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Research Article

Photosensitizer ability of 5-methoxysterigmatocystin isolated from aquatic fungus *Acremonium persicinum*

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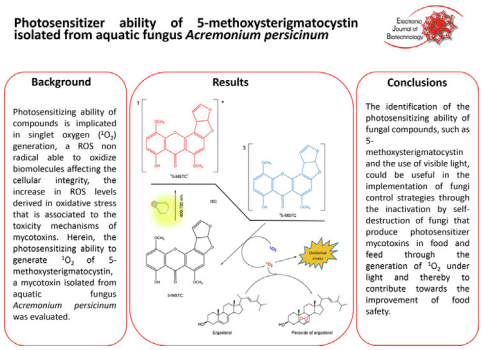
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Photosensitizing ability of compounds is implicated in singlet oxygen ($^1O_2$) generation, a ROS nonradical able to oxidize biomolecules affecting the cellular integrity, the increase in ROS levels derived in oxidative stress that is associated to the toxicity mechanisms of mycotoxins. Herein, the photosensitizing ability to generate $^1O_2$ of 5-methoxysterigmatocystin, a mycotoxin isolated from aquatic fungus *Acremonium persicinum*, was evaluated. Photosensitizing ability was determined through photooxidation of ergosterol method and corroborated by theoretical studies using Gaussian 16 programs, the M06-2X functional and the 6-311++G (d,p) basis set.

Results: 5-methoxysterigmatocystin exhibited efficient photosensitizing ability because it transformed 100% of ergosterol into ergosterol peroxide. Theoretical studies confirmed the experimental results showing that 5-methoxysterigmatocystin satisfied the energy requirements and is able to generate $^1O_2$ corroborating their photosensitizing ability.

Conclusions: The identification of the photosensitizing ability of fungal compounds, such as 5-methoxysterigmatocystin and the use of visible light, could be useful in the implementation of fungi control strategies through the inactivation by self-destruction of fungi that produce photosensitizer mycotoxins in food and feed through the generation of $^1O_2$ under light and thereby to contribute toward the improvement of food safety.

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1. Introduction

5-methoxysterigmatocystin (5-MSTC) is a sterigmatocystin (STC) derivative and both are considered as polyketide mycotoxins that can be detected from several damp environments colonized by fungi mainly belonging to *Aspergillus* genera [1,2]. STC can be metabolized by cytochrome P450 system, sulfotransferases and UDP-glucoronosyltransferases in liver and in epithelial cells from respiratory tract, leading to the formation of intermediary metabolites that act as aflatoxin-biochemical precursors [3]. Thereby, the presence of 5-MSTC could be higher due to the fact that they do not lead to biochemical precursors as SCT does, a reason why SCT in rare cases bioaccumulates [4]. Recent reports show that 5-MSTC exhibit a cytotoxic effect on solid tumor cell lines A549 (lung) and HepG2 (liver) that is 10-fold greater than the STC cytotoxic effect because of the additional methoxy group present in 5-MSTC which increase their bioavailability [5,6]. Regarding toxicity mechanisms associated with STC, oxidative stress is of particular interest owing to the fact that it has been reported that the formation of reactive oxygen species (ROS) increases in a dose-dependent manner, causing lipid peroxidation [4,7]. However, to the best of our knowledge, no previous reports exist regarding oxidative stress and ROS associated with the possible toxicity mechanisms of 5-MSTC.

