Deanship of Graduate Studies

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Phytochemical Screening Of Wild *Ephedra Foeminea* And *Smilax Aspera* Fruits And Their *In-Vitro* Pharmacological Activities

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Phytochemical screening of wild *Ephedra foeminea* and *Smilax aspera* fruits and their in-vitro pharmacological activities

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Jerusalem–Palestine
1439/2018

Dedication

I would like to dedicate this work to my beloved parents who have always believed in me, lighten my road, and empower my ambitions. My father, Khaled, who I always consider my example. My mother, Hend, the strong and gentle soul who had always graced me with her unconditional love and drive me to believe that I am the one who will change the world to the best.

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To my Precious son, Ahmad, all the persistence comes from a look into your eyes.

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To my husband's family, for their warm love and endless support.

To all my dear supportive friends during this journey.

To all people who work hard and faithfully to gain knowledge and discover ways to serve humans and our planet for the sake of Allah.

Maryam
July,2018

Declaration

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

Signed: 

Name :Maryam Khaled Mohammad Sawalha

Date: July 21, 2018

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List of Abbreviations

NPs: Natural products

UV: Ultraviolet

Vis: Visible

TPC: Total Phenolics content

TFC: Total Flavonoids content

GAE: Gallic acid equivalent

CEQ: Catechin equivalent

E: *Ephedra*

S: *Smilax*

TCM: Traditional Chinese Medicine

USA: United States

DNA: Deoxyribonucleic acid

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

FRAP: Ferric ion Reducing Antioxidant Power

NCI: National Cancer Institute

HPLC: High Performance Liquid Chromatography

PDA: Photo Diode Array detection

MS: Mass Spectrometry

ESI: Electrospray ionization

PIS: Product ion scanning

TIC: Total ion chromatogram

COSY: Correlation Spectroscopy

NMR: Nuclear Magnetic Resonance

HMQC: Heteronuclear Multiple-Quantum Coherance

$R_{t:}$ Retention time

DW: Distilled Water

EtOH:	Ethanol
µ:	Micro
V:	Very
C:	Carbon
g:	gram
mg:	milligram
mm:	millimeter
ppm:	part per million
m.wt.:	molecular weight
M^+:	molecular ion
Da:	Dalton
mol:	mole

Abstract

The present work explores two wild Palestinian plants, *Ephedra foeminea* and *Smilax aspera* fruits for the first time. Both plants acquired their attractive red color due to chromophores functionalities of phenolic secondary metabolites abundance. The fruits were collected upon their ripening season in October of 2016 and 2017; *Ephedra* from Bani Naem and *Smilax* from Beit-Jala. After being air-dried, extracted separately in 99% ethanol, 80% ethanol and 100% distilled water, subjected to ultra-sonication and kept at 4^0C for analysis. For both plants, the total phenolic content (TPC) and total flavonoids content (TFC) were calculated. The highest content of phenolics was observed in water extracts of *E. foeminea* and *S. aspera* with values of 3.22 and 8.21 mg gallic acid per gram sample respectively. Similarly, TFC showed highest flavonoids content in the water extract for *S. aspera* with a value of 1.22 mg catechin/ g sample. However, *E. foeminea* extract has a higher percentage in 99% ethanolic extract with a value of 1.0433 mg catechin per g sample. The antioxidant activity was investigated by two methods: Ferric ion reducing antioxidant power (FRAP) method and 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging assay. Using DPPH, the best values obtained for *E. foeminea* and *S. aspera* was found in ethanolic extracts with values of 9.5 and 5.9 mg trolox/ g sample respectively, with 91.3% of radical inhibition for *E. foeminea*. The antioxidant activity was further indicated by the quiet good ability to reduce the FRAP reagent for both plants with high values in water extracts (0.57 and 0.71 mg Fe+2 /g of dry sample in *E. foeminea* and *S. aspera* respectively). The antimicrobial activity was measured using well diffusion technique. *S. aspera* fruit extract showed minor positive influence against gram positive bacteria (*Staphylococcus aureus*) when compared to Penicillin G; the positive control, also it showed positive effect against yeast (*Candida albicans*). *E. foeminea*, however, showed no effects on both. When examined against colon cancer cell lines (HT-29), the two plants extracts did not show anticancer activities.

Phytochemical screening of crude and pure isolated fractions using analytical reversed phase HPLC-PDA, preparative-HPLC-PDA, full scan LC-MS (positive and negative ESi modes) and LCMSMS (product ion scanning) of active 80% EtOH extract of both plants was performed to reveal the identities of the plants phenolic secondary metabolites. Injection of 1000 µl (100mg) of fruit extracts at 350 nm to the inch C18 preparative HPLC column gave seven major pure isolates form *E. foeminea* and eight major pure compounds form *S. aspera*. Full scan LC-MS and LC-MSMS experiments revealed the presence Quercetin, Kaempferol, Lucenin 2, Vicenin 2, Quercetin 3-O-rhamnoside-7-O-glucoside and Quercetin-3-O-rhamnoside in *E. foeminea*. Whereas, five compounds were identified in *S. aspera*: apigenin 7-glucuronide, luteolin derivative, lucenin 2, quercetin-3-glucoside and isorhamnetin-3-O-rutinoside.

Keywords: *Ephedra foeminea*, *Smilax aspera,* total flavonoid content, total phenolic content, Antioxidant, FRAP, DPPH, in-vitro antimicrobial, anticancer, HPLC-PDA, LC-MSMS.

Chapter One

Introduction

Chapter One:

Introduction:

1. Background

Since ancient times, people have been exploring the nature particularly natural plants in search to treatment human diseases. This has resulted recently in the use of large number of medicinal plants and ultimately new drugs have emerged (Verpoorte, 1999).

Compounds derived from natural sources such as plants, animals, and micro-organisms that have biological activities, are called natural products (NPs), were globally extracted and used (Baker, Chu, Oza, & Rajgarhia, 2007; Newman, Cragg, & Snader, 2000). Despite the competition from other drug discovery methods such as synthesis, NPs from plant sources still have their fair share of new clinical lead candidates and drugs, as they nowadays represent central theme of research in the drug discovery (Grabley, Thiericke, & Sattler, 2000).

Knowledge of the separation, isolation and structural elucidation of the chemical constituents of plants is desirable to facilitate the synthesis of any complex chemical substances.

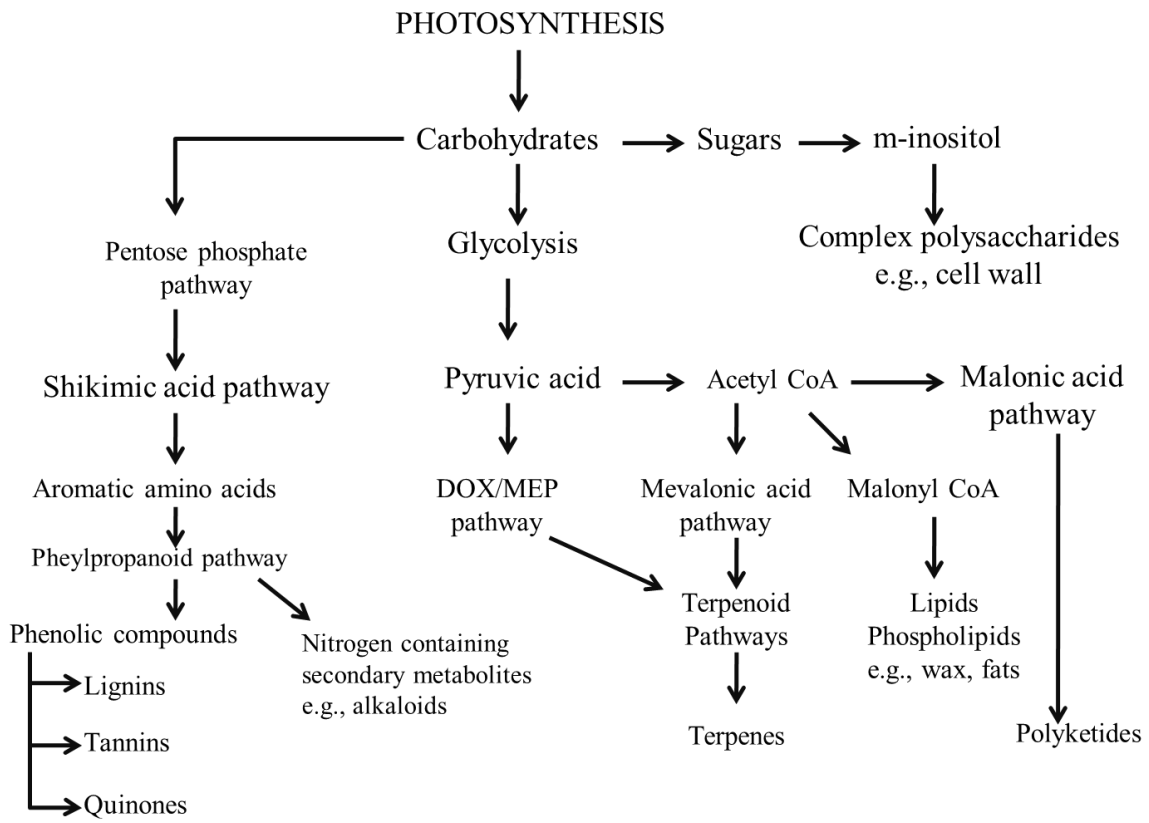
In Palestine, the use of plants in traditional medicine has proved its usefulness over the years. Despite its small area, the distinctive geographical location of Palestine at the crossroads of the African, Asian and European continents have graced it with rich biodiversity, especially when compared to other countries in the region (Fifth National Report on Biodiversity Conservation, 2015).

According to the Environmental Quality Authority of Palestine; Palestine contains five bio-geographical zones (Ecosystems) which associated with their climate and biodiversity

(Central Highlands, Semi -Coastal Region, Eastern Slopes, Jordan Rift Valley, Gaza Strip), in addition to four phytogeographical regions (Mediterranean, Irano, Turanian, Saharo, Arabian and Sudanese/Ethiopian) (Fifth National Report on Biodiversity Conservation, 2015). There are about 51,000 living species in Palestine, constituting approximately 3% of the global biodiversity. Its flora consists of over than 2,000 species including 54 endemic plants that do

not exist in any other part of the world (Ali-Shtayeh, Salameh, Abu-Ghdeib, Jamous, & Khraim, 2002). The most dominant families are the Asteraceae with 96 genera and 260 species, Poaceae with 87 genera and 198 species, Fabaceae with 62 genera and 268 species, Brassicaceae with 63 genera and 124 species, Lamiaceae which is famous as a medicinal plants, with 23 genera and 99 species. Moreover, Smilacaceae is known for its beautiful flowers, with 23 genera and 97 species, the Trifolium genus which is used as a forage plant contains 40 species, Medicago genus contains 22 species, and Trigonella genus, which contains 18 species (Bregheith, 1995).

NPs are usually restricted to purified compounds isolated from natural sources. Plant cells produce two types of metabolites, primary and secondary. Primary metabolites are components of basic metabolic pathways that are required for life. They are associated with essential cellular functions such as nutrient assimilation, energy production, and growth/ development. These include carbohydrates, lipids, amino acids, and nucleic acids that are the basic building blocks of life (Demain & Fang, 2000). Secondary metabolites, in contrast, are not absolutely required for survival. Tracking their biosynthesis life, they originate mostly from the primary metabolites [Scheme 1].



Scheme 1: Principle biosynthetic pathways leading to synthesis of secondary metabolites (Demain & Fang, 2000).

Carbohydrates are important compounds that have the most significant effects on flavonoids and phenolics production and partitioning in plant organs. Plant phenolics (flavonoid and phenols) are biosynthesized via several routes and thus constitute a heterogeneous group from the metabolic point of view. The two basic pathways involved are the shikimic acid and the malonic acid pathways. The shikimic acid pathway participates in the biosynthesis of most plant phenolics. Through this pathway, soluble carbohydrates are the basic component used to produce phenolic component. The shikimic acid pathway is able to convert simple carbohydrate precursors derived from glycolysis and pentose phosphate pathway to aromatic amino acids (Herrmann & Weaver, 1999).

Therefore, from metabolic point of view, as plant phenolics are biosynthesized through several routs, they are a diverse group of metabolites.

Plant secondary metabolites are categorized in three main branches based on their biosynthesis pathways: Phenolic compounds, terpenes and nitrogen-containing compounds (Parsaeimehr, Sargsyan, & Vardanyan, 2011).

The function of these compounds to plant itself is to act as a defense mechanism; secondary metabolites can be considered as the immune system in the plant, as many of which are cytotoxic. On the other side, they attract and interact with other living beings; insects and microbes, as they have a major role in plant pollination and seed dispersion regarding their attractive colors (Kårlund, Moor, Sandell, & Karjalainen, 2014).

Considerably, herbivorous animals and insects use the plants as a source for their existence while the plants staidly express these metabolites as a defensive mechanism, or even carnivorous plants have the ability to trap insects in the specialized organs, obtaining additional nitrogen and phosphorus in nutrient-poor environments (Stotz, Kroymann, & Mitchell-Olds, 1999).

1.1. Phenolic compounds

Phenolic compounds are secondary metabolites widely distributed in the plant kingdom. As mentioned earlier; they are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways (Randhir, Lin, & Shetty, 2004). For the plant, these compounds play an important role in growth and reproduction, providing protection against pathogens and predators (Lewis, 2017), may affect the sensory characteristics of fruits with important impacts on color, flavor, and astringency. An adequate consumption of phenolic compounds may also offer health benefits (Cosme et al., 2017) (Alasalvar, Grigor, Zhang, Quantick, & Shahidi, 2001). Structurally, phenolic compounds comprise an aromatic ring, bearing one or

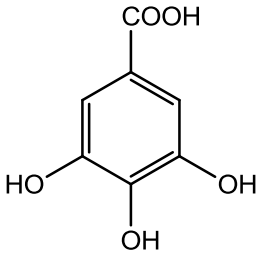
more hydroxyl substituents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). This structural diversity gives a wide range of phenolic compounds that occur in nature, they are basically categorized into several classes as shown in [Table 1] (Cosme et al., 2017).

Table 1: classes of phenolic compounds in plants.

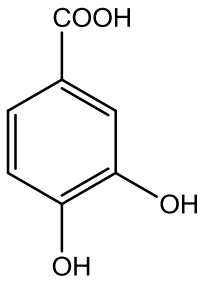
Class	**Basic skeleton**
Simple phenolics, bezoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acids	C_6-C_2
Hydroxyinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stillbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6$-C_3-$C_6)_n$

Of these, phenolic acids, flavonoids and tannins are regarded as the main dietary phenolic compounds (King & Young, 1999). Phenolic acids consist of two subgroups: the hydroxybenzoic and hydroxycinnamic acids [Figure 1].

a

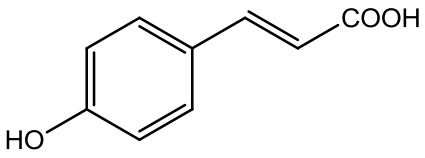


gallic acid

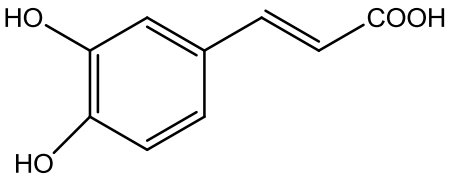


protocatechuic acid

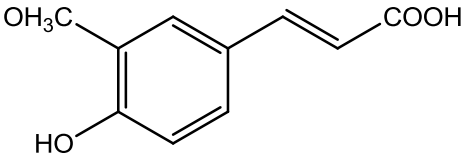
b



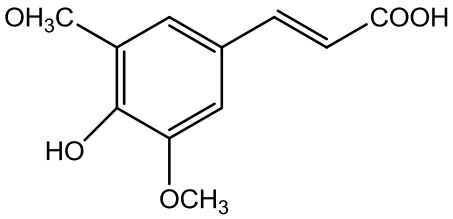
p-coumaric acid



caffeic acid



ferulic acid



sinapic acid

Figure 1: Examples of hydroxybenzoic (a) and hydroxycinnamic (b) acids.

Several interesting studies reported the significance inhibitory influences of these fabulous compounds on pathogens and insects, besides their risen concentration when going through abiotic stresses like UV or Ozon. For example the phenolics increased significantly in blueberries under UV treatments and the elevated Ozone increased the concentrations of phenolics and antioxidant in red clover (Trifolium pratense) leaves (Morales et al., 2010; Saviranta, Julkunen-Tiitto, Oksanen, & Karjalainen, 2010). Regard to the plant and fungi interactions there are sufficient evidences that different phenolic compounds are over

synthesized and expressed by plants under fungus attacks (Lattanzio, Lattanzio, & Cardinali, 2006).

To quantify the total phenolic content (TPC) in certain plant extract, a reliable method have been widely applied. This method established by (Singleton & Rossi, 1965), uses Folin-Ciocalteu reagent that should be mixed with the plant extracts in certain quantities along with sodium bicarbonate, after certain time the absorbance is measured at 765nm using UV-Spectrophotometer. Aqueous solution of known gallic acid concentrations are used for calibration. Results of this assay are expressed as milligram (mg) gallic acid equivalent (GAE) per gram (g) dry weight of sample.

1.2. Flavonoids

Flavonoids represent the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne, 1999). They are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Middleton, 1998), many of which are responsible for the attractive colors of flowers, fruit, and leaves (Groot & Rauen, 1998). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C [figure 2].

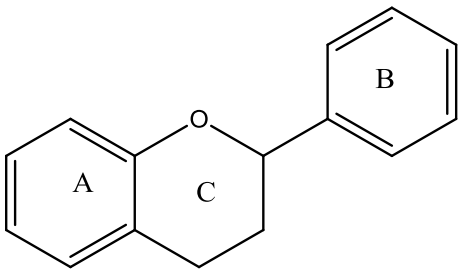


Figure 2: Generic structure of a flavonoid molecule

The aromatic ring A is derived from the acetate/ malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway (Bohm, 1998; Merken & Beecher, 2000). Variations in substitution patterns to ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins [figure 3] (Pérez-Jiménez & Saura-Calixto, 2015). Of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne, 1999). Substitutions to rings A and B give rise to the different compounds within each class of flavonoids (Pietta, 2000).

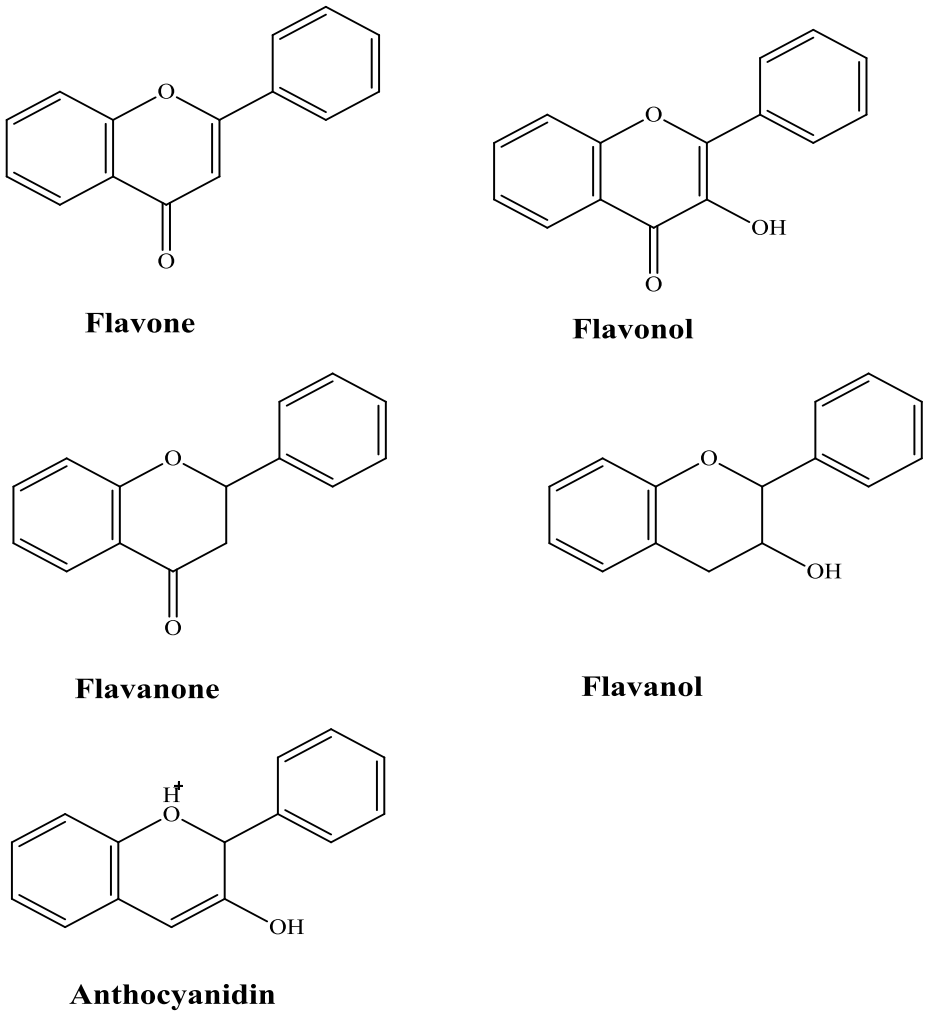


Figure 3: Generic structure of major classes Flavonoids

To quantify the total flavonoid content (TFC) in any plant extract, a reliable method have

been widely applied. This method established by (Choi, Hwang, & Kim, 2003) adds sodium nitrite solution, aluminum chloride solution, sodium hydroxide and distilled water to the plant extract, in specific quantities of each, then the absorbance is measured at 510 nm using UV-Spectrophotometer. Aqueous solutions of known Catechin concentrations are used for calibration and the results are expressed as mg catechin equivalents (CEQ)/ g sample.

1.3. Phenolics and flavonoids health benefits

Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables (F. Shahidi & Ambigaipalan, 2015). Despite their wide distribution among plant kingdom, the health effects of dietary polyphenols have gain the attention of nutritionists recently. This interest has arisen due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases. Several studies indicated that flavonoids possess anti-inflammatory, anti-allergic, anti-viral, and anti-carcinogenic properties (Kabera, Semana, Mussa, & He, 2014).

The phenolic composition of the red/dark-colored fruits depends on cultivar, maturity, growing environment, cultural practices, postharvest conditions, and processing techniques (Kårlund et al., 2014). Therefore, the phytochemical composition differs, as these factors differ, and plant of the same species will vary in constituents from one region to another.

1.4. Palestinian plants rich in phenolics

Palestine is rich in biodiversity which has not been yet fully investigated. In the current work, the attention will be focused on two wild Palestinian plant fruits, namely, *Ephedra foeminea* (*E. foeminea*) that belongs to Ephedraceae family and *Smilax aspera* (*S. aspera*) that belongs to Smilacaceae family.

1.4.1. *Ephedra foeminea* (*E. foeminea*)

The genus of Ephedra, which contains over 50 species, is a member of the famous family Ephedraceae, a genus of non-flowering seed plants belonging to the Gnetales, the closest living relative of the Angiosperms (Caveney, Charlet, Freitag, Maier- Stolte, & Starratt, 2001; Friedman, 1996). The shrubs, which reach approximately one meter in height, grow in semiarid and desert conditions in both hemispheres across six continents (Price, 1996)

Ephedra in general is one of the oldest medicinal herbs known to Traditional Chinese Medicine (TCM); *E. sinica* is the primary species that has been used in *Ephedra* preparations and extracts all around the world to treat allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion; as it has been a natural source of alkaloids such as ephedrine, pseudoephedrine, norpseudoephedrine (K. Chen & Schmidt, 1926; Konar & Singh, 1979; Nawwar, Barakat, Buddrust, & Linscheidt, 1985; O'Dowd et al., 1998). Ephedra has also been used as a dietary supplement in United States (USA) and Europe during 20th century, but, misuse or abuse of ephedrine-containing dietary supplement for the purpose of weight loss or euphoric stimulation has resulted in various adverse effects. Therefore, USA and many western countries have banned the sale of ephedrine-containing dietary supplements since 1994 (Kitani et al., 2009).

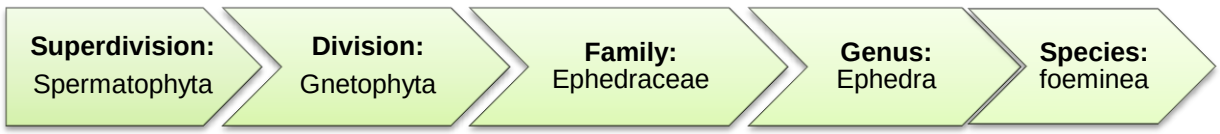
Phenolic compounds which are present in *Ephedra*, are of great importance because of their potential antioxidant and antimicrobial properties. Therefore, this prodigious plant shows significant antimicrobial activity.

E. foeminea (Alanda in Arabic) [figure 4], is a wild, light green, densely branched, monogenic, small and perennial climber shrub, the twigs appear leafless and the leaves reduced to small scales, cones sessile shaped, clustered in the axils or at branch tips, the red fruits are fully matured upon October. *E. foeminea* grows in the Mediterranean region, its

exact taxonomy is shown in [scheme 2], its geographical distribution among Palestine along with its level of existence (very common, common, rare and very rare) is shown in [figure 5]. *E. foeminea* was addressed in traditional Arab medicine as a stimulant in treatment of anxiety and skin rash, also as a deobstruent, to treat kidney, bronchi, circular system, digestive system disorders and to relief asthma attacks (Philips, 1958); The dried stems are the used parts of the plant in traditional medicine, they are used as hot drink decoction.

But in Palestine only recently it has been popularized as a treatment of cancer; when a Palestinian shepherd has talked about his story in defeating cancer in October 2013 using the decoction of this plant. Despite the never mentioned anticancer use of Alanda in traditional medicine nor the existence of any scientific based approval of this use, the oncology patients in Palestine and even broader started seeking this plant as a "wonder cure" of their hopeless disease.

Figure 4: Photo of *E. foeminea* growing wild in Al- Mgaier Wadi of Bani Na'em village in Hebron (photo captured in October 2017)



Scheme 2: *E. foeminea* taxonomy.

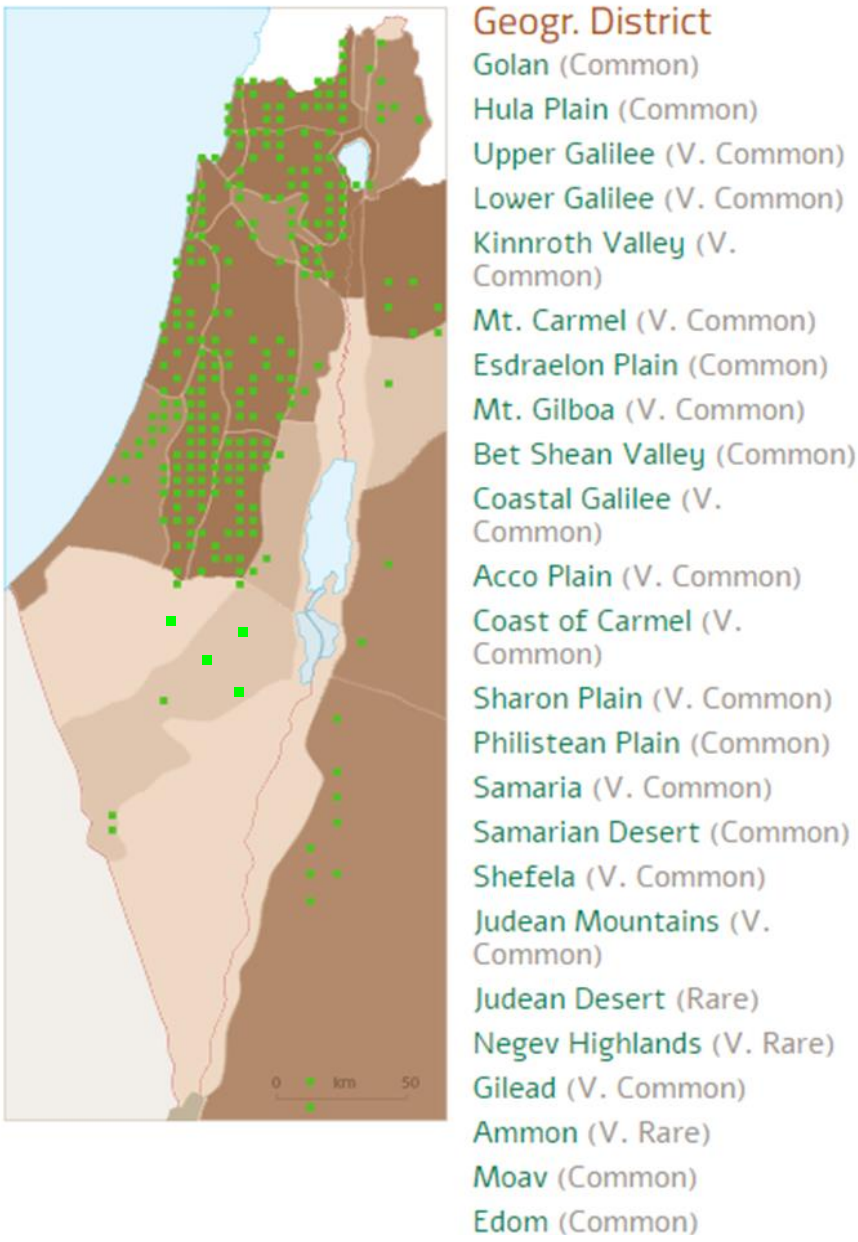


Figure 5: geographical distribution of *E. foeminea* in Palestine (Danin, 2016).

1.4.2. *Smilax aspera* (*S. aspera*)

The genus *Smilax* (Smilacaceae), commonly called sarsaparilla, consists of about 350 species. About 79 species are natives of China, 24 species are from India and 29 species are from Central America, others are distributed among Mediterranean regions. The plants of this genus are climbers, have long, thin, thorny stems and have tendrils which attach to other plants or objects to climb steadily.

S. aspera plant [figure 6] is an evergreen, creeping, and extremely tough shrub of the family Smilacaceae, typical of the Mediterranean region. Its full taxonomy is illustrated in [scheme 3]. Leaves and roots of this plant are edible: the roots are used as ingredient of soft drink and young shoots can be cooked and used as an asparagus substitute (Longo & Vasapollo, 2006). *S. aspera* has also been used traditionally for treatment of syphilis, diabetes, and rheumatism, and as an antioxidant and for treatment of symptoms of menopause in women (Harba, abu Zargab, & Abdallaa, 2009). It finds application in herbal medicine for its depurative, diaphoretic, diuretic, stimulating and tonic action(Longo & Vasapollo, 2006).

S. aspera geographical distribution among Palestine along with its level of existence (very common, common, rare and very rare) is shown in [figure 7]. The fruits of which are ripened on October are bright red, (Raúl, Beatriz, Joseoziel, & Francenia, 2017) had reported that anthocyanins are the responsible compounds of their color.

Preparations from the roots of *S. aspera* plant are used traditionally in Palestine to treat inflammatory skin diseases, including psoriasis, along with rheumatic complaints and inflammation of the urinary tract (Amira, Dade, Schinella, & Ríos, 2012).

Figure 6: Photo of *S. aspera* growing wild in Al-Makhrour Wadi in Beit-Jala. (photo captured in October 2017)

Scheme 3: *S. aspera* taxonomy.

