

Photophysical, photochemical and photobiological properties of anthraquinones isolated from a phototoxic vegetal species: *Heterophyllaea pustulata*

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ABSTRACT

Studies undertaken on isolated photosensitizing agents from *Heterophyllaea pustulata*, a phototoxic plant, which natural habitat is the North-western part of Argentina, are presented here. The carried out work involved several steps that were initiated with the chemical-structural study of their most important secondary metabolites. As a result, three flavonoids, one iridoid glycoside (asperuloside) and ten aglycone anthraquinones were identified. Through the study of the photophysical, photochemical and photobiological properties of the anthraquinone derivatives, we concluded that they show good photosensitizing (type I and type II) characteristics. The performed toxicological assays *in vivo* showed a direct cause-effect relation between these metabolites and the reported phototoxicity for *H. pustulata*. Finally, a few words about the potential application of these photosensitizing compounds as antibacterial agents will be given.

KEYWORDS: photosensitizers, reactive oxygen species, anthraquinones, *Heterophyllaea pustulata*,

phototoxicity, photodynamic antimicrobial chemotherapy

INTRODUCTION

The interest in studying toxic plants has as main objective the knowledge of the chemical characteristics of the active principles as well as the determination of the mechanisms through which they produce these toxic effects. This knowledge is of fundamental importance to identify both, the symptomatology and the treatment, or preventive actions, which should be undertaken in order to avoid intoxications, irrespective of whether the target is a human or an animal.

Nevertheless, the metabolite having toxic effects should not be considered always deleterious since, many of them, when are administered in adequate doses, do show beneficial effects in therapeutic treatments. For this reason, many toxic plants are considered an important natural resource that provides bioactive substances, which in turn, form the basis of molecular models to improve the development of new medicaments.

In the particular case of phototoxic plants, the deleterious effect is triggered by light acting upon the photosensitizing agents, which chemical identification and photophysical, photochemical and photobiological properties must be known before any attempt to study their pharmacological activities.

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Photosensitizing agents are chemical compounds that show the capacity of absorbing a photon to reach an excited state, from which several different phenomena can occur to give place to a broad definition between photodynamic or not photodynamic photosensitizers, depending upon the requirement of oxygen (O₂) to carry out the reaction [1].

Nonphotodynamic photosensitizers act through one of the following mechanisms: a) bonding on DNA by a photoaddition process to obtain covalent photoadducts that interfere, both in replication and transcription; b) giving a photoproduct that alters the cell's membrane structure [1].

Photodynamic photosensitizers give rise to reactive oxygen species (ROS) such as hydroxyl radical (OH[•]) and superoxide anion radical (O₂^{•-}) through the so called Type I mechanism, and singlet oxygen (¹O₂) through the Type II mechanism [2]. ROS can react with several components of cellular membrane, producing different injuries that ultimately lead to cell death. Thus, the unsaturated fatty acids, chief components of the cellular membranes [3], suffer peroxydation that triggers a free radicals process, which ends with the membrane lysis. Cholesterol and certain amino acids, such as tryptophan and histidine, can be attacked by ROS, provoking a functional disruption of the membrane [4-6]. In addition, singlet oxygen is capable of reacting with DNA, facilitating the single-strand and double-strand breaks that also lead to cell death [7].

Although cellular damages can occur, it is important to note that photosensitizing agents, used in controlled doses, can produce pharmacologically directed effects as, for example, by acting against microorganisms or pathogen cells with minimum or null effect on the host cells. Used thus, they become an important therapeutic tool as antiviral, antibacterial, antifungal and/or anticarcinogenic agent.

A great variety of plant metabolites are photosensitizing agents. Among the structures known there are thiophens, coumarines, benzofurans, furanocoumarines and some alkaloids [8, 9]. Interest has been centered in anthraquinone derivatives (AQs), which are chemotaxonomic features in certain families of plants [10]. These

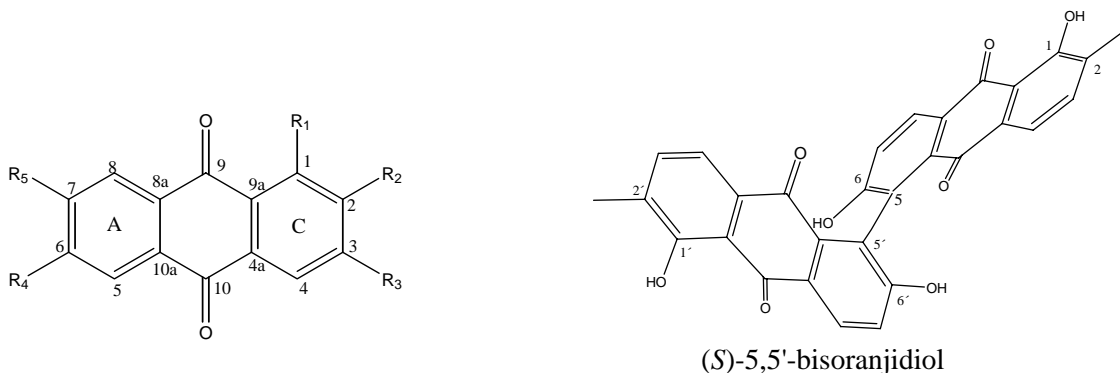
derivatives have been extensively studied with respect to their UV/Vis absorption characteristics and their photosensitizing properties in photodynamic reactions [11-13]. Particularly, amino-AQs, are used for the treatment of human neoplasia [14], whereas other photosensitizing AQs showed good activity as antibacterial and antiviral agents [15-17].

***Heterophyllaea pustulata* Hook. f. (Rubiaceae)**

H. pustulata 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 "cegedera" (blindness), which causes significant economic losses in the livestock of its habitat region [18]. Natural intoxication by "cegedera" has been reported for equine, bovine and goat cattle, causing dermatitis and loss of sight in severe cases, without confirmation of animals' death [19, 20]. Toxic effects were experimentally proved by Hansen and Martiarena (1967) [19], demonstrating that the ingestion of different parts of the plant at different vegetative development periods produces dermatitis and keratoconjunctivitis, especially in animals with white coats. These authors also established that the described effects are reversible if toxins have worked only in a short time 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.

Chemical components

The first chemical investigations of *H. pustulata* were performed by our researcher group [21, 22], where the presence of several chemical components (anthraquinones, flavonoids and iridoids) was established, with a significant predominance of aglycone-9,10-anthraquinones (AQs). Thus, from the aerial parts of *H. pustulata* three flavonoids (quercetin, isoquercitrin, quercetin-3-*O*- α -D-glucosyl-6"-acetate), an glycoside iridoid (asperuloside), ten aglycone AQs: soranjidiol, soranjidiol 1-methyl ether, rubiadin, rubiadin 1-methyl ether, damnacanthol, damnacanthol, 2-hydroxy-3-methyl anthraquinone, heterophylline, pustuline and 5,5'-bisoranjidiol (Fig. 1) were isolated. It is necessary to highlight

Anthraquinones

AQs	R ₁	R ₂	R ₃	R ₄	R ₅
soranjidiol	OH	CH ₃	H	OH	H
soranjidiol 1-methyl ether	OCH ₃	CH ₃	H	OH	H
rubiadin	OH	CH ₃	OH	H	H
rubiadin 1-methyl ether	OCH ₃	CH ₃	OH	H	H
damnacanthal	OCH ₃	CHO	OH	H	H
damnacanthol	OCH ₃	CH ₂ -OH	OH	H	H
2-hydroxy-3-methyl AQ	H	OH	CH ₃	H	H
heterophylline	OH	CH ₃	H	OH	OCH ₃
pustuline	H	OH	OCH ₃	H	CH ₃
nordamnacanthal	OH	CHO	OH	H	H

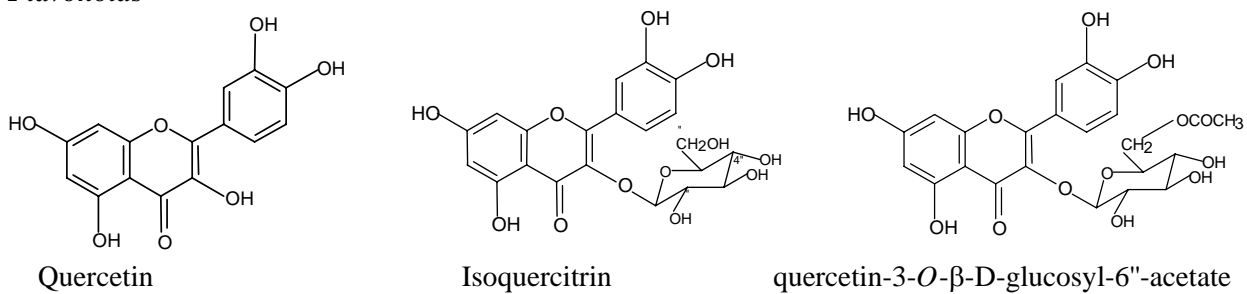
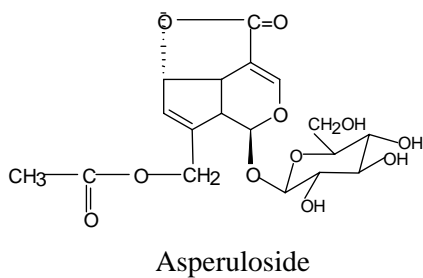
Flavonoids**Glycoside iridoid**

Fig. 1. Structures of anthraquinones, flavonoids and iridoid isolated from *Heterophyllaea pustulata*.

that the last three compounds were new chemical structures reported by us for this compound's family [22].

