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Ascorbic acid potentiates the cytotoxicity of the naphthoquinone 5-methoxy-3,4-dehydroxanthomegnin

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ABSTRACT

The interaction of ascorbic acid with 5-methoxy-3,4-dehydroxanthomegnin, an 1,4-naphthoquinone, was investigated using the cytotoxic index for McCoy cells by neutral red assay. The synergstic effect was observed when such compounds were added simultaneously, most probably due to hydrogen peroxide being generated by ascorbate – driven 5-methoxy-3,4-dehydroxanthomegnin redox cycling. Incubation of cells in the presence of 5-methoxy-3,4-dehydroxanthomegnin/ascorbic acid/catalase, an enzyme that destroys H_2O_2, resulted in an increase of cell survival, reinforcing the involvement of hydrogen peroxide generated as an important oxidizing agent that kills McCoy cells.

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1. Introduction

Mass screening programs of natural products by the National Cancer Institute have identified the quinone moiety as an important pharmacophoric element for cytotoxic activity that makes it possible to apply them as antitumor and antibacterial agents (Pérez-Sacau et al., 2007). Among molecular mechanisms of quinones cytotoxicity and pharmacological activities suggested in the literature, quinone-redox cycling is believed to be the most universal (Cape et al., 2006; Verma, 2006; O'Brien, 1991). Since the higher redox potential of quinone molecules has been correlated with enhanced cellular deleterious effects, the rationale is that the reduction of quinones should be dependent on the quinone-redox potential (Verrax et al., 2004). Nearly 300 naphthoquinones of different structural types have been isolated from plants, bacteria and fungi. These natural occurring compounds have long been used in folk medicine, and more recent studies have proved the therapeutic value of both natural and synthetic naphthoquinones, particularly as antiparasitic and anticancer agents (Munday et al., 1995). The 1,4-naphthoquinone pharmacophore is known to impart anticancer activity in a number of drugs for example, streptonigrin, actinomycins, mitomycins, etc. (Tandon et al., 2004). 5-Methoxy-3,4-dehydroxanthomegnin (**2**) an 1,4-naphthoquinone, was isolated from the capitula *Paepalanthus latipes*, a plant of the Eriocaulaceae family (see Fig. 1 for structures). This species is widespread in the region of the Serra do Cipó, State Minas Gerais, Brazil, and showed a significant cytotoxic index when compared to cisplatin (Kitagawa et al., 2004). The purpose of this study was to explore to what extent ascorbic acid (**1**) affects the basal cytotoxicity of this naphthoquinone.

2. Results and discussion

In the current study we evaluated the ability of the association of ascorbic acid (**1**)/5-methoxy-3,4-dehydroxanthomegnin (**2**) to cause cell death in the same cell line using the neutral red assay (NR). The dose–response curves showed a significant effect of ascorbic acid (**1**) on 5-methoxy-3,4-dehydroxanthomegnin (**2**) cytotoxicity (Fig. 2) and the CI_{50} index was employed to evaluate synergism (Table 1). The synergic effect of ascorbic acid (**1**) on 5-methoxy-3,4-dehydroxanthomegnin (**2**) resulted in cytotoxic index 7 times lower (CI_{50} of 5.9 μM) than when 5-methoxy-3,4-dehydroxanthomegnin (**2**) was added alone to McCoy cell line. This information led us to study the chemical interaction between these species. The spectral change expressed in Fig. 3 is an indication of the reduction of the 5-methoxy-3,4-dehydroxanthomegnin (**2**). A similar result was reported to the reduction of rifampicin-quinone