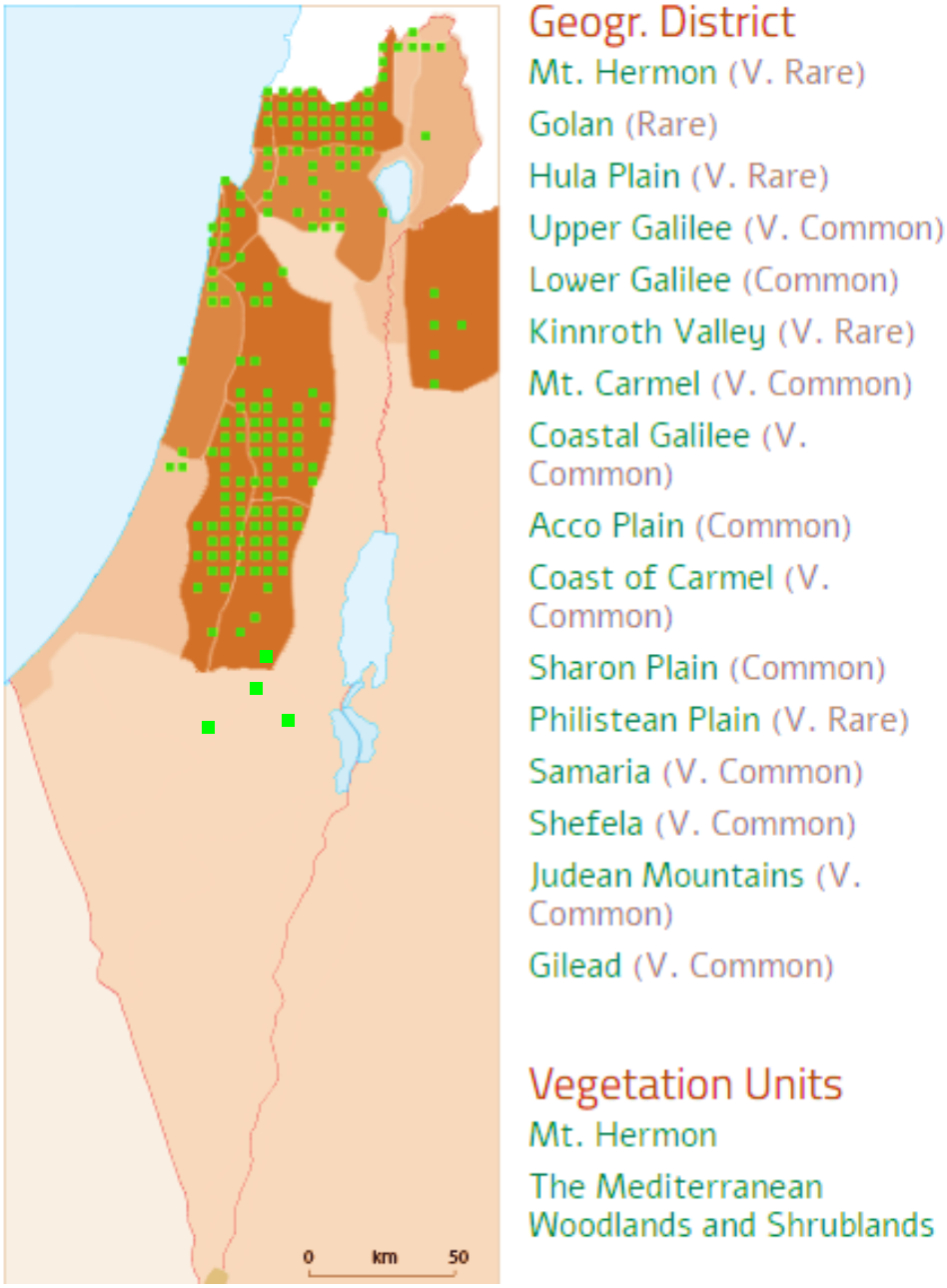


Figure 7: geographical distribution of *S. aspera* in Palestine (Danin, 2016).

1.5. In-vitro chemical assays of plant extracts:

1.5.1. Antioxidant activity

There is an increased evidence for the participation of free radicals in the etiology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, aging etc. A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS is composed of superoxide anion ($O_2\cdot$), hydroxyl (OH·), hydroperoxyl (OOH·), peroxyl (ROO·), alkoxyl (RO·) radicals non free radicals are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) singlet oxygen ($1O_2$). RNS are mainly nitric oxide (NO·), peroxynitrite (ONOO·) and nitrogen dioxide (NO2). Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Parr & Bolwell, 2000).

The antioxidant capacity is the capability of scavenging the oxygen-derived free radicals. It has often been correlated with the phenolic content (Cantin, Moreno, & Gogorcena, 2009).

The structure of phenolic compounds is a key determinant of their radical scavenging activity; for example the antioxidant activity of phenolic acids increases with increasing the degree of hydroxylation (Balasundram, Sundram, & Samman, 2006).

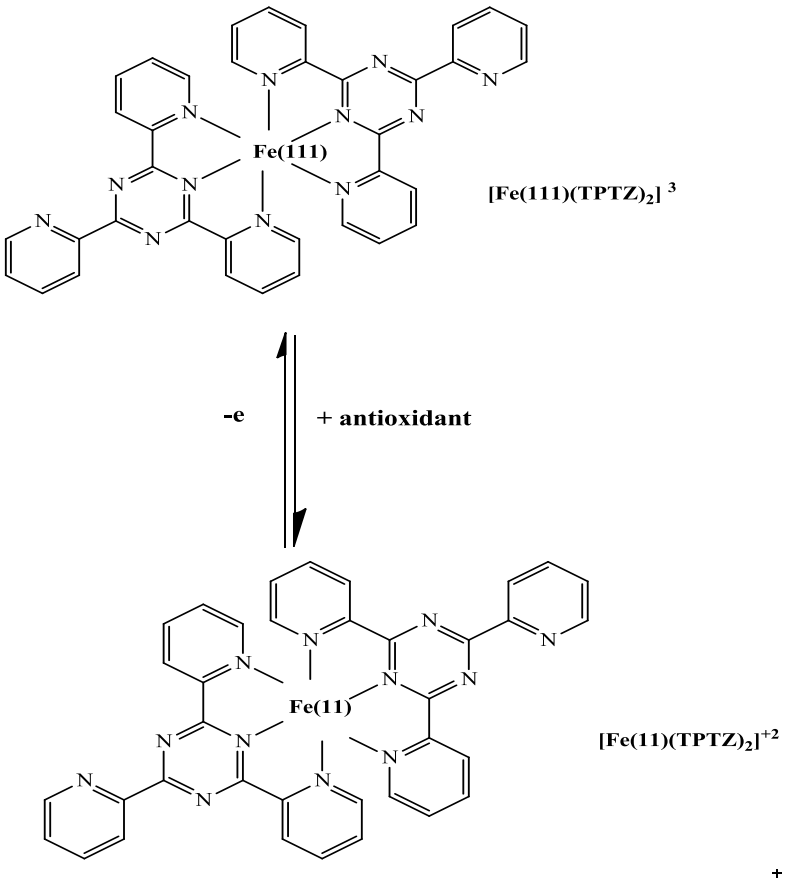
The food matrices have antioxidant properties, due to presence of a complex mixture of compounds of varying polarity, such as vitamin C, vitamin E, carotenoids, and polyphenols. Due to the potential synergistic action of all these bioactive compounds

present in food (Serafini, Bellocco, Wolk, & Ekström, 2002).

It is important to select and employ a stable and rapid method to assess antioxidant activity, to make the determination of plant extract activity less time consuming and more accurate. Several methods have been developed to assay free radical scavenging capacity and total antioxidant activity of plant extracts. The most common and reliable method involves the determination of the disappearance of free radicals using a spectrophotometer, such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). Another reliable, widely used method is measuring the reducing antioxidant power; called FRAP assay. These two selected methods have advantages over the disadvantages of other methods as CUPRAC and ABTS (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016; Badarinath et al., 2010).

1.5.1.1. FRAP method

One of the most important methods used to measure antioxidant activity of plants extract is Ferric Ion Reducing Antioxidant Power Assay (FRAP). It is simple, fast, inexpensive, robust and does not required specialized equipment. In the FRAP method the yellow Fe^{3+} TPTZ complex (2, 4, 6-tri (2-pyridyl)-1,3,5-triazine) is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances (such as phenolic compounds) under acidic conditions (Benzie & Strain, 1996). Any electron donating substances with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+} TPTZ will drive the reaction and the formation of the blue complex forward (Singh, Guizani, Essa, Hakkim, & Rahman, 2012) as shown in [Figure 8].



Figur e 8: 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, Wu, & Schaich, 2005).

1.5.1.2. DPPH method

DPPH is free radical that is stable at room temperature, which produces a violet solution in methanol or ethanol and has a lambda max at 517nm. 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, which can be quantified by its decrease of absorbance at wavelength of 517 nm **[**Figure 9] (Xie & Schaich, 2014). Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution loses color stochiometrically depending on the number of electrons taken up (Sannigrahi, Mazumder, Pal, & Mishra, 2009).

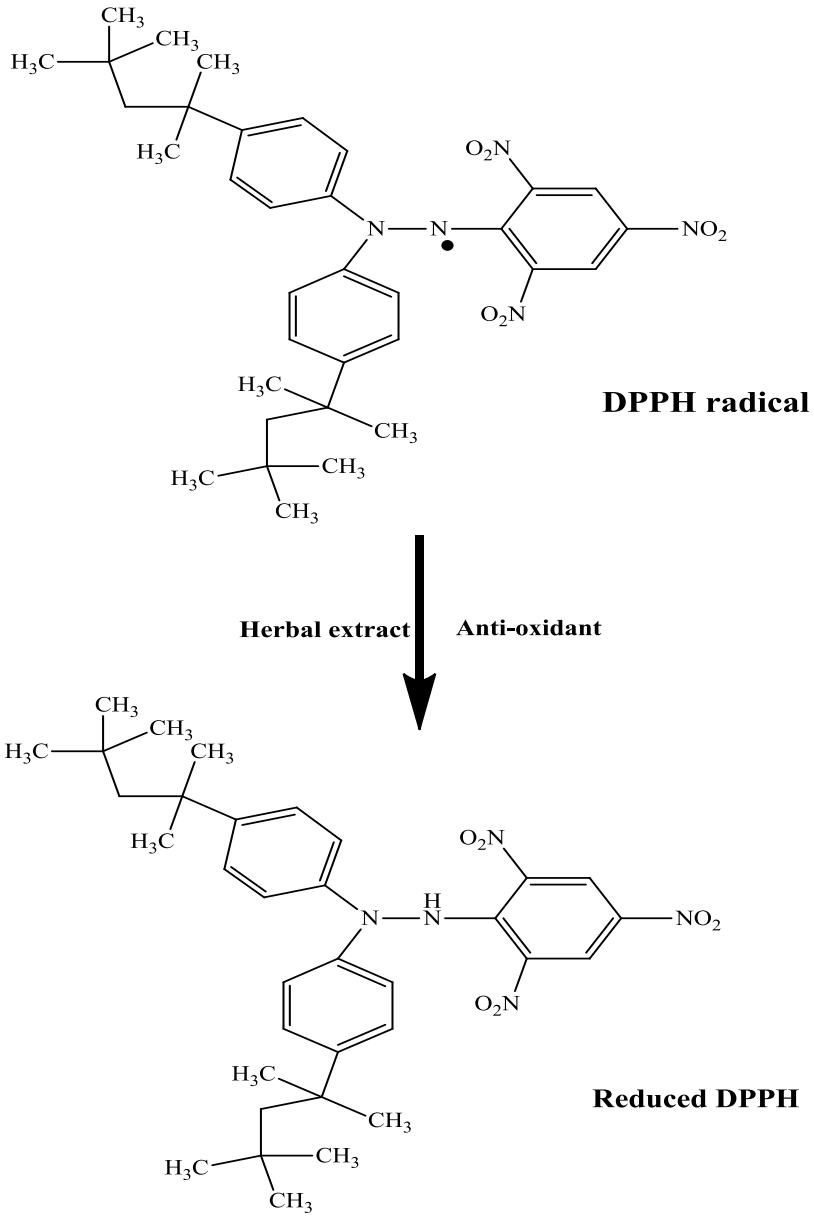


Figure 9: Mechanism of DPPH $^{\bullet}$ with an antioxidant having transferable hydrogen radical

1.5.2. Anti-bacterial activity

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their emergance. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses, not only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing countries, traditional medicine is one of the primary healthcare systems (Abdallah, 2011). Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (S.-Y. Pan et al., 2014).

According to statistics, about 61% of new drugs developed between 1981 - 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer (Cragg & Newman, 2005). Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action (Runyoro, Matee, Ngassapa, Joseph, & Mbwambo, 2006; B. G. H. Shahidi, 2004). The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world (Bala et al., 2015; Nabavi et al., 2015; Pagliarulo et al., 2016).

Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found to have antimicrobial properties in vitro (Hardoim et al., 2015; Ludwig-Müller, 2015).

Herbal medicines have been known to man for centuries. Therapeutic efficacy of many indigenous plants for several disorders has been described by practitioners of traditional medicine(Ramasamy & Manoharan, 2004). Antimicrobial properties of medicinal plants are

being increasingly reported from different parts of the world. The World Health Organization (WHO) estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population (Ekor, 2014). The harmful microorganisms can be controlled with drugs and these resulted in the emergence of multiple drug-resistant bacteria and it has created alarming clinical situations in the treatment of infections. The pharmacological industries have produced a number of new antibiotics, but the resistance to these drugs by microorganisms has increased. In general, microbial pathogens have the genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents (Towers, Lopez, & Hudson, 2001).

1.5.3. Anti-cancer activity

Despite the advancement in all fields of science and medicine, cancer still is the ambiguous, tricky, and mostly hard to treat disease. So far, there is no extremely effective drugs to treat most cancers; furthermore many cancer treatments are very expensive. Since the 1960s the National Cancer Institute (NCI) began to screen plant extracts with antitumor activity (Monks et al., 2002). Natural compounds isolated from medicinal plants, as rich sources of novel anticancer drugs, have been of increasing interest since then. For example, The five antineoplastic lead compounds: vinblastine [vincaleukoblastine], vincristine [leurocristine], podophyllotoxin, paclitaxel [taxol], and camptothecin, that represent significant contributions to natural products isolation accomplished by pioneering natural product chemists, and these have all led subsequently to considerable advances in cancer chemotherapy(L. Pan, Chai, & Kinghorn, 2012).

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, Haber, & Settleman, 2010). HeLa was the first cultured cancer line. It was derived from cervical cancer cells taken from Henrietta Lacks in 1951 (Scherer, Syverton, & Gey, 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, Bak, Hahn, & Volm, 1988).

1.5.4. HPLC-PDA and LCMS 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. The separation of different classes of phenolic compounds is achieved through the introduction of a reverse phase column, and water-acetonitrile mobile phase. Usually, photo diode array detector (PDA) is used for food phenolic compounds detection since phenolics possess strong chromophore. HPLC coupled with mass spectrometry (MS) has commonly been used for structural characterization of phenols. Electrospray ionization mass spectrometry (ESI/MS) for both the positive and negative modes has been employed for the structural confirmation of phenols in matrices (Naczk & Shahidi, 2004).

Preparative HPLC-PDA can be used to separate pure isolates out of the crude powder of the natural product. The column large capacity enable great loadability of solution to isolate (10-100 mg) of each compound separated. Further spectroscopic techniques can be utilized such as ^{1}H-NMR and 2D-NMR in the COSY and HMQC mode to further the structure of phenolic phytochemicals.

1.6. Hypotheses and Research Questions

The hypothesis of this study declares the existence of variations in TPC, TFC, antimicrobial and antioxidant activity in *E. foeminea* and *S. aspera* from Palestine and same species studied in other countries. Both plants can be used in different applications and fields, such as pharmaceutical and food.

1. Are *E. foeminea* and *S. aspera* rich in phenolic and flavonoid compounds?
2. Do *E. foeminea* and *S. aspera* extracts possess antioxidants, antimicrobial and anticancer activities?
3. What phenolics are present in the plants as per the HPLC-PDA and LCMS analysis?
4. What are the pharmaceutical applications that can be designed based on the plants in-vitro activities?

1.7. Objectives

1- To measure TPC and TFC of *E. foeminea* and *S. aspera* fruits extracts in different solvents with different polarities spectrophotometrically.

2- To measure some *in-vitro* pharmacological activities such as the antioxidants, antimicrobial, and anticancer effects of *E. foeminea* and *S. aspera* fruits extracts using different solvents with different polarities.

3- To screen the phytochemical components of both plants fruits using HPLC-PDA, LC-MS and LC-MSMS.

4- To scale up some fractions on pure isolates of the extracts that showed promising anticancer activity by using preparative chromatography.

5- Further structural elucidation of pure isolated compounds will be investigated using spectroscopic techniques.

1.8. Significance of the study

E. foeminea and *S. aspera* the two plants were chosen and collected from Palestine to study their TPC and TFC, and to investigate their anti-cancer effect, anti-microbial and anti-oxidant activities. The study will utilize state of the art instruments such as HPLC-PDA, LC-MS and LC-MSMS to acquire their identity and quantity.

In the literature, the antioxidant, antimicrobial and anticancer activities were investigated separately besides some other tests including TPC and TFC but for other genus species and from different origins.

A comprehensive work that includes all of the above mentioned studies have never been investigated collectively in one package. More importantly, it is the first time to study these plants from Palestinian wild origin. Our study will use the red-colored fruits whereas the previous studies mostly used the stems and roots parts of the plant.

1.9. Aim of the study

The main aim of the current study is to analyze and characterize the phytochemical constituents of *E. foeminea* and *S. aspera* of Palestinian origin, working specifically on the red-colored fruits, thus, excluding other parts of both plants.

Spectrophotometric method will be used to determine the identity of their total phenolic and flavonoid content. Screening of secondary metabolites types and composition will be accomplished by using cutting-edged hyphenated chromatographic methodologies such as HPLC-PDA and LC-MS and/ or LC-MSMS in the positive and negative ESi modes.

Possible pharmacological activities will be investigated. Including antioxidant, anti-microbial and anti-cancer activities.

Chapter Two

Literature review

Chapter Two:

Literature review:

2.1. Previous studies

2.1.1. *E. foeminea* plant

Antimicrobial activity of different *Ephedra* species have been studied. For example, *E. major* plays an important role as inhibitor to fungus growth (Bagheri-Gavkosh, Bigdeli, Shams-Ghahfarokhi, & Razzaghi-Abyaneh, 2009). *In vitro* antibacterial activity of *E. gerardiana* against various bacterial species such as *Staphylococcus aureus, Bacillus anthracis, B. diphtheriae, B. dysenteriae, B. typhosus* and *Pseudomonas aeruginosa* were reported. Its volatile oil showed inhibitory activity against Asian influenza virus (Ramawat & Arya, 1976; Soltan & Zaki, 2009). Moreover, Kwon *et al.* reported the antimicrobial activity of *E. sinicia* extracts against bacteria, such as *Vibrio parahaemolyticus, Clostridium perfringens, Bacillus subtilis and Staphylococcus aureus* (Kwon et al., 2001).

An anti-yeast activity of *E. intermedia* against three yeast species (*Saccharomyces cerevisiae, Candida albicans* and *C. utilis*) were also investigated (Bonjar, 2004). The growth inhibition activity of materials derived from the stems of *E. pachyclada* against intestinal bacteria was examined. Purification of the active constituent from *E. pachyclada* stems was performed and the active component was identified as quinaldic acid. Quinaldic acid derived from *Ephedra* showed a strong inhibition against *Clostridium difficile* and *Clostridium perfringens* (Lee & Lee, 2009). Another study showed that *E. strobiliacea*, *E. procera* and *E. pachyclada* have antibacterial and antifungal activity, most highly against *Pseudomonas aeruginosa (*Parsaeimehr, Sargsyan, & Javidnia, 2010).

It is worthwhile mentioning that the antimicrobial activities of all the above mentioned reports does not include *E. Foeminea species*, and the parts studied were roots and stems rather than the flavonoid-rich red fruits.

Several studies worldwide investigated the TPC and TFC of several Ephedra species including *E. campylopoda, E. major, E. distachya subsp. helvetica, E. monosperma, E. fragilis, E. foeminea, E. alata, E. altissima* and *Ephedra foliate* (Ibragic & Sofić, 2015; Mohamad et al.). Only two studies published recently in 2015 and 2017 have evaluated the antioxidant activity of *E. alata* in Palestine and determined their total flavonoid and phenol contents (Al-Rimawi et al., 2017; Jaradat, Hussen, & Al Ali, 2015).

Some studies indicated that *Ephedra* have positive influence in cancer therapy including *E. sinica* but not the *E. foeminea* (Oshima et al., 2016). Hyuga *et al* excluded the benefit of ephedrine and pseudoephedrine active ingredient of ephedra from the anticancer properties. In the contrary, they claimed it may cause side effects because ephedrine alkaloids stimulate both sympathetic and parasympathetic nerves; adverse effects may include palpitations, hypertension, insomnia, and dysuria. They concluded that the anticancer activity is produced by the non-alkaloid fraction of ephedra which contains herbacetin-glycosides and some other bioactive molecules that produce activity by synergistic effects(Hyuga et al., 2016). Linking this observation to a study published in 2015 which investigated alkaloids content of several ephedra species using UPLC-PDA emphasized that *E. foeminea* does not include ephedrine nor pseudoephedrine compounds (Ibragic & Sofić, 2015). Thus, *E. foeminea* plant could contain a promising non-alkaloid anticancer candidates with no interruption of side effects.

However, the use of Alanda (*E. foeminea*) decoction by Palestinian oncology patients was scientifically investigated by (Ben-Arye et al., 2016); who had tested Alanda decoction on

breast cancer cell lines, the results reveal that Alanda extract alone has no effect on cancer cell but its concomitant use with chemotherapy agents as carboplatin and cisplatin resulted in a dose-dependent reduction in these agents cytotoxic ability.

The anticancer effect of *E. foeminea* was further investigated by (Mendelovich et al., 2017), who concluded that the leaf ethanol extract and fruit juice of *E. foeminea* have a significant ability to reduce cancer cell viability when tested on variable cell lines with varying experimental conditions.

2.1.2. *S. aspera* plant

The TPC and TFC of *S. aspera* has been studied by (Amira et al., 2012); who have also measured the antioxidant activity using DPPH and FRAP assays besides its cytotoxic and potential anti-inflammatory activity.

The isolation of steroidal saponins, trans-resveratrol and anthocyanins from *S. aspera* has been reported (Belhouchet, Sautour, Miyamoto, & Lacaille-Dubois, 2008). The steroidal saponins possess properties such as forth formation, hemolytic activity, toxicity to fish, and complex formation with cholesterin (Kang et al., 2007). During recent years, they have attracted a growing interest owing to the range of their biological actions including anti-diabetic, anti-tumor, anti-tussive, and anti-dementia and as platelet aggregation inhibitors (Sparg, Light, & Van Staden, 2004). The genus *Smilax* approved exhibition of antioxidant and pro-apoptotic anticancer activities (Gao et al., 2011; Xu, Li, Zhang, Li, & Wang, 2005).

The antioxidant property mainly attributed to its secondary metabolites phenolic compounds such as stilbenes, flavones, flavanones, flavonols, smilasides, smiglasides and helionosides, among others, expressed as DPPH radical scavenging activity approved of species of the genus *Smilax*, as *S. bockii*(Rugna et al., 2003), *S. campestris* (T. Chen et al., 1999), *S. glabra*

(Laitonjam & Kongbrailatpam, 2010), *S. lanceifolia* (Cheng, Zhang, & Yu, 2004), *S. perfoliata* (Sun, Zhang, Han, Dong, & Wang, 2012), *S. riparia* (Zhang et al., 2014), *S. scobinicaulis* (Ao, Higa, Khanh, Upadhyay, & Tawata, 2011) and *S. sebeana* (Wu et al., 2010).

Chapter Three

Experimental Work

Chapter Three:

Experimental Work:

3.1. Chemicals, Reagents and Plant materials

3.1.1. Chemicals

Solvents and standards used were 99% ethanol, 95% methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl- S-triazine (TPTZ), ferric chloride hexahydrate, catechin, gallic acid, agar, sodium hydroxide, hydrolic acid, acetic acid, sodium nitrite, aluminum chloride, quercetin, quercetin 3-D-galactose were all purchased from Sigma-Aldrich, Israel. HT29 colon cancer cell line, Dulbecco's modified Eagle's Medium (DMEM) (10% FBS, 1% glutamin, 1% pen/strep), 6 well plates, 10% FBS (Gibco), 1% glutamine, and penicillin/streptomycin from (Biological industries, Israel). Phenolic and flavonoids standards that were used as markers in the plant identification based on their retention and UV-Vis spectra are 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 were from Sigma.

LCMS grade of formic acid, acetonitrile (ACN), methanol (MeOH) and water (H_2O) were purchased from Sigma-Aldrich, Israel. Membrane filters (0.45 μm pore size) were purchased from Sigma-Aldrich, Israel. Ultrapure water (>18 MΩ cm-1) for analytical and preparative HPLC experiments was generated from an Ultrapure Water system, Millipore, Merck-Israel. Ethanol (EtOH) solvent was purchased from Merckm Israel. The acetonitrile and water were of an HPLC grade from Sigma, Israel.

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 pH 3.6. 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 0.04 M HCl was prepared by diluting 21.8 ml of stock HCl solution (0.0917M) to 50 ml with water. Ferric chloride hexahydrated (20mM, Mwt = 270.3 g/mol) was prepared by dissolving 540mg of it 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.

3.1.3. Plant materials

The fruits of *E. foeminea* and *S. aspera* were collected. The former was from Al-Mgaier Wadi of Bani Na'em village in Hebron. The latter was from Al-Makhrour Wadi in Beit Jala upon their maturity season on the middle of October of 2016 and 2017, respectively. The two plants were botanically identified and authenticated by Botanist Dr. Khaled Sawalha, Biology Department, Al-Quds University, confirmed with the aid of The Plant List website and flora of Israel website (Danin, 2016).

3.2. Instrumentation

Specord 40 UV VIS spectrum, versatile single-beam spectrophotometer for the measurement of 190-1100 nm conforms to Ph.Eur. quality, made by analytikjena company, Rotary evaporator, inverted fluorescent microscope (Olympus CKX 41), laboratory water bath, ultrasonic homogenizer and autoclave.

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). The full scan UPLC-MS analysis via a chromatographic column or direct to the API of the crude samples using in the Electrospray Ionization (ESI) was carried out using a TSQ Endura™ Triple Quadrupole Mass Spectrometer LCMSMS system (Thermo Fisher Scientific). All data acquisition and analysis were performed using Trace finder software, version 4.1, TSQ-Endura and TSQ Quantiva, Xcalibur Quan browser software version 3.0 (Thermo Fisher Scientific). The Preparative High-Pressure Liquid Chromatography (Prep-HPLC) system consisted of 3535 quaternary gradient module equipped with 996 PDA detectors.

3.3. Methodology

3.3.1. Preparation of plant materials and extraction

The fruits of both plants samples were air dried for nearly one month, grinded with a blender. Ten grams of the dried powder of both plants were macerated with 100 ml of different solvents (distilled water (D.W.), 80% ethanol (EtOH), 99% EtOH), extracted on a ultra-sonication bath for 90 min at 37^{o}C, and then filtrated using disposable filter of PTFE membrane, pore size 0.45 µm. Then the crude extracts were stored in Refrigerator at 4^{o}C until analysis.

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

Total phenolics were determined using Folin-Ciocalteu reagents (Singleton & Rossi, 1965). The extracts (40) µl were mixed with 1.8 ml of Folin-Ciocalteu reagent (pre-diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) were added to the mixture. After standing for 60 min at room

temperature, absorbance were 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 GAE/ g sample (Shui & Leong, 2006). This experiment was performed in duplicate to ensure the accuracy of the results and the average absorbance for each extract was taken for calculations.

3.3.3. Total flavonoids content (TFC)

The determination of flavonoids was performed according to the colorimetric assay (Choi 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. This experiment was performed in duplicate to ensure the accuracy of the results and the average absorbance for each extract was taken for calculations.

3.3.4. Measurement of antioxidant activity by FRAP assay

The antioxidant activity of the extracts was determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of (Benzie & Strain, 1996). 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) (FeSO4.6H2O) were used for calibration. This experiment was performed in duplicate to ensure the accuracy of the results and the average absorbance for each extract was taken for calculations.

3.3.5. Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of extracts of fruits of *E. foeminea* and *S. aspera* plants were measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) (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:

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

where A_0 is the Absorbance of control reaction and A_1 is the Absorbance in presence of test or standard sample. This experiment was performed in duplicate to ensure the accuracy of the results and the average absorbance for each extract was taken for calculations.

3.3.6. Antimicrobial activity

The antimicrobial activity was tested against different types of microorganisms:

Staphylococcus aureus, E. coli and *Candida albicans*. Positive control used (Penicillin & Gentamicin) for bacteria but no positive control for fungus.

Antibacterial activities of the extracts were studied by using well diffusion technique which depends on diffusion of the sample tested from a vertical cylinder through a solidified agar layer in a plate.

In this method, the Muller Hinton media was prepared by mixing 11.4 g agar in 300mL distilled water, and nutrient broth prepared for each microorganism. The media boiled and then sterilized at 121 °C for 15 minutes. After sterilization, the media cooled, then at 45 °C the suspension of each microorganism added separately to the nutrient broth. Both gram positive (Staphylococcus aureus) and gram negative (Escherichia coli) bacteria and yeast (candida albicans) were tested using UV-Spectrophotometer until suitable concentration is reached. The Muller Hinton media was then distributed to plates (20 mL/plate), after the media solidified, four holes with a diameter of 1 cm were made using sterile cylinder. 100 µL of each extract was placed in each hole for each plate. A positive control disk was placed on the agar as well. The plates incubated at 37 ± 0.5 °C for 24 hours. After incubation period, the zone of inhibition was measured by a caliper.

3.3.7. Anticancer activity

To test the possible anticancer effect of *E. foeminea* and *S. aspera* , colon cancer cells (HT-29) cell lines were treated with four different concentrations (100, 50, 10 and 1 microgram) from the stock solution of 80% EtOH extract of both plants. The procedure was as follows: 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, and mixed by hand. 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 45^0C) 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. The cells was counted under microscope.

3.3.8. HPLC & LCMS analysis of phenolic compounds

3.3.8.1. Sample preparation for analytical HPLC analysis

The plant extracts were filtered using suction filtration, and then the solvents were evaporated under reduced pressure at 40^0C using Rotary evaporator. The resulting crude extracts were dissolved in the respective solvents (water, ethanol, and 80% ethanol) at a concentration of 5 mg/mL, and 20 µL were injected into the HPLC chromatograph, and analyzed for their phenolics and flavonoids. Seventeen phenolic and flavonoid standards were injected and separated simultaneously to identify the presence of any of these compounds in the crude extracts. Calibration curve of each individual standard was also prepared at three concentration levels namely 50, 100 and 250 ppm.

3.3.8.2. 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).

3.3.8.3. HPLC chromatographic condition

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

3.3.8.4. Preparative HPLC-PDA conditions

The crude extract was obtained by macerating (1:10) wt/vol of dried plant fruits absolute ethanol, sonicated for 2 hrs at room temperature. The resultant extract was evaporated using rotary evaporator at 40°C under reduced pressure. The final extract was stored in amber vial and kept in the fridge until fractionated by preparative HPLC. Prior injection, the solution (~0.1g/10 ml 80% EtOH) was filtered using disposable Polytetrafluoroethylene PTFE filters of 0.45 μm. The preparative HPLC experiments were run on Atlantis® T_3 Prep OBD™, 100 A^o, 10 μm, 19 x 250 mm column supported with Atlantis® T_3 Prep, 5 μm, 19 x 20 mm guard column. The gradient program started from 98% acidic water (0.5% acetic acid): 2% acetonitrile to 98 % acetonitrile in 58 minutes and back to the starting mobile phase composition in 2 minutes. The flow rate was 15 ml/minutes, the injection volume was 1000

µl, the column temperature was set at room temperature and the PDA wavelengths ranged from 210-600 nm.

3.3.8.5. LC-MS chromatographic condition

The UHPLC-MS chromatography were performed under reverse phase conditions using a TSQ Quantum Access MAX (Thermo Scientific, San Jose, CA, USA) which includes a Dionex Pump with a degasser module, an Accela PDA detector and an Accela Autosampler. The chromatographic separation were performed on a Kinetex™ column (C8, 2.6 µm particle size, 100 Å pore size, 100 x 2.1 mm) from Phenomenex, USA. A UHPLC SecurityGuard™ cartridge (C8, for 2.1 mm ID column) was from Phenomenex, USA. The injection volume was 2 µL, the oven temperature was maintained at 35°C.

