

# Homeostatic unbalance via *HSPA5* gene expression in HepG2/C3A cells exposed to $\alpha$ -tomatine

## Desequilíbrio homeostático através da expressão do gene *HSPA5* em células HepG2/C3A exposto a $\alpha$ -tomatine

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### Abstract

The  $\alpha$ -tomatine is a glycoalkaloid found in immature tomatoes (*Lycopersicon esculentum*). Currently,  $\alpha$ -tomatine has shown anticancer effects due to its anti-proliferative property. Stressors are one of the factors contributing to the antiproliferative activity of  $\alpha$ -tomatine that can modify cellular homeostasis. Among the cell stressors are the endoplasmic reticulum stress response elements, which can be altered leading to cell death. In the course of this study, we verified the expression of genes involved in the stress response of the endoplasmic reticulum in HepG2/C3A cells. The  $\alpha$ -tomatine reduced the viability of HepG2/C3A cells in a dose-dependent manner. Thus, we selected 2 $\mu$ g/mL of  $\alpha$ -tomatine (62% in cell viability) to evaluate the gene expressions. After 24 hours of exposure to  $\alpha$ -tomatine, the level of HSPA5 transcripts was reduced. The HSPA5 chaperone reduced marker is an indicative of homeostasis unbalance with the consequent lack of cellular resistance and, probably, cell death. Our results indicate the involvement of oxidative stress mechanisms in the death of HepG2/C3A cells exposed to  $\alpha$ -tomatine

**Keywords:**  $\alpha$ -Tomatine. Antiproliferative. HSPA5 chaperone

### Resumo

A  $\alpha$ -tomatina é um glicoalcaloide encontrado no tomate imaturo (*Lycopersicon esculentum*). Atualmente, a  $\alpha$ -tomatina tem mostrado efeito anticancerígeno devido sua propriedade antiproliferativa. O estresse celular é um dos fatores que contribui para a atividade antiproliferativa da  $\alpha$ -tomatina que pode modificar a homeostase celular. Entre os estressores celulares esta os elementos de resposta ao estresse do retículo endoplasmático, que podem ser alterados, levando à morte celular. No decorrer deste estudo, verificamos que a expressão de genes envolvidos na resposta ao estresse do retículo endoplasmático em células HepG2/C3A. A  $\alpha$ -tomatina reduziu a viabilidade das células HepG2/C3A de forma dose-dependente. Assim, selecionamos a concentração de 2 $\mu$ g/mL de  $\alpha$ -tomatina (viabilidade celular de 62%) para avaliar a expressão gênica. Após 24 horas de exposição a  $\alpha$ -tomatina, o nível de transcrição de HSPA5 foi reduzido. A redução de HSPA5 é um indicativo de desequilíbrio da homeostase, com a consequente falta de resistência celular e, provavelmente, a morte celular. Nossos resultados indicam o envolvimento de mecanismos de estresse oxidativo na morte de células HepG2/C3A exposto a  $\alpha$ -tomatina e mostram a eficácia do sistema como um futuro candidato para os estudos de terapia de câncer.

**Palavras-chave:**  $\alpha$ -Tomatine. Antiproliferativo. HSPA5 chaperone

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## Introduction

Glycoalkaloids are chemical compounds naturally produced by the secondary metabolism of some plant species, such as tomatoes (*Lycopersicon esculentum*), which belong to the genus *Solanum* (BARCELOUX, 2008). Tomatoes produce glycoalkaloids, among which  $\alpha$ -tomatine is the most common.  $\alpha$ -tomatine is present in immature tomatoes at an average of 4-7 mg per 100g of fresh fruit and degrades according to the levels of fruit ripening (KOZUKUE et al., 2004).

Structurally,  $\alpha$ -tomatine (C<sub>50</sub>H<sub>83</sub>BO<sub>21</sub>) consists of tomatidine (aglycon) attached by an O-glycosidic bond to the tetrasaccharide moiety of  $\beta$ -lycotetraose (composed of D-galactose, D-xylose and 2 molecules of D-glucose) (SHIH et al., 2009).

