Hebron University

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Determination of the Phytochemical, Antioxidant and Antibacterial activities of *Arbutus andrachne* L. Methanolic Leaf and Fruit Extract

By

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M.Sc. Thesis

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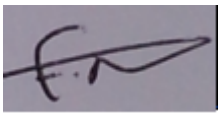
Prof. Dr. Fuad Al- Rimawi	**Supervisor**	
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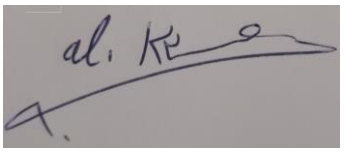
Dr. Alaa Khraiwesh	**Internal examiner**	

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Prepared by:

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Declaration

I certify that this thesis submitted for the degree of the master is the result of my research, except where otherwise acknowledged, and this thesis has not been submitted for a higher degree to any other university or institute.

Acknowledgment

I am deeply indebted to the generous Allah, who always bestows upon me the power of striving for knowledge and success. I would like to thank all the people who contributed in some way to facilitate the success of the work described in this thesis. First and foremost, I'm extremely grateful to my Supervisor Professor **Fuad Rimawi** for supervision, useful comments, and continuous support. I appreciate your giving me the intellectual freedom to engage new ideas while demanding high-quality work in my research. To my colleagues factually of pharmacy and medical sciences, the faculty of agriculture, and my doctors, my deepest respect and thanks for their valuable help and for supporting me there Dr. Abdel Qader Qawasmeh and MSc. Seema Al-Falah. Also, Eng. Majed Eiswed who helped me during the sampling process. Special thanks to my cousins, Shehadeh for their support and encouragement. Lastly, I would be remiss in not mentioning my family, especially my hero mother, Fatimah and my friendly brother, Lotfy. Their belief in me has kept my spirits and motivation high during this process. I would also like to thank my friends for all the entertainment and emotional support.

Dedication

This thesis is dedicated:

To the spirit of my father who always accompanies me because I am the hope that life has given him, and my brave mother who drew the path for me from her precious days. A special feeling of gratitude to my loving brother, and his words of encouragement and urging perseverance to finish this dissertation.

Signed.....
Amal Fawzi Arar

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List of Abbreviations

$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl hydrate
$ABTS^{\bullet+}$	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
TPC	Total phenolic content
GC-MS	Gas chromatography-mass spectrometry
HPLC-PDA	High-performance liquid chromatography–photodiode array
Rt	Retention time
Min	Minute
mg GAE/g	Milligrams of gallic acid equivalents per gram
S. aureus	*Staphylococcus aureus*
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
P. acnes	*Propionibacterium acnes*
WHO	World Health Organization
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
CT	Condensed tannin
kcal/g	Kilocalories per gram
DNA	Deoxyribonucleic acid
etc.	et cetera
XO	Xanthine oxidase
TICE	Transintestinal cholesterol excretion
CVD	Cardiovascular diseases
LDL	Low-density lipoprotein
LC-MS	Liquid chromatography-mass spectrometry
αTOC	α-Tocopherol
FRAP	Ferric reducing antioxidant power assay
SOD	Superoxide dismutase
ROS	Reactive oxygen species
GPx	Glutathione peroxidase
GSSG	Oxidized glutathione
CUPRAC	CUPric reducing antioxidant capacity
MCF-7	Michigan cancer foundation-7
A549	Adenocarcinomic human alveolar basal epithelial cells
Hep	Hepatocellular carcinoma
AST	Aspartate aminotransferase
ALT	Alanine transaminase
MDA	Malondialdehyde
PGE2	Prostaglandin E2

IL-6	Interleukin-6
Mm	Millimeter
nmole/g	Nanomoles per gram
°C	Celsius
v/v	Volume/Volume
GA	Gallic acid
Na_2CO_3	Sodium carbonate
Ml	Milliliter
DW	Distilled water
Ml	Microliter
Nm	Nanometer
Mmol	Millimole
$K_2S_2O_8$	Potassium persulfate
µM	Micron or micrometer
Ms	Millisecond
cm/s	Centimeter per second
amu/s	Atomic mass unit per second
Mv	Intensity of refraction index signals
EMB	Eosin Methylene Blue agar
MSA	Mannitol salt agar
M.H	Muller Hinton
Abs	Absorbance
CFU	Colony forming units
UV/Visible spectrophotometer	Ultraviolet-visible spectrophotometer
F.C	Folin-Ciocalteu reagent
No.	Number
M/Z	Molecular mass
MW	Molecular weight
CB1	Cannabinoid receptor-interacting protein1

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Abstract

Kotlob (*Arbutus andrachne* L., Ericaceae) is a vital plant for the Palestinian economy and folkloric culture. Kotlob is used traditionally as a diuretic, blood tonic, antiseptic, antidiabetic, anti-inflammatory, antidiarrheal, depurative, and laxative. In this study, antioxidant activity, total phenols, and phytochemical characteristics were determined in the leaves and fruits of kotlob. Mature fruits and well-developed leaves were collected from Mahmiyat Wadi Al-Quff, in Hebron city from late September to late October 2021. The antioxidant activities were assessed by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging assays; the total phenolic content (TPC) was determined using the Folin-Ciocalteau method; and the volatile compounds were determined using GC-MS equipped with electron impact mode. HPLC with PDA detector was used for analysis of phytochemicals.

Kotlob leaves exhibited higher antioxidant capacity compared with fruits. Leaves showed 91.41%, fruits showed 31.67%, scavenging as assessed by $DPPH^{\bullet}$ and 94.86%, 87.82% scavenging as assessed by $ABTS^{\bullet+}$, respectively. Moreover, leaves showed the highest value of total phenolic content 391.25 mg GAE/g, while fruits 64.16 mg GAE/g. Phytochemical analysis of kotlob samples including screening of saponins, steroids, tannins, terpenoids, and other secondary metabolites revealed that kotlob fruits and leaves are a good source of phenolic groups, anthocyanin and tannins. On the other hand, saponins steroids, terpenoids and flavonoids were only found in the leaves.

GC-MS analysis showed hydroquinone at *Rt*=10.702 in the leaves, while penten-1-ol was presented in fruits at *Rt*=2.043, so low molecular weight volatile compounds can partially participate in the overall aroma of leaves and fruits, but overall there is no major volatile compound under our experimental conditions. On the other hand, in HPLC rutin was identified at *Rt* =47.4 min in leaves, while gallic acid was detected in fruits at 7.78 min.

Methanolic extracts of leaves and fruits of kotlob were investigated for their antimicrobial activity against two bacterial strains, one gram-positive *S. aureus* and one gram-negative *E. coli*. The results revealed that the methanolic extract of leaves displayed antibacterial activity against *S. aureus* bacteria with a zone of inhibition of 17mm compared with the positive control

(cefoxitin), and the methanolic extract of fruits displayed antibacterial activity against *E. coli* bacteria with a zone of inhibition of 10mm compared with the positive control (meropenem).

This study was the first to screen and evaluate phytochemical compounds in selected Palestinian kotlob for antioxidant and antibacterial activity. The studied kotlob extract demonstrated exceptional bioactivities. For the development of natural products, these extracts have presented outstanding potential. Future research needs to be done to verify these actions in various matrices.

Keywords: *Arbutus andrachne* L., antioxidant, phytochemical, GC-MS, HPLC, antibacterial.

Chapter 1: Introduction

Chapter 1: Introduction

Arbutus andrachne L. known as Greek strawberry and kotlob in Arabic, it is an evergreen small tree belongs to the family Ericaceae, extending from the eastern Mediterranean to the northern Black Sea region (Davis, 1970; Jaradat et al., 2016). The tree's scarlet twisted stems and branches, ornamental flowers, decorative fruits and evergreen leaves make it particularly appealing and valuable in the landscape (Facciola, 1990; HEDRICK, 1972; Karam & Al-Salem, 2001). Kotlob is self-fertile reproduction. In Palestine, kotlob thrives on rocky hills with high clay content and minimal ventilation. Its fruits ripen in Autumn, the flowers ripen from March to April and are white or yellowish green and the seeds from September to October. Bees pollinate the hermaphrodite flowers (Sakar et al., 1991; Serçe et al., 2010). Additionally, fruits become sweet with good taste when ripe and can be eaten fresh, dried or as jam (Facciola, 1990; Hedrick, 1972). The leaves are large, round and straight. Their tops are black, while their bottoms are pale green and lint-free. Only young plants have geared leaves (Kayacik, 1982).

In recent decades, populations of *A. andrachne* have faced very poor restoration of in natural habitats maybe because of the difficult germination of seeds in natural conditions and the slowness of plant growth (Karam & Al-Salem, 2001).

The leaves and fruits of *A. andrachne* have traditionally been used as an astringent and urinary antiseptic, as well as for urinary system, blood tonic and laxative, cancer treatment, aching joints and wound healing and depurative properties treatment for diarrhea and hemorrhoids (Fonseca et al., 2015; Sakar et al., 1991; Sakar et al., 1992; Şeker & Toplu, 2010). Additionally, it is the most functioning plant to eliminate gallstones (Dingil, 1990).

Arbutus andrachne has medicinal benefits because of its secondary metabolites; phenolics, flavones, aromatic hydroxyacids (Tawaha et al., 2007), anthocyanin, tannin (Baskan et al., 2019), iridoids, fat-soluble antioxidant (Tenuta et al., 2019). Triterpens, sterols in the fruits (Grishkovets et al., 1979) and triterpenoides, sterols, and lipids are isolated from bark, leaves, and fruits. Arbutin, monotropeins, unedoside, and catechin were also isolated from bark and leaves (Sakar et al., 1991).

In Palestine, a few studies have been conducted to assess the antioxidant, phytochemical and antibacterial properties of kotlob fruit and leaf extracts. In addition, kotlob has become rare and almost extinct. Therefore, the main goal of the present research is to identify the antioxidant, phytochemical and antibacterial properties of kotlob to indicate the importance of preserving its cultivation and realizing its pharmaceutical importance as a medical product for the treatment of many health problems.

Chapter 2: Literature Review

Chapter 2: Literature Review

2.1. The importance of therapeutic plants

The World Health Organization (WHO) has acknowledged the value of traditional medicine and the promising outcomes of its development through the testing of medicinal plants. Plants perform a vital part in the discovery of new drugs (Organization, 2013). Clinical studies of plant-derived compounds investigated to treatment of various animal and human diseases through health applications like infectious, cardiovascular, cancer and inflammatory diseases (Kunduhoglu et al., 2011; Saklani & Kutty, 2008). Medicinal plants are considered a plentiful resource of antioxidant activity (Tlili et al., 2013). In addition, herbal bioactive compounds have antimicrobial, anti-diabetic and anti-proliferative effects (Tenuta et al., 2019). Bioactive products include phenolic compounds (Thaipong et al., 2006), flavonoids (Özgen et al., 2009) anthocyanins, triterpens, fatty acids (Tenuta et al., 2019) and more. Furthermore, medicinal plants are used extensively in developing nations due to their unique qualities, adaptability, ease of use, affordability, low levels of technological input, and growing economic significance (Aslantürk et al., 2021).

2.2. Palestinian herbal medicine

Palestine has been considered to be a richly diverse land with abundant flora; due to the diversity of the soil and climate (e.g. *Asphodeline lutea* L. (Liliaceae), *Clematis cirrhosa* L. (Ranunculaceae), *Eryngium creticum* (Umbilliferae), *Juglans regia* L. (Juglandaceae), *Lycium europeum* L. (Solanaceae), *Pistacia lentiscus* L. (Anacardiaceae), *Salvia fruticosa* L. (Labiatae) (Ali-Shtayeh et al., 1998). On the other hand, in Palestine, many plant populations that are important for food and medicine are in danger of disappearing and are being eroded genetically. For example, *Arbutus andrachne* L. numbers are suffering from seed germination difficulties, slow growth, dangerous habitat fragmentation, overexploitation, extensive agriculture and human activity-related destruction (Karam & Al-Salem, 2001). Climate change and global warming scenarios have changes in natural distribution that must be taken into account (Sarikaya & Orucu, 2021).

2.3. *Arbutus andrachne* L.

2.3.1. General aspects

2.3.1.1. Kotlob classification (Riedl & Davis, 1978; Sicak & Eliuz, 2019)

Family	**Ericaceae**
Genus	***Arbutus***
Species	***andrachne***

2.3.1.2. Origin and distribution

Arbutus andrachne L. is found naturally in the Mediterranean and southwestern Asia, the Black Sea Regions are enclaved by kotlob, too (Eminağaoğlu & Anşin, 2003; Markovski, 2017). According to POWO, (2024), all of Albania, Bulgaria, Cyprus, East Aegean Is., Greece, Iraq, Kriti, Krym, Lebanon-Syria, Palestine, Transcaucasus, Turkey, Turkey-in-Europe are the native of kotlob, green-colored in the **Figure (2.1) (**POWO; 2024).

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Figure (2.1): Distribution of kotlob, green-colored areas represent its' native.

