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In-vitro anticancer, antioxidant activities of *Ephedra alata* and *Polygonum bellardii* plants growing wild in Palestine

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In-vitro anticancer, antioxidant activities of *Ephedra alata* and *Polygonum bellardii* plants growing wild in Palestine

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Dedication

This thesis is dedicated to:

The sake of Allah, Our Creator and Master,

The teacher of teachers, Prophet of humanity Mohammad -peace be upon him,

My beloved parents and family, and my future wife, Iman, who never stop giving of themselves in countless ways and for their endless support and encouragements.

Finally this humble work is dedicated for cancer suffering patients, whom I ask Allah to pave the way of their fast and complete healing.

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……

Ayman Abd Al-Majeed Ata Alamarneh

Date: 28 /10 / 2017

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Abstract:

Ephedra alata and *Polygonum bellardii* are among Palestinian medicinal plants that are traditionally used in folkloric medicine for treating many diseases. The goal of this study is to evaluate the antioxidant activity, total phenolic and flavonoids content of different solvent extracts from two plants growing wild in Palestine. Moreover, analysis of their phenolic and flavonoid constituents by using HPLC/PDA and UPLC/MS, and testing their anticancer activity were also investigated. Samples of the two plants were collected from Bethlehem city in March 2016, dried at room temperature under shade and grinded to powder and were extracted with three different solvents namely, water, 80% ethanol, and 99% ethanol at 40^{o} C for about 4 hours with sonication. The crude extracts were then filtered and the solvents were evaporated using rotary evaporator under vacuum and the aqueous extract were freeze dried to dry the crude extract.

The extracts were analyzed for their total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (AA), anticancer activity, as well as phenolic and flavonoids content by UPLC/PDA/MS. The results revealed that the polarity of the extraction solvent affects the TPC, TFC, and AA of extracts. It was found that both TPC and AA are highest for plant extracted with 80% ethanol (TPC is 101.2, 70.4mg/g for *Ephedra alata* and *Polygonum bellardii*, respectively) , followed by 99% ethanol (40.9, 20.3 mg/g) , and finally with water (30.9, 12.1 mg/g). TFC, however, was highest in the following order: 99% ethanol (19.5 *Ephedra alata*, 16.4 *Polygonum bellardii* mg/g) > 80% ethanol (9.8 *Ephedra alata*, 6.1 *Polygonum bellardii* mg/g) > water (4.2 *Ephedra alata*, 2.1 *Polygonum bellardii* mg/g). Pearson correlation indicated that there is a significant correlation between AA and TPC, but there is no correlation between AA and TFC. Simultaneous HPLC-PDA and UHPLC-MS analysis of the ethanolic plant extracts of *Ephedra alata* revealed the presence of Luteolin-7-O-glucuronide flavone, Myricetin 3-rhamnoside and some other major polyphenolic compounds that share myricetin skeleton. The in-vitro anticancer test of *Ephedra alata* and *Polygonum bellardii* extracts revealed that they have an effect on MDA MB231 breast and HCT116 colon cancer cell lines, and the effect of *Ephedra alata* extract is more potent than that of *Polygonum bellardii* extract on both cancer cell lines.

On the basis of these findings, it is concluded that *Ephedra alata* and *Polygonum bellardii* constitute a natural source of potent antioxidants that may prevent many diseases and could be potentially used in food, cosmetics, and pharmaceutical products.

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List of Acronyms

Abbreviation	Description
DNA	Deoxyribo Nucleic Acid
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
MDA	Malondialdehyde
HNE	Hydroxynonrnal
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine)
AA	Antioxidant activity
TPC	Total Phenolic Content
TFC	Total Flavonoids Content
HPLC	High-performance liquid chromatography
UPLC	Ultra High-performance liquid chromatography
DPPH	2,2-diphenyl-1-picrylhydrazyl
NO	Nitric Oxide
UV	Ultraviolet
MS	Mass Spectrometry
ESI/MS	Electrospray ionization mass spectrometry
IC_{50}	half maximal inhibitory concentration
GAE	Gallic acid equivalence
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
UV-Vis spectrophotometer	Ultraviolet – visible spectrophotometer
EtOH	Ethanol
D.W	Deionized water
Ppm	part per million
CEQ	Catechin equivalent
FRAP	Ferric Ion Reducing Antioxidant Power Assay
CUPRAC	Cupric reducing antioxidant capacity
PDA	Photo Diode Array
TAC	Total Antioxidant Capacity

pH	Power of hydrogen
DMSO	Dimethyl sulfoxide
RPMI medium	Roswell Park Memorial Institute medium

CHAPTER ONE
INTRODUCTION

1. Introduction:

1.1. Background

Throughout history, man used various natural materials as a remedy for various diseases. In the past few decades, many natural products were replaced with synthetic drugs that were based on modern chemistry and biotechnology. However, we are recently witnessing a vastly growing and renewed interest in natural medicines in western countries. In particular, the herbal medicine market has distributed and became prosperous in pharmacies and many stores (Eisenberg. D.M et al, 1998).

Recent years have witnessed a renewed interest in plants as an alternative avenue to discover new pharmaceuticals particularly at the 1990s up to now. This interest is driven by both academia and pharmaceutical industry and has led to the espousal of crude extracts of plants for self-medication by the general public. Plants used in traditional medicine; therefore, have an important role to play in the maintenance of health worldwide, and in the introduction of new treatments (Zaid H. et al, 2010).

Plants often contain wide variety of antioxidant molecules, such as phenolic compounds (e.g., phenolic acids, flavonoids, quinones, and tannins), tocopherols, carotenoids and ascorbic acid. These natural antioxidants are distributed in different parts of the plants such as wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen and seeds (Huang WY *et al*, 2008)(WHO, 2008).

Phenolic compounds are plant secondary metabolites possessing aromatic ring with one or more hydroxyl groups. The presence of phenolic compounds enables plants to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Previous epidemiological studies have shown that intake of natural antioxidants is associated with reduced risks of cancer, cardiovascular disease, diabetes and other diseases associated with aging (Cragg, GM *et al*,1997), (Soejarto DD, 1996), (Hamilton AC, 2004).

Some researchers suggest that two-thirds of the world's plant species have medicinal value; in particular, many medicinal plants have great antioxidant potential. Antioxidants reduce the oxidative stress in cells and are therefore useful in the treatment of many human diseases, including cancer, cardiovascular diseases and inflammatory diseases (Eddouks M *et al* 2002), (Ali-Shtayeh *et al*, 2000), (Said O *et al*, 2002).

Cancer is a disease of multicellular organisms characterized by uncontrolled multiplication of subtly modified normal human cells. Cancer is a leading cause of death all over the world and represents a major public health burden. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries and the burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, and physical inactivity (Said O et al, 2002).

There are many types of cancers infect humans like lung, breast, prostate, ovarian, bladder, neck, blood, colorectal, stomach, esophageal, pancreatic, liver, brain cancer, and other neoplasm. Cancer is a complex disease involving numerous temper spatial changes in cell physiology, which ultimately lead to malignant tumors. Abnormal cell growth (neoplasia) is the biological endpoint of the disease. Tumor cell invasion of surrounding tissues and distant organs is the primary cause of morbidity and mortality for most cancer patients. The biological process by which normal cells are transformed into malignant cancer cells has been the subject of a large research effort in the biomedical sciences for many decades. Despite this research effort, cures or long term management strategies for metastatic cancer are as challenging today as they were 44 years ago when President Richard Nixon declared a war on cancer (Anand P. et al, 2008),(Bailar J.C, Gornik H.L, 1997). When cancer spreads to other parts of the body, this is called metastasis. Metastases can occur when cancer cells enter the blood stream or lymph system, these systems circulate all over the body and allow the cells to travel to another part of the body, which increases the seriousness of the disease (Husemann Y. et al, 2008).

There are four main types of cancer:

1) Carcinomas – cancers of the organs.

2) Sarcomas – cancers of the muscles, bone, cartilage, and connective tissue.

3) Lymphomas – cancers of the lymphatic system.

4) Leukemia's – cancers of the blood-making system.

Anticancer activity is the effect of natural and synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression. Several synthetic agents are used to cure the disease but they have their toxicity and hence the research is going on to investigate the plant derived chemotherapeutic agents (Chanda, S. and Nagani, K. , 2013).

Unfortunately the use of Complementary and Alternative medicine "CAM" therapies is still not acknowledged and the research is still slow. CAM therapies need to be evaluated with the same long and careful research process used to evaluate standard treatments. Standard cancer treatments have generally been studied for safety and effectiveness through an intense scientific process that includes clinical trials with large numbers of patients (Blendon RJ *et al*, 2001).

Islamic medicine, or Arab-Islamic medicine, refers to medicine developed in the Golden Age of the Arab-Islamic civilization, which extended from Spain in the west to Central Asia and India in the east.(Zaid H *et at*, 2010)

A lot of work has been going on to revive both traditional herbal medicine and Prophetic medicine in many Arab and Islamic countries. There is, however, a great need to improve on traditional medicine and integrate it with the national medical system. The majority of physicians and pharmacists, trained in Western medicine, lack even basic knowledge of traditional medicine and are therefore hostile towards it (Albar, M., 1996). This negative attitude will not disappear unless the traditional and Prophetic medicine is well understood and used by professional scientists.

In Palestine, traditional remedies are usually part of its culture and religious life, still, however, few herbal products used in folk medicine were investigated and recorded. It has a rich and prestigious heritage of herbal medicines; in addition, more than 700 species of medicinal plant are known to exist, and approximately 63 of these are activity used for the preparation of traditional medicines (Alzeer J. *et al*, 2014).

1.2 Polyphenolic compounds and flavonoids:

Polyphenolic 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. 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 1.1 illustrates the different groups of polyphenols and their chemical structures (Rodríguez *et al*, 2015).

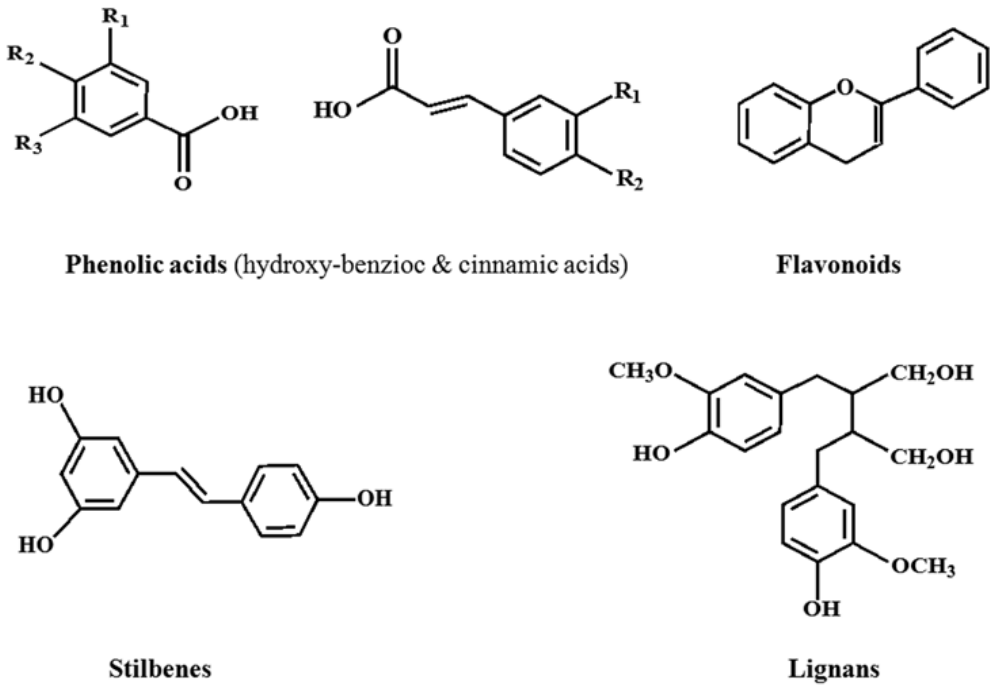


Figure 1.1: Chemical structures of the different classes of polyphenols, where R_1, R_2 and R_3 are H, OH or OCH_3.

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 (Shahidzi & Naczk, 1995). The hydroxycinnamic acids are more common than hydroxybenzoic acids and consist chiefly of *p*-coumaric, caffeic, ferulic and sinapic acids.

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 (Figure1.2). 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.