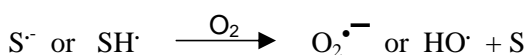
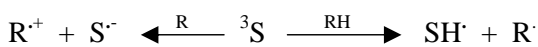
Isolation and purification of the compounds were done by repeated combination of several chromatographic techniques. The identification of each metabolite was made by means of the application of different spectroscopic/spectrometric techniques (UV-V, IR, $^1\text{H-RMN}$, $^{13}\text{C-RMN}$, HRMS) [21, 22].

Having in mind that the flavonoids and the iridoid isolated from *H. pustulata* have not been reported like photosensitizer, and quercetin derivatives are antioxidants [23, 24], we center our attention to study the photosensitizing properties of the AQs and their possible involvement in photooxidation reactions.

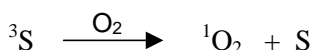
STUDY OF THE PHOTOCHEMICAL, PHOTOPHYSICAL AND PHOTOBIOLOGICAL PROPERTIES OF *H. PUSTULATA* ANTHRAQUINONES

ROS production

A photosensitizer (S) absorbs light to become a short-lived electronically excited species in a singlet state (^1S), which can be converted into its corresponding triplet state (^3S) by intersystem crossing. Two competing reactions of the long-lived triplet sensitizer (^3S) can occur in the presence of molecular oxygen to produce ROS [12]. On the one hand, ^3S may react with a reducing substrate (R or RH) by the transfer of a hydrogen atom or electron and the resulting sensitizer radical may then react with O_2 to produce $\text{O}_2^{\bullet-}$ or OH^\bullet (Type I mechanism) [9].



On the other hand, ^3S may transfer energy directly to O_2 to form the electronically excited singlet state of $^1\text{O}_2$ (Type II mechanism) [13].



In our laboratory the production of $\text{O}_2^{\bullet-}$ (Type I mechanism) was detected and quantified with an indirect bioassay that measures the reduction of

Nitroblue Tetrazolium (NBT) by $\text{O}_2^{\bullet-}$ generated within human leukocytes when an oxidizer (AQ) is present. The nitroblue diformazan (reduced NBT) produced is proportional to the $\text{O}_2^{\bullet-}$ generated and it was measured spectrophotometrically by the increase in absorption at 575 nm with regard to the basal situation (in absence of oxidizer) [25, 26]. This procedure was performed in darkness and under continuous UV radiation (290-400 nm or UVR) in order to evaluate the photosensitization of the different AQs. The solutions of AQs were prepared at selected concentrations so that a specified volume added in the NBT assay provided a final concentration of either 10 or 20 $\mu\text{g/mL}$. In the NBT assay, the leukocytes were independently incubated with each AQ solution and NBT at 37°C for 30 min in both working conditions (darkness and irradiation).

Results proved that at 10 $\mu\text{g/mL}$, soranjidiol, damnacanthal, damnacanthol, pustuline and 5,5'-bisoranjidiol increase $\text{O}_2^{\bullet-}$ production with respect to basal situation only when they were irradiated and damnacanthal stood out as the highest producer of $\text{O}_2^{\bullet-}$ (Table 1). Whereas rubiadin and heterophylline increase $\text{O}_2^{\bullet-}$ production with respect to basal situation in darkness, soranjidiol 1-methyl ether and rubiadin 1-methyl ether do not. Nevertheless, all four AQs at the 10 $\mu\text{g/mL}$ dose do not present a photosensitizing effect since $\text{O}_2^{\bullet-}$ production does not increase under irradiation (Table 1). Having in mind that the $\text{O}_2^{\bullet-}$ production is dose-dependent [27], we increased the concentration twofold (20 $\mu\text{g/mL}$) and measured an increase in $\text{O}_2^{\bullet-}$ production in darkness, which was further intensified by exposition to UVR (Table 1). According to the NBT results, the best producer of $\text{O}_2^{\bullet-}$ in leukocytes under UVR at 10 $\mu\text{g/mL}$ was damnacanthal followed by damnacanthol, pustuline, 5,5'-bisoranjidiol and soranjidiol. For those AQs whose dose was doubled, soranjidiol 1-methyl ether showed a better capacity to generate $\text{O}_2^{\bullet-}$ than rubiadin 1-methyl ether, heterophylline and rubiadin.

It is necessary to point out that the $\text{O}_2^{\bullet-}$ generation was mainly intracellular since the

Table 1. Increase in percentage of $O_2^{\bullet-}$ in leucocytes with respect to basal situation for every AQ. Assay of NBT reduction in darkness and under UV irradiation.

Concentrations	Compounds	Darkness	UVR
10 $\mu\text{g/mL}$	damnacanthal	0.0	79
	damnacanthol	0.0	58.6
	rubiadin	15.4	14.6
	rubiadin 1-methyl ether	0.0	0.0
	soranjidiol	0.0	6.8 ± 3.7
	soranjidiol 1-methyl ether	0.0	0.0
	heterophylline	27.9 ± 3.7	28.4 ± 3.0
	pustuline	0.0	32.7 ± 3.9
20 $\mu\text{g/mL}$	5,5'-bisoranjidiol	0.0	29.5 ± 2.9
	rubiadin	31.6	42.1
	rubiadin 1-methyl ether	71.4	81.4
	soranjidiol 1-methyl ether	50	161.4
	heterophylline	33.0 ± 5.8	45.7 ± 8.1

optical density value at 575 nm of the supernatant from the leucocytes, treated and not treated with AQs, was similar. Should the $O_2^{\bullet-}$ had been generated outside the cells, we should have noticed an increase in absorbance. Thus, it was necessary to break the leucocytes membrane with DMSO in order to measure the reduced nitroblue diformazan into cells.

The ability to generate 1O_2 (Type II mechanism) when each AQ is excited by pulsed laser radiation (N_2 laser -337.1 nm-) was determined by measuring the direct luminescence of the 1O_2 produced. The quantum yield of singlet oxygen (Φ) showed by each AQ was measured in chloroform ($CHCl_3$), using a comparative method based on the use of perinaphthenone (PN, with a known Φ of 0.95 ± 0.05) as a reference photosensitizer [25, 26]. The intensity of the luminescence of the 1O_2 generated, extrapolated to zero time (I_0), was measured at different laser input energies (E_L) for each solution of AQ as well as for PN. The Φ were calculated by comparing the slopes of the linear plots I_0 vs. E_L , for the reference and sample.

Eight out of the nine AQs studied generated 1O_2 . In Fig. 2, the detector response, taken at the maximum of the production-decay curve (I_0), is shown as a function of the laser input energy (E_L) for the reference PN and two selected AQs. As it can be seen, good straight lines are obtained evidencing that no saturation effects are involved. Although only two AQs are shown, the remaining six also gave good straight slopes for energies below 0.9 mJ/pulse. The figure clearly depicts that the most intense signal comes from the reference PN, so the Φ for each compound resulted lower than unity.

We determined six (five neutral density filters and the unfiltered laser beam) quantum yields values for each AQ by rationing the intensities of the reference and the particular AQ at the same energy. The dispersion of the results is within the error associated to any single measurement, therefore only the average value is listed in Table 2.

An analysis of the data in Table 2 in comparison with the structures of the AQs (Fig. 1) allows the following arguments to be put forward.

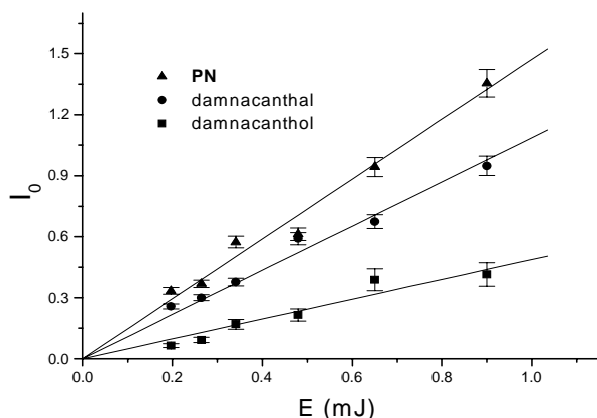


Fig. 2. Laser fluence dependence of the amplitude at zero-time I_0 of the $^1\text{O}_2$ phosphorescence signal in CHCl_3 for PN, damnacanthal and damnacanthol.

