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ORIGINAL PAPER

Determination of the Antimicrobial and Antioxidative Properties and Total Phenolics of Two "Endemic" Lamiaceae Species from Turkey: *Ballota rotundifolia* L. and *Teucrium chamaedrys* C. Koch

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Abstract This study was designed to examine the *in vitro* antimicrobial and antioxidant activities and the amount of total phenolics of the methanol extracts of *Ballota rotundifolia* L. and *Teucrium chamaedrys* C. Koch. In the case of antimicrobial activity tests, polar sub-fractions of the methanol extracts of plant species exhibited weakest antimicrobial activity when compared with the non-polar ones. While, non-polar sub-fraction of *B. rotundifolia* showed moderate activity against *A. lwoffii*, *C. perfringens* and the yeasts, *T. chamaedrys* performed excellent activity pattern against all of the tested microorganisms. The sub-fractions were also screened for their possible antioxidant activities by two complementary tests, namely DPPH free radical-scavenging and β-carotene/linoleic acid assays. Non-polar extracts of the plant species remained inactive in both test systems. On the other hand polar extracts showed remarkable antioxidant activities. In DPPH system, free radical scavenging effect of *T. chamaedrys* was measured as 18.00±1.42 $\mu g.mg^{-1}$. It is extremely important to point out that, polar sub-fraction of *T. chamaedrys* is found as effective as the positive control BHT. Non-polar sub-fraction of *T. chamaedrys* found to have the highest total phenolic amount (97.12±1.28 μg/mg). Results obtained from this experiment confirm the relationship between the amount of phenolics and biological activities.

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Introduction

Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries [1, 29]. Plants still continue to be almost the exclusive source of drugs for the majority of the world's population [33]. According to world health organization (WHO), more than 80% of the world's population relies on traditional plant medicines for their health care. The medicinal value of plants lies in some chemical substances that contain some of the most important bioactive compounds. The phytochemical research based on ethno-pharmacological information is considered an effective approach in the discovery of new agents from higher plants [6, 12]. Higher plants have been shown to be a potential source for the new antimicrobial agents. The screening of plant extracts has been of great interest to scientists for the discovery of new drugs effective in the treatment of several diseases, and about 20% of the plants or their extracts in the world have been submitted to biological or pharmacological tests [26, 30]. A number of reports concerning the antibacterial screening of plant extracts of medicinal plants have been appeared in the literature, but the vast majority have not been adequately evaluated. This is also particularly valid for the Turkish flora which has one of the "most extensive floras in continental Europe" [28] with more than 9,000 flowering plant species [9]. Owing to its strategic position, the accumulation of the knowledge of traditional medicine

from the West and the East enabled this region to have a rich tradition in terms of the uses of medicinal plants [13].

The reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radical and hydrogen peroxide are well-known inducers of cellular and tissue pathogenesis leading to various diseases including cancer, neurodegenerative and cardiovascular diseases [19, 31]. The cellular radical-scavenging systems include antioxidant enzymes and non-cellular radical scavenging molecules that can counteract ROS include glutathione, flavanoids, ubiqinon-10 and albumin [14]. The increase body of evidence suggests that many plants have antioxidant activities that could be therapeutically beneficial.

There is strong interest in the discovery of substances that posses the ability to protect the body from damage caused by free radical-induced oxidative stress. Natural antioxidants are considered useful agents for the prevention of cardiovascular disease and several cancers [18].

Chemical constituents with antioxidant activity found in high concentrations in various plants [32] have been determined to have considerable role in prevention of various degenerative diseases [16]. Besides the fruits and vegetables that are recommended at present as optimal sources of such components, the supplementation of human diet with herbs, containing especially high amounts of compounds capable of deactivating free radicals may have beneficial effects [20].

Halvorsen et al. [15] demonstrated over a 1,000 fold difference between total antioxidants in dietary plants and stressed the need for investigations on the role of processing on storage of raw materials. Among the important constituents participating in the cell defense system against free radicals are phenolic compounds and also ascorbic acid and carotenoids.

The aim of this study was to evaluate the *in vitro* antimicrobial and antioxidant activities and the amount of total phenolics of the methanol extracts of two "endemic" Lamiaceae species named as *B. rotundifolia* and *T. chamaedrys* from Turkish flora.

Materials and Methods

Collection of Plant Material

Herbarium information of the plant species, which are individually numbered, are listed below;

1. *Ballota rotundifolia*: B6, Divrigi, Sivas-Turkey; 18th July 2004.
2. *Teucrium chamaedrys*: A9, Horasan to Kararkurt, in Aras gorge, Erzurum-Turkey; 21th August 2004.

