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Original scientific paper

Antimicrobial activity of secondary metabolites isolated from *Centaurea spruneri* Boiss. & Heldr.

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Abstract: Two coumarins, scopoletin (**1**) and isoscopoletin (**2**), two simple phenolic acids, protocatechuic acid (**3**) and isovanillic acid (**4**) and one flavonoid, eriodictyol (**5**) were isolated from the aerial parts of *Centaurea spruneri*. The structure of the compounds was established by spectroscopic methods. The *in vitro* antimicrobial activity of the isolated compounds was tested against eight bacteria and eight fungal species, using a microdilution method. All compounds tested showed moderate antibacterial and antifungal activities. Their minimum inhibitory concentrations were in the range 0.655–2.38 μmol ml^{-1} and their minimal bactericidal concentrations ranged from 0.694 to 4.15 μmol ml^{-1} against the tested bacterial species. All compounds showed fungistatic activity at 0.259–2.38 μmol ml^{-1} and fungicidal at 0.69–2.6 μmol ml^{-1} against all fungi tested.

Keywords: Centaurea spruneri; polyphenols; antibacterial activity; antifungal activity.

INTRODUCTION

The genus *Centaurea* L., with nearly 500 species, is mainly distributed around the Mediterranean area and in western Asia.[1] It has been the subject of many chemical investigations, which led to the isolation of various types of compounds, such as sesquiterpene lactones, being the most abundant group, flavonoids, lignans, nor-isoprenoids and volatile constituents. Previous investigations revealed a large number of *Centaurea* species possessing biologically active

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compounds.[2–15] Sesquiterpene lactones isolated from *C. aschaia*,[2] *C. thessala* subsp. *drakiensis*,[3] *C. attica*,[3] *C. deusta*,[4] *C. chilensis*,[5] and *C. nicolai*[6] were found to possess strong antifungal activity, while those isolated from *C. spinosa* possessed a slight inhibitory effect against Gram-positive bacteria.[7] In addition, flavonoids from *C. raphanina* spp. *mixta* showed moderate antifungal activity,[8] while flavonoids from *C. floccosa*[9] were effective against Gram-positive bacteria.

As a continuation of research on *Centaurea* species,[2–4,7,8,10–17] the secondary metabolites of *C. spruneri* are reported herein.

C. spruneri Boiss. & Heldr. is a perennial species flowering rather late in summer, which grows wild in Greece and Albania. It is classified in the section *Acrocentron* (Cass.) DC.[1] Hitherto, the volatile constituents of the aerial parts were investigated, which were mainly represented by terpenoids and homologous series of alkanes, alkenes, aliphatic alcohols and related aldehydes, as well as fatty acids and fatty acid methyl esters.[16] The present study concerns the non-volatile constituents of the plant. The evaluation of the antimicrobial activity of the isolated compounds was also investigated.

EXPERIMENTAL

Plant material

The plant was collected from the margins of cultivated fields on the foothills of Mt. Pateras (Attiki), 38° 6' N, 23° 14' E, c. 400–500 m, in July, 1998. A voucher specimen of the plant is kept in the Herbarium of the University of Patras (UPA) under the number: Constantinidis 2216.

General procedure

The structures of the isolated compounds were deduced by spectroscopic methods. The UV spectra were recorded on a Shimadzu UV-160A spectrophotometer, according to Mabry *et al.*[18] The IR spectra were obtained on a Perkin-Elmer Paragon 500 FT-IR spectrophotometer. The 1D and 2D NMR spectra were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers. COSY, HMQC, HSQC, HMBC, and NOESY (mixing time 950 ms) were performed using standard Bruker micro programs. Vacuum liquid chromatography (VLC) was performed on silica gel (Merck, Art. 9385) and column chromatography on silica gel (SDS, Art. 2050044), using solvent mixtures indicated in each case. Reversed-phase chromatography was carried out on JASCO HPLC system equipped with a UV detector; preparative HPLC was performed using a C_{18} 25 cm×10 mm Kromasil column. Fractionations were always monitored by ^{1}H-NMR and TLC on silica gel 60 F-254 (Merck, Art. 5554) and cellulose (Merck, Art. 5552) with visualization under UV radiation (254 and 365 nm) and spraying with anisaldehyde–sulfuric acid reagent on silica gel and Neu' s reagent on cellulose.[19]

Extraction and isolation of the bioactive compounds

The fresh plant material (2.26 kg) was ground finely and extracted successively at room temperature with cyclohexane–Et_2O–MeOH (1:1:1) and MeOH–H_2O (5:1). The non-polar extract was washed with brine; the aqueous layer was re-extracted with EtOAc and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. The latter residue (6.73 g) was pre-fractionated by VLC on silica gel (10.0 cm×8.0 cm), using cyclohexane––EtOAc–Me_2CO mixtures of increasing polarity as eluents to give 12 fractions (**A1**–**A12**).

