Phytochemistry 137 (2017) 94–100

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Photosensitizing anthraquinones from *Heterophyllaea lycioides* (Rubiaceae)

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ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form
1 February 2017
Accepted 3 February 2017
Available online 11 February 2017

Keywords:
Heterophyllaea lycioides
Rubiaceae
Structural elucidation
Chlorinated anthraquinones
Lycionine
7-Chlorobisoranjidiol
5-Chlorosoranjidiol
Type I photosensitizers

ABSTRACT

Seven anthraquinones were isolated from aerial parts of *Heterophyllaea lycioides* (Rusby) Sandwith (Rubiaceae), including three derivatives that have not been described before: a hetero-bianthraquinone identified as (*R*)-2-hydroxymethyl-2′methyl-1,1′,6,6′-tetrahydroxy-5,5′ bianthraquinone (lycionine), and two mono-chlorinated derivatives related to soranjidiol. One of them is a homo-bianthraquinone: (*R*)-7-chloro-2,2′-dimethyl-1,1′,6,6′-tetrahydroxy-5,5′ bianthraquinone (7-chlorobisoranjidiol), whereas the second halogenated derivative corresponds to a monomeric structure: 5-chloro-1,6-dihydroxy-2-methyl anthraquinone (5-chlorosoranjidiol). The four known compounds were already isolated from another species of this genus, *H. pustulata*, and they were identified as 5,5′-bisoranjidiol, soranjidiol, pustuline and heterophylline. Structural elucidation was performed by means of an extensive spectroscopic analysis, including 1D and 2D NMR data as well as by HRMS analysis. Chemical structures of 7-chlorobisoranjidiol and 5-chlorosoranjidiol were confirmed by their synthesis from 5,5′-bisoranjidiol and soranjidiol, respectively. Type I photosensitizing properties (superoxide anion radical generation, $O_2^{\bullet -}$) were assessed by using the nitroblue tetrazolium assay. When lycionine and chlorinated derivatives were irradiated, they enhanced the $O_2^{\bullet -}$ production with respect to the control; 7-chlorobisoranjidiol stood out by generating an increase of 20%, whereas the other anthraquinones only produced a slight increase of 7%.

1. Introduction

Heterophyllaea J.D. Hook. (Rubiaceae) is a South American genus formed by two species, *H. pustulata* Hook f. and *H. lycioides* (Rusby) Sandwith (Taylor, 2010). Both species are renowned as toxic plants, since they generate progressive blindness in cattle. Thus, they are known as “cegaderas” (blindness), together with other popular names that vary according to each region (Bacigalupo, 1993; Atahuachi Burgos, 2005). Only *H. pustulata* has been described as a phototoxic species that produces discomfort and skin-irritation in those animals that ingest it and receive sunlight. Dermatitis and blindness (keratoconjunctivitis) can occur with regular consumption of the plant, but it has been shown that these effects are reversible (Aguirre and Neumann, 2001).

H. pustulata is native to Argentina and Bolivia, with a habitat restricted to the Andean region (Bacigalupo, 1993). Chemical composition and biological activities of this plant species have been widely studied. 9,10-anthraquinone aglycones (AQs) are the predominant compounds (Núñez-Montoya et al., 2006), which have photosensitizing properties (Núñez-Montoya et al., 2005; Comini et al., 2007) that give them potentiality to be used in photodynamic therapy (PDT) (Kharkwal et al., 2011).

H. lycioides is a shrub that reaches up to 1 m in height. It inhabits the mountain regions of central Bolivia and southern Peru, between 2200 and 3300 m altitude, where it is also known as “ceguera” and “Q'api” (Atahuachi Burgos, 2005; Brako and Zarucchi, 1993). Although this vegetal species is mentioned in the literature as a toxic plant (Atahuachi Burgos, 2005), there are no prior chemical or toxicological studies to justify this assertion. Therefore, chemical components of the aerial parts of *H. lycioides* were examined in this study.

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http://dx.doi.org/10.1016/j.phytochem.2017.02.003
0031-9422/

Considering that AQs isolated from *H. pustulata* exhibited photosensitizing properties, it was evaluated as whether the new AQ derivatives could increase the production of superoxide anion radical ($O_2^{\bullet -}$) under irradiation (photosensitizing activity Type I). This effect would be related not only to the phototoxic activity of this plant species, but also to obtaining new photosensitizers, which would be of significant importance due to their potential use in PDT.

2. Results and discussion

2.1. Anthraquinones structures

Seven aglycone-AQs were obtained from aerial parts of *H. lycioides* (**1**–**7**) (Fig. 1). Compounds **4**–**7** (5,5′-bisoranjidiol, soranjidiol, heterophylline and pustuline) were previously isolated from *H. pustulata* and were identified by comparing their experimental spectroscopic data (UV–Vis, 1D and 2D NMR, and IR spectroscopy) with those previously reported in the literature (Núñez-Montoya et al., 2006; Wijnsma and Verpoorte, 1986). The structural elucidation of the new AQs (**1**–**3**) is reported herein, on the basis of the analysis of their spectroscopic and spectrometric properties.

