Research, Society and Development, v. 12, n. 1, e16012139541, 2023
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Evaluation of chalcones derivatives in lipid peroxidation reduction induced by Fe^{2+}/EDTA *in vitro*

Avaliação de derivados de chalconas na redução da peroxidação lipídica induzida por Fe2+/EDTA in vitro

Evaluación de derivados de chalconas en la reducción de la peroxidación lipídica inducida por Fe2+/EDTA in vitro

Received: 12/19/2022 | Revised: 12/31/2022 | Accepted: 01/04/2023 | Published: 01/06/2023

Jadyellen Rondon e Silva
ORCID: https://orcid.org/0000-0002-4564-4614
Federal University of Mato Grosso, Brazil
E-mail: jrondon586@gmail.com
Anderson de Oliveira Souza
ORCID: https://orcid.org/0000-0002-3067-380X
Federal University of Mato Grosso, Brazil
E-mail: anderson.souza@ufmt.br
Italo Curvelo dos Anjos
ORCID: https://orcid.org/0000-0001-5575-6102
Federal University of Mato Grosso, Brazil
E-mail: italocurvelo@gmail.com
Geilly Mara Silva de Pádua
ORCID: https://orcid.org/0000-0002-5738-1022
Federal University of Mato Grosso, Brazil
E-mail: geilly_mara@hotmail.com
Lucas Campos Curcino Vieira
ORCID: https://orcid.org/0000-0002-1138-9117
Federal University of Mato Grosso, Brazil
E-mail: lucas.vieira@ufmt.br
Bibiana Mozzaquatro Gai
ORCID: https://orcid.org/0000-0002-3523-3010
Federal University of Mato Grosso, Brazil
E-mail: bibianagai@yahoo.com.br

Abstract
Reactive oxygen species (ROS), when in excess, cause damage to biomolecules (DNA, lipids, carbohydrates, and proteins), which are related to several pathologies. There is a growing study of substances that act to inhibit or reduce the action of these ROS. Chalcones are aromatic ketones with a naturally occurring and easily synthesized α,β-unsaturated system. In this work, we investigated the *in vitro* protective action of a new class of chalcones functionalized by the thiobarbituric acid reactive substances (TBARS) assay. The most promising molecule (Chalcone E) was submitted to the DPPH radical scavenging assay, and the MTT cell viability test evaluated its cytotoxicity. In addition, Chalcone E reduced lipid peroxidation, with maximum inhibition values of 73.05% for the brain, 81.42% for the liver, and 87.23% for the kidney, demonstrating a potential antioxidant effect. Still, the DPPH test did not observe this effect, suggesting further investigation of this molecule.
Keywords: Chalcones; Reactive species; Antioxidant; Hepatoprotective; Lipid peroxidation.

Resumo
As espécies reativas do oxigênio (EROs), quando em excesso, ocasionam danos a biomoléculas (DNA, lipídeos, carboidratos e proteínas) que estão relacionadas a diversas patologias. Diante disso, aumentou-se a busca por substâncias capazes de reduzir ou inibir a ação destas espécies. Chalconas são cetonas aromáticas com sistema α,β- insaturado, de ocorrência natural e facilmente sintetizada. Neste trabalho, investigamos a atividade protetora *in vitro* de uma nova classe de chalconas funcionalizadas pelo teste de Substâncias Reativas ao Ácido Tiobarbitúrico (TBARS). A molécula mais promissora (Chalcona E) foi submetida ao teste de sequestro do radical DPPH e toxicidade através da viabilidade celular pelo método de MTT. Ademais, a Chalcona E foi eficiente em reduzir a peroxidação lipídica com valores de inibição máxima de 73,05% para o cérebro, 81,42% para o fígado e 87,23% para o rim, demonstrando um potencial efeito antioxidante. Ainda assim, o teste DPPH não observou esse efeito, sugerindo uma investigação mais aprofundada dessa molécula.
Palavras-chave: Chalconas; Espécies reativas; Antioxidante; Hepatoprotetora; Peroxidação lipídica.

