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Cytoprotection assessment against mycotoxins on HepG2 cells by extracts from *Allium sativum* L

Ana Juan-García [a,*], Fojan Agahi [a], Maria Drakonaki [b], Paola Tedeschi [c], Guillermina Font [a], Cristina Juan [a,**]

[a] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100, Burjassot, València, Spain
[b] Department of Food Technology, Faculty of Food Technology and Nutrition, University of West Attica, Greece
[c] Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121, Ferrara, Italy

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ABSTRACT

Cytoprotection effects of *Allium sativum* L garlic extract from a local garlic ecotype from Ferrara (Italy) on hepatocarcinoma cells, HepG2 cells, is presented in this study. This garlic type is known as Voghiera garlic and has been characterized as PDO (Protected designation of Origin) product. Voghiera garlic extract (VGE) was evaluated against beauvericin (BEA) and two zearalenone (ZEA) metabolites (α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL))-induced cytotoxicity on HepG2 cells by the MTT (3–4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, over 24 h and 48 h. Direct treatment, simultaneous treatment and pre-treatment strategies at the dilution 1:16–1:00 for VGE and at the concentration range from 0.08 to 2.5 μM for BEA and from 1.6 to 50 μM for both α-ZEL and β-ZEL were tested. Individual IC_{50} values were detected at all times assayed for BEA (>0.75 μM) and VGE (dilution upper 1:8) while this was not observed for ZEA's metabolites. When simultaneous strategy of VGE + mycotoxin was tested, cytoprotection with increases of viability (upper 50%) were observed. Lastly, in pre-treatment strategy with VGE, viability of HepG2 cells was significantly protected when α-ZEL was tested. As a result, the greatest cytoprotective effect of VGE in HepG2 cells is obtained when simultaneous treatment strategy was performed.

1. Introduction

Garlic extract (*Allium sativum* L) has been used for many centuries to treat infections, heart disease, and cancer. Its complex phytochemistry has been the subject of several studies as reported in the literature; confirming the beneficial effects of garlic on the cardiovascular system, immunomodulation and cancer; among that, antioxidant properties of garlic and the conclusion that raw garlic has health benefits is gaining momentum (Mansingh et al., 2018). Direct antibacterial and antiviral properties have also been described been proposed as a pesticide option, with allicin being regarded as responsible for such effects (Mylona et al., 2019).

Most of the natural compounds presents in garlic belong to the family of allyl and sulfur compounds (organosulfur compounds (OSCs)) (Tedeschi et al., 2007, 2011) and properties associated have been related to inner organic sulfur compounds (dithiosulfinates and sulfoxides) (OSCs) responsible to its strong smell (especially allicin). Recently, Quesada et al. (2020) have compiled garlic extracts' bioactivities to organosulfur compounds which interfere against inflammation, oxidative stress, obesogenic effects, and mitochondrial dysfunction. Nonetheless, promising pharmacological properties and biological properties associated correspond to natural compounds which have demonstrated to be stable when cooking (sapogenins, saponins and flavonoids). *Allium sativum* L variety from Voghiera (Ferrara, Italy), registered as Protected Designation Origin (PDO) has been analyzed through chemotype analyses and random amplified polymorphic DNA genomic analyses (Brandolini et al., 2005). Voghiera garlics have a unique composition according to the soil where they grow and differ genetically with other garlics as a good clustering differentiation was reported with low intravarietal polymorphisms of Voghiera samples in several studies (Brandolini et al., 2005; Tedeschi et al., 2007, 2011). Among that, micronutrients and macronutrient of Voghiera garlic are characterized by having high nitrogen content, low fat content, low toxic heavy metals (Cd, Cr and Pb) and high concentration of potassium and sodium

* Corresponding author.
** Corresponding author.
E-mail addresses: ana.juan@uv.es (A. Juan-García), cristina.juan@uv.es (C. Juan).

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(Brandolini et al., 2005). The polar extract of Voghiera garlic extract (VGE) contains several furostanol saponins (40 mg/kg) which have demonstrated to have antifungal activity against *Botrytis cinerea* and *Trichoderma harzianum* (de Falco et al., 2018; Lanzotti et al., 2012). Among these saponins, two aglycones were the first time found in *Allium* spp., which reveals the unique of this Italian Voghiera garlic spp (Brandolini et al., 2005; de Falco et al., 2018).

Allicin constitutes the major compound as essential functional substance (Schultz et al., 2020) without effect in *in vitro* proliferation in intestinal cells (INT-407) (Mansingh et al., 2018); while for other cell lines increases in cell viability have been reported (in L929 and HT29 cells) (Ghazanfari et al., 2011) and even protection against ROS production (in vascular smooth muscle cells) (Torres-Palazzolo et al., 2020). With this opposite and diverse results there is no doubt that even if effects differ, a positive tendency is observed.

