

**DEVELOPMENT OF *IN VITRO* CULTURE PROTOCOL AND
EVALUATION OF BIOLOGICAL ACTIVITIES OF *BELLIS PERENNIS* L.
(COMMON DAISY), A MEDICINAL PLANT**

**by
FATMA PEHLİVAN KARAKAŞ**

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Prof. Dr. Yaşar Dürüst
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

Assoc. Prof. Dr. Mustafa ÜNAL
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 Doctor of Philosophy.

Assoc. Prof. Dr. Arzu UÇAR TÜRKER
Supervisor

Examining Committee Members

1. Prof. Dr. Rukiye TIPIRDAMAZ
2. Prof. Dr. Ekrem GÜREL
3. Prof. Dr. Akçahan GEPDİREMEN
4. Prof. Dr. Nusret ZENCİRCİ
5. Assoc. Prof. Dr. Arzu UÇAR TÜRKER

ABSTRACT

DEVELOPMENT OF *IN VITRO* CULTURE PROTOCOL AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *BELLIS PERENNIS* L. (COMMON DAISY), A MEDICINAL PLANT

Pehlivan Karakaş, Fatma
Ph.D., Department of Biology
Supervisor: Assoc. Prof. Dr. Arzu Uçar Türker

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Bellis perennis L. (common daisy) is a medicinal plant in the family Asteraceae. It has been used in the treatment of common cold, wounds, rheumatism and inflammation in traditional medicine. A highly efficient and rapid regeneration system was developed for common daisy. Field-grown plants and *in vitro*-derived plantlets were compared in terms of their regeneration capacity. The field-grown plants were found to be much more responsive than the *in vitro*-derived plantlets when used as explant source. Leaf, pedicel and root explants obtained from field-grown plants, and leaf, petiole and root explants obtained from *in vitro*-derived plantlets were cultured on MSMO medium with various plant growth regulator combinations. The best shoot proliferation was obtained with pedicel explants on media containing 0.5 mg/l TDZ and 0.5 mg/l IAA. Regenerated shoots were rooted on MSMO medium containing different concentrations of IAA, IBA, 2,4-D and NAA. IAA (1.0 mg/l) was determined as the most effective auxin for rooting. Flowering of regenerated plants was also observed. Chemical composition of *B.*

perennis was determined by using GC-MS, TLC and LC-MS/MS methods. Biological activities of common daisy extracts were assessed using selected bioassays such as antibacterial, antitumor, toxicity, anticancer, anti-inflammatory and antioxidant [radical scavenging activity (DPPH), total phenolic content, total flavonoid content, oxygen radical absorbing capacity (ORAC) and cell-based (DCFH-DA) assays]. Methanol and ethyl acetate extracts of flowers exhibited a broad-spectrum of antibacterial activity against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterobacter cloacea*. *Agrobacterium tumefaciens*-induced tumors in potato tissue were inhibited by all tested flower extracts. Results of brine shrimp toxicity bioassay showed that extracts of daisy were toxic at higher doses. The anticancer activity of extracts was evaluated against human lung carcinoma (A-549), colon adenocarcinoma (DLD-1) and breast adenocarcinoma (MCF-7). *In vitro*-derived leaf extracts showed the best anticancer activity against selected cell lines. Moreover, *n*-butanol and ethyl acetate fractions of flowers exerted high levels of anticancer activity. Metanol extract of flowers showed strong anti-inflammatory activity on RAW 264.7 macrophages. The ethyl acetate fraction of flower was found to be a strong antioxidant, with total phenolic content and ORAC value. The results of this thesis showed that *B. perennis* has a great potential to be used in phytotherapy, pharmacy and food industry.

Keywords: *Bellis perennis*, common daisy, *in vitro* culture, extraction methods, GC-MS, TLC, LC-MS/MS, bioassay, antibacterial, antitumor, toxicity, anticancer, cytotoxicity, anti-inflammatory, antioxidant.

ÖZET

TIBBİ BİR BİTKİ OLAN *BELLIS PERENNIS* L. (ÇAYIR PAPATYASI) 'İN İN VİTRO KÜLTÜR PROTOKOLÜNÜN GELİŞTİRİLMESİ VE BİYOLOJİK AKTİVİTESİNİN DEĞERLENDİRİLMESİ

Pehlivan Karakaş, Fatma
Ph.D., Biyoloji Bölümü
Tez Danışmanı: Doç. Dr. Arzu Uçar Türker

Temmuz 2012, 178 Sayfa

Bellis perennis L. (çayır papatyası) Asteraceae familyasına ait olan tıbbi bir bitkidir. Halk arasında soğuk algınlığı, yaralar, romatizma ve iltihapların tedavisinde kullanılmaktadır. Çayır papatyası için oldukça etkili ve hızlı rejenerasyon sistemi geliştirilmiştir. Doğada yetişen bitkiler ile *in vitro* yetiştirilen bitkiciklerin rejenerasyon kapasiteleri karşılaştırılmıştır. Eksplant kaynağı olarak, doğada yetişen bitkiler kullanıldığında, rejenerasyon yeteneklerinin *in vitro* yetiştirilen bitkiciklerden daha başarılı olduğu görülmüştür. Doğada yetişen bitki eksplantları (pedisel, yaprak ve kök) ile *in vitro* yetiştirilen bitkicik eksplantları (petiyol, yaprak ve kök), çeşitli bitki büyüme düzenleyici kombinasyonlarını içeren MSMO ortamında kültüre alınmışlardır. En iyi sürgün gelişimi, pedisel eksplantlarından, 0.5 mg/l TDZ ve 0.5 mg/l IAA içeren ortamda elde edilmiştir. Rejenere olmuş sürgünler, IAA, IBA, 2,4-D ve NAA' nın farklı konsantrasyonlarını içeren MSMO ortamında köklendirilmiştir. Köklendirme için en etkili oksinin IAA (1.0 mg/l) olduğu tespit

edilmiştir. Ayrıca, rejenere olmuş bitkilerin çiçeklendiği de görülmüştür. *B. perennis*'in kimyasal kompozisyonu GC-MS, TLC ve LC-MS/MS metodları ile belirlenmiştir. Çayır papatyası özütlерinin, antibakteriyel, antitümör, toksisite, antikanser, antiinflamatuvar ve antioksidan [radikal süpürücü aktivite (DPPH), toplam fenol içeriği, toplam flavonoid içeriği, oksijen radikali yakalama kapasitesi (ORAC) ve hücre temelli (DCFH-DA) testler] gibi biyolojik aktiviteleri, çeşitli biyolojik testler kullanılarak değerlendirilmiştir. Çiçeğin metanol ve etil asetat özütleri, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* ve *Enterobacter cloacea*'ya karşı geniş spektrumlu antibakteriyel etki göstermişlerdir. Bütün test edilen çiçek özütleri, patates dokusunda *Agrobacterium tumefaciens* tarafından oluşturulan tümörleri inhibe etmiştir. Brine shrimp biyolojik testinin sonuçları, çayır papatyası çiçek özütlерinin yüksek dozlarda toksik olduğunu göstermiştir. Çayır papatyası özütlерinin antikanser aktivitesi insan akciğer kanseri (A-549), kolon kanseri (DLD-1) ve meme kanseri (MCF-7) hücre dizilerinde değerlendirilmiştir. Seçilmiş kanser dizileri üzerinde en iyi antikanser aktiviteyi *in vitro* yetiştirilmiş yaprak özütleri göstermiştir. Bununla birlikte, çiçeğin *n*-butanol ve etil asetat fraksiyonlarının da yüksek potansiyelde antikanser aktiviteye sahip olduğu görülmüştür. Çiçeğin metanol özütlünün, RAW 264.7 makrofajları üzerinde güçlü antiinflamatuvar aktiviteye sahip olduğu gösterilmiştir. Çiçeğin etil asetat fraksiyonunun, oksijen radikalini yakalama kapasitesi ve toplam fenolik içeriği bakımından, yüksek antioksidan aktiviteye sahip olduğu belirlenmiştir. Bu tezin sonuçları, *B. perennis*'in fitoterapi, ilaç ve gıda endüstrisinde kullanılmak üzere büyük bir potansiyele sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Bellis perennis* L., ayır papatyası, *in vitro* kltr, ekstraksiyon metodları, GC-MS, TLC, LC-MS/MS, biyolojik test, antibakteriyel, antitmr, toksisite, antikanser, sitotoksisite, antiinflamatuvar, antioksidan.

DEDICATION

I dedicate this thesis to my son, Tuna Kaan Karakaş; my daughter, Elanur Karakaş; my husband, Alper Karakaş; my mother and father, Neziha and Osman Pehlivan; my sisters, Nazan, Derya, Hümeyra, Hatice and Melikenur; and my mother-in-law, Ayşe Karakaş for their love, helping and encouragement.

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
2,4-D	2,4-dichlorophenoxyacetic acid
A-549	Human lung carcinoma
ANOVA	Analysis of variance
BA	Benzyladenine
BFH	Hexane extract of <i>B. perennis</i> - flowers (soxhlet)
BFD	DCM extract of <i>B. perennis</i> - flowers (soxhlet)
BFM	MeOH extract of <i>B. perennis</i> - flowers (soxhlet)
BFW	Water extract of <i>B. perennis</i> - flowers (soxhlet)
BFHW	Hot water extract of <i>B. perennis</i> - flowers (water-bath)
BFCW	Cold water extract of <i>B. perennis</i> - flowers (water-bath)
BFEt	EtOH extract of <i>B. perennis</i> - flowers (water-bath)
BFMe	MeOH extract of <i>B. perennis</i> - flowers (water-bath)
BFA	Acetone extract of <i>B. perennis</i> - flowers (water-bath)
BF-dec.	Decoction extract of <i>B. perennis</i> - flowers
BF-inf.	Infusion extract of <i>B. perennis</i> - flowers
BFN	<i>n</i> -Butanol fraction of <i>B. perennis</i> - flowers
BFE	Ethyl acetate fraction of <i>B. perennis</i> - flowers
BIH	Hexane extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BID	DCM extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BIM	MeOH extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BIW	Water extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BLH	Hexane extract of <i>B. perennis</i> - leaves (soxhlet)
BLD	DCM extract of <i>B. perennis</i> - leaves (soxhlet)
BLM	MeOH extract of <i>B. perennis</i> - leaves (soxhlet)
BLW	Water extract of <i>B. perennis</i> - leaves (soxhlet)
GA ₃	Giberellic acid
GC-MS	Gas Chromatography-Mass Spectrometry
DCFH-DA	2',7'-dichlorofluorescein-diacetate
DCM	Dichloromethane
DLD-1	Human colon adenocarcinoma
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl

Abbreviations	Full Name
EtOAc	Ethyl acetate
EtOH	Ethanol
FDA	Food and Drug Administration
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LPS	Lipopolysaccharide
MCF-7	Human breast adenocarcinoma
MeOH	Methanol
MS-222	Tricaine methane sulfonate
MSMO	Murashige and Skoog Minimal Organic
M-H	Mueller-Hinton
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	Naphthalene acetic acid
<i>n</i> - BuOH	<i>n</i> -butanol
NCCLS	National Committee for Clinical Laboratory Standards
ORAC	Oxygen radical absorbing capacity
PBS	Phosphate buffered saline
RAW 264.7	Murine monocyte-macrophage
TDZ	Thidiazuron
TLC	Thin Layer Chromatography
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV-Vis	Ultraviolet visible
YEM	Yeast extract media

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Bellis perennis L. (common daisy) is a member of the cosmopolitan family Compositae (Asteraceae), which encompasses 1.100 genera and 25.000 species. The genus *Bellis* comprises about 15 European and Mediterranean species. Some of them are medicinal and some are cultivated ornamentals, especially forms of *B. perennis* (Mitich, 1997). Common daisy is a herbaceous perennial plant of meadows, lawns and other grassy areas (Panda, 2004). It is native to western, central, and northern Europe, but is commonly found as an invasive plant in North America (Davis, 1975; Tutin et al, 1976). *Bellis* species are diversely distributed in Anatolia and represented by three different species, namely *Bellis annua* L., *B. sylvestris* Cyr. and *B. perennis* L. (Davis, 1975; Kavalcıoğlu et al., 2010). *B. perennis* is the most common and well known as “koyungözü, koyun çiçeği and çayır papatyası” in Anatolia. This plant is used equivalent to chamomile. The plant is used against common cold, eczema, eye diseases, stomach ache, tonsillitis, skin boils, as a diuretic, diaphoretic and laxative in folk medicine in Turkey. The aerial parts of *B. perennis* are also used as a vegetable in Antakya in southern parts of Turkey (Kavalcioglu et al., 2010; Baytop, 1994; Baytop, 1999; Şahin and Kılıç, 2009).

1.2 Literature Review

1.2.1 Botany

B. perennis is usually a rosulate herb. Leaves are spatulate, $2-30 \times 0.5-1.5$ cm, obtuse or broadly acute at apex, rather abruptly attenuate into winged petiolar base, entire or with up to 5 shallow teeth on each side near apex, sparsely pubescent on both surfaces. Capitula is found on 1.5-10 cm of the scapes. Involucre is 0.5-1 cm broad; phyllaries are ovate-lanceolate, $3-6 \times 0.75-2.5$ mm, obtuse or acute at apex. Ray flowers are 30-50, ligules are 5-7 mm, white and often pink beneath. Disc corollas are 1.5-2 mm. Achenes are rounded at apex, $1.3-1.5 \times 0.7-0.8$ mm, weakly pubescent (Davis, 1975).

This plant is usually found in moist places, often in forests. *B. perennis* has long flowering season, with flowers being produced mainly from about March to September in Bolu. Daisy successively produces several generations of flowers. The solitary flowerheads have white to pinkish, ligulate ray-florets and yellow tubular disc-florets that close in the evening. Bees find the flowers very attractive. All parts of the plant are sparsely hairy. The fruit is an oval, downy achene without a pappus (Panda, 2004). The signature of this species is a flower head that closes at night and in wet weather (Mitich, 1997).

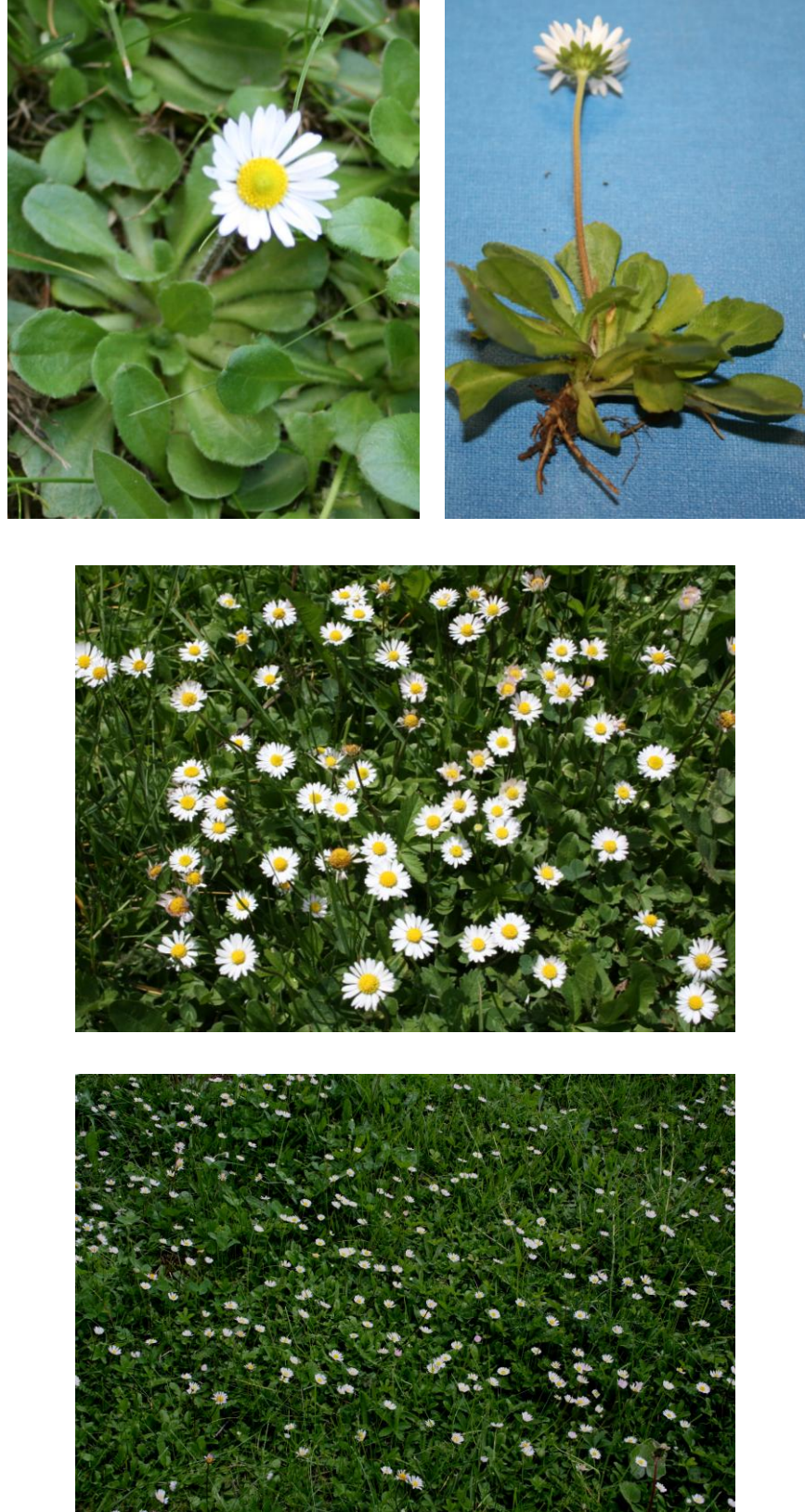


Figure 1. Pictures of *Bellis perennis* L. (by Fatma Pehlivan Karakaş, 2012)

1.2.2 History and medicinal uses of *B. perennis*

The generic name, *Bellis*, is said to come from the Latin word *bella* (= beautiful) or from a dried called *Belidis* (Panda, 2004). *Perennis* means “through the years, continuing, or perennial” (Mitich, 1997). The common name, Daisy, is derivated from the Anglo-Saxon name *daeges eage* (= day’s eye), a reference to the plant’s resemblance to a ‘small sun’ as it opens and folds mornings and closes evenings (Panda, 2004). Other common names for English daisy include daisy, dogdaisy, day’s eye, herb Margaret (because it was the emblem of Queen Margaret, wife to King Henry VI of England), bruisewort and bairnwort because children made daisy chains by slitting the stems and threading the flowers through them (Mitich, 1997). Gerard mentions the Daisy, under the name of Bruisewort, as an unfailing remedy in all kinds of paines and aches, besides curing fevers, inflammation of the liver. In 1771, Dr. Hill suggested that an infusion of the leaves was excellent against Hectic Fevers. The common daisy was an ingredient of an ointment much used in the fourteenth century for wounds, gout and fevers. The plant also good for heavy menstruation, in the form of a decoction. Chewing the fresh leaves will help to cure mouth ulcers (Higgins, 1980; Mitich, 1997). Another notion, eating three heads of daisies after having a tooth pulled will alleviate toothaches forever (Durant, 1976). Common daisy is a traditional wound herb and has been used for the treatment of bruises, broken bones and wounds by Crusaders in the middle Ages (Mitich, 1997). The daisy once had a great reputation as a cure for fresh wounds (Grieve, 1982). An ointment made from the leaves is applied externally to wounds and bruises (Grieve, 1982; Leporatti and Ivancheva, 2003; Panda, 2004; Al-Douri and Al-Essa, 2010).

In Scotland it is the bairnwort, testifying to the joy of the children in gathering it for daisy-chains. There is a common proverb associated with the flower and its abundance in spring and early summer: When you can put your foot on seven daisies, summer is coming (Grieve, 1982). Folklore maintains that if you dream of daisies in spring or summer, it's a sign of good luck, but it is a sign of bad luck to dream of them in fall or winter (Mitich, 1997).

Since the ancient times, *B. perennis* has been used as a medicinal plant for the treatment of rheumatism, diuretic (Nazaruk and Gudej, 2001), anti-inflammatory (Schöpke and Hiller, 1992; Grabias et al., 1995; Nazaruk and Gudej, 2001), vulnerary (Tava, 1996), mildly anodyne, antispasmodic, antitussive, demulcent, digestive, emollient, expectorant (Schöpke and Hiller, 1992; Nazaruk and Gudej, 2001), astringent, antipyretic, laxative, ophthalmic, purgative, tonic, catarrh, arthritis, homeostatic, liver and kidney disorders, and as a blood purifier (Hansel et al., 1992; Ozarowski et al., 1989; Grieve, 1982; Bown, 1995; Baytop, 1999; Duke, 2002; Panda, 2004). *B. perennis* has also been used in the treatment of sore throat (Uysal et al., 2010), headache (Uzun et al., 2004), common cold, stomachache, eye diseases, eczema, skin boils, gastritis, enteritis, diarrhea, bleeding, rheumatism, inflammation, and infections of the upper respiratory tract in traditional medicine in Turkey (Cakilcioglu et al., 2010). Furthermore, booster and diaphoretic properties have been recorded (Grieve, 1982; Baytop, 1999; Duke et al., 2002). The whole flowering plant has been used for bruises, bleeding, muscular pain, purulent skin diseases, and rheumatism in European folk medicine (Morikawa et al., 2008).

1.2.3 Active ingredients of *B. perennis*

Essential oils (Tava, 1996; Avato et al., 1997), several triterpenoid saponins, (Li et al., 2005; Hiller et al., 1988; Schöpke et al., 1990; Schöpke et al., 1991; Schöpke et al., 1992; Schöpke and Hiller, 1996; Schöpke et al., 1994; Glensk et al., 2001; Yoshikawa et al., 2008) and polyacetylenes (Avato and Tava 1995; Avato et al., 1997) have been isolated from the roots and flowers of *B. perennis*.

The chemical investigation of the essential oils from the aerial organs of *B. perennis* has been showed that polyacetylenes were one of the dominant classes of compounds. The major constituents, methyl deca-4,6-diynoate and deca-4,6-diynoic acid, and their structural analogues, deca-4,6-diyne, dimethyl octa-3,5-diyne-1,8-dioate and deca-4,6-diyne-1,10-dioic acid were determined (Avato et al., 1997).

The phenolic constituents of *B. perennis* include flavonoids (Nazaruk and Gudej, 2001; Gudej and Nazaruk, 1997; Nazaruk and Gudej, 2000; Toki et al., 1991), anthocyanins (three glucuronylated and malonylated cyanidin-3-glucosides) (Toki et al., 1991), tannins (Hegi, 1979), and phenolic acids (e.g., caffeic, ferulic, sinapic, p-coumaric, and salicylic acids) (Grabias et al., 1995). The following flavonoids were described in daisy flowers: quercetin, apigenin, kaempferol (Gudej and Nazaruk, 1997), isorhamnetin (Nazaruk and Gudej, 2001), apigenin-7-O- β -D-glucoside, apigenin-7-O- β -D-glucuronide (Nazaruk and Gudej, 2000), apigenin-7-O-(6'-E-caffeoyl)- β -D-glucoside, apigenin-7-O- β -D-methylglucuronide (Gudej and Nazaruk, 1997), isorhamnetin-3-O- β -D-galactoside, isorhamnetin-3-O- β -D-(6'-acetyl)-galactoside, and kaempferol-3-O- β -D-glucoside (Gudej and Nazaruk, 2001). The contents of flavonoids after acid hydrolysis were determined spectrophotometrically

according to Christ and Müller (0.25% or 1.32%, expressed as quercetin or apigenin, respectively) and by HPLC (sum of flavonoids: 0.23%; derivatives of quercetin, apigenin, kaempferol, and isorhamnetin: 0.013%, 0.10%, 0.007%, and 0.019%, respectively) (Nazaruk and Gudej, 2001; Siatka and Kasparova, 2010).

1.2.4 Biological activity studies of *B. perennis*

Wound healing activity (Karakaş et al., 2012), anxiolytic properties (Karakas et al., 2011), antitumor activity (Karakas et al., 2010), antibacterial (Avato et al., 1997; Kavalcioglu et al., 2010), antifungal (Desevedavy et al., 1989; Avato et al., 1997; Kavalcioglu et al., 2010), antihyperlipidemic (Morikawa et al., 2008; Morikawa et al., 2010a), antioxidant (Siatka and Kasparova, 2010; Kavalcioglu et al., 2010), postpartum antihemorrhagic (Oberbaum et al., 2005), pancreatic lipase inhibitor (Morikawa et al., 2010b) and cytotoxic activity against HL-60 human promyelocytic leukemia cells (Li et al., 2005), A-549 human lung cancer and DLD-1 human colon cancer cells (Karakas et al., 2011) of *B. perennis* have also been shown in some controlled laboratory studies.

1.3 Objectives of the Study

1) To develop an efficient *in vitro* plant regeneration protocol for *B. perennis* via adventitious shoot regeneration from leaf, pedicel, petiole and root explants cultured on medium containing different concentrations and combinations of various plant growth regulators.

2) To evaluate the effects of different organic solvents and extraction methods on the extraction quality; to analyze the chemical composition of a hydrodistilled essential oil of *B. perennis* flowers collected from Bolu by using Gas Chromatography-Mass Spectrometry (GC-MS) system; to analyze the phenolic and saponin constituents in field-grown flowers, leaves and *in vitro*-grown leaves by using Thin Layer Chromatography (TLC) method; to define the phenolic profile of the 22 different phenolic standards in field-grown flowers, leaves and *in vitro*-grown leaves by using Liquid Chromatography-Electrospray Tandem Mass Spectrometry (LC-MS/MS) method.

3) To screen and compare the biological activities of different extracts of *B. perennis* obtained from field-grown flowers, field-grown leaves and *in vitro*-grown leaves using specific bioassays (antibacterial, antitumor, toxicity, anticancer, anti-inflammatory and antioxidant).

CHAPTER 2

***IN VITRO* REGENERATION OF *BELLIS PERENNIS* L. (COMMON DAISY)**

2.1 Introduction

Plant tissue culture is an alternative method of commercial propagation and is being used widely for propagation of a large number of plant species including many medicinal plants (Rout et al., 2000). 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 not only allows rapid propagation or mass production of identical clones of plant species but also has the following uses: the elimination of viruses and other pathogens; the storage of essential germ plasm instead of conventional seeds; the embryo rescue; and the production of haploids by anther and ovary culture (gametoclonal variants) (Smith, 2004). *In vitro* micropropagation of medicinal plants provides pesticide free plants and produces large numbers of vegetative planting stock easily. In addition, with an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year and genetically uniform plant materials (less genetic diversity) can be produced easily that will be higher with seed germination (Turker et al., 2008). *In vitro* micropropagation can also 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).

All normal living cells within the plant body possess the potential capacity to regenerate an entire plant. This potentiality has been exploited through the culture of protoplasts, cells, tissues and organs *in vitro*. Cells and tissues, which are mitotically quiescent or already committed to some function or pathway of development, can be (re) directed into organ or embryo formation. 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). The earliest report of controlled shoot formation *in vitro* was by White (1939). In the same year, the first observation of root formation from callus was reported by Nobecourt (1939), using carrot callus. White's observation was confirmed and extended by Skoog (1944), who showed that auxins could stimulate root formation and inhibit shoot formation. A similar conclusion on the role of auxin in rooting also was made by Gautheret (1945). In addition, Skoog (1944) found that the inhibitory effect of auxin on shoot formation could be partially overcome by increasing the concentration of sucrose and inorganic phosphate in the medium. Skoog and Tsui (1948) demonstrated that adenine sulfate was active in promoting shoot formation and counteracted the inhibitory action of auxin. They further reported (Skoog and Tsui, 1951) that the extent of organ

formation was dependent on both the concentration and proportion of these additives. The discovery of kinetin (Miller et al., 1956) led to the now classic finding of Skoog and Miller (1957) that a basic regulatory mechanism underlying organogenesis involved a balance between auxin and cytokinin. Both of these substances are required for cell division and enlargement in tobacco callus, but a relatively high level of auxin to cytokinin favored root formation and the reverse favored shoot formation. These observations led them to suggest that quantitative interactions between diverse growth factors rather than specific morphogenetic substances provided a common mechanism for the regulation of all types of morphogenetic phenomena in plants that is basic concept on the regulation of organogenesis *in vitro* (Thorpe, 1980).

Christianson and Warnick (1988) established 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. Plant hormones are centrally involved in control of organogenic processes. In this manner, the destination of meristemoids can be administered by manipulation of exogenous supply of hormones or by insertion of T-DNA genes specifying synthesis of auxins or cytokinins (Hicks, 1993). Initially, in a state of organogenic competence (or which become competent after dedifferentiation), react *in vitro* to exogenous inducers, usually auxins or cytokinins or both. Furthermore, the exogeneous phytohormones can reverse the T-DNA-induced morphogenesis, suggesting that indeed the phytohormones play a direct role in organized development (Thorpe, 1990). Consequently, responding cells enter a state of determination fixing them along a

particular developmental pathway. Competence is lost and organogenesis follows (Hicks, 1993).

Biotech crops are grown commercially on a large scale and also being increasingly used for the production of vaccines and many pharmaceuticals (Vasil, 2008). The secondary metabolites of plants include such compounds as pigments and aromas which aid pollination by attracting insects to flowers, poisons which make the plant inedible, compounds which control invasion by pathogens and grazers and compounds whose function we do not understand. Many of these secondary compounds have uses beneficial to man. A large number of plant secondary metabolites are used in the food, fragrance, flavorings, cosmetics and pharmaceutical industries. Many medicines were initially isolated from natural plant sources-a gene pool that is diminishing (Burbidge, 1993). Therefore, plant tissue culture and *in vitro* protocol of plants especially medicinal plants have high value.

Micropropagation via direct or indirect organogenesis from different plant organs has been used for clonal propagation of many species, with different auxins and cytokinins used to induce organogenesis (Vasil and Thorpe, 1994). Both types of organogenesis (direct and indirect) can occur in the same culture, and callus proliferation in the cut zone is usually unavoidable (Bigot et al., 1977). De novo induction of shoot buds is commonly promoted by phytohormones, and a combination of cytokinins and auxins is usually necessary (George et al., 2008). Cytokinins play an important role in plant growth and development (Werner et al., 2001), and participate in the maintenance of meristem function and in the modulation of metabolism and morphogenesis in response to environmental stimuli (Sakakibara et al., 2006). However, appropriate plant genotype and physiological status of

explants are crucial for an organogenic response (Bianchi et al., 1999; Lin et al., 1998; Magyar-Ta'bori et al., 2010).

Common daisy is a valuable medicinal herb, but there is no study about *in vitro* propagation of this species. The main objective of this study was to establish an *in vitro* culture protocol of *B. perennis* via adventitious shoot formation from pedicel, leaf, petiole and root explants cultured on medium containing different concentrations and combinations of various plant growth regulators (PGRs).

2.2 Materials and Methods

Plant materials (leaf, pedicel, root and seed) of *B. perennis* were collected from Abant Izzet Baysal University Campus, Bolu, Turkey in May-August of 2010. Fresh field-grown plant parts (leaf, pedicel and root) were washed under running tap water for 30 min and after treating with Tween-20 (5-10 drops per 100 ml water) for 15 min, rinsed again with running tap water. Plant parts were surface sterilized by shaking in 0.1% HgCl₂ (mercuric chloride) for 10 min, rinsed well with sterile distilled water 4-5 times and then dipped into 70 % EtOH (ethanol) for 1-2 min, finally followed by washing with sterile distilled water three times.

After surface sterilization, 3 different explants (leaf, pedicel and root) were used for shoot regeneration. Leaf (36 mm² in diameter), pedicel (5-6 mm) and root (7-8 mm) explants were placed into sterile disposable petri dishes (90 × 15 mm) containing Murashige and Skoog Minimal Organics (MSMO) medium (4.43 g/l MSMO, Sigma Chemical Co., St. Louis, MO; Murashige and Skoog, 1962) with 30 g/l sucrose and 8.0 g/l Bacto-agar (Difco). Media were adjusted to pH 5.7 before being autoclaved for 20 min at 121 °C and 105 kPa. All cultures were incubated at

23 °C under a 16 h photoperiod. After a 1 week incubation on this medium, uncontaminated explants were transferred to petri plates containing MSMO with different combinations and concentrations of plant growth regulators (PGRs): benzyladenine (BA; 0.5, 1.0 or 3.0 mg/l) and indole-3-acetic acid (IAA; 0, 0.5 or 1.0 mg/l); BA (0.5, 1.0 or 3.0 mg/l) and indole-3-butyric acid (IBA; 0, 0.5 or 1.0 mg/l); thidiazuron (TDZ; 0.1, 0.25, 0.5, 1.0, 1.5 or 2.0 mg/l) and IAA (0, 0.25, 0.5 or 1.0 mg/l); BA (3.0 or 5.0 mg/l) and NAA (1.0, or 3.0 mg/l); kinetin (KIN; 1.0 or 1.5 mg/l) and naphthalene acetic acid (NAA; 1.0 mg/l).

To break the dormancy, seeds were exposed to cold at 0 and -12 °C, and again 0 °C respectively, for one week each. The treated seeds were then sterilized by immersing in 50% H₂SO₄ (sulphuric acid) for 20 minutes followed by several rinses with sterile distilled water. Then, seeds were kept in 70% EtOH for one min before being soaked in a 1% HgCl₂ for 4 min. Surface sterilized seeds were placed into sterile disposable petri dishes containing MSMO medium with or without 0.5 mg/l and 1 mg/l gibberellic acid (GA₃). In order to test regeneration efficiency, experiment was employed using different explant types including; leaf (about 36 mm² in diameter), petiole (5-6 mm) and root (7-8 mm) segments excised from regenerated plantlets (2 months old) (Figure 7). Explants were placed into MSMO medium supplemented with different combinations and concentrations of PGRs as follows: TDZ (0.05, 0.1, 0.5 or 1.0 mg/l) and IAA (0, 0.25, 0.5 or 1.0 mg/l); TDZ (0.05, 0.1, 0.5 or 1.0mg/l) and IBA (0, 0.25, 0.5 or 1.0 mg/l); BA (0.1, 0.5, 1.0 or 3.0 mg/l) and IAA (0, 0.25, 0.5 or 1.0 mg/l).

