Deanship of Graduate Studies

Al-Quds University

"Chemical Constituents and Its Bioactivity of Extract from Paeonia lactiflora Roots"

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M.Sc. Thesis

Jerusalem, Palestine

1444/2022

“Chemical Constituents and Its Bioactivity of Extract from Paeonia lactiflora Roots”

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A thesis submitted in partial fulfillment of requirements for the degree of Master of Pharmaceutical Industry, Al-Quds University- Palestine.

1444/2022

Al-Quds University

Deanship of Graduate Studies

Industrial Technology Program

Thesis Approval

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Master thesis Submitted and Accepted, Date: 3/9/2022

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Jerusalem–Palestine
1444 - 2022

Declaration

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

Signed: Ragad

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Date: Aug 1, 2022

Acknowledgments

First and foremost, I praise and thank God, the almighty, for his showers of blessings throughout my research work to complete the research successfully. I would like to express my deep and sincere gratitude to my research supervisor, Dr. Fuad Rimawi for giving me the opportunity to do research and providing invaluable guidance throughout this research. His dynamism, vision, sincerity and motivation have deeply inspired me, he has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under his guidance. I am extremely grateful for what he has offered me. I am extending my heartfelt thanks to Dr. Mahmoud Alkhatib for his support and help all the time for the past years. I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. Also I express my thanks to my sister and brothers for their support. Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

Raghad I. Salameh

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List of Abbreviations

AC: antioxidant capacity

ACE: angiotensin-converting enzyme

ACN: acetonitrile

AGEs : advanced glycation end products (Antiglycation end products)

BHT: Butylated hydroxytoluene

BSA: glucose-bovine serum albumin

C: Carbon

CAT: catalase

Da: Dalton

DMSO: dimethyl sulfoxide

DPPH: 2, 2-diphenyl-1-picryl-hydrazyl-hydrate

DW: distilled water

E-coli Escherichia coli

EGC: epigallocatechin

ESI: Electrospray ionization

EtOH: ethanol

FRAP: ferric ion Reducing Antioxidant Power

g: gram

GAE: gallic acid equivalent

GSH-Px: glutathione peroxidase

HMPs: herbal medicinal products

HPLC-PDA: High Performance Liquid Chromatography- photodiode array detector

M^+: molecular ion

mg: milligram

mm: millimeter

mol: mole

MRSA: methicillin-resistant Staphylococcus aureus

MS: mass Spectrometry

m.wt.: molecular weight

Pae: paeoniflorin

PCs: phenolic compounds

PDA: The photodiode array

PIS: Product ion scanning

P.L: Paeonia lactiflora

ppm: part per million

$R_{t:}$ retention time

SOD: superoxide dismutase

TCM: traditional Chinese medicine

TFC: total flavonoids content

TPC: total Phenolics content

TGP: total glycoside of paeony

USA: United States

UV:	ultraviolet
V:	very
Vis:	visible
WHO:	World Health Organization (WHO)
µ:	micro

Abstract

A traditional Chinese herbal plant (Paeonia lactiflora pall) was analyzed to study its bioactivity and chemical constituents using different analysis techniques. Paeonia lactiflora is a widely used herb in medicine, food, and cosmetics. A dried root was purchased from Al-shaleh traditional herbal market from Al-Irsal Street-Ramallah-Palestine. The crude extract was prepared using 99% ethanol (EtOH) as extraction solvent, then exposing it to ultrasonication, and then saving it at 4C° for different analysis. The plant roots are known, where it proved its health protective effects for many diseases. Non-enzymatic glycation generates a diverse set of compounds known as advanced glycation end products (AGEs) that accumulate in the body and cause the development of chronic diseases in humans, such as type-2 diabetes, atherosclerosis, and Alzheimer's. Therefore, the development of a natural AGEs inhibitor needs extensive research investigation. Therefore, the anti-glycation production of the end product was assessed with the extract by using an in vitro glucose-bovine serum albumin (BSA) test. The obtained results of the extract sample indicate an inhibition action of AGE-formation, whereas the root extract is rich in antioxidants like flavonoids and phenols which have shown significant promise as natural diabetes regulators. Total phenolic content of the plant extract (TPC) was measured by using the Folin-Ciocalteu test. Total flavonoid content (TFC) was determined using aluminum chloride colorimetric assay. Two different methods were used to determine antioxidant capacity (AC): 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging assay, and ferric ion reducing antioxidant power (FRAP). The antimicrobial activity of the extract was conducted by a well-diffusing technique to test the plant extract activity against different types of gram

positive and gram negative bacteria. Major positive influence against gram positive bacteria methicillin resistant staphylococcus aureus (MRSA) was shown when compared to Penicillin G (the positive control), also a minor positive influence against another gram positive bacteria staphylococcus aureus was recorded. However, a negative influence was recorded for three different types of bacteria (Shigella, Escherichia coli, Pseudomonas). The results of different concentrations of the extract (120, 300) ppm shows an inhibitory percent of (48.2%, 69.0%) respectively. This potency in suppressing AGE end products is due to the existence of the phytochemicals in the plant extract. TPC assays was 193.9 mg / g of plant extract, TFC was 55.9 mg/ g of plant sample. Using the DPPH scavenging method, the plant extract gave 56.8% inhibition, while in the FRAP assay, the antioxidant activity (mg BHT/ g of plant sample) was calculated to be 1524. The results above demonstrate the ability of Paeonia lactiflora root extract to inhibit the growth of different types of bacteria. HPLC-PDA was used to identify polyphenolic compounds and flavonoids of the ethanolic extract and results showed the presence of gallic acid and 3,4-Dihydroxybenzoic. Paeonia lactiflora can be used to treat different diseases, because it contains a large group of phytochemicals.

Chapter One: Introduction

1. Background

From ancient times to the present days, plants have played a major role in the medical field all over the world and may even be considered the origin of modern medicine. This role was a result of the exploration of new biological effects through the use of plant extracts by experiments of the general public for self-medication [Houghton, 1995]. Plants have always been known for their curative properties for many diseases, which are the reasons for the emergence of the pharmaceutical industry. In the last century, the adoption of active compounds in plants using different methods, which led to an increase in the number of effective drugs that are widely used today, but these results were not effective enough in the treatment of some complex diseases such as cancer, diabetes and others. However, it has contributed to reducing the symptoms associated with these diseases [Weng et al., 2012]. While the role of the plant kingdom continues in its important source for chemical entities that contribute to the discovery of medicines, through trial and error on living organisms. Phytochemicals are substances that plants create ("phyto" means "plant"). Fruits, vegetables, grains, beans, and other plants all contain them. Some of these phytochemicals may shield cells from harm that can cause cancer. Phytochemicals consist of secondary metabolites are substances created in other metabolic pathways and, while they are significant, are not absolutely necessary for a plant to operate. Primary metabolites are substances that directly contribute to a plant's growth and development. There is still some information that has not been disclosed until

now, and it is awaiting disclosure by modern research methods. In this research, scientific and historical evidence will be presented on the main role of plants in the manufacture of medicines for humans, through studying the chemical activity of the medicinal Paeonia lactiflora plant, which is widely used in traditional medicine [Li et al., 2017]. Paeonia lactiflora has been well known in traditional medicine in China, Korea and other countries, and it's still used until today. P.L is a herbaceous plant of the buttercup family, consist of two parts roots and flowers, the roots extract used to treat a various of diseases includes rheumatoid arthritis, fever, muscle cramping, migraine, relieve the pain, treatment of liver disease, to treat inflammatory disorders in addition to treat myriad of maladies. As a result of many studies that have been done on this plant, the biological data for Paeonia plant constituents were evaluated and classified according to pharmacological-like activity, with emphasis on compounds of greatest concentration Total glycoside of paeony (TGP) [Parker et al., 2016]. TGP is extracted from the dried root of Paeonia lactiflora, it has been widely used for treatment of many diseases because of its pharmacological effects. A number of biologically active compounds are called glycosides, by exploiting the medicinal properties of glycosides which have been extensively used for the treatment of heart diseases and as antibiotics. Flavonoid C-glycosides showed significant antioxidant activity, anticancer and antitumor activity, hepato-protective activity, anti-inflammatory activity, anti-diabetes activity, antiviral activity, antibacterial and antifungal activity, and other biological effects. Where the dietary flavonoids, especially their glycosides, are the most vital phytochemicals in diets and are of great general interest due to their diverse bioactivity [Xiao et al., 2016].

Paeoniflorin (Pae) is the major active component of TGP, about more than 40% of TGP, Pae is a chemical compound consisting of one of the major constituents of an herbal medicine derived from Paeonia lactiflora. Pae has an analgesic (pain-relieving) effect, used to prevent thrombosis, in addition it helps in prevention of hair loss, fever, wrinkles, besides to all cases mentioned before Pae has proven its effectiveness in treating many symptoms and diseases [Ngo et al., 2019].

Monoterpenes [figure 1], another major component of TGP, with ten carbon atoms [Irina et al., 2018], which are made up of two isoprene units, are the most common type of naturally occurring organic compounds. Natural monoterpenes and their synthesized derivatives have been demonstrated to have antifungal, antibacterial, antioxidant, anticancer, antiarrhythmic, anti-aggregating, local anesthetic, antinociceptive, anti-inflammatory, antihistaminic, and antispasmodic characteristics [Koziol et al., 2014].

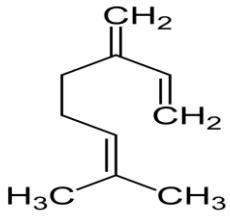


Figure 1: Generic structure of Monoterpene

Every type of terpene, which consists of a five-carbon molecule called an isoprene, has a basic building block made of hydrocarbons. On the other hand, isoprene does not occur naturally as free molecules. Instead, chemical bonding joins numerous isoprene units to build molecules with 10, 15, 20, and so on carbons. The scientific name of this class of molecule, which usually ends in -ene, can be used to identify it. A 10-carbon molecule known as a monoterpene is formed when two isoprene units create a chemical bond.

Monoterpenes come in a variety of forms, including cyclic, straight, and branching [O´Connor and Maresh 2006].

1.1. Phenolic Compound

Molecules having a hydroxyl group directly attached to a benzene ring are called phenolic compounds (PCs)

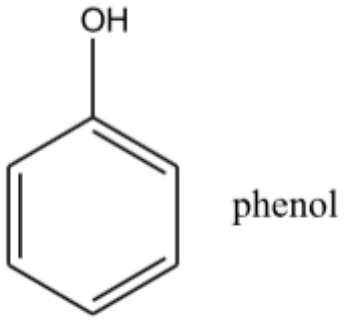


Figure 2: Phenolic compound.

PCs are phytochemicals that are present in practically every plant tissue, as well as the tissues of fruits and vegetables. They come from the phenylpropanoid and shikimic acid metabolic pathways, respectively, and are secondary metabolites. Because PCs contain several bioactive qualities and, despite the fact that they are not nutrients, their consumption has health-protective effects [Saxena et al., 2013].

Table 1: The Major Classes of Phenolic Compounds in Plants [Saxena et al. 2013].

