IN VITRO CULTURE, BIOLOGICAL ACTIVITY AND ESSENTIAL OIL ANALYSIS OF YELLOW LOOSESTRIFE (*Lysimachia vulgaris* L.)

by

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

JANUARY 2011

Approval of the Graduate School of Natural Sciences

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ABSTRACT

IN VITRO CULTURE, BIOLOGICAL ACTIVITY AND ESSENTIAL OIL ANALYSIS OF YELLOW LOOSESTRIFE (*Lysimachia vulgaris* L.)

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January 2011, 131 Pages

Lysimachia vulgaris L. (Yellow loosestrife) is a medicinal plant that has been used in the treatment of fever, ulcer, diarrhea and wounds in traditional medicine. It has also analgesic, expectorant, astringent and anti-inflammatory activity. A reliable an *in vitro* culture protocol for yellow loosestrife was established. Explants (leaf, stem and root segments) were cultured on Murashige and Skoog minimal organics (MSMO) medium with various plant growth regulator combinations. Of the tested combinations, those that involved BA with either IAA or IBA were the most effective for

all used explants in shoot production. Best shoot proliferation was obtained from leaf explant cultured on media containing 0.5 mg/l BA and 0.1, 0.5 or 1 mg/l IBA, from stem explant cultured on media containing 1 mg/l BA and 0.5 mg/l IBA or 0.01 mg/l TDZ and 0.5 mg/l IAA, and from root explant cultured on media containing 0.5 mg/l BA and 0.5 mg/l IAA. Regenerated shoots were rooted on MSMO medium containing different concentrations of IAA, IBA, 2,4-D and NAA. IBA was determined as the most effective auxin for rooting. Most shoots developed roots on medium with 0.5 mg/l IBA. After rooting, plantlets were transferred to Magenta containers including vermiculate for acclimatization. After 3 weeks, they were transferred to soil for hardening. Essential oil analyses were also conducted with *in vitro*-grown and field-grown plant materials of *L. vulgaris*. Moreover, three different bioassays (antibacterial, antitumor and antioxidant) were performed to show the biological activities of yellow loosestrife extracts. Field-grown and *in vitro*-grown plants parts were extracted with water, ethanol and acetone and their biological activities were evaluated. For antibacterial test, disc diffusion method (Kirby-Bauer) and 10 different pathogenic bacteria were used. Generally, yellow loosestrife extracts showed antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *S. epidemidis* and *Streptococcus pyogenes*). Strong antitumor activity of yellow loosestrife was observed via potato disc diffusion bioassay. Furthermore, the free radical scavenging potency of *L. vulgaris* extracts was determined with DPPH method. The phenolic and flavonoid contents of yellow loosestrife extracts were also investigated.

Keywords: *Lysimachia vulgaris*, yellow loosestrife, *in vitro* culture, auxine, cytokinin, antibacterial, antitumor, essential oil, GC-MS, antioxidant, DPPH method, Folin-Ciocalteau method, Aluminum chloride ($AlCl_3$) colorimetric assay

ÖZET

KARGA OTU (*Lysimachia vulgaris* L.) BİTKİSİNİN DOKU KÜLTÜRÜ, BİYOLOJİK AKTİVİTELERİ VE UÇUCU YAĞ ANALİZİ

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Ocak 2011, 131 Sayfa

Lysimachia vulgaris L. (Karga otu) halk arasında yüksek ateş, ülser, ishal ve yaraların tedavisinde kullanılan tıbbi bir bitkidir. Bitkinin analjezik, ekspektorant, astringent ve anti-enflamatuvar aktiviteleri de vardır. Karga otu için güvenilir bir *in vitro* kültür protokolü geliştirilmiştir. Eksplantlar (yaprak, gövde ve kök parçaları) çeşitli bitki büyüme düzenleyici kombinasyonlarını içeren Murashige ve Skoog minimal organic (MSMO) ortamında kültüre alınmışlardır. Test edilen kombinasyonlar arasından sürgün üretiminde BA' nın IAA veya IBA kombinasyonları kullanılan bütün eksplantlar içinde en etkili olmuştur. En iyi sürgün gelişimi yaprak eksplantından 0.5 mg/l BA ve 0.1, 0.5 veya 1 mg/l IBA içeren ortamlarda, gövde eksplantından 1 mg/l BA ve 0.5 mg/l IBA veya 0.01 mg/l TDZ ve 0.5 mg/l IAA içeren ortamlarda ve kök

eksplantından 0.5 mg/l BA ve 0.5 mg/l IAA içeren ortamlarda elde edilmiştir. Rejenere olmuş sürgünler, IAA, IBA, 2,4-D ve NAA' nın farklı konsantrasyonlarını içeren MSMO ortamında köklendirilmiştir. Köklendirme için en etkili oksinin IBA olduğu tespit edilmiştir. Sürgünlerden en fazla kök 0.5 mg/l IBA içeren ortamlarda gelişmiştir. Köklendirme işleminden sonra, bitkicikler vermikülit içeren magenta kaplarına transfer edilmişler ve 3 hafta sonra da toprağa aktarılmışlardır. Doğal ve *in vitro* ortamda yetişen *L. vulgaris* bitki materyallerinin uçucu yağ analizleri de yapılmıştır. Ayrıca karga otu özütlerinin biyolojik aktivitelerini görmek için 3 farklı biyolojik test (antibakteriyel, antitümor ve antioksidant) uygulanmıştır. *In vitro* ve doğal ortamında yetişen bitki parçalarından su, etanol ve aseton ile özütler hazırlanmış ve biyolojik aktiviteleri değerlendirilmiştir. Karga otu özütlerinin antibakteriyel aktivitelerini görmek için disk difüzyon metodu (Kirby-Bauer) ve 10 farklı patojen bakteri kullanılmıştır. Genellikle, karga otu özütleri Gram-pozitif bakterilere (*Staphylococcus aureus*, *S. epidemidis* ve *Streptococcus pyogenes*) karşı antibakteriyel etki göstermiştir. Karga otunun güçlü antitümor etkisi patates disk difüzyon biyolojik testi ile gözlemlenmiştir. Ayrıca, özütlerin serbest radikal süpürme gücü DPPH metodu ile belirlenmiştir. Karga otu özütlerinin fenolik ve flavonoid içeriği de araştırılmıştır.

Anahtar Kelimeler: *Lysimachia vulgaris*, karga otu, *in vitro* kültür, oksin, sitokinin, antibakteriyel, antitümör, uçucu yağ, GC-MS, antioksidant, DPPH metodu, Folin-Ciocalteau metodu, Alüminyum klorit ($AlCl_3$) kolorimetrik analiz

To the super woman who has made everything a real in my life, mom,

Azime GÜNER, thanks to you.

ACKNOWLEDGEMENTS

I wish to acknowledge the indispensable assistance afforded me by so many people throughout my life and this experience. I would like to express my appreciation and gratitude to my supervisor Assoc. Prof. Dr. Arzu Uçar Türker for her guidance, support, patience and encouragement. I would like to thank also Assoc. Prof. Dr. Hakan Türker with my deepest appreciation. My special thanks go to my co-supervisor Assoc. Prof. Dr. Belgin Coşge Şenkal for her guidance, advices, helps and supports and I would like to thank my committee members Prof. Dr. Tekin Babaç, Prof. Dr. Ekrem Gürel, and Assist. Prof. Dr. Adem KARATAŞ for their teaching doctrine, sincerities and helps. Also so much thanks to Assist Prof. Dr. Arif İpek for his helps. I am indebted to all of the Professors, faculty members and staff at Abant İzzet Baysal University.

This research would not be completed without reference to my fellow grad students in Dr. Türker's lab. I am grateful to my lab-mates, Arzu Birinci Yıldırım, Fatma Karakaş, Günce Şahin and Buhara Yücesan for their assistance and conversation in the lab. To Murat Şen, I offer sincere thanks for his unshakable faith in me, for his love and perseverance. My parents because of whom I am today: the affection, encourage, love and care shown by them have give me strength to work constantly. I thank my parents for understanding and I would like to say

sorry to them for my frequent absences. I would like to thank my friends Demet Aşık for her every kinds helps, Günnur İpek for her technical helpings and sharing private and enjoyable times, Nesrin Demirdügen for her comments and conversation and specially Sandeep Kumar Verma for sharing the stressful moments with me and for helping me whenever I needed. Finally, special thanks are due to financial supports of the Scientific and Technological Research Council of Turkey (TÜBİTAK). I apologize to those not mentioned- they are, however, not forgotten.

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LIST OF ABBREVIATIONS

ANOVA : Analysis of Variance

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

BA : Benzyladenine

DMSO : Dimethyl Sulfoxide

DPPH : 2,2-diphenyl-1-picrylhydrazil

EtOH : Ethanol

GA_3 : Gallic acid

GAE : Gallic acid equivalent

GC-MS : Gas chromatography – Mass Spectroscopy

IAA : Indole-3-acetic acid

IBA : Indole-3-butyric acid

KIN : Kinetin

MSMO : Murashige and Skoog's Minimal Organics

NAA : Naphthalene acetic acid

PBS : Phosphate Buffered Saline

TDZ : Thidiazuron

Zea : Zeatin

CHAPTER 1

1. Introduction and Literature Review

1.1. Introduction

It is predicted that there are 250,000 to 500,000 plant species on Earth. A relatively small percentage (1 to 10%) of these is used as foods by both humans and other animal species. It is possible that even more are used for medicinal purposes. Hippocrates, Dioscorides, Paracelsus, even the all holly books offered many healing plants. During the Dark Ages, the Arab world, and Asian cultures were also busy compiling their own pharmacopoeia (Cowan, 1999). Interest in medicinal plants reflects the recognition of the validity of many traditional claims regarding the value of natural products in healthcare (Duraipandiyan and Ignacimthu, 2007). Plant products are also known to possess potential for food preservation (Tepe *et al*., 2005).

The genus *Lysimachia* L., contains approximately 180 species, and about 150 species are widely distributed in the south, southwest and central China (Zheng *et al*., 2009).

Lysimachia vulgaris L. is attractive plant that is called as loosestrife, yellow loosestrife, garden loosestrife, golden loosestrife, willow herb, willow-wort, yellow rocket, wood pipernel (Dobelis, 1990; Chevallier, 1996; Grieve, 1982), in Turkish name is karga otu and adi karga otu (Baytop, 1999). The plant is native of the European herbal medicine for some 2000 years or more (Dobelis, 1990).

In Turkey, 6 species of *Lysimachia* can be found easily (Akman *et al*., 2007). The genus was traditionally placed in the Primulaceae family, but upon review of molecular phylogenetic research, it has now been assigned in the much enlarged family Myrsinaceae (Oh *et al*., 2008; Hao *et al*., 2004; Huang *et al*., 2009). Genus *Lysimachia*, which has always been placed within the family Primulaceae was shifted to the Myrsinaceae based on the results of recent botanical investigations (Podolak and Strzalka, 2008).

Yellow loosestrife (*Lysimachia vulgaris* L.), a member of the Primulaceae family, is a rhizomatous perennial herb (Davis, 1978), that is used in fever, ulcers, diarrhea, healing wounds and an analgesic, expectorant and anti-inflammatory agent since ancient time by folk because of having these effects on human health (Baytop, 1999). It is also useful plant for phytopurification of wastewater (Borin, 2003).

It is native to Eurasia that is widely distributed across the northern United States and southern Canada. It is found growing wild throughout most of Europe and Asia and thrives especially in moist mountainous grassland, wetland and riparian areas (Messick and Kerr, 2007; Podolak *et al*., 1998).

Many plants of the Primulaceae are widely used in the treatment of various diseases. Within the genus *Lysimachia* several species were or still

are renowned by traditional medicine. The total flavones, obtained from these plants have been proven to be anticancer agents. Apart from their medicinal value, the *Lysimachia* plants have ornamental significance; dozens of them have been grown as ground cover or hanging basket plants in North America, East Asia and Europe (Podolak *et al*., 1998 and Zheng *et al*., 2009). Many species of Primulaceae family are attractive and flashy. They are cultivated as horticultural because of having vivid color, especially those belonging to the largest genus, *Primula* (Akman *et al*., 2007; Cullen, 1997; Chevallier, 1996). Moreover, *Lysimachia vulgaris* L. has also attractive bright yellow flowers. Therefore, it is gathered when in flower in summer (Chevallier, 1996).

The main objectives of this study were a) to obtain an *in vitro* culture protocol for *L. vulgaris* using plant tissue culture methods; b) to screen and compare the biological activities of water, ethanol and acetone extracts of field grown and *in vitro*-grown *L. vulgaris* plants using specific bioassays (antibacterial, antitumor, and antioxidant); c) essential oil isolation and identification by gas chromatography from *in vitro* grown and wild population of *L. vulgaris* and comparing these results each other.

Figure 1. 1 Pictures of *Lysimachia vulgaris* L.

1.2. Literature Review

1.2.1. Medicinal and Historical Uses

Both the scientific and popular names of the Loosestrife have interesting origins. Pliny (AD 23-79) recorded that *Lysimachia*, the plant's Latin name, was a tribute to King Lysimachus of Sicily, who was the first to discover its medicinal benefits then introduced it to people. The common name "loosestrife" refers to the plant's reputed power to prevent conflict, particularly between animals, and to repel insects. There is also different belief that the plant would quieten savage beasts. The plant appears to be obnoxious to oxen and also horses that is placing it under the yoke, relieved the beasts of their tormentors, thus making them quiet and tractable (Grieve, 1982). The Greek physician Dioscorides (AD 40-90) recommended loosestrife for nosebleeds and to staunch wounds, and noted that its smoke would drive snakes and flies (Chevallier, 1996), also he reported that the juice of the leaves administrated as a drink or an enema was an effective treatment for persons who had dysentery or were vomiting blood (Dobelis, 1990).

An astringent herb, yellow loosestrife is principally used to treat gastrointestinal conditions such as diarrhea and dysentery, to stop internal and external bleeding, and to cleanse wounds. Loosestrife proves useful in checking bleeding of the mouth, nose and wounds, restraining profuse hemorrhage of any kind. It makes a serviceable mouthwash for sore gums and canker sores, and may be used to treat nosebleeds and heavy menstrual bleeding. Yellow loosestrife has also been taken as an expectorant

(Chevallier, 1996; Dobelis, 1990; Grieve, 1982). In addition to these, *L. vulgaris* is used to treat fever and ulcers, and shows anti-inflammatory and analgesic properties (Janik *et al*., 1994). Hence, in present-day herbalists recommend a tea herbal tradition infusions made from the plant as an astringent, which helps stop minor bleeding by causing the skin and capillaries contract; as an expectorant, for bringing up phlegm; and as a gargle it finds use in relaxed throat and quinsy (Dobelis, 1990; Grieve, 1982). It has demulcent and astringent virtues which render it useful in obstinate diarrhea. For the cure of sore eyes, this herb has been considered equal, if not superior to Eyebright. For wounds, an ointment was used made of the distilled water of the herb. The distilled water was also recommended for cleansing ulcers and reducing their inflammation, and also, applied warm, for removing 'spots', marks and scabs in the skin (Grieve, 1982). In Hawaii, and China, the indigenous women use maceration of *L. vulgaris* for their skin to protective, smoothing and renovate the skin. Moreover, some endemic species of *Lysimachia* genus are used as diuretic and anti-lithic (Ulusoylu *et al*., 2002).

Furthermore, related species from *Lysimachia* genus have similar effects for example*, L. nemorum* is also astringent and staunches blood, *L. christinae* is a diuretic used to treat urinary pain so the latter Chinese trial showed, and *L. christinae* is also effective in treating both kidney stones and gallstones (Chevallier, 1996). *L. foenum-graecum* has been commonly used as a perfume and pest repellent (Park *et al*., 2008). The ethanol extract of whole plant of *L. clethroides* was examined on antithrombotic activity. This extract significantly inhibited collagen, ADP-induced platelet aggregation and

effectively decreased the mortality rate of mice from thromboembolism (Lee *et al*., 2010a). Experiments were undertaken to determine whether hydro-alcoholic extract obtained from *L. clethroides* has vasorelaxant activity in the rat aorta rings and, if so, to elucidate the underlying mechanism. In addition, this extract decreased oxidative stress in aorta by inhibition of NADPH oxidase activity (Lee *et al*., 2010b)

The taste of the dried herb is astringent and slightly acid (Chevallier, 1996; Grieve, 1982). The rhizome (underground stem) of this herb yields a brown dye and the leaves or stalks a yellow one. Because of this reason also it is used to dye. The concentrated extract of the plant sometimes serves as hair bleach (Dobelis, 1990). As well, when the plant is burned, it gives off sharp-smelling fumes because of driving off snakes and kill flies (Dobelis, 1990). *L. foenum-graecum* was used an experiment that was in vivo fungicidal activity against six phytopathogenic fungi. Methanol extract of *L. foenum-graecum* showed potent fungicidal activity against pyhtopathogens (Kim *et al*., 2008).

Scientists have not investigated the plant for its medicinal values that were explained below, and can neither confirm nor refute any these claims (Dobelis, 1990).

1.2.2. Botany

Primulaceae family includes 28 genera and approximately 500-600 species. Fourteen genera are native to Europe, 10 to North America (Cullen, 1997). They are generally cosmopolitan but they are mainly present in temperate and cold regions of the Northern Hemisphere (Takhtajan, 1997;

Akman *et al*., 2007). There are 9 genus and 40 species in Turkey. This family is perennial or less often annual herbs or herbaceous, sometimes aquatic, rarely subshrubs. In some genera, particularly in *Lysimachia*, occur secretory cells or cavities containing red or reddish substance (as in Myrsinaceae) (Takhtajan, 1997).

Lysimachia L. Kargaotu, in Turkish has 80 species and this genus is cosmopolitan. In Turkey, 6 species of *Lysimachia* are common. Some of them are *L. vulgaris*, *L. punctata*, *L. dubia*, *L. nummularia*. *L. vulgaris* is very prevalent near river or stream (Akman *et al*., 2007). In recent research, a new alien for the flora of Turkey from the North East Anatolia that is Japanese species has been recorded which is *L. japonica* Thunb (Primulaceae) (Terzioglu and Karaer, 2009).

L. vulgaris has botanical properties that, the yellow loosestrife is a tall, handsome plant, found as a rule on shady banks or crowning the herbage of the stream-side vegetation. It has a creeping root, which persists year after year, and every spring throws up afresh the tall, golden-topped stems, whose flowers are at their best in July and August (Grieve, 1982).

L. vulgaris (yellow loosestrife) has following properties as botanical: erect and attractive perennial, growing to 35-120 cm in height with broad sparsely to densely hairy and glandular. *L. vulgaris* produces stolons (runners) that send up erect, branched stems. Narrowly oval, pointed leaves grow in clusters of three or in opposite pairs and are almost stalkless. Leaves place opposite or whorled; petiole can reach 2-8 mm; having ovate to ovate-lanceolate, entire. Inflorescence of terminal and axillary panicles. Corolla has bright yellow, densely glandular inside, glabrous outside, lobes ovate-oblong.

The natural habitats are streamside, marshy ground, along roadsides etc. 1-2150 m. The yellow flowers (June-September) are borne in long-stalked clusters from the upper leaf axils (Davis, 1978; Chevallier, 1996; Dobelis, 1990), but it has no odour (Grieve, 1982).

It seems slightly branched and covered with a soft, fine down. Closely set upon them are a number of nearly stalk-less leaves, sometimes in pairs, sometimes three or four springing from the same spot. They are rather large and broad, 3-6 inches long, oblong or lance –shaped and sharply tapering at the top. Their edges are unbroken. The undersurfaces are downy with soft, spreading hairs, especially on the veins, and the upper surfaces are marked with black dots which are glands. Whatever arrangement is found in any given plant holds throughout: in the same plant some of the leaves in pairs are not found and others in three. When the leaves are in pairs, the stem is quadrangular and the angles increase as the leaves increase in number. At the top of the stem arise the flower –buds, in the axils of the leaves. Each becomes a short stalk carrying a terminal flower, below which other flowers on smaller stalks arise- the ends of the main stem thus becoming covered with a mass of golden blossoms. The flower stalks are somewhat viscid, or sticky, to the touch.

Each flower is about ¾ inch in diameter, forming a cup of five petals, quite distinct at their tips, but joined together near the base. When the flowers droop, five-pointed calyx, whose edges are fringed with the fine red hairs, are seen at the back of the petals. The five stamens look quite separate, but are joined together at the bottom by a fleshy band attached to the petals, so that they seem to stand on little glandular tube. This tube has not, as one would

expect, any honey, and, in fact, there is neither honey nor scent in any part of the flower. Nevertheless, the plant is visited by one particular kind of bee, *Macropsis labiata*, which will visit no other flower, hence where the Loosestrife does not grow the *Macropsis* does not seem to exist. Self-fertilization also takes place in smaller, less attractive-looking flowers, sometimes found among the others. As a result of fertilization, whether self or effected by insects, the ovary develops into a rounded capsule, which when dried opens at the top by five valves. The swaying of the stems by the wind jerks out the minute seeds (Grieve, 1982).

It has widespread distribution in the world; Native to Europe, North West and South of Europe, North and East of Turkey, North West of Africa and Caucasia, North of Iraq, North and West of Iran to China and North of America (Davis, 1978; Chevallier, 1996; Dobelis, 1990).

1.2.3. Constituent and Bioactivity

Yellow loosestrife contains a benzoquinone, saponins, phenolic acids, sterols, flavonoids and tannins (Chevallier, 1996). A benzoquinone pigment, 2,5-dihydroxy-3-pentadecyl-benzoquinone, was isolated for the first time from the dried rhizomes of *L. vulgaris*, and also its structure was elucidated from chemical and spectral analyses by Janik *et al*. (1994). Furthermore, Rzadkowskabodalska and Olechnowiczstepien (1975) have been reported the flavonoids of Yellow loosestrife. They identified six glycosides and structure of the three compounds was established partially, one of these was triglycoside of quercetin, the other two were the higher-molecular glycosides of quercetin and myricetin. It has also some biological activities such as

cytotoxic and antifungal activity. Podolak *et al*. (1998) reported that isolated a benzoquinone pigment and triterpene saponosides (saponoside B) from underground parts of yellow loosestrife has a cytotoxic activity *in vitro* against several cancer cell lines (human and mouse melanoma cells) and inhibit the growth of *Candida albicans* strains.

L. vulgaris and other *Lysimachia* species were analyzed as qualitative and quantitative by reversed-phase high-performance liquid chromatography (LC). Qualitative and quantitative determination of the pharmacologically active benzoquinones, embelin and rapanone in different organs of eight *Lysimachia* species has been conducted by Podolak and Strzalka (2008). Embelin and raponin are closely related compounds belonging to the class of simple alkyl benzoquinones and display a wide range of interesting biological activities, including cytotoxic effects, which are characteristic of quinones due to their capacity to undergo redox reactions. Many embelin- and rapanone-rich plants have well recorded ethnomedical uses and in most cases their pharmacological action may be attributed to these constituents. Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) was reported to possess antimicrobial, trypanocidal, wound-healing, chemopreventive, cytotoxic, antipyretic, anti-inflammatory, central analgesic properties. The compound has received much attention as a potential male-antifertility agent, which also shows fertility regulating activity in female mammals. Recent investigations indicate that embelin may serve as a promising initial lead for developing new anticancer drugs to target XIAP. Activities reported for rapanone (2,5-dihydroxy-3-tridecyl-1,4-benzoquinone) include inhibition of acute and chronic inflammatory responses, antiovulatory and antispermatogenic effects

(Podolak and Strzalka, 2008). The distribution of embelin and rapanone is scattered. The compounds were reported in members of Oxalidaceae family—embelin in *Oxalis erythrorhiza* and rapanone in *O. purpurata*. The latter was also detected in *Connarus* (Connaraceae). Both the embelin and radonine compounds were reported in members of Myrsinaceae, a family characteristic of tropical and subtropical regions (Podolak and Strzalka, 2008)

Yasukawa and Takido (1987, 1988) have found new compounds and flavones as 3. quercetin 3-rhamnosyl (1→2) galactoside from *L. vulgaris* var *davurica*. Bengang *et al.* (2007) determined the total flavones in *L. clethroide* Duby by spectrophotometer. Ulusoylu *et al*. (2002) isolated 7 constituents from *L. atropurpurea* L. and exhibited the antioxidant activity of this species. Moreover, toxicological activity of ethanol extracts of the aerial parts of *L. atropurpurea* was tested with Brine Shrimp method (Ulusoylu *et al*., 2002). Shen *et al*. (2005) isolated five triterpene glycosides from *L. foenum-graenum* and determined their effects on the arachidonic acid metabolizing enzyme. Yasukawa *et al*. (1997) reported that four flavonol glycosides were isolated from the ethyl acetate fraction of whole plant of *L. thyrsiflora* by column chromatography. These compounds were identified as quercetin 3-rhamnoside, quercetin 3-galactoside, isorhamnetin 3-rhamnoside and isorhamnetin 3-galactoside. Quercetin and isorhamnetin glycosides were only isolated from *L. thyrsiflora.* The gland cells were collected from the stems of *L. fordiana* Oliver, and the homologous pigments of fordianin A, fordianin B, fordianaquinone A and fordianaquinone B were detected in the glands by HPLC (Huang *et al*., 2009b). Liu *et al*. (2010) showed that total

flavonoids of *L. clethroides* Duby exerted potential anticancer activity through growth inhibition and apoptosis in K562 cells.