3.3.8.6. Direct injection to LC-MS

A direct injection LC-MS analysis of all the preparative HPLC collected aliquots (pure compounds) were performed in both the positive and negative electrospray ionization (ESi) modes using the full scan mode and the product ion scanning mode. The mobile phase consisted of 70% acidic water (0.1% formic acid): 30% acetonitrile at flow rate of 50µl/minute. The MS conditions were as follows:

1. For the negative ESi experiments, the ion voltage was 2500 volts, ion transfer tube temperature was 320^0 C, full scan range was 150-750 Dalton (Da), quadrupole resolution was 0.7 full width at half maximum (FWHM), sheath gas 25 L/min, aux gas 10 L/min and scan rate was 1000 Da per second.

2. For the positive ESi, the ion voltage was 3500 volts, ion transfer tube temperature was 325^oC, vaporizer temperature 275^0 C only for the product ion scanning experiments, full scan

range was 150-750 Dalton (Da), quadrupole resolution was 0.7 full width at half maximum (FWHM), sheath gas 35 L/min, aux gas 10 L/min, sweep gas 1L/min and scan rate was 1000 Da per second.

3. For the product ion scanning (PIS) experiments in the the negative ESi mode, the ion voltage was 2500 volts, ion transfer tube temperature was 320^0 C, vaporizer temperature 320^oC, full scan range was 150-750 Dalton (Da), quadrupole resolution was 0.7 full width at half maximum (FWHM), sheath gas 25 L/min, aux gas 10 L/min, sweep gas 2 L/min, scan rate was 1000 Da per second and collision energy of 15, 25 and 35 volts with CID gas (in Torr) of 1.5.

Chapter Four
Results and Discussion

Chapter Four:

Results and Discussion:

4.1. Ultrasonic extraction

To the best of our knowledge, there is no optimal extraction protocol that has been developed in the past for phenolic extraction from *E. foeminea* nor *S. aspera* fruits.

In this work, in order to maximize the recovery yield of the phenolic compounds from the fruits extracts, the dried fruits were grinded prior extraction into a fine powder to ensure the reduction of particle size, and three different ratios of ethanol to water were selected. Ethanol and water was chosen because both are safer and less toxic when compared to acetone, methanol or any other organic solvents. Moreover, ethanol/ water binary mixture can easily extract phenolics of polar to semi-polar nature

It is believed that extended extraction time would lead to exposure of more oxygen and thus increase the chances for occurrence of oxidation on phenolic compounds (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007; Naczk & Shahidi, 2004), and thus would affect antioxidant capacity when measured using DPPH and FRAP assays.

Further investigation performed by (Chew et al., 2011) indicated no significant change in (p<0.05) after 180 min. This had explained this phenomenon of Fick's second law of diffusion which states that final equilibrium will be attained between the solution concentrations in the solid matrix and solvent after a particular duration (Pinelo, Sineiro, & Núñez, 2006). Therefore, as crucial as it is to minimize energy and cost of extraction process our extraction time was within a range of 120-180 min.

Sonication was the method of choice since it rupture the cell membrane hosting the secondary metabolites of the fruits. It is simply based on applying sound energy to which the frequency

of the sound used breaks the cell walls and facilitate the release of secondary metabolites to the solvent.

4.2. Total phenolic content (TPC)

Many studies of phenolic compounds have reported that the environmental, climatic, or geographical factors as well as extraction techniques may significantly influence the quality and the quantity of phenolic components present (Ibragic & Sofić, 2015; Ozkan, Yumrutas, Saygideger, & Kulak, 2011; Pourmorad, Hosseinimehr, & Shahabimajd, 2006).

TPC of different extracts of *E. foeminea* and *S. aspera* was determined by using the Folin-Ciocalteu reagent and were expressed as mg gallic acid equivalents (GAE) per gram of plant extract. The TPC of the test fractions were calculated using the standard curve of gallic acid. Linear fit was obtained with an equation $y = 0.0027x + 0.1172$ and R^2 value of 0.9988 [Figure 10].

Results showed that the TPC for both plants extracts using different solvents (D.W, 99% EtOH, 80% EtOH) have different quantities [table 2]. The results were found to follow the following trend: D.W > 80% EtOH > 99% EtOH for both plants.

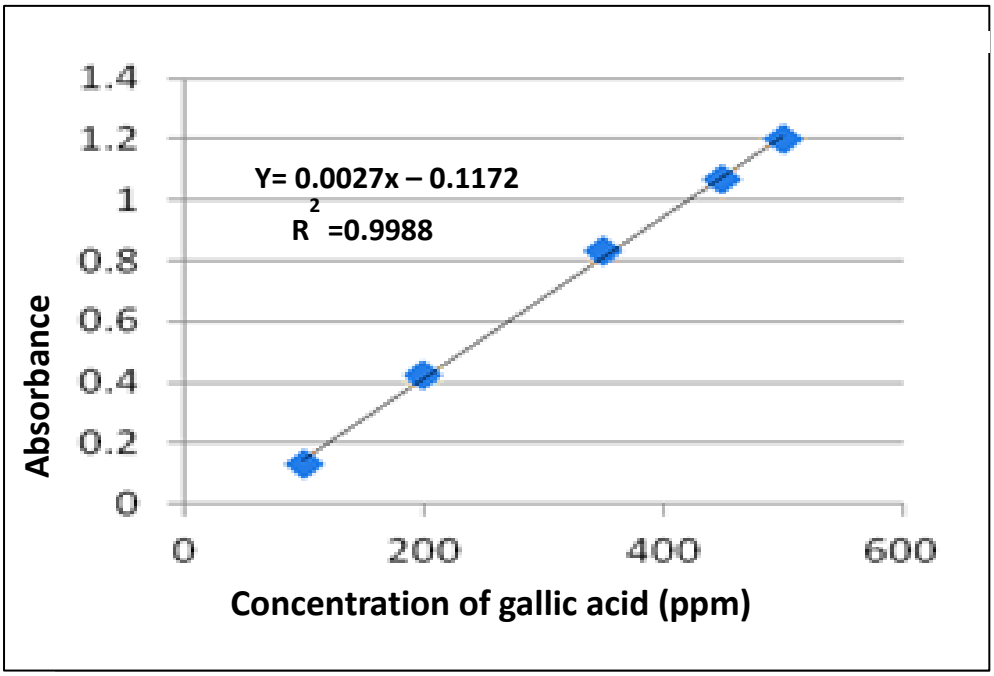


Figure 10: Calibration curve of TPC (absorbance of different concentrations of gallic acid vs. concentration (ppm))

Table 2: TPC (mg Gallic acid/ g of dry sample) of *E. foeminea* and *S. aspera* samples.

Sample		mg Gallic acid/ g sample
E. foeminea	99% EtOH*	1.5
	80% EtOH	1.87
	D.W**	3.22
S. aspera	99% EtOH	4.56
	80% EtOH	5.36
	D.W	8.21

* EtOH: ethanol. ** D.W.: distilled water

From the results in [table 2], it is evident that water percent played a key role in extracting phenolics; which indicate the polar nature of the phenols present in the fruits.

4.3. Total flavonoid content (TFC)

The calorimetric assay was used to determine the TFC of different extracts of *E. foeminea* and *S. aspera* plants. Total flavonoid contents was calculated using the standard curve of Catechin. Linear fit was obtained with an equation y = 0.0048x + 0.0034 and R^2 value of 1 [Figure 11], and were expressed as mg Catechin per gram of the plant extract.

The TFC results of *E. foeminea* and *S. aspera* are represented in [table 3]. The values are not following the same trend as in TPC. For *E. foeminea*, the highest concentration was found in 99% EtOH and the lowest was in D.W., while for *S. aspera* the highest concentration was found in 80% EtOH and the lowest was in 99% EtOH.

This observation indicates the polar nature of the flavonoids in both fruits to which *E. foeminea* comprises more apolar compounds in comparison to more polar of *S. aspera*.

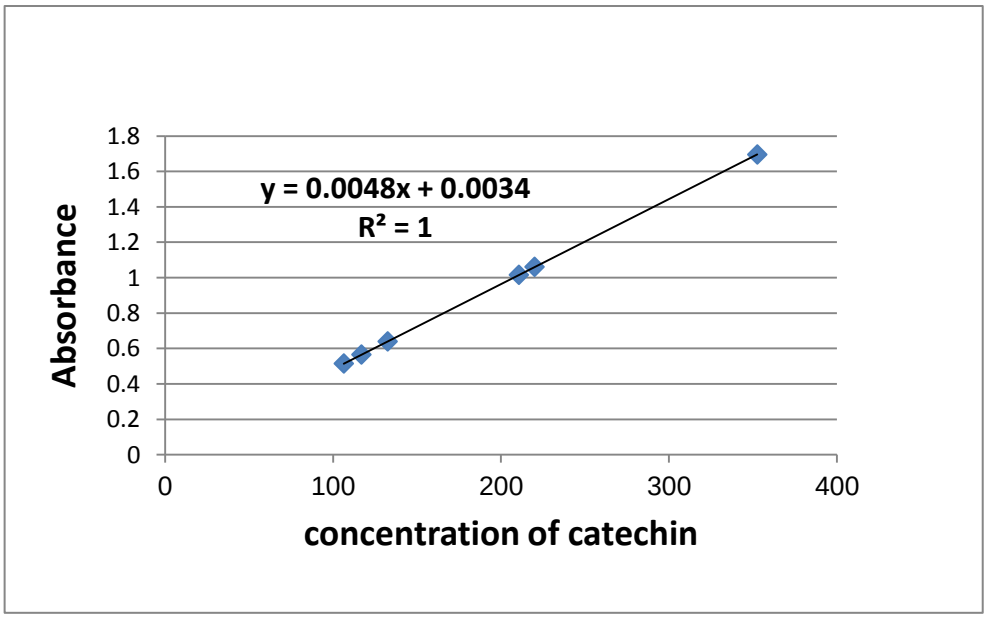


Figure 11: Calibration curve of TFC (absorbance of different concentrations of Catechin vs. concentration (ppm).

Table 3: TFC (mg Catechin/g of dry sample) of *E. foeminea* and *S. aspera* samples

Sample		mg catechin/ g sample
E. foeminea	99% EtOH*	1.0433
	80% EtOH	0.1527
	D.W.**	0.217
S. aspera	99% EtOH	0.736
	80% EtOH	1.2245
	D.W.	0.8287

* EtOH:ethanol. ** D.W.:distilled water.

In comparison with previous studies in the literature surveys, concerning the TPC and TFC in individual *Ephedra* species and *Smilax* species, the results were variant. For example, (Ibragic & Sofić, 2015) determined the TPC and TFC in ten of *Ephedra* species including *E. foeminea* using the same method, but using different extracting technique. They determined the highest and lowest TPC and TFC among the studied species and *E. Foeminea* was among the lowest with TPC of 6.8 mg GAE/g sample and TFC of 0.6 mg CE / g sample.

4.4. Antioxidant activity

The anti-oxidant capacity of plant extracts largely depends on their composition and the conditions of the testing system used. Because many factors play a role, the effects of the extracts cannot be wholly described with one single method. It is thus necessary to perform more than one type of antioxidant capacity measurement to get a full understanding of the various mechanisms of antioxidant action. In this study, the antioxidant activity of the extracts was evaluated on the basis of the following methods: the analysis of their scavenging effects with regard to the DPPH and the testing of their ability to reduce ferric (III) iron to ferrous (II) iron in the FRAP reagent.

4.4.1. Free radical scavenging activity by DPPH

The free radical scavenging activity is studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple color dye having maximum absorption of 515 nm and upon reaction with a hydrogen donor, the purple color fades or disappears due to conversion to 2, 2-diphenyl-1-picryl hydrazine resulting in a dramatic decrease in absorbance. The DPPH scavenging activity of the fruits extracts was calculated using the standard curve of concentration of Trolox (ppm) [Figure 12]. Linear fit was obtained with an equation $y = -0.0026x + 0.7803$ and R^2 value of 0.9991)

For *E. foeminea,* results of mg trolox\ g sample were found to increase as follows: 99% EtOH > 80% EtOH > D.W. with values of 9.512, 9.188 and 2.62 respectively. For *S. aspera*, however, 99% EtOH > D.W. > 80% EtOH with values of 5.956, 1.62 and 2.772 respectively. It was shown that the 99% EtOH extract has the highest values in *E. foeminea* and *S. aspera* alike as shown in [table 4].

The same trend for both plants was achieved regarding the percent inhibition, with quiet high values for *E. foeminea* 99% and 80% EtOH extracts to be 91.3% and 90.2% respectively.

In general, the ethanolic extracts of both plants showed a high radical scavenging activity than the aqueous extracts; as *S. aspera* had its highest percent of inhibition in 99% EtOH of 78.96%.

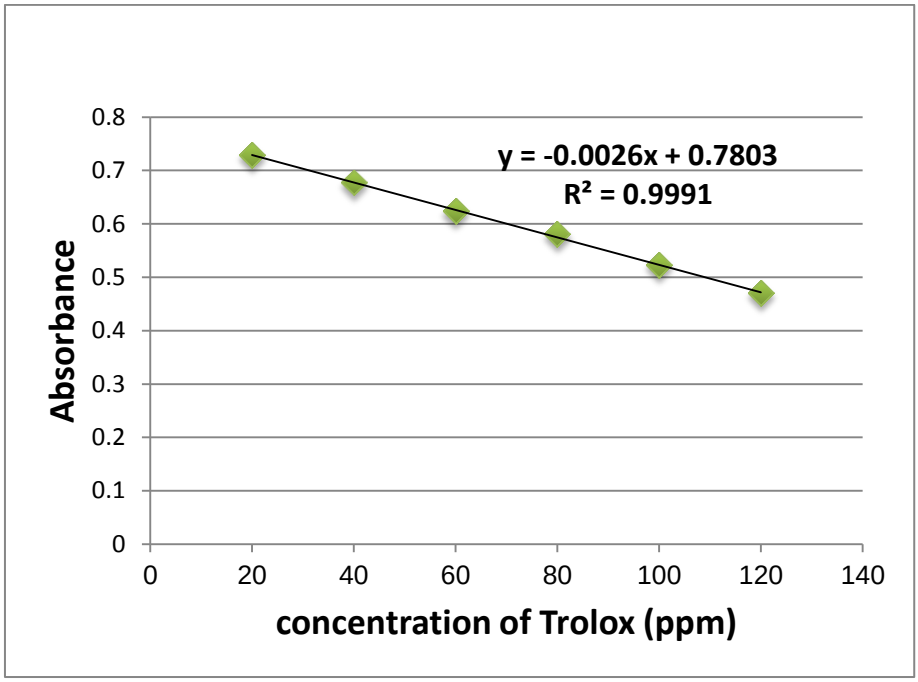


Figure 12: Calibration curve of DPPH (Absorbance of different concentrations of Trolox vs. concentration (ppm))

Table 4: DPPH free redical scavenging activity (mg trolox /g of dry sample) and % inhibition of *E. foeminea* and *S. aspera* samples

Sample		mg Trolox \ g sample	% inhibition
E. Foeminea	99% EtOH*	9.512	91.3%
	80% EtOH	9.188	90.2%
	D.W.**	2.62	67.34%
S. aspera	99% EtOH	5.956	78.96%
	70% EtOH	1.62	63.86%
	D.W.	2.772	67.88%

* EtOH: ethanol. ** D.W.: distilled water

Comparing our results of DPPH of ***S. aspera*** with that found by (Amira et al., 2012); who extracted their ***S. aspera*** fruits in D.W. the values are almost the same 2.77 mg trolox /g of dry sample and 3 mg trolox /g of dry sample respectively.

To our knowledge, no study in the past has measured the antioxidant activity for this species of *Ephedra*, the *foeminea*.

4.4.2. **Antioxidant activity by FRAP assay**

Ferric Ion Reducing Antioxidant Power Assay (FRAP) is simple, fast, inexpensive, and robust method, and does not require specialized equipment. In the FRAP method the yellow Fe^{3+} TPTZ complex (2,4,6-tri (2-pyridyl)-1,3,5-triazine) is reduced to the blue Fe2+ TPTZ complex by electron-donating substances (such as phenolic compounds) under acidic conditions.

The antioxidant activity of ethanol and aqueous plant extracts of *E. foeminea* and *S. aspera* were evaluated by FRAP method and were expressed as mg Fe^{+2} per gram of plant extract. It was calculated using the standard curve of concentration of Fe^{+2} [Figure12]. Linear fit was obtained with an equation $y = 0.2019x - 0.1766$ and R^2 value of 0.9822.

Both plants, the *E. foeminea* and *S. aspera* results were found to increase in the following pattern: D.W. > 80% EtOH > 99% EtOH with values of 0.64, 0.57 and 0.49 mg Fe+2/ g sample for *E. foeminea* respectively and 0.61, 0.67 and 0.71 mg Fe+2/ g sample for *S. aspera* respectively as shown in [table 5].

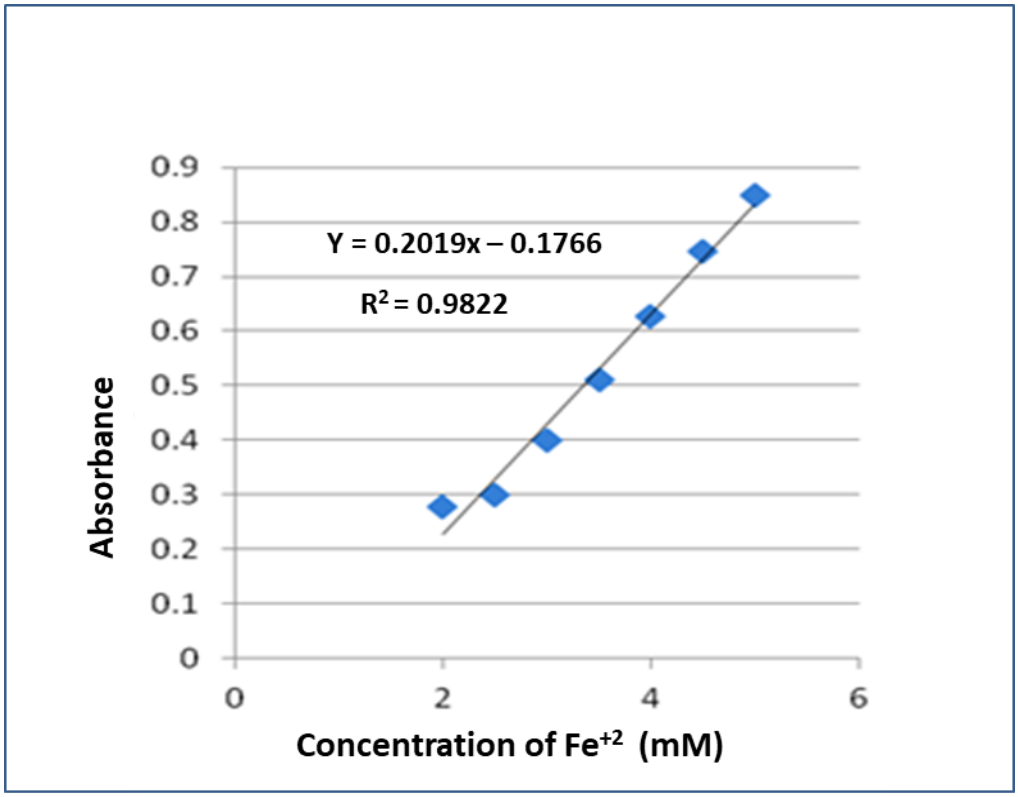


Figure 12: Calibration curve of Fe^{+2} Standard

Table 5: Antioxidant activity FRAP (as mg Fe^{+2} /g of dry sample) of *E. foeminea* and *S. aspera* samples

Sample		mg Fe^{+2}/ g sample
E. foeminea	99% EtOH*	0.49
	80% EtOH	0.57
	D.W.**	0.64
S. aspera	99% EtOH	0.61
	80% EtOH	0.67
	D.W.	0.71

* EtOH:ethanol. ** D.W.:distilled water

Several studies have evaluated the relationships between the anti-oxidant activities of plant products and their phenolic contents. However, these relationships are difficult to explain on the basis of quantitative analysis alone. In our work, the extract of *S. aspera* as an example, showed a high scavenging activity for DPPH, and a quiet good ability to reduce the FRAP reagent with relatively low concentrations of total phenols and flavonoids. This relatively high total antioxidant activity for an extract with low phenolic content suggests that the type of phenolics may play the key role rather than their amounts. Flavonoids and anthocyanins described by (Montoro et al., 2006) have been reported to exert higher anti-oxidant capacity than the phenolics described for the other species. Our results are also in agreement with (F. Shahidi & Naczk, 2003) who reported that differences in the anti-oxidant activities of plant extracts could be due to different qualitative and quantitative compositions of their phenolic constituents.

4.5. Antimicrobial activity by well diffusion technique

The antibacterial activity of *E. foeminea* and *S. aspera* was studied against gram positive bacteria (Staphylococcus aureus), gram negative bacteria (Escherichia coli), and yeast (candida albicans) in different extractions. The test on each species was performed in duplicate to insure the reliability of the results. The zone of inhibition for each plant in different solution is depicted in [table 6] and the photos of the test results is in [table 7].

Results show that *S. aspera* has an activity against gram positive bacteria (Staphylococcus aureus) with a zone of inhibition 20 mm with the 99% EtOH extract and the same with 80% EtOH extract, but no effect was noticed with *S. aspera* water extract nor with the three extracts of *E. foeminea*. When *S. aspera* results are compared with the positive control (Penicillin G), a huge difference is obvious. This significant difference can be due to the minor antibacterial effect of *S. aspera*, or it could be due to the un-unified experimental conditions; that is the use of the positive control "disk" within the well diffusion method of the plant extracts solution. Each method has its own distinct conditions (Balouiri, Sadiki, & Ibnsouda, 2016). Experimental conditions for test and control should be as close as possible so that the conclusion of results can be valid; therefore the diffusion of the positive control into the agar is likely to be greater than that of the extracts solutions.

S. aspera show a good inhibition zone against the yeast Candida albicans in all the extracts (99% EtOH, 80% EtOH, and D.W) with values of 20mm, 19mm, 13mm respectively. No comparison with positive control was performed as we intended to use Nystatin but it was not available in the laboratory.

On the other hand, *E. foeminea* extracts (99% EtOH, 80% EtOH, and D.W) did not show any effect against the yeast Candida albicans. As the 15mm zone of inhibition shown in plate B is surely the ethanol effect (16mm) according to the noticeable poor candida growth on that

plate, the other plate indicated the result that (no anti-yeast activity of *E. foeminea*) [photo 2 in table 7].

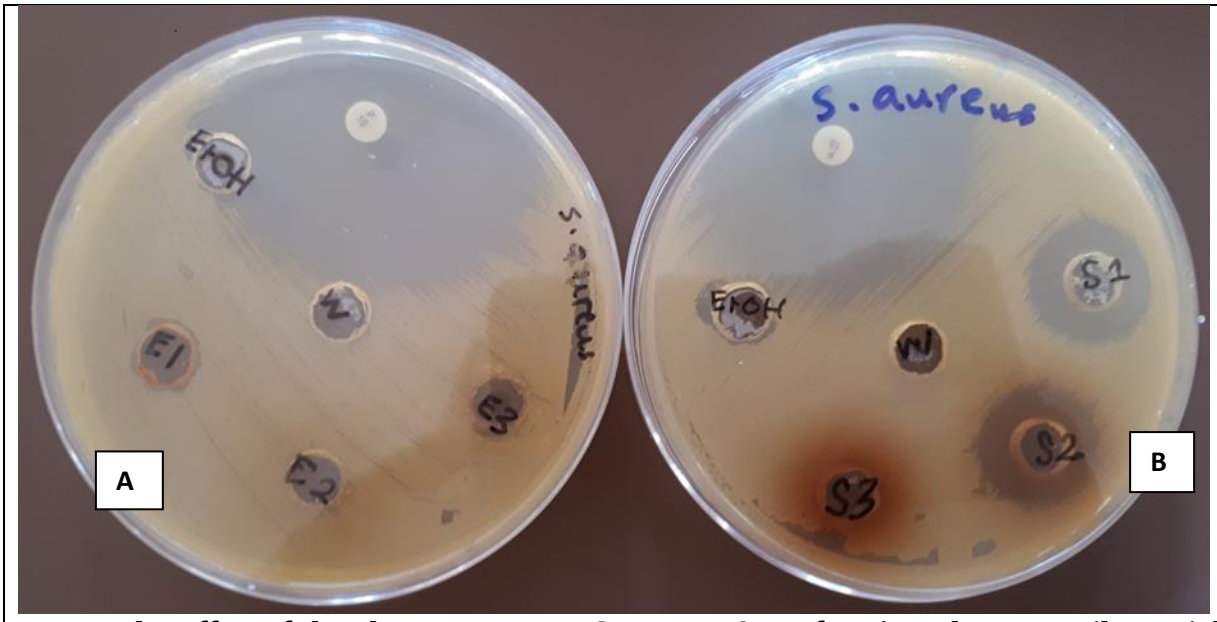
None of the extracts of both plants show positive effect against gram negative bacteria (*E.Coli*), which means that the *S. aspera* fruit are effective only against gram positive bacteria in specific extracts, while Gentamicin inhibit in a zone of 24mm as shown in table 6. Lastly, it should be noticed that solubility and diffusion of active components in agar media could play a major role in evaluating the antimicrobial capability of plants (Vlietinck, 1991).

Table 6: Zone of inhibition of *E. foeminea* and *S. aspera* samples against Staphylococcus aureus, Candida albicans, and E.Coli, corresponding to positive control [Penicillin G, Gentamicin, and Nystatin].

Bacterium type	**Zone of inhibition**			
	Solvent	***E. foeminea***	***S. aspera***	**Ref. (std.)**
Staphylococcus aureus	99% EtOH	ND*	20 mm	Penicillin G 50 mm
	80% EtOH	ND	20 mm	
	100% D.W.	ND	ND	
	Blank (EtOH)	ND	ND	
	Blank (D.W.)	ND	ND	
Candida albicans	99% EtOH	15 mm	20mm	Nystatin NT**
	80% EtOH	ND	19 mm	
	100% D.W.	ND	13 mm	
	Negative control (EtOH)	16 mm	ND	
	Negative control (D.W.)	ND	ND	
E.Coli	99% EtOH	ND	ND	Gentamicin 24 mm
	80% EtOH	ND	ND	
	100% D.W.	ND	ND	
	Blank (EtOH)	ND	ND	
	Blank (D.W.)	ND	ND	

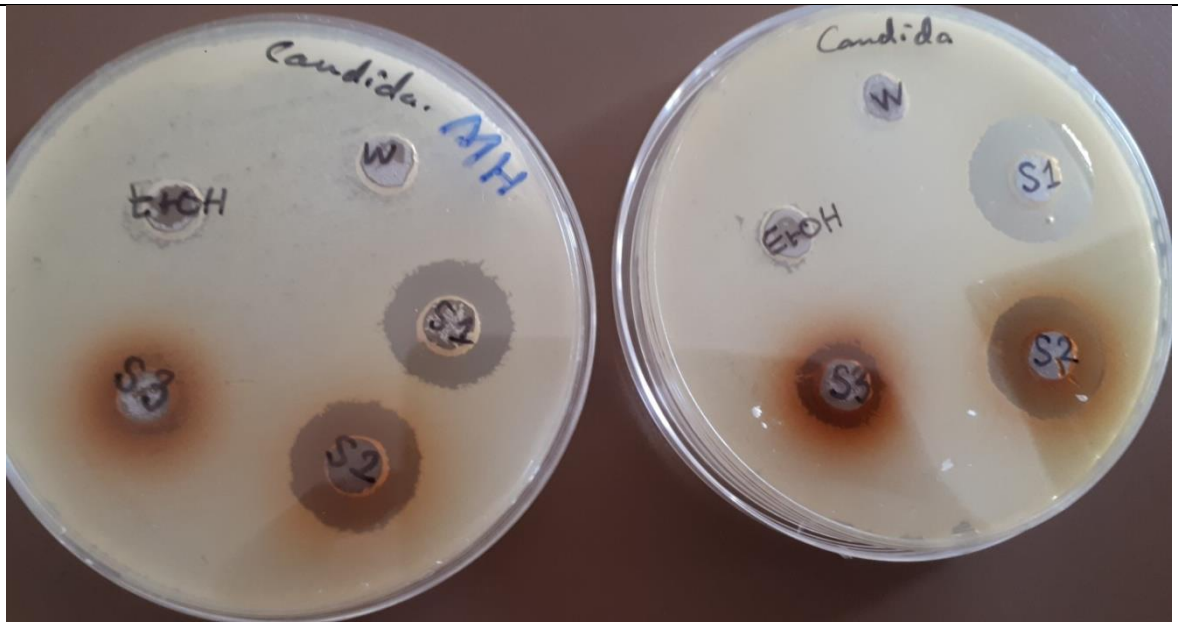
***ND= Not Detected (no effect), **NT= Not Tested.**

Table 7: The antibacterial test of *E. foeminea* and *S. aspera* fruit extracts against Staphylococcus aureus, Candida albicans, and E.Coli. The abbreviations on plates refers to: E1, E2 and E3: *E. foeminea* extracts (99% EtOH, 80% EtOH, and D.W) respectively. S1, S2 and S3: *S. aspera* extracts (99% EtOH, 80% EtOH, and D.W) respectively

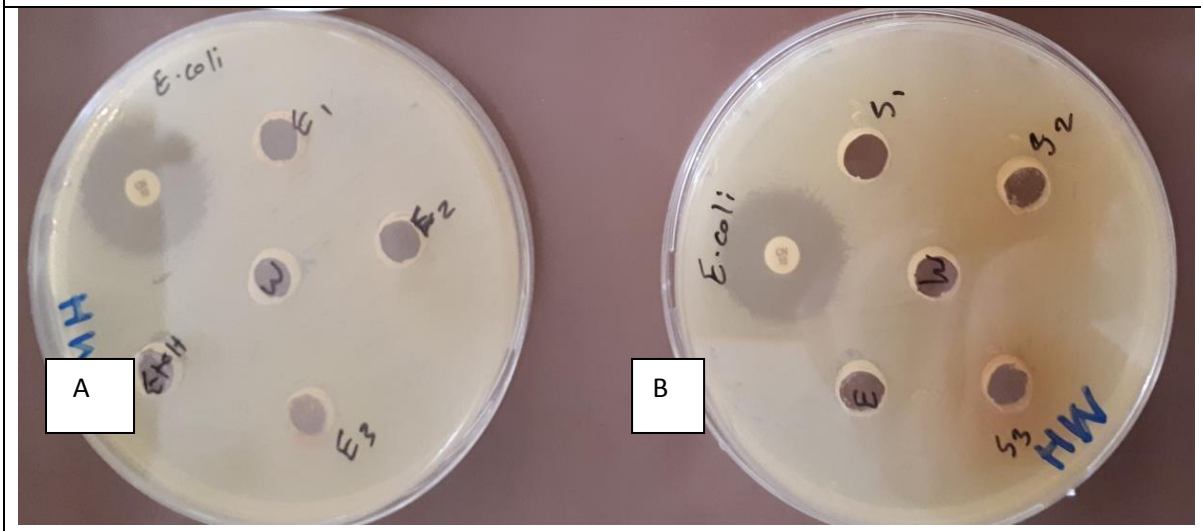


1- **The Effect of the plant extracts on *S. aureus*. A: *E. foeminea* has no antibacterial effect, B: Two extracts of *S. aspera* (S1 and S2) have antibacterial effect while the (S3) has no effect.**

2- **The effect of *E. foeminea* extracts on *Candida albicans*: B refers to False positive result due to poor candida growth, A is the right result.**



3- The Effect of *S. aspera* on Candida albicans.