Oxidative stress is caused by increased levels of ROS, from which singlet oxygen ($^1O_2$) is of our particular interest. Due to the complexity of biological systems, chemical antioxidant activity assays are increasingly being replaced by the cellular ones that consider the issues of bioavailability and metabolism [8]. However, due to the risk that certain antioxidants with light can act as photosensitizers and act as prooxidants to generate $^1O_2$, our group has implemented a simple and efficient method for the detection of $^1O_2$ through ergosterol photooxidation [9]. $^1O_2$ is a non-radical ROS that can be generated through a type II photosensitizing mechanism, in which a photosensitizing compound absorbs energy from UV–Visible light and transfers it to molecular oxygen ($^3O_2$); if the absorbed energy is enough, will lead to the formation of a singlet state, known as $^1O_2$. The reactivity of $^1O_2$ toward biomolecules has been widely described, causing the oxidation of components of cell membranes such as sterols and fatty acids. Photosensitizing compounds have a great relevance in photosensitizing the generation of $^1O_2$, thereby we have focused on identifying $^1O_2$-photosensitizing compounds. Recently, the efficient photosensitizing ability to generate $^1O_2$ of cosmetic dyes belonging to the xanthene group was reported through photo-oxidation of ergosterol method [10,11]. Likewise, the efficient photosensitizing ability of xanthones and their derivatives has been reported previously [12,13,14].

Consequently, in the search of photosensitizing compounds, our work group evaluated 5-MSTC, which possess a xanthone core merged to an ortho-bisfuran group, highly related to STC differing only to the methoxy group in position 5 (Fig. 1). 5-MSTC was obtained from *in vitro* culture of *Acremonium persicinum* fungus, isolated from Atexcac alkaline crater-lake, Puebla, Mexico; structural elucidation of 5-MSTC was made by Nuclear Magnetic Resonance experiments (NMR $^1H/^{13}C$). Subsequently, the photosensitizing ability in 5-MSTC to generate $^1O_2$ was evaluated through photo-oxidation of ergosterol method, the final purpose being to provide information related to the possible mechanisms by which 5-MSTC could cause oxidative damage mediated by $^1O_2$.

2. Materials and methods

2.1. Apersicinum culture and isolation of 5-methoxysterigmatocystin

Apersicinum was obtained from the strain collection of Centro de Investigación en Micología Aplicada (Universidad Veracruzana), which was isolated and molecularly identified by Franceschy et al. [15]. The 5-MSTC was obtained from a liquid fermentation (50 L) using a modified Wickerham medium at pH 8.5, inoculated with a suspension of mycelium and conidia from a culture of 7 d of growth from a culture medium formulated for the isolation of marine fungal strains [16] adjusted to pH 8.5; the liquid culture was incubated during 7 d at 110 rpm followed by 7 d under static conditions at 25 ± 2°C with natural lighting cycles of 12 h. After the incubation period, the culture broth and the biomass produced were separated by vacuum filtration. Subsequently, both parts were subjected to a lyophilization process (LABCONCO FreeZone Plus 6). The dehydrated biomass (160.5 g) was extracted 5 times by maceration in 500 mL of ethanol ($CH_3CH_2OH \geq 99.5\%$, Sigma-Aldrich Química S de RL de CV) and alternating ultra-sonification bath cycles (30 min). Thus, the ethanolic extract (80 g) of the biomass of *A. persicinum* was fractioned (F1) with 500 mL of chloroform ($CHCl_3 \geq 99.5\%$, Sigma-Aldrich Química S de RL de CV), performing 3 successive extractions in separating funnel, using 250 mL of distilled water as the aqueous phase. All extracts were evaporated under reduced pressure to dryness using a rotary evaporator (Büchi B-485). Subsequently, the purification of F1 (8.7 g) was made through a column (49 × 700 mm) packed with silica gel (0.043–0.060 mm) as a stationary phase. Compounds were eluted using an ascending polarity gradient of Hexane and Ethyl Acetate (Hx:AcOEt) as mobile phase. The eluted fractions were monitored by thin-layer chromatography (Merck 60 GF254 of 0.2 mm thickness), using mixtures of Hx:AcOEt as the mobile phase, revealed with short and long-wave UV light (254 and

