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RESEARCH ARTICLE

In vitro and *in vivo* evaluation of genotoxicity, cytotoxicity, and protective effects of synthetic chalcones (E)-3-(4-chlorophenyl)-1-phenyl-2-propen-1-one (4-CL) and (E)-3-(3,4-dimethoxyphenyl)-1- phenyl-2-propen-1-one (DMF)

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Abstract: The chalcones (E)-3-(4-chlorophenyl)-1-phenyl-2-propen-1-one (4-CL) and (E)-3-(3,4-dimethoxyphenyl)-1-phenyl-2 -propen-1-one (DMF) are versatile and easily synthesized into low-cost compounds that have a wide spectrum of biological activities. In this study, the cytotoxic, genotoxic and modulatory activities of 4-CL and DMF were evaluated using the Ames test and the mouse micronucleus assay. The results of the Ames test revealed that both chalcones did not show mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100, and demonstrated significant antimutagenicity ($p < 0.05$) when co-administered with sodium azide (SA) in strain TA100. In the micronucleus assay, both showed a significant increase in the frequency of micronucleated polychromatic erythrocytes (MNPCE) at 24 h and 48 h, revealing a genotoxic effect. In the co-treatment with mitomycin C (MMC) there was a significant decrease ($p < 0.05$) in the frequency of MNPCE both in chalcones at 24 h and in the less concentrated dose of DMF at 48 h, demonstrating its antigenotoxic activity. 4-CL showed a significant decrease in the polychromatic / normochromatic erythrocyte (PCE / NCE) ratio at 24 and 48 h ($p < 0.05$), indicating cytotoxicity. However, 4-CL and DMF when co-administered with MMC showed a significant increase in the PCE/NCE ratio within 24 hours, demonstrating anticytotoxicity. Furthermore, a biphasic dose-response behavior was observed in both chalcones, 4-CL in the co-administration with SA, in the Ames test and DMF in the co-treatment with MMC, at 48 hours of exposure, in the micronucleus assay. In this study, 4-CL and DMF showed genotoxic, cytotoxic, antigenotoxic, anticytotoxic and no mutagenic properties.

Keywords: *Salmonella typhimurium*, mutagenicity, genotoxicity, chlorochalcone, dimethoxychalcone, micronucleus, OECD

1 Introduction

Chalcones are precursors of the biosynthesis of flavonoids and chemically, consists of an open-chain molecule with two aromatic rings (A and B) interconnected by a three-carbon α, β-unsaturated carbonyl system [1,2]. A classical method of chalcone synthesis involves base catalyzed Claisen-Schmidt condensation of an acetophenone and a benzaldehyde [2]. The introduction of different phenyl substituents makes chalcone a versatile compound and resulting in a wide spectrum of bioactivities and potential applications [2–10].

The chalcones (E)-3-(4-chlorophenyl)-1-phenyl-2-propen-1-one (4-CL) and (E)-3-(3,4-dimethoxyphenyl)-1-phenyl-2-propen-1-one (DMF) exhibit pharmacological properties, such as antifungal [11,12]; antibacterial [13,14]; inhibitory properties against enzymes [15]; *in vitro* cytotoxicity in cancer cell lines and no hemolytic activities [16]. Recently, according to Chen *et al*. [17], DMF exhibited cardioprotective and potential anticancer effects in mice thought the activation of pro-autophagic transcription factors. The chalcone 4-CL has presented also others activities, such as antioxidant [18], antiprotozoal [19], antinociceptive [20], anti-inflammatory [18],

larvicidal [21, 22], osteogenic [23], antiplasmodial activity [24] and no neurotoxic effect [25]. According to Sahin *et al*. [26], chalcone 4-CL can be also considered as good candidate for liver cancer therapeutics. Considering the importance of chalcones, including chalcones 4-CL and DMF for further therapeutic development, it is relevant to evaluate the genotoxic, mutagenic and antigenotoxic activities of these compounds.

In vitro and *in vivo* genotoxicity tests are mandatory by regulatory agencies and recommended by scientific committees worldwide prior to marketing of new drugs, since they can be used for screening mutagens and potential carcinogens in human environments, as well as for the identification of antimutagens and anticarcinogens [27–29].

Several, toxicogenetic assays have been associated to detect different endpoints of mutagenicity and genotoxicity, such as chromosomal alterations and gene mutations. In this sense, two common test systems employed for genotoxicity evaluation, following OECD (Organisation for Economic Co-operation and Development) guidelines are the Ames test (OECD 471) and the micronucleus assay in bone marrow (OECD 474). The first one is widely as initial standard assay to determine the mutagenic potential of new drugs, chemicals even the environmental samples [27, 29]. The Ames test uses histidine auxotrophic *Salmonella typhimurium* strains having different mutational specificities. In this study, two strains, TA98 and TA100, which detect frameshift mutations and base-pair substitutions, respectively, were used to evaluate chemical-induced point mutation activity [30, 31].

