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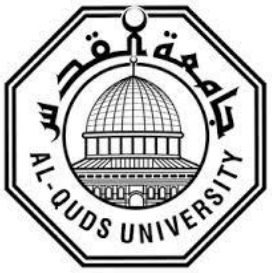

Phenolic compounds and antioxidant activity of *Urtica dioica* and *Sarcopoterium spinosum* in Palestine and their Biological activities

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M.Sc. Thesis

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Phenolic compounds and antioxidant activity of *Urtica dioica* and *Sarcopoterium spinosum* in Palestine and their Biological activities

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Thesis Approval

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Dedication

To my parents

You are the reason I am where I am today. Thank you for your continuous support and unconditional love.

To my siblings.

You have all been my motivation to do my best and encourage me always. For that, I will forever be grateful.

To my dear friends

To my supervisor Dr. Fuad Rimawi

Declaration:

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

Signed: 

Mirna Yacoub Sami Abu Abara

Date: 19 / 3 / 2017

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Finally, I thank my family and my friends for their help and encouragement.

Abstract

Leaves from *Urtica dioica* and *Sarcopoterium spinosum* plants were collected from West Bank in January 2016, air dried at 30□ C, grinded, and extracted with four solvents (99% ethanol, 70% ethanol, 50% ethanol, and D.W) for 90 min at 37□ C and filtered. The crude extracts were then analyzed using standard assay methods for: total phenolic content (TPC) by the Folin-Ciocalteau method and total flavonoid content (TFC) by colorimetric assay method. Antioxidant activity (AA) was recorded by four different assays, two of them measures the ability of the plant extract for free radical scavenging: DPPH, and ABTS, and two others to measure the reducing ability of plant extract: Ferric ion reducing Antioxidant Power (FRAP), and CUPRAC assay. Their biological activities were analyzed using the *In Vitro* Tyrosinase assay and their antimicrobial activity by Cylinder plate technique. HPLC was used to analyze the polyphenolic compounds in the extracts of each plant. Both samples were independently analyzed in each sampling, and all of the determinations were carried in triplicate.

TPC values were determined in different solvents (99% ethanol, 70%ethanol, 50% ethanol, and D.W) as mg Gallic acid equivalent (GAE)/ per gram of plant extracts, the values of *Urtica dioica* were found to be 47.4±1.5, 81.1±1.7, 66.1±0.6, and 63.2±0.6 respectively, and the values of *Sarcopoterium spinosum* were found to be 173.1±11.3, 310.8±16.6, 252.5±5.6, and 70.6±1.3 respectively. TFC values were subjected as mg Catechin/ g of dry sample in the same solvents, results of *Urtica dioica* were found to be 11.3±0.8, 15.7±0.3, 12.6±0.3, and 6.1±0.1 respectively, and the values of *Sarcopoterium spinosum* were found to be 19.8±0.4, 24.0±0.2, 19.3±0.7 and 6.6±0.01 respectively.

The antioxidant activity was also evaluated by different assays for both plants with the same extracts. It was expressed by FRAP method as mmole Fe^{+2}/ g sample, and results were found to be 1.8±0.02, 1.9±0.01, 0.5±0.13, and 0.9±0.01 respectively for *Urtica dioica* and 1.8±0.04, 2.1±0.04, 0.7±0.03, and 0.9±0.08 respectively for *Sarcopoterium spinosum*. CUPRAC method of total antioxidant capacity was evaluated and expressed as mg Trolox/ g sample; results were found to be 86.8±0.6, 158.3±0.3, 27.4±4.1, and 54.2±7.7

respectively for *Urtica dioica* and 120.2±5.0, 349.8±3.4, 66.7±16.3, and 62.8±11.0 respectively for *Sarcopoterium spinosum*. Regarding the radical scavenging measurements, DPPH method was expressed as µmole Trolox/ g sample, results showed a high radical scavenging activity of the 70% ethanol extracts with an inhibition of 80.65% of *Urtica dioica* and 86.20% of *Sarcopoterium spinosum*. Moreover, the plant extracts have also the ability to inhibit ABTS radical, this assay was expressed as µmole Trolox/ g sample, results showed that the 70% ethanol of *Urtica dioica* and *Sarcopoterium spinosum* have the highest inhibition percentage of ABTS radical (92.9%, and 98.4% respectively).

The antimicrobial activity was also studied for both plants extracts against gram positive bacteria (*Staphylococcus aureus*), gram negative bacteria (*Escherichia coli*), and yeast (*Candida albicans*) in different extractions (D.W, 70% ethanol, 99% ethanol). Results generally showed that the zone of inhibition of the microbial activity of *Urtica dioica* is significant against *Staphylococcus aureus,* and against *Candida albicans*, with no effect against *Escherichia coli*. While, the zone of inhibition of the microbial activity of *Sarcopoterium spinosium* is significant against *Staphylococcus aureus* only in the 70% ethanol, not effective against *Escherichia coli* and against *Candida albicans.*

The inhibition of tyrosinase in skin was also studied to evaluate the effective skin whitening agent of the plant extracts, results showed a high inhibition percentage of melanin formation.

Different phenolic compounds were detected using HPLC for the 99% ethanol extract of *Urtica dioica*, 70% ethanol and water extracts of *Sarcopoterium spinosum.*

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List of Acronyms

Abbreviation	Description
AA	Antioxidant activity
AAI	Antioxidant activity index
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BT-474	Breast tumor cell line
CEQ	Catechin equivalent
CUPRAC	Cupric reducing antioxidant capacity
DNA	Deoxyribo Nucleic Acid
DW	Distilled water
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI/MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
FRAP	Ferric Ion Reducing Antioxidant Power Assay
GAE	Gallic acid equivalence
HPLC	High-performance liquid chromatography
HQ	Hydroquinone
HNE	Hydroxynonrnal
IC_{50}	half maximal inhibitory concentration
LP 229v	Lactobacillus Plantarum 299v
L-DOPA	L-3,4- dihydroxyphenylalanine
MDA	Malondialdehyde
MS	Mass Spectrometry
MTT	Microculture Tetrazolium Assay
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
NO	Nitric Oxide
ODS	Octadecyl-silica
Ppm	part per million
PDA	Photo Diode Array

PTEF	Polytetrafluoroethylene
pH	Power of hydrogen
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSD	Relative standard deviation.
SD	Standard Deviation.
TAC	Total Antioxidant Capacity
TFC	Total Flavonoids Content
TPC	Total Phenolic Content
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine)
TEAC	Trolox equivalent antioxidant capacity
UV	Ultraviolet
UV-Vis spectrophotometer	Ultraviolet – visible spectrophotometer

CHAPTER ONE
INTRODUCTION

1. Introduction:

1.1. Background

1.1.1. Polyphenols

Polyphenols are secondary metabolites of plants of which 8,000 polyphenolic compounds have been identified in various plant species. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid as shown in figure 1.1.

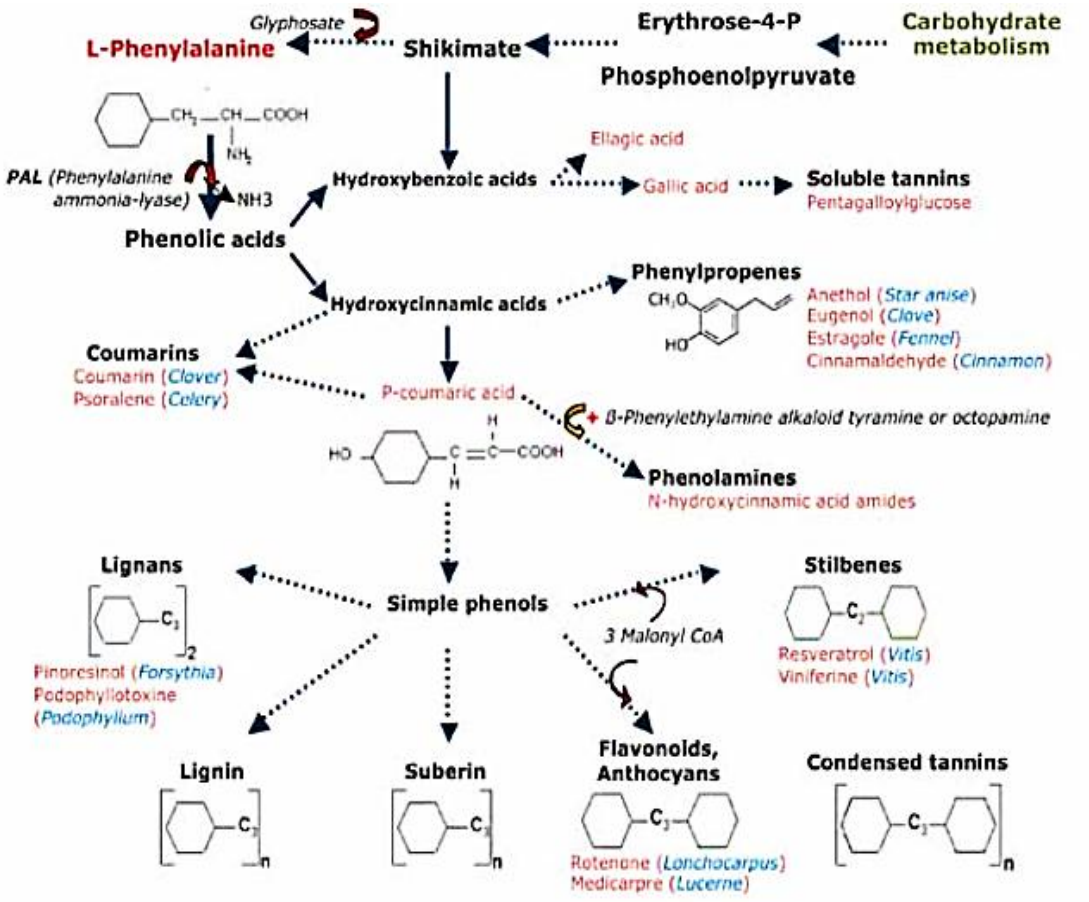


Figure 1.1: Simplified pathway of phenolic compound synthesis (Roland Douce, 2005)

Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist. Association with other

compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common (Kondratuk *et al*, 2004). Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans. Figure 1.2 illustrates the different groups of polyphenols and their chemical structures (Rodríguez *et al*, 2015).

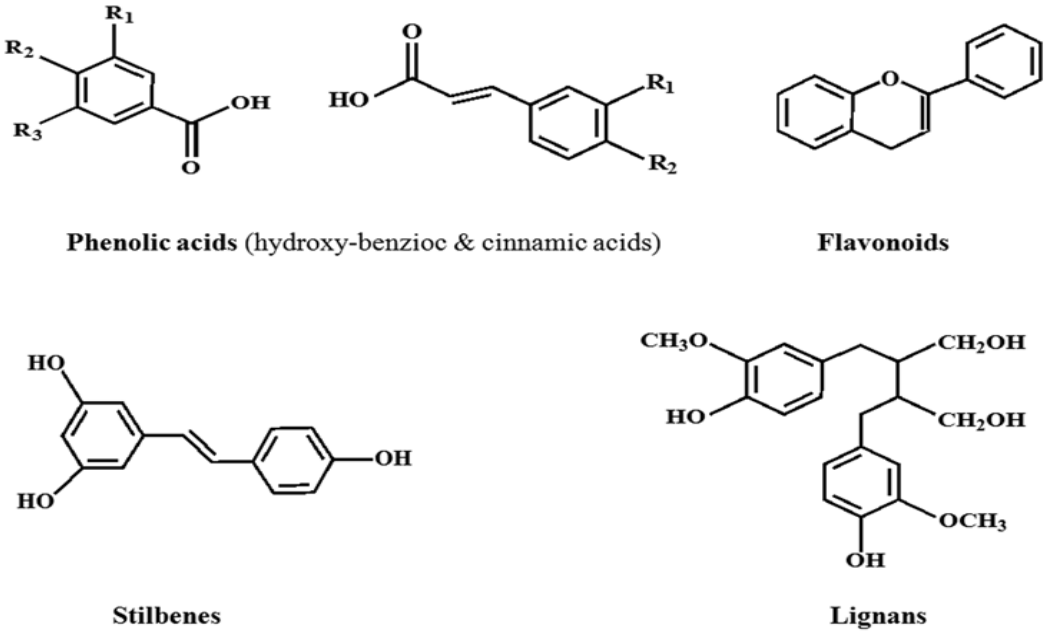


Figure 1.2: Chemical structures of the different classes of polyphenols, where R_1, R_2 and R_3 are H, OH or OCH_3 (Rodríguez *et al*, 2015).

1.1.2. Phenolic Acids

Phenolic acids are found abundantly in foods and divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid. The hydroxybenzoic acid content of edible plants is generally low, with the exception of certain red fruits, black radish and onions, which can have concentrations of several tens of milligrams per kilogram fresh weight (Shahidi & Naczk, 1995). The hydroxycinnamic acids are more common than hydroxybenzoic acids and consist chiefly of *p*-coumaric, caffeic, ferulic and sinapic acids.

1.1.3. Flavonoids

Flavonoids comprise the most studied group of polyphenols. This group has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (Figure 1.2). More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the attractive colours of the flowers, fruits and leaves (Groot *et al*, 1998). Based on the variation in the type of heterocycle involved, flavonoids may be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones (Figure 1.3). Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Quercetin, myricetin, catechins, etc., some most common flavonoids.

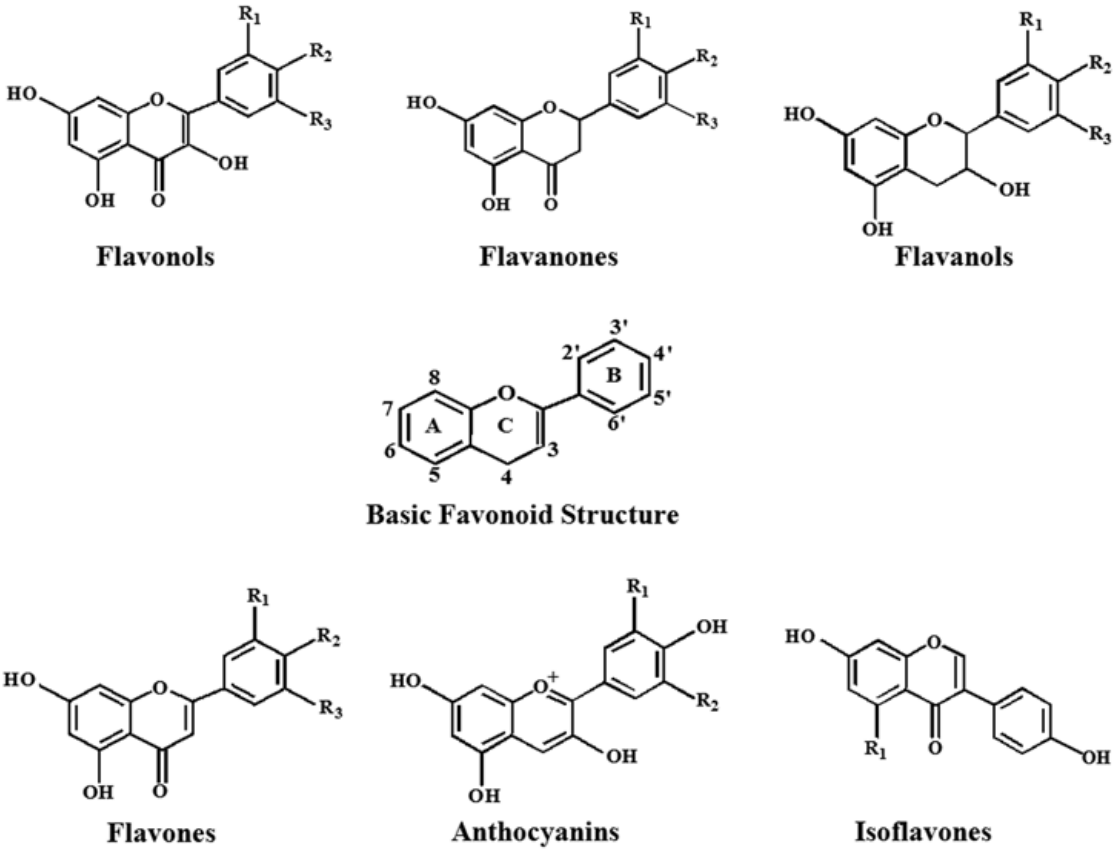


Figure 1.3: Chemical structures of the different classes of flavonoids, where R_1, R_2 and R_3 are H, OH or OCH_3 (Pandey *et al*, 2009)

1.1.4. Biological role of polyphenols in plants

Both natural phenols and the larger polyphenols play important roles in the ecology of most plants. Their effects in plant tissues can be divided into the following categories (Lattanzio *et al*, 2006):

1. Release and suppression of growth hormones such as auxin.
2. UV screens to protect against ionizing radiation and to provide coloration (plant pigments).
3. Deterrence of herbivores (sensory properties) and microbial infections (phytoalexins).
4. Signaling molecules in ripening and other growth processes.

1.2. Overview of the Study Plants:

1.2.1 Urtica dioica

Urtica dioica is a perennial plant in the family Urticaceae, which is commonly known as stinging nettle in English language and Qurais in Arabic language. It occurs in moist sites along streams, on mountain slopes, on deep rich, soils and in distributed areas (Bassett *et al*, 1977) (Woodland, 1982). The plant is available in many South Asian countries and Indian subcontinent. It has been known in the world as a medicinal herb for a long time. The plant is used traditionally as diuretic, stomachache, emmenagogue, blood purifier, anthelminthic, rheumatic pain and for

colds and cough. It is also used in nephritis, haematuria, jaundice and menorrhagia. The plant has been reported to contain lectins, linolenic acid, lutein, lutein isomers, b-carotene and b-carotene isomers, neoxanthin, violaxanthin and lycopene (Joshi *et al*, 2015). In some studies, the plant is reported to have anti-diabetic, hepatoprotective, anti-inflammatory, antihypertensive activity, diuretic and natriuretic effects (Joshi *et al*, 2014).

1.2.2 Sarcopoterium spinosum

Sarcopoterium spinosum, which is commonly known as thorny burnet in English language and Natesh in Arabic language, is a spiny rosaceous dwarf shrub in the family Rosaceae, 30-60 cm in height, with branches ending in dichotomous and leafless thorns (Litav & Orshan, 1971).

Sarcopoterium spinosum appears in a wide range of habitats and on soils overlaying different substrates, including soft chalk, hard limestone and sand-stone (Litav & Orshan, 1971). It dominates many of the hilly parts of the eastern Mediterranean region and common in Greece, Italy, Tunisia and Turkey (Henkin Z *et al*, 2014).

In Arab villages the whole bush is used as fuel for making fences and sheep pens, for making brooms from its branches and as stuffing for mattresses. In addition the branches are used to cover tender young plants to protect them from birds and animals (Dafni *et al*, 1984).

According to ethnopharmalogical studies *Sarcopoterium spinosum* extract is used for the treatment of several disorders. The primary use, mentioned in most surveys,

is of an aqueous extract prepared from the root bark for the treatment of diabetes (Hamdan & Afifi, 2004) (Said *et al*, 2002) (Friedman *et al*, 1986) (Al-Qura'n, 2009) (Yaniv *et al*, 1987) (Steinmetz, 1965) (Yaniv, 2007).

Antidiabetic activity might be mediated by several mechanisms; inhibiting intestinal digestion and absorption of carbohydrates, including insulin secretion by the pancreas or enhancing glucose disposal from the blood by target tissues such as muscles, adipose or liver tissues, either by improving insulin sensitivity or mimicking its action (Kasbari *et al*, 2011).

Secondary therapeutic applications of *Sarcopoterium spinosum* mentioned in ethnopharmacological surveys are for pain relief, mainly toothache (Yaniv, 2007), disorders of the digestive system (Friedman *et al*, 1986) (Al-Qura'n, 2009) (Ali-Shtayeh *et al*, 2000), asthma (Friedman *et al*, 1986), renal calculi (Al-Qura'n, 2009), poisoning (Yaniv *et al*, 1987) and cancer (Durodola, 1975).

