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Phototoxic effects of *Heterophyllaea pustulata* (Rubiaceae) ☆

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ABSTRACT

Heterophyllaea pustulata (Rubiaceae), a South American genus, is a phototoxic shrub that grows in the Andean mountain range of the northwest of Argentina, popularly known as "cegadera". Animals that ingest the aerial parts of this plant suffer a typical primary photosensitization reaction, clinically revealed by dermatitis and blindness in severe cases. Anthraquinone derivatives (AQs), the main metabolites of this species, are characterized as Type I and/or Type II photosensitizers according to their physicochemical properties. The natural toxicity conditions were reproduced *in vivo* assays by oral administration of soranjidiol and rubiadin, the main components of the aerial parts. By HPLC analysis, the presence of these AQs was determined in serum and quantified in the skin of experimental animals.

1. Introduction

Heterophyllaea pustulata Hook. f. (Rubiaceae) is a wild bush 2–3 m high that grows in the Andean northwest of Argentina, between 2500 and 3000 m above sea level. It is a phototoxic plant, popularly known in Spanish as "cegadera", which causes significant economic losses in the livestock of its habitat region (Bacigalupo, 1993). Natural intoxication by "cegadera" has been reported for equine, bovine and goat-like individuals as dermatitis and blindness in severe cases, without death of the animals confirmed (Hansen and Martiarena, 1967; Aguirre and Neumann, 2001).

Toxic effects were experimentally replicated by Hansen and Martiarena (1967), demonstrating that the ingestion of different parts of the plant at different vegetative development periods produces dermatitis and kerato-conjunctivitis, especially in animals with white coats. These authors also established that the effects portrayed are reversible if toxins have worked only over a short period, and they concluded that toxic action takes place provided the animals are exposed to light. Therefore, this toxicity has been pathologically defined as a photosensitization reaction, clinically presented without icterus. However, the chemical agents that generate the effects observed were not determined in that study.

The first chemical investigations of *H. pustulata* were performed by our researcher group (Núñez Montoya et al., 2003, 2006), where 9,10-anthraquinone derivatives (AQs) were found to be the predominant metabolites, whereas a minority of other structures present belongs to the flavonoid and iridoid groups. This pattern reveals taxonomic chemical properties similar to those of other

☆ *Ethical statement*: Breeding, housing and experimental procedures with animals followed guidelines established by Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) for the care and use of laboratory animals (ANMAT, 1996). In addition, we followed the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). Furthermore, the "*Comité de Ética de protocolos Experimentales en el uso de animales en los proyectos científicos*" (Facultad de Ciencas Químicas, Universidad Nacional de Córdoba, Argentina) approved the protocol for assays *in vivo* of a phototoxic vegetal species, *Heterophyllaea pustulata* Hook. f. (Rubiaceae), which is a native plant from the northwest of Argentina in the resolution no. 590/2007 of HCD (Facultad de Ciencas Químicas, Universidad Nacional de Córdoba).

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Rubiaceae species, but not all of these have shown phototoxic properties (Wijnsma and Verpoorte, 1986). In complementary studies, we have reported that AQs isolated from *H. pustulata* (soranjidiol, soranjidiol 1-methyl ether, rubiadin, rubiadin 1-methyl ether, damnacanthal, damnacanthol, heterophylline, pustuline and (*S*)-5,5′-bisoranjidiol) exhibit photosensitizing properties by generation of superoxide anion radicals ($O_2^{\bullet -}$, Type I mechanism) and/or singlet molecular oxygen ($^1O_2$, Type II mechanism) (Núñez Montoya et al., 2005; Comini et al., 2007).

Chemical, photophysical and photochemical assays performed with purified AQs from *H. pustulata* allowed us to estimate that these chemistry components would be responsible for the manifest phototoxicity of this plant. Thus, it was the objective of the present work to demonstrate *in vivo* that the predominant AQs are involved in the phototoxic process attributed to this vegetal species. Accordingly, assays were performed in order to evaluate whether these natural AQs administered orally and transported by systemic circulation would accumulate in the skin, where they would perform their photosensitizing action.

