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Extraction, isolation and preliminary structural elucidation of some anti-cancer constituents of wild *Urginea maritima* and their antioxidant activities

Aysha Ali Mahmmoud Rayyan

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Prepared By:

Aysha Ali MahmmoudRayyan

B.Sc.: Chemistry. Al -Quds University. Palestine.

Supervisor: Dr. Fuad Al-Rimawi.

Co- Supervisor: Prof. Saleh Abu-Lafi.

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Prepared by: Aysha Ali MahmmoudRayyan
Registration No: 21412313

Supervisor: Dr. Fuad Al-Rimawi.
Co-supervisor: prof. Saleh Abu-Lafi.

Master thesis submitted and accepted, Date: 14/7/2018

The names and signatures of the examining committee members are as follows:

1- Head of Committee: Dr.Fuad Al-Rimawi Signature:.....
2- Co supervisor: prof. Saleh Abu-Lafi Signature:.....
3- Internal Examiner: Prof. Ibrahim Kyalai Signature:.....
4- External Examiner: Prof. Raqi Shubietah Signature:.....

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Dedication;

To my parents

You are the reason for me being excited without you I would be nothing

of what I did reach today, thank you for being Patience, much of love.

To my brothers and sisters

Thank you for your support and believe in me and encourage me to make it happen much of love and respect.

To all of my friends

To you my friends to you my second family there is no words in the world i can express my feeling thankful to you.

Declaration:

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

Signed……

Aysha Ali Mahmmoud Rayyan

Date: 14\7\2018

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Abstract

For thousands of years, the holy land/Palestine, despite its small area, it contains colossal number of medicinal plants to which has been used as folkloric herbal medicine. *Urginea maritima (U. maritima)* is one of the medicinal plants that grows widely in Palestine. Samples of *U. maritima* were collected from Abu-Dies region in January 2017, dried in shade for two weeks until it became completely dry, grinded, and then extracted by sonication. The aim of this research is to investigate the anticancer of flowers of *U. maritima* on HCT 116 and MDA (Colon and Breast cancer cells respectively), using solvents of different polarities (Distilled water, Ethanol, Ethyl acetate, Dichloromethane, and Hexane). Antioxidant activity (AA) was studied using different tests like FRAP, DPPH, ABTS, and CUPRAC. Total phenolic content (TPC) and total flavonoids content (TFC) of the extracts was also conducted. Results showed that ethanolic extract of *U. maritima* has higher anticancer activity compared to other solvents. Therefore, ethanolic extract was utilized to HPLC-PDA analysis, semi-preparative HPLC-PDA fractionation and LCMS in both positive and negative ESi modes to elucidate some structures of the separated compounds.

All antioxidant activity tests (FRAP, DPPH, ABTS, CUPRAC), as well as TPC and TFC, was found to be higher for polar solvents like Distilled water, Ethanol, and Ethyl acetate, while it was found to be lower for non-polar solvents like dichloromethane and hexane.
The inhibition of HCT-116 and MDA cancer cells were found to be high when the concentration is 100μg/ml, which was (50% after 24 hours and 90% after 72 hours for HCT 116 cells – 90% after 24 hours and 100% after 72 hours for MDA cells). Anticancer activity of *U. maritima* extracts of 50μg/ml was found to the similar to the concentration of 100μg/ml, while (1, and 10) μg/ml has very low activity. Which mean breast cancer cells have more efficacious as anticancer activity than colon cancer cells.

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List of Acronyms

Abbreviation	**Description**
U.maritima	*Urginea maritime*
APS	Aromatic plants
Eos	Essential oils
FRAP	Ferric Ion Reducing Antioxidant Power Assay
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine)
NCI	National Cancer Institute
MCF7	Brest Cancer cells
AA	Antioxidant activity
TPC	Total Phenolic Content
TFC	Total Flavonoids Content
HPLC	High-performance liquid chromatography
DPPH	2,2-diphenyl-1-picrylhydrazyl
NO	Nitric Oxide
HQ	Hydroquinone
UV	Ultraviolet- Visible spectrophotometer
MTT	Microculture Tetrazolium blue
MS	Mass Spectrometry
MDA	Breast Cancer
IC_{50}	half maximal inhibitory concentration
GAE	Gallic acid equivalence
TEAC	Trolox equivalent antioxidant capacity
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
EtOH	Ethanol
D.W	Deionized water
Ppm	part per million
CEQ	Catechin equivalent
Abbreviation	**Description**
FRAP	Ferric Ion Reducing Antioxidant Power Assay

CUPRAC	Cupric reducing antioxidant capacity
HCT-116	Colon Cancer
PDA	Photo Diode Array
DMSO	Dimethyl Sulfoxide
TAC	Total Antioxidant Capacity
pH	Power of hydrogen
ESI	Electrospray ionization
PTFE	Polytetrafluroethylene
COSY	Correlation Spectroscopy
HMQC	Heteronuclear Multiple Quantum correlation
TIC	Total Ion Current
LC-MSMS	Liquid Chromatography- tandem mass spectrometry
FWHM	Full Width at Half Maximum
CN	Acetonitrile

Chapter One

Introduction

1. Introduction:

1.1. Background

Medicinal plants are more commercially available, cheaper, as compared to modern classical drug therapy. These plants usually contain various combinations of active components. To exploit their medicinal benefits, chromatographic isolation and spectroscopic identification can be used to discover their structure and when applied to *in-vitro* and *in-vivo* analysis, a new drug candidate could be revealed. Therefore the bioactive compounds have tangible effect on the living organism, tissue or cell (Singh, Singh et al. 2015). In the last decade, there are tremendous investigations that took place to explore new drugs to combat the resistance of antibiotics, antimalarial and Antigen(Boland 2001)

1.2. Polyphenols

Most of the promising medicinal plants contain polyphenols. Polyphenols are secondary metabolites of plants of which 8,000 polyphenolic compounds have been identified in various plant species. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist. Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common (Kondratyuk and Pezzuto 2004). Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main groups of polyphenolic compounds, the flavonoids, stilbenes and lignans are shown in figure 1.1 that illustrates the different groups of polyphenols and their chemical structures (Rodríguez-Morató, Xicota et al. 2015).

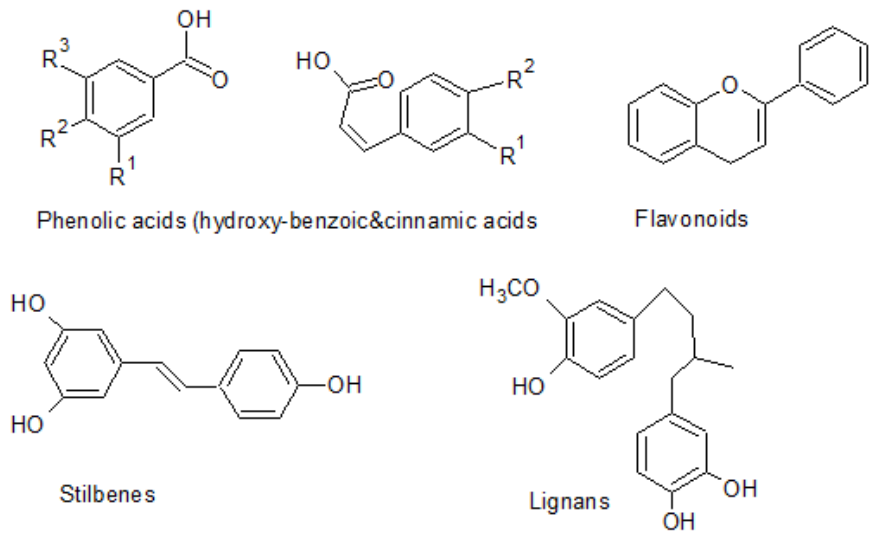


Figure 1.1: Chemical structures of the different classes of polyphenols, where R_1, R_2 and R_3 are H, OH or OCH_3 respectively.

1.2.1. Phenolic Acid

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Caffeic acid is the most abundant phenolic acid in many fruits and vegetables (Velioglu, Mazza et al. 1998).

1.2.2. Flavonoids

One of the major groups of polyphenolic compounds, the Flavonoids, is important in contributing to the flavours and colour of many fruit and vegetables (Figure1.2).

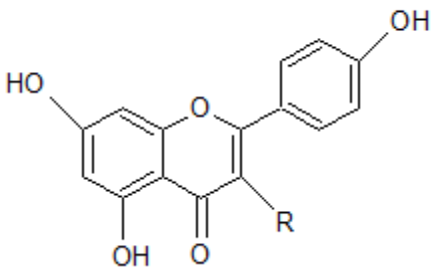


Figure1.2: Basic structure of Flavonoids (Ali 2008)

This group has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocyclic (Figure 1.3). More than 4,000 varieties of Flavonoids have been identified, many of which are responsible for the attractive colors of the flowers, fruits and leaves (Groot et al, 1998). Based on the variation in the type of heterocyclic involved, flavonoids may be divided into six subclasses: Flavonols, Flavones, Flavanones, Anthocyanins, flavan-3-ol and Isoflavones (Figure1.3). Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or Glycosylation. Quercetin, Myricetin, Catechins, etc., are some of the most common Flavonoids.

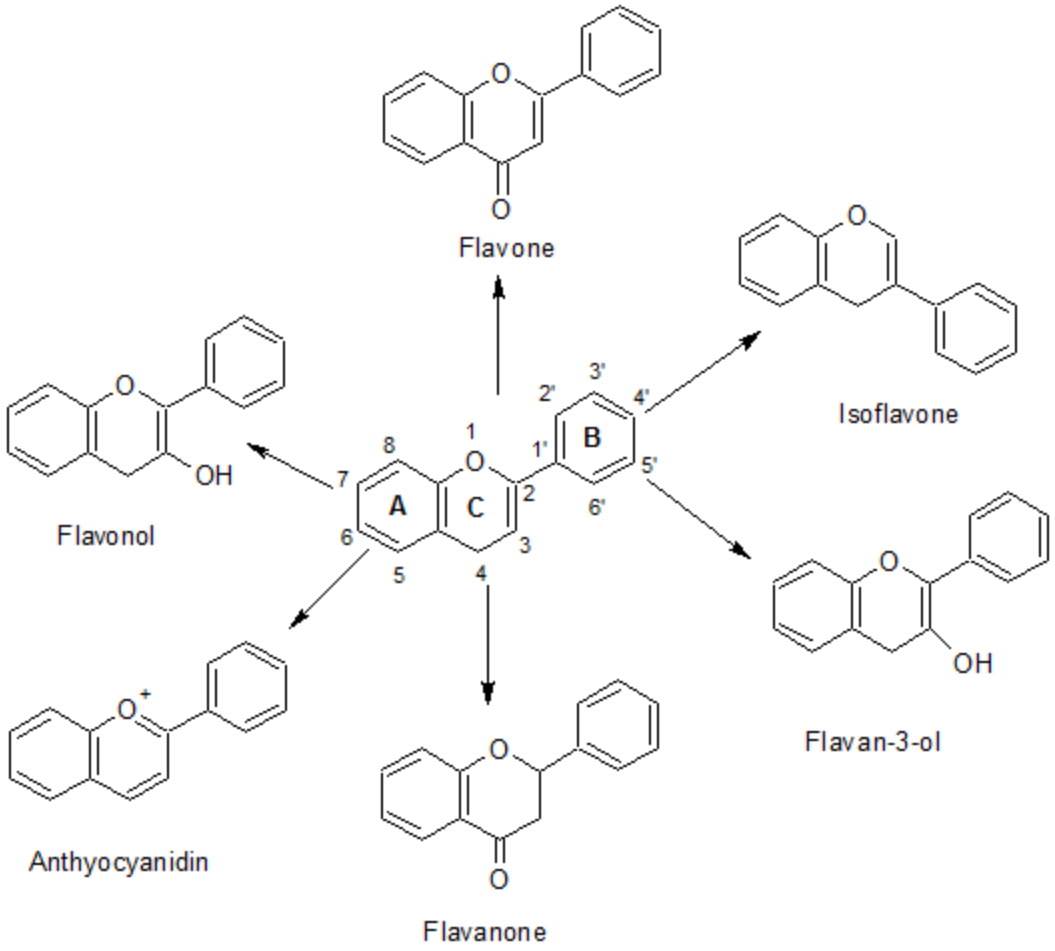


Figure 1.3: Chemical structures of the six main classes of Flavonoids (Ali 2008)

1.3. Palestinian Medicinal Plants

Throughout history, aromatic plants (APs) were used as potential source of drug discovery and development for chemoprevention and as preservatives in foods. The famous (APs), namely, chamomile, fennel, ginger, thyme, basil, and sage originate mainly in the Mediterranean area (A Al-Tamimi, Rastall et al. 2016). Palestine in particular have 2,600 reported medicinal plants where plant species that belong to 130 different families. Many of these plants are native and could be used in folk medicine (Abu-Darwish and Efferth 2018).

APs have been reported to exhibit wide range of biological effects. In particular, phenolics and essential oils (EOs) could include antioxidant, antiparasitic, antimicrobial, anti-carcinogenic, and anti-inflammatory properties. In addition, EOs are used for the management of chronic diseases like cardiovascular, diabetes, alzheimer's and cancer (A Al-Tamimi, Rastall et al. 2016).

In general EOs are part of plants volatile and semi-volatile complex composition such as terpenes, sesquiterpenes and oxygenated compounds (alcohols, aldehydes, ketones, acids, phenols, oxides, lactones, acetalse, ethers and esters) (Pourmortazavi and Hajimirsadeghi 2007). Volatile compounds are usually isolated through steam distillation. Plant essential oils are commonly used food, medicinal, pharmaceutical, perfumery, and cosmetics industries due to their attractive flavors (Ali 2008; A Al-Tamimi, Rastall et al. 2016). However, there are many other non- polar constituents in plants that may cure many diseases. These compounds are usually separated by flash and/or preparative chromatography followed by NMR analysis to elucidate their structure.

1.4. Chemical assays of plants extracts

1.4.1. Antioxidant activity (AA)

AA May be regarded as an important fundamental property for life. They have many of the biological functions, such as antimutagenicity, anticarcinogenicity, and antiaging (Velioglu, Mazza et al. 1998). Natural antioxidants are distributed in different parts of the

plants such as bulb, wood, stems, leaves and flowers (Rahiman, Tantry et al. 2013). Antioxidants inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas those natural antioxidants can be phenolic compounds such as tocopherols, flavonoids, and phenolic acids, or nitrogen compounds such as alkaloids, chlorophyll derivatives, amino acids, and amines.

The AA from natural extracts can be evaluated with different tests such as: FRAP, DPPH, CUPRAC, and ABTS.

1.4.1.1. FRAP method

The Ferric Ion Reducing Antioxidant Power Assay (FRAP) is robust, sensitive, inexpensive, and simple method used to measure antioxidant activity of plants extract. In the FRAP method reduce Fe (III)-2, 4, 6-Tri (2-pyridyl)-s-triazine (TPTZ) complex to Fe (II)-TPTZ under acidic conditions, color changed from yellow to blue. The absorbance was measured using spectrophotometer at 593 nm versus blank (Rahman, Shamsudin et al. 2018).

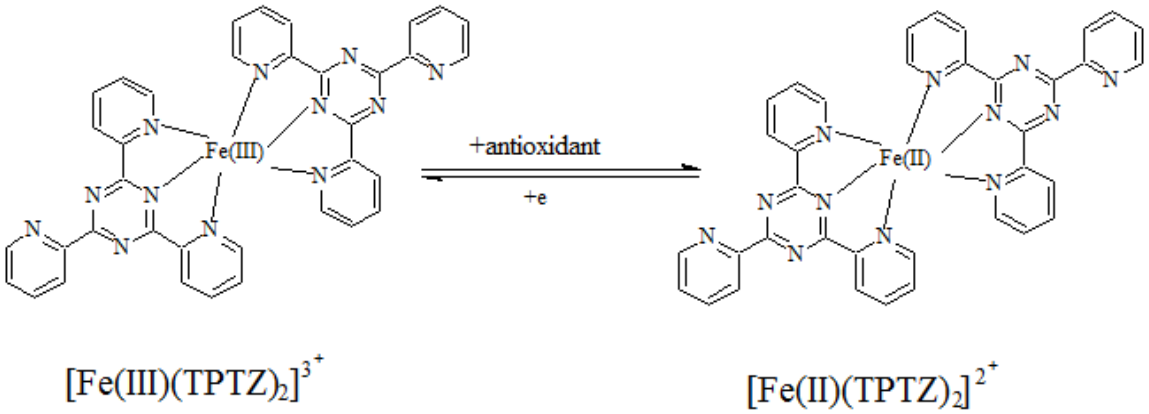


Figure 1.4: Chemical structures of reaction of yellow Fe^{3+} TPTZ complex (2, 4, 6-tri (2 pyridyl)-1, 3, 5-triazine) with antioxidants is reduced to the blue Fe^{2+} TPTZ complex by electron-donating substances (Prior, Wu et al. 2005).

1.4.1.2. Determination of 2,2 diphenyl-1-1picrylhydrazyl radical scavenging activity

DPPH is a free radical that is stable at room temperature, when reacts with sample extracts a change of color from purple to yellow is observed. This change is recorded using a spectrophotometer at 517 nm (Rahman, Shamsudin et al. 2018).

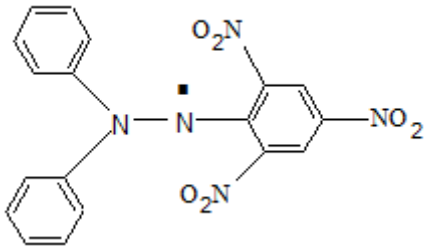


Figure 1.5: Chemical structure of DPPH.

The percentage of inhibition of DPPH radical can be calculated using the following equation:

% Antioxidant activity = (A\AC)*100

Where:

A_C = the absorbance of blank control

1.4.1.3. CUPRAC method

Cupric reducing antioxidant capacity is named of abbreviated CUPRAC method. These assays are based on the redox chemistry copper (II) to copper (I). Depending on the ability of polyphenols to reduce Copper (II). Moreover copper (II)- neoucuproine [Cu(II)-Nc] reagent was used as chromogenic oxidizing to determine the antioxidant capacity of food. The mixture consists of antioxidant solution with a copper (II) chloride solution, a neocuproine alcoholic solution (2, 9-dimethyl-1, 10-phenanthroline), and an ammonium acetate at pH 7.0 with maximum absorbance at 450 nm (Apak, Güçlü et al. 2004).

