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Tyrosinase Inhibition, Antioxidant and Antibacterial Activity of Commercial Daisy Extract (*Bellis perenn*is)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors EK, EH, SP and MI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EK, EH, ED, MI and AD performed the analyses of the study. Authors SČ and AH performed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Extracts obtained from plant material have widely applied in the chemical and pharmaceutical industries because they contain significant concentrations of biologically active substances. Commercial daisy extract (*Bellis perennis*) was used in this paper for in vitro testing of tyrosinase enzyme inhibition, and antioxidant and antimicrobial activity. Inhibition of the tyrosinase enzyme

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was determined by monitoring dopachrome formation at a wavelength of 492 nm. Antioxidant activity was tested using FRAP and DPPH methods, while antibacterial activity was tested by diffusion technique on reference strains from the ATCC collection. The results showed that daisy extract inhibits tyrosinase enzyme in a dose-dependent manner. The extract effectively neutralized DPPH radicals and also showed good reducing ability. Bacterial strains used for in vitro antimicrobial activity testing did not show sensitivity to the extract concentrations used in this study.

Keywords: Daisy extract; tyrosinase; FRAP; DPPH; antimicrobial activity.

1. INTRODUCTION

"Daisy (*Bellis perennis* L.) is a traditional medicinal plant from the Asteraceae family. It is used to treat eye diseases, eczema, gastritis, enteritis, diarrhoea, rheumatism, upper respiratory tract infections and to heal wounds" [1-4]. "It is native to western, central and northern Europe, but is commonly found as an invasive plant in North America" [5]. "The species has a very long flowering season, with flowers being produced mainly from about March to November. The dominant components in daisies are essential oils, flavonoids and triterpenoid saponins" [3,6-8]. "The phenolic constituents of *Bellis perennis*, besides flavonoids, include anthocyanins (three glucuronylated and malonylated cyanidin-3-glucosides) tannins, and phenolic acids" [9]. "The following flavonoids were described in daisy flowers: quercetin, apigenin, kaempferol, isorhamnetin, apigenin-7-O-β-D-glucoside, apigenin-7-O-β-D-glucuronide, apigenin-7-O-(6´´-E-caffeoyl)-β-D-glucoside, apigenin-7-O-β-D-methylglucuronide, isorhamnetin-3-O-β-D-galactoside, isorhamnetin-3-O-β-D-(6´´-acetyl)-galactoside, and kaempferol-3-O-β-D-glucoside" [9-11]. Daisy has a strong inhibitory effect on melanogenesis (the process of creating the pigment melanin). Inhibits tyrosinase activity. In addition to inhibiting properties, it regenerates skin cells, brightens melanin in the epidermis and protects the skin from forming new freckles and oxidative stress.

Fig. 1. *Bellis perennis*

2. MATERIALS AND METHODS

The commercial extract is accompanied by relevant physico-chemical data from the manufacturer, available in Table 1.

Table 1. Values of selected physicochemical parameters for daisy extract

Parameter	Request	Results	Measure unit
Refraction index	1.337 - 1.341	1.3392	-
Density	1.008 - 1.015	1.009	g/ml
pH	4.8 - 5.8	4.8	-
Dry residue	2 - 3	2.5	%
Color	8 - 12.5	8.6	-
2-phenoxyethanol	0.8 - 1	0.95	%
Potassium sorbate	0.2 - 0.3	0.25	%
Total aerobic bacteria	≤100	< 100	CFU/g
Total yeasts and molds	≤ 10	<10	CFU/g

All chemicals used were of analytical grade and were used as received, without further purification. The 2,2-diphenyl-1-picrylhydrazyl, sodium acetate, ferric chloride and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma-Aldrich (St. Louis, USA). Ultrapure water, prepared with a Smart2Pure device, was used to prepare the aqueous solutions. Spectrophotometric measurements in the analysis of antioxidant capacity were performed on Perkin Elmer Lambda 25 spectrophotometer [12].

