TISSUE CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITY OF *FILIPENDULA ULMARIA* (L.) MAXIM, A MEDICINAL PLANT

ARZU BİRİNCİ YILDIRIM

AUGUST 2008

TISSUE CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITY OF *FILIPENDULA ULMARIA* (L.) MAXIM, A MEDICINAL PLANT

by

ARZU BİRİNCİ YILDIRIM

THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE ABANT İZZET BAYSAL UNIVERSITY
IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
THE DEPARTMENT OF BIOLOGY

AUGUST 2008

Approval of the Graduate School of Natural Sciences

Prof. Dr. Nihat Çelebi

Director

I certify that this thesis satisfies all the requirements for the degree of Master of Science.

Prof. Dr. Ömer BOZDOĞAN

Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Arzu UÇAR TÜRKER

Supervisor

Examining Committee Members

1. Prof. Dr. Ekrem GÜREL .....

2. Assist. Prof. Dr. Belgin COŞGE .....

3. Assist. Prof. Dr. Arzu UÇAR TÜRKER .....

ABSTRACT

TISSUE CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITY OF *FILIPENDULA ULMARIA* (L.) MAXIM, A MEDICINAL PLANT

Yıldırım Birinci, Arzu

MSc, Department of Biology

Supervisor: Assist. Prof. Dr. Arzu Türker

August 2008, 70 pages

Filipendula ulmaria (L.) Maxim (meadowsweet) is a medicinal plant that has been used to treat several inflammatory diseases including gout and rheumatoid arthritis, and for the treatment of coughs, bronchitis, fevers, ulcers and colds. A reliable *in vitro* culture protocol for meadowsweet was established. Three different explants (leaf discs, petioles and roots) were cultured on Murashige and Skoog minimal organics (MSMO) medium with various hormone combinations. The best shoot formation was obtained from root explants with 0.1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3 and 0.5 mg/l TDZ+0.25 mg/l IAA+0.5 mg/l GA_3. Regenerated shoots were rooted on MSMO medium containing different concentration of IAA, IBA, 2,4-D or NAA for rooting. IBA was the most effective auxin for rooting. The greatest number of roots per explant was observed with 0.5 mg/l IBA and 0.5 mg/l IAA. Rooted plants were transferred to Magenta containers including vermiculate for acclimatization. After 3 weeks, they were transferred to soil.

Three different bioassays (antibacterial, antitumor and toxicity) were performed to show the biological activities of meadowsweet extracts. They were evaluated between field-grown plants and *in vitro*-grown plants using eight different extracts [ethanol extracts of *in vitro*-grown (IE) and field-grown (FE) plant materials, ethylacetate extracts of *in vitro*-grown (IEA) and field-grown (FEA) plant materials, hexane extracts of *in vitro*-grown (IH) and field-grown (FH) plant materials and aqueous extract of *in vitro*-grown (IW) and field-grown (FW) plant materials]. Generally antibacterial activities of field-grown plants were higher than *in-vitro* grown plants against all bacteria used. Aqueous extract of field-grown plant (FW) exhibited higher antibacterial activity than other extracts. *S.epidermidis*, *S. aureus, S. typhimurium, S. marcescens*, , *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae* and *E. cloacae* were sensitive to FW.

Antitumor activity of meadowsweet was tested with potato disc diffusion bioassay. Field-grown plants showed higher activity than *in vitro*-grown plants. But, after viability test for *Agrobacterium tumefaciens*, it was understood that inhibition of crown gall formation on potato disc is caused by decreasing the viability of the *A. tumefaciens*. It is not possible to test the antitumor activity of *F. ulmaria* with potato disc bioassay. Because meadowsweet extracts have very strong antibacterial activity and affect the viability of *A. tumefaciens*.

The brine shrimp bioassay was used to assess the general toxicity of meadowsweet extracts. All extracts were toxic at higher doses (LC_{50} > 2.000 mg/l) by comparing with MS-222 (Tricaine methane sulfonate). Aqueous extracts of field-grown and *in vitro*-grown plants were less toxic than other

extracts (ethanol, ethylacetate and hexane). Meadowsweet extracts were cytotoxic at high concentrations and they may not be very effective to kill cancer cells in cell culture.

Keywords: *Filipendula ulmaria*, meadowsweet, *in vitro* culture, GA_3, root explant, TDZ, bioassay, antibacterial, anti-tumor, toxicity, brine shrimp bioassay

ÖZET

TIBBİ BİR BİTKİ OLAN *FILIPENDULA ULMARIA* (L.) MAXIM 'İN DOKU KÜLTÜRÜ VE BİYOLOJİK AKTİVİTESİNİN DEĞERLENDİRİLMESİ

Yıldırım Birinci, Arzu

Yüksek Lisans, Biyoloji Bölümü

Tez Danışmanı: Yrd. Doc. Dr. Arzu Uçar Türker

Ağustos 2008, 70 Sayfa

Filipendula ulmaria (çayırkraliçesi) damla hastalığı ve eklem iltihabı gibi iltihaplı hastalıkların tedavisinde, öksürük, bronşit, ateş, soğuk algınlığı ve ülser tedavisinde kullanılan tıbbi bir bitkidir. Çayır kraliçesi bitkisi için güvenilir *in vitro* kültür yöntemi geliştirilmiştir. Üç farklı eksplant **(**yaprak, petiyol parçaları ve kök) çeşitli hormon kombinasyonlarının bulunduğu Murashige ve Skoog Minimal Organik (MSMO) ortamı içerisinde kültüre alınmıştır. En iyi sürgün oluşumu kök eksplantlarının 0.1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3 ve 0.5 mg/l TDZ+0.25 mg/l IAA+0.5 mg/l GA_3 içerdiği ortamlarda elde edilmiştir. Oluşan sürgünler farklı konsantrasyonlarda IAA, IBA, NAA veya 2,4-D içeren köklendirme ortamlarına aktarılmıştır. Köklendirme için en etkili oksin hormonu IBA olmuştur. En fazla kök oluşumu 0.5 mg/l IBA ve 0.5 mg/l IAA içeren ortamlarda gözlenmiştir**.** Köklendirilmiş bitkicikler vermikülat içeren Magenta kaplarına transfer edilmiş ve 3 hafta sonunda toprağa aktarılmıştır**.**

Çayırkıraliçesi özütlerinin biyolojik aktivitesinin değerlendirilmesi için üç farklı biyolojik test (antibakteriyal, antitümör ve toksisite) uygulanmıştır. Sekiz farklı özüt [*in vitro* yetiştirilmiş (IE) ve arazide yetişmiş (FE) bitkinin etanol özütü, *in vitro* yetiştirilmiş (IEA) ve arazide yetişmiş (FEA) bitkinin etilasetat özütü, *in vitro* yetiştirilmiş (IH) ve arazide yetişmiş (FH) bitkinin hekzan özütü, *in vitro* yetiştirilmiş (IW) ve arazide yetişmiş (FW) bitkinin su ile hazırlanan özütü] kullanılarak biyolojik aktivite değerlendirilmiştir. Genel olarak kullanılan bakterilere karşı, arazide yetişmiş bitkilerden elde edilen özütlerin antibakteriyal aktivitesi *in vitro* yetiştirilmiş bitkilerden elde edilen özütlerden daha iyi olmuştur. Arazide yetişmiş bitkilerden su ile elde edilen özütler (FW) diğer özütlere göre daha iyi antibakteriyal aktivite ortaya koymuştur. *S.epidermidis*, *S. aureus, S. typhimurium, S. marcescens*, , *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae* ve *E. cloacae* FW' ye karşı hassasiyet göstermiştir.

Çayırkraliçesi'nin antitümör aktivitesi patates disk yöntemi ile test edilmiştir. Arazide yetişmiş bitkiler *in vitro* yetiştirilmiş bitkilerden daha iyi antibakteriyal aktivite göstermiştir. Fakat, *Agrobacterium tumefaciens* için canlılık testi uygulandığında patates üzerindeki tümör inhibisyonunun *A. tumefaciens*'in canlılığını azalttığı gözlenmiştir. *F. ulmaria*' nın antitümör aktivitesinin patates disk yöntemi ile test edilmesi mümkün değildir çünkü bu bitki özütleri çok güçlü antibakteriyal aktiviteye sahiptir ve *A. tumefaciens*' in canlılığını etkilemektedir.

Çayırkraliçesi bitki özütlerinin genel toksisitesini değerlendirebilmek için tuzlu su karides testi kullanılmıştır. MS-222 (Trikain metan sülfonat) ile karşılaştırıldığında bütün özütlerin yüksek konsantrasyonlarda (LC_{50} > 2.000

mg/l) toksik olduğu görülmüştür. Arazide yetişmiş ve *in vitro* olarak yetiştirilmiş bitkilerden elde edilen sulu özütlerin diğer özütlere (etanol, etilasetat ve hekzan) göre toksisitesi daha düşüktür. Çayırkraliçesi özütleri yüksek konsantrasyonlarda toksiktir ve hücre kültürlerinde kanser hücrelerinin öldürülmesinde etkili olamayacağı düşünülmektedir.

Anahtar Kelimeler: *Filipendula ulmaria*, çayır kraliçesi, *in vitro* kültür, GA_3, kök eksplantı, TDZ, biyolojik test, antibakteriyal, antitümör, toksisite, tuzlu su karides testi.

DEDICATION

I dedicate this dissertation to all my family members; especially my mother, Hava Birinci.

ACKNOWLEDGMENTS

I wish to acknowledge the indispensable assistance afforded me by so many people throughout my life and this experience. I would like to express my appreciation and gratitude to my supervisor Assist. Prof. Dr. Arzu Uçar Türker for her guidance, support, patience and encouragement. I would like to thank also to Assist. Prof. Dr. Hakan Türker, with my deepest appreciation. I would like to thank my committee members Prof. Dr. Ekrem Gürel and Assist. Prof. Dr. Belgin Coşge for their sincerities and helps. I would like to express my gratitude to the entire Art and Science faculty, staff, and student body for making my work at Abant İzzet Baysal University very enjoyable and beneficial.

I am grateful to my lab-mate, Esra Cansever Mutlu without whose assistance and conversation in the lab would have made this work unbearable. She was a great friend, thank you very much for making me smile. To my husband, Muhammet, I offer my sincere thanks for his unshakable faith in me and his willingness to endure with me vicissitudes of my endeavors and to all my family members for all their moral support during my studies. My appreciation is extended to my mother, sister and all my big family for their love, encouragement and limitless support. I would also like to thank my friend Buhara Yücesan and Hilal Köylüoğlu for sharing the stressful moments with me. Finally, I apologize to those not mentioned-they are, however, not forgotten.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

List of Figures (Contiuned)

CHAPTER ONE

1. Introduction and Literature Review

1.1. Introduction

Filipendula ulmaria (L.) Maxim. is a perennial herb in the family Rosaceae growing wild all over Europe, North America and Siberia (Lindemann *et al*. 1982). It grows in damp meadows, ditches and bogs, at the edges of ponds and on river banks. The flowers, which form 10-20 cm long cream-white spikes, have a strong and attractive odor that fascinates many people (Lindemann *et al.* 1982). Common names include bridewort, dropwort, lady of the meadow, queen of the meadow, bride of the meadow, spirea, çayırkraliçesi çiçeği, su rezenesi, keçisakalı, tekesakalı (Grieve 1982; Dobelis 1990; Chevallier 1996; Baytop 1999).

The name of "*ulmaria"* means "elmlike", an odd epithet as it does not resemble the elm (*Ulmus*) in any way. However, the generic name, *Filipendula*, comes from filum, meaning "thread" and pendulous, meaning "hanging". This is possibly said to describe the root tubers that hang characteristically on the genus, on fibrous roots (Pitts 2006).

The main objectives of this study were to: a. obtain an *in vitro* culture protocol for *F. ulmaria* using plant tissue culture methods; b. screen and compare the biological activities of *F. ulmaria* using specific bioassays (antibacterial, anti-tumor and toxicity assessments).

1.2. Literature Review

1.2.1. Botany

Filipendula ulmaria is a member of the Rosaceae family. In previous botanical literature it was also called as *Spiraea ulmaria* L. (Baker and Baker 1967). It is a herbaceous, perennial shrub growing up to 2 m tall (Fig. 1.1). The stems are erect, furrowed, hollow and red-marbled. The leaves are dark green on the upper side and whitish and downy underneath, much divided, interruptedly pinnate, having a few large serrate leaflets and small intermediate ones. The toothed leaves are dark green in color. Terminal leaflets are large, 4–8 cm long and three to five-lobed. Meadowsweet has delicate, graceful, creamy-white, ornamental flowers, 5 mm in length, clustered close together in handsome irregularly-branched cymes, having a very strong, sweet smell and contain 5 petals (Davis, 1972; Duke 1989; Newall 1996; Chevallier 1996; Zeylstra 1998). The flowers are hermaphrodite (has both male and female organs) and are pollinated by bees, flies and beetles (Grieve 1982). It is in flower from June to early September (Lindemann *et al*. 1982). The natural habitats for meadowsweet are in damp meadows and moist banks. The plant prefers neutral and basic soils, and requires moist or wet soil (Grieve 1982).

Figure 1.1 Pictures of *Filipendula ulmaria.*

1.2.2. Medicinal usage and constituents of *Filipendula ulmaria*

Meadowsweet has a very long history of herbal use. It was one of the three most sacred herbs of the Druids (Vasiliauskas *et al*. 2004). The leaves and flowering stems of *F. ulmaria* are alterative, anti-inflammatory, anti-septic, analgesic, aromatic, astringent, diaphoretic, diuretic, stomachic, febrifuge and tonic (Grieve 1982; Dobelis 1990; Chevallier 1996; Baytop 1999). It has a long history of use in the treatment of inflammatory diseases including gout and rheumatoid arthritis, diarrhea, cystitis, irritable bowel syndrome, hyperacidity, heartburn, gastritis and peptic ulcers (Grieve 1982; Dobelis 1990; Chevalier 1996; Baytop 1999). A strong decoction of the boiled root is said to be effective, when used externally, in the treatment of sores and ulcers (Grieve 1982). *F. ulmaria* is rich in flavonoids (Krasnov *et al*. 2006), tannins, phenolic glycosides (salicylate), volatile oils (Lindemann *et al*. 1982; Valle *et al*. 1988) (salicylaldehyde), minerals and vitamin C (Grieve 1982; Dobelis 1990; Chevallier 1996). The flower head of meadowsweet contains salicylic acid, from which the drug aspirin can be synthesized (Chevallier 1996).

Anti-inflammatory activity is mostly predetermined by active salicylic acid derivatives: salicilin, salicylaldehyde and methyl salicylate (Jasiuleviciute *et al*. 2001). Unlike aspirin that can cause gastric ulceration at high doses, the combination of constituents in meadowsweet acts to protect the inner lining of the stomach and intestines whilst still providing the anti-inflammatory benefits of aspirin. This is provided by the salicylates contained in the plant combining with the tannins and other components (Chevallier 1996).

Analyses of volatile flower oil of *F. ulmaria* by gas chromatography and mass spectrometry methods revealed that the main compounds in the oil was salicylaldehyde, phenethyl alcohol, benzyl alcohol, 4-methoxybenzaldehyde, methyl salicylate and the significant quantity of ethyl salicylate (Lindemann *et al*. 1982).

Immunomodulatory (Halkes *et al*. 1997), anti-tumor (Bespalov *et al*. 1992), anti-carcinogenic (Bespalov *et al*. 1992; Peresunko *et al*. 1993), anti-coagulant (Kudriashov *et al*. 1990; Lyapina and Kovalchuk 1993), anti-ulcerogenic (Barnaulov and Denisenko 1980; Barnaulov *et al*. 1984; Vasiliauskas *et al*. 2004), anti-microbial (Csedo *et al*. 1993; Rauha *et al*. 2000), anti-oxidant (Sroka *et al*. 2001; Papp *et al*. 2004), cytotoxic (Spiridonov *et al*. 2005) and anti-arthritic (Jasiuleviciute *et al*. 2001) activities of *F. ulmaria* have also been reported.

Rauha *et al*. (2000) investigated the antimicrobial effects of *F. ulmaria* extracts against the selected microbes and it was observed that the extracts had a bactericidal effect on the growth of *Staphylococcus aureus* and *Escherichia coli*.

Smolarz and Sokolowska (2001) reported that the extracts of *F. ulmaria* were found to combat bacteria, e.g. *Staphylococcus aureus* as well as some viruses, particularly *Herpes simplex* and the Adenovirus and Coxsackie groups.

The whole herb possesses a pleasant taste and flavor. The green parts having a similar aromatic character to the flowers, therefore, it is used by strewing on the floors to give the rooms a pleasant aroma (Pitts 2006). The flowers are also used as a flavoring in various alcoholic

beverages and in stewed fruits (Facciola 1990) and adding them to wine or beer is said to make a very heady brew (Phillips and Foy 1990).

Some evidence suggested that meadowsweet may increase both the effect and the risk of side effects from narcotic analgesics. The heparin like and salicylate compounds in meadowsweet may increase the risk of bleeding when it is used with warfarin, other anticoagulant or anti-platelet drugs. The heparin, bound to plant proteins in meadowsweet flowers, enhances the anticoagulant and fibrinolytic properties of the nonenzymatic nature when it was administrated to animals both intramuscularly and intravenously (Kudriashov *et al*. 1990).