1.4.1.4. ABTS method

The ABTS cation radical [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt], used as decolorizing assay which absorbs at 743 nm. It is formed through the reaction between ABTS and potassium persulfate (see Figure 1.6), where of an electron is lost by the nitrogen atom of ABTS to produce blue/green (ABTS•+)

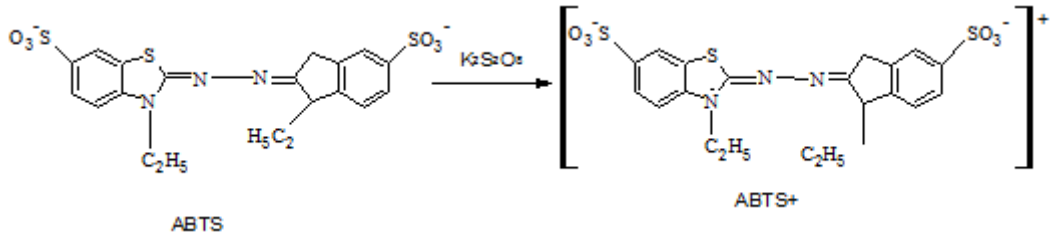


Figure 1.6: Oxidation of ABTS with $K_2S_2O_8$ and generation of ABTS+ (Miller, Rice-Evans et al. 1993).

1.5. Cancer spreading

Cancer is one of the major problems affecting public health worldwide. Cancer is the main leading causes of death globally which is characterized by rapid and uncontrolled division of abnormal cells due to their internal factors like hormones, external factors such as viruses, unhealthy and environmental change like air pollution, smoking (Aggarwal and Shishodia 2006). Currently, it is anticipated that the burden of cancer will increase to 23.6 million (Singh, Singh et al. 2015). In addition, WHO estimates that up to 19 million new cases of cancer per year will be witnessed by 2025, due to growth of global population (Jaradat, Al-Ramahi et al. 2016).

Mediterranean's use medicinal plants to fight cancer including Arabian Peninsula, Syria, Turkey, Palestinian territories, Lebanon, Iran, Egypt, Jordan and Iraq (Abu-Darwish and Efferth 2018).

Radiotherapy and chemotherapy are the most common methods of cancer treatment, but they may cause death. Some cancer patients preferred another alternative method of treatments like herbal medicine (Jaradat, Al-Ramahi et al. 2016).

There are many types of cancers infect humans like cancers of the stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas, colon lung, breast, ovarian, neck, colorectal, prostate, liver, blood, brain cancer and bladder.

In the Arab world, it was found that cancer of the lung and liver are the most common among men, and breast cancer is most common among women (Jaradat, Al-Ramahi et al. 2016). The most recent data on cancer revealed the dependence on age of patient, tumor location, and sex of patient (male or female) were in male gender diseases of the lung are (5%), liver (21%), stomach (5%) and prostate (13%). Among females, the most common cancers were those of the breast (34%), cervix (29%), ovary (11%) and endometrium (Agyare, Spiegler et al. 2018).

1.6. Secondary metabolism from natural product

Secondary metabolism, also known as photochemical can be synthesized in specialized cell types and at distinct developmental stages, this making extraction and purification more difficult. Secondary metabolism classification includes three main groups: terpenoids, alkaloids and phenolic all main groups showing a large number of natural products with pharmacology activities leading to the discovery and development of drug. In addition it can be classified on the basis of their chemical structure e.g. having rings, composition (if it contains nitrogen or not) and solubility (Waksmundzka-Hajnos, Kowalska et al. 2008; Kabera, Semana et al. 2014).

Phenolic compound is considered as one of the largest group of secondary plants which is synthesized by fruit, vegetable, teas and other plants. Besides, phenolic compound are characterized by the anti-carcinogenic, antioxidant, anti-inflammatory and other biological properties (Kabera, Semana et al. 2014).

1.7. Overview of the Studied Plant:

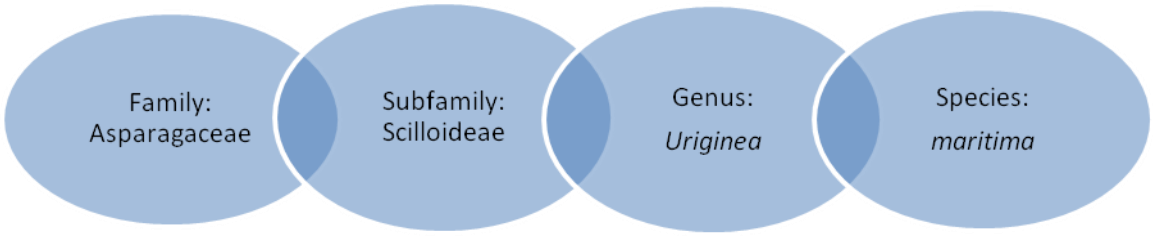
1.7.1. *U. maritima* plant (عيصلان)

U. maritima, sea onion or white squill, is a native plant found in the Mediterranean area, North Africa, and India (Bozcuk, ÖZDOĞAN et al. 2011). It belongs to Hyacinthaceae (Liliaceae) family. It used as an antibacterial, anthelmintic and abortifacient. It is also used as an expectorant in bronchitis, chronic catarrh and pneumonia. Fresh bulbs are applied to wounds to promote healing. It is useful for rheumatism, edema, gout, slowing down pulse and in the treatment of cancer (El-Seedi, Burman et al. 2013). *U. maritima* is known to exert diuretic and a powerful digitalis-like cardiac effect. It is also used as a source of natural cardiac steroid products in traditional remedial and veterinary medicine as well. Reciprocally, the acute toxicity of these compounds has been ignored in related biological studies for several decades. Recently, several works provide scientific evidence concerning the efficiency and possession of this compound's unique character to target and attack cancer cells with less severe toxicity to non-malignant cells (Elghuol, Mohamed et al. 2013). To the best of our knowledge the flowers have never been investigated either in Palestine or the rest of the world.

U. maritima comprises two main kinds of squills that differ in the color of the tunics, leaf shape, scape length and fruit size. The phenological aspects of these species all the year around are very interesting (Hassan and Khalifa).

The basic components of *U. maritima* bulb are: Glycoside, Anthocyanins, Flavonoids, Fatty acid, polysaccharides and calcium oxalate (Belhaddad, Charef et al. 2017).

Photos of *U. maritima* flower and its taxonomical subdivisions



1.8. Research Questions

1. **Does** *U. maritime* flowers have phenolic, and flavonoid compounds?
2. **Does** *U. maritima* flowers crude extracts have anti-oxidants and anticancer activities?
3. **Does** *U. maritima* of flower individual isolates have anticancer activity after purification using semi-preparative chromatography?

1.9. Objectives and aims

1. To evaluate the TPC and TFC of *U. maritima* flowers extract and to analyze the endogenous phenolic compounds using HPLC-PDA.
2. To evaluate the anticancer and antioxidant activities of *U. maritima* flowers extracts by using different solvents with different polarities (water > ethanol >ethyl acetate> dichloromethane > n-hexane).
3. To scale up some semi-preparative HPLC fractions of the extract that showed good anticancer activity.
4. To analyze the semi- preparative HPLC fractions and LC-MS, in both positive and negative ESi modes.

1.10. Hypotheses

U. maritima plant possess phenolic and flavonoids compounds and could be probably have anticancer and anti-oxidant activities.

Chapter Two

Literature Review

In the literature, there are many papers that report about the *U. maritima* plant, particularly, their antioxidant and anticancer effect. For example, (Cai, Luo et al. 2004) studied the antioxidant activity of phenolic compounds of 112 species of Chinese medicinal plants including *U. maritime* associated with their anticancer activities. AA was performed by using ABTS method Trolox equivalent antioxidant capacity, (TEAC). Results showed a positive significant linear relationship between antioxidant activity and total phenolic content (all R^2 values≥0.95) indicating that phenolic compounds were the dominant antioxidant components in the tested medicinal herbs. Moreover, *U. maritime* exhibited far stronger antioxidant activity and contained significantly higher levels of phenolics than common vegetables and fruits.

Another study conducted by (Mammadov, Makasçı-Afacan et al. 2010) investigated the antioxidant activities of extracts obtained from *U. maritima (L.) Baker* plants leaves and tubers. AA as well as TPC was determined in the plant extract obtained using different concentrations of the plant aqueous extracts. The AA and TPC was determined using DPPH, and Folin-Ciocalteu reagent (FCR).Result showed that the highest AA was found in the UTE (*Urginea* Tuber Ethanol) and the lowest AA was found UTB (*Urginea* Tuber Benzene). On the other hand, the AA was found that the ULB (*Urginea* Leaves Benzene) and the ULE (*Urginea* Leaves Ethanol) were the highest. However ULA (*Urginea* Leaves Acetone) was found to possess the lowest AA. *U. maritima (L.) Baker* plants were very rich in phenolics and flavonoids.

Third study was focused on the anticancer activities of aqueous extract of *U. maritime,* by (Obeidat and Sharab 2018). The anticancer activity of *U. maritima* fruit on breast cancer cells (MCF7), using solvents at different polarities (water, ethanol, methanol and acetone) were explored. Results showed that ethanol and acetone extracts of *U. maritima* fruits have more efficacious anticancer activity than water and methanol. Interestingly, IC50 value of acetone extracts was (6.01µg/ml). According to American National Cancer Institute (NCI), any crude extracts possessing an IC50, which is less than 20µg/ml is considered as active treatment against the tested cancer cells. Besides, ethanol and acetone extracts of *U. maritima* fruits which have IC50 less than 20µg/ml, can be considered an active treatment against breast cancer cells.

In another previous study, (Bozcuk, ÖZDOĞAN et al. 2011) investigated anticancer activity of *U. maritima* on lung cancer cells, using different concentrations of the plant aqueous extracts alone or in combination with other chemotherapeutic agents such as gemcitabine or cisplatin. They showed that *U. maritima* has a cytotoxic activity when added to A549 non-small cell lung cancer (NSCLC) cell line. The results demonstrated that *U. maritima* onion (fruit) extract was more efficacious and more cytotoxic to lung cancer cells than the conventional therapies (cisplatin and gemcitabine) as demonstrated by MTT assay and an Annexin V-FITC apoptosis detection kit. The efficacy of *U. maritima* extract was improved by the addition of an antioxidant cocktail.

Another study was involved in the cytotoxic activity determination of sixty-one Egyptian medicinal plants, demonstrated that *U. maritima* methanol and dichloromethane extract have anticancer activity against U-937 GTB human lymphoma cell line. Moreover, the authors isolated some of the bioactive cardiac glycoside proscillaridin A (El-Seedi, Burman et al. 2013).

(Merghoub, Amzazi et al. 2009) studied cytotoxic potential of seven medicinal plants from Morrocco. The result showed that *U. maritima* methanol extracts have an anticancer activity against SiHa and HeLa cell lines respectively HPV 16 and HPV 18. MTT (microcultureTetrazolium blue) colorimetric assay was used to evaluate the viability of cell cultures. Nevertheless, the same authors in (2012) studied the cytotoxic effect of the same medicinal plants from Morocco and the same extracts were determined by GC/MS.

Finally, another research, (Elghuol, Mohamed et al. 2013), investigated the cytotoxic and anti-proliferation effects of the crude plant extracts of *U. maritima* on human malignances neuroblastoma SH-SY5Y cell line, using *in-vitro* techniques . The results indicated that aqueous extracts of *U. maritima* were active on the SH-SY5Y cell line.

In addition table 2.1 which summarized the quantitative estimates of extracts of *U. maritime.*

Table 2.1: quantitative estimates of extracts of *U. maritima*

Part of *U. maritima*	Breast cancer cells	Lung cancer cells	Antioxidant activity	Reference
Leaves & Tubers	_____	_____	**+ve** AA and very rich in TPC & TFC	(Mammadov, Makasçı-Afacan et al. 2010)
Fruit(bulb)	**+ve** anticancer activity ethanolic extracts	**+ve** anticancer activity	_____	(Obeidat and Sharab 2018) and (Bozcuk, ÖZDOĞAN et al. 2011)

Chapter Three

Experimental Work

3.1. Chemicals, Reagents and Plant materials

3.1.1. Chemicals

99.9% Ethanol, n-hexane, dichloromethane, ethyl acetate, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl- S-triazine (TPTZ), 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{+}$), ferric chloride hexahydrate, Catechin, gallic acid, hydrolic acid, acetic acid, sodium nitrite, aluminum chloride, cupper chloride, Ammonium acetate, neocuproine, sodium bicarbonate are purchased from Sigma-Aldrich. HPLC grade of acetonitrile and acetic acid, LCMS grade of formic acid, acetonitrile (ACN), methanol (MeOH) and water (H2O) were purchased from Sigma-Aldrich. Membrane filters (0.45 μm pore size) were purchased from Sigma-Aldrich. Ultrapure water (>18 MΩ cm-1) for semi preparative HPLC experiments was generated from an Ultrapure Water system, Millipore, Merck. Ethanol (EtOH) solvent was purchased from Merck. The acetonitrile and water were of an HPLC grade from Sigma. Phenolic and flavonoids standards: Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin, p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose were from Sigma.

3.1.2. Reagents

The following table shows all the used reagents and the way of preparation

Number	Reagents	Preparation
1.	Ferric chloride hexahydrated (20mM)	(20mM, Mwt = 270.3 g/mol), Dissolve 540mg of it in 100ml of water.
2.	5% $NaNO_2$	Dissolve 10g of $AlCl_3$ in 100ml of water.
3.	7.5% Na_2CO_3	Dissolve 7.5g of Na_2CO_3 in 100ml of water
4.	0.3M Acetate buffer	Dissolve 16.8g of acetic acid and 0.8g of sodium hydroxide in 1000 ml of water at pH 3.6.
5.	TPTZ	(10 mM, Mwt = 312.34 g/mol), dissolve 0.312g TPTZ in 100ml HCl.40 mM HCl was prepared by diluting 3.77ml of stock HCl solution (10.6M) to 1000ml with water.
6.	DPPH	(0.1mM, Mwt= 394.32 g/mol), dissolve 19.7mg of DPPH in 500ml of 99.9% methanol.
7.	Folin–Ciocalteu reagent	10ml of folin reagent was diluted to 100ml using water.
8.	ABTS stock solution	(7mM, Mwt= 548.68 g/mol), dissolve 384mg of ABTS in 100ml water.
9.	Potassium persulfate	(2.45mM, Mwt= 270.32 g/mol), dissolve 66mg of potassium persulfate in 100ml ethanol.
10.	Neocuproine solution	(0.0075 M, Mwt= 208.26 g/mol), dissolve 156mg of neocuproine in 100ml of ethanol.
11.	Copper (II) Chloride solution	(0.01 M, Mwt= 134.45g/mol), dissolve 134.5mg of copper chloride in 100ml of water.
12.	Ammonium Acetate solution	(1M, Mwt= 77.08 g/mol) at pH 7.0, dissolve 7.7g of it in 100ml of water.
13.	10% $AlCl_3$	Dissolve 10g of $AlCl_3$ in 100ml of water.

3.1.3. Plant materials

U. maritima wild flowers samples were obtained from Abu-Dies, Palestine. *U. maritima* samples were collected in January 2017.

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, 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 Electrospray Ionization (ESI) LCMS analysis 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

The flowers of *U. maritima* samples were dried at 30 °C, grinded with a blender. Briefly, 10g of the dried powdered plant material were sonicated with 100 ml of different solvents (D.W, 99.9% Ethanol, n-hexane, dichloromethane, ethyl acetate), extracted for 180 min at 40^{o}C, and filtered. Then the crude extracts were stored in Refrigerator at 4^{o}C until analysis.

3.3.2. Total phenolics content (Folin–Ciocalteu assay)

Total phenolics were determined using Folin–Ciocalteu reagents (Singleton and Rossi 1965). The *U. maritima* extracts (40) µl were mixed with 1.8 ml of Folin–Ciocalteu reagent (pre-diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of (100 – 500 ppm) were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/ g sample (Shui and Leong 2006).

3.3.3. Total Flavonoids

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

3.3.4. Measurement of Antioxidant Activity by FRAP assay

The antioxidant activity of the *U. maritima* extracts was determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of (Benzie and Strain 1996). Freshly prepared FRAP reagent (3.0 ml) were warmed at 37◦C and mixed with 40 µl of the *U. maritima* extracts and the reaction mixtures were later incubated at 37˚C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37 ˚C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay . Aqueous solutions of known Fe (II) concentrations in the range of (2 - 5 mM) ($FeSO_4.6H_2O$) were used for calibration.

3.3.5. Cupric reducing antioxidant capacity (CUPRAC) assay

The assay was conducted as described previously (Apak, Güçlü et al. 2004). To 0.5 ml of plant extract or standard of different concentrations solution, 1 ml of copper (II) chloride solution (0.01 M prepared from $CuCl_2.2H_2O$), 1 ml of ammonium acetate buffer at pH 7.0 and 1 ml of neocaproin solution (0.0075 M) were mixed. The final volume of the mixture was adjusted to 4.1 ml by adding 0.6 ml of distilled water and the total mixture was incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against blank.

3.3.6. Antioxidant activity by DPPH radical scavenging assay

Free radical scavenging activity of extract of flower of *U. maritima* were measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) by (Shen, Zhang 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 solvents extract (D.W, 99.9% Ethanol, n-hexane, dichloromethane, and ethyl acetate). The mixture was shaken vigorously and allowed to stand at room temp for 30 min. then; absorbance was measured at 515 nm by using UV-VIS spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using following equation:

DPPH scavenging effect (%) or Percent inhibition = $(A_0 - A_1 / A_{0)} \times 100\%$.

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

3.3.7. Antioxidant activity by ABTS assay

This assay was based on the ability of different substances to scavenge 2, 2'- azino-bis (ethylbenzthiazoline-6-sulfonic acid ($ABTS^+$) radical cation (Re, Pellegrini et al. 1999). The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 14-16 h until the reaction was complete and the absorbance was stable. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9 mL of $ABTS^+$ Solution and 0.1 mL of tested samples (100 and 200 µg/mL) and mixed for 45 sec; measurements were taken at 734 nm after 15 min. The antioxidative activity of the tested samples was calculated by determining the decrease in absorbance at different concentrations by using the following equation: DPPH scavenging effect (%) or percent inhibition = $((\triangle A)/ A_o) \times 100\%$, where: A_o is the absorbance of the $ABTS^+$.

3.3.8. HPLC analysis of phenolic compounds

3.3.8.1. Chromatographic conditions

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.

3.3.8.2. Sample preparation for HPLC analysis

The plant extracts were filtered using suction filtration, and then the solvents were evaporated under reduced pressure at 40 C using Rotary evaporator. For water extract, lyophilization of the resulting crude extract was done to remove the remaining water. The resulting crude extracts were dissolved in the respective solvents (water, ethanol, and 80% ethanol) at a concentration of 5 mg/mL, and 20 μL were injected into the HPLC chromatograph, and analyzed for their phenolic and Flavonoids.

Seventeen phenolic and flavonoid standards were injected and separated simultaneously to identify the presence of any of these compounds in the crude extracts. Calibration curve of each individual standard was also prepared at three concentration levels namely 50, 100 and 250 ppm.