4- The zero effect of both plants extracts on E. coli. , A is *E. foeminea* plate, B is *S. aspera* plate

*** EtOH/ E: Ethanol as negative control. W: Distilled water as negative control. MH: Muller Hinton agar.**

4.6. Anticancer activity

The extracts of both plants were expected to induce cancer cell death therefore having an anticancer effect due to their phenolic diversity. To test this effect, HT29 cells (colon cancer) were treated with four different concentrations from both 80% EtOH of each plant (100, 50, 10 and 1 microgram from the stock solution 100 milligram per 1 ml EtOH). It was noticed, that the higher concentrations of extracts did not show any observed effect on cell density at 24 and 48 hours. Moreover, no effect were seen at the low concentration (10 microgram) at 72 hrs. No detached or floated dead cells were noticed [figure 13 &14]. This is probably attributed to the synergistic effect of certain lipophilic compounds present in the plant that could be extracted out using alcoholic solvents.

Over all, the results are considered preliminary at this stage and should be checked on other cell lines using different concentrations to reach the optimal concentration that will kill the cells.

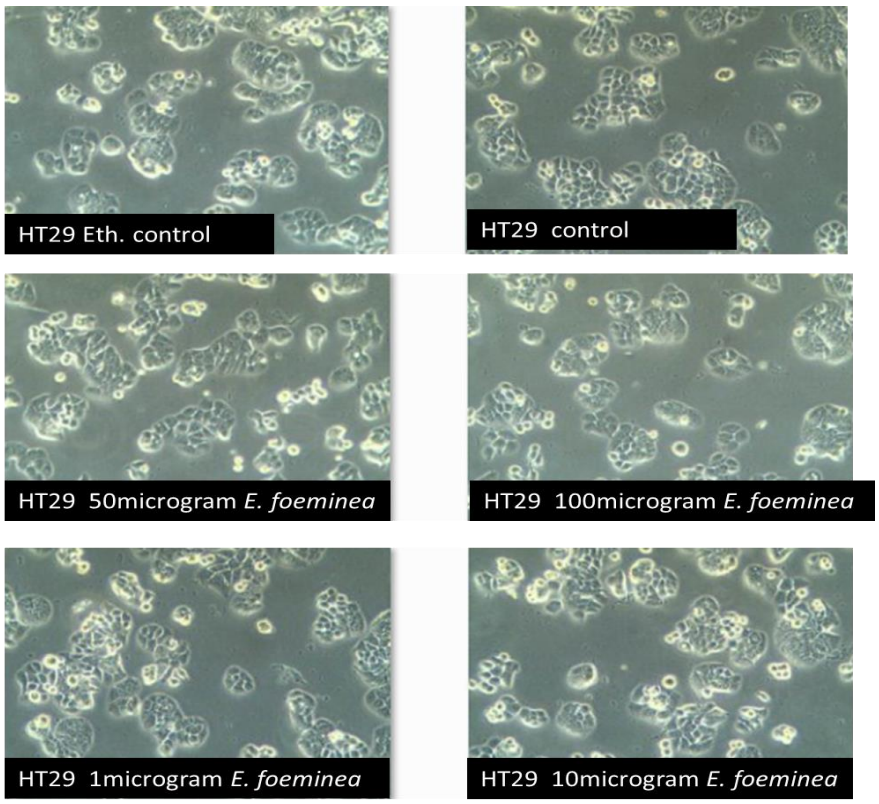


Figure 13: HT29 cells treated with *E. foeminea* extracts and control, after a period of 48 hours.

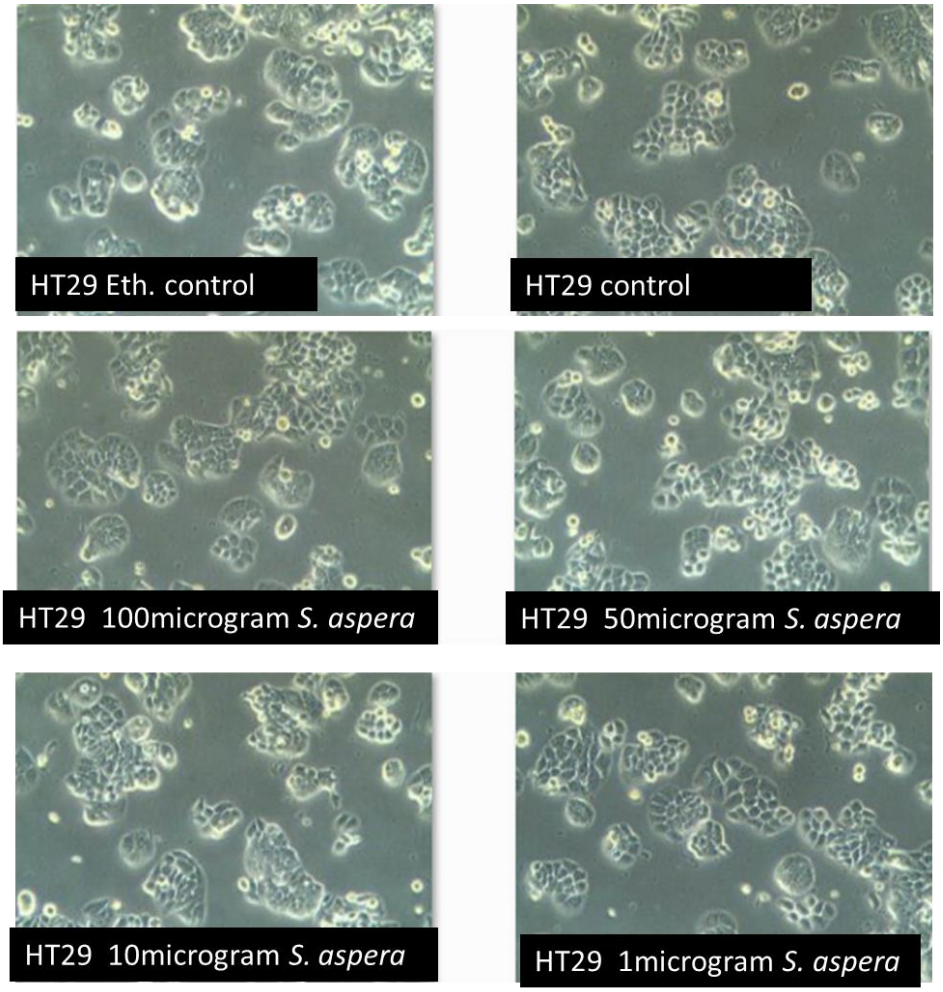


Figure 14: HT29 cells treated with *S. aspera* extracts and control, after a period of 48 hours.

4.7. HPLC Analysis

4.7.1. *E. foeminea*

Three extracts were prepared and ran into the HPLC. Figure 13 shows the chromatogram of a concentration of 5mg/ml of *E. foeminea* 80% EtOH fruit extract at 350 nm. This wavelength was selected since the main peaks showed a maximum absorption close to it. As seen from this chromatogram, different compounds were emerged in the range of 16-32 minutes. The compounds showed maximum wavelength at 216 and 255 nm. While when moving to a wavelength of 348 nm, a peak in the 31 minutes was detected [figure 15]. From the UV-Vis spectra, the compounds showed an absorption of about 255 nm and 342-348 nm. These absorptions are typical indication of flavonoid and phenolic compounds presence.

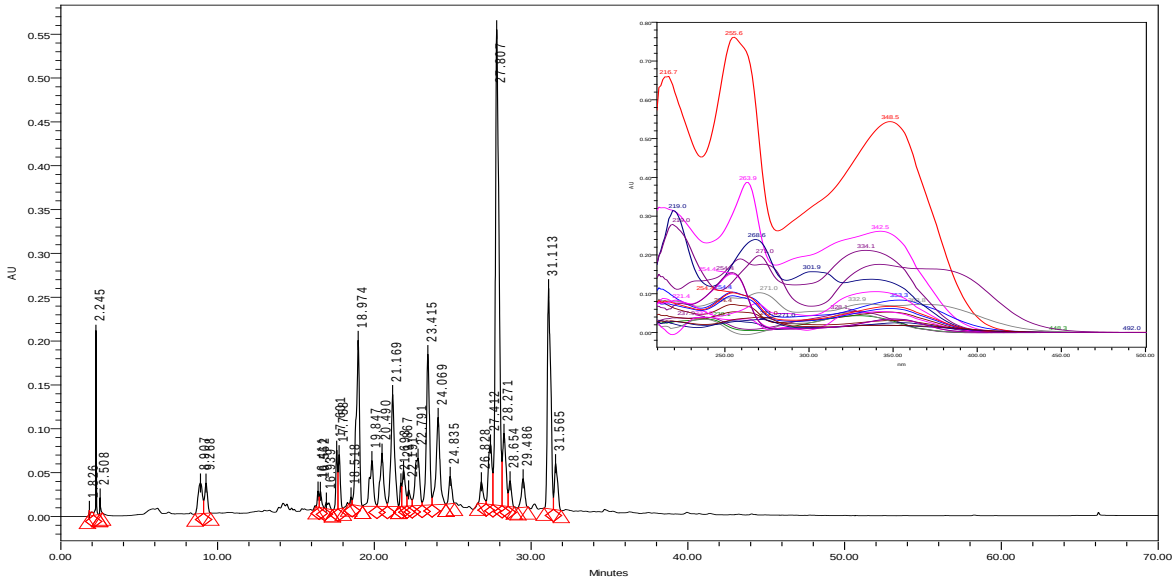


Figure 15: HPLC-PDA chromatograms of 80%EtOH crude extract of *E. foeminea* at 350 nm. The overlaid UV-Vis spectra of the main peaks are in the left side of the chromatogram.

Figure 16 shows the chromatogram of 100% water extract of *E. foeminea* with their relevant UV-Vis spectra in the range of 200-500 nm.

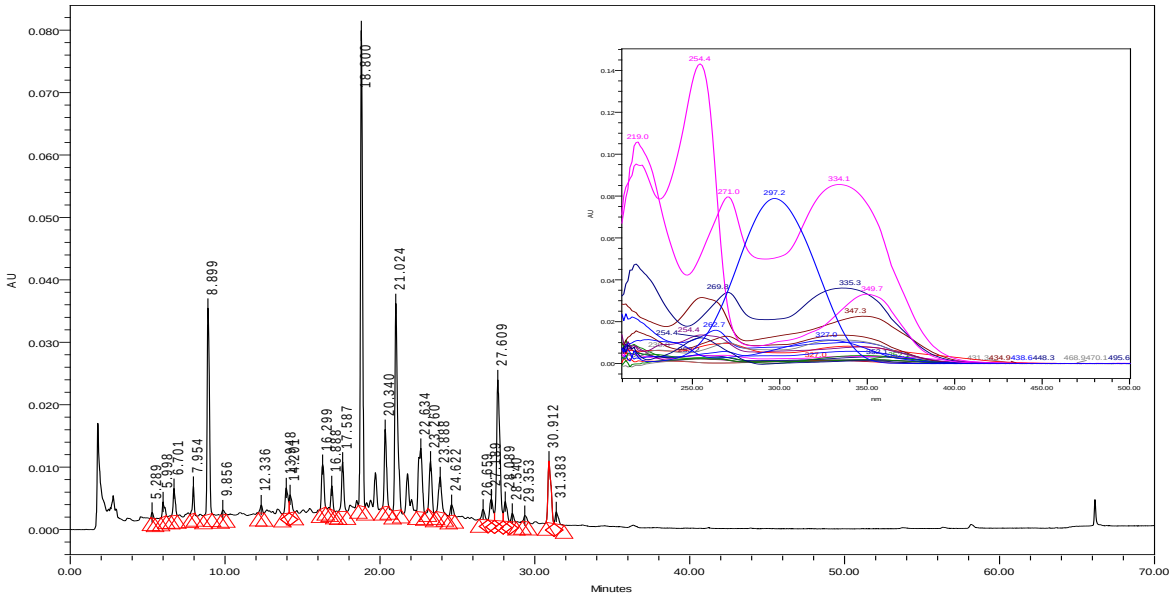


Figure 16: HPLC-PDA chromatograms of 100% water crude extract of *E. foeminea* at 350 nm. The overlaid UV-Vis spectra of the main peaks are in the left side of the chromatogram.

Figure 17 shows the chromatogram of 100% EtOH extract of *E. foeminea* with their relevant UV-Vis spectra in the range of 200-500 n.

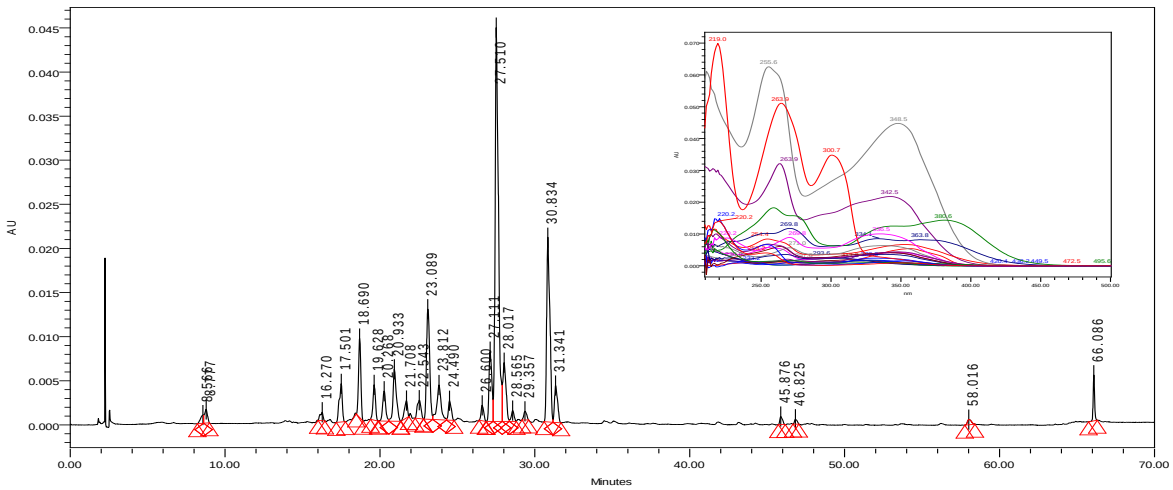


Figure 17: HPLC-PDA chromatograms of 100% EtOH crude extract of *E. foeminea* at 350 nm. The overlaid UV-Vis spectra of the main peaks are in the left side of the chromatogram.

Figure 18 shows overlaid chromatogram of three *E. foeminea* extracts**.**

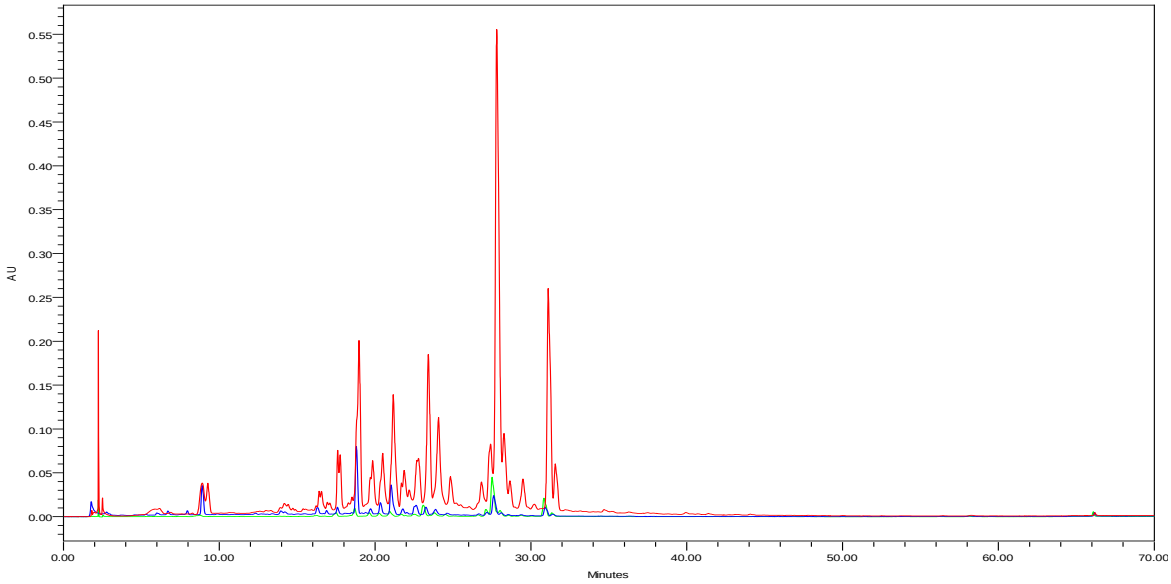


Figure 18: Overlaid chromatograms of the three *Ephedra* extracts together at the same 350 nm wavelength. Red =80% EtOH, blue = 100% water and green =100% EtOH

As it appeared from figure 18, we can conclude that the 80% EtOH is the superior solvent for extracting *Ephedra* fruits secondary metabolites.

4.7.2. *S. aspera*

Figure 19-21 shows the typical chromatograms of 5mg/ml crude *S. aspera* fruit extract sample dissolved in 80% EtOH, 100% water and 100% EtOH as an extracting solvents respectively.

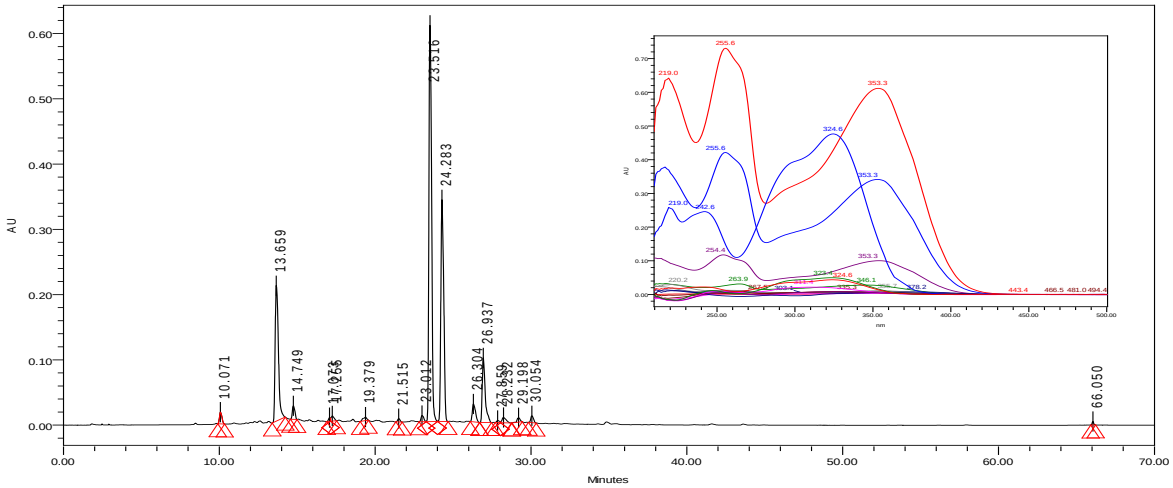


Figure 19: HPLC-PDA chromatograms of 80%EtOH crude extract of *S. aspera* at 350 nm with an overlaid UV-Vis spectra of the main peaks

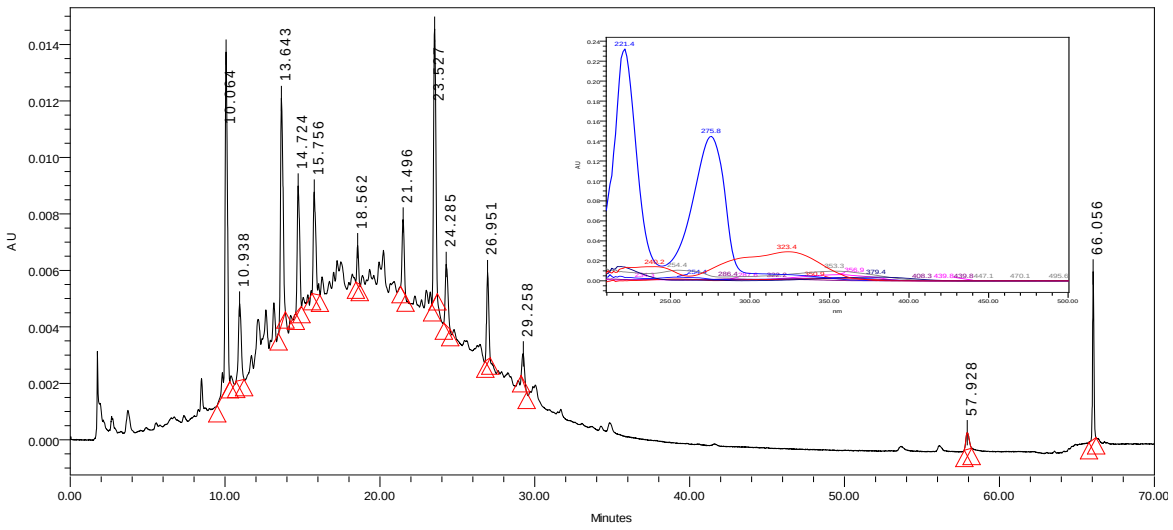


Figure 20: HPLC-PDA chromatograms of 100%water crude extract of *S. aspera* at 350 nm with an overlaid UV-Vis spectra

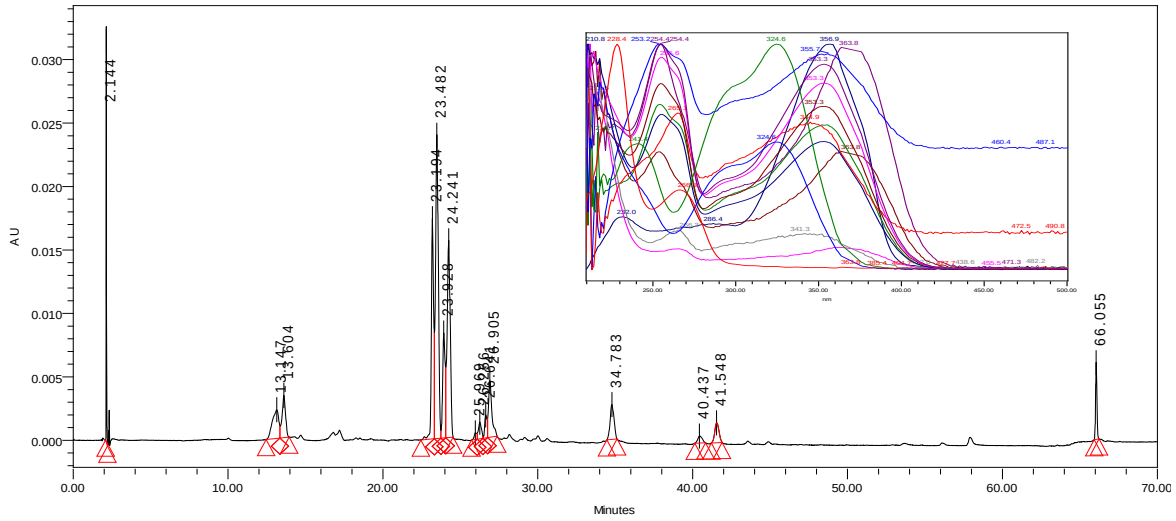


Figure 21: HPLC-PDA chromatograms of 100%EtOH crude extract of *S. aspera* at 350 nm with an overlaid UV-Vis spectra of the main peaks.

The 80% EtOH again is the superior solvent for extracting the *S. aspera* fruit containing phenolic compounds and flavonoids.

4.7.3. Samples chromatograms with standards overlay

Figure 22 shows an overlay of the chromatograms of *E. foeminea* 80% EtOH with 17 standard phenolics at 350 nm. The low absorption of the standards is probably because they absorb less at 350 nm wavelength.

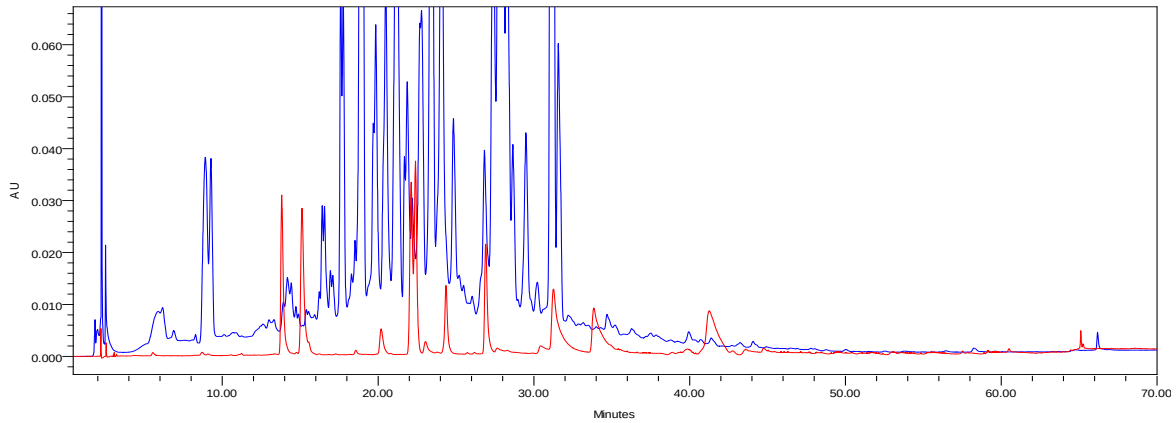


Figure 22: Overlay of *E. foeminea* 80% EtOH with the 17 standard samples at 350 nm. Red = stds and blue = *E. foeminea* 80% sample

The following chromatogram is the overlay of the chromatograms of *S. aspera* 80% EtOH with the 17 standard at 350 nm.

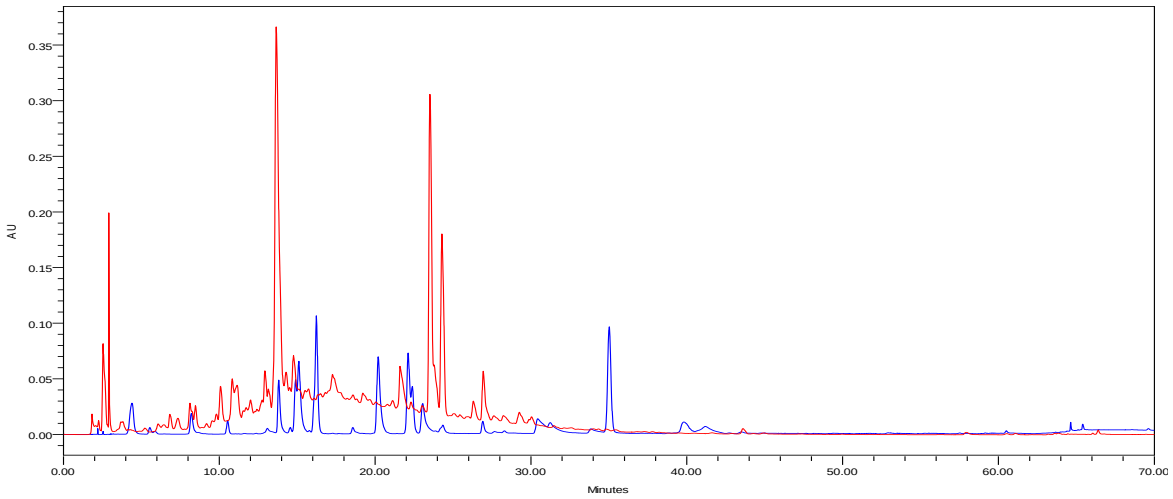


Figure 23: The overlay of the chromatograms of *S. aspera* 80% EtOH with the 17 standard samples at 350 nm. Red = stds and blue = *S. aspera* 80% EtOH.

We noticed no matching according to the stored UV-Vis spectra of all the 17 standards with the eluted peaks from both plants.

4.6. LCMS analysis

4.6.1. *E. foeminea*

To preliminary elucidate the structure and identity of the secondary metabolites of the crude extracted plants, a full scan LCMS in positive and negative ESI modes was performed based on the following TIC figure 23. Each extracted LCMS spectrum of certain retention time (R_t) showed in the TIC gave m/z corresponding to certain compound exist in *E. foeminea* fruit extract. Figure 24 is one of the MS spectrum acquired from the LCMS experiment. All the compounds identified from this test, with their relevant retention time are addressed in table 8.

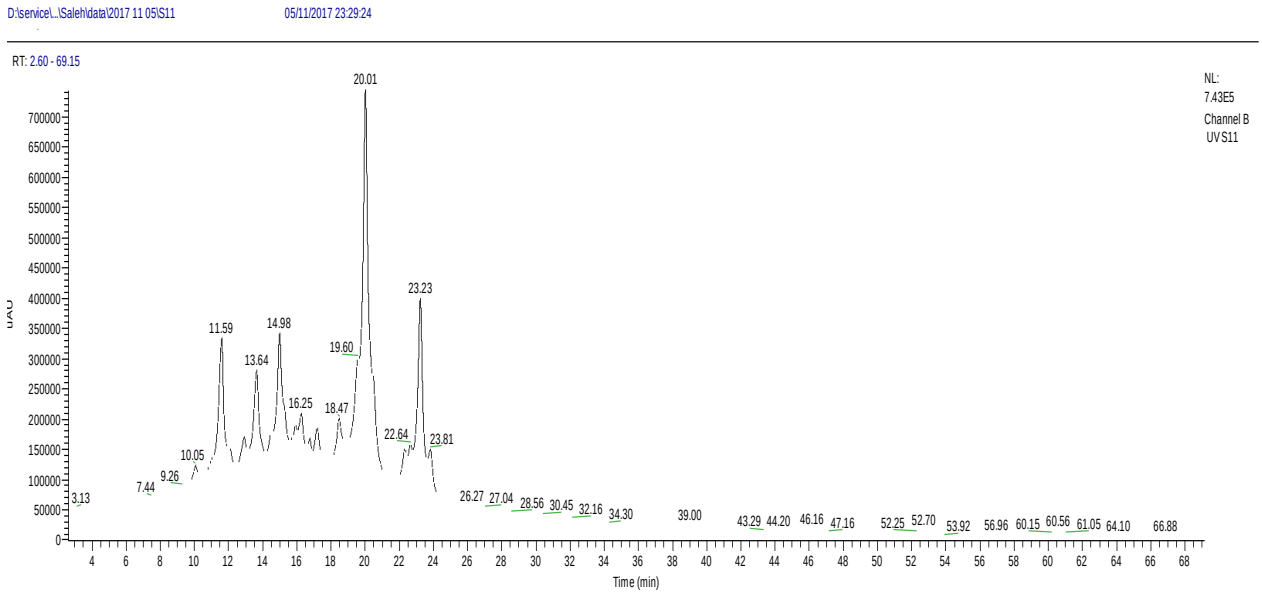


Figure 23: Full scan TIC of *E. foeminea* crude extract.

Table 8: Compounds from LCMS in +ve and –ve ESI of *E. foeminea,* and their relevant retention time appearance.

