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Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació,Toxicologia i Medicina Legal.
- Area de Toxicologia –

Doctorat en Ciències de l'Alimentaciò
Tesi Doctoral internacional

IN VITRO EVALUATION OF TOXIC EFFECTS, BIOAVAILABILITY AND BIOACCESSIBILITY OF BEAUVERICIN, ENNIATINS AND FUSAPROLIFERIN.

EVALUACIÓN *IN VITRO* DE LOS EFECTOS TÓXICOS, BIODISPONIBILIDAD Y BIOACCESIBILIDAD DE BEAUVERICINA, ENNIATINAS Y FUSAPROLIFERINA.

Presentada per: **Alessandra Prosperini**

Dirigida per: **Dra. Guillermina Font Pérez**

Dra. María José Ruiz Leal

Las Dras, Guillermina Font Pérez y María José Ruiz Leal, catedrática y profesora titular, respectivamente del Área de Toxicología del Departamento de Medicina Preventiva i Salut Pública, Ciències de l'Alimentaciò, Toxicologia i Medicina legal, de la Universitat de València,

INFORMAN QUE:

La licenciada en "Scienze dell'Alimentazione e Nutrizione Umana" ha realizado bajo nuestra dirección el trabajo que lleva por título: "*In vitro* evaluation of toxic effects, bioavailability and bioaccessibility of beauvericin, enniatins and fusaproliferin".

Y autorizamos la presentación para optar al título de Doctor Internacional.

Burjassot 24 de Junio 2013.

María José Ruiz Leal

Guillermina Font Pérez

Esta tesis Doctoral ha dado lugar a los siguientes trabajos:

1. Interaction effects of Fusarium enniatins (A, A_1, B and B_1) combinations on in vitro cytotoxicity of Caco-2 cells. Toxicology *in Vitro* (DOI: 10.1016/j.tiv.2013.06.021). Índice de impacto: 2.775.

2. Beauvericin-induced cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. Toxicology Letters (under review). Índice de impacto: 3.230.

3. Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A_1, B and B_1. Toxicology Letters (under review). Índice de impacto: 3.230.

4. Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability *in vitro* on Caco-2 cells. Food and Chemical Toxicology. Índice de impacto: 2.999.

5. Effect of polyphenols on enniatins-induced cytotoxic effects in mammalian cells. Toxicology Mechanisms and Methods. Índice de impacto: 1.033.

6. Bioaccessibility of enniatins A, A_1, B, and B_1 in different commercial breakfast cereals, cookies, and breads of Spain. Journal of Agricultural and Food Chemistry. Índice de impacto: 2.823.

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- Ha disfrutado de una beca predoctoral orientada a la formación de personal investigador estranjero en organismos de investigación de la Comunitat Valenciana, dentro del programa Santiago Grisolía, concedida por la Conselleria de Educación (Comunitat Valenciana).
- Ha conseguido el título de "Master en Calidad y Seguridad Alimentaria" (2010/2011) para poder acceder a los estudios de Doctorado.
- Ha disfrutado de una subvención para favorecer la movilidad de estudiantes en estudios de Doctorado con Mención hacia la Excelencia Curso 2011-2012. Dirección General de Universidades. Ministerio de Educación.

Además la presente Tesis Doctoral forma parte del Programa de Doctorado Internacional:

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LIST OF ABBREVIATIONS

ABC: Membrane located ATP-binding cassette transporters
ACAT: Acyl-CoA:cholesterol acyltransferase
AcDON: Monoacetyl-deoxinivalenols (3- and 5-AcDON)
Ac-FUS: Acetyl-fusaproliferin
AFBs: Aflatoxins B
AFGs: Aflatoxins G
AP: Apical compartment
BEA: Beauvericin
BL: Basolateral compartment
CA: Chromosomal aberrations
$CaCl_2$: Calcium chloride
CAT: Catalase
CCM: Cell monolayer
CI: Combination index
CIT: Citrinin
CO_2: Carbone dioxide
Da-FUS: Deacetyl-fusaproliferin
DAS: Diacetoxyscirpenol
DCF: Dichlorodihydrofluorescein
DCFH: 2',7'-dichlorodihydrofluorescein
DMSO: Dimethyl sulfoxide
DMEM: Dulbecco's modified eagle medium
DNA: Deoxyribonucleic acid
DON: Deoxynivalenol
DSB: Double strand break
$\Delta\Psi m$: Mitochondrial membrane potential
3AcDON: 3-acetyldeoxynivalenol
EC: Commission regulation
EFSA: European Food Safety Authority
EDTA: Ethylenediaminetetraacetic acid
EN/ENs: Enniatin/Enniatins
EU: European Union
FAO: Food and Agricultural Organization
FBs: Fumonisins

FC: Flow cytometry
FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FCS: Foetal calf serum
FITC: Fluorescein isothiocyanate
FHB: *Fusarium* head blight
FUS: Fusaproliferin
FUS-X: Fusarenone-X
GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced glutathione
GSSG: Oxidized glutathione
GST: Glutathione transferase
Ham-F12: F-12 nutrient mixture for CHO-K1 cells
HBSS: Hank's Balanced Salt Solution
HCl: Hydrochloric Acid
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC: High performance liquid chromatography
HPLC-DAD: High performance liquid chromatography coupled with diode array detector
HT2: HT2 toxin
H_2-DCFDA: 2',7'-dichlorodihydrofluorescein diacetate
IC_{50}: 50% inhibitory concentration
JECFA: Joint Expert Committee on Food Additives
KCl: Potassium chloride
KSNC: Potassium thiocyanate
LC-DAD: Liquid chromatography-diode array detector
LDH: Lactate dehydrogenase
LMA: Low melting point agarose
LPO: Lipid peroxidation
MDA: Malondialdehyde
MN: Micronucleus
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MON: Moniliformin
MPT: Mitochondrial permeability transition
MPTP: Mitochondrial permeability transition pore
NaCl: Sodium chloride

NaH_2PO_4: Sodium dihydrogen phosphate
$NaSO_4$: Sodium sulphate
$NaHCO_3$: Sodium bicarbonate
NEAA: Non essential amino acids
NEM: *N*-Ethylmaleimide
NEO: Neosolianol
NR: Neutral Red
NIV: Nivalenol
OPT: O-phthalaldehyde
OTA: Ochratoxin
PAT: Patulin
PBS: Phosphate-buffered saline
PIA: Penicillic acid
PI: Propidium iodide
PS: Phosphatidylserine
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SCE: sister chromatid exchange
SOD: Superoxide dismutase
SRB: Sulforhodamine B
SSB: Single strand break
TBARS: Thiobarbituric acid reactive substances
TBA: Thiobarbituric acid
TEER: Trans-epithelial electrical resistance
TIM: Dynamic *in vitro* GI-model
TMRM: Tetramethyl rhodamine methyl estere
To-Pro®-3: Quinolinium, 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]- diiodide
T2: T-2 toxin
T2-ol: T-2 tetraol
ZEA: Zearalenone
ZOH: zearalenols (α and ß)
WHO: World Health Organization

ANTECEDENTES BIBLIOGRÁFICOS

1. INTRODUCCIÓN

1.1 Las micotoxinas: una cuestión importante de seguridad alimentaria.

La contaminación de los alimentos y de los productos agrícolas por varios tipos de hongos toxigénicos (moldes) y de micotoxinas es un problema grave. A pesar de décadas de investigación, la infección por mohos sigue siendo un problema difícil (Munkvold 2003).

Con el termino "micotoxinas" se define un grupo estructuralmente diverso de compuestos, la mayoria de bajo peso molecular, producido principalmente por el metabolismo secundario de hongos filamentosos, los cuales bajo condiciones adecuadas de temperatura y humedad, pueden colonizar alimentos y piensos, causando graves riesgos para la salud humana y animal (Soriano del Castillo et al., 2007). Los principales hongos micotoxigénicos pertenecen a tres géneros: *Fusarium*, *Aspergillus* y *Penicillium*, que en determinadas condiciones favorables (temperatura, actividad del agua y humedad relativa) pueden colonizar y posteriormente contaminar con micotoxinas, los alimentos y los piensos (Paterson y Lima, 2010).

Son numerosas las matrices alimentarias expuestas a una contaminación directa por micotoxinas: oleaginosas, frutos secos, frutas secas, verduras, especias, café, cacao y cereales, éstos, en la base de la nutrición mundial (Richard, 2007).

En la Tabla 1 se indican las principales species de hongos micotoxigénicos más importantes y las micotoxinas más comunes producidas por ellos.

Tabla 1. Algunas micotoxinas más comunes relacionadas con los hongos micotoxigenicos (adaptada desde Paterson y Lima, 2010).

Hongos	Micotoxinas
Aspergillus carbonarius, A. ochraceus	Ocratoxina A (OTA)
A. flavus	Aflatoxina B_1 (AFB_1), AFB_2
A. parasiticus	AFB_1, AFB_2,AFG_1, AFG_2
A. niger	OTA, Fumonisinas (FBs)
A. terreus, A. clavatus	Patulina (PAT)
Fusarium culmorum, F. graminearum	Nivalenol (NIV), Deoxynivalenol (DON)
F. equiseti	Zearalenona (ZEA)
F. cerealis, F. poae	NIV
F. sporotrichioides	Toxina T2 (T2)
F. verticilloides (=F. moniliforme)	FB_1
Penicillium expansum, P. roqueforti	PAT
P. verrucosum	OTA

Además, la colonización fúngica y la posterior contaminación con micotoxinas puede ocurrir en las diversas etapas de la producción de un alimento o pienso: en el campo, después de la cosecha y durante el procesado, almacenamiento (depósitos, silos, etc), tratamiento y transporte (Binder, 2007).

A nivel mundial, aproximadamente el 25% de los cultivos se ven afectados por micotoxinas con enormes pérdidas económicas anuales (Gutleb et al., 2002).

Las micotoxinas pueden causar una respuesta tóxica cuando son ingeridas, inhaladas y/o absorbidas por los animales superiores (Richard, 2007). Las micotoxinas también pueden entrar en la cadena alimentaria a través de la carne u otros productos animales como la miel, los huevos, el queso y la leche de animales que han consumido alimentos contaminados por micotoxinas (http://www.efsa.europa.eu/de/topics/topic/mycotoxins.htm).