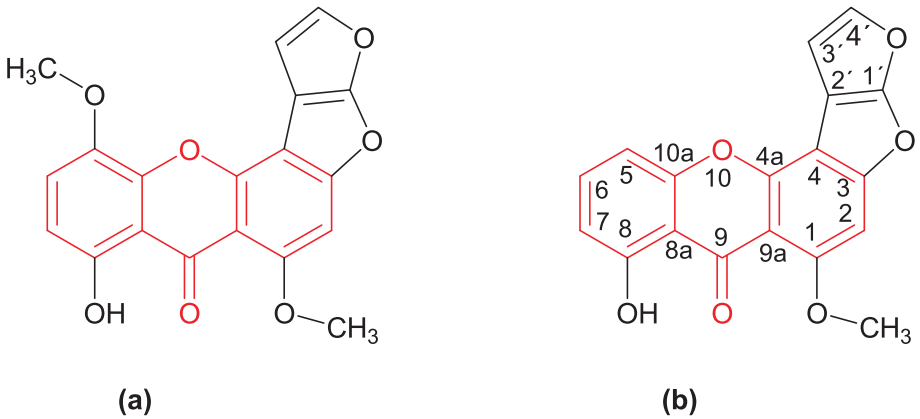

Fig. 1. Chemical structures of 5-MSTC (a) and STC (b). Xanthone core is shown in red.

365 nm) respectively as well as, with iodine and phosphomolybdic acid vapors.

2.2. Characterization of 5-methoxysterigmatocystin by NMR

From the fractions eluted with Hx:AcOEt (7:3) from the purification of F1, 4 mg of yellow crystals (melting point: 223°C) were obtained, with a retention factor of 0.3 in thin-layer chromatography (Hx:AcOEt [1:1], silica gel). The identification of 5-MSTC was performed by interpretation of ^{1}H, ^{13}C, COSY, HSQC, HMBC NMR experiments and match ^{1}H NMR spectroscopic data available in the literature^{1}H NMR (500 MHz, $CDCl_3$) δ, ppm: 12.64 (1H, s, 8-OH); 7.22 (1H, d, J = 9.0 Hz, H-6); 6.86 (1H, d, J = 7.0 Hz, H-1′); 6.72 (1H, d, J = 9.0 Hz, H-7); 6.52 (1H, dd, J = 2.0, 2.5 Hz, H-4′); 6.48 (1H, s, H-2); 5.54 (1H, dd, J = 2.5, 2.5 Hz, H-3′); 4.90 (1H, ddd, J = 7.0, 2.5, 2.0 Hz, H-2′); 4.03 (3H, s, H-15); 3.96 (3H, s, H-14). ^{13}C NMR (75 MHz, CDCl3) δ, ppm: 48.2 (C-2′, CH), 56.8 (OCH_3-1), 57.7 (OCH_3-5), 90.6 (C-2, CH), 102.6 (C-3′, CH), 106.1 (C-9a, C), 106.8 (C-4, C), 109.5 (C-7, C), 109.6 (C-8a, C), 113.3 (C-1′, CH), 120.4 (C-6, CH), 139.4 (C-5, C), 144.7 (C-4a, C), 145.3 (C-4′, CH), 154.0 (C-10a, C), 155.3 (C-8, C), 163.3 (C-1, C), 164.6 (C-3, C), 181.3 (C-9, C) [17].

