Arabian Journal of Chemistry (2020) **13**, 17–26

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

In silico and *in vitro* antioxidant and cytotoxicity evaluation of oxygenated xanthone derivatives

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Received 26 October 2016; revised 11 January 2017; accepted 11 January 2017
Available online 25 January 2017

KEYWORDS

Antioxidant activity;
Myeloperoxidase;
Scavenging effect;
Structure-activity relationships;
Xanthones

Abstract Many natural products play important roles as antioxidants and represent useful scaffolds for the development of new agents. Particularly, polyphenols in which oxygenated xanthones can be included have proven their efficacy as antioxidants for several applications. To better understand the antioxidant potential of oxygenated xanthones, a library of twenty mono and di-oxygenated xanthones was investigated. The antioxidant properties were evaluated by their 2,2-diphenyl-1-picrylhydrazyl (DPPH) and peroxyl radical scavenging effect as well as their inhibitory effect on the prooxidant enzyme myeloperoxidase (MPO). A quantitative structure-activity relationship (QSAR) model predicted the maximal atomic partial charge (Qmax) as the descriptor being implied in the antioxidant activity of the referred xanthones. From the antioxidant screening, emerged the hit compound, 1,2-dihydroxyxanthone (**10**), that was further characterized for its chelating properties and its effect on a human keratinocyte cell line. Taken together, the results suggest the possible effectiveness of xanthone derivatives as antioxidants with potential for topical administration.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2017.01.006

1. Introduction

Many natural products play important roles as antioxidants and represent useful scaffolds for the development of new agents. Particularly, polyphenols have long been recognized for their strong chain-breaking actions and ability to scavenge free radicals, protecting cells against reactive oxygen species (Ndhlala et al., 2010). Among polyphenols, xanthone (**1**, dibenzo-γ-pirone, Fig. 1) derivatives comprise an important class of the oxygenated heterocycles with a diversity of substitution patterns that have been described for their antioxidant activity (reviewed by Pinto et al. (2005) and more recently by Shagufta and Ahmad (2016)). These derivatives have shown to act as metal chelators, free radical scavengers, as well as inhibitors of lipid peroxidation. These properties have already been related to their hepatoprotective, anti-inflammatory, and cancer chemopreventive activities (Na, 2009). The use of some extracts containing xanthones with a phenolic profile such as Vimang® and Xango® in traditional medicine with antioxidant and chemopreventive actions highlights the potential of this scaffold for applications in which an antioxidant effect is desirable. Although a recent structure-antioxidant activity study was developed for a series of synthetic 2,3-diarylxanthones (Santos et al., 2010), the investigation in simple oxygenated xanthones is based mostly on naturally-occurring members and has not produced a systematic antioxidant structure-activity relationship (SAR) study for this relevant subclass of xanthones.

Thus, the aim of this study was to investigate a library of twenty mono and di-oxygenated xanthones obtained by synthesis (**2**–**21**, Table 1) for its antioxidant properties through the characterization of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and peroxyl radical (ROO•) scavenging and myeloperoxidase inhibitory properties. Moreover, a quantitative structure-activity relationship (QSAR) model to predict the antioxidant activity of xanthone derivatives was developed. In order to further analyze the mechanisms underlying the inhibition of myeloperoxidase (MPO) by three hit xanthones (**10**–**12**), these small molecules were docked into the catalytic domain of MPO. From the antioxidant screening by the referred methods emerged the hit compound, 1,2-dihydroxyxanthone (**10**), that was further investigated for its potential application in the prevention or minimization of ultraviolet (UV)-induced skin damage. Besides ascertaining biological activity, toxicological evaluation is mandatory before considering application of a new drug or cosmetic ingredient (Nohynek et al., 2010; van Meer et al., 2015). Regarding topical administration, keratinocytes are often the preferred cell line for preliminary toxicological studies since they are the predominant resident cells of the outer skin layer (epidermis). The cytotoxic assessment of the hit compound **10** was conducted in this work in a human keratinocyte cell line using four different assays.

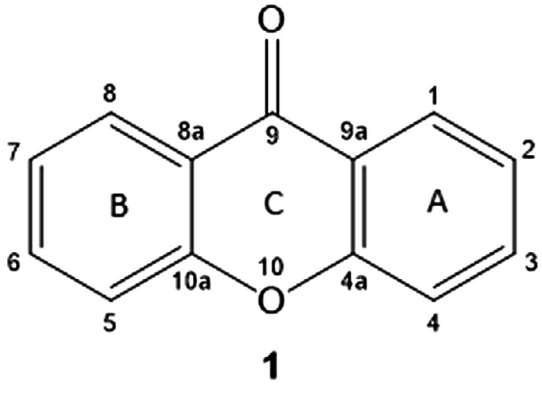

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Figure 1 Xanthone nucleus (**1**) and numbering.

