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# BIOACTIVITY ASSAYS ON *CARLINA ACAULIS* AND *C. ACANTHIFOLIA* ROOT AND HERB EXTRACTS

SOFIJA ĐORĐEVIĆ[a,*], VANJA TADIĆ[a], SILVANA PETROVIĆ[b], JELENA KUKIĆ-MARKOVIĆ[b], SILVA DOBRIĆ[c], MARINA MILENKOVIĆ[d], NIHAD HADŽIFEJZOVIĆ[e, f]

[a] *Institute for Medicinal Plant Research "Dr Josif Pančić", Tadeuška Košćuška 1, 11000 Belgrade, Serbia*

[b] *Department of Pharmacognosy, University of Belgrade - Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*

[c] *Institute for Scientific Information, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Serbia*

[d] *Department of Microbiology and Immunology, University of Belgrade - Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia*

[e] *Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, Hittorfstrasse 56, 48149 Münster, Germany*

[f] *present address: SIDROGA GfGmbH, Arzbacher Str. 78, 56130 Bad Ems, Germany*

The present study describes investigation on chemical profile and pharmacological properties of hydromethanolic extracts of *Carlina acaulis* and *C. acanthifolia* roots and herbs. In order to investigate the antioxidant properties several methods were applied: FRAP (Ferric Reducing Antioxidant Power) assay, TBA assay ($Fe^{2+}$/ascorbic induced lipid peroxidation) and scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and $OH^{\cdot}$ radicals. In assessing anti-inflammatory activity the carrageenan-induced rat paw oedema test was used, with indomethacin as a reference drug. Anti-ulcer activity of the extracts was investigated using ethanol-induced stress ulcer in rats and ranitidine as a reference drug. Antimicrobial activity was tested on five bacterial strains and one strain of fungi using the agar diffusion method. Investigated extracts exhibited considerable dose-dependent antioxidant, anti-inflammatory and anti-ulcer activities. Extracts of herbs were more active than the corresponding root extracts. All extracts exhibited moderate activity against all tested microorganisms. Herb extracts of *C. acaulis* and *C. acanthifolia*, as well as their root extracts exhibited similar bioactivity, which could be explained with their similar chemical composition revealed by HPLC analysis. The pharmacological activities of investigated *Carlina* extracts demonstrated here will serve as the basis for their further investigations in order to estimate their impact on human health and nutrition.

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## 1. Introduction

Widely distributed in Europe, species of genus *Carlina* L. (Asteraceae) were traditionally applied for their healing properties. The root of *C. acaulis* L. was used as a diuretic, diaphoretic, stomachic, and externally for treatment of skin inflammations, as well as against toothache. Phytopreparation on the basis of root extracts of *C. acaulis* are nowadays used for treating cholecystopathy and gastrointestinal disturbances [1]. The root of *C. acanthifolia* All., another

*Corresponding author: sdjordjevic@mocbilja.rs

widely distributed *Carlina* species, is very similar to the root of *C. acaulis* according to its organoleptic, morphological and anatomical features [2]. Traditionally it has been used for the same purposes and is considered as an adulterant or substitute for *C. acaulis* root. Analysis of commercial samples of the *Carlinae radix* showed that it is often consisting of *C. acanthifolia* instead of *C. acaulis* roots. Roots of these plants contain inulin (*ca*. 20%) [1], which belongs to a so-called inulin-type fructans, the most important group of prebiotics [3]. They also contain essential oil (1-2%), with carlina-oxide as the main compound (over 90%) [4, 5]. Essential oil isolated from the roots of *C. acaulis* possesses substantial antimicrobial activity [3]. Such activity has been proven for the oil isolated from the root of *C. acanthifolia*, along with other pharmacological effects: anti-inflammatory, anti-ulcer, and antioxidant [6]. Contrary to the data on traditional medicinal use of the *Carlina* roots, such data for the *Carlina* herbs are very limited. Earlier investigations showed presence of several flavonoid glycosides in *C. acaulis* and *C. acanthifolia* leaves [7].

As part of an ongoing study considering chemical and pharmacological characterization of these two species, the aim of this study was to estimate the antioxidant, anti-inflammatory, anti-ulcer and antimicrobial potential of hydromethanolic extracts of *C. acaulis* and *C. acanthifolia* roots and herbs.

## 2. Material and methods

### 2.1. Plant material and extraction

The roots and aerial blooming parts of *C. acaulis* and *C. acanthifolia* were collected on Mt. Durmitor (Montenegro), and on Mt. Suva Planina (E Serbia), respectively and identified by Dr. sc. Marjan Niketić (custodian). Once harvested, herbs were dried at room temperature. Voucher specimens (ko820033/15 and ko820035/1, respectively) were deposited in the Herbarium of Natural History Museum, Belgrade.