2. Materials and methods

2.1. Vegetal material

Plant material was collected in "La Almona", Province of Jujuy, Argentina, during the flowering period. The material was identified by Prof. Dr. Gloria Barboza (Instituto Multidisciplinario de Biología Vegetal, Consejo Nacional de Investigaciones Científicas y Técnica, Universidad Nacional de Córdoba—IMBIV, CONICET, UNC). A voucher specimen was deposited at the Museo Botánico de Córdoba (UNC) as M.E. Lázzaro s/n, CORD 305.

2.2. Preparation of AQ samples for administration

Soranjidiol (**1**) and rubiadin (**2**) (Fig. 1) are the two AQs mostly found in the aerial parts of *H. pustulata* (Núñez Montoya et al., 2003) and they stand out because they generate the highest levels of $^1O_2$ and/or $O_2^{\bullet -}$ under radiation (Núñez Montoya et al., 2005). Thus, a purified and quantified fraction of **1** and **2** was obtained in order to be used in the phototoxicity assay *in vivo*.

Air-dried aerial parts (170 g) were mechanically triturated and treated with hexane to eliminate chlorophylls and fatty components. Then, the remaining vegetal material was extracted with benzene using a Soxhlet apparatus. The resulting extract was dried *in vacuo*, yielding 2.1 g of dried extract which was submitted to column chromatography in silica gel 60 (Merck) and was eluted with benzene and increasing amounts of EtOAc until the ratio benzene–EtOAc was 2:8. The eluents were monitored by TLC on pre-coated silica gel 60 plates (Merck) with benzene–EtOAc (8:2) as the mobile phase, and four major fractions were obtained (A–D). By means of analytical TLC with authentic AQs under the same conditions as described above, **1** and **2** were identified as major components of fraction A (1.1 g). The standard AQs had been previously obtained from aerial parts of *H. pustulata* and identified by their spectroscopic properties (^{1}H NMR, ^{13}C NMR, IR, UV–Vis, MS) (Núñez Montoya et al., 2003).

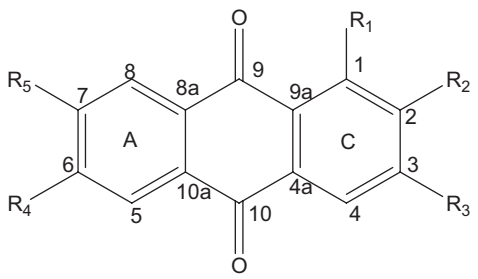
A solution of fraction A in olive oil was prepared (58.3 mg/ml) in order to be administered orally (oral sample) (United States Pharmacopeial Convention, 2000). Another aliquot of fraction A was dissolved in DMSO–H_2O (3:1) (6.8 mg/ml) to be used in subcutaneous administration (Núñez Montoya et al., 2003).

2.3. Experimental animals

Male albino Balb/c mice were used, all specimens being 2 months old and weighing 20 g approximately. The animals were maintained in standard cages with free access to tap water and under constant conditions of room temperature (22 ± 1 °C) and relative humidity (50 ± 5%) during the experiment.

2.3.1. Oral experiment

The animals ($n = 12$) were divided into two experimental groups, one problem group and another control



AQs	R_1	R_2	R_3	R_4	R_5
Soranjidiol (**1**)	OH	CH_3	H	OH	H
Rubiadin (**2**)	OH	CH_3	OH	H	H

Fig. 1. Main anthraquinones from *Heterophyllaea pustulata* assayed *in vivo*.

group. The AQ fraction (fraction A) was administered orally to the problem group ($n = 6$) using a graduated syringe in two doses of 0.4 ml each (1166.7 mg/kg each), with a period of 40 min between each dose. The same posology was used for the control group ($n = 6$), where fraction A was replaced by the vehicle (olive oil).

Between the first and second doses, three animals of each group (problem and control) were exposed to sunlight during 40 min. After the second dose, the same animals were kept under solar radiation for two further hours. The remaining animals of each group (problem and control) were kept in darkness for the same period (160 min).

