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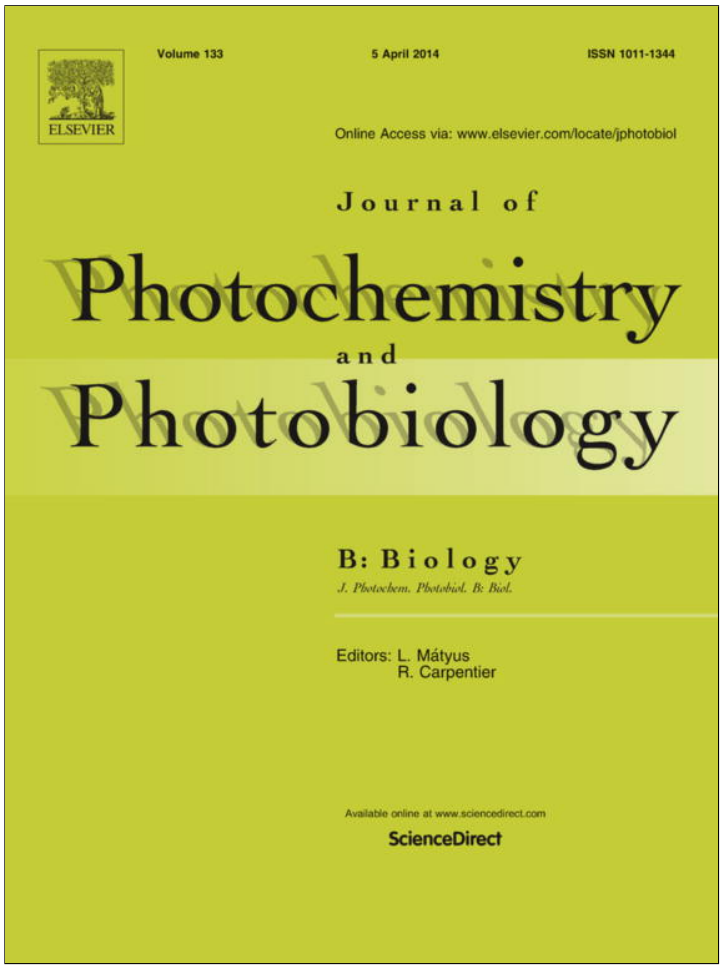

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Photodynamic inactivation of Gram-positive bacteria employing natural resources

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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.

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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 five-fold 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,

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Table 1
Ethnobotanical data of studied plants. Screening of vegetal extracts as photosensitisers of bacteria. Bacteria suspensions of *Staphylococcus epidermidis* were pre-incubated during 60 min. with the maxima non-toxic concentration of the extracts. Afterwards, the mixture was irradiated during 40 min (55 J/cm^2). The CFUs/ml were counted and the photosensitizing effects were calculated comparing to the non-irradiated controls.

Species	Family	Vernacular name	Part	Solvent
Amaranthus quitensis Kunth	Amaranthaceae	Yuyo colorado	Flower and seed	Methanol
Aristolochia esperanzae Kuntze	Aristolochiaceae		Flower	Methanol
Abutilon pictum (Hook. & Arn.) Walp.	Malvaceae		Flower	Methanol
Berberis ruscifolia Lam.	Berberidaceae	Espina amarilla	Flower	Methanol
Brugmansia arborea (L.) Lagerh.	Solanaceae	Floripón	Flower	Methanol
Brugmansia arborea (L.) Lagerh.	Solanaceae	Floripón	Flower	Water
Bromelia balansae Mez	Bromeliaceae	Caraguatá	Fruit	Methanol
Bromelia balansae Mez	Bromeliaceae	Caraguatá	Fruit	Water
Ceiba speciosa (A. St.-Hil.) Ravenna	Malvaceae	Palo borracho	Flower	Methanol
Cissus verticillata (L.) Nicolson & C.E.	Vitaceae	Cortinas del cielo	Root	Methanol
Collaea argentina Griseb	Fabaceae		Flower	Methanol
Collaea argentina Griseb	Fabaceae		Flower	Water
Colletia paradoxa (Spreng.) Escal.	Rhamnaceae	Curro, crucero, curamanuel	Flower	Methanol
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Flower	Methanol
Combretum fruticosum (Loefl.) Stuntz	Combretaceae	Cepillo de mano, cepillo	Flower	Water
Commelina erecta L.	Commelinaceae	Flor de Santa Lucía	Flower	Methanol
Cordia trichotoma (Vell.) Arráb. ex Steud.	Boraginaceae	Petiribí, loro negro	Flower	Methanol
Dicliptera tweediana Nees	Acanthaceae	Canario rojo	Flower	Methanol
Ephedra tweediana Fisch. & C.A. Mey.	Ephedraceae	Tramontana, pingo-pingo	Fruit	Methanol
Erythrina falcata Benth.	Fabaceae	Seibo de Jujuy	Flower	Methanol
Erythrina falcata Benth.	Fabaceae	Seibo de Jujuy	Flower	Water
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Flower	Methanol
Iochroma australe Griseb.	Solanaceae	Perilla, sacha pera	Flower	Water
Ipomoea bonariensis Hook.	Convolvulaceae	Campanilla	Flower	Methanol
Ipomoea cairica (L.) Sweet	Convolvulaceae		Flower	Methanol
Jacaranda mimosifolia D. Don	Bignoniaceae	Jacaranda, tarco	Flower	Methanol
Jacaranda mimosifolia D. Don	Bignoniaceae	Jacaranda, tarco	Flower	Water
Lantana camara L.	Verbenaceae	Lantana, camará	Flower	Methanol
Macfadyena unguis- cati (L.) A.H. Gentry	Bignoniaceae	Uña de gato	Flower	Methanol
Nicotiana glauca Graham	Solanaceae	Palán- palán	Flower	Methanol
Oxalis latifolia Kunth	Oxalidaceae		Flower	Methanol
Patagonula americana L.	Boraginaceae	Guayubirá	Flower	Methanol
Pavonia sepium A. St.-Hil.	Malvaceae	Malva del monte	Flower	Methanol
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Flower	Methanol
Prunus subcoriacea (Chodat & Hassl.) Koehne	Rosaceae	Persiguero bravo	Flower	Water
Pseudogynoxys benthamii Cabrera	Asteraceae		Flower	Methanol
Pyrostegia venusta (Ker Gawl.) Miers	Bignoniaceae	Flor de San Juan	Flower	Methanol
Pyrostegia venusta (Ker Gawl.) Miers	Bignoniaceae	Flor de San Juan	Flower	Water
Rivina humilis L.	Phytolaccaceae	Sangre de toro, inchiquil	Fruit	Methanol
Ruellia brittoniana Leonard	Acanthaceae	Petunia mejicana	Flower	Methanol
Ruellia elegans Poir.	Acanthaceae		Flower	Methanol
Solanum amygdalifolium Steud.	Solanaceae	Jazmin de Córdoba	Flower	Methanol
Solanum verbascifolium L.	Solanaceae	Fumo bravo	Flower	Methanol
Solidago chilensis Meyen	Asteraceae	Vara de oro	Flower	Methanol
Tabebuia impetiginosa (Mart. ex DC.) Standl.	Bignoniaceae	Lapacho rosado	Flower	Methanol
Tabebuia impetiginosa (Mart. ex DC.) Standl.	Bignoniaceae	Lapacho rosado	Flower	Water
Tabernaemontana australis (Müell. Arg) Miers	Apocynaceae	Sapirangui, horquetero, palo vibora	Flower	Methanol
Tecoma stans (L.) Juss. ex Kunth	Bignoniaceae	Tecama amarilla, guaran amarillo	Flower	Methanol