1.3 Chemical assays of plants extracts:

1.3.1 Antioxidant activity

An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals, which are natural by- products of cell metabolism (Ames *et al*, 1993) (Shenoy & Shirwaikar, 2002). Free radicals are chemical species that posse an unpaired electron in the outer (valance) shell of the molecule when oxygen is metabolized or formed in the body. This is the reason, why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. These free radicals attack the nearest stable molecules, taking its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell (Patil & Narayanan, 2003).

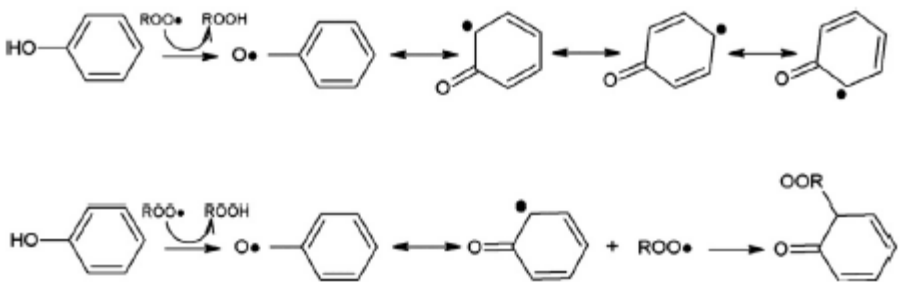


Figure 1.4: Mechanism for the antioxidant activity of phenolic compounds (Hur *et al*, 2014)

Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are O_2^- [superoxide], HO [hydroxyl], HO_2 [hydroperoxyl], ROO [peroxyl], RO [alkoxyl] as free radical and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO [nitric oxide], ONOO [peroxy nitrate], NO_2 [nitrogen dioxide] and N_2O_3 [dinitrogen trioxide] (Evas & Halliwall, 1999) (Devasagayam, 2003). In a normal cell, there are appropriate oxidants: antioxidant balance. However, this balance can be shifted, when production species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to a wide range of cytotoxic products, most of which are aldehydes, like malondialdehyde (MDA), 4- hydroxynonrnal(HNE). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's, Parkinson's, atheroscleorosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc (Peterhans, 1997).

The antioxidant activity from natural extracts can and must be evaluated with different tests which are: FRAP, CUPRAC, DPPH and ABTS.

1.3.1.1 FRAP method:

One of the most important methods used to measure antioxidant activity of plants extract is Ferric Ion Reducing Antioxidant Power Assay (FRAP). It is simple, fast, inexpensive, robust, and does not required specialized equipment. In the FRAP method the yellow Fe^{3+} TPTZ complex (2, 4, 6-tri (2-pyridyl)-1,3,5-triazine) is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances (such as phenolic compounds) under acidic conditions (Benzie *et al*, 1996). Any electron donating substances with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+} TPTZ will drive the reaction and the formation of the blue complex forward (Singh *et al*, 2012) as shown in Figure 1.5.

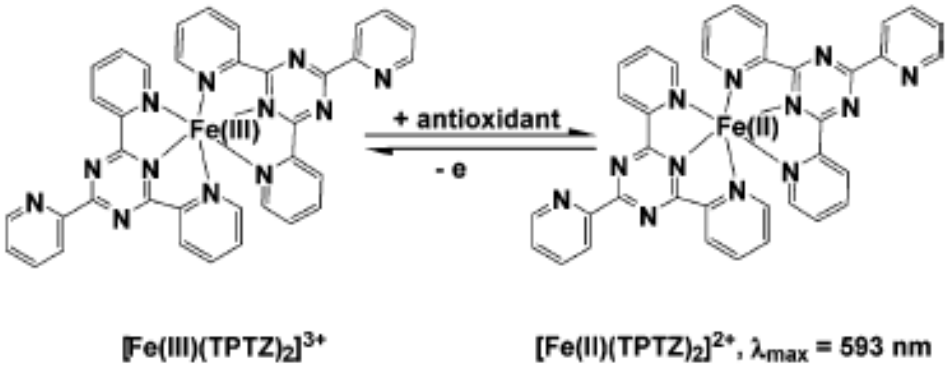


Figure 1.5: Chemical structures of reaction of yellow Fe^{3+} TPTZ complex (2,4,6-tri(2 pyridyl)-1,3,5-triazine) with antioxidants is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances (Prior *et al*, 2005).

1.3.1.2 CUPRAC method:

The putative CUPRAC method was developed by (Apak *et al*, 2006). These assays are based on the reduction of Cu^{+2} to Cu^{+} by the combined action of all antioxidants or reduction in aqueous-ethanolic medium (pH 7.0) in the presence of neocuproine (2,9-dimethyl-1,10-phenanthroline), by polyphenols, yielding a Cu^{+} complexes with maximum absorption peak at 450 nm (Lee *et al*, 2011). This method can be used for the determination of the antioxidant capacity of food constituent by the Cu^{+2}-neocuproine (Cu^{+2}-Nc) reagent as the

chromogenic oxidizing agent. The reduction of Cu^{+2} in the presence of neocuproine by a reducing agent yields a Cu^{+} complex with maximum absorption peak at 450 nm (Tütem *et al*, 1991)**.**

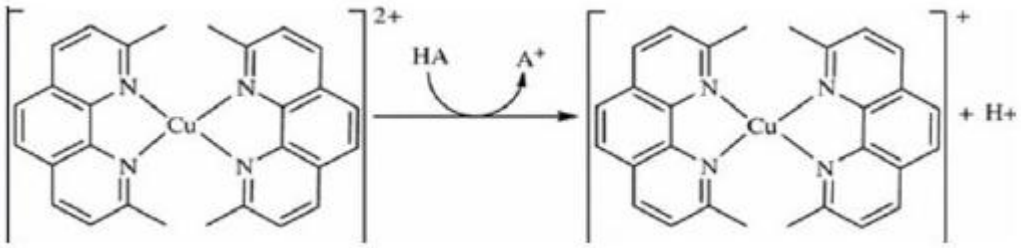


Figure 1.6: CUPRAC reaction by an oxidation molecule (HA: an antioxidant molecule, A^{+}: an oxidized antioxidant molecule) (Tütem *et al*, 1991)**.**

1.3.1.3 ABTS method:

The ABTS cation radical (ABTS•+) which absorbs at 743 nm (giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2, 2‘-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) (Marc *et al*, 2004). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization. ABTS can be oxidized by potassium persulphate (Re *et al*, 1999), (Thaipong *et al*, 2006). (Figure 1.7), giving rise to the ABTS cation radical (ABTS•+) whose absorbance diminution at 743 nm was monitored in the presence of Trolox, chosen as standard antioxidant (Pisoschi & Negulescu, 2012).

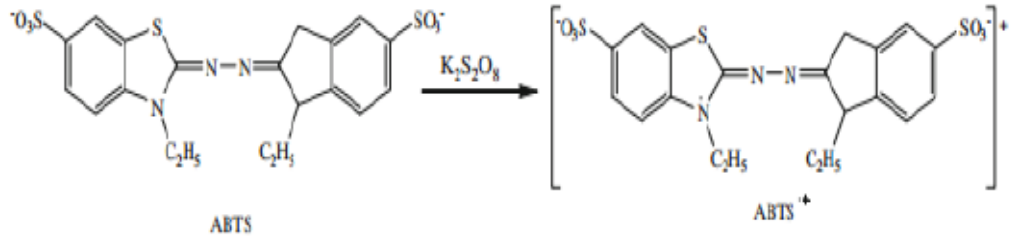


Figure 1.7: Oxidation of ABTS with $K_2S_2O_8$ and generation of ABTS+ (Miller *et al*, 1993)

1.3.1.4 DPPH method:

DPPH is a free radical that is stable at room temperature, which produces a violet solution in methanol. When the free radical reacts with an antioxidant, its free radical property is lost due to chain breakage and its color changes to light yellow (Abuja *et al*, 1997) (Figure 1.8).

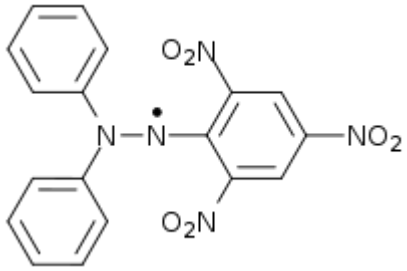


Figure 1.8: Chemical structure of DPPH (Abuja *et al*, 1997).

1.4 Antimicrobial activity

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not, only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems (Farnsworth, 1993) (Houghton, 1995). Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (Dubey *et al*, 2004).

About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer (Gragg & Newman, 2005). Natural products of higher plants may give a new source of antimicrobial agents with possibly

novel mechanisms of action (Runyoro *et al*, 2006) (Shahidi, 2004). The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world (Reddy *et al*, 2001). Much work has been done on ethnomedicinal plants in India (Maheshwari *et al*, 1986).

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have antimicrobial properties (Dahanukar *et al*, 2000) (Cowan, 1999).

Herbal medicines have been known to man for centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine (Ramasamy & Charles, 2009). Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population (Shaik *et al*, 1994). The harmful microorganisms can be controlled with drugs and these results in the emergence of multiple drug-resistant bacteria and it has created alarming clinical situations in the treatment of infections. The pharmacological industries have produced a number of new antibiotics; resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents (Towers *et al*, 2001).

1.5 Whitening effect:

One of the serious aesthetic problems in human beings is skin darkening which is more prevalent in middle aged and elderly individuals. Skin whitening is the practice of using chemical substances or traditional herbal formulations, in an attempt to lighten skin tone or provide an even skin complexion by the reduction of concentration of the pigment melanin (Jennifer *et al*, 2012).

The pigment melanin in human skin is a major defense mechanism against ultra violet light of the sun. The production of abnormal pigmentation, such as melasma, spots and other forms of melanin hyper pigmentation can be a serious aesthetic problem (Briganti *et al*, 2003). Melanin formation is also the main cause of enzymatic browning in human beings (Freidman, 1996). The most common skin lightening and depigmentation agents available commercially are kojic acid, arbutin, catechins, hydroquinone (HQ) and azelaic acid (Maeda *et al*, 1991). Some adverse effects of these synthetic compounds are irreversible. The main causes of skindarkening (skin hyper pigmentation) are auto immune conditions, sun damage (UV radiation and ionizing radiation), drug reactions (chemicals), hormonal changes, genetic factors, medications, and hormonaltherapy or birth control pills resulting in the hyper secretion of melanin from melanocytes (Maeda *et al*, 1991), (http://www.targetwoman.com/articles/skin-pigmentation.html). It can result also from skin damage, such as remnants of blemishes, wounds or rashes. This is especially true for those with darker skin tones (Jennifer *et al*, 2012).

Tyrosinase is a copper-containing, multifunctional, glycosylated, monooxygenase widely distributed in nature. It catalysis the first two steps of mammalian melanogenesis, (process leading to formation of dark macromolecular pigments melanin). This determines the color of mammalian skin and hair (Seiberg *et al*, 2000) (Nerya *et al*, 2003) (Chang, 2009) (Halder *et al*, 2004). Over-activity of this enzyme leads to overproduction of melanin in-turn leading to hyper-pigmentation of the skin (Ali *et al*, 2005). Inhibition of tyrosinase can also lead to reduced melanin production. The two step process are hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine, L-DOPA, and the oxidation of L-DOPA to dopaquinone1. This O-quinone is a highly reactive compound and can polymerize spontaneously to form melanin (Seo et al, 2003). The antityrosinase activity (skin whitening) was analyzed through inhibition ability of dopachrome formation. Tyrosinase inhibitors have become increasingly important in medication and in cosmetics to prevent

hyperpigmentation by inhibiting enzymatic oxidation. Thus the natural products containing the tyrosinase inhibiting activity are the potential sources for skin whitening (Jennifer *et al*, 2012).

1.6 HPLC analysis of phenolic compounds:

High performance liquid chromatography (HPLC) is a widely used technique for the isolation of natural products (Cannell, 1998). This technique is used nowadays in various analytical techniques as the main choice to study for the quality control of herbal plants (Fan *et al*, 2006).

Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, flow rate, suitable detectors and columns to get an optimum separation. Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, flow rate, suitable detectors and columns to get an optimum separation (Sasidharan *et al*, 2011).

In order to analyze the phenolic content in natural extracts, high performance liquid chromatography (HPLC) technique is widely applied, for both the separation and quantification of these compounds. The separation of different classes of phenolic compounds is achieved through the introduction of a reverse phase column, which enhances the process. Usually, diode array detector (DAD) is used for food phenolic compounds detection. HPLC coupled with

mass spectrometry (MS) has commonly been used for structural characterization of phenols. Electrospray ionization mass spectrometry (ESI/MS) has been employed for the structural confirmation of phenols in peaches, nectarines, olives, grape seeds, cocoa, olive oil, etc (Naczk & Shahidi, 2004).

The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation. The source material, e.g., dried powdered plant, will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extractant and following a period of maceration, solid material is then removed by decanting off the extract by filtration. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Therefore, the guard columns will significantly protect the lifespan of the analytical columns (Sasidharan *et al*, 2011).

CHAPTER TWO

LITERATURE REVIEW

2.1. Previous studies:

Bhuwan *et al*., (2015) in their article **"Antixidant potential and total phenolic content of *Urtica dioica* (Whole plant)"** reported that Whole plant of *Urtica dioica* Linn. (Urticaceae) from India- New Delhi, were subjected to extraction with different solvent according to polarity to obtain antioxidant rich extract. Different concentrations of different solvent extracts were subjected to antioxidant assay by DPPH, Nitric oxide NO scavenging method and Total phenolic contents. The IC_{50} values for different solvent extracts (Petroleum ether, ethyl acetate, n-butanol, ethanol) of *Urtica dioica* Linn. were found as μg/ml ± S.D. to be 215.96 ± 0.066, 78.99 ± 0.171, 168.24 ± 0.346 and 302.90 ± 0.141 respectively in comparison to L-Ascorbic acid as standard with IC_{50} values of 26.24 ± 0.193 respectively in DPPH model. In nitric oxide radical scavenging activity the IC_{50} values were found to be 172.38 ± 0.635, 101.39 ± 0.306, 141.23 ± 0.809, 202.26 ± 0.67 and 55.38 ± 0.56 for different extracts and L-Ascorbic acid respectively.

The highest Total phenolic content was found to be 13.06 ± 0.15 mg GAE/g in ethyl acetate extract. However, the ethyl acetate extract showed a better free radical scavenging activity as compared to other extracts.

Fattahi S *et al*., (2014) in their article "**Total Phenolic and Flavonoid Contents of Aqueous Extract of *Stinging Nettle* and In Vitro Antiproliferative Effect on Hela and BT-474 Cell Lines**" reported that Phenolic compounds including flavonoids and phenolic acids are plants secondary metabolites. Due to their ability to act as antioxidant agents, there is a growing interest to use those components in traditional medicine for cancer prevention or treatment. The aim of this study was to measure the amounts of total phenolics and flavonoids as well as anti-proliferative effect of aqueous extract of Stinging nettle from Iran on BT-474 and Hela cell lines. The amounts of phenolics content and total flavonoids were determined by folin ciocalteu and aluminium chloride methods, respectively. The free radical scavenging activity was measured by using diphenyl - picrylhydrazyl (DPPH). The reducing power of the extract was measured in the presence of potassium hexacyanoferrate and its antiproliferative activity was assessed on BT-474 and Hela cell lines using MTT assay. Total phenolic content was 322.941± 11.811 mg gallic

acid/g extract. Total flavonoid content was 133.916±12.006 mg Catechin/g. The IC50 of DPPH radical was 1.2 mg/ ml and the reducing power was 218.9± 15.582 µg ascorbic acid/ g. Cell viability of BT-474 cells decreased to less than half of the control (no added extract) at the presence of 3 mg/ ml extract while no significant changes were detected for Hela cells at similar conditions. There was no significant difference in the percentage of surviving cells between consecutive days (day 1, 2 and 3) for both BT-474 and Hela cells ($P>0.05$). Although the relatively high amount of phenolic and flavonoid contents of the aqueous extract make this plant a promising candidate for diseases treatment; however, there is not a direct relationship between the amounts of these antioxidant components and the efficiency in in vitro cancer treatment.

Bougeois *et al*., (2016) in their article **"Nettle (Urtica dioica L.) as a source of antioxidant and anti-aging phytochemicals for cosmetic applications"** reported that nettle (*Urtica dioica* L.) is a herbaceous perennial that has been used for centuries in folk medicine. More recently, nettle extracts have also been used in cosmetics because of the many benefits of their topical application for skin health. Their potential anti-aging action is of particular interest and is primarily ascribed to their antioxidant capacity. Here, using an experimental design approach and a clustering analysis, the phytochemical composition of nettle extracts were linked to their biological activities. This approach confirmed the antioxidant capacity of nettle extracts as well as providing the first evidence of another mechanism for their anti-aging potential involving the inhibition of enzyme activities, such as elastase and collagenase. The inhibitory effects were attributed to ursolic acid and quercetin present in the nettle extracts. Results also demonstrated the possibility of extracting ursolic acid, quercetin and other phenolic compounds differentially to obtain an extract with a strong antioxidant capacity and anti-aging activities toward both elastase and collagenase, which could be of particular interest for cosmetic applications of nettle extracts.

Safari *et al*., (2016) in their article **"Anti-pyretic, Anti-inflammatory and Analgesic Activities of Aqueous Leaf Extract of *Urtica dioica* (L.) in Albino Mice"** reported that *Urtica dioica* from Kenya has been used to manage several diseases including pain, inflammation and fever. However, its efficacy has not been scientifically validated. The aim of the study therefore was to investigate the analgesic, antipyretic and anti-inflammatory activities of its aqueous extracts. The plant extract was collected from Loita division, Narok County in Kenya. A total of 96 albino mice with an average weight of 20 g were used for this study. The aqueous leaf extracts of *Urtica dioica* reduced pain, inflammation and fever mostly at the dose 150 mg/kg body weight. Based on these findings it was concluded that the present study has demonstrated the analgesic, anti-inflammatory and antipyretic potential of aqueous leaf extracts of *Urtica dioica* in albino mice and will serve as good bio-resource for generating readily available herbal formulations that are more effective in the treatment of pain, inflammation and fever conditions which are cheaper than the conventional synthetic drugs and have no side effects.

Kukric *et al*. in their article "**Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica L.*)**" reported that Samples of stinging nettle or common nettle (*Urtica dioica L.)* were collected from the area of Banja Luka. The dry residue of ethanol extract was dissolved in methanol and the obtained solution was used to determine the content of total phenols, flavonoids, flavonols, as well as non-enzymatic antioxidant activity and antimicrobial activity. The non-enzymatic antioxidant activity was determined by different methods: FRAP, DPPH, and ABTS. The results were compared to those of standard substances like vitamin C, BHT, and BHA. Antimicrobial activity was screened by using macrodilution method. The total phenolic content in nettle extracts amounted to 208.37 mg GAE/g sample, the content of total flavonoids was 20.29 mg QE/g sample, and the content of total flavonols was 22.83 mg QE/g sample. The antioxidant activity determined by FRAP method was 7.50 mM Fe(II)/g sample, whereas the antioxidant activity measured by using DPPH and ABTS methods, with IC_{50} values, were 31.38 and 23.55 μg/ mL sample, respectively. These results showed the weak and

moderate antioxidant capacity of stinging nettle. Extract of *Urtica dioica* L. was tested for antibacterial activity against various Gram-positive and Gram-negative bacteria: Bacillus subtilis IP 5832, Lactobacillus plantarum 299v (Lp299v), Pseudomonas aeruginosa, and Escherichia coli isolated from food and Escherichia coli isolated fromurine samples. Ampicillin, erythromycin, ciprofloxacin, and gentamicin were used as positive control. The results showed that minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract ranged from 9.05 to more than 149.93 mg/ mL sample.

