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ABSTRACT

Food and feed commodities are often contaminated by more than one mycotoxin. Among the several combinations that frequently occur, fusariotoxins are often mentioned. The multi-mycotoxins contamination is a serious threat for health. In the present study we investigated the toxic interactions between deoxynivalenol (DON), nivalenol (NIV), and fusarenon-X (FX) in Jurkat T cells by using the MTT assay. The tested mycotoxins alone or in combination had a dose dependent effect on proliferating lymphocytes. According to IC_{50}, it could be classified in decreasing order of toxicity: FX > NIV > DON > DON+FX > NIV+FX > DON+NIV > DON + NIV + FX. The type of mycotoxin interactions was assessed by calculating the combination index (CI) and the dose reduction index (DRI). Our data indicate that an antagonistic effect was strongly observed in binary combination between DON and NIV at higher concentrations and ternary mixtures. Meanwhile, the DON and FX mixtures generated moderate antagonism, while NIV and FX provoked different interactions. The effects of tested mycotoxins on apoptosis were also determined using a FACScan flow cytometer. At IC_{75}, the percentages of apoptotic cells in all treatment groups were significantly different when compared to control group but no significant differences among treatment groups was observed. Taken together, our data suggest that immunotoxicity among multi-mycotoxins contamination cannot be predicted based on individual effects but de- pends on the mixtures, ratio and/or concentrations in the product, as well as type of target cells.

Introduction

Mycotoxins are ubiquitous secondary metabolites from filamentous fungi. These metabolites are not essential for their growth and re- production. Nowadays, about 300–400 mycotoxins are classified but around thirty are hazardous substances to humans and animals in term of chronic toxicity after prolonged exposure (Berthiller et al., 2007; Hussein and Brasel, 2001; Streit et al., 2012). Recently, mycotoxins were one of major category in border rejection in the European Union (EU) according to the annual report of the Rapid Alert System for Food and Feed (RASFF) (Marin et al., 2013). The Food and Agriculture Organization (FAO) suggested figure of one fourth of global food crop is contaminated by mycotoxins (JECFA, 2001). In fact, over 80% of agricultural commodities contained at least one mycotoxin (Kovalsky et al., 2016; Streit et al., 2013). Deoxynivalenol (DON) was most pre- valent. During an 8-year period (2004–2011), 55% of 17,316 feed and feed raw materials from all over the world was positive to DON (mean=535µg/kg) (Streit et al., 2013). According to global survey data for finished feed, maize and maize silage samples from the year 2012–2015, 79% of 1113 samples showed DON contamination with concentrations above the threshold levels (> 1.5 µg/kg) whereas 28% of samples contained nivalenol (NIV) with concentrations above 15 µg/ kg (Kovalsky et al., 2016). The co-contaminated with two or more mycotoxins were frequently observed. Alassane-Kpembi et al. (2017b) suggested that natural co-occurrence of mycotoxins

in foodstuffs occurs for at least three different reasons: (i) A complete diet consists of var- ious different commodities. (ii) Food commodities can be contaminated by various fungi simultaneously. (iii) Most fungi can simultaneously produce a number of mycotoxins. Regarding trichothecenes type B, they are mainly produced by *Fusarium graminearum*, *F. culmorum*, and other *Fusarium* and related fungi (FSCJ, 2010). Thus, their co-contamination is frequently observed in food commodities. A three-year 2006–2008) survey on commercial wheat grain in Brazil demonstrated that the co-contamination between DON and NIV was predominant (59 of 66 samples) and overall mean levels of DON and NIV were 540 and 337 μg/kg, respectively (Del Ponte et al., 2012). In Korea, the co-occurrence rates of NIV (26.1–2791.4 μg/kg in combination) and DON (10.1–655.6 μg/kg in combination) were 9.1%, 14.9%, and 41.5% for white rice, brown rice, and bran, respectively (Ok et al., 2018). In Spain, one multigrain sample most frequently consumed by Spaniard had co-occurrence of DON and fusarenon-X (FX) at concentrations of 42 μg/kg (Montes et al., 2012).

Chronic exposure to trichothecene type B can cause anorexia, emesis, growth retardation, intestinal disorder and immunomodulation (Payros et al., 2016; Pestka, 2008, 2010; Wu et al., 2012, 2013). Quickly proliferating cells with a high protein turn over such as intestinal mucosa, bone marrow, thymus, spleen and lymph nodes are especially sensitive to these mycotoxins. The immune system is considered as a primary target for trichothecenes (Bondy and Pestka, 2000). Katika et al. (2015) proposed that immune cells are more sensitive to DON than other cell types due to the induction of a T-cell activation response by increased intracellular calcium levels. Nevertheless, the effects of trichothecenes on the immunity are complex. Trichothecenes treatment at low dosage levels promotes the upregulation of many immune related genes with contributing immune stimulation, whereas high dose exposure induces leukocyte apoptosis with coordinating immune suppression (Pestka et al., 2004). For instance, Jurkat T cells exposed to DON at concentrations ranging from 62.5 to 500ng/ml significantly upregulated IL-2 and IL-8 production which may have significance in T-cell development and inflammation, respectively (Pestka et al., 2005). Likewise, a super-induction of the inflammatory cytokine IL-6 was observed in DON-treated murine mac- rophage cell line RAW264.7 at concentrations of 0.42–0.84 μM (Mayer et al., 2017). Conversely, Jurkat cells exposure to DON at concentra- tions ranging from 500 to 1000 ng/ml significantly induced apoptosis over the untreated cells (Pestka et al., 2005). The prolonged ingestion with DON-added feed (2mg/kg feed) significantly exhibited a high level of pro-inflammatory cytokine TNF-α and of IL-8 in head kidney of rainbow trout (Matejova et al., 2015). In addition, Islam et al. (2013) showed that DON-treated mice (0.5–2 mg/kg body weight) significantly decreased the population of CD19 +and CD11c +cells in the spleen and mesenteric lymph node (MLN) and of F4/80 + cells in the spleen, whereas the level of CD8+and CD4+CD25+Foxp3+ cells in the spleen and CD4+T cells in MLN was significantly increased. Orally adminis- tration with NIV at 5–15 mg/kg body weight did not change body and organ weight in NIV-treated mice but markedly increased dead lymphocytes showing characteristic of apoptosis in thymus, Peyer's patches, and spleen (Poapolathep et al., 2002). Furthermore, NIV selective damaged CD4+CD8+cells in thymus of mice receiving NIV at 15 mg/ kg body weight (Poapolathep et al., 2003) and in mouse thymocyte primary cultures treating with NIV at the dose levels of 0.25, 0.5 and 1.0 μg/ml (Poapolathep et al., 2004). An enhancement of IL-4 production was noticed in spleen cell cultures from mice exposed chronically to NIV at 8.87 mg/kg body weight (Gouze et al., 2007). Nevertheless, 10–100 μM NIV stimulates apoptosis mediated by caspase-3 and associated with a cell cycle blocking in G0/G1 phase in J774A.1 macrophages (Marzocco et al., 2009). For FX mycotoxin, apoptosis induction was observed in human promyelocytic leukemia cell line (HL-60 cells) exposed to 0.1–0.5 μg/ml FX for 24–72 h by stimulating cytochrome c release followed by the activation of multiple caspases (Miura et al., 2002). Similarly, oral exposure to FX at 0.1–0.5 mg/kg body weight for 14 days clearly induced apoptosis in lymphoid tissues of FX-treated mice (Aupanun et al., 2015) by an effect on *Bax*, *Bid*, *Trp 53*, and *Caspase-9* through mitochondrial apoptotic pathway (Aupanun et al., 2016). These data indicate that trichothecenes can act as im- munostimulatory or immunosuppressive depending on dose, exposure frequency and timing of functional immune assay (Bondy and

