ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF ERICA BOCQUETII P. F. STEVENS AND *ERICA ARBOREA* L.

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ABSTRACT

The aim of this study was to investigate *in vitro* antimicrobial, antioxidant, and cytotoxic activities of *n*-hexanoic, ethanolic, methanolic, ethyl acetate, and aqueous extracts of the aerial parts of *Erica arborea* L. and *Erica bocquetii* P.F. Stevens known to be endemic species for Turkey. The antimicrobial activity was investigated by disc diffusion method, and the minimal inhibitory concentration (MIC) by Broth Microdilution method. The antioxidant activity was determined by an improved assay based on the decolorization of the radical monocation of [2,2/-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] ($ABTS^{+}$). The cytotoxic activities were evaluated using Brine shrimp bioassay. The all extracts both species of *Erica* were also found to possess antimicrobial activity against some of the bacteria, but no activity was observed against the yeast. The antioxidant potentials of the extracts of *E. bocquetii* were obtained two times higher than those of *E. arborea. Erica* extracts did not exhibit the cytotoxic activity against Brine shrimp.

Key Words: *Erica bocquetii*, *Erica arborea*, Antioxidant Activity, Antimicrobial Activity, Cytotoxicity, Brine Shrimp

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ERICA BOCQUETII P. F. STEVENS AND *ERICA ARBOREA* L.' NIN ANTİOKSİDAN, ANTİMİKROBİYAL VE SİTOTOKSİK AKTİVİTELERİ

ÖZET

Araştırmanın amacı *Erica arborea* L. ve Türkiye için endemik olarak bilinen *Erica bocquetii* P.F. Stevens' in toprak üstü kısımlarının *n*-hekzan, etanol, metanol, etil asetat ve su ekstrelerinin *in vitro* antimikrobiyal, antioksidan ve sitotoksik aktivitelerini araştırmaktır. Antimikrobiyal aktivite disk difüzyon metodu ve Broth Tüp Dilüsyon metodunun minimum inhibitör konsantrasyonu (MIC) ile araştırılmıştır. Antioksidan aktivite [2,2/-azinobis-(3-etilbenzotiazolin-6-sülfonik asit)] ($ABTS^{+}$) radikal mono katyonunun dekolorizasyonu esas alınarak yapılan bir analiz ile belirlenmiştir. Sitotoksik aktiviteler Brine shrimp biyolojik denemeleri ile gerçekleştirilmiştir. *Erica* türlerinin tüm ekstrelerinin bazı bakterilere karşı antimikrobiyal aktiviteye sahip olduğu bulundu, fakat mayaya karşı aktivite gözlenmedi. *E. bocquetii* ekstrelerinin antioksidan potansiyelleri *E. arborea'* nınkilerden iki kat yüksek olduğu saptandı. *Erica* ekstreleri Brine shrimp' e karşı sitotoksik aktivite göstermemiştir.

Anahtar Kelimeler: *Erica bocquetii*, *Erica arborea*, Antioksidan Aktivite, Antimikrobiyal Aktivite, Sitotoksisite, Brine Shrimp.

INTRODUCTION

Medicinal plants have been well-known since antiquity to posses notable biological activity, including antioxidant, antimicrobial and cytotoxic properties (1). Antioxidants may have an important role in prevention of diseases such as cancer, aging, neurodegenerative disease, malaria, arteriosclerosis, and pathological events in living organisms (2). There is an increasing interest in the antioxidant effects of compounds derived from plants, which could be relevant in relation to their nutritional incidence and their role in health and disease (3-7). Epidemiological statistics show that cancer and infectious diseases are important causes of morbidity and mortality throughout the world (6). Many of these health problems could be avoided by improving sanitation conditions or better management of illness. The emergence of multidrug-resistant pathogens has been well-documented (9-11). Consequently, patients, infected with resistant strains are extremely difficult to cure and their treatments are much more toxic and expensive. Drug resistance is also a problem with cancer cells (12). For this reason, new anti-infective and anticancer agents need to be developed. In searching for new antioxidants and antibiotics, biological screening and bioassay/guided separation of medicinal plant extracts are well-accepted methods for determining the active constituents responsible for the effectiveness of herbal remedies. The chemodiversity offered by plants is every promising source for new compounds in the areas of cancer and infectious diseases (13).