2. Material and methods

2.1. Materials

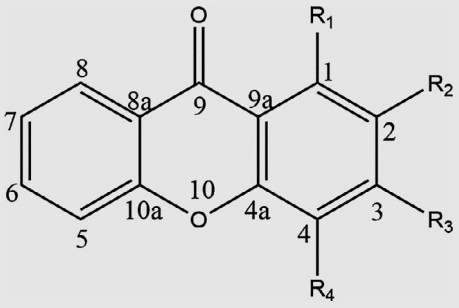
Xanthone was purchased from Sigma-Aldrich, and the oxygenated xanthones were synthesized as described before (Pedro et al., 2002b). AlamarBlue® was provided by Thermo Scientific. Cytochalasin B, dextran, dimethylsulfoxide (DMSO), lysozyme, DPPH, copper (II) chloride, iron (III) chloride, *Micrococcus lysodeikticus,* neutral red (NR) solution, N-formyl-L-methionil-L-leucyl-L-phenylalanine, tetramethylbenzidine, and 0.4% trypan blue solution were purchased from Sigma-Aldrich (USA). 2-Carboxy-2,5,7,8-tetramethyl-6-chro manol (Trolox) was purchased from Fluka. 2,2′-Azo-bis-(2-a midinopropane) dihydrochloride (ABAP) was obtained by Polysciences, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide tetrazolium (MTT) was bought to Life Technologies. Immortalized human keratinocyte (HaCaT) cell line was obtained from Cell Lines Service (CLS). Dulbecco's modified Eagles's medium (DMEM) with 4.5 g/L D-glucose and pyruvate, inactivated fetal bovine serum (FBS), Penicillin–Streptomycin solution, Dulbecco's phosphate buffered saline (DPBS) without calcium chloride and magnesium chloride, and 0.25% trypsin-Ethylene Diamine Tetraacetic Acid (EDTA) solution were supplied by Gibco®. The water used in all experiments was purified water obtained using a Direct-Q® Water Purification System (Merck Millipore) with a reverse osmosis process. For myeloperoxidase assay, human neutrophils were separated from peripheral blood of healthy volunteers, being this study performed in accordance with Declaration of Helsinki. Written informed consent was obtained from all participants.

2.2. DPPH scavenging assay

The scavenging of the stable DPPH free radical was measured by monitoring its reduction, reflected in the absorbance decrease at 515 nm, according to a described procedure (Vale et al., 2014), with modifications. For the determination of the antiradical activities of xanthones derivatives by DPPH, an ethanolic solution of DPPH (1.9 mM) was prepared to obtain the deep purple solution of DPPH•. Before the measurements, the concentration of the DPPH• solution was adjusted with ethanol in order to reach an absorbance value of 0.38 ± 0.01 at 515 nm, 25 °C, for 180 μL of the solution in the microplate reader (Synergy 2, Biotek Instruments).

For each xanthone derivative and trolox (used as a reference antioxidant) different concentrations from 1.5 μM to 15 μM were tested. The compounds were dissolved in ethanol, except for compound **19**. For its dissolution it was necessary to use a small amount of DMSO (≈0.1% in the reaction mixture, without interference in the assay). The absorbance of the aliquots (20 μL) of ethanolic samples and of the radical solutions (180 μL) was recorded every minute during 10 min and then every 5 min during the next 50 min at 25 °C. Blank control (20 μL ethanol + 180 μL of radical) was run in each assay and was set as 100% of radical (0% bleaching). Quadruplicate determinations were made at each concentration of the samples. Vis measurements were performed in a multiplate reader, with a temperature-controlled holder at 25 °C.

Table 1 ROO· scavenging activity and myeloperoxidase inhibitory effect of xanthone derivatives **1**–**21**.



					ROO· scavenging activity		Myeloperoxidase inhibitory activity
	R_1	R_2	R_3	R_4	Scavenging activity (%) at 100 μM	IC_{50} (μM)	% Inhibition at 10 μM
1	H	H	H	H	0.2 ± 1.3	> 100	–
2	OH	H	H	H	15.4 ± 0.6*	> 100	–
3	H	OH	H	H	26.6 ± 1.2*	> 100	–
4	H	H	OH	H	15.5 ± 0.9*	> 100	–
5	H	H	H	OH	69.1 ± 1.6*	59.4 ± 3.0*	–
6	OCH_3	H	H	H	0.7 ± 1.2	> 100	–
7	H	OCH_3	H	H	13.0 ± 0.6*	> 100	–
8	H	H	OCH_3	H	13.1 ± 1.0*	> 100	–
9	H	H	H	OCH_3	2.2 ± 1.4	> 100	–
10	OH	OH	H	H	100.0 ± 1.2*	23.8 ± 1.9*	73.2 ± 4.5*
11	H	OH	OH	H	75.0 ± 1.2*	37.5 ± 0.1*	73.6 ± 0.7*
12	H	H	OH	OH	92.6 ± 2.1*	35.4 ± 4.4*	47.1 ± 0.8*
13	OH	H	OH	H	28.2 ± 1.2	–	–
14	OCH_3	OCH_3	H	H	26.5 ± 1.4*	> 100	–
15	H	OCH_3	OCH_3	H	9.9 ± 5.0*	> 100	–
16	H	H	OCH_3	OCH_3	4.4 ± 0.8	> 100	–
17	H	H	OH	OCH_3	20.7 ± 0.5*	> 100	–
18	H	H	OCH_3	OH	61.2 ± 1.7*	85.2 ± 2.3	–
19	OH	CH_3	OH	H	23.2 ± 1.2*	> 100	–
20	CHO	H	OCH_3	OH	68.7 ± 2.4*,#	37.1 ± 2.5*	–
21	CHO	H	OH	OH	65.0 ± 0.4*,#	41.6 ± 0.0*	–
Propyl gallate (positive control)						33.9 ± 1.2	

Tested at 50 μM.