which provided a homogeneous fluent rate of about 23 mW/cm^2 on the surface of the sample measured with a FieldMaster power meter and a PM10 sensor (Coherent Inc., USA). Emission spectrum of the lamp was recorded on a Photon Technology International Quanta Master fluorimeter (USA). Corrected spectrum is depicted in Fig. 6. Fluence measurements and spectra were determined interposing the same water filters in order to avoid IR and UV contribution.

2.8. Absorbance and fluorescence spectra of S. verbascifolium solutions

Absorbance and fluorescence spectra of *S. verbascifolium* solutions in water were recorded on a Hewlett Packard diode array spectrophotometer model 8452A (Hewlett Packard, USA) and Perkin Elmer LS55 fluorimeter respectively using 1 cm path length quartz cuvettes at 25.0 ± 0.5 °C.

2.9. Irradiation conditions

The extracts which were considered PS, were further studied. Following a pre-irradiation incubation period in the presence of the maximal non-toxic extract concentration, 35-mm plates containing aliquots of bacteria, were exposed to the illumination system described above. During irradiation, the suspension was magnetically stirred. A standard volume of undiluted and serially diluted of irradiated samples and controls were plated in duplicate in TSA agar at time 0 and after 20, 40, 90, 120, 150 and 180 min of light exposure. After 24 h of incubation at 37 °C in the dark, the number of colonies was counted. Extracts kept in the darkness and light alone were also used as controls. Light alone did not cause either cell destruction or growth. All experiments were performed in triplicate. Toluidine blue and Chlorin e6 were also employed as control PS.

2.10. Bacterial PS binding assay

Overnight cultures of *E. coli*, *P. aeruginosa* and *S. epidermidis* were inoculated from single colonies into the appropriate culture medium. The following day, overnight cultures were diluted in fresh media and grown to mid-exponential phase, and 3×10^8 CFU/ml were employed. *S. verbascifolium* were added to

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produce a final concentration of 0.5 mg/ml upon the addition of mid-exponential phase bacteria culture. Cultures were incubated with the extracts for various time intervals and subsequently centrifuged for 5 min at 3000 g. The supernatants were removed and the remaining pellets were washed with PBS and subsequently centrifuged again for 4 min at 3000 g twice. The washed pellets were then incubated overnight in 50% EtOH/10% SDS at room temperature [37]. The next day the mixtures were centrifuged for 4 min at 3000 g and the supernatants were removed for fluorescence analysis. Fluorescence was determined at excitation wavelength set to 418 nm and the emission wavelength set to 671 nm after analysis of the emission spectrum (Perkin Elmer LS55 fluorimeter, UK). The concentration of extract was determined through the use of standard curves of fluorescence versus concentration of extract dissolved in the same solvent. This value was then divided by the concentration of cells in culture, to determine the value of pg of extract per bacterial cell.

2.11. Agar susceptibility test

Sensitivity tests were performed by a modified agar-well method. *S. verbascifolium*, Toluidine blue and Chlorin e6 solutions were prepared in sterile distilled water and added to agar at final concentrations of 0.5 mg/ml, 100 μM and 10 μM respectively, and placed into 35-mm diameter wells. Subsequently, aliquots of 15 μl of serial dilutions of *S. epidermidis* at 10^8 UFC/ml, were applied over the agar. Thirty min. afterwards, the plates were exposed for 90 min. to the Tungsten lamp device placed at a distance of 25 cm interposing water filters. After incubation at 37 °C for 24–48 h, the number of colonies formed per well were counted. A control of sterile distilled water was employed.

2.12. S. verbascifolium photobleaching in comparison with synthetic PS

Fluorescence spectra were recorded using 1 cm path length quartz cuvettes employing a Perkin Elmer LS55 fluorimeter. Emission spectra of *S. verbascifolium* solutions in DMSO:PBS (10:90) were recorded (λ ex = 418 nm). Solutions were irradiated with the light source in the same conditions described below for the treatment of bacteria. The kinetics of photobleaching of *S. verbascifolium* was studied by following the decrease of the fluorescence (F) of the 671 nm emission peak. Also, photobleaching was analyzed by following the decrease of the absorbance of the 670 nm peak employing a Hewlett Packard model 8452A diode array spectrophotometer.

