INVESTIGATION OF CHEMOPREVENTIVE PROPERTIES OF *URTICA DIOICA L.*, *IN* MCF-7 AND MDA231 BREAST CANCER CELL LINES.

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FOR
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INVESTIGATON OF CHEMOPREVENTIVE PROPERTIES OF *URTICA DIOICA L., IN* MCF-7 AND MDA231 BREAST CANCER CELL LINES.

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ABSTRACT

INVESTIGATON OF CHEMOPREVENTIVE PROPERTIES OF *URTICA DIOICA L., IN* MCF-7 AND MDA231 BREAST CANCER CELL LINES.

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September 2011, 108 pages

Cancer is a major health problem in developing world with mostly unsufficient treatment. Cancer prevention through dietary modification appears to be a practical and cost effective possibility. The aim of present study is to investigate the chemical components of *"Urtica diocia,L (U. diocia)* grown in Turkey" and the possible protective potential of its aqueous extract against breast cancer cell lines.

U. diocia was extracted by maceration method which was performed for 6,12, 24, and 36 hours, at 50°C, 37°C, and 25°C for optimization of maceration condition. Total phenol and flavon contents, and radical scavenging activity (RSA) were determined. RSA was determined by DPPH (1,1diphenyl-2-picryl-hydrazil). RSA varied from %15 to %65 in different extracts prepared under different conditions. The highest RSA was found in 12 hour at 25°C extract with %65 RSA value and the (IC50) Inhibitory concentration of 0.30 mg/mL The extract obtained by maceration for 12h at 25°C had the richest flavon content 28.9 ±0.93 mg quercetin equivalent/g lyophilized extract. Phenol content of extracts in terms of gallic acid equivalent (GaE) varied from 28.8± 0.57 to 74.2 ± 2.24mg/g lyophilized extract. 12h at 25°C *U. dioica* extract had richest phenolic content (74.2± 2.24mg GaE /g lyophilized extract).

The air dried aerial parts of the plant were extracted with methanol for 4 h at 40^0 C in sonicator. After evaporation of the combined methanol extracts of *U.dioica* under vacuum, the extract was fractionated with, chloroform, ethyl acetate and *n*-butanol by partitioning into solvents of increasing polarity. HPLC analyses of phenolic, compounds in n-butanol and ethyl acetate fractions of *U.dioica* were carried out in Varian ProStar HPLC. HPLC analyses of 12 h at 25°C *U. dioica* extract (14.4mg/mL) revealed that the extract contained 7.10^{-3} mg/mL caffeic acid and 5.10^{-3} mg/mL quercetin. $3x\ 10^{-2}$mg/mL gallic acid was found in n-butanol fraction of *U.dioica.* Caffeic and chlorogenic acid were found at $1.9X\ 10^{-2}$ mg/mL in acid macerated extract of *urtica dioica L.*

Cytotoxic effect of *urtica dioica L* in MCF-7 and MDA-231 cellls were investigated by using XTT Cell Proliferation assays which showed that *urtica diocia L* treatment for 48 hours caused a concentration dependent decrease in viable cell numbers. *In vitro* effects of *U. diocia* extracts on crude sheep liver cytosolic glutathione- S-transferase was also studied and have shown effective inhibition on cytosolic GST activity, with an IC50 of 75 nmoles/min/g protein.

Total phenol and flavon content, and radical scavenging activity (RSA) were also determined to survey antioxidant potential of *U. diocia* acid extracts**.**

Key Words; Anticancer effect, *U. dioica*, Chemoprevention, Antioxidant

ÖZ

URTICA DIOICA L., MCF-7 AND MDA231 HÜCRE HATLARI ÜZERİNDEKİ KEMOPREVENTİF ÖZELLİKLERİNİN ARAŞTIRILMASI

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Eylül 2011, 108 sayfa

Gelişmekte olan dünyanın en büyük sağlık problemi tedavide yetersiz kalınan kanser hastalığıdır. Kanserin önlenebilmesi beslenmede yapılacak düzenlemelerle uygulanabilir ve ekonomik açıdan mümkün olabilecektir. Çalışmanın amacı Türkiye'de yetişen ısırgan otunun var olabilecek kanser oluşumunu engelleyici özelliklerinin ve etki mekanizmalarının araştırılmasıdır.

Bitki özütleri 6 ve 12, 24, 36. saatler ve 50°C, 37°C, 25°C sıcaklıkta maserasyon metoduyla hazırlanmıştır. Isırgan bitki özütünde, antioksidan aktivite tayin metodlarından toplam fenol, flavon ve serbest radikal süpürücü aktivitesi çalışılmıştır. Farklı koşullarda hazırlanan bitki özütlerinde yüzde radikal süpürücü etki (RSA) değerleri %15 ile %65 arasında değişmektedir. En yüksek RSA değeri 25°C'de, 12 saatte elde edilen bitki özütünde % 65 olarak bulundu. Bu özütün % 50 radikal süpürücü konsantrasyonu (IC50) 0.30 mg/mL olarak hesaplandı. En yüksek flavon içeriği 12 saat, 25 ^{0}C'de elde edilen örnekte 28.9±0.93 mg kuarsetin eşdeğeri/g kurutulmuş özüt olarak saptandı. Özütlerde fenol miktarının gallik asit eşdeğeri (GaE) olarak 28.8 ile 74.2 mg/g kurutulmuş özüt arasında değişmektedir. En yüksek fenol içeriği 12 saat, 25 ^{0}C'de hazırlanan özütte (74.2± 2.24 mg GaE/g kurutulmuş özüt) saptandı.

U. dioica bitkisinde bulunan flavon ve fenolikler grubu aktif maddelerden kuarsetin ve kafeik asit miktarları; 12 saat, 25 ^{0}C de suyla hazırlanan özüt de, 7.10^{-3} mg/mL kafeik asit ve 5.10^{-3} mg/mL kuarsetin saptandı. 3x 10^{-2}mg/mL Gallic acid n-butanol özütünde, 1.9X 10^{-2} mg/mL kafeik ve klorojenik asit, *U. dioica* bitkisinin asitle hazırlanan özütünde saptanmıştır.

Isırgan otunun toprak üstü kısmından metanol ekstresi 4 saatte ve 40ºC'de sonikatör içerisinde hazırlanmıştır. Isırgan otu iki defa methanolde ekstre edilerek ve polarite farkından yararlanarak kloroform, etil asetat, n-butanol içerisinde fraksiyonlarına ayrılmıştır. Etilasetat ve n-butanol fraksiyonlarında içerik analizi, Varian Prostar HPLC kullanılarak yapılmıştır.

U. dioica özütünün, 48 saat süreyle, artan konsantrasyonlarda MCF7 ve MDA-231 hücre kültürleri üzerindeki sitotoksik etkisi XTT yöntemi ile tespit edildi. Isırgan sulu özütün koyun karaciğer sitozolünde *in vitro* olarak GST enzim aktivitelerine bakıldı enzim aktivite inhibisyonu IC50 of 75 nmoles/min/g protein değeriyle ifade edildi.

Asidik özütlerin total fenol, total flavon miktarları ve radikal süpürücü etkileri (RSA) tayin edilerek sulu özüt ile karşılaştırıldı.

Anahtar kelime; Antikanser etki*, U. dioica L*, kemopreventif, antioksidan

To my precious mother

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LIST OF SYMBOLS AND ABBREVIATIONS

ROS: Reactive Oxygen Species

ER: Estrogen Receptor

dH2O: Distilled Water

RT :Room Temperature

DMSO: Dimethly Sulfoxide

IC50: Inhibitory Concentration 50

SEM: Standar Error of the Means

XTT: 2,3-bis-(2-methoxy-4-nitro-5sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

MCF-7: Michigan Cancer Foundation-7

BSA: Bovine serum albumin

CDNB: 1-Chloro-2,4-dinitrobenzene

CYP: Cytochrome P450 enzyme family

GSH: Reduced Glutathione

GSSG: Oxidized Glutathione

GST: Glutathione-S-transferase

RSA: Radical Scavenging Activity

Her-2: Human epidermal growth factor receptor 2

CDK: Cell Cycle Dependent Kinase

SOD: Super Oxide Dismutase

TRAP**:** Total Radical-Trapping Antioxidant Parameter

$ABTS^{\cdot+}$Scavenging of Radical Cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)

CHAPTER 1

INTRODUCTION

1.1.What is Cancer and Molecular Mechanism of it?

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells originated from normal body cells and it is the major causes of death. Cancer disease of our genes is caused by both internal factors (mutations that occur from metabolism, inherited mutations and immune conditions) and external factors (tobacco, chemicals and radiation). Environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals cause alterations in DNAstructure, which, if left unrepaired, may lead to mutations that enhance cancer risk. Overtime, DNA accumulates changes that activate proto-oncogenes and inactivate tumour-suppressor genes. The genetic instability driving tumorigenesis is fuelled by DNA damage and by errors made by the DNA machinery (Hoeijmakers, 2001).

Transformation of normal cells in to malignant cells are capability of cells to be insensitivity to growth-inhibitory signals. In cancer the transition form G1-phase to S phase is often deregulated due to the altered gene function. As a consequence of the cell cycle will be regulated in favour of continues growth. In G1, first phase of the cell cycle, mutagenic stimulation results in activation of cell cycle dependent kinases such as Cyclin D1/ CDK4 and Cyclin E/CDK which are responsible for DNA replication and inhibit proteins that retain cells in non-dividing state. If cell is not stimulated to divide in G1 enter the G0 state so remain quiscent. Although activated cells will enter the second phase which is

called S-phase, in which DNA is duplicated. Activity of cyclin A/CDK is required for the S-phase. In different stage of cell cycle in which different interaction between CDK/ Cyclin complexes are represented in **Figure 1.1.**

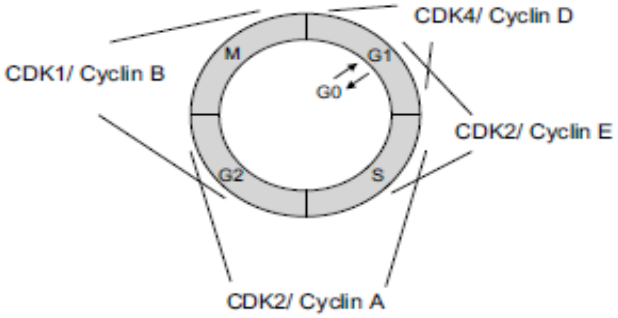


Figure 1.1 Schematic representation of the cell cycle and in which stage these different CDK/ Cyclin complexes are active. Lines show the overlapping activity ranges of the different CDK/ Cyclin complexes (Smiths and Medema,2001).

Third phase is G2 in that phase cells ensure the DNA is properly replicated and decide the final seperation of sister chromatids and cytokinesis in last M-phase or mitosis. Continues growth signalling can be a consequence of mutations in extracellular receptors or intracellular signal transducers (Smiths and Medema, 2001).

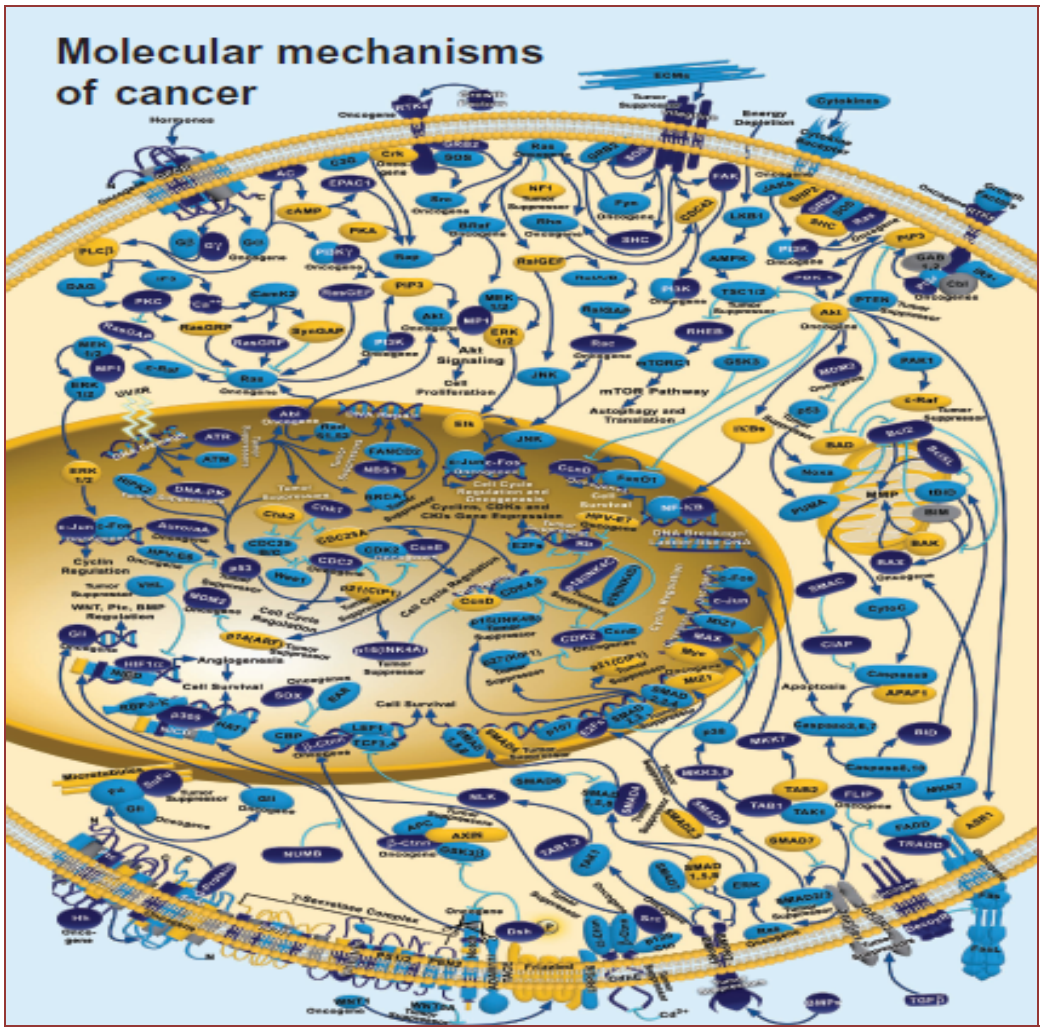


Figure 1.2 Molecular mechanism of cancer development by extracellular receptors or intracellular signal transducers (www.abcam.com/cancer).

Cancer is treated with surgery, radiation, chemotherapy, hormone therapy, biological therapy and targeted therapy (Cancer Facts& Figures, 2010).

Breast cancer is a leading cause of morbidity and mortality worldwide. Cancer that forms in tissues of the breast, usually ducts tubes that carry milk to the nipple and lobules (glands that make milk). It occurs in both men and women, although male breast cancer is rare. 40.230 breast cancer deaths (39.840

women, 390 men) are expected in 2010. Breast cancer ranks second as a cause of cancer death in women after lung cancer (Cancer Facts& Figures, 2010). The lifetime risk of developing breast cancer is 12.6 % for women. Breast cancer associated with some risk factors including; older age, higher body mass index, alcohol consumption, hormone replacement, prior radiation exposure, nulliparity, family history, gene carrier status of *BRCA1* and *BRCA2*, and prior history of breast neoplasia. There are some hormone-related risk factors for breast cancer; for example, early onset of menarche, late onset of menopause, delayed age of first pregnancy and elevated free oestradiol concentrations in post-menopausal women (Magee and Rowland, 2004).

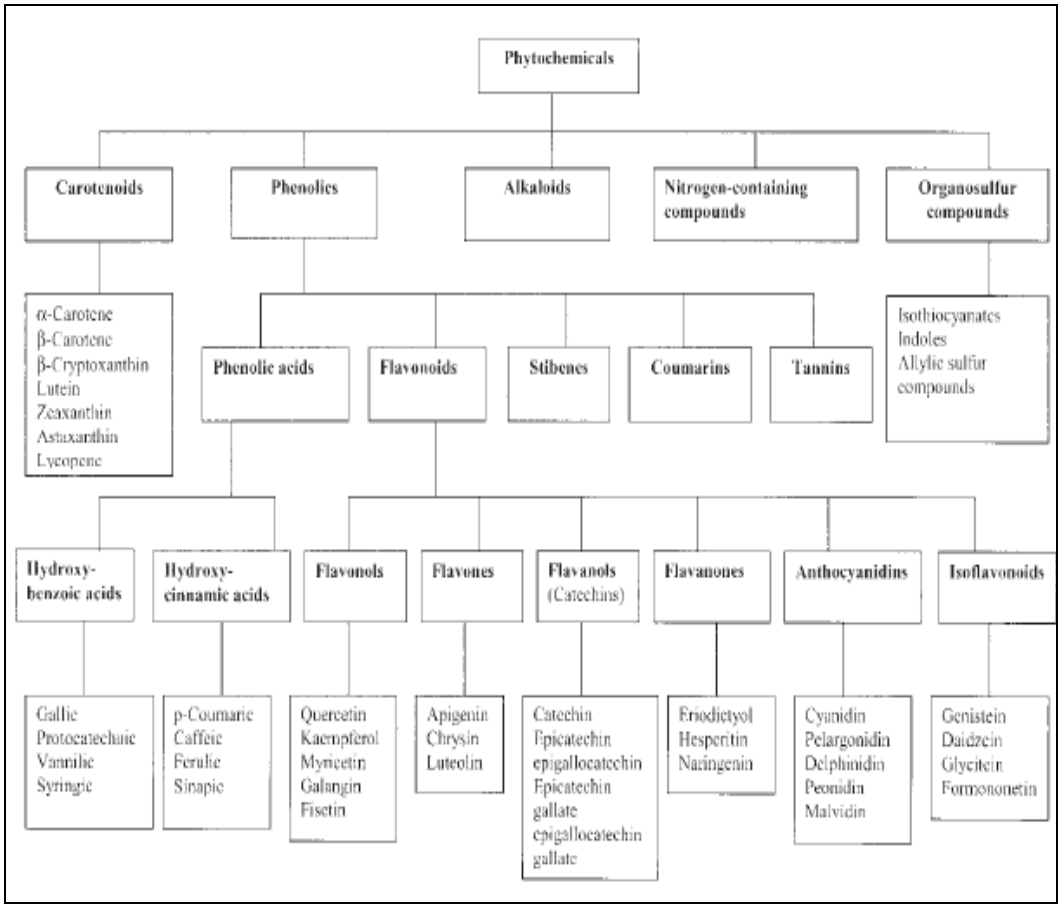
1.2. Cancer Cemoprevention by Complementary Therapies

Cancer research is very validate due to economic burden on the world economy. The National Institues of Health (NIH) estimates overall costs of cancer in 2010 at $ 263.8 billion (Cancer Facts& Figures, 2010). While modern surgery has resulted in no more than 5% reduction in the number of deaths. Cancer prevention through dietary modification appears to be a practical and cost effective possibility. Epidemiological studies have consistently shown that a high dietary intake of fruits and vegetables and whole grains is strongly associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease, which are the top 2 causes of death in most industrialized countries, In 1982 the National Academy of Sciences of the United States included guidelines in their report on diet and cancer, emphasizing the importance of fruits and vegetables (Hai.L.R, 2004).

Hippocrates and Indian physicians also reported garlic as a method to reduce tumor growth. In ancient Egypt (1550 BC) garlic was used for the treatment of tumors (World Cancer Research Fund,1997). Resveratrol and thus in found in the skins of grapes and in red wines, inhibited skin cancer in the two-stage mouse skin cancer model (Carcinogenesis, 2004) and inhibited the neoplastic progression of altered mouse mammary glands in culture (Lahiri-Chatterje et al, 1999). The researchers identified adenosine deaminaseinhibition by *U. dioica* including phytochemicals as the key effect in the prevention ofprostate cancer cell growth (Jang et al.,1997).

Phytochemicals are defined as bioactive nonnutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. It is estimated that 5000 individual phytochemicals have been founds in herbs **(Table 1.1).**

Table1.1 Classification of Dietary Phytochemicalls. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Hai, L.R,2004).



Phytoestrogens are naturally occurring hormone-like compounds found in plant foods which have an unique diphenolic structure, providing the compounds with exceptional stability (Adlercreutz et al, 1997). Due to their structural similarity to the human female hormone 17-β estradiol, phytoestrogens have the ability to bind to estrogen receptors (ER)(Setchell, 2001), having a greater affinity for ERβ than ERα (Kuiper et al., 1998). Phyto-oestrogens can therefore act as oestrogen agonists and antagonists competing for oestradiol at the receptor complex, However, this is not the only mechanism by which phyto-oestrogens exert their

effects; many of which may be unrelated to the oestrogenic properties of these compounds (Magee,et al., 2004).

Phytochemicals including antioxidant activity and scavenging free radicals; regulation of gene expression in cell proliferation, cell differentiation, oncogenes, and tumor suppressor genes; induction of cell-cycle arrest and apoptosis; modulation of enzyme activities in detoxification, oxidation, and reduction; stimulation of the immune system; regulation of hormone metabolism.

1.3.Biological Approaches to Cancer Prevention Mechanism

Cancer prevention is defined as active measures to decrease the incidence of cancer. Cancer chemoprevention includes the uses of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression, as first defined by Sporn in 1976 (Magee,et al., 2004).

This can be accomplished by avoiding carcinogens or altering their metabolism, pursuing a lifestyle or diet that modifies cancer-causing factors and/or medical intervention (chemoprevention, treatment of premalignant lesions). Chosen strategy, called chemoprevention is based on investigation of chemoprotective characteristic of our diet components.

Chemoprovention could be obtained by

(a) detoxification of the carcinogen by inducing detoxification enzymes; phase I (such as CYP 1A1 for rat hepatocytes) and phase II (such as GST).

(b) anti-oxidant activity (GSH amount, GR catalase and superoxide dismutase).

(c)Anti-inflammatory effect of compounds from natural product.

(d) immuno modulatory action.

(e)Hormon dependent mechanism.

Explanation of cancer chemoprevention mechanism which mentioned above;

1.3.1. Detoxification of carcinogens

Drug-metabolizing systems are composed of phase I and phase II enzymes. Phase I enzymes, mainly cytochrome P450, detoxify a variety of endogenous and exogenous chemicals and activate many carcinogens overwied in **Figure 1.3.**

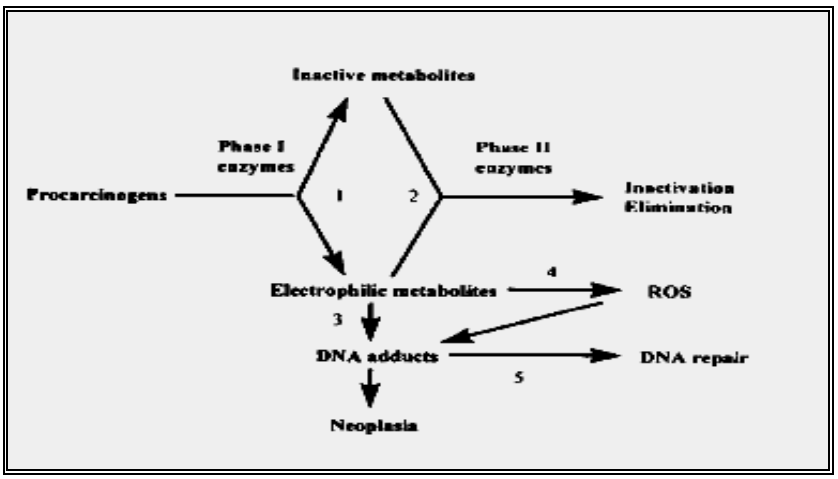


Figure 1.3. Overview of cancer chemoprevention mechanism.

Diallyl sulfide, one such compound in garlic, is an efficient inhibitor of the phase I enzyme cytochrome P450 (CYP)3 IIE1 (Brady, J et al, 1998). Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. CYP2E1 is involved in the metabolic activation of several environmental and dietary carcinogens. Diallyl disulfide significantly increases a variety of phase II enzymes, including glutathione *S*-transferase, quinone reductase and UDP glucuronosyl-transferase, which are responsible for the detoxification of procarcinogens (Wargovich, M. J, 1997).

