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DIPLOMOVÁ PRÁCE

CYTOTOXICITY OF BEAUVERICIN, CITRININ, DEOXYNIVALENOL AND T-2 TOXIN BY *IN VITRO* METHOD USING VERO CELLS

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„This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature.”

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INDEX

ABSTRACT

Mycotoxins are secondary metabolites of moulds. Contamination of food and feed by mycotoxins is a major problem for human and animal health. Ingestion of mycotoxins may cause a range of toxic responses, from acute toxicity to long term or chronic health disorders. Several mycotoxins, either from the same or from different fungal species, occur simultaneously in plant products. However, its implication for food safety assessment is generally not known, as there is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence for the toxicity. Mycotoxins with similar mode of action would be expected to have at least additive effects. Conversely, some interactions could have subtractive effects. An understanding mode of action in simple *in vitro* systems can provide a rational basses for predicting interactions between mycotoxins. The aim of this study was to obtain cytotoxicity data (EC_{50} values) of *Penicillium* and *Fusarium* mycotoxins, nominally BEA, CIT, DON and T-2 toxin. For this purpose, Vero cells viability was evaluated in the presence of these four mycotoxins using the NR assay. All mycotoxins tested diminished cell viability in a concentration and incubation time-dependent manner on Vero cells. Individual mycotoxins increase cytotoxicity as follow: CIT< BEA< DON< T-2 toxin. To determine the mechanistic interactions of BEA, DON and T-2 toxin and their influence on cellular viability of Vero cells, were used the following combinations of mycotoxins: BEA in combination with DON, BEA in combination with T-2 toxin, DON in combination with T-2 toxin and combination of all three mycotoxins. All mixtures of mycotoxins tested reduced the viability of Vero cells. Our results indicate that the most cytotoxic effect on Vero cells was observed after the tertiary mixture of BEA, DON and T-2 toxin.

Key words: mycotoxins, Neutral red assay, Vero cells, cell viability, mechanistic interactions

ABSTRAKT

Mykotoxiny jsou sekundární metabolity plísní. Jimi kontaminované potraviny a krmení jsou velkou hrozbou pro lidské zdraví a dobrý stav chovaného zvířectva. Požití mykotoxinů může způsobit celou řadu závažných zdravotních problémů, od akutní otravy až po dlouhodobé a chronické poruchy zdraví. Nicméně jejich význam z hlediska ovlivnění bezpečné výživy není všeobecně znám, protože existuje jen malé množství informací o vzájemné interakci mykotoxinů a jejich toxicitě. Předpokládá se, že mykotoxiny se stejným mechanismem účinku budou mít aditivní efekt, naopak, některé jejich interakce mohou toxicitu snižovat. Pochopení působení mykotoxinů v *in vitro* systémech nám může pomoci v předpovídání výsledku jejich vzájemného působení. Cílem této studie bylo získat cytotoxická data (hodnoty EC_{50}) toxinů z rodů *Penicillium* a *Fusarium*, jmenovitě mykotoxinů BEA, CIT, DON a T-2 toxinu. Za tímto účelem byla stanovena životaschopnost Vero buněk v přítomnosti těchto čtyř mykotoxinů za použití testu cytotoxicity pomocí neutrální červeně. Všechny testované mykotoxiny snižovaly životaschopnost buněk v závislosti na koncentraci a času inkubace. Jednotlivé mykotoxiny redukovaly životaschopnost Vero buněk v následujícím pořadí: CIT< BEA< DON< T-2 toxin. Abychom otestovaly vzájemné interakce zkoumaných mykotoxinů a jejich vliv na životaschopnost buněk, byly použity následující kombinace: BEA v kombinaci s DON, BEA v kombinaci s T-2 toxinem, DON v kombinaci s T-2 toxinem a kombinace všech tří mykotoxinů. Všechny použité směsi snižovaly životaschopnost buněk. Naše práce vedla k závěru, že nejtoxičtější účinek vykázala kombinace mykotoxinů BEA, DON a T-2 toxinu.

Klíčová slova: mykotoxin, test cytotoxicity, neutrální červeň, Vero buňky, životnost buněk, interakce mykotoxinů

ABBREVIATIONS

AFB1 – aflatoxin B1

BEA – beauvericin

CTN – citrinin

DNA – deoxyribonucleic acid

DON – deoxynivalenol

EC_{50} – half maximal effective concentration

FB1 – fumonisin B1

NR – neutral red

OTA – ochratoxin

PAT – patulin

SD – standard deviation

ZEA – zearalenone

1. INTRODUCTION

1.1 MYCOTOXINS

Mycotoxins are secondary metabolites of moulds. To date, more than 300 mycotoxins are known and their effects have, at least partially, been characterized. These toxins are of great interest with regard to human and animal health since according to the estimations of the United Nation Food and Agriculture Organization, approximately 25% of world food production is contaminated with at least one mycotoxin.[1]

All mycotoxins are low-molecular-weight natural products. These metabolites constitute toxigenically and chemically heterogeneous assemblages that are grouped together only because the members can cause a disease and death in humans' beings and other vertebrates. Not surprisingly, many mycotoxins display overlapping toxicities to invertebrates, plants, and microorganisms.[2]

Contamination of food and feed by mycotoxins is a major problem for human and animal health. Ingestion of mycotoxins may cause a range of toxic responses, from acute toxicity to long term or chronic health disorders. Acute disorders such as toxic mould syndrome, pulmonary mycotoxicosis or organic dust toxic syndrome have been described.[3]

For chronic disorders, an association exists between exposure to the mycotoxin ochratoxin A and the kidney disease Balkan endemic nephropathy, the symptoms of which are tumors of the kidney and urinary tract, as well as between the inhalation of aflatoxin and human cancer. The mycotoxins may also act as immunosuppressive agents and may increase disease susceptibility.[3]

In order to demonstrate that a disease is a mycotoxicosis, it is necessary to show a dose-response relationship between the mycotoxin and the disease. For human populations, this correlation requires epidemiological studies. Supportive evidence is provided when the characteristic symptoms of a suspected human mycotoxicosis are evoked reproducibly in animal models by exposure to the mycotoxin in question. Human exposure to mycotoxins is further determined by environmental or biological monitoring. In environmental monitoring, mycotoxins are measured in food, air, or other samples, in biological monitoring, the presence of residues, adducts, and metabolites is assayed directly in tissues, fluids, and excreta.[2]

Mycotoxins usually enter the body via ingestion of contaminated food, but inhalation of toxigenic spores and direct dermal contact are also important exposure routes. Depending on growth conditions several mycotoxins can be produced by one mould, e.g., *Aspergillus* and *Penicillium* species can simultaneously produce citrinin, gliotoxin, ochratoxin A and patulin.[3]

1.2 MYCOTOXINS PRODUCED BY PENICILLIUM AND FUSARIUM FUNGI SPECIES

1.2.1 Fusarium toxins

Aflatoxin, ochratoxin A, patulin and the *Fusarium* toxins are well–investigated mycotoxins because of their prevalence in agricultural commodities. For three decades now there has been intensive research into mycotoxins, but recently, special attention has been paid to fusarial toxins produced mostly by fungus of genus *Fusarium*. Plant fusariose is recognized as a great agricultural problem, due to the variety of mycotoxins *Fusarium* species can produce, including trichothecenes, zearalenone and fumonisins, which have been identified as important contaminants in foodstuffs.[4]

Several *Fusarium* mycotoxins are often found in combination in infested cereal grains. However, a few studies have been reported that address the toxicity of *Fusarium* toxin mixtures on human cells lines, although most of these studies were focused on binary mixtures only.[4]

Fusarium spp. may be found on cereals grown under temperate climate, and produce a number of mycotoxins of the class of trichothecenes and other toxins such as zearalenone and fumonisin. The trichothecenes and zearalenone are predominantly produced by members of the *Fusarium spp*. namely *F. graminearum*, *F. sporotrichoides* and *F. equiseti*.

