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Antimicrobial and Antioxidant Activities of *Helichrysum* Species from the Mediterranean Region of Turkey

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In this study, it was investigated the antioxidant, antiradical and antimicrobial activities of methanolic extracts from three *Helichrysum* species [*H. pamphylicum* Davis & Kupicha, *H. sanguineum* (L.) Kostel, *H. chasmolycicum* P.H. Davis (Asteraceae)]. The total phenolic contents of methanolic extracts ranged from 71.51 to 119.85 mg gallic acid/g dry extract. *H. pamphylicum* methanolic extract showed the highest antioxidant activity (173.58 mg ascorbic acid/g dry extract). Antiradical activity of *H. sanguineum* methanolic extract was the highest (IC_{50} =12.90 µg/mL) in DPPH assay. All the extracts were investigated for antimicrobial effect against 15 species of microorganisms containing thirteen bacteria and two yeasts. The methanolic extracts were inactive against *Escherichia coli* and *Proteus mirabilis*. The most sensitive bacteria were *Klebsiella pneumoniae* for *H. pamphylicum*, *Staphylococcus aureus* (B), *Proteus vulgaris* for *H. sanguineum* and *S. aureus* (B) for *H. chasmolycicum*. All of the *Helichrysum* extracts showed similar antimicrobial activities against microorganisms tested. In conclusion, this study provides the basis for the present rapidly increasing interest for the use of natural antioxidants and antimicrobials as functional food ingredients and/or as food supplements.

Key Words: *Helichrysum*, Antimicrobial, Antioxidant, DPPH, Phenolic content.

INTRODUCTION

Herbal medicines have been improved in developing countries, as an alternative solution to health problems and costs of pharmaceutical products[1]. Plants contain a diverse group of phenolic compounds, including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives

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and flavonoids. All the phenolic classes have received considerable attention because of their physiological functions, including free radical scavengers, antioxidants[2] and antimicrobial activity[3].

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance[4]. Small molecules from spices, medicinal herbs and natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases[5]. Moreover, the increasing use of plant extracts in the food, cosmetic and pharmaceutical industries suggests that, in order to find active compounds, a systematic study of medicinal plants is very important[6].

The genus *Helichrysum*, belonging to the family Asteraceae is represented by *ca.* 500 species in the world. These include 27 taxa from Turkey and 15 of them are endemics. Members of the genus *Helichrysum* are usually aromatic, perennial shrubs, having dense leaves with hardy flower heads. Their flowers come in an array of almost all colours, except blue. There are many capitula and generally flat-topped corymbs or panicles. The corolla lobes show glandular hairs at the abaxial surface[7,8].

Helichrysum species exhibited various biological properties including antioxidant[3,9-13], antimicrobial[14,15], antiinflammatory[16] and antimutagenic activity[17]. This genus are a source of many bioactive compounds[18,19]. The medicinal properties of this genus are mainly attributed to the presence of flavanoids and may be related to their antioxidant and antimicrobial activities[20]. Recently, the use of spices and herbs as antioxidants and antimicrobial agents in foods is to become of increasing importance[3,12]. Though antioxidant and antimicrobial effects of *Helichrysum* species have been investigated by some researchers in various parts of the world, there are only a few reports about phenolic content, antimicrobial and antioxidant activity of *Helichrysum* species belonging Turkish flora. The *Helichrysum* genus had endemic species in Turkish flora and commonly used as folk tea and herbal medicine in Turkey.

The aim of the present works is to study total phenolic content, *in vitro* antioxidant and antimicrobial activities of methanol extracts of *H. pamphylicum, H. sanguineum* and *H. chasmolycicum* growing in the Mediterranean region of Turkey.

EXPERIMENTAL

Aerial parts of plants were collected from the Mediterranean region of Turkey and identified by Dr. Ahmet Aksoy, Department of Biology, University of Erciyes. Voucher specimens have been deposited at the

Herbarium of the Department of Biology, Erciyes University, Kayseri, Turkey (Voucher No: 1-AAksoy 2099, 2-AAksoy 2098, 3-AAksoy 2086, respectively). *H. pamphylicum* and *H. chasmolycicum* are endemic to Turkish flora[7].