Two new oleane-type triterpene saponins, lysimachiagenoside E and lysimachiagenoside F, were isolated from the aerial parts of *L. foenum-graecum* Hance (Li *et al*., 2010). Cytotoxicity-guided phytochemical analysis on the extract of *L. heterogenea* Klatt led to the isolation of 3β,16β-12-oleanene-3,16,23,28-tetrol and its four new oligosaccharidic derivatives heterogenosides A, B, C, and D (Huang *et al*., 2009a). Seven triterpene saponins were isolated from *Lysimachia christinae* and identified as lysichriside A, lysichriside B, primulanin, lysikokianoside 1, anagallisin C, ardisiacrispin A, and ardisicrenoside B (Tian *et al*., 2008).

The isolation and purification of kaempferol pyranosides including kaempferol-3-O-beta-D-glucopyranosyl (2→1)-a-L-rhamnopyranoside, kaempferol-3-O-beta-D-glucopyranoside, and kaempferol-3-O-a-L-rhamnopyranoside from *L. christinae* Hance have been studied by Wei *et al*. (2008). One new saponin named davuricoside N was isolated from the whole plants of *L. davurica* (Zhang *et al*., 2007).

The influence of LTS-4, a saponoside from *L. thyrsiflora* L. on human skin fibroblasts and human melanoma cells was investigated. The compound, denoted LTS-4, decreased the viability and rate of cell growth of both cell types in a time-and dose-dependent manner, and proved cytotoxic against cancer cells at significantly lower concentrations than for fibroblasts (Galanty *et al*., 2008). LTS-4 also affected the morphology of the examined cells, causing vacuolization and actin cytoskeleton disintegration, and had an inhibitory effect on the tumor cell motility (Galanty *et al*., 2008).

Li *et al*. (2009) reported the antioxidant activities of water, methanol, ethyl acetate and n-BuOH extracts from *L. foenum-graecum* Hance and showed the results as ethyl acetate fraction > n-BuOH fraction > methanol extract > water extract (Li *et al*., 2009).

Park *et al*. (2008) exhibited the strong fungicidal activity of methanolic extract of *L. foenum-graecum* against some phytopathogenic fungi. Kim *et al.* (2003) reported the insecticidal activity of the whole parts of *L. davurica*.

1.2.4. Ecological and Biotechnological Studies

Graf (1996) investigated the lateral nutrient gradients and their impact on vegetation by means of transects in Zurich and showed that maximum plant height of *L. vulgaris* was suitable to indicate the soil nutrient supply. Klement and Sambraus (1999) reported that *L. vulgaris* was used as food preferences of cattle.

Traba *et al*. (2004) showed that *L. vulgaris* dominated the abandoned meadows on wet or periodically and excessively moist soils. Moreover, Auderset-Joye *et al*. (2006) reported that *L. vulgaris* exhibited both a good fit and statistical robustness. The significance for whole-plant carbon gain of plasticity in between-leaf and within-leaf partitioning of photosynthetic resources of *L. vulgaris* was investigated using an experimental and modeling approach by Pons and Anten (2004). Borin (2003) discussed in his book that *L. vulgaris* can be used in phytopurification, and is used as ornamental plant, growing in wetland ecosystems. Samecka-Cymerman and Kempers (2000) evaluated the spatial distribution of elements of *L. vulgaris* and reported the elevated levels of Co, Cd, Zn, Ni, Mn, Al, Pb, and Cu.

Significant correlations between concentrations of Cd, Zn, and Mn in water and in plant indicated the potential of these species for pollution monitoring. This plant was also used as bioindication of heavy metals (Samecka-Cymerman and Kempers, 2000). The Macropis species (are the only known oil-collecting bees) prefer only flowers of loosestrifes to collect pollen. The ranges of the Macropis species are restricted to the areas of common occurrence of *L. vulgaris* (Pekkarinen *et al*., 2003).

First tissue culture studies were reported with ornamental species of *Lysimachia* genus (*L. christinae*, *L. rubinervis* and *L. nummularia* 'Aurea') by Zheng *et al*. (2009). The regenerability of these species were investigated using *in vitro* leaves and shoot tips on Murashige and Skoog (MS) medium containing different concentrations of 6-Benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) (Zheng *et al*., 2009).

CHAPTER 2

2. *In vitro* Culture of *Lysimachia vulgaris* L.

Plant tissue culture is used for propagation of plants under sterile conditions, often to produce clones of a declared plant. Plant biotechnology is discovered on the principles of cellular totipotency and genetic transformation. Cellular totipotency (the ability of single cell to divide and produce a whole plant) is a milestone of modern plant biotechnology (Vasil, 2008).

Plant tissue culture or the aseptic culture of cells, tissues and organs, an essential component of plant biotechnology, also is an important tool in both basic and applied studies, offers novel approaches to plant production, propagation, and preservation. It has become an industrial technology (Bhojwani, 1990; Thorpe, 1990). Plant tissue culture is being used for large-scale multiplication of ornamentals and some fruit tree species. Feasibility of its applications in several other areas, such as the production of useful natural compounds, generation of useful genetic variability, and genetic transformation of crop plants has been demonstrated (Bhojwani, 1990).

Plant regeneration in tissue cultures via organogenesis or somatic embryogenesis which is a critical requirement to realize the full potential of the various techniques of plant biotechnology to crop improvement has been applied (Bhojwani, 1990). Micropropagation that is one of *in vitro* culture technique, is the true-to-type propagation of a selected genotype. Most often micropropagation is also associated with mass production (Debergh and Read, 1991).

The classical findings of Skoog and Miller (1957) continue to be the guiding principles on *in vitro* organogenesis. Organogenesis is a procedure which is a kind of tissue culture systems, including thin cell layers, leaf segments, stem explants, and variously derived calluses. Indeed, this process is forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium (Thorpe, 1994). The organogenic process begins with changes in a single or a small group of parenchyma cells, which then divide to produce a globular mass of cells or meristemoid, which is plastic and can give rise to either a shoot or root primordium. These events can occur directly in the explant or indirectly after some callus formation. These structural changes are themselves a manifestation of preceding physiological, biochemical, biophysical and molecular events which reflect selective gene activity in those cells (Thorpe, 1990).

Christianson and Warnick (1988) established that being 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 of cytokinins (Hicks, 1994).

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).

Shoot organogenesis continues to have potentially important biotechnologic applications in the development of micropropagation strategies (Lindsey and Jones 1990; Phillips and Hubstenberger, 1995).

Embryogenesis; in contrast to organogenesis, which produces a unipolar shoot or root primordium, somatic embryogenesis gives rise to a bipolar structure with a root/shoot axis. This process can be divided into 2 major processes: the induction of cells with embryogenic manipulation of the explant, medium and culture environment has led to success in a process which is very plastic, and may be structurally and/or cellularly different from zygotic embryogenesis (Thorpe, 1990).

As to callus formation, it is an unorganized or undifferentiated mass of proliferative cells produced either *in vitro* or in nature. The callus tissue in

many cases shows a high potential for organogenesis when first initiated but gradually a decline sets in as subculture proceeds with eventual loss of organogenesis response (George, 2008). Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced (Gurel and Turker, 2001). The underlying basis for organogenesis involves the interplay of a host of factors: donor plant growth, source of the explant, culture medium, supplements of growth regulators, and environmental conditions. *In vitro* organogenesis in tobacco cultures could be chemically regulated. The addition of auxin to the medium served to initiate root formation, whereas shoot initiation was inhibited. Later, it was found that adenine sulfate was active in promoting shoot initiation, and this chemical reversed the inhibitory effect of auxin. The studies of Skoog's group led to the hypothesis that organogenesis is regulated by a balance between cytokinin and auxin. A relatively high auxin: cytokinin ratio induced root formation in tobacco callus, whereas a low ratio of the same compounds favored shoot production (Dodds and Roberts, 1995).

Higher plants produce a large number of diverse organic chemicals, which are pharmaceutical and industrial interest. By 1987, there were 30 cell culture systems that were better producers of secondary metabolites than the respective plants. Industry is poised to benefit from these researches (Thorpe, 1990).

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.

Some desirable properties of plants i.e. disease resistance, increased secondary product accumulation, superior genotype characteristics, or worthwhile morphological characteristics can be gained via *in vitro* micropropagation. *In vitro* micropropagation also can ensure multiplication method for producing plantlets rapidly. Many endemic and getting extinct plants can be obtained and produced using an *in vitro* propagation protocol. Moreover, an *in vitro* propagation method would eliminate seasonal constraints and side effects with seedling by providing unlimited planting material in a year-round basis (McCoy, 1998). Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout *et al*., 2000). Although medicinal plants can easily be found in the wild, they are generally subjected to some herbicides and attacked by some insects and pathogens. For example, *L. vulgaris* can serve as viruses' infection sources (Kazinczi et al., 2004) and *Ramularia*

lysimachiae Thum. is the plant pathogen founded on *L. vulgaris* (Legg, 2004). *In vitro* micropropagation not only allows rapid propagation or mass production of identical clones of plant species but also has the following uses: (1) elimination of viruses and other pathogens; (2) storage of essential germ plasm instead of conventional seeds; (3) embryo rescue; (4) 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).

Plants are the primary source of food for the human race and only by correct management of plant agriculture can be the present human populations continue to be fed. The flow energy from sunlight through plant photosynthesis is at the heart of the importance of plants in the world economy (Smith, 2004). The increased emphasis on sustainable agriculture, and an increasing world population, coupled with the continued loss of prime agricultural land to housing and industry, signify that we will have to feed, clothe and house more people than ever existed in the history of mankind (Thorpe, 1990).

In order to have standardized formulations, the chemical constituents from plants and their parts are required to be uniform both qualitatively and quantitatively. Furthermore, an ever increasing demand of uniform medicinal plants based medicines warrants their mass cloning through plant tissue

culture strategy (Chaturvedi *et al*., 2007). This protocol can be helpful in the large scale production of certain secondary products of *L. vulgaris*. Micropropagation of *L. vulgaris* can provide a mass production of pesticide, herbicide and disease free plants on a commercial scale and unlimited, genetically uniform plant materials can consistently be obtained throughout the whole year. Moreover, this protocol can provide plant material for future pharmacological, physiological and biochemical studies.

2.2. Materials and Methods

Seeds of *L.vulgaris L.* were collected from Abant Lake, Bolu/Turkey in September of 2008. Identification of the species was made by using "Flora of Turkey and The East Aegean Island" (Davis, 1978) and voucher specimens (AUT-2008) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey.

L. vulgaris seeds were stayed for a night in tap water, washed with an anti-bacterial soap, rinsed with distilled water and surface sterilized by shaking in 20% ethanol for 20 minutes followed by rinsing well with sterilized distilled water and then dipped into 20% Domestos® (5% sodium hypochloride) for 10 minutes. Finally, seeds were washed with sterile distilled water three times. After surface sterilization of the seeds, seeds were placed in sterile, disposable petri dishes containing Murashige and Skoog's minimal organics (MSMO) medium (4.43 g/l MSMO, Sigma Chemical Co., St. Louis, MO, USA; Murashige and Skoog 1962) with 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH 5.7, autoclaved for 20 minutes at 121℃ and 105 kPa). After a two week incubation in this medium, seedlings were transferred to Magenta containers

(GA-7 Vessel, Sigma Chemical Co.) containing the same medium for an additional three weeks. For shoot regeneration, leaf (36 mm^2), petiole (4-5 mm) and root explants (6-7 mm) were excised from four weeks old sterile seedlings and placed in sterile disposable petri plates containing 4.43 g/l MSMO with different combinations and concentrations of plant growth regulators; thidiazuron (TDZ; 0.01, 0.05 and 0.1 mg/l) + indole-3-acetic acid (IAA; 0, 0.05, 0.1 and 0.5 mg/l); benzyladenine (BA; 0.1, 0.5, 1.0, 2.0 and 3 mg/l) + IAA (0, 0.1, 0.5, 1.0 and 2.0 mg/l); BA (0.1, 0.5, 1.0, 2.0 and 3 mg/l) + naphthalene acetic acid (NAA; 0, 0.1, 0.5, 1.0 and 2.0 mg/l); Kinetin (KIN; 0.1, 0.5, 1.0 and 3.0 mg/l) + IAA (0, 0.1, 0.5 and 1 mg/l); KIN (0.5, 1.0 and 2.0 mg/l) + 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 0.01, 0.1, 0.5 and1.0 mg/l); BA (0.1, 0.5, 1.0 and 2.0) + 2,4-D (0, 0.01, 0.1, 0.5 and 1.0); BA (0.1, 0.5 and 1.0) + indole-3-butyric acid (IBA; 0, 0.1, 0.5 and 1.0); Zeatin (Zea; 0.01, 0.1, 0.5 and 1.0) + IBA (0, 0.5, 1.0 and 3.0). All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$).

After three weeks, regenerated explants were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing MSMO medium containing 0.5 mg/l Gibberellic acid (GA_3) for shoot elongation for an additional two weeks. Shoots were then separated individually and placed in rooting medium containing MSMO and varying concentrations of different auxins; IAA (0.5, 1 and 3 mg/l), IBA (0.5, 1 and 3 mg/l), 2,4-D (0.1, 0.5 and 1 mg/l) and NAA (0.5, 1 and 3 mg/l) in culture jars containing 40 ml medium. Rooted explants were removed from culture jars, washed carefully with sterile distilled water to remove adhering medium and transferred to vermiculate (Agrekal®) in Magenta containers for acclimatization and after 2

weeks, they were transferred to plastic pots containing potting mixed vermiculate with soil and also added little tap water. The plants were covered with transparent polythene covers to maintain temperature and high humidity.

The shoot number and percentage of explants producing shoots were recorded after 4-5 weeks for all explants. Tests had 10 replications for each explant and the experiment was repeated three times. Moreover, after three weeks, the number of roots and the percentage of explants producing roots were recorded. There were 10 replications and the experiment was repeated three times. Results were expressed as means ± standard error of the mean. All data were analyzed by analysis of variance (ANOVA) and mean values were compared with Duncan's Multiple Range Tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA).

2.3. Results and Discussion

Although *L. vulgaris* is a very valuable medicinal and horticultural plant, there is no any study about *in vitro* propagation of this plant. We therefore aimed to develop an *in vitro* culture protocol for high frequency regeneration of loosestrife plants by adventitious organogenesis.

Three different explants (leaf lamina, stem and root) were excised from 4 or 5-weeks-old sterile seedlings and cultured on MSMO medium containing BA in combination with IAA, IBA, NAA and 2-4-D (Table 2.1, 2.2, 2.3, 2.4 and 2.5); TDZ in combination with IAA (Table 2.6); KIN in combination with IAA or 2,4-D. (Table 2.7, 2.8 and 2.9).

All 3 explants gave the best number of shoot formation with BA:IAA (Table 2.1), BA:IBA (Table 2.2) and BA:NAA (Table 2.3) combinations.

Among BA:IAA combinations, best shoot proliferation was obtained with root explant on media containing 0.5 mg/l BA + 0.5 mg/l IAA (51.2 shoots per explant; 100 % explants formed shoots); with stem explant on media containing 1 mg/l BA + 2 mg/l IAA (52.9 shoots per explant; 100 % explants formed shoots); with leaf explants on media containing 1 mg/l BA + 1 mg/l IAA (37.6 shoots per explant; 82% explants formed shoots) (Table 2.1; Figure 2.1). When root explants were cultured on media containing BA in combination with IAA, it is evident that frequency of shoot formation was closely related to concentration of the auxin (IAA) supply. Increasing concentration of auxin severely inhibited shoot development. For example, among root explants investigated, 2 mg/l IAA in combination with 0.5 mg/l BA decreased mean number of the shoot per explant to 10.6 and the number of the shoots producing explants to 83% while 0.5 mg/l IAA in combination with 0.5 mg/l BA induced shoots in all explants and the mean number of shoots per explant was 51.2. No callus formation was observed with combinations of low concentration of BA (0.1-0.5 mg/l) and IAA (0.1-0.5 mg/l) with all explants (direct organogenesis). But, higher concentrations of combinations (>0.5 mg/l BA or IAA) caused callus formation before shoot development from all 3 explants, thus suggesting indirect shoot organogenesis.

Among BA:IBA combinations, best shoot proliferation was obtained with root explant on media containing 0.5 mg/l BA or 0.5 mg/l BA + 0.1 mg/l IBA (32.7 and 30.9 shoots per explant, respectively; 74 and 91% explants formed shoots, respectively); with stem explant on media containing 1 mg/l BA + 0.5 mg/l IBA (59.1 shoots per explant; 100% explants formed shoots); with leaf explants on media containing 0.5 mg/l BA in combination with 0.1,

0.5 or 1 mg/l IBA (56.9, 50.7 and 50.3 shoots per explant, respectively; 100% explants formed shoots) (Table 2.2; Figure 2.1). Observing callus formation before shoot development from all explants suggested that adventitious shoots were of indirect origin.

Among BA:NAA combinations, best shoot proliferation was obtained with root explant on media containing only 0.5 mg/l BA (32.7 shoots per explant; 74% explants formed shoots); with stem explant on media containing 2 mg/l BA + 0.1 mg/l NAA (27.8 shoots per explant; 100% explants formed shoots); with leaf explants on media containing 0.5 mg/l BA + 0.1 mg/l NAA (38 shoots per explant; 100% explants formed shoots) (Table 2.3). Callus formation was observed before shoot formation for all explants (indirect regeneration).

BA plus 2,4-D combinations were not effective for shoot regeneration. It is evident that combinations of 2.4-D with BA lowered shoot number or caused no shoot formation (Table 2.4). BA:2,4-D combinations produced light green and/or yellow callus. Vitrification was observed in these combinations.

When TDZ was used in combination with IAA, only stem explant gave best shoot proliferation. Leaf and root explants formed fewer shoots than stem explant. With stem explants, the greatest number of shoots per explant was observed on media containing 0.01 mg/l TDZ + 0.5 mg/l IAA (63.8 shoots per explant; 100% explants formed shoots) (Table 2.6). 10.1 shoots were obtained from leaf explant with the same combination (Table 2.6). When TDZ was used alone, they were less successful for shoot regeneration. In combinations of TDZ with IAA, firstly stem, then leaf explants gave good results (Table 2.6). All explants produced yellowish and

green callus before shoot formation (indirect organogenesis). Root explants produced the most green-yellowish, watery and massive callus.

When KIN was used in combination with IAA or 2,4-D, leaf explant was not effective for shoot regeneration. Moreover, only KIN containing media were successful for shoot regeneration. Combinations of 2.4-D or IAA with KIN caused lower number of shoot or no shoot formation (Table 2.7 and 2.8). Best shoot formation was obtained with root explant on medium containing only 3 mg/l KIN (30 shoots per explant; 100% explants formed shoots). Stem explant gave the best shoot number with 1 mg/l KIN (30.2 shoots per explant; 70% explants formed shoots) (Table 2.7 and 2.8). Generally, direct organogenesis was observed with KIN:IAA combinations for all explants. But, combination of KIN with 1mg/l IAA formed callus before shoot formation for leaf explants. KIN:2,4-D combinations produced light green callus in velvet appearance. Generally, root explants produced white callus.

Zeatin:IBA combinations caused less number of microshoots than BA:IBA combinations. Best shoot proliferation was obtained with root explant on media containing 0.5 mg/l ZEA + 3 mg/l IBA (24.5 shoots per explant; 100% explants formed shoots); with stem explant on media containing only 1 mg/l ZEA (36.2 shoots per explant; 100% explants formed shoots); with leaf explants on media containing 0.1 mg/l ZEA + 3 mg/l IBA (16.9 shoots per explant; 100% explants formed shoots) (Table 2.9). Generally, leaf explants had direct shoot regenerations with ZEA:IBA combinations. But, stem and root explants had indirect organogenesis with light yellow- light green callus.

Treatments involving no plant growth regulators (control treatments) produced no shoots (Tables 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9).

Overall, among the stem explants, highest number of shoots per explant was observed on media with 0.01 mg/l TDZ plus 0.5 mg/l IAA (63.8 shoots per explant; 100% explants formed shoots) (Table 2.6). Media containing 1.0 mg/l BA plus 0.5 mg/l IBA, 1.0 mg/l BA plus 2.0 mg/l IAA, 0.1 mg/l TDZ plus 0.5 mg/l IAA and 1.0 BA plus 1.0 mg/l IBA were also effective for shoot formation with stem explants (59.1, 52.9, 47.4 and 46.9 shoots per explant, respectively; 100% explants formed shoots) (Table 2.1, 2.2, 2.5 and 2.6).

Regarding root explants, the greatest number of shoots per explant was recorded on media containing 0.5 mg/l BA plus 0.5 mg/l IAA (51.2 shoots per explant; 100% explants formed shoots). Media containing 2.0 mg/l BA plus 0.5 mg/l IAA and only 0.5 mg/l BA were also effective for shoot formation (46.2 and 32.7 shoots per explant, 78% and 74% explants formed shoots, respectively) (Table 2.1). When KIN used alone (3.0 mg/l KIN and 1.0 mg/l KIN), it produced some amounts of shoots (30 and 25.1 shoots per explant; 100% and 60% shoot inductions; respectively) (Table 2.7 and 2.8) .

Leaf explants generally formed fewer shoots than stem and root explants. In spite of this, 0.5 mg/l BA in combination with 0.1 mg/l IBA was effective to give shoot regeneration with leaf explants (56.9 shoots per explant; 100% explants formed shoots) (Table 2.2). With leaf explants, the other best plant growth regulator applications were 0.5 mg/l BA plus 0.1 mg/l NAA, 1 mg/l BA plus 1 mg/l IAA (38.0 and 37.6 shoots per explant; 100% and 82% shoot inductions; respectively) (Table 2.1 and 2.3).

Zheng *et al*. (2009) reported the *in vitro* culture protocol of three ornamental species of *Lysimachia* genus (*L. christinae*, *L. rubinervis* and *L. nummularia* 'Aurea'). They investigated the regenerability of 3 *Lysimachia* species using *in vitro* leaf and shoot tip explants on Murashige and Skoog (MS) medium containing only different concentrations of 6-Benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA). Best shoot proliferation was obtained with both shoot tip and leaf explant for *L. christinae* 3 mg/l BA+0.1 mg/l NAA or 5 mg/l BA+0.1 mg/l NAA combinations were successful for both explants of *L. christinae* (around 12.25-14.34 shoots). Only shoot tip explant of *L. rubinervis* was successful for shoot regeneration with 3 mg/l BA+0.1 mg/l NAA or 5 mg/l BA+0.1 mg/l NAA (around16.87-17.20 shoots) and no regeneration was observed with leaf explant of *L. rubinervis*. Controversy, leaf explant of *L. nummularia* 'Aurea' was successful for shoot regeneration with 1 mg/l BA+0.1 mg/l NAA (12.73 shoots) and no regeneration was observed with shoot tip explant of *L. nummularia*. Leaf explants of *L. christinae* regenerated 3.7 shoots with 1 mg/l BA+0.1 mg/l NAA and 13.01 shoots with 3 mg/l BA+0.1 mg/l NAA. Similary, leaf explant of *L. vulgaris* regenerated 4 shoots with 1 mg/l BA+0.1 mg/l NAA and 12.3 shoots with 3 mg/l BA+0.1 mg/l NAA in our study (Table 2.3). However, for BA:NAA combinations best shoot proliferation was obtained with leaf explant on media containing 0.5 mg/l BA+0.1 mg/l NAA in our study (Table 2.3). Generally, BA in combination with NAA was not as effective as BA in combination with IAA or IBA in our study (Table 2.1, 2.2, 2.3 and 2.5).