Meadowsweet flowers were observed to exhibit strong anti-oxidant activity and it can be recommended as margarine preservatives (Sroka *et al*. 2001). Toxicity of meadowsweet has not been reported. However, methyl salicylate can be absorbed through the skin, resulting in fatalities, especially in children. Bronchospasm has been documented with use of the plant; therefore, its use should be avoided in patients with asthma (Duke 1989; Newall 1996).

CHAPTER TWO

2. *In Vitro* Culture of *Filipendula ulmaria*

2.1. Introduction

Organogenesis is the process by which 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). 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 the induction phase, cells become determined and in the differentiation phase, cells form roots or shoots.

There has been a significant increase in tissue culture of medicinal plants. Because, *in vitro* culture protocols provides multiple regenerants per explant that would be very useful for mass propagation of medicinal plants. 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 pesticide or disease-free plants and produces large numbers of vegetative planting stock. In addition, with an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year and

uniform plant materials (less genetic diversity) can be produced that will be higher with seed germination (Turker *et al*. 2008).

In vitro micropropagation of *F. ulmaria* can provide a rapid multiplication method for producing plantlets exhibiting desirable characteristics. These desirable characteristics may be defined as disease resistance, increased secondary product accumulation, superior genotype characteristics, or beneficial morphological characteristics; all of which result in superior planting stock. More uniform plantlets can be obtained using an *in vitro* protocol for production, thus ensuring a more consistent crop. In addition, an *in vitro* propagation method would eliminate seasonal constraints with seedling by providing unlimited planting material on a consistent year-round basis (McCoy, 1998).

Meadowsweet is a valuable medicinal herb, but there are no reports on an *in vitro* culture protocol of this species. The present study describes, to our knowledge for the first time, an efficient *in vitro* plant regeneration protocol for *F. ulmaria* via adventitious shoot development from leaf, petiole and root explants cultured on medium containing different concentrations and combinations of various plant growth regulators.

2.2. Materials and Methods

Seeds of *F. ulmaria* were collected from Abant Lake, Bolu/Turkey in September of 2006. 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.

Seeds were washed with an anti-bacterial soap, rinsed with distilled water and surface sterilized by shaking in 20% ethanol for 10 minutes followed by rinsing well with sterilized distilled water and then dipped into 10% Domestos® (5% sodium hypochloride) for 20 minutes they were finally washed with sterile distilled water three times. After surface sterilization of the seeds, seeds were placed in sterile, disposable petri dishes containing Murashige and Skoog's minimal organics (MSMO) medium (4.43 g/l MSMO, 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). After a one week incubation in this medium, seedlings were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing the same medium for an additional three weeks. For shoot regeneration, leaf (36 mm^2), petiole (4-5 mm) and root explants (6-7 mm) were excised from four weeks old sterile seedlings and placed in sterile disposable petri plates containing 4.43 g/l MSMO with different combinations and concentrations of plant growth regulators; thidiazuron (TDZ; 0.1, 0.5 and 1 mg/l) + indole-3-butyric acid (IBA; 0.25, 0.5 and 1 mg/l); TDZ (0.1 and 0.5 mg/l) + 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1 and 0.5 mg/l); TDZ (0.01, 0.05, 0.1, 0.5, 1 and 1.5 mg/l) + indole-3-acetic acid (IAA; 0.5 and 1 mg/l); TDZ (0.1, 0.5, 1 and 1.5 mg/l) + IAA (0.25 and 0.5 mg/l) + gibberellic acid (GA_3; ; 0.5 and 1 mg/l); benzyladenine (BA; .1, 0.5, 1 and 3 mg/l) + IAA (0.5 and 1 mg/l); BA (1, 3 and 5 mg/l) + naphthalene acetic acid (NAA; 0.5, 1 and 3 mg/l); Kinetin (KIN; 1, 3 and 5 mg/l) + IBA (0.5 and 1 mg/l); KIN (1, 3 and 5 mg/l) + 2,4-D (0.1 and 0.5 mg/l). GA_3 was used after filter sterilization. All cultures were incubated at

22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$).

After three weeks, regenerated explants were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MSMO for shoot elongation for an additional two weeks. Shoots were then separated individually and placed in rooting medium containing MSMO and varying concentrations of different auxins; IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0.1, 0.5 and 1 mg/l) or NAA (0.5, 1 and 3 mg/l). Rooted explants were transferred to vermiculate (Agrekal®) in Magenta containers for acclimatization and after 2 weeks, they were transferred to plastic pots containing potting soil.

The shoot number and percentage of explants producing shoots were recorded after 3 weeks for all explants. Tests had 8 replications for each explant and the experiment was repeated three times. Moreover, after three weeks, the number of roots and the percentage of explants producing roots were recorded. There were 10 replications and the experiment was repeated three times. Results were expressed as means ± standard error of the mean. 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).

2.3. Results and Discussion

Although *F. ulmaria* is a valuable medicinal plant which has anti-inflammatory and analgesic effects especially in the treatment of rheumatic complaints of the joints, there is no study about *in vitro*

propagation of this species. We therefore aimed to develop an *in vitro* culture protocol for high frequency regeneration of meadowsweet plants by adventitious organogenesis.

Three different explants (leaf, petiole and root) and three different plant growth regulators (TDZ, BA and KIN) either alone or in combination with different auxins were used for shoot regeneration (Tables 2.1 and 2.2). TDZ was used in combination with IAA, IBA or 2,4-D and greater shoot regeneration was observed in all 3 explants with TDZ in combination with IAA. BA or KIN in combination with different auxins were not effective for shoot regeneration with all 3 explants (Table 2.2). In a second set of experiment, 0.5 or 1 mg/l GA_3 was added into different concentrations and combinations of TDZ and IAA. GA_3 along with TDZ and IAA combinations impressively enhanced multiple shoot formation (Table 2.3). The best shoot proliferation was obtained with root explants. Leaf and petiole explants formed fewer shoots than root explants (Tables 2.1; 2.2 and 2.3; Fig. 2.1).

With root explants, the greatest number of shoots per explant was observed in media containing 0.1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3 and 0.5 mg/l TDZ+0.25 mg/l IAA+0.5 mg/l GA_3 (28.6 ± 1.3 shoots per explant and 27 ± 1.5 shoots per explant, respectively; 100% explants formed shoots) (Table 2.3; Fig. 2.1; Fig. 2.3 A and B). Addition of 0.5 mg/l GA_3 into 0.1 mg/l + 0.5 mg/l IAA increased the shoot number from 19.5 to 28.6 with root explant (Tables 2.1 and 2.3). Similarly, GA_3 addition also increased the frequency of shoot formation from 91.7% to 100% (Tables 2.1 and 2.3).

Petiole and leaf explants gave the best shoot proliferation with media containing 0.1 mg/l TDZ+0.5 mg/l IAA+1 mg/l GA_3 (25.9 ± 1.3 and 22.7 ± 0.7 shoots per explant, respectively; 100% explants formed shoots) (Table 2.3; Fig. 2.1; Fig. 2.3 C and D). Addition of 1 mg/l GA_3 into 0.1 mg/l TDZ + 0.5 mg/l IAA increased the shoot number from 1.0 to 25.9 shoots per petiole explant and from 11.1 to 22.7 shoots per leaf explant. Similarly, shoot formation frequency increased from 25% to 100% with petiole explant and from 87.5% to 100% with leaf explant (Tables 2.1 and 2.3). Although leaf lamina explants produced small amounts of shoots with TDZ in combination with IBA or 2,4-D, petiole and root explants produced no shoots (Table 2.1).

When TDZ were used alone at different concentrations (0.1, 0.5, 1 and 1.5 mg/l) on the root explants, the best shoot formation was obtained with 1 mg/l TDZ (14.4 shoots per explant). 0.1 mg/l and 0.5 mg/l TDZ alone were less successful for shoot regeneration with root explants (9.6 and 6.6 shoots per explant, respectively) (Table 2.3). Addition of different concentrations of GA_3 and IAA into 1 mg/l TDZ did not increase the shoot number (Table 2.3). However, when 0.1 mg/l or 0.5 mg/l TDZ were combined with 0.5 mg/l GA_3 and 0.5 mg/l IAA or 0.25 mg/l IAA, significant increases were observed in terms of the mean number of shoots per root explant (28.6 and 27.0 shoots per explant, respectively) (Table 2.3). Addition of 0.5 mg/l GA_3 and 0.5 mg/l IAA into 0.1 mg/l TDZ also increased the percentages of the explants forming shoots from 75% to 100%.

With root explants, supplementation of 0.5 mg/l GA_3 into 0.1 mg/l TDZ slightly increased the shoot number (from 9.6 to 11.2 shoots per

explant). Addition of 0.25 mg/l IAA into this combination increased the shoot number twice (18.5 shoots). But, addition of 0.5 mg/l IAA increased the shoot number 3 times (28.6 shoots). Similarly, supplementation of 0.5 mg/l GA_3 into 0.5 mg/l TDZ increased the shoot number twice (from 6.6 to 11.7 shoots per explant). And, addition of 0.25 mg/l IAA into this combination increased the shoot number nearly 2.5 times (from 11.7 to 27.0 shoots per explant) (Table 2.3).

In the best growth hormone combination with root explants (0.1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3), if TDZ concentration was increased to 0.5, 1 and 1.5 mg/l substantially, shoot number decreased from 28.6 to 15.1, 8.6 and 0, respectively. Similar patterns were observed with leaf and petiole explants. In the best growth hormone combination with these explants (0.1 mg/l TDZ+0.5 mg/l IAA+1 mg/l GA_3), if TDZ concentration was increased to 0.5, 1 and 1.5 mg/l substantially, shoot number decreased from 22.7 to 18.5, 0 and 0 for leaf explant, and from 25.9 to 7.7, 0 and 0 for petiole explant, respectively (Table 2.3).

Treatments involving no plant growth regulators (control treatments) produced no shoots (Tables 2.1, 2.2 and 2.3). Regeneration was occurred after callus formation (green to yellowish in color) with root explants known as indirect organogenesis (Fig. 2.3 A and B). Regeneration with leaf lamina (Fig. 2.3 C) and petiole (Fig. 2.3 D) explants was not through callus formation, they were direct organogenesis.

Regenerated shoots were separated individually and cultured on MSMO medium containing different concentrations of IAA, IBA, 2.4-D or NAA. If we compare among the various auxins investigated for rooting,

IBA was better than other auxins (Table 2.4; Fig. 2.2). The greatest number of roots per explant was observed in media with 0.5 mg/l IBA (11.7 ± 1.3 roots) and 0.5 mg/l IAA (10.1 ± 0.7 roots). 0.5 mg/l IBA and IAA were more effective for root formation because all regenerated shoots formed roots (100%) (Table 2.4; Fig. 2.2; Fig. 2.3 E). Fewer regenerants produced roots as IBA and IAA concentration increased; 100% of the explants formed roots at 0.5 mg/l IBA or IAA compared to 80% and 50% at 3 mg/l IBA and IAA, respectively. Due to the low percentages of rooted regenerants, NAA and 2,4-D auxins were not effective for rooting (Table 2.4). There was rooting on control experiment (no auxin was added to the media), but the roots were longer, thicker and looked healthier with 0.5 mg/l IBA or 0.5 mg/l IAA. Similarly, IBA was also reported as the most effective auxin for rooting with some medicinal plants including *Oenothera* spp. (De Gyves *et al*. 2001), *Mucuna pruriens* (L.) DC (Faisal *et al*. 2006) and *Cassia angustifolia* Vahl (Siddique and Anis 2007).

The rooted shoots were transferred to Magenta containers including vermiculate for acclimatization for 2 weeks conditions (Fig. 2.3 F) and then finally to plastic pots containing potting soil and kept under growth room conditions (Fig. 2.3 G). Approximately 95% of the regenerated plants survived through the hardening off process.

The promoting effect of TDZ on *in vitro* development has been lately reported for many species (Ahn *et al*. 2007; Jones *et al*. 2007; Wang and Bao 2007; Yucesan *et al*. 2007). TDZ has been reported to induce adventitious shoot formation in a number of species, especially woody plants (Huetteman and Preece 1993; Lu 1993). TDZ was more successful

than BA or KIN for shoot formation in our study (Tables 2.1 and 2.2). This result corroborated the finding of Lu (1993) that TDZ was more effective than BA, KIN or zeatin for the induction of shoot regeneration from carnation and rose. Generally, with all explant types in our study, lower concentrations of TDZ were effective for shoot regeneration (0.1-0.5 mg/l) and higher concentrations of TDZ (1-1.5 mg/l) resulted in callus formation around the cut edges of the explants and less number of microshoots. Similarly, 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. Singh *et al*. (2003) also reported that in the regeneration of pigeonpea, low concentrations of TDZ (0.01-0.22 mg/l) induced multiple shoots, intermediary concentration (1.1 mg/l) produced clusters of leafy structures and higher concentrations (2.2-4.4 mg/l) induced somatic embryogenesis. 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 highlighting that when TDZ was used alone, it produced some amounts of shoots. But, if TDZ was combined with IAA, more shoot formation was obtained (Tables 2.1 and 2.3). 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. The main effect of GA_3 in plants is to cause stem elongation and flowering (Sutter 1996). Addition of GA_3 into TDZ and IAA combination in our experiments multiplied shoot

numbers and compensated the negative effects of TDZ. It is known that in tissue culture GA_3 often inhibit the induction of organogenesis, particularly adventitious root formation (Sutter 1996). But, in our study, combination of TDZ, IAA and GA_3 showed the synergistic effect causing multiple shoot formation on all explants in our experiments. Geetha *et al*. (1998) also reported that addition of GA_3 along with BA and NAA dramatically enhanced both multiple shoot proliferation and shoot elongation in all the explants of pigeonpea (*Cajanus cajan* L.). Moreover, Lata *et al*. (2002) showed that adding GA_3 into media containing BA increased shoot growth. Murch *et al*. (1997) showed that the occurrence of regenerants in TDZ-treated plants may be an adaptive reproductive mechanism to overcome the imposed stress. Murthy *et al*. (1998) hypothesized that under the influence of TDZ, a relatively high level of accumulation of minerals or other metabolites occurs in the tissues, and this causes a stress in plants (explants). To overcome this physiological stress, the plant tissue modifies their metabolic processes, resulting in the production and accumulation of various metabolites and culminating in the formation of regenerants.

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 (Schwarz and Beaty 1996). 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. In our study, when leaf, petiole and root explants were compared, root explants were much more productive for shoot formation

than leaf and petiole explants with all plant growth hormone combinations and concentrations (Tables 2.1, 2.2 and 2.3). Similarly, root explant was also reported as most responsive explant in terms of shoot organogenesis with *Spinacia oleracea* L., (Xiao and Branchard 1995), *Hypericum perforatum* L. (Zobayed and Saxena 2003), *Passiflora cincinnata* Mast. (Lombardi *et al*. 2007) and *Clitoria ternatea* L. (Shahzad *et al*. 2007).

Although there is no study about *in vitro* plant regeneration for *F. ulmaria*, some studies about the TDZ-induced regeneration of some members of Rosaceae family were recorded (Barna and Wakhlu 1995; Feyissa *et al*. 2005; Landi and Mezzetti 2006). But, to our knowledge, the effects of TDZ+IAA+GA_3 growth hormone combinations on *in vitro* regeneration of a member of the Rosaceae family were tested for the first time.

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). In order to have standardized formulations, the chemical constituents from plants and their parts are required to be uniform both qualitatively and quantitatively. Furthermore, an ever increasing demand of uniform medicinal plants based medicines warrants their mass cloning through plant tissue culture strategy (Chaturvedi *et al*. 2007). This protocol can be helpful in the large scale production of certain secondary products of *F. ulmaria*. Micropropagation of *F. ulmaria* can provide a mass production of pesticide, herbicide and disease free plants on a commercial scale and unlimited, genetically uniform 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 Shoot regeneration from leaf, petiole and root explants cultured on MSMO medium containing different concentrations and combinations of TDZ with IBA, 2,4-D or IAA. Mean values with the same letters within vertical columns are not significantly different (*P*>0.05).