The micronucleus assay can measure chromosome breaks and chromosome loss, since micronucleus are fragments formed from mutations of intact chromosomes that fail to be included in the nuclei of daughter cells at the completion of telophase. Micronucleus is easily observed in polychromatic erythrocytes (PCE) cells in the bone marrow. The greater the number of micronucleated immature erythrocytes (MNPCE), the greater is the chromosomal damage, as result of exposure to clastogenic and/or aneugenic agents [32, 33].

Given the pharmacological and therapeutic potential of chalcones 4-CL and DMF, these compounds have attracted great interest. However, the knowledge of chalcones about their effects on DNA is scarce and the detection or absence of genotoxic, mutagenic activities of 4-CL and DMF compounds remained unknown. Thus, the aim of this study was to evaluate the cytotoxicity, genotoxicity and mutagenicity of 4-CL and DMF, and their protective effects against DNA damage through the Ames test and the mouse bone marrow micronucleus assay. The present study reports relevant findings for the safety assessment in view of possible future applications of these chalcones.

2 Material and methods

2.1 Chalcones

Synthesis and structural characterization of evaluated chalcones (Figure 1) 4-CL [(*E*)-3-(4-chlorophenyl)-1-phenyl-2-propen-1-one] and DMF [(*E*)-3-(3,4-dimethoxyphenyl)-1-phenyl-2-propen-1-one] have been previously described by Ramalho *et al*. [16].

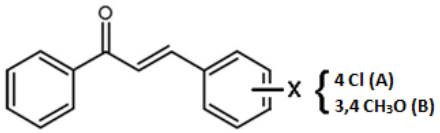

Figure 1 Chemical structure of synthetic chalcones 4-CL (A) and DMF (B)

2.2 Ames test: Salmonella mutagenicity test

2.2.1 Strains

Salmonella typhimurium strains TA98 (hisD3052) (rfa) (uvrB) (AmpR) and TA100 (hisG46) (rfa) (uvrB) (AmpR) [34] were kindly supplied by Divisão de Toxicologia, Genotoxicidade e Microbiologia Ambiental of Companhia de Tecnologia de Saneamento Ambiental do Estado de São Paulo (CETESB, São Paulo, SP, Brazil).

2.2.2 Experimental procedure and analysis

The *Salmonella* histidine point mutation assay proposed by Maron and Ames [33] was followed. A 0.1 mL aliquot of bacterial suspension (1-2 $\times$ 10^9 cells/mL) of each strain (TA98 and TA100) was incubated with doses of 1, 10, 50, 100, 500, and 1000 μg/plate of chalcones

4-Cl or DMF, previoulsy diluted in 20 μL of dimethylsulfoxide [DMSO, (CAS 67-68-5, lot no. 0900221, Vetec, Duque de Caxias, RJ, Brazil)], at 37°C for 25 min. These five doses used in our study cover a range from 1 to 1000 μg/plate in accordance to Mortelmans and Zeiger [29], which recommend a minimum of five doses and a range of at least three logs. Then, a 2.0 mL aliquot of top agar [0.6% agar (lot no. 082613209, Kasvi, Curitiba, PR, Brazil), 0.5% NaCl, 50 μM L-histidine, and 50 μM biotin] was added to the mixture at 45°C. The mixture was stirred and poured onto Petri plates over the surface of agar minimum glucose medium [1.5% agar (lot no. 082613209, Kasvi, Curitiba, PR, Brazil) 2% glucose, and Vogel-Bonner medium E ($MgSO_4H_2O$, lot no.1109865; $C_6H_8O.7H_2O$, lot no.1100795; K_2HPO_2, lot no. DCBB4100; $Na_2NH_2PO_4.H_2O$, lot no.1208265, Vetec, Duque de Caxias, RJ, Brazil)] for the detection of histidine revertant. Revertant colonies were manually counted after incubation in the dark for 48 h at 37°C. Each assay was performed three times in triplicate and included a negative control (20 µL of DMSO) and a positive control 0.5 μg 4-nitroquinoline-1-oxide [4NQO, (CAS 56-57-5, lot no. SLBD6960V, Sigma-Aldrich, Sao Paulo, Brazil)] per plate for TA98 and 3.0 µg sodium azide [SA, (CAS 26628-22-8, lot no. 26628–22–8, Merck, Cotia, SP, Brazil)] for the TA100 strain. To evaluate antimutagenicity, the same doses of chalcones 4-Cl and DMF employed in the mutagenic assessment were co-administered with the respective positive controls.

