Active Metabolites from *Dunalia spinosa* Resinous Exudates

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Dunalia spinosa, a plant used in folk medicine for toothaches, breathing problems and cleansing wounds, was found active as antimicrobial and antioxidant. A new (*E*)-aurone rutinoside (dunaurone) has been isolated from the aerial parts of the plant, and its structure was determined by spectroscopic means. Lupeol, β-sitosterol, scopoletin, quercetin and withaferin A were also found. All the extracts exhibited strong antimicrobial activity while dunaurone showed only weak antimicrobial inhibition against *Klebsiella pneumoniae;* in addition it presented a significant free radical scavenging activity.

Key words: Dunalia spinosa, (*E*)-Aurone Rutinoside, Antimicrobial Activity

Introduction

Dunalia spinosa (Meyen) Dammer is a shrub attaining a height of 2 m with colourful tubular flowers which grows in the Andes mountains between 2900 and 3700 meters above sea level (Brako and Zarucchi, 1993). In Chile it is found in the 1st Region and known with the vernacular names "chumi-chumi", " yara" or "chilca hembra" (Marticorena and Quezada, 1985). In the folk medicine the burnt leaves are used to clean wounds and to treat whooping cough. In addition, the fruits mashed with oil are used against toothache (Castro *et al.*, 1982). Children suck the flowers due to their sweet taste. No chemical or biological studies on this species are previously reported. In this paper we report the chemical composition of the resinous exudates and the antimicrobial effect detected by bioautographic techniques besides the antioxidant activity of the aerial parts of this plant.

Experimental

General experimental procedures

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR analyses were carried out on a Bruker AMX spectrometer. UV spectra were recorded on a Unicam UV3 spectrophotometer, IR spectra on a Perkin Elmer 1310 spectrophotometer. Mass spectra were obtained by electrospray ionization (ESI) on a Thermo Finnigan LCQ DECA KP Plus ion-trap mass spectrometer.

Plant material

Dunalia spinosa (Meyen) Dammer was collected at 3400 m above sea level in the Andes mountains near the town of Socoroma (1st Region, Arica, Chile) and identified by Eliana Belmonte of Tarapacá University, Chile. A voucher specimen is kept at the Herbarium of the School of Chemistry and Pharmacy, University of Chile, Santiago, Chile (SQF 22256).

Extraction and isolation of active components

Ground dried aerial parts (790 g) were immersed in CH_2Cl_2 (DMC) at room temperature for the extraction of the resinous exudate (27.9 g). The resin-free plant was dried and successively extracted with hexane, CH_2Cl_2 and MeOH, yielding 1.53 g, 12.34 g and 131.25 g of extract, respectively.

A part (17 g) of the resinous exudate was subjected to CC on silica gel 60 eluted successively with *n*-hexane (100%), *n*-hexane/DCM (1:1 v/v), DCM (100%), DCM/EtOAc (1:1), EtAOc (100%)

and MeOH (100%). Fraction DCM (100%) (2 g) was applied to CC over silica gel eluted with DCM followed by DCM with increasing percentages of EtOAc yielding lupeol [20 mg, 0.0025%, eluted with DCM/EtOAc (9:1) and DCM/EtOAc (8:2) in fractions 40–55 of 100 mL each] and β-sitosterol [30 mg, 0.004%, eluted with DCM/EtOAc (7:3) in fractions 65–77 of 100 mL each].

Fraction DCM/EtOAc (1:1 v/v) (6 g) was subjected to CC over silica gel eluted with DCM followed by DCM with increasing percentages of EtOAc yielding scopoletin [10 mg, 0.0013%, eluted with DCM/EtOAc (8:2) in fractions 20–28 of 100 mL each] and quercetin [25 mg, 0.0032%, eluted with DCM/EtOAc (6:4 v/v) in fractions 45–60 of 100 mL each].

Fraction EtOAc (100%) (5 g) was applied to CC over silica gel eluted with DCM followed by DCM with increasing percentages of EtOAc yielding crude **1**, eluted with DCM/EtOAc (9:1 v/v) and DCM/EtOAc (8:2) in fractions 82–122 of 100 mL each, and crude **2**, eluted with EtOAc/MeOH (9:1) and EtOAc/MeOH (8:2) in fractions 140–145 of 100 mL each.

Additional purification of fractions 82–122 by Sephadex LH-20 CC eluted with *n*-hexane/DCM/MeOH (2:1.5:0.25 v/v) yielded pure **1** (100 mg, 0.013%), and purification of fractions 140–145 by Sephadex CC eluted with *n*-hexane/DCM/MeOH (1:2:1) yielded compound **2** (40 mg, 0.005%). Both compounds were crystallized from DCM with drops of MeOH.

