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Cytoprotective effects of carotenoids-rich extract from Lycium barbarum L. on the beauvericin-induced cytotoxicity on Caco-2 cells

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ABSTRACT

In this work, the cytotoxicity of Beauvericin (BEA), lutein (LUT), zeaxanthin (ZEAX) and goji berries extract (GBE) rich in carotenoids, was investigated, as well as cytoprotective effects of these carotenoids against BEA induced-cytotoxicity on Caco-2 cells. Cytotoxicity was carried out using MTT and protein content (PC) assays during 24 and 48 h of exposure. Only BEA showed cytotoxic effect obtaining a reduction in cell proliferation range from 6.5 to 92.8%. Simultaneous combination of LUT and ZEAX with BEA slightly increased cell proliferation compared to BEA tested alone. LUT, ZEAX and GBE showed cytoprotective effects against cytotoxicity induced by BEA on Caco-2 cells. Pre-treatment assays showed the highest cytoprotection effect at the highest dose of BEA assayed (2.5 μM) in 29%, 31% and 35% for LUT, ZEAX and LUT + ZEAX, respectively; GBE showed a cytoprotection of 20%, for the same dose of BEA. The interaction between LUT, ZEAX and BEA studied by means of CI-isobologram method showed a synergism and antagaonism effect for all the combinations tested. These findings highlight that food containing high level of carotenoids, as goji berries, could contribute to reduce the toxicological risk that natural contaminant as BEA mycotoxin in diet can produce to the humans.

1. Introduction

Fat soluble pigments belonging to the class of carotenoids are widely distributed in nature, in fact they are found not only in bacteria, yeast and algae, but also into higher plants and animals. Main sources of carotenoids can be considered tomato and tomato-based products, guava watermelon, pumpkin, goji berries, among others (Montesano et al., 2012, 2018a; Rojas-Garbanzo et al., 2017; Oberoi and Sogi, 2017; Bertoldi et al., 2019). Currently carotenoids represent a subgroup of isoprenoid compounds comprising over 700 fully characterised structures. They are further classified based upon chemical structure as carotenes, highly conjugated C_{40} hydrocarbon chains such as β-carotene and lycopene and, as xanthophylls oxygenated carotenes such as zeaxanthin (ZEAX) and lutein (LUT) (Fig. 1) (Britton et al., 2004).

Many fruits and vegetables contain free and esterified carotenoids of great interest for human health because of their capacity to prevent human pathologies (Tapiero et al., 2004; Mayne, 1996). These molecules have been considered by several researchers as antioxidant compounds able to fight the free radicals, to reduce the risk of cancer, to prevent cardiovascular diseases and stroke, among others (Fattore et al., 2016; Montesano et al., 2008; Arathi et al., 2018; Mordente et al., 2011; Voutilainen et al., 2006; Costa-Rodrigues et al., 2018; Bahonar et al., 2017). Several studies showed that xanthophylls, such as LUT and ZEAX, were able to protect the eyes by degenerative diseases as age-related macular degeneration (Zhao and Sweet, 2008; Richer et al., 2011; Buscemi et al., 2018). Moreover, it has been demonstrated that both carotenes and xanthophylls work as protective compounds against oxidation during storage and frying (Montesano et al., 2006; Blasi et al., 2018).

Goji berries (*Lycium barbarum* L.) are considered a *superfruit* because of their high nutritional value (Montesano et al., 2018b) and biological properties (Zhang et al., 2016; Mocan et al., 2018; Wang et al., 2010). The carotenoid profile of this fruit is characterized by a high ZEAX dipalmitate content, which represents on average more than 85% of the total carotenoids and today considered one of the greatest natural sources of this xantophyll (Karioti et al., 2014). Generally, ZEAX and its LUT isomers are among the main carotenoids present in goji berries (> 88%) as reported in recent literature (Bertoldi et al., 2019; Montesano et al., 2018b; Hempel et al., 2017).

Currently, China dominates the market in terms of production, while the production capacity of the berries is very limited in North America and Europe as these berries are not native to these regions.

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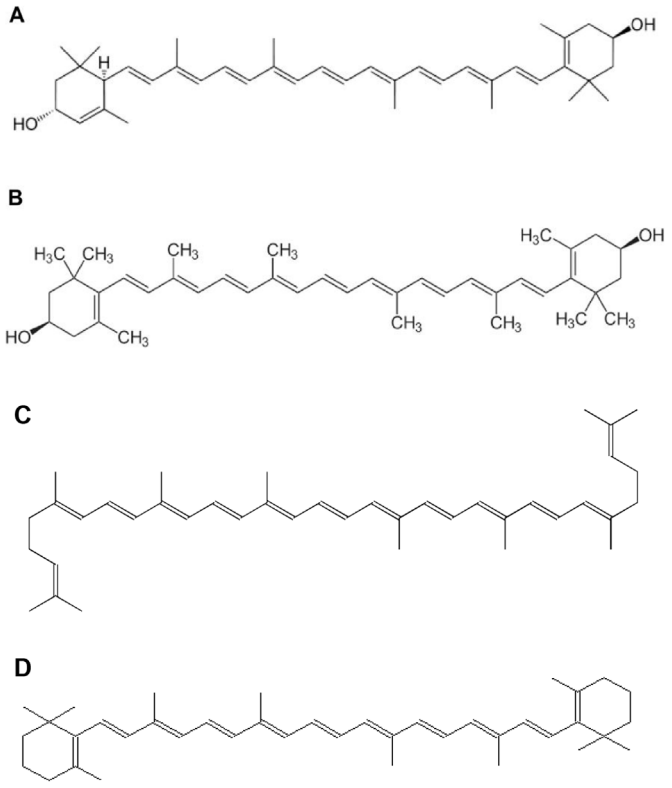

Fig. 1. Chemical structure of lutein (A), zeaxanthin (B), lycopene (C) and β-carotene (D).

However, due to the rise in the world superfruits market, several European and in particular Italian entrepreneurs have started to invest in goji as an innovative production of great nutritional value. In the last 5 years the Italian market has been at the top of the list in Europe for the cultivation and production of this precious fruit. In the southern regions there are several companies that produce goji and distribute them as a fresh, dried or processed product. In this area of Italy there is the first sustainable entirely Italian crop of fresh goji, over 150.000 plants of *Lycium barbarum* L. and 38 ha for its plantation, the biggest in Europe. Considering the increasing production trend, it is reasonable to expect to double that planted area. This year, the Italian Goji yield is expected to be about 1.5–1.8 tonnes per hectare, which is likely to mean a combined harvest of over 50 tonnes.