Mutagenicity was measured using the mutagenic index (MI), which was calculated for each concentration. MI = (number of revertant colonies per plate in the test per plate)/(number of revertant colonies per plate in the negative control), and a compound is considered mutagenic when MI $\geq$ 2 [35]. For the antimutagenicity assays was calculated the mutagenicity variation (MV), in percentage, using the formula MV = $\{[(S1\text{-}So) / (M\text{-}S0)] \text{ -1 } \} \times 100$. Where, M = number of revertant / plate induced by mutagen alone (4-NQO or SA), S0 = the number of spontaneous revertant (DMSO), S1 = the number of revertant / plate induced by chalcone plus the mutagen (4-NQO or SA). Approximately - 40% $\leq$ MV $\leq$ - 25% was defined as moderate antimutagenicity; MV $<$ - 40% was defined as strong antimutagenicity; - 25% $\leq$ MV $\leq$ 0% was defined as no antimutagenicity and MV $>$ 0% occur when there is an increase in mutagenicity, according Gulluce *et al*. [36] adapted.

2.3 Mouse bone marrow micronucleus assay

2.3.1 Animals

Healthy, male adult (8-12-weeks-old) outbred mice (*Mus musculus*, Swiss Webster), weighing 25-30 g, were provided by the central bioterium of Federal University of Goiás. Animals were randomly allocated to treatment in nine groups of five, placed in polyethylene cages (40 $\times$ 30 $\times$ 16 cm) lined with wood shavings, and kept at 24 $\pm$ 2°C and 55 $\pm$ 10% humidity, with a 12 h natural light-dark cycle. The animals were fed *ad libitum* with a standard pellet diet (commercial rodent diet Labina, Ecibra Ltda., Santo Amaro, SP, Brazil) and water. Animals were used after a minimum 5-days acclimation.

2.3.2 Ethics statement

All experimental protocols were evaluated and approved by the Comissão de Ética no Uso de Animais /CEUA of the Federal University of Goiás, Goiânia, Brazil, under CEUA/UFG no. 017/2011.

2.3.3 Experimental procedure and analysis

The experiments were carried out according to von Ledebur and Schmid [36]. To assess genotoxicity, three doses of chalcones 4-Cl or DMF (25, 50, and 100 mg.kg^{-1} body weight (b.w.)) were intraperitoneally (i.p.) administered to five animals per treatment. A solvent control group (0.1% DMSO, vehicle) and a positive control group exposed to 4 mg.kg b.w. of Mitomycin C [MMC, (CAS 50-07-7, lot no. 1K00322, Bristol-Myers Squibb, São Paulo, Brazil)] were included.

The animals were sacrificed 24 h or 48 h after the chalcones administration by cervical dislocation. Then, MN samples were flushed from the bone marrow of both femurs of each animal and immediately, suspended and homogeneized in 1 mL of fetal bovine serum (FCS, lot no. 61005001, Laborclin, Pinhais, PR, Brazil) at room temperature. Four slides of each animal were prepared. Then, they were fixed in absolute methanol (lot no. 1207433COD: 000102.06, Vetec, Duque de Caxias, RJ, Brazil), air dried, and stained with 10% Giemsa (lot no. 21101108C, Newprov, Pinhais, PR, Brazil), dibasic sodium phosphate ($Na_2HPO_4.12H_2O$, Vetec) in monobasic sodium phosphate ($NaH_2PO_4.12H_2O$ Vetec).

Genotoxicity and antigenotoxicity were assessed by the frequency of micronucleated immature erythrocytes (MNPCE). The incidence of MNPCE was calculated by counting a total of 10000 PCE per animal via light microscopy (Olympus BH-2 10 × 100, Tokyo, Japan) and results were expressed as % MNPCE. The cytotoxicity and anticytotoxicity were evaluated by polychromatic / normochromatic erythrocyte (PCE / NCE) ratio.

To analyze the genotoxic and antigenotoxic activities of chalcones 4-Cl and DMF, the frequency of MNPCE in the treated groups are presented as mean ± SD, and the analysis of variance (One-way ANOVA) was performed followed by Tukey multiple comparison test. To assess its cytotoxicity and anticytotoxicity, the PCE / NCE ratio of chalcones were compared to the negative control using the chi-square (χ^2) test. P values lower than 0.05 ($p < 0.05$) were considered indicative of significance.