Compound name	**m.wt. (g/mol)**	**M-1**	**M+1**	**R_t (min)**
Vicenin-2	594.5	593	595	11.59
Vicenin-1	564	563	564	13.64
Quercetin 3-O-arabinoside	434	433	435	16.25
Ephedradine A	492.62	491	493	16.25 20.1
Gallocatechin	306	305	307	17.3
Lucenin 2	610.52	609	611	17.3
	580.49	579	581	
cyanin (cyanidin 3,5-di-O-glucoside)	611.52	610	612	17.3
quercetin 3-O-rhamnoside	448	447	449	17.3
Quercetin 3-O-glucoside	464	463	465	18.47
Kaempferol-3-rhamnoside	431	430	432	23.23
Myricetin 3-rhamnoside	464	465	463	23.81

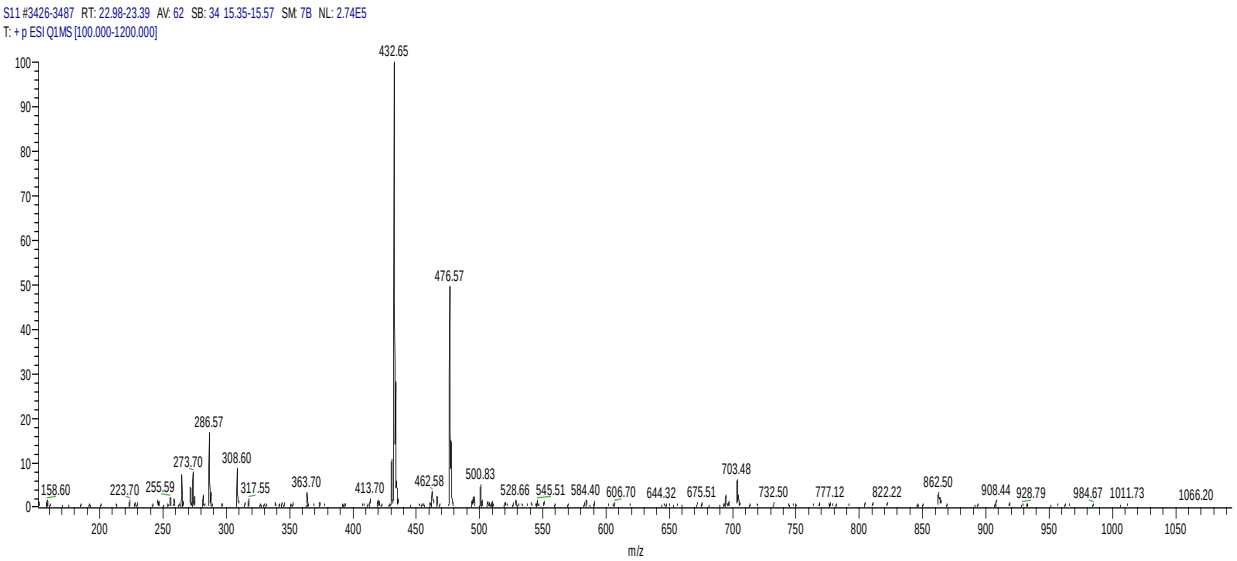


Figure 24: Example of the LCMS spectrum of Kaempferol-3-rhamnoside *from E. foeminea* (at R_t 23.23min).

4.6.2. *S. aspera*

The *S. aspera* fruits extract of LCMS (positive and negative ESI modes) was performed as seen in the TIC [figure 25]. Figure 26 is an example of the MS generated. All the expected compounds with their relevant retention time are addressed in table 9.

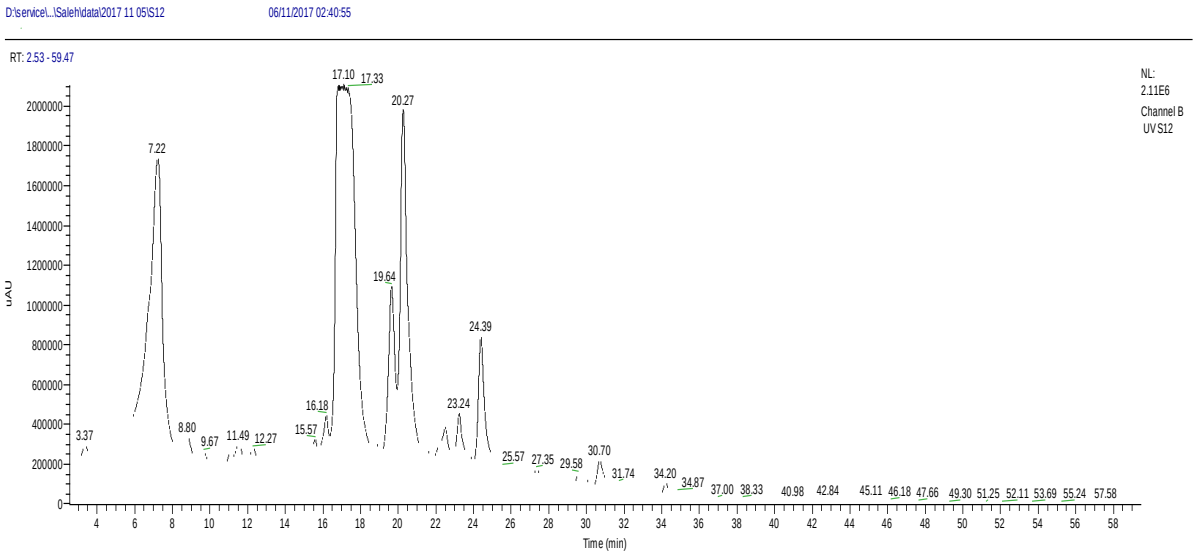


Figure 25: The chromatogram of *S. aspera*.

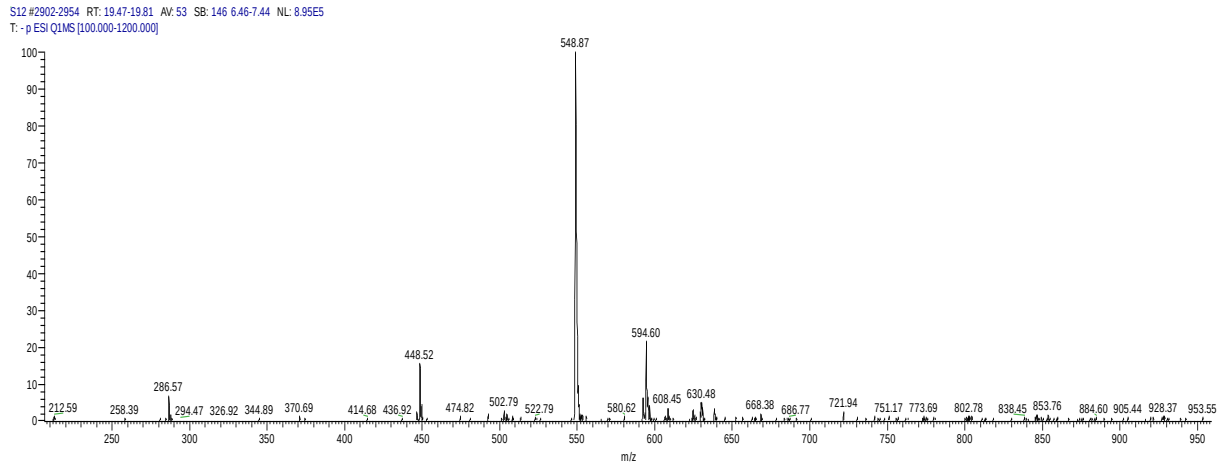


Figure 26: Example of the LCMS spectrum of Cyanidin 3-O-rutinoside of *S. aspera* (at R_t 23.23min).

Table 9: Expected compounds from LCMS in +ve and –ve ESI of *S. aspera*, and their relevant retention time appearance

Compound name	m. wt. (g/mol)	m-1	M+1	R_t (min)
Chlorogenic acid	354.31	353	355	7.22
Pelargonidin 3-O-glucoside	468.839	467	469	7.22
all-trans-β-cryptoxanthin caprate (C10:0)	706	705	707	7.22
Cyanidin 3-O-rutinoside	595	594	596	8.80 19.64
Quercetin 3-O-arabinoside	434.353	433	435	9.67 11.49
quercetin pentoside	302.238	301	303	9.67 16.18
quercetin-3-O-β-D-xylopyranoside	434.08	433	435	9.67
Lucenin 2	610.52	609	611	16.18
Cyanin (cyanidin 3,5-di-O-glucoside)	611.529	610	612	16.18
kaempferol 3-O-rhamnoside	432.381	431	433	23.24

4.7. Preparative HPLC-PDA

4.7.1. *E. foeminea*

The injection of 1000 µl of *E. foeminea* at 350 nm to the C18 preparative HPLC column equals 100-mg of crude material distributed into about seven major peaks as seen in [figure 27].

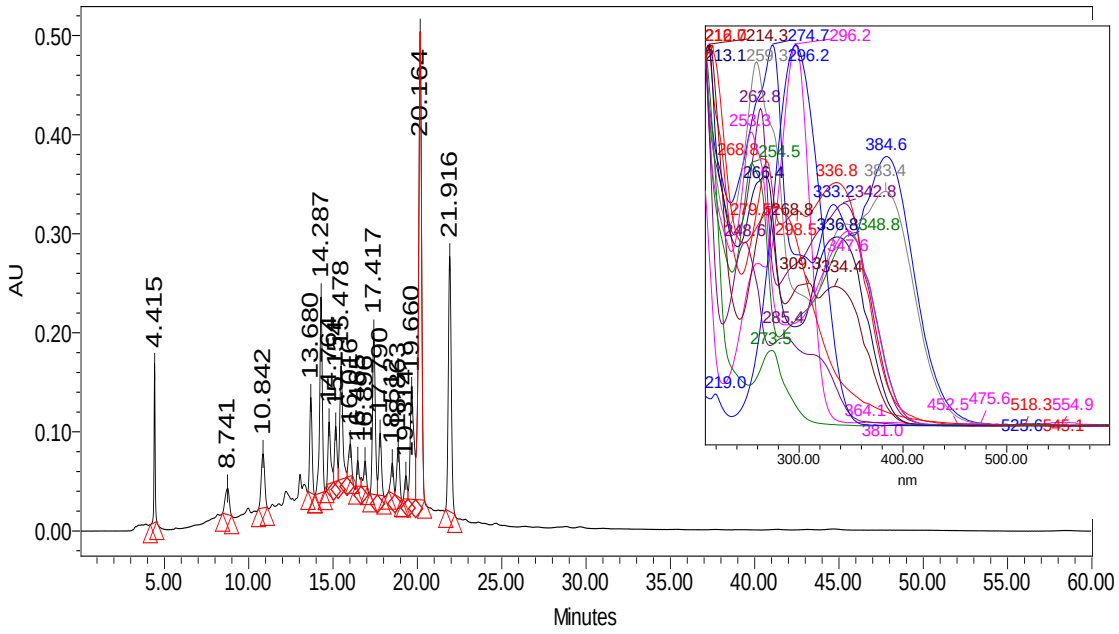
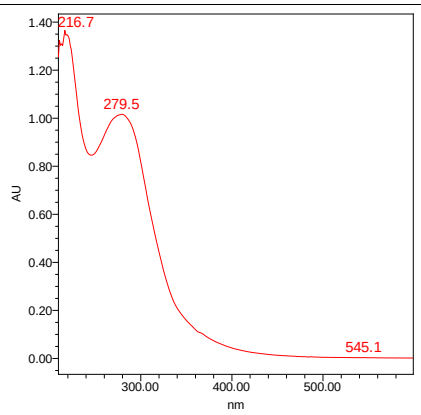
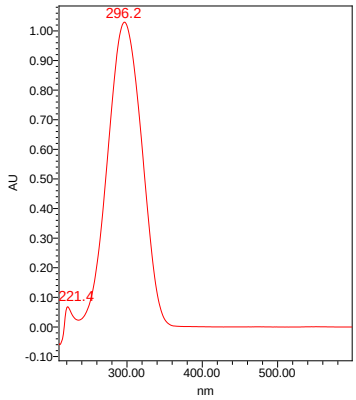
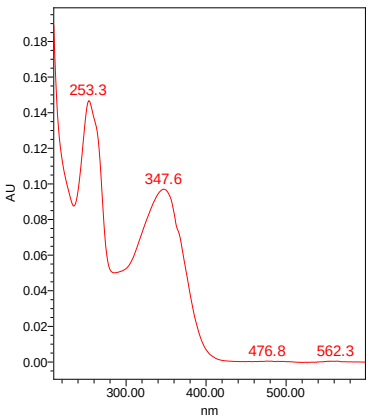


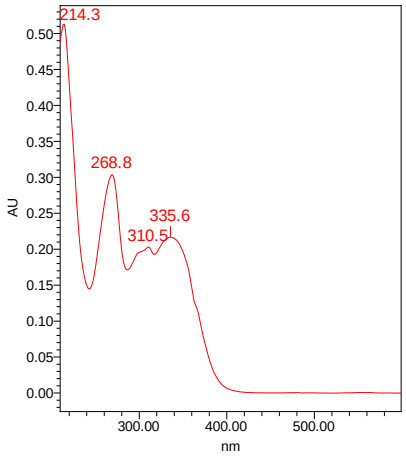
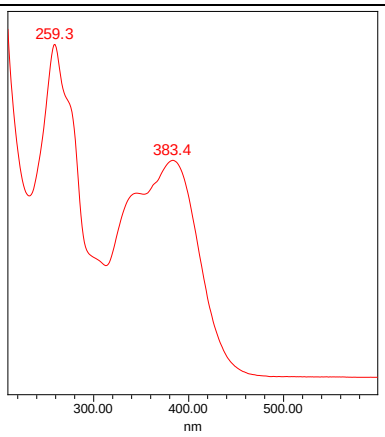
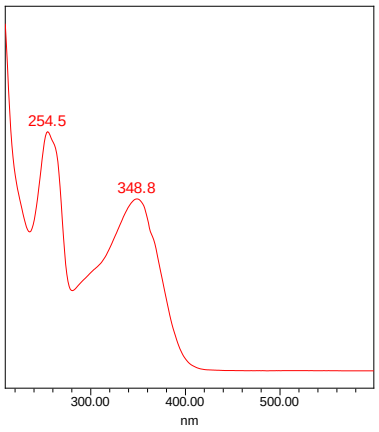
Figure 27: Typical preparative HPLC-PDA chromatogram of the 7 peaks collected from *E. foeminea* crude extract at 350 nm and their overlaid UV-Vis spectra between 210-600 nm.

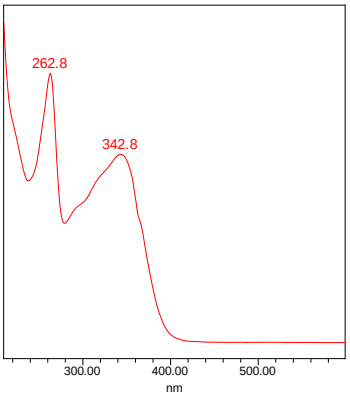
When comparing *E. foeminea* analytical to preparative HPLC chromatographic profiles, we noticed a large similarly in peak shapes and UV-Vis matching but with small shifts in retention according to the columns dimensions differences. For example, the major peaks that eluted at 27.6 and 31.1 minutes in the analytical column eluted at 20.1 and 21.9 using the preparative column. Their UV-Vis are exactly the same. Seven pure isolates were collected directly from the preparative HPLC-PDA effluent. The relevant R_t (collection time) of each

phytochemical along with their UV-Vis spectrum are shown in [table 10]. The wavelengths observed in general are close to flavonoids and phenolic compounds.

Table 10: Prep HPLC seven fractions of *E. foeminea* with their retention times and UV- Vis spectrum

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
1	4.3-4.6	
2	8.3-9.2	
3	13.4-13.9	

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
4	14-14.5	
5	17.1-17.6	
6	19.9-20.4	

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
7	21.6-22.3	

4.7.2. *S. aspera*

Typical preparative chromatogram of 100mg/ml crude *S. aspera* fruit extract sample dissolved in 80% EtOH at 350 is shown in [figure 28].

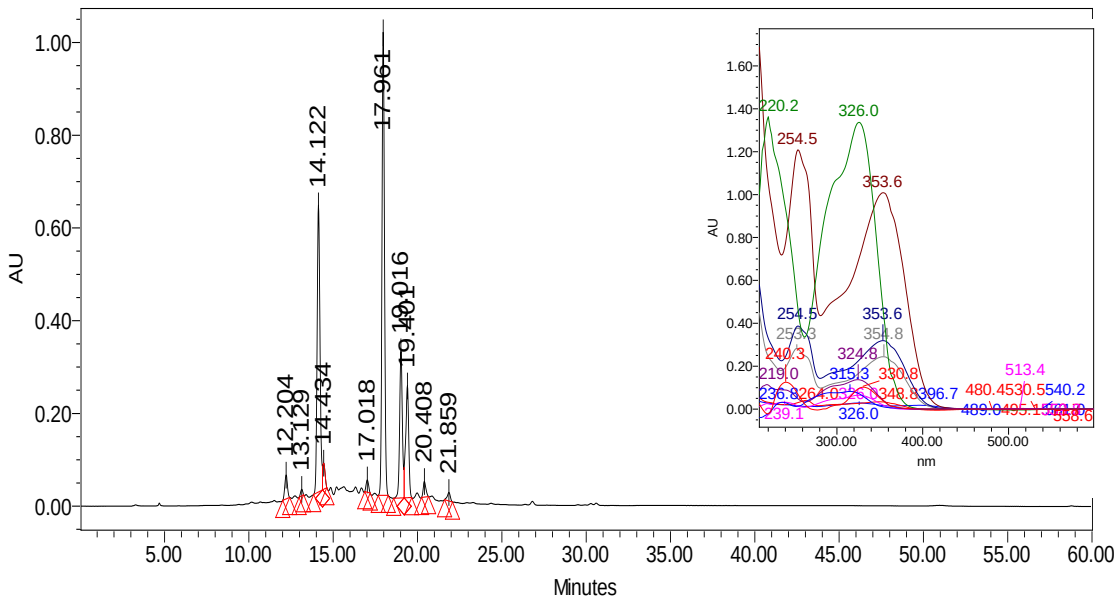


Figure 28: Typical preparative HPLC-PDA chromatogram of the 8 peaks collected from 100mg/ml crude *S. aspera* fruit extract at 350 nm and their overlaid UV-Vis spectra between 210-600 nm.

When comparing *S. aspera* analytical to preparative HPLC chromatographic profiles, we also observed similarly in peak shapes and UV-Vis matching but with small shifts in retention

according to the columns dimensions differences. For example, the major peaks that eluted at 13.6 and 23.5 minutes in the analytical column eluted at 14.1 and 17.9 using the preparative column. Their UV-Vis are exactly the same. The same chromatogram when monitored at 275 nm gave the following profile [figure 29].

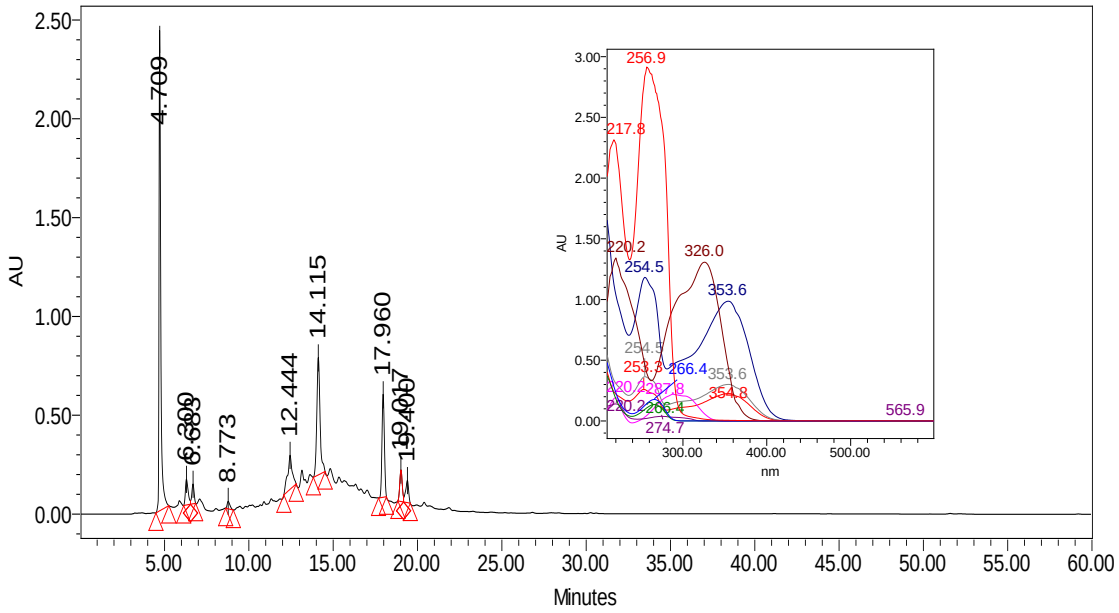
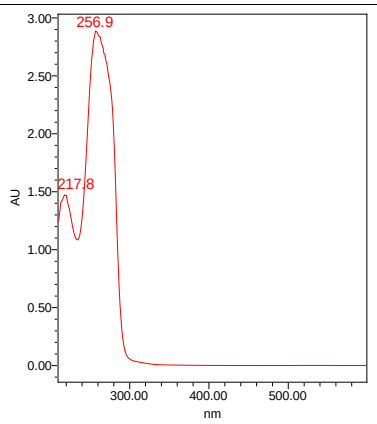
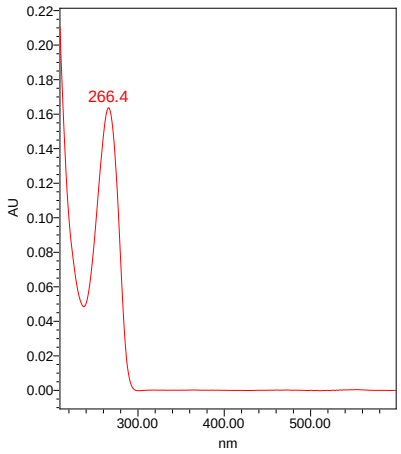
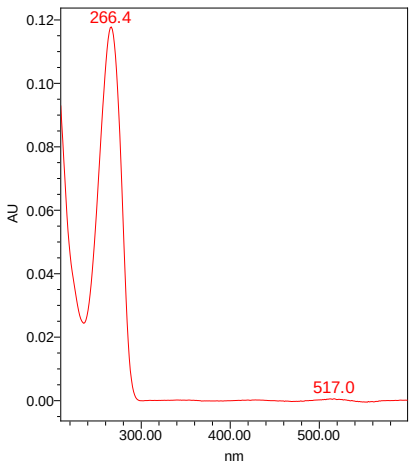
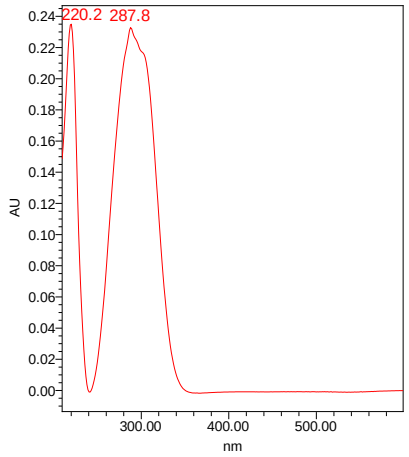
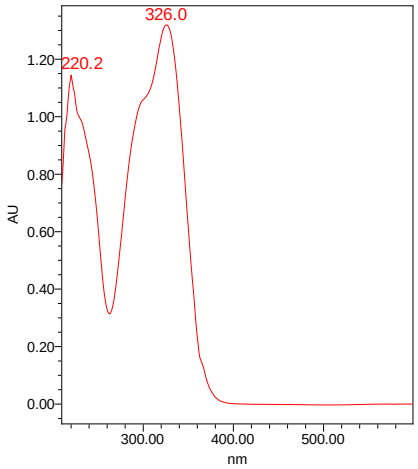
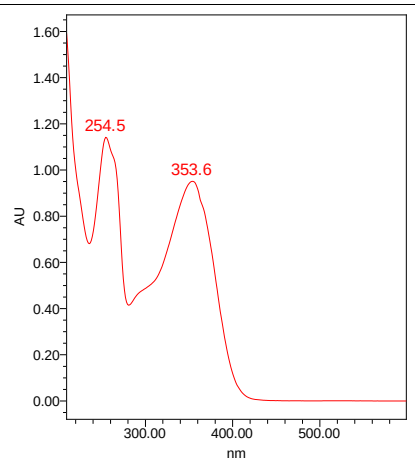


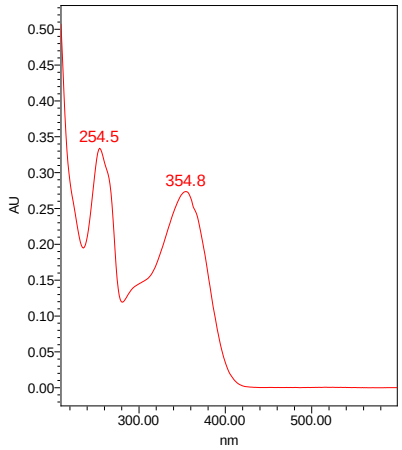
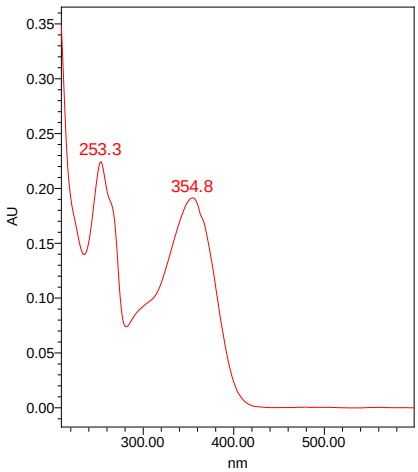
Figure 29: Typical preparative HPLC-PDA chromatogram of the 8 peaks collected from 100mg/ml crude *S. aspera* fruit extract at 275 nm and their overlaid UV-Vis spectra between 210-600 nm.

Eight pure isolates were collected directly from the preparative HPLC-PDA effluent. The relevant retention (collection time) of each phytochemical along with their UV-Vis spectrum are shown in table below. The wavelengths observed in general are close to flavonoids and phenolic compounds.

Table 11: Prep HPLC Eight fractions of *S. aspera* with their retention times and UV-Vis spectrum

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
1	4.48-5.2	
2	6.1-6.5	
3	6.5-6.87	

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
4	12.9-13.3	
5	13.9-14.3	
6	17.7-18.3	

Cpds Vial #	collection time (minutes)	UV-Vis spectrum (210-600)nm
7	18.8-19.1	
8	19.1-19.6	

Compound 2 and 3 showed a maximum wavelength at 266.4 nm. This reflects the similarity of the main chromophoric functionalities of the structurally related compounds. Similar observation was noticed in compound 6, 7 and 8 who showed clear lambda$_{max}$ at 353.6, 354.8 and 354.8 nm respectively (see table above).

4.10. Characterization of major phenolic compounds by LC-ESI-MS

Characterization of the separated peaks obtained from the preparative HPLC fractions were carried out by using LC-ESI-MS in negative and positive ion modes and MS-MS in the product ion scanning (PIS) mode. The collected pure phytochemical isolates from the two plants were directly injected to the LC-MSMS machine.

4.10.1. *E. foeminea*

Figures 30-38 show the Total Ion Current Chromatogram (TIC) generated for all the seven isolates of *E. foeminea* fruits using full scan negative ESi mode. Figure 30 shows the $[M-H]^-$ anion base peak at m/z of 302 Da (molecular formula $C_{15}H_{10}O_7$) indicative to quercetin flavonol, from the flavonoid group of polyphenols skeleton. Quercetin is the aglycone form of a number of other flavonoid glycosides, such as rutin and quercitrin, found in fruits.

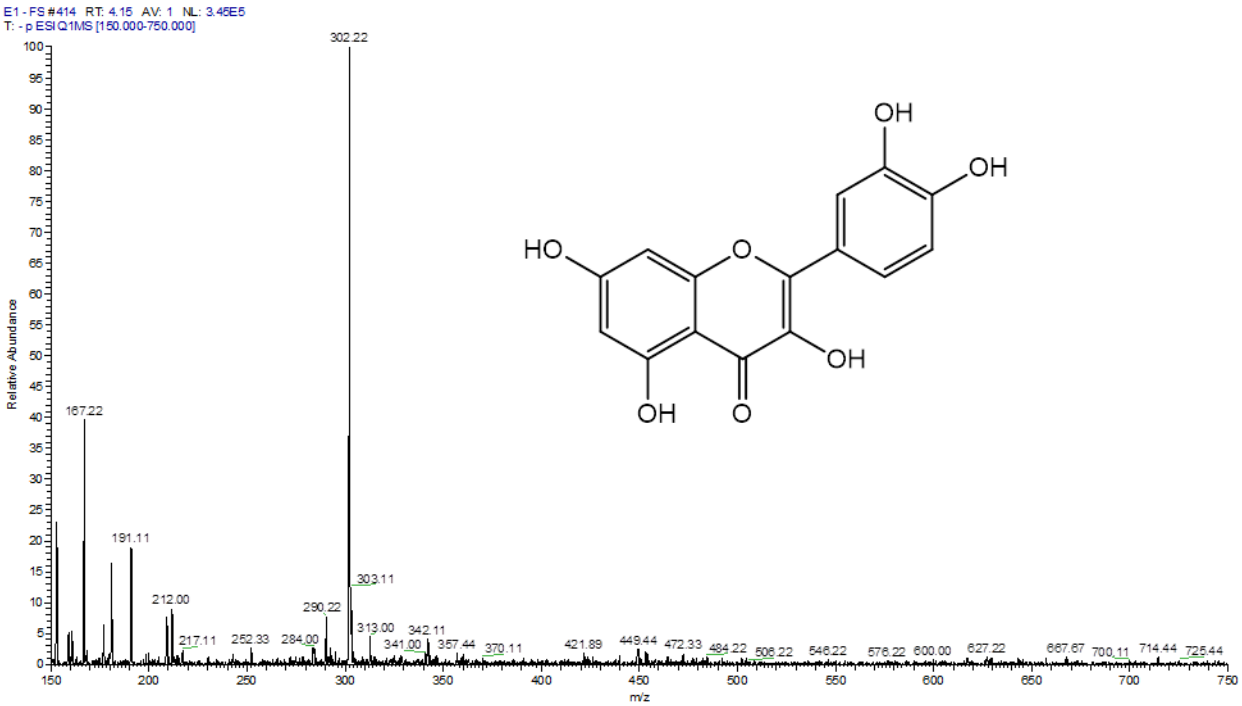


Figure 30: LCMS full scan (-ve ESi mode) of compound 1 from *E. foeminea*.

The second polyphenolic deprotonated $[M-H]^-$ anion appeared at m/z = 738 Da (molecular formula ($C_{39}H_{32}O_{15}$) is called Kaempferol 3-O-(4",6"-O-di-p-coumaroyl)-glucoside. Its structure appeared inside the mass spectrum [figure 31].

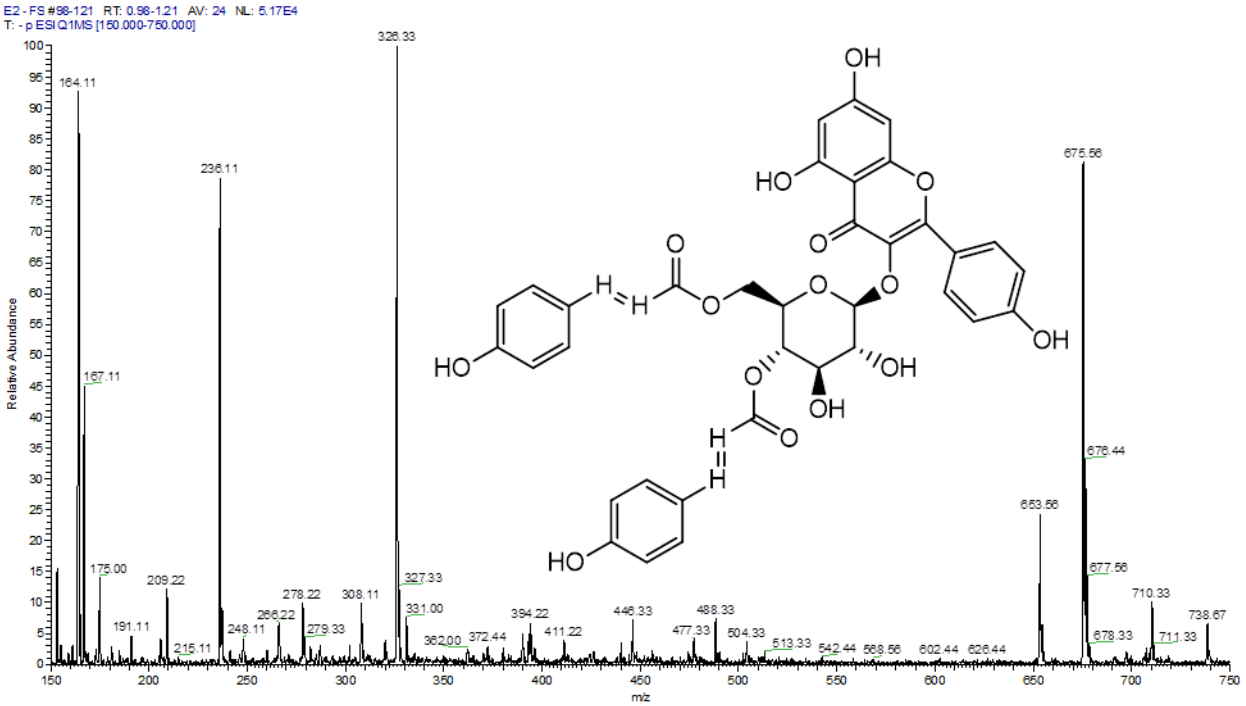


Figure 31: LCMS full scan (-ve ESi mode) of compound 2 from *E. foeminea*.

