EVALUATION OF BIOLOGICAL ACTIVITIES OF SOME ENDEMIC PLANTS IN BOLU

by

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ABSTRACT

EVALUATION OF BIOLOGICAL ACTIVITIES OF SOME ENDEMIC PLANTS IN BOLU

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Antibacterial and antitumor activities of 24 alcoholic and aqueous extracts from 8 endemic Turkish plant species (*Crocus abantensis*, *Crocus ancyrensis*, *Galanthus plicatus* subsp*. byzantinus*, *Paronychia chionaea*, *Astragalus gymnolobus*, *Trifolium pannonicum* subsp. *elongatum*, *Eryngium bithynicum* and *Convolvulus galaticus*) of 6 different families were screened.

For the antibacterial assay, *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Proteus vulgaris, Enterobacter cloacae* and *Klebsiella pneumoniae* which are Gram-negative bacteria, and *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes* which are Gram-positive bacteria were used.

Best antibacterial activity was observed with *T. pannonicum* extracts. Ethanolic extract of *T. pannonicum* was better than aqueous and methanolic extracts against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *P. vulgaris* and *K. pneumoniae*. Methanolic and ethanolic extracts of *T. pannonicum* exhibited a broad-spectrum activity against both Gram-positive and Gram-negative bacteria. All extracts of endemic plant species were not effective against *S. marcescens*, *S. typhimurium* and *E. coli* which are Gram-negative bacteria.

Best antitumor activity was observed with all extracts (water, MeOH and EtOH) of *G. plicatus*. Generally, alcoholic extracts showed better antitumor activity than aqueous extracts. Alcoholic extracts (methanolic or ethanolic) of *C. abantensis, C. ancyrensis, P. chionaea*, *C. galaticus* and *T. pannonicum* also exhibited strong antitumor activity. Antitumor activity was not observed with *E. bithynicum* and *A. gymnolobus*.

Antitumor action of *C. abantensis*, *C. ancyrensis*, *G. plicatus*, *C. galaticus* and *P. chionaea* was on the formation of tumors and not on the viability of *A. tumefaciens* bacterium. However, *T. pannonicum* extracts affected the viability of *A. tumefaciens* and its antitumor action was on the viability of the bacterium.

Keywords: *Crocus abantensis*, *Crocus ancyrensis, Galanthus plicatus*, *Convolvulus galaticus*, *Trifolium pannonicum*, *Eryngium bithynicum*, *Paronychia chionaea*, *Astragalus gymnolobus*, antibacterial, antitumor.

ÖZET

BOLU' DA BULUNAN BAZI ENDEMİK BİTKİLERİN BİYOLOJİK AKTİVİTELERİNİN DEĞERLENDİRİLMESİ

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6 farklı familyaya ait 8 adet Türk endemik bitki türünden (*Crocus abantensis*, *Crocus ancyrensis*, *Galanthus plicatus* subsp. *byzantinus*, *Paronychia chionaea*, *Astragalus gymnolobus*, *Trifolium pannonicum* subsp. *elongatum*, *Eryngium bithynicum* ve *Convolvulus galaticus*) elde edilen 24 adet alkol ve su özütlerinin antibakteriyel ve antitümor aktiviteleri araştırılmıştır.

Antibakteriyel test için Gram-negatif bakteri olarak *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Proteus vulgaris, Enterobacter cloacae ve Klebsiella pneumoniae,* Gram-pozitif bakteri olarak *Staphylococcus aureus, Staphylococcus epidermidis* ve *Streptococcus pyogenes* kullanılmıştır.

En iyi antibakteriyel aktivite *T. pannonicum* özütleri ile gözlemlenmiştir. *T. pannonicum*' un etanol özütü *S. aureus*, *S. epidermidis*, *S. pyogenes*, *P. vulgaris* ve *K. pneumoniae'* a karşı sulu ve metanolik özütlerden daha iyi sonuç vermiştir. *T. pannonicum*' un metanolik ve etanolik özütleri, Gram-pozitif ve Gram-negatif bakterilere karşı geniş spektrumlu antibakteriyel aktivite sergilemiştir. Endemik bitki özütlerinin tümü Gram-negatif bakterilerden *S. marcescens*, *S. typhimurium* ve *E. coli* 'ye karşı etkili değildir.

En iyi antitümör aktivite *G. plicatus*' un tüm özütlerinde (su, MeOH ve EtOH) gözlemlenmiştir. Genel olarak, alkol ile hazırlanan özütler su ile hazırlan özütlerden daha iyi antitümör aktivite göstermiştir. *C. abantensis, C. ancyrensis, P. chionaea*, *C. galaticus* ve *T. pannonicum*' un alkollü özütleri (metanollü veya etanollü) de güçlü antitümör aktivite sergilemişlerdir. *E. bithynicum* ve *A. gymnolobus* ile antitümör aktivite gözlemlenmemiştir.

C. abantensis, *C. ancyrensis*, *G. plicatus*, *C. galaticus* ve *P. chionaea*' nın antitümör etkisi *A. tumefaciens*' in yaşayabilirliği üzerine değil, tümör oluşumu üzerinedir. Fakat, *T. pannonicum* özütleri *A. tumefaciens*' in yaşayabilirliğini etkilemiş ve özütün antitümör etkisi bakterinin yaşayabilirliği üzerine olmuştur.

Anahtar Kelimeler: *Crocus abantensis*, *Crocus ancyrensis, Galanthus plicatus*, *Convolvulus galaticus*, *Trifolium pannonicum*, *Eryngium bithynicum*, *Paronychia chionaea*, *Astragalus gymnolobus*, antibakteriyel, antitümör.

DEDICATION

I dedicate this dissertation to my family.

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CHAPTER ONE

Introduction and Literature Review

Herbs have been used as medicines for as long as human life has existed on earth. For millennia man has utilized the properties of plants not just for food and shelter but also for health and well-being (Hamman 1991). 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 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). Plants have been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against predation or infection (Cox and Balick 1994).

A diverse or wide collection of medicinal plant species and the knowledge concerning their medicinal use function as the raw material for new drug development research. Ethnobotanical and ethnopharmacological research is very crucial in the development of drugs

from natural sources. The information obtained on identification, preparation, clinical use, gathering, and preservation of medicinal plants dramatically facilitates the search for new drugs, and the time needed for drug development programs (Aburjai *et al*. 2007).

In spite of the advances of synthetic chemistry and contribution that has been made to pharmaceuticals discovery and development during the later part of the 20th century, the use of herbal remedies is apparently growing (Fowler 2006).

The plant kingdom represents an extraordinary reservoir of novel molecules. However, only a small percentage of the estimated 400.000-500.000 plant species around the globe has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even lower. Plants contain hundreds of thousands of metabolites and any phytochemical investigation of a given plant reveal only a very narrow spectrum of its constituents. Furthermore, when a plant extract is screened for a specific activity, it will still have to be considered as uninvestigated with respect to any other pharmacological activity. The potential of higher plants as sources for new drugs is thus still largely unexplored (Hostettmann and Wolfender 1997).

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two fold in the development of new drugs: (1) they may become the base for the

development of a medicine, a natural blueprint for the development of new drugs, or; (2) a phytomedicine to be used for the treatment of disease (Iwu *et al.* 1999).

Plants seem to have served as models in drug development for 3 reasons. Firstly, at least 25% of all prescriptions contain active principles extracted from higher plants and two-thirds of people in developing countries rely on plants as sources of drugs. Secondly, they can serve as templates for synthetic modification and structure-function studies. Thirdly, many secondary, highly active, plant constituents are found to be useful in studying biological systems and disease processes (Farnsworth and Morris 1976).

Turkey has a rich plant diversity and higher endemism ratio comparing with Europe. According to last records (Vural 2003), there are 10754 native plant species and endemism ratio is about 34.8% with 3708 endemic plant species. Having various climates, geomorphology and topographic structures of Anatolia, and taking place in the intersection of the three phytogeographical regions (Euro-Siberian, Mediterranean and Irano-Turanian) caused the extraordinary variety of habitats and ecosystem in Turkey (Avci 2005). The scientific verification of biological activity of endemic plant species may be important to screen the potential value of endemics in Turkey.

1.1. *Trifolium pannonicum* Jacq. subsp. *elongatum* (Willd.) D. Zohary

1.1.1. Botany and Distribution

T. pannonicum is an erect or ascending perennial plant belonging to Fabaceae family (Figure 1.1). Stipules with linear-subulate are free portions. Leaflets are 0.5-1.2 cm and linear to oblong-lanceolate. Inflorescence is ovoid to ovoid-oblong, 1.5-1.2 cm wide and pedinculate. Calyx is cylindrical, becoming campanulate; tube is 10-nerved, spreading hairy and is closed by a 2-lipped callosity in fruit. Teeth are subulate, acute, stellate or reflexed in fruit. Corolla is white to cream and is to 25 mm, c.2 x as long as the calyx, standard c. 4 mm broad with an attenuated, subacute tip (Davis 1970).

The natural habitat for *T. pannonicum* is meadows, forest clearings and steppe at 300-2350 m. It is found in North, South and inner parts of Turkey (Bolu, Amasya, Istanbul, Çorum, Ankara, Erzurum, Gümüşhane, Kars, Kayseri, Tunceli, Isparta, Konya and Kütahya) (Figure 1.2) and blossoms from June to August (Davis 1970).

1.1.2. Medicinal usage and scientific studies of *Trifolium* spp.

Generally, *Trifolium* species has been used in folk medicine to treat skin conditions (Chevalier 1996). Gođevac *et al*. (2008) reported the antioxidant activity of *T. pannonicum*. Triterpene saponins in the seeds and flavonoid glycosides in the aerial part of *T. pannonicum* were detected (Gođevac *et al*. 2008).

1.2. *Convolvulus galaticus* Rostan ex Choisy

1.2.1. Botany and Distribution

C. galaticus is a prostrate, herbaceous, perennial with dense shining tomentum (hairs less than 0.5 mm) and creeping underground stems (Figure 1.1). It belongs to Convolvulaceae family. Leaves are petiolate, broadly ovate, obtuse or acute, base is cordate, undulate, broadly crenate to dentate. Flowers are axillary, solitary or in 2-4-flowered cymes. Outer sepals are ovate, apiculate, densely tomentose. Corolla is pink to light purple and 25-35 mm. Ovary is usually hairy at apex (Davis 1978).

