Journal of Photochemistry & Photobiology, B: Biology 160 (2016) 154–162

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Antimicrobial photodynamic effect of extracts and oxoaporphine alkaloid isomoschatoline from *Guatteria blepharophylla*

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ARTICLE INFO

Article history:
Received 28 January 2016
Accepted 11 April 2016
Available online 14 April 2016

Keywords:
Antimicrobial photodynamic therapy
Antimicrobial resistance
Guatteria blepharophylla
Isoquinoline alkaloids
Oxoaporphine alkaloid isomoschatoline

ABSTRACT

Photodynamic Therapy, a tumor therapy idealized at the beginning of the last century, emerges nowadays as a promising treatment alternative against infectious diseases. In this study we report a bioguided study of *Guatteria blepharophylla* phytoderivatives for antimicrobial PDT. Crude extracts and fraction from the species bark were obtained and further fractionated for substances isolation. All samples were evaluated in relation to their photophysical (absorbance and fluorescence) and photochemical properties (1,3-DPBF bleaching method). Then, bioassays were conducted using as biological models bacteria and yeast strains and a diode laser as a light source. Phytochemical analyses lead to the isolation of 5 isoquinoline alkaloids from oxoaporphine subclass, denominated GB1 to GB5. Photophysical and photochemical analysis showed that extracts, fraction and GB1 (isomoschatoline) presented absorption profile with bands at 600–700 nm and were positive for singlet oxygen production. Photobiological assays indicate that these samples presented photodynamic antimicrobial activity against both gram-positive and gram-negative bacterial and some *Candida* ssp. yeast strains at sub-inhibitory concentrations. The susceptibility of gram-negative bacteria was significantly enhanced when $CaCl_2$ or $MgCl_2$ were employed. Greater energy doses and double sample's dosage also decreased microbial survival. It is suggested that GB1 photodynamic activity happens through both types I and II photochemical mechanisms, but with a predominance of the latter. Phytoderivatives of *G. blepharophylla* promoted antimicrobial effect, however more detailed study concerning chemical composition of the crude extracts and fractions as also photophysical and photochemical characteristics of GB1 are necessary to ensure their potential as photosensitizers at antimicrobial photodynamic inactivation.

1. Introduction

Guatteria Ruiz et Pav. is the largest genus within the Annonaceae family and includes approximately 300 species. It is represented by shrubs and small trees, distributed along South America, especially in the lowlands of Brazilian Amazon. The species is popularly known as 'envieiras' due to the presence of long tough fibers in the bark. Some of them are used in traditional medicine for different purposes, such as the seeds of *G. ouregou* applied to treat dyspepsis and stomach-ache and the bark of *G. guianensis* is used against fever. Despite the importance of them in folk medicine genus remains virtually untouched from the phytochemical and pharmacological point view [1].

Guatteria blepharophylla Mart. *in* Mart. [synonymy *Guatteriopsis blepharophylla* (Mart.) R.E. Fr.] is one of the few species that have been chemically investigated and the studies have shown its rich content in oxoaporphine alkaloids, a subclass within the aporphine class [2–3]. Several biological activities have been described for the isoquinoline alkaloids. Among aporphine class antiparasitic, antiplatelet aggregation, vasodilating, antioxidant, antiviral [4], antimicrobial [5–7], leishmanicidal [8] and antitumor activities can be mentioned [9–10]. A more recent study shows the ability of the oxoaporphine alkaloid to produce singlet oxygen and its application as a photosensitizer in PDT [11].

