TISSUE CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITY OF *SOLANUM DULCAMARA* L., A MEDICINAL PLANT

by

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Approval of the Graduate School of Natural Sciences

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

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

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ABSTRACT

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Cansever, Esra
Master of Science, Department of Biology
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Biological activities of bittersweet extracts were assessed using selected bench-top bioassays (antibacterial and antitumor). Four different kinds of plant extracts were analyzed (IM, FM, IW and FW); methanolic extracts of *in vitro*-grown (IM) and field-grown (FM) plant materials and aqueous extract of *in vitro*-grown (IW) and field-grown (FW) plant materials.

In general, methanolic extract of field-grown leaves and stems were the most effective and showed antibacterial activity against *S.epidermidis*, *S. aureus*, *K. pneumonia*, *S. typhimurium* and *S. marcescens*. *In-vitro* grown plant materials showed less antibacterial activity than field-grown plant materials. All four extracts showed antitumor activity and the percentage inhibition was more than 40% in experiments comparing with control (water). Methanolic extracts showed better antitumor activity than water extracts and field-grown leaves and stems were better than *in vitro*-grown leaves and stems.

Keywords: *Solanum dulcamara* L., common nightshade, *in vitro* culture, bioassay, antibacterial, antitumor.

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gözlenmiştir. Köklendirilmiş bitkicikler vermikülit içeren Magenta kaplarına transfer edilmiş ve 3 hafta sonunda toprağa geçirilmiştir.

Yaban yasemini ekstraktlarının biyolojik aktiviteleri çeşitli biyolojik testler kullanılarak değerlendirilmiştir (antibakteriyal ve antitümör). Dört farklı bitki özütü analiz edilmiştir (IM, FM, IW ve FW); *in vitro* yetiştirilmiş (IM) ve araziden toplanmış (FM) bitki materyallerinin metanol ile olan özütleri ve *in vitro* yetiştirilmiş (IW) ve araziden toplanmış (FW) bitki materyallerinin su ile hazırlanan özütleri.

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Anahtar Kelimeler: *Solanum dulcamara* L., yaban yasemini, *in vitro* kültür, biyolojik test, antibakteriyal, antitümör.

DEDICATION

I dedicate this dissertation to all my family especially my uncle Mehmet Sarıbekiroğlu.

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CHAPTER 1

Introduction and Literature Review

1.1. Introduction

Bittersweet (*Solanum dulcamara* L.), a member of the Solanaceae family, is a poisonous plant that has a long history of use in the treatment of skin diseases, warts, tumours and felons (Bown, 1995). It is a slender-stemmed, woody climber growing to 4 m. It has deeply lobed oval leaves, dark purple flowers with yellow anthers, and scarlet oval berries (Figure 1.1 and 1.2) (Chevallier, 1996). Common names includes bittersweet, deadly nightshade, common nightshade, climbing nightshade, bitter nightshade, bittersweet nightshade, blue nightshade, violet-bloom, European bittersweet, felonwort, woody nightshade, poison flower, poison berry, snake berry, scarlet berry, yaban yasemini, kır yasemini, sofur, tilki üzümü, yaban asması (Grieve, 1982; Dobelis, 1990; Chevallier, 1996; Baytop, 1997, 1999).

It is native to Europe, North Africa, and Northern Asia and occurs in a very wide range of habitats, from woodlands to scrubland, hedges and marshes (Grieve, 1982; Dobelis, 1990; Chevallier, 1996).

Its generic name *Solanum* is derived from Solor, and testifies to the medicinal power of this group of plants. The second name, *dulcamara*, used to be more correctly written in the Middle Ages, Amaradulcis, signifying literally 'bittersweet,' the common country name of the plant, given to it in

reference to the fact that the root and stem, if chewed, taste first bitter and then sweet (Grieve, 1982).

The main objectives of this study were to: a. obtain an *in vitro* culture protocol for *S. dulcamara*; b. screen and compare the antibacterial and antitumor activities of field-grown and *in vitro*-grown *S. dulcamara* plants.

1.2. Literature Review

1.2.1. Medicinal and Historical Uses

In the days of belief in witchcraft, shepherds used to hang bittersweet as a charm round the necks of those of their beasts whom they suspected to be under the evil eye (Grieve, 1982).

The older physicians understand of Bittersweet and applied to many purposes in medicine and surgery, for which it is no longer used. It was in great repute as far back as the time of Theophrastus, and we know of it being in use in the thirteenth century (Grieve, 1982).

The Sweedish botanist Carolus Linnaeus (1707-1778) considered common nightshade to be a valuable remedy for fever and also for inflammatory disorders (Chevallier, 1996).

Main usage of bittersweet is for conditions that have an impact on the skin, mucous membrane and the membrane (synovial membrane) around the joints. It is considered to be an important remedy for treating herpes infections and allergies. Bittersweet was employed as an external remedy for

Figure 1.1 Leaves, flowers and berries of *Solanum dulcamara* L. (München, 2006; Kimonis and Kramer 2006)

Figure 1.2 Illustration of *Solanum dulcamara* L. (Thomé, 1885)

skin diseases and as a treatment for sores and swellings, especially felons, imflammations around the fingernails (Dobelis, 1990).

All parts of the plant are alterative, anodyne, depurative, mildly diuretic, emetic, expectorant, hepatic, mildly narcotic and purgative (Grieve, 1982; Bown, 1995; Chevallier, 1996; Baytop, 1999). The dried stem, usually collected in the autumn and preferably from the ends of branches 2-3 old, is the part that is most valued medicinally, though the leaves are also used (Grieve, 1982). The plant is mainly used as an alterative when taken internally in the treatment of a variety of skin diseases, arthritis, rheumatism, bronchial congestion, heart ailments, ulcerative colitis, jaundice and pneumonia (Grieve, 1982; Bown, 1995; Chevallier, 1996). The plant is also used externally to treat skin eruptions, ulcers, rheumatism and cellulite (Bown, 1995). Two to three year old stems are harvested in the spring, or after the leaves have fallen in the autumn, and dried for later use (Bown, 1995). The fruits are marinated in vinegar and applied to cancerous sores and other problems. A decoction of the root is used in the treatment of cancer and swellings. The root is harvested in the autumn and dried for later uses (Grieve, 1982; Bown, 1995). A homeopathic remedy is made from the fresh, green stems and leaves, harvested as the plant begins to flower. This is used in treating a variety of complaints including backaches, cough, diarrhoea, eye inflammations and joint pains (Castro, 1990). Bittersweet should be used with caution and only under the supervision of a qualified practitioner, this is a poisonous plant that, in excess, paralyzes the central

nervous system, slows the heart and respiration, and lowers temperature, causing vertigo, delirium, convulsions and death (Bown, 1995). Toxicity of bittersweet can vary widely due to environmental conditions, part of the plant or degree of maturity. Unripe berries are more toxic than ripe berries. Berries are more toxic than leaves which, in turn, are more toxic than stems or roots. Overall plant glycoalkaloid content is often higher in the autumn than in the spring (Grieve, 1982; Dobelis, 1990).

Compounds from this plant are purported to have anti-bacterial, anti-tumor, stimulant, expectorant, diuretic, and detoxifying properties (Dobelis, 1990; Chevallier, 1996).

1.2.2. Botany

Solanum dulcamara L. is a member of the Solanaceae family. It is a semi-woody herbaceous perennial vine, which scrambles over other plants, capable of reaching a height of 4 m where suitable support is available, but more often 1-2 m high (Figure 1.1 and 1.2). The flowers, which are star shape and open all summer, are of a bluish purple tint, with reflexed petals when expanded. Flowers are in loose clusters of 3-20, (1-1.5 cm) across. Its stamens are bright yellow project in a conical form around the pistil or seed bearing portion of the flower. The leaves are 4-12 cm long. It has alternate heart-shaped to oval leaves, which usually have two earlike segments at their bases. The fruit is an ovoid red berry about 1 cm long, soft and juicy, poisonous to humans and livestock but edible for birds, which disperse the seeds widely. The berries are green at first, afterwards becoming orange and

finally bright red, and are produced in constant succession throughout the summer and early autumn, many remaining on the plant long after the leaves have fallen (Davis, 1978). As most *Solanum* species, the foliage is also poisonous for humans (Davis, 1978; Grieve, 1982; Bown, 1995; Chevallier, 1996). It is in flower from June to September, and the seeds ripen from August to October. The flowers are hermaphrodite (has both male and female organs) and are pollinated by insects (Grieve, 1982).

It is found throughout the Northern part of Turkey (Davis, 1978). The natural habitats for these plants are damp places, streambanks and thickets (Dobelis, 1990) as well as wayside plant which flourishes in open areas.

The twings are collected in spring or autumn, and the root bark in autumn (Chevallier, 1996). Common nightshade is a shrubby, quite woody at the base, but throws outlong, straggling, slender branches, which trail over the hedges and bushes amoung which it grows. They are at first green and hairy, but become woody and smooth as they get older (Grieve, 1982; Dobelis, 1990).

1.2.3. Constituent and Bioactivity of *S. dulcamara* L.

Kupchan et al., (1965) reported that an alcoholic extract of *Solanum dulcamara* L. showed tumor-inhibitory activity against Sarcoma 180 in mice. Systematic fractionation of the extract has led to isolation and characterization of β-solamarine as an active principle.

Bittersweet is a rich in source of glycosides (Friedman and McDonald, 1997) and used in the commercial production of steroidal hormones (Ehmke et al., 1995). Rönsch and Schreiber (1966) isolated two new glycosides (γ_1-solamarine and δ-solamarine) in addition to α-, β-, and γ-solamarine from overground, vegetative parts of *Solanum dulcamara* L.

It contains the alkaloid solanine and amorphous glucoside dulcamarine, to which the characteristic bittersweet taste is due. Sugar, starch, gum and resin are also present. Solanine acts narcotically; in large doses it paralyses the central nervous or voluntary muscles. It slows the heart and respiration, lessens sensibility, lowers the temperature and causes vertigo and delirium, terminating in death with convulsions (Grieve, 1982). It also has steroidal saponins, and about 10 % tannins (Chevallier, 1996).

Solanine, a steroidal glycoside, is the major alkaloid of potato plant, which was toxic to insects at higher concentrations. Khanna et al., (1988) compared the callus tissues of *Solanum tuberosum* and *S. dulcamara* qualitatively and quantitatively for their solanine content.

Keeler et al., (1990) analyzed the alkaloid extracts of *S.dulcama* fruits with Gas Chromatography (GC) and reported that all alkaloidal material was apparently present as glycoside and solasodine was a principal or sole aglycone of the alkaloid glycosides. In addition to solasodine, *S. dulcamara* contains appreciable amounts of an unknown spirosolane, an aglycone provisionally identified as soladulcidine. They also reported that (Keeler et al., 1990) the induction of congenital craniofacial malformations in hamsters

by high oral doses of *S.dulcamara* that contained mainly solasodine glycosides was compared to inductions of malformations by *Solanum tuberosum*, that contained mainly solanidane glycosides. *Solanum dulcamara* fruit induced a high percentage incidence of deformed litters (16.3 %) when compered to control. Deformed litter incidence induced by sprouts of *Solanum tuberosum* was 24.0%, ($P < 0.001$).

Ceha et al. (1997) reported that a 4-yr-old girl with acute anticholinergic crisis was poisoned by woody nightshade, *S. dulcamara*. Analysis showed that sterols consistent with solanine was the reason.

Tunon et al. (1995) evaluated the water extract of *S. dulcamara* for inhibitory activity on prostaglandin biosynthesis and platelet activating factor (PAF)-induced exocytosis *in vitro*. In the PAF-test, high inhibition was obtained by *S. dulcamara.*

Baker et al. (1989) orally administered ground plant material from *S. dulcamara* to Syrian hamsters. Eight of 10 hamsters died following administration of plant material and had gastric glandular mucosal necrosis and small intestinal mucosal necrosis with little inflammation. Baker et al. (1989) reported that *S. dulcamara* contained solasodine glycoalkaloids(s), and an equal amount of other glycoalkaloids which were probably derived from soladulcidine (dihydrosolasodine). Glycoalkaloids of *S. dulcamara* were probably the agents responsible for the lesions observed.

Willuhn and Kostens (1975) reported that the following sterols were found in the roots, stems, leaves, unripe and ripe fruits of *Solanum dulcamara*: cholesterol, sitosterol, stigmasterol, campesterol, brassicasterol, isofucosterol and 24-methylenecholesterol. The most abundant components are cholesterol, sitosterol and stigmasterol (77–84%). In all parts of the plant the sterols are present in the free form and as esters, glycosides and acylated glycosides. The total sterol content and the content of combined forms were determined photometrically. In the leaves, 58% of the sterols were found in the form of glycoside (26%), acylated glycoside (29%) and ester (2%). In the roots, only 25% of the sterol were found in combined form. In the other organs the ratio of free and combined sterols was intermediate. In all cases, the ester fraction was the least.