When comparing soranjidiol ($\Phi = 0.47$) and soranjidiol 1-methyl ether ($\Phi = 0.07$), which only differ in the hydroxyl group at 1-position (OH-1), we observe that its presence strongly pushes the quantum yield up. The same conclusion can be drawn from the comparison of rubiadin ($\Phi = 0.34$) and rubiadin 1-methyl ether ($\Phi = 0.00$), which also differ in the same group at 1-position. Note that in both compounds groups, R_3 and R_4 are the same, and regardless of whether it is H or OH, it seems that the presence of the OH-1 is the responsible for the $^1\text{O}_2$ production. An inspection of the values of Φ for heterophylline ($\Phi = 0.41$) and pustuline ($\Phi = 0.11$), which were independently reported before [26], shows again that the presence of the OH-1 (adjacent to the carbonyl group) notably increases the $^1\text{O}_2$ production with respect to pustuline.

In general, it has been accepted that for 1-OH-AQs, the possibility of forming intramolecular hydrogen bonding strongly influences the radiationless deactivation processes of the electronically excited singlet state of the AQ [13]. The formation of $^1\text{O}_2$ implies that the radiationless deactivation ($^1\text{S} \rightarrow ^3\text{S}$) has to be somehow efficient, and soranjidiol, rubiadin and heterophylline do show this possibility whereas soranjidiol 1-methyl ether, rubiadin 1-methyl ether and pustuline do not, in close agreement with Gollnick *et al.* [13]. Indeed, the transition ($^1\text{S} \rightarrow ^3\text{S}$) is not the only radiationless process that can occur; but due to the low quantum efficiency for fluorescence shown

by these AQs (see results below), the participation of $^1\text{S} \rightarrow ^3\text{S}$ should be important.

However, it was observed that AQs without the OH-1, i.e. damnacanthol ($\Phi = 0.31$) and particularly damnacanthal ($\Phi = 0.70$) showed from moderate to important quantum yield values. This observation led us to postulate that the presence of aldehyde and alcohol functional groups in 2-position would be counteracting the absence of the OH-1 and would be reverting the lower quantum yields due to the OCH_3 group in 1-position.

On the basis of this analysis, it would be interesting to determine the Φ for the $R_1 = \text{OH}$, $R_2 = \text{CHO}$ derivative (nordamnacanthal). Therefore, we looked for a convenient method to obtain nordamnacanthal and we succeeded in using a semi-synthetic path starting from damnacanthal [26]. Table 2 summarizes the values of Φ for the new AQs and it is readily seen that nordamnacanthal shows the highest quantum yield for all the AQs studied.

$^1\text{O}_2$ reactive and total quenching determination

The deactivation of $^1\text{O}_2$ by chemical reaction was followed through a comparative steady-state method using CHCl_3 as solvent [25, 26]. The determination of the rate constants (k_r) required continuous irradiation of zinc tetraphenylporphine (ZnTPP) used as sensitizer. A known chemical quencher, 9,10-diphenylanthracene (9,10-DPA), ($k_r = 4.2 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$) was used as reference. The disappearance of both substrate (AQ) and reference (9,10-DPA) was followed through UV-Vis spectrophotometry. The rate constant, k_r , was obtained from the ratio of the slopes of the linear plots $\text{Ln}(\text{Abs}_0/\text{Abs})$ vs irradiation time for the reference and each AQ.

The attainment of total deactivation rate constants of $^1\text{O}_2$ by the AQs ($k_t = k_r + k_q$; reactive + physical) was performed with the time resolved infrared luminescence technique [25, 26]. In these measurements, every AQ was used simultaneously as sensitizer and quencher ($S = Q$) with the sole exception of rubiadin 1-methyl ether because its quantum yield for the production of the excited state of oxygen was zero (Table 2). In this case, an independent sensitizer was used (PN) and therefore the $^1\text{O}_2$ lifetimes were

Table 2. Quantum yields for $^1\text{O}_2$ generation upon direct 337.1 nm irradiation and quenching rate constants (k_r and k_t) in CHCl_3 .

AQ	Φ	Quenching rate constant		$\phi_r (k_r/k_t)$
		$k_r (10^5 \text{ M}^{-1} \text{ s}^{-1})$	$k_t (10^5 \text{ M}^{-1} \text{ s}^{-1})$	
soranjidiol	0.47 ± 0.04	2.79 ± 0.19	13.40 ± 0.03	0.208
soranjidiol 1-methyl ether	0.07 ± 0.01	<0.1	5.72 ± 0.23	0.017
rubiadin	0.34 ± 0.04	4.20 ± 0.13	19.35 ± 0.08	0.217
rubiadin 1-methyl ether	0.00	2.62 ± 0.04	10.81 ± 0.02	0.242
damnacanthal	0.70 ± 0.03	3.81 ± 0.15	3.97 ± 0.16	0.957
damnacanthol	0.31 ± 0.01	1.89 ± 0.09	6.31 ± 0.19	0.299
heterophylline	0.41 ± 0.02	1.01 ± 0.02	6.50 ± 0.02	0.155
pustuline	0.11 ± 0.01	2.15 ± 0.10	2.69 ± 0.03	0.799
5,5'-bisoranjidiol	0.18 ± 0.01	<0.1	5.45 ± 0.13	0.018
nordamnacanthal	0.84 ± 0.10	NA	NA	NA

NA: Not available.

evaluated in the absence (τ_0) and in the presence (τ) of the quencher, and their ratio plotted as a function of AQ concentration using the standard Stern-Volmer treatment (Eq. 1) [12].

$$\text{Eq. 1. } \tau_0/\tau = 1 + k_t(k_r + k_q) \tau [Q]$$

The method used for the Aqs that act both as sensitizers and quenchers requires the progressive increase in the concentration of AQ following every decay recording, until the luminescence is completely quenched and therefore we are forced to use a slightly modified Stern-Volmer treatment due to the lack of a lifetime measurement free from the presence of the quencher [25, 26]. Every individual $^1\text{O}_2$ lifetime (τ) was determined by averaging 36 independent decay traces (36 successive laser flashes). Since the concentration of AQ changes negligibly over the reaction period, a first-order kinetics results, with an observed rate constant for the decay of $^1\text{O}_2$ of $1/\tau$ ($1/\tau = k_d + (k_r + k_q) [Q]$). The slope and intercept, of a plot $1/\tau$ vs $[Q]$, give values of $(k_r + k_q)$ for the quencher and k_d for the solvent, respectively.

Table 2 shows the absolute values of k_r . They do not resemble particularly good reactive quenchers.

As an example, Fig. 3 shows the linear plot $\text{Ln}(\text{Abs}_0/\text{Abs})$ vs irradiation time for the reference used (9,10-DPA) and one particular AQ, namely, damnacanthal.

Although no attempts were made to study the products of the reaction between the Aqs and $^1\text{O}_2$, it is accepted that the initial attack of the oxygen molecule should be on a double bond in the molecule [28] to give rise either to a hydroperoxide or an endoperoxide. Being so, there are several possibilities with every AQ and therefore it is difficult to give a trend in the absolute values of the quenching rate constants, as was done for the variation in quantum yields.

We have also measured the total quenching of the $^1\text{O}_2$ emission (k_t) with the time resolved system for these Aqs. Fig. 4 corresponds to the plot of $1/\tau$ vs $[Q]$ for heterophylline in a typical experiment. In addition, the k_t of rubiadin 1-methyl ether was evaluated according to a simple Stern-Volmer treatment; the plot of τ_0/τ vs $[Q]$ for this AQ is shown in Fig. 5.

The absolute values for the total rate constants are also listed in Table 2. As can be seen, the great majority of the Aqs are poor or moderate quenchers. Whereas the overall constants do not

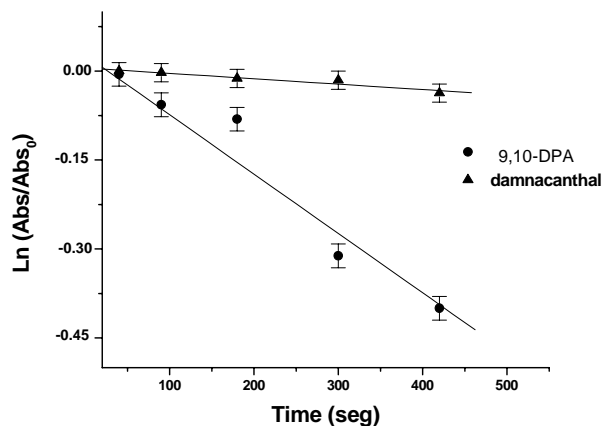


Fig. 3. Reactive quenching rate constant derived for 9,10-DPA and damnacanthal.

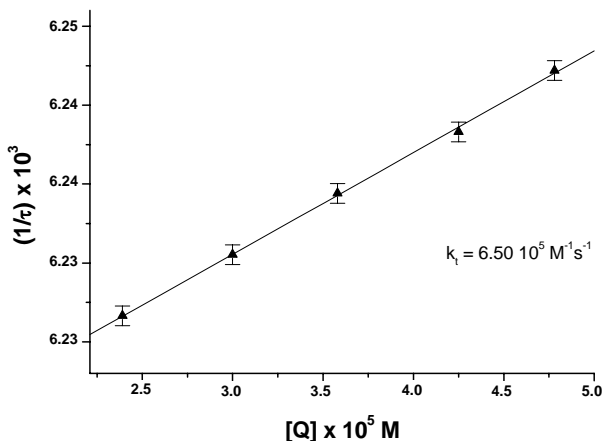


Fig. 4. Total quenching rate constant derived for heterophylline.

change dramatically between different partners, some of them show a degree of self-protection (defined as (k_r/k_t)), [29] against photo-oxidation. Particularly, soranjidiol 1-methyl ether and 5,5'-bisoranjidiol present a very good self-protection ratio mainly due to very low reactive rate constants. Within the same order of magnitude for k_t , damnacanthal and pustuline present the worst case for self-protection with a ratio close to unity.