Compound **1** was isolated as a yellowish amorphous powder. Its HRESIMS data showed a quasi-molecular ion $[M\text{-}H]^-$ at *m/z* 521.0899, which corresponds to the molecular formula of $C_{30}H_{18}O_9$ (calcd. for $C_{30}H_{17}O_9$ 521.0867). Also considering its NMR spectroscopic data, this compound would have the structure of a bianthraquinone (Núñez-Montoya et al., 2006). Its ^{1}H NMR spectrum (Table 1) indicated the presence of 8 aromatic protons, all doublet signals with a characteristic *ortho* spin coupling constant (H-3/H-4; H-7/H-8; H-3′/H-4′; H-7′/H-8′). Typical signals for a methyl group (CH_3), two free phenolic hydroxyls (OH), and two OH groups in the *peri*-position, hydrogen bonded to a carbonyl group (C=O) with a characteristic deshielding effect that moved their signals downfield (ca. 13 ppm) as compared to free-OH resonances (ca. 10 ppm) (Wijnsma and Verpoorte, 1986; Schripsema and Dagnino, 1996), were also noted. In addition, the doublet at δ_H 4.80 ($J = 5.4$) indicated a methylene group (CH_2) and the triplet at δ_H 4.42 ($J = 5.3$) was attributed to a primary alcohol that was coupled with the CH_2

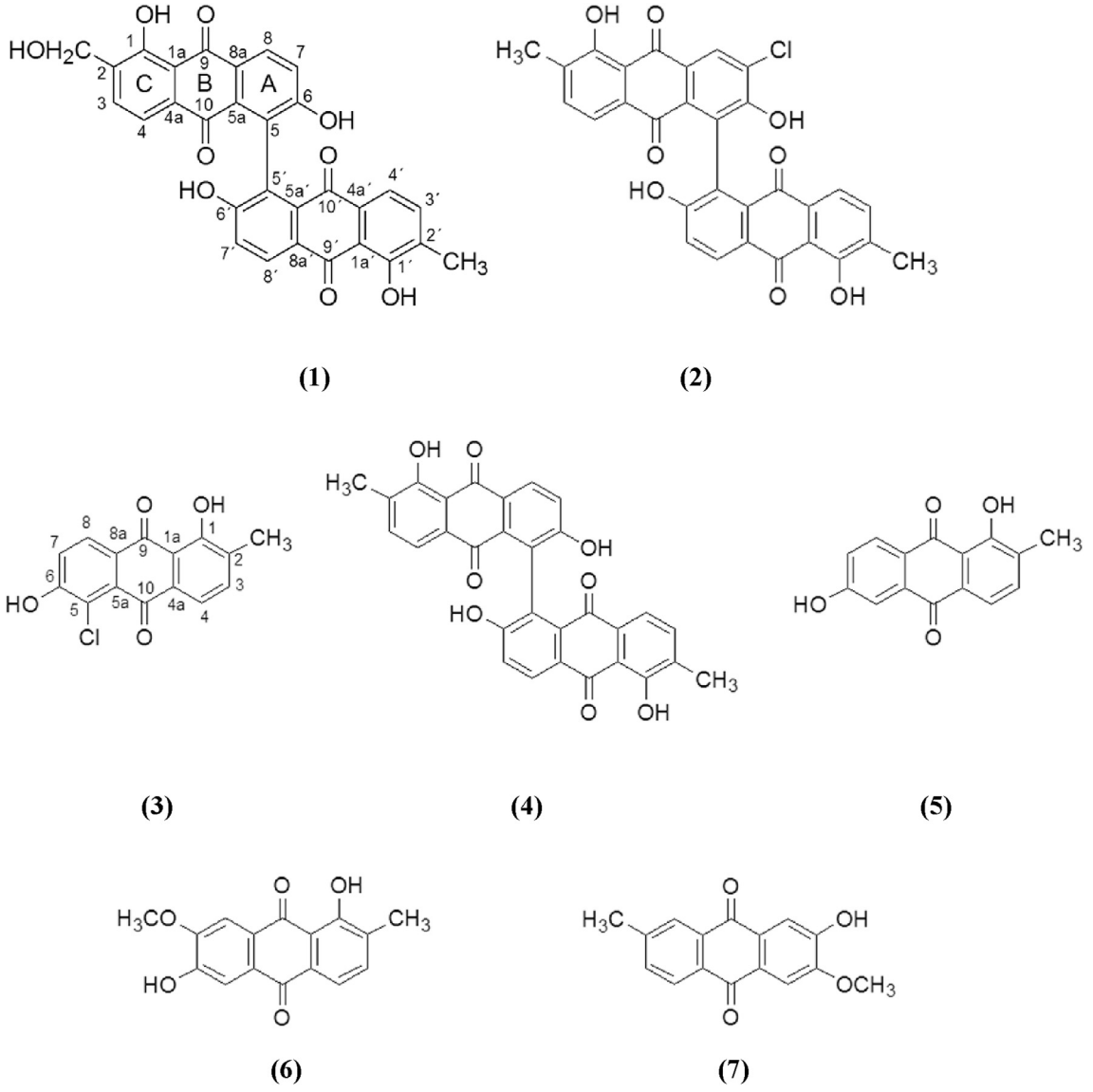

Fig. 1. Anthraquinones isolated from aerial parts of *Heterophyllaea lycioides*.

Table 1
Summary of ^{13}C NMR and ^{1}H NMR data (400 and 100 MHz) for compounds **1**–**3**.