2.3.2. Subcutaneous experiment

Subcutaneous administration was carried out on the depilated back of the animals, exactly 2 cm above the tail. A volume of 100 μl of fraction A (34.1 mg/kg) was injected in each problem animal ($n = 6$) and the same volume of vehicle (DMSO–H_2O) in each control animal ($n = 6$). Whereas three animals of each group (problem and control) were radiated with solar light during 160 min, the others were kept in darkness for the same period.

2.4. Evaluation of the animals

While experiments were being carried out, changes in the animals' behavior such as restlessness, irritability, photophobia and itching were evaluated. In order to observe typical symptoms of skin toxicity such as erythema, inflammation, edema and rash, a portion of the back of the animals was depilated.

2.5. Preparation of biologic samples

Animals were anesthetized with a sterile aqueous solution of ketamine chlorhydrate (Holliday Scott)–xylazine chlorhydrate (Alfasan)–acepromazine (Holliday Scott) (85.5–17.0–3.0 mg/kg) by intraperitoneal (i.p.) injection before the animals were sacrificed by decapitation (Alvarez et al., 2004).

Serum and skin samples were taken from the animals used in the oral experiment in order to determine the presence of AQs at the systemic and dermal levels.

Serum samples were obtained from the blood of each animal by centrifugation at 1500*g* for 15 min (Presvac centrifuge). A portion of skin (200 mg) from the back of each animal was immediately removed after its decease. Serum samples as well as skin samples were stored and preserved at −20 °C.

Breeding, housing and experimental procedures with animals followed guidelines established by Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) for the care and use of laboratory animals (ANMAT, 1996; Institute of Laboratory Animal Resources et al., 1996).

2.6. HPLC analysis

2.6.1. Sample preparation

A solution of fraction A (0.09 mg/ml) in methanol HPLC grade (MeOH, Merck) was used for its qualitative and quantitative analysis by HPLC.

In order to identify and/or quantify the AQs in serum and skin samples, these components had to be extracted from biological material. Whereas serum samples were extracted from buffer pH = 2 with ethyl ether (Merck) in a proportion of 1:10 volume (Jackson, 1978), skin samples were malaxed with buffer pH = 2 and then extracted under the same conditions as described above (Chamberlain, 1985). For both biological materials, this procedure was repeated until depletion of material and ether fractions were evaporated to dryness; finally, they were dissolved with MeOH, using 200 μl for serum samples and 160 μl for skin samples. All samples were filtered through a 0.2 μm cellulose acetate membrane filter (Micro Filtration System) before HPLC analysis.

2.6.2. HPLC instruments and chromatographic conditions

HPLC analysis (qualitative and quantitative) was performed on a Varian Pro Star chromatograph (model 210, series 04171), equipped with a UV–Vis detector and a Microsorb-MV column 100-5 C_8 (250 × 4.6 mm i.d., Varian).

The mobile phase was MeOH–H_2O (8:2) at constant flow (1 ml/min) and the injection volume was 20 μl. The detection was performed at the wavelength of 269 nm. Data analyses were carried out using Origin 6.0.

2.6.3. AQs identification and quantification

Identification was carried out by comparison of the HPLC retention times (t_R) with the corresponding standards under the same chromatography conditions.

The AQs were quantified using the external calibration method (Cunico et al., 1998). Primary stock solutions of each standard AQ were prepared in MeOH: soranjidiol (1×10^{-3} M) and rubiadin (3.62×10^{-3} M). Then, they were diluted quantitatively with MeOH to give seven working solutions within a range of concentrations which allowed quantifying the identified AQs in fraction A and in skin samples.

Calibration curves (Fig. 2) were obtained by graphing the area under each peak (AUP) as a function of AQ concentration. The seven pointed curves constructed ($n = 3$) were linear (correlation coefficients > 0.99) (Fig. 2). Using the calibration curves, the concentration of each AQ (soranjidiol and rubiadin) in fraction A and in each skin sample was calculated by interpolating the AUP for each compound (Marinova and Ribarova, 2007).