Powdered plant material was twice extracted with 70% methanol (drug:solvent ratio 1:10, w/v) in the water bath under reflux for 30 min. Extracts were filtered and further evaporated under reduced pressure. Yields of dry extracts were: 21.3% (*C. acaulis* root), 20.0% (*C. acaulis* herb), 23.8% (*C. acanthifolia* root) and 20.8% (*C. acanthifolia* herb).

### 2.2. Determination of total phenolics content

Total phenolics content (TPC) in *Carlina* herb extracts was determined using Folin-Ciocalteu (FC) reagent and expressed as gallic acid (GA) equivalents (mg GA/mg of dry extract) [8].

### 2.3. HPLC analysis

Analysis of the samples was performed using HP 1090 Liquid chromatograph (Hewlett Packard) with UV detector (DAD), equipped with the LiChrospher®-100, RP-18e (150 × 4.6 mm i.d., 5 μm particle size). Samples were dissolved in methanol (10 mg/mL) and further filtered through a 0.45 μm membrane filter. Gradient elution was performed at 20 °C, for 60 min, at a flow rate of 0.8 mL/min, using acetonitrile (solvent A) and 3% *o*-$H_3PO_4$ in $H_2O$ (solvent B). Phenolic compounds were identified at 280 nm by comparison of their retention times and UV data with those of authentic standard compounds (chlorogenic acid, apigenin, apigenin 7-O-glucoside, vitexin, orientin, homoorientin) (Roth, Karlsruhe, Germany) and schaftoside (isolated from *Ruscus aculeatus* L.) [9]. Solutions of standard compounds were prepared at a final concentration of 0.12 mg/mL in methanol. For the purpose of the phenolic compounds identification and determination, mixtures of the standards in already mentioned concentration were prepared: mixture 1 with chlorogenic acid and schaftoside, mixture 2 with orientin, mixture 3 contained the rest of investigated phenolic compounds. The volume injected was 4 μL, the same as the investigated extract. The identification was carried out thanks to retention time and spectra matching. Once spectra matching succeeded, results were confirmed by spiking with respective standards to achieve a complete identification by means of the so-called peak purity test. Those peaks not fulfilling these requirements were not quantified. Quantification was performed by the external standard method. All the samples were analyzed in triplicate. The amount of constituents was calculated taking into account the response factor of corresponding standards to considered

constituent and purity of the authentic reference samples. The content of the constituents was calculated by the following equation:

$$\text{content (\%)} = \frac{RF_{sample} \times c_{st} \times purity_{st}}{c_{sample}}$$

where *RF* is response factor for the investigated sample, $c_{st}$ is concentration of corresponding standard and $c_{sample}$ is concentration of the investigated sample.

### 2.4. Antioxidant activity

#### *2.4.1. Thin-layer chromatography*

Twenty microlitres of each extract dissolved in 70% methanol (1%, w/v) was applied on silica gel plates (Merck, Darmstadt, Germany) and developed using ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:26, v/v/v/v) system. Components of the extracts were detected by spraying with NP/PEG reagent [10]. TLC plates were also sprayed with 0.2% (w/v) 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol, which revealed contribution to the antioxidant activity of different compounds separately [11].

#### *2.4.2. FRAP assay*

Total antioxidant activity (TAA) was investigated using Ferric Reducing Antioxidant Power (FRAP) assay, which is based upon reduction of $Fe^{3+}$-2,4,6-tris-(2-pyridyl)-*s*-triazine complex ($Fe^{3+}$-TPTZ) in acidic conditions. Calibration curve of ferrous sulfate (100-1000 μM) was used, and results were expressed in μmol $Fe^{2+}$/mg dry weight extract (FRAP value). The relative activity of the samples was compared to L-ascorbic acid [12].

#### *2.4.3. DPPH Radical Assay*

Extracts were dissolved in 70% methanol (0.5%, w/v), mixed with 1 ml of 0.5 mM DPPH in methanol, and final volume adjusted up to 5 ml. Scavenging of DPPH radical was calculated using the equation: $S(\%)=100\times(A_0-A_s)/A_0$, where $A_0$ is the absorbance of the control (containing all reagents except the test compound), and $A_s$ is the absorbance of the tested sample. The $SC_{50}$ value represented the concentration of the extract that caused 50% scavenging [12].

#### *2.4.4. 2-Deoxyribose Assay*

The 2-deoxyribose method was used for determining the scavenging effect on hydroxyl radical. Extracts were applied in different concentrations (6.25-250 μg/ml). Inhibition of 2-deoxyribose degradation in percents was calculated using the equation: $I(\%)=100\times(A_0-A_s)/A_0$, where all symbols have the same meaning as in DPPH radical assay [12].