The natural habitat for *C. galaticus* is *Pinus* woods, open steppe, stony slopes, meadows, cultivated and fallow fields which are usually calcareous at 880-2000 m. It is found in the inner and rarely in the North part of Turkey (Bolu, Amasya, Ankara, Burdur, Denizli, Elazığ, Eskişehir, Gaziantep, Gümüşhane, Kayseri, Konya, Kütahya, Malatya, Mardin, Niğde, Şanlıurfa and Van) (Figure 1.2) and blossom time for this species is from May to August (Davis 1978).

1.2.2. Medicinal usage and scientific studies of *Convolvulus* spp.

Roots and leaves of *Convolvulus* species are cholagogue, laxative and strongly purgative in traditional medicine (Grieve 1982; Baytop 1999). The aerial parts, roots and flowers of *C. arvensis* extracts have moderate

diuretic, tranquillizing, hypoglycemic, antihemorrhagic, antibacterial, antifungal (Awaad *et al*. 2006) and antitumor activities (Habibinya *et al*. 1993).

1.3. *Paronychia chionaea* Boiss

1.3.1. Botany and Distribution

P. chionaea, a member of Illecebraceae family, is a prostrate caespitose herb and often woody at base (Figure 1.1). Adventitious roots are conspicuous. Leaves are 3-5 x 1.25-2 mm, obovate-spathulate, obtuse, adpressed-pubescent to puberulent and often recurved. Stipules are mostly shorter than leaves. Glomerules are 10-15 mm in diameter, terminal and congested. Bracts are broadly ovate to rounded, has an exceeding and concealing flowers. Flowers are 2.5-3.25 mm, adpressed-pubescent. Sepals are narrowly oblong, obtuse, incurved at apex. Fruit has almost equaling sepals (Davis 1967).

The natural habitat for *P. chionaea* is rocky places at high altitudes and is found in North, West and Central parts of Turkey (Bolu, Adana, Amasya, Antalya, Burdur, Bursa, Denizli, Konya, Kütahya, Manisa, Kahramanmaraş, Muğla and Niğde) (Figure 1.2). Blossom time for *P. chionaea* is from June to August (Davis 1967).

1.3.2. Medicinal usage and scientific studies of *Paronychia* spp.

Traditionally, an infusion of the *Paronychia* leaves is known as aphrodisiac and diuretic. It is also used in the treatment of tuberculosis in folk medicine (Grieve 1982).

Inhibitory effect of *P. argentea* was found against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Al-Bakri and Afifi 2007).

1.4. *Astragalus gymnolobus* Fischer

1.4.1. Botany and Distribution

A. gymnolobus is a dwarf, cushion-forming shrub (Figure 1.1) belonging to Fabaceae family. Stems are erect, 5-30 cm, bifurcate-sericeous. Leaves are up to 15 cm, imparipinnate, rachis is spiny with early loss of terminal leaflet; leaflets are 5-15 mm, ovate to elliptic, acute or obtuse, mucronate, both surfaces are sparsely bifurcate-sericeous, sometimes glabrescent above with 7-10 paired. Stipules are 5-10 mm, triangular-lanceolate, glabrous but ciliate. Peduncles are sparsely adpressed-hairy, exceeding leaves. Flowers are in a 6-18-flowered raceme, dense at first, elongating with age. Bracts are 4-10 mm and linear- lanceolate. Calyx is 8-11 mm, tubular with very sparse, black and white adpressed-bifurcate hairs; teeth are 1.5-4 mm. Corolla is white, cream or pale yellow, often flushed pink with age and 18-25 mm. Legumes are 13-15 x 4-5 mm, ovate-oblong, glabrous, wrinkled and mucronate (Davis 1970).

The natural habitat for *A. gymnolobus* is steppe, fields, woods, cliffs at 1000-1800 m. It is found in North West, Central, South and East parts of Turkey (Bolu, Afyon, Ankara, Antalya, Isparta, Konya, Kütahya, Malatya, Niğde and Şanlıurfa) (Figure 1.2) and blossoms from May to June (Davis 1970).

1.4.2. Medicinal usage and scientific studies of *Astragalus* spp.

Astragalus species have been used in folk medicine to raise immune resistance, improve physical endurance and lower blood pressure (Chevalier 1996). The roots of various *Astragalus* spp. are used as antiperspirant, diuretic and tonic agents. They have been used in the treatment of diabetes mellitus, nephritis, leukemia and uterine cancer, but also for their hepatoprotective, antioxidative, immunostimulant and antiviral properties (Rios and Waterman 1997; Shirataki *et al.* 1997; Toda and Shirataki 1999; Pistelli *et al*. 2002). Anti-inflammatory, analgesic, hypotensive, sedative and cardiotonic activities are also reported (Rios and Waterman 1997; Cuéllar *et al.*1998; Pistelli *et al*. 2002).

Rittenhouse *et al.* (1991) reported that *A. membranaceous* may exert its antitumour activity by abolishing tumor-associated macrophage suppression. Tin *et al*. (2007) proposed that members of *Astragalus* may possess anti-tumorigenic potential in certain cancer cell types. The anti-carcinogenic effects of *Astragalus* saponin extracts were investigated in HT-29 human colon cancer cells and the results indicated that this extracts could be an effective chemotherapeutic agent in colon cancer treatment.

Cho and Leung (2007a) isolated bioactive fractions from the roots of *A. membranaceous.* One of the fractions exhibited potent anti-tumor effects both *in vitro* and *in vivo*. Cho and Leung (2007b) also reported the immunomodulating and immunorestorative effects of same fraction. They concluded that A. *membranaceous* could exhibit both *in vitro* and *in vivo* anti-tumor effects, which might be achieved through activating the anti-tumor immune mechanism of the host (Cho and Leung 2007a; 2007b).

Yang *et al.* (1987) reported the antiviral activity of *A. membranaceus* against Coxsackie viruses. The extracts of *A. siculus* showed antibacterial effects against Gram-negative and Gram-positive bacteria isolated from urinary tract infections (Bisignano *et al*. 1994).

There are some reports about antibacterial activities of some members of *Astragalus* species: *A. siculus*, *A. gummifer*, *A. membranaceus*, *A. malanophrurius* and *A. verrucosus* exhibit moderate antibacterial affects against Gram-positive and Gram-negative tested strains (Pistelli *et al*. 2002).

Figure 1.1 Pictures of endemic plant species (*T. pannonicum*, *C. galaticus*, *P. chionaea* and *A. gymnolobus*) screened for antibacterial and antitumor activities.

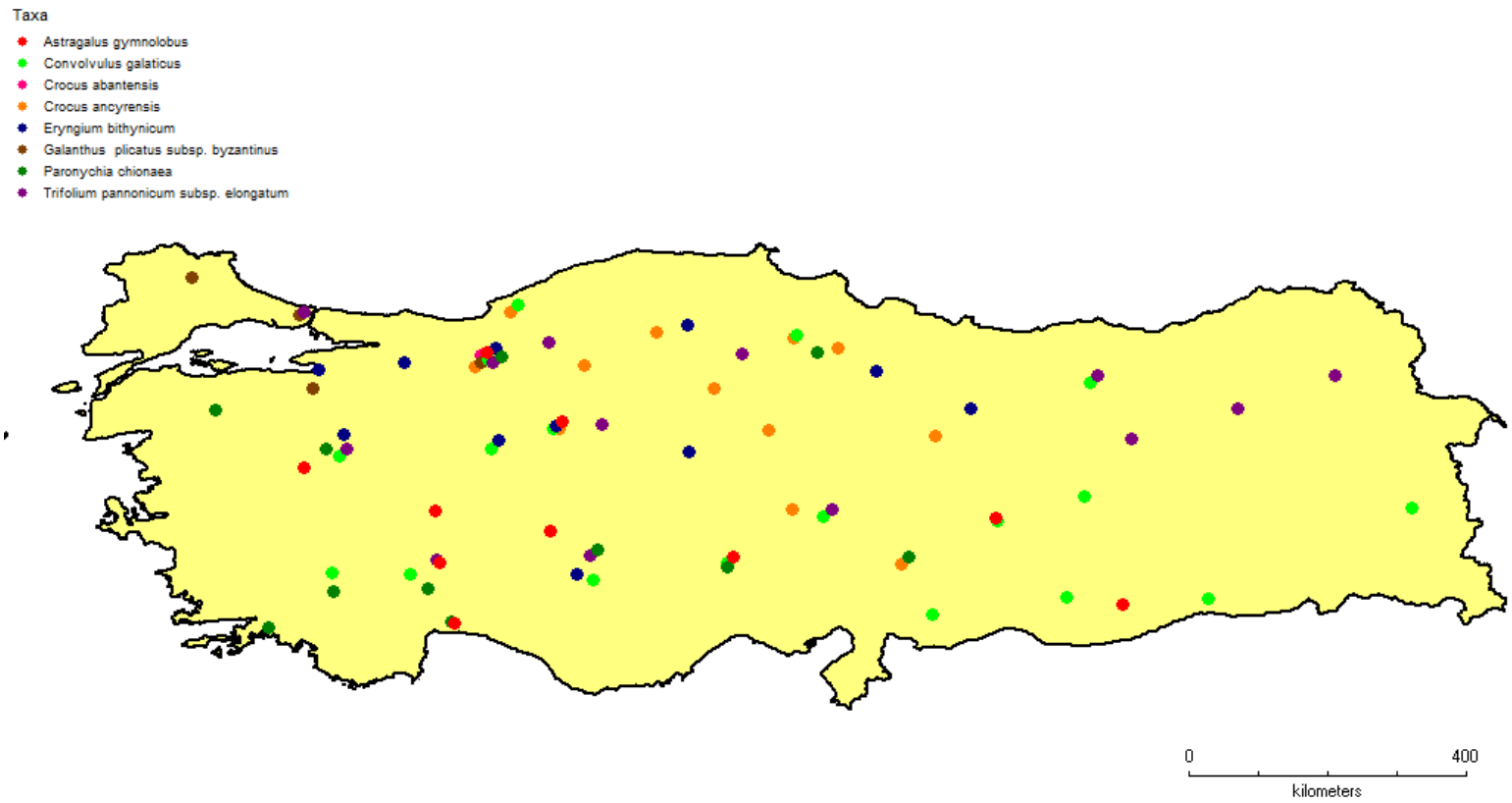

Figure 1.2 Map of the distribution of studied endemic plant species in Turkey.