The trichothecenes are a group of tricyclic sesquiterpenes, consisting of a shared 12,13-epoxy-trichothec-9-ene ring system. The diversity of these particular mycotoxins are derived by an initial cyclisation of the head to tail linkage of three isoprene units, farnesyl pyrophosphate to trichodiene, followed by a numerous species specific oxygenations, isomerisations, cyclisations and esterifications, which gives rise to both type A and type B trichothecenes. The type A trichothecenes included T-2 toxin and HT-2 toxin. The type B trichothecenes are deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON, nivalenol (NIV) and 4-acetyl NIV.[5]

1.2.1.1 Deoxynivalenol (DON)

One of the trichothecenes – DON produced abundantly by *Fusarium graminearum,* when ingested, can induce a decrease in food intake or refusal to eat food, vomiting, and digestive disorders, with subsequent looses of weight gain in animals how ingest this mycotoxin. The gastrointestinal system is the target organ of the toxin. *In vitro* toxicity studies on trichothecenes revealed that DON binds to the ribosomal peptidyltransferase site and inhibits protein synthesis, resulting in decreased cell proliferation.[4]

The toxin has also a haemolytic effect on erythrocytes. Both effects, which are also seen with other trichothecene toxins, are thought to be mediated by affecting the serotonergic activity in the CNS or via peripheral actions on serotonin receptors.[6]

1.2.1.2 T-2 toxin

T-2 toxin belongs to a group of mycotoxins synthesized by *Fusarium* fungi that are widely encountered as natural contaminants of certain important agricultural commodities particularly, cereals. Among trichothecenes, T-2 toxin is the most toxic compound. Upon exposure, T-2 toxin causes several human and animal diseases. It is considered to be a major causative agent in fatal alimentary toxic aleukia in humans affecting the mucosa and the immune system. Leucopenia and necrotic lesions of the oral cavity, oesophagus and stomach are the main pathological findings. T-2 toxin is rapidly absorbed after ingestion in most animal species and it is distributed in the organism with little or no accumulation in any specific organs.[7]

1.2.1.3 Fumonisin B1 (FB1)

The fumonisins (FNs) are a group of structurally related polar metabolites produced by *Fusarium moniliforme, Fusarium verticillioides, Fusarium proliferatum* and other species to a lacer extent. Their structures are based on a hydroxylated hydrocarbon chain which contains methyl and either amino or acetyl groups. FB1 was first isolated in 1988. It is the most abundantly produced of the FNs in contaminated foods and feeds, found primarily as a contaminant of the corn constituent, and it is the most toxicologically significant.[8]

FB1 causes equine leukoencephalomalacia, porcine pulmonary oedema, nephrotoxicity, hepatotoxicity, and hepatocarcinogenicity in laboratory animals and of oesophageal carcinoma in humans, reported hepatotoxicity and nephrotoxicity in lambs, rabbits, and mink, as well as an increased rate of apoptosis in liver and kidney. Due to its structural similarity with sphingosine, FB1 inhibits the aramide synthase activity leading to an intercellular accumulation of sphingoid basis, which

mediate several key biological processes including inhibition of protein, DNA synthesis.[4]

In addition, FB1 also induces lipid peroxidation (prevented by vitamin E) in Vero cells, in primary rat hepatocytes and in C6 glioma cells.[4]

1.2.1.4 Zearalenone (ZEA)

ZEA is a non-steroidal estrogenic mycotoxin, mainly produced by *Fusarium graminearum* and *Fusarium culmorum* found in a variety of host plants and soil debris around the world. ZEA is usually non-lethal to animals, but it is important to livestock producers because its hyperestrogenic effects adversely influence the reproductive performance of animals. ZEA has high binding affinity for the intra-cellular estrogen receptor and can enhance the proliferation of estrogen responsive tumor cells. There can be suggestions of the involvement of ZEA in human cervical cancer and premature initial breast development.[9]

ZEA induces lipid peroxidation, cell death and inhibits protein and DNA syntheses. It has been shown to be genotoxic, and to induce DNA-adduct formation.[10]

1.2.1.5 Beauvericin (BEA)

BEA is a cyclic hexadepsipeptide, which shows antimicrobial, insecticidal, cytotoxic, ionophoric, apoptotic and immunosuppressive activity. It is the most potential specific inhibitor of cholesterol acyltransferase. BEA increases ion permeability in biological membranes by forming a complex with essentials cations (Ca^{2+}, Na^{+}, K^{+}) and/or cation-selective channels in lipid membranes, which may affect ionic homeostasis.[11]

1.2.2 Penicillium toxins

The most investigated mycotoxins produced by genus *Penicillium* are citrinin (CTN), ochratoxin A (OTA), patulin (PAT) and gliotoxin.[12]

Among different mycotoxins, OTA and CTN are quite common contaminants that can occur jointly in a wide range of food commodities, both are in fact, produced by *Penicillium* and *Aspergillus* families, which are worldwide in distribution. OTA and CTN constitute one of the most frequently occurring combinations of mycotoxins in different plant products. This mycotoxin combination is particularly relevant since although both OTA and CTN have many toxic effects in humans and animals, they share an important nephrotoxic potential. Indeed, OTA and CTN have been identified to be nephrotoxic and associated with alterations of renal functions and the development of renal pathologies *in vivo* studies.[12]

1.2.2.1 Citrinin (CTN)

CTN was first isolated from *Penicillium citrinum* priory to World War II, subsequently; it was identified in over a dozen species of *Penicillium* and several species of *Aspergillus*, including certain strains of *Penicillium camemberti* and *Aspergillus oryzae*. More recently, CTN has also been isolated from *Monascus ruber* and *Monascus purpureum*, industrial species to produce pigments.[2]

CTN has been associated with yellow rice disease in Japan. It has also been implicated as a contributor to porcine nephropathy.[2]

CTN acts as a nephrotoxin in all animal species tested, but its acute toxicity varies in different species. CTN acts synergistically with OTA to depress RNA synthesis in murine kidneys.[2]

Wheat, oats, rye, corn, barley, and rice have all been reported to contain CTN. With immunoassays, CTN was detected in certain vegetarian food colored with *Monascus* pigments.[2]

Although CTN is regularly associated with human foods, its significance for human health is unknown.[2]

CTN has been also associated with hepatotoxicity in cell cultures and animal models. Although CTN was proposed for use as an antibiotic, cytotoxicity studies showed that the compound had nephrotoxic properties and that it induced renal adenomas in rats.[13]

Furthermore, CTN cytotoxicity has been associated with alterations in mitochondrial calcium ion fluxes and membrane permeability in kidney and liver cells and with chromosomal abnormalities and aneuploidy.[13]

A recent study showed that CTN triggers programmed cell death (apoptosis) through an oxidative stress-independent mitochondrial pathway in HL-60 cells.[13]

Although previous studies showed that CTN has relatively small effects on rat embryonic development and the mechanisms or mode of action are not known. Apoptosis plays an important role in development and disease. Although previous studies demonstrated that apoptosis may play a role in normal embryonic development and mechanistically diverse teratogenes have been shown to induce excessive amounts of apoptosis in early embryos, leading to developmental injury.[13]

1.2.2.2 Patulin (PAT)

PAT is a frequent contaminant of apples and apple products. Many other fruits, including grapes, pears, peaches, and berries, have also been shown to contain PAT.[14]

Animal tissues affected by PAT administration included kidney and intestine.[14]

In addition, it is considered to be a carcinogen and teratogen in certain animal models.[14]

