabilis* Inhibition (mm)	*A. baumannii* Inhibition (mm)	*P. aeruginosa* Inhibition (mm)
Ranya	29 b	18 b	17 b	15 b	10 a
Hawar	35 a	25 a	23 a	21 a	10 a

* Different letters indicate significant differences among them according to Duncan's Multiple Range Test at $p \leq 0.01$.

4.3.1.2 Molecular detection of multidrug resistant *Acinetobacter baumannii*

Identification of *A. baumannii* was done using 16S rRNA gene and then confirmed by targeting the $bla_{\text{Oxa-51}}$ gene. Moreover, *A. baumannii* were characterized by PCR technique for the detection of $bla_{\text{Oxa-23}}$ and bla_{TEM} genes as shown in (Fig. 4.15). *A. baumannii* was identified to species level using Vitek 2 system, 16S rRNA, and $bla_{\text{OXA-51}}$ gene. The 16S rRNA gene is the most conserved among the rRNA genes, the sequencing of this gene considered as an important tool for the identification and taxonomic classification of bacterial species (Rossi-Tamisier *et al.*, 2015). These results were in agreement with other studies that reported the existence of blaoxa-51-like gene in all clinical *A. baumannii* isolates but not found in other *Acinetobacter* spp. (Abdullah and Merza, 2019; Anane *et al.*, 2020). Furthermore, *A. baumannii* was characterized by PCR technique for the detection of $bla_{\text{Oxa-23}}$ and bla_{TEM} genes, which are

known genes coding for β-lactam antibiotic resistance. The major source of carbapenem resistance in *A. baumannii* is *bla*$_{\text{Oxa-23}}$ (Smith *et al.*, 2013). In addition, *bla*$_{\text{TEM}}$ is another antibiotic-resistant gene in *A. baumannii* that is resistant to broad-spectrum cephalosporins (Al-Khafaji and Aljelehawy, 2021). The current findings were consistent with previous studies (Mohamed *et al.*, 2022; Shali *et al.*, 2022) which also recorded a16S rRNA and *bla*$_{\text{Oxa-51}}$ as a target for identification of *A. baumannii* and *bla*$_{\text{Oxa-23}}$ and *bla*$_{\text{TEM}}$ gene was also successfully amplified in *A. baumannii.*

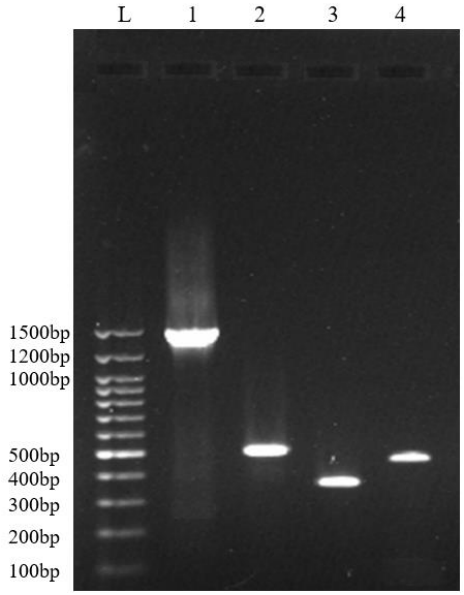


Figure 4.15 Gel electrophoresis of PCR products of *A. baumannii* (L) Ladder. (1) 16S rRNA. (2) *bla*$_{\text{Oxa-23}}$. (3) *bla*$_{\text{Oxa-51}}$. (4) *bla*$_{\text{TEM}}$.

4.3.1.3 Antigrowth activity of *Pistacia eurycarpa* essential oil from Hawar gum against *Acinetobacter baumannii*

The antibacterial activity of various concentrations of *P. eurycarpa* essential oil from Hawar (PEEO-H) (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10, 20, 40 µl/ml) against *A. baumannii* was determined by the presence of inhibition zones. The Diameters Inhibition Zone values (DIZ) for *A. baumannii* increased significantly along with the increasing of PEEO-H concentration. The highest antimicrobial activity observed against *A. baumannii* was 19 mm at a concentration of 40 µl/ml (Fig. 4.16). The DIZ values for *A. baumannii* were shown with range from 10.5 mm to 19 mm.

The action of PEEO-H on multidrug-resistant *A. baumannii* was carried out by comparing growth rate under control and test conditions (Fig. 4.17). Growth of the bacterium was monitored by microplate spectrophotometer and compared to the growth of the bacterium without PEEO-H. The results illustrated that PEEO-H was significantly effective and inhibited

growth of *A. baumannii* completely at concentration 2.5 µl/ml. The minimum inhibitory concentration (MIC) of PEEO-H against *A. baumannii* was equivalent to the minimum bactericidal concentration (MBC) value which was 2.5 µl/ml. The results indicated that PEEO-H have statically highly effective and inhibited growth. To our knowledge, this study is the first reported investigation of the antibacterial activity of PEEO-H against MDR Gram-negative *A. baumannii*. The findings here support the hypothesis that PEEO can inhibit MDR strains with equal or greater efficacy as compared to other drugs. Furthermore, we observed successful antigrowth of PEEO-H by evaluating it against *A. baumannii*. The MIC of PEEO-H against *A. baumannii* was equivalent to the MBC value, indicating a bactericidal behavior. Hawar-grown *P. eurycarpa* gum contains 81.4% alpha-pinene as per GC-MS analysis of its essential oil. As claimed by Memariani *et al.* (2017), bactericidal activity originates from high concentrations of Alpha-Pinene that disrupt the integrity of the bacterial cell membrane. Comparable to our findings, some EOs from plants that contain major components of terpenoids exhibit inhibitory effects against various bacteria (Aljaafari *et al.*, 2021).

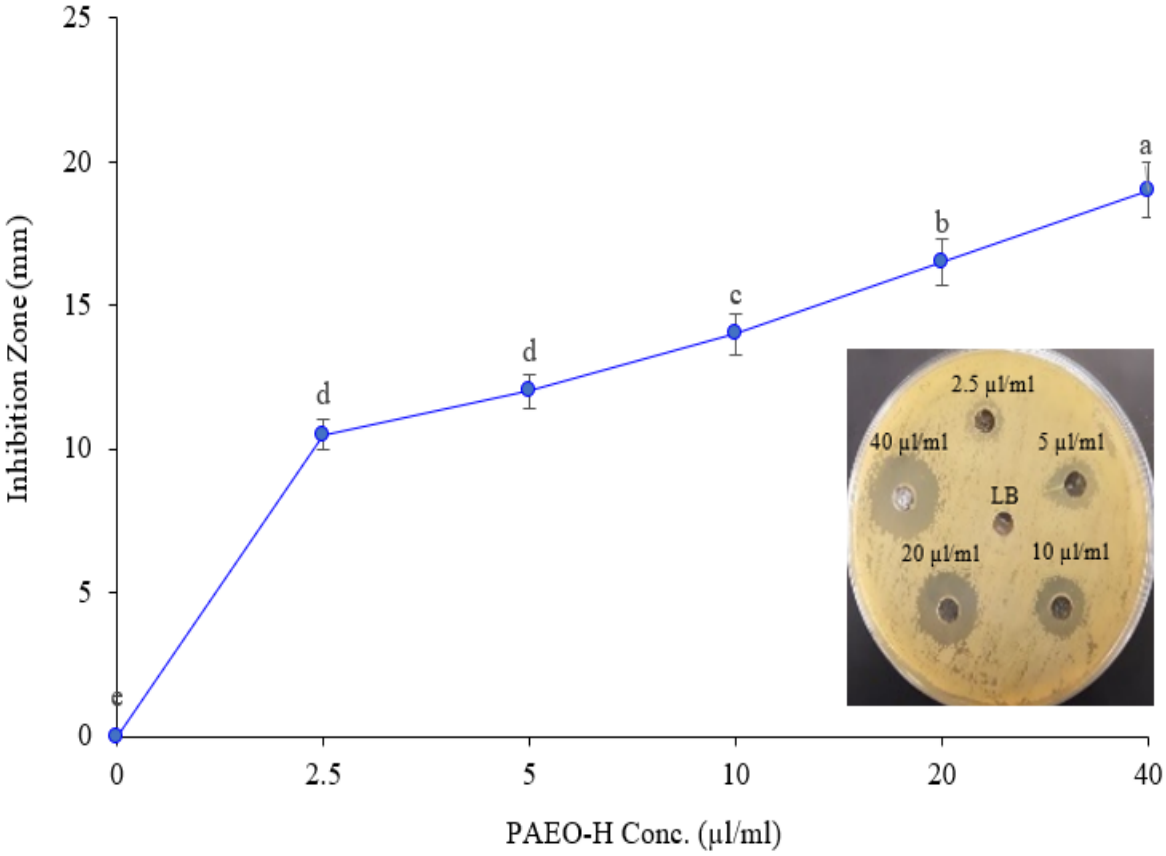


Figure 4.16 Bacterial inhibition zone assay of *A. baumannii* by PEEO-H. Inset: Muller-Hinton agar plate with 100 µl PEEO-H for each well at different concentration. Different letters indicate significant differences among them according to Duncan's Multiple Range Test at p≤ 0.01.

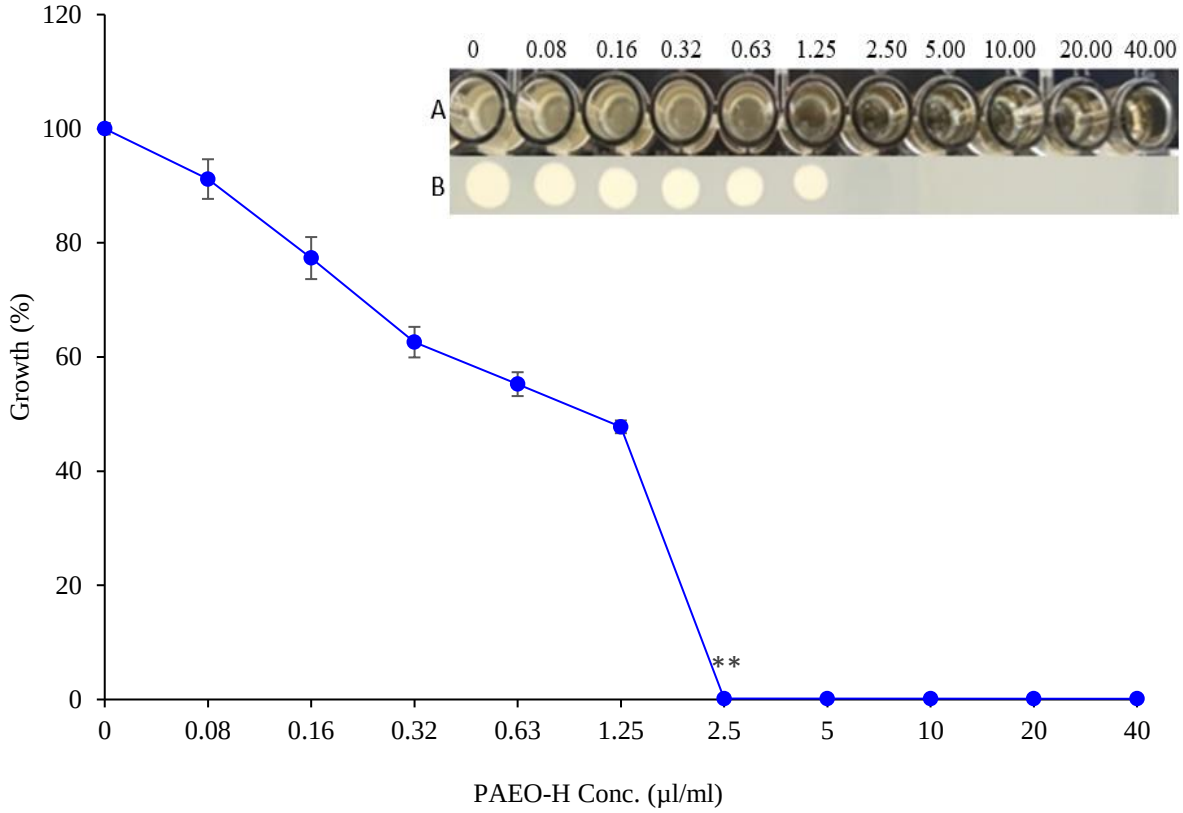


Figure 4.17 Effect of different concentrations of PEEO-H on the growth of *A. baumannii* using microtiter plate technique. Inset: (A) Growth column with addition of different concentration of essential oil. (B) Five µl was taken from each well and spotted onto LB agar plates. ** Highly significant $p \leq 0.01$ t stat.: 172.876 $t_{0.01}$ (4): 4.604.

4.3.1.4 Antibiofilm activity of *Pistacia eurycarpa* essential oil from Hawar gum against *Acinetobacter baumannii*

The inhibitory effect of PEEO-H at various concentrations (0.08, 0.16, 0.32, 0.63, 1.25, 2.5, 5, 10, 20, 40 µl/ml) on *A. baumannii* biofilm formation was examined. As shown in (Fig. 4.18), the bacterial biofilm production decreased as PEEO-H concentrations increased, and significant inhibition was observed when bacterial cells were exposed to 2.5 µl/ml of PEEO-H. *A. baumannii* is one of the most problematic MDR species discovered in hospitals, which is particularly resistant to a wide variety of antibiotic classes (Lukovic *et al*., 2020). The severity of the issue concerning *A. baumannii* is noteworthy, particularly in consideration of its notable inclination towards biofilm formation (Upmanyu *et al*., 2022). Thus, the development of novel antibacterial strategies has become imperative. It has been reported that EO can reduce bacterial adhesion and inhibit biofilms since it possesses promising functional compounds that could be used as an alternative antibacterial strategy in the face of emerging bacterial infections resistant to conventional antibiotics (Swamy *et al.*, 2016). Moreover, the antibiofilm activity of other plant's EO was also examined against MDR *A. baumannii* such as the essential oil of

Pennyroyal (Tutar *et al*., 2016) and Cinnamon (Ganić *et al*., 2022) that exhibited strong anti-biofilm and antimicrobial activities.