2.3.1.3. Description of the plant

The kotlob tree is growing up to 5 m high (Bamidele et al., 2014), a thickly branched tree. The tree has a beautiful orange-brown bark with a wide and rounded crown. The entomophilous, bisexual flowers are white or yellowish-green when they bloom in the spring. The oval-shaped leaves have dark upper faces, but light green lower sides. The fruit which ripens in autumn is a red-colored with a roundish shape (Abidi, Habib, Mahjoub, et al., 2016; Melia et al., 2012; Şeker & Toplu, 2010; Serçe et al., 2010) **Figure (2.2) (**POWO; 2024).

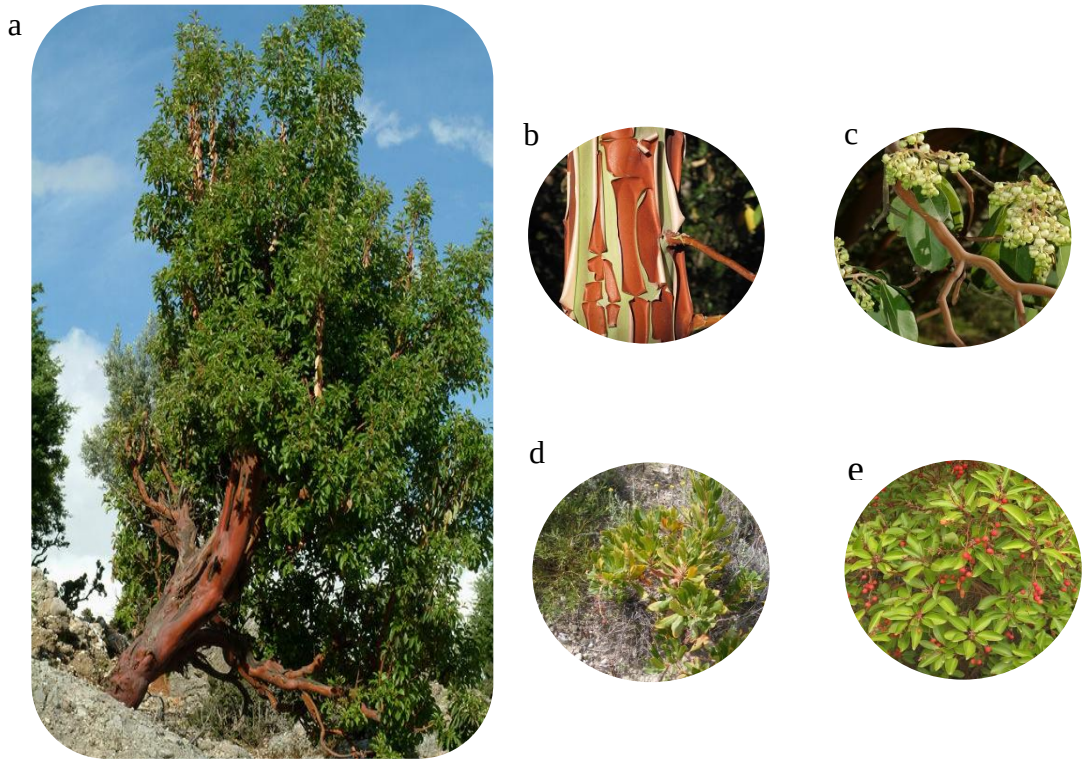

Figure (2.2): *A. andrachne* tree (a), bark (b), flower (c), leaves (d), fruit (e).

2.3.1.4. Environmental requirements:

Kotlob grows slowly, it develops in well-drained, nitrogenous and fertile, alkaline soil with high clay content. Also, it grows in stony, mountainous habitats (Gungor et al., 2002).

2.3.2. Food and industry

2.3.2.1. Kotlob as food

Some reports were published that sweet ripened fruits are edible as fresh or dried (Cavuşoğlu et al., 2015; Molina et al., 2011) or changed into an assortment of dietary products including, jelly, jam (Dönmez, 2018), marmalade, and alcoholic beverages (spirits drinks and wine) (Baskan et al., 2019). Kotlob fruits are harvested from the wild, not cultivated (Ayaz et al., 2000).

2.3.2.2. Kotlob and industry

Kotlob wood is utilized for packaging, stools, making chairs and other furniture like curved spindles, as well as producing biofuel. Wood-derived oil is widely used in both medicine and the fragrance industry (Gultekin, 2004). Furthermore, bakeries use the plant's wood as charcoal. Because kotlob is a barren species with a strange nature and acceptable soil demand, it is an important species in studies on reforestation. Its long-lasting red berries and evergreen leaves make it a good decorative plant for greenery. Recently, green shoots in the Turkish provinces of Isparta and Antalya have been used in the production of tourism goods. Several countries receive these decorative items as exported goods (Sarikaya & Orucu, 2021). The powdered dried leaves are used as a skin-whitening compound in facial masks (Issa et al., 2008).

2.3.3. Traditional uses as medicine

Kotlob leaves and fruits are traditionally used as laxative, urinary antiseptic, blood tonic, diuretics (Abidi, Habib, Yassine, et al., 2016; Oran), they are also used as anti-diarrheal, astringent, and anticancer agents (Sakar et al., 1992; Şeker & Toplu, 2010). In Jordanian traditional medicine, flowers, barks, and leaves of kotlob are used for the treatment of asthma (Amro et al., 2013) and diabetes mellitus (DM) (Hamdan & Afifi, 2008).

2.3.4. Nutritional value of kotlob

The study of nutrition science examines how food ingredients and diets as a whole interact with human and other biological systems, which are necessary to maintain function, prevent disease and supply energy in both individuals and communities (Beauman et al., 2005). In addition to water, humans need five types of nutrients from food, which are carbohydrates, proteins, and fat, which are relatively required in large amounts and are known as macronutrients. The other

two types, vitamins and minerals, are required in small amounts and are called micronutrients (Martiniakova et al., 2022).

Kotlob with its edible fruits, which can be eaten fresh or dried or used as jam, are good providers of protein; ash; minerals (aluminum, arsenic, boron, beryllium, cadmium, calcium, chromium, copper, gallium, iron, potassium, and magnesium, manganese, nickel, lithium, phosphorus, vanadium, and zinc) (Şeker & Toplu, 2010); vitamins (mainly vitamin C (ascorbic acid) and vitamin E (α-tocopherol)); total sugars (malic acid, fumaric acid, fructose, glucose, sucrose) and dietary fiber (Kivçak et al., 2001; Serçe et al., 2010). Concerning kotlob leaves, it is well documented that they're an excellent source of ash; fibers, neutral detergent fiber (NDF) and acid detergent fiber (ADF) (Karabulut et al., 2006); protein; and condensed tannin (CT) (Kamalak et al., 2010).Kotlob leaves have natural sources of fatty acids, that determined through the (LC-MS) by Jaffal et al. (2020) who revealed the presence of linoleic acid, lauric acid and myristic acid.

2.3.5. Phytochemistry

Phytochemicals are a class of naturally occurring compounds, primarily found in fruits, vegetables, legumes, beans, nuts, and whole grains, include many different compounds such as phytosterols, saponins, flavonoids, terpenes, triterpenoids, iridoids glucoside and others, which are what give plant-based foods and beverages their protective health benefits (Brindha, 2016; Şeker & Toplu, 2010; Serçe et al., 2010). The taste, color, and smell of plant foods, like the strong smell of garlic and the vibrant color of blueberries, are all attributed to phytochemicals, which are a broad and diverse class of chemical compounds. Because of its significant biological qualities and antioxidant activity, it is also regarded as a multipurpose food ingredient.

Phytochemical research revealed that kotlob with all its different parts has flavonoids, tannin, anthocyanin, aromatic hydroxyacids, phenolic compounds, non-volatile components, and some other classes of secondary metabolites. These phytochemicals are found in leaves, fruit, bark, wood and root. Also, it supports kotlob with remarkable pharmacological properties such as anticancer, antioxidant, anti-inflammatory effects, and others (Baskan et al., 2019; Einbond et al., 2004; Tawaha et al., 2007).

2.3.5.1. Phenolic compound

Phenolic compounds that are mainly found in fruits and vegetables have a variety of biological advantages, such as improving β-cells and relieving oxidative stress and inflammatory response (Lin et al., 2016).

2.3.5.1.1. Flavonoids

Flavonoids have strong antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties and are potent inhibitors of several enzymes, including xanthine oxidase (XO), cyclo-oxygenase, and lipoxygenase, they primarily have unique health-promoting effects and are regarded as one of the most significant compositions of nutraceutical, pharmaceutical, medicinal, and cosmetic application (Panche et al., 2016).

Kotlob leaf has the potential to play a significant health-promoting role, due to its anti-inflammatory, anti-microbial, and antioxidant effects, that mainly related to flavonoids as major compounds such as myricetin-3-O-rhamnopyranoside, quercetin-3-O-rhamnopyranoside, quercetin-3-O-arabinoside (Sakar et al., 1992), catechin gallate (Legssyer et al., 2004), and isoquercitrin (Maleš et al., 2006). According to phytochemical studies, kotlob fruit and leaves are a good supply of arbutin (Sakar et al., 1992).In addition, kotlob fruit, leaves and roots include rutin, catechins, quercetin, myricetin (Ergun et al., 2014) and flavones (Bertsouklis et al., 2021).

2.3.5.1.2. Anthocyanins

Anthocyanins are naturally red, purple, and blue pigments found in plants mainly fruits, vegetables, and flowers. Anthocyanins are phenolic phytochemicals with a variety of applications. Although it has long been used as a natural food coloring and dye, it has also been used as a medication to prevent several illnesses, including DM, cardiovascular disease (CVD), and cancer, as well as to improve neurological and visual health. This is achieved through a variety of mechanisms and pathways, such as the scavenging of free radicals, which lowers oxidative stress, and indirect pathways, which include the inhibition of cell proliferation and apoptosis by lowering lipid peroxidation and oxidative stress. Of the anthocyanin pigments found in plants, cyanidin-3glucoside is the main one. However, cyanidin, delphinidin,

pelargonidin, malvidin, peonidin, and petunidin are the most prevalent forms of anthocyanidins found in plants (Khoo et al., 2017).

Consumption of fresh kotlob fruits provides a health benefit due to the antioxidant potential that is mainly related to the anthocyanins found in fruits, especially cyanide-3-glucoside, which scavenges on reactive oxygen species (Bertsouklis et al., 2021; Güzel et al., 2015) and delphinidin 3-O galactoside (Pawlowska et al., 2006).

2.3.5.1.3. Tannins

Chung et al. (1998) reported, tannins are water-soluble polyphenols found in a variety of plant-based diets. Tannins have the potential to be both antimutagenic and anticarcinogenic due to their antioxidative properties, which are crucial in preventing oxidative damage to cells, including lipid peroxidation. Tannins inhibit the growth of numerous yeasts, bacteria, viruses, and fungi. We have also discovered that foodborne bacteria, aquatic bacteria, and microorganisms that produce off-flavors were inhibited by tannic acid and propyl gallate. Tannin's antibacterial qualities can also be utilized in food processing to extend the shelf life of specific foods, like catfish fillets. Tannins have also been shown to have additional physiological effects, including the induction of liver necrosis, lowering of blood pressure, lowering of serum lipid levels, and control of immune responses. For these effects to occur, the type and dosage of tannins are necessary.

Kotlob fruit can help to relieve stomach, and bowel laziness, reduce high blood pressure, relieve liver bloating, and have antipyretic properties due to tannin (Sarikaya & Orucu, 2021). It also has therapeutic effects on urinary tract infections as well as anticancer, antibacterial, and antioxidant activity (Baskan et al., 2019).

2.3.5.1.4. Saponins

Triterpene glycosides or surface-active sterols are known as saponins. There are many different types of them found in plants, but only about 28 of them are regularly consumed by humans. Among these, spinach, peanuts, chickpeas, and soybeans are the most often consumed. Even within a single plant species, there are numerous distinct saponins. Although saponins are toxic to fish and insects and have antibiotic properties, they seem to be essentially non-toxic to

humans and stay in the gastrointestinal tract. In some mammalian species, dietary saponins either as food plants that contain them or as isolated compounds reduce plasma cholesterol levels. As a result, their importance in human diets to lower the risk of coronary heart disease is probably justified (Oakenfull, 1981).

Phytochemical analysis revealed that kotlob fruit and leaves are a good source of monotropein, monotropein methyl ester, stilbericoside, unedoside, pomolic acid, and ursolic acid (Cirva et al., 1980; Grishkovets et al., 1979; Jaffal et al., 2022; Sakar et al., 1991). Also Tawaha et al. (2007) investigated that the bark and fruits of kotlob contain triterpenoids. Linalool is presented in the leaves, demonstrated antinociceptive activity (Jaffal et al., 2020). The major compounds of kotlob wood as essential oils are isobornyl acetate, cinnamyl alcohol, and 4-tert-butylcyclohexyl acetate (Sicak & Eliuz, 2019).

2.3.5.1.5. Phytosterols

Plant sterols and plant stanols are the two main categories of phytosterols, which are bioactive compounds that resemble cholesterol and are naturally present in foods originating from plants. The three plant sterols that are most frequently found in diets are stigmasterol, beta-sitosterol, and campesterol. Because phytosterol can decrease the intestinal absorption of cholesterol (30–50%), increase the rate of cholesterol removal by transintestinal cholesterol excretion (TICE), and decrease the rate of cholesterol esterification in the enterocyte, daily consumption of phytosterol is linked to a significant reduction in low-density lipoprotein (LDL) cholesterol. Therefore, to lower the risk factors for CVD in people with hypercholesterolemia, phytosterols are thought to be a useful supplement to pharmacological therapy (Cabral & Klein, 2017).