$\alpha$ -Tomatine travels through the digestive tract and reaches the bloodstream without being transformed (MORROW; YANG; SHEIK, 2004). Early studies showed that the  $\alpha$ -tomatine activity was able to reduce the levels of LDL (Low-Density Lipoprotein), stimulating T lymphocytes and potentiating the anti-inflammatory immune response (CHIU; LIN, 2008; FILDERMAN; KOVACS, 1969; FRIEDMAN et al., 2010; MORROW; YANG; SHEIK, 2004; WAKKARY et al., 1967).

Current related studies have shown that  $\alpha$ -tomatine has anticancer effects. These investigations revealed that its molecule has an antiproliferative property in the HT29 human colon carcinoma cells, HepG2 liver cancer cell lines, A549 and NCI-H460 human lung cells, PC3 human prostate cancer adenocarcinoma cells, EL4 (mouse lymphoma cells) and MCF-7 breast adenocarcinoma cells (LEE et al., 2004; LEE et al., 2011; SHI et al., 2013; SHIEH et al., 2011; SHIH et al., 2009; YANG; WU; MORROW, 2004).

The antiproliferative effect of  $\alpha$ -tomatine in some cell models has been attributed to cell death. However, the  $\alpha$ -tomatine antiproliferative mechanism appears to be cell type-specific. Some studies report cell death by apoptosis (LEE et

al., 2011). Other works have reported caspase-independent cell death, including the induction of p53 (CHAO et al., 2012; KÚDELOVÁ et al., 2013).

Stressors that may induce alterations in p53 were observed. The study conducted by Sucha et al. (2013) detected p53 induction in MCF-7 cells exposed to  $\alpha$ -tomatine. Another type of alteration in homeostasis was the loss of ATP in MCF-7 cells exposed to  $\alpha$ -tomatine observed by Kúdelová et al. (2013). Nevertheless, different forms of cellular stress, including endoplasmic reticulum stress response elements, can change and lead the cells to death.

In our study, we selected the HepG2/C3A cell line (human hepatocellular carcinoma), which is a model for genetic toxicological studies. This cell line comprises phase I and phase II enzymes essential to the metabolism of drugs and carcinogens (DAWSON; ADAMS; WOLF, 1985). Moreover, being a metabolizing cell, one of its key functions is sensitivity or response to the environmental changes through homeostasis (DECAENS et al., 2008). Thenceforth, we evaluated the gene expression and integrated stress response in HepG2/C3A cells related to the cellular response. The results revealed that there is homeostatic unbalancing indicated by the stress response elements of the endoplasmic reticulum (HSPA5), indicative of cell death and absence of resistance of HepG2/C3A cells to glycoalkaloid.

## Material and Methods

### *Human hepatocellular carcinoma (HepG2/C3A) cells and chemicals*

The human hepatocellular carcinoma cell line (HepG2/C3A) was obtained from the Cell Bank of Rio de Janeiro, Brazil. The cells were grown in 25 cm<sup>2</sup> tissue culture flasks in 5 mL of DMEM (Dulbecco's Modified Eagle Medium) medium (Gibco, BRL, USA) supplemented with 10% fetal bovine serum (Gibco, BRL, USA) and antibiotics/

antimycotics solution (Gibco, BRL, USA). Under this condition, the cells underwent incubation at 37°C in an atmosphere of 5% CO<sub>2</sub>.

The synthetic  $\alpha$ -tomatine (CAS17406-45-0) was prepared in 1% dimethyl sulfoxide (DMSO) solution and added to the culture flasks. The control flasks were treated with DMSO (1%). The damage-inducing agent used was doxorubicin (10 $\mu$ g/mL) (CAS 25316-40-9; Adriblastin, Pharmacia, Italy). The concentration of the 10  $\mu$ g/mL was selected based on preliminary experiments.