2.3. Scavenging of peroxyl radicals

ROO· were generated by thermal decomposition of ABAP. The ROO· scavenging activity was assessed by monitoring the decay in turbidity at 450 nm of *M. lysodeikticus* suspensions, due to the inhibition of lysozyme by ROO· (Vale et al., 2014). The reaction was carried out at 45 °C for 90 min in reaction mixtures (1 mL) of 50 mM KH_2PO_4-KOH pH 7.4, 0.68 mM lysozyme, different concentrations of xanthones and 10 mM ABAP. Aliquots of 50 μL were added to 950 μL of a suspension of *Micrococcus lysodeikticus* (0.3 mg/mL) in Dulbecco's buffer and the change of absorbance was followed at 450 nm (Cary 1E Varian Spectrophotometer, Australia) during the first minute (Vale et al., 2014). Controls containing DMSO instead of xanthone solutions and blanks containing KH_2PO_4-KOH buffer (50 μM, pH 7.4) instead of ABAP solution were also made. No direct effect was observed between the tested samples and lysozyme activity. The scavenging activity (%) was calculated according to the following formula: Peroxyl scavenging activity (%) = [1 – (dA/min of blank – dA/min of sample)/(da/min blank – dA/min control)] × 100. The results were expressed as IC_{50}, representing the concentration required to capture 50% of peroxyl radicals, i.e. the concentration required to inhibit 50% of lysozyme activity. All tests were performed in triplicate and propyl gallate was used as positive control.

2.4. Inhibition of myeloperoxidase

2.4.1. Preparation of human neutrophils

Human neutrophils were separated from peripheral blood of healthy volunteers. The citrated blood was centrifuged at 200 g for 15 min at room temperature. The platelet-rich plasma was removed and the neutrophils that are contained in the residual blood were isolated by sedimentation with 2.0% dextran in 0.9% NaCl. The supernatant was centrifuged at 1200 g for 10 min at 4 °C. Contaminating erythrocytes were lysed by hypotonic treatment and neutrophils were purified by ficoll-hypaque sedimentation and resuspended in phosphate-buffered saline (PBS) containing 1.26 mM Ca^{2+} and

0.9 mM Mg^{2+}. Viability was greater than 95% by trypan blue exclusion test.

2.4.2. Myeloperoxidase assay

Effects on MPO were assessed using supernatants of human neutrophils prepared as described in **2.4.1.** Neutrophils (2.5×10^6/mL) were stimulated with cytochalasin B (10 μM) and *N*-formyl-*L*-methionil-*L*-leucyl-*L*-phenylalanine (10 μM) for 10 min at 37 °C. After centrifugation at 1200 g at 4 °C, supernatants were used for MPO determination according to the described procedure (Cerqueira et al., 2008). Briefly, supernatants were incubated with 10 μM of each tested xanthone, H_2O_2 (0.013%), NaH_2PO_4/Na_2HPO_4 and PBS buffer for 5 min at 37 °C. After the addition of tetramethylbenzidine (18 mM) in DMF 8% reaction mixture was incubated for 3 min at 37 °C, sodium acetate (1.97 M, pH 3) was added and the enzyme activity was determined by reading the absorbance at 620 nm (Synergy 2, Biotek Instruments).

2.5. QSAR model

Twenty-one xanthonic derivatives were used to construct QSAR models using the biological data obtained in *in vitro* studies (% inhibition, Table 1). % Inhibition was adopted as a dependent variable in the QSAR analysis. The 21 xanthones were randomly distributed into a training set (16 molecules) and a test set (5 molecules). CODESSA software (Comprehensive Descriptors for Structural and Statistical Analysis, version 2.7.10, University of Florida, USA) was used to calculate more than 500 constitutional, topological, geometrical, electrostatic, quantum-chemical, and thermodynamical molecular descriptors (Katritsky et al., 2004). The heuristic multilinear regression procedures available in the framework of the CODESSA program were used to perform a complete search for the best multilinear correlations with a multitude of descriptors of the training set (Ćwik and Koronacki, 1998). The heuristic method proceeds with a preselection of descriptors by eliminating the following: those descriptors that are not available for each structure; descriptors having a small variation in magnitude for all structures; descriptors found to be correlated pairwise; and descriptors found to be of no statistical significance. The heuristic method is a very useful tool for searching the best pool of descriptors. It is a quick method and presents no restrictions on the size of the data set (Dunn and Hopfinger, 2002). The two dimensional (2D)-QSAR model with the best correlation coefficient (R^2), F-test (F), and standard error (s) was selected. The final model was further validated using the test set.

2.6. UV spectrum and metal chelating effect

The ultraviolet/visible (UV/Vis) spectra were recorded in ethanol using a path length of 1 cm, at room temperature on a spectrophotometer (JASCO V-650). Qualitative analysis of spectra was made in the wavelength range of 200–400 nm, satisfying the Lambert–Beer law. 1,2-Dihydroxyxanthone (**10**): UV λ_{max} ethanol (nm): 261, 243, 232, 203 (ε resp. 33300, 28800, 14200, 11000).

The metal chelating effect was evaluated by described procedures (Moradkhani et al., 2012) with 1,2-dihydroxyxanthone (**10**) stock solution (1.0×10^{-4} M) in methanol. Iron (III) and cupper (II) solutions were prepared in concentration of 1.0×10^{-3} M from $FeCl_3$ and $CuCl_2$. Briefly, sequential additions of 33 μL of metal ion solution were performed to 3 mL of compound **10**. UV/Vis spectra were recorded on a JASCO V-650 spectrophotometer at room temperature.