1.5. *Eryngium bithynicum* Boiss

1.5.1. Botany and Distribution

E. bithynicum is an annual or biennial species belonging to Apiaceae family (Figure 1.3). It has stem which is angled and sulcate, 15-20 cm, branched from below the middle. Basal leaves often wither at flowering time, subcoriaceous, ovate-oblong to trilobed, crenate-muticous. Median cauline leaves are sessile, pinnatifid to palmatifid into lanceolate spinulose-margined lobes. Inflorescense is glaucous or bluish, richly paniculate and domed; capitula is numerous, less than 15-flowered, hemispherical and 6-10 mm in diameter. Bracts are 5(-6), lanceolate, flat, pungent, usually spinulose-margined, 2-4 x capitulum. Bracteoles are tricuspidate (Davis 1972).

The natural habitat for *E. bithynicum* is steppe, fallow fields, eroded banks at 100-1400 m. It is found in central, adjacent North and South parts of Turkey (Bolu, Ankara, Bursa, Çankırı, Eskişehir, Kastamonu, Kırşehir, Konya, Kütahya, Sakarya, Sivas and Tokat) (Figure 1.2) and blossom time for this species is the from June to September (Davis 1972).

1.5.2. Medicinal usage and scientific studies of *Eryngium* spp.

Eryngium species have been used in folk medicine as antispasmodic, aromatic, diaphoretic, diuretic, expectorant, stimulant, nervine and aphrodisiac (Baytop, 1999). The crushed leaves of *E.*

foetidum are placed in the ear to treat pain, and are used for the local treatment of arthritic processes (García *et al*. 1999).

Küpeli *et al*. (2006) reported the anti-inflammatory and antinociceptive activities of some members of *Eryngium* species in Turkey. Antibacterial activity of *E. foetidium* against *Helicobacter pylori* was recorded (Ndip *et al*. 2007).

1.6. *Galanthus plicatus* M.Bieb. subsp. *byzantinus* (Baker) D.A. Webb

1.6.1. Botany and Distribution

G. plicatus subsp. *byzantinus* has a bulb which is subglobose to ovoid and belongs to Amaryllidaceae family (Figure 1.3). Leaves are narrowly oblanceolate to strap-shaped, margins are explicative in bud, sometimes only slightly revolute at maturity, 5-21 x 1-1.5 cm at anthesis, to 29-30 x 2 cm and upright at maturity, apex is obtuse, cucullate, glaucous, often with a paler central band on upper surface. Scape is 9-22 cm. Outer perianth segments are convex, elliptic to obovate and are 17-28 x 6-12 mm, inner segments are flat, not flared at apex, oblong-spathulate, 8 x 4-7 mm, with separate green patches at base and apex, sometimes joined in centre. Filaments are 0.5-1 mm, anthers are 6-7 mm. Capsule is ellipsoid to subglobose, 16 x 12 mm (Davis 1984).

The natural habitat for *G. plicatus* is open turf or margins of *Abies* and *Fagus* forest in deep leaf-mould at 45-1100 m. It is found in the North West part of Turkey (Bolu, Bursa, Istanbul and Kırklareli) (Figure 1.2) and

blossoms at early spring between months of January and April (Davis 1984).

1.6.2. Medicinal usage and scientific studies of *Galanthus* spp.

Some *Galanthus* species has been used traditionally in Bulgaria and Turkey for neurological conditions (Howes *et al*. 2003) besides as emmenagogue, digestive, resolutive and hearth strengthener (Grieve 1982; Baytop 1999).

Galantamine (trade name Reminyl®) is a natural product discovered through an ethnobotanical lead and first isolated from *Galanthus woronowii* in Russia in the early 1950's. Galantamine is approved for the treatment of Alzheimer's disease, slowing the process of neurological degeneration (Balunas and Kinghorn 2005).

1.7. *Crocus abantensis* T. Baytop & Mathew

1.7.1. Botany and Distribution

C. abantensis has a corm tunic which is strongly reticulate-fibrous and belongs to the Iridaceae family (Figure 1.3). The leaves are 5-8, synanthous and 0.5-0.8 mm broad. The prophyll is absent and bracteole is present which is subequal to bract. Throat of the perianth is yellow, glabrous; segments are 2-2.7 x 0.7-1.3 cm, obtuse, has intense color of blue to lilac-blue and yellow at base inside. The filaments are 5 mm, yellow, sparsely scabrid; anthers are 0.8-1.4 cm and yellow. The style is divided into 3 slender orange branches (Davis 1984).

The natural habitat for *C. abantensis* is mountain meadows. It is found only in Abant, Bolu (Figure 1.2) and blossoms in April (Davis 1984).

1.8. *Crocus ancyrensis* (Herbert) Maw

1.8.1. Botany and Distribution

C. ancyrensis has a corm tunic which is coarsely reticulate-fibrous (Figure 1.3). The leaves are 2-6, synanthous and are 0.5-2 mm broad. Prophyll is absent. Bracteole is present and throat of the perianth is yellow, glabrous; segments are 1.5-3 x 0.9-1.3 cm, obtuse to rounded, has color of bright yellow, often stained purplish at base and on perianth tube. Filaments are 2-4 mm, glabrous; anthers are 6-13 mm and yellow. Style is divided into 3 orange or reddish-orange branches (Davis 1984).

The natural habitat for *C. ancyrensis* is open rocky places, scrub and *Pinus* woods. It is found in the North and Central parts of Turkey (Bolu, Amasya, Ankara, Çorum, Kastamonu, Zonguldak, Kayseri, Kahramanmaraş, Samsun, Sivas and Yozgat) (Figure 1.2) and blossoms at early spring (Davis 1984).

1.8.2. Medicinal usage and scientific studies of *Crocus* spp.

Flowers of some *Crocus* species are consumed as a snack food and recorded as an antiseptic (Pieroni 2000). Saffron harvested from the dried stigmas of *Crocus sativus* flowers is used mainly as a spice for flavoring and coloring food, in alcoholic and non-alcoholic beverages, as a perfume and as a herbal medicine. Since ancient times saffron has been

considered to have a number of therapeutic properties. It has been used as a sedative, a tonic, a stimulant of the stomach and an expectorant in traditional medicine (Assimopoulou *et al*. 2005). It has also been used to treat several human health conditions, including ailments such as dysentery, measles, enlargement of the liver and gall bladder, urological infections, cough, stomach disorders, asthma and cardiovascular disorders (Abdullaev 2003).

Recent studies indicate its potential as an anticancer, antitumoral, cytotoxic, hypolipidaemic, anti-inflammatory and oxygenation enhancement agent (Assimopoulou *et al*. 2005; Hosseinzadeh and Ghenaati 2006; Rios *et al*. 1996).

Characteristic compounds of saffron include crocins, crocetin, picrocrocin and β-carotene, while the main biologically active metabolites of saffron are crocins, red-colored water-soluble carotenoids. Safranal is the main component of the essential oil and is responsible for the characteristic saffron aroma (Assimopoulou *et al*. 2005). Crocin derivatives and mainly crocin suppress tumour growth, while safranal and crocetin were proved to possess antileukaemic activity (Assimopoulou *et al*. 2005). Tumor incidence and histopathological studies proves crocetin is a potent antitumour agent (Magesh *et al.* 2006).

Figure 1.3 Pictures of endemic plant species (*E. bithynicum, G. plicatus, C. abantensis and C. ancyrensis*) screened for antibacterial and antitumor activities.

CHAPTER TWO

Antibacterial Bioassay

Medicinal plants have been widely used for the treatment of common animal and human infectious diseases since antiquity (Rios and Recio 2005). It is estimated that there are 250.00 to 500.000 species of plants on Earth. A relatively small percentage (1 to 10%) of these is used as foods by both humans and other animal species. It is possible that even more are used for medicinal purposes (Cowan 1999). The use of plant extracts, as well as other alternative forms of medical treatments, is enjoying great popularity in the late 1990's. It was reported that in 1996, sales of botanical medicines increased 37% over 1995 (Cowan 1999).

2.1. Introduction

Clinical microbiologists have two reasons to be interested in the topic of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by physicians; several are already being tested in humans. It is reported that, on average, two or three antibiotics derived from microorganisms are launched each year. Worldwide spending on finding new anti-infective agents (including vaccines) is expected to increase 60% from the spending levels in 1993. Second, the public is becoming

increasingly aware of problems with the over prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. A multitude of plant compounds is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is commonplace (Cowan 1999).

During the second half of the 20th century, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the classical antibiotics led researchers to investigate the antimicrobial activity of several medicinal plants. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Al-Bakri and Afifi 2007).

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. The substances that can either inhibit the growth of microorganisms or kill them are considered candidates for developing new drugs for treatment of various infectious diseases. The use of medical plants as traditional medicines is well known in rural areas of many developing countries (Catalano *et al*. 1998; Martinez *et al.* 1996; Sundar 1996; Taylor *et al*. 1996).

Infectious diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in the understanding of microbiology and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns. Historically, plants have provided a good source of anti-infective agents; emetine, quinine and berberine remain highly effective instruments in the fight against microbial infections (Iwu *et al.* 1999).

In general, phenolics are the predominant active chemical in antibacterial plants, with Gram positive bacteria being the most sensible germs (Rios and Recio, 2005).

A method frequently used to screen plant extracts for antimicrobial activity is the agar disc diffusion technique (Cowan 1999). At the end of the 1950's, antimicrobial susceptibility testing was marked by lack of an acceptable standardized procedure, led to variable results depending on which laboratory was used (Atlas 1988). The study of medicinal plants as antimicrobial agents is necessary for gaining insight into medicinal flora and their real value, but the use of a standard method for investigation is essential (Rios and Recio 2005).

The disc diffusion procedure (Kirby-Bauer method) has been accepted by the Food and Drug Administration (FDA) and as a standard by the National Committee for Clinical Laboratory Standards (NCCLS). It was developed by Kirby and Bauer in the 1960's (Prescott *et al.* 1990).

Reasonably accurate and precise results can be obtained with Kirby-Bauer procedure, which all-procedural details are carefully standardized and controlled (Barry and Thornsberry 1985). 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℃ for 18-24 hours. In such agar diffusion methods antibiotics diffuse into the agar, establishing a concentration gradient. At the end of the incubation period, plates are observed and clear zones around the disc are measured. A clear area (zone of inhibition) indicates inhibition of microbial growth around the antibiotic disc. The diameter of the zone of inhibition reflects the solubility properties of the particular antibiotic; that is the concentration gradient established by diffusion of the antibiotic into the agar and the sensitivity of the given microorganism to the specific antibiotic. Kirby-Bauer agar diffusion test procedure is designated for use with rapidly growing bacteria. It is not directly applicable to filamentous fungi, anaerobes or slow-growing bacteria (Atlas 1988). The 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 2.1.