Position	**1**[a]		**2**[a]		**3**[b]	
	δ_c, type	δ_H, mult. (*J* in Hz)	δ_c, type	δ_H, mult. (*J* in Hz)	δ_c, type	δ_H, mult. (*J* in Hz)
1	158.8, C		162.0, C		159.8, C	
1a	114.9, C		114.8, C		114.1, C	
2	136.7, C		134.1, C		135.7, C	
3	133.3, CH	7.84, *d (7.6)*	137.5, CH	7.55, *d (7.6)*	137.3, CH	7.64, *d (7.7)*
4	118.8, CH	7.49, *d (7.8)*	119.1, CH	7.39, *d (7.6)*	119.0, CH	7.55, *d (7.7)*
4a	132.6, C		132.0, C		132.8, C	
5	126.5, C		125.8, C		132.9, C	
5a	133.3, C		131.3, C		131.5, C	
6	160. 3, C		156.8, C		158.5, C	
7	120.7, CH	7.44, *d (8.6)*	134.6, C		121.2, CH	7.27, *d (8.6)*
8	129.1, CH	8.36, *d (8.6)*	128.2, CH	8.41, *s*	128.6, CH	8.11, *d (8.6)*
8a	130.7, C		126.6, C		119.5, C	
9	188.2, C		188.3, C		189.3, C	
10	183.1, C		182.9, C		180.1, C	
1′	160.4, C		160.1, C			
1a′	114.9, C		114.8, C			
2′	133.8, CH		134.0, C			
3′	136.7, CH	7.54, *d (7.7)*	137.5, CH	7.54, *d (7.6)*		
4′	118.8 CH	7.38, *d (7.7)*	118.7, CH	7.37, *d (7.6)*		
4a′	132.3, C		132.4, C			
5′	126.5, C		125.8, C			
5a′	133.3, C		133.4, C			
6′	160,3, C		160.1, C			
7′	120.7, CH	7.44, *d (8.6)*	120.8, CH	7.46, *d (8.6)*		
8′	129.1, CH	8.35, *d (8.6)*	128.2, CH	8.38, *d (8.6)*		
8a′	129.5, C		129.3, C			
9′	188.2, C		188.3, C			
10′	183.1, C		183.0, C			
1-OH		13.22, *s*		12.98, *s*		13.06, *s*
2-$\underline{CH_3}$			16.1, CH_3	2.32, *s*	16.0, CH_3	2.28, *s*
2-$CH_2\underline{OH}$		4.80, *d (5.4)*				
2-$\underline{CH_2}OH$	64.1, CH_2	4.42, *t (5.3)*				
6-OH		9.56, *br*		(10.53-10.92), *br*		9,56, *br*[a]
1-OH′		13.18, *s*		13.17, *s*		
2′-$\underline{CH_3}$	15.0, CH_3	2.32, *s*	16.1, CH_3	2.32, *s*		
6-OH′		9.56, *br*		(10.53-10.93), *br*		

Multiplicity: *s* (singlet), *d* (doublet), *t* (triplet), *br* (brouser).
The numbers in italics and between parentheses are the coupling constant values.
The underlined atoms or groups indicate that are the informed signals in the table.
[a] Recorded in Acetone-d_6.
[b] Recorded in DMSO-d_6.

protons. Therefore, the last substituent was a hydroxy-methyl moiety (CH_2OH), which was confirmed by COSY correlations (Data in supplementary material). Bearing in mind the substituents proposed and the observed differences in δ_H values between H-3/H-3′ and H-4/H-4′, which were not observed between H-7/H-7′and H-8/H-8′, it was concluded that this dimer was formed by two different monomers that respond to the substitution pattern of a 1,6-dihydroxy anthraquinone with different substituents in ring C. This assertion was confirmed by the deshielding of the hydrogen of the OH group at C-1/1′, due to its bonding with the adjacent carbonyls (C=O), which resulted in a ^{13}C NMR resonance at δ 188.2 for C-9/9′ (Table 1). The IR spectrum showed characteristic signals for free C=O (1673 cm^{-1}) and C=O hydrogen-bonded (1630 and 1625 cm^{-1}) groups (Wijnsma and Verpoorte, 1986; Schripsema and Dagnino, 1996). The COSY correlations confirmed placement of the CH_2OH group at the 2-position, and the CH_3 group at the 2′-position, together with the presence of four spin systems. The absence in the ^{1}H NMR spectrum of a *meta*-coupled doublet for H-5 (Table 1) indicated that the two anthraquinone units would be linked through the 5-5′-positions. This was corroborated by the HMBC (Fig. 2) between H-7/C-5 and H-7′/C-5′. Since this C5-C5′ biphenyl bond is a chiral stereogenic unit, a CD spectrum was measured. A positive Cotton effect centered at 255 nm was observed, which correlated with an intense π-π* UV absorption. This indicated a (*P*)-axial chirality and, hence, an *R*-configuration of the biphenyl bond in **1** (Bringmann et al., 2005; Talapatra and Talapatra, 2015). The chemical structure of compound **1** was, therefore, assigned as (*R*)-2-hydroxymethyl-2′methyl-1,1′,6,6′ tetrahydroxy-5,5′ bianthraquinone (lycionine).

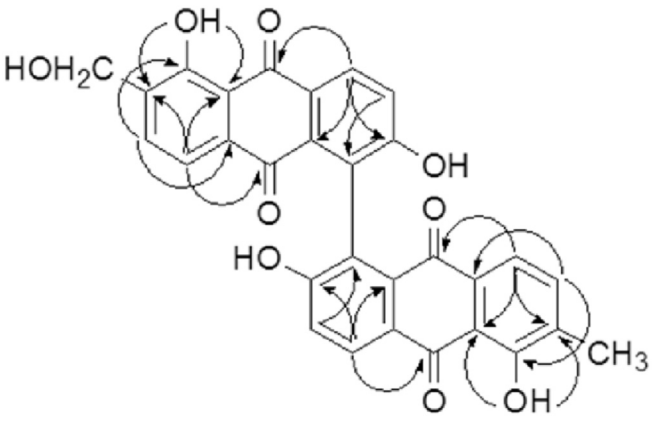

Fig. 2. HMBC correlations observed for lycionine.