In addition, photobleaching of Chlorin e6 and Toluidine blue aqueous solutions were recorded by their decrease of fluorescence (F) peaks (λ ex = 405 nm, λ em = 650 nm and λ ex = 606 nm, λ em = 657 nm respectively).

The observed rate constants (k_{obs}) of photobleaching were obtained by a linear least-quares fit of the semilogarithmic plot of Ln F_0/F versus irradiation time of the linear part of the fluorescence decay, according to Caminos and Durantini [38].

2.13. Photochemical mechanism studies

Suspensions of bacteria (10^8 CFU/ml) were incubated at room temperature and illuminated for 120 min. in the presence of 0.05 mg/ml *S. verbascifolium* solution with NaN_3 or mannitol at 100 mM concentrations in pH 7.4 PBS. Controls were carried out illuminating the suspension containing the quencher in the absence of *S. verbascifolium*. At the completion of illumination aliquots of the bacterial suspensions were taken to determine CFU as described above. A control group of bacteria was treated with *S. verbascifolium* in the absence of light. NaN_3 and mannitol were prepared in distilled water immediately before experiments. To induce anaerobic conditions, the bacterial sample was deaerated by bubbling nitrogen gas for 5 min before illumination and then for 120 min of PDI. A control of bacteria exposed to nitrogen bubbling and illumination was included.

2.14. Photosensitization experiments on sunlight

Aliquots of 5 ml of bacteria (3×10^8 CFU/ml) were placed on 25 cm^2 flasks and were pre-incubated for 10 min with *S. verbascifolium* solutions at a concentration of 0.05 mg/ml. Toluidine blue and Chlorin e6 were employed at 100 μM and 10 μM respectively. The plates were exposed to direct summer sunlight (60–120 mW/cm^2, as measured by a FieldMaster power meter and a PM10 sensor, Coherent Inc., USA). To prevent excessive heating, the light was filtered through 1-in. water filters, and fluence measurements were carried out interposing the same water filters in order to avoid IR and UV contribution. Samples were also placed on a tray with clamps and the bottom was covered with water. Water temperature was monitored and maintained at 25 °C. At different times of illumination, unirradiated and irradiated bacteria were serially diluted with PBS and each dilution was plated on TSA. After 24 h incubation of the plates at 37 °C, the CFU/ml were counted.

2.15. Statistical analysis

Values are reported as the mean ± standard error of the mean (SEM) of three independent experiments. Each experimental condition was performed in duplicate. Differences between two means were assessed for significance by the two-tailed Student's t test, assuming equal or unequal variances of the standard deviations as appropriate. A value of $p < 0.05$ was considered significant.

3. Results

A collection of 48 flowers, fruits and roots of Argentinean plants were screened for phototoxicity upon the Gram-positive species *S. epidermidis* (Table 1). Leaves and stems were discarded to avoid possible chlorophyll interference. Previously to the screening, the maxima non-bactericidal concentration of each extract was determined.

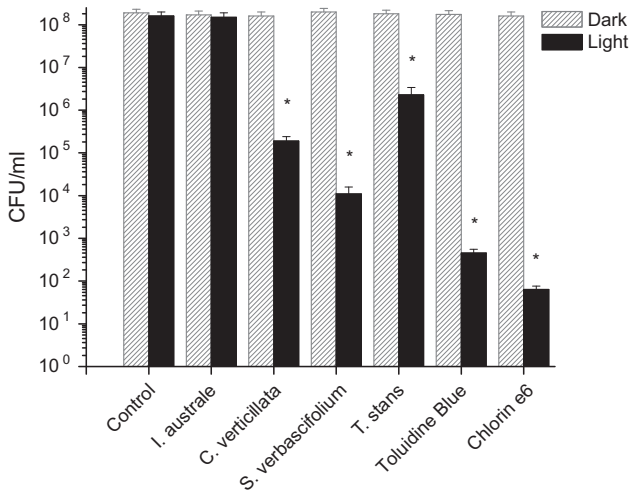

Fig. 1. PDI of *S. epidermidis* induced by illumination of plant extracts employed at their maximum non-toxic concentrations (0.5 mg/ml) upon 55 J/cm^2 (40 min of irradiation). The number of CFUs/ml was determined. Dark incubation: 60 min. Chlorin e6 was employed at 0.6 μg/ml (10 μM) and Toluidine blue at 30 μg/ml (100 μM). * $p<0.01$.

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Upon illumination with 55 J/cm^2, methanolic extracts of three plant species probed to be photosensitizers: *S. verbascifolium* (BAA 26663) flower, *T. stans* (BAA 15487) flower and *C. verticillata* (BAA 27069) root (Fig. 1). The three extracts were employed at non toxic concentrations (0.5 mg/ml), and whereas *T. stans* and *C. verticillata* induced 2 and 3 logs decrease in bacteria survival respectively, *S. verbascifolium* was the most photoactive extract, inducing 4 logs decrease. Positive controls of two pure and synthetic PS (Chlorin e6 (neutral charge PS) and Toluidine blue (cationic PS) lead to a reduction of approximately 6 and 7 logs respectively. *Iochroma australe* flower (BAA 26662) was employed as a negative control. *S. verbascifolium,* the most photoactive extract, was employed to carry out further studies.

None of the extracts exhibited photoactivity against *E. coli* and *P. aeruginosa* but these bacteria were slightly responsive (<1 log) to PDI with Chlorin e6 and Toluidine blue (data not depicted).

Different *S. verbascifolium* concentrations ranging from 0.005 to 1 mg/ml were employed (Fig. 2A) to phososensitize *S. epidermidis.* Using a concentration of 0.005 mg/ml, the colony forming ability was reduced 2 logs, whereas from 0.05 mg/ml onwards, the level of reduction was increased to 4 logs. A concentration of 0.05 mg/ml was fixed to carry out the rest of the experiments.