Pestka, 2000; Pestka et al., 2004). Thus, the immunomodulatory function of trichothecenes is regulated by a balance between cell-survival and death-signalling pathways (Wu et al., 2017). At molecular level, ribotoxic stress response has been considered as central to trichothecene effects (Pestka et al., 2004; Pierron et al., 2016). This capacity causes the inhibition of protein synthesis in eu- karyotic cells (Ueno, 1983). Trichothecenes binding to ribosomes ra- pidly activate mitogen-activated protein kinases (MAPKs) (Pestka et al., 2004). There are three MAPK subfamilies: (i) extracellular signal regulated protein kinase 1 and 2 (ERK1 and ERK2), (ii) p54, p46 and c- Jun N-terminal kinase 1 and 2 (JNK1/2), and (iii) p38 (Pestka, 2008). These mechanisms are important transducers of downstream signalling events related to immune response and apoptosis (Pestka et al., 2004). Furthermore, DON can induce ER stress by increasing protein expres- sion of two major ER stress markers ATF3 and DDIT3 (Katika et al., 2015). The overexpression of ATF3 and DDIT3 could result in cell cycle arrest and/or apoptosis (Liao et al., 2018). Several investigators have documented the generation of oxidative stress as a result of DON treatment, which may result in damage to proteins, lipids, and DNA (Mishra et al., 2014; Katika et al., 2015). In addition, the transcriptome analysis revealed that FX and DON down-regulate the peroxisome proliferator-activated receptor (PPAR) and liver X receptor - retinoid X receptor (LXR-RXR) signalling pathways that control lipid metabolism (Alassane-Kpembi et al., 2017a). The interference of lipid homeostasis is linked to immunity. LXR regulates ATP-binding cassette (ABC) A1 expression in macrophages which relates to the formation of foam cells and the subsequent onset of atherosclerotic lesions (Venkateswaran et al., 2000; Alassane-Kpembi et al., 2017a).

Regarding to natural co-incidence, toxic interaction between my- cotoxins is of increasing concern. The toxicity of mixtures is complex and cannot be predicted based on their individual effects (Alassane- Kpembi et al., 2013, 2015). Combination of toxins may result in ad- ditive, synergistic, or antagonistic effects. Furthermore, the legal limits of exposure have been established taking into account only the presence of individual mycotoxins. In the EU, guidance values for DON in feed materials are 0.9–12 mg/kg (EC, 2006). Nevertheless, the regulatory limits have yet been set for NIV, FX, and multi-mycotoxin. For these reasons, it is necessary to determine the risk of toxicological interaction derived from mycotoxins to human. The aim of the present study was to determine the nature of toxicological interaction among three major trichothecenes type B (DON, NIV, and FX) on the immune cells. Indeed, due to commonly contaminated in foodstuffs, DON was assessed its toxicological interaction with FX and/or NIV which provoke high toxic potency in animal models. For this purpose, interactions between two- or three-toxin mixtures were examined using the calculation of com- bination index and the dose reduction index by Chou-Talalay method (Chou, 2006). In addition, the induction of apoptosis was also assessed in single, binary and ternary toxins treated groups when compare to control. Although the studies of toxin interaction in animal models are more reliable than that of *in vitro* tests but they require large sample size and time consumption. In the present study, human Jurkat T cells were representative as target immune cells which further extrapolate the toxic interaction of trichothecenes type B in animal models and predict potential adverse human health effects.

Materials and methods

Reagents

Standard of DON (296.319 g/mol, purity: 98.0+% HPLC), NIV (312.318 g/mol, purity: 98.0+% HPLC), and FX (354.355 g/mol, purity: 95.0+% HPLC) were purchased from Wako Pure Chemical Industries Ltd. (Kyoto, Japan), dissolved in 10% dimethyl sulfoxide (DMSO) to 1 mM of stock solutions, and maintained at −20 °C in the dark. Working dilutions were prepared in growth medium and kept at 4 °C in the dark until used. The final concentration of dimethyl sulf- oxide (DMSO) in the cell culture media was less than or at a maximum of 0.4% (v/v). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Annexin V-FITC apoptosis detection kit were ob- tained from Sigma-aldrich (Missouri, USA).

Cell culture

Jurkat T cells (clone E6-1 (ATCC® TIB-152TM), passage 15) were kindly provided by Associate Professor Dr. Sutatip Pongcharoen, Faculty of Medicine, Naresuan University (Phitsanulok, Thailand). The cells were maintained in 75-cm^2 culture flasks in growth medium consisted of RPMI-1640 medium with L-glutamine supplemented with 10% (v/v) fetal bovine serum (Gibco Life Technologies, Rockville, MD), 100 U/ml penicillin and 100µg/ml streptomycin (Gibco Life Technologies, Rockville, MD), at 37 °C in an atmosphere of 5% CO_2/ 95% air.

Determination of cell viability

Jurkat T cells were seeded in flat bottom 96-well microplates at a density of 1×10^5 cells/well. Cells were treated with five dilutions of each individual mycotoxin, DON (0.25, 0.5, 1, 2, and 4 µM), NIV (0.15, 0.3, 0.6, 1.2, and 2.4 µM) and FX (0.05, 0.1, 0.2, 0.4, and 0.8 µM). In the present study, ranged concentrations were selected from pre- liminary experiments. The control group was treated with 0.4% DMSO in cell culture media. Jurkat T cells were exposed to five dilutions of each binary and tertiary mycotoxin combinations. The mycotoxin mixtures were prepared with a fixed constant ratio as follows: FX + NIV, ratio = 1:3; FX + DON, ratio = 0.2:1; NIV + DON, ratio = 0.6:1; FX + NIV + DON, ratio = 0.2:0.6:1. These ratios were calculated from preliminary individual cytotoxicity studies which pro- vided a similar toxicity to be obtained for each toxin. Five dilutions of individual mycotoxin, the combinations and control were carried out on three independent experiments in triplicate.

MTT assay was performed for determination of cell viability. After 48 h, cells of each well received 10 µl of MTT solution, 5 mg/ml MTT in RPMI-1640 without phenol red, and incubated for 3 h at 37 °C under darkness. The resulting MTT formazan crystals were dissolved in 0.1 N HCl in anhydrous isopropanol. The absorbance was measured at a wavelength of 595 nm using a Bio-Rad iMarkTM microplate reader.