PAT is a potent inducer of chromatide-type aberrations in Chinese hamster V79-E cells, but does not increase sister-chromatide exchange frequency. Induction of chromosome damage and micronuclei formation in mammalian cells suggests a potential clastogenic property of PAT. PAT is believed to exert its cytotoxic effects mainly by forming covalent adducts with essential cellular thiols in proteins and amino acids. Nevertheless, the precise mode of action leading to genotoxicity and clastogenicity associated with PAT in human cells is poorly understood.[14]

1.3 MECHANISTIC INTERACTIONS OF MYCOTOXINS

Studies of combined toxic effects for exposure against mixtures of mycotoxins have been performed using animal models. Fewer studies reported the use of *in vitro* test systems for the analysis of mycotoxin mixture toxicity. Different toxicological parameters and experimental procedures were used for the analysis of the combined effects *in vivo*. For example using a mouse model, synergistic effects of a combination of CTN and OTA were observed with respect to renal carcinogenesis and nephrotoxicity. Additive effects on embryotoxicity manifestations and organ affliction for the same mixture were found in chicken embryos. Different

immunobiological effects provoked by the mixture of aflatoxin B1 (AFB1) and fumonisin B1 (FB1) in comparison to the individual action of the same toxins were detected in rats. For example, the peritoneal macrophages of animals fed with AFB1 released less hydrogen peroxide, similar to what happened with the FB1 intake. However, the consumption of the AFB1–FB1 mixture reverted this decrease in the release of hydrogen peroxide. Thus, the changes in hydrogen peroxide production were not an addition of the effects produced individually by the individual toxins. None of these studies have provided an explicit theory about what is meant by additivity or synergism.[3]

Typically, combined effect assessments should be based on the comparison of an expected effect of a mixture based on knowledge about the individual components behavior and the observed effects in a mixture experiment.[3]

As, however, several models for calculating expected mixture effects, e.g., additivity exist, for the same mixture data different assessments as to their combined effects may prevail. From the literature survey, it is apparent that the necessity of mixture assessment is appreciated.[3]

In vivo studies on combined effects, due to a lack of analytical power focus on more observation of specific mixtures. Due to the large number of possible mixtures occurring, this typically, however, lacks a predictive scope. For systematic investigations we therefore need to establish tools on the biological assay side as well as on the prediction modeling side. However, explicit concepts as to what entails an expected combined effect are necessary in order to compare findings from different assays and to utilize results to predict untested concentrations in prospective risk assessment of mycotoxin mixtures. Only a few studies provide the experimental design suitable for coupling an individual and a combined effect.[3]

Several mycotoxins, either from the same or from different fungal species, occurs simoultaneously in plant products. However, its implication for food safety assessment is generally not known, as there is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence for the toxicity.[15]

The available data show that adequate studies to establish antagonistic, additive or synergistic effects after combined exposure to mycotoxins are rare, and it is known that the issue of combined toxicity is very complicated.[15]

Mycotoxins with similar mode of action would be expected to have at least additive effects. Conversely, some interactions could have subtractive effects. An understanding mode of action in simple in vitro systems can provide a rational basses for predicting interactions between mycotoxins. In this manner, it can also help setting priorities for specially designed studies to established interactions, preferably those suspected to reveal synergistic actions.[15]

The theoretical consideration based on the cellular mode of action what interaction between mycotoxins can be predicted is not a final answer. The toxicokinetic behavior, metabolism and the toxicodynamic aspects are on influence on the final outcome when man or experimental animals are exposed to a mixture of some mycotoxins. What happens if different types of cells/issues are involved, in other words, what is the result of the interaction between different tissues? Similarly, what will be the final result if more organ systems are affected by the mycotoxins? With respect to the toxicokinetic part the application of physiologically based pharmaco-kinetic models could be useful.[15]

In practice, the outcome of combined exposure to mycotoxins might either quantitatively or even qualitatively be different from what would be predicted. The result can also depend on the species or the type of endpoint studied. Therefore,

further specific experiments, which are complicated and include a lot of effort, are needed. These studies need carefully planned experimental designs for more accurate statistical analysis. At present, there is an increase in the successful application of such especially designed studies for chemical mixture, including ray design, response-surface design and fractionated factorial design.[15]

1.4 IN VITRO ACUTE TOXICITY TESTS

The development of *in vitro* methods as alternatives to animal experimentation is of great relevance for the detection of the potential toxicity of mycotoxins in man. The *in vitro* techniques have a great potential in the research work.[16]

In vitro techniques have many practical advantages over *in vivo* models. The advantages of *in vitro* techniques include: rapid effective assay, control over test conditions which are not possible *in vivo*, utilization of large number of cells per test, allowing intrinsically continuous dose-response relationships, reduction of variability between experiments, the ability to obtain repeated samples during the course of one experiment allowing to follow sequence of events during toxicological response, significantly smaller quantities of testing chemicals than *in vivo* tests, the possibility of inducing many *in vitro* test simultaneously. Finally, and most important advantages of *in vitro* assay is that, they offer opportunities to study and describe mechanism of the harmful effects of chemicals.[17]

Cell cultures represent an alternative method to animal experiment and allow to evaluate and to compare the toxic effects of different compounds from cytotoxicity criteria.[18]

In cell culture, established cell lines derived from primary cultures by transformations processes which are spontaneous or induced are used. They have a limited life span or continue life-span. The life-span of a cell line is up, 400-500 cell

cycles, and the cells are usually normal ones that are undergoing the process of senescence in vitro. Established cell lines are used for basal cytotoxicity studies. The general cellular response to a toxic attack, in spite the great variety of mechanism and molecular targets that may be involved, can be identified in a restricted number of cellular alterations that eventually cause the death of cells.[19]

In vitro cytotoxicity assay are designed to evaluate the intrinsic ability of a chemical to kill cells, inhibition of cell proliferation, cell density in culture, quantification of DNA, proteins, enzymatic activity, alteration of metabolic activity, etc.

For *in vitro* toxicity assessment it was necessary to develop a battery of assay. Many *in vitro* cytotoxicity tests were developed over last years to evaluate the intrinsic ability of a mycotoxin to injure cells. The Neutral red (NR) is a colorimetric based assay used to measure toxicity of mycotoxins.

NR assay is a good cytotoxicity assay used for identification of viability of cells in cultures. In damaged or dead cells, NR is no longer retained in the cytoplasmatic vacuoles and the plasma membrane does not act as a barrier to retain the NR within cells. Therefore, they loose ability to retain the NR during the wash.[19]

This assay quantifies the number of viable and uninjured cells after their exposure of toxicants. NR assay is relatively simple to perform, can be semi-automated, are rapid, sensitive and adaptable. Sensitivity of this assays is based on a small amount of cells needed and, therefore, less consumable are required, compared to many other systems. Thus, it provides a relatively inexpensive method to screen a large number of compounds over a wide range of concentrations.[19]

1.5 AIMS OF THE STUDY

1. To determine cytotoxicity of individual mycotoxins: beauvericin, deoxynivalenol and T-2 toxin (from genus *Fusarium*), citrinin (from genus *Penicillium*) by *in vitro* method Neutral red assay using Vero cells at the different times of exposure.

2. To determine cytotoxicity of the mixtures of two and three mycotoxins: beauvericin+deoxynivalenol, beauvericin+T-2 toxin, deoxynivalenol+T-2 toxin and beauvericin+deoxynivalenol+T-2 toxin by *in vitro* method Neutral red assay using Vero cells at the different times of exposure.