Ahmed Al Mustafa and Osama Al-Thunibat, (2008), reported in their article **"Antioxidant activity of some Jordanian medicinal plants used traditionally for treatment of diabetes"** that Medicinal plants are being used extensively in Jordanian traditional medicinal system for the treatment of diabetes symptoms. Twenty one plant samples were collected from different Jordanian locations and used for antioxidant evaluation, *Sarcopoterium spinosum* was one of these studied plants. The level of antioxidant activity was determined by DPPH and ABTS assays in relation to the total phenolic contents of the medically used parts. The most frequently used plant parts as medicines were fruit, shoot and leaves. The total phenolic contents of methanol and aqueous extracts, from plants parts, ranged from 6.6 to 103.0 and 3.0 to 98.6 GAE mg/ g sample of plant part dry weight, respectively. DPPH-TEAC of the methanol extracts of plants parts were varied from 4.1 to 365.0 mg/ g sample of plant dry weight versus 0.6 to 267.0 mg/ g sample in aqueous extracts. Moreover, the mean values of ABTS*- (IC50) varied from 6.9 to 400.0 microg dry weight mL(-1) ABTS in methanol extracts versus 9.8 to 580.5 micro g/ ml in aqueous extracts. According to their antioxidant capacity, the plants were divided into three categories: high (DPPH-TEAC > or = 80 mg/ g sample), (i.e., Punica granatum peel, Quercus calliprinos leave, Quercus calliprinos fruit, Cinchona ledgeriana and Juniperus communis leave), moderate (DPPH-TEAC range 20-80 mg/ g sample) (i.e., Salvia fruticosa shoot, Crataegus azarolus stem, Crataegus azarolus leave, Varthemia iphionoides shoot, Artemisia herba-alba shoot, Thymus capitatus shoot, Morus nigra leaves and Arum palaestinum leaves) and low antioxidant plants (DPPH-TEAC < 20 mg g(-1)), (i.e.,

Matricaria aurea shoot, Artemisia judaica shoot, Teucrium polium shoot, Pinus halepenss pollen grains, *Sarcopoterium spinosum* root, Crataegus azarolus fruit, Inula viscose shoot and Achillea fragrantissima shoot). The antioxidant activity of these plant's extracts and their potential role in radical scavenging agreed with their potential use by Jordanian population as a traditional anti-diabetic agents.

Seham *et al*., (2016) in their article "**Evaluation of the Phenolic and Flavonoid Contents, Antimicrobial and Cytotoxic Activities of Some Plants Growing in Al Jabal Al-Akhdar in Libya**" studied the phenolic and flavonoid content, the antimicrobial and cytotoxic activities of the methanolic extract of the aerial part of two Libyan medicinal plants *Arbutus pavarii*. *Pampan* (Ericaceae) and *Sarcopoterium spinosium*. L. (Rosaceae) growing in El-Jabal Al Akhdar area. Total polyphenol contents ranged from 61.7±2.7to 163.6±0.85 μg gallic acid equivalent / g (*A. pavarii Pampan* and *S. spinosium*. L.) and total flavonoid contents ranged from 126.9±2.98 to 206.1±1.09 μg rutin equivalent (*A. pavarii Pampan* and *S. spinosium* L.). Qualitative and quantitative analysis of major phenolic and flavonoids in the extracts were conducted by high-performance liquid chromatography (HPLC). Finally, antimicrobial activities of the two plants were measured using the disc diffusion method. While, cytotoxic properties (quality of being toxic to cell) were tested against the HEPG2 and T47D cell lines. *Arbutus pavarii* extract proved to be the most cytotoxic extract in this study with IC50 19.7±2.8 and 19±0.65 (μg/ml) on HEPG2 and T47D respectively.

M.S. Ali-Shtayeh *et al*., (1997) in their article **"Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area"** reported that Ethanolic and aqueous extracts of 20 Palestinian plant species from Nablus and Jenin areas – *Sarcopoterium spinosum* was one of these plants- used in folk medicine were investigated for their antimicrobial activities against five bacterial species (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa*) and one yeast (*Candida albicans*). The plants showed 90% of antimicrobial activity, with significant

difference in activity between the different plants. The most antimicrobially active plants were Phagnalon rupestre and Micromeria nervosa, whereas, the least active plant was Ziziphus spina-christi. Only ten of the tested plant extracts were active against *Candida albicans*, with the most active from M. nervosa and Inula viscosa and the least active from Ruscus aculeatus. Of all extracts the ethanolic extract of M. nervosa was the most active, whereas, the aqueous extract of Phagnalon rupestre was the most active of all aqueous extracts tested. The ethanolic extracts (70%) showed activity against both Gram positive and negative bacteria and 40% of these extracts showed anticandidal activity, whereas, 50% of the aqueous extracts showed antibacterial activity and 20% of these extracts showed anticandidal activity.

Luisa Rizza, (2012) reported in her article **"Skin-whitening effects of Mediterranean herbal extracts by in vitro and in vivo models"** that several plant extracts are able to protect skin against ultraviolet-light-induced damage and hyperpigmentation in a safe way. The anti-melanogenic effect of herbal extracts seems to be related to their antioxidant activity and their polyphenolic content. In this study, the skin-whitening effect of some Mediterranean species, already known for their strong antioxidant and radical scavenger activity, has been evaluated by in vitro and in vivo models. The results obtained showed that herbal extracts possessed an inhibitory effect on tyrosinase enzyme. Each extract showed a similar inhibiting activity even though it was less intensive than kojic acid and hydroquinone. Otherwise, a significant higher activity than kojic acid and hydroquinone was observed when the herbal extracts were combined. Furthermore, the anti-melanogenic activity and an evaluation of skin tolerance were affected by in vivo methods.

2.2. Hypotheses and Research Questions

The hypothesis of this study declares the existence of variations in TPC, TFC, antimicrobial and antioxidant activity in *Urtica dioica* from Bethlehem-Palestine and *Sarcopoterium spinosum* from Ramallah-Palestine. Both plants can be used in different applications and fields, such as pharmaceutical, and food. Regards the high percentage of

inhibition of melanin formation of both plants, these plants can be used in whitening creams.

1. Are *Urtica dioica* and *Sarcopoterium spinosum* rich with anti-oxidants, Phenolic, and flavonoid compounds?
2. Do *Urtica dioica* and *Sarcopoterium spinosum* extracts have whitening effect, and antimicrobial activities?
3. According to their activity, what applications can each plant used for?

2.3. Significance of the study

Due to the importance of these plants and depending on the previous studies, *Urtica dioica* and *Sarcopoterium spinosum* were chosen and collected from Palestine to study their phenolic contents, flavonoid contents, whitening effect, antimicrobial and antioxidant activity. Some articles around the world discussed the antioxidant activity, others discussed the antimicrobial activity, or TPC and TFC, but none of them investigated all of these tests together in one study and none of these studies were conducted in Palestine, which make this study important and significant.

2.4. Objectives

1. Evaluate and analyze the phenolic and flavonoids contents of *Urtica dioica* and *Sarcopoterium spinosum* plant extracts in different solvents.

2. Evaluate the antioxidants, antimicrobial, and whitening effect of *Urtica dioica* and *Sarcopoterium spinosum* plant extracts in different solvents.

CHAPTER THREE
EXPERIMENTAL WORK

3.1. Chemicals, Reagents and Plant materials

3.1.1. Chemicals

The chemicals used for analyzing the antioxidant compounds (TPC, AA, and TFC) are: 99% ethanol, 95% methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl- S-triazine (TPTZ), 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{+}$), ferric chloride hexahydrate, Catechin, gallic acid, Agar, sodium hydroxide, hydrolic acid, acetic acid, sodium nitrite, aluminum chloride, cupper chloride, Ammonium acetate, neocuproine, sodium bicarbonate, L-tyrosine, monopotassium phosphate, mushroom tyrosinase, acetonitrile, Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin , p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose are purchased from Sigma-Aldrich.

3.1.2. Reagents

Folin–Ciocalteu reagent. FRAP reagent was prepared according to Benzie and Strain, 1999 by the addition of 2.5 ml of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer at pH3.6.

Acetate buffer (0.3 M) at pH 3.6 was prepared according to British Pharmacopeia by dissolving 16.8g of acetic acid and 0.8g of sodium hydroxide in 1000 ml of distilled water.

TPTZ (10 mM, Mwt = 312.34 g/mol) was prepared by dissolving 0.312g TPTZ in 100ml HCl.40 mM HCl was prepared by diluting 3.77ml of stock HCl solution (10.6M) to 1000ml with distilled water.

Ferric chloride hexahydrated (20mM, Mwt = 270.3 g/mol) was prepared by dissolving 540mg of it in 100ml of distilled water.

5% $NaNO_2$ was prepared by dissolving 5g of $NaNO_2$ in 100ml of distilled water.

10% $AlCl_3$ was prepared by dissolving 10g of $AlCl_3$ in 100ml of distilled water.

7.5% Na_2CO_3 was prepared by dissolving 7.5g of Na_2CO_3 in 100ml of distilled water.

DPPH (0.1mM, Mwt= 394.32 g/mol) was prepared by dissolving 19.7mg of DPPH in 500ml of 99.9% methanol.

ABTS stock solution (7mM, Mwt= 548.68 g/mol) was prepared by dissolving 384mg of ABTS in 100ml distilled water.

Potassium persulfate (2.45mM, Mwt= 270.32 g/mol) was prepared by dissolving 66mg of potassium persulfate in 100ml ethanol.

Neocuproine solution (0.0075 M, Mwt= 208.26 g/mol) was prepared by dissolving 156mg of neocuproine in 100ml of ethanol.

Copper (II) Chloride solution (0.01 M, Mwt= 134.45g/mol) was prepared by dissolving 134.5mg of copper chloride in 100ml of distilled water

Ammonium Acetate solution (1M, Mwt= 77.08 g/mol) at pH 7.0 was prepared by dissolving 7.7g of it in 100ml of distilled water.

L-tyrosine (0.244mM, Mwt= 181.19 g/mol) was prepared by dissolving 44 mg of L-tyrosine in 1L of monopotassium phosphate buffer solution.

Monopotassium phosphate buffer solution (10mM, mwt= 136.086 g/mol) at a pH of 6.8 was prepared by dissolving 1.4g of KH_2PO_4 in 1L of water, adjusting pH to 6.8.

Mushroom tyrosinase (312.5 U/mL) was prepared by dissolving 10mg in 100ml of distilled water.

3.1.3. Plant materials

The leaves of *Urtica diocia* plant were collected from Bethlehem, Palestine, in January 2016.

The leaves of *Sarcopoterium spinosum* plant were collected from Ramallah, Palestine, in January 2016.

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 analytikjena company, Rotary evaporator, HPLC, laboratory water bath, ultrasonic homogenizer, autoclave.

3.3. Methodology

3.3.1. Preparation of plant materials

The leaves of both plants samples were dried at 30 °C for one week, grinded with a blender. Briefly, 10g of the dried powdered of both plants were mixed with 100 ml different solvents (D.W, 70% EtOH, 99% EtOH, 50% EtOH), extracted for 90 min at 37^{o}C, and filtrated. Then the crude extracts were stored in Refrigerator at 4^{o}C until analysis.

3.3.2. Total phenolics content (Folin–Ciocalteu assay)

Total phenolics were determined using Folin–Ciocalteu reagents (Singleton & Rossi, 1965). The extracts (40) µl were mixed with 1.8 ml of Folin–Ciocalteu reagent (pre-diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then

1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of (100 – 500 ppm) were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/ g sample (Shui & Leong, 2006).

3.3.3. Total flavonoids

The determination of flavonoids was performed according to the colorimetric assay of Kim *et al*, 2003. Distilled water (4 ml) was added to (1 ml) of the extracts. Then, 0.3 ml of 5% sodium nitrite solution was added, followed by 0.3 ml of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature (25◦C) for 5 min, and then 2 ml of 1 M sodium hydroxide were added to the mixture. Immediately, the volume of reaction mixture was made to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink color developed was determined at 510 nm. Aqueous solutions of known Catechin concentrations in the range of (50 – 100 ppm) were used for calibration and the results were expressed as mg Catechin equivalents (CEQ)/ g sample.

3.3.4. Measurement of Antioxidant Activity by FRAP assay

The antioxidant activity of the extracts was determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of Benzie and Strain, 1999.Freshly prepared FRAP reagent (3.0 ml) were warmed at 37◦C and mixed with 40 µl of the leaf extract and the reaction mixtures were later incubated at 37°C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37 °C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay . Aqueous solutions of known Fe (II) concentrations in the range of (2 - 5 mM) ($FeSO_4.6H_2O$) were used for calibration.

3.3.5. Cupric reducing antioxidant capacity (CUPRAC) assay

The assay was conducted as described previously Resat *et al*, 2004. Two 0.5 ml of plant extract or standard of different concentrations solution, 1 ml of copper (II) chloride

solution (0.01 M prepared from $CuCl_2.2H_2O$), 1 ml of ammonium acetate buffer at pH 7.0 and 1 ml of neocuproine solution (0.0075 M) were mixed. The final volume of the mixture was adjusted to 4.1 ml by adding 0.6 ml of distilled water and the total mixture was incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against blank.

3.3.6. Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of extracts of leaves of *Urtica dioica* and *Sarcopoterium spinosum* plants were measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) by shen *et al*, 2010. In brief, 0.1 mM solution of DPPH in ethanol was prepared. This solution (3.9 ml) was added to 0.1 ml. of different extracts in ethanol at different concentrations (50%, 70%, and 99%) and DI. The mixture was shaken vigorously and allowed to stand at room temp for 30 min. Then, absorbance was then measured at 515 nm by using UV-VIS spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using the following equation (Shen *et al*, 2010):

DPPH scavenging effect (%) or Percent inhibition = $A_0 - A_1 / A_0 \times 100\%$.

Where A_0 was the Absorbance of control reaction and A_1 was the Absorbance in presence of test or standard sample.

The results were also presented as antioxidant activity index (AAI) (Scherer & Godoy, 2009)

$$AAI = \frac{C \text{ final DPPH } (\mu mole/g)}{IC50 \ (\mu mole/g)}$$

3.3.7. Antioxidant activity by ABTS assay

This assay was based on the ability of different substances to scavenge 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$) radical cation by Re *et al*, 2000. The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4-16 h until the reaction was complete and the absorbance was stable. The $ABTS^+$ solution was diluted with ethanol to an absorbance

of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9mL of $ABTS^+$ Solution and 0.1mL of tested samples (100 and 200 μg/mL) and mixed for 45 sec; measurements were taken immediately at 734 nm after 15 min. The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation: DPPH scavenging effect (%) or percent inhibition = $((A_o - A_{sample})/ A_o) \times 100\%$, where: A_o is the absorbance of the $ABTS^+$.

3.3.8. Antibacterial Activity by Well Diffusion Method

3.3.8.1. Media Preparation

In this method, the Muller Hinton agar media was prepared by mixing 38g of powder media with 1000ml of distilled water, boiled and sterilized at 121□ C for 15min. After sterilization, the media cooled, and then at 45□ C the media was poured into sterile petri dishes and let to solidify. Wells were done in the media using sterile pipette with a diameter of 1cm and emptied using sterile forceps.

3.3.8.2. Preparation of Inocula

Bacterial and Candidal specimens were brought from diagnostic microbiological lab in Life Sciences Faculty in Al-Quds university.

Part of an isolated bacterial or *Candida albicans* colony was inoculated into a 5ml Muller-Hinton broth tube and incubated for 4-18 hrs at 37□ C. The growth turbidity in Muller-Hinton broth was adjusted by further incubation or dilution with sterile physiological saline, after comparison with that of a MacFarland nephlometer tube no. 0.5 (10^8 cfu/ml) using a spectrophotometer at 625 nm (Optical density 0.08-0.1). An inoculum of 10^6 cfu/ml of bacterial suspension was prepared by diluting 0.1ml of the prepared bacterial broth culture with 9.9 ml sterile saline. Candida specimens were used undiluted (10^8 cfu/ml).

3.3.8.3. Antimicrobial Activity Screening Methods by Well Diffusion Method

With a sterile cotton applicator 10^6 cfu/ml of bacterial suspension of 10^8 cfu/ml of *Candida albicans* culture was swabbed on the surface of Muller-Hinton agar as follows: The cotton applicator was dipped into the bacterial or *Candida* suspension, rotated several times and pressed against the inside was of tube to remove excess inoculum. The agar plate was then streaked in three different directions and around the agar margin to ensure even distribution of inoculum. The plates were left to dry for 3-5 minutes. The selected extracts (D.W, 99% ethanol, and 70% ethanol) were then poured into the wells; where well no.1 has the D.W extracts, well no.2 has the 70% ethanol extracts and well no.3 has the 70% ethanol extracts. Each plate has two negative controls (Distilled water and ethanol). Positive controls (Reference antibiotics) were added to the culture. For *Escherichia coli* Gentamicin 10mcg (CN 10) (Bioanalyse) antibiotic was used, for *Staphylococcus aureus* Penicillin G 10units (Mastdiscs) antibiotic was used, and for *Candida albicans* Novobiocin 30mcg (NV30) (Bioanalyse) was used. The plates were incubated at 37□ C for 24 hrs. for bacteria and 48 hrs. for *Candida albicans.* Each test was done in triplicates. The inhibition zone around each well was measured using a transparent ruler.

3.3.9. *In vitro* whitening effect property of the skin cream by Tyrosinase assay

Tyrosinase catalyses the transformation of L-tyrosine into L-DOPA by hydroxylation and into Odopaquinone by oxidation. Then, through a series of non-enzymatic reactions, O dopaquinone is rapidly transformed into melanin, which is measured at 492 nm in a spectrophotometer. The skin cream LPR1 and LP3 was assayed for tyrosinase inhibition by measuring its effect on tyrosinase activity using a 96-well reader. The reaction was carried out in a 50 mM potassium phosphate buffer (pH 6.8) containing 20 mM L-tyrosine and 312.5 U/mL mushroom tyrosinase at 30 °C warmed in a water bath. The reaction mixture was pre-incubated for 10 min before adding the enzyme. The reaction mixture without the enzyme serves as blank. The Kojic acid serves as control. The change of the absorbance at

492 nm was measured. The percent inhibition of tyrosinase was tested in triplicates and calculated according to (Naraysnaswamy *et al*, 2011) as follow:

Tyrosin as inhibition (%) = ((OD of control – OD of test) / OD of control) ×100

Where OD (Optical Density) = Absorbance

3.3.10. HPLC analysis of phenolic compounds

3.3.10.1. HPLC Instrumentation systems

The analytical HPLC used was Waters Alliance (e2695 separations model), quipped with 2998 Photo diode Array (PDA). Data acquisition and control were carried out using Empower 3 chromatography data software (Waters, Germany).

3.3.10.2. Chromatographic conditions

The HPLC analytical experiments of the crude water, 80% ethanol and 100% ethanol extracts were run on ODS column of Waters (XBridge, 4.6 ID x 150 mm, 5 μm) with guard column of Xbridge ODS, 20 mm x 4.6mm ID, 5 μm. The mobile phase is a mixture of 0.5% acetic acid solution (A) and acetonitrile (B) ran in a linear gradient mode. The start was a 100% (A) that descended to 70% (A) in 40 minutes. Then to 40% (A) in 20 minutes and finally to 10% (A) in 2 minutes and stayed there for 6 minutes and then back to the initial conditions in 2 minutes. The HPLC system was equilibrated for 5 minutes with the initial acidic water mobile phase (100 % A) before injecting next sample. All the samples were filtered with a 0.45 μm PTFE filter. The PDA wavelengths range was from 210-500. The flow rate was 1 ml/min. Injection volume was 20 μl and the column temperature was set at 25◦C. The HPLC system was then equilibrated for 5 minutes with the initial mobile phase composition prior injecting the next sample. All the samples were filtered via 0.45 μm micro porous disposable filter.

