

***IN VITRO* REGENERATION, EVALUATION OF BIOLOGICAL
ACTIVITIES AND DETERMINATION OF PHENOLIC COMPOUNDS IN
WILD STRAWBERRY (*FRAGARIA VESCA* L.)**

ARZU BİRİNCİ YILDIRIM

JULY 2012

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by
ARZU BİRİNCİ YILDIRIM

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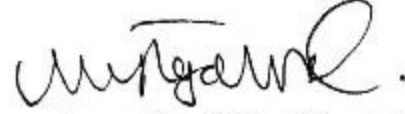
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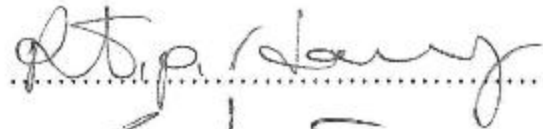


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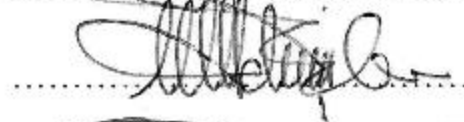
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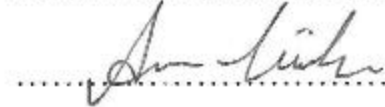
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ABSTRACT

***IN VITRO* REGENERATION, EVALUATION OF BIOLOGICAL ACTIVITIES AND DETERMINATION OF PHENOLIC COMPOUNDS IN WILD STRAWBERRY (*FRAGARIA VESCA* L.)**

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Fragaria vesca L. is a perennial herb in the family Rosaceae that has been used in the treatment of liver and kidney complaints, as well as in the treatment of rheumatism in folk medicine. A highly efficient *in vitro* culture protocol of *F. vesca* was developed via multiple shoot formation. Leaf and petiole explants obtained from field-grown plants and sterile seedlings were cultured on MS medium containing different concentrations and combinations of various plant growth regulators and regeneration enhancers (silver nitrate, tween 20, tween 80 or pluronic F-68). Shoot regeneration was observed with explants obtained from sterile seedlings. However, field-grown plant materials were not responsive for shoot regeneration. The regeneration efficiency was found to be higher in leaf explants than petiole explants. Supplementing media with pluronic F-68 significantly increased shoot number and frequency of shoot formation. Among leaf explants, the best shoot proliferation was obtained with 1.0 mg/l TDZ in combination with 0.5 mg/l IBA and 0.1 % pluronic. Among petiole explants, best shoot proliferation was obtained with 1.5 mg/l TDZ in combination with 0.5 mg/l IBA and 0.1% pluronic. Regenerated shoot

were rooted on MS medium containing different concentration of IAA, IBA, 2,4-D or NAA. Best root formation was observed with MS basal medium (control). Comparing various auxins for rooting, IBA and IAA were more effective. After rooting, plantlets were transferred to soil for hardening.

The phenolic contents in the methanolic extracts of field-grown leaves, fruits and *in vitro*-grown leaves and callus of *F. vesca* were analysed using LC-ESI-MS/MS. According to the results, concentrations of phenolic compounds in field-grown leaf extracts (NFM and NM) were at least five times higher than in other extracts. Five different biological assays (antibacterial, anti-tumor, toxicity, anticancer and antioxidant activity) were used for assessing the biological activities of *F.vesca* extracts. Extracts obtained from field-grown leaves and fruits indicated better antibacterial activity than extracts of *in vitro* grown leaves and callus against *E. cloacae*. The best antitumor activity was observed with aqueous (NW) and methanolic (NM) extracts of field-grown leaves and hot ethanolic extract (FFHE) of fresh fruit via potato disc diffusion assay. In brine shrimp toxicity assay, extracts of field-grown leaves and fruits were more toxic than in the extracts of *in vitro*-grown leaves and callus. The best anticancer activity on MCF-7 cell lines was observed with extracts of field-grown leaves and fruits. Furthermore, the free radical scavenging potency of methanolic extracts of *F. vesca* was determined with DPPH method. The total phenolic and flavonoid contents were also investigated.

Keywords: *Fragaria vesca* L., *In vitro* regeneration, LC-ESI-MS/MS, Biological activities, Antibacterial, Antioxidant, Toxicity, Antitumor, Anticancer.

ÖZET

DAĞ ÇİLEĞİNDE (*FRAGARIA VESCA* L.) *IN VITRO* REJENERASYON, BİYOLOJİK AKTİVİTE DEĞERLENDİRİLMESİ VE FENOLİK BİLEŞİKLERİN BELİRLENMESİ

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Fragaria vesca L., geleneksel tıpta karaciğer ve böbrek rahatsızlıklarının tedavisinde ve ayrıca romatizma tedavisinde kullanılan gülgiller ailesine ait çok yıllık bir bitkidir. Çoklu sürgün oluşumu yoluyla, yüksek verimli bir *in vitro* kültür yöntemi geliştirilmiştir. Doğal ortamda yetişen bitkiden ve steril fideden alınan yaprak ve yaprak sapı eksplantları, farklı konsantrasyonlarda ve kombinasyonlarda bitki büyüme düzenleyicisi ve rejenerasyon arttırıcı (gümüş nitrat, tween 20, tween 80 ve pluronic F-68) içeren MS ortamlarında kültüre alınmıştır.

Sürgün oluşumu steril fideden alınan eksplantlarla elde edilmiştir. Bununla birlikte, doğal ortamda yetişen bitki materyalleri sürgün oluşumunda etkili olmamıştır. Rejenerasyon verimi, yaprak eksplantlarında, yaprak sapı eksplantlarına göre daha yüksek bulunmuştur. Ortama pluronic F-68 eklenmesi, sürgün sayısını ve sürgün oluşum frekansını arttırmıştır. Yaprak eksplantları arasında, en iyi sürgün gelişimi 1.0 mg/l TDZ'nin, 0.5 mg/l IBA ve % 0.1 pluronic ile kombinasyonundan elde edilmiştir. Yaprak sapı eksplantlarında ise, en iyi sürgün gelişimi 1.5 mg/l TDZ'nin, 0.5 mg/l IBA ve % 0.1 pluronic ile kombinasyonundan elde edilmiştir.

Rejenere olmuş sürgünler, IAA, IBA, 2,4-D veya NAA' nın farklı konsantrasyonlarını içeren MS ortamında köklendirilmiştir. En iyi kök oluşumu, MS (kontrol) ortamında gözlenmiştir. Köklendirme için kullanılan oksin çeşitlerini kendi içinde karşılaştırsak, IBA ve IAA köklendirmede daha etkili olmuştur. Köklendirme işleminden sonra, elde edilen bitkicikler toprağa aktarılmıştır.

LC-ESI-MS/MS yöntemi kullanılarak, doğal ortamda yetişen bitkilerin, meyvelerin ve *in vitro* yetişen bitki ve kallusların metanolik ekstraktlarının fenolik içerikleri analiz edilmiştir. Sonuçlara göre, doğal ortamda yetişen bitki ekstraktlarında (NFM ve NM) fenolik bileşiklerin konsantrasyonu, diğer ekstraktlara göre en az beş kat daha yüksek bulunmuştur. *F. vesca* ekstrelerinin biyolojik aktivitelerinin değerlendirilmesi için, beş farklı biyolojik test (antibakteriyal, anti-tümör, toksisite, antikanser ve antioksidant aktivite) kullanılmıştır. Doğal ortamda yetişen yaprak ve meyvelerden elde edilen ekstreler, *in vitro* yetişen yaprak ve kallus ekstrelerine göre, *E. cloacae*'ya karşı daha iyi antibakteriyal aktivite göstermiştir. En iyi antitümör aktivite ise, patates disk difüzyon yöntemi kullanılarak, doğal ortamda yetişen yaprakların sulu (NW) ve metanollü (NM) ekstreleri ve taze meyvelerin sıcak etanollü ekstreleri ile gözlenmiştir. Tuzlu su karidesi toksisite testinde, doğal ortamda yetişen yaprak ve meyvelerden elde edilen ekstreler, *in vitro* yetişen yaprak ve kallus ekstrelerine göre daha toksik bulunmuştur. En iyi antikanser aktivite ise, MCF-7 hücreleri üzerinde doğal ortamda yetişen yaprakların ve meyvelerin ekstreleri ile gözlenmiştir. Ayrıca, *F. vesca* bitkisinin metanolik ekstrelerinin serbest radikal sönmüleme potansiyeli, DPPH metodu ile belirlenmiştir. Toplam fenolik ve flavonoid içerikleri de araştırılmıştır.

Anahtar kelimeler: *Fragaria vesca* L., *In vitro* rejenerasyon, LC-ESI-MS/MS, Biyolojik aktivite, Antibakteriyal, Antioksidant, Toksisite, Antitümör.

To my lovely daughter Defne and my husband Muhammet,

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TABLE OF CONTENTS

ABSTRACT	iii
ÖZET	v
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	ix
LIST OF TABLES	xiv
LIST OF FIGURES	xvi
ABBREVIATIONS	xv
CHAPTER	
1. INTRODUCTION AND LITERATURE REVIEW	1
1.1. Introduction	1
1.2. Literature Review	3
1.2.1. Botany	3
1.2.2. Folkloric usage of <i>F. vesca</i>	3
1.2.3. Active constituents of <i>F. vesca</i>	5
1.2.4. Biological activity studies of <i>F. vesca</i>	7
1.2.5. Biotechnological studies of <i>F. vesca</i>	9
1.2.6. Strawberry cultivars	10
1.3. Objectives of the Study	12
2. <i>IN VITRO</i> CULTURE OF <i>FRAGARIA VESCA</i>	13
2.1. Introduction	13
2.2. Materials and Methods	16
2.3. Results and Discussion	21
2.3.1. Field-grown plants as explant source	22
2.3.1. Sterile seedlings obtained from germinated seeds as explant source	23
3. PLANT EXTRACTION LC-ESI-MS/MS ANALYSES OF THE EXTRACTS	44
3.1. Plant Extraction	44
3.1.1. Introduction	44
3.1.2. Plant Materials and Methods.....	47
3.1.2.1. Direct extraction method	48
3.1.2.2. Gradual extraction method (starting from non-polar to polar solvent)	48
3.2. LC-ESI-MS/MS Analysis of the Methanolic Extracts for Phenolic Compounds.....	54
3.2.1. Introduction	54
3.2.2. Materials and Methods	57
3.2.2.1. Standard phenolic compounds	57
3.2.2.2. Method of LC-ESI-MS/MS analysis	59
3.2.3. Results and Discussion	60

4. BIOLOGICAL ACTIVITY STUDIES	68
4.1. Introduction	68
4.1.1. Antibacterial Activity	69
4.1.2. Antitumor Activity	72
4.1.3. Brine Shrimp Toxicity Assay	73
4.1.4. Anticancer Activity	75
4.1.5. Antioxidant Activity	77
4.2. Materials and Methods	80
4.2.1. Antibacterial Assay	80
4.2.2. Anti-tumor Assay	82
4.2.2.1. Bacterial Viability Testing	84
4.2.3. Brine Shrimp Toxicity Test	84
4.2.4. Anticancer Assay	86
4.2.5. Antioxidant Assay	88
4.2.5.1. Free radical scavenging activity (DPPH (2,2-diphenyl-picrylhydrazil) method)	88
4.2.5.2. Total phenolic assay (Folin-ciocalteau method for total phenolic content)	89
4.2.5.3. Total flavonoid assay (Aluminium chloride (AlCl ₃) colorimetric assay for total flavonoids)	90
4.3. Materials and Methods	91
4.3.1. Antibacterial Activity	91
4.3.2. Anti-tumor Activity	102
4.3.3. Brine Shrimp Toxicity	108
4.3.4. Anticancer Activity	112
4.3.5. Antioxidant Activity	119
4.3.5.1. Free radical scavenging activity-DPPH (2,2-diphenyl-1-picrylhydrazil) method	119
4.3.5.2. Total phenolic assay (Folin-ciocalteau method for total phenolic content)	124
4.3.5.3. Total flavonoid assay (Aluminium chloride (AlCl ₃) colorimetric assay for total flavonoids)	125
5. CONCLUSIONS	130
REFERENCES	138
APPENDICES.....	152

LIST OF TABLES

Table

1.1.	Geographical origin of the cultivars cultivated in Turkey.	11
2.1.	Effects of different concentrations and combinations of TDZ and IBA supplemented with or without GA ₃ or AgNO ₃ on shoot regeneration	34
2.2.	Effects of different concentrations and combinations of surfactants (Pluronic F-68, Tween 20 and Tween 80) in combination with TDZ and IBA on shoot regeneration	35
2.3.	Effects of different concentrations and combinations of TDZ and 2,4-D supplemented with or without GA ₃ , AgNO ₃ or surfactant (PluronicF-68) on shoot regeneration	36
2.4.	Effects of different concentrations and combinations of BA and IBA supplemented with GA ₃ , AgNO ₃ or surfactant (PluronicF-68) on shoot Regeneration	37
2.5.	Effects of the tested auxins on root formation from regenerated shoots ...	39
3.1.	Designation of extracts in bioassays, used plant materials and their extraction procedures and yields	50
3.2.	Designations of the extracts in the text	53
3.3.	Main classes of phenolic compounds in higher plants	55
3.4.	Identified Phenolic Compounds and Their Amounts in the Methanolic Extracts of Fruit, Field-grown plant, <i>In vitro</i> -grown plant and callus	64
4.1.	Bacteria used in antibacterial assay and their representative diseases, habitats and treatments	71
4.2.	Antibacterial activity of <i>F.vesca</i> extracts and controls (antibiotics) against used bacteria	96
4.3.	Mean number of tumors observed with extracts of <i>F. vesca</i> extracts	105
4.4.	LC ₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of <i>F. vesca</i> methanolic extracts and MS-222 (a positive control) ..	110

4.5.	The percentage of cell viability of different extracts from <i>F. vesca</i> L. on the human breast adenocarcinoma (MCF-7) cell line for 24h	116
4.6.	The percentage of cell viability of different extracts from <i>F. vesca</i> L. on the human breast adenocarcinoma (MCF-7) cell line for 48h	117
4.7.	Free-radical (DPPH●) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, <i>in vitro</i> grown plant and callus from <i>F.vesca</i> and ascorbic acid	121
4.8.	Total phenol and total flavonoid contents of methanolic extracts of <i>F. vesca</i> L	128

LIST OF FIGURES

Figure

1.1.	Pictures of <i>Fragaria vesca</i> L.	6
2.1.	<i>In vitro</i> culture of <i>Fragaria vesca</i> L.	20
2.2.	Effects of different concentrations and combinations of TDZ and IBA supplemented with or without Pluronic F-68 (Pl) on shoot regeneration	38
2.3.	Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots	40
2.4.	Callus formation in leaf explant of <i>F. vesca</i> on MS medium containing 0.5 mg/l TDZ + 0.1 mg/l 2,4-D	41
2.5.	Shoot regeneration of leaf explant of <i>F. vesca</i> on MS medium containing 1.5 mg/l TDZ + 0.5 mg/l IBA + 0.1% Pluronic F-68	42
2.6.	Rooting of the regenerated shoots on medium containing 0.5 mg/l IBA	43
2.7.	Regenerated plants in Magenta containers including vermiculate for acclimatization; Regenerated plants transferred to plastic pots containing sterile potting soil under growth room conditions	43
3.1.	Detection of natural products by LC-MS/MS system in ESI mode	57
3.2.	Phenolic compounds used as standard in LC-ESI-MS/MS analyses	59
3.3.	Total Ion Chromatogram (TIC) for the mixture of 22 authentic standards by LC-ESI-MS/MS analysis	65
3.4.	Total Ion Chromatogram (TIC) of CFM extract in comparion with those of standards by LC-ESI-MS/MS analysis	66
3.5.	Total Ion Chromatogram (TIC) of NFM extract in comparion with those of standards by LC-ESI-MS/MS analysis	67
4.1.	Antibacterial activity of <i>F. vesca</i> extracts and controls (antibiotics) against <i>S. aureus</i> and <i>S. epidermidis</i>	97

4.2.	Antibacterial activity of <i>F. vesca</i> extracts and controls (antibiotics) against <i>S. pyogenes</i> and <i>E. cloaceae</i>	98
4.3.	Antibacterial activity of NFW and FFW (gradually prepared aqueous extracts of field-grown plant and fruit) extracts and NA (acetone extract of field grown plant) extract against <i>E. cloaceae</i> by comparing with positive control (ampicillin).....	99
4.4.	Antibacterial activity of ILM (methanolic extract of <i>in vitro</i> -grown plant) extract and NFM and FFM (methanolic extracts of field grown plant and fruit) extracts against <i>E. cloaceae</i> by comparing with positive control (erythromycin)	100
4.5.	Antibacterial activities of NA (left) and NM (acetone and methanolic extracts of field grown plant) against <i>S. epidermidis</i> by comparing with positive controls (tetracycline and ampicillin)	101
4.6.	The percentage of tumor inhibition of <i>F. vesca</i> extracts and controls ...	106
4.7.	Tumors produced by <i>A. tumefaciens</i> with NW (aqueous extract of field-grown plants) and its closed version	107
4.8.	Tumors produced by <i>A. tumefaciens</i> with distilled water (control I) and camptothecin (positive control)	107
4.9.	LC ₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of <i>F. vesca</i> methanolic extracts and MS-222 (positive control)..	110
4.10.	LC ₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of <i>F. vesca</i> methanolic extracts	111
4.11.	The dose-response curves of the effects of gradually prepared extract of field-grown leaves on cell growth in MCF-7 cells for 48 h	118
4.12.	The dose-response curves of the effects of gradually prepared extract of fruits on cell growth in MCF-7 cells for 48 h	118
4.13.	Free-radical (DPPH [•]) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, <i>in vitro</i> grown plant and callus from <i>F.vesca</i> and ascorbic acid (positive control)	122
4.14.	Free-radical (DPPH [•]) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, <i>in vitro</i> grown plant and callus from <i>F.vesca</i> and ascorbic acid (positive control)	123
4.15.	Calibration curve for gallic acid standards	124
4.16.	Calibration curve for catechol standards	126
4.17.	Total phenol and flavonoid contents of methanolic extracts of <i>F.vesca</i>	129

ABBREVIATIONS

2,4-D: 2,4-dichlorophenoxyacetic acid

AgNO₃: Silver nitrate

AlCl₃: Aluminium chloride

ANOVA : Analysis of Variance

APCI: Atmospheric Pressure Chemical Ionization

BA : Benzyladenine

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CC: Column Chromatography

CE: Catechol equivalent

CFD: Gradually prepared dichloromethane extract of *in vitro*-grown callus

CFH: Gradually prepared hexane extract of *in vitro*-grown callus

CFM: Gradually prepared methanol extract of *in vitro*-grown callus

cfu: colony forming unit

CFW: Gradually prepared water extract of *in vitro*-grown callus

CM: Methanol extract of *in vitro*-grown callus

DFCE: Cold ethanol extract of dry fruit

DFCW: Cold water extract of dry fruit

DFHE: Hot ethanol extract of dry fruit

DFHW: Hot water extract of dry fruit

FFCE: Cold ethanol extract of fresh fruit

DMSO : Dimethyl Sulfoxide

DPPH : 2,2-diphenyl-1-picrylhydrazil

ESI: Electrospray Ionization

FCC: Flash Column Chromatography

FFCW: Cold water extract of fresh fruit

FFHE: Hot ethanol extract of fresh fruit

FFHW: Hot water extract of fresh fruit

FFD: Gradually prepared dichloromethane extract of fruit

FFH: Gradually prepared hexane extract of fruit

FFM: Gradually prepared methanol extract of fruit

FFW: Gradually prepared water extract of fruit

GA3 : Gibberellic acid

GAE : Gallic acid equivalent

GC-MS: Gas Chromatography-Mass Spectrometry

HPLC: High-Performance Liquid Chromatography

IAA : Indole-3-acetic acid

IBA : Indole-3-butyric acid

ILM: Methanol extract of *in vitro*-grown plant

KIN : Kinetin

LC: Liquid Chromatography

LC-ESI-MS/MS: Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

LC-MS/MS : High Performance Liquid Chromatography – Mass Spectroscopy

LC-NMR: Liquid Chromatography-Nuclear Magnetic Resonance

MS : Murashige and Skoog's Basal Salt

MS: Mass Specrometry

NA: Acetone extract of field-grown plant

Na₂CO₃: Sodium carbonate

NAA : Naphthalene acetic acid

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NaNO₂: Sodium nitrite

NaOH: Soidum hydroxide

NE: Ethanol extract of field-grown plant

NFD: Gradually prepared dichloromethane extract of field-grown plant

NFH: Gradually prepared hexane extract of field-grown plant

NFM: Gradually prepared methanol extract of field-grown plant

NFW: Gradually prepared water extract of field-grown plant

NM: Methanol extract of field-grown plant

nm: nanometer

NW: Water extract of field-grown plant

PTLC: Preparative Thin-Layer Chromatography

TDZ : Thidiazuron

TEAC: Trolox equivalent antioxidant capacity

TLC: Thin-Layer Chromatography

TRAP: Total radical-trapping antioxidant parameter

UV-VIS: Ultraviolet-visible

WHO: World Health Organization

CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Fragaria vesca L. is a low-growing perennial herb in the family Rosaceae, which grows in meadows, woods and along roadsides (Davis, 1972; Grieve, 1982; Dobelis, 1990; Chevallier, 1996). *F. vesca*, commonly called wild strawberries or woodland strawberry, is a plant that grows in the temperate to subtropical zones of the Northern hemisphere, exclusive of the tropics (Grieve, 1982; Heywood, 2007). It is native to Europe and temperate regions of Asia. (Davis, 1972; Hancock, 1999; Marta et al., 2004). In Turkey, it is found in North West of Turkey and North, South and East parts of Anatolia (Davis, 1972). Other names for this species include Alpine Strawberry, Fraises des Bois, Wild Strawberry and European Strawberry. It is called as Dağ çileği, Orman çileği and Yabani çilek in Turkish. *Fragaria* species are represented by three different species, namely *F. vesca* L., *F. viridis* Duchesne and *F. X ananassa* Duchesne in Turkey (Davis, 1972).

In 1966, there were 11 recognized species in the genus *Fragaria* (Darrow, 1996), however, in the 1990s, recognized species number reached approximately 15 (Hancock, 1990) and at the end of 1999, totally 19 species of genus *Fragaria* were recognized (Hancock, 1999). The present *Fragaria* taxonomy includes 20 named wild species and many hybrids and cultivars (Hummer et al., 2011). The taxonomy and phylogenetic relationships of the Rosaceae is complex

and difficult due to size of the family, its range of morphological diversity, and the role of hybridization, polyploidy and agamospermy. This diversity has been recognized by its division into four subfamilies: Rosoideae, Spiraeoideae, Maloideae and Amygdaloideae, primarily diagnosed by the structure of the fruits (Heywood et al., 2007). Strawberry, genus *Fragaria*, is a member of the Rosoideae subfamily and includes 20 named wild species. Some of them are *F. viridis* Duch, *F. chiloensis* (L.) Miller, *F. X ananassa* Duch and *F. vesca* L. (Hummer et al., 2011). Therewithal, the taxonomic classification of the genus is still evolving (Munger, 2006). *Fragaria* species exist as a natural polyploidy series from diploid through decaploid. There are seven basic types of chromosomes that they all have in common. However, they exhibit different polyploidy. *F. vesca* is a diploid ($2n=2x=14$, having two sets of the seven chromosomes). Others are tetraploid ($2n=4x=28$ chromosomes total), hexaploid ($2n=6x=42$ chromosomes), octoploid ($2n=8x=56$ chromosomes), or decaploid ($2n=10x=70$ chromosomes) (Hummer et al., 2011).

Evidence from archaeological excavations suggests that *F. vesca* has been consumed by humans since the Stone Age. The woodland strawberry was first cultivated in ancient Persia where farmers knew the fruit as Toot Farangi. Its seeds were later taken along the Silk Road towards the far East and to Europe where it was widely cultivated until the 18th century, when it began to be replaced by the garden strawberry, (*Fragaria X ananassa*), which has much larger fruit and showed greater variation, making them better suited for further breeding (Grieve, 1982; Turhan and Kargi, 2007) .

1.2. Literature Review

1.2.1. Botany

F. vesca produces scaly rhizome and long, slender trailing stolons (runners). It spreads by runners. It has small and scarlet berries in the form of cone-shaped. Its seeds (or achenes) uniformly scattered over the fruits, and projecting from the receptacle (Grieve, 1982). *F. vesca* has a fragrance and flavor more delicate even than the cultivated strawberry. It prefers a slightly sheltered position, and being very small, considerable labor goes to the collection of its fruit Great Britain. (Grieve, 1982). Leaves and fruits of *F. vesca* are gathered in early summer (Chevallier, 1996).

Basal leaves of *F. vesca* are in a rosette and trifoliate. The leaflets are ovate, obovate with straight, prominent veins, sparsely hairy and no individual leaf stalks. Leaflets are 1-6 cm. Scape is (5-) 10-30 cm, usually longer than leaves. *F. vesca* is in flower from April to July. It produces 2-7 white flowers. Flower is about 12-18 mm diameter and has five petals. It is usually hermaphrodite (has both male and female organs). Sepals are 3-4 mm, lanceolate- cuspidate, slightly longer than epicalyx. Segments are patent or deflexed in fruit. Petals are white, 4-5 mm, obovate or rounded. Receptacles are red, glabrous, in fruit up to 1 cm. (Davis, 1972).

1.2.2. Folkloric Usages of *F. vesca*

The berries, leaves, and roots of *F. vesca* have all been used medicinally in the past. The roots of *F. vesca* were once a popular household remedy for diarrhea and the stalks were remedy for wounds. They are also used as laxative, diuretic, astringent, antidiarrheic, antiseptic and antirheumatismal (Lamari et al., 2008)

Antioxidant properties have recently been discovered in the fruit, making them a valuable preventive for cancer. The leaves are gently astringent. One can make a tea with the leaves for diarrhea, digestive upsets, and to stimulate the appetite (Alpine strawberry, 2008). In traditional phytotherapy, leaves of wild strawberry are used as diuretic and in gastrointestinal and skin diseases. Both the leaves and the fruit of *F. vesca* were in early pharmacopoeias, though the leaves were mostly used. Strawberry vitamins are of value in sprue which is inflammation of the lining of the small intestine. Culpepper declares the plant to be “Singularly good for the healing of many ills,” but Linnaeus was the first to discover and prove the efficacy of the berries as a cure for rheumatic gout (Grieve, 1982). The leaves of *F. vesca* have the same property, and a tea made from them checks dysentery. The stalks of *F. vesca* only entered into the composition of the once-famous Antioch drink and vulnerary (Grieve, 1982).

The strawberry is a useful dentifrice and cosmetic. Fresh fruit of *F. vesca* removes discoloration of the teeth if the juice is allowed to remain on for about five minutes and the teeth are then cleansed with warm water, to which a pinch of bicarbonate of soda has been added. A cut of strawberry rubbed over the face immediately after washing will whiten the skin and remove slight sunburn. For a badly sunburnt face, it is recommended to rub the juice well into the skin, to leave it on for half an hour, and then wash off with warm water to which a few drops of simple tincture of benzoin have been added; no soap should be used (Grieve, 1982).

1629 is the date assigned to the introduction of the Scarlet Strawberry from Virginia, and the earliest mention of the strawberry in English writings is in a Saxon plant list of the tenth century. The common idea that the word strawberry is derived

from the habit of placing straw under the cultivated plants when the berries are ripening is quite erroneous (Grive, 1982).

1.2.3. Active constituents of *F. vesca*

F. vesca leaves (woodland strawberry) contains flavonoids, tannins, quercetin, volatile oils (Chevallier, 1996) and also trace amount of alkaloids (Creasy and Swain, 1966) and silicium acids which are thought to be important antioxidant agents (Garcia et al., 2001; Lara et al., 2004). The fruits contain malic and citric acids, a volatile matter, sugar, mucilage, pectin, woody fibre and water. It easily digested and is not subjected to acetius fermentation in the stomach (Grive, 1982).

The fruits of *F. vesca* contain fruit acids and a volatile oil with methyl salicylate and borneol (Chevallier, 1996). The fruits are beneficial in the treatment of liver and kidney complaints, as well as in the treatment of rheumatism and gout due to its content salicylic acid (Phillips and Foy, 1990).

Fruits of *F. vesca* have vitamin C otherwise the fresh fruits contain vitamin B and provitamin A. The fruits are reported to be rich in etheric oil, aroma esters, phosphorus salt, and sugars (Garcia et al., 2001; Lara et al., 2004) and high total anthocyanin content and cyanidin derivatives are found in the achenes of *F. vesca* (Cheel et al, 2007).



Figure 1.1. Pictures of *Fragaria vesca* L. growing in A.İ.B.U. Campus area (by Arzu Birinci Yıldırım).

1.2.4. Biological Activity Studies of *F. vesca*

Antioxidant (Cao et al., 1998; Heinonen et al., 1998; Wang and Jiao, 2000; Vinson et al., 2001; Scalzo et al., 2005; Kiselova et al., 2006), anticoagulant (Pawlaczyk et al., 2009), antimutagenic (Howell, 2002), vasodilatory (Mudnic et al., 2009), antithrombotic (Naemura et al., 2005) anti-inflammatory (Howell, 2002), antibacterial (Yurdugul, 2008), analgesic (Kanodia and Das, 2008) cardiovascular (Mudnic et al., 2009) and anticarcinogenic (Carlton et al., 2001; Wedge et al., 2001; Howell, 2002) activities of *F. vesca* have also been reported.

Ethanollic extracts of fruits and whole plant of *F. vesca* were examined for analgesic activity. The results showed that ethanolic extracts produced significant central and peripheral analgesia (Kanodia and Das, 2008).

Yurdugul (2008) reported that rehydrated alpine strawberry juice exhibited an antimicrobial activity towards an important foodborne pathogen, *Enterobacter faecium* ATCC 6057.

Mudnic et al. (2009) investigated vasodilatory potential of aqueous extract of the wild strawberry leaves against isolated guinea pig hearts and rat aortic rings, and it was observed that extract had a direct, endothelium-dependent vasodilatory action without increasing heart rate and contraction. Furthermore, they also determined the phenolic compounds of the water extract by using Reverse Phase HPLC technique. In the extract, flavonoid monomers, catechin, epicatechin and epigallocatechin were found in significant amounts. Epicatechin-3-gallate, astringin, quercetin-40-glucoside, trans-resveratrol, piceid, procyanidins (procyanidin B1 and B2) were also found, but in smaller quantities.

Marinova and Ribarova (2007) determined the carotenoid content such as luteolin, zeaxanthin, β -cryptoxanthin, lycopene, α - and β -carotene in the strawberry (*F. vesca*) by using HPLC and reported that strawberry had β -carotene and lutein.

Pawlaczyk et al. (2009) measured the anticoagulant activity of the substances extracted from *F. vesca* by using APTT (activated partial thromboplastin time) and PT (prothrombin time) test. They reported that *F. vesca* had the strong anticoagulant activity of the extract and also the high content of hexuronic acids as well as phenolic glyco-conjugates seemed to be responsible for the observed anticoagulant activity.

Kiselova et al. (2006) studied the antioxidant activity of aqueous extract of *F. vesca* leaf in relation to its polyphenol content by using ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] cation radical decolorization assay. They reported that the extract had high antioxidant activity and also positive relation between antioxidant activity and polyphenol content of aqueous extracts of *F. vesca*.

Buricova et al. (2011) investigated the total phenolic content (Folin-Ciocalteu method), free radical scavenging activity (DPPH), ferric reducing antioxidant capacity (FRAP) and oxygen radical absorbance capacity (ORAC) of the aqueous extract of *F. vesca* leaf. Also 15 different compounds (gallic acid, rutin, ellagic acid, caffeic acid, p-coumaric acid, quercetin, kaempferol, myricetin, quercetin-3-D-glucoside, ascorbic acid, (+)-catechin, (-)-epicatechin, epicatechingallate, procyanidin B1) were studied by HPLC-ECD and their antioxidant capacities were compared to the antioxidant capacity of the extract. They reported that the studied compounds in the extract moderately contributed to the antioxidant capacity.

1.2.5. Biotechnological Studies of *F. vesca*

F. vesca has been used in molecular and genetic studies over the past 15 years. Especially, it has been used in the transformation studies mediated by *Agrobacterium tumefaciens* (Wadl, 2005). *Fragaria X ananassa* (garden strawberry) is a natural hybrid of *F. chiloensis* and *F. virginiana* that is cultivated worldwide for its fruits (Davis, 1972; Hummer et al., 2011). *F. x ananassa* is an octoploid ($2n=8x=56$ chromosome) species and high ploidy levels makes molecular and genetic studies difficult (Davis, 1972; Wadl, 2005). *F. vesca* is amenable to transformation than the other species in the Rosaceae family since it has small genome size and a short life cycle around 12 – 16 weeks (El-Mansouri et al., 1996; Haymes and Davis, 1998; Alsheikh et al., 2002; Zhao et al., 2004).

Haymes and Davis (1998) achieved *Agrobacterium*-mediated transformation of β -glucuronidase (*gus*) and neomycin phosphotransferase (*nptII*) genes into *F. vesca* Alpine plants with the vector pBI121 and demonstrated that Alpine *F. vesca* is suitable for transgenic strawberry research.

El-Mansuri et al. (1996) reported that after regeneration of *F. vesca* leaf explants with BA and IBA combinations, transformed plants were obtained using *Agrobacterium tumefaciens* as gene vector. The efficiency of transformation was 7.7%. In a similar study of Alsheikh et al. (2002), the efficiency of the transformation rised to 15% with the same explant in the same combination of BA/IBA.

Zhoa et al. (2004) reported the transformation efficiency ranging from 47% to 76% for *F. vesca* Alpine accessions FRA197 and FRA198 when leaf and petiole

explants were transformed on media containing MS basal salt and B5 vitamins with the combinations of TDZ and IBA.

Oosumi et al. (2006) investigated fourteen different *F. vesca* accessions to obtain high efficiency transformation using *in vitro* leaves. They reported that the transformation efficiency was 100% when leaf explants were transformed on MS basal medium containing the combinations of BA and IBA. This efficiency was achieved for 6 of 14 accessions tested.

Wadl (2005) investigated the shoot regeneration of Alpine *F. vesca* to develop more efficient transformation protocol than others performed. He reported that the regeneration frequency of *in vitro* leaf or petiole explants on media containing the combinations of BA and IBA was 8% when compared on media containing the combinations of TDZ and 2,4-D was 27%.

Greene (1988) reported that nearly 100% shoot formation was observed on MS basal medium containing the combinations of BA and IAA with leaf explants of *F. vesca* 'Baron solemacher'. However, when Baron solemacher leaf explants were infected via *A. tumefaciens*, only transformed callus was recovered.

1.2.6. Strawberry Cultivars

There are diploid species of strawberry (*Fragaria vesca* and *Fragaria viridis*) in Turkey's flora and these wild species are considered genetic resources available for the improvement of the octoploid *F. X ananassa*. *F. X ananassa* is derived from natural crosses between the American octoploids *F. chiloensis* and *F. virginiana* (Turhan and Kargi, 2007). Most of the cultivated varieties have a long flowering period (and have been considered by botanists as belonging to *Fragaria*

vesca var. *vesca* ssp. *semperflorens*). They are usually called alpine strawberries. *Fragaria X ananassa* is an economically important world berry fruit whose production continues to increase throughout the world. A wide range of cultivars with different origins is available. Among the most important are ‘Oso Grande’, ‘Camarosa’, ‘Chandler’ from California; and ‘Sweet Charlie’ from Florida. Recently ‘Camarosa’ has become more popular in Turkey but testing is underway with ‘Gaviota’, ‘Maya’, ‘Paros’ and ‘Diamante’. Hybridization is being carried out between local and foreign cultivars at Cukurova University (Turhan and Kargi, 2007). The strawberry cultivars used in Turkey are shown in Table 1.1.

Table 1.1. Geographical origin of the cultivars cultivated in Turkey.

Cultivar	Origin
♦ Ottoman	Turkey
♦ Yalova 110	Turkey
♦ Maya	Italy
♦ Miranda	Italy
♦ Paros	Italy
♦ Cruz	USA
♦ Tioga	USA
♦ Camaraso	USA, California
♦ Chandler	USA, California
♦ Diamante	USA, California
♦ Dauglas	USA, California
♦ Fern	USA, California
♦ Gaviota	USA, California
♦ Oso Grande	USA, California
♦ Seascape	USA, California
♦ Selva	USA, California
♦ Pocahontas	USA, Maryland
♦ Sweet Charlie	USA, Florida
♦ 216 (Dorit)	Israel, Volcani

1.3. Objectives of the study

- A. To obtain an efficient *in vitro* plant regeneration protocol for *F. vesca* via adventitious shoot development from leaf and petiole explants cultured on medium containing different concentrations and combinations of various plant growth regulators and regeneration enhancers.
- B. To analyse the phenolic contents in the methanolic extracts of field-grown leaves, fruits and *in vitro*-grown leaves and callus of *F. vesca* by using Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS).
- C. To screen and compare the biological activities of different extracts of *F. vesca* obtained from field-grown leaves and fruits, and *in vitro*-grown leaves and callus using specific bioassays (antibacterial, antitumor, toxicity, anticancer and antioxidant).