Nowadays, in a recent survey, Italy is first among the major European countries for the consumption of Goji berries (16% of respondents vs. less than 6% in Germany, Great Britain and Spain). Seven Italians out of ten (68%) are convinced that these berries can be used for healing purposes.

Mycotoxins are considered to be one of the most common food and feed contaminants in the world and nowadays represent an important risk factor for human and animal health (da Silva and BracarenseOswald, 2018; Pereira et al., 2018). Beauvericin (BEA) is a cyclohexadepsipeptide mycotoxin produced by many species of fungus *Fusarium* and by *Beauveria bassiana* (Fig. 2). BEA is considered as a natural contaminant of cereals and cereals based products (Juan et al., 2017; OueslatiBerradaMañesJuan, 2018; Stanciu et al., 2017) and it has been reported as the most frequently detected mycotoxin in Chinese medicinal herbs, with a concentration in the range of < 1.2–124.8 μg/kg (Hu and Rychlik, 2014). BEA mycotoxic properties have been established in the last years by *in vitro* studies on several cell lines (Mallebrera et al., 2018; Prosperini et al., 2013a; Juan-García et al., 2015). In particular, the inhibition of cell proliferation by exposure to BEA has been reported in several researches on human cell lines (Ruiz et al., 2011; Lin et al., 2005; Juan-García et al., 2015) using different

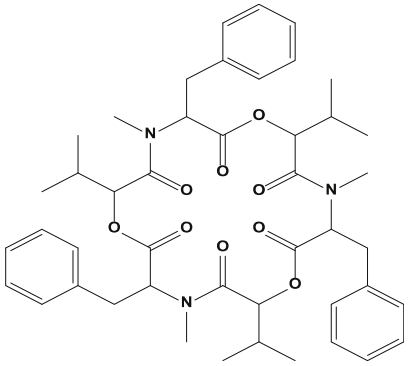

Fig. 2. Chemical structure of beauvericin.

determination assays mostly the tetrazolium salt (MTT) and the neutral red dye exclusion (Juan-García et al., 2016; Ferrer et al., 2009; Klaric et al., 2006; Calo et al., 2003).

Today it is possible to register a widespread presence of BEA in both food and feed. European Food Safety Authority (EFSA) constantly monitors this problem although to date it appears that there are no real dangers to an acute exposure risk, while there are still very few data on chronic exposure toxicity, and then a risk assessment for BEA food exposure cannot yet be calculated (EFSA, 2014). Although not much data are available on the toxic effects of BEA on humans and animals, it is certain, however, that it causes cytotoxicity in various cell lines and is able to produce oxidative stress at the molecular level. Furthermore, BEA is genotoxic (produces DNA fragmentation, chromosomal aberrations and micronucleus). This could certainly represent a risk to human health especially as a result of chronic exposures (Mallebrera et al., 2018). Furthermore, in a very recent study performed on sheep, it appears that exposure to BEA is able to cause oocyte mitochondrial dysfunction and affects embryo development (Mastrorocco et al., 2019).

In the present study, Caco-2 cells were used to evaluate cytotoxic effects of (a) BEA and (b) ZEAX, LUT and a goji berries extract (GBE). Moreover, to assess the cytoprotective effects of ZEAX, LUT and GBE, Caco-2 cells were pre-treated with this compounds and simultaneously treated with BEA. Finally, the interaction mixtures of ZEAX + BEA, LUT + BEA, ZEAX + LUT + BEA were evaluated in order to find out whether they could interact among themselves and produce synergistic, additive or antagonistic effect.

To the best of our knowledge, this is the first work using goji berries as carotenoids-rich fruit matrix in order to mitigate BEA induced-cytotoxicity on Caco-2 cells.

2. Materials and methods

2.1. Fruit material

Goji berries (*Lycium barbarum* L.) were provided from Italian farm Favella Group - Sud Rienergy srl by Nicola Rizzo (Corigliano C., Italy) and were taxonomically identified by Antonella Montanucci (University of Perugia). A representative specimen was deposited at Orto Botanico, Centro di Ateneo per i Musei Scientifici, University of Perugia (Italy). Goji sample was frozen at −20 °C until the time of the experimental procedures in order to preserve it from degradation.

2.2. Chemicals and reagents

Lutein (≥95.0%) and all-*trans*-zeaxanthin (≥98.0%) analytical standards were purchased from Extrasynthese (Lyon, France). Anhydrous sodium sulphate and disodium hydrogen phosphate and dimethylsulfoxide (DMSO), potassium dihydrogen phosphate were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). All solvents tetrahydrofurane (THF), aceitc acid-ethanol, water (proteomic grade),

hexane, acetone and methanol (LCMS grade) were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Cell culture

Caco-2 cells (ATCC HTB-37) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS, 25 mM HEPES buffer, 1% Non-Essential Amino acids (NEAA), 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM sodium pyruvate. Cells were grown near confluence in 75 cm^2 plastic flasks at 37 °C in an atmosphere containing 5% of CO_2 at 95% of relative humidity. The cells were subcultivated after trypsinization (trypsin- EDTA) once or twice per week and resuspended in complete medium in a 1:3 split ratio. Cell were subculture routinely with only a small number of sub-passages (< 70 subcultures) in order to maintain the genetic homogeneity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (SigmaeAldrich, St. Louis, MO, USA).

2.4. Preparation of goji berries extract rich in carotenoid

The carotenoid extraction from *L. barbarum* fruits was performed according to Blasi et al. (2018) with some modifications. Briefly, about 7.0 g of goji berries was treated with distilled water (80 mL) in a kitchen blender for 3 min and then centrifuged for 15 min at 5000 rpm in order to remove the more polar compounds. The water was discarded and the residue washed with absolute methanol (10 mL) under hand shaking for 1 min. After that, the residue was recovered after discarded the water/alcohol fraction. Successively, the residue was extracted by sonication for 10 min with 80 mL of a hexane-acetone mixture (3:2, v/v) and filtered under vacuum. The procedure was repeated three times or until the residue became colorless. The extracted fractions were pooled and dried over anhydrous sodium sulphate and then filtered by means of a paper filter and concentrated to dryness by means of a rotary evaporator (Büchi Rotavapor R-200, Germany) and finally submitted to a gentle nitrogen flow. The final residue was dissolved in 1 ml of THF and stored in glass-stoppered bottle at − 20 °C until the successive experimental procedure.