There are no reports on antibacterial activities of endemic plants with either veterinary or human origin pathogens in Turkey. The aim of this study is to describe antibacterial activities of eight endemic plant species found in Bolu against commonly occurring human pathogens.

Table 2.1 Bacteria used in antibacterial assay and their representative diseases (Levinson and Jawetz 2002).

	Pathogens	Representative Diseases	Drugs of Choice
Gram Positive	***Staphylococcus aureus***	Abscesses, Pyogenic infections (endocarditis and osteomyelitis), Food poisoning, Toxic shock syndrome, Hospital-acquired pneumonia, Septicemia (sepsis), Surgical-wound infections, skin infections (impetigo, furuncles, paroncyhia, cellulitis), pospartum breast infections (mastitis), Eyelid infections, Scalded-skin syndrome	Ciprofloxacin, Trimethoprimsulfamethoxazole, Cefazolin, Amoxicillin, Vancomycin, Nafcillin, Mupirocin
	Staphylococcus epidermidis	Sepsis in neonates, Peritonitis, Cerebrospinal fluid shunt infections, Infections of intravenous catheter sites and prosthetic devices	Vancomycin, Rifampin
	Streptococcus pyogenes	Pyogenic skin infections (impetigo and cellulitis), Pharyngitis, Sepsis, Scarlet fever, Toxic shock, Rheumatic fever, Acute glomerulonephritis	Penicillin G, Vancomycin
Gram Negative	***Escherichia coli***	Urinary tract infections, neonatal meningitis, diarrhea	Ampicillin + cefotaxime, Trimethoprimsulfamethoxazole
	Pseudomonas aeruginosa	Sepsis, Pneumonia, Urinary tract infections, Wound infections	Tricarcillin, piperacillin + gentamicin or amikacin
	Salmonella typhimurium	Enterocolitis, Enteric fevers, Septicemia, Typhoid	Ceftriaxone or Ciprofloxacin
	Serratia marcescens	Pneumonia, Urinary tract infections, Nosocomial infections	Ampicillin, Gentamicin, Cefotaxime
	Proteus vulgaris	Pneumonia, Wound infections, Septicemia, Urinary tract infections	Cefotaxime, Trimethoprim-sulfamethoxazole
	Enterobacter cloacaea	Pneumonia, Urinary tract infections, Nosocomial infections	Gentamicin, Cefotaxime, Imipenem + Gentamicin
	Klebsiella pneumoniae	Pneumonia, Urinary tract infections, Nosocomial infections	Gentamicin, Cefotaxime

2.2. Materials and Methods

2.2.1. Plant Material and Extraction

Five endemic plant species [*Crocus abantensis*, *Crocus ancyrensis*, *Galanthus plicatus* subsp. *byzantinus*, *Paronychia chionaea* and *Astragalus gymnolobus*] were collected from Abant Lake, Bolu, Turkey and three endemic species [*Trifolium pannonicum* subsp. *elongatum*, *Eryngium bithynicum* and *Convolvulus galaticus*] were collected from Abant İzzet Baysal University, Gölköy Campus. Identification of species was made by using "Flora of Turkey and the East Aegean Islands" (Davis 1967; 1970; 1972; 1978; 1984) and voucher specimens were deposited at the Abant İzzet Baysal University (AİBU) Herbarium, Bolu, Turkey. All plant samples, collection numbers and dates were presented in Table 2.1.

All collected plants were oven dried at 40°C and extracted with different solvents [water, methanol (MeOH) and ethanol (EtOH)]. For aqueous extraction, twenty grams from each powdered plant sample were extracted with 200 ml water at 80°C in a waterbath for 12 hours and then filtered. Water was evaporated using lyophilizator. For alcoholic extraction (MeOH and EtOH), twenty grams of plant sample were Soxhlet extracted with 350 ml MeOH or EtOH at 60°C for 12 hours and liquid portion was evaporated under vacuum. For antibacterial assay, each residue was dissolved in sterile distilled water in order to obtain a final concentration of 100 mg/ml. Plant materials, designation of treatments and yield (%) for each extraction were summarized in Table 2.1.

Table 2.2 Designation of studied plant extracts, their family and botanical names, used parts, collection numbers and dates.

Family and plants species	Turkish name	Part used	Designation	Extract	Yield (%)	Collection number	Collection date
IRIDACEAE							
Crocus abantensis T. Baytop & Mathew	Abant çiğdemi	Aerial	Ex 1a	Water	20		
			Ex 1b	MeOH	61	AUT-2010	April 07, 2007
			Ex 1c	EtOH	*22*		
Crocus ancyrensis (Herbert) Maw	Ankara çiğdemi	Aerial	Ex 2a	Water	20		
			Ex 2b	MeOH	*36*	AUT-2011	April 07, 2007
			Ex 2c	EtOH	*34*		
AMARYLLIDACEAE							
Galanthus plicatus Bieb.			Ex 3a	Water	*27*		
subsp. *byzantinus* (Baker) D.A. Webb.	Kardelen	Aerial	Ex 3b	MeOH	*17*	AUT-2012	April 07, 2007
			Ex 3c	EtOH	17		
CONVOLVULACEAE							
Convolvulus galaticus Rostan ex Choisy	Sarmaşık	Aerial	Ex 4a	Water	26		
			Ex 4b	MeOH	10	AUT-2013	June 08, 2007
			Ex 4c	EtOH	7		
FABACEAE							
Trifolium pannonicum Jacq.			Ex 5a	Water	20		
subsp. *elongatum* (Willd.) Zoh.	Yonca	Aerial	Ex 5b	MeOH	9	AUT-2014	June 08, 2007
			Ex 5c	EtOH	5		
APIACEAE							
Eryngium bithynicum Boiss	Boğa dikeni	Aerial	Ex 6a	Water	17		
			Ex 6b	MeOH	12	AUT-2015	July 11, 2007
			Ex 6c	EtOH	11		
ILLECEBRACEAE							
Paronychia chionaea Boiss	Dolama otu	Aerial	Ex 7a	Water	24		
			Ex 7b	MeOH	10	AUT-2016	July 11, 2007
			Ex 7c	EtOH	39		
FABACEAE							
Astragalus gymnolobus Fischer	Geven	Aerial	Ex 8a	Water	26		
			Ex 8b	MeOH	16	AUT-2017	June 04, 2007
			Ex 8c	EtOH	14		

2.2.2. Antibacterial Bioassay

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

BD-Microtrol discs (Becton Dickinson Laboratories, France) containing different bacterial strains were transferred to test tubes containing 2 ml of Tryptic Soy Broth (TSB) and incubated for 3 hours at 37°C. After 3 hours, 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, 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 0.2 ml from each culture was transferred into 0.8 ml TSB in eppendorf tubes. A sterile cotton swab dipped into the bacterial suspension and 0.1 ml from each strand was transferred into Mueller-Hinton (M-H) agar plate (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. Agar plates were streaked four times, each time turning the plate at a 90° 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 paper discs (Glass Microfibre filters, Whatman®; 6 mm in diameter) were soaked in the extract and placed onto inoculated plates. The inoculum for each bacterium was prepared from broth cultures. There were 3 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 (100 µg) (CB-100), Tetracycline (30µg) (TE-30) and Chloramphenicol (30 µg) (C-30). One antibiotic disc was 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 hours of incubation, the results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. 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).

2.3. Results and Discussion

Twenty-four different extracts with three kinds of solvent (water, MeOH and EtOH) of eight different endemic plant species were tested in order to screen and show their potential as antibacterial agents (Table 2.2; Figure 2.1) .

Best inhibitory activity was obtained with *T. pannonicum*. This plant exhibited strong antibacterial activity against *S. epidermidis* (Figure 2.2) and *S. pyogenes* (Figure 2.3). *S. epidermidis* was susceptible to all 3 kinds of extracts (water, MeOH and EtOH) of *T. pannonicum*. Antibacterial activity of *T. pannonicum* may explain why *Trifolium* spp. is used in folk medicine to treat skin conditions (caused by *S. epidermidis*).

Generally, ethanolic extract of *T. pannonicum* was better than aqueous and methanolic extracts of this plant against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *P. vulgaris* and *K. pneumoniae*. Although *E. cloacae* was susceptible to methanolic extract of *T. pannonicum*, this bacterium did not susceptible to aqueous and ethanolic extracts of *T. pannonicum* (Figure 2.1; 2.2). Only ethanolic extract of *T. pannonicum* showed inhibition against *K. pneumoniae* (Figure 2.4).

Antibacterial activity of aqueous, methanolic and ethanolic extracts of *T. pannonicum* were greater than reference antibiotic Tetracycline against *S. epidermidis* (Table 2.2; Figure 2.1).

Bacterial growth was generally sensitive to the reference antibiotics tested (Table 2.2). Inhibition zones varied from 46.25 mm for Ampicillin and *S. pyogenes* to 9.25 mm for the Tetracycline and *S. epidermidis*. Since final concentrations of all extracts were adjusted with distilled water, it was used as a negative control and there was no inhibition with this control solvent (Table 2.2; Figure 2.1).

T. pannonicum extracts were active against both Gram-positive and Gram-negative bacteria. Especially, methanolic and ethanolic extracts of *T. pannonicum* exhibited of a broad-spectrum activity against both Gram-positive and Gram-negative bacteria. (Table 2.2; Figure 2.1). This activity against both the types of bacteria may be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins (McCutcheon *et al.* 1992).

All extracts of endemic plant species were not effective against *S. marcescens*, *S. typhimurium* and *E. coli* which are Gram-negative bacteria (Table 2.2; Figure 2.1). In our study, Gram-positive bacteria (*S. aureus*, *S. epidermidis* and *S. pyogenes*) were more susceptible to plant extracts than Gram-negative bacteria (Table 2.2). 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).

C. abantensis did not show any inhibitory activity against all used pathogens. Similarly, Ilcim *et al*. (1998) reported that *Crocus chrysanthus* was not active against their bacteria species (*Bacillus megaterium*, *B. brevis*, *K. pneumoniae*, *E. coli*, *Enterobacter aerogenes*, *P. aeruginosa*, *S. aureus*, and *Listeria monocytogenes*). However, ethanolic extract of *C. ancyrensis* showed just a little activity (7 and 7.5 mm) against only *S. aureus* and *K. pneumoniae* (Table 2.2).