In Fig. 2B we tested a range of dark incubation periods of *S. verbascifolium* extract with *S. epidermidis* bacteria. We found that, as soon as 10 min after incubation, and ulterior illumination, killing of 3.5 logs was observed. Further incubation only increased 0.5 logs of killing. Conditions of dark incubation were fixed at 1 h for subsequent experiments.

Fig. 3 shows that photodynamic treatment of *S. epidermidis* employing 0.05 mg/ml of *S. verbascifolium* extract, induced a light dependant reduction of viable bacteria. Such reduction was 4 logs upon 40 min illumination (55 J/cm^2) and 6 logs upon 180 min illumination, as compared to the non-illuminated controls ($p < 0.001$). Photosensitization with the positive controls Toluidine blue and Chlorin e6 induced total eradication of bacteria after 150 and 60 min of light exposure respectively. Dark controls exposed to the PS, as well as light alone controls did not induce decreased bacteria viability (data not depicted).

We quantified the amount of extract bound and/or taken up by the bacteria, by fluorescence emission of the portion bound to the bacteria, as well as fluorescence microscopy (Fig. 4). The inset depicts the steady-state fluorescence emission spectra of *S. verbascifolium* showing a peak in the red spectral region (λ max = 671 nm). Suspensions of *S. epidermidis* were incubated with *S. verbascifolium* extract in the dark. The figure shows that the extract quickly binds to the bacteria membrane. The amount of extract bound/incorporated to the bacteria after 5 min. incubation is 12 ± 1.5 pg extract/CFU. Fluorescence of *P. aeruginosa* and *E. coli* incubated with *S. verbascifolium* was negligible.

In Fig. 5 we depicted the susceptibility of *S. epidermidis* to PDI employing *S. verbascifolium,* Toluidine blue and Chlorin e6 on an agar test, where the agar was embedded on the PS and after plating the bacterial suspension, it was subsequently irradiated. We obtained around 5 logs decrease on colony formation after PDI with *S. verbascifolium* and Toluidine blue, and 6 logs decrease employing Chlorin e6 and 90 min. illumination. This experiment is a modification of typical antibiotic susceptibility tests, and since sensitivity of bacteria to PDI is much pronounced as compared to irradiation in planktonic state, it is a good model for screening of new potential PS.

Photobleaching of *S. verbascifolium* extract in comparison with the synthetic PS Toluidine blue and Chlorin e6 was evaluated through the decay of fluorescence upon illumination (Fig. 7). The observed constants of photobleaching (k_{obs}) were 4.01×10^{-4}/seg,

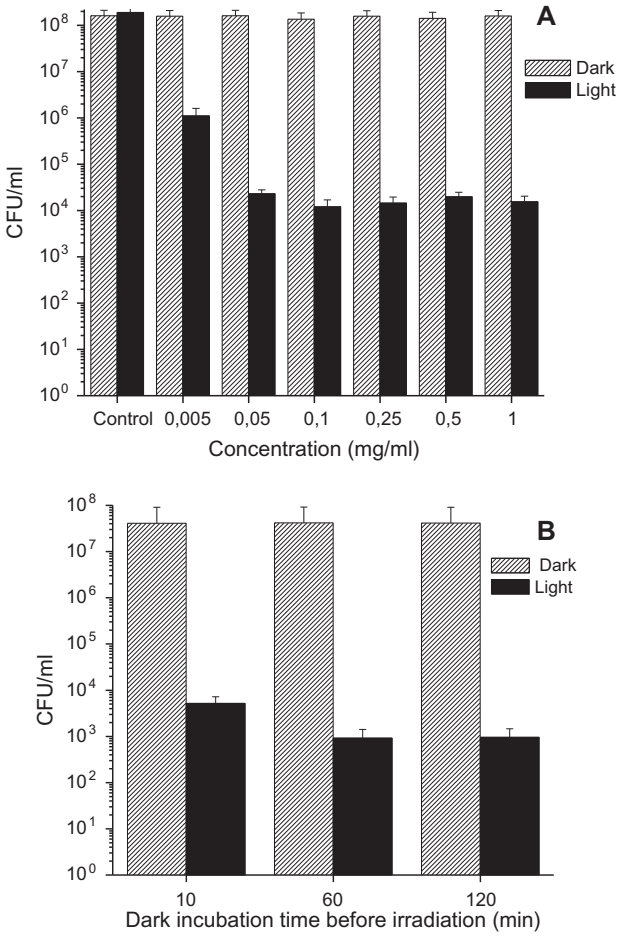

Fig. 2. PDI of *S. epidermidis* employing different *S. verbascifolium* concentrations ranging from 0.005 to 1 mg/ml (1 h dark incubation). Light dose: 55 J/cm^2 illumination (A), and different dark incubation periods employing *S. verbascifolium* at 0.05 mg/ml and 55 J/cm^2 light dose (B). The number of the CFUs/ml was determined.

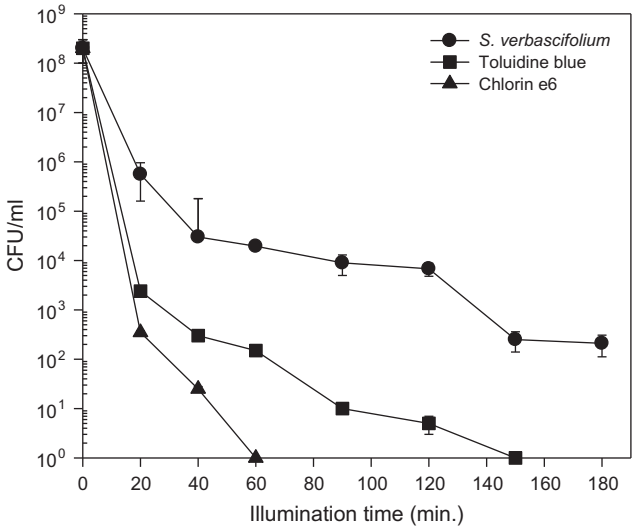

Fig. 3. PDI of *S. epidermidis* employing *S. verbascifolium* extract and different light fluence rates. The bacterial suspension was dark incubated for 60 min with 0.05 mg/ml of *S. verbascifolium* flower methanol extract. Afterwards, the bacteria were illuminated with different fluence rates. The number of CFUs/ml was determined. Toluidine blue (100 μM) and Chlorin e6 (10 μM) were employed as a positive control.