2. EXPERIMENTAL

2.1 CHEMICALS AND MATERIALS

Materials and mycotoxins

The reagent grade chemical dimethylsulfoxide (DMSO) and cell culture components used, namely DMEM (Dulbecco´s modified eagle´s medium), penicillin-streptomycin (Gibco BRL®), trypsin/EDTA solution (Sigma-cell culture™), (N-[2-hydroxyethyl]piperazine-N'-[zethanesulfonic acid)] (HEPES), 3-amino-7-dimethylammino-2-methylphenazine hydrochloride (neutral red dye), sodium pyruvate, acetic acid, ethanol, methanol, $CaCl_2$, ethylenediaminetetraacetic acid (EDTA) and formaldehyde 37-40 % were obtained from Sigma Chemical Co. (St. Louis Mo. USA). Fetal bovine serum (FBS) was from Cambrex Company (Belgium).

Mycotoxins, DON, T-2 toxin, BEA and citrinin with purity up to 99% were supplied by Sigma Chemical Company (St. Louis, MO, USA). The primary stock solution of each mycotoxin was completely dissolved in distilled water or sterilized methanol (when it was not possible in water).

Equipments

- Incubator THERMO SCIENTIFIC HEPA CLASS 100, model 371,USA
- Microscope NIKON eclipse TE2000-S, Japan
- Beckman Coulter Z1 particle counter, Germany.
- Centrifuge 5810R eppendorfs AG, Hamburg
- Laminar Cabin: TELSTAR BIO-II-A, Valtek Nova, Spain
- Microtiter plate reader Wallace Victor, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland.
- Analytical weight METTER AJ100L (δ=0,0001), Spain

2.2 CELL CULTURE

2.2.1. Cell lines and maintenance

Vero cells are derived from epithelial cells of kidney from African green monkey *(Cercopithecus aethiops)*. The Vero lineage was isolated on 27 March 1962, by Yasumura and Kawakita at the Chiba University, Chiba, Japan. The Vero cell lineage is continuous and aneuploid. A continuous cell lineage can be replicated through many cycles of division and not become senescent.[20]

Vero cells were grown in polystyrene tissue culture dishes at 37ºC, in a humidified 5% CO_2 atmosphere in DMEM medium supplemented with 10% FCS and 100 U/ml penicillin and 100µg/ml streptomycin.

The cells were subcultured as they reached confluence. For this process the medium was removed and the cells were gently washed two times with 5 ml of medium. The cells were dissociated by addition of 1 ml of trypsin (0,005%)/EDTA(0,002%). One minute of exposure removed the cells, longer results in cell death. 1 ml of culture medium was added to each tube and homogenized gently to disrupt cell clumps. The cells were then either passed directly to the new culture dish to continue with passage, or counted the number of cells per ml with a Beckman coulter (Z1 coulter particle counter) and then seeded to each wells of the 96-well plates to start with the cytotoxicity assay selected. All these procedures were performed under sterile conditions; manipulations were carried out in a laminar flow hood.

2.2.2. Determination of cytotoxicity with individual mycotoxins and their mixtures

The cytotoxicity of individual and combined mycotoxins was estimated in Vero cells using the NR assay. The NR assay method was performed and described by Ruiz et al. (2006)[19]. Vero cells (passages 24-58) were harvested in 96-well tissue culture microtiter plate at a density of 2 x 10^4 cell/well in DMEM medium with 10% FCS. Cells in 200µl medium were seeded to each of 96 wells in a microtriter plate to form a monolayer of about 65% of confluence. It is important that confluence has not

been reached at the time of testing so that cells are fully exposed to toxic agents during cell division, since confluent monolayer cultures are less sensitive to toxic agents.

After the cells reached 65% confluence, the medium was removed and replaced with 200 µl of fresh medium containing decanally increasing concentrations of mycotoxins. After preliminary studies, test concentration was selected from 1.56 to 50 µM for BEA, from 0.5 to 50 µM for CIT, from 0.1 to 20 µM for DON, and from 0.1 to 30 nM for T-2 toxin. Normally at least 8 control wells were used in each experiment. Each concentration tested, medium was distributed over 8 wells. All additions to the cells were performed with care, using an Eppendorfs pipette, to avoid detaching the cells. During all the experiments the cells were scanned under a light microscope to check for bacterial/fungal contamination.

After the plates were incubated at 37 °C for 24, 48 and 72 hours the medium with mycotoxins, was replaced with fresh medium containing 50µg/ml NR dye. The fresh medium containing NR had previously been incubated overnight at 37°C and was filtered, using a 0.25 µm filter, before addition to remove fine precipitation and dye crystals formed. Deposit of such precipitated crystals into the cell cultures during incubation would interfere with the assay.

Once, NR dye was added to cells, the microtiter plate was returned to the incubator for another 3 hours to allow for the uptake of the supravital dye into lysosomes of viable, uninjured cells. After this period the plates were again scanned, by light microscope to ensure no crystals had formed.

After 3 h, cells were washed 200µl of a solution of 0.5% (v/v) formaldehyde and 1% $CaCl_2$ to remove unincorporated dye and simultaneously promote adhesion of the cells to the substratum. The formaldehyde was left only briefly in contact with the cells (10 seconds), since longer exposure would result in extraction of the dye. After removal, cells were fished with 200µl of a solution of 1% acetic acid in 50% ethanol. After approximately 30 minutes at incubator, the 96 well plates were rapidly agitated for a five minute on a microtiter plate shaker and NR was quantified using a microtiter plate reader (Wallace Victor, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) at 490 nm.

2.2.3. Statistical analysis of data

To evaluate the cytotoxicity of the tested compounds, seven different concentrations of each mycotoxin were tested. All experiments were performed at least three times, in separate days.

The EC_{50} values, defined as the concentration of test mycotoxins that modified the cell viability by 50% in comparison with the appropriate untreated control cultures, were calculated from the dose-response curve. The results thus obtained are expressed in the Fig. 1 to 8, as percentage change compared with unexposed cells. EC_{50} values were determined by linear interpolation. Statistical analysis was performed using analysis of variance (ANOVA) and a comparison of means with a Student's t-test (two tails) was applied out to determine the differences between cytotoxicity by Vero cells after incubation with different mycotoxin amounts. Data were expressed as means ± SD from at least three experiments. *P*-values<0.05 were considered statistically significant. Slopes and regression coefficients were estimated from each toxicity regression line using the Sigma Plot program.

3. RESULTS AND DISCUSSION

3.1 CYTOTOXICITY ASSAY OF INDIVIDUAL MYCOTOXINS

The aim of this study was to obtain cytotoxicity data (EC_{50} values) of *Penicillium* and *Fusarium* mycotoxins, nominally BEA, CIT, DON and T-2 toxin. For this purpose, Vero cells viability was evaluated in the presence of these four mycotoxins using the NR assay.

Cellular viability was conduced on confluent Vero cells monolayers in 96-well microculture plates. The 65% confluent monolayer was exposed to mycotoxin at different concentration range and incubation periods (24, 48, and 72 h). Figures 1 to 5 show the concentration-response curves for the individual mycotoxins, at 24, 48, and 72 h. Significant differences were observed between the compounds tested. All toxins, BEA, CIT, DON and T-2 toxin, tested individually diminish cell viability (Table 1).