Regenerated shoots were cultured on shoot elongation medium containing 0.5 mg/l GA_3 for additional 2 weeks (Figure 2.2). After 2 weeks,

regenerated shoots were separated individually and cultured on MSMO medium containing IAA, IBA, 2.4-D or NAA. If we compare among the various auxins investigated for rooting, IBA was better than other auxins (Table 2.10; Figure 2.2). They formed roots in 4-5 weeks. The greatest number of roots per explant was observed on media with 0.5 and 1 mg/l IBA (38.35 and 28.45 roots per explant; 100% explants formed roots). Medium containing 0.1 mg/l 2,4-D was also effective for root formation (22.9 roots per explant; 100% explants formed roots). The data presented in Table 2.10 clearly indicate that rooting was even possible on basal medium, which produced a mean of 6.8 roots per shoot explant. However, adding IBA at 0.5 or 1.0 mg/l not only increased the mean number of roots per shoot explant and % shoots forming roots (from 80% to 100%) significantly but also produced longer, thicker and healthier shoots (Figure 2.2). Higher concentrations of both IBA and 2,4-D caused significant reductions in terms of mean number of roots per shoot explant. Similarly, IBA was reported as the most effective auxin for rooting with some medicinal plants including *Oenothera* spp. (De Gyves *et al*., 2001), *Mucuruna pruriens* (L.) DC (Faisal *et al*., 2006) and *Cassia angustifolia* Vahl (Siddique and Anis, 2007). Generally, high concentrations of auxines induced to decrease number of roots.

Zheng *et al*. (2009) used full strength or half strength MS medium containing only 0.1 mg/l NAA for *in vitro* rooting of regenerated three ornamental species of *Lysimachia* genus (*L. christinae*, *L. rubinervis* and *L. nummularia* 'Aurea'). Half strength MS medium containing 0.1 mg/l NAA was the best medium for rooting in these *Lysimachia* species. Generally, NAA cocentrations were not as effective as other used auxins (IAA, IBA and 2,4-

D) for rooting of regenerated shoots of *L. vulgaris* in our study (Table 2.10). Three different concentrations of NAA (0.5, 1 and 3 mg/l) were used and more root formation was obtained with 0.5 mg/l NAA (6 roots) in our study. Similarly, Zheng *et al*. (2009) obtained 4.26 roots for *L. christinae*, 7.56 roots for *L. rubinervis* and 8.17 roots for *L. nummularia* 'Aurea' on full strength MS medium containing 0.1 mg/l NAA.

The rooted plants were transferred to Magenta containers including vermiculate for acclimatization (Figure 2.3). After 3 weeks, they were transferred to soil and kept under growth room conditions. Approximately 85% of the generated plants survived through the hardening off process (Figure 2.3).

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 (Yucesan *et al*., 2007). In this study, every plant growth regulators were more effective on different tissues. For example, leaf lamina which had very less response according to other explants, were fairly good with together BA:IAA combinations. Moreover, stem explants gave more shoots in TDZ:IAA combinations although, the other explants did not form shoots or very few. In the preceding studies, the promoting effect of TDZ has been reported for many species as it has property to induce adventitious shoot formation in a number of species, especially woody plants (Ahn *et al*., 2007;

Jones *et al*., 2007; Wang and Bao, 2007; Yücesan *et al*., 2007; Huetteman and Preece 1993; Lu 1993). TDZ was more successful for only stem explants than the other plant growth regulators in this study. Similarly, Heutteman and Preece (1993) reported that low concentrations of TDZ have been found to be useful for micropropagation and higher concentrations of TDZ increased callus formation in woody plants. Singh *et al*. (2003) also reported that in the regeneration of pigeonpea, low concentrations of TDZ (0.01-0.22 mg/l) induced multiple shoots, intermediary concentration (1.1 mg/l) produced clusters of leafy structures and higher concentrations (2.2-4.4 mg/l) induced somatic embryogenesis. De Gyves *et al*. (2001) and Huetteman and Preece (1993) emphasized the potential use of TDZ in the regulation of adventitious shoot production and synergism existing between TDZ and both endogenous and some exogenous auxins. This finding is consistent with our data highlighting that when TDZ was used alone, it produced some amounts of shoots. But, if TDZ was combined with IAA, more shoot formation was obtained.

This protocol offers a potential system for micropropagation and genetic improvement of this medicinal and ornamental species in the future. This study provides not only a rapid clonal 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). The usage medicinal plants from wild may create extinction problem. Another driving factor for the renewed interest in plant antimicrobials, antitumor or any properties in the past 20 years has been the rapid rate of (plant) species extinction (Cowan, 1999).

Therefore, the plant tissue culture, propagation of plants, and plant biotechnology possesses a great value.

Thus, the present study is the first to describe an efficient *in vitro* plant regeneration protocol for *L. vulgaris* through adventitious shoot regeneration from leaf, stem and root segment explants of aseptically germinated seedlings. 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 yellow loosestrife plants and can provide plant material for future pharmacological, physiological and biochemical studies.

Table 2. 1 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of BA and IAA. Means with the same letter within columns are not significantly different at P>0.05.

		EXPLANTS					
Plant growth regulators		**Leaf**		**Stem**		**Root**	
BA (mg/l)	**IAA (mg/l)**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	6.6 ± 2.3^{gijk}	32	17.6 ± 3.6^{def}	63
0.1	0.1	1.0 ± 0.5^{h}	18	2.9 ± 1.3^{ijk}	31	12.8 ± 3.4^{defg}	47
0.1	0.5	0.3 ± 0.2^{h}	13	5.8 ± 1.7^{ijk}	41	4.3 ± 0.9^{fg}	58
0.1	1.0	1.7 ± 0.7^{h}	24	4.9 ± 1.4^{ijk}	44	11.2 ± 2.1^{defg}	79
0.1	2.0	0.1 ± 0.1^{h}	6	0.5 ± 0.3^{k}	25	3.5 ± 0.8^{fg}	72
0.5	0.0	2.6 ± 0.9^{gh}	40	15.1 ± 4.1^{cdefgijk}	44	32.7 ± 4.4^{bc}	74
0.5	0.1	12.7 ± 2.2^{defg}	63	5.2 ± 1.9^{ijk}	32	45.8 ± 5.2^{ab}	97
0.5	0.5	17.1 ± 4.4^{cde}	68	18.0 ± 3.8^{cdefgij}	76	51.2 ± 6.4^{a}	100
0.5	1.0	15.5 ± 2.7^{cdef}	76	28.8 ± 5.0^{bc}	91	22.9 ± 3.4^{cde}	95
0.5	2.0	19.2 ± 4.1^{bcd}	83	25.9 ± 8.8^{bcde}	50	10.6 ± 1.8^{defg}	83
1.0	0.0	6.7 ± 2.2^{fgh}	50	4.4 ± 2.1^{ijk}	17	24.6 ± 4.7^{cd}	66
1.0	0.1	17.6 ± 3.4^{cde}	74	10.5 ± 3.2^{efgijk}	58	33.4 ± 4.9^{bc}	76
1.0	0.5	25.0 ± 4.9^{bc}	84	35.5 ± 6.1^{b}	82	19.6 ± 3.9^{cde}	74
1.0	1.0	37.6 ± 4.9^{a}	82	23.0 ± 4.3^{bcdef}	89	8.6 ± 1.3^{g}	84
1.0	2.0	27.6 ± 8.2^{b}	78	52.9 ± 9.9^{a}	100	23.8 ± 5.3^{cde}	89
2.0	0.0	9.8 ± 4.6^{defgh}	56	3.4 ± 2.0^{ijk}	21	21.6 ± 6.5^{cde}	72
2.0	0.1	11.1 ± 3.1^{defgh}	67	13.1 ± 6.1^{defgijk}	36	44.2 ± 11.0^{ab}	67
2.0	0.5	4.3 ± 2.1^{gh}	44	11.4 ± 5.0^{efgijk}	47	46.2 ± 11.3^{ab}	78
2.0	1.0	1.1 ± 0.4^{h}	39	21.9 ± 6.1^{bcdefg}	93	1.2 ± 0.5^{g}	33
2.0	2.0	8.3 ± 2.7^{efgh}	61	19.9 ± 4.5^{cdefgi}	93	1.2 ± 0.8^{g}	17
3.0	0.0	- -	-	7.6 ± 2.8^{fgijk}	43	1.1 ± 0.4^{g}	39
3.0	0.1	0.3 ± 0.2^{h}	11	7.8 ± 3.5^{fgijk}	38	3.9 ± 1.5^{fg}	61
3.0	0.5	0.3 ± 0.2^{h}	11	18.2 ± 7.2^{cdefgij}	56	8.9 ± 2.4^{efg}	67
3.0	1.0	3.6 ± 1.7^{gh}	28	28.6 ± 10.0^{bcd}	50	2.8 ± 1.2^{fg}	50
3.0	2.0	2.1 ± 0.8^{h}	50	22.6 ± 7.5^{bcdef}	44	4.0 ± 1.6^{fg}	50

Table 2. 2 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of BA and IBA. Means with the same letter within columns are not significantly different at P>0.05.

Plant growth regulators		EXPLANTS					
		Leaf		Stem		Root	
BA (mg/l)	IBA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	6.6 ± 2.3^{fg}	32	17.6 ± 3.6^{abc}	63
0.1	0.1	3.7 ± 3.3^{cd}	30	39.9 ± 9.1^{bc}	100	1.36 ± 0.5^{c}	64
0.1	0.5	6.1 ± 2.8^{bcd}	60	19.7 ± 2.4^{def}	100	2.3 ± 0.8^{c}	80
0.1	1.0	12.9 ± 2.2^{bc}	100	8.6 ± 2.3^{fg}	90	1.1 ± 0.5^{c}	50
0.5	0.0	2.6 ± 0.9^{cd}	40	15.1 ± 4.1^{efg}	44	32.7 ± 4.4^{a}	74
0.5	0.1	56.9 ± 12.3^{a}	100	7.8 ± 4.4^{fg}	40	30.9 ± 8.3^{a}	91
0.5	0.5	50.7 ± 6.4^{a}	100	27.7 ± 6.6^{cde}	90	12.9 ± 4.6^{bc}	80
0.5	1.0	50.3 ± 5.8^{a}	100	33.5 ± 5.9^{bcd}	100	2.0 ± 0.8^{c}	60
1.0	0.0	6.7 ± 2.2^{bcd}	50	4.4 ± 2.1^{fg}	17	24.6 ± 4.7^{ab}	66
1.0	0.1	11.0 ± 4.5^{bcd}	60	40.0 ± 11.3^{bc}	90	12.8 ± 7.5^{bc}	80
1.0	0.5	12.2 ± 4.2^{bc}	80	59.1 ± 5.2^{a}	100	2.8 ± 1.7^{c}	40
1.0	1.0	16.1 ± 2.9^{b}	100	46.9 ± 7.5^{ab}	100	2.8 ± 1.5^{c}	60

Table 2. 3 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of BA and NAA. Means with the same letter within columns are not significantly different at P>0.05.

		EXPLANTS					
Plant growth regulators		Leaf		Stem		Root	
BA (mg/l)	NAA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	6.6 ± 2.3^{bcde}	32	17.6 ± 3.6^{b}	63
0.1	0.1	5.6 ± 0.8^{bcd}	100	1.4 ± 0.5^{e}	88	3.1 ± 1.3^{c}	50
0.1	0.5	- -	-	- -	-	3.9 ± 1.1^{c}	90
0.1	1.0	- -	-	- -	-	- -	-
0.1	2.0	- -	-	- -	-	0.4 ± 0.3^{c}	20
0.5	0.0	2.6 ± 0.9^{cd}	40	15.1 ± 4.1^{bcd}	44	32.7 ± 4.4^{a}	74
0.5	0.1	38.0 ± 7.8^{a}	100	6.3 ± 3.1^{bcde}	63	0.4 ± 0.2^{c}	30
0.5	0.5	- -	-	- -	-	0.3 ± 0.1^{c}	30
0.5	1.0	- -	-	- -	-	0.4 ± 0.2^{c}	40
0.5	2.0	- -	-	- -	-	- -	-
1.0	0.0	6.7 ± 2.2^{bcd}	50	4.4 ± 2.1^{cde}	17	24.6 ± 4.7^{ab}	66
1.0	0.1	4.0 ± 1.2^{cd}	70	17.5 ± 3.8^{ab}	100	- -	-
1.0	0.5	- -	-	- -	-	0.5 ± 0.3^{c}	30
1.0	1.0	- -	-	- -	-	- -	-
1.0	2.0	- -	-	- -	-	- -	-
2.0	0.0	9.8 ± 4.6^{bc}	56	3.4 ± 2.0^{de}	21	21.6 ± 6.5^{ab}	72
2.0	0.1	8.4 ± 3.6^{bc}	80	27.8 ± 9.2^{a}	100	- -	-
2.0	0.5	7.8 ± 1.9^{bcd}	100	3.0 ± 1.1^{de}	63	- -	-
2.0	1.0	2.3 ± 0.7^{cd}	70	0.5 ± 0.3^{e}	25	- -	-
2.0	2.0	0.3 ± 0.3^{d}	10	- -	-	- -	-
3.0	0.0	- -	-	7.6 ± 2.8^{bcde}	43	1.1 ± 0.4^{c}	39
3.0	0.1	12.3 ± 2.3^{b}	100	15.8 ± 3.7^{bc}	88	- -	-
3.0	0.5	0.3 ± 0.2^{d}	20	7.9 ± 2.1^{bcde}	100	0.3 ± 0.3^{c}	10
3.0	1.0	0.1 ± 0.1^{d}	10	0.9 ± 0.4^{e}	50	- -	-
3.0	2.0	0.4 ± 0.3^{d}	20	- -	-	- -	-

Table 2. 4 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of BA and 2,4-D. Means with the same letter within columns are not significantly different at P>0.05.

		EXPLANTS					
Plant growth regulators		**Leaf**		**Stem**		**Root**	
BA (mg/l)	**2,4-D (mg/l)**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	6.6 ± 2.3[b]	32	17.6 ± 3.6[b]	63
0.1	0.01	1.6 ± 0.5[b]	60	- -	-	- -	-
0.1	0.1	- -	-	- -	-	- -	-
0.1	0.5	- -	-	- -	-	- -	-
0.1	1.0	- -	-	- -	-	- -	-
0.5	0.0	2.6 ± 0.9[b]	40	15.1 ± 4.1[a]	44	32.7 ± 4.4[a]	74
0.5	0.01	- -	-	- -	-	0.1 ± 0.1[c]	10
0.5	0.1	- -	-	- -	-	- -	-
0.5	0.5	- -	-	- -	-	- -	-
0.5	1.0	- -	-	- -	-	- -	-
1.0	0.0	6.7 ± 2.2[ab]	50	4.4 ± 2.1[b]	17	24.6 ± 4.7[ab]	66
1.0	0.01	- -	-	- -	-	- -	-
1.0	0.1	- -	-	- -	-	- -	-
1.0	0.5	- -	-	- -	-	- -	-
1.0	1.0	- -	-	- -	-	- -	-
2.0	0.0	9.8 ± 4.6[a]	56	3.4 ± 2.0[b]	21	21.6 ± 6.5[ab]	72
2.0	0.01	- -	-	- -	-	- -	-
2.0	0.1	- -	-	- -	-	- -	-
2.0	0.5	- -	-	- -	-	- -	-
2.0	1.0	- -	-	- -	-	- -	-

Table 2. 5 Shoot development from leaf, stem and root explants incubated on MSMO medium containing BA in combination with IAA, IBA and NAA. Means with the same letter within columns are not significantly different at P>0.05.

Plant growth regulators		EXPLANTS					
		Leaf		Stem		Root	
BA (mg/l)	IAA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	6.6 ± 2.3^{ijk}	32	17.6 ± 3.6^{cdefg}	63
0.1	0.1	1.0 ± 0.5^{j}	18	2.9 ± 1.3^{ijk}	31	12.8 ± 3.4^{defg}	47
0.1	0.5	0.3 ± 0.2^{j}	13	5.8 ± 1.7^{ijk}	41	4.3 ± 0.9^{efg}	58
0.1	1.0	1.7 ± 0.7^{ij}	24	4.9 ± 1.4^{ijk}	44	11.2 ± 2.1^{defg}	79
0.1	2.0	0.1 ± 0.1^{j}	6	0.5 ± 0.3^{k}	25	3.5 ± 0.8^{efg}	72
0.5	0.0	2.6 ± 0.9^{ij}	40	15.1 ± 4.1^{fghijk}	44	32.7 ± 4.4^{bc}	74
0.5	0.1	12.7 ± 2.2^{defghij}	63	5.2 ± 1.9^{ijk}	32	45.8 ± 5.2^{ab}	97
0.5	0.5	17.1 ± 4.4^{cdefg}	68	18.0 ± 3.8^{efghijk}	76	51.2 ± 6.4^{a}	100
0.5	1.0	15.5 ± 2.7^{cdefghi}	76	28.8 ± 5.0^{defg}	91	22.9 ± 3.4^{cd}	95
0.5	2.0	19.2 ± 4.1^{cde}	83	25.9 ± 8.8^{defghi}	50	10.6 ± 1.8^{defg}	83
1.0	0.0	6.7 ± 2.2^{efghij}	50	4.4 ± 2.1^{jk}	17	24.6 ± 4.7^{cd}	66
1.0	0.1	17.6 ± 3.4^{cdef}	74	10.5 ± 3.2^{ghijk}	58	33.4 ± 4.9^{bc}	76
1.0	0.5	25.0 ± 4.9^{cd}	84	35.5 ± 6.1^{bcde}	82	19.6 ± 3.9^{cdef}	74
1.0	1.0	37.6 ± 4.9^{b}	82	23.0 ± 4.3^{defghij}	89	8.6 ± 1.3^{defg}	84
1.0	2.0	27.6 ± 8.2^{bc}	78	52.9 ± 9.9^{ab}	100	23.8 ± 5.3^{cd}	89
2.0	0.0	9.8 ± 4.6^{efghij}	56	3.4 ± 2.0^{jk}	21	21.6 ± 6.5^{cde}	72
2.0	0.1	11.1 ± 3.1^{efghij}	67	13.1 ± 6.1^{ghijk}	36	44.2 ± 11.0^{ab}	67
2.0	0.5	4.3 ± 2.1^{ghij}	44	11.4 ± 5.0^{ghijk}	47	46.2 ± 11.3^{ab}	78
2.0	1.0	1.1 ± 0.4^{j}	39	21.9 ± 6.1^{defghij}	93	1.2 ± 0.5^{fg}	33
2.0	2.0	8.3 ± 2.7^{efghij}	61	19.9 ± 4.5^{efghijk}	93	1.2 ± 0.8^{fg}	17
3.0	0.0	- -	-	7.6 ± 2.8^{ijk}	43	1.1 ± 0.4^{fg}	39
3.0	0.1	0.3 ± 0.2^{j}	11	7.8 ± 3.5^{ijk}	38	3.9 ± 1.5^{efg}	61
3.0	0.5	0.3 ± 0.2^{j}	11	18.2 ± 7.2^{efghijk}	56	8.9 ± 2.4^{defg}	67
3.0	1.0	3.6 ± 1.7^{ghij}	28	28.6 ± 10.0^{defg}	50	2.8 ± 1.2^{fg}	50
3.0	2.0	2.1 ± 0.8^{ij}	50	22.6 ± 7.5^{defghij}	44	4.0 ± 1.6^{efg}	50
BA (mg/l)	IBA (mg/l)						
0.1	0.1	3.7 ± 3.3^{ghij}	30	39.9 ± 9.1^{bcd}	100	1.36 ± 0.5^{fg}	64
0.1	0.5	6.1 ± 2.8^{efghij}	60	19.7 ± 2.4^{efghijk}	100	2.3 ± 0.8^{fg}	80
0.1	1.0	12.9 ± 2.2^{defghij}	100	8.6 ± 2.3^{hijk}	90	1.1 ± 0.5^{fg}	50
0.5	0.1	56.9 ± 12.3^{a}	100	7.8 ± 4.4^{ijk}	40	30.9 ± 8.3^{bc}	91
0.5	0.5	50.7 ± 6.4^{a}	100	27.7 ± 6.6^{defgh}	90	12.9 ± 4.6^{defg}	80
0.5	1.0	50.3 ± 5.8^{a}	100	33.5 ± 5.9^{cdef}	100	2.0 ± 0.8^{fg}	60
1.0	0.1	11.0 ± 4.5^{efghij}	60	40.0 ± 11.3^{bcd}	90	12.8 ± 7.5^{defg}	80
1.0	0.5	12.2 ± 4.2^{defghij}	80	59.1 ± 5.2^{a}	100	2.8 ± 1.7^{fg}	40
1.0	1.0	16.1 ± 2.9^{cdefgh}	100	46.9 ± 7.5^{abc}	100	2.8 ± 1.5^{fg}	60
BA (mg/l)	NAA (mg/l)						
0.1	0.1	5.6 ± 0.8^{efghij}	100	1.4 ± 0.5^{k}	88	3.1 ± 1.3^{efg}	50
0.1	0.5	- -	-	- -	-	3.9 ± 1.1^{efg}	90
0.1	2.0	- -	-	- -	-	0.4 ± 0.3^{fg}	20
0.5	0.1	38.0 ± 7.8^{b}	100	6.3 ± 3.1^{ijk}	63	0.4 ± 0.2^{fg}	30
0.5	0.5	- -	-	- -	-	0.3 ± 0.1^{g}	30
0.5	1.0	- -	-	- -	-	0.4 ± 0.2^{fg}	40
1.0	0.1	4.0 ± 1.2^{fghij}	70	17.5 ± 3.8^{efghijk}	100	- -	-
1.0	0.5	- -	-	- -	-	0.5 ± 0.3^{fg}	30
2.0	0.1	8.4 ± 3.6^{efghij}	80	27.8 ± 9.2^{defgh}	100	- -	-
2.0	0.5	7.8 ± 1.9^{efghij}	100	3.0 ± 1.1^{jk}	63	- -	-
2.0	1.0	2.3 ± 0.7^{ij}	70	0.5 ± 0.3^{k}	25	- -	-
2.0	2.0	0.3 ± 0.3^{j}	10	- -	-	- -	-
3.0	0.1	12.3 ± 2.3^{defghij}	100	15.8 ± 3.7^{fghijk}	88	- -	-
3.0	0.5	0.3 ± 0.2^{j}	20	7.9 ± 2.1^{ijk}	100	0.3 ± 0.3^{g}	10
3.0	1.0	0.1 ± 0.1^{j}	10	0.9 ± 0.4^{k}	50	- -	-
3.0	2.0	0.4 ± 0.3^{j}	20	- -	-	- -	-

Table 2. 6 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of TDZ and IAA. Means with the same letter within columns are not significantly different at P>0.05.

Plant growth regulators		EXPLANTS					
		Leaf		Stem		Root	
TDZ (mg/l)	IAA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots
0.0	0.0	- -	-	- -	-	- -	-
0.01	0.00	5.9 ± 4.3^{b}	30	22.8 ± 5.5^{cd}	80	- -	-
0.01	0.05	3.6 ± 0.7^{bc}	90	27.3 ± 6.6^{c}	100	- -	-
0.01	0.10	2.1 ± 0.9^{bc}	60	13.4 ± 3.4^{cdef}	70	0.7 ± 0.3^{cd}	40
0.01	0.50	10.1 ± 1.2^{a}	100	63.8 ± 9.8^{a}	100	- -	-
0.05	0.00	- -	-	24.6 ± 6.5^{cd}	90	1.2 ± 0.6^{bcd}	40
0.05	0.05	5.8 ± 1.7^{b}	80	9.6 ± 2.5^{def}	80	0.3 ± 0.2^{d}	20
0.05	0.10	0.7 ± 0.5^{c}	30	11.9 ± 4.7^{cdef}	70	2.1 ± 1.1^{abc}	40
0.05	0.50	2.3 ± 0.8^{bc}	50	19.7 ± 5.9^{cde}	100	- -	-
0.10	0.00	- -	-	5.2 ± 2.4^{ef}	60	2.4 ± 0.9^{abc}	70
0.10	0.05	- -	-	10.3 ± 2.1^{def}	80	2.8 ± 1.4^{ab}	30
0.10	0.10	2.1 ± 1.2^{bc}	30	21.3 ± 4.0^{cde}	100	1.5 ± 0.4^{bcd}	70
0.10	0.50	0.5 ± 0.3^{c}	20	47.4 ± 9.6^{b}	100	3.7 ± 0.9^{a}	80

Table 2. 7 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of KIN and IAA. Means with the same letter within columns are not significantly different at P>0.05.