1.2.4. Physiological and Biotechnological Studies

A haploid clone of *S. dulcamara* has been obtained by anther culture. The haploid status has been conserved over a period of more than 4 years. Haploid plants were recovered from isolated protoplasts of shoot tip (Binding and Mordhorst, 1984).

Kumar et al., (1992) reported the potential value of non-ionic surfactant, Pluronic F-68, as growth-stimulating additives to plant culture media and studied it on the growth of transformed roots, callus and protoplasts of *S. dulcamara* L. Root growth was stimulated by addition of 0·001–0·05% (w/v) of freshly-prepared, commercial grade Pluronic to culture

medium, with maximum increases in root fresh and dry weights at 0·01%. Higher concentrations (0·5–1·0% w/v) of freshly-prepared Pluronic inhibited growth. Callus growth was also stimulated by the addition of freshly-prepared, commercial grade Pluronic F-68 to medium, with maximum increases at 0·1% (w/v); in contrast, 1·0% (w/v) Pluronic was inhibitory to callus growth.

Emke and Eilert (1986) established callus and cell suspension cultures of *S. dulcamara* and compared their soladulcidine variety. Plantlets were regenerated from undifferentiated callus. Soladulicidine, solasodine and the corresponding neutral spirostanes tigogenin and diosgenin were isolated from callus as well as suspension cultures of *S. dulcamara* and identified by thin layer chromatography and mass spectrometry. Total alkaloid concentrations were about 0.2 mg/g dry weight in callus and 0.1 mg/g dry weight in green suspension cultures. Alkaloid accumulation in callus of *S. dulcamara* could be enhanced by the induction of organogenesis. The shoots of the regenerated plants from the callus contained soladulcidine (1.6 mg/g dry weight) and tigogenin. Thus, in concentration and composition the regenerated plants equaled the source plant.

S. dulcamara has been shown to be susceptible to infection by *Agrobacterium rhizogenes* (Davey et al., 1987; McInnes et al., 1991), producing transformed `hairy roots' from wounded decapitated stems.

Curtis et al., (2000a) described a transformation system for *Solanum dulcamara* using the supervirulent *Agrobacterium tumefaciens* strain 1065,

carrying both the ß-glucuronidase (*gus*) and neomycin phosphotransferase II (*npt* II) genes adjacent to the right and left T-DNA borders, respectively. Leaf explants were more efficient for the production of transformed plants when compared to stem explants on medium containing 50 mg $l^{-1}$of kanamycin sulphate.

Curtis et al. (2000b) described the consequences of over-expressing the pumpkin gibberellin (GA) 20-oxidase gene in *S. dulcamara*. They reported that the presence of the pumpkin enzyme resulted in a reduction in bioactive GAs due to the normal GA biosynthetic pathway being diverted to the inactive tricarboxylic acids and this could be used to control the stature of ornamental plants as well as other important crop species.

Urrita et al., 1992 showed that *S. dulcamara* had low levels of thermal hysteresis activity, but activity was absent in summer. Proteins which produce a thermal hysteresis (i.e. lower the freezing point of water below the melting point) are common antifreezes in cold adapted poikilothermic animals, especially fishes from ice-laden seas and terrestrial arthropods. This suggests that thermal hysteresis proteins may be fairly common for winter adaptation in angiosperms. Winter stem fluid from S. *dulcamara* also showed the recrystallization inhibition activity characteristics of the animal thermal hysteresis proteins (THPs), suggesting a possible function for the THPs in this freeze tolerant species.

CHAPTER 2

In Vitro Culture of *Solanum dulcamara* L.

2.1. Introduction

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

In vitro culture protocol providing multiple regenerants per explant is very useful for mass propagation of medicinal plants. Also micropropagation of medicinal plants are important when a source of plant material free of adulterated bulk material is needed, or when a source of material is needed that is free of any pesticide residues or disease infestation (Turker et al., 2001).

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, namely a shoot or root primordium, whose vascular system is often connected to the parent tissues (Thorpe, 1980; Thorpe, 1994). Christianson

and Warnick (1988) stated that there are three phases of organogenesis as follow: dedifferentiation (which sometimes involves callus production and ends when cells become competent), induction (cells become determined), and differentiation (cells from roots and shoots). Skoog and Miller (1957) showed that shoot organogenesis in tobacco callus and stem segments can be controlled experimentally using various growth regulator concentrations and combinations. The first observation of controlled shoot formation *in vitro* was by White (1939) and root formation from callus was by Nobecourt (1939). Also role of auxin in root elongation was reported by Gautheret (1945). In 1948, Skoog and Tsui experienced adenine sulfate and concluded that shoot generation was releated with this compound and its concentration. Miller discovered the Kinetin in 1956 and he proved that higher concentration of cytokinin than auxin provide shoot generation and higher concentration of auxin than cytokinin provide root generation and same concentrations induce the callus formation (Gurel and Turker, 2001).

The main objective of this study was to establish an *in vitro* culture protocol for *S. dulcamara*.

2.2. Materials and Methods

Seeds of *S. dulcamara* were collected from Abant Lake, Bolu/Turkey in September of 2005. Identification of the species was made by using “Flora of Turkey and the East Aegean Islands” (Davis, 1978) and voucher specimens (AUT-1438) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. Seeds were washed with anti-bacterial

soap, rinsed with distilled water and surface disinfected by shaking for 20 min in 20% EtOH, for 20 min in 20% Domestos® (1.05% sodium hypochloride), and then rinsed with sterile distilled water three times. Seeds were placed in sterile, disposable petri dishes (60 x 15 mm) containing 15 ml of Murashige and Skoog Minimal Organics 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 min at 121°C and 105 kPa). Thirty-day-old seedlings were transferred to Magenta containers (GA-7 Vessel, Sigma Chemical Co.) containing liquid MSMO. Explants excised from 50-60 day old plants (3cm in height) were leaf segments (5-6 mm) and petiole segments (5-6 mm). Explants were placed in sterile disposable Petri plates containing 20 ml MSMO with combinations of auxin and cytokinin: Kinetin (1,3 and 5mg/l) and 2,4-D (0; 0,1 and 0,5 mg/l); BA (0,1; 0.5; 1and 3 mg/l) and IAA (0; 0,5 and 1 mg/l); BA (0,5; 1and 3 mg/l) and NAA (0;1; 3 and 5); TDZ (0,1; 0,5; 1and 3 mg/l) and IAA (0; 0.5 and 1 mg/l); TDZ (0,1; 0,5 and 1 mg/l) and 2,4-D (0,1; 0,5 and 1 mg/l); TDZ (0,1; 0,5;1 and 3 mg/l) and IBA (0,1; 0,5;1 and 3 mg/l). All cultures were incubated at 22 °C under a 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$). Shoot number and percentage of explants producing shoots were recorded after 3 weeks for leaf and petiole segments. 10 replications for each explant and each experiment was repeated three times.

Well-developed shoots were excised from the various explants and subcultered for 10 days on the same iniation medium. Shoots were then

seperated individually and placed on rooting medium containing MSMO and various levels of either IAA (0,5; 1 and 3 mg/l) ; IBA (0,5; 1 and 3 mg/l) ; 2,4-D (0.5; 0.1 and 0,5 mg/l) or NAA (0,5; 1 and 3 mg/l). After four weeks, the number of roots and percentage of explants producing roots were recorded. There were 10 replications and experiment was replicated three times. Rooted explants were transferred to vermiculate (Agrekal) in Magenta containers for acclimatization and after 3 weeks they were transferred to plastic disposable pots containing potting soil.

Data were subjected to ANOVA and Duncan's Multiple Range Tests (SAS, 1999).

2.3. Results and Discussion

S. dulcamara is a valuable medicinal herb, but there has been almost no interest in the *in vitro* propagation of this species. We, therefore, aimed of developing an efficient protocol for *in vitro* regeneration of *S. dulcamara* and thus examined the effects of several combinations of plant growth regulator using different explants. Two different explants (leaf and petiole) were used for the experiment. When leaf and petiole explants were cultured on MSMO medium containing BA or Kinetin in combination with 2,4-D, IAA or NAA, generally all explants tested formed shoots with BA in combination with IAA and Kinetin in combination with 2,4-D.

Best shoot proliferation was obtained with leaf explants. Petiole explants formed fewer shoots than leaf explants (Table 2.1; 2.2 and Figure 2.1). Overall, number of shoots per explant was lower for all explants on

media with BA plus NAA combinations when compared to BA plus IAA combinations (Table 2.1 and Figure 2.1).

Regarding leaf explants, the greatest number of shoots per explant was observed on media containing 3 mg/l BA plus 0.5 mg/l IAA (40 ± 7.07 shoots per explant; 100 % explants formed shoots) (Figure 2.2). Media containing 3 mg/l Kinetin (33.88 ± 1.95 shoots per explant; 100 % explants formed shoots) and 3 mg/l BA (33.13 ± 1.62 shoots per explant; 100 % explants formed shoots) were also effective for shoot formation in leaf explants (Table 2.1 and Figure 2.1).

With petiole explants, the greatest number of shoots per explant was observed on media containing 3 mg/l Kinetin plus 0.1 mg/l 2,4-D (28 ± 0.82 shoots per explant; 100 % explants formed shoots) and 3 mg/l Kinetin (10.38 ± 0.61 shoots per explant; 100 % explants formed shoots) (Table 2.1 and Figure 2.1).

When leaf or petiole explants were cultured on media containing BA or Kinetin in combination with 2,4-D, IAA or NAA, it is evident that the frequency of shoot formation was closely related to concentrations of the auxin supply (Table 2.1 and Figure 2.1). Increasing concentrations of auxin severely inhibited shoot development. For example, among leaf explants investigated, 1 mg/l IAA in combination with 3 mg/l BA decreased mean number of shoots per explant to 1.38 and the number of the shoots producing explants to 62% while 0.5 mg/l IAA in combination with 3 mg/l BA induced shoots in all explants and mean number of shoots per explant was 40 (Table 2.1 and

Figure 2.1). Similarly, among petiole explants investigated, although 0.5 mg/l 2,4-D in combination with 3 mg/l Kinetin did not form shoots, 0.1 mg/l 2,4-D in combination with 3 mg/l Kinetin was the most effective combination for shoot formation.

In addition, some root formation was observed in the treatments which were used higher NAA (3 and 5 mg/l), 2,4-D (0.5 mg/l) or IAA (1 mg/l). Such effects of auxin on shoot and root developments are in agreement with the classical findings of Skoog and Miller (1957); a low ratio of auxin to cytokinin induces shoots, while the reverse situation induces roots.

Callus was observed for leaf and petiole explants before shoot formation (indirect organogenesis). In control experiments (no hormones added to media) none of the explants formed shoots (Figure 2.1).

Although leaf and petiole explants did not form shoots on media containing TDZ in combination with IBA, 2,4-D or NAA hormones, only 0.1 mg/l TDZ formed shoots on leaf and petiole explants (Table 2.2 and Figure 2.1). TDZ was not an effective growth regulator for shoot induction of *S. dulcamara* explants.

Regenerated shoots were separated individually and put on rooting medium. They formed roots in 3-4 weeks (Figure 2.3). IAA and IBA were better than 2,4-D and NAA in response to rooting (Table 2.3 and Figure 2.6). The greatest number of roots per explant was observed on media with 0.5 mg/l IBA (Table 2.3 and Figure 2.3; 2.6). Media containing 3 mg/l IAA (9.2 ± 4.43 roots per explant; 80 % explants formed roots) and 1 mg/l IBA (7.7 ±

1.51 roots per explant; 90 % explants formed roots) were also good for root formation (Table 2.3 and Figure 2.6). Fewer explants produced roots as IBA concentration increased, 100% of the explants formed roots at 0.5 mg/l IBA compared to 10% at 3 mg/l IBA. Similar pattern was observed with NAA concentrations on rooting. Although 60% of the explants formed roots at 0.5 mg/l NAA, root formation was not observed with 3 mg/l NAA (Table2.3; Figure 2.6). In control experiments (no auxin hormone added to the media) none of the explants formed roots (Table2.3; Figure 2.6). Rooted plants were transferred to Magenta containers including vermiculate for acclimatization (Figure 2.4). After 3 weeks, they were transferred to soil (Figure 2.5).

Some of the regenerated shoots on media with 3 mg/l Kinetin plus 0.5 mg/l 2,4-D, 0.5 mg/l BA plus 1 mg/l IAA and 3 mg/l BA plus 3 mg/l NAA hormone combinations formed roots and these regenerants directly transferred to vermiculate and then to pots without rooting procedure.

Bittersweet has a history of medical use and Ancient Romans used bittersweet to treat pneumonia, absent menstruation, jaundice, rheumatism, cramps, eczema, psoriasis, asthma, and phlegm (Kupchan et al., 1965; Grieve, 1982; Dobelis, 1990; Bown, 1995; Chevallier, 1996). An *in vitro* culture protocol was established, which provides plant material for future physiological and biochemical studies. These studies include developing extraction and analytical procedures for the active ingredients.