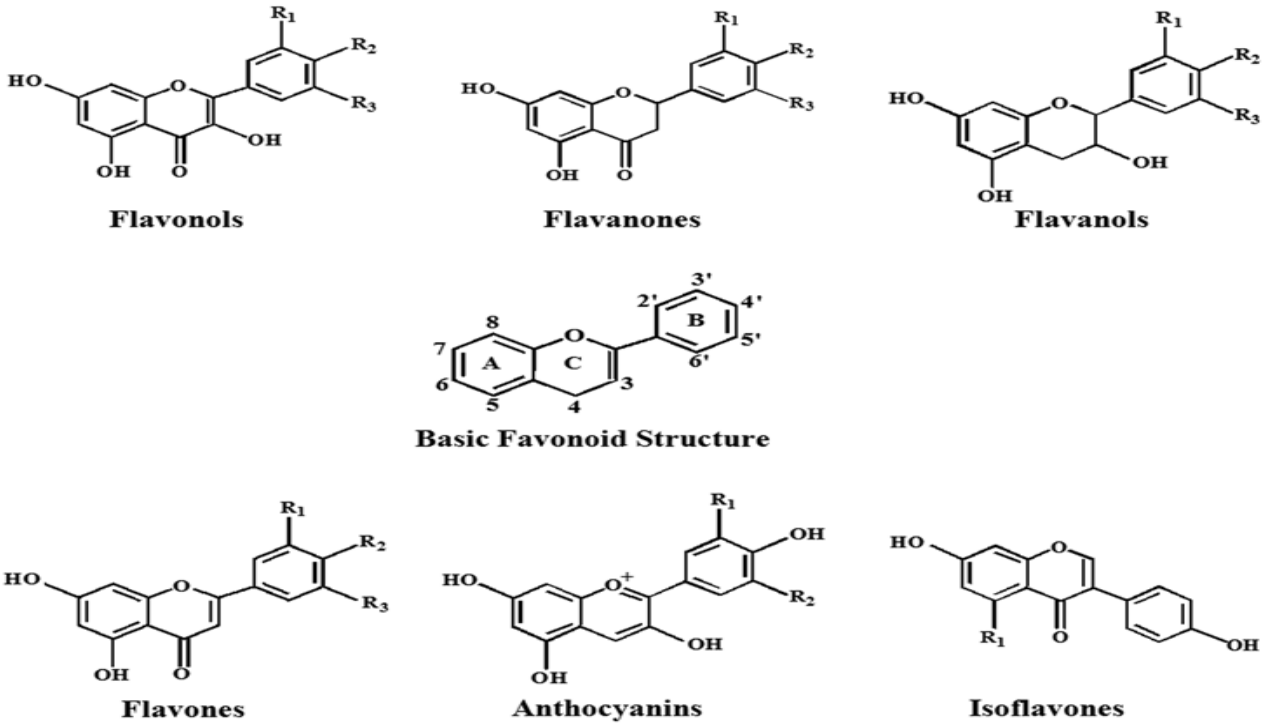


Figure 1.2: Chemical structures of the different classes of flavonoids, where R_1, R_2 and R_3 are H, OH or OCH_3.

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: release and suppression of growth hormones such as auxin, and UV screens to protect against ionizing radiation and to provide coloration (plant pigments), deterrence of herbivores (sensory properties) and microbial infections (phytoalexins), signaling molecules in ripening and other growth processes (Lattanzio *et al*, 2006).

1.3 Overview of the Studied Plants:

Palestine, despite of its small area, it is rich in biodiversity which has not been yet fully investigated. In addition, they have not been yet fully analyzed and used, not only in Palestine, but worldwide. In the current study, the attention has been focused on two plants, namely *Ephedra alata* and *Polygonum bellardi.*

1.3.1 *Ephedra alata* plant

Ephedra alata (Arabic name is Alanda, it belongs to family *Ephedraceae*) is a perennial genus of non flowering seed herb belonging to the Gnetales plant, the closest living relative of the angiosperm (Friedman W, 1996). The native land for this species is Iran, Algeria, Iraq, Chad, Egypt, Palestine, Lebanon, Jordan, Saudi Arabia, Morocco,

Syrian Arab Republic, Libya, Mauritania, Mali, Somalia and Tunisia (Abourashed E. *et al*, 2003). This plant is light green densely branched dioecious small and perennial stiff shrub, about 50-100 cm tall, the twigs appear leafless and the leaves reduced to small scales, cones sessile shaped, clustered in the axils or at branch tips. *Ephedra alata* grows wildly on the gravely rocky, sandy and clay soil in arid environments often near shifting sand dunes (Jaradat N *et al*, 2015).

Figure 1.3: Photo of *Ephedra alata* (Alanda)

The name "*Ephedra*" is from ancient times, dating back to Pliny's description of Equisetum. Three thousand years before Pliny the Chinese realized that species of *Ephedra* had medicinal properties for treating respiratory ailments. *Ephedra* is taken orally to stimulate the body in a manner similar to injected adrenaline. *Ephedra* is now synthetically produced under the name of "ephedrine" and is one of the leading over-the-counter and prescription treatments for allergies, congestion, asthma, etc.

Ephedra alata stem is used in the folk medicine as decoction, as a stimulant, a deobstruent, to treat kidney, bronchi, circular system, digestive system disorders and to relief asthma attack as well as used for treatment of cancer. Also the plant stems are chewed for treatment of bacterial and fungal infections. Nawwar *et al*. (1985); O'Dowd *et al*. (1998).

Although *Ephedra* has many uses, intake of *Ephedra* (or any medicinal plant) should be under medical care, to avoid some expected adverse effects.

1.3.2 Polygonum belladrii

The second wild plant that was investigated is Polygonum bellardi (in Arabic Quddab) (Dardona A, 2016) or Assa Al-ra'e (Sincich F. 2002)). Other names*: Polygonum aviculare* L (USDA, NRCS. 2009), *Polygonum equisetiforme* Sibth (Bedevian, A. K. 1936) It is very similar in shape to *Ephedra alata*, and therefore people sometimes miss recognize the difference between these two plants. Therefore, serious consequences may arise in the case of misuse of the required plant.

Figure 1.4: Photo of *Ephedra alata (Alanda*) (A)
And *Polygonum bellardii* (*Quddab*) (B) From Beit-Jala,

It is a herbaceous glabrous perennial, 50-100 cm, rhizome thickened, stems numerous and branched, prostrate, leaves up to 5x0.5-1.5 cm, deciduous, inflorescences long, terminal, white. The habitat is around waste areas and roadsides. Their distribution is mainly in the Mediterranean area (including Palestine).

In the literature many people used it to treat: diarrhoea, kidney and bladder troubles, blood in the urine (by infuse), vulnerary (by decoction), wounds, brush burns (smoke of green grass when there is no water to prepare a decoction for washing the burn); astringent for wounds (leaves) (Sincich, F. 2002).

Figure 1.5: Photos of *Polygonum bellardii* (Quddab), source: apatite.com website

1.4 Chemical assays of plants extracts:

1.4.1 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 form when oxygen is metabolized or formed in the body and are chemical species that poses an unpaired electron in the outer (valance) shell of the molecule. 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).

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 yield 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).

In the literature, there are as ten distinctly different methods of determining antioxidant activity. We will try different spectrophotometric antioxidant measurement methods; FRAP, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), and CUPRAC (2,4,6-tripyridyl-s-triazine).

1.4.1.a 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, and robust 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) see. 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 1.6.

The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and fast. The FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.

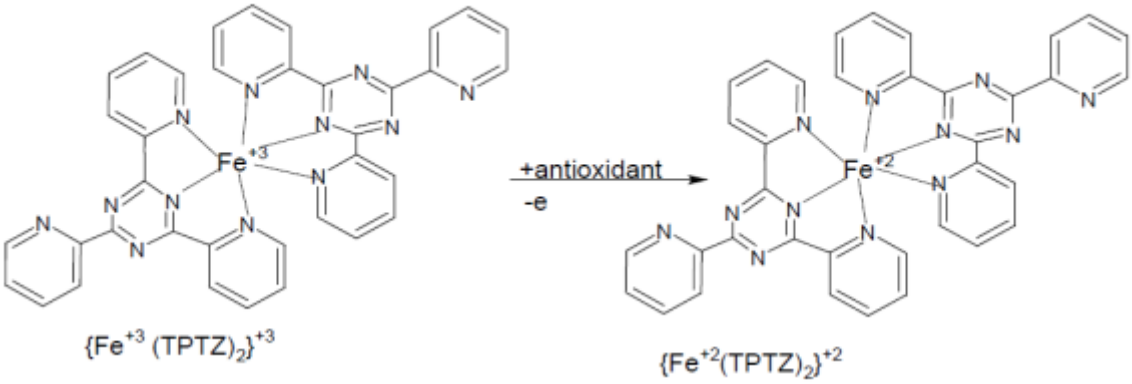


Figure 1.6: Chemical structures of reaction of yellow Fe^{3+} TPTZ complex (2,4,6-tri(2 pyridyl)-1,3,5-triazine) with antioxidants is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances (Prior *et al*, 2005).

1.4.1.b CUPRAC method:

The putative CUPRAC method was developed by (Apak *et al*, 2006). This assays is based on the reduction of Cu^{+2} to Cu^{+} by the combined action of all antioxidants or reducing 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)**.**

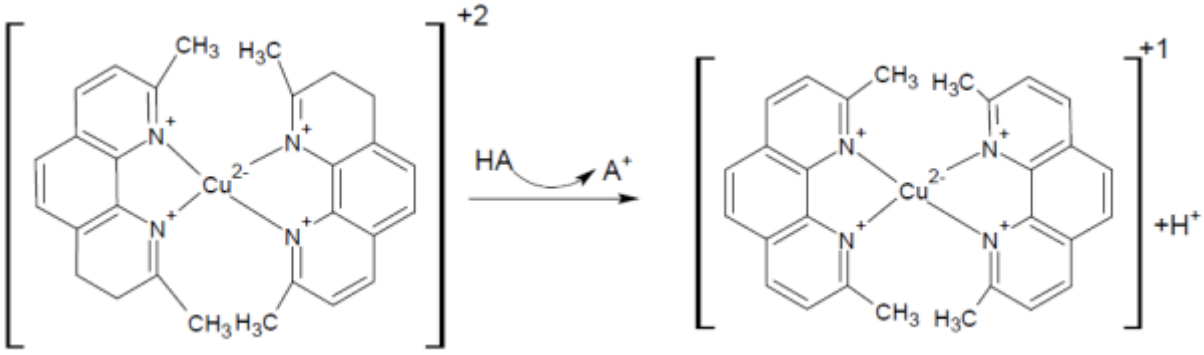


Figure 1.7: CUPRAC reaction by an oxidation molecule (HA: an antioxidant molecule, A^+: an oxidized antioxidant molecule). Protons liberated in the reaction are neutralized by the ammonium acetate buffer (Tütem *et al*, 1991)**.**

1.4.1.c 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 oxidized by potassium persulphate (Re *et al*, 1999) (Thaipong *et al*, 2006). See (Figure 1.8), 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).

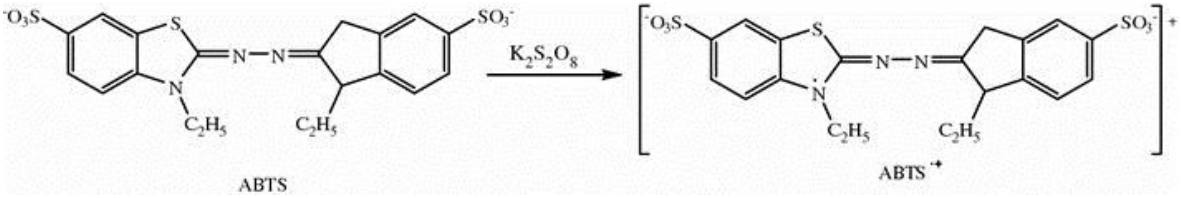


Figure 1.8: Oxidation of ABTS with $K_2S_2O_8$ and generation of ABTS+ (Miller *et al*, 1993)

1.4.1.d DPPH method:

DPPH is really the most straightforward assay. The bleaching of a dark blue-purple methanolic solution of DPPH is measured with a spectrophotometer.

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) as shown in Figure 1.9.

The problem with DPPH is that it tends to take a while to stabilize after the antioxidant is added. Usually, readings are taken up to an hour after mixing. ABTS takes a little preparation because you have to generate the radical with something like $K_2S_2O_8$

(potassium persulfate). The reagent works best if you mix it and let it sit overnight before using it.

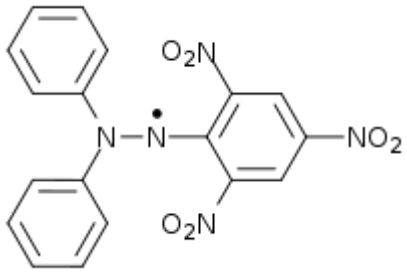


Figure 1.9: Chemical structure of DPPH.

1.5 HPLC analysis of phenolic compounds:

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. HPLC was used for the separation of the crude raw extracts of the two plants. The HPLC is an excellent tool used to separate the nonvolatile compounds. The advantage of utilizing the PDA which has multiple photodiodes (~1000) is to obtain information over a wide range of wavelengths at virtually one time. Spectra are measured at intervals of less than 1 second during separation. Moreover, based on standard HPLC injections, UV-Vis spectral libraries can be created to match spectra generated during separation for identification and predicting coelutions. Reversed phase columns (C18) was used along with water and acetonitrile or methanol as a mobile phase. Buffers could be also used to maintain the pH for weak acids or basic compounds present in the medicinal plant. The photodiode array detector was utilized to extract the maximum wavelength of each separated compound. Standards was the first injected and their retention and spectrum shall be compared to the peaks of interest.

