Antimicrobial activity of natural photosensitizing anthraquinones

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It was demonstrated that anthraquinone-rich extracts, obtained from the phototoxic vegetal species *Heterophyllaea pustulata* Hook f. (Rubiaceae), exhibited bacteriostatic activity on *Micrococcus luteus* ATCC 9341, selectively inhibiting both oxacillin-sensitive and resistant *Staphylococcus aureus*, and antifungal activity against important opportunism microorganisms and against those involved in superficial mycosis, all from nosocomial origin. The acute in vitro cytotoxicity evaluation of each anthraquinone (AQ) isolated from these bioactive extracts, on a mammalian eukaryotic cell line (Vero cells), allowed us to establish the non-cytotoxic concentration range, which was used to evaluate the antimicrobial effect. Four from nine AQs tested, soranjidiol, rubiadin, dammacanthal and (S)-5,5’-bisoranjidiol, showed in vitro bacteriostatic/bactericide activity against *S. aureus*. The action mechanism seems to involve an increase in the levels of superoxide anion and/or singlet oxygen molecular. Moreover, the effect of actinic irradiation as a boosting agent for the production of both reactive oxygen species as well as its influence on antibacterial effect was assessed.

**Keywords** anthraquinones; antimicrobial activity

**1. Introduction**

Throughout history, natural products have been a rich source of compounds that have found many applications in the field of medicine. In microbiology, particularly, several plant-derived compounds have been studied with this aim, including alkaloids, flavonoids, tannins, quinones, essential oils and other secondary metabolites [1]. Among them, anthraquinone derivatives (AQ) have aroused special interest since they have demonstrated potential therapeutic uses as antibacterial, antiviral, antifungal agents and other biological activities [1-5]. Within this family of compounds, several AQs have been thoroughly studied in relation to their photosensitizing properties in photodynamic reactions [6, 7]. On the basis of this, some of them show good antibacterial and antiviral effects, by producing reactive oxygen species (ROS) such as superoxide anion (O$_2^-$), hydroxyl radical (OH) and singlet molecular oxygen (1O$_2$), in the presence of light, with subsequent oxidative damage [8, 9]. Hypericin, a photosensitizing AQ found in certain members of the genus *Hypericum*, is a clear case showing significant antimicrobial activity by means of a photodynamic photosensitization, acting mainly through the generation of ROS, particularly 1O$_2$ [10-12]. It is widely accepted that substances with photosensitizing characteristics have become particularly relevant due to their potential applications in photodynamic antimicrobial chemotherapy (PACT), which involves photosensitizers and visible or ultraviolet light. This therapeutic (PACT) has been proposed in the treatment of local infections, especially those from caries, periodontal diseases, oral candidiasis as well as wounds [9, 13].

In this context, bearing in mind the many potential applications of photosensitizers and the need for new chemical structures with this particular feature, we started a series of chemical, physical and biological studies on photosensitizing AQs isolated from a phototoxic plant species, *Heterophyllaea pustulata* Hook f. (Rubiaceae). This vegetal species grows in the Andean mountain range in the northwest of Argentina [14] and the animals that ingest the aerial parts of this plant experience a typical primary photosensitization reaction, clinically revealed by dermatitis and blindness in severe cases [15, 16]. The chemical investigation of this plant revealed the presence of several constituents (AQs, flavonoids and iridoids) with an evident predominance of aglicone-9,10-AQs. From leaves and stems, nine AQs were isolated and purified by using repeated combination of several chromatographic techniques. The identification of each metabolite was made by applying different spectroscopic/spectrometric techniques (UV-V, IR, $^1$H-RMN, $^{13}$C-RMN, HRMS) [17, 18]. In previous studies we demonstrated that these AQs also exhibit photosensitizing properties by generation of O$_2^*$ (Type I mechanism) and/or 1O$_2$ (Type II mechanism) [19, 20], which are directly involved in the phototoxic effects that *H. pustulata* produces on cattle [21].

Following previous studies, we have focused our attention on studying the in vitro antibacterial and antifungal activity of enriched AQ extracts obtained from *H. pustulata*. To analyze further the results obtained, we evaluated the antibacterial activity of each isolated AQ, centering our interest on determining whether this effect was solely due to a photodynamic process or to a concurrent combination of light-driven and dark processes.
In addition, the minimal bactericidal concentration (MBC) was determined [17]. Cylindrical pieces (4 mm diameter) measuring the inhibition-zone diameter observed.