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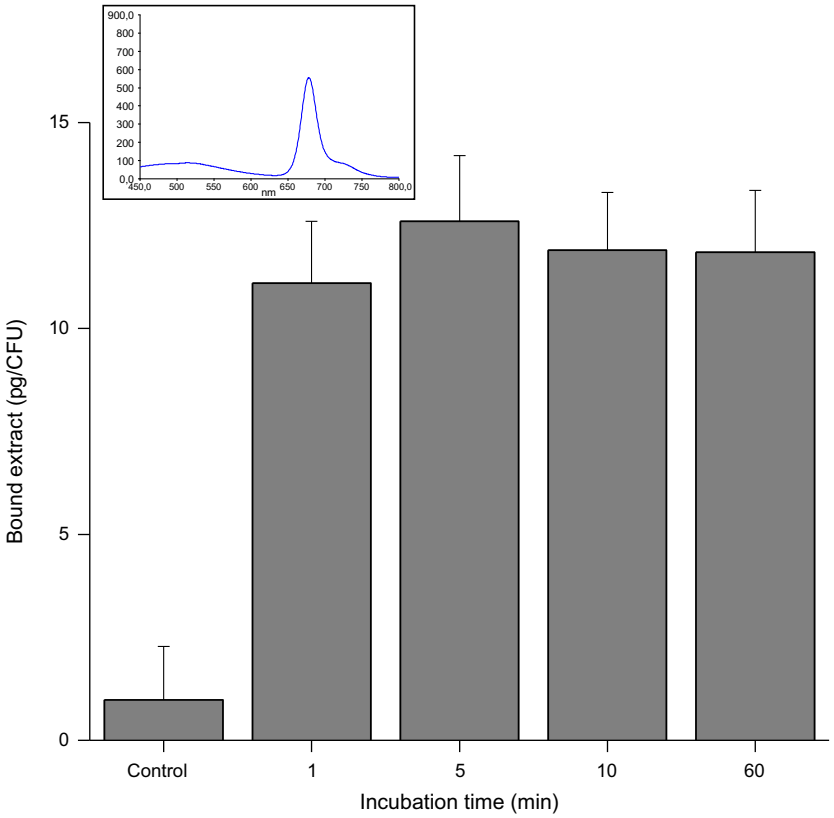

Fig. 4. Binding and/or uptake of *S. verbascifolium* extract to *S. epidermidis* bacteria. Suspensions of *S. epidermidis* were incubated in presence of *Solanum*, and the amount of extract bound and/or incorporated to bacteria was quantified by fluorescence (inset shows *S. verbascifolium* fluorescence emission peak).

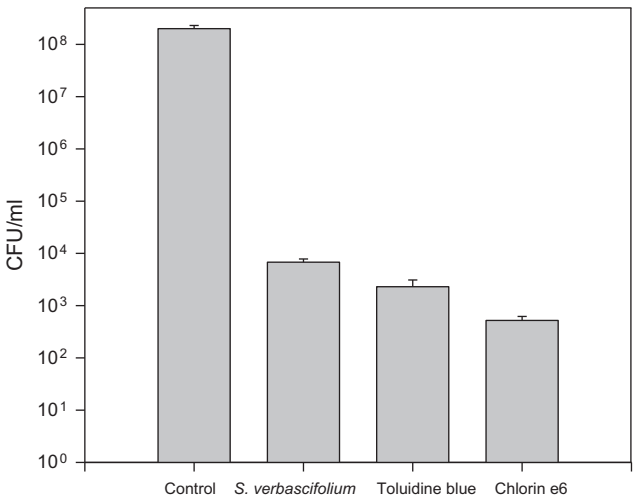

Fig. 5. Susceptibility of *S. epidermidis* to PDI on agar. *S. verbascifolium*, Toluidine blue and Chlorin e6 were added to TSA at final concentrations of 0.05 mg/ml, 100 μM and 10 μM respectively. Subsequently, aliquots of PS solutions were applied and illuminated for 90 min. The number of colonies formed per well were counted.

$5{,}01 \times 10^{-4}$/seg and 2.94×10^{-4}/seg for *Solanum*, Toluidine blue and Chlorin e6 respectively, showing that *S. verbascifolium* decay of fluorescence is of the same order of magnitude comparing to commercial PS. The photoactive principle of the extract is even less photolabile than Chlorin e6. A slight shift in 671 nm peak to 669 nm was observed in photobleached *S. verbascifolium* solutions. In a parallel set of experiments, we followed the decrease of absorbance peaks of *S. verbascifolium* (670 nm), Chlorin e6 (406 nm) and Toluidine blue (606 nm) showing slight differences on the k_{obs} (data not depicted).

Photobleached solutions at the end of the experiment induced significative decreases on bacteria inactivation as compared to the controls ($p < 0.001$). Photobleached extracts of *S. verbascifolium* and Toluidine blue induced 40% and 70% impairment of photoactivity respectively as compared with the non-illuminated extracts,

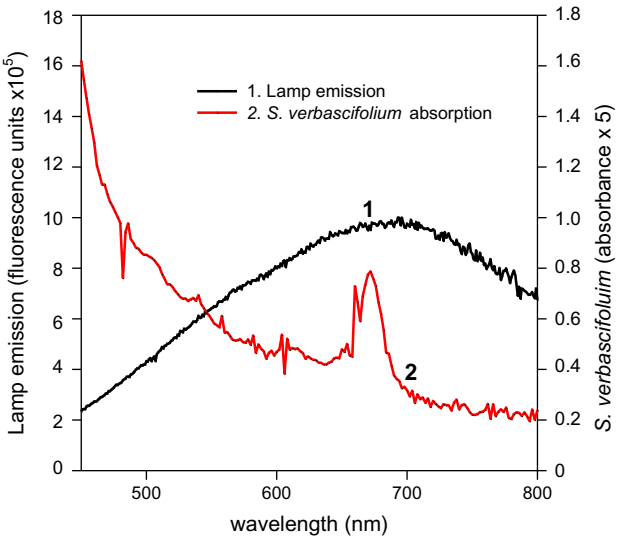

Fig. 6. Absorbance spectrum of *S. verbascifolium* extract in DMSO: PBS (10:90) and lamp emission spectrum.