2.7. Cytotoxicity assays

2.7.1. Cell culture conditions

Cells of immortalized human keratinocyte cell line (HaCaT) with less than 40 passages, were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin (growth medium). For the cytotoxicity experiments, HaCaT cells were incubated with compound **10** solutions in DMSO (12.5, 25, 50, 100, 200 μM) in growth medium for 24 h. For trypan blue assay, a smaller set of concentrations was tested (12.5, 50, 200 μM). DMSO concentration was in all cases lower than 1%. DMSO (10%) was used as positive control and cells treated with the solvent were used as negative control. The cell viability was calculated with regard to negative control, which was set to 100% viability. IC_{50} values were calculated by linear regression. All absorbance measurements were performed in a microplate reader (Synergy HT, Biotek Instruments). On each assay, the cell density, and test reagent concentration and incubation time (when appropriate) were optimized for HaCaT cells.

2.7.2. MTT reduction assay

HaCaT cells (1×10^4 cells/well, 96-well microplates) were incubated with compound **10**. After 24 h incubation, cells were washed with DPBS and 5 mg/mL MTT solution (serum free DMEM) was added. After 2 h incubation, DMSO was added to dissolve the formazan crystals and absorbance was measured at 570 nm.

2.7.3. Neutral red uptake assay

HaCaT cells (2×10^4 cells/well, 96-well microplates) were incubated with compound **10**. After 24 h incubation, cells were washed with DPBS and 50 μg/mL NR solution (DMEM) was added. After 3 h of incubation cells were washed with DPBS and the solution formed by 50% ethanol/1% acetic acid was added to extract the NR dye captured within the cells. The plates were then placed in a microplate shaker for 10 min, at room temperature and protected from light. Finally, absorbance was measured at 540 nm.

2.7.4. AlamarBlue® reduction assay

HaCaT cells (2×10^4 cells/well, 96-well microplates) were incubated with compound **10**. After 24 h incubation, 10% AlamarBlue® was added to each well and incubated for 4 h. Finally, the absorbance at 570 nm and 600 nm was determined.

2.7.5. Trypan blue exclusion assay

HaCaT cells (1×10^5 cells/well, 12-well microplates) were incubated with compound **10**. After 24 h incubation, cells were trypsinized and counted after addition of 0.4% trypan blue vital dye (1:1).

2.8. Statistical analysis

Statistical evaluation of cytotoxicity results was carried out using One-way analysis of variance (ANOVA) followed by the Dunnett post hoc test. Significance level was 0.05. Statistical analyses were performed using the SPSS software (v 23.0; IBM, Armonk, NY, USA) and graphs were generated by GraphPad Prism for Windows (version 6.0; GraphPad Software, Inc., USA).

3. Results and discussion

3.1. Scavenging activity

The simple oxygenated xanthones (**2**–**21**) were obtained by classical methods (Sousa and Pinto, 2005) and their synthesis are described elsewhere (Pedro et al., 2002a). The xanthones **2**, **6**, **13**, and **19** were obtained by the Grover, Shah, and Shah reaction with the condensation of an *ortho*-oxygenated benzoic acid with the appropriated phenol, in phosphorous oxychloride and zinc chloride. To obtain xanthones **3**, **4**, **8**, **12**, and **16**–**18**, a Friedel–Crafts acylation of methoxybenzene derivatives with benzoyl chloride afforded the desirable 2,2′-dioxygenated benzophenones that were cyclized by a base-catalyzed elimination. Selective demethylations and Duff formylation allowed to obtain compounds **20** and **21**. An Ullmann condensation was carried out via suitable biphenyl ether intermediates followed by the intramolecular acylation to afford xanthones **5**, **9**, **11**, and **15** or by *ortho*–metalation to afford xanthones **10** and **14**. The screening of the scavenging activity of xanthone (**1**) and its derivatives **2**–**21** was firstly estimated by a decolorization assay using DPPH$^{\cdot}$ as free radical. Antiradical activity was defined as the maximum inhibition reached using a concentration until 15 μM. Among all tested xanthone derivatives (**1**–**21**), 1,2-dihydroxyxanthone (**10**), 2,3-dihydroxyxanthone (**11**), 3,4-dihydroxyxanthone (**12**), and 1-formyl-3,4-dihydroxyxanthone (**20**) revealed DPPH scavenging ability (Fig. 2). Derivatives **10** and **11** presented the maximum inhibition for the concentration of 15 μM (Fig. 2).

For these derivatives the DPPH radical scavenging activity was evaluated at lower concentrations to determine the IC_{50} values. Both xanthones **10** and **11** revealed a potent DPPH scavenging activity, showing IC_{50} values of 11.3 ± 0.2 μM and 11.1 ± 0.4 μM, respectively, similar to the positive control trolox (14.9 ± 0.9 μM). In this scavenging method, only the dihydroxyxanthone derivatives with an *ortho*-dihydroxy group revealed good antioxidant activity at 15 μM. Xanthones with methoxy groups and monohydroxyxanthones were not able to transfer a hydrogen atom to reduce the DPPH radical.