*Withaferin A (**1**):* M.p. 243–245 °C [249–259 °C (Kupchan *et al.*, 1965)]. – IR: ν_{max} = 3400, 2940, 1675, 1230, 1030, 1010, 930, 810 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. ESI-MS: m/z = 964 $[2M+Na]^+$, 1433 $[3M+Na]^+$.

*Dunaurone (**2**):* M.p. 249–250 °C. – UV (MeOH): λ_{max} (log ε) = 216 (3.76), 256 (3.51), 296sh, 358 (3.55) nm; (+NaOMe) 232, 272, 398 nm, unaltered in the time; (+NaOAc) 262, 372 nm; (+NaOAc + H_3BO_3) 264, 372 nm; (+$AlCl_3$) 270, 394 nm; (+$AlCl_3$ + HCl) 270, 360 nm. – ^1H and ^{13}C NMR: see Table II.

Table I. NMR data (DMSO-d_6) of withaferin A (**1**).

Position	δ_H	δ_C	Long-range connectivities
1		203.3	H-2, H-3, Me-19
2	6.01d (9.8)	132.8	H-4
3	6.94dd (9.8; 6.3)	146.7	
4	3.42dd (6.3; 4.1)	70.1	H-2, H-6
4-OH	5.53d (4.1)		
5		64.7	H-3, Me-19, OH-4
6	3.05br s	60.2	H-4
7	2.30/1.20m	30.5	H-6
8	1.90m	30.9	H-6
9	0.65m	44.9	Me-19
10		48.6	Me-19, H-2, H-6
11	1.40/1.20m	22.2	
12	1.87/1.15m	32.9	
13		43.9	Me-18
14	1.00m	52.6	Me-18
15	1.45/0.95m	25.5	
16	1.65/1.15m	28.0	
17	0.80m	56.7	Me-18
18	0.53s	12.8	
19	1.14s	17.7	
20	1.70m	39.9	Me-21
21	0.79d (6.6)	16.4	
22	4.17dt (13.1; 2.0)	79.0	Me-21
23	2.30m	40.1	
24		156.3	Me-28, H-17
25		126.9	H-27, Me-28, H-27
26		166.9	H-27
27	4.02dq (16.6; 5.3)	56.0	OH-27
27-OH	4.52t (5.3)		
28	1.88s	21.4	

Table II. NMR data (DMSO-d_6) of dunaurone (**2**).

Position	δ_H	δ_C	Long-range connectivities
2		156.6	H-β
β	7.53s	116.2	
3		177.3	H-7
3a		103.9	OH-4, H-7, H-5
4		161.2	OH-4, H-5
4-OH	12.60s		
5	6.19d (1.6)	98.7	OH-4, H-7
6		164.1	H-7, H-5
7	6.39d (1.6)	93.6	H-5
7a		156.4	H-7
1′		121.1	H-5′
2′		144.7	H-5′, H-6′
3′		133.2	H-1″
4′		148.4	H-5′, H-6′
5′	6.84d (8.6)	115.2	
6′	7.56d (8.6)	121.6	
1″	5.34d (7.0)	101.1	
2″	3.23–3.23[a]	74.0	
3″	3.23–3.23[a]	76.4	
4″	3.23–3.23[a]	70.5	
5″	3.23–3.23[a]	75.8	
6″	3.71d (9.9); 3.23–3.23[a]	67.0	
1‴	4.38s	100.7	
2‴	3.42–3.42[b]	70.3	
3‴	3.23–3.23[a]	68.2	
4‴	3.07–3.07[b]	71.8	
5‴	3.07–3.07[b]	69.9	
6‴	0.99d (6.6)	17.7	

[a, b] Overlapped.

Klebsiella pneumoniae (clinical isolated), *Salmonella aviatum* (ATCC 2228), *Pseudomonas aeruginosa* (ATCC 14207), *Staphylococcus aureus* (ATCC 6538P), *Micrococcus flavus* (ATCC 10290), *Bacillus subtilis* (ATCC 6633), *Candida albicans* and *Saccharomyces cerevisiae* (clinical isolated). The extracts were dissolved in DMSO. Dilutions of 100 and 200 μg/mL were added to a fixed volume of Plate Count Agar (PCA, Merck). They were then superficially inoculated with an overnight culture of the different microorganisms and incubated at 37 °C for 24 h for bacteria and 28 °C for 48 h for fungi and yeasts. Results were recorded as growth or growth inhibition at each extract concentration (Erazo *et al.*, 2006).