Fluorescence quantum yields and photolysis

The relative fluorescence quantum yields (ϕ_f) for the AQs were measured in CHCl_3 [26]. On the basis of the excitation spectra of each AQ and depending on its excitation wavelength (λ_{exc}),

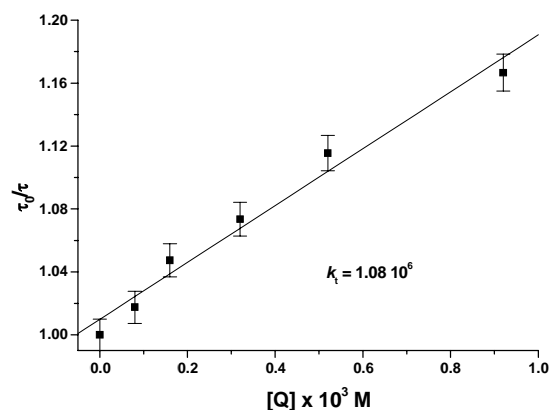


Fig. 5. Stern-Volmer plot for the quenching of rubiadin 1-methyl ether.

two different references were used: 9,10-DPA ($\lambda_{\text{exc}} = 350 \text{ nm}$, $\phi_f = 1$) for soranjidiol, rubiadin, rubiadin 1-methyl ether, damnacanthol, heterophylline, pustuline and 5,5'-bisoranjidiol and naphthalene (NP) ($\lambda_{\text{exc}} = 270 \text{ nm}$, $\phi_f = 0.23$) for soranjidiol 1-methyl ether and damnacanthal and the relative quantum yield was evaluated according to:

$$\phi_f(\text{AQs}) = I_f(\text{AQs}) \times \phi_f(\text{Ref}) / I_f(\text{Ref})$$

The fluorescence intensities (I_f) were recorded at the maximum of emission for both, reference and AQs [26].

On the other hand the photochemical studies (photolysis) were carried out in steady state conditions by irradiating the AQS dissolved in CHCl_3 under two different conditions [26]. Monochromatic light corresponding to the absorption maximum for each AQ was used with one set and the other set of samples was irradiated with the whole output of the lamp. A high-pressure SP 200 Bausch & Lomb mercury light source was used. Irradiation times varied between 15 and 7200 s. The course of the reaction was followed by UV-Vis spectrophotometry.

The results obtained (Table 3) show very low fluorescence yields (around 10^{-2} - 10^{-4}) for most of the AQS and a particular one (damnacanthol) whose quantum yield is below our detection limit. We should note that the decomposition of the illuminated AQS is negligible within the total irradiation time.

Table 3. Quantum yields of fluorescence of the AQs.

AQ	Reference	λ_{exc}	ϕ_f
soranjidiol	9,10 DPA	395.5	0.0050 \pm 0.0002
soranjidiol 1-methyl ether	Naphtalene	276	0.0145 \pm 0.0009
rubiadin	9,10 DPA	376	0.0180 \pm 0.0010
rubiadin 1-methyl ether	9,10 DPA	376	0.0011 \pm 0.0001
damnacanthal	Naphtalene	276	0.0202 \pm 0.0002
damnacanthol	9,10 DPA		---
heterophylline	9,10 DPA	395.5	0.0052 \pm 0.0003
pustuline	9,10 DPA	374	0.0076 \pm 0.0004
5,5'-bisoranjidiol	9,10 DPA	395.5	0.0008 \pm 0.0001

Conclusions

On the basis of the above results, we could say that all AQs isolated from *H. pustulata* photosensitize the generation of $O_2^{\bullet-}$ within leukocytes. With the exception of rubiadin 1-methyl ether, all of them show the ability to produce 1O_2 when they are irradiated; all of them show low k_{ts} and k_{fs} . Moreover, the fluorescence quantum yields are in general low. Thus, these AQs should show a good performance as Type II photosensitizers.

PHOTOTOXIC EFFECTS OF HETEROPHYLLAEA PUSTULATA

The chemical, photophysical and photochemical performed assays with AQs from *H. pustulata* allowed us to estimate that these chemical components would be responsible for the manifest phototoxicity of this plant. Thus, our objective was demonstrated *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 AQs administered orally and transported by systemic circulation would accumulate in the skin, where they would perform their photosensitizing action [30].

Thus, a purified and quantified fraction (fraction A) of soranjidiol (43.8 \pm 1.8 %) and rubiadin (14.7 \pm 0.4 %) was obtained in order to be used in the phototoxicity assay *in vivo*. A solution of

fraction A in olive oil was prepared (58.3 mg/ml) in order to be administered orally (oral sample).

Male albino Balb/c mice were used, all specimens being two month old and weighing 20g approximately. The animals (n = 12) were divided into two experimental groups, one problem group and another control 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 minutes 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 dose and the second, three animals of each group (problem and control) were exposed to sunlight during 40 minutes. 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 minutes).

While experiments were being carried out, changes in the animal's 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.

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.

After evaluation of symptoms as a result of the oral administration of the AQs, the animals were anesthetized with a sterile aqueous solution of ketamine chlorhydrate - xylazine chlorhydrate - acepromazine (85.5-17.0-3.0 mg/kg) by intraperitoneal (i.p.) injection before the animals were sacrificed by decapitation [30].

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 level.

In order to identify and/or quantify the AQs in serum and skin samples, these compounds had to be extracted from biological material. Whereas serum samples were extracted from buffer pH =2 with ethyl ether, skin samples were malaxed with buffer pH = 2 and then extracted under the same conditions as described above [30]. Then, ether fractions were evaporated to dryness; finally, they were dissolved with MeOH to be used for HPLC analysis. This analysis (qualitative and quantitative) was performed according to reported literature [30]. 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 [30].

The Fig. 6 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 (soranjidiol and rubiadin) are identical as 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. 7 shows the chromatograms obtained for skin samples from animals treated with AQs in darkness and under irradiation together with the control chromatogram and compared with fraction A. Their analysis allows the conclusion that soranjidiol and rubiadin accumulate at 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 4).

Data are expressed as means \pm 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 $p < 0.01$.

Discussions and conclusions

Our previous studies had demonstrated that it is characteristic of these AQs (soranjidiol and rubiadin) to be effective photosensitizing agents *in vitro* as well as in homogeneous media (Tables 1 y 2). However, it had not been ascertained that this characteristic would be manifested *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.

It was observed that, as a result of the oral administration of soranjidiol and rubiadin 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 analysis shown in Figs. 6 and 7.

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 4). This difference could be due to the increase in the animal's body heat when kept

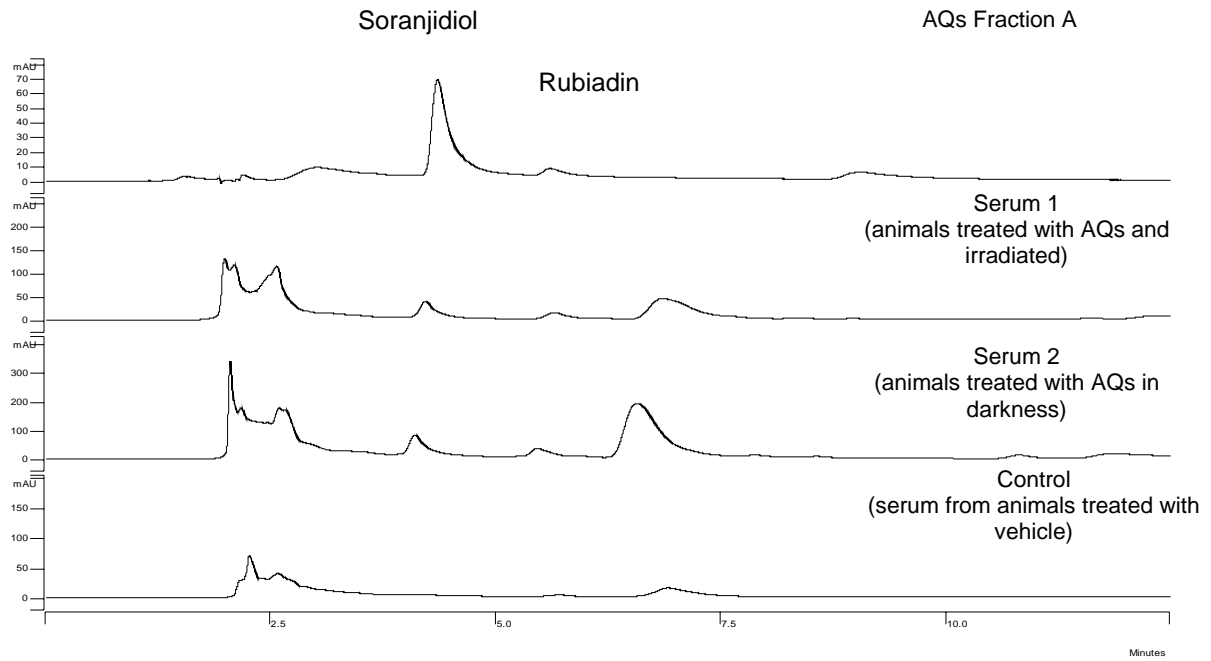


Fig. 6. Identification of AQs administered orally in serum samples.

