IN VITRO CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *DİGİTALİS TROJANA* I.

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IN VITRO CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *DIGITALIS TROJANA* I.

by

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ABSTRACT

IN VITRO CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITY OF *DIGITALIS TROJANA* IVAN

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The genus *Digitalis* involves poisonous plants that has a long history of use in the treatment of heart diseases including myocardial infarction, edema, angina, cardiac dysfunction, hypertrophy, and arterial hypertension due to their rich cardiac glycosides content. *Digitalis trojana* (Helen of troy foxglove) is a Turkish endemic plant which is found in Kazdağı, Edremit/Balıkesir. In this study, callogenesis potential of *D. trojana* was determined and a protocol for callus induction was established. Collected *D. trojana* seeds were surface sterilized in 40 % commercial bleach with a few drops of tween-20 for 15 minutes using a sonicator, and finally rinsed with sterile distilled water for three times. Surface sterilized seeds of *D. trojana* were germinated on Murashige and Skoog's medim (MS) containing 0.3 mg/l

gibberellic acid (GA_3). Two different explants (leaf lamina and petiole segments) were obtained from 7 weeks-old sterile seedlings and cultured on MS medium containing TDZ and IAA combinations. Best callus formation was obtained with 1.0 mg/l TDZ and 0.5 mg/l IAA (100 % frequency of callusing). Leaf lamina explants were found to be more productive in terms of callus production than petiole explants.

Two different bioassays (antibacterial and antitumor) were performed to show the biological activities of *D. trojana* extracts. They were evaluated between field-grown plant materials (leaf, stem, fruit) and *in vitro*-grown plant materials (calli and *in vitro* seedlings) using ten different extracts [ethanol extracts of *in vitro*-grown (IE1, IE2) and field-grown (FE1, FE2, FE3) plant materials, and aqueous extract of *in vitro*-grown (IW1, IW2) and field-grown (FW1, FW2, FW3) plant materials].

Best antibacterial activity was observed with water (IW1) and ethanol extracts (IE1) of *in vitro* seedlings against *Streptococcus pyogenes* which is a gram-positive bacterium. Antitumor activity was evaluated with potato disc diffusion bioassay**.** All *D. trojana* extracts showed antitumor activity and % tumor inhibition was more than 50 % for all extracts. Water extract of field-grown leaf (FW1) exhibited better % tumor inhibition than other extracts. It was proved that antitumor action of extracts was on the formation of tumors and not on the viability of *Agrobacterium tumefaciens*.

Keywords : *Digitalis trojana*, Helen of troy foxglove, *in vitro* culture, callus, bioassay, antibacterial, antitumor.

ÖZET

DIGITALIS TROJANA IVAN' IN DOKU KÜLTÜRÜ VE BİYOLOJİK AKTİVİTESİNİN DEĞERLENDİRİLMESİ

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Digitalis kalp glikozitleri bakımından zengin olması sebebiyle uzun yıllardan beri miyokard enfarktüsü, ödem, anjin, kalp kasının fonksiyonel bozuklukları, kalp dokusu büyümesi, arteriyal hipertansiyon gibi kalp rahatsızlıklarının tedavisinde kullanılan, zehirli bitkileri içeren bir cinstir. *Digitalis trojana* (troya/truva yüksükotu) Kazdağı, Balıkesir/Edremit 'te bulunan Türkiye'ye endemik bir bitkidir. Bu çalışmada *D.trojana*'nın *kallus* oluşturma potansiyeli saptanmış ve bir *kallus* üretim protokolü geliştirilmiştir. Toplanan *D.trojana* tohumlarının yüzey sterilizasyonu % 40 ticari çamaşır suyu ve birkaç damla tween-20 içeren çözelti içinde 15 dakika süreyle sonikatör kullanılarak yapılmış, son olarak tohumlar steril distile su ile üç kez durulanmıştır. Steril tohumlar 0.3 mg/l giberellik asit (GA_3) içeren Murashige ve

Skoog (MS) ortamında çimlendirilmiştir. Iki farklı eksplant (yaprak ve yaprak sapı parçaları) 7 haftalık steril fidelerden elde edilmiş ve TDZ/IAA kombinasyonlarını içeren MS ortamında *kültür*e alınmıştır. En iyi *kallus* oluşumu 1.0 mg/l TDZ ve 0.5 mg/l IAA içeren ortamda gözlenmiştir (% 100 *kallus* oluşum frekansı). Yaprak eksplantları, yaprak sapı eksplantlarından *kallus* üretimi bakımından daha verimli bulunmuştur.

Troya yüksükotu ekstraktlarının biyolojik aktiviteleri iki farklı *biyolojik test* kullanılarak değerlendirilmiştir (*antibakteriyal ve antitümör*). Araziden toplanmış bitki materyalleri (yaprak, gövde, meyve) ve *in vitro* yetiştirilmiş bitki materyalleri (*kallus, in vitro* fide) arasındaki değerlendirme on farklı ekstrakt [*in vitro* yetiştirilmiş bitki materyallerinin (IE1, IE2) ve araziden toplanmış bitki materyallerinin (FE1, FE2, FE3) etanol ile olan özütleri ve *in vitro* yetiştirilmiş bitki materyallerinin (IW1, IW2) ve araziden toplanmış bitki materyallerinin (FW1, FW2, FW3) su ile hazırlanan özütleri] kullanılarak yapılmıştır.

En iyi antibakteriyel aktivite bir gram-pozitif bakteri olan *Streptococcus pyogenes'e* karşı *in vitro* fidelerin su ve etanol ile hazırlanan özütlerinde gözlenmiştir. Antitümör aktivite patates disk difüzyon testi kullanılarak değerlendirilmiştir. Tüm *D. trojana* özütleri antitümör aktivite göstermiş ve tüm özütler için % tümör inhibisyonu % 50' den fazla olmuştur. Araziden toplanan yaprakların su özütleri (FW1) diğer özütlere göre daha iyi % tumor inhibisyonu göstermiştir. Özütlerin *Agrobacterium tumefaciens*' in tümör oluşumu üzerinde etkili olduğu ve bakterinin yaşayabilirliği üzerinde herhangi bir etkisi olmadığı kanıtlanmıştır.

Anahtar Kelimeler: *Digitalis trojana*, troya yüksükotu, *in vitro* kültür, kallus, biyolojik test, antibakteriyel, antitumor

DEDICATION

I dedicate this dissertation to my mother, Necla Gürel.

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CHAPTER ONE

Introduction and Literature Review

Digitalis is a genus of about 20 species of herbaceous perennials, shrubs, and biennials that are commonly called foxgloves. Other common names for *Digitalis* species include dead man's bells, fairy cap, fairy finger, lady's thimble, lion's mouth and witch's bells (Baytop 1999). Turkey has 5 endemic species of *Digitalis* (Table 1.1) (Davis 1978). The genus was traditionally placed in the figwort family Scrophulariaceae, but upon review of phylogenetic research, it has now been placed in the much enlarged family Plantaginaceae (Olmstead *et al*. 2001). The genus is native to Europe, western and central Asia, and northwestern Africa. The scientific name means "finger-like" and refers to the ease with which a flower of *Digitalis* can be fitted over a human fingertip (Grieve 1982; Chevalier 1996). *Digitalis* flowers are produced on a tall spike, are tubular, and vary in color with species, from purple to pink, white, and yellow. The genus *Digitalis* involves poisonous plants that has been has a long history of use in the treatment of heart diseases including myocardial infarction, edema, angina, cardiac dysfunction, hypertrophy, and arterial hypertension due to their rich cardiac glycosides content (Grieve 1982; Chevalier 1996; Baytop 1999). *Digitalis* species may contain several deadly physiological and chemically related cardiac and steroidal glycosides (Baytop 1999).

Table 1.1 Distribution areas of best known *Digitalis* species.

Species name	Geographical distribution
D. cariensis Boiss.	Turkey
D. ferruginae L. subsp. *ferruginea*. subsp. *schischkinii* (Ivan) Werner	Turkey, Italy, Greece, Australia
D. schischkinii (Ivan.) Werner	Minor Asia, Turkey
D. grandiflora Miller	Europe, Balkan
D. lamarckii Ivan	Turkey
D. lanata Ehrh.	Eastern Europe
D. purpurea L.	Europe
D. davisiana Heywood	Turkey
D. viridiflora Lindley	Balkan
D. trojana Ivan	Turkey
D. ciliate Trautv	Caucasus
D. dubia Rodrig.	Spain
D . laevigata Waldst. & Kit.	Southern Europe
D. leucophaea Sm. subsp. ikarica	Turkey
D. obscura L.	Spain
D. parviflora Jacq.	Northern Spain
D .thapsi L.	Portugal, Spain
D. lutea L.	South Germany

1.1. Botany

Digitalis trojana Ivan is a Turkish endemic plant which is found in Çanakkale, Balıkesir, Kazdağları and Edremit (Figure 1.1) and blossoms from May to June (Davis 1978). Used common name for *D. trojana* is “Helen of Troy Foxglove” (Truva Yüksükotu in Turkish). The natural habitat for *D. trojana* is bushy slopes, limestone cliffs and fields (Davis 1978). This plant is biennial or perennial with few or no sterile basal shoots. It has dark green lanceolate leaves and hairy spikes with cream white flowers with a large lower lip and dark brown spots inside (Davis 1978).