Fig. 1. Figure of cytotoxicity study on Vero cells of the exposure to BEA for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

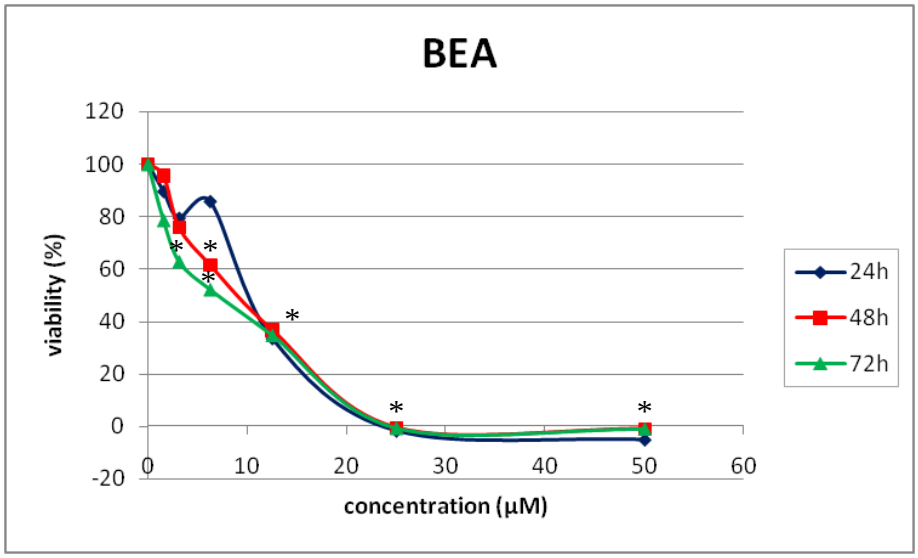


Fig. 2: Figure of cytotoxicity study on Vero cells of the exposure to CIT for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

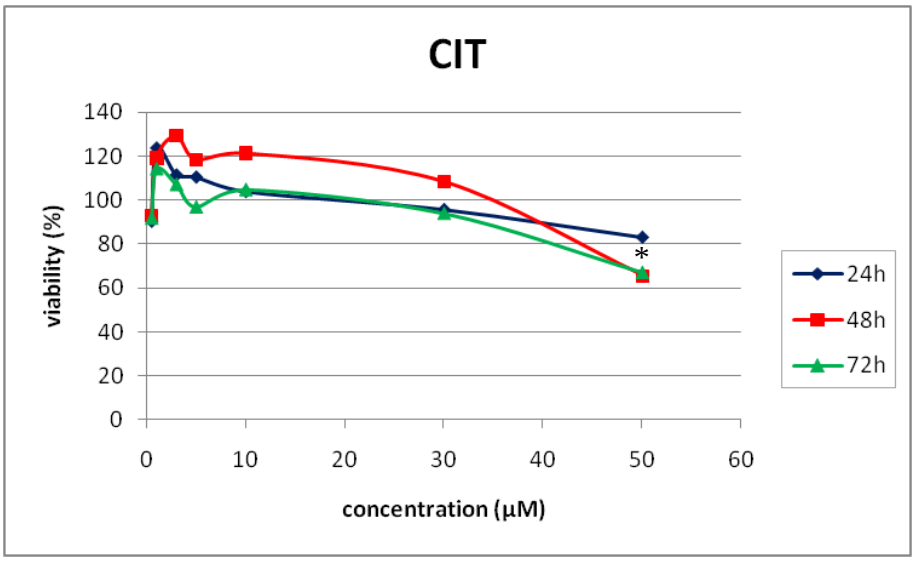


Fig. 3. Figure of cytotoxicity study on Vero cells of the exposure to DON for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

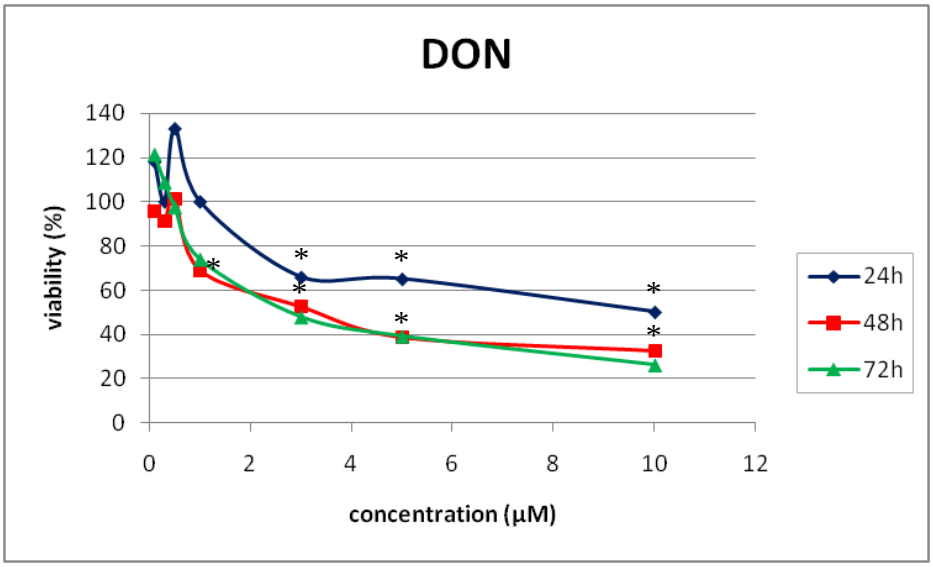


Fig. 4. Figure of cytotoxicity study on Vero cells of the exposure to T-2 toxin for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values (P < 0.05).

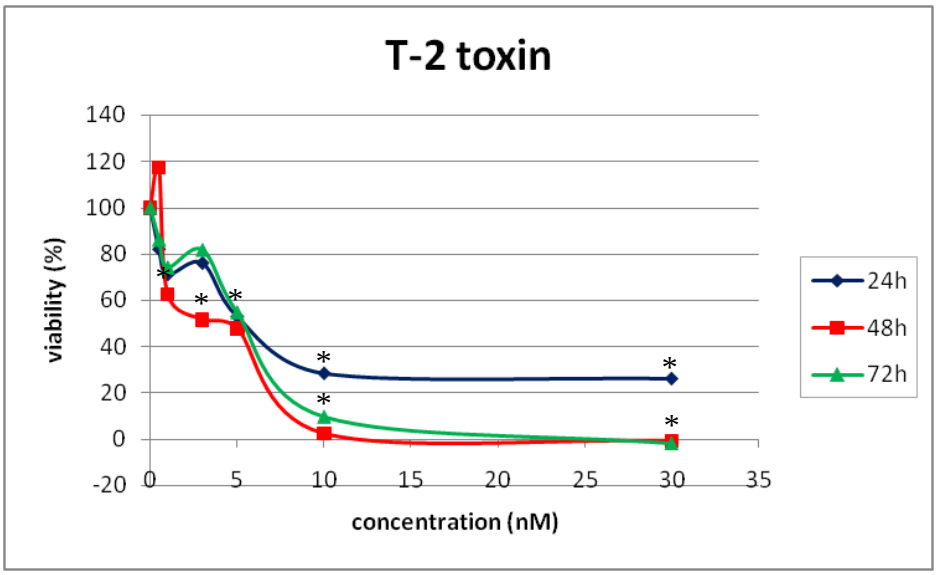


Table 1 shows the cytotoxicity (EC_{50}) of individual BEA, CIT, DON and T-2 toxin exposition on Vero cells after 24, 48 and 72 hours exposure using NR assay.

Table 1: Cytotoxicity values (EC_{50}) and standard deviation (SD) of the individual mycotoxins tested after 24, 48 and 72 hours exposure, on Vero cells.

