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A STUDY ON ANTIMICROBIAL, ANTIOXIDANT AND ANTIMUTAGENIC ACTIVITIES OF *ELAEAGNUS ANGUSTIFOLIA* L. LEAVES

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Abstract

Backround: The aim of this work was to investigate the antimicrobial, antioxidant, and antimutagenic potentials of methanol extracts from *E. angustifolia*.
Materials and Methods: Methanol extracts were screened for antimicrobial activity against different species of 4 Gram positive and 3 Gram negative bacteria and one fungus. These bacteria included food pathogens. The leaf extract was tested using disc diffusion assay.
Results: The methanol extract of *E. angustifolia* showed maximum inhibition zone of 16 mm against *Yersinia enterocolitica*. Whereas, the inhibition zone was not determined by methanol extract against *Escherichia coli* ATCC 1122 and *Candida albicans* RSKK 02029. The MIC was evaluated on plant extracts as antimicrobial activity. All of bacterial strains showed the lowest sensitivity to methanol extract of E. *angustifolia* (3.5 mg/mL), except *Yersinia enterocolitica* NCTC 11174. In addition, the plant extracts were tested against the stable DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free-radical. Finally, the methanol extract displayed a strong antioxidant activity (Trolox equivalent = 1.49 mM). Also, *E. angustifolia* methanol extracts were screened for their antimutagenic activity against sodium azide by Ames test in absence of rat microsomal liver enzyme (-S9). The results showed that *E. angustifolia* methanol extracts can inhibit mutagenic agents of sodium azide. The plant leaf extracts with the inhibition of 36% sodium azide showed moderate potential in decreasing mutagenic agents in *Salmonella typhimurium* TA100.
Conclusion: *E. angustifolia* methanol extracts have antimicrobial, antioxidant and antimutagenic potential.

Keywords: *Elaeagnus*, antimicrobial activity, antioxidant activity, antimutagenic activity

Introduction

Medicinal plants are natural resources, yielding valuable herbal products which are often used in the treatment of various ailments (Grabley and Thiericke, 1999). *Elaeagnus angustifolia* (Russian olive, Russian silverberry, Oleander) is one of these herbs (Farahbakhsh *et al*., 2011). *E. angustifolia* is a member of *Elaeagnaceae* family. *E. angustifolia* contains flavonoids compounds, sitosterols, cardiac glycosides, terpenoids (Burgess, 2008; Beigom Taheri *et al*., 2010). Flavonoids, a group of low molecular weight polyphenolic substances including flavone, flavonols, isoflavone, flavanol, flavanone, anthocyanin and proanthocyanidin, widely exist in fruits and vegetables. In fact, these flavonoid compounds have attracted the attention of food and medical scientists because of their strong *in vitro* and *in vivo* antioxidant activities and their ability to scavenge free radicals, break radical chain reaction and chelate metals (Liu and Yao, 2007). Up to now, nine flavonoids including (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, kaempferol, quercetin, luteolin, isorhamnetin and isorhamnetin-3-*O*-β- D--galactopyranoside have already been isolated and identified from this plant (Si *et al*., 2009; Wang and Wei, 2010). Medicinal plants represent a rich source of antimicrobial agents. These plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava *et al*., 1996). According to the World Health Organisation, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al*., 2000).

Many plants have been used due to their antimicrobial traits, due mainly to compounds synthesised in the secondary metabolism of the plant. The antimicrobial activity of *E. angustifolia* leaves has not been studied. The *in vitro* antimicrobial activity of leaves parts of the plant growing in Mugla was evaluated using disc diffusion method. Additionally, antioxidant and antimutagenic activities of *E. angustifolia* have not been reported. In this study, the plant leaves methanol extracts were investigated for antimicrobial, antioxidant and antimutagenic activities.

Material and Methods

Plant material

E. angustifolia samples were collected from Mugla Sıtkı Kocman University Campus on July in 2012. The plant material was authenticated by Olcay Ceylan, and a specimen was deposited in the herbarium of the Biology Department of the Mugla Sıtkı Kocman University, Turkey. The identification of these specimens was carried out using the Flora of Turkey (Davis, 1965). The leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water. Fresh plant material was air-dried. The dried leaves were powdered in a blender. All samples were stored at ambient temperature until initial sample preparation, after which they were stored at 4°C until required for analysis.

Plant extraction

The air dried and powdered leaves of the plant samples (10 g) were extracted with methanol (100 mg/mL) using the Soxhlet apparatus. The extract was evaporated and then extracted in metanol and then kept in small sterile opac bottles under refrigerated conditions until used.