Treatments (mg/l)	Leaf Lamina Explants		Petiole Explants		Root Explants	
	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots
Control	**0**	**0**	**0**	**0**	**0**	**0**
0.1 TDZ + 0.25 IBA	2.3 ± 0.9 ^{de}	50	0	0	0	0
0.1 TDZ + 0.5 IBA	0	0	0	0	0	0
0.1 TDZ + 1.0 IBA	8.9 ± 3.4 ^{ab}	50	0	0	0	0
Means	**3.4 ± 1.4**	**33.5**	**0**	**0**	**0**	**0**
0.5 TDZ + 0.25 IBA	0	0	0	0	0	0
0.5 TDZ + 0.5 IBA	5.3 ± 2.1 ^{cd}	50	0	0	0	0
0.5 TDZ + 1.0 IBA	1.9 ± 1.9 ^{de}	12.5	0	0	0	0
Means	**2.4 ± 1.3**	**22.2**	**0**	**0**	**0**	**0**
1.0 TDZ + 0.25 IBA	4.5 ± 1.0 ^{cd}	75	0	0	0	0
1.0 TDZ + 0.5 IBA	0	0	0	0	0	0
1.0 TDZ + 1.0 IBA	0	0	0	0	0	0
Means	**1.5 ± 0.3**	**25**	**0**	**0**	**0**	**0**
0.1 TDZ + 0.1 2,4-D	0	0	0	0	0	0
0.1 TDZ + 0.5 2,4-D	0	0	0	0	0	0
Means	**0**	**0**	**0**	**0**	**0**	**0**
0.5 TDZ + 0.1 2,4-D	1.0 ± 1.6 ^{e}	25	0	0	0	0
0.5 TDZ + 0.5 2,4-D	2.25 ± 0.6 ^{de}	75	0	0	0	0
Means	**1.6 ± 1.1**	**50**	**0**	**0**	**0**	**0**
0.01 TDZ + 0.5 IAA	0	0	0	0	4.7 ± 1.8 ^{def}	50
0.01 TDZ + 1.0 IAA	0.6 ± 0.3 ^{e}	41.7	3.1 ± 1.6 ^{b}	50	4.9 ± 1.7 ^{def}	75
Means	**0.3 ± 0.2**	**20.9**	**1.6 ± 0.8**	**25**	**4.8 ± 1.8**	**62.5**
0.05 TDZ + 0.5 IAA	0	0	0	0	3.4 ± 0.2 ^{efg}	100
0.05 TDZ + 1.0 IAA	0	0	0.5 ± 0.3 ^{c}	25	2.0 ± 1.7 ^{fg}	25
Means	**0**	**0**	**0.3 ± 0.2**	**12.5**	**2.7 ± 1.0**	**62.5**
0.1 TDZ + 0.5 IAA	11.1 ± 1.8 ^{a}	87.5	1.0 ± 0.6 ^{c}	25	19.5 ± 3.2 ^{a}	91.7
0.1 TDZ + 1.0 IAA	5.1 ± 1.6 ^{cd}	66.7	5.5 ± 0.9 ^{a}	87.5	0	0
Means	**8.1 ± 1.7**	**77.1**	**3.3 ± 0.8**	**56.3**	**9.8 1.6**	**45.9**
0.5 TDZ + 0.5 IAA	6.8 ± 1.0 ^{bc}	83.3	6.2 ± 1.4 ^{a}	79.2	10.5 2.7 ^{b}	75
0.5 TDZ + 1.0 IAA	0	0	6.1 ± 1.4 ^{a}	79.2	8.5 1.7 ^{bcd}	83.3
Means	**3.4 ± 0.5**	**41.7**	**6.2 ± 1.4**	**79.2**	**9.5 2.2**	**79.2**

Table 2.2 Shoot regeneration from leaf, petiole and root explants cultured on MSMO medium containing different concentrations and combinations of BA with IAA or NAA and KIN with IBA. Mean values with the same letters within vertical columns are not significantly different (P>0.05).

Treatments (mg/l)	Leaf Lamina Explants		Petiole Explants		Root Explants	
	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots
Control	**0**	**0**	**0**	**0**	**0**	**0**
0.1 BA	0	0	0	0	0	0
0.1 BA + 0.5 IAA	0	0	0	0	0	0
0.1 BA + 1.0 IAA	0	0	0	0	0	0
0.5 BA	0	0	0	0	1.2 ± 0.5 ^{c}	50
0.5 BA + 0.5 IAA	0	0	0	0	6.1 ± 0.6 ^{a}	100
0.5 BA + 1.0 IAA	0	0	0	0	1.9 ± 0.7 ^{cd}	50
1.0 BA	0	0	0	0	1.7 ± 0.7 ^{cd}	50
1.0 BA + 0.5 IAA	2.7 ± 1.0 ^{b}	50	0	0	0	0
1.0 BA + 1.0 IAA	0	0	0	0	0	0
3.0 BA	0	0	0	0	0	0
3.0 BA + 0.5 IAA	0	0	0	0	0	0
3.0 BA + 1.0 IAA	0	0	0	0	0	0
Means	**0.2 ± 0.1**	**4.2**	**0**	**0**	**0.9 ± 0.2**	**20.8**
1.0 BA + 0.5 NAA	4.5 ± 1.0 ^{a}	75	0	0	0	0
1.0 BA + 1.0 NAA	0	0	0	0	0	0
1.0 BA + 3.0 NAA	0	0	0	0	0	0
3.0 BA + 0.5 NAA	0	0	0	0	0	0
3.0 BA + 1.0 NAA	0	0	0	0	0	0
3.0 BA + 3.0 NAA	0	0	0	0	0	0
5.0 BA + 0.5 NAA	0	0	0	0	0	0
5.0 BA + 1.0 NAA	0	0	0	0	0	0
5.0 BA + 3.0 NAA	0	0	0	0	0	0
Means	**0.5 ± 0.1**	**8.3**	**0**	**0**	**0**	**0**
1.0 KIN + 0.5 IBA	2.2 ± 0.9 ^{b}	50	0	0	0	0
1.0 KIN + 1.0 IBA	0	0	0	0	0	0
3.0 KIN + 0.5 IBA	0	0	0	0	0	0
3.0 KIN + 1.0 IBA	0	0	0	0	0	0
5.0 KIN + 0.5 IBA	0.5 ± 0.3 ^{c}	25	0	0	0	0
5.0 KIN + 1.0 IBA	0	0	0	0	0	0
Means	**0.5 ± 0.2**	**12.5**	**0**	**0**	**0**	**0**

Table 2.3 Shoot regeneration from leaf, petiole and root explants cultured on MSMO medium containing different concentrations and combinations of TDZ, IAA and GA_3. Mean values with the same letters within vertical columns are not significantly different (P>0.05).

Treatments (mg/l)	Leaf Lamina Explants		Petiole Explants		Root Explants	
	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots	Mean number of shoots per explant ± SE	% explants forming shoots
Control	**0**	**0**	**0**	**0**	**0**	**0**
0.1 TDZ	0	0	0	0	9.6 ± 2.9 ^{def}	75
0.1 TDZ + 0.5 GA_3	2.4 ± 1.2 ^{de}	37.5	3.2 ± 1.6 ^{efg}	33.3	11.2 ± 1.8 ^{cde}	91.7
0.1 TDZ + 1.0 GA_3	0	0	2.4 ± 0.6 ^{fg}	62.5	8.5 ± 0.5 ^{def}	100
0.1 TDZ + 0.25 IAA + 0.5 GA_3	6.1 ± 4.1 ^{cd}	25	6.2 ± 1.9 ^{cde}	66.7	18.5 ± 0.9 ^{b}	100
0.1 TDZ + 0.25 IAA + 1.0 GA_3	0	0	0	0	12.6 ± 2.6 ^{cd}	100
0.1 TDZ + 0.5 IAA + 0.5 GA_3	0	0	10.0 ± 3.9 ^{b}	50	28.6 ± 1.3 ^{a}	100
0.1 TDZ + 0.5 IAA + 1.0 GA_3	22.7 ± 0.7 ^{a}	100	25.9 ± 1.3 ^{a}	100	8.5 ± 0.5 ^{def}	100
Means	**4.5 ± 0.9**	**23.2**	**6.8 ± 1.3**	**44.6**	**13.9 ± 1.5**	**95.2**
0.5 TDZ	0	0	8.7 ± 0.4 ^{bc}	100	6.6 ± 1.0 ^{fgh}	100
0.5 TDZ + 0.5 GA_3	9.6 ± 3.9 ^{c}	50	0	0	11.7 ± 0.7 ^{cde}	100
0.5 TDZ + 1.0 GA_3	5.9 ± 0.9 ^{cd}	87.5	0	0	5.9 ± 0.4 ^{fgh}	100
0.5 TDZ + 0.25 IAA + 0.5 GA_3	0	0	0.6 ± 0.3 ^{g}	33.3	27.0 ± 1.5 ^{a}	100
0.5 TDZ + 0.25 IAA + 1.0 GA_3	3.4 ± 3.4 ^{de}	16.7	8.2 ± 2.7 ^{bcd}	62.5	3.0 ± 2.1 ^{hij}	29.2
0.5 TDZ + 0.5 IAA + 0.5 GA_3	5.5 ± 3.6 ^{cd}	25	2.1 ± 1.0 ^{fg}	41.7	15.1 ± 3.9 ^{bc}	75
0.5 TDZ + 0.5 IAA + 1.0 GA_3	18.5 ± 1.3 ^{b}	100	7.7 ± 1.8 ^{bcd}	100	8.2 ± 0.4 ^{def}	100
Means	**6.1 ± 1.9**	**39.9**	**3.9 ± 0.9**	**48.2**	**11.1 ± 1.4**	**86.3**
1.0 TDZ	0	0	0	0	14.4 ± 1.1 ^{bc}	100
1.0 TDZ + 0.5 GA_3	0	0	0	0	13.5 ± 0.8 ^{c}	100
1.0 TDZ + 1.0 GA_3	0	0	0	0	3.5 ± 1.5 ^{ghij}	50
1.0 TDZ + 0.25 IAA + 0.5 GA_3	0	0	5.0 ± 0.8 ^{def}	83.3	5.7 ± 0.9 ^{fgh}	83.3
1.0 TDZ + 0.25 IAA + 1.0 GA_3	0	0	0	0	6.6 ± 2.3 ^{fgh}	75
1.0 TDZ + 0.5 IAA + 0.5 GA_3	0	0	0	0	8.6 ± 2.7 ^{def}	66.7
1.0 TDZ + 0.5 IAA + 1.0 GA_3	0	0	0	0	0	0
Means	**0**	**0**	**0.7 ± 0.1**	**11.9**	**7.5 ± 1.3**	**67.9**
1.5 TDZ	0	0	0.5 ± 0.3 ^{g}	25	8.9 ± 0.9 ^{def}	100
1.5 TDZ + 0.5 GA_3	0	0	0.5 ± 0.3 ^{g}	25	8.0 ± 1.0 ^{efg}	100
1.5 TDZ + 1.0 GA_3	0	0	0	0	0	0
1.5 TDZ + 0.25 IAA + 0.5 GA_3	2.7 ± 1.4 ^{de}	41.7	0	0	4.9 ± 0.4 ^{fghi}	100
1.5 TDZ + 0.25 IAA + 1.0 GA_3	0	0	0	0	1.1 ± 1.1 ^{ij}	16.7
1.5 TDZ + 0.5 IAA + 0.5 GA_3	2.5 ± 1.7 ^{de}	25	0	0	0	0
1.5 TDZ + 0.5 IAA + 1.0 GA_3	0	0	0	0	0	0
Means	**0.7 ± 0.4**	**6.2**	**0.1 ± 0.1**	**7.1**	**3.3 ± 0.5**	**45.2**

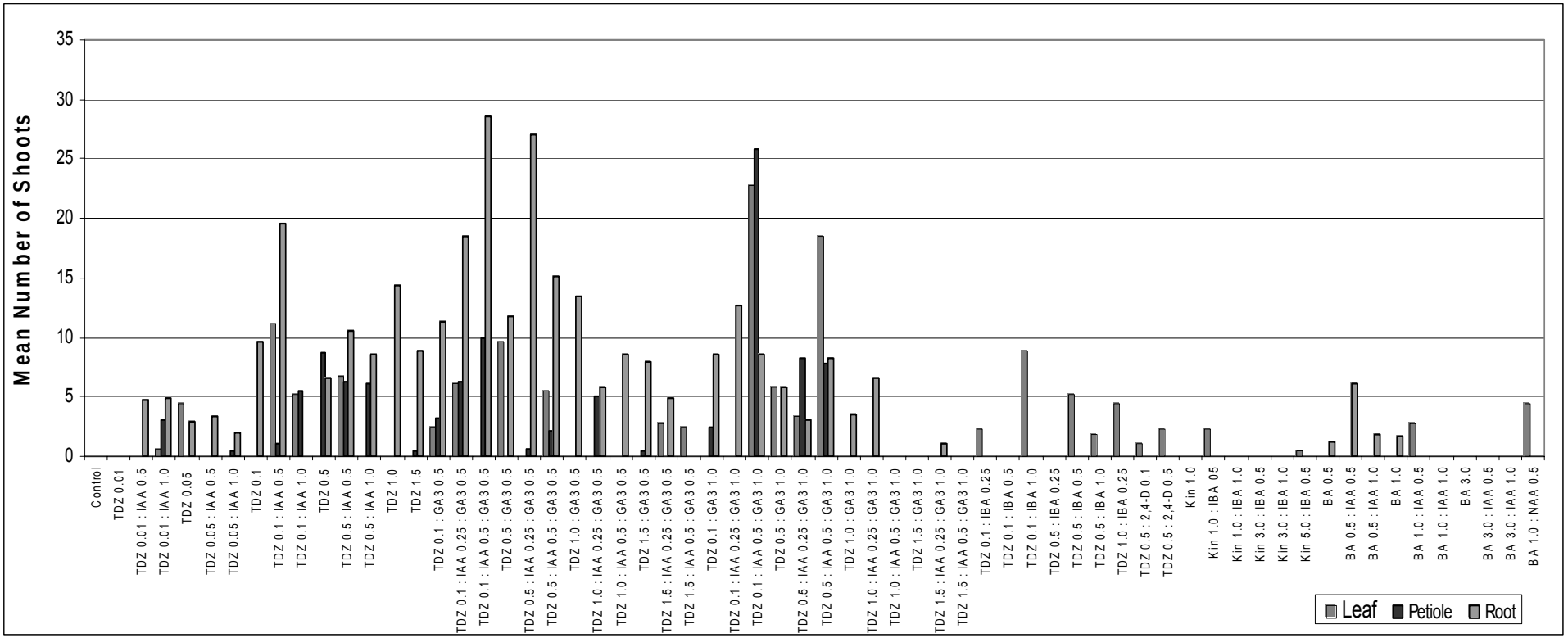


Figure 2.1 Shoot development from leaf, petiole and root explants incubated on media containing different hormone combinations.

Table 2.4 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.

Auxins (mg/l)	Mean number of roots per shoot ± SE	% of shoots that rooted
Control	**8.1 ± 1.5** ^{bc}	**100**
IAA 0.5	10.1 ± 0.7 ^{ab}	100
IAA 1.0	8.8 ± 1.1 ^{abc}	90
IAA 3.0	6.1 ± 2.1 ^{cd}	50
Means	**8.3 ± 1.3**	**80**
IBA 0.5	11.7 ± 1.3 ^{a}	100
IBA 1.0	8.7 ± 1.6 ^{abc}	80
IBA 3.0	5.7 ± 1.5 ^{cd}	80
Means	**8.7 ± 1.5**	**86.7**
2.4-D 0.1	1.5 ± 0.6 ^{e}	50
2.4-D 0.5	0.5 ± 0.3 ^{e}	30
2.4-D 1.0	0.3 ± 0.1 ^{e}	30
Means	**0.8 ± 0.3**	**36.7**
NAA 0.5	3.0 ± 0.9 ^{de}	70
NAA 1.0	1.1 ± 0.5 ^{e}	50
NAA 3.0	0.4 ± 0.3 ^{e}	20
Means	**1.5 ± 0.6**	**46.7**

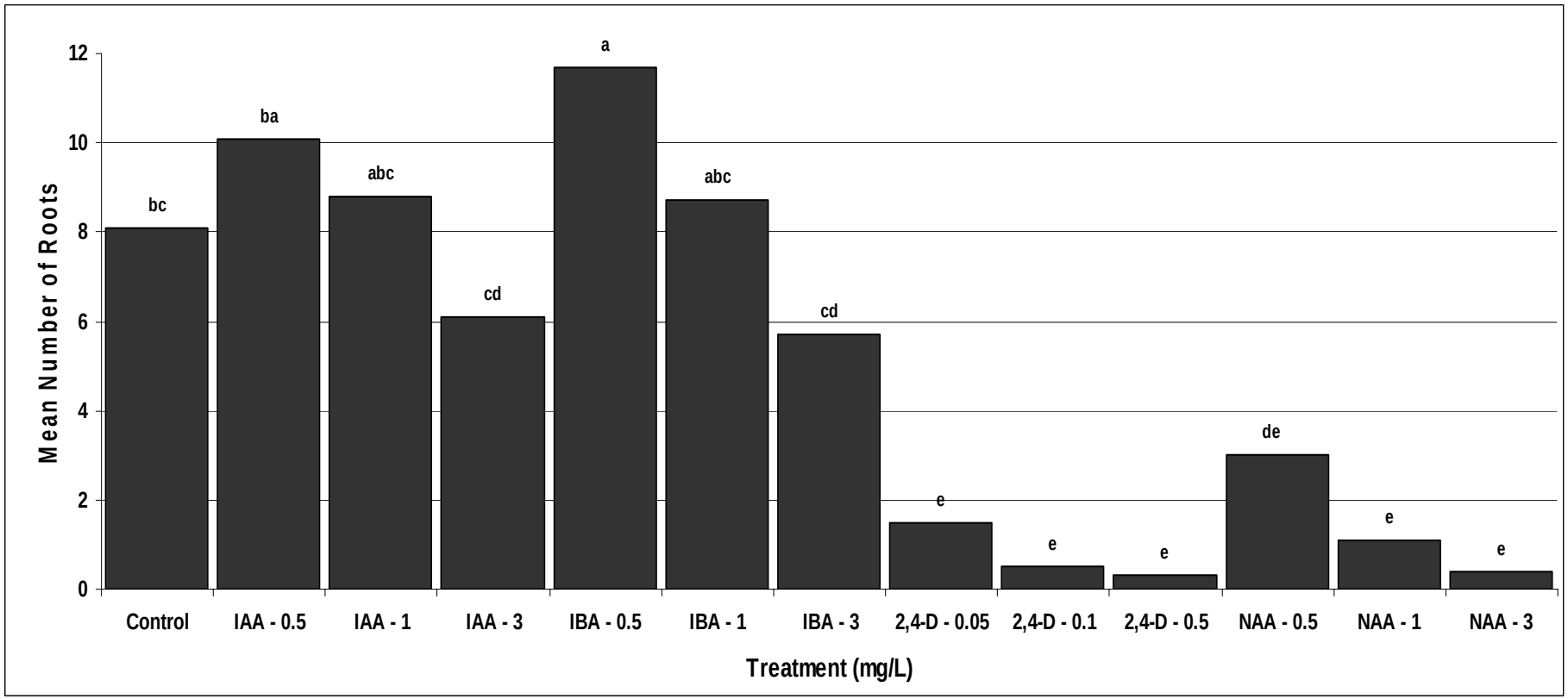


Figure 2.2 Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots.