The antimicrobial activity of enriched AQ extracts was determined using the agar-well diffusion method modified according to the following experimental conditions, using nutrient agar for bacteria or Sabouraud agar for fungi [17]. Six-millimeter-diameter wells were punched into the agar and filled with different extract dilutions of a stock solution (1 mg/ml in EtOH) and solvent blanks or standard antibiotic solutions, according to the sensitivity of the microorganism tested [24]. The antimicrobial activity was evaluated by measuring the inhibition-zone diameter observed.

In addition, the minimal bactericidal concentration (MBC) was determined [17]. Cylindrical pieces (4 mm diameter) were extracted from the inhibition-zones of S. aureus produced by the highest concentration of the different H. pustulata extracts. The pieces were transferred to sterile tubes containing tryptose broth. The tubes were incubated at 26 °C for 24 h. A 100 ml aliquot of this broth was spread over Petri plates containing sterile nutrient agar. This was

<table>
<thead>
<tr>
<th>AQs</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
</tr>
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<tbody>
<tr>
<td>soranjiol</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>soranjiol 1-methyl ether</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>rubiadin</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>rubiadin 1-methyl ether</td>
<td>OCH₃</td>
<td>CH₃</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>dammacanthal</td>
<td>OCH₃</td>
<td>CHO</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>dammacanthol</td>
<td>OCH₃</td>
<td>CH₂-OH</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>2-hydroxi-3-methyl AQ</td>
<td>H</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>heterophylline</td>
<td>OH</td>
<td>CH₃</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
</tr>
<tr>
<td>pustuline</td>
<td>H</td>
<td>OH</td>
<td>OCH₃</td>
<td>H</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

Fig. 1 Anthraquinones structures used in this study.

2. In vitro antibacterial and antifungal activity of enriched AQ extracts obtained from Heterophyllaea pustulata.

Since the AQ-rich extracts obtained from several plant species have shown significant antimicrobial activity [22], we decided to evaluate the in vitro antibacterial and antifungal properties of enriched AQ extracts obtained from H. pustulata [17].

In order to prepare extracts, the air-dried plant material was separated into stems and leaves. Both were ground separately and extracted with benzene in a Soxhlet apparatus. Phytochemical studies have shown that benzenic stem extract has damnacanthal, damnacanthol, rubiadin, soranjidiol and rubiad in 1-methyl ether. Benzenic leaf extract has a similar chemical composition except for the absence of damnacanthal and damnacanthol, and the presence of soranjidiol 1-methyl ether, heterophylline, pustuline and 5,5'-bisoranjiol.

A screening with the benzenic stem and leaf extracts was performed in relation to their in vitro antibacterial activity against the reference strain Micrococcus luteus ATCC 9341, oxacillin-sensitive and resistant Staphylococcus aureus, coagulase-negative (c.n.). S. saprophyticus, Escherichia coli (two different strains), Proteus mirabilis, Pseudomonas aeruginosa (two different strains) and the fungal species Candida albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Cryptococcus neoformans, Aspergillus fumigatus, A. flavus, Trichophyton mentagrophytes, T. rubrum, and T. floccosum [17]. The clinical importance of these pathogenic species (oxacillin-resistant and sensible S. aureus; the later, a Beta-lactamase producer) should be stressed, since they participate in diverse infections such as abscesses, endocarditis, pneumonia, meningitis and osteomyelitis. In addition, Candida is the fourth germ isolated from hospital hemocultures, associated with 38% of mortality. C. albicans participates in cutaneous and deep candidiasis. C. krusei offers natural resistance to fluconazole, an antibiotic commonly administered in numerous treatments. C. parapsilosis represents the main cause of candidemia in children and catheter-bearing patients. Cryptococcosis and aspergillosis produce mortality rates higher than 95% and are transmitted by the inhalatory route, frequently affecting AIDS patients. T. mentagrophytes is associated with superficial skin, nail and hair mycosis [17].