Table 2.1 Shoot development from leaf and petiole explants incubated on media containing combinations of BA or Kinetin with 2,4-D, IAA or NAA. Means with the same letter within columns are not significantly different at P>0.05.

Treatments (mg/l)	Leaf					Petiole				
	Mean # of shoots per explant		SE		% explants forming shoots	Mean # of shoots per explant		SE		% explants forming shoots
0 (Control)	-				-	-				-
Kinetin : 2,4-D										
1.0:0	26.63	±	1	cd	100	9	±	0.38	bc	100
1.0:0.1	23.75	±	2.65	de	100	7.63	±	0.82	c	100
1.0:0.5	-				-	-				-
3.0:0	33.88	±	1.95	b	100	10.38	±	1.61	b	100
3.0:0.1	13.88	±	0.55	fg	100	28	±	0.82	a	100
3.0:0.5	22.5	±	3.66	de	100	-				-
5.0:0	11.88	±	0.91	g	100	-				-
5.0:0.1	10.38	±	0.75	gh	100	4.88	±	0.67	d	100
5.0:0.5	-				-	-				-
BA : IAA										
0.1:0	-				-	-				-
0.1:0.5	21.5	±	3.44	de	100	4.25	±	1.25	d	75
0.1:1	4.88	±	0.61	hij	100	3.5	±	0.19	d	100
0.5:0	18.63	±	5.04	ef	100	1.5	±	0.19	ef	100
0.5:0.5	20.13	±	3.33	de	100	8.38	±	2.09	e	100
0.5:1	11	±	2.1	g	100	9	±	1.35	bc	100
1.0:0	23.25	±	6.11	de	100	4.38	±	1.24	d	100
1.0:0.5	19	±	3.12	ef	100	1.75	±	0.37	ef	87.5
1.0:1	2.25	±	0.88	ij	75	1	±	0.44	ef	50
3.0:0	30	±	6.55	bc	100	8.5	±	0.57	c	100
3.0:0.5	40	±	7.07	a	100	4.88	±	1.19	d	100
3.0:1.0	1.38	±	0.53	j	62.5	-				-
BA : NAA										
0.5:0	21.38	±	2.98	de	62.5	2	±	0.63	e	62.5
0.5:1	4.63	±	1.43	hij	62.5	-				-
0.5:3	-				-	-				-
0.5:5	-				-	-				-
1.0:0	21.25	±	3.3	de	100	-				-
1.0:1.0	8.13	±	0.74	ghi	100	-				-
1.0:3.0	-				-	-				-
1.0:5.0	-				-	-				-
3.0:0	33.13	±	1.62	b	100	-				-
3.0:1.0	22.5	±	4.91	de	100	-				-
3.0:3.0	4	±	1.09	ij	87.5	-				-
3.0:5.0	-				-	-				-

Table 2.2 Shoot development from leaf and petiole explants incubated on media containing combinations of TDZ with IAA, 2,4-D or IBA. Means with the same letter within columns are not significantly different at P>0.05.

Treatments (mg/l)	Leaf					Petiole				
	Mean # of shoots per explant		SE		% explants forming shoots	Mean # of shoots per explant		SE		% explants forming shoots
0 (Control)	-				-	-				-
TDZ : IAA										
0.1:0	8	±	2.43	ghi	100	2	±	1.23	e	25
0.1:0.5	-				-	-				-
0.1:1	-				-	-				-
0.5:0	-				-	-				-
0.5:0.5	-				-	-				-
0.5:1	-				-	-				-
1.0:0	-				-	-				-
1.0:0.5	-				-	-				-
1.0:1	-				-	-				-
3.0:0	-				-	-				-
3.0:0.5	-				-	-				-
3.0:1.0	-				-	-				-
TDZ : 2,4-D										
0.1:0.1	-				-	-				-
0.1:0.5	-				-	-				-
0.1:1	-				-	-				-
0.5:0.1	-				-	-				-
0.5:0.5	-				-	-				-
0.5:1	-				-	-				-
1.0:0.1	-				-	-				-
1.0:0.5	-				-	-				-
1.0:1.0	-				-	-				-
TDZ : IBA										
0.1:0.1	-				-	-				-
0.1:0.5	-				-	-				-
0.1:1	-				-	-				-
0.1:3	-				-	-				-
0.5:0.1	-				-	-				-
0.5:0.5	-				-	-				-
0.5:1	-				-	-				-
0.5:3	-				-	-				-
1.0:0.1	-				-	-				-
1.0:0.5	-				-	-				-
1.0:1.0	-				-	-				-
1.0:3.0	-				-	-				-
3.0:0.1	-				-	-				-
3.0:0.5	-				-	-				-
3.0:1.0	-				-	-				-
3.0:3.0	-				-	-				-

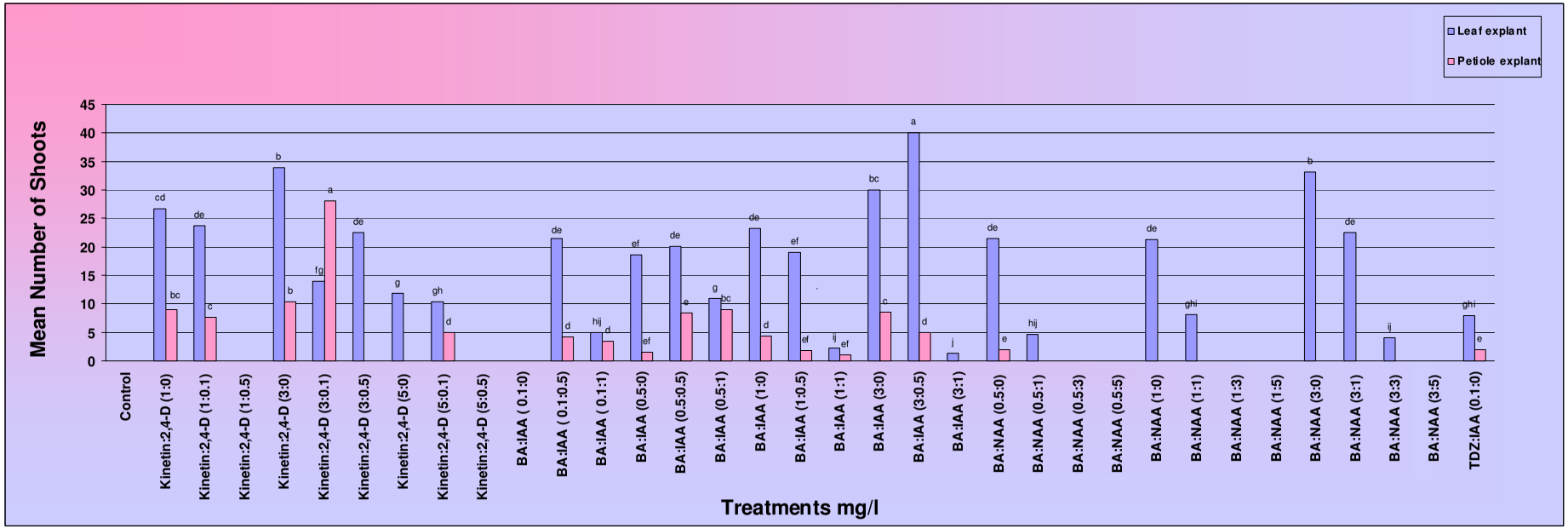

Figure 2.1 Shoot development from leaf and petiole explants incubated on media containing different hormone combinations.

Figure 2.2 Shoot formation from leaf explants with 3mg/l BA: 0.5 mg/l IAA.

Figure 2.3 Root formations from regenerated shoots with 0.5 mg/l IBA.

Figure 2.4 Regenerated plants in vermiculate for acclimatization.

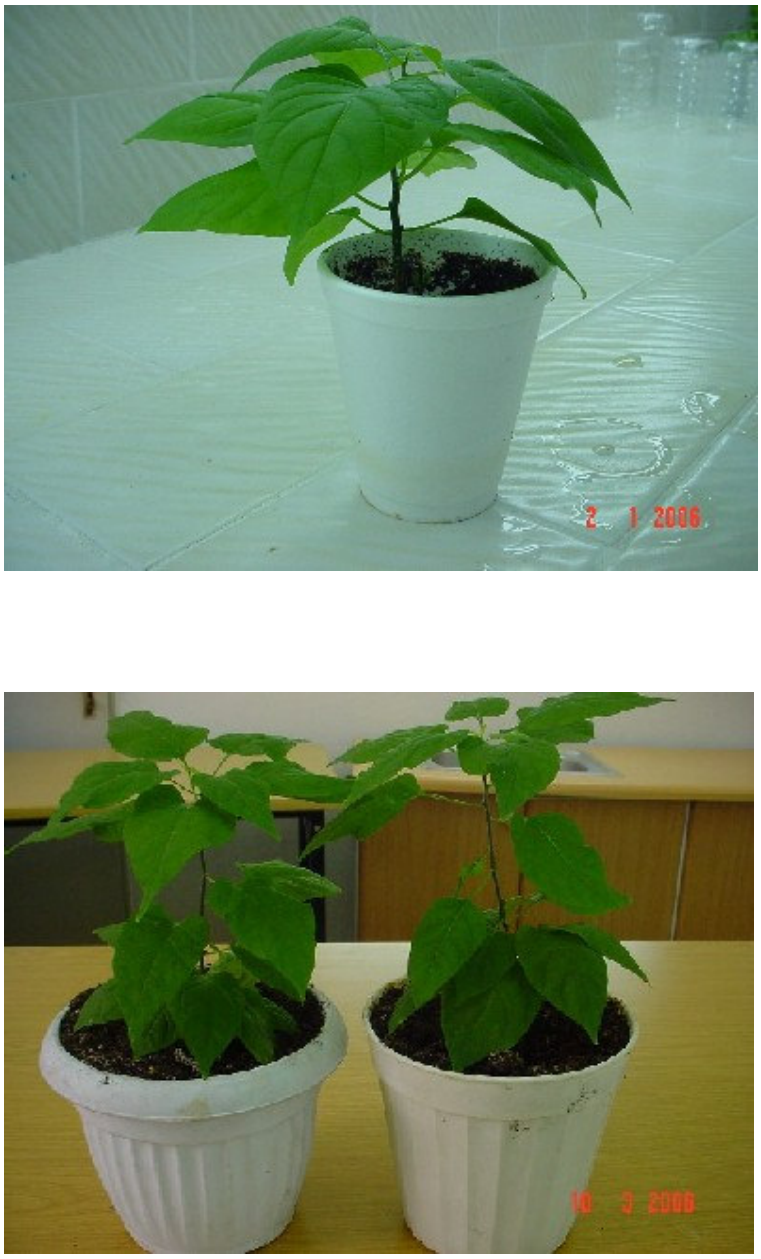

Figure 2.5 Regenerated plants in soil.

Table 2.3 Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots. Means with the same letter within columns are not significantly different at P>0.05.