The use of DPPH$^{\cdot}$ decolorization assay provides an easy and rapid way to evaluate the antiradical activity of compounds, but once DPPH$^{\cdot}$ is an unnatural radical, this assay can only be considered a model to test antioxidant activity. Therefore, we have also examined the effect on peroxyl radical (ROO$^{\cdot}$) scavenging activity. The ROO$^{\cdot}$ scavenging activity of xanthone (**1**) and derivatives **2**–**21** was determined at the maximum concentration of 100 μM, except for xanthones **20** and **21**, which were tested at 50 μM, because of their low solubility. Xanthones having at this concentration a scavenger activity higher than 50% were tested at lower concentrations (100, 75, 50, 40, 30, and 20 μM for xanthones **5**, **10**, **11**, **12**, and **18** and 50, 40, 30, 20 μM for xanthones **20** and **21**) to determine the IC_{50} values (Table 1). No appreciable activity was shown for xanthone **1** and methoxyxanthone derivatives **6**–**9** and **14**–**16**. Similar results were obtained for monohydroxyxanthone derivatives, except for 4-hydroxyxanthone (**5**) and 4-hydroxy-3-methoxyxanthone (**18**), which exhibited IC_{50} values of 59.4 ± 3.0 μM and 85.2 ± 2.3 μM, respectively. As for the DPPH radical assay, the dihydroxyxanthone derivatives possessing a catechol group (**10**, **11**, **12**, and **21**) showed maximum scavenging properties, being 1,2-dihydroxyxanthone (**10**) the most effective to scavenge ROO$^{\cdot}$, while xanthone **13** possessing two hydroxyl groups at *meta* position showed no appreciable effect. Interestingly, the comparison of IC_{50} values of xanthones **18** and **20** suggests that the presence of a formyl group at C-1 is associated with an increase of ROO$^{\cdot}$ scavenging activity (Table 1). These results are in agreement with the results described for antioxidant activity of other structure related xanthone derivatives. In fact, it has been proposed that the number and the position of hydroxyl groups in the xanthone scaffold influence their scavenging activity, being the presence of catechol groups favorable to this activity (Panda et al., 2013).

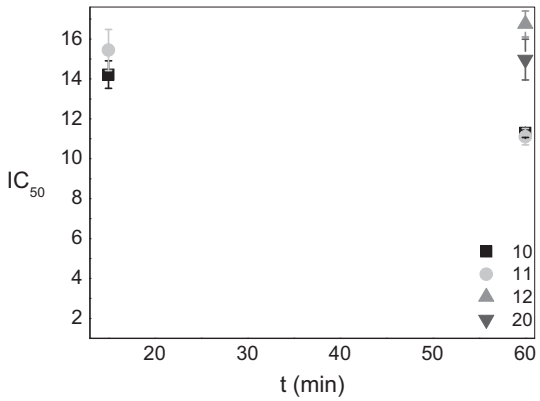


Figure 2 DPPH free radical scavenging activity of xanthone derivatives **10**–**12** and **20** expressed as IC_{50} values (mean ± SD) after 15 and 60 min of reaction.

3.2. Myeloperoxidase inhibitory effect

Taking into account that dihydroxyxanthone derivatives **10**–**12** revealed to be the most potent DPPH, and ROO$^{\cdot}$ scavengers, their effect on the prooxidant enzyme MPO was also evaluated. All dihydroxyxanthones at 10 μM revealed MPO inhibitory effect, being 1,2- and 2,3-dihydroxyxanthones (**10** and **11**, respectively) the most potent inhibitors (Table 1).

Subsequently, a docking study was performed to allow a visual inspection of oxygenated xanthones on MPO target and to bring insights into the putative mechanism of action (supplementary data 1). The results suggest that the aromatic ring A of xanthones and the hydroxyl groups may be important for interaction with the active site of MPO through pi-stacking interactions and H-bond interactions, respectively. MPO is one of the key players in the first line of the nonspecific immune defense system (Lazarevic-Pasti et al., 2015). In the presence of hydrogen peroxide and halide ions, MPO catalyzes the synthesis of the oxidizing compound hypochlorous acid

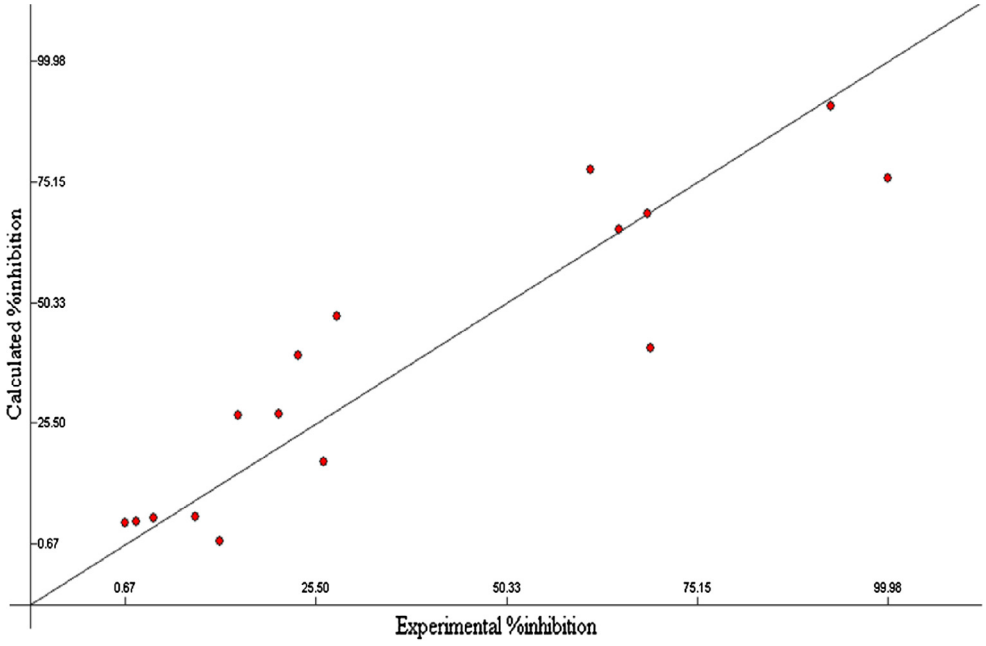
(HOCl), which destroys foreign invaders (Kato, 2016; Pattison et al., 2012). Although MPO is released as a means of killing invading bacteria, activated immune cells have been reported to release MPO even in the absence of infection, which induces oxidative injury of host tissue triggering severe inflammatory disorders (Klebanoff, 2005; Lau and Baldus, 2006; Nussbaum et al., 2013; van der Veen et al., 2009). In addition, the topical exposure to many occupational and environmental chemicals is often accompanied by the accumulation of MPO. Increase in MPO activity has also been reported after UV exposure indicating an influx of leukocytes into the inflamed skin (Katiyar and Mukhtar, 2001).