The antimicrobial activity of benzenic stem and leaf extracts was determined using the agar-well diffusion method modified according to the following experimental conditions, using nutrient agar for bacteria or Sabouraud agar for fungi [17]. Except for M. luteus, all bacterial microorganisms were pathogenic strains isolated from patients with different infections. All fungal microorganisms were isolated from different lesions of patients from the Hospital de Clínicas (Buenos Aires), as described by Pérez et al. [23]. Six-millimeter-diameter wells were punched into the agar and filled with different extract dilutions of a stock solution (1 mg/ml in EtOH) and solvent blanks or standard antibiotic solutions, according to the sensitivity of the microorganism tested [24]. The antimicrobial activity was evaluated by measuring the inhibition-zone diameter observed.

In addition, the minimal bactericidal concentration (MBC) was determined [17]. Cylindrical pieces (4 mm diameter) were extracted from the inhibition-zones of S. aureus produced by the highest concentration of the different H. pustulata extracts. The pieces were transferred to sterile tubes containing tryptose broth. The tubes were incubated at 26 °C for 24 h. A 100 ml aliquot of this broth was spread over Petri plates containing sterile nutrient agar. This was
incubated at 36 °C for 24 h and the development of microorganisms was checked. The minimal extract concentration that reduces the viable bacteria to 1:1000 or less is considered MBC.

The benzenic stem extract shows selective activity on *Micrococcus luteus* ATCC 9341, oxacillin-sensitive and resistant *Staphylococcus aureus*, without acting against other Gram + as *S. saprophyticus* c.n. neither Gram – as *Escherichia coli* (two different strains), *Proteus mirabilis* nor *Pseudomonas aeruginosa* (two different strains). In this aspect the leaf extract showed a similar activity to that of the stem [17]. A bacteriostatic effect of the different *H. pustulata* extracts against oxacillin-sensible and resistant *S. aureus* is implied by the MBC/MIC (minimum inhibitory concentration) ratio, whose results are clearly higher than 1 [17].

Moreover, the antimicrobial spectrum of the benzenic stem extract proves interesting because of its activity against fungal species from nosocomial origin. Indeed, it was useful against fungal cultures isolated from different corporal lesions such as several strains of *C. albicans, C. krusei, C. parapsilosis, C. tropicalis, C. neoformans, A. fumigatus, A. flavus* and *T. mentagrophytes* (Table 1).

**Table 1** Antifungal activity of *Heterophyllaea pustulata* stem extract (1mg/ml)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Source material</th>
<th>Inhibition Diameter (mm)</th>
<th>I.A. (u)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract</td>
<td></td>
<td>Mz</td>
</tr>
<tr>
<td><strong>Opportunistic mycosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans I</em></td>
<td>hemoculture</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td><em>Candida albicans II</em></td>
<td>urineculture MC</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td><em>Candida albicans III</em></td>
<td>hemoculture 37203</td>
<td>18</td>
<td>46</td>
</tr>
<tr>
<td><em>Candida albicans IV</em></td>
<td>hemoculture 37204</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td><em>Candida albicans V</em></td>
<td>hemoculture 15564</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td><em>Candida albicans VI</em></td>
<td>hemoculture 16655</td>
<td>22</td>
<td>60</td>
</tr>
<tr>
<td><em>Candida albicans VII</em></td>
<td>hemoculture 1999</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td><em>Candida glabrata I</em></td>
<td>penis swab (candidemia by <em>C. glabrata</em>) 35234</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Candida glabrata II</em></td>
<td>hemoculture 35202</td>
<td>-</td>
<td>38</td>
</tr>
<tr>
<td><em>Candida glabrata III</em></td>
<td>hemoculture 36293</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td><em>Candida krusei I</em></td>
<td>laryngeal prothesis</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Candida krusei II</em></td>
<td>urineculture 38495</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Candida parapsilosis I</em></td>
<td>hemoculture 35416</td>
<td>22</td>
<td>40</td>
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<tr>
<td><em>Candida parapsilosis II</em></td>
<td>hemoculture 15760</td>
<td>25</td>
<td>30</td>
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<tr>
<td><em>Candida tropicalis</em></td>
<td>hemoculture 16819</td>
<td>18</td>
<td>35</td>
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<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>CSF (HIV patient) 20772</td>
<td>15</td>
<td>25</td>
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<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>nasal polyps (fungal sinusitis)</td>
<td>13</td>
<td>40</td>
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<tr>
<td><em>Aspergillus flavus</em></td>
<td>nasal swab (in leukemic patient)</td>
<td>21</td>
<td>40</td>
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<tr>
<td><strong>Superficial mycosis</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Trychophyton rubrum</em></td>
<td><em>Tinea pedis</em> (male patient)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Trychophyton mentagrophytes</em></td>
<td>toe nail</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td><em>Epidermophyton floccosum</em></td>
<td><em>Tinea pedis</em> (a year evolution in HIV patient)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Control: miconazole (MZ) (1 mg/ml). I.A.: inoculum absorbance at 580 nm. Inoculum dilution in the culture medium was 1/8 v/v. Values are means of 2 to 4 data that differ in less than 10 % from the mean. - = no activity detected