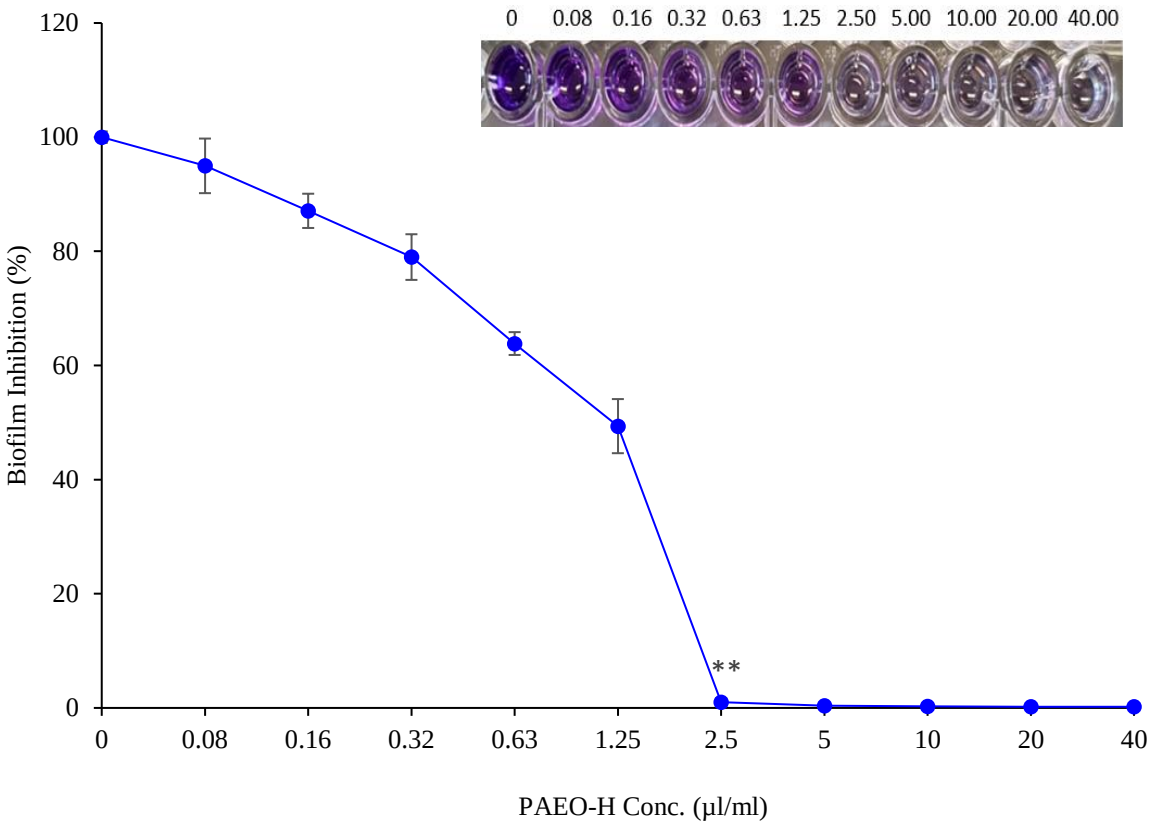


Figure 4.18 Effect of different concentrations of PEEO-H on the biofilm inhibition of *A. baumannii* using microtiter plate technique. Inset: Biofilm column with addition of different concentration of essential oil. ** Highly significant $p \leq 0.01$ t stat.: 155.885 $t_{0.01}(4)$: 4.604.

4.3.2 Mode of action of *Pistacia eurycarpa* essential oil from Hawar gum against *Acinetobacter baumannii*

4.3.2.1 Protein leakage assay

A. baumannii cells were treated with various concentrations (0.08, 0.16, 0.32, 0.63, 1.25, 2.5 µl/ml) of PEEO-H; then protein concentration was estimated in the supernatants. When *A. baumannii* was treated with 0.08 µl/ml PEEO-H, a sharp increase in protein leakage was observed as shown in (Fig. 4.19). *A. baumannii* treated with PEEO-H showed increased leakage of protein as the PEEO-H concentration increased, and protein concentration in the supernatants of treated cells at MIC concentration (2.5 µl/ml) was greatly elevated to 60.66 µg/ml in comparison with untreated cells (5.33 µg/ml). The results indicated that PEEO-H have statistically significant effect in protein leakage. In EOs, a large number of different groups of chemical compounds is present; their antibacterial activity targets multiple areas in the cell

(Ellahi *et al.*, 2019). The protein concentration excreted out of the cells treated with essential oil is considerably high, indicating critical damage to the cytoplasmic membranes. It has been reported that essential oil can increase the permeability of the bacterial membrane, resulting in the leakage of intracellular substances (Li *et al.*, 2019).

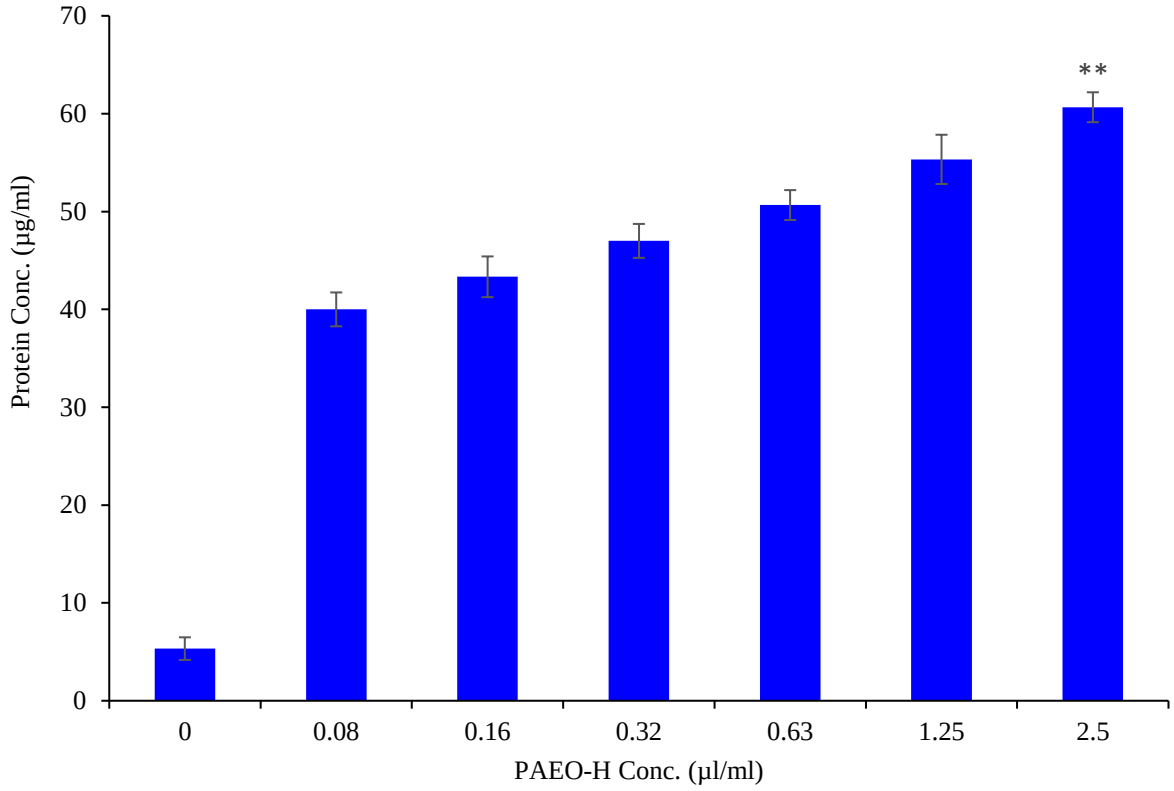


Figure 4.19 Effect of PEEO-H on protein leakage of *A. baumannii* cells. ** Highly significant $p \leq 0.01$ t stat.: 41.651 $t_{0.01}$ (4): 4.604.

4.3.2.2 Phosphate and potassium ions' leakage

Further, antibacterial mode of action of PEEO-H against *A. baumannii* was confirmed at concentrations (0.08, 0.16, 0.32, 0.63, 1.25, 2.5 µl/ml) using the assay for phosphate and potassium ions leakage from the treated cells of *A. baumannii*. In this assay, leakage of phosphate and potassium ions from the bacterial cells occurred immediately after the addition of PEEO-H. The phosphate and potassium ions values increased with increasing concentrations of PEEO-H as shown in (Fig. 4.20). The highest values of phosphate and potassium ion leakages were 61.37 and 190 µmol/l, respectively at MIC (2.5 µl/ml) concentration. No leakage of phosphate and potassium ions were observed in control cells of *A. baumannii*. The results indicated that PEEO-H have statistically significant effect in phosphate and potassium ions' leakage.

The antimicrobial mode of action of PEEO-H was also confirmed by the leakage of the phosphate and potassium ions from *A. baumannii* cells. Phosphates are most commonly found

in the form of adenosine phosphates like AMP, ADP, and ATP as well as in DNA and RNA; they can be released through the hydrolysis of ATP or ADP. As these chemical compounds coalesce and influence the structural integrity of the cell membrane, cell metabolism is hindered. The consequence of such interaction could be cell death (Sharma *et al.*, 2013). The results of our study showed a significant increase in the leakage of ions that is consistent with (Zhang *et al.*, 2017). The internal environment of cells is generally rich in potassium ions; their presence in the extracellular medium is an indication of cytoplasmic membrane damage (Carson *et al.*, 2002). The bacterial plasma membrane provides a permeability barrier to the passage of electrolytes necessary to facilitate cell membrane functions, retain proper enzyme activity, and maintain normal metabolism (Diao *et al.*, 2014). Previous studies indicate that EOs form channels across the membrane by pushing apart the fatty acid chains of the phospholipids and allowing ions to exit the cytoplasm (Bajpai *et al.*, 2013).

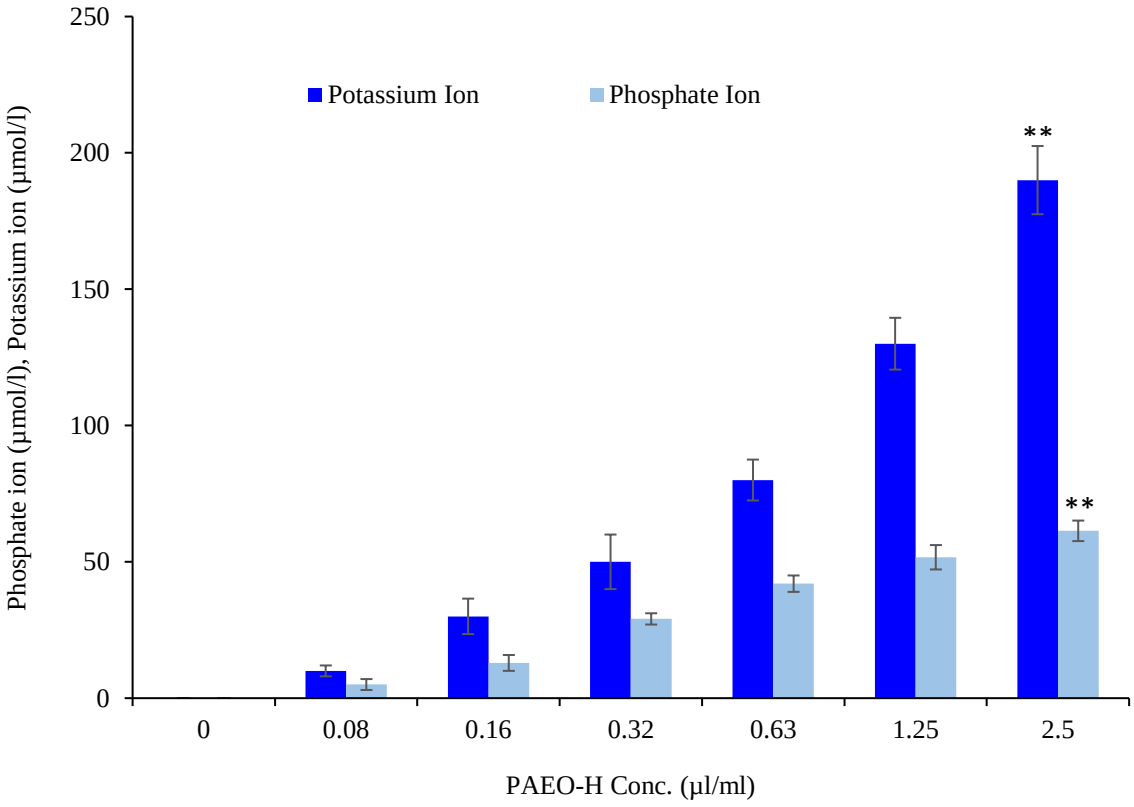


Figure 4.20 Effect of PEEO-H on phosphate and potassium ions leakages of *A. baumannii* cells. ** Highly significant $p \leq 0.01$ t stat. (potassium): 109.697 t stat. (phosphate): 25.926 $t_{0.01}$ (4): 4.604.

4.3.2.3 Transmission electron microscopy

To further examine the morphological changes of *A. baumannii* cells after treatment with PEEO-H, transmission electron microscopy (TEM) was used. The intact (control) cells of the bacteria were shown in (Fig. 4.21 A), in which the inner and outer membranes of the *A.*

baumannii envelope were smooth. The cytoplasmic content of bacterial cells was evenly distributed and cell aggregation was not detected. Significant morphological changes were observed in PEEO-H treated with *A. baumannii* cells compared to control cells (Fig. 4.21 B and C). A large number of cells appeared with damaged cell membranes and the cytoplasmic contents leaked into the extracellular medium. In addition, coagulated materials were observed in the cytoplasm. The changes were more evident with an increase in concentration of PEEO-H.

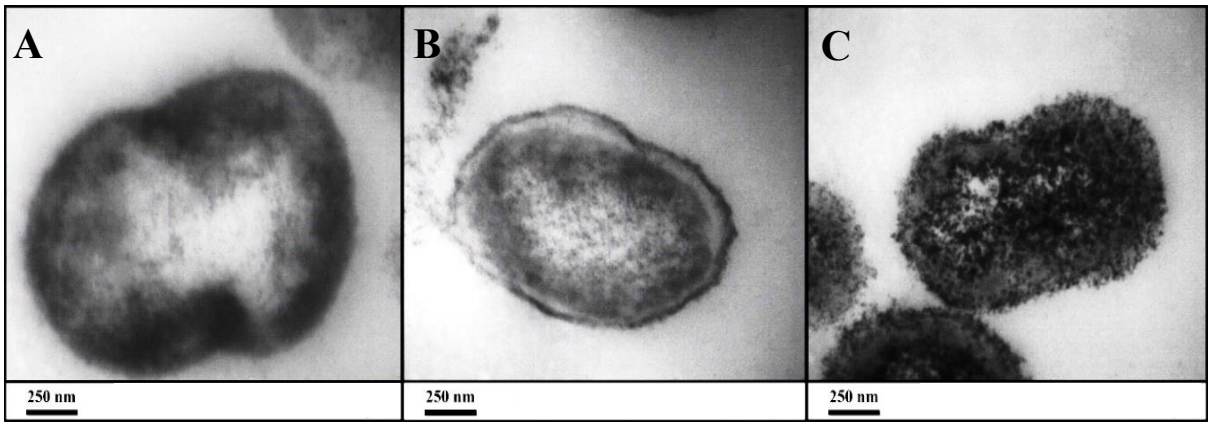


Figure 4.21 TEM photography of *A. baumannii* cultured in LB medium with different PEEO-H concentration treatments. (A) Untreated cells. (B) Cells were treated with PEEO-H at 1/4 MIC (0.63 µl/ml). (C) Cells were treated with PEEO-H at 1/2 MIC (1.25 µl/ml).

4.3.3 Applications of *Pistacia eurycarpa* essential oil from Hawar gum with Band-Aids

The anti-adhesion activity of PEEO-H was assessed against selected adherent *A. baumannii* bacterial isolate on the surfaces of Band-Aids (Fig. 4.22). Adhered bacterial cells (CFU/ml) were compared before and after treatment with PEEO-H, with significant reduced adhesion seen in the case of treated cells on the surface of Band-Aids at 15 µl. The results shown statistically significant reduced bacterial adhesion on the surface of Band-Aids.

For additional applications, Band-Aids were incorporated with PEEO-H to investigate the PEEO's ability in the reduction of bacterial adhesion. The ability of bacterial pathogens to attach to host tissue is usually one of the first steps in biofilm formation and contributes overall to the pathogenicity of the microorganism (Barawi *et al.*, 2021). Preventing initial bacterial attachment to host surfaces is an effective strategy to prevent biofilm formation and thereby bacterial infections (Song *et al.*, 2019). In our study, PEEO-H was investigated for its anti-adhesion activity on the surfaces of Band-Aids. Additionally, PEEO-H efficiently reduced bacterial adhesion on the surface of Band-Aids.