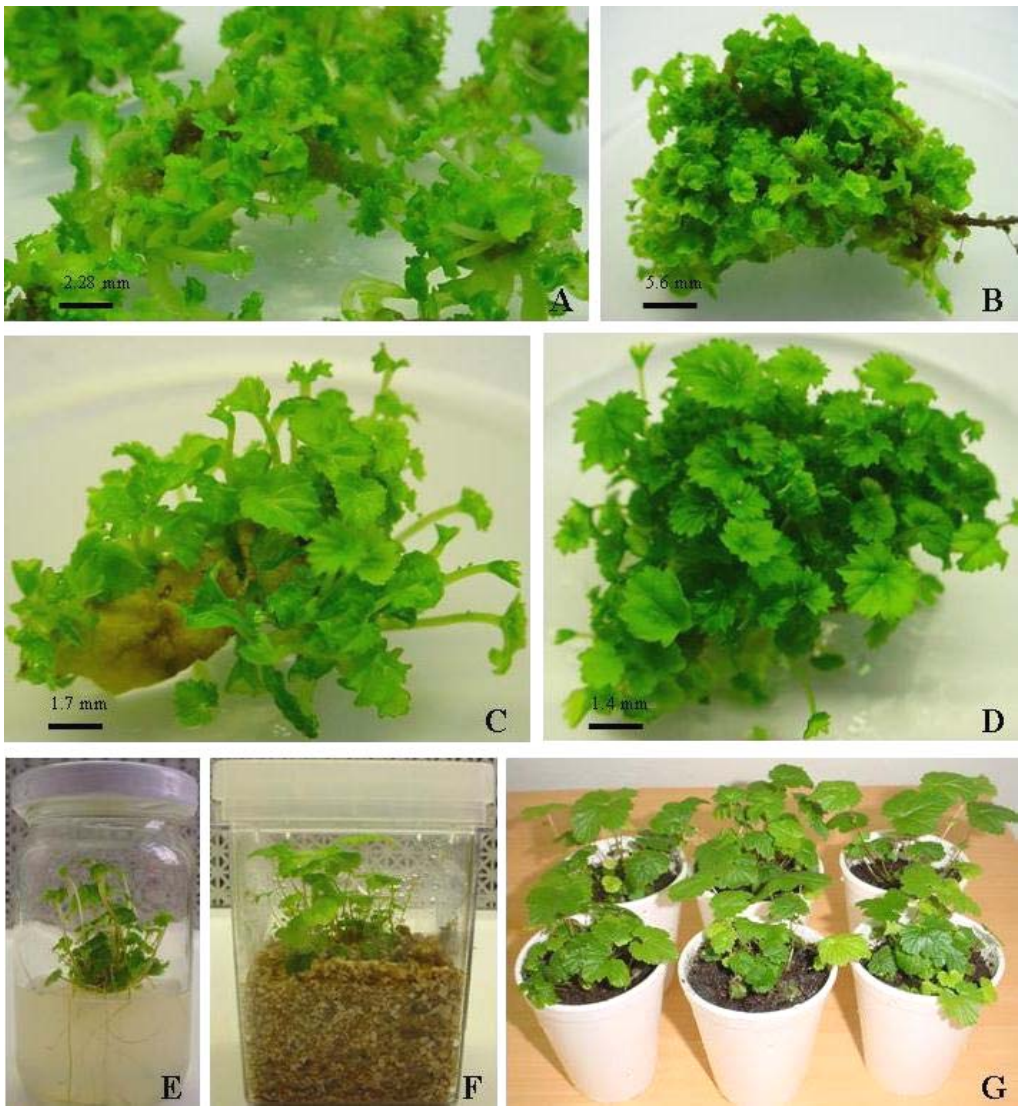


Figure 2.3 *In vitro* regeneration of meadowsweet (*F. ulmaria*): Shoot regeneration through callus formation (indirect organogenesis) from root explants cultured on MSMO medium containing 1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3 after 2 weeks (**A**) and 4 weeks (**B**); Direct organogenesis from leaf lamina (**C**) and petiole explants (**D**) cultured on MSMO medium containing 0.1 mg/l TDZ+0.5 mg/l IAA+1 mg/l GA_3; Rooting of the regenerated shoots in medium containing 0.5 mg/l IBA (**E**); Regenerated plants in Magenta containers including vermiculate for acclimatization (**F**); Regenerated plants transferred to plastic pots containing sterile potting soil under growth room conditions (**G**).

CHAPTER THREE

3. Biological Activities of *Filipendula ulmaria*

3.1. Introduction

Herbs have been used as medicines for as long as human life has existed on earth. The World Health Organization estimates that 80 percent of people in developing countries (65 percent of Earth's population) still rely on traditional medicine (Starbuck 1999). Plants have been a rich source of 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 are adaptable for the purpose of screening and testing plant extracts (McLaughlin *et al*. 1998).

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.

The Kirby-Bauer Disc Diffusion 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). It was developed by

Kirby and Bauer in the 1960's (Prescott *et al*. 1990). The Kirby-Bauer Disc Diffusion Assay (Barry and Thornsberry 1985) has been accepted by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS). In this standardized assay, a culture is inoculated onto a Mueller-Hinton agar plate, followed by the addition of antibiotic impregnated discs to the agar surface. Inoculated plates are incubated at 37°C for 18-24 hours. At the end of the incubation period, a clear area (zone of inhibition) around the disc is measured and this area indicates the inhibition of microbial growth around the disc. 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 the particular antibiotic. This method is not directly applicable to filamentous fungi, anaerobes,or slowly-growing bacteria (Atlas 1988).

Agrobacterium tumefaciens is the causal agent of Crown Gall disease (the formation of tumors) in over 140 dicot species. Symptoms are caused by the insertion of a small segment of DNA (known as the T-DNA, for 'transfer DNA') into the plant cell, which is incorporated at a semi-random location into the plant genome. This bacterium contains a plasmid which upon infection of plant material is transferred into the plant's DNA where it transforms normal cells into tumor cells (Ferrigini 1982; Agrios 1997).

Galsky et al. (1980) demonstrated that the inhibition of crown gall tumors on discs of potato tubers showed an apparent correlation with

compounds and plant extracts known to be active in the 3PS (*in vivo,* murine leukemia) antitumor assay (McLaughlin *et al*. 1998). In the 3PS *in vivo* tumor assay, leukemic mice are treated with possible antitumor agents (McLaughlin 1991; McLaughlin *et al*. 1993; McLaughlin *et al*. 1998). Differences in life span of the leukemic mice compared to healthy mice are used as a measure of antitumor activity. Later, Ferrigini *et al*. (1982) modified the Galsky potato disc method for several plant extracts, including an extract from periwinkle (*Vinca major*) called vincristine.

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). Finally, Ferrigini *et al*. (1982) reported that crown gall tumors on potato discs could routinely be used as a comparatively rapid, inexpensive, safe and statistically reliable prescreen for *in vivo* 3PS antitumor activity.

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).

3.2. Materials and Methods

3.2.1. Plant Material and Extraction

Stem and leaves of *F. ulmaria* (field-grown plants) were collected from Abant Lake, Bolu/Turkey in September of 2006. Identification of the species was made by using “Flora of Turkey and the East Aegean Islands” (Davis 1972) and voucher specimens (AUT-2001) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Leaves and stems of *in vitro* cultured plants were collected from meadowsweet plants

that were previously micropropagated in our laboratory. Plant materials were extracted with different solvents (ethanol (EtOH), ethylacetate (EtOAc), hexane and water) and their biological activities evaluated. Two different sources of plant (field-grown and *in vitro*-grown) were used for extractions. Collected plants were dried in oven at 45 °C and then ground into a powder. Twenty grams from each plant sample were extracted with 300 ml water, ethanol, ethylacetate and hexane at 45 °C for 12 hours and then filtered. For water extraction, frozen filtrate was lyophilized by using freeze-dryer at -65 °C and for ethanol, ethylacetate and hexane extractions, they were evaporated under vacuum using rotary evaporator at 60 °C. Each residue was then dissolved in sterile distilled water to produce a final concentration of 100 mg/ml. Treatments, plant materials and extraction procedures are summarized in Table 3.1.

Table 3.1 Designation of extracts in bioassays and their extraction procedures.

Test Materials	Designations	Materials in Treatment	Extraction Procedure
Water extract	FW	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.
	IW	*In vitro*- grown leaves and stems	20 g *in vitro* -grown leaves and stems were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.
Ethanol extract	FE	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with EtOH at 45 ºC for 12 hr. EtOH was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
	IE	*In vitro*- grown leaves and stems	20 g *in vitro* -grown leaves and stems were extracted with EtOH at 45 ºC for 12 hr. EtOH was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
Hexane extract	FE	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with hexane at 45 ºC for 12 hr. Hexane was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
	IE	*In vitro*- grown leaves and stems	20 g *in vitro* -grown leaves and stems were extracted with hexane at 45 ºC for 12 hr. Hexane was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
Ethylacetate extract	FEA	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with EtOAc at 45 ºC for 12 hr. EtOAc was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
	IEA	*In vitro*- grown leaves and stems	20 g *in vitro* -grown leaves and stems were extracted with EtOAc at 45 ºC for 12 hr. EtOAc was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.

3.2.2. Antibacterial Assay

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* (ATTC 13883) which are Gram-Negative bacteria and *Streptococcus pyogenes* (ATTC 19615), *Staphylococcus aureus* (ATTC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are Gram-Positive bacteria (Table 3.2). BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated for 3 hr at 37°C. After 3 hr, 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, 4-5 loops of pure culture were transferred to 10 ml of TSB in a test tube for each bacterial strain and incubated overnight at 37°C; then 0.25 ml from each culture was transferred into 0.75 ml TSB in eppendorf tubes. 0.1 ml from each strand was transferred into Mueller-Hinton (M-H) agar plates. (One eppendorf tube was used for each agar plate). The plates were inoculated with a microorganism suspension at a density of 10^6 cells / mL.

Table 3.2 Bacteria used in antibacterial assay and their representative diseases (Vazquezt and Archer 1980; Atlas 1988; Prashanth *et al*. 2001; Ciftci *et al*. 2003; Büyükbaba-Boral *et al*. 2004; Uzun *et al*. 2004; Sahayara *et al*. 2006).

	Pathogen	Representative Diseases	Drugs of Choice
Gram Pozitif	***Streptococcus pyogenes***	Tonsillopharyngeal cellulitis or abscess, Otitis media, Sinusitis, Necrotizing fasciitis, Streptococcal bacteremia, Meningitis or brain abscess.	Penicillin, ampicillin,cefazolin, cefuroxime, ceftriaxone, ofloxacin, vancomycin.
	Staphylococcus aureus	Boils and pimples (folliculitis), Pneumonia, Food poisoning (emesis or vomiting), Septicemia (invasion of the bloodstream), Osteomyelitis (invasion of bone), Toxic shock syndrome, Surgical wound infections, Scalded skin syndrome (analogous to scarlet fever).	Methicillin, oxacillin, penicillin, amoxicillin.
	Staphylococcus epidermidis	Wound infections, Cystitis (Urinary tract infection - catheterized patients), Septicemia, Endocarditis, Endopthalmitis, Catheter infections, Prosthetic implant infection.	Vancomycin, gentamicin or rifampin.
Gram Negatif	***Escherichia coli***	Urinary tract infections, Sepsis/meningitis and Infectious diarrhea.	Ampicillin, ampicillin + sulbactam, cephalothin, cefuroxime, ceftriaxone, amikacin, ciprofloxacin, sulfamethoxazole and trimethoprim.
	Pseudomonas aeruginosa	Septicemia, Urinary tract infections, Pneumonia, Chronic lung infections, Endocarditis, Dermatitis, and Osteochondritis.	Ticarcillin, piperacillin, ticarcillin-clavulanate, piperacillin-tazobactam.
	Salmonella typhimurium	Diarrhea, Abdominal cramps, Vomiting and Nausea. Pains in joints, irritation of the eyes, and painful urination (Reiter's syndrome), Chronic arthritis.	Cephalosporins, fluoroquinolones.
	Serratia marcescens	Conjunctivitis, keratitis, Endophthalmitis, and Tear duct infections.	Cephalosporins, fluoroquinolones, tetracycline, trimethoprim/sulfamethoxazole and synthetic penicillins.
	Proteus vulgaris	Urinary tract infection, Bacteremia, Pneumonia and Focal lesions.	Carbenicillin.
	Enterobacter cloacea	Cloacae pneumonia, Acute necrotizing, Myocarditis.	Tetracycline or amoxicillin and metronidazole.
	Klebsiella pneumoniae	Pneumonia, Septicemia, Wound infection, Burn infection, Urinary tract infection (cystitis), Ankylosing spondylitis(autoimmune disease).	Gentamicin and cephalothin.

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 each agar plate). Agar plates were streaked three times, each time turning the plate at a 60° angle and finally rubbing the swab through the edge of the plate. All 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 50 μl of extract. There were five replicates in each plate and two plates for each extract tested for each bacterium. Positive controls consisted of five different antimicrobial susceptibility test discs (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, inhibition zone diameter (mm) was measured. All experiments were repeated two 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).

3.2.3. Anti-tumor Assay

Antitumor activity of Meadowsweet 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. Camptothecin (Sigma) (tumor suppressant) served as a positive control. All extracts were aqueous; therefore, water was used as a negative control. Seven to eight loops of *A. tumefaciens* were added to 10 ml Phosphate buffered saline (PBS) (pH=7.2). Bacteria concentrations were adjusted to absorbance (600 nm) values of 0.96 ± 0.02 which equaled to 1.0 X 10^9 bacteria.

All extracts, controls and solutions were filter sterilized (sterile 0.22 μm filter, Pal-Gelman Laboratory). The following design was followed:

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

Solvent control I: 600 μl control (water) + 150 μl sterile distilled water + 750 μl *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 scrubbed with a brush under running water and surface sterilized by immersion in 10% Domestos® for 20 min. Tubers were then placed on sterile Petri dishes and cut along either side revealing the largest flat surface area available; tubers were immersed in 20% Clorox for 15 min and then placed on sterile Petri dishes. Cylinders (8 mm diameter) were cut from the center of potato tissue (skin portion was eliminated) and placed in sterile distilled water.

Cylinders were rinsed twice more. 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 room temperature (25°C) in the dark for 2 weeks. After 2 weeks, discs were stained with Lugol's reagent and tumors on each disc were counted. 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.

3.2.4. Bacterial viability testing

A. tumefaciens (strain B6) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28 °C. Six to seven loops of *A*. *tumefaciens* were added to 10 ml phosphate buffered saline (PBS). Bacterial concentration was adjusted to absorbance (600 nm) values of 0.96 ± 0.02, which equaled to 1.0 X 10^9 colony forming units (CFU) (standardized value). This suspension was serially diluted with PBS to 1 X 10^3 cells/ml (CFU). 1 ml of meadowsweet extract was mixed with 1ml of *A. tumefaciens* culture

diluted to 1 X 10^3 cells/ml and left for 30 min. 30-min exposure was chosen as attachment of bacterial cells to the plant tissue, the initial step in crown gall formation occurs within 15 min of inoculation (Lippincott and Lippincott 1969; Glogowski and Galsky 1978). After 30 min, 0.1 ml of inoculum (bacteria + extract) was inoculated on YEM media with spread plate technique and colony counts were made after 24 hours.

3.2.5. Brine Shrimp Bioassay

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 º 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. Twenty–four 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 anesthesizer, was used at concentrations of 1, 10, 100, 1000 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).

3.3. Results and Discussion

Eight different extracts [EtOH extract of field-grown plants (FE) and i*n vitro*-grown plants (IE), EtOAc extract of field-grown plants (FEA) and i*n vitro*-grown plants (IEA), hexane extract of field-grown plants (FH), and i*n vitro*-grown plants (IH), aqueous extract of field-grown plants (FW) and *in*

vitro-grown plants (IW)] were used to screen for antibacterial, antitumor activity and toxicity effects of *F. ulmaria* (Table 3.3; 3.4; 3.5).