3 Results

3.1 Ames test: Salmonella mutagenicity assay

The results of the chalcones 4-CL and DMF mutagenicity and antimutagenicity carried out using the bacterial reverse mutation test (Ames test) are shown in Table 1. All the tested doses of both chalcones (0.1, 1, 10, 100, and 1000 μg/plate) - did not increased significantly ($p > 0.05$) the number of histidine revertant (His+) colonies in TA98 and TA100 strains from that of the control group. In contrast, the positive control chemicals markedly increased the corresponding mutant counts over the vehicle control group in each *Salmonella* strain, demonstrating the sensitivity of our testing system and in agreement with the guidance established by Maron and Ames [33] and Mortelmans and Zeiger [29]. These results indicated that 4-CL and DMF did not induce gene mutation in prokaryotic cells in the Ames test showing a mutagenicity index (MI) of less than 2 (MI < 2) for both compounds in all treatments.

In addition, samples resulting in > 80 % viable cells compared with the negative control or MI > 0.8 in the Ames test are considered non-toxic [37]. Thus, 4-CL and DMF did not affect bacteria's viability, suggesting no cytotoxicity in the both strains at the tested doses, except for the treatment with DMF at a dose of 1000 μg/plate in the TA98 strain which showed a toxic effect corroborated by the decrease in the number of His+ revertants.

Table 1 Evaluation of mutagenicity and antimutagenicity of the chalcones 4-CL and DMF using two strains of *Salmonella typhimurium* by Ames test

Treatment		Mutagenicity				Antimutagenicity			
Compound	Dose (μg/plate)	TA98		TA100		TA98		TA100	
		$\overline{X}$ + SD	MI	$\overline{X}$ + SD	MI	$\overline{X}$ + SD	MV (%)	$\overline{X}$ + SD	MV (%)
DMSO*	**	30.92 ± 4.21	1.0^{i}	181.66 ± 60.66	1.0^{i}	30.92 ± 4.21	-100	181.66 ± 60.66	-100
4-NQO***	0.5	398.75 ± 74.83	12.90^{j}	-	-	398.75 ± 74.83	0	-	-
SA***	3.0	-	-	1652.62 ± 169.86	9.10^{j}	-	-	1652.62 ± 169.86	0
4-CL	0.1	42.55 ± 3.51^{a}	1.38^{i}	169.99 ± 30.80^{a}	0.94^{i}	402.87 ± 38.22^{c}	1.12^{h}	776.81 ± 04.30^{d}	-59.54^{g}
4-CL	1.0	34.90 ± 6.93^{a}	1.13^{i}	159.08 ± 28.82^{a}	0.88^{i}	398.38 ± 33.33^{c}	-0.10^{e}	483.65 ± 12.57^{d}	-79.47^{g}
4-CL	10	36.90 ± 5.57^{a}	1.19^{i}	152.74 ± 27.68^{a}	0.84^{i}	373.69 ± 25.00^{c}	-6.81^{e}	1354.90 ± 64.69^{c}	-20.24^{e}
4-CL	100	26.26 ± 2.08^{a}	0.85^{i}	166.44 ± 30.18^{a}	0.92^{i}	372.20 ± 24.61^{c}	-7.22^{e}	1321.51 ± 65.10^{c}	-22.51^{e}
4-CL	1000	30.25 ± 2.52^{a}	0.98^{i}	163.65 ± 29.97^{a}	0.90^{i}	335.16 ± 21.69^{c}	-17.29^{e}	2114.51 ± 66.15^{d}	31.40^{h}
DMF	0.1	27.79 ± 4.15^{a}	0.90^{i}	165.77 ± 38.57^{a}	0.91^{i}	415.04 ± 54.02^{c}	4.43^{h}	1388.44 ± 148.80^{c}	-17.96^{e}
DMF	1.0	26.09 ± 2.88^{a}	0.84^{i}	151.91 ± 42.65^{a}	0.84^{i}	384.25 ± 33.17^{c}	-3.94^{e}	1447.87 ± 626.17^{c}	-13.92^{e}
DMF	10	29.49 ± 3.77^{a}	0.95^{i}	168.31 ± 35.61^{a}	0.93^{i}	386.39 ± 33.31^{c}	-3.36^{e}	1456.55 ± 298.99^{c}	-13.33^{e}
DMF	100	30.06 ± 3.81^{a}	0.97^{i}	166.38 ± 39.93^{a}	0.92^{i}	390.62 ± 46.74^{c}	-2.21^{e}	1239.28 ± 279.28^{d}	-28.10^{f}
DMF	1000	20.98 ± 5.12^{a}	0.68^{i}	177.79 ± 33.49^{a}	0.98^{i}	386.02 ± 36.56^{c}	-3.46^{e}	1232.67 ± 252.87^{d}	-28.55^{f}

*Negative control: dimethylsulfoxide (DMSO); **20 μL per plate. ***Positive control: 4-nitroquinoline-1-oxide (4-NQO) for TA98 or sodium azide (SA) for TA100. MI, mutagenic index; MV, mutagenicity variation. ^{a}No significant difference compared with the negative control ($p > 0.05$); ^{b}Significant difference compared with the negative control ($p < 0.05$); ^{c}No significant difference compared with the positive control ($p > 0.05$); ^{d}Significant difference compared with the positive control ($p < 0.05$); ^{e}No antimutagenic effect (- 25% < MV < 0%); ^{f}Moderate antimutagenicity (- 40% < MV < - 25%); ^{g}Strong antimutagenicity (VM < - 40%); ^{h}Increment in mutagenicity (MV > 0%); ^{i}no mutagenic (MI < 2); ^{j}mutagenic (MI > 2). All values are resulting of three independent experiments carried out in triplicate. Statistical analysis: one-way ANOVA and Tukey's test.