Jaffal et al. (2020) identified some phytosterols from kotlob leaf extract via the (LC-MS) namely; β-Sitosterol and ursolic acid which exert analgesic effects due to (Jaffal et al., 2022). Also, Sakar et al. (1992) reported, b-amyrin, a-amyrin, and lupeol.

2.3.5.1.6. Organic acids

Organic acids are most commonly found in fruits. Furthermore, the kind and concentration of organic acids vary among species, developmental phases, and tissue types; these variations impact the flavor and acidity of fruits and vegetables as well as other organoleptic properties.

Because of their antioxidant activity, organic acids are thought to be important protective agents against a variety of diseases, and they also play a critical role in regulating pH (Vallarino & Osorio, 2019).

Sakar et al. (1992) showed that the organic acids profile of kotlob fruits and leaves mainly contains citric and malic acid.

2.3.5.1.7. Phenolic acid

Aromatic secondary metabolites found in many different parts of the plant kingdom are called phenolic acids. For instance, the concentration of hydroxycinnamic acids was greater than that of hydroxybenzoic acids. In terms of consumption, hydroxycinnamic acids contribute more to the total amount of polyphenols consumed than flavonoids or derivatives of benzoic acid. Coffee is the food that consumes the most phenolic acids due to its high hydroxycinnamic acid concentrations. Furthermore, some epidemiological and experimental investigations document the protective effects of phenolic acids against a range of degenerative illnesses. The interest in phenolic acids biological roles as secondary metabolites, their effects on food quality, and their organoleptic qualities led to the development of current analytical techniques for these compounds. The potential protective effect of phenolic acids against oxidative damage diseases (such as cancer, stroke, and coronary heart disease) through fruit and vegetable consumption has sparked renewed interest in these compounds (Lafay & Gil-Izquierdo, 2008; Robbins, 2003).

Jaffal et al. (2020) proved a strong anti-nociceptive effect of kotlob leaf extract because of phenolic compounds such as gallic acid via improved an important mechanism of action of it.

2.3.5.2. Non-phenolic compound

Carotenoids

Carotenoids are pigments that dissolve in lipids and give a wide range of foods the color they have. They can be separated into two categories: carotenes, which are non-oxygenated molecules like lycopene and α-carotene, and xantophylls, which are oxygen-containing molecules like lutein and zeaxanthin (Shen et al., 2009). Some of them are pro-vitamin A carotenoids, which are then converted into vitamin A and can help prevent serious eye

conditions like night blindness, infection susceptibility, rough, scaly skin, and delayed development of teeth and bones. To varying degrees, almost all carotenoids exhibit scavenging abilities against an excess of free radicals that can be generated during a cell's life cycle (Santocono et al., 2007). The most studied of the carotenoids' properties is their antioxidant capacity, which has been proposed as their main mechanism of action (Amorim-Carrilho et al., 2014).

The presence of carotenoid in kotlob fruit and leaf investigated by (Baskan et al., 2019).

2.3.6. Biological activities

The biological properties of multiple Arbutus species, such as their antitumor, antimicrobial, hypoglycemic, hypocholesterolemic, cardiovascular, anti-diarrheal, and antioxidant have been studied. Herein, we report the main investigated activities useful to prospect a potential use of kotlob parts.

2.3.6.1. Antioxidant activity

Because oxidative strain is the cause of many medical conditions, the concept of "antioxidant" means chemicals, secondary metabolites (terpenes, flavonoids), or molecules that can stall or even prohibit permanent harm such as paralysis and cancer, thereby promoting health benefits because of the protection role of antioxidants against oxidative stress of reactive oxygen species on biomolecules such as genetic material, lipids, and protein. Antioxidant biological activity procedures demand distinct methodologies beyond an exhaustive explanation. This is because different kinds of chemicals, from the well-known vitamin C to the relatively new but frequently discovered peptides sourced from either animals or plants, might show this form of activity. Thus, quantifying antioxidant activity entails quantifying the rate of reaction or the potential impact of antioxidants on the rate of autoxidation of the substrate they are known to shield (Amorati & Valgimigli, 2018; Çol Ayvaz et al., 2018; Gul et al., 2017; Özgen et al., 2009). Plants and humans both contain antioxidant enzymes, such as glutathione peroxidases (GPx), superoxide dismutases (SOD), and catalase (Andre et al., 2010). Antioxidant enzymes like peroxidase and catalase rise in stressed plants. Catalase activity was highest in kotlob leaf extract, and ascorbate peroxidase activity was highest in kotlob flower extract (Ergun et al., 2014). Abidi, Habib, Mahjoub, et al. (2016) found that the kotlob roots have the highest in vitro

antioxidant activity after studying the antioxidant capacity of various extracts from the bark roots, leaves, and fruits of kotlob. In recent years, antioxidant capability (DPPH and FRAP methods) and total phenol and flavonoid contents of kotlob fruits and flowers were studied (Saral et al., 2017). Flowers exhibited greater contents of both phenols and flavonoids and a better ferric-reducing capacity strength than the fruits. In an identical direction, Okmen (2015) found that kotlob flowers had greater ABTS radical scavenging activity than leaves. Alzoubi et al. (2018) was postulated that the antioxidative qualities of the methanolic fruit kotlob extract would prevent chronic sleep deprivation-induced impairment of hippocampal memory via normalized catalase, SOD, GPx, and oxidized glutathione (GSSG) antioxidant enzymes. The β-carotene/linoleic acid assay results showed that the essential oil extracted from the wood of kotlob had lipid peroxidation inhibitory activity than standard α-tocopherol (α-TOC). Compared to standard α-TOC, essential oil demonstrated superior cation radical scavenging activity in the ABTS˙+ assay. In DPPH-free scavenging activity, the essential oil showed a higher value than standard. The essential oil demonstrated superior CUPRAC activity compared to αTOC when used as a pharmaceutical standard (Sicak & Eliuz, 2019).

2.3.6.2. Anti-proliferative activity

One of the primary causes of death in the world is cancer. Problems with cancer therapy are present at the same time as the global incidence of cancer is steadily expanding. Several factors have restricted the use of anticancer agents in medical centers, including the rise of resistance, their high toxic effects, and their expensive price (Ferlay et al., 2014). To address these problems, a significant portion of research is currently devoted to developing novel anticancer drugs. The cytotoxicity of kotlob leaves and stems against breast adenocarcinoma (MCF-7) cell lines was examined by (Abu-Dahab & Afifi, 2007). Ethanol extracts of the leaves and stems revealed survival rates of 103.48 and 111.05%, respectively. Methanol, chloroform, and n-hexane extracts were tested against lung carcinoma (A549) and MCF-7 cell lines to determine their anti-proliferative effects (Alsabri et al. 2013). The proliferation of MCF-7 cells was significantly inhibited by both the methanol and chloroform extracts. The n-hexane extract was determined to have a lower activity. It's interesting to note that the chloroform extract was effective against A549 cells. However, Abu-rish et al. (2016) reported that the aerial parts of kotlob proved weak antiproliferative activity. Aslantürk et al. (2021) hepatocellular carcinoma

(Hep3B) and (HepG2) are not susceptible to the cytotoxic effects of kotlob stem methanol extract.

2.3.6.3. Hypoglycaemic activity

Diabetes mellitus (DM) is a chronic illness caused by insulin resistance or an autoimmune disease. Including two types: type I DM, also known as autoimmune disorder, arises due to the inability of β-cells to secrete insulin (Aja et al., 2015), and type II DM means impaired in the insulin receptors causes a halted signal transduction pathway and increased blood glucose levels (hyperglycemia) (Bamidele et al., 2014). According to WHO, DM causing a lot of abnormalities such as abnormal levels of liver enzymes and inflammatory markers, dyslipidemia, and hemodynamic deviations. It has been reported that several species of plants may reduce the severe symptoms of diabetes. The use of the ethanol extract of the entire kotlob plant demonstrated the action against the enzyme that hydrolyzes carbohydrates. In actuality, the extract's IC50 value against a-amylase was 0.44 mg/mL. An improvement in the blood sugar was identified (maximum rise 65%) in contrast to acarbose (25%) and the control group (88%), demonstrated this action in vivo. The first hour following sucrose loading was when the greatest hypoglycemic effect appeared, and it seemed to last for at least five hours (Hamdan & Afifi, 2008). The results of Abu Zaiton et al. (2019) indicated that kotlob ethanol extract has hypoglycemic and hypolipidemic effects on diabetic Wistar albino rats induced by streptozotocin. Furthermore, based on the various phytochemicals present in kotlob, the induction of streptozotocin has resulted in decreased liver damage and normalization of the hepatic enzymes, aspartate aminotransferase (AST) and alanine transaminase (ALT) (Issa et al., 2008).

2.3.6.4. Antibacterial and antifungal activity

According to the WHO, antimicrobial compounds are commonly identified in huge numbers in medicinal herbs that would be a good source to obtain a wide range of drugs. Baskan et al. (2019) found that ethanolic extracts had the greatest effectiveness against *S. aureus* and *Bacillus cereus*. In a similar vein, kotlob extracts were shown to inhibit the growth of five bacterial species in another study carried out in Turkey by (Ergun et al., 2014), with the inhibition zones ranging from 8 to 17 mm. The *S. aureus* (17 mm) bacteria showed the

strongest antibacterial activity. The results of Sicak & Eliuz. (2019) investigation into the antioxidant and antimicrobial properties of essential oil extracted from Turkish kotlob wood indicate that the oil has the potential to be used as an antibiotic in food. Arbutus spp. leaves have the most promising action against Gram-positive bacteria, according to (Tenuta et al., 2019). On the other hand, the paucity of information regarding kotlob oil's antibacterial potency in the literature (Sicak & Eliuz, 2019). Additionally, they demonstrated a moderate antimicrobial effect against *Salmonella typhimurium* and *Escherichia coli*, as well as strong antibacterial action against *Pseudomonas aeruginosa*, *Bacillus subtilis*. Abidi, Habib, Mahjoub, et al. (2016) revealed the antibacterial properties of the root extracts from the kotlob bark for the first time, with a focus on Gram-positive bacteria. According to Amro et al. (2013) *Propionibacterium acnes*, which overgrows in the pilosebaceous unit, contributes to the development of acne vulgaris, one of the most prevalent skin diseases. The potential use of kotlob leaves, flowers, and bark as a treatment for acne was examined, due to their effectiveness against *P. acnes*. In some studies, Ergun et al. (2014) kotlob leaves and flowers exhibited intriguing activity against both Gram-positive and Gram-negative bacteria, but not against yeasts like *Candida albicans* (Assaf et al., 2016).

2.3.6.5. Anti-inflammatory and antipyretic effect

Due to Eze et al. (2019), inflammation is a normal biological pathway that happens in response to tissue damage. Within inflammation, several inflammatory cytokines are released at the site of injury to promote vasodilation in addition releasing of plasma, fluids, and leukocytes into the inflammation location to initiate the regeneration of tissue. As Blomqvist & Engblom (2018) mentioned another typical reaction in the body to combat infection is called pyrexia (fever). It is a multifaceted process defined as an increase in body temperature brought on by pyrogens, which are endogenous or exogenous stimuli. In particular, certain common markers, such as prostaglandin E2 (PGE_2) and interleukin-6 (IL-6), contribute to the development of fever and inflammation. Jaffal et al. (2021) showed a decrease in the level of PGE_2 and IL-6, because of the effect of kotlob leaves extracts.

2.3.6.6. Analgesic properties

Pain is a health issue that hurt society and the economy requires treatment with analgesic which have side effects (Cazacu et al., 2015). So, a large number of medicinal plants have safer effective analgesic properties(de Cássia da Silveira e Sá et al., 2017). Jaffal et al. (2020) included a strong anti-nociceptive effect of kotlob leaf extract in thermal and chemical pain models, this result can open an option for the development of safer powerful analgesic.

2.3.6.7. Effects on cardiovascular diseases

Recent research Abidi, Habib, Mahjoub, et al. (2016) examined the effects of kotlob methanol extracts of leaves, fruits, and roots on the cardiodynamics of separated perfused rabbit hearts. Results showed that root extracts reduced left ventricular pressure by 32%. The tested extracts did not show any discernible effects on heart rate. On the coronary flow, the methanol extracts of leaves, fruits, and roots had little or no impact. Also, over one minute, the roots raised the coronary flow at concentrations of one and two mg/mL. Malondialdehyde (MDA) levels in heart tissue treated with root extracts dropped from 70.51 to 48.58 nmole/g of tissue after electrolysis. In addition, the extract from roots had an antihypertensive effect. This final action could result from the ability to lower heart tissue's MDA content, protect against the production of free radicals, and lower left ventricular pressure.