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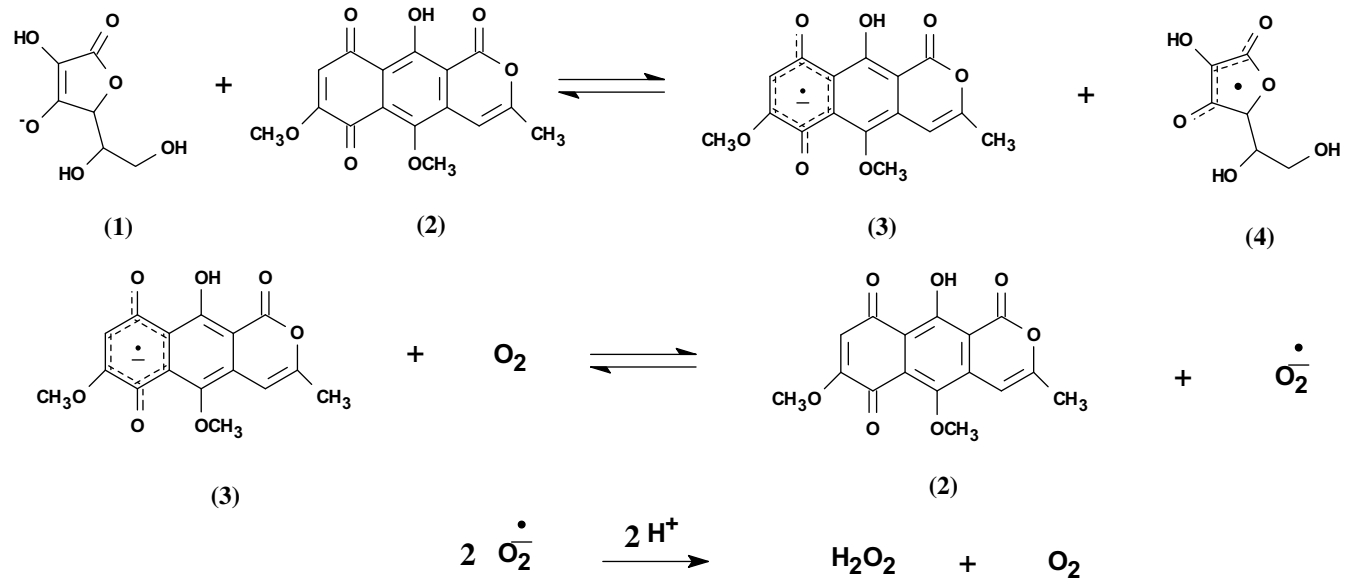


Fig. 1. The proposed effect of ascorbic acid (**1**) on 5-methoxy-3,4-dehydroxanthomegnin (**2**), ascorbate (**1**), 5-methoxy-3,4-dehydroxanthomegnin (**2**), 5-methoxy-3,4-dehydroxanthomegnin semiquinone (**3**), and semidehydroascorbatec (**4**).

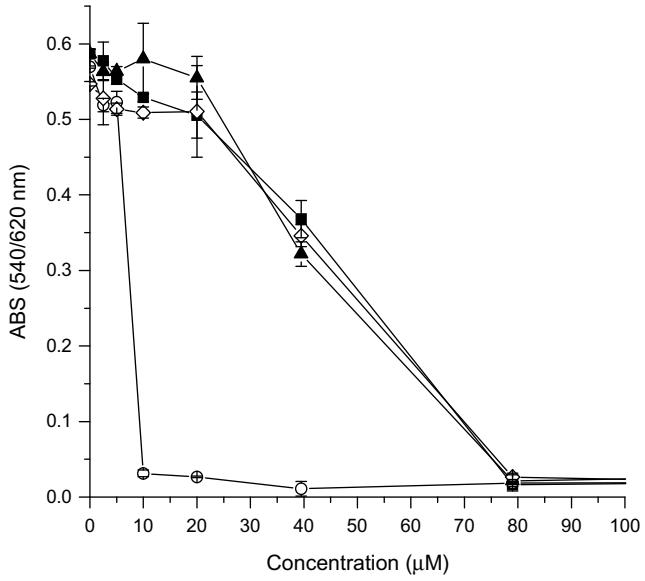


Fig. 2. Dose–effect curve of 5-methoxy-3,4-dehydroxanthomegnin (**2**) (-■-) and its association with ascorbic acid (**1**) (-○-), ascorbic acid (**1**)/catalase (-▲-) and catalase (-◇-). Cells were plated at a density of 10^4 cells/mL per well using 96-well plates. The cell growth was assessed by the NR assay. Each point represents the average of at least three independent experiments, each with four replicates. Errors bar represent the SEM.