Treatments (mg/l)	Mean # of roots per explant		SE		% explants forming roots
Control	-	-	-	-	-
IAA - 0.5	5.2	±	1.27	bc	90
IAA - 1	4.1	±	1	bc	80
IAA - 3	9.2	±	4.43	ab	80
IBA - 0.5	11.6	±	1.42	a	100
IBA - 1	7.7	±	1.51	ab	90
IBA - 3	0.5	±	0.5	c	10
2,4-D - 0.05	3.5	±	1.38	bc	50
2,4-D - 0.1	4.5	±	1.28	bc	80
2,4-D - 0.5	0.3	±	0.3	c	10
NAA - 0.5	6.4	±	3.26	ab	60
NAA - 1	0.2	±	0.13	c	20
NAA - 3	-	-	-	-	-

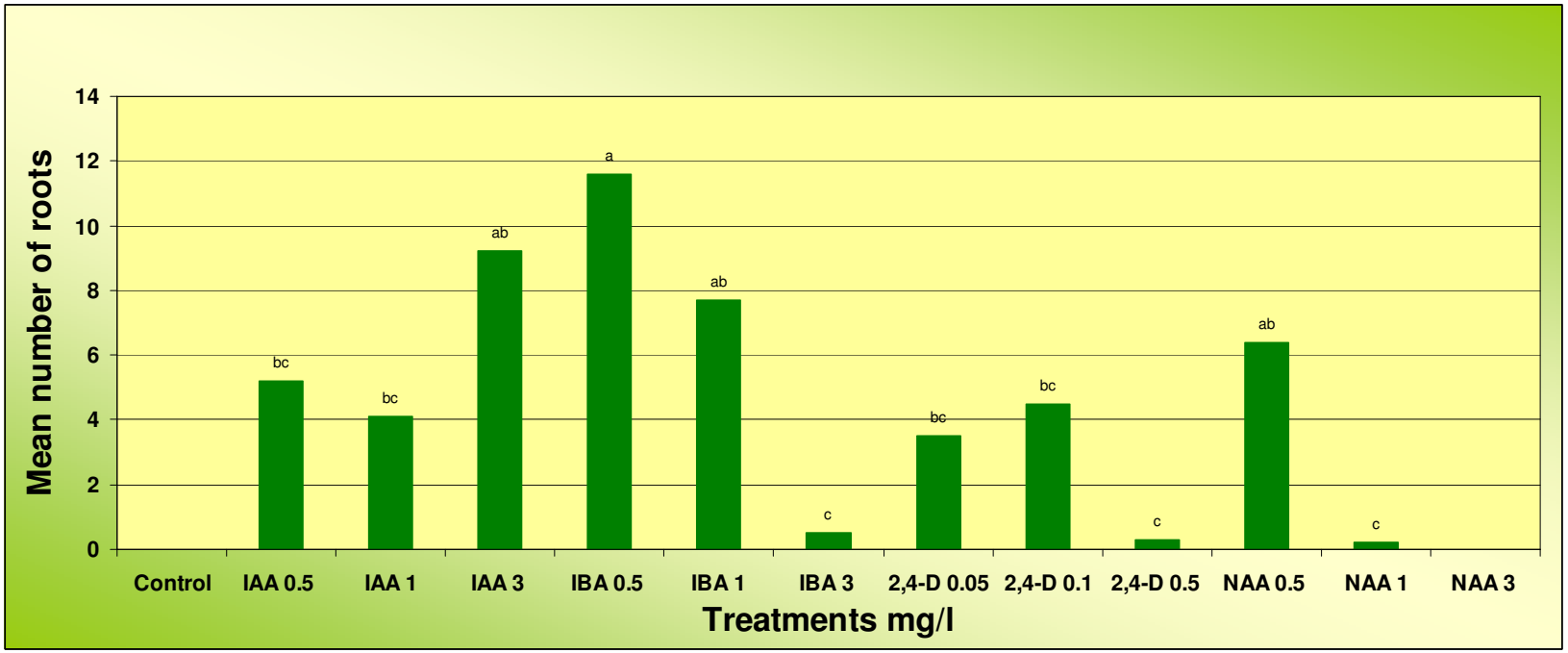

Figure 2.6 Effects of IAA, IBA, 2,4-D and NAA on root formation from regenerated shoots.

CHAPTER 3

Biological Activity of *Solanum dulcamara* L.

3.1. Introduction

The Kirby-Bauer Disc Diffusion Assay measures antimicrobial activity based on bacteriostatic/bacteriocidal properties. It was developed by Kirby and Bauer in the 1960's (Prescott et al., 1990). In this assay a pathogenic microbe is inoculated onto a Mueller-Hinton agar plate. Filter paper discs impregnated with antibiotics are placed onto the dried, inoculated plate, then incubated at 37°C for 18-24 hours. At the end of the incubation period, plates are observed and clear zones around the disc are measured. 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, immediately sensitive, or resistant to the particular antibiotic. Kirby-Bauer agar diffusion test procedure is designated for use with rapidly growing bacteria (Atlas, 1988).

The disc diffusion method for screening antimicrobial activity has been used for plant extracts. In these assays the test substance saturated on a filter paper disc replaces the antibiotic impregnated discs.

Ferrigini et al. (1982) used a potato disc tumor assay to determine possible antitumor activity of several plant extracts (e.g., members of the Euphorbiaceae) with *Agrobacterium tumefaciens* as the tumor inducer.

Agrobacterium tumefaciens is the causative agent inducing crown gall disease (Agrios, 1997). 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. Tumorigenisis in plants and animals involve similar mechanisms and common nucleic acid components (Ferrigini, 1982; Agrios, 1997). Ferrigini et al. (1982) established anti-tumor activity for several plant extracts, including an extract from periwinkle (*Vinca major*) called vincristine. Vincristine is a Federal Drug Administration currently (FDA) approved Vinctistine for pediatric leukemia problem. In 1993, Jerry McLaughlin used the potato tumor disc assay to evaluate several other plant extracts. In this study, as well as in the Ferrigini study, the potato tumor disc assay was compared to the 3PS in vivo tumor assay, the standard test for new antitumor agents. In the 3PS in vivo tumor assay, leukemic mice are treated with possible antitumor agents (McLaughlin, 1991; McLaughlin et al., 1993; McLaughlin and Rogers, 1998). Differences in life span of the leukemic mice compared to healthy mice are used as a measure of antitumor activity. A major problem in using the 3PS in vivo tumor assay is that high concentrations of antitumor agent often prove fatal to the subjects. The potato tumor assay eliminates this problem (Galsky et al., 1980; Ferrigini et al., 1982; McLaughlin, 1991; McLaughlin et al., 1993; Coker, 1999; Turker and Camper, 2002; Boonkaew et al., 2003; Coker et al., 2003).

Crude extract of aeral parts from *Solanum dulcamara* L. (Common Nightshade) has been reported in the past to have stimulant, narcotic, sedative, expectorant, diuretic, and detoxifying properties which are used to treat skin problems such as eczema, itchiness, psoriasis, warts, scrofula as an external astringent and be taken to relative asthma, chronic bronchitis, rheumatic conditions, including gout (Chevallier,1996). On the other hand it has been much employed for jaundice as a medicinal internal astringent herb (Grieve, 1982). Pharmacologist has found little evidence to validate any of these uses. Although antibacterial and antitumor activities of bittersweet have been mentioned in herbal books (Dobelis, 1990; Chevallier, 1996), there is no any scientific research carried out to determine the antibacterial action and just one research (Kupchan et al., 1965) about antitumor activity of *S. dulcamara*.

The objective of this experiment was to evaluate the antibacterial and antitumor activities of *in vitro*-grown and field-grown plant extracts (MeOH and water) of *S. dulcamara*.

3.2. Materials and Methods

3.2.1. Plant Material and Extraction

Stem and leaves of *S. dulcamara* (field-grown plants) were collected from Abant Lake, Bolu/Turkey in September of 2006. Identification of the species was made by using “Flora of Turkey and the East Aegean Islands” (Davis, 1978) and voucher specimens (AUT-1438) were deposited at the Abant Izzet Baysal University (AIBU) Herbarium, Bolu/Turkey. For

extractions, two different sources of plant (field-grown, *in vitro* cultured) were used. Leaves and stems of *in vitro* cultured plants were collected from bittersweet plants that were previously micropropagated in our laboratory (Cansever and Turker, 2006). Collected plants were dried in oven at 40 °C and then ground into a powder. Twenty grams from each plant sample were extracted with 200 ml water or MeOH (methanol) at 45 °C for 12 hours and then filtered. For water extraction, frozen filtrate was lyophilized by using freeze-dryer at -65 °C and for MeOH extraction, MeOH was evaporated under vacuum using rotary evaporator at 60 °C. Each residue was then dissolved in sterile distilled water to produce a final concentration of 100 mg/ml. Treatments, plant materials used and extraction procedures are summarized in Table 3.1.

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

Test Material	Designation*	Materials in Treatment	Extraction Procedure
MeOH Extract	FM	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with MeOH at 45 ºC for 12 hr. MeOH was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
	IM	*In vitro*-grown leaves and stems	20 g *in vitro*-grown leaves and stems were extracted with MeOH at 45 ºC for 12 hr. MeOH was evaporated under vacuum and residue was dissolved in water to final concentration of 100 mg/ml.
Water Extract	FW	Field-grown leaves and stems	20 g field-grown leaves and stems were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.
	IW	*In vitro*-grown leaves and stems	20 g *in vitro*-grown leaves and stems were extracted with water at 45 ºC for 12 hr. Water was freeze-dried and residue was dissolved in water to final concentration of 100 mg/ml.

* FM: MeOH extract of field-grown leaves and stems

IM: MeOH extract of i*n vitro*-grown leaves and stems

FW: Water extract of field-grown leaves and stems

IW: Water extract of *in vitro*-grown leaves and stems

3.2.2. Antibacterial Bioassay

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott et al., 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATTC 13883) which are gram-negative bacteria and *Streptococcus pyogenes* (ATTC 19615), *Staphylococcus aureus* (ATTC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are gram-positive bacteria (Table 3.2).

BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated for 3 hours at 37°C. After 3 hr, one bacteriological loop from each broth was streaked on Tryptic Soy Agar (TSA) plates and incubated for 2 days at 37°C. After 2 days, a single colony was removed and streaked on a new TSA plate and incubated 37°C for 2 additional days. After 2 days, 4-5 loops of pure culture were transferred to 20 ml of TSB in a test tube for each bacterial strain and incubated overnight at 37°C; then 0.1 ml from each culture was transferred into 0.9 ml TSB in eppendorf tubes. 0.1 ml from each strand was transferred into Mueller-Hinton (M-H) agar plates. (One eppendorf tube was used for each agar plate). The plates were inoculated with a microorganism suspension at a density of 10^6 cells/ml.

Table 3.2 Bacteria used in antibacterial assay and their representative diseases (Vazquezt and Archer, 1980; Atlas, 1988; Prescott and Harley, 1990; Prashanth et al., 2001; Ciftci et al., 2003; Büyükbaba-Boral et al., 2004; Uzun et al., 2004; Sahayara et al., 2006).

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

A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface of a (60 X 15 mm) Mueller-Hinton (M-H) agar plate (One test tube was used for each agar plate). Agar plates were streaked three times, each time turning the plate at a 60° angle and finally rubbing the swab through the edge of the plate. All extracts were sterilized by filtering through a 0.22 μm filter (Pal-Gelman Laboratory) and sterile filter paper discs (Glass Microfibre filters, Whatman®; 7 mm in diameter) were impregnated with 50 μl of extract. There were five replicates in each plate and two plates for each extract tested for each bacterium. Positive controls consisted of five different antimicrobial susceptibility test discs (Bioanalyse®): Erythromycin (15 μg) (E-15), Ampicillin (10 μg) (AM-10), Carbenicillin (100μg) (CB-100), Tetracycline (30 μg) (TE-30) and Chloramphenicol (30 μg) (C-30). Four antibiotic discs were used for each plate and run in duplicate. Negative control consisted of water. Inoculated plates with discs were placed in a 37°C incubator. After 16 to 18 hrs of incubation, inhibition zone diameter (mm) was measured. All experiments were repeated three times.

The results obtained from antibacterial bioassays were statistically analyzed using ANOVA and Duncan's Multiple Range Test (SAS, 1999).

3.2.3. Anti-tumor Assay

Antitumor activity of Bittersweet extracts was assessed with the potato disc method as modified by McLaughlin's group (Ferrigini et al., 1982). *Agrobacterium tumefaciens* (strain A 281) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28°C. Camptothecin (Sigma) (tumor suppressant) served as a positive control. All extracts were aqueous; therefore, water was used as a negative control. Six to seven loops of *A. tumefaciens* were added to 10 ml Phosphate buffered saline (PBS) (pH=7.2). Bacteria concentrations were adjusted to absorbance (600 nm) values of 0.96 ± 0.02 which equaled to 1.0×10^9 bacteria.

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

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

Solvent control I: 600 μl control water + 150 μl sterile distilled water + 750 μl *A. tumefaciens* in PBS.

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

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

Potatoes (*Solanum tuberosum* L.) were scrubbed with a brush under running water and surface sterilized by immersion in 10% Domestos® for 20

min. Tubers were then placed on sterile Petri dishes and cut along either side revealing the largest flat surface area available; tubers were immersed in 20% Clorox for 15 min and then placed on sterile Petri dishes. Cylinders (10 mm diameter) were cut from the center of potato tissue (skin portion was eliminated) and placed in sterile distilled water. Cylinders were rinsed twice more. Each cylinder was cut into 0.5-cm discs after excluding 1-cm end pieces. These discs were transferred to 24-well culture plates containing water-agar (15g/L). One well plate (24 cell wells) was used for each experiment; each experiment had 24 replicates. Each disc was overlaid with 50 µl of appropriate inoculum. No more than 30 min elapsed between cutting the potato discs and inoculation (McLaughlin, 1991). Plates were incubated at room temperature (25°C) in the dark for 2 weeks. After 2 weeks, discs were stained with Lugol's reagent and tumors on each disc were counted. Experiments were repeated three times. Percent inhibition of tumors was calculated (McLaughlin, 1991; McLaughlin et al., 1993; McLaughlin and Rogers, 1998).

