Modulation Of Fumonisin B_1 Toxic Action-Induced By Zeralenone In Human Intestinal Cells Caco-2

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Abstract: The natural co-occurrence of *Fusarium* toxins fumonisin B_1 (FB_1) and Zearalenone (ZEA) in cereal grains is well known. However, a few studies have been reported that address to the toxicity of *Fusarium* toxins mixtures. Thus, the aim of the present study was to investigate in the interactive effect of binary *Fusarium* toxins FB_1 and ZEA on intestinal cells line Caco-2 using several cellular endpoints such as caspace-3 activity modulation, malonedialdehyde (MDA) production, lactate dehydrogenase (LDH) leakage as necrosis measure and cells viability as evaluated by lysosome and mitochondria integrities. As results, ZEA + FB_1 led to an antagonistic effect on lysosome and mitochondria damage but, the necrosis-induced was more potent when compared to ZEA or FB1 alone showing an additive effect. Moreover, MDA production induced by ZEA+FB_1 was higher than additive effect but not synergistic effect. At last, the combined effect of toxins on key apoptosis enzyme caspace-3 kinetic activity was an additive effect after 6 h and 24 h but, after 3h, ZEA tended to exert its anti-apoptotic action by reducing the enzyme activation by FB_1. Taken together**,** the results were contrasted and results suggested that combined effects of binary *Fusarium* toxins ZEA and FB_1 in cell line Caco-2 were unpredictable and varied according to several parameters such as the cellular endpoints and the duration of cells incubation with toxins.

Index Terms: *Fusarium*-toxins, interactive-effect, caspace-3-kinetic-activity, cytotoxicity, oxidative-stress

1 INTRODUCTION

Zearalenone (ZEA) and Fumonisin B1 (FB1) are secondary metabolites of some toxigenic species of *Fusarium* genera. They pose a health risk not only to humans but also to livestock and, as a consequence, may cause economical losses either by unfavorable effects on domestic animals themselves or by an increased potential for health effects in human beings from consuming mycotoxin-contaminated edible animal products. Fumosinsin B1 causes liver and kidney cancer, and neural tube defects in rodents, leukoence-phalomalacia in horses and pulmonary oedema in pigs [1]. Of major concern is the association of FB1 with elevated incidence of human oesphageal cancer in parts of South Africa, North Eastern Iran and China, upper gastrointestinal tract cancer in Northern Italy [2, 3] and neural tube defects in human babies [4]. The structures of FB_1 and sphingolipids show marked similarities [5], which may be the reason why FB1 drastically disrupts the normal sphingolipid metabolism leading to an intracellular accumulation of sphingoid bases (mainly sphinganine relative to sphingosine), which mediate several key biological processes including inhibition of protein, DNA synthesis and apoptosis caspace-3 dependant [5-9]. In addition, FB1 also induces lipid peroxidation in Vero cells [6], in primary rat hepatocytes [8] in C6 glioma cells [9] and human intestinal Caco-2 cells [10]. On the other hand, ZEA have estrogenic and anabolic activities in several species (rodents, pigs and monkeys), being able to cause alterations in the reproductive tract of laboratory animals [11]. ZEA is associated with outbreaks of precocious pubertal changes in children in Puerto Rico, and has been suggested to have a possible involvement in human cervical cancer [11]. Cellular mechanism of ZEA has been described by its high binding affinity to oestrogen receptors [12] and DNA adducts formation in female mousse tissues and carcinogenic disorders [11]. It has also been reported that ZEA inhibits DNA and protein synthesis and induces oxidative stress mediated cell death [11-14]. Consequently, the intracellular generation of reactive oxygen species (ROS) by ZEA is likely responsible for its cytotoxic and genotoxic effects [11, 13]. ZEA causes cells death by apoptosis via caspase-independent and mitochondria /AIF-mediated pathways [15] with a key role of activations of p53 and JNK/p38 [16]. ZEA and FB1 could be found in the same commodities as secondary metabolites of different *Fusarium* species [17]. For example, co-occurrence of *Fusarium* mycotoxins FB1 and Zearalenone ZEA had been found in rice, maize and peanut from West Africa countries such as Côte d'Ivoire [18]. Thus, in preliminary study on possible interactive effect of combination of FB1 and ZEA, we had reported an antagonistic cytotoxic effect as measured only by cell lysosome integrity but an additive effect on lipid peroxydation [10]. In order to understand the best possible interactive effect of these *Fusarium* toxins, we investigated in their combined effect regarding several cellular endpoints including the key apoptosis enzyme caspase-3 kinetic activity, lactate dehydrogenase (LDH) release as necrosis measure, lipid peroxydation, lysosome and mitochondria damage.