by ascorbic acid (**1**) (dos Santos et al., 2005). Evidence of the reduction of 5-methoxy-3,4-dehydroxanthomegnin (**2**) was obtained by mass spectroscopic analysis as demonstrated in Figs. 4 and 5. The mass spectrum depicted in Fig. 5 shows a new base peak of 319 ($M+H^+$) which correlates with the molecular weight of the reduced 5-methoxy-3,4-dehydroxanthomegnin (**2**). To clarify the mass spectroscopy results, Fig. 6 demonstrates the structure of the 5-methoxy-3,4-dehydroxanthomegnin (**2**) and its hydroquinone derivative (two units of mass higher) obtained after non-enzymatic two electron reduction with ascorbate (**1**). The mechanistic proposal for the formation of hydrogen peroxide is given in Fig. 1, which could be responsible for the enhanced cytotoxicity observed

Table 1
Basal cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin (**2**) and its association to McCoy cells determined with the neutral red (NR) assay after 24 h exposure with the compound

Compound(s)	Cytotoxicity Cl_{50} (μM)[a]
5-Methoxy-3,4-dehydroxanthomegnin (**2**)	42.47 ± 1.25
5-Methoxy-3,4-dehydroxanthomegnin (**2**)/ascorbic acid (**1**)	5.9 ± 0.19
5-Methoxy-3,4-dehydroxanthomegnin (**2**)/ascorbic acid (**1**)/catalase	42.67 ± 0.38
5-Methoxy-3,4-dehydroxanthomegnin (**2**)/catalase	42 ± 0.8
Ascorbic acid	3534 ± 18.58

[a] Data represent the means ± SEM of the Cl_{50} obtained from at least three independent experiments, each with four replicates.

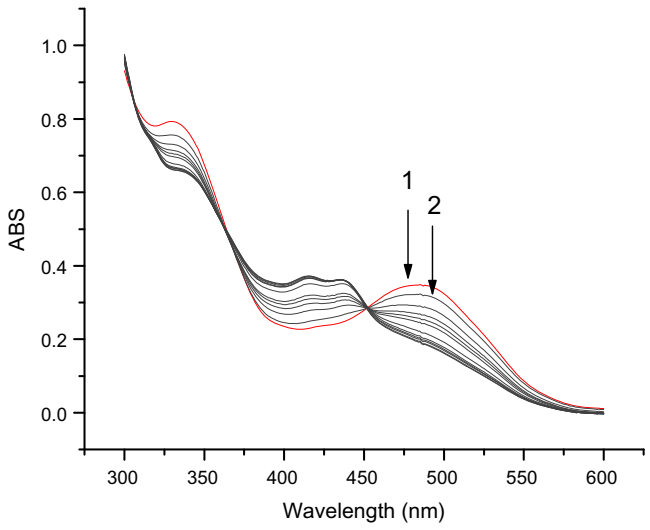


Fig. 3. Spectral changes upon reaction of ascorbic acid with 5-methoxy-3,4-dehydroxanthomegnin. The cuvette contained 0.316 mM 5-methoxy-3,4-dehydroxanthomegnin in 10 mM phosphate buffer pH 7.0 and 4.96 mM of ascorbic acid. The reaction was triggered by adding ascorbic acid. The arrows indicate the direction of the spectral changes. The scans were taken at 1 min intervals.

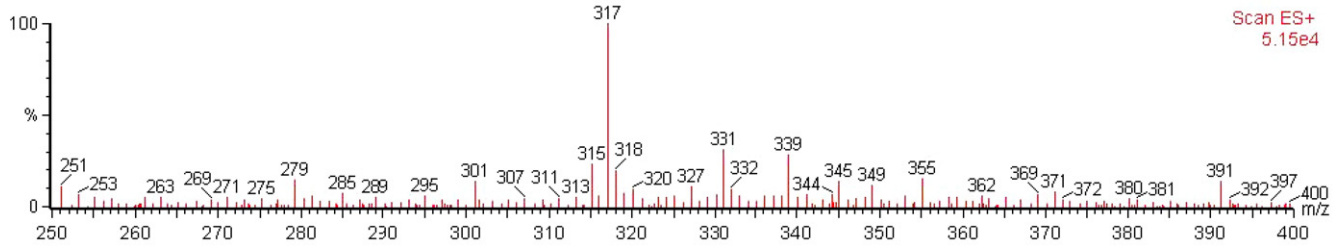


Fig. 4. Mass spectra of 5-methoxy-3,4-dehydroxanthomegnin 3 μM in methanol. MW: 316.