3. Results

3.1. AQs identification and quantification from fraction A

Fig. 3 shows the HPLC analysis of fraction A administered orally and subcutaneously. The chromatogram allowed one to corroborate the identification of soranjidiol

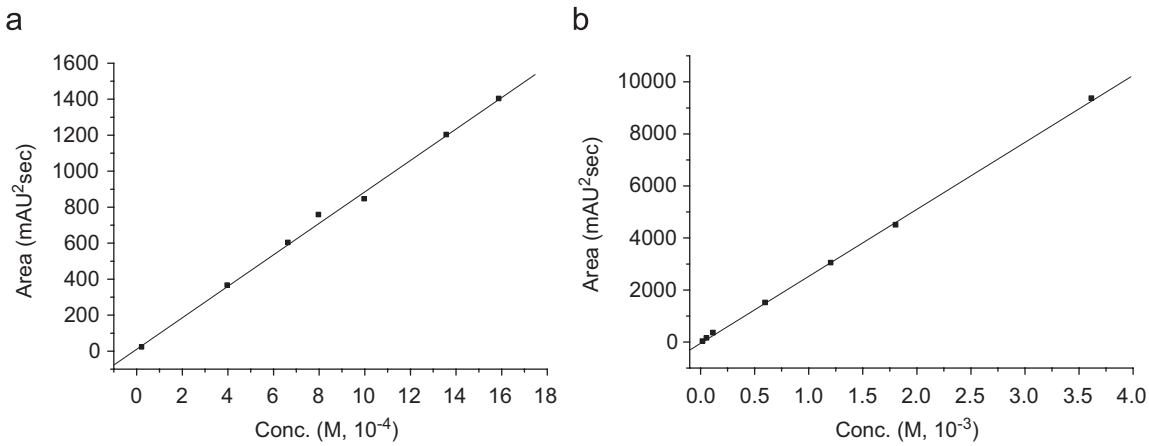


Fig. 2. HPLC calibration curves for standard anthraquinones of soranjidiol (**1**) and rubiadin (**2**). A: Soranjidiol ($\lambda = 269$ nm); B: Rubiadin ($\lambda = 269$ nm).

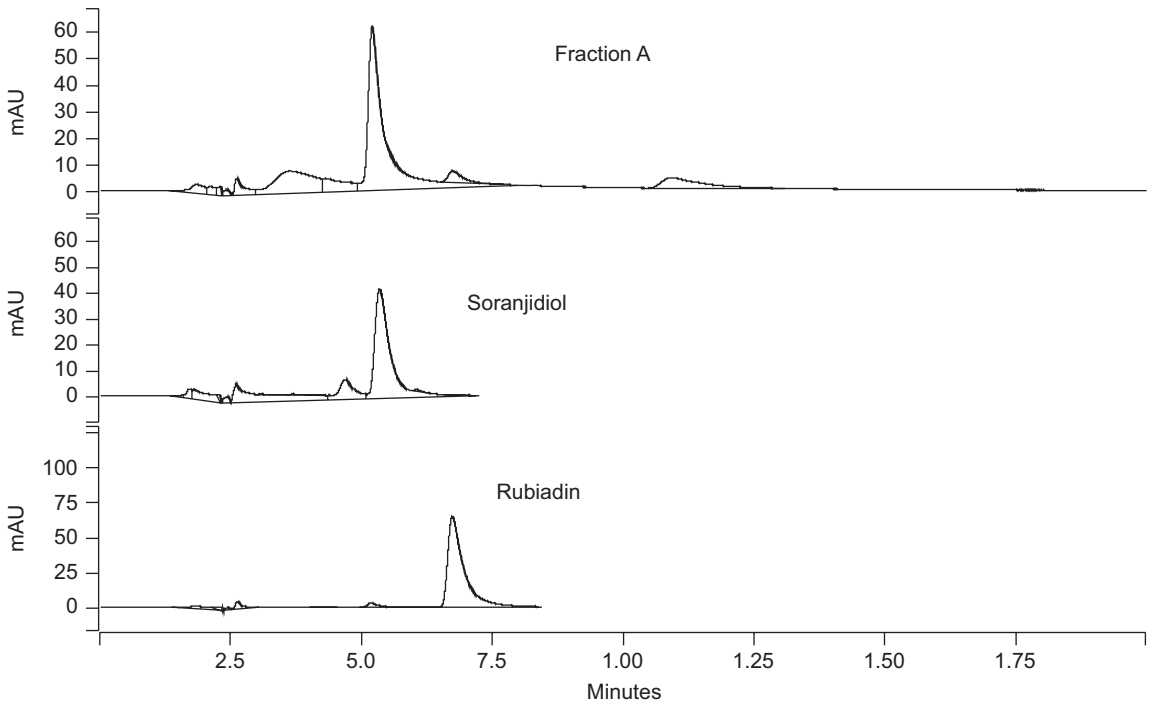


Fig. 3. Qualitative HPLC analysis of fraction A (administered orally and subcutaneously).