Collection information of the three *Helichrysum* species which are individually numbered is listed below: (i) *H. pamphylicum* Davis & Kupicha Manavgat, Aspendos-Cakis Village, 40 m, Antalya-Turkey, 30 May, 2005. (ii) *H. sanguineum* (Linnaeus) Kostel Between Antakya Rubbish-head and Beyza Chicken Farm, 420 m, Antakya-Turkey, 26 May, 2006. (iii) *H. chasmolycicum* P.H. Davis In vicinity of Kovada Power-Station, 890 m, Isparta, Turkey, 22 June, 2006.

Preparation of the plant extracts: Dried plant at room temperature was ground to fine powder with a grinder. Then the powdered plant material (10 g) was extracted in a Soxhlet extractor with 100 mL methanol at 60 ºC for 6 h. The extract was filtered and concentrated to dryness under reduced pressure at 40 ºC with a rotary evaporator. After determining the yield, the extract was dissolved in methanol for further study.

Determination of total phenolic content: The Folin-Ciocalteu colorimetric method was used to determine total phenolic content of methanolic extract[21]. Concentration of methanol extract was adjusted to 1 mg/mL. The sample of each plant extract solution (40 µL) was transferred into a test tube and then mixed thoroughly with 2.4 mL distilled water. This solution was then mixed with 200 µL Folin-Ciocalteu reagent. After 30 s, 600 µL sodium carbonate (20 % Na_2CO_3) and 760 µL distilled water were added and mixed. Methanol was used as a blank instead of extract. The absorbance of reaction mixture was measured at 765 nm against a blank after incubation at room temperature for 2 h in dark. The total phenolic content was determined using a standard curve with gallic acid as the standard. The mean of three readings was used and expressed as mg of gallic acid (GAE) equivalents/g extract.

Determination of antioxidant activity: The antioxidant activity of plant extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.*[22]. 0.4 mL of plant extract was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The effective concentration of extract was 1 mg/mL in the reaction mixture. The tubes were capped and incubated in a incubator at 95 ºC for 1.5 h. The samples was cooled down to room temperature and the absorbance of mixture was measured at 695 nm. A typical blank solution contains 4 mL reagent solution and the appropriate volume of the same solvent used for the extract. The antioxidant activity was determined using a standard curve with ascorbic acid as the standard. The mean of three readings was used and expressed as mg of ascorbic acid equivalents (AAE)/g extract.

Determination of antiradical activity: The scavenging activity of plant extract for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described by Lee *et al.*[23] with some modifications. A series of extract concentration in methanol, *i.e.*, 2, 1, 0.5, 0.25, 0.1 mg/mL were prepared. 50 µL of each concentration plant extract was mixed with 450 µL *Tris*-HCl and 1000 µL of 0.1 mM DPPH in methanol. Methanol was used as a control instead of extract. The mixtures were left for 0.5 h at room temperature in the dark and the absorbance at 517 nm measured using methanol as blank. IC_{50} (concentration causing 50 % inhibition) values of each extract was determined graphically. The same procedure was repeated with synthetic antioxidant butylated hydroxytoluene (BHT) as positive control. The measurements were performed in triplicate and the results were averaged.

Antiradical activity was expressed as percentage inhibition of DPPH radical and was calculated by following equation:

I (%) = 100 × (1-Absorbance of sample/Absorbance of control)

Bacterial cultures: The test organisms used in this study were as follows: *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* RSKK 863, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* FMC 5, *Morganella morganii*, *Mycobacterium smegmatis* RUT, *Proteus mirabilis* BC 3624, *Proteus vulgaris* RSKK 96026, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 (A), *Staphylococcus aureus* ATCC 25923 (B), *Yersinia enterocolitica* ATCC 1501, *Candida albicans* ATCC 1223, *Saccharomyces cerevisiae* BC 5461.