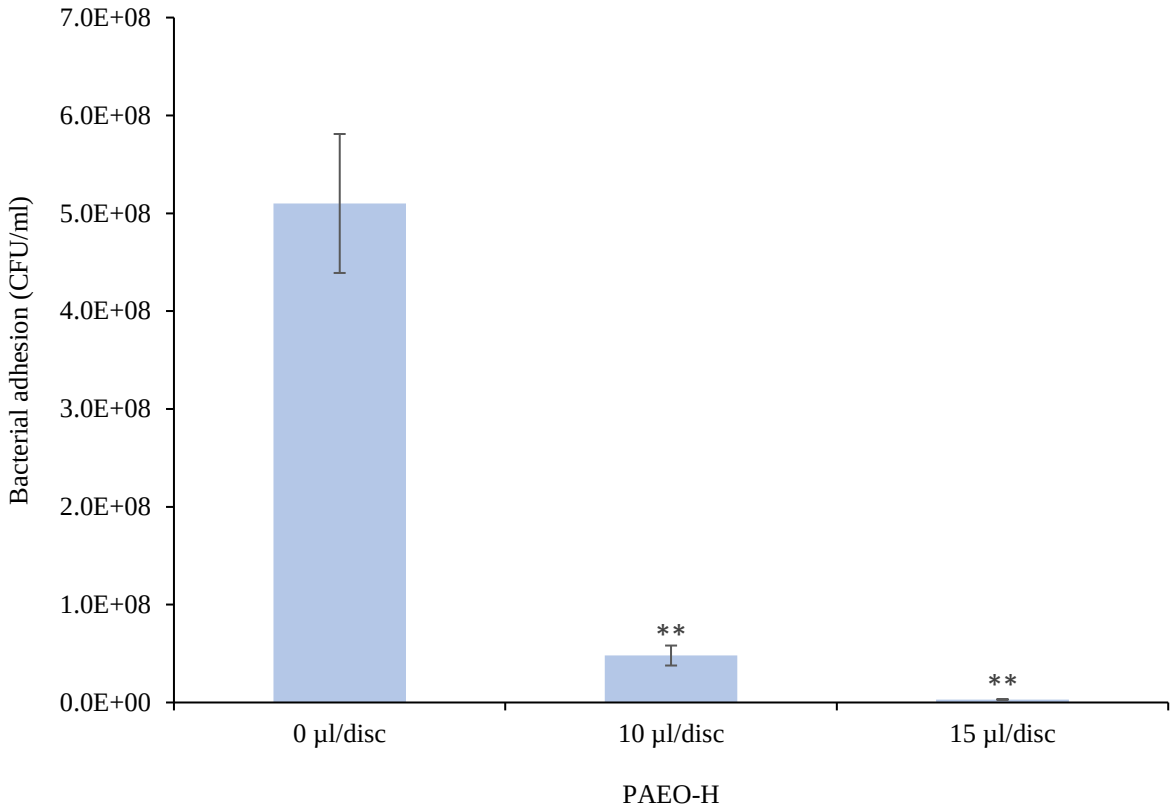


Figure 4.22 Anti-adhesion effect of PEEO-H on *A. baumannii* isolates on Band-Aids. ** Highly significant $p \leq 0.01$ t stat. (10 µl/disc): 79.624 t stat. (15 µl/disc): 87.793 $t_{0.01}(4)$: 4.604.

Conclusions

The most important conclusions of this study can be stated as follows:

- **Regarding the molecular identification of *Pistacia* species**

1. A novel primer set was used in molecular part of this study.
2. According to the molecular study, there are two wild species (*Pistacia atlantica* and *Pistacia khinjuk*) in Sulaymaniyah province.

- **Regarding the chemical constituent of plant parts**

3. The chemical profile of *P. eurycarpa* is affected by plant parts and location.
4. Gum collected from Sharbazher had higher essential oil.
5. Gum and autumn rachis EO contain Alpha-Pinene more than other components at all locations, the highest concentration of Alpha-Pinene was found in gum EO collected from Hawar.
6. Autumn fruit in Qaradagh had higher fixed oil.
7. Autumn fruit fixed oil contained approximately four times more unsaturated fatty acids than saturated fatty acids.
8. Spring leaves of *P. eurycarpa* were found to be high in phenolic and flavonoid compounds at all locations.
9. Rutin and cinnamic acid were shown to appear in all parts collected at all locations. While quercetin has the highest concentration among the phenolic compounds in various parts of plants except bark and gum at different locations.

- **Regarding the biological activity of essential oil**

1. In our study, the essential oils of *P. eurycarpa* showed better activity against Gram-positive bacteria than against Gram-negative bacteria.
2. The *P. eurycarpa* essential oils collected from Hawar (PEEO-H) possessed excellent antibacterial activity against *Acinetobacter baumannii*. The PEEO-H revealed its inhibitory effect as confirmed by severe morphological alterations in the cell wall and membrane of *A. baumannii*.
3. According to our knowledge, this study is the first reported investigation of the use of PEEO-H against multidrug-resistant *A. baumannii* in vitro.
4. PEEO-H shows promising antibiofilm and anti-adhesion agents.

Recommendations

Based on the conclusions mentioned previously, the following important recommendations can be noted:

1. Conducting further studies in Kurdistan region of Iraq to determine the best location containing plant parts with the highest concentrations of active compounds.
2. Qaradagh spring leaves of *P. eurycarpa* were also found to be high in phenolic compounds. Therefore, more studies are needed to investigate the biological activity of phenolic compounds isolated from *P. eurycarpa* Qaradagh spring leaves.
3. Study of antibacterial activity of PEEO against pandrug-resistant (PDR) and extensively drug-resistant (XDR) bacteria.
4. Future studies aimed to assess the mode of action of PEEO following the application of authenticated cancer cell lines, as well as producing ample and clinically feasible sources of PEEO for testing in larger rodents and small animal studies, are required to translate these findings to clinical use.

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APPENDICES

Appendix 1. CTAB buffer preparation.

CTAB Buffer

- 10 ml 1 M Tris HCl pH 8.0.
- 28 ml 5 M NaCl.
- 4 ml of 0.5 M EDTA.
- 2 g of CTAB (cetyltrimethylammonium bromide).
- Brought total volume to 100 ml with ddH_2O.

Tris HCl (1 M) pH 8.0

- 12.1 g Tris dissolved in about 70 ml of dH_2O.
- Brought pH down to 8.0 by added concentrated HCl.
- Brought total volume to 100 ml with dH_2O.

NaCl (5 M)

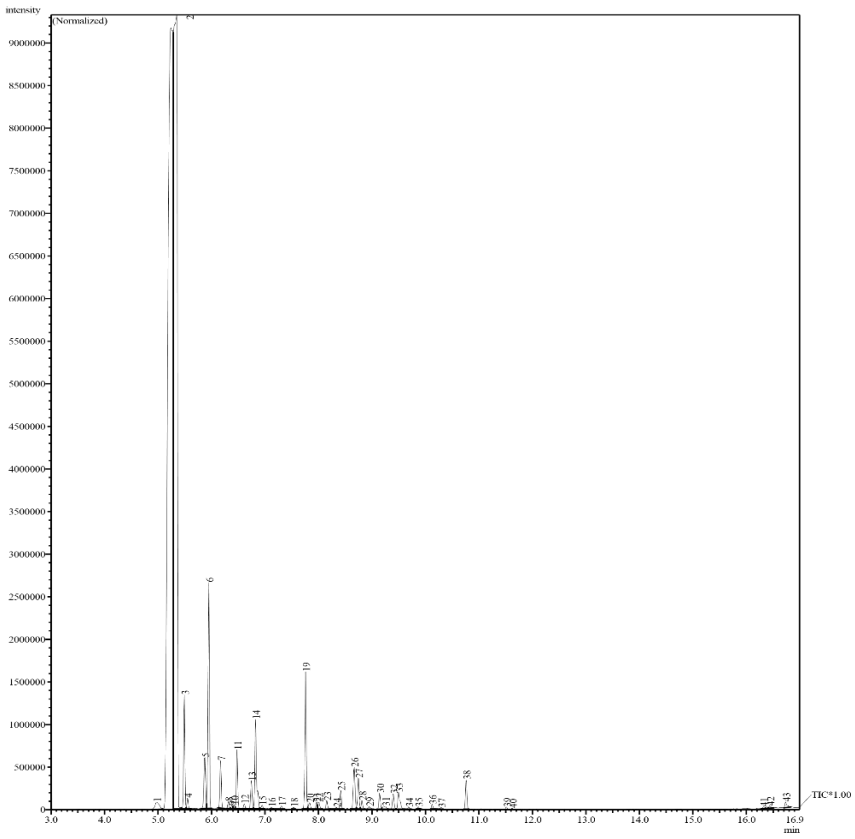
- 29.2 g of NaCl.
- 70 ml dH_2O.
- Dissolved and brought to 100 ml.

EDTA (0.5 M)

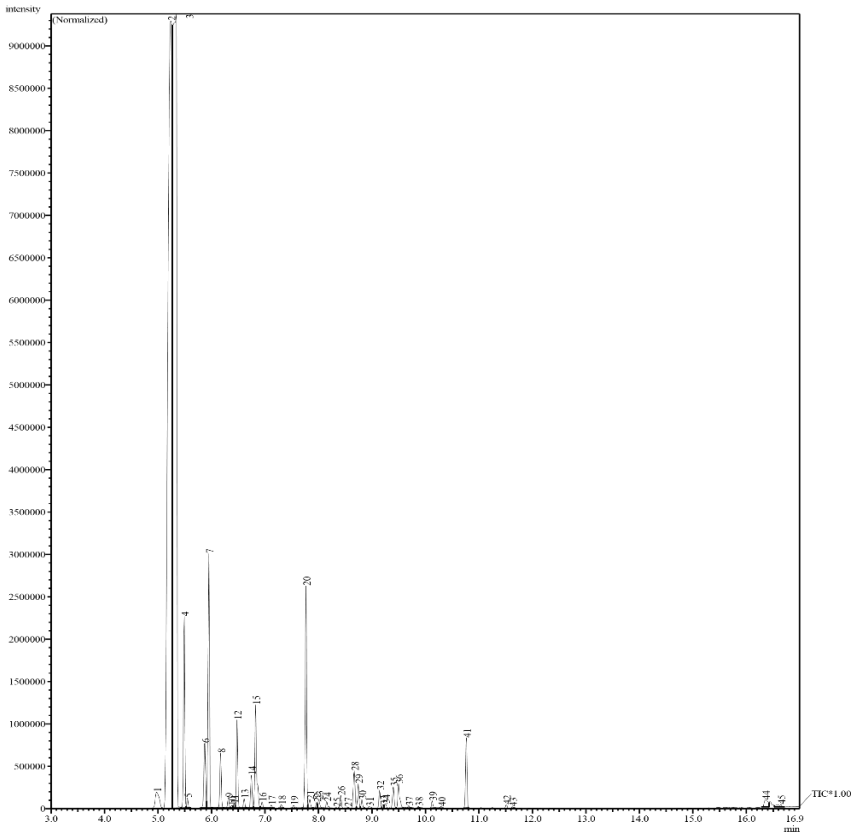
- (14.7g)18.6 g EDTA Added to nearly 70 ml dH_2O.
- Added 1.6-1.8 g of NaOH pellets.
- Adjust pH to 8.0 by with a few more pellets.
- Brought total volume to 100 ml with dH_2O.

Ammonium acetate (7.5 M)

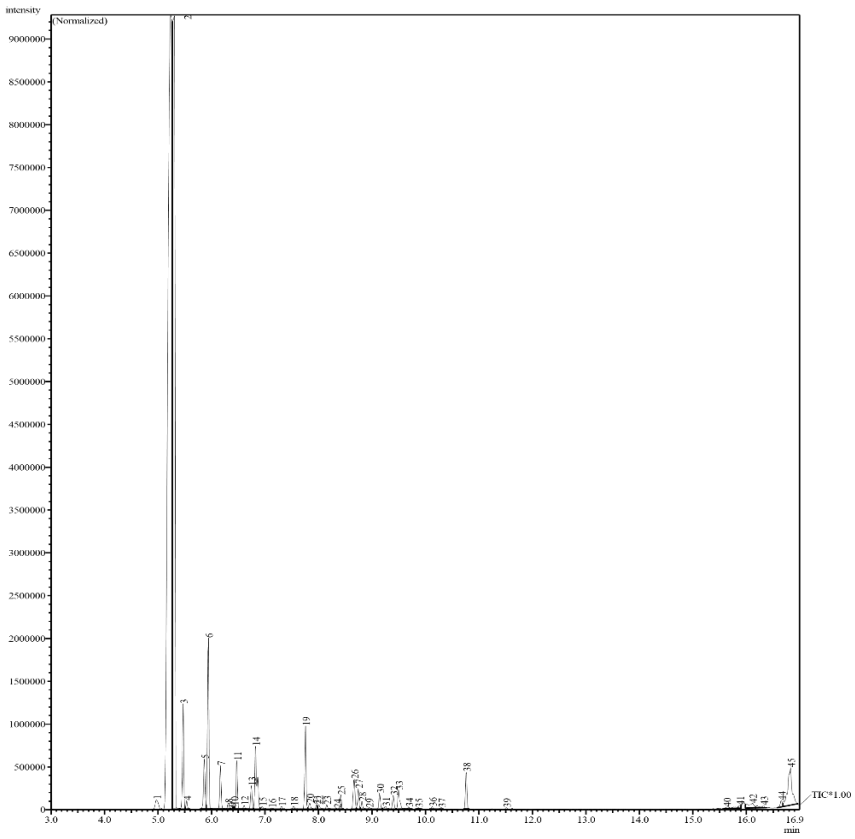
- 57.81 g ammonium acetate.
- 50 ml of dH2O.
- Brought to 100 ml total volume.