Table 1
Anthraquinone composition of fraction A administered orally and subcutaneously

AQs	Fraction A (%)*
Soranjidiol (**1**)	43.8 ± 1.8
Rubiadin (**2**)	14.7 ± 0.4

* Data were expressed as means ± SD.

(**1**) and rubiadin (**2**) as the predominant AQs in fraction A as well as to establish its quantitative composition (Table 1).

3.2. Observed toxicity

3.2.1. Oral experiment

A few minutes after the first oral administration, those animals that were exposed to sunlight showed uneasiness, aggressiveness and looked for a place protected from radiation (photophobia). At skin level, it could be observed that inflammation, erythema, itching and petechiae appeared on the depilated part of the animals' backs after the second dose.

On the other hand, animals treated with AQs and kept in darkness showed neither abnormal behavior nor symptoms at skin level. Control animals

both in darkness and under radiation showed no symptoms.

3.2.2. Subcutaneous experiment

Edema and strong inflammation were observed to appear immediately on the back of animals exposed to radiation, followed by erythema. After 20 min of exposure to direct sunlight, petechiae and slight skin eruptions appeared. These symptoms slowly receded during the last 2 h of exposure to sunlight, and normal behavior was observed in the animals during this period; erythema, inflammation and petechiae gradually disappeared, whereas skin eruption continued.

The above-described symptoms were not observed either in those animals treated with AQs and kept in darkness or in control animals.

3.3. AQs identification and quantification in biological material

Serum and skin samples from animals administered orally were used in order to determinate the presence of AQs in systemic circulation. Fig. 4 exhibits chromatograms corresponding to serum from animals treated with AQs in sunlight (serum 1) and darkness (serum 2), as compared with fraction A. The control chromatogram corresponding to the serum sample taken from animals treated with the vehicle is also included; it proved to be identical to both controls for irradiated animals and controls for those kept in darkness. As can be seen, the AQs administered orally (**1** and **2**) are identical to serum under either set of experimental conditions (sunlight and darkness).

The skin of the animals was also analyzed in order to determine the capacity of these metabolites to accumulate in it. Fig. 5 shows the chromatograms obtained for skin samples from animals treated with AQs in darkness and under radiation together with the control chromatogram and compared with fraction A. Their analysis allows the conclusion that **1** and **2** accumulate at the skin level. In addition, HPLC determination showed the amount of AQs accumulated on 200 mg skin under either set of conditions, as well as the corresponding percentages of recovery (amount of AQ present on the skin as compared with the initially administered amount) (Table 2). Data are expressed as means ± standard deviation (SD). The *t*-test was used to determinate the degree of statistical difference between animals kept in darkness and those exposed to light, after treatment with AQ. Differences between means were considered significant at $p < 0.01$.

4. Discussions

Our previous studies had demonstrated that it is characteristic of the AQs studied in the present work to be effective photosensitizing agents *in vitro* (Núñez Montoya et al., 2005; Comini et al., 2007). However, it had not been ascertained that this characteristic would be manifest *in vivo*, where metabolic and pharmacokinetic processes occur that might affect both the chemical structures of the AQs and their availability to produce a toxic effect.