On the other hand, acute toxicity studies were performed by administration schedules using unique doses on female mice of CF1 strain, 3-month old and weight ranging between 31 and 40 g [17]. Because of the low solubility characteristics of the benzenic stem and leaf extract, a mixture of dimethylsulfoxide (DMSO)/H2O (3:1) (v/v) was selected among several solvents. The extract was administered subcutaneously in doses ranging from 60 to 4,000 mg/kg. The intravenous administration was performed through the tail veins with doses within 70 to 280 mg/kg range. In both cases, the control mice were injected with the corresponding vehicle. The toxicity signs, including death, were observed during 8 days following the extract administration. The LD<sub>50</sub> was estimated through the up-and-down method for small samples by Dixon [25].
Upon subcutaneous administration, the mice tolerated doses of up to 4,000 mg/Kg body wt. without signs of toxicity, indicating a LD<sub>50</sub> equal or higher than that level. With the aim of determining LD<sub>50</sub> values and taking into account the difficulty of obtaining the extract, acute toxicity following intravenous administration was studied. We established a LD<sub>50</sub> value of 123 mg/Kg body wt. without higher signs of toxicity in the surviving mice. In both cases, the benzenic extract of stem and leaf appears to exhibit similar patterns [17].

In conclusion, the AQ-rich extracts have proved antibacterial and antifungal effect, without a manifest toxicity in experimental animals at the concentration assayed.

Some of these experiments were performed in the Department of Pharmacology, School of Dentistry, University of Buenos Aires, under the direction of Dr. Cristina Pérez.

3. Evaluation in vitro of biological effects of each AQ isolated from H. pustulata.

3.1 In vitro antibacterial activity of each AQ

Considering the above results of the antimicrobial activity of AQ-rich extracts, obtained from H. pustulata, we evaluated the in vitro antibacterial activity of each AQ against a Gram (+) bacterium with the aim of establishing the metabolites responsible for the effect shown by the extracts.

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 according to international standards from the Clinical and Laboratory Standards Institute (CLSI) by means of broth macrodilution method in Mueller-Hinton medium (MH, Britania) [26]. The strain used for this purpose was S. aureus ATCC 29213 (1x10<sup>5</sup> colony-forming units (cfu)/ml). The working solutions of each AQ were prepared by means of serially diluted from 256 µg/ml up to 0.125 µg/ml. MIC determination was carried out after 24 h of incubation at 37 ºC by observing turbidity.

Our studies led to the conclusion that only soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol showed susceptibility to MICs within a 32-64 µg/ml range.

Bearing in mind that for many substances having antibacterial activity there are mechanisms that elicit a physiological response in bacteria by producing reactive oxygen species (ROS) [27-29], our objective is demonstrated if the antibacterial effect shown by soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol (through the determination of the MIC) is directly linked to the increase of O_2<sup>-·</sup> and/or 1O_2 levels. Nevertheless, it should be noted that some photosensitizers also generate bacterial photoinactivation mainly by means of the generation of ROS, particularly singlet molecular oxygen (1'O_2), but through a photodynamic photosensitization [13]. Therefore, as the AQs studied are photosensitizing agents, we also assessed whether the irradiation causes photosensitization by these AQs, which consequently increases the production of ROS and its antibacterial effect.

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 µg/ml), specifically was selected the subtoxic doses of 10 µg/ml because higher concentrations proved to be toxic for normal mammal cells (African green monkey kidney cells –Vero) (see results below) [30].