#### *Cytotoxicity assay*

For the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, 5x10<sup>3</sup> HepG2/C3A cells were incubated for 24 h in 96-well plates at 37°C in a 5% CO<sub>2</sub> atmosphere. The next step consisted of a 24-hour exposure of the cells to  $\alpha$ -tomatine 1.0, 2.0, 2.5, 3.0 and 4.0  $\mu$ g/mL. Only HepG2/C3A cells were used as negative control and exposed to 10  $\mu$ g/mL of doxorubicin as DNA damage agent. The selection of the  $\alpha$ -tomatine concentration followed the criterion established by Lee et al. (2004). The protocol used for the MTT assay was described by Mosmann (1983). Cell viability was expressed as the percentage relative to the control group (HepG2/C3A cells): Cell viability =  $(A_{\alpha\text{-tomatine + HepG2/C3A}} - A_{\text{blank}}) / (A_{\text{HepG2/C3A}} - A_{\text{blank}}) \times 100$ , where A = absorbance. We performed all experiments on three independent biological replicates and three technical replicates. The statistical analysis of the data considered the values of measured parameters compared by ANOVA followed by the Dunnet's multiple comparison test using the GraphPadPrism®5 software ( $p < 0.05$ ).

#### *Real-time reverse transcriptase quantitative polymerase chain reaction*

The quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) was

developed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (BUSTIN et al., 2009). The HepG2/C3A cells (3.0x10<sup>6</sup> cells/flask) underwent pre-incubation for 24h and a subsequent exposure to  $\alpha$ -tomatine. The conditions of treatment were: HepG2/C3A cells, HepG2/C3A cells exposed to 2g/mL of  $\alpha$ -tomatine for 1h only and HepG2/C3A cells exposed to 2 $\mu$ g/mL of  $\alpha$ -tomatine for 24 h. The total RNA was extracted using TRIzol LS reagent (Invitrogen, Life Technologies®).

The First-strand cDNA synthesis was performed with 200U/ $\mu$ l reverse transcriptase (M-MLV, Invitrogen, Life Technologies®), according to the manufacturer's protocol using 1  $\mu$ g of total RNA as template. The qRT-PCR reactions were accomplished using the LightCycler® Nano thermocycler (Roche, Diagnostics). Table 1 shows the oligonucleotides used in these experiments. We used the Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Life Technologies®) (10  $\mu$ L) as the reaction mixture with the addition of 0.4  $\mu$ M of each primer and 2  $\mu$ L of template cDNA. The final reaction volume was 20  $\mu$ L. The PCR thermal cycling conditions included an initial step at 95 °C for 3 min; 30 cycles at 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 20 sec; followed by another step at 95 °C for 10 sec; and, finally, 40 °C for 1 min. A thorough melting curve analysis was performed at the end of the reaction with temperatures ranging from 50 °C to 90 °C. The transcripts assessed were *MARK2* (MAP/microtubule affinity-regulating kinase 2), *HSPA5* (glucose-regulated protein), *HSPA14* (heat shock 70kDa protein 14), *TRAF2* (TNF receptor-associated factor 2), *EIF2AK3* (eukaryotic translation initiation factor 2- $\alpha$  kinase 3) and *GADD153* (growth arrest and DNA damage-inducible gene 153) (Table 1). The data were normalized against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The entire analysis was performed with three independent biological replicates and three

technical replicates. We obtained the relative expression data using the Pfaffl method.

Data variability was expressed as  $\pm$  standard deviation (SD) of the mean. We analyzed

the data by the Pair-Wise Fixed Reallocation Randomization Test in REST<sup>®</sup> 2002 (PFAFFL; HORGAN; DEMPFLER, 2002) software, and values of  $p < 0.05$  were considered significant.

**Table 1** - Sequence of primers used in RT-qPCR.

Name	Sequence 5'-3'	Reference
<i>GAPDH</i>	F- 5' GAAGGTGAAGGTCGGAGTC R- 5' GGAAGATGGTGATGGGATTT	Sugaya, Nakanishi and Tanzawa, 2005 with modifications
<i>MARK2</i>	F- 5' TCCGCGAAGTAAGAATAATGAA R- 5' GCCACTAGCGTACTCCATGA	Present study
<i>HSPA5</i>	F- 5' GTGGAATGACCCGTCTGTG R- 5' TGCCACCTCCAATATCAAC	Song, Yan and Cai, 2006 with modifications
<i>HSPA14</i>	F-5' CCAGCTGTTGTTGCTTACTCA R-5' CTGCCCAGGATCTGCTTTAC	Present study
<i>EIF2AK3</i>	F-5' CAACCGAGAGGTATTTTGGAA R- 5' GAAAGGAACTGTTTCCATGCT	Present study
<i>GADD153</i>	F- 5' AGCAGATGTGCTTTTCCAGAC R- 5' GTCCTCATACCAGGCTTCCA	McCullough et al., 2001 with modifications
<i>TRAF2</i>	F- 5' GCCCCAAGTCCCCTTAACT R- 5' GAATCTGCAAGGGACTCGAC	Present study