The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas-Turkey (CUFH-Voucher No: 1-AA 3510 and 2-AA 3515, respectively).

Preparation of the Methanolic Extracts

The air-dried and finely ground samples were extracted by using the method as described elsewhere [29]. Briefly, the sample, weighing about 100 g, was extracted in a Soxhlet with methanol (MeOH) at 60°C for 6 h (extract yields; 11.37, and 16.60%, *w/w*, respectively). Then, the crude methanol extract was further fractionated to obtain polar (water-soluble, yields; 2.49 and 1.73% *w/w*, respectively) and non-polar (water-insoluble, yields; 8.64 and 13.27% *w/w*, respectively) extracts. Polar extracts were freeze-dried under vacuum at −50°C and kept in the dark at +4°C until analysis.

Antimicrobial Activity

Microbial Strains

The sub-fractions of the methanol extracts were individually tested against a panel of microorganisms including *Streptococcus pneumoniae* IK3, *Bacillus cereus* RK75, *Acinetobacter lwoffii* ATCC 19002, *Escherichia coli* Hak59, *Klebsiella pneumoniae* A137, *Clostridium perfringens* KUKENS-TURKEY, *Candida albicans* A117 and *Candida krusei* ATCC 6258. Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA), with the exception of *S. pneumoniae* (MHA containing 50 ml citrate blood/L) and *C. perfringens* (in anaerobic conditions). Yeasts were cultured overnight at 30°C in Sabouraud dextrose agar.

Antimicrobial Screening

Agar-well diffusion method was employed for the determination of antimicrobial activities of the extracts [22]. The MIC's of the extracts against the test microorganisms were determined by broth micro dilution method [21]. All tests were performed in duplicate.

Agar-Well Diffusion Method

The water-soluble extracts were weighed and dissolved in phosphate buffer saline (PBS; pH 7.0–7.2), 10 mg/ml, water-insoluble parts were dissolved in dimethylsulphoxide (DMSO), 10 mg/ml. Both extracts were filter-sterilized using a 0.45 μm membrane filter. Each microorganism was suspended in sterile saline and diluted at ca. 10^6 colony forming unit (cfu)/ml. They were "flood-inoculated" onto the surface of MHA. The wells (eight mm in diameter) were cut from the agar and 0.06 ml of extract solution was delivered into them. After incubation for 24 h at 37°C, all plates were examined for any zones of growth inhibition,

and the diameter of these zones were measured in millimetres [4].

Determination of Minimum Inhibitory Concentration (MIC)

A broth microdilution susceptibility assay was used, as recommended by NCCLS, for the determination of the MIC [22]. All tests were performed in Mueller Hinton Broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5% (*v*/*v*)), with the exception of the yeasts (Sabouraud dextrose broth-SDB+Tween 80). Bacterial strains were cultured overnight at 37°C in MHA and the yeasts were cultured overnight at 30°C in SDB. Test strains were suspended in MHB to give a final density of 5×10^5 cfu/ml and where confirmed by viable counts. Geometric dilutions ranging from 0.036 mg/ml to 72.00 mg/ml of the extracts were prepared in a 96-well microtiter plate, including one growth control (MHB+Tween 80) and one sterility control (MHB+Tween 80+test oil). Plates were incubated under normal atmospheric conditions at 37°C for 24 h for bacteria and at 30°C for 48 h for the yeasts. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom.

Antioxidant Activity

DPPH Assay

Hydrogen atoms or electrons donation ability of the corresponding extracts was measured from the bleaching of purple colored methanol solution of DPPH. This spectrophotometric assay uses stable radical 2,2'-diphenylpicrylhydrazyl (DPPH) as reagent [3]. Fifty microliters of various concentrations (0.5–50 mg/ml) of the extracts in the methanol extracts were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in following way:

$$I\% = \left(A_{blank} - -A_{sample}/A_{blank}\right) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate.

β-Carotene-Linoleic Acid Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [8]. A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade), 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen (30 min 100 ml/min) was added with a vigorous shaking. 2.5 ml of this reaction mixture was dispersed to test tubes and 350 µl portions of the oils prepared at 2 gl^{-1} concentrations were added, and emulsion system was incubated up to 48 h at room temperature. Same procedure was repeated with synthetic antioxidant, buthylated hydroxytoluene (BHT), ascorbic acid

Table 1 Antimicrobial activities of the extracts of *B. rotundifolia* and *T. chamaedrys* [1]