The four AQs that showed susceptibility to S aureus (soranjidiol, rubiadin, damnacanthal and 5,5'-bisoranjidiol) were examined to ascertain whether this activity was associated with an increased generation of O_2<sup>-·</sup> with respect to the basal production. The O_2<sup>-·</sup> determination was based on the NBT assay [26].

Two assays were simultaneously performed in darkness. One was carried out in the absence of AQs to measure the basal level of the O_2<sup>-·</sup> production in the bacteria (control). The second preformed by adding AQs to the culture medium, in order to establish whether these compounds induce an increase in the O_2<sup>-·</sup> 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<sup>9</sup> cfu/ml). In the NBT assay, the bacterial suspension was incubated with each AQ solution and NBT at 37 ºC in darkness. 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 measured as a function of time [26].

Moreover, the same set of AQs used in the preceding paragraph was examined to evaluate an increase of 1O_2 generation 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 [31]. This compound reacts chemically with the generated 1O_2 with a rate constant (k<sub>r</sub>) of 2.1 x 10<sup>7</sup> L mol<sup>-1</sup>s<sup>-1</sup> [32]. 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 1O_2 basal production generated by bacteria (control), we incubated bacterial suspension (10<sup>9</sup> 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.

By using sodium azide (NaN$_3$) as a $^{1}\text{O}_2$ physical quencher ($k_q = 5.8 \times 10^8$ L mol$^{-1}$s$^{-1}$) we can confirm whether the consumption of MET is a direct consequence of the $^{1}\text{O}_2$ generation in the medium [33]. Due to its large quenching constant, NaN$_3$ was added in a concentration high enough 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. 2 shows the increase in percentage of $\text{O}_2^{-\cdot}$ in $\text{S. aureus}$ 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. As observed, there is no clear indication of $\text{O}_2^{-\cdot}$ at 10 min, neither for darkness nor for irradiation. At 20 and 40 min, all the AQs induce an increase in $\text{O}_2^{-\cdot}$ production with respect to the basal situation in darkness. Except for damnacanthal, this effect is more noticeable at longer times. Under actinic irradiation, there is a further increase in $\text{O}_2^{-\cdot}$ production with respect to basal situation with the same exception shown previously.

Results are given as mean ± SD, n=3.

a $p<0.05$ measured with respect to darkness

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

Fig. 2 NBT assay. Increase in percentage of $\text{O}_2^{-\cdot}$ in $\text{S. aureus}$ ATCC 29213 with respect to basal situation for every AQ and time measured.

Fig. 3 shows the MET consumption caused by the $^{1}\text{O}_2$ generated in $\text{S. aureus}$ under the conditions outlined in i, ii, iii (evaluation of the $^{1}\text{O}_2$ generation), both for darkness and under actinic irradiation for 5,5'-bisoranjidiol. As can be observed, in the absence of AQ, small amounts of $^{1}\text{O}_2$ are produced due to the normal breathing process. 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 control experiments. These results confirm that MET consumption is a direct consequence of the $^{1}\text{O}_2$ generation. The preceding discussion is 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.
Fig. 3 Consumption of MET caused by $^{1}\text{O}_2$ in *S. aureus* ATCC 29213 treated with 5,5'-bisoranjidiol (10 µg/ml), in darkness and under irradiation. Average of three experiments.

Fig. 4 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 comparison of 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.

Lastly, bearing in mind that, while those compounds that can reduce a minimum of $10^3$ cfu/ml (3.0 Log$_{10}$) are considered bactericidal agents, those below that range (which merely inhibit growth) are considered bacteriostatic agents [27, 34, 35], we carried out assays with the purpose of establishing whether the AQs show any of such characteristics. With this objective, *S. aureus* ATCC 29213 ($10^8$ cfu/ml) was incubated in triplicate with each AQ (damnacanthal,
rubadin, soranjidiol and 5,5'-bisoranjidiol) at 37 °C independently in darkness and under irradiation, replicating also the whole set to be measured at 10, 40 and 90 min [26]. 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 1O2 production by a chemical quencher changes the reduction in the number of cfu/ml. A bacterial suspension (108 cfu/ml) with 5,5'-bisoranjidiol under irradiation was treated with MET, after 40 min of incubation, allowing the system to proceed normally and measuring the cfu/ml at 90 min.