2 MATERIAL AND METHODS

2.1 Material

Chemicals: ZEA and FB_1 were obtained from Sigma Chemical Company (St Louis, MO, USA) and were dissolved in ethanol/water (90:10). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS) and neutral red (NR) solution were provided from Sigma-Aldrich (Saint Quentin Falavier, France). All other chemicals used were of analytical grade.

Cells culture and treatment: Caco-2 cells, a human colon cancer cell line, were obtained from Dr. Jing Yu, Tufts School of Medicine (Medford, MA, USA) [19]. The cells were grown as monolayer culture in a high glucose concentration (4.5 g/l) DMEM medium supplemented with 10% foetal calf serum (FCS), 8mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100µg/ml) incubated at 37 1C in an atmosphere of 5% CO2—95% air mixture. For cell counting and subculture, the cells were dispersed with a solution of 0.05% trypsin and 0.02% EDTA.

2.2 Methods

Cytotoxicity assay by NR test: The NR test was performed to assess cell viability, as previously described [10, 13]. Viable cells actively transport this dye across their cell membrane by the system Endosome-lysosome; therefore, after subsequent lyses absorbance can be used as a measure of cell viability. The solution stock of NR (3.3 g/l) was diluted to 1/100 in the cell culture medium and the extract solution consisted of 50%

(v/v) ethanol in Milli-Q water with 1% (v/v) acetic acid. After 72 h of incubation in presence of each mycotoxin alone and their mixture (ZEA+FB_1) or the vehicle, 150 ml of freshly prepared NR solution pre-warmed to 37°C was added to each well and all plates returned to the incubator at 37°C for 4 h. The cells were washed two times and 150 µl of the extract solution were added in each well and plates were shaken for 15 min. The absorbance at 540 nm was determined using a Microplate Reader DYNATECH MR 4000.

Cytotoxicity assay by MTT test: MTT test was second test used to assess cell viability based on the capacity for viable cells to metabolise a tetrazolium colourless salt to a blue formazan in mitochondria [10, 13]. After 72 h of incubation in presence of toxins ZEA, FB_1 and their mixture or the vehicle, 100 µl of 0.5% solution of thiazolyl blue tetrazolium bromide (MTT) were added to each well and 2 h later the medium was eliminated. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.

Cytotoxicity assay by Lactate dehydrogenase measure: The LDH assay was performed to assess cytotoxicity or cell death by necrosis, as previously described [20]. Caco-2 cells (1 x 105 cells/ml/well) were preincubated in 24-well multidishes for 24 h at 5% CO2 – 95% air at 37°C. Cell viability was assessed by LDH leakage through the membrane into the medium. After 48 h of incubation in presence of mixture of toxins (ZEA and FB1) or the vehicle, cells supernatant were used for the presence of LDH by LDH assay kit (Biomerieux, Lyon, France). In this test, three wells were used for each mycotoxin (ZEA and FB_1) and their mixture. The amount of LDH measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford [21]. Leakage was expressed as percentage difference from controls.