The genus *Erica* L. (Ericaceae) is represented in the Turkish flora by four species. *Erica bocquetii* P.F. Stevens is an endemic taxon of Turkey (14). *Erica arborea* L. and *E. bocquetii* P.F. Stevens, commonly known as “funda”, are commonly used in Turkish medicine as diuretic, urinary antiseptic and against constipation (15, 16). The flowers of *Erica arborea* L. (Ericaceae) have traditionally been used in the Canary Islands as hypotensor, anti-inflammatory, urinary antiseptic, and diuretic (17) used to treat the wounds in Spain (18).

The constituents of *Erica* are mainly flavonoids (19), phenolic acids (20) and triterpenoids (21), the latter including tannins (22, 23).

Therefore, the aim of this study was to evaluate, the antioxidant, antimicrobial, and cytotoxic activities of the *n*-hexane, ethanol, methanol, ethyl acetate and water extracts of the aerial parts extracts of *E. bocquetii*, and *E. arborea*.

MATERIALS AND METHODS

I. Collection of Plants

Collection sites, dates and the herbarium numbers of *Erica bocquetii* P.F. Stevens known to be endemic species for Turkey and of *Erica arborea* L. (Ericaceae) were listed in Table 1. Samples were identified by S.Gökhan ŞENOL. Voucher specimens have been deposited in the herbarium of the Department of Pharmacognosy, Ege University.

Table 1. Collection Site, Date And The Herbarium Numbers Of The *Erica* Species

Plant	Colection Site	Collection Data	Herbarium No
Erica bocquetii	Elmalı- Akçam (Antalya)	06/2003	1322
Erica arborea	Gümüldür- (İzmir)	08/2003	1324

II. Preparation of Extracts

The aerial parts of the plants were dried at room temperature and then reduced to coarse powder. 20 g of each sample was extracted with 100 mL of *n*-hexane, ethanol, methanol and ethyl acetate at room temperature, with stirring for 2 days. The water extracts were prepared by % 2 decoction. The resultant extract solution is evaporated to dryness. Sample solutions were prepared by dissolving the dried extracts in their solvents (5 mg/mL).

III. Test Microorganisms and Artemia salina (Leach)

Escherichia coli ATCC 29998, *Escherichia coli* ATCC11230, *Salmonella thyphimurium* CCM 5445, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228 were used as bacteria and *Candida albicans* ATCC 10239 as yeast-like fungi for testing antimicrobial activity. Lyophilized bacteria and the yeast were obtained from the Department of Basic and Industrial Microbiology, Faculty of Science, Ege University.

The cytotoxic assays were performed with *Artemia salina* (Leach) larvae (Brine shrimp Eggs, San Francisco, CA, USA).

IV. Antimicrobial Activity

A.Disc Diffusion Assay

The disc diffusion method, known as the Kirby Bauer method, was used to determine antimicrobial activities (24-26). Overnight cultures containing 10^8 CFU (Colony Forming Unit) /mL of bacterial strains were used and diluted with sterile distilled water to obtain equivalent to 0.5 Mc Farland's standards of turbidity. Overnight cultures of the yeast were prepared in Sabouraud Dextrose Broth to obtain 10^7 CFU / mL. The 40 μL of reconstituted crude extracts were absorbed onto sterile 6 mm discs (Oxoid Antibacterial Susceptibility Blank Tests Disc) under aseptic conditions to obtain 100 μg extract/disc and dried at 50ºC. The discs were transferred onto plates containing test organisms with sterile forceps. Negative control discs was prepared with 40 μL of sterile 10% aqueous DMSO (dimethyl sulphoxide). Agar plates containing bacteria were incubated at 37 °C for 24 h and those containing yeast at 27 °C for 48 h. The standard antibacterial agent Ceftazidime (30 μg/disc) was used as a positive control for bacteria and the standard antifungal agent Nystatin (25 μg/disc) was used as the positive control for yeast. Also, antimicrobial activity of each solvent used for extraction was tested.