Zearalenone (ZEA), a mycotoxin produced by several *Fusarium* spp., is most commonly found as a contaminant in stored grain. ZEA derivatives (α-zearalenol (α-ZEL), β-zearalenol (β-ZEL)) can also be produced by *Fusarium* spp. in corn stems infected by fungi in the field. Also, following oral exposure, zearalenone is metabolized in various tissues, particularly in the liver, the major metabolites being α-ZEL and β-ZEL. ZEA is known to be an estrogen-like non-steroidal mycotoxin and jointly its metabolites it has demonstrated harmful effects of exposure as endometrial adenocarcinoma, breast cancer, reduced testicular germ cells, etc. On the other hand, beauvericin (BEA) is a mycotoxin produced by many species of fungus *Fusarium* and *Beauveria bassiana*. BEA is a natural contaminant of cereals and cereal based products which can be found in a great variety of food commodities. The co-exposure of cells to mixture of a combination of mycotoxins may cause an increase of toxicity produced by these mycotoxins (Agahi et al., 2020b). Moreover, it has been demonstrated that it can cause cytotoxicity and genotoxicity in various cell lines and it is also capable to produce oxidative stress at molecular level (Juan-García et al., 2019a, 2019b, 2020). A study of cytoprotection with goji berry extract was reported by Montesano et al. (2020).

Garlic can reduce and kill microorganisms and fungal species which highlight the possibility not only in reducing the mycotoxins effects but also the occurrence of such natural compounds as the origin of its production, is attacked (Mylona et al., 2019; Ozcakmak et al., 2017). Interestingly epidemic fungal disease affecting garlic by *Fusarium* spp. has supposed a problem during the early growth stages of the crop (Palmero et al., 2012). Tonti et al. (2012) reported that these fungi is present in the majority of the garlic bulb processed for human consumption from China, France, Italy and Spain reporting necrotic spots on cloves. Such fungi, at the same time, are the responsible of mycotoxins' production of: fumonisins (B1 (FB1), B2 (FB2) and B3 (FB3)), moniliformin (MON), beauvericin (BEA), fusaproliferin (FUS), fusaric acid (FA) and enniatins (ENNs). Studies of mycotoxins on commercial garlics did not report substantial contamination indicating the absence of risk associated for the population (Tonti et al., 2017). However, isolates of the mycotoxigenic fungi *Fusarium proliferatum* present in garlic was used to study the capacity of producing FB1, FB2, FB3, MON and BEA (Gálvez et al., 2017).

Fumonisins were among all mycotoxins the ones more abundant, but 88.61% of isolates were BEA-producers at levels from 3.51 to 995.37 μg/g which describes presence of BEA in garlic (Gálvez et al., 2017) and opens the possibility of studying the interactive effects when both mycotoxins and natural compounds coincide.

In this study, Voghiera garlic extract (VGE) from Italy with Protected Designation of Origin was assayed in a human hepatocarcinoma cell line, HepG2. Its effect was evaluated by MTT assay following three different strategies of treatment (direct, pre-treatment and simultaneous treatment) and with three mycotoxins: BEA, α-ZEL and β-ZEL.

2. Materials and methods

2.1. Chemicals and reagents

The reagent grade chemicals and cell culture components used, Dulbecco's Modified Eagle's Medium (4.5 g/L glucose) (DMEM, Sigma-Aldrich, St. Louis, USA), penicillin and streptomycin (Sigma-Aldrich, St. Louis, USA), trypsin (Trypsin–EDTA, Sigma-Aldrich, St. Louis, USA), Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS) and phosphate buffer saline (PBS) were supplied by Thermofisher, Gibco ™ (Paisley, UK). Methanol (MeOH, HPLC LS/MS grade), was obtained from VWR International (Fontenay-sous-Bois, France).

Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific Co, Fisher BioReagnts ™ (Geel, Belgium). [3- (4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] (MTT) for MTT assay, penicillin, streptomycin and Trypsin–EDTA was purchased from SigmaAldrich (St. Louis, MO, USA). Deionized water (<18, MΩcm resistivity) was obtained in the laboratory using a MilliQSP® Reagent Water System (Millipore, Beadford, MA, USA). The standard of BEA (MW: 783.95 g/mol), α-ZEL and β-ZEL (MW: 320,38 g/mol) were purchased from SigmaAldrich (St.Louis Mo. USA). Stock solutions of mycotoxins were prepared in MeOH (α-ZEL and β-ZEL) and DMSO (BEA) and maintained at −20 °C in the dark. The final concentration of either methanol or DMSO in the medium was ≤1% (v/v) as per established. All other standards were of standard laboratory grade.

2.2. Plant material

For performing this study Voghiera garlics (*Allium sativum* L ecotype) have been generously supplied by Cooperativa Agricola Voghierese (founding member of Voghiera garlic Producer Consortium), Voghiera (Ferrara, Italy). Voghiera garlic identification was carried out using RAPD-PCR technique by University of Ferrara. Garlic samples were kept in the fridge 4 °C until the experimental procedures in order to preserve it from maturation.