#### *2.4.5. TBA Test*

Lipid peroxidation (LP) was measured using preparation of liposomes containing 3% (w/v) lecithin. Liposomes were prepared from the commercial preparation "Lipotech 10", containing 10% (w/v) lecithin, diluted with distilled water in ultrasonic bath for 30 min. All extracts were dissolved in 70% methanol in different concentrations (0.5-10%, w/v). Inhibition of LP was calculated in the same way as described in DPPH radical assay [12].

### 2.5. Anti-inflammatory activity

The carrageenan-induced rat paw oedema test has been used as an experimental model for screening the anti-inflammatory activity as reported earlier [13]. The investigated *Carlina* extracts, dissolved in DMSO were administered *p.o.* in doses of 25-200 mg/kg. Indomethacin (Sigma Chemical Co., St. Louis, USA) dissolved in DMSO, was used as a reference drug in doses of 1-8 mg/kg *p.o.* The control animals were given the vehicle (DMSO) in a dose of 1 mL/kg *p.o.* One hour after the oral administration of the extracts or indomethacin, carrageenan-saline solution (0.5%, w/v) and saline were injected in a volume of 0.1 mL into the plantar surface of the right and left hind paw, respectively. Left paw served as the control (non-inflammed) paw. The animals were sacrificed 3 h after the carrageenan and saline injection and paws were cut off for weighing. Difference in weight between right and left paw, active drug-treated *versus* vehicle-treated

(control) rats, served as an indicator of the anti-inflammatory activity of drugs tested (the extracts and indomethacin). The anti-inflammatory effect was calculated using the equation:

$$\text{Anti-inflammatory effect } (\%) = \frac{k-e}{k} \times 100$$

where $k$ is difference in the paw weight in the control group, and $e$ is difference in the paw weight in the treatment group.

### 2.6. Anti-ulcer activity

In order to study the anti-ulcer activity an experimental model of acute gastric mucosa damage induced by absolute ethanol (1 mL/rat *p.o.*) was used. The investigated *Carlina* extracts, dissolved in DMSO, were administered *p.o.* in doses of 25-200 mg/kg 60 min prior to ethanol. Ranitidine (Ranisan®ampoules, 10 mg/mL, Zdravlje Actavis Company, Leskovac, Serbia) given in doses of 5-20 mg/kg *p.o.*, was used as a reference drug. The control animals were given the vehicle in a dose of 1 mL/kg *p.o.*, also 60 min before ethanol. The animals were sacrificed 1 h after giving ethanol and their stomachs were removed and opened along the greater curvature. Lesions were examined under an illuminated magnifier (3×). The intensity of gastric lesions was assessed according to a modified scoring system of Adami et al. [14].

### 2.7. Animals

Adult male Wistar rats, weighing 200-250 g, were used in both the carrageenan-induced rat paw oedema and the ethanol-induced stress gastric ulcer tests. Experimental groups consisted of 6-10 animals each. The animals were deprived of food for 18–20 h before the beginning of experiments with free access to tap water. Animal studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care in the European Community guidelines (Directive 86/609/EEC) adopted by Ethical Committee of Military Medical Academy, Belgrade.

### 2.8. Antimicrobial activity

Antimicrobial activity of investigated *Carlina* extracts was assayed using the agar diffusion method [6], against: Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212; Gram-negative bacteria *Klebsiella pneumoniae* NCIMB-9111, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853; and yeast *Candida albicans* ATCC 24433 (Institute of Immunology and Virology, Torlak, Belgrade, Serbia). The overnight cultures of tested strains were diluted in saline in order to adjust the turbidity of the microbial suspension to 0.5 McFarland standard (approximately $10^8$ CFU/mL). Agar diffusion method was performed on Müller-Hinton and Sabouraud agar to test sensitivity of bacteria and *C. albicans*, respectively. Extracts were dissolved in absolute ethanol (2 and 4%, w/v) and one drop of each solution was poured on the agar prepared as required. After incubation (18 h at 37 °C for bacteria and 48 h at 26 °C for *C. albicans*) diameters of zones of inhibition (in mm) were measured. Ampicillin (10 μg/disk), amikacin (30 μg/disk) and nystatin (100 units/disk) were used as appropriate controls of sensitivity of tested microorganisms.

### 2.9. Statistical analysis

Results were expressed as means ± standard deviations (SD). Statistical analysis was performed by the Mann–Whitney *U*-test and ANOVA. Differences were accepted as statistically significant when $P<0.05$.