Generally, field-grown plants showed better antibacterial activities than *in vitro*-grown plants. Among field-grown plants extracts, ethanol and aqueous extracts were better than hexane and ethylacetate extracts. Best inhibitory activity was observed with ethanol and aqueous extracts of field-grown plants and they exhibited a broad-spectrum activity against both Gram-negative and Gram-positive bacteria. This activity against both types of bacteria may be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins (McCutcheon *et al.* 1992).

Aqueous extracts of field-grown plants showed better antibacterial activity than ethanol extracts of field-grown plants against *S. epidermidis, S. aureus, S. typhimurium, S. marcescens, P. aeruginosa, P. vulgaris, K. pneumoniae* and *E. cloacae* (Table 3.3; Fig. 3.6 and 3.7). Although *S. pyogenes* was susceptible to ethanol extract of field-grown plants, this bacterium was not susceptible to aqueous extracts of field-grown plants (Table 3.3; Fig. 3.1; 3.2; 3.3; 3.4; 3.5).

Antibacterial activity of water extracts against *S. epidermidis*, *S. marcescens*, *S. aureus*, *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae* and *E. cloacae* may explain why meadowsweet is used in folk medicine to treat arthritis, rheumatism and joints pains (caused by *S. aureus*, *K. pneumoniae* and *S. marcescens*), urinary tract infections (caused by *S. epidermidis*, *P. aeruginosa*, *P. vulgaris* and *K. pneumoniae*), chronic lung infections and bronchitis (caused by *P. aeruginosa* and *S. aureus*) (Table 3.2; 3.3 and Fig. 3.1; 3.2; 3.3; 3.4; 3.5; 3.6; 3.7).

Water extracts of plant materials may contain active components such as antocynanines, starches, tannins, saponins, terpenoids, polypeptides and lectines groups (Kaul *et al*. 1985; Scalbert 1991; De Pasquale *et.al.* 1995). The effectiveness of water extracts of *F. ulmaria* may be explained by the inhibitory effect of these compounds on bacteria.

Generally, the Gram-positive bacteria (*S. pyogenes, S. epidermidis* and *S. aureus*) seem to be more susceptible to the inhibitory effects of the plant extracts than Gram-negative bacteria (*S. typhimurium*, *S. marcescens*, *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae*, *E. cloacae* and *E. coli*). Susceptibility of Gram-positive bacteria may come from their cell wall structure consisting of a single layer. However, the Gram-negative cell wall is a multi-layered structure and quite complex (Essawi and Srour 2000).

Bacterial growth was generally sensitive to the reference antibiotics tested (Table 3.3). Aqueous extract of field-grown plants (FW) showed greater antibacterial activity than reference antibiotic tetracycline and similar activity with ampicillin against *S. epidermidis* (Table 3.3; Fig. 3.1 and 3.6).

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 (Table3.3).

Generally, antibacterial activities of field-grown plants were better than *in-vitro* grown plants against used bacteria (Table 3.3). Sometimes the level of the detected secondary products in shoot cultures is lower than donor plants (Stafford 1991). For example, production of steroids obtained from shoot tip cultures of *Digitalis* species was much lower than

those found in the donor plant (Seidel and Reinhard 1987). If the secondary product synthesis is low, there are some procedures for enhancing productivity. Optimization of hormone regime is often effective. The type and concentration of phytohormones available to cultured cells is probably the most important factor influencing their potential for secondary product synthesis. For example 2,4-D can stimulate both cell division and cell expansion in many systems. However, it can also bring about a dramatic suppression of secondary product synthesis in cell cultures. Alterations in other environmental factors such as nutrient levels, light regime and temperature may also be effective in increasing productivity and reduced phosphate levels often stimulate product accumulation (Parr 1989).

Csedo et al. (1993) examined the antibacterial activity of 70% aqueous ethanol extract of meadowsweet and revealed that antibiotic effects were obtained with *Staphylococcus aureus haemolyticus*, *Streptococcus pyogenes haemolyticus*, *E. coli*, *Shigella flexneri*, *K. pneumoniae* and *Bacillus subtilis*. Moreover, Rauha *et al*. (2000) reported that 80% aqueous methanol extract of meadowsweet exhibited antibacterial activity against *S. aureus* and *E. coli* but no activity against *S. epidermidis*. In our study, meadowsweet extracts showed strong antibacterial activity against *S. aureus* (up to 18 mm), *S. epidermidis* (up to 25.3 mm) and *K. pneumoniae* (up to 12.4 mm). But, only hexane extract of field-grown meadowsweet showed just a little activity (8.4 mm) against *E. coli*.

It can be conceivable that strong antibacterial activity may come from salicylic acid constituent of meadowsweet. But, an extract of goat willow (*Salix caprea*) which contains high amount of salicylic acid, showed very little activity against *S. aureus* and *E. coli* and no activity against *S. epidermidis* (Rauha *et al.* 2000). Flavone, quercetin and morine were found to be active against *S. epidermidis* (Nishino *et al.* 1987). The growth of *S. aureus* was inhibited very effectively by flavone, flavonoids, flavonones, flavonols and naringenin (Mori *et al*. 1987; Rauha *et al*. 2000).

Although several kinds of biological activities of *F. ulmaria* were reported, antitumor activity of *F. ulmaria* was not mentioned in the literature. Strong antitumor activity was observed with all extracts of meadowsweet (Table 3.4; Fig. 3.8). When compared with control (water), the percentage inhibition of all extracts was more than 40% in three separate experiments (Fig. 3.9). No tumor formation was observed with camptothecin (100 % inhibition) (Table 3.4 and Fig. 3.9). Antitumor activities of field-grown plant extracts were better than *in vitro*-grown plant extracts and among all extracts, best antitumor activity was observed with ethanol and hexane extracts of field-grown plants (Fig. 3.8; 3.9).

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 extracts to distinguish between these possibilities. Bacterial viability was determined by incubating meadowsweet extracts with 1 X 10^3 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension. Meadowsweet extracts affected on the viability of bacterium. Because of the strong antibacterial activity of

the extracts, they killed the *A. tumefaciens* and tumors were not formed on potato discs. So, we concluded that the action of tested extracts was not on the formation of tumors and on the viability of bacterium. Although the results herein did not show an anti-tumor effect for the extracts of *F. ulmaria*, Sroka *et al*. (2001) and Papp *et al*. (2004) recorded the antioxidant activity of *F. ulmaria*.

Ethanol and hexane 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 strong antibacterial activity against all bacteria including *A. tumefaciens*.

Toxicity of meadowsweet extracts was tested with brine shrimp bioassay (Table 3.5; Fig. 3.10). Results of this bioassay showed that extracts of meadowsweet were toxic at higher doses (around 2.000-7.000 mg/l) by comparing with MS-222 (positive control). Among all extracts, aqueous extracts (FW and IW) were less toxic than other extracts with higher LC_{50} values (70103 mg/l and 62243 mg/l, respectively). Moreover, hexane extracts (FH and IH) were more toxic than other extracts with lower LC_{50} values (2151 mg/l and 2275 mg/l, respectively). Brine shrimp bioassay determines the toxicity of materials toward brine shrimp larvae and can predict the ability to kill cancer cells in cell cultures, kill 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.

Meadowsweet extracts were cytotoxic at high concentrations and they may not be very effective to kill cancer cells in cell culture.

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).

A variety of medicinal plants were discovered and used traditionally but the effects of them have not been supported by scientific studies and information up to now. Using the simple “bench-top” bioassays, initial screening could be provided. However, bioassays must be rapid, convenient, reliable, inexpensive, sensitive, require little material, and be able to be applied in-house by chemists, botanists, and other scientists who lack the resources or expertise to carry out more elaborate bioassays (Ghisalberti 1993).

The leaves and flowering stems of meadowsweet are alterative, anti-inflammatory, antiseptic, astringent, diuretic and stomachic (Vasiliauskas *et al*. 2004). The plant is used in traditional medicine as a febrifuge and for the treatment of several inflammatory diseases, including gout and rheumatoid arthritis (Halkes *et al*. 1997). Results obtained herein confirm the antibacterial activity and toxicity of meadowsweet extracts. Definitive clinical studies are needed to fully understand the many medicinal uses of meadowsweet. Future studies should focus on fractionation of the extracts in hopes of identifying active components.

Table 3.3 Antibacterial activity of *F. ulmaria* extracts. Data presented as zone of inhibition of bacterial growth in mm. Means with the same letter within columns are not significantly different at *P*>0.05.

Treatments	Mean diameter of inhibitory zones (mm ± SE)																																							
	S.pyogenes			*S.epidermidis*			*S.aureus*			*S. marcescens*			*S.typhimurium*			*P.aeruginosa*			*P. vulgaris*			*K.pneumonia*			*E. cloacae*			*E. coli*											
FW	-			25.3	±	0.2	c	18.0	±	0.6	f	10.9	±	0.3	d	8.0	±	0.2	e	10.1	±	0.2	d	15.6	±	0.2	d	12.4	±	0.3	c	17.9	±	0.2	d	-		
FE	14.7	±	0.2	e	20.4	±	0.6	e	14.0	±	0.3	g	8.8	±	0.1	e	-			9.5	±	0.2	d	13	±	0.2	e	10.6	±	0.5	d	15.8	±	0.5	e	-		
FH	8.6	±	0.2	g	18.8	±	0.4	f	13.4	±	0.3	gh	8.1	±	0.2	e	7.1	±	0.1	g	-			11.1	±	0.5	f	8.9	±	0.3	fg	15.4	±	0.3	e	8.4	±	0.2	e
FEA	-			-			-			-			-			-			-			-			-			-			-			-			-		
IW	-			10.9	±	0.3	i	-			-			-			-			-			-			8.2	±	0.3	g	9.6	±	0.4	g	-		
IE	9.5	±	0.2	f	17.2	±	0.4	g	12.6	±	0.2	gh	-			-			-			-			12.1	±	0.1	ef	8.2	±	0.4	g	14.4	±	0.4	f	-		
IH	-			9.2	±	0.4	j	-			-			-			-			-			-			-			10.2	±	0.4	g	-		
IEA	-			-			-			-			-			-			-			-			-			-			-			-			-		
Chloramphenicol (30 mg)	33.8	±	1.3	d	35.9	±	1.0	a	27.5	±	0.5	c	25.1	±	0.6	a	32.4	±	0.4	a	15.0	±	0.4	b	24	±	1.1	c	28.0	±	0.4	a	29.6	±	0.4	a	26.1	±	0.8	a
Tetracycline (30 mg)	43.8	±	2.4	ab	13.4	±	0.3	h	22.9	±	0.2	e	19.8	±	0.5	b	19.6	±	0.3	c	13.4	±	0.4	c	26	±	0.3	b	16.9	±	0.6	b	20.7	±	0.5	c	20.4	±	0.2	c
Ampicillin (10 mg)	46.3	±	2.4	a	22.5	±	0.8	d	33.0	±	0.3	b	16.0	±	1.2	c	26.2	±	0.3	b	-			25	±	0.3	bc	9.9	±	0.2	de	25.0	±	0.4	b	20.1	±	0.3	c
Carbenicillin (100mg)	43.0	±	2.6	b	26.0	±	0.9	b	37.1	±	0.6	a	24.2	±	0.7	a	26.8	±	0.6	b	28.1	±	1.0	a	36.2	±	0.5	a	-			30.4	±	0.3	a	23.0	±	0.5	b
Erythromycin (15 mg)	38.3	±	1.7	c	35.9	±	0.7	a	26.0	±	0.4	d	-			14.4	±	0.2	d	12.9	±	0.9	c	14.6	±	1.0	d	9.4	±	0.2	ef	10.0	±	0.0	g	11.2	±	0.4	d
Water	-			-			-			-			-			-			-			-			-			-			-			-			-		

FW: Water extract of field-grown plants

FE: Ethanol extract field-grown plants

FH: Hexane extract of field-grown plants

FEA: Ethylacetate extract of field-grown plants

IW: Water extract of *in vitro*-grown plants

IE: Ethanol extract of *in vitro*-grown plants

IH: Hexane extract of i*n vitro* -grown plants

IEA: Ethylacetate extract of i*n vitro*-grown plants

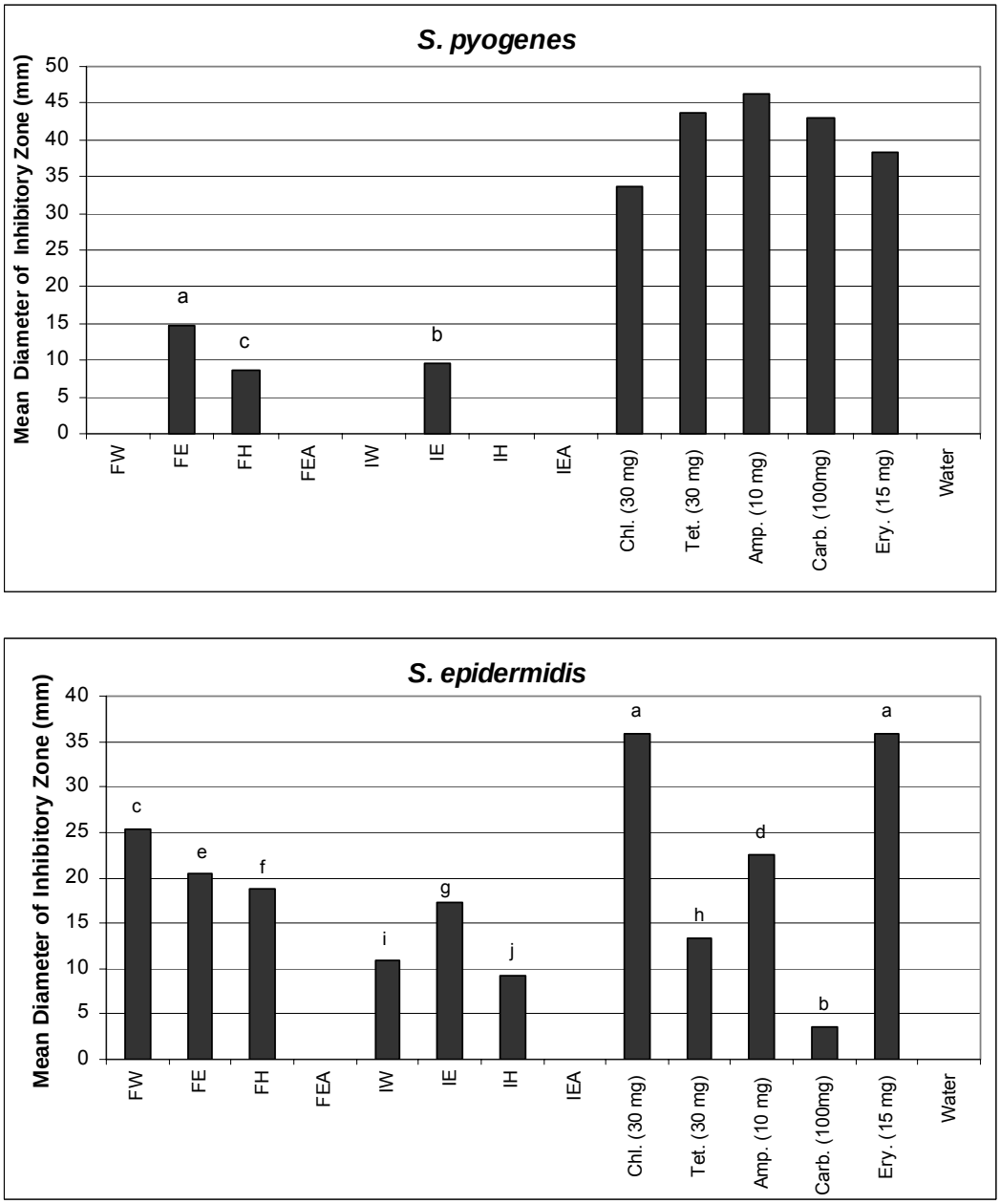


Figure 3.1 Antibacterial activity of *F. ulmaria* extracts and controls against *S. pyogenes* and *S. epidermidis*

FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants

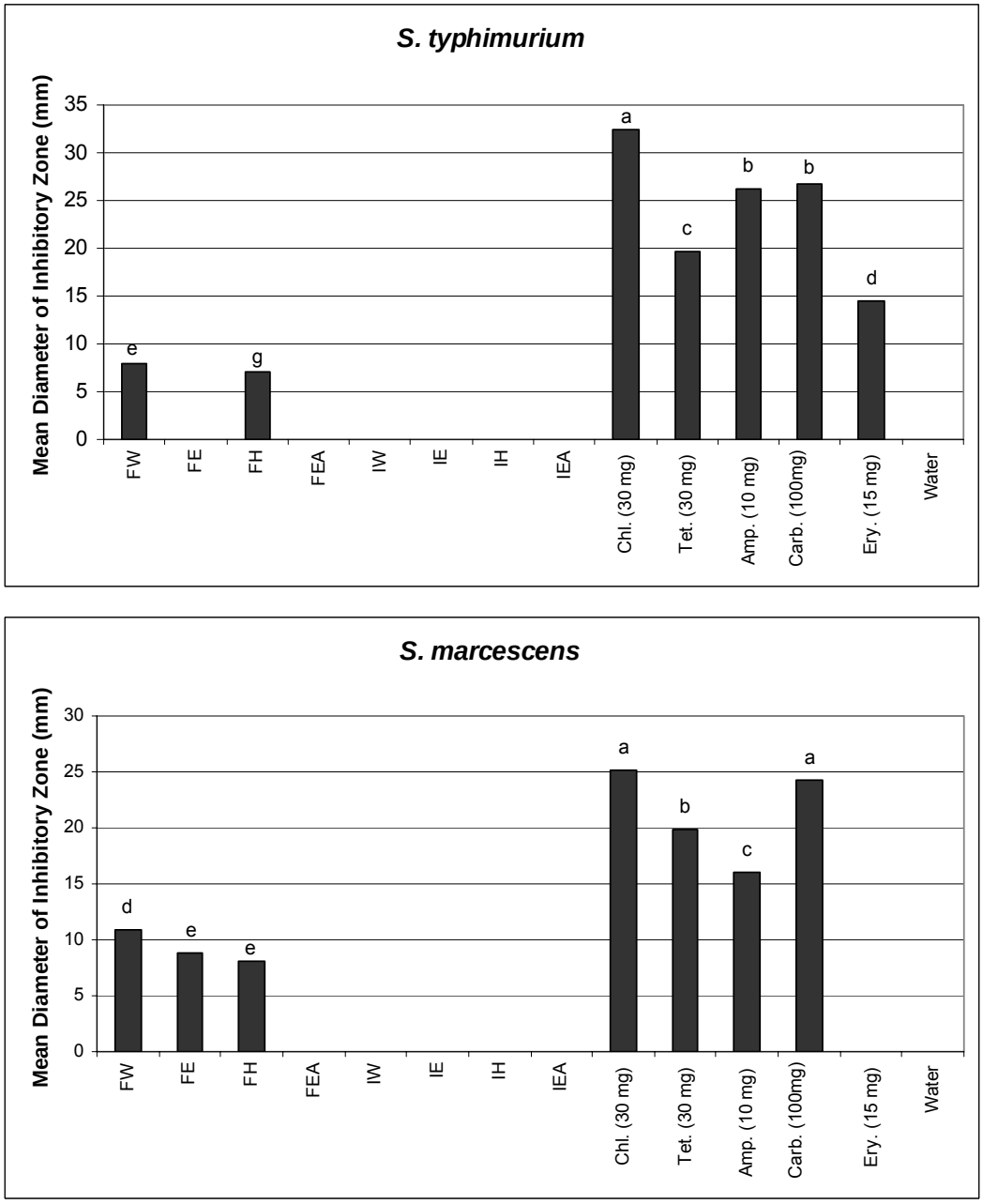
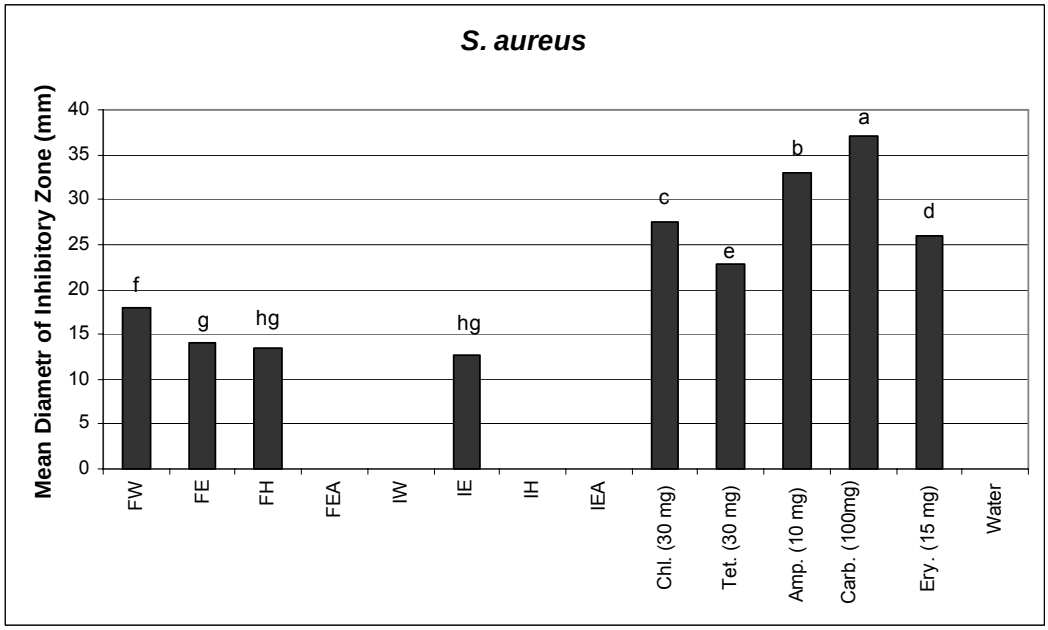


Figure 3.2 Antibacterial activity of *F. ulmaria* extracts and controls against *S. typhimurium* and *S. marcescens*.

FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants



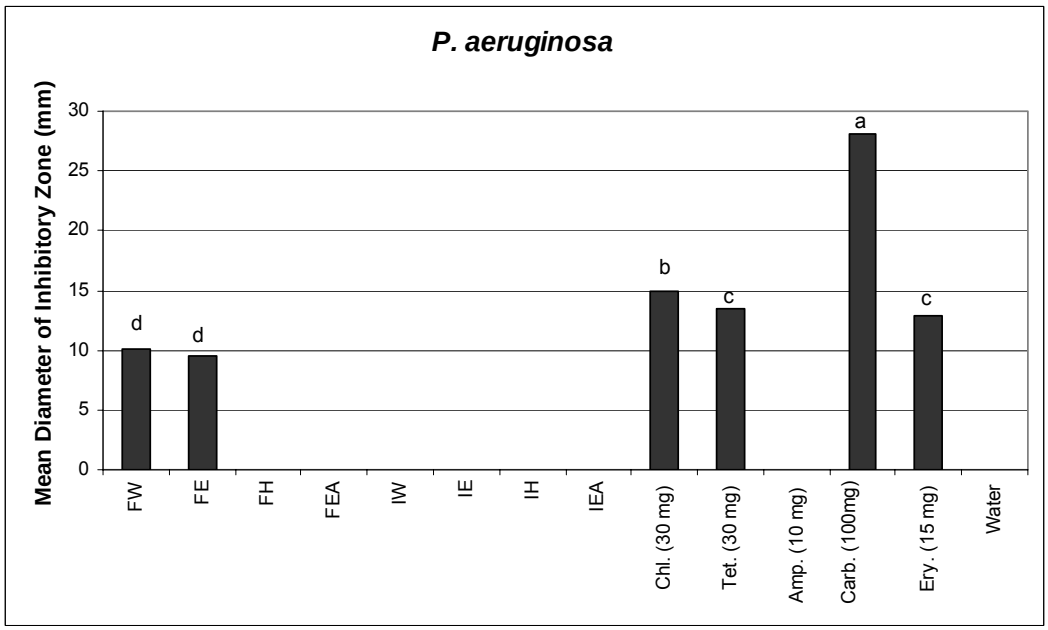


Figure 3.3 Antibacterial activity of *F. ulmaria* extracts and controls against *S. aureus* and *P. aeruginosa*.

FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants

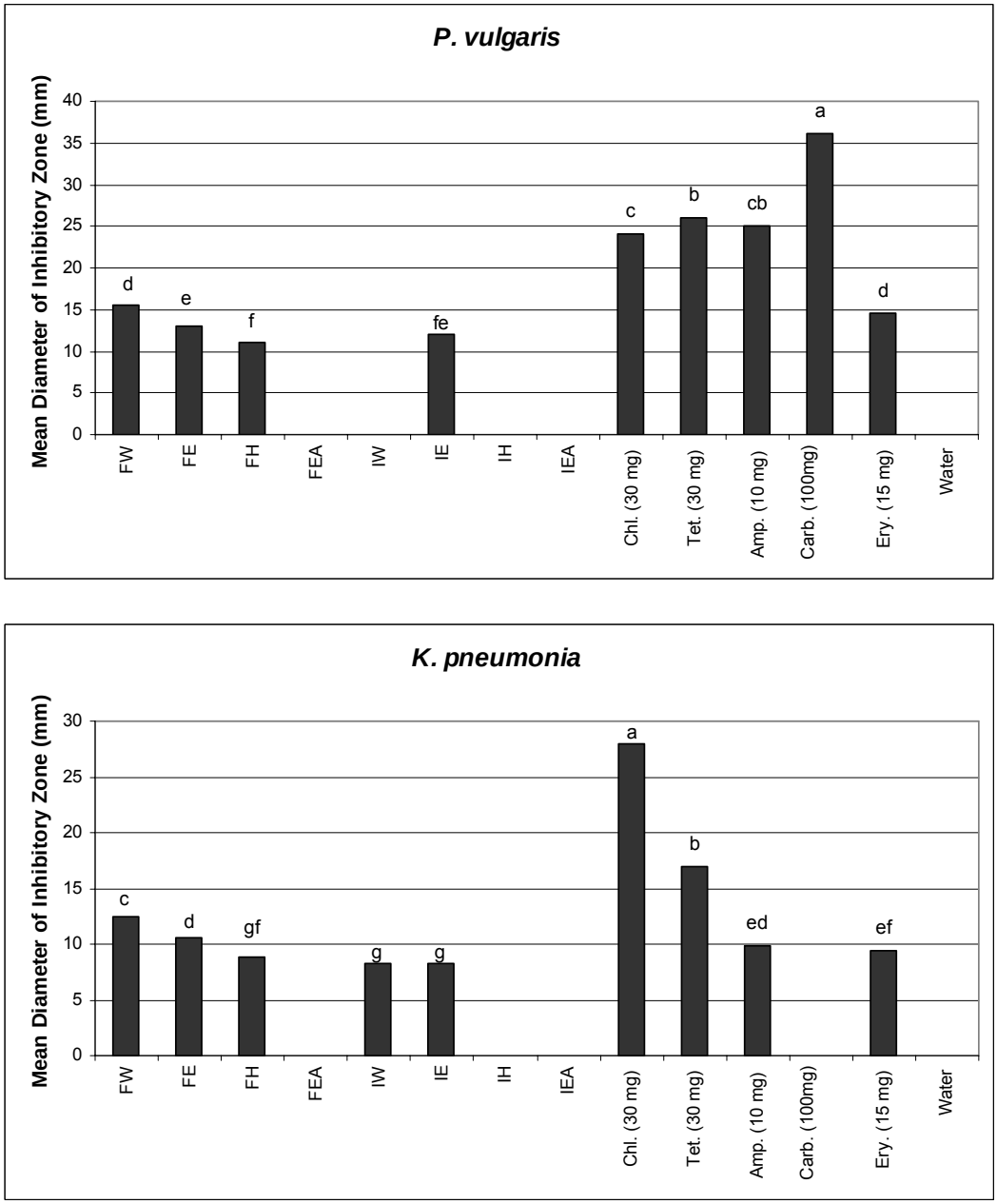
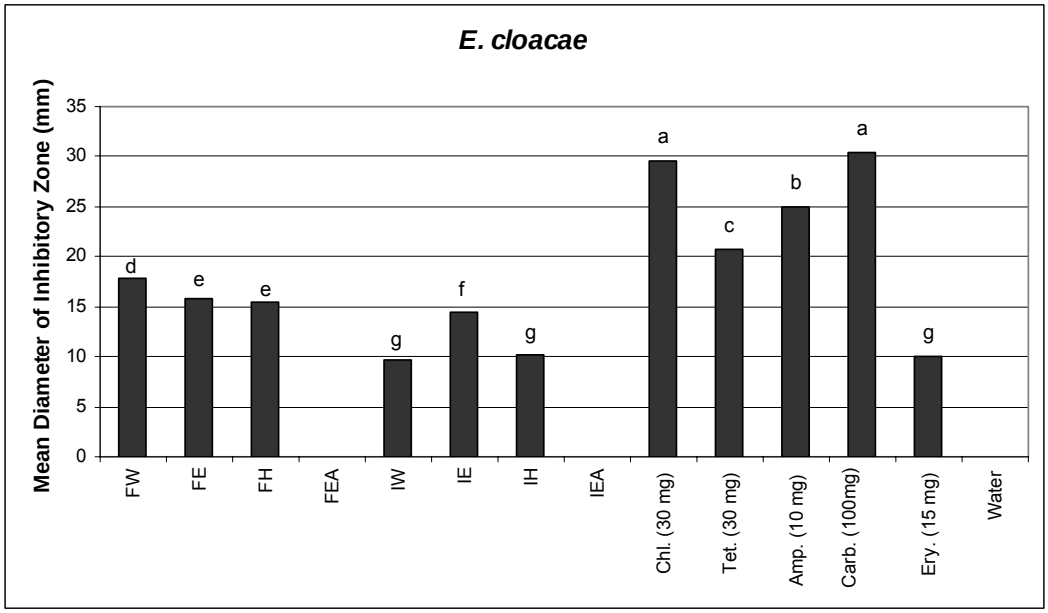


Figure 3.4 Antibacterial activity of *F. ulmaria* extracts and controls against *P. vulgaris* and *K. pneumoniae*.

FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants



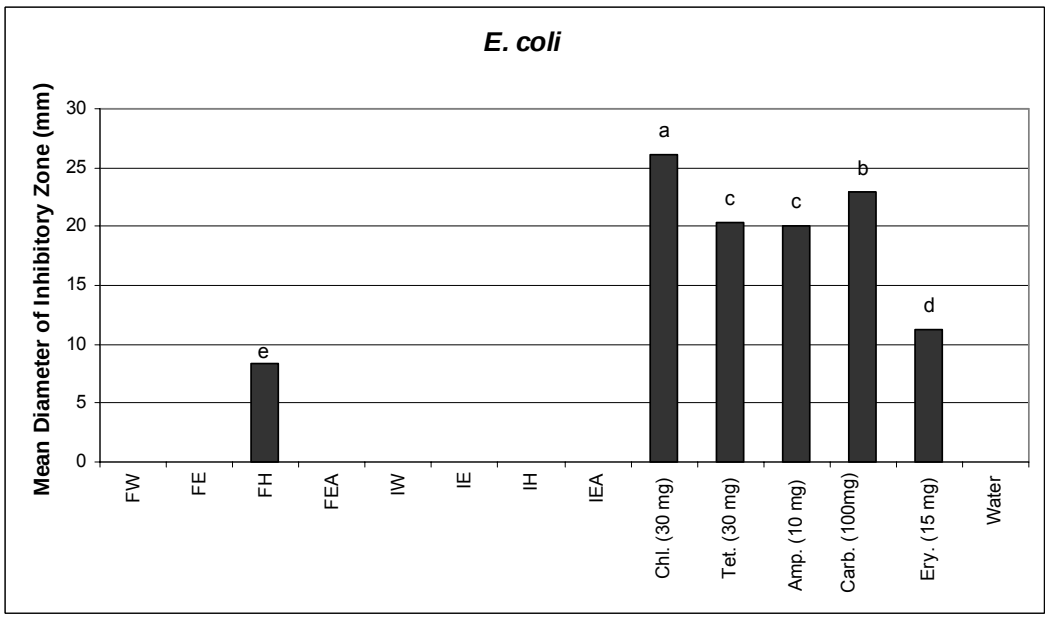


Figure 3.5 Antibacterial activity of *F. ulmaria* extracts and controls against *E. cloacae* and *E. coli*.

FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants

Figure 3.6 Antibacterial activity of FW (water extract of field-grown plants) against *S. aureus* (above), *K. pneumoniae* (middle) and *E. cloacae* (below) by comparing with control.