2.3.6.8. Anti-tyrosinase effect

In terms of surface area, the skin is the largest organ in the body. It makes up 15% of the body weight and covers the whole surface of the human body (Petit & Pierard, 2003). Depending on the type of skin, melanocytes located in the basal layer of the epidermis synthesize different amounts of melanin, the skin's natural pigment. Several variables impact and control this complex biochemical process, including environmental, hormonal, and genetic influences. Melanocyte proliferation and differentiation can be influenced by a variety of inflammatory mediators, cytokinase, and growth factors in the epidermis, all of which can affect pigment synthesis (Briganti et al., 2003; Issa et al., 2005; Perez-Bernal et al., 2000). Tyrosinase is the essential enzyme for the synthesis of melanin, also affects a few intermediate phases of the formation of pigments (Sturm et al., 2001; Zuidhoff & Van Rijsbergen, 2001). Kotlob aqueous extract is applied topically as a skin-lightening agent in some regions of Jordan. In order to compare the effectiveness of various plant extracts with the reference inhibitors, IC50 was

measured in (Issa et al., 2008) study. The highest concentration of arbutin and ursolic acid was found in the stem of kotlob, compared to the leaves, fruits, and other parts that were tested. The most effective extract with the lowest IC50 value was the methanolic extract, according to the results. Tyrosinase activity was 97.49% inhibited by 9 mg of this extract.

2.4. Analytical techniques

One of the most important bioanalytical methods utilized in many areas of chemistry and the life sciences is chromatography. It enables the qualitative and quantitative separation, identification, and purification of compounds with different origins, classes, and nature from a complex mixture. Based on a compound's ability to bind specificity, it can be separated into groups and have the appropriate shape, size, and charge. Due to its widespread application in separation science, it is one of the many flexible techniques with numerous variations that are effectively used for separation on an industrial and laboratory scale (Kumari et al., 2022). The foundation of chromatography is the idea that mixtures of molecules applied to surfaces or solids, as well as fluid stationary phases (stable phases), separate from one another while moving with the help of a mobile phase. The molecular properties associated with adsorption (liquid-solid), partition (liquid-liquid), affinity, or variations in their molecular weights are the factors that have an effective effect on this separation process (Cuatrecasas et al., 1968).

2.4.1. Gas Chromatography-Mass Spectrometry (GC-MS)

Numerous metabolites can be measured using GC-MS (Villas-Bôas et al., 2005). This includes a variety of volatiles that can all be measured directly, including lipids, ketones, aldehydes, alcohols, heterocyclic compounds, isocyanates, isothiocyanates, sulfides, and hydrocarbons with up to 12 carbons. Furthermore, after derivatization, a variety of non-volatile or semi-volatile metabolites are accessible, such as sugars, sugar-phosphates, sugar alcohols, organic acids, amino acids, lipids, peptides, long-chain alcohols, alkaloids, amines, amides, etc. (Wittmann, 2007).

2.4.2. High-performance liquid chromatography (HPLC)

This chromatography technique allows for the quick purification, structural and functional analysis of numerous molecules. This method produces flawless results when it comes to

identifying and separating proteins, steroids, lipids, carbohydrates, nucleic acids, and other physiologically active molecules. The mobile phase in HPLC flows through columns at a high flow rate of 0.1–5 cm/sec while operating at an atmospheric pressure between 10–400. This method improves the separation power of HPLC and completes the analysis quickly by using small particles and applying high pressure to the solvent flow rate. A high-pressure pump, solvent depot, commercially prepared column, detector, and recorder are necessary parts of an HPLC apparatus. A computerized system helps control the duration of the separation, and material is accumulated (Regnier, 1983).

2.5. Problem statement and motivation of the study

A few studies about Palestinian herbs are available, but their composition, efficacy, and safety are still unexplored. In addition, due to the undesirable side effects of orthodox synthetic medications such as toxicity and carcinogenicity and the emerging microbial resistance to available antimicrobial agents, attention has considerably increased to finding naturally occurring antioxidant and antimicrobial compounds suitable for use in food and/or medicine.

Palestinian kotlob (*Arbutus andrachne* L.) was chosen for this study because of its medicinal reputation among researchers. The lack of phytochemical composition of its volatiles, semi-volatiles, and minerals and the scarcity of pharmacological studies on indigenous kotlob in Palestine motivated this research.

2.6. Aim of the study

The main purpose of this study is to reveal phenolic content, antioxidant and antibacterial biological activities of the grown kotlob in Palestine. For this purpose, the total phenolic content of kotlob samples were calculated using Folin-Ciocalteu's. The antioxidant activities of *A. andrachne* plant extracts were investigated using radical scavenging activity $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods. To screen secondary metabolites, by using GC-MS and HPLC-PDA. Finally, the antibacterial effects of kotlob specimens were determined by the disk diffusion method.

2.7. Objectives of the study

1. To assess the relation between *A. andrachne* fruits and leaves concerning the contents and biological activities.
2. To evaluate the antioxidant activity of *A. andrachne* using $ABTS^{\bullet+}$ & $DPPH^{\bullet}$ assays.
3. To identify the chemical components of the phytochemical and volatile compounds from *A. andrachne* L. fruit and leaves using GC-MS and HPLC-PDA.
4. To determine the inhibitory effects of *A. andrachne* leaves and fruits on the growth of selected gram-negative bacterium strain namely *Escherichia coli,* and a gram-positive bacterium *Staphylococcus aureus* in comparison with positive controls.

Chapter 3: Materials and Methods

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3.1. Experimental site and sampling

A. andrachne ripen fruits and leaves were collected from Mahmiyat Wadi Al-Quff, which is located near Tarqumiayah, from late September to late October 2021.

3.2. Sample Preparation

The fruits and leaves were washed thoroughly 2-3 times with tap water. The samples were dried in the shade at room temperature for three weeks. Kotlob leaves and fruits were ground to a fine powder using a grinder (Morphy Richards, MR9100, British). All samples were stored in a glass jar at ambient temperature until initial sample preparation and protected from light until the analyses (Okmen, 2015).

3.3. Total phenols

3.3.1. Preparing the extract

Ten milliliter of methanol (80% v/v) was used to soak 1g of the powdered fruits and leaves with shaking at 25°C for 24 hours, as reported in (Labtech, Model No. LSI-3016R, Daihan Labtech India Pvt. Ltd., Hyderabad, India) (Dowek et al., 2020). The extracts were filtered by Whatman filter paper (Lot No. CB162201, 150mm, Germany) to determine total phenols. The extract was kept in an air-tight container in the freezer, as reported by (Qawasmeh et al., 2012).

3.3.2. Preparing Gallic acid as a stock solution

The preparation of gallic acid (GA) required dissolving 250 mg of GA (Batch No. GA/104/18) in 5 ml of methanol (80% v/v), then diluted with distilled water up to 50 ml. A cuvette was filled with five different volumes of GA (50, 100, 150, 250, and 500 µl), and then distilled water up to 10 ml was added, this created variable concentrations of GA.

3.3.3. Preparing Sodium carbonate (Na_2CO_3)

Sodium carbonate (Na_2CO_3) was made by combining 20g of Na_2CO_3 (Lot No. 04/2014) with 80 ml of distilled water (DW) in a beaker, placing the mixture in a magnetic stirrer and hot plate for rapid mixing and homogenizing, then cooling, filtering and adding up to 25 ml of DW.

3.3.4. Total phenols procedure

The total phenolic content (TPC) of fruits and leaves was determined by using the Folin-Ciocalteu (F.C) assay (Slinkard & Singleton, 1977). In plastic macro-cuvettes, 20 µl of variously diluted kotlob extracts (1:50 diluted leaves and fruit extracts) were combined with 1.58 ml DW, 100 µl F.C reagent, then the mixture was mixed. After eight-minute incubation, 300 µl of Na_2CO_3 was added. After vortexed, each cuvette was left in the dark for one hour. At 760 nm, the resulting solution absorbance was checked. For measuring the GA stock solution, 20 microliters of GA (from each concentration in cuvettes) were added instead of the extract. GA milligrams of per gram of extracted samples were used to represent data (mg GAE/g) (Dowek et al., 2020). The blank is water and the assay was carried out in four replications and the results were expressed as averages± SD (standard deviation).

3.4. Antioxidant

According to Dowek et al. (2020), using the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radical scavenging assays to assess the anti-oxidant capacity of kotlob -extracted samples (leaves and fruits) *in vitro*.

3.4.1. Extract preparation

The extraction of kotlob samples followed the provided procedure in (Qawasmeh et al., 2012), where 1g of each dried sample was extracted with 10 ml methanol (80% v/v) for 24 hours at 25 °C in a shaking incubator. Then the extracts were filtered through a filter paper and used to estimate the total phenols.

3.4.2. 2, 2'-diphenyl-1-picrylhydrazyl stable radical ($DPPH^{\bullet}$) assay

The 2,2-diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$), a molecule containing a stable free radical, was used to examine the extract's capacity to scavenge free radicals (Sharma & Bhat, 2009), $DPPH^{\bullet}$ scavenging capacity of extracted solutions were assayed based on the methods reported in (Barros et al., 2007) with minor modification. A stock solution of $DPPH^{\bullet}$ was prepared by dissolving 6 mg of $DPPH^{\bullet}$ (Sigma Aldrich-STBD4146V) with 15 ml of methanol (80 %v/v). Then, 2000 µl of $DPPH^{\bullet}$ of the stock solution was mixed with 30 µl of different diluted plant extracts (1:10, samples). Methanol (80% v/v) was used as a blank, while methanol with $DPPH^{\bullet}$ solution was used as a control, continuously all cuvettes were mixed and kept in the dark for 1 hour at room temperature. Finally, the absorbance of the samples and the control

were measured at 517 nm using a Genway UV-visible spectrophotometer (Cole-Parmer Ltd, UK). All trials were conducted simultaneously with four replications and the results were expressed as averages ± SD.

The radical scavenging activity was calculated as a percentage of $DPPH^{\bullet}$ discoloration using the following equation: $DPPH^{\bullet}$ Scavenging (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100\%$.

3.4.3. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{\bullet+}$) assay

The $ABTS^{\bullet+}$ solution was prepared by following the protocol described by (Qawasmeh et al., 2012), mixing 3 ml of $ABTS^{\bullet+}$ (Sigma Aldrich, Canada) stock solution (7mmol, prepared by dissolving 11.2 mg in 3 ml DW (each 18 mg of $ABTS^{\bullet+}$ need 5ml DW), with 3 ml of potassium persulfate ($K_2S_2O_8$) solution (2.45 mmol, prepared by dissolving 8.8 mg of $K_2S_2O_8$ with 13 ml DW(each 6.6 mg of $K_2S_2O_8$ need 10 ml DW). Then keep in the dark for 24 hours at room temperature.

To get the final absorbance of 0.7000±0.02 at 734 nm, the working solution of $ABTS^{\bullet+}$ was prepared by diluting the $ABTS^{\bullet+}$ solution with DW. A 30 µl of diluted extracts (1:10, samples) was mixed with 2000 µl $ABTS^{\bullet+}$ working solution in micro cuvettes. For control, 30 µl DW was mixed with 2000 µl $ABTS^{\bullet+}$. After mixing, each cuvette was left at room temperature for one hour in the dark. The absorbance of plant extracts (A sample) and the $ABTS^{\bullet+}$ with distilled water (A control) were measured at 734 nm using the Genway UV/Visible spectrophotometer (Dowek et al., 2020). All trials were conducted simultaneously with four replications and the results were expressed as averages ± SD.

The percentage scavenging of $ABTS^{\bullet+}$ was calculated according to the equation: $ABTS^{\bullet+}$ Scavenging (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100\%$.

3.4.4. Data analysis

The average of the four values (n=4) for each sample was calculated. Results were presented as mean ± SD. The statistical significance of difference between the averages of total phenolic and antioxidant values of fruit and leaf extracts was assessed by T-Test using Microsoft Excel. $P < 0.05$ was considered very significant.

3.5. Phytochemicals

Phytochemicals tests of the kotlob samples were examined according to the procedure described by (Velavan, 2015).

3.5.1. Extract preparation

Kotlob samples (3g) were extracted in 60ml methanol 80% for 24 hours at 25°C in a shaking incubator. Then the extracts were filtered through a filter paper and used to screen the phytochemicals content in each sample as the following:

3.5.2. Tests methods:

3.5.2.1. Test for phenolic groups

A few drops of 10% $FeCl_3$ were combined with 2 ml of DW. 1 ml of the extract was used. The phenolic group is indicated by a blue or black color.

3.5.2.2. Test for anthocyanins

Two milliliters extract was heated for 5 min with 1 ml of NaOH (2N) added. Anthocyanin was detected by the use of a bluish-green color.

3.5.2.3. Test for coumarins

One milliliter of extract was mixed with 1 ml of NaOH in a test tube, and after a few minutes in a boiling water bath, the presence of yellow color indicates the presence of coumarins.

3.5.2.4. Test for saponins

Two milliliters of extract and 5 ml of DW were shaken in a test tube, the production of foam signifies the presence of saponins.

3.5.2.5. Test for anthraquinones

One milliliter of a 10% NH_3 solution was added to 2 ml of extract that had been combined with benzene in a test tube. The presence of red, pink, or violet color indicates the presence of anthraquinones.

3.5.2.6. Test for quinones

One milliliter of extract and 1 ml of concentrated H_2SO_4 were combined in a test tube. A positive for quinines is indicated by the presence of red color.