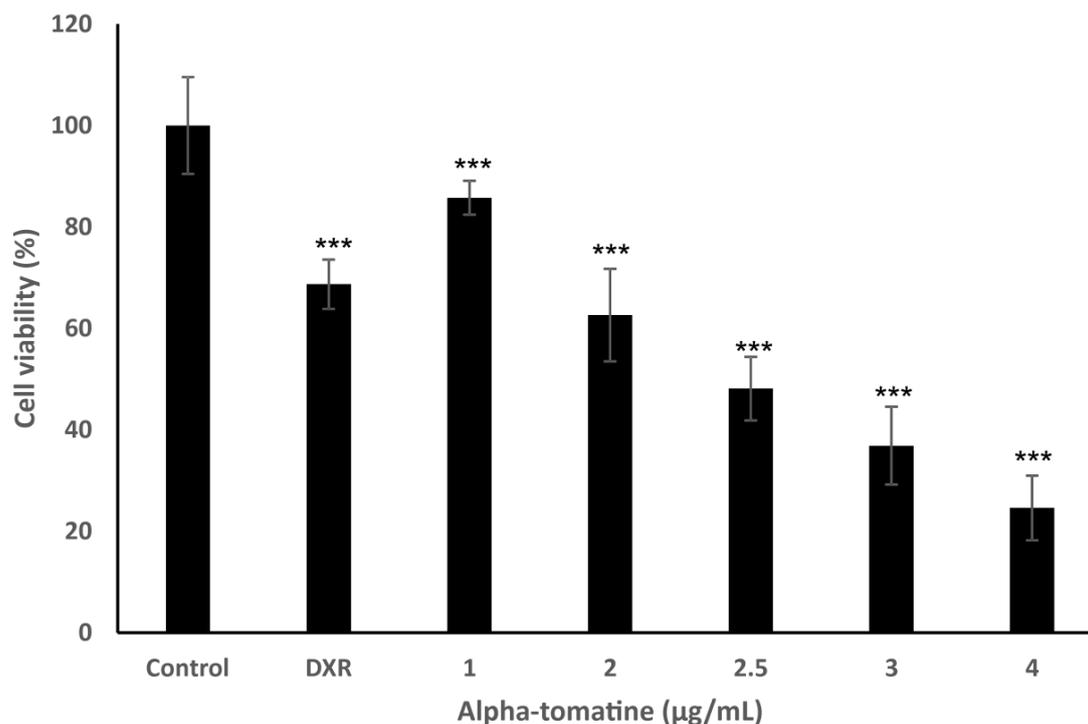
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## Results and Discussion

Cytotoxicity assays were performed to verify the effects of the exposure of  $\alpha$ -tomatine on HepG2/C3A cell viability. After 24h of exposure, all quantities of  $\alpha$ -tomatine (1.0, 2.0, 2.5, 3.0 and 4.0  $\mu\text{g/mL}$ ) reduced the HepG2/C3A cell viability (85%, 62%, 48%, 37%

and 24%, respectively) (Figure 1). Several studies have described the  $\alpha$ -tomatine as an antiproliferative agent on cell lines *in vitro* (KÚDELOVÁ et al., 2013; LEE et al., 2004; LEE et al., 2011; SHI et al., 2013; SHIH et al., 2009; SHIEH et al., 2011; SUCHA et al., 2013; YANG; WU; MORROW, 2004).

**Figure 1** - Percent cell viability of HepG2/C3A cells obtained according to the evaluation of cytotoxicity after 24 hours of  $\alpha$ -tomatine exposition. The percentage of cell viability was calculated from the absorbance values obtained in cytotoxicity assay. The damage inducing agent was doxorubicin (10  $\mu\text{g/mL}$ ) (DXR, Adriblastin, Pharmacia). Control: HepG2/C3A cells + DMEM culture medium. The bars represent the standard deviations obtained from three independent experiments. Statistical differences: \*  $p < 0.05$  compared to control HepG2/C3A cells by Dunnett's test.