3.3.9. Semi-preparative HPLC-PDA and LCMS

3.3.9.1. Semi-preparative HPLC-PDA conditions

The crude extract was obtained by macerating (1:10) wt/vol of dried plant flowers absolute ethanol, sonicated for 2 hours at room temperature. The resultant extract was evaporated using rotary evaporator at 70°C under reduced pressure. Then, it was lyophilized using freeze drier until constant weight was achieved. The final dried extract was stored in amber vial and kept in the fridge until fractionated by semi-preparative HPLC. Prior injection, the solution (0.1g/10 ml EtOH and one drop of acetic acid) was filtered using disposable Polytetrafluoroethylene PTFE filters of 0.45 μm. The semi-preparative HPLC experiments were run on ODS column (Cosmosil packed column 5C18-MS-II, 10 μm ID x 250 mm) supported with pre-column (Cosmosil packed column 5C18-MS-II, 10 μm ID x 20 mm). The gradient program started from 99% acidic water (0.5% acetic acid): 1% acetonitrile to 95 % acetonitrile in 60 minutes and back to the starting mobile phase composition in 2 minutes. The flow rate was 4 ml/minutes, the injection volume was 50 μl, the column

temperature was set at room temperature and the PDA wavelengths ranged from 200-500 nm.

3.3.9.2. Direct injection to LCMS

A direct injection LCMS analysis all the semi-preparative HPLC collected aliquots were performed in both the positive and negative electrospray ionization (ESi) modes. The mobile phase consisted of 70% acidic water (0.1% formic acid):30% acetonitrile. The MS conditions were as follows:

1. For -ESi, the ion voltage was 2500 volts, ion transfer tube temperature was 250oC, vaporizer temperature 200oC, full scan range was 100-600 Dalton (Da), quadrupole resolution was 0.7 full width at half maximum (FWHM), sheath gas 25 L/min, aux gas 5 L/min and scan rate was 1000 Da per second.
2. For +ESi, the ion voltage was 3500 volts, ion transfer tube temperature was 325oC, vaporizer temperature 275oC, full scan range was 100-1000 Dalton (Da), quadrupole resolution was 0.7 full width at half maximum (FWHM), sheath gas 35 L/min, aux gas 10 L/min and scan rate was 1000 Da per second.

Chapter Four

Results & Discussion

The flowers of *U. maritima* were collected, dried at room temperature, milled, extracted in different solvents then filtrated. Samples of crude extracts were analyzed for TPC, TFC, AA, and anticancer activity.

4.1. Total phenolic content (TPC)

Total phenolic content of the different extracts of *U. maritima* was determined by using the Folin-Ciocalteu reagent and were expressed as mg gallic acid equivalents (GAE) per gram of plant flower extract. The TPC of the tested fractions were calculated using the standard curve of gallic acid. Linear equation was generated y = (0.0027x + 0.1172) with high coefficient of determination R^2 = 0.9988 (Figure 4.1).

Results showed that the TPC for *U. maritima* extracts using different solvents with different polarities (D.W, absolute ethanol, ethyl acetate, CH_2Cl_2, hexane) have different quantities. As shown in (Table 4.1), high TPC value was found using polar solvents (D.W, ethanol, ethyl acetate) compared to the non -polar ones (CH_2Cl_2, hexane). The highest TPC was obtained in water extract and the lowest was in CH_2Cl_2 extract.

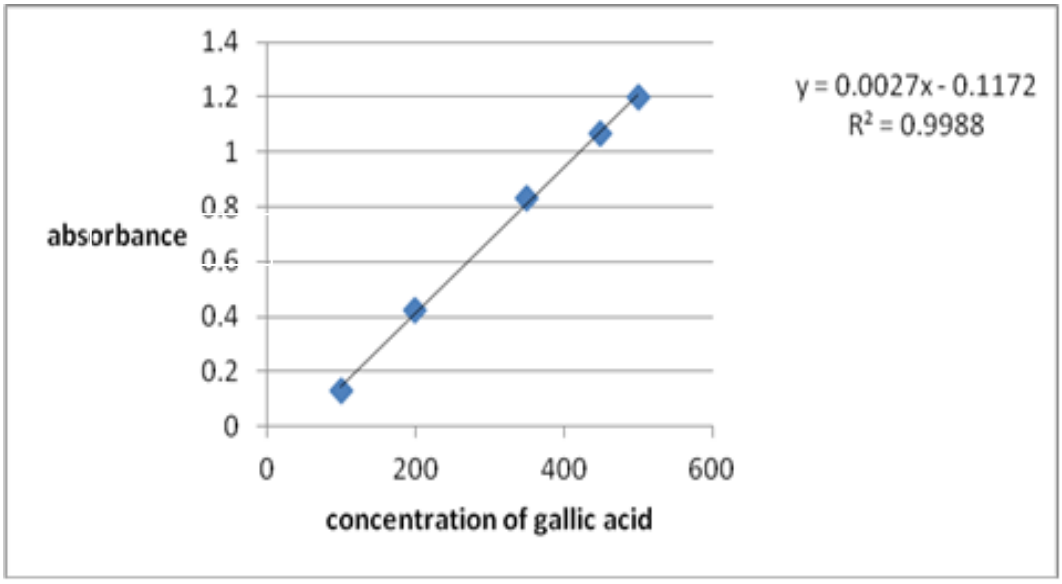


Figure 4.1: Calibration curve of Gallic acid standard.

Table 4.1: TPC in (mg Gallic acid/ g dry sample) of *U. maritima* extracted with different solvents polarities.

Flowers *U. maritima*	mg Gallic acid/ g sample
D.W	85.3
Absolute ethanol	78.8
Ethyl acetate	73.5
CH_2Cl_2	45.26
Hexane	57.16

4.2. Total flavonoid content (TFC)

This method was used to determine the TFC of the different extracts of *U. maritima* TFC was calculated based on standard curve of Catechin. Linear equation was generated y = (0.0048x + 0.0034) with high coefficient of determination $R^2 = 1$ (Figure 4.2).

The TFC values were found to be higher for intermediate polar solvents (ethyl acetate and ethanol) compared to polar solvents (water) or non-polar solvents (hexane and CH_2Cl_2). The highest TFC was found to be for ethyl acetate extract and the lowest TFC was for hexane extract (see table 4.2).

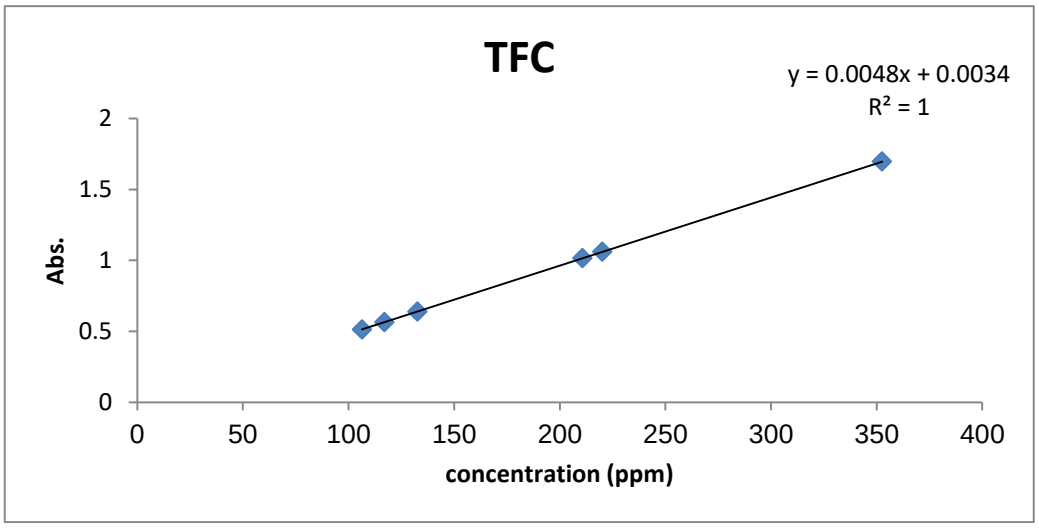


Figure 4.2: Calibration curve Catechin standard.

Table 4.2: TFC in (mg Catechin/g of dry sample) of *U. maritima* extracted with different solvents polarities.

Flowers *U. maritia*	mg catechin / g sample
D.W	5.2
Absolute ethanol	6.6
Ethyl acetate	29.7
CH_2Cl_2	1.3
Hexane	1.1

4.3. DPPH free radical scavenging assay

The free radical in-vitro scavenging activity of plant extracts were evaluated by examining the ability to reduce DPPH, a stable free radical reagent that has a purple color, with maximum absorption wavelength of 515 nm. Upon reaction with a hydrogen donor DPPH, the purple color fades away due to conversion of DPPH to 2, 2-diphenyl-1-picryl hydrazine resulting in a decrease in absorbance. Linear equation was generated y = (-0.0026x + 0.7803) with high coefficient of determination R^2 = 0.9991(Figure 4.3).

Figure 4.3 showed that the calibration curve for DPPH (Abs Vs Conc. of Trolox) and results were expressed as $\mu Mole$ Trolox / g sample of powder dry plant material. Table 4.3 shows DPPH free radical scavenging assay of the extracts. DPPH values were found to be higher for water, ethanol, and ethyl acetate extracts compared to hexane and CH_2Cl_2 solvents. Percent inhibition of DPPH by plant extracts was also calculated (table 4.3) and was found to be in the range of 35-81 %.

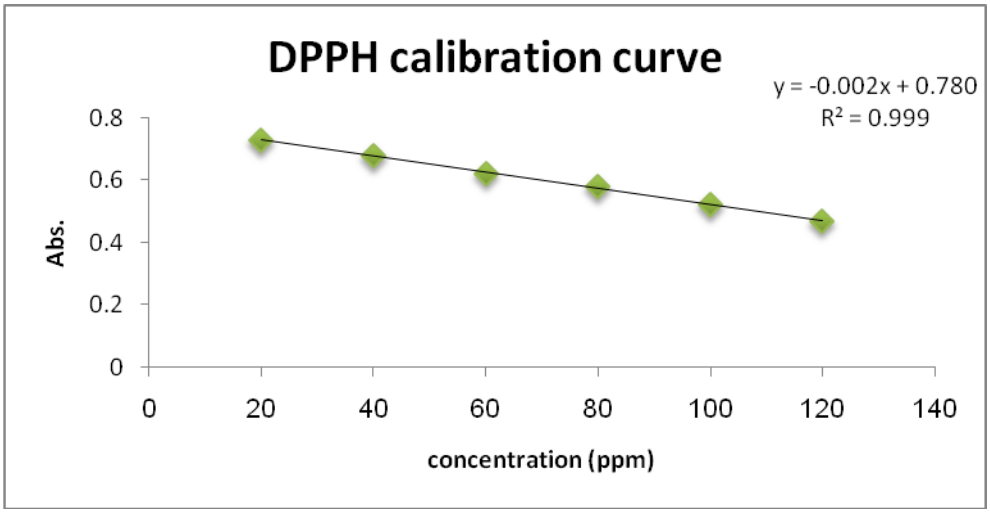


Figure 4.3: Calibration curve of Trolox standard (DPPH).

The % inhibition was calculated by:

$(A_o - A_{sample})/ A_o) \times 100\%$

Where:

A_0: Absorbance of control sample

A_{sample}: Absorbance of sample examined

Table 4.3: DPPH content in $\mu Mole$ Trolox / g dry sample of *U. maritima* extracted with different solvents polarities.

Flowers *U. maritia*	$\mu Mole$Trolox / g sample	% inhibition
D.W	15.1	81
Absolute ethanol	9.4	70
Ethyl acetate	13.7	74
CH_2Cl_2	5.7	59
Hexane	4.0	35

4.4. Ferric reducing antioxidant power (FRAP)

This method evaluated the free radical scavenging activity of plant extracts. FRAP is simple, fast, inexpensive, and robust method, 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).

The AA of solvents plant extracts of *U. maritima* was evaluated by FRAP method and were expressed as mmole Fe^{2+} per gram of plant extract. It was calculated based on standard curve of concentration of Fe^{2+}. Linear equation was generated y = (0.2019x – 0.1766) with high coefficient of determination R^2 = 0.9822 (Figure 4.4).

Table 4.4 showed FRAP of *U. maritima* plant extracts. As shown in this table, D.W extracts found to be higher FRAP followed by ethanol and ethyl acetate, while it was found that FRAP is low for non-polar solvents (CH_2Cl_2 and hexane).

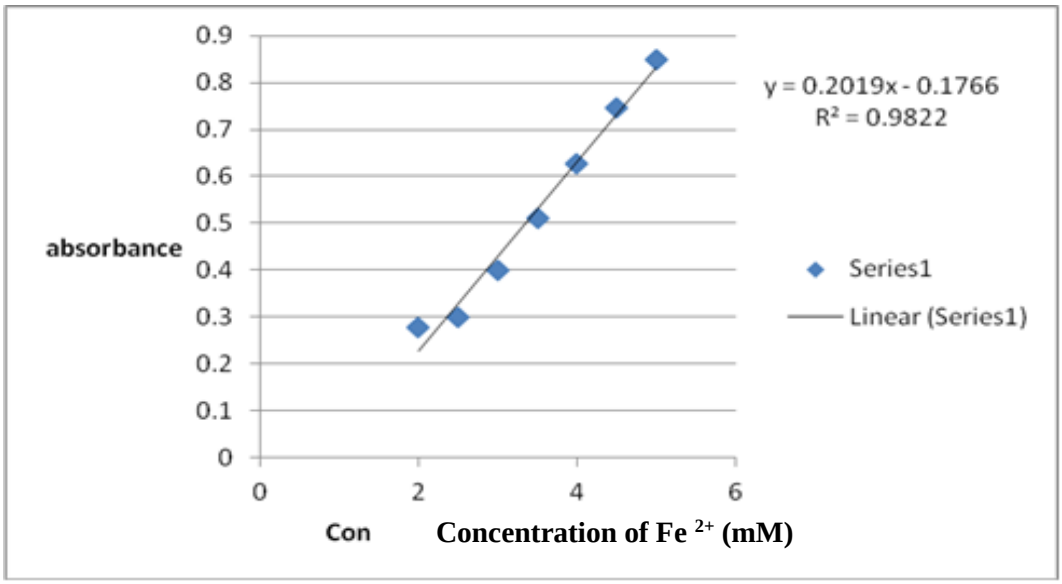


Figure 4.4: Calibration curve of Fe^{2+} Standard.

Table 4.4: FRAP content in mM Fe^{2+} / g of dry sample of *U. maritima* extracted with different solvents polarities.

Flowers *U. maritima*	mg Fe^{2+} / g sample
D.W	0.89
Absolute ethanol	0.42
Ethyl acetate	0.36
CH_2Cl_2	0.06
Hexane	0.04

4.5. Cupric reducing antioxidant power

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

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

This assay was expressed as mg Trolox /g sample by the standard curve of CUPRAC (Figure 4.5) measured based on the equation (y= 0.001x + 0.011, R^2 = 1). Results in (Table 4.5) showed cupric of *U. maritima* plant extracts. As shown in this table, D.W extracts

found to be highest for water followed by ethanol and ethyl acetate, while it was found lowest for non-polar solvents (CH_2Cl_2 and hexane).

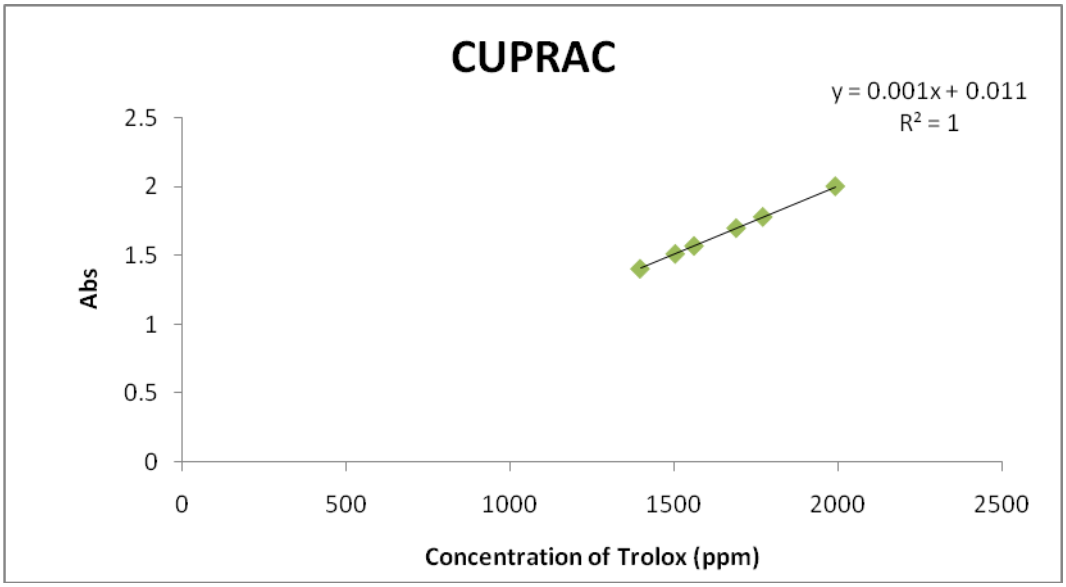


Figure 4.5: Calibration curve of Trolox standard (CUPRAC).

Table 4.5: cupric reducing content in mg Trolex / g dry sample of *U. maritima* extracted with different solvents polarities

Flowers *U. maritima*	mg Trolex/ g sample
D.W	72.4
Absolute ethanol	62.7
Ethyl acetate	59.7
CH_2Cl_2	24.4
Hexane	15.4

4.6 Free radical scavenging activity using ABTS

This method was used to evaluate the FRAP of plant extracts. The plant extracts have the ability to inhibit ABTS radical and this method is used to measure it. This assay was expressed as µmole Trolox /g sample. This assay was expressed as µmole Trolox /g sample by the standard curve of ABTS (Figure 4.6) measured based on the linear equation was generated y= (-0.0154x + 0.6578) with high coefficient of determination R^2= 0.9971.

Table 4.6 showed ABTS of *U. maritima* plant extracts. As shown in this table, D.W extracts was found to be highest for water followed by ethanol and ethyl acetate, while it was found lowest for non-polar solvents (CH_2Cl_2, and hexane).

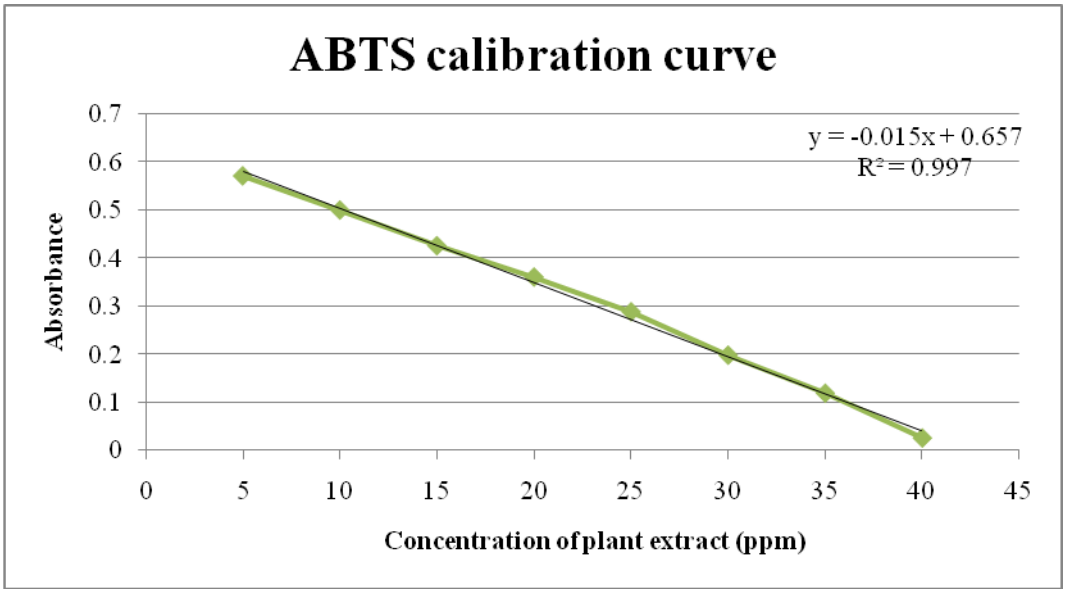


Figure 4.6: Calibration curve of Trolox standard (ABTS).