As seen in figure 32, there is two unit (Da) difference between the positive and negative ions of the base peak fragment backbone. In the positive mode, the m/z was 328 Da while in the negative mode a fragment at m/z of 326 Da was seen.

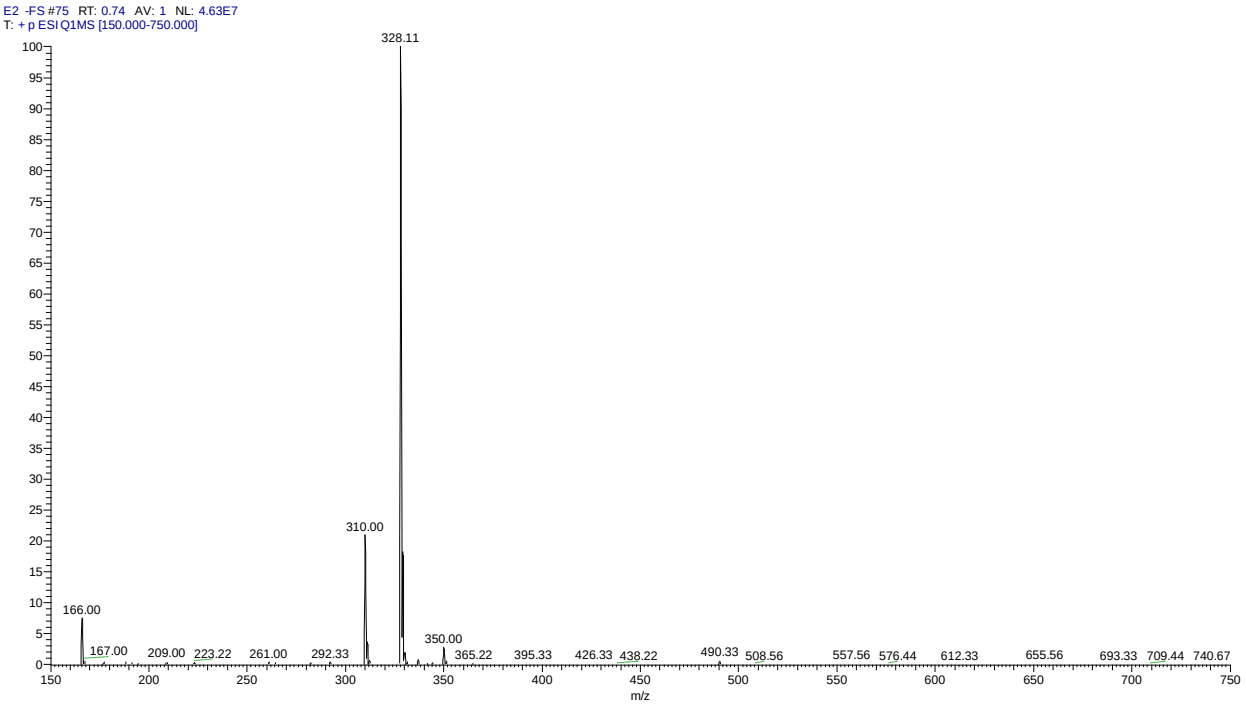


Figure 32: LCMS full scan (+ve ESi mode) of compound 2 from *E. foeminea*.

Figure 33 shows the negative ESi MS of compound 3. A deprotonated peak at 609 Da, typical to Lucenin 2 phytochemical (molecular formula: $C_{27}H_{30}O_{16}$, molecular weight:610.521 g/mol).

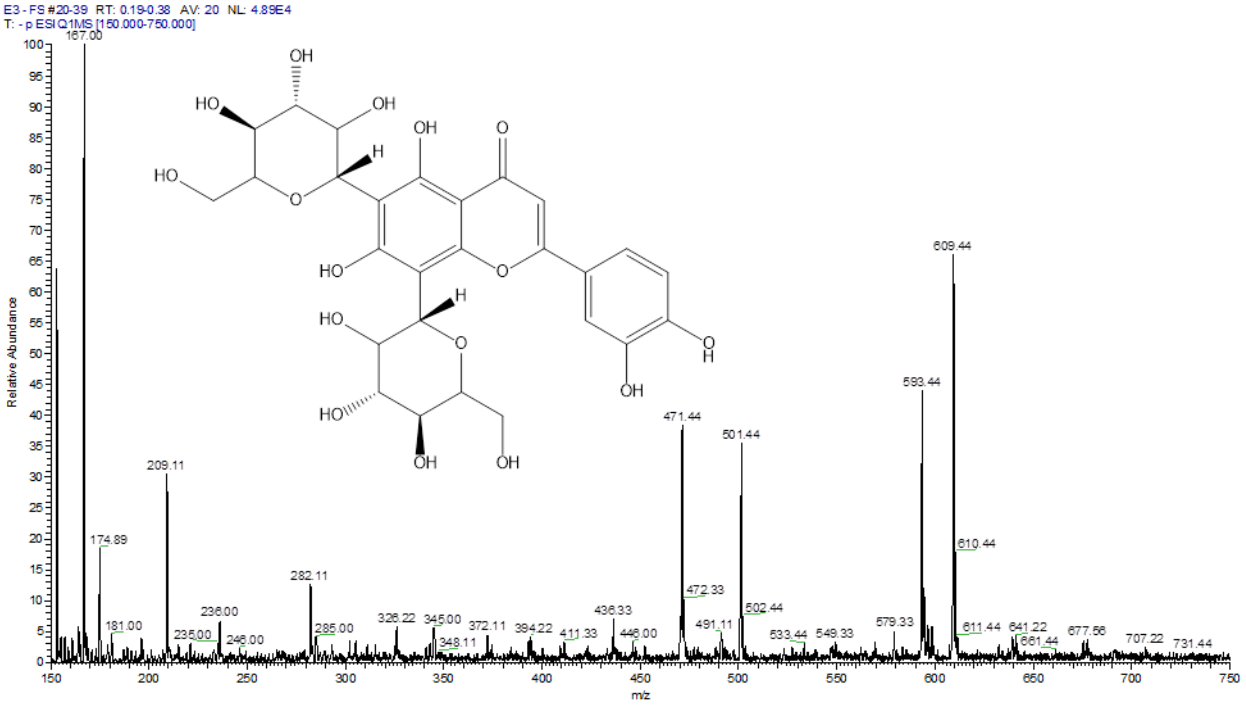


Figure 33: LCMS full scan (-ve ESi mode) of compound 3 from *E. foeminea*.

Figure 34 shows the negative ESi MS of compound 4. A deprotonated peak at 593 Da, most probably due to Vicenin 2 phytochemical (molecular formula: $C_{27}H_{30}O_{15}$, molecular weight: 594.52 g/mol).

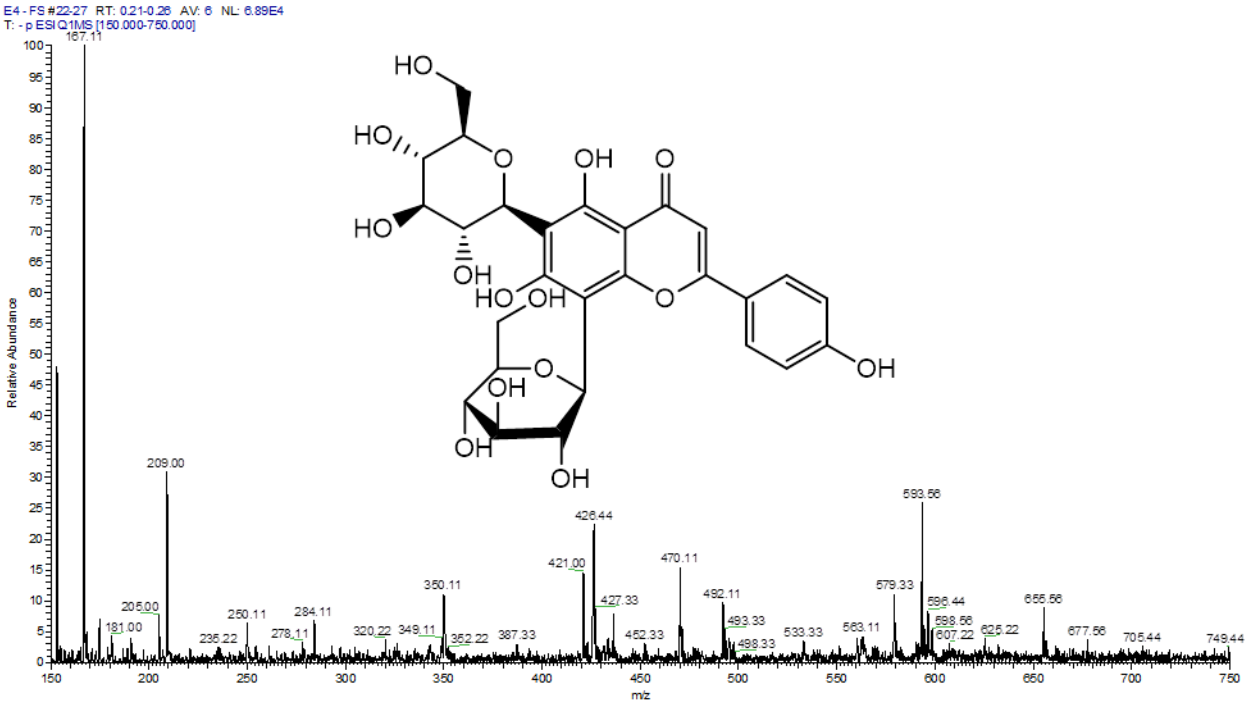


Figure 34: LCMS full scan (-ve ESi mode) of compound 4 from *E. foeminea*.

Figure 35 shows the negative ESi MS of compound 5. A deprotonated peak at 609 Da, most probably due to Quercetin 3-O-rhamnoside-7-O-glucoside phytochemical (molecular formula: $C_{27}H_{30}O_{16}$, molecular weight: 610.521 g/mol).

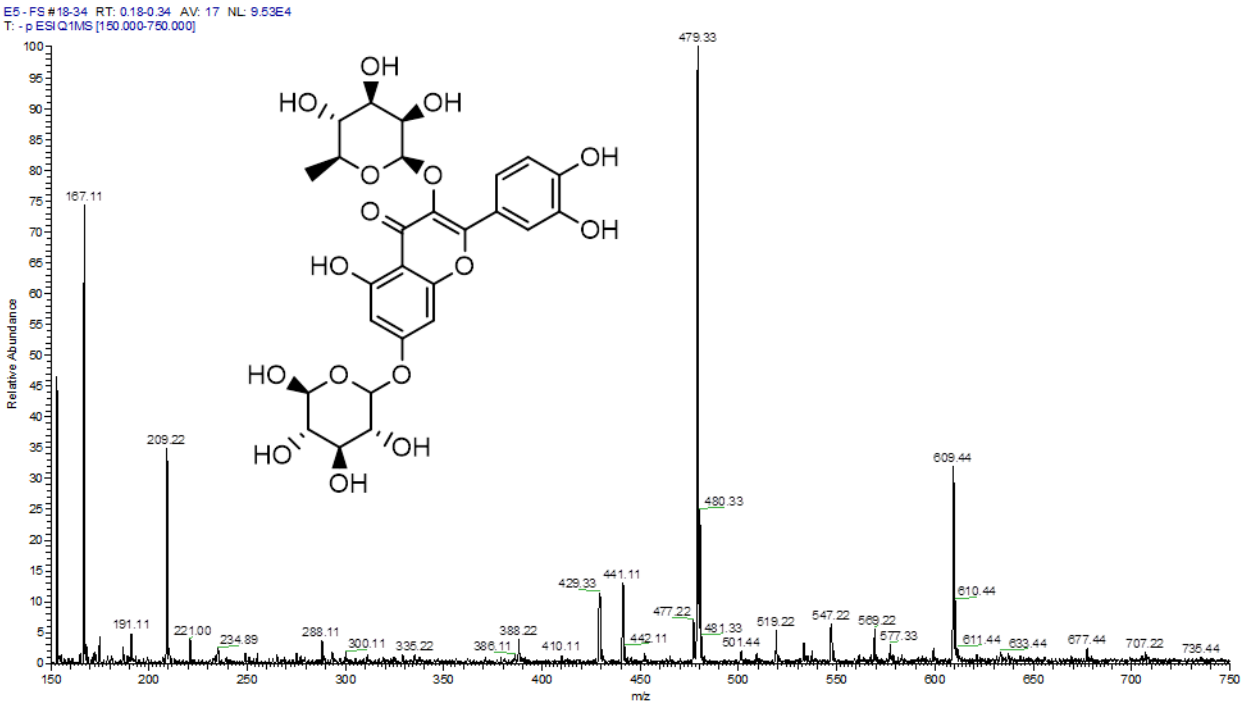


Figure 35: LCMS full scan (-ve ESi mode) of compound 5 from *E. foeminea*.

Figure 36 shows the negative ESi MS of compound 6. A typical deprotonated peak at 447 Da, is due the presence of Quercetin-3-O-rhamnoside phytochemical (molecular formula: $C_{21}H_{20}O_{11}$, molecular weight: 448.38 g/mol).

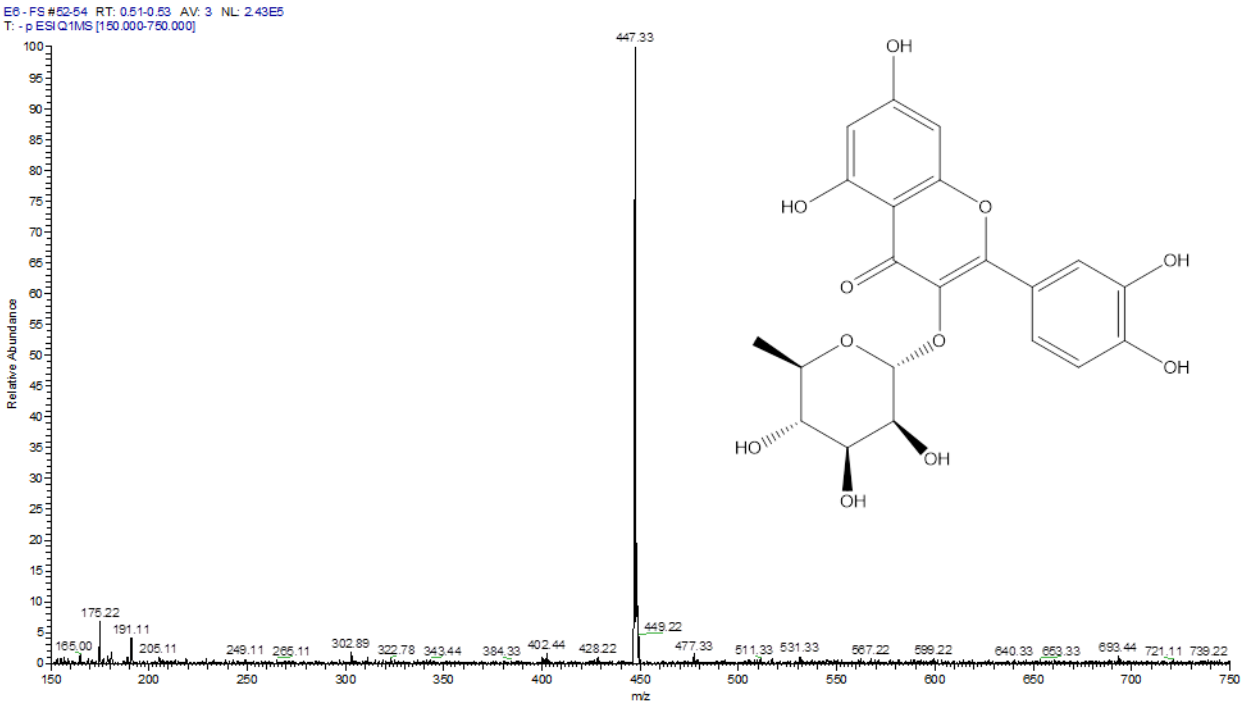


Figure 36: LCMS full scan (-ve ESi mode) of compound 6 from *E. foeminea.*

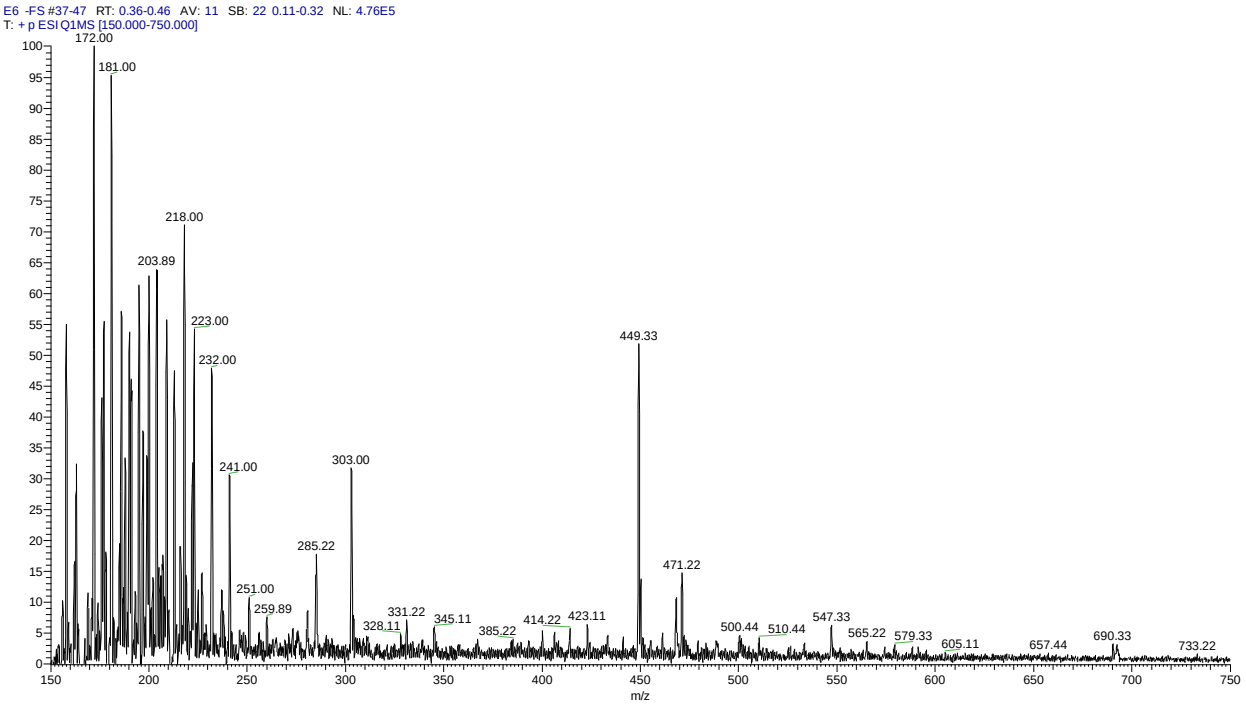


Figure 37: LCMS full scan (-ve ESi mode) of compound 6 from *E. foeminea.*

As seen in figure 37, there is a two unit (Da) difference between the positive and negative ions of the protonated and deprotonated molecular ions. In the positive mode, m/z was 449

Da while in negative mode gave a fragment at m/z of 447 Da. Since the ionization parameters used LCMS is seems to be too soft, only the deprotonated $[M-H]^-$ peak appeared. To see the degradation daughter peaks, product ion scanning experiment was done. As in figure 38, the quercetin-3-O-rhamnoside compound was broken down to give a peak at m/z of 301 Da, the quercetin backbone.

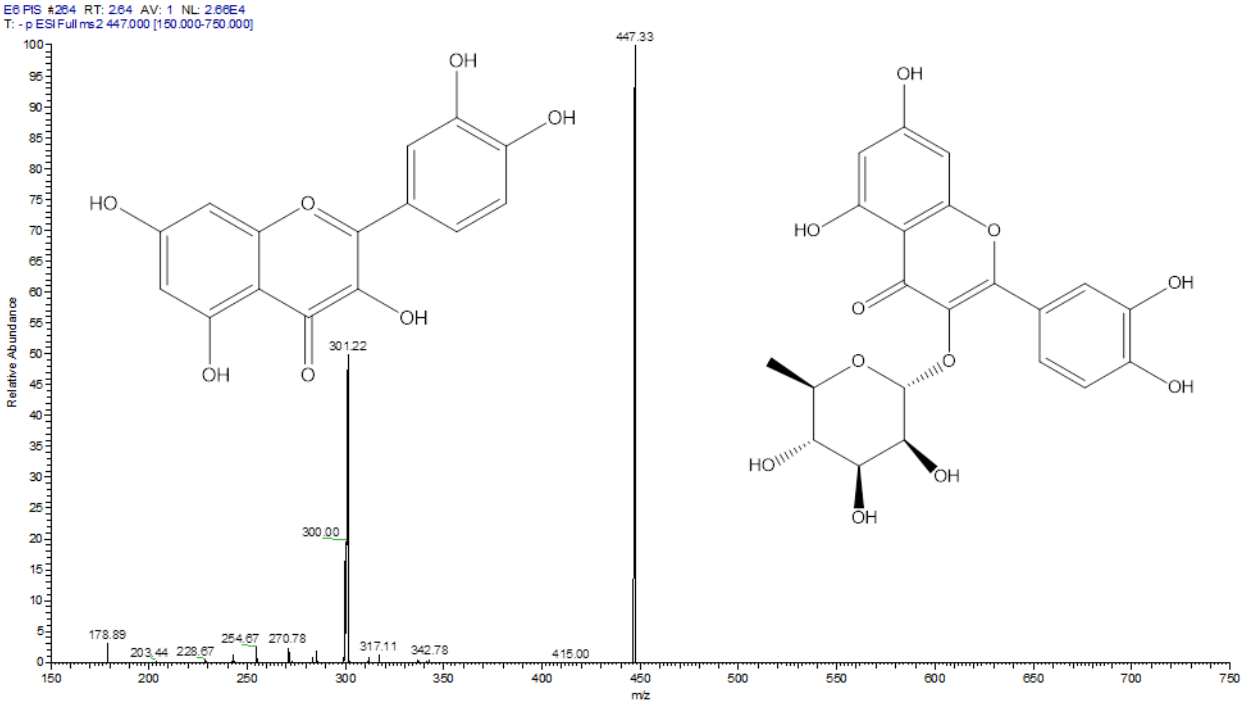
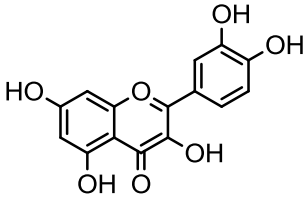
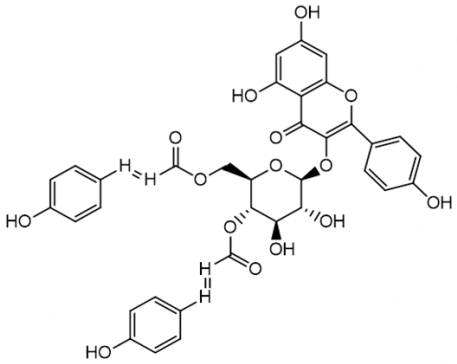
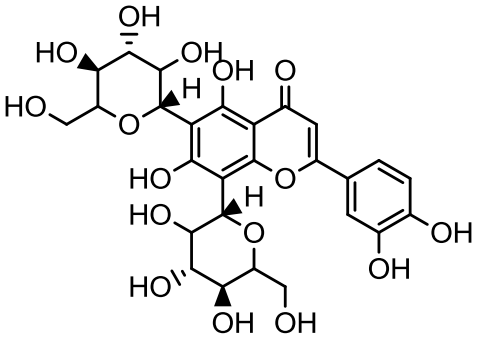


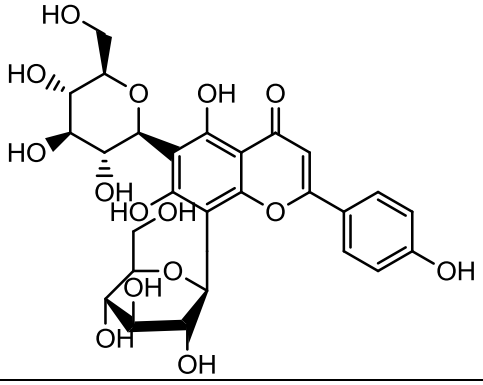
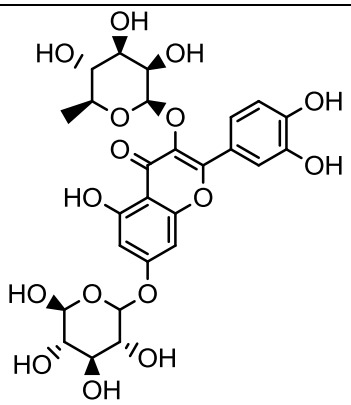
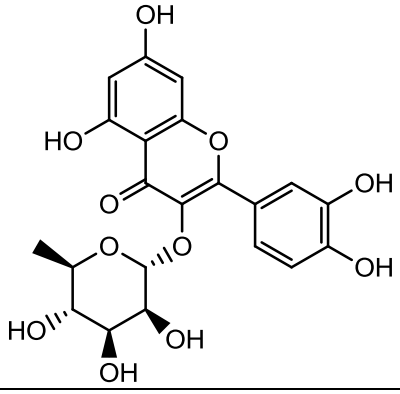
Figure 38: LCMSMS (product ion scanning in the -ve ESi mode) of compound 6 from *E. foeminea*

The last peak collected from the preparative HPLC gave a very noisy MS spectrum probably due to the dominance of acetonitrile solvent on water.

Table 12 represents the six compounds isolated from *E. foeminea* with the molecular formula, molecular weight, +ve / –ve values on the ESI and the structure of each.

Table 12: Summary of the isolates of *E. foeminea* fruits and their relevant information.

Compound number & mass spectrum figure	Compound name & molecular formula & m.wt	Compound appeared at		Structure
		+ve ESI m/z (Da)	-ve ESI m/z (Da)	
Compound 1 Figure 30	Quercetin $C_{15}H_{10}O_7$ 302.238 g/mol	304	302	
Compound 2 Figure 31	Kaempferol $C_{39}H_{32}O_{15}$ 740.67 g/mol	740	738	
Compound 3 Figure 33	Lucenin 2 $C_{27}H_{30}O_{16}$ 610.521 g/mol	611	609	

Compound number & mass spectrum figure	Compound name & molecular formula & m.wt	Compound appeared at		Structure
		+ve ESI m/z (Da)	-ve ESI m/z (Da)	
Compound 4 Figure 34	Vicenin 2 $C_{27}H_{30}O_{15}$ 594.522 g/mol	595	593	
Compound 5 Figure 35	Quercetin 3-O-rhamnoside-7-O-glucoside $C_{27}H_{30}O_{16}$ 610.521 g/mol	611	609	
Compound 6 Figure 36	Quercetin-3-O-rhamnoside $C_{21}H_{20}O_{11}$ 448.38 g/mol	449	447	

4.10.2. *S. aspera*

The same experimental LCMS parameters were applied on *Smilax Aspera* plant pure compounds from the preparative HPLC. Figures 39-49 show the TIC generated for all the eight isolates of *S. Aspera* fruits using full scan negative ESi mode. Figure 39 showed [M-

$H]^-$ anion at m/z of 445 Da (molecular formula: $C_{21}H_{18}O_{11}$, molecular weight: 446.364 g/mol) indicative most likely to apigenin 7-glucuronide backbone presence. Figure 40 showed the $[M+H]^+$ cation at m/z of 447 Da, typical two units difference as expected. Very similar MS profiles were obtained for the second and third collected compounds from the preparative HPLC, which may indicate to different isomeric forms or to another precursor that lead to apigenin 7-glucuronide daughter ion skeleton.

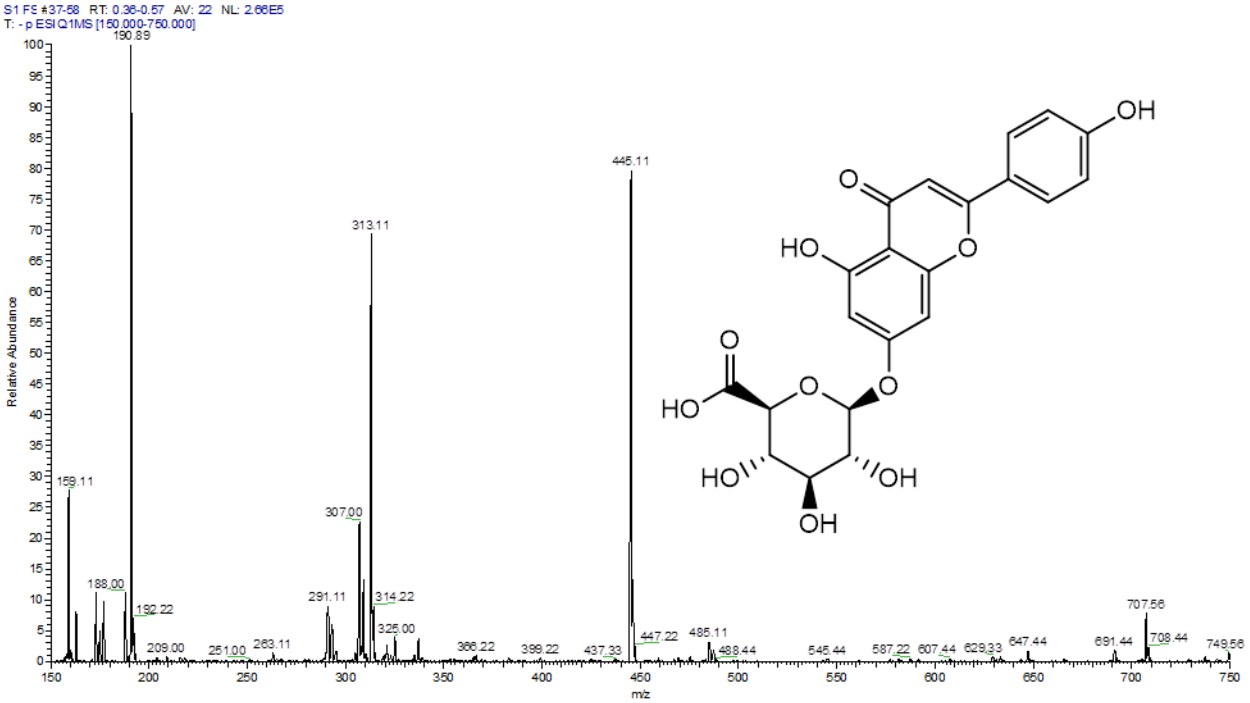


Figure 39: LCMS (-ve ESi mode) of compound 1 from *Smilax Aspera*.

