Active Metabolites from *Dunalia spinosa* Resinous Exudates

Silvia Erazo[a,*], Giovanna Rocco[a], Mercedes Zaldivar[a], Carla Delporte[a], Nadine Backhouse[a], Consuelo Castro[a], Eliana Belmonte[b], Franco Delle Monache[c], and Rubén García[a]

[a] Department of Pharmacological and Toxicological Chemistry, School of Chemical and Pharmaceutical Sciences, University of Chile, P. O. Box 233, Santiago 1, Chile. Fax: 56-2-2227900. E-mail: serazo@uchile.cl
[b] Department of Anthropology, Faculty of Social Sciences, University of Tarapacá, Arica, Chile
[c] Department of Chemistry and Technology of the Biologically Active Substances, University of Rome, Rome, Italy

* Author for correspondence and reprint requests

Z. Naturforsch. **63c**, 492–496 (2008); received December 10, 2007/February 11, 2008

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%)

0939–5075/2008/0700–0492 $ 06.00 © 2008 Verlag der Zeitschrift für Naturforschung, Tübingen · http://www.znaturforsch.com · D

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: υ_{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.

Acid hydrolysis of **2**: 10 mg of **2** were mixed with 6% aqueous hydrochloric acid (25 mL) using a minimum of methanol to effect complete solution. The solution was heated on a steam bath for 45 min and then cooled and extracted with diethyl ether. Evaporation of the aqueous layer yielded rhamnose and glucose. The diethyl ether layer, after drying over sodium sulfate, yielded the aglycone **3** on evaporation (Mabry *et al.*, 1970).

*Aglycone **3**:* M.p. 281–283 °C. – UV: λ_{max} (log ε) = 216 (3.92), 256 (3.96), 296sh, 370 (3.97) nm; (+NaOMe) 232, 286, 322, 426 degrading to 212, 322 nm after 15 min; (+$AlCl_3$) 268, 436 nm; (+$AlCl_3$ + HCl) 264, 426 nm. – ^{1}H NMR (DMSO-d_6): δ = 12.46 (s, OH-4), 10.90 (br s, OH-3′), 9.64 (br s, OH-6), 9.33 (br s, OH-2′ and OH-4′), 7.65 (d, J = 1.8 Hz, H-β), 7.50 (dd, J = 8.5 and 1.8 Hz, H-6′), 6.83 (d, J = 1.6 Hz, H-5′), 6.43 (d, J = 1.6 Hz, H-7), 6.19 (d, J = 1.6 Hz, H-5). – ^{13}C NMR (DMSO-d_6): δ = 175.2 (C-3), 163.4 (C-8), 160.1 (C-4), 155.5 (C-2), 147.1 (C-4′), 146.2 (C-7a), 145.5 (C-2′), 135.1 (C-3′), 121.4 (C-1′), 119.4 (C-6′), 115.0 (C-β), 114.5 (C-5′), 102.4 (C-3a), 97.6 (C-5), 92.8 (C-7).

Structure determination

Known compounds, lupeol, β-sitosterol, scopoletin and quercetin, were purified and identified on the basis of their physical data and the structures confirmed by comparison with standards, previously isolated in our laboratory. NMR data of compound **1**, $C_{28}H_{38}O_6$, are reported in Table I, as the result of mono- (^{1}H and ^{13}C) and bi-dimensional experiments, and allowed to assign structure **1**. This structure was previously attributed to withaferin A, a withanolide isolated from *Acnistus arborescens*, Solanaceae (Kupchan *et al.*, 1965).

The complete NMR data of compound **2** are reported in Table II. Preliminary examination of UV and NMR data suggested that the compound is a flavonoid diglycoside. In particular, in the UV spectrum the maxima at 256 and 370 nm were consistent with an aurone more than a flavone or isoflavone (Markham and Mabry, 1975). Accordingly, among the NMR data (Table II) there was a sharp singlet at δ_H 7.53 correlated to the signal at δ_C 116.2 which agrees with an *E*-type aurone (Brady *et al.*, 1979; Pelter *et al.*, 1979; Sharma and Chibber, 1979).

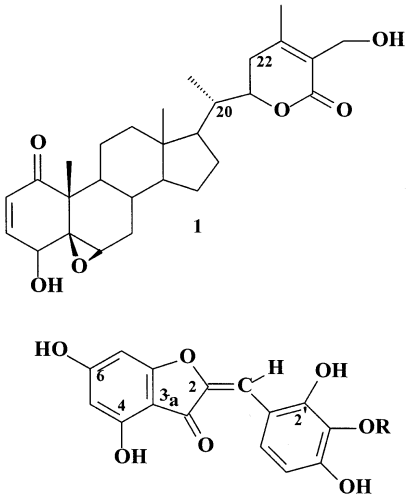

Fig. 1. Chemical structures of withaferin A (**1**), dunaurone (**2**) and its aglycone **3**.

In addition, the presence of hydroxy groups at positions 4 and 6 of the aurone moiety may be inferred by the bathochromic shifts of the UV maxima after adding $AlCl_3$ and $NaHCO_3$, respectively. The 4,6-di-hydroxy substitution of the A ring was corroborated by two *meta*-coupled proton signals (δ 6.39 and 6.19) in the ^{1}H NMR spectrum.