Compound **2** was obtained as an orange amorphous powder. Its quasi-molecular ion $[M-H]^-$ at m/z 539.0562 in its HRESIMS allowed inference that this compound is also a bianthraquinone. An isotopic ion intensity at 541.0553 $[M-H+2]^-$, in a ratio of 1:3 in comparison with the quasi-molecular ion, showed the presence of a chlorine atom. Subsequently, its molecular formula was established as $C_{30}H_{17}ClO_8$ (calcd. for $C_{30}H_{16}ClO_8$, 539.0528). Its NMR spectroscopic data (Table 1) were very similar to those of 5,5′-bisoranjidiol (**4**), a bianthraquinone previously reported (Núñez-Montoya et al., 2006). The only difference was the presence of a chlorine atom that was placed at C-7, considering the signal pattern of seven aromatic protons: six doublets attributed to *ortho*-positioned aromatic protons (H-3/H-4, H-3′/H-4′ and H-7′/H-8′) and a singlet signal (H-8). This was confirmed by COSY (Data in supplementary material) and HMBC spectra (Fig. 3). Correlations between the singlet at δ_H 8.41 with C5a, C6 and C=O in the 9-position indicated the presence of a proton at C-8. The absolute configuration of the stereogenic center at C-5/C-5′ was assigned as *R* on the basis of the CD spectrum of **2**, which was in agreement with compound **1**. In conclusion, compound **2** was identified as (*R*)-7-chloro-2,2′-dimethyl-1,1′,6,6′-tetrahydroxy-5,5′-bianthraquinone (7-chlorobisoranjidiol). By using the method of Cohen and Neil Towers (1995) (See Experimental Section), compound **2** was obtained by chlorination of **4** as the main product. The ^1H NMR spectroscopic data of the semi-synthetic compound were identical to those found for the natural compound, and thus the presence of chlorine in the proposed structure was confirmed.

The HRESIMS of compound **3** displayed a quasi-molecular ion $[M-H]^-$ at *m/z* 287.0149 and an isotopic ion at 289.0115 $[M-H+2]^-$, which allowed us to infer that this compound is an AQ with a chlorine atom in its structure. Thus, its molecular formula was determined as $C_{15}H_9ClO_4$ (calcd. for $C_{15}H_8ClO_4$, 287.0106). It was isolated as an orange amorphous powder and its NMR spectroscopic data (Table 1) were similar to those of soranjidiol (**5**) (Wijnsma and Verpoorte, 1986). Except for a chlorine, both compounds showed the same substituents with characteristic signals of a 1,6-dihydroxy-2-methyl anthraquinone. Considering the aromatic proton signals: two pairs of doublets ($J = 7.7$ and 8.6) attributable to *ortho*-positioned aromatic protons (H-3/H-4 and H-7/H-8), this compound was identified as a chlorinated derivative of soranjidiol at the 5-position. The COSY correlations (Data in supplementary material) confirmed the presence of two spin systems H/H and placement of the CH_3 at the 2-position. HMBC correlations (Data in supplementary material) confirmed an OH group at C-1, and the correlation between H-8 with a quaternary carbon signal at δ_C 158.5 (C-6) corroborated the position of the free OH at the 6-position. Thus, compound **3** was identified as 5-chloro-1,6-dihydroxy-2-methyl anthraquinone (5-chlorosoranjidiol). By chlorination of **5**, the main compound obtained (75%) had the same ^1H NMR spectroscopic data as **3**.

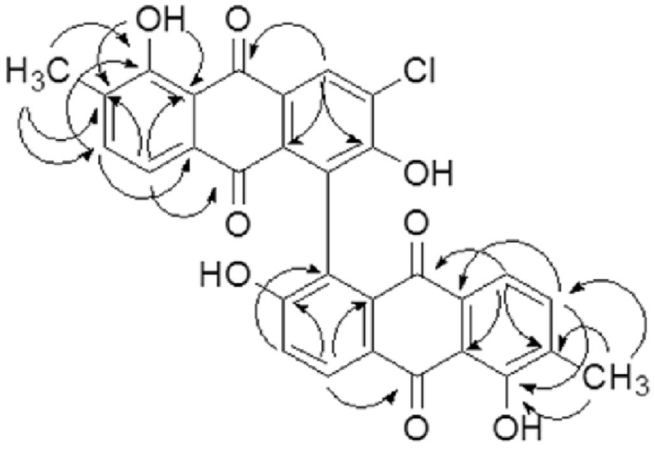

Fig. 3. HMBC correlations observed for 7-chlorobisoranjidiol (2).

2.2. $O_2^{\bullet -}$ generation

The increase in $O_2^{\bullet -}$ production by each AQ, in absence and presence of irradiation, was measured under aerobic conditions using the nitrobule tetrazolium assay (NBT). This test measures the NBT reduction to formazan blue (FB) spectrophotometrically; this process is caused by the generated $O_2^{\bullet -}$ in the biologic system as a consequence of the action of each AQ (Núñez-Montoya et al., 2005). The stimulation of this ROS in human neutrophils by **1**, **2** and **3** at 10 and 20 μg/ml, under darkness and irradiation conditions, is shown in Fig. 4. It was also demonstrated here that light does not affect neutrophils (irradiation control). The new AQs (**1**–**3**) only increased $O_2^{\bullet -}$ production under irradiation at the highest concentration tested (20 μg/ml). Compound **2** was the most active AQ with the greatest production of this ROS, generating an increase of 20% compared to control. The other two AQs (**1** and **3**) produced only a slight rise of 7%.

