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Studies on the photostability and phototoxicity of aloe-emodin, emodin and rhein

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Aloe-emodin (**1**), emodin (**2**) and rhein (**3**) were found to be photolabile by visible (390–500 nm) light under aerobic conditions. The drugs **1**, **2** and **3** were phototoxic *in vitro* when examined by the photohemolysis test under both oxygen and argon atmospheres, although the photohemolysis rate was markedly lower under anaerobic conditions. The experiments were also carried out in the presence of butylated hydroxyanisole (BHA), reduced glutathione (GSH), sodium azide (NaN_3) and superoxide dismutase (SOD). Based on the inhibition of this process on addition of BHA, GSH, SOD and NaN_3, there would seem to be involvement of free radicals (type I mechanism) and singlet oxygen in the process (type II mechanism). The *in vitro* phototoxicity of this anthraquinone series was also verified in a lipid-photoperoxidation test with linoleic acid. In summary, this anthraquinone series is phototoxic *in vitro*. This behavior can be explained through the involvement of singlet oxygen and stable photoproducts.

1. Introduction

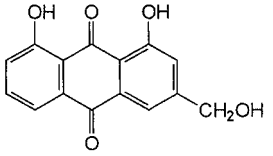
Anthraquinones and dianthrones (1,8-dihydroxyanthraquinones, DHAs) are present in several plants mainly as glycosides. Humans usually ingest DHAs as non-prescription drugs, and once DHAs arrive at the intestine the sugar moiety is removed by intestinal bacteria giving DHA-aglycons, which have strong laxative effects [1]. Although, anthrones are supposed to be the active drugs, they are probably oxidized to anthraquinones [2]. Despite this beneficial use, the chronic ingestion of laxatives has some adverse effects, such as development of colorectal cancer [3]. Nonetheless, these compounds have both therapeutic and cytotoxic properties related to different types of human diseases. For example, aloe-emodin (1,8-dihydroxy-3-hydroxy-methylanthraquinone, **1**) is a compound present in *Aloe vera* leaves, which exhibits antifungal [4], mutagenic [5], and tumorigenic properties [6], while in a recent report aloe-emodin was postulated as a new lead antitumor drug [7]; emodin (1,3,8-trihydroxy-6-methylanthraquinone, **2**) possesses anticancer, diuretic, antibacterial, vasorelaxant [8] and anti-inflammatory effects [9], and rhein (1,8-dihydroxyanthraquinone-3-carboxilic acid, **3**) is the active metabolite of diacerein, a drug for the treatment of patients with osteoarthritis. Diacerein is entirely converted into rhein before reaching the systemic circulation [10]. However, this drug has been considered as a possible culprit in a case of toxic epidermal necrosis [11].

On the other hand, some anthraquinones, both synthetic and of vegetable origin, have been shown to be able to produce highly cytotoxic free radicals and singlet oxygen, when they are irradiated with visible light [12]. Specifically, indications of phototoxicity have been detected *in vitro* in chinese hamster V79 cells irradiated with UVA and UVB light in the presence of emodin [13]. These findings suggest, in general terms, that the irradiation of DHAs can lead to the production of reactive oxygen species able to produce toxic effects on various cellular systems. Also, some *in vivo* evidence exists that confirms the importance of the *in vitro* discoveries. For example, two cases of photodermatitis have been reported: the first [14], caused by anthraquinone (9,10-anthracenedione) and the other by a preparation from *Aloe vera* [15].

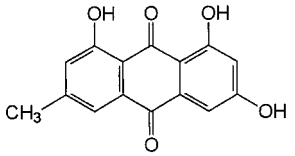
However, it ought to be emphasized that in some circumstances the phototoxic effects of DHAs can have beneficial effects. For example, some bis(aminoalkyl)-anthraquinones act as photosensitizers able to generate singlet oxygen [16, 17] in a way that can be used in the treatment known as photodynamic therapy. By means of this treatment, tumor cells can be destroyed by the action of the drugs. Additionally, using photosensitizing reactions, it has been possible to inactivate viruses and bacteria in platelet concentrates as well as in whole blood in order to use them safely in transfusions. In that sense, the anthraquinoid pigment hypericin has been used as a photosterilizing agent in human erythrocytes [18].

Recent photochemical studies have shown that emodic acid may be potentially phototoxic through photogeneration of reactive oxygen species or oxidation of nucleic acids and amino acids by the excited triplet state species [19]. Phototoxicity due to occasional or therapeutic contact with chemicals is attracting clinical and research interest because of its increasing relevance. Progress in this field has probably been hindered by its intrinsic complexity, the need for an interdisciplinary approach, and the lack of reliable *in vitro* tests for assaying the photosensitizing capability of xenobiotics.