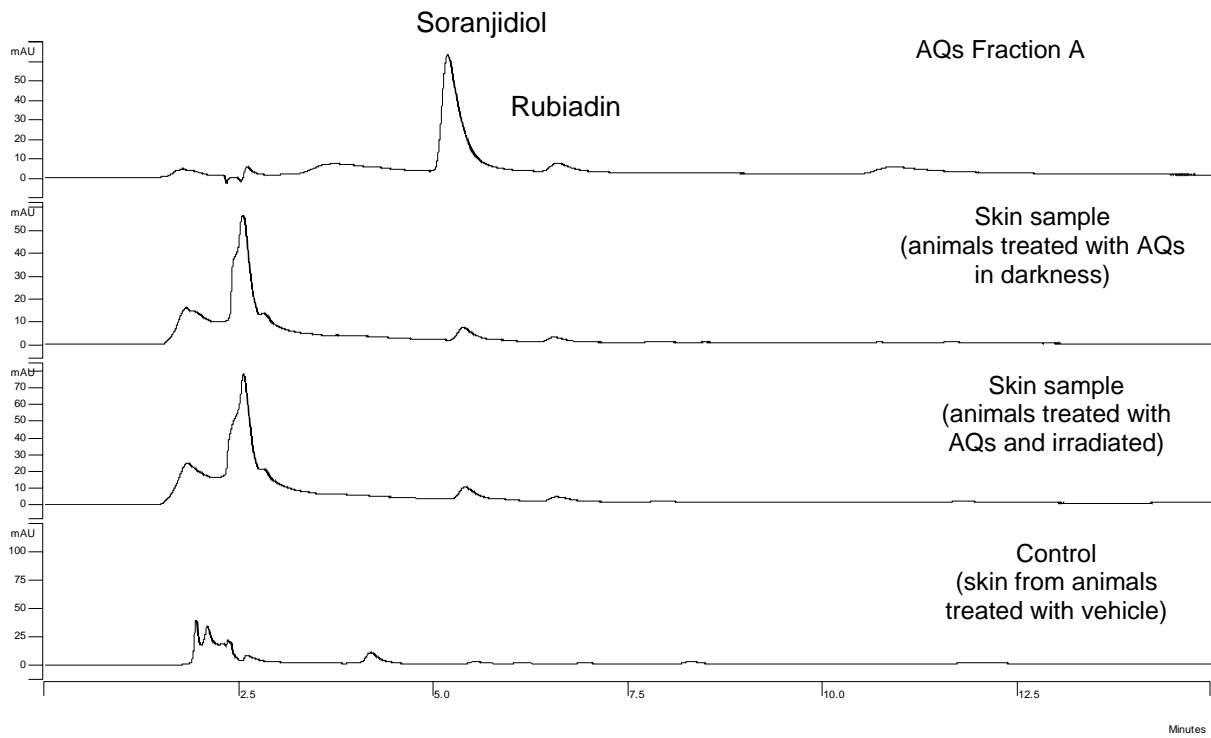


Fig. 7. Identification of AQs administered orally in skin samples.

Table 4. Quantification and percentages of AQs recovery in skin samples.

AQs	Darkness		Radiation	
	Amount of AQs in 200 mg of skin (μg)	Recovery %	Amount of AQs in 200 mg of skin (μg)	Recovery %
soranjidiol	$1.2 \pm 0.1^*$	$0.0059 \pm 0.0003^*$	1.8 ± 0.1	0.0085 ± 0.0005
rubiadin	$0.42 \pm 0.02^*$	$0.0062 \pm 0.0002^*$	0.47 ± 0.02	0.0069 ± 0.0001

Animals treated with AQs in darkness, n = 3.

Animals treated with AQs and irradiated, n = 3.

*p<0.01 with respect to irradiated animals.

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 that, 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.

Another factor to be considered in order to understand the toxicity of *H. pustulata* is related to the biosynthetic way by which it produces its AQs. It is well known that natural AQs are generally found in plants as glycosides [31], 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 [32]. 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 [31].

Some of these experiments were developed in the Molecular Biology Department of National University of Río Cuarto, under the direction of Dr. Viviana A. Rivarola.

ANTIBACTERIAL ACTIVITY OF ANTHRAQUINONE DERIVATIVES

Substances with photosensitizing characteristics have become particularly important in the field of pharmacology due to their potential applications [8]. The photodynamic antimicrobial chemotherapy (PACT) involves photosensitizers and visible or ultraviolet light and has been proposed in the treatment of locally occurring infections, especially of caries, periodontal diseases, oral candidosis as well as infected wounds [33].

Some photosensitizers, like psoralen derivatives, produce bacterial photoinactivation through a mechanism that allows their intercalation between the nucleic acid bases. Others, have the same final output but this is reached through a photodynamic photosensitization acting mainly through the generation of ROS, particularly singlet molecular oxygen ($^1\text{O}_2$) [33].

Nevertheless, it should be noted that for many substances having antibacterial effects, there are other mechanisms acting without the presence of light that elicit a physiological response in bacteria by producing ROS [34-36] and therefore this put stringent experimental conditions making it necessary a good differentiation between dark and irradiated experiments.

Within the photodynamic framework, it is worth mentioning that several AQs have been thoroughly studied in relation to their photosensitizing properties in photodynamic reactions [12, 13]. Thus, for instance, some of them show good antibacterial and antiviral effects, producing ROS such as $\text{O}_2^{\bullet-}$, OH^\bullet and $^1\text{O}_2$ with subsequent oxidative damage [15, 16, 37].

We demonstrated, as previously detailed, that the AQs isolated from *H. pustulata* exhibit photosensitizing properties by generation of $O_2^{\bullet-}$ and/or 1O_2 . Thus, our objective was demonstrated that these AQs have antibacterial effect against Gram (+) bacteria: *Staphylococcus aureus* and this effect is directly linked to the increase of $O_2^{\bullet-}$ and/or 1O_2 levels and this is not due solely to a photodynamic process but to a concurrent combination between light driven and dark processes.

In order to reach this conclusion, the experimental model was carried out according to the following guidelines: The AQs were dissolved in phosphate buffer solution (PBS) and the concentrations used throughout were below the minimal inhibitory concentration (MIC) (32-64 $\mu\text{g/mL}$ -see results above-) and specifically selected at the subtoxic doses of 10 $\mu\text{g/mL}$ because higher concentrations, proved to be toxic for normal mammal cells (African green monkey kidney cells –Vero) [38].

Routine susceptibility tests, including the determination of MIC, are an estimate of bacteriostatic action. In this case, measurement of the MIC for each AQ was carried out in accordance with international standards from the Clinical and Laboratory Standards Institute (CLSI) by means of broth macrodilution methods in Mueller-Hinton medium (MH, Britania) [39]. The strain used for this purpose was *S. aureus* ATCC 29213 (1×10^5 colony forming units (cfu)/mL). The working solutions of each AQ were prepared by means of seriated dilutions from 256 $\mu\text{g/mL}$ up to 0.125 $\mu\text{g/mL}$. MIC determination was carried out after 24 h of incubation at 37 °C by observing turbidity.

Our studies led to the conclusion that only soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol showed a susceptibility with MICs below the 100 $\mu\text{g/mL}$ range (Table 5).

Only those AQs which showed antibacterial activity (through the determination of the MIC), were treated to evaluate whether this activity was linked to an $O_2^{\bullet-}$ generation increase with respect to the basal production. The $O_2^{\bullet-}$ determination, as usual, was based on the NBT assay [39].

Table 5. Susceptibility of *S. aureus* ATCC 29213 to each AQ.

AQs	MIC ($\mu\text{g/mL}$)
soranjidiol	32-64
soranjidiol 1-methyl ether	> 256
rubiadin	32-64
rubiadin 1-methyl ether	> 256
damnacanthal	32-64
damnacanthol	> 256
heterophylline	> 256
pustuline	> 256
5,5'-bisoranjidiol	32-64

Two assays were performed in darkness simultaneously. One was carried out in the absence of AQs to determine the basal level of the $O_2^{\bullet-}$ production in the bacteria (control). The second was carried out by adding AQs to the culture medium, in order to determine whether these compounds induce an increase in the $O_2^{\bullet-}$ generation through a physiological response. Both assays were independently repeated under actinic irradiation to evaluate the photosensitized response. The strain used was *S. aureus* ATCC 29213 (10^9 cfu/mL). In the NBT assay, the bacterial suspension was incubated with each AQ solution and NBT at 37 °C in darkness (n=3). Again, the control experiments were carried out under the same conditions but without adding the AQ. Triplicate sets were incubated under actinic irradiation. All these experiments were independently repeated measuring at 10, 20 and 40 min of incubation.