3.3.10.3. Sample preparation for HPLC analysis

The plant extracts were filtered using suction filtration, and then the solvents (99% ethanol, 70% ethanol, distilled water extracts) were evaporated under reduced pressure at 40 C using Rotary evaporator. The resulting crude extracts were dissolved in the respective solvents (water, ethanol, and 80% ethanol) at a concentration of 5 mg/mL, and 20 µL were injected into the HPLC chromatograph, and analyzed for their phenolic and flavonoids. Seventeen phenolic and flavonoid standards were injected and separated simultaneously to identify the presence of any of these compounds in the crude extracts. Calibration curve of each individual standard was also prepared at three concentration levels namely 50, 100 and 250 ppm.

3.3.11. Statistical Analysis

The data were analyzed using the SPSS program (Statistical Package for Social Sciences), then filled at the program under 13 variables, 12 of them were the tests for both plants and one is the concentration with 12 different cases. After that, ANOVA procedure was used to test the significant difference at yield means depending on the used solvents, then post-hoc shaffe was used to test the significant difference between the means at a significant difference ($\alpha \leq 0.05$), and a pearson test was used to examine the correlation between the tests at significant difference ($\alpha \leq 0.05$), which gives the values of the coefficient from -1 to 1, so that -1 mean complete negative relation between the variables, and 1 means complete positive relation between the variables, and the relation is strong when the coefficient is close to 1 or -1.

CHAPTER FOUR

RESULTS & DISCUSSION

The leaves of *Urtica dioica* and *Sarcopoterium spinosum* were collected, air dried at room temperature, grinded, extracted in different solvents, and filtered. The crude extracts of samples were then analyzed for their total phenolic content, total flavonoid content, antioxidant activity, and their biological activities as following.

4.1. Total phenolic content (TPC)

Many studies of phenolic compounds have reported that the environmental, climatic, or geographical factors as well as extraction techniques may significantly influence the quality and the quantity of phenolic components present (Ozkan *et al*, 2011) (Pourmorad *et al*, 2006) (Semih & Buket, 2012).

Total phenolic content of the different extracts of *Urtica dioica* and *Sarcopoterium spinosum* was determined for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) by using the Folin-Ciocalteu reagent and were expressed as mg gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve regression equation of Gallic acid ($y = 0.0027x + 0.1172$; $R^2 = 0.9988$) (Figure 4.9).

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference except between D.W and 50% ethanol, it showed that the higher mean as mg Gallic acid/ g sample was found to be in the 70% ethanol extract and the lower is for the 99% ethanol extract. For *Sarcopoterium spinosum* there is a significant mean difference between every two concentrations and that the 70% ethanol extract was the higher mean and the D.W was the lowest as shown in appendix A (Table 2 and Table 3).

The TPC results of *Urtica dioica* showed that extraction using the 70% ethanol gave the highest amount of mg Gallic acid/ g sample (81.1±1.7 mg GAE/g sample), and the 99% ethanol extract gave the lowest amount of Gallic acid/g sample (47.4±1.5 mg GAE/g sample) according to the following trend: 70% ethanol > 50% ethanol > D.W > 99% ethanol to be 81.1±1.7 mg GAE/g sample, 66.1±0.6 mg GAE/g sample, 63.2±0.6 mg GAE/g sample, and 47.4±1.5 mg GAE/g sample respectively; while for *Sarcopoterium*

spinosum results showed that the 70% ethanol extract gave the highest amount of mg Gallic acid/ g sample (310.8±16.6 mg GAE/g sample) and the D.W extract gave the lowest amount (70.6±1.3 mg GAE/g sample according to the following trend: 70% ethanol > 50% ethanol > 99% ethanol > D.W to be 310.8±16.6 mg GAE/g sample, 252.5±5.6 mg GAE/g sample, 173.1±11.3 mg GAE/g sample and 70.6±1.3 mg GAE/g sample respectively as shown in Table 4.1. The results strongly suggest that phenolic compounds are important components of the tested plant extracts.

In comparison with previous studies in literature surveys, Bhuwan *et al*. in their article "Antioxidant potential and total phenolic content of *Urtica dioica* (whole plant)" using the same assay (Folin-Ciocalteau) showed that the *Urtica dioica* extracts as mg GAE/ g sample were found to be highest in ethyl acetate extract (13.06±0.15 mg GAE/g sample), while the results obtained from this study showed higher results to be in a range of 47.4±1.5 mg GAE/g sample to 81.1±1.7 mg GAE/g sample of *Urtica dioica* and less concentrations of GAE/g sample in comparison with the study of Fattahi *et al*. "Total phenolic and Flavonoid contents of aqueous extract of stinging nettle and *In vitro* anti proliferative effect on Hela and BT-474 cell lines" to be 322.9±11.8 mg gallic acid/g sample. While, the total phenolic content of *Sarcopoterium spinosum* of the methanolic extracts in the study of Fattahi *et al* was studied as µg GAE/g sample and showed a range between 61.7±2.7 to 163.6±0.85 µg GAE/g sample, which is more than 1000 times less than the obtained result of this study which ranged between 70.6±1.3 to 310.8±16.6 mg GAE/g sample.

The findings showed that both plants are rich in phenolics, and this may provide a good source of antioxidants which play an important role in inhibiting mutagens and diseases.

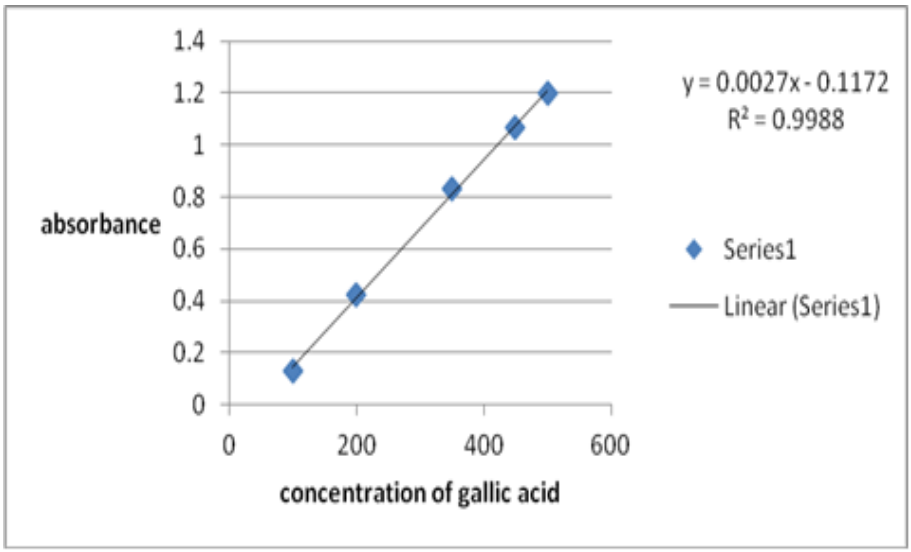


Figure 4.9: Calibration curve of Total phenolic content (TPC) (absorbance of different concentrations of Gallic acid vs. concentration (ppm))

Sample		mg Gallic acid/ g sample
Urtica dioica	99% ethanol	47.4±1.5[c]
	70% ethanol	81.1±1.7[a]
	50% ethanol	66.1±0.6[b]
	D.W	63.2±0.6[b]
Sarcopoterium spinosum	99% ethanol	173.1±11.3[c]
	70% ethanol	310.8±16.6[a]
	50% ethanol	252.5±5.6[b]
	D.W	70.6±1.3[d]

Table 4.1: Total phenolic content (TPC) (as mg Gallic acid/g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

4.2. Total flavonoid content (TFC)

This method was used to determine the total flavonoid contents of the different extracts of *Urtica dioica* and *Sarcopoterium spinosum* plants for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) using the calorimetric assay and were expressed as mg Catechin per gram of plant extract. Total flavonoid contents was calculated using the standard curve regression equation of Catechin ($y = 0.0048x + 0.0034$; $R^2 = 1$) (Figure 4.10) and was expressed as mg Catechin per gram of the plant extract.

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference except between 50% ethanol and 70% ethanol, and we can see that the higher mean as mg Catechin/ g sample was found to be in the 70% ethanol extract and the lower is for the D.W extract. For *Sarcopoterium spinosum* there is a significant mean difference between every two concentrations except between the 50% ethanol extract and the 99% ethanol extract, and that the 70% ethanol extract was the higher mean and the D.W was the lowest as shown in appendix B (Table 5 and Table 6).

The TFC results of *Urtica dioica* showed that extraction using the 70% ethanol gave the highest amount of mg Catechin/ g sample (15.7±0.3 mg Catechin/g sample), and the water extract gave the lowest amount of mg Catechin/g sample (6.1±0.1 mg Catechin/g sample) according to the following trend: 70% ethanol > 50% ethanol > 99% ethanol > D.W to be 15.7±0.3 mg Catechin/g sample, 12.6±0.3 mg Catechin/g sample, 11.3±0.8 mg Catechin/g sample, and 6.1±0.1 mg Catechin/g sample respectively; while for *Sarcopoterium spinosum* results showed that extraction using the 70% ethanol gave the highest amount of mg Catechin/ g sample (24.0±0.2 mg Catechin/g sample), and the water extract gave the lowest amount of mg Catechin/g sample (6.6±0.01 mg Catechin/g sample) according to the following trend: 70% ethanol > 99% ethanol > 50% ethanol > D.W to be 24.0±0.2 mg Catechin/g sample, 19.8±0.4 mg Catechin/g sample, 19.3±0.7 mg Catechin/g sample and 6.6±0.01 mg Catechin/g sample respectively. The results strongly suggest that phenolic compounds are important components of the testes plant extracts as shown in Table 4.2. Flavonoids play an important role in antioxidant system in plants.

In comparison with previous studies in literature surveys, Fattahi *et al*. in their article "Total phenolic and Flavonoid contents of aqueous extract of stinging nettle and *In vitro* anti proliferative effect on Hela and BT-474 cell lines" using the same calorimetric assay by aluminum chloride methods showed that the *Urtica dioica* extracts as mg Catechin/ g sample was found to be (133.916±12.006 mg Catechin/g sample), while the results obtained from this study showed that the highest reading was for the 70% ethanol extract (15.7±0.3 mg/g). While, the total flavonoid contents of *Sarcopoterium spinosum* of methanolic extracts in seham *et al*. in their article "Evaluation of the phenolic and flavonoid contents, antimicrobial and cytotoxic activities of some plants growing in Al Jabal Al-Akhdar in Libya" using the spectrophotometric method based on the intensity of the color developed by different types of flavonoids expressed as mg Catechin/g sample to be 0.182 mg Catechin/ g sample, which was studied in this study as mg Catechin/ g sample in my study to be its highest in the 70% ethanol extract (24.0±0.2 mg Catechin/ g sample) and its lowest concentration in the water extract (6.6±0.01 mg Catechin/g sample).

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation (Benavente-Garcia *et al*, 1997).

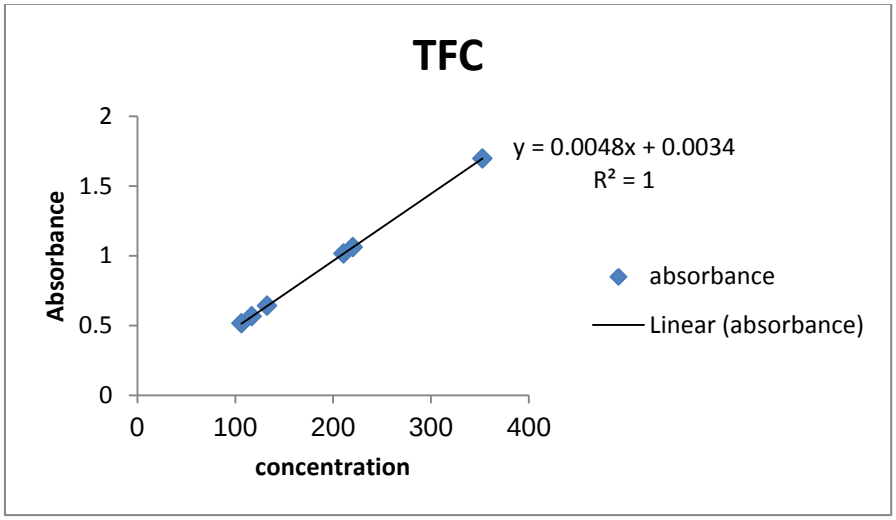


Figure 4.10: Calibration curve of Total Flavonoid content (TFC) (absorbance of different concentrations of Catechin vs. of dry sample

Sample		mg Catechin/ g sample
Urtica dioica	99% ethanol	11.3 ± 0.8^{b}
	70% ethanol	15.7 ± 0.3^{a}
	50% ethanol	12.6 ± 0.3^{a}
	D.W	6.1 ± 0.1^{c}
Sarcopoterium spinosum	99% ethanol	19.8 ± 0.4^{b}
	70% ethanol	24.0 ± 0.2^{a}
	50% ethanol	19.3 ± 0.7^{b}
	D.W	6.6 ± 0.01^{c}

Table 2: Total flavonoid content (TFC) (mg Catechin/g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

4.3. Antioxidant activity (AA)

To evaluate the antioxidant activity, one method is not sufficient since many factors can affect the evaluation. It is required to take more than one measurement and also to take in consideration different mechanisms of antioxidant activity as follow:

4.3.1 Reducing ability of plant extracts:

4.3.1.1. Antioxidant activity by FRAP assay

Ferric Ion Reducing Antioxidant Power Assay (FRAP) is simple, fast, inexpensive, and robust method, and does not require specialized equipment. In the FRAP method the yellow Fe^{3+} TPTZ complex (2,4,6-tri (2-pyridyl)-1,3,5-triazine) is reduced to the blue Fe^{2+}

TPTZ complex by electron-donating substances (such as phenolic compounds) under acidic conditions (Benzie & Strain, 1996). The yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

The antioxidant activity of ethanol and aqueous plant extracts of *Urtica dioica* and *Sarcopoterium spinosum* plants for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) using FRAP method and were expressed as mmole Fe^{+2} per gram of plant extract. It was calculated using the standard curve regression equation of Fe^{+2} concentration ($y = 0.2019x - 0.1766$; $R^2 = 0.9822$) (Figure 4.11).

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference except between 99% ethanol and 70% ethanol, and we can see that the higher mean as mmole Fe^{+2}/ g sample was found to be in the 70% ethanol extract and the lower is for the D.W extract. For *Sarcopoterium spinosum* there is a significant mean difference between every two concentrations, and that the 70% ethanol extract was the higher mean and the D.W was the lowest as shown in appendix C (Table 8 and Table 9).

The results of *Urtica dioica* showed that the extraction using the 70% ethanol and the 99% ethanol gave the highest amounts of mm Fe^{+2}/g sample (1.9±0.01 mM Fe^{+2}/g sample) according to the following trend: 70% ethanol , 99% ethanol > D.W > 50% ethanol to be 1.9±0.01 mM Fe^{+2}/g sample, 1.8±0.02 mM Fe^{+2}/g sample, 0.9±0.01 mM Fe^{+2}/g sample, and 0.5±0.13 mM Fe^{+2}/g sample respectively; while for *Sarcopoterium spinosum* results showed that the 70% ethanol extract gave the highest amount of mM Fe^{+2}/g sample (3.0±0.05 mM Fe^{+2}/g sample) and the water extract gave the lowest amount of mM Fe^{+2}/g sample (1.2±0.1 mM Fe^{+2}/g sample) according to the following trend: 70% ethanol > 99% ethanol > 50% ethanol > D.W to be 3.0±0.05 mM Fe^{+2}/g sample, 2.6±0.1 mM Fe^{+2}/g sample, 2.3±0.05 mM Fe^{+2}/g sample and 1.2±0.1 mM Fe^{+2}/g sample respectively as shown in Table 4.3.

In comparison with previous studies in literature surveys, Kukric *et al*. in their article "Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica* L.)" using the same FRAP method, showed that the *Urtica dioica* extracts expressed as mM Fe^{+2} per g sample were found to be 7.50±0.43 mM Fe^{+2}/ g sample which showed a weak antioxidant activity compared to the used control antioxidants (Vitamin C (143.09±11.29 mM Fe^{+2}/ g sample) and BHA (147.28±13.87 mM Fe^{+2}/ g sample)) that were 20 times more powerful than the *Urtica dioica* extract, and as compared to BHT (16.64±0.30 mM Fe^{+2}/ g sample) control results showed to have two times higher than the ethanol extract of *Urtica dioica*, while the results obtained in this study showed a very weak antioxidant activity ranged from (1.9±0.01 mM Fe^{+2}/ g sample to 0.5±0.13 mM Fe^{+2}/ g sample) compared to the results obtained from Kukric *et al*. study. While, none of the studies before discussed the antioxidant activity of *Sarcopoterium spinosum* by FRAP method, but as compared to the control antioxidants (vitamin C, BHT and BHA) in previous studies, results showed very weak antioxidant activity.

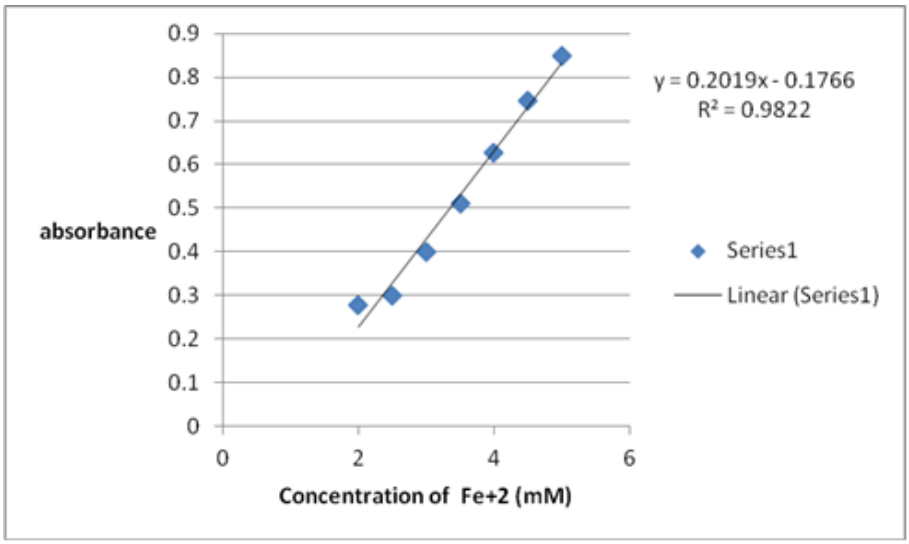


Figure 4.11: Calibration curve of concentration of Fe^{+2}(mM) (Absorbance of different concentrations of Fe^{+2} vs. concentration (mM))

Sample		mM Fe^{+2}/ g sample
Urtica dioica	99% ethanol	1.8±0.02[a]
	70% ethanol	1.9±0.01[a]
	50% ethanol	0.5±0.13[b]
	D.W	0.9±0.01[c]
Sarcopoterium spinosum	99% ethanol	2.3±0.05[c]
	70% ethanol	3.0±0.05[a]
	50% ethanol	2.6±0.1[b]
	D.W	1.2±0.1[d]

Table 4.3: Antioxidant activity (AA) FRAP (as mmole Fe^{+2} /g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

4.3.1.2 Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC method of total antioxidant capacity (TAC) assay uses bis(2,9-dimethyl-1,10-phenanthroline: neocuproine) Cu(II) chelate cation as the chromogenicoxidant, which is reduced in the presence of antioxidants to the cuprous neocuproine chelate [Cu(I)–Nc] showing maximum light absorption at 450 nm. Color development in the CUPRAC method is based on the following reaction (Karaman *et al*, 2010):

$$nCu(Nc)_2^{+2} + n\text{-e reductant} \leftrightarrow nCu(Nc)_2^{+} + n\text{-e oxidized product} + nH^{+}$$

The antioxidant activity of ethanol and aqueous plant extracts of *Urtica dioica* and *Sarcopoterium spinosum* plants for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) using CUPRAC method and were expressed mg Trolox per gram sample of plant extract. It was calculated using the standard curve of CUPRAC (Figure 4.12),

using the regression equation (y= 0.001x + 0.011, R^2= 1). Results in (Table 4.4) show good antioxidant activity.

