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Photodynamic activity of natural anthraquinones on fibroblasts

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ABSTRACT

Natural anthraquinones (AQs) isolated from *Heterophyllaea lycioides* (Rusby) Sandwith (Rubiaceae) demonstrated to have photodynamic properties: soranjididol (Sor), 5-Chlorosoranjidiol (5-ClSor), bisoranjidiol (Bisor), 7-Chlorobisoranjidiol (7-ClBisor) and lycionine (Lyc). Sor, 5-ClSor and Bisor exhibited photodynamic inactivation on bacteria and parasites. As they could be used in topical application, the aim of this work was to study their photodynamic activity on fibroblasts.

AQs were tested at 2.5 μM in darkness and under irradiation conditions. They were photoactivated with violet-blue LED ($\lambda = 410 \pm 10$ nm; fluence rate =50 mW/cm^2) and exposure time corresponded to a fluence of 27 J/cm^2. Negative and positive control (–C and +C, respectively) were included. Mitochondrial activity was determined by using MTT assay that is a measure of the cell viability and it was expressed as a percentage respect to –C (% CV).
Results showed that AQs in darkness conditions showed similar metabolic activity as –C, except for 5-ClSor (about 75% CV). Under irradiation, AQs exhibited dissimilar results. Sor and 7-ClBisor maintained cell viability at approximately 100%, Bisor and Lyc around 70%, whereas 5-ClSor reduced cell viability by 90%. Taken together, our results suggest that Sor could mediate photodynamic therapy (PDT) in cutaneous infections since no toxicity was observed in fibroblasts. On the other hand, 5-ClSor could be used for topical PDT of keloids and hypertrophic scars.

Keywords: natural anthraquinones, photosensitizers, fibroblast, violet-blue LED.

1. INTRODUCTION

Photodynamic therapy (PDT) arises as an innovative and alternative method for the treatment of various malignant and nonmalignant skin diseases as acne, psoriasis and other cutaneous infections [1,2]. Topical PDT refers to the topical application of a non-toxic drug or dye known as a photosensitizer (PS) at the injured site, followed by irradiation with a light source of specific wavelength. The mechanism in PDT involves the production of reactive oxygen species (ROS) by two mechanisms: Type I, production of superoxide anion radical ($O_2^{\cdot-}$) and Type II, $^1O_2$ generation [3,4]. These cytotoxic species cause cell death by oxidative stress [1].

Anthraquinone derivatives (AQs) are an important family of natural compounds that are known because of their biological properties as well as their application as dyes in industry [5]. In addition, they are characterized to present a maximum absorption (λ_{max}) in the visible region (410-420 nm) [6]. Study of natural AQs obtained from *Heterophyllaea pustulata* Hook f. demonstrated they have the ability to produce efficiently ROS in presence of light [7,8]. Until the present, several *in vitro* studies have been conducted to determine their potential photodynamic effects on cancer cells [9,10], bacteria [11], fungus [12,13] and virus [14] with promising results.

Taking into account those results, chemical study of *H. lycioides*, another species that belong to *Heterophyllaea* genus [15] was carried out. Seven AQs were isolated, and three of them were reported as novel for this family of compounds: 5-Chlorosoranjidiol (5-ClSor), 7-Chlorobisoranjidiol (7-ClBisor) and lycionine (R-2-hydroxymethyl-2´methyl-1,1´,6,6´-tetrahydroxy-5,5´-bianthraquinone, Lyc) [16]. It was demonstrated that they produce an increase in $O_2^{\bullet-}$ levels under irradiation, showing the same mechanism as soranjidiol (Sor) and 5,5´-bisoranjidiol (Bisor), AQs obtained from *H. pustulata* [8].

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Thinking in a topical treatment, we found interesting to study their photodynamic effect on dermal fibroblasts. In this work, five anthraquinones were tested: Sor, Bisor, 5-ClSor, Lyc and 7-ClBisor (Fig. 1) that were photoactivated using violet-blue LED.

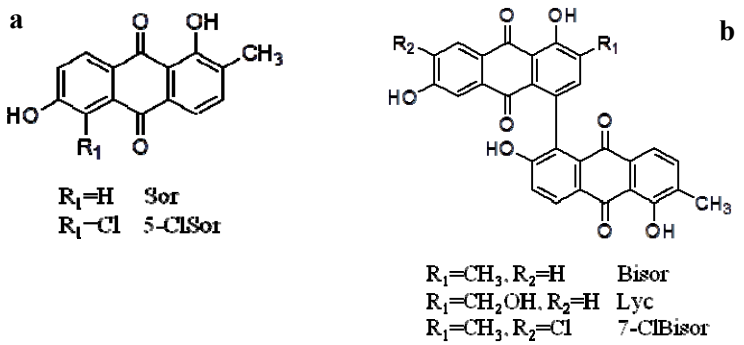

Fig. 1 Natural anthraquinones structure: monomer AQ (a) and bianthraquinones (b).