The percentage of viable cells was obtained by the following formula:

$$Cell\ viability\ (\%) = 100\ x\ \frac{Mean\ OD\ of\ mycotoxin\ (s)\ treated\ samples}{Mean\ OD\ of\ vehicle\ treated\ samples}$$

Mycotoxin interactions by Chou Talalay method

The dose-response relationships for individual and combined my- cotoxins were established simultaneously by using the Median-Effect Equation of the Mass Action Law (Chou, 2006) which is provided by

$$f_a/f_u = (D/D_m)^m$$

where D is the concentration of the toxin, D_m is the median-effect concentration (e.g., IC50), f_a is the fraction affected by D, f_u is the fraction unaffected (i.e., $f_u = 1 - f_a$), and m is the coefficient signifying the shape of the dose-effect relationship ($m = 1$, $m > 1$, $m < 1$ in- dicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curve, respectively).

The combination index (CI) and dose reduction index (DRI) values were used to determine the types of interaction when DON, NIV, and FX are in combination. The Computer software Compusyn was used to clarify the mycotoxin interactions (Combosyn Inc., Paramus, NJ, USA). The CI value is a parameter to quantify the degree of mycotoxin interaction. The CI index was calculated according to Chou (2006), as follows:

$$^n(CI)_x = \sum_{j=1}^{n} \frac{(D)j}{(Dx)j} = \frac{(Dx)1-n\ \{[D]j/\sum_{j=1}^{n}[D]\}}{(Dm)j\left\{\frac{(fax)j}{[1-(fax)j]}\right\}1/mj}$$

where $^n(CI)_x$ is the combination index for n toxins at x% inhibition, $(D_x)_{1\text{-}n}$ is the sum of the concentration of n toxins that exerts x% inhibition in combination, $\{[D]j/\sum_{j=1}^{n}[D]\}$ is the proportionality of concentration of each of n toxins that exerts x% inhibition in combination, and $(D_m)_j\{(f_{ax})_j/[1 - (f_{ax})_j]\}^{1/mj}$ is the concentration of each toxin alone that exerts x% inhibition. A CI near 1 indicates an additive effect, CI < 1 indicates a synergism, and CI > 1 indicates antagonism of the combined mycotoxins.

The DRI is a measure of how many-fold the dose of each drug in a synergistic combination may be reduced at a given effect level com- pared with the doses of each drug alone. The DRI value is given by converting the CI equation:

$$^{n}(\mathrm{CI})_{x}= \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

FACScan flow cytometer analysis

Jurkat cells were seeded at a density of 1 × 10^6 cells/ml in 6-well tissue culture plate. Regarding to the induction of apoptosis by trichothecenes depends on dose, cells were exposed to each individual mycotoxin or mycotoxin mixtures at IC_{75} as follows; 59 μM for DON, 28.5 μM for NIV, 14.8 μM for FX, 67 μM for FX + DON, 109.2 μM for NIV + DON, 188.8 μM for FX+NIV, and 352 μM for FX + NIV + DON. In the present study, the concentration was selected based on preliminary study. Cells treated with 0.4% DMSO in cell culture media served as control. After 48 h of treatment, the cell sus- pensions were collected into 15 ml centrifuge tube, centrifuged at 3000×*g* for 5 min at 4 °C to pellet cells, and then washed twice with PBS and re-suspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/ml. Each cell suspension was incubated in 5 μl of Annexin V-FITC conjugate, and 10μl of Propidium Iodide solution at room temperature for 10 min. After incubation, the cells' fluorescence was immediately determined by flow cytometry using a BD FACSCantoTM II flow cytometer (Becton Dickinson). Thirty thousand events were collected per sample.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) of three independent experiments in triplicate. The differences between groups were compared using one-way ANOVA analysis and the Tukey test for pairwise multiple comparisons. Statistical analysis of data was carried out using GraphPad Prism version 5.0. (GraphPad Software, Inc. CA, USA). A *p*-value less than 0.05 was considered statistically significant. The CI and DRI values were calculated by the Computer software Compusyn.

Results

Cytotoxicity of individual and combined mycotoxins on Jurkat T cells

The inhibition of cell viability was determined by MTT assay after 48 h of exposure of DON, NIV, and FX alone or in combination. Fig. 1 show the viability (%)-concentration (μM) plot of binary and ternary mycotoxin combinations in Jurkat T cells at 48 h of exposure, compared to the concentration-response curve of each mycotoxin when applied individually in the same assay. At 48 h, DON, NIV and FX decreased proliferation of Jurkat T cells in concentration-dependent manner. Similarly, binary and ternary combinations of DON, NIV, and FX also decreased cell viability with increasing mycotoxin concentrations (Fig. 1). At low concentrations [DON (0.25–1 μM), NIV (0.15–0.6 μM), FX (0.05–0.2 μM)], mycotoxin combinations had a higher inhibition of cell viability than that of mycotoxin alone. The combination of DON + NIV exhibited more cytotoxicity than NIV alone, while the DON + FX mixtures resulted in greater cytotoxic effects than FX. The binary combination between NIV and FX also exhibited a higher toxi- city to Jurkat cells than individual mycotoxin. Moreover, the three- toxin mixtures inhibited cell viability stronger than single FX. Never- theless, all combinations were not significantly reduced cell viability when compared to individual mycotoxin at the highest concentrations [DON (4 μM), NIV (2.4 μM), FX (0.8 μM)].

The computer software Combosyn was used to calculate the para- meters of the dose-effect relationships for the toxicity of the tested mycotoxins. The parameter D_m, *m*, and *r* of the mycotoxin combinations are presented in Table 1. In the present study, all the concentration-response curves showed good linear correlation coefficients ($r > 0.9$). Following the shape parameter, the *m* values indicated flat sigmoidal shape for all the dose-effect plots ($m < 1$). The slopes of individual mycotoxins had steeper dose-response curves than that of mycotoxin combinations. Regarding to dose-effect curves and *m* values, mycotoxin combinations tend to be less cytotoxicity than single toxins when concentration increases. According to IC_{50} (D*m*) in Table 1, the cyto- toxic effect decreased in the following order: FX > NIV > DON > DON+FX > NIV+FX > DON+NIV > DON+NIV+FX.

Analysis of mycotoxin interaction

The combination index/fraction affected (*fa*-CI) curves for DON, NIV, and FX in combination are presented in Fig. 2. The straight lines on the graphs display an additive effect, with points above and below the lines indicating antagonistic and synergistic effects, respectively. The combination of DON and NIV was associated with nearly additive to strong antagonism for higher doses. DON and FX interacted with moderate antagonism whatever the cytotoxic degrees. For NIV and FX, the type of interaction varied according to tested concentrations. For the combination of the three toxins, an antagonism was observed at all cytotoxic effects (Fig. 2).

The DRI values estimate how many-fold the dose of each mycotoxin in combination may be reduced at a desired cytotoxic level compared with the doses of each toxin alone. The values > 1 and < 1 indicate supportable and not supportable dose-reduction, respectively. Nevertheless, DRI equal to 1 represents no dose-reduction. In order to discern the activity between DON, NIV, and FX, DRI values were pre- sented for 10%, 20%, and 30% cytotoxicity in Table 2. For binary combination between DON and NIV, the DRI values were more than 1 at 10%–30% cytotoxicity indicating a dose reduction for a given ef- fect. For NIV and FX mixtures, the DRI values decreased ranging from 12.28 to 1.82at 10–30% cytotoxicity which corresponded to the CI values. For the tertiary mixture, the reduction indices of FX decreased from 3.69 to 2.94 whereas DRI values for DON and NIV were less than 1 at 10%–30% cytotoxicity levels indicating a favorable dose-reduction of FX in the three-toxin mixture.