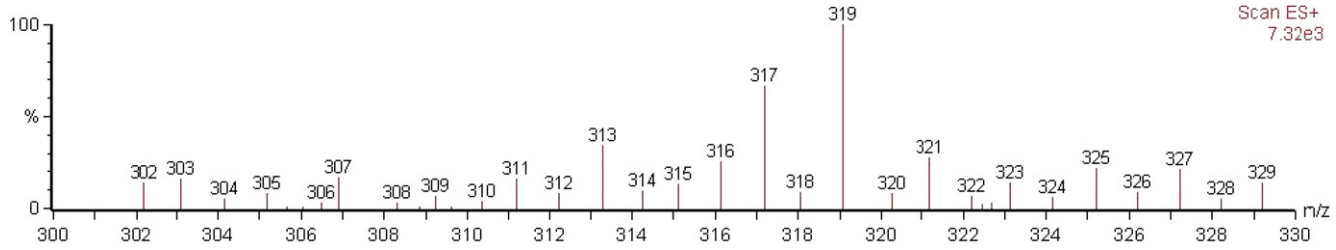
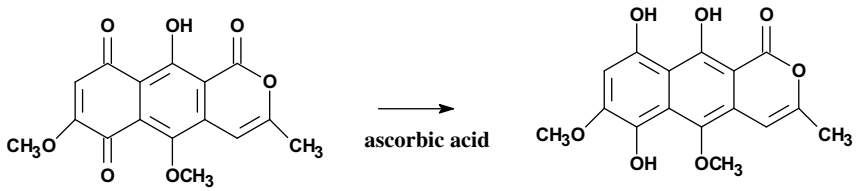


Fig. 5. Structural change of 5-methoxy-3,4-dehydroxanthomegnin by ascorbic acid.



(M+H$^+$) = 317 **(M+H$^+$) = 319**

Fig. 6. Structural change of 5-methoxy-3,4-dehydroxanthomegnin by ascorbic acid.

when ascorbic acid (**1**) was added. In agreement, it is well established that a semiquinone is the intermediate during the reduction of quinone compounds by ascorbate (**1**) and that, in aerobic systems, part of this semiquinone can be oxidized back by molecular oxygen leading to superoxide anion ($O_2^{\cdot-}$), and, subsequently, to hydrogen peroxide (H_2O_2). Among different reactive oxygen species that may be formed during redox cycling between ascorbate and quinones, H_2O_2 is likely the major oxidizing agent involved in the cytotoxicity process (Verrax et al., 2004, 2005). The incubation of cells in the presence of 5-methoxy-3,4-dehydroxanthomegnin (**2**)/ascorbic acid (**1**)/catalase, an enzyme that destroys H_2O_2, canceled the effect of ascorbic acid (**1**), reinforcing that the hydrogen peroxide generated by ascorbic acid (**1**) driven 5-methoxy-3,4-dehydroxanthomegnin (**2**) redox cycling as an important agent that kills McCoy cells. Although catalase canceled the effect of the addition of ascorbic acid (**1**), it had no effect upon the cytotoxic property of 5-methoxy-3,4-dehydroxanthomegnin (**2**) itself (Table 1). It seems to indicate that the enhanced cytotoxicity caused by ascorbic acid (**1**) was an extracellular event, since catalase could not inhibit generation of intracellular H_2O_2. On the other hand, for the native cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin (**2**), a cell membrane permeable molecule, a semiquinone free radical intermediate generated into the intracellular medium could be not discarded. In fact, semiquinone free radicals have been proposed to be able to bind to nucleophilic small molecules (such cysteine and reduced glutathione) or (biological) macromolecules and induce cell death (Tayama and Nakagawa, 1991).