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

3.3. Results and Discussion

Four different extracts [MeOH extract of field-grown leaves and stems (FM), MeOH extract of i*n vitro*-grown leaves and stems (IM), water extract of field-grown leaves and stems (FW) and water extract of *in vitro*-grown leaves

and stems (IW)] were used to screen for antibacterial and antitumor activity of *S. dulcamara*. Methanolic extract of field-grown leaves and stems gave the best inhibitory activities against *S.epidermidis*, *S. aureus*, *K. pneumonia*, *S. typhimurium* and *S. marcescens* (Table 3.3; Figure 3.1; 3.2; 3.3; 3.4; 3.5; 3.6; 3.7; 3.8; 3.9; 3.10; 3.11; 3.12; 3.13). Antibacterial activity of methanolic extract against *S.epidermidis*, *S. aureus*, *K. pneumonia*, *S. typhimurium* and *S. marcescens* may explain why bittersweet is used in folk medicine to treat skin diseases (caused by *S. epidermidis*, *S. aureus* and *K. pneumonia*), arthritis, rheumatism, backaches and joints pains (caused by *S. aureus*, *K. pneumonia* and *S. typhimurium*), urinary tract infections (caused by *S. epidermidis* and *K. pneumonia*), diarrhea (caused by *S. typhimurium*), eye inflammations (caused by *S. marcescens*, S. epidermidis and *S. typhimurium*) and pneumonia (caused by *S. aureus* and *K. pneumonia*) (Table 3.2; 3.3 and Figure 3.1; 3.2; 3.4; 3.7; 3.8).

Generally antibacterial activity of field-grown leaves and stems was better than *in-vitro* grown leaves and plants against bacteria. Sometimes the level of the detected secondary products in shoot cultures is lower than donor plants (Stafford, 1991). For example, in *Catharanthus roseus* shoot cultures, the dimeric alkaloids have been detected, although at lower levels than those found in the leaves of the plant, while cell suspension cultures contained no trace of these complex structures. Although tropane alkoloids were obtained from multiple shoot cultures of shoot tip, lateral bud or leaf discs of *Atropa belladonna*, detected level was about 7% of donor plant

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

Biological evaluation of extracts showed that they were active against both Gram-positive and Gram-negative bacteria. This may indicate the presence of broad-spectrum antibiotic compounds or simply general metabolic toxins (McCutcheon et al., 1992). Generally the Gram-positive bacteria seem to be more susceptible to the inhibitory effects of the plant 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).

Bacterial growth was generally sensitive to the reference antibiotics tested (Table 3.3). No inhibition was observed with the extraction solvent (water). Methanolic extract was the most effective extract. Antibacterial activity of MeOH extract of field-grown leaves and stems was greater than reference antibiotic ampicillin against *S. epidermidis* (Table 3.3; Figure 3.1; 3.13). Similarly, this extract showed better antibiotic activity than reference antibiotic tetracycline against *S. aureus* (Table 3.3; Figure 3.2; 3.11) and reference antibiotic ampicillin, carbenicillin and erythromycin against *K. pneumonia* (Table 3.3; Figure 3.4; 3.12).

All extracts showed antitumor activity (Table 3.4 and Figure 3.14; 3.15; 3.16). The percentage inhibition was more than 40% in experiments comparing with control (water) (Figure 3.17). No tumor formation was observed with camptothecin (100 % inhibition) (Table 3.4 and Figure 3.14; 3.17). Methanolic extracts showed better antitumor activity than water extracts and field-grown leaves and stems were better than *in vitro*-grown leaves and stems (Table 3.4 and Figure 3.14; 3.15; 3.16).

Although antitumor activity of *S. dulcamara* was mentioned in herbal books, there is just one scientific study on antitumor activity of alcoholic extract of this plant in 1965. According to Kupchan et al. (1965), β-solamarine was the active principal for tumor inhibition against Sarcoma 180 in mice.

The inhibition of *A. 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). Crown gall is a neoplastic plant disease caused by *A. tumefaciens*. The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Coker et al., 2003). It has been shown that the inhibition of crown gall tumor initiation on potato discs and subsequent growth showed good correlation with compounds and extracts active in the 3PS leukemic mouse assay (Galsky et al., 1980). Ferrigini et al. (1982) showed that the potato disc tumor assay was statistically more predictive of 3PS activity than either the 9KB or the 9PS cytotoxicity assays. According to Ferrigini et al. (1982), there is a good agreement between potato disc assay and 3PS assay *in vivo* antileukemic activity in mice and they suggested that the potato disc assay is a safe, simple, rapid, in-house, low cost prescreen for 3PS antitumor activity.

The use of most medicinal plants discovered by traditional societies has not been supported by scientific data. Simple “bench-top” bioassays can provide initial screening data. 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).

Bittersweet has been used as a remedy for skin diseases, arthritis, rheumatism, backaches, joints pains, urinary tract infections, diarrhea, eye inflammations, pneumonia and to inhibit tumor formation (Kupchan et al.,

(1965; Grieve, 1982; Dobelis, 1990; Bown, 1995; Chevallier, 1996). Results obtained herein confirm the antibacterial and antitumor activities of bittersweet extracts. Future studies should focus on fractionation of the extracts in hopes of identifying active components.

Table 3.3 Antibacterial activity of *S. dulcamara* 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.

Treatment	Mean diameter of inhibitory zones (mm ± SE)									
	S.epidermidis	***S.auerus***	***S.pyogenes***	***K.pneumonia***	***E.coli***	***P.aeruginosa***	***S.typhimurium***	***S. marcescens***	***P. vulgaris***	***E. cloacae***
FM	26.7 ± 0.2 b	23.0 ± 1.1 d	7.8 ± 0.2 f	13.8 ± 0.5 c	8.0 ± 0.0 e	7.8 ± 0.5 b	9.5 ± 0.2 d	8.5 ± 0.2 d	8.0 ± 0.0 f	7.0 ± 0.0 c
FW	7.0 ± 0.0 e	8.0 ± 0.0 e	- - - -	8.0 ± 0.0 e	7.5 ± 0.6 f	- - - -	- - - -	7.0 ± 0.0 f	8.0 ± 0.0 f	- - - -
IM	7.0 ± 0.0 e	- - - -	- - - -	9.0 ± 0.0 d	7.4 ± 0.5 f	8.0 ± 0.0 b	7.0 ± 0.0 e	7.0 ± 0.0 f	8.0 ± 0.0 f	- - - -
IW	9.0 ± 0.0 d	8.0 ± 0.0 e	- - - -	8.0 ± 0.0 e	- - - -	8.0 ± 0.0 b	- - - -	8.0 ± 0.0 e	9.0 ± 0.0 e	- - - -
Chloramphenicol (30 µg)	- - - -	26.0 ± 0.0 c	28.0 ± 0.2 d	29.0 ± 0.0 a	25.0 ± 0.0 a	- - - -	28.0 ± 0.0 a	30.0 ± 0.0 a	26.0 ± 0.0 b	- - - -
Tetracycline (30 µg)	- - - -	22.0 ± 0.0 d	25.4 ± 0.1 e	16.0 ± 0.0 b	16.0 ± 0.0 d	- - - -	15.0 ± 0.0 c	15.0 ± 0.0 c	25.0 ± 0.0 c	13.0 ± 0.0 b
Ampicillin (10 µg)	22.0 ± 0.0 c	27.0 ± 0.0 c	32.5 ± 0.1 b	8.0 ± 0.0 e	20.0 ± 0.0 c	- - - -	28.0 ± 0.0 a	- - - -	25.0 ± 0.0 c	13.0 ± 0.0 b
Carbenicillin (100 µg)	31.0 ± 0.0 a	37.0 ± 0.0 a	34.6 ± 0.1 a	8.0 ± 0.0 e	22.0 ± 0.0 b	19.0 ± 0.0 a	26.0 ± 0.0 b	20.0 ± 0.0 b	30.0 ± 0.0 a	24.0 ± 0.0 a
Erythromycin (15 µg)	- - - -	30.0 ± 0.0 b	29.1 ± 0.2 c	8.0 ± 0.0 e	- - - -	- - - -	- - - -	- - - -	15.0 ± 0.0 d	- - - -
Water	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -

FM: MeOH extract of field-grown leaves and stems

IM: MeOH extract of i*n vitro*-grown leaves and stems

FW: Water extract of field-grown leaves and stems

IW: Water extract of *in vitro*-grown leaves and stems

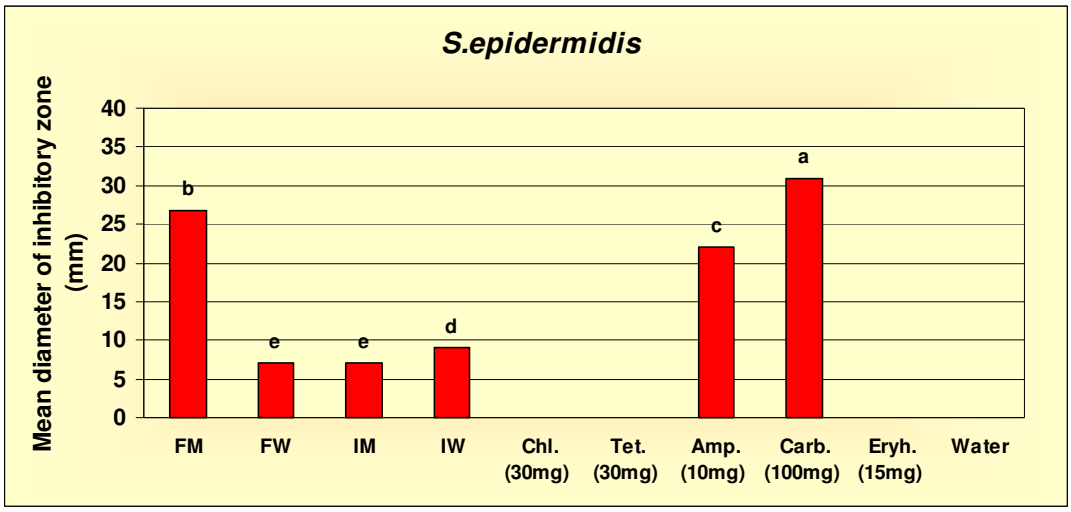

Figure 3.1 Antibacterial activity of *S. dulcamara* extracts and controls against *S. epidermidis*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-grown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

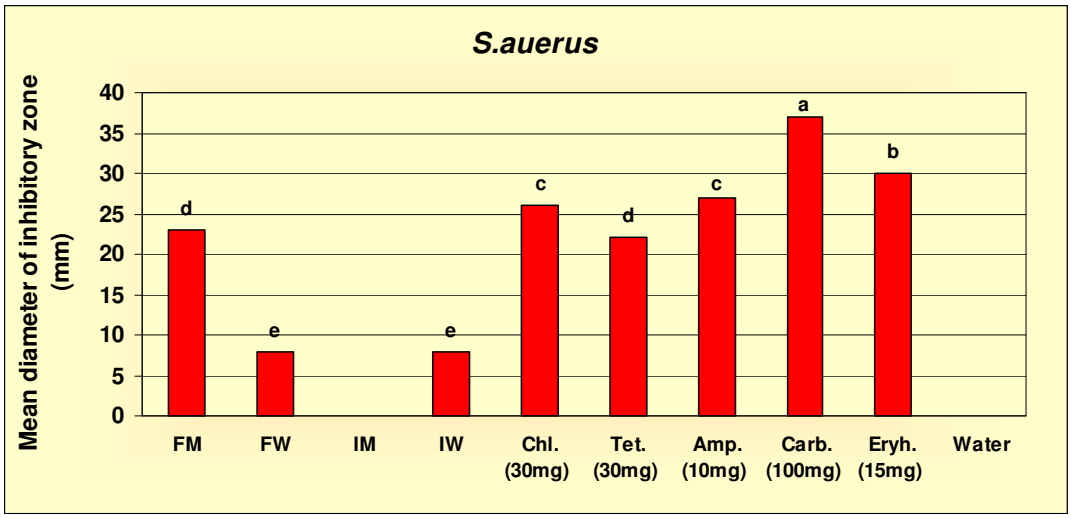

Figure 3.2 Antibacterial activity of *S. dulcamara* extracts and controls against *S. aureus*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

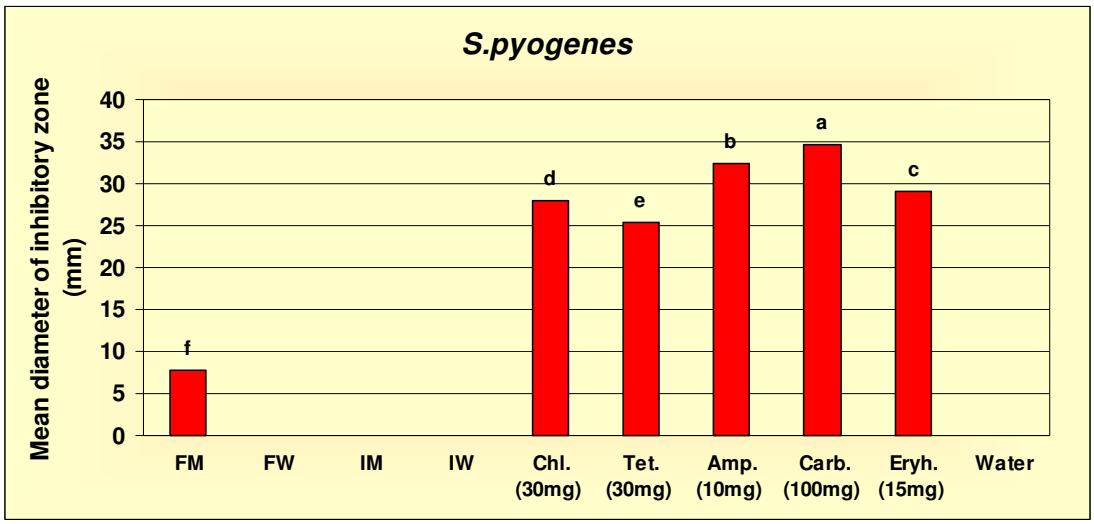

Figure 3.3 Antibacterial activity of *S. dulcamara* extracts and controls against *S. pyogenes*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