D. trojana has 30-80 cm stem, raceme long, many flowered and moderately dense. Median leaves linear lanceolate, glabrous or rarely sparsely ciliate. Bracts narrowly oblong-lanceolate, acuminate, glandular and ciliate, lower bracts shortly cuspidate and usually longer than lower flowers. Calyx lobes 6-9 mm, linear, very acute, usually cuspidate, densely glandular-pubescent or even shortly villosulous without a margin of furry white hairs. Corolla reddish-brown, (16-) 20-25 mm, tube ventricose, dark veined inside, lower lobe 7-13 mm, oblong, obtuse, whitish with reddish-brown veins. Capsule 13-15 mm long (Davis 1978). The entire plant is poisonous including the roots and seeds (Baytop 1999).

Figure 1.1 Pictures of *D. trojana* (Photographs: İsmail Eker, June 2007).

1.2. Medicinal uses and active constituents

Digitalis has been used from early times in heart cases. It increases the activity of all forms of muscle tissue, but more especially that of the heart and arterioles, the all-important property of the drug being its action on the circulation. The first consequence of its absorption is a contraction of the heart and arteries, causing a very high rise in the blood pressure (Grieve 1982; Chevalier 1996). After the taking of a moderate dose, the pulse is markedly slowed. *Digitalis* also causes an irregular pulse to become regular. Added to the greater force of cardiac contraction is a permanent tonic contraction of the organ, so that its internal capacity is reduced, which is a beneficial effect in cases of cardiac dilatation, and it improves the nutrition of the heart by increasing the amount of blood (Grieve 1982). It has also been employed in the treatment of internal haemorrhage, in inflammatory diseases, in delirium tremens, in epilepsy, in acute mania and various other diseases, with real or supposed benefits (Grieve 1982; Chevalier 1996; Baytop 1999).

The action of *Digitalis* in all the forms in which it is administered should be carefully watched, and when given over a prolonged period it should be employed with caution, as it is liable to accumulate in the system and to manifest its presence all at once by its poisonous action, indicated by the pulse becoming irregular, the blood-pressure low and gastro-intestinal irritation setting in. The constant use of *Digitalis*, also, by increasing the activity of the heart, leads to hypertrophy of that organ. In large doses, the action of *Digitalis* on the circulation will cause various cerebral symptoms,

such as seeing all objects blue, and various other disturbances of the special senses. In cases of poisoning by *Digitalis*, with a very slow and irregular pulse, the administration of atropine is generally all that is necessary. In the more severe cases, with the very rapid heart-beat, the stomach pump must be used, and drugs may be used which depress and diminish the irritability of the heart, such as chloral and chloroform (Grieve 1982; Chevalier 1996; Baytop 1999).

Among all *Digitalis* species, *D. purpurea* is a widely used herbal medicine with a recognized stimulatory effect upon the heart. *D. ferruginea*, *D. grandiflora, D. laevigata*, *D. lanata* and *D. lutea* are also mentioned in the literature for the treatment of cardiac disorders (Grieve 1982; Chevalier 1996; Baytop 1999). All of them have a profound tonic effect upon a diseased heart, enabling the heart to beat more slowly, powerfully and regularly without requiring more oxygen. At the same time it stimulates the flow of urine which lowers the volume of the blood and lessens the load on the heart. The plant contains cardiac glycosides (including digoxin, digitoxin and lanatosides). Digitoxin rapidly strengthens the heartbeat but is excreted very slowly. Digoxin is therefore preferred as a long-term medication (Dobelis 1990; Chevalier 1996; Baytop 1999). A group of pharmacologically active compounds are extracted mostly from the leaves of the second year's growth, and in pure form are referred to by common chemical names such as *digitoxin* or *digoxin*, or by brand names such as *Crystodigin* and *Lanoxin*, respectively. The two drugs differ in that digoxin has an additional hydroxyl group at the C-3 position on the B-ring (adjacent to the pentane). Both

molecules include a lactone and a triple-repeating sugar called a glycoside (Grieve 1982; Dobelis 1990; Chevalier 1996; Baytop 1999).

The leaves are cardiac, diuretic, stimulant and tonic. The leaves should only be harvested from plants in their second year of growth, picked when the flowering spike has grown and about two thirds of the flowers have opened. Harvested at other times, there is less of the medically active alkaloid present. The seed has also been used in the past. The leaves also have a very beneficial effect on the kidneys, they are strongly diuretic and are used with benefit in the treatment of dropsy. Great care should be exercised in the use of this plant, the therapeutic dose is very close to the lethal dose (Grieve 1982; Dobelis 1990; Baytop 1999; Chevalier 1996). Lethal doses of *Digitalis* spp. found in Turkey against cats were given in Table 1.2 (Baytop 1999).

Table 1.2 *Digitalis* species that grown in Turkey and their lethal doses (Baytop 1999).

Species	Lethal doses for cats (g/kg)
D. davisiana	0.99
D.ferruginea	0.053
D.grandiflora	0.033
D.lamarckii	0.025
D.schishkinii	0.072
D.viridiflora	0.087

1.3. Biological activities of *Digitalis* spp.

Cardiac glycosides have played a prominent role in the therapy of congestive heart failure since William Withering codified their use in his late 18th century monograph on the efficacy of the leaves of the common foxglove plant (*Digitalis purpurea*) (Hauptman and Kelly 1999; Hauptman *et al*. 1999).

Cardiac glycosides are a diverse family of naturally derived compounds that bind to and inhibit Na+/K+-ATPase. Members of this family have been in clinical use for many years for the treatment of heart failure and atrial arrhythmia, and the mechanism of their positive inotropic effect is well characterized. Exciting recent findings have suggested additional signaling modes of action of Na+/K+-ATPase, implicating cardiac glycosides in the regulation of several important cellular processes and highlighting potential new therapeutic roles for these compounds in various diseases. Perhaps most notably, the increased susceptibility of cancer cells to these compounds supports their potential use as cancer therapies, and the first generation of glycoside-based anticancer drugs are currently in clinical trials (Prassas and Diamandis 2008).

Haux (1999) reported that digitalis in non-toxic concentrations has been shown to induce apoptosis in different malignant cell lines. Johansson *et al*. (2001) evaluated the saponin digitonin, the aglycone digitoxigenin and five cardiac glycosides for cytotoxicity using primary cultures of tumor cells from patients and a human cell line panel (representing different cytotoxic

drug-resistance patterns). Of these seven compounds, proscillaridin A was the most potent (IC50: 6.4-76 nM), followed by digitoxin, and then ouabain, digoxin, lanatoside C, digitoxigenin and digitonin. Digitoxin and digoxin expressed selective toxicity against solid tumor cells from patients, while proscillaridin A expressed no selective toxicity against either solid or hematological tumor cells. Some forms of digitalis, such as digitoxin, inhibits proliferation and induces apoptosis in cancer cells in clinically relevant concentrations (Haux 2002). Different extracts of *D. purpurea* subsp. *heywoodii* have been assessed for cytotoxic activity against three human cancer cell lines. All of them showed high cytotoxicity, producing IC_{50} values in the 0.78-15 mug/mL range with the methanolic extract being the most active, in non toxic concentrations (Lopez-Lazaro *et al*. 2003). Lopez-Lazaro (2007) showed that the cardiac drug digitoxin might be used in cancer therapy. Because, digitoxin can inhibit the growth and induce apoptosis in cancer cells at concentrations commonly found in the plasma of cardiac patients treated with this drug.

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. Cardiotonic steroids, known ligands of the sodium pump have been widely used for the treatment of heart failure. However, early epidemiological evaluations and subsequent demonstration of anti-cancer activity *in vitro* and *in vivo* have indicated the possibility of developing this class of compound as chemotherapeutic agents in oncology. Their development to date as anti-

cancer agents has however been impaired by a narrow therapeutic margin resulting from their potential to induce cardiovascular side-effects (Mijatovic *et al*. 2007).