Extraction and determination of malonedialdehyde (MDA)–thiobarbituric acid (TBA) adduct: Cells (105 cells/ml) were cultured in 24-well multidishes (Polylabo, France) for 24 h at 37°C as described above, and then cultures were incubated in the presence of each mycotoxin alone and their mixture ZEA+FB_1 for 24 h at 37°C. After this incubation, cells were trypsinised, centrifuged and resuspended in SET buffer (0.1M NaCl, 20mM EDTA, 50mM Tris–HCl, pH 8.0). As previously described [6, 13], extraction and determination of the malonedialdehyde (MDA)–thiobarbituric acid (TBA) adduct by HPLC and fluorimetric detection after extraction in n-butanol (50 µl injected for analysis) was performed. The amount of MDA measured is related to the protein content of cellular homogenates, determined using the colorimetric method of Bradford [21].

Caspace-3 activity assay: The assay was performed according to the manufacturer's instructions, ***Promega, USA.*** After 3, 6 or 24 h of incubation at 37°C in the presence of each mycotoxin alone or their mixture ZEA+FB_1, cells (106 cells/ml) were disrupted by incubation ice-cold lysing buffer for 10 min and then centrifuged at 15,000 x g for 20 min. Supernatants (cell extracts containing caspase-3) were retrieved and 50 µl aliquots (100-200 µg total protein) along with Ac-DEVD substrate labeled with the chromophore p-nitrianiline (*p*NA) were added in a 96-well flat bottomed microplate. In presence of active caspase-3, cleavage and release of *p*NA from substrate occurs. Free *p*NA produced a yellow color that can be detected by spectrophotometer Microplate Reader DYNATECH MR 4000 at 405 nm. Additional controls, some free from cell lysates and others lacking substrate, were included. The results were expressed as caspase-3 specific activity (IU/mg protein).

Statistical analysis of data: The data are expressed as mean standard deviation (SD) for at least three independent determinations in triplicate or quadruplicated for each experimental point. The statistical analyze was carried out using a non- parametric statistical test, Mann–Whitney test for significance of differences. Acceptable limit is set from $p < 0.05$* to $p < 0.01$** [22].

3 Results

3.1 Cells viability and necrosis:

Cells viability: Fig.1 showed the viability of Caco-2 cells measured by MTT test after incubation of either individual mycotoxin FB_1 or ZEA or their mixture and the results were diminishing of cell viability. ZEA (10µM) reduced slightly cell viability by about 20–25%, which is lower than 10µM FB_1 does (35-38%). Thus, ZEA appears weakly cytotoxic. Strikingly, FB_1 and ZEA mixed did not affect cell viability higher than FB_1 alone. Indeed, combined effect observed of these toxins is lower than expected additive effect of their mixture. Fig.2 showed Caco-2 cells viability by Neutral Red test. Cells incubation with mycotoxin FB_1 or ZEA alone or their mixture revealed reduction of cell viability. But, the induction of cell viability induced by FB_1 (10µM) is low i.e. only by about 10–15%. ZEA (10µM) reduced Caco-2 cells viability by about 23-25%. Surprisingly, addition of FB_1 to ZEA leads to reduction of cell viability similarly to those induced by FB_1 alone.

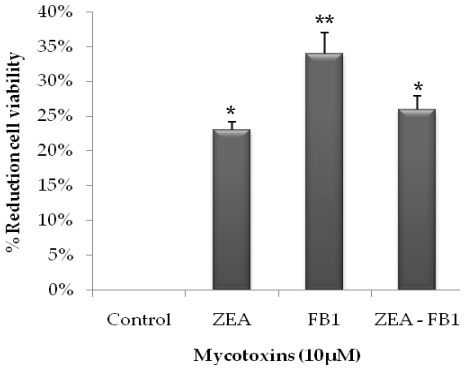

Fig. 1. Cytotoxicity effect of ZEA (10µM) or FB_1 (10µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by MTT test. Results are given as mean ± SD from three independent experiments. .**Different from control at p < 0.05 and **p < 0.001*.