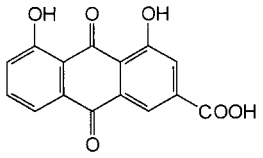
In this work we aim to study the effects of UV-A and visible irradiation on the structurally related DHAs aloe-emodin, emodin and rhein in the presence of cellular and acellular systems through the photohemolysis test, the acetylcholinesterase activity of human erythrocytes, and photoinduced lipid peroxidation, as these are systems sensitive to reactive oxygen species and radicals generated by those treatments.



Aloe-emodin



Emodin



Rhein

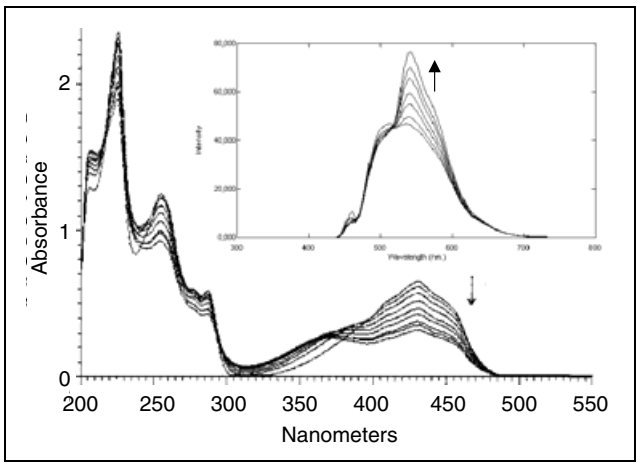


Fig. 1: UV-Vis and emission spectra monitoring of the photolysis of aloe-emodin (**1**) (10^{-4} M in ethanol) under aerobic conditions at regular intervals of 20 min of irradiation

2. Investigations and results

2.1. Photostability of anthraquinones 1–3

There is sufficient evidence that ethanolic solutions of **1**–**3** are photolabile under aerobic conditions by irradiation with visible light (340−500 nm). The photolysis of drugs **1**–**3** was carried out under these conditions and was followed by monitoring the changes of their absorption and emission bands. The course of the photolysis reactions for ethanolic solutions (1×10^{-4} M) of **1**–**3** are shown in Figures **1**–**3**. A gradual decrease and transformation of the original UV spectra and increase of fluorescence in the emission spectra of these compounds were observed during the irradiation.

In general, no net isobestic points were observed for compounds **1** and **2**, which indicated formation of complex reaction mixtures. From the fluorescence spectra it is apparant that the photoproducts of the three anthraquinones studied have a higher emission than the parent compounds, principally due to aloe-emodin photoproducts.

From the fluorescence spectra of aloe-emodin (**1**) with the maxima wavelength $\lambda_{exc} = 451$ nm and $\lambda_{emis} = 537$ nm we could determine the fluorescence quantum yield $\Phi_{fl} = 0.16$.

From the fluorescence spectra of emodin (**2**) with the maxima wavelength $\lambda_{exc} = 465$ nm and $\lambda_{emis} = 535$ nm we could determine the fluorescence quantum yield $\Phi_{fl} = 0.14$.

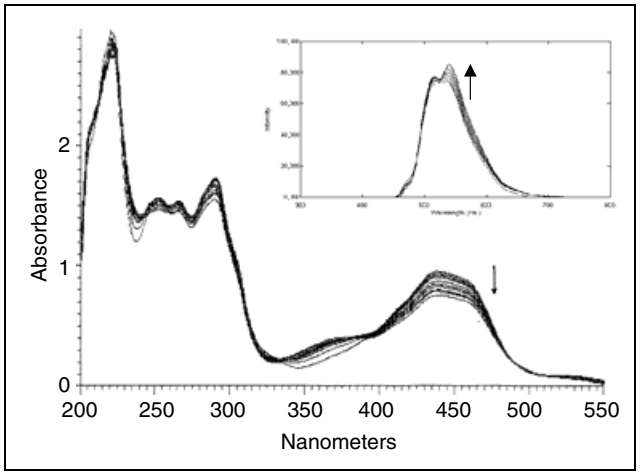


Fig. 2: UV-Vis monitoring of emodin photolysis (**2**) (10^{-4} M in ethanol) under aerobic conditions at regular intervals of 20 min of irradiation