2.3. Preparation of Voghiera garlic extract

The extracts from *Allium sativum* L garlics was performed according to Petrovic et al. (2018) with some modifications. 35 g of garlic cloves were crushed in 25 mL 40% ethanol using a bench-top blender. The mixture was transferred to a glass jar with an air-tight lid, and stored in darkness at 4 °C for 5 days. The dry mass was discarded and the liquid was collected after centrifugation at 5000 rpm for 10 min. Finally, the extract obtained was placed in a vial and stored at −20 °C until its use. The final extract obtained from the described procedure was evaluated if it was able to influence the HepG2 proliferation cells, using the MTT assay as described in detail in section 2.6.

2.4. Cell culture

Human hepatocarcinoma cells, HepG2 cells (ATCC HB-8065), were maintained in DMEM supplemented with antibiotic-free 10% newborn calf serum (NCS; Invitrogen), 100 U/ml penicillin (5%), and 100 mg/mL streptomycin (5%). Cells were grown near confluence in 75 cm^2 plastic flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37 °C in an atmosphere containing 5% of CO_2 at 95% of relative humidity. A small number of sub-passages (from 12 to 20 passages) was routinely controlled in order to maintain the genetic homogeneity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit.

2.5. Treatment of HepG2 cells with beauvericin, α-zearalenol, β-zearalenol and Voghiera garlic extract

HepG2 cells were cultured into 96-well tissue-culture plates by

adding 200 μl/well of a suspension of 2 × 10^6 cells/ml. After cells reached 80% confluence, the culture medium was replaced and cells were treated through direct treatment during 24 and 48 h to 200 μl of fresh medium containing different concentrations of i) BEA (from 5 to 0.05 μM, 1:2 dilutions); or ii) α-ZEL and β-ZEL (from 50 to 0.2 μM, 1:2 dilutions); or iii) VGE (extract of 35gr, 1:2 dilutions). Dilution series of VGE with medium was performed: from 1:0 to 1:16.

Two more treatments were assayed: simultaneous and pre-treatment. In both cases combinations of BEA, α-ZEL, β-ZEL and VGE were performed. For pre-treatment studies, HepG2 cells were exposed to VGE with dilution according to previous cell proliferation assays (VGE was no-toxic from 1:06 to 1:16 dilutions and no saturation of the culture media) during 24 h. Successively, the medium containing VGE was removed and cells were exposed to mycotoxins for 24 h and 48 h of incubation at different concentration ranges: i) BEA from 2.5 to 0.08 μM (1:2 dilutions); ii) αZEL and β-ZEL from 50 to 1.6 μM (1:2 dilutions). Also to perform studies of simultaneous treatment, concentrations described above were used, starting for BEA at 2.5 μM, α-ZEL and β-ZEL at 50 μM and VGE directly at extract concentrated at 1:06 and following the 1:2 dilutions for all scenarios proposed.

2.6. MTT assay

The MTT assay determines the viability of cells by the reduction of yellow soluble tetrazolium salt, only in the metabolically active cells, via a mitochondrial-dependent reaction to an insoluble purple formazan crystal. The MTT viability assay was performed as reported previously in Juan-García et al. (2019b). Briefly, after treatment studies, the medium containing these compounds was removed and cells of each well received 200 μl fresh medium plus 50 μl of MTT (at 5 μg/mL). The plates were wrapped in foil and incubated for 4 h at 37 °C. Afterwards, the medium containing the MTT was removed and the resulting formazan salt was solubilized in DMSO. The absorbance was measured at 570 nm using an ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA).

Cell viability was expressed in percent relative to control cells (1% DMSO for BEA, 1% MeOH for α-ZEL and β-ZEL and VGE). Mean inhibition concentration (IC_{50}) values were calculated from full dose–response curve by using four parameters logistic equation with the SigmaPlot program. Three independent experiments were performed with eight replicates each.

2.7. Statistical analyses of data

Statistical analysis of data was carried out using IBM SPSS Statistic version 24.0 (SPSS, Chicago, Il, USA) statistical software package. Data were expressed as mean ± SD of four independent experiments. The statistical analysis of the results was performed by Student's *t*-test for paired samples. Differences between groups were statistically analyzed using ANOVA followed by the Tukey HDS post hoc test for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effects of beauvericin, α-zearalenol, β-zearalenol and Voghiera garlic extract in HepG2 cells by direct treatment strategy

Fig. 1 collects HepG2 cells viability evaluated by MTT assay after 24 and 48 h of treatment with α-ZEL, β-ZEL, BEA and VGE.