	Number of carbon atoms	Basic skeleton	Class
1.	6	C_6	Simple phenols and benzoquinones
2.	7	C_6-C_1	Phenolic acids
3.	8	C_6-C_2	Benzophenone and tyrosine derivatives
4.	9	C_6-C_3	Hydrocinnamic acid and coumarines
5.	10	C_6-C_4	Naphtoquinones
6.	13	C_6-C_1-C_6	Xanthones
7.	14	C_6-C_2-C_6	Stilbenes
8.	15	C_6-C_3-C_6	Falvonoids
9.	18	$(C_6$-$C_3)_2$	lignans
10	30	$(C_6$-C_3-$C_6)_2$	bioflavonoids
11.	n	$(C_6$-C_3-$C_6)_n$	Condensed tannins

Chronic diseases, also known as non-communicable diseases, are long-term health problems with a gradual course that account for 63 percent of all fatalities globally. Numerous chronic diseases are caused by excessive oxidant production and persistent inflammation, and experimental and clinical studies have demonstrated that plant antioxidants can both prevent and treat these ailments. Plant-derived antioxidants, especially phenolic compounds, can reduce oxidative stress in the body while maintaining a balance between oxidants and antioxidants because of their reducing, free radical scavenging, or metal chelating properties. [Zhang et al., 2019]. Hydrogen can be readily donated by phenolic compounds to lipids or other biomolecules via hydroxyl groups positioned along the aromatic ring, and the unpaired electron can be stabilized and delocalized inside the aromatic phenolic ring. [Zhang et al., 2019].
To date, over 8000 distinct phenolic structures have been found [Jiang et al., 2009]. The amount of phenol rings and the structural components that connect these rings to one another categorize phenolics into distinct groups [Zhang et al., 2011]. Flavonoids, phenolic acids, tannins, stilbenes, and lignans are the primary families of phenolic chemicals [Zhang et al., 2011].
The biological effect of phenolic chemicals is mediated by a variety of mechanisms, both nonspecific and specific [Stryjewska et al., 2014]. Nonspecific processes include phenolic compounds' ability to scavenge free radicals and sequester metals, as well as their interactions with membranes. Specific mechanisms, on the other hand, take into account how phenolics interact with receptors, transcription factors, and enzymes. Because of the intricacy of some diseases, researchers have been looking for new therapeutic approaches based on multifunctional compounds and combination therapeutic

procedures. As a result, phenolic compounds, which, through different mechanisms, have a wide range of biological effects, have a lot of promise for application in the prevention and treatment of a variety of human disorders[Gonçalves and Romano 2017]. The total phenolic content (TPC) of particular plant extracts has been determined using a trusted method that has been applied frequently. This method, developed by [Gonçalves and Romano 2017], employs the Folin-Ciocalteu reagent, which is mixed with plant extracts in specific amounts coupled with sodium bicarbonate, the absorbance is calculated at 765 nm using a UV-Spectrophotometer after a period of time. An aqueous solution with known gallic acid concentrations is used for calibration. Gallic acid equivalent (GAE) milligrams (mg) per gram (g) of dry sample weight are used to express the test results. [Gonçalves and Romano 2017]

1.2. Flavonoid Compound

Flavonoids are secondary metabolites found in plants. They are divided into four primary categories: flavanols, flavones, anthocyanidins, and isoflavonoids as shown in the figure below. They are also classified into several subcategories. They are found in dietary foods and are used to treat a variety of illnesses. Flavonoids are compounds found in plants and spices that have been utilized in traditional medicine from ancient years. In many nations, flavonoids have been employed in clinical trials. One of them, baicalein and its glycosides, has been studied in clinical trials. Flavonoids have a significant mechanism for controlling cell division and proliferation. Anticancer qualities are among their therapeutic benefits [Gonçalves and Romano 2017]

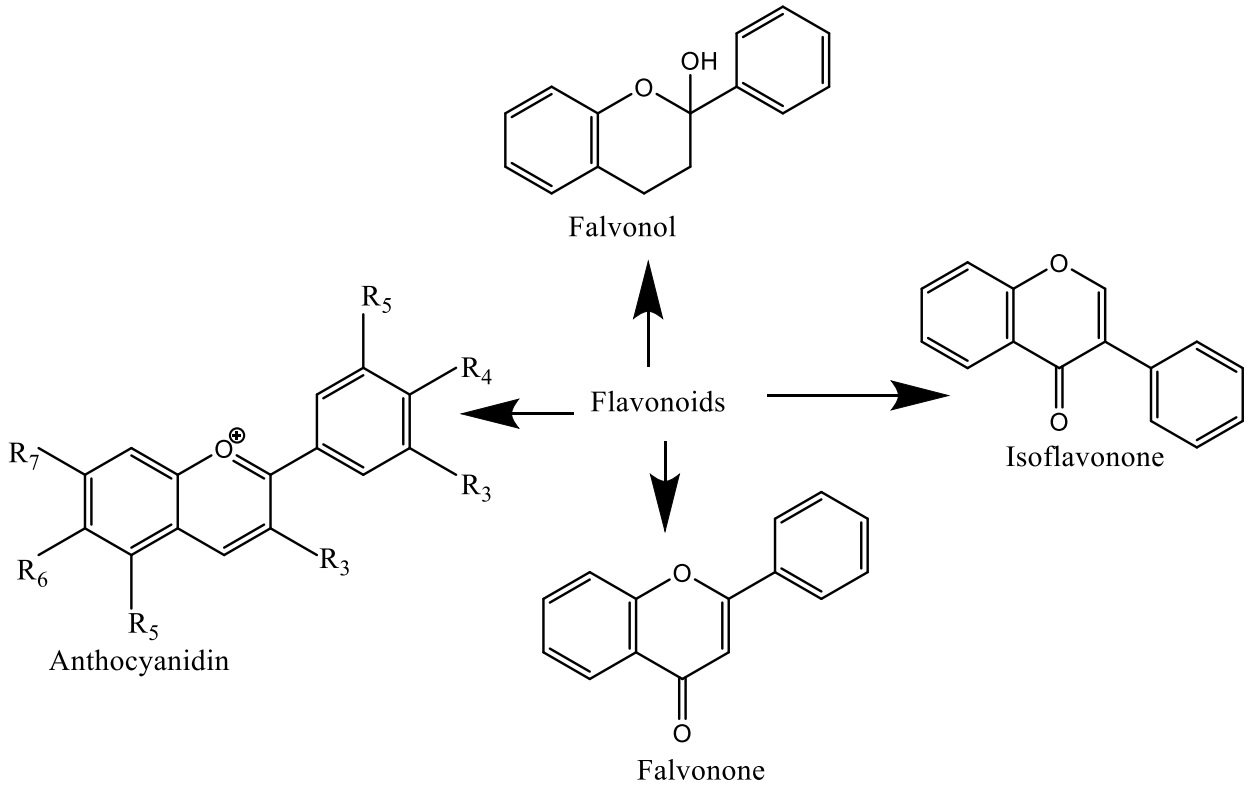


Figure 3: flavonoids classification

Flavonoids can be divided into four types: flavanols, flavones, anthocyanidins, and isoflavonoids. The primary categories of flavonoids are presented in the following text. For each flavonoid group, a chemical structure of the molecule is drawn. Compounds from diverse flavonoid subclasses are grouped together in their flavonoid groups. Figure 4 depicts the major categories of flavones and anthocyanidins. They are also classified into several subcategories. Catechin, gallocatechin, catechin-3-gallate, epicatechin, and epigallocatechin are flavan-3-ols (EGC). The flavonol subclass includes kaempferol, myricetin, quercetin, and rutin [Batra et al., 2013]. Other substances are likewise categorized as flavonoids, but in different subclasses.

Any plant extract's total flavonoid content (TFC) can be determined using a reliable method. Sodium nitrite solution, aluminum chloride solution, sodium hydroxide, and

distilled water are added to the plant extract in prescribed amounts by [Kim and Lee 2003], and a UV-Spectrophotometer is used to detect the absorbance at 510 nm. Calibration is done with aqueous solutions of known quercetin concentrations, and the results are given in mg quercetin/g sample.

1.3. Benefits of Phenolics and flavonoids Compounds for human health

Many chronic diseases are caused by an excess of oxidants and chronic inflammation, and studies in both experimentation and epidemiology have shown that plant antioxidants can help prevent and treat these diseases. Due to their ability to reduce free radical scavenging, or metal chelating activities, Antioxidants from plants, especially phenolic compounds, can reduce oxidative stress while keeping a balance between oxidants and antioxidants in the body. [Aguilera et al., 2016]. Phenolic compounds can easily donate hydrogen to lipids or other biomolecules via hydroxyl groups positioned along the aromatic ring, and the aromatic phenolic ring can stabilize and delocalize the unpaired electron inside its aromatic ring [Dey et al., 2016]. Hypertension is a common and typically progressive condition that puts people at risk for heart disease and other consequences [Chalmers et al., 2009]. Around the world, hypertension is a significant and growing public health problem, with some studies placing the rate of the condition at one-fourth of the adult population. [Van et al., 2013]. It is believed that inhibiting the angiotensin-converting enzyme (ACE) is a potential area for the discovery of new antihypertensive medications and an essential therapeutic strategy for the management of high blood pressure. [Ji et al., 2005]. ACE catalyzes the conversion of angiotensin I into angiotensin II, a peptide that causes vasoconstrictive effects, as well as the degradation of bradykinin, a strong

vasodilator. Some of the most often used ACE inhibitors, such as captopril, benazepril, enalapril, and others [Sleiman et al., 2001], have shown some drawbacks, such as susceptibility to proteolytic degradation, which can lead to side effects. As a result, scientists have been looking for novel ACE inhibitors in natural sources, notably those derived from plants. Many studies have found that polyphenol-rich foods are useful in preventing and treating hypertension, mostly by inhibiting ACE [Hügel et al., 2016]. Patten et al. [Patten et al., 2016] described 74 plant groups with substantial ACE inhibitory action in a recent review. Field and Newton [Field et al., 2013] It was also found that bioavailable cocoa polyphenols have antihypertensive effects, including ACE inhibition.

1.4. Chinese medicine plant and their phenolic content

Traditional Chinese medicine (TCM) has been practiced in China for thousands of years [Liu et al., 2018] and was the first medical treatment used to cure wounds and diseases by the ancient Chinese. With time, Chinese people began to examine and document the pharmacological actions of the herbs they were consuming, based on their own personal experiences. They created the first TCM system by classifying medicinal herbs into five flavors: pungent, sweet, sour, bitter, and salty. The therapeutic effects of TCM, as with all herbal medicinal products (HMPs), are influenced by a variety of elements that affect the quality of the beginning materials, such as seed stock quality and age, climate, soil, humidity, temperature, and sunlight. Storage, contamination, and contaminants can all have an impact on material quality [WHO., 2007].

1.5. Paeonia lactiflora roots

Traditional Chinese medicine (TCM) has been used for centuries, particularly herbal therapy, as described in the Yellow Emperor's Inner Canon and the Essential Prescriptions from the Golden Cabinet, has been used to treat cardiovascular diseases. [Jin Gui Yao Lue 2012]. Chinese herbal medicine is widely used in Taiwan to treat cardiovascular disorders such as hypertension, dyslipidemia, and stroke as a supplemental and alternative therapy. As a result, research into the drugs used to prevent and cure atherosclerosis has gotten a lot of attention in recent years. The cellular and molecular aspects of Chinese herbal medicine's underlying effective processes in treating atherosclerosis are only beginning to be explored [Hung et al., 2021]. For over 1200 years, One of the most well-known herbs in China, Korea, and Japan has been Paeoniae Radix. The tree Peony (also known as Paeonia Moutan) and the herbaceous Peonies are separated into two categories. Paeonia lactiflora Pall. (commonly known as Chinese Peony) is a herbaceous perennial blooming plant which has no persistent woody stem above ground. It stands 60–100 cm tall and has 20 – 40 cm long compound leaves.The enormous, spherical flower buds open to reveal massive flowers with five to ten white, pink, or crimson petals and golden stamens that measure 8–16 cm in diameter (Figure 5). It grows on dry open stony slopes, riverbanks, and sparse woodland borders in its native East Asia [He et al., 2011].