On the other hand, the same set of AQs used in the preceding paragraph was treated to evaluate 1O_2 generation increase with respect to the basal production. The 1O_2 production in the reaction medium was followed by spectrophotometrical determination of the consumption of methionine (MET) at 236 nm as a function of time [39]. This compound reacts chemically with the generated 1O_2 with a rate constant (k_t) of $2.1 \times 10^7 \text{ L mol}^{-1} \text{ s}^{-1}$ [40]. Here again, two sets of experiments were

done; one in darkness and the other under actinic irradiation. For each set, the procedure is described below.

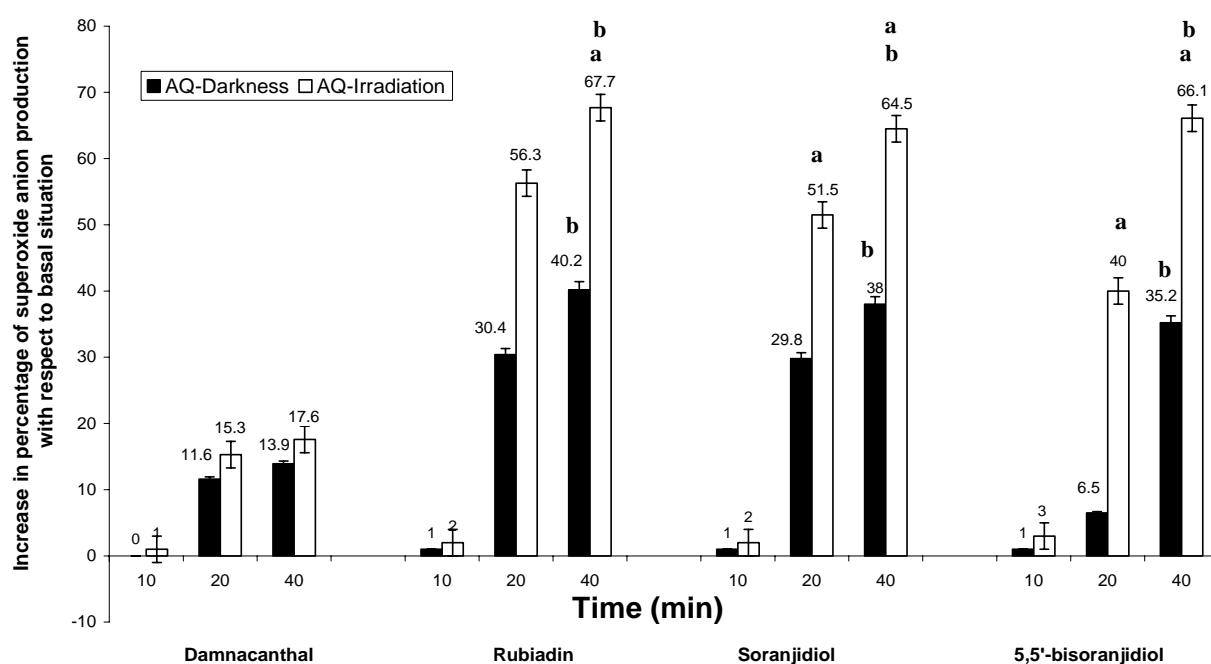
- i) In order to determine the $^1\text{O}_2$ basal production generated by bacteria (control), we incubated bacterial suspension (10^9 cfu/mL), MET and PBS.
- ii) The production of $^1\text{O}_2$ in the presence of AQs was measured incubating bacterial suspension (10^9 cfu/mL), MET, 1 mL of AQ and PBS.

Whether the consumption of MET is a direct consequence of the $^1\text{O}_2$ generation in the medium, is confirmed using sodium azide (NaN_3) as a $^1\text{O}_2$ physical quencher ($k_t = 5.8 \cdot 10^8 \text{ L mol}^{-1}\text{s}^{-1}$) [41]. Due to its large quenching constant, NaN_3 was added in a high enough concentration to suppress the consumption of $^1\text{O}_2$ by MET as described below:

- iii) The bacterial suspension (10^9 cfu/mL) was incubated with MET, 1 mL of AQ, NaN_3 and PBS.

Fig. 8 shows the increase in percentage of $\text{O}_2^{\bullet-}$ in *S. aureus* with respect to basal situation when this strain was treated with each AQ in darkness and under actinic radiation at 10, 20 and 40 min. It is worth mentioning that control experiments in darkness and under irradiation gave no statistical differences. As it can be observed in Fig. 8, there is no clear indication of $\text{O}_2^{\bullet-}$ at 10 min, neither for darkness nor irradiation. At 20 and 40 min, all the AQs induce an increase in $\text{O}_2^{\bullet-}$ production with respect to the basal situation in darkness. With the exception of damnacanthal this effect raises at longer times. Under actinic irradiation, there is a further increase in $\text{O}_2^{\bullet-}$ production with respect to basal situation with the same exception as before.

On the other hand, Fig. 9 shows the MET consumption caused by the $^1\text{O}_2$ generated in *S. aureus* in the conditions outlined in i, ii, iii (evaluation of the $^1\text{O}_2$ generation) both, for darkness and under actinic irradiation for



Results are given as mean \pm SD, $n=3$.

^a $p < 0.05$ measured with respect to darkness

^b $p < 0.05$ measured with respect to 20 min time

Fig. 8. NBT assay. Increase in percentage of $\text{O}_2^{\bullet-}$ in *S. aureus* ATCC 29213 with respect to basal situation for every AQ and time measured.

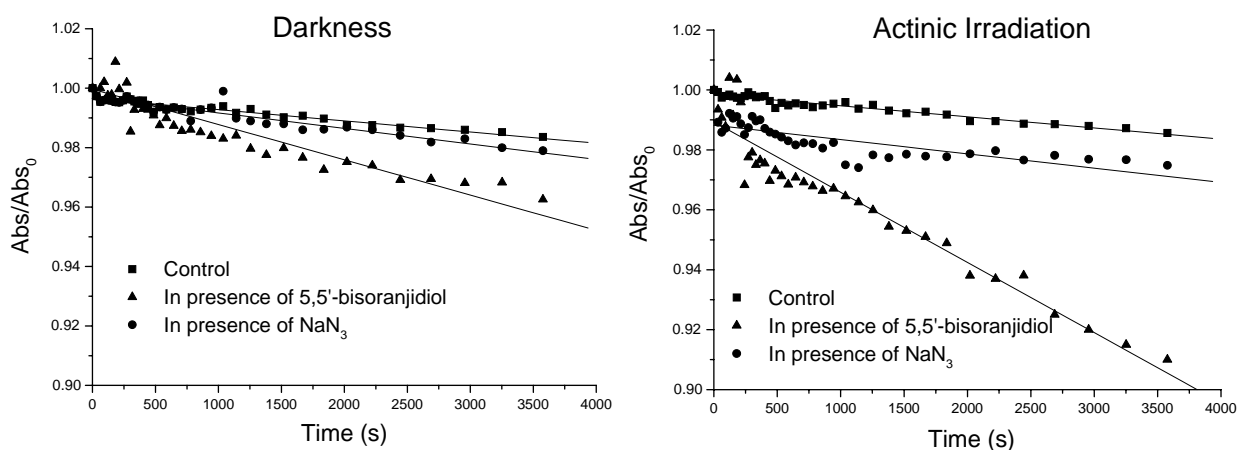


Fig. 9. Consumption of MET caused by $^1\text{O}_2$ in *S. aureus* ATCC 29213 treated with 5,5'-bisoranjidiol (10 $\mu\text{g}/\text{mL}$), in darkness and under irradiation. Average of three experiments.

5,5'-bisoranjidiol. As it can be observed, in the absence of AQ, small amounts of $^1\text{O}_2$ due to the normal breathing process are produced. MET consumption was higher when 5,5'-bisoranjidiol was present which means that the $^1\text{O}_2$ production increased. This phenomenon is observed under both working conditions (darkness and actinic irradiation). The $^1\text{O}_2$ generated was counteracted by addition of NaN_3 . This physical quencher efficiently competes with MET for the deactivation of $^1\text{O}_2$ and consequently, the trend line is similar to the control experiments. These results confirm that MET consumption is a direct consequence of the $^1\text{O}_2$ generation.

The preceding discussion referred only to 5,5'-bisoranjidiol. The other Aqs tested also show a similar behavior, though with a smaller increase in $^1\text{O}_2$ generation. As an example, Fig. 10 shows the corresponding results for rubiadin.

Fig. 11 shows the MET consumption caused by the $^1\text{O}_2$ generation in bacteria treated with each AQ (see ii: evaluation of the $^1\text{O}_2$ generation), in darkness vs. irradiation. Its analysis (by comparisons of the trend lines) shows that, the $^1\text{O}_2$ production is higher in the presence of actinic radiation with respect to darkness because MET consumption increased, except for damnacanthal. The straight lines drawn in Figs. 9, 10, 11 and 14 (see below) only intend to show the trend and by no means represent a least squares analysis.