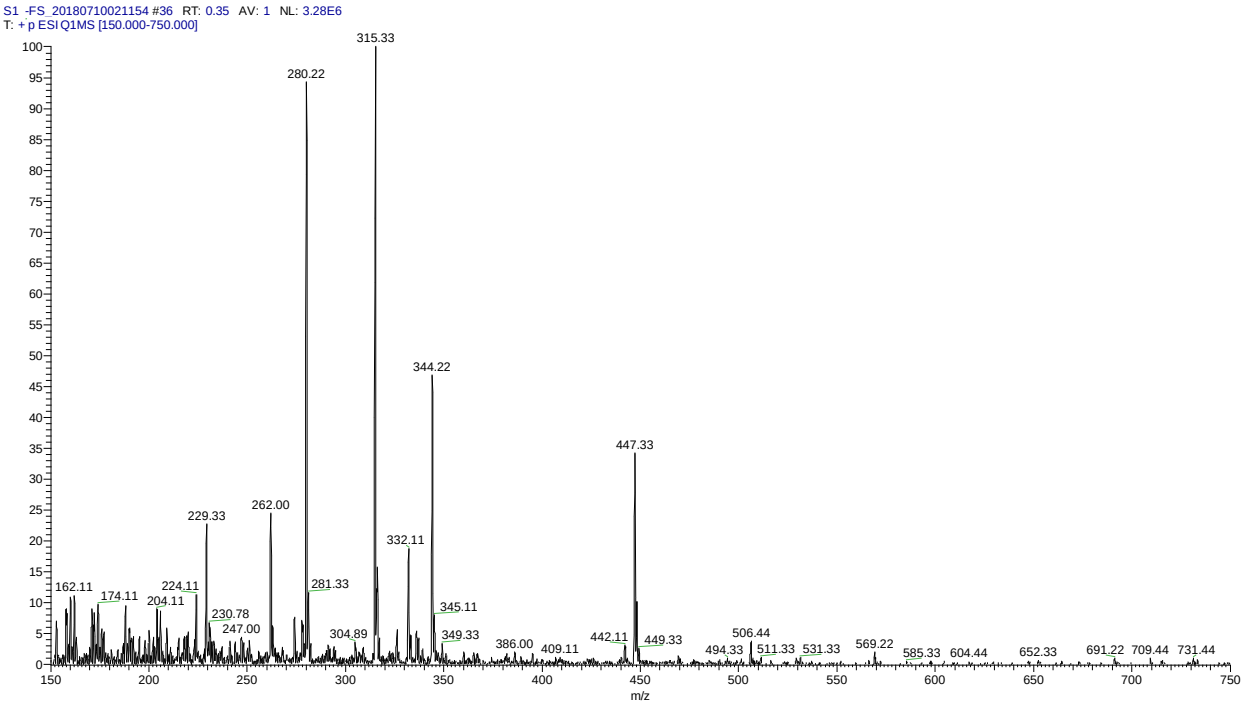


Figure 40: LCMS (+ve ESi mode) of compound 1 from *Smilax Aspera*.

Moreover, a deprotonated m/z of 707 Da could probably indicate to 3-hydroxy-3-methylglutaroyl derivative of 2''-*O*-pentosyl-C-hexosyl-apigenin (MW 708 Da) (Pereira, 2012). An unidentified peak 4 MS spectrum (-ve ESi) showed precursor ion at m/z 567 $[M-H]^-$ and base peak signal at 355 Da.

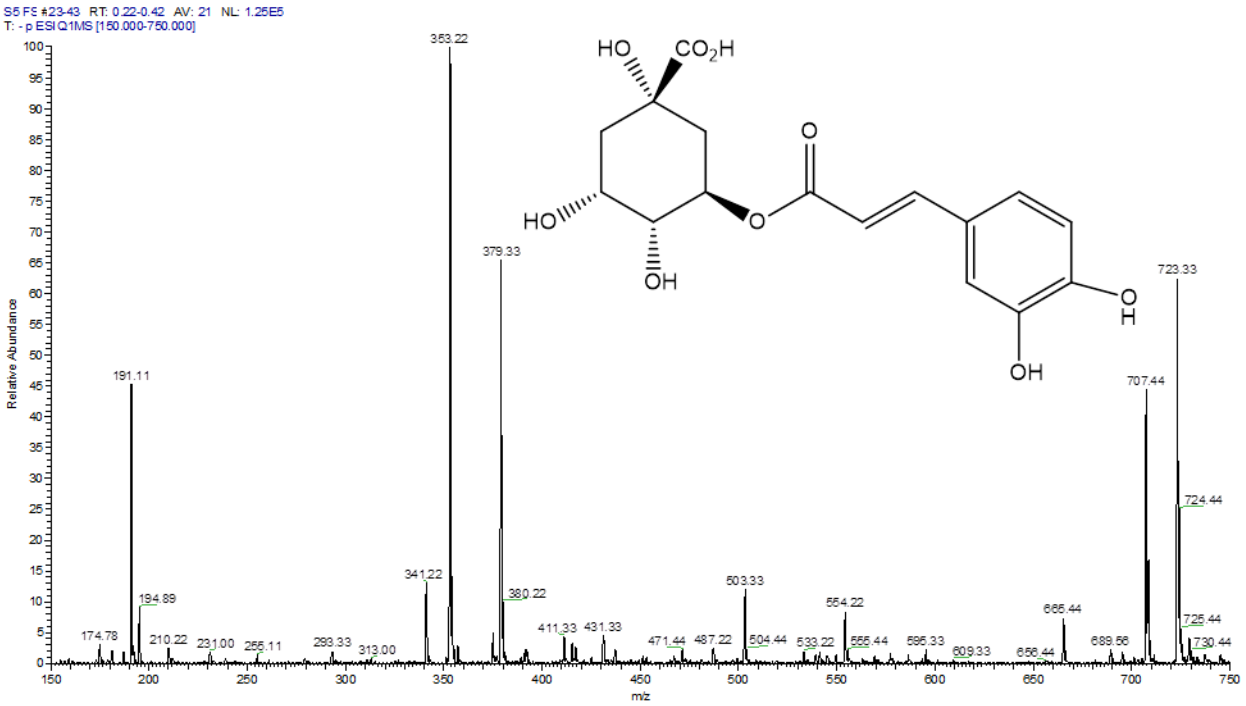


Figure 41: LCMS (-ve ESi mode) of compound 5 from *Smilax Aspera.*

Negative ESi m/z gave a signal at 723 Da, due to luteolin derivative in fraction 5 (MW 724 Da), the presence of 3-hydroxy-3-methylglutaric acid moiety was proposed based on literature data (Di Donna, 2009). As in figure 41, the base peak showed a precursor of chlorogenic acid ($C_{16}H_{18}O_9$, molar mass 354.31 g·mol^{-1}) ion at m/z 353 $[M-H]^-$ ($C_{27}H_{30}O_{16}$). The positive ESi mode gave a protonated $[M+H]^+$ signal at m/z of 725 Da, a typical two units more to the molecular weight of compound 5 [figure 42]. Moreover, a protonated chlorogenic acid base peak was shown at 355 Da as expected [figure 42].

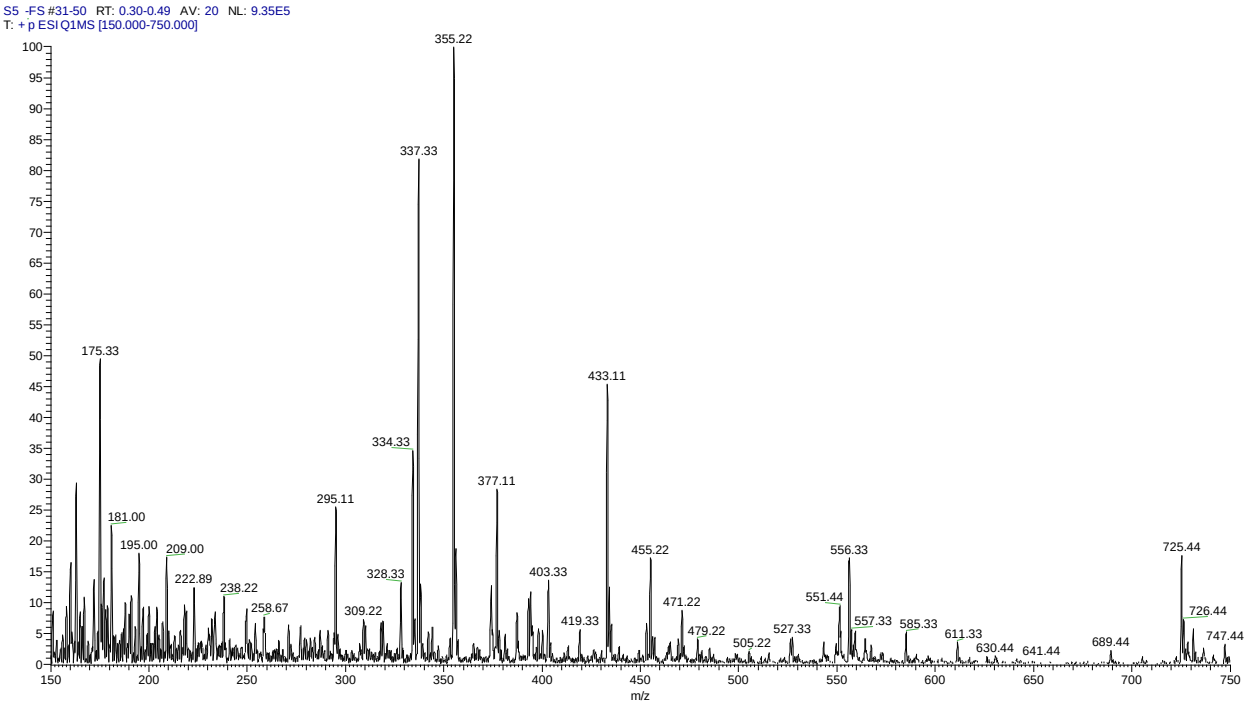


Figure 42: LCMS (+ve ESi mode) of compound 5 from *Smilax Aspera.*

Compound 6 was assigned as lucenin 2 phytochemical (molecular formula: $C_{27}H_{30}O_{16}$, molecular weight: 610.521 g/mol). The LCMS spectrum in the negative ionization mode showed a typical parent deprotonated ions at m/z 609 Da while the positive mode showed a typical protonated signal at 611 Da (figure 43 and 44). This identification was also confirmed by comparison with the literature. In order to see the daughter ions of the lucenin 2 parent, the collision energy increased from 12 to 25 volts. As in figure 45, the lucenin 2 precursor was broken down to give a peak at m/z of 301 Da, the quercetin backbone.

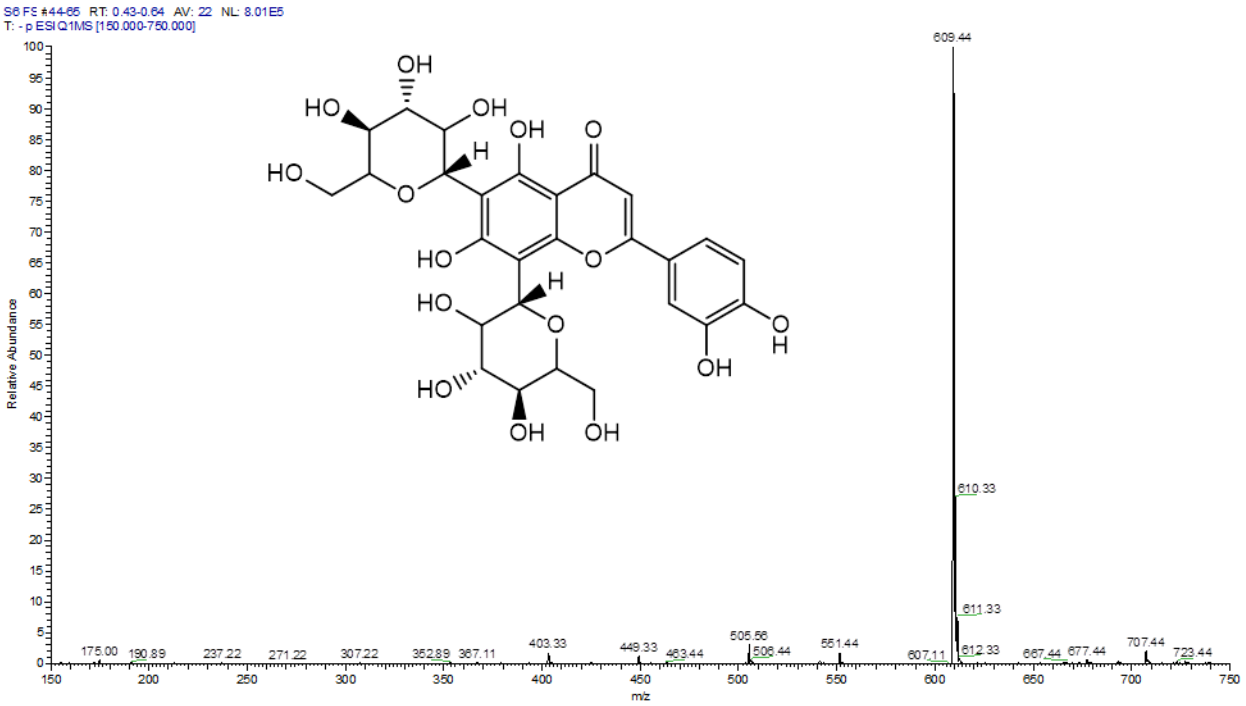


Figure 43: LCMS (-ve ESi mode) of compound 6 from *Smilax Aspera.*

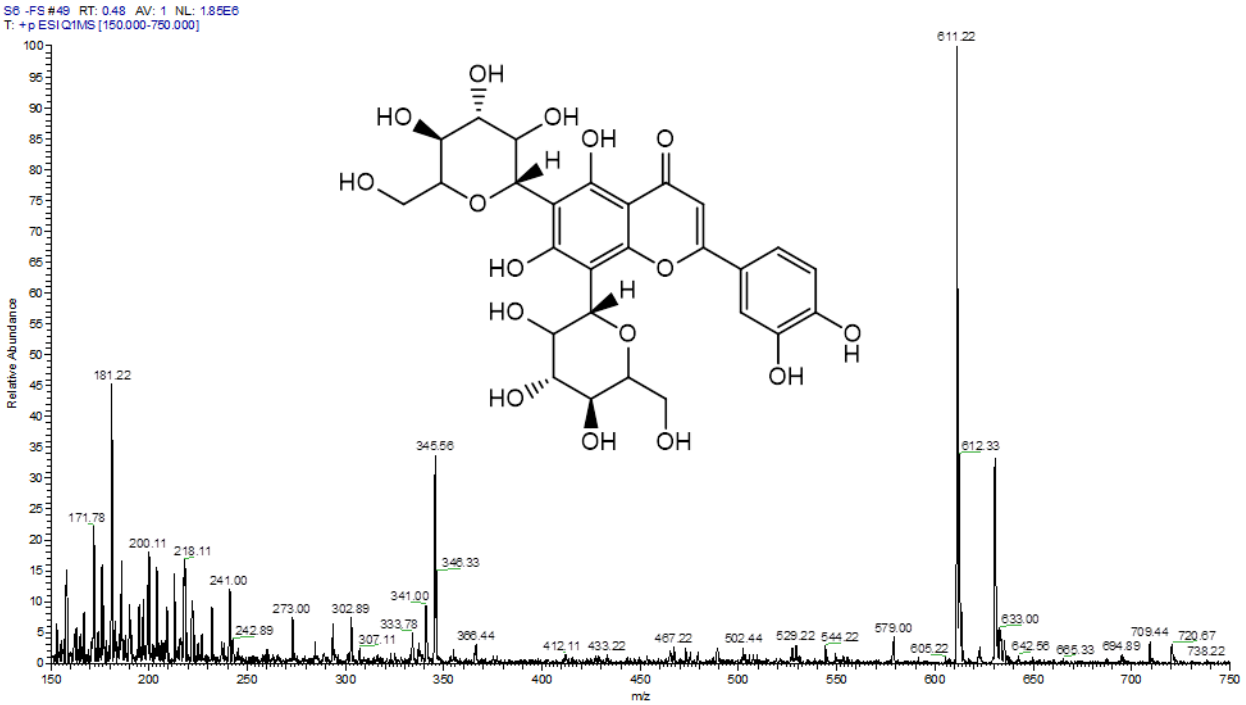


Figure 44: LCMS (+ve ESi mode) of compound 6 from *Smilax Aspera*

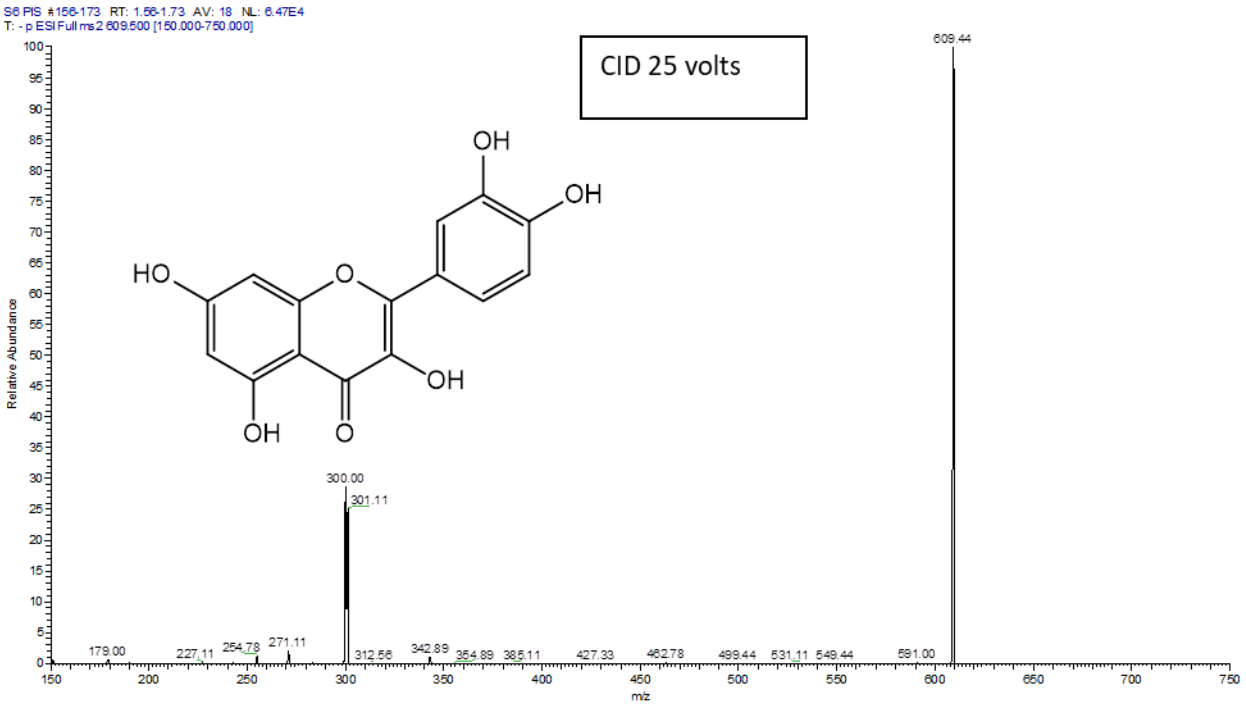


Figure 45: LCMSMS (-ve ESi product ion scanning mode) of compound 6 from *Smilax aspera.*

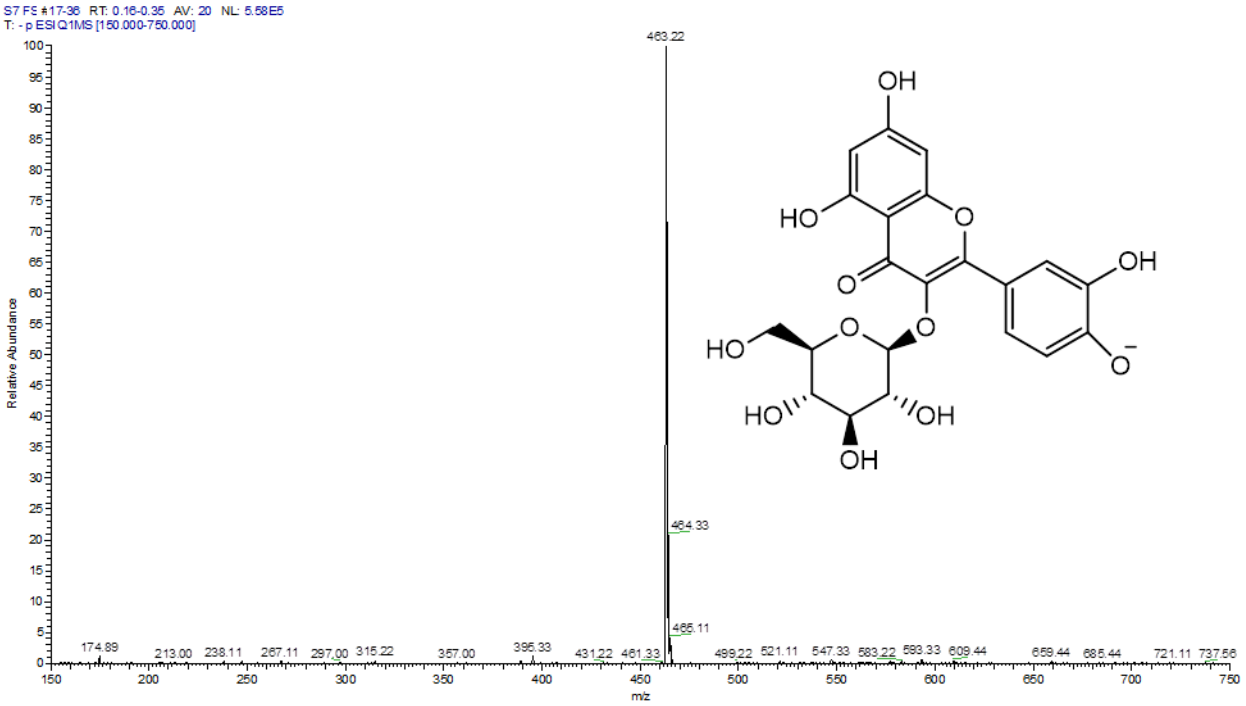


Figure 46: LCMS (-ve ESi mode) of compound 7 from *Smilax Aspera.*

Figure 46 depicted compound 7 which was identified as quercetin-3-glucoside (molecular formula: $C_{21}H_{19}O_{12}$, molecular weight: 463.371 g/mol). The LCMS positive mode showed 465 Da signal [figure 47].

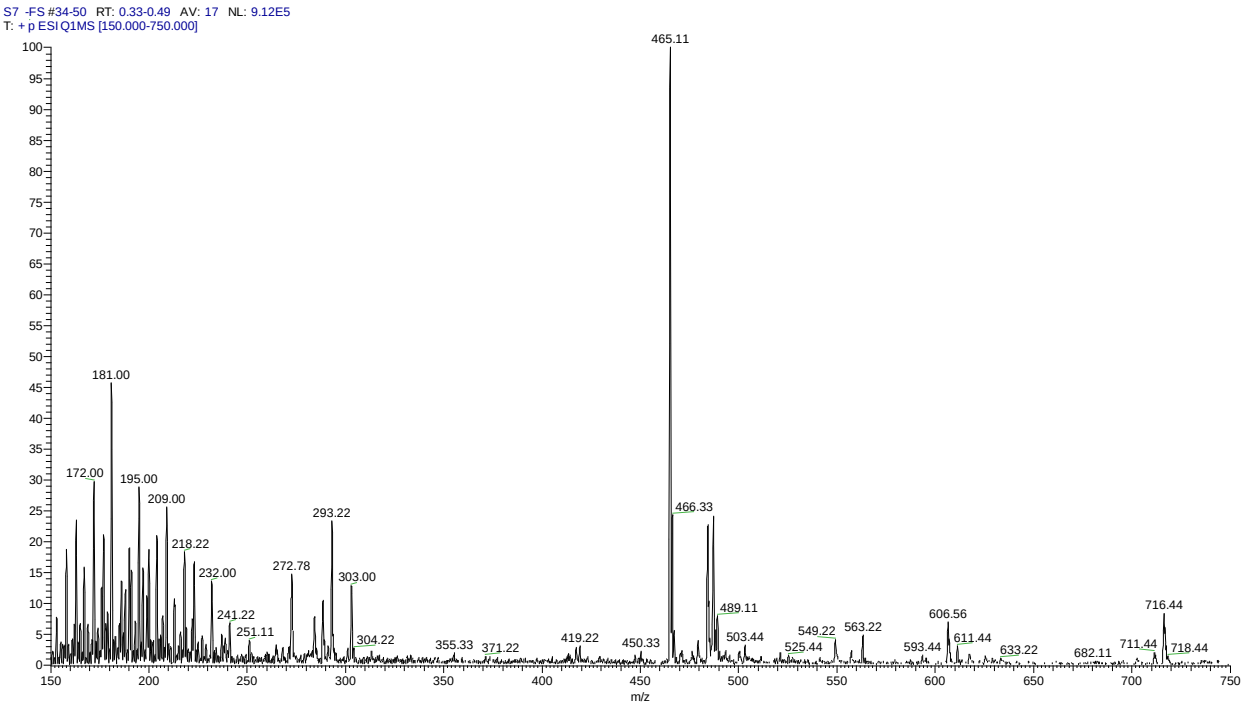


Figure 47: LCMS (+ve ESi mode) of compound 7 from *Smilax Aspera.*

Figure 48 showed compound 8 which was identified as isorhamnetin-3-O-rutinoside (molecular formula: $C_{28}H_{32}O_{16}$, molecular weight: 624.548 g/mol). The LCMS positive mode showed 625 Da signal [figure 49].

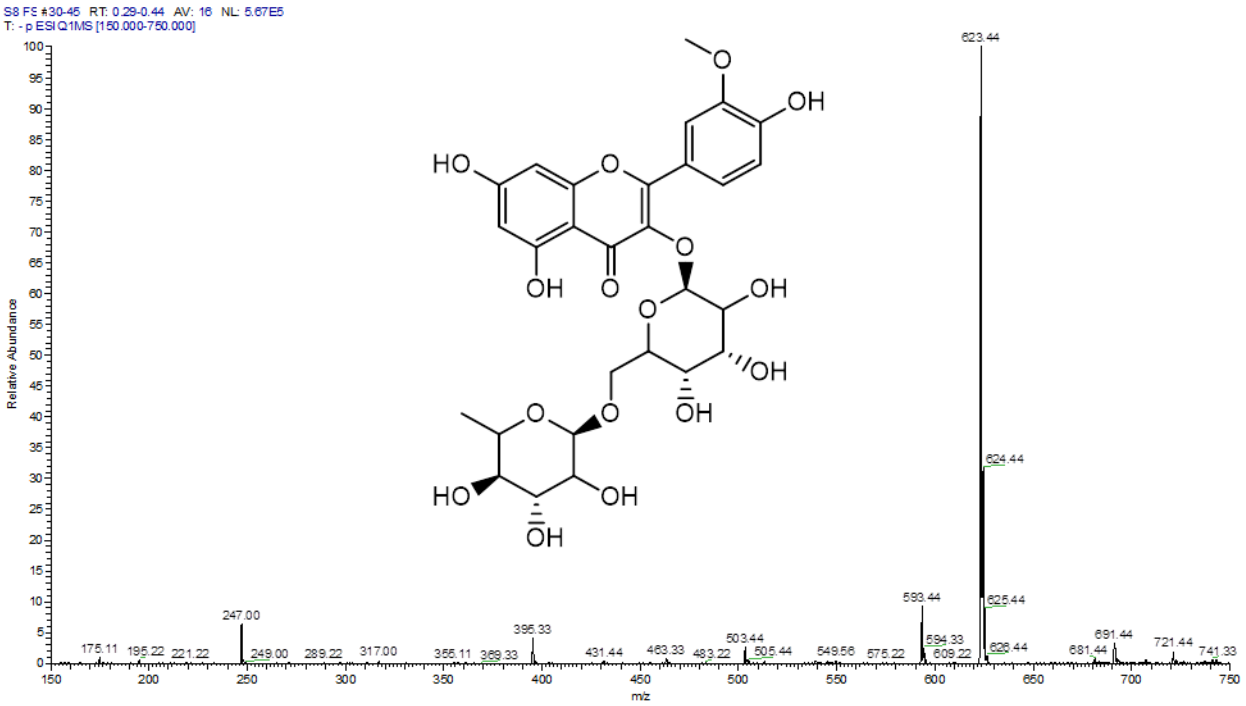


Figure 48: LCMS (-ve ESi mode) of compound 8 from *Smilax Aspera.*

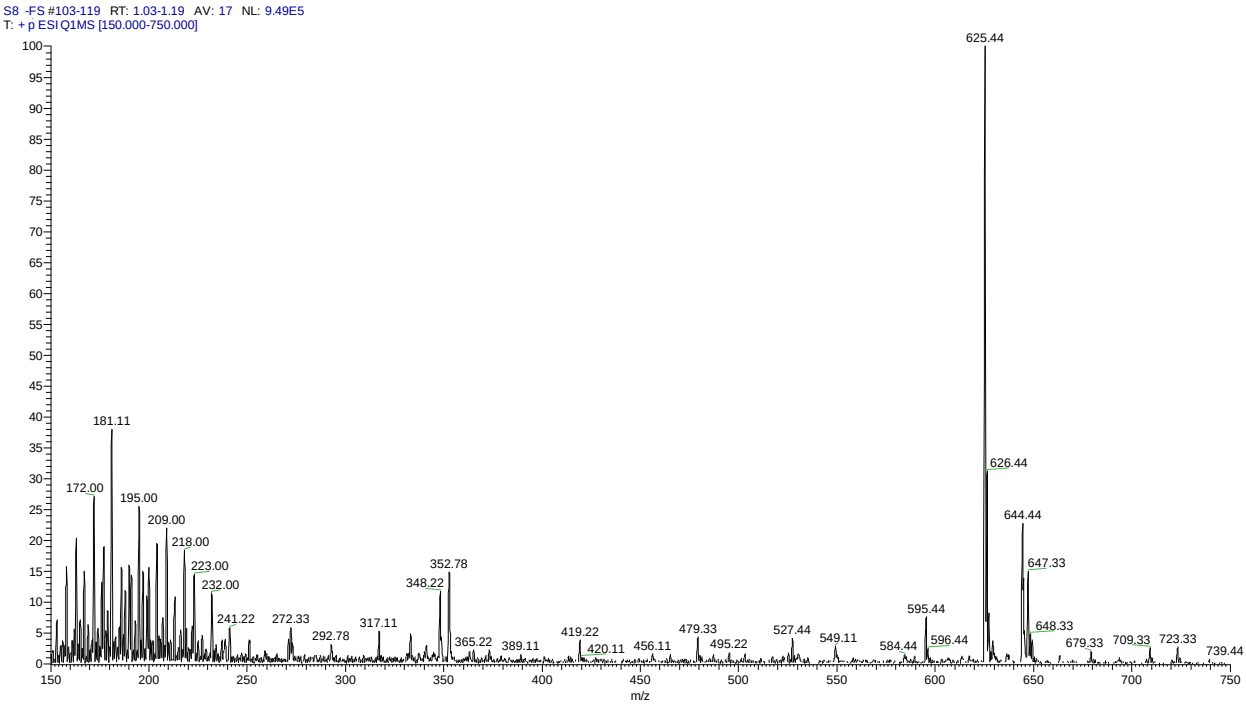
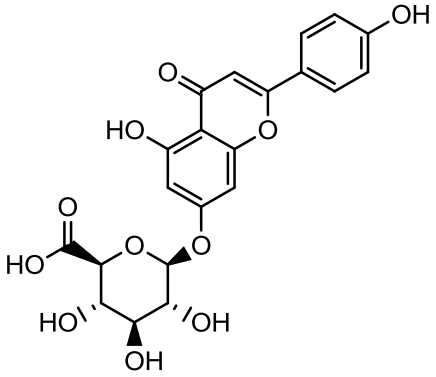
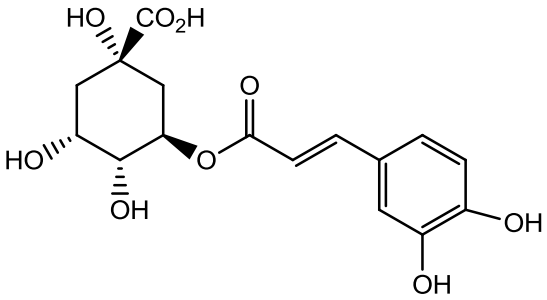
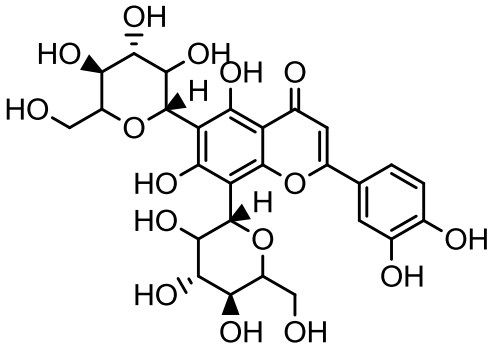
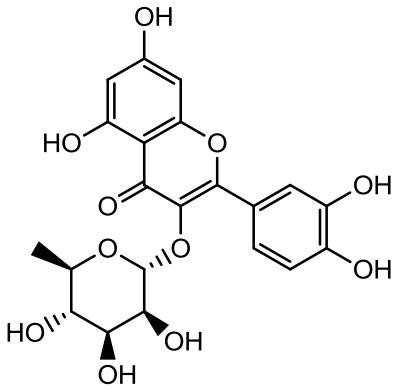
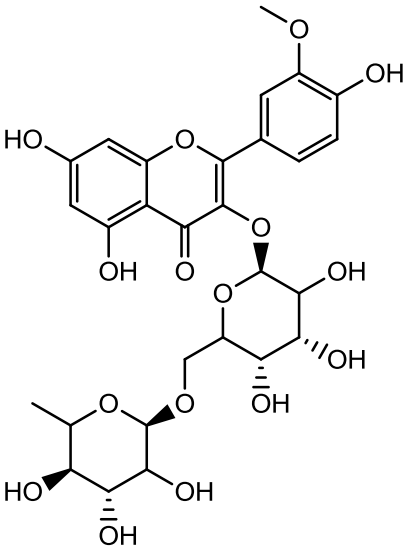


Figure 49: LCMS (+ve ESi mode) of compound 7 from *Smilax Aspera*

Table 13 is a summary of all the isolates from *S. aspera* with the molecular formula, molecular weight, +ve / –ve values on the ESI and their structures

Table 13: Summary of the five isolates *S. aspera* fruits and their relevant structures

Compound number & mass spectrum figure	Compound name & molecular formula & m.wt	Compound appeared at		Structure
		+ve ESI m/z (Da)	-ve ESI m/z (Da)	
Compound 1 Figure 39	apigenin 7-glucuronide $C_{21}H_{18}O_{11}$, 446.364 g/mol	447	445	
Fraction of Compound 5 Figure 41	Chlorogenic acid $C_{16}H_{18}O_9$ 354.31 g/mol		353	
Compound6 Figure 43 & Figure 44	lucenin 2 $C_{27}H_{30}O_{16}$, 610.521 g/mol	611	609	

Compound number & mass spectrum figure	Compound name & molecular formula & m.wt	Compound appeared at		Structure
		+ve ESI m/z (Da)	-ve ESI m/z (Da)	
Compound 7 Figure 46 & Figure 47	quercetin-3-glucoside $C_{21}H_{19}O_{12}$, 463.371 g/mol	465	463	
Compound 8 Figure 48 & Figure 49	isorhamnetin-3-O-rutinoside $C_{28}H_{32}O_{16}$, 624.548 g/mol	625	623	

Chapter 5
Conclusions and Recommendations

Chapter 5:

Conclusions and Recommendations:

5.1. Conclusions

Wild Palestinian plant fruits (80%EtOH extract) of *Ephedra foeminea* and *Smilax aspera* which are rich in phenolics were fully investigated for the first time. Combination of several chromatographic and spectroscopic methodologies (analytical HPLC-PDA, preparative-HPLC-PDA, full scan LC-MS in the +ve and -ve ESi modes and LCMSMS-PIS mode) were utilized to separate, isolate and structurally elucidate the crude extracts secondary metabolites of both plants. In *E. foeminea,* Quercetin was found to be the backbone of most of the major compounds present in the extact such as Kaempferol, Lucenin 2, Vicenin 2, Quercetin 3-O-rhamnoside-7-O-glucoside and Quercetin-3-O-rhamnoside. In *S. aspera*, however, apigenin 7-glucuronide, luteolin derivative, lucenin 2, quercetin-3-glucoside and isorhamnetin-3-O-rutinoside was identified.