Figure 4: Paeonia lactiflora Pall

The dried root of P. lactiflora, also known as Bái Sháo (literally, "Red Peony"), has been used for thousands of years as a medicinal plant in traditional Chinese medicine. The root is dug in the summer or autumn from 4–5-year-old cultivated plants and cleansed with water. It is boiled in water for a brief time after the bark and rootlets have been removed, then dried in the sun before being sliced. An extract of the root has been used to treat rheumatoid arthritis hepatitis, dysmenorrhea, cramps and spasms in the muscles, and chronic fever.[He et al., 2011].

1.6. In-vitro chemical assay of plant extract

1.6.1. Antioxidant Activity

Superoxide anion radicals, hydroxyl radicals, lipoxyl radicals, nitric oxide radicals, and other radicals are examples of uncoupled electron groups or atoms that can live independently. Free radicals are the natural metabolic products of an organism, and they

also serve a crucial purpose in the redox process of substance metabolism, such as transferring energy. However, light, heat, radiation, and other conditions may boost the formation of free radicals. Excessive free radicals in the body are unstable, capable of capturing electrons and exhibiting a strong oxidative ability, ultimately destroying the cell membrane, proteins, DNA, RNA, and other components, resulting in aging and other diseases. The oxidation property of oxygen free radicals produced by the oxidation respiratory chain is strong [Kim et al., 2014]. The body's endogenous free radical scavenging system, which comprises superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and other antioxidants, is the first line of defense against oxygen free radicals.
The following are the relevant reactions [Zhao et al., 1999]:

- SOD catalyzes the formation of hydrogen peroxide and oxygen from superoxide anion radicals (Figure 6).

Figure 5: SOD catalysis.

- CAT catalyzes the reaction of hydrogen peroxide with oxygen and water to create oxygen and water (Figure 7).

Figure 6: CAT catalysis.

- GSH-Px catalyzes the formation of oxidized GSH (GSSG) and two molecular

waters from reduced GSH (Figure 8).

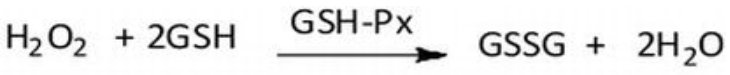


Figure 7: GSH-Px catalysis.

In order to speed up and improve the accuracy of the measurement of plant extract activity, it is critical to choose and use a reliable and quick approach to test antioxidant activity. Plant extracts' free radical scavenging capability and total antioxidant activity have been measured using a variety of approaches. The most frequent and reliable way is to use a spectrophotometer to measure the loss of free radicals, such as the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [Miller et al., 1999]. The Measure Ferric Antioxidant Power (FRAP) assay, which measures the reducing antioxidant power, is another reliable and commonly used approach.

1.6.1.1. DPPH method

The free radical scavenging method, α, α-diphenyl-β-picrylhydrazyl (DPPH), is the first technique for figuring out whether a chemical, an extract, or other biological sources have antioxidant properties. The most simple approach includes combining the desired chemical or extract with a DPPH solution and measuring the absorbance after a predetermined period of time. [Kedare et al., 2011] .DPPH is a room-temperature stable free radical that generates a violet solution in methanol or ethanol and has a lambda maximum of 517 nm. When a free radical combines with an antioxidant, it loses its free radical property due to chain breakage, and its color changes to light yellow, which may be measured by a drop in absorbance at 517 nm [Figure 9], [Xie et al., 2014]. With

increasing percentages of free radical inhibition, radical scavenging activity increased. By their hydrogen donating capacity, the level of discoloration shows the antioxidant's capacity to scavenge free radicals. The electrons become paired off, and the color of the solution fades stoichiometrically as the quantity of electrons taken up decreases [Saxena et al., 2021].

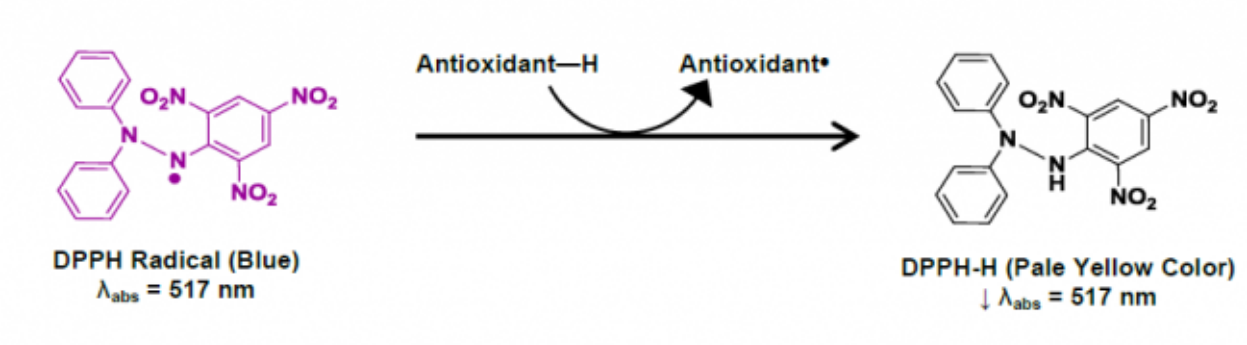


Figure 8: DPPH Antioxidant Assay Kit (Colorimetric) [biovision, 2021]

1.6.1.2. FRAP method

Ferric Ion Reducing Antioxidant Power Assay is one of the most widely used methods for determining the antioxidant activity of plant extracts (FRAP). It's simple, quick, cheap, and durable, and it doesn't require any specialist equipment. Under acidic circumstances, electron-donating chemicals (such as phenolic compounds) convert the yellow Fe^{3+} TPTZ complex (2, 4, 6-tri (2-pyridyl)-1,3,5-triazine) to the blue Fe^{2+} TPTZ complex in the FRAP technique [Benzie, and Strain 1996]. Any electron donating substance having a lower redox potential half reaction than Fe^{3+}/Fe^{2+} TPTZ will accelerate the reaction and increase the amount of blue complex produced. [Singh et al., 2012].

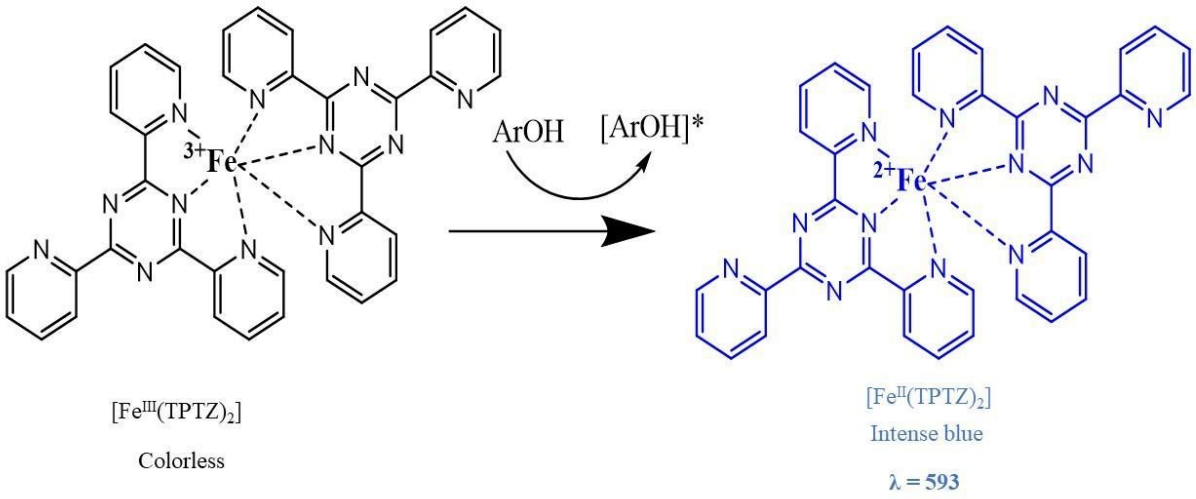


Figure 9: Ferric reducing antioxidant power (FRAP) reaction mechanism [Sadeer et al., 2020].

1.6.2. Antibacterial activity

Bacterial infections are a major medical problem due to the indiscriminate use of antibiotics, which select resistant germs, which then multiply [Rodrigues et al., 2019]. Among the microorganisms that cause infections include Pseudomonas aeruginosa (Pseudomonadaceae), Escherichia coli (Enterobacteriaceae), and Staphylococcus aureus (Staphylococcaceae).[Santos et al., 2019]. Bacterial resistance strategies have been found to include efflux pumps, which expel the antibiotic; also, bacteria are capable of changing the antibiotic's target for mutation or enzymatic inactivation, as well as altering the bacterium's permeability to the medication [Veras et al., 2017]. Antibiotics cannot limit bacterial growth on their own, thus chemicals that modify their effect are required to augment the drug's action [Costa et al., 2017, Coutinho et al., 2008]. These molecules capable of influencing standard medications can be found in plants,

which have antibacterial ingredients produced from their secondary metabolism, particularly aromatic herbs, whose essential oils have a wide range of biological and pharmacological activity [Bezerra et al., 2017, Duarte et al., 2017]. So the goal of this study was to see how effective paeonia lactiflora extract was against pathogenic strains. As a result, we predict that the species contains a high concentration of phytochemical elements with biological action against harmful microorganisms like bacteria. Herbal medicines have been used by humans for millennia. Traditional medicine practitioners have highlighted the therapeutic usefulness of several indigenous plants for a variety of ailments [Ramasamy et al., 2004]. Antimicrobial properties of medicinal plants are being reported more frequently from throughout the globe. According to the World Health Organization (WHO), 80 percent of people worldwide use plant extracts or their active substances as folk medicine in conventional treatments [Ekor et al., 2014]. Drugs can suppress hazardous microbes. However, this has led to the creation of several germs that are resistant to drugs. posing a serious clinical problem in the treatment of infections. The pharmaceutical industry has developed a variety of novel antibiotics, yet bacterial resistance to these medications has increased. Microbial pathogens generally have the genetic ability to acquire and spread resistance to synthetic therapeutic drug substances [Rates et al., 2001].

1.6.3. HPLC-PDA and LCMS analysis of phenolic compounds

High performance liquid chromatography (HPLC) is a technology that is frequently used for both the isolation and quantification of these chemicals in order to assess the phenolic content in natural extracts. The use of a reverse phase column and a water-acetonitrile

mobile phase allows for the separation of various kinds of phenolic compounds. Since phenolic compounds have potent chromophores, a photodiode array detector (PDA) is typically utilized for phenolic chemical detection in food. For structural characterisation of phenols, mass spectrometry (MS) in conjunction with HPLC has commonly been used. For the structural verification of phenols in matrices, electrospray ionization mass spectrometry (ESI/MS) in both the positive and negative modes has been used. [Naczk et al., 2004].

The natural product's raw powder can be separated into pure isolates using preparative HPLC-PDA. The huge capacity of the column allows for excellent solution loadability to isolate each chemical (10–100 mg) independently. To learn more about the structure of phenolic phytochemicals, further spectroscopic methods can be used. These include 1H-NMR and 2D-NMR in the COSY and HMQC modes.