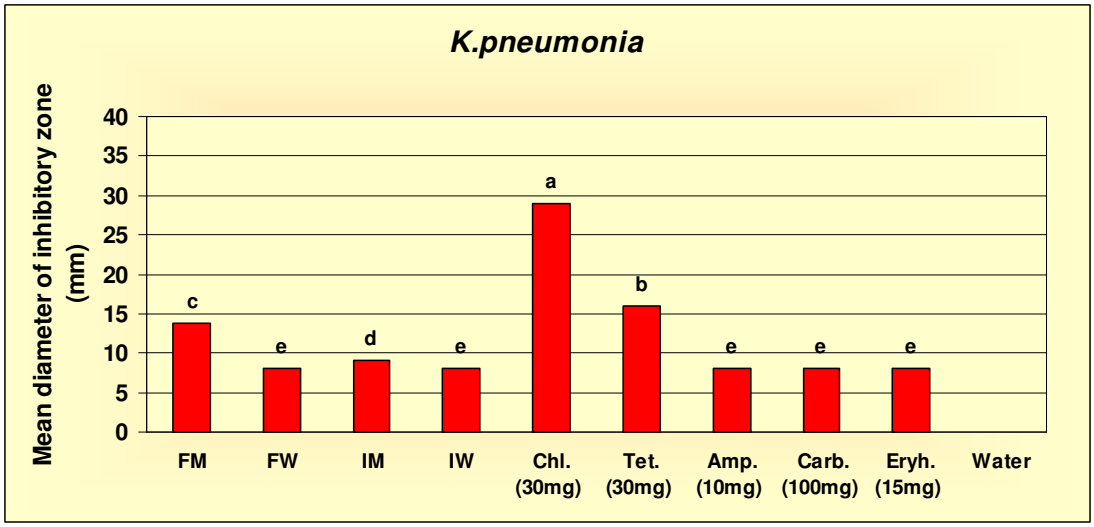

Figure 3.4 Antibacterial activity of *S. dulcamara* extracts and controls against *K. pneumonia*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

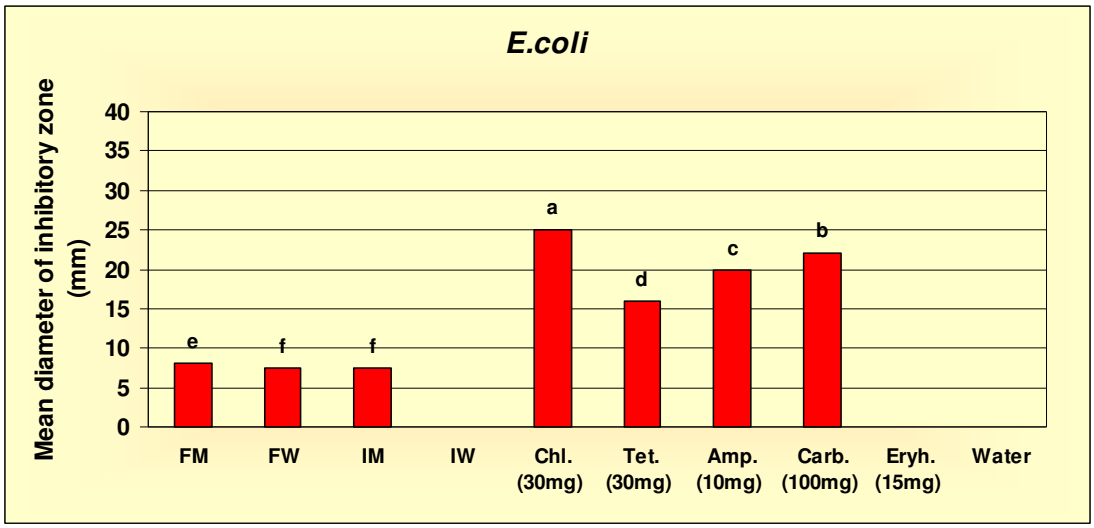

Figure 3.5 Antibacterial activity of *S. dulcamara* extracts and controls against *E. coli*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

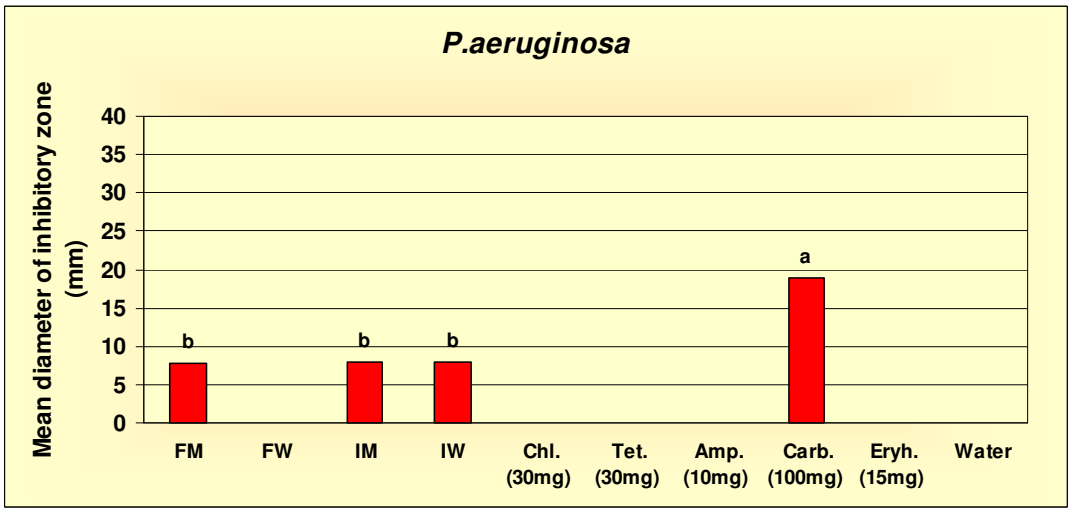

Figure 3.6 Antibacterial activity of *S. dulcamara* extracts and controls against *P. aeruginosa*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

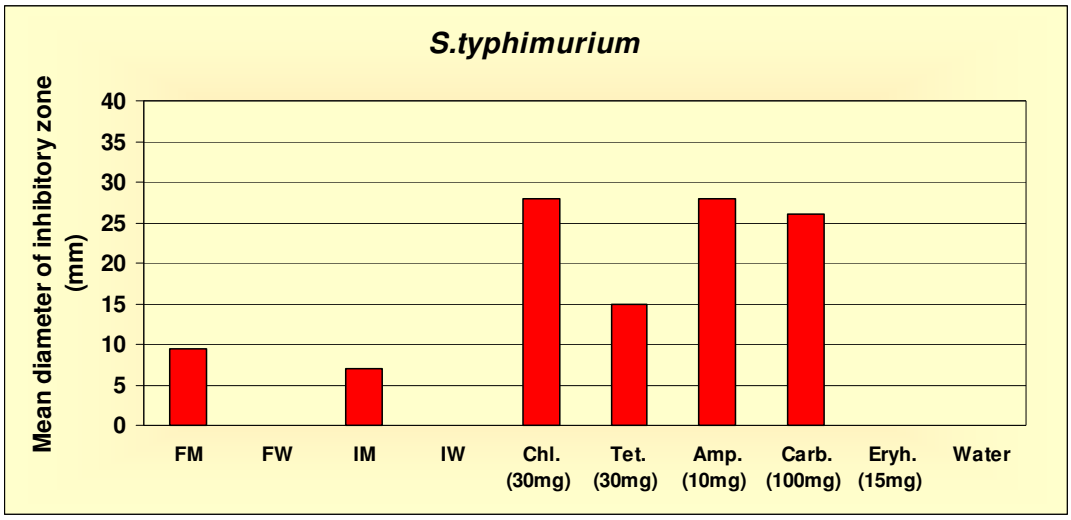

Figure 3.7 Antibacterial activity of *S. dulcamara* extracts and controls against *S. typhimurium*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

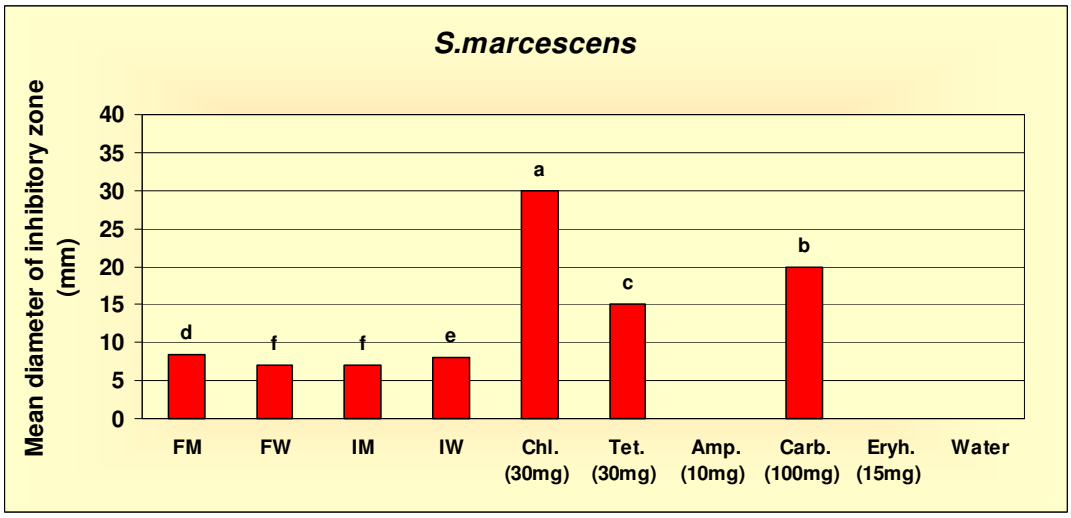

Figure 3.8 Antibacterial activity of *S. dulcamara* extracts and controls against *S. marcescens*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

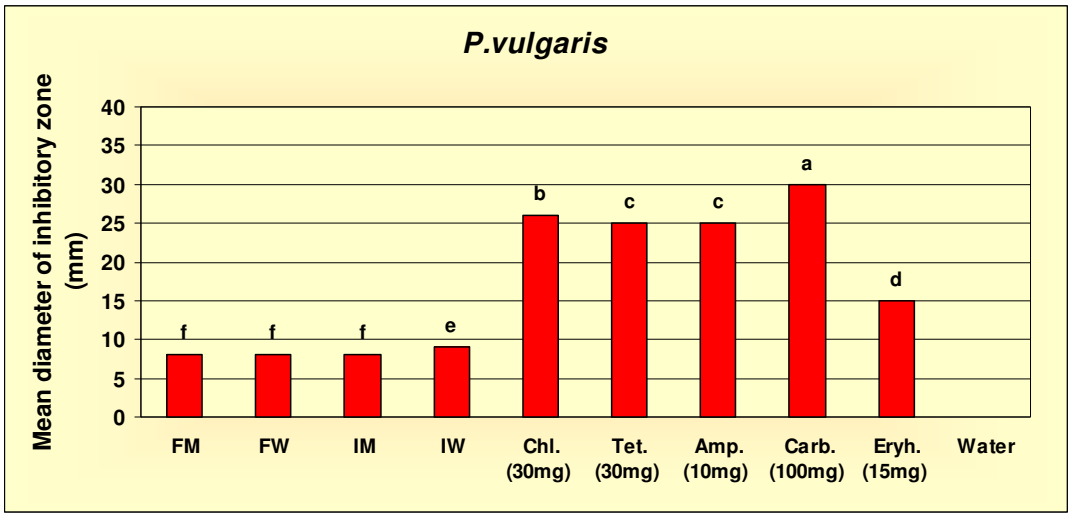

Figure 3.9 Antibacterial activity of *S. dulcamara* extracts and controls against *P. vulgaris*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