Column chromatography over silica gel (15 cm×1.0 cm) of fraction **A5** (92.7 mg; eluted with EtOAc, 100 %) afforded 12 fractions (**B1**–**B12**). Fraction **B11** (26.7 mg; eluted with cyclohexane–CH_2Cl_2, 30:70) was subjected to HPLC (MeOH–H_2O with 2 % AcOH, 4:6) and afforded compounds **4** (1.1 mg; t_R 11.4 min) and **5** (0.9 mg; t_R 89.1 min). Fraction **A6** (116.6 mg; eluted with EtOAc–Me_2CO, 90:10) was subjected to column chromatography over silica gel (12 cm×1.5 cm) and afforded 11 fractions (**C1**–**C11**). Further purification of fraction **C8** (64.4 mg; eluted with cyclohexane–CH_2Cl_2, 50:50) by HPLC (MeOH–H_2O with 2 % AcOH, 4:6) yielded compounds **1** (3.1 mg; t_R 22.6 min) and **5** (0.6 mg; t_R 49.1 min). Column chromatography over silica gel (12 cm×1.5 cm) of fraction **A7** (274.3 mg; eluted with EtOAc––Me_2CO, 75:25) afforded 10 fractions (**D1**–**D10**). Fraction **D8** (64.0 mg; eluted with cyclohexane–EtOAc, 30:70) was subjected to HPLC (MeOH–H_2O with 2 % AcOH, 4:6) and afforded compounds **2** (3.0 mg; t_R 22.8 min), **3** (2.0 mg; t_R 13.9 min) and **5** (2.7 mg; t_R 78.3 min).

Antimicrobial activity

The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 35210), *Proteus mirabilis* (clinically isolated), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311); and the following Gram-positive bacteria: *Bacillus cereus* (clinically isolated), *Micrococcus flavus* (ATCC 10240), *Listeria monocytogenes* (NCTC 7973) and *Staphylococcus aureus* (ATCC 6538). For the antifungal bioassays, eight fungi were used: *Aspergillus niger* (ATCC6275), *Aspergillus versicolor* (ATCC11730), *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (clinical strain), *Candida albicans* (clinical strain) *Penicillium funiculosum* (ATCC10509), *Penicillium ochrochloron* (ATCC9112) and *Trichoderma viride* (IAM5061). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia and the Institute of Public Health of Serbia "Dr Milan Jovanović Batut". The micromycetes were maintained on malt agar (Torlak, Serbia) and the cultures were stored at 4 °C and sub-cultured once a month.[20] In order to investigate the antibacterial and antifungal activity of the isolated compounds, a modified microdilution technique was used for both activities.[21,22] Bacterial species were cultured overnight at 37 °C in tryptic soy broth medium (Biolife, Italy). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 spores in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on Müller–Hinton agar for bacteria and solid malt agar for fungi to verify the absence of contamination and to check the validity of the inoculum. The compounds investigated were dissolved in DMSO (0.1–1 μg mL^{-1}) and added in the broth medium (bacteria)/broth malt medium (fungi) with inocula. The minimum inhibitory concentration (*MIC*) values were determined by a serial dilution technique using 96-well micro titer plates. The micro plates were incubated for 48 h at 37 °C (bacteria) or 72 h at 28 °C (fungi). For the fungi, the lowest concentration without visible growth (using a binocular microscope) was defined as the *MIC*. For the bacteria, the following day, 50 μL of 0.2 mg mL^{-1} solution of INT (*p*-iodonitrotetrazolium violet) was added, and the plates were returned to the incubator for at least 30 min to ensure adequate color reaction. Inhibition of growth was indicated by a clear solution or a definite decrease in the color reaction. This value was taken as the *MIC* value for the bacteria.[23] The minimum bactericidal concentration (*MBC*) and minimum fungicidal concentration (*MFC*) were determined by serial sub-cultivation of a 2 μL sample into microtiter plates containing 100 μL of broth per well and further incubation for 48 h at 37 °C or 72 h at 28 °C for the bacteria and fungi, respectively. The lowest concentration with no visible growth was defined as the *MBC* or *MFC*, respectively, indicating 99.5 % killing of the original inoculum.[20] DMSO was used as the negative control, while the antibiotics strepto-

mycin and amoxicillin and the commercial fungicides bifonazole and ketokonazole were used as the positive controls (0.1–5 μL mL^{-1}). All experiments were performed in triplicate.