Shoot number and percentage (%) of explants producing shoots were recorded after 5 weeks from initial inoculation for all explants. The tests had 15

replications for each explants and the experiment was repeated 3 times. After 3 weeks, regenerated explants were transferred to Magenta containers (GA-7 Vessels, Sigma Chemical Co., St. Louis, MO) containing MSMO and 0.5 mg/l GA₃ for shoot elongation for an additional 2 weeks (Figure 7).

Shoots were then separated individually and placed on rooting medium containing 4.43 g/l MSMO with 30 g/l sucrose, 8.0 g/l agar (pH 5.7, autoclaved for 20 min at 121 °C and 15 psi) MSMO and varying concentrations of different auxins; IAA (0.5, 1.0 and 3.0 mg/l), IBA (0.5, 1.0 and 3.0 mg/l), naphthalene acetic acid (NAA; 0.5, 1.0 and 3.0 mg/l) or 2,4-dichlorophenoxyacetic acid (2,4-D; 0.05, 0.5 and 1.0 mg/l). After 3 weeks, the number of roots and percentage of the regenerated shoots producing roots were recorded (Figure 7). There were 20 replications and the experiment was repeated three times. Rooted shoots, namely regenerants were transferred to vermiculate (Agrekal[®]) for acclimatization in Magenta containers (Figure 7). Two weeks later, regenerants were transferred to plastic pots containing commercial potting soil (Premium[®] containing peat moss) and maintained under high humidity with subsequent transfer to the greenhouse (Figure 8).

Flow chart of the experiments is given in Figure 2. Results were expressed as means $\pm$ standard error of the mean. All data were analyzed by one way analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA).

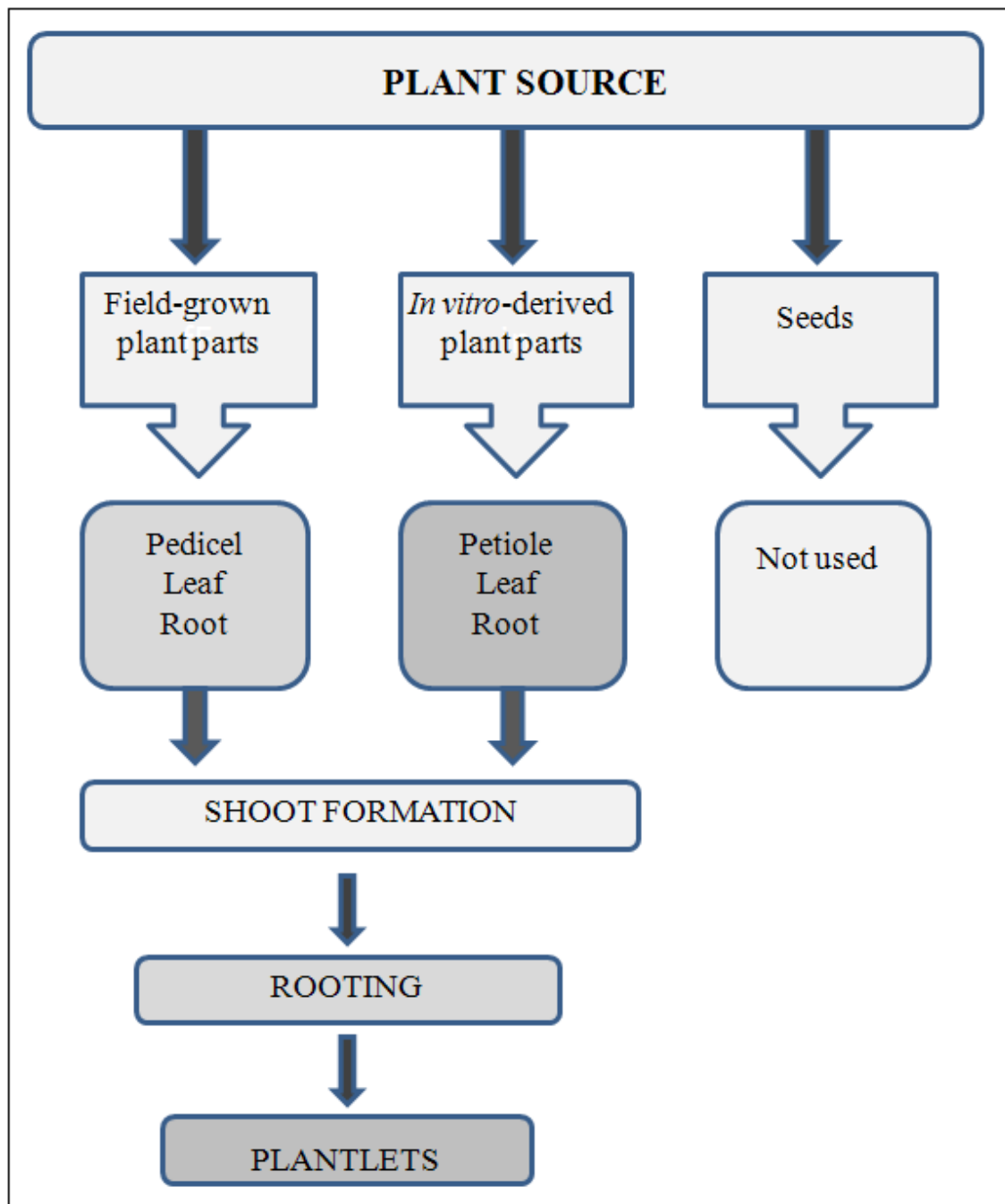


Figure 2. Protocol for *in vitro* culture of *B. perennis* (common daisy).

2.3 Results and Discussion

Although *B. perennis* is a valuable medicinal plant, there is no study about *in vitro* propagation of this plant in the literature. We, therefore, aimed to develop an *in vitro* culture protocol for high frequency regeneration of common daisy plants by adventitious organogenesis.

Our initial attempts for obtaining sterile explants from sterile seedlings were unsuccessful due to low seed viability. Germination rate of *B. perennis* seeds was low (5% seed germination). Furthermore, seeds were germinated at least in 2-3 months of incubation and obtained seedlings were weak to become a donor plant. Therefore, explants were not taken from sterile seedlings for regeneration experiments.

In this study, two different explant sources (field-grown plants and *in vitro*-grown plantlets) were compared in terms of their regeneration capacity. Firstly, the explants were obtained from field-grown plants, and then regenerated plantlets (2 months-old) were used as explant source (Figure 7). It was observed that field-grown plants were more responsive (Table 1) than *in vitro*-grown plantlets when used as explant source (Table 2).

Three different explants (leaf, pedicel and root) were obtained from field-grown plants and cultured on MSMO medium containing various concentrations of BA in combination with IAA and/or IBA or TDZ in combination with IAA. Best shoot proliferation was obtained with pedicel explants (Figure 5). Leaf explants formed less number of shoots than pedicel explants (Figure 6). Root explants did not form any shoot regeneration on any media (Table 1). Overall, number of shoots per

explants was lower for all explants on media with BA plus IAA or BA plus IBA combinations when compared to TDZ plus IAA combinations (Table 1).

Of the tested combinations, different concentrations of TDZ with IAA were the most effective for pedicel and leaf explants in shoot production. With pedicel explants, the greatest number of shoots per explant was observed on MSMO media containing 0.5 mg/l TDZ plus 0.5 mg/l IAA (20.13 ± 2.19 shoots per shooting explant; 100% explants formed shoots) (Table 1; Figure 3). With leaf explants, the best shoot proliferation was obtained with media containing 0.25 mg/l TDZ plus 1.0 mg/l IAA (12.13 ± 1.18 shoots per explant; 100% explants formed shoots) (Table 1; Figure 6). When pedicel explants were cultured on media containing 0.5 mg/l TDZ in combination with IAA, it is evident that the shoot formation and frequency were closely related to concentrations of IAA supply. For example, increasing concentration of IAA from 0.25 mg/l to 0.5 mg/l increased shoot number from 3.4 shoots to 20.13 shoots. Similarly, shoot frequency was increased from 86.6% to 100%, respectively. On the other hand, increasing concentration of IAA from 0.5 mg/l to 1.0 mg/l severely inhibited shoot development (no shoot formation was observed) (Table 1). Callus was observed in leaf and pedicel explants before shoot formation (indirect organogenesis) and microshoots appeared 15 days after culture initiation.

KIN in combination with NAA and BA in combination with NAA were also tested for shoot regeneration for all explants obtained from field-grown plants. None of the tested treatments or explant types, however, was able to produce adventitious shoots.

Three different explants (petiole, leaf and root) were obtained from *in vitro*-grown plantlets of *B. perennis* and cultured on MSMO medium containing TDZ in combination with IAA or IBA and BA in combination with IAA. Best shoot proliferation was obtained with petiole explants. Leaf explants formed less number of shoots than the petiole explants and root explants did not form any shoot regeneration on any media (Table 2). Overall, number of shoots per explants was lower for all explants on media with BA plus IAA or TDZ plus IBA combinations when compared to TDZ plus IAA combinations (Table 2). In consistent with our results, Wildi et al. (1998) demonstrated that petiole explants of *Petasites hybridus* L. (Asteraceae) had a higher morphogenetic potential than leaf explants.

Among the combinations used, TDZ with IAA were the most effective for petiole and leaf explants in shoot production. With petiole explants, the greatest number of shoots per explant was observed on media containing 0.5 mg/l TDZ plus 0.5 mg/l IAA (7.9 ± 0.98 shoots per shooting explant; 100% explants formed shoots). With leaf explants, the best shoot proliferation was obtained with media containing 0.1 mg/l TDZ plus 0.25 mg/l IAA, 0.5 mg/l TDZ plus 1.0 mg/l IAA and 0.1 mg/l TDZ plus 0.5 mg/l IBA (5.0 ± 0.44 , 4.73 ± 0.99 and 5.3 ± 0.7 shoots per shooting explant; 100%, 100%, 86.66% explants formed shoots, respectively) (Table 2). When petiole explants were cultured on media containing 0.5 mg/l TDZ in combination with IAA, it is evident that the shoot formation and frequency were closely related to concentrations of IAA supply. For example, increasing concentration of IAA from 0.25 mg/l to 0.5 mg/l increased shoot number from 4.7 shoots to 7.9 shoots. Similarly, shoot frequency was increased from 86.6% to 100%, respectively. On the other hand, increasing concentration of IAA from 0.5 mg/l to 1.0 mg/l inhibited

shoot development from 7.9 shoots to 6.1 shoots (Table 2). Callus was observed in leaf and petiole explants before shoot formation (indirect organogenesis). None of the root explants from *in vitro*-derived and field-grown plants formed shoots on any of the shoot initiation media.

Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray, 2000). Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success (Turker et al., 2008). The origin and the nature of the plant material play an important role in the success of micropropagation. In our study, when pedicel, petiole, leaf and root explants were compared, it was clear that pedicel explants were much more productive for shoot formation than leaf, petiol and root explants (Table 1 and 2). Similarly, pedicel explant was also reported as the most responsive explant in terms of shoot organogenesis with *Lycopersicon esculentum* L. (Compton and Veilleux 1991), *Phaseolus vulgaris* L. (Mohamed et al., 1993) and *Pulsatilla koreana* Nakai (Lin et al., 2011). On the other hand, Dhar and Joshi (2005) reported that leaves of *Saussurea obvallata* (DC.) Edgew. (Asteraceae) were the best plant material for adventitious shoot induction rather than roots, hypocotyls and cotyledons. Mallon et al. (2011) showed that roots of *Centaurea ultreiae* Silva Pando (Asteraceae) were more efficient than leaves as explants.

In fact, both endogenous regulators and exogeneous PGRs supplied in the culture medium determine the results obtained with *in vitro* plant cultures (Pintos et al., 2002). In the present study, when there is no auxin or TDZ was used alone, the

shoot proliferation was not observed. There is evidence that, the presence of auxins is necessary in order to promote shoot proliferation, because auxins can regulate cytokinin biosynthesis and reduce cytokinin levels in plants (Nordstrom et al., 2004). Thus, a good balance between PGRs supplied to the culture medium and endogenous levels of regulators of plant material is required (Mallon et al., 2011). 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 1 and 2). This result corroborated the finding of Yucesan et al. (2007) that TDZ was more effective than BA, KIN or zeatin for the induction of shoot regeneration from *Cichorium intybus* L. (Asteraceae). The effects of TDZ on regeneration rate of common daisy combined with IAA, supported the results confirming the promoting effect of TDZ previously observed in our laboratory for several other medicinal species, including *Digitalis davisiana* Heywood (Gurel et al., 2011), *Cichorium intybus* L. (Yucesan et al., 2007), *Achillea millefolium* L. (Turker et al. 2009a) and *Lythrum salicaria* L. (Turker et al., 2009b).

All these studies indicated that different species need different PGRs combinations and concentrations. In all cases, the regeneration pathway may be either somatic embryogenesis, shoot organogenesis, or both. TDZ is able to alter the pathway of morphogenesis from shoot organogenesis to somatic embryogenesis through a simple change in TDZ concentration (Mithila et al., 2003; Ma et al., 2011).

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. Generally, with all explant types in our study, lower concentrations of TDZ were effective for shoot regeneration (0.5 mg/l) and higher concentrations of TDZ (1.5-2 mg/l) resulted in callus formation around the cut edges of the explants and less number of microshoots. For example, with pedicel explants, 0.5 mg/l TDZ in combination with 0.5 mg/l IAA gave best shoot proliferation (20.13 shoots). But, increasing of TDZ concentration to 1 mg/l in combination with 0.5 mg/l IAA decreased shoot number to 3.8 shoots (Table 1).

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, no shoot formation was observed. But, if TDZ was combined with IAA, more shoot formation was obtained (Tables 1 and 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. Therefore, regenerated shoots were transferred to elongation medium including 0.5 mg/l GA₃ for two weeks in this study. The main effect of GA₃ in plants is to cause stem elongation and flowering (Sutter, 1996).

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.

Regenerated shoots were separated individually and cultured on MSMO medium containing different concentrations of auxins; IAA, IBA, NAA or 2,4-D. They formed roots in 3 weeks. In terms of the most efficient auxin for rooting stage, IAA was found the most effective auxin (Table 3; Figure 4). The highest number of roots per explant was observed on MSMO media containing 1.0 mg/l IAA (19.65 ± 2.55 roots per explant; 100% explants formed roots) (Figure 4). Similarly, IAA was reported as the most effective auxin for rooting with *C. intybus* belonging to Asteraceae family (Yucesan et al., 2007). When IAA concentration was increased from 0.5 to 1.0 mg/l, more root formation was observed. However, when IAA concentration was increased from 1.0 to 3.0 mg/l, no root formation was observed and frequency of rooting was decreased from 100% to zero (Figure 4). Auxin is generally considered necessary for the acquisition of the meristematic competence of the responsive cells. Once this competence has been established, excessive auxin concentrations were often found to be inhibitory for further embryonic (Charriere et al., 1999) or adventitious root (Gurel and Wren, 1995) development. Increasing concentrations of IBA did not change the root numbers. But, when IBA concentration was increased from 0.5 to 3.0 mg/l, frequency of rooting decreased from 85% down to 45%. Similar pattern was observed with NAA or 2,4-D concentrations on rooting. Although 55% of the explants formed roots at 0.5 mg/l NAA, the root formation was not observed with 3.0 mg/l NAA. All regenerants formed roots at 0.05 mg/l 2,4-D (100%), however, root formation was not observed with 1.0 mg/l 2,4-D (Table 3). In control experiments (no auxin added to the media),

none of the explants formed roots (Table 3; Figure 4).

The rooted shoots were transferred to Magenta containers including vermiculate for acclimatization (Figure 7). After 2 weeks, they were transferred to small plastic pots containing commercial soil (Figure 8). More than 90% of the regenerated plants survived through the hardening off process when the rooted shoots (regenerated plantlets) were transferred to bigger pots and kept under growth room conditions for four weeks before finally moving to room conditions at low humidity (20-30%) (Figure 9). Approximately 3 months after transferring to room condition, flowering of regenerated plants was observed (Figure 10).

The protocol that we developed for *B. perennis* has a potential for micro propagation and genetic improvement of this medicinal and ornamental species in the future. This study provides not only a rapid clonally propagation but also an excellent platform for gene transformation studies since it is known that rapid shoot bud development helps to ensure more successful and stable genetic transformation (Dandekar and Fisk, 2005). This protocol can be helpful in the large scale production of certain secondary products of *B. perennis*. Micropropagation of *B. perennis* 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.

The present study is the first to describe an efficient *in vitro* plant regeneration protocol for *B. perennis* through adventitious shoot regeneration of pedicel explants from field-grown plants. It is believed that the protocol will have an important contribution to the future efforts for a large scale production of certain

secondary metabolites from common daisy plants and can provide plant material for future pharmacological, physiological and biochemical studies.

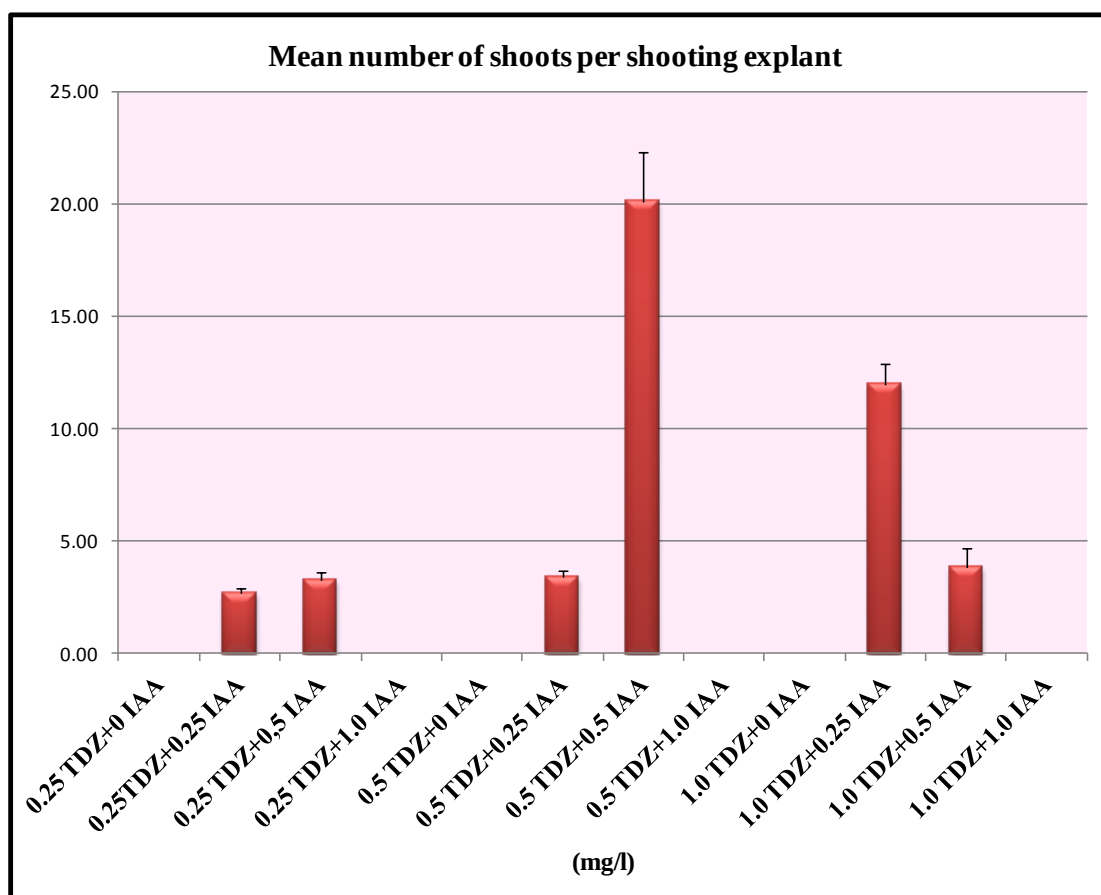


Figure 3. Mean number of shoots per shooting pedicel explant obtained from field-grown plants when cultured on MSMO medium containing different combinations of TDZ with IAA.

Table 1. Shoot regeneration from leaf, pedicel and root explants obtained from field-grown plants cultured on MSMO medium containing different combinations of TDZ or BA with IAA or IBA.

Treatments		EXPLANTS					
		Leaf		Pedicel		Root	
		Mean number of shoots per shooting explant ($\pm$ SE)	% explants forming shoots	Mean number of shoots per shooting explant ($\pm$ SE)	% explants forming shoots	Mean number of shoots per shooting explant ($\pm$ SE)	% explants forming shoots
Control		-	-	-	-	-	-
TDZ (mg/l) IAA (mg/l)							
0.1	0.0	-	-	-	-	-	-
0.1	0.25	-	-	-	-	-	-
0.1	0.5	-	-	-	-	-	-
0.1	1.0	-	-	-	-	-	-
0.25	0.0	-	-	-	-	-	-
0.25	0.25	-	-	2.66 $\pm$ 0.21 ^f	60	-	-
0.25	0.5	-	-	3.26 $\pm$ 0.33 ^{ef}	86.6	-	-
0.25	1.0	12.13 $\pm$ 1.18 ^a	100	-	-	-	-
0.5	0.0	-	-	-	-	-	-
0.5	0.25	-	-	3.4 $\pm$ 0.27 ^{ef}	86.6	-	-
0.5	0.5	8.13 $\pm$ 0.47 ^c	86.6	20.13 $\pm$ 2.19 ^a	100	-	-
0.5	1.0	9.53 $\pm$ 1.19 ^b	100	-	-	-	-
1.0	0.0	-	-	-	-	-	-
1.0	0.25	5.06 $\pm$ 0.46 ^{def}	80	11.93 $\pm$ 0.96 ^b	100	-	-
1.0	0.5	-	-	3.8 $\pm$ 0.87 ^{ef}	86.6	-	-
1.0	1.0	-	-	-	-	-	-
1.5	1.0	-	-	-	-	-	-
2.0	0.5	-	-	-	-	-	-
BA (mg/l) IAA (mg/l)							
0.5	0.0	-	-	-	-	-	-
0.5	0.5	-	-	3.2 $\pm$ 0.26 ^{ef}	66.6	-	-
0.5	1.0	-	-	13.26 $\pm$ 1.88 ^b	60	-	-
1.0	0.0	-	-	-	-	-	-
1.0	0.5	-	-	4.33 $\pm$ 0.93 ^{ef}	73.33	-	-
1.0	1.0	-	-	5.2 $\pm$ 0.85 ^{de}	60	-	-
3.0	0.0	-	-	-	-	-	-
3.0	0.5	-	-	2.6 $\pm$ 0.52 ^f	20	-	-
3.0	1.0	5.53 $\pm$ 0.49 ^{de}	20	7.93 $\pm$ 1.2 ^c	73.33	-	-
BA (mg/l) IBA (mg/l)							
0.5	0.0	-	-	-	-	-	-
0.5	0.5	3.07 $\pm$ 0.33 ^g	60	4.26 $\pm$ 0.62 ^{ef}	86.6	-	-
0.5	1.0	4.27 $\pm$ 0.45 ^f	60	7.4 $\pm$ 1.02 ^c	60	-	-
1.0	0.0	-	-	-	-	-	-
1.0	0.5	4.66 $\pm$ 0.52 ^{ef}	46.6	5.06 $\pm$ 0.63 ^{de}	80	-	-
1.0	1.0	5.86 $\pm$ 0.32 ^d	40	6.6 $\pm$ 1.02 ^{cd}	60	-	-
3.0	0.0	-	-	-	-	-	-
3.0	0.5	1.86 $\pm$ 0.3 ^h	46.6	6.86 $\pm$ 0.81 ^{cd}	60	-	-
3.0	1.0	1.53 $\pm$ 0.64 ^h	26.6	3.8 $\pm$ 0.61 ^{ef}	100	-	-

^a ,^b ,^c ,^d ,^e ,^f ,^g Mean-values with the same letters within vertical columns are not significantly different ($P > 0.05$) (i.e., comparison of growth regulator combinations for each explants). Control means that no PGRs were added to the MSMO medium.

Table 2. Shoot regeneration from leaf, petiole and root explants obtained from *in vitro*-grown regenerants cultured on MSMO medium containing different combinations of TDZ or BA with IAA or IBA.

Treatments	EXPLANTS					
	Leaf		Petiole		Root	
	Mean # of shoots per shooting explants	% explants forming shoots	Mean # of shoots per shooting explants	% explants forming shoots	Mean # of shoots per shooting explants	% explants forming shoots
Control	-	-	-	-	-	-
TDZ (mg/l) IAA (mg/l)						
0.05 0.0	-	-	-	-	-	-
0.05 0.25	1.2 ± 0.1 ^{hi}	13	2.6 ± 0.3 ^{hij}	53	-	-
0.05 0.5	2.6 ± 0.46 ^{defg}	73	2.1 ± 0.28 ^{hij}	60	-	-
0.05 1.0	1.9 ± 0.3 ^{efgh}	73	2.3 ± 0.34 ^{hij}	60	-	-
0.1 0.0	-	-	-	-	-	-
0.1 0.25	5.0 ± 0.44 ^a	100	6.6 ± 1.1 ^b	86.66	-	-
0.1 0.5	2.0 ± 0.33 ^{efgh}	53	5.1 ± 0.64 ^{cd}	60	-	-
0.1 1.0	1.73 ± 0.31 ^{fgh}	66.66	4.4 ± 0.6 ^{def}	100	-	-
0.5 0.0	-	-	-	-	-	-
0.5 0.25	4.9 ± 0.56 ^{ab}	73.33	4.7 ± 0.93 ^d	86.66	-	-
0.5 0.50	2.5 ± 0.42 ^{defg}	53	7.9 ± 0.98 ^a	100	-	-
0.5 1.0	4.73 ± 0.99 ^{ab}	100	6.1 ± 0.72 ^c	100	-	-
1.0 0.0	-	-	-	-	-	-
1.0 0.25	3.9 ± 0.84 ^{bc}	73	4.4 ± 0.57 ^{def}	100	-	-
1.0 0.5	-	-	-	-	-	-
1.0 1.0	3.3 ± 0.33 ^{cd}	60	5.5 ± 0.66 ^{bcd}	87	-	-
TDZ (mg/l) IBA (mg/l)						
0.05 0.0	-	-	-	-	-	-
0.05 0.25	1.9 ± 0.16 ^{efgh}	53.3	3.3 ± 0.64 ^{efgh}	47	-	-
0.05 0.5	2.4 ± 0.32 ^{defg}	80	2.7 ± 0.12 ^{hij}	47	-	-
0.05 1.0	3.0 ± 0.56 ^{cde}	53	4.4 ± 0.32 ^{def}	60	-	-
0.1 0.0	-	-	-	-	-	-
0.1 0.25	2.7 ± 0.4 ^{defg}	100.0	4.3 ± 0.57 ^{efg}	100	-	-
0.1 0.5	5.3 ± 0.7 ^a	87	4.5 ± 0.66 ^{de}	93	-	-
0.1 1.0	2.7 ± 0.20 ^{defg}	73	4.6 ± 0.34 ^{de}	80	-	-
0.5 0.0	-	-	-	-	-	-
0.5 0.25	2.9 ± 0.65 ^{cde}	87	3.1 ± 0.4 ^{ghij}	73.33	-	-
0.5 0.5	1.9 ± 0.21 ^{efgh}	73	3.2 ± 0.31 ^{fghi}	80	-	-
0.5 1.0	-	-	2.9 ± 0.32 ^{hij}	20	-	-
1.0 0.0	-	-	-	-	-	-
1.0 0.25	2.8 ± 0.34 ^{def}	73.33	3.1 ± 0.33 ^{ghij}	46.66	-	-
1.0 0.5	3.5 ± 0.42 ^{cd}	73	5.5 ± 0.51 ^{bcd}	60	-	-
1.0 1.0	-	-	-	-	-	-
BA (mg/l) IAA (mg/l)						
0.1 0.0	-	-	-	-	-	-
0.1 0.25	-	-	-	-	-	-
0.1 0.5	-	-	-	-	-	-
0.1 1.0	-	-	-	-	-	-
0.5 0.0	-	-	-	-	-	-
0.5 0.25	-	-	-	-	-	-
0.5 0.5	2.1 ± 0.33 ^{efgh}	27	3.1 ± 0.5 ^{fghij}	13	-	-
0.5 1.0	5.1 ± 0.74 ^a	47	5.27 ± 0.6 ^{cd}	67	-	-
1.0 0.0	-	-	-	-	-	-
1.0 0.25	-	-	-	-	-	-
1.0 0.5	3.3 ± 0.34 ^{cd}	40	2.2 ± 0.2 ^{hij}	27	-	-
1.0 1.0	1.6 ± 0.21 ^{gh}	33.33	1.8 ± 0.4 ^j	27	-	-
3.0 0.0	-	-	-	-	-	-
3.0 0.25	-	-	-	-	-	-
3.0 0.5	2.47 ± 0.23 ^{defg}	33.33	1.93 ± 0.3 ^{ij}	6.66	-	-
3.0 1.0	0.2 ± 0.8 ⁱ	6.66	-	-	-	-

a,b,c,d,e,f,g,h,i,j Mean-values with the same letters within vertical columns are not significantly different ($P > 0.05$) (i.e., comparison of growth regulator combinations for each explants). Control means that no PGRs were added to the MSMO medium.

Table 3. Effects of the auxins tested on root formation from regenerated shoots.

Treatments	Mean number of roots per shoot ($\pm$ SE)	% explants forming roots
Control	0 $\pm$ 0	0
IAA (mg/l)		
0.5	14.70 $\pm$ 1.87 ^b	100
1	19.65 $\pm$ 2.55 ^a	100
3	0 $\pm$ 0	0
IBA (mg/l)		
0.5	5.1 $\pm$ 1.12 ^{cd}	85
1	3.35 $\pm$ 0.99 ^{cde}	55
3	4.90 $\pm$ 1.81 ^{cd}	45
NAA (mg/l)		
0.5	4.5 $\pm$ 1.17 ^{cd}	55
1	3.60 $\pm$ 1.17 ^{cde}	45
3	0.00 $\pm$ 0	0
2,4-D (mg/l)		
0.05	7.15 $\pm$ 0.77 ^c	100
0.5	1.65 $\pm$ 0.35 ^{de}	60
1	0 $\pm$ 0	0

a, b, c, d, e Mean-values with the same letters within vertical columns are not significantly different ($P > 0.05$). Control means that no auxin was added to the medium.

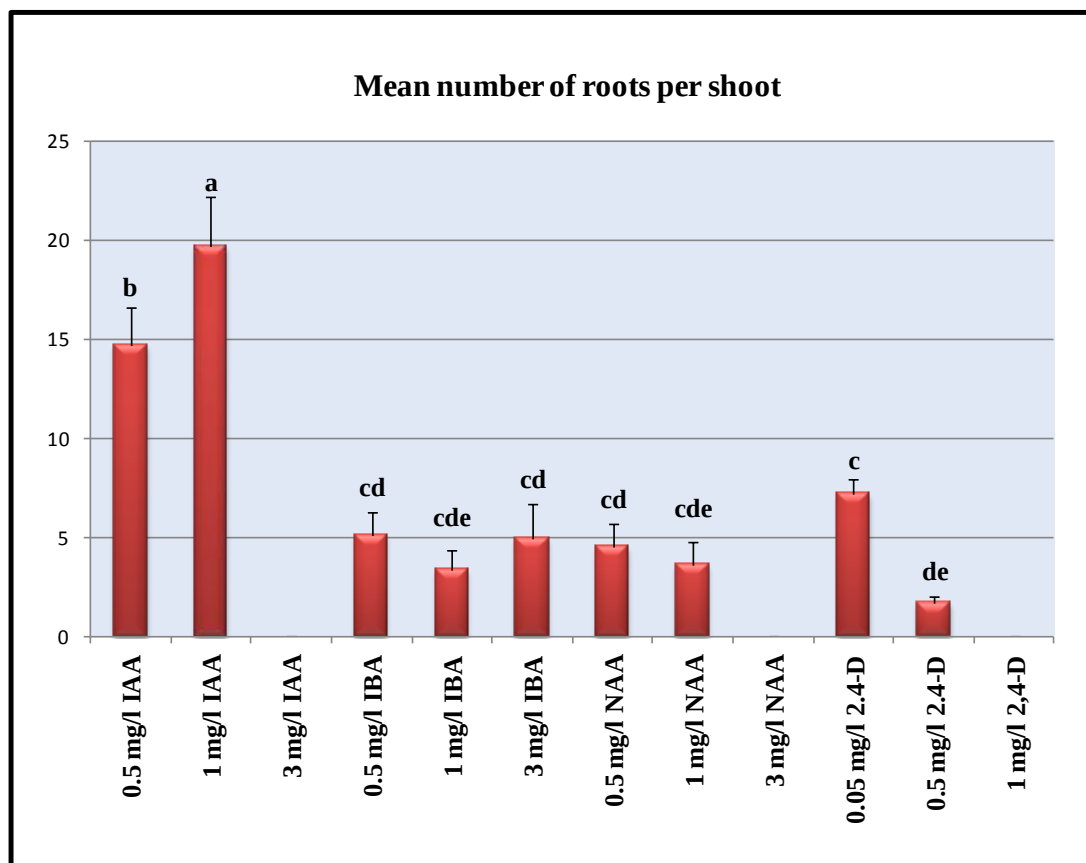


Figure 4. Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots.



Figure 5. Shoot formation from pedicel explants on MSMO medium containing 0.5 mg/l TDZ + 0.5 mg/l IAA after 3-4 weeks in culture.