Resumen
Las especies reactivas de oxígeno (EROs), en exceso, provocan daños en biomoléculas (ADN, lípidos, carbohidratos y proteínas), las cuales se relacionan con diversas patologías. Existe un creciente estudio de sustancias que actúan para inhibir o reducir la acción de estas EROs. Las chalconas son cetonas aromáticas con un sistema α,β-insaturado natural y fácilmente sintetizado. En este trabajo, investigamos la acción protector in vitro de una nueva clase de chalconas funcionalizadas por el ensayo de sustancias reactivas al ácido tiobarbitúrico (TBARS). La molécula más prometedora (Chalcone E) se sometió al ensayo de eliminación de radicales DPPH y la prueba de viabilidad celular MTT evaluó su citotoxicidad. Además, Chalcone E redujo la peroxidación lipídica, con valores máximos de inhibición del 73,05 % para el cerebro, 81,42 % para el hígado y 87,23 % para el riñón, demostrando un potencial efecto antioxidante. Aún así, la prueba DPPH no observó este efecto, lo que sugiere una mayor investigación de esta molécula.
Palabras clave: Chalconas; Especies reactivas; Antioxidante; Hepatoprotector; Peroxidación lipídica.

1. Introduction

Reactive oxygen species (ROS) naturally occur in the body as part of inflammatory defense, cell signaling, and ATP production, among other physiological processes. Additionally, ROS can be produced from external sources such as ultraviolet radiation (UV), stress, diet, alcohol, and drugs (Barreiros et al., 2006; Ribeiro et al., 2005). The imbalance between ROS and antioxidant agents is known as "oxidative stress." It is responsible for damage to biomolecules (proteins, carbohydrates, lipids and nucleic acids) related to processes such as mutagenesis, carcinogenesis, and neurodegenerative diseases, among others already reported in the literature (Sies, 1993).

Natural products can be obtained from the diet, constituting a non-enzymatic antioxidant defense from an exogenous source (Xu et al., 2017). In the history of natural products, widely used medicinal herbs for treatments became the basis for synthesizing new drugs. So, the union of different areas of knowledge and chemistry allowed a significant advance in the pharmaceutical industry (Lourenço et al., 2019; Barros, 2020). The interest in molecules with simple structures and pharmacological actions is based on cost reduction, easy obtaining, and flexibility in changing structures to enhance their effect, and chalcones have met this requirement.

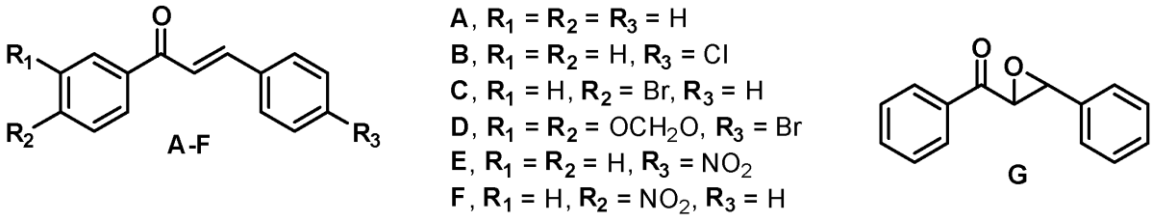
Chalcones (1,3-diaryl-2-propen-1-ones) are a class of compounds derived from flavonoids, found naturally in roots, fruits, and flowers, responsible for their yellowish color (Bohm, 1975). This class is considered a privileged structure because it has a broad spectrum of biological activities and is easy to synthesize (Zhuang et al., 2017). Their biological activities included: anti-inflammatory, anti-cancer, bactericidal, antiviral, antimalarial, anxiolytic, and antioxidant (Sahu, 2012; Ferreira et al., 2018). In this way, the chalcones have great drug potential. In addition, the structural modification of chalcones has improved their biological profile (Ouyang et al., 2021), further increasing interest in this class and reinforcing its title of privileged structure. This study aimed to investigate the *in vitro* protection against lipid peroxidation of seven synthetic chalcones.

2. Methodology

Synthesis of Chalcone Derivatives

The chalcone derivatives A-G (Figure 1) were prepared according to Vieira et al (2012), Vieira et al (2017), and Targanski et al (2021).

Figure 1 - Chemical structure of chalcone derivatives A-G.



Source: Authors.

Animals

Swiss albino mice weighing 25 to 30 g were used, conditioned in temperature conditions of 22 ± 2ºC, and maintained in a 12h light/12h dark cycle, fed with commercial chow. The experiments were conducted in accordance with the Ethics Committee for Animal Use-UFMT (Nº23108.151789/2016-39). The animals were euthanized, and tissues (brain, liver, and kidneys) were removed to assess lipid peroxidation.