Microorganims	*B. rotundifolia*				*T. chamaedrys*			
	Polar extract		Non-polar extract		Polar extract		Non-polar extract	
	AWD[2]	MIC[3]	AWD	MIC	AWD	MIC	AWD	MIC
Escherichia coli	NA[4]	> 72.00*	NA	> 72.00*	NA	> 72.00*	13.00±1.68*	72.00*
Streptococcus pneumoniae	NA	> 72.00*	NA	> 72.00*	NA	> 72.00*	14.00±1.26*	72.00*
Bacillus cereus	NA	> 72.00*	NA	> 72.00*	NA	> 72.00*	8.00±1.05**	>72.00**
Clostridium perfringens	NA	> 72.00*	8.00±1.12*	> 72.00*	NA	> 72.00*	16.00±0.19***	36.00***
Candida albicans	NA	> 72.00*	10.00±0.25*	> 72.00*	8.00±0.50*	> 72.00*	19.00±1.76****	36.00***
Candida krusei	NA	> 72.00*	12.00±0.76**	72.00**	8.00±1.05*	> 72.00*	23.00±1.22*****	18.00****
Acinetobacter lwoffii	8.00±0.73	> 72.00*	14.00±1.19**	72.00**	9.00±0.24*	> 72.00*	26.00±1.73******	9.00*****
Klebsiella pneumoniae	NA	> 72.00*	NA	> 72.00*	NA	> 72.00*	10.00±1.28**	>72.00**

[1] Results are means of three different experiments, Statistical superscripts (asterisks) given in each column are different from the other columns

[2] *AWD* agar-well diffusion, diameter of inhibition zone including disc diameter of 8 mm

[3] *MIC* minimum ınhibitory concentration (as mg/mL)

[4] *NA* not active

Table 2 Free radical scavenging capacities and the inhibition ratio of linoleic acid oxidation by extracts measured in DPPH and β-carotene/linoleic acid assays[1]

Samples	DPPH (as μg/mg)		β-carotene/linoleic acid assay (Inhibition, %)	
	Polar extract	Non-polar extract	Polar extract	Non-polar extract
Ballota rotundifolia	138.00±1.26*	NA [2]	35.97±2.73*	NA
Teucrium chamaedrys	18.00±1.42**	NA	66.73±1.78**	NA
BHT	18.80±1.21**	18.80±1.21*	96.00±0.23***	96.00±0.23*
Ascorbic acid	3.80±0.10***	3.80±0.10**	94.50±2.14***	94.50±2.14*
Curcumin	7.80±0.30****	7.80±0.30***	89.30±1.86***	89.30±1.86*

[1] Results are means of three different experiments, Statistical superscripts (asterisks) given in each columns are different from the other columns

[2] *NA* not active

and curcumine as positive controls, and a blank. After this incubation period absorbance of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those of BHT and blank.

Assay for Total Phenolics

The content of the total phenolic constituents of aforesa extracts were determined employing the literature methods involving Folin-Ciocalteu reagent and gallic acid as standard [5]. 0.1 ml of extract solution containing 1,000 μg extract was taken in a volumetric flask, 46 ml distilled water and 1 ml Folin-Ciocalteu reagent were added and flask was shaked thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated to all standard gallic acid solutions (0–1,000 mg 0.1 ml^{-1}) and standard curve was obtained with the equation given below:

$$\text{Absorbance} : 0.0012 \times Gallic\tilde{}acid(\mu g) + 0.0033$$

Statistical Analysis

Data were presented as the mean±SD (standard deviation) and analyzed by repeated measures of ANOVA followed by the Tukey test for post hoc pair wise comparisons. The difference was considered significant when the P value was<0.05.

Results and Dıscussıon

Antimicrobial Activity

As it can be seen from the Table 1, polar sub-fractions of the methanol extracts of the plant species above mentioned exhibited the weakest antimicrobial activity when compared with the non-polar ones. *A. lwoffii* was found to be sensitive to the polar sub-fractions of both species. Additionally, polar sub-fraction of *T. chamaedrys* showed activity against the yeasts (*C. albicans* and *C. krusei*). These findings are further supported by the MIC values (>72.00 mg/ml).

In the case of the non-polar sub-fraction, results from agar-well diffusion method, followed by measurements of minimal inhibition concentration (MIC) indicate that, *B. rotundifolia* showed moderate activity against *A. lwoffii*, *C. perfringens* and the yeasts. On the other hand, *T. chamaedrys* performed excellent activity pattern against to all of the tested micro-organisms. Among the microorganisms, the most sensitive was *A. lwoffi* with a MIC value of 9.00 mg/ml (inhibition zone was 26.00±1.73). Furthermore, this activity showed by yeasts was of 36.00 and 18.00 mg/ml of yeasts. *B. cereus* and *K. pneumoniae* were the most resistant microorganisms with the MIC value of (>72.00 mg/ml).