B.Broth Microdilution Assay

The minimal inhibitory concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to the extracts in disc diffusion assay. The inocula of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to a 0.5 McFarland standard turbidity. All the extracts dissolved in 10% dimethyl sulphoxide (DMSO) were first diluted to highest concentration (2.5 mg/mL) to be tested, and then serial two-fold dilution were made in a concentration range of 0.0195-2.5 mg/mL in sterile water. MIC values of the extracts against bacterial strains were determined based on a microwell dilution method (27) and described with some modifications as follows.

The 96-well plates were prepared by dispensing into each well 95 μL of Mueller-Hinton broth and 5 μL of the inocula. 100 μL from extracts initially prepared at the concentration of 2.5 mg/mL was added into the first wells. Then, 100 μL from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μL of Mueller- Hinton broth without compound and 5 μL of the inoculum on each strip was used as negative control. The final volume in each well was 200 μL. Ceftazidime at the concentration range of 400-0.39 μg/mL was prepared in sterile water and used as standard drug for positive

control, and the DMSO was maintained as negative control. MIC values were defined as the lowest extract concentration that prevents visible bacterial growth after 24 h of incubation at 37 °C. Nystatin was used as a reference, and appropriate controls without extracts and solvents were used. Each experiment was repeated at least three times.

V.Assay for Cytotoxic Activity

Cytotoxicity was evaluated by the brine shrimp lethality bioassay (28). The sea salt (3.8 g) was dissolved in 100 mL water and filtrated. Brine shrimp (*Artemia salina*) eggs were placed seawater and allowed to incubate for 48 h at 28 ^{o}C in a small tank. Each extract was tested at 1000, 100 and 10 ppm. 20 mg plant extract was dissolved in 2 mL of chloroform (10 mg/1mL). 500, 50 and 5 µl of this solutions are transferred to final concentrations of 1000, 100 and 10 ppm, respectively. Also, the vials including chloroform and extraction solvents (500 µL) were prepared as controls. After incubation, 10 brine shrimp larvae (nauplii) were introduced into vials containing graded concentrations (ranging from 10 to1000 ppm) of the test extracts. After 24h, the number of surviving shrimps at each concentration of the extracts was counted and data analyzed with Finney Computer Program to determine the LC_{50} at 95% confidence interval. Cytotoxic activity of all extracts was compared with umbelliferone and colchicine as the cytotoxic active substances (29, 30).

VI. Antioxidant Activity

The trolox equivalent antioxidant capacity (TEAC) of plant extracts was determined by an improved assay based on the decolorization of the radical monocation of [2,2$^{/}$-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] ($ABTS^{\cdot+}$) (31). Trolox was used as an antioxidant standard. ABTS stock solution (7 mM concentration) and 2.45 mM potassium persulfate were left at room temperature for 16 h to produce ABTS radical cation ($ABTS^{+}$). This solution was diluted with ethanol to an absorbance value of 0.700($\pm$ 0.02) at 734 nm and pre-incubated at 30^{o}C prior to use.

1 g of sample was homogenized in 50 mL of absolute methanol and resulting homogenate was filtered. After addition of 1.0 mL of diluted $ABTS^{+}$ solution to 10 µl of *Erica* extracts or trolox standards in ethanol, the decrease in absorbance at 734 nm was monitored at 30 ^{o}C exactly 6 min after initial mixing. The solvent blanks were run in each assay. All determinations were performed in triplicate. A dose-response curve was plotted for

trolox, and antioxidant ability was expressed as the trolox equivalent antioxidant capacity (TEAC).

VII. Phytochemical Screening

Yields and results of the phytochemical screening are given in Table 2 (32, 33).

Table 2. Yields and phytochemical screening of *E. bocquetii* and *E. arborea*

Plants	Extracts	Yields (%)	Alkaloids	Flavonoids	Tannins	Saponins (Steroidal)	Saponins (Triterpenic)	Starch
E.bocquetii	hexane	2.6	-	-	-	-	-	-
	EtOAC	4.2	-	+	-	+	-	-
	EtOH	6.2	-	+	+	+	+	-
	MeOH	9.8	-	+	+	+	+	-
	Water	9.2	-	+	+	+	+	-
E. arborea	hexane	5.8	-	-	-	-	-	-
	EtOAC	7.8	-	+	-	+	-	-
	EtOH	12.0	-	+	+	+	+	-
	MeOH	7.9	-	+	+	+	+	-
	Water	12.6	-	+	+	+	+	-

+, present; and -, absent

RESULTS AND DISCUSSION

Table 3, 4 and 5 show the antimicrobial, cytotoxic, and antioxidant activities of the *n*-hexanoic, ethanolic, methanolic, ethyl acetate, and aqueous extracts of the aerial parts extracts of *E. bocquetii* and *E. arborea*, respectively. In vitro antimicrobial activities of the extracts of *Erica* species were evaluated by using two different methods at the same time and the results were displayed in Table 3.