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference between D.W and the 50% ethanol, between the D.W and the 70% ethanol, and between the D.W and the 90% ethanol. It showed that the higher mean as mg Trolox/ g sample was found to be in the 70% ethanol extract and the lower is for the 50% ethanol extract. For *Sarcopoterium spinosum* there is a significant mean difference between D.W and the 50% ethanol, between the D.W and the 70% ethanol, and between the D.W and the 90% ethanol, and that the 70% ethanol extract was the higher mean and the D.W was the lowest as shown in appendix D (Table 11 and Table 12).

The CUPRAC test results of *Urtica dioia* showed that extraction using the 70% ethanol gave the highest amount of mg Trolox/ g sample (158.3±0.3 mg Trolox/ g sample), and the 50% ethanol extract gave the lowest amount of mg Trolox/ g sample (27.4±4.1 mg Trolox/ g sample) according to the following trend: 70% ethanol > 99% ethanol > D.W > 50% ethanol to be 158.3±0.3 mg Trolox/ g sample, 86.8±0.6 mg Trolox/ g sample, 54.2±7.7 mg Trolox/ g sample, and 27.4±4.1 mg Trolox/ g sample respectively; while for *Sarcopoterium spinosum* results showed that that extraction using the 70% ethanol gave the highest amount of mg Trolox/ g sample (349.8±3.4 mg Trolox/ g sample), and the water extract gave the lowest amount of mg Trolox/ g sample (62.8±11.0 mg Trolox/ g sample) according to the following trend: 70% ethanol > 99% ethanol > 50% ethanol > D.W to be 349.8±3.4 mg Trolox/ g sample, 120.2±5.0 mg Trolox/ g sample, 66.7±16.3 mg Trolox/ g sample and 62.8±11.0 mg Trolox/ g sample respectively. It was shown that the 70% ethanol extract of *Urtica dioica* and *Sarcopoterium spinosum* have the highest results 158.3±0.3 mg Trolox/g sample, and 349.8±3.4 mg Trolox/g sample respectively as shown in Table 4.4, probably due to the mixed polarity of this concentration (30% polar water, and 70% non-polar ethanol) that facilitated e-transfer in ionizing solvents.

In comparison with previous studies in literature surveys, Bourgeois *et al*. in their article "Nettle (*Urtica dioia* L.) as a source of antioxidant and anti-aging phytochemicals for

cosmetic applications" using the CUPRAC and FRAP assays showed that the antioxidant capacity of *Urtica dioica* extracts given by these tests (FRAP, and CUPRAC) revealed a strong antioxidant ranging from 0.53 to 2.71 Trolox C equivalent antioxidant capacity (TEAC) using CUPRAC assay measured with a standard 1mM concentration of Trolox, and 0.15 to 0.73 TEAC using FRAP assay. Results of Bourgeois *et al*. study also indicated the presence of potential antioxidant compounds such as flavonoids or lignans in the nettle extracts (Orčić *et al*, 2014) (Otles & Yalcin, 2012), while the results obtained in this study were expressed as TEAC with a standard 7.5 mM concentration of Trolox and showed higher results in both plants extracts (*Urtica dioica* and *Sarcopoterium spinosum*) than the results obtained in Bourgeois *et al*. study.

The difference observed in the TEAC determined by FRAP and CUPRAC could be explained by the physiochemical nature of the antioxidant evidenced by these two tests, where the CUPRAC test is applied to lipophilic and hydrophilic antioxidants (Ratz-Lyko *et al*, 2012).

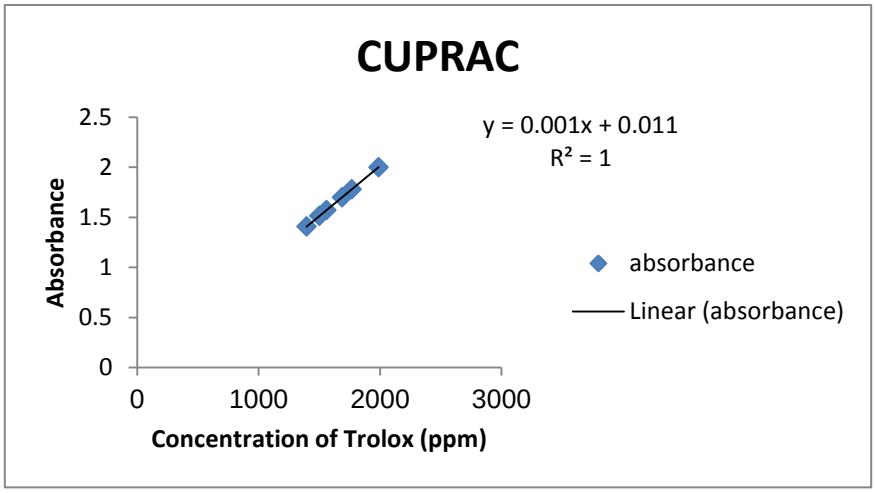


Figure 4.12: Calibration curve of CUPRAC (absorbance of different concentrations of Trolox vs. concentration (ppm))

Sample		mg Trolox/ g sample
Urtica dioica	99% ethanol	86.8 ± 0.6^{b}
	70% ethanol	158.3 ± 0.3^{a}
	50% ethanol	27.4 ± 4.1^{d}
	D.W	54.2 ± 7.7^{c}
Sarcopoterium spinosum	99% ethanol	120.2 ± 5.0^{b}
	70% ethanol	349.8 ± 3.4^{a}
	50% ethanol	66.7 ± 16.3^{c}
	D.W	62.8 ± 11.0^{d}

Table 4.4: CUPRAC (as mg Trolox /g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

4.3.2. Scavenging ability of plant extracts:

4.3.2.1. Free radical scavenging activity by DPPH

The free radical scavenging activity is studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple color dye having absorption maxima of 515 nm and upon reaction with a hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance (Mahuya Hom Choudhury *et al*, 2014).

The antioxidant activity of ethanol and aqueous plant extracts of *Urtica dioica* and *Sarcopoterium spinosum* plants for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) using DPPH method and were expressed µmole Trolox per gram sample of plant extract. It was calculated using the standard curve regression equation of Trolox (ppm) ($y = -0.0026x + 0.7803$; $R^2 = 0.9991$) (Figure 4.13).

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference except between 99% ethanol and 70% ethanol, and we can see that the higher mean as µmole Trolox per gram was found to be in the 70% ethanol extract as well as the 99% ethanol and the lower is for the D.W extract as shown in appendix E (Table 14 and Table 15). For *Sarcopoterium spinosum* there is no significant difference between the extracts.

The DPPH results of *Urtica dioica* showed that extraction using the 70% ethanol (117.8±0.7 µmole Trolox/ g sample) and the 99% ethanol (118.2±0.5 µmole Trolox/ g sample) gave the highest amount of µmole Trolox/ g sample and the water extract gave the lowest amount of µmole Trolox/ g sample (24.0±3.4 µmole Trolox/ g sample) according to the following trend: 70% ethanol, 99% ethanol > 50% ethanol > D.W to be 118.2±0.5 µmole Trolox/ g sample, 117.8±0.7 µmole Trolox/ g sample, 94.3±3.6 µmole Trolox/ g sample, and 24.0±3.4 µmole Trolox/ g sample respectively; while for *Sarcopoterium spinosum* results showed no significant difference in all extracts as µmole Trolox/ g sample according to the following trend: 99% ethanol > 50% ethanol > 70% ethanol > D.W to be 185.5±1.8 µmole Trolox/ g sample, 158.3±0.1 µmole Trolox/ g sample, 152.0±0.8 µmole Trolox/ g sample and 100.0±1.9 µmole Trolox/ g sample respectively.

Whereas, the inhibition percentage of antioxidant were studied, results showed that the 70% ethanol extract of *Urtica dioica* and *Sarcopoterium spinosum* has the highest concentration of Trolox per gram sample with an inhibition% of 80.65% and 86.20% respectively as shown in (Table 4.5). In general, the ethanolic extracts of both plants showed a high radical scavenging activity than the aqueous extracts.

The AAI has been used to identify the antioxidant activity are classified as weak, when AAI < 0.5, moderate AAI between 0.5-1.0, strong, when AAI between 1.0-2.0, and very strong, when AAI > 2.0. (Scherer & Godoy, 2009)

In comparison with previous studies in literature surveys, Kukric *et al*. in their article "Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica* L.)" using the same DPPH method, showed that the AAI (Antioxidant activity index) values of *Urtica dioica* extracts showed moderate antioxidant activity (0.85±0.003) compared to the used control antioxidants (Vitamin C (4.97±0.01), BHA (3.96±0.17), and BHT (1.15±0.04)) that was 17.2 times lower than vitamin C, 13.7 times lower than BHA and 3.8 times lower than BHT, while the results of AAI obtained in this study regarding the *Urtica dioica* extract showed very strong antioxidant activity ranged from (2.73 to 3.07) compared to the results of the extract and the reference antioxidants obtained from Kukric *et al*. study. While, the antioxidant activity of *Sarcopoterium spinosum* by DPPH method as compared to the control antioxidants (vitamin C, BHT and BHA) in Kukric *et al*., results of AAI showed very strong antioxidant activity ranged from (2.56 to 4.33).

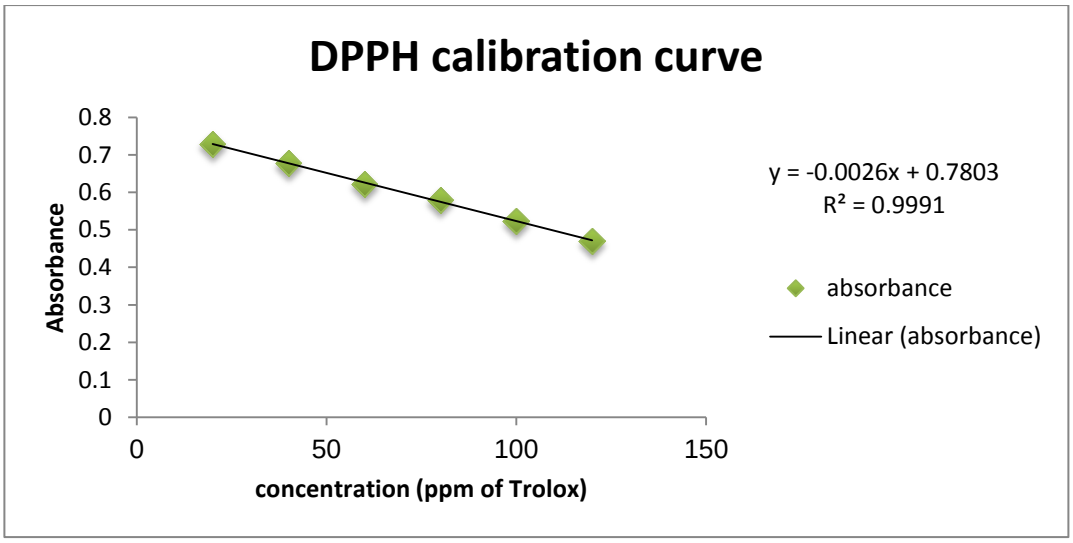


Figure 4.13: Calibration curve of DPPH (Absorbance of different concentrations of Trolox vs. concentration (ppm))

Sample		µmole Trolox /g sample	Inhibition %	AAI
Urtica dioica	99% ethanol	117.8±0.7[a]	76.67%	3.07
	70% ethanol	118.2±0.5[a]	80.65%	2.93
	50% ethanol	94.3±3.6[b]	68.99%	2.73
	D.W	24.0±3.4[c]	16.35%	2.93
Sarcopoterium spinosum	99% ethanol	185.5±1.8[a]	85.63%	4.33
	70% ethanol	152.0±0.8[a]	86.20%	3.52
	50% ethanol	158.3±0.1[a]	85.21%	3.71
	D.W	100.0±1.9[a]	77.95%	2.56

Table 4.5: Antioxidant activity (AA) DPPH content (as µmole Trolox /g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

4.3.2.2. Free radical scavenging activity by ABTS

This method was used to evaluate the free radical scavenging activity of plant extracts. The antioxidant activity of ethanol and aqueous plant extracts of *Urtica dioica* and *Sarcopoterium spinosum* plants for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol) using ABTS method and were expressed µmole Trolox per gram sample of plant extract. The plant extracts have the ability to inhibit ABTS radical and this method is used to measure it. This assay was expressed as µmole Trolox /g sample by the standard curve of ABTS (Figure 4.14), it was measured using the regression equation ($y = -0.0154x + 0.6578$, $R^2 = 0.9971$).

The statistical analysis showed for the *Urtica dioica* that there is a significant mean difference except between 50% ethanol and the distilled water extract, and results showed

higher mean as µmole Trolox /g sample was found to be in the 70% ethanol extract and the lower is for the distilled water extract. For *Sarcopoterium spinosum* there is a significant mean difference between every two concentrations, and that the 70% ethanol extract was the higher mean and the D.W was the lowest as shown in appendix F (Table 17 and Table 18).

The ABTS method results of *Urtica dioica* showed that extraction using the 70% ethanol gave the highest amount of µmole Trolox/g sample (3.6±0.4 µmole Trolox/g sample), and that the distilled water extract showed the lowest amount of µmole Trolox/g sample (0.4±0.2 µmole Trolox/g sample), according to the following trend: 70% ethanol > 99% ethanol > 50% ethanol > D.W to be 3.6±0.4 µmole Trolox/g sample, 3.8±0.07 µmole Trolox/g sample, 1.1±0.1 µmole Trolox/g sample, and 0.4±0.2 µmole Trolox/g sample respectively; while for *Sarcopoterium spinosum* results showed that the 70% ethanol extract gave the highest amount of µmole Trolox/g sample (5.9±0.03 µmole Trolox/g sample) and that the distilled water extract showed the lowest amount of µmole Trolox/g sample (2.4±0.07 µmole Trolox/g sample) according to the following trend: 70% ethanol > 50% ethanol > 99% ethanol > D.W to be 5.9±0.03 µmole Trolox/g sample, 5.6±0.03 µmole Trolox/g sample, 5.0±0.02 µmole Trolox/g sample and 2.4±0.07 µmole Trolox/g sample respectively. It was shown that the 70% ethanol extract of *Urtica dioica* and *Sarcopoterium spinosum* have the highest results 3.6±0.4 µmole Trolox/g sample, and 5.9±0.03 µmole Trolox/g sample respectively.

Organic solvent is needed to obtain high ABTS scavenging ability as appeared in (Table 4.6) that the 99% ethanol extract of *Urtica dioica* and *Sarcopoterium spinosum* have the highest inhibition % of ABTS radical 92.9%, and 98.4% respectively. In general the *Sarcopoterium spinosum* extracts show a high percentage of inhibition comparing to the nettle extracts.

In comparison with previous studies in literature surveys, Kukric *et al*. in their article "Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica* L.)" using the same ABTS method showed that the nettle leaves have the ability to inhibit

the ABTS radical and that the ethanol extract of nettle leaves had significantly lower antioxidant activity measured according to ABTS than other compared to the standard control antioxidants such as vitamin C, BHA and BHT, while the results in this study regarding the *Urtica dioica* extract showed high antioxidant activity ranged with an inhibition percentage ranged from 17.9% to 92.9%). While, the antioxidant activity of *Sarcopoterium spinosum* by ABTS method as compared to the control antioxidants (vitamin C, BHT and BHA) in Kukric *et al*., showed high antioxidant activity ranged from (65.3% to 98.4%) and higher results than the nettle extract.

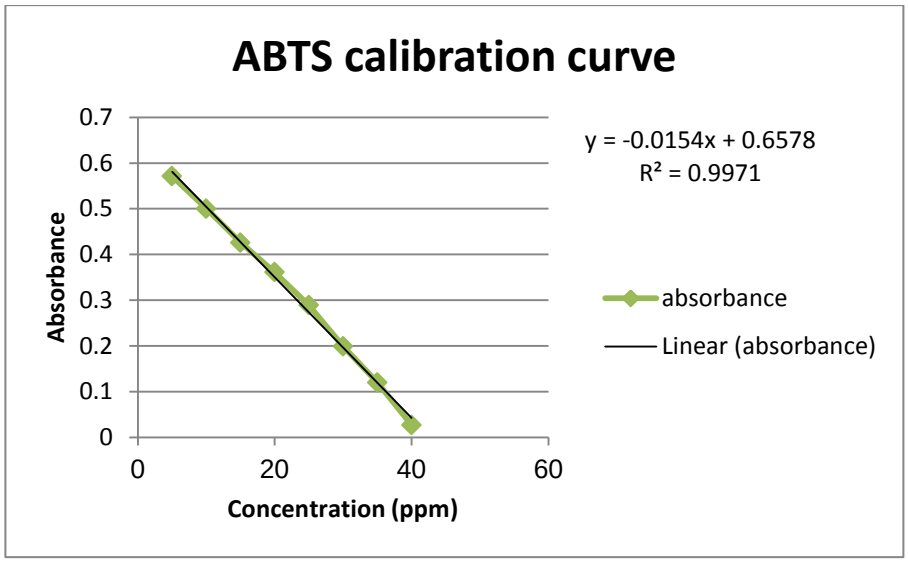


Figure 4.14: Calibration curve of ABTS (absorbance of different concentrations of Trolox vs. Concentration (ppm))

Sample		µmole Trolox /g sample	Inhibition %
Urtica dioica	99% ethanol	3.8 ±0.07[b]	92.9%
	70% ethanol	3.6 ±0.4[a]	86.0%
	50% ethanol	1.1 ±0.1[c]	70.7%
	D.W	0.4 ±0.2[c]	17.9%
Sarcopoterium spinosum	99% ethanol	5.0 ±0.02[c]	98.4%
	70% ethanol	5.9 ±0.03[a]	98.1%
	50% ethanol	5.6 ±0.03[b]	98.3%
	D.W	2.4 ±0.07[d]	65.3%

Table 4.6: Antioxidant activity (AA) ABTS content (as µmole Trolox /g of dry sample) of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water. Results are expressed as average ± SD with the same letters are not statistically different from one another by ANOVA followed by Tukey's post hoc test ($p \leq 0.05$). RSD is relative standard deviation of three samples of each extract).

In Figure 4.15 below, results of different tests (CUPRAC, TPC, DPPH, TFC, ABTS, FRAP), showed that the 70% ethanol extracts have the higher estimated mean probably due to the mixed polarity of this concentration (30% polar water, and 70% non-polar ethanol) which showed the higher solubility of phenolic compounds and antioxidants and that the D.W extracts have the lowest estimated mean also due to the high polarity of water that don't have the ability of dissolution of many phenolic compounds and antioxidants, while the 50% ethanol extracts and the 99% ethanols extract concentrations are nearlly similar to each other. The descending order for the means with respeect for the tests:

CUPRAC > TPC > DPPH > TFC > ABTS > FRAP

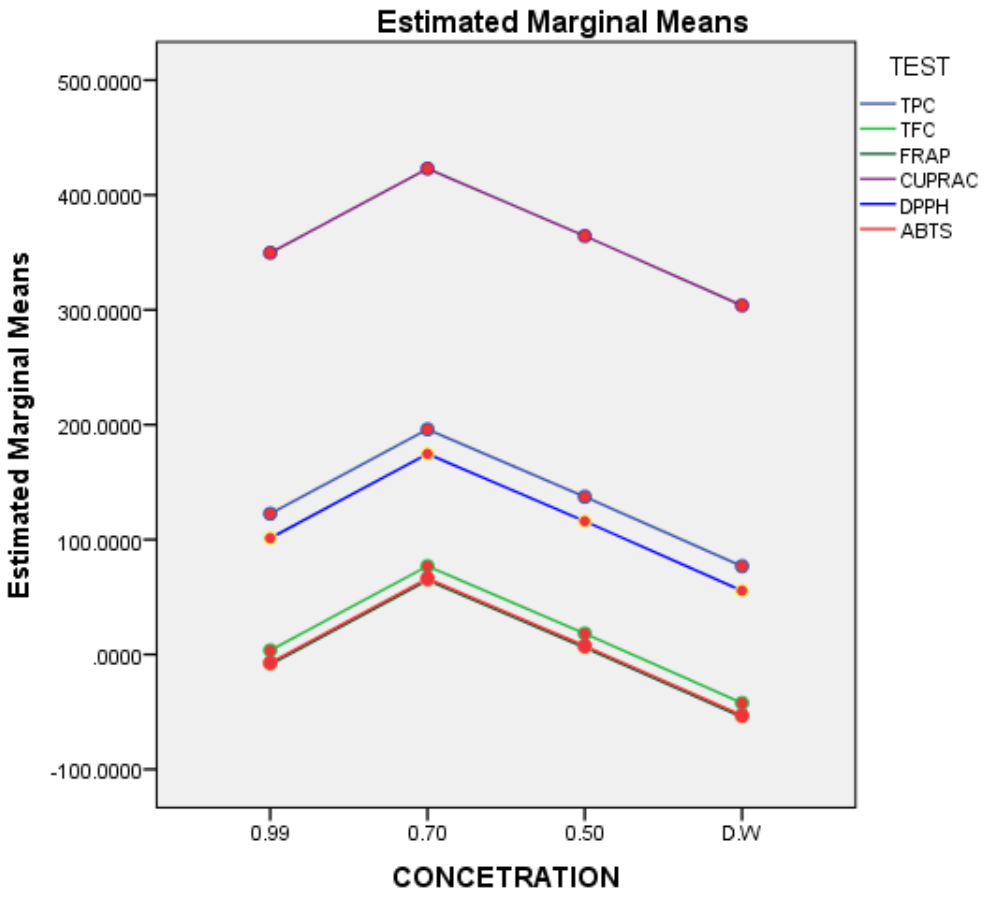


Figure 4.15: Estimated marginal means vs. concentration weight figure for each test where every single line represent certain test, and every nod represesnt certain concentration.