Desde el descubrimiento de las AF en 1960 y los efectos perjudiciales sobre la salud animal y humana, las normativas de los de organismos competentes a nivel mundial, como el FAO/WHO, Comité Mixto de Expertos en Aditivos Alimentarios (Joint Expert Committee on food Additivies-JECFA) y europeos como la Agencia Europea de Seguridad Alimentaria (EFSA, European Food Safety Authority; van Egmond et al., 2007) han establecido niveles máximos permitidos de micotoxinas en alimentos y piensos. En la actualidad, se conocen entre 300 y 400 micotoxinas de las cuales la Comisión Europea ha fijado el contenido máximo únicamente de unas 20 micotoxinas en varios productos alimenticios y piensos (EC 1881/2006).

1.2 *Fusarium* spp. and fusarotoxins

Several *Fusarium* species are widespread pathogens on small-grain cereals (soft and durum wheat, barley, oats, rye and triticale) around the world, including all European cereal-growing areas.

Fusarium spp. are responsible of roots and crowns desease, known as *Fusarium* head blight (FHB), which affects kernels, single ear spikelets or entire heads and scab of the kernels. The reduction in crop yield is often estimated between 10% and 40% (Bottalico and Perrone, 2002).

Mycotoxigenic species isolated from FHB of wheat in Europe and main mycotoxins produced are shown in Table 2.

Table 2. Mycotoxigenic species and their produced mycotoxins, isolated from FHB of wheat in Europe.

Species	Geographical Identification (Europe)		Mycotoxins
	North/Centre	South	
F. graminearum	+++	+++	DON, NIV, ZEA, AcDON, FUS
F. avenaceum	+++	++	MON, BEA, ENS
F. culmorum	+++	++	DON, ZEN, ZOH,NIV
F. poae	++	+	NIV, BEA, DAS, FUS-X, ENS
F. equiseti	++	+	DAS, ZEN, ZOH
F. tricinctum	+	+	MON, BEA, ENs
F. cerealis	+	±	NIV, FUS-X, ZEA, ZOH
F. sporotrichioides	+	±	T2, HT2, T2ol, NEO
F. acuminatum	±	±	T2, NEO
F. subglutinans	±	-	MON, FUS
F. solani	±	-	-
F. oxysporum	±	-	ENs
F. proliferatum	+	+	FUS
F. temperatum	+	+	ENs

AcDON = Monoacetyl-deoxynivalenols (3-AcDON, 15-AcDON); BEA = Beauvericin; DAS = Diacetoxyscirpenol; DON = Deoxynivalenol (Vomitoxin); ENS = Enniatins; FUS-X = Fusarenone-X (4-Acetyl-NIV); HT2 = HT-2 toxin; MON = Moniliformin; NEO = Neosolaniol; NIV = Nivalenol; T2 = T-2 toxin; T2ol = T-2 tetraol; ZEA = Zearalenone; ZOH = zearalenols (α and β isomers); data collected form Gäumann et al. (1947), Bottalico and Perrone (2002), Logrieco et al. (2002), Scauflaire et al. (2012).

DON, NIV, other trichothecene derivatives, FBs and ZEA are the most common *Fusarium* mycotoxins, which can frequently occur at biologically significant concentrations in European cereals. These compounds can occur naturally in cereals, individually or in combination, depending on the producing fungal species (Jelinek et al., 1989). Maize is the most susceptible crop to *Fusarium* mycotoxins (particularly important are FBs) contamination, while wheat and barley

are subjected to contamination of DON, NIV and, at lesser extent ZEA, T-2 toxin and related trichothecenes.

Fusarium spp. are capable to produce, under determinate environmental condition, hexadepsipeptidic structurally related mycotoxins, such as beauvericin (BEA) and enniatins (ENs) and an isoprenoid mycotoxin, fusaproliferin (FUS). These mycotoxins have been called by Jestoi et al. (2008) "emerging" mycotoxins since, there are limited data worldwide about their toxicity, occurrence, and contamination levels. Nowadays, "emerging" mycotoxins are called also "minor" mycotoxins to distinguish them to the majors or "traditional" *Fusarium* mycotoxins, like trichothecenes, ZEA or FB.

In the last three decades, data about their toxicity and contamination have been continuously growing up. Thus, due to the fact that an intense toxicological evaluation of certain mycotoxins (DON, T2, HT2, ZEA, FB_1+FB_2, AFs, and OTA) have been carried out, the European Union (EU) has set (or will set in the near future) the maximum permitted level of these mycotoxins in food and feed (Jestoi et al., 2004b). As far as the minor mycotoxins (BEA, ENs, FUS) are concerned, maximum permitted levels have not been established yet due to the lacking data related to their occurrence, contamination level, and toxicity (Jestoi et al., 2008). However, it is one of the main topics in progress by EFSA (http://www.efsa.europa.eu/en/topics/topic/mycotoxins.htm).

1.3 Minor *Fusarium* mycotoxins

1.3.1 Beauvericin

BEA is a cyclic hexadepsipeptide that was originally isolated from the soil-borne entomopathogenic fungus *Beauveria bassiana* (Wang and Xu, 2012) and produced by several strains of *Fusarium* (Logrieco et al., 1998).

BEA consists of three D-hydroxyisovaleryl and three N-methylphenylalanyl residues in an alternating sequence. In the BEA structure the three aromatic amino acids are linked by peptide bonds that form alternate links to intra-molecular ester lactones to form a depsipeptide structure (Figure 1).

BEA affects human and animal health, due to its contamination of food commodities like corn, rice and wheat (Leslie and Summerell, 2006). Main strains of *Fusarium* fungi that are capable to syntethize BEA are shown in Table 2.

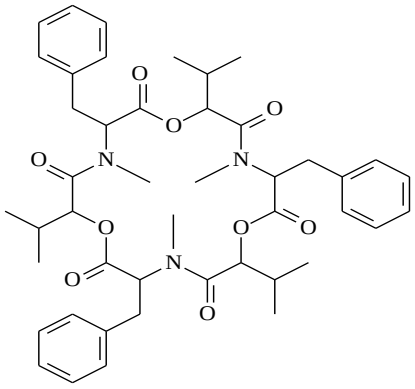


Figure 1. Chemical structure of beauvericin.

1.3.1.1 Biological properties

BEA possess a wide range of biological properties. BEA is a well-known insecticidal compound. Insecticidal activity was investigated against several model organisms such as: *Calliphora erytrocephala*, *Aedes aegypti* (Grove and Pople, 1980), *Spodoptera frugiperda* (Fornelli et al., 2004), *Leptinotarsa decemlineata* (Gupta et al., 1991) and *Schizaphis graminum* (Ganassi et al., 2002).

BEA is an antibacterial compound. This biological activity have been demonstrated against human, animal and plant pathogenic bacteria, both Gram-positive and Gram-negative (Castelbury et al., 1999; Nilanonta et al., 2000; Meca et al., 2010a; Xu et al., 2010). Human gram-positive bacteria sensitive to BEA are *Bacillus pumilus* LACB101, *B. cereus*, *B. mycoides*, *B. sphaericus*, *Paenibacillus alvei*, *P. azotofixans*, *P. macquariensis*, and *P. pulvifaciens*, *P. validus*, *Enterococcus faecium*, *Yersinia enterocolitica, Listeria monocytogenes, Clostridium perfringens, Mycobacterium tuberculosis,* and the anaerobes *Eubacterium biforme*, *Peptostreptococcus anaerobius*, *P. productus* and *Bifidobacterium adolescentis*. The gram-negative bacteria sensitive to BEA are *Escherichia coli, Salmonella enterica, Shigella dysenteriae* and *Pseudomonas aeruginosa* (Castelbury et al., 1999; Meca et al., 2010a). Moreover, antibacterial activity of BEA was reported by Xu et al. (2010) against six test bacteria (*B. subtilis*, *Staphylococcus haemolyticus*, *P. lachrymans*, *Agrobacterium tumefaciens*, *E. coli* and *Xanthomonas vesicatoria*) by *in vitro* methods.

BEA is an antihelmintic compound (Pleiss et al., 1996) and inhibits *Plasmodium falciparum* proliferation (Nilanonta et al., 2000). The toxicity have been reported also against *Artemisia salina* (Hamill et

al., 1969). Antifungal activity have been proved. BEA in combination with miconazole and ketoconazole has been considered to have synergistic antifungal effects against *Candida albicans* and *C. parapsilosis*. However, *C. albicans* showed flucanozole-resistant effect (Fukuda et al., 2004; Zhang et al., 2007). Moreover, phytotoxic activity of BEA was reported in tomato protoplasts (Paciolla et al., 2004) and maize roots of tolerant cv. Lucia (Pavlovkin et al., 2006).

1.3.1.2 Biochemical activity

The ionophoric property seems to be the cause of BEA toxicity. Ionophores are compounds that are able to transport small ions across lipid membranes. They can be carrier type (Ojicius et al., 1991) or incorporated into both mammalian cells and synthetic membranes forming a cation-selective channel (Kouri et al., 2003) and uncouple oxidative phosphorylation (Wallace and Starkov, 2000). BEA, as ionophore, is capable to promote the transport of mono- and divalent cations through membranes leading to toxic actions via disturbances in their normal physiological concentrations. The ability to create pores is not, however, excluding the ability of BEA to act also as a carrier (Jestoi, 2008).

These ionophoric complexes usually consist of one cation and one ionophore (1:1); in addition, BEA forms a sandwich-structure with a molecular ratio of 2:1 with cations, enabling the complex to span the membrane (Kouri et al., 2003).

Moreover, it have been proposed that BEA interacts with cell membranes (ionophoric activity) which causes an increase in the intracellular Ca^{2+} concentration. In some mammalian cells, BEA induced

cell death involved Ca^{2+}-dependent pathway, by which BEA induced a significant increase in intracellular [Ca^{2+}] that leads to a combination of cellular apoptosis and necrosis responses (Jow et al., 2004).