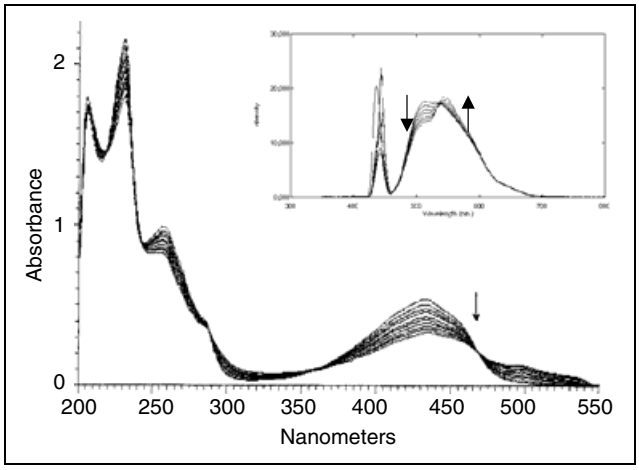


Fig. 3: UV-Vis monitoring of rhein photolysis (**3**) (10^{-4} M in ethanol) under aerobic conditions at regular intervals of 20 min of irradiation

On the other hand, compound **3** showed some defined points in its photodegradation pattern.

From the fluorescence spectra of rhein (**3**) with the maxima wavelength $\lambda_{exc} = 437$ nm and $\lambda_{emis} = 513$ nm we could determine the fluorescence quantum yield $\Phi_{fl} = 0.04$.

From these studies we can conclude that the photolability of the ethanolic solutions of aloe-emodin and emodin is higher than for rhein under visible light and aerobic conditions. Fig. 4 shows a comparison of the photodegradation rates of the three anthraquinones studied.

2.2. Phototoxic effects of anthraquinones 1–3

Red blood cell lysis was first studied. Aloe-emodin and emodin were able to induce photohemolysis in human erythrocytes (Fig. 5). Compound **1** produced massive cell lysis (>50%) within 70 min after irradiation of the erythrocyte suspension, while compound **2** produced photohemolysis (>40%) after 100 min. The *in vitro* studies of photohemolysis are of obvious significance as a model for the study of diseases involving photoreactive processes. A probable mechanism of photohemolysis, based on the intervention of reactive oxygen species photogenerated from aleo-emodin and emodin (type II mechanism) is confirmed by the efficient inhibition of the process in the presence of sodium azide (singlet oxygen quencher) (Figs. 6, 7). Lysis occured neither in the controls in the dark nor with rhein (**3**).

On the other hand, GSH (a well-established free radical scavenger) and SOD showed a very low protective effect

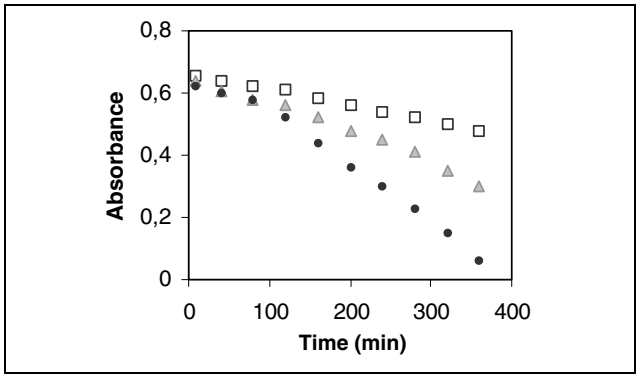


Fig. 4: Comparative photodegradation rates of the anthraquinones **1**–**3** ($\lambda = 430$ nm). □ Rhein, △ Emodin, ● Aloe-emodin

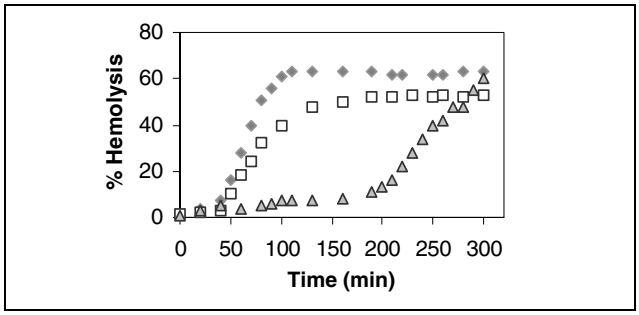


Fig. 5: RBC photohemolysis induced by aloe-emodin, emodin and rhein at 0.5×10^{-4} M. ($[\mathbf{RBC}] = 3.3 \times 10^6$ cells $\cdot$ ml^{-1}), ($\lambda = 650$ nm). Each point represents the mean $\pm$S.E.M. derived from 3 observations, SEM was always less than 8%. ◆ Aloe-emodin, □ Emodin, ▲ Rhein

on the photohemolytic process. Figs. 6 and 7 show the relative inhibition of photohemolysis by the addition of specific antioxidants.