Apoptosis assessment

To assess the apoptosis of Jurkat T cells in control and all treatment groups (individual, binary or ternary combinations), annexin V-FITC/PI double staining was performed by FACscan flow cytometry. Treated cells were exposed to mycotoxins, at IC_{50} and IC_{75} for 48 h. At IC_{50}, all mycotoxin treatment, except for binary mixtures between DON and NIV, increased the proportion of both early and late apoptotic cells (data not shown). An increased proportion of early and late apoptotic cells was clearly observed after mycotoxin treatment at IC_{75} (Fig. 3). The percentages of early apoptotic cells in all treatment groups were significantly different when compared to control group. However, there was no significant difference in the proportion of apoptotic cells be- tween the different treated groups (Fig. 4).

4. Discussion

The immune system is a primary target for trichothecenes (Bondy and Pestka, 2000). When compared to other tested cell lines, Jurkat T cells showed the highest sensitivity to DON (Nielsen et al., 2009). Katika et al. (2015) proposed that immune cells are more sensitive than other cell types when treated with DON due to calcium leakage from the ER leads to induction of T-lymphocyte activation responses. They were thus selected as *in vitro* model to observe the toxicological inter- action among three major members of Type B trichothecenes (DON, NIV, and FX).

MTT assay was used to estimate the proliferation of Jurkat T cells after 48 h of exposure to the toxins alone or in combination. The test is based on enzymatic reduction of tetrazolium by cytosolic dehy- drogenases and reducing agents results in the formation of water in- soluble formazan product. The generation of tetrazolium crystals is the proportionality of reductive activity of living cells. However, various reports proposed that MTT assay might not be the best cell enumeration assay because of its limitations such as interference with certain gly- colysis inhibitors, interactions with many phytochemicals and poly- phenols, and correlation between the glucose concentration of the cell culture medium and the reductive rate of MTT (van Tonder et al., 2015; Pascua-Maestro et al., 2018). Nevertheless, the test is a popular tool in assessing the cell proliferation, viability and cytotoxicity, due to being sensitive, fast, and simple screening procedure. Nowadays, a variety of tetrazolium salts were developed in order to enable more reliable, continuous measurements of the progress of their reduction (Grela et al., 2018).

For the median-effect dose, individual mycotoxin displayed the low IC_{50} when compared to binary and ternary combinations. FX showed the lowest IC_{50} values (1.90 ± 1.51 μM). These

findings suggest that Jurkat T cells were sensitive to single toxin and FX was the most cytotoxic agent. These finding are in accordance with those obtained previously. Alassane-Kpembi et al. (2013) revealed that FX is more toxic than NIV and NIV is higher toxic than DON in human colon adenocarcinoma (Caco-2) cells. In addition, NIV is more powerful than DON in inducing cytotoxic effects on J774A.1 macrophage (Marzocco et al., 2009).

The toxicity of mycotoxin mixtures is complicated and cannot be accurately predicted on the basis of the effect of mycotoxin alone (Alassane-Kpembi et al., 2013, 2015; Wan et al., 2013). The interaction of mycotoxin mixtures on different cell types are presented in Table 3. In the present study, the combined toxicity for DON and NIV in Jurkat T cells was nearly additive to strong antagonistic effects for higher my- cotoxin doses. Conversely, synergistic effect was noticed in the mixtures of DON and NIV in IPEC-1 and Caco-2 cells (Alassane-Kpembi et al., 2013, 2015), but no interaction in J774.A1 macrophages (Marzocco et al., 2009) and porcine whole blood (Luongo et al., 2008). The binary mixtures by addition DON and FX was moderate antagonism in Jurkat T cells which was in agreement with the previous studies in IPEC-1 cells (Alassane Kpembi et al., 2015). However, DON and FX mixtures ex- hibited synergistic effects in Caco-2cells (Alassane-Kpembi et al., 2013). For NIV and FX, we observed different interactions based on cytotoxic levels in Jurkat T cells. These observations are in accordance with those reported in Caco-2 cells (Alassane-Kpembi et al., 2013), but additivity was presented in IPEC-1 cells (Alassane-Kpembi et al., 2015). In the tertiary combination of DON, NIV, and FX, strong antagonism were observed in Jurkat T cells at all affected levels, whereas the pre- vious studies in Caco-2cells showed antagonistic to additive effect based on tested concentrations (Alassane-Kpembi et al., 2013). These finding supported that the effects of mycotoxin mixtures differ in the mixtures of mycotoxins, ratio and/or concentrations for each myco- toxin combination, type of cell lines, as well as the method used to determine the dose-effect relationship.

Trichothecenes can act as immunostimulatory or im- munosuppressive depending on dose, exposure frequency and timing of functional immune assay (Bondy and Pestka, 2000; Pestka et al., 2004). In this study, the comparison of apoptosis induction after individual, binary, or ternary combinations of DON, NIV, and FX exposure on Jurkat T cells was also assessed at IC_{50} and IC_{75} levels by FACscan flow cytometry. At IC_{50}, all mycotoxin treatment groups, except the combi- nations between DON and NIV, clearly increased early apoptotic cells when compared to control (data not shown). Nevertheless, the per- centages of early apoptotic cells in all treatment groups at IC_{75} were significantly different when compared to control group but there were no significantly different of early apoptotic cell proportion among treatment groups. These finding indicated high concentrations of tri- chothecenes alone or in combination increase leucocytes apoptosis.

The mechanisms underlying mycotoxins interaction are complex. In the case of our study, we observed the interactions between three members of 8-ketotrichothecenes. Their structures consist of epoxy- trichothecene nucleus with the presence of hydroxyl or acetyl groups at appropriate positions. Nevertheless, we noted the different types of interaction among varied mixtures on different cytotoxicity levels. Synergistic effects can occur when one toxin increases the absorption or decreases the elimination of the other (Cavaliere et al., 2005; Wan et al., 2013). In the present study, synergism was observed in the combinations of NIV and FX at low levels of cytotoxicity. This action may explain that NIV or FX alter the absorption or elimination of an- other at lower graded toxicity. These hypotheses deserve to be further investigated. By contrast, antagonism may occur when toxins compete each other for the same target or receptor site (Ruiz et al., 2011; Wan et al., 2013). The involvement of cellular active transport systems could be speculated the differences of toxicological interactions among type B trichothecenes mycotoxins (Alassane-Kpembi et al., 2015). In our ex- periment, the most interactive effects of binary and ternary combina- tions of DON, NIV, and FX in tested concentrations were antagonism. Although they have a similar structure, these three toxins may complete each other for the same transport protein on Jurkat T cells. However, the further investigation is needed.