There is no much report about antibacterial activities of *Digitalis* spp. Dogruoz *et al*. (2008) reported that ethanol and aqueous extract besides cardiac glycosides of *D. purpurea* did not show antibacterial activity against *E. coli*, *P. aeruginosa*, *B. subtilis*, *K. pneumoniae*, *S. auresu*, *S. epidermidis* and *Aeromonas* spp.

1.4. *In vitro* culture of *Digitalis* spp.

Although there are some reports on the tissue culture of certain *Digitalis* spp, no studies were found on *D. trojana*.

Corduan and Spix (1975) obtained haploid callus and achieved regeneration of plants from anthers of *D. purpurea*. Li (1981) reported the plantlet regeneration from mesophyll protoplasts of *D. lanata*. Brisa and Segura (1987) investigated the isolation, culture and plant regeneration from mesophyll protoplasts of *D. obscura***.** Arrillaga *et al*. (1987) studied somatic embryogenesis and plant regeneration from hypocotyl cultures of *D. obscura*.

Reinbothe *et al*. (1990) regenerated *D. lanata* from suspension cultures via somatic embryogenesis. Cacho *et al*. (1991) investigated the effects of the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) either alone or in combination with kinetin or benzyladenine (BA) to assess the morphogenetic potential of leaf, root and hypocotyl explants of *D. thapsi*. Calli were obtained from all the three explant types in basal medium without the addition of

growth regulators and in leaves, the calli formed roots. Application of 2,4-D, NAA or BA increased callus formation. The presence of NAA induced root formation and that of BA induced shoot formation via callus interphase. IAA alone only induced the generation of roots in the hypocotyl callus. Kinetin was ineffective in all the explants tested. Combinations of NAA with kinetin or BA were more effective in inducing organogenesis in leaf explants. Optimum responses were obtained in hypocotyl and root explants by using IAA in combination with BA, the highest rate of shoot regeneration being observed in hypocotyl explants (Cacho *et al*. 1991).

Onisei *et al*. (1992) reported that stem tips of 3-month-old plants and 0.5-1.0 cm long explants of young spikes (15-months-old) of *D. lanata* and *D. purpurea* were excised and cultured on MS medium supplemented with various growth regulators. Culture response by the 2 species was similar but *D. purpurea* explants exhibited a stronger and faster organogenetic capacity, and survival of regenerated plants exceeded 95%. *D. lanata* and *D. purpurea* regenerated, respectively, 10 and 15 shoots/explant on MS medium containing 2 mg benzyladenine/litre. Rooting capacity of *D. lanata* explants was lower than that of *D. purpurea* and survival following soil acclimatization was only 65-70 %, compared with 95-98 % for *D. purpurea* (Onisei *et al*. 1992).

Yürekli and Baba (1995) studied on an endemic plant *Digitalis cariensis* and produced green callus from *in vitro* seedlings cultured on MS medium containing 3 mg/l BA and 0.4 mg/l NAA. However, regeneration was not achieved.

Bosila *et al*. (2003) investigated that explants (shoot tip, leaf, hypocotyl, and root) of *D. lanata*, which is a major source of digoxin and digitoxin, were cultured on aseptic MS basal solid medium containing 5.0 mg 2.4-D and 0.5 mg benzyladenine/litre. On the other hand, callus derived from different explants was recultured (in initial amounts of 49-63 mg) on the same new sterilized MS medium to increase the mass of callus. The cultures were then maintained in a growth chamber at 26 ℃ ± 2 ℃ and subjected to four different light photoperiods as follows (in hours): 10 light/14 dark, 14 light/10 dark, 16 light/8 dark, and 18 light/6 dark. The greatest callus production and the highest amount of glycosides occurred in callus derived from 2- or 20-week-old leaf explants among all the examined types and ages of explants. Repeating the subculturing three times decreased significantly the dry weight of callus, but it significantly favored digoxin and digitoxin content. The optimal callus growth was obtained at 16 h light per day but the best glycosidal content was achieved when callus was exposed to 18 h light per day (Bosila *et al*. 2003).

CHAPTER TWO

In vitro *culture of* D. trojana

2.1. Introduction

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. *In vitro* micropropagation of medicinal plants provides pesticide or disease 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).

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 component), induction (cells become determined) and differentiation (cells from roots and shoots).

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 organogenic 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). Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant regeneration studies such as, studies in nutrition requirement of plants, cell and organ differentiation and morphogenesis, somaclonal variations and its exploitation, developing cell suspension cultures and protoplasts cultures, genetic transformation using biolistic particle gun technology, production of secondary metabolism and plant material for various bioassay studies (Kayser 2007).

Plant growth regulators in the tissue culture medium can be manipulated to induce callus formation and subsequently changed to induce embryos to form from the callus. Somatic embryogenesis is a process by which somatic (nongametic) cells undergo differentiation to form bipolar structure containing both root and shoot axes. These somatic embryos are similar to zygotic embryos and can mature and germinate (Smith 1992). Asexual embryos develop from small embryogenic cell clusters (proembryos) that are growing in a medium containing the synthetic auxin 2,4-

dichlorophenoxyacetic acid (2,4-D). Embryo development is initiated by removing very large cell clusters and single differentiated cells by filtration from a suspension culture and then transferring the small cell clusters at a low cell density to a medium lacking the 2,4-D. Embryos develop from these clusters and pass successively through globular, heart, and torpedo stages, which are similar to their zygotic counterparts. In the globular stage, the embryo develops radial patterning through a series of cell divisions, with the outer layer of cells differentiating into the 'protoderm.' The globular embryo can be thought of as two layers of inner cells with distinct developmental fates; the apical layer will go on to produce cotyledons and shoot meristem, while the lower layer produces the hypocotyl and root meristem. Bilateral symmetry is apparent from the heart stage; provascular cells will also differentiate at this stage. In the subsequent torpedo and cotyledonary stages of embryogenesis, the embryo completes its growth by elongating and enlarging (Bhojwani and Razdan 1992).

The main objective of this study is to produce callus cultures of *D. trojana* using different plant growth regulator concentrations and combinations.

2.2. Materials and Methods

Seeds of *D. trojana* were collected from different altitudes (309 m and 345 m) of Edremit, Kazdağı, Balıkesir /Turkey in June of 2007. Identification of the species was made by using “Flora of Turkey and the East Aegean

Islands" (Davis 1978) and voucher specimens (IE-1906-1907) were deposited at the Abant Izzet Baysal University Herbarium, Bolu, Turkey.

Surface sterilization of the seeds was achieved by a sonicator emitting millions of shock sound waves through seed surfaces in 40 % Domestos® (containing 5% sodium hypochloride) with a few drops of Tween-20 for 15 minutes. Secondly, as a different method, sterilization was repeated using a magnetic stirrer at 250 rpm for 15 min with same chemical constituents. Surface sterilization was completed by rinsing seeds three times with sterile distilled water.

For germination medium, 4.43 g/l Murashige and Skoog's medim (MS; Duchefa, Netherland; Murashige and Skoog 1962) containing 30 g/l sucrose, 8 g/l Difco Bacto-agar (pH adjusted to 5.7, autoclaved for 20 minutes at 121℃ and 105 kPa) with or without 0.3 mg/l of gibberellic acid (GA_3) was used. Surface sterilized seeds were placed on germination medium for 7-8 weeks. At the end of the germination period, seedlings were kept for either callus induction or as the whole plantlets that would later be used for dried (ground) material for the determination of antibacterial bioassay of *D. trojana*.

For callus induction medium, MS basal medium containing different concentrations of thidiazuron (TDZ; 0.05, 0.1, 0.5 or 1.0 mg/l) and indole-3-acetic acid (IAA; 0.05, 0.1, 0.5 or 1.0 mg/l) supplemented with 3 % sucrose and 0.8 % agar were used. For callusing, leaf–lamina segments (5-6 mm) and petiole segments (5-6 mm) were excised from 7 weeks-old sterile seedlings.

As a different treatment, after callus formation, massive callus produced from lamina explants were transferred to the suspension cultures for embryo induction. Suspension cultures was prepared in 250 ml flask containing 100 ml of MS basal medium, supplemented with 3% sucrose and for embryo induction 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg/l benzyladenine (BA) (pH at 5.7-5.8). Each flask containing 4 calli were placed on an orbital shaker and shaken continuously at 135 rpm. Every 10 days of cultivation period, 50 ml of the medium used for embryo induction was replenished by another fresh medium for 30 days.

All cultures were incubated at 23 ℃ under 16-h photoperiod (cool-white fluorescent lights, 22-28 µmol $m^{-2}s^{-1}$). The explants that produced callus were recorded after 2-3 weeks cultivation. Experiments were repeated thrice. After 4 times subculturing, well-developed calli were dried (at 40 ℃) and powdered for the biological activity studies.