The antimutagenicity results, shown in Table 1, demonstrated that all tested doses of 4-CL and DMF (1, 10, 50, 100, 500 and 1000 μg/plate) co-administered with 4-NQO did not cause a significant decrease ($p > 0.05$) in the number of revertant histidine colonies (His +) in the TA98 strain colonies compared to the positive control (4-NQO). In contrast, in strain TA100 there were strong and moderate protective effects of 4-CL and DMF, respectively, against the mutagenic effects of SA with a significant decrease ($p < 0.05$) in the number of revertant colonies. Co-treatment with 4-CL at 0.1 and 1 μg/plate reached MV of -59.54% and -79.47%

(strong antimutagenicity), respectively, while DMF at 100 and 1000 μg/ plate reached MV of -28.10% and -28.55% (moderate antimutagenicity), respectively. Hence, at these doses, the tested chalcones were able to protect DNA against base-pair substitution induced from the mutagen SA. In contrast, at 1000 μg/plate, chalcone 4-CL increased significantly ($p > 0.05$) the mutagenicity in the same system by 31.40% compared to the positive control (SA).

3.2 Mouse bone marrow micronucleus assay

The frequencies of MNPCE and PCE / NCE ratio of the groups treated with 4-CL and DMF alone and co-treated with MMC after 24 and 48 h of treatments are shown in Table 2. In this study, the negative control group (DMSO) exhibited low MNPCE values, and the positive control group (MMC) exhibited a significant increase in MNPCE compared to the negative control group ($p < 0.05$), validating the sensitivity of the micronucleus assay.

Table 2 Frequency of MNPCE and (PCE / NCE) in bone marrow cells of mice

Treatment ($mg.kg^{-1}$ b.w)			24 Hours			48 Hours		
			MNPCE / 2000 PCE	MNPCE	PCE / NCE	MNPCE / 2000 PCE	MNPCE	PCE / NCE
MMC**	4-CL	DMF	$\overline{X} \pm$ SD	variation (%)	$\overline{X} \pm$ SD	$\overline{X} \pm$ SD	variation (%)	$\overline{X} \pm$ SD
0	0*	0*	3.00 ± 1.00	-	1.07 ± 0.03	3.00 ± 1.00	-	1.07 ± 0.03
0	25	0	7.00 ± 0.67^{b}	-	0.93 ± 0.26^{b}	3.60 ± 0.52^{a}	-	0.89 ± 0.24^{b}
0	50	0	21.80 ± 1.55^{b}	-	1.08 ± 0.15^{a}	11.40 ± 1.55^{b}	-	1.01 ± 0.20^{a}
0	100	0	16.20 ± 3.00^{b}	-	0.93 ± 0.07^{b}	7.40 ± 1.05^{b}	-	0.75 ± 0.26^{b}
0	0	25	7.80 ± 0.42^{b}	-	1.28 ± 0.27^{b}	4.60 ± 1.26^{a}	-	1.10 ± 0.10^{a}
0	0	50	8.00 ± 1.13^{b}	-	1.35 ± 0.31^{b}	5.60 ± 0.51^{b}	-	1.02 ± 0.79^{a}
0	0	100	8.80 ± 0.41^{b}	-	1.51 ± 0.17^{b}	6.80 ± 0.77^{b}	-	0.99 ± 0.14^{a}
4	0	0	39.00 ± 1.19	0.00	0.55 ± 0.13	18.20 ± 1.76	0.00	0.83 ± 0.38
4	25	0	26.20 ± 2.44^{d}	- 35.56	0.86 ± 0.10^{d}	27.60 ± 2.37^{f}	61.84	0.54 ± 0.18^{f}
4	50	0	23.60 ± 2.13^{d}	- 42.78	0.95 ± 0.21^{d}	23.80 ± 1.66^{f}	36.84	0.70 ± 0.28^{f}
4	100	0	23.20 ± 4.42^{d}	- 43.89	1.02 ± 0.12^{d}	22.00 ± 3.49^{f}	25.00	1.00 ± 0.58^{f}
4	0	25	11.80 ± 0.95^{d}	- 75.56	0.95 ± 0.12^{d}	9.00 ± 0.71^{f}	- 60.53	0.85 ± 0.03^{e}
4	0	50	23.40 ± 1.40^{d}	- 43.33	1.04 ± 0.20^{d}	16.80 ± 1.48^{e}	- 9.21	0.58 ± 0.05^{f}
4	0	100	36.80 ± 5.33^{c}	- 6.11	1.72 ± 0.43^{d}	26.60 ± 2.61^{f}	55.26	0.76 ± 0.26^{e}