Measures of the photohemolytic effect of **1** were carried out *in vitro* in the presence of serum proteins. A similar photohemolytic effect was observed but approx 20% less (data not shown).

Pre-irradiated solutions of aloe-emodin, emodin and rhein were assayed in the same range of concentration. Neither emodin nor rhein solutions had significant photohemolytic

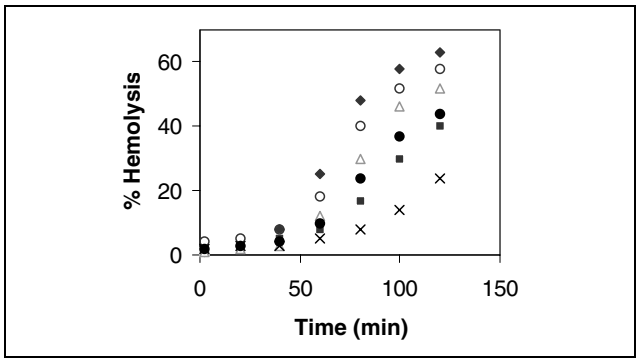


Fig. 6: RBC photohemolysis induced by aloe-emodin at 0.5×10^{-4} M [**1**], 0.5×10^{-5} M, also in the presence of GSH, BHA, NaN_3 and SOD 0.5×10^{-4} M respectively. ($[\mathbf{RBC}] = 3.3 \times 10^6$ cells $\cdot$ ml^{-1}), ($\lambda =$ 650 nm). Each point represents the mean $\pm$S.E.M. derived from 3 observations, SEM was always less than 8%. ◆ 0.5×10^{-4} M [1], ■ 0.5×10^{-5} M, ○ [1] + SOD, △ [1] + GSH, ● [1] + BHA, × [1] + NaN_3

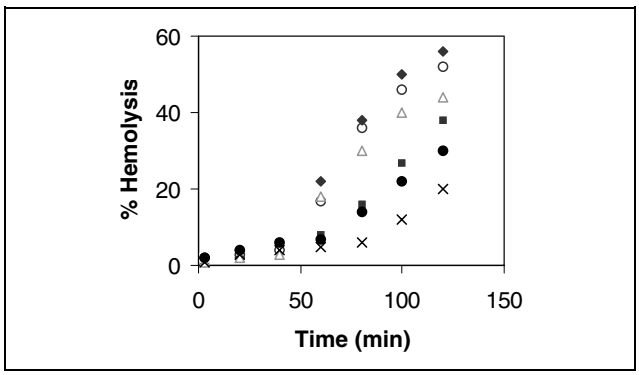


Fig. 7: RBC photohemolysis induced by Emodin at 0.5×10^{-4} M [**2**], 0.5×10^{-5} M, also in the presence of GSH, BHA, NaN_3 and SOD 0.5×10^{-4} M respectively. ($[\mathbf{RBC}] = 3.3 \times 10^6$ cells $\cdot$ ml^{-1}), ($\lambda =$ 650 nm). Each point represents the mean $\pm$S.E.M. derived from 3 observations, SEM was always less than 8%. ◆ 0.5×10^{-4} M [2], ■ 0.5×10^{-5} M, ○ [2] + SOD, △ [2] + GSH, ● [2] + BHA, × [2] + NaN_3

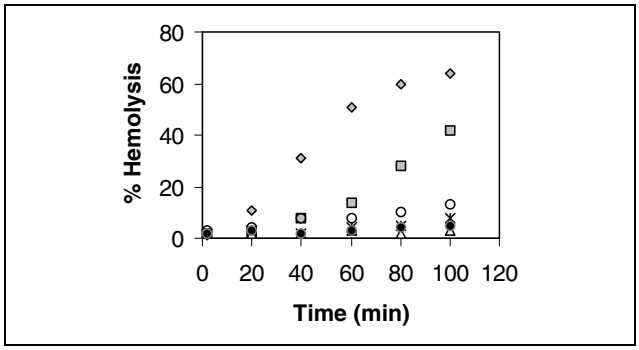


Fig. 8: RBC photohemolysis induced by pre-irradiated solutions of aloe-emodin (P1), emodin (P2) and Rhein (P3) at 0.5×10^{-4} M. ($[\mathbf{RBC}] = 3.3 \times 10^6$ cells $\cdot$ ml^{-1}), ($\lambda = 650$ nm). Each point represents the mean $\pm$S.E.M. derived from 3 observations, SEM was always less than 8%. ◇ P1, □ P2, △ P3, ○ P1 (darkness), ✳ P2 (darkness), ● P3 (darkness)

effects on erythrocytes. Only the photoproducts of aloe-emodin showed a phototoxic effect in this test. (Fig. 8). These solutions were not toxic in the dark. It is important to emphasize the phototoxicity of photoproducts of compounds **1** and **2** on erythrocytes. These products are responsible for 60% hemolysis during the 80 min that follow after RBC irradiation in a 0.5×10^{-4} M pre-irradiated solution of compound **1** and 30% hemolysis with compound **2**.