Plant growth regulators		EXPLANTS					
		Leaf		Stem		Root	
KIN (mg/l)	IAA (mg/l)	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots	Mean number of shoots per explant (±SE)	% explants forming shoots
0.0	0.0	- -	-	- -	-	- -	-
0.1	0.0	- -	-	2.7 ± 2.2^{b}	20	1.8 ± 0.6^{ef}	70
0.1	0.1	- -	-	2.3 ± 0.8^{b}	50	2.5 ± 0.6^{ef}	90
0.1	0.5	0.1 ± 0.1^{c}	10	5.1 ± 1.3^{b}	90	2.9 ± 0.7^{ef}	80
0.1	1.0	3.0 ± 1.1^{b}	60	3.0 ± 0.6^{b}	90	2.4 ± 0.8^{ef}	70
0.5	0.0	- -	-	1.2 ± 0.9^{b}	30	13.3 ± 7.3^{bcde}	70
0.5	0.1	- -	-	3.6 ± 1.3^{b}	60	4.4 ± 1.0^{def}	100
0.5	0.5	0.9 ± 0.4^{c}	50	0.3 ± 0.3^{b}	10	0.6 ± 0.3^{ef}	30
0.5	1.0	4.3 ± 1.6^{b}	70	2.0 ± 1.8^{b}	20	2.4 ± 1.3^{ef}	50
1.0	0.0	- -	-	30.2 ± 12.9^{a}	70	25.1 ± 11.2^{ab}	60
1.0	0.1	- -	-	- -	-	7.0 ± 2.5^{cdef}	80
1.0	0.5	7.0 ± 1.8^{a}	80	0.5 ± 0.5^{b}	10	9.3 ± 1.8^{cdef}	100
1.0	1.0	4.5 ± 1.4^{b}	70	5.3 ± 2.1^{b}	60	15.6 ± 2.8^{bcd}	100
3.0	0.0	- -	-	1.8 ± 1.8^{b}	10	30.0 ± 4.8^{a}	100
3.0	0.1	- -	-	- -	-	17.2 ± 7.5^{bc}	40
3.0	0.5	- -	-	0.9 ± 0.6^{b}	30	2.4 ± 1.2^{ef}	50
3.0	1.0	0.2 ± 0.1^{c}	20	0.9 ± 0.9^{b}	10	6.2 ± 4.7^{cdef}	30

Table 2. 8 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of KIN and 2,4-D. Means with the same letter within columns are not significantly different at P>0.05.

		EXPLANTS					
Plant growth regulators		**Leaf**		**Stem**		**Root**	
KIN (mg/l)	**2,4-D (mg/l)**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**
0.0	0.0	- -	-	- -	-	- -	-
0.5	0.0	- -	-	1.2 ± 0.9[b]	30	13.3 ± 7.3[b]	70
0.5	0.01	- -	-	- -	-	- -	-
0.5	0.1	- -	-	- -	-	- -	-
0.5	0.5	- -	-	- -	-	- -	-
0.5	1.0	- -	-	- -	-	- -	-
1.0	0.0	- -	-	30.2 ± 12.9[a]	70	25.1 ± 11.2[a]	60
1.0	0.01	- -	-	- -	-	- -	-
1.0	0.1	- -	-	- -	-	- -	-
1.0	0.5	- -	-	- -	-	- -	-
1.0	1.0	- -	-	- -	-	- -	-
2.0	0.0	- -	-	0.30 ± 0.2[b]	20	7.8 ± 2.5[bc]	70
2.0	0.01	- -	-	- -	-	- -	-
2.0	0.1	- -	-	- -	-	- -	-
2.0	0.5	- -	-	- -	-	- -	-
2.0	1.0	- -	-	- -	-	- -	-

Table 2. 9 Shoot development from leaf, stem and root explants incubated on MSMO medium containing different concentrations of Zea and IBA. Means with the same letter within columns are not significantly different at P>0.05.

		EXPLANTS					
Plant growth regulators		**Leaf**		**Stem**		**Root**	
Zeatin (mg/l)	**IBA (mg/l)**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**	**Mean number of shoots per explant (±SE)**	**% explants forming shoots**
0.0	0.0	- -	-	- -	-	- -	-
0.01	0.0	- -	-	7.8 ± 1.9^{efg}	80	- -	-
0.01	0.5	4.9 ± 1.6^{c}	90	17.8 ± 1.7^{bc}	100	0.7 ± 0.3^{c}	40
0.01	1.0	12.9 ± 3.2^{ab}	90	17.7 ± 2.1^{bc}	100	0.1 ± 0.1^{c}	10
0.01	3.0	9.3 ± 3.9^{b}	70	20.7 ± 2.2^{b}	100	0.3 ± 0.2^{c}	20
0.1	0.0	- -	-	16.8 ± 5.9^{bcd}	60	1.3 ± 1.3^{c}	10
0.1	0.5	0.1 ± 0.1^{d}	10	13.3 ± 2.6^{bcde}	100	3.4 ± 1.0^{c}	100
0.1	1.0	4.0 ± 1.4^{cd}	80	11.4 ± 2.6^{cdef}	100	10.2 ± 3.5^{b}	80
0.1	3.0	16.9 ± 1.7^{a}	100	13.5 ± 1.6^{bcde}	100	24.5 ± 4.1^{a}	100
0.5	0.0	- -	-	8.4 ± 4.6^{defg}	90	- -	-
0.5	0.5	0.1 ± 0.1^{d}	10	4.4 ± 0.8^{fg}	90	11.9 ± 2.3^{b}	100
0.5	1.0	1.0 ± 0.5^{cd}	40	3.6 ± 0.3^{fg}	100	13.5 ± 1.7^{b}	100
0.5	3.0	2.8 ± 0.7^{cd}	70	8.3 ± 1.7^{defg}	100	2.4 ± 0.8^{c}	70
1.0	0.0	- -	-	36.2 ± 5.9^{a}	100	- -	-
1.0	0.5	0.8 ± 0.6^{cd}	30	6.4 ± 2.2^{efg}	100	4.4 ± 1.5^{c}	90
1.0	1.0	4.5 ± 2.2^{cd}	80	14.9 ± 3.0^{bcde}	100	1.3 ± 0.8^{c}	30
1.0	3.0	13.8 ± 0.6^{a}	100	10.8 ± 2.4^{cdef}	100	4.1 ± 1.1^{c}	100

Figure 2. 1 *L. vulgaris* shoot regeneration from leaf explant on medium containing 0.5 mg/l BA + 0.1 mg/l IBA(left); from stem explant on medium containing 1 mg/l BA + 0.5 mg/l IBA (middle); from root explant on medium containing 0.5 mg/l BA + 0.1 mg/l IAA (right).

Figure 2. 2 Shoot elongation of regenerated shoots on medium containing 0.5 mg/l GA_3 (left); Rooting of the regenerated shoots on medium containing 0.5 mg/l IBA (right).

Figure 2. 3 Regenerated plants in Magenta containers including vermiculate for acclimatization (left); Regenerated plants transferred to plastic pots containing sterile potting soil under growth room conditions (right).

Table 2. 10 Effects of the tested auxins on root formation from regenerated shoots. Means with the same letter within columns are not significantly different at P>0.05.

Treatments	Mean number of roots per explant (±SE)	% explants forming roots
Control	6.80 ± 1.2^{fg}	80
IAA (mg/l)		
0.5	11.20 ± 1.8^{ef}	95
1	18.25 ± 1,8^{cd}	100
3	14.40 ± 2.3^{de}	100
IBA (mg/l)		
0.5	38.35 ± 3.7^{a}	100
1	28.45 ± 3.4^{b}	100
3	2.20 ± 0.7^{g}	75
2,4-D (mg/l)		
0.1	22.90 ± 3.1^{bc}	100
0.5	7.60 ± 1.1^{fg}	100
1	3.85 ± 1.9^{g}	90
NAA (mg/l)		
0.5	6.00 ± 1.4^{fg}	85
1	1.80 ± 0.5^{g}	70
3	2.40 ± 0.7^{g}	75

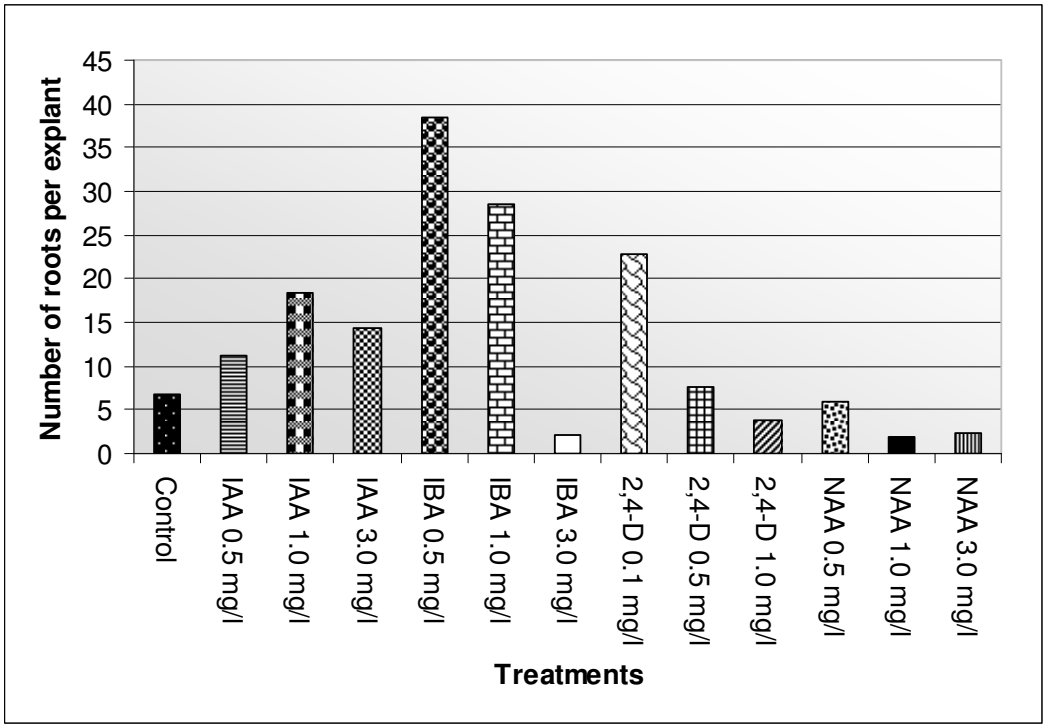

Figure 2. 4 Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots**.**

CHAPTER 3

3. Essential Oil Analyses of *Lysimachia vulgaris L.,* by Gas Chromatography

3.1. Introduction

Plant secondary metabolites, including volatile essential oils and non-volatile alkaloids, quinones, lactones, etc., played an important role in the life of human beings. Thus the localization of sites of their biosynthesis and accumulation was of interest for researchers. The volatile essential oils, valued for its medicinal of fragrant properties, can be used in pharmacy, perfume and cosmetics industries (Huang *et al*., 2009b).

Essential oils are volatile natural complex secondary metabolites characterized by a strong odor and have a generally lower density than that of water (Bruneton, 1999; 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 (Franzios *et al*., 1997; 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. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999).

Essential oils are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, wood, fruits, and roots). They can be accumulated by expression, fermentation, enfleurage or extraction but the method of stream 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). There is a different usage in China. After the leaves of *Lysimachia foenum-graenum* are dried, they exhibit a smoky, spicy, green, maple, woody and caramel odour. Thus, the local people of China use a smoking method to increase the smoky odour.

Extracts of *L. foenum-graenum* can be used as smoky fragrances and widely used in the cigarette flavour industry in China (Shu and Shen, 2008).

The plant kingdom has more abundant diversities in chemical structures with extraordinary potent pharmacological activities and rather low toxicities. However, there is only very limited knowledge about their chemical compositions, pharmacokinetics, and metabonomics. This is mainly due to the fact that plants consist of multi-component systems with mostly unknown ingredients. The definition and investigation of folk medicine usage applications in biochemical, pharmaceutical and clinical research is important. 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.

Based on a literature survey, no report concerning *in vitro* or *ex vitro* culture of *L. vulgaris* or analyses of essential oil in *in vitro*, hardening, magenta and plants from natural population was found, therefore, the aim of this study were: 1) to develop an efficient regeneration system for *in vitro* cultured materials 2) to analyze the variations of essential oil compounds in *in vitro* regenerated plants (magenta), seed and hardening, as well as in natural population.

3.2. Material and Method

The aerial parts of *L. vulgaris* L. (W) were collected during flowering stage in July to August, 2008, seeds of *L. vulgaris* L. (SE) were collected during seed time in October 2008, near Lake of Abant. In addition, yellow

loosestrife seedlings cultures were gotten from *in vitro* studying of *L. vulgaris*. These samples were dried in incubator within two days at 30ºC. These seedlings were separated as after magenta (M) (grown only *in vitro* conditions) and after hardening (H) (transferred to soil after magenta and grown in soil). For each of two *in vitro* cultures, material used for essential oil isolation was collected periodically i.e. about 10 times over a period of about one year after stabilization of cultures had occurred.

Steam Distillation

All material types of *L. vulgaris* were powdered. For all dried materials were weighted as 100g. Then, they were 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 -20ºC before analysis. The oil samples isolated by hydrodistillation were used to estimate the oil yields.

GC-MS Analysis

All GC-MS analyses of essential oil of yellow loosestrife were recorded in Faculty of Agticulture of Ankara University. All gas chromatography (GC) analyses were 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℃ to 150℃ at an initial rate of 3℃/min. The injector and detector temperatures were programmed at 220℃ and 290℃, 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℃ and then heated gradually to 150℃ with a 3℃/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). Relative percentages of the separated compounds were calculated from FID chromatograms.

3.3. Results and Discussion

The yellowish-golden color essential oil isolated from the natural populations (W), both studying *in vitro* cultures that were in magenta (M) and that were grown in soil after it's tissue culture (H) were obtained in a yield of 0.01 % (v/w). However, the essential oil color of seeds of *L. vulgaris* L. (S) was transparent but little turbidity. Similarly, 0.01% (v/w) was obtained. They were dissolved in n-hexane. The oils were obtained by water steam distillation of the above samples of *L. vulgaris*, and were analyzed as described above. Table (3.1) shows the results of the qualitative and quantitative oil analyses listed in order of elution in the capillary column.

In total, 38 compounds were identified, from seed (18), magenta (19), hardening (11) and natural population (10). In fact, results obtained from

above samples showed a highly chemical variability within the oil's in *L. vulgaris*. The oils of above samples *L. vulgaris* were separated by gas chromatography and the three major components eicosane, 2-pentadecanone and palmitic acid (Figure 3.1; 3.2; 3.3; 3.4; 3.5; 3.6; 3.7; 3.8; 3.9).

Ten components of the oil from the W were identified, amounting to 90.43% of the essential oil. The major compounds of the oil palmitic acid (63.37%), n–tetracosane (11.51%) and 2-pentadecanone (8.25%) were attained. The minor components were found as docosane (0.29%), 3-buten-2-one (0.47%), nonadecane (0.65%) and 1,2-benzenedicarboxylic acid (0.75%). Eleven components were identified in the oil isolated from the H, amounting to 65.28% of total oil Table 3.1. The main components, again 2-pentadecanone (29.35%) and palmitic acid (17.18%) represented a significant part of the major components of the oil. In addition, neophytadiene (5.18%) and pentacosane (4.52%) also were identified distinctly. The minor substances were beta-ionone (2.42%), 1,2-benzenedicarboxylic acid (2.40%), Ethyl palmitate (0.64%) and octanal was trace with amounting 0.44 %. The most components were gained from seeds and Magenta. Nineteen components of oil isolated from the M and eighteen components also from the S were identified, amounting to 92.66% and 96.86%, respectively. Palmitic acid (31.73%), eicosane (10.79%), pentadecanone (8.60%), 9,12-octadecatrienoic acid (8.38%) and neophytadiene (6.71%) were dominant in the oil of M, being the main components.

Also 18 components of the oil isolated from the seeds of yellow loosestrife were identified, amounting to 96.86%. Eicosane (42.71%),

docosane (21.69%), palmitic acid (7.21%), naphthalene (6.16) and valencene (4.80%) were abundant ones. Ethyl palmitate was trace amount with 0.13%. In addition this heptadecane (0.20%) were little quantity.

Among major components of essential oils, eicosane ranged from 42.71% in seed to 10.79% in magenta, while 2- pentadecanone ranged from 29.35% in hardening to 2.81% in seed. For palmitic acid ranged from 63.37% in natural population to 7.21% in seed. However, 2- pentadecanone as a major constituents has already been reported in many medicinal plants in natural population (ranged from 6.5 to 29.9%) 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) and result from the present study confirm a similar ranged in *L. vulgaris*. Working with *L. vulgaris* we found highest amount of 2-pentadecanone in hardening areal plant followed by 8.25% in natural population. For, eicosane observation in aerial part of plant have been reported in other plants such as *Tanacetum densum* (Bagci, 2009), *Achilla gypsicola* (Korsali *et al*., 2009), *Artemisia scoparia* (Kapoor *et al*., 2004). Working with *L. vulgaris* we found eicosane only in seed sample and magenta samples, fourfold higher amount.

Table 3. 1 The chemical constituents (%) of the oil of *Lysimachia vulgaris*. RT : retention time

RT	Constituents	Natural Population	Hardening	Seed	Magenta
20.48	Isoborneol	–	–	–	2.83
21.68	alpha-Terpineol	0.85	–	–	0.76
30.95	n-Tetradecane	–	–	0.44	–
33.16	Geranylacetone	–	–	–	1.88
34.52	beta-ionone	–	2.42	–	1.28
34.53	3-Buten-2-one	0.47	–	–	–
35.22	Aromadendrene	–	–	3.83	–
35.58	Phenol, 2,6-bis(1,1-dimethyletheyl)	–	–	0.36	–
38.14	1H-Cycloprop[e]azulene-7-ol	–	–	0.31	–
38.65	Valencene	–	–	4.80	–
39.20	E-2-Tetradecen-1-ol	–	–	0.36	–
41.23	Heptadecane	–	–	0.20	–
41.52	Lauric aldehyde	–	–	1.08	–
41.52	Tetradecanal	–	–	–	0.50
41.90	3-Tetradecene	–	1.13	–	–
42.14	Octanal	–	0.44	–	–
42.15	alpha-Hexylcinnamic aldehyde	–	–	–	0.56
43.62	2-Pentadecanone	8.25	29.35	2.81	8.60
43.98	1,2-Benzenedicarboxylic acid	0.75	2.40	0.68	1.59
44.56	Nonadecane	0.65	–	–	0.32
44.61	Farnesyl acetone C	–	–	–	1.87
44.64	Hexadecanoic acid	–	1.16	–	–
44.66	Pentadecanoic acid	–	–	–	0.75
45.17	Palmitic acid	63.37	17.18	7.21	31.73
45.46	Ethyl palmitate	–	0.64	0.13	1.17
46.33	Naphthalene	–	–	6.16	–
46.57	Heneicosane	1.48	–	–	–
46.63	9,12,15-Octadecatrienoic acid		0.86	–	3.11
46.74	Neophytadiene	2.81	5.18	–	6.71
46.89	n-Tetracosane	11.51	–	–	4.86
46.96	9,12-Octadecadienoic acid	–	–	–	8.38
47.06	1-Octadecene	–	–	–	4.97
47.21	Pentacosane	–	4.52	–	–
47.21	Eicosane	–	–	43.56	10.79
47.30	3-Methylheneicosane	–	–	1.52	–
47.39	Docosane	0.29	–	21.69	–
47.55	n-Octadecane	–	–	1.29	–
48.04	Heptacosane	–	–	0.43	–
	Total	90.43	65.28	96.86	92.66

Data presented in Table (3.1) show that all analyzed in *L.vulgaris*, contain varying amounts of minor components such as 1,2-Benzenedicarboxylic acid, ethyl palmitate, α-terpineol, neophytadiene, beta-ionone. Among major components of essential oils, ethyl palmitate ranged from 0.13% in seed to 1.17% in magenta, was not found in natural population and 1,2- Benzenedicarboxylic acid was found in all samples ranged from 0.68 % in seed to 2.40% in hardening. While α-terpineol was found only in two samples, magentas and natural population, ranged from 0.76% to 0.85% respectively. For, neophytadiene ranged from 2.81% in natural population 6.71% in magenta, was not found in seed. For, beta-ionone ranged from 1.28% in magenta to 2.42% in natural population, was not found in seed as well as natural population. However, beta-ionone as minor constituents has already been reported in many medicinal plants in natural population such as *Genista tinctoria* (Rigano *et al*., 2010), *Nelumbo nucifera* (Huang *et al*., 2010), *Equisetum palustre* (Stojanovic *et al*., 2008), *Humulus lupulus* (Eyres *et al*., 2007). For, neophytadiene observation in aerial part of plant have been reported in other plants such as *Elatostema laetevirens* (Miyazawa *et al*., 2009), Serebian tobacco types (Radulovic *et al*., 2006). For, ethyl palmitate, α-terpineol and 1,2- Benzenedicarboxylic acid observation in aerial part of plant have been reported in other plants such as *Swertia densifolia* (Naik *et al*., 2010), *Centaurea drabifolia* (Ugur *et al*., 2009), *Cleome viscosa* (Olatunji *et al*., 2005), *Eugenla catharianensis* (Apel *et al*., 2002), *Agathis phillippiensis* (Lassak and Brophy, 2008) *Laurus nobilis* (Ozcan and Cholchat, 2005), *Salvia schimperi* (Endeshaw *et al*., 2000), *Salvia lavandulaefolia* (Perry *et al*., 2000), *Hypercum perforatum* (Cirak *et al*., 2010) and *Marchantia convoluta*

(Yan *et al*., 2008), *Ceratophyllum demersum* and *Vallisneria spiralis* (Xian *et al*., 2006), *Dolycoris baccarum* (Durak, 2008) respectively. Also some of the components of the oil isolated from the above samples were only found in seed (12), magenta (15), natural population (2), and hardening (3). Among the components two components was found in n-tetracosane (Natural population and Magenta) amount with 11.51% and 4.86%, Docosane (Natural population and Seed) amount with 0.29% and 21.69%, respectively. Similar result was (n-tetracosane, 11.6%) found in natural population in *Ficus carica* (Ayoub *et al*., 2010). Ayoub *et al*. (2010) found that Docosane amount with 7.7%, working with *L. vulgaris,* we found very less amount in natural population. The chemical composition of essential oils depends on climatic, seasonal and geographic conditions, harvest period and distillation technique.

Unlike, yellow loosestrife (*L. vulgaris*) has never been studied for essential oil until this time but *Lysimachia foenum-graecum* from *Lysimachia* genus has searched to analyze essential oil. In addition, around 50 volatile compounds have been identified (Shu and Shen, 2008). In Shu and Shen (2008) study is related to aroma impact of *L. foenum-graecum*. Only naphthalene is same constituent with this search.

Dionysia diapensifolia Boiss. which is member of Primulaceae family was reported essential oil search (Javidnia *et al*., 2010). According to this study; *L. vulgaris* has 6 same compounds with *D. diapensifolia*. These are geranyl acetone, beta-ionone, heptadecane, heneicosane, docosane, tetracosane. Javidnia *et al*. (2010) found geranyl acetone, beta-ionone and heptadecane as trace compounds but in this study these compounds were

found as geranyl acetone 1.88% in magenta samples, beta-ionone 2.42% in hardening and 1.28% in magenta, heptadecane 0.20% in seed. Heneicosane and docosane were found as 0.1% in Javidnia *et al*. (2010) study. However, in this study these were found 1.48% in natural populations for heneicosane, 0.29% in natural populations 21.69% in seed for docosane. The last same compound with this study is tetracosane. It was obtained as 0.3% in Javidnia *et al.* (2010) study. In this research, it was obtained as 11.51% in natural plants, 4.86% in magenta samples. The GS-MS method was little different than our method. Therefore, the amounts of percentages of compounds can be different.