*Negative control: dimethylsulfoxide (DMSO) 10 $mL.Kg^{-1}$ body weight (b.w.). **Positive control: mitomycin C (MMC) 4 $mg.kg^{-1}$ b.w. (80% LD50). [a]No significant difference compared with the negative control ($p > 0.05$). [b]Significant difference compared with the negative control ($p < 0.05$). [c]No significant difference compared with the positive control at 24 h ($p > 0.05$). [d]Significant difference compared with the positive control at 24 h ($p < 0.05$). [e]No significant difference compared with the positive control at 48 h ($p > 0.05$). [f]Significant difference compared with the positive control at 48 h ($p < 0.05$). All values are means ($\overline{X}$) $\pm$ SD (media $\pm$ standard deviation). Data for the groups (n = 5) were analysed using one-way ANOVA, Tukey's test, and chi-square test.

The genotoxic results, shown in Table 2, indicate that the groups treated with doses of 25 $mg.kg^{-1}$ b.w. of 4-CL and DMF showed significant genotoxicity ($p < 0.05$) at 24 h compared with the negative control group. The groups treated with 50 and 100 $mg.kg^{-1}$ b.w. of 4-CL and DMF showed genotoxicity at both exposure times compared to the negative control group, however, the genotoxic effects at these same doses decreased by up to 50% in the period from 24 to 48 h. The PCE / NCE ratio assesses the cytotoxic potential of the compounds tested. The groups treated with dosages of 25 and 100 $mg.kg^{-1}$ b.w. of 4-CL showed significant cytotoxicity ($p < 0.05$) compared to the negative control group at both treatment times.

Regarding the protective effects of both chalcones against MMC-induced DNA damage (4 $mg.kg^{-1}$ b. w., 80% LD_{50}), all co-treated groups showed a significant decrease in the frequency of MNPCE / 2000 PCE and cytotoxicity induced by MMC at 24 h ($p < 0.05$), except for the group co-treated with 100 $mg.kg^{-1}$ b. w. of DMF, which did not show an antigenotoxic effect. In the 24-hour treatment time, the antigenotoxicity of both chalcones at the doses tested reduced from 35.56% to 75.56% the frequency of MNPCE induced by MMC.

On the other hand, after 48 h, 4-CL at all doses tested and DMF at 100 $mg.kg^{-1}$ bw co-administered with MMC, showed a significant increase in genotoxicity in all groups ($p < 0.05$) when compared with the positive control group. Thus, at the 48-hour treatment time, there was an increase in the percentage of MNPCE in all groups, indicating no antigenotoxicity effect of 4-CL and DMF chalcones against MMC-induced genotoxicity (Table 2). In this same treatment period, only 4-CL at 100 $mg.kg^{-1}$ b. w. co-treated with MMC, showed anticytotoxic activity (PCE / NCE ratio not statistically different from the negative control), while the other treatments induced an increase in cytotoxicity co-administered with MMC when compared to the positive control group.

In addition, at 48-hour treatment time, only the chalcone DMF, at 25 $mg.kg^{-1}$ b. w. co-treated with MMC, showed significant antigenotoxic activity ($p < 0.05$). A biphasic dose-response

behavior seems to be associated, as an antigenotoxic effect can be observed at the lowest dose (25 mg.kg^{-1} b.w.), followed by a decrease in this action at 50 mg.kg^{-1} b.w. and an increase in genotoxicity at the highest dose (100 mg.kg^{-1} b.w.).

4 Discussion and conclusion

The chalcones 4-CL and DMF have been shown to have diverse biological activities and pharmaceutical applications [11–19, 21–23]. However, the toxicogenetic risk information about them is mandatory to ensure their safe use [27, 28]. In this sense, this study evaluated the cytotoxicity, genotoxicity and mutagenicity of 4-CL and DMF, and their chemoprotective effects against DNA damage induced by 4-nitroquinoline-1-oxide (4-NQO) and sodium azide (SA) using Ames test, and mitomycin C (MMC) via micronucleus assay.