The mechanism(s) underlying BEA-induced [Ca^{2+}] increase and the direct evidence of BEA as ionophore affecting extracellular Ca^{2+} influx have not yet been clearly understood, however the cytotoxicity related to Ca^{2+} flux have been demonstrated (Jow et al., 2004; Lin et al., 2005; Chen et al., 2006).

The potential mechanism of action of BEA is reported in Figure 2.

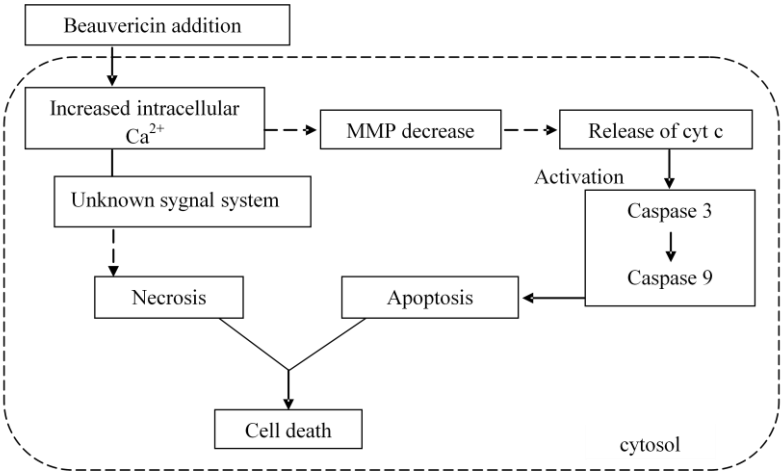


Figure 2. Mechanism of action of BEA. Dashed arrow indicate those mechanism that are still unclear. MMP = mitochondrial membrane potential.

The influence of BEA on physiological cell ion balance involve several ions, as indicated by the electomechanical and –physiological observations carried out on isolated smooth and heart muscle

preparations of guinea pig: BEA affects the calcium current with effects on contractility and interacts with the Na^+ inward and K^+ outward currents (Lemmens-Gruber et al., 2000). In isolated rat mitochondria, BEA induced a concentration dependent drop of mitochondrial membrane potential ($\Delta\Psi m$), uncoupled oxidative phosphorylation, induced mitochondrial swelling and decreased calcium retention capacity of the mitochondria. The mitochondrial effects were strongly connected with the potassium (K^+) ionophoric activity (Tonshin et al., 2010).

BEA also formed non-covalent products, i.e. interchelate with DNA with different stoichiometrics (1:1–1:10) with no preferred base sequences (Pocsfalvi et al., 1997). It have been also proposed that when the membrane damage is large enough, BEA-molecules can enter the nucleus and gain access to DNA. DNA-BEA–adduct formation takes place, and damaged areas of DNA are fragmentable by calcium-dependent endonuclease (Pocsfalvi et al., 1997).

In rat liver microsomes, BEA is one of the most powerful and specific inhibitors of the ACAT enzyme (acyl-CoA: cholesterol acyltransferase). ACAT enzyme catalyzes the conversion of cellular cholesterol and long chain fatty acyl-CoA to cholesteryl ester, playing an important role in cholesteryl ester accumulation in atherogenesis and in cholesterol absorption from intestines (Tomoda et al., 1992).

1.3.1.3 Toxicological activity

In vivo studies about BEA toxicity are scarce. Table 3 shows *in vivo* studies with BEA collected in the published literature.

Table 3. *In vivo* studies of beauvericin.

Animal	Dosage/Route	Observed effects	Reference
Mouse	Oral Intraperitoneal	LD_{50} ≥ 100 mg/Kg b.w. LD_{50} ≥ 10 mg/Kg b.w.	Omura et al., 1991
Broiler	Oral 1.43 mg/Kg (BEA) + 9.8 mg/Kg (DON) + 1.04 mg/Kg (MON) + 0.105 (FB_1) feed	No negative effect on live weight gain, feed conversion rate, blood parameters, growing and meat quality. Increase heart weight	Leitgeb et al., 1999
Duckling	Gastric intubation/100 mg/Kg b.w.	No 7-day LC_{50} obtained at doses up to 100 mg/Kg b.w.	Vesonder et al., 1999
Turkey	Oral 0-2.5 mg/Kg (BEA) + 0-2.4 mg/Kg (MON)	No effects on growth, carcass and meat of quality	Leigeb et al., 2000
Broiler	Oral 12 mg/Kg (BEA) + 2.7 mg/Kg (MON)	No effects on growth, carcass traits and composition. No residues in selected internal organs and meat quality	Zollitsch et al., 2003
Pig	Oral bolus 0.05 mg/Kg b.w.	-	Devreese et al., 2013

b.w. = body weight.

BEA exerts a cytotoxic activity against several human and animal cell lines. Different endpoints and different cell lines have been chosen to assess BEA cytotoxicy. Table 4 summarizes the type of cells, the toxicity endpoint, the time of exposure and the median inhibition concentration (IC_{50}) determined by *in vitro* methods.

Table 4. *In vitro* cytotoxicity studies on beauvericin, as determined by different toxicity endpoint, times of exposure and cell types.

Cell lines	Cell types	Assay	Time of exposure (h)	IC_{50} (μM)	Reference
A549	Human lung carcinoma	SRB	72	1.43 ± 0.27	Lee et al., 2008
		MTT	24	4.5 ± 0.35	Lin et al., 2005
BC-1	Human breast cancer	SRB	24	10 > 20 μg/mL	Nilanonta et al., 2002
CHO-K1	Chinese hamster ovary	NR	24 48 72	17.22 ± 1.20 6.20 ± 0.06 3.80 ± 0.18	Ruiz et al., 2011b
		MTT	24 48 72	similar to NR IC_{50} (not shown)	
		NR MTT	24	17.22 12.08	Ferrer et al., 2009
Caco-2	Human colon carcinoma	MTT	24 48	24.6 12.7	Prosperini et al., 2012
CCRF-CEM	Human leukemia	MTT	24	2.46 ± 0.12	Jow et al., 2004
GLC-4	Breast adenocarcinoma	MTT	72	1.88 ± 0.03	Dornetshuber et al., 2009a
GLC-4/adr	ABCC1-, MVP-overexpressing subline	MTT	72	2.06 ± 0.29	Dornetshuber et al., 2009a
HCT-15	Human colon carcinoma	SRB	72	1.86 ± 0.12	Lee et al., 2008

Table 4. Continued.

Cell lines	Cell types	Assay	Time of exposure (h)	IC_{50} (μM)	Reference
Hep-G2	Human hepatic carcinoma	Alamar Blue	24	0.6 - 1.1	Ivanova et al., 2006
		BrdU		1.4 - 4.0	
HL-60	Promyelocityc leukaemia carcinoma	MTT	72	2.27 ± 0.04	Dornetshuber et al., 2009a
		Trypan blue	24	≈15	Calò et al., 2004
HL-60/vinc	ABCB1- overexpressing subline	MTT	72	2.19 ± 0.02	Dornetshuber et al., 2009a
HL-60/adr	ABCC1- overexpressing subline	MTT	72	3.23 ± 0.74	Dornetshuber et al., 2009a
HT-29	Human colon carcinoma	MTT	24	15.0	Prosperini et al., 2012
			48	9.7	
KB-3-1	Epidermal carcinoma	MTT	72	2.85 ± 1.02	Dornetshuber et al., 2009a
		SRB	24	10 > 20 μg/mL	NIlanonta et al., 2002
KBC-1	ABCB1- overespressing subline	MTT	72	3.79 ± 0.20	Dornetshuber et al., 2009a
MDA-MB-231	Alveolar epithelial cell	MTT	72	2.4 ± 0.79	Dornetshuber et al., 2009a
MDA-MB-231/adr	ABCG2- overexpressing	MTT	72	4 ± 0.76	Dornetshuber et al., 2009a
MRC-5	Human fetal lung fibroblast	Alamar Blue	24	4.7 - 5.0	Ivanova et al., 2006
		BrdU		0.6 - 22.2	
NSCLC A549	Human non-small cell lung cancer	MTT	24	4.5 ± 0.35	Lin et al., 2005

Table 4. Continued.

Cell lines	Cell types	Assay	Time of exposure (h)	IC_{50} (µM)	Reference
PK15 cells	Porcine kidney	MTT	24	5.0 ± 0.6	Klarić et al., 2010
		LDH	48	6	Klaric et al., 2008
		Trypan blue	24	6	Klaric et al., 2007
		Alamar blue	24	12	Uhlig et al., 2005
SK-OV-3	Human ovarian carcinoma	SRB	72	1.39 ± 0.16	Lee et al., 2008
SK-MEL-2	Human skin melanoma	SRB	72	1.47 ± 0.17	Lee et al., 2008
SW1537	Small cell lung carcinoma	MTT	72	3.17 ± 0.03	Dornetshuber et al., 2009a
SW1537/2R120	ABCC1-, MVP-overexpressing subline	MTT	72	3.53 ± 0.74	Dornetshuber et al., 2009a
SW1537/2R160	ABCB1- overexpressing	MTT	72	3.00 ± 1.1	Dornetshuber et al., 2009a
U-937	Monocytic lymphoma	Trypan blue	24	≈30 (CC_{50})	Caló et al., 2004
Vero cells	African green monkey kidney fibroblast	MTT NR	24, 48, 72	6.25 - 10.02 6.77 - 11.08	Ruiz et al., 2011a
		SRB	24	10->20 µg/mL	Nilanonta et al., 2002
XPA, XPAcorr, XPD and XPDcorr	Skin fibroblasts	MTT	72	2.0	Dornetshuber et al., 2009b

NR = Neutral Red; MTT = tetrazolium salt; SRB = Sulforhodamine B; LDH = Lactate dehydrogenase; IC_{50} = 50% inhibitory concentration; CC_{50} = 50% cytotoxic concentration.