S. pyogenes was the most susceptible bacterium, except *C. abantensis* and C. *ancyrensis*, generally all alcoholic extracts (MeOH and EtOH) showed inhibition against *S. pyogenes* (Table 2.2; Figure 2.1) .

Alcoholic extracts (MeOH and EtOH) of *E. bithynicum*, *P. chionaea* and *A. gymnolobus* showed inhibition against only *S. pyogenes* in our study (Table 2.2). Ndip *et al*. (2007) recorded the antibacterial activity of *E. foetidium* against *Helicobacter pylori*. Inhibitory effect of *P. argentea* was also found against *Bacillus subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* (Al-Bakri and Afifi 2007). Moderate antibacterial activities of some members of *Astragalus* spp. (*A. siculus*, *A. gummifer*, *A. membranaceus*, *A. malanophrurius* and *A. verrucosus*) were recorded against Gram-positive and Gram-negative bacteria (Bisignano *et al*. 1994; Pistelli *et al*. 2002). *A. siculus* showed antibacterial activity against *S. aureus, S. epidermidis, Streptococcus faecalis, Proteus mirabilis, Citrobacter freundii, P. aeruginosa* and *Klebsiella oxytoca* (Bisignano *et al*. 1994).

Alcoholic extracts of *C. galaticus* exhibited antibacterial activity against *S. pyogenes*, *P. aeruginosa*, *P. vulgaris* and *E. cloacae* in our study (Table 2.2). Similarly, antibacterial activity of *Convolvulus arvensis* was recorded by Awaad et al. (2006).

Results obtained herein revealed the strong antibacterial activities of *T. pannonicum*. Future studies should focus on fractionation of the extracts of *T. pannonicum* in hopes of identifying active components.

Table 2.3 Antibacterial activity of used plant 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.

	Mean diameter of inhibitory zones (mm ± SE)									
Treatments	***S. aureus***	***S. epidermidis***	***S. pyogenes***	***S. marcescens***	***S. typhimurium***	***P. aeruginosa***	***P. vulgaris***	***K. pneumonia***	***E. cloacae***	***E. coli***
Ex 1a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 1b	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 1c	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 2a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 2b	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 2c	7.00 ± 0.00 *e*	- -	- -	- -	- -	- -	- -	7.50 ± 0.50 *c*	- -	- -
Ex 3a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 3b	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 3c	- -	7.25 ± 0.25 *g*	12.50 ± 0.50 *f*	- -	- -	- -	8.25 ± 0.62 *d*	7.25 ± 0.25 *c*	- -	- -
Ex 4a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 4b	- -	- -	7.00 ± 0.00 *h*	- -	- -	- -	- -	- -	- -	- -
Ex 4c	- -	- -	11.00 ± 1.15 *fg*	- -	- -	7.00 ± 0.00 *d*	7.75 ± 0.47 *d*	- -	7.50 ± 0.28 *e*	- -
Ex 5a	- -	10.75 ± 1.03 *ef*	8.50 ± 0.28 *gh*	- -	- -	- -	- -	- -	- -	- -
Ex 5b	7.00 ± 0.00 *e*	10.75 ± 0.25 *ef*	18.00 ± 0.40 *e*	- -	- -	- -	7.00 ± 0.00 *d*	- -	7.50 ± 0.28 *e*	- -
Ex 5c	7.50 ± 0.28 *e*	11.50 ± 0.86 *e*	19.75 ± 0.75 *e*	- -	- -	- -	7.25 ± 0.25 *d*	7.75 ± 0.75 *c*	- -	- -
Ex 6a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 6b	- -	- -	9.50 ± 0.28 *gh*	- -	- -	- -	- -	- -	- -	- -
Ex 6c	- -	- -	8.25 ± 0.25 *gh*	- -	- -	- -	- -	- -	- -	- -
Ex 7a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 7b	- -	- -	7.75 ± 0.75 *h*	- -	- -	- -	- -	- -	- -	- -
Ex 7c	- -	- -	7.00 ± 0.00 *h*	- -	- -	- -	- -	- -	- -	- -
Ex 8a	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
Ex 8b	- -	- -	7.00 ± 0.00 *h*	- -	- -	- -	- -	- -	- -	- -
Ex 8c	- -	-	7.00 ± 0.00 *h*	- -	- -	- -	- -	- -	- -	- -
Chloramphenicol (30 µg)	26.00 ± 0.57 *d*	29.75 ± 1.18 *b*	33.75 ± 1.31 *d*	27.75 ± 0.62 *a*	27.75 ± 1.31 *a*	10.75 ± 1.49 *c*	20.50 ± 1.25 *b*	28.50 ± 0.95 *a*	30.50 ± 0.64 *b*	27.25 ± 0.85 *b*
Tetracycline (30 µg)	31.50 ± 0.28 *c*	9.25 ± 0.25 *f*	43.75 ± 2.39 *ab*	23.00 ± 0.91 *c*	26.25 ± 1.31 *b*	18.50 ± 1.32 *b*	30.75 ± 1.88 *a*	27.75 ± 1.31 *a*	28.75 ± 1.03 *c*	29.00 ± 0.70 *a*
Ampicillin (10 µg)	39.00 ± 2.48 *b*	19.00 ± 1.00 *d*	46.25 ± 2.39 *a*	14.25 ± 1.25 *d*	26.25 ± 0.75 *b*	- -	21.00 ± 2.38 *b*	- -	27.00 ± 1.08 *d*	20.75 ± 0.47 *d*
Carbenicillin (100 µg)	40.50 ± 0.28 *a*	23.75 ± 0.62 *c*	43.00 ± 2.64 *b*	25.25 ± 0.47 *b*	24.00 ± 1.29 *c*	23.25 ± 2.49 *a*	31.75 ± 1.65 *a*	- -	32.50 ± 1.70 *a*	22.75 ± 1.03 *c*
Erythromycin (15 µg)	25.25 ± 0.25 *d*	32.50 ± 2.25 *a*	38.25 ± 1.65 *c*	10.75 ± 1.18 *e*	11.50 ± 0.28 *d*	18.50 ± 5.54 *b*	11.00 ± 0.57 *c*	12.75 ± 0.85 *b*	- -	15.25 ± 2.13 *e*
Water	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

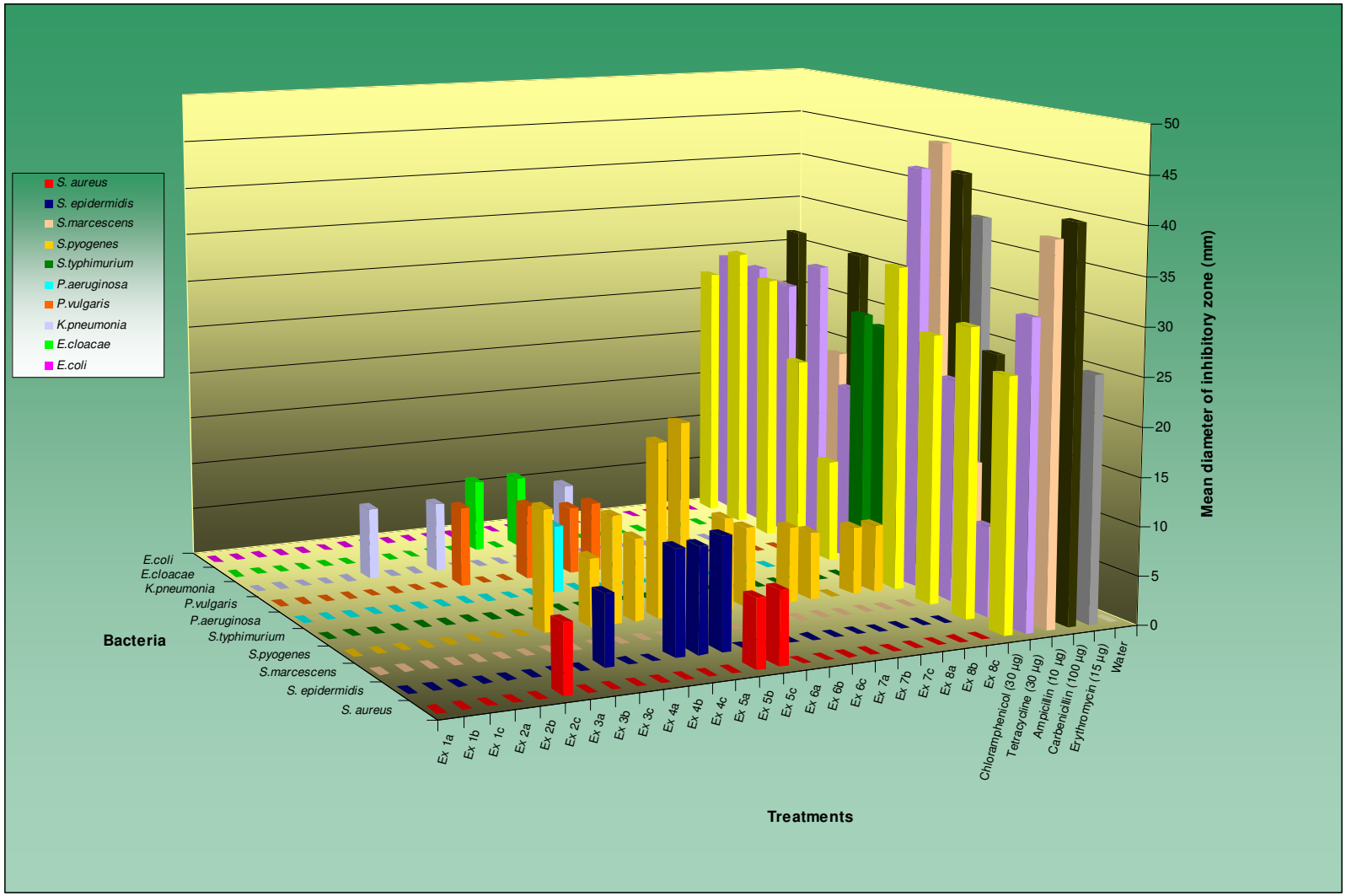

Fig 2.1 Antibacterial activity of used plant extracts and controls against ten different kinds of bacteria.