2.5. Exposure to beauvericine, zeaxanthin, lutein and goji berries extract

Caco-2 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 90% confluence, the culture medium was replaced and cells were exposed during 24 and 48 h to 200 µl of fresh medium containing different concentrations of (i) BEA (from 25 to 0.1 µM, 1:2 dilutions); or (ii) LUT (from 3 to 0.01 µM, 1:2 dilutions); or (iii) ZEAX (from 6 to 0.02 µM, 1:2 dilutions) or, (iv) GBE (extract of 7gr, 1:2 dilutions). Dilution series of GBE with medium was performed: from 1:0 to 1:131072.

Combinations of BEA and carotenoids (ZEAX and LUT) were performed in pre-treatment and in simultaneous-treatment studies. For pre-treatment studies cells were exposed to LUT 3 µM or ZEAX 6 µM (fixed doses) and to a combination of LUT 3 µM + ZEAX 6 µM during 24 h, while for GBE, the experiments were performed without dilution in Caco-2 cells. Afterwards, the medium containing carotenoids was removed and cells were exposed to BEA (from 2.5 to 0.01 µM, 1:2 dilutions) for 24 h of incubation. For mixtures of simultaneous treatment studies, concentrations used were the same as those described above starting for BEA at 25 µM, LUT at 3 µM, ZEAX at 6 µM and GBE directly as the highest concentration and 1:2 dilutions for all scenarios proposed. The plates were incubated for 24 h at 37 °C, and the MTT assay was performed.

2.6. MTT and protein content (PC) assays

The MTT assay determines the viability of cells by the reduction of yellow soluble tetrazolium salt (MTT), only in the metabolically active cells, via a mitochondrial-dependent reaction to an insoluble purple formazan crystal. The MTT viability assay was performed as Ruiz et al. (2006). Briefly: after exposure of BEA, ZEAX, LUT and GBE pre-treatment or simultaneous-treatment studies, the medium containing these compounds was removed and cells of each well received 200 µl fresh medium plus 50 µl of MTT. 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 solubilised in DMSO. The absorbance was measured at 570 nm using an ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA).

The PC assay was carried out according to Davoren et al. (2007). The PC gives data about cell damage independently of the toxic mechanism involved. PC was analyzed after 24 h *in situ*, in the same 96-well culture plates in which MTT took place. Briefly, plates were washed with 100 µL of PBS and cells fixed with 100 µL of acetic acid-ethanol solution. Afterwards, solution was removed and 100 µL of Coomassie Brilliant Blue dye was added and kept for 10 min shaking at 240 rpm. Commassie exceding was removed and cells were washed with 100 µL of acetic-ethanol solution. Dye was extracted from cells with 100 µL of potassium dihydrogen phosphate and shaking the plate during 10 min at 240 rpm at room temperature. Absorbance at 570 nm was measured using an automatic ELISA plate reader Multiscan Ex (Thermo Scientific). Cell viability (for MTT and PC assays) was expressed in percent relative to control cells (1% DMSO for BEA, 1% THF for carotenoids and GBE). 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. Cytoprotective effects of lutein, zeaxanthin and goji berries extract

To determine the interactive effect between BEA and carotenoids or GBE, the MTT and PC assays were used as described in section 2.6. The concentrations were selected considering the margin of carotenoids/goji berries intake (3–12 mg) according to food balance sheets published by FAOSTAT (2011), while the concentrations used for BEA are in agreement with the results obtained by Mallebrera et al. (2017) and Prosperini et al. (2012), and considering the results published by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2011). On the basis of the concentration–response function of individual BEA and carotenoids, predictions of effect concentration were calculated for mixtures containing BEA and carotenoids in the following relation: for BEA + LUT 2.5:1 ratio, for BEA + ZEAX 4:1 ratio and for BEA + LUT + ZEAX 8:1:2 ratios.

The isobologram analysis (Chou-Talalay model) was used to determine the type of interaction (synergism, addition or antagonism effect) that occurs when compounds studied are combined. This model allows characterizing the interactions induced by combinations of mycotoxins, in this case, in different cell lines and with different mycotoxins; but it does not allow elucidation of mechanisms by which these types of interaction are produced. Originally the median-effect/combination index (CI)-isobologram equation by Chou (2006) and Chou and Talatay, 1984 permitted analyzing drug combination effects. The isobologram analysis involves plotting the dose–effect curves for each compound and its combinations in multiple diluted concentrations. Parameters as *Dm* (the median-effect dose), *fa* (fraction affected by concentration), and *m* (coefficient signifying the shape of the dose–effect relationship) are relevant in the equation (Chou and Talatay, 1984). Therefore, the method takes into account both the potency (*Dm*) and shape (*m*) parameters.

Chou and Talatay, 1984 introduced the term combination index (CI). The CI < 1, = 1, and > 1 indicates synergism, additive and antagonism effect of the combination, respectively. CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007) was used to study the types of interactions assessed by isobologram analysis. The CI_{25}, CI_{50},