Several approaches have been used to inhibit MPO-mediated cell injury, being the directly inhibition of MPO activity the most effective (Lazarevic-Pasti et al., 2015). Although several scaffolds have been studied for myeloperoxidase-inhibition (Aldib et al., 2012; Bensalem et al., 2014; Roth et al., 2014; Soubhye et al., 2010), there is still no information regarding xanthones. However, several studies show that phenol and/or indole-like compounds are capable of competing with halides to prevent conversion of the halides into toxic hypohalous acids (Heinecke, 2002; Kettle and Candaeis, 2000).

3.3. QSAR model

With the overall antioxidant results, a QSAR model was built to establish a relationship between the oxygenated xanthone structures and their antioxidant properties, which will allow speeding the design of new active compounds (Dudek et al., 2006). The correlation coefficient (R^2) (a statistical measure of how close the data are to the fitted regression line), standard error (s) (which consists of an absolute measure of the quality of fit), and Fisher's value (F) (which represents the F-ratio between the variance of actual and predicted activity), were employed to judge the validity of regression equation (Kubinyi, 1993). A major point in developing QSAR model is the number of descriptors used to elaborate the equation. Laws of QSAR establish that it should be one descriptor for each five molecules (Kubinyi, 1993); therefore, as the training set was composed of 16 molecules, 3 descriptors were used to build the QSAR models. The multilinear regression analysis using Heuristic method for 16 compounds in the three-parameter model is given in Fig. 3.

The best training model had a coefficient of determination (R^2) of 0.835, Fisher value of 20.23, and S of 15.04, which demonstrate that the proposed model has satisfactory statistical stability and validity in spite of the small group of molecules used to build the model. The squared correlation coefficient R^2 is a relative measure of quality of fit by regression equation. Correspondingly, it represents more than 80% of the total variance (R^2 = 0.835) in antioxidant activity exhibited by xanthone derivatives. Its value is close to 1.0 which represents the better fit to the regression line (Alexander et al., 2015). The F-test reflects the ratio of the variance explained by the model and the variance due to the error in the regression. High value of the F-test indicates that the model is statistically significant. The QSAR model is signif-



Nr	X	ΔX	t-test	Name of the descriptor
0	-146.59	31.26	-4.69	Intercept
1	3369.5	564.43	5.97	Maximum partial charge (Qmax)
2	-3.19	0.67	-4.78	RPCS Relative positive charged surface area (SAMPOS*RPCG) [Zefirov's PC]
3	-28.88	10.4	-2.78	Count of H-acceptor sites [Zefirov's PC]

Figure 3 QSAR model obtained with the heuristic method for 16 xanthones with the CODESSA software (R^2 = 0.835, F = 20.23, and s = 15.04). X, ΔX, and t-test are the regression coefficient of the linear model, standard errors of the regression coefficient, and the t significance coefficient of the determination, respectively.

icant at 95% level as shown by their Fisher ratio values which exceed the tabulated values (4.96) as desired for a meaningful correlation (Veerasamy et al., 2011). Standard deviation expresses the variation of the residuals or the variation about the regression line. Thus, standard deviation is an absolute measure of quality of fit and should have a low value for the regression to be significant (Liu and Long, 2009).

Maximal (most positive) atomic partial charge (Qmax) is the descriptor predicted as being implied in the antioxidant activity of hydroxylated xanthones. This electrostatic parameter is associated with the presence of covalent bonds between two atoms with unequal electronegativities, resulting in unequal sharing of electrons (dipole). This parameter indicates the importance of less electronegative atom that becomes partially positively charged, such as carbons. Relative positive charged surface area (SAMPOS/RPCG) is the second most important descriptor controlling the attained QSAR model, which is the solvent accessible surface area of the most positive atom divided by the relative positive charge. Count of H-acceptor site is the third descriptor controlling the QSAR model. Hydrogen bonds occur when a donor atom donates its covalently bonded hydrogen atom to an electronegative acceptor atom. The lone pairs of hydroxyl or amino groups, as well as those of carbonyl oxygens, for example, can serve as H-bond acceptor sites. The presence of H-acceptor sites makes possible the binding of the ligands to polar groups in the target. External (test set) predictivity is used as validation criteria (Supplementary data 2) (Golbraikh et al., 2003). This model adds new predictors to a previous theoretical study with naturally-occurring xanthones in which a relationship between the ionization potential and the electron affinity was proposed to predict the thermochemical viability of the single electron transfer mechanism processes (Martinez et al., 2012) with their derivatives. Previously, by means of quantum chemical calculation, the differences in antioxidant activity of xanthones were attributed to their different O-H bond dissociation enthalpies and were further explained in terms of electronic effect and intramolecular hydrogen bond effect of substituents (Ji et al., 2005). In this study, xanthones with a catechol group revealed high radical–scavenging activity, which indicates that the radical derived from catechol could be well stabilized by the hydroxyl group and an intramolecular hydrogen bond (Ji et al., 2005). The importance of this hydroxylation pattern on the aromatic ring (vicinal hydroxyl groups) has also been recognized on other series of compounds, such as flavonoids (Silva et al., 2002) and phenolic derivatives (Bendary et al., 2013). Herein, not only the position of the hydroxyl groups, but also its location in the aromatic ring (vicinal) was found to play a role on the structure–antioxidant relationship activity due to its ability to form intramolecular hydrogen bonding. Accordingly, the cooperative effects of hydroxyl groups, especially vicinal, are reflected in increasing hydrogen bond strength between the xanthone and its target (as hypothesized for myeloperoxidase on supplementary data 1). Therefore, QSAR studies are congruent with the docking studies on myeloperoxidase, where compounds **10**, **11,** and **12** showed high binding affinity (supporting information 1). However, further testing using myeloperoxidase *in vitro* will be performed in the future in order to test the myeloperoxidase direct inhibition hypothesis.