1.7 Antiglycation and diabetes complications

Protein, lipid, and nucleic acid amino acids interact non-enzymatically with reducing sugars to generate the glycation process, which results in the creation of anti glycation end products (AGEs). The production of AGEs has a significant influence on health, causes organ damage, and affects the function of various organs. such as the heart, eyes, blood vessels, kidney and nerves. Aging-related poor glycemic control and elevated glucose lead to increased AGE production, which in turn heightens the oxidation process. Studies employing natural substances, such as flavonoids from various plants, were conducted in vitro and in vivo to examine the effects of these substances on the production of AGEs. The mechanism of inhibition uses two different approaches: first,

type A inhibitors (sugar competitors), which alter amino acids and peptides to prevent sugar attachment; and second, type B inhibitors, which interact with the ketose or aldose groups of sugars to prevent their binding to proteins. [Abbas et al., 2016].

1.8. Hypothesis and Research Question

The study's premise asserts that there are differences in TPC, TFC, antibacterial, and antioxidant activities between Paeonia lactiflora and similar species tested in other countries. Plant extracts can be employed in a variety of disciplines and applications, including pharmaceuticals and food.

1. Are phenolic and flavonoid compounds abundant in Paeonia lactiflora?
2. Does Paeonia lactiflora extract possess antibacterial and antioxidant activity?
3. According to the HPLC/PDA and LC/MS analysis, what phenolics are found in the plant?
4. What biological applications based on plant in-vitro activities can be created?

1.9. Objectives

_ To characterize and analyze the phytochemical constituents of Paeonia lactiflora, focusing on the roots and omitting other parts of the plant.

_ To determine TPC and TFC of Paeonia lactiflora root extract.

_ To determine various in-vitro pharmacological activities of paeonia lactiflora root extract, such as antioxidants and antibacterial activity.

_ Utilizing HPLC/PDA to screen the phytochemical elements of plant root extracts.

1.10. significant of the study

The traditional Chinese herb Paeonia lactiflora roots were chosen to analyze their TPC and TFC, as well as to look into their antibacterial and antioxidant capabilities. The antioxidant and antibacterial activity, along with several additional tests like TPC and TFC but for other genus species and from various origins, were studied separately in the literature.

Chapter Two

Literature Review:

Paeonia lactiflora has been well established in traditional medicine since ancient times. The peony's origins go back thousands of years. It has been grown in China and Europe for hundreds of years. Originally cultivated for their medicinal properties, it was well-known for its significance. Gout, osteoarthritis, fever, respiratory tract infections, and cough are all treated with peony [Gong et al., 2017].

Whereas Paeonia lactiflora Pallas' dried root extract demonstrated strong anti-allergic properties [Shi et al., 2016], By directly regulating the gene expression of cardiomyocytes, P. lactiflora protects heart function. Meanwhile, It may indirectly affect other cardiac function pathways, assisting in the therapy of heart disease. [Liu et al., 2018].

The impact and mechanisms of peony total glucosides on rat collagen-induced arthritis-related joint damage were investigated by Zhu. TGP (25, 50, and 100 mg/kg/d) was given to rats orally from day 14 to day 28 after immunization. TGP's therapeutic effect may be

attributed to its ability to improve synoviocyte secretion and metabolism while also inhibiting the abnormal proliferation[Zhu et al., 2005].

Wei et al. (2019) gave rabbits with antigen-induced arthritis in New Zealand 60 mg/kg bw TGP orally for three months. Total glucosides of peony avoid juxta-articular bone loss and subchondral bone damage in experimental arthritis, according to the scientists [Wei et al., 2019].

The herbal drug, according to Blaschek et al. (2013), includes monoterpenes and monoterpene glycosides (such as paeoniflorin, albiflorin, and oxi paeoniflorin), gallotannins, triterpenoids (such as ß-sitosterol, daucosterol, and benzoic acid), and carbohydrates.

Investigated was paeoniflorin's ability to break the vicious cycle of inflammation between adipocytes and macrophages. by Jiang B et al. (2012). According to the scientists, paeoniflorin has anti-inflammatory properties.

In a mouse model of passive cutaneous anaphylaxis, Lee B (2008) investigated the anti-allergic activity of the root of Paeonia lactiflora (80 percent ethanolic extract) and its constituents paeoniflorin and paeonol. The research agents were taken orally for 5 days. Paeonia lactiflora extract, paeoniflorin, and paeonol all prevented scratching and passive cutaneous anaphylaxis in mice.

Im et al. (2012) investigated the methanol extract and fractions from Paeoniae radix for their inhibitory effect on nitric oxide development and antibacterial activity. As a result, the ethyl acetate fraction of the methanol extract had the strongest antibacterial activity and the strongest inhibitory effect on nitric oxide development. Polyphenols have been examined extensively over the world as an antioxidant agent. Antioxidants are commonly

found in fruits, vegetables, drinks, and Traditional Chinese medicine [Cai et al. 2004, Song et al. 2010]. For a very long time, traditional Chinese medicine (TCM) has been utilized extensively in Asia. Plants provide the majority of TCM sources, including leaves, stems, roots, and whole plants. Plants have a lot of polyphenols, and TCM has a lot of them.

Chapter Three: Experimental work

3.1. Chemicals, Reagents and Plant materials

3.1.1. Chemicals

Ethanol (HPLC-grade), methanol (HPLC-grade), acetonitrile (ACN) (HPLC-grade), water (HPLC-grade), dimethyl sulfoxide (DMSO), trichloroacetic acid, ferric chloride hexahydrate, gallic acid, agar, sodium hydroxide, acetic acid, LC-MS grades of formic acid, sodium phosphate, potassium ferricyanide, aluminum chloride, quercetin, ferrous sulfate, ferric chloride, glucose & fructose were all purchased from Sigma-Aldrich, Israel. Six well plates, Gentamicin, penicillin from (Biological industries, Israel). Based on their retention and UV-Vis spectra, phenolic and flavonoid standards were employed as markers in the identification of plants are Quercetin, Gallic acid, were from Sigma. Sodium phosphate monobasic monohydrate buffer, Bovine Serum Albumin (BSA) preparing by dissolving 100.0 mg albumin in 100 ml phosphate buffer. Membrane filters (0.45 µm pore size) were purchased from Sigma-Aldrich. Ultrapure water (>18 MΩ cm-1) for analytical and preparative HPLC experiments was generated from an Ultrapure Water system, Millipore.

3.1.2. Reagents

Folin-Ciocalteu reagent. Ferric chloride hexahydrated (20 mM, Mwt = 270.3 g/mol) was prepared by dissolving 540 mg of it in 100 ml of water. 10% $AlCl_3$ was prepared by dissolving 10 g of $AlCl_3$ in 100 ml of water. 7.5% Na_2CO_3 was prepared by dissolving 7.5g of Na_2CO_3 in 100ml of water. DPPH (0.1mM, Mwt= 394.32 g/mol) was prepared by dissolving 4 mg of DPPH in 100 ml of 99.9% methanol. sodium phosphate monobasic monohydrate buffer, Bovine Serum Albumin (BSA), albumin, phosphate buffer.

3.1.3. Plant materials

The roots of P. lactiflora pall were purchased from Al-sha'leh traditional herbal market (Ramallah, Palestine) and authenticated by Dr. Nidal Jaradat, professor in pharmacognosy at Al-Najah University. The root was already dried, therefore, the mixer was used in order to ground the roots then prepare them for extraction.

3.2. Instrumentation

Specord 40 UV- VIS spectrum, versatile single-beam spectrophotometer for the measurement of 190-1100 nm conforms to Ph.Eur. quality, made by analytik jena company, Rotary evaporator, laboratory water bath, ultrasonic homogenizer and autoclave. The analytical HPLC is the Waters Alliance (e2695 separations module), equipped with 2998 Photodiode Array (PDA). Data acquisition and control were carried out using Empower 3 chromatography data software (Waters, Germany). The full scan UPLC-MS analysis via a chromatographic column or direct to the API of the crude samples using in the Electrospray Ionization (ESI) was carried out using a TSQ EnduraTM Triple Quadrupole Mass Spectrometer LCMS system (Thermo Fisher

Scientific). All data acquisition and analysis were performed using Trace finder software, version 4.1, TSQ-Endura and TSQ Quantiva, Xcalibur Quan browser software version 3.0 (Thermo Fisher Scientific). The Preparative High-Pressure Liquid Chromatography (Prep-HPLC) system consisted of 3535 quaternary gradient modules equipped with 996 PDA detectors.

3.3. Methodology

3.3.1. Preparation of plant materials and extraction

P. lactiflora roots were dried, so a blender was used to grind it, Paeonia extracts were obtained by maceration from dried roots using ethanol as the extraction agent, this was done by weighing 22.5 g of Paeonia lactiflora, added to 600 ml ethanol (EtOH) 99% EtOH in 1000 ml volumetric flask, then put the flask in the sonicator for 3 hours. Thereafter the sample was filtered using suction filtration. To obtain the final extract, the solvent was then evaporated using a rotary evaporator, and the extraction yield was then determined. The recovered extract was then put in storage until we could use it for another type of analysis.

3.3.2. Extraction Yield

The extraction yield was measured by weighing the dry extract sample, then measure the yield by the equation:

$$\% \text{ Extract yield} = \text{weight of dried extract} / \text{weight of dried plant} * 100\%$$

3.3.3. Fluorescence-based assay of the inhibition of Advanced glycation end products (AGEs) formation

Fluorescence technique was used to conduct the assay of the samples' ability to suppress the development of AGEs(Harris et al., 2011), with a few modifications from what was previously published as follows:

1. **Preparation of reagents:**

a. (100mM) sodium phosphate monobasic monohydrate buffer (pH 7.4) was prepared.

b. Bovine Serum Albumin (BSA) stock solution (1.0 mg/ml) was prepared by dissolving 100.0 mg albumin in a 100 ml phosphate buffer (pH 7.4).

c. Stock solution (1.0 mg/ml) of mixed glucose & fructose (1:1) was prepared in a phosphate buffer pH 7.4.

2. Preparation of samples and standard:

Each sample was dissolved and diluted with 95% ethanol to prepare two concentrations (300 & 120 ppm).

a. Test samples:

1. Preparing extract samples:

0.5 ml of each sample was mixed with 0.3 ml phosphate buffer pH 7.4, then 0.1 ml solution of sugar and 0.1 ml of BSA solution was added.

2. Preparing negative control:

0.3 ml phosphate buffer was mixed with 0.1 ml solution sugar, 0.1 ml BSA solution and 0.5 ml of ethanol (995).

Preparing positive control:

0.5 ml of standard solution (quercetin at 100 ppm concentration in ethanol) was mixed with 0.3 ml phosphate buffer pH 7.4, 0.1 ml solution sugar and 0.1 ml of BSA solution.

All test samples and standards were prepared in a glass test tube & cover then incubated in an incubator shaker at 37 **º**C for 7 days.

b. Fluorescence-based assay of the inhibition of AGEs formation:

After 7 days of incubation, quantitative analysis of fluorescent advanced glycation end products (AGEs) was performed for each sample by using a spectrofluorometer at excitation and emission wavelengths of 375 nm and 455 nm, respectively. The percentage of inhibition of AGEs formation was determined by the following equation:

$$\%\ inhibition = \frac{(F\ negative\ control - F\ experimental\ corrected\ )}{F\ negative\ control} * 100\ \% \quad (1)$$

Where F negative control is the fluorescence reading of negative control against blank as the base line & F experimental correction is the fluorescence reading of sample control against blank as the base line.

3.3.4. Total phenolic content (TPC) (Folin-Ciocalteu assay)

The content of the phenolic compounds was determined by using the Folin-Ciocalteu reagent [Singleton et al., 1965], which is a method based on electron transfer (ET) assay, which is done by reducing capacity to phenolic content.