RESULTS AND DISCUSSION

The structures of the isolated compounds are given in Fig 1. Their spectroscopic data were according to the literature.[18,24,25]

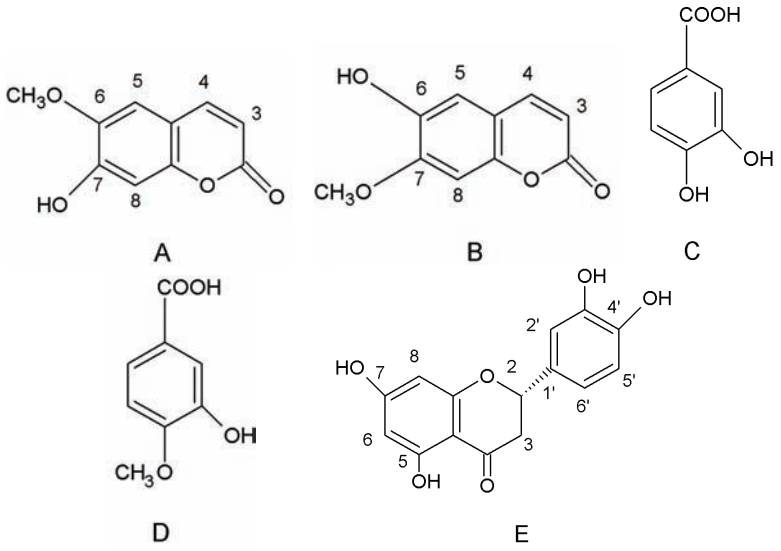

Fig. 1. Structures of the isolated compounds **1**–**5** (A–D, respectively).

The results for the antibacterial activity of compounds **1**–**5** are shown in Table I. The tested compounds possessed moderate antibacterial activity against all the tested bacterial species. The best results were obtained with compound **3**, which showed the strongest antibacterial activity with *MIC* values of 0.65–1.3 μmol ml^{-1} and *MBC* values of 1.3–2.6 μmol ml^{-1}. Compound **4** exhibited the lowest antibacterial activity, with MIC and MBC values in the range 1.19–2.38 μmol ml^{-1}. *B. cereus* was the most sensitive bacterial species (*MIC* 0.65–1.19 μmol ml^{-1}), while *L. monocytogenes* was the most resistant species (*MIC* 1.30––2.38 μmol ml^{-1}).

The results from the antifungal assay are summarized in Table II. Compound **5** possessed the best antifungal activity, with *MIC* and *MFC* values in the range 0.694–1.388 μmol ml^{-1}, while compound **3** showed the lowest antifungal activity. This latter compound inhibited fungal growth at 0.65–1.3 μmol ml^{-1}, while fungicidal activity was achieved at 1.3–2.6 μmol ml^{-1}. The majority of the compounds showed the worst activity against *A. flavus*, while *C. albicans* was the most sensitive species. The commercial drugs possessed lower or similar antifungal activity as the tested compounds, with *MIC* values of 0.322–0.644 μmol

ml^{-1} and *MFC* values of 0.483–0.806 μmol ml^{-1} for bifonazole, and *MIC* values of 0.376–4.70 μmol ml^{-1} and MFC values of 0.64–5.64 μmol ml^{-1} for ketokonazole. Although compound **3** showed the lowest antifungal activity, it exhibited the strongest antibacterial activity. In an effort to explain the different activity profiles of this compound, it was found that phenolic compounds with more hydroxyl groups possess better antibacterial activity,[26] while the antifungal activity was ascribed to the absence of polar groups in the molecule.[27,28]

TABLE I. Minimum inhibitory and bactericidal concentrations (*MIC* and *MBC* / 10^{-2} μmol ml^{-1}, 1st and 2nd row for each bacterium, respectively) of compounds **1**–**5**