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The antiproliferative effect of  $\alpha$ -tomatine has been attributed to its binding with cholesterol in biological membranes unbalancing its polarity and changing the flow of ions. Consequently, the homeostasis leads the subsequent cell death (BLANKEMEYER et al., 1997; GEE et al., 1996; KEUKENS et al., 1995; MORROW; YANG; SHEIK, 2004; YANG; WU; MORROW, 2004).

Researchers agree that  $\alpha$ -tomatine acts as an antiproliferative agent and the effect is due to cell death. However, the antiproliferative mechanism of  $\alpha$ -tomatine appears to be cell type-specific. Some authors corroborate that the  $\alpha$ -tomatine antiproliferative effect induces cell death via apoptosis (CHAO et al., 2012; LEE et al., 2011).

However, recent studies report that  $\alpha$ -tomatine inhibits cell proliferation and induce caspase-independent cell death. Furthermore, cell death can occur by different antiproliferative mechanisms, such as stressors, including p53-induced apoptosis (CHAO et al., 2012; KÚDELOVÁ et al., 2013; SUCHA et al., 2013).

After confirming the antiproliferative effect of  $\alpha$ -tomatine on the HepG2/C3A cells, we selected 2 $\mu\text{g/mL}$  of  $\alpha$ -tomatine to check the expression of genes involved in endoplasmic reticulum oxidative stress. The HepG2/C3A cell viability was reduced to 62%. Under these conditions, HepG2/C3A cells were exposed to  $\alpha$ -tomatine for 1 hour and 24 hours to verify the involvement of elements of

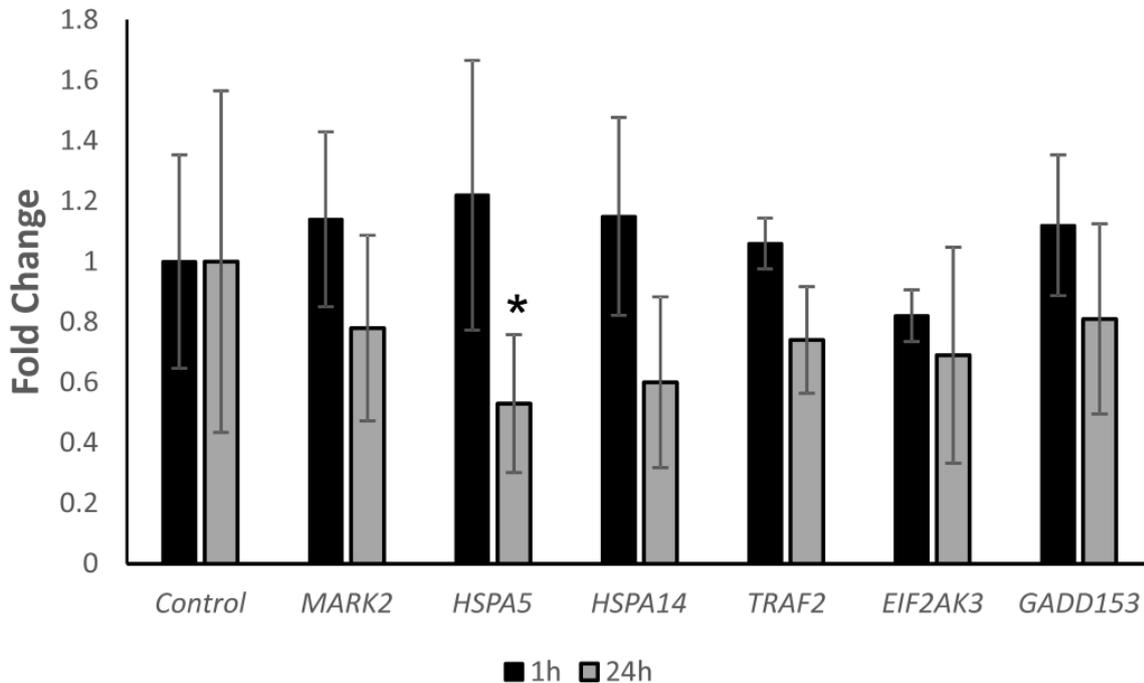
the endoplasmic reticulum stress-related oxidative form of an early or a late stage.