Fig. 5 shows Log10 change in cfu/ml of S. aureus treated with each AQ in darkness, including the control. As noted, a decrease, more pronounced for 5,5'-bisoranjidiol, is seen after an initial increase. This reduction could relate to the capability of producing a physiological response in bacteria that generate O2•− (Fig. 2) as well as 1O2 (Fig. 3) 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 Log10 of cfu/ml at 90 min. Rubadin and soranjidiol produce a reduction of 2.2 and 2.0 Log10 of cfu/ml, respectively. The increase in O2•− production for rubadin and soranjidiol is similar to that of 5,5'-bisoranjidiol (Fig. 2). Nevertheless, the increase in 1O2 generation is lower than the bianthraquinone. For damnacanthal, only an inhibitory effect without producing death is noticed.

![Fig. 5 Log10 change in colony-forming units per milliliter (cfu/ml) of S. aureus ATCC 29213 treated with each AQ (10 µg/ml) in darkness.](image)

![Fig. 6 Log10 change in colony-forming units per milliliter (cfu/ml) of S. aureus ATCC 29213 treated with each AQ (10 µg/ml) under irradiation.](image)

On the other hand, the actinic radiation and the consequent O2•− and 1O2 increase by means of a photosensitization phenomenon (Figs. 2 and 4), further increased the reduction of cfu/ml for all AQs, except damnacanthal (Fig. 6).

These results clearly demonstrate that 5,5'-bisoranjidiol shows higher antibacterial activity than that of rubadin and soranjidiol, although the three increase O2•− production at about the same level as a function of time (Fig. 2) regardless that they are acting in darkness or under irradiation. In contrast, the 1O2 production for 5,5'-bisoranjidiol is high irrespective of conditions (darkness or irradiation) [26]. This would suggest that, among the different species that comprise ROS, 1O2 is the one mainly involved in the bactericidal effect, as already reported by Becerra et al. [31] using precisely S. aureus. This becomes evident since, after 40 min of incubation, the addition of MET to a sample having 5,5'-bisoranjidiol shows that even under irradiation, the cfu/ml reduction is completely suppressed at 90 min (open squares, Fig. 6).

In conclusion, these assays allowed us to identify the AQs: rubadin, soranjidiol, damnacanthal and 5,5'-bisoranjidiol as the compounds responsible for the antibacterial effects shown by AQ-rich extracts obtained from the phototoxic vegetal species, H. pustulata. In addition, our results suggest that the antibacterial effect on S. aureus, found for these AQs, is closely linked to the increase in O2•− and/or 1O2 levels. This increase could result from the interaction between bacteria and the AQs without needing light, i.e., without a photosensitizing process to produce a physiological response eliciting O2•− and 1O2. Actinic irradiation, on the other hand, generates photosensitization for rubadin, soranjidiol and 5,5'-bisoranjidiol, consequently increasing their antibacterial effects (particularly bactericide). This effect is, in turn, suppressed when a specific quencher of 1O2 is added, thus suggesting that the bactericidal activity derives mainly from that particular ROS as already proposed in other reports [36].

Most importantly, the particular bactericidal activity shown under irradiation for rubadin, soranjidiol and 5,5'-bisoranjidiol at the concentration used, which does not affect normal mammal cells (subtoxic concentration - see results above), led us to consider these AQs as potential agents for photodynamic antibacterial chemotherapy treatments.
Some of these experiments were performed interdisciplinarily with Dr. Inés Albesa (Laboratory of Pharmaceutical Microbiology, Department of Pharmacy) and Dr. Gustavo A. Argüello (Department of Physical Chemistry), School of Chemical Sciences, National University of Cordoba.

3.2 Cytotoxic evaluation in vitro of each AQ

Prior to the antibacterial tests of each AQ, it was necessary to evaluate the cytotoxicity of these compounds on a mammalian eukaryotic cell line (Vero cells) [37], with the aim of establishing the non-cytotoxic concentration range for evaluating the antimicrobial effect of each AQ.

