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Photodynamic inactivation of Gram-positive bacteria employing natural resources

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ABSTRACT

The aim of this paper was to investigate a collection of plant extracts from Argentina as a source of new natural photosensitizers (PS) to be used in Photodynamic Inactivation (PDI) of bacteria. A collection of plants were screened for phototoxicity upon the Gram-positive species *Staphylococcus epidermidis*.

Three extracts turned out to be photoactive: *Solanum verbascifolium* flower, *Tecoma stans* flower and *Cissus verticillata* root. Upon exposure to a light dose of 55 J/cm^2, they induced 4, 2 and 3 logs decrease in bacterial survival, respectively.

Photochemical characterisation of *S. verbascifolium* extract was carried out. PDI reaction was dependent mainly on singlet oxygen and to a lesser extent, on hydroxyl radicals, through type II and I reactions. Photodegradation experiments revealed that the active principle of the extract was not particularly photolabile.

It is noticeable that *S. verbascifolium* –PDI was more efficient under sunlight as compared to artificial light (total eradication vs. 4 logs decrease upon 120 min of sunlight). The balance between oxidant and antioxidant compounds is likely to be masking or unmasking potential PS of plant extracts, but employing the crude extract, the level of photoactivity of *S. verbascifolium* is similar to some artificial PS upon exposure to sunlight, demonstrating that natural resources can be employed in PDI of bacteria.

1. Introduction

Photodynamic therapy (PDT) employs a nontoxic dye termed photosensitizer (PS) and visible light, which in the presence of oxygen produce cytotoxic species [1]. PDT has the advantage of dual selectivity in that the PS can be targeted to its destination cell or tissue, and in addition, the illumination can be spatially directed to the lesion.

PDT appears to be endowed with several favourable features for the treatment of infections originated by microbial pathogens, including a broad spectrum of action, the efficient inactivation of antibiotic-resistant strains, the low mutagenic potential, and the lack of selection of photoresistant microbial cells [2–4]. Proposed clinical fields of interest of antimicrobial Photodynamic Inactivation (PDI) include the treatment of chronic ulcers, infected burns, acne vulgaris, cutaneous leshmaniasis, and a variety of oral infections, as well as for the sterilization of different media such as water or disinfection of horizontal surfaces in industry and clinical purposes [5,6]. This technique is also being used to address environmental problems of high significance, such as the decontamination of wastewaters and fish-farming tanks [5].

Different porphyrins have been employed as PS in artificial models of *Staphylococci* water disinfection [7] and to inactivate fish pathogenic bacteria in aquaculture systems [8]. Porphryrins delivered in nanosystems such as immobilized on chitosan [9] or nanomagnet–porphyrin hybrids [10] have been designed for the same purpose. The preliminary evaluations show that these organic compounds and their derivatives have potential application in water treatment and other environmental purposes [11–15].

Abbreviations: DMSO, dimethyl sulphoxide; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; PDT, photodynamic therapy; PDI, photodynamic inactivation; PS, photosensitizer.

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A great challenge in the field of PDI is the development of natural non-toxic PS that are likely to be safely used in the treatment of nosocomial pathogens and water infecting bacteria. In the list of phtochemicals that have been investigated are those which are only biologically active in the presence of UVA or the ones activated by visible light, which are known as PS [16]. Chlorophyll and some derivatives have been used as PS for PDT in cancer [17,18]. Another group of photoactive compounds found in plants are the polyacetylenes, thiophenes and quinines [19]. Among the anthraquinones, *Hypericum perforatum* extract has recently been developed as a natural PS for use in PDT of cancer [20], as well as in the photochemical eradication of bacteria and fungi [21–23]. Another example of naturally occurring photosensitiser is hypocrellin-a from *Hypocrella bambusae* [24]. In addition, a few other studies on natural PS extracted from plants have been reported [25–28].

The reason for the prevalence of PS in nature is not known, and their significance or function in plants is not entirely understood, although they have been implicated as defence mechanisms against insect pests [29,30].

Argentina has an abundant and diverse flora ranging from sub-artic to sub-tropical climates. Therefore, the overall aim of this paper was to investigate a collection of regional plant extracts from Argentina as a source of new PS to be used against microbial agents. The collection of plant specimens included species obtained from the north and central regions of the country. The most photoactive extract was photochemically characterized, and PDI studies of bacteria on agar surface, bacteria binding, and use of sunlight as light source were also carried out.

2. Materials and methods

Drugs: Toluidine blue, NaN_3, and mannitol were purchased from Sigma–Aldrich (St. Louis, MO). Chlorin e6 was obtained from Frontier Scientific, Logan, Utah.