Determination of antimicrobial effect: Test yeasts namely *C. albicans* and *S. cerevisiae*, and *Y. enterocolitica* were grown in malt extract and nutrient broth at 25 ºC for 18 h, respectively. The other microorganisms were grown in nutrient broth at 35 ºC for 18 h. All test microorganisms in nutrient broth or malt extract broth were enumerated by using the serial dilution method. Their final cell concentrations were 106-107 cfu/mL. The agar diffusion method was used to detect antimicrobial activity[24]. 250 µL of each microorganism was added into a flask containing 25 mL sterile Mueller-Hinton agar or malt extract agar at 45 ºC and poured into Petri dishes (9 cm diameter). Then the agars were allowed to solidify at 4 ºC for 1 h. Four equidistant holes were made in the agar using sterile cork borers (Ø = 4 mm). The extracts (40 µL) were prepared at 1, 2.5, 5 and 10 % concentrations in absolute methanol and were applied to the holes using a pipettor and absolute methanol without herb extract was used as a control. *Y. enterocolitica, C. albicans* and *S. cerevisiae* was incubated at 25 ºC for 14-24 h in the inverted position. The other microorganisms were incubated at 35 ºC for 18-24 h. At the end of the period, inhibition zones formed on the medium were measured in millimetres (mm). All the tests were performed in duplicate and the results were presented as averages.

Statistical analyses: Data from the experiments were subjected to analysis of variance (ANOVA) using SPSS 2001 for Windows. Percentage data were transformed using arcsine √x before ANOVA. Means were separated at the 5 % significance level by the least significant difference (LSD) test.

RESULTS AND DISCUSSION

In this study, total extract yields, total phenolics, antioxidant, antiradical and antimicrobial activities of three *Helichrysum* methanolic extracts were determined. The extract yields were the following: 18.44, 29.78 and 19.17 g/100g of dry herb for *H. pamphylicum, H. sanguineum* and *H. chasmolycicum*, respectively.

The total phenolic contents of the plant extracts as determined by Folin-Ciocalteu method are reported as gallic acid equivalents (Fig. 1). All extracts contain a considerable amount of phenolic metabolites. The total phenolic contents of *H. pamphylicum*, *H. sanguineum* and *H. chasmolycicum* were 119.85 ± 2.0, 63.8 ± 0.6 and 71.51 ± 0.5 mg GAE/g dry extract, respectively. Statistically, total phenolic content of the *H. pamphylicum* extract was the highest, according to the other extracts ($p < 0.05$). The total phenolic contents of *Helichrysum* species tested in this study have been evaluated for the first time, except *H. chasmolycicum*. The present findings were similar to the observations of Ozkan *et al.*[12] reported that the total phenolic was found as 108.33 ± 0.88 mg GAE/g in *H. chasmolycicum* extract.

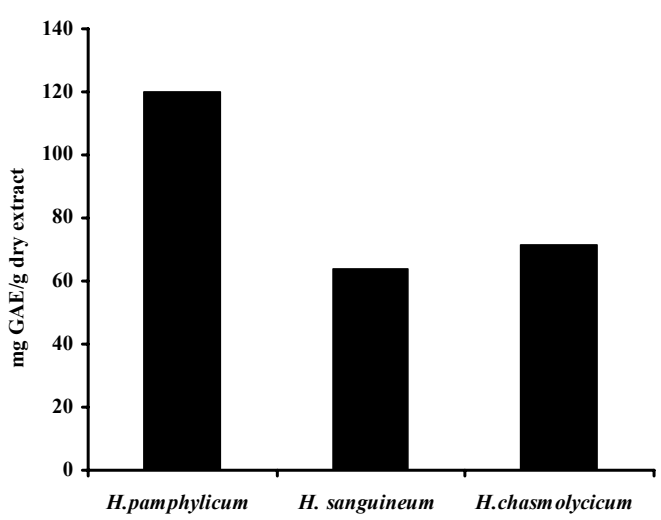

Fig. 1. Total phenolic contents of the *Helichrysum* extracts (mg GAE/g dry extract)