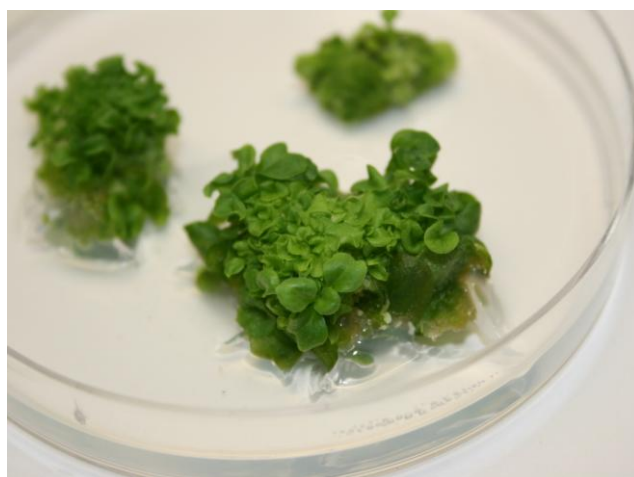


Figure 6. Shoot formation from leaf explant on MSMO medium containing 0.25 mg/l TDZ + 1.0 mg/l IAA after 3 weeks in culture.



Figure 7. Shoot elongation of regenerated shoots on MSMO medium containing 0.5 mg/l GA₃ (left). Rooting of the regenerated shoots on medium containing 1.0 mg/l IAA after 3 weeks in culture (middle). Regenerated plants in Magenta containers including vermiculate for acclimatization (right).



Figure 8. Regenerated plants transferred to foam cups containing sterile potting soil under high humidity conditions (left). Regenerated plants transferred to under growth room conditions (right).



Figure 9. Regenerated plants transferred to big plastic pots containing sterile potting soil under growth room conditions.



Figure 10. Flowering of a regenerated plant after transferring to room conditions.

CHAPTER 3

EXTRACTION AND CHEMICAL ANALYSES OF *B. PERENNIS* L.

3.1 Introduction

The plant kingdom has more abundant diversities in chemical structures with extraordinary potent pharmacological activities and rather low toxicities. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008). However, there is only very limited knowledge about their chemical compositions, pharmacokinetics, and metabolisms. This is mainly due to the fact that plants consist of multi-component systems with mostly unknown ingredients. Investigation of plants in folk medicine and their applications in biochemical, pharmaceutical and clinical researches are important.

Nowadays, bioactive compounds with potential health benefits have attracted great interest for use in several industrial areas, and researches on this topic have been strongly encouraged. Extraction is the first step in the isolation of bioactive compounds from natural sources (plant materials). Among the variety of techniques

that can be used for this purpose, solid–liquid extraction has been widely employed to extract bioactive compounds from plant materials and agro-industrial residues (Mussatto et al., 2011). However, the efficiency of this extraction process is greatly affected by the type of solvent and its concentration (Mussatto et al., 2011). Therefore, studies to define the best conditions for these variables are necessary to maximize the extraction yields to each different plant material.

Currently, conventional extraction techniques such as maceration and Soxhlet extraction have been used along with several novel extraction techniques, such as ultrasonic extraction, accelerated solvent extraction and microwave-assisted extraction, to accelerate the extraction of bioactive compounds from plants (Wang and Weller, 2006). Serial exhaustive extraction is another common method of extraction which involves successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. Some researchers employ soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Das et al., 2010). Several studies have been published regarding the variation in the biological activities of extracts obtained from different extraction techniques, emphasizing the importance of selecting the suitable extraction method (Hayouni et al., 2007).

Essential oils are volatile natural complex secondary metabolites characterized by a strong odor and have a generally lower density than that of water (Bakkali et al., 2008). There are 17,500 aromatic plant species (Bruneton, 1999) among higher plants and approximately 3,000 essential oils are known out of which

300 are commercially important for pharmaceuticals, cosmetics and perfume industries (Bakkali et al., 2008) apart from pesticidal potential (Chang and Cheng, 2002). Therefore, essential oils and extracts obtained from many plants have recently gained popularity and scientific interest (Tepe et al., 2005). The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. Major categories of essential oil are terpenoids (monoterpenes, sesquiterpenes and diterpenes) and phenylpropanoids. Terpenoid oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. Phenylpropanoids contain a benzene ring structure with an attached propane side chain (Cowan, 1999; Pengelly, 2004).

Essential oils are obtained from plant material parts such as flowers, buds, seeds, leaves, twigs, bark, wood, fruits, and roots. They can be accumulated by expression, fermentation, enfleurage or extraction but the method of steam distillation is mostly used for commercial production of essential oils (Burt, 2004). That being said, it has generally been the essential oil of plants rather than their extracts that have had the greatest use in the treatment of infectious pathologies in some vital systems, and as well as on the skin (Rios and Recio, 2005). For example, the composition of the essential oil isolated from aerial parts of *Achillea millefolium* (Asteraceae) has been a subject of extensive studies, mainly due to its medicinal properties and taxonomic value (Lourenço et al., 1999).

Phenolic compounds are one of the largest groups of secondary metabolites, consisting of four main groups divided according to the number of phenol rings and the structural elements that bind those rings, including flavonoids (e.g. anthocyanins, flavones, isoflavones), phenolic acids, tannins, stilbenes and lignans (Balasundram et al., 2006).

Flavonoids are a class of polyphenolic compounds which display a variety of biologic activities. They are derived from flavanone, include more than 4000 chemical structures and are widely distributed in the plant kingdom. They are a group of polyphenolic phytochemicals that include flavones, isoflavones, (iso) flavanones, catechins, and chalcones among other chemicals. They occur in relatively high concentrations in fruits, vegetables, nuts and grains, and in various herbs and spices. Examples are the flavanones naringenin and sakuranetin in rice (Kodama, 1996), the isoflavonoids kievitone and phaseollin in beans (Liu et al., 1995) and the catechins epicatechin and epigallocatechin in green tea (Ishikawa et al., 1997). Catechins and flavan-3,4-diols can polymerize to polyflavonoids called condensed tannins. Flavonoles are the most abundant flavonoids. The concentration of quercetin, the most important flavonole, is <10 mg/kg in the edible parts of most vegetables (Hertog et al., 1992). Like most other flavonoids, quercetin in plant tissue is found mainly in its glycosidic form, e.g., as rutin. Flavonoids are known to have widely diverse beneficial biological effects, such as anti-inflammatory (Middleton, 1998), antioxidant (Pietta, 2000), antiviral (Jassim and Naji, 2003), and anticancer (Adlercreutz, 2002; Rietveld and Wiseman, 2003).

Saponins, an important group of bioactive plant natural products, are glycosides of triterpenoid or steroidal aglycones (sapogenins) (Vincken et al., 2007). They are generally characterized by their bitter taste, foaming in aqueous solutions, and their ability to hemolyze red blood cells. The saponins are highly toxic to cold-blooded animals, their toxicity being related to their activity in lowering surface tension. Upon complete hydrolysis they yield sapogenins (aglycones), which are either steroids (C₂₇) or triterpenoids (C₃₀), and sugars (hexoses, pentoses, and

saccharic acids) (Birk and Peri, 1980; Bruneton, 1995). Saponins possess many biological activities, including conferring potential health benefits for humans. Interest in plant natural products has recently increased due to the realization of their importance for animal and human health. The name saponin is derived from the Latin word “sapo,” indicating that the plant contains a frothing agent when its extract is mixed with water. The foaming ability of saponins is caused by the combination of the hydrophobic sapogenin with the hydrophilic sugar substituent(s). Triterpenoid saponins are widely found in the Leguminosae (Suzuki et al., 2005). Their biological activities can positively or negatively impact plant traits (Dixon and Sumner, 2003). For example, saponins confer protective functions to the plant due to their antimicrobial, antifungal and anti-insect (Tava and Odoardi, 1996; Osbourn, 2003) but can be toxic to monogastric animals and reduce forage digestibility in ruminants (Oleszek et al., 1999). They also have potential health benefits for humans, and plants containing saponins have long been used in traditional medicine. Recent studies have illustrated useful pharmacological properties of saponins, including anticholesterolemic, anticancer (Haridas et al., 2001; Chen et al., 2005), anti-inflammatory, antimicrobial, immunostimulant, and antioxidant (Francis et al., 2002; Güçlü-Ustündağ and Mazza, 2007) activities.

A variety of separation techniques have been employed for quantitative determination of active components from medicinal plants in various matrices. These techniques include thin-layer chromatography (TLC), gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS), high performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS), capillary electrophoresis (CE), capillary electrophoresis coupled to laser-induced

fluorescence (CE-LIF), etc. As an important group of plants secondary metabolites, essential oils have recently attracted increasing attention. The essential oils are usually quite complex mixtures. It is very difficult to analyze such complex systems until proper baseline separation conditions are achieved, which is usually not an easy job. Fortunately, modern analytical chromatographic instruments and associated high resolution techniques are great tools in the solution of this kind of problems. GC-MS had been the most commonly used technique for the analyses of liposoluble constituents, especially volatile/semi-volatile compounds, and their metabolites in biological fluids due to its high resolution, selectivity and sensitivity. For routine analysis of volatile organics, high-resolution GC directly combined with a wide range of low-cost bench-top MS instruments remains the most attractive technique. Characteristic changes in chemical structure variations due to the toasting process were observed. Although, there are morphological differences, qualitative variations in chemical compositions as a result of different products were found generally consistent with some common characteristics both in the retention times and in the constitution of components. The results suggest that the fingerprints obtained by GC-MS can provide useful information for further interpretation of quality changes due to toasting process (Ye, 2009). GC-MS is a simple, sensitive and low-cost method for determination constituents of medicinal plants. Hence, this method was used in this research.

TLC bioautography is an ideal, convenient and simple method for the rapid detection (Gu et al., 2009; Soberon et al., 2010). Herbal drug standardization is an important area which is gaining more attention because of the indiscriminate use of herbal medicine. With regard to this, chromatographic fingerprint profiles have been

shown to be very simple, more convenient and effective for the quality assessment of herbal medicine (Chen et al., 2006).

Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). It has been shown that the LC-MS/MS (high-performance liquid chromatography coupled with tandem mass spectrometry), technology can provide a method to characterize and quantify complex herbal medicines, such as the ginsenosides, and the method greatly improves the accuracy and speed of the analysis when compared to the conventional HPLC method (Wang et al., 1999).

The objectives of this study are: i) to evaluate the effects of different organic solvents on the extraction process; ii) to analyze the chemical composition of a hydrodistilled essential oil of *B. perennis* collected from Bolu by a GC/MS system; iii) to analyze the phenolic and saponin constituents in field-grown flowers, leaves and *in vitro*-grown leaves by TLC method; iv) to define the phenolic profile of the 22 different phenolic standards in field-grown flowers, leaves and *in vitro*-grown leaves by LC-MS/MS method.

3.2 Materials and Methods

3.2.1 Plant material and extract preparation

Aerial parts (flowers and leaves) of field-grown *B. perennis* were collected from Gölköy, Bolu/Turkey in May-August of 2009–2010. *In vitro*-derived leaves of *B. perennis* were collected from *in vitro*-cultured common daisy plants that were previously micropropagated in our laboratory.

Three different sources of plant (field-grown flowers, field-grown leaves and *in vitro*-derived leaves) were dried at room temperature (Figure 11) and then grounded. Five different types of extraction methods (soxhlet, water bath, decoction, infusion and liquid-liquid extraction) were performed for flowers and soxhlet extraction method was used for field-grown leaves and *in vitro*-derived leaves (Figure 12).

3.2.1.1 Soxhlet extraction

Three different plant materials [field-grown flowers (70 g), field-grown leaves (70 g) and *in vitro*-derived leaves (55 g)] were used for soxhlet extraction method. The air-dried (1 week) powdered plant parts from *B. perennis* were successively extracted with hexane (at 65-70 °C), dichloromethane (DCM) (at 55-60 °C), methanol (MeOH) (at 60 °C) and water (at 80 °C) (from non-polar compounds to polar compounds) using a Soxhlet apparatus (700 mL each solvent for 24 hours). The extracts were filtered and pooled. Hexane, dichloromethane and methanol were evaporated under low pressure at a temperature not higher than 45 °C using rotary

evaporator (Figure 13). Aqueous extracts were evaporated using a lyophilizator at -65 °C.

3.2.1.2 Water bath extraction

The air-dried powdered flowers of *B. perennis* (20 g) were extracted with hot water (250 ml), cold water (250 ml), ethanol (EtOH) (200 ml), methanol (200 ml) or acetone (200 ml) in water bath at 45 °C for 12 hours and then filtered. Methanol, ethanol and acetone were evaporated under low pressure at a temperature not higher than 45 °C using rotary evaporator (Figure 13). Aqueous extracts were evaporated using a lyophilizator at -65 °C.

3.2.1.3 Decoction

The air-dried powdered flowers of *B. perennis* (10 g) were boiled in 100 ml of distilled water for one hour and the obtained decoction was filtered. The same procedure was repeated three times and the results of the three successive extractions were collected and combined.

3.2.1.4 Infusion

The boiling 100 ml of distilled water was added to 10 g of powdered flowers of *B. perennis* and mixed for one hour at room temperature, the resulting infusion was filtered and the procedure was repeated three times as described above. Crude aqueous extracts were lyophilized.

3.2.1.5 Liquid-liquid extraction

The air-dried and powdered flowers of *B. perennis* (400 g) were exhaustively extracted with MeOH (3 L, 60 °C, three times, 1.5 h each time) followed by MeOH-H₂O 80: 20 with heating. The extracts were filtered and pooled. After evaporation of MeOH in vacuo, the aqueous phase was extracted successively with dichloromethane (3 × 500 ml), ethyl acetate (EtOAc) (5 × 500 ml) and saturated *n*- butanol (*n*-BuOH) with H₂O (60: 40) (5 × 500ml) by using liquid-liquid extraction method (Figure 14). The *n*- BuOH and EtOAc phases were decanted, evaporated in vacuo and the residue lyophilized.

For biological activity assays (antibacterial, antitumor, toxicity, anticancer, cytotoxicity, anti-inflammatory and antioxidant), each residue were dissolved in sterile dimethyl sulfoxide (DMSO) or sterile distilled water in order to obtain a final concentration of 100 mg/ml. Plant materials and brief extraction procedures were shown in Table 4. Designation and name of the studied extracts from *B. perennis* were also shown in Table 5.



Figure 11. Plant drying process of field-grown flowers and field-grown leaves from *B. perennis*.



Figure 12. Soxhlet extraction model.



Figure 13. Rotary evaporatory.



Figure 14. Liquid- liquid extraction model.

Table 4. Designation of studied plant extracts, used parts, used extraction methods, used solvents and yields.

Plant Species	Part Used	Extraction	Heating	Solvent	Designation	Yield (%)*	
<i>Bellis perennis</i> L. (Common daisy)	Flowers (field- grown)	soxhlet (24h)	65-70 °C	Hexane	BFH	3.85	
			55-60 °C	Dichloromethane	BFD	2.52	
			60-65 °C	MeOH	BFM	29.85	
			80 °C	Water	BFW	21.77	
						Total yield	57.99
		water bath (12h)	45 °C	Hot water	BFHW	29.1	
			45 °C	Cold water	BFCW	11.5	
			45 °C	Ethanol	BFEt	22.5	
			45 °C	Methanol	BFMe	27.8	
			45 °C	Acetone	BFA	9.15	
	decoction (3×1h)	100 °C	Water	BF-dec.	30.9		
		infusion (3×1h)	100 °C	Water	BF-inf.	25.4	
			liquid-liquid	in room temperature	<i>n</i> - Butanol	BFN	6.25
					Ethyl acetate	BFE	2.62
	Leaves (field- grown)	soxhlet (24h)	65-70 °C	Hexane	BLH	2.18	
			55-60 °C	Dichloromethane	BLD	0.98	
			60-65 °C	MeOH	BLM	23.5	
			80 °C	Water	BLW	18.28	
						Total yield	44.94
	Leaves (<i>in vitro</i> - grown)	soxhlet (24h)	65-70 °C	Hexane	BIH	2.98	
			55-60 °C	Dichloromethane	BID	1.76	
			60-65 °C	MeOH	BIM	11.32	
80 °C			Water	BIW	7.6		
				Total yield	23.66		

*Yield (%) = Weight of extract (g) / powdered plant sample (g) × 100

Table 5. Designation and name of the studied *Bellis perennis* extracts.

Designation of the extracts	Name of the extracts
BFH	Hexane extract of <i>B. perennis</i> - flowers (soxhlet)
bfd	DCM extract of <i>B. perennis</i> - flowers (soxhlet)
BFM	MeOH extract of <i>B. perennis</i> - flowers (soxhlet)
BFW	Water extract of <i>B. perennis</i> - flowers (soxhlet)
BFHW	Hot water extract of <i>B. perennis</i> - flowers (water-bath)
BFCW	Cold water extract of <i>B. perennis</i> - flowers (water-bath)
BFEt	EtOH extract of <i>B. perennis</i> - flowers (water-bath)
BFMe	MeOH extract of <i>B. perennis</i> - flowers (water-bath)
BFA	Acetone extract of <i>B. perennis</i> - flowers (water-bath)
BF-dec.	Decoction extract of <i>B. perennis</i> - flowers
BF-inf.	Infusion extract of <i>B. perennis</i> - flowers
BFN	<i>n</i> -BuOH fraction of <i>B. perennis</i> - flowers
BFE	Ethyl acetate fraction of <i>B. perennis</i> - flowers
BLH	Hexane extract of <i>B. perennis</i> - leaves (soxhlet)
BLD	DCM extract of <i>B. perennis</i> - leaves (soxhlet)
BLM	MeOH extract of <i>B. perennis</i> - leaves (soxhlet)
BLW	Water extract of <i>B. perennis</i> - leaves (soxhlet)
BIH	Hexane extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BID	DCM extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BIM	MeOH extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)
BIW	Water extract of <i>B. perennis</i> - <i>in vitro</i> (soxhlet)

3.2.2 GC-MS analysis

3.2.2.1 Steam distillation

Field-grown flowers of *B. perennis* were powdered. For the dried material was weighted as 100 g. Then, it was steam distilled for 3 h using a Clevenger-type apparatus. In this way, the essential oils were isolated. The essential oils were accumulated with hexane solvent and stored at -20°C before analysis. The oil samples isolated by hydrodistillation were used to estimate the oil yields.

3.2.2.2 Gas chromatography

Gas chromatography (GC) analysis was carried out on a Hewlett Packard 6890 N GC instrument, fitted with a HP 5MS 30 m×0.25 mm×0.25 µm film thickness capillary column and FID detector. The column temperature was programmed from 50 °C to 150 °C at an initial rate of 3 °C/min. The injector and detector temperatures were programmed at 220 °C and 290 °C, respectively. Helium was used as the carrier gas at a flow rate 1 ml/min. The gas chromatography-mass spectrometry (GC/MS) analyses were performed using a Hewlett Packard 5973 (mass selective detector)-6890 GC/MS system operating in the electron ionization system with ionization energy of 70 eV (equipped with a HP 5MS 30 m × 0.25 mm × 0.25 µm film thickness capillary column), using He (1 ml/min) as the carrier gas. The initial temperature of the column was 50 °C and then heated gradually to 150 °C with a 3 °C/min rate, held for 10 min and finally raised to 250 °C/min. Diluted samples (1/200 in hexane, v/v) of 1.0 µl were injected automatically and in the splitless mode. The identification of chemical compounds obtained from our study was performed by matching their retention times and mass spectra with those obtained from the

Flavor2.L, Wiley7n.1 and NIST98.L spectral and literature data (Sahin et al., 2004). The results were expressed as FID response area in relative percentages. Each reported result is given as the average value of three GC analyses. The results are presented as means.

3.2.3 Qualitative analysis of phenolics and saponins by TLC

Thin layer chromatography (TLC) was performed on TLC plates (20 × 20 cm silica gel 60W, Merck, Germany) after being loaded 25 µl of each sample. TLC was run in a glass chamber containing mobile phase as follows chloroform: methanol (25:1) for hexane and DCM extracts. TLC was run in a glass chamber containing mobile phase as follows chloroform: metanol: water (26: 14: 3) for methanol and water extracts. After 10-25 min, the plates were taken out from the chamber and sprayed with natural products-polyethylene glycol reagent (NP/PEG) to identify the phenolic groups or sprayed with 20% sulphuric acid (H₂SO₄) in MeOH to identify the terpenoids. The plate was heated at 100 °C for 3-5 min, then the plate was evaluated in visible light for terpenoids. The plate was heated at 100 °C for 1-2 min, then the plate was evaluated in 254 and 365 nm UV light for phenolic compounds.

3.2.4 LC-MS/MS analysis of the selected phenolics

The Agilent 6460 series liquid chromatograph, consisting of vacuum degasser, binary pump (Agilent BinPump-SL, G1312B9), autosampler (Agilent h-ALS-SL+, G1367D) and thermostated column compartment (Agilent G1316B 1200 series) was used for separation of all analyses, whose detection was carried out by means of Agilent series 6410B triple-quadrupole mass spectrometer with

electrospray ionization (ESI). MassHunter optimizer software (Agilent G3793AA) was used for instruments control and data analysis.

The injection volume for all samples was 5 μ l. The binary mobile phase consisted of 0.05% formic acid + 5 mM ammonium formate (milliQ water) (A) and methanol (MS grade, Merck) (B) and was delivered at a flow rate of 0.3 ml/min. Gradient elution was performed. The eluate was forwarded, without flowsplitting, into an ESI ion source with following settings: drying gas (N_2) temperature, 350 $^{\circ}$ C; flow, 10 ml/min; nebulizer gas pressure, 40 psi; capillary voltage, 4 kV, negative polarity. All compounds were quantified in dynamic MRM mode (multiple reaction monitoring mode).

Extracts used for LC-MS/MS quantification were dissolved in starting methanol (solvent B) to the concentration of 50 mg/ml. All used standards were dissolved in methanol to prepare stock solutions of 1 mg/ml. The mix of stock solutions was prepared, with concentration of each compound being 25 μ g/ml. The mix was subsequently serially diluted, giving working standard solutions with concentration ranging from 25.0 to 0.01 μ g/ml that were used for construction of the calibration curves. Concentrations of standard compounds in extracts were determined from the peak areas by using the equation for linear regression obtained from the calibration curves (R^2 ; 0.995).

3.3 Results and Discussions

3.3.1 Plant extractions

The extraction yields of different extraction methods and solvents are shown in Table 4. Extraction yield is dependent up on the solvent and method of extraction (Goli et al., 2004). The extraction method must enable complete extraction of the compounds of interest and must avoid their chemical modification (Zuo et al., 2002).

Soxhlet extraction method was found to be the best method in obtaining the maximum yield (57.99%) from the plant and methanol was the most effective solvent in obtaining the maximum yield. Better yield was obtained with field-grown flowers (57.99%) of the common daisy than the field-grown leaves (44.94%) and *in vitro*-derived leaves (23.66%).

In a previous study, Kavalcioglu et al (2010) dried the aerial parts of the *B. perennis* and then extracted either with water or methanol at room temperature for overnight. They obtained the maximum yield with water extraction (11%). In consistent with this study, 11.5% yield was obtained with cold water in water bath extraction in our study. The highest yield was obtained with decoction method among aqueous extracts (30.9%). Extraction with high temperature was the main factor for obtaining higher yields. But, this method can not be used for thermolabile compounds as prolonged heating may lead to degradation of compounds (Sun and Ho, 2005; Tiwari et al. 2011).

3.3.2 GC-MS Analysis

The chemical composition of hydrodistilled oil of *B. perennis* growing in Bolu was analyzed by GC-MS for the first time. The essential oil of air-dried flowers was obtained by hydrodistillation using a Clevenger type apparatus. The yellowish-golden color essential oil isolated and obtained in a yield of 0.01 % (v/w). It was dissolved in *n*-hexane. Table (6) shows the results of the qualitative and quantitative oil analyses listed in order of elution in the capillary column.

Nineteen constituents (*alpha*-pinene, *beta*-pinene, myrcene, *p*-cymene, *beta*-caryophyllene, *gamma*-himachalene, *alpha*-copaene, *cis*-calamenene, *delta*-cadinene, *beta*-bisabolene, ethyl myristate, 2-pentadecanone, 1,2-benzenedicarboxylic acid, hexadecanoic acid, palmitic acid, ethyl palmitate, neophytadiene, linoleic acid ethyl ester and ethyl linoleolate) were identified that represented 88.71% of the total oil (Table 6). The major compounds were *beta*-bisabolene (27.42%), 2-pentadecanone (6.98%), palmitic acid (6.74%), and *gamma*-himachalene (6.57%) (Figure 15). The GC-MS analysis showed that the oil was rich in sesquiterpenes. The minor components were found as hexadecanoic acid (0.77%), 1,2-benzenedicarboxylic acid (1.38%), *p*-cymene (1.66%), *alpha*-copaene (1.68%) and myrcene (1.77%).

Kavalcioğlu et al. (2010) analyzed the hydrodistilled *B. perennis* samples (flower and leaf) by GC and GC/MS. They found that the major constituents were *gamma*-himachalene (5.2-17.4%), phytol (3.5-30.4%), tetradecanal (0.1- 8.6%), hexadecanoic acid (14.0- 87.6%), nonacosane (0- 3.2), *cis*-chrysanthenyl acetate (0- 2.2%) and germacrene D (0.2-6.4%). The amount of the compound *gamma*-

himachalene (6.57%) was consistent with our results. On the other hand, while the hexadecanoic acid (14.0- 87.6%) was the biggest group of compound in their analysis, this was a minor compound (0.77%) in our sample. This difference may be a result of where the plant samples were collected. They collected their samples (herba) from Rize (15.6%) and Antalya (40.1%) regions of Turkey. The chemical composition of essential oils depends on climatic, seasonal and geographic conditions, harvest period and distillation technique. In addition, steam distillation, as a traditional preparation method, is widely used to prepare essential oils. However, unstable compounds decompose at high temperatures, which may produce cooked notes in the extract. Moreover, not all classes of compounds can be entrained in the steam (Shu and Shen, 2008). The yield and composition of essential oils obtained in different seasons can differ in quantity and quality (Ye, 2009).

Different examples of plants belonging to Asteraceae family can be given. For instance, *Eremanthus erytropappus* (D.C.) Mc Leish is a small tree found in the savannah areas of the Brazilian Central Plateau. Determination of chemical composition of *Eremanthus erytropappus* oil by GC/MS and bioguided chemical fractionation led to the identification of β -bisabolene (2.7%), as the main active compound. The antibacterial activity of β -bisabolene (sesquiterpene) with ampicillin against *Staphylococcus aureus* was shown previously (Nascimento et al., 2007). The 2-pentadecanone was a major compound of our sample. It has been reported in many medicinal plants in natural populations such as *Ajuga austro-iranica* Rech F. (Javidnia et al., 2010), *Stachys byzantica* (Manafi et al., 2010), *Centaurea pullata* (Dob et al., 2009), three *Ficus* species (Ogunwande et al., 2009), and *Messerschmidia sibirica* L. (Morteza-Semnani et al., 2008). The amount of this

compound in these plants (ranged from 6.5 to 29.9%) and in *B. perennis* (6.98%) was similar.

Neophytadiene was another compound in our sample. This compound was also determined in aerial parts of some plants such as *Elatostema laetevirens* (Miyazawa *et al.*, 2009) and Serebian tobacco types (Radulovic *et al.*, 2006).

The ethyl palmitate and 1,2-Benzenedicarboxylic acid which were determined in our sample has been observed also in aerial parts of another plants such as *Salvia lavandulaefolia* (Perry *et al.*, 2000), *Centaurea drabifolia* (Ugur *et al.*, 2009), *Swertia densifolia* (Naik *et al.*, 2010) and *Hypericum perforatum* (Cirak *et al.*, 2010).

Palmitic acid and linoleic acid (omega-6) were determined as fatty acids in our sample (Table 6). Palmitic acid (6.74%) was as a major compound in *B. perennis*. Palmitic acid was also the major saturated fatty acid in *Centaurea* species (Asteraceae family) and the amount of fatty acids was found to be between 17.83% and 25.31% in this species (Tekeli *et al.*, 2010). Linoleic acid (4.73%) was determined in flowers of *B. perennis*. The fatty acid composition of oil seeds grown in Turkey has been investigated previously, and the linoleic acid content was determined between 3.3% and 70.1% (Tekeli *et al.*, 2010). Linoleic acid, one of the essential fatty acids, is very important for the nutritional value of oils (Eromosele and Eromosele, 2002). The linoleic acid content makes the oil of *B. perennis* nutritionally valuable.

Table 6. The essential oil composition (%) of *B. perennis* flowers in Bolu.

Retention Time	Compound	%
9.85	alpha-Pinene	2.12
11.67	beta -Pinene	2.73
12.37	Myrcene	1.77
13.86	p -Cymene	1.66
31.8	beta -Caryophyllene	3.91
34.2	gamma -Himachalene	6.57
35.71	alpha -Copaene	1.68
36.02	cis - Calamenene	4.37
40.03	delta - Cadinene	4.23
41.01	beta - Bisabolene	27.42
42.88	Ethyl myristate	1.84
43.63	2 -Pentadecanone	6.98
43.98	1,2 -Benzenedicarboxylic acid	1.38
44.67	Hexadecanoic acid	0.77
45.09	Palmitic acid	6.74
45.47	Ethyl palmitate	3.42
46.75	Neophytadiene	3.94
47.23	Linoleic acid ethyl ester	4.73
47.3	Ethyl linoleolate	2.45
Total %		88.71

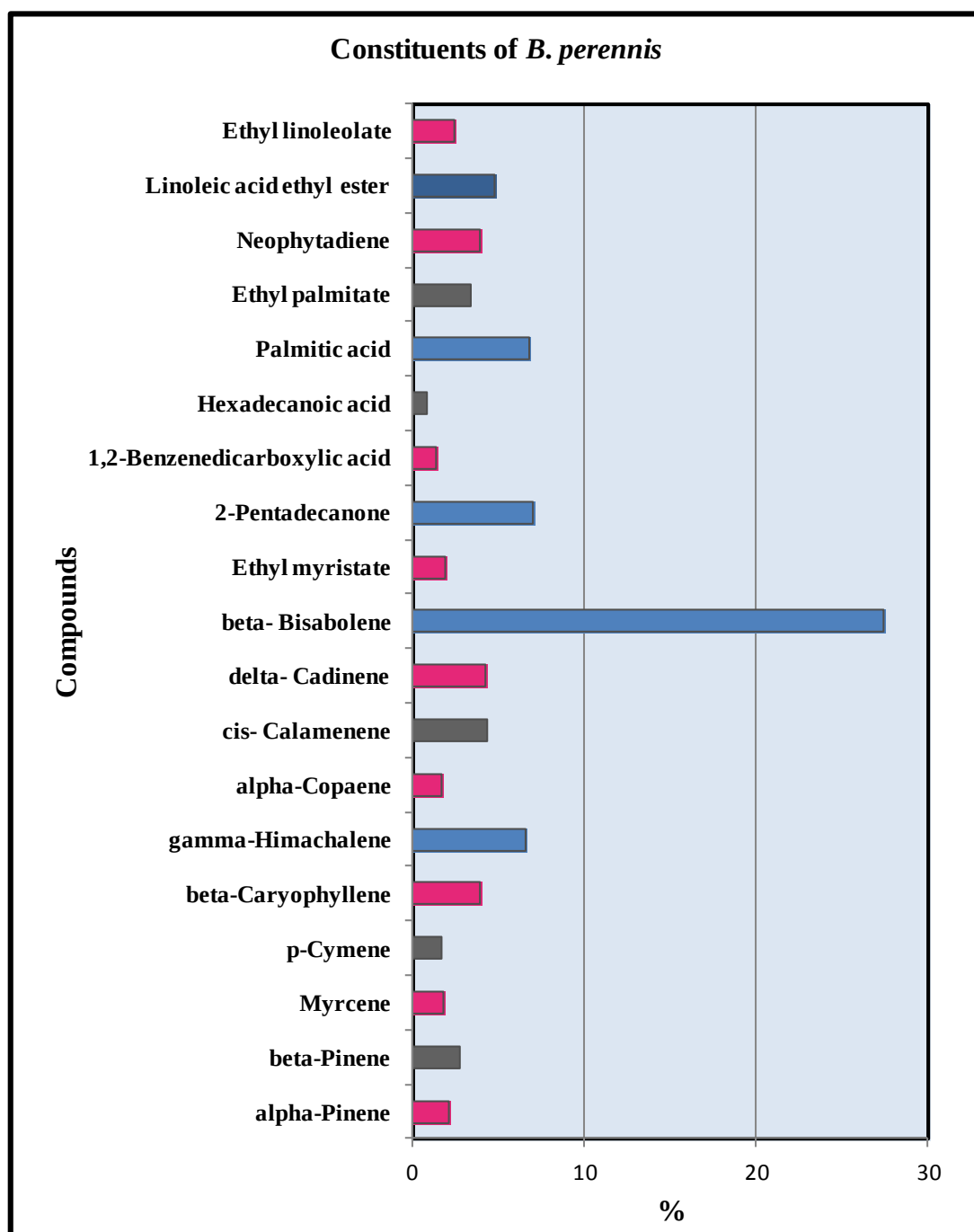


Figure 15. The essential oil constituents (%) of *B. perennis* flowers.

3.3.2 TLC analysis for saponins and phenolic compounds

Thin layer chromatography (TLC) has the advantage of speed of analysis and comparison of many samples simultaneously, versatility of supports, solvent systems and detection reagents (Stahl, 1969). These attributes make TLC an ideal classic tool for first stage of phytochemical analysis as well as for monitoring of column chromatography fractions during purification of natural plant products. The common method for detection of saponins are color reactions (Kerem et al., 2005; Muetzel et al., 2003). Analysis of saponins using high performance thin layer chromatography (HPTLC) has also been reported (Chaicharoenpong and Petsom, 2009). However, the HPTLC instrumentation is expensive. A simple procedure for detection of saponins would be of immense utility to natural products chemists and biologists for screening plant sources for saponins and for monitoring bioactivity guided purification. The protocol is simple, convenient and inexpensive.