## 3. Results

HPLC chromatograms of *Carlina* herb extracts revealed presence of flavone glycosides and several phenolic acids, mostly caffeic acid derivatives. Extracts showed very similar composition: homoorientin, orientin, apigenin and chlorogenic acid were determined in both extracts in similar quantity. Vitexin and apigenin 7-O-glucoside were detected only in *C. acaulis* herb extract, and schaftoside only in *C. acanthifolia* extract (Table 1). This is the first report on

presence of apigenin and chlorogenic acid in herbs of these two *Carlina* species, while the presence of other components is known from earlier investigations [7].

*Table 1. Flavonoids and phenolic acids content[a] in Carlina herb extracts determined by HPLC[b]*

| No. | Rt (min) | Compounds | *C. acaulis* herb | *C. acanthifolia* herb |
|---|---|---|---|---|
| 1 | 18.12 | Chlorogenic acid | 1.94 ± 0.02 | 1.81 ± 0.03 |
| 2 | 29.87 | Schaftoside | - | 0.75 ± 0.01 |
| 3 | 30.05 | Homoorientin | 0.92 ± 0.01 | 0.91 ± 0.02 |
| 4 | 30.12 | Orientin | 0.32 ± 0.02 | 0.26 ± 0.02 |
| 5 | 31.35 | Vitexin | 0.61 ± 0.06 | - |
| 6 | 33.29 | Apigenin 7-O-glucoside | 0.45 ± 0.01 | - |
| 7 | 39.38 | Apigenin | 0.15 ± 0.06 | 0.22 ± 0.01 |

[a] in %; [b] means of three replicates ± SD

HPLC chromatograms of investigated *Carlina* root extracts indicated similar chemical pattern in both samples. Analysis of main peaks in these chromatograms showed presence of several phenolic acids, which are caffeic acid derivatives, but neither of peaks could be assigned to the standard compounds used.

All investigated *Carlina* extracts showed moderate antioxidant activity. Total antioxidant activity (TAA) was in correlation with total phenolics content (TPC) (Table 2), but lower than the activity of L-ascorbic acid used as standard (7.41 μmoL $Fe^{2+}$/mg). Both values for herb extracts were *ca*. 2.5-3 times higher than those for root ones.

*Table 2. Total phenolics content (GA equvalents) and total antioxidant activity (FRAP value) of Carlina extracts.*

| Extract | GA equvalents[a] | FRAP value[b] |
|---|---|---|
| *C. acaulis* root | 0.032 ± 0.005 | 0.207 ± 0.000 |
| *C. acaulis* herb | 0.091 ± 0.002 | 0.592 ± 0.001 |
| *C. acanthifolia* root | 0.031 ± 0.007 | 0.251 ± 0.003 |
| *C. acanthifolia* herb | 0.071 ± 0.001 | 0.579 ± 0.006 |

[a] mg of galic acid (GA)/mg of dry extract; [b] μmol $Fe^{2+}$/mg of dry extract

Quenching of DPPH radical was concentration-dependent. Extracts of *Carlina* herbs were more potent scavengers of DPPH radical than the extracts of the roots. Herb extracts of *C. acaulis* and *C. acanthifolia* reached 50% of DPPH neutralisation at 72.00 and 87.33 μg/mL, respectively, while their root extracts expressed much lower activity ($SC_{50}$ values at 208.00 and 155.00 μg/mL) (Table 3). TLC-DPPH test showed that in all extracts flavonoids and phenolic acids were the main antioxidant compounds.

*Table 3. Scavenging activity of Carlina extracts against DPPH radical.*

| Concentration (μg/mL) | *C. acaulis* root | *C. acaulis* herb | *C. acanthifolia* root | *C. acanthifolia* herb |
|---|---|---|---|---|
| 5 | 2.43±1.14 | 3.65±0.78 | 3.12±0.45 | 3.11±0.62 |
| 10 | 2.97±0.62 | 5.40±1.34 | 4.50±1.63 | 5.75±0.95 |
| 50 | 9.71±0.11 | 32.14±0.73 | 16.28±0.51 | 28.57±1.72 |
| 100 | 21.09±0.40 | 61.28±0.38 | 31.90±1.53 | 54.77±0.67 |
| 250 | 60.92±0.74 | 94.51±0.10 | 81.00±0.00 | 94.71±0.12 |
| **$SC_{50}$** | **208.00±1.56** | **72.00±3.28** | **155.00±1.95** | **87.33±1.25** |

Scavenging of $OH^{\cdot}$ radicals by extracts was concentration-dependent (Table 4). Extract of *C. acaulis* herb was the most pronounced "scavenger" among all extracts ($IC_{50}$=8.00 μg/mL). Activity of *C. acanthifolia* herb extract was lower, but still substantial ($IC_{50}$=9.59 μg/mL). Neither of *Carlina* root extracts reached $IC_{50}$ value.