1.3.2. Antioxidant activity (GSH amount, GR and catalase and superoxide dismutase).

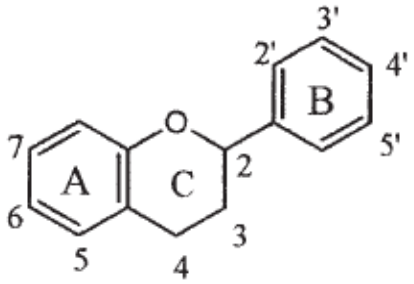
Reactive oxygen species (ROS) are known to play a major role in either the initiation or progression of carcinogenesis by inducing oxidative stress (Sun, 1990).

Peroxides and superoxide anions (•O2−) produce cytotoxicity/genotoxicity in cellular systems.

Dietary estrogen 'phytoestrogen' are found in wide variety of food products including herbs, even though the level varies depending on the source. Flavanoid and Lignan phytoestrogens have antioxidant activity (Thompson et al.,2006). The most studied of the phytochemicals are the phenolics and carotenoids.

Phenolics; Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally are categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins. Phenolics that are usually the products of secondary metabolism in plants, providing essential functions in the reproduction and the growth of the plants; acting as defense mechanisms against pathogens, parasites (Hai, L.R,2004).

Flavonoids; Flavonoids are the group of phenolic compounds with antioxidant activity that have been identified in fruits, vegetables, and other plant foods. Flavonoids have been linked to reducing the risk of cancer. More than 4000 flavonoids have been identified. They commonly have a generic structure consisting of two aromatic rings (A and B rings) linked by 3 carbons which are usually in an oxygenated heterocycle ring, or C ring was shown in **Figure 1.4**



The generic structure of flavonoids.

Figure1.4 The generic structure of flavonoids with A,B aromatic ring an one heterocycle ring C (Hai, L.R,2004).

Differences in the generic structure of the heterocycle C ring classify them as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids are represented in **Figure 1.5.**

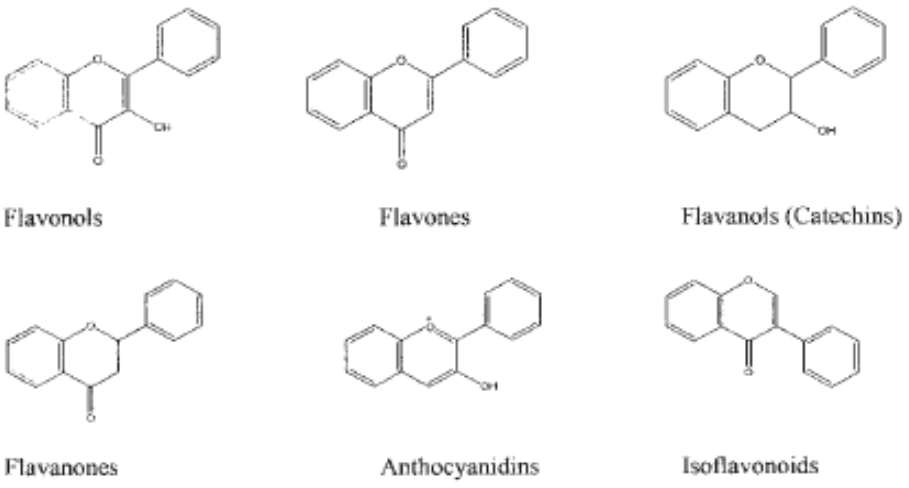
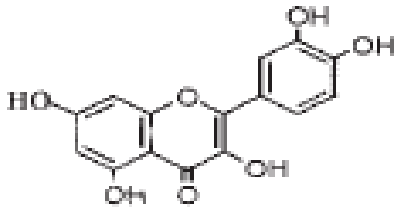


Figure1.5 Structure of main classes of dietary flavonoids (Hai, L.R,2004).

Flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate

and epigallocatechin gallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein) are common flavonoids in the diet **(Figure 1.6).**



Quercetin

Figure1.6 Chemical Structure of Quercetin as an flavonols.

Chlorogenic acids and curcumin including two ferrulic acid, are also major derivatives of hydroxycinnamic acids present in plants. Chlorogenic acids are the ester of caffeic acids and are a substrate for enzymatic oxidation (Hai, L.R , 2004).

Because of the antioxidative property, it is suggested that flavonoids may delay or prevent or reverse the onset of diseases (such as cancer) induced by free radicals.They also inhibit low density lipoprotein (LDL) oxidation by free radicals (Sellappan et al., 2002).

*(*c) another major parameter is evaluation of anti-inflammatory effect of compounds from natural product as an chemopreventive agent. Nearly all nonsteroidal anti-inflammatory drugs (NSAID) modify preferentially both isoforms of the cyclooxygenase (COX) enzyme responsible for the production of prostaglandins, COX-1 and COX-2. Strong inhibition of COX-1, whose products are involved in platelet aggregation, cytoprotection of the stomach lining and kidney function and ulcers.

Anti-inflammatory drug use, especially use of NSAID, has been associated with reduced risk for colon and breast cancer (Brady et al.,1998).

In order to achieve a more rational design of plant therapy, it is necessary to clarify the mechanism of the plant effects comprehensively.

d) *immuno modulatory action.*
Immunomodulation is defined as a procedure which alters the immune system by interfering with its function.

e) hormon dependent action

A few nonpolar signal molecules such as estrogens and other steroid hormones are able to diffuse through the cell membranes and, hence, enter the cell (Figure 1.7). Once inside the cell, these molecules can bind to proteins that interact directly with DNA and modulate gene transcription. Thus, a chemical signal enters the cell and directly alters gene expression patterns.

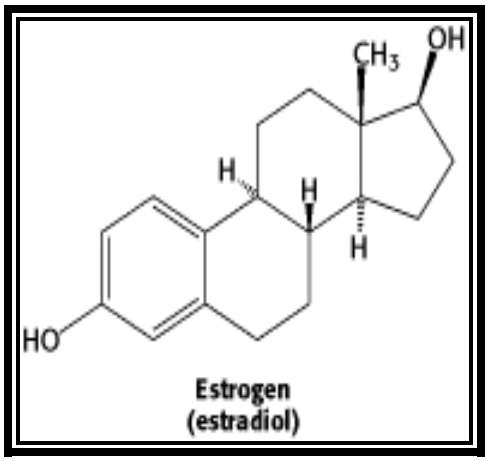


Figure1.7 Chemical Structure of Estrogen (estradiol) (Berg et al., 2002)
Estrogens play important roles in growth, development, reproduction, and maintenance of a diverse range of mammalian tissues. The physiological effects

of estrogens are mediated by the intracellular ERs, which regulate transcription of target genes through binding to specific DNA target sequences.

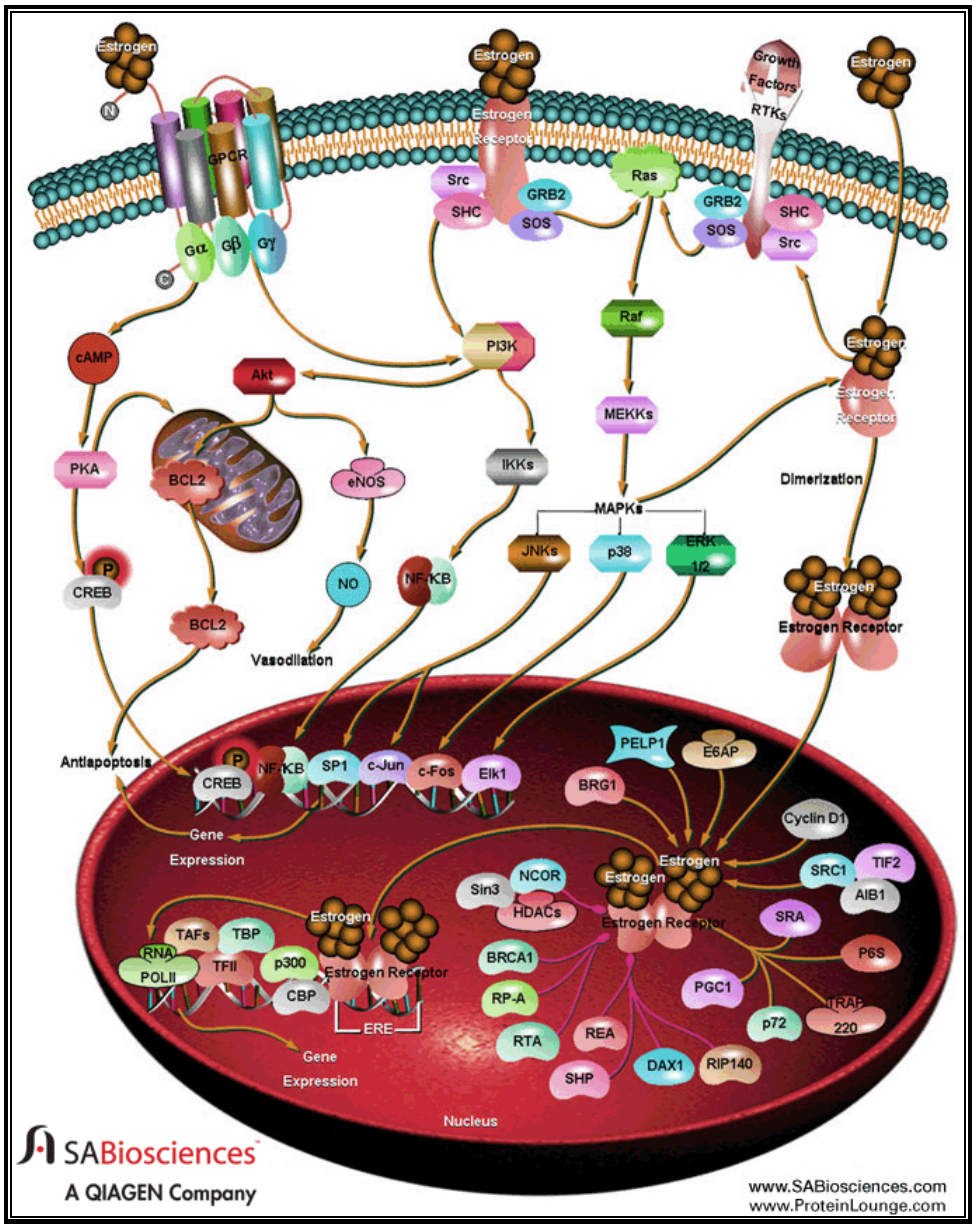


Figure 1.8 The ERs orchestrate both transcriptional and non-genomic functions in response to estrogens, xenoestrogens and signals emanating from growth factor signalling pathways (www.ProteinLounge.com).

The pleiotropic and tissue-specific effects of estrogens are mediated by the differential expression of two distinct ER subtypes: ER-α and ER-β, and their coregulators (Moggs et al., 2001). The activities of a plethora of ER-interacting proteins converge to confer distinct functionalities on ERs, including the activation and repression of transcription, the integration of intracellular signaling pathways and the control of cell cycle progression. Both ERs are distributed widely in the body in both genders. ER-Alpha predominates in the uterus and mammary gland, whereas ER-Beta has significant roles in the central nervous, cardiovascular, and immune systems; urogenital tract, bone, kidney, and lungs (Gustafsson JA, 2000). Typically, the majority of either ER-Alpha or ER-Beta is found in the cytoplasm and nucleus.

Endogenous estrogens are categorized as; E1 (Estrone), E2 (Estradiol/17-beta Estradiol) and E3 (Estriol); and, various syntehetic forms. Developmental exposure to high doses of exogenous E2 induces multiple persistent structural and functional abnormalities in the accessory sex glands. These include reduction in overall gland size; focal epithelial hyperplasia, metaplasia, and dysplasia; altered hormonal sensitivity; altered expression of ERs and AR (Androgen Receptor); alterations in stromal cell growth and function; disturbance of TGF-Beta (Transforming Growth Factor-Beta) signaling system; induction of protooncogenes; and inflammatory changes (Driggers et al., 2002).

Dietary estrogen (phytoestrogen) are found in wide variety of food products including herbs. The major phytoestrogen groups are isoflavones, flavones, coumestans and lignans.
Some studies indicate that phytoestrogens have health benefits including potential reduction in breast cancer, prostate cancer and cardiovascular disease risks, possible protection against osteoporosis and menopausal symptoms (Sellappan et al., 2002).

1.4. Traditional Turkish Plant *'U. Dioica'*

*U. dioica.*is a plant belonging to the plant family Urticaceae as shown in **Figure 1.9.**

Family*: Urticaceae*

Scientific Name;*Urtica diocia L,*

Common Name; Stringing Nettles

Turkish Name; ISIRGAN

Figure 1.9 *U. dioica* is a plant belonging to family Urticaceae (http://plant _life .org/urticaceae/large/urtica-dioica2_lg.htm)

1.4.1. General Distribution of *U. dioica*

Moist shaded lowland or montane slopes, always in deep rich soil or near moisture, often on disturbed ground, from the plains to subalpine, in all parts of Mediterranian. Widespread at Anatolia.

1.4.2. General Description of *U. dioica*

General Description; erect perennial, 50-300 cm tall with 4-sided stems, armed with stinging hairs (http://montana.plant-life.org /species/urtica_dioica.htm).

Leaves: opposite, 7-15 cm long, the stalks from about 1/10 as long to nearly 1/2 as long as the blades, depending on variety. The stipules prominent, mostly 10-15 mm long.
Flowers: numerous in hanging clusters from upper leaf axils, greenish, sometimes tinged pinkish, inconspicuous, 1-2 mm long, with 4 tiny sepals and no petals
Fruits: achenes, lens-shaped, flattened, about 1.5 mm long, enclosed by the 2 inner sepals. Leaves opposite, stipulate, dentate, with stinging hairs =Urtica Dioecious perennial; fruiting female perianth segments pilose over entire surface =dioica (http://montana.plant-life.org/species/urtica_dioica.htm) ,

1.4.3. Active Component of *U. dioica*

More than 24 chemical components have been identified in nettles. The primary structure of *U. dioica*agglutinin has been determined. Compounds isolated from the roots and flowers include scopoletin, sterylderivatives, lignan glucosides and flavonol glycosides.

There are two main classes of phyto-oestrogens; the isoflavones, and the lignans, which are found in a wide variety of foods including flaxseed, cereals, fruits and berries (Thompson et al., 1996).

The major lignans, which occur in the glycosidic form in foods, are matairesinol and seco-isolariciresinol **(Figure1.10).**

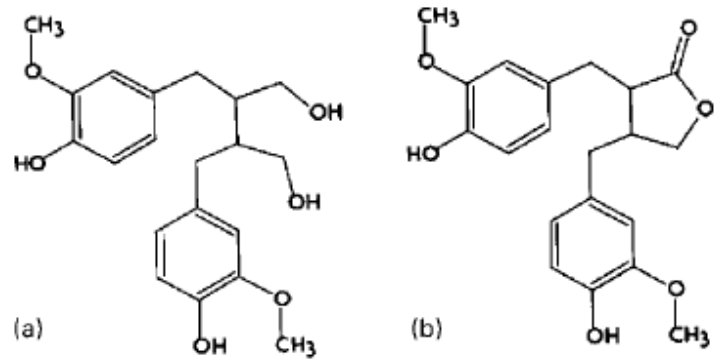


Figure 1.10 Structure of the lignans seco-isolariciresinol (a) and matairesinol (b) (Magee et al., 2004).

Nine flavonoid compounds have been isolated and identified. Phenylpropanes and lignans from the roots have been isolated. The plant also contains vitamins C, B-group and K. Glucokinin (responsible for “antidiabetic activity”) has been reported. In addition to sitosterol, at least six other related steroids have been identified. The young shoots are rich in carotene and vitamin C. The stinging trichomes of nettle contain amines, such as histamine, serotonin and choline.

HPLC, GC have determined specific lectin found only in*U. dioica*roots, which may help to standardize preparations (Kanter M, 2005). Polar extract of the *U. dioica*contains lignans +)-neoolivil, (-)-secoisolariciresinol,Dehydrodiconiferyl alcohol, isolariciresinol, pinoresinol, and 3,4divanillyltetrahydrofuran, and has antiinflammatory effects and stimulates the proliferation of human lymphocytes (Kanter M, 2005).

Table 1.2 Active components of a *U. dioica* characteristics of these components.

Scientific Name	***U. dioica***	
Common Name	**Stringing Nettles**	
Active Component	**scopoletin, steryl derivatives, lignan glucosides, flavonolglycosides, sitosterol**	**SURVEY OF CHEMOPROTECTIVE PROPERTIES**
Characteristics of components	Antiinflammatory effects Antiproliferative effects Binding to cystolic & nuclear receptors. Inhibits sodium potassium ATP-as activity.	**Obertreis et al., 1996, Riehemann et al., 1999** **Kanter M, Coskun O, Budancamanak M. 2005** **Durak I, et al.**,**Cancer Biol 2004;3:855-7**. **(Konrad *et al.*, 2000)**

1.4.4. Why Traditional Turkish Plant '*U. dioica*' is a Candidate to Study Chemoprevention

U. dioica is a plant belonging to the plant family Urticaceae. Its seeds are widely used in folk medicine in many parts of Turkey, particularly in the therapy of advanced cancer patients. Some actions of this plant such as anti-inflammatory effects (Obertreis et al.,1996) andstimulation of proliferation of human lymphocytes (Wagner et al., 1989) were reported.

A recent study found that a 20% extract of *U. dioica*, had significant antiproliferative effects on human prostatic epithelial and stromal cells (Harput et al.,2005). The researchers identified adenosine deaminase inhibition as the key effect in the prevention of prostate cancer cell growth (Prostate Cancer Fund, 2009).

The effects of *U. dioica*its main activity appears to be the interference of dihydrotestosterone binding to both cystolic and nuclear receptors (Durak I et al., 2004).This suggests a role in the prevention of prostate cancer. The most common uses for stinging nettle are treatment of benign prostatic hyperplasia (BPH).

In vitro inhibition of prostate cancer cell proliferation by *U. dioica* extract has been reported. This effect may be related to flavonoids such as caffeic malic acid, caffeic acid, chlorogenic acid and quercetin (Konrad *et al.*, 2000).Benign prostatic hyperplasia A possible mechanism may be caused by a hydrophobic constituent (eg, steroidal), which inhibits the sodium-potassium ATP-ase activity of the prostate, leading to suppressed cell growth in this area. Another report explains a different mechanism but suggests the aqueous extract is the active component in BPH therapy to inhibit the sex hormone-binding globulin to its receptor.

Leaves of this plant have been reported to show hypotensive (Garnier et al., 1961) and anti-inflammatory effects, to be useful in the therapy of prostatic hyperplasia (Krzeski et al., 1993; Lichius and Muth, 1997), to show diuretic (Tahri et al., 2000) and immunomodulatory activity (Özen et al., 2003).*(U. dioica*) could prevent the damage caused by TCA (chemical carcinogen trichloroacetic acid exposure), decrease the content of MDA and increase the antioxidant defense system in rats. Flavonoids present in *U. dioica*had strong anticancer, chemoprotective, antioxidant and metal-chelating properties and therefore might

protect cells and tissues against free oxygen radicals (Lou *et al.*, 1999; Mukhtar and Ahmad, 2000).The preventive potential of the plant infusions was evaluated by measuring the level of serum marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), acid phosphatase (ACP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH);antioxidant defense systems, reduced glutathione (GSH), glutathione reductase (GR), superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT); and lipid peroxidation (malondialdehyde, MDA) content in various organs of rats (Celik et al,. 2007).

The CCl_4 treatment for 60 days increased the lipid peroxidation and liver enzymes, and also decreased antioxidant enzyme levels.*U. dioica* treatment for 60 days decreased the elevated lipid peroxidation and liver enzyme levels and increased the reduced antioxidant enzyme levels (Kanter M., 2005).

U. dioica water extract was investigated for cytotoxic activity against KB, B16, HeLa and HLA tumor cell lines. Cells were incubated with the extract in the concentration range 800–1µg/mL for 48 h and the cytotoxicity was determined using the MTT assay (Harput, et al., 2005).

In recent study (Özen et al., 2003), *U. dioica* augmented the levels of all the measured components of cyt b5 and cyt b5 R. The activities of cytochrome b5 (cyt b5), NADH-cytochrome b5 reductase (cyt b5 R), glutathione S-transferase (GST), DT-diaphorase (DTD), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) showed a significant increase in the liver at both dose levels of extract. Both extract-treated showed significantly lower activity of cytochrome P450 (cyt P450), lactate dehydrogenase (LDH), NADPH-cytochrome P450 reductase (cyt P450 R), total sulfhydryl groups (T-SH), nonprotein sulfhydryl groups (NP-SH) and proteinbound sulfhydryl groups (PB-SH).BHA-treated Swiss albino mice showed

a notable increase in levels of cyt b5, DTD, T-SH, PB-SH, GPx, GR, and SOD in the liver while, LDH, cyt P450, cyt P450 R, Cyt b5 R, GST, NP-SH, and CAT levels were reduced significantly as compared to control values. The extract was effective in inducing GST, DTD, SOD and CAT activity in the forestomach and SOD and CAT activity in the lung at both dose levels. BHA-treated Swiss albino mice induced DTD, GST and all antioxidative parameters in the kidney, lung and forestomach.

1.4.5 Pharmocognostic Collection Guidelines of *U. dioica*

Pharmacognostic Collection Guidelines modified to medicinal plant "*U. dioica"* (Proposed Guidelines for Commercial Collection of Medicinal Plant Material., 2000*).*

I- Pick up just aerial part of nettle, above the soil surface
II- Dried under shadow (up to 35 ^{o}C) with no rain or sun at 48-72 h.
III- Don't use press.
IV- Preserved direct sunlight
V- Harvested leaves are cut than put on the drying racks
VI- Collected in to cardboard boxes.

U. dioica should not be stuffed in to bags (Harnischfeger et al.,2003). Plant material *U. dioica* must be harvested according to pharmacognostic collection guidelines.

1.4.6. Distribution of *U. dioica* in Anatolia

Widespread but scattered Istanbul; Kavaali,Elazığ; Hasarbabadağı, Erzurum; B-9,Kars: Akova gorge, Gaziantep: gaziantep, Hakkari: Sat Da., above

Yüksekova, 2700m. also scattered at Balıkesir, Kavaali, Boytop, Bolu, Ankara, İzmir, Giresun: Tamdere to Yavuzkemal, Marmara Adası (Davis PH, ED, 1982). Flowering Time of *U. dioica* is May- September (late june for eastern part of Turkey).

1.4.7. Drying Conditions for Medicinal Plants After the Collection

The cells of living plants contain enzymes when the plant dies, the barriers are quickly broken down and the enzymes then get the opportunity to promote chemical changes, e.g. by oxidation or hydrolysis. To stop the enzymic processes, the water content must be brought down to about 10 %. Freeze-drying (lyophilization) is a very mild method. Frozen material is placed in an evacuated apparatus which has a cold surface maintained at -60 to -80 °C. Water vapor from the frozen material then passes rapidly to the cold surface. The method requires a relatively complicated apparatus and is much more expensive than hot-air drying. For this reason, it is not used as a routine method, but it is very important for drying heat-sensitive substances, e.g. antibiotics and proteins.

1.5. Methods of Extraction

Extracts can be defined as preparations of crude drugs which contain all the constituents which are soluble in the solvent used in making the extract. In dry extracts all solvent has been removed. Softextracts and fluid extracts are prepared with mixtures of water and ethanol as solvent. Tinctures an alcoholic extractare prepared by extraction of the crude material five to ten parts of ethanol of varying concentration, without concentration of the final product. The ideal solvent for a certain pharmacologically active constituent should:

1. Be highly selective for the compound to be extracted.
2. Have a high capacity for extraction in terms of coefficient of saturation of the compound in the medium.
3. Not react with the extracted compound or with other compounds in the plant material.
4. Have a low price.
5. Be harmless to man and to the environment.
6. Be completely volatile.