Bacterium	**1**	**2**	**3**	**4**	**5**	Streptomycin[a]	Amoxicillin[a]
Bacillus	103.8±0.6	103.8±0.6	65.0±0	119.0±1.5	69.4±0.3	4.3±0.3	27.4±0.6
cereus	103.8±0.6	103.8±0.6	130.0±0.6	119.0±1.5	69.4±0.3	8.6±1.2	54.8±1.7
Micrococcus	103.8±0.6	103.8±0.6	130.0±0.6	119.0±1.0	69.4±0.3	8.6±1.2	6.9±0.3
flavus	207.6±1.2	207.6±1.2	260.0±1.0	238.0±1.0	277.6±1.2	17.2±0.3	13.7±0.3
Staphylococcus	103.8±0.6	207.6±1.2	130.0±0.6	119.0±1.5	69.4±0.3	17.2±0.3	6.9±0.3
aureus	207.6±1.2	415.2±1.7	260.0±1.0	238.0±1.0	277.6±1.2	34.4±0.3	13.7±0.3
Listeria	207.6±1.2	207.6±1.2	130.0±0.6	238.0±2.0	138.8±0.6	34.4±0.3	27.4±0.6
monocytogenes	207.6±1.2	415.2±1.7	260.0±1.0	238.0±2.0	277.6±1.2	68.8±0.6	54.8±0.6
Escherichia	103.8±0.3	103.8±0.6	130.0±0.6	119.0±0.6	69.4±0.3	8.6±1.2	27.4±0.6
coli	103.8±0.3	103.8±0.6	130.0±0.6	119.0±0.6	277.6±1.2	17.2±0.3	54.8±1.7
Pseudomonas	103.8±0.6	103.8±0.3	65.0±0	119.0±1.0	69.4±0.3	17.2±0.3	13.7±0.6
aeruginosa	103.8±0.6	103.8±0.3	130.0±0.6	119.0±1.0	277.6±2.1	34.4±0.3	27.4±1.2
Proteus	103.8±0.6	207.6±1.7	130.0±0.6	238.0±2.0	138.8±1.2	34.4±0.3	27.4±1.2
mirabilis	207.6±1.7	207.6±1.7	260.0±1.0	238.0±2.0	277.6±2.1	68.8±1.2	54.8±1.7
Salmonella	103.8±0.3	103.8±0.3	65.0±0	119.0±1.5	69.4±0.3	17.2±1.7	13.7±0.6
typhimurium	103.8±0.3	103.8±0.3	130.0±0.6	119.0±1.5	277.6±2.1	34.4±1.2	27.4±1.2

[a]Positive control, standard antibiotics

Previous investigations revealed that the antimicrobial potential of *Centaurea* species is mainly due to their high content of sesquiterpene lactones.[2–7,13,14] Thus, sesquiterpene lactones such as cnicin, 4'-acetylcnicin, 8-(3-hydroxy-4-acetoxy-2-methylenebutanoyloxy)-4-*epi*-sonchucarpolide, 8*α*-*O*-(4,5-dihydroxytigloyloxy) esters of salonitenolide, 11,13-dihydromelitensin and 9-*O*-acetyl and 3--*O*-deacetyl-9-O-acetyl derivatives of salograviolide A isolated from various of *Centaurea* species exhibited high or moderate antifungal activities against all the tested fungi with exception of *Trichoderma viride*, for which no inhibition was observed or with the worst activity.[2,3,7] The sesquiterpene lactones: 8*α*-*O*-(4-hydroxy-2-methylenebutanoyloxy)melitensine, melitensin, 11*β*,13-dihydrosalonitenolide, 8*α*-hydroxy-11*β*,13-dihydro-4-*epi*-sonchucarpolide and 8*α*-hydroxy-11*β*,13-dihydroonopordaldehyde isolated from the aerial parts of *C. pullata* showed greater antibacterial and antifungal activities than the positive controls used.[14] The flavonoids, 6-methoxyapigenin, apigenin, 6-methoxyluteolyn-4',7--dimethylether, 6-methoxyluteolyn-3'-methylether, 6-methoxyluteolyn-3',4',7-

trimethylether and C-glycosyl flavonoids characterized from the *C. virgata*, *C. kilea* and *C. intermis* exhibited inhibitory activities on fungal species, Gram-positive and Gram-negative bacteria. None of them was effective against *Staphylococcus aureus* and *S. epidermidis*.[29] The present study revealed that *C. spruneri* is a species poor in sesquiterpene lactones but rich in polyphenols, which were shown to possess antimicrobial activities against all the tested fungi, Gram-positive and Gram-negative bacteria. With respect to the antifungal potential of this latter group, the results obtained in the present study are in agreement with those previously reported for *C. raphanina* ssp. *mixta*[8] and *C. floccosa*.[9]