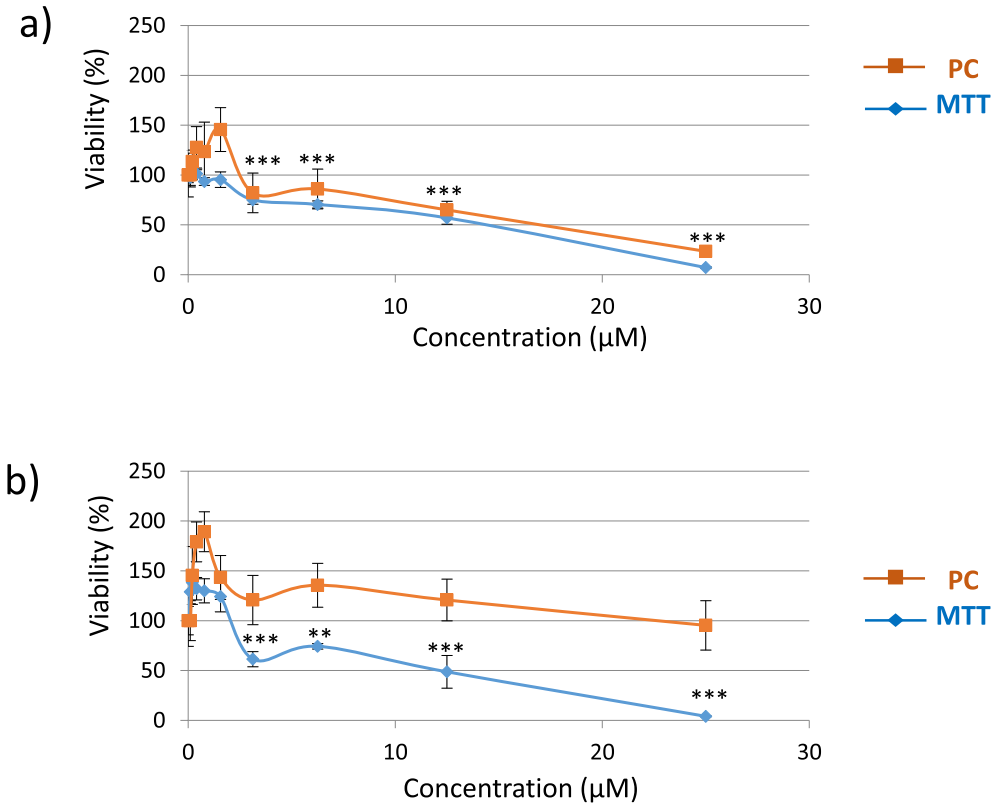

Fig. 3. Concentration–effect curves of BEA in Caco-2 cells after (a) 24 h and (b) 48 h of exposure by MTT and PC assays. All values are expressed as mean ± SD of 8 replicates. (*) $p \leq 0.05$ represents significant difference as compared to control values.

CI_{75} and CI_{90} are the doses required to inhibit proliferation at 25%, 50%, 75% and 90%, respectively.

2.8. 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 ± SEM 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 at $P \leq 0.05$.

3. Results

3.1. Cytotoxicity of beauvericin, zeaxanthin, lutein and goji berries extract

The effects of BEA, ZEAX, LUT and GBE in cell viability were evaluated by the MTT and PC assays after 24 and 48 h of exposure (Figs. 3–5).

The results clearly indicated that BEA has a cytotoxic effect on Caco-2 cells in a dose dependent manner as reported in Fig. 3. It is shown that after 24 h and 48 h of BEA incubation on Caco-2 cells a significant reduction in cell viability was obtained at the highest concentration tested (25 μM); such reduction went from 96 to 92%. In fact, the molar concentrations of BEA, ZEAX and LUT that reached 50% inhibition of cellular proliferation (IC_{50}) was possible to calculated only for BEA. At 24 h IC_{50} value was (12.5 ± 1.5) μM, whereas at 48 h it was (14.5 ± 0.8) μM.

The main carotenoids present in the goji berries, ZEAX and LUT, were evaluated for their cytotoxicity on Caco-2 cells (Fig. 4). The results of this study showed that the viability of Caco-2 cells by these carotenoids was not affected at the concentrations tested, both at 24 h and 48 h (Fig. 4).

In accordance with previously published data (Bertoldi et al., 2019), the carotenoid fraction in goji berries used for this work was 0.4% dw, of which 85% is represented by ZEAX. The maximum final concentrations of ZEAX and LUT in the medium were 170 and 11 μM respectively. These concentrations then underwent scalar dilutions as shown in Fig. 5. The GBE was also tested following the previous described procedure for MTT and PC (section 2.6). Also in this case the viability of Caco-2 cells was not affected at all the dilutions tested, both at 24 h and 48 h (Fig. 5). Viability was always higher than 100%, for both 24 h and 48 h, while PC assay, denoted a not fall in viability below 70% (Fig. 5). In particular at 24 h no diluted GBE (1:0) significantly increased ($p \leq 0.05$, 10%) cell proliferation using MTT assay while by means of PC assay the increasing was 25%; however, for other dilutions (from 1:2 to 1:32) the increase of cell proliferation ranged from 7 to 15% by means of MTT and from 2 to 38% by PC assay. For tests at 48 h, no diluted GBE (1:0) and all dilutions assayed (1:2 and 1:32) showed a significant ($p \leq 0.05$) reduction in cell proliferation by PC assay from 16 to 27%; while by MTT assays an increasing from 13 to 27% was recorded. It was noticeable that no IC_{50} values were reached for GBE dilutions.

3.2. Cytoprotection of zeaxanthin and lutein

To investigate whether ZEAX and LUT can inhibit BEA-induced cytotoxic effect in Caco-2 cells, fresh medium containing simultaneously carotenoids (1:2 dilutions LUT from 3 to 0.01 μM and, ZEAX from 6 to 0.02 μM) and BEA (1:2 dilutions from 25 to 0.1 μM) and solvent control (1% DMSO) were assayed for 24 h by using MTT and PC assays (Fig. 6). As shown in Fig. 6a and b no significant differences were observed when BEA was simultaneously exposed with 0.16 μM LUT or 0.32 μM ZEA respect to BEA tested alone. Nevertheless, when Caco-2 cells were simultaneously incubated with BEA at highest