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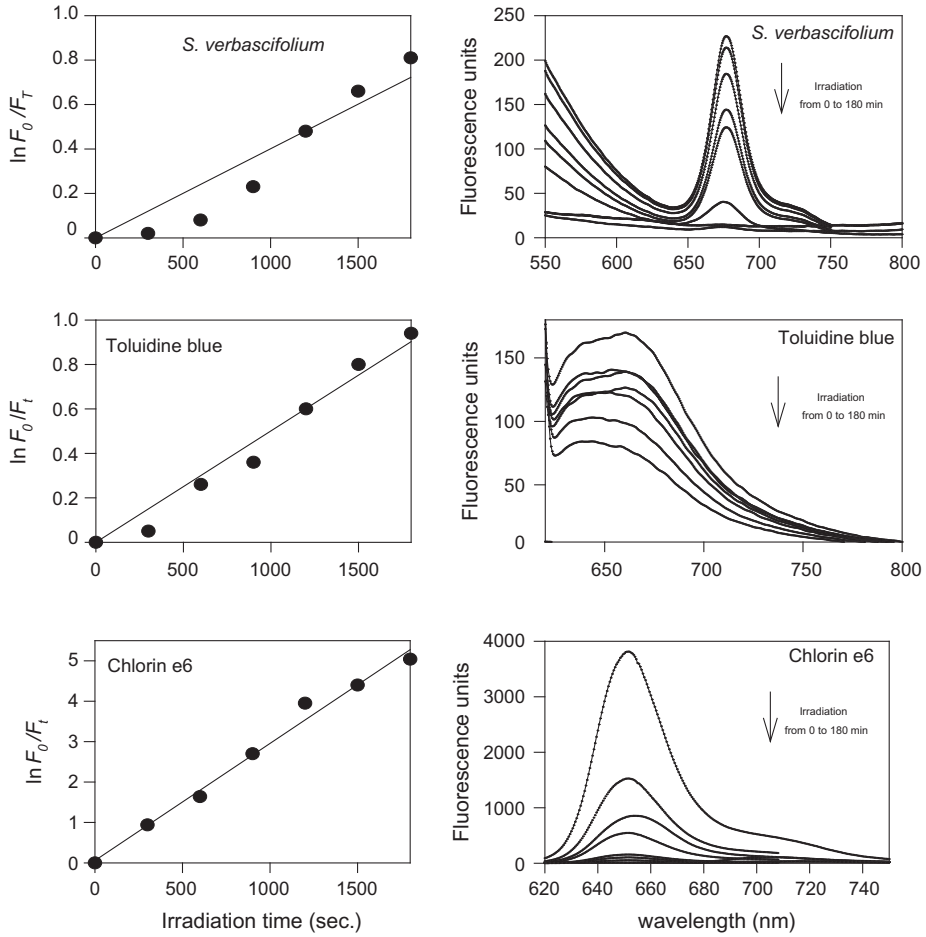

Fig. 7. Photobleaching of *S. verbascifolium*, Toluidine blue and Chlorin e6. Fluorescence decay of *S. verbascifolium* (0.5 mg/ml), Toluidine blue (100 μM) and Chlorin e6 (10 μM) solutions induced by Tungsten lamp illumination. Right panel: spectra after different illumination times. Left panel: ln F_0/F_t of the linear part of the decay of fluorescence major peaks.

whereas photobleached Chlorin e6 increased 4 logs of CFU as compared to the non-irradiated PS (Fig. 8).

To gain insight into the mechanism of photosensitization involved, *S. verbascifolium*-PDI was also studied in presence of azide ion, mannitol and nitrogen atmosphere (Fig. 9). The addition of sodium azide (an efficient quencher of singlet oxygen) produces

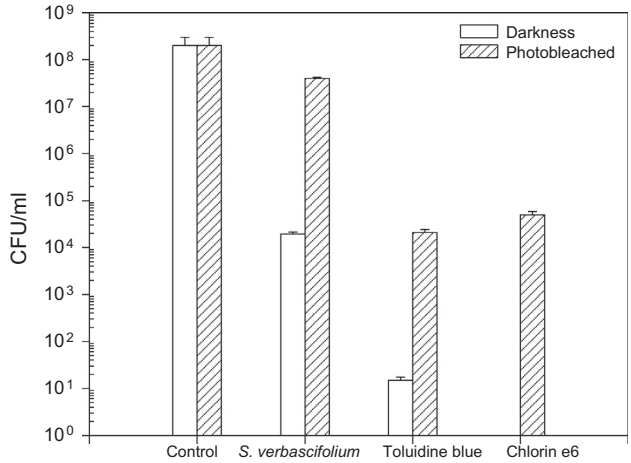

Fig. 8. PDI of *S. epidermidis* employing photobleached PS. Solutions of *S. verbascifolium* (0.05 mg/ml), Toluidine blue (100 μM) and Chlorin e6 (10 μM) exposed 180 min to the illumination system, were employed to photosensitize *S. epidermidis* as explained in Fig. 3. The number of CFUs/ml was determined. Controls were carried out with the same PS concentrations kept in the dark.

a high reduction in the inactivation of *S. epidermidis* sensitized by *S. verbascifolium*. Under this condition, no more than 1 log was decreased in the survival of the Gram-positive bacteria after 120 min irradiation, showing the involvement of the reactive oxygen species $^1O_2$. In contrast, only small but significant protective effect of mannitol (quencher of hydroxyl radical) was observed (2.5 logs reduction of CFU/ml vs. 4 logs reduction of PDI + *Solanum* control, $p < 0.01$).