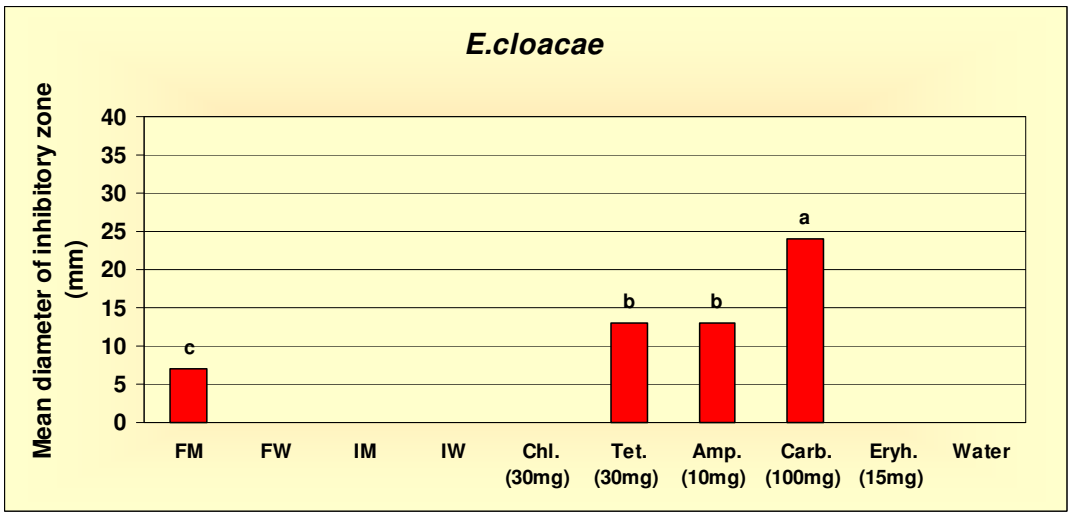

Figure 3.10 Antibacterial activity of *S. dulcamara* extracts and controls against *E. cloacae*. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

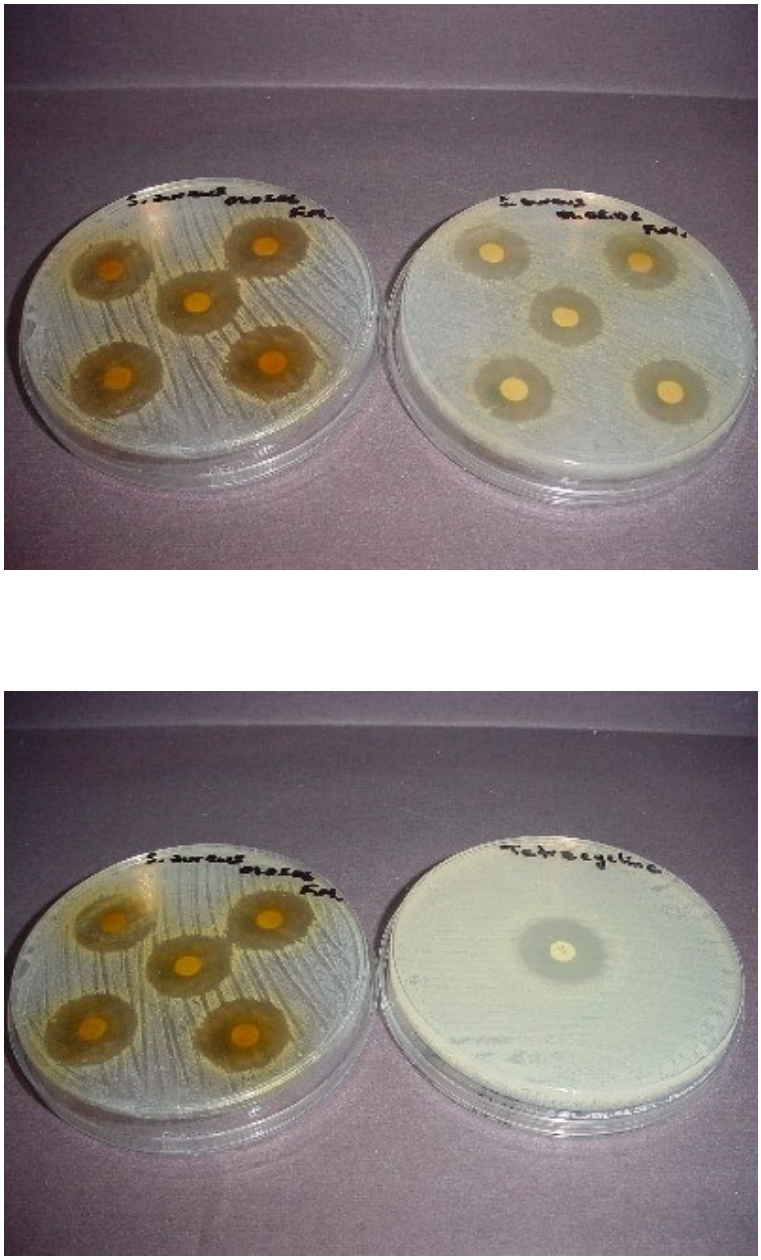

Figure 3.11 Antibacterial activity of FM (MeOH extract of field-grown leaves and stems) against *S. aureus* (above) and comparison with control (below).

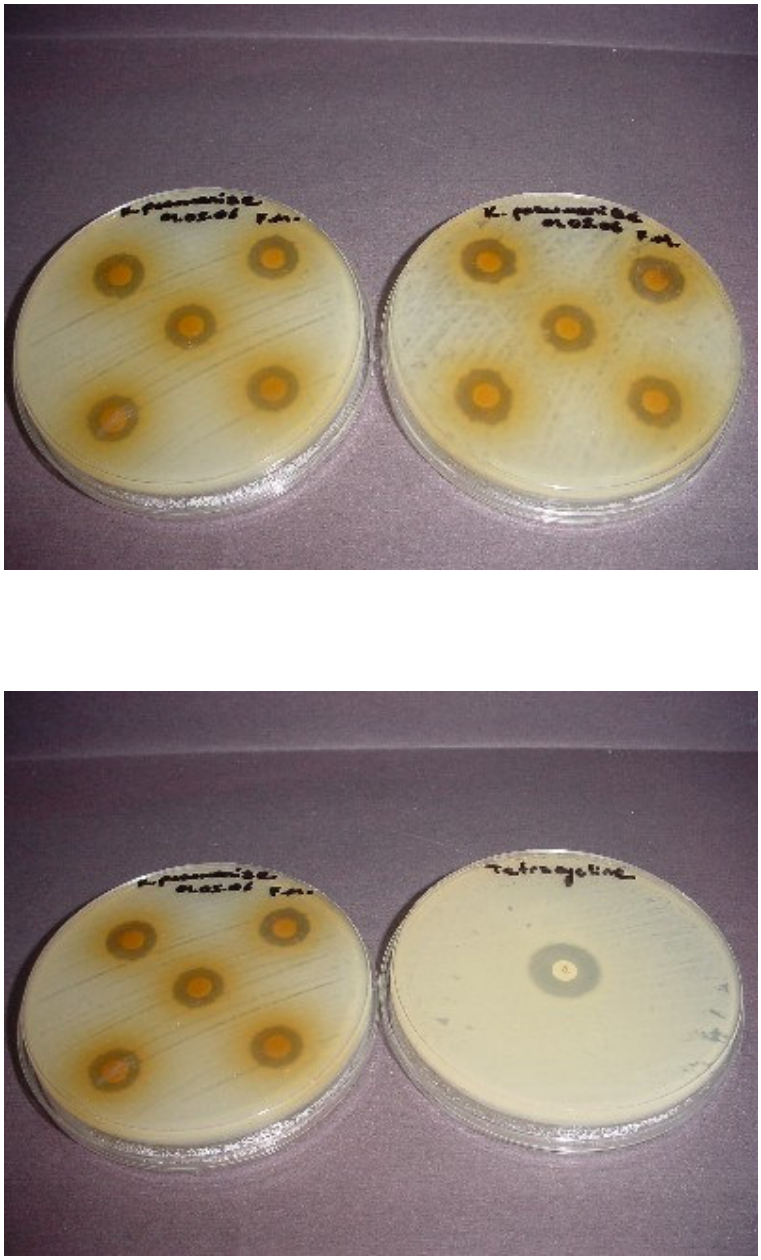

Figure 3.12 Antibacterial activity of FM (MeOH extract of field-grown leaves and stems) against *K. pneumonia* (above) and comparison with control (below).

Figure 3.13 Antibacterial activity of FM (MeOH extract of field-grown leaves and stems) against *S. epidermidis* (above) and comparison with control (below).

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

Treatment	Mean Number of Tumors (±SE)				% Tumor Inhibition
Water	36.42	±	1.93	a	-
Camptothecin	0	±	0		100
FM	3.63	±	0.74	d	88.9
FW	6.75	±	1.01	d	80.6
IM	15.54	±	1.72	c	55.6
IW	21.33	±	1.78	b	41.7

FM: MeOH extract of field-grown leaves and stems
IM: MeOH extract of i*n vitro*-grown leaves and stems
FW: Water extract of field-grown leaves and stems
IW: Water extract of *in vitro*-grown leaves and stems

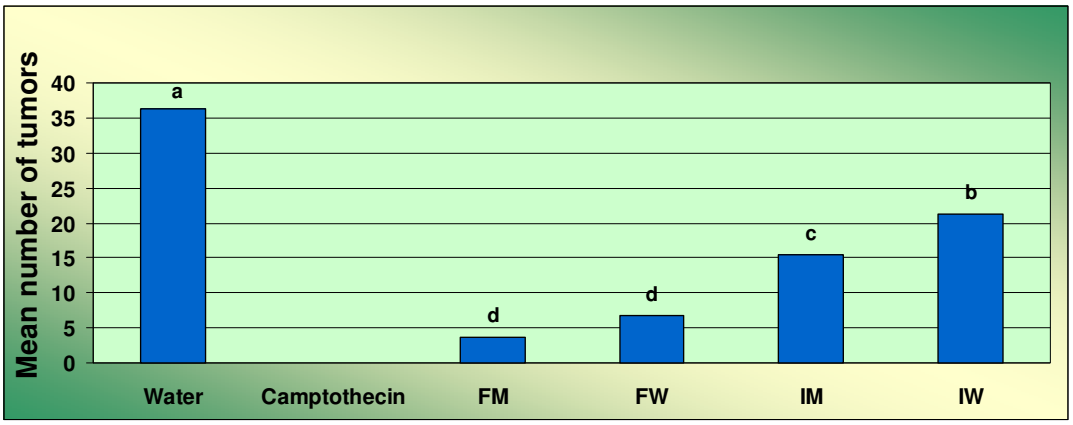

Figure 3.14 Mean number of tumors observed with *S. dulcamara* extracts and controls. FM: MeOH extract of field-grown leaves and stems; IM: MeOH extract of i*n vitro*-grown leaves and stems; FW: Water extract of field-rown leaves and stems;IW: Water extract of *in vitro*-grown leaves and stems.

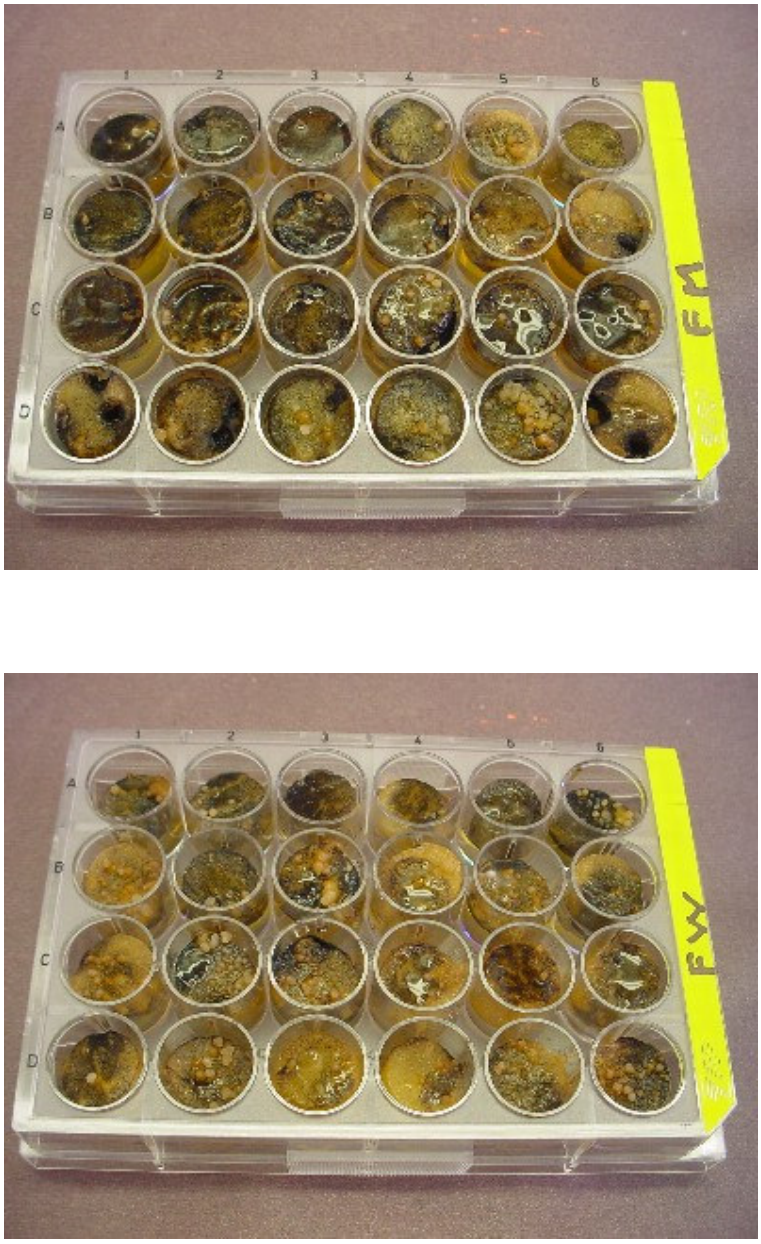

Figure 3.15 Tumors produced by *A. tumefaciens* with FM (above) and FW (below). FM: MeOH extract of field-grown leaves and stems; FW: Water extract of field-rown leaves and stems.

