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IN VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF EXTRACTS OF BULGARIAN *MALVA SYLVESTRIS* L.

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Abstract: The widespread use of culinary plants for health purposes has increased dramatically due to their great importance to a balanced diet. In this study, different extracts of fresh *Malva sylvestris* leaves and flowers were investigated for their level of total phenolics, antimicrobial activity against Gram-positive (*Bacillus cereus, Staphylococcus aureus* ATCC 6538-P), Gram-negative (*Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp. (clinical isolate), *Proteus vulgaris* J) bacteria, molds (*Aspergillus niger, Penicillium* sp., *Rhizopus* sp.) and yeasts (*Saccharomyces cerevisiae*); and their antioxidant activities using various methodologies: ABTS, DPPH, FRAP, and CUPRAC assays. The total phenolic content was established to be 2.35 ± 0.01 and 0.83 ± 0.05 mg GAE/g fresh plant weight in the decoction extracts of leaves and flowers, respectively. The flowers were found to be more active toward pathogenic microorganisms. The present study revealed that the consumption of this plant would exert several beneficial effects by virtue of their antioxidant and antimicrobial activities.

INTRODUCTION

Humans have developed a broad knowledge of useful plants over time through continuous contact with their environment. Cultivated plants are widely used today, although edible plants have significant medical properties. Antioxidants are important components because they protect against free radicals, such as reactive oxygen species in the human body. Free radicals are known to be the major contributors to degenerative diseases of aging and are recognized as major factors causing cancer. The human can use antioxidants either as dietary, food

supplements or as a medicine. Nowadays, there is an increasing interest both in industry and scientific research in vegetables, fruits, medicinal plants and spices because of their antioxidative phytochemicals and antimicrobial properties.

Malva sylvestris L., usually known as common mallow, is native to Europe and its traditional use has been documented since a long-time ago, although little clinical evidence is available. Roots, leaves, flowers, fruits, and seeds are applied in infusions, decoctions, liniments, lotions, baths and gargles (Camejo-Rodrigues et al., 2003; Novais et al., 2004; Carvalho, 2005; Natali and Pollio, 2007; Quave et al., 2008; Neves et al., 2009). Traditionally, the medicinal applications of the common mallow treat specified disorders of several systems of the body, such as the respiratory, the digestive system, the muscular and skeletal system, as well as skin injuries and disorders. The common mallow is considered to have diuretic, laxative, spasmolytic, lenitive and choleretic effects. It is also used as bronchodilator, expectorant, antitussive, anti-diarrheal and highly recommended for acne treatment and skin care, and as antiseptic, emollient and demulcent (Carvalho, 2005; Quave et al., 2008; DellaGreca et al., 2009; Neves et al., 2009). Edible uses are concerned with folk gastronomy and nourishment (Guarrera, 2003; Carvalho 2005). Young leaves are eaten raw in salads, leaves and shoots are consumed in soups and as boiled vegetables. Immature fruits are sucked or chewed by children, shepherds and hunters (Pardo de Santayana, 2004; Carvalho, 2005; Neves et al., 2009). The biological activity of this plant may be attributed to antioxidants, such as polyphenols, vitamin C, vitamin E, β-carotene, and other important phytochemicals. Polyphenols are secondary plant metabolites, widely distributed in plants and foods of plant origin. Their health benefits (vasodilatatory, anti-inflammatory, anti-cancerogenic, antiviral and antibacterial effects) arise from the antioxidative effects of these phytochemicals, which are based on their ability to scavenge different free radicals leading to the protection of biological molecules against oxidation (Rackova et al., 2009).

Thus, it is important to study the antimicrobial and antioxidant potential of different edible parts of the common mallow. The aim of this report is to contribute to the knowledge of the beneficial effect of *M. sylvestris* as well as to enhance an interest in the consumer and to provoke a frequent use as an ingredient in culinary recipes.

MATERIALS AND METHODS

Plant material

Plant samples were collected in May 2014 from their natural habitat in Plovdiv region, Bulgaria.

Extract preparation

Fresh plant material of *M. sylvestris* was subjected of three different types of extractions:

- decoction – extraction by boiling of the plant material for 30min with distilled water;

- infusion – extraction by boiling water and then pouring it over the plant material, allowing it to steep in the liquid for 20min.

- microwave assisted extraction (MAE) – the experiments were performed with water as solvent in a domestic microwave oven (LG MB4047C) with frequency of the waves 2450 MHz and output power- 800W;

The resulting extracts solutions were filtered before analyses.

Determination of total polyphenolic content (TPhC)

A modified Kujala et al. (2010) method with Folin - Ciocalteu's reagent was used for the determination of the total polyphenolic content (TPC). Gallic acid was employed as a calibration standard and the results were expressed as mg gallic acid equivalents (mg GAE) per gram of plant fresh weight.