Mycotoxin	*Cytotoxicity (EC_{50}; μM) ± SD*		
	24h	**48h**	**72h**
Beauvericin	11.08 ± 0.02	9.03 ± 0.24	6.77 ± 0.08
Deoxynivalenol	10.00 ± 0.35	4.24 ± 0.03	3.30 ± 0.06
T-2 toxin	5.31×10^{-3} ± 0.01	4.69×10^{-3} ± 0.01	3.75×10^{-3} ± 0.01
Citrinin	>50	>50	>50

Data observed in Table 1 indicate that all mycotoxins tested reduced Vero cells viability as expected. T-2 toxin is the most effective in reducing cell viability, whereas no significant differences over control were detected when Vero cells were exposed to CIT at any of the concentration and incubation times tested using EC assay. No higher concentration was assayed because of the high cytotoxicity values obtained from the literature as seen from the results presented below**.** Golli et al. (2006) obtained a cytotoxicity value of 260 μM for CIT in cultured Vero cells after 24 h incubation using MTT assay.[21] Similarly, Heussner et al. (2006) demonstrated that CIT cytotoxicity assays using porcine kidney cells (PKC) yield $EC_{50} > 400$ μM.[1]

All mycotoxins tested diminished cell viability in a concentration and incubation time-dependent manner on Vero cells (Figures 1 to 4). As shows in Table 1, individual mycotoxins increase cytotoxicity as follow: CIT< BEA< DON< T-2 toxin. Our results obtained with individual mycotoxins are similar to those obtained by other authors. Bouaziz et al. (2006) determined T-2 toxin cytotoxicity after 24 h exposure, using NR and MTT assay. They found a dose-dependent increase of the percentage of growth inhibition by the two tested assays. The corresponding IC_{50} found were 4 nM by the NR assay and 60 nM in the MTT assay.[7] On the other hand,

Golli et al (2006) obtained an IC_{50} value for T-2 toxin, after 24 h exposure by MTT, 21 times higher that our results.[21]

3.2 CYTOTOXICITY ASSAY OF MYCOTOXINS MIXTURES

To determine the mechanistic interactions of BEA, DON and T-2 toxin and their influence on cellular viability of Vero cells, were used the following combinations of mycotoxins: BEA in combination with DON, BEA in combination with T-2 toxin, DON in combination with T-2 toxin and combination of all three mycotoxins. However, due to not EC_{50} was obtained from individual assay of CIT, this mycotoxin was not considered to include in the binary and tertiary combination assays.

Tables 2 and 3, show the cytotoxicity (EC_{50}) of mycotoxins mixtures on Vero cells after 24, 48 and 72 hours exposure using NR assay. Figures 5 to 7 show the concentration-response curves for the mixture of binary mixture of mycotoxins, at 24, 48, and 72 hours incubation time on Vero cells.

Table 2: Cytotoxicity values (EC_{50}) and SD of mycotoxins mixture tested after 24, 48 and 72 hours exposure, on Vero cells

Mycotoxins	***Cytotoxicity (EC_{50}) ± SD***					
BEA+DON	**BEA (µM)**			**DON (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	4.39 ± 0.90	1.97 ± 0.02	2.88 ± 0.09	1.81 ± 0.08	0.61 ± 0.02	0.91 ± 0.05
T-2+DON	**T-2 toxin (nM)**			**DON (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	8.21 ± 0.82	6.92 ± 0.11	7.31 ± 0.01	2.59 ± 0.20	2.19 ± 0.15	2.39 ± 0.31
T-2+BEA	**T-2 toxin (nM)**			**BEA (µM)**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
	5.00 ± 0.54	4.50 ± 0.31	4.38 ± 0.20	3.18 ± 0.32	2.88 ± 0.12	2.72 ± 0.25

As can be observed in Table 2, all the mixture of mycotoxins tested reduced the viability of Vero cells. Significant differences were observed between the

mycotoxins tested. When compared mean neutral red concentration obtained with BEA and DON alone (Table 1), with those data obtained with mixture of BEA and DON, a high reduction value in cell viability was observed for BEA and DON (Table 2). However, T-2 toxin shown similar cytotoxicity effect as compared to individual EC_{50} value (Table 1) obtained using NR assay.

When DON was combined with BEA, higher decreasing in cell viability was observed than when it was combined with T-2 toxin. On the opposite, when compared mean neutral red concentration obtained with T-2 toxin combined with DON, an increase in cell viability of DON was observed respect to the DON and BEA combination. On the other hand, T-2 toxin demonstrated higher cytotoxicity when was assayed alone (Table 1) in relation to binary combination with DON (Table 2). However, similar cytotoxicity effect was observed in combination with BEA.

The EC_{50} obtained from tertiary combination of mycotoxins is shown in Table 3. The mixture of the three mycotoxins (BEA, DON and T-2 toxin) at 24, 48 and 72 h exposure significantly reduces cell viability as expected. The tertiary mixture shows the highest cytotoxic effect on Vero cells, compared to either individual mycotoxins or mixtures of two of them.

Table 3. Cytotoxicity values (EC_{50}) and SD of tertiary mycotoxins mixture tested after 24, 48 and 72 hours exposure, on Vero cells

Mycotoxin	*Cytotoxicity (EC_{50}; μM) ± SD*		
	24 h	**48 h**	**72 h**
Beauvericin	1.21 ± 0.03	0.98 ± 0.01	1.06± 0.02
Deoxynivalenol	0.41 ± 0.01	0.35± 0.01	0.36 ± 0.02
T-2 toxin	$2.31x10^{-3}$ ± 0.01	$1.54x10^{-3}$ ± 0.03	$1.79x10^{-3}$ ± 0.02

Fig. 5. Figure of cytotoxicity study on Vero cells of the exposure to BEA and DON for a) 24 h, b) 48 h, and c) 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).

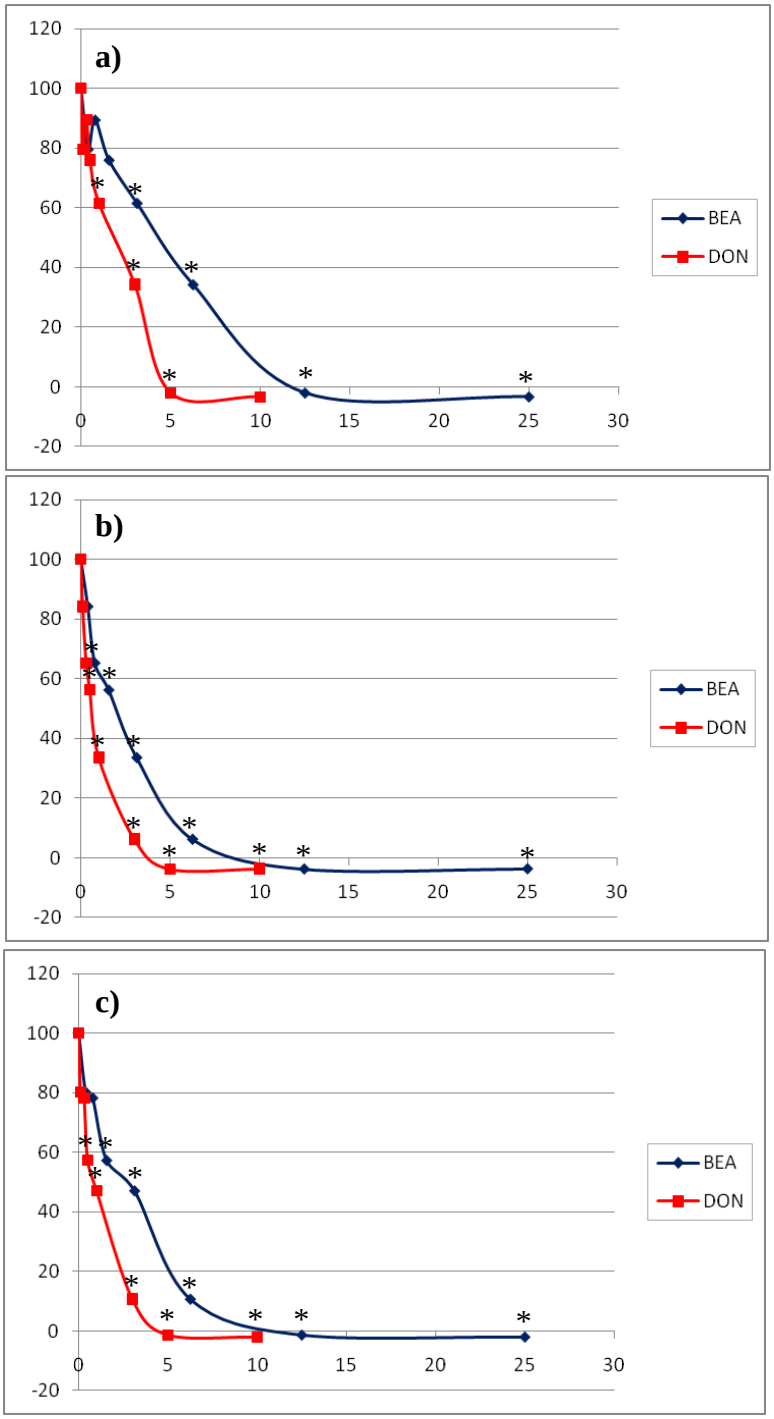
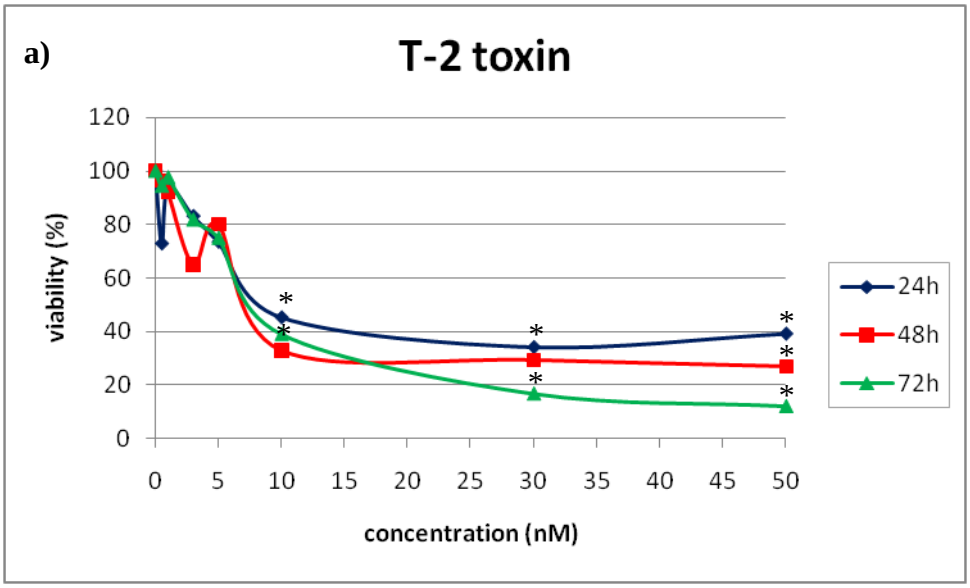