According to the pharmacopoeias, ethyl alcohol is the solvent of choice for obtaining classic extracts such as tinctures and fluid, soft and dry extracts

There are many procedures for obtaining extracts like:

1. Infusion
2. Maceration
3. Percolation
4. Digestion
5. Decoction
6. Continuous hot extraction
7. Solvent-solvent precipitation
8. Liquid-liquid extraction
9. Distillation
10. Specific procedures

Infusion:

This might be hot or cold, depending on whether decomposition of ingredients could occur at higher temperatures. In this method, the plant material (herbal tea) is placed in a pot and wetted with cold water for about fifteen minutes after which the tea is poured off.

Percolation:

In this method, the ground plant material is subjected to a slow flow of fresh solvent. Percolation concerns also the movement and filtering of fluids through porous materials. Examples include the movement of solvents through filter paper

Digestion:

This method is similar to maceration and in some cases even considered as maceration. But digestion is simply carried out at higher temperatures. This is the main difference from maceration method. The temperature of the extracting medium is generally between 35-40°.This method is particularly used for barks of the plants and woods.

Decoction:

If the plant material is boiled for ten minutes or if boiling water is poured over it and allowed to stand for thirty minutes, the result is called decoction.

Continuous hot extraction method:

In this method the sample is dried, ground into small particles and placed in a porous cellulose thimble. The thimble is placed in an extraction chamber, which is suspended above a flask containing the solvent and below a condenser. The flask is heated and the solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. The extraction chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. At the end of the extraction process, which lasts a few hours, the flask containing the solvent and lipid is removed. In some device a funnel allows to recover the solvent at the end of the extraction after closing a stopcock between the funnel and the extraction chamber. The solvent

in the flask is then evaporated and the mass of the remaining lipid is measured. The percentage of lipid in the initial sample can then be calculated.

Solvent-solvent precipitation:

This method need two immiscible liquids. It separates according to solubility (polarity OR charge). It is especially useful forseparating alcoholic extractsinto more polar/less polar fractions and to concentrate to low volume. One example to this method is to add water + e.g. ethyl acetate, butanol, dichloromethane and performing alkaloid separation from other lipophilic compounds. Extraction is done with dilute acid by making alkaline. Extract is also possible with lipophilic solvents like DCM.

Liquid-liquid extraction:

Liquid-Liquid extraction is a mass transfer operation in which a liquid solution (the feed) is contacted with an immiscible or nearly immiscible liquid (solvent) that exhibits preferential affinity or selectivity towards one or more of the components in the feed. Two streams result from this contact: the extract, which is the solvent rich solution containing the desired extracted solute, and the raffinate, the residual feed solution containing little solute. Depending on the nature of the extraction process, the temperature, pH and residence time could have an effect on the yield and selectivity.

Decantation

Decantation is a process for the separation of mixtures, carefully pouring a solution from a container, leaving the precipitate in the bottom of the container.

1.5.1. Maceration

This method is used frequently for water soluble active constituents. It consists of macerating the plant material in cold water (15-20°C) for several hours. In Latin, *maceratus* means “to soften.” The term is used in a wide range of professions, from chemistry to medicine, but all of the uses refer in some way or another to softening. Preparation of an extract by soaking material (such as animal skins or parts of fibrous plants) in water, vegetable oil or some organic solvent.

Maceration of essential oils: With the maceration extraction method, the flowers are soaked in hot oil to have their cell membranes ruptured and the hot oil then absorbs the essence. The oil is then cleared of the botanical and decanted. (This is very much the same technique used in solvent extractions, where solvents are used instead of the hot oil as used in maceration).

In the literature, There are various reports about the example of maceration techniques from different studies such as;

i) In a *U. dioica* maceration study; 10 g of the dried powdered plant material was extracted in 100 mL ethanol: water (4:1) for 30 min, then the extract was filtered, the volume concentrated under vacuum and finally freezedried (A. Pieroni, 2002).

ii)The other study shows that; Aqueous extract of dried and powdered aerial parts (10 g) was obtained by maceration in preboiled hot water (1l) with occasional stirring for 20 min, decantation and filtration. Petroleum ether extract of dried and powdered plant material (100 g) was obtained by maceration in the solvent (1 l) for 48 h under shaking, filtration and evaporation in vacuo. Effect of *U. dioica* extract intake upon blood lipidprofile in the rats (Daher et al., 2006).

iii) 5 g of *U. dioica* dried material was inundated in 100 mL of sterile cold water and remained under covering over 24 hours in a temperature of 20°C.

iv)Fifty grams of each plant powder was extracted in 500 mL of methanol by maceration (48 h). The solvent was removed under the vacuum at temperature below 0°C and the extracts were freeze-dried (Nickavar, B, 2003).

v)The coarse powder of Brahmi (30g) was extracted using different maceration methods. The dried plant material was macerated in 180 mL of 95% ethanol for 3 days at room temperature and the resulting extract was filtered through filter paper (Whatman no.1). The residue from the filtration was extracted again twice using the same procedure. The filtrates obtained were combined and then evaporated to dryness under reduced pressure.

vi))Nigerian medicinal plats 50.0 g weight was taken and soaked in 200 mL of ethanol or cold water at room temperature with intermittent shaking for 24–48 h or in 200 mL of hot water (at about 100 ◦C) for 2 h also with intermittent shaking. Each extract was filtered through a Whatman no. 1 filter paper and the filtrate was evaporated to dryness in a steady air current for about 24 h in a previously weighed Petri dish (Benzien et al., 1996).

1.6. Methodologies for Testing Radical Scavenging Species

Nowadays it is well known that phenolic compounds are highly responsible for the health effects derived from consumption of plant origin food. They play a key role as antioxidants due to the presence of hydroxyl substituents and their aromatic structure, which enables them to scavenge free radicals (Villano et al., 2007).

Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic

acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

Antioxidant effectiveness is measured by monitoring the inhibition of oxidation of a suitable substrate. After the substrate is oxidized under standard conditions, the extent of oxidation (an end-point) is measured by chemical, instrumental or sensory methods. So, the essential features of any test are a suitable substrate, an oxidation initiator and an appropriate measure of the end-point. A wide variety of in vitro methods to assess radical scavenging ability have been set up.

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods. The malondialdehyde (MDA) or thiobarbituric acid-reactive-substances (TBARS) assays have been used extensively since the 1950's to estimate the peroxidation of lipids in membrane and biological systems.

1.6.1. TRAP assay (Total Radical-Trapping Antioxidant Parameter)

The total radical-trapping antioxidant parameter (TRAP) assay was introduced by Ingold group for the determination of the antioxidant status of human plasma. This method was based on the measurement of the time period in which oxygen uptake was inhibited by plasma during a controlled ROO• peroxidation reaction.

One of the major problems with the original TRAP assay lies in the utilization of the oxygen electrode as detector, since it may not maintain its stability over the period of time required. To overcome this limitation, this assay was later

improved using β-phycoerythrin (β-PE) as the fluorescent target/ probe, and the ability of the plasma to protect β-PE from peroxyl radical oxidation was fluorimetrically monitored.

The main shortcoming of TRAP assay is the use of the lag phase for quantifying antioxidant capacity, where not every antioxidant possesses an obvious lag phase. Also the antioxidant capacity profile after the lag phase is totally ignored. Another important limitation of this assay is that the oxidative deterioration and antioxidant protection of fluorescent target/probe does not necessarily mimic a critical biological substrate.

1.6.2. ORAC assay (Oxygen-Radical Absorbance Capacity)

The oxygen radical absorbance capacity, or ORAC assay, is one of the most common methods for assessing ROO• scavenging capacity. The principle of this assay is based on the intensity of fluorescence decrease of the target/probe along time under reproducible and constant flux of peroxyl radicals which are generated from the thermal decomposition of AAPH (a peroxyl radical generator) in aqueous buffer. In the presence of a sample that contains chain-breaking antioxidants, the decay of fluorescence is inhibited. The synthetic, non-protein fluorescein is used as the fluorescent target/probe, which react with ROO• to form a non-fluorescent product.

1.6.3.Methods of Cationic Radical Scavenging ABTS Assay

The TEAC assay involves the generation of the long-lived radical cation chromophore 2,2_-azinobis-(3-ethylbenzothiazoline-6 sulphonate) ($ABTS^{\cdot+}$)

which has absorption maxima at 414, 645, 734, and 815 nm. The original TEAC assay, developed by Miller et al.,1993 was based on the activation of metmyoglobin, acting as peroxidase, with H_2O_2 to generate ferrylmyoglobin radical, which then reacted with ABTS to form the $ABTS^{\cdot+}$ radical cation. In this strategy, the sample to be tested is added previously to the formation of the ABTS•+. The test compounds/samples reduce the ABTS•+ radicals formed and the lag phase, which corresponds to the delay time in radical formation, is measured.

1.6.4 Determination of Free Radical Scavenging Activity by DPPH Method

DPPH free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002).

A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of a stable free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years.

The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods.

Basis of the Method by Blois 1958.

1. DPPH - free radical and reduced form

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl; DPPH) is a stable free radical with ability of the delocalization of the spare

electron over the molecule as a whole, as a result the molecules do not dimerise, as would be the case with most other free radicals. The delocalization results in the deep violet color, characterized by an absorption band in ethanol solution centered at about 520 nm.

When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, DPPH is reduced to the corresponding hydrazine and the color turns from violet into a residual pale yellow color which would be expected from the picryl group still present in **Figure 1.11.**

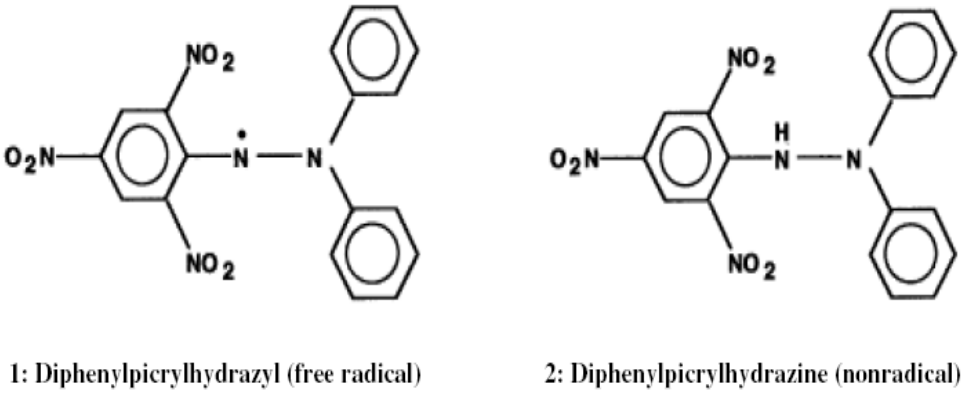


Figure1.11 Structures of free radical and nonradical form of DPPH (Koleva *et al.,*2002).

Showing the DPPH radical by $Z^{\bullet}$ and the donor molecule by AH, the primary reaction is

$Z^{\bullet} + AH = ZH + A^{\bullet}$ [1]

where ZH corresponds to the reduced form and $A^{\bullet}$ corresponds to the free radical produced in this first step. This latter radical will then undergo further reactions which regulate the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant. The reaction [1] provides the link with the reactions taking place in an oxidizing

system. So the DPPH molecule $Z^{\bullet}$ is intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH.

The parameter IC50 (“Effective concentration” value) IC50 is inversely related to the antioxidant capacity of a compound, as it expresses the amount of antioxidant needed to decrease the radical concentration by 50%. The lower IC50, the higher the antioxidant activity of a compound is.

The steric accessibility of DPPH• radical is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity. On the other hand, many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay. In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample. Finally, the DPPH• assay is not suitable for measuring the antioxidant capacity of plasma, because proteins are precipitated in the alcoholic reaction medium.
The percentage of inhibition or percentage of decolouration was calculated as follows:

% Inhibition = A(blank) - A (sample) / A (blank) x 100
Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

1.7. Metabolism of Active Compounds in *U. dioica*

Antioxidant capacity of *U. dioica* needs further extensive investigation for it’s gastrointestinal tract absorption and metabolism. One reason for this uncertainty

is that dietary active parent compounds in *U. dioica* are metabolized extensively in the body into partly conjugated compounds and their contribution to the antioxidant defense in vivo is uncertain. Therefore, In vitro tests of antioxidant activity of the parent phenols might be less relevant to the in vivo situation. This implies that the stomach acid (pH2) and colonic microflora (pH 7.4) convert most of these dietary phenols or flavon are a group of polyphenolic compounds into metabolites that then reach the circulation.

Two important groups of phenols in*U. dioica*are flavonoids and cinnamic acids The major representative of dietary cinnamic acids is caffeic acid. Caffeic acid is conjugated mainly with quinic acid, which yields chlorogenic acid (5-caffeoylquinic acid) reaches the colon, where the colonic microflora probably first hydrolyze chlorogenic acid into caffeic acid and quinic acid as shown in the **Figure 1.12.** The quinic acid moiety is dehydroxylated into cyclohexane carboxylic acid and then aromatized into benzoic acid by the colonic microflora (Adamson et al.,1969) (Cotran, et al., 1960). The benzoic acid formed is conjugated with glycine and excreted in urine as hippuric acid.

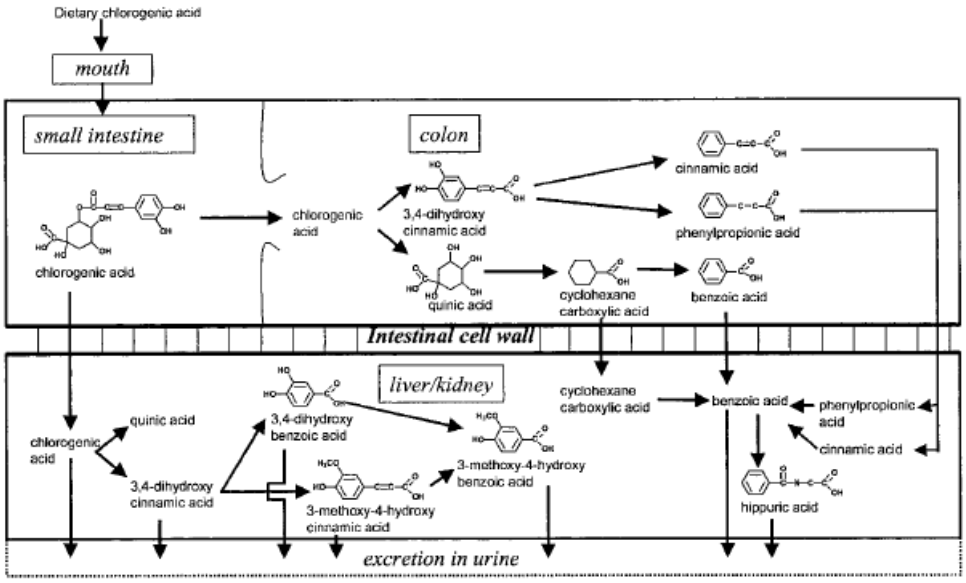


Figure 1.12 Proposed metabolic pathway of chlorogenic acid in human. (Hollman et al., 1998).

Gallic acid (3,4,5-trihydroxybenzoic acid) are present in urine. It probably originated from gallic acid present in *U. dioica* or from its esters with catechins. Gallic acid and Quercetin are not well absorbed in the small intestine of humans, and Quercetin nearly 83% will be transported into the colon and metabolized. Phenylacetic acids, the major metabolites of quercetin-3-rutinoside, have antioxidant activity in vitro that is similar to that of vitamin E, but can be lower than that of the parent compound quercetin were given in **Figure 1.13.** The breakdown of flavonoids and phenolic acids into smaller molecules through ring cleavage and β-oxidation in the colon and liver unfortunately lowers their antioxidant activity (Rice-Evans et al,1996). Subsequently, phenols and their metabolites are conjugated with glucuronic acid, sulfates or glycine, which also lowers their antioxidant activity (Olthof et al., 2011).

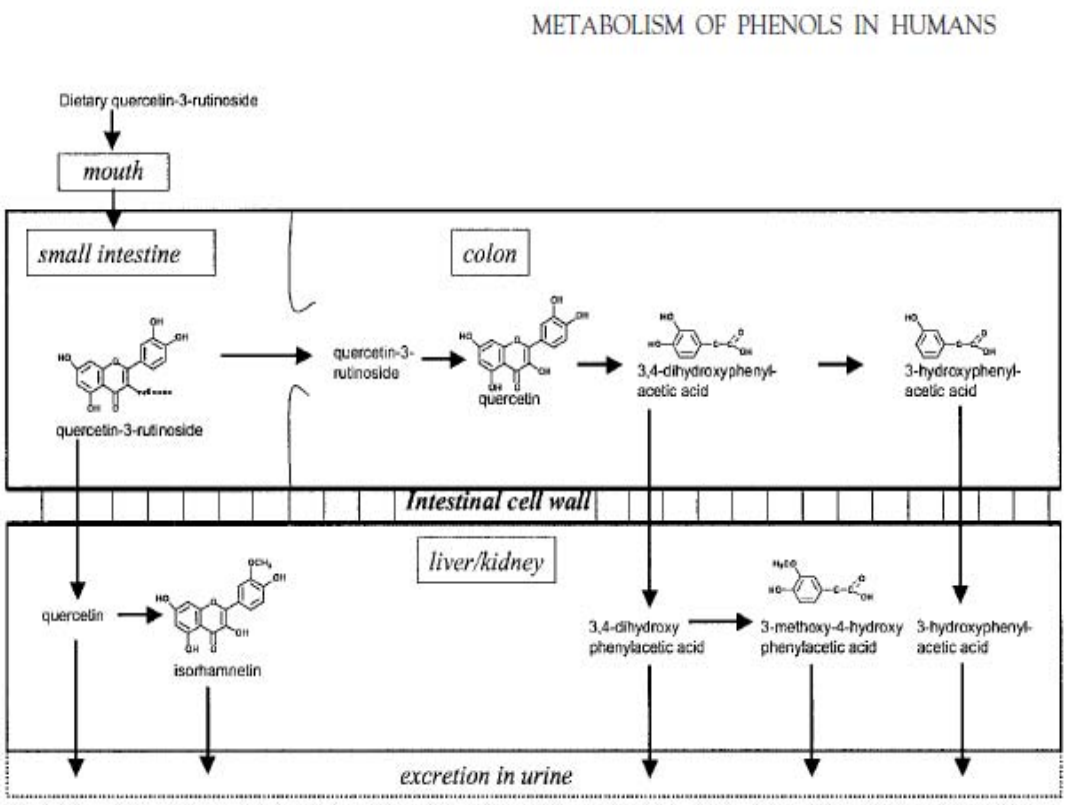


Figure 1.13 Proposed metabolic pathway of quercetin in humans. (Hollman et al., 1998).

1.8 Cytotoxicity Assay (XTT)

XTT Assay was a colorimetric method based on the reduction tetrazolium salt, XTT(sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate) to colored formazan products, was used as first described by Scudiero et al (1988). The Antiproliferative and cytotoxic effects of *U. dioica* in MCF-7 cells by mitochondria of live cells were evaluated

by using XTT Kit of Biological Industries according to manufacturer' s instructions.

The cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by the activity of mitochondrial enzymes in metabolic active cells **(Figure 1.14)**.

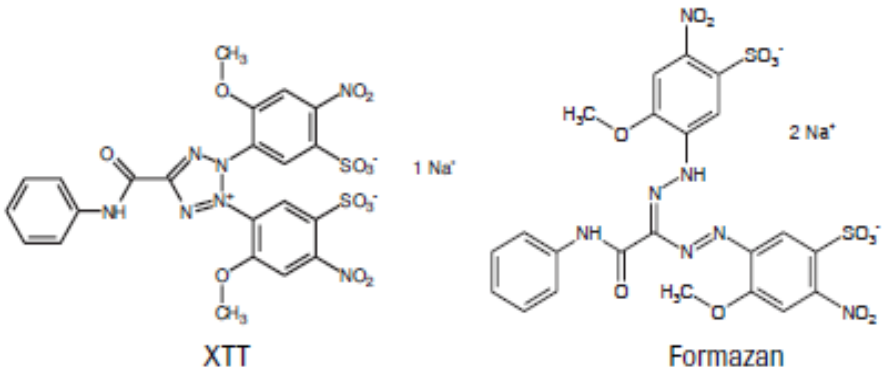


Figure 1.14 Metabolization of XTT to a water soluble formazan salt by viable cells.(Scudiero *et al*.,1988)

1.9 High Pressure Liquid Chromatography (HPLC)

High-performance liquid chromatography is a chromatographic technique can separate a mixture of compounds (drug substances, coloring agents, vitamins, pesticides, organic acids, saccharides ect) to identify, quantify and purify the individual components of the mixture. HPLC utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the sample (**Figure 1.15)**.

Figure1.15 HPLC analysis performed on Varian ProStar HPLC at METU Research Center.

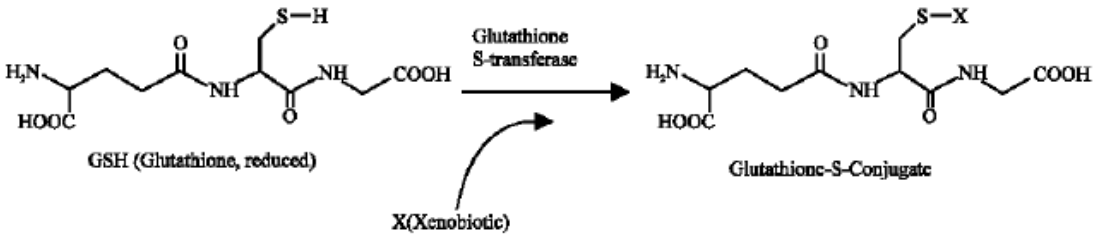
The flow rate of the mobile phase determined on the nature of the sample and compositions of the stationary (column) phase. The time at which a specific sample elutes is called the retention time; the retention time is considered an identifying characteristic of a the given analyte. The use of smaller particle size column packing that creates higher backpressure increases the linear velocity giving the components less time to diffuse within the column, improving the chromatogram resolution (Lloyd R, 2006). The column is filled with tiny silica particles, and the solvent is non-polar (hexane), for example. A typical column has an internal diameter of 4.6 mm which may be less than, and a length of 150 to 250 mm.

Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times (Xiang, Y, 2006).

Methanol-water mixture can be prefered as the solvent, having a greater wavelength than 205 nm, false readings should be avoide from the solvent. Methanol absorbs light at wavelengths below 205 nm, and water below.

1.10 Glutathione-S-Transferases

The glutathione-S-transferases (GSTs) (EC.2.5.1.18) are dimeric, mainly cytosolic, complex family of multifunctional enzymes that involved in cellular detoxification of endogenous as well as foreign electrophilic compounds, function in the cellular detoxification systems. GSTs composed of two polypeptide are evolved to protect cells against reactive oxygen metabolites by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly) As can be seen in **Figure 1.16**. (Dixon et al., 2002).



Glutathione S-Transferase catalyzed conjugation reaction of xenobiotics

Figure 1.16 GST catalyzed conjugation reaction of xenobiotics.

All GSTs have the ability to conjugate GSH with compounds containing an electrophilic center. The compounds that contain electrophilic center are arene oxides, aliphatic and aromatic halides,α,β-unsaturated carbonyls, organic nitrate esters, organic thiocyanates olefins, organic peroxides, quinines and sulfate esters. GST s are able to bind non catalytically, their non-catalyting roles, binding flavonoid natural products in the cytosol prior to their deposition in the vacuole (Edwards et al., 2000).

The availability of largescale genome data, expressed sequence tag (EST) databases, and the determination of three-dimensional structures by X-ray crystallography novel sequence alignment procedures, have greatly extended knowledge of structure and function relationships in GST enzyme family (Dirr et al, 1994, Mann, M. A.,1996).

Most of the mammalian GSTs that have been purifed and characterized are localized and synthesized within the cytosol, where they exist as homodimers or heterodimers of subunits with molecular masses ranging from 24.5 to 28.5 kDa. (Mannervik, B.et al., 1985 and 1992).