The antioxidant activities of *Helichrysum* methanolic extracts were measured spectrophotometrically through phosphomolybdenum method. The differences among antioxidant activities of three *Helichrysum* extracts were statistically significant ($p < 0.05$). Among the three species, *H. pamphylicum* extract showed maximum antioxidant activity as 173.58 ± 1.1 mg AAE/g extract followed by *H. sanguineum* as 159.94 ± 0.3 mg AAE/g extract and then *H. chasmolycicum* as 147.88 ± 0.9 mg AAE/g extract. Similarly, the antioxidant activity of *H. chasmolycicum* was reported as 246.83 ± 1.23 mg AAE/g by Ozkan *et al.*[12].

The antiradical activities of the extracts from three *Helichrysum* species at different concentrations were tested by DPPH method (Fig. 2). Statistically, significant difference ($p < 0.05$) was obtained for antiradical activities of the extracts. Percentage inhibition rates of *Helichrysum* methanolic extracts and BHT is possible carcinogen were very similar to each other at the highest concentration examined. The concentrations of methanolic extracts required to scavenge 50 % of the DPPH radicals, IC_{50} values, are depicted in Fig. 3. Each data point represents the average of triplicate analyses. Maximum antiradical capacity was observed in the *H. sanguineum* extract (IC_{50} = 12.90 µg/mL) whereas minimum antiradical capacity was observed in the *H. chasmolycicum* extract (IC_{50} = 25.33 µg/mL).

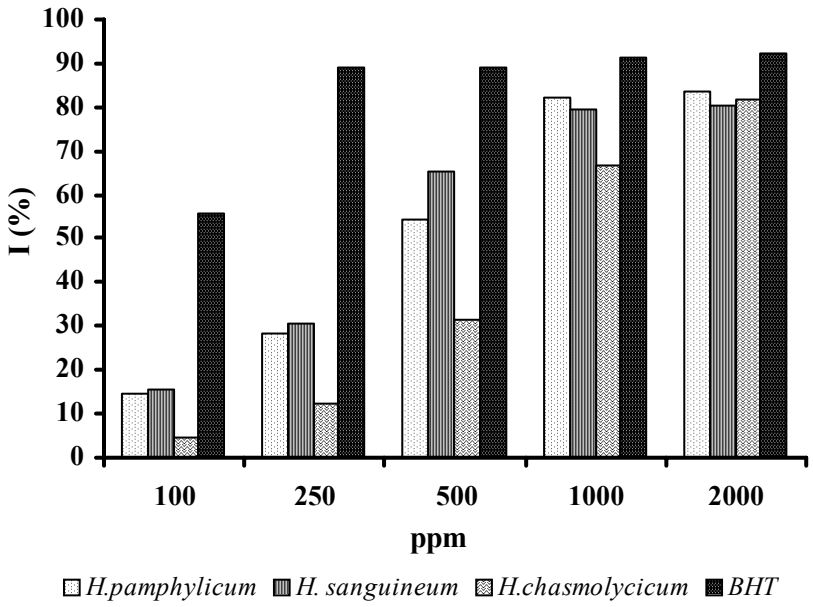

Fig. 2. Antiradical activities of three Helichrysum extracts and BHT at different concentrations (ppm)