A bioautographic agar overlay assay by TLC of the resinous exudates and compounds **1** and **2** was carried out on silica gel 60 G F_{254} plates developed with DCM/EtOAc (9:1 v/v) for **5** and EtOAc/MeOH (8:2) for **2** (Rahalison *et al.*, 1991).

The turbidimetric method (Balow *et al.*, 1991) was used with serial dilutions of the extract in 4 mL of Plate Count Broth or Tryptic Soy Broth (Merck). Both media were used to assay the MIC values of compound **1** against *S. aureus* and *E. coli*.

Xanthine oxidase activity

Both xanthine and xanthine oxidase (XO) were purchased from Sigma Co., and the standard inhibitor allopurinol was obtained from Laboratorios Saval (Santiago, Chile). The global methanol extract was evaluated at 50 μg/mL and further tested for IC_{50} determination with an inhibition value > 50% (Noro *et al.*, 1983). The inhibition of XO activity using xanthine as the substrate was spectrophotometrically measured in relation to the amount of uric acid, which was determined at 290 nm.

The IC_{50} value of allopurinol was 0.035 μg/mL (0.267 μM). For XO activity, the drug-induced changes were statistically estimated using the Wilcoxon test for independent data (Hollander and Wolfe, 1973). Effects were significant for $p \leq 0.05$.

Superoxide anion generation

The enzyme xanthine oxidase is able to generate O_2 *in vivo* by oxidation of reduced products from the intracellular ATP metabolism. The superoxide (SO) generated in this reaction sequence reduce the nitro blue tetrazolium dye, leading to a chromophore with a maximum at 560 nm.

Superoxide anion scavengers reduce the generation of the chromophore. The activity was measured spectrophotometrically as reported previously (Payá *et al.*, 1992; Masaki *et al.*, 1995). Compounds isolated were evaluated at 50 μg/mL.

DPPH decolouration assay

The quenching of free radicals by isolated compounds was evaluated spectrophotometrically at 517 nm through the residual absorbance of the DPPH radical (Sigma). The scavenging activity of substances was assessed by the decolouration of a methanol solution of DPPH (Feresin *et al.*, 2002). A freshly prepared DPPH solution (20 mg/L) was used for the assays. Samples were dissolved in methanol and the DPPH solution served as a control. The degree of decolouration indicates the free radical scavenging efficiency of the samples. Quercetin, a free radical scavenger, was used as reference. The percentage of DPPH decolouration was calculated as follows: decolouration (%) = [1 − (absorbance of compound with DPPH − absorbance of blank sample)/(absorbance of DPPH control)] × 100.

Compounds were assayed starting at a maximum concentration ten times higher (33 μM) than the IC_{50} value of quercetin (3.3 μM). The IC_{50} value was calculated according to the scavenging efficiency.

Results and Discussion

Six compounds were isolated and characterized from *Dunalia spinosa*: lupeol, β-sitosterol, scopoletin, quercetin, withaferin A (**1**) and dunaurone (**2**).

The antimicrobial activity tests showed that all *Dunalia* extracts were active, the resinous exudate being the most active extract against *S. aureus, B. subtilis, M. flavus, E. coli* and *K. pneumoniae*. Bioguided fractionation led to the isolation of withaferin A (**1**), active against all this microorganisms; the new dunaurone (**2**) and its aglycone **3** were weakly active against *K. pneumoniae* and inactive against all other microorganisms tested. The extracts and isolated metabolites were inactive against the fungi. The minimal inhibitory concentration (MIC) of **1** determinated against the most sensibile microorganisms was 80 μg/mL for *S. aureus* (MIC for ampicillin as reference antibiotic was 5 μg/mL) and 30 μg/mL for *E. coli* (> 5 μg/mL for chloramphenicol).

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No significant results were obtained in the XO assay at 50 μg/mL of crude extract. Only the dichloromethane extract showed a weak inhibitory activity of 14.7%. Dunaurone at 22 μM showed an important activity of 45.7% in the SO assay compared with withaferine A at the same concentration, which showed an activity of 9.1%. None of them showed XO inhibition activity.

In the DPPH assay the free radical scavenging efficiency of dunaurone was significant (CE_{50} = 27.9 μM) compared with the standard quercetin (CE_{50} = 3.3 μM).

In conclusion, no previous studies have been reported for this species, and the results obtained in this work agree with the cleansing wounds properties attributed by the folk medicine for this plant. The results of this study are a contribution to the scientific knowledge of our flora.

Acknowledgements

This research has been performed under the auspices of Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile.

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