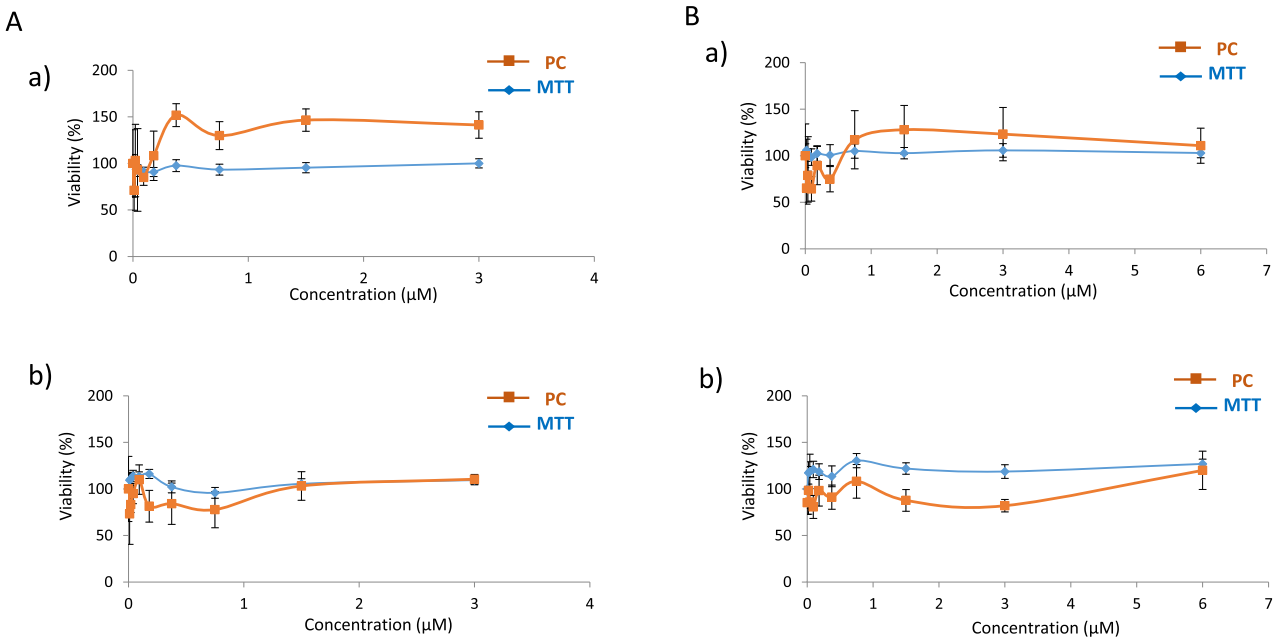

Fig. 4. Concentration–effect curves of (A) Lutein and (B) Zeaxanthin in Caco-2 cells after 24 (a) and 48 h (b) of exposure by MTT and PC assays. All values are expressed as mean ± SD of 8 replicates.

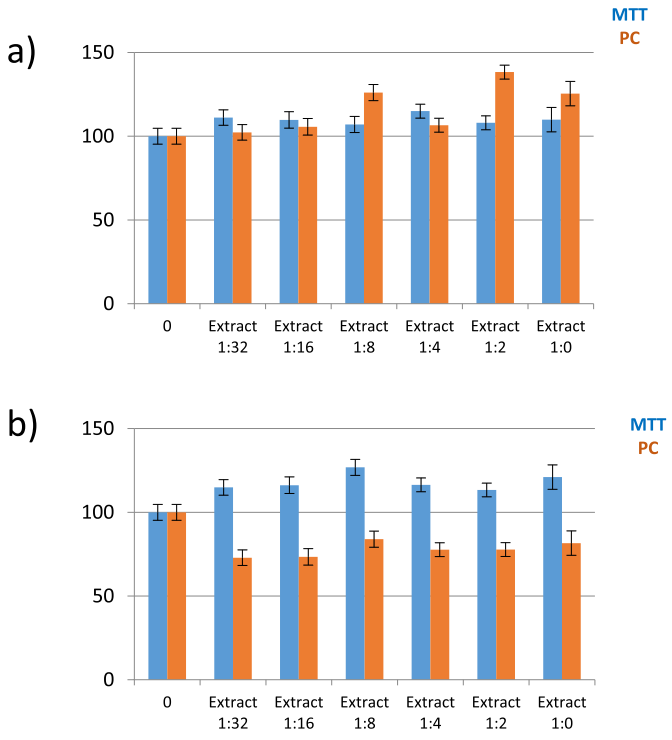

Fig. 5. Goji extracts–effect relationship in Caco-2 cells after 24 h (a) and 48 h (b) of exposure by MTT and PC assays. Serial goji extract dilutions (from 1:0 to 1:32) were obtained with distilled water. All values are expressed as mean ± SD of 8 replicates.

concentrations (12.5 and 25 μM) and higher concentrations of ZEAX or LUT, a slight cytoprotection of 13.2% and 9.8% for LUT (Fig. 6a) and ZEAX (Fig. 6b), respectively was observed. Regarding triple combination (LUT + ZEAX + BEA), only at the lowest concentrations assayed a slight cytoprotection (within 10%) was detected; although no cytotoxicity increase respect to the BEA tested alone was detected (Fig. 6c).

Regarding pre-treatment assays at concentrations described in section 2.5, the highest cytoprotection effect was observed for the highest BEA dose assayed (2.5 μM) with 29%, 31% and 35% for LUT, ZEAX and LUT + ZEAX, respectively (Fig. 7).

3.3. Cytoprotection of goji berries extract

Cytoprotection of GBE on Caco-2 cells due to BEA exposure was observed (Fig. 8) by MTT assay. Fig. 8a shows cell viability increased after all dilutions of GBE tested simultaneously with BEA. The major protective effect was obtained by 1:32 and 1:64 dilutions, recording an increasing of viability of 48% and 43%, respectively. Regarding pre-treatment assays, cytoprotection of 20% was obtained for BEA 2.5 μM (Fig. 8b).

3.4. Lutein, zeaxanthin, and beauvericin interaction effect in Caco-2 cells

In order to investigate the type of the interaction between LUT, ZEAX and BEA in combinations described in section 2.5, the CI-isobologram method was applied. The parameters *Dm, m* and *r* of binary and triple combination as well as CI values are shown in Table 1. CI values were calculated automatically by the computer software CalcunSyn. The CI/*fa* curve for combination tested on Caco-2 cells is shown in Fig. 9. As assessed by CI-isobologram equation, antagonism (CI > 1) and synergism (CI < 1) effects were produced by all the combinations tested except for the triple combination (Table 1; Fig. 9). *fa* for BEA + LUT + ZEAX (8:1:2), CI values indicated antagonism effect in all cases (Fig. 9).

4. Discussion

Actually, several researchers agree in asserting that mycotoxin toxicity is expressed through molecular mechanisms involving free radicals and the consequent oxidative stress (Adhikari et al., 2017; Juan-García et al., 2019; Wang et al., 2016). Mycotoxins, in fact, once in contact with living organisms, are able to promote an imbalance between the antioxidant protective shield and free radicals, causing chemical damage to protein, lipid and DNA structures, changes in cell cycle distribution, mitochondrial membrane potential disturbances, micronucleus induction and cell death (Prosperini et al., 2013b; Juan-García et al., 2016, 2015; Mallebrera et al., 2016; Tapiero et al., 2004; Assi,