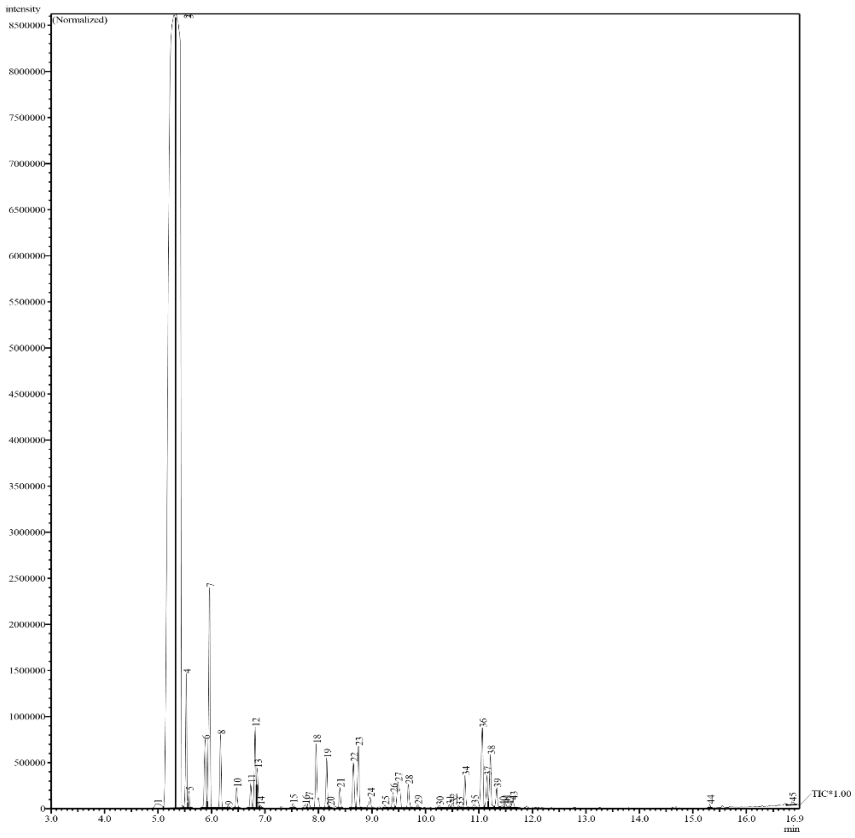
Appendix 2. Chromatogram of GC-MS analysis of the gum essential oil in Qaradagh



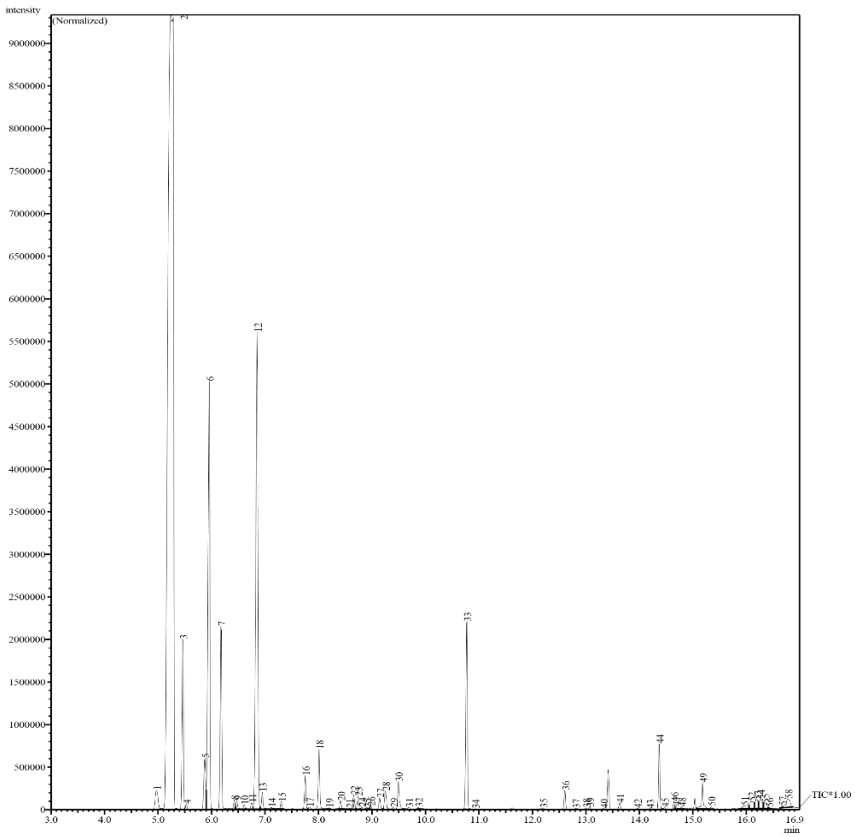
Appendix 3. Chromatogram of GC-MS analysis of the gum essential oil in Ranya.



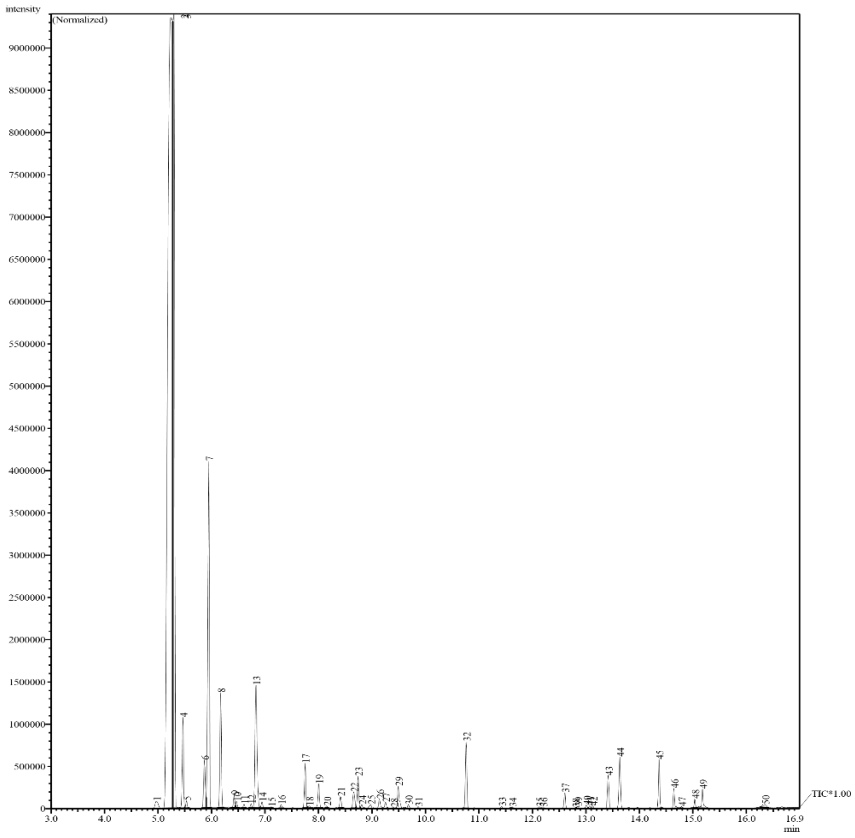
Appendix 4. Chromatogram of GC-MS analysis of the gum essential oil in Sharbazher.



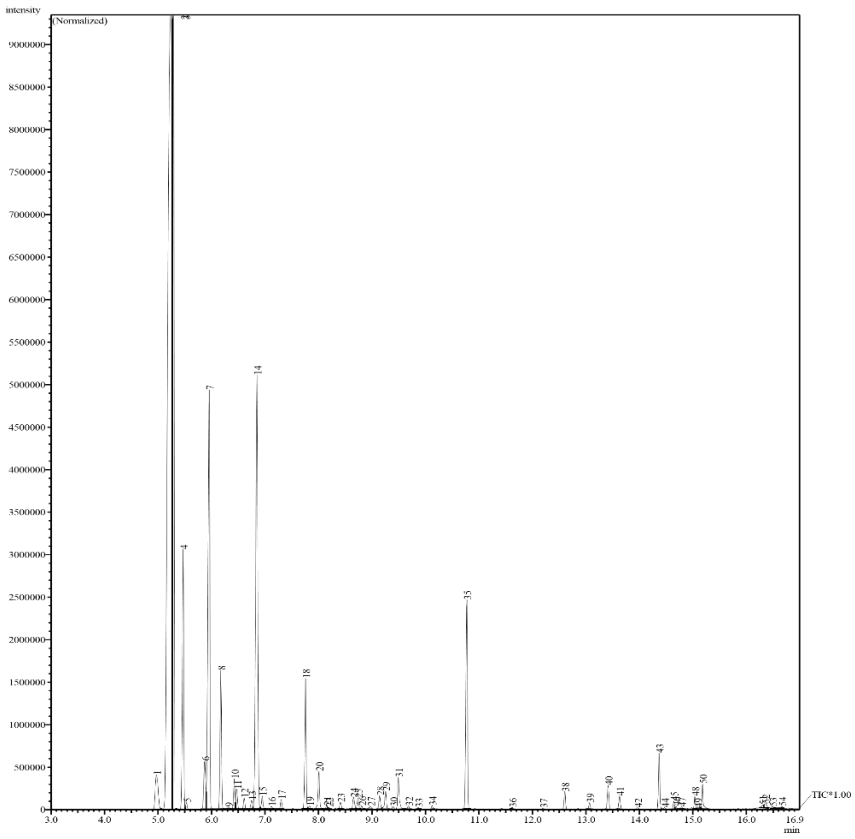
Appendix 5. Chromatogram of GC-MS analysis of the gum essential oil in Hawar.



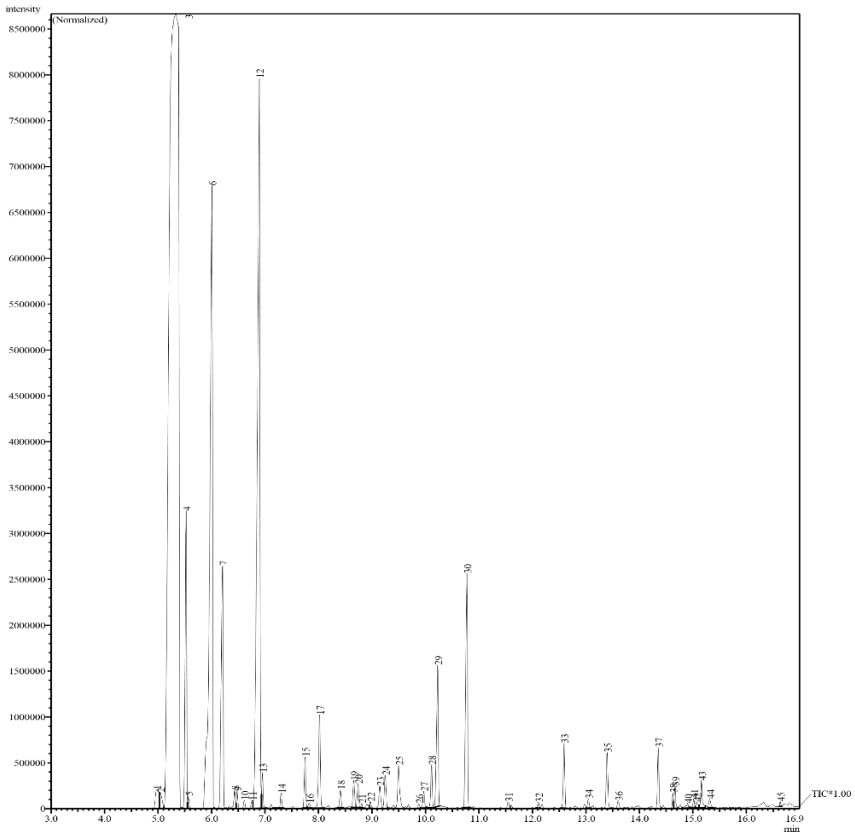
Appendix 6. Chromatogram of GC-MS analysis of the autumn rachis essential oil in Qaradagh.



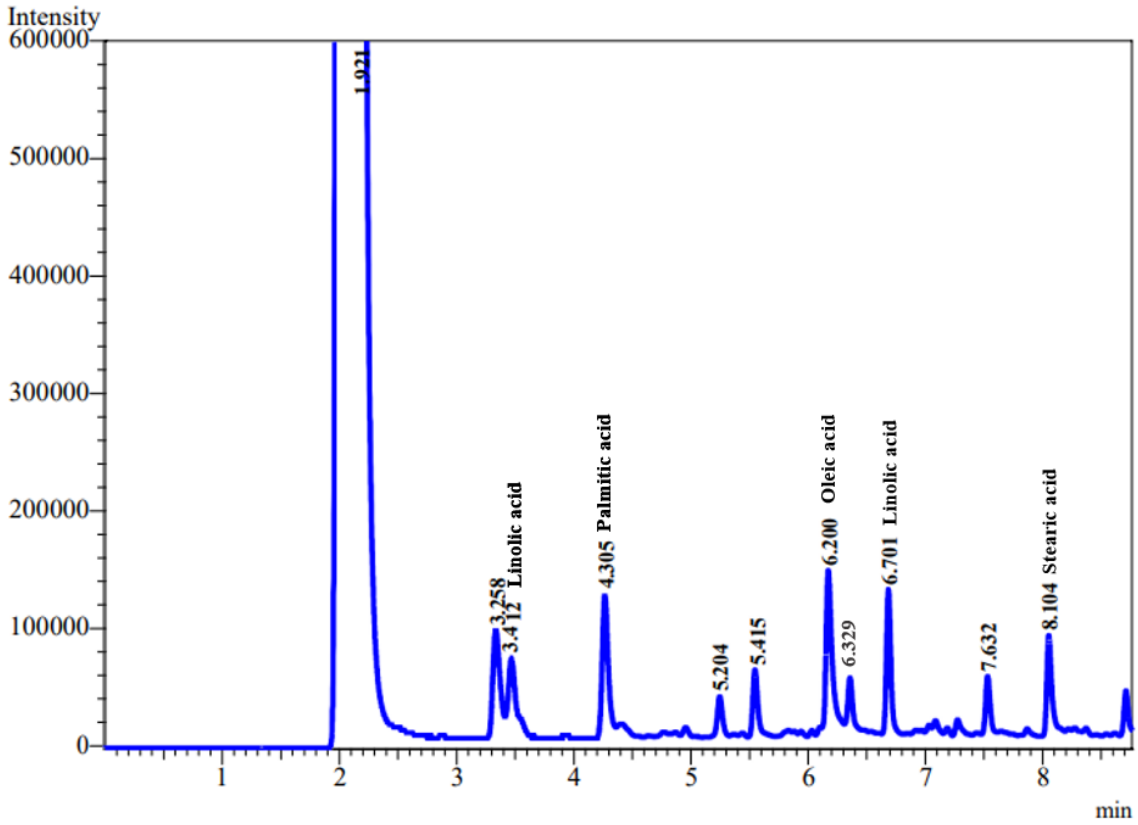
Appendix 7. Chromatogram of GC-MS analysis of the autumn rachis essential oil in Ranya.



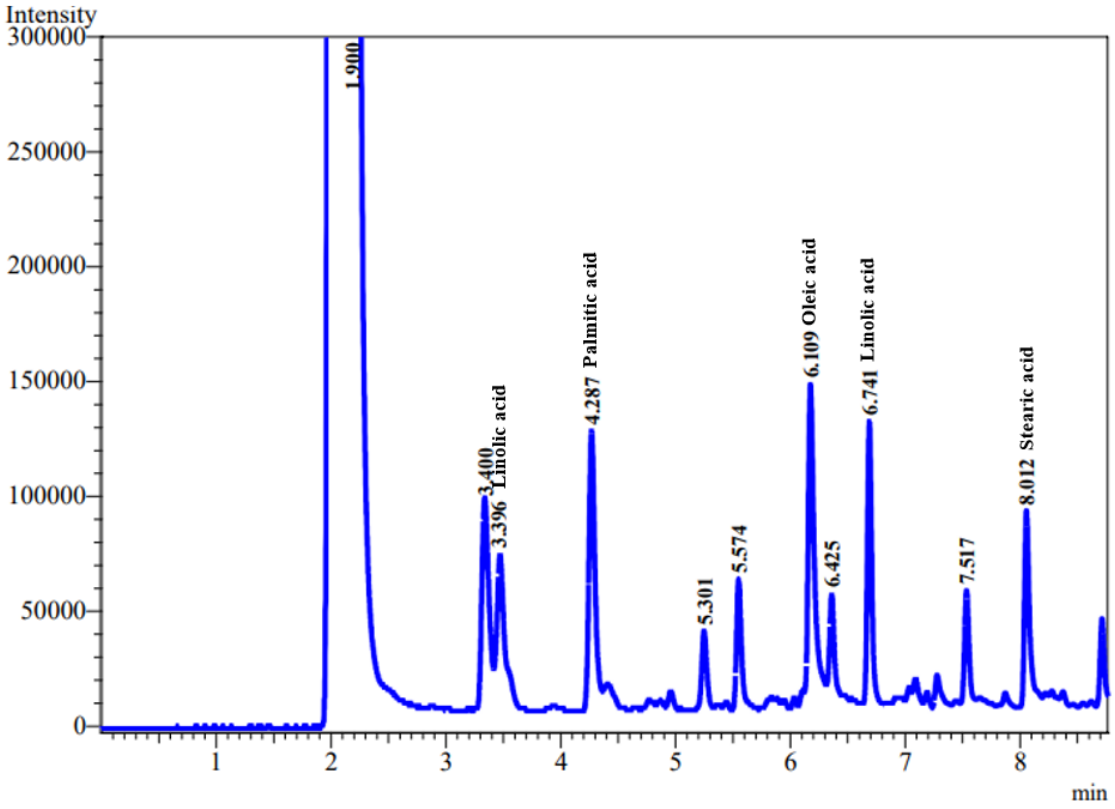
Appendix 8. Chromatogram of GC-MS analysis of the autumn rachis essential oil in Sharbazher.