The minimal inhibition concentration (MIC) values were studied for the extracts by disc diffusion assay. All of the extracts of *E. bocquetii* and *E. arborea* inhibited *Escherichia coli* ATCC 11230 G. The MIC fluctuated in a range of 31.25-62.50 µL/mL of extracts. However, the growth of *Escherichia coli* ATCC 29998 was inhibited by all of the extracts except to *n*-hexane extracts of *E. bocquetii* and *E. arborae*. The various extracts of *E. bocquetii* and *E. arborea* were determined to be similarly effective to that of Ceftazidime used

as comparison antibiotic against *Escherichia coli* ATCC 11230 G and *Escherichia coli* ATCC 29998 (Tablo 3).

Table 3. Antimicrobial Activity Of *E. bocquetii* And *E. arborae* Extracts [a]

Microorganisms[b]	***E. bocquetii***					***E. arborae***					**Standards**		
	A	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Escherichia coli G(-) ATCC 29998	7	-	7	7	7	7	-	7	8	7	15	NT	-
Escherichia coli G(-) ATCC 11230 G	8	8	8	7	7	8	8	8	8	8	18	NT	-
Staphylococus aureus G(+) ATCC 6538P	-	-	8	-	7	7	-	-	-	-	12	NT	-
Staphylococcus epidermidis G(+) ATCC 12228	-	-	-	-	-	-	-	-	-	-	15	NT	-
Salmonella typhimirium G(-) CCM 5445	7	-	7	-	-	-	-	-	-	-	15	NT	-
Enterobacter cloacae G(-) ATCC 13047	-	-	-	-	-	-	-	-	-	-	13	NT	-
Enterococcus faecalis G(-) ATCC 29212	-	-	-	-	-	-	-	-	-	-	10	NT	-
Pseudomonas aeroginosa G(-) ATCC 27853	-	-	-	-	-	-	-	-	-	-	20	NT	-
Candida albicans ATCC 10239	-	-	-	-	-	-	-	-	-	-	NT	17	-

A : Ethanol extract ; B : *n*-Hexane extract ; C : Ethyl acetate extract ; D : Methanol extract ; E : Water extract (20 µg/disc) .; "- ": no inhibition; NT:not tested.[a] Values (mean of three replicates) indicate zone of inhibition in millimeters and include disc diameter (6 mm).[b] Incubation period : 24h at 37°C for bacteria; 48h at 27°C for the yeast..[c] F : Ceftazidime (30 µg/disc); G : Nystatine(25 µg/disc) ; H : Control (DMSO) .

The ethyl acetate and water extracts of *E. bocquetii* and the ethanol extract of *E. arborea* were found to be similarly effective to that of the control antibiotic against *Staphylococus aureus* ATCC 6538P. The MIC fluctuated in a range of 15.62-62.50 µL/mL of extracts. Therefore, the ethanol and ethyl acetate extracts of *E. bocquetii* showed high antibacterial activity against *Salmonella typhimirium* CCM 5445. The MIC fluctuated in a range of 31.25-62.50 µL/mL of extracts. As listed in Table 1, none of the extracts were active against *Staphylococcus epidermidis, Enterobacter cloacae, Enterococcus faecalis* and

Pseudomonas aeroginosa. The extracts of both the *Erica* species showed no activity against the fungus, *Candida albicans*. In the previous study, in vitro antimicrobial activity of *E. arborea, E. manipuliflora, E. bocquetii* and *E. sicula* were investigated. Ethyl acetate extracts of the four species of *Erica* showed activity against *Staphylococus aureus* (ATCC 25923 and MRSA), *Proteus mirabilis* and *Staphylococcus epidermidis* (MRSE), this extracts of these were not effective against *Escherichia coli, Pseudomonas aeroginosa,* and *Candida albicans* (34).