CHAPTER 2

2. *IN VITRO* CULTURE OF *FRAGARIA VESCA*

2.1. Introduction

Plant tissue culture is an essential component of plant biotechnology. Generally, *in vitro* tissue culture or micropropagation is defined as the capability to regenerate and propagate plants from different somatic cells, tissues or organs of plants under sterile and controlled *in vitro* conditions. Besides, the aim of tissue culture methods is to produce a large number of progeny plants, which will be genetically identical to the stock plant in a relatively short time compared to conventional propagation. Otherwise, cells and tissues are forced to undergo changes, which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissues (Thorpe, 1994). This implies *in vitro* cloning based on the fact that different plant parts, buds, meristems, tissues and cells are capable of regeneration into whole plants under adequate *in vitro* conditions. A special characteristic of plant cells and meristems in which they retain a latent capacity to produce a whole plant is called totipotency (Reinert and Backs, 1968; Vasil and Vasil, 1972; Verdeil et al., 2007; George, 2008).

Christianson and Warnick (1988) determined that there are three phases of organogenesis: dedifferentiation, induction, and differentiation. Dedifferentiation involves callus production and ends when cells become competent. During induction

phase, cells become determined and in the differentiation phase, cells form roots or shoots.

There has been a significant increase in tissue culture studies of medicinal plants. Because, *in vitro* culture protocol provides multiple shoots per explant that would be very useful for mass propagation of the medicinal plants. The micropropagation of elite or selected plants showed good results, which benefit the agriculture, horticulture and forestry (Conger, 1981). Although medicinal plants can easily be found in the wild, they are generally subjected to some herbicides and attacked by some insects and pathogens. *In vitro* micropropagation of medicinal plants provides a rapid multiplication method for producing plantlets exhibiting desirable characteristics. These desirable characteristics may be defined as increased production of secondary metabolites, production of pharmaceuticals and also some novel compounds not found in the whole plant. Other characteristics are the regeneration of plants resistant to herbicides, diseases, pests and plants with different morphological traits, and transgenic plants for the production of vaccines etc. *In vitro* vegetative propagation has important benefits to produce stable lines in plants (less genetic diversity) (Conger, 1981). In addition, with an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year (Turker et al., 2008) whereby an *in vitro* propagation method would eliminate seasonal constraints and site effects with seedling by providing unlimited planting material on a consistent year-round basis (McCoy, 1998).

Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either

from a callus culture or directly from an explant. Alternatively, the third method is by axillary bud formation and growth, which can also be used to regenerate whole plants from some types of tissue culture (Thorpe, 1990). The underlying basis for organogenesis involves the interplay of a host of factors: donor plant growth, source of the explant, culture medium, environmental conditions, and supplements of growth regulators. For example, *in vitro* organogenesis in tobacco cultures could be chemically regulated. In other words, *in vitro* organogenesis can be controlled experimentally using various growth regulator concentrations and combinations. The studies of Skoog's group led to the hypothesis that organogenesis is regulated by a balance between cytokinin and auxin (Dodds and Roberts, 1995). A high auxin to cytokinin ratio generally induces root formation, whereas a high cytokinin to auxin ratio induces shoot formation. An intermediate ratio induces callus production (Gurel and Turker, 2001).

Recently, apart from plant growth regulators, non-ionic and copolymer surfactants such as Pluronic F-68 (Poloxamer 188) have been used in plant tissue culture studies. It has been using for over 50 years in animal cell culture both to aid cell protection but also for their cell protecting and growth stimulatory properties (Lowe et al., 1993). In plant cell culture, *in vitro* supplementation of surfactants (Pluronic F-68) to the culture media was shown to have a number of beneficial effects. It confers cryoprotection to suspension-cultured cells, growth-promoting effects on callus, increased cell proliferation of isolated protoplasts. In addition, it stimulates growth and differentiation of tissues and organs from several plant species (Khatun et al., 1993 and Khehra et al., 1995). Several non-ionic surfactants can be separated by their hydrophilic-hydrophobic balance number (HLB) (Helenius and Simons, 1975). Tween 20 (HLB number, 16.7) and Triton X-100 (HLB number,

13.5) have low HLB number. Pluronic F-68 (HLB number, 29.0) has high HLB number. Surfactants with a high HLB number have low capacity to interact with the lipid fraction of the cell membranes. Surfactants with a low HLB number have higher capacity to interact with the lipid fraction of the cell membranes and also demonstrated beneficial effects in plant cultures. Despite the precise mechanism of how pluronic F-68 interacts with plant cells is unknown, it is considered that at low concentrations, the surfactant increases the permeability of the plasma membrane and so facilitates the movement of culture nutrients into the cell. However, when surfactant (pluronic F-68) is used at higher concentrations, the surfactant may cause detrimental, irreversible changes to the plasma membrane and so impair cell growth (Curtis and Mirkov, 2012).

2.2. Materials and Methods

Field-grown plant parts (leaves, petioles and fruits) of *F. vesca* were collected from Campus area of AIBU in May and June. Identification of the species was made by using “Flora of Turkey and The East Aegean Island” (Davis, 1972) and voucher specimens (AUT-2001) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey.

In vitro regeneration of *F. vesca* was attempted by using two different explant sources (field-grown plants and sterile seedlings obtained from germinated seeds). A diagram for *in vitro* culture of *F. vesca* is illustrated in Figure 2.1.

Seeds obtained from *F. vesca* fruits were washed with an anti-bacterial soap, rinsed with sterile distilled water and surface sterilized by shaking in 70% ethanol for 5 minutes followed by rinsing well with sterilized distilled water 3 times

and then dipped into 2% Domestos® (5% sodium hypochloride) for 5 minutes and finally washed with sterile distilled water 4 times. After surface sterilization of the seeds, they were placed in sterile, disposable petri dishes containing Murashige and Skoog's Basal Salt (MS) medium (4.3 g/l MS, Sigma Chemical Co., St. Louis, MO, USA; Murashige and Skoog 1962) with 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121°C and 105 kPa) for seed germination. After a two week incubation in this medium, plantlets were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing the same medium for an additional three weeks to obtain sterile seedlings.

Field-grown plant parts (leaves and petioles) were washed under running tap water for 30 minutes, and then treated with water containing 5-10 drops Tween 20 (5-10 drops per 100 ml) for 15 minutes, washed again running tap water. Surface disinfected by shaking in 0.1 % Mercuric Chloride (HgCl₂) for 15 minutes followed by rinsing well with sterile distilled water three times and dipped into 70 % Ethanol for 1-2 minutes. Finally, field-grown plant parts were washed again with sterile distilled water for 4 times.

For shoot regeneration, leaf (36 mm²) and petiole explants (4-5 mm) were excised from five weeks-old sterile seedlings and disinfested field-grown plant materials. Excised explants were placed in sterile disposable petri plates containing 4.3 g/l MS Basal Salt with different combinations and concentrations of plant growth regulators (PGRs); thidiazuron (TDZ; 0.1, 0.5, 1, 1.5 and 2.5 mg/l) + indole-3-butyric acid (IBA; 0.1, 0.5, 1, 2 and 2.5 mg/l); TDZ (0.5 and 1 mg/l) + IBA (0.5 and 1 mg/l) + gibberellic acid (GA₃; 0.5 mg/l); TDZ (1 and 1.5 mg/l) + IBA (0.5 and 1 mg/l) + Silver nitrate (AgNO₃; 1, 2 and 5 mg/l); TDZ (0.1, 0.5 and 1 mg/l) + 2,4-

dichlorophenoxyacetic acid (2,4-D; 0.1, 0.5 and 1 mg/l); TDZ (0.1, 0.25, 0.5 and 1 mg/l) + 2,4-D (0.05, 0.1, 0.5 and 1 mg/l) + GA₃ (0.5 mg/l); TDZ (1.5 mg/l) + 2,4-D (0.5 mg/l) + AgNO₃ (1 and 5 mg/l); TDZ (0.1, 0.5 and 1 mg/l) + indole-3-acetic acid (IAA; 0.5 and 1 mg/l); benzyladenine (BA; 0.1, 0.5 and 1 mg/l) + IAA (0.1, 0.5 and 1 mg/l); BA (0.5 and 1 mg/l) + naphthalene acetic acid (NAA; 1, 3 and 6 mg/l); BA (0.5 and 1 mg/l) + IBA (0.1, 0.5 and 1 mg/l); BA (0.5 and 1 mg/l) + IBA (0.5 and 1 mg/l) + GA₃ (0.5 mg/l); BA (0.5 mg/l) + IBA (0.5 mg/l) + AgNO₃ (1 and 5 mg/l). GA₃ was used after filter sterilization. All above combinations were also prepared by adding surfactants (pluronic F-68, tween 20 and tween 80) or AgNO₃. All surfactants were used after filter sterilization. All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 $\mu\text{mol m}^{-2}\text{s}^{-1}$).

After six weeks, regenerated explants were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MS medium containing 0.5 mg/l Gibberellic acid (GA₃) for shoot elongation for an additional two weeks. Shoot number and percentage of explants producing shoots were recorded after 8 weeks. There were 10 replications for each explant and the experiment was repeated three times. Shoots were then separated individually and placed in rooting medium containing MS and varying concentrations of different auxins; IAA (0.5 and 1 mg/l), IBA (0.5 and 1 mg/l), 2,4-D (0.5 and 1 mg/l) or NAA (0.5 and 1 mg/l). Rooted plants were transferred to vermiculate (Agrekal[®]) in Magenta containers for acclimatization. After 3 weeks, they were transferred to plastic pots containing potting soil and maintained under high humidity with subsequent transfer to the greenhouse.

Results were expressed as means $\pm$ standard error of the mean. Mean differences of all data were detected by one-way ANOVA and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA). Experiments were repeated three times.

A diagram for *in vitro* culture of *F. vesca* is illustrated in Figure 2.1.

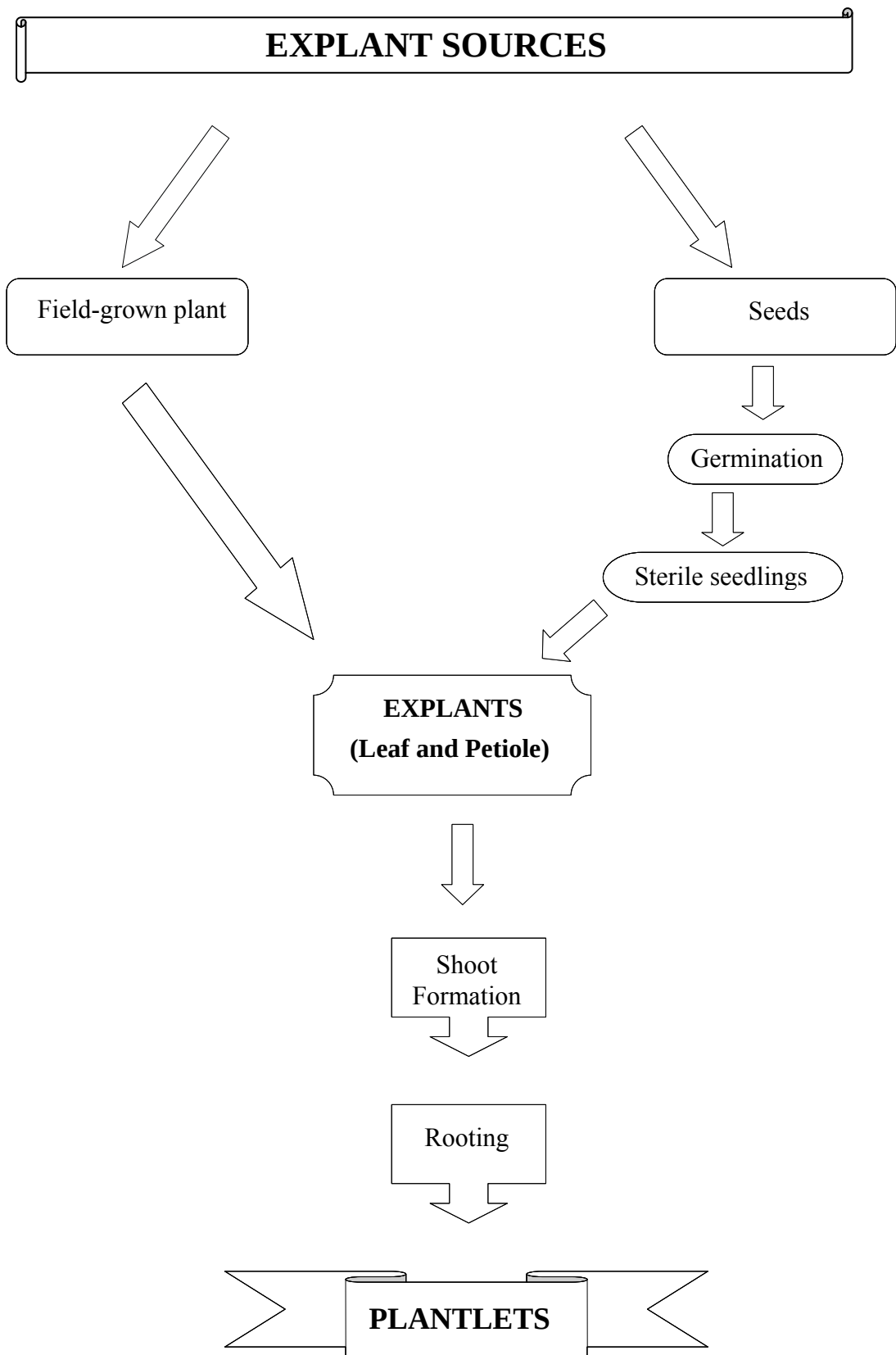


Figure 2.1. *In vitro* culture of *Fragaria vesca* L.

2.3. Results and Discussion

Although *F. vesca* is a valuable medicinal herb, there are no detailed regeneration studies involving different concentrations and combinations of plant growth regulators in relation with different regeneration enhancers such as silver nitrate (AgNO_3) or surfactants (Tween 20, Tween 80 and Pluronic F-68). Generally, *Agrobacterium*-mediated transformation has been used to generate transgenic strawberry plants and only some of the regeneration media were employed to optimize transformation protocols mediated by *Agrobacterium tumefaciens* that will facilitate gene manipulation on this species (El Mansouri, 1996). Wild strawberry (*F. vesca*), that contains a diploid genome ($2n=14$), represents an ideal model to study foreign gene expression in *Fragaria*. Moreover, transformed plants could be used to transfer introduced genes to the octoploid cultivated strawberry by conventional breeding techniques (El Mansouri, 1996). *F. vesca* is an attractive model for functional genomic research in Rosaceae. Its small genome size and short reproductive cycle make *F. vesca* a promising candidate for genetic experiments (Oosumi et al. 2006). In spite of the advantages, not much effort has been dedicated to set up an efficient system for regeneration of *F. vesca*. We therefore aimed to develop an efficient protocol for *in vitro* regeneration of *F. vesca* and thus examined the effects of several combinations of plant growth regulators and different regeneration enhancers (AgNO_3 and surfactants) using different explants.

Two different explants (leaf and petiole) obtained from sterilized field-grown plants and sterile seedlings from germinated seeds were cultured on MS medium containing TDZ, BA or KIN in combination with different auxins (IAA,

IBA, 2,4-D, NAA) supplemented with or without some surfactants (Tween 20, Tween 80 and Pluronic F-68), AgNO₃ or GA₃ for shoot regeneration.

2.3.1. Field-Grown Plants as Explant Source

Explants obtained from field-grown plant materials were not responsive for shoot regeneration. None of the tested treatments or explant types was able to produce adventitious shoots.

Generally, the browning of the explants was the major problem. Browning was believed to be the result of oxidation of polyphenols exuded from the cut surfaces of the explants which could be overcome by adding substances such as PVP (polyvinylpyrrolidone), citric acid or ascorbic acid (Rout et al. 1999; Pati et al. 2006). To minimise browning, Murashige (1974) has suggested the pre-soaking of explants in ascorbic and citric acid solutions and adding them to the culture medium. Browning of the tissue and the adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinones which are toxic to the tissues (Zaid 1984). Supplementation of the medium with PVP, activated charcoal, citric acid or ascorbic acid didn't circumvent the browning problem of the explants. Supplementation of medium with surfactants also was not effective for shoot formation. Interestingly, regeneration was not observed with all explants obtained from field-grown plant materials.

Red colored callus formation was observed with some combinations of TDZ and 2,4-D (Figure 2.4.). More callus formation was obtained with leaf explants on media containing 0.5 mg/l TDZ + 0.1 mg/l 2,4-D; 1.0 mg/l TDZ + 0.1 mg/l 2,4-D; 0.5 mg/l TDZ + 0.5 mg/L 2,4-D and 0.1 mg/L TDZ + 0.1 mg/L 2,4-D. With leaf

explants, when TDZ in combination with 0.1 mg/l 2,4-D, increasing concentration of TDZ from 0.1 mg/l to 1.0 mg/l decreased red colored callus formation. On the other hand, when TDZ in combination with 0.5 mg/l or 1.0 mg/l 2,4-D, increasing concentration of TDZ from 0.1 mg/l to 1.0 mg/l increased red callus formation. Furthermore, high concentration of 2,4-D (2.0 and 3.0 mg/l) in combination with all concentration of TDZ did not lead to red callus formation.

With petiole explants, red callus formation was lower than leaf explants, only 50% of petiole explants induced callus formation. When 2,4-D in combination with 1.0 mg/l TDZ, increasing concentration of 2,4-D from 0.1 to 1.0 mg/l increased density of red colored callus. On the other hand, when 2,4-D in combination with 0.1 or 0.5 mg/l TDZ, increasing concentration of 2,4-D from 0.1 to 1.0 mg/l decreased callus formation intensity.

2.3.2. Sterile Seedlings Obtained from Germinated Seeds as Explant Source

All used explants (leaf and petiole) obtained from sterile seedlings produced shoots with TDZ:IBA (Table 2.1.), TDZ:IBA:GA₃ (Table 2.1.), TDZ:IBA:AgNO₃ (Table 2.1.), TDZ:2,4-D (Table 2.3.) and BA:IBA (Table 2.4.) combinations. Supplementation of media with pluronic F-68 significantly increased shoot number [TDZ:IBA:pluronic (Table 2.2.) and BA:IBA:pluronic (Table 2.4.)]. Callus formation was observed before shoot formation for both leaf and petiole explants with all experimented combinations (indirect regeneration). Control treatments involving no plant growth regulators produced no shoots in both explants (Table 2.1., 2.2., 2.3. and 2.4.). Generally, shoot regeneration along the periphery of the explants was visually detected within 4-6 weeks. Well developed shoots were observed within 8 weeks.

Of the tested combinations, different concentrations of TDZ with IBA were effective for leaf and petiole explants in shoot production. Leaf explants showed better shoot formation than petiole explants. With leaf explants, the greatest number of shoots per shooted explant was observed on MS media containing 1.5 mg/l TDZ + 0.5 mg/l IBA (13.5 shoots per shooted explant; 54% explants formed shoots) (Table 2.1. and Figure 2.2). When leaf explants were cultured on media containing 1.5 mg/l TDZ in combination with IBA, it is evident that the shoot formation and frequency were closely related to concentrations of IBA supply. For example, decreasing IBA concentration of from 2.0 mg/l to 0.5 mg/l increased shoot number from 6.0 shoots to 13.5 shoots. Similarly, shoot frequency was increased from 25% to 54%, respectively. All combinations of 0.1 mg/l and 2.5 mg/l TDZ with IBA were not effective for shoot formation with leaf explants.

With petiole explants, the best shoot proliferation was obtained with media containing 1.5 mg/l TDZ + 2.0 mg/l IBA (10.5 shoots per shooted explant; 25% explants formed shoots) (Table 2.1. and Figure 2.2). When petiole explants were cultured on media containing 1.5 mg/l TDZ in combination with IBA, contrary to leaf explants, shoot number increased with increasing concentration of IBA (Table 2.1.). All combinations of 0.1 mg/l, 0.5 mg/l and 2.5 mg/l TDZ with IBA were not effective for shoot formation with petiole explants.

When TDZ concentration was held constant at 0.5 mg/l and concentration of IBA was increased from 0.5 to 2.0 mg/l, the number of shoots decreased from 8.5 to 6.3 shoots per shooted leaf explant. When IBA concentration was held constant at 0.5 mg/l and the concentration of TDZ was increased from 0.5 to 1.0 mg/l, the number of shoots decreased from 8.5 to 5.3 shoots. With leaf explants, Although addition of 0.5

mg/l GA₃ into 0.5 mg/l TDZ + 0.5 mg/l IBA combination decreased the number of shoots from 8.5 to 4.8 shoots, shoot frequency increased from 33% to 80%, respectively (Table 2.1.). Supplementation of 1.0 or 5.0 mg/l AgNO₃ into 1.5 mg/l TDZ + 0.5 mg/l IBA combination decreased the number of shoots from 13.5 to 5.2 or 4.5 shoots, respectively and also slightly decreased the percentage of shoot formation from 54% to 40% (Table 2.1.) with leaf explants. Similarly, addition of 1.0 mg/l AgNO₃ to 1.5 mg/l TDZ + 0.5 mg/l IBA did not change the shoot formation number (4.5 and 4.8 shoots, respectively). Moreover, the addition of 5.0 mg/l AgNO₃ to this combination decreased the number of shoots from 4.5 to 3.0 shoots (Table 2.1.).

Mohiuddin et al. (1997) reported that the addition of AgNO₃ to the medium showed positive effect on plant tissue culture. Supplementation of AgNO₃ to the medium improved the regeneration frequency and also reduced callus formation (Anantasaran and Kanchanapoom, 2008). However, the addition of AgNO₃ into the medium lowered shoot formation or no shoot formation was observed in our study. AgNO₃ is a potent inhibitor of ethylene action, and ethylene is considered to suppress shoot organogenesis *in vitro* (Zhang et al., 1998). This inhibitory effect of AgNO₃ may have suppressed callus formation, thereby ceasing shoots regeneration via callus formation.

Addition of some surfactants plays an important role to improve plant regeneration and frequency of shoot formation (Lowe et al., 1994). For this purpose, some surfactants such as pluronic F-68, tween 20 and tween 80 were used as an additive in varying concentrations. Different concentrations of these surfactants were added into the combinations that previously induced the shoot formation.

Supplementation of different concentration of pluronic F-68 (0.005, 0.05, 0.1, 0.2 and 0.4%) with different combinations and concentrations of PGRs were tested for shoot formation. TDZ in combination with IBA and pluronic were the most effective in terms of shoot regeneration number and frequency for both leaf and petiole explants (Table 2.2.). The regeneration efficiency was found to be higher in leaf explants than petiole explants with regard to both the mean number of shoots per shoot explant and the percentages of explants forming shoots (Table 2.2.). Regarding the leaf explants, best shoot proliferation was obtained with 1.0 mg/l TDZ in combination with 0.5 mg/l IBA and 0.1 % pluronic (22.8 shoots; 90% explants formed shoots) (Table 2.2.). Addition of 0.1 mg/l pluronic into 1 mg/l TDZ and 0.5 mg/l IBA increased shoot number 4 times (from 5.3 to 22.8 shoots). Percentage of explants regenerating shoots was also increased from 20% to 90% (Table 2.1. and 2.2.). Although other two combinations (1.5 mg/l TDZ + 0.5 mg/l IBA + 0.05% pluronic and 1.5 mg/l TDZ + 0.5 mg/l IBA + 0.1% pluronic) also gave best results for shoot number (23.7 and 24.5 shoots, respectively), shoot frequencies were lower for these two combinations (20% and 75%, respectively). Addition of 0.05% or 0.1% pluronic into 1.5 mg/l TDZ + 0.5 mg/l IBA increased shoot number from 13.5 to 23.7 or 24.5 shoots, respectively. Although addition of 0.1% pluronic increased shoot formation frequency from 54% to 75%, addition of 0.05% pluronic decreased shoot frequency from 54% to 20% (Table 2.1. and 2.2.).

Regarding the petiole explants, best shoot proliferation was obtained with 1.5 mg/l TDZ in combination with 2.5 mg/l IBA and 0.05 % pluronic (12.0 shoots; 40% explants formed shoots) Addition of 0.1% pluronic into 1.5 mg/l TDZ and 0.5 mg/l IBA increased shoot number from 4.5 to 11.6 shoots. But, just a little increase

was observed in percentage of explants regenerating shoots (from 20% to 25%) (Table 2.1. and 2.2.).

Curtis and Mirkov (2012) reported that, at low concentrations, the surfactants increase the permeability of the plasma membrane of plant cells. Pluronic F-68 has a low capacity to interact with the lipid fraction of the cell membrane, therefore, at very low concentrations, pluronic F-68 could not interact with cell membrane and also do not perform movement of culture nutrients into the cell. If pluronic F-68 was used at higher concentrations (greater than 0.1 % w/v), the surfactant may cause detrimental, irreversible changes to the plasma membrane and so impair cell growth (Curtis and Mirkov 2012). Similarly, it is evident that the number of shoots obtained was closely related to the concentrations of the surfactant (Pluronic F-68) supply in our study. Low concentrations of pluronic (0.05% and 0.1%) significantly increased shoot number. But, higher (0.2% and 0.4%) or lower (0.005%) concentrations did not affect on shoot formation in our study. Increasing concentration of pluronic inhibited shoot development. When the concentration of pluronic was decreased from 0.05 % to 0.005 % in 1.5 mg/l TDZ + 0.5 mg/l IBA combination, the number of shoots decreased from 23.7 to 3.0 shoots with leaf explants.

Addition of tween 20 (0.005%, 0.05% and 0.1%) or tween 80 (0.005%, 0.05% and 0.1%) into TDZ and IBA combinations were not effective for shoot regeneration of leaf or petiole explants. Conversely, these surfactants prevented shoot formation. Although 13.5 shoots from leaf explants and 4.5 shoots from petiole explants were obtained with 1.5 mg/l TDZ and 0.5 mg/l IBA, no shoot formation was

observed with the addition of different concentrations of tween 20 or 80 into this combination for both explants (Table 2.1. and 2.2.).

When TDZ was used in combination with 2,4-D, the best shoot formation was obtained with leaf explants on media containing 1.0 mg/l TDZ + 0.1 mg/l 2,4-D (9.4 shoots; 40% explants formed shoot); with petiole explants on media containing only 0.5 mg/l TDZ + 0.1 mg/l 2,4-D (4.0 shoots; 30% explants formed shoots) (Table 2.3.). When leaf explants were cultured on media containing TDZ in combination with 2,4-D, it is clear that the frequency of shoot formation was closely related to the concentration of 2,4-D supply (Table 2.3.). For example, increasing concentration of 2,4-D from 0.1 mg/l to 1.0 mg/l decreased shoot regeneration number from 9.4 to 3.6 shoots. Supplementation of medium containing TDZ and 2,4-D combinations with different concentrations of AgNO₃, GA₃ or pluronic did not induce any shoot formation in both explants (Table 2.3.). More callus formation was obtained with TDZ and 2,4-D combinations than other tested PGR combinations. Furthermore, leaf explants produced greater amount of callus than petiole explants. In leaf explants, yellowish and red colored callus were observed in media containing 0.5 mg/l TDZ + 0.1 mg/l 2,4-D, 0.5 mg/l TDZ + 0.5 mg/l 2,4-D, 1.0 mg/l TDZ + 0.1 mg/l 2,4-D and 1.0 mg/l TDZ + 0.5 mg/l 2,4-D. When TDZ in combination with 0.1 mg/l 2,4-D, increasing concentration of TDZ from 0.1 mg/l to 0.5 mg/l increased red colored callus formation. On the other hand, when TDZ in combination with 0.5 mg/l or 1.0 mg/l 2,4-D, increasing concentration of TDZ from 0.1 mg/l to 1.0 mg/l decreased red callus formation.

Different combinations and concentrations of BA and IBA were not as effective as TDZ and IBA combinations for shoot regeneration. Among BA and IBA

combinations, leaf and petiole explants gave shoots with media containing only 0.5 mg/l BA+ 0.5 mg/l IBA (6.0 and 5.5 shoots, respectively) (Table 2.4.). Shoot formation frequency was also very low for both explants (10% explants formed shoot). Addition of 0.1 % pluronic into BA and IBA combinations slightly increased the number of regenerated shoots and percentage of shoot formation in leaf explants. However, no shoot formation was observed by the addition of 0.1% pluronic into this combination in petiole explants. When 0.1% pluronic was added into 0.5 mg/l BA + 0.5 mg/l IBA, the number of shoots increased from 6.0 to 9.3 shoots with leaf explants. Also, shoot formation frequencies raised from 10% to 40%. Furthermore, although no shoot formation was observed at 1.0 mg/l BA + 0.5 mg/l IBA concentration, the addition of 0.1% pluronic into this combination induced shoot regeneration (6.8 shoots). Supplementation of medium with GA₃ or AgNO₃ into BA and IBA combinations did not induce any shoot formation for both explants (Table 2.4.).

When TDZ or BA was used in combination with IAA or NAA, no shoot formation was observed in all explants (leaf and petiole) (data not shown).

El Mansouri et al. (1996) investigated the shoot regeneration of *F. vesca* using leaf explants on MS medium containing only different concentrations of BA and IBA. Best shoot proliferation was obtained with 4 mg/l BA and 0.25 mg/l IBA (4.6 shoots) and they used this combination to obtain transformed plants using *Agrobacterium tumefaciens* as gene vector (El Mansouri et al., 1996). Oosumi et al. (2006) developed a highly efficient transformation protocol for *F. vesca* ‘Alpine’ and used MS basal medium supplemented with 3 mg/l BA and 0.25 mg/l IBA for regeneration studies. Landi and Mezzetti (2006) investigated the *in vitro* organogenic

(adventitious shoots and roots) and callus formation competence of leaves from 14 *Fragaria* spp. genotypes (*F. vesca* cultivars) in response to TDZ and auxin [IBA, 2,4-D or 3-benzoselenienyl acetic acid (BSAA)] combinations. The highest overall percentage (43.9%) of leaves with regenerated shoots was detected with the medium supplemented with 1 mg/l TDZ and 0.2 mg/l IBA for all *F. vesca* cultivars. Similarly, efficient shoot proliferation was obtained with TDZ in combinations with IBA in our study. In the study of Barcelo et al. (1998), the effect of BA and IBA concentration on percentage of shoot formation of leaf explants from strawberry cultivar chandler (*Fragaria x ananassa*) were investigated. Best results were obtained in the presence of 2.0 mg/l BA and 0.5 mg/l IBA (47% explants formed shoots). On the other hand, among the tested BA and IBA combinations in our study, only 0.5 mg/l BA and 0.5 mg/l IBA gave response with leaf explants (6.0 shoots and 10% explants formed shoots). Wadl (2005) investigated on *Agrobacterium*-mediated transformation on *F. vesca* 'Alpine' and he reported that regeneration frequency for leaf and petiole explants on medium supplemented with BA/IBA was 8% compared to 27% on medium supplemented with the combination of TDZ/2,4-D.

Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray, 2000). Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success (Turker et al., 2008). The origin and the nature of the plant material play an important role in the success of micropropagation. In our study, when leaf and petiole explants were compared, it was clear that leaf explants were much more productive for shoot formation than petiole explants (Table 2.1., 2.2., 2.3.

and 2.4.). Similarly, leaf explant was also reported as the most responsive explant in terms of shoot regeneration for *F. vesca* cultivars (Park and Lim, 1999).

TDZ was more successful than BA for shoot formation in our study (Tables 2.1. and 2.4.). TDZ has been reported to induce adventitious shoot formation in a number of species, especially woody plants (Huetteman and Preece 1993; Lu, 1993). Heutteman and Preece (1993) reported that low concentrations of TDZ have been found to be useful for micropropagation and higher concentrations of TDZ increased callus formation in woody plants. On the other hand, moderate concentrations of TDZ (0.5 – 1.5 mg/l) were more effective for shoot formation in all explant types in our study (Table 1 and 2). Similarly, Ledbetter and Preece (2004) reported that the lower concentration of TDZ stimulated the formation of fewer shoots but they elongated. The moderate concentration of TDZ increased significantly the number of shoots but strongly inhibited shoot elongation. Mendoza De Gyves et al. (2001) and Huetteman and Preece (1993) emphasized the potential use of TDZ in the regulation of adventitious shoot production and synergism existing between TDZ and both endogenous and some exogenous auxins. This finding is consistent with our data emphasizes that when TDZ was used alone, the formation of shoots was not observed (Table 2.1.). However, the combination of TDZ, IBA and Pluronic F-68 showed synergistic effects and more shoot formation was obtained. Heutteman and Preece (1993) and Lu (1993) reported some problems with conversion of TDZ-induced shoots into complete plantlets, poor elongation of shoots and inadequate rooting. Therefore, regenerated shoots were transferred to elongation medium including 0.5 mg/l GA₃ for two weeks in this study.

After 8 weeks in regeneration medium, regenerated shoots were cultured on shoot elongation medium containing 0.5 mg/l GA₃ for additional 2 weeks (Figure

2.5.(D)). After that period, regenerated shoots were separated individually and cultured on MS medium containing different concentrations of IAA, IBA, 2,4-D or NAA (Table 2.5., Figure 2.3. and Figure 2.6.). Best root formation was observed with MS basal medium (control). Mean number of roots (7.9 roots) was higher with basal medium than medium including different concentrations of auxins and roots were longer, thicker and seemed healthier. Supplementation of basal medium with IBA or IAA decreased root formation. If we compare among the various auxins investigated for rooting, IBA and IAA were effective for rooting. But, root formation was not observed with NAA and 2,4-D. Similarly, El-Mansouri et al. (1996) reported that shoots were rooted in MS basal medium and the presence of IBA (1 mg/l) caused a decrease in the number of rooted shoots from 87.5% to 37.5%. Some root formation also appeared in shoot regeneration medium in our study, so transfer of regenerants to basal medium or medium including auxins may not be necessary. Similarly, El-Mansouri et al. (1996) observed root formation during shoot regeneration.

The rooted shoots were transferred to Magenta containers including vermiculate for acclimatization for 2 weeks conditions and then finally to plastic pots containing potting soil and kept under growth room conditions (Figure 2.7.). Approximately 95% of the regenerated plants survived through the hardening off process.

The present study is the first report to describe an efficient *in vitro* plant regeneration protocol for *F. vesca* through adventitious shoot regeneration with pluronic F-68 in combination with TDZ and IBA. This protocol is expected to contribute to the future genetic manipulation studies of *F. vesca* as well as to the

attempts to develop a large scale production of secondary metabolites from *in vitro* propagated material. Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout et al. 2000). Chemical constituents of medicinal plants and their parts are required to be uniform both qualitatively and quantitatively to have standardized formulations (Chaturvedi et al., 2007). Micropropagation of *F. vesca* can provide a mass production of pesticide, herbicide and disease free plants on a commercial scale and unlimited plant materials can consistently be obtained throughout the whole year. Moreover, this protocol can provide plant material for future pharmacological, physiological and biochemical studies.

Table 2.1. Effects of different concentrations and combinations of TDZ and IBA supplemented with or without GA₃ or AgNO₃ on shoot regeneration. Means with the same letter within columns are not significantly different at P>0.05. Mean number of shoots for Table 2.1 and Table 2.2 were statistically analyzed together.

Plant Growth Regulators			EXPLANTS			
			Leaf		Petiole	
			Mean number of shoots per shoot explant ± SE	% explants forming shoots	Mean number of shoots per shoot explant ± SE	% explants forming shoots
TDZ (mg/l)	IBA (mg/l)					
-	-		-	-	-	-
0.1	-		-	-	-	-
0.1	0.1		-	-	-	-
0.1	0.5		-	-	-	-
0.1	1.0		-	-	-	-
0.1	2.5		-	-	-	-
0.5	-		-	-	-	-
0.5	0.1		-	-	-	-
0.5	0.5		8.5 ± 0.3 ^{bc}	33	-	-
0.5	1.0		7.7 ± 0.9 ^{bcd}	20	-	-
0.5	2.0		6.3 ± 1.8 ^{bcde}	20	-	-
0.5	2.5		-	-	-	-
1.0	-		-	-	-	-
1.0	0.1		-	-	-	-
1.0	0.5		5.3 ± 1.8 ^{cde}	20	-	-
1.0	1.0		-	-	-	-
1.0	2.5		-	-	-	-
1.5	0.5		13.5 ± 1.1 ^a	54	4.5 ± 0.3 ^{bc}	20
1.5	1.0		9.2 ± 1.3 ^b	12.5	6.3 ± 1.2 ^b	30
1.5	2.0		6.0 ± 2.3 ^{bcde}	25	10.5 ± 1.4 ^a	25
2.5	0.1		-	-	-	-
2.5	0.5		-	-	-	-
2.5	1.0		-	-	-	-
2.5	2.5		-	-	-	-
TDZ (mg/l)	IBA (mg/l)	AgNO ₃ (mg/l)				
1.0	0.5	1.0	-	-	-	-
1.0	0.5	2.0	-	-	-	-
1.0	0.5	5.0	-	-	-	-
1.5	0.5	1.0	5.2 ± 0.7 ^{cde}	40	4.8 ± 0.7 ^{bc}	20
1.5	0.5	2.0	-	-	-	-
1.5	0.5	5.0	4.5 ± 1.0 ^{de}	40	3.0 ± 0.0 ^c	10
1.5	1.0	1.0	-	-	-	-
1.5	1.0	5.0	-	-	-	-
TDZ (mg/l)	IBA (mg/l)	GA ₃ (mg/l)				
0.5	-	0.5	-	-	-	-
0.5	0.5	0.5	4.8 ± 0.4 ^{cde}	80	-	-
0.5	1.0	0.5	-	-	-	-
1.0	0.5	0.5	3.0 ± 1.5 ^e	37	-	-
1.0	1.0	0.5	-	-	-	-

Table 2.2. Effects of different concentrations and combinations of surfactants (Pluronic F-68, Tween 20 and Tween 80) in combination with TDZ and IBA on shoot regeneration. Means with the same letter within columns are not significantly different at $P>0.05$. Mean number of shoots for Table 2.1 and Table 2.2 were statistically analyzed together.