Conclusion

Our results clearly demonstrate the toxicological interaction among three major members of type B trichothecenes to immune cells. The mainly antagonistic effects have been observed. Their simultaneous presence in agricultural commodities may be less immunotoxicity than the basis of individual mycotoxin. Nevertheless, toxicity of the toxins to immune cells is complex and single cell line is insufficient to address their toxic interaction. Thus, further investigation in multi cell types is needed to understand the correlation and molecular mechanisms underlying these interactions. Meanwhile, the finding in the present study should be benefit to enable an appropriate assessment of health risk effects in the co-occurrence of mycotoxins in foodstuffs.

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References

Alassane-Kpembi, I., Gerez, J.R., Cossalter, A.M., Neves, M., Laffitte, J., Naylies, C., Lippi, Y., Kolf-Clauw, M., Bracarense, A.P.F.L., Pinton, P., Oswald, I.P., 2017a. Whole-transcriptome analysis reveals specific signaling pathways for the type B trichothe- cene mycotoxin fusarenon-X. Sci. Rep. 7, 7530.

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F.A., Oswald, I.P., Puel, O., 2013. New insights into mycotoxin mixtures: the toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. Toxicol. Appl. Pharmacol. 272, 191–198.

Alassane-Kpembi, I., Oswald, I.P., Puel, O., 2015. Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetylated derivatives in intestinal epithelial cells. Arch. Toxicol. 89, 1337–1346.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O., Oswald, I.P., 2017b. Mycotoxins co-contamination: methodological aspects and biological relevance of combined toxicity studies. Crit. Rev. Food Sci. Nutr. 16, 3489–3507.

Aupanun, S., Phuektes, P., Poapolathep, S., Sutjarit, S., Giorgi, M., Poapolathep, A., 2016. Apoptosis and gene expression in Jurkat human T cells and lymphoid tissues of fusarenon-X-treated mice. Toxicon 123, 15–24.

Aupanun, S., Poapolathep, S., Imsilp, K., Prapong, T., Poapolathep, A., 2015. Oral ex- posure of fusarenon-X induced apoptosis in Peyer's patches, thymus, and spleen of mice. Res. Vet. Sci. 102, 217–222.

Berthiller, F., Sulyok, M., Krska, R., Schuhmacher, R., 2007. Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. Int. J. Food Microbiol. 119, 33–37.

Bondy, G.S., Pestka, J.J., 2000. Immunomodulation by fungal toxins. J. Toxicol. Environ. Health B 3, 109–143.

Cavaliere, C., Foglia, P., Pastorini, E., Samperi, R., Laganà, A., 2005. Development of a multiresidue method for analysis of major *Fusarium* mycotoxins in corn meal using liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 19, 2085–2093.

Chou, T.C., 2006. Theoretical basis, Experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681.

Del Ponte, E.M., Garda-Buffon, J., Badiale-Furlong, E., 2012. Deoxynivalenol and niva- lenol in commercial wheat grain related to *Fusarium* head blight epidemics in southern Brazil. Food Chem. 132, 1087–1091.

European Commission, 2006. Commission regulation no 576/2006 of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fu- monisins in products intended for animal feeding. Off. J. Eur. Union L 229.

Food Safety Commission of Japan (FSCJ), 2010. Risk Assessment Report Deoxynivalenol and Nivalenol (Mycotoxin). Risk Assessment Report – Veterinary Medicines FS/872/ 2010.

Gouze, M.E., Laffitte, J., Pinton, P., Dedieux, G., Galinier, A., Thouvenot, J.P., Loiseau, N., Oswald, I.P., Galtier, P., 2007. Effect of subacute oral doses of nivalenol on immune and metabolic defence systems in mice. Vet. Res. 38 (4), 635–646 Epub 2007 Jun 13.

Grela, E., Kozłowska, J., Grabowiecka, A., 2018. Current methodology of MTT assay in bacteria – a review. Acta Histochem. 120, 303–311.

Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology 167, 101–134.

Islam, M.R., Roh, Y.S., Kim, J., Lim, C.W., Kim, B., 2013. Differential immune modulation by deoxynivalenol (vomitoxin) in mice. Toxicol. Lett. 221, 152–163.

Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2001. Safety Evaluation of Certain Mycotoxins in Food. Food and Agriculture Organization, Rome, Italy, pp. 281–320.

Katika, M.R., Hendriksen, P.J., van Loveren, H., Peijnenburg, A.C.M., 2015. Characterization of the modes of action of deoxynivalenol (DON) in the human Jurkat T-cell line. J. Immunot. 12, 206–216.

Kovalsky, P., Kos, G., Nährer, K., Schwab, C., Jenkins, T., Schatzmayr, G., Sulyok, M., Krska, R., 2016. Co-occurrence of regulated, masked and emerging mycotoxins and secondary metabolites in finished feed and maize—an extensive survey. Toxins 8, E363. https://doi.org/10.3390/toxins8120363.

Liao, Y., Peng, Z., Chen, L., Nüssler, A.K., Liu, L., Yang, W., 2018. Deoxynivalenol, gut microbiota and immunotoxicity: a potential approach? Food Chem. Toxicol. 112, 342–354.

Luongo, D., de Luna, R., Russo, R., Severino, L., 2008. Effects of four *Fusarium* toxins (fumonisin B1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation. Toxicon 52, 156–162.

Marin, S., Ramos, A.J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60, 218–237.

Marzocco, S., Russo, R., Bianco, G., Autore, G., Severino, L., 2009. Pro-apoptotic effects of nivalenol and deoxynivalenol trichothecenes in J774A.1 murine macrophages. Toxicol. Lett. 189, 21–26.

Matejova, I., Vicenova, M., Vojtek, L., Kudlackova, H., Nedbalcova, K., Faldyna, M., Sisperova, E., Modra, H., Svobodova, Z., 2015. Effect of the mycotoxin deox- ynivalenol on the immune responses of rainbow trout (*Oncorhynchus mykiss*). Vet. Med. Czech 60, 515–520.

Mayer, E., Novak, B., Springler, A., Schwartz-Zimmermann, H.E., Nagl, V., Reisinger, N., Hessenberger, S., Schatzmayr, G., 2017. Effects of deoxynivalenol (DON) and its microbial biotransformation product deepoxy-deoxynivalenol (DOM-1) on a trout, pig, mouse, and human cell line. Mycotoxin Res. 33, 297–308.

Mishra, S., Dwivedi, P.D., Pandey, H.P., Das, M., 2014. Role of oxidative stress in Deoxynivalenol induced toxicity. Food Chem. Toxicol. 72, 20–29.

Miura, K., Aminova, L., Murayama, Y., 2002. Fusarenon-X induced apoptosis in HL-60 cells depends on caspase activation and cytochrome c release. Toxicology 172, 103–112.

Montes, R., Segarra, R., Castillo, M.A., 2012. Trichothecenes in breakfast cereals from the Spanish retail market. J. Food Comp. Anal. 27, 38–44.

Nielsen, C., Casteel, M., Didier, A., Dietrich, R., Märtlbauer, E., 2009. Trichothecenes- induced cytotoxicity on human cell lines. Mycotox. Res. 25, 77–84.

Ok, H.E., Lee, S.Y., Chun, H.S., 2018. Occurrence and simultaneous determination of nivalenol and deoxynivalenol in rice and bran by HPLC-UV detection and im- munoaffinity cleanup. Food Control 87, 53–59.