In the present study, saponin and phenolic contents of hexane, DCM, MeOH and water extracts obtained from field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* were investigated using TLC method. The saponins appears on the chromatogram in visible light as blue, blue-violet, violet, green and sometimes red or yellow-brown zones (Wagner and Bladt, 2001). In this study, when hexane and DCM extracts were compared on TLC plates, the DCM extract of *in vitro*-derived leaves contained the most saponins qualitatively (Figure 16). When we examined the saponin contents of the methanol and water extracts, the biggest saponin content was found in the methanol extract of field-grown flowers (Figure 17). The least amount of saponin content was observed in field-grown leaf extracts.

The spots appear on the chromatogram at 365 nm UV light as yellow, orange, green or blue bands refer to phenolic compounds (Jork et al., 1990). The TLC plates for phenolics represented that DCM, methanol and water extracts of field-grown flowers contained more phenolics than field-grown leaves of *B. perennis* (Figure 18 and 19). Moreover, methanol extract of field-grown leaves was found to be including more phenolics than methanol extract of *in vitro*-derived leaves. However, DCM extract of *in vitro*-derived leaves was including more phenolics than the field-grown leaves. In general, it can be concluded that the extracts from *in vitro*-derived leaves have a low phenolic content (Figure 18; Figure 19). When *n*-butanol and ethyl acetate fractions of the flowers were compared, it was found that, the ethyl acetate fraction was including more phenolics (Figure 20). All these results coming from our TLC plates were in good correlation with our results of LC-MS/MS analysis (Table 7).

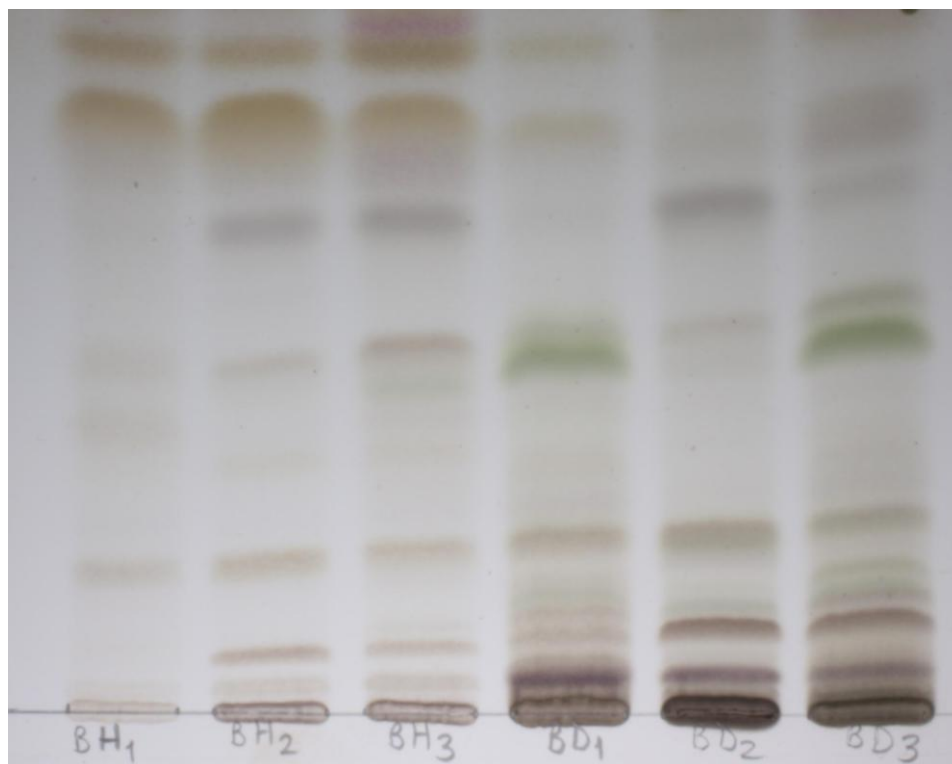


Figure 16. Thin Layer Chromatography (TLC) of hexane and DCM extracts of field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* for saponin determination.

Injection volume: 25 μ l

Solvent system: Chloroform: MeOH (25: 1 v/v)

Spray: 20% H₂SO₄ in MeOH

Detection: Visible light

BH1: Hexane extract of *B. perennis*-flowers

BH2: Hexane extract of *B. perennis*-leaves

BH3: Hexane extract of *B. perennis-in vitro*-leaves

BD1: DCM extract of *B. perennis*-flowers

BD2: DCM extract of *B. perennis*-leaves

BD3: DCM extract of *B. perennis-in vitro*-leaves

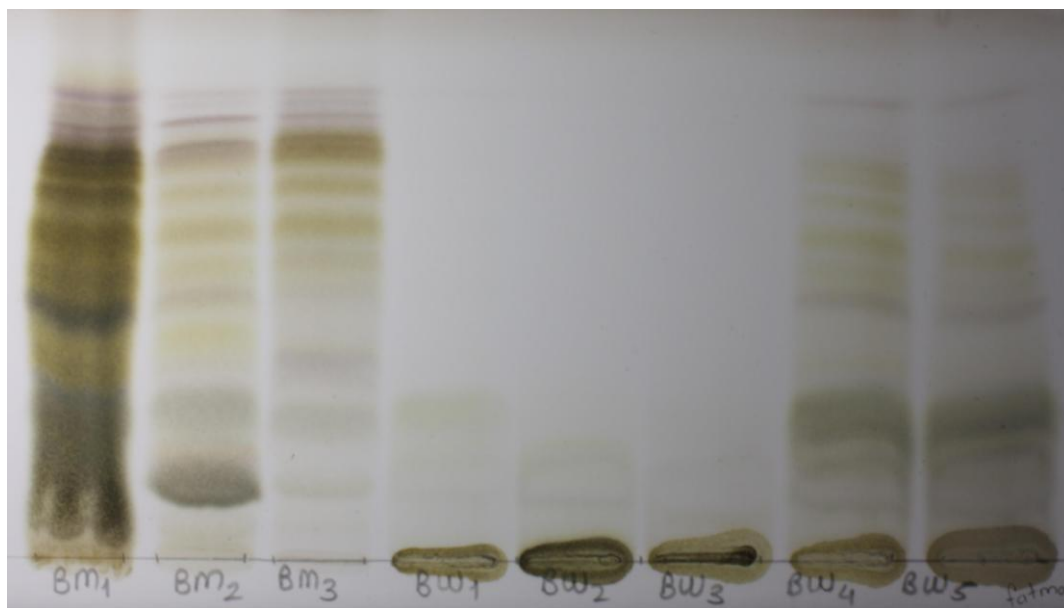


Figure 17. Thin Layer Chromatography (TLC) of MeOH and water extracts of field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* for saponin determination.

Injection volume: 25 μ l

Solvent system: Chloroform: MeOH: Water (26: 14: 3 v/v)

Spray: 20% H₂SO₄ in MeOH

Detection: Visible light

BM1: MeOH extract of *B. perennis*- flowers

BM2: MeOH extract of *B. perennis*- leaves

BM3: MeOH extract of *B. perennis*- *in vitro*- leaves

BW1: Water extract of *B. perennis*- flowers

BW2: Water extract of *B. perennis*- leaves

BW3: Water extract of *B. perennis*- *in vitro*- leaves

BW4: Water extract (decoction) of *B. perennis*- flowers

BW5: Water extract (infusion) of *B. perennis*- flowers

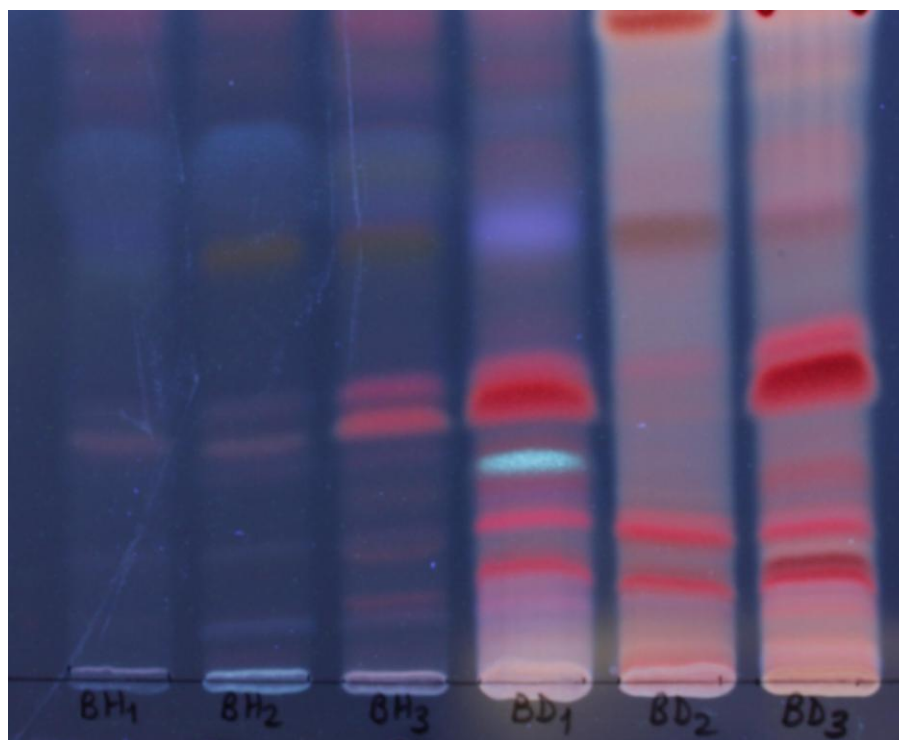


Figure 18. Thin Layer Chromatography of hexane and DCM extracts of field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* for phenolics determination.

Injection volume: 25 μ l

Solvent system: Chloroform: MeOH (25: 1 v/v)

Spray: NP + PEG

Detection: 365 nm UV light

BH1: Hexane extract of *B. perennis*-flowers

BH2: Hexane extract of *B. perennis*-leaves

BH3: Hexane extract of *B. perennis*- *in vitro*-leaves

BD1: DCM extract of *B. perennis*-flowers

BD2: DCM extract of *B. perennis*-leaves

BD3: DCM extract of *B. perennis*- *in vitro*-leaves

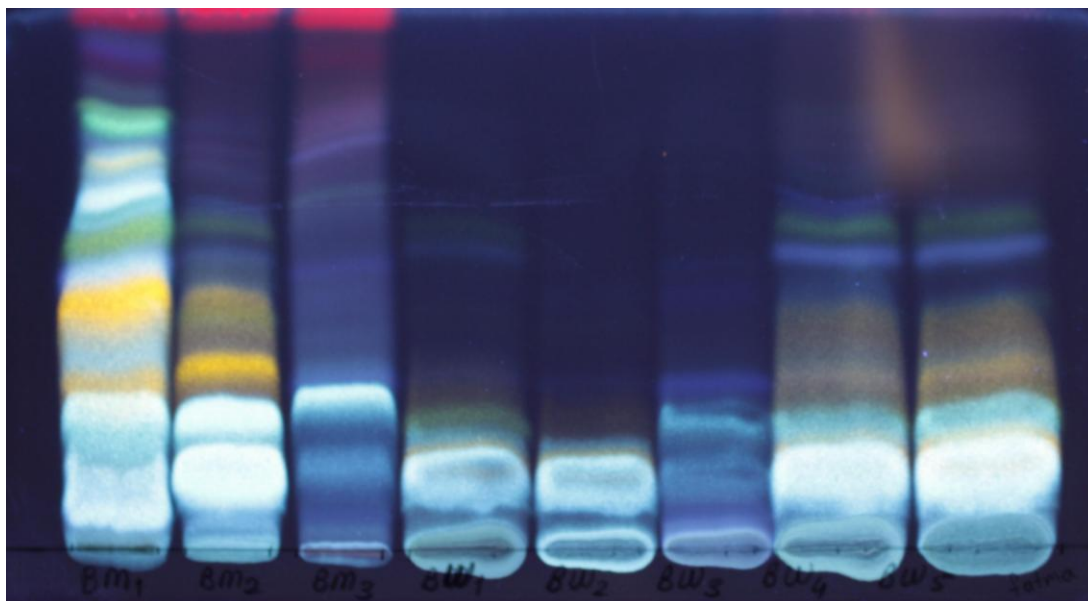


Figure 19. Thin Layer Chromatography of MeOH and water extracts of field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* for phenolics determination.

Injection volume: 25µl

Solvent system: Chloroform: MeOH: Water (26: 14: 3 v/v)

Spray: NP + PEG

Detection: 365 nm UV light

BM1: MeOH extract of *B. perennis*- flowers

BM2: MeOH extract of *B. perennis*- leaves

BM3: MeOH extract of *B. perennis*- *in vitro*- leaves

BW1: Water extract of *B. perennis*- flowers

BW2: Water extract of *B. perennis*- leaves

BW3: Water extract of *B. perennis*- *in vitro*- leaves

BW4: Water extract (decoction) of *B. perennis*- flowers

BW5: Water extract (infusion) of *B. perennis*- flowers

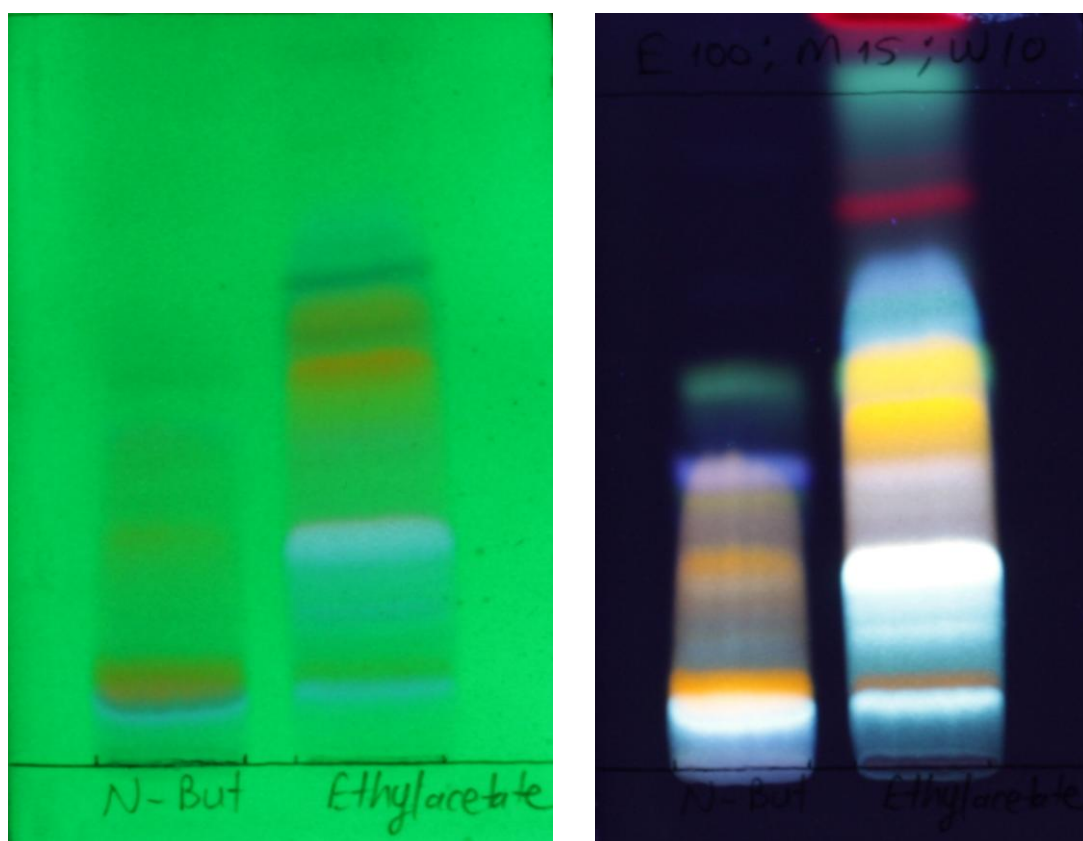


Figure 20. Thin Layer Chromatography of *n*-butanol and ethyl acetate fractions of flowers of *B. perennis* for phenolics determination.

Injection volume: 25 μ l

Solvent system: Ethyl acetate: MeOH: Water (100: 15: 10 v/v)

Spray: NP + PEG

Detection: 254 (left) and 365 (right) nm UV light

3.3.3. LC-MS/MS analysis of the selected phenolics

In the present study, quantification of the chosen phenolics in field-grown flowers, leaves, *in vitro*-derived leaves extracts (DCM and MeOH) and field-grown flower fractions (*n*-butanol and ethyl acetate) was performed using LC-MS/MS technique. The MRM mode was applied as the preferred acquisition method for accurate quantification. This type of analysis provides a high sensitivity and specificity, due to the fact that only ions which are specific to targeted analytes are monitored. The chromatogram of the standards used in the experiment were shown in Figure 21, the retention times of the standarts were also shown in Table 7 while overall data concerning the content of the standard compounds are presented in Table 8.

The results of analysis showed that the flower extracts and fractions are rich in phenolic compounds. Considerable amounts of kaempferol, rutin hydrate, caffeic acid, apigenin, quercetin, *p*-coumaric acid, luteolin-7-O- β -D glucoside, gallic acid and myricetin were found in methanol extract and ethyl acetate fraction of flowers. The most dominants were kaempferol (0.4-14605.2 $\mu\text{g/g}$ of dw) and rutin hydrate (2.7-6252.9 $\mu\text{g/g}$ of dw) in all tested extracts. In accordance with previous studies of *B. perennis* (Nazaruk and Gudej, 2001), our study also detected the presence of quercetin, apigenin, kaempferol in field-grown flower and leaf extracts. Our research was focused on phenolic compounds. Because, the analysis of flavonoid profile points out that *B. perennis* mainly contains flavonoids (Nazaruk and Gudej, 2001). Consequently, the most dominant flavonoids in both methanol extracts of field-grown flower and leaf were rutin hydrate and kaempferol. Results obtained for kaempferol in *B. perennis* was in agreement with the data previously reported with

HPLC method by Nazaruk and Gudej (2001). However, the presence of rutin in *B. perennis* was not detected in their study (Nazaruk and Gudej, 2001).

When the extracts were compared according to the results of LC-MS/MS, the highest total phenolic content was determined in methanolic extract of field-grown flowers ($12444 \mu\text{g/g} \pm 0.6$) (Table 8 and Figure 22). The phenolic content of field-grown leaves ($2250 \mu\text{g/g} \pm 0.33$) was found to be higher than *in vitro*-derived leaves ($70 \mu\text{g/g} \pm 0.01$) in methanol extracts. The reason of high phenolic content of field-grown leaves may be attributed to the stress conditions in the natural environment. An enhancement of the amount of phenolic compounds can be observed under different environmental factors and stress conditions (Sakihama and Yamasaki, 2002). The synthesis of some flavonoids is induced when plants are infected or injured (Takahama and Oniki, 2000), or under low temperatures and low nutrient conditions (Ruiz et al., 2003). On the other hand, DCM extract of *in vitro*-derived leaves ($260 \mu\text{g/g} \pm 0.08$) had more phenolics than field-grown leaves ($20 \mu\text{g/g} \pm 0.06$). When we compared the fractions of ethyl acetate and *n*-butanol of flowers, ethyl acetate ($21340 \mu\text{g/g} \pm 0.31$) fraction had more phenolic content than *n*-butanol ($3560 \mu\text{g/g} \pm 0.32$) fraction as in the TLC plates. *B. perennis* includes apigenin, caffeic acid, *p*-coumaric acid, coumarin, gallic acid, genistein, kaempferol, luteolin-7-O- β -D glucoside, myricetin, procyanidin C1, quercetin, rutin hydrate, taxifolin hydrate and vanillic acid. (Table 8 and Figure 22). Fourteen of total twenty-two samples were found on the limit of detection. Catechin, daidzein, epicatechin, epigallocatechin, procyanidin B1, procyanidin B2, pyrocatechol and resveratrol were lower than the detection limit of all tested extracts. It has been previously shown that phenolic constituents of *B. perennis* include flavonoids (Nazaruk and Gudej, 2001;

Gudej and Nazaruk, 1997; Nazaruk and Gudej, 2000; Toki et al., 1991), anthocyanins (three glucuronylated and malonylated cyanidin-3-glucosides) (Toki et al., 1991), tannins (Hegi, 1979), and phenolic acids (e.g., caffeic, ferulic, sinapic, *p*-coumaric, and salicylic acids) (Grabias et al., 1995). The following flavonoids were described in daisy flowers: quercetin, apigenin, kaempferol, isorhamnetin (Nazaruk and Gudej, 2001), apigenin-7-O- β -D-glucoside, apigenin-7-O- β -D-glucuronide (Nazaruk and Gudej, 2000), apigenin-7-O-(6''-E-caffeoyl)- β -D-glucoside, apigenin-7-O- β -D-methylglucuronide (Gudej and Nazaruk, 1997), isorhamnetin-3-O- β -D-galactoside, isorhamnetin-3-O- β -D-(6''-acetyl)-galactoside, and kaempferol-3-O- β -D-glucoside (Gudej and Nazaruk, 2001). In the present study, some phenolic substances were determined in *B. perennis* for the first time. These phenolic compounds are coumarin, genistein, luteolin-7-O- β -D glucoside, myricetin, procyanidin C1, rutin hydrate, taxifolin hydrate and vanillic acid. All tested extracts included apigenin, caffeic acid, *p*-coumaric acid, coumarin, kaempferol, luteolin-7-O- β -D glucoside, rutin hydrate and vanillic acid as dominant compounds in our study. In consistent with Nazaruk and Gudej (2001), flavonoid contents were higher in the flowers than leaves (field-grown or *in vitro*-grown) in our study.

Considerable content of determined flavonoids, which are natural polyphenol products with particularly wide and potent biological activity, can implicate antioxidant, antispasmodic, cytotoxic or anti-inflammatory activity of investigated species (Havsteen, 2002; Beara et al., 2009). The presence of foremost phenolics in flowers of *B. perennis*, such as kaempferol, apigenin, rutin or luteolin-7-O- β -D-glycoside detected in this study can be particularly valuable as a base for further

research of this species and its content can implicate numerous potential applications of the extracts examined here.

Flavonoids have shown antitumor activity for a number of cancer cell types (Fotsis et al., 1997). Genistein, a naturally occurring isoflavone, has shown antitumor activity in pancreatic cancer models (Buchler et al., 2004). Apigenin has been shown to possess anti-inflammatory effects, free radical scavenging properties, and anti-carcinogenic effects (Kim et al., 1998). Apigenin has been shown to possess growth inhibitory properties in several cancer lines, including breast, colon, skin, thyroid, pancreatic cancer and leukemia cells (Ujiki et al., 2006). Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonoid found in many edible plants (e.g. tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries and grapes) and in plants or botanical products commonly used in traditional medicine (e.g. *Ginkgo biloba*, *Tilia* spp, *Equisetum* spp, *Moringa oleifera* and *Sophora japonica*). Some epidemiological studies have found a positive association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases. Numerous preclinical studies have shown that kaempferol and some glycosides of kaempferol have a wide range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic (Calderon-Montano et al., 2011). Buckwheat flour is well known for its highly antioxidative ingredient, rutin. Rutin also demonstrated hydroxyl radical scavenging activity and antioxidative activity against hydroxyl radicals in a DNA protection assay. (Awatsuhara et al., 2010).

Table 7. Retention times of tested phenolic compounds by LC-MS/MS method.

Compound	Retention Time (minute)
Apigenin	6.945
Caffeic acid	3.072
(+) Catechin	2.539
<i>p</i> -Coumaric acid	3.878
Coumarin	4.706
Daidzein	5.832
(-) Epicatechin	3.310
(-) Epigallocatechin	2.477
Gallic acid monohydrate	0.920
Genistein	6.425
Kaempferol 3- β -D-glucopyranoside	5.416
Luteolin-7-O- β -D glucoside	4.809
Myricetin	5.260
Procyanidin B2	2.846
Procyanidin C1	3.181
Procyanidin B1	2.215
Pyrocatechol	1.892
Quercetin	6.063
Resveratrol	4.954
Rutin hydrate	4.895
($\pm$) Taxifolin hydrate	4.070
Vanillic acid	3.043

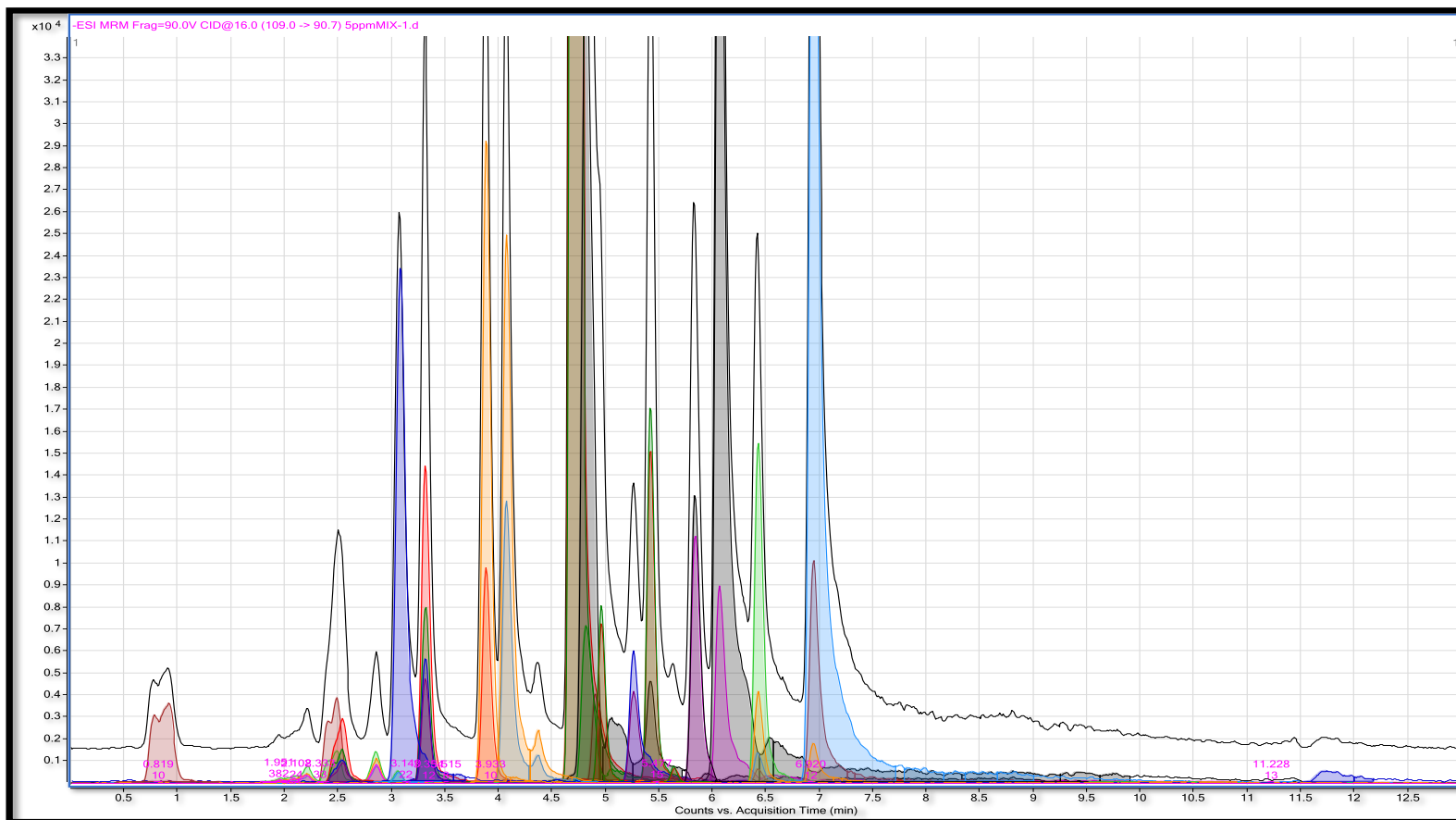


Figure 21. Total Ion Chromatogram (TIC) for the mixture of the 22 phenolic standart compounds by LC-MS/MS.

Table 8. Determined concentrations of selected phenolics in examined *B. perennis* extracts. Values are means $\pm$ SD of three measurements. Nd; not detected.

Standart Compounds	Content of selected phenolics in examined <i>B. perennis</i> extracts and fractions ($\mu\text{g/g}$ of dry extract)							
	BFD	BFM	BFN	BFE	BLD	BLM	BID	BIM
Apigenin	572.3 $\pm$ 0.5	270.7 $\pm$ 0.01	Nd	737.9 $\pm$ 0.3	0.2 $\pm$ 0.000	Nd	77.9 $\pm$ 0.02	Nd
Caffeic acid	10.2 $\pm$ 0.003	373.3 $\pm$ 0.30	8.2 $\pm$ 0.006	1341.3 $\pm$ 0.02	Nd	66.8 $\pm$ 0.04	Nd	35.5 $\pm$ 0.01
(+) Catechin	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
<i>p</i> - Coumaric acid	161.9 $\pm$ 0.07	75.1 $\pm$ 0.07	2.6 $\pm$ 0.006	378.0 $\pm$ 0.01	6.9 $\pm$ 0.02	36.7 $\pm$ 0.02	30.4 $\pm$ 0.007	15.2 $\pm$ 0.001
Coumarin	0.4 $\pm$ 0.000	0.3 $\pm$ 0.001	Nd	4.7 $\pm$ 0.009	0.7 $\pm$ 0.000	0.8 $\pm$ 0.006	Nd	Nd
Daidzein	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
(-) Epicatechin	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
(-) Epigallocatechin	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Gallic acid monohydrate	Nd	17.0 $\pm$ 0.007	2.6 $\pm$ 0.000	148.5 $\pm$ 0.02	Nd	0.7 $\pm$ 0.000	Nd	0.7 $\pm$ 0.000
Genistein	26.1 $\pm$ 0.06	Nd	Nd	Nd	3.1 $\pm$ 0.007	0.1 $\pm$ 0.000	10.9 $\pm$ 0.001	Nd
Kaempferol 3- β -D-glucopyranoside	9.2 $\pm$ 0.009	5207.4 $\pm$ 0.5	426.4 $\pm$ 0.03	14605.2 $\pm$ 0.1	Nd	249.1 $\pm$ 0.009	Nd	0.4 $\pm$ 0.000
Luteolin-7-O- β -D glucoside	0.4 $\pm$ 0.000	70.1 $\pm$ 0.07	32.1 $\pm$ 0.05	232.4 $\pm$ 0.01	Nd	2.7 $\pm$ 0.002	0.9 $\pm$ 0.001	0.1 $\pm$ 0.000
Myricetin	Nd	2.4 $\pm$ 0.02	Nd	7.5 $\pm$ 0.01	Nd	Nd	4.0 $\pm$ 0.01	0.3 $\pm$ 0.002
Procyanidin C1	Nd	Nd	Nd	Nd	Nd	Nd	3.1 $\pm$ 0.001	Nd
Procyanidin B1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Procyanidin B2	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Pyrocatechol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Quercetin	19.5 $\pm$ 0.10	174.6 $\pm$ 0.1	0.4 $\pm$ 0.001	775.2 $\pm$ 0.09	Nd	8.1 $\pm$ 0.01	1.0 $\pm$ 0.002	Nd
Resveratrol	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Rutin hydrate	38.9 $\pm$ 0.06	6252.9 $\pm$ 0.17	3073.7 $\pm$ 0.3	3029.1 $\pm$ 0.1	9.4 $\pm$ 0.03	1879.5 $\pm$ 0.4	2.7 $\pm$ 0.002	8.3 $\pm$ 0.01
($\pm$) Taxifolin hydrate	Nd	0.1 $\pm$ 0.000	Nd	5.1 $\pm$ 0.005	Nd	Nd	Nd	Nd
Vanillic acid	87.6 $\pm$ 0.3	0.7 $\pm$ 0.005	15.2 $\pm$ 0.04	29.4 $\pm$ 0.09	3.7 $\pm$ 0.007	1.5 $\pm$ 0.001	129.1 $\pm$ 0.05	5.1 $\pm$ 0.01
Total Phenolics	930 $\pm$ 0.60	12440 $\pm$ 0.60	3560 $\pm$ 0.32	21295 $\pm$ 0.31	20 $\pm$ 0.06	2250 $\pm$ 0.33	256 $\pm$ 0.08	70 $\pm$ 0.01

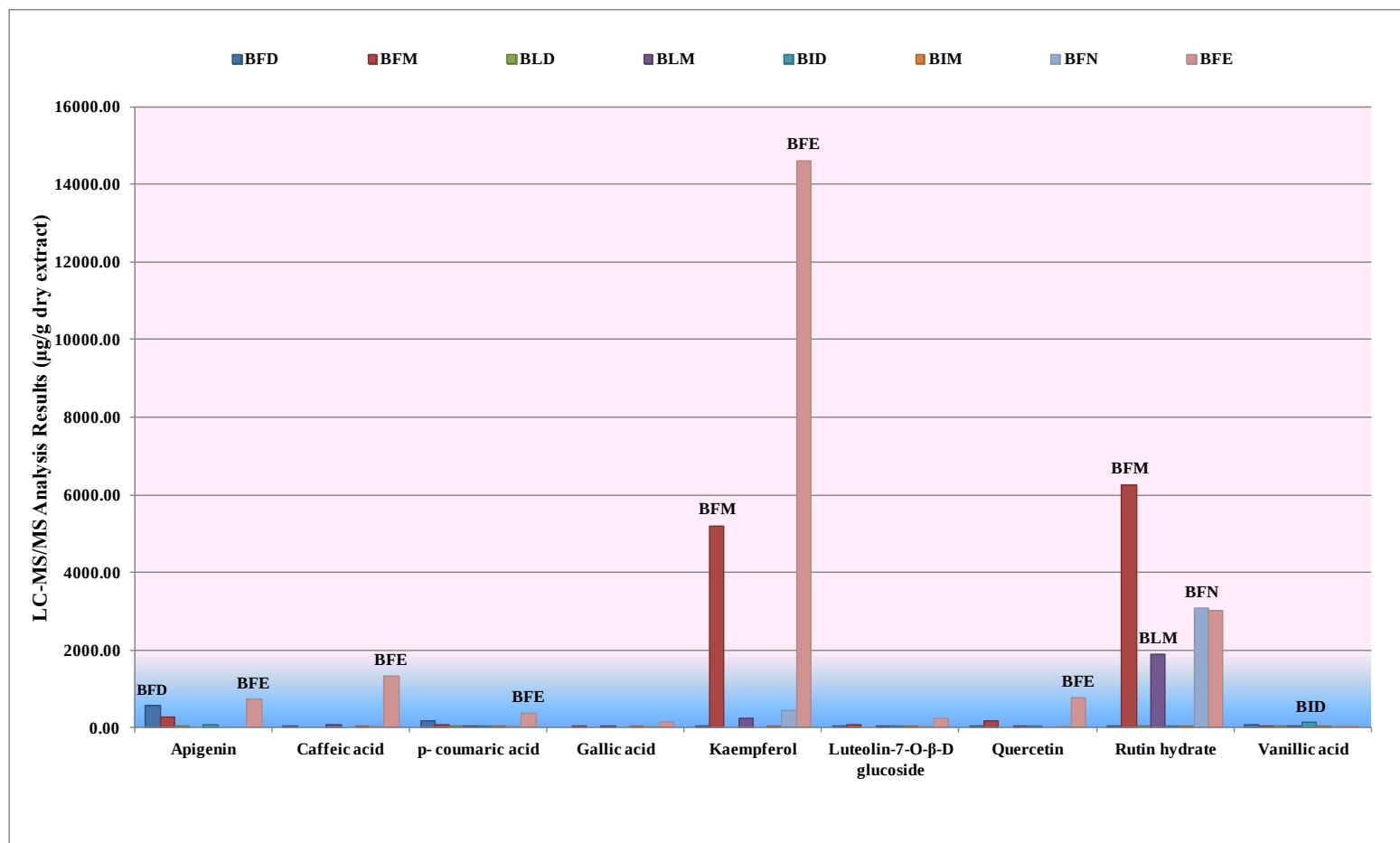


Figure 22. The LC-MS/MS analysis results of selected major phenolic compounds in *B. perennis* extracts and fractions.