In the Ames test, reversion to histidine prototrophy occurs because of mutations that restore gene function and allow the cells to synthesize histidine [30, 35]. In this study, the chalcones 4-CL and DMF did not show mutagenicity. A similar condition was found in other studies with the Ames test using substituted chalcones with other different groups [38–41]. Furthermore, Rashid *et al*. [42] analyzed the mutagenicity of several ρ-monosubstituted chalcones, by the Ames test, and using multiple factorial analysis revealed that the increase in hydrophilicity and resonance electronic were the most important factors associated with mutagenicity. Thus, the low hydrophilicity may be associated with absence of mutagenicity in *S. typhimurium* of 4-CL and DMF.

In this study, animals treated with 4-CL at 25 and 100 mg.kg^{-1} b.w, exhibited statistically significant cytotoxicity within 24 h, which increased within 48 h. This cytotoxic activity is also identified in other animal models, in which 4-CL has shown toxicity in brine shrimp lethality assay [18, 43] and in several tumor cell lines using the MTT assay [16, 25, 44]. A similar cytotoxic condition can be observed in the Ames test when the MI results from the treatment with doses of 4-CL and DMF was lower than that of the negative control (IM = 1.00) in strains TA98 and TA100. However, in the latter case, lethality may be related to the antibacterial effect of these chalcones, since in previous studies, 4-CL and DMF compounds showed antibacterial activity [12–14, 45–49] .

Several studies have suggested that chalcones exert cytotoxicity through the inhibition and/or induction of metabolic enzymes [50], inhibition of tubulin polymerization [51], induction of apoptosis [52], induction of mitochondrial uncoupling and membrane collapse [53], interfering with the p53 interaction [54], producing ROS [55], blocking the cell cycle [53], and acting as antimitotic agents [56].

Microtubules of the mitotic spindle play an important role in cellular processes, including cell division, and the tubulins are the target of antimitotic drugs [57]. Studies have revealed that chalcones can also inhibit tubulin polymerization, prevent the formation of the mitotic spindle, or make it defective inhibiting the mitotic activity [51]. Structure-activity relationship (SAR) studies have shown that tubulin polymerization can be inhibited via hydrophobic interaction. However, when inhibition is associated with other types of cell damage induced by genotoxic processes, cells can evade apoptosis through of the formation of micronuclei cells [58].

Cabrera *et al*. [38] evaluated the potential of 4-CL and other chalcones to induce liver enzymes in rats and observed a significant reduction in the activity of glutathione S-transferases (GST) ($p < 0.05$). GST detoxifies cytotoxic substrates and protects cells from damage induced by oxidative stress. They can be inhibited by a variety of hydrophobic compounds, through the conjugation of the chalcone portion to a glutathione (GSH) molecule, generating a less active and more hydrophilic conjugate, which can be expelled from the cell via transport proteins [59, 60]. This conjugation can also occur between GSH and an α, β-unsaturated carbonyl entity (weak electrophile) through the addition of Michael. Substituents that withdraw electrons in the B ring, such as chlorine and methoxyl, can increase the reactivity of chalconic compounds, favoring their reactivity to thiol groups present in proteins such as *N*-acetyl-*L*-cysteine and o-acetylserine. Acetylation can impair the detoxification system and induce DNA adduct formation and chromosomal instability that can lead to apoptosis and growth arrest (cytotoxicity), or the formation of MN in clastogenic and aneugenic processes [54, 61]. This mechanism may be responsible for the formation of micronuclei in 4-CL and DMF.

Iftikhar *et al*. [54] demonstrated that 4-CL can induce the accumulation of p53 in a tumor cell (HCT116), similar to a positive control. Tumor suppressor protein p53 induces cell cycle arrest and cell death by apoptosis. The increased cytotoxicity of 4-CL from 24 to 48 h during co-treatment with MMC may be associated with the observed cumulative DNA damage resulting in cell death. Furthermore, micronucleated cells can be eliminated by apoptosis, which may

explain the decrease in the frequency of MNPCE observed from 24 hours to 48 hours of treatment with both chalcones.

The physical state of the surrounding lipids and the composition of the lipid bilayer can modulate membrane transport proteins such as P-glycoprotein (P-gp) [4]. Hydrophobicity and other factors, such as the size and shape of molecules, can interfere with the ability of chalcones to interact with P-gp and change the inflow / efflux pump, reducing its absorption or promoting its bioaccumulation [62]. Di Pietro *et al*. [63] investigated the binding capacity of flavonoids, including chalcones, to the P-gp domain and found that halogenated chalcones have high affinity for P-gp. Furthermore, Ivanova *et al*. [62] demonstrated that 4-chloro-substituted chalcones in combination with cytotoxic compound have similar properties to the response observed with the highest concentration of 4-CL co-treated with SA, in the Ames test, and all doses co-administered with MMC in 48 h, in the micronucleus test, where an increase in mutagenicity and genotocicity was observed in relation to the positive controls SA and MMC, respectively. Some authors also observed that the cytotoxic effect of the association, chalcone and cytotoxic compounds, such as MMC, were greater than that presented by each compound alone, due, at least in part, to the decrease in cell membrane fluidity [64, 65].