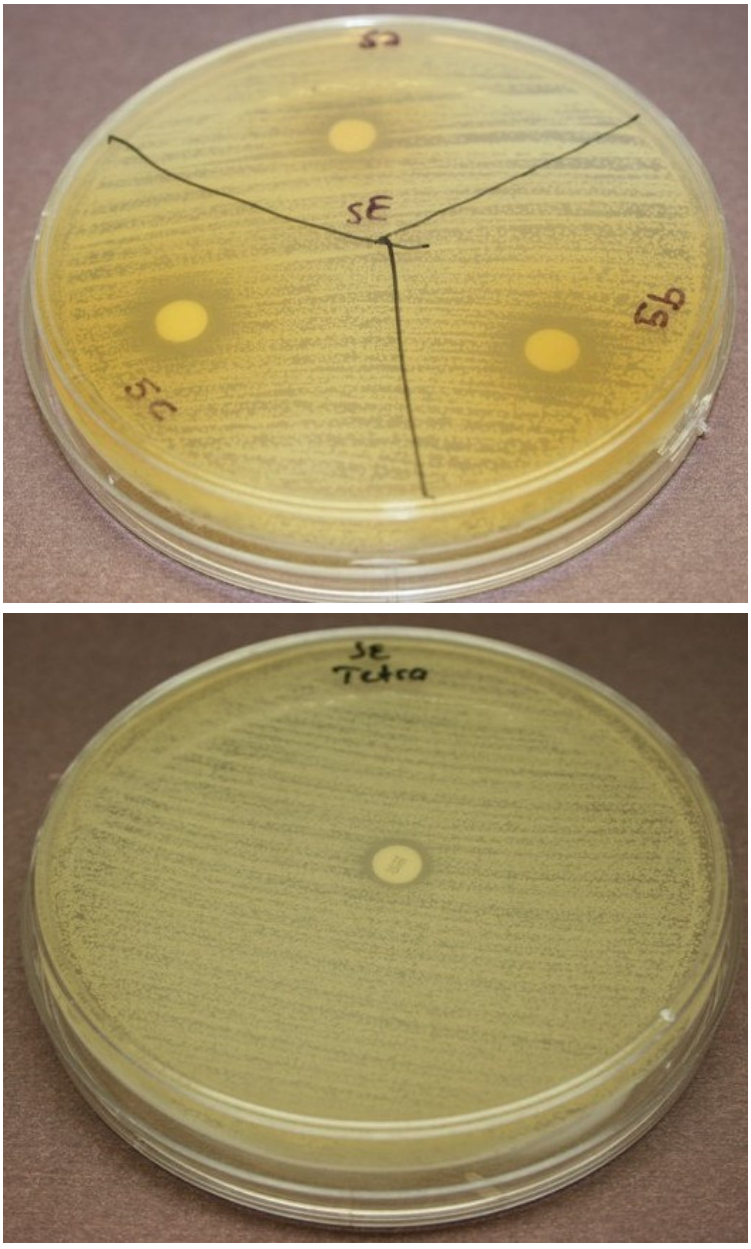

Figure 2.2 Antibacterial activity of *Trifolium pannonicum* extracts (5a: aqueous, 5b: MeOH, 5c: EtOH) against *Staphylococcus epidermidis* (above) and comparison with positive control Tetracycline (below).

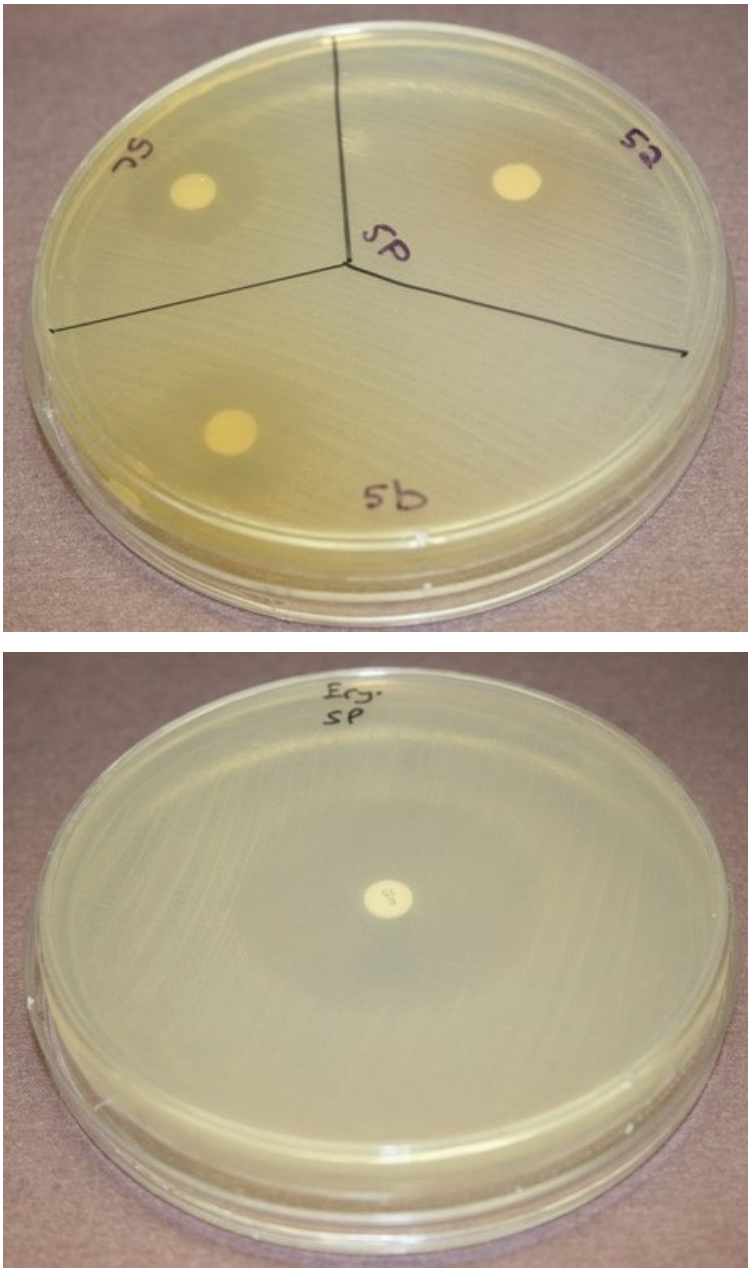

Figure 2.3 Antibacterial activity of *Trifolium pannonicum* extracts (5a: aqueous, 5b: MeOH, 5c: EtOH) against *Streptococcus pyogenes* (above) and comparison with positive control Erythromycin (below).

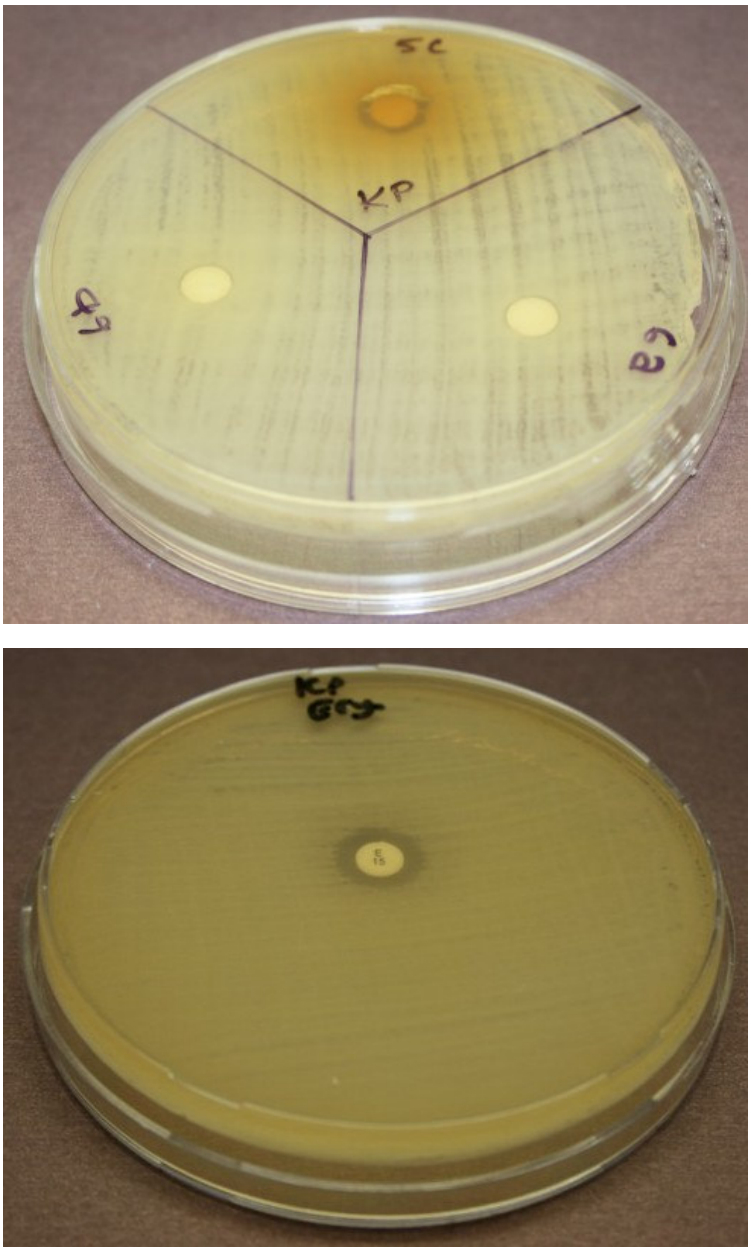

Figure 2.4 Antibacterial activity of *Trifolium pannonicum* ethanol extracts (5c) against *Klebsiella pneumoniae* (above) and comparison with positive control Erythromycin (below).

CHAPTER THREE

Antitumour Bioassay

Plants have a long history of use in the treatment of cancer. Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. While the actual compounds isolated from the plant frequently may not serve as the drugs, they provide leads for the development of potential novel agents (Cragg and Newman 2005).

3.1. Introduction

Cancer, the top killer in the world, has threatened human for centuries of years. Yet western medical treatments for cancer, including surgery, radiotherapy, chemotherapy and immunotherapy are either hard to achieve complete remission or produce severe adverse effects. In order to counteract such problems, some attentions have shifted to the discovery of new anti-cancer strategy, such as the use of oriental medicine, in the hope of producing anti-tumor effect without too many serious side effects or developing tolerance in cancer cell (Cho and Leung 2007a).