3. Concluding remarks

On the basis of the biological and structural properties, 1,4-naphthoquinones are considered important structures in medicinal chemistry (Pérez-Sacau et al., 2007). The biological activity of 1,4-naphthoquinone is mainly due to the presence of two carbonyl groups that have the ability to accept one and/or two electrons to form the corresponding radical anion or di-anion species (Verma, 2006). Quinone and quinone derivates redox cycling with participation of ascorbate mimics to some degree the enzymatic redox cycling since both processes have nearly the same elementary stages (Roginsky et al., 1999). Given that cancer cells are lacking antioxidant defenses (Borovic et al., 2007; Verrax et al., 2004) they are expected to be more sensitive towards oxidative stress by ascorbic acid (**1**)/5-methoxy-3,4-dehydroxanthomegnin (**2**) than healthy cells.

4. Experimental

4.1. Chemicals

Ascorbic acid (**1**), catalase (EC 1.11.1.7) and dimethylsulfoxide (DMSO) were purchase from Sigma (St. Louis, MO, USA). 5-Methoxy-3,4-dehydroxanthomegnin (**2**), isolated and characterized as previously described from *P. latipes* (Kitagawa et al., 2004), was stored as stock solution at 10.0 mg/ml in DMSO. Eagle medium

with various concentrations of DMSO was used in the experimental design. The final concentration of DMSO (2%) used does not interfere cell viability.

4.2. Cytotoxicity assay

Cytotoxicity activity was evaluated with McCoy mouse fibroblast cell line (ATCC CRL-1696b). Stock culture was maintained in Eagle medium with 7.5% fetal bovine serum. After trypsinization, 200 μL of medium containing approximately 10^4 cells/ml were seeded into 96-well tissue-culture plates and incubated at 37 °C. After 24 h, the Eagle medium was removed and the cells were exposed to unmodified medium (control) or to medium modified with various concentrations of 5-methoxy-3,4-dehydroxanthomegnin (**2**) in the presence or absence of ascorbic acid (**1**) (1:20) and catalase (1160 U). After incubating for another 24 h, the medium was removed and the plates were prepared for microculture neutral red (NR) assay, which is based on the uptake of NR in the lysosomes of living cells (Borenfreund and Puerner, 1985). After brief agitation, the plates were transferred to a microplate reader (Spectra and Rainbow (Shell) Readers – Tecan, Austria) and the optical density of each well was measured using a 540 nm filter and 620 nm reference wavelength. All experiments were performed at least four times, using three wells for each concentration of 5-methoxy-3,4-dehydroxanthomegnin (**2**) alone or associated with ascorbic acid (**1**) and catalase. The cytotoxicity data was standardized by determining absorbance and calculating the 5-methoxy-3,4-dehydroxanthomegnin (**2**) concentration. Linear regression analysis with 95% confidence limit was used to define dose-response curve and to compute the concentration of sample needed to reduce absorbance of the NR by 50%, the so called cytotoxic index (CI_{50}) (Barile, 1994).

4.3. Spectrophotometric analysis

An UV/visible spectrophotometry using a Hewlett–Packard diode-array spectrophotometer (HP8452) was use. Reactions contained 0.316 mM 5-methoxy-3,4-dehydroxanthomegnin (**2**), 4.96 mM ascorbic acid (**1**) and 10 mM phosphate buffer pH 7.0 at 37 °C.

4.4. Mass spectrometry

The reduction of 5-methoxy-3,4-dehydroxanthomegnin (**2**) by ascorbic acid (**1**) was also analyzed by mass spectrometry using a Quattro II Micro™, triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source. The mass spectrometer was operated with positive ionization in the full-scan mode (scan range 300–350 m/z). Spray voltage was set at 4.5 kV, the capillary temperature at 300 °C and desolvation gas flow at 300 L/h. The mass spectra were obtained by direct infusion of the reaction mixture between 5-methoxy-3,4-dehydroxanthomegnin (**2**) and ascorbic acid (**1**) as described above.

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