In the last spectrum further doublets (J = 8.6 Hz) at δ 7.56 and δ 6.84 were assigned to two *ortho*-coupled aromatic protons and allowed to complete the hydroxy substitution of the aurone moiety as 4,6,2′,3′,4′. The remaining NMR signals (Table II) were easily attributed to a rutinose residue by comparison with other compounds containing such substituent (Agrawal and Bansal, 1989). Accordingly, acid hydrolysis of **2** gave glucose, rhamnose and the corresponding aglycone **3**.

About the location of the sugar chain on position 3′, it was firstly suggested by the long-rang connectivity (Table II) of the C-3′ signal with the anomeric proton of the glucose moiety at δ_H 5.34, than confirmed by the behaviour of UV maxima on addition of NaOMe in **2** and **3**. While the UV spectrum of **2** was stable under alkaline conditions, that of the aglycone **3** showed rapid decomposition due to vicinal hydroxy groups (Mabry *et al.*, 1970).

Antimicrobial assays

The antimicrobial activity of the extracts was determined against *Escherichia coli* (ATCC 8739),

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)] $\times$ 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).

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.

Agrawal P. K. and Bansal M. C. (1989), Carbon-13 NMR of Flavonoids (Agrawal P. K., ed.). Elsevier, Amsterdam, Chapter 6.

Balow A., Hausler Jr. W., Hermann K. L., Isenberg H. D., and Shadomy H. J. (1991), Manual of Clinical Microbiology, 5th ed. American Society for Microbiology, Washington DC, pp. 1105–1112.

Brady B. A., Kennedy J. A., and O'Sullivan W. I. (1979), The configuration of aurones. Tetrahedron **29**, 359–363.

Brako L. and Zarucchi J. (1993), Catálogo de Angiospermas y Gimnospermas del Perú. Mo. Bot. Gard. **45**, 1–1286.

Castro M., Villagrán C., and Kalin M. T. A. (1982), Estudio etnobotánico en la precordillera *y* altiplano de los Andes de Arica (18°–19° S). In: El ambiente natural y las poblaciones humanas de los Andes de Arica del norte grande de Chile (Arica, latitud 18°–28° S) (Veloso A. and Bustos E., eds.). Vol. de síntesis-Proyecto MAB-6-UNEP-UNESCO No. 1105–77–01). Santiago, Chile, Vol. 2, pp. 133–205.

Erazo S., Delporte C., Negrete R., García R., Zaldivar M., Iturra I., Caballero E., López J. L., and Backhouse N. (2006), Constituents and biological activities of *Schinus polygamus*. J. Ethnopharmacol. **107**, 395–400.

Feresin G. E., Tapia A., Gutiérrez R. A., Delporte C., Backhouse N., and Schmeda-Hirschmann G. (2002), Free radical scavengers, anti-inflammatory and analgesic activity of *Acaena magellanica*. J. Pharm. Pharmacol. **54**, 835–844.

Hollander M. and Wolfe D. A. (1973), Nonparametric Statistical Methods. J. Wiley and Sons, New York, pp. 68–70.

Kupchan S. M., Doskotch R. W., Bollinger P., Mc Phail A. T., Sim G. A., and Saenz Renauld J. A. (1965), The isolation and structural elucidation of a novel steroidal tumor inhibitor from *Acnistus arborescens*. J. Am. Chem. Soc. **87**, 5805–5806.

Mabry T. J., Markham K. R., and Thomas M. B. (1970), The Systematic Identification of Flavonoids. Springer-Verlag, New York, Heidelberg, Berlin, p. 24.

Markham K. R. and Mabry T. J. (1975), Ultraviolet and proton magnetic resonance spectroscopy of flavonoids. In: The Flavonoids (Harborne J. B., Mabry T. J., and Mabry H., eds.). Academic Press, New York, p. 45.

Marticorena C. and Quezada M. (1985), Flora vascular de Chile. Gayana Bot. **42**, 71.

Masaki H., Sakaki S., Atsumi T., and Sakurai H. (1995), Active-oxygen scavenging activity of plant extract. Biol. Pharm. Bull. **18**, 162–166.

Noro T., Oda Y., Miyase T., Ueno A., and Fukushima S. (1983), Antinociceptive properties of steroids isolated from *Phyllanthus corcovadensis* in mice. Chem. Pharm. Bull. **31**, 3984–3987.

Payá M., Halliwell B., and Hoult R. S. (1992), Interactions of a series of coumarins with reactive oxygen species. Scavenging of superoxide, hypochlorous acid and hydroxyl radicals. Biochem. Pharmacol. **44**, 205–214.

Pelter A., Ward R., and Keller H. G. (1979), ^{13}C NMR spectra of (*Z*)- and (*E*)-aurones. J. Chem. Soc. Perkin Trans. I, 328–331.

Rahalison L., Hamburger M., Hostettman K., Monod M., and Fronk E. (1991), A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. Phytochem. Anal. **2**, 199–203.

Sharma A. and Chibber S. S. (1979), Carbon-13 NMR spectroscopy of methoxy and acetoxyaurones. J. Heterocycl. Chem. **18**, 275–278.