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# Methods for the detection of reactive oxygen species employed in the identification of plant photosensitizers

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ABSTRACT

Over the past ten years, alternative methods for the rapid screening of PSs have been developed. In the present work, a study was undertaken to correlate the phototoxicity of plant extracts on either prokaryotic or eukaryotic cells, with the total oxidation status (TOS) as well as with their ability to produce $^1O_2$.

Results demonstrated that the extracts containing PSs that were active either on eukaryotic cells or bacteria increased their TOS after illumination, and that there was a certain degree of positive correlation between the extract phototoxic efficacy and TOS levels.

The production of $^1O_2$ by the illuminated extracts was indirectly measured by the use of the fluorescence of "singlet oxygen sensor green", which is a method that has proved highly sensitive for such measurement. $^1O_2$ was detectable only upon illumination of the most active extracts.

In addition, the oxidation of tryptophan and was employed as a method capable of measuring ROS generated by both type I and II ROS reactions. However, it turned out to be not sensitive enough to detect the species generated by plant extracts.

Results demonstrated that the TOS method, initially developed to measure the oxidant status in plasma, can be readily applied to plant extracts. Unlike the method used to detect $^1O_2$, the method employed for the detection of TOS proved to be accurate, since all the extracts that displayed a high phototoxic activity on either prokaryotic or eukaryotic cells, presented high TOS levels after illumination.



## 1. Introduction

Photoactive compounds have proved to have many biological and pharmaceutical applications in many areas, including photomedicine. One of the branches of photomedicine includes photodynamic therapy (PDT), which for over 40 years has been successfully used to treat different medical conditions including cancer. PDT combines a non-toxic photoactivatable dye or photosensitizer (PS) which undergoes excitation of its triplet state and generates cytotoxic reactive oxygen species (ROS) upon exposure to a visible light source of the appropriate wavelength. These ROS can be classified into those produced by the Type I photochemical mechanism such as the hydroxyl radical ($OH^{\cdot}$), the superoxide anion ($O_2^{-\cdot}$); and the hydrogen peroxide ($H_2O_2$) and those produced by the Type II photochemical mechanism, this is the case of singlet oxygen ($^1O_2$) [1–3]. These ROS are able to destroy either tumour cells (photodynamic therapy, PDT) [4,5] or microorganisms (photodynamic antimicrobial chemotherapy, PACT) [6,7]. These ROS can damage macromolecules, such as proteins, lipids and DNA. PDT has the advantage of having dual selectivity, that is, PSs can be targeted to a cell type or tissue, and at the same time, the illumination can be spatially directed to the lesion.

Plants have been a useful source of PSs, among which, psoralens present in certain fruits and seeds, are commonly used in the treatment of psoriasis. The role of the PSs in the plant metabolism are not entirely understood; however, they are thought to be involved in plant defence mechanisms against insect pests.

Apart from the abundant cyclic tetrapyrrolic compounds (mainly chlorophylls), other naturally occurring chromophores such as quinones derivatives, coumarins, polyacetylenes and thiophenes were also found to be active PSs. Examples of

*Abbreviations:* ALA, aminolevulinic acid; $OH^{\cdot}$, hydroxyl radical; $H_2O_2$, hydrogen peroxide; PACT, photodynamic antimicrobial chemotherapy; PDT, photodynamic therapy; PMNT, poly[3-(3′-*N*,*N*,*N*-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride]; PS, photosensitizer; ROS, reactive oxygen species; $^1O_2$, singlet oxygen; SOSG, singlet oxygen sensor green; $O_2^{-\cdot}$, superoxide anion; TOS, total oxidant status; Trp, L-tryptophan.

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non-tetrapyrrolic natural PSs are hypericin, a perylquinone which was first isolated from *Hypericum perforatum* and hypocrellin-A isolated from *Hypocrella bambusae*.

In the search for novel PSs from natural sources, several programs aimed at the systematic screening of plant biodiversity have been carried out in several countries [9–14]. The *in vitro* study on the interactions of PSs with cells is difficult, since even natural light can activate these photoactive compounds leading to their photodegradation. For these reasons, most experimental manipulations of living cells or tissues have to be carried out under special illumination conditions. In addition, due to their varied chemical nature, these compounds may emit intrinsic fluorescence over a broad range of excitation and emission wavelengths. The latter chemical feature hinders the use conventional assays that are broadly used for drug evaluation. Moreover, since the *in vivo* toxicity of these PSs is dependent on the presence of light, the drug and the oxygen concentrations, the interpretation of data has to be carried out with great caution [15].

In recent years, alternative methods for the rapid screening of PSs have been approached. Zhang et al. [16] have developed a method employing scanning electrochemical microscopy for the detection of new porphyrinic synthetic PSs in a cell-free system. In addition, Vaz et al. [15] have developed an automated high-throughput screening of PS candidates by means of high content analysis technologies to be used in cancer treatment and which employ morphology parameters such as cell area, nuclear area and cell roundness.