3. Conclusions

From a phenotypic and chemotaxonomic point of view, *H. lycioides* is shown as a species very close to *H. pustulata* (Taylor, 2010). Chemically, both species contain anthraquinone derivatives with little variations in skeletal structure, but in *H. lycioides* the chlorinated compounds are especially noteworthy (**2** and **3**). These AQs belong to a group of chlorinated natural compounds that are rarely found in higher plants. Among natural halogenated organic derivatives, the chlorinated AQs are more common in fungi and lichens, with a substitution pattern dictated by the acetate/malonate biosynthetic pathway (1,8-dihydroxy-AQ) (Gribble, 2010). In contrast, the halogenated AQs isolated from *H. lycioides* show a different substitution pattern, resulting from the shikimic acid/mevalonate pathway, which is the biosynthetic route present in the Rubiaceae family (Wijnsma and Verpoorte, 1986; Martins and Nunez, 2015). To our knowledge, this is the first time that halogenated AQs are reported for a species of this family. The interaction between endophytic microorganisms with their host plant achieved during long periods of coevolution could be an explanation for the modification in the biosynthesis of its metabolites (Golinska et al., 2015; Martinez-Klimova et al., 2016). However, there are no antecedents in the genus to suggest it. Therefore, this and other conjectures, including other external factors, should be studied in depth to attain a consistent response.

The three new AQs isolated from *H. lycioides* possess Type I photosensitizing properties, as evidenced by an increase in the $O_2^{\bullet -}$ production under the action of light. Compounds **1**–**3** may, therefore, contribute to the toxicity attributed to this plant species, along with the other isolated AQs (**4**–**7**) that also demonstrated photosensitizing properties (Núñez-Montoya et al., 2005; Comini et al., 2007). Moreover, the discovery of these new natural anthraquinones implicates an important contribution to PDT, since they have proved to be photosensitizing compounds that should be evaluated for their potential use in this promising therapy.

4. Experimental section

4.1. General experimental procedures

Optical rotations were measured on a Jasco P-1010 polarimeter (Tokyo, Japan). UV spectra were recorded on a Cary Win UV-VIS

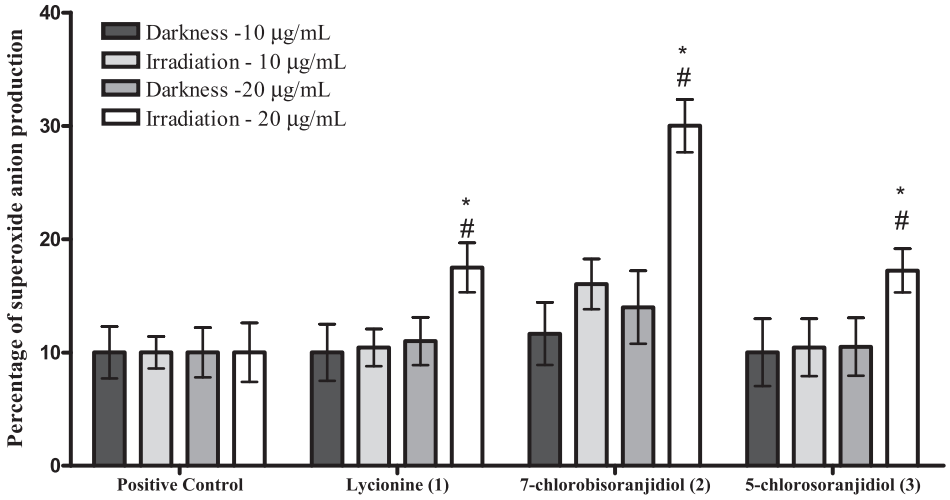

Fig. 4. Stimulation of $O_2^{\bullet -}$ generation in neutrophils produced by natural anthraquinones (**1**–**3**). Assay of nitroblue tetrazolium reduction in dark condition and with irradiation.

spectrophotometer (Santa Clara, United States). The CD spectra were recorded on a JASCO Model J-810 spectropolarimeter (Tokyo, Japan). IR spectra were obtained with a Nicolet 5-sxc-FTIR infrared spectrophotometer (Madison, WI, USA). NMR spectra were acquired in DMSO-d_6 and acetone-d_6 on a Bruker Advance II 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer (Rheinstetten, Germany). Chemical shifts (δ) were reported in ppm relative to TMS as internal standard and coupling constants (*J* value) in Hz. All carbon atoms were unequivocally assigned from the HMBC spectra because some signals corresponding to quaternary carbons were missing in the ^{13}C NMR spectra. HR-ESI-MS analysis were performed by direct infusion in a MicroTOF QII (Bruker Daltonics, USA) spectrometer, equipped with a qTOF analyzer, which was operated with an electrospray ion source (ESI) in the negative ion mode, using N_2 as nebulizing and drying gas. Column chromatography (CC) was performed on Sephadex LH-20 (Sigma Aldrich, St. Louis, MO, USA) and silica gel 60 (0.063–0.200 mm) (Merck, Darmstadt, Germany). TLC utilized either pre-coated plates of silica gel 60 (Macherey-Nagel, Düren, Germany) or reversed phase TLC with pre-coated plates of silica gel 60 RP-18 $F_{254}S$ (Merck). AQs were detected under UV light with NH_4OH vapors. Prep. TLC were developed on the same pre-coated plates described above, but applying between 5 mg and 10 mg of sample; bands were detected under UV light and recovered from the stationary phase with acetone.