TABLE II. Minimum inhibitory and fungicidal concentrations (*MIC* and *MFC* / 10^{-2} μmol ml^{-1}, 1st and 2nd row for each fungus, respectively) of compounds **1**–**5**

Fungus	**1**	**2**	**3**	**4**	**5**	Bifonazole[a]	Ketoconazole[a]
Penicillium	103.8±0.3	103.8±0.3	130.0±1.0	119.0±1.0	69.4±0.6	64.4±0.3	37.6±0.6
funiculosum	103.8±0.3	103.8±0.3	130.0±1.0	119.0±1.0	138.8±1.2	80.6±0.6	64.0±1.5
Penicillium	103.8±0.6	103.8±0.6	130.0±0.6	238.0±2.0	138.8±1.2	48.3±0.6	188.0±1.0
ochrochloron	207.6±0.6	207.6±0.6	260.0±1.5	238.0±2.0	138.8±1.2	64.4±1.7	188.0±1.0
Trichoderma	51.9±0.6	51.9±0.6	65.0±0	59.0±0.6	69.4±0.6	64.4±1.2	470.0±2.0
viride	103.8±0.6	103.8±0.6	130.0±1.0	238.0±1.0	138.8±0.6	80.6±0.6	564.0±1.5
Aspergillus	103.8±0.6	103.8±0.6	130.0±0.6	238.0±1.0	138.8±0.6	48.3±0.6	37.6±0.6
fumigatus	103.8±0.6	103.8±0.6	260.0±1.0	238.0±2.0	138.8±0.6	64.4±1.7	94.0±1.0
Aspergillus	103.8±0.6	103.8±0.6	130.0±1.0	238.0±1.0	138.8±0.6	48.3±0.6	37.6±0.6
niger	207.6±1.2	207.6±1.2	260.0±1.5	238.0±1.0	138.8±0.6	64.4±1.7	94.0±1.0
Aspergillus	207.6±1.7	207.6±1.7	130.0±0.6	238.0±2.0	138.8±0.6	48.3±0.6	282.0±2.0
flavus	207.6±1.7	207.6±1.7	260.0±1.0	238.0±2.0	138.8±0.6	64.4±1.7	376.0±1.0
Aspergillus	51.9±0.6	51.9±0.6	65.0±0	59.0±0.6	69.4±0.3	32.2±0.3	37.6±0.6
versicolor	207.6±1.2	207.6±1.2	260.0±1.5	238.0±1.5	138.8±1.2	64.4±1.2	94.0±1.0
Candida	25.9±0.3	51.9±0.6	65.0±0	59.0±0.6	69.4±0.6	32.2±0.3	37.6±0.6
albicans	103.8±0.3	103.8±0.6	130.0±0.6	119.0±1.0	69.4±0.6	48.3±0.6	94.0±1.0

[a]Positive control, standard antibiotics

Together with previously published data, the presented results indicate that *Centaurea* species possess biologically active compounds with different degrees of action. It can be seen that the growth of the tested microorganism responded differently to the investigated compounds, suggesting that different components may have different modes of action or that the metabolism of some microorganisms are better able to overcome the effect of the compound or adapt to it.

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ИЗВОД

АНТИМИКРОБНА АКТИВНОСТ СЕКУНДАРНИХ МЕТАБОЛИТА ИЗОЛОВАНИХ ИЗ *Centaurea spruneri* Boiss & Heldr.

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Из надземног дела *Centaurea spruneri* изолована су два кумарина скополетин (**1**) и изоскополетин (**2**), две просте фенолске киселине протокатехуинска киселина (**3**) и изованилинска киселина (**4**) и флавоноид ериодиктиол. Хемијске структуре познатих једињења су утврђене коришћењем 1D и 2D NMR, MS и UV спектроскопских анализа. У *in vitro* тесту за одређивање антимикробне активности изолованих једињења коришћена је микродилуциона метода. Добијена једињења тестирана су на по осам бактеријских и гљивичних врста. Сва једињења су имала умерену антибактеријску и антифунгалну активност. Минимална инхибиторна концентрација тестираних једињења се кретала од 0,655 до 2,38 μmol ml^{-1}, а минимална бактерицидна концентрација 0,694–4,15 μmol ml^{-1}. Тестирана једињења су показала фунгицидну активност од 0,259–2,38 μmol ml^{-1} и фунгистатичку 0,694–2,60 μmol ml^{-1}, на све тестиране гљиве.

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