4.4. Antimicrobial activity by Well diffusion method

The antibacterial activity of *Urtica dioica* and *Sarcopoterium spinosum* was studied against gram positive bacteria (*Staphylococcus aureus*), gram negative bacteria (*Escherichia coli*), and yeast (*Candida albicans*) in different extractions (D.W, 70% ethanol, and 99% ethanol) using the well diffusion method. Penicillin, Gentamicin, and Novobiocin were used as positive controls respectively for *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. Distilled water and ethanol were used as negative controls. The zone of inhibition for each plant in different solution is represented in Table 4.7.

The effect of D.W extract of *Sarcopoterium spinosum* plant against *Staphylococcus aureus* bacteria (The zone of inhibition 26mm) showed higher effect than that of the positive control Penicillin (20mm) and the effect of the 70% ethanolic extract for the same plant (22mm), while the 99% ethanolic extract has no effect. While for *Urtia dioica*, the only effect was for the 70% ethanolic extract on *Staphyloccus aureus* and was higher than it for the positive control.

The effect of D.W extract of *Sarcopoterium spinosum* plant against *Candida albicans* yeast (The zone of inhibition 20mm) showed a similar effect as the positive control Novobiocin (20mm) and the effect of the 70% ethanolic extract for the same plant (22mm) showed higher effect than the positive control, and the 99% ethanolic extract was the highest (25mm). While for *Urtia dioica*, none of the extracts show any effect against *Candida albicans.*

None of the extracts of any of the plants showed any effect against *Escherichia coli* bacteria, as shown in Table 4.7.

In comparison with previous studies in literature surveys, Kukric *et al*. in their article "Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica* L.)" using the macro-dilution method with slight modification. The nettle extract leaves were diluted with methanol and incubated at 37□ C for 24 hours against the cultures *B. Subitilis* IP 5832, *Escherichia coli*, *P. aeruginosa*, and *L. plantarum*. Results showed that the nettle leaves extracts have a weak antibacterial activity against *B. Subitilis* IP 5832 and *Escherichia coli*, while the other tested bacteria strain of *Escherichia coli* isolated from urine, *P. aeruginosa*, and *L. plantarum* didn't exhibit any antibacterial activity of the tested extract. The results in this study regarding the *Urtica dioica* ethanol and water extracts also showed weak antimicrobial effect against *Staphylococcus aureus* with no bacterial inhibition against *Escherichia coli* and *Candida albicans*. Whereas, M.S. Ali-Shtayeh *et al*., in their article "Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area" studied the antimicrobial activity of different plants including *Sarcopoterium spinosum* that showed 90% of antimicrobial activity. Moreover, Seham *et al*. in their article "Evaluation of the Phenolic and Flavonoid Contents, Antimicrobial and Cytotoxic Activities of Some Plants Growing in Al Jabal Al-Akhdar in Libya" studied the antimicrobial of *Sarcopoterium spinosium*. L. (Rosaceae) against *Staphylococcus aureus*, *Candida albicans*, and *Escherichia coli*, results showed that *Sarcopoterium spinosium* has lower zone of inhibition against *Candida albicans* (18.3 mm± 1.2) than the standard Amphotericin B (26.4 mm± 0.72), lower zone of inhibition against *Staphylococcus aureus* (16.3 mm± 1.5) than the standard Ampicillin (28.9 mm± 1.2), and higher zone of inhibition against *Escherichia coli* (26.3 mm± 0.58) than the standard Gentamycin (25.3 mm± 0.18). While results obtained in this study showed higher zone of inhibition against *Staphylococcus aureus* (22mm for the 70% ethanol and 26 mm for the D.W extract) than the standard Penicillin G with no effect for the 99% ethanol, similar to higher zone of inhibition (20mm to 25mm) against *Candida albicans* than the standard Novobiocin, and no effect was observed against *Escherichia coli* probably due to personal error.

Bacterium type	Zone of inhibition			
	Solvent	***Sarcopoterium spinosum***	***Urtica dioica***	**Positive control (std.)**
Staphylococcus aureus	D.W	26 mm	No effect	Penicillin G 10 units (Mastdisc) 20 mm
	70% EtOH	22 mm	22 mm	
	99% EtOH	No effect	No effect	
Candida albicans	D.W	20 mm	No effect	Novobiocin 30mCg (NV30) (Bioanalyse) 20 mm
	70% EtOH	22 mm	No effect	
	99% EtOH	25 mm	No effect	
Escherichia coli	D.W	No effect	No effect	Gentamicin 10mCg (CN10) (Bioanalyse) 24 mm
	70% EtOH	No effect	No effect	
	99% EtOH	No effect	No effect	

Table 4.7: Zone of inhibition of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water against *Staphylococcus aureus*, *Candida albicans*, and *Escherichia coli*, corresponding to positive control [Penicillin G20, Nonobiocin, and Gentamicin]

4.5. *In vitro* whitening effect property of the skin cream by tyrosinase assay

Skin is the important external defense organ of the body in living organisms. Hence, it is prone to environmental factors including UV light, drugs, pesticides, ozone, industrial waste, chemical solvents and pollutants. The exposure of skin to these environmental factors causes aging, hyperpigmentation, inflammation etc. Skin aging and hyperpigmentation pose an aesthetic problem in socioeconomic status. Hyperpigmentation is caused by the key enzyme tyrosinase. It is a copper-containing monooxygenase that catalyses melanin synthesis in melanocytes. The accumulation of excessive epidermal pigmentation leads to various dermatological disorders such as freckling, age spots, and

sites of actinic damage (Narayanaswamy *et al*, 2011). Hence, it has become essential for any plant extract to inhibit tyrosinase to be an effective skin whitening agent (Lalitha *et al*, 2014).

Skin whitening products are commercially available for cosmetic purposes in order to obtain a lighter skin appearance. They are also utilized for clinical treatment of pigmentary disorders such as melasma or post-inflammatory hyperpigmentation. Whitening agents act at various levels of melanin production in the skin (Smit *et al*, 2009).

Many of them are known as competitive inhibitors of tyrosinase, the key enzyme in melanogenesis. Others inhibit the maturation of this enzyme or the transport of pigment granules (melanosomes) from melanocytes to surrounding keratinocytes (Smit *et al*, 2009).

In vitro whitening effect by inhibition of tyrosinase enzyme of *Urtica dioica* and *Sarcopoterium spinosum* was studied for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol). Results in table 4.8 showed that the 70% ethanol of *Urtica dioica* gave the highest inhibition percentage of tyrosinase enzyme, where the 99% ethanol extract has an inhibition of 76.67%, the 50% ethanol with an inhibition of 68.99%, and then the water extract with an inhibition of 16.35%. Whereas, the 70% ethanol extract of *Sarcopoterium spinosum* gave the highest inhibition percentage of tyrosinase enzyme with an inhibition of 86.20%, 85.63% for the 99% ethanol extract, 85.21% for the 50% ethanol extract, and 77.95% for the water extract. All results were compared to kojic acid as reference which has an inhibition percentage of 100%. And as compared to the study of Luisa Rizza in her article “Skin-whitening effects of Mediterranean herbal extracts by in vitro and in vivo models”, results showed that herbal extracts possessed an inhibitory effect of tyrosinase enzyme and less intensive than kojic acid and hydroquinone, the study of Luisa Rizza highly suggest the usage of herbs with kojic acid and hydroquinone for a significant higher whitening effect than can be applied in vivo and used in creams to inhibit the melanin formation in the skin.

Sample		Inhibition %
Urtica dioica	99% ethanol	76.67%
	70% ethanol	80.65%
	50% ethanol	68.99%
	D.W	16.35%
Sarcopoterium spinosum	99% ethanol	85.63%
	70% ethanol	86.20%
	50% ethanol	85.21%
	D.W	77.95%
Kojic Acid		100%

Table 4.8: Inhibition of Melanin formation of *Urtica dioica* and *Sarcopoterium spinosum* samples obtained in January 2016, extracted with different concentrations of Ethanol (50%, 70%, and 99%) and with distilled water.

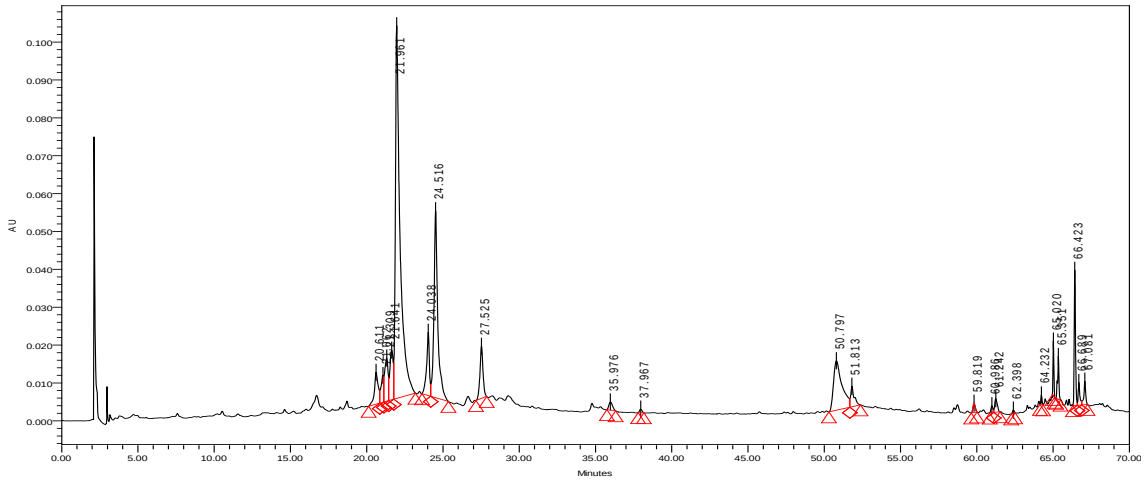
4.6 HPLC Analysis of phenolic compounds

4.6.1. *Urtica dioica* (99% ethanol)

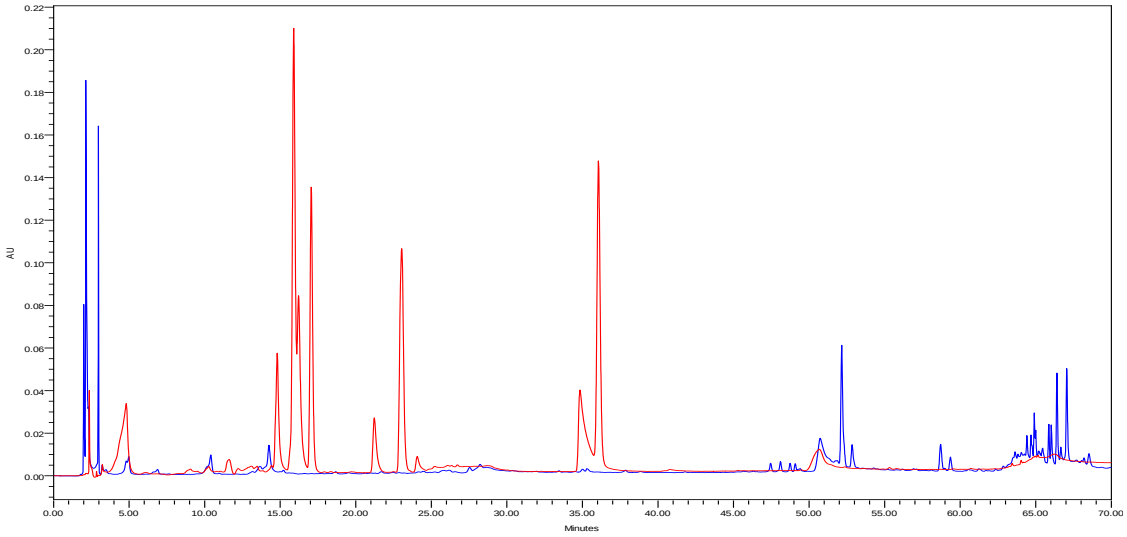
Figure 4.16 shows chromatograms of the crude extract of *Urtica Dioica* (99% ethanol) at 254 nm (A) and overlaid chromatogram with the standards (B). This wavelength was selected since the main peaks showed a maximum absorption close to it. As seen from Figure 1A, different phenolic compounds were detected in the range of 20-40 minutes and compared to different standards, but none of these compounds were part of the phenolic and flavonoids standards (Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin , p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose) injected as per their retention and UV-Vis spectra tells. Additionally other lipophilic compounds in the range of 50-70 minutes were also detected. Bourgeois *et al*, studied the presence of ursolic acid in nettle root extracts and quercetin in leaf extracts, has been described using an HPLC method and separated on a C18-grafted reverse phase column using an HPLC linear gradiant composed of a mixture of acetonitrile and 0.1% (v/v)

formic acid acidified water during one hour at a rate flow of 0.6 ml per minute, and detected at 254nm, results of this study confirmed the presence of these molecules in nettle (Retention time (min) for: quercetin (39 min) and ursolic acid (58 min)) with a possibility of their simultaneous extraction from the whole plant.

(A)



(B)



(C)

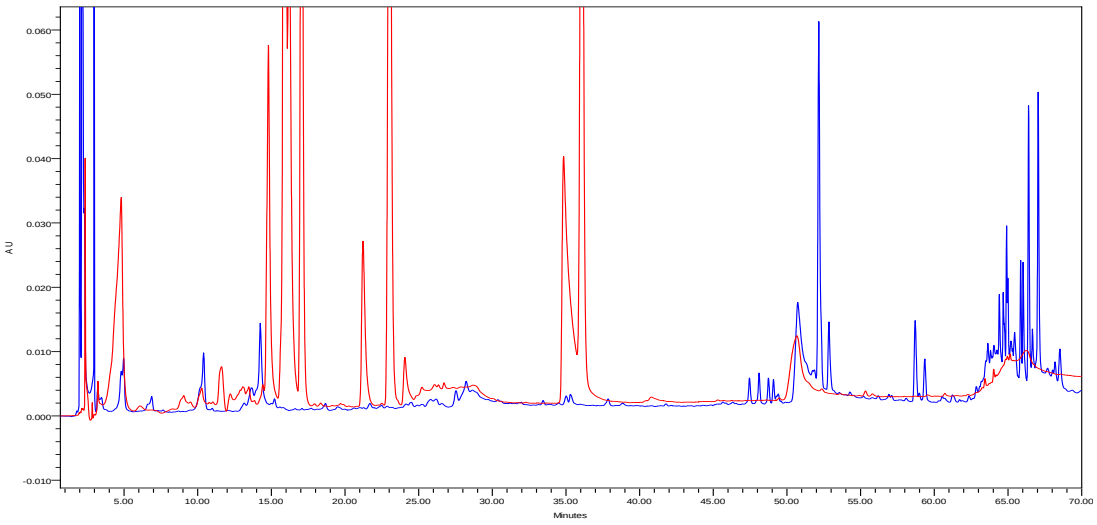


Figure 4.16: (A) HPLC-PDA chromatograms of crude ethanolic (99%) extract of *Urtica dioica*, and (B) an overlaid chromatogram with the standards at 254 nm. Figure 1C is the zoomed chromatogram of B.

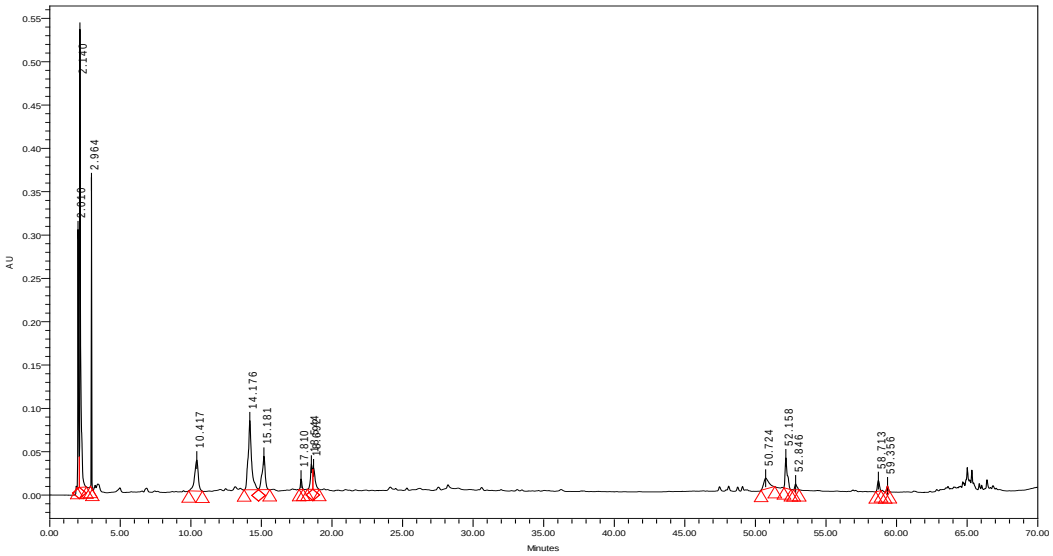
4.6.2. *Sarcopoterium spinosum*

4.6.2.3 70% ethanol extract:

After Reher et al, in 1991, deduced the presence of catechin, α-tocopherol content of *Sarcopoterium spinosum* was studied by Sarikaya in 2010 (Reher et al., 1991) (Sarıkaya & Kayalar, 2010) . However, no detailed study exists in the literature regarding the content of *Sarcopoterium spinosum* extract. So, in this study it was tried to analyze the 70% ethanol extract of *Sarcopoterium spinosum*, Figure 4.17 showed chromatogram of the crude extract (70% ethanol) at 254 nm. This wavelength was selected since the main peaks showed a maximum absorption close to it. As seen from this figure, different polar compounds (with retention times of 2-3 minutes) and other phenolic compounds with retention times from 10-20 minutes were detected and compared to different standards, but none of these compounds were part of the phenolic and flavonoids standards (Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin , p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose) injected as per their retention and UV-Vis

spectra tells. Additionally other lipophilic compounds in the range of 50-70 minutes were also detected.