2.3. Evaluation of the photosensitizing ability of 5-methoxysterigmatocystin by experimental methods

The evaluation of the photosensitizing ability of 5-MSTC to generate $^{1}O_2$ was evaluated by the ergosterol photo-oxidation method, using ergosterol as a $^{1}O_2$ trapper. The $^{1}O_2$ generated was indirectly detected by the formation of ergosterol peroxide, an oxidation product of ergosterol by $^{1}O_2$ [9]. A solution of ergosterol 1 mM (Sigma-Aldrich, Corp., St. Louis, Mo., U.S.A.) was prepared in $CHCl_3$ (reagent grade) to which 5-MSTC was added at an initial concentration of 150 µM. The solution was irradiated for 1 h using four compact fluorescent lamps (CFL-T4-GE20W) with an emission light at the visible light spectrum (19,623 ± 129 lux) maintaining a continuous flow of medicinal grade oxygen (75 mL/s). The temperature inside the photochemical chamber was 32 ± 2°C. After the reaction time, the solution was analyzed by UV–vis light spectrophotometry to detect the presence of ergosterol peroxide and the depletion of ergosterol. Subsequently, the solvent was evaporated under reduced pressure in a rotary evaporator (Büchi B-485) and the photosensitizing ability was determined by mixtures ^{1}H NMR analysis using $CDCl_3$ as solvent and TMS as internal reference (Bruker Avance HD III Spectrometer, 500 MHz) through the integration of the signals of the H-6 and H-7 protons of ergosterol peroxide ($\delta_{H\text{-}6}$ = 6.50 ppm, d, 1H; $\delta_{H\text{-}7}$ = 6.25 ppm, d) and ergosterol ($\delta_{H\text{-}6}$ = 5.57 ppm, dd, 1H; $\delta_{H\text{-}7}$ = 5.38 ppm, dd).

2.4. Theoretical methodology

All electronic calculations were performed using the Gaussian 16 system of programs. Geometry optimization and frequency calculations have been carried out using the M06-2X functional and the 6-311++G (d,p) basis set.

The First singlet excited state ($^{1}S^{*}$) geometry optimization, frequencies calculations and solvent effects were performed by TD-DFT at TD-M06-2X/6-311++G (d,p) level.

3. Results

3.1. Characterization of 5-methoxysterigmatocystin by NMR

5-MSTC (1) (Fig. 1) crystallized in the form of needles (4 mg) with a yellowish color and melting point of 223°C, obtained in 70:30 Hx:AcOEt fractions from chloroform extract (F1) presented an Rf = 0.3 (Hx:AcOEt 50:50), revealed with UV light (254 nm) and iodine vapor. Identification of 5-MSTC was through comparison of ^{1}H NMR shift spectroscopic data reported by Shao et al. [17]. However, we corroborate the structural identification through two-dimensional NMR experiments, which are summarized in Fig. 2.

3.2. Photosensitizing ability of 5-methoxysterigmatocystin

The presence of EP in the mixture reaction was confirmed by ^{1}H NMR mixtures analysis, where two double signals were detected attributed to H-6 and H-7 of EP at 6.25 and 6.50 ppm, respectively. Likewise, signals attributed to H-6 and H-7 of E at 5.57 ppm and 5.38 ppm were not detected in ^{1}H NMR spectrum (Fig. 3). The formation of EP in the reaction occurs due to the oxidation that ergosterol suffers after $^{1}O_2$ was generated by the photosensitizing ability of 5-MSTC. Our results showed that 5-MSTC acts as an efficient photosensitizer because 100% of E is transformed to EP, while in the control reaction without a photosensitizer (negative control), only a 5% of EP was formed, calculated from signal integration of H-6 and H-7 of E:EP. Furthermore, the photosensitizing ability of 5-MSTC was compared with eosin Y (D&C red 22), an efficient dye previously reported [9,10].

3.3. Theoretical study of the photosensitizing ability of 5-methoxysterigmatocytin

The study on the photosensitizing ability of 5-MSTC in chloroform by theoretical methods was carried out, and the results are shown in Fig. 4. The ΔG between the oxygen molecules $^{1}O_2$ and $^{3}O_2$ was 37.77 kcal/mol in chloroform at 298.15 K, which is the minimum energy to transform $^{3}O_2$ to $^{1}O_2$ in chloroform media. The results indicated that the first excited state of 5-MSTC ($^{1}S^{*}$) had a Gibbs free energy difference of 66.44 kcal/mol from the basal state in chloroform; in the same sense, it was observed that the triplet state of 5-MSTC (^{3}S) had a Gibbs free energy difference of 47.92 kcal/mol, relative to the basal state in chloroform. The difference between the triplet and basal states of 5-MSTC is greater than