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).

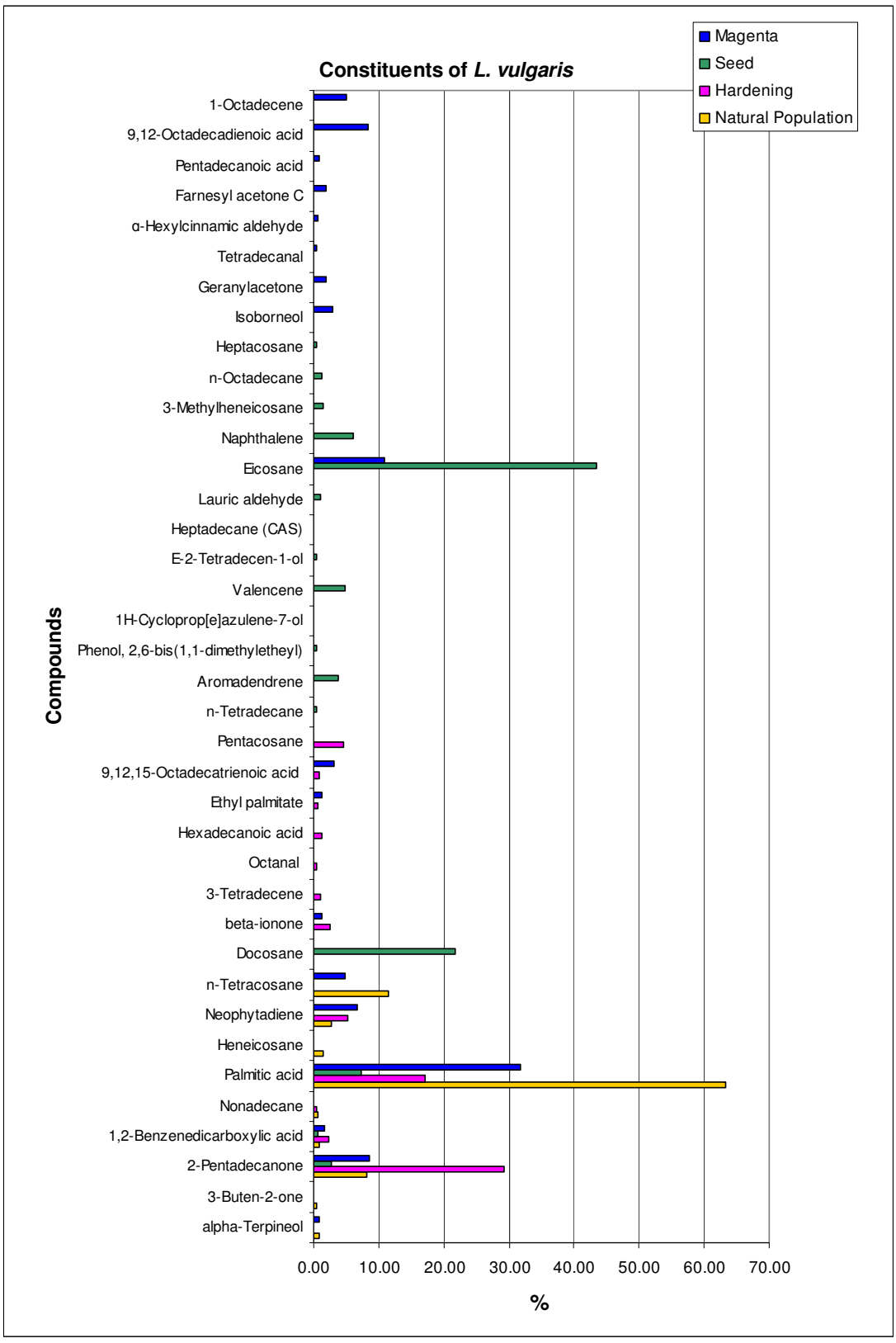

Figure 3. 1 Essential oil constituents of samples of *L. vulgaris.*

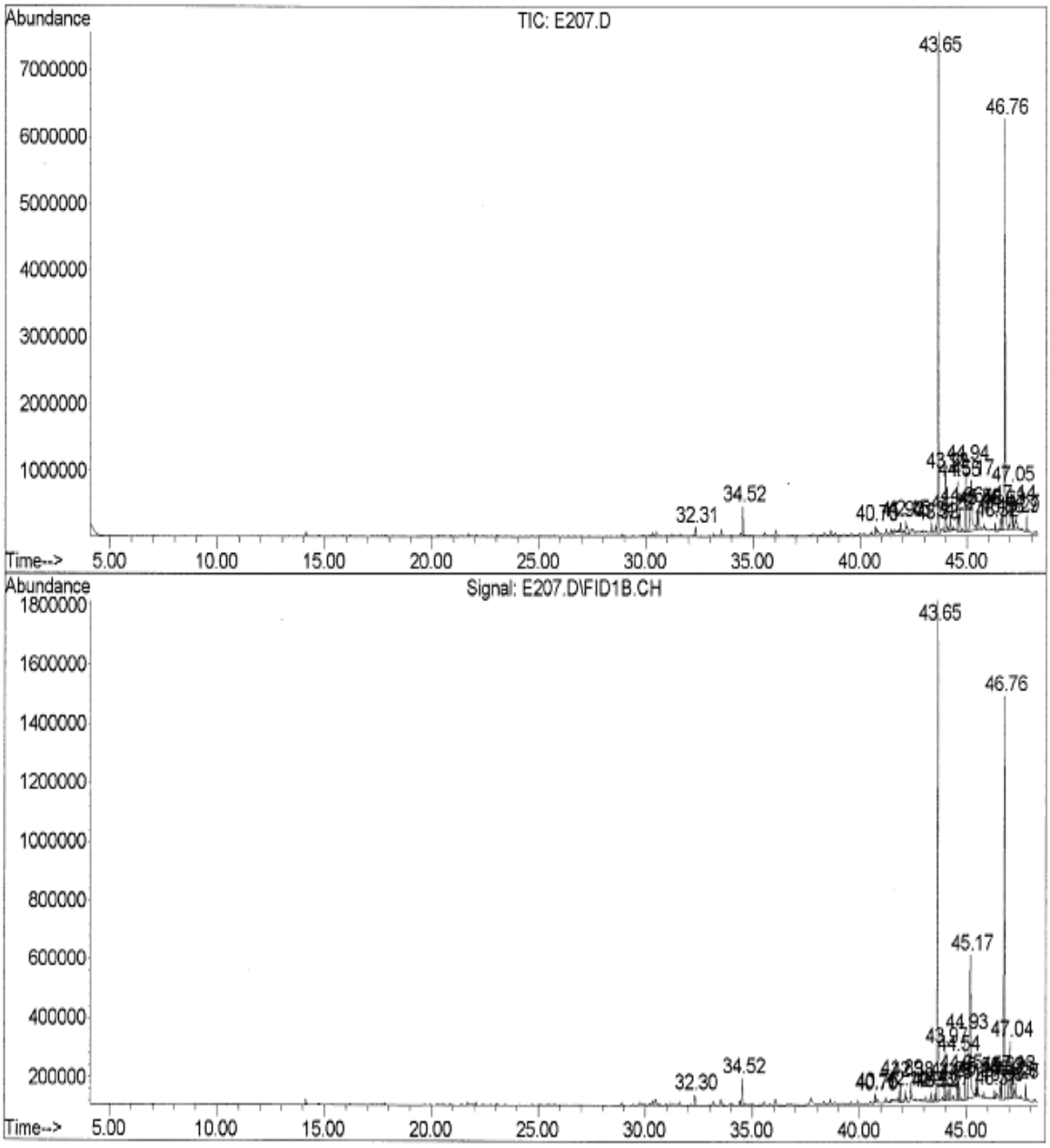

Figure 3. 2 The GC and FID graphs obtained from Hardening samples of *L. vulgaris*.

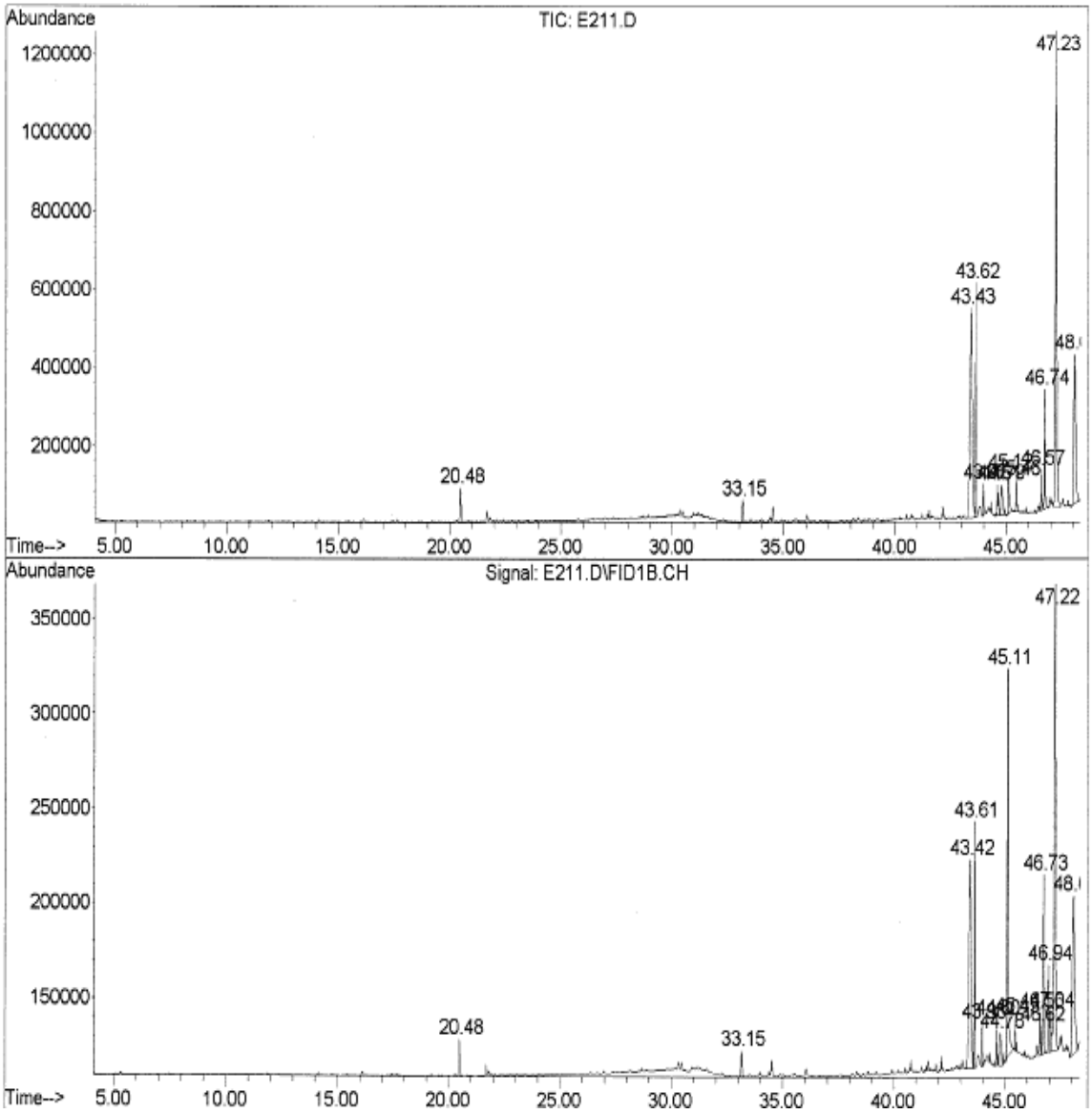

Figure 3. 3 The GC and FID graphs obtained from Magenta samples of *L. vulgaris*.

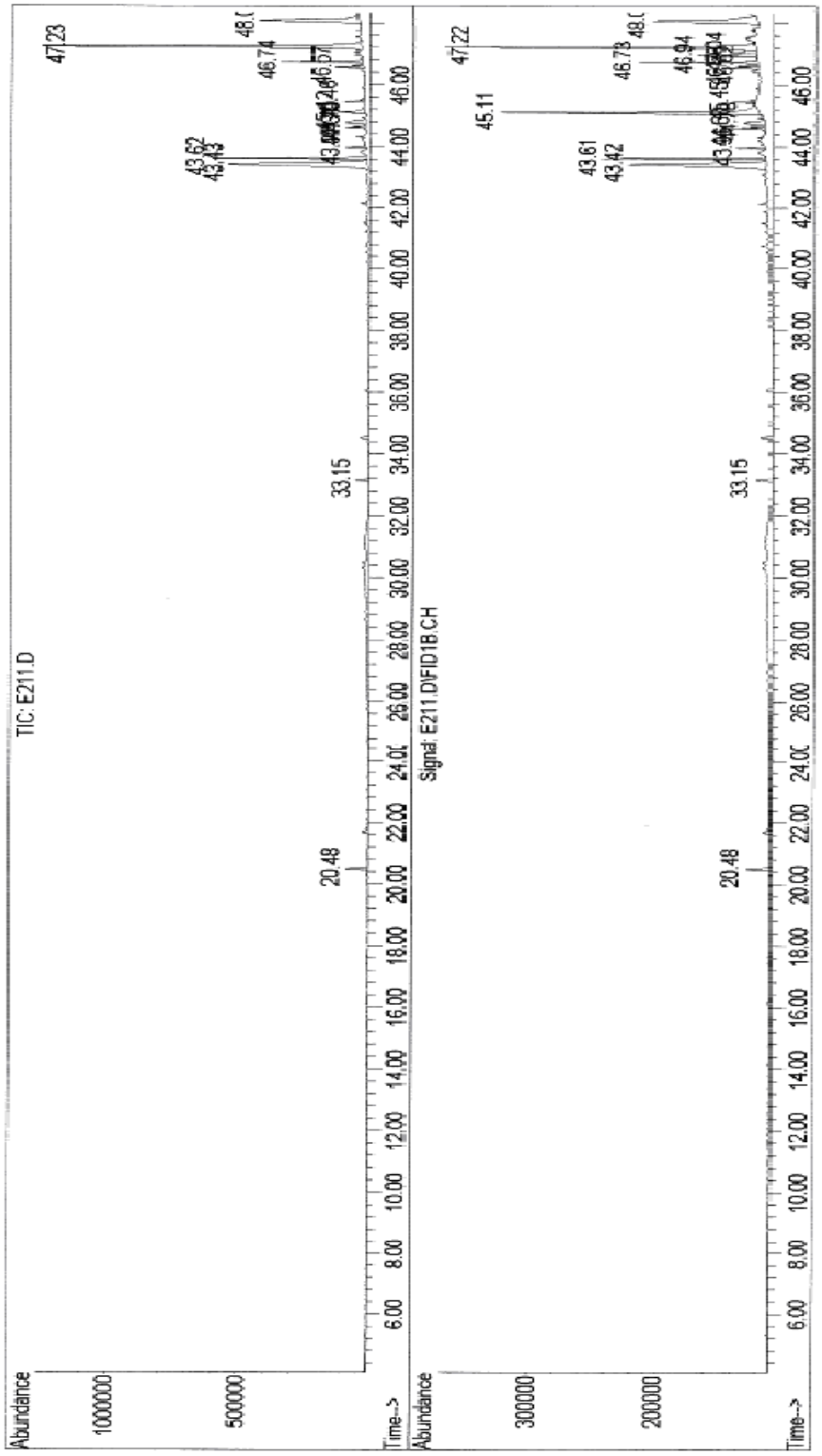

Figure 3. 4 The GC and FID graphs obtained from Magenta samples of *L. vulgaris*.

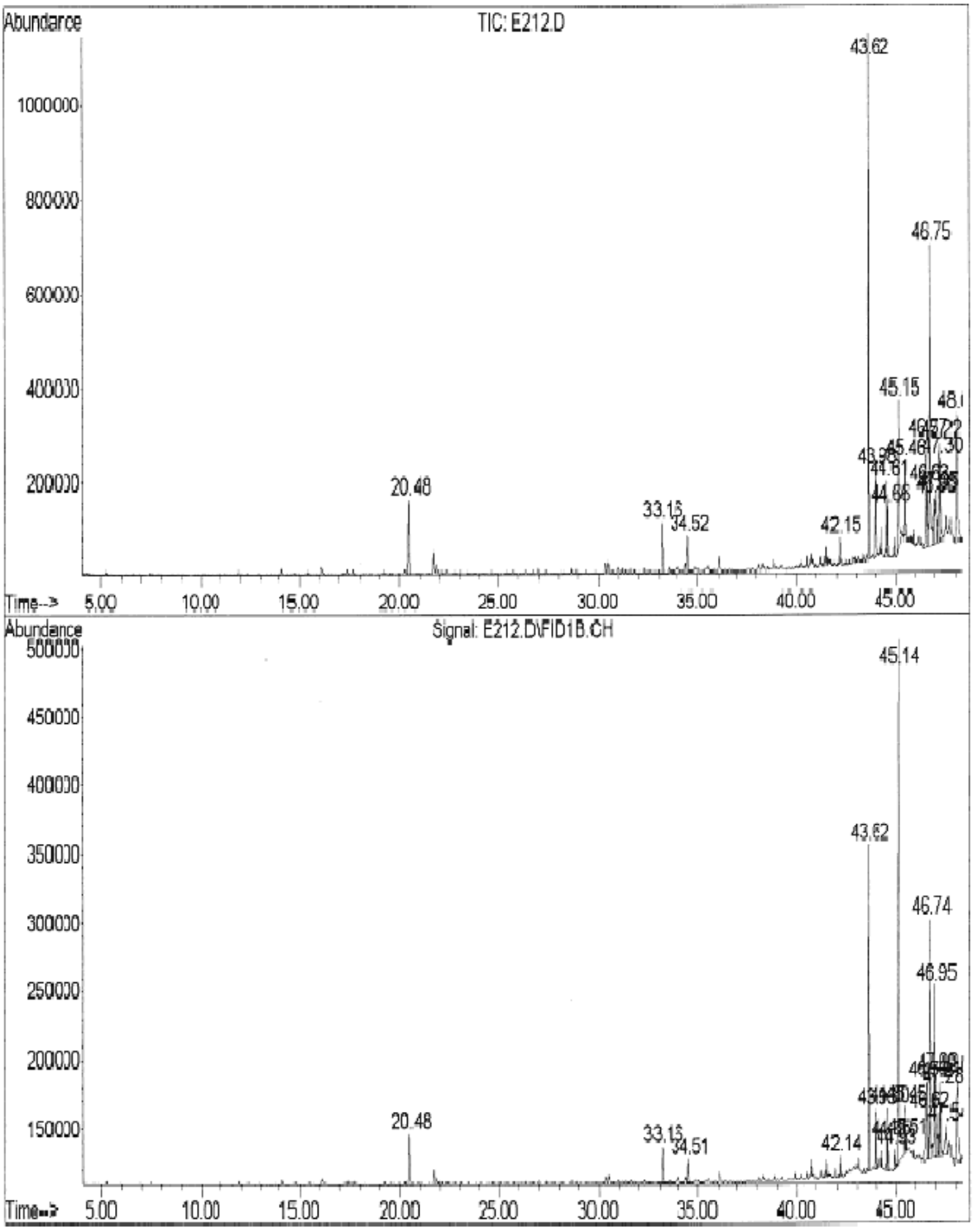

Figure 3. 5 The GC and FID graphs obtained from Magenta samples of *L. vulgaris*.

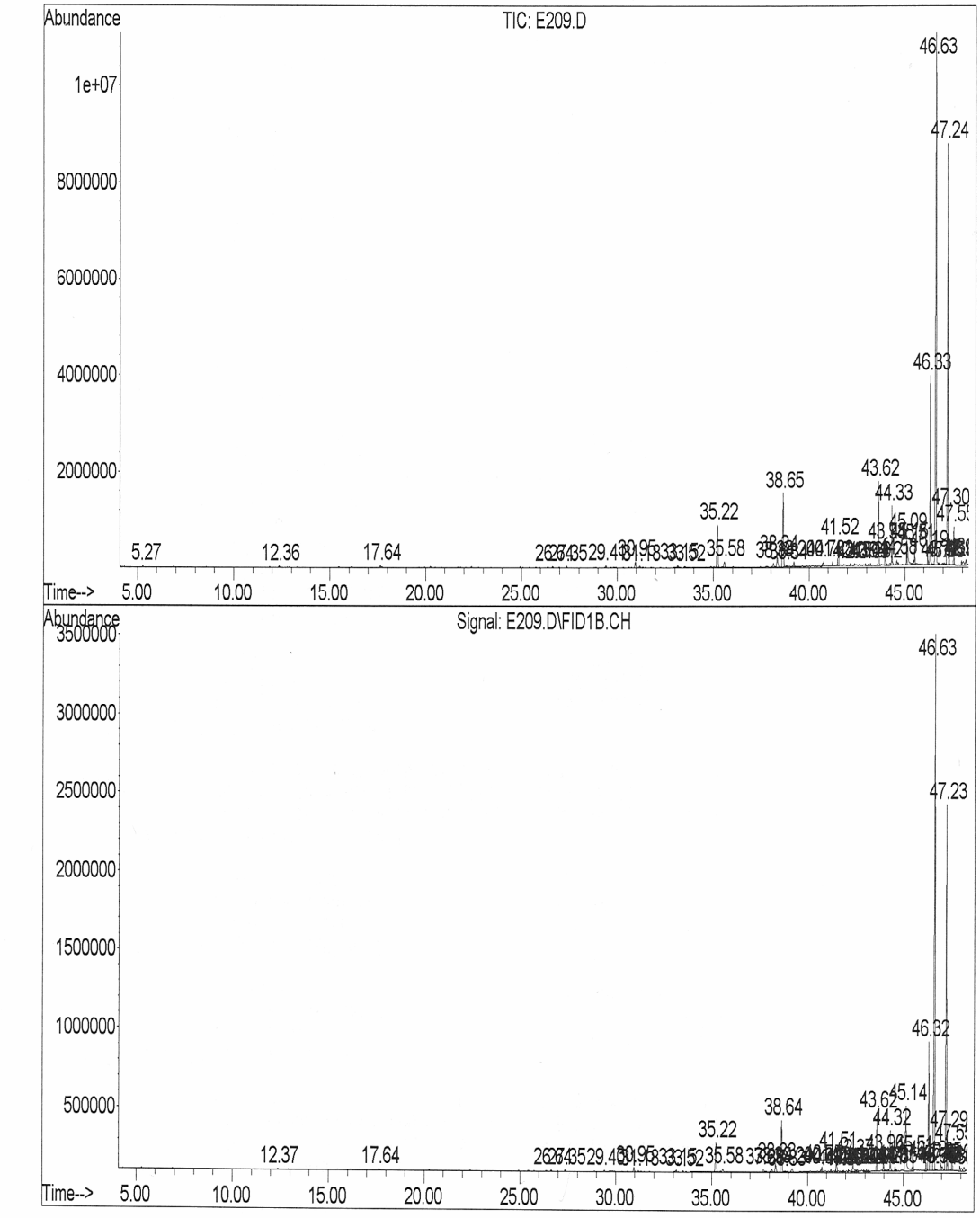

Table 0-1

Figure 3. 6 The GC and FID graphs obtained from Seed samples of *L. vulgaris*.