Table 4.7: ABTS content in $\mu Mole$ trolex / g dry sample of *U. maritima* extracted with different solvents polarities.

Flowers *U. maritima*	$\mu Mole$Trolox / g sample	% inhibition
D.W	0.52	63
Absolute ethanol	0.12	37.2
Ethyl acetate	0.11	18.6
CH_2Cl_2	0.09	7.1
Hexane	0.10	4.2

4.7 HPLC analysis of the extracts

Figure 4.7, shows the chromatogram of the crude ethanolic extract of *U. maritima* with a concentration of 5mg/ml at 430 nm at 10µl injection. This wavelength was selected since the main peaks showed a maximum absorption close to it. As seen from this chromatogram, different lipophilic compounds were detected in the range of 62-68 minutes. The compounds showed maximum wavelength at 407 and 408 nm with shoulders at 436 nm (figure 4.7). While when moving to a wavelength of 335 nm, bunch of peaks in

the range of 17-35 minutes as well as from 45-55 minutes was detected (figure 4.8). From the UV-Vis spectra, the compounds showed an absorption of about 268 nm and 317-357 nm. These absorptions are typical indication of flavonoid and phenolic compounds presence. However, all the separated compounds that have been detected either on 430 or 335 nm are not part of the standards injected under the same experimental conditions as per their retention times and their corresponding UV-Vis spectral matchings (figure 4.9).

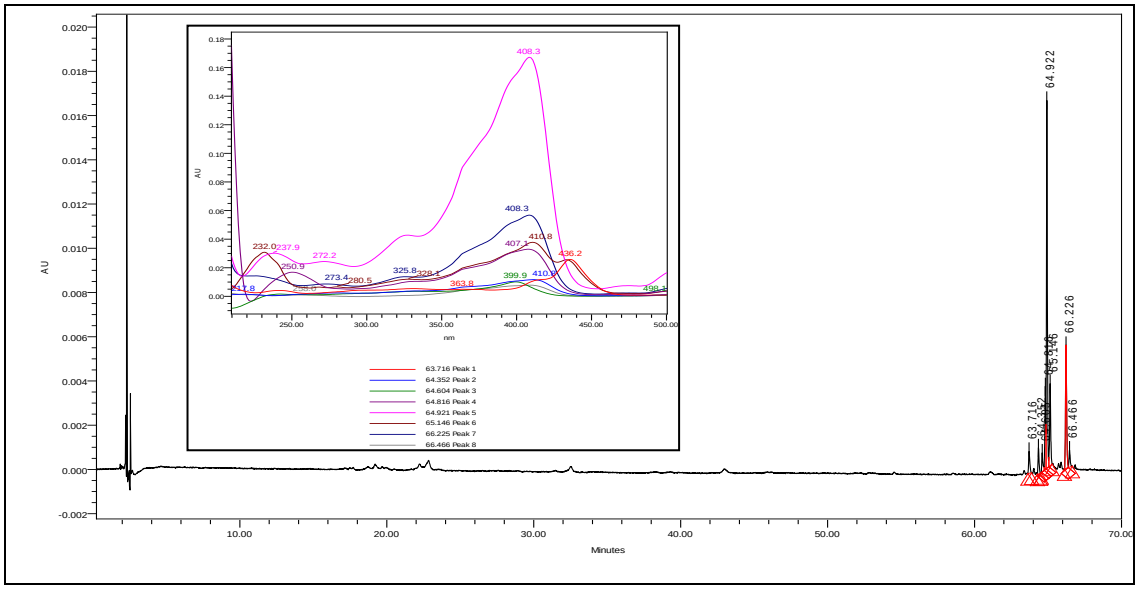


Figure 4.7: HPLC-PDA chromatograms of crude extract at 430 nm. The overlaid UV-Vis spectra of the main peaks are in the left side of the chromatogram.

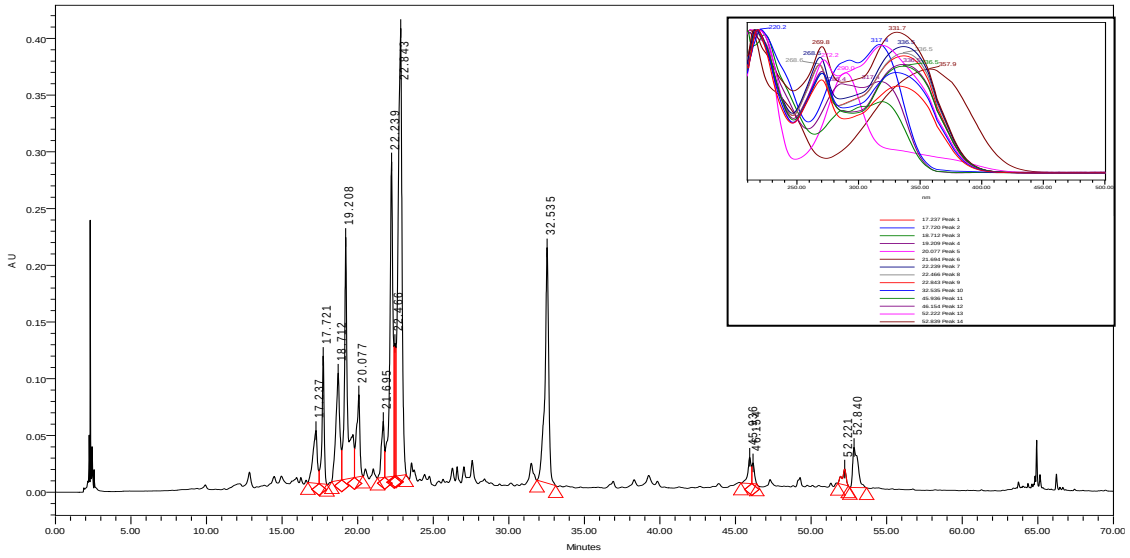


Figure 4.8: HPLC-PDA chromatograms of standard at 335 nm. The overlaid UV-Vis spectra of the separated compounds are seen in the right side of the chromatogram.

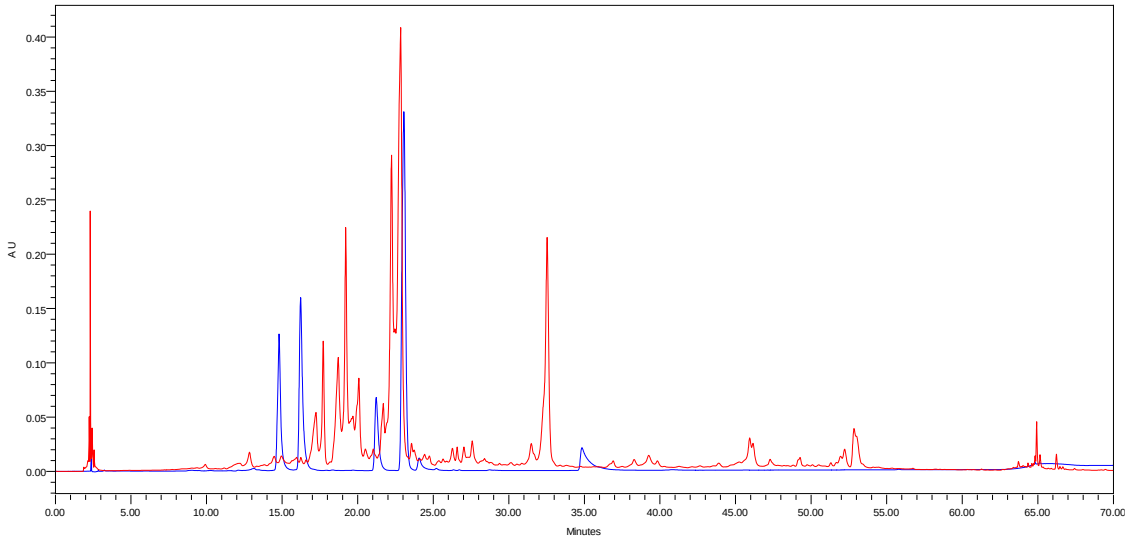


Figure 4.9: Overlaid HPLC-PDA chromatograms of plant extract (red) and standards (blue) at 335 nm.

4.8 Semi-preparative HPLC-PDA and LCMS

The injection of 50 µl to the semi-preparative HPLC column equals nearly 0.5 mg of crude material distributed about 15 major peaks as seen in figure 4.10. The corresponding overlaid UV-Vis spectra is shown inside the HPLC profile.

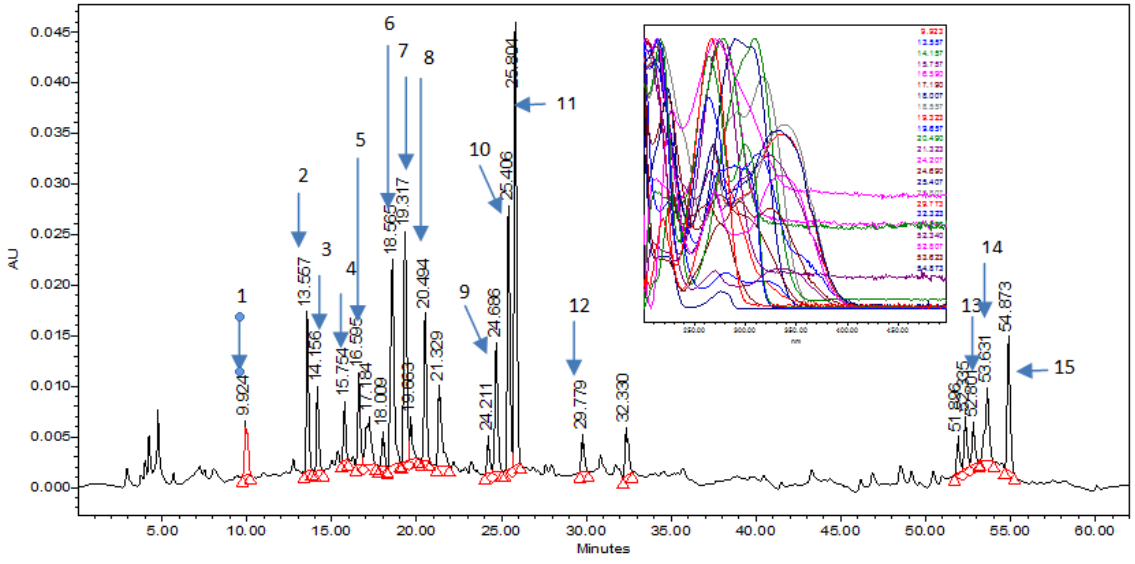


Figure 4.10: Typical semi-preparative HPLC-PDA chromatogram of all the peaks (1-15) present in crude absolute ethanol extract at 283 nm and their overlaid UV-Vis spectra between 200-500 nm.

The semi-preparative chromatograms showed peaks that sometimes share the same maximum of UV-Vis wavelengths (Table4.7).

Fifteen pure isolates were collected directly from the semi-preparative HPLC-PDA effluent holding with it the mobile phase constituent at the collection time. The relevant retention (collection time) of each phytochemical along with their UV-Vis spectrum are shown in table 4.7. The wavelengths observed in general are close to flavonoids and phenolic compounds.

Table 4.8: Semi-prep HPLC fractions with retention times

Cpds Vial #	**Retention time (minutes)**
1	9.92
2	13.55
3	14.15
4	15.75
5	16.59
6	18.55
7	19.32
8	20.49
9	24.69
10	25.4
11	25.8
12	29.77
13	52.34
14	53.62
15	54.87

Compound 1 showed a maximum wavelength at 266.4 nm while compound 2 and 3 were very similar in their overlaid UV-Vis spectra having two maximum wavelengths at 264 and 299.7 respectively. This reflects the similarity of the main chromophoric functionalities of the structurally related compounds. Similar observation was noticed in compound 4 and 5 who showed clear two wavelength maxima's at 270 and 335.6 nm

respectively. Compounds 6 and 7 were also similar to 4 and 5 with slight shift of both maxima's (table 4.7).

Compound 8 showed a maximum wavelength at 309.3 nm while compound 9 showed low absorption at 270 nm indicative to very low concentration or weak chromophore. Compounds 10 possess maximum absorption at 290.2 nm while compound 11 at 317.2 nm and compound 12 at 278 nm. Compounds 13, 14 and 15 share almost the same absorption at 274.7, 275.9 and 275.9 nm respectively.

The collected pure phytochemical isolates were directly injected to the LC-MSMS machine. Figures 4.11 to 4.34 show the Total Ion Chromatogram (TIC) generated for all the 15 isolates using both the negative and positive ESi modes. Figure 4.12 (+ve ESi mode of compound 1) showed a peak at m/z of 345 Da indicating to the presence of bufadienolide steroidal skeleton. This compound may form many bufadienolide glycosides which is believed to act as cardiac glycosides. The backbone of peak 345 Da has been seen in all the +ve ESi modes of the 15 phytochemicals examined. This indicate to an important observation that most of the phytochemicals are based on bufadienolide added to it some glucose moieties. The structure of bufadienolide skeleton is shown inside figure 4.12.

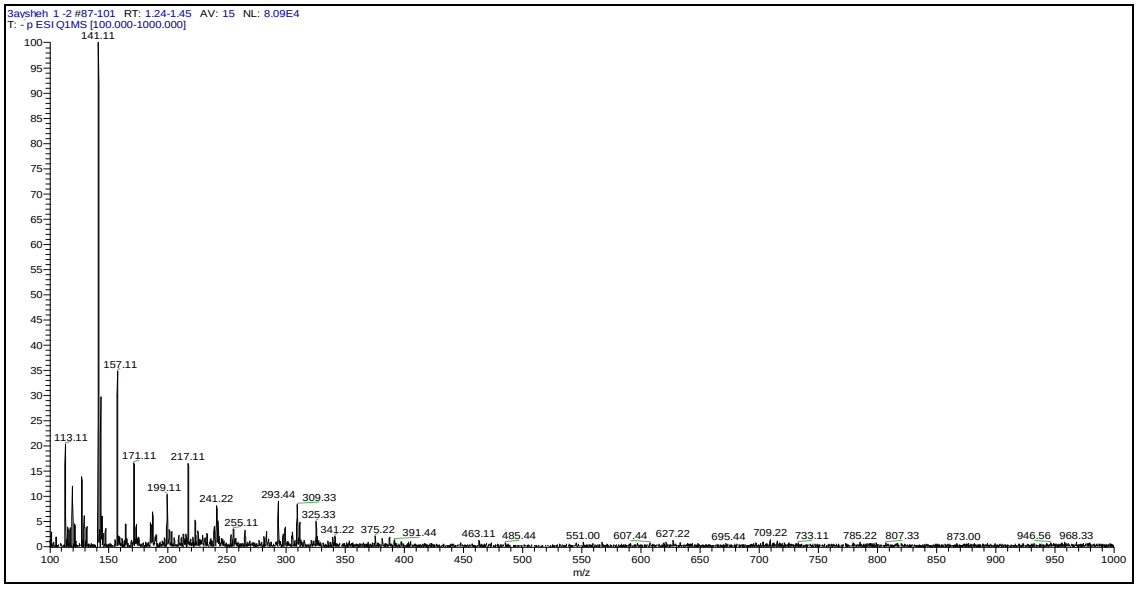


Figure 4.11:–ve ESi mode LCMS for compound 1

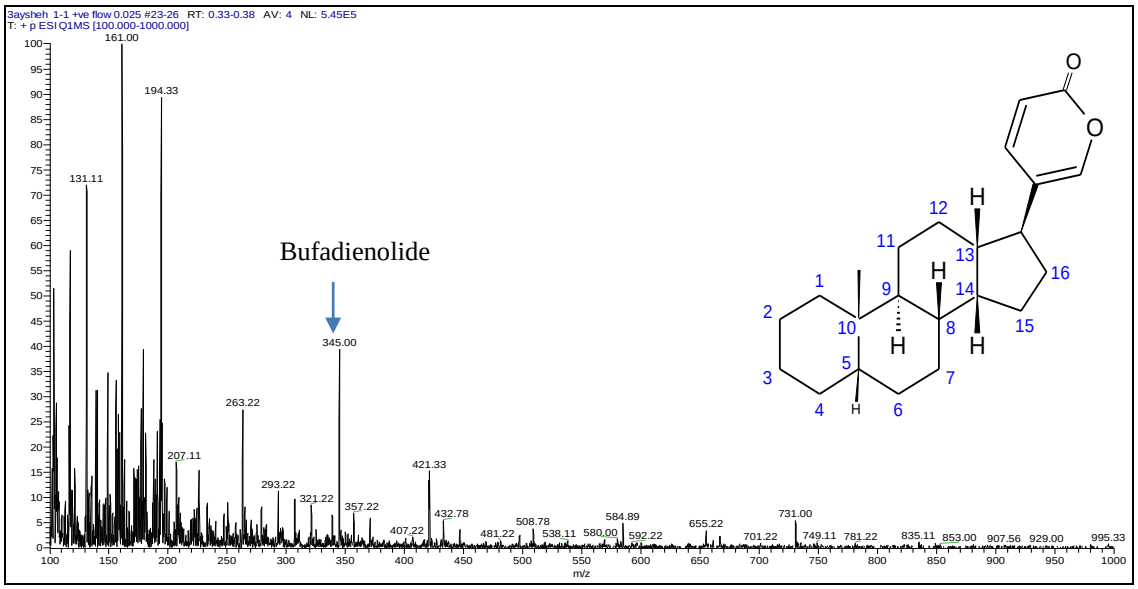


Figure 4.12: +ve ESi mode LCMS for compound 1

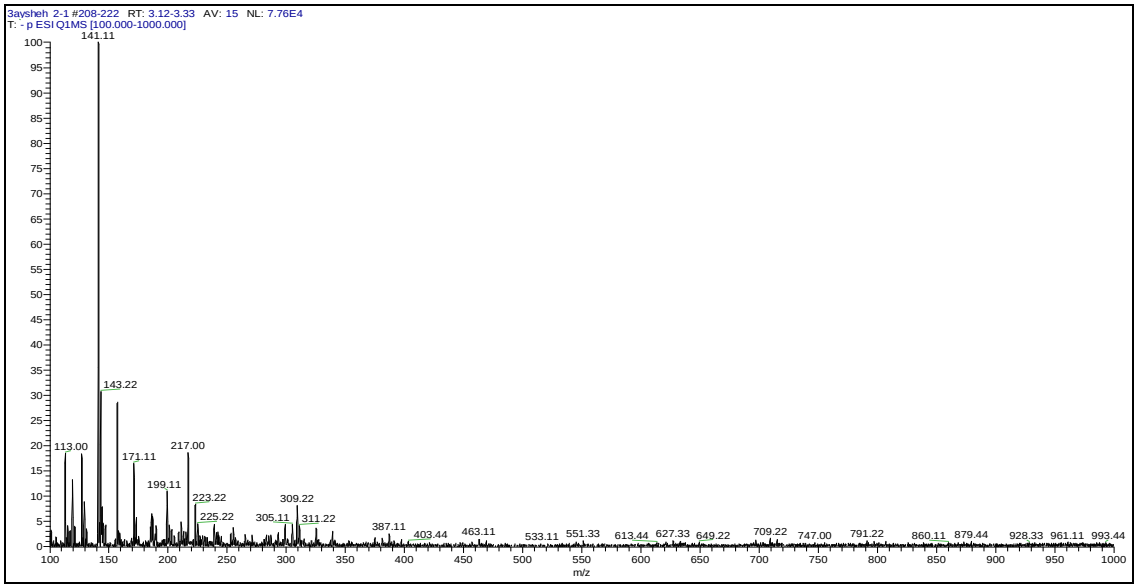


Figure 4.13:–ve ESi mode LCMS for compound 2

Figure 4.14 (compound 2) showed a peak at m/z of 591 Da probably indicating to Scillarenin-glucoside existence.