Determination of antioxidant activity

DPPH radical scavenging activity

The ability of the extracts to donate an electron and scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams, Cuvelier, and Berset (1995). Freshly prepared $4x10^{-4}$ M methanolic solution of DPPH was mixed with the samples and a standard solution in a ratio of 2:0.5 (v/v). The light absorption was measured at 515 nm and the percentage of inhibition of DPPH• by the obtained extracts was calculated for each sample using the following formula:

$$\% \text{ Inhibition} = [(A_B - A_E)/A_B] \times 100$$

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with DPPH.

The DPPH radical scavenging activity was presented as a function of the concentration of Trolox. The unit of Trolox equivalent antioxidant capacity (TEAC) was defined by the concentration of Trolox having equivalent antioxidant activity expressed as μM TE/g FW.

ABTS radical scavenging assay

The radical scavenging activity of the ethanol extract against radical caption ($ABTS^{\bullet+}$) was estimated according to Re et al. (1999) with some modifications. $ABTS^{\bullet+}$ was produced by reacting 7 mM of $ABTS^{\bullet+}$ solution with 2.45 mM of potassium persulphate, and the mixture was kept in the dark at room temperature (20 - 22°C) for 12-16 h. At the moment of use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. Each sample (0.01 ml) was added to 1 ml of $ABTS^{\bullet+}$ solution and mixed vigorously. After reaction at 30°C for 6 min, the absorbance at 734 nm was measured. The percentage of inhibition of $ABTS^{\bullet+}$ by the obtained extracts was calculated for each sample using the following formula:

$$\% \text{ Inhibition} = [(A_B - A_E)/A_B] \times 100,$$

Where: A_B = absorbance of the control without sample; A_E = absorbance of the test sample with $ABTS^{\bullet+}$.

The TEAC value was defined as the concentration of Trolox having equivalent antioxidant activity expressed as μM TE per gram fresh weight (μM TE/g FW).

CUPRAC assay

The CUPRAC assay was carried out according to the procedure of Apak et al., 2008. To a test tube were added 1 ml of $CuCl_2$ solution (1.0×10^{-2}M), 1 ml of neocuproine methanolic solution (7.5×10^{-3}M), and 1 ml NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 ml of herbal extract (sample) followed by 1 ml of water were added (total volume = 4.1 ml), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of extracts was expressed as μM TE/g FW.

FRAP assay

The FRAP assay was carried out according to the procedure of Benzie & Strain (1996) with slight modification. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe (II) -tripyridyltriazine compound from colourless oxidized Fe (III) form of the action of electron donating antioxidants. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C in a water bath prior to use. One hundred and fifty microliters of plant extracts were allowed to react with 2850 μl of the FRAP reagent solution for 4 min at 37°C. The absorbance of the reaction mixture was recorded at 593 nm. The results were expressed as μM TE/g FW.

Antimicrobial activity

The agar diffusion test was used to determine the antibacterial activity of extracts of Malva sylvestris. Melted and cooled to the temperature at about 45°C selective media were inoculated with the tested microorganisms and after setting of media, small amount of the extract was placed in sterile metal rings (Ø 6 mm). Plates were incubated at 37°C for required incubation periods (24h or 48h) according to the strain type and then the distinct zone of growth inhibition around the rings was measured. The antimicrobial activity is tested against the following saprophytic test microorganisms: bacteria - *Bacillus cereus*; yeasts - *Saccharomyces cerevisiae*, molds - *Aspergillus niger, Penicillium* sp., *Rhizopus* sp. A suspension of each of the test microorganisms (10^6-10^7 cfu/cm^3) is used to inoculate a Petri dish with agar medium and after the hardening of the agar discs impregnated with the samples (6 mm) are placed on the solid media. The pathogenic test microorganisms are *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Salmonella* sp. (clinical isolate), *Staphylococcus aureus* ATCC 6538-P and *Proteus vulgaris* J.

Statistical analysis

All measurements were carried out in triplicates. The results were expressed as mean ± SD and statistically analysed using MS-Excel software.

RESULTS AND DISCUSSION

Naturally, occurring substances in fruits and vegetables such as phenolic compounds has antioxidant activity. These substances have the ability to scavenge free radicals by single electron transfer (Re et al., 1999). Total phenolic contents of *Malva sylvestris* L. samples are presented in Table 1. Phenolic compounds ranged from 0.29 to 2.35 mg GAE/g FW in the infusion/MAE of flowers and the decoction of leaves, respectively.