3.5.2.7. Test for steroids

One milliliter extract was mixed with 2 ml of $CHCl_3$ and 50% H_2SO_4. The presence of steroids is indicated by a reddish-brown ring.

3.5.2.8. Test for tannins

Two milliliters of extract, 1ml of DW and 1-2 drops of $FeCl_3$ were added. Tannins are indicated by a green or blue-black color.

3.5.2.9. Test for terpenoids,

The extract was combined with 2 ml of $CHCl_3$ and 3 ml of concentrated H_2SO_4. Terpenoids are indicated by a layer that is reddish-brown in color.

3.5.2.10. Test for flavonoids,

Two milliliters of the extract, a few drops of 1% NH_3 solution were added. The color yellow signifies the existence of flavonoids.

3.5.2.11. Test for phlobatannins,

Two milliliters of the extract, 1 ml of 10% NaOH solution was added. Phlobatannins are indicated by a yellow color.

3.6. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

One gram of leaves and fruits *A. andrachne* was extracted with 10 ml methanol (80% v/v) overnight on the shaker, the suspension was filtered using Whatman filter paper by syringe filters with pore size of 0.45 µM (Millex, Sigma Aldrich, Palestine). All compounds in methanolic extracts were analyzed using Clarus SQ 8S, Perkin Elmer, USA GC-MS equipped with column (BD-5ms, 30m, 0.25 µm film thickness, 0.25mm capillary diameter) as described by (Qwasmeh et al., 2011). The injection volume was 1 µl. The oven temperature was maintained at 80 °C for 2 min and raised to 280 °C at the rate of 8 °C /min. Helium was used as the carrier gas, and the total gas flow and velocity were maintained at 134.3ml/min and 43.1 cm/s, respectively. MS scan speed was 1000amu/s. the molecular mass of the compounds between 50-500 in which M/Z were acquired at 70mv. Each sample's analysis was conducted

twice. The compounds were identified using the NIST05 mass spectral library, and finally, their mass spectra were compared with those published in the literature review (Mujeeb et al., 2014).

3.7. HPLC – PDA detection of phytochemicals

One gram of leaves and fruits *A. andrachne* was extracted with 10 ml methanol (80% v/v) overnight on the shaker, the suspension was filtered using Whatman filter paper.

The following standards: gallic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, 4-hydroxyphenylacetic acid, vanillic acid, caffeic acid, syringic acid, isovanillic acid, *p*-coumaric acid, ferulic acid, sinapic acid, rutin, verbascoside, quercetin, *trans*-cinnamic acid, and kaempferol were prepared using a solvent of 20% ethanol with a concentration of 25 mg/ 100 mL. A standard mixture was made by mixing 1.0 mL of each standard solution into a 25 mL volumetric flask that was made up to volume with the same solvent.

Table (3.1) shows the mobile phase composition and the gradient elution method used for the detection of main components, where (A) is 0.5% acetic acid and (B) is acetonitrile. RP BDS Hypersil C18 column (Thermo Scientific, 250 x 4.6 mm, 3 µm) was used, with a flow rate of 0.6 mL/minute. The PDA range was set from 210 to 400 nm, while the column temperature was set to 25°C. The injection volume was set to 20 µL. All samples were filtered through a 0.45 µm disposable filter.

Table (3.1): Mobile phase composition of HPLC – PDA.

Time (minutes)	A%	B%
0	95	5
50	80	20
65	65	35
70	40	60
75	10	90
78	95	5
80	95	5

3.8. Antibacterial

3.8.1. Study Design, Location, and Ethical Considerations:

The proposed quantitative experimental study has been conducted by ethical guidelines approved by the Ethical and Protocol Review Committee of Hebron University.

3.8.2. Extract preparation:

To conduct the extraction of plant material, one gram of previously crushed and dried samples of kotlob leaves and fruits were weighed out using an analytical weighing scale. It was mixed with 10 ml of methanol (80% v/v) (Lot No. TRD0069814) and macerated overnight at 25°C in a shaking incubator. After 24 hours, the methanol (80% v/v) was decanted and filtered through a Whatman filter paper (Lot No. CB162201, 150mm, Germany) and subjected to antibacterial analysis.

3.8.3. Media preparing

Eosin methylene blue (EMB) (HIMEDIA, 0000535149): based on 35,96 g/ 1000 mL, 26.97 g EMB were dissolved in 750 ml distilled water. Mannitol salt agar (MSA) (HIMEDIA, 0000527813) base: 111,02 g/ 1000ml, for 750 ml, approximately 83.265 g needed. Blood agar (HIMEDIA, 0000367056): 40 g/1000ml, for 750 ml, approximately 30 g needed. Prepared small amounts of each type of media for identification of isolated bacteria and prepared one bottle of Muller Hinton (M.H) (HIMEDIA, 0000381656) media to be used for culturing the bacteria and testing the sensitivity of the plant extract with positive and negative controls. Approximately 28.5 g of M.H powder was weighed in an autoclavable bottle, and then 750 mL of DW was added. The bottles were then heated on the hot plate until they became clear (using a magnetic stirrer), wait 20 minutes, close the bottle cover it with aluminum foil, and autoclave (Labtech, Model No. LAC-5065SP, Daihan Labtech Korea) the media (EMB, MSA, Blood agar) for an hour, and a half at 121 °C to become sterile. After an hour and a half of sterilization in the autoclave, the media was poured into sterile petri dishes (90 X 16 mm) as follows, where 25 mL of M.H was poured into each sterile petri dish and the quantity was filled about 30 petri dishes. In addition, differential media was poured into petri dishes until the circle was covered. Finally, all the dishes were stored in the refrigerator at 4°C for later use.

3.8.4. Bacterial subculture

At the Microbiology Department of the University of Hebron, bacterial isolates were obtained from the microbiology laboratory. The grown bacteria were subcultured into different differential media as the following: *Staphylococcus aureu*s (*S. aureus*) was cultured on MSA, and *Escherichia coli* (*E. coli*) was cultured on EMB. All the plates were incubated at 37 °C for 24 hours in Thermo Fisher Scientific, Heratherm incubator (OGS60, Germany). Isolates were characterized phenotypically using colonial morphology and Gram staining. The characterization of the isolates is described in detail below:

a. *E. coli:* appearance of greenish metallic sheen colonies of EMB agar.
b. *S. aureus*: appearance of golden-yellow colonies on MSA.

3.8.5. Antibacterial Activity Evaluation of Plant Extracts by Disc diffusion method

Normal saline (Batch No. 203548143) was added into two test tubes about 2 ml in each one, then by cotton swap (Lot No. 092818), one touch of bacterial colony that was isolated previously and identification was taken and added to the normal saline and shacked. The density/turbidity of the inoculum and the absorbance should be about 0.07 Abs by using a spectrophotometer with lambda max 450 nm, adjusted to 0.5 McFarland turbidity standard (Lot No. 0000497800), resulting in a suspension of 1.5×10^8 CFU colony forming units. Then Whatman filter paper (Lot No. 17387931, 90mm, China), was cut as a disk (about 6 mm in diameter) and put in the extract of kotlob leaves and fruits until the next step.

Muller Hinton agar plates were seeded with the test organisms and the plates left for five minutes to dry. Then, Whatman filter paper discs, containing the extract of kotlob leaves and fruits at a desired concentration, were placed on the agar surface after labeled the petri dish, positive disk control (Meropenem disk was used as a positive control of *E. coli*, while Cefoxitin disk used for *Staphylococcus aureus*) and negative control disk methanol (80% v/v) were placed. The plates were then incubated at 37 °C for 24 hours and the zones of inhibition were measured in millimeters. Analysis was done in triplicates (Balouiri et al., 2016).

Chapter 4: Results

Chapter 4: Results

4.1. Total phenolic assay

Gallic acid was used to estimate TPC, which was expressed as mg/L of Gallic acid equivalent (GAE) (**Table (4.1)**; **Figure (4.1)**). There were significant differences among the experimental groups in the level of quantitative analysis of TPC (P=4.6731E-09; P < 0.05, **Figure (4.2)**).

Table (4.1): Absorbance values of Gallic acid standard (mg/L), n=4

GA mg\l	A1 mg\l	A2 mg\l	A3 mg\l	A4 mg\l	Mean
50	0.049	0.041	0.042	0.047	0.04475
100	0.06	0.05	0.064	0.058	0.058
150	0.064	0.073	0.068	0.068	0.06825
250	0.112	0.095	0.139	0.138	0.121
500	0.34	0.361	0.289	0.34	0.3325

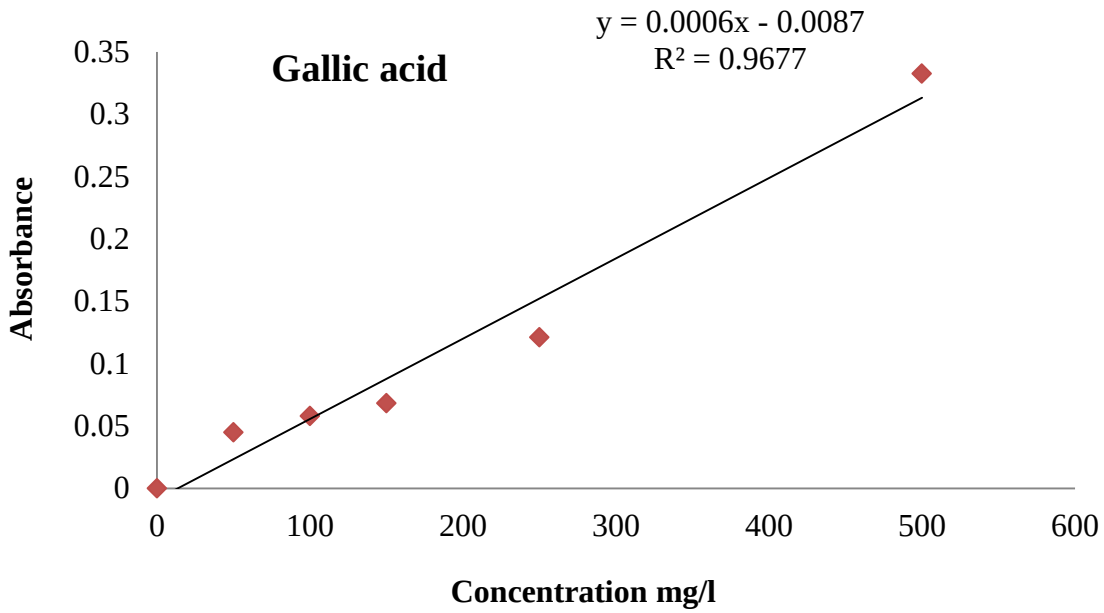

Figure (4.1): Calibration curve of Gallic acid. Each point represents the mean of quadruplicate.

Furthermore, kotlob parts diluted (1:50) in methanol showed pronounced phenols as assessed by F.C reagent. According to **Figure (4.2)**, leaves and fruits of kotlob were (391.25 ± 10.48 mg GAE/g), (64.16 ± 9.47 mg GAE/g), respectively.

A high value of TPC found in the kotlob leaves, compared to kotlob fruit.

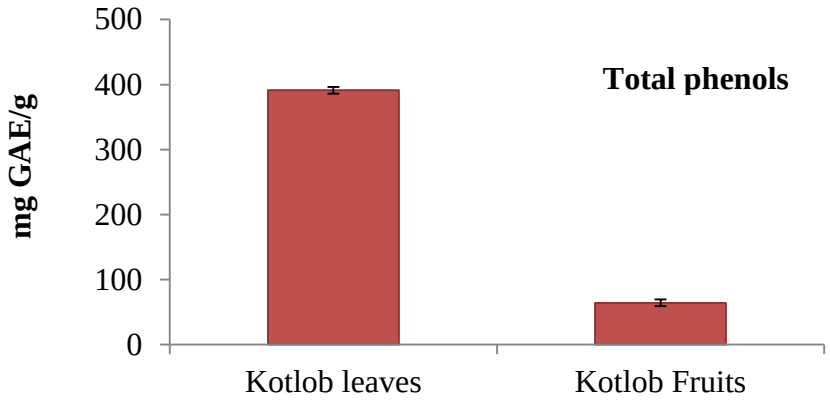

Figure (4.2): Total phenols contents of kotlob leaves and fruits methanolic extracts produced by Folin-Ciocalteu method, n=4.

4.2. Antioxidant

4.2.1. DPPH• scavenging capacity

Diluted methanol extracts (1:10) for selected kotlob parts leaves and fruits showed pronounced antioxidant activity as assessed by DPPH• free radical scavenging assay. The percentage of scavenging of diluted methanolic extracts of leaves, and fruit were (91.41 ± 0.15%) and (31.67 ± 0.65%), respectively. There were significant differences among the experimental groups in the level of scavenging capacity (P=1.0923E-12; P < 0.05, **Figure (4.3)**).

A high percentage of antioxidants was found in the kotlob leaves extract, whereas the antioxidant activity was lower in the fruit extract.