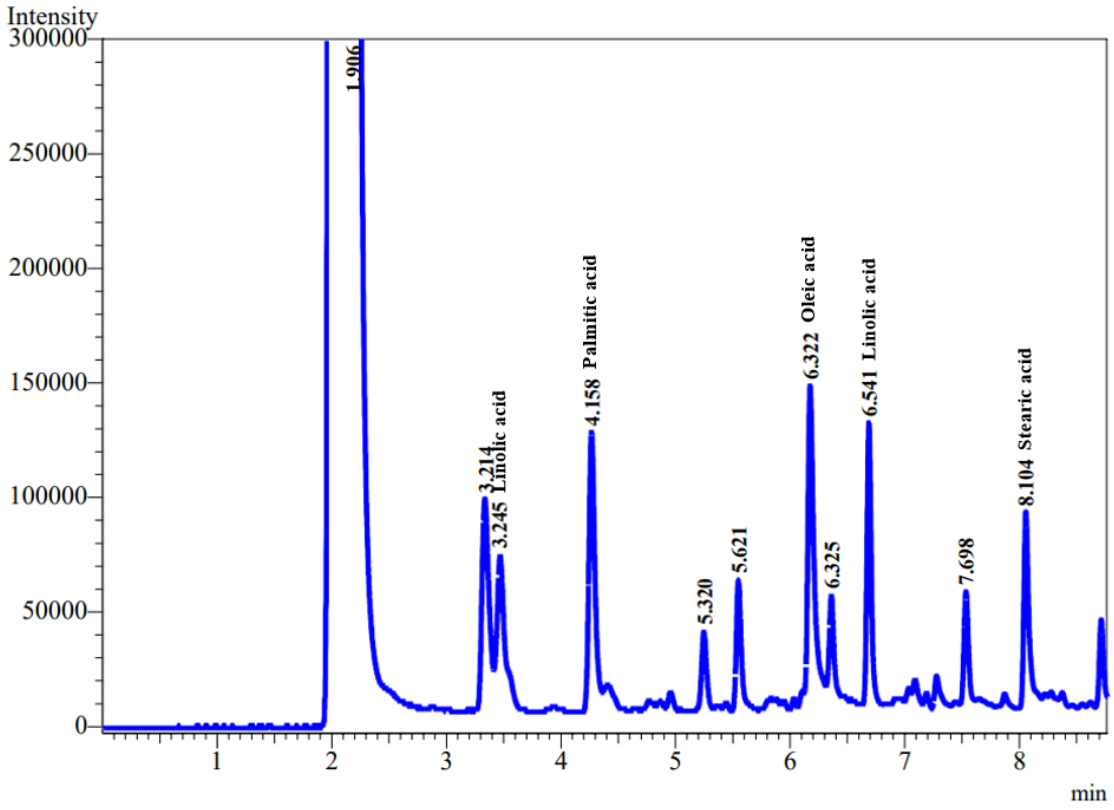
Appendix 9. Chromatogram of GC-MS analysis of the autumn rachis essential oil in Hawar.



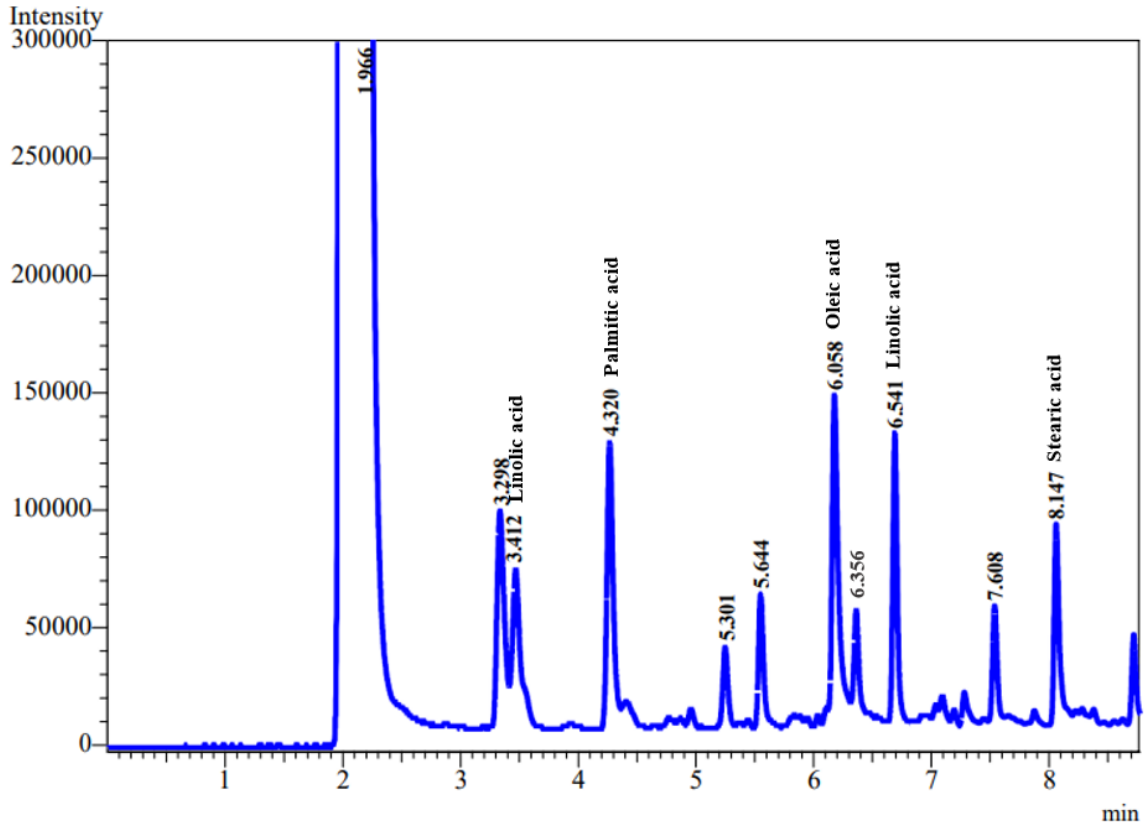
Appendix 10. GC chromatogram of different fixed oil compounds of the autumn fruit oil in Qaradagh.



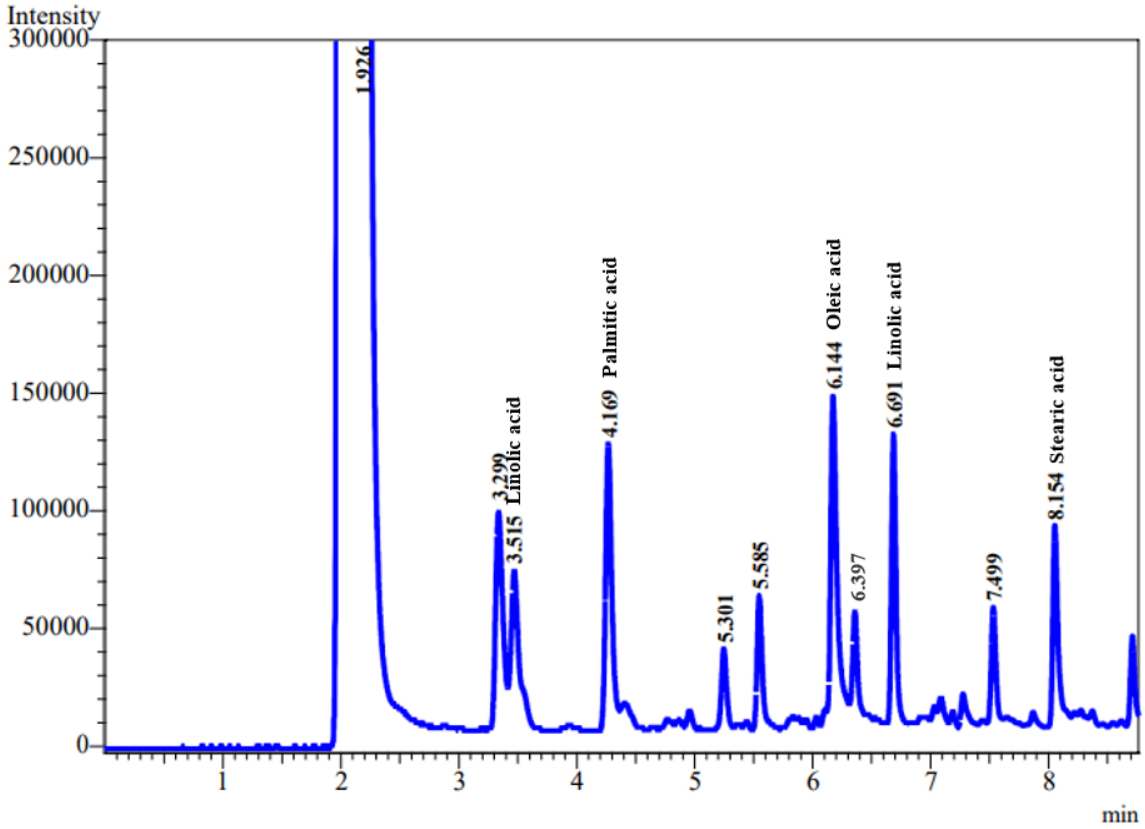
Appendix 11. GC chromatogram of different fixed oil compounds of the autumn fruit oil in Ranya.



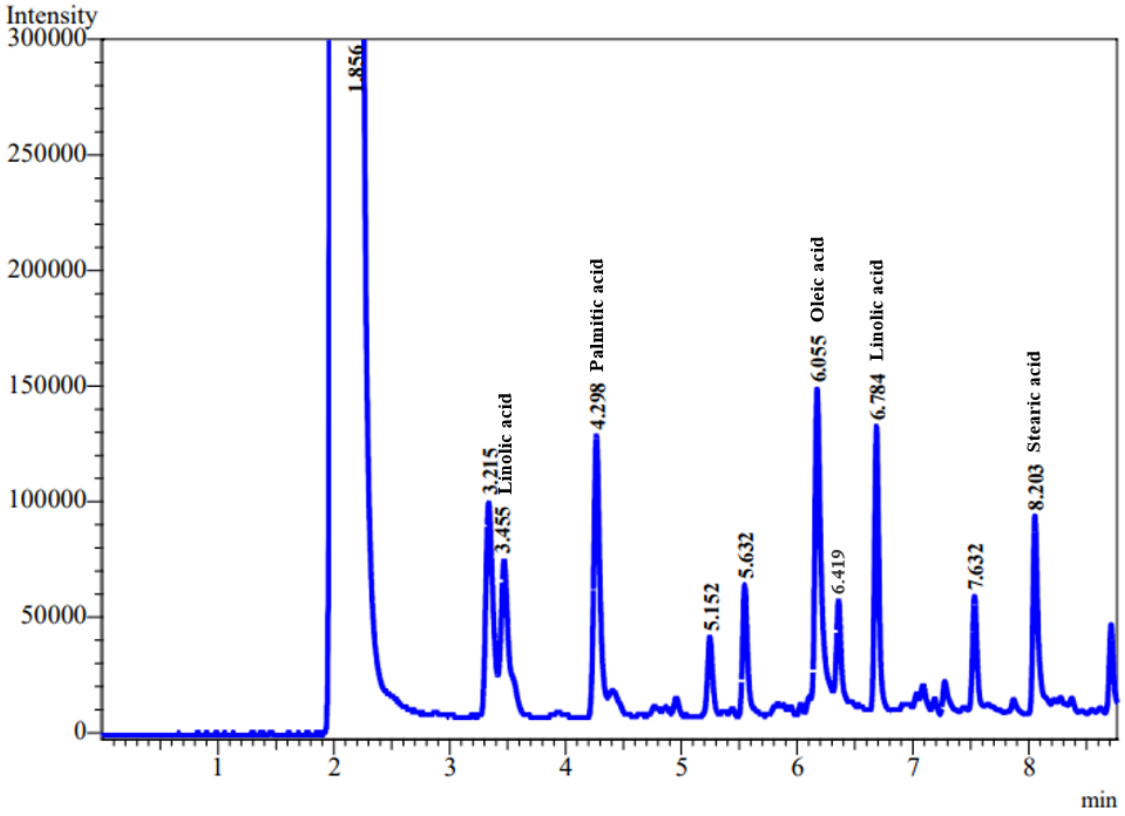
Appendix 12. GC chromatogram of different fixed oil compounds of the autumn fruit oil in Sharbazher.



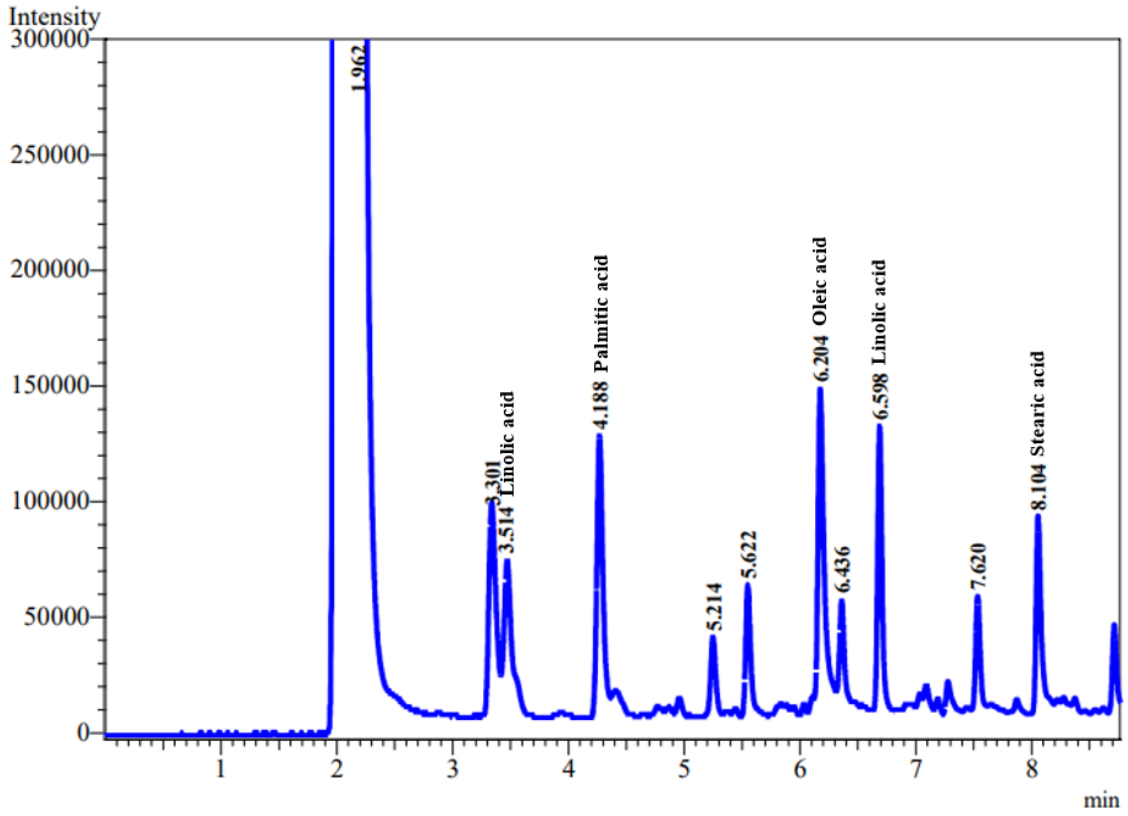
Appendix 13. GC chromatogram of different fixed oil compounds of the autumn fruit oil in Hawar.