Antibacterial effects of different extracts of *E. bocquetii* and *E. arborea* against *Escherichia coli* ATCC 11230 G., *Escherichia coli* ATCC 29998, *Staphylococus aureus* ATCC 6538P and *Salmonella typhimirium* CCM 5445 suggest that they may posses therapeutic action in the treatment of gastrointestinal, urinary infections and skin diseases (35). However, the plant extracts were unable to inhibit *Candida albicans* which implies that they could not to be used to treat fungal diseases. The high potency of *E. arborea* against these bacteria gives scientific basis for it uses in folk medicine in the treatment of wounds and as diuretic and urinary antiseptic. The brine shrimp bioassay used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds (28). No cytotoxic activity was observed in all extracts of the *Erica* species (Table 4). The results obtained provide the safety of Herba Ericae from prepared *Erica* species in traditional uses.

Table 4. Cytotoxicity of *E. bocquetii* and *E. arborea* extracts

Plants	Tested material	ppm	LC_{50} (ppm)
E. bocquetii	*n*-Hexane	1000 : 100 :10	>1000
	EtOAc	1000 : 100 :10	>1000
	EtOH	1000 : 100 :10	>1000
	MeOH	1000 : 100 :10	>1000
	Water	1000 : 100 :10	>1000
E. arborea	*n*-Hexane	1000 : 100 :10	>1000
	EtOAc	1000 : 100 :10	>1000
	EtOH	1000 : 100 :10	>1000
	MeOH	1000 : 100 :10	>1000
	Water	1000 : 100 :10	>1000
Colchicine		500 : 50 : 5	0.0009
Umbelliferon		500 : 50 : 5	377.02

On the other hand, the ethanolic, methanolic, and aqueous extracts of both the *Erica* species showed antioxidant activity based on scavenging of $ABTS^{+}$ radical cation (Tablo 5)

Table 5. Trolox equivalent antioxidant capacity (TEAC) of *E. bocquetii* and *E.arborea* extracts [a]

Plants	Extracts	TEAC (mM) [b]
E. bocquetii	*n*-Hexane	$0.32_{\pm}$ 0.06
	EtOAc	$0.75_{\pm}$ 0.03
	EtOH	$2.36_{\pm}$ 0.01
	MeOH	$2.65_{\pm}$ 0.00
	Water	$2.6_{\pm}$ 0.03
E. arborea	*n*-Hexane	$0.43_{\pm}$ 0.001
	EtOAc	$0.57_{\pm}$ 0.00
	EtOH	$1.63_{\pm}$ 0.07
	MeOH	$1.92_{\pm}$ 0.06
	Water	$1.92_{\pm}$ 0.12

[a] Values are means $_{\pm}$ S.D. of three replicate analysis.

[b] TEAC is the 1mM Concentration of a trolox solution having the antioxidant capacity equivalent to 500µg/mL solution of the extracts

The antioxidant potentials of the ethanolic, methanolic and aqueous extracts of the aerial parts extracts of *E. bocquetii* were two times higher than those of *E. arborea.* This observation suggests that these extracts of *E. bocquetii* possess more polyhydroxy phenolics such as tannins and flavonoids, which may be acting synergistically together. On the other hand, interestingly, the hexane and ethyl acetate fractions were found to be a very weak antioxidant as compared to all other extracts in the polar fraction. Also this result suggests that polar extracts possess more antioxidant phytomolecules in them, than in the non-polar extracts. In the previous study antioxidant activity of methanol extract and ethyl acetate, buthanol, and water soluble fractions of *E. arborea* collected from Kazdağı-Çanakkale were investigated. The ethyl acetate extract was found to be the richest for phenolic and flavonoid content which showed the highest activity (36).

Phytochemical analysis showed that these extracts contained flavonol glycosides and tannins. Tannins and flavonoids are very valuable plant constituents in the free radical scavenging action, due to their several phenolic hydroxyl groups (37). Therefore, flavonol glycosides and tannins might have been the active principles responsible for the antioxidant activity of *E. bocquetii* and *E. arborea* extracts.

In addition, the data may also suggest that the extracts of *Erica* species tested posses compounds with antimicrobial properties as well as antioxidant activity, which requires further studies to determine antimicrobial agents in new drugs for theraphy of infectious diseases in human and plant diseases.

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