On the other hand, high concentrations of some hydrophobic compounds can result in microcrystallization [42], increasing the generation of ROS during biotransformation from hydrophobic to a hydrophilic compound to improve clearance and therefore an increase in mutagenicity [66], such as occurred with the highest concentration of 4-CL co-treated with SA, in the Ames test.

Arora *et al*. [67] demonstrated that flavonoids and isoflavonoids are preferentially partitioned in the hydrophobic core of membranes and interact with the polar head groups of phospholipids at the lipid-water interface and stabilize the membrane through a decrease in lipid fluidity. This interaction may contribute to protection against lipid peroxidation, making the membrane less susceptible to ruptures caused by oxidative mechanisms. In previous studies on membrane stabilization, 4-CL and DMF were reported to have hemolytic activity in erythrocytes [16, 18]. This fact suggests that membrane stabilization may be responsible for the increase in the PCE / NCE ratio observed during co-treatment with MMC at all doses tested after 24 h, for both chalcones and at 100 $mg.kg^{-1}$ of 4-CL after 48 h when compared to the respective positive control, generating a protective effect against MMC cytotoxicity in mouse bone marrow.

The antimutagenic activity of 4-CL and DMF in S. typhimurium was evaluated in co-treatment with two mutagenic agents, 4-NQO and SA. 4-NQO induces intracellular oxidative stress, generating reactive oxygen species (ROS) and metabolic products that can bind to guanine DNA causing damage (frameshift mutations) in the TA98 strain [68]. In this study, the results showed that both compounds were unable to reduce the mutagenicity induced by 4-NQO (MV $< 25\%$). However, 4-CL was evaluated by Kumar *et al*., [18] for its antioxidant effect and weak activity against ROS was observed when compared to their respective controls. This data can justify its statistically significant decrease in the mutagenicity of the TA98 strain.

However, in the TA 100 strain, 4-CL strongly reduced the mutagenic effect induced by SA, mainly at doses of 0.1 and 1 μg/plate, while DMF showed mild antimutagenic effect at doses of 100 and 1000 μg/plate. SA induces the generation of ROS and organic metabolites, which interact with [69]. The mutagenicity of SA is mediated by the production of the metabolite azidoalanine through the action of o-acetylserine sulfhydrylase [70]. The antimutagenicity of the evaluated chalcones may occur due to the inactivation of this enzyme as a function of the chalcone - thiol enzyme group interaction. Furthermore, Torigoe *et al*. [71] found that chalcones with methoxy substituent groups in the para-position showed antimutagenic activity in TA100 strains in co-treatment with benzopyrene.

The treatments of DMF co-treated with MMC, at 48 hours of exposure, in micronucleus test, and treatments of 4-CL co-treated with SA, in the TA100 strain showed protective and damage effects to DNA, depending of the dose applied. This biphasic dose-response behavior was observed in many other compounds [72–75]. These data are very important for risk assessments, since they can be used to avoid potential toxic effects and to maximize benefits in the design of pre-clinical and clinical studies [76].

Although the results of this study may be useful for understanding these small molecules and their interaction with DNA, the mechanism by which 4-CL and DMF exert their effects remains unknown and may involve multiple and complex actions.

Finally, in flavonoid mutagenicity research, activity reports are largely conflicting, likely due to variable characteristics of the chemical structure based on inter- and intra-assay variation [77]. However, similar results for 4-CL and DMF were observed with other compounds, such as hydroquinone, taxol, chloramphenicol and azidothymidine, without mutagenicity found in bacterial experimental systems and clastogenic / aneugenic activity in mammals [78, 79]. Furthermore, the balance between the therapeutic and toxicological effects of a compound is an

important and necessary measure of a therapeutic agent's usefulness.

Finally, in flavonoid mutagenicity research, reports of activity are widely conflicting, probably owing to varying characteristics of the chemical structure based on inter- and intra-assay variation [78]. However, similar results for 4-CL and DMF were observed with other compounds, such as hydroquinone, taxol, chloranfenicol, and azidothymidine, with no mutagenicity found in bacterial experimental systems and clastogenic/aneugenic activity in mammals [78, 79]. Furthermore, the balance between therapeutic and toxicological effects of a compound is an important and required measure of the usefulness of a therapeutic agent.

Conflict of Interest and Funding

The authors declare that there are no conflicts of interest. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa de Goiás (FAPEG) and Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior - Brazil (CAPES) under Grant number 88881.189920/2018-01.

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