*Table 4. Scavenging activity of Carlina extracts against OH radical obtained in 2-deoxyribose test.*

| Concentration (μg/mL) | *C. acaulis* root | *C. acaulis* herb | *C. acanthifolia* root | *C. acanthifolia* herb |
|---|---|---|---|---|
| 6.25 | 8.67±4.91 | 40.43±0,18 | 21.66±5.19 | 46.94±0.58 |
| 12.5 | 25.37±1.39 | 54.20±1.55 | 33.71±0.58 | 54.71±0.35 |
| 25 | 34.70±4.51 | 59.89±0.27 | 43.04±1.80 | 57.36±1.40 |
| 50 | 39.77±1.31 | 60.16±0.82 | 43.70±2.72 | 57.68±0.35 |
| 100 | 40.75±1.43 | 58.86±2.96 | 44.84±1.62 | 56.69±1.06 |
| 200 | 36.82±2.06 | 55.39±0.81 | 41.08±0.00 | 46.94±1.52 |
| **$IC_{50}$** | **-** | **8.00±0.35** | **-** | **9.59±0.36** |

As for inhibition of LP, neither extract reached 50% of inhibition. In this case *C. acanthifolia* herb extract was the most active inhibitor of LP (47.26% of inhibition at 125 μg/mL) (Table 5).

*Table 5. Inhibitory effect of Carlina extracts on lipid peroxidation (LP)*

| Concentration (μg/mL) | *C. acaulis* root | *C. acaulis* herb | *C. acanthifolia* root | *C. acanthifolia* herb |
|---|---|---|---|---|
| 25.0 | 16.53±7.47 | 29.65 ± 3.48 | 5.70±1.15 | 32.02±4.04 |
| 62.5 | 18.54±7.26 | 37.48 ± 0.00 | 8.16±1.78 | 39.90±0.35 |
| 125 | 20.16±0.57 | 38.82±0.27 | 14.97±6.06 | 47.26±6.78 |
| 250 | 25.73±2.31 | 36.87±0.00 | 19.34±9.89 | 42.58±10.81 |

Results of the experiment showed that in all applied doses the extracts of *C. acaulis* and *C. acanthifolia* roots and herbs reduced carrageenan-induced rat paw oedema in dose-dependent manner, achieving high degree of anti-inflammatory activity. Extract of herbs were more active than the corresponding root extracts (Table 6). This effect was comparable with that of indomethacin used as a reference drug. The most active was the extract of *C. acanthifolia* herb given in a dose of 200 mg/kg *p.o*. (average anti-inflammatory activity 61.24%).

*Table 6. Anti-inflammatory activity of Carlina extracts.*

| | Anti-inflammatory effect (%) | | | | | |
|---|---|---|---|---|---|---|
| **Dose[a]** | ***C. acaulis* root** | ***C. acaulis* herb** | ***C. acanthifolia* root** | ***C. acanthifolia* herb** | **Indomethacin** | **Dose[a]** |
| 25 | 29.69 ± 5.94* | 43.74 ± 7.36** | 27.23 ± 2.88* | 44.84 ± 6.77** | 27.14 ± 11.83* | 1 |
| 50 | 38.50 ± 9.95** | 48.22 ± 11.89** | 41.15 ± 7.38** | 51.12 ± 6.26** | 50.37 ± 5.96** | 2 |
| 100 | 40.77 ± 8.67** | 53.24 ± 8.93** | 42.53 ± 1.54** | 56.65 ± 6.57** | 58.06 ± 13.87** | 4 |
| 200 | 52.37 ± 7.03** | 58.03 ± 15.04** | 57.28 ± 11.60** | 61.24 ± 15.89** | 74.32 ± 15.70** | 8 |

[a] mg/kg *p.o*.; *p<0.05; **p<0.001 vs. control DMSO, 1 mL/kg (0.00 ± 20.57)

In the ethanol-induced stress gastric ulcer test in rats it was shown that all tested extracts produced significant and very similar dose-dependent gastroprotective activity, comparable to that of the well-known anti-ulcer drug ranitidine. The most active one was herb extract of *C. acaulis* (Gastric damage score of 1.98) (Table 7).