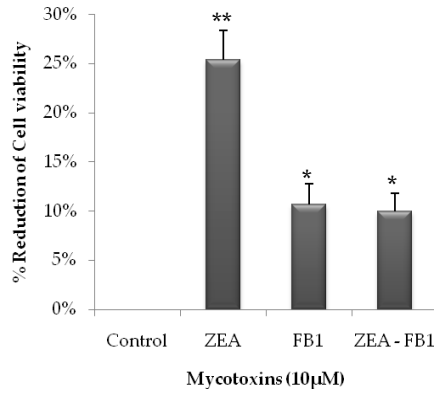

Fig. 2. Cytotoxicity effect of ZEA (10µM) or FB_1 (10µM) or their mixture on Caco-2 cells after 72 h incubation evaluated by Neutral Red test. Results are given as mean ± SD from three independent experiments. .**Different from control at p < 0.05 and **p < 0.001*.

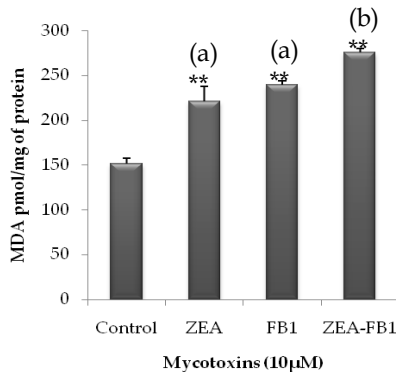

Fig.4. Increasing of MDA production after 24 h incubation of ZEA (10µM) or FB_1 (10µM) or their mixture with Caco-2 cells. Results are given as mean ± SD from three independent experiments. .**Different from control at p < 0.05 and **p < 0.001*.

Cell necrosis: Fig. 3 showed necrosis resulted from incubation of cells Caco-2 with mycotoxins FB_1 or ZEA alone or their mixture. All toxins and their mixture increased leakage of LDH into the culture medium compared with controls. Effects produced by both *Fusarium* toxins were similar and the mixture induced higher increasing of leakage of LDH probably as additive effect.

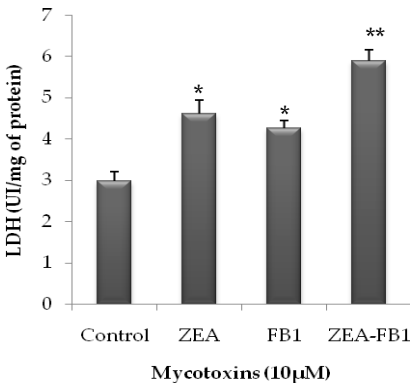

Fig. 3. Increasing of LDH leakage into the cell culture medium after incubation of Caco-2 cells with ZEA (10µM) or FB_1 (10µM) or their mixture by 24h. Results are given as mean ± SD from three independent experiments. .**Different from control at p < 0.05 and **p < 0.001*.

3.2 Oxydative stress:

Fig.4 showed the oxidative damage induced by ZEA, FB_1 or their mixture as measured by MDA production resulting from lipid peroxydation. ZEA and FB_1, all at a concentration of 10 µM, increased MDA production in Caco-2 cells by 33%, 36%, respectively. Their mixture also increased MDA production by about 80%. A rapid comparison between experimental values of MDA increases (80%) and theoretical values calculated from observed individual ones (33% + 36%) showed that the addition of ZEA and FB_1 increased MDA production in a more additive way.

3.3 Caspace-3 kinetic activity:

Fig.5 revealed the modulation of caspase-3 activation after ZEA (10µM) or FB_1 (10µM) or their mixture exposure on Caco-2 cells according to variable duration namely 3 h, 6 h and 24 h. ZEA (10µM) or FB_1 (10µM) or their mixture increased activity of caspase-3. But, the modulation of caspase-3 activity varied for the same toxin according to the duration of its incubation with Caco-2 cells. Thus, the activation of caspase-3 by ZEA increased proportionally to the duration of Caco-2 cells exposure. Indeed, ZEA was more potent at 24h > 6h > 3h. FB_1 also increased caspase-3 activity proportionally to the duration of cells exposure until 6h but decreased caspase-3 activation at 24h. The mixture of ZEA and FB_1 was more potent at 6 h and 24 h where compared to ZEA or FB_1 alone but at 3h of cells incubation, FB_1 alone appeared stronger than the mixture. Thus, the mixture of toxins leaded to additive effect at 6h and 24h of cells exposure but not at 3h where ZEA tended to reduce FB_1 effect-induced.