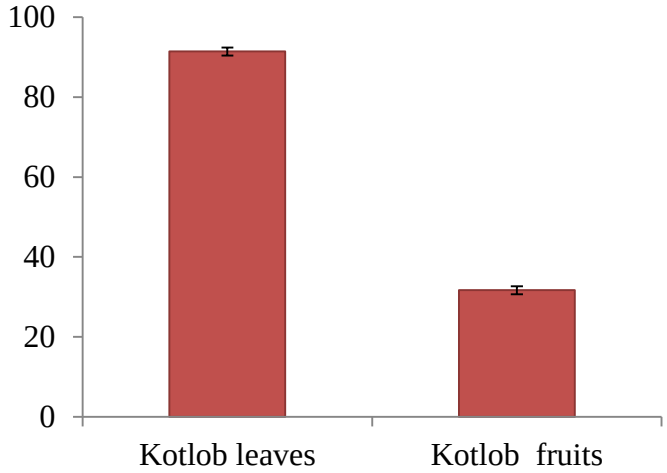

Figure (4.3): Mean radical scavenging effect of kotlob leaves and fruits, assayed by DPPH• radical scavenging method using methanolic extracts, n=4.

4.2.2. ABTS$^{\bullet+}$ scavenging capacity

Diluted methanol extracts (1:10) for selected kotlob parts showed pronounced antioxidant activity as assessed by ABTS$^{\bullet+}$ free radical scavenging assay. The percentage of scavenging of diluted methanolic extracts of leaves, and fruit were 94.86 ± 0.85% and 87.82 ± 2.23%, respectively. There were significant differences among the experimental groups in the level of scavenging capacity (P=0.00053546; P< 0.05, **Figure (4.4)**).

A high percentage of antioxidants was found in the kotlob leaves, whereas the lowest percentage were found in the fruit according to ABTS$^{\bullet+}$ scavenging.

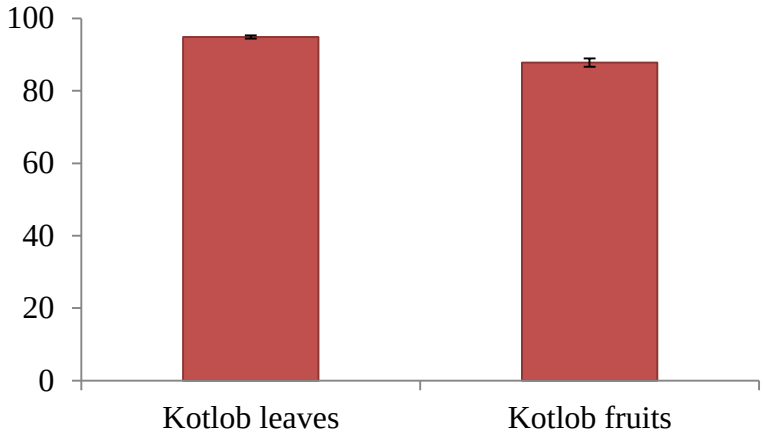

Figure (4.4): Mean radical scavenging effect of kotlob leaves and fruits, assayed by ABTS$^{\bullet+}$ radical scavenging method using methanolic extracts, n=4.

4.3. Phytochemical screening

The phytochemical screening assays for the methanolic extracts of kotlob samples revealed the presence of a wide range of phytochemical groups such as coumarins, saponins, steroids, terpenoids, and flavonoids. Other groups such as anthraquinone, quinones and phlobatannins were not detected as summarized in **Table (4.2)**. Interestingly, phenolic groups, anthocyanins and tannins were found in the two examined kotlob parts.

Table (4.2): Phytochemical screening for the methanolic extracts of kotlob samples, n=3

Kotlob Parts	Phytochemical screening tests										
	Phenolic groups	Anthocyanins	Coumarins	Saponins	Anthraquinone	Quinones	Steroids	Tannins	Terpenoids	Flavonoids	Phlobatannins
Leaves	+	+	-	+	-	-	+	+	+	+	-
Fruits	+	+	+	-	-	-	-	+	-	-	-

4.4. GC-MS analysis

The GC-MS analysis of a methanolic extract of fruits and leaves of kotlob is illustrated in the **Figure (4.6) and (4.7)**. The GC-MS analysis revealed few interesting bioactive compounds in leaves and fruits of kotlob including hydroquinone and penten-1-ol. These identified compounds with their retention time and molecular weight are shown in **Table (4.3) and (4.4)**.

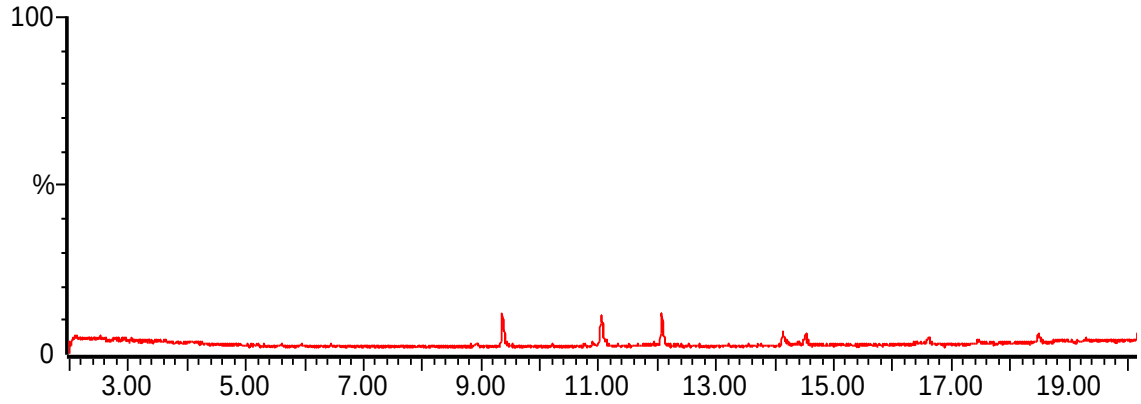

Figure (4.5): Representative GC-MS total ion mass chromatograms of the methanol.

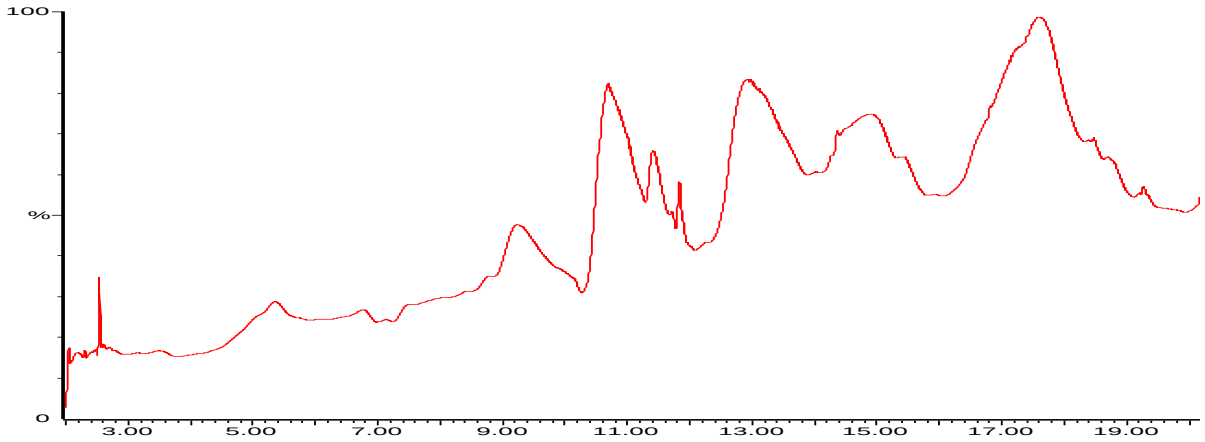

Figure (4.6): Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of kotlob leaves.

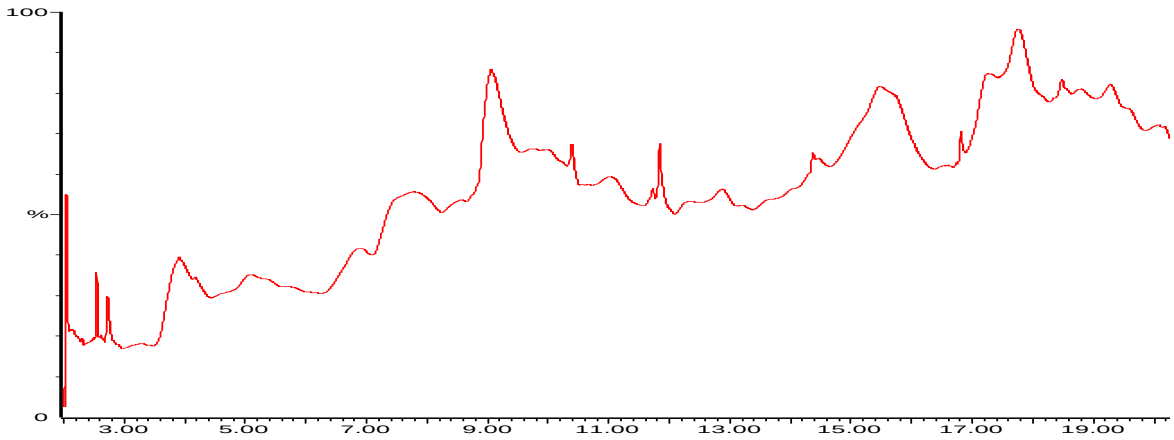

Figure (4.7).: Representative GC-MS total ion mass chromatograms of the volatile compounds detected in the methanolic extracts of kotlob fruits.

Table (4.3): Major compounds detected in kotlob leaf extracts with their retention time (*Rt*), molecular masses (M/Z), molecular weight (MW), and molecular formula (MF).

Rt	**M/Z**	**Compound name**	**MW**	**Formula**
10.702	109, 110, 111	Hydroquinone	110	$C_6H_6O_2$

Table (4.4): Major compounds detected in kotlob fruit extracts with their retention time (*Rt*), molecular masses (M/Z), molecular weight (MW), and molecular formula (MF).

Rt	**M/Z**	**Compound name**	**MW**	**Formula**
2.043	53, 55, 58	Penten-1-ol	86	$C_5H_{10}O$

4.5. HPLC

Seventeen standards of flavonoids and phenolic compounds were separated in different retention times and each has its corresponding number in Figure 1. This optimal chromatogram was chosen because it revealed all the standards at 280 nm, although each has a different maximum wavelength.

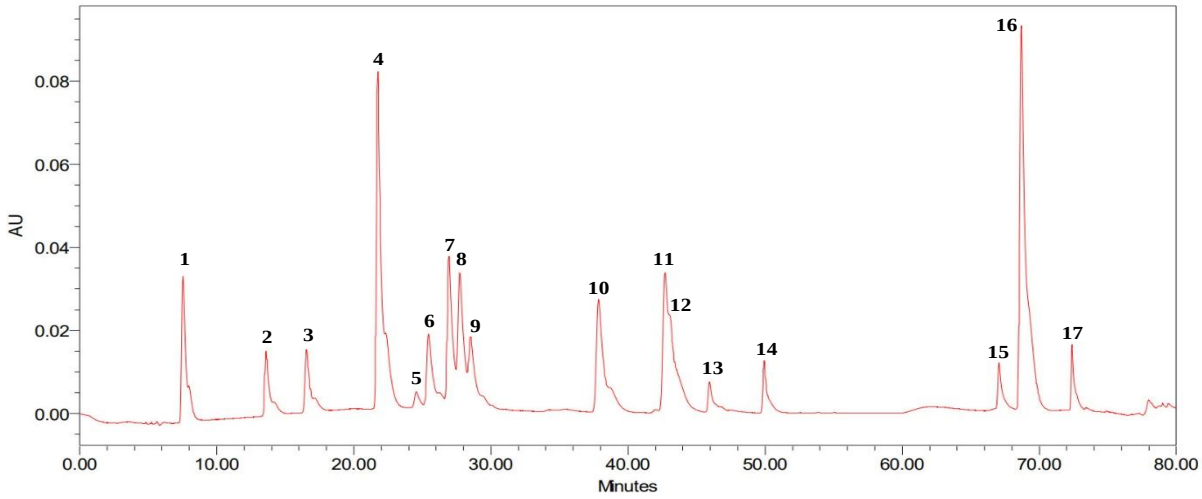

Figure (4.8): HPLC chromatogram for standards used at 280 nm: 1. Gallic acid, 2. 3,4-dihydroxybenzoic acid, 3. 3,4-dihydroxyphenylacetic acid, 4. Chlorogenic acid, 5. 4-hydroxyphenylacetic acid, 6. Vanillic acid, 7. Caffeic acid, 8. Syringic acid, 9. Isovanillic acid, 10. *p*-coumaric acid, 11. Ferulic acid, 12. Sinapic acid, 13. Rutin, 14. Verbascoside, 15. Quercetin, 16. *trans*-cinnamic acid, and 17. Kaempferol.

HPLC results were expressed in these selective chromatograms. The identification was done through the retention time and wavelengths for both standards and samples. Accordingly, rutin was identified at 47.40 minutes in leaves while gallic acid was detected in fruits at 7.78 minutes **Figure (4.9) and (4.10)**.