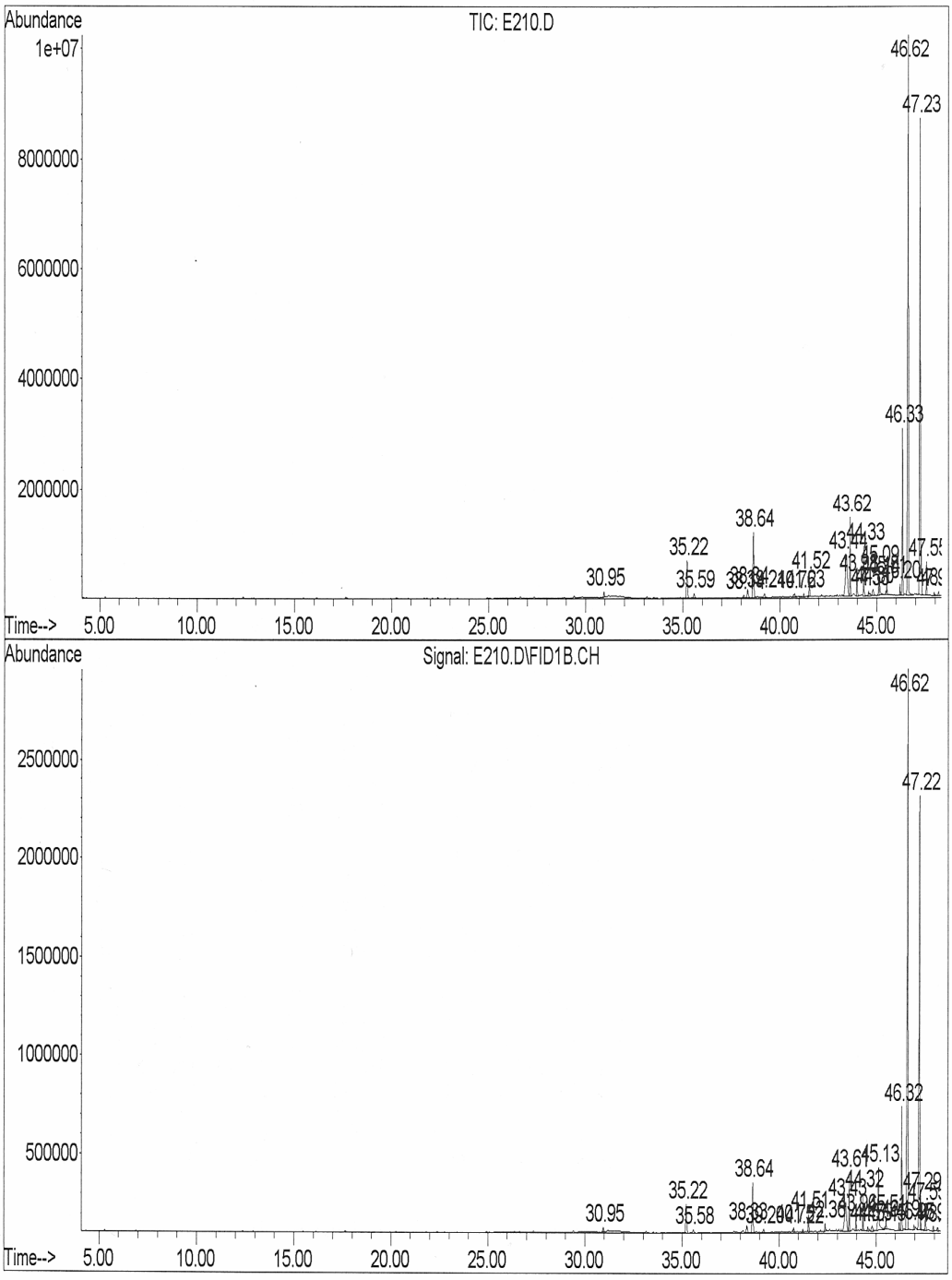

Figure 3. 7 The GC and FID graphs obtained from Seed samples of *L. vulgaris*.

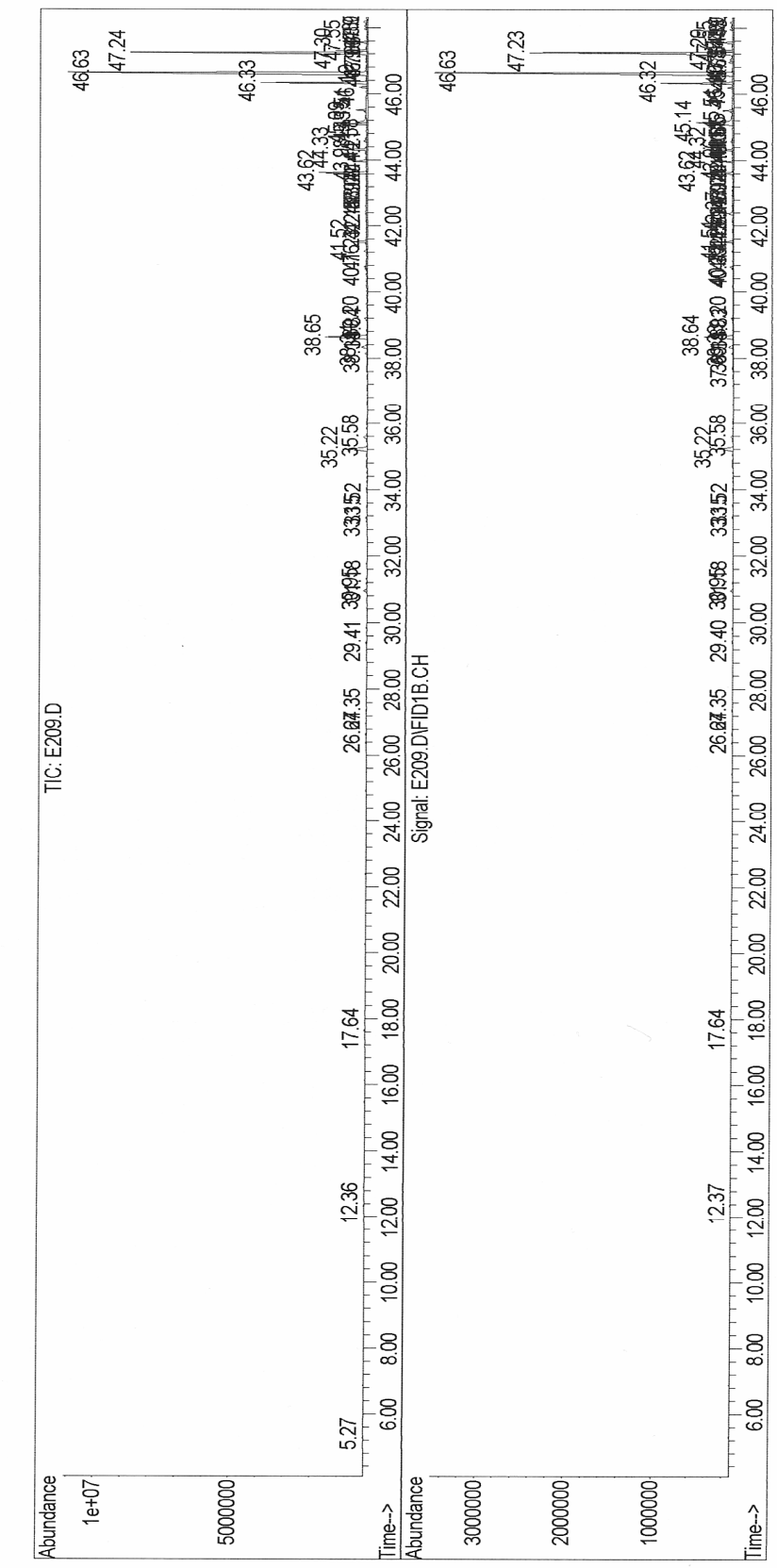

Figure 3. 8 The GC and FID graphs obtained from Seed samples of *L. vulgaris*.

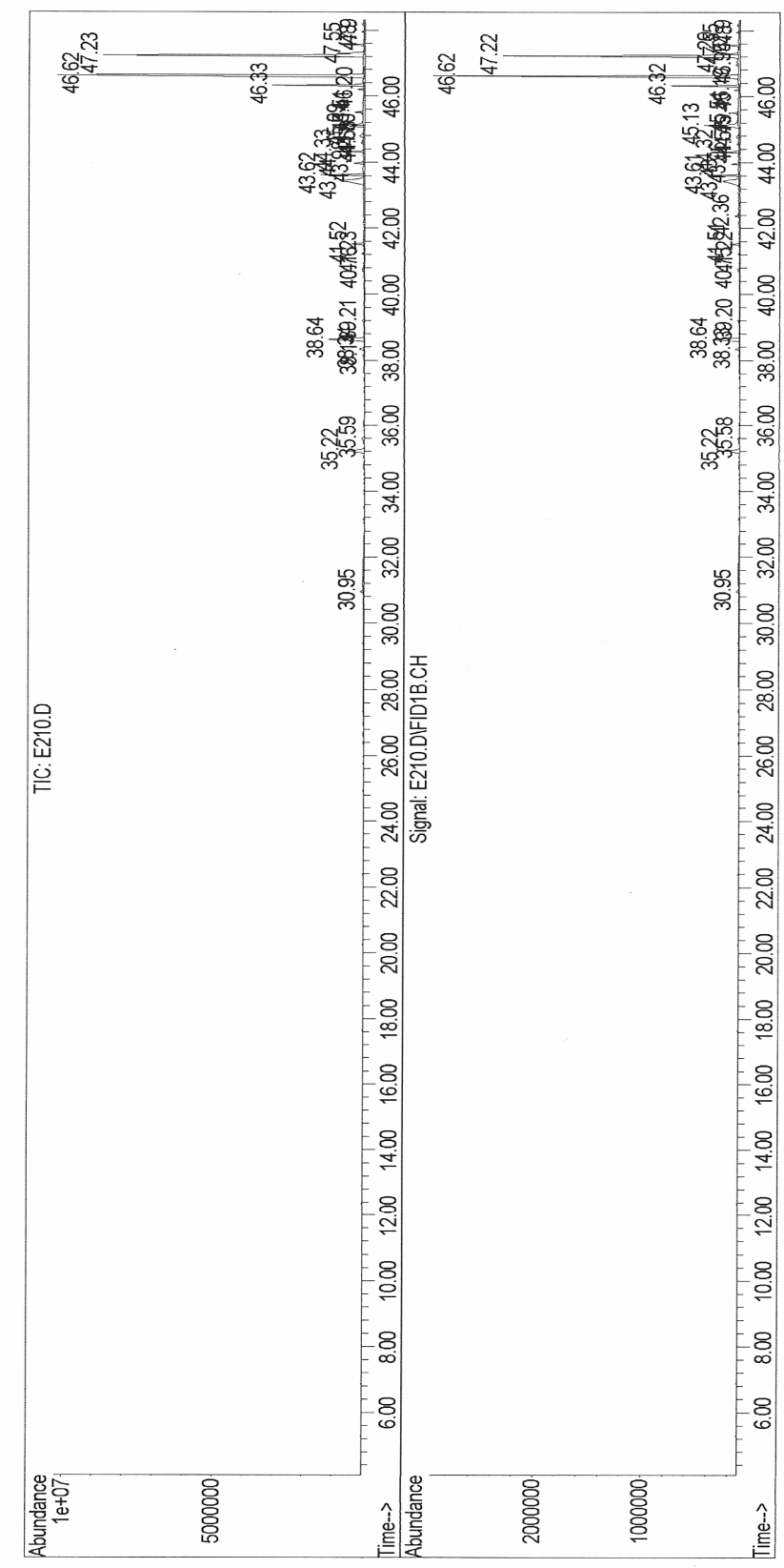

Figure 3. 9 The GC and FID graphs obtained from Hardening samples of *L. vulgaris*.

CHAPTER 4

4. Biological Activities of *Lysimachia vulgaris L*.

4.1. Introduction

Throughout the history plants have been the most important source of medicines for human health (Balandrin *et al*., 1993). The mankind has used herbs as remedy for as long as human life on earth. The World Health Organization forecasts that 80 percent of people in developing countries (65 percent of Earth's population) still rely on folk medicine for their primary health care (Starbuck, 1999; Farnsworth *et al*., 1985). A total of 122 biologically active compounds have been identified and these compounds were derived from only 94 species of plants. A conservative estimate of the number of flowering plants occurring on the planet is 250,000. Of these, only about 6 % have been screened for biologic activity, and a reported 15% have been evaluated phytochemically. There should be an abundance of drugs remaining to be discovered in these plants (Fabricant and Farnsworth, 2001). Because of production a host of bioactive molecules, plants have been a rich source of medicines. Most of which probably evolved as chemical defenses

against predation or infection (Cox and Balick, 1994). Hence, to test plant extracts and screen bioactive molecules, bioassays are adaptable (McLaughlin *et al*., 1998).

Finding healing powers in plants is an ancient idea. People on continents have long applied poultices and imbibed infusions of hundreds of indigenous plants, dating back to prehistory. Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new, particularly viral, diseases remain intractable to this type of drug. Scientists from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. It is advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated (Cowan, 1999). Researchers have been interested in biologically active compounds isolated from plant species in order to eliminate and eradicate of pathogenic microorganisms because of the antibiotic resistance that microorganisms have built against antibiotics (Tepe *et al*., 2005; Benli *et al*., 2008). In addition, chemotherapeutic agent must kill or inhibit the microbial pathogen while damaging the host as little as possible (Prescott *et al*., 1990).

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 medicinal plants studies are important because folkloric usages of these plants gain some scientific justification.

A number consideration is involved in selecting an appropriate antimicrobial agent to treat an infection. The concentrations of antimicrobial agents required to inhibit or kill organisms *in vitro* (Thornsberry and Sherris, 1985).

The *Lysimachia* genus comprises over a hundred Eurasian species, several of which are known for their antibacterial, cytostatic and molluscicidal activities. Phenolic compounds, flavonoids and triterpene saponins are considered characteristic chemical constituents of this genus (Podolak and Strzalka, 2008).

The disc diffusion assay (Kirby-Bauer Method) is used to screen for antibiotic activity and measures antimicrobial activity based on bacteriostatic/bacteriocidal properties. In 1940, Heatley introduced the use of adsorbent paper for carrying antimicrobial solutions. At the end of the 1950's, antimicrobial susceptibility testing was marked by lack of acceptable standardized procedures (Atlas, 1988). It was developed by William Kirby, A. W. Bauer, and their colleagues in the early 1960's (Prescott *et al*., 1990). The procedure, which is accepted by the National Committee for Clinical Laboratory Standards (NCCLS) is a modification of that described by Bauer, Kirby, Sherris, and Turck (commonly known as the Kirby-Bauer test) (Barry and Thornsberry, 1985; Atlas 1988; Prescott *et al*., 1990).

The most widely used procedure is still the disk diffusion method as a supplemental procedure or the routine method. More experience has been

gained over the years with this diffusion procedure than with any other test. The Kirby-Bauer Disc Diffusion Assay (Barry and Thornsberry, 1985) has been carefully standardized by the Food and Drug Administration (FDA) and the National Committee for Clinical Laboratory Standards (NCCLS) and if performed precisely according to the protocol, will provide data that can reliably predict the vivo effectiveness of the drug or plant extracts in question. 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℃ 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 of inhibition correspond to the degree of microbial resistance and 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. 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 4.1.

Table 4. 1 Bacteria used in antibacterial assay and their representative diseases (Levinson and Jawertz, 2002).

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

Crown gall is a neoplastic disease of plants induced by specific strains of the Gram-negative bacterium, *Agrobacterium tumefaciens. 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). 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.

Seven species of *Lysimachia* genus found in Turkey have been used as a febrifuge and vulnerary in traditional medicine (Ulusoylu *et al*., 2002). Some endemic *Lysimachia* species in Hawaii and China have been used as diuretic and antilithic in folklore medicine. Moreover, water maceration of *L. vulgaris* has been used as skin protectant and renovator in these regions (Ulusoylu *et al*., 2002).

Rzadkowskabodalska and Olechnowiczstepien (1975) determined the flavonoids campherol, quercetin, hyperoside and mircetin in *L. vulgaris.* Yasukawa and Takido (1988) reported the flavonoid quercetin in *L. vulgaris* var. *davurica*. Janik *et al*. (1994) isolated a benzoquinone pigment, 2,5-dihydroxy-3-pentadecyl-benzoquinone from the dried rhizomes of *L. vulgaris.* Antifungal, cytotoxic and insecticidal activities of *Lysimachia* species have

been reported (Podolak *et al*., 1998; Kim *et al*., 2007; Park *et al*., 2008). Podolak et al. (1998) isolated a benzoquinone pigment and triterpene saponosides from the underground parts of *L. vulgaris*. Cytotoxic and antifungal activity of these compounds were tested *in vitro* against human and mouse melanoma cells and the yeast *Candida albicans* respectively. The results showed that saponoside B exerted cytotoxicity especially towards human melanoma cells. The pigment was more active as an antifungal agent (Podolak *et al*., 1998)**.** There are several reports on cytotoxic and antifungal activity of benzoquinones and triterpene saponosides (Janik *et al*., 1994; Kinoshita *et al*., 1992). Park *et al*., (2008) reported the very strong fungicidal activity by extracts of *Lysimachia foenum-graecum* Hance. Kim *et al*. (2007) indicated the acaricidal activity of *Lysimachia davurica* leaf extracts.

A major benzoquinone pigment, embelin, was isolated from the underground parts of *Lysimachia punctata*. It showed a significant cytotoxic activity *in vitro* against B 16 and XC cell lines with ED50 values of 13 mu g/ml and 8 mu g/ml, respectively (Podolak *et al*., 2005).

Benzodilactones of fordiana A, fordiana B, quinones of fordianaquinone A and fordianaquinone B have isolated from leaves of *L. fordiana* and these pigments have antifungal activity, and fordianaquinone B can strongly inhibit the activity of DNA topoisomerase I (Huang *et al*., 2009b). More than 50 species in *Lysimachia* genus are exploited as medicinal herbs. The total flavones, obtained from these plants, have been proven to be anticancer agents (Zheng *et al*., 2009).

Oxidation of lipids, which occurs during raw material storage, processing, heat treatment and further storage of final products, is one of the basic processes causing rancidity of food products, leading to their deterioration. Due to undesirable influences of oxidized lipids on the human organism, it seems to be essential to decrease contact with products of lipid oxidation in food. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. But, according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have already been documented. For example, these substances can show carcinogenic effects in living organisms. From this point of view, governmental authorities and consumers are concerned about the safety of their food and about the potential effects of synthetic additives on health (Tepe *et al*., 2005).

There is widely used method for the analysis of total phenolics. The Folin-Ciocalteau method has the advantage of a fairly equivalent response to different phenols. The Folin-Ciocalteau colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. Singleton adapted this method to food phenolic analysis and has written two major reviews on its use. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols (Waterhouse, 2002). The differential sensory effect of phenolics aside, a major advantage of the Folin-Ciocalteau procedure is that it has a fairly equivalent response to different phenolic substances. In

general, the response of total phenol tests is comparable to antioxidant tests, with better correlations for antioxidant tests (Waterhouse, 2002).

There are no reports about antibacterial, antitumor and antioxidant activities of *L. vulgaris* in the literature. The main objective of this study was to evaluate and compare the biological activities of *in vitro*-grown and field-grown plant (aerial parts, flower parts and leaves) extracts (ethanol, acetone and water) of yellow loosestrife using some selected bioassays including antibacterial, antitumor and antioxidant.

4.2. Materials and Methods

4.2.1. Plant Material and Extraction

Stem and leaves of *L. vulgaris* (field-grown plants) were collected from Abant Lake, Bolu/Turkey in August of 2009. Identification of the species was made by using “Flora of Turkey and the East Aegean Islands” (Davis, 1978) and voucher specimens (AUT-2008) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Leaves and stems of *in vitro* cultured plants were collected from yellow loosestrife plants that were previously micropropagated in the laboratory. Plant materials were extracted with water, ethanol (EtOH) and acetone. Two different sources of plant (field-grown and *in vitro*-grown) were used for extractions. All plant materials were dried in a room avoiding sun light and then ground into a powder, and 300 ml ethanol or acetone.

For aqueous extraction, 20 grams from each plant sample were extracted with 200 ml water in waterbath at 45°C for 12 hours and then filtered. Frozen filtrate was lyophilized by using freeze-dryer at -65 °C. For

ethanol and acetone extraction, 20 grams from each plant sample were soxhlet extracted with 300 ml solvent (ethanol or acetone) at 75°C for ethanol and 50°C for acetone for 12 hours and then filtered. Filtrates were evaporated under vacuum using rotary evaporator. Each residue of all extractions was then dissolved in sterile distilled water or dimethyl sulfoxide (DMSO) to produce a final concentration of 100 mg/ml. Plant materials, their designations and extraction yields are represented in Table 4.2.

Table 4. 2 Designation, plant material and yield of extracts.

*Yield (%) = Weight of extract (g) / 20 gr of powdered plant sample * 100

Extract	Designation	Part used	Yield* (%)
Water	NLW	Field-grown leaves	12
	NFW	Field-grown flowers	18
	NAW	Field-grown aerial parts	59
	IVW	In vitro-grown leaves and stems	22
	SW	Field-grown seeds	6
Ethanol	NLE	Field-grown leaves	9
	NFE	Field-grown flowers	12
	NAE	Field-grown aerial parts	7
	IVE	In vitro-grown leaves and stems	10
	SE	Field-grown seeds	7
Acetone	NLA	Field-grown leaves	*6*
	NFA	Field-grown flowers	*12*
	NAA	Field-grown aerial parts	*7*
	IVA	In vitro-grown leaves and stems	10
	SA	Field-grown seeds	7

4.2.2. Antibacterial Assay

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott *et al*., 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATTC 13883) which are Gram-negative bacteria and *Streptococcus pyogenes* (ATTC 19615), *Staphylococcus aureus* (ATTC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are Gram-positive bacteria (Table 4.1).

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 ($1.5x10^8$ cfu/ml). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface of a (60 X 15 mm) Mueller-Hinton (M-H) agar plate (One test tube was used for each agar plate). Agar plates were streaked three times, each time turning the plate at a 60° angle and finally rubbing the swab through the edge of the plate. All extracts were sterilized by filtering through a 0.22 μm

filter (Pal-Gelman Laboratory) and sterile filter paper discs (Glass Microfibre filters, Whatman®; 6 mm in diameter) were impregnated with 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).

4.2.3. Anti-tumor Assay

Antitumor activity of all *L. vulgaris* extracts was assessed with the potato disc method as modified by McLaughlin's group (Ferrigini *et al*., 1982). *Agrobacterium tumefaciens* (strain A 281) was cultured on Yeast Extract Media (YEM) for 2-3 days at 25°C. Camptothecin (Sigma) (tumor suppressant) served as a positive control. Extracts were dissolved in water or DMSO (12.5%) (Ferrigini *et al*., 1982), so water and DMSO (12.5%) were used as a negative control. NLA, NFE, NFA, NAA, SE, SA and IVA extracts were dissolved in DMSO (12.5%) to a final concentration of 1000 mg/l. NLW,

NLE, NFW, NAW, NAE, SW, IVW, and IVE extracts were dissolved in water to a final concentration of 1000 mg/l or 100.000 mg/l. 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 X 10^9 bacteria.

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

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

Solvent control I: 600 µl control (water) + 150 µl sterile distilled water + 750 µl *A. tumefaciens* in PBS (For aqueous solutions).

150 µl filtered sterile DMSO + 600 µl sterile distilled water + 750 µl *A. tumefaciens* in PBS (For DMSO solutions).

Solvent control II: 750 µl sterile distilled water + 750 µl PBS.

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

150 µl test extract dissolved in DMSO + 600 µl sterile distilled water + 750 µl *A.tumefaciens* in PBS.

Potatoes (*Solanum tuberosum* L.) were scrubbed with a brush under running water and surface sterilized by immersion in 10% Domestos® for 20 min. Tubers were then placed on sterile Petri dishes and cut along either side revealing the largest flat surface area available; tubers were immersed in 20% Clorox for 15 min and then placed on sterile Petri dishes. Cylinders (8 mm diameter) were cut from the center of potato tissue (skin portion was

eliminated) and placed in sterile distilled water. Cylinders were rinsed twice more. Each cylinder was cut into 0.5-cm discs after excluding 1-cm end pieces into sterile glass petri dishes. These discs were transferred to 24-well culture plates containing water-agar (15g/l). One well plate (24 cell wells) was used for each experiment; each experiment had 24 replicates. Each disc was overlaid with 50 µl of appropriate inoculum. No more than 30 min elapsed between cutting the potato discs and inoculation. 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 % inhibition= [(solvent control mean - tested extract mean) / solvent control mean] X 100.

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

4.2.4. Bacterial Viability Testing

A. tumefaciens (strain A 281) was cultured on Yeast Extract Media (YEM) for 2 days at 25°C. Bacterial viability was determined by incubating each extracts with $1x10^9$ colony formed units of bacterial suspension contained in phosphate buffered saline (PBS). Therefore, six to seven loops of *A. tumefaciens* were added to 10 ml PBS. Bacterial concentration was adjusted to absorbance (600 nm) values of 0.96 ± 0.02, which equaled to

$1.0x10^9$ colony forming units (CFU) (standardized value). 1 ml of yellow loosestrife extract was mixed with 1ml of *A. tumefaciens* culture adjusted to $1x10^9$ cells/ml in Eppendorf tube (four tubes per test). At 10, 20, 30 and 60 minutes after inoculation, 0.1 ml of inoculum (bacteria + extract) of each solution was removed and placed on Yeast Extract Medium (YEM) plates with spread plate technique, and incubated for 24 h. Colony of *A. tumefaciens* were made after 24 hours. 30-min exposure was chosen as attachment of bacterial cells to the plant tissue, the initial step in crown gall formation occurs within 15 min of inoculation (Lippincott and Lippincott, 1969; Glogowski and Galsky, 1978). Bacterial growth was evidenced by growth across the plates.