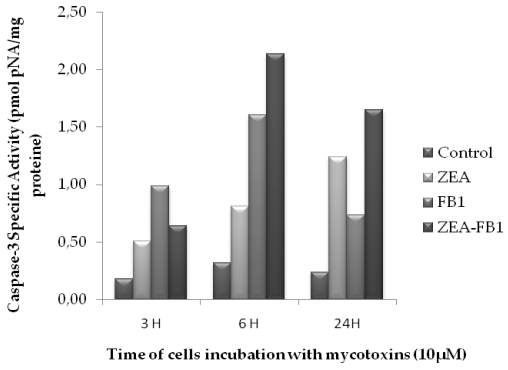

Fig. 5. Activation of caspase-3 after ZEA (10µM) or FB1 (10µM) or their mixture exposure on Caco-2 cells for variable duration namely 3 h, 6 h and 24 h.

4 Discussion

Concentrations of toxins ZEA and FB_1 used in the present study were 10µM for each mycotoxin and are those that allow distinguishing synergistic or antagonistic effects of toxins when used in mixture following previous data [10, 13]. Additionally, these concentrations are the concentrations that could be reached in animals or possibly in human tissues following ingestion of 2–4 mg/kg BW of FB_1 or ZEA in foods or feed. In previous studies focused on interactive effect of *Fusarium* toxins ZEA, FB_1 and Deoxynivalenol (DON), it had been reported that the cytotoxic effect as evaluated by DNA synthesis and cell membrane transport integrity of combination of ZEA and FB_1 seemed to be antagonistic effect in contrast to additive effect observed on lipid peroxydation and protein synthesis [10]. Such interactive effect must be clarified by using several cellular specific endpoints. Thus, the binary toxins had been tested on mitochondrial succinate dehydrogenase activity and the results revealed ZEA undoubtedly reduced FB_1 inhibition mitochondria enzyme-induced. Similarly, results in the present study have confirmed the antagonistic effect of ZEA and FB_1 mixture on cell lysosome damage [10]. In contrast to both anterior specific cell endpoints, the combination of ZEA and FB_1 produced an additive effect on leakage of lactate dehydrogenase (LDH), an enzyme marker of cell membrane damage or cells necrosis [23, 24]. These findings were surprising because the three cells endpoints namely lysosome integrity, mitochondrial succinate dehydrogenase activity and leakage of LDH have been always considered as makers of cells viability or cytoxicity [25-27]. But, the differences between cell damage pathways evaluated or measured in the three assays could explain such findings. Indeed, cell membrane leakage of LDH is known as a marker of necrosis which represents passive cell death without an underlying regulatory mechanism or activation of executive caspaces [28]. Thus, the amount of LDH release resulted from cell disorders induced by individual effect of each mycotoxin leading necrotic cell death. In contrast, membrane transport integrity and mitochondrial succinate dehydrogenase activity represent cell specific vital functions implying regulatory or organized mechanisms which also could be targets of toxicants [26, 27]. Previously, it had been reported that ZEA and FB_1 targeted the same cellular organelles namely mitochondria and/or lysosomes consequently authors have hypothesized that combinations of these toxins would lead to additive or synergistic effects [13]. Surprisingly, in the present study, it has been observed an antagonistic effect. In fact, the concentrations of mycotoxins could modulate their interactive effect leading to unexpected findings as previously reported on growth of yeast [29]. These authors [29] reported antagonism for low concentrations but synergy for high concentrations for mixture of ZEA and DON. Concerning lipid peroxydation, association of ZEA and FB_1 leaded to more additive effect as reported previously [10]. These findings traduce the capability of ZEA or FB_1 to produce reactive oxygen species (ROS) but the nature of ROS and the mechanism involved in their production remains unclear. However, it appears probable that FB_1 or ZEA induces ROS production in mitochondria and/or by inflammatory disorders involving TNF-α [7, 8] and the intracellular generation of ROS is likely or partly responsible for their cytotoxic and genotoxic effects [10, 14]. Although, both ZEA and FB_1 target the same cellular organelle i.e. mitochondria in the production of ROS [13], but any chemical interaction is not at stake. On the other hand, since ZEA or FB_1 had been shown to induce apoptosis caspace-3 dependent [7], it appeared interesting to test their possible interactive effect on caspase-3 activity modulation. The present study confirmed clearly modulation of caspase-3 activity induced by ZEA or FB_1 [9]. It had been reported that FB_1 modules caspase-3 activity by several pathways involving mitochondria disturbs following by cytochrome-c release and cell membrane TNF-R1 receptor activation by TNF-α [30]. Concerning ZEA, studies reported that ZEA induced apoptosis caspase-3 dependant or not [14, 16] and apoptosis caspase independent induced by ZEA could be related to AIF-mediated and ROS-dependent pathways, in which p53 and JNK/p38 MAPK play crucial roles as upstream effectors [31]. In the present study, FB_1 has been showed to induce early apoptosis caspase-3 dependent in contrast to ZEA which was very potent later until after 24 h of cells incubation. In contrast to FB_1, ZEA could module caspase-3 activity by long processes. The mixture of toxins leaded to additive effect on caspase-3 activation at 6h and 24h of cells exposure in contrast to 3h of incubation where FB_1 alone is stronger than the mixture. In fact, ZEA tended to counterbalance FB_1-caspase-3 activity modulation by a probable mechanism of functional antagonism which contrasted with additive effect observed subsequently. The effect of mixture of ZEA and FB_1 on caspase-3 activity was unpredicted or in the present study, this effect is linked to the duration of cells incubations with mycotoxins.