From all the above, it can be concluded that the QSAR model is applicable for antioxidant xanthones, which suggests that the model may have predictive capacity for more antioxidant hits.

3.4. UV spectrum and metal chelating activity

From the antioxidant screening assays, emerged the hit compound, 1,2-dihydroxyxanthone (**10**) that showed ability to scavenge DPPH and alkylperoxyl radicals with IC_{50} values of 11.3 μM and 23.8 μM, respectively and inhibitory effect on MPO. The ability of compound **10** to absorb UV radiation was also investigated in the present study. The UV spectrum of the hit compound **10** (Fig. 4) revealed high absorbance for UVB range (280–320 nm). UVB radiation is an inherent component of sunlight and is responsible for a series of damage effects on the skin. Short-term UV exposure leads to erythema and burns, while long-term exposure can promote photocarcinogenesis (Svobodova et al., 2006). The sequential additions of Fe (III) and Cu (II) solutions to compound **10** (Fig. 4A and B, respectively) resulted in significant change of the absorbance spectrum with bathochromic shifts from the original band. As expected, due to the presence of a hydroxyl group *peri* carbonyl in compound **10**, a complex was formed (bathochromic shift in UV–Vis spectra) in the presence of Fe (III) and Cu (II) irons, highlighting the potential of this xanthone derivative to chelate metals. These results indicate that the putative ability of this xanthone derivative to protect the skin against UV damage might be dual, combining antioxidant/metal chelating properties with UV-filter capacity.

Skin oxidative stress occurs due to the continuous exposure to a number of external aggressors such as ultraviolet radiation (UV), ozone and atmospheric pollution. Psoriasis (Yildirim et al., 2003), atopic dermatitis (Niwa et al., 2003), skin cancer (Sander et al., 2003), and photoaging (Wenk et al., 2001) are some examples of skin conditions associated with cutaneous oxidative stress. Direct evidence for the UV radiation-mediated generation of reactive species in skin is growing (Herrling et al., 2003, 2006), which constitutes a rationale for the use of antioxidants in topical formulations. Topical administration of antioxidants has proved to be an efficient way to enrich the endogenous cutaneous protection system (Dreher and Maibach, 2001; Lin et al., 2003), which can contribute to the minimization of UV radiation-mediated oxidative damage in skin. Polyphenols are a structural class of compounds characterized by the presence of phenol structural units as is the case of 1,2-dihydroxyxanthone (**10**) and, among antioxidants, have gained special interest over the past years (Almeida et al., 2008; Ndhlala et al., 2010; Sevin et al., 2007; Svobodova et al., 2003).

3.5. Cytotoxicity evaluation

Considering all the cytotoxicity assays, 1,2-dihydroxyxanthone (**10**) did not promote a significant cytotoxic effect up to 50 μM (Fig. 5). Even at this concentration this xanthone only decreased HaCaT cells viability (to 83%) in the Neutral red assay. Noteworthy, cell viability above 70% can be considered non-cytotoxic (Standardization, 2009). The positive control (DMSO 10%) induced a decrease in HaCaT viability for all assays, falling in the range 7 to 15%. When comparing the four cytotoxicity assays used (Fig. 5), some differences were noted, being the MTT reduction and NR uptake assays the most sen-

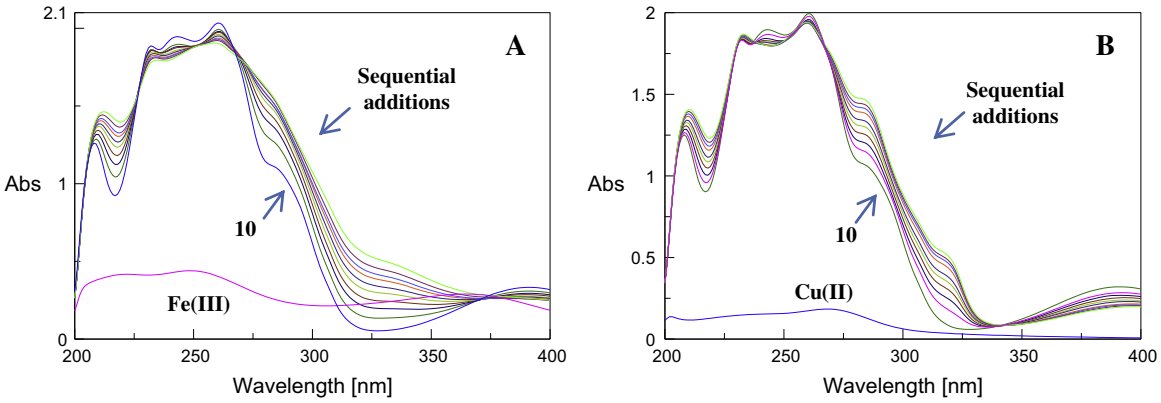


Figure 4 UV/Vis spectra of 1,2-dihydroxyxanthone (**10**) in methanolic solution with sequential additions of Fe (III) (**A**) and Cu (II) (**B**) solutions.