Microorganisms and cultivation

The extracts of plant leaves were individually tested against food pathogenic strains such as *Bacillus subtilis* RSKK 245, *Staphylococcus aureus* RSKK 2392, *Salmonella typhimurium* RSKK 19, *Enterococcus faecalis* ATCC 8093, *Escherichia coli* ATCC 11229, *Listeria monocytogenes* ATCC 7644, *Yersinia enterocolitica* NCTC 11174 and *Candida albicans* RSKK 02029. The bacteria were grown for 24h at 37°C

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in Mueller-Hinton Broth (Merck). *C. albicans* was grown for 24- 48 h at 30°C in Sabouraud Dextrose Broth (Merck). These strains of bacteria and *C. albicans* were obtained from ATCC (American Type Culture Collection, USA), RSKK (Refik Saydam National Type Culture Collection, Turkey) or NCTC (National Collection of Type Cultures).

Antimicrobial activity assay

Bauer-Kirby method was applied for antimicrobial activity. The leaf methanol extract was tested using disc diffusion assay. The bacteria were maintained on Mueller-Hinton agar plates (MHA, Merck) at 37°C and fungus was maintained on Sabouraud Dextrose agar plates (SDA, Merck) (Bauer *et al.*, 1966). Bacteria and *C. albicans* cultures adjusted to 0.5 McFarland. This experiment was performed in triplicate. Incubations were at 37°C for 24h for bacteria. *C. albicans* incubated at 30°C for 24h. After incubation, the inhibition zones formed and were measured. Methanol was used as negative control. Tetracycline (30µg), chloramphenicol (30µg), vancomycin (30 µg), nystatin (100µg), penicillin (10µg), ampicillin (10µg), and erythromycin (15µg) antibiotics were used as positive control.

Determination of minimum inhibitory concentration (MIC)

The MIC was evaluated on plant extracts as antimicrobial activity. The MIC was taken as the lowest concentration that inhibited growth after incubation. The serial dilution assay was performed as described in the CLSI standards (CLSI, 2003; CLSI, 2006). This test was performed at final concentrations of each extract (6.5; 3.25; 1.625; 0.812; 0.406 mg/mL).

***In vitro* Antioxidant Activity**

The antioxidant activities were determined using DPPH as a free radical. Extract (0.1 ml) was added to 3.9 mL of a 0,1 mM methanol DPPH solution. After incubation for 30 minutes, absorbance of extract was determined at 515 nm using spectrophotometer. DPPH in methanol was used as control (Brand-Williams *et al*., 1995). DPPH radical scavenging activity was determined using the following formula:

DPPH radical scavenging activity (%) = [Abs (control) – Abs (extract)] × 100.

***In vitro* Antimutagenic Activity**

Antimutagenic activity was evaluated by the *Salmonella*-microsome assay, using the *Salmonella typhimurium* tester strains TA98, and TA100, kindly provided by Dr. B.N. Ames (Berkeley, CA, USA), without (−S9) metabolisation by the pre-incubation method (Maron and Ames, 1983). The *Salmonella* histidine point mutation assay of Maron and Ames (1983) was used to test the antimutagenic activity of extracts without S9 mix. In this study, two different tester strains were employed to measure the antimutagenicity of *E. angustifolia* methanol extracts. These strains included *Salmonella typhimurium* TA 98 and TA 100. The calculation percentage of inhibition was done according to the formula given by Ong et al (1986): Antimutagenic Percentage = [1-T/M] ×100; where T is number of revertants per plate in presence of mutagen and test sample and M is number of revertants per plate in positive control (Ong *et al*., 1986).

Results

The antimicrobial activity of *E. angustifolia* leaf methanol extracts was evaluated *in vitro* against 8 microorganisms test species, which are known to cause some diseases in foods. Results of antimicrobial activity of methanol extract of used plant against the test bacteria are shown in Table 1. Besides, the inhibition zone diameters of the reference antibiotics to the test microorganisms are shown in Table 2.

Results show that the methanol extract of plant leaf inhibited the growth of six bacteria and the inhibition zones ranged between 9-16 mm. In addition, the methanolic extract of this plant did not determine anticandidal effect against used yeast. Furthermore, methanol extract of *E. angustifolia* did not show antimicrobial effects against used one Gram negative bacterium. Results also show that the methanol extracts of the plant leaves inhibited the growth of 3 bacteria with 9 mm inhibition zones.