The effect of ZEA's metabolites, α-ZEL and β-ZEL in HepG2 cells is presented in Fig. 1A and B, respectively. The results clearly indicated that the viability of HepG2 cells was affected for α-ZEL at the highest concentration assayed; while for β-ZEL metabolite at the concentrations 12.5 μM for 24 h with decreases of 18% and 33% for the highest concentration studied; also at 48 h that decrease was observed at 12.5 μM and above from 42% to 57%. It was possible to reach an IC_{50} value at 18 μM for β-ZEL at 48 h.

For BEA, viability effect after 24 h and 48 h was significantly reduced at concentration of 1.25 μM and above; such reduction went from 60% to 93% and from 71% to 94%, respectively. In fact, the molar concentrations of BEA that reached IC_{50} was possible to calculated and at 24 h IC_{50} which was 1.15 μM, whereas at 48 h it was 1 μM (Fig. 1C).

By applying the same procedure described above (MTT assay), the effect of VGE was also evaluated (Fig. 1D). Equally to the above for BEA and β-ZEL, also for VGE IC_{50} value was reachable. Viability was below 50%, for both 24 h and 48 h after 1:06 dilution. In particular, at 24 h VGE dilutions, from 1:06 to 1:0, significantly decreased cell proliferation

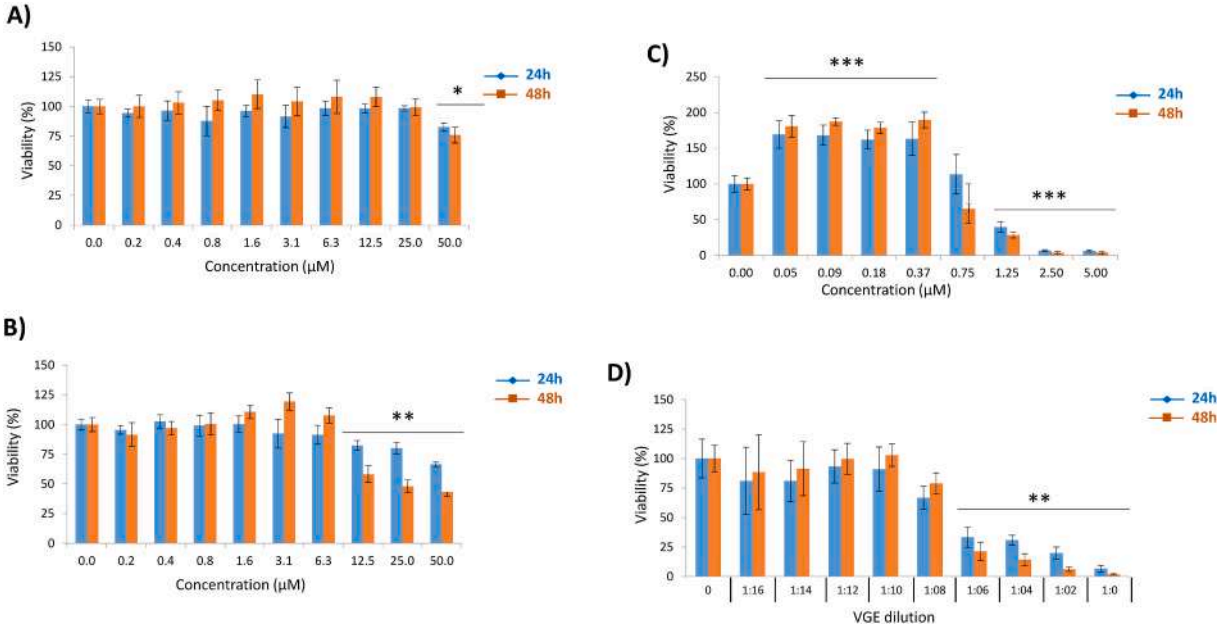

Fig. 1. Concentration–effect bars in HepG2 cells of α-ZEL (A), β-ZEL (B), BEA (C) and Voghiera garlic extract (VGE) (D), after 24 h and 48 h of exposure by MTT assay. All values are expressed as mean ± SD of 8 replicates. (*) p ≤ 0.05, (**)p ≤ 0.01 and (***)p ≤ 0.001 represents significant difference as compared to control values.

from 67% to 93%, respectively in HepG2 cells; however, from 1:16 to 1:08 the cell proliferation was reduced only from 18% to 33%, respectively. For tests at 48 h, the significant decrease in dilution range from 1:06 to 1:0 was from 79% to 98%, respectively; while for all other dilutions assayed (1:08 and 1:16) a slight reduction in cell proliferation was recorded.

3.2. Effects of simultaneous strategy treatment in HepG2 cells exposed to beauvericin, α-zearalenol, β-zearalenol and Voghiera garlic extract

The effect in HepG2 cells exposed to the concentrations described above with VGE and mycotoxins simultaneously are described for α-ZEL, β-ZEL and, BEA in Figs. 2–4, respectively.