HPLC coupled with mass spectrometry (MS) has commonly been used for structural characterization of phenols. Electrospray ionization (Esi); both positive and negative has been employed for the structural confirmation of phenols in peaches, nectarines, olives, grape seeds, cocoa, olive oil, etc (Naczk & Shahidi, 2004).

1.6 Anti-cancer test at different cancer cells:

Cancer cell line is cancer cells that keep dividing and growing over time under certain conditions in a laboratory. Human cancer-derived cell lines are fundamental models used in laboratories to study the biology of cancer, and to test the therapeutic efficacy of anticancer agents (Sharma SV *et al*, 2010). HeLa was the first cultured cancer line. It was derived from cervical cancer cells taken from Henrietta Lacks in 1951 (Scherer WF *et al*, 1953). Since then, hundreds of cancer cell lines have been established and propagated either in vitro as monolayer cultures or in vivo as xenografts in mice (Mattern J, 1988). No model is perfect and the caveats of these models are well known(Borrell B, 2010) (Weinstein JN, 2012). Therefore, cancer cell lines have been marked by both success and failure (Borrell B, 2010) .

1.7 Objectives of the Study:

This study aims to fulfill the following three main objectives:

- To determine Total Phenolic Compounds (TPC) and total flavonoids content (TFC) of the two plants extracts spectrophotometrically and to analyze their phenolic compounds using HPLC-PDA and UPLC-MS.
- To investigate the differences between the plants in terms of their constituents, total phenolic content, total flavonoid content, and antioxidant activity.
- To evaluate the anticancer activity of both plant extracts using the ethanolic solvent over different cell lines (HCT-116 colon cancer cells and MDA breast cancer cells).

CHAPTER TWO

LITERATURE REVIEW

2.1. Previous studies:

There are limited studies concerning the aforementioned two plants from Palestine, for example, the estimation of total flavonoids, total phenols and antioxidant activity of *Ephedra alata* was investigated by Nidal Jaradat *et al*. This study was designed to evaluate the antioxidant activity, screen the existence of phytogenic chemical compounds and to determine the total flavonoid and phenol contents of the *Ephedra alata* to prove its utilization in the Palestinian folk medicine for treatment of cancer. Total flavonoid contents of the plant were determined by using rutin reference standard method and total phenols determined by using Folin Ciocalteu method while antioxidant activity evaluated by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay. Phytochemical qualitative analyses indicated the presence of cardiac glycosides, reducing sugars, flavonoids, phenolic compounds and alkaloids. The total phenolic content in the ethanolic extract was 19.17 mg gallic acid while the highest in the methanolic extract which 47.62 mg gallic acid equivalent/g of extract powder. The total flavonoid content of the plant was 0.51 mg RU/g in the aqueous extract and 5.44 mg RU/g in the ethanolic extract while the highest in the methanolic extract was 54.66 mg RU/g.

Recently, another study was reported on the assessment of antimicrobial activity of flavonoids extract from *Ephedra alata* by (Elyacout Chebouat *et al*, 2014). The study of the antibacterial activity of *Ephedra alata* extract was carried out on Gram positive and Gram negative pathogenic bacteria. The results exhibited variable of microorganisms for different concentrations of flavonoid extracts. The activity was associated with high concentration. Using plate methods, the extracts of *Ephedra* alata displayed relatively important effects with a variable diameter of growth inhibition zones in most types of bacteria. However no effect was recorded against Serratiamarcescens ATCC 13880 with butanol extracts of flowers and leaves and ethyl acetate and dichloromethane extracts of leaves as well as with butanol, ethyl acetate, and dichloromethane extracts of leaves against Enterococcus faecalis ATCC 29212.

Yoshiaki Amakura *et al*, 2013 worked on the characterization of phenolic Constituents from *Ephedra* Herb Extract. Nine known compounds were isolated from a Ephedra herb extract. Six of them are known and one new flavonoid glycoside was characterized as herbacetin 7-*O*-neohesperidoside.

Recently, Al-Quds University, Makhamra, S, and Abu-Lafi, S., 2016, worked on *Ephedra alata* and other plants. They measured the anticancer effect of it and the results was as follow: *Ephedra alata* plant methanol extract is prepared to disclose the effectiveness of inhibit growth of tumors. The human liver cancer cell-line (HepG2)_was exposed to *Ephedra* alata crude methanol extract (0-800 µg/mL) for 24 h, and cytotoxicity was determined using the MTT assays. The EC50 values obtained by the MTT assay was 25 ± 1.2 µg/ml.

Polygonum bellardi was studied by Nafady, A *et al*. 2013. They isolated five compounds from the methanolic extract of the aerial parts of *Polygonum bellardii* growing in Egypt. The total extract and its fractions showed significant antioxidant potential by $DPPH^{\cdot}$ scavenging activity technique.

Another research is recently done by El-Toumy, S. *et. al.* 2016. They worked on the isolation and identification of secondary metabolites from *Polygonum equisetiforme* and evaluation of antioxidant activity of its extract using DPPH method. The extract showed significant antioxidant activity (IC50 = 37.45 µg/ml). The total phenolic and flavonoid content was found to be 130.79±5.502 and 45.8±1.63 µg/ml, respectively.

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:** almost 99% ethanol (it's not critical for our experiment the presence of some water in the absolute 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,

Standard phenols and flavonoids used (in HPLC experiment): 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.

For the anticancer activity test, chemicals and biochemical materials used were: Human HCT-116 colon cancer cells and MDAmB231gfp breast cancer cells.
Cultured on 6 well plates using RPMI media (10% FBS, 1% glutamin, 1% pen/strep).
Incubated at 37^{0}C, 5% CO_2, for 24 hrs.

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

- 5% $NaNO_2$ was prepared by dissolving 5g of $NaNO_2$ in 100ml of water.
- 10% $AlCl_3$ was prepared by dissolving 10g of $AlCl_3$ in 100ml of water.
- 7.5% Na_2CO_3 was prepared by dissolving 7.5g of Na_2CO_3 in 100ml of water.
- DPPH (0.1mM, Mwt= 394.32 g/mol) was prepared by dissolving 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 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 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 water.

3.1.3 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, sonicator, refrigerator, HPLC (Waters, Alliance c2695 module) with PDA detector, and UPLC_MS (Thermo Fisher scientific), laboratory water bath, ultrasonic homogenizer, autoclave, labofuge 200.

3.1.4. Plant materials

3.1.4.1 Plant materials, collection and drying:

The stems of *Ephedra alata* plant and *Polygonum bellardi* were collected from Bethlehem in March 2016, and shade dried at room temperature for two weeks. The plant species is properly authenticated by Professor Khalid Sawalha, the director of biodiversity research laboratory, Al-Quds University.

3.1.4.2 Grinding of the plant materials

The Dried samples of the plants were grinded into fine powders using simple blender.

3.1.4.3 Extraction of plant materials

3.1.4.3.a By Sonication

10 g of the powdered plant materials were extracted with three solvents (D.W, 80% EtOH, and 99% EtOH), with sonication at 40^{o}C for 3 hours, and then left overnight until extraction process is complete. Then the solutions were be filtered (Whatman filter paper).

3.1.4.3.b Isolation of the sonicated Aqueous Extract By Centrifuge:

After sonication, aqueous extract become viscous and hard to be filtered (by filter paper nor by vacuum). We used centrifuge machine at speed of 5300 r/m for four minutes to isolate the aqueous phase from the solid particles of Ephedra alata.

The aqueous layer was withdrawn by a pipette and the clear solution was kept in the refrigerator for the next extraction steps.

3.1.4.5.d Evaporation of the solvents, and freeze-drying:

The solvents were evaporated under reduced pressure using rotary evaporator at 50 ^{0}C. The aqueous extracts were lyophilized to get rid of residual water remained in the extracts. The crude extract were stored in the fridge at 4^{o}C for further analysis and tests.

3.2. Methodology

3.2.1 Total phenolic content (Folin–Ciocalteu assay)

Total phenolic content 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).

3.2.2. 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.

3.2.3. 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.

3.2.4 Cupric reducing antioxidant capacity (CUPRAC) assay

The assay was conducted as described previously Resat *et al*, 2004. To 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 neocaproin 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.

3.2.5 Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of extracts of leaves of *Ephedra alata* and *Polygonum bellardii* 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 D.W. The mixture was shaken vigorously and allowed to stand at room temp for 30 min. then, absorbance was 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 following equation:

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.

3.2.6 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 0.9 mL of $ABTS^+$ Solution and 0.1 mL 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 HPLC analysis of phenolic compounds

3.3.1 HPLC Instrumentation systems

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

3.3.2 HPLC and UHPLC Instrumentation Systems

The analytical HPLC is Waters Alliance (e2695 separations module), equipped with 2998 Photo diode Array (PDA). Data acquisition and control were carried out using Empower 3 chromatography data software (Waters, Germany). The chromatography was performed under reverse phase conditions using a TSQ Quantum Access MAX (Thermo Scientific, San Jose, CA, USA) which includes a Dionex Pump with degasser module, an Accela PDA detector and an Accela Autosampler.

3.3.3 Chromatographic Conditions

The HPLC analytical experiments of the crude water, 80% ethanol and 99% 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 nm. 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.

The UHPLC chromatographic separations were performed on a Kinetex™ (Phenomenex, Torrance, CA, USA) column (C8, 2.6 μm particle size, 100 Å pore size, 100 x 2.1 mm), protected by a UHPLC SecurityGuard™ (Phenomenex, Torrance, CA, USA) cartridge (C8, for 2.1 mm ID column). The injection volume was 10 μL, the oven temperature was maintained at 35°C. The chromatographic separation was achieved using the same HPLC linear gradient program using formic acid instead of acetic acid at a constant flow rate of 0.4 mL/min over a total run time of 70 min. The samples were detected by a TSQ Quantum Access Max mass spectrometer in positive ion mode using Electron Spray ionization (ESI) and full scan acquisition. Air was produced (SF 2 FF compressor, Atlas Copco, Belgium). Purified nitrogen was used as source and exhaust gases. Samples of the crude extracts were prepared at a concentration of 5 mg/ml by dissolving 50 mg of crude extract in 10 ml of respective solvent (water, 80% ethanol, or 99% ethanol).

3.3.4 Sample preparation for HPLC analysis

The crude extracts were dissolved in the respective solvents (water,99% 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.

3.4 Evaluation of anticancer activity

The plant extracts were tested using different cancer cell lines. The procedure was as follow: The specific media for cell and trypsin solution was withdrawn from freezer, then put in water bath at 37^0C. The old media was aspirated from the plate by pump (in sterilized condition on hood). 1ml of trypsin was put in the plate by pipette for few seconds. Then trypsin solution in the plate was aspirated before the cells move, then it was mixed by hand & left right. Afterwards it was incubated in CO_2 incubator at 37^0C for 3-5 min. After cells move, 2.5 ml of media was added to the plate (tilted at 45^0) then mixed well by pipette. 0.5 ml of the mixture was added to new 10 ml plate & 8ml of media was added. Then it was checked under microscope and put in CO_2 incubator at 37^0C for 3-4 days. Plant extracts were diluted with DMSO (5mg/1ml) and tested against cancer cell lines. 40-50 % ≈ confluent of cells were seeded in 6 cm plate before 24h of treatment. 100 mg of powder extract were dissolved in 1 ml of absolute ethanol (Stock concentration 100mg/ml) 100µg/ml , 330µg/ml and 1000µg/ml of extract were used as final concentration to treat the cells for 48h

The cells was counted under microscope.

3.5 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

4.1 HPLC-PDA Profiles of *Ephedra* alata Extracts

To enrich the active ingredients present in dried *Ephedra alata*, two extractions methods were adopted and the extract components were directly examined on HPLC-PDA at different wavelengths. The first extract method is based on soaking the stems of *Ephedra* alata separately in water, 80% ethanol and in 99% ethanol. The second technique however utilized sonication of the same weight of the herb stems using the same solvent volume for three hours. Eighteen phenolic and flavonoid standards mixture 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. It was noticed that extraction by sonication is much more efficient in comparison to typical infusion procedure (Figure 4.1).