When photohemolysis tests were carried out under argon atmosphere, a marked decrease of the photoactivity induced by **1** and **2** was observed. The photohemolysis values obtained were approximately 54% lower than those obtained in aerobic conditions.

Due to the detrimental effects of photoperoxidation to cell membranes, this process is thought to play an important role in skin phototoxicity. The photohemolysis observed to be induced by compounds **1** and **2** might reflect extensive photoperoxidation of the membrane lipids (Fig. 9). When PBS solutions of linoleic acid were irradiated in the presence of aloe-emodin (**1**) and emodin (**2**) significant amounts of dienic hydroperoxides were formed. They were confirmed spectrophotometrically by the appearance of a new UV-absorption band at 233 nm [20].

It should be pointed out that photoinduced lipid peroxidation experiments *in vitro* have an obvious significance as a model for the study of diseases involving photoreactive processes. This suggests a probable mechanism, based on a radical chain process caused by radical species and singlet oxygen photogenerated from compounds **1** and **2** (type I and II mechanism) [21], and in fact, this is confirmed by the efficient inhibition of the process in presence of BHA, GSH and NaN_3 which are well-established

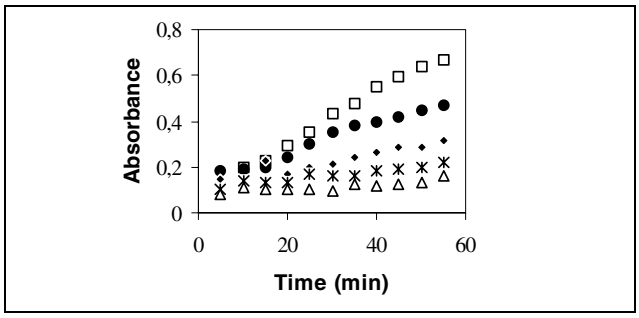


Fig. 9: Photoperoxidation of linoleic acid (10^{-3} M) sensitized by aloe-emodin (**1**), emodin (**2**) and rhein (**3**). □ Linoleic acid/hv + 1, ● Linoleic acid/hv + 2, ◆ Linoleic acid/hv + 3, ✳ Linoleic acid/hv, △ Linoleic acid (dark)

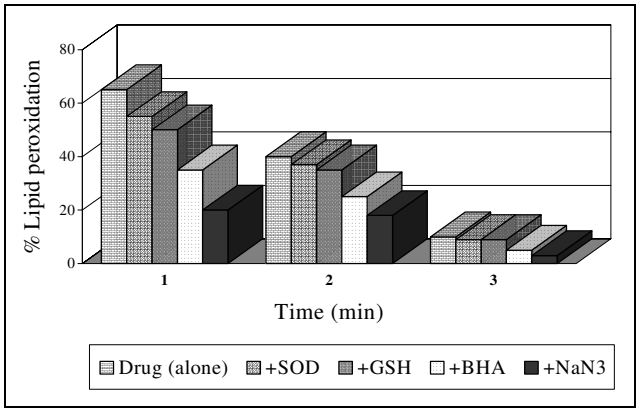


Fig. 10: Photoperoxidation of linoleic acid (10^{-3} M) sensitized by aloe-emodin (**1**), emodin (**2**) and Rhein (**3**) in presence of SOD, GSH, BHA and NaN_3 1.0×10^{-5} M respectively

as free radical scavengers. On the other hand, SOD (superoxide scavenger) showed a lower protective effect in this process. Fig. 10 shows the relative inhibition of photo-induced lipid peroxidation by the addition of specific anti-oxidants.

When the photohemolysis and lipid peroxidation tests were carried out under argon atmosphere, a decrease in the photoactivity induced by **1**–**3** was observed. The values obtained were approximately 34% and 36% lower for photohemolysis and for lipid peroxidation, respectively, than those obtained in aerobic conditions.

The effects on the acetylcholinesterase (ACE) activity of human erythrocyte membranes were also studied. This activity was inhibited when the membrane preparation was irradiated with visible light in presence of the anthraquinones **1**–**3** at a 5×10^{-6} M concentration (Fig. 11).