Plant Growth Regulators			EXPLANTS			
			Leaf		Petiole	
TDZ (mg/l)	IBA (mg/l)	Pluronic (%)	Mean number of shoots per shoot explant $\pm$ SE	%explants forming shoots	Mean number of shoots per shoot explant $\pm$ SE	%explants forming shoots
-	-	-	-	-	-	-
0.5	0.5	0.1	-	-	-	-
0.5	1.0	0.1	-	-	-	-
1.0	0.5	0.1	22.8 $\pm$ 3.8 ^a	90	-	-
1.0	1.0	0.1	-	-	-	-
1.5	0.5	0.005	3.0 $\pm$ 1.1 ^c	50	-	-
1.5	0.5	0.05	23.7 $\pm$ 4.5 ^a	20	-	-
1.5	0.5	0.1	24.5 $\pm$ 1.8 ^a	75	11.6 $\pm$ 1.9 ^a	25
1.5	0.5	0.2	-	-	-	-
1.5	0.5	0.4	-	-	-	-
1.5	1.0	0.05	-	-	-	-
1.5	1.0	0.1	9.0 $\pm$ 2.4 ^b	12.5	-	-
1.5	1.0	0.2	-	-	-	-
1.5	2.5	0.05	5.8 $\pm$ 1.0 ^{bc}	66	12.0 $\pm$ 1.5 ^a	40
1.5	2.5	0.1	7.3 $\pm$ 0.9 ^{bc}	50	-	-
1.5	2.5	0.2	4.5 $\pm$ 0.3 ^{bc}	10	-	-
1.5	2.5	0.4	-	-	-	-
2.0	0.5	0.1	-	-	-	-
2.0	1.0	0.1	-	-	-	-
TDZ (mg/l)	IBA (mg/l)	Tween 20 (%)				
1.5	0.5	0.005	-	-	-	-
1.5	0.5	0.05	-	-	-	-
1.5	0.5	0.1	-	-	-	-
TDZ (mg/l)	IBA (mg/l)	Tween 80 (%)				
1.5	0.5	0.005	-	-	-	-
1.5	0.5	0.05	-	-	-	-
1.5	0.5	0.1	-	-	-	-

Table 2.3. Effects of different concentrations and combinations of TDZ and 2,4-D supplemented with or without GA₃, AgNO₃ or surfactant (PluronicF-68) on shoot regeneration. Means with the same letter within columns are not significantly different at P>0.05.

Plant Growth Regulators			EXPLANTS			
			Leaf		Petiole	
			Mean number of shoots per shooted explant ± SE	%explants forming shoots	Mean number of shoots per shooted explant ± SE	%explants forming shoots
TDZ (mg/l)	2,4-D (mg/l)					
-	-		-	-	-	-
0.1	-		-	-	-	-
0.1	0.1		-	-	-	-
0.1	0.5		-	-	-	-
0.1	1.0		-	-	-	-
0.5	-		-	-	-	-
0.5	0.1		6.2 ± 0.9 ^b	30	4.0 ± 0.6 ^a	30
0.5	0.5		5.6 ± 0.9 ^b	20	-	-
0.5	1.0		-	-	-	-
1.0	-		-	-	-	-
1.0	0.1		9.4 ± 1.1 ^a	40	-	-
1.0	0.5		4.7 ± 0.5 ^{bc}	40	-	-
1.0	1.0		3.6 ± 0.4 ^c	33	-	-
TDZ (mg/l)	2,4-D (mg/l)	AgNO ₃ (mg/l)				
1.5	0.5	1.0	-	-	-	-
1.5	0.5	5.0	-	-	-	-
TDZ (mg/l)	2,4-D (mg/l)	GA ₃ (mg/l)				
0.1	-	0.5	-	-	-	-
0.1	0.05	0.5	-	-	-	-
0.1	0.1	0.5	-	-	-	-
0.25	-	0.5	-	-	-	-
0.25	0.05	0.5	-	-	-	-
0.25	0.1	0.5	-	-	-	-
0.5	-	0.5	-	-	-	-
0.5	0.05	0.5	-	-	-	-
0.5	0.1	0.5	-	-	-	-
0.5	0.5	0.5	-	-	-	-
0.5	1.0	0.5	-	-	-	-
1.0	-	0.5	-	-	-	-
1.0	0.05	0.5	-	-	-	-
1.0	0.1	0.5	-	-	-	-
1.0	0.5	0.5	-	-	-	-
1.0	1.0	0.5	-	-	-	-
TDZ (mg/l)	2,4-D (mg/l)	Pluronic (%)				
1.0	0.1	0.1	-	-	-	-
1.0	0.5	0.1	-	-	-	-
1.5	0.5	0.1	-	-	-	-

Table 2.4. Effects of different concentrations and combinations of BA and IBA supplemented with GA₃, AgNO₃ or surfactant (PluronicF-68) on shoot regeneration. Means with the same letter within columns are not significantly different at P>0.05.

Plant Growth Regulators			EXPLANTS			
			Leaf		Petiole	
BA (mg/l)	IBA (mg/l)		Mean number of shoots per explant ± SE	%explants forming shoots	Mean number of shoots per explant ± SE	%explants forming shoots
-	-		-	-	-	-
0.5	-		-	-	-	-
0.5	0.1		-	-	-	-
0.5	0.5		6.0 ± 1.6 ^{ab}	10	5.5 ± 0.5 ^a	10
0.5	1.0		-	-	-	-
1.0	-		-	-	-	-
1.0	0.1		-	-	-	-
1.0	0.5		-	-	-	-
1.0	1.0		-	-	-	-
BA (mg/l)	IBA (mg/l)	AgNO ₃ (mg/l)				
0.5	0.5	1.0	-	-	-	-
0.5	0.5	5.0	-	-	-	-
BA (mg/l)	IBA (mg/l)	GA ₃ (mg/l)				
0.5	0.5	0.5	-	-	-	-
0.5	1.0	0.5	-	-	-	-
1.0	0.5	0.5	-	-	-	-
1.0	1.0	0.5	-	-	-	-
BA (mg/l)	IBA (mg/l)	Pluronic (%)				
0.5	0.5	0.1	9.3 ± 2.0 ^a	40	-	-
1.0	0.5	0.1	6.8 ± 1.6 ^{ab}	25	-	-

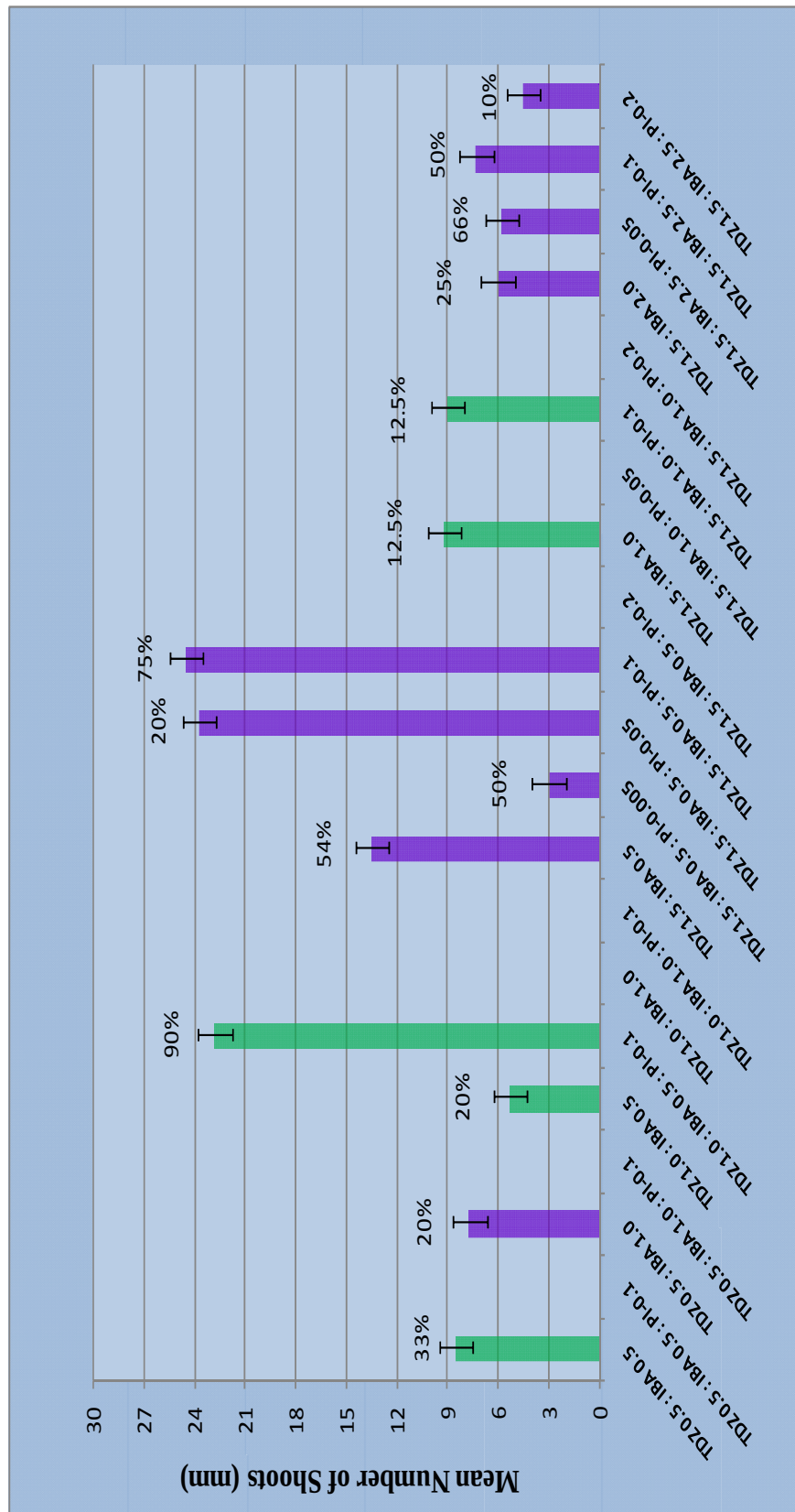


Figure 2.2. Effects of different concentrations and combinations of TDZ and IBA supplemented with or without Pluronic F-68 (PI) on shoot regeneration. Percentages of explants forming shoots were indicated above each bar representing mean number of shoot formation.

Table 2.5. Effects of the tested auxins on root formation from regenerated shoots. Means with the same letter within columns are not significantly different at $P>0.05$.

Treatments (mg/l)	Mean number of roots per shoot $\pm$ SE	% explants forming roots
Control	7.9 $\pm$ 1.8^a	100
IAA 0.5	5.1 $\pm$ 0.9 ^b	80
IAA 1.0	4.3 $\pm$ 0.9 ^b	100
Means	4.7 $\pm$ 0.9	90
IBA 0.5	5.7 $\pm$ 0.6 ^b	90
IBA 1.0	4.5 $\pm$ 0.6 ^b	80
Means	5.1 $\pm$ 0.6	85
2,4-D 0.1	-	-
2,4-D 0.5	-	-
Means	-	-
NAA 0.5	-	-
NAA 1.0	-	-
Means	-	-

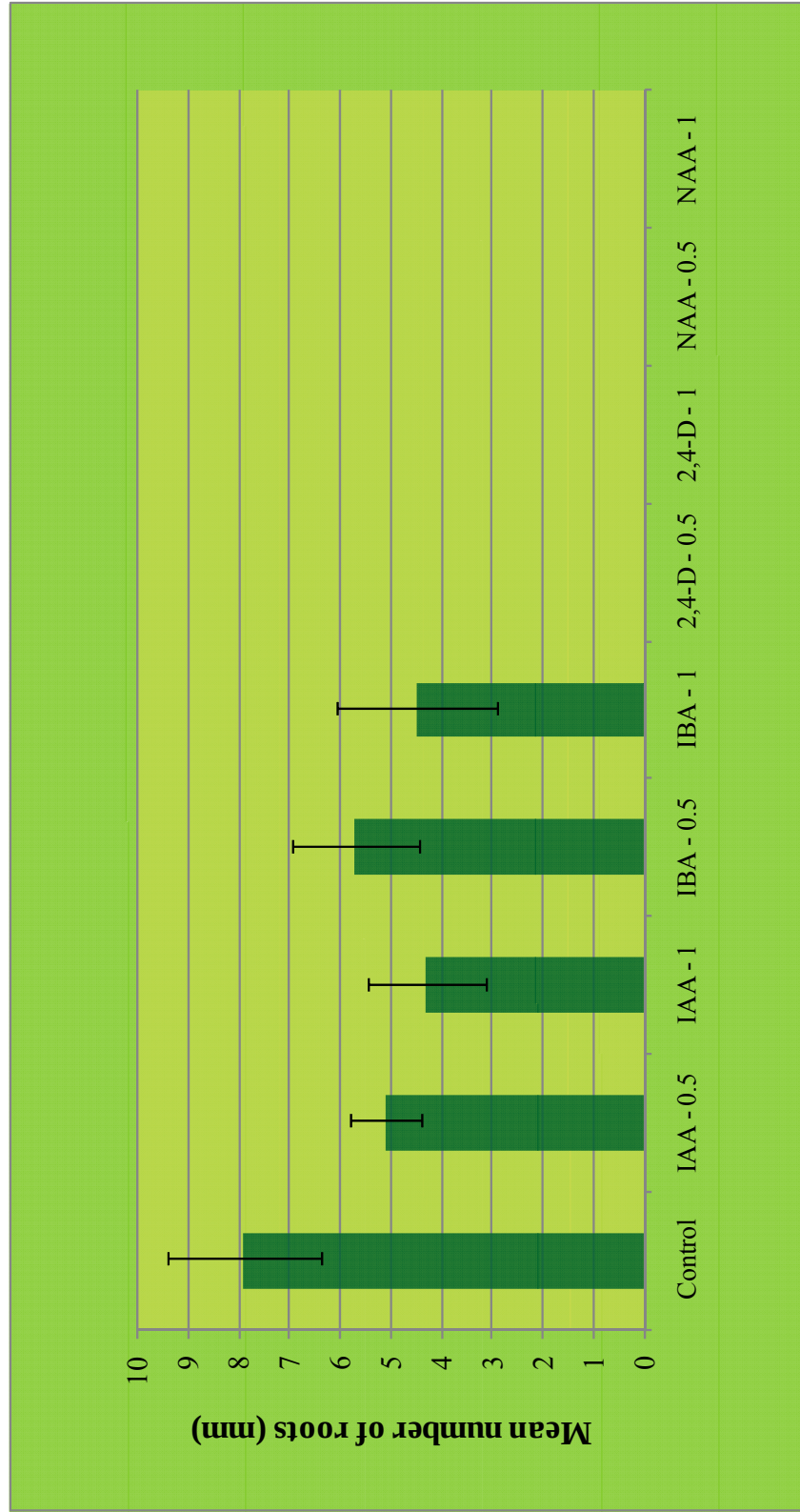


Figure 2.3. Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots.

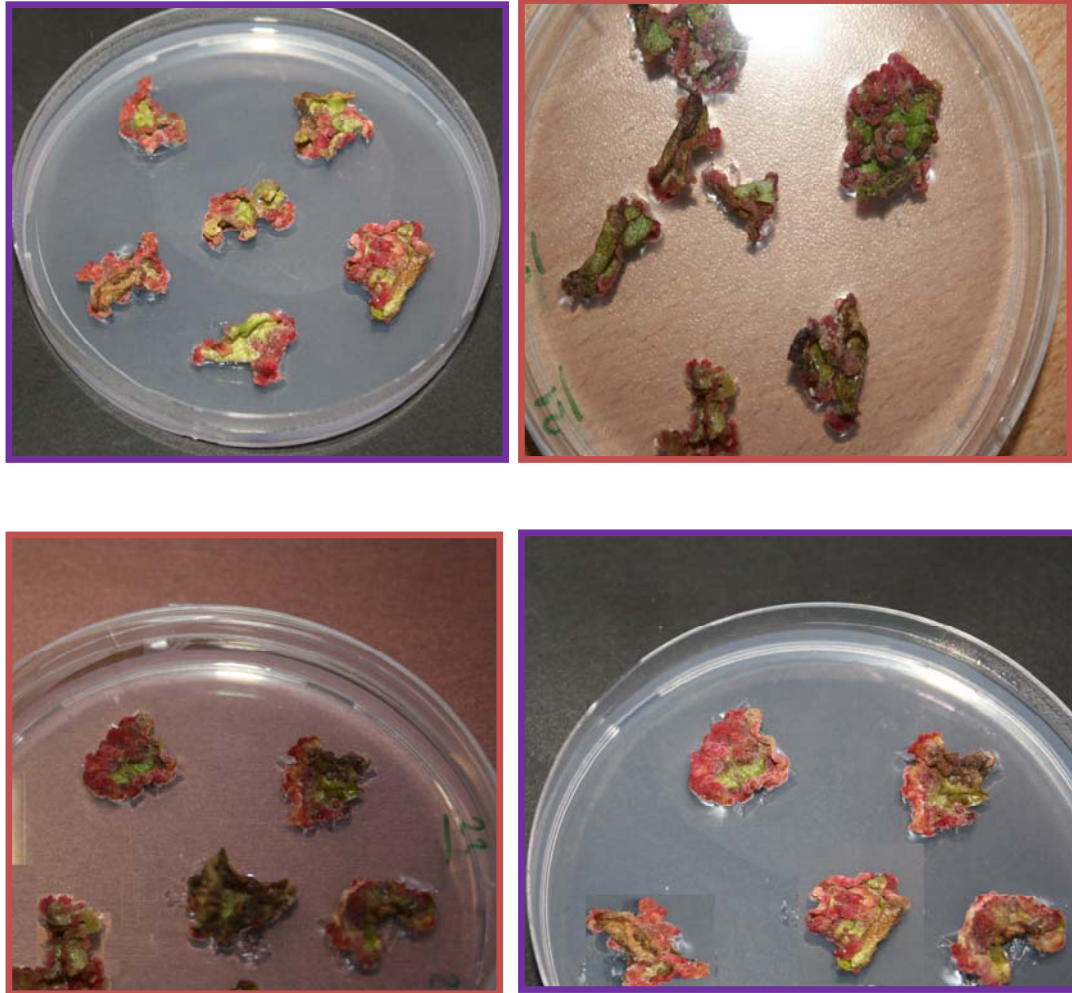


Figure 2.4. Callus formation in leaf explant of *F. vesca* on MS medium containing 0.5 mg/l TDZ + 0.1 mg/l 2,4-D.

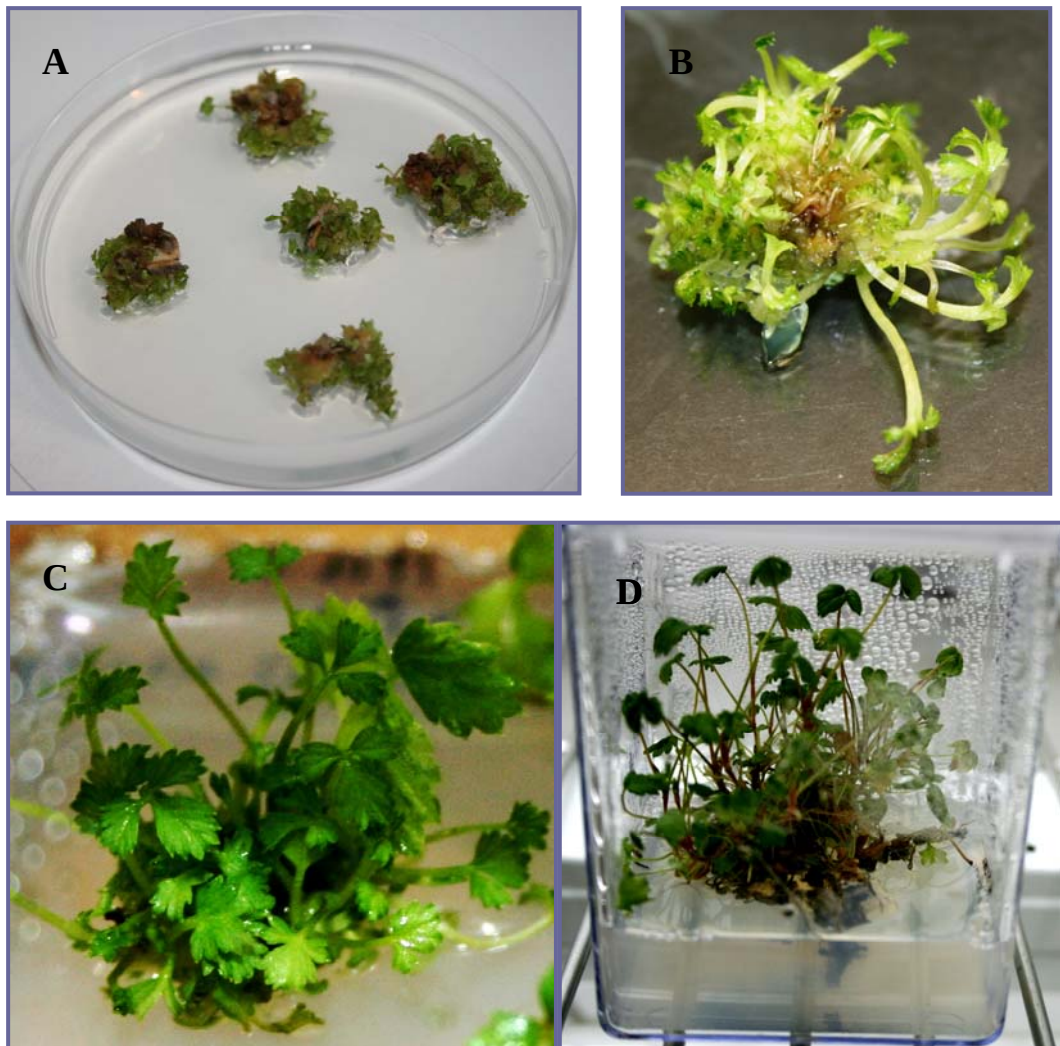


Figure 2.5. Shoot regeneration of leaf explant of *F. vesca* on MS medium containing 1.5 mg/l TDZ + 0.5 mg/l IBA + 0.1% Pluronic F-68. **A:** Regenerated shoots after 4 weeks in culture, **B:** Regenerated shoots after 6 weeks in culture, **C:** Regenerated shoots after 8 weeks in culture and **D:** Shoot elongation of regenerated shoots on medium containing 0.5 mg/l GA₃.



Figure 2.6. Rooting of the regenerated shoots on medium containing 0.5 mg/l IBA.



Figure 2.7. Regenerated plants in Magenta containers including vermiculate for acclimatization (left); Regenerated plants transferred to plastic pots containing sterile potting soil under growth room conditions (right).

CHAPTER 3

3. PLANT EXTRACTION AND LC-ESI-MS/MS ANALYSIS OF THE EXTRACTS

3.1. Plant Extraction

3.1.1. Introduction

Plants have an almost limitless ability to synthesize aromatic substances which have no direct function in growth and development. They are called as secondary metabolites, secondary products or natural products. The secondary metabolites can be divided into several categories with respect to their structures and functions (Taiz and Zeiger, 2002). These substances have a variety of important roles. Phenolic compounds (phenols and phenolic acids, flavonoids, flavonols, tannins, quinones etc.) which are readily recognized by their hydrophilic nature and by their common origin from the aromatic precursor shikimic acid. They usually serve as defensive compounds against herbivores and pathogens, and also absorb harmful ultraviolet (UV) radiation. Terpenoids (essential oils, diterpenoids, triterpenoids, carotenoids etc.), all share lipid properties and a biosynthetic origin from isopentenyl pyrophosphate and especially give plants their odor. Organic acids (fatty acids), lipids, polyacetylenes derived from biosynthetically from acetate. Nitrogen compounds of plants (amino acids, alkaloids, indoles, purines, chlorophylls etc.) are produced either from nitrogen fixation in the root or from enzymatic reduction of absorbed nitrate in shoot and leaf. Various natural alkaloids (taxol, reserpine and morphine) and their derivatives have been used to treat various

diseases. Other important groups are high molecular weight compounds such as sugars (monosaccharides, oligosaccharides) nucleic acids and proteins (Harborne, 1998).

For the analysis of these phytochemicals in the plants, fresh or dried plant tissues should be used. In general, plant materials should be dried at temperature below 30 °C to avoid decomposition of thermolabile compounds. Likewise, it should be protected from sunlight because of the potential for chemical transformations resulting from exposure to ultraviolet radiation (Sarker et al., 2006).

Grinding of plant materials improves the efficiency of the extraction by increasing the surface area of the plant material. If the particles are too fine, solvents can not flow easily around them and the extraction will be more effective (Sarker et al., 2006).

A range of techniques may be used for extraction of plant materials: maceration, percolation, soxhlet extraction, pressured solvent extraction, ultrasound-assisted solvent extraction, extraction under reflux, and steam distillation. Methods of solvent extraction can be classified as continuous or discontinuous. In continuous methods (percolation and soxhlet extraction), solvent flows through the plant material continuously. As constituents diffuse from the plant material into the surrounding solvent, the solvent becomes increasingly saturated, but because the solvent is continually flowing, the saturated solvent is replaced with less-saturated solvent. In the discontinuous methods, the solvent is added and removed in batches. Hence, once equilibrium is reached between the concentration of solute inside the plant material and the concentration in the solvent, extraction essentially stops until the solvent is decanted and replaced with new solvent (Sarker et al., 2006).

A convenient and frequently used procedure is sequential solvent extraction (with Soxhlet apparatus) to obtain organic compounds from dried plant tissue (dried seeds, root or leaf). A first step with hexane will remove lipids, and then with dichloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid glycosides and more polar constituents (Andersen and Markham, 2006).

Flavonoids (particularly glycosides) can be degraded by enzyme action when collected plant material is fresh or none dried. It is thus advisable to use dry, lyophilized, or frozen samples. For extraction, the solvent is chosen as a function of the type of flavonoid required. Polarity is an important consideration here. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable. The bulk of extractions of flavonoid-containing material are still performed by simple direct solvent extraction (Andersen and Markham, 2006).

Certain flavanone and chalcone glycosides are difficult to dissolve in methanol, ethanol, or alcohol–water mixtures. Flavanone solubility depends on the pH of water-containing solutions (Andersen and Markham, 2006).

Flavan-3-ols (catechins, proanthocyanidins, and condensed tannins) can often be extracted directly with water. However, the composition of the extract does vary with the solvent — whether water, methanol, ethanol, acetone, or ethyl acetate.

For example, it is claimed that methanol is the best solvent for catechins and 70% acetone for procyanidins (Andersen and Markham, 2006).

Anthocyanins are extracted with cold acidified methanol. The acid employed is usually acetic acid (about 7%) or trifluoroacetic acid (TFA) (about 3%). The use of mineral acid can lead to the loss of attached acyl groups (Andersen and Markham, 2006).

After the fractionation of crude extracts with suitable solvents, they are usually clarified by filtration and concentrated using a rotary evaporator (*in vacuo*) at temperatures below 30-40°C. Then, the fractions were subjected to several biological activity studies (antibacterial, antifungal, anticancer, anti-HIV etc.). The fractions which give positive results with those tests are analyzed or separated into main classes of compounds from each other using chromatographic techniques (Harborne, 1998).

3.1.2. Plant Materials and Methods

Leaves and fruits of *F. vesca* obtained from field-grown plants were collected from Campus, Bolu/Turkey in May-June. Identification of the species was made by using “Flora of Turkey and the East Aegean Islands” (Davis, 1972) and voucher specimens (AUT-1919) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Leaves and callus of *in vitro*-cultured plants were collected from *F. vesca* plants that were previously micropropagated in the laboratory. All used leaves included petioles.

Different extracts were prepared from four different sources of plant (field-grown leaves and fruits, and *in vitro*-grown leaves and callus). Collected plants

(field-grown leaves, *in vitro*-grown leaves and callus) were dried in a room avoiding sun light and then ground into a powder. Collected fruits were freeze-dried and then lyophilized by using freeze-dryer at -65 °C and then ground into a powder, and also fresh fruit samples were used. Powdered plants materials have been extracted with different solvents by using two extraction methods:

3.1.2.1. Direct Extraction Method

-Twenty grams of each field-grown leaves have been extracted with 300 ml water, ethanol, methanol or acetone at 45 °C in a water bath for 24 hours (Table 3.1.).

- Twenty grams of *in vitro*-grown leaves and callus have been extracted with 300 ml methanol at 45 °C in a water bath for 24 hours (Table 3.1.).

-Fifty grams of dry or fresh fruits have been extracted with either 200 ml of cold water and ethanol or hot water and ethanol separately at 45 °C in a water bath for 18 hours (Table 3.1.).

3.1.2.2. Gradual Extraction Method (starting from non-polar to polar solvent)

-Fifty grams of field-grown leaves and fruits, and *in vitro*-grown callus have been gradually extracted by using hexane (at 65-70 °C), dichloromethane (at 55-60 °C), methanol (at 60 °C) and then water (at 80 °C) , respectively in soxhlet extractor for 24 hours (Table 3.1.).

For aqueous extracts, frozen filtrate was lyophilized by using freeze-dryer at -65 °C. For hexane, dichloromethane, ethanol, methanol and acetone extracts, they were evaporated under vacuum using rotary evaporator at 40 °C. When it was used for biological studies, each residue was then dissolved in sterile distilled water to

produce a final concentration of 100 mg/ml. Plant materials, extraction procedures, their designation and extraction yields are summarized in Table 3.1.

Phenolic content of methanolic extracts were analyzed by using LC-ESI-MS/MS (Table 3.4.) and prepared extracts were used for biological activity studies (antibacterial, antitumor, toxicity, anticancer and antioxidant activity) (Table 3.1.).

Table 3.1. Designation of extracts in bioassays, used plant materials and their extraction procedures and yields. Yield (%) = Weight of extract (g) / 20 gr of powdered plant sample * 100

Plant Parts	Extraction	Solvents	Extraction Procedure (Direct)	Designation	Yield (%) *
Field-grown leaves		Water	20 g field-grown leaves were extracted with water at 45 °C for 18 h. Water was freeze-dried and residues was dissolved in water to final concentration of 100 mg/ml.	NW	14.3
		Ethanol	20 g field-grown leaves were extracted with ethanol at 45 °C for 18 h. Ethanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NE	2.9
		Methanol	20 g field-grown leaves were extracted with methanol at 45 °C for 18 h. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NM	20.0
In vitro -grown leaves	Water Bath (18 h)	Acetone	20 g field-grown leaves were extracted with acetone at 45 °C for 18 h. Acetone was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NA	18.2
		Methanol	20 g <i>in vitro</i> -grown leaves were extracted with methanol at 45 °C for 18 h. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	ILM	36.0
In vitro -grown callus	Water Bath (18 h)	Methanol	20 g <i>in vitro</i> -callus were extracted with methanol at 45 °C for 18 h. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	CM	9.0

Continued (Table 3.1.)

Plant Parts	Extraction	Solvents	Extraction Procedure (Direct)	Designation	Yield (%) *
Fruits	Dried	Water	Cold 50 g dry fruit were extracted with cold water at 25 °C for 18 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	DFCW	30.0
			Hot 50 g dry fruit were extracted with hot water at 45 °C for 18 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	DFHW	25.5
		Ethanol	Cold 50 g dry fruit were extracted with ethanol at 25 °C for 18 h. Ethanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	DFCE	7.5
			Hot 50 g dry fruit were extracted with ethanol at 45 °C for 18 h. Ethanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	DFHE	15.0
Fresh	Water Bath (18 h)	Water	Cold 50 g fresh fruit were extracted with cold water at 25 °C for 18 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	FFCW	3.5
			Hot 50 g fresh fruit were extracted with hot water at 45 °C for 18 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	FFHW	5.6
		Ethanol	Cold 50 g fresh fruit were extracted with ethanol at 25 °C for 18 h. Ethanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	FFCE	3.3
			Hot 50 g fresh fruit were extracted with ethanol at 45 °C for 18 h. Ethanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	FFHE	7.1

Continued (Table 3.1.)

Plant Parts	Extraction	Solvents	Extraction Procedure (Gradual)	Designation	Yield (%) *
Field-grown leaves	Soxhlet Extractor (24 h)	Hexane	50 g field-grown leaves were extracted with hexane at 65-70 °C for 24 h. Hexane was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NFH	8.0
		Dichloromethane	50 g field-grown leaves were extracted with DCM at 55-60 °C for 24 h. DCM was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NFD	2.0
		Methanol	50 g field-grown leaves were extracted with methanol at 60-65 °C for 24 h. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	NFM	20.0
		Water	50 g field-grown leaves were extracted with water at 80 °C for 24 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	NFW	2.5
		Hexane	50 g fruits were extracted with hexane at 65-70 °C for 24 h. Hexane was evaporated under vacuum and residue was dissolved in water to final concentration of 100	FFH	7.0
		Dichloromethane	50 g fruits were extracted with DCM at 55-60 °C for 24 hr. DCM was evaporated under vacuum and residue was dissolved in water to final concentration of 100	FFD	1.0
Fruits	Soxhlet Extractor (24 h)	Methanol	50 g fruits were extracted with methanol at 60-65 °C for 24 h. Methanol was evaporated under vacuum and residue was dissolved in water to final	FFM	35.0
		Water	50 g fruits were extracted with water at 80 °C for 24 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	FFW	12.0
		Hexane	50 g <i>in vitro</i> -grown callus were extracted with hexane at 65-70 °C for 24 h. Hexane was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	CFH	1.4
		Dichloromethane	50 g <i>in vitro</i> -grown callus were extracted with DCM at 55-60 °C for 24 h. DCM was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	CFD	4.0
		Methanol	50 g <i>in vitro</i> -grown callus were extracted with methanol at 60-65 °C for 24 h. Methanol was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.	CFM	42.7
		Water	50 g <i>in vitro</i> -grown callus were extracted with water at 80 °C for 24 h. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.	CFW	7.0
<i>In vitro</i> -grown callus					

3.2. LC-ESI-MS/MS Analysis of the Methanolic Extracts for Phenolic Compounds

3.2.1. Introduction

Plant secondary metabolites, including terpenes, alkaloids and phenolic compounds play important roles in plants and also have significance in pharmacology and human health (Fabricant and Farnsworth, 2001). Thus the localization of sites of their biosynthesis and accumulation was of interest for researchers. Phenolic compounds such as polyphenols, tannins and flavonoids where are ubiquitously found across the plant kingdom and represent an important component of a normal human diet. Epidemiological studies have suggested associations between consumption of phenolic-rich foods or beverages and various diseases, such as stroke, neurodegenerative and cardiovascular diseases, and cancer (Steffen, 2006).

Phenolic compounds can be grouped into different classes according to their basic chemical structures (type and number of phenol rings) and into different subclasses, according to specific substitutions in basic structures (Table 3.2) (Adriana and Donangelo, 2006).

In earlier times, TLC, PTLC, CC and FCC were the major separation techniques for phenolic compounds. Of these methods, TLC is still the workhorse of flavonoid analysis. It is used as a rapid, simple, and versatile method for the polyphenolics in plant extracts and in fractionation work. However, the majority of published work now refers to qualitative and quantitative applications of liquid chromatographic techniques (LC, HPLC) and hyphenated (online-combination)

techniques (HPLC-PDA, LC-MS, GC-MS, LC-NMR, LC-MS/MS) for analysis. LC is a fundamental separation technique in the life sciences and related fields of chemistry. LC is suitable for nonvolatile and thermally fragile molecules and can safely separate a very wide range of organic compounds, from small molecules to the proteins. However, HPLC is becoming by far the most popular technique for the separation of phenolic compounds, both on preparative and analytical scales. Improvements in instrumentation, packing materials, and column technology are being introduced all the time, making the technique more and more attractive (Andersen and Markham, 2006).

Table 3.2. Main classes of phenolic compounds in higher plants

Classes and subclasses	Example
Non-flavonoid compounds	
Phenolic acids	
Benzoic acids	Gallic acid,p-hydroxybenzoic acid.
Hydroxycinnamic acids	Coumaric acid,caffeic acid.
Hydrolyzable tannins	Pentagalloylglucose.
Stilbenes	Resveratrol.
Lignans	Matairesinol,pinoresinol.
Flavonoid compounds	
Flavonols	Kaempferol,quercetin,myricetin.
Flavones	Apigenin,luteolin.
Flavanones	Naringenin, hesperetin.
Flavanols	Catechins,gallocatechins.
Antocyanidins	Pelargonidin,cyanidin,malvidin.
Condensed tannins (proanthocyanidins)	Trimeric procyanidin.
Isoflavones	Daidzein,genistein,glycitein.

