



Original Article

Evaluation of mutagenicity and metabolism-mediated cytotoxicity of the naphthoquinone 5-methoxy-3,4-dehydroxanthomegnin from *Paepalanthus latipes*



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ABSTRACT

A large number of quinones have been associated with antitumor, antibacterial, antimalarial, and antifungal activities. Results of previous studies of 5-methoxy-3,4-dehydroxanthomegnin, a naphthoquinone isolated from *Paepalanthus latipes* Silveira, Eriocaulaceae, revealed antitumor, antibacterial, immunomodulatory, and antioxidant activities. In this study, we assessed the mutagenicity and metabolism-mediated cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin by using the Ames test and a microculture neutral red assay incorporating an S9 fraction (hepatic microsomal fraction and cofactors), respectively. We also evaluated the mutagenic activity in *Salmonella typhimurium* strains TA100, TA98, TA102, and TA97a, as well as the cytotoxic effect on McCoy cells with and without metabolic activation in both tests. Results indicated that naphthoquinone does not cause mutations by substitution or by addition and deletion of bases in the deoxyribonucleic acid sequence with and without metabolic activation. As previously demonstrated, the *in vitro* cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin to McCoy cells showed a significant cytotoxic index (CI₅₀) of 11.9 μg/ml. This index was not altered by addition of the S9 fraction, indicating that the S9 mixture failed to metabolically modify the compound. Our results, allied with more specific biological assays in the future, would contribute to the safe use of 5-methoxy-3,4-dehydroxanthomegnin, compound that has showed in previous studies beneficial properties as a potential anticancer drug.

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Introduction

"For many centuries, plants have provided a rich source of therapeutic agents and bases for synthetic drugs. Despite the development of organic synthesis, 25% of the drugs prescribed worldwide are derived from plant sources, showing that plant species are still an important source of new drugs" (Sacoman et al., 2008). Much research has been conducted on plants in popular use, with the objective of identifying natural products with therapeutic potential (Balunas and Kinghorn, 2005; Gurib-Fakim, 2006; Newman and Cragg, 2012).

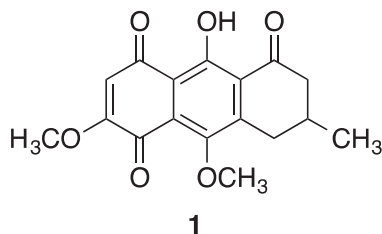
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The Eriocaulaceae family, commonly found in the States of Bahia and Minas Gerais, Brazil, has been the source of several biologically active compounds. *Paepalanthus*, with about 500 species, is one of its principal genus. A number of studies have demonstrated that almost all species of *Paepalanthus* subgenus *Platycaulon* possess naphthopyranone derivatives, including paepalantine and 8,8'-paepalantine dimer isolated from *Paepalanthus bromelioides* (Vilegas et al., 1990; Coelho et al., 2000), planifolin isolated from *Paepalanthus planifolius* (Santos et al., 2001; Varanda et al., 2006), and 5-methoxy-3,4-dehydroxanthomegnin isolated from *Paepalanthus latipes* Silveira, Eriocaulaceae, (Kitagawa et al., 2004, 2008).

The compound 5-methoxy-3,4-dehydroxanthomegnin (**1**) is a naphthoquinone with potential therapeutic applications. Previous studies have shown that this compound has antitumor and immunomodulatory effects (Kitagawa et al., 2011), as well as anti-*Helicobacter pylori* and antioxidant properties

(Kitagawa et al., 2012). The antitumor effect of 5-methoxy-3,4-dehydroxanthomegnin may be enhanced by association with ascorbic acid as demonstrated by a significant cytotoxic index (CI) for McCoy cells. The enhanced effect is probably due to hydrogen peroxide generated by ascorbate-driven 5-methoxy-3,4-dehydroxanthomegnin redox cycling (Kitagawa et al., 2008).



Currently, interest is focused on cytotoxic compounds that appear to exert a beneficial effect on key mechanisms involved in the pathogenesis of cancer and infection diseases. Many active compounds that work by interfering with the function of DNA seem to play a decisive role in antitumor activity (Harvey, 2008; Ma and Wang, 2009; Sudan and Rupasinghe, 2014).

Short-term tests that detect genetic damage have allowed evaluation of the carcinogenic risks of chemicals to humans. The Ames assay, which is recommended for testing the mutagenicity of chemical compounds with potential pharmacological applications (Varanda et al., 2006; Resende et al., 2012; Aardema, 2013), was used in the present study to evaluate *in vitro* the mutagenic effect of 5-methoxy-3,4-dehydroxanthomegnin (1).