*Table 7. Anti-ulcer activity of Carlina extracts*

| Gastric damage score (GDS)[a] | | | | | | |
|---|---|---|---|---|---|---|
| **Dose[b]** | ***C. acaulis* root** | ***C. acaulis* herb** | ***C. acanthifolia* root** | ***C. acanthifolia* herb** | **Ranitidine** | **Dose[b]** |
| 50 | $2.92 \pm 1.39^{***}$ | $2.70 \pm 2.07^{**}$ | $3.30 \pm 2.33^{**}$ | $2.68 \pm 2.43^{*}$ | $3.08 \pm 0.8^{***}$ | 5 |
| 100 | $2.75 \pm 1.47^{***}$ | $2.50 \pm 2.00^{**}$ | $2.45 \pm 2.33^{**}$ | $2.57 \pm 2.38^{*}$ | $2.83 \pm 1.03^{***}$ | 10 |
| 200 | $2.05 \pm 1.44^{***}$ | $1.98 \pm 1.52^{***}$ | $2.00 \pm 1.64^{***}$ | $2.07 \pm 1.83^{***}$ | $1.77 \pm 1.08^{***}$ | 20 |

[a] **0 =** no lesions; **0.5 =** slight hyperaemia or ≤5 petechiae; **1 =** ≤5 erosions ≤5 mm in length; **1.5 =** ≤5 erosions ≤ 5 mm in length and many petechiae; **2 =** 6-10 erosions ≤5 mm in length; **2.5** = 1-5 erosions >5 mm in length; **3** = 5-10 erosions >5 mm in length; **3.5** = >10 erosions >5 mm in length; **4** = 1-3 erosions ≤5 mm in length and 0.5-1 mm in width; **4.5** = 4-5 erosions ≤5 mm in length and 0.5-1 mm in width; **5** = 1-3 erosions >5 mm in length and 0.5-1 mm in width; **6 =** 4 or 5 grade 5 lesions; **7 =** ≥6 grade 5 lesions; **8** = complete lesion of the mucosa with haemorrhage (according to Adami et al., 1964); [b] mg/kg *p.o.*; *p<0.05; **p<0.01; ***p<0.001 vs. control DMSO, 1 mL/kg (GDS = 5.90 ± 1.14)

Results presented in this study demonstrated considerable antimicrobial activity of all investigated extracts against all tested microorganisms (Table 8). Extract of *C. acaulis* root was the most active against *K. pneumoniae* (97.1% activity of ampicillin, for 2% ethanol solution of extract) and against *E. coli* (76.7% activity of ampicillin, for 4% ethanol solution). Activity of *C. acanthifolia* root extract against these bacteria was also pronounced: 85.3% activity of ampicillin against *K. pneumoniae*, and 66.7% activity of ampicillin against *E. coli*, for 4% ethanol solutions, respectively. Investigated *Carlina* root extracts showed strong antifungal activity. The most expressed activity was that of 2% solution of *C. acaulis* root extract (90.9% of the activity of nystatin, for 2% ethanol solution). *Carlina acaulis* herb extract was most active against *K. pneumoniae* (82.3% of the activity of ampicillin, for 2% ethanol solution), and against *E. coli* (69.4% of the activity of ampicillin, for 2% ethanol solution). The highest activity of *C. acanthifolia* herb extract was detected against *K. pneumoniae* (88.2% of the activity of ampicillin, for 2% ethanol solution of extract) and *E. faecalis* (75.0% of the activity of ampicillin, for 4% ethanol solution). Antifungal activity of tested extracts was also evident. *Carlina acanthifolia* herb extract (4% solution) exhibited the highest activity against yeast *C. albicans* (72.7% of the activity of nystatin).

*Table 8. Antimicrobial activity of Carlina extracts against standard microorganisms*

| Microorganism | Inhibition zones in mm | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***C. acaulis* root[a]** | | ***C. acaulis* herb[a]** | | ***C. acanthifolia* root[a]** | | ***C. acanthifolia* herb[a]** | | **AMP** | **AMK** | **NYS** |
| | **2%** | **4%** | **2%** | **4%** | **2%** | **4%** | **2%** | **4%** | | | |
| *Staphylococcus aureus* ATCC 25923 | 12.50 ±0.60 | 14.50 ±0.80 | 15.00 ±0.82 | 11.00 ±0.82 | 11.50 ±0.60 | 11.50 ±1.30 | – | 10.00 ±0.82 | 27.00 | 26.00 | n.t. |
| *Enterococcus faecalis* ATCC 29212 | 12.50 ±0.90 | 12.00 ±0.80 | 11.00 ±1.26 | 9.30 ±0.82 | 7.00 ±0.80 | 11.00 ±1.40 | 10.00 ±1.91 | 12.00 ±0.82 | 16.00 | n.t. | n.t. |
| *Klebsiella pneumoniae* NCIMB-9111 | 16.50 ±0.60 | 11.50 ±1.26 | 14.00 ±0.82 | 8.50 ±2.87 | 13.00 ±1.80 | 14.50 ±0.80 | 15.00 ±1.53 | 12.00 ±1.63 | 17.00 | n.t. | n.t. |
| *Escherichia coli* ATCC 25922 | 13.00 ±0.80 | 13.80 ±0.80 | 12.50 ±0.82 | 12.00 ±0.82 | 11.00 ±1.40 | 12.00 ±1.40 | 14.00 ±2.31 | 12.00 ±1.00 | 18.00 | 24.00 | n.t. |
| *Pseudomonas aeruginosa* ATCC 27853 | 10.00 ±0.80 | 9.00 ±1.63 | 12.00 ±0.96 | 12.00 ±0.96 | 13.50 ±2.20 | 14.00 ±0.80 | 13.50 ±1.00 | 9.00 ±2.08 | n.t. | 26.00 | n.t. |
| *Candida albicans* ATCC 24433 | 20.00 ±0.80 | 14.50 ±0.60 | 13.50 ±2.52 | 8.00 ±1.71 | 13.00 ±2.50 | 13.50 ±1.70 | 15.50 ±0.58 | 16.00 ±3.56 | n.t. | n.t. | 22.00 |