Secondary product synthesis by disorganized callus or cell suspension is typically low in un-manipulated cultures. In contrast to disorganized cultures the behavior of differentiated cultures is much more predictable (Parr, 1989). Shoot cultures often produce higher product levels and more conventional product profiles than their corresponding callus or suspension cultures (Stafford, 1991). But, sometimes the level of the detected secondary products in shoot cultures is lower than donor plants. For example, in *Catharanthus roseus* shoot cultures, the dimeric alkaloids have been detected, although at lower levels than those found in the leaves of the plant, while cell suspension cultures contained no trace of these complex structures. Although tropane alkaloids were obtained from multiple shoot cultures of shoot tip, lateral bud or leaf discs of *Atropa belladonna*, detected level was about 7% of donor plant (Benjamin et al., 1987). Also, 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).

Future studies will focus on isolation of essential oils, phenolics and saponins from *B. perennis* with subsequent elucidation of their structures. Perhaps this information can assist in standardization of commercial samples and elucidation of active ingredients and their mode of action.

CHAPTER 4

BIOLOGICAL ACTIVITIES OF *B. PERENNIS* L.

4.1 Introduction

For many years plants have been used as therapeutic resources either as herbal teas or other home made remedies, as crude extracts or as “standard enriched fractions” in pharmaceutical preparations such as tinctures, fluid extracts, powders, pills and capsules (Dzhambazov et al., 2002; Rates, 2001). The World Health Organization estimates that 80 % of people in developing countries (65 % of World’s population) still rely on traditional medicine (Starbuck, 1999). Plants have been rich sources of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against 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.

4.1.1 Antibacterial activity

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. Kirby-Bauer agar diffusion test procedure is designated for use with rapidly growing bacteria. It is not directly applicable to filamentous fungi, anaerobes, or slow-growing bacteria (Atlas, 1988). The disk diffusion method has been employed by using plant extracts in antibacterial susceptibility testing (Caceres et al., 1991; McCutcheon et al., 1992; Mousa, 1994; Mongelli, 1995; Essawi and Srour, 2000; Kelmanson, 2000; Samy and Ignacimuthu, 2000).

The pathogenic organisms were selected for the study on the basis of their clinical and pharmaceutical importance. A list of bacteria used in the antibacterial study and their representative diseases were given in Table 9.

In the present study, we investigated the antibacterial activity of *B. perennis* extracts and fractions against to ten different bacterial strains [*Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883) which are Gram-negative bacteria and *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are Gram-positive bacteria] by using the disc diffusion assay (Kirby-Bauer Method).

Table 9. Bacteria used in antibacterial assay and their representative diseases, habitats and treatments (Levinson and Jawertz, 2002).

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

4.1.2 Antitumor activity

Crown gall is a neoplastic disease of plants induced by specific strains of the Gram-negative bacterium, *Agrobacterium tumefaciens*. The bacterium contains a large Ti (tumor-inducing) plasmid, which carries genetic information (T-DNA) that transforms normal, wounded, plant cells into autonomous tumor cells (McLaughlin, 1991). *A. tumefaciens* is the causal agent of Crown Gall disease (the formation of tumors) in over 140 species of dicot. 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).

The inhibition of *Agrobacterium tumefaciens*-induced tumors (or crown gall) in potato disc tissue is an assay based on antimitotic activity and can detect a broad range of known and novel antitumor effects (McLaughlin and Rogers, 1998). Tumorigenesis in plants and animals involve similar mechanisms and common nucleic acid components. The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Coker et al., 2003; Agrios, 1997).

Galsky et al. (1980) demonstrated that the inhibition of crown gall tumor initiation discs of potato tubers and subsequent growth showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (P388) (*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. Jerry McLaughlin used the potato tumor disc assay to evaluate several other plant extracts (e.g., members of the Euphorbiaceae) with *A. tumefaciens* as the tumor initiator (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; Turker et al., 2012). 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.

B. perennis has been used as a medicinal herb against to cancer, breast cancer and uterus cancer (Duke, 2002). Li et al. (2005) isolated six triterpenoid saponins from the roots of *B. perennis*. The cytotoxic activities of these saponins against HL-60 human promyelocytic leukemia cells were reported. However, no antitumor activity has been shown for field-grown flower extracts of *B. perennis*. In the present study, we investigated the antitumor activity of 4 different concentrations (100.000, 10.000, 1.000, 100 mg/l) of field-grown flower extracts of *B. perennis* (hot water, cold water, ethanol and methanol).

4.1.3 Toxicity

Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose, or toxicology is simply pharmacology at a higher dose. Thus, *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. The eggs of brine shrimp, *Artemia salina* (Leach) are readily available in pet shops at low cost and remain viable for years in the dry state. Upon being placed in seawater, the eggs hatch within 48 hours to provide large numbers of larvae (nauplii) for experimental use. A positive correlation was found between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity, and now the brine shrimp test has been used as a prescreen for a panel of six human solid tumor cell lines at the Cell Culture Laboratory of Purdue Cancer Center (McLaughlin et al., 1998). It is possible to detect and then monitor the fractionation of cytotoxic, as well as 3PS (*in vivo* murine leukemia) active extracts using the brine shrimp lethality bioassay rather than more tedious and expensive *in vitro* and *in vivo* antitumor assays. The brine shrimp assay has the advantages of being rapid (24 hours), inexpensive, and simple. 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). Active compounds thus obtained could then be subjected to more elaborate bioassays for specific pharmacologic activities (Meyer et al., 1982). Furthermore, it does not require animal serum as is needed for cytotoxicities. Animal rights advocates have not yet objected to the use of these invertebrates in experimental work (McLaughlin et al., 1991).

In the present study, we investigated the toxicity of 4 different concentrations of field-grown flower extracts of *B. perennis* (hot water, cold water, ethanol and methanol).

4.1.4 Anticancer activity

A high number of new drugs derived from plant secondary metabolites have been applied towardly in the treatment and/or prevention of cancer (Balunas and Kinghorn, 2005; Newman et al., 2003). Investigations about natural products have recently regained prominence with the increasing understanding of their biological significance and increasing recognition of the origin and function of their structural diversity. Since 1990, there has been a 22% increase in cancer incidence and mortality, with over 10 million new cases and over 6 million deaths worldwide in 2000 (excluding non-melanoma skin cancers) (Parkin et al., 2001). Important progress has been made in cancer chemotherapy, a considerable portion of which can be attributed to plant-derived drugs (Newman et al., 2003). Anticancer agents from plants currently in clinical use comprise four main compound classes (including synthetic and/or semi-synthetic derivatives): Vinca (or Catharanthus) alkaloids from *Catharanthus roseus* L. (G. Don) (Apocynaceae; formerly *Vinca rosea* L.) (Okouneva et al., 2003), epipodophyllotoxins from *Podophyllum peltatum* L. (Berberidaceae) (Gordaliza et al., 2004), taxanes from *Taxus brevifolia* Nutt. (Taxaceae) (Oberlies and Kroll, 2004), and camptothecins from *Camptotheca acuminata* Decne. (Nyssaceae) (Wall and Wani, 1996; Conforti et al., 2008). Moreover, many of the active molecules sold for the treatment of cancer, are highly expensive, mutagenic, carcinogenic and teratogenic. Hence, there is a need to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and

accessible to common man. A need is therefore felt to search newer remedies, which are cheaper economically and do not have severe side effects of the pure compounds (Yue-Zhang, 1998).

Resazurin is a colorimetric (fluorescent) assay for cell viability. It is used as an indicator for cell viability in mammalian cell cultures (Anoopkumar-Dukie et al., 2005). The oxidized, blue dye, itself nonfluorescent resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is reduced to a pink colored and highly fluorescent resorufin dye in the medium by cell activity (likely to be by oxygen consumption through metabolism). Resazurin is effectively reduced in mitochondria, making it useful also to assess mitochondrial metabolic activity. Usually, in the presence of diaphorase as the enzyme, NADPH or NADH is the reductant that converts resazurin to resorufin (O'Brien et al., 2000). Resazurin based assays show excellent correlation to reference viability assays such as formazan-based assays (MTT/XTT) and tritiated thymidine based techniques, while being much easier and safer to use for the user. It also allows for longer studies (minimally toxic to living cells), works for adherents cells and bacteria/fungi. Besides standard applications as cell culture assays (cell counting, cell proliferation assays) and cytotoxicity testing, it also can be multiplexed with several chemiluminescent assays, such as cytokine assays, caspase assays to measure apoptosis, or reporter assays to measure a gene or a protein expression (De Fries and Mistuhashi, 1995; O'Brien et al., 2000).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells (Mosmann, 1983). A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric

acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. The MTT assay and the MTS assay are colorimetric assays for measuring the activity of enzymes that reduce MTT or close dyes (XTT, MTS, WST) to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth (Mosmann, 1983).

B. perennis has been used as a medicinal herb against to cancer, breast cancer and uterus cancer (Duke, 2002). Li et al., (2005) isolated six different triterpenoid saponins from the roots of *B. perennis*. The cytotoxic activities of these saponins against HL-60 human promyelocytic leukemia cells were reported. However, no anticancer and cytotoxic activity has been shown for field-grown flowers, leaves and *in vitro*-derived leaf extracts of *B. perennis*.

In the present study, we investigated the anticancer activity (cytotoxic activity) of *B. perennis* extracts and fractions against to human lung cancer (A-549), human colon cancer (DLD-1) and human breast cancer (MCF-7) cell lines with the resazurine reduction test (O'Brien et al., 2000) and/ or MTT assay (Mosmann, 1983).

4.1.5 Anti-inflammatory activity

Inflammation is a complex mechanism developed as an immunological response to a viral, bacterial or even parasitic infection. During the ultimate phase of

a series of signaling events, macrophages induce the expression of pro-inflammatory genes such as inducible nitric oxide synthase (iNOS). This enzyme is up-regulated by secretion of pro-inflammatory cytokines, and produces nitric oxide (NO) from L-arginine. Large amounts of such a biological mediator can cause severe or collateral tissue injury because NO can form peroxynitrite (ONOO^-), a cytotoxic molecule. The regulation of NO production is therefore an important target for inflammatory diseases (Guzik et al., 2003; Jonville et al., 2011).

Lipopolysaccharide (LPS) is a gram-negative bacterial endotoxin that could stimulate macrophage and monocytes through the binding of the LPS-binding protein (LBP) to CD14, a membrane glycoprophosphatidylinositol anchors protein. RAW 264.7, a murine macrophage-like cell lines, often play an important role in secreting inflammatory mediators and especially associate with endotoxin effects (MacMicking et al., 1997). Pro-inflammatory genes are activated via signal transduction pathways that lead to the production of proinflammatory parameters such as NO, tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) (Lahti et al., 2000; Tracey and Cerami, 1994; Dinarello, 1996). NO is produced by activating inflammatory cells. NO is synthesized from L-arginine through the action of constitutively expressed NO synthase (eNOS) and an inducible NO synthase (iNOS) (Nathan and Xie, 1994; Liang et al., 1999). The augmentation of NO production by LPS is solely dependent on the expression of iNOS. Once iNOS is induced, it produces excessive amounts of NO which profoundly influence cell and tissue function and damage (Moncada et al., 1991). In the biological system, NO has many functions such as vasodilation, neurotransmission, and inflammatory response (Lo et al., 2002). TNF- α is produced in the early phase of inflammatory cells, such as

macrophages. TNF- α mediates early-stage responses of inflammation by regulating the production of other cytokines, including IL-1 β and IL-6. Therefore, it is suggested as an important therapeutic approach to regulate the production of proinflammatory mediators for the treatment and prevention of inflammatory diseases (Wu, 2012).

Reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide, hydroxyl radicals and hypochlorous acid, as well as reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, contribute significantly to tissue injury in asthma, rheumatisms and burns (Bauerova and Bezek, 1999; Horton, 2003; Dufour et al., 2007). Moreover, several studies suggest that antioxidant and anti-inflammatory agents could be beneficial in the prevention and treatment of these pathologies (Bauerova and Bezek, 1999; Horton, 2003; Dufour et al., 2007).

Oxidative stress is linked to inflammation playing together an important role in the pathogenesis of cancer (Maeda and Omata, 2008), cardiovascular diseases (Montecucco et al., 2011), type 2 diabetes and obesity (Sell and Eckel, 2009). Oxidative stress is an imbalance between production of reactive oxygen species and antioxidant defences (Betteridge, 2000). The redox stress triggers the activation of immune cells which release pro-inflammatory cytokines, reactive oxygen and nitrogen species causing damage to biological molecules and inducing imbalances in physiological and pathological pathways (Lonkar and Dedon, 2011). Epidemiological and *in vivo* studies have provide evidence that dietary intake of antioxidant and anti-inflammatory compounds is a key strategy for health promotion by lowering oxidative stres and inflammation (Martinez-Villaluenga et al., 2012).

B. perennis has been used as an anti-inflammatory, diuretic, vulnerary, expectorant and homeostatic in traditional medicine (Grieve, 1982; Bown, 1995; Baytop, 1999; Duke et al., 2002). However, anti-inflammatory effect of *B. perennis* has not been studied before. In the present study, anti-inflammatory activities of twelve different *B. perennis* extracts (hexane, dichloromethane, methanol and water) of field-grown flowers, leaves and *in vitro*-derived leaves were evaluated by measuring their capacity to inhibit cellular NO generation.

4.1.6 Antioxidant activity

Reactive oxygen species (ROS) are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals ($OH\cdot$), and non free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Gülçin, 2006a). ROS attack to the biological molecules, leading to cell or tissue injury. ROS induce some oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. Their damage causes ageing, cancer, and many other diseases (Aruoma, 1994).

All aerobic organisms have antioxidant defenses, including antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and antioxidant food constituents to remove or repair the damaged molecules. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage (Halliwell, 1997). Antioxidants can protect the human body from free radicals and ROS effects. Many

small molecules are also involved in ROS detoxification, such as glutathione (GSH), α -tocopherol (vitamin E) and ascorbic acid (vitamin C) (Sies, 1997). They retard the progress of many chronic diseases as well as lipid peroxidation. Hence, a need has appeared to identify alternative natural and safe sources of food antioxidants, and the search for natural antioxidants, especially of plant origin, has notably increased in recent years. Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation steps, leading to the termination of the reaction and delaying the oxidation process (Shahidi, et al., 1992; Gülçin, 2002a).

When the mechanism of antioxidant protection becomes unbalanced by exogenous factors such as smoking, ionising radiation, certain pollutants, organic solvents, pesticides and endogenous factors such as normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes may occur, resulting in some diseases such as malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and cancer (Hertog, et al., 1993; Tanizawa et al., 1992; Duh, 1998; Yıldırım et al., 2001) with accelerating ageing (Büyükokuroğlu et al., 2001). However, antioxidant supplements or foods rich in antioxidants may be used to help the human body in reducing oxidative damage by free radicals and active oxygen (Halliwell and Gutteridge, 1984; Mau et al., 2001; Gülçin et al., 2002b). Recently, various phytochemicals and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs have been intensively studied (Ho et al., 1994).

Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Yen and Duh, 1994; Duh,

1998). Recently, interest has considerably increased in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito et al., 1983; Zheng and Wang, 2001). Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (Kinsella, 1993; Pryor, 1991; Lai et al., 2001). Hence, the studies on natural antioxidant have gained increasingly greater importance.

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

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

Flavonoids belong to a large group of plant-derived low-molecular-weight polyphenolic compounds biosynthesized from both shikimic acid and acetic acid pathways (Bruneton, 1999). They deserve attention because of their biological properties, such as vascular protective and venous tonic, anti-inflammatory, diuretic, antispasmodic, hepatoprotective, and hypocholesterolemic (Bruneton, 1999;

Cazarolli et al., 2008). There is a lot of interest in the antioxidant activity of flavonoids and other plant phenolic compounds due to their potential in health promotion and disease prevention (Dillard and German, 2000; Su et al., 2007, Pereira et al., 2009; Parr and Bolwell, 2000). Antioxidants are able to scavenge free radicals by various mechanisms (Pereira et al., 2009; Parr and Bolwell, 2000), and thus to contribute to the protection of biologically important cellular components, such as DNA, proteins, and membrane lipids, from free radical attacks leading to cell damage, which has been linked to aging, inflammation, atherosclerosis, ischemic injury, and cancer (Bruneton, 1999; Su et al., 2007; Stanojevic et al., 2009; Siatka and Kasparova, 2010).

Diets rich in foods containing antioxidant compounds, such as fruits and vegetables, could help prevent pathologies caused by oxidative stress (Lampe, 1999). Therefore, it is important to evaluate the antioxidant potential of fruits and vegetables in order to be certain of their quality and to develop methods for their optimal conservation. Several *in vitro* methods such as ORAC assay (oxygen radical absorbing capacity), TRAP method (total radical-trapping antioxidant parameter) and TEAC method (trolox equivalent antioxidant capacity) are used for the evaluation of the antioxidant activities of fruit and vegetable extracts (MorenoSanchez, 2002; Pellegrini et al., 2003; Proteggente et al., 2002; Wu et al., 2004). *In vitro* tests such as the ORAC assay do not however take into account the physiological conditions of the cell, the bioavailability of the antioxidant molecule as well as general cellular metabolism (Liu and Finley, 2005). Moreover, antioxidant molecules present in fruits and vegetables are heterogenous and multifunctional (Frankel and Meyer, 2000). Many factors can affect their antioxidant potential, such as the affinity of the

molecules for the aqueous or lipid phase, the oxidation conditions in the cell as well as the nature of the oxidizable substrate used in the assay. Therefore, a one-dimensional assay protocol cannot be used alone to test all relevant parameters (Frankel and Meyer, 2000). A complementary method used to evaluate the antioxidant activities of plant extracts directly in live mammalian cells could be useful.

Kavalcioğlu et al. (2010) showed that antioxidant activities of methanolic and aqueous extracts of *B. perennis* by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging (Blois method), reducing power (method of Oyaizu) and total antioxidant capacity (thiocyanate method). Siatka and Kasparova (2010) also showed that seasonal variation in total phenolic and flavonoid contents and DPPH scavenging activity of *B. perennis* flowers.

In the present study, we investigated the antioxidant activities of twelve different *B. perennis* extracts (hexane, dichloromethane, methanol and water) of field-grown flowers, leaves and *in vitro*-derived leaves by using five different antioxidant methods; free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteu), total flavonoid content (aluminum chloride colorimetric), oxygen radical absorbing capacity (ORAC) and direct antioxidant assays [cell-based assay using 2',7'-dichlorofluorescein-diacetate (DCFH-DA)].

4.2 Materials and Methods

4.2.1 Antibacterial activity 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* (ATCC 13883) which are Gram-negative bacteria and *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are Gram-positive bacteria. BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes containing 5 ml of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. 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 0.9% saline suspension in a test tube for each bacterial strain and the turbidity of the suspension is adjusted to 0.5 McFarland standard (1.5×10^8 cfu/ml). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface of a (90 × 15 mm) Mueller-Hinton (M-H) agar plate (One test tube was used for each agar plate). Agar plates were streaked four 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 13 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 and DMSO. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hrs of incubation, diameter (mm) of inhibition zone was measured. All experiments were repeated three times.

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

4.2.2 Anti-tumor activity assay

4.2.2.1 Antitumor assay

Antitumor activity of flower extracts of *B. perennis* 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 25 °C. Camptothecin (Sigma) (tumor suppressant) served as a positive control. Extracts were dissolved in water, so water was used as a negative control. BFHW(*Bellis*-flower-hot water), BFCW (*Bellis*-flower-cold water), BFEt

(*Bellis*-flower-EtOH) and BFMe (*Bellis*-folwer-MeOH) extracts were dissolved in water to a final concentration of 100, 1.000, 10.000 or 100.000 mg/ml. 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 equivalent to 1.0×10^9 bacteria.

All extracts, controls and solutions were filter sterilized through 0.2 μ m syringe filter (Acrodisc, Pal-Gelman Laboratory). The following design was followed:

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

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

Solvent control II: 750 μ l sterile distilled water + 750 μ l PBS.

Test extracts: 600 μ l test extract + 150 μ l sterile distilled water + 750 μ l *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 into sterile glass petri dishes. These discs were transferred to 24-well culture plates containing water-agar (15 g/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. These were done in the laminar hood (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 (iodine/potassium iodide solution) 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;

$$\% \text{ inhibition} = [(\text{solvent control mean} - \text{tested extract mean}) / \text{solvent control mean}] \times 100.$$

Significant activity was indicated when two or more independent assays gave consistently negative values of approximately 20% or greater inhibition.

4.2.2.2 Bacterial viability testing

Standardized bacterial suspension (1×10^9 CFU of *A. tumefaciens* in PBS) was serially diluted with PBS to 1×10^3 CFU. Bacterial viability was determined by incubating 1 ml of each plant extract with 1 ml of bacterial suspension (1×10^3 CFU of *A. tumefaciens* in PBS) in microcentrifuge tubes (4 tubes per extract) and left for 30 min. At 30 min after inoculation, 0.1 ml of inoculum (bacteria + extract) was removed and inoculated on YEM media with spread plate technique. After 24 h

incubation of inoculated plates at 28 °C, colony counts were made. Also, bacterial growth was evidenced by growth across the plates (Coker et al., 2003).

4.2.3 Brine shrimp bioassay

Brine shrimp (*Artemia salina* Leach) eggs (San Francisco Bay Brand, Inc.) were purchased from a pet store. Seawater was prepared by dissolving 36 g sea salt (Instant Ocean Salt Mix, Aquatic Eco-Systems, Inc. Florida) in 1 liter of distilled water and put in V-shaped, Plexiglas hatching container. Oxygen was supplied and 60-watt lamp was positioned near the container to provide direct light and heat (~ 27-28 °C). One-teaspoon brine shrimp eggs were placed in 1 liter of seawater. After 10 to 12 hr, cysts began hatching. Two days was allowed for the shrimp to hatch and mature as nauplii (Figure 23) (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 were prepared by suspending dried extract in saltwater to prepare a 10.000 mg/l solution. The suspension was mixed for 5 min; then, 1.000, 100 and 10 mg/l solutions were prepared by dilution. Twenty-four-well culture plates were used. A suspension of nauplii was removed and 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, 1,000, 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, Aquatic Eco-Systems, Inc. Florida), a common fish anesthesizer, was used at concentrations of 1, 10, 100, 1,000 mg/l.

After 24 hrs, numbers of dead nauplii were counted. Live nauplii were then killed by addition of a few drops of MeOH and the total dead were 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 Reed Muench method (Sam, 1993).



Figure 23. Brine shrimp egg (right) and mature nauplii (left) used in the experiment.

4.2.4 Anticancer activity assay

4.2.4.1 Human cancer cell lines and culture conditions

Human lung carcinoma A-549 (ATCC #CCL-185), colon adenocarcinoma DLD-1 (ATCC #CCL-221) and normal skin fibroblast WS-1 (ATCC #CRL-1502) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human breast adenocarcinoma MCF-7 cell line was obtained from Histology and Embryology Department of Istanbul Medical School. All cell lines were grown in Dulbecco's minimum essential medium (DMEM) with Earle's salts (Mediatech Cellgro, Herndon, USA). Culture medium was supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA), a solution of vitamins, sodium pyruvate and non-essential amino acids (all at 1:100 v/v dilution of supplied solutions), penicillin (100 I.U./ml) and streptomycin (100 µg/ ml) (Mediatech Cellgro, VA). Cells were cultured at 37 °C in a humidified environment containing 5% CO₂ (Figure 24).

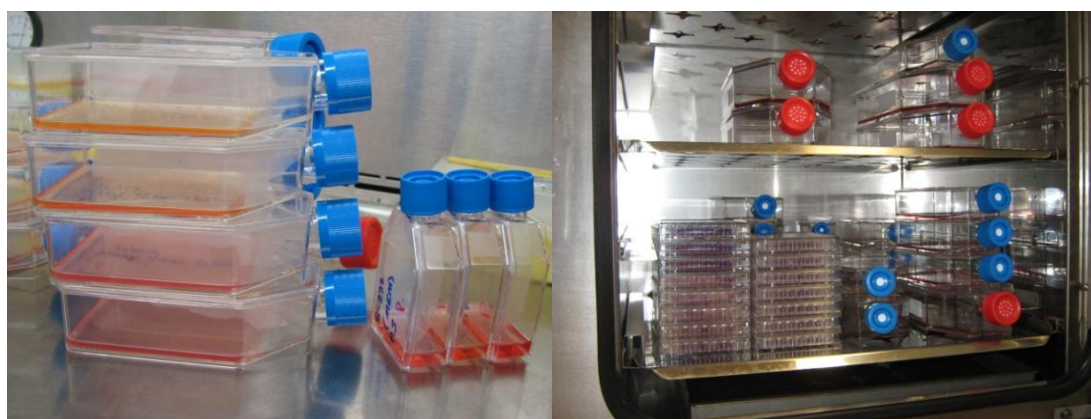


Figure 24. Culture flaks and culture conditions.

4.2.4.2 Cytotoxicity by Resazurin assay

Exponentially growing cells were plated in 96-well microplates (Costar , Corning Inc.) at a density of 5×10^3 cells per well in 100 μ L of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of each compound in DMSO (Sigma-Aldrich) were then added (100 μ L per well) and the cells were incubated for 48 h. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cytotoxicity was assessed using resazurin (O'Brien et al., 2000) on an automated 96-well Fluoroskan Ascent F1TM plate reader (Labsystems) using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Fluorescence was proportional to the cellular metabolic activity in each well. Survival percentages were defined as the fluorescence in experimental wells compared to that in control wells after subtraction of blank values. Etoposide was used as positive control. Cytotoxicity results were expressed as means $\pm$ standard deviation and represent the concentration inhibiting 50% of cell growth (IC₅₀). Each experiment was carried out three times in triplicate.

4.2.4.3 Cell viability by MTT assay

The MCF-7 (human breast adenocarcinoma) cells were seeded in a 96-well plates at a density of 10×10^3 cells per well in 100 μ L of culture medium (DMEM). After 24 h of cells incubation, increasing concentrations of each extract in DMSO were then added (100 μ L per well) and the cells were incubated at 37 °C in 5% CO₂ for 24 h. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in

living cells. The MTT assay was performed according to the method of Mosmann (1983). The proliferation test is based on the color reaction of mitochondrial dehydrogenase in living cells by MTT. The culture medium was removed and replaced with 90 μ l of fresh culture medium. Ten microlitres of sterile filtered MTT solution (5 mg/ml) in phosphate buffered saline (PBS, pH 7.4) were added to each well, reaching a final concentration of 0.5 mg MTT/ ml which was then incubated at 37 °C in 5% CO₂ for 4 h. After 4 h, 100 μ l/well of DMSO were added to all samples for dissolving of formazan that is the final product of MTT reaction and were allowed to incubate at 37 °C, in a 5% CO₂ humidified incubator for a night. After incubation, absorbance of formazan was measured spectrophotometrically in a Multiskan FC microplate photometer reader (Thermo Fisher Scientific Inc.) at 570 nm. Each experiment was carried out three times in triplicate. The relative cell viability (%) related to control wells containing cell culture medium without samples was calculated as:

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

4.2.5 Anti-inflammatory activity assay

To investigate the anti-inflammatory activity of *B. perennis* extracts, nitric oxide (NO) production in LPS-stimulated RAW 264.7 murine monocyte-macrophage (ATCC #TIB-71) cells was examined. Exponentially growing cells were plated in 96-well microplates (BD Falcon) at a density of 75×10^3 cells per well in 100 μ l of culture medium (DMEM) and were allowed to adhere overnight. Each extract sample was prepared in a series of eight twofold dilutions (final concentrations ranging from 0 to 160 μ g/ml) in DMSO and medium culture. Cells were treated or not with

positive control N(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME, $\geq 98\%$, Sigma–Aldrich), or increasing concentrations of plant extracts on three rows. Cells were then stimulated with 100 $\mu\text{g/ml}$ of lipopolysaccharide (LPS) and incubated at 37 °C, 5% CO₂ for 24 h. After 24 h, cell-free supernatants were collected and NO concentration was immediately determined using the Griess reaction with minor modifications (Green et al., 1990). Briefly, 100 μl aliquots of cell supernatants were incubated with 100 μl of a mix of 1% sulfanilamide in 2.5% phosphoric acid (H₃PO₄) and of 0.1% N-1-naphtylethylenediamine dihydrochloride in water at room temperature for 20 min. Absorbance at 550 nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron) and the presence of nitrite was quantified by comparison with an sodium nitrate (NaNO₂) standard curve. Macrophage survival was assessed using the resazurin reduction test.

4.2.6 Evaluation of antioxidant activity

4.2.6.1 DPPH radical scavenging assay

Radical scavenging activity of methanolic extracts from *B. perennis* against stable DPPH• (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 517 nm on a UV/visible light spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200V, JAPAN). Radical scavenging activity of extracts was measured by slightly modified method of Brand-Williams (1995), as described below. Extract solutions were prepared by dissolving 0.003 g of dry extract in 3 ml of methanol. Finally, this stock

solution was prepared as 1.000 µg/ml concentration. The extract solutions were diluted with methanol (12.5, 25, 50, 100 µg/ml) from the stock extract solutions. The solution of DPPH• in methanol (1.5×10^{-5} M) was prepared daily, before UV measurements. 0.5 ml of this solution were mixed with 1.5 ml extract solution in 10 cm path length glass tube (final mass ratio of extracts with DPPH• was approximately 3:1). The samples were kept in the dark for 30 min at room temperature and then the decrease in absorption was measured. Absorption of blank sample (positive control) containing the same amount of methanol and DPPH• solution (1.5 ml of the MeOH and 0.5 ml of the 1.5×10^{-5} M DPPH) was prepared and measured daily. The experiment was carried out in triplicate. Radical scavenging activity of *B. perennis* methanolic extracts was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A) / A_B] \times 100 \text{ (Gülçin et al., 2003),}$$

where: A_B - absorption of blank sample (control);

A_A - absorption of tested extract solution.

4.2.6.2 Total phenolic assay (Folin-Ciocalteu method)

In the first part of the experiment, total phenolic content was determined using the Folin- Ciocalteu reagent according to the procedure reported by Singleton and Rossi (1965) with some modifications. Briefly, a volume of 50 µl containing growing concentrations of extract ranging from 0.39 to 50 mg/ml were mixed with 25µl of a Folin-Ciocalteu reagent in 96-well plates. After 2 min, 125 µl of sodium bicarbonate (NaHCO_3) solution (0.27 g/ml) was added to each well. The plate was shielded from the light and stored for 2h at room temperature. Its absorbance was

then measured at 758 nm using an automated 96-well Varioskan Ascent plate reader (Thermo Electron). Analysis was performed in triplicate, and the results were expressed in tannic acid equivalents (TAE).

In the second part of the experiment, the amount of total phenolic content was determined using the Folin- Ciocalteu reagent according to the procedure reported by Slinkard and Singleton (1977) with some modifications. Gallic acid is accepted standard that was used as a reference phenol. It is a particularly good standard because it is relatively inexpensive in pure form and is stable in its dry form (Waterhouse, 2002). Gallic acid stock solution: In a 100 ml volumetric flask, 0.5 g of dry gallic acid was dissolved in 10 ml of pure ethanol and was diluted to required volume with water. This stock solution was used as phenol control. To prepare a calibration curve, 0, 50, 100, 150, 200, 250 and 500 mg/l gallic acid concentrations were prepared. Preparation of extract solution (stock solution): 2ml of distilled water was added to 0.01g plant extracts (5 mg/ml) of *B. perennis* (BFM, BLM, BIM, BFN and BFE). Prepared stock solution was then diluted to 1 mg/ml. Sodium carbonate (Na_2CO_3) solution: 20 g of anhydrous sodium carbonate was dissolved in 100 ml of water in a 100 ml volumetric flask. Twenty μl from each calibration solution, sample, or blank was placed into separate cuvettes. 1.58 ml water and 100 μl Folin-Ciocalteu reagent (Sigma[®]) was added to each, and then mixed well. After 2 minutes, 300 μl Na_2CO_3 solution was added and was shaken very well. The solutions were incubated at 20 ± 2 °C for 2 hours and measured the absorbance of each solutions at 765 nm against the blank (the “0 ml” solution) using the spectrophotometer (Hitachi U-1900, UV-VIS Spectrophotometer 200V, JAPAN). After the created calibration curve with standards, phenol levels in the samples were determined. The total phenol content of

methanolic extracts, *n*-butanol and ethyl acetate fractions from *B. perennis* was expressed as g gallic acid equivalents (GAE)/100 g dried extract and analysis was performed in triplicate.