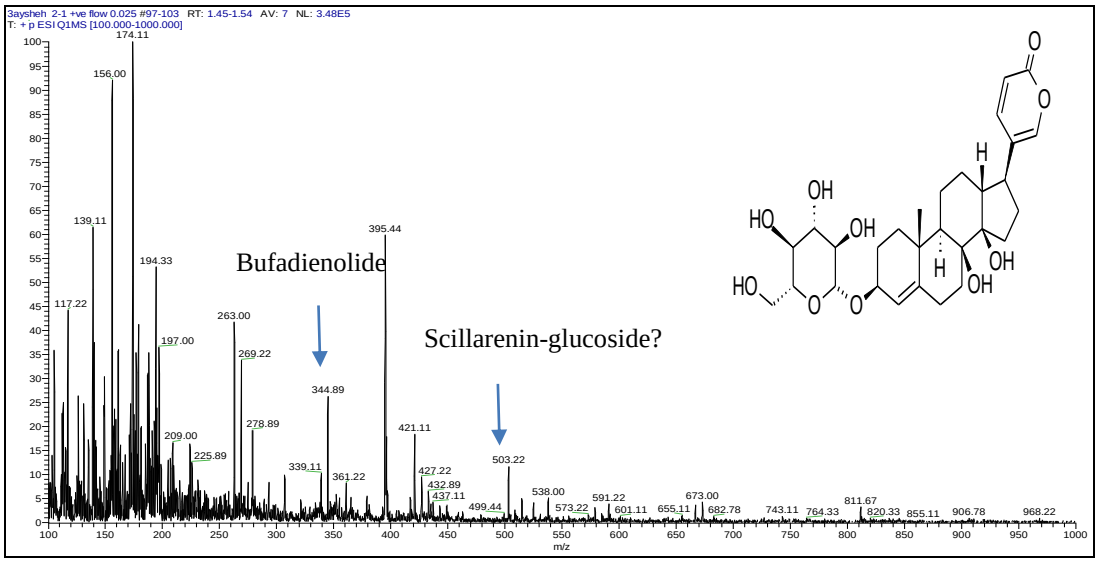


Figure 4.14: +ve ESi mode LCMS for compound 2

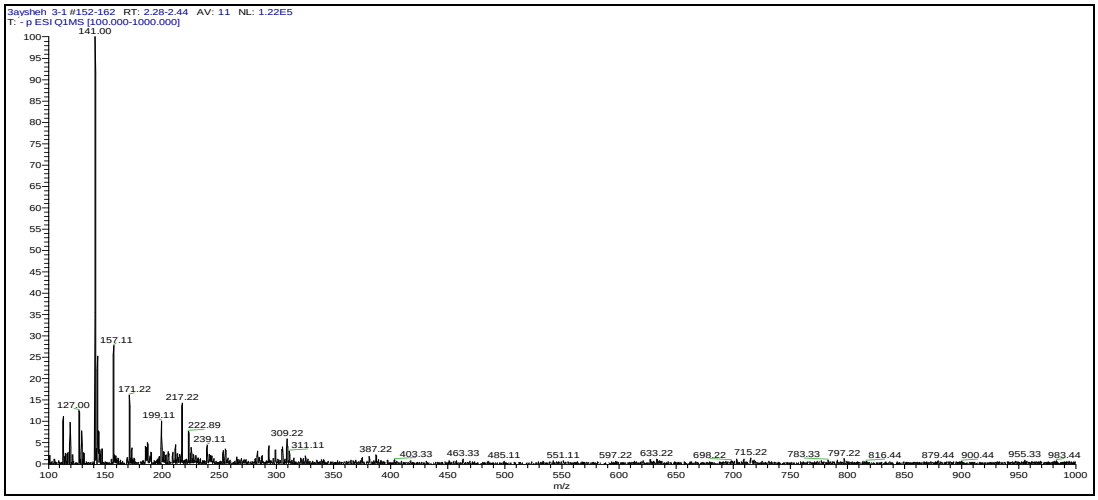


Figure 4.15: –ve ESi mode LCMS for compound 3

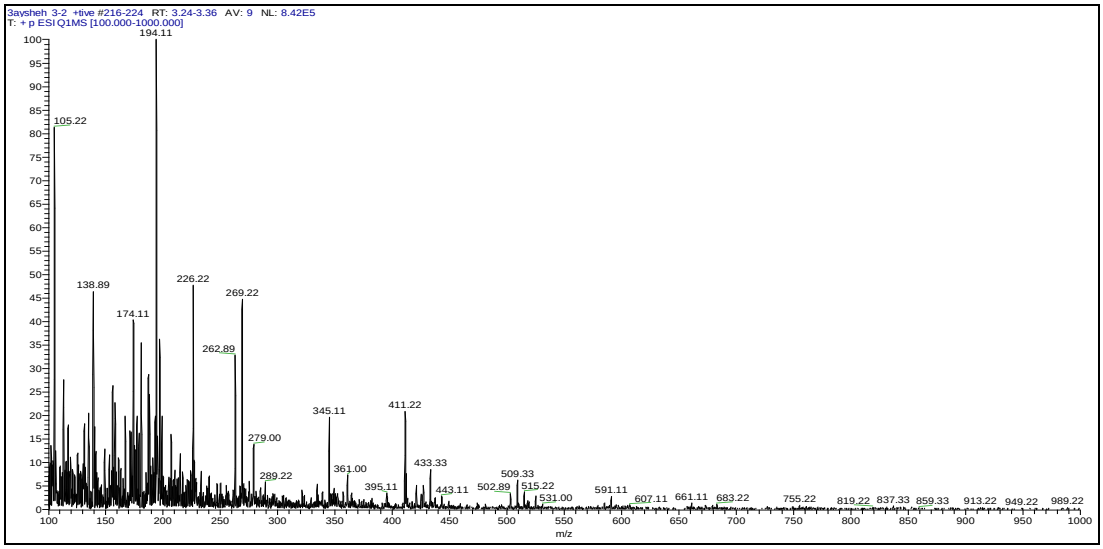


Figure 4.16: +ve ESi mode LCMS for compound 3

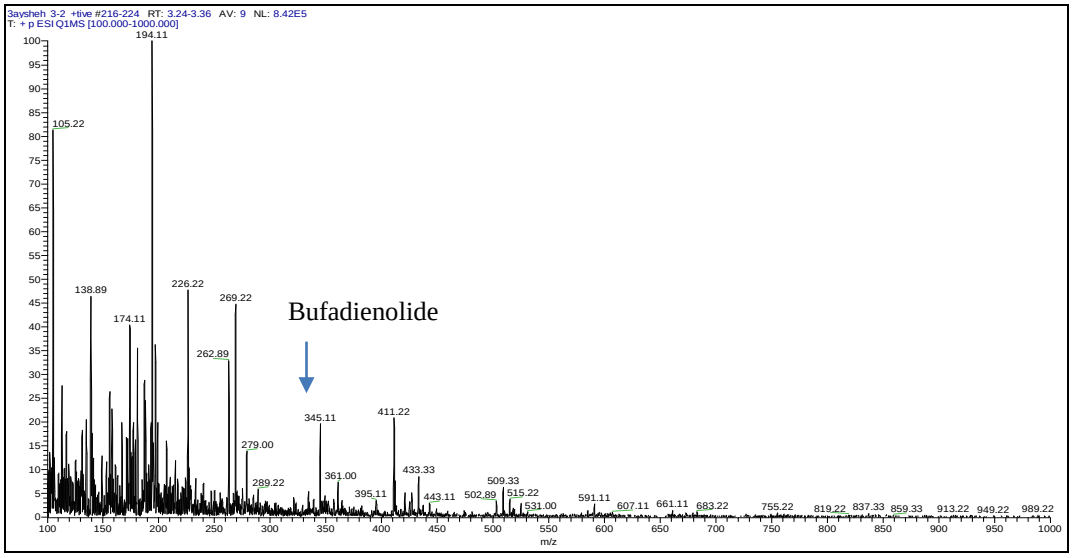


Figure 4.17: +ve ESi mode LCMS for compound 4

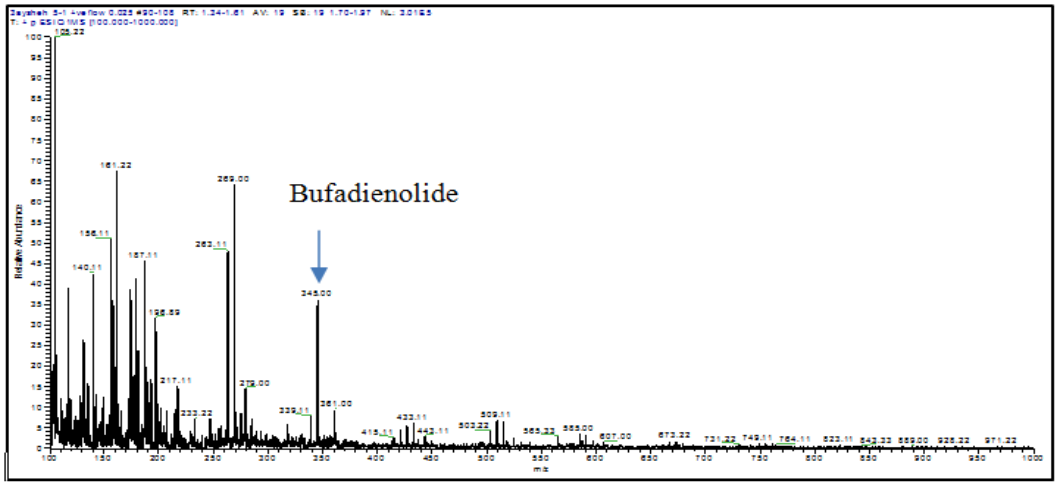


Figure 4.18: +ve ESi mode LCMS for compound 5

Figure 4.19 (compound 5) showed a peak at m/z of 563 Da indicating to Scillirubroside existence.

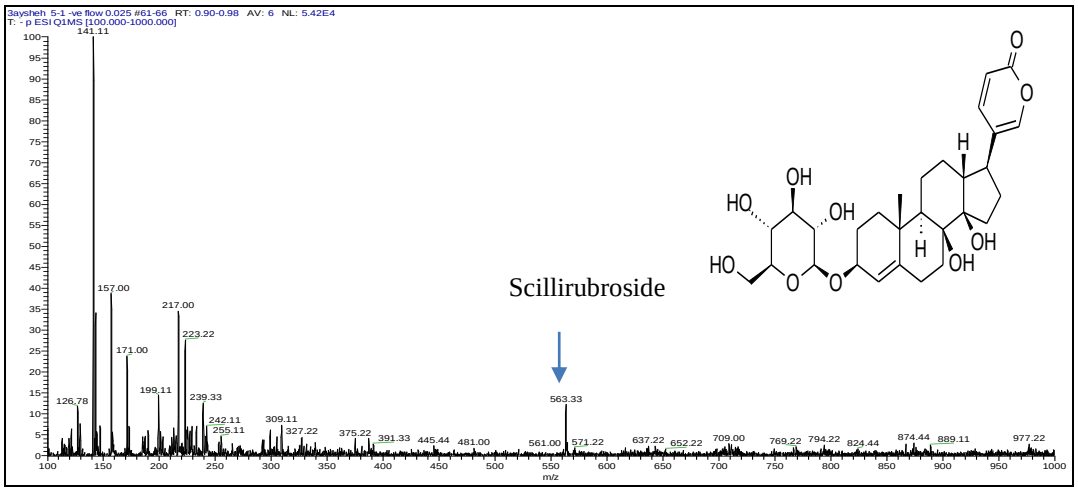


Figure 4.19: -ve ESi mode LCMS for compound 5

Figure 4.20 (+ve ESi compound 6) showed a peak at m/z of 579 Da indicating to deacetylscilliroside existence. While in Figure 4.21 (-ve ESi compound 6) showed a peak at m/z of 577 Da, difference in 2 units typical to deacetylscilliroside compound.

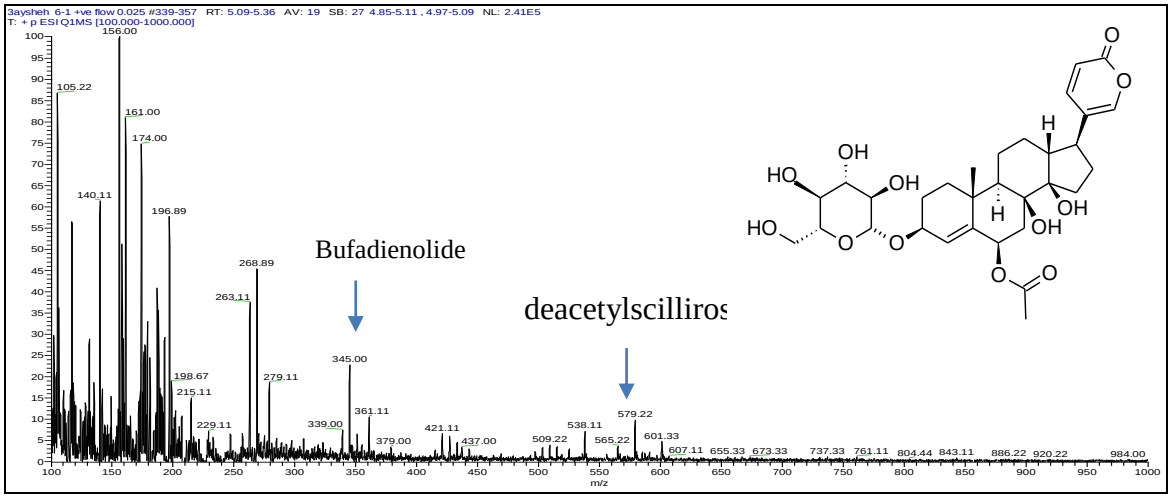


Figure 4.20: +ve ESi mode LCMS for compound 6

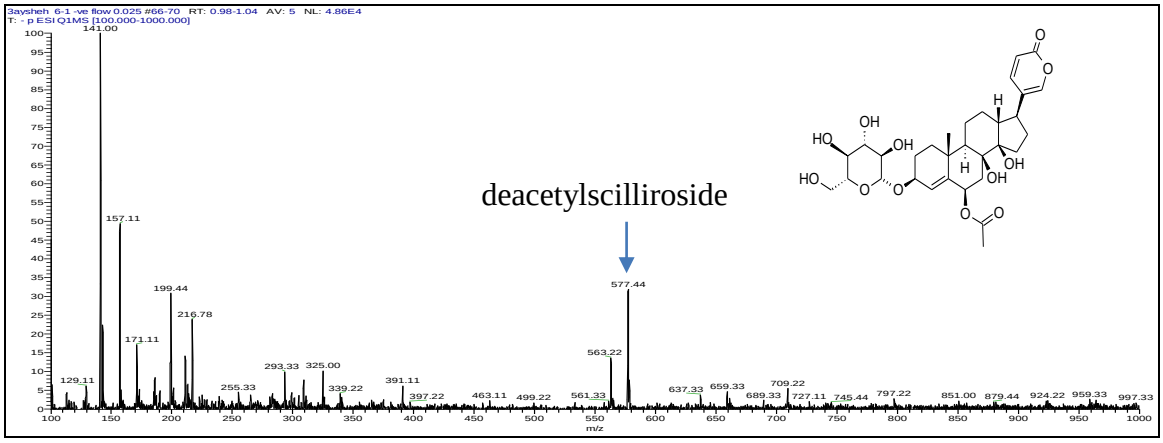


Figure 4.21: -ve ESi mode LCMS for compound 6

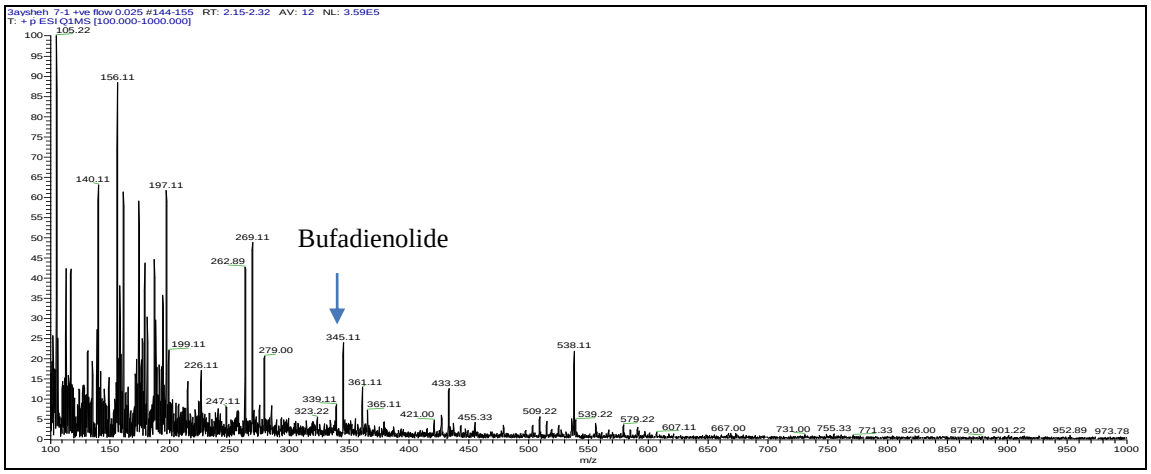


Figure 4.22: +ve ESi mode LCMS for compound 7

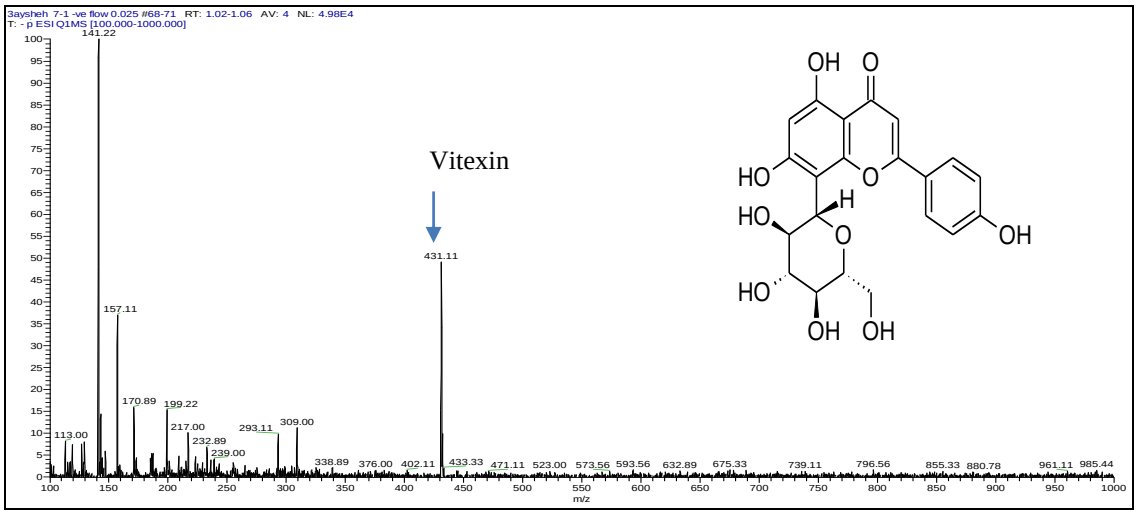


Figure 4.23: -ve ESi mode LCMS for compound 7

Figure 4.23 (-ve ESi compound 7) showed a peak at m/z of 431 Da indicating to Vitexin. While in Figure 4.22 (+ve ESi compound 7) showed a peak at m/z of 433 Da, difference in 2 units indicating to Vitexin compound presence.