4.2.5. Antioxidant Assay

4.2.5.1 Free Radical Scavenging Activity-DPPH (2,2-diphenyl-1-picrylhydrazil) Method

DPPH is a free radical and when it was dissolved in ethanol, it has a blue-violet color. The solution loses color which depends upon the number of electrons taken up. Hence, the loss of color indicates radical scavenging activity of test material (Angayarkanni *et al*., 2010).

DPPH free radical scavenging activities of the extracts were determined according to the method described by Brand-Williams *et al*. (1995) with some modifications such as 80% ethanol instead of methanol and molarity of DPPH concentration ($1.5x10^{-5}$ M instead of $6x10^{-5}$M) (Cai *et al*., 2003). The free radical-scavenging activities of the samples were

measured in terms of hydrogen donating or radical-scavenging ability to the stable radical DPPH, as a reagent.

The samples of extracts were weighted as 0.003 g (NLW, NLE, NFW, NFE, NAW, NAE, IVW and IVE) and they were dissolved in 3 ml of 80% ethanol. Finally, this stock solution was prepared as 1000 µl/ml concentration.

The extract solutions were diluted with 80% ethanol (25, 50, 100, 200 µl/ml) from the stock extract solutions. 1.5 ml of the diluted samples was mixed vigorously with 0.5 ml of $1.5x10^{-5}$ M DPPH in 80% ethanol. After 30 min at room temperature in the dark, absorbance was measured against blank (ethanol of 80%) at 517 nm with Hitachi U-1900, UV-VIS Spectrophotometer 200V. The solution which includes only 1.5 ml of the 80% EtOH and 0.5 ml of the $1.5x10^{-5}$ M DPPH in 80% ethanol were positive control (Milardovic *et al*., 2006; Brand-Williams *et al*., 1995). All analyses were made in triplicate.

The capability of *L. vulgaris* samples to scavenge the DPPH· radical was calculated using the following equation:

DPPH· Scavenging Effect (%) (% inhibition) = $[(A_0–A_1/A_0) \times 100]$ (Gülçin *et al*., 2003) where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample of *L. vulgaris* extracts.

4.2.5.2 Total Phenolic Assay (Folin-Ciocalteau Method for Total Phenolic Content)

Preparation of extract solution (stock solution): 2ml of distilled water was added to 0.01g plant extracts of *L. vulgaris* (NLW, NLE, NAW, NAE, NFW, NFE, IVW and IVE). Prepared stock solution was then diluted to 1mg/ml.

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. The accepted standard is gallic acid. It is a particularly good standard because it is relatively inexpensive in pure form and is stable in its dry form (Waterhouse, 2002).

Sodium Carbonate (Na_2CO_3) Solution: 20g of anhydrous sodium carbonate was dissolved in 100 ml of water in a 100 ml volumetric flask.

To prepare a calibration curve: The phenol concentrations of 0, 50, 100, 150, 200, 250 and 500 mg/l gallic acid was prepared (Figure 4.8).

20 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 20^0C for 2 hours and measured the absorbance of each solutions at 765 nm against the blank (the “0 ml” solution) using the spectrophotometer. The absorbance vs. concentration was plotted. After the created calibration curve with standards, phenol levels in the samples were determined. The results were reported at Gallic Acid Equivalent, GAE. The

phenol content was expressed as GAE calibrated (Jeong *et al*., 2010). The total phenol content of samples of *L. vulgaris* was expressed as mg gallic acid equivalents (GAE)/100g dried mass. All analyses were made in triplicate.

4.2.5.3 Total Flavonoid Assay-Aluminum Chloride ($AlCl_3$) Colorimetric Assay for Total Flavonoids

The amount of total flavonoid 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 (Figure 4.7).

Preparation of extract solution: 0.005 g of each *L. vulgaris* 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% $NaNO_2$ 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. Samples were analyzed in three replications (Marinova *et al*., 2005; Chang *et al*., 2002). The total

flavonoid content of *L. vulgaris* samples were expressed as mg catechol equivalents (CE)/100g dried mass.

4.3. Results and Discussion

Fifteen different extracts (EtOH, acetone and aqueous extracts of field-grown and *in vitro*-grown plants) and essential oil obtained from field-grown aerial parts of *L. vulgaris* were used to screen for antibacterial activity (Table 4.3; Figure 4.1).

Generally, tested Gram-positive bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) seem to be more susceptible to the inhibitory effects of the *L. vulgaris* extracts than the Gram-negative bacteria. 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 and DMSO. Therefore, they were used as a negative control and no inhibition was observed with them (Table 4.3).

Generally, field-grown plant extracts indicated better antibacterial activities than *in vitro*-grown plant extracts. Among field-grown plant extracts, ethanol and acetone extracts were better than aqueous extracts.

Best antibacterial activity was obtained with ethanolic extract of field-grown flowers (NFE) against *S. aureus* (17.7 mm) and *S. epidermidis* (19.1

mm), and ethanolic extract of field-grown aerial parts against *S. pyogenes* (16.1 mm) (Table 4.3). NFE showed greater antibacterial activity than reference antibiotic tetracycline against *S. epidermidis* (Table 4.3, Figure 4.2).

Generally ethanol extracts of field-grown plant materials (leaves, flowers and aerial parts) exhibited a broad-spectrum of activity against both Gram-positive and Gram-negative bacteria.

S. typhimurium did not show sensitivity against to used extracts. In addition, *E. coli* and *E. cloacae* were inhibited by only ethanol extract of field-grown flowers (NFE). Ethanol and water extract of *in vitro*-grown plant materials (IVW and IVE) did not show any inhibitory activity against used bacteria. Only, acetone extract of *in vitro*-grown plant materials (IVA) exhibited moderate antibacterial activity against *S. epidermidis*, *S. aureus*, and *S. pyogenes* (Table 4.3). Ethanol and acetone extracts of field-grown seeds displayed moderate inhibition against only *S. pyogenes* and *S. epidermidis* (Table 4.3).

Essential oil of *L. vulgaris* did not show any inhibitory activity against used bacteria (Table 4.3).

Generally, antibacterial activities of field-grown plants were better than *in-vitro* grown plants against used bacteria. Sometimes the level of the detected secondary products in shoot cultures is lower than donor plants (Stafford, 1991). For example, production of steroids obtained from shoot tip cultures of *Digitalis* species was much lower than those found in the donor plant (Seidel and Reinhard, 1987). If the secondary product synthesis is low, there are some procedures for enhancing productivity. Optimization of

hormone regime is often effective. The type and concentration of phytohormones available to cultured cells is probably the most important factor influencing their potential for secondary product synthesis. 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).

It can be conceivable that antibacterial activity may come from benzoquinone, triterpene saponaside and tannin constituents of *L. vulgaris* (Chevallier, 1996; Janik *et al*., 1994; Rzadkowskabodalska and Olechnowiczstepien, 1975; Podolak *et al*., 1998). 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). Ethanol extracts of plant materials may contain active components such as tannins, polyphenols, polyacetylenes, flavonol, terpenoids, alkaloids, sterols and propolis. Also, acetone extracts of plant materials may contain flavonols (Cowan, 1999). Recio *et al*. (1989a) showed that 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 ethanol and acetone extracts of *L. vulgaris* may be explained by the inhibitory effect of these compounds on bacteria.

Strong antibacterial activity of *L. vulgaris* extracts against *S. epidermidis*, *S. aureus*, and *S. pyogenes* may explain why yellow loosestrife is used in folk medicine as anti-diarrheal, anti-pyretic, anti-inflammatory and vulnerary.

Antitumor activity of yellow loosestrife extracts was tested with the potato disc method as modified by McLaughlin's group (Ferrigni *et al*., 1982). For preparation of the final concentration of extracts, they were dissolved in water or DMSO (12.5%). There was a dissolving problem of some extracts (NLA, NFE, NFA, NAA, SE, SA and IVA) so; they were dissolved in DMSO (12.5%) (Ferrigini *et al*., 1982). DMSO does not affect bacteria viability. But, it inhibits tumor inhibition. According to Ferrigini *et al*. (1982) 12.5% concentration of DMSO compensated this inhibition and is safe for using as a dissolving solvent at antitumor assays. Final concentration of extracts (NLA, NFE, NFA, NAA, SE, SA and IVA) dissolved in DMSO were 1000 mg/l. On the other hand, other extracts (NLW, NLE, NFW, NAW, NAE, SW, IVW, and IVE) were dissolved in water easily and final concentrations of them were prepared as 100.000 mg/l or 1000 mg/l (Table 4.4; 4.5; 4.6, Figure 4.3; 4.4; 4.5).

High concentration (100.000 mg/l) of all extracts that dissolved in water showed strong antitumor activity (Table 4.4 and Figure 4.3). Best antitumor activity was observed with water extract of field-grown flowers (NFW) at 100.000 mg/l (Table 4.4; Figure 4.6). Low concentration (1000 mg/l) of extracts that dissolved in water also showed moderate antitumor activity (Table 4.5 and Figure 4.4). Best antitumor activity was observed with ethanol extract of *in vitro*-grown leaves and flowers (IVE) at 1000 mg/l (Table 4.5). On the other hand, although water extract of field-grown leaves (NLW) showed 82.7% tumor inhibition at 100.000 mg/l, tumor inhibition of NLW was not observed at 1000 mg/l (Table 4.4 and 4.5).

Extracts that dissolved in DMSO showed moderate antitumor activity (Table 4.6 and Figure 4.5). Best antitumor activity was observed with acetone extract of field-grown leaves (NLA) and aerial parts (NAA) (Table 4.6 and Figure 4.5).

Generally, when compared with control (water or DMSO), the percentage inhibition of all extracts was more than 29% in three separate experiments (Table 4.4; 4.5; 4.6, Figure 4.3; 4.4; 4.5). No tumor formation was observed with camptothecin (100 % inhibition) (Table 4.4; 4.5; 4.6; Figure 4.6).

Cytotoxic activity of underground parts of *L. vulgaris* was reported by Podolak *et al*. (1998). This study showed the antitumor activity of aerial parts of *L. vulgaris* for the first time. Future studies should be focused on fractionation of the extracts to identify active components for antitumor activity.

Inhibition of crown gall formation on potato discs is caused by two effects: by anti-tumorogenesis or decreasing the viability of the *A. tumefaciens*. Viability tests were carried out with extracts to distinguish between these possibilities. Bacterial viability was determined by incubating yellow loosestrife extracts with 1 X 10^9 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension. Yellow loosestrife extracts did not affect on the viability of bacterium. Because, the extracts of yellow loosestrife did not show antibacterial activity against *A. tumefaciens*. Therefore, they could not kill *A. tumefaciens* and tumors formed on potato discs. Hence, we concluded that the action of tested extracts worked on the formation of tumors and were not on the viability of bacterium.

Ethanol and acetone 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*.

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).

In DPPH assay; IVE has little tendency to scavenge the free radicals according to other extracts. IVW has also low ability but when its concentration increased, the free radical scavenge tendency increased. The other extracts have high active radical scavenge capability as much as ascorbic acid that was used as control antioxidant (Table 4.7).

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups. In water and ethanol extracts of *L. vulgaris* (NLW, NLE, NFW, NFE, NAW, NAE and IVW), 280.3, 585.9, 188.4, 260.9, 266.2, 483.8 and 40.9 mg gallic acid equivalent of phenols was detected. The most phenolic content is included by NLE among extracts. Ethanol extracts include more phenolics than water extracts (Table 4.8; Figure 4.8).

If these extracts were compared depending on having flavonoid content, the result was NLE>NLW>NAE>NAW>NFW>IVW. Leaf parts contain more flavonoids and phenols than the other parts (Table 4.9; Figure 4.7).

The leaves and flowering stems of yellow loosestrife are alterative, anti-inflammatory, astringent, diuretic and cicatrisant (Dobelis, 1990; Grieve, 1982; Baytop, 1999). The plant is used in traditional medicine as a febrifuge and for the treatment of several inflammatory diseases, including antineoplastic and rheumatoid pains (Baytop, 1999; Podolak *et al*., 1998). On the basis of the results of this study, it is clearly indicated that extracts of yellow loosestrife have a powerful antioxidant activity oxidative systems *in vitro* and are 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. Phenolic compounds appear to be responsible for the antioxidant activity of extracts of yellow loosestrife. 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 analgesic activities. Analgesic activities of yellow loosestrife may be related to antioxidant activity.

Phenolic lipids are a much diversified group of compounds derived from mono and dihydroxyphenols, i.e., phenol, catechol, resorcinol, and hydroquinone. Due to their strong amphiphilic character, these compounds can incorporate into erythrocytes and liposomal membranes. The ability of these compounds to inhibit bacterial, fungal, protozoan and parasite growth seems to depend on their interaction with proteins and/or on their membrane-disturbing properties (Stasiuk and Kozubek, 2010).

One new saponin, named capilliposide D (1), and a known saponin, candidoside (2), were isolated from the whole plants of *Lysimachia capillipes*. Compound 1 and 2 were examined against human A2780 cells. Compound 1 showed significant cytotoxic activity with an IC_{50} value of 0.2 μg/ml. Compound 2 showed no cytotoxic activity (Tian *et al*., 2006a). Tian *et al*. (2006b) also isolated capilliposide A (1), capilliposide B (2) and capilliposide C (3) from ethanol extract of *L. capillipes*. They proofed that Capilliposide B showing significant cytotoxicity against human A-2780 cells (Tian *et al*., 2006b).

From the whole plants of *Lysimachia japonica* Thumb., two Na^+-K^+-ATPase inhibitors were isolated by chromatographic procedures. Shoji *et al*. (1984) have found that methanolic extract of *L. japonica* markedly inhibited the Na^+-K^+-ATPase activity. The sodium pump, Na+/K+-ATPase, could be an important target for the development of anti-cancer drugs as it serves as a versatile signal transducer. It is a key player in cell adhesion and its aberrant expression and activity are implicated in the development and progression of different cancers (Mijatovic *et al*., 2007).

The chemical structures of the purified material can be analyzed via chromatographic techniques for chemical analysis. However, the contents of the pigments are low in the plant and the plant is rarely distributed, which makes it difficult to collect enough amounts of samples for in vivo experiments. The study on the biosynthesis site of these pigments may be helpful for related research in future (Huang *et al*., 2009b).

The study of medicinal plants as antimicrobial, antitumor and antioxidant agents is necessary for gaining insight into medicinal flora and real value. These researches can be carried on until the agent responsible for the activity has been determined or, as the case may be, the most active fraction or extracts have been discovered. The mechanisms of action, interactions with antibiotics or medicinal plants or compounds, and the pharmokinetic profile of the extracts should be given high priority.

Results obtained herein confirm the antibacterial, antitumor and antioxidant activity of yellow loosestrife extracts. Definitive clinical studies are needed to fully understand the many medicinal uses of yellow loosestrife. Future studies should focus on fractionation of the extracts in hopes of

identifying active components. This plant may be a source of drugs that could improve the treatment of infections caused by Gram- positive bacteria or also can be a source food additive as antioxidant due to preventing the liposomal oxidation and prevention cancer in diet.

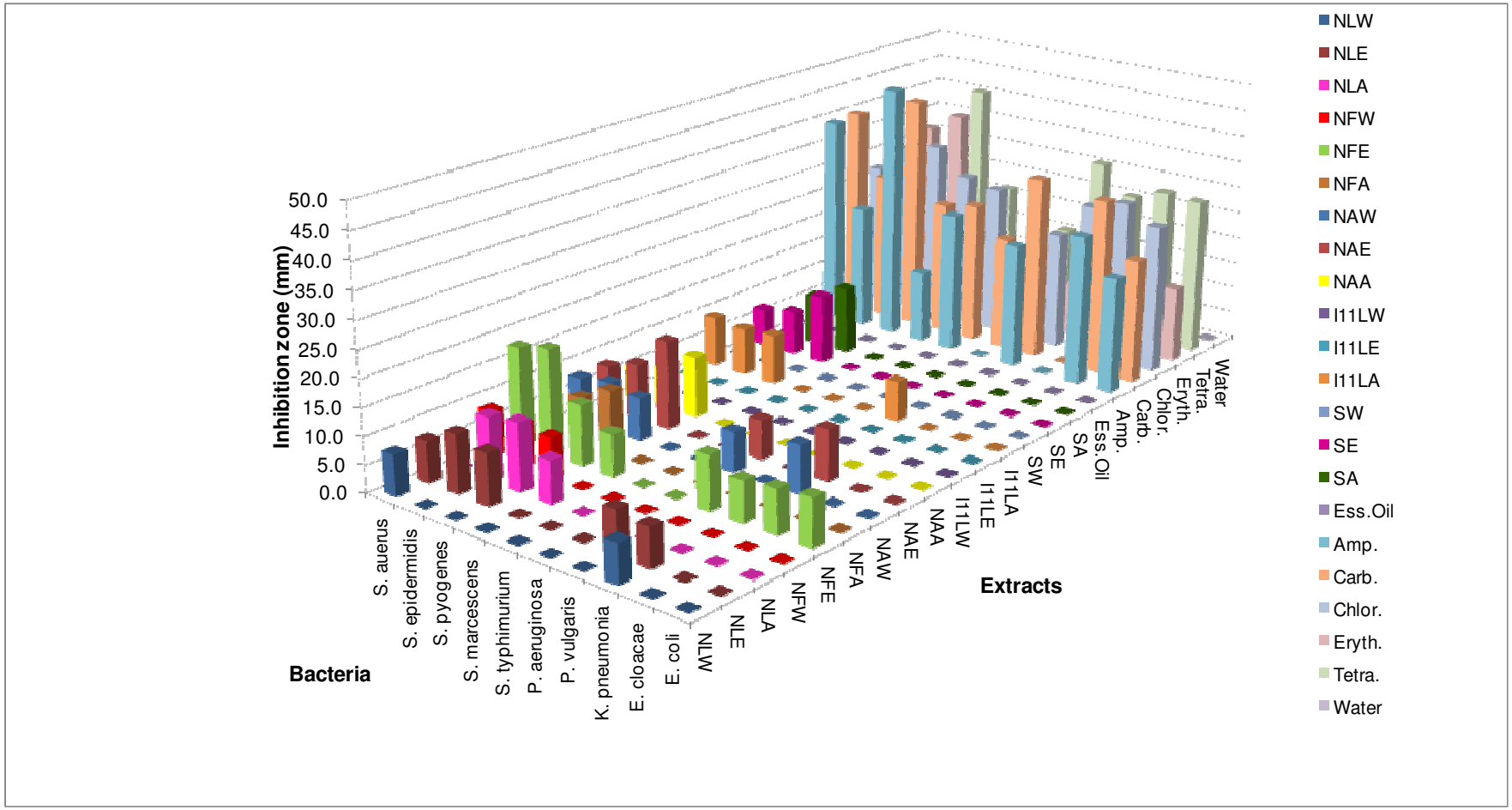

Figure 4. 1 Antibacterial activity of *L. vulgaris* extracts and controls (antibiotics) against used bacteria.

Table 4. 3 Antibacterial activity of *L. vulgaris* extracts. Data presented as zone of inhibition of bacterial growth in mm. Means with the same letter within columns are not significantly different at $P>0.05$.

Treatments	Mean diameter of inhibitory zones (mm ± SE)																					
	S. auerus		*S. epidermidis*		*S. pyogenes*		*S. marcescens*		*S. typhimurium*		*P. aeruginosa*		*P. vulgaris*		*K. pneumonia*		*E. cloacae*		*E. coli*	
NLW	7.4 ± 0.2fg		-	-	-	-	-	-	-	-	-	-	-	-	7.0 ± 0e		-	-	-	-
NLE	7.5 ± 0.2fg		10.7 ± 0.5fg		9.3 ± 0.2hij		-	-	-	-	-	-	7.9 ± 0.2f		7.0 ± 0e		-	-	-	-
NLA	-	-	11.4 ± 0.14f		12.3 ± 0.8g		7.6 ± 0.3f		-	-	-	-	-	-	-	-	-	-	-	-
NFW	8.4 ± 0.2ef		7.7 ± 0.1hi		7.4 ± 0.1j		-	-	-	-	-	-	-	-	-	-	-	-	-	-
NFE	17.7 ± 0.3d		19.1 ± 0.4e		10.9 ± 0.2ghi		7.6 ± 0.3f		-	-	-	-	9.8 ± 0.4e		7.4 ± 0.1e		8.0 ± 0.2e		8.6 ± 2f	
NFA	-	-	8.2 ± 0.1hi		11.3 ± 0.7g		-	-	-	-	-	-	-	-	-	-	-	-	-	-
NAW	7.9 ± 0.3fg		8.8 ± 0.2ghi		7.8 ± 0.3j		-	-	-	-	7.3 ± 0.1e		-	-	8.8 ± 0.1d		-	-	-	-
NAE	7.9 ± 0.3fg		10.0 ± 0.4fgh		16.1 ± 0.5f		-	-	-	-	7.1 ± 1.0e		-	-	9.3 ± 0.1d		-	-	-	-
NAA	7.0 ± 0.7g		7.0 ± 0i		11.1 ± 0.3gh		-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVA	9.2 ± 0.12e		8.6 ± 0.3ghi		9.1 ± 0.2ij		-	-	-	-	-	-	7.3 ± 0.1f		-	-	-	-	-	-
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SE	7.0 ± 0g		8.1 ± 0.3hi		12.8 ± 0.7g		-	-	-	-	-	-	-	-	-	-	-	-	-	-
SA	-	-	9.3 ± 0.3fghi		12.4 ± 0.7g		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Essential oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin	39.2 ± 1.9a		23.2 ± 4.3d		48.0 ± 2.6a		13.4 ± 1.3d		26.2 ± 0.6b		-	-	23.2 ± 2.9c		-	-	28.0 ± 1.3d		21.6 ± 0.9d	
Carbenicillin	39.6 ± 0.9a		28.0 ± 4.3c		44.4 ± 2.5b		25.2 ± 0.4b		26.4 ± 2.6b		21.2 ± 2.8a		34.4 ± 2.9a		-	-	33.0 ± 1.4a		23.2 ± 0.9c	
Chloramphenicol	27.0 ± 1.1c		32.4 ± 2.8b		34.0 ± 1.1e		29.2 ± 1.5a		28.0 ± 1.1a		8.6 ± 2.4d		21.8 ± 1.6d		28.8 ± 0.8a		30.8 ± 0.6b		27.8 ± 0.9b	
Erythromycin	26.6 ± 1.4c		35.0 ± 3.1a		38.6 ± 1.3d		9.4 ± 1.6e		10.8 ± 0.7c		10.4 ± 1.4c		10.4 ± 0.8e		11.8 ± 1.2c		-	-	14.0 ± 2.1e	
Tetracycline	32.6 ± 1.1b		9.4 ± 0.3fgh		42.2 ± 2.4c		23.6 ± 0.9c		26.0 ± 1.1b		17.8 ± 1.2b		32.6 ± 2.4b		27.4 ± 1.1b		29.6 ± 1.2c		29.2 ± 0.6a	
DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NLW: Water extract of field-grown leaves
NLE: Ethanol extract of field-grown leaves
NLA: Acetone extract of field-grown leaves
NFW: Water extract of field-grown flower
NFE: Ethanol extract of field-grown flower
NFA: Acetone extract of field-grown flower
NAW: Water extract of field-grown aerial parts
NAE: Ethanol extract of field-grown aerial parts
NAA: Acetone extract of field-grown aerial parts

IVW: Water extract of *in vitro*-grown leaves and stem
IVE: Ethanol extract of *in vitro* -grown leaves and stem
IVA: Acetone extract of *in vitro* -grown leaves and stem
SW: Water extract of field-grown seed
SE: Ethanol extract of field-grown seed
SA: Acetone extract of field-grown seed
DMSO: Dimethyl Sulfoxide

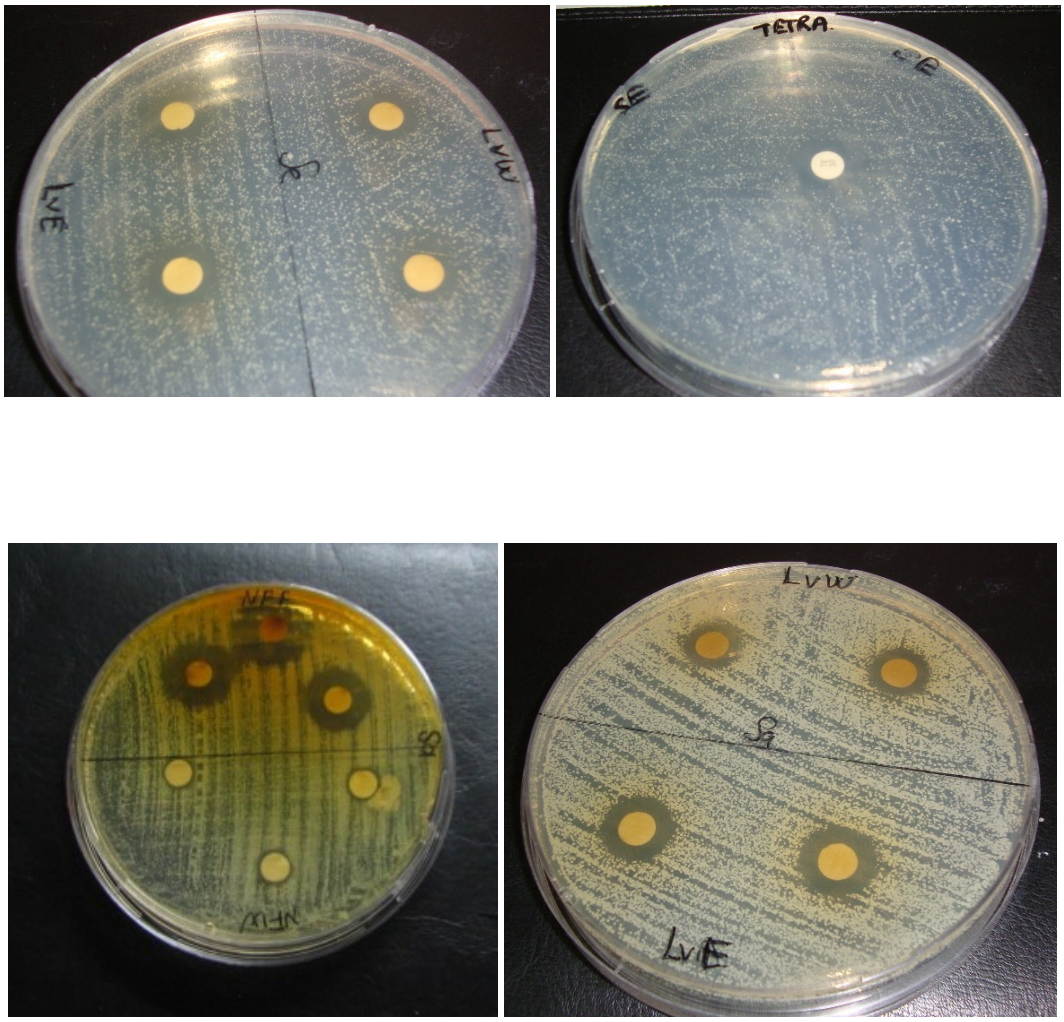

Figure 4. 2 Antibacterial activity of NFE (ethanol extract of field-grown flowers) and NFW (water extract of field-grown flowers) against *S. epidermidis* by comparing with control (tetracycline) (above); antibacterial activity of NFE and NFW against *S. aureus* (belove).