2.3. Results and Discussion

Although genus *Digitalis* contains valuable medicinal plants and active constituents, there is no study on *in vitro* propagation of *D. trojana*. We therefore aimed to develop an efficient protocol for callus and young seedling production in *D. trojana*.

Surface sterilization of the seeds with sonicator was more effective on disturbing the possible contaminations than shaker. The mean percentage of germinated seedlings was 36.64 % in sonicator, it was while 29.17 % in shaker. The effect of GA_3 on germination was better in either sterilization

methods. Germination frequency was 45.20 % with sonicator and 37.80 % with shaker (Table 2.1).

Table 2.1 Different methods for surface sterilization of D*. trojana* seeds and their germination frequencies after 3 weeks.

	STERILIZATION METHODS	
MEDIUM	**SONICATOR**	**SHAKER**
MS Basal Medium	28.08 %	20.54 %
MS + 0.3mg/l GA_3	45.20 %	37.80 %
MEAN	**36.64 %**	**29.17 %**

Combinations of TDZ and IAA were effective for callus formation and leaf lamina explants were found to be more productive in terms of callus production than petiole explants. There was no callus formation on medium containing 0.05 mg/l TDZ in combination with 0.05, 0.1, 0.5 and 1 mg/l IAA for leaf and petiole explants (Table 2.2). Herein, necrosis or tissue browning occurred after 3 weeks cultivation.

The frequency of callus formation was closely related to concentrations of the TDZ (Table 2.2). Increasing concentrations of TDZ (from 0.1 to 1.0 mg/l) caused more callusing. Best callus formation was obtained with 1.0 mg/l TDZ and 0.5 mg/l IAA for leaf lamina explants (100 % frequency of callusing) (Table 2.2; Figure 2.1A). Similarly, petiole explants gave best callusing response (40 % frequency of callusing) with the same combination (Table 2.2 and Figure 2.1B). Green friable callus was observed with TDZ and IAA combinations. Having more venation on leaf lamina explant may promote the callusing due to the abundance of endogenous hormones which are effective in dedifferentiation of excised tissues. There was no regeneration after callus formation.

Perez-Bermudez *et al*. (1984) investigated the callusing response of *D. obscura*, a Spanish endemic plant. They reported that the synthetic auxins 2,4-D or NAA (both at concentrations equal or greater than 0.5 mg/l) promoted callus formation. The IAA by itself produced insignificant results, however, with the inclusion of BA callus induction was observed. They

pointed that BA showed a clear synergism with the auxins, not only in the callus induction but also in their growth.

Higher IAA concentrations produced less callus production in lamina explants and in some cases explants promoted root differentiation (rhizogenesis) from lamina segments in medium containing 0.05 mg/l TDZ in combination with 0.5 or 1.0 mg/l IAA (Figure 2.1C and D). Similarly, Perez-Bermudez *et al*. (1984) reported that root differentiation was promoted from leaf segments of *D. obscura* (rhizogenesis) with the optimal concentration being 1 mg/l IAA.

Massive calli which were effectively produced from lamina explants were transferred to the suspension cultures for the assessment of the embryogenesis (Figure 2.2A). After 30 days of the cultivation, embryogenic callus did not produce whole seedling on germination medium (Figure 2.2B and C), although globular and heart shape stages of the embryogenesis were observed (Figure 2.D).

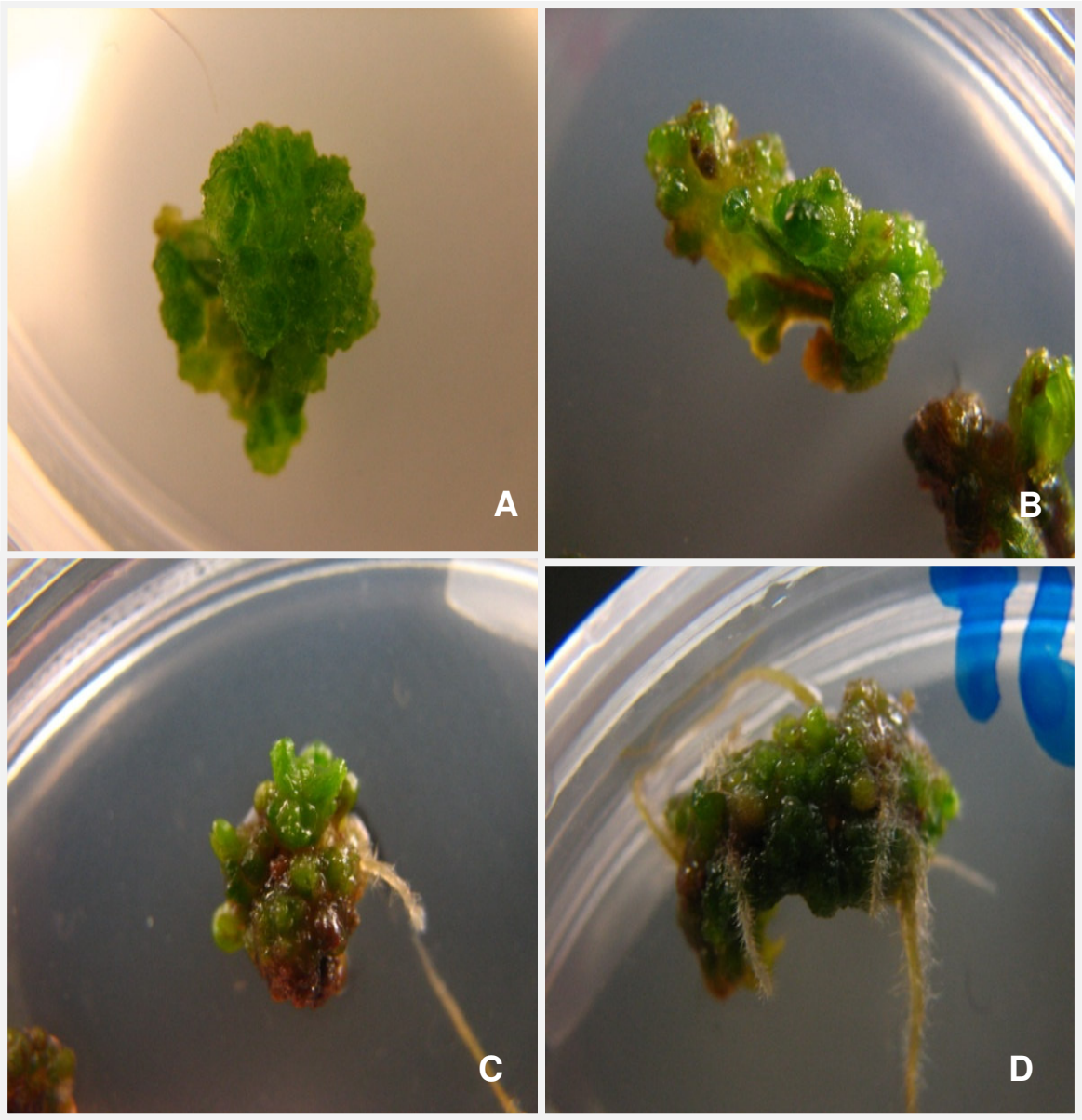


Figure 2.1 Callus formation from leaf lamina explants (A), petiole explants (B), root formation at higher IAA/TDZ ratio on petiole explants (C) and lamina explants (D).