Worldwide, over ten million new cases of cancer (all sites excluding non-melonoma skin), with over six million deaths, were estimated in the year 2000. Since 1990, there has been a 22% increase in cancer

incidence and mortality with the four most frequent cancers being lung, breast, colorectal, and stomach and the four most deadly cancers being lung, stomach, liver, and colorectal (Parkin *et al.* 2001). Of all available anticancer drugs between 1940 and 2002, 40% were natural products per se or natural product-derived with another 8% considered natural product mimics (Newman *et al.* 2003).

Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves initiation, typically from DNA damaging agents, promotion, during which cell proliferation is increased, and progression involving additional genetic alterations. Chemoprevention strategies target each of these steps including anti-initiation strategies (eg., DNA repair, detoxification, free-radical scavenging and carcinogen metabolism) and anti-promotion/anti-progression strategies (eg. free-radical scavenging, proliferation suppression, differentiation induction, immunity enhancement, inflammation reduction, increase in apoptosis, altered gene expression and decrease in angiogenesis) (Balunas and Kinghorn 2005).

Herbal medicines, botanicals, dietary supplements and edible plants have all been suggested as potentially important in cancer chemoprevention due to their long history of human consumption (Park and Pezzuto 2002).

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

Crown gall tumor is a neoplastic disease of plants, which occurs in more than 60 families of dicotyledons and many gymnosperms. The disease is characterized by the transformation of normal plant cells into autonomous tumor cells in a short period of time. Once the tumor is initiated, it possesses the capacity for autonomous growth independent of the normal control mechanism of the host. The causative agents of this disease are specific strains of the Gram negative bacterium *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Binns *et al*. 1992). These bacteria are found in soil and can induce aberrant growths on a wide variety of dicotyledonous plants. *A. tumefaciens* initiates unorganized growth, termed Crown gall tumours, by causing a stable change in the cellular heredity of the affected plant cells, resulting in their uncontrolled proliferation, whereas *A. rhizogenes* initiates root forming tissues, often referred to as hairy roots, at infected wounded sites on susceptible plants (Binns and Thomashow 1988).

Crown gall is a disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. These masses (tumors) may be spongy or hard, and may or may not have a deleterious effect on the plant. The tumors produced are histologically similar to those tumors found in humans and animals. During infection of plant material

with *A. tumefaciens*, a tumor-producing plasmid (Ti-plasmid), found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. When plant tissue is wounded, it releases phenols, etc., which will activate the Ti plasmid in A. *tumefaciens*. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (Agrios 1997). Crown gall has been described as a form of genetic colonization in which the transfer and expression of a suite of *Agrobacterium* genes in a plant cell causes uncontrolled cell proliferation and the synthesis of nutritive compounds that can be metabolized specifically by the infecting bacteria. Thus, infection effectively creates a new niche suited to *Agrobacterium* survival (Escobar and Dandekar 2003).

Agrobacterium pathogenesis requires two basic elements: (1) delivery of tumorigenic DNA into the plant genome (transformation); and (2) the resultant alteration of plant cell metabolism, resulting in cell proliferation and the synthesis of nutritive compounds that provide a selective advantage for *Agrobacterium* (tumorigenesis) (Escobar and Dandekar 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 (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.

The aim of this study was to evaluate the antitumour activities of eight endemic plant species using potato disc bioassay.

3.2. Materials and Methods

3.2.1. Potato disc tumor induction assay

Antitumour activity of extracts was assessed with 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℃. Camptothecin (Sigma) (tumour suppressant) served as a positive control and 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). Suspensions of *A. tumefaciens* in PBS were standardized to 1.0 X 10^9 Colony Forming units (CFU) as determined by an absorbance value of 0.96 ± 0.02 at 600 nm.

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

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

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

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

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

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). 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 (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.

3.2.2. Bacterial viability testing

A. tumefaciens (strain B6) was cultured on Yeast Extract Media (YEM) for 2-3 days at 28 ℃. 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 (Ex 1b, 2b, 2c, 3a, 3b, 3c, 4b, 4c, 5b, 5c, 7b) 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

Twenty-four different extracts with three kinds of solvent (water, MeOH and EtOH) of eight different endemic plant species were tested in this assay in order to screen and show their potential as antitumour agents (Table 3.1; Figure 3.1).

Best antitumor activity was observed with all extracts (water, MeOH and EtOH) of *G. plicatus* (Table 3.1; Figure 3.1; 3.2). Methanolic extracts of *C. abantensis* (1b) and *P. chionaea* (7b), methanolic and ethanolic extracts of *C. ancyrensis* (2b and 2c), *C. galaticus* (4b and 4c) and *T. pannonicum* (5b and 5c) also showed strong antitumor activity (Table 3.1; Figure 3.1). Generally, alcoholic extracts (MeOH and EtOH) of all endemic plants exhibited better % tumor inhibition than aqueous extracts (Table 3.1; Figure 3.1). *E. bithynicum* and *A. gymnolobus* did not show any antitumor activity (Table 3.1).

First, all extracts' antitumor activity were tested at 100.000 mg/l. Then, extracts having best tumor inhibition (1b, 2b, 2c, 3a, 3b, 3c, 4b, 4c, 5b, 5c and 7b) were tested at 10.000 mg/l, 1000 mg/l and 100 mg/l, respectively (Table 3.2). Decreasing concentrations (from 10.000 mg/l to 100 mg/l) of all extracts of *G. plicatus* (3a, 3b and 3c) showed antitumor activity (Figure 3.2). As the concentration of all *G. plicatus* extracts decreased from 10.000 mg/l to 100 mg/l, % tumor inhibition was decreased from 100%, 96.6% or 97.2% to 0 (Table 3.2).

No tumor formation was observed with positive control camptothecin (100% inhibition) (Table 3.1; Figure 3.1; 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*) (Table 3.1; Figure 3.3).

Bacterial viability was determined by incubating extracts having best tumor inhibition (1b, 2b, 2c, 3a, 3b, 3c, 4b, 4c, 5b, 5c and 7b) with 1 X 10^3 colony-forming units (CFU) of *A. tumefaciens* bacterial suspension. Except 5b and 5c, other extracts did not affect the viability of the bacterium (Table 3.3). Thus the action of tested extracts (1b, 2b, 2c, 3a, 3b, 3c, 4b, 4c and 7b) was on the formation of tumors and not on the viability of bacterium. But, methanolic and ethanolic extracts of *T. pannonicum* affected on bacterial viability. Because of the antibacterial activity of the alcoholic extracts of *T. pannonicum*, they killed the *A. tumefaciens* and tumors were not formed on potato discs (Table 3.1; 3.2; 3.3). Although the results herein did not show an anti-tumor effects for the extracts of *T. pannonicum*, Godevac *et al*. (2008) recorded the antioxidant activity of *T. pannonicum*.

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.

Among the studied extracts of endemic plant species, *G. plicatus* extracts (3a, 3b and 3c) showed the best antitumor activity. But, there are no records about anticancer activity of *Galanthus* spp. up to now. Generally, *Galanthus* spp. has been used traditionally in Bulgaria and Turkey for neurological conditions (Howes *et al*. 2003).

Anticancer, antitumoral and antioxidant activities of *Crocus sativus* (saffron) have been reported by some studies (Assimopoulou *et al*. 2005; Hosseinzadeh and Ghenaati 2006; Magesh *et al.* 2006). Similarly, strong antitumor activity was observed with alcoholic extracts of *C. abantensis* (1b) and *C. ancyrenis* (2b and 2c) in our study (Table 3.1; 3.2; Figure 3.1).

Although antitumor activity of *A. membranaceous* was observed with some studies (Rittenhouse *et al.* 1991; Cho and Leung 2007a; Tin *et al*. 2007), we did not observe strong antitumor activity with *A. gymnolobus* (8a, 8b and 8c) (Table 3.1).

Alcoholic extracts of *C. galaticus* (4b an 4c) exhibited strong antitumor activity (Table 3.1). Similarly, Habibinya *et al*. (1993) recorded the antitumor activity of *C. arvensis.*

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; Coker *et al.* 2003). The validity of this bioassay is predicted

on the observation that certain tumorigenic mechanisms are similar in plants and animals.

Results obtained herein revealed the strong antitumor activities of *G. plicatus*. Future studies should focus on fractionation of the extracts of *G. plicatus* in hopes of identifying active components.

Table 3.1 Mean number of tumors observed with used plant extracts at a concentration of 100.000 mg/l 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	76.81	±	2.91	fg	-
Camptothecin	0	±	0		100
Ex 1a	68.64	±	2.72	f	10.6
Ex 1b	10.04	±	1.86	b	86.9
Ex 1c	33.33	±	2.49	c	56.6
Ex 2a	57.10	±	2.31	e	25.7
Ex 2b	0	±	0	a	100
Ex 2c	0.06	±	0.04	a	99.9
Ex 3a	0.12	±	0.12	a	99.8
Ex 3b	0	±	0	a	100
Ex 3c	0	±	0	a	100
Ex 4a	69.77	±	2.97	f	9.2
Ex 4b	0	±	0	a	100
Ex 4c	0.97	±	0.43	a	98.7
Ex 5a	71.08	±	3.96	f	7.5
Ex 5b	13.08	±	1.31	b	83.0
Ex 5c	10.50	±	1.96	b	86.3
Ex 6a	80.10	±	3.42	g	0.0
Ex 6b	74.70	±	3.34	fg	2.7
Ex 6c	57.12	±	2.88	e	25.6
Ex 7a	72.35	±	3.70	fg	5.8
Ex 7b	6.37	±	1.31	a	91.7
Ex 7c	59.27	±	3.40	e	22.8
Ex 8a	89.33	±	3.99	h	0.0
Ex 8b	44.45	±	3.88	d	42.1
Ex 8c	47.41	±	4.28	d	38.3

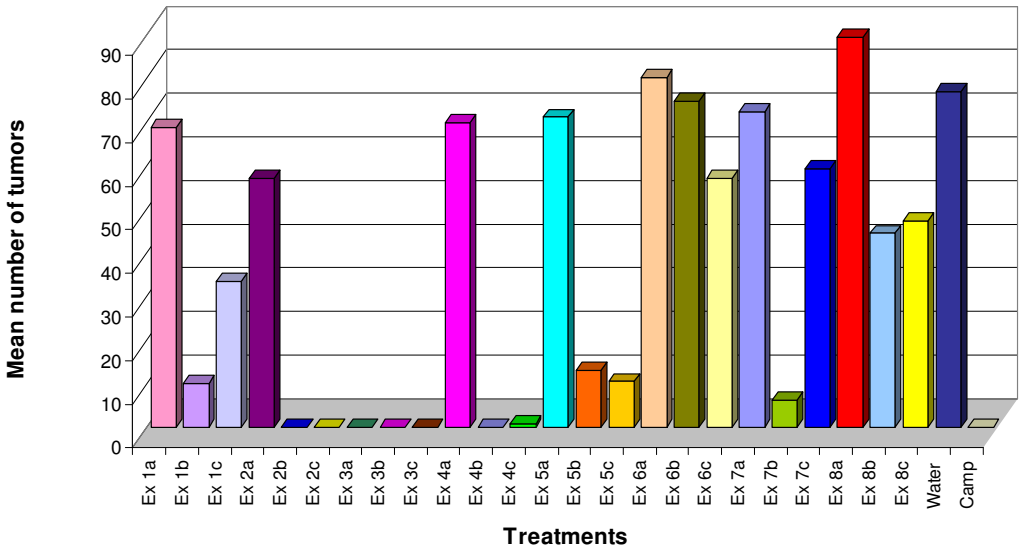

Figure 3.1 Mean number of tumors observed with used plant extracts at a concentration of 100.000 mg/l and controls (water and Camptothecin).