Lastly, bearing in mind that those compounds that can reduce a minimum of 10^3 cfu/mL (3.0 Log_{10}) are considered bactericide agents whereas those below that range (which merely inhibit growth) are considered bacteriostatic agents [34, 42, 43], we carried out assays with the purpose of establishing whether the Aqs show any of such characteristics.

With this purpose, *S. aureus* ATCC 29213 (10^8 cfu/mL) was incubated by triplicate with each AQ (damnacanthal, rubiadin, soranjidiol and 5,5'-bisoranjidiol) at 37°C independently in darkness and under irradiation and also replicating the whole set to measure at 10, 40 and 90 min totaling an amount of 90 different assays (72 + 18 controls) [39]. After incubation time, bacteria were serially 10-fold diluted with PBS and each dilution was placed on plate count agar MH and incubated for 24 h at 37°C . Bactericidal activity was determined by means of plate recount of colony-forming units per milliliter (cfu/mL).

A particular experiment was carried out to prove whether the suppression of $^1\text{O}_2$ production by a chemical quencher changes the reduction in the number of cfu/mL. A bacterial suspension (10^8 cfu/mL) with 5,5'-bisoranjidiol under irradiation was treated, after 40 min of incubation, with MET allowing the system to proceed as usual and measuring the cfu/mL at 90 min.

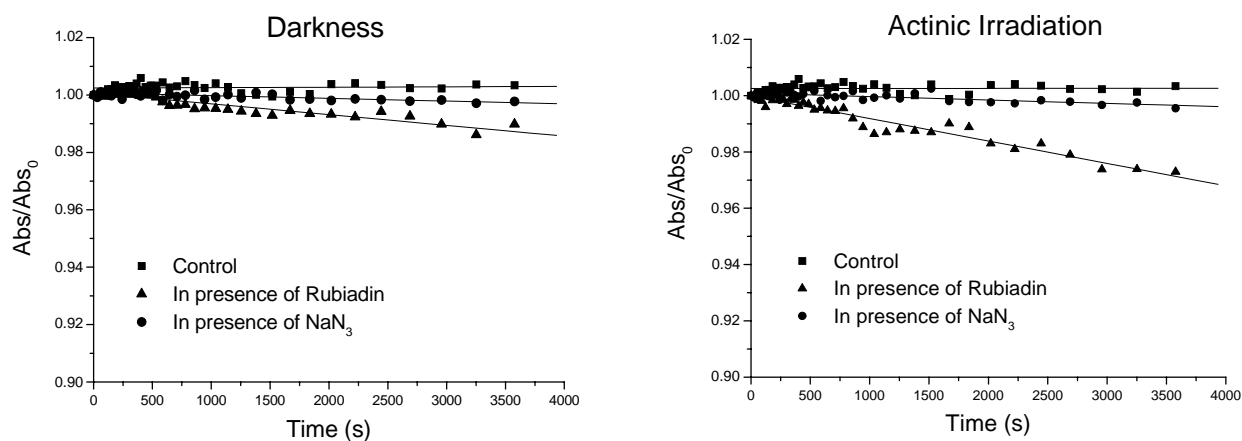


Fig. 10. Consumption of MET caused by $^1\text{O}_2$ in *S. aureus* ATCC 29213 treated with rubiadin (10 $\mu\text{g/mL}$), in darkness and under irradiation. Average of three experiments.

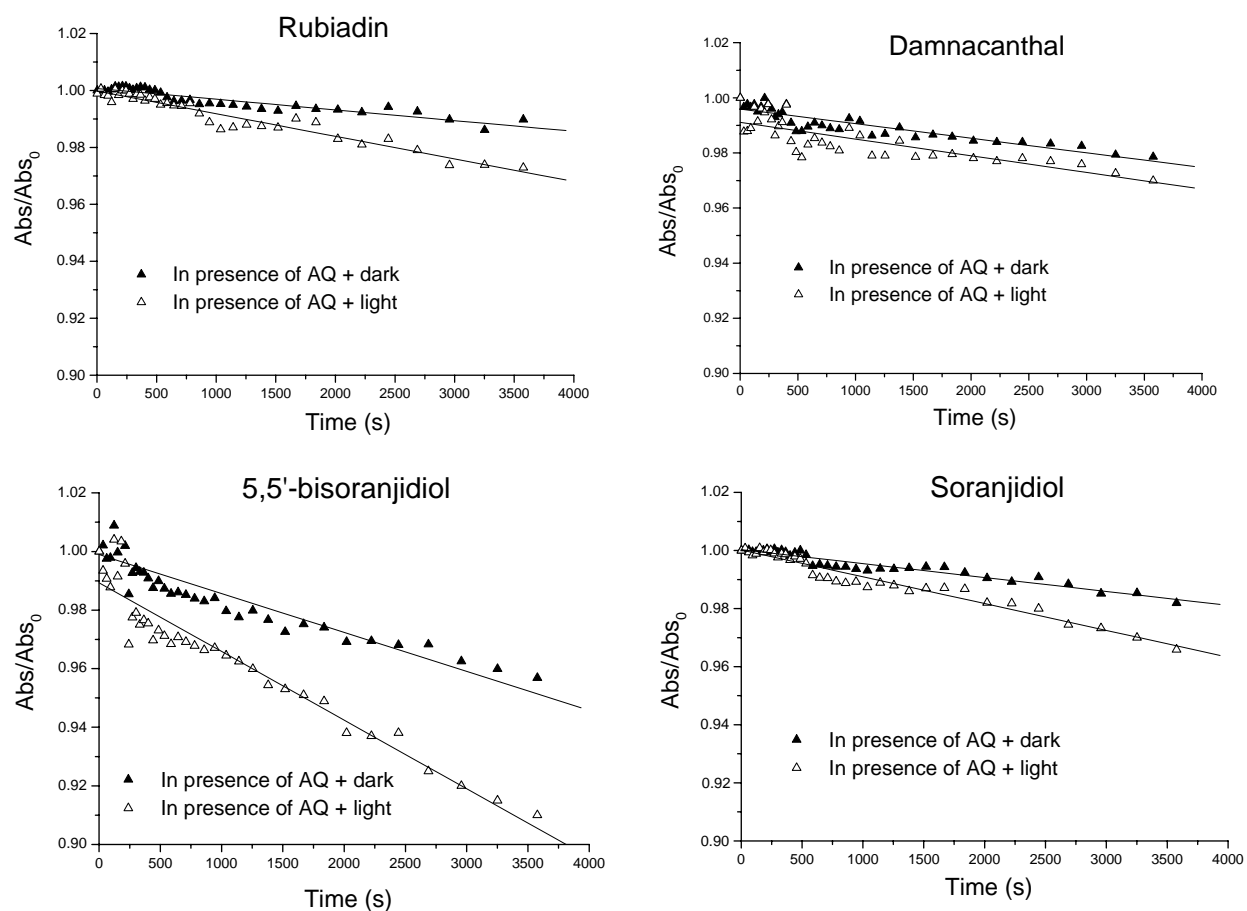


Fig. 11. Consumption of MET caused by $^1\text{O}_2$ in *S. aureus* ATCC 29213 treated with each AQ (10 $\mu\text{g/mL}$), comparing process occurring in darkness and under actinic irradiation. Average of three experiments.

Fig. 12 shows Log_{10} change in cfu/mL of *S. aureus* treated with each AQ in darkness, as well as the control. As it can be observed, after an initial increase there is a reduction, which is more pronounced for 5,5'-bisoranjidiol. This reduction could be related to the capability of producing a physiological response in bacteria that generate $\text{O}_2^{\bullet-}$ (Fig. 8) as well as $^1\text{O}_2$ (Figs. 9 and 10) in the absence of light. 5,5'-bisoranjidiol was the only AQ that showed bactericide effect on this microorganism with a reduction of 3.0 Log_{10} of cfu/mL at 90 min. Rubiadin and soranjidiol,

produce a reduction of 2.2 and 2.0 Log_{10} of cfu/mL, respectively. For them, the increase in $\text{O}_2^{\bullet-}$ production is similar to 5,5'-bisoranjidiol (Fig. 8), but the increase in $^1\text{O}_2$ generation is lower (Fig. 10). For damnacanthal there is only an inhibitory effect without producing death.

On the other hand, the actinic radiation and the consequent $\text{O}_2^{\bullet-}$ and $^1\text{O}_2$ increase by means of a photosensitization phenomenon (Figs. 8 and 11), further increased the reduction of cfu/mL for all AQs but damnacanthal (Fig. 13).