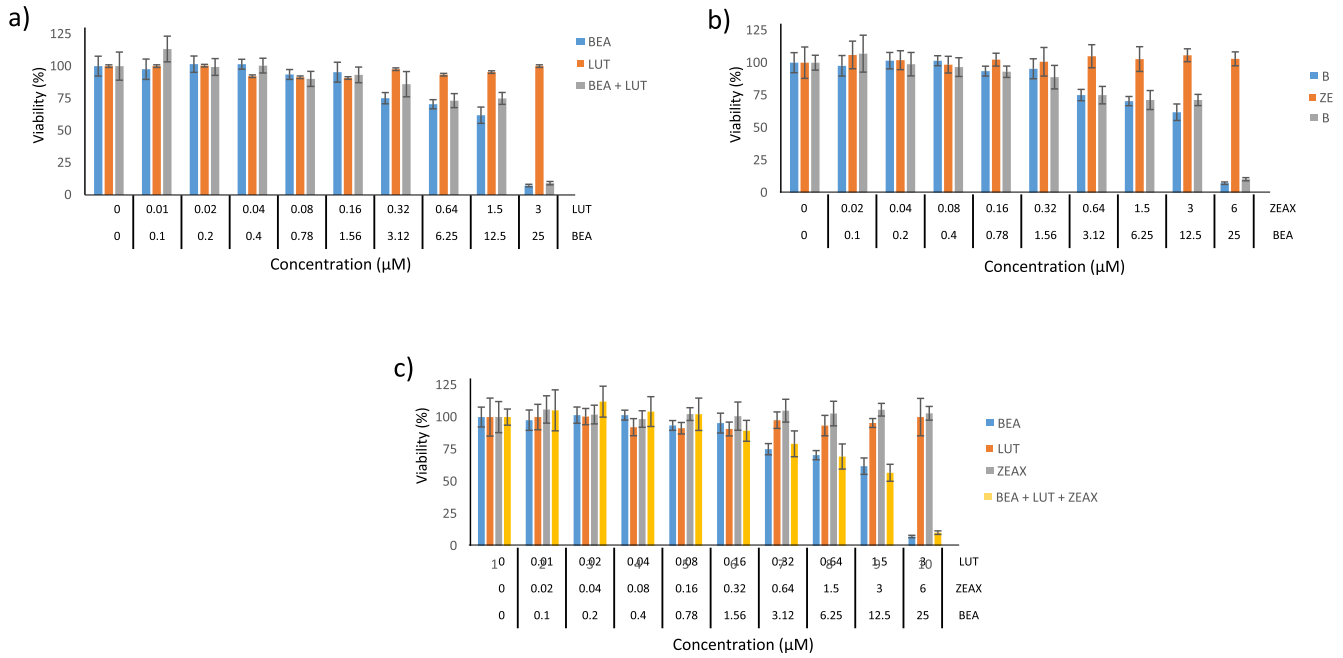

Fig. 6. Concentration–effect curves obtained after simultaneous combination of BEA + LUT (a), BEA + ZEAX (b) and BEA + LUT + ZEAX (c) in Caco-2 cells during 24 h of exposure by MTT assay. All values are expressed as mean ± SD of 8 replicates.

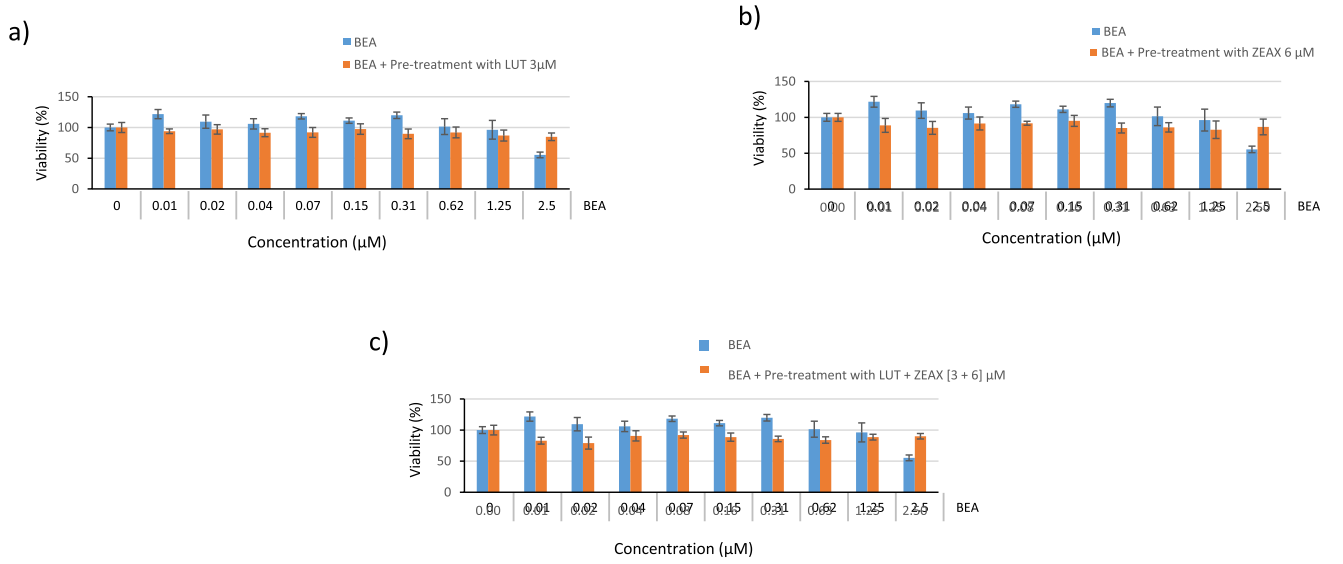

Fig. 7. Concentration curves obtained after pre-treatment of carotenoids (a) LUT 3 μM, (b) ZEAX 6 μM and (c) LUT + ZEAX [3 + 6] μM during 24 h, and subsequent addition of fresh medium with serial dilutions of BEA (0.01–2.5 μM) during 24 h in Caco-2 cells by MTT assay. All values are expressed as mean ± SD of 8 replicates.

2017).

For these reasons the use of natural antioxidants could be useful in limiting and/or preventing the toxic effects of mycotoxins (Mallebrera et al., 2017; Cano-Sancho et al., 2015; Sorrenti et al., 2013) and then it is reasonable to think that the intake of antioxidants can represent a concrete strategy to completely inhibit or at least reduce the toxicity of mycotoxins.