2.1. Plant material

The species were collected in the Botanical Garden *Lucien Hauman* of the Agronomy School, University of Buenos Aires, and were identified by Ing. Agr. Juan José Valla.

Voucher specimens are kept in the Herbarium at the Botanical Garden of the Agronomy School (BAA), University of Buenos Aires. The ethnobotanical information of the plants assayed is presented in Table 1. Plant nomenclature (scientific names) is largely according to Cabrera and Zardini [31], Hunziker [32], Zuloaga and Morrone [33,34], and Zuloaga et al. [35]. Vernacular names were taken from de la Peña and Pensiero [36].

2.2. Extraction procedure

Fresh material (100–200 g) was washed with distilled water, air-dried to lower moisture content, and homogenized in absolute methanol or water and blended to macerate the plant material for 3 min at high speed. When lower quantities of plants were available (20–50 g) they were blended employing an Ultra Turrax T-50 homogenizer (IKA, Germany). Aqueous mixtures were centrifuged for 10 min and the supernatant decanted and filtered to remove particulate matter and lyophilized. Methanol extracts were filtered and evaporated under reduced pressure using a rotary evaporator and lyophilized afterwards to remove any traces of solvent. The obtained yields were 4–5%, and the resulting powders were stored at −20 °C. To avoid possible interferences of chlorophylls, flowers, fruits and roots were selected, whereas leaves were discarded.

2.3. Preparation of extract solutions

Stock solutions of Toluidine blue in water and Chlorin e6 in DMSO:water (10:90), were filtered before use. Lyophylised extracts of *Solanum verbascifolium* flower, *Tecoma stans* flower and *Cissus verticillata* root were dissolved in DMSO:water (10:90), to obtain stock solutions. All the solutions were prepared in dim laboratory light and kept in the darkness.

2.4. Bacterial strains, media and culture conditions

Clinical isolates from *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* were employed. *S. epidermidis* and *P. aeruginosa* single colonies were routinely streaked on tryptein soya agar (TSA) from Laboratorios Britania S.A., Buenos Aires, Argentina and *E. coli* on Luria agar. Cultures were incubated at 37 °C and broth cultures were incubated with shaking. Overnight cultures were routinely grown in tryptein soya broth (TSB) (*S. epidermidis* and *P. aeruginosa*) or Luria broth (*E. coli*) to aid in synchronization of growth, in subsequent culture, to mid-exponential phase. Cells in the logarithmic phase of growth were harvested by centrifugation, washed three times with 10 mM phosphate buffered saline (PBS) at pH 7.4, and diluted in the same buffer to a final concentration of 2×10^8 colony forming units per millilitre (CFU/ml). The value of cells per milliliter of culture was determined at 600 nm and compared with a standard curve for cell concentration versus optical density.

2.5. Determination of dark toxicity of the plant extracts

In a first stage, all the extracts were tested on their *per se* toxicity. *S. epidermidis* bacteria (10^8 CFU/ml) were exposed to different concentrations of the extracts for 40 min. Cells were serially fivefold diluted with PBS and each dilution was plated on TSA agar. After 24 h incubation of the plates at 37 °C, the CFU/ml were counted, and the maximal non-toxic concentration of the extracts was determined.

2.6. Screening procedure of photosensitizers on S. epidermidis

For the photosensitization screening, bacteria suspensions were incubated with the extract and exposed at the same time to the light source. Aliquots of 500 μl of bacterial cell suspensions containing 1.5×10^8 CFU were dark incubated 60 min. in the presence of the plant extracts at non-toxic concentrations, prepared as the maximal value determined previously in the dark toxicity studies. The 60 min. dark period of incubation was chosen as an estimated time to allow binding of the extracts to the bacteria cells, under the assumption that all PS bind in a few minutes to bacteria [5]. Immediately, and without washing the unbound extract, the bacteria suspensions were placed in the wells of 24-well plates, and the plate was placed on a glass slide and exposed to the light source at 20 °C for 40 min. To prevent excessive heating, the light was filtered through 1-in. water filters inserted between the sample and the light source. A fluence rate of 55 J/cm^2 was used for the screening. Unirradiated and irradiated bacteria were serially diluted with PBS and each dilution was plated on a solid growth medium. After 24 h incubation of the plates at 37 °C, the CFU/ml were counted. When bacterial counts were reduced 50% under these conditions, the extracts were considered as potential PS and were further studied.

2.7. Light source

The radiation source was a set of two ELH tungsten halogen GE Quartzline lamps with a reflector (500 W, General Electric Co., Cleveland, OH, USA) placed at 25 cm distance from the sample,