Mass spectrometer is used as a detector in liquid chromatographic analyses and separations. It generates mass spectral data that can provide valuable information about the molecular weight, structure, identity, quantity, and purity of a sample.

Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analyses (Agilent, 2001). Most frequently used MS instruments are single- and triple-quadrupole, ion trap and time-of-flight (TOF) mass spectrometers.

Both LC-MS and GC-MS are the most popular hyphenated techniques in use today. LC-MS (or HPLC-MS) refers to the coupling of an LC with a mass spectrometer (MS). The separated sample emerging from the column can be identified on the basis of its mass spectral data (Wilson et al., 2003). The ionization techniques used in LC-MS are generally soft ionization techniques that mainly display the molecular ion species with only a few fragment ions. The two most widely used ionization techniques, especially in relation to natural product analysis, are ESI and APCI. Hence, the information obtained from a single LC-MS run, on the structure of the compound, is rather poor. However, this problem has now been tackled by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced.

LC-ESI-MS/MS has been recognized as extremely powerful analytical tool in natural product analysis with its high sensitivity, short run time and less use of toxic organic solvents used as mobile phase compared to reversed phase stand alone HPLC coupled with Diode-Array Detector (HPLC-DAD) (Liu et al., 2005). It permits the fast screening of crude natural product extracts for detailed information about metabolic profiles, with a minimum amount of material (Herderich et al., 1997).

For the precise identification of individual flavonoid in a crude extract, more often LC-MS/MS is used. In the ESI mode, in addition to the pseudomolecular

ion for the individual flavonoid present in the crude extract or fraction, formation of $[M+H_2O]^+$, $[M+Na]^+$, and $[M+MeOH]^+$ has been common in flavonoid analyses. (Oleszek, 2002).

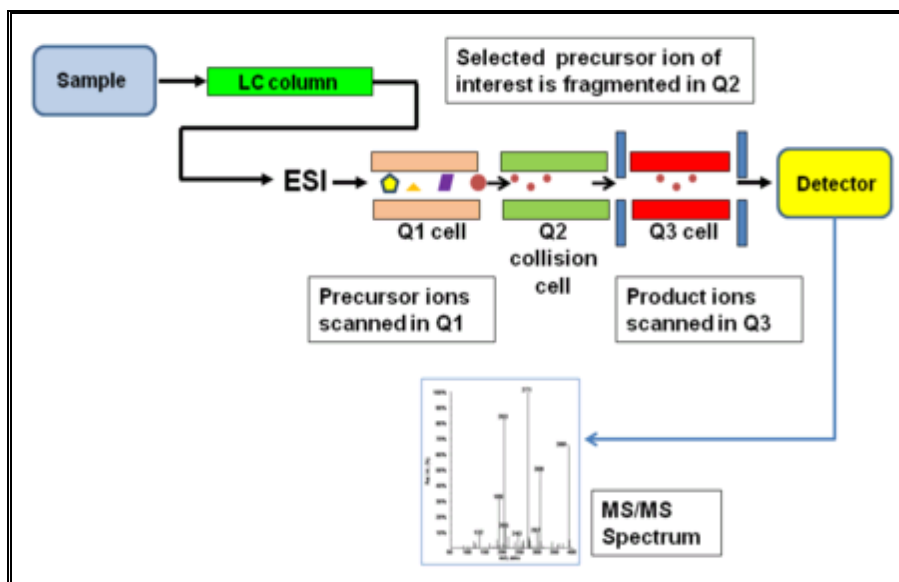
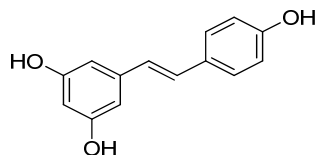
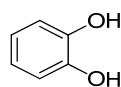


Figure 3.1. Detection of natural products by LC-MS/MS system in ESI mode

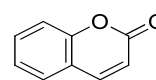
3.2.2. Materials and Methods

3.2.2.1. Standard Phenolic Compounds

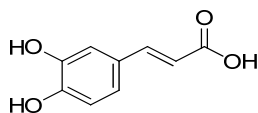
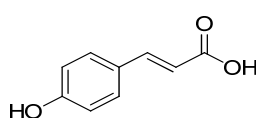
In current study, all the phenolic compounds were chosen as HPLC grade (>98% purity) and bought from Sigma-Aldrich Chemical Company. Totally, twenty-two phenolic compounds were used as standards in the analysis of extracts by LC-ESI-MS/MS) technique (Figure 3.2).

Resveratrol (**Stilbenes**)Pyrocatechol (**Phenols**)

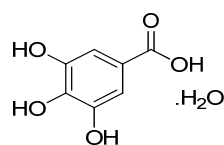
Coumarin

**(Hydroxycinnamic acids)**

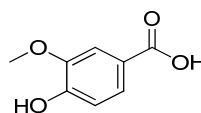
Caffeic acid

*p*-coumaric acid

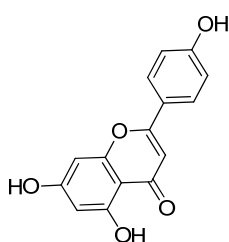
Gallic acid monohydrate



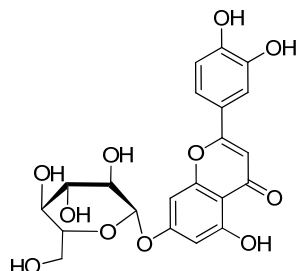
Vanillic acid

**(Flavones and Flavone glucosides)**

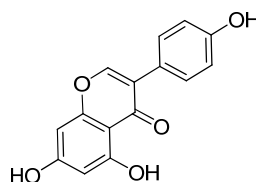
Apigenin



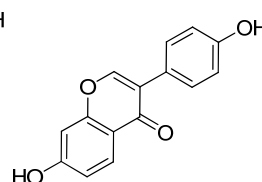
Luteolin-7-O-beta-D-glucoside



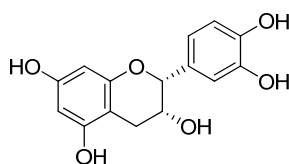
Genistein



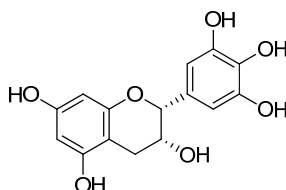
Daidzein

**(Flavanols)**

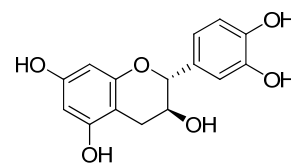
(-)-epicatechin



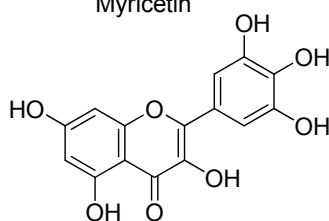
(-)-epigallocatechin



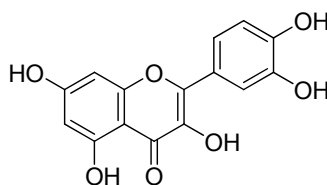
(+) -catechin

**(Flavonols and Flavonol glucosides)**

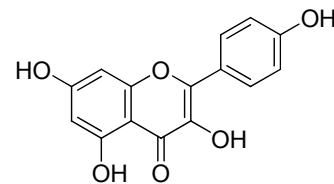
Myricetin



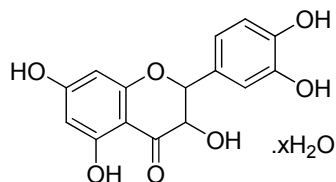
Quercetin



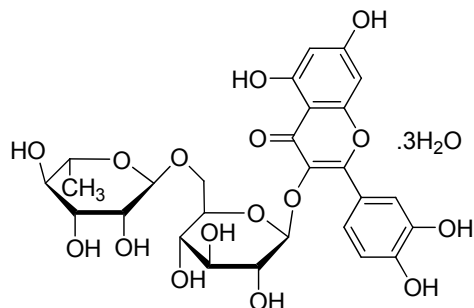
Kaempferol



Taxifolin hydrate



Rutin hydrate



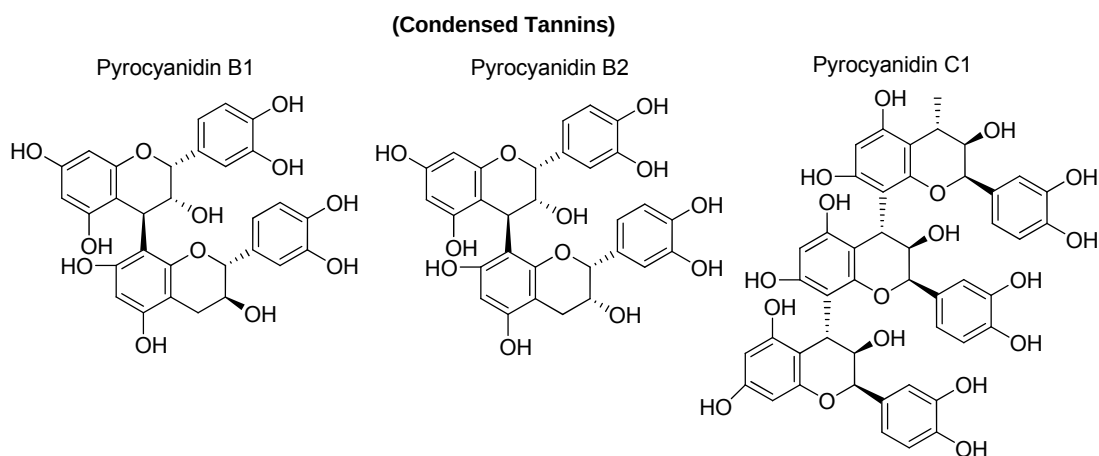


Figure 3.2. Phenolic compounds used as standard in LC-ESI-MS/MS analyses

3.2.2.2. Method of LC-ESI-MS/MS Analysis

LC-MS analysis was performed on a Triple Quadrupole LC-MS system with an Agilent 1290 Infinity LC (Agilent Technologies, Inc. Headquarters, Santa Clara, CA, USA) in Central Laboratory, METU, Ankara, Turkey. Triple Quadrupole LC-MS system is equipped with a Jet Stream ESI source. Prior to analysis, 1-5 mg of each standard compound was weighed and diluted with 1 ml of methanol and 100 mg of each sample was weighed and diluted with 2 ml of methanol. 5 μ l of each sample extract was injected for LC-ESI-MS/MS. Compounds were separated on an Zorbax SB-C18 column (Agilent Technologies, Inc. Headquarters, Santa Clara, CA, USA, 50 mmx2.1 mm; 1.8 μ m particle size) using 0.05% formic acid+5mM ammonium formate (MilliQ water) (solvent A) and methanol (MS Grade, Merck) (solvent B). Column temperature was maintained at 40 °C. A stepwise gradient from 5% to 95% solvent B was applied at a flow rate of 0.3 ml/min for 13 min. Electrospray mass spectra data were recorded on either positive or negative ionisation mode for a mass range m/z 100 to m/z 500. Capillary voltage and cone voltage were set at ± 4 kV and

30 V, respectively. Collision induced fragmentation (CID) of the analytes was achieved using 12 eV to 20 eV energy with nitrogen as the collision gas.

3.2.3. Results and Discussion

Six different methanolic extracts of *F. vesca* were subjected to Liquid Chromatographic Analyses:

-Methanolic extracts of field-grown leaves (NM), *in vitro*-grown leaves (ILM) and callus (CM) with direct extraction method.

-Methanolic extracts of field-grown leaves (NFM) and fruits (FFM), and *in vitro*-grown callus (CFM) with gradual extraction method.

A mixture of twenty-two standard phenolic compounds (apigenin, caffeic acid, (+)- catechin, *p*-coumaric acid, coumarin, daidzein, (-) epicatechin, (-) epigallocatechin, gallic acid monohydrate, genistein, kaempferol, luteolin-7-O- β -D glucoside, myricetin, procyanidin C1, pyrocyanidin B1 + B2, pyrocatechol, quercetin, resveratrol, rutin hydrate, ($\pm$) taxifolin hydrate and vanillic acid) were eluted using liquid chromatography and a chromatogram for the standards were obtained. Then, the phenolic content of the above extracts were compared with standard chromatogram and also identified with mass spectrometer (MS) and finally, the concentrations (ppm) of each phenolic compound in the extracts were found. The results were converted into mg compound/g dry weight and given in Table 3.3. Corresponding mass spectra showing *m/z* ratio and chromatograms (for quantification) for each standard compound were given in appendices.

According to LC-ESI-MS/MS results, concentrations of phenolic compounds in field-grown leaf extracts (NFM and NM) were at least five times higher than the other extracts. The phenolic compounds found in high concentrations in NFM and NM were (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, gallic acid monohydrate, kaempferol, procyanidin C1, pyrocyanidin B1 + B2 and quercetin. The concentration of these compounds in field-grown leaf extract obtained with gradual extraction method (NFM) was higher than field-grown leaf extract obtained with direct extraction method (NM). Probably, in gradual extraction, moderately polar solvents such as dichloromethane (DCM) might have removed some other compounds that would be soluble in methanol. So the concentration of the phenolic compounds was found higher in NFM extract (Table 3.3.).

Mudnic et al. (2009) analyzed the some phenolics of water extract of *F. vesca* leaf. They found that the amount of (+)-catechin, (-)-epicatechin and epigallocatechin were as 4.1 mg/g, 4.3 mg/g and 5.4 mg/g, respectively. Quantities of these compounds were higher than our results. They also determined the quantity of procyanidin B1 and procyanidin B2 as 2.9 mg/g and 0.25 mg/g, respectively. However, in our study, the total amount of procyanidins (B1 + B2) was 4.6 mg/g in NFM and the total procyanidin content was found higher than those found in Mudnic et al. (2009) study. The differences in (+) catechin, (-) epicatechin, epigallocatechin and procyanidins (B1 + B2) content of extracts used in two different studies may be due to the extraction solvents, procedures and collection from different localities.

It was observed that the quantity of kaempferol in field-grown fruit extract (FFM) was one half of field-grown leaf extract obtained with direct method (NM) and five times lower than in field-grown leaf extract obtained with gradual extraction

(NFM). The quantity of kaempferol was more than in the study of Milivojevic et al. (2010), where kaempferol amount was 0.00235 mg/g of water extract of *F. vesca* fruit. In the same study, the amount of myricetin and quercetin were found three and four times lower than in our study (Table 3.3.).

Luteolin-7-O- β -D glucoside and gallic acid were dominant compound in *in vitro*-grown callus extract (CFM) (0.2939 mg/g and 0.2725 mg/g, respectively), whereas lower and quite similar amounts were obtained in NM extract (0.2052 mg/g and 0.2452 mg/g, respectively). Also, the amount of gallic acid monohydrate in *in vitro*-grown leaf extract (ILM) (0.1156 mg/g) was half of CFM extract. The amount of apigenin and ($\pm$) taxifolin were found high in CFM extract (0.0110 mg/g and 0.0376 mg/g, respectively). The amount of vanillic acid was found high in CFM and CM extracts (0.1240 mg/g and 0.1115 mg/g, respectively) (Table 3.3.).

When the results of phenolic content and antioxidant activity were compared with each other, there is a significant relation between the phenolic contents and antioxidant activity of the only NFM and NM extracts. It is observed that the antioxidant activity results are high where the concentrations of phenolic compounds were found higher.

When the results of phenolic contents and antioxidant activities of the other extracts (FFM, CFM, ILM and CM) were compared, there is no relation between their phenolic contents and antioxidant activities. The antioxidant activity of the plant extracts is not dependent only to phenol (or polyphenol) content, however, other plant components (vitamins E and C, and caretonoids) contribute to the antioxidant activity of the plant extracts (Kiselova et al., 2006).

Table 3.3. Identified phenolic compounds and their amounts in the methanolic extracts of fruit, field-grown plant, *in vitro*-grown plant and callus of *Fragaria vesca* L.

STANDARD COMPOUNDS (mg/g dry extract)	Retention time (min)	EXTRACTS				
		NM	CM	ILM	NFM	CFM
Apigenin	6,945	0.0021	0.0043	0.0011	0.0021	0.0110
Caffeic acid	3,092	0.1943	0.0006	0.0028	0.2029	0.1764
(+) catechin	2,539	4.0416	0.016	1.134	4.524	0.0225
p-coumaric acid	3,884	0.1842	0.0862	0.0973	0.2201	0.1136
Coumarin	4,716	0.0714	≤ 0.0005	0.0067	0.0155	≤ 0.0005
Daidzein	5,829	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002
(-) epicatechin	3,32	1.8053	≤ 0.0002	≤ 0.0002	1.801	≤ 0.0002
(-) epigallocatechin	2,497	0.1200	0.0005	≤ 0.0002	0.2034	≤ 0.0002
Galic acid monohydrate	0,92	0.2452	0.0262	0.1156	0.8603	0.2725
Genistein	6,425	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002
Kaempferol	5,42	0.6155	≤ 0.001	0.0822	1.6355	0.0110
Luteolin-7-O-β-D glucoside	4,809	0.2052	0.0057	0.0064	0.0105	0.2939
Myricetin	5,27	0.0455	0.0085	0.0038	0.0187	≤ 0.0002
Procyanidin B1	2,225	3.2296	≤ 0.001	0.5137	3.6712	≤ 0.001
Procyanidin B2	2,886	3.1202	≤ 0.001	0.0309	3.6632	≤ 0.001
Procyanidin C1	3,216	0.7893	≤ 0.001	0.0014	2.2386	≤ 0.001
Pyrocatechol	1,902	0.0127	≤ 0.0005	≤ 0.0005	≤ 0.0005	≤ 0.0005
Quercetin	6,073	0.1029	0.0146	0.0058	0.3763	0.0270
Resveratrol	4,956	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002	≤ 0.0002
Rutin hydrate	4,898	0.0460	0.0013	≤ 0.0002	0.0102	0.0129
(±) Taxifolin hydrate	4,07	0.0021	0.0051	0.0004	0.0072	0.0376
Vanillic acid	3,063	0.0189	0.1115	0.0232	0.0675	0.1240

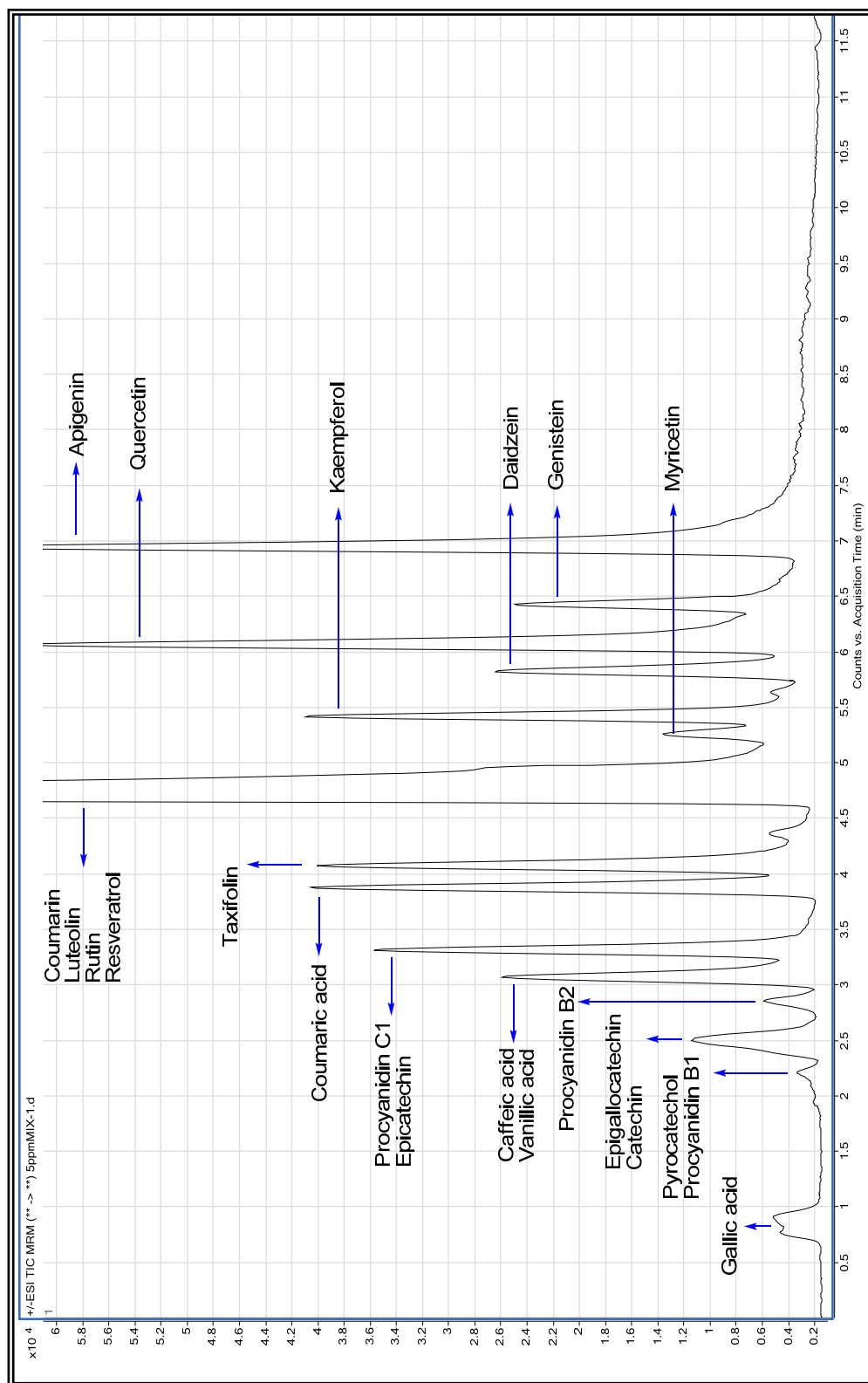


Figure 3.3. Total Ion Chromatogram (TIC) for the mixture of 22 authentic standards by LC-ESI-MS/MS analysis.

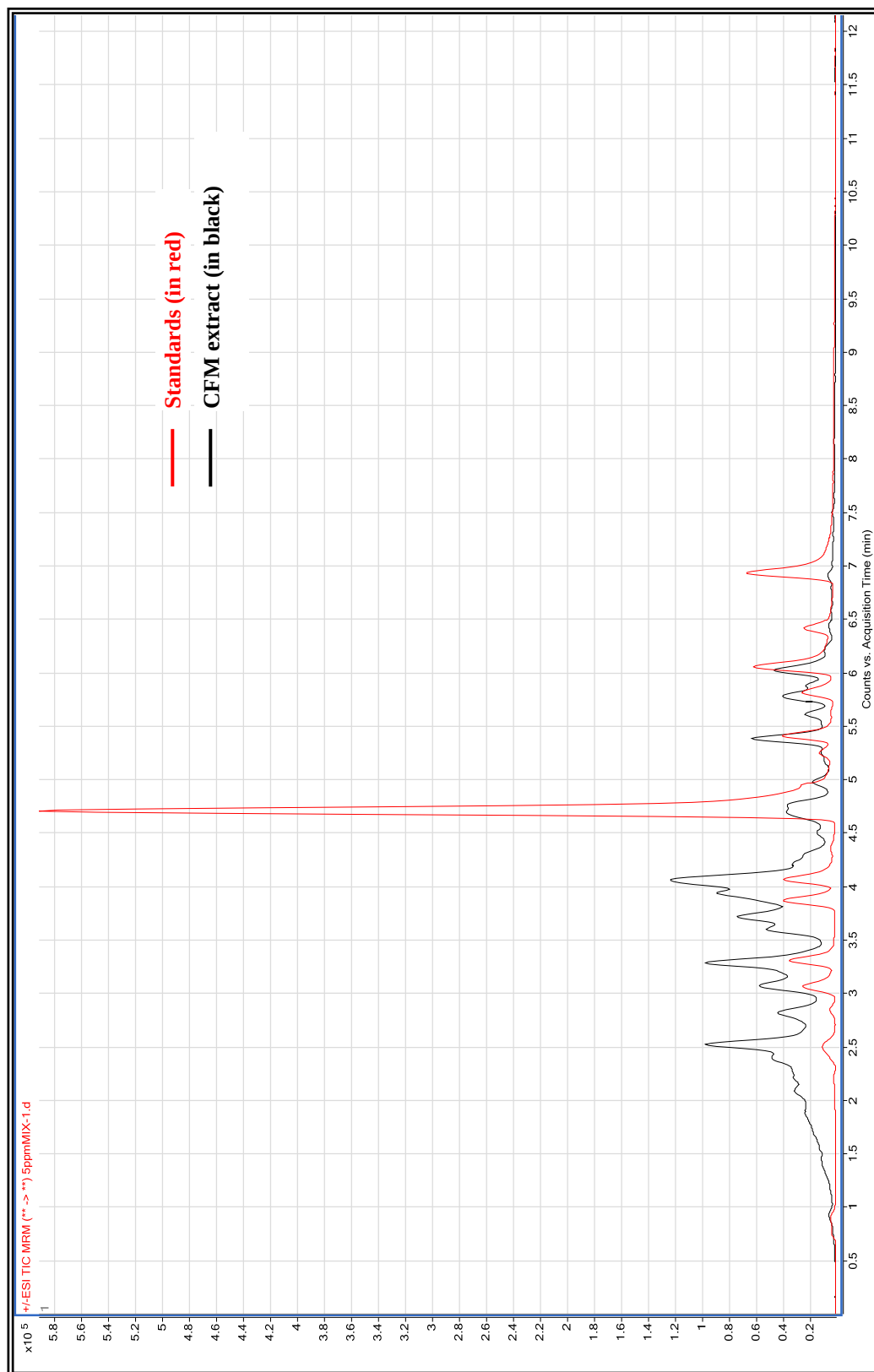


Figure 3.4. Total Ion Chromatogram (TIC) of CFM extract in comparison with those of standards by LC-ESI-MS/MS analysis.

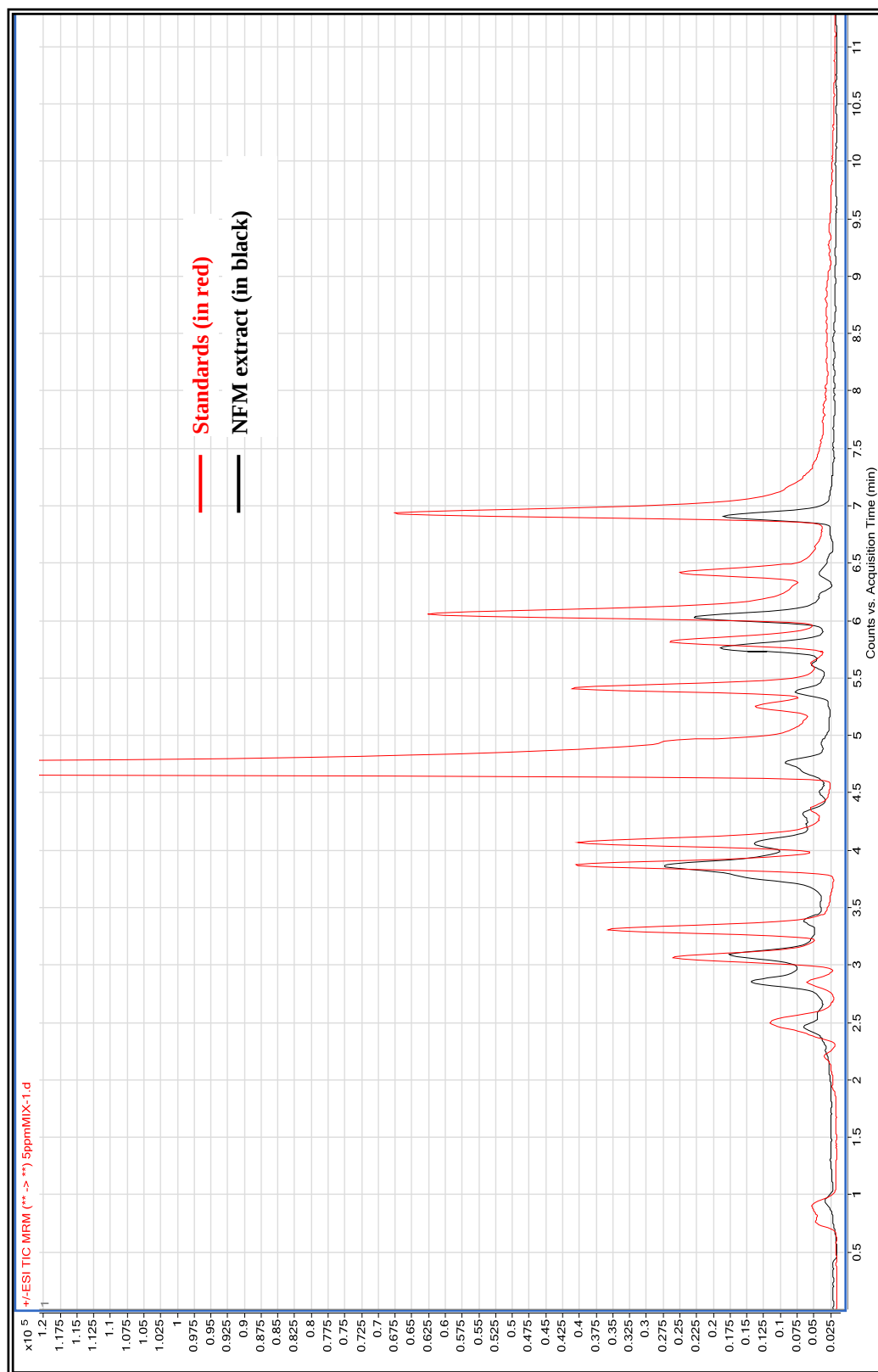


Figure 3.5. Total Ion Chromatogram (TIC) of NFM extract in comparison with those of standards by LC-ESI-MS/MS analysis.

CHAPTER 4

4. BIOLOGICAL ACTIVITY STUDIES

4.1. Introduction

Plants and their products have been used as medicines for as long as human life has existed on earth. Medicinal plants are widely and successfully used on every continent (Balandrin et al., 1993). The World Health Organization estimates that 80 percent of people in developing countries (65% of Earth's population) still rely on traditional medicine (Starbuck, 1999). The use of traditional medicine has increased in developed countries, mainly due to the failure of modern medicine to provide effective treatment for chronic diseases and emergence of multi-drugs resistant bacteria and parasites (WHO, 2002). The number of higher plant species on this planet is estimated at about 250.000. Of these, only about 6% have been screened for biologic activity, and reported 15% have been evaluated phytochemically. A total of 122 compounds were identified; 80% of these compounds were used for the same ethnomedical purposes. Further, it was discovered that these compounds were derived from only 94 species of plants. There should be an abundance of drugs remaining to be discovered in higher plants (Fabricant and Farnsworth, 2001). Plants have been a rich source of effective and safe medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against predation or infection (Cox and Balick, 1994).

Bioassays offer a special advantage in the standardization and quality control of heterogeneous botanical products. Such products can be “heterogeneous” due to the presence of mixture of bioactive components either from the same or from purposefully mixed botanical sources (McLaughlin et al. 1998). Screening studies for medicinal plants are important because folkloric usage of these plants gains some scientific justification. Bioassays are adaptable for the purpose of screening and testing plant extracts (McLaughlin et al., 1998).

4.1.1. Antibacterial Activity

The Disc Diffusion Assay (Kirby-Bauer Method) is a convenient antibiotic susceptibility test and used to screen for antibiotic activity, meaning this assay measures antimicrobial activity based on bacteriostatic/bacteriocidal properties. At the end of the 1950's, antimicrobial susceptibility testing was marked by lack of acceptable standardized procedures (Atlas, 1988). In the 1960's, it was developed by Kirby and Bauer (Prescott et al., 1990). Reasonably accurate and precise results can be obtained with Kirby-Bauer procedure, which all-procedural details are carefully standardized by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS) (Barry and Thornsberry, 1985; Atlas, 1988; Prescott et al., 1990).

In this disc-diffusion susceptibility test, a pathogenic microbe is inoculated onto a Mueller-Hinton agar plate. Filter paper discs containing known amounts of antimicrobial agents or antibiotics are placed on the agar surface. Inoculated plates are incubated at 37°C for 18-24 hours. The antimicrobial agents or antibiotics diffuse into the agar, causing a zone of inhibition of growth of the strain around the disc

corresponding to the susceptibility of the strains to the agents. At the end of the incubation period, a clear area (zone of inhibition) around the disc is measured. These zones are compared to known values obtained with standard drugs (Prescott et al., 1990). Standardized zones for each antibiotic disc have been established to determine whether the microorganism is sensitive (S), intermediately sensitive (IS), or resistant (R) to an antimicrobial agent or particular antibiotic. This method is not directly applicable to filamentous fungi, anaerobes, or slowly-growing bacteria (Atlas, 1988). A list of bacteria used in the antibacterial study and their representative diseases were given in Table 4.1.

Table 4.1. Bacteria used in antibacterial assay and their representative diseases, habitats and treatments (Levinson and Jawertz, 2002).

Pathogen		Representative Diseases	Habitat	Drugs of choice
Gram (-) Bacteria	<i>Escherichia coli</i>	Urinary tract infection (UTI), sepsis, neonatal meningitis, “traveler’s diarrhea’	Human colon, colonize the vagina and urethra.	Ampicilin or sulfonamides for UTI, Cephalosporin for meningitis and sepsis, trimethoprim- sulfamethoxazole for diarrhea
	<i>Proteus vulgaris</i>	Wound infection, UTI and sepsis	Human colon and environment (soil and water)	Trimethoprim-sulfamethoxazole or ampicillin, cephalosporin for serious infections.
	<i>Enterobacter cloacae</i>	Hospital-acquired pneumonia, Urinary Tract Infection, ophthalmic infections, and sepsis.	Enteric tract	Highly antibiotic-resistant.
	<i>Salmonella typhimurium</i>	Enterocolitis, diarrhea	Enteric tract	Ampicillin, ciprofloxacin or ceftriaxone
	<i>Serratia marcescens</i>	Conjunctivitis, Nosocomial infections and hospital-acquired pneumonia, Urinary Tract Infections, wound infections, and sepsis.	Enteric tract also soil and water	Gentamicine and cephalosporin
	<i>Klebsiella pneumoniae</i>	Pneumonia, Urinary Tract Infections (cytitis), wound infections, and sepsis.	Human upper respiratory and enteric tracts.	Cephalosporin or with aminoglycosides
	<i>Pseudomonas aeruginosa</i>	Wound infections, Urinary Tract Infections, pneumonia, eye infections, and sepsis.	Environmental water sources, hospital respirators and humidifiers and also skin, upper respiratory tract and colon	Antipseudomonal penicilin and aminoglycoside eg. gentamicin or amikacin
Gram (+) Bacteria	<i>Streptococcus pyogenes</i>	Suppurative (pus-producing) diseases, cellulitis, immunologic diseases such as rheumatic fever and acute glomerulonephritis	Human throat and skin	Penicilin G
	<i>Staphylococcus aureus</i>	Abscesses of many organs, endocarditis, gastroenteritis, toxic shock syndrome, sepsis, hospital-acquired pneumonia, and wound infections	Human nose and also human skin.	Penicilin G, Nafcillin
	<i>Staphylococcus epidermidis</i>	Endocarditis, neonatal sepsis, prosthetic hip infection, wound infections, and cytitis (UTI)	Human skin and mucous membranes.	Vancomycin plus either rifampin or an aminoglycoside.

4.1.2. Antitumor Activity

Potato disc tumor assay is used to determine a broad range of known and novel antitumor effects of drugs and plant extracts with *Agrobacterium tumefaciens* (*A. tumefaciens*) as the tumor initiator (Ferrigini et al., 1982). *A. tumefaciens* is the gram-negative bacteria that are the causative agent of Crown Gall disease (the formation of tumors) in over 140 species of dicot (Agrios, 1997). The bacteria contain large Ti (tumor-inducing) plasmids which carry genetic information (T-DNA) into plant's DNA where transforms normal, wounded plant cells into autonomous tumor cells (Ferrigini, 1982; Agrios, 1997).

The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals. It has been shown that the inhibition of Crown Gall tumor initiation on potato discs and subsequent growth showed good correlation with compounds and extracts active in the 3PS leukemic mouse assay (Galsky et al., 1980).

In preliminary studies in 1980–1983, the authors modified the Galsky potato disc method and tested the effectiveness, with appropriate statistical evaluation, of the modified assay as a prescreen for 3PS activity in crude plant extracts. The modified assay was initially performed on a series of natural antitumor compounds, on plant extracts, including an extract from periwinkle (*Vinca major*) called vincristine, suspected to have 3PS activity (Ferrigini et al., 1982).

A major problem in using the 3PS *in vivo* tumor assay is that high concentrations of antitumor agent often prove fatal to the subjects. The potato tumor assay eliminates this problem (Galsky et al., 1980; Ferrigini et al., 1982; McLaughlin

1991; McLaughlin et al., 1993; Coker, 1999; Turker and Camper 2002; Boonkaew et al., 2003; Coker et al., 2003). Crown Gall tumors on potato discs could routinely be employed as a comparatively rapid, inexpensive, safe, animal-sparing, and statistically reliable prescreen for *in vivo* 3PS antitumor activity (Ferrigini et al., 1982).