Li et al. [17] have proposed a method that is based on the fluorescence quenching of the cationic conjugate poly[3-(3′-*N*,*N*,*N*-trie thylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT) for high-throughput screening of PSs to be used in PACT. With this method, the bacterial number can be detected quantitatively by PMNT via fluorescence quenching efficiency. Exponentially growing bacteria are heavily coated by PMNT through electrostatic and hydrophobic interactions, resulting in aggregates and fluorescence quenching of PMNT. Conversely, after active PSs-mediated PACT, bacterial growth is abrogated, thus preserving the original strong PMNT fluorescence.

In cell-free systems certain performance indicators of putative good PS candidates may be used, among which, the singlet oxygen quantum efficiency has proved to be useful, since the production of $^1O_2$ is directly proportional to the effectiveness of Type II processes.

Plant metabolism must be highly regulated in order to allow effective integration of a diverse spectrum of biosynthetic pathways that are reductive in nature. This regulation does not completely avoid photodynamic or reductive activation of molecular oxygen to produce ROS, particularly $O_2^{-\cdot}$, $H_2O_2$ and $^1O_2$ [18,19]. However, in many cases, the production of ROS is genetically programmed, induced by developmental events and by environmental factors. Besides, ROS have complex downstream effects on both the primary and the secondary metabolism. Plant cells produce ROS, particularly $O_2^{-\cdot}$ and $H_2O_2$, as second messengers in many processes associated with plant growth and development [20].

Singlet oxygen is a highly reactive molecule that is potentially damaging to biological systems. It can rapidly oxidize molecules containing carbon-carbon double bonds to form hydroperoxides or endoperoxides. In proteins, $^1O_2$ oxidizes cysteine, histidine, methionine, and tryptophan residues. In addition, it can stimulate rapid membrane lipid peroxidation [21].

The photodynamic action of PSs on the aminoacids via $^1O_2$ is well known [22]. The photo-oxidation of L-tryptophan (Trp) by several PSs has been reported [23,24]. In addition, Trp oxidation is capable of measuring ROS generated by both type I and II ROS reactions [25–27].

The commercially available reagent, singlet oxygen sensor green (SOSG) [28] is highly selective for $^1O_2$, and does not show any appreciable response to the hydroxyl radical or the superoxide anion [29]. It is a dyad composed of fluorescein and anthracene moieties [30]. It has been applied to a range of biological systems that are known to generate $^1O_2$ [31–33]. Among other applications, SOSG has been used to measure $^1O_2$ production after illumination of synthetic PSs [34,35].

SOSG exhibits weak blue fluorescence with excitation peaks at 372 nm and 393 nm and emission peaks at 395 nm and 416 nm. In the presence of $^1O_2$, SOSG emits a green fluorescence with excitation and emission peaks at 504 nm and 525 nm, respectively which can be readily detected [28].

Erel et al. [36] have developed a colorimetric and automated method for the measurement of the total oxidation status (TOS) in serum. This method is based on the oxidation of the ferrous ion to the ferric ion in the presence of various oxidant species in acidic medium followed by the quantification of the resulting xylenol orange-chelated ferric ion. This method has proved to be a good indicator of ROS levels in patient plasma samples [36].

In a previous work, we screened 40 plant species for new PSs that can be used for the treatment of cancer and infectious diseases. We found that, upon illumination with low light doses (0.2 J/cm$^2$), methanol extracts of *Combretum fruticosum* (10 μg/ml) and *Scutia buxifolia* (100 μg/ml) were highly phototoxic for LM2 mammary tumour cells [11].

In addition, upon illumination with a much higher light dose (55 J/cm$^2$) methanol extracts of flowers of *Tecoma stans* and roots of *Cissus verticillata*, employed at 500 μg/ml, induced a 2 and 3 log decrease in *Staphylococcus epidermidis* viability, respectively. *Solanum verbascifolium* was the most photoactive extract, inducing a 4 log decrease of *Staphylococcus aureus* growth. Positive controls carried out with two pure and synthetic PSs, Chlorin e6 (neutral charge) and Toluidine blue (cationic), led to a reduction of approximately 6 and 7 logs, respectively.

The aim of the present study was to the correlate the phototoxicity of plant extracts, in either prokaryotic or eukaryotic cells, with the total oxidation status. Besides, the ability of such extracts to produce $^1O_2$ upon illumination was also assessed. For that purpose, we adapted a method for the measurement of $^1O_2$ and one for the determination of TOS. The relevance of these putative indicators of photodynamic activity are also discussed.

## 2. Material and methods

### 2.1. Chemicals

Xylenol orange, *o*-dianisidine dihydrochloride and L-tryptophan were purchased from Sigma-Aldrich (Poole, UK). SOSG was purchased from Invitrogen (Eugene, Oregon, USA). The rest of the chemicals employed were of analytical grade.

### 2.2. Photoactive plant extracts

The species employed herein were collected in the Botanical Garden Lucien Hauman of the Agronomy School, University of Buenos Aires, and were identified by AE Juan José Valla. Voucher specimens are kept in the Herbarium at the Botanical Garden of the Agronomy School, University of Buenos Aires.

Fresh material (100–200 g) was washed with distilled water, air-dried to lower moisture content, and homogenized in absolute methanol and blended for maceration for 3 min with a tissue homogeniser at high speed. Extracts were filtered and evaporated under reduced pressure using a rotary evaporator and lyophilized to remove any traces of solvent. The resulting powders were stored at 20 °C. All the extracts were dissolved in PBS immediately before use.