4.2.6.3 Total flavonoid assay [aluminum chloride (AlCl₃) colorimetric method]

The amount of total flavonoid content of methanolic extracts, *n*-butanol and ethyl acetate fractions from *B. perennis* was measured by aluminum chloride (AlCl₃) colorimetric assay. Catechol was used as a reference flavonoid.

Preparation of catechol solution: 0.0125 g catechol was dissolved in 25 ml of 80% ethanol and this stock solution was adjusted to concentration as 500 mg/ml. In order to obtain calibration curve of catechol, 20, 40, 60, 80 and 100 mg/ml concentrations were prepared.

Preparation of extract solution: 0.005 g of each *B. perennis* extracts was dissolved in 2 ml of 80% ethanol (2500 mg/ml). Later, this stock solution was diluted to 1250 mg/ml and 500 mg/ml concentrations. 500 µl of extract solution or standard solution of catechol was added to a 10 ml test tube containing 2 ml distilled water. At zero time, 150 µl 5% NaNO₂ was added to the test tubes. After 5 min, 150 µl of 10 % AlCl₃ was added. At 6 min, 1000 µl of 1M NaOH was added to the mixture. Immediately, the reaction tube was diluted to volume 5 ml with the addition of 1200 µl distilled water and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm versus a blank (Chang et al., 2002). Samples were analyzed in three replications. The total flavonoid content of methanolic extracts, *n*-butanol and ethyl acetate fractions from *B. perennis* was expressed as g catechol equivalents (CE)/100 g dried extract.

4.2.6.4 ORAC assay (oxygen radical absorbing capacity)

The procedure was performed as described by Ou et al., (2001) with some modifications. Briefly, ORAC assay was carried out in black round bottom 96-well microplates (Costar) on a Fluoroskan Ascent FLTM plate reader (Labsystems) equipped with an automated injector. Four concentrations of 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) (the control standard, a vitamin E analogue) were used (1.56; 3.13; 6.25 and 12.5 μ M) in quadruplicate, and a gradient of 16 concentrations of the samples were prepared without replication. Experiment was conducted at 37.5 °C and in pH 7.4 phosphate buffer, with a blank sample in parallel. The fluorimeter was programmed to record the fluorescence (λ ex.: 485 nm/em.: 530 nm) of fluorescein every minute after addition of 375 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (free radical generator), for a total of 35 min. The final results were calculated using the net area under the curves of the sample concentrations for which decrease of at least 95% of fluorescence was observed at 35 min and which also was presented a linear dose-response pattern. ORAC values were expressed in micromoles of Trolox equivalents (TE) per gram (μ mol TE/g).

4.2.6.5 Antioxidant cell-based assay using 2',7'-dichlorofluorescein-diacetate (DCFH-DA)

Antioxidant activity was evaluated using DCFH-DA (2',7'-dichlorofluorescein-diacetate) assay as described by Legault et al. (2003), with some modifications. The intracellular ROS generation of cells can be investigated using the DCFH-DA as a well-established compound to detect and quantify intracellular produced H₂O₂ (Cathcart et al., 1983). The conversion of the nonfluorescent 2',7'-

dichlorofluorescein-diacetate (DCFH-DA) to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) happens in several steps. First, DCFH-DA is transported across the cell membrane and deacetylated by esterases to form the nonfluorescent 2',7'-dichlorofluorescein (DCFH). This compound is trapped inside of the cells. Next, DCFH is converted to DCF through the action of peroxidase catalyzed by the presence of peroxidase (LeBel et al., 1992).

Briefly, WS-1 cells were plated in 96 microwell plates at 10,000 cells per well and were incubated for 24 h at 37 °C and 5% CO₂. The cells were washed with 150 µl Hank's balanced salt solution (HBSS) at pH 7.4 and incubated for 30 min with 100 µl HBSS (pH 7.4) containing 5 µM DCFH-DA (Sigma–Aldrich). The cells will be then washed again with 150 µl HBSS. To assess antioxidant activity, the cells were incubated either with a growing concentration of extracts from *B. perennis*, or quercetin, in the absence or presence of 200 µM *tert*-butyl hydroperoxide (*t*-BuOOH). In our cell-based assay, *tert*-butylhydroperoxide (*t*-BuOOH) was chosen as the intracellular oxidizing agent. Fluorescence was measured immediately after *t*-BuOOH administration and also 90 min later on the automated 96-well plate reader (Fluoroskan Ascent FLTM, Labsystems) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. IC₅₀ was calculated using the logarithmic regression of the dose–response curve after subtraction of both blank and intrinsic sample fluorescence values. In all cases, the coefficients of determination of the regression (R^2) was greater than 0.95. IC₅₀ was the means ± standard deviations of three determinations.

4.3 Results and Discussion

4.3.1 Antibacterial activity

Antibacterial activity of 19 different extracts of different plant parts (flower, leaf and *in vitro*-derived leaf) of *B. perennis* was tested with the disc diffusion assay (Kirby-Bauer Method) (Prescott et al., 1990). Generally, tested Gram-positive bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) seem to be more susceptible to the inhibitory effects of *B. perennis* extracts than the Gram-negative bacteria (Table 10). Susceptibility of Gram-positive bacteria may come from their cell wall structure consisting of a single layer, but the Gram-negative cell wall is a multi-layered structure and quite complex (Essawi and Srour, 2000). Usually most of the Gram-negative bacteria are more resistant than Gram-positive bacteria (Duraipandian and Ignacimuthu, 2007). Positive controls (reference antibiotics) generally showed antibacterial activity to our test organisms. Since final concentrations of all extracts were adjusted with distilled water or DMSO, they were used as a negative control and no inhibition was observed with them (Table 10).

In the present study, field-grown plant extracts indicated better antibacterial activities than *in vitro*-derived plant extracts. Among all plant extracts, methanol extracts were better than hexane, DCM and water extracts. The best antibacterial activity was obtained with methanolic extract of field-grown flowers (BFMe) against *S. aureus* (16 mm) and *S. epidermidis* (23.2 mm), and ethyl acetate extract of field-grown flowers against *S. pyogenes* (12.4 mm) and *E. cloacae* (15.9 mm) (Table 10). BFMe (23.2 mm), BFA (20.3 mm), BFN (19.9 mm), BFCW (19.6 mm), BFEt (13.9

mm), BFE (12.9 mm), BFM (12.3 mm), BFHW (11.1 mm), BLM (8.8 mm), BIM (8.6 mm) and BFW (8.5 mm) extracts showed greater antibacterial activity than reference antibiotic tetracycline (8 mm) against *S. epidermidis* (Table 10; Figure 25; Figure 26). The BFE (15.9 mm) and BFMe (11.6 mm) extracts showed higher antibacterial activity than reference antibiotic erythromycin (9.3 mm) against *E. cloacae* (Table 10; Figure 27; Figure 28).

In this study, methanol and ethyl acetate extracts of field-grown flowers exhibited a broad-spectrum of activity against both Gram-positive (*S. pyogenes*, *S. aureus* and *S. epidermidis*) and Gram-negative bacteria (*E. cloacea*). However, *S. marcescens*, *S. typhimurium*, *P. aeruginosa*, *P. vulgaris*, *K. pneumonia* and *E. coli* did not show any sensitivity against to used extracts (Table 10).

Table 10. Antibacterial activity of *B. perennis* extracts and fractions. Data presented as zone of inhibition of bacterial growth in mm.

Treatments	Mean diameter of inhibitory zones (mm ± SE)										
	Gram (+) bacteria				Gram (-) bacteria						
	<i>S.pyogenes</i>	<i>S.aureus</i>	<i>S.epidermidis</i>		<i>S.marcescens</i>	<i>S.typhimurium</i>	<i>P.aeruginosa</i>	<i>P.vulgaris</i>	<i>K.pneumonia</i>	<i>E.cloacae</i>	<i>E.coli</i>
BFCW	-	-	12.3 ± 0.1 ^g	19.6 ± 0.3 ^f	-	-	-	-	-	8.1 ± 0.9 ^g	-
BFHW	-	-	10.4 ± 0.1 ^{hi}	11.1 ± 0.2 ⁱ	-	-	-	-	-	-	-
BFEt	10.9 ± 0.2 ^g	13.3 ± 0.3 ^f	13.9 ± 0.3 ^g	-	-	-	-	-	-	-	-
BFMe	-	-	16.0 ± 0.3 ^e	23.2 ± 0.4 ^e	-	-	-	-	-	11.6 ± 0.3 ^e	-
BFA	11.1 ± 0.2 ^g	9.4 ± 0.2 ^j	20.3 ± 0.5 ^f	-	-	-	-	-	-	-	-
BFH	8.5 ± 0.3 ^h	-	-	-	-	-	-	-	-	-	-
BFD	-	-	-	-	-	-	-	-	-	-	-
BFM	8.2 ± 0.1 ^h	-	12.3 ± 0.4 ^h	-	-	-	-	-	-	-	-
BFW	-	-	8.5 ± 0.2 ^j	-	-	-	-	-	-	-	-
BFN	-	-	9.7 ± 0.4 ^{ij}	19.9 ± 0.4 ^f	-	-	-	-	-	-	-
BFE	12.4 ± 0.4 ^f	10.7 ± 0.3 ^h	12.9 ± 0.6 ^h	-	-	-	-	-	-	15.9 ± 0.4 ^d	-
BLH	8.7 ± 0.4 ^h	-	-	-	-	-	-	-	-	-	-
BLD	-	-	-	-	-	-	-	-	-	-	-
BLM	-	-	-	8.8 ± 0.2 ^j	-	-	-	-	-	-	-
BLW	-	-	-	-	-	-	-	-	-	-	-
BIH	-	-	-	-	-	-	-	-	-	-	-
BID	-	-	-	-	-	-	-	-	-	-	-
BIM	-	-	-	8.6 ± 0.2 ^j	-	-	-	-	-	-	-
BIW	-	-	-	-	-	-	-	-	-	-	-
Chloramphenicol (30 µg)	32.2 ± 0.6 ^e	26 ± 0.3 ^d	33.2 ± 0.4 ^c		25.0 ± 0.5 ^b	27.5 ± 0.2 ^a	8.6 ± 0.2 ^c	25.0 ± 1 ^c	28.6 ± 0.3 ^a	30.0 ± 0.7 ^b	29.0 ± 0.8 ^a
Tetracycline (30 µg)	35.9 ± 0.5 ^d	31.1 ± 0.5 ^c	8.0 ± 0.1 ^j		22.2 ± 0.2 ^c	23.5 ± 0.2 ^c	14.2 ± 0.5 ^b	31.0 ± 0 ^b	26.6 ± 0.4 ^b	29.3 ± 0.4 ^b	26.8 ± 0.5 ^b
Ampicillin (10 µg)	43.2 ± 1.0 ^b	39.6 ± 0.7 ^b	30.5 ± 0.2 ^d		14.2 ± 0.13 ^d	27.0 ± 0.3 ^b	-	25.0 ± 0 ^c	10.0 ± 0.2 ^d	26.4 ± 0.3 ^c	22.5 ± 0.5 ^d
Carbenicillin (100 µg)	44.8 ± 0.9 ^a	42.6 ± 0.4 ^a	37.2 ± 0.6 ^b		28.4 ± 0.3 ^a	22.0 ± 0.6 ^d	20.0 ± 0.9 ^a	34.0 ± 1 ^a	8.0 ± 0.4 ^e	34.0 ± 0.9 ^a	25.0 ± 0.3 ^c
Erythromycin (15 µg)	38.9 ± 1.5 ^c	30.9 ± 0.9 ^c	39.0 ± 0.4 ^a		11.6 ± 0.4 ^e	12.4 ± 0.7 ^e	-	14.6 ± 1 ^d	13.0 ± 0.2 ^c	9.3 ± 0.1 ^f	12.9 ± 0.4 ^e
DMSO	-	-	-	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-	-	-	-

a, b, c, d, e, f, g, h, i, j Mean-values (± standart error) with the same letters within vertical columns are not significantly different (P> 0.05).

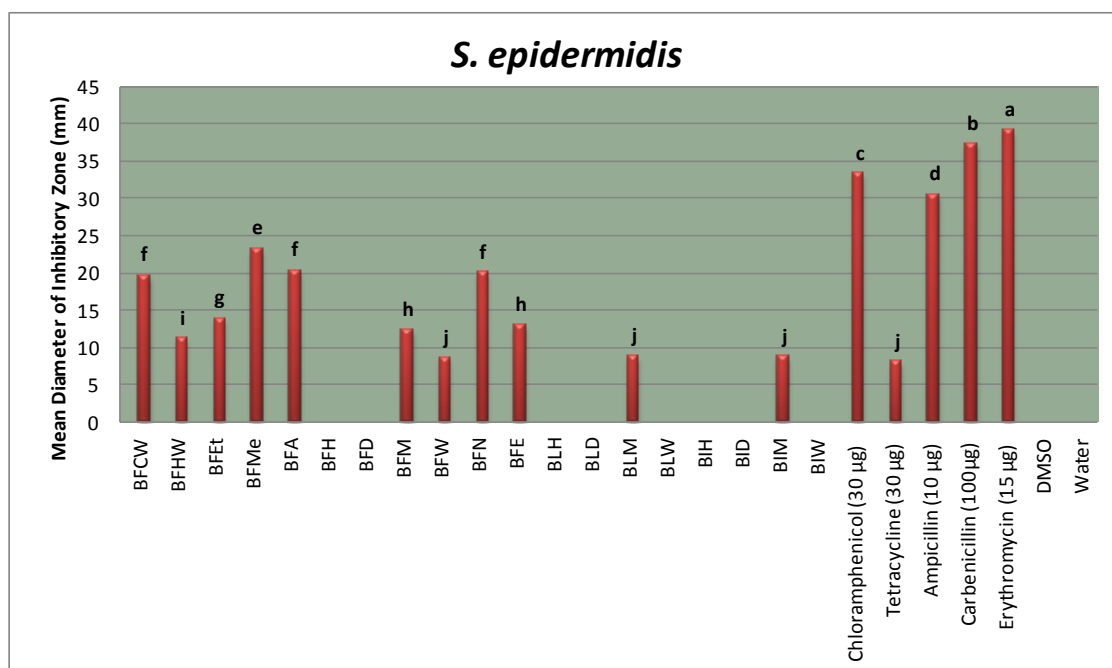


Figure 25. Antibacterial activity of *B. perennis* extracts and controls against *S. epidermidis*.



Figure 26. Antibacterial activity of BFCMe (methanol extract of field-grown flowers) (left) against *S. epidermidis* by comparing with positive control (tetracycline) (right).

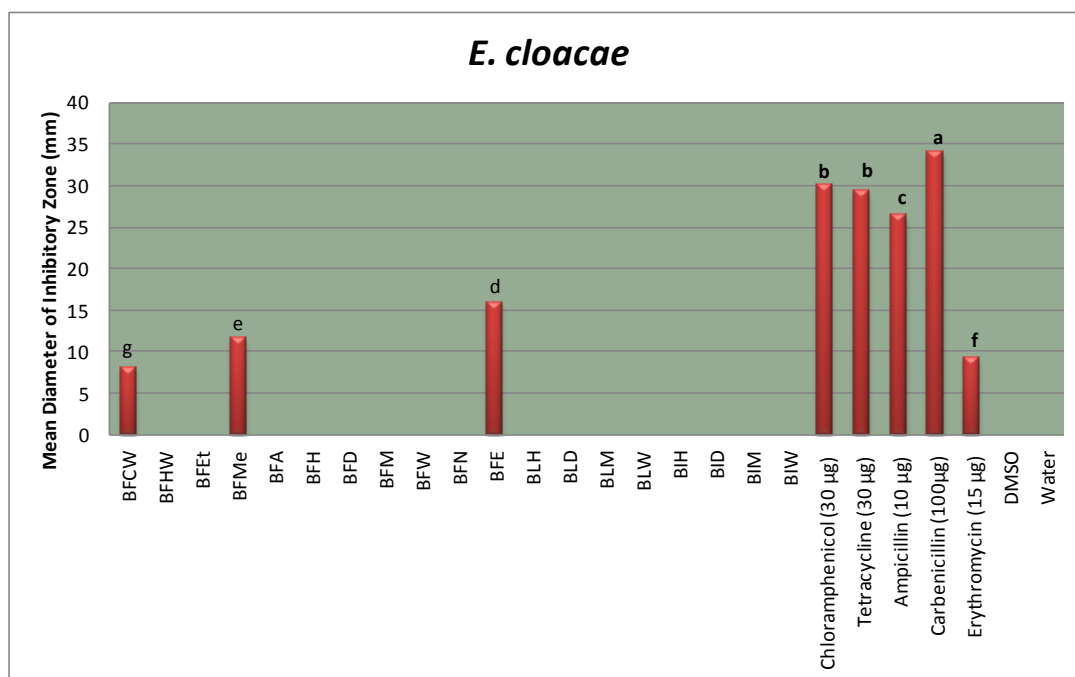


Figure 27. Antibacterial activity of *B. perennis* extracts and controls against *E. cloacae*.

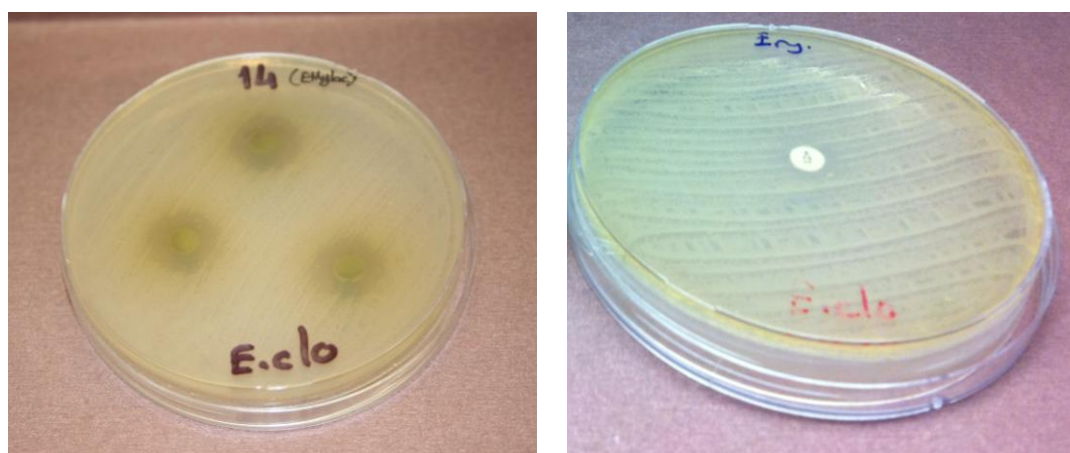


Figure 28. Antibacterial activity of BFE (ethyl acetate extract of field-grown flowers) (left) against *E. cloacae* by comparing with positive control (erythromycin) (right).

Kavalcioğlu et al. (2010) showed the antimicrobial activity of only methanolic extract and volatiles of *B. perennis* against 6 bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Proteus vulgaris* and *Salmonella typhimurium*) by using Broth microdilution method. The MICs (Minimum Inhibitory Concentration) of methanol extract of *B. perennis* was found as > 2.0 mg/ml, with moderate to low antimicrobial activity (Kavalcioğlu, 2010). Avato et al. (1997) showed the antimicrobial activity of essential oils (methyl deca-4,6-diynoate and deca-4,6-diynoic acid, and their structural analogues, deca-4,6-diyne, dimethyl octa-3,5-diyne-1,8-dioate and deca-4,6-diyne-1,10-dioic acid) from the aerial organs of *B. perennis*. Only deca-4,6-diynoic acid and deca-4,6-diyne-1,10-dioic acid showed antimicrobial activity, being the two compounds mainly effective against Gram-positive (*Micrococcus luteus*, *Bacillus subtilis*, *Enterococcus faecalis* and *S. aureus*) and Gram-negative (*Acinetobacter calcoaceticus* and *E. coli*) bacteria, respectively.

Antibacterial activities of field-grown flowers were better than field-grown leaves and *in vitro*-derived leaves against the used bacteria. There is evidence that 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). The antibacterial activity of *B. perennis* may be attributed to the phenolics, triterpene saponins and essential oil constituents. Previously 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). Methanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, and flavonoids. A general procedure for extracting of flavonoid-rich fraction was based on liquid–liquid extraction of total extract with ethyl acetate (Samsam-Shariat, 1992). The ethyl acetate fraction of *B. perennis* has good inhibition activity of bacterial growth against *E. cloae* may due to its flavonoid contents (Table 8). The phenolics were the predominant active chemicals in plants, with Gram positive bacteria being the most sensible germs (Rios and Recio, 2005). The effectiveness of methanol extracts of *B. perennis* may be explained by the inhibitory effect of these compounds on bacteria. Strong antibacterial activity of the flower extracts may be attributed to high flavonoid contents (Table 8). In consistent with our results, Nazaruk and Gudej (2001) demonstrated higher flavonoid contents of flowers than leaves of *B. perennis*. Strong antibacterial activity of *B. perennis* extracts against *S. epidermidis* and *E. cloacea* may explain why common daisy is used in folk medicine as vulnerary and anti-inflammatory.

4.3.2 Antitumor activity

Antitumor activity of *B. perennis* flower extracts was tested with potato disc method as modified by McLaughlin's group (Ferrigni et al., 1982). Extracts (BFHW, BFCW, BFEt and BFMe) were dissolved in water to prepare final concentrations of extracts (100, 1.000, 10.000 and 100.000 mg/l). Antitumor activity of all flower extracts was depended on concentration value. High concentration (100.000 mg/l) of all tested extracts showed strong antitumor activity (100% tumor inhibition) (Table 11 and Figure 29). Low concentrations (100 and 1.000 mg/l) of extracts showed moderate antitumor activity (Table 11 and Figure 29). Generally, when compared

with negative control (water), the percentage inhibition of all extracts was more than 35% in three separate experiments (Table 11; Figure 29 and Figure 30). No tumor formation was observed with positive control (camptothecin) (100 % inhibition) (Table 11; Figure 31).

Table 11. Mean number of tumors and % tumor inhibition of *B. perennis* flower extracts at 100, 1.000, 10.000 and 100.000 mg/l, camptothecin (positive control) and water (negative control).

Treatments	Mean number of tumors ($\pm$ SE)				% Tumor Inhibition
Water	40	$\pm$	1.7	^g	-
Camptothecin	0	$\pm$	0	^a	100
BFHW (100000mg/l)	0	$\pm$	0	^a	100
BFHW (10000mg/l)	16	$\pm$	1.7	^{cd}	60
BFHW (1000mg/l)	22	$\pm$	1.6	^{ef}	45
BFHW (100mg/l)	25	$\pm$	2.1	^f	37.5
BFCW (100000mg/l)	0	$\pm$	0	^a	100
BFCW (10000mg/l)	20	$\pm$	1.3	^{de}	50
BFCW (1000mg/l)	22	$\pm$	1.9	^{ef}	45
BFCW (100mg/l)	26	$\pm$	2.7	^f	35
BFEt (100000mg/l)	0	$\pm$	0	^a	100
BFEt (10000mg/l)	8	$\pm$	1.3	^b	80
BFEt (1000mg/l)	12	$\pm$	1.1	^{bc}	70
BFEt (100mg/l)	15	$\pm$	1.9	^c	62.5
BFMe (100000mg/l)	0	$\pm$	0	^a	100
BFMe (10000mg/l)	9	$\pm$	1.5	^{bc}	77.5
BFMe (1000mg/l)	21	$\pm$	2	^{ef}	47.5
BFMe (100mg/l)	22	$\pm$	1.8	^{ef}	45

a, b, c, d, e, f, g Mean values ($\pm$ standart error) with the same letter are not significantly different at $P>0.05$.

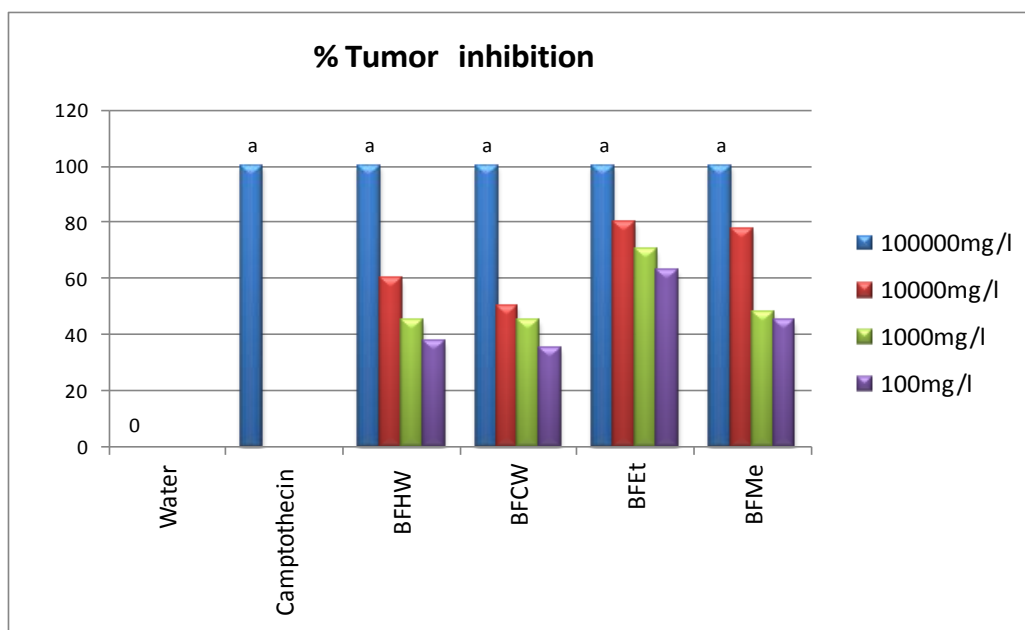


Figure 29. Percentage of tumor inhibition of different concentrations (100, 1.000, 10.000 or 100.000 mg/l) of *B. perennis* flower extracts and controls (water and camptothecin).



Figure 30. Tumors produced by *A. tumefaciens* with distilled water (solvent control I-negative control) (left) and negative control closed vision (right).



Figure 31. Tumors produced by *A. tumefaciens* with BFMe (methanol extract of field-grown flowers) (left), camptothecin (positive control) (right).

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

Ethanol and methanol 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 antitumor activity against *A. tumefaciens* (Figure 17; Table 8). This bioassay has the advantage of being rapid, inexpensive, safe and statistically reliable prescreens for 3PS antitumor activity. 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). Extracts are screened initially for biological activity and may be fractionated with subsequent identification of active ingredients (Boonkaew and Camper, 2005).

Cytotoxic activity of underground parts (roots) of common daisy was reported by Li et al., (2005). Potato disc tumor assay was applied as a preliminary study in order to test whether *B. perennis* flower extracts have an antitumor activity or not. Our study revealed the strong antitumor activity of *B. perennis* flower extracts for the first time. This study guided us to carry out some anticancer and cytotoxicity experiments using different cancer cell lines with different extracts of *B. perennis*.

4.3.3 Toxicity of *B. perennis* (brine shrimp bioassay)

Toxicity of common daisy flower extracts was tested with brine shrimp bioassay. Brine shrimp bioassay determines the toxicity of materials toward brine shrimp larvae and can predict the ability to kill cancer cells in cell cultures, various pests, and exert a wide range of pharmacological effects (McLaughlin et al., 1991). Meyer et al. (1982) found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. Results of this assay (Table 12; Figure 32) showed that extracts of common daisy were low toxic at higher doses

(around 1.000-2.500 mg/l). Among the tested extracts, methanol and cold water extracts was more toxic than ethanol and hot water extracts (Table 12). As shown in Table 11, methanol and cold water extracts can be regarded as containing more toxic compounds than other extracts ($LC_{50} > 1.000$ mg/l). In addition, they showed significant activity in the potato disc bioassay (Table 11; Figure 29). However, it is interesting to note that, although all extracts showed significant activity in tumor inhibition, they displayed no toxicity in the brine shrimp test ($LC_{50} > 1.000$ mg/l) (Meyer et al., 1982). This bioassay has the advantage of being rapid (24 hr 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). Obtained results of brine shrimp toxicity assay were correlated with resazurin cytotoxic assay in our study. For example, the least toxicity was obtained with BFHW extract (infusion extract) of *B. perennis* in the brine shrimp toxicity assay (Table 12). Similarly, the least toxicity was obtained with BF-inf extract in the resazurin cytotoxic assay (Table 13). Moreover, the methanolic extract of *B. perennis* flowers exhibited the highest toxicity in both brine shrimp and resazurin toxicity assays.

Table 12. LC₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values of *B. perennis* flower extracts and MS-222 (a positive control). MS-222: Tricaine methane sulfonate.

Treatments	LC ₅₀ (mg/l)	95% Confidence Intervals
BFHW (<i>Bellis</i> -flower-hot water)	2418	1530 - 3822
BFCW (<i>Bellis</i> -flower-cold water)	1087	688 - 1717
BFEt (<i>Bellis</i> -flower-EtOH)	1334	844 - 2109
BFMe (<i>Bellis</i> -flower-MeOH)	1008	638 - 1593
MS-222	47	30 - 74

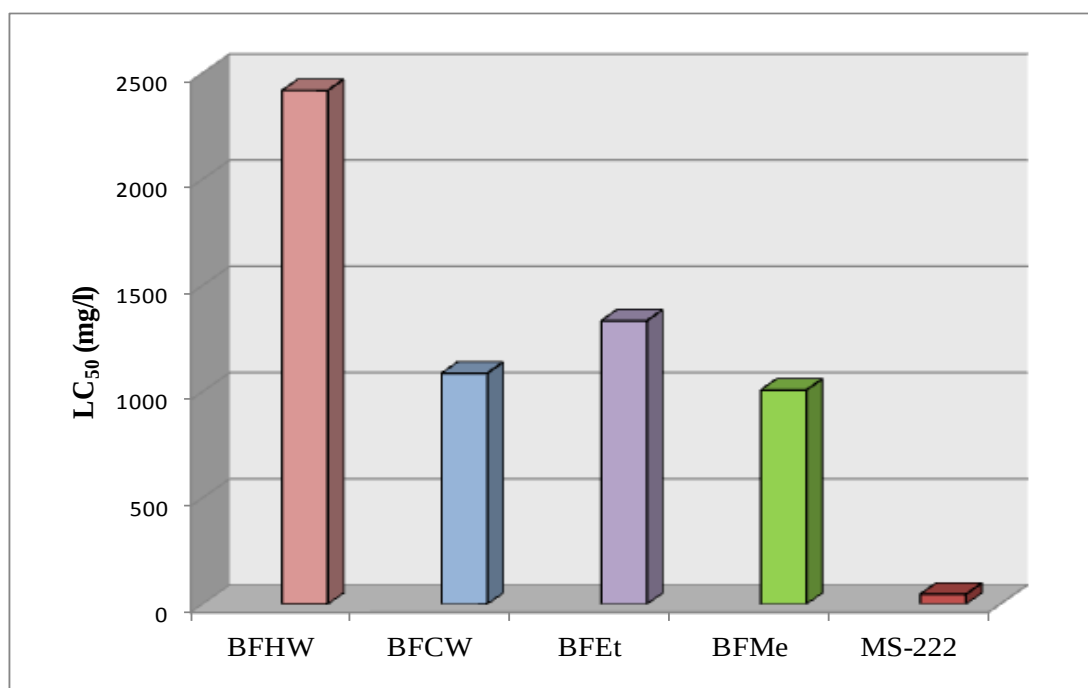


Figure 32. LC₅₀ (lethal concentration for 50% mortality after 24 hr exposure) values for common daisy extracts and MS-222 (a positive control). MS-222: Tricaine methane sulfonate.

4.3.3 Anticancer and cytotoxicity

There are no reports about anticancer activity of aerial parts of *B. perennis* extracts on human lung carcinoma (A-549), human colon adenocarcinoma (DLD-1) and human breast adenocarcinoma (MCF-7) up to now. Six saponins isolated from the roots of *B. perennis* were found to be cytotoxic against only human promyelocytic leukemia (HL-60) cells (Li et al., 2005).

Cytotoxicity of hexane, DCM, MeOH and water extracts of field-grown flowers, field-grown leaves and *in vitro*-derived leaves, and two fractions (*n*-butanol and ethyl acetate) of *B. perennis* was evaluated against to human lung carcinoma (A-549) and human colon adenocarcinoma (DLD-1) cell lines with resazurin test. Inhibition of the cell proliferation was assessed using the resazurin test as the vital strain (Figure 33; Figure 34). Moreover, anticancer activity of these extracts was evaluated against to human breast adenocarcinoma (MCF-7) cell line with MTT assay. Percentage of the cell viability was measured by MTT assay. A plant extract is usually regarded as interesting for *in vitro* cytotoxic activity when $IC_{50} < 100 \mu\text{g/ml}$ (Boyd, 1997).