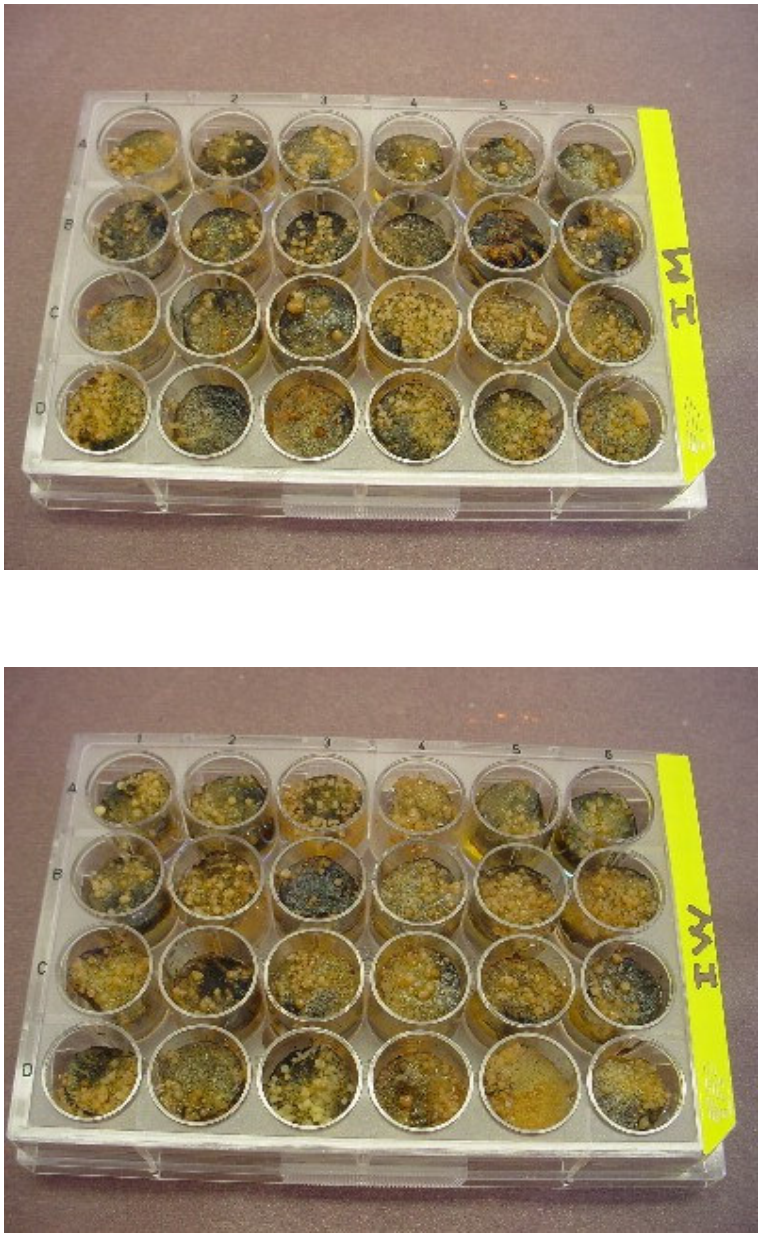

Figure 3.16 Tumors produced by *A. tumefaciens* with IM (above) and IW (below). IM: MeOH extract of i*n vitro*-grown leaves and stems; IW: Water extract of *in vitro*-grown leaves and stems.

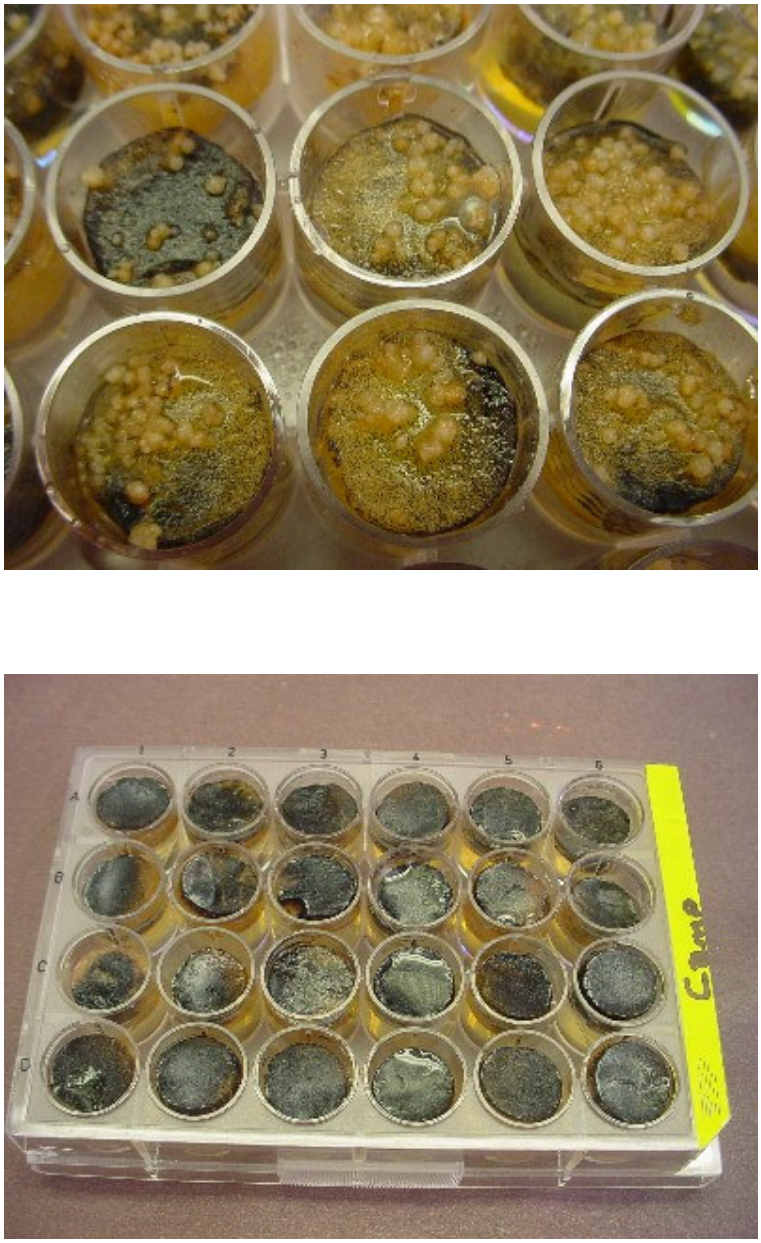

Figure 3.17 Tumors produced by *A. tumefaciens* with distilled water (above) and no tumor formation with camptothecin (below).

CHAPTER 4

Conclusions

Bittersweet (*Solanum dulcamara* L.), a member of the Solanaceae family, is a poisonous plant that has a long history of use in folk medicine in the treatment of skin diseases, warts, tumors, felons, arthritis, rheumatism, bronchial congestion, heart ailments, ulcerative colitis, eye inflammations, jaundice and pneumonia (Grieve, 1982; Dobelis, 1990; Bown, 1995; Chevallier, 1996).

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. Two different explants (leaf and petiole) were used for tissue culture experiments. Best shoot proliferation was obtained with leaf explants. The greatest number of shoots per explant was observed on media with 3 mg/l BA plus 0.5 mg/l IAA with leaf explants. Media containing 3 mg/l Kinetin and 3 mg/l BA were also good for shoot formation with leaf explants. Petiole explants formed fewer shoots than leaf explants. The greatest number of shoots per explant was observed on media containing 3 mg/l Kinetin plus 0.1 mg/l 2,4-D and 3 mg/l Kinetin with petiole explants. There was indirect organogenesis because callus was observed for leaf and petiole explants before shoot formation. Leaf and petiole explants did not form shoots with media containing TDZ in combination with IBA, 2,4-D or NAA hormones, only 0.1

mg/l TDZ formed shoots on leaf and petiole explants. TDZ was not effective in inducing shoot formation.

Regenerated shoots were separated individually and put on rooting medium. They formed roots in 3-4 weeks. IAA and IBA hormones were better than 2,4-D and NAA. The greatest number of roots per explant was observed on media with 0.5 mg/l IBA, 3 mg/l IAA and 1 mg/l IBA. In control experiments (no auxin hormone added to the media) none of the explants formed roots. Rooted plants were transferred to Magenta containers including vermiculate for acclimatization. After 3 weeks, they were transferred to soil.

An *in vitro* culture protocol for this medicinal plant was established which provides plant material for future physiological and biochemical studies. These studies include developing extraction and analytical procedures for the active ingredients.

In recent years, multiple drug resistance has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases. In addition to this, antibiotics are sometimes associated with adverse effects on the host-like hypersentivity. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from other sources, such as plants. Natural products of higher plants may be a new source of antimicrobial agents possibly with novel mechanisms of action.

Antibacterial and antitumor activities of *S. dulcamara* were evaluated and compared by using four different extracts [methanolic extracts of *in vitro*-grown (IM) and field-grown (FM) plant materials and aqueous extract of *in vitro*-grown (IW) and field-grown (FW) plant materials].

The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibiotic activity (Prescott et al., 1990). Ten bacterial strains were used in the bioassay: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, *Enterobacter cloacae* and *Klebsiella pneumoniae* which are gram Gram-negative bacteria and *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis* which are gram-positive bacteria. Methanolic extract of field-grown leaves and stems gave the best inhibitory activity against *S.epidermidis*, *S. aureus*, *K. pneumonia*, *S. typhimurium* and *S. marcescens*. Antibacterial activity of methanolic extract against *S.epidermidis*, *S. aureus*, *K. pneumonia*, *S. typhimurium* and *S. marcescens* may explain why bittersweet is used in folk medicine to treat skin diseases (caused by *S. epidermidis*, *S. aureus* and *K. pneumonia*), arthritis, rheumatism, backaches and joints pains (caused by *S. aureus*, *K. pneumonia* and *S. typhimurium*), urinary tract infections (caused by *S. epidermidis* and *K. pneumonia*), diarrhea (caused by *S. typhimurium*), eye inflammations (caused by *S. marcescens*, S. epidermidis and *S. typhimurium*) and pneumonia (caused by *S. aureus* and *K. pneumonia*).

Generally antibacterial activity of field-grown leaves and stems was better than *in-vitro* grown leaves and plants against bacteria. Biological evaluation of extracts showed that they were active against both Gram-positive and 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). Bacterial growth was generally sensitive to the reference antibiotics tested and no inhibition was observed with the extraction solvent (water).

All four extracts showed antitumor activity. The percentage inhibition was more than 40% in experiments comparing with control (water). No tumor formation was observed with camptothecin (100 % inhibition). Methanolic extracts showed better antitumor activity than water extracts and field-grown leaves and stems were better than *in vitro*-grown leaves and stems.

Tumor inhibition was observed well in ethanol extract of field grown material on potato discs. Development of a simple antitumor prescreen using a convenient and inexpensive plant tumor assay systems can offer numerous advantages as alternatives to extensive animal testing in the search for new anticancer drugs. The potato disc assay demonstrates the inhibition of tumor formation on potato discs; materials that inhibits these plant tumors have a high predictability of showing activity the 3PS (P388) (methylcholanthrene-induced) leukomia mice (Ferrigini et al., 1982). This

bioassay is a sensitive, bench-top antitumor assay for chemicals that disrupt the cell cycle (mitosis, S phase, etc.) (Cooker et al., 2003).

The bioassay results herein confirmed the popular utilization of bittersweet extracts in folk medicine as remedy for skin diseases, arthritis, rheumatism, backaches, joints pains, urinary tract infections, diarrhea, eye inflammations, pneumonia and tumor formation. With these results, *S. dulcamara* L. has some scientific justification as a medicinal herb. In the future more different bioassays related to biological activity of bittersweet can be performed and more scientific rationale for this plant may be obtained.

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