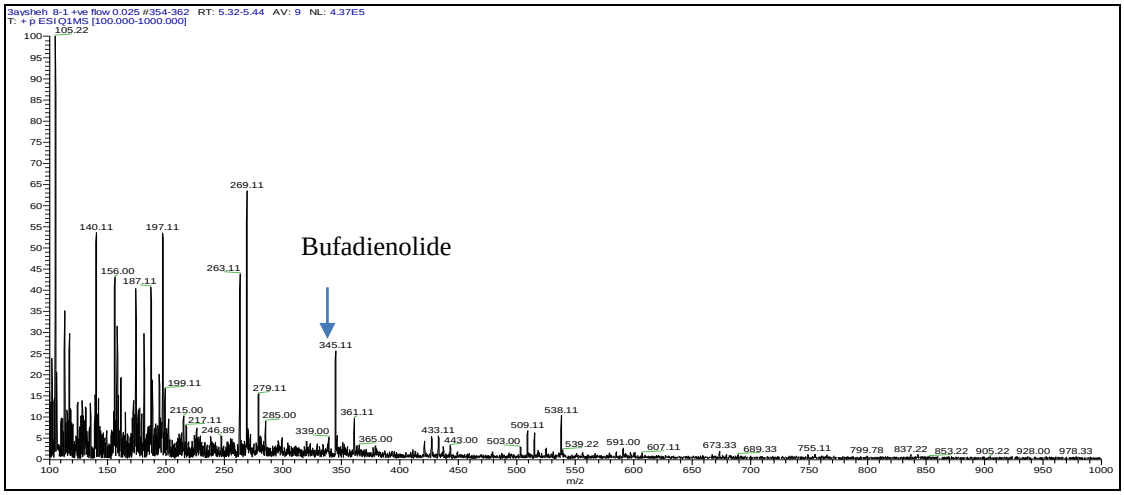


Figure 4.24: +ve ESi mode LCMS for compound 8

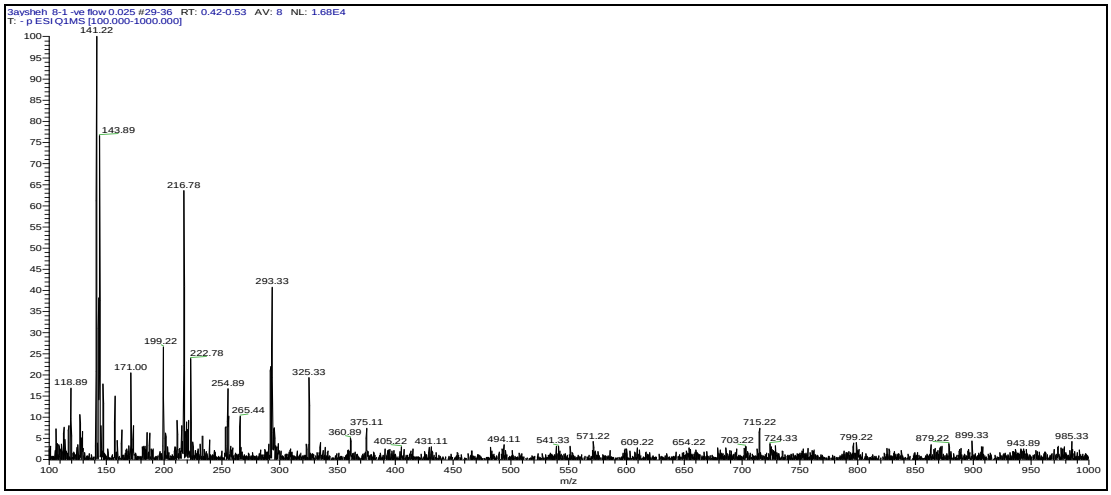


Figure 4.25: -ve ESi mode LCMS for compound 8

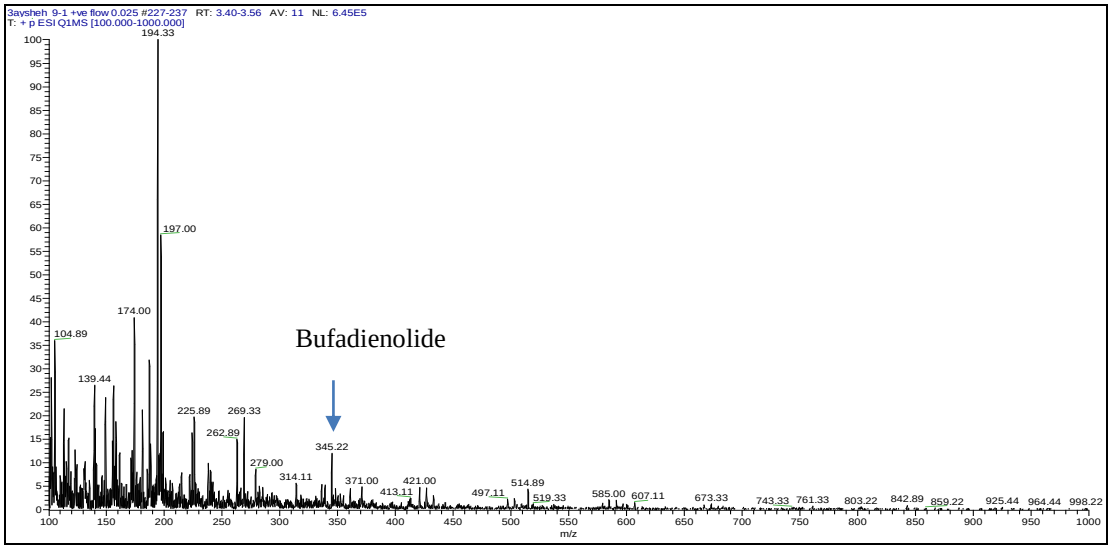


Figure 4.26: +ve ESi mode LCMS for compound 9

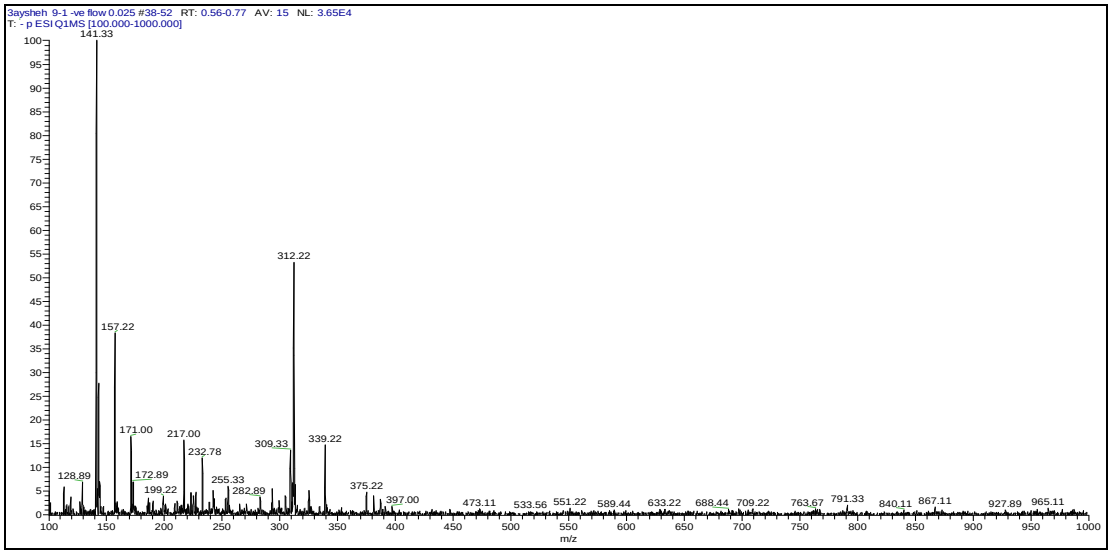


Figure 4.27: -ve ESi mode LCMS for compound 9

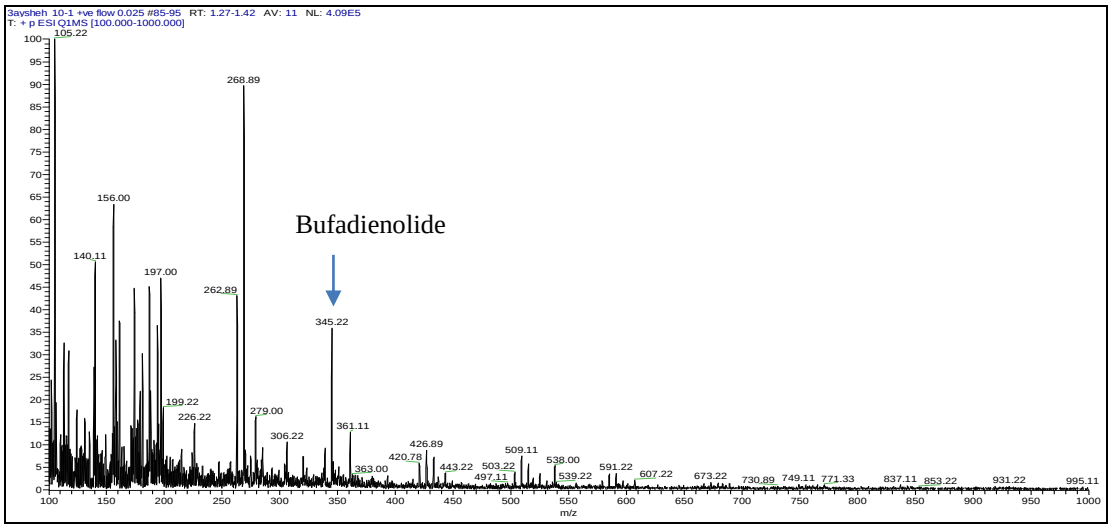


Figure 4.28: +ve ESi mode LCMS for compound 10

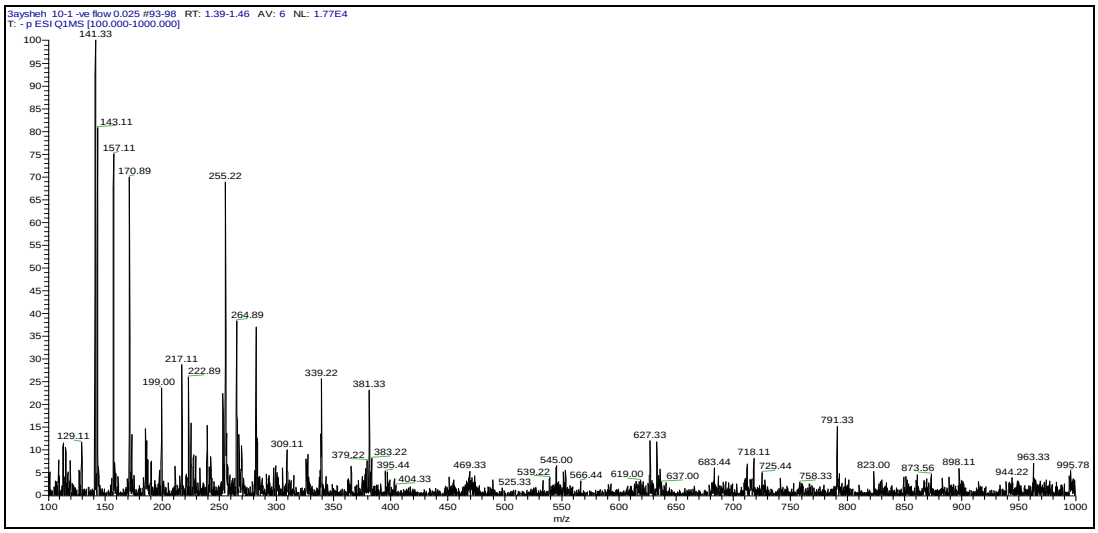


Figure 4.29: -ve ESi mode LCMS for compound 10

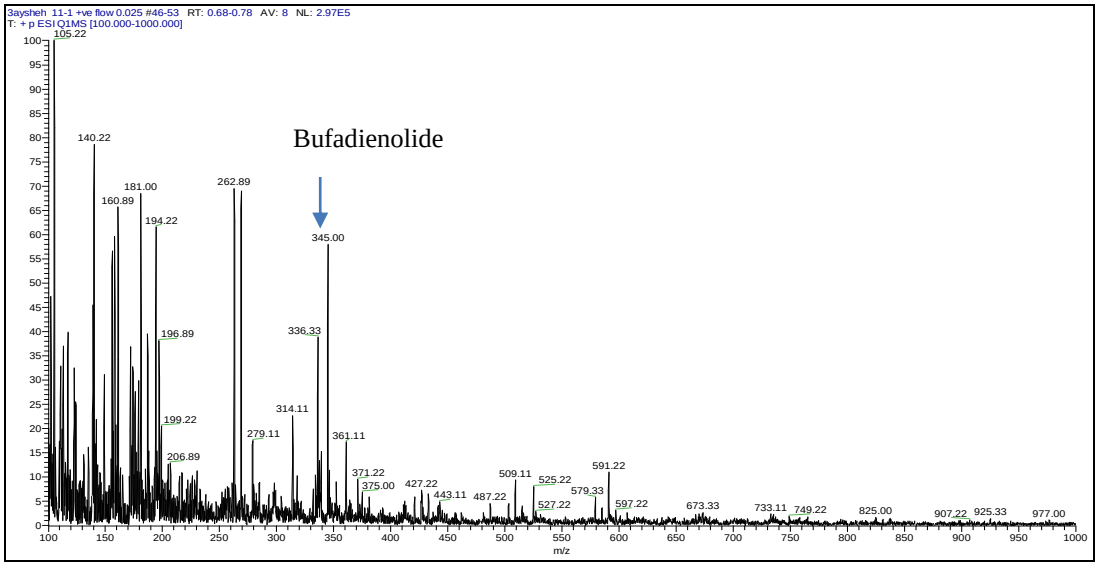


Figure 4.30: +ve ESi mode LCMS for compound 11

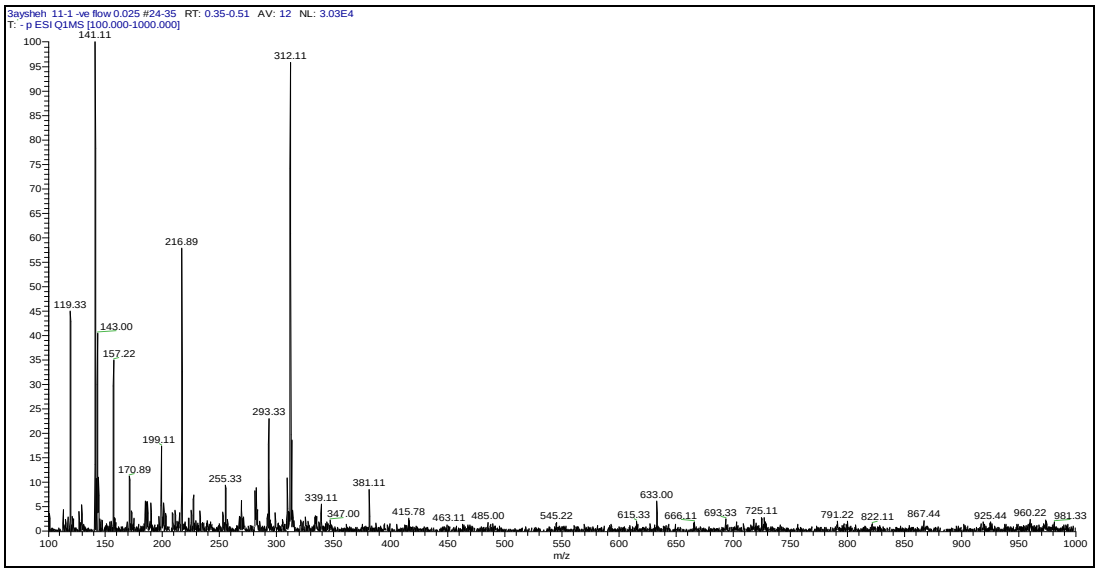


Figure 4.31: -ve ESi mode LCMS for compound 1

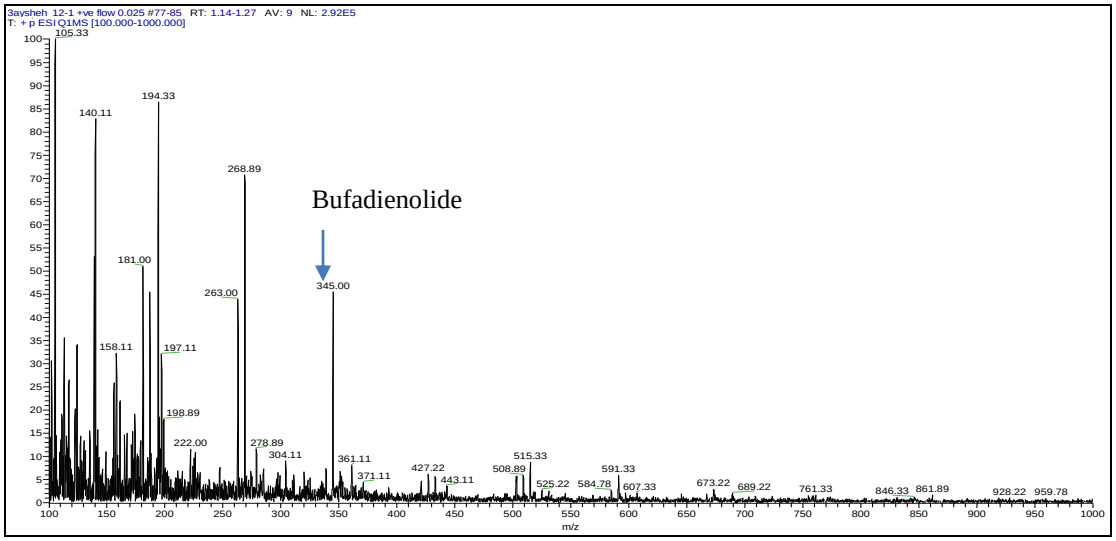


Figure 4.32: +ve ESi mode LCMS for compound 12

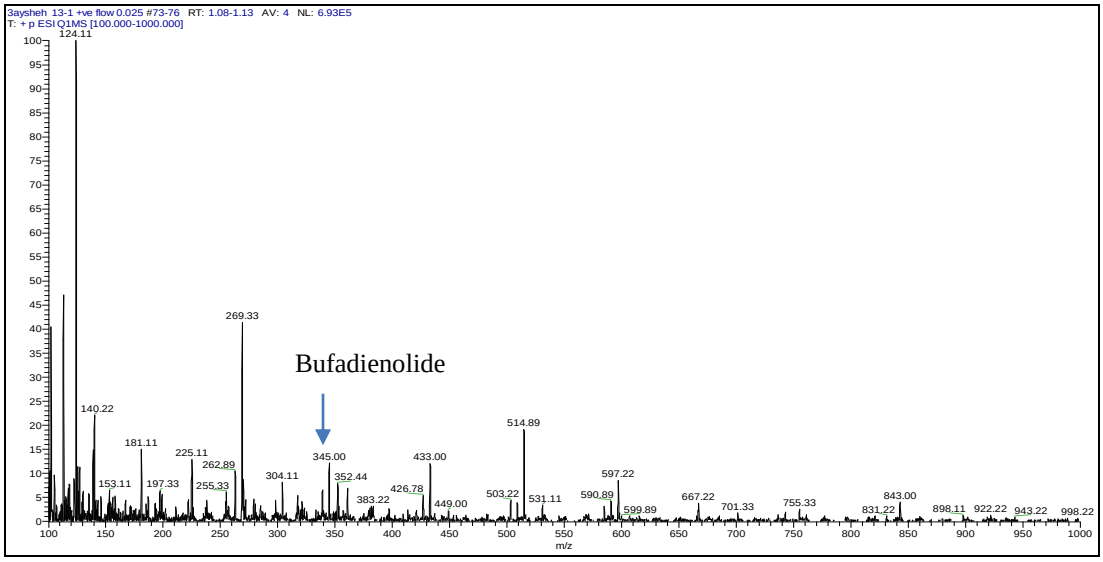


Figure 4.33: +ve ESi mode LCMS for compound 13

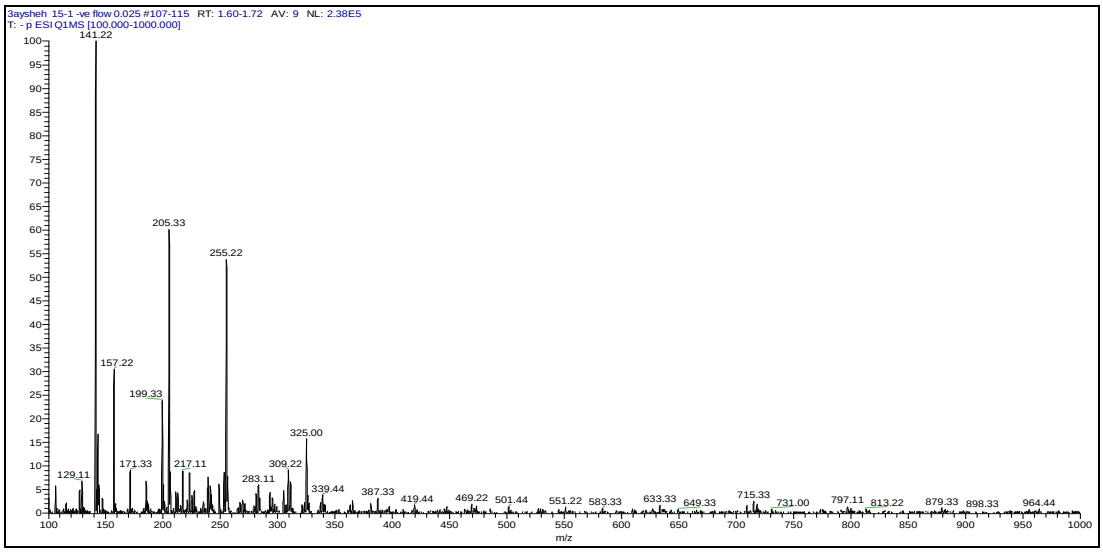


Figure 4.34: Compound14 and 15 using the +ve ESi modes were much diluted.

Table 4.9 shows the major fragments

Table 4.9: Major m/z fragments in Daltons (Da)

# phytochemical	+ve Esi mode	-ve Esi mode
1	131, 161 (base), 194, 207, 263, 293, 321, 345, 357, 421, 584, 655, 731	141 (base), 157, 171, 199, 217, 241, 293,309, 325
2	117, 139, 156, 174, 194, 197, 209, 225, 263, 269, 278, 339, 344, 395, 421, 427, 503, 538, 591, 673, 811	141 (base), 143, 171, 199, 217, 223, 225, 305, 309, 311, 387
3	105, 138, 174, 194 (base), 226, 262, 269, 279, 345, 411, 433, 509, 515, 591	141 (base), 157, 171, 199, 217, 222, 239, 309
4	139, 156, 161, 194, 197, 226, 262, 269, 279, 344, 361, 427, 509, 591	
5	140, 156, 161, 187, 196, 217, 233, 263, 269, 279, 345, 361, 433, 509, 585	126, 141 (base), 157, 171, 199, 217, 223, 239, 309, 375, 445, 563
6	105, 140, 156, 161, 174, 196, 198, 215, 263, 268, 279, 339, 345, 361, 421, 509, 538, 579, 601	129, 141 (base), 157, 171, 199, 216, 255, 293, 325, 391, 445, 563, 577
7	140, 156, 197, 199, 226, 262, 269, 279, 345, 361, 433, 538	113, 141 (base), 157, 170, 199, 217, 293, 309, 431
8	140, 156, 187, 197, 199, 226, 262, 269, 279, 345, 361, 433, 538	119, 141 (base), 144, 171, 199, 217, 223, 255, 265, 293,325, 360
9	104, 139, 174, 194 (base), 197, 225, 262, 269, 279, 314, 345, 371, 421, 514	129, 141(base), 157, 171, 173, 199, 217, 233, 255, 283, 309, 312, 339, 375, 397
10	140, 156, 197, 226, 262, 268 base), 279, 306, 345, 361, 426, 509, 538, 591	129, 141(base), 143, 157, 171, 199, 217, 223, 255, 265, 309, 339, 379, 381, 383, 395, 404, 469, 525, 539, 545, 566, 619, 627, 637, 683, 718, 725, 758, 791, 823, 874, 898, 944, 963, 996
11	140, 160, 181, 194, 262, 268, 279, 314, 336, 345, 361, 427, 509, 525, 538, 591	119, 141(base), 143, 157, 171, 199, 217, 255, 293, 312, 339, 347, 381,
12	140, 158, 181, 197, 263, 268, 278, 345, 361, 427, 508, 515, 591	
13	124 (base), 140, 153, 181, 197, 225, 262, 269, 304, 345, 352, 433, 514, 597, 667	
14	Very diluted no MS spectrum	
15	131, 161 (base), 179, 194, 207, 263, 345, 421, 584, 731	

Although LC-MS experiments were utilized to determine the preliminary phytochemical isolate structures, however, the full structures of the isolates were yet to be elucidated. Extra spectroscopic techniques such as ^{1}H-NMR and 2D-NMR are needed to determine their exact structures. Extra amounts of pure isolates (10-100mg) are needed to achieve this goal by using preparative HPLC experiments.