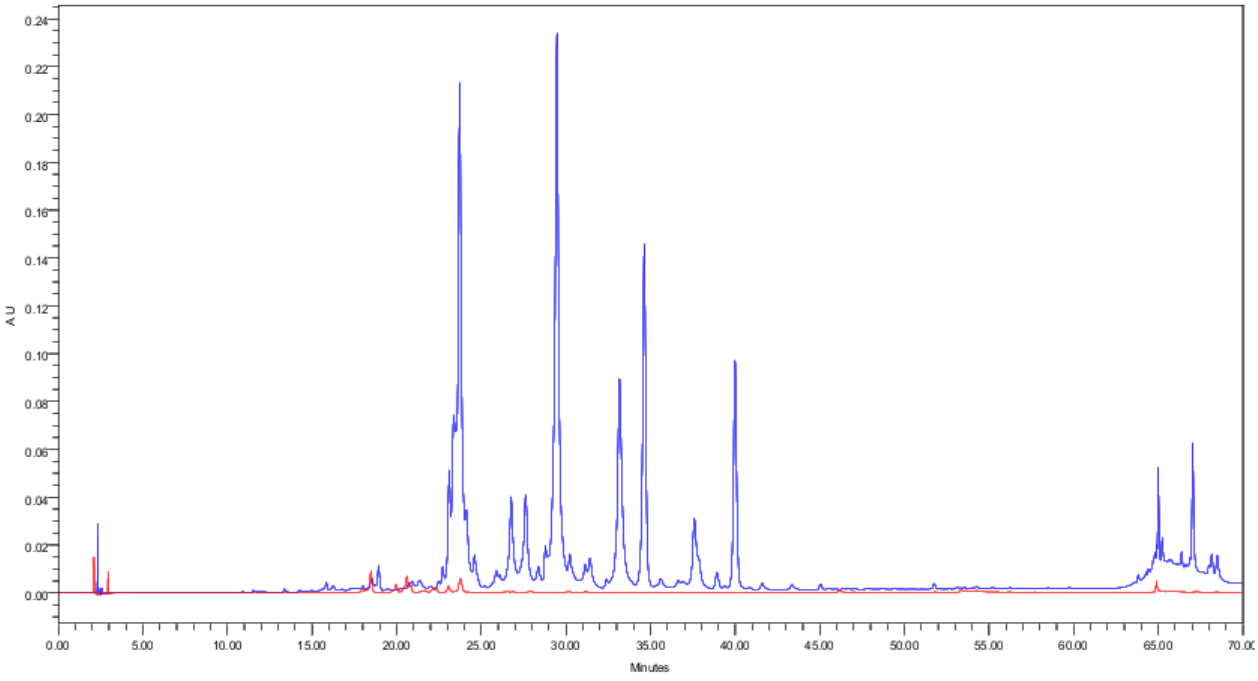


Figure 4.1: Overlaid HPLC-PDA chromatograms of crude 99% ethanol *Ephedra* alata extract using infusion (red) and sonication (blue) methods at 350 nm.

Moreover, the solvents extraction power using sonication was in the order of 99% ethanol, 80% ethanol and water respectively. Figure 2 showed overlaid chromatograms of the three crude extracts at 350 nm. This wavelength was selected since the main *Ephedra* alata peaks showed a maximum absorption close to it.

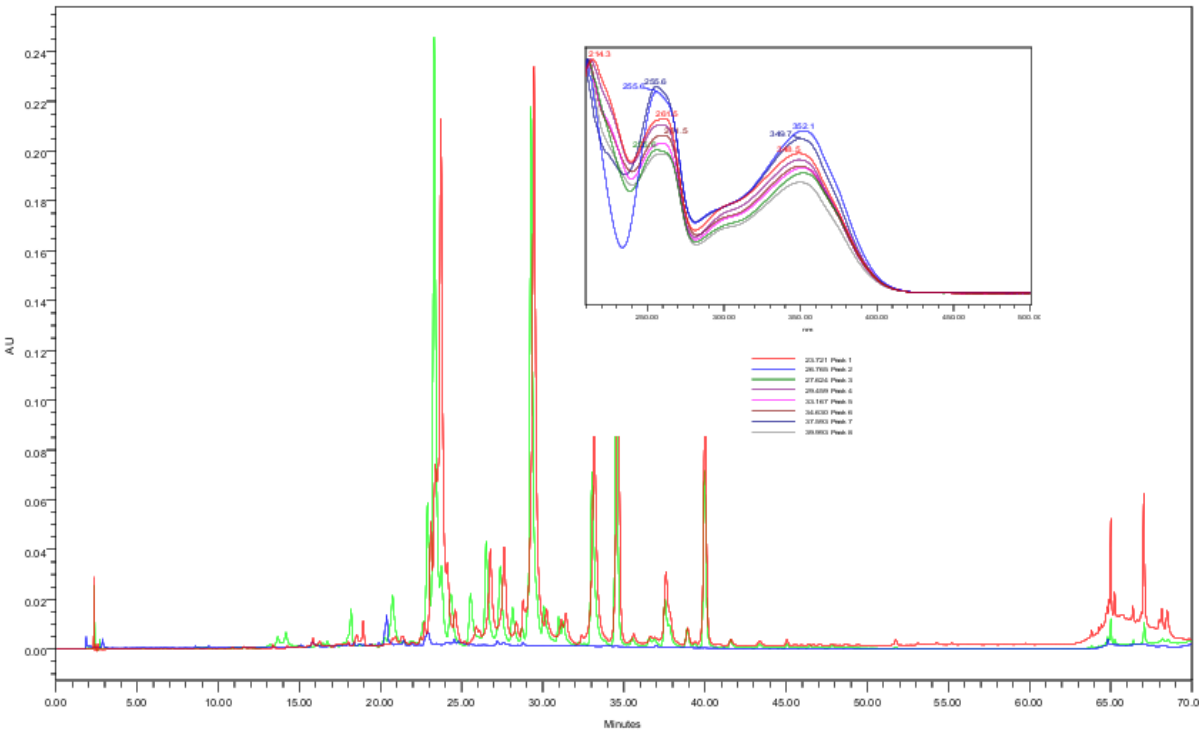


Figure 4.2: Overlaid HPLC-PDA chromatograms of crude water (blue), 80% ethanol (green) and 99% ethanol (red) extracts of *Ephedra* alata at 350 nm. The overlaid UV-Vis spectra of the main peaks are depicted at the right corner.

When the monitoring wavelength was set at two channels of 442 nm or 472 nm, the 99% ethanol extract showed few lipophilic peaks that eluted late between 64-68 minutes. These peaks were less pronounced in 80% ethanol and did not exist in the water extract (Figure 4.3).

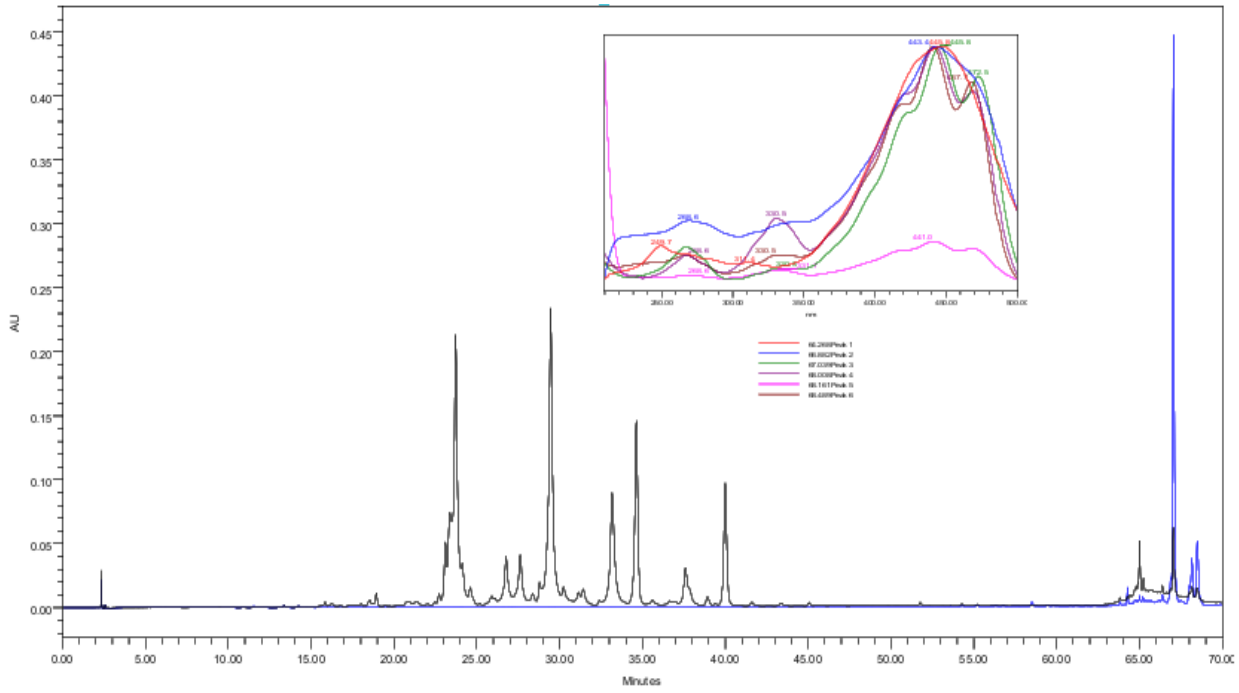


Figure 4.3: Overlaid HPLC-PDA chromatograms of *Ephedra* alata crude 99% ethanol at 350 nm (black) and 472 nm (blue). The overlaid UV-Vis spectra of the main peaks eluted later between 64-68 minutes are depicted at the right corner.

Figure 4.4 portrays the phenolic and flavonoids standards mixture and *Ephera alata* 99% ethanol extract at two channels of 272 nm (A) and 350 nm (B) respectively. As in figure 4, most of the compounds seen in the 99% ethanol extract does not match any of the standards injected as seen from their retention and UV-Vis spectra. However, almost all the main peaks shared maximum wavelengths of 348.5 nm-352.1 nm. These types of compounds are very close to isomeric flavonoid glycosides.

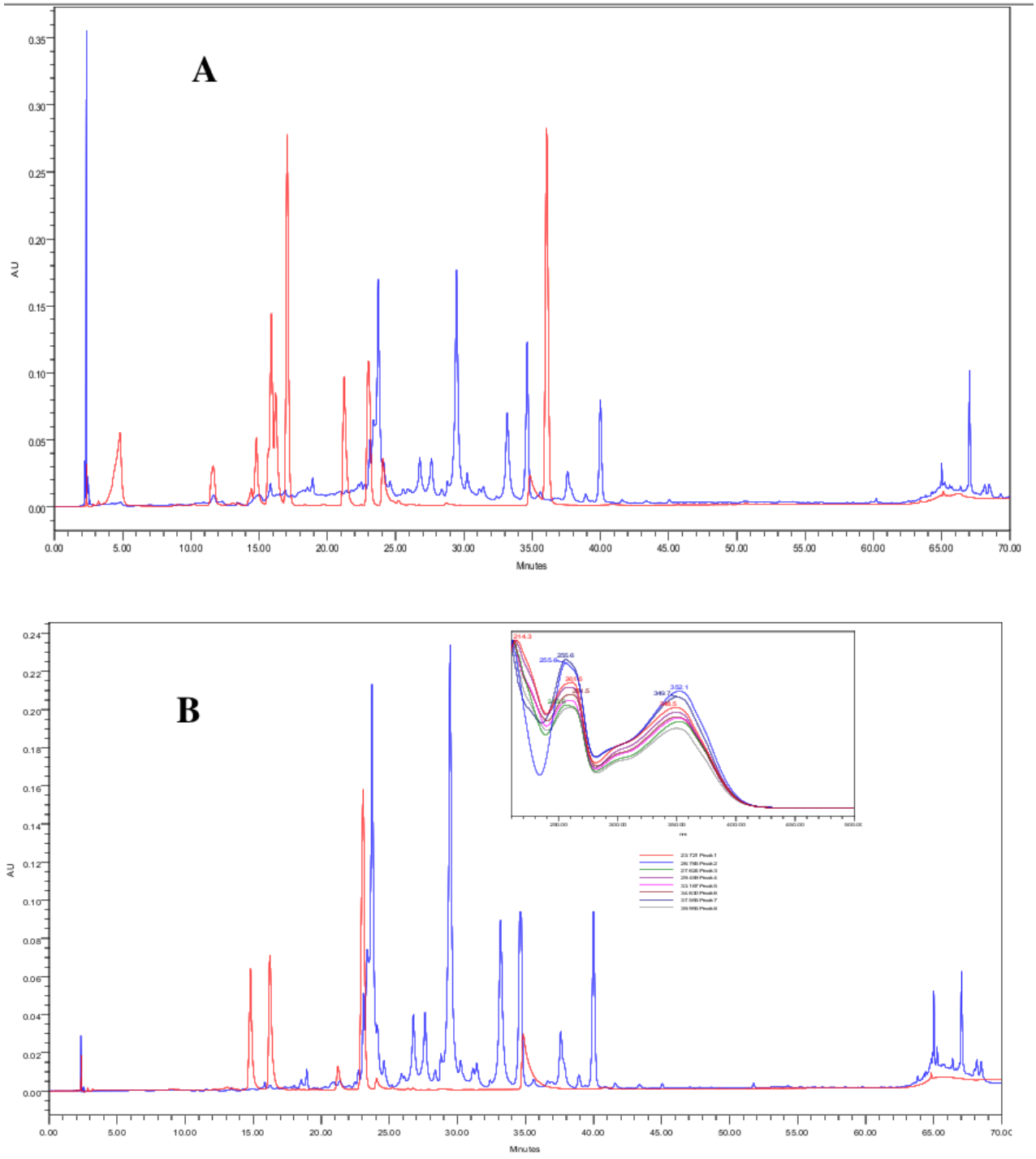
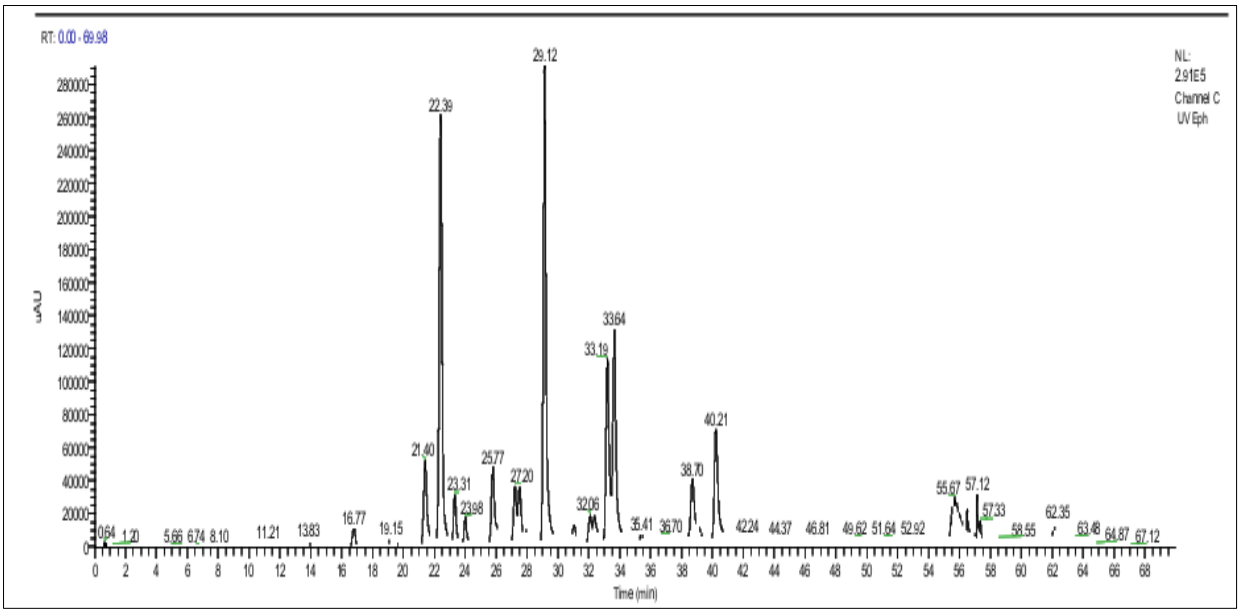