Fig. 6. Figure of cytotoxicity study on Vero cells of the exposure of a combination of a) T-2 toxin (nM) and b) DON (µM) for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).



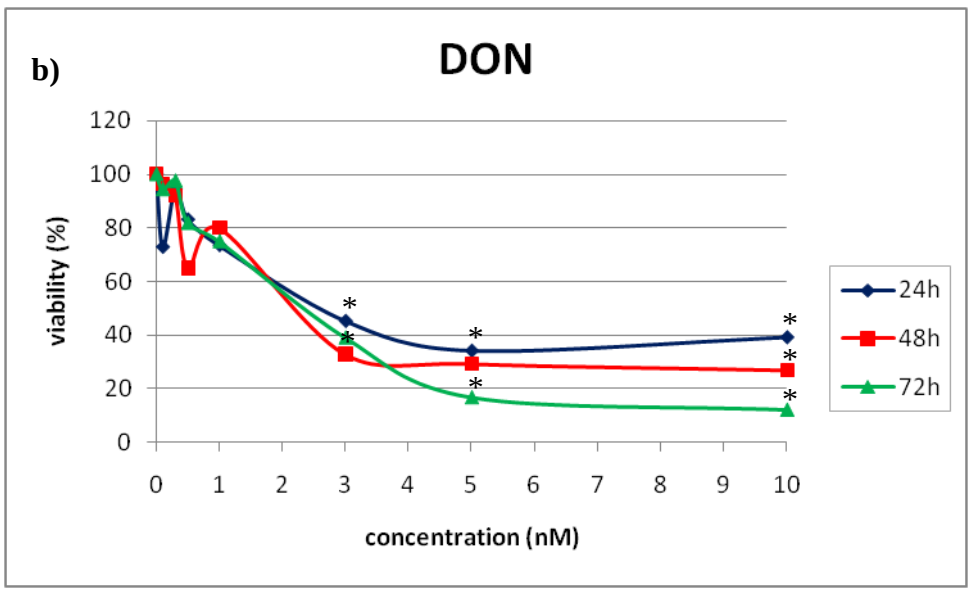


Fig. 7. Figure of cytotoxicity study on Vero cells of the exposure of a combination of a) T-2 toxin (nM) and b) BEA (µM) for 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).