(A)



(B)

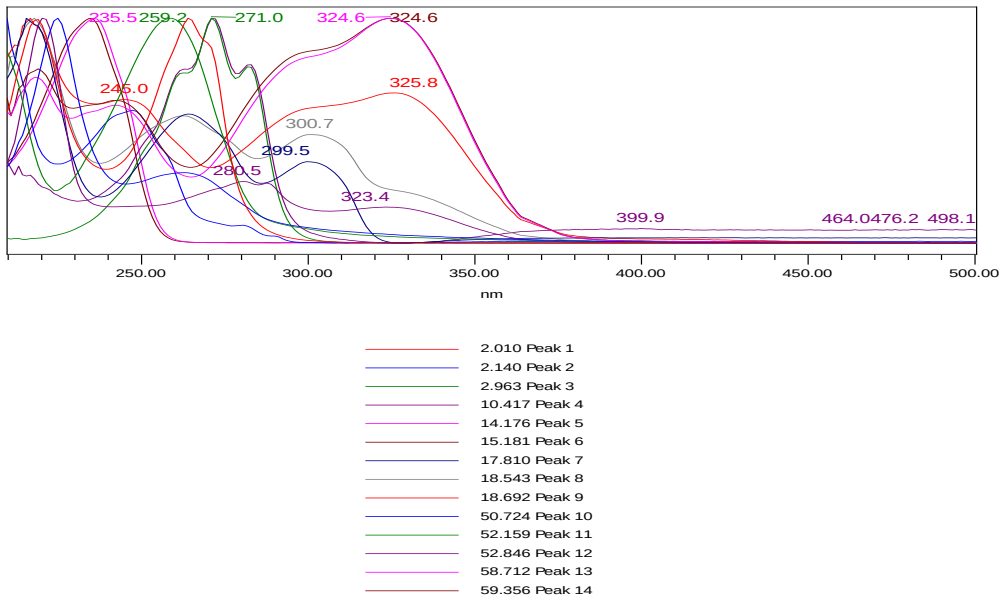
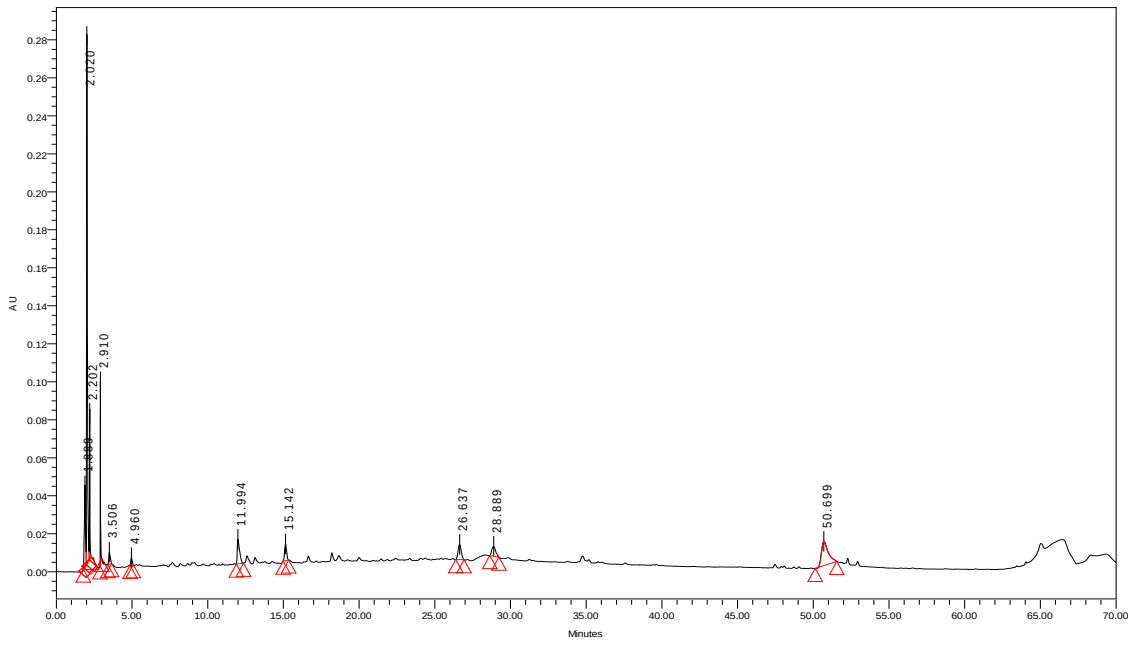


Figure 4.17: (A) HPLC-PDA chromatograms of crude ethanolic (70%) extract of *Sarcopoterium spinosum*. (B) The overlaid UV-Vis spectra of the main peaks.

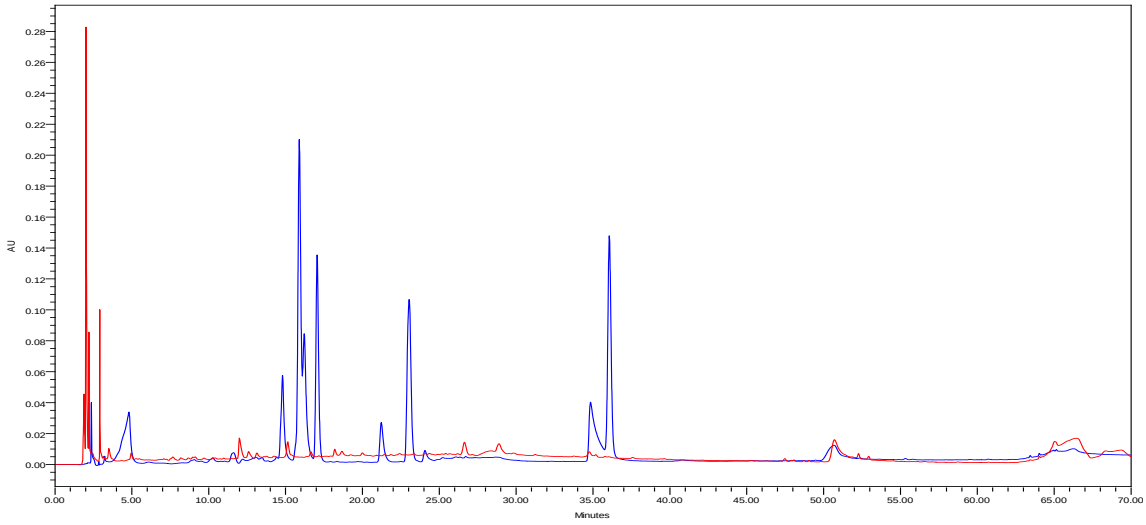
4.6.2.4 Water Extract

Figure 4.18 showed chromatogram of the crude extract (water extract) at 254 nm. As seen from this figure, different polar compounds (with retention times of 2-5 minutes) and other phenolic compounds with retention times from 10-30 minutes were detected and compared to different standards, but none of these compounds were part of the phenolic and flavonoids standards (Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin , p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose) injected as per their retention and UV-Vis spectra tells.

(A)



(B)



(C)

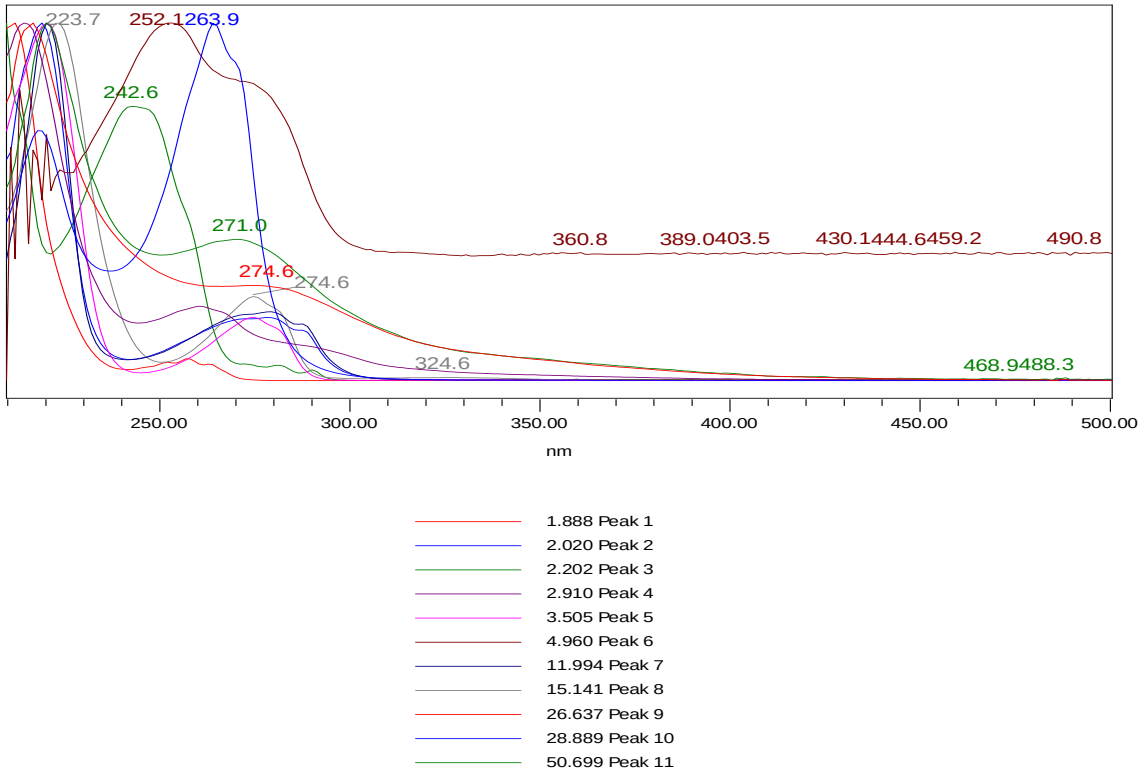


Figure 4.18: HPLC-PDA chromatograms of crude water extract (A). and an overlaid chromatogram with the standards at 254 nm (B). The overlaid UV-Vis spectra of the main peaks are depicted in Figure C.

CHAPTER FIVE

CONCLUSION
&
RECOMMENDATIONS

Conclusion

The *Urtica dioica* and *Sarcopoterium spinosum* leaves from flora of Palestine are rich in phenolic, flavonoid compounds and constitutes a natural source of potent antioxidants that may prevent serious diseases, and disorders, and could be used in further considered for future applications such as food, pharmaceuticals, preservatives, and cosmetics.

The total phenolic content, the total flavonoid content, the antioxidant activities and the biological activity of these plants were studied for different solvents (D.W, 70% ethanol, 99% ethanol, and 50% ethanol). It has been found generally in several tests that the 70% ethanol extract of both plants gave the highest TPC, TFC and AA values probably due to the mixed polarity of this concentration (30% polar water, and 70% non-polar ethanol) which showed the higher solubility of phenolic compounds and antioxidants and that the D.W extracts have the lowest estimated mean also due to the high polarity of water that don't have the ability of dissolution of many phenolic compounds and antioxidants, while the 50% ethanol extracts and the 99% ethanols extract concentrations are nearlly similar to each other.

Moreover, the whitening effect property of the skin by tyrosinase enzyme was studied in vitro for both plants. Results showed that *Urtica dioica* and *Sarcopoterium spinosum* have high inhibition percentage of tyrosinase enzyme as compared to kojic acid that inhibits the formation of melanin pigmentation which could be a good candidate to be used in whitening creams that can be tested in vivo.

The antibacterial activity of *Urtica dioica* and *Sarcopoterium spinosum* was studied against gram positive bacteria (*Staphylococcus aureus*), gram negative bacteria (*Escherichia coli*), and yeast (*candida albicans*) in different solvents (99% ethanol, 70% ethanol, and distilled water). Results showed that the *Urtica dioica* ethanol and water extracts showed weak antimicrobial effect against *Staphylococcus aureus* with no bacterial inhibition against *Escherichia coli* and *Candida albicans*. While results obtained in this study showed higher zone of inhibition against *Staphylococcus aureus* than the standard Penicillin G with no effect for the 99% ethanol, similar to higher zone of inhibition against

Candida albicans than the standard Novobiocin, and no effect was observed against *Escherichia coli* probably due to personal error.

HPLC analysis for phenolic compounds for *Urtica dioica* were detected showed shromatogram (99% ethanol extract) at 245nm. This wavelength was selected since the main peaks showed a maximum absorption close to it. Results showed that in the range of 20-40 minutes and compared to different standards, but none of these compounds were part of the phenolic and flavonoids standards (Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin , p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose) injected as per their retention and UV-Vis spectra tells. Moreover, *Sarcopoterium spinosum* phenolic compounds were also detected showed chromatogram of the crude extract (70% ethanol extract and water extract) at 254 nm. Results showed that different polar compounds (with retention times of 2-3 minutes) and other phenolic compounds with retention times from 10-20 minutes were detected and compared to different standards, but none of these compounds were part of the phenolic and flavonoids standards injected as per their retention and UV-Vis spectra tells. Additionally results showed other lipophilic compounds in the range of 50-70 minutes were also detected for both plants.

Recommendations:

It would be very interesting to accomplish this study by other interventions to determine the anti-inflammatory activity of these plants, the anti-glycation activity, know more about different compounds responsible for the antioxidant activity, find the correlations between these activities and the chemical contents in attempt to identify more active compounds, and investigate the mechanism of the whitening effect in vivo and formulate an effective whitening cream.

References

References

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APPENDICES

Appendix A: Total phenolic contents

Concentration of gallic acid (ppm)	Absorbance at λ= 765 nm
100	0.132
200	0.426
350	0.830
450	1.070
500	1.199

Table 1: Absorbance of different concentration of Gallic acid

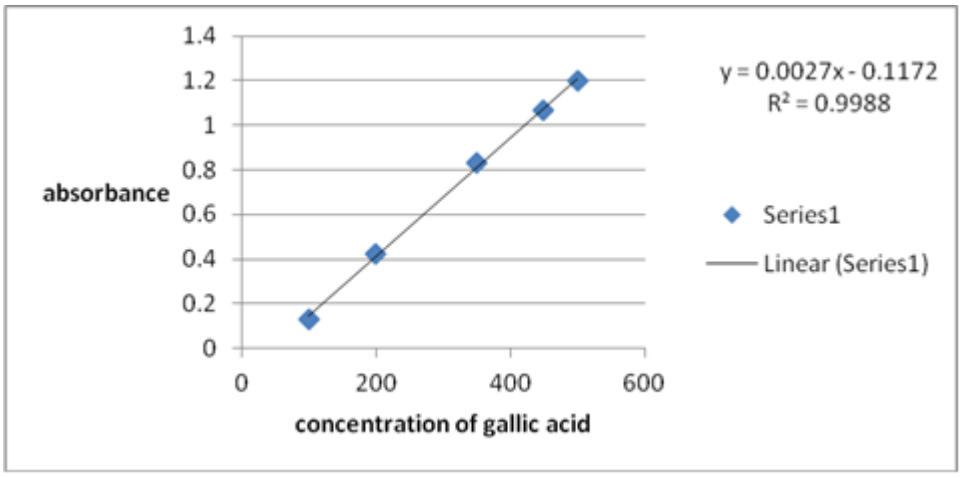

Figure 1: Calibration curve of total phenolic content

ANOVA Table

		Sum of Squares	Df	Mean Square	F	Sig.
TPC *Urtica dioica*	Between Groups	1722.103	3	574.034	388.179	.000
	Within Groups	11.830	8	1.479		
	Total	1733.933	11			
TPC *Sarcopoterium spinosum*	Between Groups	97466.891	3	32488.964	297.834	.000
	Within Groups	872.672	8	109.084		
	Total	98339.563	11			

Table 2: ANOVA table (as mg gallic acid/g sample) of TPC

Dependent Variable	(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.
TPC(*Urtica dioica*)	D.W	.50	-2.8690733333	.9929037977	.110
		.70	-17.8963333333*	.9929037977	.000
		.99	15.8600000000*	.9929037977	.000
	.50	D.W	2.8690733333	.9929037977	.110
		.70	-15.0272600000*	.9929037977	.000
		.99	18.7290733333*	.9929037977	.000
	.70	D.W	17.8963333333*	.9929037977	.000
		.50	15.0272600000*	.9929037977	.000
		.99	33.7563333333*	.9929037977	.000
	.99	D.W	-15.8600000000*	.9929037977	.000
		.50	-18.7290733333*	.9929037977	.000
		.70	-33.7563333333*	.9929037977	.000
TPC (*Sarcopoterium spinosum*)	D.W	.50	-181.8305566667*	8.5277603641	.000
		.70	-240.1877781000*	8.5277603641	.000
		.99	-102.3246333333*	8.5277603641	.000
	.50	.00	181.8305566667*	8.5277603641	.000
		.70	-58.3572214333*	8.5277603641	.001
		.99	79.5059233333*	8.5277603641	.000
	.70	.00	240.1877781000*	8.5277603641	.000
		.50	58.3572214333*	8.5277603641	.001
		.99	137.8631447667*	8.5277603641	.000
	.99	.00	102.3246333333*	8.5277603641	.000
		.50	-79.5059233333*	8.5277603641	.000
		.70	-137.8631447667*	8.5277603641	.000

Table 3: Scheffe test for mean differences of concentrations of TPC

Appendix B: Total flavonoid contents

Concentration of Catechin (ppm)	Absorbance at λ= 510 nm
50	0.255
60	0.282
75	0.353
86	0.396
100	0.496

Table 4: Absorbance of different concentration of Catechin.

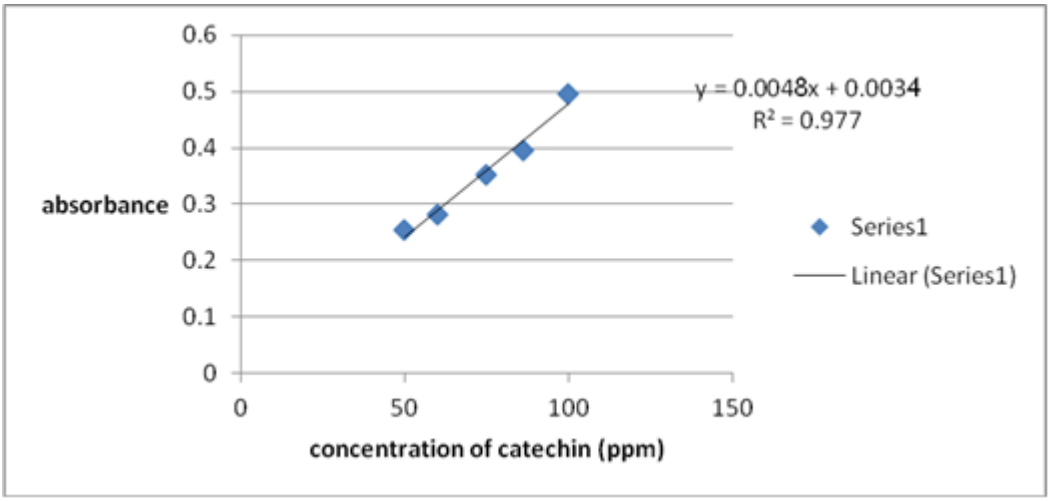

Figure 2: Calibration curve of total flavonoid content

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
TFC *Urtica dioica*	Between Groups	102.943	3	34.314	188.022	.000
	Within Groups	1.460	8	.183		
	Total	104.403	11			
TFC *Sarcopoterium spinosum*	Between Groups	490.983	3	163.661	652.729	.000
	Within Groups	2.006	8	.251		
	Total	492.989	11			

Table 5: ANOVA table (as mg gallic acid/g sample) of TFC

Dependent Variable	(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.
TFC (*Urtica dioica*)	D.W	.50	-6.5294999987*	.3488088914	.000
		.70	-7.6486110987*	.3488088914	.000
		.99	-5.2225749987*	.3488088914	.000
	.50	.00	6.5294999987*	.3488088914	.000
		.70	-1.1191111000	.3488088914	.072
		.99	1.3069250000*	.3488088914	.036
	.70	D.W	7.6486110987*	.3488088914	.000
		.50	1.1191111000	.3488088914	.072
		.99	2.4260361000*	.3488088914	.001
	.99	D.W	5.2225749987*	.3488088914	.000
		.50	-1.3069250000*	.3488088914	.036
		.70	-2.4260361000*	.3488088914	.001
TFC (*Sarcopoterium spinosum*)	.D.W	.50	-12.3943533333*	.4088465902	.000
		.70	-17.0447866667*	.4088465902	.000
		.99	-13.1604533333*	.4088465902	.000
	.50	D.W	12.3943533333*	.4088465902	.000
		.70	-4.6504333333*	.4088465902	.000
		.99	-.7661000000	.4088465902	.380
	.70	D.W	17.0447866667*	.4088465902	.000
		.50	4.6504333333*	.4088465902	.000
		.99	3.8843333333*	.4088465902	.000
	.99	.00	13.1604533333*	.4088465902	.000
		.50	.7661000000	.4088465902	.380
		.70	-3.8843333333*	.4088465902	.000

Table 6: Scheffe test for mean differences of concentrations of TFC

Appendix C: FRAP method

Concentration of Fe^{+2} (mM)	Absorbance at λ= 593 nm
2	0.279
2.5	0.299
3	0.400
3.5	0.511
4	0.627
4.5	0.745
5	0.848

Table 7: Absorbance of different concentration of Ferric ion.