4.1.3. Brine Shrimp Toxicity Assay

Bioactive compounds in plants (secondary plant metabolites) are compounds produced by plants having pharmacological or toxicological effects in man and animals. Globally, there is a gradual increase in medicinal plant sales. However, till now no much is known about the dose-related toxicity of the medicinal plants, particularly at the histological side. Bioactive compounds can be useful but might have serious dose-related side effects (Taylor et al., 2001). Therefore, in view of their wide spread use of medicinal plants in alternative medicine, toxicological assessment becomes imperative in order to arrive at potencies that can be considered as safe formulations for clinically efficient remedies (Chaudhary et al., 2010).

The *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. One of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise is the toxicology which is simply pharmacology at a higher dose, thus if we find toxic compounds as a lower, or non-toxic, dose might elicit a useful on a physiologic system (McLaughlin, 1991).

The preclinical toxicological evaluation, carried out routinely in mice, is of great importance for validation of the traditional use of medicinal plants. The current tendency is the substituting of the use of laboratory animals in toxicological tests, due to the high cost and animals' suffering caused by these tests (Parra et al. 2001). Also, since most active plant principles are toxic at elevated doses, a possible approach to developing an effective general bioassay might be simply to screen for substances that are toxic to zoological systems. A tiny crustacean, brine shrimp has been using as a rapid, in-house bioassay tool for screening and fractionation monitoring of physiologically active plant extracts. The brine shrimp, *Artemia salina* (Leach), is an invertebrate component of fauna of saline aquatic and marine ecosystems and it is readily available at low cost in pet shops and remains viable for years in the dry state. The eggs hatch within 48 hours, providing large number of larvae (nauplii) upon being placed in brine solution (Meyer et al., 1982). Brine shrimps have been previously utilized in various bioassay systems and it can be used in a laboratory bioassay in order to determine toxicity through the estimation of the medium lethal concentration (LC_{50} value) (Lewan et al., 1992), which have been reported for a series of toxins and plant extracts (Meyer et al., 1982). The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the microwell scale. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (50 mg for crude extracts). Moreover, it does not require animal serum as is needed for cytotoxicities (McLaughlin et al., 1991).

4.1.4. Anticancer Activity

Cancer is a group of illnesses that result from cells in the body growing abnormally. These cells divide and produce new cells in an uncontrolled way that can spread throughout the body and cause damage to essential organs. Cancer is classified into various types of cancer by their original locations, such as lung, colon, breast, or prostate (Dervan, 1999). Normal cells grow and divide to form new cells as the body needs them. When normal cells grow old or get damaged, they die, and new cells take their place. Sometimes, this process goes wrong. New cells form when the body doesn't need them, and old or damaged cells don't die as they should. The buildup of extra cells often forms a mass of tissue called a tumor. Not all tumors are cancerous. Tumors that aren't cancer are called benign and are not as harmful as malignant tumors. Cancer cells can spread by breaking away from the original tumor. They enter blood vessels or lymph vessels, which branch into all the tissues of the body. The cancer cells may attach to other tissues and grow to form new tumors that may damage those tissues. The spread of cancer to the other parts of the body is called metastasis (American cancer society, 2012).

Cancer is one of the leading causes of death in developed and developing countries, and responsible for about 25% of all deaths with 30 % of these from lung cancer. On a yearly basis, 0.5% of the population is diagnosed with cancer (Jemal et al., 2008). Cancer is the second largest cause of death after heart diseases in the worldwide, where one in four deaths is due to cancer (Rath, 2001). The most common fatal cancers in men are lung and bronchus, prostate, and colon and rectum and in women are lung and bronchus, breast, and colon and rectum (Jemal et al.,

2008). To date, most common form of cancer-related death among women is breast cancer (Rath, 2001).

Although the incidence of many types of cancer has declined in the United States over the last thirty years, the incidence of breast cancer globally has increased (Bray et al., 2004). For many years, breast cancer incidence and mortality rates have been the highest in North America and Northern Europe (Verkooijen et al., 2003), intermediate in Southern Europe and Latin America, and the lowest in Asia and Africa (Parkin et al., 2005).

Women with breast cancer have many treatment options. These options are surgery, radiation therapy, hormone therapy, chemotherapy, and targeted therapy. Surgery and radiation therapy are types of local therapy. They remove or destroy cancer in the breast. Hormone therapy, chemotherapy, and targeted therapy are types of systemic therapy. The drug enters the bloodstream and destroys or controls cancer throughout the body. Natural products, especially plant-derived compounds have a long history of use in the treatment of cancer (Shoeb, 2006). Indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied toward combating cancer (Newman, 2000 and Butter, 2004). More than 3000 plant species have reportedly been used in the treatment of cancer (Hartwell, 1982). Of commercially available anticancer drugs, 60 % are natural origin which is derived from plants, marine organisms or microorganisms (Shoeb, 2006).

MCF-7 is the human breast carcinoma cell lines isolated in 1970 from a 69-year-old Caucasian woman's pleural effusion which is the most commonly used breast cancer cell line *in vitro* cancer research in the world (Burdall et al., 2003).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate) assays are used in cell proliferation and cytotoxicity assays but most widely used one is MTT assay. These assays are tetrazolium salt colorimetric assays which detect living cells, not dead cells (Berridge et al., 1996). In MTT assay, MTT enters the cells and passes into mitochondria where mitochondrial succinate dehydrogenase in living cells reduces yellow tetrazolium salt (MTT) to water insoluble crystalized purple formazan via the cleavage of the tetrazolium ring. Unlike MTT, WST-1 is efficiently reduced by NADH and NADPH and also occurs at the cell surface (Berridge et al., 1996).

4.1.5. Antioxidant Activity

Antioxidant is a molecule, capable of slowing or inhibiting the oxidation of other molecules which are damaged by unstable molecules known as free radicals. Antioxidant molecules interact with free radicals and prevent the process of oxidation by neutralizing free radicals (Moon and Shibamoto, 2009).

Reactive oxygen is a product of normal metabolism and attacks biological molecules, leading to cell or tissue injury. Reactive oxygen species (ROS) induce some oxidative damage to biomolecules like lipids, carbohydrates, proteins and nucleic acids (DNA) which can initiate a chain of events resulting in the onset of variety of disease such as aging, cancer and many other diseases (Aruoma, 1994).

ROS are continuously produced as a consequence of normal aerobic metabolism such as respiration and some cell mediated immune functions. ROS include free radicals which have unpaired electrons such as superoxide anion radicals

(O_2^-), hydroxyl radicals ($OH\cdot$), and non free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gülçin et al., 2006).

There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. When there is imbalance between ROS and antioxidant defense mechanisms, ROS lead to oxidative modification in cellular membranes or intracellular molecules. In addition, under pathological conditions or oxidative stress, ROS are overproduced and result in oxidation of membrane lipids, leading to the accumulation of lipid peroxides (Gülçin et al., 2003). Lipid peroxidation is believed to be associated with some diseases such as carcinogenesis, mutagenesis, ageing, and arteriosclerosis (Gülçin et al., 2004).

In other words, exogenous factors such as smoking, ionising radiation, certain pollutants, organic solvents and pesticides, and endogenous metabolic processes in the human body such as normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages and peroxisomes might produce highly reactive free radicals, especially oxygen derived radicals which are capable of oxidizing biomolecules. Oxidative damage plays a significantly pathological role in human disease such as acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer and accelerating ageing. However, antioxidant supplements or foods containing antioxidants can protect the human body from free radicals and ROS effects, and can retard the progress of many chronic diseases as well as lipid peroxidation (Gülçin et al., 2004).

Many naturally occurring antioxidant compounds, especially plant origin, have been identified as free radical or active oxygen scavengers. At the present time, BHA, BHT, propyl gallate, and tert-butylhydroquinone are the most commonly used

antioxidants. But, recently, interest has considerably increased in finding naturally occurring antioxidants such as vitamin E (α -tocopherol), vitamin C, and polyphenols or flavonoids instead of synthetic ones which are being restricted due to their side effects such as carcinogenicity. Natural antioxidants can protect the human body from free radicals and reduce the incidence of chronic diseases including heart disease. Hence, the free radical scavenging activity studies on natural antioxidants have gained increasingly greater importance (Gülçin et al., 2003).

Plant tissues synthesize a wide variety of phenolic compounds (Loliger, 1991). Many kinds of antioxidative components that contain polyphenolic compounds, flavonoids, chlorophylls, carotenoids, tocopherol derivatives, lignan, and related isoprenoids have been isolated from different kinds of plants, such as oilseeds, cereal crop, vegetables, leaves, roots, spices, herbs, and seaweeds, for use as antioxidants (Wettasinghe and Shahidi, 1999, Gülçin et al., 2006).

Natural antioxidants are known to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic, and vasodilatory activity. In fact, a fundamental property important for life is antioxidant activity and this property may give rise to anticarcinogenicity, anti-mutagenicity, and anti-aging activity, among others (Cook and Samman, 1996; Liyana- Pathirana and Shahidi, 2006).

Several *in vitro* methods are used for the estimation of efficiency of natural or synthetic antioxidants, like ORAC assay (oxygen radical absorbing capacity), the TRAP method, the TEAC method and DPPH method (free radical scavenging activity) (Albayrak et al., 2010). It is very difficult to select a suitable antioxidant assay method. Antioxidants act by several mechanisms and no one assay can capture

the different modes of action of antioxidant. Conventional cuvette assay of radical scavenging activity is replaced by 96-well plate titer assay from past couple of years. Cuvette assay method uses UV-VIS spectrophotometry to see the absorbance, whereas 96-well plate method uses ELISA plate reader for absorbance. The conventional method is very tedious, time consuming method, allows only one sample to read a time and requires high quantity of reagent, whereas the 96-well plate method is time saving and it reads about 96 samples at a time, with small amount of reagent.

4.2. Materials and Methods

4.2.1. Antibacterial Assay

Antibacterial activities of 17 different extracts (NW, NE, NM, NA, NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW and ILM) (Table 3.2.) of *F. vesca* were evaluated.

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott et al. 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883) which are gram-negative bacteria and *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are gram-positive bacteria.

Each lyophilized bacteria discs (BD, Microtrol Discs - Becton Dickinson Laboratories, France) were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. After incubation, one bacteriological

loop from each broth was streaked on Tryptic Soy Agar (TSA) plates and incubated for 2 days at 37°C. After 2 days, a single colony was removed and streaked on a new TSA plate for pure culture and incubated 37°C for 2 additional days. After 2 days, 1-2 loops of pure culture were transferred to 10 ml of 9 % saline suspension in a test tube for each bacterial strain. The turbidity of the each suspension is adjusted to obtain turbidity visually comparable to that of a 0.5 McFarland standard (1.5×10^8 cfu/ml). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface of a (60 X 15 mm) Mueller-Hinton (M-H) agar plate (One test tube was used for 10 agar plate). Agar plates were streaked four times, each time turning the plate at a 90° angle and finally rubbing the swab through the edge of the plate.

All extracts were dissolved in sterile distilled water in order to obtain a final concentration of 100 mg/ml. Then, all dissolved extracts were sterilized by filtering through a 0.22 µm filter (Pal-Gelman Laboratory) and sterile filter paper discs (Glass Microfibre filters, Whatman®; 6 mm in diameter) were impregnated with 13 µl of extract. There were five replicates in each plate and two plates for each extract tested for each bacterium. Five different antimicrobial susceptibility test discs were used as positive controls which consist of (Bioanalyse®): Erythromycin (15 µg) (E-15), Ampicillin (10 µg) (AM-10), Carbenicillin (100µg) (CB-100), Tetracycline (30 µg) (TE-30) and Chloramphenicol (30 µg) (C-30). Four antibiotic discs were used for each plate and run in duplicate. Negative control consisted of water. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hrs of incubation, diameter (mm) of inhibition zone was measured. All experiments were repeated three times.

All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc. Chicago, IL, USA).

4.2.2. Anti-tumor Assay

Antitumor activities of 11 different extracts (NW, NE, NM, DFCW, DFHW, DFCE, DFHE, FFCW, FFHW, FFCE and FFHE) (Table 3.2.) of *F. vesca* were evaluated.

Antitumor activity of *F. vesca* extracts was assessed with the potato disc method as modified by McLaughlin's group (Ferrigini et al., 1982). *Agrobacterium tumefaciens* (strain A 281) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28°C. Seven to eight loops of *A. tumefaciens* were added to 10 ml Phosphate buffered saline (PBS) (pH=7.2). Bacteria concentrations were standardized to 1.0×10^9 Colony Forming Units (CFU) as determined by an absorbance value of 0.96 ± 0.02 at 600 nm. Camptothecin (Sigma[®]) (tumor suppressant) was used as a positive control. All extracts were dissolved in sterile distilled water (100 mg/ml); therefore, water was used as a negative control. All extracts, controls and solutions were filter sterilized through 0.22 μ m syringe filter (Pal-Gelman Laboratory). The following design was followed:

Positive control: 600 μ l camptothecin stock + 150 μ l sterile distilled water + 750 μ l *A.tumefaciens* in PBS.

Solvent control I: 600 μ l control (water) + 150 μ l sterile distilled water + 750 μ l *A. tumefaciens* in PBS.

Solvent control II: 600 µl control (water) + 150 µl sterile distilled water + 750 µl PBS.

Test extracts: 600 µl test extract + 150 µl sterile distilled water + 750 µl *A.tumefaciens* in PBS.

Potatoes (*Solanum tuberosum* L.) were washed and scrubbed with a brush under running water and surface sterilized by immersion in 10% Domestos® for 20 minutes. Tubers were then placed on sterile Petri dishes and cut along either side revealing the largest flat surface area available. The trimmed tubers were then immersed in 20% bleach (Domestos®) for 15 minutes and then placed on sterile Petri dishes. Cylinders (8 mm diameter) were cut from the center of potato tissue (skin portion was eliminated) using a cork borer on sterile paper towels and placed in sterile distilled water. Cylinders were rinsed twice more using sterile distilled water. Each cylinder was cut into 0.5 cm discs after excluding 1 cm end pieces. These discs were transferred to 24-well culture plates containing water-agar (15g/l). One well plate (24 cell wells) was used for each experiment; each experiment had 24 replicates. Each disc was overlaid with 50 µl of appropriate inoculum. No more than 30 min elapsed between cutting the potato discs and inoculation (McLaughlin 1991). Plates were incubated at 28°C in the dark for 2 weeks. After 2 weeks, discs were stained with Lugol's reagent (I2-KI; 5% I2 plus 10% KI in distilled water) and tumors on each disc were counted. Lugol's reagent stains the starch in potato tissue to dark blue to dark brown color, but the tumors do not take up the stain and appear creamy to orange. Experiments were repeated three times. Percent inhibition of tumors was calculated (McLaughlin 1991; McLaughlin et al. 1993; McLaughlin et

al. 1998) using the formula “% inhibition= [(solvent control mean - tested extract mean) / solvent control mean] x 100”.

Significant activity was indicated when two or more independent assays gave consistently negative values of approximately 20% or greater inhibition. Data obtained with this assay have correlated well with various other antitumor assay including small animal systems (McLaughlin et al., 1993; McLaughlin et al., 1998).

4.2.2.1. Bacterial Viability Testing

Standardized bacterial suspension (1×10^9 CFU of *A. tumefaciens* in PBS) was serially diluted with PBS to 1×10^3 CFU. Bacterial viability was determined by incubating 1 ml of each plant extract with 1 ml of bacterial suspension (1×10^3 CFU of *A. tumefaciens* in PBS) in microcentrifuge tubes (4 tubes per extract) and left for 30 minutes. At 30 minutes after inoculation, 0.1 ml of inoculum (bacteria + extract) was removed and inoculated on YEM media with spread plate technique. After 24 h incubation of inoculated plates at 28 °C, colony counts were made. Also, bacterial growth was evidenced by growth across the plates (Coker et al., 2003).

4.2.3. Brine Shrimp Toxicity Test

The brine shrimp lethality test was used to predict the cytotoxic activity of the extracts (Meyer et al., 1982)

Brine shrimp (*Artemia salina* Leach) eggs were purchased from a pet store. Seawater was prepared by dissolving 36 g sea salt in 1 liter of distilled water and put in V-shaped, plexiglass hatching container. Oxygen was supplied and 60-watt lamp was positioned near the container to provide direct light and heat (~ 27-28 ° C). One-

teaspoon brine shrimp eggs were placed in 1 liter of seawater. After 10 to 12 hr, cysts began hatching. Two days was allowed for the shrimp to hatch and mature as nauplii (shrimp can be used 48-72 hrs after the initiation of hatching). After 72 hr they should be discarded (McLaughlin et al., 1991). Nauplii were harvested by turning off the aeration and letting the culture settle for about 10 min. Hatched, empty shells floated on the surface and unhatched cysts sank to the bottom. Newly hatched nauplii concentrated just above the unhatched cysts on the bottom. Since the nauplii are positively phototropic (attracted to light), shining a light at the middle of the container and shading the container at the bottom helped direct them to an area where they can be easily harvested by siphoning or draining (Hoff and Snell, 1987). Stock solutions of extracts and other commercial products were prepared by suspending dried extract in salt water to prepare a 10,000 mg/ml solution. The suspension was mixed for 5 min; then, 1000, 100 and 10 mg/l solutions were prepared by dilution. Nine well culture plates were used. 10 nauplii were placed into each well and 2.5 ml of appropriate concentration of extract+ salt mixture was added. Uncovered plates were incubated for 24 hr at room temperature under illumination. There were three replicates for each concentration (10,000, 1000, 100, 10 mg/l) and experiments were repeated three times. The same saline solution used to prepare the stock test sample solution was used as a negative control. As a positive control, MS-222 (tricaine methane sulfonate), a common fish anesthetizer, was used at concentrations of 1, 10, 100, 1000 and 10,000 mg/l.

After 24 hrs, numbers of dead nauplii were counted. Live nauplii were then killed by addition of few drops of MeOH and the total dead was counted to confirm the number of animals in each well. LC_{50} (lethal concentration for 50 % mortality

after 24 hr exposure) and 95% confidence intervals were determined according to the Read Muench method (Sam, 1993).

4.2.4. Anticancer Assay

Anticancer activities of 14 different extracts (NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW, NM and ILM) (Table 3.2.) were evaluated.

Anticancer activity test of *F. vesca* extracts were carried out on human breast cancer (MCF-7) cell line. MCF-7 cell line was obtained from Istanbul University, Faculty of Medicine, Department of Histology and Embryology. This cell line was continuously maintained from the stock cultured at Abant Izzet Baysal University, Faculty of Medicine, Department of Pharmacology. The cells were maintained in Dulbecco's modified eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) and 100 ng/ml of penicillin and streptomycin (Sigma). Cells were allowed to grow in tissue culture flasks and were kept in CO₂ incubator at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For experimental purpose, cells from exponentially growing culture were used. All experiments were repeated three times.

The MTT (3-[4-5 dimethylthiazol-2-yl] 2-5 diphenyl-tetrazolium bromide) assay was used in this study to assess growth inhibition. For the assay, the MCF-7 cells from exponential-phase culture were detached with 0.1% trypsin-EDTA (Sigma Chemical Co.) to make single-cell suspensions, and viable cells were counted using a Coulter counter. About 1×10^4 viable cells in 100 µl of culture medium (DMEM) were added to each well in of a 96-well cell culture plate and incubated for 24 h at 37

°C under 5 % CO₂ in a humidified incubator to allow cells to attach to wells of 96 well cell culture plates. After 24 h or 48 h, the cell attachment was checked and the cells were treated with the serial concentrations of *F. vesca* extracts and then incubated for 24 h or 48 h.

Hexane, dichloromethane and methanolic extracts were initially dissolved in an amount of 100 % DMSO. Aqueous extracts were initially dissolved in an amount of water (80 mg/ml) and further diluted in medium to produce six concentrations. 100 µl per well of each concentration was added to the plates to obtain final concentration of 200, 100, 50, 25, 12.5 and 6.25 µg/ml for the extracts. The final concentration of DMSO for the tested dilutions was not greater than 0.25 % (v/v) to avoid solvent toxicity, the same as in solvent control wells. The final volume in each well was 200 µl. The plates were incubated for 24 h or 48 h.

After incubation of 24 h or 48 h, the culture medium was removed and replaced with 90 µl fresh culture medium, DMEM. Then, 10 µl of MTT solution (5 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was added to each well and the final concentration of MTT reached 0.5 mg/l. The culture was incubated at 37 °C in 5% CO₂ for additional 4 h. After 4 h, 100 µl DMSO/well were added to all samples for dissolving of formazan that is the final product of MTT reaction and were allowed to incubate at 37 °C, in a 5% CO₂ humidified incubator for a night. After incubation, absorbance of formazan was measured spectrophotometrically in a Multiskan FC microplate photometer reader (Thermo Fisher Scientific Inc.) at 570 nm. Each experiment was carried out three times in triplicate. The percent cytotoxicity of extracts (cell viability) was calculated according to their control groups as;

$$100 \times A_{570 \text{ nm}} (\text{sample}) / A_{570 \text{ nm}} (\text{control}).$$

Quantitative data were expressed as mean absorbance $\pm$ S.E.M. The significance of differences between the means was assessed using one-way ANOVA using SPSS 15 software.

4.2.5. Antioxidant Assay

4.2.5.1. Free Radical Scavenging Activity (DPPH (2,2-diphenyl-picrylhydrazil) Method)

Free radical scavenging activity of the methanolic extracts of *F. vesca* was determined spectrophotometrically by monitoring the disappearance of 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma-Aldrich Chemie, Steinheim, Germany) at 517 nm, according to the method described by Brand-Williams et al. (1995).

All methanolic extracts of *F. vesca* were weighted as 0.003 g (NFM, FFM, CFM, ILM, CM and NM) (Table 3.2.) and they were dissolved 3 ml of methanol. Finally, this stock solution was composed as 1000 µg/ml concentration. The extract solutions were diluted with methanol (25, 50, 100, 200 µg/ml) from the stock extract solutions. Ascorbic acid was used and a standard curve was prepared using different concentrations of ascorbic acid (200, 100, 50, 25 and 12.5 µg/ml).

0.1 mM solution of DPPH• was prepared in methanol by daily before UV measurements. 1.5 ml of the diluted samples or ascorbic acid (200, 100, 50, 25 and 12.5 µg/ml) was mixed vigorously with 0.5 ml of 0.1 mM DPPH in methanol. These solutions were shaken vigorously and incubated at room temperature in dark for 30 minutes. After 30 minutes, the decrease in the absorbance of these solutions was measured at 517 nm with Hitachi U-1900, UV-VIS Spectrophotometer 200V against

blank samples. The blank sample includes only 1.5 ml of the methanol and 0.5 ml of the 0.1 mM DPPH which was used as a positive control (Milardovic et al., 2006; Brand-Williams et al., 1995). All analyses were made in triplicate. The DPPH• scavenging capacity of the extracts was calculated using the following equation:

DPPH• Scavenging Effect (% inhibition) = $[(A_0 - A_1)/A_0] \times 100$ (Gülçin et al., 2003), where A_0 is the absorbance of the positive control
 A_1 is the absorbance of tested extracts.

4.2.5.2. Total Phenolic Assay (Folin-Ciocalteu Method For Total Phenolic Content)

Total soluble phenolics in the methanolic extracts of *F. vesca* were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound.

Preparation of extract solution: 0.01 g each methanolic extract of *F. vesca* (NFM, FFM, CFM, ILM, CM and NM) (Table 3.2.) was weighted. Then, 2 ml of distilled water was added to extracts. These extract solutions were stock solution (5 mg/ml). Later, these solutions were diluted as 1 mg/ml.

Preparation of gallic acid stock solution: Gallic acid was used as phenol control. In a 100 ml volumetric flask, 0.5 g of dry gallic acid was dissolved in 100 ml distilled water (5 mg/ml) and diluted as 0, 50, 100, 150, 200, 250 and 500 mg/ml gallic acid. Then, a calibration curve was obtained by using these gallic acid solutions to determine phenol concentration of *F. vesca* extracts.

Na₂CO₃ Solution: 20 g of anhydrous sodium carbonate was dissolved in 100 ml of water in a 100 ml volumetric flask.

20 µl from each calibration solution (gallic acid), sample, or blank (water) was added into separate centrifuge tubes, and 1.58 ml water was added into each, and then 100 µl of the Folin-Ciocalteu reagent was added and mixed well. After 2 minutes, 300 µl of Na₂CO₃ solution was added and shaken very well. The solutions were incubated at 20⁰C for 2 hours and the absorbance of each solutions were measured at 765 nm against the blank (all reagents without sample) using the spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200V, Japan). The absorbance vs. concentration was plotted. After the created calibration curve with standard gallic acid, the total concentration of phenolic compounds in the samples was determined as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph. The results were expressed in mg of gallic acid per g (GEA) of dry weight of plant. All analyses were made in triplicate.

4.2.5.3. Total Flavonoid Assay (Aluminum chloride (AlCl₃) Colorimetric Assay For Total Flavonoids)

The total flavonoid content in methanolic extracts of *F. vesca* (NFM, FFM, CFM, ILM, CM and NM) (Table 3.2.) was measured by aluminum chloride (AlCl₃) colorimetric assay. Catechol was used as a reference flavonoid.

The preparation of extract solution: 0.005 g each methanolic extract of *F. vesca* was weighted and dissolved in 2ml ethanol (2500 mg/ml). Later, this stock solution will be diluted to 1250 mg/ml and 500 mg/ml concentrations.

The catechol solution preparation: 0.0125 g of catechol was weighted. Then, it was dissolved in 25 ml ethanol. Thus, this stock solution was adjusted as 500 mg/ml concentration. Different concentrations of catechol (20, 40, 60, 80 and 100 mg/ml) were prepared in order to obtain standard calibration curve of catechol.

500 µl of extract solution or standard solution of catechol was added to a 10 ml test tube containing 2 ml distilled water. Then, 150 µl 5% NaNO₂ was added to the test tubes. After 5 minutes, 150 µl of 10 % AlCl₃ was added. 1 minute later, 1000 µl of 1M NaOH was added to the mixture. Immediately, the reaction tube was diluted to volume 5 ml with the addition of 1200 µl distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm versus a blank. Samples were analyzed in three replications (Chang et al., 2002). The total flavonoid content of samples of *F. vesca* was expressed as mg catechol equivalents (CE) of dried weight of plant.

4.3. Results and Discussion

4.3.1. Antibacterial Activity

Seventeen different extracts (NW, NE, NM, NA, NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW and ILM) (Table 3.1.) of *F. vesca* were used to screen for antibacterial activity (Table 3.1. and Table 4.2.).

Bacterial growth was generally sensitive to the tested reference antibiotics (Table 4.2.). Inhibition zones varied from 50.4 mm for carbenicillin and *S. pyogenes* to 8.0 mm for tetracycline and *S. epidermidis* (Table 4.2.). Since final concentrations of extracts were adjusted with distilled water or DMSO, they were used as a negative control and there was no inhibition with these control solvents. For positive control

(quality control), results of antimicrobial susceptibility test discs were consistent with expected susceptibility for each bacterial strain tested.

Among all tested extracts, hexane and dichloromethane extracts of fruit (FFH and FFD), field-grown leaves (NFH and NFD) and *in vitro*-grown callus (CFH and CFD) did not show any antibacterial activity against tested bacteria.

In our study, both gram positive and gram negative bacteria seemed to be susceptible to the inhibitory effects of the *F. vesca* extracts. None of the tested extracts showed antibacterial activity against *S. marcescens*, *S. typhimurium* and *E. coli* (gram negative bacteria).

Among gradually prepared extracts of *in vitro*-grown callus, only aqueous extract (CFW) exhibited good antibacterial activity against only *E. cloacea* (15.7 ± 0.2 mm). The remaining extracts did not show antibacterial activity against all tested bacteria (Table 4.2. and Figure 4.2.).

Among gradually prepared extracts of field-grown leaves, the best antibacterial activity was obtained with methanolic (NFM) and aqueous extracts (NFW) against *E. cloacae* (18.1 ± 0.2 and 18.9 ± 0.6 mm, respectively). NFW exhibited of a broad spectrum activity against both gram positive and gram negative bacteria (Table 4.2.). This activity against both types of bacteria may be indication of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins (Srinivasan et al, 2001). NFW extract showed better antibacterial activity against *S. epidermidis* (10.4 ± 0.4 mm) than NFM extract (9.2 ± 0.2 mm). However, NFM extract showed better antibacterial activity against *S. pyogenes* (10.2 ± 0.8 mm) than NFW extract (8.5 ± 0.1 mm). Although *S. aureus*, *P. aeruginosa*, *P.*

vulgaris and *K. pneumonia* were susceptible to NFW extract, these bacteria were not susceptible to NFM extract (Table 4.2.).

Among gradually prepared extracts of fruits, aqueous (FFW) extract showed the best antibacterial activity against *E. cloacae* (20.8 ± 0.3 mm) and followed by methanolic extract (FFM) against *E. cloacae* (16.2 ± 0.7 mm) (Table 4.2. and Figure 4.2.). Although FFW exhibited moderate antibacterial activity against *P. aeruginosa* and *P. vulgaris*, FFM did not show antibacterial activity against these bacteria. FFW extract showed better antibacterial activity against tested gram positive bacteria than FFM extract and also *S. epidermidis* was very sensitivity to FFW extract (13.2 ± 0.8 mm) (Figure 4.1.).

Methanolic extract (ILM) of *in vitro*-grown leaves showed the best inhibitory activity against *E. cloacae* whose inhibition zone was 16.3 ± 0.4 mm. ILM extract exhibited weak antibacterial activity against *S. pyogenes* and *P. vulgaris* (8.3 ± 0.2 and 9.0 ± 0.0 mm, respectively).

Aqueous (NW), ethanolic (NE), methanolic (NM) and acetone (NA) extracts of field-grown leaves showed moderate antibacterial activity against tested gram positive bacteria. Among these extracts, against *S. aureus* and *S. epidermidis*, NA extract showed the best antibacterial activity. Average zone of inhibition was 12.8 ± 0.5 mm and 14.5 ± 0.3 mm, respectively. Against *S. pyogenes*, NE extract showed the best antibacterial activity (13.5 ± 0.9 mm). Against *P. vulgaris*, NA (12.3 ± 0.6 mm) and NM (12.3 ± 0.3 mm) extracts showed the best antibacterial activity. Observed antibacterial activities of these extracts against *S. epidermidis* were better when compared with reference antibiotic tetracycline (8.0 ± 0.0 mm). Among the tested bacteria with these extracts (except NE extract), the strongest antibacterial

activity was obtained against *E. cloacae*. For NA, NW and NM extracts, inhibition zone was 19.5 ± 0.6 mm, 16.0 ± 0.0 mm, and 14.5 ± 0.3 mm, respectively.

When we made a comparison within all used bacteria, *E. cloacae* was the most susceptible one to the extracts having antibacterial activity. The best antibacterial activity was obtained with FFW, NA, NFW and NFM extracts. *E. cloacae* is highly resistant organism against widespread use of broad spectrum antibiotics. Once many of the drugs were used to kill this organism, they won't be no longer effective against it due to the fact that the organism has acquired genetically derived resistance mechanisms over time (Pickering, 1996-1997). The antibacterial activity of *F. vesca* extracts against *E. cloacae* may be explained why *F. vesca* is used in folk medicine as vulnerary.

Against *S. aureus*, the best antibacterial activity was obtained with NA extract and followed by NE and NM extracts. The growth of *S. aureus* was inhibited very effectively by flavone, flavonoids, flavonones, flavonols and naringenin (Mori et al., 1987; Rauha et al., 2000). Against *S. epidermidis*, the best antibacterial activity was obtained with NA, FFW, NM and NE extracts, respectively. Flavone, quercetin and morine were found to be active against *S. epidermidis* (Nishino et al., 1987). Correspondingly, in our study, quercetin was detected in NM extract by LC-MS/MS (Table 3.3.). Against *S. pyogenes*, the best antibacterial activity was obtained with NE extract. Ethanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, alkaloids, sterols and propolis. Also, acetone extracts of plant materials may contain flavonols (Cowan, 1999). The effectiveness of the ethanol and acetone extract of *F. vesca* may be explained by the inhibitory effect of these compounds on bacteria.

The antibacterial activity of *F. vesca* extracts against *S. aureus*, *S. epidermidis* and *S. pyogenes* may be explained why *F. vesca* is used in folk medicine to treat diarrhea, rheumatism and skin diseases.

It can be conceivable that strong antibacterial activity may come from phenolic constituent of *F. vesca*. Polyphenols, such as tannins and flavonoids, are important antibacterial substances. Ellagitannin (punicalagin) in pomegranate (*P. granatum*) is the active substance responsible for its antimicrobial activity (Machado et al., 2002). Shan et al. (2007) reported many plant flavonoids (e.g., epigallocatechin, catechin, myricetin, quercetin) have antimicrobial activity. They also reported a highly positive relationship between antibacterial activity and total phenolic content in a large number of herb extracts. They suggested that the phenolic compounds might significantly contribute to their antibacterial activity (Shan et al. 2007). They also demonstrated that many of the extracts contained high levels of phenolics and possessed strong antibacterial activity. In parallel with the explanations above, we can say that there is a positive relation between antibacterial activity and epigallocatechin, catechin, myricetin content of the methanolic extracts (Table 3.3.).

Table 4.2. Antibacterial activity of *F. vesca* extracts and controls (antibiotics) against used bacteria. Data presented as inhibition zone of bacterial growth in mm. Means with the same letter within columns are not significantly different at $P>0.05$.