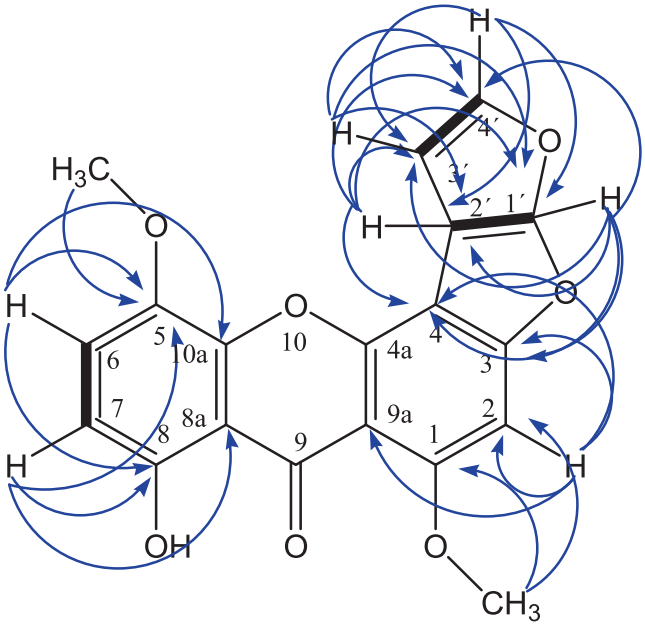

Fig. 2. COSY and HMBC correlations of 5-methoxysterigmatocystin (1). Bold line for COSY correlations, and blue arrows from proton to carbon HMBC correlations.

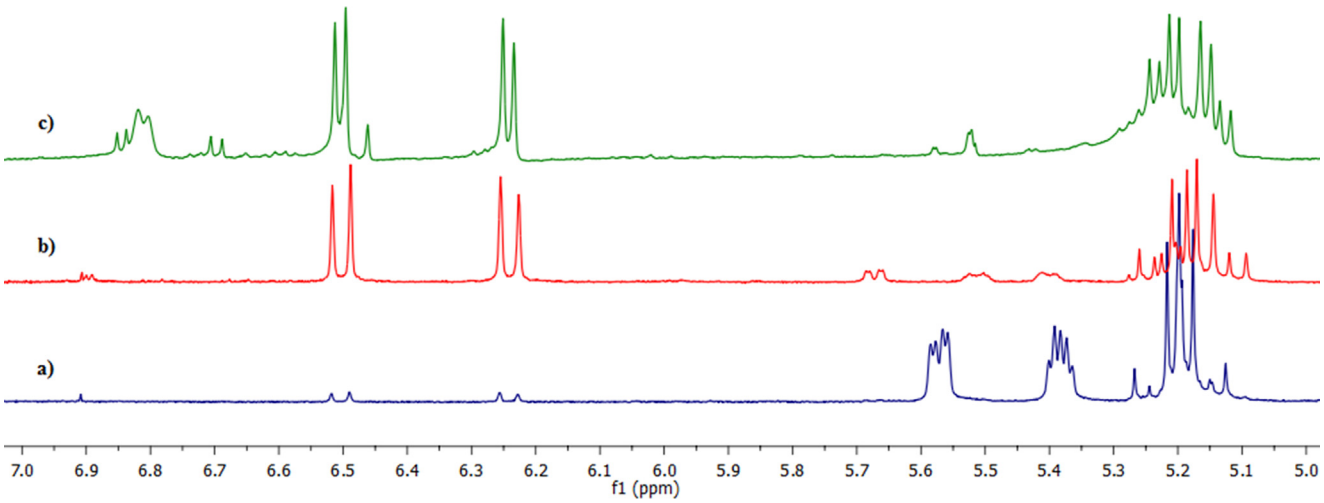

Fig. 3. ^{1}H NMR mixture analysis of photo-oxidation of ergosterol reactions ($CDCl_3$, 500 MHz). a) negative control: reaction without photosensitizer; b) positive control: reaction with eosin Y; c) reaction with 5-MSTC.