In vitro cytotoxicity tests simulate injury to cells from the tested substances, which may be caused by a number of incomplete mechanisms, during periods of exposure that are realistic for acute toxicity (Benbow et al., 2010). The central point regarding *in vitro/in vivo* comparisons refers to xenobiotic-metabolizing capacity. Bioactivation is an important consideration in *in vitro* cytotoxicity assays, since *in vivo* the test agent may be bio-transformed. The incorporation of the S9 microsomal fraction has been used in the study of metabolic activation of chemicals in a variety of cell culture models (Soares et al., 2006; Ponsoni et al., 2013). The test is applicable to the analysis of toxic ranges, for the detection of biotransformation of parent compounds, and for the evaluation of the cytotoxic effects of chemotherapeutic agents (Borenfreund and Puerner, 1987). In this study, we investigated *in vitro* metabolism-mediated cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin using the S9 system. For comparison, we evaluated cyclophosphamide, an indirect-acting cytotoxicant employed in antineoplastic therapy, as the control of biotransformation induction by the hepatic S9 fraction (Hill and Hill, 1984).

Material and methods

Plant material

Paepalanthus latipes Silveira, Eriocaulaceae, was collected at Serra do Cipó in the Cadeia do Espinhaço, Minas Gerais, Brazil, and authenticated by Professor Paulo Takeo Sano from the Institute of Biosciences, University of São Paulo. The voucher specimen (CFSC, 13846) is on file at the Herbarium in the Department of Botany, Institute of Biosciences, University of São Paulo, Brazil.

Chemicals and culture media

Eagle medium was purchased from Adolfo Lutz (São Paulo, Brazil), and fetal bovine serum from Cultilab (Campinas, Brazil). Dimethyl sulfoxide (DMSO), nicotinamide adenine dinucleotide

phosphate sodium salt (NADP), D-glucose-6-phosphate disodium salt, magnesium chloride (MgCl₂), L-histidine monohydrate, D-biotin, sodium azide, 2-anthramine, and 2-aminofluorene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oxoid Nutrient Broth No. 2 (Oxoid; Basingstoke, UK) and Bacto Agar (BD Bacto™; Sparks, MD, USA) were used as bacterial media. D-glucose, magnesium sulfate, citric acid monohydrate, anhydrous dibasic potassium phosphate, sodium ammonium phosphate, monobasic sodium phosphate, dibasic sodium phosphate, and sodium chloride were purchased from Merck (Whitehouse Station, NJ, USA). Neutral red (NR) was obtained from Riedel-de-Haën AG (Seelze, Hannover, Germany). The 5-methoxy-3,4-dehydroxanthomegnin, isolated and characterized as previously described (Kitagawa et al., 2004), was stored as stock solution at 10 mg/ml in DMSO.

Metabolic activation system (S9 mixture)

The S9 fraction, prepared from livers of Sprague-Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg/kg), was purchased from Molecular Toxicology, Inc. (Boone, NC, USA). The metabolic activation system consisted of S9 fraction (4%), 0.4 M MgCl₂ (1%), 1.65 M KCl (1%), 1 M D-glucose-6-phosphate disodium (0.5%), 0.1 M NADP (4%), 0.2 M phosphate buffer (50%), and sterile distilled water (39.5%) (Maron and Ames, 1983).