Subunits of GSTs have been grouped into seven distinct classes as: pi (π), alpha (α), mu (μ), sigma (σ), omega, theta (θ) and zeta (δ). This classification is in accordance with the substrate specificity, chemical affinity, structure, amino acid sequence and kinetic behavior of the enzyme. GSTs alpha (α), mu (μ), pi (π) form are active in drug metabolism. GST Sigma is responsible from of the functioning in prostaglandin synthesis. (Landi, 2000). Much emphasis tends to be placed on the primary structure at the N-terminus tends to be better conserved than others, as it includes an important part of the active site for placing a GST in a particular class. This region contains a catalytically essential tyrosine, serine or cysteine residue that interacts with the thiol group of GSH, thus lowering its p*K*a to a value of approx. 6±7 from its normal value of around 9.0. The N-terminal domain 1 (approx. residues 1±80) adopts a topology similar to that of the thioredoxin fold, consisting of four β-sheets with three flanking α-helices**(Figure 1.17)**.

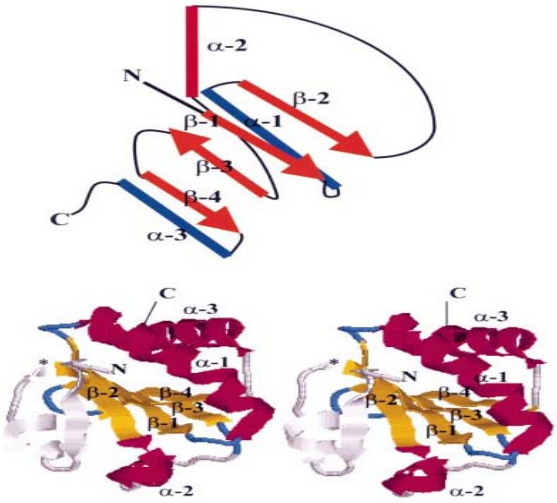


Figure 1.17 A schematic diagram representing the thioredoxin fold is shown above a RasMol depiction of the thioredoxin dimer.

GST classes representative crystal structures are available with the exception of the kappa class. Including those structures of traditional mammalian GSTs are from classes [Alpha, Mu, Pi, Theta, Sigma and MAPEG]; α [hGSTA1-A from human liver (Sinning et al.,1993); μ [rGSTM1-1 from rat liver (Ji, 1992); π [pGSTP1-1 from pig lung (Reinemer, 1991); hGOTP1-1 from human placenta (Reinemer,et al.,1992); δ [s GSTS1-1 from squid digestive gland (Ji, 1995) and θ [from *Lucilia cuprina* (Wilce, 1995), from*Arabidopsisthaliana* (Reinemer,1996), human (Rossjohn *et al.*, 1998) and a GST from *Schistosoma japanicum* (SjGST) (Lin, 1994), providing the structural basis for investigations of the enzyme active site. A comparison of some structures is provided in **Figure 1.18.**

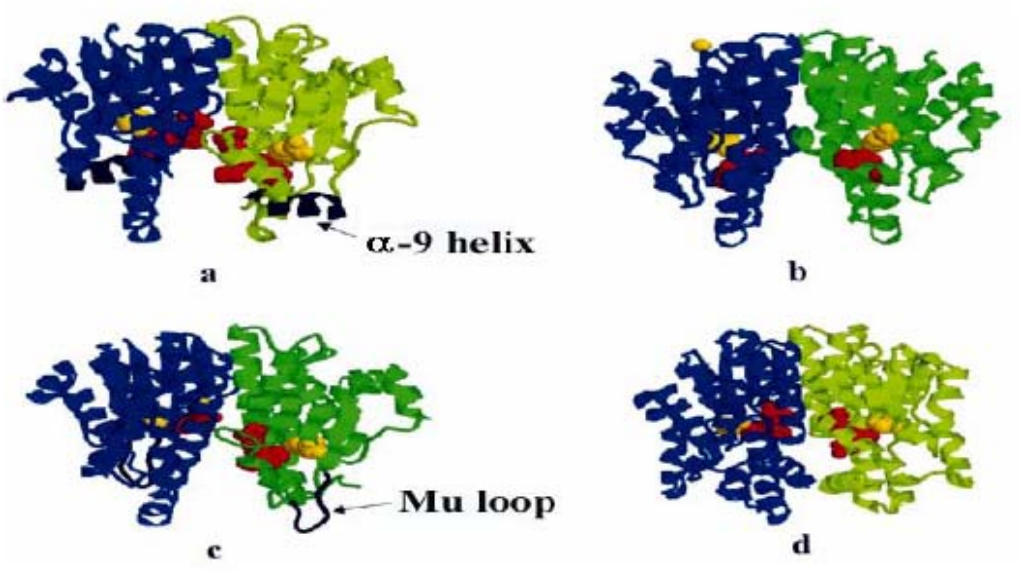


Figure 1.18 Traditional Mammalian GST structures (Sheehan, 2001) Structures are visualized using theRasMol program [RasMol (http ://www.bernstein-plus sons.Com /software/ rasmol/).

GSH is synthesized within the cytosol and depleted in it by conjugation reactions, and by the reaction of H_2O_2 and generated radicals across the cell membrane. Oxidation of GSH results in the formation of glutathione disulphide (GSSG), this is rapidly returned to the reduced state by glutathione reductase, thus maintaining the GSH: GSSG ratio around 99:1 (Kearns et al., 1998)(**Figure 1.19).**

GSTs can catalyse nucleophilic aromatic substitutions, Michael additions to a,b unsaturated ketones and epoxide ring-opening reactions, all of which result in the formation of GSH conjugates and the reduction of hydroperoxides, resulting in the formation of oxidized glutathione (GSSG).

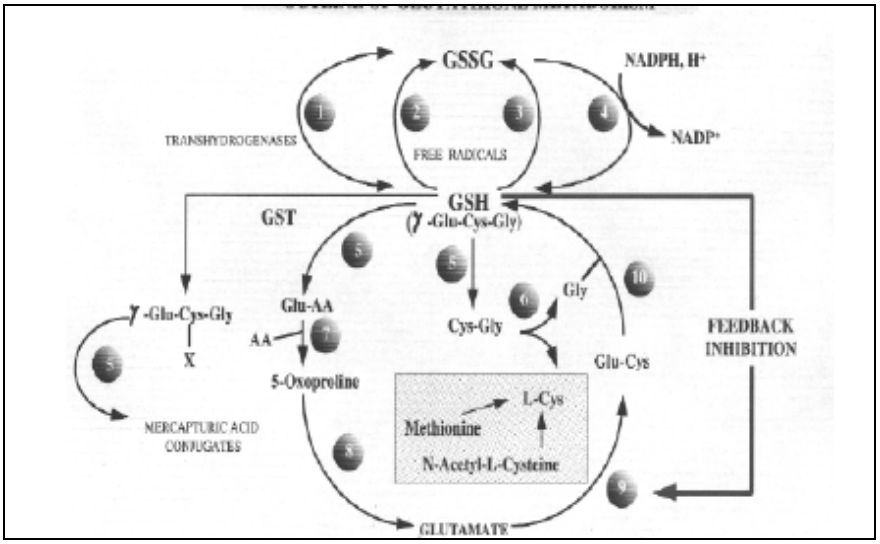


Figure 1.19 Outline of Glutathione metabolism (McLellan 1999).
1-GSH thioltransferase;2-reaction of free radicals with GSH;3-GSH peroxidases 4-Glutathione disulphide reductase; 5- Gama-glutamyl transpeptidases
6- dipeptidases 7-Gama-glutamyl cyclotransferase 8- 5-oxoprolinase 9-Gama-glutamyl cysteine synthetase 10- GSH-synthetase

GSTs have promising therapeutic target because specific isozymes are overexpressed in a wide variety of tumors and may play a role in the etiology of other diseases, including neuro -degenerative diseases, multiple sclerosis and asthma.

A marked increase in GST activity has been observed in tumor cells resistant to anticancer drugs (Daniel,1993).It has also been shown that alterations in GSH and GST levels are related not only in vitro drug resistance but also to clinical response to chemotherapy (Kearns *et al.,*1998) (Figure 1.20).

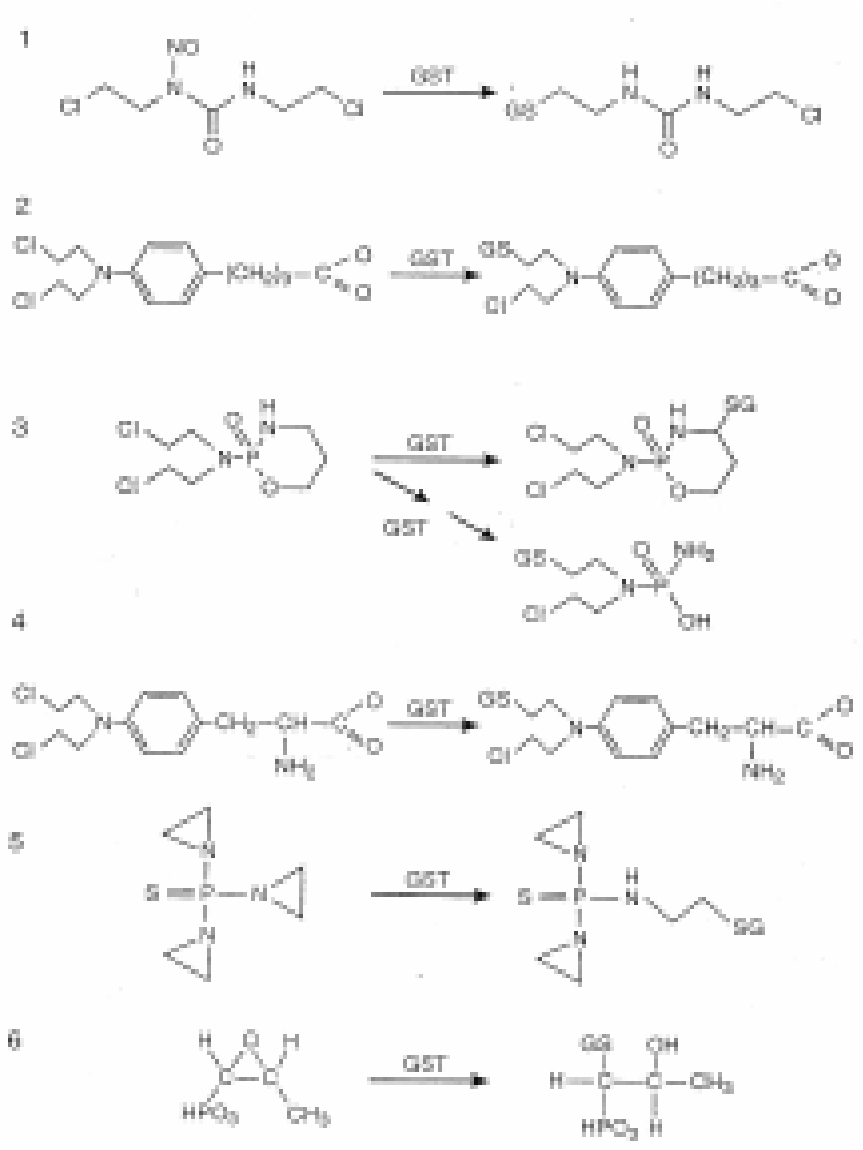


Figure 1.20 Examples of chemotherapeutic agents that are GST substrates: (1) BCNU; (2) chlorambucil; (3) cyclophosphamide; (4) melphalan; (5) thiotepa; (6) fosfomycin (Hayes and Pulford, 1995).

The GST isoenzymes display marked differences in their abilities to conjugate GSH with various electrophiles.1-chloro-2,4-dinitrobenzene (CDNB) is known as the universal substrate for GSTs since it is used for the demonstration of multiple forms of GSTs in various biological species.

When conjugated with GSH it gives *S*-(2,4-dinitrophenyl) glutathione, a compound possessing an absorbance spectrum sufficiently different from that of CDNB to allow a simple spectrophotometric assay at 340 nm (Habig *et.al.,*1974).

1.11 Scope of the Study

Aim of present research is to check traditional Turkish herb "*U. dioica"* which seems to provide promising agents with the potential protection against cancer and search for their mechanism of action.

Epidemiologic (preventive medicine) studies have associated certain food plants with pronounced reductions in cancer risk. Cancer chemopreventive potential of naturally occuring phytochemicals is of great interest. It appears that*U. dioica*traditional herbs may be a candidate for dietary anticarcinogens in cancer prevention (Cancer and Metastasis, 2002).

Additional studies are needed to assess the potential usefulness of *U. dioica* in treating or preventing for human cancer. In particular, its mechanism of action remains unclear.

Total phenol and flavon and radical scavenging activity measurement (RSA) were used to survey antioxidant potential of*U. dioica.* The effects of *U. dioica* extracts on glutathione-S-trans ferase (GST) activities were also investigated along with their antioxidant capacities. MCF-7 and MDA-231 breast cancer cell lines can be used in anticancer and antiproliferation research. XTT (Tetrazolium blue) antiproliferation assay was used to evaluate the reduction of viability of MCF-7 and MDA-231 cell cultures in the presence or absence of the extracts.

In study has been proposed that the additive and synergistic effects of phytochemicals in *U. dioica* are responsible for these potent antioxidant and anticancer activities. Phytochemical extracts exhibit strong antioxidant and antiproliferative cancer activities and that the major part of total antioxidant activity is from the combination of phytochemicals. Lack of quantitative data and proper evaluation on phytochemical metabolites in the *U. dioica* that reach to the stomach and the colon convert most of these dietary phytochemical into metabolites that then reach the circulation.

The aim of this study was to evaluate biological effect of metabolites of parent compounds (chlorogenic acid, quercetin ect) and their contribution to the antioxidant defense in vivo and correlate the Antioxidant capacity of these compounds with high performance liquid chromatography (HPLC) findings.

CHAPTER 2

MATERIALS AND METHODS

2.1. Collection of Plant Materials

Plant material *U. dioica* were harvested according to pharmacognostic collection guidelines and taken the their GPS records (Appendix A).

*U. dioica*were collected according to Pharmacognostic Collection Guidelines from Erzurum, Şenkaya (22.07.2008), Ardahan (27.07.08), Ankara, Eymir (2009-2010) locations at their flowering time shown in Figure 2.1. Samples were washed by soaking in distilled water.

Samples were protected from direct sunlight. Special hood racks designed for this purposes.

Figure 2.1 Collected *U. dioica* from Ankara, Eymir (2009-2010) locations.

2.2. Drying Conditions

Drying conditions can be critical to consequential herb quality. Dry herbs never

stayed outside. Samples are protected from direct sunlight. Also outside nocturnal rehydration of drying herbs can cause significant deterioration of medicinal herb quality. Literature recommend drying herbs on screened racks. We used special hood racks for this purpose (Drum, R,1999).

The amount of a constituent is usually not constant throughout the life of a plant. The stage at which a plant is collected or harvested is, therefore, very important for maximizing the yield of the desired constituent. Medicinal plants must be largely collected by hand. This is especially true in the case of wild plants.

2.3. Extraction Methods by Maceration

Before applying the maceration method to aerial part of *U. dioica,* first the plant materials were crushed into small pieces by simply breaking the plant one by one by hand. 4g of *U. dioica*were immersed in 40 mL of distilled water. Dried plant to distilled water ratio was 1:10 (w/v)(g/mL) in a beaker. The beakers were closed with parafilm and alimunium folio and incubated at different temperatures for varying periods of time for the optimization of maceration conditions.

4 Four sets of samples were prepared for extraction to optimize the maceration condition;

I-Maceration was performed for 6 hours at 50°C, 37°C, and room temperature. Beakers were kept in shaking water bath

II-Maceration was performed for 12 hours at 50°C, 37°C, and room temperature.

III-Maceration was performed for 24 hours at 50°C, 37°C room temperature.

IV-Maceration was performed for 36 hours at 50°C, 37°C room temperature.

The extract was separated from the plant material by filtration through filter paper. Then lyophilization (freeze-drying) of these samples were carried out in order to obtain plant material in the powder form The clear extracts were cooled

to -80°C at SANYO freezer for overnight and dried in HETO, Lyophilizator for 24 -36 hour to obtain the extract in the powder form was shown in Figure 2.2

Figure 2.2 Lyophilization of Plant material in Heto, lyophilizator.

2.4. Antioxidant Activity

2.4.1.Determination of Free Radical Scavenging Activity by DPPH Method

DPPH is a free radical, stable at room temperature, which produces a purple solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured or yellow ethanol solutions. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability.

An ethonolic solution (0.05 mg/mL) of the radical DPPH• was prepared daily and protected from light.0.05mg/mL DPPH• was prepared by dissolving 5mg of DPPH in 100mL %99.5 ethanol. Five different concentrations of extract mg powder/mL are prepared in water. These were 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL and 6 mg/mL, 8 mg/mL. Then 100 µl from each were added onto 1400 µl of the DPPH solution to give a total volume of 1500 µl. Following an incubation

of 30 min, the absorbance values were measured at 517 nm at room temperature. From these recorded data, the Final concentration vs. Absorbance (571 nm) graph is plotted. The percentage of inhibition or percentage of decolouration was calculated as follows:

% Inhibition = A(blank) - A (sample) / A (blank) x 100 . [2.4.1]

2.4.2. Determination of Total Phenol Content

The Folin-Ciocalteu (FC) method (McDonald *et al.,*2001) based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products was used to determine the phenolic compounds.

Aqueous solution (1mg/mL or 2mg/mL) of lyophilized extract (100µl) or gallic acid (standard phenolic compound) wasmixed with Folin Ciocalteu reagent (1mL, 1:9 diluted with distilledwater) and aqueous Na_2CO_3 (800 µl,1 M). The mixtures wereallowed to stand for 15 min and the absorbance values were measured at 765 nm at room temperature. The standard curve was prepared using0, 25, 50, 75, 100, and 150 mg/L solutions of gallic acid in distilled water. Total phenol values are calculated using the standard curve and expressed in terms of gallic acid equivalent (mg per gof dry mass of*U. dioica* water extract). Samples were analysed in dublicates.

2.4.3. Determination of Total Flavonoid Content

Aluminum chloride colorimetric method was used for flavonoid determination (Chang et al., 2002). Lyophilized*U. dioica*extracts (0.5 mL, 2 mg /mL) were separately mixed with 1.5 mL of dH_2O, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room

temperature for 30 min; the absorbance of mixture was monitored at 415 nm with a single beam Schimadzu 1240 UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 µg/mL, 50 µg/mL up to 100 µg/mL in ethanol.

2.4.4.UV-VIS Absorbance Spectra of *U. dioica*

The absorbance spectrum of 1mg/mL aqueous solution of *U. dioica*of macerated which performed for 6 and 12, 24, 36 hours at 50°C,37°C, 25°C were determined. Absorbance spectrum of 1 mg *U. dioica* solution absorbance values were recorded between 250-800nm wavelengths.

2.5.Extraction and Fractionation of *U. dioica*

U. dioica was collected from Eymir, Ankara city in August 2010. The air dried aerial parts of the plant (30g) were extracted with methanol (150mL) for 4 h 40^0 C in Cole-parmer 8851,sonicator. The liquid extract was separated from the plant material by filtration through filter paper. The final volume of methanol extract reduced to 120 mL. The liquid extract (120mL) was put in to seperatory funnel.

After filtration,100 mL 70% methanol was added on the plant material for 2^{nd} extraction for 4 h under the same conditions. Filter through filter paper and collect the filtrate again in the separator funnel. Fill the 100mL methanol extract into separatory funnel and add the 220 mL Chloroform. 2 times in a 15 minutes separator funnel was shaked to seperate the methanol and chloroform phase. Gaseous phase was released with opening tap of seperatory funnel. Compounds solved in the chloroform isolated from aqueous phase. Stayed

extract until the phases were separated. Lower chloroform phase and upper aqueous phase separately collected without disturbing and mixing the phases. 2nd Chloroform extraction was perfomed under the same conditions. 205 mL chloroform phase concentrated by evaporating in a vacuum rotary evaporator at 40^0C. 130 mL Ethyl acetate added in a remaning portion of 130mL aqueous phase and shaked.2nd Ethyl acetate extraction was obtained with a yield of 125mL. Upper ethylacetate phase has been collected seperately using tap of seperatory funnel. Then Powder extract obtained by evaporating in a vacuum rotary evaporator.

Last aqueous phase was extracted with 125 mL n-butanol. Remaning fraction of n-butanol phases was extracted second times with 120mL n-butanol. N-butanol extracts were kept to -80°C at SANYO freezer for overnight and dried in HETO, lyophilizator (HETO) for 24 h to obtain the extract in the powder form. Aqueous phase was obtained at the end of the extraction and was dried in lyophilizator.

2.6 HPLC -DAD Analyses

HPLC-DAD analyses were performed onan Varian Prostar HPLC equipped with a ProStar 330 Photodiode Array Detectormanaged between UV-VIS-NIR regions (190- 700 nm). Phenolic compounds were separated using a4.6 × 150 mm pursuit C18 (5 µm) column (Varian, Germany) operatingat 35 °C. A four step linear gradient solvent system was used, startingfrom 2.5% formic acid (HCOOH) to methanol (H_3COH) in water during a 73-min period, at the flowrate of 1mL min-1 (Saracini, E,*2005*). Determinations of phenolic contents were carried out in duplicate, andresults are given as means (standard deviation (SD). HPLCanalyses of phenolic, compounds in aerial partsof *U. dioica* was carried

out in Varian ProStar HPLC. Phenolic compound in*U. dioica* identified and quantified were provided in Table 2.1.

Table 2.1 Chromatography conditions for the determination of phenolic compounds in *U. dioica* extracts.

Chromatographic conditions	
Injected volume	10µl
Column	Pursuit C18 (150X 4,6mm i.d, 5µm)
Mobile phase	A(formic acid in water, 2,5% v/v): B (methanol)
	0-7 min: 0:100 (elution step)
	7-42 min: 20:80 (elution step)
	42-67 min: 60:40 (elution step)
	68-73 min: 0:100 (flushing step)
Flow rate	1mL/min
Temperature	35 °C
Detection conditions	
Detection wavelenght	Gallic acid 280nm, Caffeic acid 320nm, Quercetin 360nm.

2.7 Cell Culture

2.7.1 Growth Condition of MCF-7&MDA-231 Cells

The parental MCF-7 cell line was from ATCC (American Type Culture Collection). MCF-7 is a model cell line for 69 years old human, caucasian,

adenocarcinoma which exhibits some features of differentiated mammary epithelium. MDA was an highly agressive and metastatic, ER negative breast adenomacarsinoma cell, The monolayer cells were maintained as an attached type monolayer culture in complete RPMI 1640 Cell Culture Medium with phenol red containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biochrom AG). Cell culture media were supplemented with 0.2 % (v/v) gentamycin (Biological Industries). Incubation conditions at 37°C in a 95% (v/v) humidified atmosphere of 5% (v/v) CO2 were maintained in a Heraeus incubator (Germany).

Cell line was handled in a Type Biolagical flow cabinet by using appropriate cell culture techniques.

2.7.2 Cell Proliferation by XTT Assay

Antiproliferative effects *U. dioica* on MCF-7 and MDA 231 cells were evaluated by means of the Cell Proliferation Kit (Biological Industries) according to manufacturer's instructions. Assay was a colorimetric test based on the reduction tetrazolium salt, XTT to colored formazan products by mitochondria of live cells. In brief, cells were seeded to 96-well microtiter plates (Greiner) at a concentration of (10.000cells/well) and incubated for 48 h in order to let them attach and grow in CO_2 incubator at 37^0C. After incubation, medium was changed, 50 µl fresh medium was added and cells were treated with 50 µl XTT reagent (0,1mL activation reagent and 5 mL XTT reagent) in order to reach final *U. dioica*concentration; 5,7, 14.6, 19.5, 29.2, 49, 73 µg/mL).

In each plate assay was performed with a column of blank medium control and a cell control column.