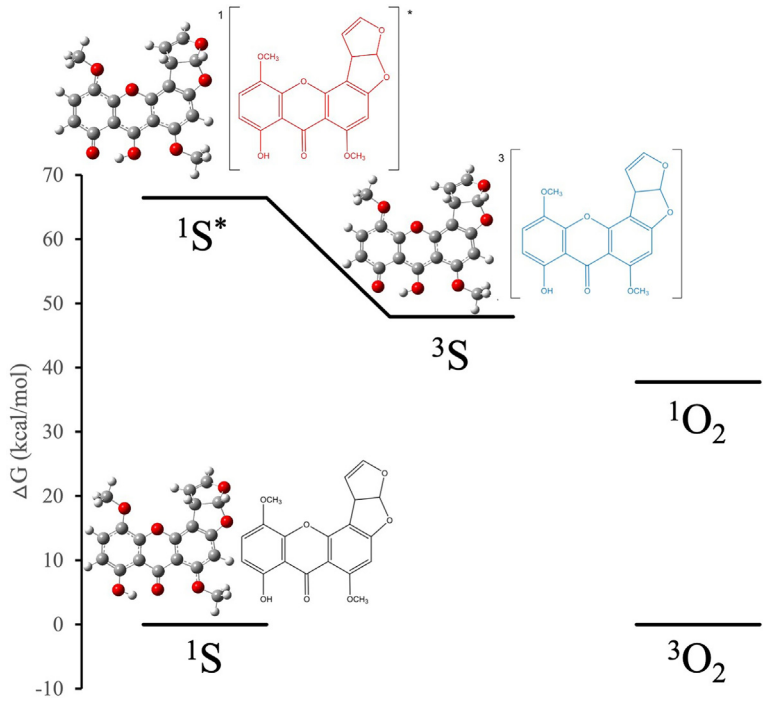

Fig. 4. Geometries and energies from basal and excited states from 5-MSTC and oxygen molecule in chloroform media.

the difference between the $^{1}O_2$ and the $^{3}O_2$ molecules; according to this result, the 5-MSTC satisfied the energy requirements for carrying out the conversion of oxygen molecules in triplet states to singlet states.

In addition, the thermodynamic calculation on the reaction of 5-MSTC with the oxygen molecule (Equation 1) on the photosensitizing transformation in chloroform at 298.15 K was carried out.

$$^{3}S + {}^{3}O_2 \rightarrow {}^{1}S + {}^{1}O_2 \quad \Delta G = -10.15 \text{ kcal/mol} \qquad \text{Equation1}$$

The thermodynamic study showed that the generation reaction of $^{1}O_2$ is feasible in chloroform at 298.15 K; the reaction between 5-MSTC and the oxygen molecule has a Gibbs free energy of reaction of −10.15 kcal/mol. These theoretical results support the photosensitizing ability of 5-MSTC in chloroform media, and these are accorded with the experimental results.

4. Discussion

The presence of mycotoxins in foods such as grains and cereals, as well as feeds, represents a health risk since chronic exposure to mycotoxins is associated with genotoxic, carcinogenic and mutagenic effects [18,19,20,21]. However, the understanding of toxicity mechanisms of mycotoxins involved in disease development still remains a challenge. In this sense, aflatoxins have been, until now the mycotoxins that have received the greatest number of studies in determining the hepatotoxic chronic effect associated with liver cancer development, focused to understand the metabolic reactions of aflatoxins in the organism and the identification of reactive intermediate metabolites that are formed during elimination processes [22,23,24]. Particularly in relation to the aflatoxin B1 (AFB1) has been shown that the formation of reactive

intermediate metabolite AFB1-exo-8,9 epoxide causes biomolecules oxidation, affecting the cellular integrity; besides, has been shown that oxidative stress associated to AFB1 metabolism is implicated in this process, being recognized as a toxicity mechanism associated with chronic and acute effects of mycotoxins [25].