[a] % (w/v) in absolute ethanol; n.t. = not tested; AMP = ampicillin (10 μg/disk); AMK = amikacin (30 μg/disk) ); NYS = nystatin (100 units/disk)

## 4. Discussion and conclusion

Obtained activities of *Carlina* herb extracts were in correlation with the presence of phenolic compounds identified in both extracts using HPLC. As previously shown, apigenin, luteolin and their glycosides are powerful antioxidants [15, 16]. Flavone C-glycosides (orientin, homoorientin, and vitexin) are capable of blocking chain reactions of lipid auto-oxidation, chelating metal ions of transient state, scavenging nitric compounds and blocking the synthetic reaction of nitrosamine [17]. Orientin expresses strong radical scavenging activity and thus, high radioprotective efficacy [18].

Some compounds identified in the investigated *Carlina* herb extracts demonstrated anti-inflammatory properties earlier. Apigenin has COX-2 inhibitory activity comparable to NSAIL [19]. Vitexin and schaftoside inhibit LPS-induced mouse lung inflammation [20]. Luteolin inhibits lipopolysaccharide-induced TNF-α production. Flavones resembling luteolin in structure (with 3',4',5,7-tetrahydroxy substitution) also resemble its anti-inflammatory activity [21]. Isoorientin has been shown to possess significant anti-nociceptive and anti-inflammatory activities [22]. Chlorogenic acid inhibits SE-induced T-cell proliferation and production of various pro-inflammatory compounds (e.g. IL-1β, TNF-α, IL-6, INF-γ, MCP-l, MIP-lα and MIP-lβ) by human peripheral blood mononuclear cells and may serve as a potent anti-inflammatory agent [23].

Antioxidant activity of the investigated extracts could be, at least partly, responsible for the observed anti-inflammatory effects, since it was earlier shown that antioxidants may mediate anti-inflammatory activity by direct scavenging of ROS [24], by influencing polymorphonuclear neutrophils oxidative metabolism [25, 26] and also by preventing induction of the cytokine cascade and up-regulating the expression of adhesion molecules [27].

The antioxidant and free radical scavenging activity might be also considered as one of the possible mechanisms of gastroprotective effects of the various plant products, since oxygen derived radicals and agents with antioxidant properties have been implicated in the pathogenesis of

ethanol-induced gastric ulcers and in their prevention and healing, respectively [28, 29]. Many flavonoids, known as strong free-radical scavengers, have been reported to have gastric ulcer protective effects [30].

The presence of the compounds identified could also explain the observed moderate antimicrobial activity. Zhang and co-workers (2005) showed that homoorientin possesses antimicrobial activity against some bacterial strains. The presence of apigenin, apigenin 7-O-glucoside, luteoline and other flavone derivatives for which the antimicrobial activity has been reported previously [31, 32], should not be neglected, nor the possibility of interactions between the others components of the extracts.

In all experiments, *C. acaulis* and *C. acanthifolia* herb extracts, as well as their root extracts, showed high resemblance with each other, for both their activities, and their composition of phenolic compounds. The pharmacological activities of investigated *Carlina* extracts demonstrated here will serve as the basis for their further investigations in order to estimate their impact on human health and nutrition.