Natural compounds of different chemical nature have been used to mitigate the toxicity of mycotoxins due mainly to oxidative stress, and carotenoids in addition to ascorbic acid, tocopherol, and flavonoids must certainly be included among these substances (Strasser et al., 2013; Salem et al., 2015; Salem et al., 2001Sorrenti et al., 2013). Among the carotenoids, surely the most representative are the crocins and lycopene as reported by several researchers (Salem et al., 2015; Aydin et al., 2013; Hedayati et al., 2019), as well as it is very interesting the detoxifying action of curcumin (Verma and Mathuria, 2008, 2009). In particular, there are several *in vivo* studies that report the detoxifying action of lycopene against some mycotoxins. Boeira et al. (2015) reported that mice treated with lycopene 20 mg/kg were protected against acute zearalenone-induced oxidative, endocrine, inflammatory and reproductive damages concluding that this carotenoid can be considered a potential therapeutic nutrient in protecting against male reproductive toxicity induced by zearalenone. Aydin et al. (2013) showed that rats treated with lycopene (5 mg/kg for given orally both 7 and 14 days) were protected against DNA damage induced by ochratoxin A, evaluated by means of COMET assay in the kidney and liver cells. Moreover, high-intake of lycopene (5–100 mg/kg) has shown to be effective in decreasing the risk of several kinds of natural toxins including mycotoxins (Hedayati et al., 2019).

Few researchers have reported interesting results related to the

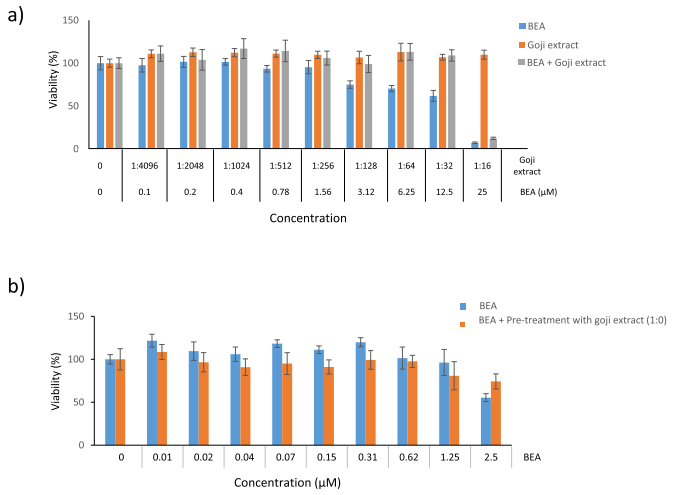

Fig. 8. Concentration–effect curves obtained after simultaneous combination of BEA + Goji extract (a) and pre-treatment with Goji berries extract (1:0) (b) in Caco-2 cells during 24 h of exposure by MTT assay. All values are expressed as mean ± SD of 8 replicates.

biological and pharmacological properties of different carotenoids at the most suitable concentrations, by means of *in vitro* studies on various cell lines. Shyu et al. (2008) reported that lycopene (5–20 μM) significantly inhibited lipopolysaccharide (LPS)-induced NO release in primary cultured microglia. Moreover, this carotenoid also concentration-dependently diminished the LPS-induced production of proinflammatory cytokines such as TNF-α and IL-1β in microglia. Reddy et al. (2006) evaluated the chemoprotective effects of lycopene and β-carotene on HepG2 cells exposed to different concentrations of aflatoxin B1 (AFB1). Cell protection was ascertained both at the cellular and molecular level, in fact increases in mitochondrial activity, cell survival and cell-cell communication were recorded, in addition to a reduction in ultrastructural damage. In light of the results obtained from this study, the authors conclude that β-carotene and lycopene can prevent the toxic effects of AFB1 by interfering with mitochondrial activity, preventing DNA damage and increasing programmed cell death.

The antioxidant properties of lutein, lycopene and apocarotenoic ester *in vitro* regarding their ability to reduce the cytotoxic effect of T-2 toxin on chicken hepatocytes has been evaluated (Leal et al., 1998). Results revealed that for simultaneous treatment of LUT + T-2 toxin a partial protective effect was produced. In the same way, lycopene was able to reduce the cytotoxic effect of the mycotoxin when both were in contact with the cells, either simultaneously or in pre-treatment assays; while, apocarotenoid treatment was unable to produce the same protective effect. Krishnaswamy et al. (2010) showed that LUT was able to reduce DON-induced oxidative stress in HT29 cells and downregulated expression of two inflammatory genes (NF-kappaB and COX-2). In that study it was demonstrated that LUT prevented DON-induced migration of NF-kappaB into the nucleus. Moreover, thanks to morphological studies by electron microscopy and the analysis of the cell cycle by flow cytometry it was concluded that LUT was able to prevent DON-induced apoptosis, demonstrating that this carotenoid exerts a cytoprotective role in DON-induced toxicity. Grudzinski et al. (2018) reported the effects of ZEAX and LUT at 1.5, 5 and 10 μM on human colon epithelial cells (CCD 841 CoTr) and colon adenocarcinoma cells (HT-29). Both carotenoids resulted to be non-cytotoxic to normal cells but effective in decreasing the viability of tumor cells. According to this work, both 6 and 3 μM for ZEAX and LUT, respectively, were selected for this study, which correspond to the physiological levels in human blood plasma.

In this work, then, ZEAX and LUT at the aforementioned concentrations and GBE were tested in order to mitigate the toxicity expressed by the BEA on Caco-2 cells.

The IC_{50} evidenced by BEA in the Caco-2 cell line was 12.5 and 14.5 μM at 24 and 48 h, respectively. BEA toxicity tested on different cell lines have reported IC_{50} values of 11 μM after 24 h on mouse macrophages (J774) (Tapiero et al., 2004) and similar results have been obtained on U-937 and HL-60 cell (Calo et al., 2003). In a study conducted on CHO–K1 the cytotoxicity of BEA (Ferrer et al., 2009), by MTT and analysis by neutral red (NR) assay at 24 h of exposure, showed an IC_{50} value of 17.22 mM and 12.08 mM by MTT assay. These cases are very similar to those reported in this study; however, in an interesting work carried out on human leukemia cell line (CCRF-CEM) BEA IC_{50} value was 2.46 mM after 24 h of exposure (Jow et al., 2004) which corresponds to 6-fold lower than those for Caco-2 cells here reported.