Lipid peroxidation

The lipid peroxidation assay was performed by the TBARS method, according to Okawa (1984). Different concentrations of chalcones **A-G** (1-100 µM) were added to the reaction mixture containing tissue homogenate (brain, liver, and kidney), 500 µM of EDTA, and 1.4 mM of iron sulfate (Fe_2SO_4). After incubation for 1 hour at 37º C, 200 µL of sodium dodecyl sulfate (SDS) 8.1%, 500 µL of thiobarbituric acid (TBA) solution 0.8%, and 500 µL of acetic acid buffer pH 3.4 were added. After incubation for 2 hours at 95º C, performed for reading in a UV-Vis spectrophotometer (Shimadzu) at a wavelength of 532 ηm. MDA concentration was expressed as nmol MDA/mg tissue. For calculations, we constructed a standard MDA curve.

Radical scavenger activity (2,2-diphenyl-1-picrylhydrazyl) DPPH

In vitro, antioxidant activity was evaluated by the DPPH radical reduction method as standardized by Sharma and Bhat (2009). Different concentrations of chalcone were added to the reaction mixture containing 50 µM of the DPPH radical diluted in ethanol. The samples were incubated at room temperature for 30 minutes and measured in a spectrophotometer at 517 ηm. Ascorbic acid (0.01; 0.1; 0.25 and 1 µM) was used as a standard.

Cell Viability Test

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, which is based on the reduction of MTT to formazan in metabolically active cells. Chinese hamster lung fibroblast cells (V79-4, ATCC® CCL-93™) were treated for 24 hours with a culture medium containing chalcone E of 1, 10, and 100 µM. After this period, the cells were washed and received MTT, incubated for 2 hours, and washed again for the addition of DMSO and consequent solubilization of formazan. The absorbance quantification was determined in a microplate reader (Spectramax® 190, Molecular Devices) at 540 ηm. The absorbance values obtained were converted to % cell viability, with 100% representing control cells grown only with the appropriate medium.

Statistical analysis

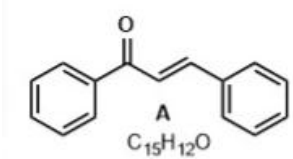
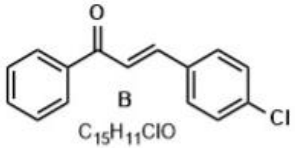
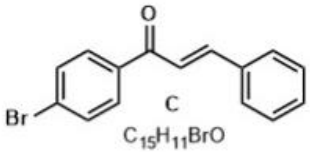
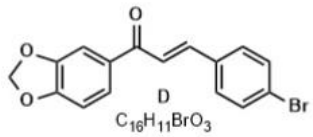
Data are expressed as mean ± standard error of the mean (S.E.M.). Data normality was analyzed using the D'Agostino-Pearson test. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test and T-test. *P* values

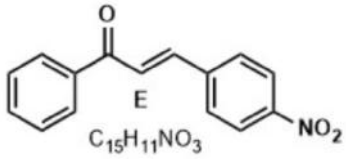
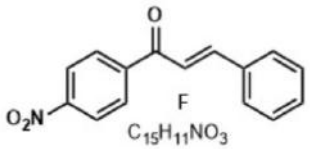
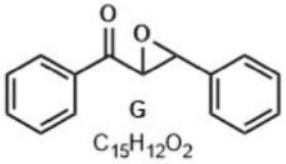
< 0.05 were considered significant.

3. Results

Herein, we show the effects of chalcones on iron sulfate-induced lipid peroxidation in Swiss albino mice (brain, liver, and kidney). Also, the statistical analysis showed that chalcones **A-C**, and **G** did not change the iron sulfate-induced lipid peroxidation in the mouse brain, liver, and kidney, in none of the concentrations evaluated compared to the induced group (Table 1).

Table 1 - Effect of functionalized chalcones on Fe^{2+}/EDTA-induced lipid peroxidation in mouse brain, liver, and kidney *in vitro*.