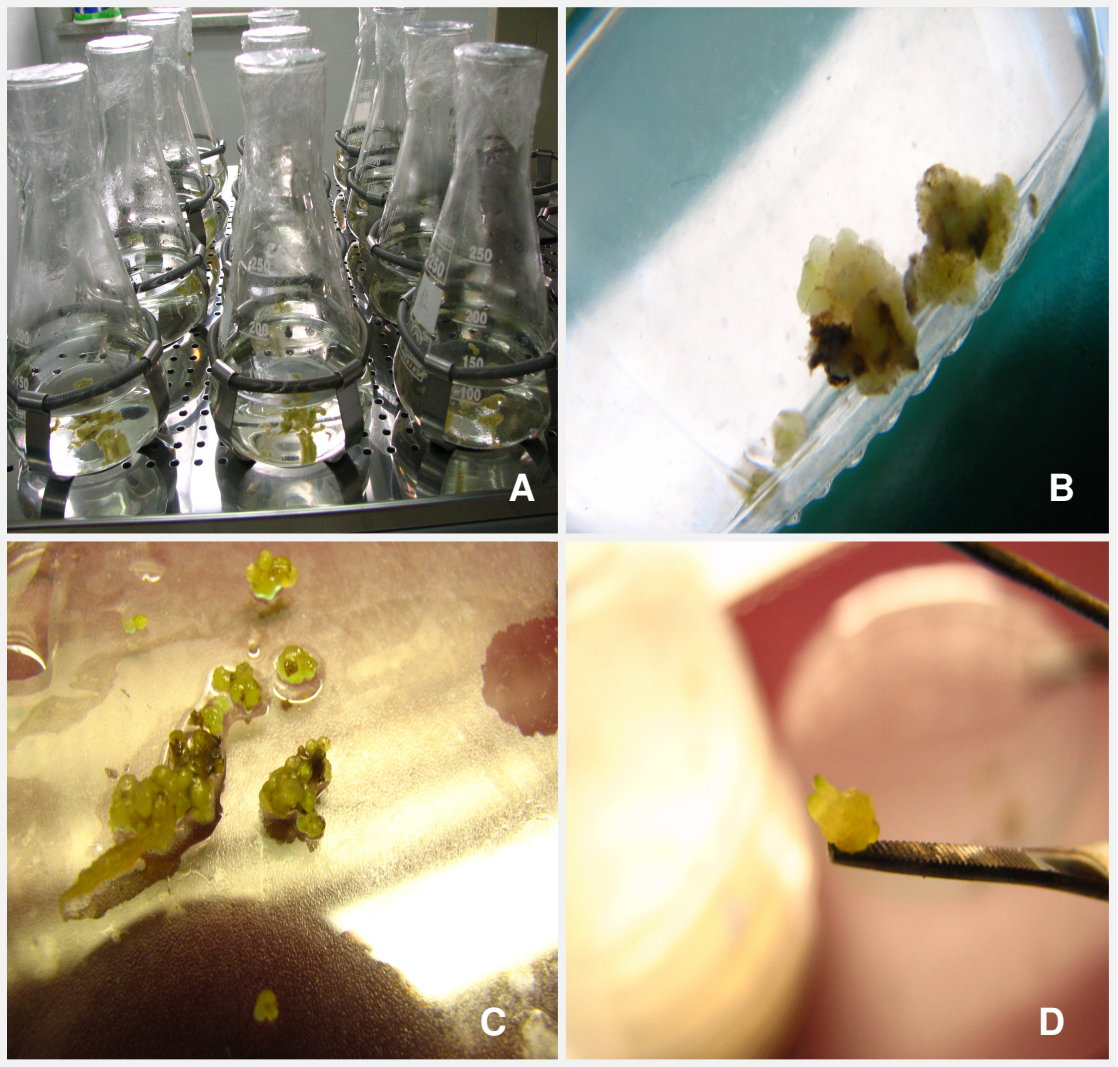


Figure 2.2 Suspension culture of *D. trojana* callus. (A) Flask containing embryo induction medium in shaker at 135 rpm, (B) globular callus initiation, (C) transferring of globular callus to the germination medium, (D) heart shaped embryonic callus.

Table 2.2 Percentage of callusing (A) and callus response of lamina and petiole explants (B) excised from 7-8 weeks old seedlings at different concentrations of TDZ and IAA ("N" represents necrosis, "R" rhizogenesis, "+"minimal, "++" intermediate, "+++" massive callus growth).

		TDZ (mg/l)															
		0.05				**0.1**				**0.5**				**1.0**			
		Leaf		**Petiole**		**Leaf**		**Petiole**		**Leaf**		**Petiole**		**Leaf**		**Petiole**	
		A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
IAA(mg/l)	**0.05**	0	N	0	N	15%	+	0	N	55%	+	0	N	60%	+	10%	+
	0.10	0	N	0	N	45%	+	35%	+	90%	+++	40%	+	95%	+++	40%	+
	0.50	0	R	0	N	N	N	0	N	75%	++	35%	+	100%	+++	40%	++
	1.0	0	R	0	N	N	N	0	N	45%	+	10%	+	100%	++	0	N

CHAPTER THREE

Biological activities of *D. trojana*

3.1. Introduction

Throughout the history plants have been the most important source of medicines for human health (Balandrin *et al*. 1993). World Health Organization estimates that up to 80 % of the world's people rely on plants for their primary health care (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).

Diffusion method of antimicrobial susceptibility test was developed in 1940s. In 1940, Heatley introduced the use of adsorbent paper for carrying antimicrobial solutions. 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 Kirby-Bauer Disc Diffusion Assay measures antimicrobial activity based on bacteriostatic / bacteriosidal properties. It was redeveloped 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, and then incubated at 37 ℃ for 18-24 hours. At the end of the incubation period, plates are observed and clear zones around the discs 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 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 3.1.

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). The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Coker *et al*. 2003).

Table 3.1 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.

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 (P388) (*in vivo* murine leukemia) leukemic mouse assay (Galsky *et al*. 1980; Coker *et al.* 2003). Ferrigini *et al*. 1982 showed that crown gall tumors on potato discs could routinely be employed as comparatively rapid, inexpensive, safe, and statistically reliable prescreen for 3PS antitumor activity.

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 *A. tumefaciens* as the tumor initiator. *A. tumefaciens* is the causative agent of 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. Tumorigenesis in plants and animals involve similar mechanisms and common nucleic acid components (Agrios 1997). Ferrigini *et al*. (1982) established anti-tumor activity for several plants extracts, including an extract from periwinkle (*Vinca major*) called vincristine. Vincristine is a Federal Drug Administration (FDA) approved drug currently used for pediatric leukemia. 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 were 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 and Camper 2003; Coker *et al*. 2003).

There are no reports about antibacterial and antitumor activities of *D. trojana* in the literature. The main objective of this study is to evaluate and compare antibacterial and antitumor activities of *in vitro* grown and field grown plant materials of *D. trojana*.

3.2. Materials and Methods

3.2.1. Plant material and extraction

Leaves, fruits and stems (field-grown plant materials) of *D. trojana* were collected from Kazdağı, Edremit/Balıkesir in June of 2007. Identification of the species was made by using "Flora of Turkey the East Aegean Islands" (Davis 1978). Calli and *in vitro* seedlings (*in vitro*-grown plant materials) of *D. trojana* were previously micropropagated in our laboratory (Figure 3.1A and B). *In vitro* seedlings were obtained with germination of *D. trojana* seeds on MS + 0.3mg/l GA_3 (Figure 3.1A) and used calli were obtained from leaf explants cultured on media containing 1.0 mg/l TDZ and 0.5 mg/l IAA (Figure 3.1B). All plant materials (field-grown and *in vitro*-grown) were oven dried at 40°C and extracted with water and ethanol (EtOH). Plant extracts were prepared using *in vitro*-grown (*in vitro* seedlings and calli) or field-grown (leaf, fruit or stem) plant materials (Table 3.2).

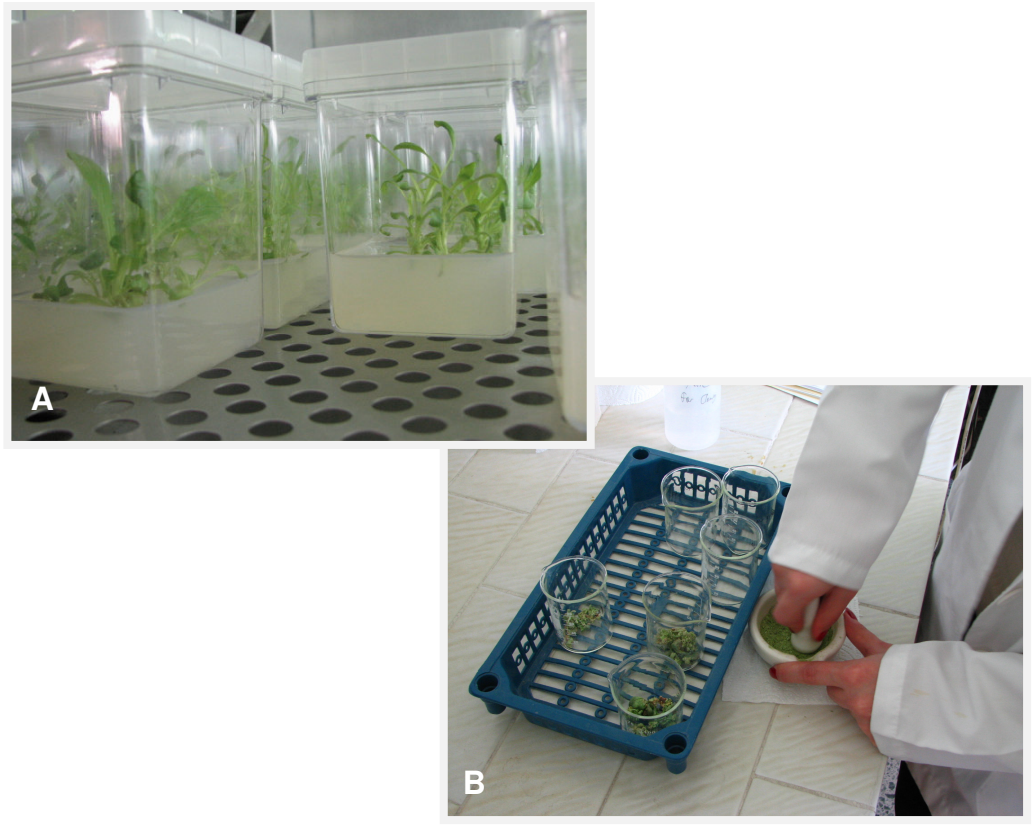


Figure 3.1 Eight weeks old *in vitro* seedlings of *D. trojana* (A) and dried calli while being powdered (B).