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (ROS), and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage to important biomolecules, such as DNA, proteins and lipids and plays a role in the initiation or progression of numerous disorders, including atherosclerosis, neurodegeneration and even cancer (Finkel and Holbrook, 2000). It has been hypothesized that cellular damage produced by BEA could involve also oxidative stress. ROS generation and membrane lipid peroxidation (LPO) have been observed in cell culture of mammalian CHO-K1 and PK-15 cells exposed to BEA (Klarić et al., 2007; Ferrer et al., 2009). Moreover, the involvement of the major cellular antioxidant, the enzyme glutathione (GSH) has been reported in PK 15 cells (Klarić et al., 2007).

Compounds that interact with the DNA of living cells and cause the loss of structural and functional integrity of DNA, involving a single gene or a group of genes, are defined genotoxic. The damage can be chromosomal, which includes the formation of micronucleus (MN), chromosome aberration (CA), sister-chromatid exchange (SCE), or DNA lesions such as DNA adduct formation, single-strand (SSB) and double-strand breaks (DSB), alkali labile sites, cross-link and oxidative damage by ROS production. One of the most dangerous DNA lesions is the DSB. A single DSB can cause apoptosis (Barzilai and Yamamoto, 2004).

To evaluate DNA damage induced by substances in cell cultures *in vitro*, several genotoxicity tests have been developed. Single cell gel electrophoresis or Comet assay is a rapid and sensitive method for detection of DNA SSB, DSB and alkali labile sites, which are the

consequence of direct DNA damage or they occur as intermediates formed during the repair of DNA lesions (Singh et al., 1988).

Genotoxic activity of BEA has been evidenced by Klarić et al. (2010). Çelik et al. (2010) observed that BEA induced CA (chromatid, chromosome breaks and fragment), SCE and MN. Negative results were obtained by the Ames mutagenicity assay; however it was moderately toxic by the bacterial bioluminescence assay (Fotso and Smith, 2003; Kouvelis et al., 2011).

1.3.1.4 Occurrence in food

The natural occurrence of BEA in food and feed is reported in Table 5.

Table 5. Natural occurrence of BEA, ENs A, A_1, B and B_1 and other mycotoxins in food and feed.

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Barley	22/22	<3.8 - 9760	BEA, DON, 3AcDON, NIV, HT-2, T-2, ZEN, ENs A, A_1, B, B_1, MON	Finland	Jestoi et al., 2004a
	73/73	<3 - 270	BEA, ENs A, A_1, B, B_1	Norway	Uhlig et al., 2006
	27/29	<4 - 81	BEA, ENs A, A_1, B, B_1, MON, DON, 3AcDON, NIV, HT-2, T-2, ZEN	Finland	Yli-Mattila et al., 2006
	2/4	4870 - 361570	BEA, ENs A_1, B, B_1	Spain	Meca et al., 2010b
	4/5	27500 - 149000	ENs A, A_1, B, B_1	Tunisia	Oueslati et al., 2011
	7/8	18000 - 220000	EN A, A_1, B, B_1, FUS	Morocco	Zinedine et al., 2011
	4/15	19.4 - 2029	DON, ENs A_1, B, B1, HT-2, T-2	Czech Republic	Rubert et al., 2012
Cereal based products	36/61	0.1 – 30	BEA, EN A, A_1, B, B_1	Portugal	Blesa et al., 2012
	5/9	510 - 268540	BEA, ENs A_1, B_1	Spain	Meca et al., 2010b
	21/68	100 - 795000	BEA, ENs A, A_1, B, B_1, FUS	Morocco	Manhine et al., 2011
	27/27	25100 - 464300	ENs A, A_1, B, B_1	Tunisia	Oueslati et al., 2011

Table 5. Continued.

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
	113/116	6 - 2532	ENs A, A_1, B, B_1, DON, DON-3-ß-D-glucoside, NIV	Czech Republic	Malachova et al., 2011
Grain-based products	30/30	<0.6 - 170	BEA, ENs A, A_1, B, B_1, MON, DON, 3AcDON, NIV, T-2, HT-2	Finland, Italy	Jestoi et al., 2004b
Maize	13/14	5000 - 60000	BEA, MON	Poland	Logrieco et al., 1993
	12/12	1800 - 36890	BEA, MON	Poland	Kostecki et a., 1995
	4/6	5000 – 10000	BEA, FB_1	Italy	Bottalico et al., 1995
	6/22	<1000 – 520000	BEA, FB_1, FUS	Italy	Ritieni et al., 1997b
	2/5	500	BEA, FB_1, FUS	USA	Munkvold et al., 1998
	10/10	8 - 1734	BEA, FUS	South Africa	Shephard et al., 1999
	4/4	7.6 - 238.8	BEA, FUS	South Africa	Sewram et al., 1999
	1/22	3000	BEA, FB_1, FB_2, FUS	Slovakia	Šrobárová et al., 2002
	3/99	120 - 450	BEA, FB_1, FB_2, MON, ZEN	Switzerland	Noser et al., 2001
	25/28	2470 - 813010	BEA, ENs A_1, B, B_1	Spain	Meca et al., 2010b
	6/31	8000 - 445000	BEA, ENs A_1, B, B_1, FUS	Morocco	Zinedine et al., 2011
	2/3	17000 - 29600	ENs A, A_1, B, B_1	Tunisia	Oueslati et al., 2011

Table 5. Continued.

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Maize and maize-based products	19/209	13 - 1864	BEA, FB_1, FB_2, OTA	Croatia	Jurjevic et al., 2002
Fresh maize and grains	76/80	nd - 2598	BEA, ENs A, A_1, B, B_1	Denmark	Sorensen et al., 2008
3-month old maize silage	18/20	nd - 218	BEA, ENs B, B_1	Denmark	Sorensen et al., 2008
Nuts and dried fruits	15/74	nd - 23300	BEA, ENs A, A_1, B, B_1	Spain	Tolosa et al., 2013
Oats	73/73	<3 - 270	BEA, ENs A, A_1, B, B_1	Norway	Uhlig et al., 2006
	1/1	<4 - 23	BEA, DON, 3AcDON, NIV, HT-2, T-2, ZEN, ENs A, A1, B, B1, MON	Finland	Jestoi et al., 2004a
Oats flour	171	4180 - 388380	BEA, A_1, B_1	Spain	Meca et al., 2010d
Rice	1/1	3170 - 814420	BEA, ENs A_1, B, FUS	Spain	Meca et al., 2010b
	53/70	3600 - 448700	ENs A, A_1, B, B_1, BEA, FUS	Morocco	Sifou et al., 2011
Rye	13/13	<1.3 - 6900	ENs A, A_1, B, B_1	Finland	Logrieco et al., 2002
Sorghum	3/3	72450 - 480000	ENs A, A_1, B, B_1	Tunisia	Oueslati et al., 2011

Table 5. Continued.

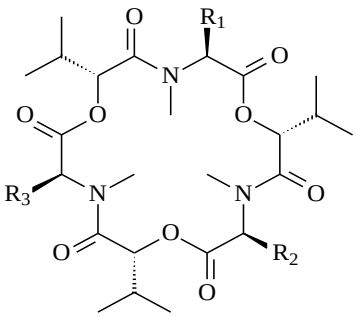
Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Wheat	14/14	1 - 18300	DON, 3AcDON, NIV, HT-2, T-2, ZEN, BEA, ENs A, A_1, B, B_1, MON	Finland	Jestoi et al., 2004a
	80/80	<3 - 5800	BEA, ENs A, A_1, B, B_1	Norway	Uhlig et al., 2006
	9/9	<0.6 - 170	BEA, ENs A, A_1, B, B_1, MON, DON, 3AcDON, NIV, T-2, T-2, ZEN	Finland	Yli Mattila et al., 2006
	13/21	2300 - 225370	BEA, EN A_1, FUS	Spain	Meca et al., 2010d
	13/13	30600 - 180600	ENs A, A_1, B, B_1	Tunisia	Oueslati et al., 2011
	12/25	2000 - 105000	ENs A, A_1, B, B_1, FUS	Morocco	Zinedine et al., 2011
Baby food	16/23	< 1 (LQ) – 1100	BEA, ENs A, A_1, B, B_1, B_4	Italy	Juan et al., 2012
	18/45	nd - 149600	ENs A, A_1, B, B_1, FUS	Spain	Serrano et al., 2012
Whole egg	85/112	nd - <1.12	BEA, EN B, B_1	Finnish residue control programme	Jestoi et al., 2009
Egg yolk	169/367	nd - 7.5	BEA, EN A, A_1, B, B_1	Finland	Jestoi et al., 2009
Feed	3/3	100 - 3000	FB_1, FUS	USA	Munkvold et al., 1998
Feed	14/52	10 - 18000	-	South Korea	Lee et al., 2010

Table 5. Continued.

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Pasta	11/25	<1 (LQ) - 106	BEA, ENs A, A_1, B, B_1, B_4	Italy	Juan et al., 2012
Organic and conventional, fresh and dried pasta	103/144	0.5 - 979.56	BEA, ENs A, A_1, B, B_1, FUS	Spain, Germany, Italy, Portugal	Serrano et al., 2013a,b
Tiger nuts	8/47	21.6 - 4440 µg/g	BEA, ENs A, A_1, B, B_1	Spain	Sebastià et al., 2012
	11/35+48	2.5 - 161	BEA, DON, AFB_1, AFB_2, AFG_1, AFG_2, OTA	Spain and unknown origin	Rubert et al., 2012

1.3.2 Enniatins

Enniatins (ENs) are cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, commonly valine (Val), leucine (Leu), and isoleucine (Ile), and three hydroxy acids, typically hydroxyisovaleric acid (Hiv). In Figure 3 the chemical structure of ENs object of this study is reported. ENs are produced by several strains of *Fusarium* (Table 2). However some species of *Verticillum* (Nilanonta et al., 2003), *Halosarpheia* (Lin et al., 2002), and *Alternaria* (McKee et al., 1997) can also produce ENs.



EN	R1	R2	R3
A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
A_1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
B_1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Figure 3. Chemical structure of enniatins.