For α-ZEL (Fig. 2A and B) it was revealed a remarkable cytoprotection at 24 h ranging from 70% to 30% when mixtures of α-ZEL + VGE were: [6.25 μM + 1:12], [12.5 μM + 1:10] and [25 μM + 1:08]; however, no cytoprotection was revealed at 48 h for the mixtures assayed. When β-ZEL was tested (Fig. 3A and B) with this strategy of simultaneous treatment with VGE no cytoprotection was observed for the mixtures assayed in HepG2 cells. Finally, simultaneous treatment of VGE + BEA (Fig. 4A and B) in HepG2 cells cytoprotection was observed at 24 h and 48 h for [1.25 μM + 1:08] and [25 μM + 1:06] with 60% and 54%, respectively for 24 h and with 40% and 35%, respectively for 48 h compared to BEA directly treated.

3.3. Effects of pre-treatment strategy in HepG2 cells exposed to beauvericin, α-zearalenol, β-zearalenol and Voghiera garlic extract

The effect of pre-treatment strategies in HepG2 cells for the scenarios described above with VGE and afterwards exposed to mycotoxins are described for α-ZEL in Fig. 2, for β-ZEL in Fig. 3 and, for BEA in Fig. 4.

In detail, for α-ZEL (Fig. 2C and D) scenarios reporting VGE slight protection was reported at 24 h correspond to a very diluted extract (1:16–1:14) with oscillation of 5–3%; while at 48 h this effect was not observed, indicating that VGE was not able to protect or ameliorate the cytotoxic α-ZEL effect in HepG2 cells. For β-ZEL (Fig. 3C and D), at 24 h the higher protection reported was 20% and 14% for the scenario of VGE diluted at 1:16 and 1:14 respectively; while at 48 h the highest cytoprotection was for pre-treatment of HepG2 cells with pre-treatment with VGE 1:10 dilution and 12.5 μM β-ZEL. Lastly for BEA (Fig. 4C and D), at 24 h it was noticed a protection of VGE in HepG2 cells when BEA concentrations were above 0.62 μM and 1:10 VGE dilution, from 55% respect to the direct treatment of the mycotoxin. However, cytoprotection at 48 h was observed when BEA concentrations were above 0.31 μM and 1:12 VGE dilution oscillating between 70% and 56% at 48 h respect to the direct treatment of the mycotoxin.

4. Discussion

The present work deals with the study of the capacity of VGE extracts of PDO from Italy in exerting protection in HepG2 cells α-ZEL, β-ZEL and BEA-induced toxicity.

The effects associated to garlic and its components have been widely studied besides there is a high demand of using natural products to counteract the detrimental toxic effects mediated by activation of routes and subsequently as potential preventers of multiple human diseases (AbidEsseffi et al., 2012). *In vivo,* garlic oil extract has demonstrated to mitigate the adverse effects of T-2 toxin in broiler chickens and, although at the lowest dose (0.3 mg/kg) a slight weight gain was noticed compared to the reduction of weight in T-2 toxin exposed, reduced glutathione was high in blood plasma which reduced the red blood cell haemolysate associated to T-2 toxin as well as redox parameters (Ancsin et al., 2013). On the contrary, when doses were high (1.5 g/kg), adverse effects were associated to the high amount of organic sulfur compounds (Ancsin et al., 2013).

The capacity of garlic extract and/or its components in the mycotoxins production because of its effect in fungi growth as promoters of mycotoxins' producers has been carried out in different studies (Ozcakmak et al., 2017; Gorna et al., 2016; Mylona et al., 2019). Ozcakmak et al. (2017) studied the antifungal activity of garlic essential oil on the growth of *Penicillium verrucosum* and the production of OTA. It was demonstrated that the presence of natural compounds in food can prevent the effect of toxic metabolites of natural origin as well as mycotoxins and for OTA with a prevention of production oscillating between 80 and 100% (Ozcakmak et al., 2017). The presence of saponins from VGE (Lanzotti et al., 2012) could also interfere the effects associated to mycotoxins as reported for those from lentils and studied against alternariol mycotoxins (Vila-Donat et al., 2015). Opposite to this intention, garlic extract in *Fusarium proliferatum* induced the biosynthesis of fumonisin (Gorna et al., 2016). Finally, Mylona et al. (2019),