In resazurin test, among the tested extracts, *in vitro*-derived leaf extracts showed the best anticancer activity against to selected cell lines (Table 13). The DCM extract of *in vitro*-derived leaves showed a strong anticancer activity against to human lung carcinoma (A-549) cells, with IC_{50} values of $15.6 \pm 0.5 \mu\text{g/ml}$ and showed a moderate anticancer activity against to human colon adenocarcinoma (DLD-1) cells, with IC_{50} values of 69 ± 14 (Table 12). MeOH extracts of *in vitro*-derived leaves showed a moderate anti-cancer activity against to A-549 and DLD-1

cell lines, with IC_{50} values of 61 ± 6 and 62 ± 7 $\mu\text{g/ml}$, respectively (Table 13). However, hexane and water extracts were inactive against to A-549, and DLD-1 with an IC_{50} value higher than 100 $\mu\text{g/ml}$ (Table 13). In addition, *n*-butanol fraction exerted the most potent cytotoxic activity (with IC_{50} values of 16 ± 3 $\mu\text{g/ml}$ and 10 ± 2 $\mu\text{g/ml}$), showing stronger activity than ethyl acetate fraction (with IC_{50} values of 25 ± 8 $\mu\text{g/ml}$ and 20 ± 4 $\mu\text{g/ml}$) towards the lung carcinoma (A-549) and colon adenocarcinoma (DLD-1) human cell lines (Table 13). MeOH extracts of field-grown flowers and leaves showed a moderate anti-cancer activity against to A-549 and DLD-1 cell lines, with IC_{50} values of 78 ± 7 ; 88 ± 8 and 75 ± 2 ; 93 ± 12 $\mu\text{g/ml}$, respectively (Table 13). However, hexane, DCM and water extracts of field-grown flowers and leaves were inactive against to A-549, and DLD-1 with an IC_{50} value higher than 100 $\mu\text{g/ml}$ (Table 13).

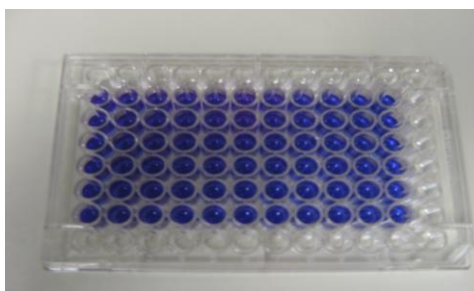


Figure 33. Resazurine test plate at zero time.

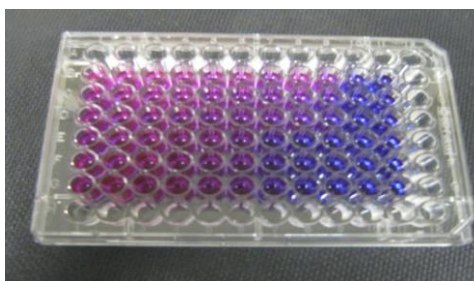


Figure 34. Resazurine test plate of DCM and methanol extracts of field-grown flowers after incubation.

Table 13. *In vitro* anticancer results of *B. perennis* extracts and fractions on human lung carcinoma (A-549) and colon adenocarcinoma (DLD-1) cell lines.

Treatments	IC ₅₀ (μg ml ⁻¹) ^a	
	Resazurine	
	A-549	DLD-1
BFH (<i>Bellis</i> -flower-hexane)	>200	>200
BFD (<i>Bellis</i> -flower-DCM)	117 ± 9	164 ± 8
BFM (<i>Bellis</i> -flower-MeOH)	78 ± 7	75 ± 2
BFW (<i>Bellis</i> -flower-water)	>200	>200
BF-dec. (<i>Bellis</i> -flower-decoction)	>200	>200
BF-inf. (<i>Bellis</i> -flower-infusion)	>200	>200
BFN (<i>Bellis</i> -flower-<i>n</i> -butanol)	16 ± 3	10 ± 2
BFE (<i>Bellis</i> -flower-ethyl acetate)	25 ± 8	20 ± 4
BLH (<i>Bellis</i> -leaf-hexane)	>200	>200
BLD (<i>Bellis</i> -leaf-DCM)	>200	>200
BLM (<i>Bellis</i> -leaf-MeOH)	88 ± 8	93 ± 12
BLW (<i>Bellis</i> -leaf-water)	>200	>200
BIH (<i>Bellis</i> -<i>in vitro</i> -hexane)	128 ± 14	178 ± 7
BID (<i>Bellis</i> -<i>in vitro</i> -DCM)	15.6 ± 0.5	69 ± 14
BIM (<i>Bellis</i> -<i>in vitro</i> -MeOH)	61 ± 6	62 ± 7
BIW (<i>Bellis</i> -<i>in vitro</i> -water)	>200	>200
Etoposide^b	8.9 ± 0.9 μM	6.7 ± 0.2 μM

Note: Mean values (± standart deviation) for triplicate assays.

^a Concentration of extract that cause 50% inhibition of cell proliferation.

^b Positive control.

In MTT assay, among the tested extracts, methanolic extracts of *B. perennis* showed the best antiproliferative activity against to selected cell line (MCF-7) at 200 µg/ml concentration (Table 14). MeOH extract of *in vitro*-derived leaves showed a strong anticancer activity against to human breast adenocarcinoma (MCF-7) cells, with the percentage of the cell viability values of 7.5% at 200 µg/ml and 14.2% at 100 µg/ml concentrations (Table 14). MeOH extract of field-grown flowers also showed a strong anticancer activity against to human breast adenocarcinoma (MCF-7) cells, with the percentage of cell viability values of 8.9% at 200 µg/ml and 23.6% at 100 µg/ml concentrations (Table 14). MeOH extract of field-grown leaves showed a high anticancer activity against to human breast adenocarcinoma (MCF-7) cells, with the percentage of the cell viability value of 19.3% at 200 µg/ml but it showed weak anticancer activity with the percentage of the cell viability value of 82.7% at 100 µg/ml concentrations (Table 14). Other tested extracts except DCM extract of *in vitro*-derived leaves did not show any antiproliferative activity against to MCF-7 cell line at all used concentrations. Shape of dose-response curves indicated a significant inhibition of cell growth in dose-dependent manner (Figure 35). Generally, methanolic extracts were more active on MCF-7 cell line. Methanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, and flavonoids (Samsam-Shariat, 1992). In present study, TLC plates also showed that methanolic extracts contain more phenolic and saponin compounds qualitatively than other tested extracts (Figure 17 and Figure 19).

Table 14. Percentage of antiproliferative activity of *B. perennis* extracts and fractions on human breast adenocarcinoma (MCF-7) cell line. Cells were treated with various concentrations (6.25, 12.5, 25, 50, 100, 200 µg/ml) of extracts, after 24h of exposure. % of cell viability (MCF-7) was measured by MTT assay. Results were expressed as means $\pm$ SD for three independent determinations.

Treatments	% MCF-7 Cell Viability					
	Concentrations					
	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
BFH	102.7 $\pm$ 0.5	101.0 $\pm$ 1.1	106.0 $\pm$ 1.9	106.9 $\pm$ 2.5	106.2 $\pm$ 4.3	106.3 $\pm$ 2.9
BFD	90.2 $\pm$ 2.0	91.3 $\pm$ 2.1	90.2 $\pm$ 1.5	92.2 $\pm$ 2.3	92.5 $\pm$ 2.7	94.7 $\pm$ 1.0
BFM	89.3 $\pm$ 2.2	88.4 $\pm$ 2.2	84.0 $\pm$ 0.6	84.0 $\pm$ 1.9	23.6 $\pm$ 3.6	8.9 $\pm$ 0.2
BFW	96.0 $\pm$ 2.8	90.8 $\pm$ 3.7	91.7 $\pm$ 2.4	86.2 $\pm$ 2.0	83.7 $\pm$ 0.6	25.4 $\pm$ 1.9
BLH	107.9 $\pm$ 1.0	108.5 $\pm$ 1.1	108.5 $\pm$ 2.3	109.5 $\pm$ 0.4	112.5 $\pm$ 0.3	117.9 $\pm$ 2.5
BLD	96.7 $\pm$ 1.9	95.5 $\pm$ 2.7	97.8 $\pm$ 1.4	99.1 $\pm$ 1.3	100.4 $\pm$ 1.3	97.1 $\pm$ 0.7
BLM	98.1 $\pm$ 0.6	94.1 $\pm$ 2.2	93.9 $\pm$ 2.2	89.9 $\pm$ 0.4	82.7 $\pm$ 1.6	19.3 $\pm$ 0.6
BLW	102.6 $\pm$ 1.5	99.3 $\pm$ 1.3	96.3 $\pm$ 1.4	97.8 $\pm$ 1.6	98.4 $\pm$ 2.3	98.3 $\pm$ 1.5
BIH	109.2 $\pm$ 2.4	103.5 $\pm$ 3.3	93.1 $\pm$ 3.5	71.9 $\pm$ 1.9	70.5 $\pm$ 1.8	103.9 $\pm$ 2.7
BID	98.4 $\pm$ 0.8	99.7 $\pm$ 1.4	101.2 $\pm$ 2.0	99.6 $\pm$ 2.4	101.5 $\pm$ 3.3	62.9 $\pm$ 3.6
BIM	99.6 $\pm$ 1.9	95.3 $\pm$ 2.5	92.2 $\pm$ 2.2	86.4 $\pm$ 2.5	14.2 $\pm$ 2.5	7.5 $\pm$ 0.3
BIW	106.0 $\pm$ 0.8	101.7 $\pm$ 1.0	99.0 $\pm$ 1.3	102.0 $\pm$ 1.3	98.8 $\pm$ 3.0	102.1 $\pm$ 0.5

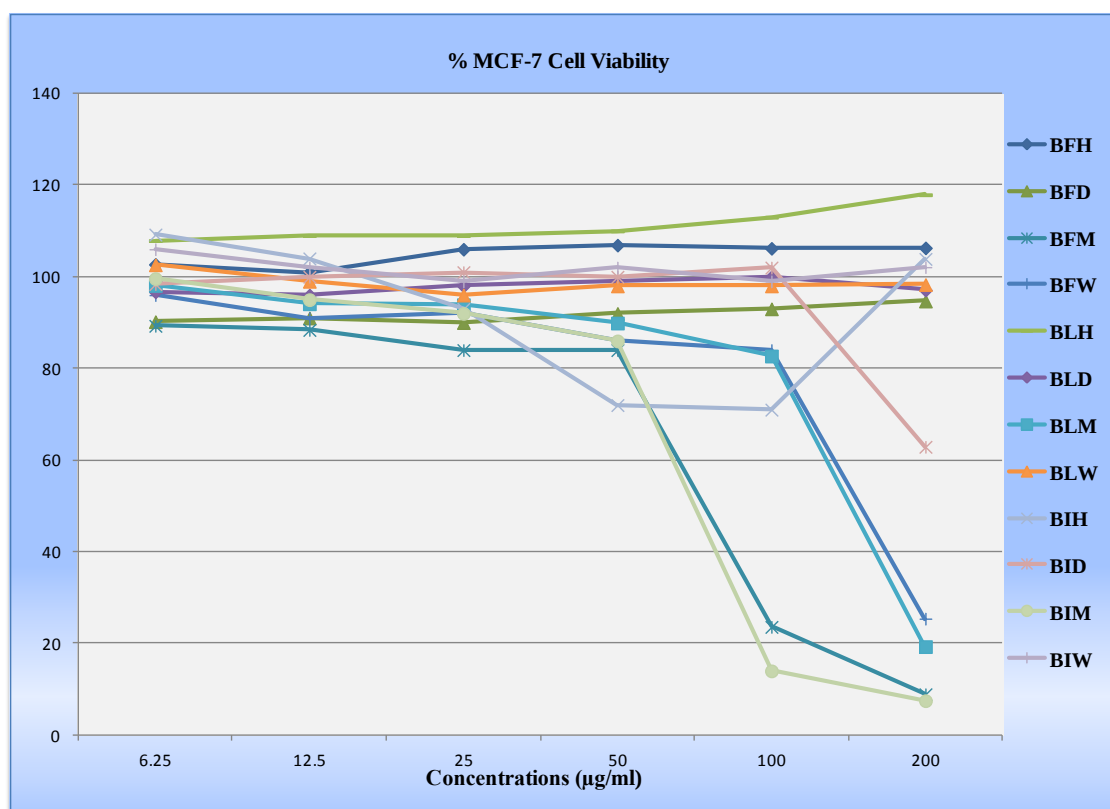


Figure 35. Dose-response curves of the effects of *B. perennis* extracts on cell growth in MCF-7 cells. Cells were treated with various concentrations (6.25, 12.5, 25, 50, 100, 200 µg/ml) of extracts, after 24h of exposure. Antiproliferative effects were measured by MTT assay. Results were expressed as means $\pm$ SD for three independent determinations.

Natural products have been shown to be an excellent and reliable source for the development of new drugs (Newman and Cragg, 2007). Triterpenoids are the major constituents of some medicinal herbs and are widely present in all parts of a variety of plants (Connolly and Hill, 2005). Thousands of structures have been reported with hundreds of new derivatives being described each year (Connolly and Hill, 2007). The biological activities of triterpenoids have recently attracted more attention in the biochemical and medical fields because of their immunomodulatory

and antitumor effects (Dzubak et al., 2006). Saponins are a class of natural compounds, which possess many bioactivities, such as anti-spasmodic (Barile et al., 2005), anti-inflammatory, ion channel-blocking, immune-stimulating, anti-fungal (Lanzotti, 2005), anti-fertility (Pant et al., 1988), and anti-tumor activity (Sparg et al., 2004; Sharma et al., 2012). Recently, some triterpenoids have been shown to promote specific and non-specific immune response in different ways, and their antitumor activity might be achieved by improving immunity (Wu et al., 1990; Li et al., 2000; You et al., 2001; Shishodia et al., 2003; Feng et al., 2006; Ikeda et al., 2007). Natural products play a significant role in human health in relation to the prevention and treatment of inflammatory conditions. Among them, terpenoids (also referred to as terpenes), are the largest and most widespread class of secondary metabolites. Some terpenoids have been used for therapeutic purposes for centuries as antibacterial, anti-inflammatory, antitumoral agents, and in recent decades research activity into the clinical potential of this class of compounds has increased continuously as a source of pharmacologically interesting agents (Heras and Hortelano, 2009).

Saponins are well known compounds in *B. perennis* and they have anticancer activity against human promyelocytic leukemia (HL-60) cells (Li, et al., 2005). In the present study, TLC results showed that DCM extract of *in vitro*-derived leaves of *B. perennis* have more saponins than other extracts, quantitatively (Figure 16). Saponin contents of DCM extract of *in vitro*-derived leaves were found to be higher than in the field-grown flowers and leaves of *B. perennis*. In addition, MeOH extracts of flowers, leaves and *in vitro*-derived leaves of *B. perennis* have more phenolic compounds and saponins than other extracts, quantitatively (Figure 17). Thus, in our

present study, the high levels of anticancer activity of DCM extract of *in vitro*-derived leaves and MeOH extracts of field-grown flowers, leaves and *in vitro*-derived leaves may be attributed to this high saponin and phenolic contents. Extracts of different parts (field-grown flowers, field-grown leaves and *in vitro*-derived leaves) of *B. perennis* have anti cancer activity against the lung carcinoma (A-549), colon adenocarcinoma (DLD-1) and breast adenocarcinoma (MCF-7) human cell lines. Altogether, these results support the traditional use of *B. perennis* in the treatment of cancer and breast cancer.

4.3.5 Anti-inflammatory

Anti-inflammatory activities of twelve different *B. perennis* extracts (hexane, dichloromethane, methanol and water) of field-grown flowers, leaves and *in vitro*-derived leaves were evaluated by measuring their capacity to inhibit cellular NO generation. Nitric oxide is an endogenous free radical species that is synthesized from L-arginine by nitric oxide synthase (NOS) in various tissues. This radical is an important regulator of physical homeostasis, whereas large amounts have been closely correlated with the pathophysiology of a variety of diseases and inflammations (Marletta, 1993). Therefore, the inhibition of NO production may be a useful strategy for the treatment of various inflammatory disorders (Choi et al., 2007).

A variety of *in vitro* and *in vivo* experiments have shown that several phenolic compounds possess anti-inflammatory activities (Middleton, 1998). Phenolic compounds have been reported to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of nitric oxide (NO) (Jiang

and Dusting, 2003). The anti-inflammatory activities of many plants have been attributed to their triterpene (Ghannadi et al., 2005) or flavonoid contents (Ahmad et al., 1983). It has been also showed that various flavonoids (such as rutin, quercetin, and luteolin), biflavonoids, and triterpenoids (such as ursolic acid) produced significant antinociceptive and/or anti-inflammatory activities (La Casa et al., 2000; Silva et al., 2005; Sabu and Kuttan, 2002; Çadırcı et al., 2012).

In the inflammation process, NO is produced from L-arginine by the inducible NO synthase (iNOS). Peroxynitrite, formed by the rapid reaction between superoxide and NO, is a toxic substance that contributes to tissue injury in inflammatory diseases (Szabo, 2003). Some terpenoids have demonstrated to possess anti-inflammatory properties (Fernandes et al., 2007). The presence of phenolic and terpenoid compounds in *B. perennis* extracts prompted us to investigate their anti-inflammatory activity.

The anti-inflammatory activity of *B. perennis* extracts was evaluated using LPS-stimulated RAW 264.7 macrophages. Stimulation of RAW 264.7 macrophages by LPS induces iNOS and overproduction of NO. NO released from cells can be detected and quantified photometrically as its stable product, nitrite, by a simple colorimetric reaction (Griess reaction) as described in materials and methods. L-NAME, a NO synthase inhibitor, prevents the formation of NO in LPS-stimulated RAW 264.7 macrophages (Paul et al., 1997) and thus was used as positive control. As presented in Table 15 and Figure 36, the MeOH extract of field-grown flowers from *B. perennis* showed stronger inhibitory effect on LPS-induced NO secretion with 96.3% inhibition than DCM (86.4% inhibition), hexane (25.3% inhibition) and water (14.2% inhibition) extracts at 20 µg/ml concentration (Table 15 and Figure

36). DCM extract of field-grown leaves from *B. perennis* showed stronger inhibitory effect on LPS-induced NO secretion with 31.9% inhibition than hexane (15.7% inhibition), MeOH (19% inhibition) and water (7.1% inhibition) extracts at 20 µg/ml (Table 15 and Figure 36). DCM extract of *in vitro*-derived leaves from *B. perennis* showed stronger inhibitory effect on LPS-induced NO secretion with 87.5% inhibition than MeOH (43.7% inhibition), hexane (26.9% inhibition) and water (7.2% inhibition) extracts observed at 20 µg/ml (Table 15 and Figure 36). Comparatively, L-NAME used as positive control inhibited NO release by 65.3% at 250.0 µM (67.4 µg/ml).

Cytotoxic effect of the extracts was evaluated on macrophages (RAW 264.7 cells) using resazurin to ensure that the anti-inflammatory activity was not due to cytotoxicity. All extracts were not found to be significantly cytotoxic at doses ranging from 1.25 to 40 µg/ml (data not shown).

A relationship between the inhibition of cancer cell proliferation and inflammatory mediator production by extracts has been hypothesized. Indeed, inflammatory mediators, such as NO and cytokines, have been reported to contribute to mutagenesis (Marletta, 1993). It has been shown that the activity of the enzyme NOS is consistent in human cancer and its selective modulation has been suggested as a potential strategy for chemoprevention and reduction of cancer cell proliferation (Ahmad et al., 1997; Nishikawa et al., 2004; Bourgou et al., 2010). In this study, the effect of *B. perennis* extracts was also evaluated on the growth of cancer cell lines.

The strong anti-inflammatory activity of MeOH extract of field-grown flowers may be attributed to the reported phenolic, flavonoid and terpenoid

constituents of the plants. Altogether, these results support the traditional use of *B. perennis* in the treatment of inflammation diseases implicating NO and peroxynitrite.

Table 15. Effects of *B. perennis* extracts (20.0 µg/ml) and L-NAME (250 µM) on NO production in LPS-stimulated RAW-264.7 macrophages. Values are mean ± S.D of three replications.

Treatments	NO Inhibition (%) ± Standart Deviation
BFH (<i>Bellis</i> -flower-hexane)	25.3 ± 0.19
BFD (<i>Bellis</i> -flower-DCM)	86.4 ± 0.29
BFM (<i>Bellis</i> -flower-MeOH)	96.3 ± 0.11
BFW (<i>Bellis</i> -flower-water)	14.2 ± 0.4
BLH (<i>Bellis</i> -leaf-hexane)	15.7 ± 3.12
BLD (<i>Bellis</i> -leaf-DCM)	31.9 ± 1.26
BLM (<i>Bellis</i> -leaf-MeOH)	19 ± 0.3
BLW (<i>Bellis</i> -leaf-water)	7.1 ± 1.69
BIH (<i>Bellis</i> -in vitro -hexane)	26.9 ± 1.1
BID (<i>Bellis</i> -in vitro -DCM)	87.5 ± 0.55
BIM (<i>Bellis</i> -in vitro -MeOH)	43.7 ± 0.2
BIW (<i>Bellis</i> -in vitro -water)	7.2 ± 1.3
L-NAME (positive control)	65.3 ± 5.97

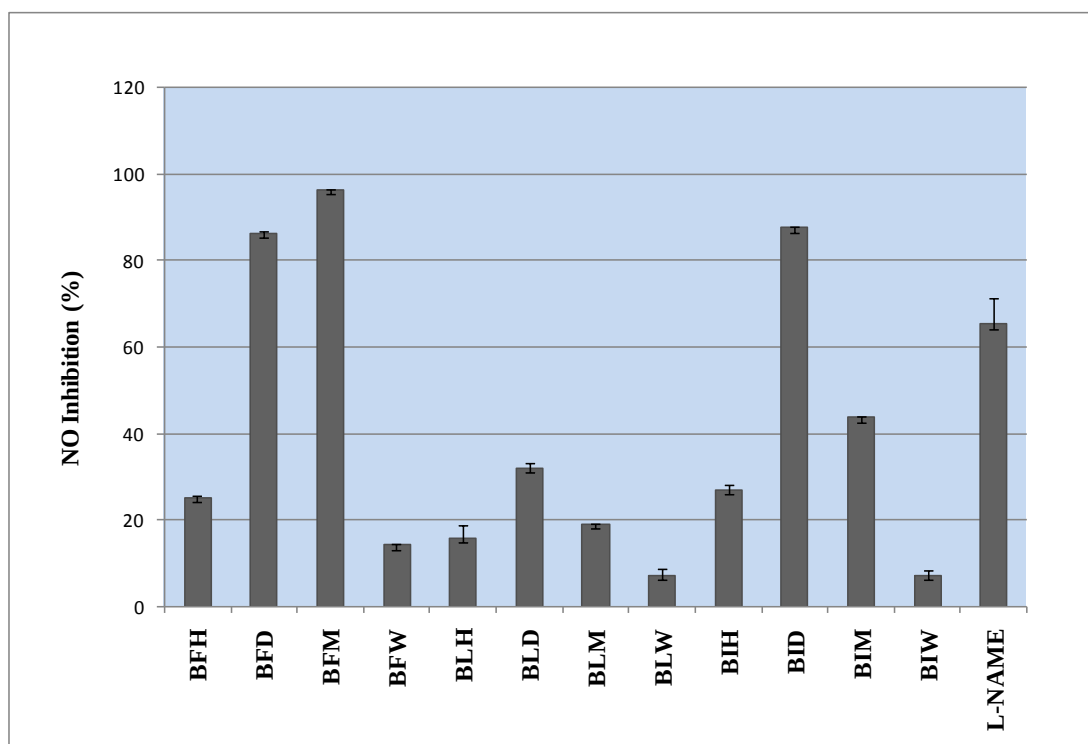


Figure 36. Effects of *B. perennis* extracts (20.0 μ g/ml) and L-NAME (250 μ M) on NO production in LPS-stimulated RAW-264.7 macrophages. Values are mean $\pm$ S.D of three replications.

4.3.6 Antioxidant activity

In the previous studies (Siatka and Kasparova, 2010; Kavalcioglu et al., 2010), it has been reported that flower extracts of *B. perennis* possessed some indirect antioxidant activity. The DPPH radical scavenging activity, reducing power, total antioxidant capacity (thiocyanate method), total phenolic and total flavonoid contents of flower extracts of *B. perennis* were also shown (Siatka and Kasparova, 2010; Kavalcioglu et al., 2010). However, there is no information about the antioxidant activity of field-grown leaf and *in vitro*-derived leaf extracts and the direct antioxidant activity of *B. perennis* extracts.

In the present study, the antioxidant activity of hexane, DCM, methanol and water extracts of field-grown flowers, leaves and also *in vitro*-derived leaves was assessed. The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteu), total flavonoid content (aluminum chloride colorimetric), oxygen radical absorbing capacity (ORAC) and direct antioxidant assays (cell-based assay using 2',7'-dichlorofluorescein-diacetate) were used in this assessment.

In DPPH assay, methanol extracts of *B. perennis* showed a concentration dependent antiradical scavenging activity (%) by scavenging DPPH radical. Free radical scavenging activity elevated when concentrations increased from 12.5 to 100 µg/ml. Methanol extracts of leaves (95.15% at 100 µg/ml) and flowers (94% at 100 µg/ml) showed higher DPPH radical scavenging activity compared to the methanol extract of *in vitro*-derived leaves (58.2% at 100 µg/ml). The field-grown leaf and flower extracts had high active radical scavenge capability as much as ascorbic acid (97.21% at 100 µg/ml) that was used as control antioxidant (Table 16; Figure 37 and Figure 38). Similarly, DPPH radical scavenging activity of aqueous and methanol extracts of aerial parts of *B. perennis* was shown by Kavalcıoğlu et al., (2010). Their results were 85.8%, 79.6% at 102.5 µg/ml, respectively.

Table 16. Free-radical (DPPH) scavenging activity (%) of MeOH extracts of field-grown flowers (BFM), leaves (BLM) and *in vitro*-derived leaves (BIM) of *B. perennis* at different concentrations and ascorbic acid (positive control).

Treatments	% Inhibition of DPPH $\pm$ SD			
	Concentrations			
	12.5 μ g/ml	25 μ g/ml	50 μ g/ml	100 μ g/ml
Ascorbic acid	48.77 $\pm$ 0.07	62.35 $\pm$ 0.02	97.01 $\pm$ 0.03	97.21 $\pm$ 0.04
BFM	38.80 $\pm$ 0.02	55.66 $\pm$ 0.05	78.75 $\pm$ 0.06	94.00 $\pm$ 0.09
BLM	40.88 $\pm$ 0.04	60.83 $\pm$ 0.03	81.11 $\pm$ 0.02	95.15 $\pm$ 0.05
BIM	12.93 $\pm$ 0.02	26.10 $\pm$ 0.02	43.65 $\pm$ 0.05	58.20 $\pm$ 0.03

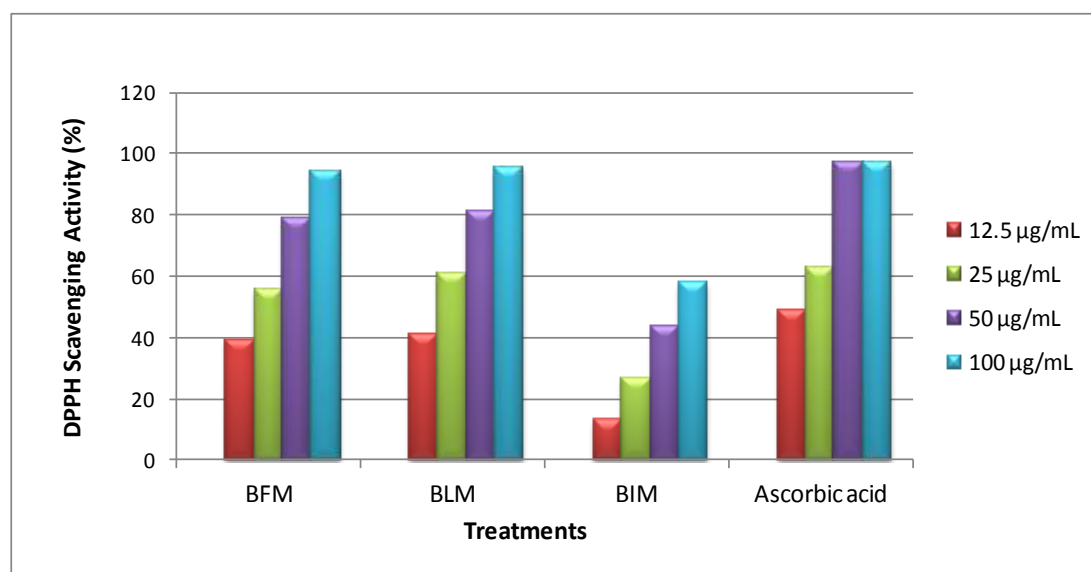


Figure 37. Free-radical scavenging activity (%) of MeOH extracts of field-grown flowers (BFM), leaves (BLM) and *in vitro*-derived leaves (BIM) of *B. perennis* at different concentrations and ascorbic acid (positive control).

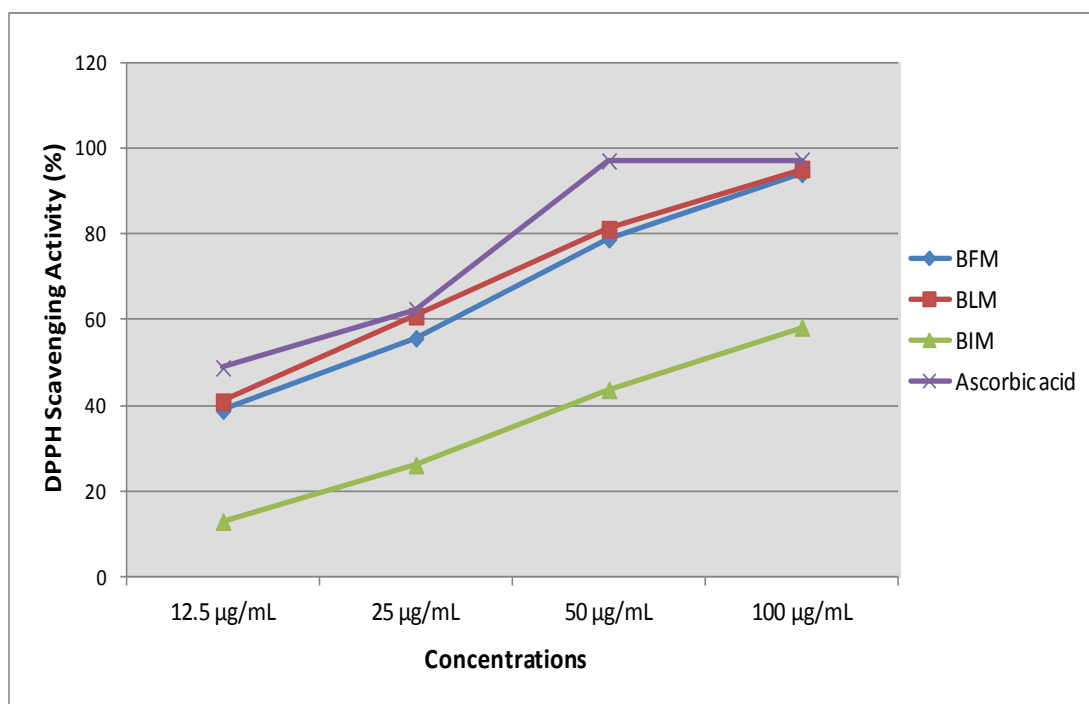


Figure 38. Free-radical scavenging activity (%) of MeOH extracts of field-grown flowers (BFM), leaves (BLM) and *in vitro*-derived leaves (BIM) of *B. perennis* at different concentrations and ascorbic acid (positive control).

Total phenolic content of common daisy extracts was assessed using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965; Slinkard and Singleton, 1977). The results were expressed as g gallic acid equivalents (GAE)/100 g dried mass (Table 17; Figure 39) (method 1) and g tannic acid equivalents (TAE)/100 g dried mass (Table 18) (method 2).

In the first method, methanolic extracts of field-grown flowers, leaves and *in vitro*-derived leaves, and *n*-BuOH and EtOAc fractions of *B. perennis* flowers contained 3.3, 4.5, 0.9, 2.4 and 6.4 g GAE/100 g dried mass, respectively (Table 17). In the second method, the results presented in Table 17 indicated that the methanolic extracts of field-grown flowers, leaves and DCM extract of *in vitro*-derived leaves

contained high concentrations of phenolic compounds, with, respectively, 5.20, 10.60 and 4.20 g TAE/100 g of extract. The contents of total phenolics varied from 0.40 to 10.60 g TAE/100 g dried mass. There was a significant correlation in between the radical scavenging activity and the total phenolics. Our results were consistent with the results of Siatka and Kasparova (2010) which they investigated the seasonal variation in total phenolic and flavonoid contents of *B. perennis* flowers. They found that total phenolics ranged from 2.81 to 3.57 mg GAE/100 mg dry weight (Siatka and Kasparova, 2010).

The total flavonoid content in common daisy methanolic extracts (BFM, BLM and BIM) and two fractions (BFN and BFE) were determined spectrophotometrically by aluminium chloride method (Christ and Müller, 1960). The content of flavonoids was expressed as g catechol equivalents (CE)/100 g dried mass (dm) (Figure 40). The results presented in Table 17 indicated that the methanolic extract of field-grown flowers contained higher concentration of flavonoid content than methanolic extracts of field-grown leaves and *in vitro*-derived leaves (1.46, 0.52 and 0.36 g CE/100 g dm, respectively). Field-grown flowers contained more flavonoids than field-grown leaves and *in vitro*-derived leaves (Table 17; Figure 41). Moreover, the highest flavonoid content was found in EtOAc fraction (3.91 g CE/100 g dm) of flowers. *n*-BuOH fraction (1.38 g CE/100 g dm) also had high level of flavonoid content (Table 17; Figure 42).