Pascua-Maestro, R., Corraliza-Gomez, M., Diez-Hermano, S., Perez-Segurado, C., Ganfornina, M.D., Sanchez, D., 2018. The MTT-formazan assay: complementary technical approaches and *in vivo* validation in *Drosophila* larvae. Acta Histochem. 120, 179–186.

Payros, D., Alassane-Kpembi, I., Pierron, A., Loiseau, N., Pinton, P., Oswald, I.P., 2016. Toxicology of deoxynivalenol and its acetylated and modified forms. Arch. Toxicol. 90, 2931–2957.

Pestka, J.J., 2008. Mechanisms of deoxynivalenol-induced gene expression and apoptosis. Food Addit. Contam. 25, 1128–1140.

Pestka, J.J., 2010. Toxicological mechanisms and potential health effects of deox- ynivalenol and nivalenol. World Mycotoxin J. 3, 323–347.

Pestka, J.J., Uzarski, R.L., Islam, Z., 2005. Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: role of mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. Toxicology 206, 207–219.

Pestka, J.J., Zhou, H.R., Moon, Y., Chung, Y.J., 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol. Lett. 153, 61–73.

Pierron, A., Mimoun, S., Murate, L.S., Loiseau, N., Lippi, Y., Bracarense, A.P.F.L., Schatzmayr, G., He, J., Zhou, T., Moll, W.D., Oswald, I.P., 2016. Microbial biotransformation of DON: molecular basis for reduced toxicity. Sci. Rep. 6, 29105.

Poapolathep, A., Kumagai, S., Suzuki, H., Doi, K., 2004. Development of early apoptosis and changes in T-cell subsets in mouse thymocyte primary cultures treated with ni- valenol. Exp. Mol. Pathol. 77, 149–152.

Poapolathep, A., Nagata, T., Suzuki, H., Kumagai, S., Doi, K., 2003. Development of early apopotosis and changes in lymphocyte subsets in lymphoid organs of mice orally inoculated with nivalenol. Exp. Mol. Pathol. 75, 74–79.

Poapolathep, A., Ohtsuka, R., Kiatipattanasakul, W., Ishigami, N., Nakayama, H., Doi, K., 2002. Nivalenol – induced apoptosis in thymus, spleen and Peyer's patches of mice. Exp. Toxicol. Pathol. 53, 441–446.

Ruiz, M.J., Macáková, P., Juan-García, A., Font, G., 2011. Cytotoxic effects of mycotoxin combinations in mammalian kidney cells. Food Chem. Toxicol. 49, 2718–2724. Streit, E., Nährer, K., Rodrigues, I., Schatzmayr, G., 2013. Mycotoxin occurrence in feed
and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. J. Sci. Food Agric. 93, 2892–2899.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P., 2012. Current situation of mycotoxin con- tamination and co-occurrence in animal feed-focus on Europe. Toxins 4, 788–809.

Ueno, Y., 1983. Trichothecenes - Chemical, Biological and Toxicological Aspects. Kodansha and Elsevier Science, Tokyo, Japan.

van Tonder, A., Joubert, A.M., Cromarty, A.D., 2015. Limitations of the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when com- pared to three commonly used cell enumeration assays. BMC Res. Notes 8 (47), 1–10.

Venkateswaran, A., Laffitte, B.A., Joseph, S.B., Mak, P.A., Wilpitz, D.C., Edwards, P.A., Tontonoz, P., 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. Proc. Natl. Acad. Sci. U.S.A. 97 (22), 12097–12102.

Wan, L.Y.M., Turner, P.C., El-Nezami, H., 2013. Individual and combined cytotoxic ef- fects of *Fusarium* toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1) on swine jejunal epithelial cells. Food Chem. Toxicol. 57, 276–283.

Wu, Q., Wang, X., Nepovimova, E., Miron, A., Liu, Q., Wang, Y., Su, D., Yang, H., Li, L., Kuca, K., 2017. Trichothecenes: immunomodulatory effects, mechanisms, and anti- cancer potential. Arch. Toxicol. 91, 3737–3785.

Wu, W., Bates, M.A., Bursian, S.J., Link, J.E., Flannery, B.M., Sugita-Konishi, Y., Watanabe, M., Zhang, H., Pestka, J.J., 2013. Comparison of emetic potencies of the 8-ketotrichothecenes deoxynivalenol, 15-aetyldeoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, and nivalenol. Toxicol. Sci. 131 (1), 279–291.

Wu, W., Flannery, B.M., Sugita-Konishi, Y., Watanabe, M., Zhang, H., Pestka, J.J., 2012. Comparison of murine anorectic responses to the 8-ketotrichothecenes 3- acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X and nivalenol. Food Chem. Toxicol. 50, 2056–2061.

Table 1. The Parameters of Dose-Effect Relationship for DON, NIV, FX and Their Combinations in Jurkat T Cells

Mycotoxin	Dose-effect parameters		
	D_m (μM)	*m*	*r*
DON	4.70 ± 6.15	0.79	0.9247
NIV	3.35 ± 3.57	0.92	0.9668
FX	1.90 ± 1.51	0.99	0.9922
DON + NIV	6.98 ± 4.71	0.67	0.9469
DON + FX	5.56 ± 9.14	0.80	0.9269
NIV + FX	5.28 ± 8.46	0.50	0.9827
DON + NIV + FX	24.19 ± 8.66	0.46	0.9909

D_m (median-effect concentration or IC_{50}), *m* (slope), *r* (coefficient of linear correlation). D_m and *m* are used for calculating the CI values.

Table 2. The Combination Index and Dose Reduction Index Values for Viability Inhibition by DON, NIV, and FX in Jurkat T Cells

Mycotoxin	Combination ratio	10% cytotoxicity		20% cytotoxicity		30% cytotoxicity	
		CI	DRI	CI	CRI	CI	DRI
DON	1:0.6	0.98	1.80	1.17	1.60	1.34	1.49
NIV			2.34		2.06		1.90
DON	1:0.2	1.65	0.83	1.47	0.85	1.38	0.87
FX			0.97		1.48		1.95
NIV	3:1	0.46	6.17	0.63	2.96	0.79	1.82
FX			12.28		5.56		3.29
DON	1:0.6:0.2	2.29	0.62	2.2	0.69	2.19	0.74
NIV			0.81		0.89		0.95
FX			3.69		3.21		2.94

CI = 0.9–1.1, CI < 0.9, and CI > 1.1 indicate and additive effect, a synergism, and an antagonism, respectively. DRI > 1 and < 1 indicate supportable and not supportable dose-reduction; DRI = 1 represents no dose-reduction. All CI and DRI values are calculated using a computer software.

Table 3. Interaction between DON, NIV, and FX on different cell types.