Table 3. 2 Plant materials, designation of treatments and yield (%) for each extraction.

	Solvents	Designations	Parts Used	Yield (%)
Field-grown plant materials	Water	FW1	leaf	23
	Water	FW2	fruit	6.25
	Water	FW3	stem	16.5
	EtOH	FE1	leaf	21.75
	EtOH	FE2	fruit	5.5
	EtOH	FE3	stem	8
***In vitro*-grown plant materials**	Water	IW1	seedling	10.5
	Water	IW2	callus	28
	EtOH	IE1	seedling	6.75
	EtOH	IE2	callus	16

For aqueous extraction, four grams of plant materials were extracted with 200 ml of water in hot waterbath at 45 ℃ for 12 hr and then filtered. Water was evaporated using lyophilizator. For ethanolic extraction, four grams of plant materials were extracted with 200 ml of pure ethanol in hot water bath at 45 ℃ for 12 hr. Ethanol was evaporated under vacuum using rotary evaporator at 60 ℃ and then filtered. Each residue was dissolved in sterile distilled water in order to obtain a final concentration of 100 mg/ml for antibacterial bioassay and 10 mg/ml for antitumor bioassay. Plant materials, designation of treatments and yield (%) for each extraction were summarized in Table 3.2.

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), *Solmanella typhimurum* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883) which are gram-negative bacteria and S*treptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are gram-positive bacteria.

BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes including 2 ml of tryptic soy broth (TSB). They were incubated for 3 hours at 37 ℃. After incubating period, one bacteriological loop from each broth was streaked on

tryptic soy agar (TSA) plates and incubated for 2 days at 37 ℃. After 2 days, single colony was removed and streaked on a new TSA plate and incubated at 37 ℃ for 2 additional days. After this duration, 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 ℃, and then 0.2 ml from each culture was transferred into 0.8 ml TSB in eppendorf tubes. 0.1 ml from each strand was transferred into Mueller-Hinton (M-H) agar plates. The plates were inoculated with microorganism suspension at density of 10^6 cells/ml. A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly streak the entire surface a Mueller-Hinton agar plate (60x15 mm).

All extracts were sterilized by filtering through a 0.22 µm filter (Pal-Gelman Laboratory) and sterile paper discs (glass microfibre filters, Whatman; 6 mm in diameter) were impregnated with 13 µl of extract. Positive controls consisted of five different antimicrobial susceptibility test discs (Bioanalyse®): Erythomycin (15 µg) (E-15), Ampicillin (10 µg) (AM -10), Carbenicillin (100 µg) (CB-100), Tetracycline (30µg) (TE-30) and Chloramphenicol (30µg) (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 ℃ incubator. After 24 hours of incubation, inhibition zone diameter (mm) was measured. All experiments were repeated three times.

The results obtained from antibacterial bioassays 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 vers. 15 (SPSS Inc., Chicago, IL, USA).

3.2.3 Anti-tumor bioassay

Antitumor activity of all extracts was assessed with the potato disc method as modified by McLaughlin's group (Ferrigini *et al*. 1982). *Agrobacterium tumefaciens* (strain B6) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28°C. Camptothecin (Sigma) (tumor suppressant) served as a positive control. Water was used as a negative control. Eight loops of *A. tumefaciens* were added to 10 ml Phosphate Buffered Saline (PBS) (pH=7.2). Bacteria concentrations were adjusted to absorbance (600 nm) values of 0.96 ± 0.02 which equaled to 1.0 X 10^9 bacteria.

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

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

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

Solvent control II: 600 μl control ((water or EtOH) + 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.

White potatoes (*Solanum tuberosum* L.) were washed and 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 surface area available; tubers were immersed in 20 % Domestos® for 15 min and then placed on sterile paper towel. They were cut into cylinders using a cork borer and placed in sterile

distilled water with lactic acid (pH=4.0). Cylinders (10 mm diameter) were cut from the center of potato tissue (skin portion was eliminated). Cylinders were rinsed twice more using sterile distilled water with lactic acid. 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 (15 g/L). 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 (I_2KI; 5% I_2 plus 10% KI in distilled water) and tumors on each disc were counted. Lugol's reagent stains the starch in potato tissue to dark blue to dark brown color, but the tumors do not take up the stain and appear creamy to orange. Experiments were repeated three times. Percent inhibition of tumors was calculated (McLaughlin 1991; McLaughlin *et al.* 1993; McLaughlin and Rogers 1998) using the formula % inhibition= [(solvent control mean - tested extract mean) / solvent control mean] X 100.

3.2.4. Bacterial viability testing

A. tumefaciens (strain B6) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28 °C. Six to seven loops of *A. tumefaciens* were added to 10 ml phosphate buffered saline (PBS). Bacterial concentration was adjusted to absorbance (600 nm) values of 0.96 ± 0.02, which equaled to 1.0 X 10^9 colony forming units (CFU) (standardized value). This suspension was serially diluted with PBS to 1 X 10^3 cells/ml (CFU). 1 ml of extracts having anti-tumor activity was mixed with 1ml of *A. tumefaciens* culture diluted to 1 X 10^3 cells/ml and left for 30 min. 30-min exposure was chosen as attachment

of bacterial cells to the plant tissue, the initial step in crown gall formation occurs within 15 min of inoculation (Lippincott and Lippincott 1969; Glogowski and Galsky 1978). After 30 min, 0.1 ml of inoculum (bacteria + extract) was inoculated on YEM media with spread plate technique and colony counts were made after 24 hours.

3.3. Results and Discussion

Ten different extracts [EtOH extract of field-grown fruits, leaves and stems (FE1, FE2, FE3), EtOH extract of *in vitro*-grown seedling and callus, (IE1,IE2), water extract of field-grown fruits, leaves and stems (FW1, FW2, FW3) and water extract of *in vitro* cultured seedlings and callus (IW1, IW2)] were used to evaluate the antibacterial and antitumor activities of *D. trojana* (Table 3.3; Table 3.4).

Among studied plant materials of *D. trojana*, best antibacterial activity was observed with *in vitro*-grown seedlings. Both aqueous and ethanol extracts of *in vitro*-grown seedlings gave the best inhibitory activity against *S. pyogenes* (19.25 mm for aqueous extract of seedlings and 17.00 mm for ethanol extract of seedlings). Also, *S. typhimurium* showed weak sensitivity with these extracts (Table 3.3; Figure 3.2; Figure 3.3).

In our study, Gram-negative bacteria were more susceptible to plant extracts than Gram-positive bacteria (Table 3.3). However, 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 their reference antibiotics tested (Table 3.3). No inhibition was observed with the extraction solvent (water).

Aqueous and ethanol extracts of field-grown leaf and stem showed antibacterial activity against *E. coli*. But, none of the extracts of field-grown fruit showed antibacterial activity against all used bacteria. *S. typhimurium, P. vulgaris, K. pneumonia and E. coli* were susceptible to ethanolic extracts of field-grown leaf. Although aqueous and ethanol extracts of *in vitro*-grown seedling showed antibacterial activity against *S. pyogenes* and *S. typhimurium*, both extracts of *in vitro*-grown callus did not exhibit antibacterial activity against all used bacteria (Table 3.3; Figure 3.2).

Dogruoz *et al*. 2008 reported that aqueous and ethanol extracts of *D. purpurea* did not show activity against *E. coli*, *P. aeruginosa*, *B. subtilis*, *K. pneumoniae*, *S. auresu*, *S. epidermidis* and *Aeromonas* spp. Also, cardiac glycosides obtained from *D. purpurea* did not exhibit any activity against these bacteria.