For the plant extract, the content of phenol depends on the solvent used in the extraction [Falleh et al., 2013]. The calibration was done by using different known gallic acid concentrations (20, 30, 40, 50, 60, 70, 90 and 100 ppm).

In brief, we weighed 20 mg of paeonia lactiflora extract (dissolved with 2 ml 99% DMSO) in a volumetric flask of 100 ml, marked up with D.W. Thereafter, 0.5 ml of the plant extract with 2.5 ml of Follin reagent (10 %, v\v), 2.5 ml sodium carbonate (7.5 %, w\v) At the end, then the mixture was allowed to stand for 30 min at room temperature (dark condition). Eventually, At 760 nm, the absorbance was measured. Gallic acid standard at various concentrations (20–110 ppm) for calibration curve. Gallic acid equivalents (GAE) were used to express results as mg/g of sample. In order to ensure the precision of the result, the test was done three times, then the average of the absorbance was taken for calculation.

3.3.5. Total flavonoids content (TFC)

TThe flavonoids assay was conducted using a colorimetric technique. [Liu et al., 2007]. In a 100ml volumetric flask, the sample is prepared introducing 0.0217 g of plant extract dissolved in 2 ml DMSO), followed by 2 ml (95% EtOH), and 0.1 ml aluminum chloride (10% w/v), 2.8 ml distilled water, and and 0.1 ml of 1M sodium acetate, Test tubes were incubated at ambient temperature (25°C) for 30 min. The mixture was completely vortexed, and the samples' absorbance at 415 nm was measured. The calibration was done by using different known concentrations of standard quercetin (1, 2, 5, 10, 100, 200 ppm). In order to ensure the precision of the result, the test was done three times, then the average of the absorbance was taken for calculation.

3.3.6. Antioxidants Assay

3.3.6.1. Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of paeonia lactiflora root extract was measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) (Shen et al., 2010). In brief, the DPPH solution was prepared by weighing 4 mg of DPPH mixed with 100 ml 80% methanol in a volumetric flask, then covering the flask with Al foil and saving it in a cool place. The sample was prepared by weighing 0.04 g of the plant extract, mixed with 2 ml of DMSO then put it in a volumetric flask of 25 ml and marked up with DW. Different concentration series (80, 100, 150 and 300 ppm) were prepared using the extracted solution and DW. Eventually, 1.5 ml of DPPH solution was added to 0.5 ml of each sample, then marked up each sample with DW up to 10 ml. TThe mixture was vigorously mixed and left to stand for 30 minutes at room temperature. Then, a UV-VIS spectrophotometer was used to measure absorbance at 517 nm.. In order to ensure the precision of the result, the test was done three times, then the average of the absorbance was taken for calculation. Then, the percent of DPPH scavenging effect was calculated by using the following equation:

DPPH scavenging effect (%) or Percent inhibition

$$\%\ inhibition = \frac{(A\ control - A\ test\ )}{A\ control} * 100\ \% \qquad (2)$$

where $A_{control}$ is the Absorbance of the control reaction (only DPPH), A_{test}: the Absorbance in the presence of a standard of extract's sample.

3.3.6.2. Measurement of antioxidant activity by FRAP assay

0.04 g of BHT was dissolved in 100 ml of distilled water. Dilutions of this solution with distilled water were prepared to give the concentrations of 100, 200 and 300 µg/ml, which

are used to construct a calibration curve.

To prepare stock solutions of the sample, 0.02 g of paeonia lactiflora extract mixed with 2 ml DMSO was dissolved in 10 ml of methanol to give a concentration of 1mg/ml. Then sample concentrations of 25, 50, 200 and 400 µg/ml were prepared.

According to this method, aliquots of various concentrations of the standard and test sample extracts (100, 200 and 300 µg/ml) was mixed with 0.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of potassium ferricyanide (1% w/v) and incubated at 50°C for 20 minutes before adding 2 ml of 10% trichloroacetic acid and then centrifuged at 40 rpm for 10 minutes. A solution of BHT with a range of concentrations (100, 80, 60, and 40 ppm) was prepared as a positive control. At the end, the absorbance was measured at 700 nm by using a UV-VIS spectrophotometer.

3.3.7. Antimicrobial activity

Testing of the antimicrobial activity involved many types of microorganisms: Staphylococcus aureus, E. coli, MRSA, pseudomonas, and Shigella. Penicillin & Gentamicin are antibiotics used as positive controls against bacteria.

The extract's antibacterial properties were investigated using the well diffusion method, which depends on sample diffusion through a layer of solidified agar in a plate from a vertical cylinder.. This test is done by preparing Muller Hinton media, via mixing 11.4 g agar in 300 ml distilled water, and nutrient broth prepared for each microorganism. The media is boiled then sterilized by using an autoclave at 121 °C for 15 minutes. Then, the media is cooled, after that at 45 °C the suspension of each microorganism is added separately to the nutrient broth. Both gram positive (Staphylococcus aureus, MRSA) and gram negative (*Escherichia coli*, *Pseudomonas, Shigella*) bacteria were tested using UV-

Spectrophotometer until a suitable concentration was reached. Following the distribution of the Muller Hinton media to the plates (20 ml/plate), four 1 cm-diameter holes were made using a sterile cylinder. 100 µL of each extract was placed in each hole for each plate. A positive control disk was placed on the agar as well. The plates incubated at 37 ± 0.5 °C for 24 hours. After the incubation period, the zone of inhibition was measured by a caliper.

3.3.8 RP-HPLC analysis of phytochemicals

For the analysis of bioactive chemicals, an HPLC Waters Alliance e2695 equipped with a 2998 PDA detector was employed. Empower 3 chromatography data software was used for data collecting and control.

3.3.8.1 Quantification of phenolic compounds

Reversed phase HPLC method was employed for analysis of different polyphenolic compounds and flavonoids using C18 column (25 cm with 3.6 µm inner diameter) and a mixture of 0.5% acetic acid (solution B) and Acetonitrile (solution C) with a linear gradient mode according to the table below (Table 2), with an injection volume of 20 µL, a flow rate of 0.5 mL/min, and a column temperature of 25 ºC. A disposable 0.45 µm filter was used to filter all samples.A photodiode array detector with a wavelength range of 210 - 400 nm was employed.

Table 2: The gradient mobile phase conditions used for RP-HPLC analysis of the polyphenolic compounds and flavonoids in the plant extracts 0.5% acetic acid (solution B) and acetonitrile (solution C).

Time (minutes)	B %	C %
0.0	95%	5%
50.0	80%	20%
65.0	65%	35%
70.0	40%	60%
75.0	10%	90%
78.0	95%	5%
80.0	95%	5%

3.3.8.2. Preparation of standard solutions

The followings are the standards that used in HPLC analysis: gallic acid, caffeic acid, trans cinnamic acid, syringic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, rutin, ferulic acid, quercetin, vanillic acid, isovanillic acid, kaempferol, verbascoside, chlorogenic acid, Sinapic acid, and p-coumaric acid. 5mg of each standard was dissolved in 5 ml of 20% ethanol and volume was made up to 25 ml by distilled water. The mixture of the standards were injected (20 µL) into the HPLC chromatography and analyzed using the RP-phase method described above.

3.3.8.3 Preparation of Samples of the plant extracts for HPLC test:

A 100mL volumetric flask was filled with about 100 mg of dry extract that had been precisely weighed. 20mL of 95% ethanol was added and sonicated and volume was made up to 100mL by distilled water.

Chapter Four

4. Results and discussion:

4.1. Ultrasonic extraction

Ultrasonic extraction or sono-extraction method is a process used in this study to intensify the recovery yield of the phenolic compounds from the root extract. The dried root was ground to a fine powder before the extraction process in order to insure the reduction of particle size and increase in surface area. Methanol and Ethanol were used as a solvent for the extraction, where in [Falleh et al., 2013] studies, its shown that in order to optimize the extraction of the polyphenols, polar solvents used instead of non-polar solvents [Liu et al., 2007], ethanol was chosen because it's safer and less toxic, in addition ethanol can easily extract phenolics of polar to semi polar nature. In this part of this work, the extraction time was between 90-120 min, at 30°C for the extraction of polyphenolic from Paeonia lactiflora roots, where as in studies of (Ju et al. 2018 and Chirinos et al. 2007) showed that long time of extraction can increase the chance of occurrence of the oxidation on phenolic compounds by exposure of more oxygen atom, which will affect the capacity of antioxidants in the root extract, then the mixture was evaporated at 50-57 °C using rotary evaporator [figure 10]. Sonication was the method of choice, due to the relatively low cost, faster extraction time, and the need for small solvent volumes to speed the dissolution of a solid (root) into a liquid (ethanol).
The extraction yield was obtained by dividing the weight of the dried extract after evaporation (1.43 g) to the mass of dried plant material to give a yield of 6.4 %.

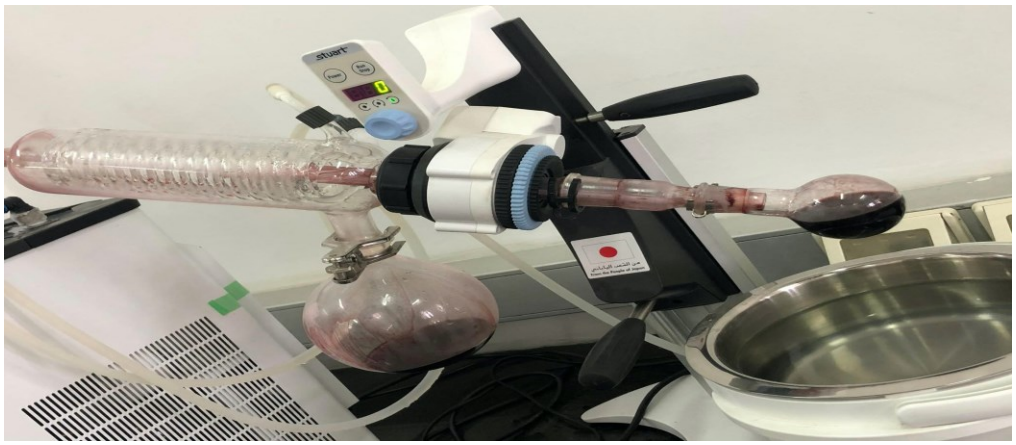


Figure 10: Rotary evaporator

4.2. Anti-glycation End Products formation (AGEs) Assay:

In this study, Paeonia lactiflora root extract was found to have positive effects on the reduction of AGEs. Results showed that the percentage of AGEs inhibition is 48.2 and 69.0% for 120 and 300 ppm concentration of Paeonia lactiflora roots extract, respectively, (Table 3). Quercetin was used as a positive control for inhibition of AGEs at 30-300 ppm (Table 4) which shows that the percentage inhibition ranges from 35.7 to 83.4%. Additionally, concentration-dependent percentage inhibition of AGEs was observed using the quercetin standard for the concentration range of 30-300 ppm, Figure 11.

Table 3: Percentage inhibition of AGEs using Paeonia Lactiflora Roots extracts using fluorescence method. (Fluoresce reading of negative control is 5.6).

concentration (ppm)	Fluorescence Response	Average	% inhibition	Standard error
300 ppm	1.75, 1.68 and 1.78	1.74	69.0 %	±0.53
120 ppm	2.9, 2.8 and 3.1	2.9	48.2%	±0.81

Table 4: percentage inhibition of AGEs for different concentrations of quercetin using fluorescence method. (Fluoresce reading of negative control is 5.6).