1.3.2.1 Biological properties

ENs have been showed to exhibit a wide array of biological activities. Several studies investigated the insecticidal activity of ENs (individually and in combination; Grove and Pople, 1980; Strongman et al., 1988; Mulè et al., 1992; Ciancio 1995; Pleiss et al., 1996). This activity has been confirmed in the blowfly *C. erythrocephala,* in the mosquito larvae (*Aedes aegypti*), in the spruce budworm (*Choristoneura fumiferana*) and against *Meloidogyne javanica* (Grove and Pople, 1980; Strongman et al., 1988; Ciancio et al., 1995). However, no insecticidal activity of EN B was found by Mulè et al. (1992) against larvae of *Galleria mellonella* L. EN A showed an anthelmintic activity against *Nippostrongylus brasiliensis, Trichinella spiralis, Heterakis spumosa* and *Haemonchus contortus* Rudolphi (Pleiss et al., 1996; Jeschke et al., 2006; 2007).

ENs exhibit antibacterial activities against *Mycobacterium tuberculosis, M. tuberculosis H37Ra, M. phlei, M. paratuberculosis, Bacillus subtilis, S. aureus, S. aureus* CECT 976 and CECT 240, *E. coli*, *E. coli* CECT 4782, *Clostridium perfringens* CECT 4647, *C. perfringens* CECT 4647, *Enterococcus faecium* CECT 410, *Salmonella enterica* CECT 554, *Listeria monocytogenes* CECT 935, *Yersinia enterocolitica* CECT 4054, *Pseudomonas aeruginosa* CECT 4628, *Shigella dysenteriae* CECT 584 (Gäumann et al., 1947; 1950; Vesonder and Golinski, 1989; Supothina et al., 2004; Meca et al., 2011a; Sebastià et al., 2011).

Growth inhibitory activity was reported in human malaria parasite (*Plasmodium falciparum* K1; Supothina et al., 2004).

The antifungal activity of EN B have been confirmed by Meca et al. (2010c) on several strains of *Beauveria bassiana* (CECT 20499, CECT 20191, CECT 20412) and *Trichoderma harzianum* T22.

ENs produced necrotic lesions in potato tuber tissue (Herrmann et al., 1996) and on knapweed leaves (*Centaurea maculosa*) when exposed with acetamido-butenolide (Hershenhorn et al., 1992). Combination of EN A + EN B showed decreased level of leaf and root development, wilting of shoots, necrosis of leaves and loss of turgor (Gäumann et al., 1960; Burmeister and Plattner, 1987).

1.3.2.2 Biochemical activity

A similar pattern for ion transporting through membranes reported for BEA was proposed also for ENs. The primary action is the ionophoric property, which enable ENs to form stable complexes with cations, and transport them into the lipophilic phase (Logrieco et al., 2008). The ability of the ENs to form complexes with alkali metal ions and increase the cationic permeability of membranes has been previously documented (Benz, 1978; Ivanov et al., 1973). In particular, cations transport by ENs in liposome seems to involve a mobile carrier mechanism which is selective for K+ versus Na+, requires two EN molecules and is realized by a "sandwich"model (Kamyar et al. 2004). ENs form both 1:1 and 2:1 EN:cation complexes with alkali, alkaline earth, and various transition metal ions. The probability of the 3:2 conformation is much less than the two other conformations (Ovchinnikov et al., 1974). It is suggested that electronic, inductive or steric effects could indirectly stabilize the 2:1 complex. Cation selectivity was ranked $K^+ > Ca^{2+} \geq Na^+ > Mg^{2+} > Li^+$. In addition, the

transport efficiency appears to be related to the hydrophobic trait of the EN molecules (Kamyar et al., 2004).

The inhibition of the activity of ACAT by ENs has been demonstrated (Tomoda et al., 1992). Recently, it was shown that ENs inhibit one of the major multidrug efflux pumps such as Pdr5p in *Saccharomyces cerevisiae* cells at non-toxic concentrations (Hiraga et al., 2005). The inhibition mechanism is clearly different from its function as ionophores (Hiraga et al., 2005). This property of the ENs may be important for the clinical use in combination with chemotherapeutic drugs.

ENs and BEA interaction with membrane- located ATP-binding cassette (ABC) transporters, especially with ABCB1 and ABCG2 transporter suggests influences on bioavailability of xenobiotics and pharmaceuticals (Dornetshuber et al., 2009a).

The mitochondriotoxic properties of ENs have recently been demonstrated in isolated rat mitochondria (Tonshin et al., 2010). The mitochondrial effects were strongly connected with the K^+ ionophoric activity. ENs induced K^+ uptake by mitochondria. Moreover, they decreased the calcium retention capacity of the mitochondrion matrix leading to the $\Delta\Psi m$ collapse via permeability transition pore (PTP) opening (Hoornstra et al., 2003; Tonshin et al., 2010).

1.3.2.3 Toxicological activity

Few studies have been developed *in vivo*. Table 6 illustrate *in vivo* studies carried out with ENs A, A_1, B and B_1.

Table 6. *In vivo* studies of enniatins.

Animal	Dosage/Route	Observed effects	Reference
Guinea pig, rabbit	3 × 50 mg (EN A)/subcutaneous	no changes in blood, urine or organs; EN A was not metabolized but remained intact under the skin	Gäumann et al., 1950
Mouse	6 × 1.0 or 0.5 mg/Kg bw. (EN A)/ oral and Subcutaneous	No toxic effects	Gäumann et al., 1950
Rat	2 mg	No toxic effects	Bosch et al., 1989
Mouse	1.25–40 mg/Kg b.w., (every 8.hour)/intraperitoneal	Death in 2–5 days (10–40 mg/Kg bw.), reduced weight, no anti-HIV activity	MCKee et al., 1997
Pig	0.05 mg/Kg b.w. Oral bolus	Absorption EN B>B_1>A_1>A>BEA 73.4, 35.2, 11.6 and 6.8 ng/mL, respectively.	Devreese et al., 2013

Cytotoxic activity has also been observed in several cell lines exposed to ENs. The type of cells, the toxicity endpoint, the time of exposure and the IC_{50} determined are summarized in Table 7.

Table 7. *In vitro* cytotoxicity studies on ENs A, A_1, B and B_1, as determined by different toxicity endpoint, times of exposure and cell types.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
A427	Human lung cancer	MTT	72	1.61 ± 0.14	Dornetshuber et al., 2007
A549				4.08 ± 1.04	Dornetshuber et al., 2007
Caco-2	Human colon adenocarcinoma	NR	3, 24	EN B: 10 ± 3.8 - 2.1 ± 0.4	Ivanova et al., 2012
		MTT	24	EN A: >30 EN A1: 12.3 EN B: >30 EN B1: 19.5	Meca et al., 2012a
		MTT	24, 48	EN B: > 30	Meca et al., 2011a
		MTT	24, 48	EN A: - ; 9.3 ± 0.6 EN A1: 12.3 ± 4.3; 2.7 ± 0.8 EN B: - EN B1: 19.5 ± 4.1; 11.5 ± 5.3	Meca et al., 2011b
		MTT	24	EN A: >30 EN A1: 12.3 EN B: >30 EN B1: 19.5	Meca et al., 2010d
		MTT	72	EN_{mix}: 1.99 ± 0.09	Dornetshuber et al., 2007

Table 7. Continued.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
CHO-K1	Chinese hamster ovary	MTT	24, 48, 72	EN A: >7.5 - 3.33 ± 0.22 EN A_1: 8.8 ± 2.29 - 1.65 ± 0.06 EN B: 11.0 ± 2.65 - 2.80 ± 0.16 EN B_1: 4.53 ± 1.23 - 2.47 ± 0.29	Lu et al., 2013
C6	Rat glioma	MTT	3, 24	EN_{mix}: 2.5 – 10	Watjen et al., 2009
GBL1	Human Glioblastoma	MTT	72	EN_{mix}: 2.65 ± 0.3	Dornetshuber et al., 2007
GBL2		MTT	72	EN_{mix}: 2.29 ± 0.05	Dornetshuber et al., 2007
GBL3		MTT	72	EN_{mix}: 2.55 ± 0.14	Dornetshuber et al., 2007
GBL4		MTT	72	EN_{mix}: 2.33 ± 0.3	Dornetshuber et al., 2007
GLC-4	Breast adenocarcinoma	MTT	72	EN_{mix}: 2.4 ± 1.53	Dornetshuber et al., 2007; Dornetshuber et al., 2009a
GLC-4/adr	ABCC1-, MVP-overexpressing subline	MTT	72	EN_{mix}: 1.41 ± 0.83	Dornetshuber et al., 2009a
HepG2	Human hepatic cancer	MTT	24, 48	EN A: 26.2 ± 7.6; 11.4 ± 4.6 EN A_1: 11.6 ± 5.7; 2.6 ± 0.6	Meca et al., 2011b

Table 7. Continued.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
				EN B: - EN B_1: 24.3 ± 6.3; 8.5 ± 3.7	
		MTT	3, 24	ENs A_1, B, B_1 10	Watjien et al., 2009
		Alamar Blue BrdU	24	EN A: 7.3-9.4; 1.6-2.5 EN A_1: 11.7-18.1; 2.6-4.2 EN B: 206.7-435.9; 0.9-1.1 EN B_1: 9.2-36.0; 2.8-3.5	Ivanova et al., 2006
HL60	Promyelocityc leukaemia carcinoma	MTT	72	EN_{mix}: 1.74 ± 0.2	Dornetshuber et al., 2007, 2009a
HL60/vinc	ABCB1 – overespressing subline	MTT	72	EN_{mix}: 2.4 ± 0.14	Dornetshuber et al., 2009a
HL60/adr	ABCC1 Overespressing subline	MTT	72	EN_{mix}: 2.1 ± 0.12	Dornetshuber et al., 2009a
HT 29	Human colon adenocarcinoma	MTT	24, 48	EN A: - ; 8.2 ± 1.8 EN A_1: 9.1 ± 2.2; 1.4 ± 0.7 EN B: - ; 2.8 ± 0.9 EN B_1: 16.8 ± 4.3; 3.7 ± 0.7	Meca et al., 2011b
HUVEC	Human	MTT	72	EN_{mix}: 7.89 ± 0.214	Dornetshuber et al.,