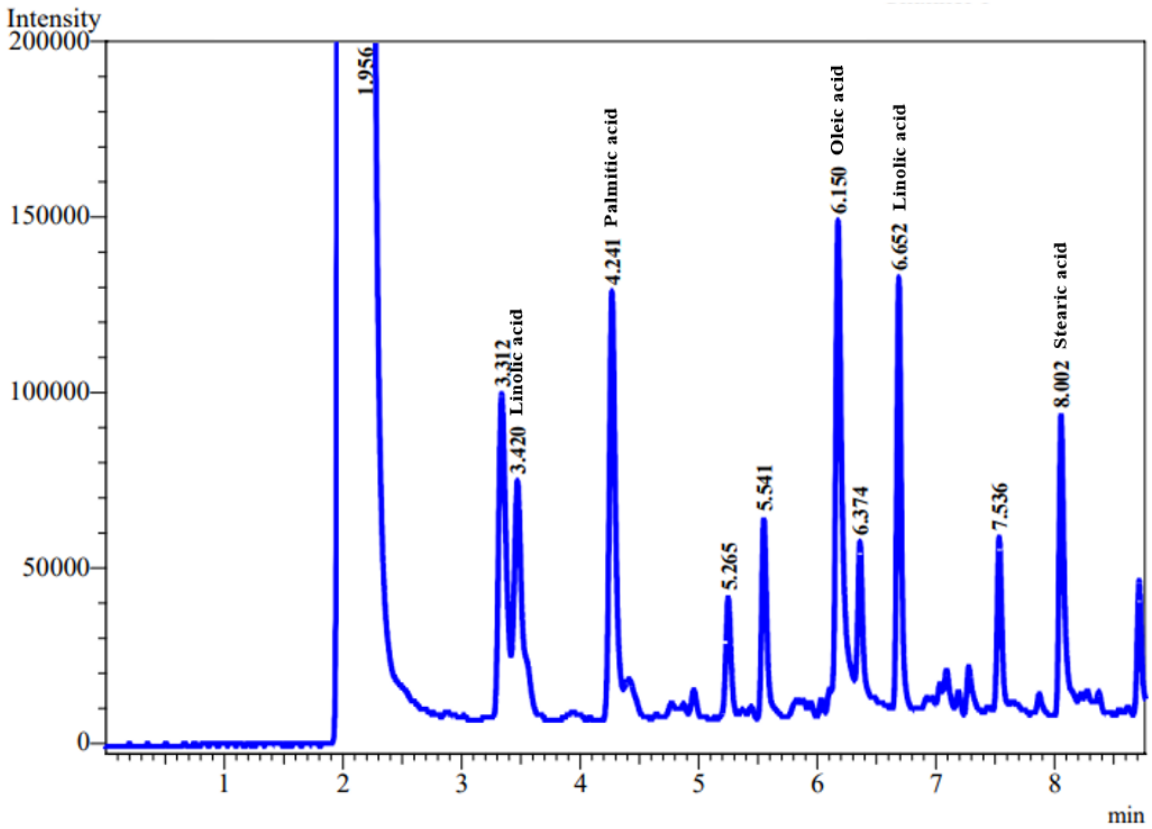
Appendix 14. GC chromatogram of different fixed oil compounds of the bark oil in Qaradagh.



Appendix 15. GC chromatogram of different fixed oil compounds of the bark oil in Ranya.



Appendix 16. GC chromatogram of different fixed oil compounds of the bark oil in Sharbazher.



Appendix 17. GC chromatogram of different fixed oil compounds of the bark oil in Hawar.

Appendix 18. Preparation of solutions to total phenolic content determination.

Folin–Ciocalteu phenol reagent (10 x) for 100 ml:

10 mL of Folin–Ciocalteu phenol reagent are added to 90 ml of dH_2O.

Na_2CO_3 10% for 100 ml:

10 of Na_2CO_3 are dissolved in 90 ml dH_2O. Store at 15-27 ºC.

Appendix 19. Preparation of solutions to total flavonoids content determination.

Methanol 80% for 100 ml:

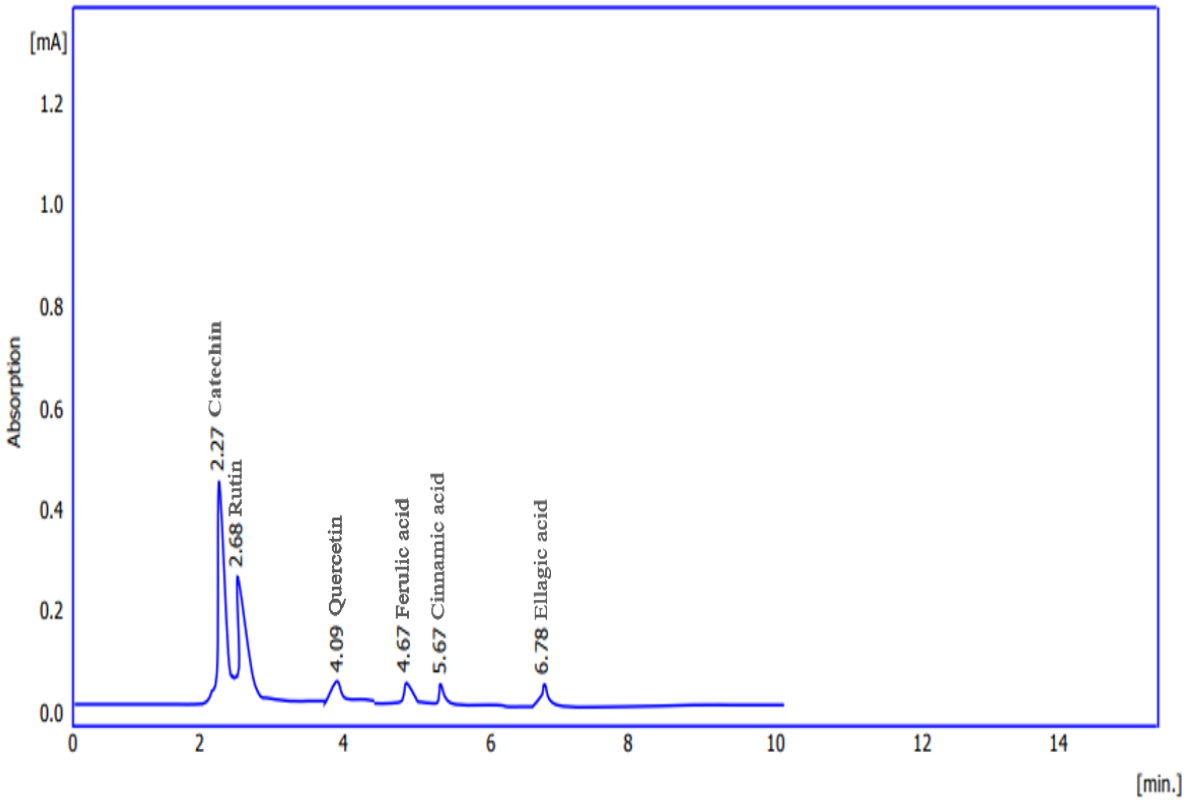
80 ml of methanol is added to 20 ml of dH_2O. Store at 5-27 ºC

Aluminum chloride hexahydrate ($AlCl_3$) 2% for 100 ml:

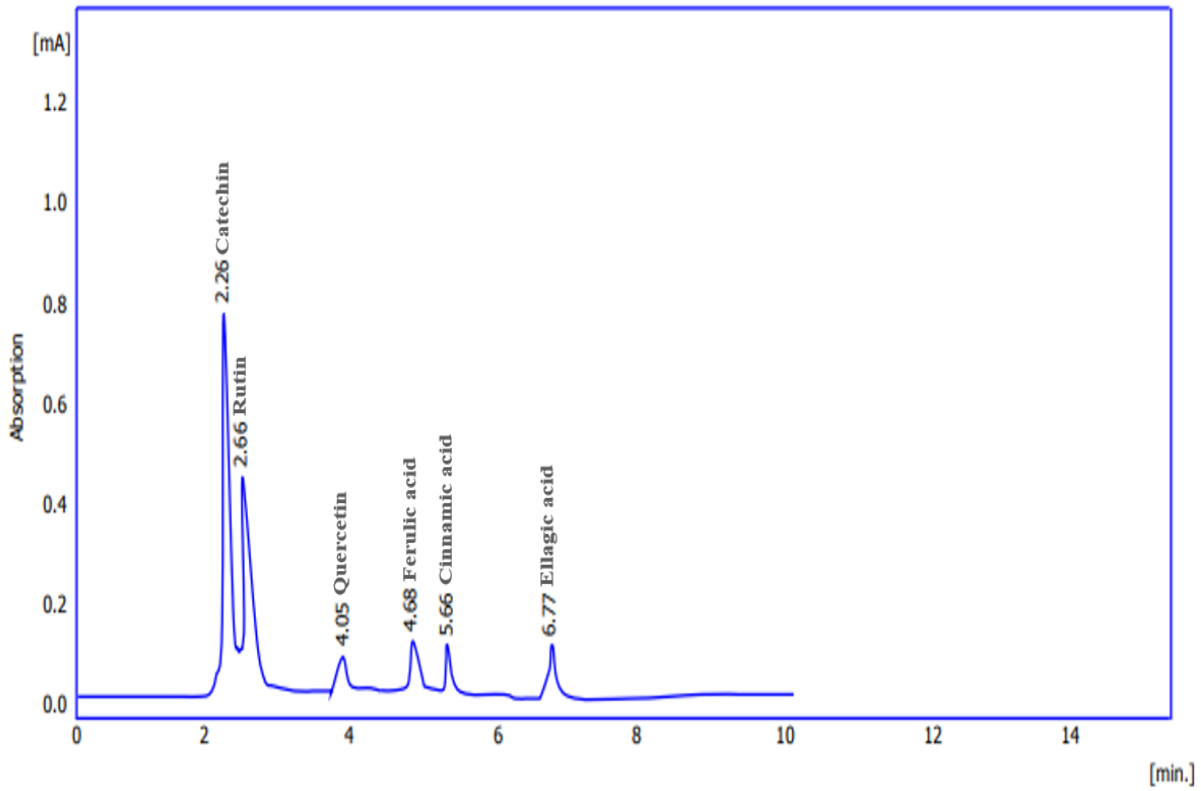
2 g of $AlCl_3$ are dissolved in dH_2O and the volume is made up to 100 ml. Store at 15-27 ºC

Potassium acetate (CH_3COOK) 1 M for 100 ml:

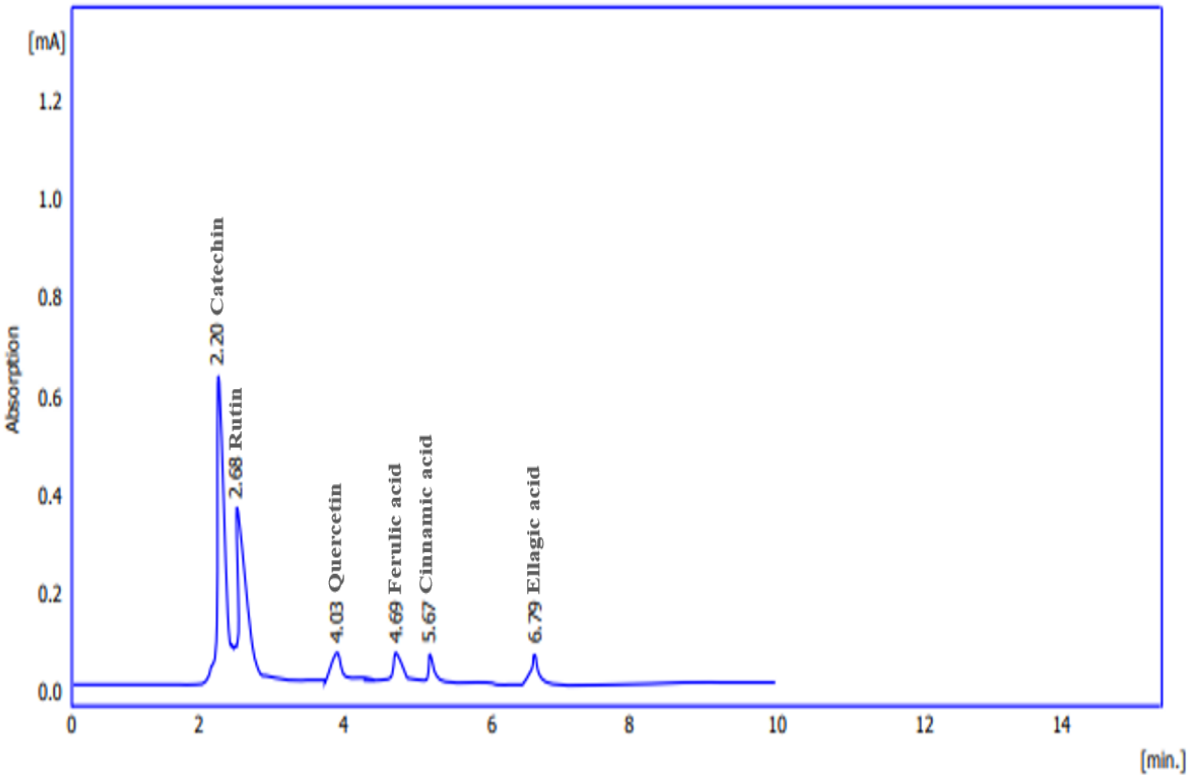
9.815g of CH3COOK are dissolved in distilled water and the volume is made up to 100 ml to give a 1 M CH3COOK solution. Store at 15-27 ºC



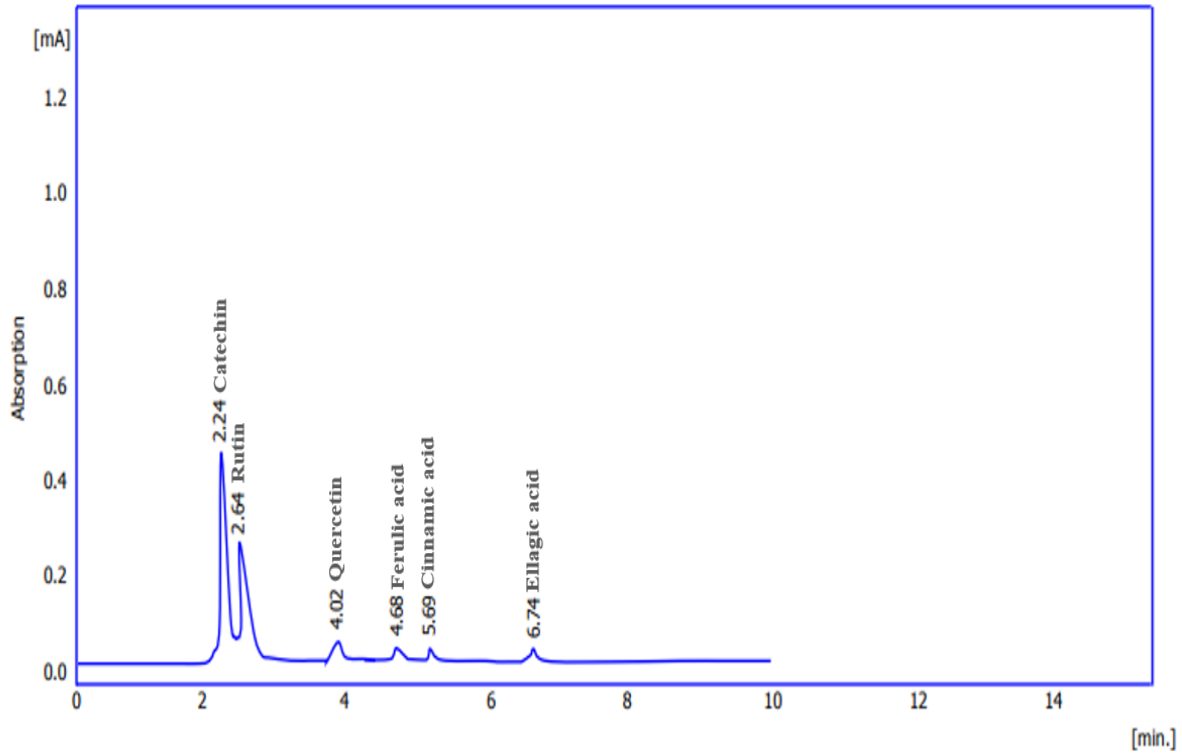
Appendix 20. HPLC chromatogram of different phenolic compounds in the spring leaves of Qaradagh.