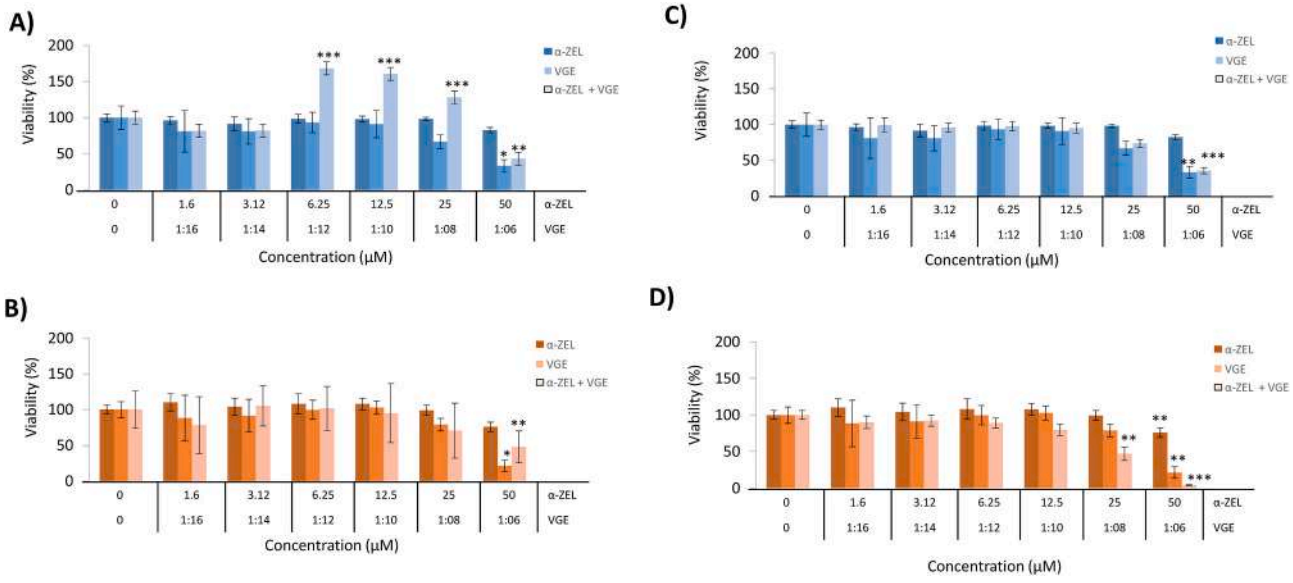

Fig. 2. Concentration curves obtained after simultaneous (A and B) and pre-treatment (C and D) of VGE (dilution 1:16–1:6) during 24 h (A and C) and 48 h (B and D), and serial dilutions of α-ZEL (0–50 μM) in HepG2 cells by MTT assay. All values are expressed as mean ± SD of 8 replicates. *(*) $p \leq 0.05$, (**)$p \leq 0.01$ and (***)$p \leq 0.001$* represents significant difference as compared to α-ZOL tested alone.

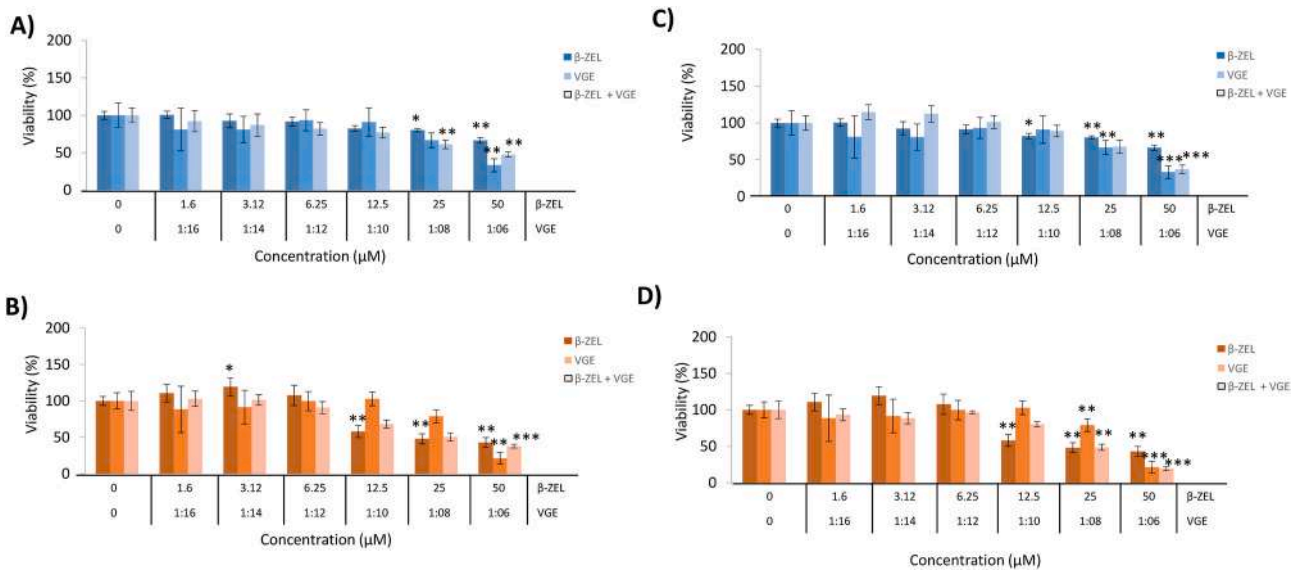

Fig. 3. Concentration curves obtained after simultaneous treatment (A and B) and pre-treatment (C and D) of VGE (dilution 1:16–1:6) during 24 h (A and C) and 48 h (B and D), and with serial dilutions of β-ZEL (0–50 μM) in HepG2 cells by MTT assay. All values are expressed as mean ± SD of 8 replicates. *(*) $p \leq 0.05$, (**)$p \leq 0.01$ and (***) $p \leq 0.001$* represents significant difference as compared to β-ZEL tested alone.