Salmonella mutagenic assay

Mutagenic activity was tested by *Salmonella*/microsome assay, using the *Salmonella typhimurium* tester strains TA97a, TA98, TA100, and TA102 (kindly provided by B.N. Ames; Berkeley, CA, USA), with and without metabolism by the preincubation method (Maron and Ames, 1983). The strains from frozen cultures were grown overnight for 12–14 h in Oxoid Nutrient Broth No. 2. The metabolic activation mixture (S9) was freshly prepared before each test. Various concentrations of 5-methoxy-3,4-dehydroxanthomegnin dissolved in DMSO were tested: 62.5, 125.0, 250.0, 500.0, and 750.0 µg/plate for strains TA98, TA97a, and TA100; 6.25, 12.5, 25.0, 50.0, and 75.0 µg/plate for strain TA102. These concentrations were selected based on a preliminary toxicity test. In all subsequent assays, the upper limit of the dose range tested was either the highest nontoxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was apparent as a reduction in the number of His⁺ revertants or as an alteration in the auxotrophic background (*i.e.*, background lawn). The various concentrations of 5-methoxy-3,4-dehydroxanthomegnin were added to 0.5 ml of 0.2 M phosphate buffer (pH 7.4) or to 0.5 ml of 4% S9 mixture combined with 0.1 ml of bacterial culture and then incubated at 37 °C for 20–30 min. After this time, 2 ml of top agar was added to the mixture and poured on to a plate containing minimal agar. The plates were incubated at 37 °C for 48 h and the revertant colonies were counted manually. All experiments were analyzed in triplicate. The results were analyzed with the Salanal statistical software package, adopting the Bernstein et al. (1982) model. The data (revertants/plate) were assessed by analysis of variance (ANOVA), followed by linear regression. The mutagenic index (MI) was also calculated for each concentration tested, this being the average number of revertants per plate with the test compound divided by the average number of revertants per plate with the negative (solvent) control. A sample was considered mutagenic when a dose–response relationship was detected and a 2-fold increase in the number of mutants (MI ≥ 2) was observed with at least 1 concentration (Santos et al., 2006). The standard mutagens used as positive controls in experiments without the S9 mixture were 4-nitro-*O*-phenylenediamine (10.0 µg/plate) for TA98

Table 1
Mutagenic activity and mutagenic index (MI) of 5-methoxy-3,4-dehydroxanthomegnin (naphthoquinone) at various concentrations in strains TA100, TA98, and TA97a of *Salmonella typhimurium* in the presence (+S9) and absence (–S9) of metabolic activation.

Naphthoquinone ($\mu\text{g}/\text{plate}$)	Number of revertants/plate and (MI)					
	TA100		TA98		TA97a	
	–S9	+S9	–S9	+S9	–S9	+S9
0	147 \pm 22	121 \pm 13	22 \pm 3	26 \pm 1	218 \pm 2	343 \pm 6
62.5	151 \pm 9 (1.08)	199 \pm 33(1.32)	21 \pm 4 (1.2)	28 \pm 2 (1.27)	304 \pm 28 (1.2)	332 \pm 27 (1.05)
125	151 \pm 8 (1.08)	176 \pm 9 (1.17)	18 \pm 3(1.05)	25 \pm 2 (1.13)	277 \pm 12 (1.1)	399 \pm 10 (1.26)
250	154 \pm 12 (1.10)	185 \pm 30(1.23)	17 \pm 2 (1.0)	26 \pm 2 (1.18)	309 \pm 22 (1.25)	476 \pm 16 (1.51)
500	131 \pm 14 (0.94)	185 \pm 19 (1.23)	17 \pm 3 (1.0)	27 \pm 3 (1.22)	301 \pm 10 (1.21)	398 \pm 26 (1.26)
750	124 \pm 10 (0.88)	216 \pm 53 (1.44)	19 \pm 2 (1.1)	27 \pm 4 (1.22)	294 \pm 16 (1.19)	431 \pm 25 (1.36)
Control+	957 \pm 24	973 \pm 10	1492 \pm 28	2002 \pm 60	1484 \pm 32	1521 \pm 31

0 = negative control: 75.0 μl DMSO.

Positive controls: +S9 \rightarrow 2-anthramine (2.5 $\mu\text{g}/\text{plate}$) for TA100, TA98 and TA97a strains. –S9 \rightarrow sodium azide (1.25 $\mu\text{g}/\text{plate}$) for TA100; 4 nitro-*o*-phenylenediamine (10.0 $\mu\text{g}/\text{plate}$) for TA98 and TA97a. The results are reported as means \pm SD.

* $p < 0.05$ (ANOVA) 5-methoxy-3,4-dehydroxanthomegnin concentrations compared with negative control.

and TA97a, sodium azide (1.25 $\mu\text{g}/\text{plate}$) for TA100, and daunomycin (3.0 $\mu\text{g}/\text{plate}$) for TA102. In the experiments with metabolic activation, 2-anthramine (2.5 $\mu\text{g}/\text{plate}$) was used with TA98, TA97a, and TA100, and 2-aminofluorene (10.0 $\mu\text{g}/\text{plate}$) with TA102. DMSO served as the negative (solvent) control (75.0 $\mu\text{l}/\text{plate}$).