2.1 Tyrosinase Inhibition Assay

Tyrosinase enzyme activity was examined in 96 wells of microtiter plates, monitoring the formation of dopachrome at a wavelength of 492 nm, using a microtiter plate reader (Tecan Sunrise). The prepared stock solution of daisy extract in phosphate buffer (0,1 M, pH=6,8) was mixed with phosphate buffer, 40 µl of substrate (L-DOPA, 0,1 mM) and 40 µl of enzyme (mushroom tyrosinase 35 U/Ml-Sigma-Aldrich in phosphate buffer) to optain final daisy extract concentrations (5% - 183,5 mM, 2%- 73,4 mM, 1%- 36,7 mM, 0,5%- 18,3 mM and 0,2%- 7,2 mM) in 200 µl of reaction mixture. The assay mixture containing daisy extract, phosphate buffer and substrate was first incubated at 25°C for 10 minutes. After incubation, 40 µl of enzyme was added to the reaction mixture, and the amount of dopachrome production in the reaction mixture was determined spectrophotometrically at 492 nm every 60 seconds for 30 minutes in a microplate reader.

The extent of inhibition by the tested daisy samples was expressed as the concentration of sample needed to inhibit 50% of the enzymatic activity (IC_{50}). Kojic acid (90 µmol/L) dissolved in phosphate buffer was used as a positive control. Blank tests with and without tyrosinase enzyme were also performed. Each measurement was performed at least in triplicate.

The percentage inhibition of the tyrosinase enzyme is calculated according to the equation:

$$\% \text{ tyrosinase inhibition} = (A-B) - (C-D) / (A-B) \times 100 \quad (1)$$

Where A is the absorbance of the blank with enzyme, B is the absorbance of the blank without enzyme, C is the absorbance of samples with enzyme and D is the absorbance of samples without enzyme.

2.2 DPPH Radical Scavenging Activity

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to the earlier described method [13]. Different extract volumes were mixed with methanol to 2 ml, after which 0.5 ml of 0.5 mM DPPH radical solution was added. The absorbance was measured at 517 nm with methanol as a blank sample. Then, 0.5 ml of 0.5 mM DPPH dilution, diluted with 4 ml of methanol, was used as a control sample.

The radical scavengingeffect (%) or percent inhibition of DPPH radical was calculated according to the equation:

$$[(Ac - As) / Ac] \times 100 \quad (2)$$

Where As is the absorbance of the solution containing the sample at 517 nm and Ac is the absorbance of the DPPH solution.

2.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of the extract, which reflects the antioxidant activity, was determined following the protocol [14]. 3 ml of prepared FRAP reagent was mixed with 100 µl of diluted extract. Absorbance at 593 nm was recorded after a 30 min incubation at 37°C. The FRAP value was calculated from the iron(II) sulfate heptahydrate calibration curve.

2.4 *In vitro* Antibacterial Activity Testing

The *in vitro* antibacterial activities of daisy extract were investigated using two Gram-positive bacteria (*S. aureus* and *E. faecalis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) by applying the diffusion technique. Pure extract and dilutions in the 3-5% range were used to test the susceptibility of bacterial strains. The sample volumes applied to the agar wells were 100 µl. Ciprofloxacin was used as a control.

2.5 Heavy Metal Content

The content of bioelements in such prepared parent sample was determined using ICP OPTIMA 2100 DV (Perkin Elmer) spectrometer. Daisy extracts were directly analysed, without prior preparation.