Displacement of oxygen by a nitrogen atmosphere abolished completely the photoinduced inactivation of bacteria driven by the plant extract.

Controls of mannitol, sodium azide and nitrogen irradiated did not induce any significant inactivation of bacteria.

Fig. 10 reveals an extensive drop in cell survival upon exposure of *S. epidermidis* to sunlight in the presence *S. verbascifolium*. The decrease in microbial population was 4 logs after 30 min ($p < 0.001$), 5 logs after 60 min., and eradication of bacteria after 120 min of sunlight exposure. Controls of Toluidine blue and Chlorin e6 were also carried out, and whereas at short illumination times (30 min), the photoactivity of *Solanum* was equal to Toluidine, Chlorin e6 was more effective (5 logs decrease of CFUs), at longer illumination periods (120 min), *Solanum* appears as a better PS as compared to Toluidine blue.

4. Discussion

We employed 48 plant species from Argentina, to carry out a screening study comprising mainly flowers. Some fruits and aerial

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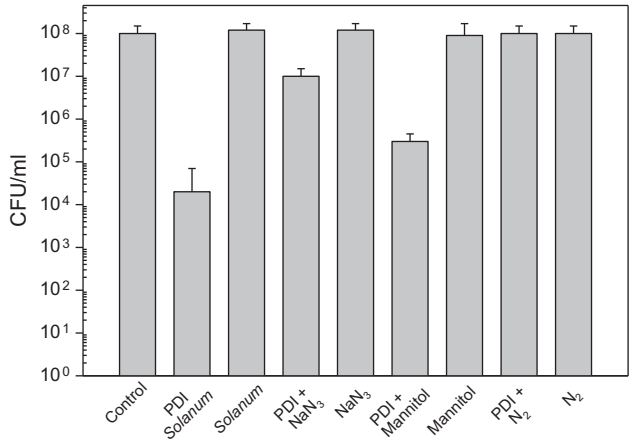

Fig. 9. Dependance of *S. verbascifolium*-PDI on oxygenation, and effect of singlet oxygen and hydroxyl radical quenchers. PDI was carried out in the presence of 0.05 mg/ml *S. verbascifolium* and irradiated 120 min with NaN_3 or mannitol (100 mM) at pH 7.4 in PBS. Controls were carried out by illuminating the suspension containing the quencher in the absence of *S. verbascifolium*. To induce anaerobic conditions, the bacterial sample was deaerated by bubbling nitrogen gas. CFUs/ml were determined at the end of the experiment.

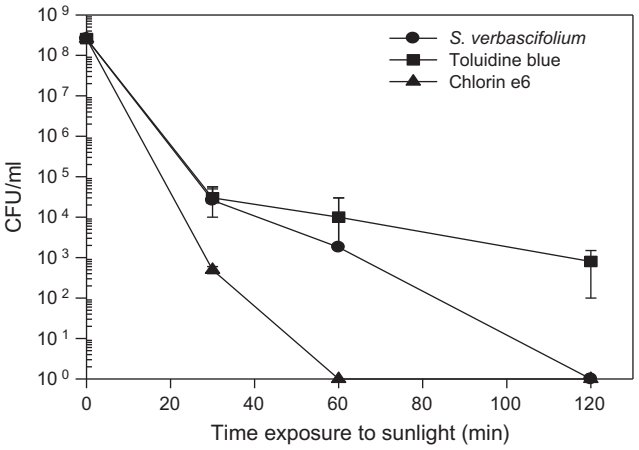

Fig. 10. PDI of *S. epidermidis* employing sunlight. *S. epidermidis* were pre-incubated with different PS (*S. verbascifolum* 0.05 mg/ml, Toluidine blue 100 μM and Chlorin e6 10 μM) and exposed to direct sunlight. At different times of illumination, unirradiated and irradiated bacteria were serially diluted and plated on TSA. The number of he CFUs/ml was determined.

roots were included. We found that methanol extracts of *S. verbascifolium* flower, *T. stans* flower and *C. verticillata* root were the most photoactive against the Gram-positive bacteria *S. epidermidis*. We obtained 2 and 3 logs reduction of CFU employing *T. stans* flower extract and *C. verticillata* root respectively upon sensitization with artificial light. The most photoactive extract was *S. verbascifolium* flower, which induced a reduction of 4 logs of killing at 55 J/cm^2. Positive controls of known PS such as Toluidine blue and Chlorin e6 were more effective upon artificial illumination against *S. epidermidis* strains, although concentrations cannot be comparable since we have employed non-purified plant extracts.

Gram-negative bacteria *E. coli* and *P. aeruginosa* were not responsive to PDI with any of the plant extracts employed, but they were slightly responsive to PDI with Chlorin e6 (neutral PS) and Toluidine blue (cationic PS). It is known that Gram-negative bacteria are resistant to PDI with many commonly used PS that will readily lead to phototoxicity in Gram-positive species. Gram-negative bacteria cause differences in the ability of various antimicrobials to reach the bacterial cell membrane, as well as antibacterial PDI [3,39]. It is believed that cationic PS have a greater affinity for the negatively charged phospholipids of Gram-negative bacteria and enter more easily via self-promoted uptake in comparison with anionic and neutral PS, a notion in accord with the dependence of antimicrobial peptide action on cationic charge [40,41]. Based on these evidences, we assume that *S. verbascifolium* contain probably either neutral or slightly cationic PS molecules, since we neither found photodynamic action against Gram-negative species, nor binding to its bacteria surface.

The actual identity of the particular reactive oxygen species involved in PDI, have been extensively studied. The use of $^1O_2$ quenchers such as sodium azide and of free radical scavengers represents a simple approach to determine which pathways are involved in PDI.