Mycotoxin	Cell model	Mycotoxin Concentration (μM)	Combination Effects	Reference
DON + NIV	Caco-2	DON (0.25–4), NIV (0.2–3.2); ratio 1:0.8	Synergism	Alassane-Kpembi et al (2013)
	J774.A1	DON (10–100), NIV (10–100); ratio 1:1	No interaction	Marzocco et al (2009)
	IPEC-1	DON (0.2–15), NIV (0.2–15); ratio 1:1	Synergism	Alassane-Kpembi et al (2015)
	Porcine whole blood	DON (0.0625–1), NIV (0.0625–1); ratio 1:1	No interaction	Luongo et al (2008)
DON + FX	Caco-2	DON (0.25–4), FX (0.0075–0.12); ratio 1:0.3	Synergism	Alassane-Kpembi et al (2013)
	IPEC-1	DON (0.2–15), FX (0.12–9); ratio 1:0.8	Antagonism	Alassane-Kpembi et al (2015)
NIV + FX	Caco-2	NIV (0.2–3.2), FX (0.0075–0.12); ratio 1:0.04	Synergism to additive	Alassane-Kpembi et al (2013)
	IPEC-1	NIV (0.2–15), FX (0.16–12); ratio 1:0.8	Additive	Alassane-Kpembi et al (2015)
DON + NIV + FX	Caco-2	DON (0.25–4), NIV (0.2–3.2), FX (0.0075–0.12); ratio 1/0.8/0.03)	Antagonism to additive	Alassane-Kpembi et al (2013)

FIGURES

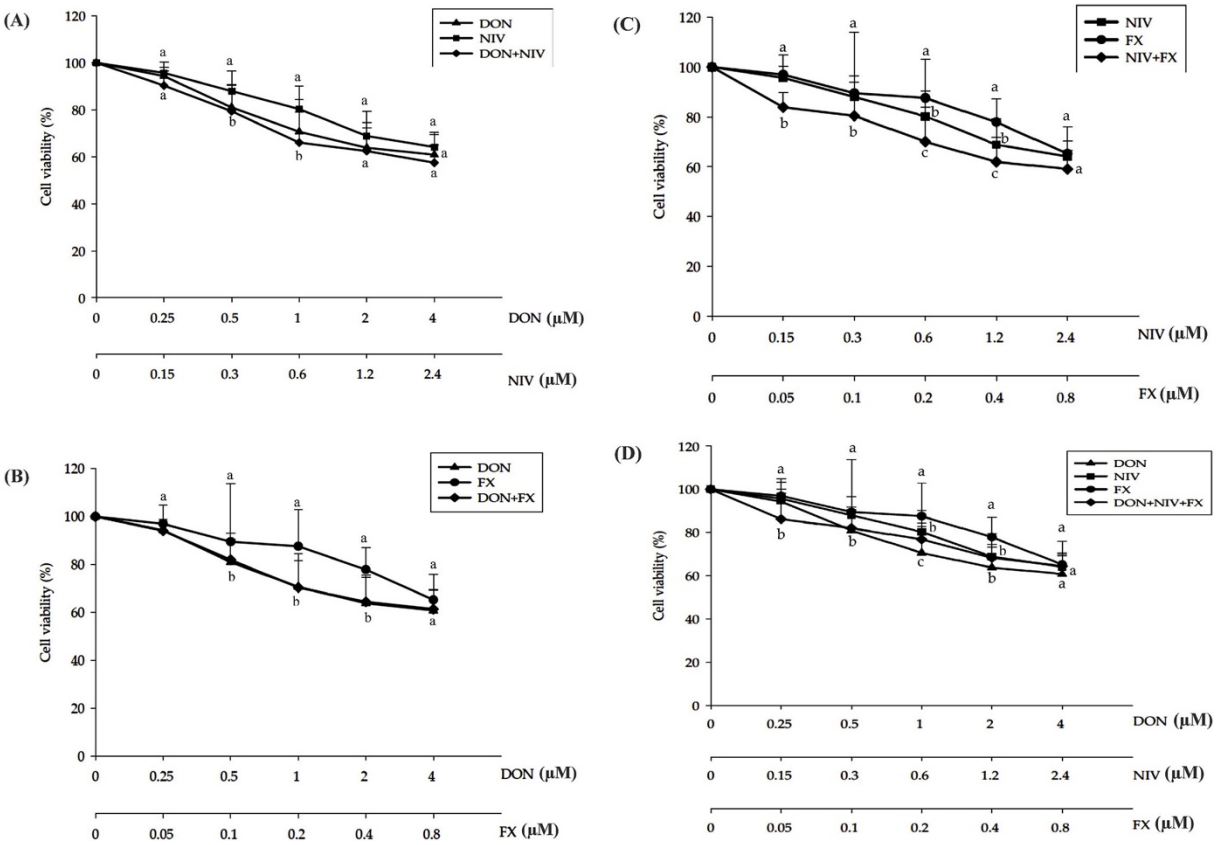

Fig. 1. Dose-response curves of individual and combinations of DON, NIV, and FX after 48 h exposure in Jurkat T cells. DON (—▲—), NIV ( ), FX (—●—), and mycotoxin combinations (—◆—). FX + NIV, ratio = 1:3; FX + DON, ratio = 0.2:1; NIV + DON, ratio = 0.6:1; FX + NIV + DON, ratio = 0.2:0.6:1. Values are expressed as the percentage of cell viability of control cells and represent the mean ± SD of three independent experiments. Data in the same combinations labeled with different letters represent significant difference ($p < 0.05$).