Our results indicated that emodin, aloe-emodin and rhein could inhibit this activity up to 75%, 50% and 8% with respect to the control, respectively. In other experiments, suspensions of fragmented membranes were incubated for 10 min at 20 °C with different reactive oxygen scavengers and thereafter irradiated in the presence of emodin or aloe-emodin (Table).

In these experiments, we could not use DL-cysteine or GSH because ACE activity was measured by the Ellman method which depends on thiol formation from acetylthiocholine rupture. Nonetheless, because of the protecting effects of both BHA, a potent chain-breaking antioxidant, and DABCO, a singlet oxygen quencher, our data suggest that radical species and singlet oxygen production and subsequent peroxyl radical formation are involved in the photoinhibitory effects of both DHAs on ACE activity.

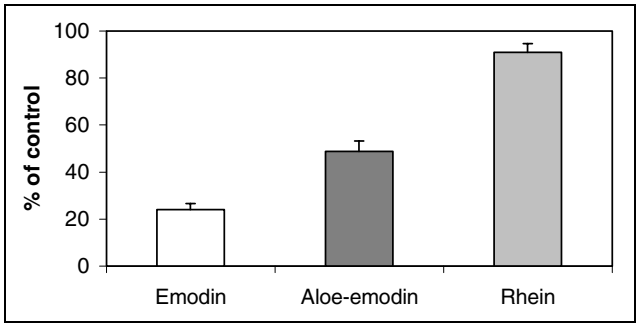


Fig. 11: Red cell acetylcholinesterase activity inhibition photosensitized by 5 μM anthraquinones **1**–**3**. Data are calculated as percentage of irradiated fragmented membranes in absence of quinones. In the darkness, acetylcholinesterase activity was not affected by anthraquinones **1**–**3**. Values represent the mean ± SEM of triplicates

Table: ACE recovery activity by reactive oxygen scavengers in fragmented membranes of human erythrocytes irradiated in presence of emodin and aloe-emodin

Scavenger	% of recovery (Emodin)	% of recovery (Aloe-emodin)
BHA 1 mM	34.6 ± 10*	33.1 ± 5.4*
Sodium azide 1 mM	4.4 ± 2.0 (n.s.)	21.0 ± 4.8*
DABCO 1 mM	14.5 ± 5.1*	21.9 ± 3.8*
SOD 0.01 mg/ml	4.6 ± 2.4 (n.s.)	3.8 ± 3.0 (n.s.)

Data are expressed as means ± S.E.M. of the difference of ACE activity in the presence of scavenger minus those in its absence (N = 3 assays). Statistical significance was assessed by Student's t-test for unpaired data. n.s. = non significant.
* Statistically significant ($P < 0.05$)

3. Discussion

In this paper we report some photochemical and phototoxic properties of the 1,8-dihydroxyanthraquinones aloe emodin (**1**), emodin (**2**) and rhein (**3**). UV-monitoring compounds **1**–**3** irradiated in diluted ethanol solution allowed their photolability under aerobic conditions to be established. These solutions of aloe-emodin (**1**) and emodin (**2**) are photolabile under visible light and aerobic conditions, while the photolability of rhein (**3**) is lower under the same conditions. Although a previous study showed that the related compounds danthron (1,8-dihydroxyanthraquinone) and chrysophanol (1,8-dihydroxy-3-methylanthraquinone) can generate superoxide anion when illuminated with broad spectrum light in the presence of dissolved oxygen, such activity required the presence of an added reducing agent, suggesting a photomediated redox cycling mechanism. On the contrary, **2** inhibited this activity under the same conditions [22]. More recently, although **2** showed a potent *in vitro* phototoxic activity in chinese hamster V79 cells [13], the identity of the radical species involved was not investigated.

The main finding of this study is to demonstrate that compounds **1** and **2** are strong photosensitizing agents under aerobic conditions, especially in the ACE assay. For example, under similar conditions of irradiation, fragmented RBC membranes treated for 30 min with 5×10^{-6} M emodin caused a ~ 75% decrease in the ACE activity, whereas it was necessary to irradiate for 90 min with 5×10^{-5} M emodin to provoke ~ 40% of hemolysis. Indeed, it has been shown previously that irradiation (near ultraviolet light) of red cells containing high protoporphyrin levels leads to a decrease in ACE activity, and loss in activity precedes hemolysis [23].