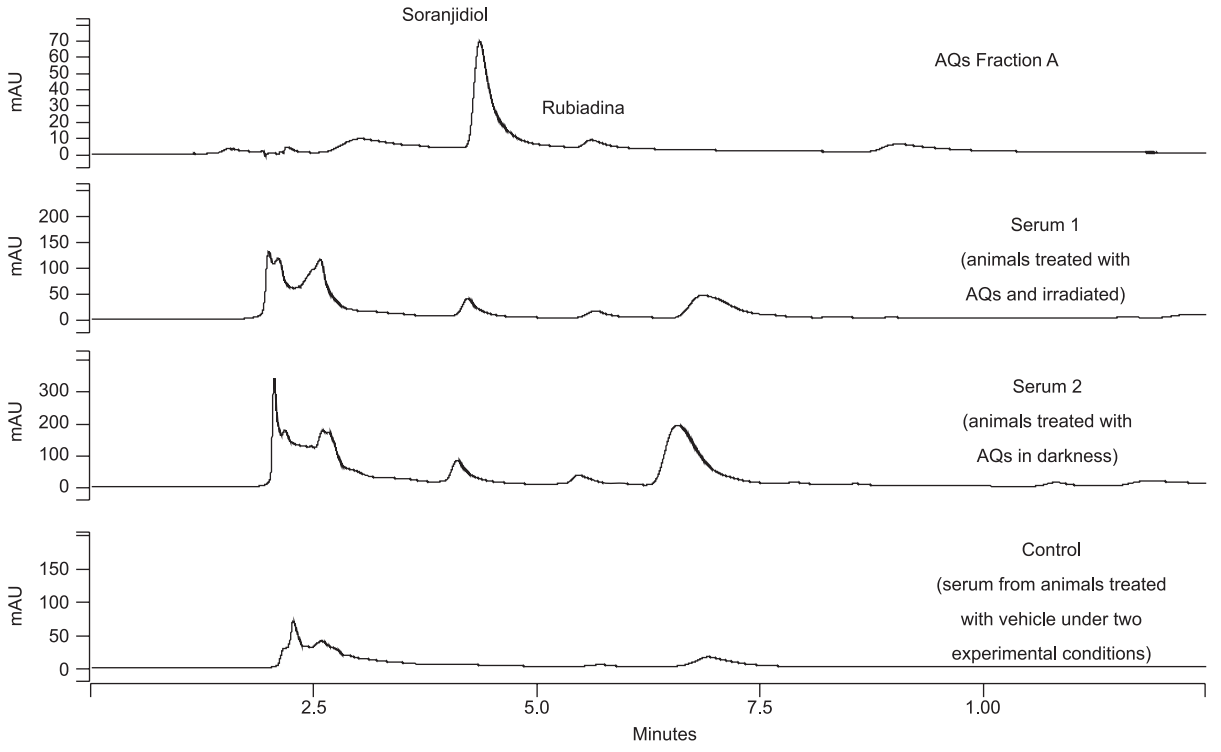


Fig. 4. Anthraquinones identification in serum samples from animals treated with fraction A under two experimental conditions (sunlight and darkness).