The results of antimicrobial activity, recorded as zone of inhibition in mm for all the materials used, are as follows: Methanol extract of the leaf was found to be highly effective against all of the Gram positive and negative bacteria, except *E. coli* ATCC 1122 (Table 1). The maximum zone of inhibition was produced by methanol extract against *Yersinia enterocolitica* NCTC 11174 (16 mm). Whereas, the inhibition zone was not determined by methanol extract against *Escherichia coli* ATCC 1122 and *Candida albicans* RSKK 02029. *Escherichia coli* ATCC 1122 and *Candida albicans* RSKK 02029 were found resistant to the methanol extract. The extract was found equally effective in inhibiting the growth of *Bacillus subtilis* RSKK 245, *Staphylococcus aureus* RSKK 2392 and *Salmonella typhimurium* RSKK 19.

Tetracycline (30µg), chloramphenicol (30µg), vancomycin (30 µg), nystatin (100µg), penicillin (10µg), ampicillin (10µg), and erythromycin (15µg) antibiotics were used as positive control. Tetracycline and chloramphenicol very strongly inhibited the growth of *Yersinia enterocolitica*, whereas chloramphenicol exhibited a very big zone of inhibition against *Salmonella typhimurium* and *E. coli* (Table 2).

Table 1: Antimicrobial activity of *Elaeagnus angustifolia* leaf extracts

Microorganisms	İnhibition zone (mm)
Bacillus subtilis RSKK 245	9
Staphylococcus aureus RSKK 2392	9
Listeria monocytogenes ATCC 7644	11
Enterococcus faecalis ATCC 8093	10
Escherichia coli ATCC 1122	-
Yersinia enterocolitica NCTC 11174	16
Salmonella typhimurium RSKK 19	9
Candida albicans RSKK 02029	-

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Table 2: Inhibition zone diameters of the reference antibiotics to test microorganisms

Microorganisms	Antibiotics (inhibition zone- mm)						
	TE	**C**	**VA**	**NS**	**P**	**AM**	**E**
Bacillus subtilis RSKK 245	(nt)	(nt)	(nt)	(nt)	10	10	(nt)
Staphylococcus aureus RSKK 2392	(nt)	(nt)	(nt)	(nt)	(nt)	10	(nt)
Listeria monocytogenes ATCC 7644	(nt)	(nt)	(nt)	(nt)	10	12	(nt)
Enterococcus faecalis ATCC 8093	(nt)	(nt)	(nt)	(nt)	(-)	(-)	(nt)
Escherichia coli ATCC 11229	14	20	(nt)	(nt)	10	(nt)	(nt)
Yersinia enterocolitica NCTC 11174	20	30	(-)	(nt)	(nt)	(nt)	(nt)
Salmonella typhimurium RSKK 19	14	21	(-)	(nt)	(nt)	(nt)	(nt)
Candida albicans RSKK 02029	(nt)	(nt)	(nt)	7	(nt)	(nt)	(nt)

TE: Tetracycline (30µg); C: Chloramphenicol (30µg); VA: Vancomycin (30 µg); NS: Nystatin (100µg);
P: Penicillin (10µg); AM: ampicillin (10µg); E: Erythromycin (15µg); (-) : zone did not occur; (nt) : not tested

Table 3 shows MICs of E. *angustifolia* leaf methanol extracts obtained by using the serial dilution method. All of bacterial strains showed the lowest sensitivity to methanol extract of E. *angustifolia* (3.25 mg/mL), except *Yersinia enterocolitica* NCTC 11174 (6.5 mg/mL).

Table 3: Minimum inhibitory concentration of methanolic extracts of E. *angustifolia*

Bacteria	**MIC (mg/mL)**
Bacillus subtilis RSKK 245	3.25
Staphylococcus aureus RSKK 2392	3.25
Salmonella typhimurium RSKK 19	3.25
Enterococcus faecalis ATCC 8093	3.25
Escherichia coli ATCC 11229	3.25
Listeria monocytogenes ATCC 7644	3.25
Yersinia enterocolitica NCTC 11174	6.5
Candida albicans RSKK 02029	3.25

The antioxidant activity of plant extract was evaluated by the DPPH radical scavenging capacity. Table 4 shows the percent of DPPH radical scavenging capacity with trolox as reference. The extract showed 57% inhibition at 100 mg/mL concentration (Table 4).

Table 4: DPPH radical scavenging capacity of *E. angustifolia* leaf extract

Plant material (100 mg/mL)	Trolox equivalent (mM)
57 ± 0.08	1.49

The results obtained from studies on the antimutagenic potential of *E. angustifolia* leaves are presented in Table 5. To evaluate the antimutagenicity of *E. angustifolia*, diagnostic mutagens were incorporated in the sample plates. In this study, two different tester strains were employed to measure the antimutagenicity of the plant leaves. *E. angustifolia* leaf extract (100 mg/plate) showed a weak positive inhibitory effect (36%) for *Salmonella typhimurium* TA 100. On the other hand, the extract determined mutagenic effect for *Salmonella typhimurium* TA 98 (Table 5).