2. METHODOLOGY

2.1 Natural anthraquinones

AQs were obtained from *H. lycioides* as was described in Dimmer et al., 2017 [16]. They were purified by applying successive chromatographic techniques in order to obtain a purity above 90% that was determined by high-performance liquid chromatography (HPLC). For that, a Varian Pro Star chromatography apparatus (model 210, Agilent, CA, USA), equipped with an UV-Vis detector and a Microsorb-MV column 100-5 C-18 (250 × 4.6 mm i.d.) was used at 25 °C. The mobile phases consisted of ultrapure water (solvent A) and MeOH (solvent B) both with 0,62% V/V of formic acid. The following gradient elution program was used: 0-3 min, 50%; 3-5 min, 50- 80% B; 5-46 min, 80%; 46-47 min, 80-100% B; 47-61 min, 100%, 61-63 min, 50% B. A flow rate of 0.4 mL/min was used and the detector was set at 269 nm. Samples were dissolved in HPLC-MeOH, filtered through cellulose (Merck Millipore, Sao Paulo, Brazil) and manually injected (20 μL). Data analysis was performed using the Varian software (Star Chromatography Workstation 6.41).

For the assay, AQs were dissolved in phosphate buffered saline (PBS) solution and dymethyl sulfoxide (DMSO) as co-solvent, making sure that the final concentration of DMSO was below 1 %, that is considered nontoxic for cells and parasites. Solutions were filtered through a sterile 0.22 μm filter membrane.

2.2 Fibroblast culture and conditions

Experiments were performed with human fibroblasts FN1 kindly provided by Prof. Durvanei Augusto Maria (Butantan Institute - Brazil). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEN, Gibco, USA), supplemented with 10 % fetal bovine serum (Sigma, USA) 100 μ/mL penicillin and 100 μ/mL streptomycin (Sigma, USA) at 37 ºC with 5 % CO_2 humidified air. The cells were passaged using 0.25 % trypsin (Sigma, USA) in PBS solution. After cells reached confluence, they were centrifuged at 1500 rpm and plated with $5x10^6$ cells.

2.3 PDT assay in fibroblast line

AQs were tested at 2.5 μM in darkness (D AQ) and under irradiation conditions (LED + AQ). A pre-irradiation time of 10 min was used. For irradiation, a violet-blue LED (λ = 410 ± 10 nm) with a radiant exposure of 27 J/cm^2 was placed above the samples (Fig. 2). An outpower of 157 mW was measured by a power meter (Fieldmate, PM10, Coherent, CA,

USA) to give a constant irradiance of 50 mW/cm^2. A non-treated group (negative control, -C) and cells treated with sodium dodecyl sulfate (SDS) 2% (positive control, +C) were included. Once finished, solution of each well was removed and fresh medium with 10 % of fetal bovine serum was added.

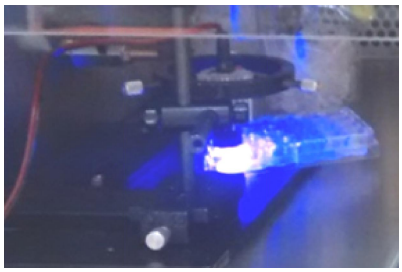

Fig. 2 Samples irradiated with violet-blue LED

2.4 MTT assay

After 24 h of incubation, mitochondrial activity (MA) was measured by using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a colorimetric assay. In each experimental condition, medium was removed from each well and 40 µL of MTT solution (5 mg/mL-Sigma, USA) was added. After four hours of incubation at 37 °C, the solution was removed and then 100 µL of DMSO was added. Thirty minutes later, each well was homogenized and the optical density (OD) was measured with a microplate reader (SPECTRAMAX-M4, Molecular Devices, Sunnyvale, CA – USA) at 570 nm. Results were expressed as the mean percentage of mitochondrial activity (% MA) respect to –C (100 %), as a measure of cell viability (CV).

2.5 Statistical analysis

Data are presented as mean values ± standard deviation (SD). All data obtained were submitted to ANOVA test using GraphPad Prism 5.03 software. Mean comparisons were carried out with Bonferroni post-test and the overall significance level was set at $p < 0.05$.