Then, XTT reagent was added to each well and soluble product was measured at 415 nm with an Spectromax 340 96-well plate reader (Molecular Devices, USA). MCF-7 and MDA 231 cells (10^4 cells/well) were cultivated in RPMI 1640 for 24h, and then varying concentrations (0-73 µg/mL) of extracts were added and continued incubation for 48h.The same concentrations of extracts were also added in the wells without cells as sample blanks. XTT reagent were added and incubated for 4 hours. The intensity of orange colour formed was measured with an ELISA plate reader using filter at 415 nm.
% Cell Viability was calculated as follows:

$$\frac{(OD_{415} \text{ with [cells]}) - (OD_{415} \text{ w/o [cells]})}{(OD_{415} \text{ with [cells] DMSO control}) - OD_{415} \text{ w/o [cells] DMSO control})}$$

2.8 Preparation of Sheep Liver Cytosolic Fraction

The livers of male sheeps older than 4 months are obtained from slaughter house (Kazan-Ankara), All subsequent steps are carried out in cold room at 0-4 ^{0}C. Livers are cut into medium size cubes, connective and fatty tissue are removed, and washed with ice cold distilled water twice and then in 1.15 % KCl (0.154 M) containing 1.0 mM EDTA to remove blood as much as possible (İşcan and Arınç, 1988).

Liver cubes are dried on coarse tissue paper and cut in small pieces by scissors. 60 g of the resulting tissue mince is homogenized in pre-cooled tissue blender run at 2,400 rpm by adding 180 mL ice-cold homogenization solution (0.1 M potassium phosphate buffer, pH 7.4, containing 1.15 % KCl and 0.1 mM EDTA). Homogenization is carried out with five times 15 seconds passes, with 1 min cooling intervals in ice bath in between passes in the cold room. The homogenate is centrifuged at 12,000xg by a Sorvall by using SS-34rotor for 25

minutes at 4 ^{0}C to remove cell debris, nuclei and mitochondria. Supernatant is collected and filtered through double layered sterile cheese-cloth in the cold room. Then, the supernatant is centrifuged at 105,000xg for 40 minutes in Hitachci Himac CP 100 WX preparative ultra centrifuge, rotor model P50AT2(Rav 8.13 cm). The supernatan solution is saved as the cytosol fraction, 0.5 mL aliquot is removed for protein determination. Most frequently, the protein determination, and enzyme activity measurements are started immediately after the preparation of the cytosol without any further storage. Otherwise, the cytosol in small aliquots of 0.5 mL were stored at -80°C to be used later (Cristobal L et al.,1998).

2.9 Determination of Cytosolic Protein Amount by Lowry Assay

The protein concentrations are determined according to Lowry method (1951) adjusted to measurement by Elisa Plate Reader with crystalline Bovine serum Albumin (BSA) as the standard. Cytosolic fractions prepared from Sheep Liver are diluted as 1/50 and aplied to 96 well plate as triples as a volume of 40µl. 200 µl of Lowry alkaline copper reagent (ACR) is prepared (composed of 2 % copper sulfate($CuSO_4.5H_2O$);2%sodium-potassium tartarate and 0,1N NaOH containing 2 % sodium carbonate in the ratio of 1:1:100). First copper sulfate and sodium potassium tartarate is mixed and then NaOH containing 2 % sodium carbonate is added and mixed. After 10 minutes incubation at room temperature ,Folin Ciolcateu, Phenol Solution is added as 20 µl with immediateley mixing in seconds. After 45 minutes incubation at room temperature, plate was read at 650nm by Elisa plate reader (Yılmaz, 2006). The intensity of the color is directlty proportional to the protein concentration. Protein concentration in each well caculated by the software (KC Junior) after providing necessary dilution factors. Standard curve is prepared by using BSA solutions.

2.10. Determination of Glutathione-S transferase Activity

GSTs activity was determined spectroscopically by monitoring the thioether formation at 340 nm using CDNB as the substrate and GSH as cofactor (Habig et al., 1974). Sheep liver cytosolic fractions were used as the enzyme source to measure GST activity toward CDNB.

To a reaction mixture contained 50 µl 500 mM potassium phosphate buffer at pH:7.4 with 10µl 25 mM GSH, 162.5µl dH_2O, 12.5 µl 20 mM CDNB and 1-4 mg/mL cytosolic protein in a final volume of 250 µl in 96 well plate. 25 µl enzyme source were added. After sample addition solution was mixed and reaction started. The blank wells contained 25 µl water except cytosol as enzyme source. After machine mixing, reading is started automatically at every 20 seconds for 10 minutes. The slopes of the best lines drown for each well separately by the software of the instrument were used as the rate of reaction (dA/dt) and the further calculations were completed (Figure 2.3).

$$SpecificActivity = \left(\frac{OD}{\varepsilon} \times DilutionFactor\right) \Big/ ProteinConcentration$$

$$SA = \left[\frac{mOD(slope\ value\ given\ by\ software) \times 10^{-3}}{9,6 \times mM^{-1} \times cm^{-1}} \times 400(outside) \times 10(in\ well)\right] \Big/ 25,668 mg/ml$$

$$\frac{\left[\left(400 \times 10 \times 10^{-3}\right) / 9,6\right]}{25,668} = 0,0162$$

$$SA = \frac{0,0162 \times \frac{1}{mM^{-1}}}{mg/ml} = 0,0162 \times \frac{mM \times ml}{mg} = 0,0162 \times \frac{\mu mole}{mg}$$

Figure 2.3 The GSTs activities were expressed as unit/mg protein. One unit is equal to one nmole of substrate consumed or product formed per minute.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Free Radical Scavenging Activity of '*U. dioica*'

The extraction of water soluble active constituents of *U. dioica* was performed by maceration of dry *U. dioica*leaves in water. The conditions of maceration were optimized.The experimental design for optimization studies was also shown in Table 3.1.The extract obtained under different maceration conditions were lyophlized and their radical scavenging activity (RSA)were calculated.

The results of the present study established that RSA varied from 15% to 65% in the different extracts.

The highest radical scavenging activity was observed as %65 RSA (as can be seen in Table 3.1) from an aqueous extract of macerated *U. dioica* under room temperature at 12 hour time point.

T**able 3.1** The Radical Scavenging Activity (RAS) of the aqueous extracts of *U. dioica* obtained under different maceration conditions.

Maceration Conditions	**Radical Scavenging Activity (RSA)%**			
Incubation Periods / **Temperature**	6hour	12 hour	24 hour	36 hour
25°C RT	**37%**	**65%**	**15%**	**34%**
37°C	**33%**	**26%**	**50%**	**55%**
50°C	**53%**	**37%**	**56%**	**55%**

All measurements were performed in duplicate. Five different concentrations of the *U. Dioica* extract have been assayed in order to check the linearity of response and to establish the antioxidant activity values in the adequate linear range.

Percent Radical Scavenging Activity was calculated by using Blois (1958) method. The calculation of the highest radical scavenging activity (%65) obtained by 12 hour at room temperature macerated *U. dioica was* shown in the following, Table 3.2. And can be seen in Figure.3.1 and Figure 3.2.Absorbance values of 0.03-0.53 mg mL^{-1} solutions of final concentration of 12 hour at room temperature macerated *U. dioica* were measured 517 nm at room temperature.

Table 3.2 The Radical Scavenging Activity (RSA) of aqueous extract of *U. dioica* macerated for 12 hour at room temperature.

Concentration of urtica dioica L extract (mg/mL)	Final Concentration (mg/mL)	FRSA (%)
8	0,53	49
6	0,40	65
4	0,27	59
2	0,13	40
1	0,06	29
0,5	0,03	20

The IC50 values of percent DPPH scavenging were determined from the percent inhibition versus final extract concentration curve for the aerial parts of *U. dioica* shown in **Figure 3.1** and **Figure 3.2,** respectively. The IC50 value was found to be 300 mg/L.

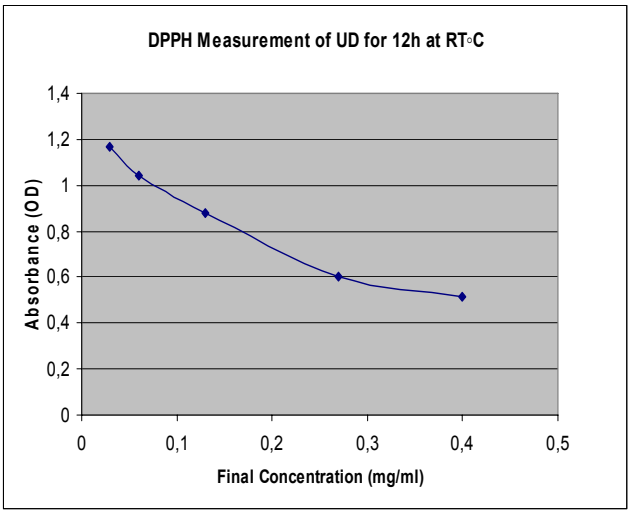


Figure 3.1DPPH Measurements of 12 hour at room temperature macerated *U. dioica*.

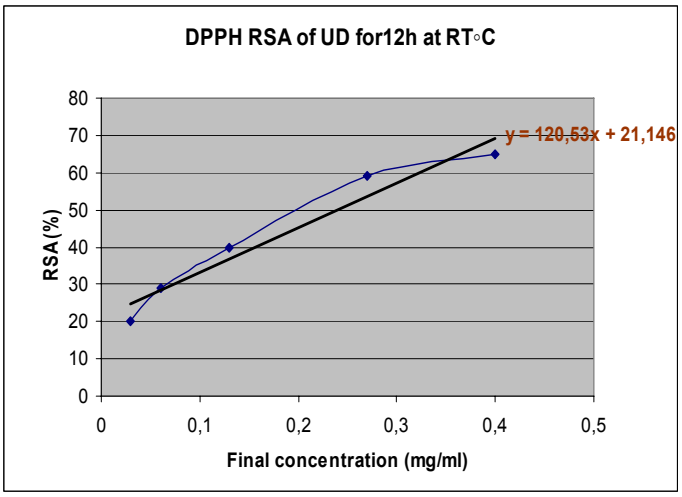


Figure 3.2 Free Radical Scavenging % of 12 hour at room temperature macerated *U. dioica*.

In literature (Mavi et al.,2004),IC_{50} value of methanolic extract of *U. dioica* was found as 335 mg/L. This study confirms that IC_{50} value of methanolic extract of *U. dioica* was quite similar to IC_{50}value of aqueous extract of *U. dioica* and highlights the positive correlation between these two studies.

The slight difference (35mg/L) among IC_{50}values of aqueous and methanol extracts of*U. dioica* was probably related to solvent polarity. The solubility differences of active content in *U. dioica* in methanol versus water can, gradually, effect the radical scavenging activity.

The standart used in present assay was quercetin. The IC_{50} value for quercetin was found to be 30µg/mL which wasabout ten folds more than that of aqueous *U. dioica* extracts.

Mohamad and coworkers(Mohamad et al.,2004) previously reported that the IC_{50} value of quercetin was 15.8 µg/mL which was also higher than DPPH inhibitory activities of *U. dioica* extract.

3.2. Total Phenol Content of *U. dioica* Aqueous Extract

Total phenols were determined by Folin Ciocalteu reagent (McDonald *et al.*, 2001). Phenol content of extracts in terms of GaE equivalent varied from 28.8± 0.57 to 74.2± 2.24 mg g^{-1}.12h at room temperature macerated *U. dioica* have the richest phenolic content (74.2± 2.24mgGaE/g extract).The standard curve was prepared using0-150 mg L^{-1} solutions of gallic acid in distilled water, as shown in Figure 3.3 below.

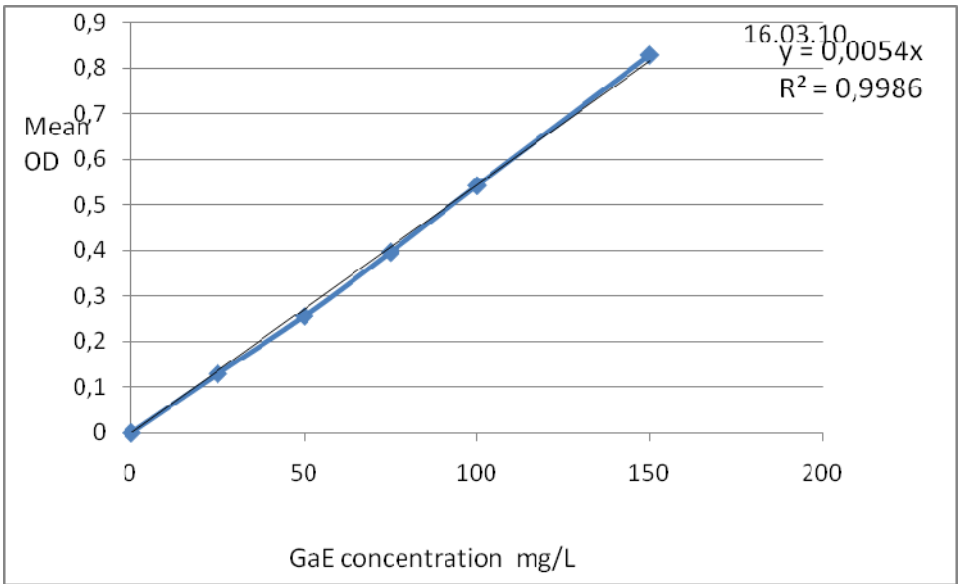


Figure 3.3 Gallic acid Standard Curve.

Absorbance values ofGallic acid in varyingconcentrations weremeasured at 765 nm. From these recorded data, the Final concentration vs. Absorbance graph was plotted with the standard curve equation: y = 0.0054x +, r^2= 0.9986 as Figure 3.3 shows.

As can be seen in Table 3.3, total phenol values are calculated using this standard curve equation and expressed in terms of gallic acid equivalent (mg g^{1}) of dry mass which is lyophilized water extract of *U. dioica*.

Table 3.3 Total phenol content of *U. dioica* extracts as gallic acid equivalents in mg/ L.The experiments were performed in quadrublicate.

Maceration Conditions	**Total phenol content (mg GaE/gextract)**			
Incubation Periods / Temperature	**6 hour**	**12 hour**	**24 hour**	**36 hour**
RT °C	**57.9±** 1.19	**74.2±** 2.24	**28.8±** 0.57	**67.5±** 1.89
37 °C	**56.3±**1.05	**50.1±** 1.31	**36.9±** 1.89	**56.2±** 1.57
50 °C	**66.9±**0.84	**68.7±**3.63	**42.8±**0.92	**68.5±**1.18

Total Phenol Amount of *U. dioica*extractwhich macerated at 12Hroom temperature has (4.2 mg g^{-1}**±** 2.24mg GaE) and this was approximately 2,6 folds more than 24 h atroom temperaturemacerated *U. dioica* extract (28.8± 0.57 mg GaE/g extract) (Figure 3.4).

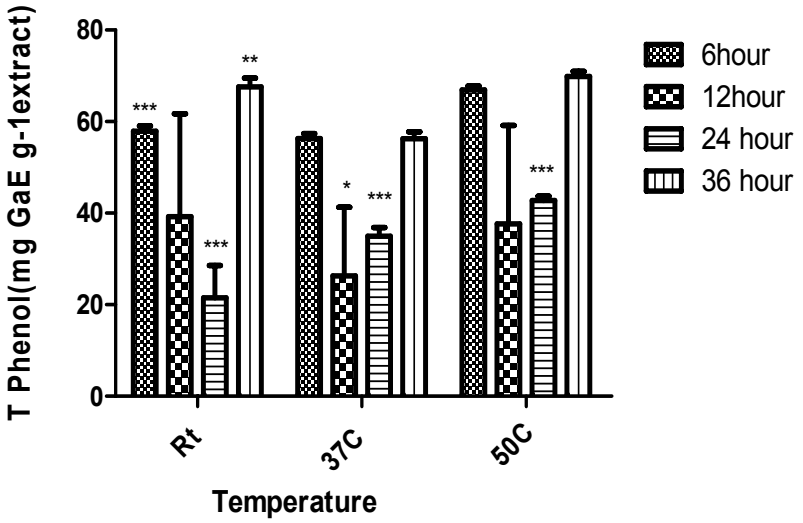


Figure 3.4 The total phenol content of *U. dioica* extracts prepared under different conditions. In this graph, * , ** and *** represent statistically significant difference at ($p < 0.05$), ($p < 0.001$) and ($p < 0.01$), respectively. The graph was drawn using Graph Pad Prism program.

The highest total phenolic was found in 12 h atroom temperaturemacerated *U. dioica*extract, followed by 12 h at 50^{o}C and 36h at 50^{o}C macerated *U. dioica*extract (p<0,05).

3.3.Total Flavonoid Content of *U.dioica* Extracts

Aluminum chloride colorimetric method was used for flavonoid determination (Chang et al., 2002). According to the results of the present study the richest flavonoid content is found in aqueous *U. dioica* extract obtained from maceration at room temperature for 12 hour.The flavonoid contents of extracts in terms of quercetin equivalent were between14.5±1.33 and 28.9±0.93 mg quercetin per g of extract.

The calibration curve was drawn by preparing quercetin solutions at concentrations 12.5 –100 µg/mL in ethanol are shown in Figure 3.5.

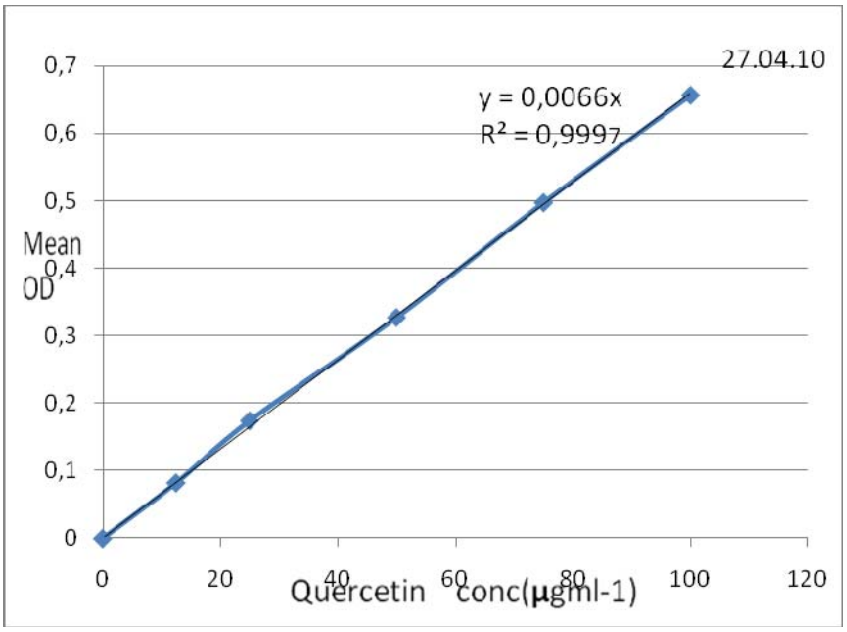


Figure 3.5 Quercetin Standard Curve.

Total flavon values are calculated using the standard curve and expressed in terms of gallic acid equivalent (mg g^{-1}) of dry mass which is lyophilized water extract of *U. dioica*) are given in **Table 3.4.**The experiments were performed in quardublicate.

Table 3.4 Total flavonoid content of *U. dioica* extracts prepared under different conditions, expressed as mg quercetin equivalents per gram extract. The values were the average of four different sets of experiments in duplicates.

Incubation Conditions	**Total flavonoid content (mg quercetin/gextract)**			
Hour / Temperature	**6 Hour**	**12 Hour**	**24 Hour**	**36 Hour**
RT °C	**17.4**±1.78	**28.9**±0.93	**14.5**±1.33	**20.1**±1.33
37 °C	**20.2**±0.92	**24.8**±1.80	**16.1**±1.09	**21.4**±1.13
50 °C	**27.3**±1.07	**12.3**±0.80	**17.4**±0.58	**22.5**±1.09

12H at room temperature macerated *U. dioica* sample has the richest flavonoid content (28.9±0.93mg quercetin/gextract).As a significant change in flavonoid content, a maximum of 2.4 fold change was observed ($p<0.05$) by using one way ANOVA **(Figure 3.6).**

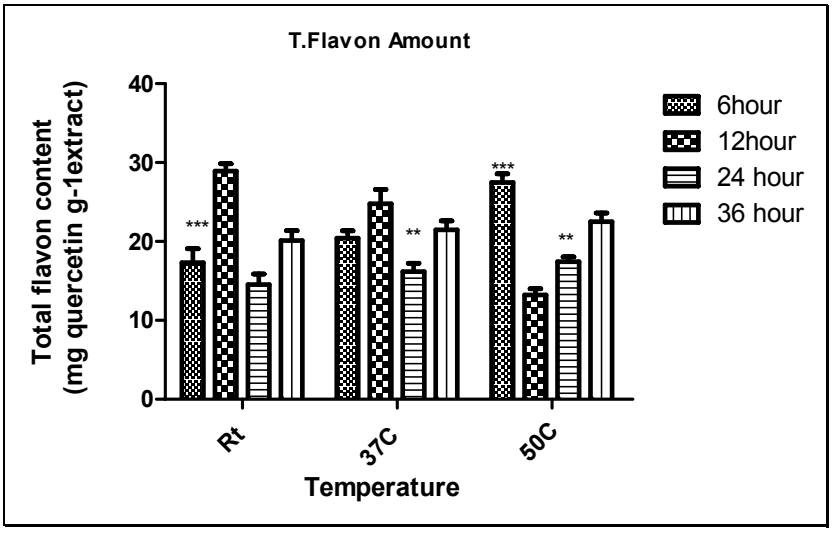


Figure 3.6 The total flavonoid content of *U. dioica* extracts prepared under different conditions. In this graph, * , ** and *** represent statistically significant difference at ($p < 0.05$), ($p < 0.001$) and ($p < 0.01$), respectively. The graph was drawn using Graph Pad Prism program.

3.4 Free Radical Scavenging Activity of *U. dioica* Acidic and Aqueous Extracts

The extraction of soluble active constituents of *U. Dioica* was performed by maceration of dry *U. dioica* leaves in acidified water at pH 2.0. The pH of the extraction water was adjusted to two by using 1 N HCl, and maceration was carried out for 12 h at room temperature. The extracts was separated from the plant material by filtration through filter paper.

The free radical scavenging activity of acidic extract was performed as described before. Samples were analysed in quardublicate. Absorbance values of 0.5-8 mg/mL of concentration of extracts were measured. The Final

concentration vs. Absorbance (571 nm) graph is plotted and Radical Scavenging Activitiy values were calculated are listed in the Table 3.5.

Table 3.5.5 Radical Scavenging Activity (RAS) of acidic and aqueous extracts of *U. dioica dioica* macerated for12 hour at room temperature.

Maceration **Condition** Period / **Temperature**	AQUEOUS Maceration	ACID Maceration
25°C RT 12 Hour	**% 62**	**%66**

Similar Radical Scavenging Activity (RAS) was observed in Acidic and Aqueous Extracts of *U. dioica* at the optimum maceration condition. While the RSA value of 12 hour atroom temperatureaqueous macerated *U. dioica* was62%, that of acid macerated U. dioica was 66%, which is not a statistically significant difference.

3.5. Total Flavonoids and Phenol Content of *U. Dioica* Acidic and Aqueous Extracts.

12H incubation atroom temperaturemacerated *U. dioica* extract have the richest flavanoid content (28.9 ±0.93 mg quercetin) are shown in the Table 3.6.

Table 3.6 Total flavon and phenol content of acidic and aqueous extracts of 12 hour at 25°C, expressed as quercetin and GaE equivalents per gram of extract.The values were the average of four different sets of experiments in duplicates.