Discussion

Medicinal plants have traditionally been used worldwide for the treatment of various human diseases (Chitme *et al*., 2004). They have proved to be abundant sources of biologically active compounds, many of which have been used as compounds to develop new pharmaceuticals (Palombo, 2011). *E. angustifolia* was selected based on its relevant ethnomedical use (Baytop, 1999; Bulut *et al*., 2010).

In the present study, the extract of plant leaf obtained in methanol was tested against 8 types of the test organisms. The antimicrobial activity was compared with the standard antibiotics. However, the methanol extract showed maximum inhibition against *Yersinia enterocolitica* (Table 1). Although the antibacterial activities of essential oils from many plant species have been extensively surveyed, antimicrobial mechanism of this plant has not been reported in great detail. Since the active antimicrobial compounds of essential oils are phenolics and terpenes in nature (Jansen *et al*., 1987; Saxena *et al*., 1994), it seems reasonable that their mode of action might be similar to that of other phenolic compounds. Flavonoids and phenolic compounds have already been reported in plants (Chopra *et al*., 1986). These compounds have antibacterial and antifungal activities. In this study, methanol extracts of the leaves were found to be effective against *S. typhimurium* RSKK 19

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Table 5: Antimutagenic effect of E. angustifolia leaf extract (100 mg/plate)

Test subtances	*Salmonella typhimurium* TA 98 + S9$^-$		*Salmonella typhimurium* TA 100 + S9$^-$	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Control	42±3		42±3	
Positive control (sodium azid)	80±5		251±5	
Negative control (metanol)	43±1		43±1	
E. angustifolia leaf extract	66±1	Mutagenic	161±35	36

(Table 1). Ratha et al. (2012) reported that plant roots extracts of *Hemidesmus* sp. and *Vetiveria* sp. exhibited highest antibacterial activity on the growth of *Salmonella typhi.* In this study, leaf methanol extract was found to be lowest effective against *S. aureus* RSKK 2392 (Table 1). Similar results with *Eclipta alba* leaf extract were obtained by Peraman *et al*. (2011).

Phenolic compounds have been extensively investigated in the past 30 years. They have one or more aromatic rings bearing hydroxyl groups that are potentially able to act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Rice-Evans *et al*., 1996; Mattei *et al*., 1998). Phytochemical studies show that aqueous fruit extract of *E. angustifolia* contains flavonoids compounds, such as sitosterols, cardiac glycosides, terpenoids (Burgess, 2008; Beigom Taheri *et al*., 2010). Flavonoids play different roles in biological systems. The flavonoids, due to their phenolic hydroxyl groups and their capacity to chelate metals, have antioxidant activities, and can reduce levels of free radicals and reduce lipid peroxidation. Also, this extract has antioxidant activity which are believed to be responsible for the therapeutic effects of this plant (Robak and Gryglewski, 1996). In this study, the methanol extract showed 57% free radical inhibition at 100 mg/ml concentrations (Table 4). Similar results with *Alseodaphne semecarpifolia* leaf extract were obtained by Charles *et al*. (2012). Another research found that guarana seed methanol extract had high antibacterial activity, as well as possessing high phenolic contents (Majhenic *et al*., 2007). The screening of plant extracts using the DPPH free radical method proved to be effective for the selection of those which could have an antioxidant activity.

These extracts may be rich in radical scavengers, such as flavonoids known as antioxidants. It has been reported that free radical scavenging and antioxidant activities of many medicinal plants are responsible for their therapeutic effect against cancer, tissue inflammatory, cardiovascular disease (Cai *et al*., 2004).

In our study, *E. angustifolia* leaf extract (100 mg/plate) showed a positive inhibitory effect (42%) for *Salmonella typhimurium* TA 100 (Table 5). In general, compounds that strongly inhibit the mutagenicity of indirectly and directly acting mutagens also have a high free radical scavenging activity (Chung *et al*., 2007; Chung *et al*., 2008). From the above results, it can be concluded that plant extracts have great potential as antimicrobial, antioxidant and antimutagenic compounds against food pathogens. The significant antimutagenic activity showed by *E. angustifolia* provides a scientific validation for the traditional use of this plant. Our work suggests that the *E. angustifolia* leaf may be utilised as effective and safe antioxidant, antimutagen and antibacterial source. However, searching for further bioactive compounds which are responsible for the biological activities of *E. angustifolia* is needed.

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