Although *Digitalis* spp. have been used for the treatment of cardiac problems for centuries, anticancer activities of them have been reported by some studies recently (Haux 1999; Johansson *et al*. 2001; Haux 2002; Lopez-Lazaro *et al*. 2003: Lopez-Lazaro 2007, Mijatovic *et al*. 2007; Prassas and Diamandis 2008). Similarly, in our study antitumor activity was observed with all extracts of *D. trojana* (Table 3.4; Figure 3.4). Tumor inhibition percentages were more than 50 %. Water extract of field-grown leaf exhibited better % tumor inhibition than other extracts (Table 3.4).

No tumor formation was observed with positive control camptothecin (100% inhibition) (Table 3.4; Figure 3.4). Final concentrations of all extracts were adjusted with distilled water. Therefore, water was used in solvent control I. There were many tumor formation with solvent control I (water) and no tumor formation with solvent control II (PBS without *A. tumefaciens*) (Figure 3.4).

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 extracts having best tumor inhibition with 1 X 10^3 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension. All extracts did not affect the viability of the bacterium (Figure 3.5). Thus the action of extracts was on the formation of tumors and not on the viability of bacterium.

The initial step in the formation of *A. tumefaciens*-induced tumors involves attachment of the bacterium to a tumor-binding site (Glogowski and Galsky 1978). The attachment of the bacterium to the tissue is complete within 15 min following inoculation (Coker *et al*. 2003). Galsky *et al*. (1980) examined the effects of several compounds and plant extracts on crown gall tumor formation and found no effects on bacterial viability or on the attachment process.

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 antitumour 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 tumour initiation on potato discs and subsequent growth showed good correlation with compounds and extracts active in 3PS leukemic mouse assay (Galsky *et al.* 1980).

Future studies should focus on fractionation of the extracts of *D. trojana* in hopes of identifying active components.

Table 3.3 Antibacterial activity of *D. trojana* 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*
FW1	- -	- -	- -	- -	- -	- -	- -	- -	- -	9.00 ± 1.15 c
FE1	- -	- -	- -	- -	7.25 ± 0.25 d	- -	8.75 ± 0.47 c	8.25 ± 0.50 b	- -	8.00 ± 0.57 c
FW2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
FE2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
FW3	- -	- -	- -	- -	- -	- -	- -	- -	- -	8.50 ± 0.28 c
FE3	- -	- -	- -	7.50 ± 0.28 d	- -	- -	- -	- -	- -	8.00 ± 0.00 c
IW1	- -	- -	19.25 ± 0.47 f	- -	7.50 ± 0.28 d	- -	- -	- -	- -	- -
IE1	- -	- -	17.00 ± 2.34 f	- -	8.00 ± 0.57 d	- -	- -	- -	- -	- -
IW2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
IE2	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ampicillin	42.00 ± 0.57 a	23.00 ± 1.54 b	53.50 ± 2.02 a	18.00 ± 1.15 c	28.00 ± 1.15 a	- -	27.50 ± 1.44 b	10.50 ± 0.28 b	27.50 ± 0.86 c	22.50 ± 0.86 b
Carbenicillin	40.00 ± 0.00 a	30.00 ± 0.00 a	46.00 ± 0.00 b	28.00 ± 0.00 a	29.00 ± 0.00 a	22.00 ± 0.00 a	33.00 ± 0.00 a		34.00 ± 0.00 a	29.00 ± 0.00 a
Chloramphenicol	28.50 ± 0.28 c	30.00 ± 0.57 a	33.00 ± 0.00 e	29.50 ± 1.44 a	26.50 ± 0.86 b	10.00 ± 0.00 b	24.50 ± 0.28 b	26.50 ± 0.28 a	29.00 ± 0.00 b	29.50 ± 0.28 a
Erythromycin	26.00 ± 0.00 c	31.00 ± 0.57 a	38.00 ± 1.15 d	8.50 ± 0.86 d	10.50 ± 0.28 c	8.00 ± 0.57 b	11.00 ± 1.15 c	11.50 ± 0.28 b	9.00 ± 1.15 d	9.00 ± 0.00 c
Tetracycline	35.00 ± 0.57 b	9.50 ± 0.28 c	42.00 ± 1.15 c	27.00 ± 0.57 b	28.00 ± 1.15 a	21.00 ± 0.57 a	34.50 ± 0.86 a	27.50 ± 0.28 a	28.50 ± 0.86 b	28.50 ± 0.86 a
Water	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

FW1: water extract of field-grown leaf

FE1: ethanol extract of field-grown leaf

FW2: water extract of field-grown fruit

FE2: ethanol extract of field-grown fruit

FW3: water extract of field-grown stem

FE3: ethanol extract of field-grown stem

IW1: water extract of in-vitro grown seedling

IE1: ethanol extract of in-vitro grown seedling

IW2: water extract of callus

IE2: ethanol extract of callus

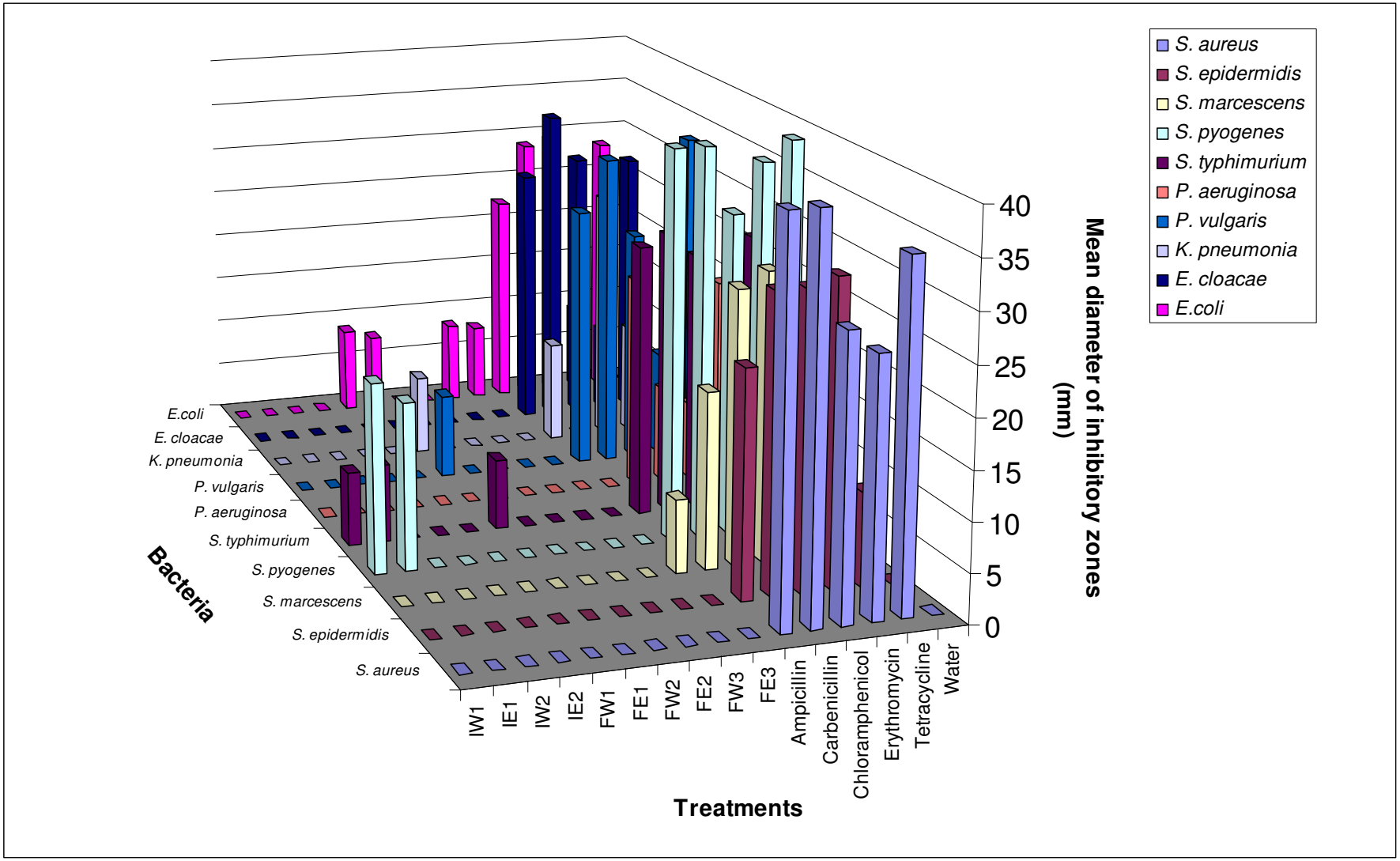


Figure 3.2 Summary of the antibacterial activity of *D. trojana* extracts and controls against used bacteria.