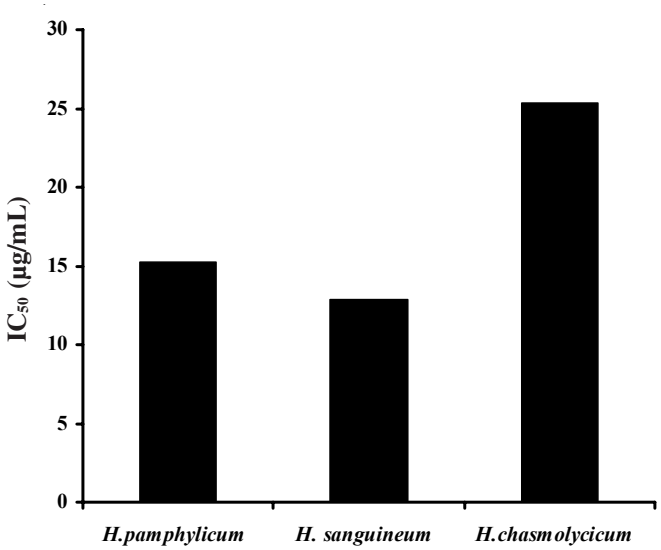

Fig. 3. DPPH IC_{50} values (µg/mL) for the extracts of three *Helichrysum* species

As far as we know, antioxidant, antiradical and antimicrobial activities of Turkish *Helichrysum* species studied in this research have been reported here for the first time, except *H. chasmolycicum*. In a previous paper, Tepe *et al.*[13] showed that non polar subfractions of the methanol extracts of *Helichrysum* species tested did not show any antiradical activity, while the extract of *H. chionophilum* (IC_{50} = 40.5 µg/mL) was the most active among the polar subfractions. Lourens *et al.*[25] also showed that, the methanol and acetone extracts of four *Helichrysum* species were active in the DPPH assay and the acetone extract of *H. dasyanthum* was the most active free radical scavenger (IC_{50} = 9.53 µg/mL). Poli *et al.*[11] reported that *H. italicum* extract showed antiradical activity in the DPPH assay. The present findings were similar to the observations of some previous researchers who studied on antioxidant properties of the other *Helichrysum* species.

At 1, 2.5, 5 and 10 % concentrations, the antimicrobial effects of three *Helichrysum* species against 15 microorganisms were shown in Table-1. The extracts caused different inhibition zones on the tested microorganisms. Pure methanol (control) used as solvent had no inhibitory effects on the 15 microorganism tested. Statistically, significant differences ($p < 0.05$) were obtained between microorganism. No activity was found with all the methanolic extracts against *E. coli* and *P. mirabilis*. *M. morganii* was inhibited by only *H. pamphylicum* while this species does not exhibited antimicrobial effect against *Y. enterocolitica*. The most sensitive bacteria were *K. pneumoniae* for *H. pamphylicum* extract, *S. aureus* (B), *P. vulgaris* for *H. sanguineum* extract and *S. aureus* (B) for *H. chasmolycicum* extract. The antimicrobial

TABLE–1
ANTIMICROBIAL ACTIVITIES OF THE EXTRACTS OF THREE *Helichrysum* SPECIES AT DIFFERENT CONCENTRATIONS (INHIBITION ZONES, mm)