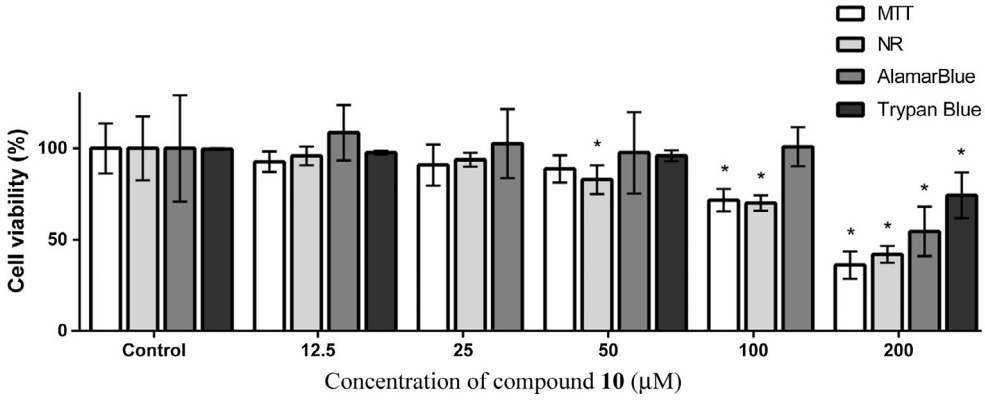


Figure 5 Cell viability of HaCaT cell line exposed to compound **10**, determined by the MTT reduction assay, NR uptake assay Alamar Blue reduction assay and Trypan Blue exclusion. Results were calculated as percentage of negative control (cells treated with the solvent). Data are presented as mean ± SD (n = 3–9). Data were analyzed using One-way ANOVA with Dunnett post hoc test. *Data are significantly different from the control value $p < 0.05$.

sitive tests. IC_{50s} were for these assays 155.43 ± 8.15 and 172.40 ± 14.37 μM, respectively (Table 2). These remarks further reinforce the importance of using different *in vitro* cytotoxicity tests, which rely on different mechanisms of cell death. The cytotoxicity assays used herein assess the lysosomal integrity (NR uptake), cell membrane damage (trypan blue) and metabolic activity (MTT and AlamarBlue®).

The biological activity was observed for concentrations well below the cytotoxic concentrations for keratinocytes which is favorable for the putative application of this compound in topical formulations.

In conclusion, this study highlighted important features in the antioxidant activity of simple oxygenated xanthones, such as a vicinal diols and paves the way to the potential application of the antioxidant hit compound 1,2-dihydroxyxanthone (**10**). Nevertheless, further studies are necessary to assess 1,2-dihydroxyxanthone (**10**) potential as an antiaging or chemopreventive topical ingredient in human volunteers, after UV radiation exposure. More toxicological tests, including *in vivo* skin compatibility should be carried out to meet regulatory requirements before considering the use of the tested compound in cosmetic or pharmaceutical products. To highlight,

Table 2 IC_{50} values and the values of the lowest concentration that promote a statistically significant decrease in cell viability for different cytotoxicity assays.

MTT reduction		AlamarBlue® reduction		Neutral Red uptake		Trypan Blue exclusion	
IC_{50} (μM) [#]	[§]	IC_{50} (μM) [#]	[§]	IC_{50} (μM) [#]	[§]	IC_{50} (μM) [#]	[§]
155.43 ± 8.15	100	> 200	200	172.40 ± 14.37	50	> 200	200

[#] Results expressed as mean ± SD of at least six independent experiments.
[§] Denotes the lowest concentration that promotes a statistically significant decrease in cell viability in comparison with control ($p < 0.05$).

the association of antiradical properties with MPO inhibitory activity observed for the hit xanthone **10** might be valuable in the minimization of UV radiation-mediated oxidative damage in skin.

Acknowledgments

This research was partially supported by the Structured Program of R&D&I INNOVMAR –Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, Research Line NOVELMAR), funded by the Northern Regional Operational Programme (NORTE2020) through the European Regional Development Fund (ERDF) and by Foundation for Science and Technology (FCT) – Portugal, and COMPETE under the projects PTDC/MAR-BIO/4694/2014 (POCI-01-0145-FEDER-016790), PTDC/DTP-FTO/1981/2014 (POCI-01-0145-FEDER-016581), and PTDC/AAGTEC/0739/2014 (POCI-01-0145-FEDER-016793). Helena Ferreira acknowledges POPH/FSE for co-financing and FCT for the fellowship SFRH/BPD/38939/2007. The authors acknowledge CESPU – Portugal, for financial support (04-GCQF-CICS-2011N and COXANT-CESPU 2016).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2017.01.006.

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