Chalcone		Brain	Liver	Kidney
 A $C_{15}H_{12}O$	Control	1.070 ± 0.0751	0.3600 ± 0.0379	0.3175 ± 0.0239
	Induced	1.830 ± 0.1501*	0.6267 ± 0.0449*	0.5600 ± 0.0638*
	Vehicle	1.967 ± 0.0722	0.6300 ± 0.0458	0.5567 ± 0.0769
	1 μM	2.223 ± 0.2165	0.5200 ± 0.0851	0.5067 ± 0.0612
	10 μM	1.957 ± 0.0780	0.5333 ± 0.0555	0.5067 ± 0.0467
	100 μM	1.910 ± 0.0693	0.5800 ± 0.0656	0.5133 ± 0.0811
 B $C_{15}H_{11}ClO$	Control	0,8533 ± 0.0433	0.5900 ± 0.0635	0.3100 ± 0.0289
	Induced	2.630 ± 0.0231***	0.9467 ± 0.0549*	0.6200 ± 0.0692*
	Vehicle	2.630 ± 0.0289	0.8767 ± 0.0240	0.5500 ± 0.0635
	1 μM	2.527 ± 0.0033	0.9100 ± 0.0520	0.6367 ± 0.1068
	10 μM	2.570 ± 0.0520	0.9000 ± 0.0231	0.6500 ± 0.0751
	100 μM	2.520 ± 0.0289	0.9600 ± 0.0116	0.5200 ± 0.0231
 C $C_{15}H_{11}BrO$	Control	0.8617 ± 0.0929	0.2510 ± 0.1089	0.2677 ± 0.0568
	Induced	1.923 ± 0.2509*	0.6990 ± 0.0995*	0.7557 ± 0.1223*
	Vehicle	1.749 ± 0.1056	0.7040 ± 0.1448	0.7277 ± 0.1137
	1 μM	1.800 ± 0.2445	0.6907 ± 0.1157	0.7443 ± 0.1729
	10 μM	1.930 ± 0.2420	0.6913 ± 0.1294	0.7533 ± 0.1233
	100 μM	2.586 ± 0.4194	0.7020 ± 0.1385	0.7440 ± 0.1305
 D $C_{16}H_{11}BrO_3$	Control	0.8233 ± 0.1557	0.2492 ± 0.0386	0.2867 ± 0.0449
	Induced	2.349 ± 0.1688**	0.6100 ± 0.0468***	0.6560 ± 0.0223**
	Vehicle	2.374 ± 0.1998	0.6050 ± 0.0309	0.6080 ± 0.0287
	1 μM	2.467 ± 0.1194	0.5970 ± 0.0377	0.6473 ± 0.0321
	10 μM	2.414 ± 0.1208	0.6654 ± 0.0482	0.5597 ± 0.0308

	100 μM	2.303 ± 0.1920	0.4022 ± 0.0553$^{\#}$	0.4600 ± 0.068$^{\#}$
	Control	0.7047 ± 0.1247	0.2027 ± 0.0337	0.17426 ± 0.0766
	Induced	1.451 ± 0.0810**	0.3840 ± 0.0471*	0.5120 ± 0.0443*
	Vehicle	1.429 ± 0.0277	0.3793 ± 0.0310	0.4854 ± 0.0574
	1 μM	1.414 ± 0.1023	0.3213 ± 0.0170	0.4272 ± 0.0575
	10 μM	1.298 ± 0.0448	0.1437 ± 0.0537$^{\#\#}$	0.3034 ± 0.0323$^{\#}$
	100 μM	0.3910 ± 0.1704$^{\#\#\#}$	0.07133 ± 0.0323$^{\#\#}$	0.06534 ± 0.0266$^{\#\#}$
	Control	0.6015 ± 0.0678	0.1939 ± 0.0156	0.3643 ± 0.0107
	Induced	2.311 ± 0.2466***	0.3258 ± 0.0261*	0.5650 ± 0.0375**
	Vehicle	2.242 ± 0.2336	0.2839 ± 0.0302	0.5843 ± 0.0427
	1 μM	2.376 ± 0.3220	0.2665 ± 0.0370	0.6100 ± 0.0263
	10 μM	2.055 ± 0.3765	0.1738 ± 0.0345$^{\#}$	0.5523 ± 0.0270
	100 μM	1.424 ± 0.1552	0.1248 ± 0.0340$^{\#\#}$	0.4757 ± 0.0288
	Control	1.513 ± 0.1047	0.3000 ± 0.0245	0.5025 ± 0.0239
	Induced	2.773 ± 0.1392**	0.7000 ± 0.0327***	0.7475 ± 0.0544**
	Vehicle	2.717 ± 0.1922	0.6175 ± 0.0347	0.8275 ± 0.0680
	1 μM	2.597 ± 0.0606	0.5767 ± 0.0788	0.7400 ± 0.0855
	10 μM	2.727 ± 0.1392	0.5867 ± 0.0555	0.6525 ± 0.0473
	100 μM	2.383 ± 0.0601	0.5533 ± 0.0384	0.7000 ± 0.0687

Legend: The results were expressed in ηmol malondialdehyde (MDA)/mg protein as mean ± standard error of the mean (S.E.M.). The statistical comparison between the control and induced groups was performed using the Student's t-test and asterisks represented the statistical differences between these groups (*P<0.05; **P<0.01; ***P<0.001). The comparison between the induced groups and the different concentrations of chalcones (0-100 μM) was performed by one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test. and sharps represent the statistical difference to the induced group ($^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001). Source: Authors.