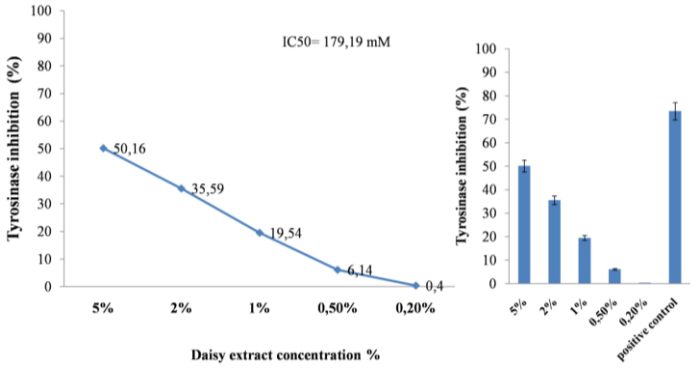

Fig. 2. Results of tyrosinase inhibition analysis with daisy extracts

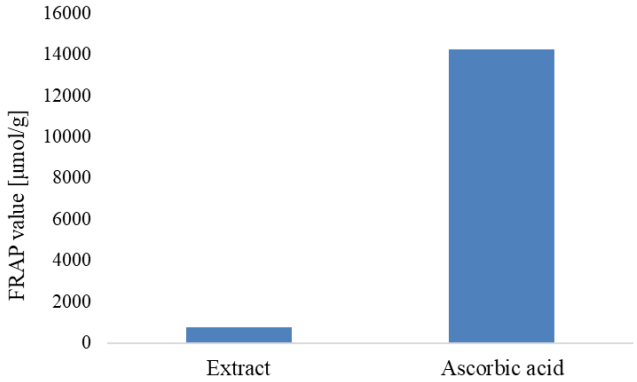

Fig. 3. Graphical comparison of FRAP values of extract and ascorbic acid

3. RESULTS AND DISCUSSION

3.1 Tyrosinase Inhibition

Tyrosinase enzyme plays an important role in melanogenesis. It converts L-tyrosine to DOPA and oxidizes DOPA to dopachtome which is responsible for melanin pigment production [15]. In this study L-DOPA was used as the substrate to evaluate diphenolase activity. All analyzed daisy extracts showed inhibitory potential. The tyrosinase inhibitory potential of the extracts increased as the concentrations were increased from 7,2 mM to 183,5 mM. The calculated concentration of sample needed to inhibit 50% of the enzymatic activity (IC50) was 179,19 mM. The obtained results were comparable to that of the standard tyrosinase inhibitor kojic acid, used as a positive control in a tyrosinase inhibitory assay.

3.2 *In vitro* Antioxidant Activity

FRAP and DPPH methods were used in the antioxidant capacity test, which showed that daisy extract has a high antioxidant capacity in vitro. A FRAP value of 742.11 µmol/g indicates a good reducing ability of the sample, but is still significantly weaker than the ascorbic acid used as a control, whose FRAP value is 14250 µmol/g. The reducing power of bioactive compounds is associated with their electron donating capacity, which is reflected in their antioxidant activity.

Daisy extract has a high power to neutralize DPPH radicals. Therefore, a calibration series was used to construct a calibration direction from which the IC_{50} value for the extract, which was 0.097 mg/ml, was determined. The obtained value is significantly higher than the IC_{50} value of the control, which is 0.03 mg/ml, which indicates a weaker antioxidant capacity of the sample.

Siatka and Kašparova [10] examined seasonal variations in the total phenol and flavonoid content and the activity of removing DPPH daisy radicals, collected at three sites in the Czech Republic. Compared to our study, a slightly better ability to neutralize DPPH radicals was found. It was also found that the content of phenols and flavonoids, as well as the radical activity of cleaning daisy flowers vary relatively little during the year and do not depend on the time of collection.