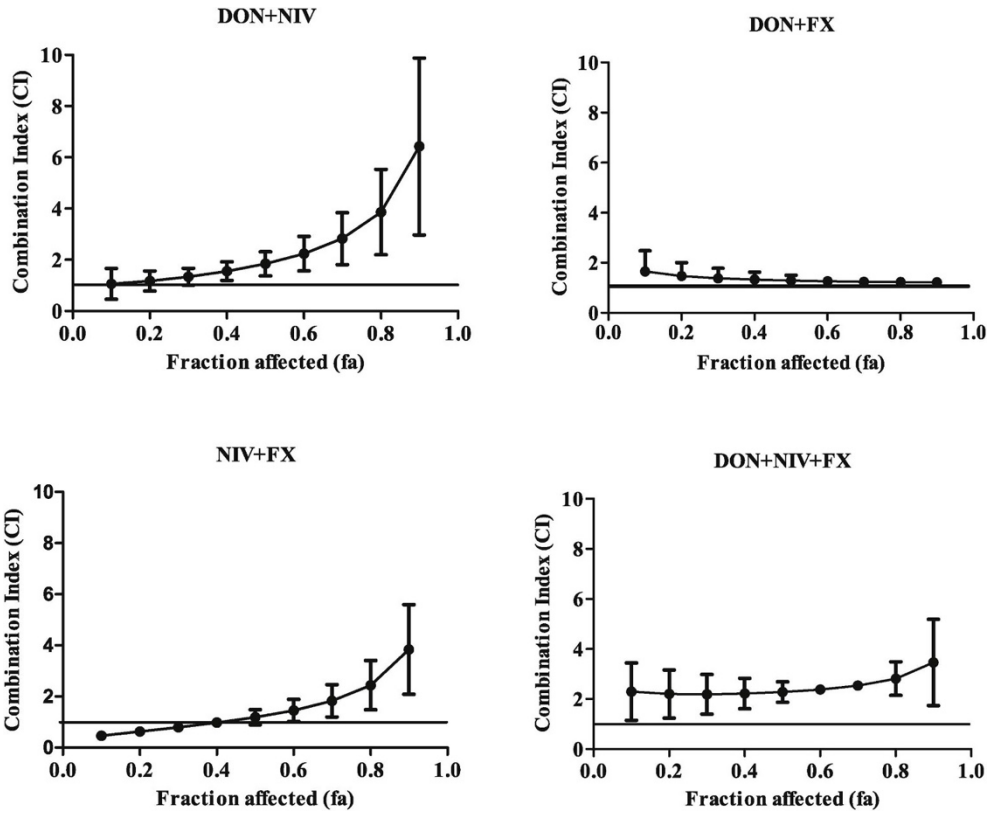

Fig. 2. Combination index/fraction affected (*fa*-CI) curve as described by Chou and Talalay model (Chou, 2006) for Jurkat T cell exposed DON, NIV, and FX binary and ternary mixtures. Each point represents the CI ± SD at a fractional effect as determined in our experiments. The line (CI=1) indicates additive effect, the area above the line antagonism, and the area under the line synergism.

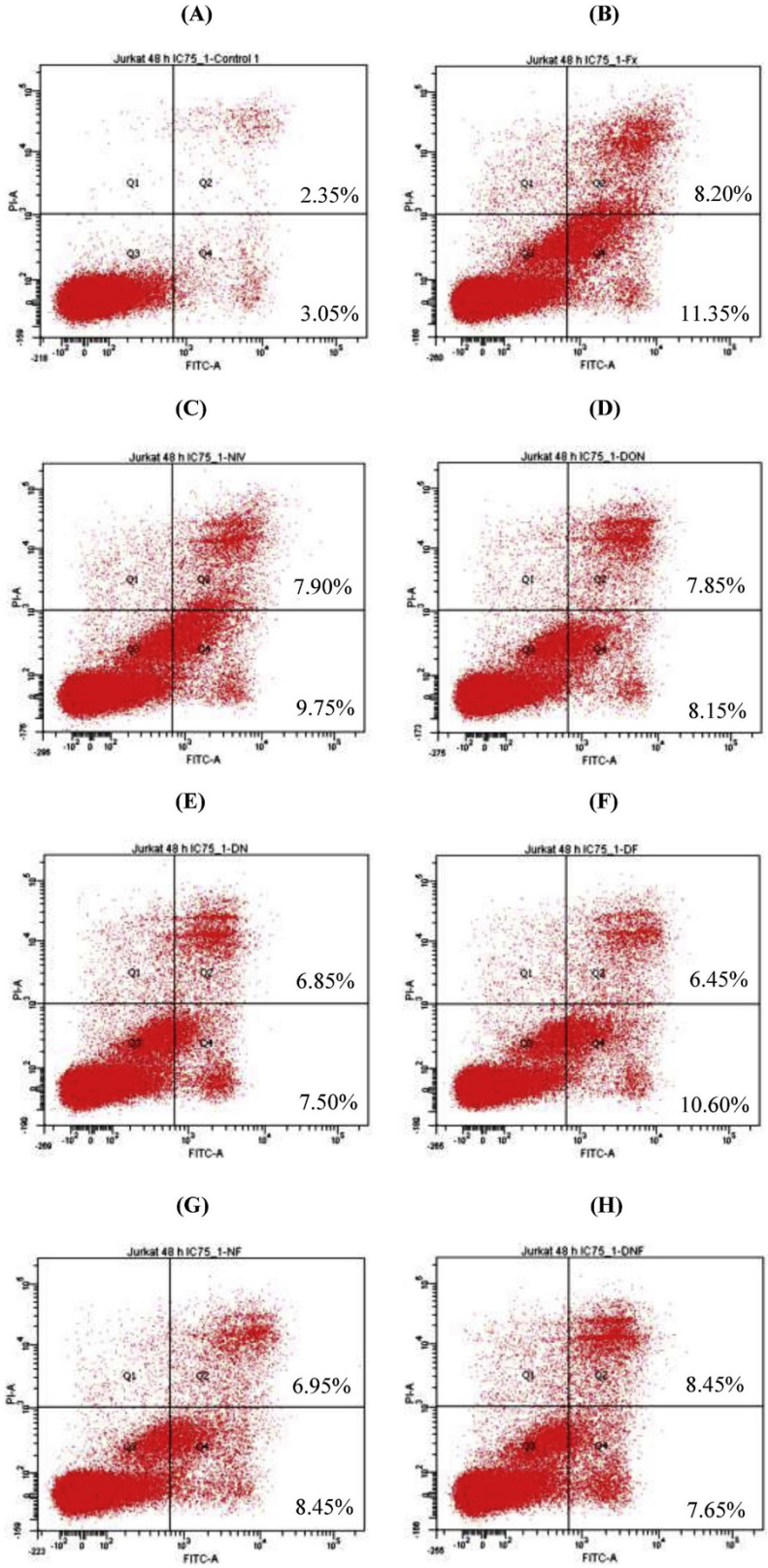

Fig. 3. Apoptosis assessment by flow cytometry in Jurkat cells after mycotoxin exposure at IC75 for 48 h (A) control (B) FX (C) NIV (D) DON (E) DON + NIV (F) DON + FX (G) NIV + FX (H) DON + NIV + FX.

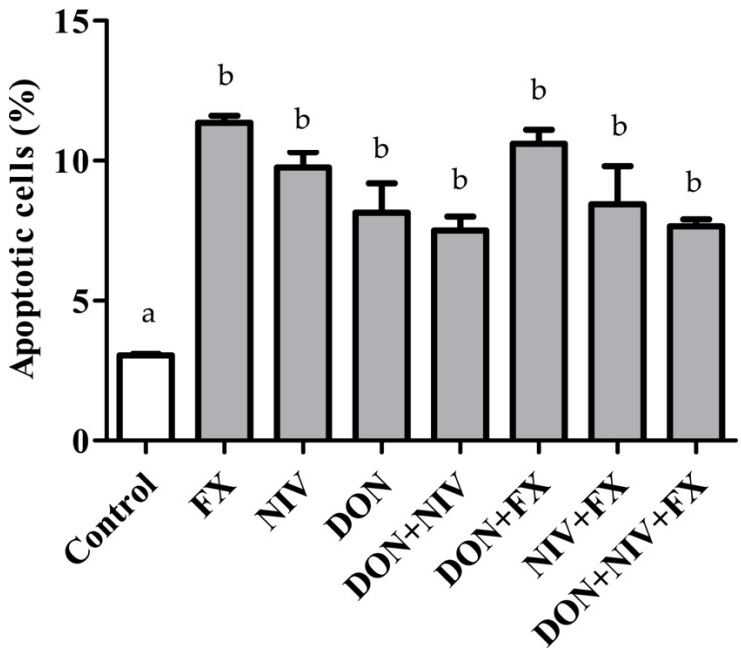

Fig. 4. The percentage of early apoptotic cells (Q4) after single, binary, or ternary treatment at IC_{75} when compared to control group. These results are mean ± SD of data reported in Q4 of Fig. 3. Data labeled with different letters represent significant difference ($p < 0.05$).