5 Conclusion

Fusarium toxin ZEA or FB_1 alone had been found to cause cytotoxicity, oxidative damage as measured by MDA production and key apoptosis enzyme caspase-3 kinetic activity modulation. That indicated their capability to target the same cell endpoints but caspase-3 activity modulation was precocious with FB_1 when compared to ZEA. Despite the same targets, the combined effect of ZEA and FB_1 on cells Caco-2 seemed to be unpredictable. ZEA antagonized FB_1 toxic action on mitochondria and lysosome integrities but enhanced its effect in necrosis, apoptosis and oxidative damage. Since ZEA reduced or enhanced FB_1 caspase-3 activation-induced according to the duration of cells incubation with toxins, it was plausible that the interactive of binary ZEA and FB_1 varied according to several parameters such as the cellular endpoints and the duration of cells incubation with toxins.

References

[1] M.F. Dutton, “Fumonisins, mycotoxins of increasing importance: Their nature and their effects”, *Pharmacology and Theorapeutics,* vol. 70, no. 2, pp. 137–161, Oct. 1996.

[2] F.S. Chu and G.Y. Li, “Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People’s Republic of China in regions with high incidences of esophageal cancer,” *Applied and Environmental Microbiology*, vol. 60, no. 3, pp. 847-852, Mar. 1994.

[3] J.P. Rheeder, W.F.O. Marasas, P.G. Thiel, E.W. Sydenham, G.S. Shephard and D.J. Van Schalkwyk, “*Fusarium moniliforme* and fumonisins in corn in relation to human oesophageal cancer in Transkei,” *Phytopathology,* vol. 82, no. 3, pp. 353-857, Jan. 1992.

[4] K. Hendricks “Fumonisins and neural tube defects in South Texas,” *Epidemiology,* vol. 10, no. 2, pp. 198-200, Mar. 1999.

[5] S.C. Bezuidenhout, W.C.A. Gelderblom, C.P. Gorst-Allman, R.M. Horak, W.F.O Marasas, G. Spiteller and R. Vleggaar R, “Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme,” Journal of the Chemical Society,* Chemical Communications, 11, pp. 743–745, 1988.

[6] K. Abado-Bécognée, T.A. Mobio, R. Ennamany, F. Fleurat-Lessart, W.T. Shier, F. Badria and E.E. Creppy, “Cytotoxicity of fumonisin B1/implication of lipid peroxidation and inhibition of protein and DNA syntheses,” *Arch. Toxicol.*, vol. 72, no. 4, pp. 233–236, Mar. 1998.