Figure 4.4: Overlaid chromatograms of phenolic and flavonoids standards mixture and 99% ethanol extract of *Ephera alata* at 272 nm (A) and 350 nm (B) respectively. The overlaid UV-Vis spectra of the main peaks are depicted at the right corner of chromatogram (B).

The full scanned LC-MS using the positive and negative electrospray ionization modes revealed the presence of Luteolin-7-O-glucuronide flavonoid (molecular ion $[M+H]^+$ at m/z of 463.27 Da) at 22.39 minutes with a fragment ion at 319.37 Da signifying to the myricetin antioxidant skeleton (figure 4.5). The peak at 23.98 minutes showed a

deprotonated molecular ion $[M-H]^-$ of 463.22 Da presumably indicating Myricetin 3-rhamnoside existence. Other major peaks appeared at retention of 29.12 minutes showed molecular ion $[M-H]^-$ at m/z of 505.33 Da. Ranged peaks from 33.19 to 33.64 minutes showed peaks at 557.73, 533.26 and 477.30 Da suggesting flavonoid-like structures with myricetin fragment backbone. Another peak at a retention time of 40.21 minutes showed a deprotonated peak molecular ion $[M-H]^-$ at 519.38 Da. The UHPLC-MS spectra for the latter few compounds were not sufficient to be deconvoluted. However, preparative HPLC collection of pure flavonoids from *Ephedra* plant along with NMR experiments would assist in the exact determination of their structure.

It is evident that all the major peaks showed a fragment at m/z of 319 Da which indicates that the isomeric flavonoids shared myricetin polyphenolic flavonoid compound which is known for its antioxidant and anticancer activities.



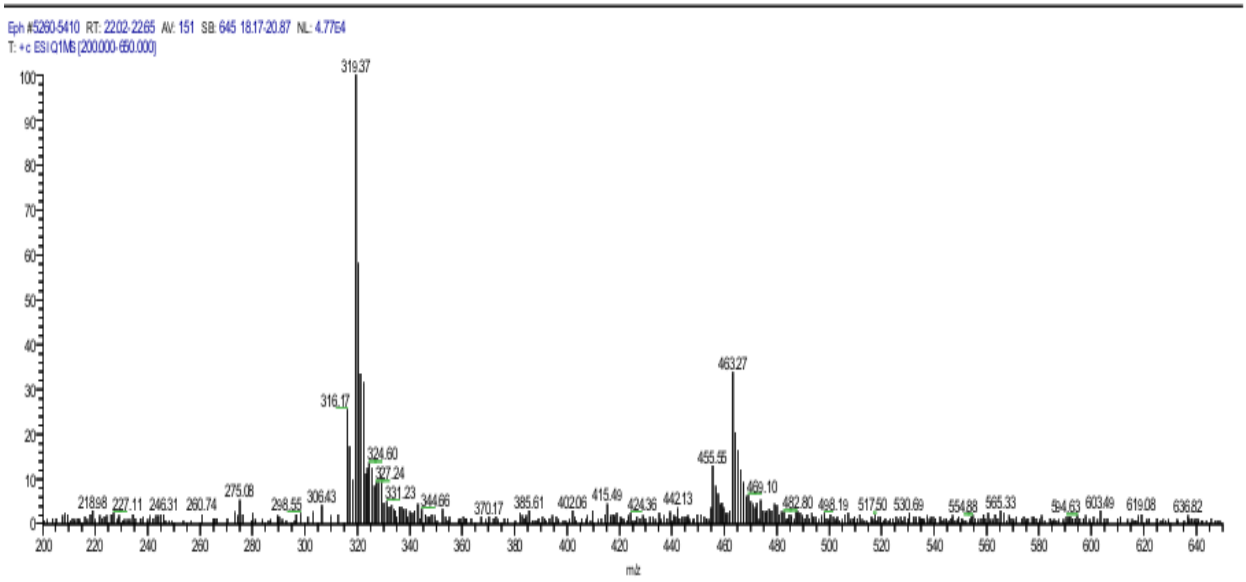


Figure 4.5: UHPLC of the *Ephedra* alata ethanol extract (A) and the (+)-ESI mass spectrum of Luteolin-7-O-glucuronide flavonoid.

4.1.2 HPLC-PDA Profiles *of Polygonum bellardii* Extracts

Six samples of *Polygonum bellardii* extracts were prepared. Three solvents were used, namely 99% ethanol, 80% ethanol and water. These solvents were used on the stem and leaves of the plant. The 80% ethanol leaves extracts was only one to give reasonable results. This may advocate that the active phenolic compounds exist in the leaves of the plant.

Injecting the 80% ethanol extract to the HPLC-PDA gave the many compounds as shown in figure 4.6

Figure 4.6: Overlaid HPLC-PDA chromatograms, 80% ethanol extract of *Polygonum bellardii* at 270 nm. The overlaid UV-Vis spectra of the main peaks are depicted at the right corner.

Many of the separated compounds are sharing the same λ maximum, as shown if figures 4.6 and 4.7.

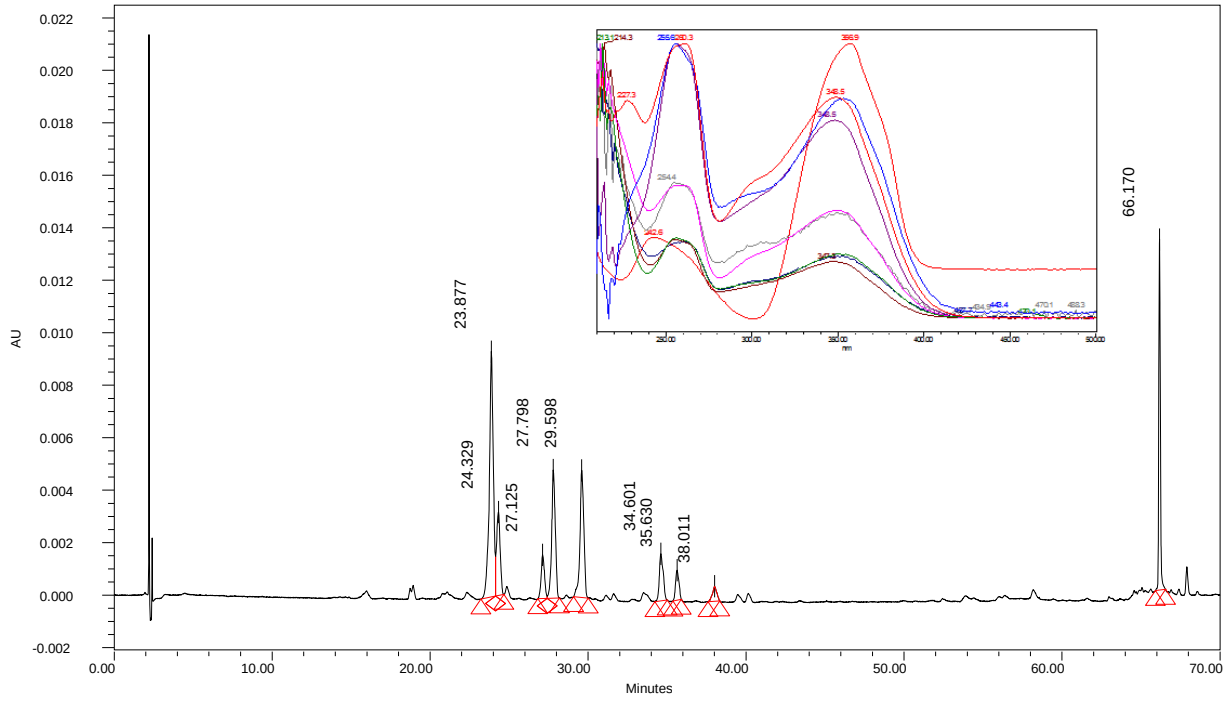


Figure 4.7: Overlaid HPLC-PDA chromatograms, 80% ethanol extract of *Polygonum bellardii* at 360 nm. The overlaid UV-Vis spectra of the main peaks are depicted at the right corner.

4.2 Total Phenolic contents, Total Flavonoid Content, and antioxidant activity :

4.2.1 *Ephedra alata*:

4.2.1.1 Total Phenolic contents

TPC of *Ephedra alata* plant extracts using three different solvents is shown in Table 4.1. As it is obvious from this table, the extraction solvent has an effect on the TPC of the *Ephedra* extracts where significant differences ($p < 0.05$) between the TPC of the three extracts are indicated by different small letters (a, b, and c). The highest TPC was found for the plant material when extracted with 80% ethanol (101.2 ± 0.9 mg/g), followed by plant material extracted with 99% ethanol (40.9 ± 0.2 mg/g) and finally with water (30.9 ± 0.5 mg/g). These results show that TPC were only 40% and 30% when the plant material was extracted by 99% ethanol and distilled water respectively as compared with the TPC extracted with 80% ethanol indicating the higher solubility of the phenolic compounds in 80% ethanol.

The results showed that *Ephedra* plant investigated in this study are richer with phenolic compounds (101.2 mg/g using the best extraction solvent) than that of Guava and Plum

fruits reported earlier (1.26-2.47mg/g in guava, 1.25-3.73 mg/g in plums) (Thaipong *et al*. (2006)). As plant phenolics have multifunctional properties and can act as singlet oxygen quenchers and scavenge free radicals, the presence of substantial amounts of these compounds in Palestinian *Ephedra* promotes the latter as an important source of antioxidants which if properly consumed may reduce risk associated with degenerative diseases and provide health promoting advantage.

It is interesting to compare TPC of Palestinian *Ephedra* with *Ephedra* from other countries. For example, *Ephedra alata* from Jordan was analyzed for TPC and was found to have 16.2 and 11.9 mg GA/g for aqueous and methanolic extracts, respectively (Alali et al. (2007)) which is lower than TPC of *Ephedra alata* investigated in this study.