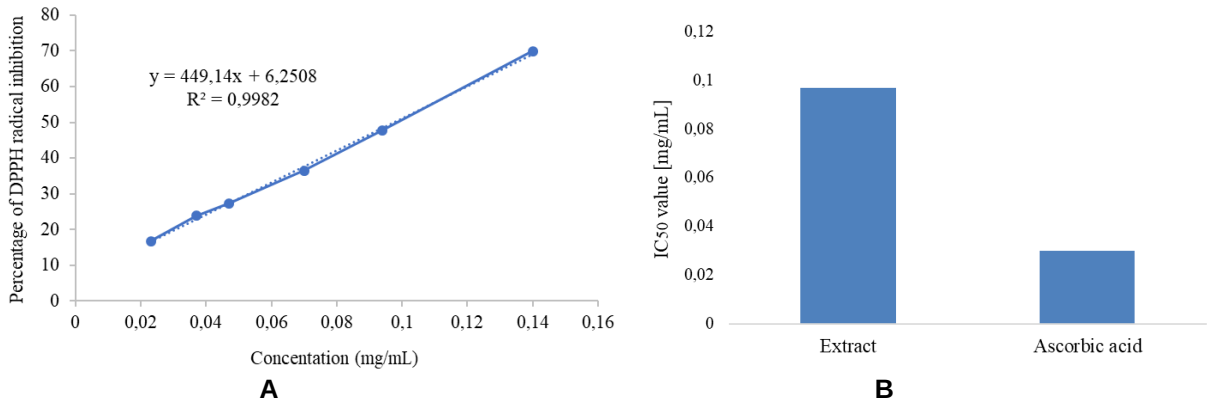

Fig. 4. Results of DPPH radical inhibition by daisy extract (A) and graphical comparison of IC_{50} values for extract and ascorbic acid (B)

Table 2. Content of heavy metals in daisy extract

Heavy metal	Content (mg/l)
As	0,000
Cd	0,000
Cu	0,083
Cr	0,028
Co	0,000
Fe	0,545
Ni	0,273
Sn	0,031
Pb	0,002
Mn	1,267
V	0,000
Sr	0,335
Zn	0,000
Se	0,000

3.3 *In vitro* Antibacterial Activity

The parent extract as well as its dilutions did not inhibit the growth of the bacteria used in this study. The reason for this may be the geographical origin of the sample from which the extract was obtained, as well as the method of production and storage conditions. The antibacterial activity of extracts of different parts of daisies was investigated by the diffusion technique of Karakas et al. [1]. Gram-positive bacteria (*S. aureus*, *S. pyogenes* and *S. epidermidis*) showed greater sensitivity to extracts of *B. perennis* flowers than Gram-negative bacteria. The susceptibility of Gram-positive bacteria may depend on the structure of their cell wall consisting of a single layer. In contrast, the cell wall of Gram-negative bacteria is a multilayered formation.

3.4 Heavy Metal Content

The content of heavy metals in the commercial daisy extract is shown in Table 2. The presence of arsenic, cadmium, cobalt, vanadium, zinc and selenium was not detected in the sample, while other elements were found in very small quantities. The most common element is manganese, with a content of 1.267 mg/l. Typically, manganese can enter the environment from the industries of steel, fireworks, dry cell batteries, fertilizers and paints. Heavy metals are also present in various concentrations in spices and herbs. The share of some heavy metal in the plant, i.e. extracts and essential oils of these plants certainly depends on the locality where the plant is grown, the way the plant is treated with various fertilizers and pesticides, as well as environmental contamination by industrial activities.

4. CONCLUSION

Daisy extract has shown significant effectiveness in neutralizing DPPH radicals and good reducing ability, characterized by an effective natural antioxidant. Antibacterial activity was not recorded on the reference bacterial strains used in this study. Still, a more detailed analysis should certainly be done on other reference and clinical strains, which will certainly be the subject of further research. Daisy extract in all concentrations showed inhibitory activity on the enzyme tyrosinase. As the concentration of daisy extract increased, so did the percentage of inhibition. Based on the chemical composition, it was determined that daisy extract is safe but also effective in inhibiting the enzyme tyrosinase. As such, it could be found in emulsions intended for treating hyperpigmentation.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests, or personal relationships that could have appeared to influence the work reported in this paper.

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