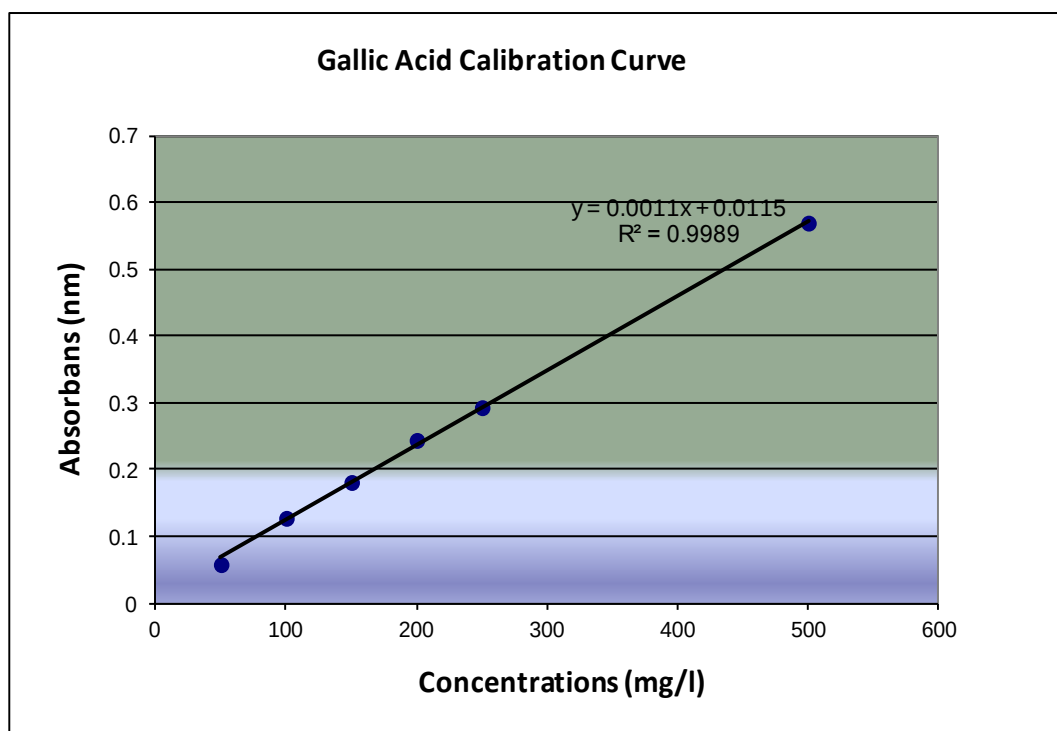


Figure 39. Gallic acid calibration curve.

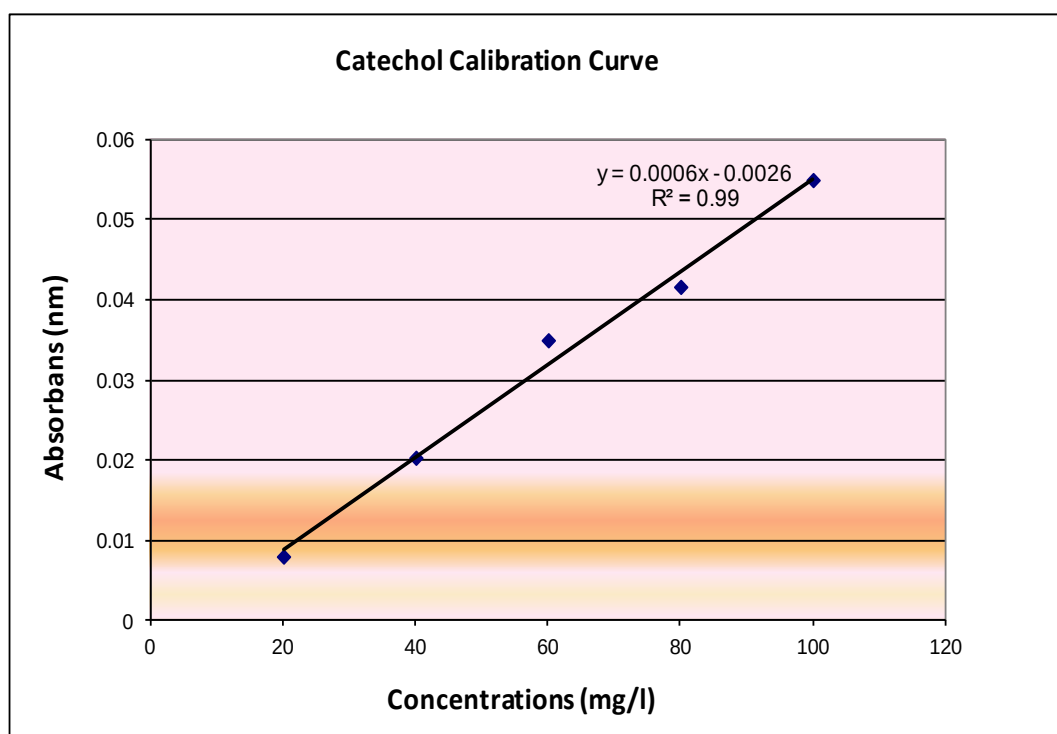


Figure 40. Catechol calibration curve.

Table 17. Total phenolics and total flavonoids contents of methanol extracts and two fractions of *B. perennis*. Gallic acid equivalent was used as g GAE/100 g of dried mass of plant material. Catechol equivalent was used as g CE/100g of dried mass of plant material. Results were expressed as means $\pm$ SD of three different samples.

Treatments	Total amounts of phenolic content (g GAE/100 g dried mass)	Total amounts of flavonoid content (g CE/100 g dried mass)
BFM (Flower-MeOH)	3.3 $\pm$ 0.05	1.46 $\pm$ 0.04
BLM (Leaf- MeOH)	4.5 $\pm$ 0.01	0.52 $\pm$ 0.03
BIM (<i>In vitro</i> - MeOH)	0.9 $\pm$ 0.02	0.36 $\pm$ 0.01
BFN (Flower-<i>n</i> -BuOH)	2.4 $\pm$ 0.04	1.38 $\pm$ 0.02
BFE (Flower- EtOAc)	6.4 $\pm$ 0.02	3.91 $\pm$ 0.04

In the present study, in consistent with Nazaruk and Gudej (2001) the flavonoid contents in the flowers were higher than in the leaves of *B. perennis*. Siatka and Kasparova (2010) measured total flavonoid with the same method but they used quercetin as a standart flavonoid. They found the contents of flavonoids varied from 0.31 to 0.44 mg quercetin equivalent/100 mg dry weight. There was no correlation between total determined content of flavonoid compounds of our study and the results of the Siatka and Kasparova (2010). In the present study, phenols were found to be more effective than flavonoids in the radical scavenging activity possibly because of their hydroxyl groups. There was not also a correlation between

total phenolic and flavonoid contents (Figure 3.26). Phenolic content of extracts depends on the solvents used for extraction. It should be pointed out that some non-phenolic reducing compounds, such as sugars (Singleton and Rossi, 1965), organic acids (Fu et al., 2010), ascorbic acid (Singleton and Rossi, 1965), and amino acids (Roura et al., 2006) could interfere with the determination of total phenolics in the Folin-Ciocalteu assay and lead to an overvaluation of the phenolic content (Prior et al., 2005). Moreover, different phenolics present different responses with the Folin-Ciocalteu reagent, for example, while gallic acid, rutin and caffeic acid have a similar behavior, several flavonoids showed low absorption, which leads to an underestimation of various compounds (Fu et al., 2010).

Antioxidant potential of *B. perennis* extracts was also assessed using the ORAC_{FL} assay (oxygen radical absorbing capacity). In Table 18, the results indicated that ethyl acetate fraction of flower was strong antioxidant, with ORAC value, 9.05 ± 0.38 μmol of TE/mg of dry mass. In comparison, ORAC values of standard phenolic compound were 20.65 ± 2.01 μmol TE/mg for quercetin. In the present study, there was a correlation between total phenolics, flavonoid contents and ORAC values. Methanol extracts of flower and leaf, and DCM extract of *in vitro*-derived leaves were moderate antioxidant, with ORAC values, respectively, of 1.61 ± 0.05 , 2.71 ± 0.17 and 1.21 ± 0.08 μmol of TE/mg of dry mass (Table 18).

Antioxidant activity of flower, leaf and *in vitro*-derived leaf extracts was also assessed *ex vivo* using a cellular based-assay (using the DCFH-DA) (Legault et al., 2003; Girard-Lalancette et al., 2009). DCFH-DA is a useful indicator of reactive oxygen species (ROS) and oxidative stress.

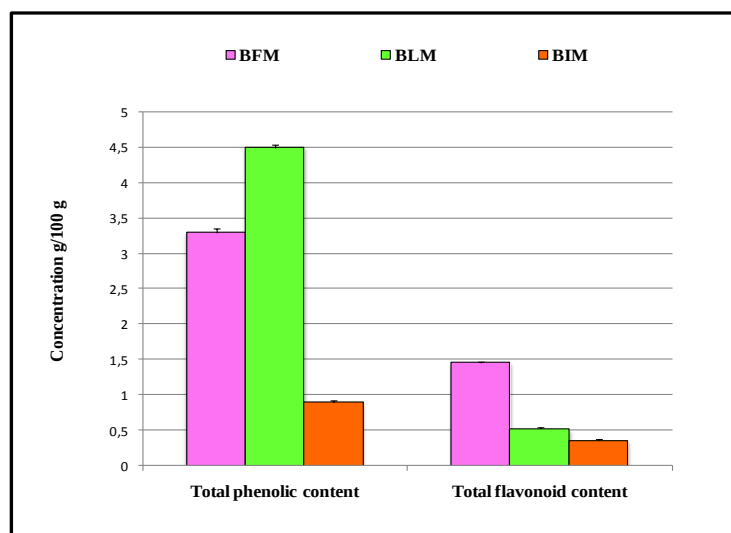


Figure 41. Total phenolic and flavonoid contents of methanol extracts (BFM, BLM and BIM) of *B. perennis* (field-grown flowers, field-grown leaves and *in vitro*-derived leaves). Gallic acid equivalent was used as g GAE/100 g of dried mass of plant material. Catechol equivalent was used as g CE/100 g of dried mass of plant material. Results were expressed as means $\pm$ SD of three different samples.

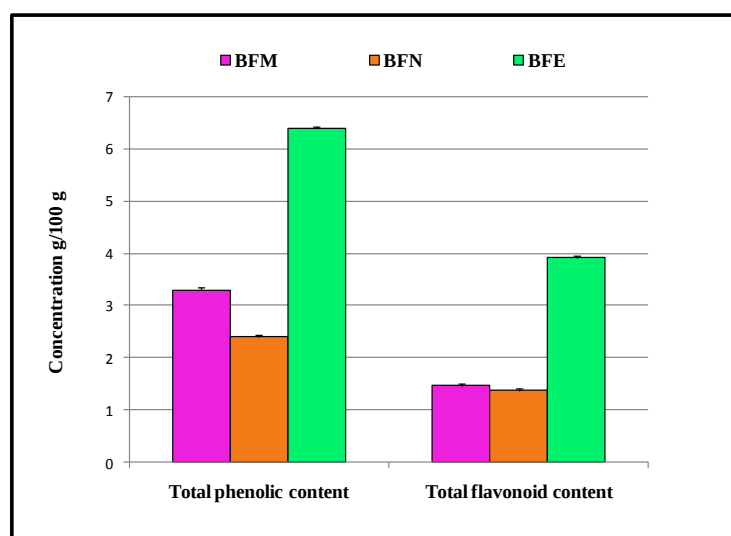


Figure 42. Total phenolic and flavonoid contents of MeOH (BFM) extract, *n*- BuOH (BFN) and EtOAc (BFE) fractions of *B. perennis* flowers. Gallic acid equivalent was used as g GAE/100 g of dried mass of plant material. Catechol equivalent was used as g CE/100 g of dried mass of plant material. Results were expressed as means $\pm$ SD of three different samples.

Nonpolar and non-ionic DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescin (DCFH). In the presence of ROS such as hydrogen peroxide (H_2O_2), lipid hydroperoxides and peroxinitrite, DCFH is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF). The oxidation is amplified by intracellular ferrous iron (Fe^{2+}). The reaction of H_2O_2 with Fe^{2+} can generate hydroxyl radicals ($\text{HO}\cdot$) which can oxidize DCFH. Superoxide anions do not directly oxidize DCFH, however, in biological systems; superoxide dismutase (SOD) transforms superoxide anions into H_2O_2 which can then oxidize DCFH. In addition, DCFH can be oxidized by intracellular oxidases and oxidants formed during the reduction of H_2O_2 . Altogether, these observations indicated that the oxidation of DCFH may be derived from several ROS intermediates (Wang and Joseph, 1999). Therefore, DCFH is useful to indirectly measure the effect of intracellular antioxidant activities in scavenging the ROS and in protecting the DCFH from the oxidation.

Results presented in Table 18 showed that *n*-BuOH ($1 \pm 6 \mu\text{g/ml}$), methanol ($1.8 \pm 0.2 \mu\text{g/ml}$) and ethyl acetate extracts of field-grown flowers ($3.5 \pm 0.3 \mu\text{g/ml}$), methanol extract of field-grown leaves ($3 \pm 0.6 \mu\text{g/ml}$) and DCM extract of *in vitro*-grown leaves ($13 \pm 1 \mu\text{g/ml}$) inhibited the *t*BH-induced oxidation of DCFH by 50%. In comparison, Trolox ($0.15 \mu\text{g/ml}$) and quercetin ($0.25 \mu\text{g/ml}$) inhibited DCFH oxidation by 50%. These results showed that flower extracts of *B. perennis* possessed a strong *ex vivo* antioxidant activity.

Table 18. Antioxidant activities of *B. perennis* extracts and fractions of field-grown flowers, leaves and *in vitro*-grown leaves and positive controls (quercetine, trolox and tannic acid) with using ORAC, Folin-Ciocalteu and Cell-based assays.

Samples	ORAC value ($\mu\text{mol Trolox/mg}$) ^a	Total phenolic compounds (g/100 g) ^b	Cell-based assay WS1 IC ₅₀ ($\mu\text{g/ml}$) ^c
BFH (flower-hexane)	0.07 $\pm$ 0.00	0.40 $\pm$ 0.1	11 $\pm$ 2
BFD (flower-DCM)	0.73 $\pm$ 0.03	4.30 $\pm$ 0.6	55 $\pm$ 2
BFM (flower-MeOH)	1.61 $\pm$ 0.05	5.20 $\pm$ 1.4	1.8 $\pm$ 0.2
BFW (flower-water)	1.23 $\pm$ 0.03	1.10 $\pm$ 0.2	119 $\pm$ 14
BFN (flower-<i>n</i>-butanol)	4.88 $\pm$ 0.12	nd	1.00 $\pm$ 6.00
BFE (flower-ethyl acetate)	9.05 $\pm$ 0.38	nd	3.50 $\pm$ 0.30
BLH (leaf-hexane)	0.17 $\pm$ 0.02	1.30 $\pm$ 0.3	122.00 $\pm$ 36.00
BLD (leaf-DCM)	0.30 $\pm$ 0.04	3.00 $\pm$ 0.9	47.00 $\pm$ 14.00
BLM (leaf-MeOH)	2.71 $\pm$ 0.17	10.60 $\pm$ 0.3	3.00 $\pm$ 0.60
BLW (leaf-water)	0.73 $\pm$ 0.05	3.50 $\pm$ 0.8	7.10 $\pm$ 0.60
BIH (<i>in vitro</i> -hexane)	0.52 $\pm$ 0.05	1.30 $\pm$ 0.3	101.00 $\pm$ 30.00
BID (<i>in vitro</i> -DCM)	1.21 $\pm$ 0.08	4.20 $\pm$ 1.2	13.00 $\pm$ 1.00
BIM (<i>in vitro</i> -MeOH)	0.86 $\pm$ 0.08	3.10 $\pm$ 0.4	19.00 $\pm$ 2.00
BIW (<i>in vitro</i> - water)	0.51 $\pm$ 0.04	1.60 $\pm$ 0.6	43.00 $\pm$ 4.00
Quercetine	20.65 $\pm$ 2.01	nd	0.25 $\pm$ 0.02
Trolox	nd	nd	0.15 $\pm$ 0.05
Tannic Acid	nd	93.10 $\pm$ 10.6	nd

nd: not determined.

^a ORAC: oxygen radical antioxidant capacity.

^b g total phenolic compounds/100 g extract: tannic acid equivalents.

^c Concentration inhibiting 50% of cell growth (mean $\pm$ S.D.; *n* = 3).

On the basis of the results of this study, it was clearly indicated that extracts of common daisy had a powerful antioxidant activity oxidative systems *in vitro* and *ex vivo* and were rich from the point of view of phenolics and flavonoids. They can be used as accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. The antioxidant mechanisms and their phenols or flavonoids may be attributed to strong hydrogen donating ability, and their effectiveness as scavengers of free radicals (Gülçin et al., 2004). Phenolic and flavonoid compounds appear to be responsible for the antioxidant activity of extracts of *B. perennis*. In addition, free radicals have been demonstrated to be a contributing factor in the tissue injury and modulation of the pain. Some studies have revealed that the antioxidants melatonin and β -carotene potentiate the antinociceptive responses. It was indicated that vitamin E (tocopherols) has beneficial effects in improvement of rheumatic disease, intermittent claudication or angina pectoris due to its antioxidant activity (Gülçin et al., 2004). According to this knowledge, there is a relationship between antioxidant and anti-inflammatory activities. Anti-inflammatory activities of *B. perennis* may be related to antioxidant activity.

CHAPTER 5

CONCLUSIONS

B. perennis (common daisy) is a herbaceous perennial herb in the family Asteraceae (Panda, 2004). It has been used in the treatment of sore throat (Uysal et al., 2010), headache (Uzun et al., 2004), common cold, wounds, bruises, broken bones (Mitich, 1997), stomachache, eye diseases, eczema, skin boils, gastritis, enteritis, cancer, diarrhea, bleeding, rheumatism, inflammation and infections of the upper respiratory tract in traditional medicine (Grieve, 1982; Baytop, 1999; Duke, 2002; Cakilcioglu et al., 2010). It is also used as an astringent, expectorant, diuretic, booster, purgative and anti-inflammatory agent since ancient time in folk medicine (Grieve, 1982; Baytop, 1999; Duke, 2002).

An *in vitro* culture protocol was established for this medicinal plant. In the present study, two different explants excised from field-grown plants or *in vitro*-derived plantlets were compared in terms of their regeneration capacity. Plants collected from the field were successfully disinfested. After surface sterilization, three different explants (leaf, pedicel and root) were used for tissue culture experiment. Various auxins and cytokinins were attempted and also combinations of them. These were BA and TDZ, either alone or in combinations with IAA and IBA as well as the basal medium (control treatment). It was observed that field-grown plants were the best plants for explant source. However, regeneration rate decreased

when *in vitro*-derived plantlets were used as explant source. Generally, all explants tested formed shoots with TDZ in combination with IAA. The best shoot proliferation was obtained with pedicel explants. Leaf explants generally formed fewer shoots than pedicel explants. Root explants did not form any shoot regeneration on any media. Among the pedicel explants, the highest number of shoots per explant was observed on MSMO media containing 0.5 mg/l TDZ plus 0.5 mg/l IAA (20.13 ± 2.19 shoots per shooting explant; 100% explants formed shoots).

Three different explants (petiole, leaf and root) obtained from *in vitro*-derived plantlets were cultured on MSMO medium containing TDZ in combination with IAA or IBA and BA in combination with IAA. Among the combinations used, TDZ with IAA were the most effective for all explants used in shoot production. The best shoot proliferation was obtained with the petiole explants. Leaf explants formed fewer shoots than petiole explants and root explants did not form any shoot regeneration on any media. With petiole explants, the greatest number of shoots per explant was observed on media containing 0.5 mg/l TDZ plus 0.5 mg/l IAA (7.9 ± 0.98 shoots per shooting explant; 100% explants formed shoots).

Regenerated shoots formed roots in 3 weeks. Four different auxins were used for rooting (IBA, IAA, NAA and 2,4-D). IAA was found the most effective auxin for rooting stage. The highest number of roots per explant was observed on media containing 1.0 mg/ml IAA. The rooted plants were transferred to Magenta containers and subsequently to pots in greenhouse. Approximately 6.5 months after initiating to culture, flowering of regenerated plants were observed. With this efficient protocol, disease and herbicide free bulk plant material can be supplied throughout the year for pharmaceutical purposes.

Five different types of extraction methods (soxhlet, water bath, decoction, infusion and liquid-liquid extraction) were performed for flowers, and soxhlet extraction method was used for field-grown leaves and *in vitro*-derived leaves. Different solvents (hexane, dichloromethane, methanol, water, ethyl acetate, *n*-buthanol, ethanol and acetone) were utilized for extraction of plant materials. Soxhlet extraction method was found to be the best method in obtaining the maximum yield from the plant and methanol was the most effective solvent in obtaining the maximum yield. Better yield was obtained with field-grown flowers of the common daisy than the field-grown leaves and *in vitro*-derived leaves.

Chemical composition of hydrodistilled oil of *B. perennis* flowers growing in Bolu was analyzed by GC-MS for the first time. Nineteen constituents (*alpha*-pinene, *beta*-pinene, myrcene, *p*-cymene, *beta*-caryophyllene, *gamma*-himachalene, *alpha*-copaene, *cis*-calamenene, *delta*-cadinene, *beta*-bisabolene, ethyl myristate, 2-pentadecanone, 1,2-benzenedicarboxylic acid, hexadecanoic acid, palmitic acid, ethyl palmitate, neophytadiene, linoleic acid ethyl ester and ethyl linoleolate) were identified in essential oil of flowers. The major compounds were *beta*-bisabolene, 2-pentadecanone, palmitic acid, and *gamma*-himachalene.

Saponin and phenolic contents of hexane, DCM, MeOH and water extracts obtained from field-grown flowers, leaves and *in vitro*-derived leaves of *B. perennis* were investigated using TLC method. When hexane and DCM extracts were compared on TLC plates, the DCM extract of *in vitro*-derived leaves contained the most saponins qualitatively. When we examined the saponin contents of the methanol and water extracts, the biggest saponin content was found in the methanol extract of field-grown flowers. The TLC plates for phenolics represented that DCM,

methanol and water extracts of field-grown flowers contained more phenolics than field-grown leaves and *in vitro*-derived leaves of *B. perennis*. In general, it can be concluded that the extracts from *in vitro*-derived leaves have a low phenolic content. When *n*-butanol and ethyl acetate fractions of the flowers were compared, it was found that, the ethyl acetate fraction was including more phenolics.

Quantification of the chosen phenolics in field-grown flowers, leaves, *in vitro*-derived leaves extracts (DCM and MeOH) and field-grown flower fractions (*n*-butanol and ethyl acetate) was performed using LC-MS/MS technique. The results of analysis showed that the flower extracts and fractions are rich in phenolic compounds. Considerable amounts of kaempferol, rutin hydrate, caffeic acid, apigenin, quercetin, *p*-coumaric acid, luteolin-7-O- β -D glucoside, gallic acid and myricetin were found in methanol extract and ethyl acetate fraction of flowers. The most dominants were kaempferol and rutin hydrate in all tested extracts.

Six different biological assays (antibacterial, antitumor, toxicity, anticancer, anti-inflammatory and antioxidant) were used for assessing biological activities of common daisy extracts. The Kirby-Bauer Disc Diffusion Assay was used to evaluate the antimicrobial activity with ten different kinds of bacteria. Bacteria tested were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, *Enterobacter cloacae* and *Klebsiella pneumoniae* as Gram-negatives and *Streptococcus pyogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* as Gram-positives. Generally, flower extracts indicated better antibacterial activities than field-grown and *in vitro*-derived leaf extracts from common daisy. With antimicrobial assay, methanolic extract (water bath) of flower gave the best results and showed inhibitory

activity against *S. epidermidis* and *S. aureus*, which are associated with urinary tract infection, eye infection, wound infections, rheumatism and inflammatory complaints in the human body. The best antibacterial activity was also obtained with ethyl acetate fraction of flowers against *S. pyogenes* and *E. cloacae*, which are associated with sore throat (pharyngitis), and enteric tract infections in the human body.

The antitumor activity of common daisy was tested with the potato disc method. All flower extracts from common daisy showed antitumor activity and the percentage inhibition was more than 35% in three separate experiments. The antitumor activity of all extracts of flower was depended on concentration value. High concentration (100.000 mg/l) of all tested extracts showed strong antitumor activity (100% tumor inhibition). Low concentrations (100 and 1.000 mg/l) of extracts showed moderate antitumor activity. The ethanolic and methanolic extracts of flowers were more effective in inhibited tumor formation than the water extracts at lower concentrations.

The brine shrimp bioassay was used to assess the general toxicity of different extracts of flowers from common daisy. Results showed that extracts of flower were toxic at higher doses (LC_{50} around 1000- 2500 mg/l). The methanolic extract was more toxic (LC_{50} = 1008 mg/l) than the ethanolic extract (LC_{50} = 1334 mg/l). In addition, the cold water extract was more toxic (LC_{50} = 1087 mg/l) than the hot water extract (LC_{50} = 2418 mg/l).

The anticancer activity of crude hexane, DCM, MeOH and water extracts of field-grown flowers, field-grown leaves, *in vitro*-derived leaves and two fractions (*n*-butanol and ethyl acetate) of field-grown flowers from *B. perennis* was evaluated

against to human lung carcinoma (A-549), human colon adenocarcinoma (DLD-1), and human breast adenocarcinoma (MCF-7) cell lines. Inhibition of the cell proliferation was assessed using the resazurin test for human lung carcinoma (A-549) and human colon adenocarcinoma (DLD-1) cell lines. MTT assay was used for human breast adenocarcinoma (MCF-7) cell line. In resazurin test, among the tested extracts, the DCM extract from *in vitro*-derived leaves showed a strong anticancer activity against to human lung carcinoma (A-549) cells, with IC_{50} value of 15.6 ± 0.5 $\mu\text{g/ml}$. The *n*-butanol fraction exerted the most potent cytotoxic activity (with IC_{50} values of 16 ± 3 $\mu\text{g/ml}$ and 10 ± 2 $\mu\text{g/ml}$), towards the lung carcinoma (A-549) and colon adenocarcinoma (DLD-1) human cell lines. In addition, the ethyl acetate fraction showed the strong anticancer activity on A-549 and DLD-1 cells, with IC_{50} values of 25 ± 8 $\mu\text{g/ml}$ and 20 ± 4 $\mu\text{g/ml}$, respectively. In MTT assay, among the tested extracts, all methanolic extracts of common daisy showed the best antiproliferative activity against to MCF-7 cell line. MeOH extract from *in vitro*-derived leaves showed a strong anticancer activity against to human breast adenocarcinoma (MCF-7) cells, with the percentage of the cell viability values of 7.5% at 200 $\mu\text{g/ml}$ and 14.2% at 100 $\mu\text{g/ml}$ concentrations. Depending on the results of the present study, we can suggest that DCM and MeOH extracts of *in vitro*-derived leaves have the most potent anticancer activity towards to human lung, colon and breast cancers. The results of the present study is also supporting the ethnobotanical usage of *B. perennis* against various cancer types. High anticancer activities of DCM and MeOH extracts obtained from *in vitro*-derived leaves revealed the quality of *in vitro*-derived materials of *B. perennis*.

The anti-inflammatory activities of twelve different *B. perennis* extracts (hexane, dichloromethane, methanol and water) of field-grown flowers, field-grown leaves and *in vitro*-derived leaves were evaluated by measuring their capacity to inhibit cellular NO generation. The MeOH extract of field-grown flowers from *B. perennis* showed a strong inhibitory effect on LPS-induced NO secretion with 96.3% inhibition at 20 µg/ml concentration. Comparatively, the L-NAME, used as positive control inhibited NO release by 65.3% at 250.0 µM (67.4 µg/ml). The strong anti-inflammatory activity of the MeOH extract of field-grown flowers may be attributed to the reported phenolic, flavonoid and terpenoid constituents of *B. perennis*. Altogether, these results support the traditional use of *B. perennis* in the treatment of inflammation diseases implicating NO and peroxinitrite.

The antioxidant activity of hexane, DCM, methanol and water extracts of field-grown flowers, leaves and also *in vitro*-derived leaves was assessed. The free radical scavenging activity (DPPH), total phenolic content (Folin-Ciocalteu), total flavonoid content (aluminum chloride colorimetric), oxygen radical absorbing capacity (ORAC) and direct antioxidant assays (cell-based assay using 2',7'-dichlorofluorescein-diacetate) were used in this assessment. The methanol extracts of *B. perennis* showed a concentration dependent antiradical scavenging activity (%) by scavenging DPPH radical. The field-grown leaves and flowers showed higher DPPH radical scavenging activity compared to the *in vitro*-derived leaves. The field-grown leaf and flower extracts had high active radical scavenge capability as much as ascorbic acid that was used as control antioxidant. The total phenolic content of common daisy extracts was assessed using the Folin-Ciocalteu reagent. The results indicate that the methanolic extracts of field-grown flower, leaf and DCM extract of

in vitro-derived leaf contain high concentrations of phenolic compounds. The MeOH extract of field-grown leaves contain the highest concentration phenolic compounds as gallic acid and tannic acid equivalent. The total flavonoid content in common daisy methanolic extracts were determined spectrophotometrically by the aluminium chloride method. The content of flavonoids were expressed as g catechol equivalents (CE)/100 g dried mass. Results indicated that the methanolic extract of field-grown flowers contained higher concentration of flavonoid content than the methanolic extracts of field-grown leaves and *in vitro*-derived leaves. Field-grown flower parts included more flavonoids than the field-grown leaf and *in vitro*-derived leaf parts. The antioxidant potential of *B. perennis* extracts was also assessed using the ORAC_{FL} assay (oxygen radical absorbing capacity). The *n*-butanol and ethyl acetate fractions of flowers were strong antioxidant, with ORAC values, respectively, of 4.88 ± 0.12 and 9.05 ± 0.38 μmol of TE/mg of dry mass. In comparison, the ORAC value of standard phenolic compound was 20.65 ± 2.01 μmol TE/mg for quercetin. In addition, the methanol extracts of flowers, leaves and DCM extract of *in vitro*-derived leaves were moderate antioxidant activity. The antioxidant activity of flower, leaf and *in vitro*-derived leaf extracts was also assessed *ex vivo* using a cellular based-assay (using the DCFH-DA). Results show that *n*-BuOH (1 ± 6 $\mu\text{g/ml}$), methanol (1.8 ± 0.2 $\mu\text{g/ml}$) and ethyl acetate extracts of field-grown flowers (3.5 ± 0.3 $\mu\text{g/ml}$), methanol extract of field-grown leaves (3 ± 0.6 $\mu\text{g/ml}$) and DCM extract of *in vitro*-grown leaves (13 ± 1 $\mu\text{g/ml}$) inhibited the *t*BH-induced oxidation of DCFH by 50%. In comparison, Trolox (0.15 $\mu\text{g/ml}$) and quercetin (0.25 $\mu\text{g/ml}$) inhibited DCFH oxidation by 50%. These results showed that flower extracts of *B. perennis* possessed a strong *ex vivo* antioxidant activity. On the basis of the results of this study, it was clearly indicated that extracts of *B. perennis* had a powerful

antioxidant activity oxidative systems *in vitro* and *ex vivo*, and were rich from the point of view of phenolics and flavonoids. They can be used as accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

Results obtained herein revealed the bioactivity (antibacterial, antitumor, toxicity, anticancer, anti-inflammatory and antioxidant) of common daisy extracts and fractions. Furthermore, the total phenolic, flavonoid content colorimetric methods, thin layer chromatography plates (for saponins and phenolics), GC and LC-MS/MS results confirmed the activities of antibacterial, antitumor, anticancer, anti-inflammatory and antioxidant. The results of all studies indicated that different parts of *B. perennis* had different levels of biological activity depending on the type of the solvent used in the extraction procedure. The bioassay results confirmed the popular utilization of common daisy extracts in folk medicine as remedy for respiratory disorders, urinary tract infection, eye infection, rheumatism, certain skin diseases, inflammatory and cancer. With these results, *Bellis perennis* L. has some scientific justification as a medicinal herb.

Definitive clinical studies are needed to fully understand the many medicinal uses of common daisy. In the future more different bioassays related to biological activity of common daisy can be performed and more scientific rationale for this plant may be obtained. Furthermore, future studies should focus on fractionation of the extracts having anticancer, antiinflammatory and antibacterial in hopes of identifying active molecules.

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CURRICULUM VITAE

Name : Fatma PEHLİVAN KARAKAŞ
Permanent Address : Abant İzzet Baysal University, Faculty of
Arts and Sciences, Department of Biology,
14280 BOLU / TURKEY
Degree and date to be conferred : Ph.D., 2012
Place Date of Birth : Istanbul, 06 August 1978
Secondary education : Fatsa Lisesi, Ordu, 1995

<u>Collegiate institutions attended</u>	<u>Date</u>	<u>Degree</u>	<u>Date of degree</u>
Canakkale Onsekiz Mart University, Dept of Biology	1996-2000	B.S.	15/06/2000
Abant İzzet Baysal University, Dept of Biology	2001-2003	M.S.	10/09/2003
Abant İzzet Baysal University, Dept of Biology	2009-2012	Ph.D.	02/07/2012

Publications (SCI papers) :

Pehlivan Karakaş, F., Karakaş, A., Boran, Ç., Uçar Türker, A., Yalçın, F.N., Bilensoy, E. “The evaluation of topical administrations of *Bellis perennis* fractions on circular excision wound healing in Wistar albino rats”. *Pharmaceutical Biology*, 50 (8): 1031-1037, 2012.

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Türker H., Birinci Yıldırım A., **Pehlivan Karakaş F.** “Sensitivity of bacteria isolated from fish to medicinal plants”. Turkish Journal Fish Aquatic Science, 9: 181-186, 2009.

Publications (not SCI):

Ucar Turker A., Birinci Yildirim A., **Pehlivan Karakas F.** “Antitumor and antibacterial activities of *Viscum album* L. grown on different host trees”. Journal of Herbs, Spices and Medicinal Plants, (in press), 2012.

Pehlivan F., Karakaş A., Gündüz B. “Leptin hormone profile in Syrian hamster during estrous cycle.” Genel Tıp Dergisi, 13 (2, Ek), p: 62, 2003.

Positions held: Expert in department of Biology, AIBU since 2004.