Table 4.1: Total phenolic content (TPC as mg Gallic acid/g DW[*]), total flavonoids contents (TFC as mg catechin/g DW), FRAP (mmol Fe^{+2}/g DW), CUPRAC (µmol Trolox/g DW), DPPH (µmol Trolox/g DW), ABTS (µmol Trolox/g DW), DPPH % inhibition, and ABTS % inhibition of *Ephedra Alata* plant extracted with water, 80% ethanol, and 99% ethanol.

	TPC[**] (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)	ABTS (µmol/g)	DPPH % inhibition	ABTS % inhibition
Water	30.9^{c} ± 0.5	4.2^{c} ± 0.10	7.1^{c} ± 0.1	2182^{c} ± 25	305.7^{c} ± 3.4	40.5^{c} ± 1.0	88.7^{c} ± 0.5	81.9^{c} ± 0.6
Ethanol (80 %)	101.2^{a} ± 0.9	9.8^{b} ± 0.1	21.3^{a} ± 0.4	6442^{a} ± 52	482.5^{a} ± 1.7	66.0^{a} ± 1.5	95.3^{a} ± 0.6	91.0^{a} ± 0.6
Ethanol (100 %)	40.9^{b} ± 0.2	19.5^{a} ± 0.3	11.1^{b} ± 0.2	3272^{b} ± 30	351.7^{b} ± 1.2	47.5^{b} ± 1.0	91.5^{b} ± 0.6	87.0^{b} ± 0.3

[*] DW: Dry weight

[**] Results are expressed as average of three samples of *Ephedra Alata* shoots. Different small letters within column indicate significant difference ($p < 0.05$, $n = 3$).

4.2.1.2 Total Flavonoid Content:

The results of ferric chloride colorimetric test for determining flavonoids content are presented in Table 4.1. The same statistical analyses as for TPC were performed for total flavonoids content (TFC), and the results (Table 4.1) showed that significant differences between total flavonoids content of the plant materials extracted with the three solvents were obtained, where significant differences ($p < 0.05$) indicated by small letters (a, b, and c). The highest TFC was found for the plant material when extracted with 99% ethanol (19.5 ±0.4 mg/g) which is two times significantly higher than that extracted with 80%

ethanol (9.8 ±0.1 mg/g) and the later was two times significantly higher than the TFC extracted with water (4.2 ±0.1 mg/g). Comparing the trend of solvent effect on TFC and TPC, there is a difference in the two trends where the highest content of TPC was obtained when the plant was extracted with 80% ethanol while the TFC was obtained when the plant material was extracted with 99% ethanol. This can be attributed to the polarity of the extraction solvent and the flavonoids, where flavonoids need less polar solvent (or higher amount of ethanol e.g. 99% ethanol). Apparently, mixed solvents of intermediate polarities (99% or 80% ethanol) are the most suitable extracting solvents for recovering the highest amounts of phenolic and flavonoid compounds which have both polar and nonpolar functional groups.

These results demonstrated that the Ephedra plant extracts are rich with flavonoids (range: 4.2-19.5 mg/g). Comparing TFC of Ephedra plant analyzed in this study with Ephedra from other countries revealed that the Ephedra grown in Palestine is richer with flavonoids, for example according to the study of Harisaranraj et al. (2009) of Ephedra vulgaris from India, total flavonoids was found to be 1.48±0.20 mg/100 g (Harisaranraj et al. (2009)).

4.2.1.3 Antioxidant Activity (AA):

Evaluation of AA is becoming increasingly relevant in the field of nutrition as it provides useful information with regard to health promoting and functional quality of raw materials whether they are fruits, vegetables, or medicinal plants (Scalfi et al. (2000)). This parameter accounts for the presence of efficient oxygen radical scavengers, such as phenolic compounds. The antioxidant activity of phenolics is mainly due to their redox properties, which make them acting as reducing agents, hydrogen donors, and singlet oxygen quenchers.

There are two types of antioxidant assays used to evaluate the antioxidant activity of plant extracts. The first category measures the potential of plant extracts to reduce ions or oxidants (to act as reducing agents) like ferric ion, cupric ion. The main two assays of this antioxidant activity category are FRAP (measures the reduction potential of ferric to ferrous ion), and CUPRAC (measures the reduction of cupric to cuprous ion). The second category of antioxidant activity measures the ability of plant extracts to scavenge free radicals. DPPH and ABTS assays (where DPPH and ABTS are stable free radicals) are the two main examples of this category. These assays are used because they are quick and

simple to perform, and reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present.

4.2.1.3.a FRAP assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe3+–TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe2+–TPTZ). The reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. The reduction of Fe3+–TPTZ complex to blue-colored Fe2+–TPTZ occurs at low pH.

The antioxidant test based on FRAP assay of Ephedra plant extracts using three different solvents are presented in Table 4.1 (expressed as mmol Fe+2/g of dry plant material). Statistical analyses showed that there are significant differences between total flavonoids content as a function of extraction solvent (Table 4.1), where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Table 4.1 revealed that antioxidant activity (FRAP) of the Ephedra plant increased as the polarity of solvent changes (80% ethanol > 99% ethanol > water), where FRAP values were found to be about two and three times significantly higher when extracted with 80% ethanol compared to 99% ethanol and water, respectively.

The trend of extraction solvent on the FRAP values was found to be the same as for TPC but different from TFC. This suggest that there is a correlation between AA (expressed as FRAP) and TPC, reflecting the fact that total phenolics are the major determinant of AA. Pearson correlation revealed that FRAP (as well as other antioxidant activities under study) were highly and significantly correlated to TPC but were not correlated with TFC, see Table 4.2.

As in the case of TPC and TFC, ethanol (99% or 80%) gives higher amounts of AA (FRAP) compared with water as extraction solvent of Ephedra plant.

Table 4.2: Pearson coefficients between quality indices (TPC, TFC, FRAP, CUPRAC, and DPPH).

	TPC (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)
TPC (mg/g)					
TFC (mg/g)	-0.022				
FRAP (mmol/g)	0.989***	0.1269			
CUPRAC (µmol/g)	0.993***	0.0937	0.999***		
DPPH (µmol/g)	0.992***	0.0984	0.999***	0.999***	
ABTS (µmol/g)	0.989***	0.125	0.999***	0.999***	0.999***

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, n = 9.

4.2.1.3.b CUPRAC Assay

Although FRAP antioxidant assay has been very popular among researchers, CUPRAC assay is a relatively new assay developed by Apak et al. (2006). It utilizes the copper(II)–neocuproine [Cu(II)–Nc] reagent as the chromogenic oxidizing agent and is based on the cupric reducing ability of reducing compounds to cuprous.

Table 4.1 shows the CUPRAC antioxidant activity (expressed as µmole Trolox/g) of Ephedra plant extracts using three different solvents. Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that CUPRAC antioxidant activity of the Ephedra plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as FRAP antioxidant activity, and TPC but different from TFC, which suggests that there is a correlation between CUPRAC AA and TPC. Pearson correlation confirms the correlation between CUPRAC antioxidant activity and total phenolic content but no correlation with total flavonoids content (TFC), see Table 4.2.

4.2.1.3.c Free Radical Scavenging Ability of Plant Extracts DPPH Assay

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Sakanaka et al. (2005)). It is a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character (Naik et al. (2003)). The color changed from purple to yellow and the absorbance at wavelength 517 nm decreased.

DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenolics. The bleaching of DPPH solution increases linearly with increasing amount of extract in a given volume.

Table 4.1 shows the percentage of inhibition of DPPH free radicals by the Ephedra plant extracted with the three solvents. Statistical analyses showed that there are significant differences between percentage of inhibitions using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c), see Table 4.1.

Table 4.3 shows the percentage of inhibition of DPPH at different concentrations of the crude extract (from 10 to 150 µg/mL). This data shows that the extracts exhibited a dose dependent scavenging activity (a linear relationship between percentage of DPPH inhibition and concentration ($y = 0.594x + 2.216$, with R2 of 0.997), where y is the percentage of inhibition and x is the concentration). From this linear relationship, IC50 which is the concentration required to quench 50% of the DPPH free radicals was determined and was found to be 78 µg/mL. DPPH antioxidant activity of Ephedra plant extracts using three different solvents was expressed as µmole Trolox/g (Table 4.1). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c)(Table4.1) .

Results showed that DPPH antioxidant activity of the Ephedra plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as TPC, FRAP, and CUPRAC antioxidant activity. Correlation studies showed a significant correlation between DPPH and TPC but not with TFC (Table 4.2).

Table 4.3: % inhibition of DPPH and ABTS free radicals by different concentrations of *Ephedra Alata* plant extract.

Concentration of DPPH (µg/mL)	% inhibition of DPPH *	Concentration of ABTS (µg/mL)	% inhibition of ABTS *
10	7.1 ± 0.5	10	9.2 ± 0.3
20	13.6 ± 1.2	20	18.9 ± 0.7
40	26.2 ± 1.0	40	37.1 ± 0.9
80	53.6 ± 1.4	80	66.0 ± 1.1
150	94 ± 2.1	100	88.4 ± 1.5

*Results are expressed as Average ± standard deviation of three samples.

4.2.1.3.d ABTS Assay

The ABTS assay measures the relative antioxidant ability of extracts to scavenge the radical-cation ABTS+. produced by the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate.

Table 4.1 shows the % inhibition of ABTS free radicals by the plant extracted with the three solvents. Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c), see Table 4.1.

Table 4.2 shows the % inhibition of ABTS at different concentrations of the crude extract (from 10 to 100 µg/mL). This data shows that the extracts showed a dose dependent scavenging activity (a linear relationship between % of ABTS inhibition and concentration ($y = 0.849x + 1.47$, with R2 of 0.995), where y is the % of inhibition and x is the concentration). From this linear relationship, IC50 was determined and was found to be about 57 µg/mL.

ABTS antioxidant activity of *Ephedra* plant extracts using three different solvents was expressed as µmol Trolox/g (Table 4.1). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that ABTS antioxidant activity of the *Ephedra* plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as FRAP, CUPRAC, and DPPH antioxidant activities. Additionally, this trend is the same as TPC but different from TFC, which suggests that there is a correlation between ABTS and TPC. As it is obvious from Table 4.2, there is a correlation between ABTS and TPC but not with TFC.

It is interesting to compare AA (ABTS) of Palestinian *Ephedra* with *Ephedra* from other countries. For example, *Ephedra alata* from Jordan was analyzed for ABTS and was found to have 46.6 and 60.2 µmol Trolox/ g DW for aqueous and methanolic extracts, respectively,(Alali et al. (2007)) which is comparable to *Ephedra* investigated in this study.

The comparison between our results and results from other researches is in the following table:

Researcher/Test	TPC	TFC	FRAP test	CUPRAC test	DPPH (µmol/g)	ABTS (µmol/g)
Alquds University (2017)	30.9-101.2[a]	4.2-19.5[b]	7.1-21.3[a]	2182-6442[a]	305.5-482.5[a]	40.5-66.0[a]
Jaradat et al (Alnajah university)	19.17-47.62[c]	5.44-54.66[c]	----------	----------	----------	----------
Alali et al (Jordan)	11.9-16.2[c]	----------	--------	-------	-------	46.6-60.2[c]

a) Aqueous and 80% ethanolic extract ,respectively.

b) Aqueous and 99% ethanolic extract.

c) Aqueous and methanolic extract, respectively.

4.2.1.4 Pearson Correlation Analyses

A correlation between antioxidant activity (each of FRAP, CUPRAC, DPPH, and ABTS) and total phenolic content or total flavonoid content, as well as between total phenolic content and total flavonoid content was performed (Table 4.2). Additionally, correlations between the four antioxidant assays (FRAP, CUPRAC, DPPH, and ABTS) were also performed (Table 4.2).

Pearson correlation revealed that all antioxidant activities under study were highly and significantly correlated to TPC but were not correlated with TFC. All antioxidant activities were also highly and significantly correlated with each other.

4.2.2 *Polygonum bellardii*

4.2.2.1 Total Phenolic Contents (TPC)

TPC of *Polygonum bellardii* plant extracts using three different solvents is shown in Table 4.4. As it is obvious from this table, the extraction solvent has an effect on the TPC of the *Polygonum bellardii* extracts where significant differences ($p < 0.05$) between the TPC of the three extracts are indicated by different small letters (a, b, and c). The same as *Ephedra*, the highest TPC was found for the plant material when extracted with 80% ethanol (70.4 ± 0.8 mg/g), followed by plant material extracted with 99% ethanol (20.3 ± 1.2 mg/g) and finally with water (12.1 ± 0.4 mg/g). These results show that TPC were only

29% and 17% when the plant material was extracted by 99% ethanol and distilled water respectively as compared with the TPC extracted with 80% ethanol indicating the higher solubility of the phenolic compounds in 80% ethanol.

Table 4.4 Total phenolic content (TPC as mg Gallic acid/g DW[*]), total flavonoids Trolox/g DW), DPPH (µmol Trolox/g DW), ABTS (µmol Trolox/g DW), DPPH % inhibition, and ABTS % inhibition of *Polygonum bellardii* plant extracted with water, 80% ethanol, and 99% ethanol.