3. RESULTS

Results established that AQs in darkness conditions exhibit similar MA as –C (100%), except for 5-ClSor that showed about a 75% MA. Under irradiation, AQs exhibited dissimilar results. On the one hand, both monomeric AQs tested showed opposite results: Sor maintained the 100% MA, whereas 5-ClSor reduced CV at 90% (Fig. 2a). On the other hand, one of the bianthraquinones (biAQs) resulted to be less toxic for fibroblast cells; 7-ClBisor kept the CV at 100%, whereas Bisor and Lyc reduced the MA at about 30% (Fig. 2b).

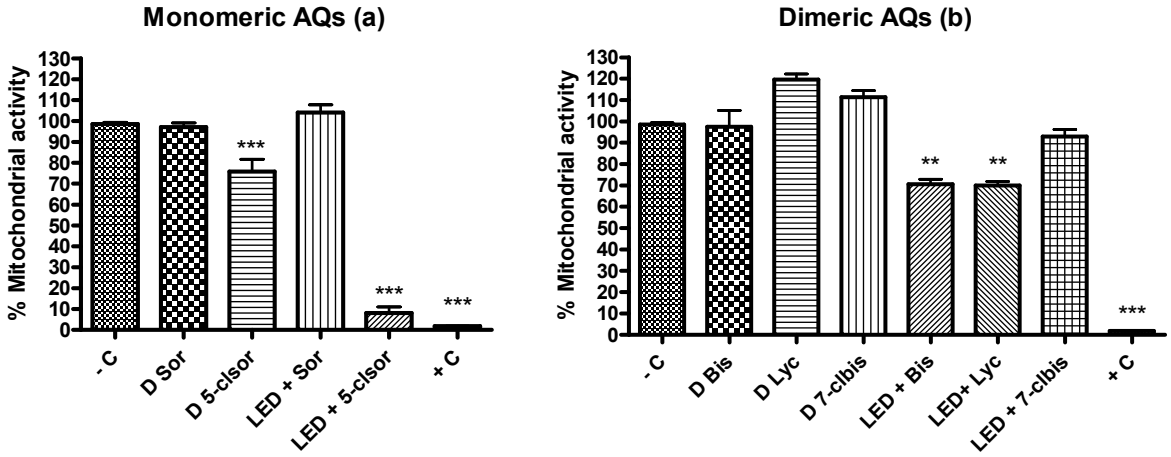

Fig. 3 Fibroblast cell line treated with natural monomeric (a) and dimeric (b) AQs. **p<0,01/***p<0,001 *vs.* negative control.

4. DISCUSSION

Antimicrobial PDT is an emerging approach to manage cutaneous leishmaniasis (CL) since no side effects, contraindications and parasite resistance have been reported. Thus, in this work we investigated the photodynamic activity of natural AQs on fibroblasts aiming their use in CL.

Considering the AQ structures, two of them are monomer (Sor and 5-ClSor) whereas the other three are biAQs (Bisor, Lyc, and 7-ClSor) constituted by a monomer of Sor linked in position 5-5´ with another monomer different from Sor. It is known that monomeric compounds are more efficient structures in the production of $^1O_2$ since biAQs present a deactivation mechanism that is given by the rotation of the 5,5' bridge [17].

Interestingly, regarding the monomeric compounds, Sor and 5-ClSor showed dissimilar results. Sor did not affect the metabolic activity of fibroblasts while 5-ClSor showed high cytotoxicity in the same way as +C. In this case, we hypothesized that the presence of chlorine atom increases the photodynamic effect on fibroblasts, because this moiety produces an increase in $^1O_2$ production [18].

On the other hand, biAQs exhibited more similar activity. In fact, 7-ClBisor did not show any cytotoxic effect on fibroblasts. In comparison, Bisor and Lyc could be also considered non-cytotoxic since they promoted less than 30% of reduction of fibroblast viability [19].

Recent study by Dimmer showed that Sor, 5-ClSor, and Bisor associated to blue LED were able to inactivate *Leishmania* amazonensis [20]. In contrast, Lyc and 7-ClBisor did not exhibit any leishmanicidal activity. Taking together, our results indicate that Sor is a promising agent for CL due to its significant killing of parasites and no activity on fibroblasts.

It is also important to notice that 5-ClSor decreased the fibroblast viability significantly. Although further studies are needed, we hypothesize that 5-ClSor could be a promising photosensitizer to mediate PDT in abnormal skin scarring since PDT has been reported as therapeutic strategy for keloids and hypertrophic scars [21,22].

5. CONCLUSIONS

Taken together, our results suggest that Sor could mediate antimicrobial PDT in skin infections since no toxicity on dermal fibroblasts was observed. In addition, the chlorinated monomer could be a novel photosensitizer to be used in the photodynamic treatment of fibrosis skin diseases.

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