In our assays, we demonstrated that emodin and aloe-emodin photosensitized linoleic acid peroxidation. In addition, the chain-breaking antioxidant BHA and the singlet oxygen quencher sodium azide could partially recover both ACE activity and erythrocyte hemolysis, suggesting that the two drugs in the lipid phase may photoreact by type I and II mechanisms. Since ACE is inactivated by singlet oxygen and there is also evidence that suggests that ACE is relatively insensitive to other reactive intermediates that may be generated in photosensitization reactions [24], it is possible that singlet oxygen production and subsequent peroxyl radical formation mediates the photoinhibitory effects of emodin and aloe-emodin on ACE activity.

Because protein kinase C (PKC) activation can facilitate the formation of tumors [25], the photosensitizing ability of anthraquinones such as emodin may have important implications in pharmacology, because these substances, as well as related antiretroviral anthraquinoid compounds, have been reported as rat brain PKC inhibitors [26], and

this effect is increased by exposure to UV-visible light. In this respect, it has been suggested that PKC photoinhibition by these compounds includes: binding of quinones to the PKC regulatory domains; photobinding between −SH groups of PKC cysteine residues and the quinone moiety and quinone-sensitized photodamage of PKC via type I and/or type II photosensitization [27]. Interestingly, emodin is a selective inhibitor of protein kinase CK2 (CK2 activity is always abnormally high in solid tumors [28]), because of its penetration capacity inside the active site of the enzyme, partially overlapping the position occupied by ATP [29]. This competitive binding to the ATP binding site appears to be the mechanism of action of emodin on several Ser/Thr protein kinases including PKC [30]. Following these ideas, we are at present evaluating the effects of UV-visible irradiation on inhibition by anthraquinones of the respiratory burst of human neutrophils subsequently activated by various stimuli, including the phorbol ester PMA (phorbol myristate acetate), a potent activator of PKC.

Our investigation on red blood cells shows that rhein and its photoproducts are not phototoxic. The phototoxicity mechanism for aloe-emodin and emodin most probably involves reactions of radical species, reactive oxygen intermediates, and stable photoproducts of **1** and **2** with cellular components. Further studies are required in order to gain a deeper understanding of the phototoxicity of these anthraquinones. These would include the development of irradiation devices allowing discrimination between effects due to stable photoproducts and short-lived intermediates, and also, the use of cultures and co-cultures of various human cells mimicking the human skin. We are currently investigating the toxic effects of the isolated photoproducts (also their isolation and spectroscopic identification) using *in vitro* as well as *in vivo* models together with the efficiencies of production of reactive intermediates such as the superoxide ion and singlet oxygen.

4. Experimental

4.1. Chemicals

Aloe-emodin (**1**), emodin (**2**) and rhein (**3**) were provided by Sigma (St. Louis, MO, USA). The purity was 99.2% as determined by ^{1}H NMR-spectroscopy (Bruker Aspect 3000, 300 MHz) and UV-vis spectrometry (Milton-Roy 3000). Reduced glutathione (GSH), butylated hydroxyanisole (BHA), dipyridamole, acetylthiocholine iodide, dithiobisnitrobenzoic acid and superoxide dismutase (SOD) were also purchased from Sigma, while sodium azide (NaN_3) and 1,4-diazabicyclo [2.2.2] octane (DABCO) from Aldrich. All analytical or HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

4.2. Photolysis

Drugs **1**–**3** (1×10^{-4} M) were irradiated under an oxygen atmosphere at room temperature, in methanol, in a Rayonet photochemical reactor which is equipped with 16 phosphor lamps with an emission range between 340–500 nm with a total irradiance of 18 mW/cm^2 as measured with a model of UVX Digital Radiometer after 1 h continuous illumination. The distance between the light source and the test aliquots was 10 cm. The temperatures detected in the cuvette during a standard 1 h irradiation were no higher than 27 °C. A parallel experiment was carried out under argon atmosphere (data not shown). The course of the photodegradation was followed by UV-Vis spectrophotometry using a Milton-Roy 3000 instrument and also by TLC until the drugs were completely consumed. The fluorescence spectra were registered with a Shimadzu RF 1501 spectrofluorophotometer.

4.3. Photohemolysis experiments

A red blood cell (RBC) suspension from freshly obtained human erythrocytes was prepared by washing them four times with tenfold volume of a phosphate-buffered saline solution (PBS) pH 7.4 (0.01 M phosphate buffer and 0.135 M NaCl), centrifuging the cells each time at 2500 g for 15 min and carefully removing the supernatant. For the hemolysis experiments, RBC were diluted in PBS containing the individual compounds **1**–**3** so that the resultant suspensions had an optical density (OD) of 0.4–0.8 at 650 nm. An OD value of 0.5 corresponded to 3.3×10^6 cell ml^{-1}. This was read on a Milton-Roy 3000 spectrophotometer.