PDT is a treatment technique that relies on light association with photoactivatable drugs (photosensitizer) and has proved versatile for the treatment of various pathological conditions such as cancer, infectious diseases (bacterial, fungal, parasitic and viral), psoriasis, dermatosis and against macular degeneration [12–17]. Once activated by the light, the photosensitizer reaches its triplet state, which in turn transfers the energy to molecular oxygen present in the target cell leading the

Abbreviations: 1,3-DPBF, 1,3-diphenylisobenzofuran; CFM, confocal fluorescent microscopy; GBCH, bark hexane extract; GBCM, bark methanol extract; GBFA, alkaloid fraction; HMBC, Heteronuclear Multiple Bond Correlation; HMQC, Heteronuclear Multiple Quantum Coherence; TLC, thin layer chromatography; TMS, Tetramethylsilane; SEM, Scanning Electron Microscopy.

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http://dx.doi.org/10.1016/j.jphotobiol.2016.04.010
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formation of ROS (Reactive Oxygen Species), such as superoxide and hydroxyl radicals (type I mechanism) and singlet oxygen (type II mechanism) [18]. These highly reactive chemical species can react with proteins, lipids, nucleic acids and other cellular components damaging their structures and therefore, resulting in the target cell death.

Compared to conventional antimicrobial treatments, antimicrobial PDT (also called Photodynamic Antimicrobial Chemotherapy or even Photodynamic Inactivation) presents several advantages, such as, promotion of rapid cell death; ability to inactivate antibiotic-resistant strains; prevention to the development of new antimicrobials and antifungals resistant forms (due to multiplicity of target molecules); the possibility to design pharmaceutical formulations, with specific action, on the infected area and the absence of undesirable drug interactions [19–20].

Within this context, the aim of this study was to perform Bioactivity-Guided Study of hexane and methanol extracts from the bark of *G. blepharophylla* to evaluate their photosensitizing potential in antimicrobial PDT, as well as isolate and identify the substances responsible for the photochemical effect. This research is part of a larger study focused on the identification of bioactive extracts and natural photosensitizers in Brazilian plants.

2. Material and Methods

2.1. Chemicals

Solvents (analytical grade) and reagents used for plant extraction, compounds isolation, photophysical and photochemical analyses were purchased from Merck (Darmstadt, Germany). Silica gel (60 Å, mesh 70-230, 0.063–0.200 mm) was purchased from Fluka® Analytical (Sigma Aldrich, St. Louis, MO, USA) and TLC plates from Macherey-Nagel (Düren, Germany). Syringe filters (22 μm) were obtained from EDM Millipore Chemicals (Billerica, MA, USA). 1,3-dimetilisobenzofuran (1,3-DPBF) was purchased from Sigma Aldrich (Saint Louis, MO, USA). A stock solution was prepared in Dimetylsulfoxide (DMSO) a few minutes before use and manipulated in the dark to avoid photobleaching. Microorganism culture media were purchased from Becton, Dickinson and Company (Sparks, Maryland, USA) and storage at 25 °C.

2.2. Plant Material

Stem Bark of *G. blepharophylla* was collected from plants at Federal University of Amazonas (UFAM) campus, Manaus, Amazonas, Brazil. A voucher specimen was deposited in the herbarium of UFAM under the register number 7340.

2.3. Extraction and Isolation

The bark was dried at 40 °C, grinded and stored at room temperature. Then, the powder (200 g) underwent repeated maceration with hexane and methanol, successively, at a mass/solvent ratio of 1:20 (mass/volume). The resulting biomass for each solvent was filtered and the liquid was removed on a rotary evaporator (below 40 °C) under reduced pressure, to yield hexane (GBCH, 3.11 g) and MeOH (GBCM, 45.58 g) crude extracts. Alkaloid (GBFA, 1.79 g) and neutral (GBFN, 1.5 g) fractions were obtained from the methanol crude extract through acid-basic partition [2]. Then, GBFA (1.0 g) was subjected to an open silica gel (0.063–0.200 mm 20.0 g) chromatography column (H φ x = 42.0 × 3.5 cm), treated with 10% $NaHCO_3$ solution and eluted with hexane ($CH_3(CH_2)_4CH_3$), dichloromethane (CH_2Cl_2), ethyl acetate ($C_4H_8O_2$) and methanol (CH_4O) at increasing polarity mixtures, yielding 157 fractions of 20 mL each. These fractions were analyzed through thin layer chromatography (TLC, in silica gel) and visualized by UV light (254 and 365 nm). Those that presented the same spots profile were pooled resulting in 11 new fractions. Then, each of these fractions was subjected to preparative TLC (dichloromethane: methanol 95:05, 3×) affording the isolation of five substances, GB1, GB2, GB3, GB4 e GB5.