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## References

[1] M. Wichtl, Teedrogen und Phytopharmaca, Stuttgart, Wissenschaftliche Verlagsgesellschaft, mbH Stuttgart (2002).
[2] S. Đorđević, B. Lakušić, S. Petrović, M. Niketić, Arch. Pharm. **54**, 773-783 (2004).
[3] S. Đorđević, S. Lukić, M. Mraović, S. Žižić, Pharm. Pharmacol. Lett. **8**, 126-128 (1998).
[4] J.C. Chalchat, S. Đorđević, M. Gorunović, J. Essent. Oil Res. **8**, 577-578 (1996).
[5] S. Đorđević, S. Petrović, M. Ristić, D. Đoković, Chem. Nat. Comp. **41**, 410-412 (2005).
[6] S. Đorđević, S. Dobrić, S. Petrović, M. Milenković, D. Vučićević, S. Žižić, J. Kukić, J. Ethnopharmacol. **109**, 458-463 (2007).
[7] H. Meusel, A. Kästner, Lebensgeschichte der Gold-und Silberdisteln, Monographie der mediterran-mitteleuropäischen Compositen-Gattung *Carlina*, Band I, Springer-Verlag, Wien, New York, 1990.
[8] V. M. Tadić, S. Dobrić, G. M. Marković, S. M. Đorđević, I. A. Arsić, N. R. Menković, T. Stević, J Agric. Food Chem. **56**, 7700-7709 (2008).
[9] N. Hadžifejzović, PhD Thesis, University of Münster, Germany (2006).
[10] H.Wagner, S. Bladt, Plant Drug Analysis. A Thin Layer Chromatography Atlas, second ed., Springer-Verlag, Berlin-Heidelberg, Germany, 1996.
[11] M. Cuendet, K. Hostettmann, O. Potterat, Helv Chim Acta **80**, 1144-1152 (1997).
[12] J. Kukić, S. Petrović, M. Niketić, Biol. Pharm. Bull. **29**, 725-729 (2006).
[13] S. D. Petrović, S. Dobrić, D. Bokonjić, M. Niketić, A. Garcia-Piñeres, I. Merfort, J. Ethnopharmacol. **87**, 109-113 (2003).
[14] E. Adami, E. Marazzi-Uberti, C. Turba, Arch. Int. Pharmacodyn. Ther. **147**, 113-145 (1964).
[15] Y. S. Kwon, E. Y. Kim, W. J. Kim, W. K. Kim, C. M. Kim, Arch. Pharm. Res. **25**, 300-305 (2002).
[16] S. D. Müller, S. B. Vasconcelos, M. Coelho, M. W. Biavatti, J. Pharm. Biomed. Anal. **37**, 399-403 (2005).
[17] Y. Zhang, B. Bao, B. Lu, Y. Ren, X. Tie, Y. Zhang, J. Chromatogr. A **1065**, 177-185 (2005).
[18] B. Vrinda, P. Uma Devi, Mutat. Res. **498**, 39-46 (2001).
[19] M. A. Kelm, M. G. Nair, G. M. Strasburg, D. L. DeWitt, Phytomedicine **7**, 7-13 (2000).
[20] G. O. de Melo, M. F. Muzitano, A. Legora-Machado, T. A. Almeida, D. B. de Oliveira, C. R. Kaiser, V. L. Koatz, S. S. Costa, Planta Med. **71**, 362-363 (2005).

[21] H. Ueda, C. Yamazaki, M. Yamazaki, Biosci. Biotechnol. Biochem. **68**, 119-125 (2004).
[22] E. Kupeli, M. Aslan, I. Gurbuz, E. Yesilada, Z. Naturforsch. [C] **59**, 787-790 (2004).
[23] T. Krakauer, Immunopharmacol. Immunotoxicol. **24**, 113-119 (2002).
[24] C. D. Sadik, H. Sies, T. Schewe, Biochem. Pharmacol. **65**, 773-781 (2003).
[25] S. Sahpaz, N. Garbacki, M. Tits, F. Bailleul, J. Ethnopharmacol. **79**, 389-392 (2002).
[26] J. Y. Lee, E.-R. Woo, K. W. Kang, J. Ethnopharmacol. **97**, 561-566 (2005).
[27] M. Menegazzi, R. Di Paola, E. Mazzon, C. Muià, T. Genovese, C. Crisafulli, H. Suzuki, S. Cuzzocrea, Free Rad. Biol. Med. **40**, 740-753 (2006).
[28] A. Terano, H. Hiraishi, S. Ota, J. Shi Tega, T. Sugimoto, Gastroenterol Jpn. **24**, 488-493 (1989).
[29] A. S. Salim, Digestion **47**, 24-28 (1990).
[30] C. La Casa, I. Villegas, C. A. de la Lastra, V. Motilva, M. J. Martín Calero, J. Ethnopharmacol. **71**, 45-53 (2000).
[31] I. Aljančić, V. Vajs, N. Menković, I. Karadžić, N. Juranić, S. Milosavljević, S. Macura, J. Nat. Prod. **62**, 909-911 (1999).
[32] T. E. Tshikalange, J. J. M. Meyer, A. A. Hussein, J. Ethnopharmacol. **96**, 515-519 (2005).