On the other hand, the oxidative stress is caused by an imbalance between the oxidizing species and antioxidants in favor of the former. One of the oxidizing species implicit in oxidative stress is $^1O_2$, a ROS nonradical able to lead lipid peroxidation when generated through light-activated photosensitizer [26,27,28,29]. Our results show that 5-MSTC exhibit an efficient photosensitizing ability to generate $^1O_2$ under visible light. The $^1O_2$ generated during photo-oxidation reaction was trapped by ergosterol and was transformed into ergosterol peroxide. ^1H NMR mixture analysis allowed us to confirm the above through the identification of characteristic signals of ergosterol peroxide and the absence of ergosterol signals in the reaction mixture, confirming that 5-MSTC transformed a 100% into ergosterol peroxide. The photosensitizing ability of 5-MSTC is related to the xanthone core in the molecule structure, which possess a system of double conjugated bonds that acts as a chromophore group and is responsible of the efficient energy absorption in the UV–vis spectrum [13,30]. The experimental results obtained were corroborated by theoretical methods and it was possible to confirm the photosensitizing ability of 5-MSTC, which was able to absorb energy from visible light and be energetically activated into an excited singlet state ($^1S^*$) subsequently to form a triplet state (^3S) keeping enough energy to satisfy the energetic requirements to transfer it to $^3O_2$ and favor $^1O_2$ generation. This energy difference ($\Delta G \neq$) has been reported by theoretical studies as a common mechanism associated to oxidative stress caused by $^1O_2$ [31].

Several studies have demonstrated that mycotoxins metabolism by cytochrome P450 (CYT450) induces oxidative stress through ROS generation. Regarding STC, it has been reported that this triggers oxidative stress by an increase of hydroxyl radicals and hydrogen peroxide, which are responsible for causing lipid peroxidation [5,7,32]. However, to the best of our knowledge, there are no previous reports that show a correlation between 5-MSTC and an increase in ROS and oxidative stress. Nevertheless, it has been established that 5-MSTC is produced in association with STC and CYP450 and is implicated in both as a detoxifying metabolism in tracheal epithelial cells. During this elimination process, STC forms a reactive intermediate metabolite, while for 5-MSTC, the formation of reactive intermediates was not detected, but an increase in the expression of CYP1A1 mRNA was detected [3].

The results obtained allow us to suggest the photosensitizing ability to generate $^1O_2$ as a possible mechanism by which 5-MSTC can induce oxidative stress by an increase of $^1O_2$ levels, added to the possible increase of ROS radicals that can be derived from metabolic processes. The above acquire relevance if alternatives are to be considered in the search for the decontamination of fungi and mycotoxins in foods, where treatments that employ electromagnetic radiation for fungi and mycotoxins inactivation are being used [33,34,35,36]. In this sense, the efficiency of visible light (405 nm) has been shown in the inactivation of fungi potentially mycotoxin producers in foods such as *Aspergillus niger*, without adding an exogenous photosensitizer [37]. Likewise, the efficiency of blue light (470 nm) has been reported in the inactivation of *Fusarium graminearum* without adding an exogenous photosensitizer, but the inactivation of *Penicillium digitatum* is only achieved when an exogenous photosensitizer as erythrosine is added [38]. In line with this, previously our research group reported the fungal inactivation of *Papalauspora immersa* through a photosensitizing mechanism in which a plasma membrane disruption was caused by $^1O_2$ [39]. Consequently, the identification of the photosensitizing ability of fungal compounds, such as 5-MSTC and the use of visible light, could prove useful in the implementation of fungi control strategies or inactivation by the self-destruction of fungi that produce photosensitizer mycotoxins in food and feed through the generation of $^1O_2$ under light and could thereby contribute to the improvement of food safety.

Author contribution

- Study conception and design: C Espinoza; A Trigos.
- Data collection: C Espinoza; C Francheschy; I Lagunes; ME Medina; G Mendoza.
- Analysis and interpretation of results: C Espinoza; I Lagunes; ME Medina.
- Draft manuscript preparation: C Espinoza; I Lagunes.
- Revision of the results and approval of the final version of the manuscript: A Trigos; C Espinoza; I Lagunes; ME Medina.

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Conflict of interest

None.

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