Quercetin standard solution					
concentration (ppm)	Fluorescence Response	% of inhibition	Average % of inhibition	Standard Deviation	Standard error
300	0.92	83.5	83.4	0.12	±0.21
	0.93	83.3			
	0.92	83.5			
150	1.803	66.5740	66.2	1.06	±0.61
	1.779	67.0189			
	1.888	64.9981			
90	2.296	57.4342	59.12	1.63	±0.94
	2.199	59.2325			
	2.120	60.6971			
30	3.451	36.0215	35.7	1.9	1.15±
	3.577	33.6856			
	3.363	37.6529			

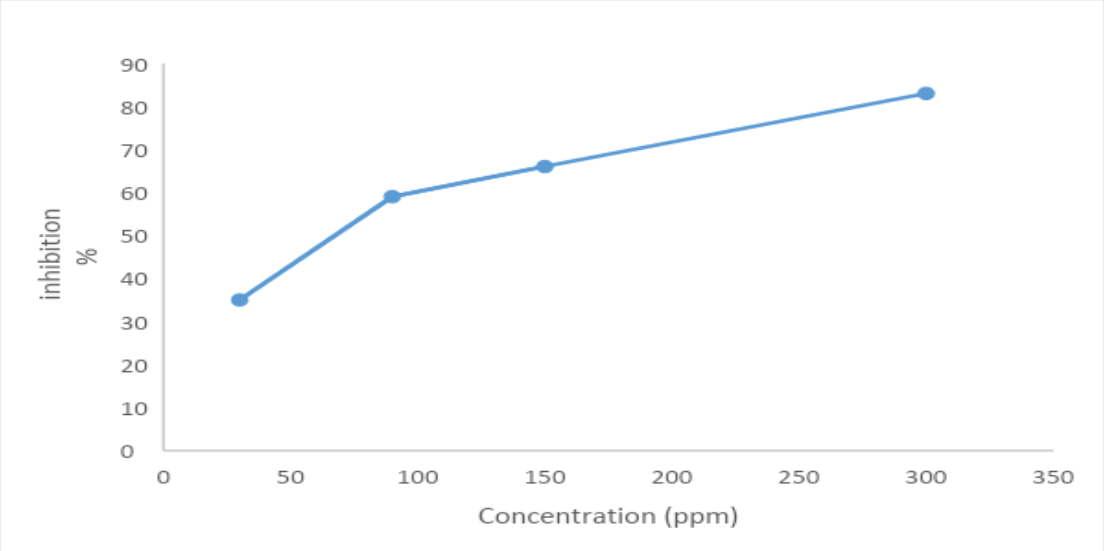


Figure 11: Concentration dependent effects of Quercetin positive control on in vitro formation of fluorescent AGEs.

The studied extract was found to have positive effects on the reduction of AGEs. In this simulation, higher concentrations of each extract and a positive control imply a higher inhibition percent of glycation products between glucose and fructose as mixed with albumin serum in the simulation as in the human body. The percentage inhibition of AGEs is increasing with increasing the concentration of plant extracts.

4.3. Total Phenolic Content (TPC)

In plants, the phenolic compounds can be found in the cell membrane, fruits, roots, and leaves. Therefore, the amount of the phenolic compounds can be affected by the environment and the climate. Thus the quality and quantity of the phenolic compound can be affected.

The TPC of the Paeonia lactiflora roots extract was determined by using a Folin-cioalea reagent, and was expressed as mg gallic acid equivalents (GAE), per gram of plant extract. The TPC of the test fractions were measured using the standard curve of gallic

acid, linear fit was obtained with an equation y = 0.0096x + 0.0836 and R^2 value of 0.9983 [figure 12].

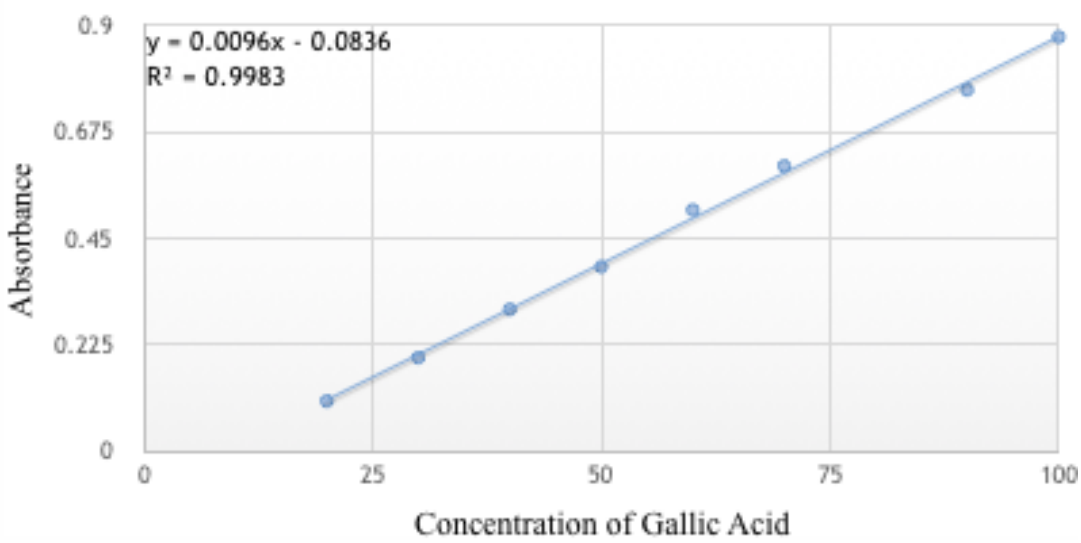


Figure 12: Calibration curve of TPC (absorbance of different concentrations of gallic acid vs. concentration (ppm))

TPC in this study was calculated to be 193.9 ± 1.54 mg/g of Paeonia lactiflora extract. It is less than reported by Zhou et al., 2019 (323.19 ± 10.19). This can be explained due to using ethanol for extraction. Ethanol plays a key role in extracting phenolic compounds; which indicate the polar nature of the phenols present in the plants.

4.4. Total Flavonoid Content (TFC)

The total flavonoid content of paeonia lactiflora extract was measured using a colorimetric assay. TFC was calculated using the standard curve of quercetin. Linear fit was obtained with an equation y = 0.0094x + 0.0205 and R^2 value of 0.9993 [figure 13], and were expressed as mg quercetin per gram of the plant extract. The TFC was calculated to be 55.9 ± 0.34 mg Quercetin/ g of dry sample of Paeonia lactiflora.

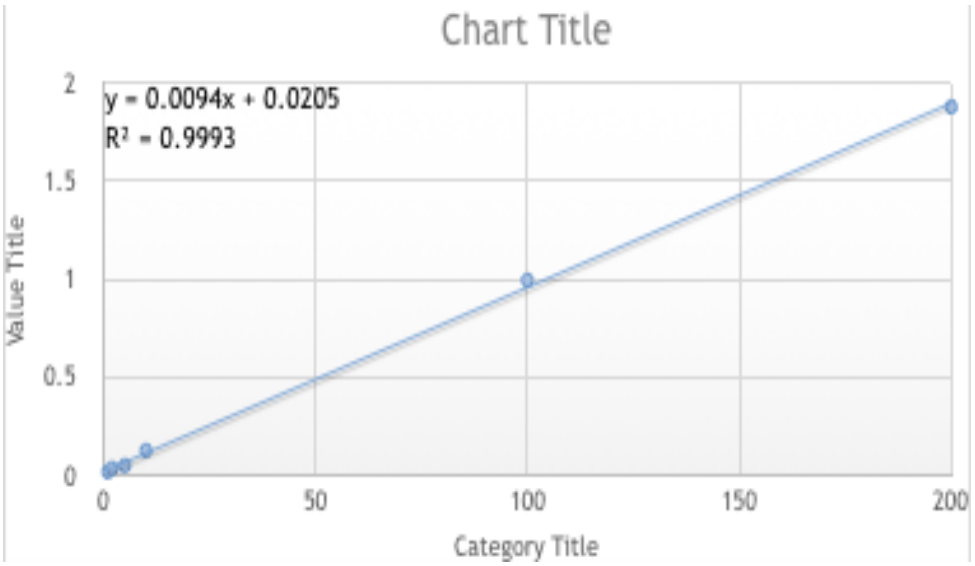


Figure 13: Calibration curve of TFC (absorbance of different concentrations of Catechin vs. concentration (ppm).

4.5. Antioxidant Activity

The antioxidant ability of plant extracts is largely determined by their composition and the testing circumstances. Because there are so many variables at play, the effects of the extracts can't be fully characterized by a single approach. To fully comprehend the diverse processes of antioxidant action, it is required to perform multiple types of antioxidant capacity measurements. The antioxidant activity of the extracts was assessed in this work using the following methods: investigation of their DPPH scavenging effects and testing of their ability to reduce ferric (III) iron to ferrous (II) iron in the FRAP reagent.

4.5.1. Antioxidant activity by DPPH radical scavenging assay

Oxidation reactions can form free radicals which can damage different cells. The principle of the antioxidants are to these reactions first, by removing the free radicals, then prevent

other oxidation reactions. In this test, DPPH, which is a stable purple free radical with maximum absorbance at 515 nm, was used, which can react with hydrogen donors, which lead to color change from purple (strong absorption) to yellow (low absorption).

The concentration of plant extract and inhibition curve was used to calculate the DPPH scavenging activity of the plant extract %; linear fit was obtained with an equation y=-6.7503x + 1.4397 and R^2 value = 0.976. As a result, the plant extract showed a high radical scavenging activity as shown in table [5]:

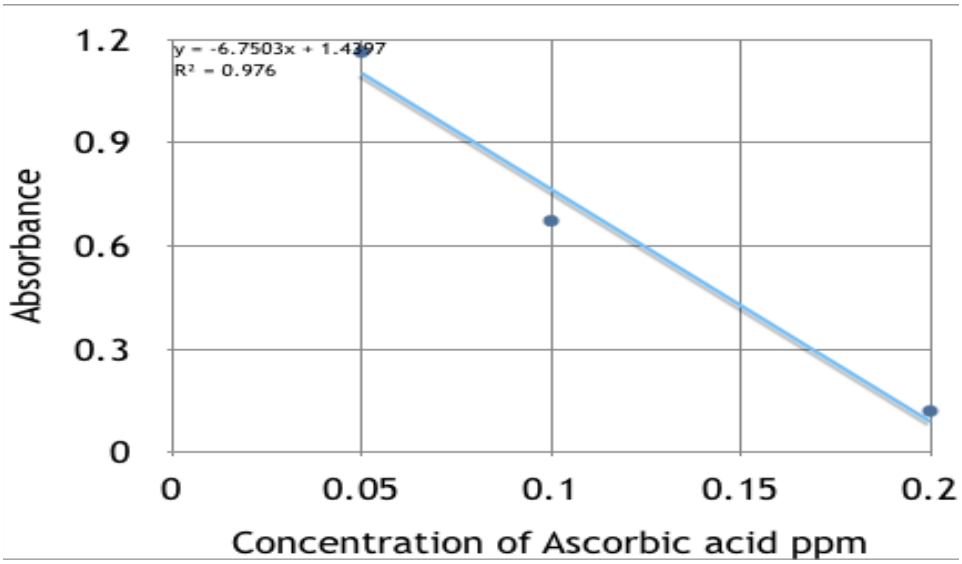


Figure 14: Calibration curve of DPPH (Absorbance of different concentrations of Ascorbic acid vs. concentration (ppm)).

Table 5: DPPH free radical scavenging activity (mg ascorbic acid /g of dry sample) and % inhibition of Paeonia lactiflora sample.