Treatment	Mean diameter of inhibitory zones (mm $\pm$ SE)									
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>S. pyogenes</i>	<i>S. marcescens</i>	<i>S. typhimurium</i>	<i>Paeruginosa</i>	<i>P. vulgaris</i>	<i>K. pneumonia</i>	<i>E. cloacae</i>	<i>E. coli</i>
NW	10.0 $\pm$ 0.0 ^h	11.0 $\pm$ 0.6 ^{fg}	10.5 $\pm$ 0.3 ^f	-	-	-	-	8.0 $\pm$ 0.0 ^d	16.0 $\pm$ 0.0 ^f	-
NE	10.8 $\pm$ 0.5 ^g	12.0 $\pm$ 1.2 ^{ef}	13.5 $\pm$ 0.9 ^e	-	-	-	-	-	9.0 $\pm$ 0.0 ^h	-
NM	10.8 $\pm$ 0.3 ^g	12.5 $\pm$ 0.3 ^e	9.5 $\pm$ 0.3 ^f	-	-	8.3 $\pm$ 0.3 ^e	12.3 $\pm$ 0.6 ^e	-	14.5 $\pm$ 0.3 ^g	-
NA	12.8 $\pm$ 0.5 ^f	14.5 $\pm$ 0.3 ^d	10.5 $\pm$ 0.3 ^f	-	-	9.8 $\pm$ 0.5 ^{cd}	12.3 $\pm$ 0.3 ^e	-	19.5 $\pm$ 0.6 ^{de}	-
NFH	-	-	-	-	-	-	-	-	-	-
NFD	-	-	-	-	-	-	-	-	-	-
NFM	-	9.2 $\pm$ 0.2 ^{hi}	10.2 $\pm$ 0.8 ^f	-	-	-	-	-	18.1 $\pm$ 0.2 ^e	-
NFW	9.1 $\pm$ 0.2 ⁱ	10.4 $\pm$ 0.4 ^{gh}	8.5 $\pm$ 0.1 ^{fg}	-	-	9.9 $\pm$ 0.3 ^c	9.8 $\pm$ 0.2 ^f	9.2 $\pm$ 0.2 ^c	18.9 $\pm$ 0.6 ^e	-
FFH	-	-	-	-	-	-	-	-	-	-
FFD	-	-	-	-	-	-	-	-	-	-
FFM	7.6 $\pm$ 0.1 ^j	8.9 $\pm$ 0.2 ⁱ	7.4 $\pm$ 0.1 ^g	-	-	-	-	-	16.2 $\pm$ 0.7 ^f	-
FFW	9.8 $\pm$ 0.2 ^{hi}	13.2 $\pm$ 0.8 ^e	10.2 $\pm$ 0.2 ^f	-	-	9.2 $\pm$ 0.2 ^d	10.1 $\pm$ 0.1 ^f	-	20.8 $\pm$ 0.3 ^d	-
CFH	-	-	-	-	-	-	-	-	-	-
CFD	-	-	-	-	-	-	-	-	-	-
CFM	-	-	-	-	-	-	-	-	-	-
CFW	-	-	-	-	-	-	-	-	15.7 $\pm$ 0.2 ^{fg}	-
ILM	-	10.7 $\pm$ 0.2 ^{fg}	8.3 $\pm$ 0.2 ^{fg}	-	-	-	9.0 $\pm$ 0.0 ^f	-	16.3 $\pm$ 0.4 ^f	-
Ampicillin (10 mg)	39.2 $\pm$ 0.7 ^b	30.6 $\pm$ 0.2 ^c	48.0 $\pm$ 2.5 ^b	15.0 $\pm$ 0.5 ^d	27.4 $\pm$ 0.2 ^c	-	28.0 $\pm$ 1.2 ^c	8.4 $\pm$ 1.0 ^{cd}	27.0 $\pm$ 0.0 ^c	21.4 $\pm$ 0.6 ^c
Carbenicillin (100 mg)	43.2 $\pm$ 0.7 ^a	38.2 $\pm$ 1.3 ^a	50.4 $\pm$ 0.7 ^a	29.2 $\pm$ 0.7 ^a	30.0 $\pm$ 0.0 ^a	20.8 $\pm$ 0.5 ^a	38.0 $\pm$ 1.6 ^a	8.6 $\pm$ 1.1 ^{cd}	33.4 $\pm$ 1.4 ^a	24.8 $\pm$ 0.4 ^b
Chloramphenicol (30 mg)	25.8 $\pm$ 0.5 ^e	33.6 $\pm$ 0.6 ^b	32.4 $\pm$ 1.1 ^d	27.2 $\pm$ 1.0 ^b	28.4 $\pm$ 0.7 ^b	8.6 $\pm$ 0.2 ^e	28.0 $\pm$ 1.3 ^c	29.8 $\pm$ 0.7 ^a	30.0 $\pm$ 1.1 ^b	28.4 $\pm$ 0.6 ^a
Erythromycin (15 mg)	29.6 $\pm$ 0.2 ^d	39.2 $\pm$ 0.5 ^a	36.4 $\pm$ 2.9 ^c	11.6 $\pm$ 0.6 ^e	11.6 $\pm$ 0.7 ^e	-	13.6 $\pm$ 0.2 ^d	13.0 $\pm$ 0.0 ^b	9.4 $\pm$ 0.2 ^h	13.2 $\pm$ 0.7 ^d
Tetracycline (30 mg)	30.8 $\pm$ 0.7 ^c	8.0 $\pm$ 0.0 ⁱ	36.8 $\pm$ 0.7 ^c	23.6 $\pm$ 0.7 ^c	24.6 $\pm$ 0.6 ^d	14.8 $\pm$ 0.7 ^b	35.8 $\pm$ 1.8 ^b	29.2 $\pm$ 0.9 ^a	30.0 $\pm$ 0.5 ^b	28.0 $\pm$ 0.8 ^a
DMSO	-	-	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-	-	-

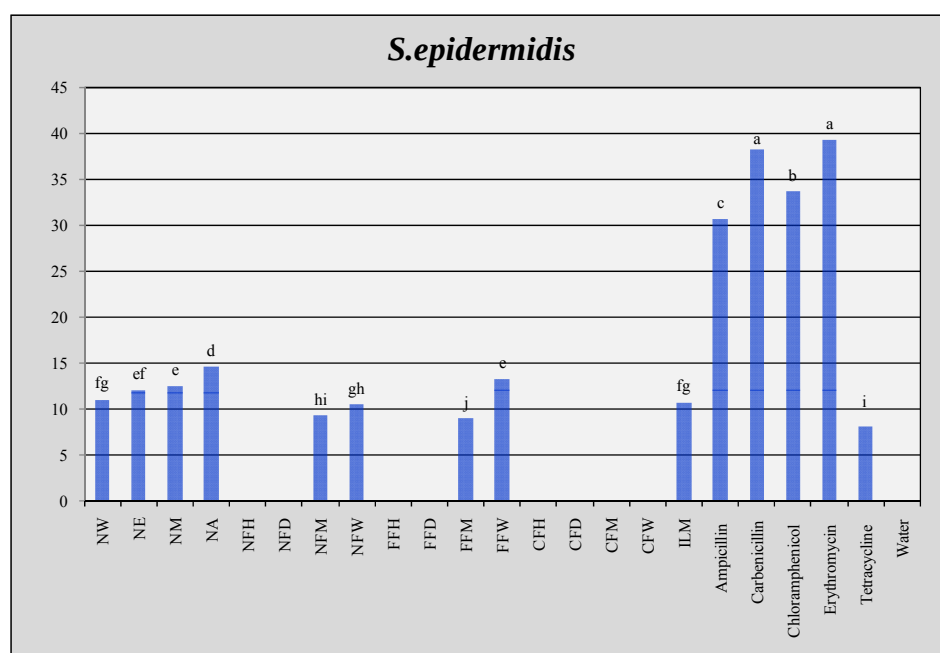
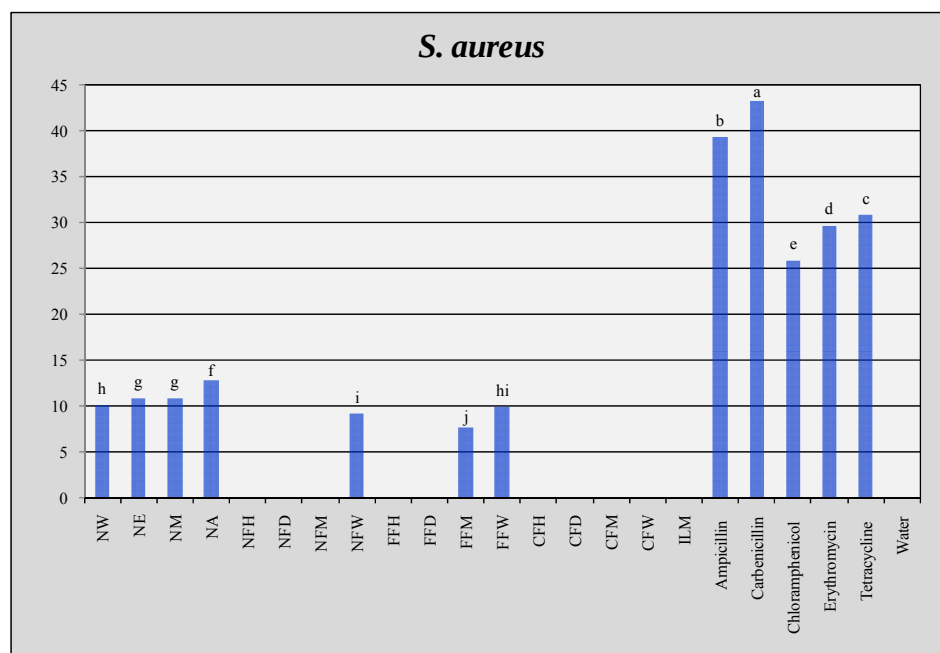


Figure 4.1. Antibacterial activity of *F. vesca* extracts and controls (antibiotics) against *S. aureus* and *S. epidermidis*.

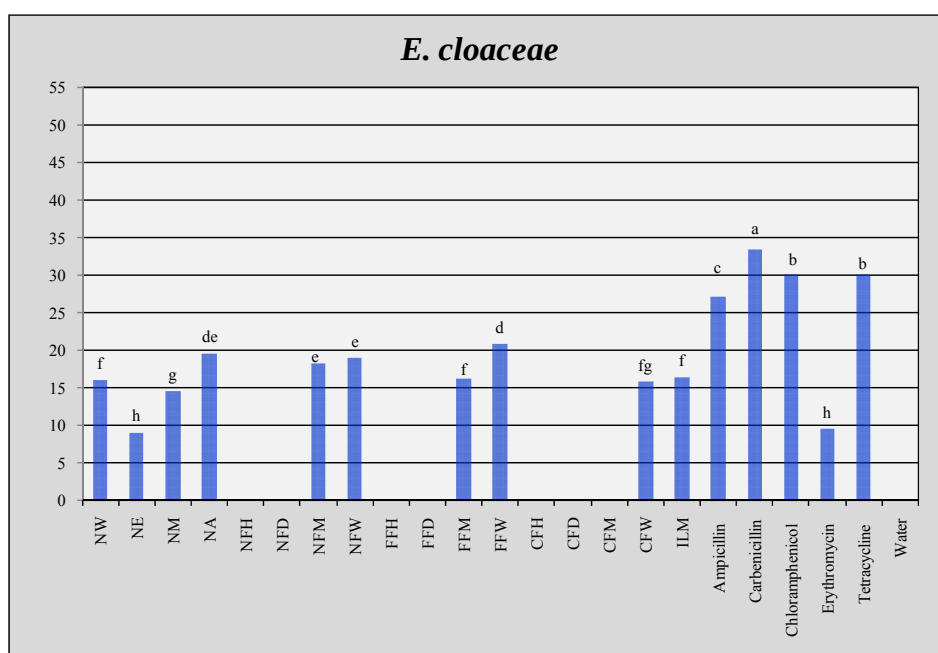
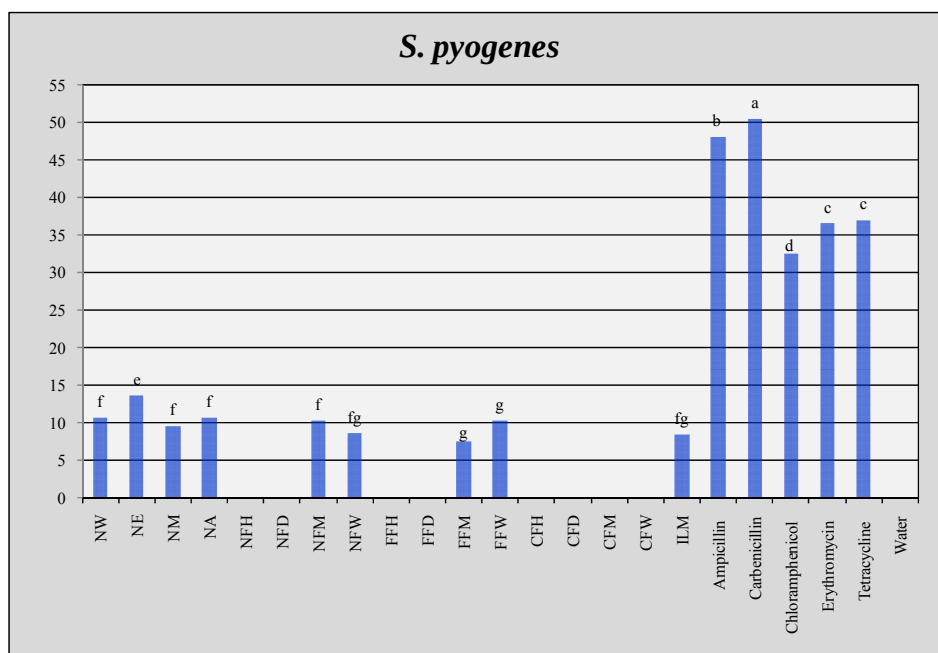


Figure 4.2. Antibacterial activity of *F. vesca* extracts and controls (antibiotics) against *S. pyogenes* and *E. cloacae*.

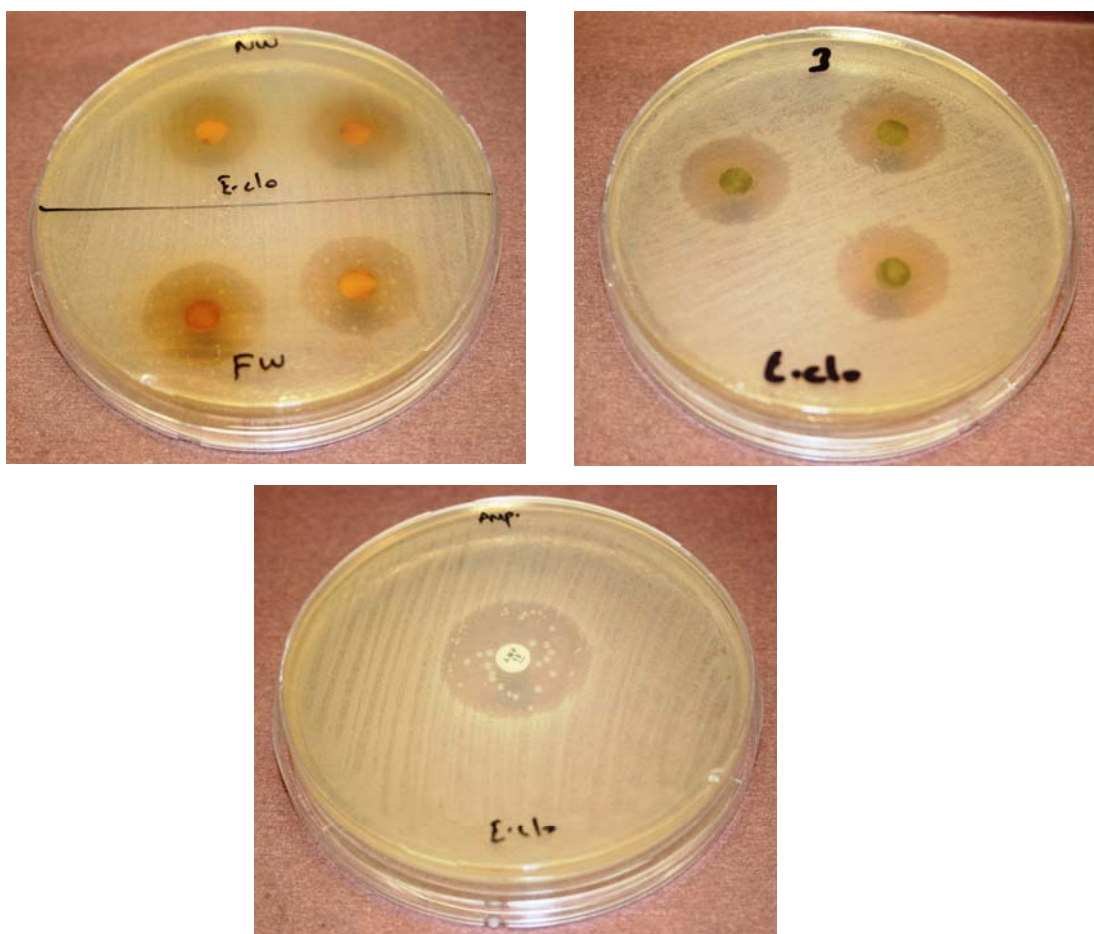


Figure 4.3. Antibacterial activity of NFW and FFW (gradually prepared aqueous extracts of field-grown plant and fruit) extracts (left) and NA (acetone extract of field grown plant) (right) extract against *E. cloaceae* by comparing with positive control (ampicillin) (below).

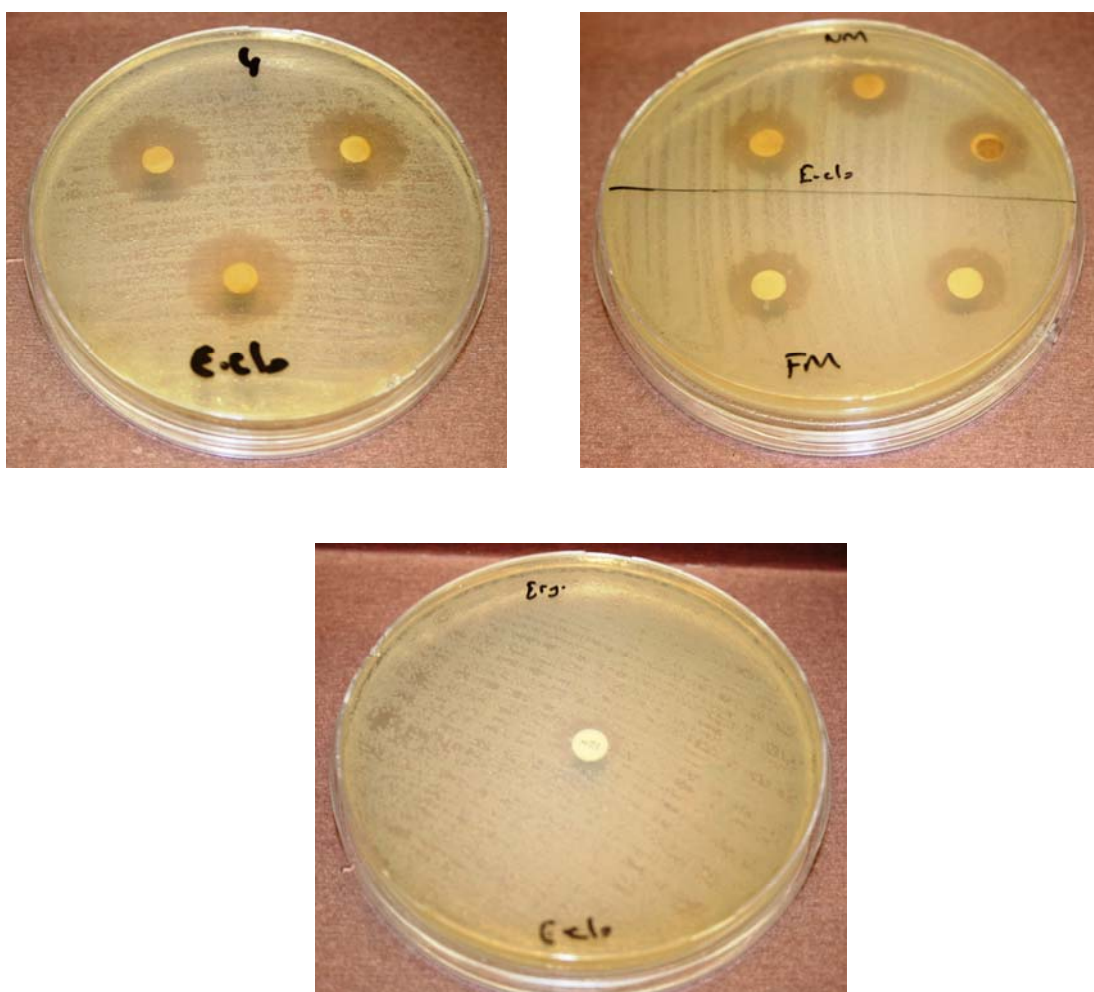


Figure 4.4. Antibacterial activity of ILM (methanolic extract of *in vitro*-grown plant) extract (left) and NFM and FFM (methanolic extracts of field grown plant and fruit) (right) extracts against *E. cloaceae* by comparing with positive control (erythromycin) (below).

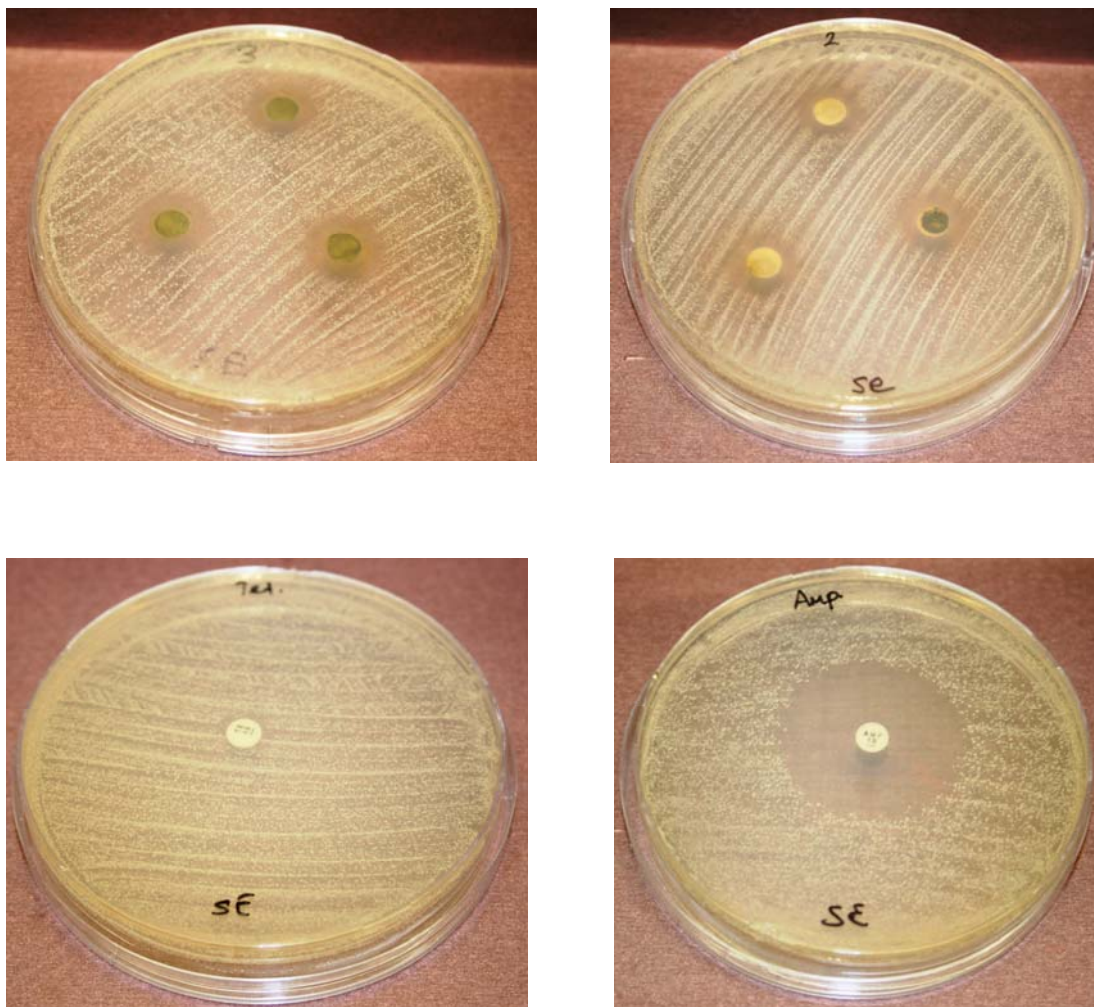


Figure 4.5. Antibacterial activities of NA (left) and NM (right) (acetone and methanolic extracts of field grown plant) against *S. epidermidis* by comparing with positive controls (tetracycline and ampicillin) (below).

4.3.2. Anti-tumor Activity

Eleven different extracts (NW, NE, NM, DFCW, DFHW, FFCW, FFHW, DFCE, DFHE, FFCE and FFHE) (Table 3.1.) were tested in this assay in order to screen and show their potential as antitumour agents (Table 4.3.; Figure 4.6.).

Although several kinds of biological activities of *F. vesca* were reported, antitumor activity of *F. vesca* was not mentioned in the literature.

The best antitumor activity was observed with aqueous (NW) and methanolic (NM) extracts of field-grown leaves (98.1% and 97.1% tumor inhibition, respectively) and hot ethanolic extract (FFHE) of fresh fruit (93.2% tumor inhibition) (Table 4.3. and Figure 4.6.). When compared with control (water), the percentage inhibition of all extracts except cold aqueous extract of fresh fruit (FFCW) and hot aqueous extract (DFHW) of dry fruit was more than 40% in three separate experiments. Since final concentrations of all extracts were adjusted with distilled water, it was used as a negative control and no inhibition was observed with water. No tumor formation was observed with camptothecin (100% tumor inhibition) (Table 4.3.; Figure 4.6. and Figure 4.8.).

Among prepared extracts from field-grown leaves, NW and NM extracts showed quite similar and strong antitumor activity. Ethanolic extracts (NE) of field-grown leaves exhibited good antitumor activity (84.1% tumor inhibition).

Among prepared extracts from dry fruit, the best antitumor activity was obtained with hot ethanol (DFHE) extract whose percentage of inhibition is 82.1% and least antitumor activities (less than 50% tumor inhibition) were obtained with hot

(DFHW) and cold aqueous (DFCW) extracts as 39.1% and 47.3% tumor inhibition, respectively. Cold ethanol extracts of dry fruit showed moderate antitumor activity (64.7% tumor inhibition) (Table 4.3. and Figure 4.6.).

Among prepared extracts from fresh fruit, the best antitumor activity was obtained with FFHE extracts. Hot aqueous and cold ethanol extracts of fresh fruit showed good antitumor activity (71.5% and 83.1% tumor inhibition, respectively) (Table 4.3. and Figure 4.6.).

In summary, all extracts (methanol, ethanol and water) of field-grown leaves (NM, NE and NW) exhibited strong antitumor activity. On the other hand, ethanolic extracts of both dry and fresh fruits (DFCE, DFHE, FFCE and FFHE) exhibited better antitumor activity than those of aqueous extracts.

Ethanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, sterols, alkaloids and propolis groups (Kaul et al., 1985; Scalbert, 1991; De Pasquale et al., 1995). These components may cause antitumor activity.

Some studies have reported that certain dietary flavonoids possess antitumor activity. In another study, Molnar et al. (1981) reported the antitumor activity of polyhydroxylated flavonoids towards NK/LY ascites tumors in mice. Quercetin and apigenin also inhibited tumor development in other animal models. The polyhydroxylated flavonoid, quercetin exerted potent growth inhibitory effects on several malignant tumor cell lines *in vitro*. Flavones and flavonols, such as 3-hydroxyflavone, 3',4'- dihydroxyflavone, 2',3'-dihydroxyflavone, apigenin and luteolin, also inhibited tumor cell growth (Kanadaswami et al., 2005).

Correspondingly, in our study, flavonoid content in methanolic extract of field-grown leaves (NM) was high (Table 4.6), and the flavonoids such as apigenin, quercetin and luteolin were detected in NM extract by LC/MS-MS analysis (Table 3.3.). Thus, the antitumor activity of the methanolic extract (NM) may be attributed to these contents.

A prerequisite for potato disc tumor induction assay is that the extract or substance being tested should not have antibacterial activity toward *A. tumefaciens* (Coker et al., 2003). Inhibition of crown gall formation on potato discs is caused by two effects: by anti-tumorigenesis or decreasing the viability of the *A. tumefaciens*. Viability tests were carried out with all extracts to distinguish between these possibilities. Bacterial viability was determined by incubating plant extracts with 1×10^3 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension and left for 30 min. As the attachment of the bacterium to a tumor-binding site is complete within 15 min following inoculation (Glogowski and Galsky, 1978; Lippincott and Lippincott, 1969), 30 min exposure was chosen in the experiment. There was no difference in bacterial growth across the plates between control (only *A. tumefaciens*) and tested extracts (*A. tumefaciens* + plant extracts) in terms of colony counts (ranged from 9.2×10^3 to 13×10^3 CFU). All tested extracts did not affect the viability of the bacterium. Thus, observed inhibition of tumor formation for these extracts was on the formation of tumors. Potato disc tumor assay was applied as a preliminary study in order to test whether *F. vesca* extracts have an antitumor activity or not. Our study revealed the strong antitumor activity of *F. vesca* extracts for the first time. This study guided us to carry out some anticancer and cytotoxicity experiments using cancer cell line.

Table 4.3. Mean number of tumors observed with extracts of *F. vesca* extracts that dissolves in water at 100.000 mg/l and controls (water and camptothecin). Means with the same letter are not significantly different at $P>0.05$.

Treatment	Mean Number of Tumors ($\pm$SE)				% Tumor Inhibition
Water	20.66	$\pm$	2.0	f	0.0
Camptothecin	0.0	$\pm$	0.0	a	100
NW	0.4	$\pm$	0.3	a	98.1
NE	3.3	$\pm$	0.6	ab	84.1
NM	0.6	$\pm$	0.2	a	97.1
DFCW	10.9	$\pm$	1.1	d	47.3
DFHW	12.6	$\pm$	1.5	d	39.1
DFCE	7.3	$\pm$	1.2	c	64.7
DFHE	3.7	$\pm$	1.2	ab	82.1
FFCW	16.3	$\pm$	2.1	e	21.3
FFHW	5.9	$\pm$	1.3	bc	71.5
FFCE	3.5	$\pm$	0.6	ab	83.1
FFHE	1.4	$\pm$	0.5	a	93.2

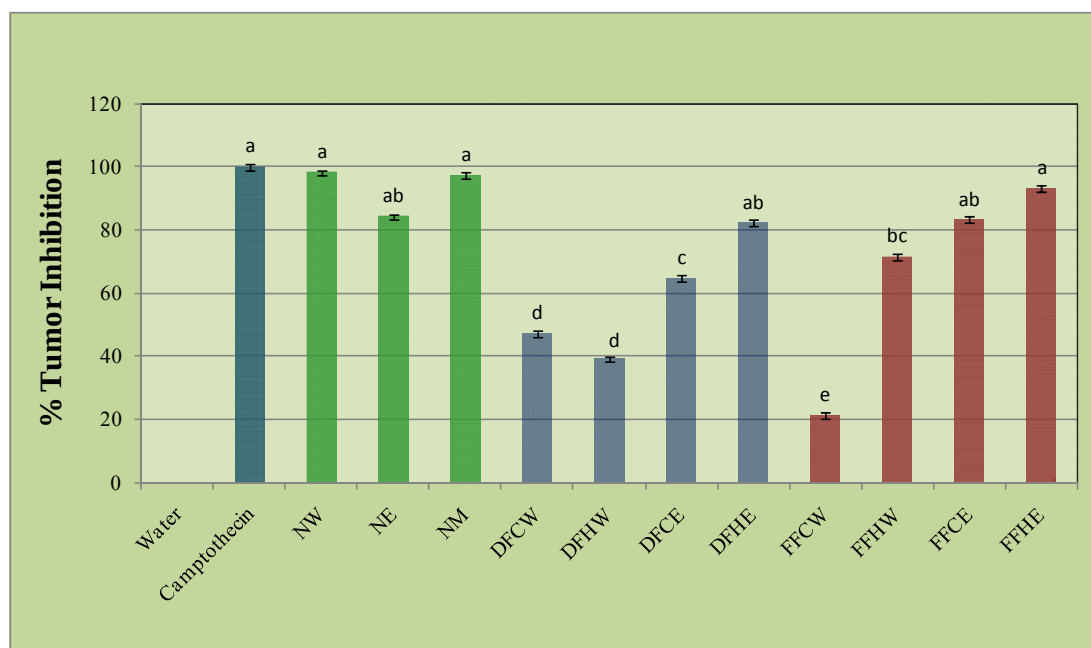


Figure 4.6. The percentage of tumor inhibition of *F. vesca* extracts and controls.



Figure 4.7. Tumors produced by *A. tumefaciens* with NW (aqueous extract of field-grown plants) (left) and its closed version (right).



Figure 4.8. Tumors produced by *A. tumefaciens* with distilled water (solvent control I) (left) and camptothecin (positive control) (right).

4.3.3. Brine Shrimp Toxicity

Toxicity of six different *F. vesca* extracts (NM, NFM, FFM, CFM, CM and ILM) (Table 3.1.) was tested with brine shrimp bioassay (Table 4.4.). This bioassay determines the toxicity of materials toward brine shrimp larvae and can predict the ability to kill cancer cells in cell cultures, various pests, and exert a wide range of pharmacological effects (McLaughlin et al., 1991). Meyer et al. (1982) found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. Results of this assay showed that extracts of *F. vesca* were low toxic at higher doses (around 400-1500 mg/l) by comparing with MS-222 (positive control). Among the tested extracts, gradually prepared methanol extracts of field-grown leaves (NFM) and fruits (FFM) were more toxic than other tested extracts with higher LC₅₀ values (696 mg/l and 465 mg/l, respectively) (Table 4.4). Moreover, methanol extracts of *in vitro*-grown leaves (ILM) and callus (CM) were less toxic (1246 mg/l and 1407 mg/l, respectively).

In summarily, extracts of field-grown leaves and fruits were more toxic than in the extracts of *in vitro*-grown leaves and callus. When we made a comparison within extraction types, gradually prepared methanolic extracts were more toxic than methanolic extracts prepared by direct extraction. This means that gradually prepared methanolic extracts might be regarded as containing more toxic compounds (Table 4.4. and Figure 4.9.). This bioassay has the advantage of being rapid (24 hrs following introduction of shrimp), inexpensive and simple (e.g., no aseptic techniques are required). With this assay, it is possible to monitor fractionation of cytotoxic and 3PS (*in vivo* murine leukemia) active extracts rather than more tedious and expensive *in vitro* and *in vivo* antitumor assays (McLaughlin et al., 1998).

It is obvious that, in our study, there is a good relation between the brine shrimp toxicity and anticancer activity of the tested extracts. In brine shrimp toxicity assay, the least toxicity was obtained with *in vitro*-grown leaves and callus (Table 4.4). Similarly, in anticancer assay, the least anticancer activity was obtained with *in vitro*-grown leaves and callus (Table 4.6.).

Table 4.4. LC₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of *F. vesca* methanolic extracts and MS-222 (a positive control).

Treatments	LC ₅₀ (mg/l)	% 95 Confidence Intervals		
NM	1002	633	-	1583
NFM	696	440	-	1099
FFM	465	294	-	735
CFM	1054	667	-	1665
ILM	1246	789	-	1970
CM	1407	891	-	2225
MS-222	33	21	-	52

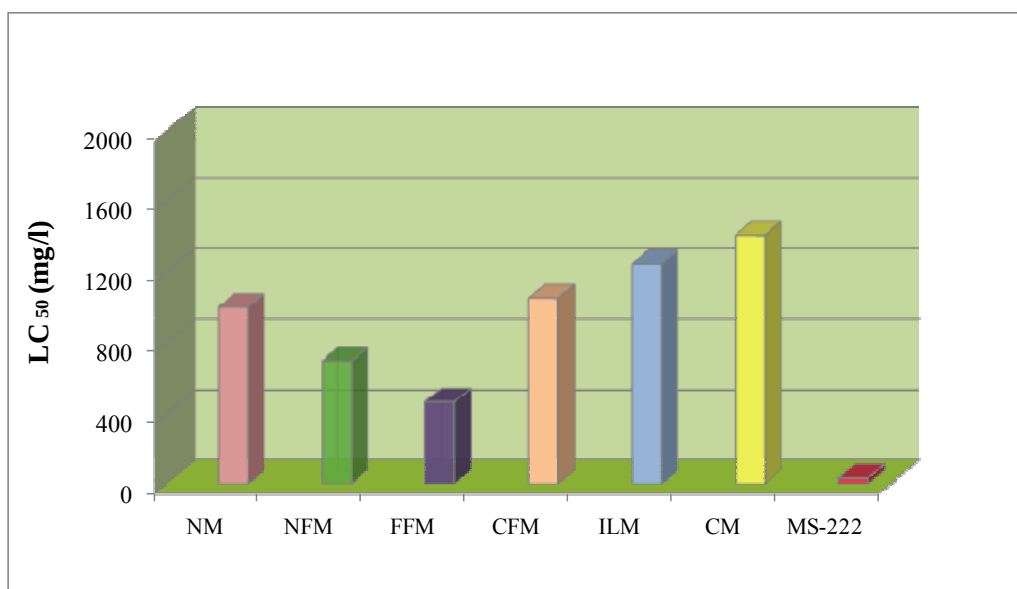


Figure 4.9. LC₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of *F. vesca* methanolic extracts and MS-222 (a positive control).

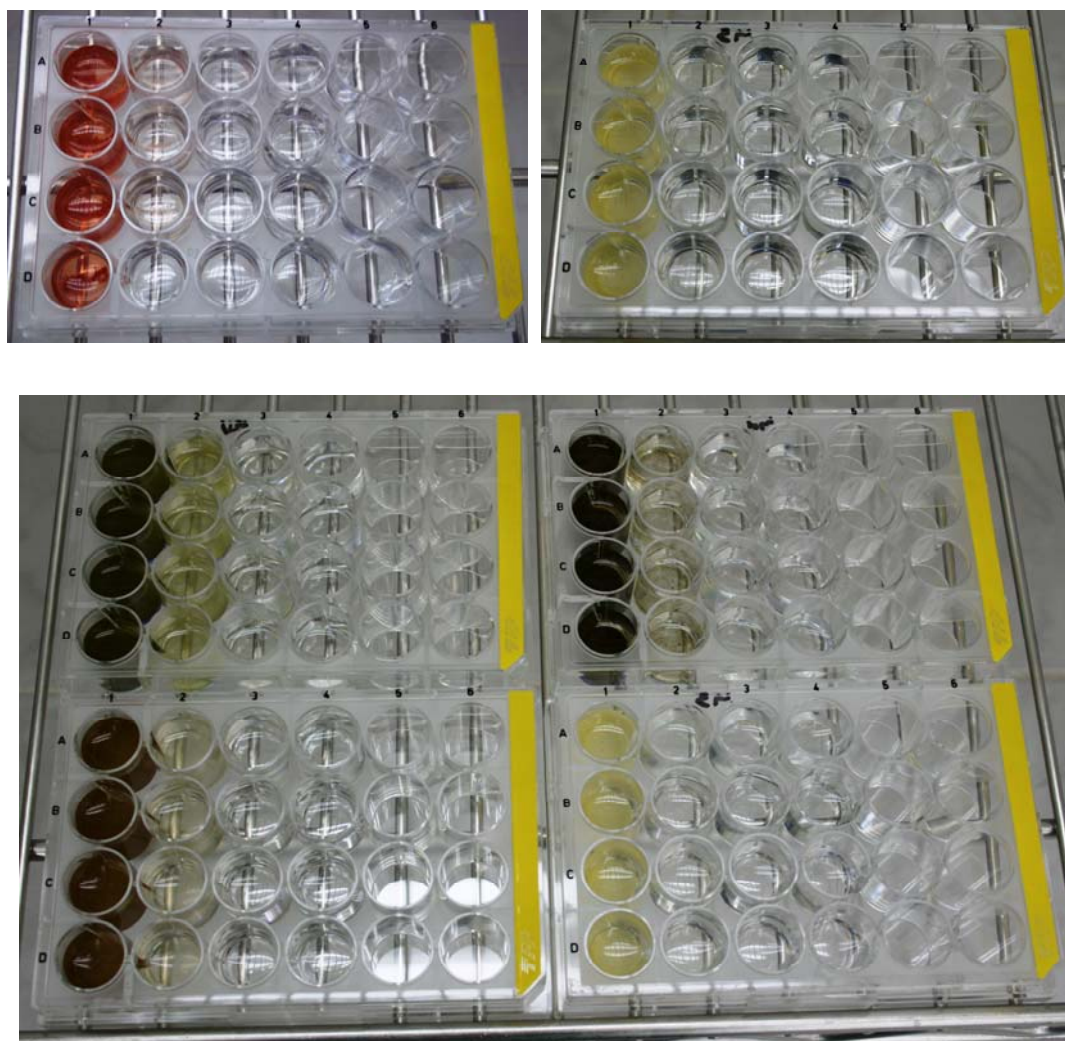


Figure 4.10. LC_{50} (lethal concentration for 50% mortality after 24 hr exposure) values of *F. vesca* methanolic extracts.