Microorganisms	Concentration of the extract (%)											
	H. pamphylicum				*H. sanguineum*				*H. chasmolycicum*			
	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0	1.0	2.5	5.0	10.0
A. hydrophila	–	7.5±0.7	8.5±0.7	9.5±0.7	15.0±0.0	21.0±1.4	24.0±1.4	25.0±1.4	18.5±0.7	20.0±0.0	24.5±0.7	26.5±0.7
B. cereus	7.0±0.0	8.0±0.0	9.0±0.0	10.0±0.0	16.0±1.4	19.5±0.7	23.5±0.7	26.0±1.4	12.5±0.7	16.0±1.4	19.0±1.4	22.0±1.4
B. subtilis	–	9.0±1.4	10.0±1.4	11.0±0.0	11.5±0.7	13.0±0.0	14.0±0.0	22.5±0.7	14.5±0.7	19.5±0.7	22.5±0.7	25.5±0.7
E. coli	–	–	–	–	–	–	–	–	–	–	–	–
K. pneumoniae	7.5±0.7	12.0±1.4	14.0±0.7	16.0±0.7	15.5±0.7	21.0±0.0	23.5±0.7	26.0±1.4	14.5±0.7	16.5±0.7	20.5±0.7	27.0±0.0
M. morganii	–	–	6.0±0.0	8.0±0.0	–	–	–	–	–	–	–	–
M. smegmatis	6.5±0.7	8.0±0.0	8.5±0.7	11.0±0.7	13.5±0.7	15.5±0.7	18.0±1.4	20.0±0.0	12.0±1.4	13.5±0.7	16.0±0.0	18.5±0.7
P. mirabilis	–	–	–	–	–	–	–	–	–	–	–	–
P. vulgaris	7.0±1.4	9.0±0.0	9.5±0.7	12.0±0.7	14.0±1.4	20.0±1.4	28.0±1.4	29.5±0.7	17.0±0.0	20.0±1.4	22.0±1.4	26.0±1.4
P. aeruginosa	7.0±0.0	8.0±0.0	9.5±0.7	9.5±0.7	18.0±0.0	20.0±0.0	24.0±0.0	27.0±0.0	19.5±0.7	25.0±0.0	27.0±0.0	29.5±0.7
S. aureus (A)	0.0	6.0±0.0	7.0±0.0	7.0±0.0	7.5±0.7	9.0±0.0	10.0±0.0	11.5±0.7	7.5±0.7	9.5±0.7	11.5±0.7	13.5±0.7
S. aureus (B)	0.0	7.0±1.4	8.0±1.4	10.0±1.4	19.0±1.4	24.0±1.4	26.0±1.4	29.0±1.4	21.0±1.4	25.0±1.4	28.5±0.7	32.0±1.4
Y. enterocolitica	–	–	–	–	–	–	–	6.0±0.0	–	–	7.0±0.0	7.0±0.0
C. albicans	–	–	–	–	–	–	–	–	–	6.0±0.0	7.0±0.0	7.0±0.0
S. cerevisiae	–	–	–	–	–	–	–	–	–	–	6.0±0.0	6.0±0.0

–: not detected
Values expressed are mean ± standard deviation of two experiments.

effects of three *Helichrysum* species were statistically not significant at $p > 0.05$. All the methanol extracts showed similar antimicrobial activities against microorganisms tested.

Among extracts obtained 3 *Helichrysum* species, only *H. chasmolycicum* showed low effects against two yeasts. It was previously determined that the methanolic extract of *H. chasmolycicum* had antimicrobial activity[12]. In this study, the findings similar to the observations of some other researchers that studied on antimicrobial properties of the other *Helichrysum* species. It was reported that *Helichrysum compactum* inhibited the growth of moulds, yeast and bacteria[3]. Nostro *et al.*[14] reported that *H. italicum* diethyl ether extract had an inhibitory effect on *S. aureus* strains. The same researchers also indicated that inhibition of Staphylococcal growth appeared with 250-125 μg/mL of *H. italicum* diethyl extract in culture medium[15]. It was showed that a new compound isolated from *H. caespititium* inhibited growth of many bacteria and moulds[19]. Meyer and Afolayan[26] indicated that the methanol extract of *H. aureonitens* had antimicrobial activity against *Bacillus cereus, B. pumilus* and *Micrococcus kristinae*. Cushine and Lamb[27] determined that *H. aureonitens* had antibacterial activity against *S. aureus*. The methanol extract of *H. plicatum* subsp. plicatum showed inhibitory effect against 15 different bacteria and strains except *Corynebacterium xerosis* UC 9165[28]. Similarly, the methanol extracts of vary *Helichrysum* species were active against Gram (+) bacteria[25]. The inhibiting effect of *H. foetidum* methanolic extract on the growth of *Mycobacterium tuberculosis* and *S. aureus, Streptococcus pyogenes, E. coli* and *P. aeruginosa* was showed by Steenkamp *et al.*[29].

Conclusion

The present results suggest that the methanolic extracts of *Helichrysum* species drunk as tea in Turkey contain a considerable amount of phenolic metabolites and had antioxidant, antiradical and antimicrobial activities. Therefore, these extracts may be used as antioxidant and antimicrobial agents. In conclusion, this study provides the basis for the increasing interest for the use of natural antioxidants and antimicrobials as functional food ingredients and/or as food supplements.

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