The TPC experiments enabled us to calculate the amount of phenolics from each extraction solvent and water extract of *E. foeminea* and *S. aspera* was found to be superior. TFC showed better flavonoids content in the 99% ethanolic extract for *E. foeminea* while water was better solvent for *S. aspera*. This reflects the nature of the polarity of the separated compounds in each plant. Therefore, as expected, the antioxidant activities as revealed by FRAP and DPPH methods were high with 91.3% of radical inhibition for *Ephedra*. Moreover, the antimicrobial activity was for *S. aspera* fruit extract showed positive influence against gram negative bacteria (*Escherichia. coli*) and yeast (*Candida albicans*). Although *E. foeminea* (*Alanda* in Arabic) represents an example of a widely popularized anticancer herbal Palestinian traditional medicine, however, on colon cancer cell lines (HT-29), the extract gave negative

results in combating cancer. This is probably attributed to the synergistic effect of certain lipophilic compounds present in the plant that could be extracted out using alcoholic solvents.

5.2. Recommendations and future work

Further studies concerning both plants are recommended as follows:

1. To use preparative HPLC-PDA machine to collect more concentrated isolates of pure compounds (>100-mg each) in order to run 2D-NMR spectroscopy experiments particularly using COSY and HMQC modes to fully elucidate the exact structures of the separated phytochemicals along with the use of high resolution mass spectroscopy.

2. In parallel, to test all the separated isolates anticancer activities on other numerous cell lines and to test their antioxidant activities in comparison to positive control.

3. To investigate the combination of different isolates on cell lines to explore their synergistic effect.

4. To examine other extraction methods using other solvents such as ethyl acetate, butanol, etc and to identify their exact separated compounds.

Referances:

Abdallah, E. M. (2011). Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science, 1*(6), 16-20.

Al-Rimawi, F., Abu-Lafi, S., Abbadi, J., Alamarneh, A. A., Sawahreh, R. A., & Odeh, I. (2017). Analysis of phenolic and flavonoids of wild Ephedra alata plant extracts by LC/PDA and LC/MS and their antioxidant activity. *African Journal of Traditional, Complementary, and Alternative Medicines, 14*(2), 130.

Alasalvar, C., Grigor, J. M., Zhang, D., Quantick, P. C., & Shahidi, F. (2001). Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *Journal of Agricultural and Food Chemistry, 49*(3), 1410-1416.

Ali-Shtayeh, M., Salameh, A., Abu-Ghdeib, S., Jamous, R. M., & Khraim, H. (2002). Prevalence of tinea capitis as well as of asymptomatic carriers in school children in Nablus area (Palestine). *mycoses, 45*(5), 188-194.

Amira, S., Dade, M., Schinella, G., & Ríos, J.-L. (2012). Anti-inflammatory, anti-oxidant, and apoptotic activities of four plant species used in folk medicine in the Mediterranean basin. *Pak J Pharm Sci, 25*(1), 65-72.

Ao, C., Higa, T., Khanh, T. D., Upadhyay, A., & Tawata, S. (2011). Antioxidant phenolic compounds from Smilax sebeana Miq. *LWT-Food Science and Technology, 44*(7), 1681-1686.

Apak, R. a., Ӧzyürek, M., Güçlü, K., & Ҫapanoğlu, E. (2016). Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *Journal of Agricultural and Food Chemistry, 64*(5), 997-1027.

Badarinath, A., Rao, K. M., Chetty, C. M. S., Ramkanth, S., Rajan, T., & Gnanaprakash, K. (2010). A review on in-vitro antioxidant methods: comparisions, correlations and considerations. *International Journal of PharmTech Research, 2*(2), 1276-1285.

Bagheri-Gavkosh, S., Bigdeli, M., Shams-Ghahfarokhi, M., & Razzaghi-Abyaneh, M. (2009). Inhibitory effects of Ephedra major host on Aspergillus parasiticus growth and aflatoxin production. *Mycopathologia, 168*(5), 249.

Baker, D. D., Chu, M., Oza, U., & Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. *Natural product reports, 24*(6), 1225-1244.

Bala, N., Saha, S., Chakraborty, M., Maiti, M., Das, S., Basu, R., & Nandy, P. (2015). Green synthesis of zinc oxide nanoparticles using Hibiscus subdariffa leaf extract: effect of temperature on synthesis, anti-bacterial activity and anti-diabetic activity. *RSC Advances, 5*(7), 4993-5003.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry, 99*(1), 191-203.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis, 6*(2), 71-79.

Belhouchet, Z., Sautour, M., Miyamoto, T., & Lacaille-Dubois, M.-A. (2008). Steroidal Saponins from the Roots of Smilax aspera subsp. mauritanica. *Chemical and Pharmaceutical Bulletin, 56*(9), 1324-1327.

Ben-Arye, E., Mahajna, J., Aly, R., Ali-Shtayeh, M. S., Bentur, Y., Lev, E., Den, G., Samuels, N. (2016). Exploring an herbal "wonder cure" for cancer: a multidisciplinary approach. *Journal of cancer research and clinical oncology, 142*(7), 1499-1508.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry, 239*(1), 70-76.

Bohm, B. A. (1998). *Introduction to flavonoids*: Harwood academic publishers.

Bonjar, G. S. (2004). Anti yeast activity of some plants used in traditional herbal medicine of Iran. *J Biol Sci, 4*(212), 5.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews, 56*(11), 317-333.

Bregheith, A. (1995). *Forests of Palestine between 1950-1995*. Palestinian Ministry of Agriculture. West Bank.

Cantin, C. M., Moreno, M. A., & Gogorcena, Y. (2009). Evaluation of the antioxidant capacity, phenolic compounds, and vitamin C content of different peach and nectarine [Prunus persica (L.) Batsch] breeding progenies. *Journal of Agricultural and Food Chemistry, 57*(11), 4586-4592.

Caveney, S., Charlet, D. A., Freitag, H., Maier-Stolte, M., & Starratt, A. N. (2001). New observations on the secondary chemistry of world Ephedra (Ephedraceae). *American journal of botany, 88*(7), 1199-1208.

Chen, K., & Schmidt, C. F. (1926). The action and clinical use of Ephedrine: An alkaloid isolated from the chinese drug Ma Huang. *Journal of the American Medical Association, 87*(11), 836-842.

Chen, T., Li, J., Cao, J., Xu, Q., Komatsu, K., & Namba, T. (1999). A new flavanone isolated from rhizoma smilacis glabrae and the structural requirements of its derivatives for preventing immunological hepatocyte damage. *Planta Medica, 65*(01), 056-059.

Cheng, Y.-B., Zhang, D.-M., & Yu, S.-S. (2004). Chemical constituents of Smilax perfoliata. *ACTA BOTANICA SINICA-ENGLISH EDITION-, 46*(5), 618-620.

Chew, K., Khoo, M., Ng, S., Thoo, Y., Wan Aida, W., & Ho, C. (2011). Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of Orthosiphon stamineus extracts. *International Food Research Journal, 18*(4).

Chirinos, R., Rogez, H., Campos, D., Pedreschi, R., & Larondelle, Y. (2007). Optimization of extraction conditions of antioxidant phenolic compounds from mashua (Tropaeolum tuberosum Ruíz & Pavón) tubers. *Separation and Purification Technology, 55*(2), 217-225.

Choi, S.-J., Hwang, J. M., & Kim, S. I. (2003). A colorimetric microplate assay method for high throughput analysis of lipase activity. *Journal of biochemistry and molecular biology, 36*(4), 417-420.

Cosme, F., Gonçalves, B., Bacelar, E. A., Inês, A., Jordão, A. M., & Vilela, A. (2017). Genotype, Environment and Management Practices on Red/Dark-Colored Fruits Phenolic Composition and Its Impact on Sensory Attributes and Potential Health Benefits *Phenolic Compounds-Natural Sources, Importance and Applications*: InTech.

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100*(1-2), 72-79.

Danin, A. O. F.-S. (2016). Flora of Israel Online. http://flora.org.il/en/plants/. D.O.U. 8/2017.

Demain, A. L., & Fang, A. (2000). The natural functions of secondary metabolites *History of Modern Biotechnology I* (pp. 1-39): Springer.

Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in pharmacology, 4*, 177.

. *Fifth National Report on Biodiversity Conservation*. (2015). EQA (Environmental Quality Authority-Palestine).

Friedman, W. E. (1996). Introduction to biology and evolution of the Gnetales. *International Journal of Plant Sciences, 157*(S6), S1-S2.

Gao, Y., Su, Y., Qu, L., Xu, S., Meng, L., Cai, S.-Q., & Shou, C. (2011). Mitochondrial apoptosis contributes to the anti-cancer effect of Smilax glabra Roxb. *Toxicology letters, 207*(2), 112-120.

Grabley, S., Thiericke, R., & Sattler, I. (2000). Tools for drug discovery: natural product-based libraries *The Role of Natural Products in Drug Discovery* (pp. 217-252): Springer.

Groot, H. d., & Rauen, U. (1998). Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundamental & clinical pharmacology, 12*(3), 249-255.

Harba, A. H., abu Zargab, M., & Abdallaa, S. (2009). Effects of Trans-Resveratrol, Isolated from Smilax Aspera, on Smooth Muscle, Blood Pressure, and Inflammation in Rats and Nociception in Mice.

Harborne, J. B., and Herbert Baxter. (1999). *The handbook of natural flavonoids.* (Vol. Volume 1 and Volume 2): John Wiley and Sons.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Doring, M., Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews, 79*(3), 293-320.

Herrmann, K. M., & Weaver, L. M. (1999). The shikimate pathway. *Annual review of plant biology, 50*(1), 473-503.

Hyuga, S., Hyuga, M., Oshima, N., Maruyama, T., Kamakura, H., Yamashita, T., Yoshimura, M., Amakura, Y., Hakamatsuka, T., Odaguchi, H. (2016). Ephedrine alkaloids-free Ephedra Herb extract: a safer alternative to ephedra with comparable analgesic, anticancer, and anti-influenza activities. *Journal of natural medicines, 70*(3), 571-583.

Ibragic, S., & Sofić, E. (2015). Chemical composition of various Ephedra species. *Bosnian journal of basic medical sciences, 15*(3), 21.

Jaradat, N., Hussen, F., & Al Ali, A. (2015). Preliminary Phytochemical Screening, Quantitative Estimation of Total Flavonoids, Total Phenols and Antioxidant Activity of Ephedra alata Decne. *Journal of Materials and Environmental Science, 6*(6), 1771-1778.

Kabera, J. N., Semana, E., Mussa, A. R., & He, X. (2014). Plant secondary metabolites: biosynthesis, classification, function and pharmacological properties. *J Pharm Pharmacol, 2*, 377-392.

Kang, L. P., Liu, Z. J., Zhang, L., Tan, D. W., Zhao, Y., Zhao, Y., Chen, H.-B., Ma, B. P. (2007). New furostanol saponins from Allium ascalonicum L. *Magnetic Resonance in Chemistry, 45*(9), 725-733.

Kårlund, A., Moor, U., Sandell, M., & Karjalainen, R. O. (2014). The impact of harvesting, storage and processing factors on health-promoting phytochemicals in berries and fruits. *Processes, 2*(3), 596-624.

King, A., & Young, G. (1999). Characteristics and occurrence of phenolic phytochemicals. *Journal of the American Dietetic Association, 99*(2), 213-218.

Kitani, Y., Zhu, S., Omote, T., Tanaka, K., Batkhuu, J., Sanchir, C., Fushimi, H., Mikage, M., Komatsu, K. (2009). Molecular analysis and chemical evaluation of Ephedra plants in Mongolia. *Biological and pharmaceutical bulletin, 32*(7), 1235-1243.

Konar, R., & Singh, M. (1979). Production of plantlets from the female gametophytes of Ephedra foliata Boiss. *Zeitschrift für Pflanzenphysiologie, 95*(1), 87-90.

Kwon, Y.-b., Lee, J.-d., Lee, H.-j., Han, H.-j., Mar, W.-c., Kang, S.-k., Beitz, A. J., Lee, J.-h. (2001). Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. *Pain, 90*(3), 271-280.

Laitonjam, W. S., & Kongbrailatpam, B. D. (2010). Studies on the chemical constituents and antioxidant activities of extracts from the roots of Smilax lanceaefolia Roxb. *Natural product research, 24*(12), 1168-1176.

Lattanzio, V., Lattanzio, V. M., & Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochemistry: Advances in research, 661*, 23-67.

Lee, C.-H., & Lee, H.-S. (2009). Growth inhibiting activity of quinaldic acid isolated from Ephedra pachyclada against intestinal bacteria. *Journal of the Korean Society for Applied Biological Chemistry, 52*(4), 331-335.

Lewis, N. G. (2017). Plant phenolics *Antioxidants in higher plants* (pp. 135-169): CRC press.

Longo, L., & Vasapollo, G. (2006). Extraction and identification of anthocyanins from Smilax aspera L. berries. *Food Chemistry, 94*(2), 226-231.

Ludwig-Müller, J. (2015). Plants and endophytes: equal partners in secondary metabolite production? *Biotechnology letters, 37*(7), 1325-1334.

Mattern, J., Bak, M., Hahn, E. W., & Volm, M. (1988). Human tumor xenografts as model for drug testing. *Cancer and Metastasis Reviews, 7*(3), 263-284.

Mendelovich, M., Shoshan, M., Fridlender, M., Mazuz, M., Namder, D., Nallathambi, R., Selvaraj, G., Kumari, P., Ion, A., Wininger, S. (2017). Effect of Ephedra foeminea active compounds on cell viability and actin structures in cancer cell lines. *Journal of Medicinal Plants Research, 11*(43), 690-702.

Merken, H. M., & Beecher, G. R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: a review. *Journal of Agricultural and Food Chemistry, 48*(3), 577-599.

Middleton, E. (1998). Effect of plant flavonoids on immune and inflammatory cell function *Flavonoids in the living system* (pp. 175-182): Springer.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical science, 84*(4), 407-412.

Mohamad, N., Falah, A.-s., Fatima, J., Hussein, K., Akram, H., Ali, C., & Hassan, R. International Reseach Journal of Pharmacy.

Monks, N. R., Bordignon, S. A., Ferraz, A., Machado, K. R., Faria, D. H., Lopes, R. M., Mondin, C., Souza, I., Lima, M., da Rocha, A. B. (2002). Anti-tumour screening of Brazilian plants. *Pharmaceutical biology, 40*(8), 603-616.

Montoro, P., Tuberoso, C. I., Piacente, S., Perrone, A., De Feo, V., Cabras, P., & Pizza, C. (2006). Stability and antioxidant activity of polyphenols in extracts of Myrtus communis L. berries used for the preparation of myrtle liqueur. *Journal of pharmaceutical and biomedical analysis, 41*(5), 1614-1619.

Morales, L. O., Tegelberg, R., Brosché, M., Keinänen, M., Lindfors, A., & Aphalo, P. J. (2010). Effects of solar UV-A and UV-B radiation on gene expression and phenolic accumulation in Betula pendula leaves. *Tree Physiology, 30*(7), 923-934.

Nabavi, S. M., Marchese, A., Izadi, M., Curti, V., Daglia, M., & Nabavi, S. F. (2015). Plants belonging to the genus Thymus as antibacterial agents: From farm to pharmacy. *Food Chemistry, 173*, 339-347.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*(1-2), 95-111.

Nawwar, M., Barakat, H., Buddrust, J., & Linscheidt, M. (1985). Alkaloidal, lignan and phenolic constituents of Ephedra alata. *Phytochemistry, 24*(4), 878-879.

Newman, D. J., Cragg, G. M., & Snader, K. M. (2000). The influence of natural products upon drug discovery. *Natural product reports, 17*(3), 215-234.

O'Dowd, N., McCauley, P., Wilson, G., Parnell, J., Kavanagh, T., & McConnell, D. (1998). X Ephedra Species: In Vitro Culture, Micropropagation, and the Production of Ephedrine and Other Alkaloids *Medicinal and Aromatic Plants X* (pp. 154-193): Springer.

Oshima, N., Yamashita, T., Hyuga, S., Hyuga, M., Kamakura, H., Yoshimura, M., Maruyama, T., Hakamatsuka, T., Amakura, Y., Hanawa, T. (2016). Efficiently prepared ephedrine alkaloids-free Ephedra Herb extract: a putative marker and antiproliferative effects. *Journal of natural medicines, 70*(3), 554-562.

Ozkan, A., Yumrutas, O., Saygideger, S. D., & Kulak, M. (2011). Evaluation of antioxidant activities and phenolic contents of some edible and medicinal plants from Turkey's flora. *Advances in Environmental Biology*, 231-237.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P., & Volpe, M. G. (2016). Inhibitory effect of pomegranate (Punica granatum L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic Staphylococcus aureus and Escherichia coli. *Food Chemistry, 190*, 824-831.

Pan, L., Chai, H.-B., & Kinghorn, A. D. (2012). Discovery of new anticancer agents from higher plants. *Frontiers in bioscience (Scholar edition), 4*, 142.

Pan, S.-Y., Litscher, G., Gao, S.-H., Zhou, S.-F., Yu, Z.-L., Chen, H.-Q., . . . Ko, K.-M. (2014). Historical perspective of traditional indigenous medical practices: the current renaissance and conservation of herbal resources. *Evidence-Based Complementary and Alternative Medicine, 2014*.

Parr, A. J., & Bolwell, G. P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture, 80*(7), 985-1012.

Parsaeimehr, A., Sargsyan, E., & Javidnia, K. (2010). A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra. *Molecules, 15*(3), 1668-1678.

Parsaeimehr, A., Sargsyan, E., & Vardanyan, A. (2011). Expression of secondary metabolites in plants and their useful perspective in animal health.

Pérez-Jiménez, J., & Saura-Calixto, F. (2015). Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries. *Food Research International, 74*, 315-323.

Philips, H. J. (1958). *Lebanese folk cures.* Columbia University.

Pietta, P.-G. (2000). Flavonoids as antioxidants. *Journal of natural products, 63*(7), 1035-1042.

Pinelo, M., Sineiro, J., & Núñez, M. a. J. (2006). Mass transfer during continuous solid–liquid extraction of antioxidants from grape byproducts. *Journal of Food Engineering, 77*(1), 57-63.

Pourmorad, F., Hosseinimehr, S., & Shahabimajd, N. (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African journal of biotechnology, 5*(11).

Price, R. A. (1996). Systematics of the Gnetales: a review of morphological and molecular evidence. *International Journal of Plant Sciences, 157*(S6), S40-S49.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*(10), 4290-4302.

Ramasamy, S., & Manoharan, A. C. (2004). Antibacterial effect of volatile components of selected medicinal plants against human pathogens. *Asian Journal of Microbiology Biotechnology and Enviromental Sciences, 6*(2), 209-210.

Ramawat, K., & Arya, H. (1976). Growth and morphogenesis in callus cultures of Ephedra gerardiana. *Phytomorphology*.

Randhir, R., Lin, Y.-T., & Shetty, K. (2004). Stimulation of phenolics, antioxidant and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors. *Process Biochemistry, 39*(5), 637-646.

Raúl, S.-C., Beatriz, H.-C., Joseoziel, L.-G., & Francenia, S.-S. N. (2017). Phenolic Compounds in Genus Smilax (Sarsaparilla) *Phenolic Compounds-Natural Sources, Importance and Applications*: InTech.

Rugna, A., Polo, J., Evelson, P., Gurni, A., Llesuy, S., & Wagner, M. (2003). Antioxidant activity in rhizomes from Smilax campestris Griseb. Smilacaceae. *Molecular Medicinal Chemistry, 1*(1), 21-25.

Runyoro, D. K., Matee, M. I., Ngassapa, O. D., Joseph, C. C., & Mbwambo, Z. H. (2006). Screening of Tanzanian medicinal plants for anti-Candida activity. *BMC complementary and alternative medicine, 6*(1), 11.

Sannigrahi, S., Mazumder, U. K., Pal, D., & Mishra, S. L. (2009). Hepatoprotective potential of methanol extract of Clerodendrum infortunatum Linn. against CCl4 induced hepatotoxicity in rats. *Pharmacognosy magazine, 5*(20), 394.

Saviranta, N. M., Julkunen-Tiitto, R., Oksanen, E., & Karjalainen, R. O. (2010). Leaf phenolic compounds in red clover (Trifolium pratense L.) induced by exposure to moderately elevated ozone. *Environmental pollution, 158*(2), 440-446.

Scherer, W. F., Syverton, J. T., & Gey, G. O. (1953). Studies on the propagation in vitro of poliomyelitis viruses: IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *Journal of Experimental Medicine, 97*(5), 695-710.

Serafini, M., Bellocco, R., Wolk, A., & Ekström, A. M. (2002). Total antioxidant potential of fruit and vegetables and risk of gastric cancer. *Gastroenterology, 123*(4), 985-991.

Shahidi, B. G. H. (2004). Evaluation of antibacterial properties of Iranian medicinal-plants against Micrococcus luteus, Seratia mascescens, Klebsiella pneumonia and Brodetella bronchoseptica. .

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of functional foods, 18*, 820-897.

Shahidi, F., & Naczk, M. (2003). *Phenolics in food and nutraceuticals*: CRC press.

Sharma, S. V., Haber, D. A., & Settleman, J. (2010). Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. *Nature reviews cancer, 10*(4), 241.

Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X., & Li, P. (2010). Antioxidant activity in vitro of the selenium-contained protein from the Se-enriched Bifidobacterium animalis 01. *Anaerobe, 16*(4), 380-386.

Shui, G., & Leong, L. P. (2006). Residue from star fruit as valuable source for functional food ingredients and antioxidant nutraceuticals. *Food Chemistry, 97*(2), 277-284.

Singh, V., Guizani, N., Essa, M., Hakkim, F., & Rahman, M. (2012). Comparative analysis of total phenolics, flavonoid content and antioxidant profile of different date varieties (Phoenix dactylifera L.) from Sultanate of Oman. *International Food Research Journal, 19*(3), 1063.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture, 16*(3), 144-158.

Soltan, M. M., & Zaki, A. K. (2009). Antiviral screening of forty-two Egyptian medicinal plants. *Journal of ethnopharmacology, 126*(1), 102-107.

Sparg, S., Light, M., & Van Staden, J. (2004). Biological activities and distribution of plant saponins. *Journal of ethnopharmacology, 94*(2-3), 219-243.

Stotz, H. U., Kroymann, J., & Mitchell-Olds, T. (1999). Plant-insect interactions. *Current opinion in plant biology, 2*(4), 268-272.

Sun, T.-T., Zhang, D.-W., Han, Y., Dong, F.-Y., & Wang, W. (2012). Smilasides M and N, two new phenylpropanoid glycosides from Smilax riparia. *Journal of Asian natural products research, 14*(2), 165-170.

Towers, G., Lopez, A., & Hudson, J. (2001). Antiviral and antimicrobial activities of medicinal plants. *Journal of Ethno-pharmacology, 77*, 189-196.

Verpoorte, R. (1999). Chemodiversity and the biological role of secondary metabolites, some thoughts for selecting plant material for drug development *Bioassay Methods in Natural Product Research and Drug Development* (pp. 11-23): Springer.

Vlietinck, A. (1991). Screening methods for antibacterial and antiviral agents from higher plants *Methods in plant biochemistry/Hostettmann, K.[edit.]* (Vol. 6, pp. 47-69).

Wu, L.-S., Wang, X.-J., Wang, H., Yang, H.-W., Jia, A.-Q., & Ding, Q. (2010). Cytotoxic polyphenols against breast tumor cell in Smilax china L. *Journal of ethnopharmacology, 130*(3), 460-464.

Xie, J., & Schaich, K. (2014). Re-evaluation of the 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. *Journal of Agricultural and Food Chemistry, 62*(19), 4251-4260.

Xu, J., Li, X., Zhang, P., Li, Z.-L., & Wang, Y. (2005). Antiinflammatory constituents from the roots of smilax bockii warb. *Archives of pharmacal research, 28*(4), 395-399.

Zhang, C.-L., Feng, S.-X., Wang, Q., Wang, P., Xu, J., & Chen, T. (2014). Flavonoids and phenolic compounds from Smilax scobinicaulis. *Chemistry of natural compounds, 50*(2), 254-257.

فصل المركبات الاولية النقية وفحص النشاط الدوائي المضاد للفطريات والبكتيريا والاكسدة من مستخلصات النباتات البرية الفلسطينية *Ephedra foeminea* و *Smilax aspera*

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الملخص

يستكشف البحث العلمي الحالي نبتتين فلسطينيين بريتين هما *Ephedra foeminea* (العلندة) و *Smilax aspera* fruits (العُلّيق) لأول مرة. ولقد اكتسبتا كلتا النبتتين لونهما الأحمر الجذاب بسبب وجود الكروموفورز من وفرة المواد الفينولية الموجودة في النبتتين. تم جمع الثمار في موسم نضجها في أكتوبر 2016 و 2017 ؛ الإيفيدرا من بني نعيم وسميلاكس من بيت جالا بعد أن تم تجفيفهما بالهواء ، وتم استخراج المواد الفعالة بشكل منفصل في الإيثانول 99 ٪ ، والإيثانول 80 ٪ والماء المقطر 100 ٪ بواسطة سونيكيتر عند درجة اربعين مئوية. تم حساب المحتوى الكلي الفينولي (TPC) ومحتوى الفلافونويد الكلي (TFC) لكل من النبتتين. لوحظ ان أعلى محتوى للفينولات في مستخلصات الماء من *E. foeminea* و *S. aspera* هو 3.22 و 8.21 mg من حمض gallic لكل غرام عينة على التوالي. وبالمثل أظهرت نتائج ال TFC ان أعلى محتوى الفلافونيدات في مستخلص الماء لنبات *S. aspera* بقيمة 1.22 ملجم catechin لكل عينة. و مع ذلك فإن مستخلص

E. foeminea كانت له نسبة مئوية أعلى في مستخلص الايثانول 99 ٪ بقيمة 1.0433 mg catechin / g sample. تم التحقيق في نشاط مضادات الأكسدة بطريقتين: (FRAP) وطريقة (DPPH) ، وتم العثور على أفضل النتائج ل *E. foeminea* و *S. aspera* في مستخلصات الإيثانول بقيمة من 9.5 و 5.9 ملغ trolox / g على التوالي ، مع 91.3 ٪ من تثبيط جذري ل Ephedra. وتم توضيح النشاط المضاد للأكسدة كذلك من خلال القدرة على تقليل كاشف FRAP لكل من النبتتين في مستخلصات الماء بقيم عالية (0.57 و 0.71 مجم Fe + 2 / g من العينة الجافة في *E. foeminea* و *S. aspera* على التوالي). تم قياس النشاط المضاد للميكروبات وأظهرت النتائج ان مستخلص *S. aspera* له تأثير إيجابي ضد البكتيريا ـلموجبة الجرام (Staphylococcus aureus) والخميرة (Candida albicans) و *E. foeminea* ، مع ذلك ، لم تظهر أي آثار إيجابية على

البكتيريا او الخميرة. عند فحص مستخلص النبتتين ضد الخلايا السرطانية للقولون (HT-29)، كلتا النبتتين لم تظهر أي تثبيط للخلايا السرطانية.

تم فصل مركبات نقية جدا واخضاعهما للفحص التحليلي الالي باستخدام HPLC-PDA ، LC-MS (ESi الايجابي والسلبي) و LCMSMS (مسح الايون المتكون) لمستخلص EtOH الفعال 80٪ من كلا النباتين. تم التوصل الى كشف هويات المركبات الفينولية في النبتتين بعد حقن 1000 ميكرولتر (100 ملغ) من مستخلصات الفاكهة عند 350 نانومتر إلى العمود الكروماتوغرافي C18 من مثل Quercetin ، Kaempferol، Lucenin 2، Vicenin 2، Quercetin 3-O-rhamnoside-7-O-glucoside و Quercetin-3-O-rhamnoside في *E. foeminea*. في حين تم تحديد خمسة مركبات في نبات ال *S. aspera* وهي كما يلي: apigenin 7-glucuronide, luteolin derivative, lucenin 2, quercetin-3-glucoside and isorhamnetin-3-O-rutinoside.