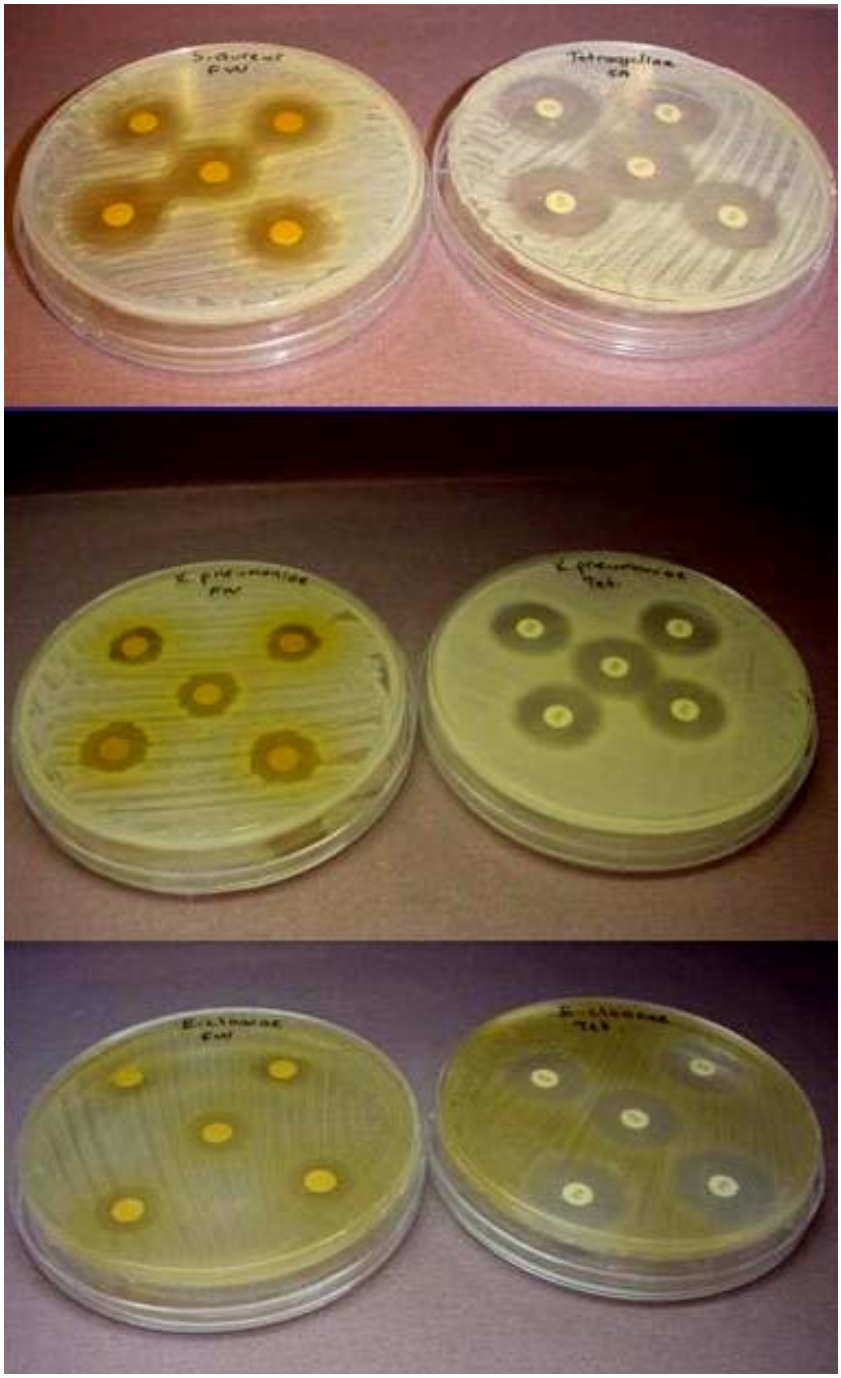


Figure 3.7 Antibacterial activity of FW (water extract of field-grown plants) against *P. vulgaris* (above), *S. epidermidis* (middle) and IE (ethanol extract of *in vitro*-grown plants) against *S. epidermidis* (below) by comparing with control.

Table 3.4 Mean number of tumors observed with *F. ulmaria* extracts and controls (water and camptothecin). Means with the same letter are not significantly different at *P*>0.05.

Treatments	Mean Number of Tumors (±SE)				% Tumor Inhibition
Water	59.3	±	3.4	e	-
Camptothecin	0.0		0.0		100.0
FW	18.8	±	2.4	b	68.3
FE	5.5	±	1.0	a	90.0
FH	7.1	±	1.3	a	88.3
FEA	36.3	±	4.2	d	40.0
IW	22.8	±	2.7	b	61.7
IE	32.2	±	3.7	cd	46.7
IH	36.2	±	2.4	bc	56.7
IEA	32.3	±	3.4	cd	46.7

FW: Water extract of field-grown plants

FE: Ethanol extract field-grown plants

FH: Hexane extract of field-grown plants

FEA: Ethylacetate extract of field-grown plants

IW: Water extract of *in vitro*-grown plants

IE: Ethanol extract of *in vitro*-grown plants

IH: Hexane extract of i*n vitro* -grown plants

IEA: Ethylacetate extract of i*n vitro*-grown plants

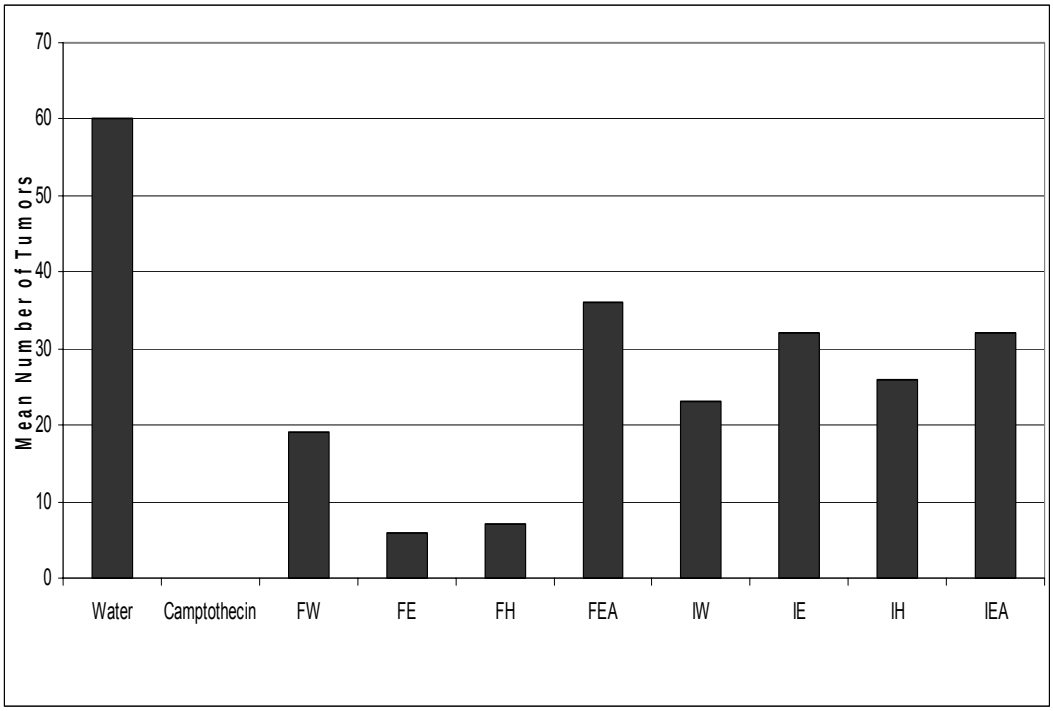


Figure 3.8 Mean number of tumors observed with *F. ulmaria* extracts and controls.

FW: Water extract of field-grown plants
FE: Ethanol extract field-grown plants
FH: Hexane extract of field-grown plants
FEA: Ethylacetate extract of field-grown plants
IW: Water extract of *in vitro*-grown plants
IE: Ethanol extract of *in vitro*-grown plants
IH: Hexane extract of i*n vitro* -grown plants
IEA: Ethylacetate extract of i*n vitro*-grown plants

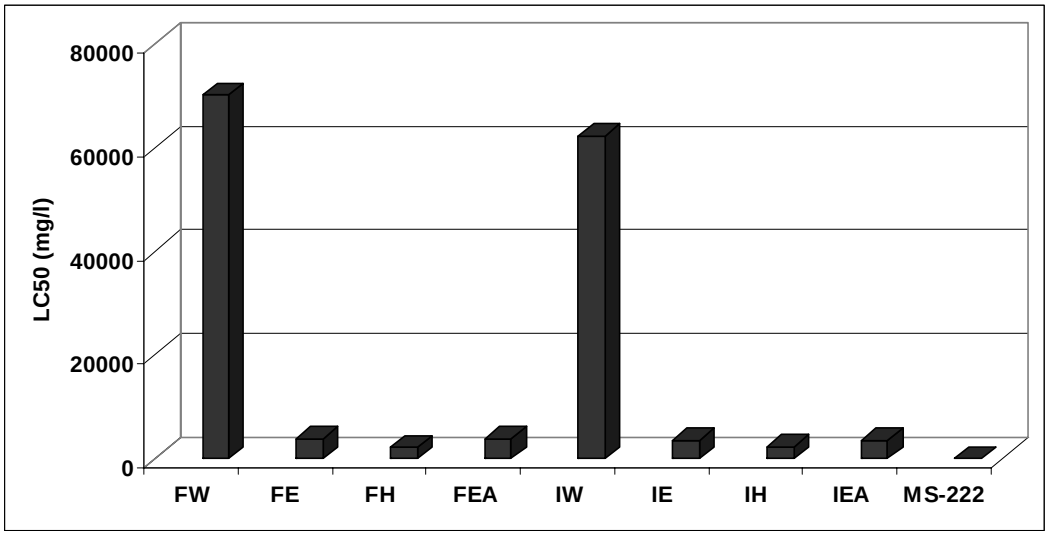
Figure 3.9 Tumors produced by *A. tumefaciens* with FE (ethanol extract of field-grown plants) (above), distilled water (solvent control I) (middle) and camptothecin (positive control) (below).

Table 3.5 LC_{50} (lethal concentration for 50% mortality after 24 hr exposure) values for meadowsweet extracts and MS-222 (a positive control).

Extractions	LC_{50} (mg/l)	Confidence Intervals		
FW	70103	44360	-	110786
FE	3877	2454	-	6128
FH	2151	1361	-	3399
FEA	3898	2466	-	6159
IW	62243	39386	-	98364
IE	3536	2238	-	5589
IH	2275	1440	-	3595
IEA	3494	2211	-	5522
MS-222	47	30	-	74

FW: Water extract of field-grown plants
FE: Ethanol extract field-grown plants
FH: Hexane extract of field-grown plants
FEA: Ethylacetate extract of field-grown plants
IW: Water extract of *in vitro*-grown plants
IE: Ethanol extract of *in vitro*-grown plants
IH: Hexane extract of i*n vitro* -grown plants
IEA: Ethylacetate extract of i*n vitro*-grown plants

Figure 3.10 LC_{50} (lethal concentration for 50% mortality after 24 hr exposure) values for meadowsweet extracts and MS-222 (a positive control).



FW: Water extract of field-grown plants; FE: Ethanol extract field-grown plants; FH: Hexane extract of field-grown plants; FEA: Ethylacetate extract of field-grown plants; IW: Water extract of *in vitro*-grown plants; IE: Ethanol extract of *in vitro*-grown plants; IH: Hexane extract of i*n vitro* -grown plants; IEA: Ethylacetate extract of i*n vitro*-grown plants

CHAPTER FOUR

4. Conclusions

Filipendula ulmaria (L.) Maxim (meadowsweet) is a perennial medicinal plant that has a long history of use in folk medicine. Meadowsweet has been used as an alterative, anti-inflammatory, antiseptic, astringent, diuretic and stomachic. The plant is used in traditional medicine as a febrifuge and for the treatment of several inflammatory diseases, including gout and rheumatoid arthritis. Also, it has been used against coughs, bronchitis, fevers and colds. It is a diuretic, usually administered as an infusion. An *in vitro* culture protocol was established for this medicinal plant. With this efficient protocol, disease- and herbicide-free bulk plant material can be supplied throughout the year for pharmaceutical purposes. Three different explants (leaf, petiole and root) were used for tissue culture experiment. Best shoot proliferation was obtained with root explants. Leaf and petiole explants formed fewer shoots than root explants. The greatest number of shoots was observed on media with 0.1 mg/l TDZ+0.5 mg/l IAA+0.5 mg/l GA_3 with root explants. TDZ was effective in inducing shoot formation. Best results were obtained by the use of TDZ hormone at minimum concentration for all types of explants. With petiole and leaf explants, best response for shoot formation was

obtained with 0.1 mg/l TDZ+0.5 mg/l IAA+1.0 mg/l GA_3 plant growth hormone combination. Callus was observed for root explants before shoot formation which means indirect organogenesis. All explants did not form shoots with media containing kinetin in combination with 2, 4-D.

Regenerated shoots were separated from each other and then cultured on MSMO medium containing different concentrations of auxin hormones. IBA and IAA are found as the most effective auxin hormones on root formation. The greatest number of roots per explant was observed on media with 0.5 mg/l IBA and 0.5 mg/l IAA. In control experiments (no auxin hormone added to the media), root formation was also observed. Rooted plants were transferred to magenta containers including vermiculate for acclimatization. After 3 weeks, they were transferred to soil.

Three different biological assays (antibacterial, anti-tumor and brine shrimp bioassay) were used for assessing biological activities of meadowsweet plant extracts. Biological activities were evaluated between field-grown plants and *in vitro*-grown plants using eight different extracts [ethanol extracts of *in vitro*-grown (IE) and field-grown (FE) plant materials, ethylacetate extracts of *in vitro*-grown (IEA) and field-grown (FEA) plant materials, hexane extracts of *in vitro*-grown (IH) and field-grown (FH) plant materials and aqueous extract of *in vitro*-grown (IW) and field-grown (FW) plant materials].

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity. Ten bacterial strains were used in the bioassay: *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. marcescens*, *P. vulgaris*, *E. cloacae*

and *K. pneumoniae* which are gram Gram-negative bacteria and *S. pyogenes*, *S. aureus* and *S. epidermidis* which are Gram-positive bacteria. Aqueous extracts of field-grown plants gave the best inhibitory activity against *S. epidermidis*, *S. marcescens*, *S. aureus*, *P. aeruginosa*, *P. vulgaris*, *K. pneumoniae* and *E. cloacae*. Antibacterial activity of aqueous extract may explain why meadowsweet is used in folk medicine to treat arthritis, rheumatism and joints pains (caused by *S. aureus*, *K. pneumoniae* and *S. marcescens*), urinary tract infections (caused by *S. epidermidis*, *P. aeruginosa*, *P. vulgaris* and *K. pneumoniae*), chronic lung infections and bronchitis (caused by *P. aeruginosa* and *S. aureus*).

Generally, antibacterial activities of field-grown plants were better than *in-vitro* grown plants against used bacteria. Bacterial growth was generally sensitive to the reference antibiotics tested and no inhibition was observed with water (control).

Results of antitumor bioassay showed that inhibition of crown gall formation on potato disc is caused by decreasing the viability of the *A. tumefaciens* inoculum. Meadowsweet extracts affected the bacterial viability. As a conclusion, we can say that anticancer activity of *F. ulmaria* can not be evaluated with potato disc bioassay. Because, medowsweet extracts have the ability to kill the *A. tumefaciens*.

The brine shrimp bioassay was used to assess the general toxicity of meadowsweet extracts. Our results showed that all extracts were toxic at higher doses (LC_{50} > 2.000 mg/l) by comparing with MS-222 (Tricaine methane sulfonate). Aqueous extracts were less toxic than ethanol,

ethylacetate and hexane extracts. Furthermore, hexane extracts were more toxic than aqueous, ethanol and ethylacetate extracts.

Results obtained herein confirm the bioactivity (antibacterial and toxicity) of meadowsweet extracts. Definitive clinical studies are needed to fully understand the many medicinal uses of meadowsweet. Future studies should focus on fractionation of the extracts in hopes of identifying active components.

REFERENCES

Ahn, YJ., Vang, L., McKeon, TA. and Chen, GQ. 2007. High-frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.). *In vitro Cellular and Developmental Biology-Plant* 43: 9-15.

Agrios, GN. 1997. Plant disease caused by prokaryotes: bacteria and mollicutes. Plant Pathology, San Diego. Academic Press. p. 407-470.

Atlas, RM. 1988. Microbiology: Fundamentals and Applications. Macmillan Publishing Company, New York. p. 632-633.

Baker, HG., and Baker, I. 1967. The cytotaxonomy of *Filipendula* (Rosaceae) and its implications. *American Journal of Botany* 54: 1027-1034.

Barna, KS., and Wakhlu, AK. 1995. Effects or Thidiazuron on micropropagation of rose. *In vitro Cellular and Developmental Biology-Plant* 31: 44- 46.

Barnaulov, OD., and Denisenko, PP. 1980. Antiulcerogenic action of the decoction from flowers of *Filipendula ulmaria* (L.) Maxim. *Farmakologiya I Toksikologiya* 43: 700- 705.

Barnaulov, OD., Manicheva, OA., Shelyuto, VL., Konopleva, MM., and Glyzin, VI. 1984. Effect of flavonoids on the development of experimental gastric dystrophies in mice, *Khimiko-Farmatsevticheskii Zhurnal* 18: 935-41.

Barry, AL., and Thornsberry, C. 1985. Susceptibility Tests: Diffusion Test Procedures. *In*: Lennette EH. (ed.): *Manual of Clinical Microbiology*. pp. 978- 987. American Association for Microbiology. Washington, D.C.

Baytop, T. 1999. Türkiye'de bitkiler ile tedavi. Nobel Tıp Kitabevleri, İstanbul.

Bespalov, VG., Limarenko, AY., Voitenkov, BO., Petrov, AS., Troyan, DN., Aleksandrov, VA., and Peresunko, AP. 1992. Anticarcinogenic, antitumor, and modulating properties of dropwort (*Filipendula ulmaria*) flower decoction. *Khimiko Farmatsevticheskii Zhurnal* 26: 59-61.

Boonkoew, T., and Camper, ND. 2003. Biological activities of Ginkgo extracts. *Phytomedicine* 12: 318-323.

Büyükbaba Boral, Ö., Gönüllü, N., Küçükbasmac, Ö., Anğ Küçüker, M., and Anğ, Ö., 2004. The Influence of urine on the *in vitro* antimicrobial activity of various antibiotics against different *Escherichia coli*

phenotypes. *International Journal of Experimental and Clinical Chemotherapy* 50: 35-39.