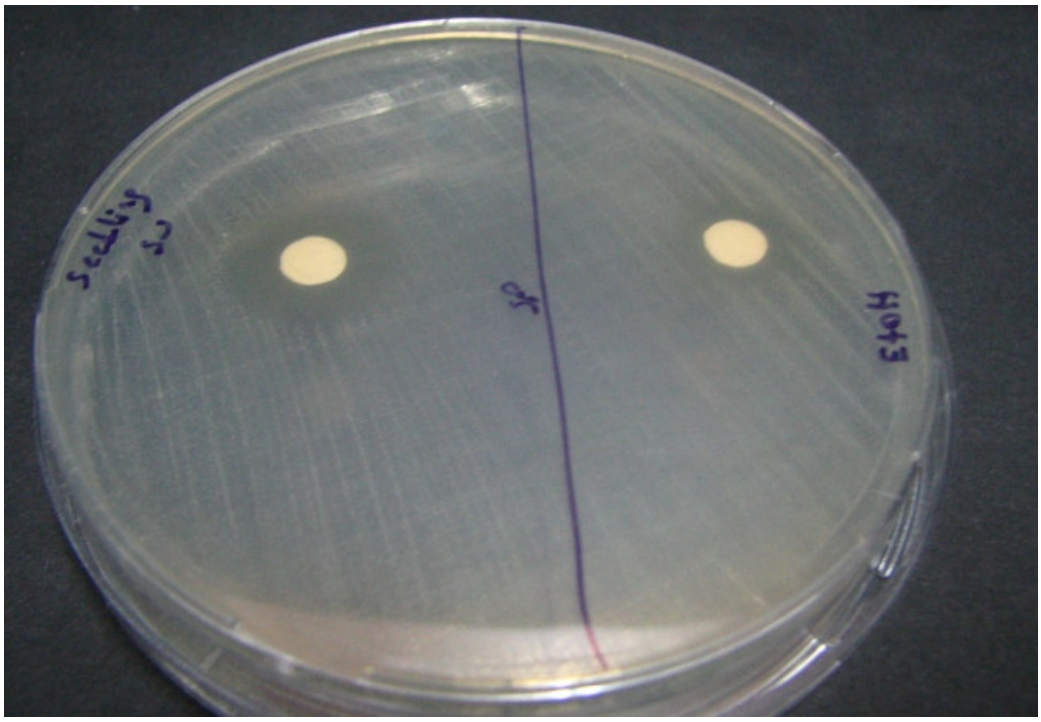


Figure 3.3 Susceptibility of *S. pyogenes* to aqueous and ethanol extract of *in vitro*-grown seedlings.

Table 3.4 Antitumor activity of *D. trojana* extracts.

Treatments	% Tumor inhibition
Water	-
Camptothecin	100
FW1	90
FE1	75
FW2	85
FE2	80
FW3	50
FE3	85
IW1	80
IE1	75
IW2	65
IE2	80

FW1: water extract of field-grown leaf

FE1: ethanol extract of field-grown leaf

FW2: water extract of field-grown fruit

FE2: ethanol extract of field-grown fruit

FW3: water extract of field-grown stem

FE3: ethanol extract of field-grown stem

IW1: water extract of in-vitro grown seedling

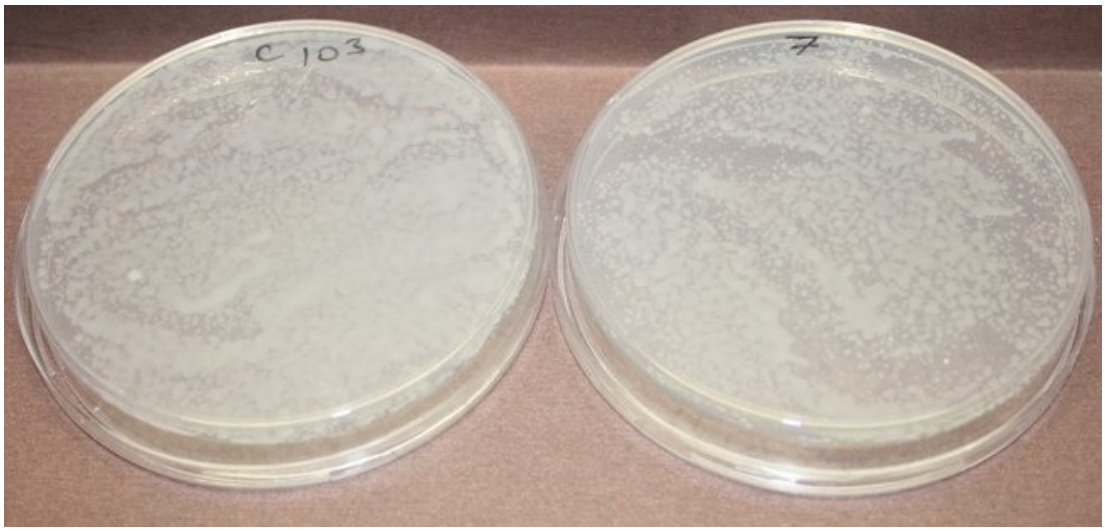
IE1: ethanol extract of in-vitro grown seedling

IW2: water extract of callus

IE2: ethanol extract of callus

Figure 3.4 Tumors produced by *A. tumefaciens* with aqueous extract of *in vitro*-grown seedlings (above), distilled water (solvent control I) (below left) and camptothecin (positive control) (below right).

Figure 3.5 Growth of *A. tumefaciens* (control) (left) and bacterial viability testing (extract+*A. tumefaciens*) (right) on YEM medium.



CHAPTER FOUR

Conclusions

Seeds of *D. trojana* were collected from Edremit, Kazdağı, Balıkesir /Turkey in June of 2007. Surface sterilization of the seeds was achieved with 40 % Domestos® (containing 5 % sodium hypochloride) with a few drops of tween-20 for 15 minutes by using a sonicator or a magnetic stirrer. Best result was obtained with the sonicator emitting millions of shock sound waves through seed surfaces. Surface sterilized seeds were placed on germination medium containing only MS medium or MS medium + 0.3 mg/l GA_3.

Two different explants (leaf lamina and petiole segments) were excised from 7 weeks-old sterile seedlings. Explants were placed on MS medium containing different concentrations of TDZ (0.05, 0.1, 0.5 or 1.0 mg/l) and IAA (0.05, 0.1, 0.5 or 1.0 mg/l). Best callus formation was obtained with 1.0 mg/l TDZ and 0.5 mg/l IAA (100 % frequency of callusing). Leaf lamina explants were found to be more productive in terms of callus production than petiole explants. Green friable callus was observed with TDZ and IAA combinations.

As a different treatment, some of the calli were transferred to the suspension cultures for embryo induction. Globular and heart shaped embryonic callus formation was observed. But, these calli did not germinate or produce whole seedling on germination medium.

Field-grown plant materials and *in vitro*-grown plant materials of *D. trojana* were evaluated for their antibacterial and antitumor activities. *In vitro* seedlings were obtained with germination of *D. trojana* seeds on MS + 0.3mg/l GA_3 and used calli were obtained from leaf explants cultured on media containing 1.0 mg/l TDZ and 0.5 mg/l IAA. Ten different extracts were obtained by extracting the field-grown plant materials (leaf, stem, and fruit) and *in vitro*-grown plant materials (calli and *in vitro* seedlings) with water and ethanol.

. The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity. Ten bacterial strains were used in the bioassay: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Solmanella typhimurum* (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Proteus vulgaris* (ATCC 13315), *Enterobacter cloacae* (ATCC 23355) and *Klebsiella pneumoniae* (ATCC 13883) which are gram-negative bacteria and S*treptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) which are gram-positive bacteria. *S. pyogenes* was susceptible to aqueous and ethanol extracts of *in vitro* seedlings. Also, *S. typhimurium* showed weak sensitivity to these extracts. However, aqueous and ethanol extracts of *in vitro*-grown callus did not exhibit antibacterial activity against all used bacteria. Aqueous and ethanol extracts of field-grown leaf and stem showed antibacterial activity against *E. coli*. But, none of the extracts of field-grown fruit showed antibacterial activity against all used bacteria. *S. typhimurium, P. vulgaris, K. pneumonia and E. coli* were susceptible to ethanolic extracts of field-grown leaf.

Antitumor activity was evaluated with potato disc diffusion bioassay. All *D. trojana* extracts showed antitumor activity. Tumor inhibition percentages were more than 50 %. Water extract of field-grown leaf exhibited better % tumor inhibition than other extracts. No tumor formation was observed with camptothecin.

Extracts having best tumor inhibition were used to test the effect of extracts on bacterial (*A. tumefaciens*) viability. Action of all extracts was on the formation of tumors and not on the viability of bacterium.

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