Of interest, the application of isobologram method was used in order to study the interaction effect among LUT, ZEAX and BEA. Generally, combined actions of two substances with similar effects are frequently expressed by pairs of doses that produce a fixed response, usually 50%, in so-called isobolograms. The data regarding the interaction among the tested carotenoids and BEA confirmed the capacity of these compounds to interact increasing the cytoprotective effect in antagonism manner, as shown by isobologram study (Fig. 9; Table 1). In Fig. 6a, is showed the improvement of the cell viability by LUT when used in simultaneous combination with BEA, specially at high concentrations (BEA 3.12–25 μM; LUT 0.32–3.0 μM), moreover, the combination BEA-LUT 2.5:1 showed an antagonism effect at low CI values and synergism at high CI values (Table 1; Fig. 9a). Similarly, ZEAX showed an improved cell viability at high concentrations (BEA 6.25 μM;

Table 1
The parameters *Dm, m* and *r* are the antilog of x-intercept, the slope and the linear correlation of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (IC_{50}), and the conformity of the data to the mass action law, respectively (Chou and Talalay, 1984; Chou, 2006). *Dm* and *m* values are used for calculating the CI value CI < 1, = 1 and > 1 indicates synergism (Syn), additive (Add) and, antagonism (Ant) effect, respectively. IC_{25}, IC_{50}, IC_{75}, and IC_{90}, are the doses required to inhibit proliferation at 25, 50, 75 and 90%, respectively. CalcuSyn Software provide automatically these values.

Mycotoxin	*Dm* (μM)	*m*	*r*	CI values							
				CI_{25}		CI_{50}		CI_{75}		CI_{90}	
BEA	22.17	0.70	0.98								
LUT	7.88	1.14	0.94								
ZEAX	1.23	0.08	0.98								
BEA + LUT (2.5:1)	8.37	136.180	0.96539	1.04 ± 0.85	Add	1.52 ± 0.87	Add	0.26 ± 0.91	Add	0.13 ± 0.97	Add
BEA + ZEAX (4:1)	18.82	0.89087	0.96287	1.17 ± 0.35	Add	0.84 ± 0.39	Add	0.61 ± 0.10	Add	0.44 ± 0.39	Syn
BEA + LUT + ZEAX (8:1:2)	30.04	0.59943	0.94024	1.23 ± 0.55	Add	1.83 ± 1.42	Add	2.04 ± 1.5	Add	5.04 ± 1.5	Add

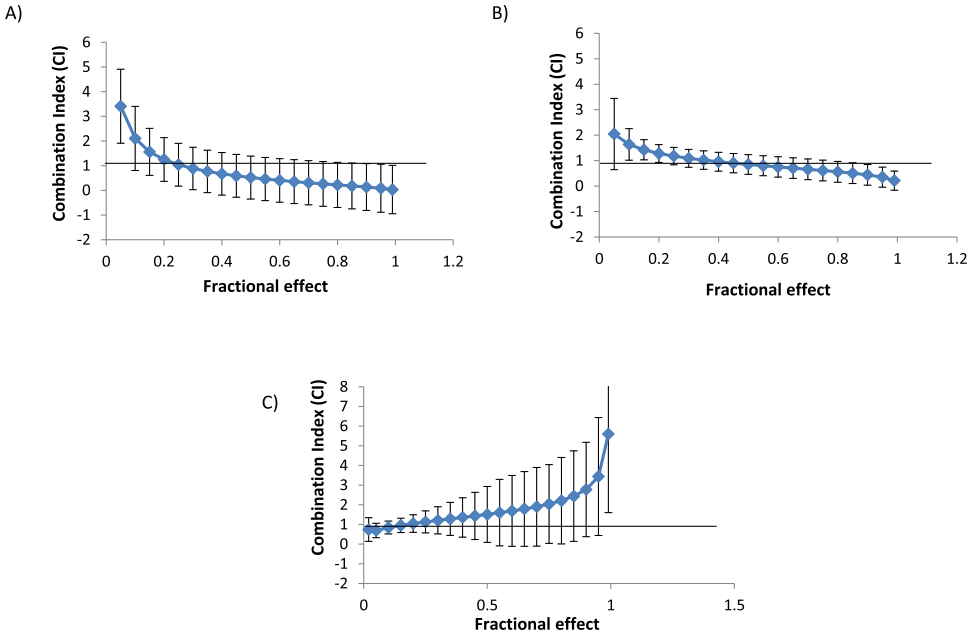

Fig. 9. Combination index (CI) vs fractional effect curve as described by Chou and Talalay model on Caco-2 cells exposed to BEA + LUT (2.5:1) (A), BEA + ZEAX (4:1) (B) and BEA + LUT + ZEAX (8:1:2) (C). Each point represents the CI ± SD at a fractional effect as determined in our experiments. The line (CI = 1) indicates additivity, the area under this line synergism and, the area above the line antagonism. Caco-2 cells were exposed during 24 h at molar ratio (equimolar proportion).

ZEAX 1.5 μM) as reported in Fig. 6b, while at elevated value of *fa*, relative to the combination BEA-ZEAX 4:1, a synergism effect was recorded. As regards the triple combination (BEA + LUT + ZEAX) instead, it is very evident, from the CI values calculated by Calcusyn, a greater antagonism effect compared to the above combinations (Table 1; Fig. 9). In fact, as shown in Fig. 6c, the triple combination was able to protect the cells both at low and high concentrations with an increase in cell viability over 30% at the maximum concentration of BEA, compared to BEA alone. The positive interaction between LUT, ZEAX and BEA is also evident in pretreatment studies (Fig. 7). In fact, the increase in cytoprotection is evident when the cells are pretreated simultaneously with both carotenoids (> 35% respect to BEA alone). However, although with less intensity, the pretreatment with the individual carotenoids also produced an improvement in cell viability, in particular 29% with LUT and 31% with ZEAX.

5. Conclusions

The aim of this study was to evaluate the cytotoxicity of BEA, LUT, ZEAX and a carotenoids-rich fruit extract from *Lycium barbarum* (GBE) as well as the cytoprotective effects of these carotenoid compounds against BEA induced-cytotoxicity on Caco-2 cells. From the data obtained it is evident that the cytoprotective effect depends on the concentration of carotenoids or GBE and BEA present in the food product, as well as the simultaneous presence of both compounds and the interaction between them. In fact, althought the individual carotenoids were able to provide cytoprotective effects, the major capacity to improve the cell viability was recorded when both were used simultaneously, as confirmed by antagonism and synergism effect recorded by studies using the isobologram method. These findings, in addition to the fact that the consumption of goji berries is strongly increasing in European countries, suggest that the consumption of goji berries could help to prevent the toxicological risk to humans that BEA can produce or mitigate the cytotoxic effects induced by this toxin. Further bioaccessibility and bioavailability studies of ZEAX and LUT from goji berries and their interactions with BEA could be useful for a better understanding of the cytoprotective effects of both carotenoids (LUT and ZEAX) and GBE.

Declaration of interests

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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