For this purpose, African green monkey kidney cells (Cercopithecus aethiops, Vero 76 ATCC CRL-587) were used. The cytopathic effect produced by each AQ on the morphology of Vero cells was observed by optical microscopy [38]. From a stock solution of each AQ (1 mg/ml in PBS with 1% DMSO as co-solvent), 15 consecutive dilutions were prepared with PBS, within a range of 1 to 30-50 µg/ml according to AQ solubility. Each dilution was inoculated in duplicate on a confluent cell monolayer (2.5 ± 0.6 x 10⁵ cells/ml, 48 h incubation). Cell controls (CC), containing only maintenance medium (MM; Eagle’s minimum essential medium (MEM) with 2% Fetal calf serum (FCS), 1% L-glutamine, gentamicine (50 µg/ml) and 1% DMSO), were included. The cells were incubated at 37 °C during 72 h, and the development of cellular alterations such as rounding, membrane retraction, cell detachment and the presence of granules in the cytoplasm was daily observed [38].

The cellular viability (CV) depending on the concentration of each AQ was measured by means of the uptake Neutral Red (NR) assay [39]. Each dilution was inoculated in triplicate on a confluent monolayer of cells (2.5 ± 0.6 x 10⁵ cells/ml). The absorbance of the NR extracted after 48 h of incubation at 37 °C was measured at 540 nm on a microplate reader (BioTek ELx800). The percentage of cellular viability (CV%) was calculated by comparison with CC (100% viability). The concentration of compound that reduces the viable cells to 50% (CC₅₀) was determined by regression (R² > 0.9) from the plot of cellular viability percentage vs. AQ concentration [39]. Maximum Non-Cytotoxic Concentration (MNCC) was defined as the maximal sample concentration showing more than 90% viable cells and exerting no cytotoxic effect as detected by microscopic monitoring [40].

Our results showed that, whereas rubiadin 1-methyl ether, dammacanthol and pustuline stand out by showing MNCC values within a 16 and 22 µg/ml range, this concentration was not higher than 10 µg/ml for the remaining AQs (Table 2). Thus, the MNCC for soranjidiol 1-methyl ether, heterophylline and 5,5'-bisoranjidiol was near 10 µg/ml, and approximately 6 µg/ml for soranjidiol, rubiadin and dammacanthal (Table 2).

Table 2 Cytotoxic Concentration to 50% (CC₅₀), Maximum Non-Cytotoxic Concentration (MNCC) and Subtoxic Concentration for each AQ tested on Vero cells.

<table>
<thead>
<tr>
<th>AQs</th>
<th>CC₅₀ (µg/ml)</th>
<th>MNCC (µg/ml)</th>
<th>Subtoxic concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rubiadin 1-methyl ether</td>
<td>34.4 ± 0.2</td>
<td>22.2 ± 0.4</td>
<td>26.8 ± 0.1</td>
</tr>
<tr>
<td>dammacanthol</td>
<td>33.7 ± 0.2</td>
<td>19.6 ± 0.2</td>
<td>23.9 ± 0.1</td>
</tr>
<tr>
<td>pustuline</td>
<td>nc</td>
<td>16.1 ± 0.3</td>
<td>22.3 ± 0.1</td>
</tr>
<tr>
<td>soranjidiol 1-methyl ether</td>
<td>27.1 ± 0.2</td>
<td>10.5 ± 0.3</td>
<td>18.4 ± 0.1</td>
</tr>
<tr>
<td>heterophylline</td>
<td>23.69 ± 0.04</td>
<td>9.7 ± 0.2</td>
<td>15.64 ± 0.04</td>
</tr>
<tr>
<td>(S)-5,5'-bisoranjidiol</td>
<td>22.7 ± 0.1</td>
<td>9.5 ± 0.2</td>
<td>13.9 ± 0.2</td>
</tr>
<tr>
<td>dammacanthal</td>
<td>20.1 ± 0.1</td>
<td>6.7 ± 0.2</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>soranjidiol</td>
<td>17.5 ± 0.2</td>
<td>6.3 ± 0.2</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td>rubiadin</td>
<td>14.9 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td>8.2 ± 0.1</td>
</tr>
</tbody>
</table>

nc: not calculated
a p < 0.05 calculated with respect to b
b p < 0.05 calculated with respect to c

Similar results were obtained by analyzing the CC₅₀ determined for each AQ (Table 2), which established that rubiadin 1-methyl ether was less cytotoxic, followed, in increasing order of cytotoxicity, by dammacanthol, pustuline, soranjidiol 1-methyl ether, heterophylline, 5,5'-bisoranjidiol, dammacanthal, soranjidiol and rubiadin. The CC₅₀ of pustuline was not estimated because concentrations higher than 30 µg/ml could not be tested due to solubility problems; however, its MNCC was established. In addition, a subtoxic concentration was determined for all AQs tested (Table 2), defined as the concentration that causes 10 - 20% cellular death [41] and produces slight morphologic changes observed by microscopy.