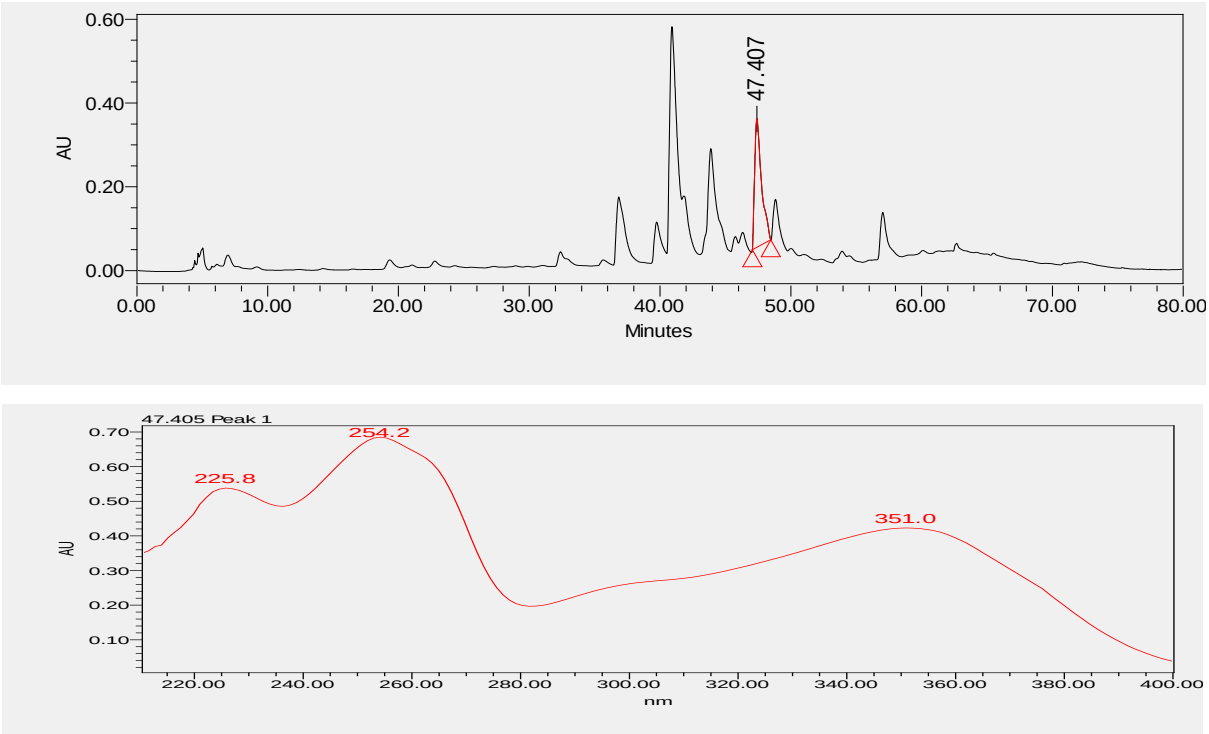

Figure (4.9): HPLC chromatogram for leaves kotlob extracted with 80% methanol at 310 nm (a) and UV spectrum for rutin.

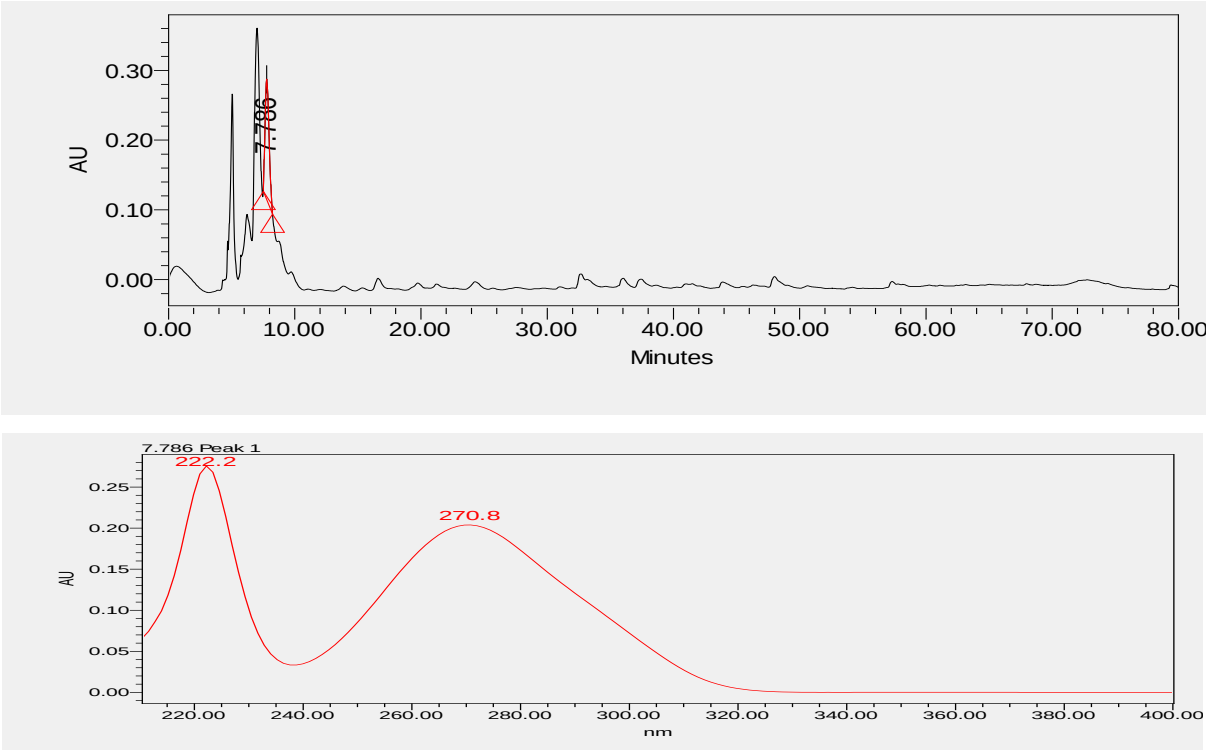

Figure (4.10): HPLC chromatogram for fruit kotlob extracted with 80% methanol at 310 nm (a) and UV spectrum for gallic acid.

4.6. Antibacterial activity

Among the examined kotlob parts (leaves and fruits), the methanolic extract of kotlob leaves displayed antibacterial activity against the gram-positive *S. aureus* bacteria with a 17mm zone of inhibition compared with the positive control (Cefoxitin) **Figure(4.11)**. In addition, the methanolic extract of kotlob fruits displayed antibacterial activity against the gram-negative *E. coli* bacteria with a 10mm zone of inhibition compared with the positive control (Meropenem) **Figure(4.12)**. On the other hand, *E. coli* was not affected by leaf extract, and *S.aureus* was not affected by fruit extract.

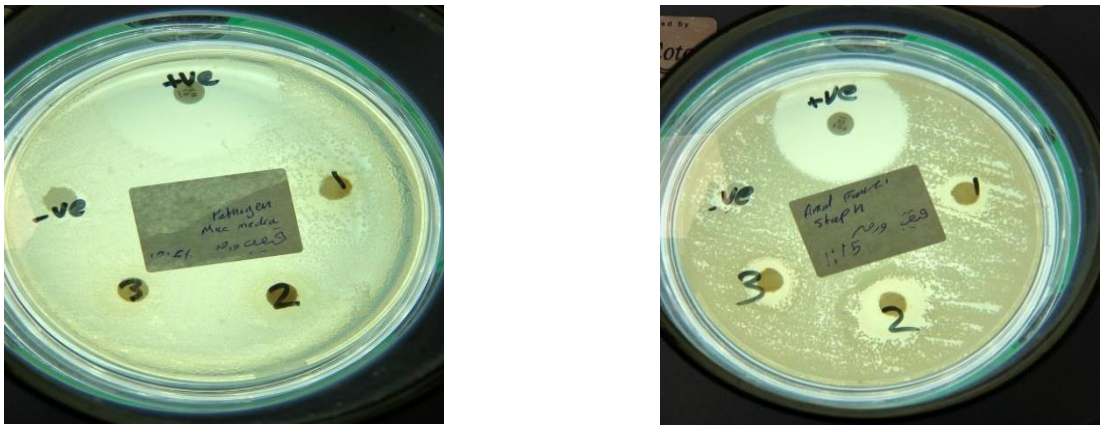

Figure (4.11): The zone of inhibition against *E. coli* bacteria (left side) and *S. aureus* (right side) for kotlob leaves.

Figure (4.12): The zone of inhibition against *E. coli* bacteria (left side) and *S. aureus* (right side) for kotlob fruits.

Chapter 5: Discussion

Chapter 5: Discussion

Plants were always a significant source of nutritional supplements and drugs. Kotlob and its different parts have an excellent nutritional profile. Kotlob is an evergreen tree belong to the Ericaceae family, the leaves and fruits of this tree are used traditionally as antiseptic, diuretic, laxative and antidiabetic (Hamdan & Afifi, 2008; Sakar et al., 1992; Şeker & Toplu, 2010). Amro et al. (2013) mentioned *A. andrachne* are used for the treatment of asthma. The fruits represent a better source of ascorbic acid (Şeker & Toplu, 2010). There are 122 species of the genus Arbutus, but *A. andrachne*, in the eastern Mediterranean region, the kotlob name is given (Torres et al., 2002). Thus, chemical composition, biological activities of kotlob which commonly grow in Palestine including different parts (leaves and fruits) were investigated.

Free radicals are extremely erratic chemical entities primarily consisting of one or more single electrons. Their primary function is to damage other molecules by removing electrons to stabilize themselves. The human body constantly produces free radicals, which are necessary for many processes, including immunological response, chemical signaling, detoxification, energy supply, and more. Free radicals, on the other hand, maybe hazardous even though the body needs them. According to Zhou et al. (2016) ionizing radiation, ultraviolet light, chemical reactions, and metabolic processes can all cause the production of reactive oxygen species (ROS). Oxidative stress, which is thought to be the primary cause of the oxidation of biomolecules like lipids, amino acids, proteins, and DNA and ultimately results in cell injury and induces several diseases (cancer, Parkinson's disease, and others), is caused by an imbalance between antioxidants and reactive oxygen species (Li et al., 2015). Numerous enzymes, including glutathione reductase, catalase, superoxide dismutase, and others, may aid in repairing the harm caused by free radicals. Likewise, owing to their capacity to neutralize or scavenge reactive oxygen species (ROS) by hydrogen donation, antioxidants (polyphenols, vitamin A, vitamin C, and others) are crucial in the treatment of several human diseases, including cancer, cardiovascular disease, and inflammatory disease (Baiano & Del Nobile, 2016). The primary sources of organic compounds like tannins, alkaloids, carbohydrates, steroids, terpenoids, and others that have specific physiological effects on humans are medicinal plants, which are mostly used as food and spice plants. The phenolic group, which is mostly found in leaves, floral tissues, and woody components like stems and bark, has a

capacity to scavenge free radicals because of their dual roles as hydrogen donors and reducing agents. As a result, according to Li et al. (2015), they may be able to stop the onset of some diseases.

The total phenolic contents of kotlob samples methanolic extracts were determined using the FolinCiocalteu method. The highest TPC was found in the leaves that TPC is 391.25 mg GAE/g. However, fruit revealed the lowest total phenolic contents. Generally, these results also are in agreement with Hmaidosh et al. (2020) who obtained that the TPC among the leaves and fruits was significantly different, following the order: leaves > fruits.

The antioxidant activity of kotlob samples was determined using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, as a stable free radical method, which is an easy, rapid, and sensitive way to examine the antioxidant activity of a specific compound of plant extracts (Zulueta et al., 2009). Interestingly, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity concurrently increased with the increase of phenolic components such as flavonoids and phenolic acids. The kotlob leaves revealed a high percentage of scavenging activity above 90% for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. Our $DPPH^{\bullet}$ results are consonant with Aslantürk et al. (2021) who obtained that the $DPPH^{\bullet}$ scavenging activity of kotlob leaves extract possess higher antioxidant activity. Tawaha et al. (2007) $ABTS^{\bullet+}$ assays of the kotlob leaves were found to be the highest among 51 other medicinal plants species in Jordan that have antioxidant content. In addition, the fruit showed high antioxidant capacity, with a percentage of scavenging activity 31.67% and 87.82% for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ respectively. According to Serçe et al. (2010) kotlob revealed that the fruits had mainly phenolic compounds as one of the major components with scavenging activity. Kotlob fruit is perhaps most comparable to blackberries, cranberry and blueberries, which are known to have the highest value of antioxidant among all fruits.

During the present work, the leaves of the kotlob possessed the strongest antioxidants activity and the fruits had the weakest activity, these results are in agreement with Hmaidosh et al. (2020) who obtained that the leaves of kotlob have the strongest antioxidant activity comparing with its fruits. This may be explained by the occurrence of the highest amounts of phenolic compounds in leaves. Interestingly, our results indicate an excellent correlation between the total phenolic contents and antioxidant activity. So, these findings are from previous studies

that also revealed the linear relation between antioxidant activity and total phenolic contents. Therefore, it can be suggested that the phenolic compounds contributed to the antioxidant potential of kotlob, and this is the main reason that always applies the leaves as the most effective part. Moreover, the results of the work indicated that phytochemicals were responsible for the medicinal effects of kotlob, and this finding agrees with (Erkekoglou et al., 2017). The finding of this study suggests that kotlob leaves could be a potential antioxidant natural source that could have great importance as therapeutic agents to prevent or slow the progress of oxidative stress-related diseases.