4.2. Plant material

H. lycioides was collected on April 2012 in Yotala, locality close to Sucre city (Bolivia, 19°9′31″ S and 65°15′51″ W). A voucher specimen was collected and identified by Eng. Ewdin Portales, and is deposited as N° 18,608 in the Herbario del Sur de Bolivia (HSB).

4.3. Extraction and isolation

Plant material was dried at room temperature and the aerial parts (89.5 g) were crushed and extracted with solvents of increasing polarity: *n*-hexane (Hex), benzene (C_6H_6), EtOAc and EtOH using a Soxhlet apparatus. Each solvent was subsequently removed under reduced pressure to yield the following crude extracts: Hex (2.28 g), C_6H_6 (2.50 g), EtOAc (1.10 g) and EtOH (12.10 g). The C_6H_6 and EtOAc extracts were suspended in an aqueous phase separately, and were partitioned following a previously described process (Núñez-Montoya et al., 2006), in which successive partitions with $CHCl_3$ and Et_2O at different pH/s were carried out. Thus, two final ethereal extracts characterized by possessing a high content of AQs without chlorophylls were obtained: Ether I (from the C_6H_6 extract) and Ether II (from the EtOAc extract).

Ether I extract (1.25 g) was subjected to Sephadex LH-20 CC, and was eluted with a step gradient of $CHCl_3$-EtOAc, EtOAc-acetone, acetoneEtOH (100:0, 8:2, 1:1, 2:8, 0:100, respectively). The eluents were analyzed by TLC (silica gel) with C_6H_6-EtOAc (8:2) and (1:1) as mobile phases, and fractions with similar TLC patterns were combined to give 5 major fractions (A-E). Fraction A (60 mg) was applied to a silica gel column with a step gradient of C_6H_6EtOAc and EtOAcEtOH (100:0, 8:2, 1:1, 2:8, 0:100, respectively) to afford four fractions (A1–A4). Sub-fraction A2 (8 mg) was subjected to prep. TLC using $CHCl_3$ as mobile phase to give compound **6** (3 mg) and **7** (1.2 mg). Compound **3** (2.5 mg) and **5** (4 mg) were purified from sub-fraction A3 (11 mg) by prep. TLC eluted with $CHCl_3$-EtOAc (9:1). Finally, sub-fraction A4 (25 mg) was separated by using prep. TLC with C_6H_6-EtOAc (1:1) to give compounds **1** (3 mg) and **4** (10 mg).

Ether II extract (0.85 g) was subjected to silica gel CC eluted with a step gradient of C_6H_6-EtOAc and EtOAc-EtOH, (100:0, 8:2, 1:1, 2:8, 0:100). Column fractions were analyzed by TLC (silica gel) and fractions with similar TLC patterns were combined to give 4 major fractions (A-D). Fraction B purification (45 mg) was achieved by using Sephadex LH-20 CC with a gradient elution of $CHCl_3$-EtOH (100:0 → 0:100, v/v) to give five fractions (B1-B5). Sub-fraction B2 was subjected to prep. TLC with $CHCl_3$ to yield compound **5** (3 mg). Finally, purification of sub-fraction B3 (23 mg) was achieved by prep. RP-TLC using MeOH-HCO_2H (100:0.61) as mobile phase to give compound **2** (5 mg) and **4** (12 mg).

4.4. (R)-2-hydroxymethyl-2′methyl-1,1′,6,6′-tetrahydroxy-5,5′-bianthraquinone (lycionine) (**1**)

Yellow amorphous powder. $[\alpha]_D^{25}$ +6 (c 0.01, MeOH). UV–Vis λ_{max} (CH_3CN) nm (log ε): 256 (1.02), 271 (1.05), 282 (sh) (0.93), 290 (sh) (0.83), 395 (sh) (0.38), 415 (0.45), 436 (sh) (0.36); λ_{max} (MeOH) nm (log ε): 254 (0.65), 271 (0.60), 281 (sh) (0.56), 401 (sh) (0.22), 415 (0.24), 427 (sh) (0.23). CD MeOH (c 1.8×10^{-4} M) λ_{max} ($\Delta\varepsilon$): 221 (−5.36), 255 (+7.57), 390 (−1.80), 442 (+2.31). IR (KBr) ν_{max} 3406 (OH free), 3290 (-CH_2OH st), 2954, 2923, 2852, 1673 (C=O free), 1630 and 1625 (C=O hydrogen-bonded), 1428, 1361, 1298, 1269 cm^{-1}. For ^{1}H NMR and ^{13}C NMR spectroscopic data see Table 1. COSY: CH_2OH-2/H-3, CH_3-2′/H-3′, H-3/H-4, H-3′/H-4′, H-7,7′/H-8,8′. HR-ESI-MS m/z 521.0899 $[M-H]^-$ (calcd. for $C_{30}H_{18}O_9$, 521.0867).