Cytotoxicity assay

McCoy cells (CCL1–ATCC/USA, from the cell culture section of the Adolfo Lutz Institute, São Paulo, Brazil) were maintained in Eagle medium with 7.5% fetal bovine serum. After trypsinization, 0.2 ml of medium containing approximately 10^4 cells/ml were seeded into 96-well microtiter tissue culture plates and incubated at 37 °C. After 24 h, the Eagle medium was removed and the cells were placed in unmodified medium (control) or in medium modified with various concentrations of 5-methoxy-3,4-dehydroxanthomegnin (5, 10, 20, 40, 50, 80, and 100 $\mu\text{g}/\text{ml}$) or cyclophosphamide (25, 50, 100, 150 $\mu\text{g}/\text{ml}$) with and without the S9 mixture at 10%. After incubating for another 24 h, the medium was removed and the plates were prepared for the NR assay (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 a 620 nm reference wavelength. All experiments were performed at least four times, using three wells for each concentration of compound tested. The cytotoxicity data were standardized by determining absorbance and calculating the corresponding chemical concentrations. Linear regression analysis with a 95% confidence limit was used to define dose–response curves and to compute the concentrations of chemical agents needed to reduce absorbance of the NR by 50% (IC_{50}), called cytotoxic midpoint (Barile, 1994).

Results

Tables 1 and 2 show the mutagenicity results for 5-methoxy-3,4-dehydroxanthomegnin which demonstrate that this compound does not possess mutagenic activity for the strains TA100, TA97a, and TA98, with MI less than 2.0 at all tested concentrations with or without metabolic activation. The TA102 strain was found to be more sensitive to the toxic effects of 5-methoxy-3,4-dehydroxanthomegnin; thus, it was necessary to decrease the concentrations used with TA102 relative to the other strains. In fact, the lowest concentration used in the experiments with TA100, TA97a, and TA98 was close to the highest concentration used with

Table 2

Mutagenic activity and mutagenic index (MI) of 5-methoxy-3,4-dehydroxanthomegnin (naphthoquinone) at various concentrations in strain TA102 of *Salmonella typhimurium* in the presence (+S9) and absence (–S9) of metabolic activation.

Naphthoquinone ($\mu\text{g}/\text{plate}$)	Number of revertants/plate and (MI)	
	TA102	
	–S9	+S9
0	400 \pm 10	301 \pm 5
6.25	355 \pm 6 (0.93)	292 \pm 17 (0.92)
12.5	401 \pm 8 (1.05)	295 \pm 38 (0.93)
25	384 \pm 5 (1.01)	307 \pm 8 (0.96)
50	389 \pm 23 (1.02)	288 \pm 15 (0.90)
75	379 \pm 30 (1.0)	282 \pm 22 (0.88)
Control +	1157 \pm 54	1348 \pm 66

0 = negative control: 75.0 μl DMSO.

Positive controls: +S9 \rightarrow 2-aminofluorene (10.0 $\mu\text{g}/\text{plate}$). –S9 \rightarrow daunomycin (3.0 $\mu\text{g}/\text{plate}$). The results are reported as means \pm SD.

* $p < 0.05$ (ANOVA) 5-methoxy-3,4-dehydroxanthomegnin concentrations compared with negative control.

TA102. Nevertheless, the TA102 strain did not display mutagenicity in the absence or presence of metabolic activation.

The concentration–response curve for cytotoxicity was established for 5-methoxy-3,4-dehydroxanthomegnin in the presence and absence of an S9 mixture as an external metabolizing system (Fig. 1). Table 3 shows the IC_{50} of 5-methoxy-3,4-dehydroxanthomegnin and cyclophosphamide (control) for McCoy cells in the presence and absence of the S9 system. The IC_{50} obtained for 5-methoxy-3,4-dehydroxanthomegnin in the presence of the S9 mixture did not differ from that attained without the metabolizing system. Exposure of the McCoy cells to cyclophosphamide with and without S9 confirms the efficacy of the hepatic microsomal fraction for *in vitro* metabolic activation assay.

Table 3

Cytotoxic midpoint ($\mu\text{g}/\text{ml}$) on McCoy cells for 5-methoxy-3,4-dehydroxanthomegnin and cyclophosphamide without and with the S9 metabolic activation.

Compound	Without S9 ^a	With S9 ^a
5-Methoxy-3,4-dehydroxanthomegnin	11.9 \pm 1.15	10.08 \pm 0.38
Cyclophosphamide	>150	21.6 \pm 1.7

^a Values are means \pm SD ($n = 3$).