[7] J.M. Soriano, L. Gonzalez and A.I. Catala, “Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B1,” *Prog Lipid Res.,* vol. 44, 6, pp. 345-356, Nov. 2005.

[8] N.V. Gopee and R.P. Sharma, “The mycotoxin fumonisin B1 transiently activates nuclear factor-kappaB, tumor necrosis factor alpha and caspase 3 via protein kinase Calpha-dependent pathway in porcine renal epithelial cells,” *Cell Biol Toxicol*. vol. 20, no. 4, pp. 197-212, Jul. 2004.

[9] T.A. Mobio, E. Tavan, I. Baudrimont, R. Anane, M.R. Carratu, A. Sanni, M.F. Gbeassor, T.W. Shier, J.F. Narbonne and E.E. Creppy, “Comparative study of the toxic effects of fumonisin B1 in rat C6 glioma cells and p53-null mouse embryo fibroblasts,” *Toxicology,* vol. 183, no. 1-3, pp. 65–75, Feb. 2003.

[10] J.H. Kouadio, D.S. Dano, S. Moukha, T.H. Mobio and E.E. Creppy “Effets of combination of Fusarium mycotoxins (deoxynivalenol, zearalenone and fumonisin B1) on Caco-2 cells viability, inhibition of macromolecules syntheses, malonaldehyde level and DNA methylation and fragmentation,” *Toxicon* vol. 49, no. 3, pp. 306 - 317, Mar. 2007.

[11] A. Zinedine, J-M. Soriano, J.C. Molto and J. Man, “Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin,” *Food and Chemical Toxicology*, vol. 45, no. 1, pp. 1–18, Jan. 2007.

[12] W.T. Shier, A.C. Shier, X. Xie and C.J. “Mirocha Structure–activity relationships for human oestrogenic activity in zearalenone mycotoxins,” *Toxicon* vol. 39, no. 9, pp. 1435–1438, Sep. 2001.

[13] J.H. Kouadio, T.H. Mobio, I. Baudrimont, S. Moukha, D.S. Dano and E.E. Creppy, “Comparative study of cytoxicity and oxidative stress induce by deoxynivalenol, zearalenone of fumonisin B1 in Human intestinal cell line Caco-2,”*Toxicology,* vol. 213, no 1-2, pp. 56-65, Feb. 2005.

[14] W. Hassen, I. Ayed-Boussema, A.A. Oscoz, A.C. Lopez and H. Bacha, “The role of oxidative stress in zearalenone-mediated toxicity in Hep G2 cells: oxidative DNA damage, gluthatione depletion and stress proteins induction,”*Toxicology,* vol. 232, no. 3, pp. 294–302, Apr. 2007.

[15] G.Y. Li and N.N. Osborne, “Oxidative-induced apoptosis to an immortalized ganglion cell line is caspase independent but involves the activation of poly (ADP-ribose) polymerase and apoptosis-inducing factor,” *Brain Research,* vol. 1188, pp. 35–43, Jan. 2008.

[16] L. Jia, Y.Y. Ji, S.L. Shin, O.S. Young, H.K. Dong, A.L. Seung, S. Xianglin and C.L. “Jeong, Cellular mechanisms of the cytotoxic effects of the zearalenone metabolites a-zearalenol and b-zearalenol on RAW264.7 macrophages,” *Toxicology in Vitro,* vol. 27, no. 3, pp. 1007–1017 , Apr. 2013.

[17] A.E. Desjardins, H.K. Manandhar, R.D. Plattner, G.G. Manandhar, S.M. Poling and C.M. Maragos, “*Fusarium* species from Nepalese rice and production of mycotoxins and gibberellic acid by selected species,” *Applied and Environ-mental Microbiology,* vol. 66, no. 3, pp. 1020-1025, Mar. 2000.