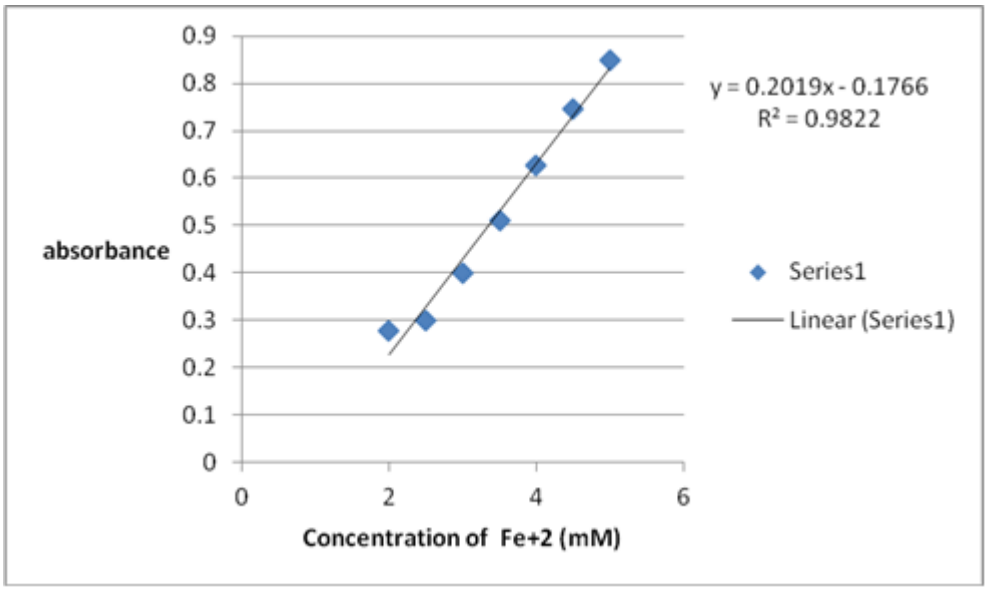

Figure 3: Calibration curve of FRAP antioxidant

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
FRAP *Urtica dioica*	Between Groups	4.069	3	1.356	324.269	.000
	Within Groups	.033	8	.004		
	Total	4.103	11			
FRAP *Sarcopoterium spinosum*	Between Groups	5.675	3	1.892	242.277	.000
	Within Groups	.062	8	.008		
	Total	5.738	11			

Table 8: ANOVA table (as mmole Fe^{+2}/g sample) of FRAP

Dependent Variable	(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.
FRAP *Urtica dioica*	D.W	.50	.4110000000*	.0528073017	.000
		.70	-.9403333333*	.0528073017	.000
		.99	-.9036666667*	.0528073017	.000
	.50	D.W	-.4110000000*	.0528073017	.000
		.70	-1.3513333333*	.0528073017	.000
		.99	-1.3146666667*	.0528073017	.000
	.70	D.W	.9403333333*	.0528073017	.000
		.50	1.3513333333*	.0528073017	.000
		.99	.0366666667	.0528073017	.920
	.99	D.W	.9036666667*	.0528073017	.000
		.50	1.3146666667*	.0528073017	.000
		.70	-.0366666667	.0528073017	.920
FRAP *Sarcopoterium spinosum*	D.W	.50	-1.4168733333*	.0721490742	.000
		.70	-1.8613400000*	.0721490742	.000
		.99	-1.1426733333*	.0721490742	.000
	.50	D.W	1.4168733333*	.0721490742	.000
		.70	-.4444666667*	.0721490742	.002
		.99	.2742000000*	.0721490742	.034
	.70	D.W	1.8613400000*	.0721490742	.000
		.50	.4444666667*	.0721490742	.002
		.99	.7186666667*	.0721490742	.000
	.99	D.W	1.1426733333*	.0721490742	.000
		.50	-.2742000000*	.0721490742	.034
		.70	-.7186666667*	.0721490742	.000

Table 9: Scheffe test for mean differences of concentrations of FRAP

Appendix D: CUPRAC method

Concentration of Trolox (ppm)	Absorbance at λ= 450 nm
20	0.032
40	0.059
60	0.077
80	0.098
100	0.118
120	0.142
140	0.168

Table 10: Absorbance of different concentration of Trolox

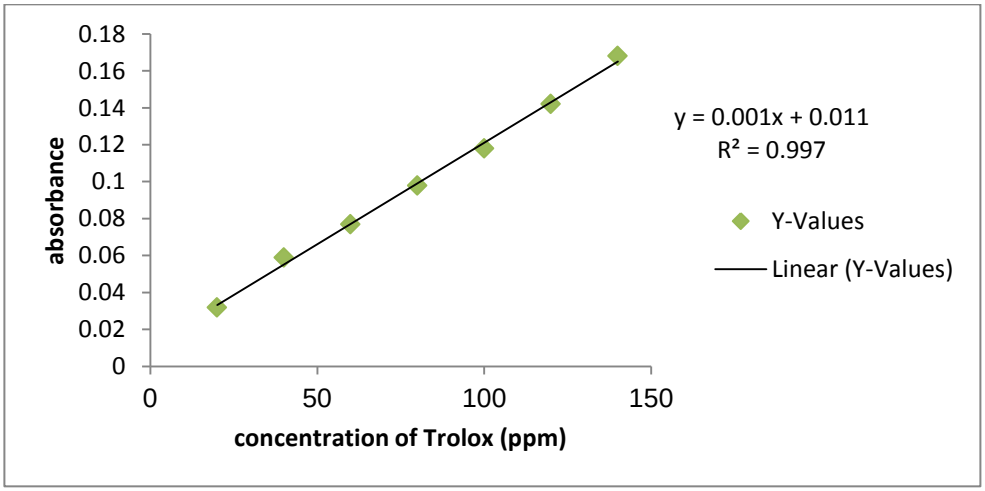

Figure 4: Calibration curve of CUPRAC antioxidant power

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
CUPRAC *Urtica dioica*	Between Groups	185042.576	3	61680.859	505.772	.000
	Within Groups	975.631	8	121.954		
	Total	186018.207	11			
CUPRAC *Sarcopoterium spinosum*	Between Groups	873191.425	3	291063.808	429.125	.000
	Within Groups	5426.177	8	678.272		
	Total	878617.602	11			

Table 11: ANOVA table (as mg Trolox/g sample) of CUPRAC

Dependent Variable	(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.
CUPRAC D U	D.W	.50	67.97356*	9.01680	.001
		.70	-263.87435*	9.01680	.000
		.99	-82.54962*	9.01680	.000
	.50	D.W	-67.97356*	9.01680	.001
		.70	-331.84791*	9.01680	.000
		.99	-150.52318*	9.01680	.000
	.70	D.W	263.87435*	9.01680	.000
		.50	331.84791*	9.01680	.000
		.99	181.32473*	9.01680	.000
	.99	D.W	82.54962*	9.01680	.000
		.50	150.52318*	9.01680	.000
		.70	-181.32473*	9.01680	.000
CUPRAC *Sarcopoterium spinosum*	D.W	.50	-456.54122*	21.26456	.000
		.70	-727.86907*	21.26456	.000
		.99	-229.67810*	21.26456	.000
	.50	D.W	456.54122*	21.26456	.000
		.70	-271.32786*	21.26456	.000
		.99	226.86312*	21.26456	.000
	.70	D.W	727.86907*	21.26456	.000
		.50	271.32786*	21.26456	.000
		.99	498.19098*	21.26456	.000
	.99	D.W	229.67810*	21.26456	.000
		.50	-226.86312*	21.26456	.000
		.70	-498.19098*	21.26456	.000

Table 12: Scheffe test for mean differences of concentrations of CUPRAC

Appendix E: DPPH method

Concentration of Trolox (ppm)	Absorbance at λ= 515 nm
20	0.729
40	0.677
60	0.623
80	0.580
100	0.523
120	0.470

Table 13: Absorbance of different concentration of Trolox

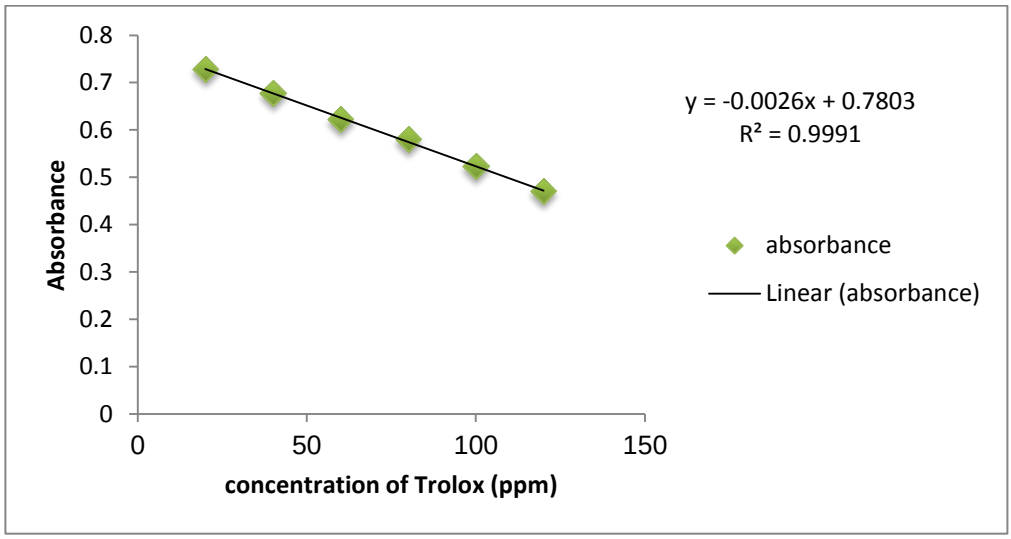

Figure 5: Calibration curve of DPPH

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
DPPH *Urtica dioica*	Between Groups	17797.210	3	5932.403	929.211	.000
	Within Groups	51.075	8	6.384		
	Total	17848.284	11			
DPPH *Sarcopoterium spinosum*	Between Groups	6230.755	3	2076.918	.887	.488
	Within Groups	18735.786	8	2341.973		
	Total	24966.541	11			

Table 14: ANOVA table (as µmole Trolox/g sample) of DPPH

(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
D.W	.50	-70.31333*	2.06306	.000	-77.5189	-63.1078
	.70	-94.12333*	2.06306	.000	-101.3289	-86.9178
	.99	-93.84000*	2.06306	.000	-101.0455	-86.6345
.50	D.W	70.31333*	2.06306	.000	63.1078	77.5189
	.70	-23.81000*	2.06306	.000	-31.0155	-16.6045
	.99	-23.52667*	2.06306	.000	-30.7322	-16.3211
.70	D.W	94.12333*	2.06306	.000	86.9178	101.3289
	.50	23.81000*	2.06306	.000	16.6045	31.0155
	.99	.28333	2.06306	.999	-6.9222	7.4889
.99	D.W	93.84000*	2.06306	.000	86.6345	101.0455
	.50	23.52667*	2.06306	.000	16.3211	30.7322
	.70	-.28333	2.06306	.999	-7.4889	6.9222

Table 15: Scheffe test for mean differences of concentrations of DPPH

* The mean difference is significant at the 0.05 level.

Appendix F: ABTS method

Concentration of Trolox (ppm)	Absorbance at λ= 734 nm
5	0.571
10	0.500
15	0.426
20	0.361
25	0.289
30	0.199
35	0.120
40	0.027

Table 16: Absorbance of different concentration of Trolox

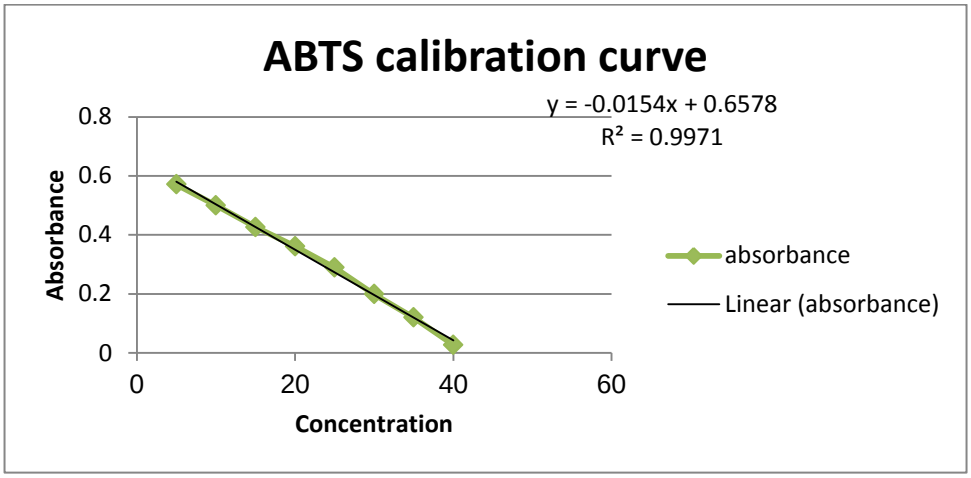

Figure 6: Calibration curve of ABTS

ANOVA Table

		Sum of Squares	df	Mean Square	F	Sig.
ABTS *Urtica dioica*	Between Groups	25.711	3	8.570	105.313	.000
	Within Groups	.651	8	.081		
	Total	26.362	11			
ABTS *Sarcopoterium spinosum*	Between Groups	20.858	3	6.953	3853.451	.000
	Within Groups	.014	8	.002		
	Total	20.872	11			

Table 17: ANOVA table (as µmole Trolox/g sample) of ABTS

Dependent Variable	(I) p	(J) p	Mean Difference (I-J)	Std. Error	Sig.
ABTS *Urtica dioica*	D.W	.50	-.77933	.23292	.061
		.70	-3.13800*	.23292	.000
		.99	-3.38133*	.23292	.000
	.50	D.W	.77933	.23292	.061
		.70	-2.35867*	.23292	.000
		.99	-2.60200*	.23292	.000
	.70	D.W	3.13800*	.23292	.000
		.50	2.35867*	.23292	.000
		.99	-.24333	.23292	.781
	.99	D.W	3.38133*	.23292	.000
		.50	2.60200*	.23292	.000
		.70	.24333	.23292	.781
ABTS *Sarcopoterium spinosum*	D.W	.50	-3.03833*	.03468	.000
		.70	-3.34800*	.03468	.000
		.99	-2.50667*	.03468	.000
	.50	D.W	3.03833*	.03468	.000
		.70	-.30967*	.03468	.000
		.99	.53167*	.03468	.000
	.70	D.W	3.34800*	.03468	.000
		.50	.30967*	.03468	.000
		.99	.84133*	.03468	.000
	.99	D.W	2.50667*	.03468	.000
		.50	-.53167*	.03468	.000
		.70	-.84133*	.03468	.000

Table 18: Scheffe test for mean differences of concentrations of ABTS

المواد الفعالة ونشاط مضادات الأكسدة والفعالية البيولوجية لنبتتي النتش و القريص في فلسطين

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إشراف: د. فؤاد الريماوي

ملخص

هدفت الرسالة الى تقييم النشاط المضاد للأكسدة والمواد الفعالة و النشاط البيولوجي لنبتة القريص التي تم جمع اوراقها من مدينة بيت لحم و نبتة النتش من مدينة رام الله في كانون الثاني من عام 2016م. اذ تم تحضير مستخلصات كل نبتة بتراكيز مختلفة من الكحول الإيثيلي (50%, 70% و 99%) و الماء المقطر. و تم عمل دراسة احصائية لايجاد العلاقة بين كل فحص و الآخر.

تم فحص مضادات الأكسدة للمستخلصات باستخدام فحوصات (FRAP، CUPRAC ، ABTS ، DPPH) بالتراكيز المذكورة اعلاه، وتم حساب TPC بطريقة (Folin- ciocalteu) و TFC بطريقة (Aluminum Chloride method)، ثم قمنا بعمل تحاليل لإيجاد تراكيز المواد الفينولية باستخدام جهاز التحليل HPLC و تبين ان النبتتين غنيتان باحتوائهما كميات جيدة من مضادات الأكسدة و المواد الفعالة، اذ تبين بأن مستخلص تركيز الكحول الإيثيلي 70% يملك اعلى نسب من هذه المواد في جميع الفحوصات و ان مستخلص الماء المقطر حصل على اقل نسب في جميع الفحوصات ايضاً.

تبين من نتائج الدراسة الإحصائية بين كل فحص و الآخر لنبتة القريص، بأن هناك علاقة و ارتباط ملحوظ بين كل من TFC مع ABTS و DPPH. تبين ايضاً ان هناك ارتباط قوي بين الفحوصات FRAP مع CUPRAC، DPPH، و ABTS. و ارتباط آخر بين ABTS مع CUPRAC و DPPH. اما بالنسبة لنبتة النتش، فلوحظ ان جميع الفحوصات لها علاقة و تؤثر فيما بينها ما عدا فحص DPPH الذي لم يظهر اي ارتباط مع غيره من الفحوصات.

تم ايضا دراسة النشاط البيولوجي للنبتتين السابق ذكرهما ضد بكتيريا سالبة غرام (*Escherichia coli*) و بكتيريا موجبة غرام (*Staphylococcus aureus*) و الخميرة (*Candida albicans*)، ولوحظ ان النبتتين تؤثران على البكتيريا موجبة غرام بينما لا تؤثران على البكتيريا سالبة غرام ،و أن نبتة النتش افضل من نبتة القريص ضد البكتيريا موجبة غرام و ضد الخميرة ايضاً.

اوضحت الدراسة الإحصائية بأنه لا يوجد اي ارتباط بنبتة القريص او النتش مع اي من الفحوصات الستة ضد بكتيريا موجبة غرام. اما بالنسبة للخميرة فالدراسة اوضحت بعدم وجود ارتباط لنبتة النتش بالفحوصات TPC، TFC،

FRAP، CUPRAC او ABTS ووجد ارتباط قوي جداً بمعامل ارتباط -1 مع فحص DPPH اي ان العلاقة خطية عكسية.

اوضحت الدراسة ايضا بعد خضوع المستخلصات من كلتا النبتتين لدراسة مخبرية لفحص فعاليتهما بتبيض البشرة، ان النبتتين لديهما فعالية كبيرة ضد تكوين طبقة الميلانين بالبشرة ، وبالتالي اوصي باستخدامهما في مراهم تبيض البشرة.

تم عمل دراسة إحصائية لإيجاد الإرتباط بين فحص تبيض البشرة و الست فحوصات الأخرى ووجد ارتباط لنبتة القريص فقط بينه و بين فحص DPPH، اما بالنسبة لنتبة النتش فهناك ارتباط بينه و بين فحص TFC و فحص ABTS.

تم ايضاً عمل مقارنات بين النتائج من هذه الدراسة مع دراسات سابقة و تبين ان نباتي النتش و القريص لهم اهمية كبيرة جداً لاحتوائهم على نسب جيدة من المواد الفعالة و مضادات الأكسدة التي لها اهمية كبيرة في محاربة العديد من الأمراض و تبين الدراسات ايضاً الفعالية البيولوجية لهذه النتباتات ضد عدة انواع من البكتيريا و الخميرة.