Incubation Conditions	Total flavonoid content (mg quercetin/ gextract)		Total phenol content GAE (mg GA gextract)	
12Hour / Temperature	Acid Macerated	Water Macerated	Acid Macerated	Water Macerated
RT °C	**20.9**±0.48	**22.7**±0.32	**77.3** ±1.91	**70.7**±1.46

Extracts of 12 hour at 25°C, expressed as quercetin and GaE equivalents per gram. The values were the average of four different sets of experiments in duplicates. The graph was drawn using Graph Pad Prism program**(Figure 3.7).**

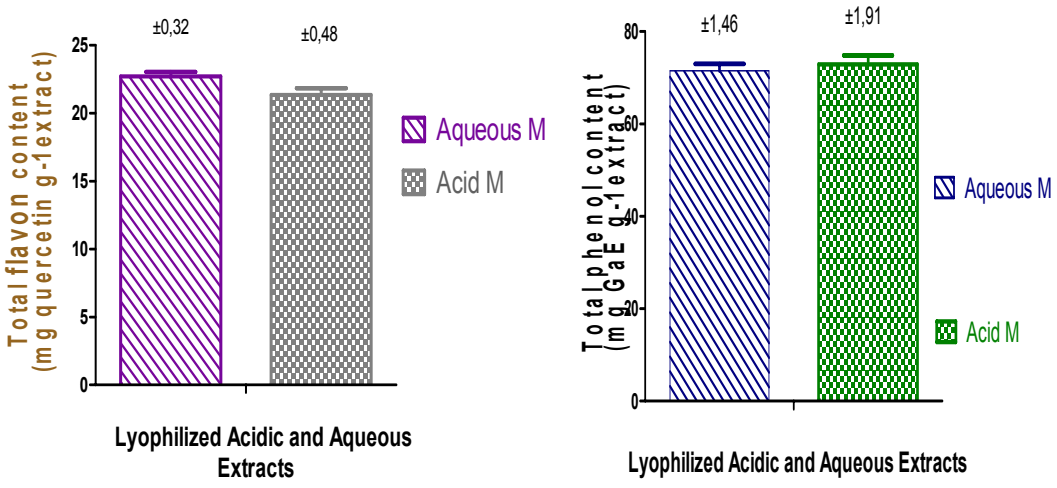


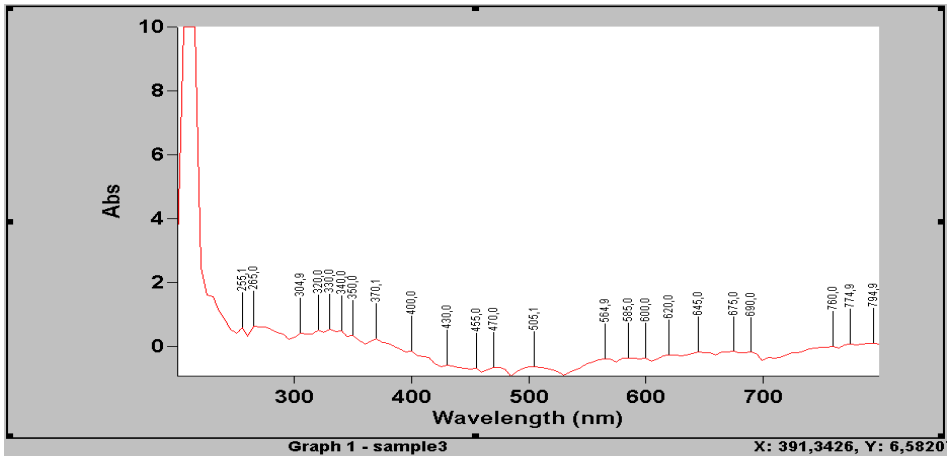
Figure 3.7 Total flavonoid and phenol content of Acid and Aqueous Macerated *U. dioica* extracts

The acid macerated *U. dioica*extracts, having higher phenolic content, with an 77.3±1.91 mg GaE/gextract Total phenol content. A relatively higher flavonoid content (22.7±0.32mg quercetin/gextract) was observed in aqueous *U. dioica* extract obtained from maceration at room temperature for 12 hour. This is slightly higher compared to the acid *U. dioica* extract obtained from maceration at room temperature for 12 hour (20.9±0.48 mg quercetin/gextract).

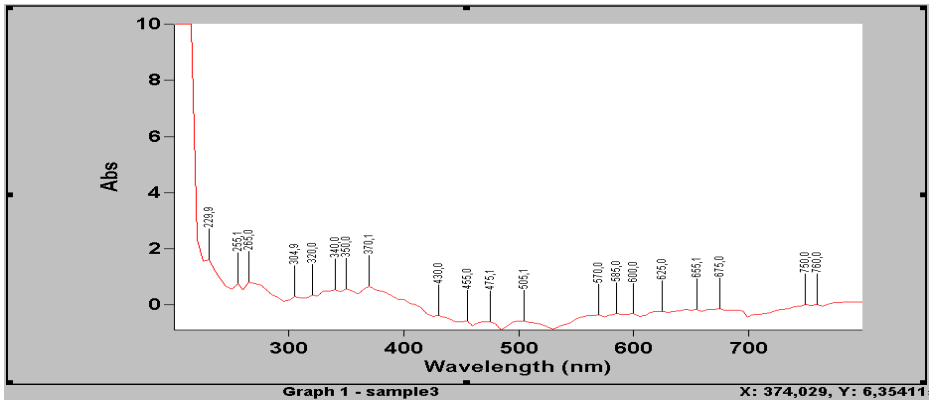
3.6. UV-VIS Spectra of *U. dioica* Extracts

UV-visible light scanning of *U. dioica* at Schimadzu S (250-800) nm were performed and shown in the (Figure 3.8, 3.9,3.10, 3.11).

a)



b)



c)

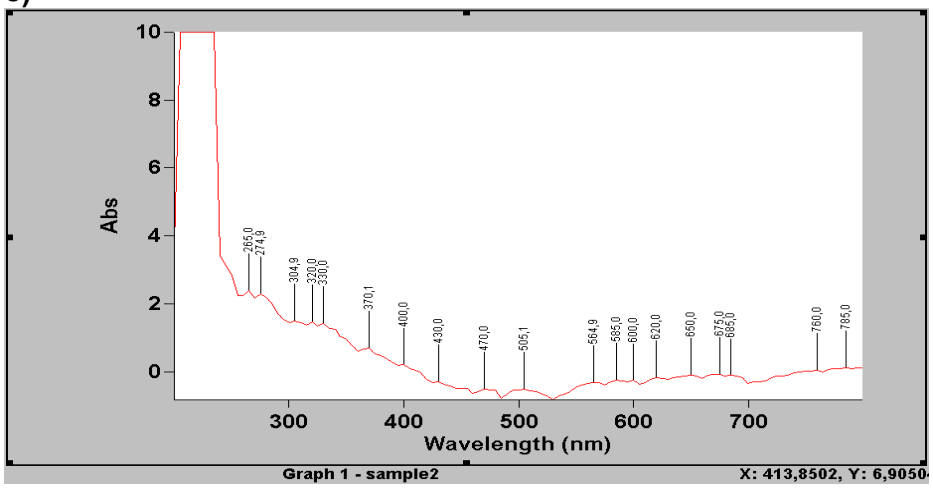
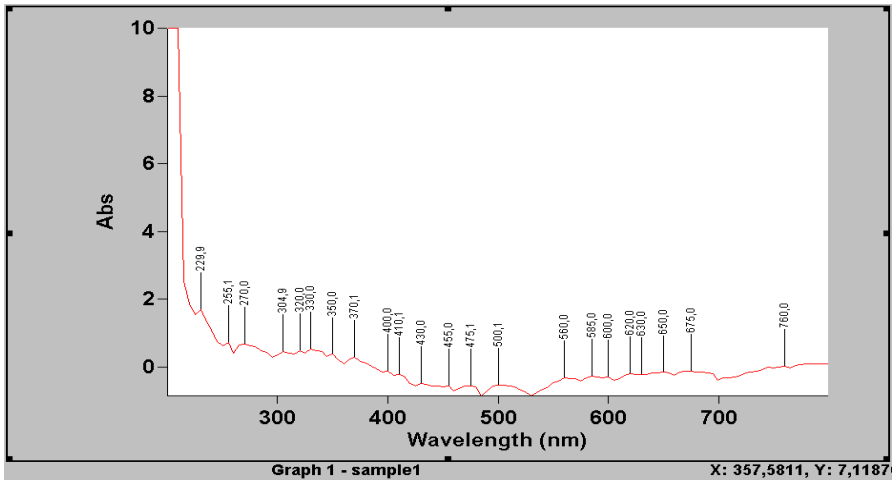
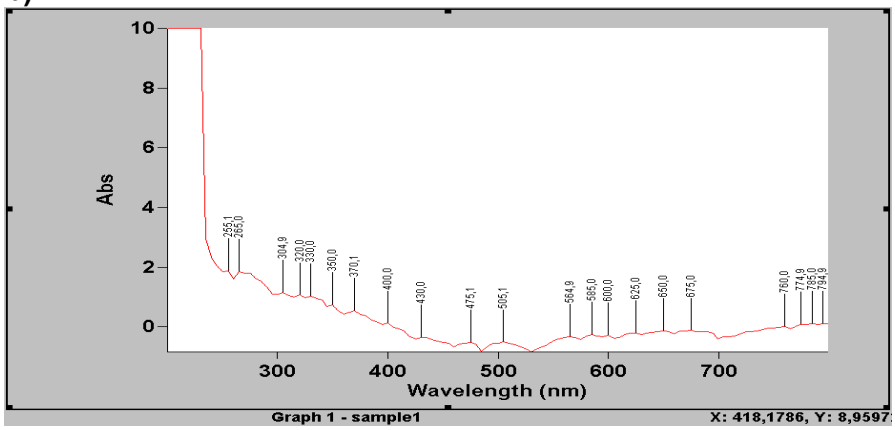


Fig 3.8 Spectra of aqueous extract of a)6 hour at 25°C b)6 hour at 37 °C, c) 6 hour at 50°C macerated *U. dioica.* [1mg extract /mL dH_2O].

a)



b)



c)

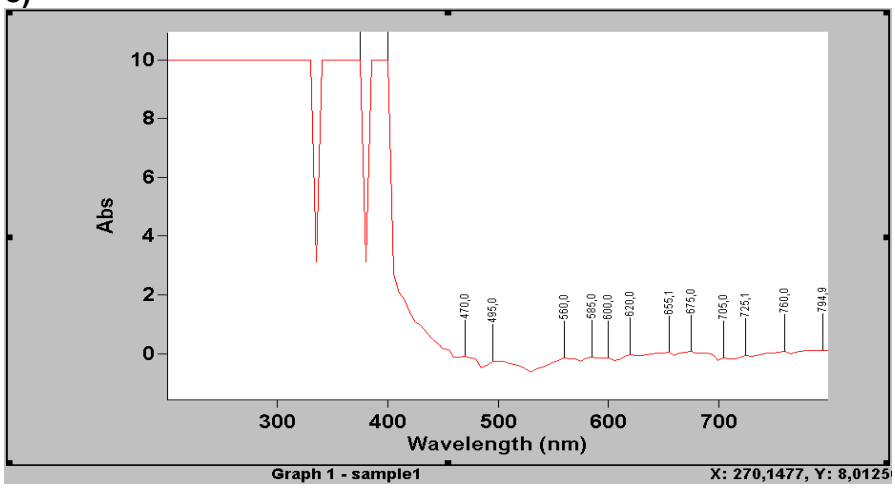


Figure 3.9 Spectra of aqueous extract of a)12 hour at 25°C,b)12 hour at 37°C c)12 hour at 50°C macerated*U. dioica*. [1mg extract /mL dH_2O].

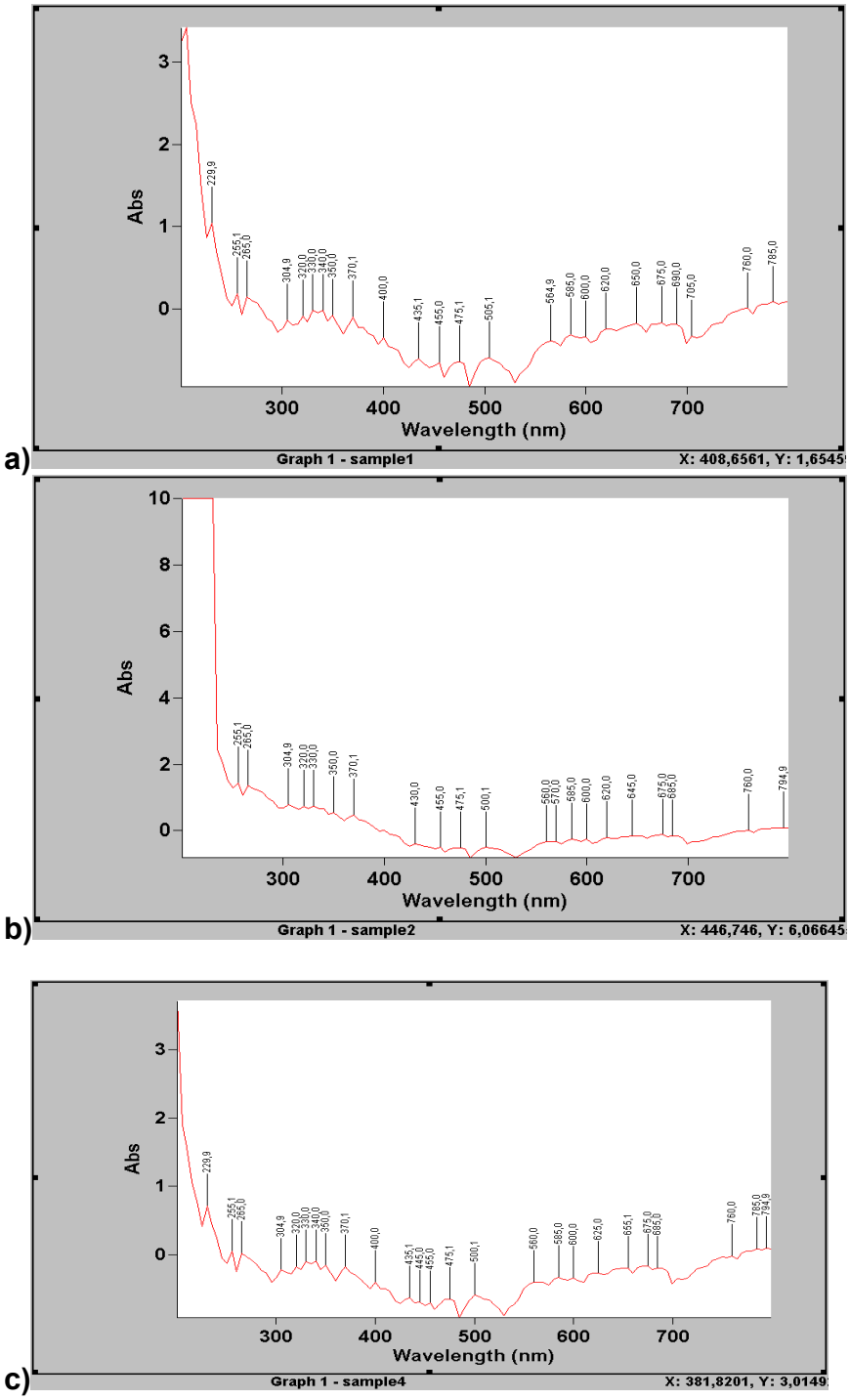


Figure 3.10 Spectra of aqueous extract of a) 24 hour at 25°C [1mg extract /mL dH_2O],b)24 hour at 37°C[0.33mg extract /mL dH_2O], c) 24 hour at 50°C[0.33mg extract /mL dH_2O]macerated *U. dioica*.

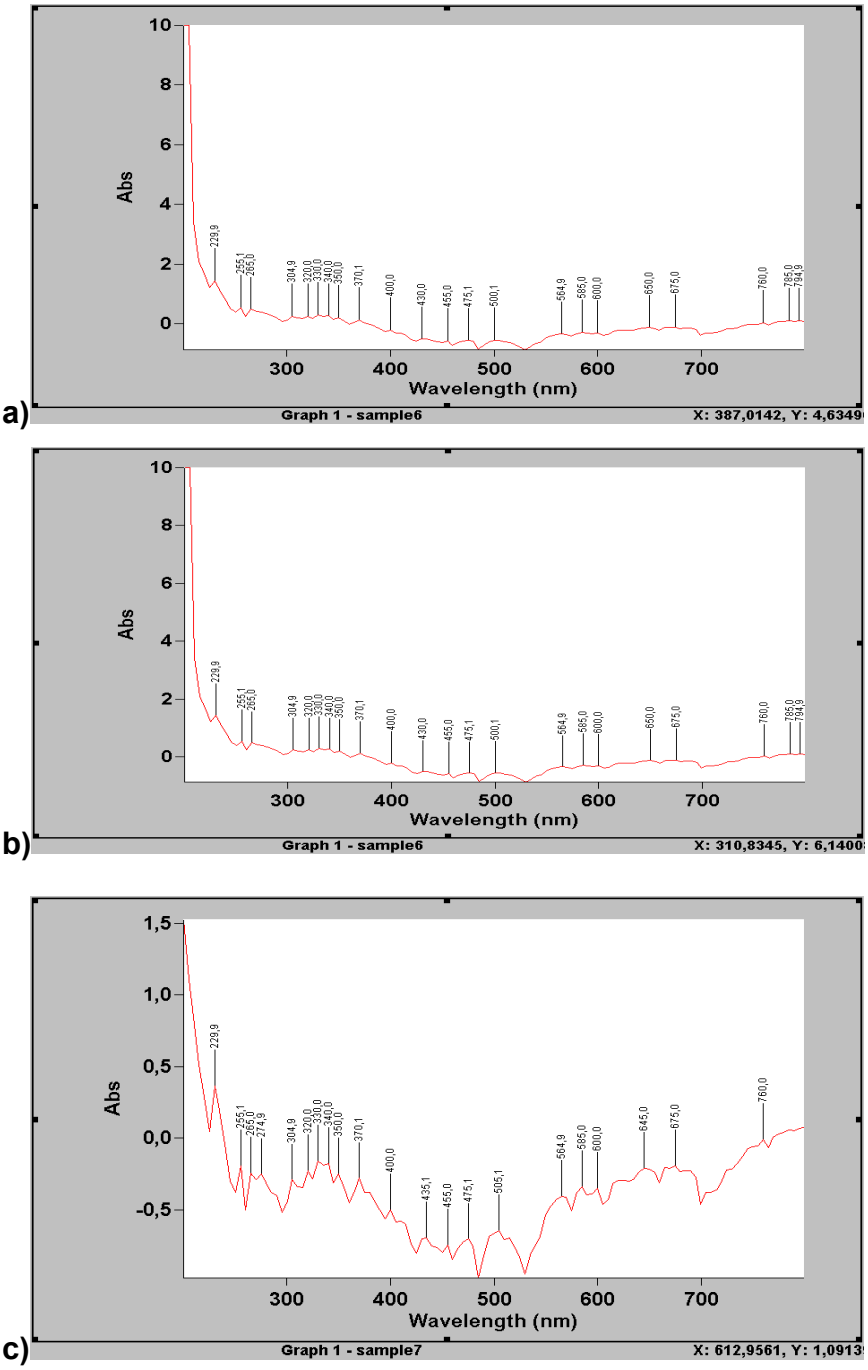


Figure 3.11 Spectra of aqueous extract of a) 36h at 25ºC [0.33mg extract /mL dH_2O], b)36 hour at 37°C [1mg extract /mL dH_2O], c) 36 hour at 50°C [0.33mg extract /mL dH_2O], macerated *U. Dioica.*

UV spectra of phenolics (flavonoids, phenolic acid), lignan and caretonoids found in aqueous solution of *U. dioica*extracts prepared by maceration under different conditions were analyzed from 250 to 800 nm as seen in the **Figure 3.12.**

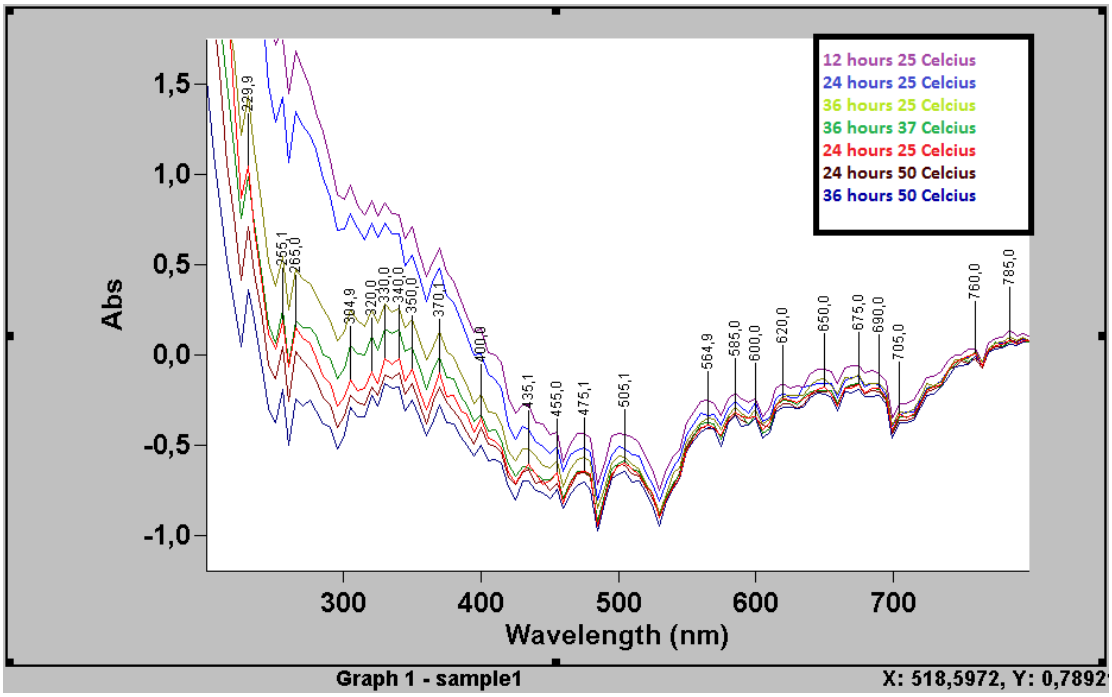


Figure 3.12 Spectra of aqueous extracts of all *U.dioica* samples macerated under varying conditions. Sample concentration was 1mg extract /mL H_2O.

*i)*Bands centered at 280 and 340 nm are presented for phenolic acids group

ii) Bands centered at 226 and 240 nm are presented for phyto-oestrogens

*iii)*The peak at 360 nm confirms flavonols group

*iv)*The peak at 350nm bands carotenoid group.

v) Bands centered at 510 and 530nm are presented for flavonoids.

The results showed that 320 nm band due to the caffeic acid belonging to hydroxycinnamic acid with bands centered at 300 and 340 nm, as chlorogenic acid in same family. Hydroxycinnamic acids which are part of a phenolic acids group are derived from phenolics. The spectrum of exracts of all H_2O macerated

U. dioica samples exhibited bands centered at 226 and 240 nm are presented for secoisolariciresinol from lignan main classes of phyto-oestrogens. The UV data showed that 280nm band due to the gallic acid shows the hydroxybenzoic acid which are part of a phenolic acid in the samples. The presence of quercetin from flavonols group derived from flavonoids in the sample was confirmed by peak at 360 nm. The band at 350nm was shown the presence of 1-Scopoletin. From spectra data, it clearly appears that the peak at 450 nm confirms the carotenoids in *U. dioica.* Anthocyanidins from flavonoids group was due to the peaks between 510 and 530nm.

The following phytochemicals were determined in all aqueous extracts prepared by maceration under different conditions by analyses of their UV-VIS spectra: caffeic acid and chlorogenic acid from the hydroxycinnamic acid subgroup, belonging to phenolic acids group. Gallic acid, which is a hydroxybenzoic acid, is also a phenolic acid.

Anthocyanidin from flavonoids, in addition to the quercetin from the flavonol group of flavonoids were observed. All of these phytochemicals determined from UV- Vis spectral scanning are categorized as phenolics.

3.7 HPLC Analysis of *U. dioica*

Phenolic compounds in *U. dioica* are usually identified by standard HPLC. HPLC analysis of aqueousextract of*U. dioica* prepared by maceration for 12 h at 25°C and fractions prepared by n-butanol and ethyl acetate extraction ofmethanol extract, after chloroform extraction, were carried out in Varian ProStar. 12 hat 25°C *U. dioica*extract (14.4mg/mL) contained 7.10^{-3} mg/mL caffeic acid and 5.10^{-3} mg/mL quercetin as separated using 4.6×150mm pursuit C18 (5 µm) column operating at 35 °C. Neither gallic acid nor the caffeic acid and quercetin were found at detectable levels in Chloroform extract of *U. dioica*.