Chalcone D did not alter lipid peroxidation in brain tissue but efficiently reduced TBARS levels in hepatic and renal tissues at the highest concentration evaluated (100 µM). Chalcone F efficiently reduced lipid peroxidation in tissue liver, producing a significant effect from the concentration of 10 µM, with an inhibition maximum of 61.7% at 100 µM. The IC_{50} value was 13.46 µM for this tissue. In turn, chalcone E reduced lipid peroxidation in all tissues studied. Chalcone E significantly affected the brain at a concentration of 100 µM and in the liver and kidney from 10 µM. IC_{50} values calculated for chalcone E were the smallest observed in this work: 49.15 µM for brain tissue, 18.42 µM for the kidney, and 13.91 µM for the liver (Table 2). Among all the chalcones studied, chalcone E was the most potent in reducing lipid peroxidation, with maximum inhibition values of 73.05% for the brain, 81.42% for the liver, and 87.23% for the kidney (Table 2).

Table 2 - IC_{50} and Imax values calculated for chalcones evaluated in the Fe^{2+}/EDTA-induced lipid peroxidation assay.

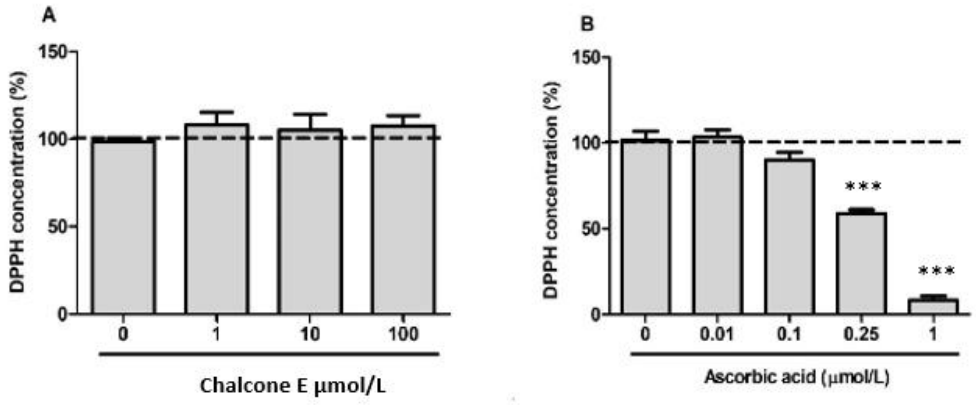
Chalcone		Brain	Liver	Kidneys
A	IC_{50}	>100 µM	>100 µM	>100 µM
	Imax	0%	7.45%	8.33%
B	IC_{50}	>100 µM	>100 µM	>100 µM
	Imax	4.18%	0%	16.12%
C	IC_{50}	>100 µM	>100 µM	>100 µM
	Imax	0%	0%	1.54%
D	IC_{50}	>100 µM	>100 µM	>100 µM
	Imax	1.95%	34.07%	28.87%
E	IC_{50}	49.15 ± 5.54 µM	13.91 ± 1.78 µM	18.42 ± 1.52 µM
	Imax	73.05%	81.42%	87.23%
F	IC_{50}	>100 µM	13.46 ± 5.20 µM	>100 µM
	Imax	38.38%	61.70%	15.98%
G	IC_{50}	>100 µM	>100 µM	>100 µM
	Imax	14.06%	20.95%	6.35%

Legend: IC_{50} represents the concentration of chalcone that inhibits 50% of tissue lipid peroxidation. Maximum inhibition (Imax) represents the percentage inhibition of tissue lipid peroxidation achieved at the highest concentration tested (100 µM). Source: Authors.

Effect of chalcone E on DPPH radical concentration *in vitro*

The effect of chalcone E on the DPPH radical concentration is demonstrated in Figure 2. According to the statistical analysis, synthetic chalcone E showed no electron scavenger activity of the DPPH radical. The ascorbic acid pattern reduced the DPPH radical concentration from 0.1 µM.