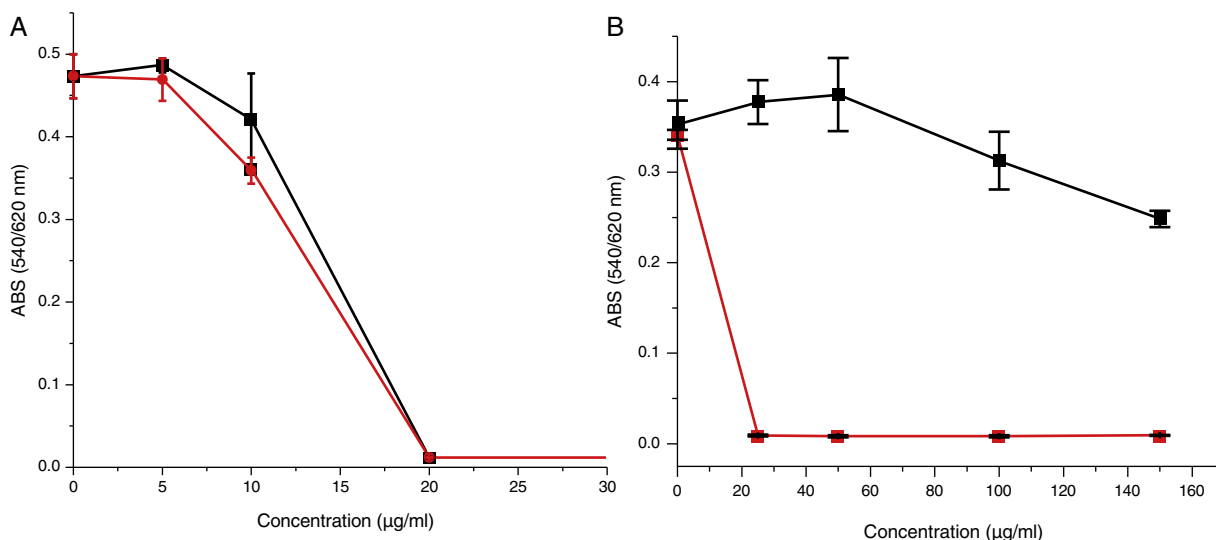


Fig. 1. Concentration-effect relationship of 5-methoxy-3,4-dehydroxanthomegnin (A) and cyclophosphamide (B) on McCoy cells with (red line) and without the S9 (black line) metabolic activation system. Each point and bar represents the mean \pm SD for at least three independent experiments carried out in triplicate. (For interpretation of the references to color in this text, the reader is referred to the web version of the article.)

Discussion

During the past few decades, plant research has revealed several chemical compounds with important pharmacological activities. Some of these compounds have been incorporated into drugs such as antineoplastics-vinblastine and vincristine isolated from *Catharanthus roseus*, camptothecin derivatives obtained from *Camptotheca acuminata*, derivatives of podophyllotoxin from the rhizomes of *Podophyllum peltatum* and *P. hexandrum*, and taxol extracted from *Taxus brevifolia* (Cragg and Newman, 2005; Srivastava et al., 2005; Basmadjian et al., 2014). However, the literature also describes many plants containing mutagenic compounds, such as furocoumarins, tannins, anthraquinones, alkaloids, and flavonoids (Rietjens et al., 2005; Nesslany et al., 2009; Guterres et al., 2013; Mininel et al., 2014). This evidence draws attention to the importance of studying the genetic risks of plant compounds, since the presence of mutagens in medicines can be dangerous to human health.

Previous studies have shown that species belonging to *Paepalanthus* subgenus *Platycaulon* possess naphthopyranone derivatives. The naphthopyranone paepalantine isolated from *P. vellozoides* exhibited strong mutagenicity and cytotoxicity (Varanda et al., 1997). The genotoxic potential of paepalantine was also demonstrated by Tavares et al. (1999) in *in vivo* assays with bone marrow cells of Wistar rats. The mutagenic activity of planifolin isolated from *P. planifolius* was tested through *Salmonella*/microsome assays, with results indicating that this naphthopyranone dimer caused mutations by substitution and by addition and deletion of bases in the DNA sequence. Moreover, its mutagenic potential increased in the presence of metabolism. These results, allied to the chemical structure, suggest that planifolin may act as an intercalating agent in the DNA molecule (Varanda et al., 2006). In the present study, we assessed the mutagenic activity of 5-methoxy-3,4-dehydroxanthomegnin (**1**) using *Salmonella*/microsome assays. The compound 5-methoxy-3,4-dehydroxanthomegnin was not mutagenic to strain TA98, which is used to detect frameshift mutations. Substitution of DNA bases in strain TA100 and oxidative DNA damage in strain TA102 were not detected. No mutagenicity was observed in the TA97a strain, which detects frameshift mutations that are sensitive to heavy metal mutagens. The results were also negative for all the *S. typhimurium* strains tested with the S9 mixture.