Table 7. Continued.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
	endothelial cells				2007
H4IIE	Rat hepatoma	MTT	3, 24	ENs A_1, B, B_1 1-1.5	Watjien et al., 2009
KB-3-1	Epidermal carcinoma	MTT	72	EN_{mix}: 1.95 ± 0.12	Dornetshuber et al., 2007; Dornetshuber et al., 2009a
KBC-1	ABCB1-overespressing subline	MTT	72	EN_{mix}: 1.77 ± 0.04	Dornetshuber et al., 2009a
MDA-MB-231	Alveolar epithelial cell	MTT	72	EN_{mix}: 2.36 ± 1.57	Dornetshuber et al., 2007; 2009a
MDA-MB-231/adr	ABCG2-overexpressing	MTT	72	EN_{mix}: 3.18 ± 1.70	Dornetshuber et al., 2009a
MGC	Human glioblastoma	MTT	72	EN_{mix}: 3.04 ± 0.58	Dornetshuber et al., 2007
MRC-5	Fibroblast-like foetal lung	Alamar Blue BrdU	24	EN A: 3-5-3.8; 0.6-0.8 EN A_1: 5.9-6.9; 1.1-1.4 EN B: 1.9-9.8; 1.9 3.6 EN B_1: 4.5-4.7; 1.2-1.4	Ivanova et al., 2006
OS 9	Human osteosarcoma	MTT	72 h	EN_{mix}: 3.55 ± 0.77	Dornetshuber et al., 2007
OS 10		MTT	72	EN_{mix}: 2.10 ± 0.15	Dornetshuber et al., 2007
PK-15	Porcine kidney cells	Alamar Blue	24	EC_{50} 41 μM (EN A+A_1+B+B_1)	Uhlig et al., 2005
SAOS	Human	MTT	72	EN_{mix} 2.13 ± 0.07	Dornetshuber et al.,

Table 7. Continued.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
	osteoscarcoma				2007
SW1537	Small cell lung carcinoma	MTT	72	EN_{mix} 2.16 ± 0.12	Dornetshuber et al., 2009a
SW1537/2 R160	ABCB1-overexpressing subline	MTT	72	EN_{mix} 2.69 ± 0.6	Dornetshuber et al., 2009a
SW480	Small cell lung carcinoma	MTT	72	EN_{mix}: 4.00 ± 1.12	Dornetshuber et al., 2007
U2-OS	Osteosarcoma	MTT	72	EN_{mix}: 1.77 ± 0.24	Dornetshuber et al., 2007
U373	Human glioblastoma	MTT	72	EN_{mix}: 4.88 ± 0.09	Dornetshuber et al., 2007
T98-G		MTT	72	EN_{mix}: >10	Dornetshuber et al., 2007
VM 8	Melanoma	MTT	72	EN_{mix}: 3.19 ± 0.85	Dornetshuber et al., 2007
VM 18		MTT	72	EN_{mix}: 2.67 ± 0.08	Dornetshuber et al., 2007
VM 22		MTT	72	EN_{mix}: 1.75 ± 0.15	Dornetshuber et al., 2007
VM 33		MTT	72	EN_{mix}: 9.65 ± 0.13	Dornetshuber et al., 2007
VM 25		MTT	72	EN_{mix}: 2.72 ± 0.11	Dornetshuber et al., 2007
VL-8	human lung cancer	MTT	72	EN_{mix}: >10	Dornetshuber et al., 2007

Table 7. Continued.

Cell lines	Cell types	Parameter	Time of exposure (h)	IC_{50} (μM)	Reference
V79	Chinese hamster fibroblast	NR	48	EN B: 4.4	Behm et al., 2009
WI-38	embryonic fibroblasts	MTT	72	EN_{mix}: >10	Dornetshuber et al., 2007

NR = Neutral Red; MTT = tetrazolim salt; SRB = Sulforhodamine B; LDH = Lactate dehydrogenase; IC_{50} = 50% inhibitory concentration; EC_{50} = 50% effective concentration. EN_{mix} = 3% A, 20% A_1, 19% B, 54% B_1.

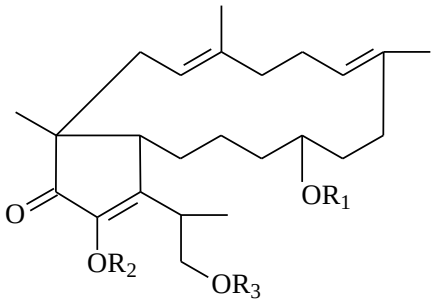
1.3.2.4 Occurrence in food

ENs A, A_1, B and B_1 have been found in food and feed, worldwide. The natural occurrence of these ENs is reported in Table 5.

1.3.3 Fusaproliferin

Fusaproliferin (FUS) is a bicyclic sesterterpene consisting of five isoprenic units, which was originally isolated from a pure culture of *Fusarium proliferatum* (Santini, 1996). FUS is produced by different *Fusarium* spp. reported in Table 2.

Fusarium-strains that produce FUS usually produce also deacetyl-FUS (Da-FUS) in a ratio of 3:1 and acetyl-FUS (Ac-FUS). The structures of FUS and related mycotoxins are shown in Figure 4.



	R_1	R_2	R_3
Da-FUS	H	H	H
FUS	H	H	CH_3CO
Ac-FUS	CH_3CO	CH_3CO	CH_3CO

Figure 4. Chemical structure of fusaproliferin, deacetyl-fusaproliferin and acetyl-fusaproliferin.

1.3.3.1 Biological and toxicological properties

The Ac-FUS form was three times more toxic than FUS. However, the deacetylated form shows limited toxicological activity compared to FUS (Ritieni et al., 1997a). Moreover, it is an inactive precursor of the FUS rather than a breakdown product (Ritieni et al., 1997a). The non-covalent interactions between single- and doublestranded DNA-oligonucleotides and FUS with 1:1 stoichiometry have been reported (Pocsfalvi et al., 2000), which may partly explain the teratogenic effects. The interchelatation was not reported to exhibit any base sequence preference in the DNA, suggesting an electrostatic type of interaction.

Cytotoxicity by *in vitro* methods was also carried out in human (Logrieco et al., 1996) and lepidopteran (Fornelli et al., 2004) cell lines, confirming the cytotoxicity of FUS (IC_{50} of 60-65 μM and approximately of 70 μM, respectively). Teratogenic effects, pathological changes and mortality was produced by FUS and Ac-FUS on chicken embryo (Ritieni et al., 1997a).

Opposite to BEA, antibacterial activity against *E. coli* and *S. aureus* was not observed with FUS (Meca et al., 2009).

1.3.3.2 Occurrence in food

Occurrence of FUS in foodstuff can be observed in Table 8.

Table 8. Occurrence of fusaproliferin and other mycotoxins in food and feed.

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Feed	2/3	500 - 30,000	FUS, BEA, FB_1, FB_2	USA	Munkvold et al., 1998
Maize	9/22	6,000 - 500,000	FUS, FB_1, BEA	Italy	Ritieni et al., 1997b
	2/5	100 - 300	FUS, BEA, FB_1, FB2	USA	Munkvold et al., 1998
	3/4	8.8 - 39.6	FUS, BEA	South Africa	Sewram et al., 1999
	9/10	7 – 62	FUS, BEA	South Africa	Shephard et al., 1999
	8/22	<25 - 8,200	FUS, BEA, FB_1, FB_2	Slovakia	Šrobárová et al., 2002
	1/31	600 (mean value)	FUS, BEA, ENs A_1, B, B_1	Morocco	Zinedine et al., 2011
Rice	3/70	300 – 19,600	FUS, BEA, ENs A_1, B, B_1	Morocco	Sifou et al., 2011
Wheat	4/31	2,000 (mean value)	FUS, BEA, ENs A_1, B, B_1	Morocco	Zinedine et al., 2011
Baby food	8/45	700 – 1,700	FUS, ENs A, A_1, B, B_1	Spain	Serrano et al., 2012
Cereals based products	5/64	2,470 – 3,170	FUS, BEA, ENs A_1, B, B_1	Spain	Meca et al., 2010b
	7/68	1,900 – 7,400	FUS, BEA, ENs A_1, B, B_1	Morocco	Mahnine et al., 2011

Table 8. Continued

Samples	Positive samples/total samples	Concentration (µg/Kg)	Co-occurring mycotoxins	Origin	Reference
Conventional and organic, dried and fresh pasta	19/144	<0.05 - 8.2	FUS, BEA, ENs A_1, B, B_1	Spain, Germany, Italy, Portugal	Serrano et al., 2013a,b

1.4 *In vitro* cytotoxicity of individual and combined mycotoxins

1.4.1 Cytotoxicity studies

In vitro methods are widely utilised for screening and ranking of mycotoxins. *In vitro* toxicology and *in vitro* data obtained from mycotoxins studies have already been considered in some instances for risk assessment purposes (Eisenbrand et al., 2002). Cells respond rapidly to toxic stress by altering, for example, metabolic rates and cell growth or gene transcription controlling basic functions. The ultimate consequence is the cytototoxicity that is considered primarily as the potential of a compound to induce cell death (Lombardi et al., 2012). Table 9 shows some of the biological endpoints for detecting cytotoxicity in cell culture.

Table 9. Biological endpoints for detecting cytotoxicity.