Chaturvedi, HC., Jain, M., and Kidwai, NR. 2007. Cloning of medicinal plants through tissue culture-A review. Indian Journal of Experimental. Biology 45: 937- 948

Chevallier, A. 1996. The Encyclopedia of Medicinal Plants. Darling Kindersley Limited, London.

Christianson, ML., and Warnick, DA. 1988. Organogenesis *in vitro* as a developmental process. *Hortscience* 23: 515-519.

Çiftçi, E., Doğru, Ü., Güriz, H., Aysev, AD., and İnce, E. 2003. Antibiotic susceptibility of *Streptococcus Pyogenes* strains isolated from throat cultures of children with tonsillopharyngitis. *Journal of Ankara Medicinal School* 25: 1.

Coker, PS. 1999. Tissue Culture, Extraction and Evaluation of Biological Activity of *Echinaceae purpurea*. Msc. thesis. Clemson University, USA.

Coker, PS., Rodecke, J., Guy, C., and Camper, ND. 2003. Potato disc tumor induction assay: A multiple mode of drug action assay. *Phytomedicine* 10:133-138.

Cox, PA., Balick, MJ. 1994. The ethnobotanical approach to drug discovery. *Scientific American* 6: 82-87.

Csedo, K., Monea, M., Sabau, M., and Esianu, S. 1993. The antibiotic activity of *Filipendula ulmaria*. *Planta Medica* 59: A 675.

Davis, PH. 1972. Flora of Turkey and the East Aegean Islands, Vol 4, Edinburgh Univercity Press.

De Gyves, EM., Sparks, CA., Fieldsend, AF., Lazzeri, PA., and Jones, HD. 2001. High frequency of adventitious shoot regeneration from commercial cultivars of evening primrose (*Oenothera* spp.) using thidiazuron. *Annals of Applied Biology* 138: 329-332.

De Pasquale, R., Germano, MP., Keita, A., Sanogo, R., and Iauk, L. 1995. Antiulcer activity of *Pteleopsis suberosa*. *Journal of Ethnopharmacology* 47: 55–58.

Dobelis, IN., 1990. Magic and Medicine of Plants. New York: The Reader's Digest Association.

Duke, J CRC. 1989. Handbook of Medicinal Herbs. Boca Raton, FL: CRC Press Inc., p. 196-197.

Essawi, T., and Srour, M. 2000. Screening of some Palestinian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology* 70: 343-349.

Facciola, S. 1990. Cornucopia - A Source Book of Edible Plants. Kampong Publications.

Faisal, M., Siddique, I., and Anis, M. 2006. *In vitro* rapid regeneration of plantlets from nodal explants of Mucuna pruriens –a valuable medicinal plant. *Annals of Applied Biology* 148: 1–6.

Ferrigini, NR., Putnam, JE., Anderson, B., Jacobsen, LB., Nichols, DE., Moore, DS., and McLaughlin, JL. 1982. Modification and evaluation of the potato disc assay and antitumor screening of Euphorbia seeds. *Journal of Natural products* 45: 679-686.

Feyissa, T., Welander, M., and Negash, L. 2005. *In vitro* regeneration of *Hagenia abyssinica* (Bruce) JF Gmel. (Rosaceae) from leaf explants. *Plant Cell Reports* 24: 392- 400.

Galsky, AG., Wilsey, TP., and Powell, RG. 1980. Crown gall tumor disc bioassay. *Plant Physiology* 65: 184-185.

Geetha, N., Venkatachalam, P., Prakash, V., and Sita, GL. 1998. High frequency induction of multiple shoots and plant regeneration from seedling explants of pigeonpea (*Cajanus cajan* L.). *Current Science* 75: 1036-1041.

Ghisalberti, EL. 1993. Detection and isolation of bioactive natural products. *In*: Colegate, SM., and Molyneux, RJ. (ed.): *Bioactive Natural Products, Detection, Isolation and Structural Determination*. P. 9-49. CRC Press.

Grieve, M. 1982. A modern herbal, Vol 2, Dover Publications, New York.

Halkes, SBA., Beukelman, CJ., Kroes, BH., Van den Berg, AJJ., Labadie, RP., and Van Dijk, H. 1997. In Vitro Immunomodulatory Activity of *Filipendula ulmaria*. *Phytotherapy Research* 7: 518–20.

Hoff, FH., and Snell, TW. 1987. Plankton Culture Manual, Florida Aqua Farms, Inc., p. 94- 99.

Huetteman, CA., and Preece, JE. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue and Organ Culture* 33: 105–119.

Jasiuleviciute, L., Keturkiene, A., and Leonaviciene, L. 2001. Relation between zinc and magnesium content in liver of rats with

adjuvant arthritis and treatment with the tincture of *Filipendula ulmaria* L. Maxim., *Acta Medica Lituanica* 8: 4.

Jones, MPA., Yi, ZJ., Murch, SJ., and Saxena, PK. 2007. Thidiazuron-induced regeneration of *Echinacea purpurea* L.: Micropropagation in solid and liquid culture systems. *Plant Cell Reports* 26: 13-19.

Kaul, TN., Middletown, Jr E., and Ogra, PL. 1985. Antiviral effect of flavonoids on human viruses. *Journal of Medical Virology*. 15: 71–79.

Kudriashov, BA., Liapina, LA., and Azieva, LD. 1990. The Content of a Heparin-like anticoagulant in the flowers of the meadowsweet (*Filipendula ulmaria*). *Farmacologia I Toxicologia* 4: 39-41.

Krasnov, EA., Raldugin, VA., Shilova, IV., and Avdeeva, E. 2006. Phenolic compounds from *Filipendula ulmaria*. *Chemistry of Natural Compounds* 42: 148-151.

Landi, L., and Mezzetti, B. 2006. TDZ, auxin and genotype effects on leaf organogenesis in *Fragaria*. *Plant Cell Reports* 25: 281-288.

Lata, H., Bedir, E., Hosick, A., Ganzera, M., Khan, I., and Moraes, RM. 2002. *In vitro* plant regeneration from leaf-derived callus of *Cimicifuga racemosa*. *Planta Medica* 68: 912-915.

Lewan, L., Andersson, M., and Morales-Gomez, P. 1992. The use of Artemia salina in toxicity in toxicity testing. *Alternatives To Laboratory Animals* 20: 297- 301.

Lindeman, A., Jounela-Eriksson, P., and Lounasmaa, M. 1982. The aroma composition of the flower of Meadowsweet (*Filipendula ulmaria* L. Maxim.), *Lebensm Wissu Technology* 15: 286-289.

Lombardi, SP., Passos, IRS., Nogueira, MCS., and Glória, BA. 2007. *In vitro* Shoot Regeneration from Roots and Leaf Discs of *Passiflora cincinnata* Mast. *Brazilian Archives of Biology and Technology* 50: 239-247.

Lu, CY. 1993. The use of TDZ in tissue culture. *In vitro Cellular and Developmental Biology-Plant* 29: 92-96.

Lyapina, LA., and Kovalchuk, GA. 1993. A comparative-study of the effects of *Filipendula ulmaria* flower and seed extracts on hemostasis. *Izvestiya Akademii Nauk Seriya Biologicheskaya* 4: 625-628.

McCoy, JH. 1988. *In Vitro* Culture of *Hypericum perforatum* and Analysis of Hypericins. Msc. thesis. Clemson University, USA.

McCutcheon, AR., Ellis, SM., Hancock, REW., and Towers, GHN. 1992. Antibiotic screening of medicinal plants of the British Columbian native peoples. *Journal of Ethnopharmacology* 37: 213 -223.

McLaughlin, JL. 1991. Crowngall tumors on potato discs and brine shrimp lethality: two single bioassays for plant screening and fraction. *In*: Hostetsman, K. (ed.): *Methods in Plant Biochemistry*. pp. 1-31. Academic Press. London.

McLaughlin, JL., Chang, CJ., and Smith, DL. 1991. "Bench-top" bioassays for the discovery of bioactive natural products: An update. *In*: Rahman, A. (ed.): *Studies in Natural Products Chemistry*, vol.9, p.383-409. Elsevier Science Publishing Company Inc. New York.

McLaughlin, JL., Chang, CJ., and Smith, DL. 1993. Simple bench-top bioassay (brine shrimp and potato discs) for the discovery of plant antitumor compounds. *In*: Kinghorn, AD., and Baladrin, MF. (eds.): *Human Medicinal Agents from Plants*. pp. 112-137. ACS Symposium Series 534. Washington.

McLaughlin, JL., Rogers, LL., and Anderson, JE. 1998. The use of biological assays to evaluate botanicals. *Drug Information Journal* 32: 513-524.

Meyer, BN., Ferrigni, NR., Putham, JE., Jacobsen, LB., Nichols, DE., and McLaughlin, JL., 1982. Brine Shrimp: A convenient general bioassay for active plant constituents. *Journal of Medicinal Plant Research* 45: 31-34.

Mori, A., Nishino, C., Enoki, N., and Tawata, S. 1987. Antibacterial activity and mode of action of plant flavonoids against *P. vulgaris* and *S. aureus*. *Phytochemistry* 8: 2231-2234.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiologia Plantarum* 15: 473-97.

Murch, SJ., Krishnaraj, S., and Saxena, PK. 1997. TDZ-induced morphogenesis of Regal Geranium (*Pelargonium domesticum*): a potential stress response *Physiologia Plantarum* 101: 183-191.

Murthy, BNS., Murch, SJ., and Saxena, PK. 1998. Thidiazuron: a potent regulator of *in vitro* morphogenesis, *In Vitro Cellular and Developmental Biology- Plant* 34: 267- 275.

Newall, CA., Anderson, LA., and Phillipson, JD. 1996. Herbal Medicines: A Guide for Health-Care Professionals.: The Pharmaceutical Press. London.

Nishino, C., Enoki, N., Tawata, S., Morim, A., Kobayashi, K., and Fukushima, M. 1987. Antibacterial activity of flavonoids against *S.epidermidis*, a skin bacterium. *Agricultural and Biological Chemistry* 1: 139-143.

Papp, I., Apati, P., Andrasek, V., Blazovics, A., Balazs, A., Kursinszki, L., Kite, GC., Houghton, PJ., and Kery, A. 2004. LC-MS analysis of antioxidant plant phenoloids. *Chromatographia* 60: 93-100.

Parr, AJ. 1989. The production of secondary metabolites by plant cell cultures. *Journal of Biotechnology* 10: 1-26.

Parra, AL., Yhebra, RS., Sardinas, IG., and Buela, LI. 2001. Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD50 value) in mice,to determine oral acute toxicity of plant extracts. *Phytomedicine* 5: 395-400.

Peresunko, AP., Bespalov, VG., Limarenko, AI., and Aleksandrov, VA. 1993. Clinico-experimental study of using plant preparations from the flowers of *Filipendula ulmaria* (L.) Maxim for the treatment of precancerous changes and prevention of uterine cervical cancer, *Voprosy Onkologii* 39: 291–295.

Phillips, R., and Foy, N. 1990. Herbs, Pan Books Ltd. London.

Pitts, M. 2006. Meadowsweet flowers in prehistoric graves. *British Archaeology* 88: 6.

Prashanth, D., Asha, MK., and Amit, A. 2001. Antibacterial activity of *Punica granatum*. *Fitoterapia* 72: 171- 173.

Prescott, LM., Harley, JP., and Klein, DA. 1990. Antimicrobial chemo- theraphy. *In*: Brown, Wm.C.: *Microbiology*. p. 253-255. Dubuque, IA.

Rout, GR., Samantaray, S., and Das, P. 2000. *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances* 18: 91-120.

Rahua, JP., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H., and Vuorela, P. 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology* 56: 3–12.

Sahayara, JK., Borgio, JF., Muthukumar, S., and Anandh, GP. 2006. Antibacterial Activity of *Rhynocoris marginatus* and *Catamirus brevipennis* venoms aganist human pathogens. *Journal of Venomous Animals and Toxins including Tropical Diseases* 12: 487-496.

Sam, TW. 1993. Toxicity testing using the brine shrimp: *Artemia salina*. *In*: Colegate, SM., and Molyneux, RJ. (eds.): *Bioactive Natural Products, Detection, Isolation and Structural Determination*. pp. 442- 454. CRC Pres.

Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30: 3875- 3883.

Schwarz, OJ., and Beaty, RM. 1996. Propagation from nonmeristematic tissues-organogenesis. *In*: Trigiano, RN., and Gray, DJ. (eds.): *In Plant Tissue Culture Concepts and Laboratory Exercises.* pp. 95-103. CRC Press. Florida.

Seidel, S., and Reinhard, E. 1987. Major cardenolide glycosides in embryogenic suspension cultures of *Digitalis lanata*. *Planta Medica* 153: 308-309.

Shahzad, A., Faisal, M., and Anis, M. 2007. Micropropagation through excised root culture of *Clitoria ternatea* and comparison between *in vitro*–regenerated plants and seedlings. *Annals of Applied Biology* 150: 341–349.

Siddique, I., and Anis, M. 2007. *In vitro* shoot multiplication and plantlet regeneration from nodal explants of *Cassia angustifolia* Vahl.: a medicinal plant . *Acta Physiologia Plantarum* 29: 233-238.

Singh, ND., Sahoo, L., Sarin, NB., and Jaival, PK. 2003. The effect of TDZ On Organogenesis and Somatic Embriyogenesis on pigeonpea (*Cajanus cajan* L. Millsp), Department of Biosciences, M.D. University, India.

Smolarz, HD., and Sokolowska-Wozniak, A. 2001. Pharmacological effects of extracts from Filipendula ulmaria and *F. hexapetala*. *Postepy Fitoterapii* 7: 16-19.

Spiridonov, NA., Konovalov, DA., and Arkhipo, VV. 2005. “Cytotoxicity of Some Russian Ethnomedicinal Plants and Plant Compounds”, *Phytotherapy Research* 19: 428–432.

Sroka, Z., Cisowski, W., Seredynska, M., and Luczkiewicz, M. 2001. Phenolic extracts from meadowsweet and hawthorn flowers have antioxidative properties. Zeitschrift fur Naturforschung. Section C. *Biosciences* 9/10: 739-744.

Stafford, A. 1991. Natural products and metabolite from plants and plant tissue cultures. *In*: Stafford, A., and Warren, G. (eds.): *Plant Cell and Tissue Culture*. pp: 125-162. Open University Press. Buckingham.

Starbuck, JJ. 1999. Herbal Use by Health care providers. *In*: Eskinazi. D. (ed.): *Botanical Medicine*. pp. 33. Mary Ann Liebert, Inc., USA.

Sutter, EG. 1996. General laboratory requirements, media and sterilization methods. *In*: Trigiano, RN., and Gray, DJ. (eds.): In *Plant Tissue Culture Concepts and Laboratory Exercises*. pp. 18. CRC Press. Florida.

Thorpe, TA. 1994. Morphogenesis and regeneration. *In*: Vasil, IK., and Thorpe, TA. (eds.): *Plant Cell and Tissue Culture*. pp. 17-36. Kluwer Academic Publishers, Netherlands.

Turker, AU., and Camper, ND. 2002. Biological activity of common Mullein, a medicinal plant. *Journal of Ethnopharmacology* 82: 117-125.

Turker, AU., Cansever, E., and Yildirim, AB. 2008. Efficient *in vitro* regeneration of Fireweed, a medicinal plant. *Acta Physiologia Plantarum* 30: 421-426.

Uzun, E., Sariyar, G., Adsersen, A., Karakoc, B., Ötük, G., Oktayoglu, E., and Pirildar, S. 2004. Traditional medicine in Sakarya province (Turkey) and antimicrobial activities of selected species. *Journal of Ethnopharmacology* 95: 287–296.

Valle, MG., Nano, GM., and Tira, S. 1988. The essential oil of *Filipendula ulmaria*. *Planta Medica* 2: 181- 182.

Vasiliauskas, A., Keturkienei, A., Leonavieiene, L., and Vaitkiene, D. 2004. Influence of herb *Filipendula ulmaria* (L.) Maxim tincture on pro-/ antioxidant status in gastric tissue with indomethacin-induced gastric ulser in rats. *Acta Medica Lithuanica* 11: 31-36.

Vazquezt, GJ., and Archer, GL. 1980. Antibiotic Therapy of Experimental *Staphylococcus epidermidis* Endocarditis *Antimicrobial Agents and Chomotheraphy* 17: 280 -285.

Wang, J., and Bao, MZ. 2007. Plant regeneration of pansy (*Viola wittrockiana*) 'Caidie' via petiole-derived callus. *Scientia Horticulturae* 111: 266- 270.

Xiao, XG., and Branchard, M. 1995. *In vitro* High-frequency Plant-Regeneration from Hypocotyl and Root Segments of Spinach by Organogenesis. *Plant Cell, Tissue and Organ Culture* 42: 239- 244.

Yucesan, B., Turker, AU., and Gurel, E. 2007. TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). *Plant Cell, Tissue and Organ Culture* 91: 243- 250.

Zeylstra, H. 1998. *Filipendula ulmaria*. *Br I Phytother 5*: 8 -12.

Zobayed, SMA., and Saxena, PK. 2003. *In vitro*-grown roots: a superior explant for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. cv 'New Stem') in a temporary immersion bioreactor. *Plant Science* 165: 463- 470.