The hemolysis rate and the hemolysis percentage were determined by measuring the decrease in OD at 650 nm, since the optical density is proportional to the number of intact RBC [31]. The individual compounds **1**–**3** were dissolved in the RBC solution. The samples containing the drugs at concentrations of 20–80 μg ml^{-1} were irradiated under aerobic conditions in a Rayonet photochemical reactor equipped with 16 phosphor lamps with an emission range between 340–500 nm or alternatively with an Osram HQL 250 Watt medium pressure Hg lamp in a Pyrex immersion-well photoreactor (radiation dose of 4.5 J/cm^2), for periods ranging between 10–200 min. Photohemolysis effects were studied. Similar experiments were performed with solutions of **1**–**3** in the presence of the radical trapping GSH and BHA, the singlet oxygen quenching NaN_3, and also SOD (a superoxide quenching). To determine the phototoxicity of the photoproducts, pre-irradiated ethanolic solutions of compounds **1**, **2** and **3** were also evaluated by this test. All the experiments were repeated three times, and the averages (arithmetic mean) of the results are reported.

4.4. Photosensitized peroxidation of linoleic acid

Linoleic acid 10^{-3} M in PBS was irradiated in the presence of compounds **1**–**3** and a pre-irradiated solution of these compounds (10^{-4} M), and the formation of dienic hydroperoxides was monitored by UV-spectrophotometry, through the appearance and progressive increase of a new band at $\lambda = 233$ nm [32, 33]. This test was also repeated in the presence of reduced gluthatione (GSH) and butylated hydroxyanisole (BHA) as radical scavengers (10^{-5} M), sodium azide (NaN_3) as a singlet oxygen quencher and superoxide dismutase (SOD) as an oxygen superoxide scavenger.

4.5. Preparation of fragmented red blood cell membranes for enzymatic assay

Blood was obtained from hematologically normal volunteers. The red cells were washed four times in 4 volumes of cool isotonic solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.6). The cells were lysed with vigorous stirring in 8 volumes of wash solution (20 mM sucrose, 10 mM NaCl, 0.005 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5) and kept on ice for at least 40 min to ensure complete lysis. The hemolysate was centrifuged at 4 °C for 15 min at $30000 \times g$ and the pellet containing the fragments was washed five times with 20 volumes of a medium, which contained 2 mM sucrose, 1 mM NaCl, 1 mM KCl, 0.005 mM $CaCl_2$, 2 mM Tris-HCl, (pH 7.7) and 50 μM β-mercaptoethanol. The fragments were finally suspended at a protein concentration of 11 μg/ml and kept at −70 °C until use.

4.5.1. Enzymatic assay

Acetylcholinesterase activity was measured in triplicate immediately following irradiation, by the colorimetric assay of Ellman et al. In this assay the enzymatic cleavage of the thiol group from acetylthiocholine is monitored spectrophotometrically at 412 nm following its reaction with dithiobisnitrobenzoic acid (DTNB) [34]. The reaction mixture contained 2990 μl of 0.01 M Na-phosphate-buffered saline (PBS) pH 8, 10 μl of 0.075 M acetylthiocholine iodide and 100 μl of 0.01M DTNB. The reaction was started by adding 50 μl of treated enzyme preparation. The changes in absorbance at 412 nm were followed for at least 6 min.

4.5.2. Irradiation conditions

The sample was diluted 1/50 to obtain a final protein concentration of 0.22 μg/ml. One aliquot of fragments was pre-incubated in the presence of different concentrations of quinones (emodin, aloe emodin and rhein) for 10 min in the dark. Later, these mixtures were illuminated with continuous stirring for 30 min under aerobic conditions at room temperature. The light source used was a slide projector with a 300 W lamp (EXR, AV/Photolamp, Wiko, Japan). The UVA-irradiance was 400 μW/cm^2 as measured by a photometer (Blak-Ray longwave meter, model J−221) (50000 lux as measured by a Broad Range Lux/FC meter, Sper Scientific). Control experiments were carried out in darkness. These experiments were repeated in the presence of several protectors of reactive oxygen species, such as 1.4 diazabicyclooctane (DABCO, 1 mM), sodium azide (NaN_3, 1 mM) and superoxide dismutase (SOD, 0.01 mg/ml), and the radical trapping butylated hydroxyanisol (BHA, 1 mM).

4.5.3. Statistical treatment of results

At least three independent experiments were performed except where indicated. Results are presented as the mean value ± SEM, n = 3. Statistical analyses were performed using the *t*-test and one-way ANOVA. A probability value of $< .05$ was considered significant.

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