4.3.4. Anticancer Activity

Fourteen different extracts (NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW, NM and ILM) (Table 3.1.) of *F. vesca* were used to evaluate the cytotoxic activities on Human Breast Adenocarcinoma (MCF-7) (Table 4.5 and 4.6.).

The results of the cytotoxicity assay (for 24-h and 48-h) of the tested extracts are shown in Table 4.5. and 4.6.

In MTT assay for 24 h, all the gradually prepared hexane extracts and also gradually prepared dichloromethane extract of *in vitro*-grown callus showed higher cell viability than in control. The cell viability of dichloromethane extracts of field-grown leaves and fruits associated with the control (Table 4.5.).

Among gradually prepared extracts of field-grown leaves, aqueous extract (NFW) showed better anticancer activity on MCF-7 cells with 82.2% cell viability at 200 µg/ml and 86.4% cell viability at 100 µg/ml concentrations. In other words, NFW extract lowered cell viability about 17.8% cell viability at 200 µg/ml and 13.6% cell viability at 100 µg/ml concentrations. Also methanolic extract (NFM) showed weak anticancer activity with 85.3% cell viability at 200 µg/ml concentrations (Table 4.5.).

Among gradually prepared extracts of fruits, aqueous extract (FFW) showed better anticancer activity than rest of the extracts at 100 and 200 µg/ml concentrations. But, FFW and methanolic (FFM) extracts showed nearly same anticancer activity at the same concentrations (Table 4.5.).

Among gradually prepared extracts of *in vitro*-grown callus, aqueous (ICW) and methanolic (ICM) extracts showed similar anticancer activity at the same concentrations. ICW and ICM extracts lowered cell viability about 12% cell viability at 200 µg/ml and 11% cell viability at 100 µg/ml concentrations (Table 4.5.). Generally, water extracts were more active on MCF-7 cell line according to the results of 24-h treatment.

In MTT assay for 48h, two additional extracts were used: Methanolic extracts of *in vitro*-grown leaves (ILM) and field-grown leaves (NM). ILM extract did not show anticancer activity and also it showed higher cell viability than in control at low concentrations (12.5 – 50 µg/ml). NM extract showed same cell viability (75.2%) at 100 and 200 µg/ml. NM extract had better anticancer activity than ILM extract (Table 4.6.).

Among gradually prepared extracts of field-grown leaves, dichloromethane extract (NFD) showed better anticancer activity with 64.3% cell viability at 200 µg/l and 68.3% at 100 µg/l than in the rest of the extracts. Aqueous extracts (NFW) at 200 µg/l concentration showed similar anticancer activity with NFD when NFD was used at 100 µg/l concentration. In these extracts, hexane extract (NFH) showed least anticancer activity (Table 4.6. and Figure 4.11.).

Among gradually prepared extracts of fruit, hexane extract (FFH) showed better anticancer activity than in the rest of the extracts. The percentage of cell viability was not changed when the concentration of FFH was used between 25 and 200 µg/l. The percentage of cell viability was about 68%. Dichloromethane (FFD), methanolic (FFM) and aqueous extracts showed similar anticancer activity when the concentrations were used between 25 and 200 µg/l (Table 4.6. and Figure 4.12.).

Among gradually prepared extracts of *in vitro*-grown callus, dichloromethane (ICD) extract showed better anticancer activity with 68.4% cell viability at 200 µg/l and 70.6% cell viability at 100 µg/l concentrations. Hexane (ICH) and aqueous (ICW) extracts showed weak anticancer activity with 90.0% and 94.4% cell viability at 12.5 µg/l concentration, respectively. When the concentrations were increased from 12.5 to 200 µg/l, the anticancer activity did not change significantly in both extracts (81.4% and 81.3% cell viability at 200 µg/l, respectively) (Table 4.6.).

As a general discussion, any increasing concentration of the extract is not effective on the anticancer activity. This means that the anticancer activity is not concentration dependent with respect to the result. However, it slightly increased by increasing time. As a final remark, extracts of field-grown leaves and fruits showed better anticancer activity than in the extracts of *in vitro*-grown leaves and callus.

Le Bail et al. (1998) reported that luteolin has been shown to inhibit a series of human breast cancer cell lines (MCF-7). In another study, luteolin-7-O-β glucoside is more active in the breast adenocarcinoma cell than luteolin (Galvez et al., 2003). In the present study, Luteolin-7-O-β-D glucoside is present in the tested methanolic extracts; however, not a strong anticancer activity was observed (Table 3.3.).

The biological selective activity of any compound might depend on the type of chemical composition and the concentration of active constituents as well as the types and developmental stages of the cancer (Alongkornsopit et al., 2011). The different activities of the extracts on the breast cancer cells may be due to the biological nature of the cell lines (Liu et al., 2001). MCF-7, the estrogen receptor

possessing cells, expressed was less sensitive to the extract than the non-possessing estrogen receptor cells, MDA-MB- 231 (Human Breast Carcinoma Cell Line) (Alongkornsopit et al., 2011). As a future work, the same extracts might be applied on different cell lines for anticancer activity assay.

Table 4.5. The percentage of cell viability of different extracts from *F. vesca* L. on the human breast adenocarcinoma (MCF-7) cell line for 24h. The cells were treated with various concentrations (6.25, 12.5, 25, 50, 100, 200 µg/ml) of extracts. Results were expressed as means ± SD for three independent determinations.

Treatments	% Cell Viability (Mean ± S.E.M)					
	Concentrations					
	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
NFH	105.2 ± 3.0 ^h	107.1 ± 1.7 ^{hi}	109.4 ± 0.7 ^{hij}	111.1 ± 2.8 ^{ij}	113.2 ± 0.6 ^j	118.2 ± 1.4 ^k
NFD	97.5 ± 1.0 ^{fg}	97.0 ± 1.2 ^{fg}	96.4 ± 1.1 ^{fg}	96.3 ± 1.6 ^{efg}	94.5 ± 2.0 ^{defg}	94.0 ± 0.5 ^{def}
NFM	97.3 ± 3.0 ^{fg}	96.8 ± 0.9 ^{fg}	93.8 ± 0.7 ^{def}	92.7 ± 1.4 ^{def}	90.0 ± 1.3 ^{bcd}	85.3 ± 0.4 ^{ab}
NFW	96.9 ± 0.9 ^{fg}	93.9 ± 1.0 ^{def}	91.1 ± 1.5 ^{cde}	89.8 ± 1.5 ^{bcd}	86.4 ± 1.2 ^{abc}	82.2 ± 1.4 ^a
FFH	105.0 ± 0.4 ^h	104.4 ± 1.6 ^h	107.1 ± 2.0 ^h	106.1 ± 1.5 ^h	107.4 ± 0.9 ^h	115.3 ± 0.5 ⁱ
FFD	96.7 ± 0.8 ^{fg}	96.0 ± 2.2 ^{efg}	94.8 ± 1.3 ^{defg}	95.6 ± 1.4 ^{defg}	93.2 ± 0.5 ^{def}	90.7 ± 1.1 ^{cde}
FFM	96.7 ± 2.7 ^{fg}	94.8 ± 1.7 ^{defg}	92.0 ± 1.4 ^{cdef}	90.2 ± 1.2 ^{cd}	87.5 ± 0.8 ^{bc}	85.1 ± 0.8 ^{ab}
FFW	96.6 ± 1.8 ^{fg}	92.1 ± 1.9 ^{cdef}	90.9 ± 2.2 ^{cde}	87.0 ± 2.6 ^{bc}	82.0 ± 1.9 ^a	80.8 ± 1.8 ^a
CFH	96.7 ± 2.0 ^{bcde}	98.1 ± 3.9 ^{cde}	100.7 ± 3.1 ^{ef}	101.0 ± 2.6 ^{ef}	105.9 ± 2.3 ^f	106.3 ± 3.9 ^f
CFD	100.4 ± 2.4 ^{ef}	101.4 ± 1.6 ^{ef}	101.8 ± 1.0 ^{ef}	103.5 ± 1.8 ^{ef}	104.0 ± 2.7 ^{ef}	106.8 ± 0.9 ^f
CFM	96.7 ± 0.4 ^{bcde}	92.9 ± 1.2 ^{abcd}	90.0 ± 2.4 ^{ab}	89.1 ± 1.5 ^a	89.0 ± 1.4 ^a	88.5 ± 2.1 ^a
CFW	96.5 ± 3.4 ^{bcde}	91.5 ± 1.9 ^{abc}	90.4 ± 0.9 ^{ab}	89.8 ± 2.2 ^{ab}	89.7 ± 0.3 ^{ab}	88.2 ± 3.4 ^a

Table 4.6. The percentage of cell viability of different extracts from *F. vesca* L. on the human breast adenocarcinoma (MCF-7) cell line for 48h. The cells were treated with various concentrations (12.5, 25, 50, 100, 200 µg/ml) of extracts. Results were expressed as means ± SD for three independent determinations.

% Cell Viability (Mean ± S.E.M)					
Concentrations					
Treatments	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
NFH	91.9 ± 0.0 ^g	91.7 ± 0.0 ^g	88.1 ± 0.0 ^{fg}	84.7 ± 0.0 ^{efg}	83.3 ± 0.0 ^{def}
NFD	80.6 ± 0.9 ^{cdef}	78.5 ± 2.1 ^{cde}	73.4 ± 2.2 ^{bc}	68.3 ± 2.4 ^{ab}	64.3 ± 0.4 ^a
NFM	87.9 ± 1.6 ^{fg}	79.6 ± 2.5 ^{cde}	76.7 ± 3.7 ^{cd}	76.3 ± 2.6 ^{cd}	76.3 ± 2.3 ^{cd}
NFW	91.9 ± 2.4 ^g	80.1 ± 2.9 ^{cde}	73.3 ± 0.9 ^{bc}	72.6 ± 0.3 ^{bc}	67.9 ± 0.9 ^{ab}
FFH	75.4 ± 1.5 ^{cd}	68.9 ± 2.6 ^{ab}	67.9 ± 0.3 ^a	67.9 ± 0.0 ^a	67.7 ± 0.1 ^a
FFD	77.7 ± 1.3 ^{cd}	77.5 ± 1.1 ^{cd}	74.7 ± 2.6 ^{bcd}	73.9 ± 0.9 ^{abcd}	73.0 ± 0.6 ^{abc}
FFM	83.7 ± 1.3 ^{ef}	79.9 ± 1.6 ^{de}	77.9 ± 1.4 ^{cd}	73.8 ± 1.5 ^{abcd}	72.6 ± 1.2 ^{abc}
FFW	99.6 ± 3.9 ^g	87.5 ± 2.8 ^f	77.2 ± 1.9 ^{cd}	74.5 ± 3.3 ^{bcd}	71.9 ± 1.7 ^{abc}
CFH	90.0 ± 1.4 ^{ef}	85.6 ± 1.7 ^{de}	83.1 ± 0.6 ^d	82.6 ± 1.6 ^d	81.4 ± 0.7 ^{cd}
CFD	76.8 ± 0.1 ^{bc}	75.6 ± 1.2 ^b	72.6 ± 1.2 ^{ab}	70.6 ± 1.6 ^a	68.4 ± 1.3 ^a
CFM	107.2 ± 0.6 ^g	93.4 ± 1.4 ^f	93.4 ± 2.3 ^f	93.3 ± 1.9 ^f	92.7 ± 2.1 ^f
CFW	94.4 ± 2.4 ^f	93.3 ± 1.8 ^f	89.4 ± 1.3 ^{ef}	83.9 ± 2.9 ^d	81.3 ± 1.2 ^{cd}
ILM	111.5 ± 2.7 ^e	104.6 ± 1.8 ^d	101.1 ± 3.1 ^d	98.4 ± 1.9 ^{cd}	98.4 ± 1.9 ^{cd}
NM	93.9 ± 1.3 ^c	83.1 ± 1.8 ^b	80.3 ± 2.6 ^{ab}	75.3 ± 1.1 ^a	75.2 ± 0.9 ^a

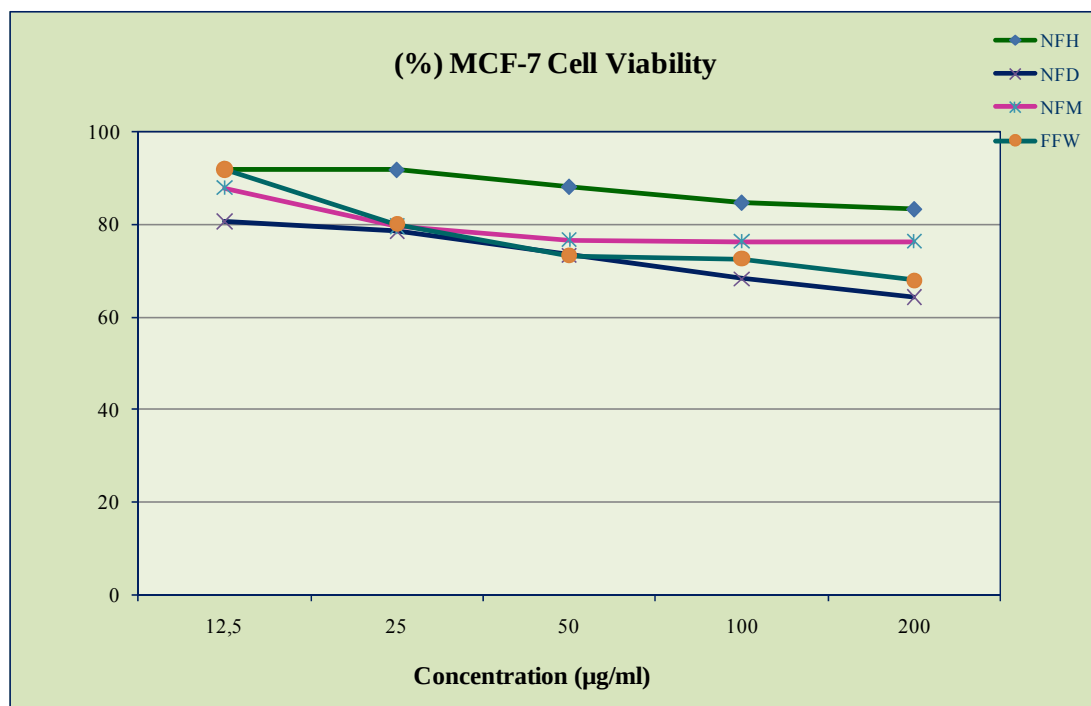


Figure 4.11. The dose-response curves of the effects of gradually prepared extract of field-grown leaves on cell growth in MCF-7 cells for 48 h.

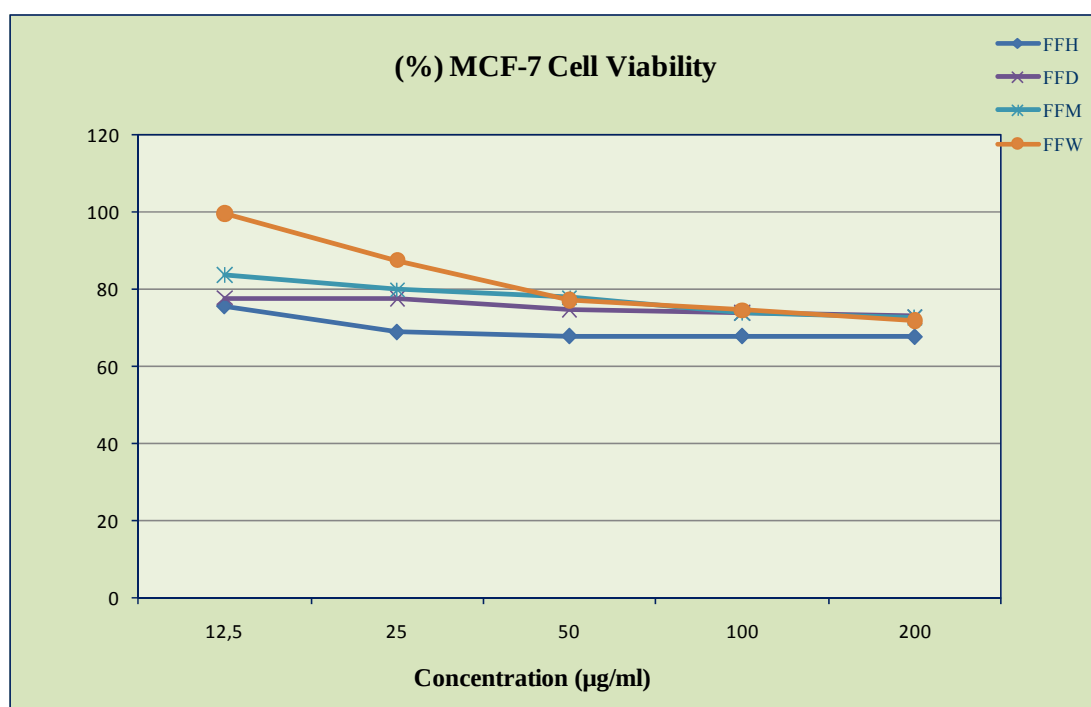


Figure 4.12. The dose-response curves of the effects of gradually prepared extract of fruits on cell growth in MCF-7 cells for 48 h.

4.3.5. Antioxidant Activity

4.3.5.1. Free radical Scavenging Activity-DPPH (2,2-diphenyl-1-picrylhydrazil)

Method

The scavenging activity of DPPH radical caused by antioxidants was determined by measuring the decrease in its absorbance at 517 nm. Hence, DPPH is often used as a substrate to evaluate the scavenging activity of antioxidant compounds. Ascorbic acid was used as the antioxidant standard in this experiment.

In the present study, antioxidant activity of methanolic extracts of field-grown leaves and fruits, and *in vitro*-grown leaves and callus was assessed. The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteu) and total flavonoid content (aluminum chloride colorimetric) were used in this assessment.

The results indicated that at minimum concentration, the best antioxidant activities were obtained by methanolic extract (NM) and gradually prepared methanolic extract (NFM) of field-grown leaves. In NM extract, DPPH radical scavenging activity was strong enough at 12.5 µg/ml concentrations (87.32 %), however, in NFM extract, DPPH radical scavenging activity was observed strong at 25 µg/ml concentrations (90.42 %). After those concentrations (12.5 µg/ml for NM and 25 µg/ml for NFM), there is no significant increase in antioxidant activity. NM and NFM extracts at 50 µg/ml concentration and over have shown the similar radical scavenging activity in that of ascorbic acid (Table 4.7; Figure 4.13. and 4.14.).

Good antioxidant activities were observed with gradually prepared methanolic extract of fruit (FFM) and methanolic extract of *in vitro*-grown leaves

(ILM). DPPH radical scavenging activities were strong at 75µg/ml (minimum concentration) for both extracts. After 75µg/ml concentration, there is no significant increase in antioxidant activity in both extracts and also they have similar antioxidant activities when compared with that of ascorbic acid (Table 4.7; Figure 4.13. and 4.14.).

Wang and Lin (2000) studied the antioxidant activities of leaves and fruits of strawberry, blackberry, and raspberry. They reported that the leaves were a richer source of antioxidants. Similarly, in our study, we found that antioxidant activity was higher in extracts prepared from field-grown leaves than in fruit extract. Furthermore, a lot of compounds possessing antioxidant activity were identified in the leaves of *F. vesca* (Mudnic et al., 2009). These are (+)-catechin, (-)-epicatechin, epigallocatechin and procyanidins (B1 and B2). Scalzo et al. (2005) investigated the antioxidant activities of *F. vesca* fruit and some cultivated strawberries by using Trolox equivalent antioxidant capacity (TEAC) assay. They found that TEAC value of *F. vesca* fruits were 2.5 times more than cultivated strawberries.

With gradually prepared methanolic extract (CFM) and methanolic extract (CM) of *in vitro*-grown callus, a dose-response relationship was found in DPPH radical scavenging activity. DPPH radical scavenging activity increased with an increase in concentration of both extracts. However, no significant antioxidant activity was observed. Also there was no significant difference in antioxidant activity among CFM and CM extracts (Table 4.7; Figure 4.13.and 4.14.).

Table 4.7. Free-radical (DPPH●) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, *in vitro* grown plant and callus from *F.vesca* and ascorbic acid (positive control).

% Inhibition of DPPH						
Treatments	Concentrations					
	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	200 µg/ml
Ascorbic acid	96.19	96.33	96.47	96.73	96.76	99.15
NM	87.32	89.96	91.55	92.34	93.96	93.99
NFM	59.01	90.42	91.54	92.99	93.28	93.77
FFM	14.53	33.51	67.05	87.66	91.85	94.28
CFM	6.75	10.40	22.38	27.32	32.56	55.17
ILM	20.81	51.41	72.29	88.70	91.32	93.11
CM	20.10	20.71	23.55	23.65	26.47	33.98

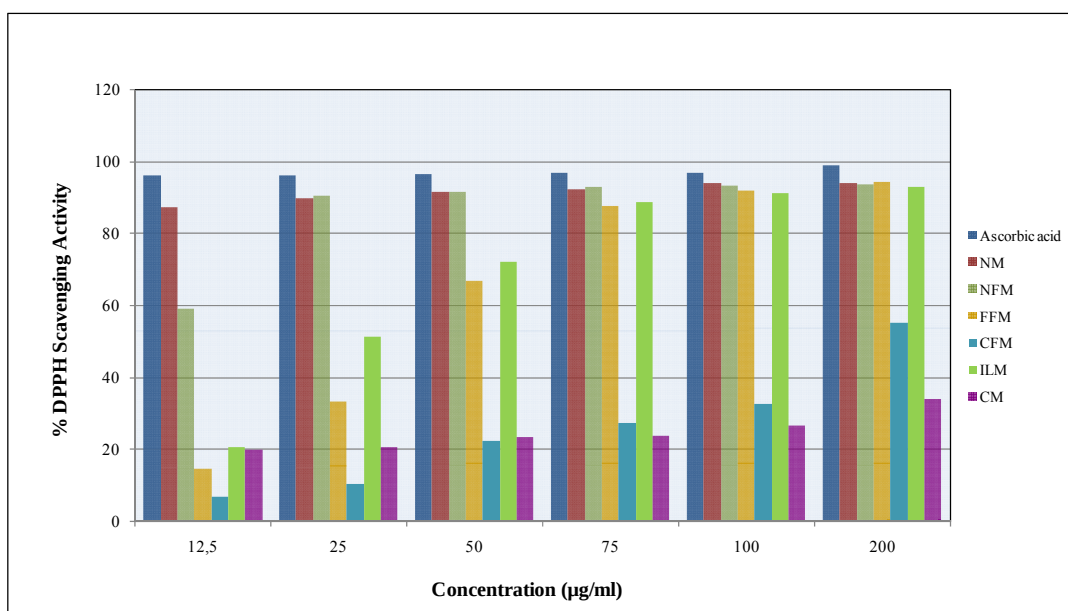
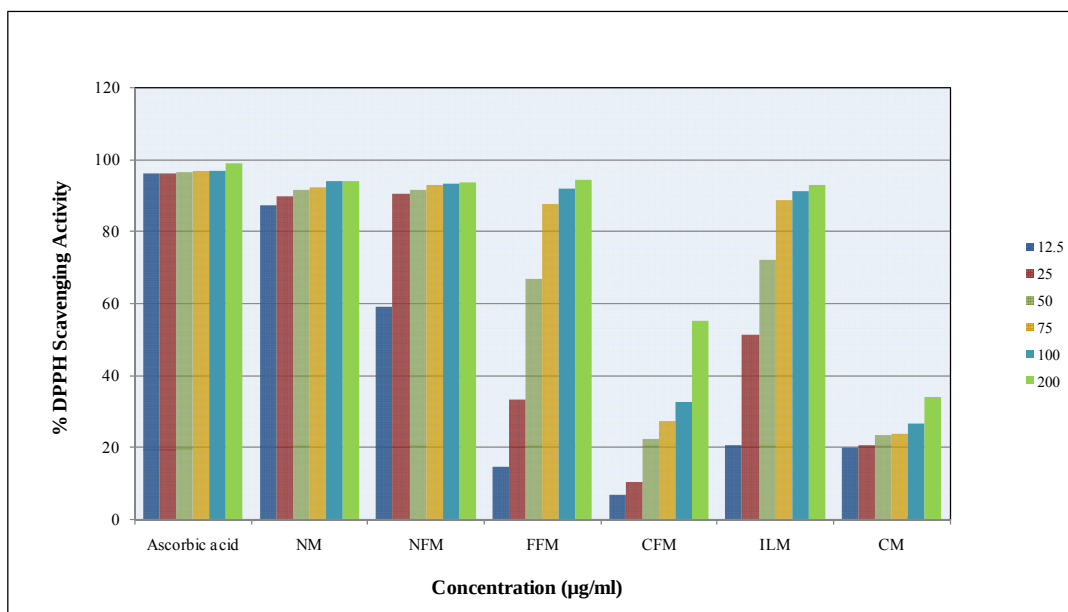


Figure 4.13. Free-radical (DPPH•) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, *in vitro* grown plant and callus from *F.vesca* and ascorbic acid (positive control).

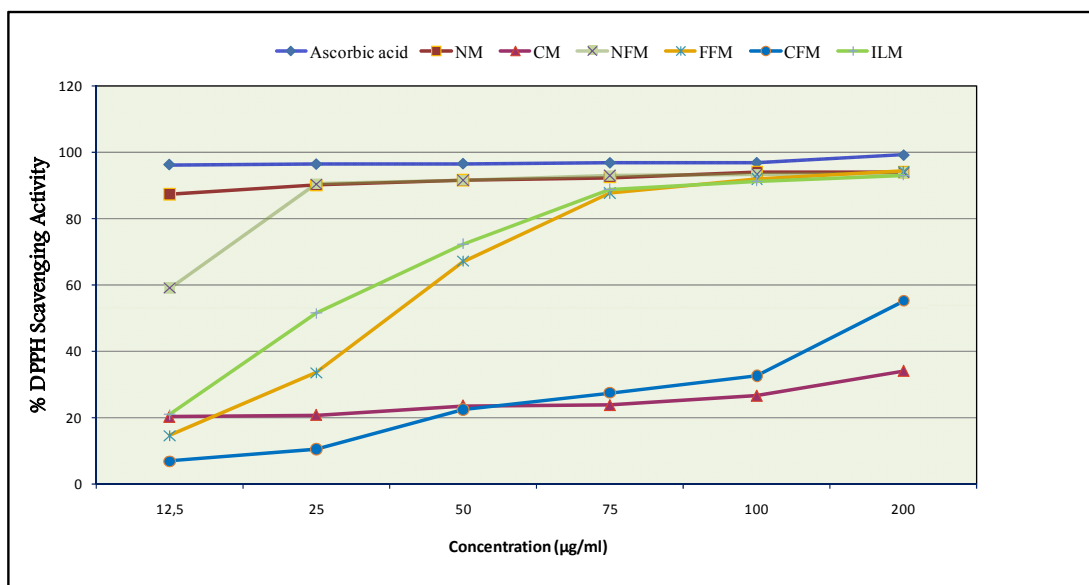


Figure 4.14. Free-radical (DPPH $\cdot$) scavenging activity (%) of methanolic extracts of fruits, field-grown plant, *in vitro* grown plant and callus from *F.vesca* and ascorbic acid (positive control).

4.3.5.2. Total Phenolic Assay (Folin-Ciocalteu Method For Total Phenolic Content)

Hydroxyl groups on phenolic compounds have scavenging ability so they are very important plant constituents. A number of studies reported a significant relation between the phenolic contents of plant extracts and their antioxidant properties (Gülçin et al., 2004).

Total phenolic contents of all methanolic extracts were shown in Table 4.6. In order to determine the total phenolic contents of these extracts, calibration curves were plotted using known quantities of standard gallic acids (Figure 4.15.).

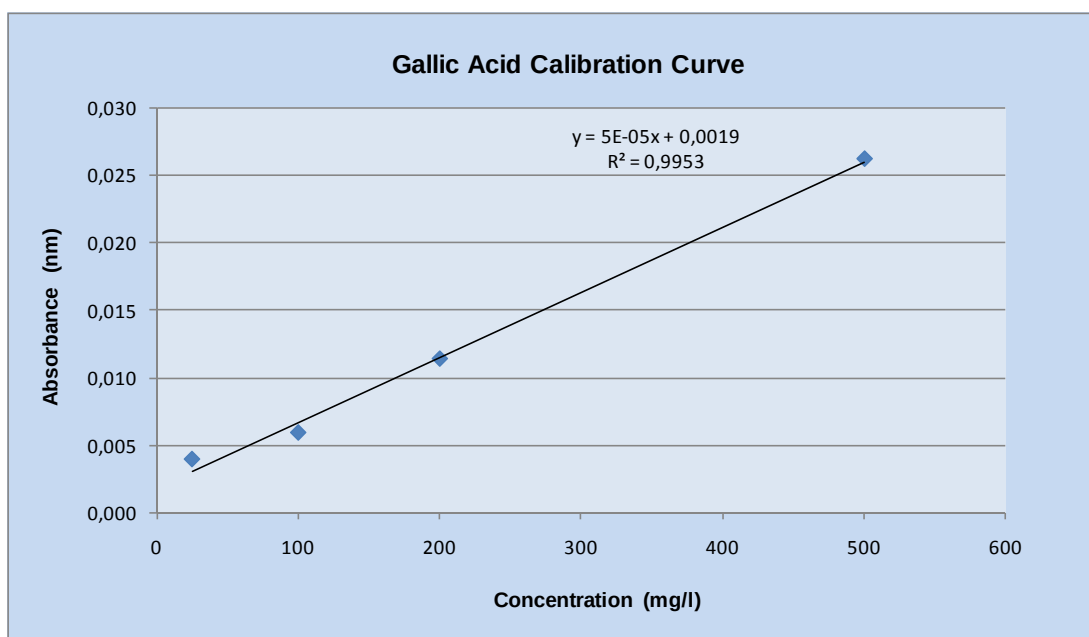


Figure 4.15. Calibration curve for gallic acid standards.

When these extracts were compared with respect to their phenol content, methanolic extract (NM) and gradually prepared methanolic extract (NFM) of field-grown leaves contained most phenolic content than other tested extracts (767.8000 ± 0.0000 mg/g and 633.0000 ± 0.0000 mg gallic acid equivalent/g dried extract,

respectively). Numerous studies have revealed that environmental stress often raise the accumulation of the phenolics, which are believed that to play a regulatory role in some metabolic process (Dixon and Paiva, 1995; Solecka 1997; Janas et al., 2000). Moreover, in these two extracts, same plant materials were used (field-grown leaves) but extraction procedure was different. Therefore, the amount of phenolic contents in NFM extract was slightly less than in NM extract (Table 4.8. and Figure 4.17.).

Gradually prepared methanolic extract of fruit (FFM) contained lower phenolic content than in NM and NFM extracts. Similarly, Wang and Lin (2000) studied leaves and berries of strawberry and they found that leaves have a greater amount of phenolic content than berries (Table 4.8. and Figure 4.17.).

The next high phenolic content was found in methanolic extract of *in vitro*-grown leaves (ILM) as 554.8610 ± 0.0000 mg gallic acid equivalent/g dried extract. ILM extract contained 2 times more phenolic content than gradually prepared methanolic extract of fruit (FFM) (251.5000 ± 0.0000 mg gallic acid equivalent/g dried extract) (Table 4.8. and Figure 4.17.).

There was no significant difference in phenolic contents among gradually prepared methanolic extract (CFM) and methanolic extract (CM) of *in vitro*-grown callus (146.0000 ± 0.0000 and 125.8333 ± 0.0000 mg gallic acid equivalent/g dried extract, respectively) (Table 4.8. and Figure 4.17.).

4.3.5.3. Total Flavonoid Assay (Aluminum chloride (AlCl₃) Colorimetric Assay For Total Flavonoids)

Aluminium chloride (AlCl₃) is used for the colorimetric determination of total flavonoid content in plant material. In order to determine the total flavonoid

content of the extracts, calibration curve of standard catechol solutions were plotted after measuring the absorbance of standards at 510 nm (Figure 4.16).

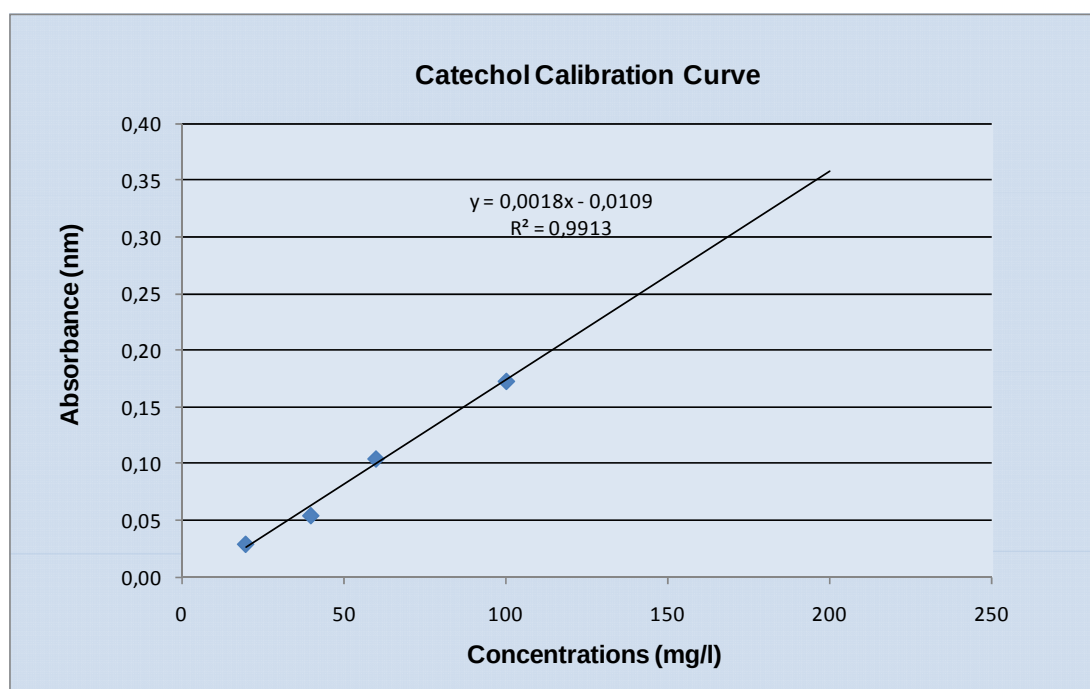


Figure 4.16. Calibration curve for catechol standards.

The results for total flavonoid content in the studied extracts indicated that methanolic extract of field-grown leaves (NM) contained the highest flavonoid contents as 184.4310 ± 0.0000 mg catechol equivalent/g dried extract and followed by ILM extract (121.4603 ± 0.0000 mg). Moreover, methanolic extract (CFM) of *in vitro*-grown callus contained the lowest flavonoid contents (29.6190 ± 0.0000 mg catechol equivalent/g dried extract) (Table 4.8. and Figure 4.17.).

There is no relation between total phenolic and flavonoid contents of the tested extracts except NM extract. Although there was no significant difference in the total phenolic contents of NM and NFM extracts, NM extract contained 2.5 times more flavonoid content than in NFM extract. The reason for this may be some

flavonoid compounds dissolved in hexane or DCM solvents until methanol extraction (Table 4.8. and Figure 4.17.).

It is obvious that, there is a good relation between the total phenolic content (Table 4.8) and LC-MS/MS analysis results (Table 3.3.) for NM and NFM extracts. However, we can not say the presence of that kind relation for the rest of the extracts.

Rice-Evans et al. (1996) reported the good relation between total phenol contents and the antioxidant activities of plant extracts. Other studies have pointed out that a high and positive relation between flavonoid content and DPPH activity of the fruits were found (Cheel et al., 2007). However, in our study, antioxidant activity was correlated with the levels of phenolic contents in the extracts. The extent of antioxidative effects produced by phenols largely depends on the structure of a given compound (or extract) and especially on the number and substitution of hydroxyl groups (-OH) on the structure. Phenolic compounds easily release hydrogen atom on -OH groups, which means that they oxidize themselves, thus acting as antioxidant (Weidner et al., 2009).

Table 4.8. Total phenol and total flavonoid contents of methanolic extracts of *F. vesca* L. Gallic acid equivalent was used as mg GAE/g of dried mass of plant material. Catechol equivalent was used as mg CE/g of dried mass of plant material. Results were expressed as means $\pm$ SD.