Table 4. 4 Mean number of tumors observed with extracts of *L. vulgaris* extracts that dissolves in water at 100.000 mg/l and controls (water and camptothecin). Means with the same letter are not significantly different at P>0.05.

Treatments	Mean Number of Tumors (±SE)	Tumor Inhibition (%)
Water (Negative Control)	77.1 ± 5.3^{a}	–
Camptothecin (Positive Control)	0.0 ± 0.0^{d}	100.0
NFW	0.3 ± 0.1^{d}	99.6
IVW	9.4 ± 2.1^{c}	87.8
IVE	10.5 ± 1.3^{c}	86.4
NAW	11.1 ± 1.6^{c}	85.6
NLW	13.3 ± 1.4^{c}	82.7
NAE	20.4 ± 2.2^{b}	73.5
SW	22.8 ± 1.8^{b}	70.4
NLE	26.7 ± 2.3^{b}	65.4

Table 4. 5 Mean number of tumors observed with extracts of *L. vulgaris* extracts that dissolves in water at 1000 mg/l and controls (water and camptothecin). Means with the same letter are not significantly different at P>0.05.

Treatments	Mean Number of Tumors (±SE)	Tumor Inhibition (%)
Water (Negative Control)	119.9±6.1^{a}	–
Camptothecin (Positive Control)	0.0±0.0^{e}	100.0
IVE	50.0± 3.4^{d}	58.3
IVW	54.3± 4.6^{cd}	54.7
NAE	65.3±5.1^{bcd}	45.5
NFW	67.4±6.0^{bcd}	43.8
SW	72.7±7.8^{bc}	39.4
NLE	78.6±4.6^{b}	34.4
NAW	84.8±6.8^{b}	29.3
NLW	131.8±10.3^{a}	–

Table 4. 6 Mean number of tumors observed with extracts of *L. vulgaris* extracts that dissolved in DMSO at 1000 mg/l and controls (DMSO and camptothecin). Means with the same letter are not significantly different at P>0.05.

Treatments	Mean Number of Tumors (±SE)	Tumor Inhibition (%)
DMSO (Negative Control)	37.2 ± 2.2^{b}	–
Camptothecin (Positive Control)	0.0 ± 0.0^{e}	100.0
NLA	13.5 ± 2.0^{d}	63.7
NAA	15.9 ± 1.6^{d}	57.3
NFA	19.0 ± 2.7^{cd}	48.9
SA	20.0 ± 2.6^{cd}	46.2
IVA	23.2 ± 2.7^{c}	37.6
SE	23.8 ± 2.3^{c}	36.0
NFE	44.2 ± 3.7^{a}	–

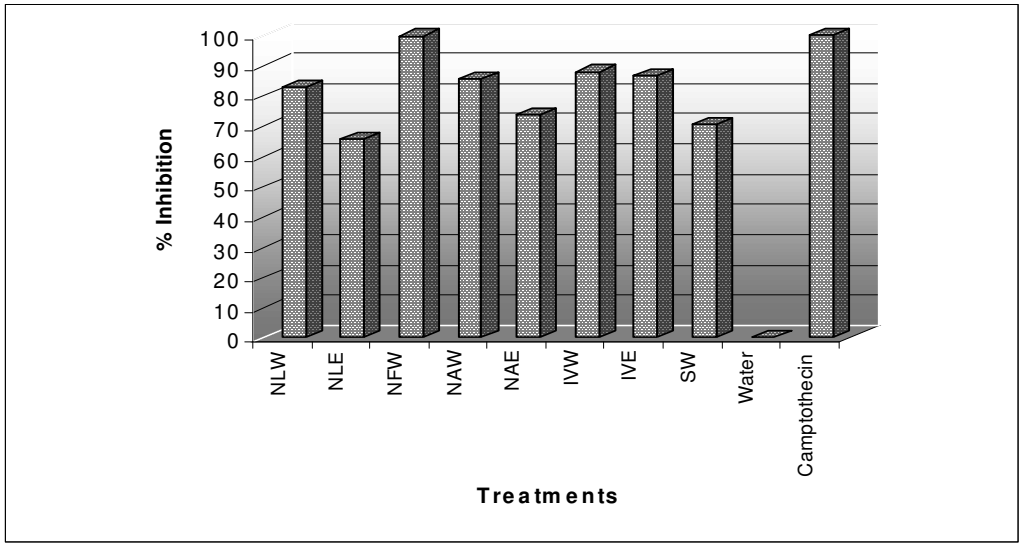

Figure 4. 3 Antitumor activity of *L. vulgaris* extracts that dissolved in water at 100.000 mg/l.

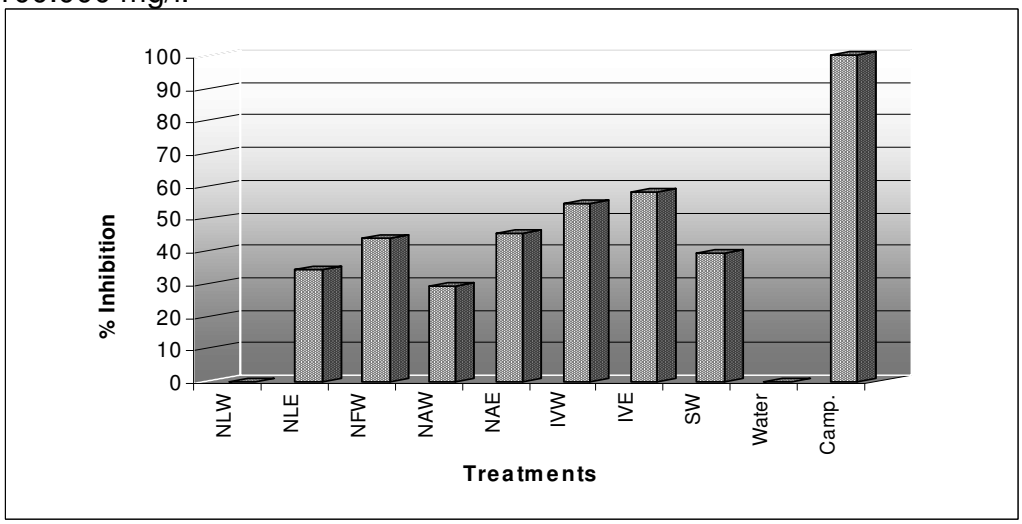

Figure 4. 4 Antitumor activity of *L. vulgaris* extracts that dissolved in water at 1000 mg/l.

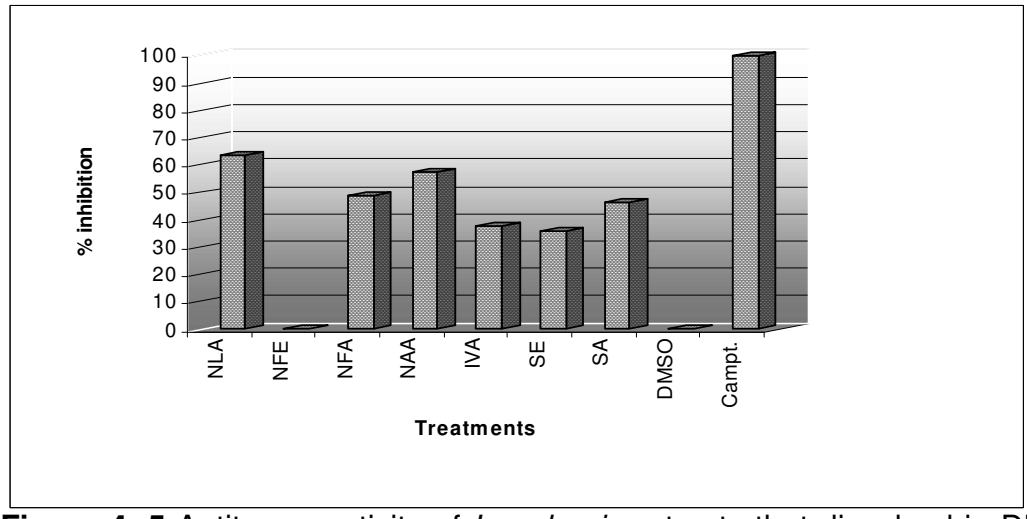

Figure 4. 5 Antitumor activity of *L. vulgaris* extracts that dissolved in DMSO at 1000 mg/l.

Figure 4. 6 Tumors produced by *A. tumefaciens* with NFW (water extract of field-grown flowers) (above) distilled water (solvent control I) (middle) and camptothecin (positive control) (below).

Table 4. 7 % inhibition of DPPH by *L. vulgaris* extracts.

	% inhibiton of DPPH Concentrations			
Treatments	**25µg/ml**	**50µg/ml**	**100µg/ml**	**200µg/ml**
Ascorbic acid	62.35	97.01	97.21	96.91
NLW	96.3	97.2	97.6	97.7
NLE	94.7	95.6	96.7	98.1
NAW	94.0	94.4	94.8	95.1
NAE	94.4	94.9	95.2	95.8
NFW	91.3	94.6	94.9	95.9
NFE	95.3	96.5	97.1	98.5
IVW	80.5	84.7	85.2	91.3
IVE	67.7	63.7	65.7	61.5

Table 4. 8 Total phenolic content of *L. vulgaris* extracts. Gallic acid equivalent was used as GAE mg/ g extract.

Treatments	Total Phenolics GAE in 1g/L of extract
NLE	585.9
NAE	483.8
NLW	280.3
NAW	266.2
NFE	260.9
NFW	188.4
IVW	40.9

Table 4. 9 Total flavonoid content of *L. vulgaris* extracts. Catechol equivalent was used mg/ g extract.

Treatments	Total flavonoids mg CE/g extract
NLE	58.5
NLW	57.8
NAE	56.9
NAW	42.3
NFE	37.1
NFW	27.8
IVW	16.4

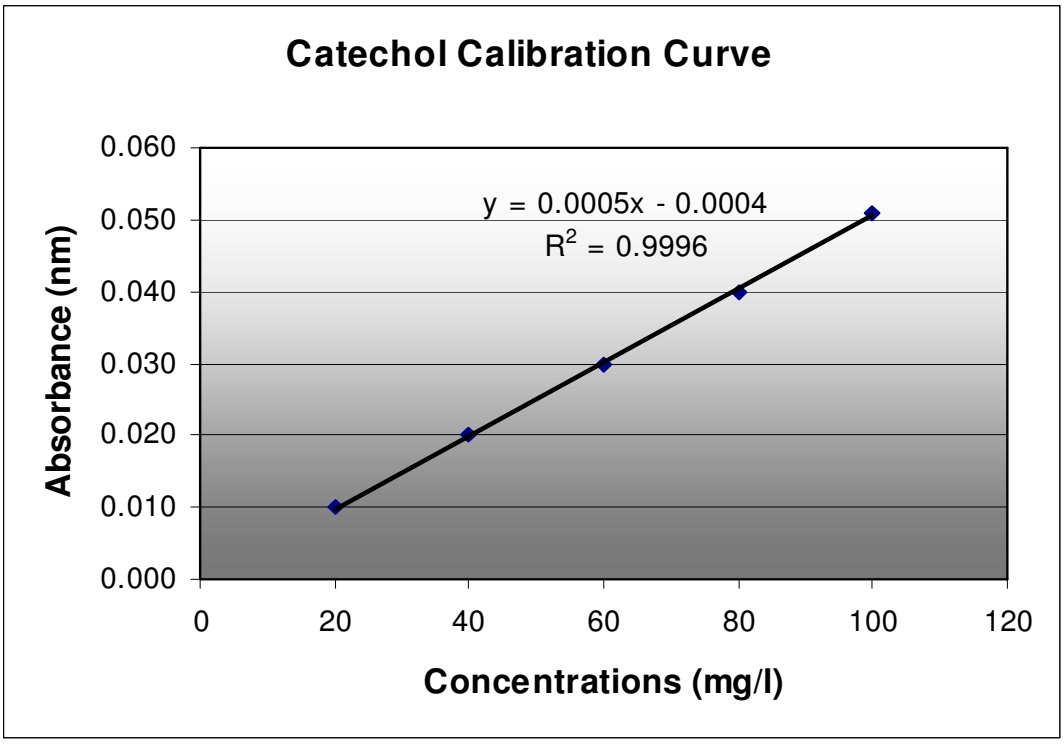

Figure 4. 7 Catechol calibration curve.

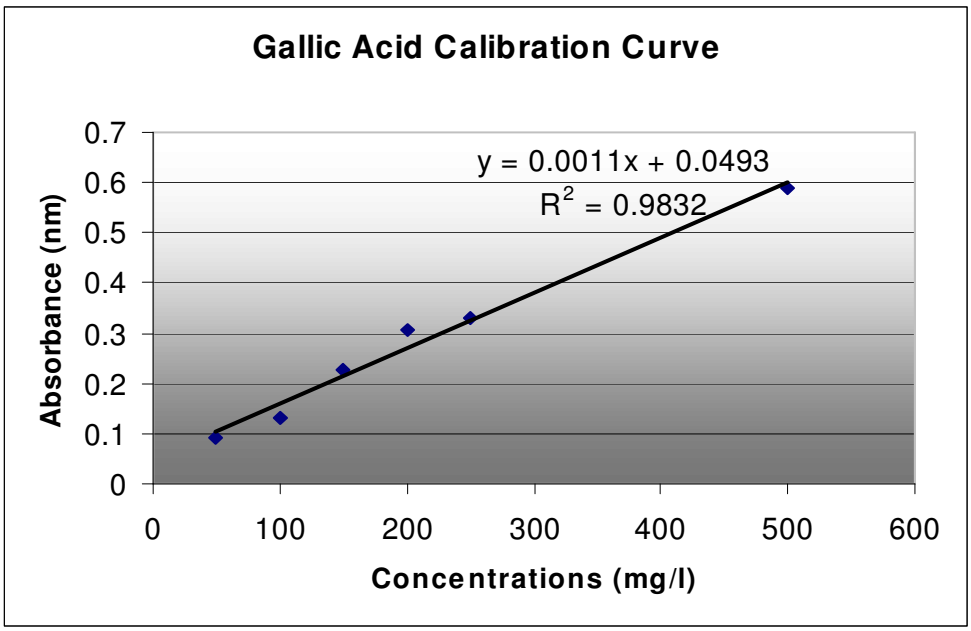

Figure 4. 8 Gallic acid calibration curve.

CHAPTER 5

5. Conclusions

Lysimachia vulgaris L. (Yellow loosestrife) is a member of the Primulaceae family but then was shifted to the Myrsinaceae based on the results of recent botanical investigations (Podolak and Strzalka, 2008).

This medicinal plant is a perennial herb (Davis, 1978) that has been used in the treatment of fever, ulcer, diarrhea and wounds in traditional medicine. It is used as an analgesic, expectorant and anti-inflammatory agent since ancient time in folk (Baytop, 1999). It is famous due to having property of astringency (Chevallier, 1996; Dobelis, 1990; Grieve, 1982).

An *in vitro* culture protocol was established for this medicinal plant. With this efficient protocol, disease-and herbicide-free bulk plant material can be supplied throughout the year for pharmaceutical purposes. Three different explants (leaf, stem and root) were used for tissue culture experiment. Various auxins and cytokinins were attempted and also combinations of them. These were BA, TDZ, KIN and Zea either alone or in combinations with IAA, 2,4-D, IBA and NAA as well as the basal medium (control treatment). Generally, all explants tested formed shoots with IAA in combination with

TDZ and BA, and IBA in combination with BA and Zea. The best shoot proliferations were obtained with stem and root explants. Among the stem explants, highest number of shoots per explant was observed on media with 0.01 mg/l TDZ plus 0.5 mg/l IAA (63.8 shoots per explant; 100% explants formed shoots). Media containing 1.0 mg/l BA plus 0.5 mg/l IBA, 1.0 mg/l BA plus 2.0 mg/l IAA, 0.1 mg/l TDZ plus 0.5 mg/l IAA and 1.0 BA plus 1.0 mg/l IBA (59.1, 52.9, 47.4 and 46.9 shoots per explant, respectively; 100% explants formed shoots) were also effective for shoot formation with stem explants.

Regarding root explants, the greatest number of shoots per explant was recorded on media containing 0.5 mg/l BA plus 0.5 mg/l IAA (51.2 shoots per explant; 100% explants formed shoots). As media containing 2.0 mg/l BA plus 0.5 mg/l IAA, 0.5 mg/l BA (46.2 and 32.7 shoots per explant, 78% and 74% explants formed shoots; respectively).

Leaf explants generally formed fewer shoots than stem and root explants. In spite of this, 0.5 mg/l BA was very effective in combination with 0.1 mg/l IBA to give shoot regeneration with leaf explants (56.9 shoots per explant; 100% explants formed shoots). With leaf explants, the other best plant growth regulator applications were 0.5 mg/l BA plus 0.1 mg/l NAA and 1.0 mg/l BA plus 1.0 mg/l IAA (38.0 and 37.6 shoots per explant; 100% and 82% shoot inductions; respectively).

Regenerated shoots formed roots in 4-5 weeks. Four different auxins were used for rooting (IBA, IAA, NAA and 2,4-D). IBA and IAA were better than 2,4-D and NAA in response to rooting. The greatest number of roots per explant was observed on media with 0.5 mg/l IBA.

In essential oil analyze research, 38 compounds were identified, from seed (18), magenta (19), hardening (11) and natural population (10). In fact, results obtained from natural populations (W), both studying *in vitro* cultures that were in magenta (M) and that were grown in soil after it's tissue culture (H) samples showed a highly chemical variability within the *L. vulgaris* essential oils. The oils were separated by gas chromatography and the three major components eicosane, 2- pentadecanone and palmitic acid which is a member of fatty acids.

To screen for antibacterial and antitumor activity of *L. vulgaris,* fifteen different extracts (EtOH, acetone and aqueous extracts of field-grown plants and i*n vitro*-grown plants) were used. Antibacterial activity of *L. vulgaris* essential oil was also determined. Generally, field-grown plants indicated better antibacterial activities than *in vitro*-grown plants. Among field-grown plants extracts, ethanol and acetone extracts were better than aqueous extracts. Best inhibitory activity was observed with ethanol and acetone extracts of field-grown plants. The antibacterial extracts showed significant zone of inhibition against "Gram-positive" bacteria that were used in this experiment. The results indicated that the tested crude extracts showed antibacterial activity towards the "Gram-positive" bacteria.

Antibacterial activity of ethanol and acetone extracts against *S. epidermidis*, *S. aureus*, and *S. pyogenes* may explain why yellow loosestrife is used in folk medicine to treat gastroenteritis, rheumatism, inflammatory complaints and fever (caused by *S. aureus*), toxic shock syndrome (caused by *S. aureus* and *S. pyogenes*), urinary tract infections, wounds and sore throat (caused by *S. pyogenes* and *P. aeruginosa*), chronic lung infections

(caused by *P. aeruginosa*), suppurative diseases, rheumatic fever, cellulitis (caused by *S. pyogenes*), neonatal sepsis, mucous membranes and skin infections (caused by *S. epidermidis*).

The antitumor activity of yellow loosestrife was tested with the potato disc method as modified by McLaughlin's group (Ferrigni *et al*., 1982). Strong antitumor activity was observed with all extracts of yellow loosestrife that dissolved in water at 100.000 mg/l. When compared with control (water), the percentage inhibition of all extracts dissolved in water at 100.000 mg/l was more than 65.4%. No tumor formation was observed with camptothecin (100% inhibition). Antitumor activities of *in vitro*-grown plant extracts were better than field-grown plant extracts and among all extracts, best antitumor activity was observed with water extracts of field-grown and *in vitro*-grown plants when compared to ethanol extracts at 100.000 mg/l. NFW showed the best antitumor activity as camptothecin.

The extracts have high active radical scavenge capability as much as ascorbic acid that was used as control antioxidant. Ascorbic acid is known as strong antioxidant.

The most phenolic content is included by NLE among extracts. Ethanol extracts include more phenolics than water extracts. Leaf parts contain more phenolics and flavanoids than the other parts.

The results obtained in this study clearly demonstrated that all the tested extracts of *L. vulgaris* showed antioxidant and radical-scavenging activities at different magnitudes of potency. The decreasing order of antioxidant and radical-scavenging activities among the extracts was found to be as NLW>NFE>NLE>NAE>NFW>IVW>IVE. This order is found as

NLE>NAE>NLW> NAW> NFW>IVW for total phenolics and NLE> NLW>NAE>NAW>NFE>NFW>IVW for total flavonoids.

Results obtained herein confirm the bioactivity (antibacterial, antitumor and antioxidant) of yellow loosestrife extracts. Furthermore, the total phenolic and flavonoid content colorometric methods gave clues for indications of antitumor, antibacterial and antioxidant activities. Definitive clinical studies are needed to fully understand the many medicinal uses of yellow loosestrife. Future studies should focus on fractionation of the extracts in hopes of identifying active components.

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