Appendix 21. HPLC chromatogram of different phenolic compounds in the spring leaves of Ranya.



Appendix 22. HPLC chromatogram of different phenolic compounds in the spring leaves of Shrabazher.



Appendix 23. HPLC chromatogram of different phenolic compounds in the spring leaves of Hawar.

Appendix 24. Identification of *S. haemolyticus* by VITEK-2 System technique.

Identification Information	**Card: GP**	**Status: Final**
Selected Organism	**98% Probability Staphylococcus haemolyticus** **Bionumber: 030002047760271**	

Susceptibility Information	**Card: AST-P641**		**Status: Final**	**Analysis Time: 12.62 hours**	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Cefoxitin Screen	POS	«	Teicoplanin	>= 32	R
Benzylpenicillin	>= 0.5	«	Vancomycin	>= 32	R
Gentamicin	>= 16	R	Tetracycline	2	S
Ciprofloxacin	>= 8	R	Tigecycline	0.25	S
Levofloxacin	>= 8	R	Fosfomycin		
Inducible Clindamycin Resistance	NEG	-	Nitrofurantoin	<= 16	S
Erythromycin			Fusidic Acid		
Clindamycin	>= 4	R	Mupirocin		
Linezolid	2	S	Trimethoprim/Sulfamethoxazole	>= 320	R
Daptomycin	1*				

+= Deduced drug *= AES modified **= User modified «= Missing required test

Appendix 25. Identification of *E. faecalis* by VITEK-2 System technique.

Identification Information	Card: GP	Status: Final
Selected Organism	99% Probability Enterococcus faecalis Bionumber: 156002421751471	

Susceptibility Information	Card: AST-P641		Status: Final	Analysis Time: 7.40 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen			Teicoplanin	<= 0.5	S
Benzylpenicillin	16	«	Vancomycin	1	S
Gentamicin			Tetracycline	>= 16	R
Ciprofloxacin	>= 8	R	Tigecycline	<= 0.12	S
Levofloxacin	>= 8	R	Fosfomycin		
Inducible Clindamycin Resistance			Nitrofurantoin	<= 16	S
Erythromycin			Fusidic Acid		
Clindamycin			Mupirocin		
Linezolid	1	S	Trimethoprim/Sulfamethoxazole		
Daptomycin	4	S			

+= Deduced drug *= AES modified **= User modified «= Missing required test

Appendix 26. Identification of *P. aeruginosa* by VITEK-2 System technique.

Identification Information	Card: GN	Status: Final
Selected Organism	99% Probability Pseudomonas aeruginosa Bionumber: 0003453103500252	

Susceptibility Information	Card: AST-N326		Status: Final	Analysis Time: 10.39 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	>= 128	R	Netilmicin	>= 32	R
Piperacillin/Tazobactam	>= 128	R	Tobramycin	>= 16	R
Ceftazidime	>= 64	R	Ciprofloxacin	>= 4	R
Cefepime	>= 32	R	Levofloxacin	>= 8	R
Aztreonam			Tetracycline		
Imipenem	>= 16	R	Tigecycline	>= 8	R
Meropenem	>= 16	R	Colistin		
Amikacin	>= 64	R	Trimethoprim/Sulfamethoxazole		
Gentamicin	>= 16	R			

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

Appendix 27. Identification of *P. mirabilis* by VITEK-2 System technique.

Identification Information	Card: GN	Status: Final
Selected Organism	99% Probability Proteus mirabilis Bionumber: 0013000340042210	

Susceptibility Information	Card: AST-N326		Status: Final	Analysis Time: 10.10 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	>= 128	R	Netilmicin	>= 32	R
Piperacillin/Tazobactam	<= 4	S	Tobramycin	>= 16	R
Ceftazidime	0.5	S	Ciprofloxacin	>= 4	R
Cefepime	2	S	Levofloxacin	>= 8	R
Aztreonam	<= 1	*R	Tetracycline	>= 16	R
Imipenem	8	I	Tigecycline	4	*R
Meropenem	<= 0.25	S	Colistin		
Amikacin	<= 2	S	Trimethoprim/Sulfamethoxazole	>= 320	R
Gentamicin	8	I			

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

Appendix 28. Identification of *K. pneumoniae* by VITEK-2 System technique.

Identification Information	Card: GN	Status: Final
Selected Organism	99% Probability Klebsiella pneumoniae Bionumber: 6607734773565010	

Susceptibility Information	Card: AST-N326		Status: Final	Analysis Time: 8.42 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	>= 128	R	Netilmicin	<= 1	S
Piperacillin/Tazobactam	<= 4	S	Tobramycin	<= 1	S
Ceftazidime	8	I	Ciprofloxacin	1	S
Cefepime	2	S	Levofloxacin	1	S
Aztreonam	16	*R	Tetracycline	<= 1	S
Imipenem	<= 0.25	S	Tigecycline	<= 0.5	S
Meropenem	<= 0.25	S	Colistin		
Amikacin	<= 2	S	Trimethoprim/Sulfamethoxazole	>= 32	R
Gentamicin	<= 1	S			

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

Appendix 29. Identification of *E. coli* by VITEK-2 System technique.

Identification Information	Card: GN	Status: Final
Selected Organism	99% Probability Escherichia coli Bionumber: 0405610450026611	

Susceptibility Information	Card: AST-N326		Status: Final	Analysis Time: 8.22 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	<= 4	S	Netilmicin	<= 1	S
Piperacillin/Tazobactam	<= 4	S	Tobramycin	<= 1	S
Ceftazidime	<= 0.12	S	Ciprofloxacin	<= 0.25	S
Cefepime	<= 0.12	S	Levofloxacin	<= 0.12	S
Aztreonam	<= 1	S	Tetracycline	>= 16	R
Imipenem	<= 0.25	S	Tigecycline	1	S
Meropenem	<= 0.25	S	Colistin		
Amikacin	<= 2	S	Trimethoprim/Sulfamethoxazole	<= 20	S
Gentamicin	<= 1	S			

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

Appendix 30. Identification of *A. baumannii* by VITEK-2 System technique.

Identification Information	Card: GN	Status: Final
Selected Organism	99% Probability Acinetobacter baumannii Bionumber: 0201010303500210	

Susceptibility Information	Card: AST-N326		Status: Final	Analysis Time: 8.45 hours	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Piperacillin	>= 128	R	Netilmicin	2	S
Piperacillin/Tazobactam	>= 128	R	Tobramycin	8	I
Ceftazidime	>= 64	R	Ciprofloxacin	>= 4	R
Cefepime			Levofloxacin	>= 8	R
Aztreonam			Tetracycline	<= 1	S
Imipenem	>= 16	R	Tigecycline	<= 0.5	S
Meropenem	>= 16	R	Colistin		
Amikacin			Trimethoprim/Sulfamethoxazole	160	R
Gentamicin	>= 16	R			

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

Republic of Iraq
Ministry of Agriculture
Directorate of seed
Testing and certification
(D.S.T.C.)

جمهورية العـراق
وزارة الزراعـة
دائرة
فحص وتصديق البذور

القسم :

العـدد : ٤٥٤
التاريخ : ٢٠٢١/٢/٤

الى/ جامعة السليمانية / كلية علوم الهندسة الزراعية

م / أبداء مساعـدة

تحية طيبـة....

أشارة الى كتابكم ذي العدد (١٠٩٤٩/٥/٣) في (٢٠٢٠/١٢/٢١) والمتضمن ابداء المساعدة لطالبة الدكتوراه (زالة محمد احمد)، وعليه نود ان نبين ان التصنيف العلمي الدقيق للعينات النباتية المرسلة رفقة كتابكم اعلاه هي:

رقم العينة	الاسم العلمي	العائلة النباتية
(١، ٢، ٣، ٤)	*Pistacia eurycarpa* Yalt.	Anacardiaceae
(٦، ٧)	*Pistacia khinjuk* Stocks.	Anacardiaceae

مع التقديـــر

المدير العام
محمد زين العابدين محمد رؤوف
٢٠٢١/٣/ ٤

نسخة منه الى:
- مكتب السيد الوزير المحترم..... للاطلاع مع التقدير
- مكتب السيد المستشار رائد الحداد المحترم..... للاطلاع مع التقدير
- مكتب السيد المدير العام المحترم..... للاطلاع مع التقدير
- قسم النبات (المعشب الوطني العراقي)

اخلاص /

Appendix 31. Morphological identification of *Pistacia* species at the Iraqi National Herbarium.

حكومة أقليم كوردستان
وزارة التعليم العالي و البحث العلمي
جامعة السليمانية
كلية علوم الهندسة الزراعية

دراسة جزيئية وكيميائية وبيولوجية للبطم البري (*Pistacia* spp.) في مواقع مختلفة في محافظة السليمانية

إطروحة

مقدمة إلى مجلس كلية علوم الهندسة الزراعية في جامعة السليمانية

كجزء من متطلبات نيل شهادة دكتوراه في البستنة

النباتات الطبية

من قبل

ژالّه محمد أحمد

بكالوريوس في البستنة (2003)، كلية الزراعة، جامعة السليمانية

ماجستير في النباتات الطبية (2010)، كلية الزراعة، جامعة السليمانية

باشراف

د. زينب صباح لازم
أستاذ المساعد

د. حيدر موسى حمزة
أستاذ

2023 م

1444 هـ

الخلاصه

أجريت هذه الدراسة في اربع مناطق في محافظة السليمانية/إقليم كردستان العراق، والتي شملت على ثلاث اجزاء. تضمن الجزء الأول الدراسة الجزيئية لتشخيص نوعين من ال *Pistacia* أحدهما *Pistacia eurycarpa* (*Pistacia atlantica* subsp. *kurdica*) و *Pistacia khinjuk*. إذ تم التعرف على أنواع ال *Pistacia* مظهرياً في المواقع التى تم جمع النباتات منها إعتماداً على الإختلاف في شكل الأوراق والساق. وقد تم جمع أوراق النبات من قرداغ و رانية و شربازير و هاوار في محافظة السليمانية في نيسان 2020. وتم تشخيص أنواع ال *Pistacia* وذلك بإستعمال برايمر يستخدم لأول مرة لتضاعف جزء من جين 18S rRNA، تم مقارنة تسلسل DNA البالغ حجمه 600 bp مع تسلسل DNA لجين 18S rRNA مع 11 نوعاً من ال *Pistacia* المستخرج من بنك الجينات التابع للمركز العالمي لمعلومات التكنلوجيا الحيوية (NCBI). وأظهرت النتائج بان أربع نباتات في قرداغ و رانية وشربازير و هاوار تعود الى *P. eurycarpa* و نباتين آخرين في قرداغ و هاوار يعودان الى *P. khinjuk*. وقد تم تسجيل جميع أنواع ال *Pistacia* في بنك الجينات التابعة ل NCBI تحت المسميات التالية: MW534226، MW534227، MW534228، MW534229، MW534230، MW534231.

أما الجزء الثاني فقد كان عبارة عن دراسة المحتوى الكيميائي لأجزاء مختلفة من نبات البطم (*P. eurycarpa*). وقد تم جمع الأوراق والثمار و حامل العنقود في الربيع والخريف، بينما تم جمع القلف والصمغ في الربيع والصيف على التوالي. وتمت عملية جمع العينات من أربع مواقع :- قرداغ و رانية و شربازير و هاوار للفترة بين نيسان- تشرين الأول، 2020. وقد تم دراسة محتوى النيتروجين والفسفور والبوتاسيوم في الاوراق ووجد ان أعلى تركيز لمحتواها من النيتروجين كان 6.70٪ في أوراق الربيع التي جمعت من قرداغ، أما أعلى تركيز للفسفور والبوتاسيوم فقد وجد في أوراق الربيع التي تم جمعها من هاوار (2.03٪ و 2.25٪) على التوالي. أما محتوى الأوراق من الكربوهيدرات فأظهرت النتائج ان أعلى محتوى كان في أوراق الربيع التي جمعت من قرداغ (30.83٪)، بينما أقل قيمة (20.31٪) فقد وجدت في أوراق الخريف التي جمعت من شاربازير. وفيما يتعلق بالزيوت الطيارة فقد تم إستخلاصها من صمغ و حامل عنقود الخريف لنبات البطم اللذين تم جمعهما من المناطق قيد الدراسة، ووجد إن

أعلى معدل للنسبة المئوية للزيوت الطيارة (29٪) كان في صمغ شاربازير وبفرق معنوي عن بقية المناطق قيد الدراسة، بينما سجلت أقل قيمة في حامل عنقود الخريف التي جمعت من هاوار (2.40٪). وقد تم تحليل هذه الزيوت الى مكوناتها الأساسية بإستعمال جهاز كروماتوجرافيا الغاز-مطياف الكتلة (GC-MS). و بينت النتائج وجود العديد من المكونات في الزيت الطيار و أعلى تركيز لمركب الألفا بينين كان في الصمغ الذي تم جمعه من هاوار (81.40٪) من الزيت الطيار. في حين كان حامل العنقود في الخريف الذي جمع من المناطق التالية يحوي على أعلى تركيز للمكونات الرئيسية الأخرى وهي بيتا بينين (12.03٪) في هاوار، والكامفين (4.42٪) في شربازير، والبيتا ميرسين (3.48٪) في قرداغ، بينما أعلى تركيز للبيتا فيلاندرين (2.32٪) كان في الصمغ الذي تم جمعه من شربازير. أما بالنسبة للزيوت الثابتة فقد تم إستخلاصها من ثمار الخريف وقلف نبات البطم اللذين تم جمعهما من المناطق قيد الدراسة. ووجد إن أعلى تركيز كان في الثمار الخريفية التي جمعت من قرداغ (32.08٪) في حين أقل تركيز كان (3.10٪) في القلف الذي جمع من هاوار. كما وجد من خلال تحليل الزيوت الثابتة بجهاز كروماتوجرافيا الغاز (GC) إن الزيوت الثابتة لثمار الخريف التي جمعت من رانية أحتوت على أعلى تركيز لحامض البالمتيك (11.69٪) والاستيريك (4.20٪) والأوليك (45.39٪) واللينوليك (15.36٪) و اللينولينيك (0.77٪). أما أقل تركيز لحامض البالمتيك (2.55٪) و الاستيريك (0.66٪) والأوليك (5.89٪) واللينوليك (3.69٪) وحمض اللينولينيك (0.14٪) فقد ظهر في الزيت الثابت للقلف الذي تم جمعه من هاوار. أما فيما يتعلق بالمركبات الفينولية فقد أعطت الأوراق الربيعية التي جمعت من قرداغ أعلى محتوى للفينول الكلي (307.057 ملغم/غم)، أما أقل محتوى للفينول الكلي فقد ظهر في صمغ شربازير (1.409 ملغم/غم). في حين أعلى تركيز للفلافونيدات الكلية كان في الأوراق الربيعية التي جمعت من رانية (101.483 ملغم/غم)، وأقل تركيز كان في قلف هاوار (0.399 ملغم/غم). وقد أظهر تحليل ال HPLC أن أعلى تركيزللكورستين والروتين والكاتكين وحمض الفيروليك وحمض الإيلاجيك كان في الأوراق الربيعية التي جمعت من رانية (168.9 و 149.7 و 124.5 و 122.4 و 97.4 ميكروغم/غم) على التوالي، في حين أعلى تركيز لحامض السيناميك و كامفيرول وحامض التانيك والستيلبين كان في الأوراق الخريفية التي تم جمعها من هاوار (142.6 و 98.7 و 97.4 و 55.2 ميكروغم/غم) على التوالي. بينما كان تركيز حامض الغاليك (10.5 ميكروغرام/غم) في الصمغ الذي تم جمعه من هاوار، و الأبجينين (10.2 ميكروغرام/غم) في القلف الذي جمع من قرداغ.