However, data reported in the literature reveal that some quinones, including naphthoquinones, present mutagenicity after metabolism. Chesis et al. (1984) concluded that the mutagenicity of quinones was mainly due to one-electron reduction of quinones to semiquinones *via* the formation of superoxide anion radical ($O_2^{\bullet-}$) and, subsequently, hydrogen peroxide (H_2O_2). Tikkanen et al. (1983) observed that naphthoquinones with 1 or 2 hydroxyl and/or methyl substituents are mutagenic with metabolic activation. Nevertheless, it seems that the position of substituents, as well as the number of substituents, is important for mutagenicity. This point seems to explain the absence of mutagenicity of 5-methoxy-3,4-dehydroxanthomegnin, which has hydroxyl and methyl substituents and did not present mutagenicity in tests with or without metabolism.

Considerable interest has been focused on short-term *in vitro* cytotoxicity assays with cultured cells for evaluation of acute toxicities of chemical agents and pilot studies in drug development. Such assays would not only curtail the use of animals for median lethal dose (LD_{50}) and similar tests, but would serve as an economical approach to the rapid screening of xenobiotics (Greene et al., 2010).

Cytotoxicity due to direct-acting chemicals is readily demonstrable *in vitro*. However, the toxicity of many chemicals is dependent upon metabolic activation, usually catalyzed by the microsomal cytochrome P-450-dependent monooxygenase system, and the majority of cell lines currently used in *in vitro* cytotoxicity tests possess little intrinsic drug-metabolic activity. Consequently, problems arise when metabolism-mediated cytotoxic events are studied *in vitro*. One possible answer to this problem is the coincubation of cultured cells with metabolically active rodent liver fractions, in a manner similar to their use in *in vitro* mutagenesis assays (Gonzalez, 2005; Li et al., 2012; Cole et al., 2014).

In previous studies, 5-methoxy-3,4-dehydroxanthomegnin showed significant *in vitro* cytotoxicity to McCoy cells in the NR microculture test compared with *cis*-diamminedichloroplatinum (cisplatin), one of the most widely used chemotherapeutic drugs (Kitagawa et al., 2004). Our results indicated that combined treatment with 5-methoxy-3,4-dehydroxanthomegnin and hepatic S9 microsomal fraction did not alter the cytotoxicity of this naphthoquinone.

One hypothesis explaining the same cytotoxic potential of 5-methoxy-3,4-dehydroxanthomegnin with and without the S9 microsomal system is that this compound does not act as a substrate for the enzymatic system. Previous studies explored redox cycling of 5-methoxy-3,4-dehydroxanthomegnin in a nonenzymatic system. In this study, we used the NR assay to evaluate the ability of ascorbic acid associated with 5-methoxy-3,4-dehydroxanthomegnin to cause cell death in the same cell line. The synergic effect of ascorbic acid on 5-methoxy-3,4-dehydroxanthomegnin (1) resulted in a CI that was seven times lower than the index for 5-methoxy-3,4-dehydroxanthomegnin alone added to the McCoy cell line. The observed synergic effect was most probably due to H₂O₂ generated by ascorbate-driven 5-methoxy-3,4-dehydroxanthomegnin redox cycling (Kitagawa et al., 2008), indicating that the association of 5-methoxy-3,4-dehydroxanthomegnin with ascorbic acid may be promising in the treatment of solid tumors that are deficient in antioxidant defenses.

The results of this study investigating the mutagenic activity and metabolism-mediated cytotoxicity of 5-methoxy-3,4-dehydroxanthomegnin, allied in the future with more specific biological assays, will contribute to the safe use of 5-methoxy-3,4-dehydroxanthomegnin, signifying its beneficial properties as a potential anticancer drug.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

R.R.K. contributed to running the laboratory work in isolation and identification of the 5-methoxy-3,4-dehydroxanthomegnin, biological studies, analysis of the data, and drafted the paper. W.V. supervised the laboratory work in the isolation and identification of the 5-methoxy-3,4-dehydroxanthomegnin and contributed to critical reading of the manuscript. E.A.V. supervised the laboratory work in Ames Test and contributed to critical reading of the manuscript. M.S.G.R. designed the study, supervised the laboratory work in the cytotoxicity test, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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