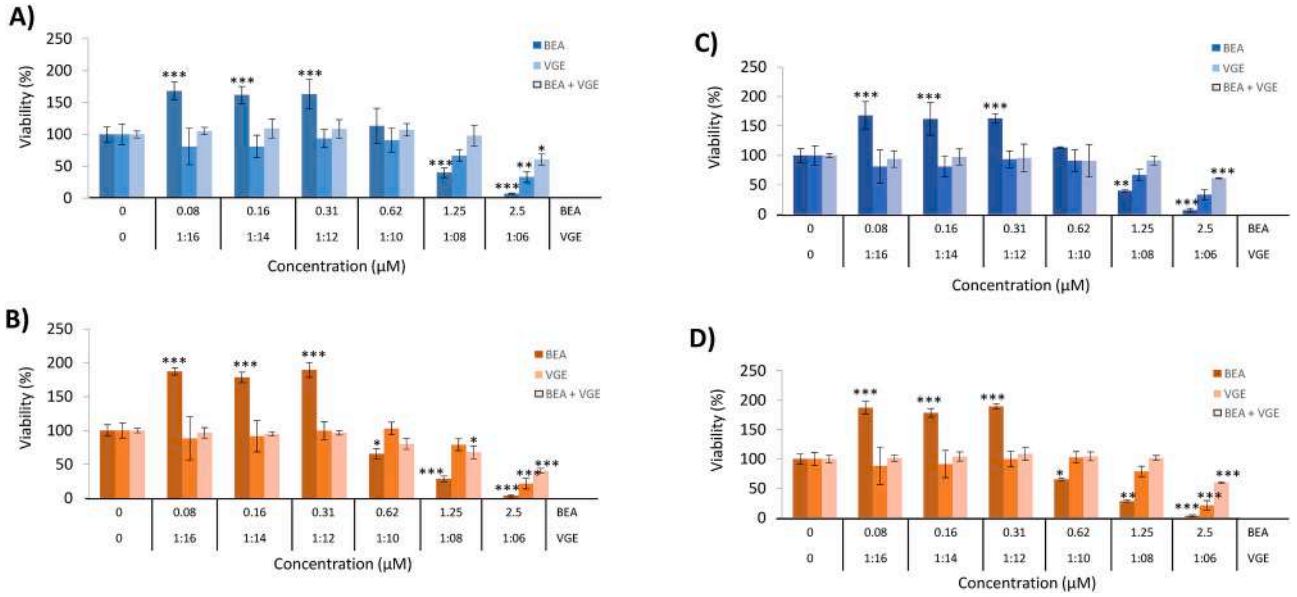

Fig. 4. Concentration curves obtained after simultaneous treatment (A and B) and pre-treatment (C and D) of VGE (dilution 1:16–1:6) during 24 h (A and C) and 48 h (B and D), and with serial dilutions of BEA (0–2.5 μM) in HepG2 cells by MTT assay. All values are expressed as mean ± SD of 8 replicates. *(*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$* represents significant difference as compared to BEA tested alone.

demonstrated the capacity of garlic extract compounds (propyl propane thiosulfonate (PTS) and propyl propane thiosulfinate (PTSO)) in reducing the production of mycotoxins by 90% specifically for deoxynivalenol (DON) and zearalenone (ZEA) demonstrating its efficacy against mycotoxigenic *Fusarium* spp. This effect has brought to propose such extracts as potential compounds for mycotoxin control in stored commodities (Mylona et al., 2019) and their protective effect for DON and ZEA; however, nothing is known about ZEA's metabolites combined with garlic extracts.

Here the cytoprotection activity of garlic extract against ZEA's metabolites (α-ZEL and β-ZEL) and BEA in HepG2 cells with different strategies of treatment have revealed that VGE increased the HepG2 cell viability above 75% at dilutions below 1:06, coinciding with the cytoprotection effect reported in previous *in vitro* studies (Soni et al., 1997; Galvano et al., 2001). Studies of garlic extract combined with mycotoxins is scarce in the literature but it has been mainly carried out with AFB1 which alleviated its cytotoxic effects (Soni et al., 1997; Galvano et al., 2001); and also some can be found *in vivo*, where it is reported that garlic enhances the detoxification of AFB1 through the suppression of CYP1A in the liver of fish (*Oreochromis niloticus*) as well as reduction in hepatic lesions produced by AFB1 (El-Barbary, 2018). OSCs from garlic extract in HepG2 cells alleviated cytotoxicity exposed to AFB1 as well as the protective effect on AFB1- induced DNA damage from the induction of glutathione S-transferase by those compounds (Guyonnet et al., 2001; Sheen et al., 2001); similarly, as anti-genotoxic in AFB1-inductor was reported for HepG2 cells by 34–47% (Belloir et al., 2006; Morales-Gonzalez et al., 2019). To notice that none of these studies report the effects of garlic extract dilutions which decrease the concentration of

OSCs from garlic which can reveal a damage in different cell lines. With our results we observed that the greatest cytoprotection was exerted at dilutions below 1:06 (Fig. 1D).