ما فيما يتعلق بالجزء الثالث فقد تم فيه دراسة النشاط البيولوجي للزيوت الطيارة من لنبات البطم. اذ تعد المواد الفعالة بايولوجياً للزيوت الطيارة مصدراً جديداً واعداً كمضادات بكتيرية. وقد أظهرت الزيوت الطيارة التي تم إستخلاصها من صمغ نبات البطم فعالية تثبيطية قوية ضد البكتيريا سالبة الغرام (*Escherichia coli*، *Acinetobacter baumannii*، *Proteus mirabilis*، *Klebsiella pneumoniae*) و البكتيريا موجبة الغرام (*Enterococcus faecalis*، *Staphylococcus haemolyticus*). إذ كانت البكتيريا موجبة الغرام أكثر حساسية للزيوت الطيارة من البكتيريا سالبة الغرام. ثم، تم اختبار الزيوت الطيارة من صمغ نبات البطم لمنطقة هاوار ضد بكتريا *A. baumannii* المقاومة للأدوية المتعددة سالبة الغرام. كما أظهرت النتائج نشاطاً ممتازاً للزيت كمضاد لبكتيريا *A. baumannii*. وقد كان الحد الأدنى لتركيز التثبيط (MIC) للزيت هو 2.5 **ميكرولتر/مل. وقد لوحظ زيادة تركيز البروتين وتدفق أيونات الفوسفات والبوتاسيوم من *A. baumannii* بعد معالجة الخلايا بـ** 2.5 **ميكرولتر/مل من للزيت. كما، أظهرت صور المجهر الإلكتروني (TEM) حدوث تشوه لشكل خلايا البكتيريا، مما أدى إلى تحلل جدار الخلية، وتسرب المكونات داخل الخلية، وبالتالي موت الخلية. وأخيراً، أظهر الزيت تطهيراً جرثومياً ونشاطاً مضاداً للالتصاق بإستخدام الضمادات اللاصقة.**

حکومەتی هەرێمی کوردستان
وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
زانکۆی سلێمانی
کۆلێجی زانستە ئەندازیارییە کشتوکاڵییەکان

لێکۆڵینەوەی گەردیلەیی و کیمیایی و بایۆلۆجی بۆ قەزوانی کێوی (*Pistacia* spp.) لە چەند شوێنێکی جیاوازی پارێزگای سلێمانی

تێزی دکتۆرایە

پێشکەش کراوە بە ئەنجومەنی کۆلێجی زانستە ئەندازیارییە کشتوکاڵییەکان لە زانکۆی سلێمانی وەک بەشێک لە پێداویستیەکانی بەدەستهێنانی بڕوانامەی دکتۆرا لە باخداری

(ڕووەکە پزیشکیەکان)

لەلایەن

ژاڵە محمد احمد

بەکالۆریۆس لە باخداری (2003)، کۆلێژی کشتوکاڵ، زانکۆی سلێمانی

ماستەر لە ڕووەکە پزیشکیەکان (2010)، کۆلێژی کشتوکاڵ، زانکۆی سلێمانی

بە سەرپەرشتی

د. زینب صباح لازم
پرۆفیسۆری یاریدەدەر

د. حیدر موسی حمزە
پرۆفیسۆر

2023 ز

2723 ك

پوختە

ئەم پرۆژەیە کە لە سێ بەش پێك هاتبوو لە هەرێمی کوردستانی عێراق جێبەجێکرا کە چوار ناوچەی سەر بە سلێمانی لە خۆگرتبوو. بەشی یەکەم بریتی بوو لە ناسینەوەی گەردیلەیی بۆ دوو جۆری *Pistacia* بە ناوەکانی (*Pistacia atlantica* subsp. *kurdica*) *Pistacia eurycarpa* و *Pistacia khinjuk*. لە مانگی نیسانی سالّی 2020 دا، جۆرەکانی *Pistacia* دیاریکران لە شوێنی کۆکردنەوەیان بەپێی جیاوازیان لە شێوەی گەلاّ و قەدەکانیاندا، پاشان نموونەی گەلاّکان لە دارستانەکانی قەرەداغ و ڕانیە و شارباژێر و هاوار لە ناوچەی سلێمانی کۆکرایەوە. بە مەبەستی ناسینەوەی ڕووەکەکان پرایمەرێکی نوێ بۆ ناوچەی 18S rRNA بەکارهێنرا. دەرکەوت کە چوار ڕووەك کە لە قەرەداغ، ڕانیە، شارباژێر و هاوار کۆکرابوەوە پەیوەندیەکی نزیکیان بە ڕەگەزێکی دیکەی تۆمارکراو (قەزوان) *P. eurycarpa* لە NCBI هەیە. دوو ڕووەکەکەی تر کە لە قەرەداغ و هاوار کۆکرایەوە پەیوەندیەکی نزیکیان بە ڕەگەزێکی دیکەی تۆمارکراو (بەنەوێشك) *P. khinjuk* لە NCBI هەیە. هەموو جۆرەکانی قەزوان کە لەم تۆێژینەوەدا لێکۆڵینەوەمان بۆ کرد تۆمارکران لە بانکی بۆهێڵی NCBI بە ژمارەکانی MW534226 و MW534227 و MW534228 و MW534229 و MW534230 و MW534231.

بەشی دووەم پێکهاتە کیمیاییەکانی بەشە جیاوازەکانی درەختی قەزوان خەملاّندووە. بەشەکانیش بریتی بوون لە گەلاّ و بەر و لاسك لە بەهار وپایزدا لەگەڵ تۆێکڵی قەد لە بەهارداو و بنێشتە تاڵ لە هاویندا. لە مانگەکانی نیسان بۆ تشرینی یەکەمی سالّی 2020 دا، بەشە جیاوازەکانی درەختی قەزوان لە ناوچەکانی قەرەداغ، ڕانیە، شارباژێر و هاوار کۆکراونەتەوە. لێکۆڵینەوە لە ڕێژەی نایترۆجین و فسفۆر و پۆتاسیۆم لە گەلاّکاندا کراو دەرکەوت کە بەرزترین ڕێژەی نایترۆجین (6.70٪) لە گەلاّکانی بەهاردا دەرکەوتووە کە لە قەرەداغ کۆکراونەتەوە، لە کاتێکدا بەرزترین ڕێژەی فسفۆر (2.03٪) و پۆتاسیۆم (2.25٪) لە گەلاّکانی بەهاردا بووە کە لە هاوار کۆکراونەتەوە. سەبارەت بە ڕێژەی کاربۆهیدراتی گەلاّکان، ئەنجامەکان دەریانخستووە کە بەرزترین ڕێژەی کاربۆهیدرات (30.83٪) لە گەلاّکانی بەهاردا کە لە قەرەداغ کۆکراونەتەوە دەرکەوتووە، لە کاتێکدا کەمترین ڕێژەی (20.31٪) لە گەلاّکانی پایزی شارباژێردا دەرکەوتووە. ڕۆنی بەهەڵم بوو لە بنێشتەتاڵ و لاسکی پایزی

قەزواندا دەرهێنرا کە لە چەند ناوچەیەکی جیاوازەوە کۆکرابونەوەو دەرکەوت بنێشتەتالّی شارباژێر بەرزترین و بەرچاوترین ڕێژەی ڕۆنی بەهەڵم بووی هەبووە (29.00٪) لە کاتێکدا نزمترین ڕێژەی ڕۆنی بەهەڵم بوو (2.40٪) لە لاسکی پایزی هاواردا دەرکەوتووە. ئەنجامی شیکاری GC-MC بۆ ڕۆنی بەهەڵم بوو دەریخستووە کە بەرزترین ڕێژەی alpha-pinene لە بنێشتەتالّدا دۆزراوەتەوە کە لە هاوار کۆکراوەتەوە (81.40٪). بەرزترین ڕێژەی پێکهاتە سەرەکیەکانی دیکە لە لاسکی پایزدا دۆزرایەوە کە بریتی بوون لە beta-pinene (12.03٪) لە هاوار و camphene (4.42٪) لە شارباژێر و beta-myrcene لە قەرەداغ (3.48٪)، بەلاّم بەرزترین ڕێژەی beta-phellandrene (2.32٪) لە ناو بنێشتەتالّی شارباژێر دۆزرایەوە. ڕۆنی جێگیر لە بەری پایز و تۆپکلّی قەدی قەزوان دەرهێنرا کە لە چەند ناوچەیەکی جیاوازەوە کۆکرابونەوە بینرا کە بەری پایزی قەرەداغ بەرزترین ڕێژەی ڕۆنی جێگیری تێدابووە (32.08٪)، لە کاتێکدا کەمترین ڕێژەی ڕۆنی جێگیری (3.10٪) لە تۆپکلّی قەدی هاواردا دەرکەوتووە. شیکاری GC بۆ ڕۆنی جێگیر دەریخستوە کە بەرزترین ڕێژەی ترشی پالّمتیك (11.69٪) و ترشی ستیریك (4.20٪) و ترشی ئۆلیك (45.39٪) و ترشی لینولیك (15.36٪) و ترشی لینولینیك (0.77٪) لە ڕۆنی جێگیری بەری پایزی ڕانیەدا دەرکەوتوە، لە کاتێکدا نزمترین ڕێژەی ترشی پالّمتیك (2.55٪) و ترشی ستیریك (0.66٪) و ترشی ئۆلیك (5.89٪) و ترشی لینولیك (3.69٪) و ترشی لینولینیك (0.14٪) لە ڕۆنی جێگیری تۆپکلّی قەدی قەزواندا دۆزراوەتەوە کە لە هاوار کۆکراوەتەوە. پاشان بڕی فینۆڵ و فلاڤۆنۆیدی گشتی لە بەشە جیاوازەکانی قەزواندا خەملّێندرا. گەلاّکانی بەهاری قەرەداغ زۆرترین بڕی فینۆڵی گشتی هەبووە (307.057 ملگم/گم)، بەلاّم کەمترین بڕ (1.409 ملگم/گم) لە بنێشتەتالّی شارباژێردا دەرکەوتووە. گەلاّکانی بەهاری ڕانیە زۆرترین بڕی فلاڤۆنۆیدی گشتی هەبووە (101.483 ملگم/گم) بەلاّم کەمترین بڕ (0.399 ملگم/گم) لە تۆپکلّی قەدی قەزوانی هاوار دەرکەوتووە. شیکاری HPLC دەریخست کە بەرزترین بڕی quercetin (168.9 مایکروگم/گم) و rutin (149.7 مایکروگم/گم) و catechin (124.5 مایکروگم/گم) و ferulic acid (122.4 مایکروگم/گم) و ellagic acid (97.4 مایکروگم/گم) لە گەلاّی بەهاری ڕانیەدا بووە. هەروەها بەرزترین بڕی مادەی cinnamic acid (142.6 مایکروگم/گم) و kaempferol(98.7 مایکروگم/گم) و tannic acid (97.4 مایکروگم/گم) و stilbene (55.2

مایکروگم/گم) لە گەڵکانی پایزی هاواردا و gallic acid (10.5 مایکروگم/گم) لە بنێشتە تاڵی هاواردا و apigenin (10.2 مایکروگم/گم) لە تووێکڵی قەردەداغدا هەبوون.

بەشی سێیەم چالاکیی بایۆلۆژی ڕۆنی بەهەڵم بووی قەزوانی لەخۆگرتبوو. مادە چالاکەکانی ڕۆنی بەهەڵم بوو سەرچاوەیەکی کاران بۆڕێگریکردن لە گەشەی بەکتریاکان. ڕۆنی بەهەڵم بووی قەزوان کاریگەرییەکی گەورەی دژی گەشەی بەکتریا گرام نێگەتیڤەکان (*Escherichia coli*، *Klebsiella pneumoniae*، *Proteus mirabilis*، *Acinetobacter baumannii*) و بەکتریا گرام پۆزەتیڤەکان (*Staphylococcus haemolyticus*، *Enterococcus faecalis*) پیشان دا، بەڵام کاریگەرییەکی کەمی دژی گەشەی *Pseudomonas aeruginosa* پیشان دا. بەکارهێنانی ڕۆنی بەهەڵم بووی قەزوان کاریگەری لەسەر بەکتریا گرام پۆزەتیڤەکان زیاتر بوو بە بەراورد بە بەکتریا گرام نێگەتیڤەکان. دواتر، ڕۆنی بەهەڵم بووی بنێشتەتاڵی قەزوان کە لە ناوچەی هاوار کۆکرابوەوە لە دژی بەکتریای *A. baumannii* تاقیکرایەوە. ڕۆنی بەهەڵم بووی بنێشتە تاڵی قەزوان چالاکی دژە بەکتریا و بایۆفیلمی نایابی لە دژی بەکتریای *A. baumannii* پیشان دا. کەمترین چڕی ڕێگریکردنی (MIC) ڕۆنی بەهەڵم بووی بنێشتەتاڵی قەزوان 2.5 مایکرولیتر/مل بوو. سەرەڕای ئەوە، میکانیزمەکانی کاریگەری ڕۆنی بەهەڵم بووی بنێشتەتاڵی قەزوان دژی بەکتریای *A. baumannii* لێکۆڵینەوەیان لەسەر کرا. ڕێژەی پرۆتین و ئایۆنەکانی فۆسفات و پۆتاسیۆم لە ناوەندی گەشەی بەکتریای *A. baumannii* زیاد بوو پاش بەکارهێنانی 2.5 مایکرولیتر/مل لە ڕۆنە بەهەڵم بووەکە. سەرەڕای ئەوەش، وێنەکانی مایکرۆسکۆبی ئەلیکترۆنی گواستنەوە (TEM) شێواندنی ڕووکەشی خانەکانی بەکتریاکەی نیشان دا پاش بەکارهێنانی ڕۆنە بەهەڵم بووەکە، کە دەبێتە هۆی شیکردنەوەی دیواری خانەکە و هاتنەدەرەوەی پێکهاتەکانی ناو خانەکە و مردنی خانەکان لە ئەنجامدا. لە کۆتایدا، ڕۆنی بەهەڵم بووی بنێشتە تاڵی قەزوان چالاکی پاککردنەوەی بەکتریا و دژە لکاندنی نیشان دا بە بەکارهێنانی لەزگەی برین.