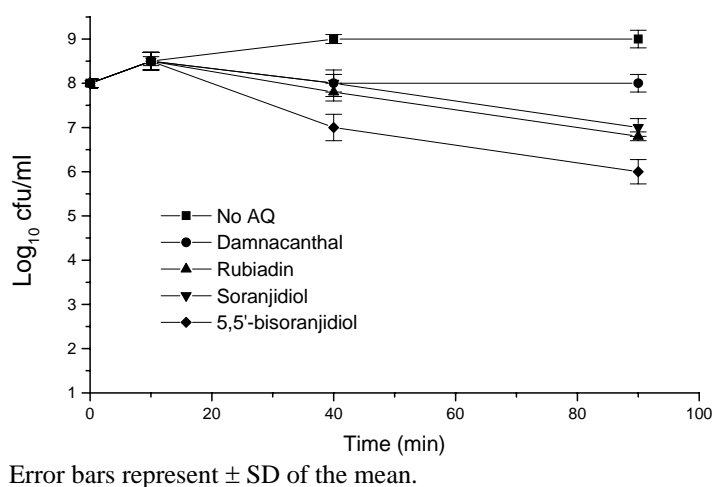


Fig. 12. Log_{10} change in colony-forming units per milliliter (cfu/mL) of *S. aureus* ATCC 29213 treated with each AQ (10 $\mu\text{g/mL}$) in darkness.

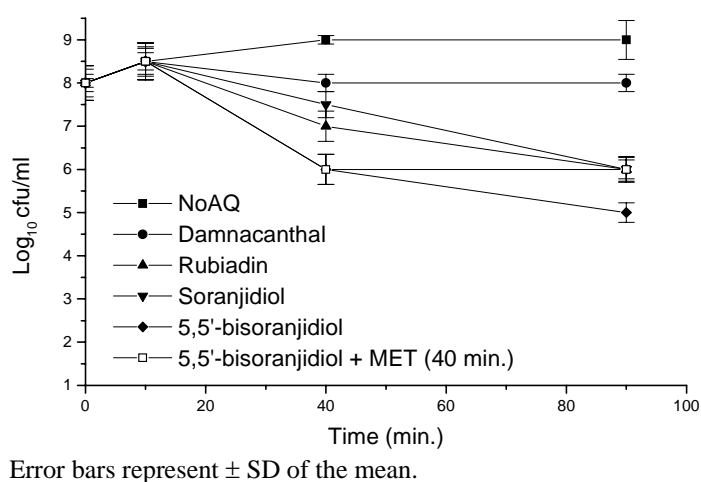


Fig. 13. Log_{10} change in colony-forming units per milliliter (cfu/mL) of *S. aureus* ATCC 29213 treated with each AQ (10 $\mu\text{g/mL}$) under irradiation.

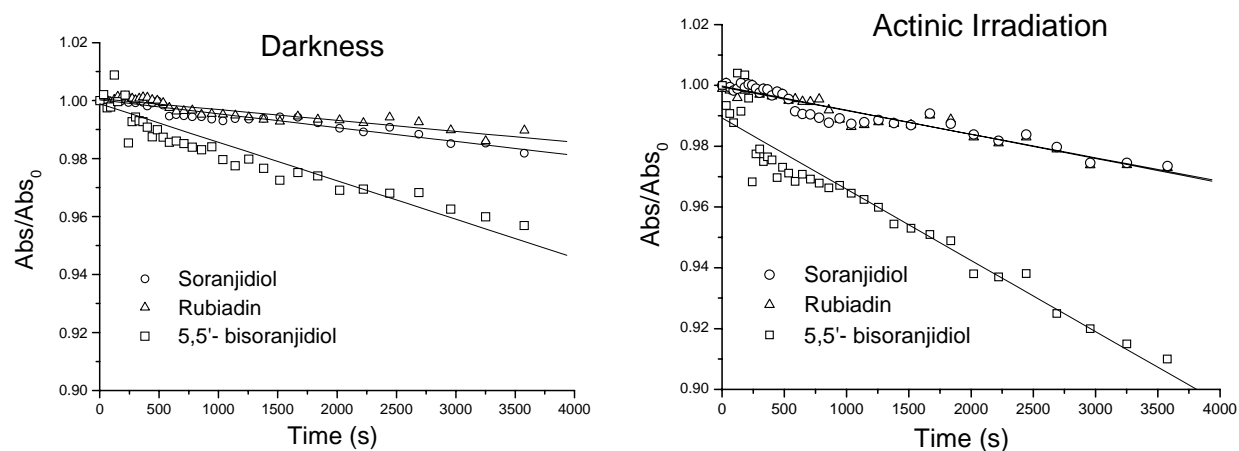


Fig. 14. Consumption of MET caused by $^1\text{O}_2$ in *S. aureus* ATCC 29213 treated with 10 $\mu\text{g/mL}$ of each rubiadin, soranjidiol and 5,5'-bisoranjidiol, in darkness and under irradiation. Average of three experiments.

These results clearly demonstrate that 5,5'-bisoranjidiol shows higher antibacterial activity than rubiadin and soranjidiol (Figs. 12 and 13), though all three increase $\text{O}_2^{\bullet-}$ production at about the same level as a function of time (Fig. 8) regardless they are acting in darkness or under irradiation.

In contrast, the $^1\text{O}_2$ production is higher for 5,5'-bisoranjidiol irrespective of conditions (darkness or irradiation) (Fig. 14). This would suggest that, among the different species that comprise ROS, $^1\text{O}_2$ is the one mainly involved in the bactericidal effect as has been already reported by Becerra *et al.* [44] using precisely *S. aureus*. This becomes evident since the addition of MET to a sample having 5,5'-bisoranjidiol after 40 min of incubation shows that even under irradiation, the cfu/mL reduction is completely suppressed at 90 min (open squares, Fig. 13).

CONCLUSIONS

It is widely accepted that ROS are toxic, and proteins, membrane lipids, and DNA constitute an important site of oxidative damage [34, 44]. It is also accepted (photodynamic therapy -PDT- for instance) that the effect is mediated by type II reactions, that is, by the production of $^1\text{O}_2$ since its electrophilic nature renders it very efficient in

producing oxidized forms of biomolecules thus initiating the damage [2].

Our results suggest that the antibacterial effect on *S. aureus*, observed for soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol is directly linked to the increase of $\text{O}_2^{\bullet-}$ and/or $^1\text{O}_2$ levels. This increase could be the result of the interaction between bacteria and the AQS without needing light, that is, without a photosensitizing process to produce a physiological response that elicit $\text{O}_2^{\bullet-}$ as well as $^1\text{O}_2$.

Actinic irradiation, on the other hand, generates a photosensitization for rubiadin, soranjidiol and 5,5'-bisoranjidiol, which consequently increases their antibacterial effects (particularly, bactericide). This effect is, in turn, suppressed when a specific quencher of $^1\text{O}_2$ is added, thus suggesting that the bactericidal activity is mainly due to that particular ROS as has been already proposed in other papers [37].

Most importantly, the particular bactericidal activity shown under irradiation for rubiadin, soranjidiol and 5,5'-bisoranjidiol at the concentration used (which do not affect normal mammal cells) led us to consider them like potential agents for photodynamic antibacterial chemotherapy treatments.

Some of these experiments were performed in Pharmacy Department of National University of Córdoba, under the direction of Dr. Inés Albesa.

ABBREVIATIONS

ROS, species reactives of oxygen; OH, hydroxy radical; $O_2^{\bullet-}$, superoxide anion radical; 1O_2 singlet molecular oxygen; AQs, anthraquinones; UV-V, ultraviolet-visible spectroscopy; IR, infrared spectroscopy; 1H -NMR, proton nuclear magnetic resonance spectroscopy; ^{13}C -NMR, ^{13}C carbon nuclear magnetic resonance spectroscopy; HRMS, high resolution mass spectrometry; S, photosensitizer; 1S , excited singlet state; 3S , long-lived triplet state; R or RH, substrate; O_2 , molecular oxygen; NBT, nitroblue tetrazolium; UVR, UV radiation; Φ , quantum yield of singlet oxygen molecular; PN, perinaphthenone (1H-phenalen-1-one); I_0 , intensity of the luminescence, extrapolated to zero time; E_L , laser input energies; OH-1, hydroxyl group at 1-position; CHO, aldehyde group; k_r , reactive rate constant for 1O_2 quenching; ZnTPP, zinc tetraphenylporphine; 9,10-DPA, 9,10-diphenylanthracene; Abs_0 , time zero absorbance; Abs , absorbance; k_t , total rate constant for 1O_2 quenching; k_q , physical rate constant for 1O_2 quenching; Q , quencher; $[Q]$, quencher concentration; τ_0 , 1O_2 lifetime in the absence of the quencher; τ^1O_2 lifetime in the presence of the quencher; k_d , rate constant for 1O_2 quenching for the solvent; ϕ_f , quantum yield of fluorescence; λ_{exc} , exciting wavelength; NP, naphthalene; I_f , fluorescence intensity; $\phi_{f(Ref)}$, quantum yield of fluorescence of reference; t_R , retention time; HPLC, High Pressure Liquid Chromatography; SD, standard deviation; PACT, Photodynamic Antimicrobial Chemotherapy; MIC, minimum inhibitory concentration; CLSI, Clinical Laboratories Standard Institute; MH, Müller-Hinton; cfu, colony forming unities; MET, methionine; PBS, phosphate buffer solution; NaN_3 , sodium azide; PDT, Photodynamic Therapy.

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