	TPC[**] (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)	ABTS (µmol/g)	DPPH %inhibition	ABTS % inhibition
Water	$12.1^{c} \pm 0.4$	$4.2^{c} \pm 0.10$	$4.3^{c} \pm 0.9$	$1015^{c} \pm 42$	$190^{c} \pm 6.5$	$32.4^{c} \pm 1.2$	$74.6^{c} \pm 0.4$	$73.4^{c} \pm 0.5$
Ethanol (80 %)	$70.4^{a} \pm 0.8$	$9.8^{b} \pm 0.1$	$16.3^{a} \pm 1.3$	$4252^{a} \pm 64$	$383.2^{a} \pm 1.9$	$53.6^{a} \pm 1.1$	$87.2^{a} \pm 0.9$	$83.1^{a} \pm 0.5$
Ethanol (99%)	$20.3^{b} \pm 1.2$	$19.5^{a} \pm 0.3$	$8.2^{b} \pm 0.4$	$2121^{b} \pm 29$	$251.3^{b} \pm 0.9$	$40.7^{b} \pm 0.8$	$82.1^{b} \pm 0.5$	$80.0^{b} \pm 1.0$

[*] DW: Dry weight

[**] Results are expressed as average of three samples of *Polygonum bellardii* shoots. Different small letters within column indicate significant difference ($p < 0.05$, $n = 3$).

4.2.2.2 Total Flavonoid Content (TFC)

The results of ferric chloride colorimetric test for determining flavonoids content are presented in Table 4.4. The same statistical analyses as for TPC were performed for total flavonoids content (TFC), and the results (Table 4.4) showed that significant differences between total flavonoids content of the plant materials extracted with the three solvents were obtained, where significant differences ($p < 0.05$) indicated by small letters (a, b, and c). The highest TFC was found for the plant material when extracted with 99% ethanol (16.4 ±0.1 mg/g) which is three times significantly higher than that extracted with 80% ethanol (6.1 ±0.9 mg/g) and the later was three times significantly higher than the TFC extracted with water (2.1 ±0.2 mg/g). Comparing the trend of solvent effect on TFC and

TPC, there is a difference in the two trends where the highest content of TPC was obtained when the plant was extracted with 80% ethanol while the TFC was obtained when the plant material was extracted with 99% ethanol. This can be attributed to the polarity of the extraction solvent and the flavonoids, where flavonoids need less polar solvent (or higher amount of ethanol e.g. 99% ethanol). Apparently, mixed solvents of intermediate polarities (99% or 80% ethanol) are the most suitable extracting solvents for recovering the highest amounts of phenolic and flavonoid compounds which have both polar and nonpolar functional groups.

4.2.2.3 Antioxidant Activity (AA)

Reducing potential of plant extracts

4.2.2.3.a FRAP assay

The antioxidant test based on FRAP assay of *Polygonum bellardii* plant extracts using three different solvents are presented in Table 4.4 (expressed as mmol Fe^{+2}/g of dry plant material). Statistical analyses showed that there are significant differences between total flavonoids content as a function of extraction solvent (Table 4.4), where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Table 4.4 revealed that antioxidant activity (FRAP) of the *Polygonum bellardii* plant increased as the polarity of solvent changes (80% ethanol > 99% ethanol > water), where FRAP values were found to be about two and four times significantly higher when extracted with 80% ethanol compared to 99% ethanol and water, respectively.

The trend of extraction solvent on the FRAP values was found to be the same as for TPC but different from TFC. This suggest that there is a correlation between AA (expressed as FRAP) and TPC, reflecting the fact that total phenolics are the major determinant of AA. Pearson correlation revealed that FRAP (as well as other antioxidant activities under study) were highly and significantly correlated to TPC but were not correlated with TFC, see Table 4.5.

As in the case of TPC and TFC, ethanol (99% or 80%) gives higher amounts of AA (FRAP) compared with water as extraction solvent of *Polygonum bellardii* plant.

Table 4.5 Pearson coefficients between quality indices (TPC, TFC, FRAP, CUPRAC, and DPPH).

	TPC (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)
TPC (mg/g)					
TFC (mg/g)	-0.029				
FRAP (mmol/g)	0.983***	0.1423			
CUPRAC (µmol/g)	0.990***	0.0823	0.998***		
DPPH (µmol/g)	0.984***	0.0824	0.997***	0.990***	
ABTS (µmol/g)	0.979***	0.215	0.982***	0.991***	0.992***

Significance indicated as * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, $n = 9$.

4.2.2.3.b CUPRAC assay

Table 4.4 shows the CUPRAC antioxidant activity (expressed as µmole Trolox/g) of *Polygonum bellardiil* plant extracts using three different solvents. Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that CUPRAC antioxidant activity of the *Polygonum bellardii* plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as FRAP antioxidant activity, and TPC but different from TFC, which suggests that there is a correlation between CUPRAC AA and TPC. Pearson correlation confirms the correlation between CUPRAC antioxidant activity and total phenolic content but no correlation with total flavonoids content (TFC), see Table 4.5.

Free radical scavenging ability of plant extracts

4.2.2.3.c DPPH assay

Table 4.4 shows the % inhibition of DPPH free radicals by the *Polygonum bellardii* extracted with the three solvents. Statistical analyses showed that there are significant differences between % inhibitions using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c), see Table 4.4.

Table 4.6 shows the % inhibition of DPPH at different concentrations of the crude extract (from 10 to 150 µg/mL). This data shows that the extracts exhibited a dose dependent scavenging activity (a linear relationship between % of DPPH inhibition and concentration ($y = 0.5667x + 2.5985$, with R^2 of 0.9919), where y is the % of inhibition and x is the concentration). From this linear relationship, IC50 which is the concentration required to quench 50% of the DPPH free radicals was determined and was found to be 93 µg/mL.

DPPH antioxidant activity of *Polygonum bellardii* plant extracts using three different solvents was expressed as µmole Trolox/g (Table 4.4). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that DPPH antioxidant activity of the *Polygonum bellardii* plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as TPC, FRAP, and CUPRAC antioxidant activity. Correlation studies showed a significant correlation between DPPH and TPC but not with TFC (Table 4.5).

Table 4.6: % inhibition of DPPH and ABTS free radicals by different concentrations of *Polygonum bellardii* plant extract.

Concentration of DPPH (µg/mL)	% inhibition of DPPH *	Concentration of ABTS (µg/mL)	% inhibition of ABTS *
10	5.3 ± 0.6	10	10.2 ± 0.9
20	18.7 ± 1.0	20	15.2 ± 0.8
40	24 ± 0.9	40	35.4 ± 0.7
80	47 ± 1.0	80	68.0 ± 1.0
150	88 ± 1.4	100	87.3 ± 1.2

*Results are expressed as Average ± standard deviation of three samples.

4.2.2.3.d ABTS assay

Table 4.4 shows the % inhibition of ABTS free radicals by the plant extracted with the three solvents. Statistical analyses showed that there are significant differences between

AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c), see Table 4.4.

Table 4.6 shows the % inhibition of ABTS at different concentrations of the crude extract (from 10 to 100 µg/mL). This data shows that the extracts showed a dose dependent scavenging activity (a linear relationship between % of ABTS inhibition and concentration ($y = 0.8645x - 0.005$, with R^2 of 0.9979), where y is the % of inhibition and x is the concentration). From this linear relationship, IC50 was determined and was found to be about 58 µg/mL.

ABTS antioxidant activity of *Polygonum bellardii* plant extracts using three different solvents was expressed as µmol Trolox/g (Table 4.4). Statistical analyses showed that there are significant differences between AA using the three extraction solvents, where significant differences ($p < 0.05$) are indicated by different small letters (a, b, and c).

Results showed that ABTS antioxidant activity of the *Polygonum bellardii* plant increased in the following order: 80% ethanol > 99% ethanol > water which is the same trend as FRAP, CUPRAC, and DPPH antioxidant activities. Additionally this trend is the same as TPC but different from TFC, which suggests that there is a correlation between ABTS and TPC. As it is obvious from Table 4.5, there is a correlation between ABTS and TPC but not with TFC.

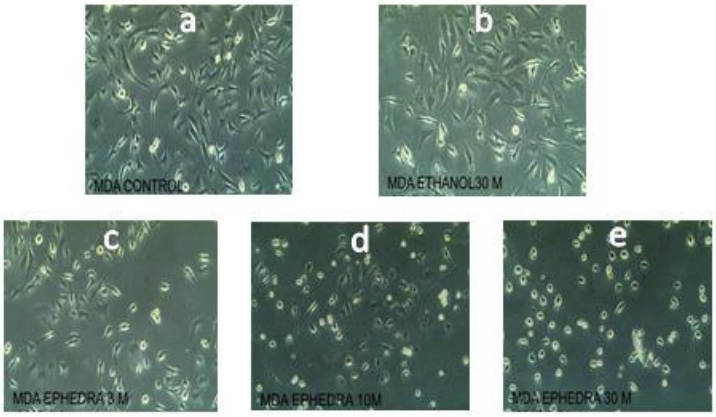
4.3 Anticancer activity of *Polygonum bellardii* and *Ephedra alata*:

The two extracts of *Polygonum bellardii* and *Ephedra alata* have a strong reputation among elderly Palestinians for their health benefits, particularly as anticancer agents. In order to validate this claim, we tested their extract anti-cancer activity on breast (MDA MB231) and colon (HCT116) cancer cell lines. For that end, both cells were treated with different concentrations of the extracts over two days, and ethanol (extraction solvent) was used as a control. To evaluate the extract effect on cells, they were observed and photographed using microscope and a camera attached to it.

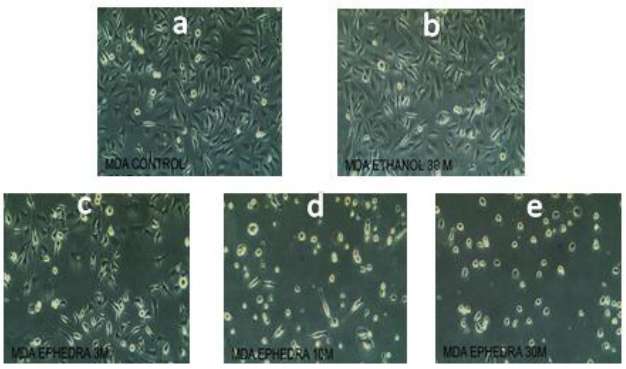
a. *Ephedra alata* plant extract

For *Ephedra alata*, our results showed that its extract has an anti-cancer activity effect on both MDA MB231 (Fig. 4.8) and HCT116 (Fig. 4.9) cancer cell lines. It was estimated that after 48hrs *Ephedra alata* extract resulted in 30, 60 and 100% MDA MB231 cell death using 100, 330 and1000 µg/mL, respectively (Fig 4.8B). Although Ephedra extract showed an anti-cancer activity against HCT116 cells also, its effect on these cells was less potent as compared to MDA MB231 cells. For example, at 100, 330 µg/mL over 24hrs, the extract had, almost, no effect on cells and only a slight effect with 1000 µg/mL

concentration (Fig. 4.9A). Moreover, even after 48 hours, the effect, although more prominent than after 24hrs, was less potent in comparison to the extract effect on MDA MB231 cells. For example, the 330 and 1000 µg/mL extract concentrations resulted in 25 and 65% cell death, respectively. (Fig. 4.9 B). Overall, our results show, here, that *Ephedra alata* plant extract has an anti-cancer activity, which is more potent against the breast cancer cell line MDA MB231 compared to the colon cancer cell line HCT116.



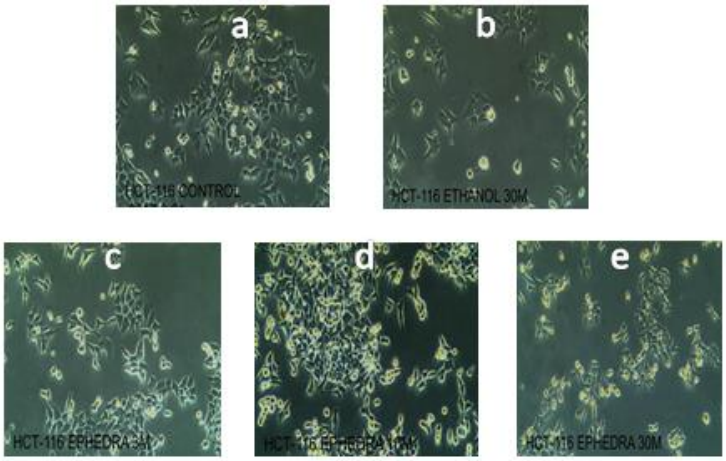
(A)



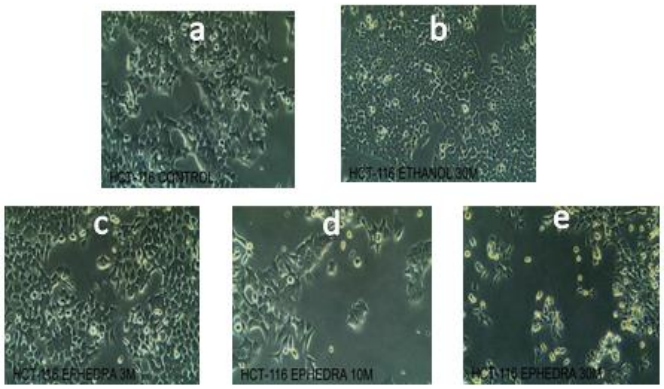
(B)

Figure 4.8: Effect of Ephedra extract on MDA MB231 breast cancer cell line. (A) Representative micrographs showing the effect of the indicated concentrations after 24 hrs. Ethanol treatment was used as control. (B). Representative micrographs showing the effect of the indicated extract concentrations after 48 hrs. Ethanol treatment was used as control. a. MDA control b. Ethanol (1000 µg/mL) c.