Chromatographic datawere processed with Chrom-Quest software and shown in (Figures **3.13, 3.14, 3.15, 3.16, 3.17, 3.18, 3.19** and **3.20**).

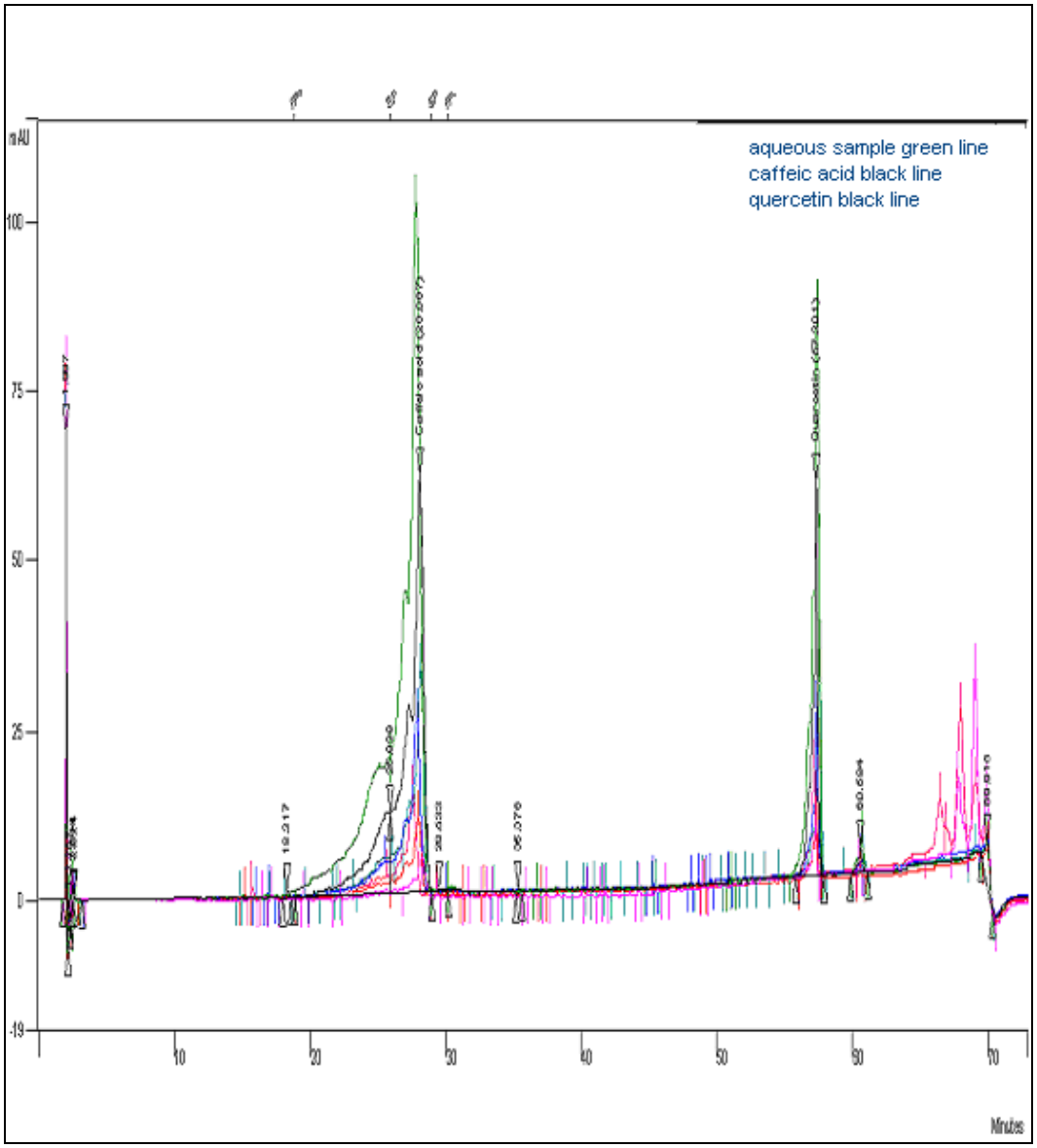


Figure 3.13 HPLC-DAD chromatograms of quercetin (57,301 min)(retention time) and caffeic acid (20,007 min) in 12 hat 25°C aqueous extracts of *U. dioica*. Aqueous macerated sample is represented by green line. Quercetin and caffeic acid are represented by black line.$7x10^{-3}$ mg/mL caffeic acid and $5x10^{-3}$ mg/mL quercetin were determined in the 12 h at 25°C *U. dioica*extract (14.4mg/mL).

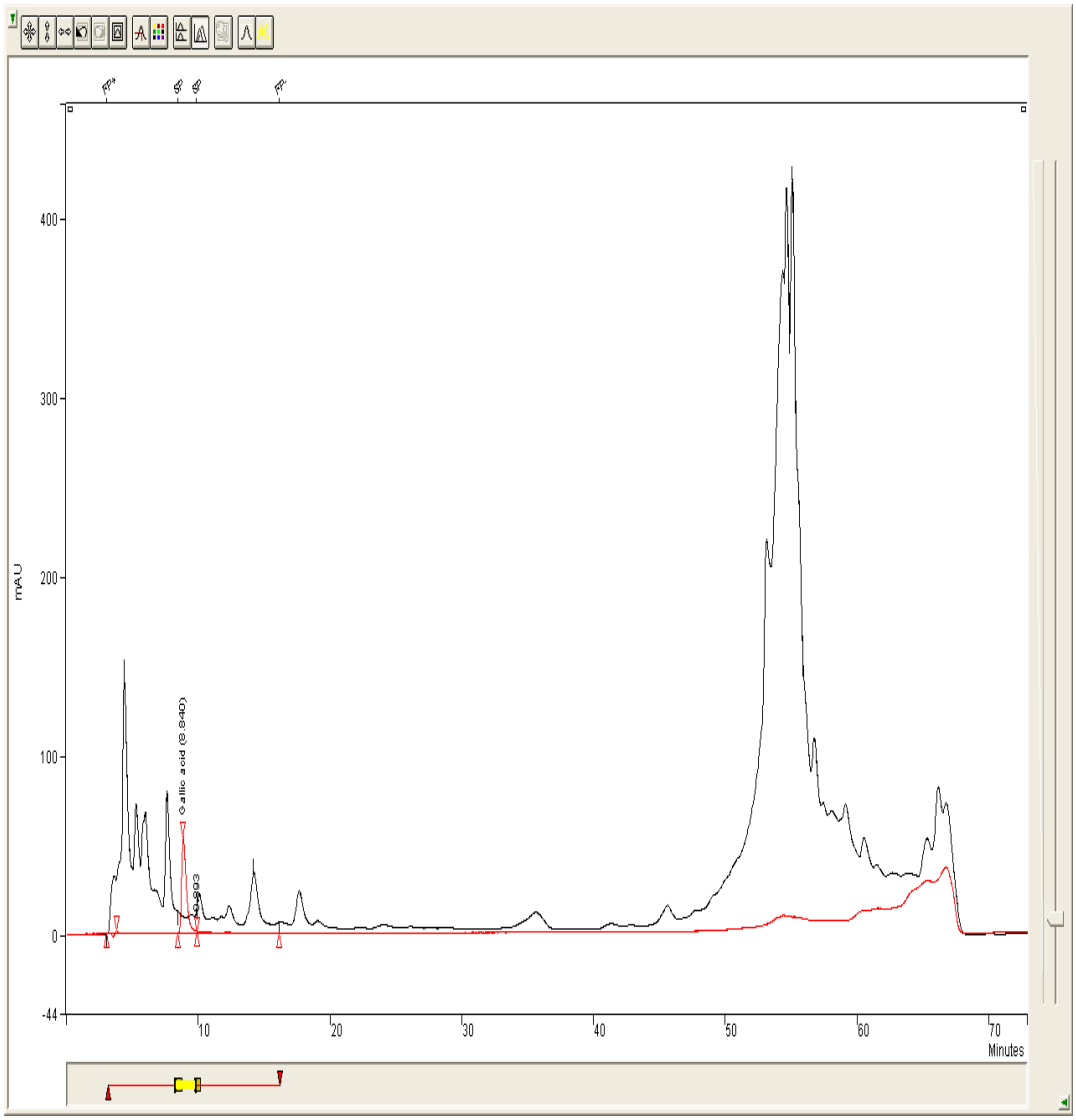


Figure 3.14 HPLC-DAD profiles of gallic acid (0.01 mg/mL) (8,840min)(retention time) standart in aqueous *U. dioica*extractprepared by maceration for 12 h at 25ºC. Gallic acid is represented by red colour, aqueous sample isrepresented by black line.

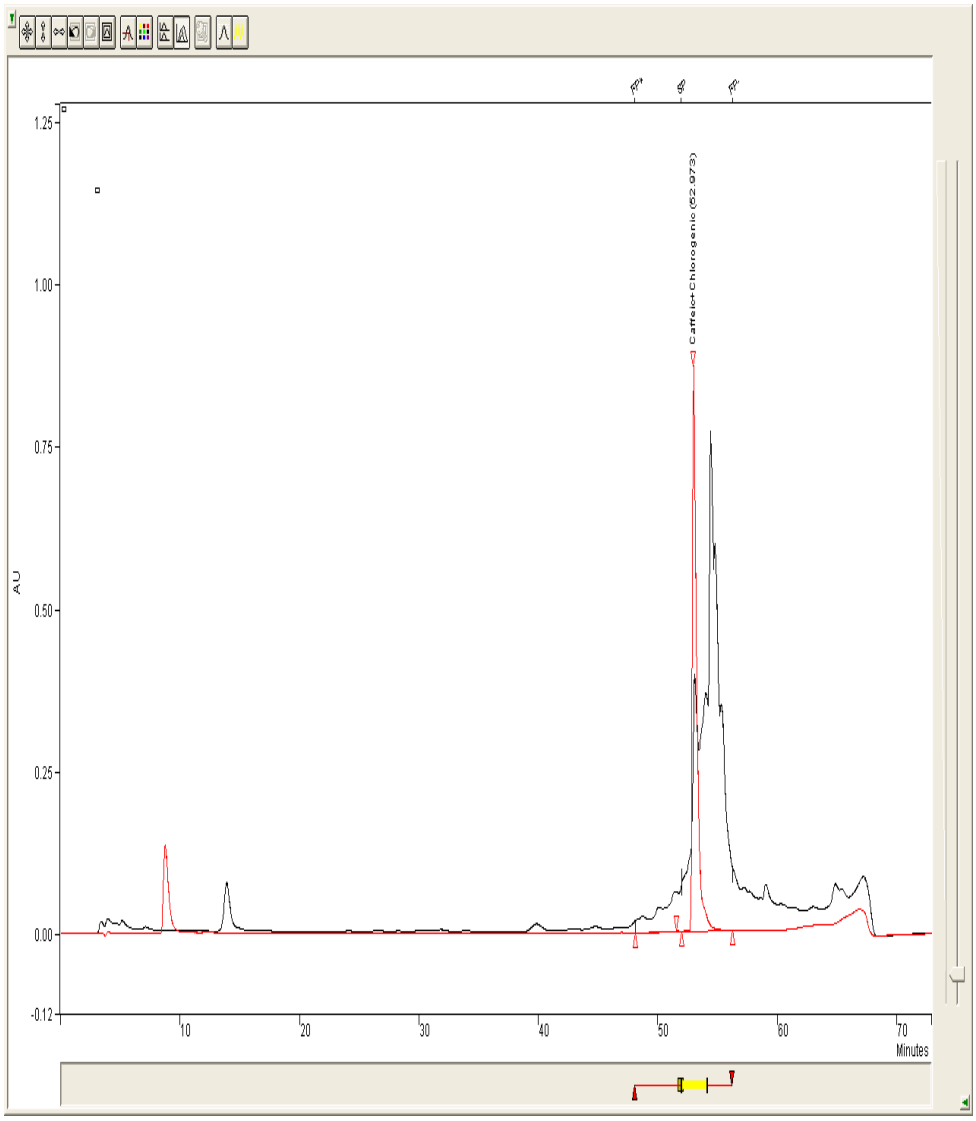


Figure 3.15 HPLC-DAD profiles of Caffeic+ Chlorogenic acid (1 mg/mL) (52,973 min) (retention time) standart in aqueous. Caffeic+ Chlorogenic acid represented by red colour, Aqueous sample represented by black line.

1X10^{-2} mg/mL chlorogenic and caffeic acid was established by aqueous macerated extract of*U. dioica*whereas gallic acid could not be identified in it.

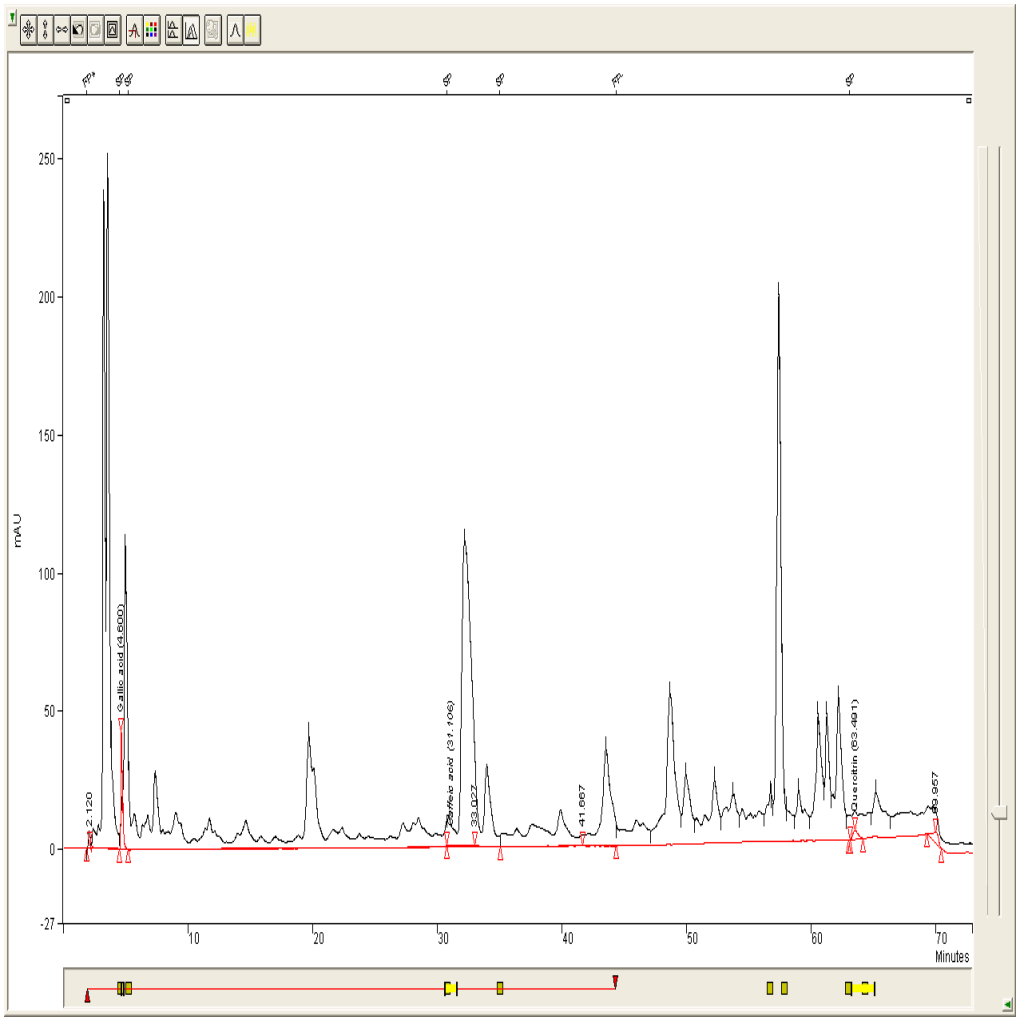


Figure 3.16 HPLC-DAD profiles of *U. dioica* ethyl acetate fraction. Ethyl acetate fraction represented by black line. Standards (gallic acid, caffeic acid and quercetin) represented by red line.

According to the results of HPLC-DAD profiles ofethyl acetate*U. dioica* fraction; Gallic acid (8x 10^{-4}mg/mL) found at lowest level in ethyl acetate fraction of *U. dioica* where ascaffeic acid and quercetin were not determined init.

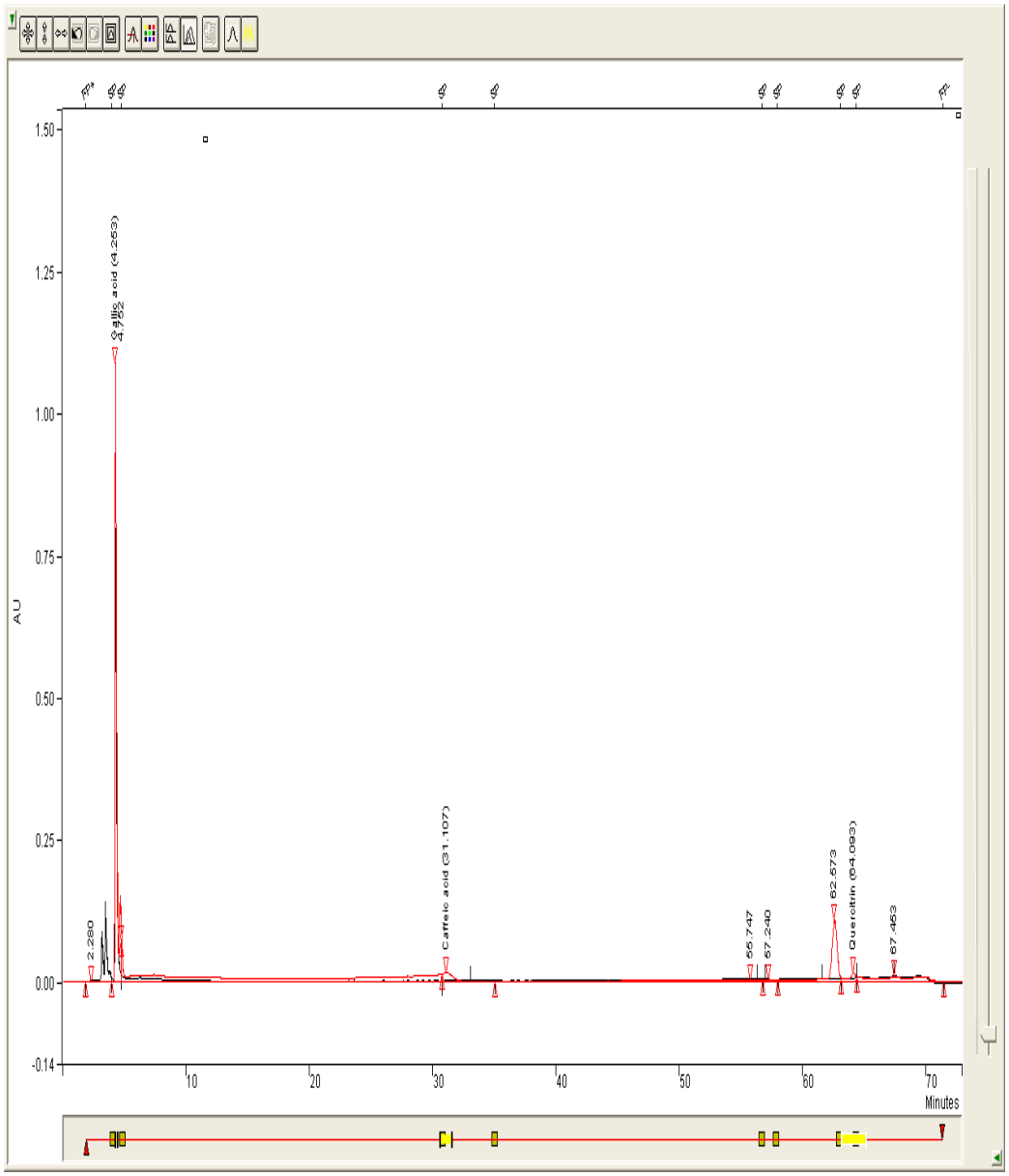


Figure 3.17 HPLC analysis of quercetin (64,003min), caffeic acid (31,107min), 0.05 mg/mL gallic acid (4,253min) in n-butanol fraction of *U. dioica* represented by black colour.

3x 10^{-2}mg/mL Gallic acid was established by n-butanol fraction of *U. dioica.*

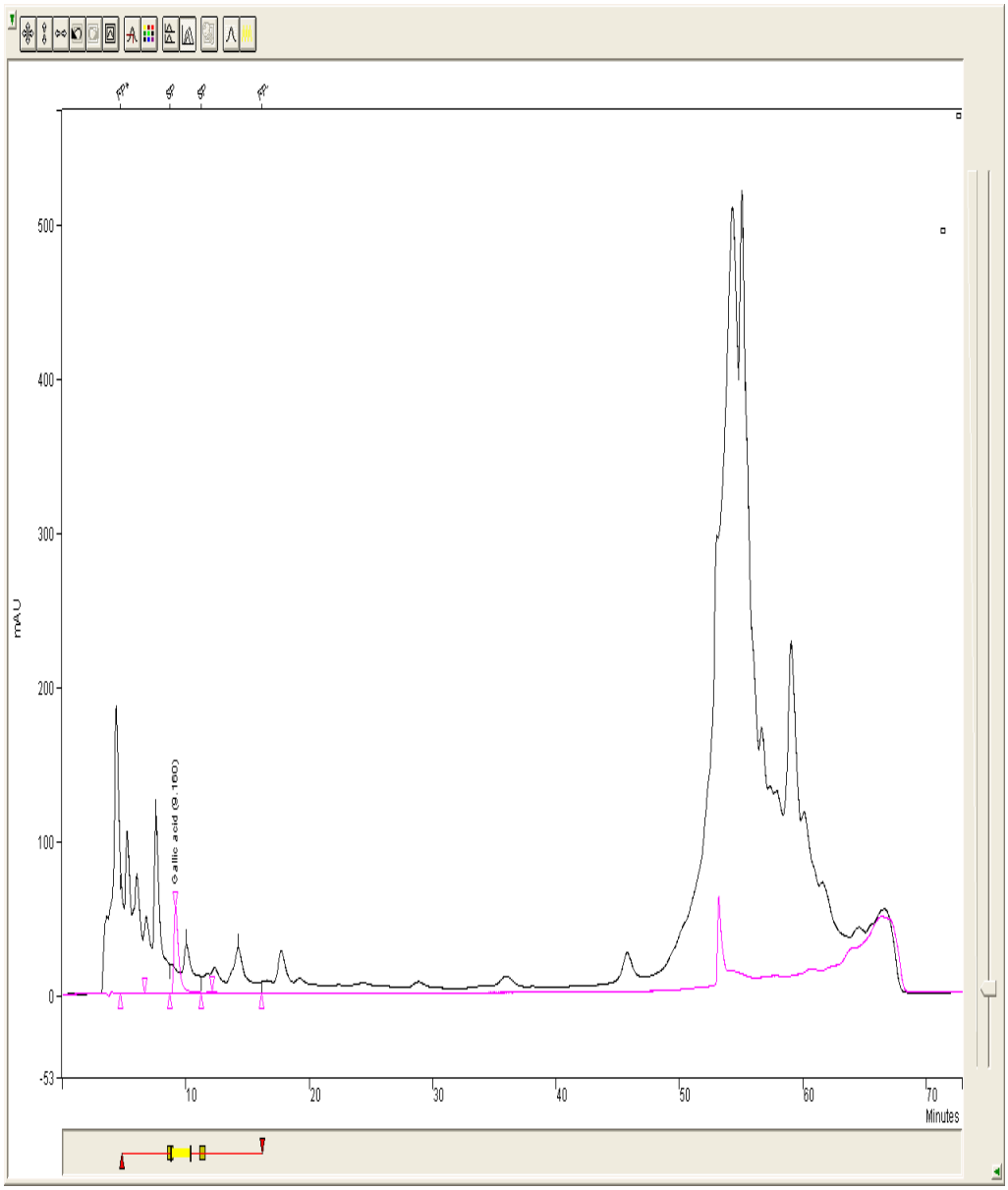


Figure 3.18 HPLC-DAD profiles of gallic acid (0.01 mg/mL) (0.100min)(retention time) standart in acid macerated *U. dioica* extract. Gallic acid represented by pink colour, Acid Macerated sample represented by black line.