-	Absorbance (a.u.)	inhibition%
control	1.250	-
sample	0.751	56.8% ±1.2%

Comparing our results of DPPH of Paaeionia lactiflora extract (**56.8%±1.2%**) with that found by (MengFei et al) the value was 94.48±0.24.

4.5.2. Reducing power FRAP assay:

The FRAP assay, a simple, automated test that measures the extract's ferric reduction activity, is offered as a novel tool for testing "antioxidant power." At low pH, ferric to ferrous ion reduction results in the formation of a colorful ferrous-tripyridyltriazine complex. The absorbance change at 700 nm in test reaction mixes is compared to those containing ferrous ions in known concentrations to produce FRAP values. With antioxidant combinations, including extract, and solutions containing one antioxidant in pure form, absorption changes are linear over a wide concentration range.

The antioxidant activity of ethanol and aqueous plant extracts of *paeonia lactiflora* was evaluated by the FRAP method and was expressed as mg BHT per gram of plant extract. It was calculated using the standard curve of concentration of BHT. Linear fit was obtained with an equation y = 0.001x + 0.2167 and R^2 value of 0.9967.

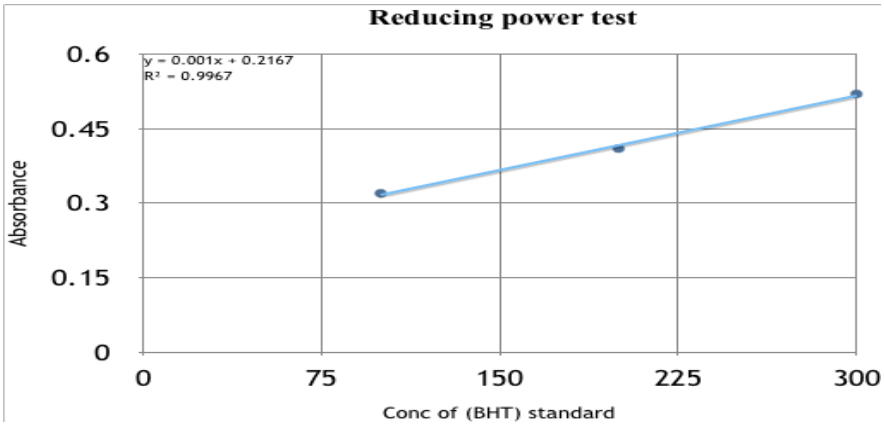


Figure 15: Calibration curve of BHT Standard

Antioxidant activity FRAP (mg BHT/ g of dry sample of Paeonia lactiflora was calculated to be 1524 ± 10.4**.** To our knowledge, no study has measured the antioxidant activity for Paeonia lactiflora using the FRAP method with the BHT standard.

4.6. Antimicrobial activity by well diffusion technique

In this part, the antibacterial activity of the plant extract (paeonia lactiflora) was studied against both gram positive and negative bacteria, gram negative like Escherichia coli and gram positive like Staphylococcus aureus. The test on each species was performed in triplicate, to ensure the reliability of the results. The zone of inhibition of the plant extract against each bacterial type is depicted in the table below (Table 6), followed by the photos of each disc of the test results.

From the result, we can conclude that In comparison to the positive control (penicillin), the paeonia lactiflora root exhibits activity against gram positive bacteria (Staphylococcus aureus) with a zone of inhibition of 10 ± 0.8 mm. Paeonia lactiflora extract has a good zone of inhibition against MRSA with a value 25 ± 1.4 mm compared to the penicillin 7mm, and gentamicin 10mm. On the other hand, paeonia lactiflora extract has no effect against both E coli, Shigella and Pseudomonas.

Table 6: Zone of inhibition of paeonia lactiflora extract against Staphylococcus aureus, MRSA, E.Coli, Shigella, and Pseudomonas Corresponding to positive control [Penicillin and Gentamicin,]. *ND= Not Detected (no effect).

Bacterium Type:	**Zone of inhibition**		
	Solvent/sample	**Distance**	**Ref. (std.)**
Staphylococcus aureus	Blank DMSO	ND	Penicillin 13 ± 1.0 mm Gentamicin 30 ± 1.5 mm
	P.L extract	10 ± 0.8 mm	
MRSA	DMSO	ND	Penicillin 7± 0.8 mm Gentamicin 10 ± 0.8 mm
	P.L extract	25 ± 1.4 mm	
E.coli	DMSO	ND	Penicillin ND Gentamicin 30 ± 1.8 mm
	P.L extract	ND	
Shigella	DMSO	ND	Penicillin ND Gentamicin 30± 1.8 mm
	P.L extract	ND	
Pseudomonas	DMSO	ND	Penicillin ND Gentamicin 30± 1.8 mm
	P.L extract	ND	

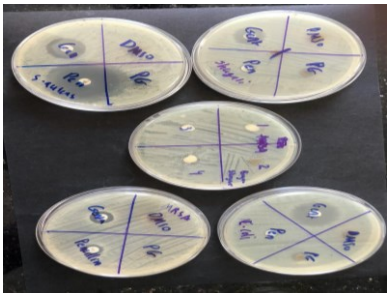


Figure 16: The Effect of the Solvent on Different Bacteria Types.

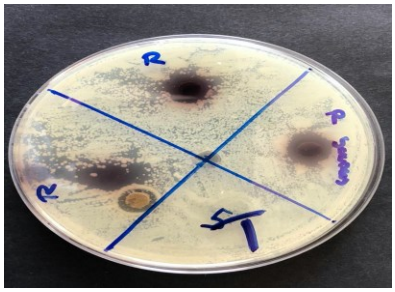


Figure 17: The effect of Paeonia lactiflora extracts on **S. aureus**

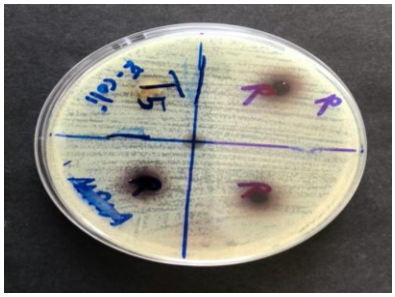


Figure 18: The effect of Paeonia lactiflora extracts on **E. coli.**

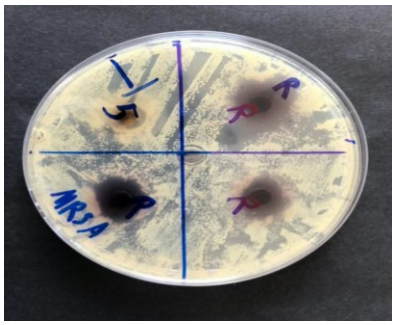


Figure 19: The effect of Paeonia lactiflora extracts on **MRSA.**

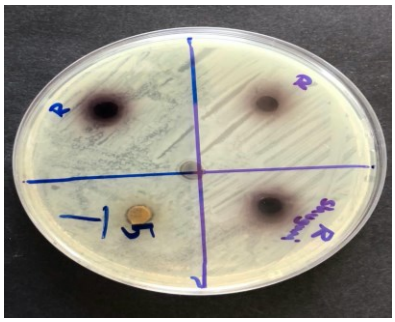


Figure 20: The effect of Paeonia lactiflora extracts on **Shigella.**

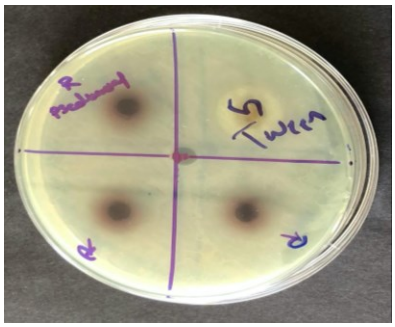


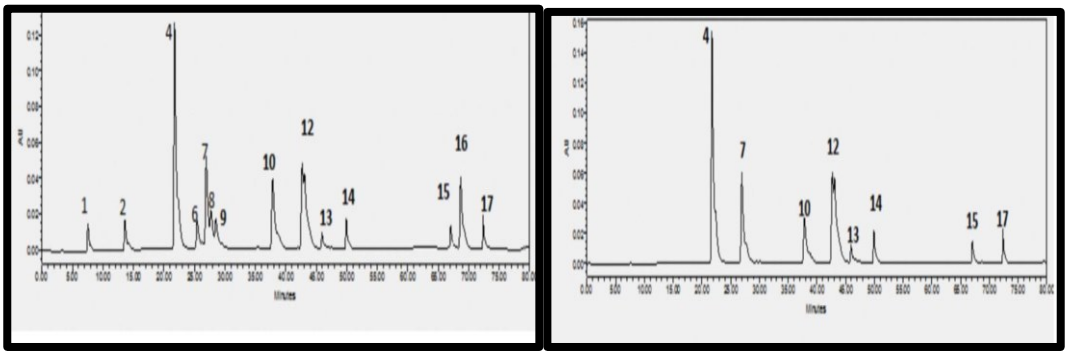
Figure 21: The effect of Paeonia lactiflora extracts on **Pseudomonas.**

Figure 16-21: The effect of paeonia lactiflora root's extract on different bacteria types.

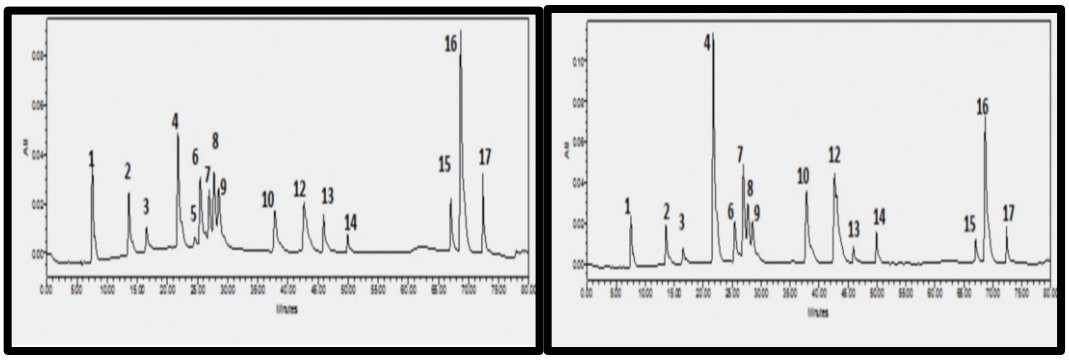
4.7. HPLC analysis

4.7.1HPLC analysis of the standards of polyphenolic compounds and flavonoids

The mixture of 17 standards were injected (20 µL) into the HPLC chromatography and analyzed using the RP-phase method described above. Different wavelengths using the photodiode array detector were used as each compound has its own wavelength of maximum absorption (Table 7). Figure 22. shows the chromatograms of the standard mixture at different wavelengths (300 nm (a), 323 nm(b), 270 nm (c), and 290 nm (d)). As it is obvious from Figure 22. (a-d), the 17 compounds were separated when different wavelengths were used. Table 7 summarizes the retention times of the standards with maximum wavelength of absorption for each standard.



a)300 nm b)323 nm



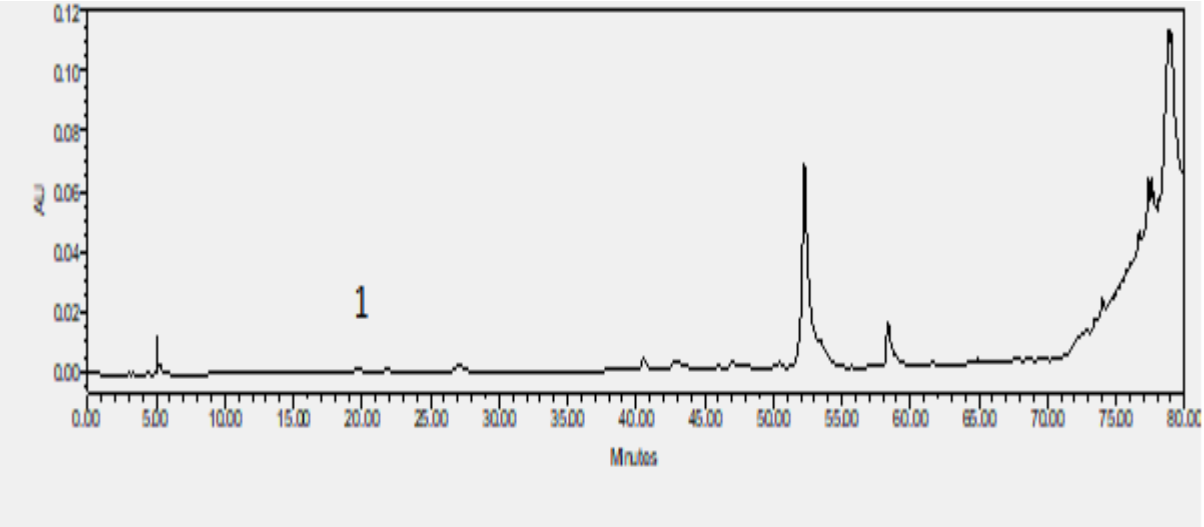
c) 270 nm d)290 nm

Figure 22. HPLC chromatogram of polyphenolic and flavonoid standards analysed using RP-HPLC method at 300 nm (a), 323 nm(b), 270 nm (c), and 290 nm (d).