Phytochemicals, as a part of a large and varied group of chemical compounds, are responsible for the color, flavor, and odor of plant food, also, considered multifunctional components of food due to their important biological properties and antioxidant activity. Phytochemical research revealed that *A. andrachne* with all its different parts have phytosterols, anthocyanins, amino acids, organic acid, fatty acids, phenolic compounds, hydrocarbons, aliphatic alcohols, volatile components, and some other classes of secondary metabolite. These phytochemicals are mainly found leaves, fruit, and root. However, these naturally-occurring secondary metabolites get widespread attention due to their ability to have remarkable pharmacological properties such as anticancer, antioxidant, and anti-inflammatory effects and others (Özgen et al., 2009). Phytochemical analysis of kotlob samples including screening of phenolic groups, saponins, steroids, tannins, terpenoids, and other secondary metabolites revealed that kotlob leaves and fruits are a good source of phenolic groups, anthocyanins, and tannins **Table (4.2)**, which improve their antioxidant activity, these results were supported by many research groups. Due to tannins, kotlob fruit can help to relieve stomach, and bowel laziness, reduce high blood pressure, relieve liver bloating, and have antipyretic properties (Sarikaya & Orucu, 2021). Some groups of tannins mainly act on arachidonic acid metabolism in leucocytes, apply an important role in reversing inflammations, treatment in fostering wound healing, and else (Okuda, 2005). On the other hand, saponins, steroids, terpenoids and flavonoids were only found in the leaves **Table (4.2)**. Sakar et al. (1992) revealed that kotlob leaves are a good source of main flavonoids . The same research group identified monotropein, monotropein methyl ester, arbutin, stilbericoside, unedoside and gardenoside, are strong antioxidants (Sakar et al., 1991).

This study may provide evidence for methanolic extracts of *A. andrachne* leaves and fruits. Therefore, these results could be of relevance to the industry, and many other medicinal and pharmaceutical uses, which makes it highly recommended in the pharmaceutical industry. The GC-MS technique was utilized and found to be precise, accurate, and reliable in the separation and identification of the components of complex volatile mixtures. Phytochemical and volatile compounds in kotlob methanolic extracts were examined by GC–MS and identified by comparing their mass spectrum with NIST05 mass spectral library. The compound's identity, retention time, molecular formula, and molecular weight values are summarized in **Tables (4.3, 4.4)**.The identified peaks of tested kotlob parts were depicted in the GC–MS TIC chromatogram **Figures (4.6, 4.7)**. Low molecular weight volatile compounds can partially participate in the overall aroma of leaves and fruits but overall there is no major volatile compound under our experimental conditions. The compounds that were found when we analyzed kotlob parts by GC-MS, which matched the GC-MS analysis and were published in various scientific papers; hydroquinone showed anti-inflammatory and analgesic properties (Fawad et al., 2018); and penten-1-ol showed antibacterial activity (Parisi et al., 2017)

HPLC – PDA detection of phytochemicals indicated the existence of rutin at 47.40 minutes as the major compound in the kotlob leaves while gallic acid in the fruits at 7.78 minutes. The findings are also consistent with research conducted by (Jaffal et al., 2020) who identified rutin of kotlob leaves by LCMS. Rutin is a glycoside made up of the disaccharide rutinose and the flavonolic aglycone quercetin. Numerous pharmacological activities have been demonstrated by it, such as antioxidant, anticarcinogenic, cytoprotective, vasoprotective, neuroprotective, analgesic, cardioprotective properties and anti-depressant effects by the upregulation of CB1(Ganeshpurkar & Saluja, 2017; Javed et al., 2012; Schwedhelm et al., 2003; Su et al., 2014). The antimicrobial activity of rutin against different bacterial strains has been thoroughly investigated. It has proven to have a significant level of inhibition on *E. coli* bacterial growth (Araruna et al., 2012). Gallic acid is one of the primary groups of phytochemicals found in the fruits of the *Arbutus* species (Bouzid et al., 2014; Mendes et al., 2011). GA has shown promise as a potent antioxidant and effective apoptosis promoter (Badhani et al., 2015). In addition Borges et al. (2013) showed GA had antimicrobial activity against the *P. aeruginosa*, *E. coli*, and *S. aureus*.

Overuse of antibiotics has led to the development of multidrug-resistant bacteria, which greatly reduces the effectiveness of treatment and is widely regarded as a major issue limiting drug efficacy. To solve these issues, it is imperative to look into novel approaches to the treatment and prevention of infectious diseases. Chemicals extracted from medicinal plants have been used as models for numerous clinically successful medications over the past ten years. These drugs are currently being reevaluated as antimicrobial agents due to a decline in the development of new antibacterial medications, an increase in the resistance to antimicrobial drugs, and the necessity of treating newly emerging pathogens (Mahady, 2005). Medicinal plants can be antibacterial through a variety of mechanisms, including disruption of the cytoplasmic membrane, inhibition of the synthesis of nucleic acids, energy metabolism, synthesis of cell walls and membranes, and others (Al-Snai, 2019). Furthermore, the discovery of natural antimicrobials may offer important solutions to combat the major global issue of antibacterial resistance. Finding fresh sources of organic antioxidants and antimicrobials is crucial. The present study investigated the antioxidant as well as antibacterial activities of kotlob, which are traditional Palestinian fruits. However, methanolic extract of kotlob leaves and fruits were investigated for their antimicrobial activity against two bacterial strains, one gram-positive (*Staphylococcus aureus*), and one gram-negative (*Escherichia coli*) using agar disk diffusion method for determining the inhibitory zone diameters. Interestingly, our results revealed that the methanolic extract of kotlob leaves displayed antibacterial activity against *S. aureus* bacteria with a reasonable zone of inhibition, compared with the positive control (cefoxitin). These findings align with Okmen (2015) who showed that the kotlob leaves extract was able to inhibit the growth of *S. aureus*. However, *E. coli* was affected by fruits kotlob samples of methanolic extract. The kotlob fruits lacks antibacterial activities against *S.aureus*. It was suggested that the antimicrobial activity of kotlob was probably due to its constituents, as the detection of methanolic extract of kotlob leaves and fruits revealed the presence of the flavonoids, organic acid (Sakar et al., 1992), saponins (Cirva et al., 1980; Grishkovets et al., 1979; Jaffal et al., 2022; Sakar et al., 1991), which possess diverse biological effects like anti-inflammatory, and antibacterial activities. Specifically, gallic acid and rutin constituents possessed remarkable toxic activity against bacteria and provide the main pharmacological importance. Gallic acid is considered a toxic compound for bacteria, due to its ability to cause irreversible changes in membrane properties by reacting with phospholipids contained in the

bacterial cell wall, triggering membrane damage and leakage of a metabolite that finally inactive the bacterial enzyme system and killing the bacteria (Borges et al., 2013). In addition, hydroquinone displayed relatively strong antibacterial activity against *S.aureus* (Ma et al., 2019). Further studies also are needed to estimate the minimum inhibitory concentration (MIC) and the safety of the kotlob methanolic extracts.

Chapter 6: Conclusion

Chapter 6: Conclusion

Based on the results obtained in this study, it was revealed that the methanolic extract of the kotlob tree commonly grown in Palestine from Mahmiyat Wadi al-Qof have the following valuable effects:

Both kotlob leaves and fruits having high content of phenolic compounds like phenolic group, anthocyanin and tannin due to total phenolic content assay and phytochemical screening tests. In addition, Phytochemical screening of the methanolic extract present hydroquinone and rutin in the leaves; penten-1-ol and gallic acid in the fruits, detected by GC-MS and HPLC analysis. These compounds exhibit anticancer, antioxidant, antibacterial, and anti-inflammatory activity.

The antimicrobial studies of kotlob showed remarkable antimicrobial activity against some gram-negative and gram-positive bacterial strains, suggesting that these Palestinian folkloric medicinal plants possess broad-spectrum antibacterial activity. Our Palestinian kotlob have an interesting zone inhibition against gram-negative and gram-positive bacteria. These results to be investigated to enhance pharmacological industry.

Recommendations

The followings are some suggestions that may be taken into consideration for future investigation:

- The current research used the compounds found in the plant while it is dried. It is recommended to examine the components in the different stages of the plant to note the differences with the application of the same protocols and compare the results.

- As kotlob is rich in minerals, it is recommended to do some comprehensive tests for the minerals. Furthermore, it recommended checking kotlob vitamins content.

- Work on more tests on these plants, for example, testing the anti-cancer and antifungal activities.

- As for the antioxidant test, it is recommended to do several tests, such as IC50, Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, and the copper reduction (CUPRAC) assay. Moreover, compare them with the results we obtained with $ABTS^{\bullet}$ & $DPPH^{\bullet}$ antioxidant activity assays. Additionally, measure the total oxidant scavenging capacity (TOSC).

- It is recommended to raise the temperature, increase the separation period of vehicles inside the GC-MS, and monitor the exit of new compounds if they appear.

- It is recommended to use the headspace to separate the volatile compounds and compare them with the results we got in this study.

- As kotlob proved to have a valuable antioxidant and antimicrobial activity, it is recommended to use these plants in combination with nanoparticles to introduce new technologies that increase the effectiveness of the plant and achieve valuable results in research.

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Abstract in Arabic

الملخص

تحديد الأنشطة الكيميائية النباتية ومضادات الأكسدة والمضادات للبكتيريا لمستخلص ميثانول ثمار وأوراق القطلب

القطلب (*Arbutus andrachne* L., Ericaceae) نبات حيوي للاقتصاد الفلسطيني والثقافة الشعبية. يستخدم القطلب تقليديا كمدر للبول، ومنشط للدم، ومطهر، ومضاد لمرض السكر، ومضاد للالتهابات، ومضاد للإسهال، ومطهر، وملين. في هذه الدراسة تم تحديد القدرة المضادة للأكسدة والفينولات الكلية والخصائص الكيميائية النباتية في أوراق وثمار القطلب. تم جمع الثمار الناضجة والأوراق الناضجة من محمية وادي القف في مدينة الخليل. تم تقييم أنشطة مضادات الأكسدة بواسطة فحوصات الكسح $DPPH^{\bullet}$ و$ABTS^{\bullet+}$؛ تم تحديد المحتوى الفينولي الكلي (TPC) باستخدام طريقة Folin-Ciocalteau ؛ وتم تحديد المركبات المتطايرة باستخدام GC-MS المجهز بوضع التأثير الإلكتروني. تم استخدام HPLC مع كاشف PDA لتحليل المواد الكيميائية النباتية. أظهرت أوراق القطلب قدرة أعلى على مقاومة الأكسدة مقارنة بالفواكه. أظهرت الأوراق 91.41%، والثمار 31.67%، والقمامة حسب تقييم $DPPH^{\bullet}$ و94.86%، و87.82% القمامة حسب تقييم $ABTS^{\bullet+}$ ، على التوالي. علاوة على ذلك، أظهرت الأوراق أعلى قيمة لمحتوى الفينول الكلي 391.25 ملغم/GAE غم، بينما الثمار 64.16 ملغم/GAE غم.

أظهر تحليل GC-MS أن hydroquinone عند Rt=10.702 في الأوراق، بينما تم تقديم penten-1-ol في الفاكهة عند Rt=2.043، لذلك يمكن للمركبات المتطايرة ذات الوزن الجزيئي المنخفض أن تشارك جزئيًا في الرائحة العامة للأوراق والفواكه، ولكن بشكل عام ليس هناك مركبًا متطايرًا رئيسيًا في ظل ظروفنا التجريبية. من ناحية أخرى، تم تحديد روتين في HPLC عند Rt = 47.4 دقيقة في الأوراق، بينما تم اكتشاف حمض الغاليك في الثمار عند 7.78 دقيقة.

تم فحص المستخلصات الميثانولية لأوراق وثمار القطلب لنشاطها المضاد للميكروبات ضد سلالتين بكتيريتين، إحداهما إيجابية الجرام *S. aureus* .وواحدة من الإشريكية القولونية سلبية الجرام. أظهرت النتائج أن المستخلص الميثانولي للأوراق أظهر نشاطاً مضاداً للبكتيريا ضد بكتيريا *S. aureus* بمساحة تثبيط 17 ملم مقارنة مع السيطرة الموجبة (السيفوكسيتين)، وأظهر المستخلص الميثانولي للثمار نشاطاً مضاداً للبكتيريا ضد بكتيريا *E. coli* بمساحة تثبيط. تثبيط 10 ملم مقارنة مع السيطرة الإيجابية (الميروبينيم).

هذه الدراسة هي الأولى التي قامت بفحص وتقييم المركبات الكيميائية النباتية في قيقاب فلسطيني مختار للنشاط المضاد للأكسدة والبكتيريا. أظهر مستخلص القطلب الذي تمت دراسته نشاطًا حيويًا استثنائيًا. ومن أجل تطوير المنتجات الطبيعية، قدمت هذه المستخلصات إمكانات متميزة. يجب إجراء أبحاث مستقبلية للتحقق من هذه الإجراءات في مصفوفات مختلفة .

الكلمات الرئيسية: *Arbutus andrachne* L.، مضاد أكسدة، نشاط كيميائي نباتي، GC-MS، HPLC، مضاد بكتيريا.