Quercetin and Gallic acid were not determined in the acid macerated *U. Dioica* fraction.

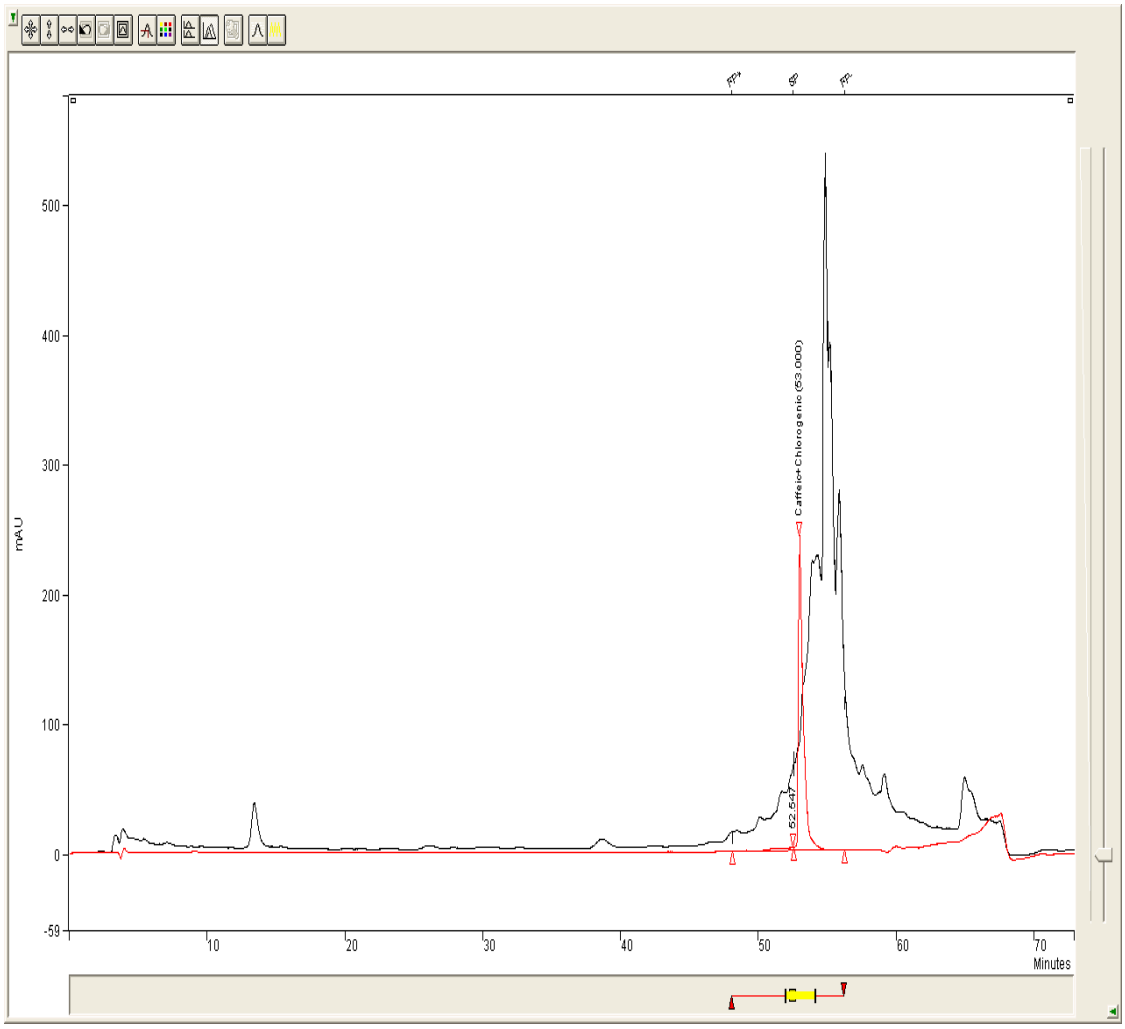


Figure 3.19 HPLC-DAD profiles of Caffeic+Chlorogenic acid (0.05 mg/mL) (53,000 min)(retention time) standart in Acid macerated *U. dioica* extract. Caffeic+Chlorogenic acid represented by red colour, Acid Macerated sample represented by black line.

Caffeic and Chlorogenic acid were at 1.9X 10^{-2} mg/mL in acid macerated extract of *U. dioica.*

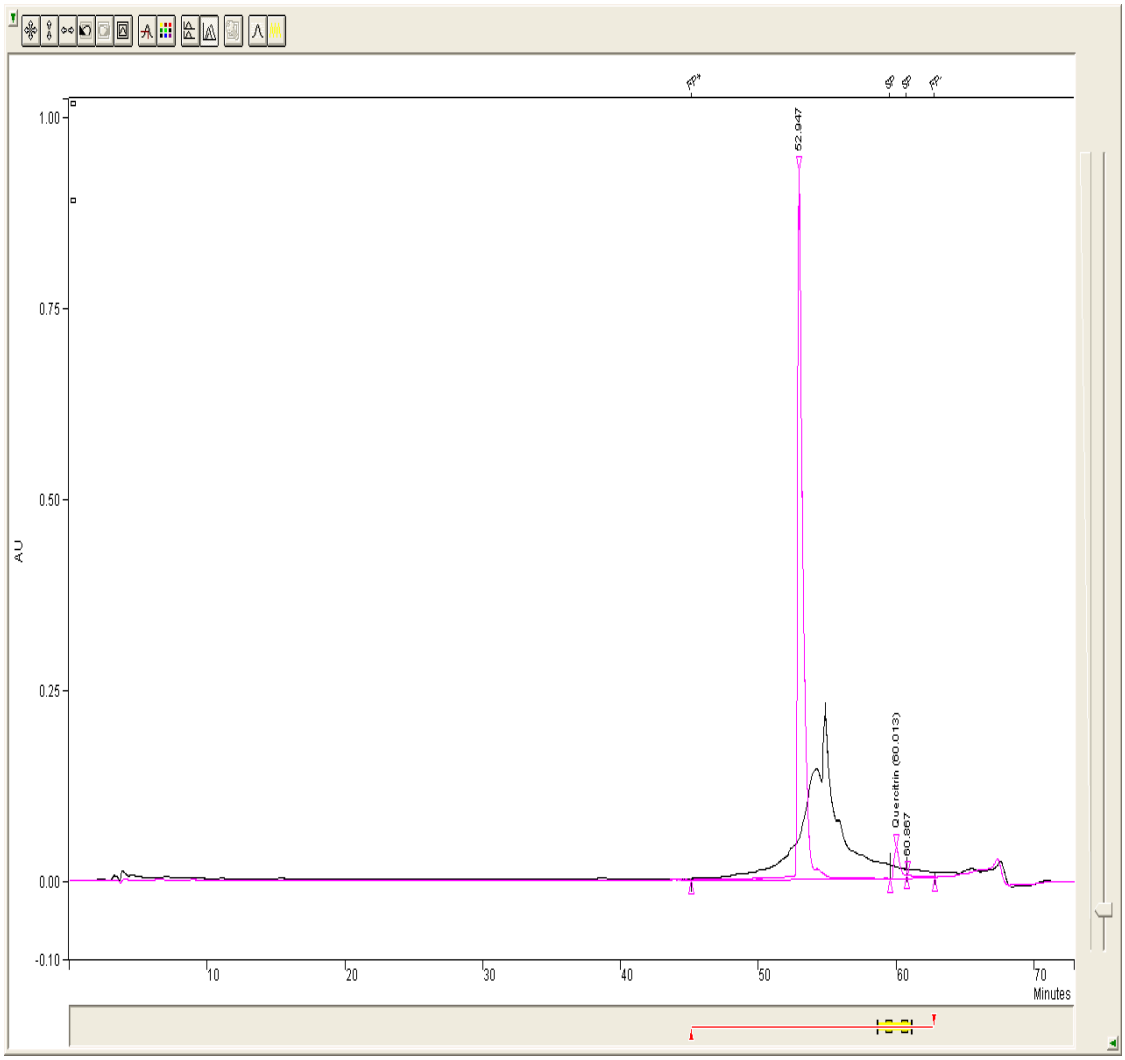


Figure 3.20 HPLC-DAD profiles of Quercetin (0.5 mg/mL) standart in Acid macerated *U. dioica* extract. Quercetin(60,013min)(retention time), represented by pink colour, Acid Macerated sample represented by black line.

3.8 XTT Cell Cytotoxicity Assay

Cytotoxic assays and determination of IC_{50} doses of aqueous extract of *U. dioica* in MCF-7 and MDA-231 cells were performed by using and XTT assay as indicated in manufacturers' instructions.

Untreated MCF-7 and MDA-231 cells were considered as control groups and compared to the treated cells with aqueous extracts of *Urtica dioica*and grouped into numerical stages Table 3.7.

Table 3.7 Effects of *U. dioica* on Viability of MCF-7 and MDA-231 cells were treated with varying concentration

Urtica dioica Concentration (µg/mL) in well	MCF-7 Cell Viability (%)	MDA-231 Cell Viability (%)
5,7	91	95
14,6	85	75
19,5	70	57
29,2	63	43
49	29	40
73	22	35

Inhibitory concentration (IC_{50} doses) value is the *Urtica dioica* concentration at which 50% of cells are viable were calculated from the XTT cell cytotoxicity assay, IC_{50} was as calculated IC_{50}: 34µg/mL for MCF-7 cells in 48 th hour as seen in **Figure 3.21.**

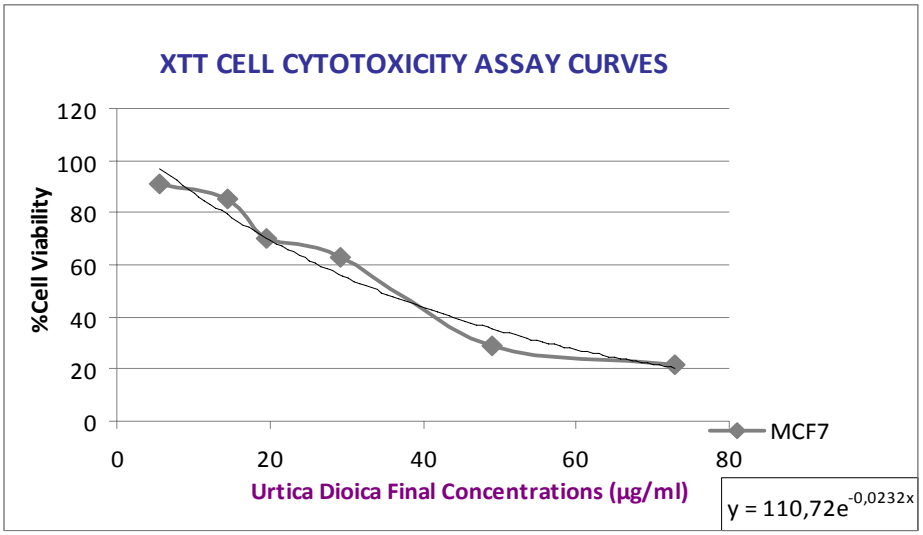


Figure3.21 Effects of varying *U. dioica* concentration on viability of MCF-7 cells which were treated with *U. dioica* for 48 hours.

Increasing the concentration of *U. dioica*aqueous extract up to 29,2µg/mL was observed to decrease MDA-231 cell viability to 43%. However, further increase of extract concentration (up to 49 and 73µg/mL) did not cause a marked decrease in percent cell viability. Since percent viable MDA-231 cell was not reduced below 35% with respect to untreated cells,in order to prevent misinterpretations, IC_{50}value of *U. dioica* extract was not cosidered as a proper indicator of cytotoxic dose of the extracts in this cell line **(Figure 3.22).**

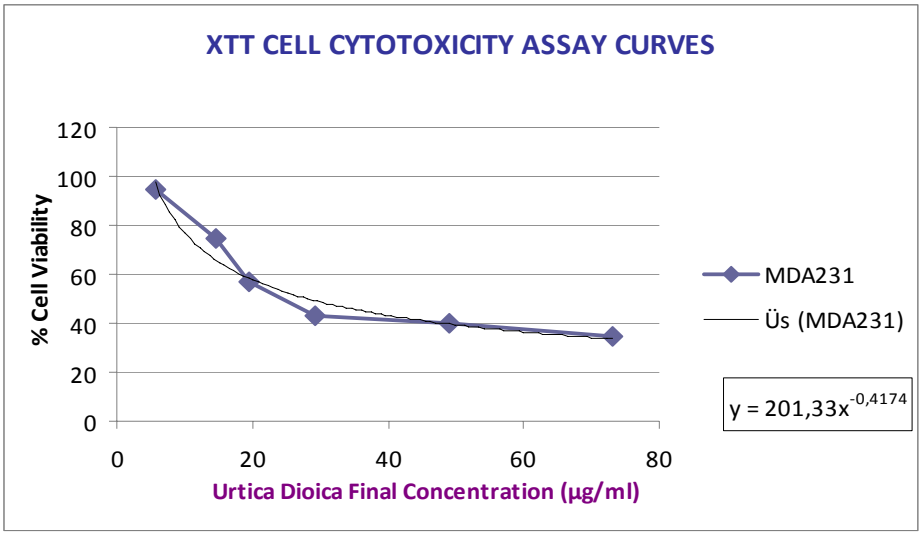


Figure 3.22 Effects of varying *U. dioica* concentration on viability of MDA-231 cells which were treated with *U. dioica* for 48 hours.

It was reported by Sebnem et al. (2005) that U. *dioica* water extract was investigated for cytotoxic activity against KB, B16, HeLa and HLA tumor cell lines. Cells were incubated with the extract in the concentration range 800–1 µg/mL for 48 h and the cytotoxicity was determined using the MTT assay. Since the extract did not affect cell viability in any tested concentrations, Immunomodulatory activity was proposed to be responsible for the anticancer usage of the plant (Harput et al.,2005).

3.9. Effects of *U. dioica* on GST Enzyme Activity

The effects of *U. dioica*extracts on glutathione-S-transferase (GST) activities were also investigated along with their antioxidant capacities. Plant extracts with high phenolic content are known to have important effects on various enzymes,

as well as glutathione-S-transferases, which are important detoxification enzymes in phase II systems with an important role in developing multi-drug resistance to chemotherapy in tumour cells. The effectiveness of many clinically useful anticancer drugs can be severely limited by drug resistance, which appears to be intrinsic to some tumors but can also arise during multiple courses of chemotherapy (Waxman et al., 1990).

The *U. dioica* extracts were used within 0.00–2.4 mg/mL final concentrations in order to calculate the percent inhibition of GST activity and respective IC_{50} values in vitro. The average value of Glutathione S-transferases (GSTs) activities in the cytosolic fractions prepared from sheep liver was calculated as 0.75 µmoles/min/mg protein.The *U. dioica* concentration was increased (up to 1.6 mg/mL) in the reaction medium, the sheep liver cytosolic GST activity decreased to about 50%. The IC50 values of GST Inhibition were determined from the percent specific activityversus final extract concentration curve shown in **Figure 3.23**.*U. dioica* extracts have shown effective inhibition of cytosolic GST activity, with an IC50 of 1.38 mg/mL.

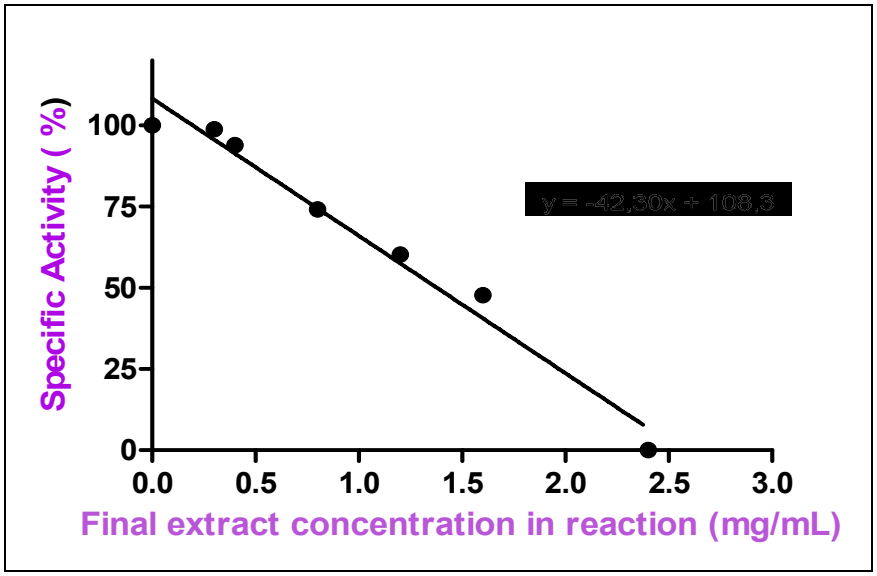


Figure 3.23 Effects of *U. dioica* aqueous extract on sheep liver cytosolic GST.

GST activity was increased about 100% with respect to 2.4 mg/mL final concentration, while it was decreased at higher *U. dioica*concentrations. The decrease at higher *U. dioica*concentrations, might be the depletion of intracellular GSH in resulting in drop in GST activity. Plant extracts with high polyphenols are known to have important inhibitory effects on glutathione-S-transferases in the literature (Coruh et al.,2007). *U. dioica* extracts which have higherphenolics content at higher doses, are also expected to be more effectivein GST inhibition was shown in Figure3.23.

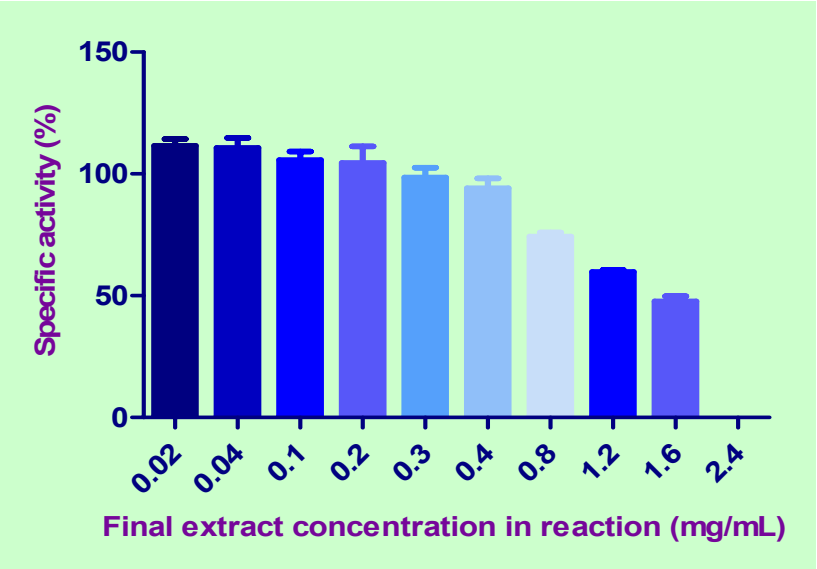


Figure 3.24 Effects of varying concentrations of *U. dioica* extract on sheep liver cytosolic GST specific activity using CDNB as substrate. Each data point was obtained by making at least 6 independent measurements.

It was seen that the increase in GST activity at low *U. dioica*concentration might be the protective response of cells against ROS generation. GSTs through covalent modification of an essential amino acid, probably a cysteine residue situated at the active site of the enzyme. It is probable that these compounds act by one or more of the following mechanisms: depletion of intracellular GSH; and

inactivation of cellular GSTs, perhaps including those involved in prostaglandin and/or leukotriene biosynthesis, by covalent modification of the transferase itself; or competition with the alkylating agent for metabolism at the GST active site.

3.10 Comparision of % RSA and total phenolic and flavon content of *U. dioica*and its effect on GST activity and inhibitory effects on MCF-7 cell line

Acid extract of *U. dioica*has higher Total PhenolAmount than its water extract. In contrast, DPPH-RSA, acid extract are lower than water extractwere provided in Table 3.8. At first glance, it can be thought as a contradiction. Peroxidation is a chain reaction which can be initiated by a reactive radical abstracting an electron from a nonradical. Thus, a radical is transformed to a nonradical. However, simultaneously a new radical can be formed and so reaction can continue. In the presence of phenolic compounds, hydrogen with an electron can be donated from phenolics, thus radical can be scavenged. Because of the resonance stability, newly formed phenoxy radical is more stable than former radical. Thus, chain reactions can be retarded. Acid extract contains more apolar compounds than water extract. Decrease in the polarity of a compound causes an increase of solubility of the compound in the apolar phase in which peroxidation occur.

Table 3.8 Comparision of % DPPH radical scavenging activitiy, and total phenolic and flavonoid content of *U. dioica,* acid and aqueous extract and their effect on GST activity and inhibitory effects on MCF-7 and MDA-231 cell line.

Maceration Condition ; 25°C ,12 hour	**Total Phenol (mg GAE/ gextract)**	**Total Flavon (mg / quercetin gextract)**	**DPPH scavenging IC_{50}(mg/mL)**	**GST Inhibition IC_{50}(µg/mL)**	**XTT (MCF7& MDA231) (µg/mL)**
Aqueous macerated UD	70.7±1.46	22.7±0.32	0,30	1.38	34
Acid macerated UD	77.3 ±1.91	20.9±0.48	0,37	ND	ND

DPPH scavenging **IC_{50}**:concentration of plant extracts for 50 percent of DPPH scavenging, Total Flavon: Total Flavoncontent mg equivalents of Quercetin/gof plant extract. Total Phenol: Total Phenolics content mg equivalents of GAE/ gof plant extract. GST inhibiton **IC_{50}**: concentration of plant extracts for 50 percent inhibiton of GST activity. Inhibitory concentration of XTT IC50 doses; the value is the *U. dioica*concentration at which 50% of cells are viable were calculated from the XTT cell cytotoxicity assay. ND: not determined.

Furthermore, significant correlations existed between phenols and flavonoids and free radical scavenging activity. The relevance of these findings discussed here, humans who consume *U. dioica* at their diet by 12 hour at room temperaturewith have more phenolics phytochemicals might benefit from their more antioxidant and antiproliferative effects.

CHAPTER 4

CONCLUSION

The data clearly outlining the richest phenolic and flavonoid compounds is 12 hour incubation at room temperature macerated *U. dioica* extract and 6 hour 50^{o}C macerated *U. dioica* samples.

The highest RSA was shown by 12 hour at room temperature macerated *U. dioica* with IC_{50}=0.24 mg mL^{-1} and %65 RSA value.

It was concluded that stomach acidity does not lead to loss in radical scavenging activity, total phenol and flavon contentof *U.dioica.*

Cytotoxicity of *U.dioica* on MCF-7 cells were calculated as IC50 value of 34µg/mL.

It was observed that the GST enzyme activity was decreased significantly in the presence of up to 1.6 mg/mL *U. Dioica* extracts with respect to control. The IC_{50} value of *U. Dioica* was calculated as1.38mg/mL extract, *in vitro.*

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APPENDIX A

COLLECTION DATA OF*URTICA DIOICA L*

Erzurum: Tortum to Narman, Kireçli pass, 40°20′528″N-41°41′788″E, 2344 m, 27.7.2008.

Erzurum: Şenkaya, 40°33′713″N-42°20′951″E, 1922 m, 27.7.2008.

Ankara: Eymir, 39° 49', 27'' N 32° 49' 39'' E

Ardahan: Ardahan to Göle, 41°07′215″N-42°48′361″E, 1800 m, 27.7.2008.

Kars: Sarıkamış, near Karakurt village, 40°09′910″N-42°36′543″E, 1463 m, 29.7.2008.

Kayseri: near Sarız, 38°26′150″N-36°40′058″E, 1800 m, 23.7.2008.

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