[18] B. Sangare-Tigori, S. Moukha, H.J. Kouadio, A.M. Betbeder, D.S. Dano and E.E. Creppy, “Co-occurrence of aflatoxin B1, fumonisin B1, ochratoxin A and zearalenone incereals and peanuts from Cote d'Ivoire” *Food Addit Contam.,* vol. 23, no. 10, pp. 1000-1007, Oct. 2006.

[19] M. Rousset, M. Laburthe, M. Pinto, G. Chevalier, C. Rouyer-Fessard, E. Dussaulx, G. Trugnan, N. Boige, J.L. Brun and A. Zweibaum, “Enterocytic differentiation and glucose utilisation in the human colon tumour cell line Caco-2: modulation by forskolin,” *J. Cell. Physiol.,* vol. 123, no. 3, pp. 377–385, Jun. 1985.

[20] A. Yusup , H. Upur , I. Baudrimont, A. Umar , T. Kader , B. Begaud , E.E. Creppy , and N. Moore, “Cytotoxicity of abnormal Savda Munziq aqueous extract in human hepatoma (HepG2) cells,” *Fundam Clin Pharmacol.*, vol. 19, no. 4, pp. 465-472, Aug. 2005.

[21] M.M. Bradford, “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding,”*Analytical Biochemistry*, vol. 72, pp. 248–254, May 1976.

[22] S.C. Gad and CS. Neil, Statistic Pharmaceutical analysis. *Pharm Biomed Anal,* vol. 33, no. , pp. 7-20, 2003.

[23] G. Fotakis and J.A. Timbrell, “In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride,” *Toxicology Letters*, vol. 160, no. 2, pp. 171–177, Aug. 2006.

[24] L. Galluzzi, S.A. Aaronson, J. Abrams, E.S. Alnemri and D.W. Andrews, “Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes,” *Cell Death Differ*, vol. 16 no. 8, pp. 1093–1107, Aug. 2009.

[25] H. Babich and F. Borenfreund, “Fathead minnow FHM cells for use in in vitro cytotoxicity assays of aquatic pollutants,” *Ecotoxicol Environ Saf.,* vol. 14, no. 1, pp. 78-87, Aug. 1987.

[26] B.E. Loveland, T.G. Jonhs, I.R. Mackay, F. Vaillant, Z.X. Wang and P.J. Hertzog, “Validation of the MTT dye assay for enumeration of cells in proliferative and antiproliferative assays,” *Biochem. Int.,* vol. 27, no. 3, pp. 501-510, Jul. 1992.

[27] S.M. Smith, M.B. Wunder, D.A. Norris and Y.A. Shellman Simple Protocol for Using a LDH-Based Cytotoxicity Assay to Assess the Effects of Death and Growth Inhibition at the Same Time. PLoS ONE vol. 6, no. 11, pp. e26908, Nov. 2011.

[28] A.H. Wyllie, J.F. Kerr and A.R. Currie, “Cell death: the significance of apoptosis,” *International Review of Cytology,* vol. 68, pp. 251–306, 1980.

[29] L.S. Boeira, J.H. Bryce, G.G. Stewart and B. Flannigan, “The effect of combinations of Fusarium mycotoxins (deoxynivalenol, zearalenon and fumonisin B1) on growth of brewing yeasts,” *J. Appl. Microbiol.,* vol.88, no. 3, pp. 388–403, Mar. 2000.

[30] A. Di Pietro, E.G. Vries, J.A. Gietema, D.C. Spierings and S. De Jong, “Testicular germ cell tumours: the paradigm of chemo-sensitive solid tumours, Review,” *Int J Biochem Cell Biol.* vol. 37, no. 12, pp. 2437-2456, Dec. 2005.

[31] J.Y. Yu, Z.H. Zheng, Y.O. Son, X. Shib, Y.O. Jang and J.C. Lee, “Mycotoxin zearalenone induces AIF- and ROS-mediated cell death through p53- and MAPK-dependent signaling pathways in RAW264.7 macrophages,” *Toxicology in Vitro,* vol. 25, no. 8, pp. 1654–1663, Dec. 2011.