Four assays evaluated the antioxidant activity of the extracts (Table 1). The use of more than one method is recommended to give a comprehensive prediction of antioxidant activity. A pattern of the highest values belonging to the leaves or flowers was not found. The ABTS scavenging capacity ranged from 0.51 to 28.87 μM TE/g FW. Tabaraki et al (2012) report 24.07-28.70 μmol Trolox /gdw ABTS antioxidant activity of mallow leaves. DPPH is a stable nitrogen-centred free radical. The DPPH values of the studied mallow samples ranged from 0.18 μM TE/g FW (flower infusion) to 34.25 μM TE/g FW (flower decoction). Tabaraki et al (2012) report IC_{50} values 0.071-0.077 mg. ml-1 for leaves (ethanol extract). Other authors (DellaGreca et al., 2009) reported, in a study with aerial parts of *Malva sylvestris*, 24% of DPPH scavenging activity at 20 μg/ml. The FRAP assay is a simple test to determine the antioxidant power. The principle of the FRAP method is based on the reduction of a ferrictripyridyltriazine complex to its ferrous coloured form in the presence of antioxidants. The reducing ability of the extracts was in the range of 4.26 ÷ 41.61 μM TE/g FW. The cupric ion (Cu^{2+}) reducing ability of various extracts of *M. sylvestris* leaves and flowers are shown in Table 1. Among all the extracts the decoction of leaves showed the highest CUPRAC value – 58.82 ± 0.28 μM TE/g FW. The results of this assay corresponded well with the already mentioned results pursuant to the FRAP assay.

Table 1. Total phenol content (mg GAE/g FW) and *in vitro* antioxidant activity (μM TE/g FW) of extracts of *M. sylvestris* flowers and leaves.

Methods/ Plant sample	TPC	$TEAC_{DPPH}$	$TEAC_{ABTS}$	$TEAC_{CUPRAC}$	$TEAC_{FRAP}$
Decoction					
-flower	0,83±0,05	34,25±0,06	9,02±0,38	32,62±0,25	16,53±0,31
-leaf	2,35±0,01	1,87±0,01	25,87±0,21	58,82±0,28	41,61±1,49
Infusion					
-flower	0,29±0,01	0,18±0,00	3,06±0,25	22,42±0,15	4,26±0,05
-leaf	0,67±0,02	4,71±0,09	0,51±0,24	24,79±0,07	8,48±0,27
MAE					
-flower	0,29±0,04	10,30±0,04	6,09±0,36	28,34±0,12	11,58±0,15
-leaf	0,62±0,07	4,82±0,07	14,68±0,12	45,27±0,25	15,88±0,08

Both flowers and leaves show antimicrobial activity against the tested microorganisms (Table 2 and 3). The flowers were found to be more active toward pathogenic microorganisms. None of the extracts showed an inhibition against *S. cearevisie, A. niger,* and *P. vulgaris* J. Thus, the *M. sylvestris* extracts showed no antifungal activity. The maximum inhibitory zone was observed by the flower infusion against *Penicillium* sp. These results are comparable to the one reported by Fatima et al (2013) considering the antimicrobial activity of *M. sylvestris* seed oil.

Table 2. Zones of inhibition (mm) of extracts of *M. sylvestris* flowers and leaves against saprophytic microorganisms.

Test-microorganism	*B.cereus*	*Penicillium* sp.	*Rhizopus* sp.
Concentration, CFU/cm^3	$1x10^{10}$	$2x10^5$	$2x10^5$
Infusion- flower	-	9,33	-
Decoction- flower	-	9,17	9
Infusion- leaf	-	9	9
Decoction- leaf	-	9	-

Table 3. Zones of inhibition (mm) of extracts of *M. sylvestris* flowers and leaves against pathogenic microorganisms.

Test-microorganism	*E. coli* ATCC 25922	*E. coli* ATCC 8739	*Salmonella* sp. (clinical isolate)	*St. aureus* ATCC 6538-P
Concentration, CFU/cm^3	1,1x10^{12}	1x10^{14}	6,3x10^{12}	1,2x10^{14}
Infusion- flower	-	-	9	9
Decoction- flower	9	9	9	-
Infusion- leaf	9	-	-	-
Decoction- leaf	-	-	9,17	-

CONCLUSION

The results of the present investigation indicate that the evaluation of the antioxidant activity of four methods, including DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP), ABTS free radical scavenging ability, and CUPRAC assay, reveal a higher level of antioxidant activity in the leaves. Aqueous extracts of flowers showed a lower radical scavenging ability compared to the leaves, contrary to being more active against the tested pathogenic microorganisms. Finally, the kind of mallow analysed here can be considered as good sources of some phenolic and antioxidant compounds.

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