Figure 2 - Evaluation of synthetic chalcone E's antioxidant activity and the ascorbic acid antioxidant pattern measured by the DPPH radical scavenging capacity.

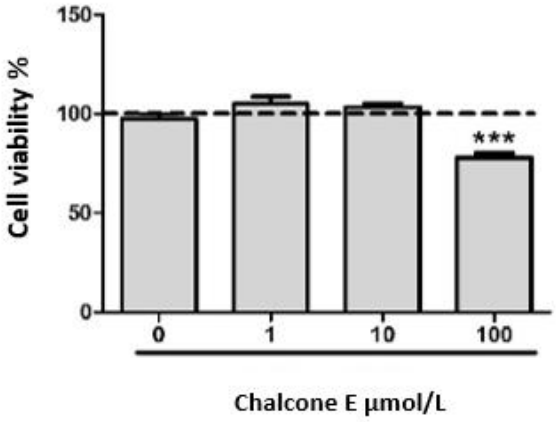


Results were expressed as % of control, as mean ± standard error of mean (S.E.M.). *** They represent statistical differences between concentrations and control. Source: Authors.

Effect of chalcone E on cell viability *in vitro*

The effect of chalcone E on cell viability is shown in Figure 3. After an incubation period of 24 hours, cell viability was significantly reduced only at the highest concentration evaluated.

Figure 3 - MTT assay for analysis of cell viability after 24 hours of exposure to chalcone E.



Results are expressed as % of control, as mean ± standard error of the mean (S.E.M.). ***They represent a statistical difference between the treated and control groups. Source: Authors.

4. Discussion

This work was primarily dedicated to the *in vitro* screening of the antioxidant effect of an unprecedented class of synthetic chalcones in an assay of lipid peroxidation induced by ferrous sulfate in different mouse tissues. Chalcones E and F, replaced with nitro grouping, stood out among the others.

In vitro studies with promising biological effects are commonly used in drug screening. The ferrous sulfate-induced lipid peroxidation assay uses the Fenton reaction to induce, *in vitro*, a situation of oxidative stress tissue, mimicking a cellular condition of disorganization and membrane damage to mitochondrial tissue, which could be the initiator of tissue damage (Friedrich, 2012; Ovalle, 2022). One of the most reactive products of the Fenton reaction is the hydroxyl radical (•OH), largely

responsible for the initiation of lipid oxidation (Ayala et al., 2014). This work studied the effect of a class of chalcones against the induced lipid peroxidation in the mouse brain, liver, and kidneys. The results demonstrated that the molecules substituted with p-nitro group, chalcones E (substitution in ring B) and F (substitution in ring A), were mainly efficient in the reduction of peroxidation in liver tissue, with IC_{50} values of about 13 µM and inhibition percentage (87.23%) higher than that of ascorbic acid (62.32%) (Dijuck *et al*, 2018).

The biological action of chalcones is attributed to the double bond conjugated to the carbonyl group (Rani, 2019) so chalcone E may have presented more significant potential due to the proximity of the NO_2 group to this double bond, whose resonance effect contributes to the movement of electrons increasing the electron density in the B ring. These results suggest that chalcones E and F are participating in some stage of LPO.

Summing up, our results suggests that the kidnapping or neutralization of negatively charged free radicals, such as the $DPPH^{\bullet}$ radicals do not appear to be involved in the mechanism of antioxidant action of chalcone E, it is likely that its structural difference from the F molecule significantly influences its effect. Even if the neutralization of direct, reactive species cannot be ruled out, chalcone E likely acts as an iron ion chelating mechanism, thus reducing the Fenton reaction and the formation of hydro and lipoperoxides. This hypothesis, however, deserves attention in future studies. A molecule with biological potential is expected to be effective at concentrations relatively low, with little or no toxic effects. In that regard, accompanied by in vitro biological effects studies, the study of the possible toxicity of new molecules becomes important. In this work, we demonstrate that chalcone E has a low potential for toxicity to healthy cells.

5. Conclusion

The results of this work demonstrate, for the first time, a potential hepatoprotective effect of chalcone E when processed at concentrations of 10-100 and low cytotoxicity. Also, our group reinforces the importance of the mechanism of action of this molecule and investigating its activity in an *ex vivo* model.

Acknowledgments

The authors are grateful to the Federal University of Mato Grosso and CNPq for the financial support. Also, thank two anonymous reviewers for their through constructive critics and corrections on this manuscript.

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