2.4. Identification of Chemical Constituents

Identification of the chemical constituents was carried out by Mass Spectrometry and Nuclear Magnetic Resonance (^1H, ^{13}C, HMQC and HMBC) spectral data analysis, as well as, by comparison with literature information [2–3,21–22]. Samples were diluted in methanol, filtered through a syringe filter (0.22 μm) and then injected (3 μL) into high performance chromatograph, provided with a UPLC BEH column (particle size particle = 2.1 × 50 mm, 1.7 mM), coupled with a mass spectrometer (Ultra-High Performance Chromatography–Mass Spectrometry, UPLC–MS/MS system, Waters, Milford, MA, USA) at 30 °C and eluted with methanol for 5 min. The mass spectrometer (Acquity TQD mass spectrometer, Micromass Waters, Milford, MA, USA) conditions were as follows: capillary 3.5 kV, Cone: 30 V, source temperature at 150 °C and desolvation temperature at 350 °C. For those samples with high purity, the ion with the m/z of interest was selected and the spectrum fragmentation was obtained (MS/MS) under the same equipment conditions and collision energy at 25 V. H^1 and C^{13}, 1D and 2D NMR data were obtained at 293 K on Bruker Avance DRX 400 equipment. For these analyses all samples were solubilized in $CDCl_3$ or $CDCl_3$ + CD_3OD. TMS was used as internal standard.

2.5. Photophysical Study and Singlet Oxygen Production

The absorption and fluorescence profiles of all samples were obtained in a Hewlett Packard 8452 A spectrophotometer and ISS-PC1 spectrofluorimeter operating with a xenon lamp, respectively. Bandwidths were fixed at 1 nm for excitation and emission. Crude extracts were analyzed at 0.5 mL/mL, fractions at 0.3 mg/mL and the isolated substance at 5 μM, all solubilized in DMSO.

The 1.3-DPBF bleaching method [23–24] with adaptations [25] was applied to measure the production of singlet oxygen of the samples.

2.6. Microorganisms and Growth Conditions

Bacterial and yeast strains applied in biological assays were: *Staphylococcus aureus* ATCC 14458, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 10799, *Candida albicans* ATCC 1023 and *Candida dubliniensis* ATCC 778157. Müller Hinton (Difco)-MHb medium and Sabouraud dextrose agar were employed for bacteria and yeast strains respectively, all of then cultivated for 24/48 h at 37 °C.

2.7. Determination of the Minimal Inhibitory Concentration (MIC)

Antimicrobial activity was analyzed by agar-well diffusion method (well technique with double layers) and MIC determinations were carried out using the method of microdilution in 96-well plates [26]. Samples were prepared in propyleneglycol/sterile distilled water (5:95). Bacitracine (2 UI/mL) and ketoconazole (0.1 mg/mL) were used as a positive controls and the diluent as a negative control. Samples were analyzed in quadruplicate and the final results were presented as arithmetic averages (mg/mL for crude extracts and fractions and μM for isolated substances).

2.8. Photodynamic Effect Against Bacteria and Yeast Strains

The photosensitizing effect was evaluated according to Gasparetto et al. (2010) [27] and Andreazza et al. (2013) [25]. Samples were tested at sub-inhibitory concentrations (herein established as MIC/2) and prepared as described above. Methylene blue (0.01 mg/mL in sterile saline solution) was applied as positive control and the samples diluent, the inoculum, and the culture medium alone, as negative controls. Bacteria and yeast inocula were prepared by incubating them in sterile saline