Treatments	Total Phenolics mg GAE/ g dried mass	Total Flavonoids mg CE/ g dried mass
NM	767.8000 $\pm$ 0.0000	184.4310 $\pm$ 0.0001
NFM	633.0000 $\pm$ 0.0000	71.6614 $\pm$ 0.0001
FFM	251.5000 $\pm$ 0.0000	46.4230 $\pm$ 0.0000
CFM	146.0000 $\pm$ 0.0000	29.6190 $\pm$ 0.0000
ILM	554.861 $\pm$ 0.0000	121.4603 $\pm$ 0.0000
CM	125.8333 $\pm$ 0.0000	55.5873 $\pm$ 0.0000

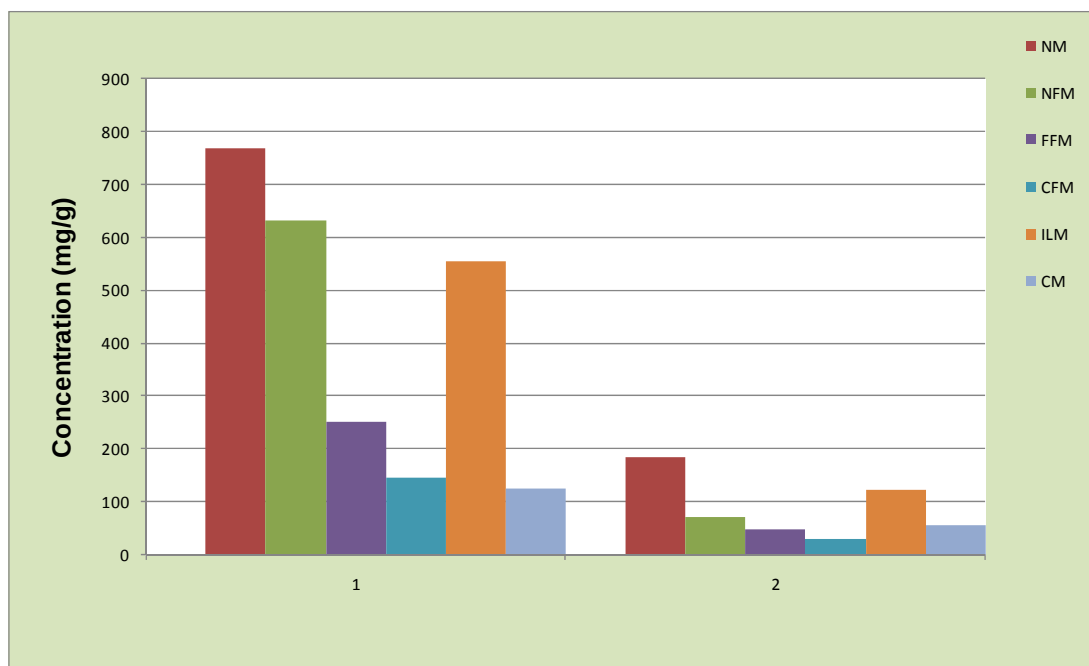


Figure 4.17. Total phenol and flavonoid contents of methanolic extracts of *F. vesca* L.

CHAPTER 5

5. CONCLUSIONS

F. vesca is a low-growing perennial herb in the family Rosaceae that has been used in the treatment of liver and kidney complaints, as well as in the treatment of rheumatism in the traditional medicine. Although *F. vesca* is a valuable medicinal herb, there are no detailed regeneration studies containing different concentrations and combinations of plant growth regulators in relation with different regeneration enhancers such as silver nitrate (AgNO_3) or surfactants (Tween 20, Tween 80 and Pluronic F-68). With this efficient protocol, disease- and herbicide-free bulk plant material can be supplied throughout the year for pharmaceutical purposes. Two different explants (leaf and petiole) obtained from sterilized field-grown plants and sterile seedlings from germinated seeds were cultured on MS medium containing TDZ, BA or KIN in combination with different auxins (IAA, IBA, 2,4-D, NAA) and supplemented with or without some surfactants (Tween 20, Tween 80 and Pluronic F-68), silver nitrate (AgNO_3) or GA_3 for shoot regeneration.

Explants obtained from field-grown plant materials were not responsive for shoot regeneration. None of the tested treatments or explant types was able to produce adventitious shoots. Red colored callus formation was observed with some combinations of TDZ and 2,4-D. More callus formation was obtained with leaf explants on media containing 0.5 mg/l TDZ and 0.1 mg/l 2,4-D.

Leaf and petiole explants obtained from sterile seedlings produced shoots with TDZ:IBA, TDZ:IBA:GA₃, TDZ:IBA:AgNO₃, TDZ:2,4-D and BA:IBA combinations. Supplementation of media with pluronic F-68 significantly increased shoot number and frequency of shoot formation. The regeneration efficiency was found to be higher in leaf explants than petiole explants with regard to both the mean number of shoots per shooted explant and the percentages of explants forming shoots.

Among the leaf explants, best shoot proliferation was obtained with 1.0 mg/l TDZ in combination with 0.5 mg/l IBA and 0.1 % pluronic (22.8 shoots; 90% explants forming shoots). Media containing 1.5 mg/l TDZ plus 0.5 mg/l IBA plus 0.05% pluronic (23.7 shoots; 20% explants forming shoots) and 1.5 mg/l TDZ plus 0.5 mg/l IBA plus 0.1% pluronic (24.5 shoots; 75% explants forming shoots) were also effective for shoot proliferation but shoot frequencies were lower for these combinations. Regarding the petiole explants, best shoot proliferation was obtained with 1.5 mg/l TDZ in combination with 2.5 mg/l IBA and 0.05% pluronic (12.0 shoots; 40% explants forming shoots). Pluronic F-68 was found as effective surfactant that induces shoot formation. Best results were obtained by the use of pluronic F-68 at minimum concentrations for all types of explants.

Callus formation was observed before shoot formation for both leaf and petiole explants with all experimented combinations (indirect regeneration). Control treatments involving no plant growth regulators produced no shoots in both explants. Generally, shoot regeneration along the periphery of the explants was visually detected within 4-6 weeks. Well developed shoots were observed within 8 weeks.

After 8 weeks in regeneration medium, regenerated shoots were cultured on shoot elongation medium containing 0.5 mg/l GA₃ for additional 2 weeks. After that period, regenerated shoots were separated individually and cultured on MS medium containing different concentrations of IAA, IBA, 2,4-D or NAA. Best root formation was observed with MS basal medium (control). If we compare among the various auxins investigated for rooting, IBA and IAA were effective for rooting. But, root formation was not observed with NAA and 2,4-D. Rooted plants were transferred to magenta containers including vermiculate for acclimatization. After 2 weeks, they were transferred to soil.

Field-grown leaves and fruits, and *in vitro*-grown leaves and callus were extracted with different solvents by using two methods; direct and gradual (starting from non-polar to polar solvent) extractions. In direct extraction, aqueous, ethanolic, methanolic and acetone extracts (NW, NE, NM and NA) of field-grown leaves; cold or hot aqueous and ethanolic extracts of both dry (CFCW, CFHW, CFCE and CFHE) and fresh fruits (FFCW, FFHW, FFCE and FFHE); methanolic extracts of *in vitro*-grown leaves (ILM) and callus (CM) were prepared. In gradual extraction, hexane, dichloromethane, methanolic and aqueous extracts of field-grown leaves (NFH, NFD, NFM and NFW) and fruits (FFH, FFD, FFM and FFW), and *in vitro*-grown callus (CFH, CFD, CFM and CFW) were prepared respectively.

The phenolic contents of methanolic extracts (NM, NFM, FFM, ILM, CM and CFM) were analysed by using Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) and twenty-two phenolic compounds were used as authentic standards. The correlation of LC-ESI-MS/MS results with

antibacterial, antitumor, toxicity, anticancer and antioxidant bioassay results were investigated.

According to LC-ESI-MS/MS results, concentrations of phenolic compounds in field-grown leaf extracts (NFM and NM) were at least five times higher than the other extracts. The phenolic compounds found in high concentrations in NFM and NM extracts were (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, gallic acid monohydrate, kaempferol, procyanidin C1, pyrocyanidin B1 + B2 and quercetin. The concentration of these compounds in field-grown leaf extract obtained with gradual extraction method (NFM) was higher than field-grown leaf extract obtained with direct extraction method (NM). Probably, in gradual extraction, moderately polar solvents such as dichloromethane (DCM) might have removed some other compounds that would be soluble in methanol. So the concentration of the phenolic compounds was found to be higher in NFM extract.

Five different biological assays (antibacterial, anti-tumor, toxicity, anticancer and antioxidant activity) were used for assessing biological activities of *F. vesca* extracts. Seventeen different extracts (NW, NE, NM, NA, NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW and ILM) of *F. vesca* were used to screen for antibacterial activity with disc diffusion assay (Kirby-Bauer Method). Both gram positive and gram negative bacteria seemed to be susceptible to the inhibitory effects of the *F. vesca* extracts. Among all tested extracts, hexane and dichloromethane extracts of fruit (FFH and FFD), field-grown leaves (NFH and NFD) and *in vitro*-grown callus (CFH and CFD) did not show any antibacterial activity against tested bacteria. Generally, field-grown leaves and fruits indicated better antibacterial activity than *in vitro*-grown leaves and callus. When we made a

comparison within all used bacteria, *E. cloacae* was the most susceptible one to the extracts having antibacterial activity and the best antibacterial activity was obtained with FFW, NA, NFW and NFM extracts. It can be conceivable that strong antibacterial activity may come from phenolic constituent of *F. vesca*.

The antitumor activity of *F. vesca* was tested with the potato disc method by using *Agrobacterium tumefaciens* as modified by McLaughlin's group. Eleven different extracts (NW, NE, NM, DFCW, DFHW, FFCW, FFHW, DFCE, DFHE, FFCE and FFHE) of field-grown leaves and fruits were tested in this assay. The best antitumor activity was observed with aqueous (NW) and methanolic (NM) extracts of field-grown leaves, and hot ethanolic extract (FFHE) of fresh fruit. When compared with control (water), the percentage inhibition of all extracts except cold aqueous extract of fresh fruit (FFCW) and hot aqueous extract (DFHW) of dry fruit was more than 40% in three separate experiments. All extracts (methanol, ethanol and water) of field-grown leaves (NM, NE and NW) exhibited strong antitumor activity. On the other hand, ethanolic extracts of both dry and fresh fruits (DFCE, DFHE, FFCE and FFHE) exhibited better antitumor activity than those of aqueous extracts. According to results, we can say that there is a positive relation between the antitumor activity of methanolic extract and LC-ESI-MS/MS results.

A prerequisite for potato disc tumor induction assay is that the extract or substance being tested should not have antibacterial activity toward *A. tumefaciens*. Inhibition of crown gall formation on potato discs is caused by two effects: by anti-tumorogenesis or decreasing the viability of the *A. tumefaciens*. Viability tests were carried out with all extracts to distinguish between these possibilities. All tested

extracts did not affect the viability of the bacterium. Thus, observed inhibition of tumor formation for these extracts was on the formation of tumors.

Toxicity of six different *F. vesca* extracts (NM, NFM, FFM, CFM, CM and ILM) was tested with brine shrimp bioassay. This bioassay determines the toxicity of materials toward brine shrimp larvae and can predict the ability to kill cancer cells in cell cultures. The extracts of *F. vesca* were low toxic at higher doses by comparing with MS-222 (positive control). Extracts of field-grown leaves and fruits were more toxic than in the extracts of *in vitro*-grown leaves and callus. Among the tested extracts, gradually prepared methanol extracts of field-grown leaves (NFM) and fruits (FFM) were more toxic than other tested extracts with higher LC₅₀ values (696 mg/l and 465 mg/l, respectively). Moreover, methanol extracts of *in vitro*-grown leaves (ILM) and callus (CM) were less toxic (1246 mg/l and 1407 mg/l, respectively).

Fourteen different extracts (NM, NFH, NFD, NFM, NFW, FFH, FFD, FFM, FFW, CFH, CFD, CFM, CFW and ILM) of *F. vesca* were used to evaluate the anticancer activity on Human Breast Adenocarcinoma (MCF-7). MTT (3-[4-5 dimethylthiazol-2-yl] 2-5 diphenyl-tetrazolium bromide) assay was used to assess growth inhibition in this study.

In MTT assay for 24 h, all the gradually prepared hexane (CFH) and dichloromethane extracts (CFD) of *in vitro*-grown callus showed higher cell viability than in control. Among tested extracts, gradually prepared aqueous extract (FFW) of fruits showed better anticancer activity than rest of the extracts at 100 and 200 µg/ml concentrations. In MTT assay for 48h, two additional extracts were used: Methanolic extracts of *in vitro*-grown leaves (ILM) and field-grown leaves (NM). ILM extract

did not show anticancer activity and it showed higher cell viability than in control at low concentrations (12.5–50 µg/ml). NM extract showed same cell viability (75.2%) at 100 and 200 µg/ml. NM extract had better anticancer activity than ILM extract. Among tested extracts, gradually prepared dichloromethane extract (NFD) of field-grown leaves showed better anticancer activity and followed by gradually prepared hexane extract (FFH) of fruits than in the rest of the extracts. As a general discussion, increasing concentrations of the extract is not effective on the anticancer activity. This means that the anticancer activity is not concentration-dependent with respect to the result. However, it slightly increased by increasing time (from 24h to 48h). As a final remark, extracts of field-grown leaves and fruits showed better anticancer activity than in the extracts of *in vitro*-grown leaves and callus. Overall, high anticancer activity was not determined for all extracts.

Antioxidant activity, total phenolic and flavonoid contents of methanolic extracts of field-grown leaves (NM and NFM), fruits (FFM) and *in vitro*-grown leaves (ILM) and callus (CM and CFM) of *F. vesca* were assessed. Antioxidant activity was determined spectrophotometrically by monitoring the disappearance of 2,2-diphenyl-1-picrylhydrazil radical (DPPH•) and ascorbic acid was used as control antioxidant compound. Total soluble phenolics were determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound. The total flavonoid content was measured by aluminum chloride (AlCl₃) colorimetric assay and catechol was used as a reference flavonoid.

The results indicated that at minimum concentration, the best antioxidant activities were obtained by methanolic extract (NM) and gradually prepared methanolic extract (NFM) of field-grown leaves. When all extracts were compared

with respect to their phenolic content, methanolic extract (NM) and gradually prepared methanolic extract (NFM) of field-grown leaves contained the most phenolic content than the other tested extracts. In our study, antioxidant capacity was correlated with the levels of phenolic contents in the extracts. The results for total flavonoid content in the studied extracts indicated that methanolic extract of field-grown leaves (NM) contained the highest flavonoid contents and followed by methanolic extract of *in vitro*-grown leaves (ILM). There is no relation between total phenolic and flavonoid contents of the tested extracts except NM extract. Although there was no significant difference in the total phenolic contents of NM and NFM extracts, NM extract contained 2.5 times more flavonoid content than in NFM extract. The reason for this may be some flavonoid compounds dissolved in hexane or dichloromethane solvents until methanol extraction. It is obvious that, there is a good relation between the total phenolic content and LC-ESI-MS/MS analysis results for NM and NFM extracts. However, the presence of that kind relation for the rest of the extracts was not observed.

In this study, obtained results revealed the biological activities (antibacterial, antitumor, toxicity, anticancer and antioxidant) of several *F. vesca* extracts. Furthermore, the total phenolic and flavonoid content, and LC-ESI-MS/MS results confirmed the activities of antibacterial, antitumor and antioxidant. Definitive clinical studies are needed to fully understand the many medicinal uses of *F. vesca*. Future studies should focus on more specific fractionation of the extracts having antibacterial, antitumor and antioxidant activities to identify more active components of different extracts of *F. vesca*.

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APPENDICES

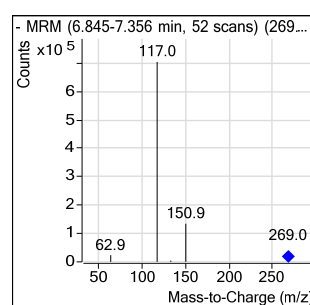
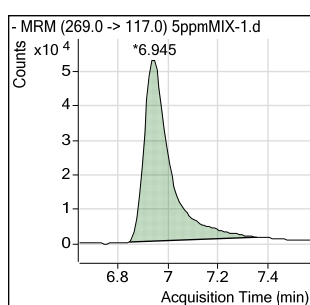
LC-ESI-MS/MS Quantifier Chromatograms and Mass Spectra for Each Standard

Phenolic Compound (Chapter 3)

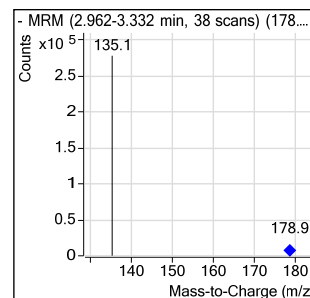
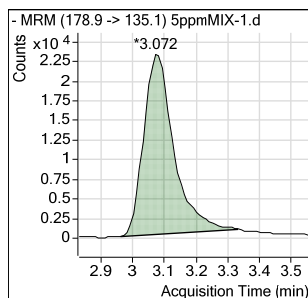
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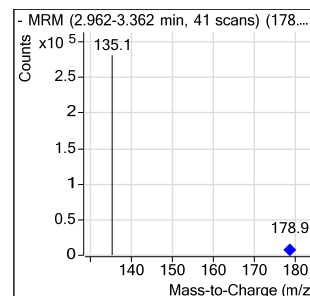
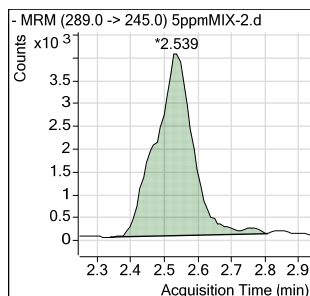
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Caffeic acid



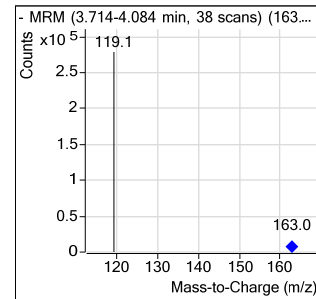
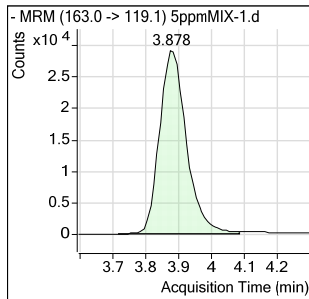
(+)-Catechin



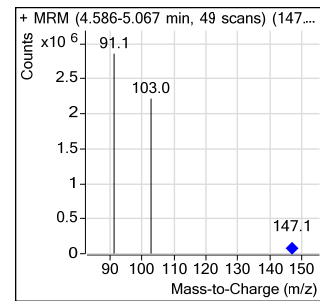
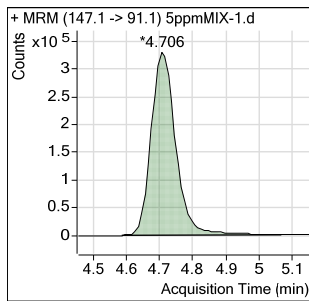
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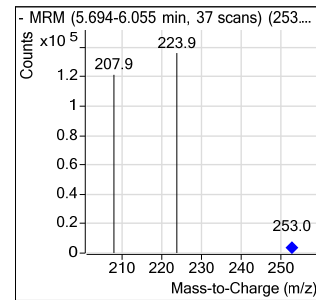
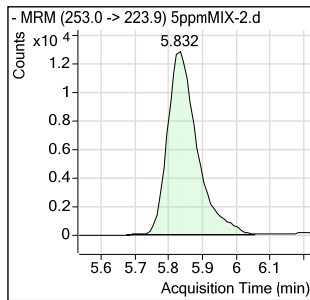
p-Coumaric acid



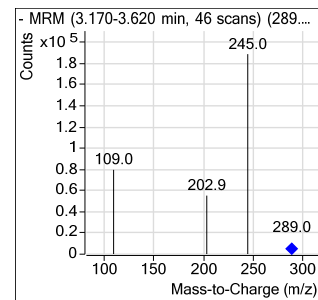
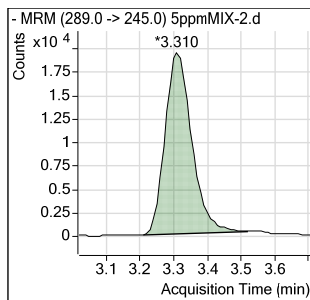
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Daidzein



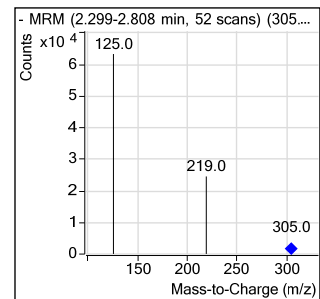
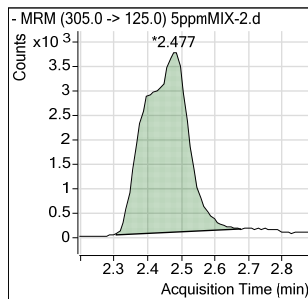
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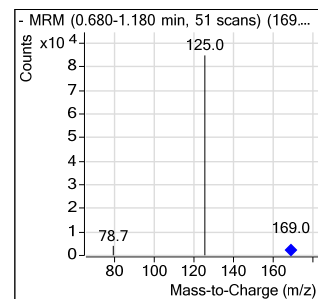
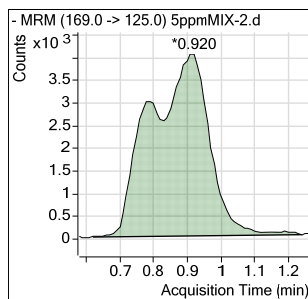
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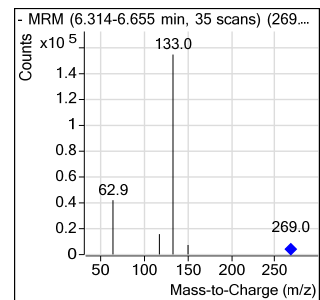
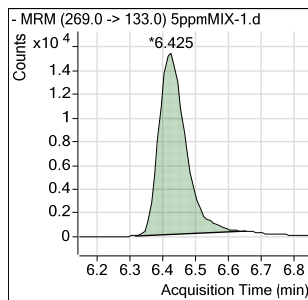
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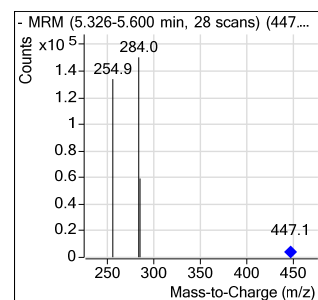
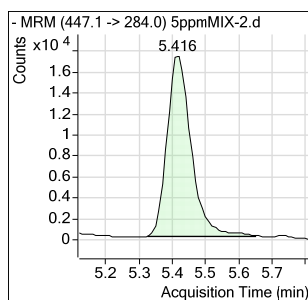
Gallic acid



Genistein



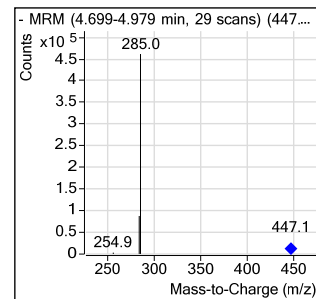
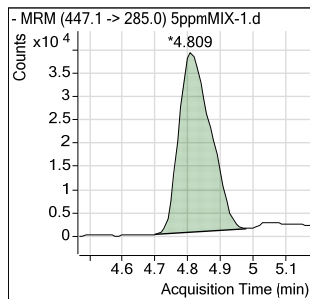
Kaempferol



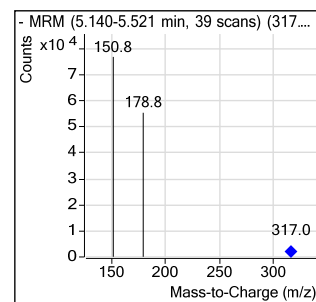
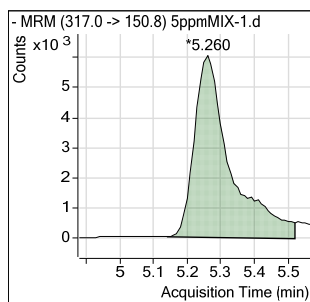
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Mass Spectrum

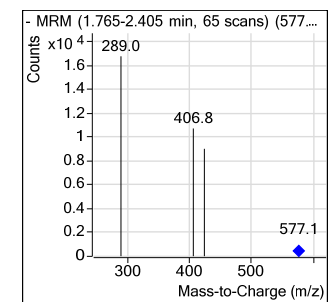
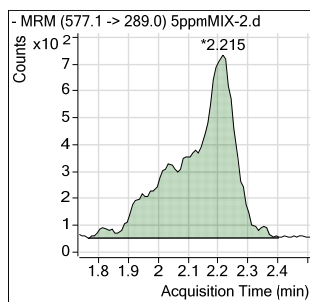
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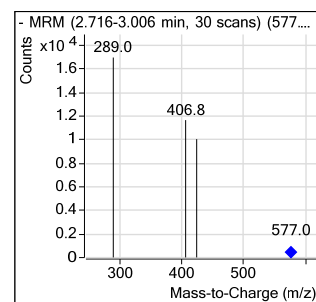
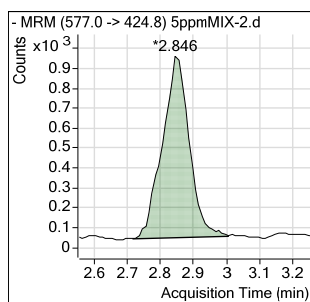
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Procyanidin B1



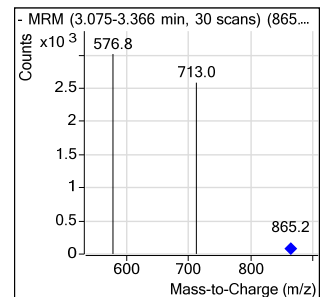
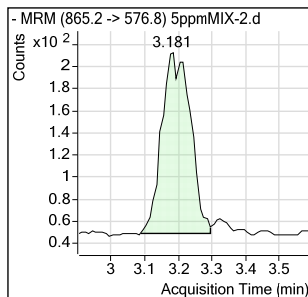
Procyanidin B2



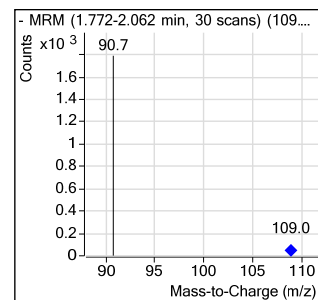
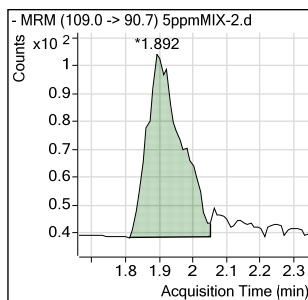
Quantifier Ion Chromatogram

Mass Spectrum

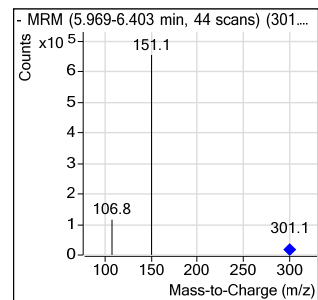
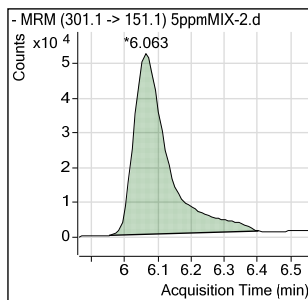
Procyanidin C1



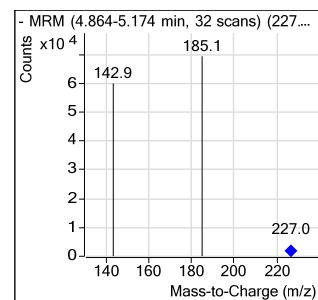
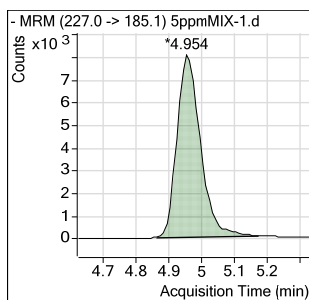
Pyrocatechol



Quercetin



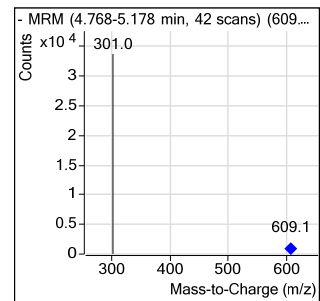
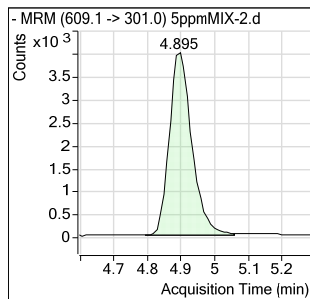
Resveratrol



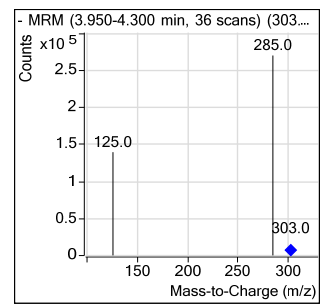
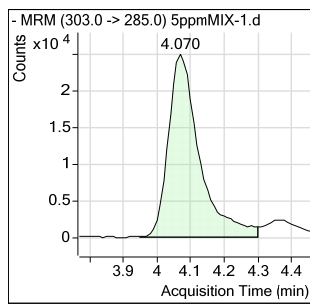
Quantifier Ion Chromatogram

Mass Spectrum

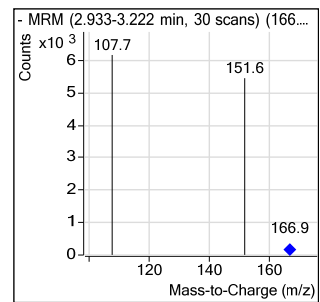
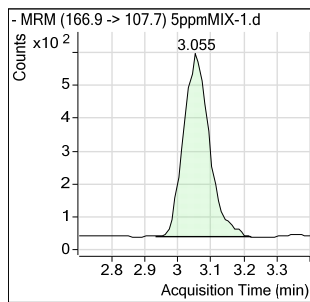
Rutin



(±)-Taxifolin



Vanillic acid



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Abant İzzet Baysal University	1999-2004	B.Sc.	June, 2004
Abant İzzet Baysal University	2005-2008	M.Sc.	Aug, 2008
Abant İzzet Baysal University	2008-2012	Ph.D.	July, 2012

Publications

- 1- Türker AU, Mutlu EC and **Yildirim AB** (2008). Efficient *in vitro* regeneration of Fireweed, a medicinal plant. *Acta Physiologiae Plantarum* 30(4): 421-426.
- 2- Hakan Türker, **Arzu Birinci Yıldırım**, Fatma Pehlivan Karakaş, Hilal Köylüoğlu (2009). Antibacterial Activities of Extracts from Some Turkish Endemic Plants on Common Fish Pathogens. *Turk J Biol*, 33: 73-78
- 3- **Yildirim AB**, Türker AU (2009). *In Vitro* Adventitious Shoot Regeneration of Meadowsweet (*Filipendula ulmaria* (L.) Maxim)- A Medicinal Plant. *In Vitro Cellular and Developmental Biology-Plant*, 45:135-144.

- 4- Hakan Turker, **Arzu Birinci Yıldırım**, Fatma Pehlivan Karakaş (2009). Sensitivity of fish pathogenic bacteria to some Turkish medicinal plants. *Turkish Journal of Fisheries and Aquatic Sciences*, 9: 181-186.
- 5- Turker, A.U, **A.B. Yıldırım**, F.P. Karakas. Antibacterial And Antitumor Activities of Some Wild Fruits Grown in Turkey. *Biotechnology and Biotechnological Equipment*, (2011).
- 6- Arzu Ucar Turker, **Arzu Birinci Yıldırım**, Fatma Pehlivan Karakas. Antitumor and Antibacterial Activities of *Viscum album* L. Grown on Different Host Trees, *in press*, 2012.
- 7- **Arzu Birinci Yıldırım**, Arzu Ucar Turker, Fatma Pehlivan Karakas. In vitro Antibacterial and Antitumor Activities of Some Medicinal Plant Extracts, Growing in Turkey, *Asian Pacific Journal of Tropical Medicine*, *in press*, 2012.
- 8- Fatma Pehlivan Karakas, **Arzu Birinci Yıldırım**, Arzu Ucar Turker. Biological Screening of Various Medicinal Plant Extracts for Antibacterial and Antitumor Activities, *in press*, 2012.

Communications to national scientific meetings

- 1- **Yıldırım AB**, Türker AU (2007) Çayır kraliçesi (*Filipendula ulmaria* L.) bitkisinin *in vitro* kültür yöntemi ile çoğaltımı. XV. Ulusal Biyoteknoloji Kongresi (Antalya) Özet kitapçığı, Sayfa 10 (Poster sunum).
- 2- Karakaş FP, **Yıldırım AB**, Köylüoğlu H, Türker AU (2007). *Vinca minor* L. (Küçük cezayir menekşesi) bitkisinin anti-tümör aktivitesi. XV. Ulusal Biyoteknoloji Kongresi (Antalya) Özet kitapçığı, Sayfa 425 (Sözlü sunum).
- 3- Türker AU, Yapıcı AF, Uğurlu E, Köylüoğlu H, **Yıldırım AB**, Karakaş FP (2007). Farklı ağaçlar üzerinde bulunan ökse otlarının toksisitelerinin karşılaştırılması. XV. Ulusal Biyoteknoloji Kongresi (Antalya) Özet kitapçığı, Sayfa 11 (Poster sunum).

- 4- Türker AU, **Yıldırım AB**, Karakaş FP (2008). Dağ çileği (*Fragaria vesca* L.) yapraklarının antimikrobiyal aktivitesi. 19. Ulusal Biyoloji Kongresi (Trabzon) Özet kitapçığı, Sayfa 340 (Poster sunum).
- 5- Karakaş FP, **Yıldırım AB**, Köylüoğlu H, Türker AU (2008). Beyaz Nilüfer (*Nymphaea alba* L.) ve Sarı Nilüfer (*Nuphar lutea* L.) bitkilerinin anti-tümör ve anti-bakteriyal aktiviteleri. 19. Ulusal Biyoloji Kongresi (Trabzon) Özet kitapçığı, Sayfa 368 (Poster sunum).
- 6- **Yıldırım AB**, Türker AU (2008). Çayır kraliçesi (*Filipendula ulmaria* (L.) Maxim.) bitkisinin antibakteriyal ve anti-tümör aktivitesi. 19. Ulusal Biyoloji Kongresi (Trabzon) Özet kitapçığı, Sayfa 368 (Poster sunum).
- 7- **Yıldırım AB**, Türker AU (2008). *Lysimachia vulgaris* L. Bitkisinin doku kültürü yöntemi ile çoğaltılması. 19. Ulusal Biyoloji Kongresi (Trabzon) Özet kitapçığı, Sayfa 436 (Poster sunum).
- 8- Türker AU, Mutlu EC, **Yıldırım AB**, (2008). *Epilobium angustifolium* L. (Yakı otu) bitkisinin *in vitro* kültür yöntemi ile çoğaltılması. 19. Ulusal Biyoloji Kongresi (Trabzon) Özet kitapçığı, Sayfa 437 (Poster sunum).
- 9- **Arzu Birinci Yıldırım**, Fatma Pehlivan Karakaş, Arzu Uçar Türker (2010). *Anemone Nemorosa* L. bitki özütlerinin *in vitro* antibakteriyel aktiviteleri. 20.Ulusal Biyoloji Kongresi (Denizli) Özet kitapçığı, Sayfa 397 (Poster sunum).
- 10- **Arzu Birinci Yıldırım**, Fatma Pehlivan Karakaş, Arzu Uçar Türker (2010). Bazı tıbbi bitkilerin *in vitro* antitümör aktiviteleri. 20.Ulusal Biyoloji Kongresi (Denizli) Özet kitapçığı, Sayfa 398 (Poster sunum).
- 11- Fatma Pehlivan Karakaş, **Arzu Birinci Yıldırım**, Arzu Uçar Türker (2010). *Lamium Crinitum* bitki özütlerinin antibakteriyel aktiviteleri. 20.Ulusal Biyoloji Kongresi (Denizli) Özet kitapçığı, Sayfa 402 (Poster sunum).

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- 1- **Yıldırım AB**, Türker AU (2011). Screening of the medicinal plant (*Prunus laurocerasus*) extracts for antibacterial activity. 1st International Symposium on Secondary Metabolites (Denizli) Özet kitapçığı, Sayfa 55 (Poster sunum).
- 2- **Yıldırım AB**, Türker AU (2011). Antitumor activity studies of *Fragaria vesca* (Woodland Strawberry) extracts. 1st International Symposium on Secondary Metabolites (Denizli) Özet kitapçığı, Sayfa 56 (Poster sunum).
- 3- Türker AU, **Yıldırım AB** (2011). Antibacterial and antitumor activities of *Viscum album* grown on different trees. 1st International Symposium on Secondary Metabolites (Denizli) Özet kitapçığı, Sayfa 63 (Poster sunum).
- 4- Türker AU, **Yıldırım AB** (2011). Antibacterial activities of some turkish endemic plants. 1st International Symposium on Secondary Metabolites (Denizli) Özet kitapçığı, Sayfa 162 (Poster sunum).

Positions held

English Teacher – Bolu Canip Baysal High School – Bolu, Turkey –2004-2005.

Biology Teacher – İzzet Baysal Industrial Vocational High School – Bolu, Turkey– September 2005 – January 2006.

Sales Representative – Tansel Elektrik A.Ş. – Bolu, Turkey – February 2006 – August 2006.