Protection against ZEA damage with garlic extracts from Tunisia on Vero cells showed a reduction of ZEA induced damages in xanthine oxidase inhibition and superoxide scavenging activity, cytotoxicity, ROS production, induction of catalase activity and a reduction of DNA fragmentation (Abid-Esseffi et al., 2012). According to our results VGE and ZEA's metabolites offered the greatest cytoprotection in simultaneous strategy treatment at 24 h when assayed with α-ZEL from 6.25 to 25 μM and VGE in the range from 1:12 to 1:06 dilution (Fig. 2A) with increases ranging from 0.35- to 0.7-folds respect to the mycotoxins' treatment in HepG2 cells. For pre-treatment this was observed for β-ZEL from 1.6 to 12.5 μM and VGE from 1:16 to 1:10 dilution also at 24 h (Fig. 3C) with increases of 0.2-folds respect to the mycotoxins treatment. Effects of ZEA's metabolites in ROS production has been reported in the literature being associated to cytotoxicity in different cell lines (Tatay et al., 2017; Agahi et al., 2020a, 2020b); however, in here, for the strategy of simultaneous treatment implies the exposure of HepG2 cells to mycotoxins and VGE at the same time, subsequently effects of cytoprotection can be associated to the favorable concentration of natural compounds in VGE which have antioxidant capacities that allow HepG2 cells to keep them alive and to continue the cell division as a consequence of association of various antioxidants compounds in the garlic extract (Fig. 2A, B, 3A and 3B). In the pre-treatment strategy, cells are being prepared to afford an unfavorable situation of stress with a high pull of antioxidant capacity, so, once exposed to ZEA's metabolites, HepG2 cells seem to be able to balance the production of ROS by mycotoxins as well as alterations in cell growth. Nevertheless, for this strategy this is maintain almost until 100% of viability for both ZEA's metabolites, until a point on which this balance is broken and once overpassed the cytoprotection decreases reaching the lowest viability, as observed in the last two scenarios assayed (either at 24 h and 48 h) (Fig. 2C, D, 3C and 3D). The capacity of cytoprotection by VGE in this strategy was observed for β-ZEL greater than for α-ZEL; this is in coincidence with the fact observed for CHO–K1 cells (Tatay et al., 2017) and SH-SY5Y cells (Agahi et al., 2020a), where β-ZEL produced less ROS than α-ZEL so that the higher cytoprotection can be observed by VGE in β-ZEL treatments than in α-ZEL treatments independently of the strategy.

Regarding BEA, simultaneous and pre-treatment with VGE revealed the greatest cytoprotection at the highest concentration of BEA tested and the most VGE concentrated extract (1:0) either at 24 h and 48 h. In a previous study carried out in our lab in SH-SY5Y cells, all three mycotoxins were studied in producing ROS (Agahi et al., 2020a) and there were noticeable effects at the same concentrations tested in here, although at different exposure time. Similarly, this was reported for BEA in Caco-2 cells (Prosperini et al., 2013). According to these effects previously published for BEA, VGE exerts its highest cytoprotection from 100% to 45% in all scenarios and strategies of treatment, simultaneous and pre-treatment.

In summary, cytoprotection of VGE in HepG2 cells has been evidenced in this study in different strategies and scenarios when exposed to mycotoxins responsible to trigger several pathways in HepG2 cells (implicated in ROS production, alterations in cell cycle and cell death). The alleviation of mycotoxins' cytotoxicity was associated to the presence of antioxidant capacity compounds present in VGE as well as the activation of protecting routes as enzymatic defense system from the inner cells. This evidences the importance of natural compounds presents in food that exert beneficial effects and a new strategy for garlic extracts in the prevention of mycotoxins' effects.

Besides all this and to have the entire puzzle complete regarding benefits from garlic compounds, bioaccessibility assays constitute an inflexion point in evaluating such final effect to be extrapolated in humans; and although some OSCs from garlic have been assayed in *in vitro* gastrointestinal digestion (Moreno-Ortega et al., 2020), further research is necessary to elucidate all its direct and indirect benefits but also to study the possibility of include them as a daily basic ingredient.

CRediT authorship contribution statement

Ana Juan-García: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Fojan Agahi:** Data curation. **Maria Drakonaki:** Data curation, Investigation. **Paola Tedeschi:** Methodology, Visualization. **Guillermina Font:** Funding acquisition, Visualization. **Cristina Juan:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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