According to our literature research antimicrobial activities of two endemic species evaluated here have not previously been reported. On the other hand, antimicrobial activity of the essential oil of *T. chamaedrys* had been studied by Kucuk et al. [17]. Chemical composition of the various extracts obtained from this plant has been established in the literature. In the light of these reports; volatiles [24], diterpenoids [27], phenylpropanoid glycosides [2] and teucrin A [10] could be assumed as the most important components for the *T. chamaedrys* extracts. Within these components, teucrin A is especially important for its interesting biological properties [10].

Table 3 Amount of total phenolic compounds of the extracts [1]

Samples	Gallic acid equivalent (μg/mg)	
	Polar extract	Non-polar extract
Ballota rotundifolia	46.13±2.89	53.82±1.39
Teucrium chamaedrys	69.75±2.62	97.12±1.28

[1] Results are means of three different experiments

It is well known that, methanol is a solvent which is capable of obtaining of the phyto-chemicals having a wide polarity range. Using this solvent, it is possible to obtain a high amount of plant constituents capable both in antimicrobial and antioxidant test systems. In this study, antimicrobial and antioxidant activity profiles of the polar and non-polar extracts have been well characterized. In the case of the antimicrobial activity, particularly, non-polar extracts are seemed to be more efficient. Due to the polar characteristics of the liquid phase, both in intra-cellular and extra-cellular area, within the organisms, in general, non-polar substances show toxic effects for the maintenance of life. This is also valid for the prokaryotic organisms.

Antioxidant Activity

The extracts obtained by Soxhlet extraction were screened for their possible antioxidant activities by two complementary tests named DPPH free radical scavenging and β-carotene/linoleic acid systems. Free radical scavenging capacities of the corresponding extracts measured by DPPH assay are shown in Table 2. According to the findings presented in the table, non-polar sub-fractions of the methanol extracts of plant species remained inactive in both test systems. On the other hand, polar extracts showed remarkable antioxidant activities. In DPPH system, free radical scavenging effect of *T. chamaedrys* was measured as 18.00±1.42 $\mu g.mg^{-1}$. In this system, free radical scavenging capacity of the polar extract of *T. chamaedrys* was statistically different in relation to those of ascorbic acid and curcumin. It is extremely important to point out that, polar sub-fraction of *T. chamaedrys* is found as effective as the positive control BHT ($p>0.05$). Inhibition value for this sub-fraction in β-carotene/linoleic acid test system was recorded as 66.73%± 1.78. In this system, polar extract of both species exhibited a statistically different activity profile than those of the standard compounds.

Antioxidant activity of *T. chamaedrys* has previously been studied by Ozgen et al. [25]. According to this report, IC_{50} value for the extract of this plant has been determined as 9.2 mu g/ml). Results obtained from this study show a strong correlation with our experiments. On the other hand, antioxidant activity of the ethanol extract of *B. rotundifolia* has been evaluated by Citoglu et al. [7]. As determined in this report, in superoxide anion formation test, IC_{50} value of this extract had been found as 0.93 mg/ml. Moreover, malondialdehyde (MDA) levels of the extract in the lipid peroxidation system, in a 2.5 mg/ml concentration, had been found as 42.11±1.32 nmol in the experimental tissue.

In different antioxidative test systems, antioxidant activities of the same extracts could be determined as variable. For example, the extracts of *Ballota larendana*, *Ballota latibracteolata*, and *Ballota rotundifolia* observed to have inhibitory effects on superoxide anion formation, whereas they were ineffective on lipid peroxidation. Distinct effects of some plant extracts and synthesized chemicals have previously been noted in two different *in vitro* systems [23]. Therefore, the observation of different effects of some plant extracts on different radical formation systems are not surprising since the mechanism of production of oxidative stress in these methods is different [11].

Amount of Total Phenolics

The systematic literature collection pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Therefore, it is worthwhile to determine their total amount in the plants chosen for the study.

Based on the absorbance values of the various extract solutions reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid equivalents as described above, results of the colorimetric analysis of total phenolics are given in Table 3. The amount of the total phenolics was high in the non-polar extracts. Within these samples, non-polar extract of *T. chamaedrys* found to have the highest total phenolic amount (97.12±1.28 μg/mg). Results obtained from this section confirm the relationship between the amount of phenolics and biological activities.

In conclusion, our study provides evidence that polar and non-polar sub-fractions of the methanol extracts of *T. chamaedrys* studied here exhibited strong antimicrobial and antioxidant activity. A strong correlation was observed between the potential activity and phenolics of the plant species studied. Therefore, these highly biologically active compounds are probably responsible from the activity observed. On the other hand, further quantitative chromatographic analyses are needed to clarify the major active substances of the extracts and their way of action.

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