In addition, in previous studies we demonstrated that some of the AQs tested had the ability to increase the O₂⁻* production in human leukocytes [19, 20]. Considering that this effect could generate the cytotoxic activity of AQs on Vero cells, the ability of each AQ to produce this ROS and its relation to the cytotoxic effect were evaluated. To this aim, the NBT reduction bioassay was performed and each AQ was evaluated at 10 µg/ml, which represents a non-cytotoxic or subtoxic concentration depending on the compound studied [42, 43].
Thus, rubiadin 1-methyl ether, damnacanthol and pustuline did not increase the production of $\text{O}_2^•$ at 10 $\mu$g/ml (Fig. 7) since it is a non-cytotoxic concentration for these AQs (approximately 95% CV) without evidencing any cytotoxic effect [30]. For the other AQs, this amount corresponds to a subtoxic value, increasing the generation of $\text{O}_2^•$ (Fig. 7). It was noted that those compounds producing a large increase in the $\text{O}_2^•$ generation at 10 $\mu$g/ml (soranjidiol and damnacanthal, Fig. 7) exhibit a low CV% (about 80%) with increased cell damage [30]. However, those AQs that at the same concentration showed a small increase in the $\text{O}_2^•$ production (soranjidiol 1-methyl ether, heterophylline, 5,5'-bisoranjidiol and rubiadin), reveal a high CV% (between 86 and 90%), except for rubiadin [30]. In general, we might conclude that an increased production of $\text{O}_2^•$ causes an important cytotoxic effect as observed by microscopy, with a concomitant decrease in cellular viability. Rubiadin is excepted from this behavior, producing a significant cytotoxic effect at 10 $\mu$g/ml, which results in a significant decrease in CV (30%), despite having a low production of $\text{O}_2^•$ (Fig 7) [30]. We may therefore assume the presence of another mechanism in the cytotoxicity of this compound. In addition, when each AQ was tested at their CC$_{50}$, the increase in $\text{O}_2^•$ production was not the same in all AQs. Therefore, the intracellular increase of this ROS would not be the sole cause for the loss of cellular viability at CC$_{50}$.

**Fig. 7** NBT assay. Increase in percentage of $\text{O}_2^•$ in Vero cells with respect to basal situation, produced by two different concentrations of every AQ.

Finally we carried out an AQ incorporation assay in Vero cells [19], which constitutes a spectrophotometric determination of intracellular content of AQs needed to stimulate $\text{O}_2^•$ production and other biologic effects. Soranjidiol was chosen to study the incorporation of AQs in Vero cells because this AQ is the predominant compound in the aerial parts of *H. pustulata*. Thus, by means of this assay, we have established that soranjidiol enters Vero cells, 29 ± 3% with respect to the initial concentration after 30 min incubation. Although only a single AQ was tested, no significant differences are expected in the rate of incorporation for the other AQs since they all have similar partition coefficients [44].

In conclusion, this work allowed us to establish the concentration range where each AQ exhibits low or no cytotoxic effect and therefore, these concentrations may be used in order to test their potential antimicrobial effects. From the nine AQs tested, we were able to identify three derivatives: rubiadin 1-methyl ether, damnacanthol and pustuline, with low or no cytotoxicity (95 ± 5% VC) in a concentration range limited by the MNCC (Table 2). The estimation of the subtoxic concentration for the other AQs (soranjidiol, soranjidiol 1-methyl ether, rubiadin, damnacanthal, heterophylline and 5,5'-bisoranjidiol) allowed us to consider that a concentration of about 10 $\mu$g/ml could be used to test different biological activities, since this concentration ensured in our experiments more than 80% CV (Table 2).

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