Many articles assume that $^1O_2$ is in fact the only species of importance in bacterial PDI [42,43], while other attribute the killing to radicals including HO· [44]. Huang et al. [45] hypothesized that Gram-negative bacteria are more susceptible to HO· while Gram-positive bacteria are more susceptible to $^1O_2$, and that differences in NaN_3 inhibition may reflect differences in the extent of PS binding to bacteria microenvironment or differences in penetration of NaN_3 into cell walls of bacteria. We found that the PDI reaction employing *S. verbascifolium* as PS was dependant on the presence of oxygen, was largely inhibited by sodium azide and mildly by mannitol, demonstrating the dependance mainly on singlet oxygen and hydroxyl radicals to a lesser extent, through the involvement of type II and I reactions respectively.

Both coherent and non-coherent light sources have been used for the inactivation of bacteria [46]. To inactivate bacteria through photodynamic effects one can use both light sources with lamps generating a continuous emission spectrum typically peaking in the visible and nearinfrared wavelengths, which is most suitable for experiments with plant extracts, in which the peak of the PS may be unknown. In the present work we have employed light doses to inactivate *S. epidermidis* of the same order of magnitude compared to those applied by other authors who employed other Tungsten lamps to photosensitize several bacteria species using porphyrins, Toluidine blue, fenothiazine, rose Bengal and Merocyanine 540 [5] [47–51].

Considering the sunlight spectrum and high fluence during summer in South America, it is noticeable that photosensitization of *S. verbascifolium* is more efficient under sunlight, inducing total eradication of *S. epidermidis* upon 120 min. of exposure, whereas artificial light induced under similar conditions, 4 logs decrease. Even the response of synthetic PS is different between artificial and solar light. Whereas *S. verbascifolium* is less effective than Toluidine blue upon lamp illumination, it is equal or better photosensitizer upon sunlight exposure. The kinetics of bacteria killing is also quite different for each PS comparing both light sources (see Figs. 3 and 10), showing that response of PS to sunlight is particular for each entity, and the advantage of using solar light in PDI is the exploitation of the entire absorbance spectra of each photosensitizing molecule.

The measured absorbance spectrum for the methanol extract of *S. verbascifolium* showed a clear peak in the spectral region between 650 and 690 nm. The lamp emission spectrum covers a broad region between 500 and 800 nm, which includes this prominent peak in the visible region. Artificial light sources appears to be more appropriate for use in bacteria-infected tissue, since the therapeutic window is located between 600 and 1000 nm due to better absorption of light by tissue.

In addition, the photobleaching constants, which were calculated in this work after illumination with an artificial light source, could be quite different when using natural light sources, and this

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may be modifying the efficacy of natural products such as *S. verbascifolium* extract upon sunlight illumination, thus changing its photoactivity as compared to other PS.

Upon illumination, all PS are chemically modified or even degraded. This results from a direct attack of ROS or singlet oxygen on the PS molecules, and these processes have a high probability of taking place, since the PS, of course, is, in the biological environment, in close proximity to the reactive molecules. In experimental spectroscopy, photodegradation and photomodification can be identified by a lowering of absorbance or fluorescence emission [52–54]. In *S. verbascifolium* extracts, photodegradation takes place at a lesser extent than Chlorin e6, a well-known PS employed in PDI, showing that the active principle of the plant is not particularly photolabile. In addition, photobleached extract is still functional, demonstrating that after 180 min of artificial light exposure, photoactive molecules are still present. It is worth to note that the photobleaching rates calculated through the decay of the 670 nm absorbance peak of *S. verbascifolium* is very similar to the one calculated through the decay in fluorescence emission intensity during irradiation, thus demonstrating that it is very likely that we are measuring the same molecule by fluorescence emission at 671 nm and absorbance at 670 nm.

Our results show that the fluorescent principle contained in *S. verbascifolium* flower methanol extract, having an emission peak at 671 nm is likely to be the photosensitizing compound. This hypothesis is reinforced by the fact that similarly to other PS, the photoactive principle quickly binds to the bacteria surface, exerting its action from the outer side of the membrane, which is in line with the short dark incubation time necessary to exert its action. In the case of purified porphyrins, several authors have addressed that the driving force for binding of cationic PS to the negatively charged functional groups on the bacterial surface is electrostatic, hence the binding process is completed within a very short time period. Several independent reports indicate that extending the preirradiation incubation from 5 min to 1–2 h has no effect on the amount of photosensitizer bound to the microbial cells [55–57].

The mixture of pro- and anti-oxidants present in plants, makes difficult the interpretation of the photoactivity of the isolated principles. We propose to use the entire plant extract as PS, since these natural extracts are more likely to be safe than artificial ones to be employed as in antimicrobial therapy, as well as in water decontamination treatment.

In the present paper, we have chosen *S. epidermidis* as a model of disinfection of water and prevention of water-borne diseases employing PDI, since it represents a common isolation from South American waters, among other *Staphylococci* [58]. The potential use of the photodynamic approach for water disinfection has already been studied, showing that PDI of bacteria in drinking and residual waters is possible under artificial and solar irradiation [11–15] [5]. A significant advantage of applying PDI to the disinfection of waterborne pathogens is that this technique has been shown to be independent of the antibiotic resistance spectrum [59]; for this reason, it could be used to treat resistant bacteria such as the several epidemic pathogens present in river, sea and swimming pool waters [60]. The strategy based on sunlight-activated natural PS could potentiate existing bacteria control measures.

As it has been addressed by Jori et al. [5] PDI employing natural resources with significant energy savings and low impact on ecosystems can be used to address environmental problems of high significance, such as the decontamination of wastewaters. The balance between oxidant and antioxidant compounds is very likely to be masking or unmasking potential PS in the extracts, but we have demonstrated that employing the crude extract of *S. verbascifolium*, the level of photoactivity is similar to some artificial PS upon exposure to sunlight. In addition, a peak of the extract within the therapeutic window for tissues, suggests its efficacy in the treatment of superficial infected tissue exposed to artificial light sources.

Acknowledgements

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