4.5. (R)-7-chloro-2,2′-dimethyl-1,1′,6,6′-tetrahydroxy-5,5′-bianthraquinone (7-chlorobisoranjidiol) (**2**)

Orange amorphous powder. $[\alpha]_D^{25}$: +23 (c 0.12, MeOH). UV–Vis λ_{max} (CH_3CN) nm (log ε): 260 (0.90), 274 (0.93), 363 (0.20), 385 (sh) (0.27), 414 (0.33), 437 (sh) (0.26), 549 (0.04); λ_{max} (MeOH) nm (log ε): 257 (0.77), 296 (sh) (0.42), 329 (0.30), 412 (0.19), 506 (0.14). CD MeOH (c 8.6×10^{-4} M) λ_{max} ($\Delta\varepsilon$): 243 (−0.79), 258 (+2.62), 392 (−0.48), 442 (+0.44). IR (KBr) ν_{max} 3406 (OH free), 2957, 2920, 2855, 1653 (C=O free), 1627 and 1620 (C=O hydrogen-bonded), 1455, 1300, 1260 cm^{-1}. For ^{1}H NMR and ^{13}C NMR spectroscopic data see Table 1. COSY: CH_3-2,2′/H-3,3′, H-3,3′/H-4,4′, H-7′/H-8′. HR-ESI-MS m/z 539.0562 $[M-H]^-$ (calcd. for $C_{30}H_{17}ClO_8$, 539.0528), 541.0553 $[M-H+2]^-$.

4.6. 5-Chloro-1,6-dihydroxy-2-methyl anthraquinone (5-chlorosoranjidiol) (**3**)

Orange amorphous powder. $[\alpha]_D^{25}$: - 5.3 (0.08, $CHCl_3$). UV–Vis λ_{max} (CH_3CN) nm (log ε): 267 (1.14), 278 (sh) (0.67), 287 (0.56), 351 (0.13), 387 (sh) (0.24), 411 (0.28), 414 (sh) (0.28); λ_{max} (MeOH) nm (log ε): 251 (0.28), 262 (sh) (0.20), 304 (sh) (0.18), 315 (0.21), 337 (0.09), 422 (0.06), 485 (0.09). IR (KBr) ν_{max} 3406 (OH free), 2957, 2920, 2855, 1664 (C=O free), 1627 (C=O hydrogen-bonded), 1455, 1300, 1260 cm^{-1}. For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1. COSY: CH_3-2/H-3, H-3/H-4, H-7/H-8. EIMS m/z 290 $[M+2]^+$ (14.9), 288 $[M]^+$ (35.5), 253 $[M\text{-}Cl]^+$ (7.1), 225 $[M\text{-}Cl\text{-}CO]^+$ (4.6), 197 $[M\text{-}Cl\text{-}2CO]^+$ (11.8), 168 $[M\text{-}Cl\text{-}2CO\text{-}HCO]^+$ (13.4), 139 $[M\text{-}Cl\text{-}2CO\text{-}2HCO]^+$ (23.3). HR-ESI-MS m/z 287.0149 $[M-H]^-$ (calcd. for $C_{15}H_9ClO_4$, 287.0106), 289.0115 $[M-H+2]^-$.

4.7. Synthesis of compound 2 and 3

Compound **4** (5.2 mg) was dissolved in dry CH_2Cl_2 (13 ml) at room temperature, and continuous stirring until complete dissolution was achieved. N-chlorosuccinimide (10.4 mg) was added in one portion to the stirred solution, and stirred for 24 h. After removing the solvent under reduced pressure, the obtained crude product was purified using the same RP-TLC procedure mentioned above. The amorphous solid thus obtained was identified by spectroscopic analysis as **2** (2.1 mg, 40%).

The same procedure was carried out to obtain compound **3** by using **5** (5 mg), dry CH_2Cl_2 (6.25 ml) and N-chlorosuccinimide (5 mg). The amorphous solid obtained was purified by the same prep. TLC used before and was identified by spectroscopic methods as **3** (4.2 mg, 75%).

4.8. NBT assay: determination of $O_2^{\bullet-}$ generation

This indirect photobiological method measures the reduction of nitroblue tetrazolium (NBT, Sigma Aldrich, St. Louis, MO, USA) to formazan blue (FB) by action of the $O_2^{\bullet-}$ generated for human neutrophils (10^6 cells/ml), when an oxidizer (AQ) is present (Becerra et al., 2001). Neutrophil suspension was obtained from voluntary and healthy donors' human blood, by following a standard procedure (Núñez-Montoya et al., 2005). Each AQ was dissolved in Hanks balanced salt solution (HBSS) with DMSO as co-solvent (<1%). The assay was adjusted to 24-well microtiter plates (Greiner Bio-One, Germany) according to the previously published procedure (Konigheim et al., 2012). Two microplates were performed simultaneously under darkness and irradiation, by using a 20W Phillips actinic lamp (380–480 nm, 0.65 mW/cm^2) with a maximum at 420 nm, placed inside a black box at 20 cm above a thermostatic bath containing the microplate. Briefly, neutrophil solution (10^6 cells/ml, pH = 7) with each AQ at two concentrations (10 and 20 μg/ml) and NBT (0.1% P/V) were incubated at 37 °C for 30 min (n = 3, for each AQ and concentration). Microwells containing neutrophil solution and NBT were included as a control (n = 3). After stopping the reaction with HCl (0.1 M), the content of each microwell was centrifuged in Eppendorf tubes at 6149 *g* for 10 min. The supernatant was discarded and the sediment was treated with DMSO to dissolve the FB contained inside neutrophils. The optical density of FB was measured at 595 nm in a microplate reader (Tecan sunrise model). The increase in $O_2^{\bullet-}$ production was expressed as a percentage with respect to controls (100%). To illustrate the increase graphically, an arbitrary value of 10% was assigned to controls. Data obtained was statistically analyzed by GraphPad Prism 4.0., and a *t*-test was used for data comparisons, in which a p-value < 0.05 was considered statistically significant.