Endpoint	Endpoint measurement
Cell morphology	Cell size and shape, cell-cell contacts, nuclear size, shape and inclusion, nuclear vacuole formation, cytoplasmatic vacuole formation
Cell viability	Vital dye uptake, trypan blue exclusion, cell number, replacing efficiency
Cell adhesion	Attachment to culture surface, cell-cell adhesion
Cell proliferation	Increase in cell number, increase in total DNA, increase in total RNA
Membrane damage	Loss of enzyme, loss of ions cofactors, leakage across cellular membrane, leakage from pre-loaded cells

Numerous bioassays using cell culture techniques have been described for the characterization of BEA, ENs and FUS, such NR assay, the MTT, the trypan blue dye exclusion assay, Alamar Blue, BrdU and SRB assays (Table 4 and 7). The *in vitro* MTT and NR assays measure cell proliferation and survival. They are the most used for preliminary and mechanistic cytotoxicity studies of BEA (Table 4) and ENs (Table 7) in a great variety of mammalian cells (Jow et al., 2004; Lin et al., 2005; Dornetshuber et al., 2009b; Ferrer et al., 2009; Watjen et al., 2009; Ruiz et al., 2011a,b; Lu et al., 2013) and insect cell line (Fornelli et al., 2004). The MTT assays is based on the capacity for viable cells to metabolize a tetrazolium colourless salt to a blue formazan in mitochondria. The amount of formazan product produced by the dehydrogenase enzymes was directly proportional to the amount of living cells in culture. The NR assay is frequently used to evaluate the viability of cells. NR is a water soluble, weakly basic, survival dye that accumulates in lysosomes of viable cells (Ferrer et al. 2009). Compounds that injure the plasma or lysosomal membrane decrease uptake and subsequent retention of the dye.

1.4.2 *In vitro* effects of combinations of mycotoxins

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. There is relatively little information on the interaction between concomitantly occurring mycotoxins and their consequence for the toxicity (Speijers and Speijers, 2004).

The Commission Regulation (EC) 1881/2006 and its amendments set maximum levels for certain contaminants in foodstuffs as for certain mycotoxins including some *Fusarium* toxins (EC 2006). However, these maximum levels have been established taking into account only the presence of individual mycotoxins. Hence, to take into consideration toxicological "mixture" effects derived from mycotoxins into implementing human risk assessment is needed (Tajima et al., 2002; Speijers and Speijers, 2004).

Toxicological studies about the effects of mycotoxins mixtures is of a great interest in order to find out whether they could interact among themselves and produce a synergistic, additive or antagonistic effect. There are considerable research works about interactions between mycotoxins in cell cultures, especially for *Penicillium* [OTA, CIT, penicillic acid (PIA), PAT)] and *Fusarium* (DON, T-2 toxin, ZEA, FB_1, BEA,…) mycotoxins. These scientific works investigated the joint action of two or more mycotoxins combined in a mixture and exposed to different types of cells. Several mathematical methods have been applied in order to assess their interaction (Bernhoft et al., 2004, Heussner et al., 2006; Kouadio et al., 2007; Klarić et al., 2008; Ruiz et al., 2011a,b). One of them is the isobologram analysis. Isobologram analysis was introduced almost 30 years ago by Chou and Talalay (1984) for drugs combination effect studies. Recently, it have been applied successfully to determine the type of interaction between two or more mycotoxins from *Penicillium* or *Fusarium in vitro* (Bernhoft et al., 2004; Luongo et al., 2006; Luongo et al., 2008; Ruiz et al., 2011a,b; Lu et al., 2013).

1.5 Mycotoxin-induced oxidative stress

1.5.1 Reactive oxygen species and oxidative stress

Oxidative stress is defined as an imbalance between production of free radicals and oxidants or reactive oxygen species (ROS), and their elimination by antioxidant mechanisms (Finkel and Holbrook, 2000). One of the key players in the production of oxidative stress are the reactive oxygen species (ROS). Molecular oxygen is involved in ROS production. Molecular oxygen is a biradical which contains two unpaired electrons in the outer shell. The reduction of molecular oxygen by one electron at a time results in reactive intermediates that can cause oxidative stress. These ROS are the superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), hydroperoxyl radical ($HOO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$; Halliwell and Gutteridge, 1984).

The generation of ROS is due to a large number of physiological and nonphysiological processes as well as is due to normal cellular metabolism (Halliwell, 2011). Over 90% of oxygen consumed by living organisms is used to produce energy by oxidative phosphorylation with operation of the electron-transport chain via a four-electron mechanism leading to ATP and water production. Addition of one by one electron to oxygen molecule leads to ROS forms which include $O_2^{\bullet -}$, $HO^{\bullet}$ and $O^{\bullet}{}_{1/2}$ (Starkov, 2008; Figure 5).

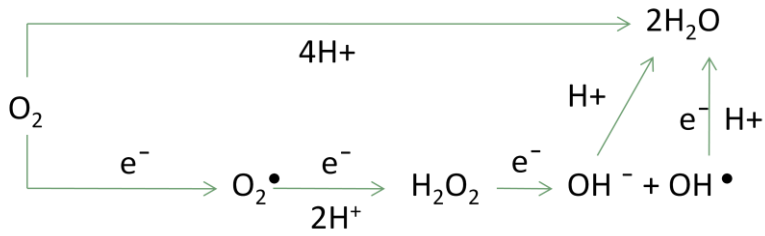


Figure 5. Stepwise reduction of molecular oxygen in biological systems leads to ROS production.

Even though the generation of ROS is an inevitable aspect of life under aerobic conditions, high levels of ROS can be generated by exogenous sources (Finkel and Holbrook, 2000; Bouaziz et al., 2008; Chaudhari et al., 2009; Ferrer et al., 2009) which perturb the normal redox balance and shift cells into a oxidative stress state. If constitutive antioxidant potential is high enough the level of reactive species quickly returns to an initial steady-state level. On the other hand if the constitutive antioxidant potential is not high enough to counteract the excess of ROS, many cellular components, such as DNA, RNA, proteins and lipids, are damaged. This leads to cellular toxicity (Ziech et al., 2011). However, the extent of oxidative needs to be excessive to damage most cells or organs by this direct pathway, as the antioxidant defense is strong and can be regulated or induced.

Oxidative stress plays important role in the etiology and/or progression of a number of human disease (Finkel and Holbrook, 2000). It is associated to atherosclerosis (Schulze and Lee, 2005), Alzheimer's and Parkinson's disease (Keane et al., 2011), cancer (Valko et al., 2006), diabetes mellitus (Houstis et al., 2006), inflammatory diseases (Koek et

al., 2011), psychological diseases (Maes et al., 2011) or aging processes (Finkel and Holbrook, 2000).

In recent years, intracellular fluorescent probes with variable specificity have been employed to assay the generation of intracellular ROS species in real-time. One of the most frequently used is the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) that facilities the rapid identification of the chronology of mycotoxins-induced injury in cells at reduced cost and time (Liu et al., 2007; Bouaziz et al., 2008; Ferrer et al., 2009).

1.5.2 Oxidative lipid damage: lipid peroxidation

Intracellular ROS generation in the hydrophobic compartment of a cell can produce lipid oxidation. As result, biomembranes may be peroxidyzed and consequently damaged. Massive cellular damage initially induced by ROS-mediated oxidative stress has been associated with LPO. LPO is one of the pathways involved in cell viability reduction induced by mycotoxins (Ferrer et al., 2009).

A general and important consequence of membrane LPO is the production of toxic aldehydes stemming from oxidative fatty acyl degradation. One of these adehydes is the malondialdehyde (MDA), which is a biomarker used to prove that LPO has occurred.

One of the most prominent assay currently being used as an index for LPO in biological system is the measurement of the thiobarbituric acid reactive substances (TBARS; Ferrer et al., 2009). This assay is based on the formation of a red adduct (absorption maximum 532 nm) between TBA and MDA raction as shown in Figure 6.

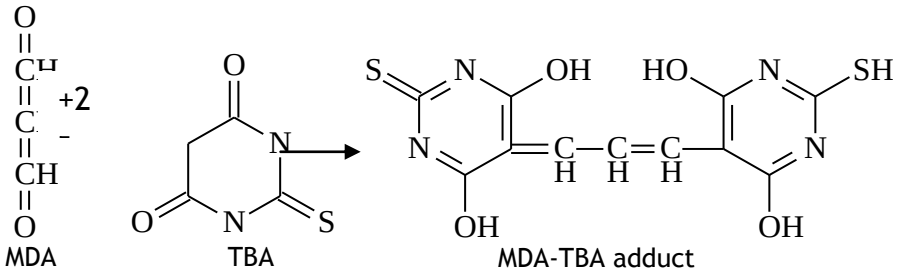


Figure 6. The chemical reaction between TBA and MDA that yields to the MDA-TBA adduct.

Mycotoxins could lead cells to the state of oxidative stress (Kouadio et al., 2005; Bouaziz et al., 2008; Chaudhari et al., 2009). No *in vivo* studies are available about the generation of ROS and induction of LPO by ENs, BEA and FUS, and very little data is available regarding *in vitro* methods.

By *in vitro* methods it have been demonstrated that BEA produced prooxidative effects increasing LPO and ROS production in porcine kidney cells (PK15; Klarić et al., 2007) and hamster ovarian cells (CHO-K1; Ferrer et al., 2009). These findings suggest an involvement of oxidative stress in BEA toxicity. Regarding to ENs, Ivanova et al. (2012) reported that EN B produced ROS generation in Caco-2 cells. On the other hand, Dornetshuber et al. (2009b) found that no ROS generation was produced after ENs mixture exposure.

1.6 Antioxidant defense systems

1.6.1 Intracellular defense system

The cellular response to the formation of ROS includes many defense mechanisms. Oxidative stress can be prevented by the action of

enzymatic and non-enzymatic antioxidant defenses. The enzymes that provide the first line of defense against superoxide and hydrogen peroxide include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx; Matés, 2000). Superoxide dismutase (SOD) and catalase (CAT) act in concert to inactivate superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), respectively. SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2, and CAT is responsible for inactivating H_2O_2 into water and oxygen. GPxs ezymes can be divided in selenium dependent and selenium independent GPxs. The Se-dependent GPxs are capable to reduce H_2O_2 and organic hydroperoxides. The Se-indipendent GPxs are inactive with H_2O_2 and only catalyzed reduction of organic hydroperoxides. GPx enzymes play a critical role in the defense against oxidative stress (Cnubben et al., 2001). If H_2O_2 escapes degradation by catalase or GPx, it can react qith iron to form an innocuous hydroxyl anion and a highly reactive $HO^{\bullet}$ via Fenton reaction (Barbosa et al., 2010). Glutathione (GSH) is an intracellular antioxidant compound that is considered as a second line of antioxidant defense (Cnubben et al., 2001).