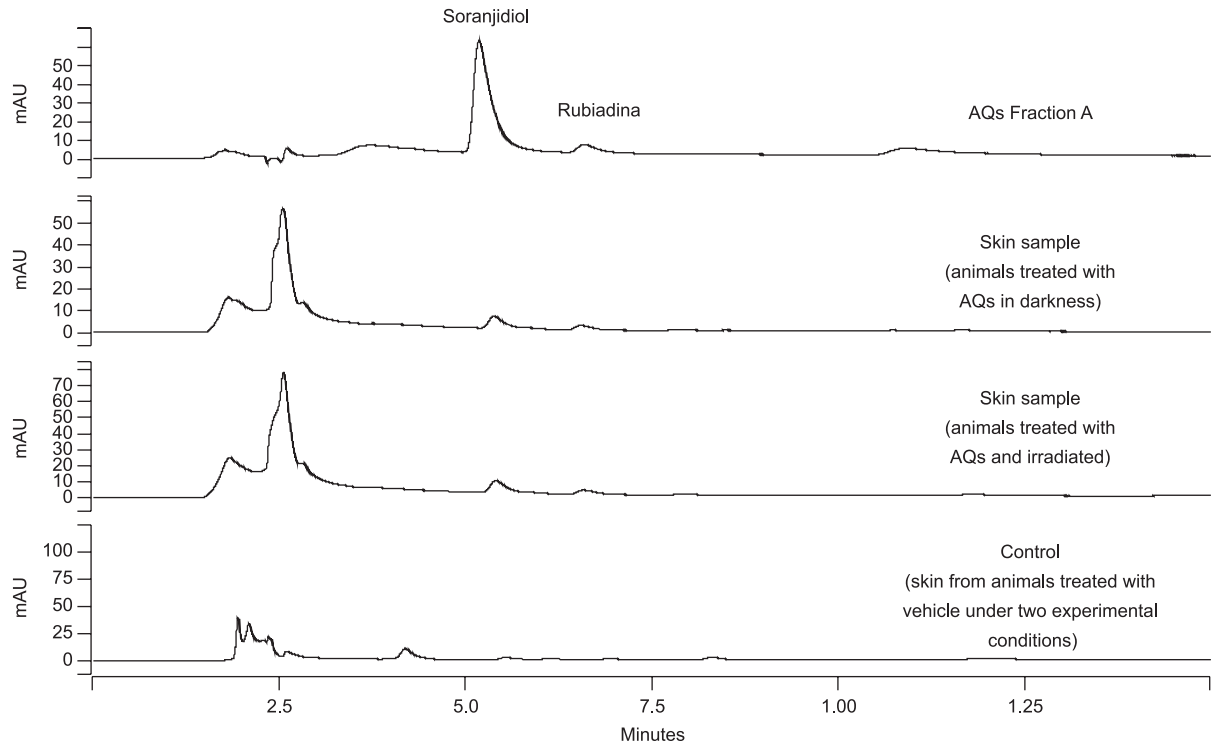


Fig. 5. Anthraquinones identification in skin samples from animals treated with fraction A under two experimental conditions (sunlight and darkness).

Table 2
Anthraquinone quantification in skin samples from animals treated with fraction A under two experimental conditions (sunlight and darkness), including percentages of AQs recovery in skin from the initially administered amount

AQs	Darkness[a]		Radiation[b]	
	Amount of AQs in 200 mg of skin (μg)	Recovery %	Amount of AQs in 200 mg of skin (μg)	Recovery %
Soranjidiol	1.2 ± 0.1*	0.0059 ± 0.0003*	1.8 ± 0.1	0.0085 ± 0.0005
Rubiadin	0.42 ± 0.02*	0.0062 ± 0.0002*	0.47 ± 0.02	0.0069 ± 0.0001

[a] Animals treated with AQs in darkness, $n = 3$.
[b] Animals treated with AQs and radiated, $n = 3$.
* $p < 0.01$ with respect to radiated animals.

It was observed that, as a result of the oral administration of **1** and **2** to experimental animals, these AQs were found after 160 min, not only at the systemic level but also in the cutaneous tissue, as evidenced by the chromatographic HPLC analyses shown in Figs. 4 and 5. It is interesting to note in these results that the recovery percentage of each AQ at skin level in radiated animals was higher than the recovery percentage obtained for animals kept in darkness (Table 2). This difference could be due to the increase in the animals' body heat when kept under sunlight over a lengthy period, which favors capillary dilation and blood irrigation in the skin, thus allowing for easier access and accumulation of AQs into the cutaneous tissue. This could also explain the behavior of those animals which, being in their habitat, looked for cover from sunlight, since not only radiation but also the amount of photosensitizing agent would decrease at the skin level. The presence of these photosensitizing agents in the cutaneous tissue, combined with exposure to light, brings about the photodynamic processes causing the toxic effects already described. This was corroborated by the results obtained in the subcutaneous assay *in vivo*, where the AQs fraction was injected using the same concentration in both sets of experimental conditions (sunlight and darkness).

Another factor to be considered in order to understand the toxicity of *H. pustulata* is related to the biosynthetic way in which it produces its AQs. It is well known that natural AQs are generally found in plants as glycosides (Sendelbach, 1989), but in the case of *H. pustulata* all of its AQs are aglycones, and are characterized not only for being photosensitizing but also for having high partition coefficients (Comini et al., 2006). This high degree of lipophilicity favors absorption at the small intestine, thus

entering a metabolic process that allows it to be transported by systemic circulation and accumulated in the cutaneous tissue. Glycosylated AQs, on the contrary, follow a different metabolic pathway, since their final destination is the large intestine (colon), where they are excreted (Sendelbach, 1989).

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