The plant extracts were analyzed using the method developed for the standards. Figure 23 shows the chromatogram for ethanolic extract of **Paeonia lactiflora root's extract** at 2 wavelengths (350 and 315 nm). At 350 nm, gallic acid was detected in the plant extract, while at 315 nm, gallic acid and 3,4-Dihydroxybenzoic acid were detected in the chromatogram of **Paeonia lactiflora root's ethanolic** extract.

Table 7. List of Standard compounds analyzed using RP-HPLC method with their retention times and maximum wavelength of absorption.

Standard #	Standard name	Retention time (min)	Wavelength (nm)
1	Gallic acid	8.26	271
2	3,4-Dihydroxybenzoic acid	13.87	259
3	3,4-Dihydroxyphenylacetic acid	16.57	280
4	Chlorogenic acid	21.64	323
5	4-hydroxyphenylacetic acid	24.55	274
6	Vanillic acid	25.42	260
7	Caffeic acid	26.92	322
8	Syringic acid	27.73	274
9	Isovanillic acid	28.55	259
10	p-Coumaric acid	37.82	309
11	Ferrulic acid	42.68	322
12	Sinapic acid	43.1	323
13	Rutin	45.99	255
14	Verbascoside	49.98	329
15	Quercetin	67.04	364
16	Trans-cinnamic acid	68.69	275
17	Kaempferol`	72.36	265



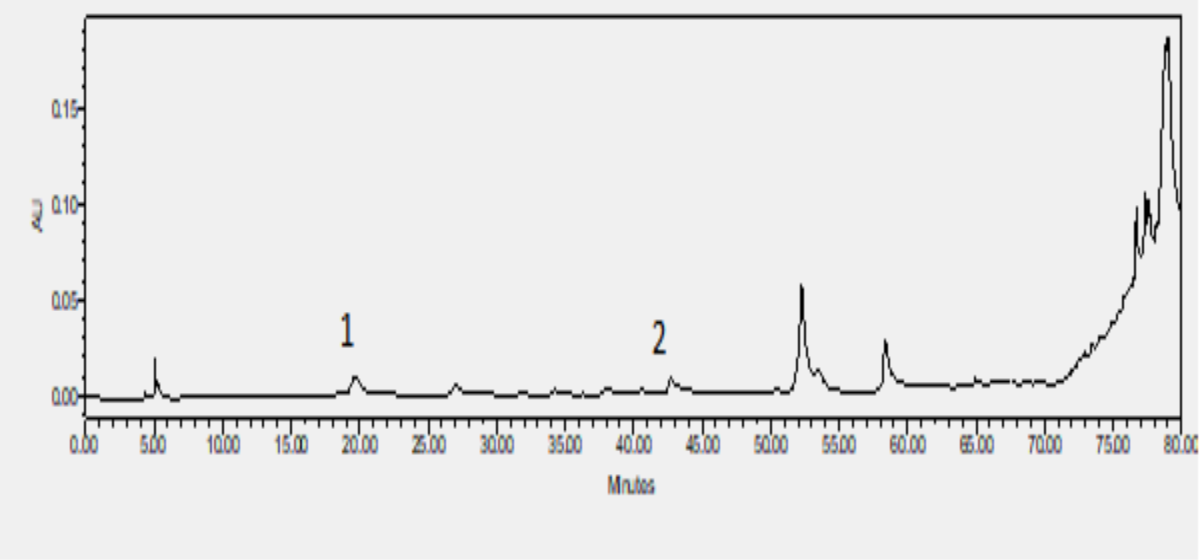


Figure 23: HPLC chromatogram of paeonia lactiflora roots analysed using RP-HPLC method at 350 (a) and 315 nm (b).

Chapter Five

5.1. Conclusion

The paeonia lactiflora roots were extracted simply and easily by using sonication with ethanol as an extracting agent. The yield of extraction was good. The present study showed the activity of biochemicals e.g. polyphenolics and flavonoids in Paeonia lactiflora extracts in the inhibition of floursense glycation end products and antibacterial effects as well as antioxidant activity, because Paeonia lactiflora extract is inexpensive, safe, and effective at preventing glycation development and, consequently, inhibiting diabetes problems, this study raises the possibility that it could be used as a food supplement.The plant extracts analyzed using the HPLC method showed the presence of gallic acid and 3,4-Dihydroxybenzoic acid, would be responsible for the activities investigated in this study. Paeonia lactiflora should be investigated further for their potential as antioxidant food supplements or antibacterial medications, etc.

5.2. Recommendations and Future work

- To investigate additional extraction procedures that use different solvents, such as butanol, ethyl acetate,and others, and to determine the particular compounds that are separated.
- To look at combining Paeonia lactiflora roots with other natural extracts to boost antibacterial action.
- To use a preparative HPLC-PDA machine to collect more concentrated isolates of pure compounds (>100 mg each) in order to run 2D-NMR spectroscopy experiments, focusing on the COSY and HMQC modes, to fully elucidate the exact

structures of the separated phytochemicals, as well as high resolution mass spectroscopy.

- to examine the P. lactiflora root's potential anti-cancer properties.
- To link between compounds isolated by HPLC and its bioactivity.

5.3ملخص البحث باللغة العربية

المكونات الكيميائية والنشاط الحيوي لمستخلص جذور شُرش الهوا.

تم تحليل نبات عشبي صيني تقليدي (شُرش الهوا) لدراسة نشاطه الحيوي ومكوناته الكيميائية باستخدام تقنيات التحليل المختلفة. شُرش الهوا هي عشبة تستخدم على نطاق واسع في الطب والغذاء ومستحضرات التجميل. تم شراء جذر جاف من متجر الشعلة للأعشاب التقليدية - شارع الارسال - رام الله - فلسطين. تم تحضير المستخلص الخام باستخدام 99٪ من الإيثانول كمذيب استخلاص ، عن طريق تعريضه للموجات فوق الصوتية (الاهتزاز)، ثم حفظه عند 4 درجات مئوية لاستخدامه في التحاليل المختلفة. تم استخدام جذور النبات، حيث أثبتت آثارها الصحية الوقائية للعديد من الأمراض مثل استخدامها لعلاج الضغط المرتفع، كمضاد للألتهابات، السكري، و غيرها. يولد الجلوكوز غير الأنزيمي مجموعة متنوعة من المركبات المعروفة باسم المنتجات النهائية للجليكيشن (AGEs) التي تتراكم في الجسم وتسبب تطور الأمراض المزمنة لدى البشر ، مثل مرض السكري من النوع 2 وتصلب الشرايين والزهايمر. لذلك ، فإن تطوير مثبطات AGEs الطبيعية تحتاج إلى تحقيق بحثي مكثف. لذلك ، تم تقييم الإنتاج المضاد للجليكشن للمنتج النهائي باستخدام المستخلص باستخدام اختبار الألبومين المصل الجلوكوز البقري (BSA) في المختبر. تشير النتائج التي تم الحصول عليها من عينة المستخلص إلى وجود تأثير مثبط لتكوين AGE ، في حين أن مستخلص الجذر غني بمضادات الأكسدة مثل الفلافونويد والفينولات التي أظهرت نتائج واعدة لتنظيم مستوى السكر. تم قياس المحتوى الفينولي الكلي للمستخلص النباتي (TPC) باستخدام اختبار Folin-Ciocalteu. تم تحديد محتوى الفلافونويد الكلي (TFC) باستخدام مقايسة كلوريد الألومنيوم اللونية. تم استخدام طريقتين مختلفتين لتحديد سعة مضادات الأكسدة: باستخدام طريقة الجذور الحرة (DPPH)، و طريقة اختزال ايونات الحديد (FRAP). تم إجراء النشاط المضاد للميكروبات للمستخلص بطريقة الانتشار القرصي لاختبار فعالية مستخلص النبات ضد أنواع مختلفة من البكتيريا موجبة وسالبة الجرام. تم إظهار التأثير الإيجابي الرئيسي ضد بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) عند مقارنتها بالبنسلين G (التحكم الإيجابي) ، كما تم تسجيل تأثير إيجابي طفيف ضد بكتريا المكورات العنقودية الذهبية Staphylococcus aureus إيجابية الجرام. ومع ذلك ، تم تسجيل تأثير سلبي لثلاثة أنواع مختلفة من البكتيريا (الشيغيلة ، الإشريكية القولونية (E-coli)، الزائفة الزنجارية (Pseudomonas)). تشير النتائج لتراكيز مختلفة من المستخلص (120 ، 300) جزء في المليون تظهر تأثير

تثبيط تكوين (AGE) بنسبة (48.2٪ ، 69.0٪) على التوالي. ترجع هذه الفعالية إلى وجود المواد الكيميائية النباتية النشطة بيولوجياً في المستخلص النباتي والتي تم اثباتها من خلال فحوصات مجموع الفينول والفلافونويد. كانت مقايسات الفينول 193.9 ميليغرام جاليك اسيد / جرام من المستخلص النباتي ، والفلافونويد 55.9 ميليغرام كوارسيتين / جرام من عينة النبات. باستخدام طريقة الجذور الحرة DPPH ، أعطى المستخلص النباتي تثبيطًا بنسبة 56.8٪ ، بينما في اختبار FRAP ، تم حساب نشاط مضادات الأكسدة (مجم BHT / جم من عينة النبات) ليكون 1524. النتائج أعلاه توضح قدرة مستخلص جذر شَرش الهوا على منع نمو أنواع مختلفة من البكتيريا. تم استخدام الاستِشراب السَّائِل الرَّفيع الإنجَاز (HPLC-PDA) للتعرف على مركبات البوليفينول والفلافونويدات من المستخلص الايثانولي وأظهرت النتائج وجود حمض الغاليك و 3،4-ثنائي هيدروكسي بنزويك. يمكن استخدام شَرش الهوا لعلاج أمراض مختلفة ، لأنها تحتوي على مجموعة كبيرة من المواد الكيميائية النباتية النشطة بيولوجياً.

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