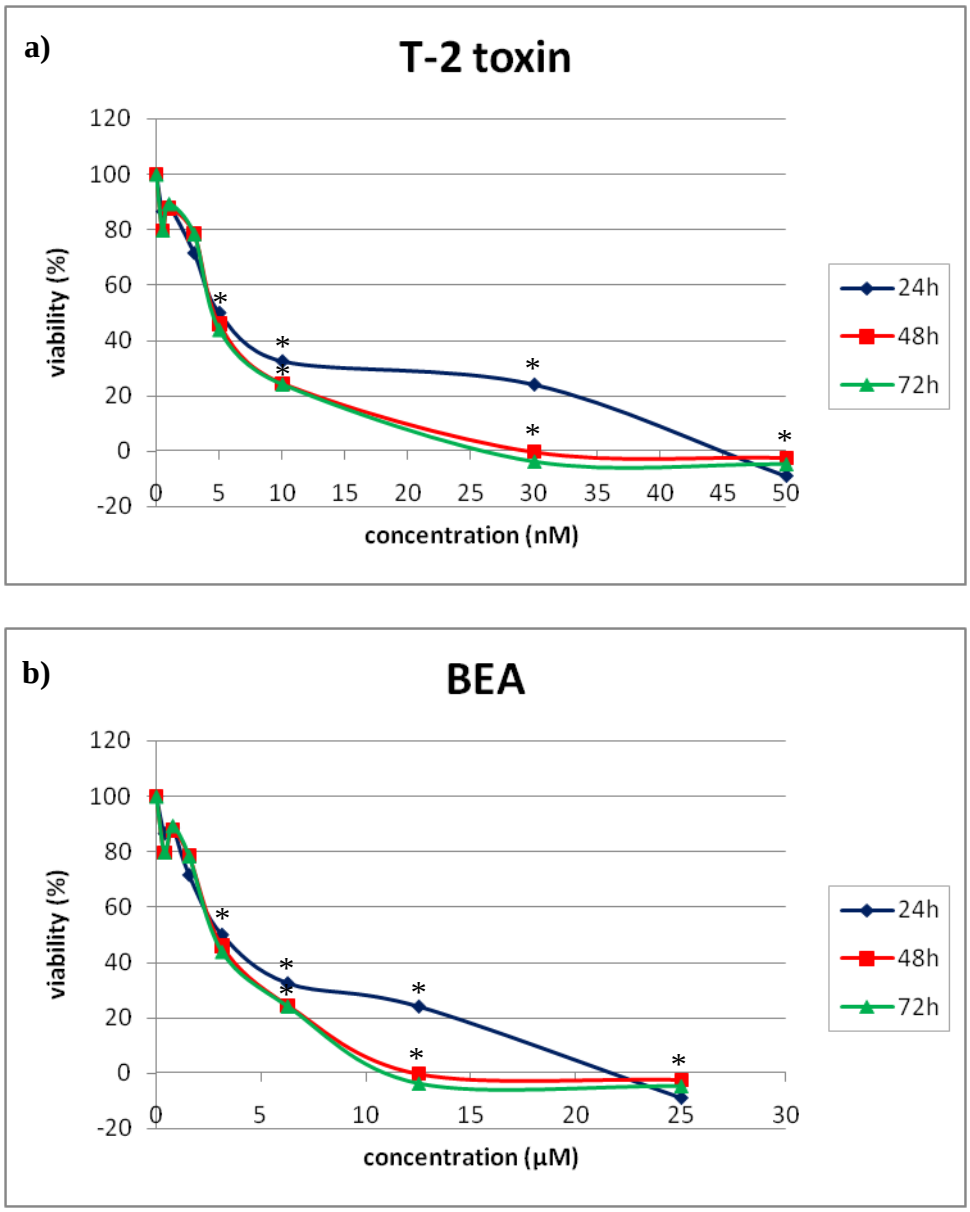
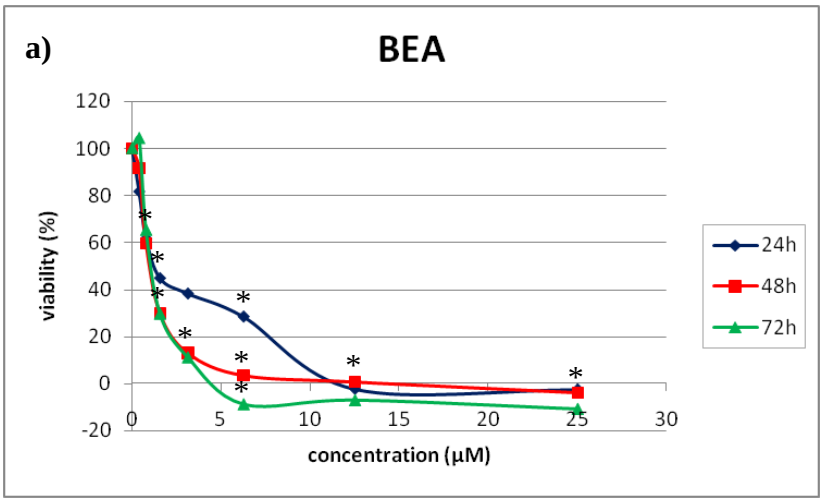
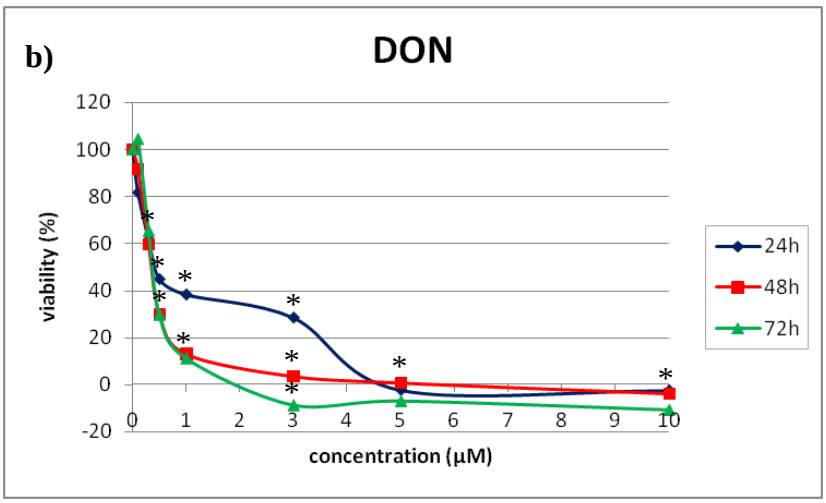
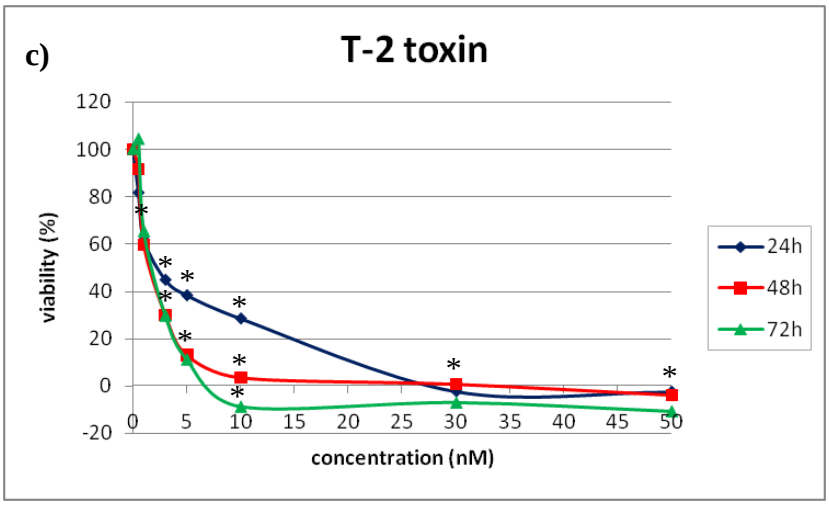


Figure 8 shows the concentration-response curves for the tertiary mycotoxins assays at 24, 48, and 72 h. All toxins, DON, BEA and T-2 toxin, significantly increased the cell viability compared to individual mycotoxins tested (Table 3).

Fig. 8. Figure of cytotoxicity study obtained from Vero cells after the exposition of a ternary combination, a) BEA, b) DON and c) T-2 toxin, during 24, 48, and 72 h. Each point represents the mean value of at least three experiments. Data expressed as percentage of the unexposed controls using eight replicates per concentration. An asterisk indicates a significant difference from control values ($P < 0.05$).







Although cytotoxic effects of individual mycotoxins have been studied; limited information exists in literature about synergistic, additive and antagonistic effects against cells exposed to mixture of mycotoxins. Data obtained from literature differ from those of this study particularly in the type of mycotoxins and in the cell line used in the mixtures. There are a few studies addressing the effects of combined exposure to several trichothecenes. Kouadio et al (2007) studied the interactive cytotoxicity effects of either binary or tertiary mixtures of *Fusarium* mycotoxins, DON, ZEA and FB1 on the intestinal cell line, Caco-2 using the NR assay.[6] They found that the mixtures of mycotoxins reduce cellular viability in the following increasing order: [FB1 + ZEA] < [FB1 + DON] < [ZEA + DON] < [FB1 + DON + ZEA]. Moreover, they observed that FB1 antagonizes the effects of ZEA, thus mixture of FB1 and ZEA improves Caco-2 cells viability in contrast to individual effects. On the contrary, mixtures of ZEA or FB1 and DON, display synergistic effects.

Our results indicate that most cytotoxic effect on Vero cells was observed after the tertiary mixture of BEA, DON and T-2 toxin. Other studies, related to cytotoxicity effects of mycotoxins were observed in literature. These studies, the great interest with regard to human and animal health, have not been commented here, because they were related to combined mycotoxins produced by a number of *Penicillium*, *Aspergillus* and other fungus genus, different from *Fusarium*.

4. CONCLUSIONS

1. Beauvericin, deoxynivalenol and T-2 toxin show cytotoxic effect on Vero cells by Neutral red assay. No significant difference over control was detected for citrinin at the concentration range tested.
2. Cell viability was influenced by the concentrations of mycotoxins and the time exposure.
3. The most cytotoxic effect among individual mycotoxins tested was obtained with T-2 toxin exposition.
4. T-2 toxin in combination with other mycotoxin (BEA or DON) obviously increases the cytotoxic effect of the second mycotoxin in mixture at any incubation time.
5. The binary mixture of BEA and DON increase the cytotoxic effect of both of them in relation to individual mycotoxin at all periods time.
6. The tertiary mixture of BEA, DON and T-2 toxins produce the highest decreasing in Vero cells viability, compared binary mixtures and individual exposition of mycotoxins.

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