4.9Anticancer Activity

Cell culture: HCT-116 colon cancer cell line was grown in DMEM, while the MDA mB231gfp breast cancer cells was grown in RPMI medium, both supplemented with 10% FBS (Gibco),1% glutamine, and penicillin/streptomycin (Biological industries, Israel). Cells were cultured in 6 well plates, incubated in CO2 incubator, and next day treated. Later after 24 hrs treatment cells were observed under inverted fluorescent microscope (Olympus CKX 41) and imaged, and left for 48 and 72 hrs to take the other 2 days after treatment result observations. We used different concentrations to test the minimum concentration that can kill cells.

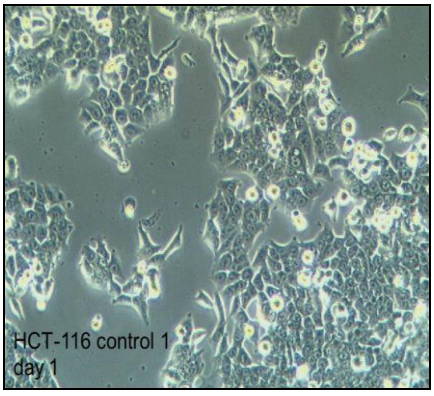
Our extract is expected to induce cancer cell death and has an anticancer effect. To test this effect, we treated HCT116 and MDA (colon and breast cells respectively) with four different concentrations (100, 50, 10 and 1 microgram from the stock solution 100 milligrams per 1 ml DMSO). As we observed, the higher concentrations lowered cell density at 24 and 72 hrs for both cell lines. Moreover, we noticed an effect in the low concentration 10 μg/ml obviously at 72 hrs. We also noticed detached and floated dead cells.

Figures (A, D, G, and J) were considered as control cells. We compared between HCT-116 cell control and cell after treatment when (100, 50, 10, and 1) μg/ml of extract was added. Results showed that the concentration of 100 μg/ml was affective against HCT lines with efficiency of 50% after 24 hours and 90% after 72 hours as shown in figures (B, C). On the other hand, the efficiency of the extract at a concentration of 50 μg/ml was almost similar to the 100 μg/ml as showed in figures (E, F). The efficiency of the extract at 10 μg/ml was observed with slight effect after 72-hour as seen in figures (H, I). Moreover, we didn't observe an effect for the extract at 1 μg/ml concentration as shown in figures (K, L).

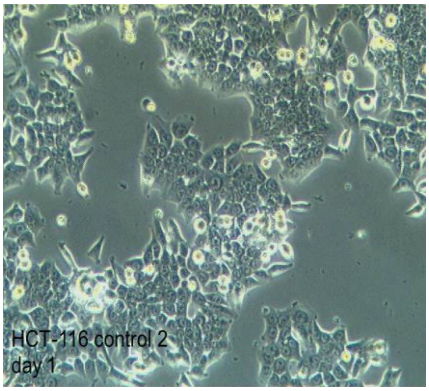
For MDA cells the efficiency of the extract at different concentrations (100, and 50) µg/ml was found to the (90-100) % against MDA cells where detached, died, and floated cells were noticed as shown in figures (3, 4, 5, and 6). However at concentration of (10, and 1) µg/ml no activity of the extract against MDA cell lines observed.

Overall, figures 4.35 and 4.36 below present's preliminary results which indicate that our plant extraction has an effect on cancer cells, as shown in the samples (A-L) and (1-10).

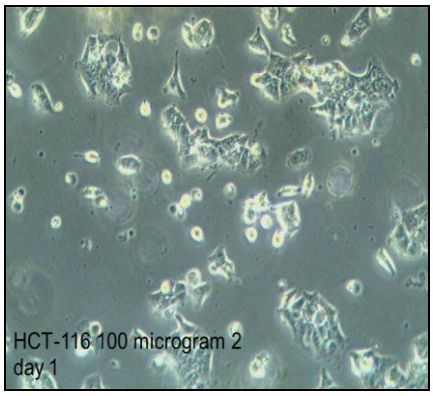
A.



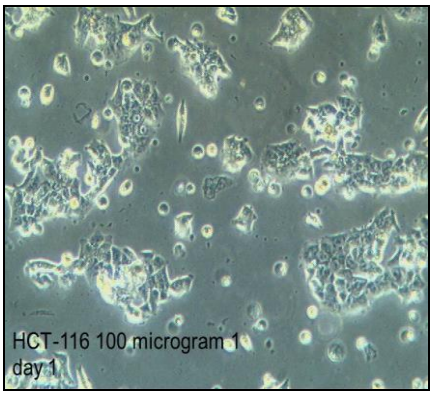
B.



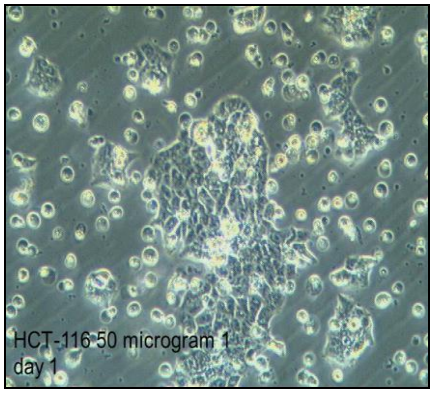
C.



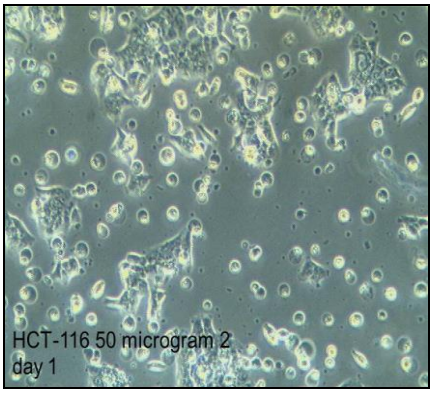
D.



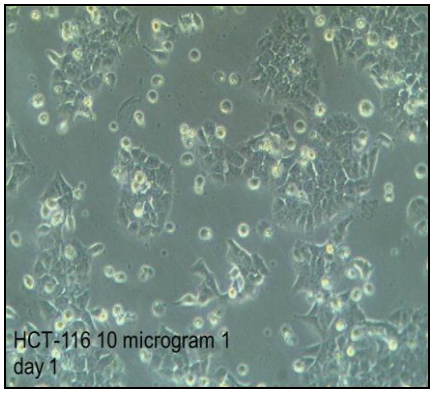
E.



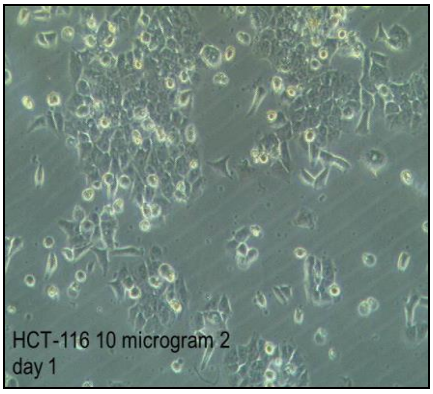
F.



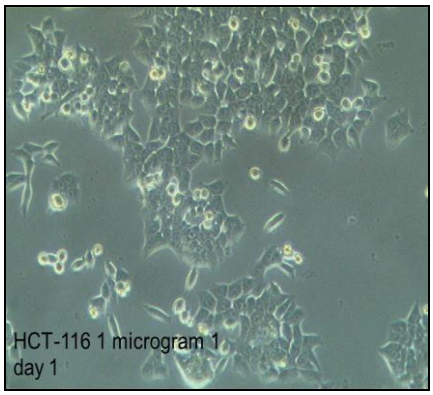
G.



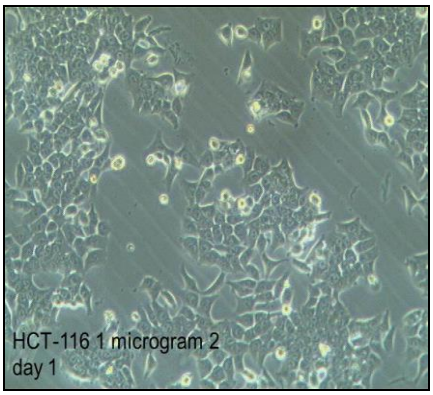
H.



I.

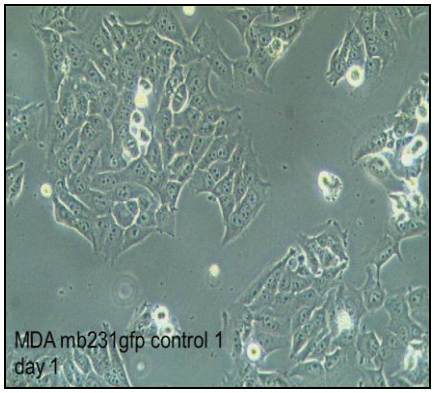


J.

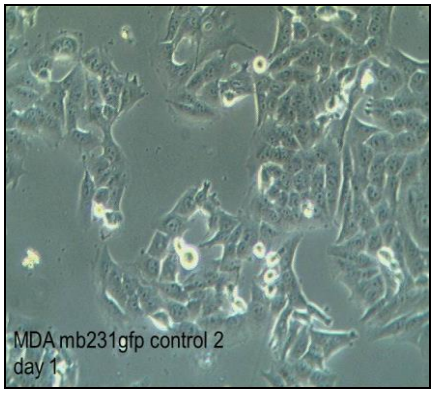


Figures 4.35: efficiency of the extract for HCT-116 cancer cells at different concentrations observed after 24 and 72hour, including figure samples (A-L).

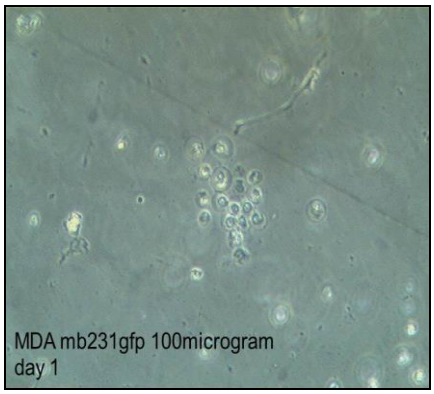
1.


MDA mb231gfp control 1
day 1

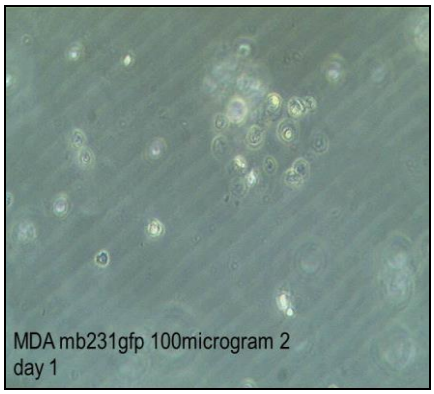
2.