The mRNA levels of the genes (Figure 2) related to the cellular response to endoplasmic reticulum stress specifically in the cell cycle arrest and/or apoptosis in *MARK2* and *GADD153*, did not show substantial differences when the HepG2/C3A cells exposed to  $\alpha$ -tomatine were compared to HepG2/C3A cells without treatment for 1h and 24h (1.14-fold and 1.12-fold for 1h of exposure, respectively, and 0.7-fold and 0.81-fold for 24h of exposure, respectively). Nevertheless, there was a reduction in the transcriptional levels, but it was not significant. The same situation occurred with the expression of the *TRAF2* gene (1.0-fold and 0.74-

fold, for 1h and 24h, respectively). The reduction in gene expression is indicative of early impaired cellular homeostatic unbalance.

The transcripts of *HSPA5*, *HSPA14* and *EIF2K3* genes have not significantly changed after 1h of exposure to  $\alpha$ -tomatine (1.2-fold, 1.1-fold and 0.82-fold, respectively) when compared to HepG2/C3A control cells. Thus, after 24h of exposure we observed a slight, non-significant reduction in gene transcripts *HSPA14* – 0.60-fold, *EIF2K3* – 0.69-fold. However, *HSPA5* (0.53-fold) was an exception and underwent a significant reduction in the transcriptional level.

**Figure 2** - Relative expression of *MARK2*, *HSPA5*, *HSPA14*, *TRAF2*, *EIF2AK3* and *GADD153* genes after 1 h and 24 h of  $\alpha$ -tomatine exposition in HepG2/C3A cells. Data were normalized with *GADPH* gene. Control: HepG2/C3A cells + DMEM culture medium. Statistically significant difference was performed as REST-384 software parameters (PFAFFL; HORGAN; DEMPFLER, 2002).



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In the event of stressor situations, the UPR (Unfolded Protein Response - UPR) pathway must be activated to maintain the cell homeostasis. The UPR pathway can be activated by the endoplasmic

reticulum chaperone *HSPA5* (GRP78), a component of the transcriptional complex IRE1 (ERN1), ATF6 and PERK (*EIF2K3*) (FORMAN; LEE; TROJANOWSKI, 2003).

Under stress, HSPA5 dissociates from the complex. Such dissociation generates a potent transcription factor for UPR gene expression activating signaling pathways to maintain homeostasis (HOTAMISLIGIL, 2010; PFAFFENBACH; LEE, 2010; TODD; LEE; GLIMCHER, 2008). Induced HSPA5 has been used as possible activation markers of the antiapoptotic and antiproliferative agents in tumor cells creating a mechanism of resistance (KIM et al., 2006; LIU et al., 2007; MILOSZEWSKA et al., 2010; ROSATI et al., 2010; ROUE et al., 2011; TAN et al., 2011). On the other hand, the induction of anti-HSPA5 functions as an apoptotic protein, forming caspase 7 and 12 complexes, keeping them inactive and blocking their pro-apoptotic function (REDDY et al., 2003).

Conversely, in this study, the *HSPA5* expression was inhibited (0.53 fold) as a result of the exposure of HepG2/C3A cells to  $\alpha$ -tomatine for 24 hours. The *HSPA5* inhibited expression indicates that the HepG2/C3A cells are probably being forwarded to cell death by a non-apoptotic mechanism, as a result of the above-described induction of HSPA5.

## Conclusions

The reduced expression of HSPA5 provided a new insight into HepG2/C3A cells, indicating that they are not resistant to 2 $\mu$ g/mL of the  $\alpha$ -tomatine when exerting their control mechanism to maintain or recover cellular homeostasis. Our results corroborate the involvement of oxidative stress mechanisms in the death of HepG2/C3A cells exposed to  $\alpha$ -tomatine and show the effectiveness of the system as a future candidate for cancer therapy studies.

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## Declaration of interest

The authors declare no conflict of interest exist.

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