Acknowledgments

This work was supported by CONICET-PIP 2013–2015 [GI 4316/2013], SeCyT-UNC [No. 203/14] and FONCYT-PICT 2010 [No. 1576] grants. J. Dimmer is a doctoral fellow at CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas). S. Núñez-Montoya and J.L. Cabrera are members of the Research Career at CONICET. The authors are thankful to Eng. Ewdin Portales (Herbario del Sur de Bolivia) for identifying the species, to Dr. G. Bonetto (Laboratorio de RMN, INFIQC, CONICET and Universidad Nacional de Córdoba) for performing the NMR experiments, to Dr. R. Di Paola (ICYTAC – CONICET and Universidad Nacional de Córdoba) for developing HRESIMS experiments, to Dr. G. Barboza (Museo Botánico de Córdoba, Fac. Cs. Exactas, Físicas y Naturales, Universidad Nacional de Córdoba) for advising about the taxonomy of the genus *Heterophyllaea*.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.02.003.

References

Aguirre, D.H., Neumann, R.A., 2001. Intoxicación por “cegadera” (*Heterophyllaea pustulata*) en caprinos del noroeste argentino. Med. Vet. 18, 487–490.
Atahuachi Burgos, M., 2005. La Guía “Darwin” de las flores de los valles bolivianos. Darwin Initiiative, La Paz.
Bacigalupo, N.M., 1993. Rubiaceae. In: Cabrera, A.L. (Ed.), Flora de la Provincia de Jujuy. Colección Científica INTA, Bs. As, pp. 375–380.
Becerra, C., Albesa, I., Eraso, A.J., 2001. Leukotoxicity of pyoverdin, production of reactive oxygen species, and effect of UV radiation. Biochem. Biophys. Res. Commun. 285, 414–418.
Brako, L., Zarucchi, J.L., 1993. Catalogue of the flowering plants and gymnosperms of Peru. Mo. Bot. Gard. 45, 1029.
Bringmann, G., Mortimer, A.J.P., Keller, P.A., Gresser, M.J., Garner, J., Breuning, M., 2005. Atroposelective synthesis of axially chiral biaryl compounds. Angew. Chem. Int. Ed. 44, 5384–5427.

Cohen, P.A., Neil Towers, G.H., 1995. The anthraquinones of *Heterodermia obscurata*. Phytochem 40, 911–915.
Comini, L.R., Núñez-Montoya, S.C., Sarmiento, M., Cabrera, J.L., Argüello, G.A., 2007. Characterizing some photophysical, photochemical and photobiological properties of photosensitizing anthraquinones. J. Photochem. Photobiol. A Chem. 188, 185–191.
Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., Rai, M., 2015. Endophytic Actinobacteria of Medicinal Plants: Diversity and Bioactivity. Antonie Van Leeuwenhoek.
Gribble, G.W., 2010. Naturally Occurring Organohalogen Compounds-a Comprehensive Update. Springer-Verlag, Wien, pp. 319–364.
Kharkwal, G.B., Sharma, S.K., Huang, Y.-Y., Dai, T., Hamblin, M.R., 2011. Photodynamic therapy for infections: clinical applications. Lasers Surg. Med. 43, 755–767.
Konigheim, B.S., Comini, L.R., Grasso, S., Aguilar, J.J., Marioni, J., Contigiani, M.S., Núñez-Montoya, S.C., 2012. Determination of non-toxic and subtoxic concentrations of potential antiviral natural anthraquinones. Lat. Am. J. Pharm. 31, 51–56.
Martinez-Klimova, E., Rodríguez-Peña, K., Sánchez, S., 2016. Endophytes as sources of antibiotics. Biochem. Pharmacol. (in press).
Martins, D., Nunez, C.V., 2015. Secondary metabolites from Rubiaceae species. Molecules 20, 13422–13495.
Núñez-Montoya, S.C., Comini, L.R., Sarmiento, M., Becerra, C., Albesa, I., Argüello, G.A., Cabrera, J.L., 2005. Natural anthraquinones probed as Type I and Type II photosensitizers: singlet oxygen and superoxide anion production. J. Photochem. Photobiol. B Biol. 78, 77–83.
Núñez-Montoya, S.C., Agnese, A.M., Cabrera, J.L., 2006. Anthraquinone derivatives from *Heterophyllaea pustulata*. J. Nat. Prod. 69, 801–803.
Schripsema, J., Dagnino, D., 1996. Elucidation of the substitution pattern of 9,10-anthraquinones through the chemical shifts of peri-hydroxyl protons. Phytochem 42, 177–184.
Talapatra, S.K., Talapatra, B., 2015. Chemistry of Plant Natural Products: Stereochemistry, Conformation, Synthesis, Biology and Medicine. Springer, Berlin, p. 184.
Taylor, C.M., 2010. Rubiaceae. Tropicos: Missouri Botanical Garden. http://www.tropicos.org/NameSearch.aspx?projectid=34.. Wijnsma
Wijnsma, R., Verpoorte, R., 1986. Anthraquinones in the Rubiaceae. In: Hill, R.A., Krebs, H.Ch, Verpoorte, R., . Wijnsma, R. (Eds.), Progress in the Chemistry of Organic Natural Products. Springer-Verlag, Wien, pp. 79–149.