Table 3.2 Mean number of tumors of extracts having best antitumor activity at different concentrations (10.000 mg/l, 1.000mg/l and 100 mg/l). Means with the same letter are not significantly different at P>0.05.

Treatments	Mean Number of Tumors (±SE)				% Tumor Inhibition
Water	47.00	±	6.62		-
Camptothecin	0	±	0		100
Ex 1b (10.000)	98.42	±	9.45	hıj	0.0
Ex 1b (1000)	108.42	±	6.31	ıj	0.0
Ex 1b (100)	111.75	±	3.25	j	0.0
Ex 2b (10.000)	3.17	±	0.80	a	93.2
Ex 2b (1000)	34.25	±	8.01	bcd	27.0
Ex 2b (100)	38.33	±	7.62	bcd	18.5
Ex 2c (10.000)	38.17	±	4.66	bcd	18.7
Ex 2c (1000)	39.85	±	8.33	bcd	15.3
Ex 2c (100)	72.75	±	12.73	efgh	0.0
Ex 3a (10.000)	0.0	±	0.0	a	100
Ex 3a (1000)	22.83	±	6.35	ab	51.5
Ex 3a (100)	53.08	±	12.91	cdef	0.0
Ex 3b (10.000)	1.58	±	1.15	a	96.6
Ex 3b (1000)	26.50	±	6.59	abc	43.6
Ex 3b (100)	91.50	±	7.36	hıj	0.0
Ex 3c (10.000)	1.25	±	0.39	a	97.2
Ex 3c (1000)	20.08	±	4.14	ab	57.2
Ex 3c (100)	48.58	±	7.79	bcde	0.0
Ex 4b (10.000)	61.75	±	8.74	defg	0.0
Ex 4b (1000)	71.75	±	13.58	efgh	0.0
Ex 4b (100)	60.92	±	12.66	defg	0.0
Ex 4c (10.000)	39.17	±	8.55	bcd	16.6
Ex 4c (1000)	85.42	±	7.99	ghıj	0.0
Ex 4c (100)	97.58	±	15.13	hıj	0.0
Ex 5b (10.000)	84.75	±	6.73	ghıj	0.0
Ex 5b (1000)	96.58	±	13.21	hıj	0.0
Ex 5b (100)	83.25	±	11.71	ghı	0.0
Ex 5c (10.000)	72.50	±	9.64	efgh	0.0
Ex 5c (1000)	77.58	±	10.59	fgh	0.0
Ex 5c (100)	56.58	±	7.31	defg	0.0
Ex 7b (10.000)	19.92	±	3.34	ab	57.7
Ex 7b (1000)	34.58	±	6.09	bcd	26.4
Ex 7b (100)	62.75	±	13.14	defg	0.0

Figure 3.2 Tumors produced by *A. tumefaciens* with 10.000 mg/l (above) and 1.000 mg/l (below) *Galanthus plicatus* ethanol extract (3c).

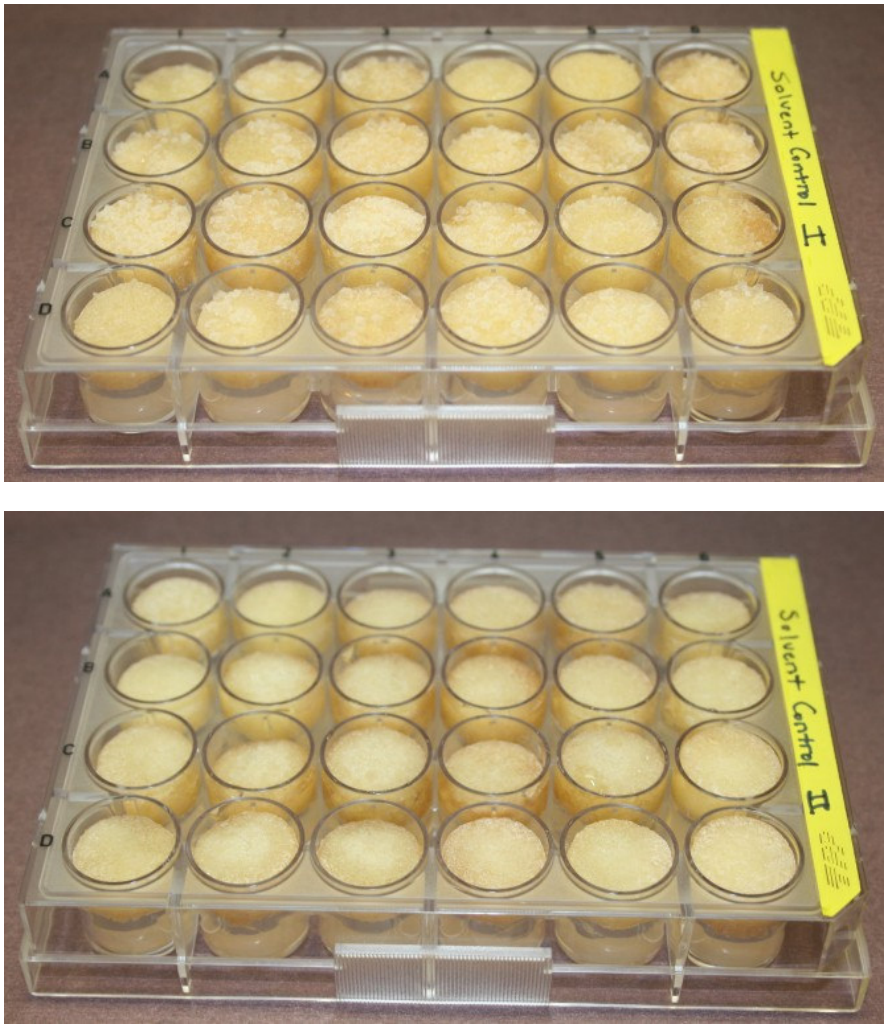

Figure 3.3 Tumors produced by *A. tumefaciens* with distilled water (solvent control I) (above) and no tumor formation with solvent control II (belove).

Figure 3.4 Tumors produced by *A. tumefaciens* with distilled water (above) and no tumor formation with positive control Camptothecin.

Table 3.3 Mean number of colony observed with some of used plant extracts having higher % tumor inhibition.

Treatments	Mean Number of colony (±SE)		
Control	33	±	7.39
Ex 1b	46	±	6.16
Ex 2b	59	±	11.72
Ex 2c	52	±	5.40
Ex 3a	49	±	10.12
Ex 3b	50	±	9.17
Ex 3c	45	±	9.71
Ex 4b	45	±	6.68
Ex 4c	28	±	5.00
Ex 5b	0	±	0
Ex 5c	0	±	0
Ex 7b	46	±	9.66

CHAPTER FOUR

Conclusions

Aqueous, methanolic and ethanolic extracts of eight endemic plant species (*Crocus abantensis*, *Crocus ancyrensis, Galanthus plicatus* subsp. *byzantinus*, *Convolvulus galaticus*, *Trifolium pannonicum* subsp. *elongatum*, *Eryngium bithynicum*, *Paronychia chionaea* and *Astragalus gymnolobus*) of Turkey were evaluated for their antibacterial and antitumour activities.

Ten pathogenic bacterial strains were selected for the antibacterial bioassay on the basis of their clinical and pharmaceutical importance: *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Proteus vulgaris, Enterobacter cloacae and Klebsiella pneumoniae* which are Gram-negative bacteria, and *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyogenes* which are Gram-positive bacteria.

Best antibacterial activity was observed with *T. pannonicum* extracts. Especially, methanolic and ethanolic extracts of *T. pannonicum* exhibited of a broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Antibacterial activity of *T. pannonicum* may explain why *Trifolium* spp. is used in folk medicine to treat skin conditions (caused by

S. epidermidis). Generally, ethanolic extract of *T. pannonicum* was better than aqueous and methanolic extracts of this plant against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *P. vulgaris* and *K. pneumoniae*.

All extracts of endemic plant species were not effective against *S. marcescens*, *S. typhimurium* and *E. coli* which are Gram-negative bacteria. *C. abantensis* did not show any inhibitory activity against all used pathogens. *S. pyogenes* was the most susceptible bacterium.

There was no inhibition with control solvent (water) and bacterial growth was generally sensitive to the reference antibiotics tested.

All extracts (water, MeOH and EtOH) of *G. plicatus* showed best antitumor activity. Generally, better antitumor activities were observed with alcoholic extracts. Alcoholic extracts (methanolic or ethanolic) of *C. abantensis, C. ancyrensis, P. chionaea*, *C. galaticus* and *T. pannonicum* also exhibited strong antitumor activity. Antitumor activity was not observed with *E. bithynicum* and *A. gymnolobus*. 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 *C. abantensis*, *C. ancyrensis*, *G. plicatus*, *C. galaticus* and *P. chionaea* was on the formation of tumors and not on the viability of bacterium. But *T. pannonicum* extracts affected the viability of *A. tumefaciens* and concluded that this plant's action was on the viability of bacterium.

The scientific verification of biological activity of endemic plant species may be important to screen the potential value of endemics in Turkey. Biological activities of studied endemic plant species have not been reported before and the results obtained herein reveal the antibacterial and antitumor activities of eight endemic plant species. Future studies may focus on fractionation of extracts having strong antibacterial and antitumor activities to identify active components. Moreover, in the future, *in vivo* anticancer studies may be conducted to test the extracts having best antitumor activities.

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