Ephedra extract (100 µg/mL) d. Ephedra extract (330 µg/mL) e. Ephedra extract (1000 µg/mL).



(A)

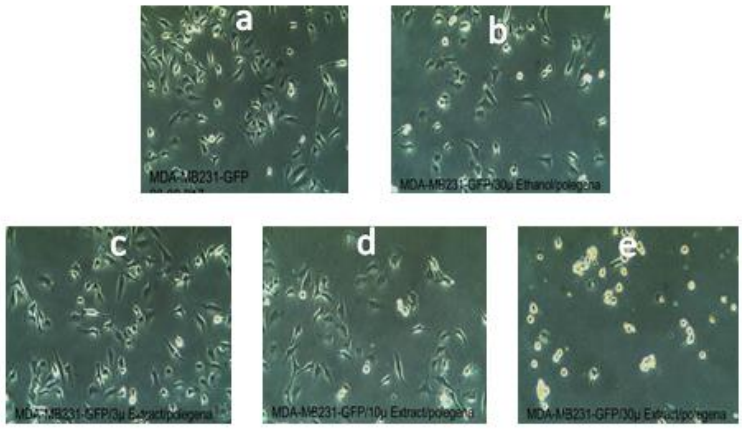


(B)

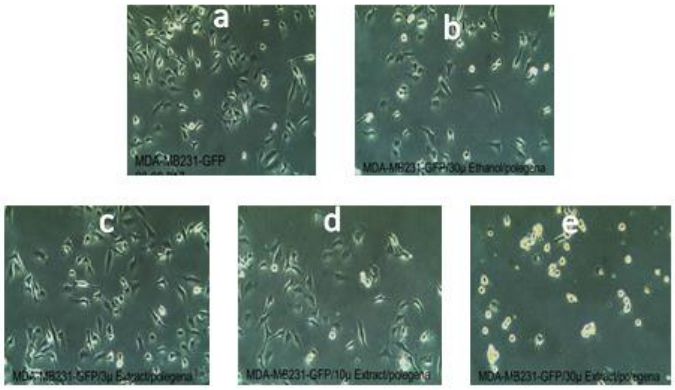
Figure 4.9: Effect of *Ephedra* extract on HCT116 colon cancer cell line. (A) Representative micrographs showing the effect of the indicated concentrations after 24 hrs. Ethanol treatment was used as control. (B). Representative micrographs showing the effect of the indicated extract concentrations after 48 hrs. Ethanol treatment was used as control. a. HCT116 control b. Ethanol (1000 µg/mL) c. Ephedra extract (100 µg/mL) d. Ephedra extract (330 µg/mL) e. Ephedra extract (1000 µg/mL).

b. *Polygonum bellardi* plant extract

The same study was conducted for *Polygonum bellardii* extract, also our results showed that its extract has an anti-cancer activity effect on bothMDA MB231 (Fig. 4.10) and HCT116 (Fig. 4.11) cancer cell lines. It was estimated that after 48 hrs *Polygonum bellardii* resulted in 20-30%, and 100% MDA MB231 cell death using 330 and1000 µg/mL, respectively (Fig 4.10B). Moreover, *Polygonum bellardii* extract showed an anti-cancer activity against HCT116 cells, and its effect on these cells was less potent as compared to MDA MB231 cells. For example, at 100, 330 µg/mL over 24hrs, the extract had, almost, no effect on cells and only a slight effect with 1000 µg/mL concentration (Fig. 4.11A). Moreover, even after 48 hours, the effect, although more prominent than after 24hrs, was less potent in comparison to the extract effect on MDA MB231 cells. For example, the 1000 µg/mL extract concentrations resulted in 55-65% cell death (Fig. 4.11B). Overall, our results showed that *Polygonum Bellardii* plant extract has an anti-cancer activity, which is more potent against the breast cancer cell line MDA MB231 compared to the colon cancer cell line HCT116.

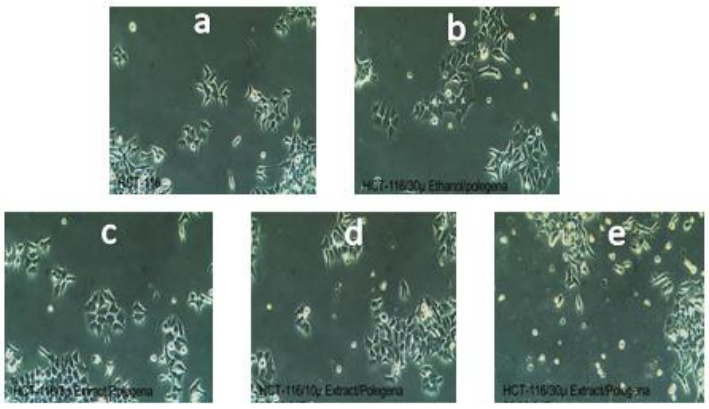


(A)

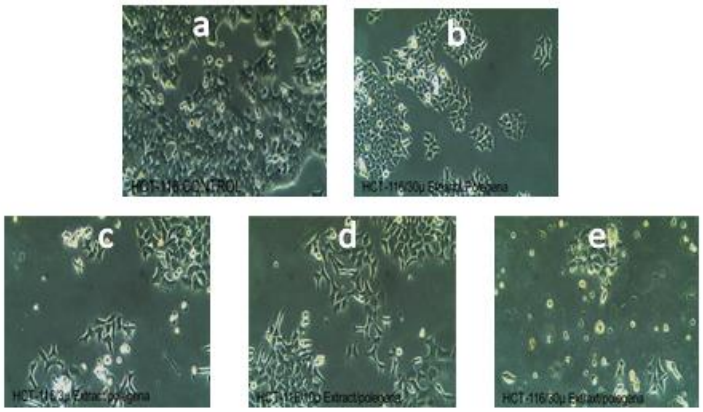


(B)

Figure 4.10: Effect of *Polygonum bellardii* extract on MDA MB231 breast cancer cell lines. (A) Representative micrographs showing the effect of the indicated concentrations after 24 hrs. Ethanol treatment was used as control. (B) Representative micrographs showing the effect of the indicated concentrations after 48 hrs. Ethanol treatment was used as control. a. MDA control b. Ethanol (1000 µg/mL) c. Ephedra extract (100 µg/mL) d. Ephedra extract (330 µg/mL) e. Ephedra extract (1000 µg/mL)



(A)



(B)

Figure 4.11: Effect of *Polygonum bellardii* extract on HCT116 colon cancer cell lines. (A) Representative micrographs showing the effect of the indicated concentrations after 24 hrs. Ethanol treatment was used as control. (B) Representative micrographs showing the effect of the indicated concentrations after 48 hrs. Ethanol treatment was used as control. a. HCT control b. Ethanol (1000 µg/mL) c. Ephedra extract (100 µg/mL) d. Ephedra extract (330 µg/mL) e. Ephedra extract (1000 µg/mL)

From these results, it is concluded that *Ephedra alata* has more anti-cancer activity than *Polygonum bellardii* on both cancer cell lines (MDA MB231 breast and HCT116).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

Conclusions:

The *Ephedra alata and Polygonum bellardii* from flora of Palestine are rich in phenolic, flavonoid compounds and constitutes a natural source of potent antioxidants that may prevent serious diseases. The total phenolic content, the total flavonoid content, the antioxidant activities and the biological activity of these plants were studied. It has been found that 80% ethanol extract for both plants gave the highest TPC and AA values probably due to the mixed polarity of this solvent combination ratio (20% water, and 80% ethanol) which afford the higher solubility of phenolic compounds and antioxidants.

Analysis of phenolics and flavonoids of Ephedra extracts by HPLC/PDA and UPLC/MS revealed that *Ephedra alata* extract is rich in potent falvonoid glycosidic compounds as reflected from their similar overlaid UV-Vis spectra and UPLC-MS results. *In-vitro* anticancer activity of the crude extract with both cancer cell lines (HCT colon and MDA Breast) was performed and results showed an observable anticancer activity of plant extracts of both plants.

On the basis of these findings, it is concluded that *Ephedra alata and Polygonum bellardii* constitutes a natural source of effective antioxidants that may prevent many diseases and could be potentially used in food, cosmetics, and pharmaceutical products.

Future recommendations:

Few steps are recommended to be investigated for the two plants in the future as follows:

1- To collect *Ephedra alata* specimens from different places all over Palestine at different harvesting times and subjecting them to different extraction methodologies for the sake of comparison on the TPC, TFC and AA values.
2- To test their anticancer activity using different cancer cell lines, and to use in-vivo systems.
3- To collect sufficient amount of individual isolated flavonoids and/or phenolic compounds and run the above test on them.
4- To study the effect of combining some compounds to investigate the synergistic effect.

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الخصائص المضادة للسرطان والمضادة للأكسدة لنبتتي العلندا والقضاب البرية في فلسطين

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الملخص

هدفت الرسالة الى تقييم نشاط نبتتي العلندا والقضاب المضاد للسرطان و المضاد للأكسدة والتي اكتسبتا شهرة بعد نجاح مستخلص نبتة العلندا البرية في شفاء حالات من السرطان ولقد تم جمع النبتتين من مدينة بيت لحم في شهر آذار من عام 2016م اذ تم تحضير مستخلصات كل نبتة بتراكيز مختلفة من الكحول الإيثيلي (80% و 99%) والماء المقطر. وبسبب تشابه نبتة العلندا (*Ephedra* alata) بنبتة القضاب (*Polygonum bellardii*)،فقد تم عمل نفس الفحوصات لهذه النبتة الشبيهة بالعلندا.

تم استخلاص النبتتين كالآتي : تم تجفيف النبتة، طحنها (بالخلاط المنزلي) ، تنخيلها ، وأخذ البودرة الناعمة، توزين 10 غم في 100 مل ماء ، أو/و 100 مل كحول 99% ، أو/ و 100 مل 80% كحول. ثم وضعها في جهاز ال "Sonicator"لمدة ما يقارب 4 ساعات على درجة حرارة 40 س^{o} وثم فلترتها. وبعد ذلك تم تبخير الماء بواسطة جهاز Rotary evaporator. تم فحص مضادات الأكسدة للمستخلصات باستخدام اربع فحوصات (FRAP، CUPRAC ، ABTS ، DPPH) ، و تم حساب المحتوى الفنولي الكلي والمحتوى الفلافونيدي الكلي للمستخلصات ثم ايضا تم عمل تحاليل لإيجاد تراكيز المواد الفينولية باستخدام جهاز ال HPLC و تبين ان النبتتان غنيتان باحتوائهما كميات جيدة من مضادات الأكسدة و المواد الفعالة، اذ تبين بأن مستخلص تركيز الكحول الإيثيلي 80%

يملك اعلى نسب من هذه المواد في جميع الفحوصات وان مستخلص الماء المقطر حصل على اقل نسب في جميع الفحوصات ايضاً. كما واظهرت نتائج ال HPLC على وجود العديد من الفلافونيدات في نبتة العلندا والقضاب. وتم تشخيص بعض المركبات الجديدة ولأول مرة باستخدام جهاز ال UPLC-ESi-MS مثل Myricetin 3-rhamnoside و Luteolin-7-O-glucuronide flavonoid

كما تم عمل فحص النشاط المضاد للسرطان لمستخلصات نبتتي العلندا والقضاب وذلك بتجريب المستخلص للنبتتين على خلايا سرطانية مخبرية من أنواع مختلفة (وبالتحديد خلايا سرطان القولون والثدي).وقد أظهرت النتائج فاعلية نبتتي العلندا والقضاب في مقاومة تلك الخلايا، وقد تبين أن لنبتة العلندا قدرة أكبر من نبتة القضاب في مقاومة الخلايا السرطانية .

وعلى أساس تلك النتائج، فقد تم التوصل إلى ان نبتتي العلندا والقضاب يمكن أن تعتبر مصدر مهم للمواد المضادة للأكسدة والتي يمكن أن تمنع الكثير من الأمراض، وبالتالي يمكن استخدامها في المستحضرات الدوائية والغذائية ومواد التجميل.