MDA mb231gfp control 2
day 1

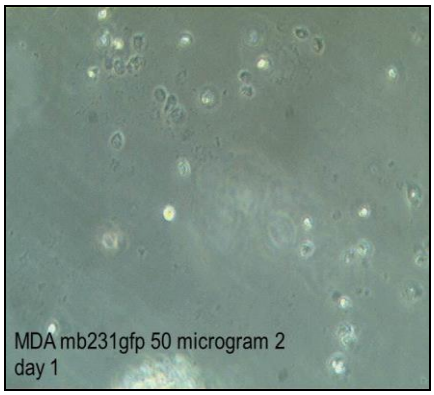
3.


MDA mb231gfp 100microgram
day 1

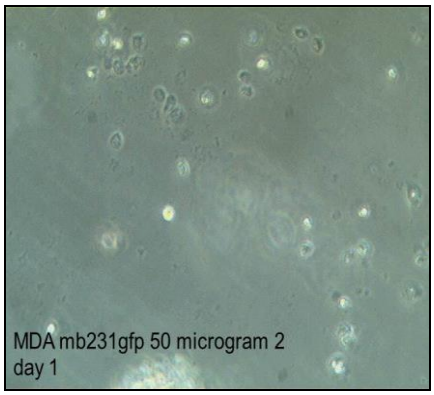
4.


MDA mb231gfp 100microgram 2
day 1

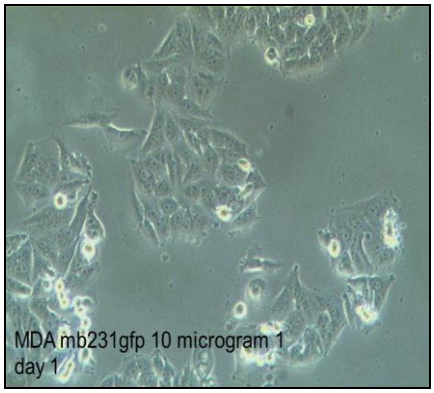
5.


MDA mb231gfp 50 microgram 2
day 1

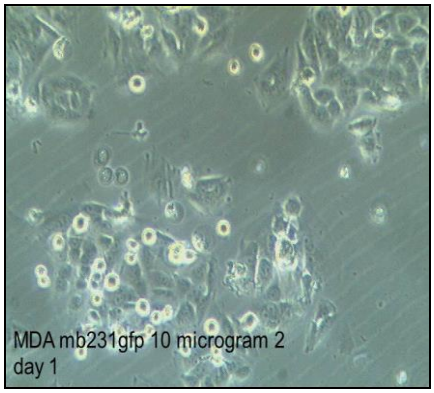
6.


MDA mb231gfp 50 microgram 2
day 1

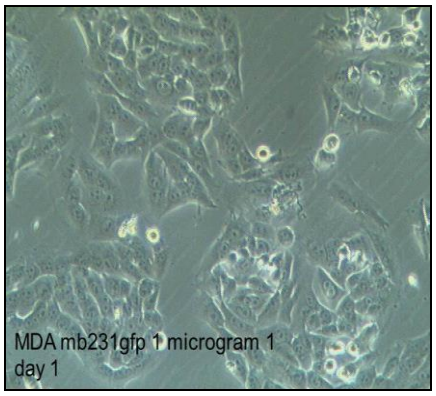
7.



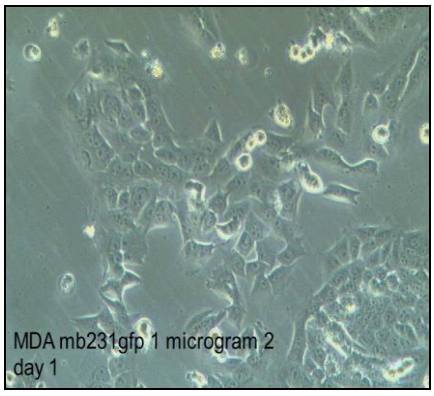
8.



9.

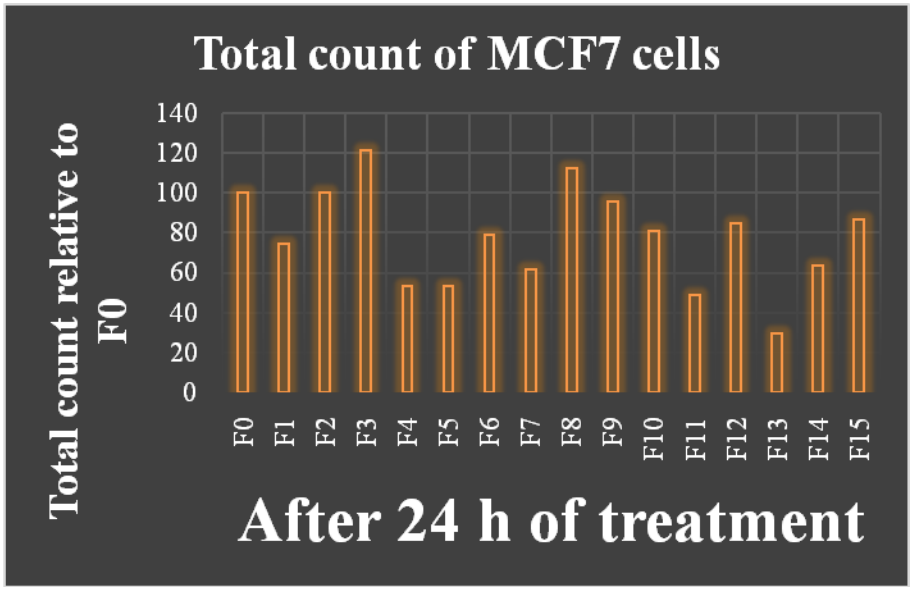


10.



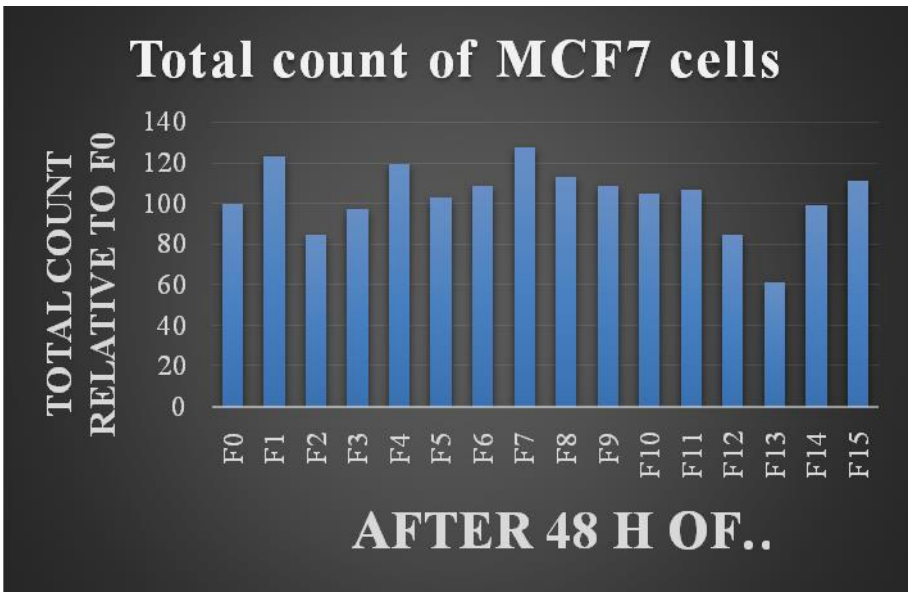
Figures 4.36: efficiency of the extract for MDA cancer cells at different concentrations observed after 24 and 72hour, including figure samples (1-10).

Semi-preparative HPLC-PDA and ESi modes were used to fractionate the sample, to which 15 isolates were tested for anti-cancer activities by using MCF7 cells that were treated with acetonitrile as control (F0) and also with 15 different types of isolates for 24 h, 48h. It was observed that isolate F13 is the major active one that prevented the growth. Moreover, F12 prevented the growth around 15% in both days as shown in figures 4.37 and 4.38 whereas: F0 is the Acetonitrile control and the F1 to F15 are the fractionations



F0	100
F1	74.40944882
F2	100
F3	121.2598425
F4	53.1496063
F5	53.1496063
F6	78.74015748
F7	61.81102362
F8	112.5984252
F9	95.66929134
F10	80.70866142
F11	48.81889764
F12	85.03937008
F13	29.76377953
F14	63.77952756
F15	87.00787402

Figure 4.37: Total count of MCF7 cells were treated with acetonitrile as control (F0) and also with 15 different types of fractionations after 24 h of treatment.



F0	100
F1	123.1560892
F2	84.39108062
F3	97.25557461
F4	119.5540309
F5	102.915952
F6	108.4048027
F7	127.787307
F8	113.0360206
F9	108.4048027
F10	104.6312178
F11	106.5180103
F12	84.39108062
F13	61.23499142
F14	99.14236707
F15	111.1492281

Figure 4.38: Total count of MCF7 cells were treated with acetonitrile as control (F0) and also with 15 different types of fractionations after 48 h of treatment

Chapter Five

Conclusions and Future Work

Conclusion

The *U. maritima* flowers indigenous to Palestine was found very rich in Phenolic, Flavanoids compounds and have a natural source of antioxidants activity that could prevent diseases, such as cancer. Ethanol extract was found to be the strongest anticancer activity against both MDA and HCT116 cancer cell lines. Moreover, ethanol extract showed a superior antioxidant activity, while Hexane and Dichloromethane have the lowest anticancer and antioxidant activities. It was found that is a positive correlation between polar solvents likes (Distilled water, Ethanol, and Ethyl acetate) and antioxidant activity tests (FRAP, DPPH, ABTS, CUPRAC), while there is negative correlation between non-polar solvents likes (Dichloromethane, and Hexane) and antioxidant activity tests.

Semi-Preparative HPLC-PDA chromatographic fractionation was used to investigate in-vitro anticancer activities. Anticancer activity against MDA (breast cancer cell) was high efficiency more than that HCT-116 (colon cancer cell).

Fraction 13 phytochemical was observed most effective fractionation, the others had an effect on day one only, this might be due to the low isolate concentration and they need to change the drug every 24 hours.

Some of fractions contain pure compound and their corresponding structure were revealed by liquid chromatography-mass spectroscopy.

Finally, they compared between *U. maritime* (for its fruit and flowers) ethanol extract on breast cancer cells. Ethanol extract showed the best efficacious as anticancer activity.

Future work

This work was mainly focused on preliminary structural elucidation out of the *U. maritima* plant. We suggest focusing on other aspects to fully explore the structure of compounds present in *U. maritima.* This can be accomplished by using Prep-HPLC on inch column. More concentrated isolates of pure compounds (~100 mg each) could be then collected. The isolated pure compounds can be subjected to LCMSMS using MRM and product ion monitoring (scan) along with H-NMR and 2D-NMR spectroscopy particularly using COSY and HMQC modes.

Moreover, all the separated phytochemicals should be tested in parallel for their anticancer activities in numerous cell line cells.

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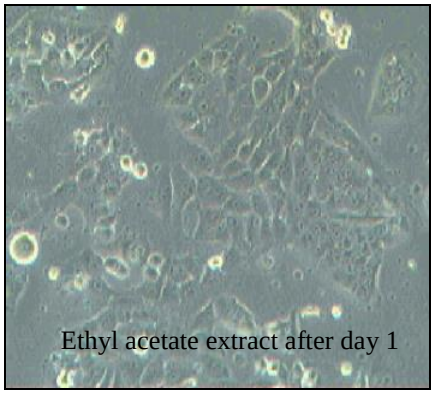
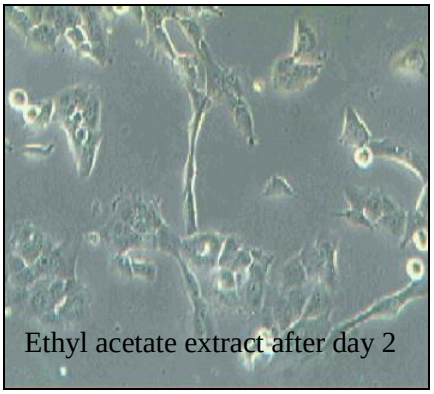
APPENDICES

1.


Ethyl acetate extract after day 1

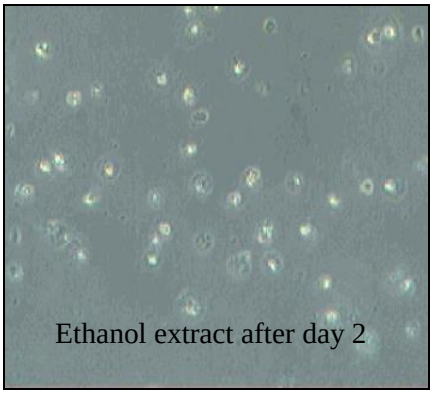
2.


Ethyl acetate extract after day 2

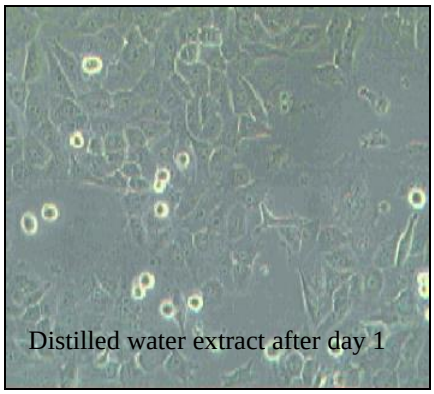
3.


Ethanol extract after day 1

4.


Ethanol extract after day 2

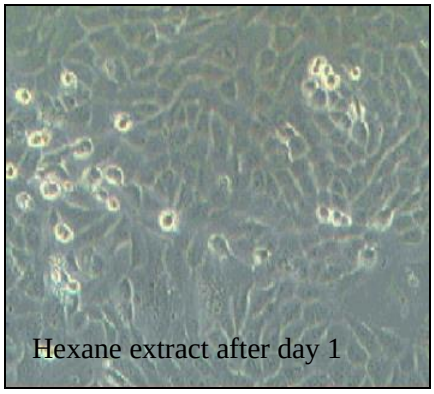
5.


Distilled water extract after day 1

6.


Distilled water extract after day 2

7.



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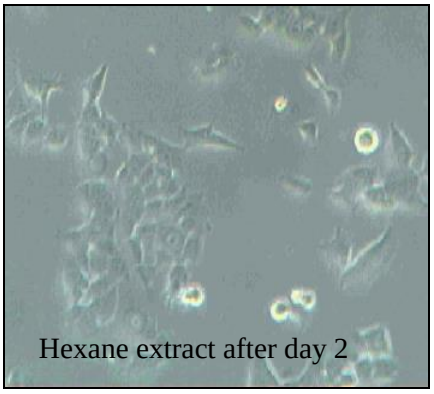

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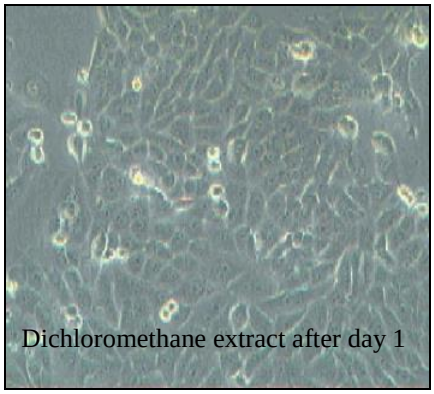

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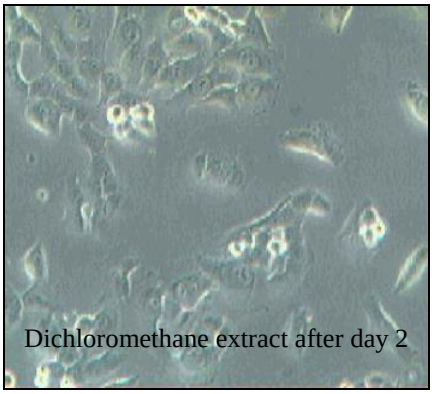

Figures 4.39: efficiency of the extract for MDA cancer cells at different solvents polarities observed after day1 and day2, including figure samples (1-10).

الاستخلاص والتنقيه ومعرفة المركبات الاولية المضادة للسرطان من نبتة *Urginea maritima* البرية الفلسطينية وأنشطتها المضادة للأكسدة

إعداد: عائشة علي محمود ريان

إشراف: د .فؤاد الريماوي و أ.د. صالح ابو لافي

الملخص:

منذ سنوات عديدة ، تشتهر ارض فلسطين المقدسة بنباتات عديدة ذات فعالية صيدلانية ومنها نبات *Urginea maritima* (*U.maritima*). تم جمع العينات من منطقة أبو ديس في يناير 2017 ، جففت في الظل لمدة أسبوعين حتى اصبحت جافة تماماً. تم طحنها وتحضير مستخلص منها بإستخدام sonication. الهدف من هذا البحث هو الكشف عن فعالية أزهار نبتة (*U.maritima*) على كل من خلايا سرطان القولون(HCT-116) والثدي(MDA) عن طريق استخدام مذيبات مختلفة القطبية (الماء المقطر ، الإيثانول ، إيثيل أسيتات ، داي كلورو ميثان ، وهكسان). تم دراسة النشاط المضاد للأكسدة باستخدام فحوصات مختلفة مثل FRAP و DPPH و ABTS و CUPRAC. تم أيضا الكشف عن إجمالي محتوى الفينول ومحتوى الفلافونويد الكلي من المستخلصات. أظهرت النتائج أن المستخلص الإيثانولي من *U.maritima* له نشاط مضاد للسرطان أعلى مقارنة بالمذيبات الأخرى. لذلك ، تم استخدام مستخلص الإيثانول لتحليل HPLC-PDA ، وتقسيم HPLC-PDA شبه التحضيري و LCMS في كل من صيغ ESi الإيجابية والسلبية لتوضيح بعض هياكل المركبات المنفصلة.

تم العثور على جميع فحوصات نشاط مضادات الأكسدة (FRAP، DPPH، ABTS، CUPRAC) ، بالإضافة إلى محتوى الفينول الكلي ومحتوى الفلافونويد الكلي أعلى عند استخدام المذيبات القطبية مثل الماء المقطر ، والإيثانول ، وأسيتات الإيثيل ، في حين تم العثور على نشاط أقل عند استخدام المذيبات الغير قطبية مثل ثنائي كلورو ميثان ، وهكسان.

وجد أن تثبيط الخلايا السرطانية HCT-116 و MDA عالية عندما كان التركيز 100 ملغ / مل ، وكان ذلك (50٪ بعد 24 ساعة و 90٪ بعد 27 ساعة للخلايا 116 HCT - 90٪ بعد 24 ساعة و 100٪ بعد 72 ساعة لخلايا MDA). تم العثور على نشاط مضاد لمستخلصات *U. maritima* من 50 ميكروغرام / مل إلى مماثلة لتركيز 100 ميكروغرام / مل ، بينما (1 ، و 10) ميكروغرام / مل له نشاط منخفض جدا. مما يعني أن خلايا سرطان الثدي أكثر فعالية كنشاط مضاد للسرطان من خلايا سرطان القولون