GSH (γ-L-Glu–L-Cys–Gly) is an endogenous tripeptide and represents the most prominent low molecular weight thiol (up to 5–10 mM) present in cells. GSH and its related enzyme, such as GPx, glutathione reductase (GR) and glutathione-S-tranferase (GST) are one of the most important antioxidants in the body. GSH, that is present in its reduced form, in presence of oxidants is oxidized to glutathione disulfide (GSSG) by the GPx. The GSSG is a substrate for GR that catalyzes its reduction by NADPH leading to the regeneration of GSH, that is available to react with free radicals and hydroperoxides. The

GSH is involved also in cellular detoxification by xenobiotics, such as mycotoxins. The GSTs are thought to play a physiological role in initiating the detoxification of potential alkylating agents (Maran et al., 2009). These enzymes catalyze the reaction of such compounds with the cystein-thiol (SH-group) of GSH, creating products that are more water soluble. The detoxification process could increase the concentration of GSSG and decreased the reduced form (GSH). The metabolic pathway of GSH is represented in Figure 7.

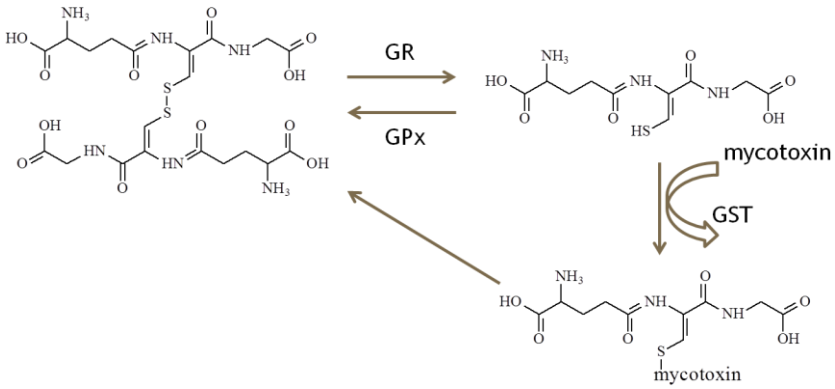


Figure 7. Metabolic pathway of glutathione.

Overall maintenance of cellular GSH and cellular redox status is a dynamic process achieved by a balance between the rate of GSH synthesis, GSH/GSSG efflux, and GSH utilization. The ability of cells to regenerate GSH (either by reduction of GSSG or new synthesis of GSH) is an important factor in efficient managing of oxidative stress within the cells (Rumora et al. 2007).

Few studies have been demonstrated on GSH depletion after BEA exposure (Klarić et al., 2007) no report data related to GSH or its related enzymes activity have been found in published scientific works.

1.6.2 Antioxidants from diet as protective system

Antioxidant compound can be provided exogenously through the diet or supplement. These antioxidant compounds include vitamins (C and E), flavonoids, carotenoids, phenolic compounds, etc. Phenolic compounds, or polyphenols constitute one of the most numerous and widely-distributed groups of substances in the plant kingdom, with more than 8,000 phenolic structures currently known. Polyphenols are products of the secondary metabolism of plants with an aromatic ring bearing one or more hydroxyl substituents (Urquiaga and Leighton, 2000). Polyphenols are widely distributed in fresh fruits, berries, black tee, red wine, purple grape juice, medicinal herbals, daily nutrition supplements, etc. constituting an integrate part of the human diet (Lombardi et al., 2012).

They exhibit a wide range of biological effects as a consecuence of their antioxidant activity, such as cancer-preventive, oxygen radical scavenging, antiproliferative and anti-inflammatory activities and stimulate the immune system. In particular, dietary polyphenols can protect cell constituents against oxidative damage limiting degenerative diseases associated to oxidative stress (Lombardi et al., 2012).

Most frequently studied polyphenols are resveratrol, quercetin and its derivates (Fig. 8); they possess antioxidant, antitumoral, anti-inflammatory, antiplatelet, and vasoprotector activities (Barcelos et al. 2011). Quercetin is a major polyphenol ingested daily by dietary intake

(20–100 mg; You et al. 2010). Pterostilbene, an analogue of resveratrol, has also been shown that has beneficial healthily properties. It possess antiproliferative, anti-inflammatory and antioxidantive effects, and presents antihyperglycemic, anticholesterol and hypolipidemic activities (Zamora-Ros et al. 2008; Chakraborty et al. 2010). The antioxidant activity of polyphenols has been widely investigated, but also their toxic and genotoxic effects. Due to the contradicting results about their ability to protect mammalian cells from cytotoxicity, studies on the level of safety are of great importance. During last decade, several studies have been carried out about the potential protective effects of polyphenols against mycotoxins effects on cell culture (Hundhausen et al. 2005; Sergent et al. 2005; Choi et al. 2010; Barcelos et al. 2011). Antioxidant effects of polyphenols and its glycosides have been previously demostrated as *in vitro* as *in vivo* after mycotoxins exposure.

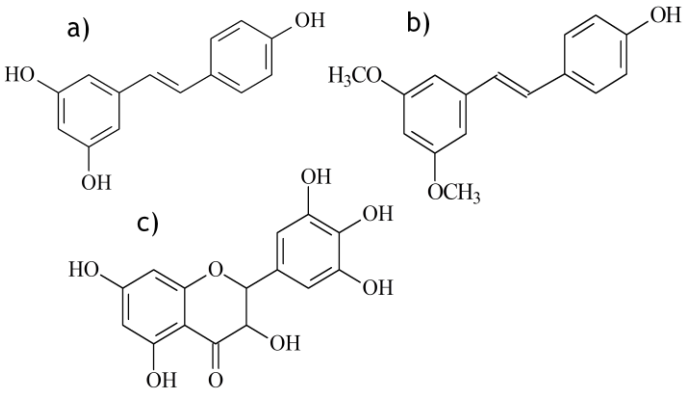


Figure 8. Chemical structure of (a) resveratrol, (b) quercetin, (c) *t*-pterostilbene.

1.7 Impairment of cell proliferation: the cell cycle distribution

The cell cycle is the entire process by which a cell undergoes cell division. It involves several processes in the nucleus and encompasses signaling pathways from plasma membrane receptors to the cytoplasm and vice versa. Cell cycle is a potentially "dangerous" process. When cells enter the cell cycle, there is no way back: they undergo cell division or they die. Therefore, if a xenobiotic compound blocks the cell cycle at one or more phases, cell can undergo apoptosis or necrosis. Cell cycle phases are: the G1 phase, where cells are preparing for DNA, RNA and protein synthesis, the S phase where DNA is synthesized, the G2 phase, where cells are preparing for mitosis and finally the M phase (mitosis) where two daughter cells are generated. Cells can remain in a quiescent phase (G0 phase) and they need growth factors to enter the G1 phase.

It have been demonstrated that mycotoxins can disturb the normal cell cycle distribution by their anti-proliferative effects on several cell types, with an accumulation of cells in one or more phases of the cell cycle (Juan-García et al., 2013a). This is due to the activation of different cell cycle checkpoint that ensure the orderly progression events and prevent aberrant mitosis in response to a range of events, including DNA damage (Visanji et al., 2004). The measure of DNA (in each phase of the cell cycle) is one of the most wide spread application of flow cytometry. It can provide useful information regarding the cytotoxic action of several substances. The most accurate method of measuring the relative number of cells in different phases of the cell cycle is to pulse-label with propidium iodide (PI) which binds to DNA by intercalating between the bases with little or no sequence

preference and with a stoichiometry of one dye per 4.5 base pairs of DNA. Cell cycle assay was performed by using PI fluorochrome in different cell lines exposed to BEA, ENs and other *Fusarium* mycotoxins demonstrating that they can alter the cell cycle distribution (Juan-García et al., 2013b).

1.8 Mechanism of apoptosis/necrosis cell death

The term apoptosis defined a genetically encoded cell death for the ordered removal of superfluous, aged or damaged cells (Kroemer et al., 1998). Necrosis is the accidental cell death that is the passive results of cellular injury (Vermes et al. 2000). Apoptosis is morphologically, biochemically and molecularly distinct from necrosis. Apoptosis is distinguished from necrosis by a highly organized mode of cell demise, followed by degradation of chromatine into internucleosomal fragments, a loss of cellular volume associated with cytoskeletal breakdown and blebbing of the plasma membrane. It also involves the activation of specific cysteine proteases (caspases) that catalyze highly selective pattern of protein degradation (Kroemer et al., 1998). Subtle changes in the plasma membrane occur before it ruptures. Thus surface exposure of phosphatidylserine residues (normally on the inner membrane leaflet) allows for the recognition and elimination of apoptotic cells by their healthy neighbors, before the membrane breaks up and cytosol or organelles spill into the intracellular space and elicit inflammatory reactions (Savill, 1996). During the process of apoptosis, mitochondria do not manifest any major ultrastructural abnormalities (Kerr et al., 1972).

In contrast to apoptosis, necrosis does not involve any regular DNA and protein degradation pattern and is accompanied by sweilling of the entire cytoplasm (oncosis) and of the mitochondrial matrix, which occur shortly before the cell membrane ruptures (Kroemer et al., 1998).

Apoptosis and necrosis have long been viewed as opposed by antinomy, but it is now generally assumed that both forms of cell death constitute two extremes of a continuum. The same xenobiotic can induce apoptosis or necrosis at a low (subnecrotic) or high dose respectively (Kroemer, 1998). Even in response to a certain dose of death-inducing agent, features of both apoptosis and necrosis may coexist in the same cell. In addition, if not engulfed by neighboring cells or in cell culture, where phagocytosis does not usually happen, dead cells in the late stages of apoptosis may present necrotic features due to the loss of cellular energy and plasma membrane integrity. This process is called “apoptotic necrosis” or “secondary necrosis” (Zong and Thompson, 2006).

BEA and ENs to induce apoptosis in several cell lines (Juan-García et al., 2013b). BEA and ENs induced apoptosis that was evidenced by DNA fragmentation and nuclear condensation, apoptotic body formation and increased caspase-3 activity (Dombrink Kurtzman, 2003; Jow et al., 2004; Lin et al., 2005; Klarić et al., 2008; Dornetshuber et al., 2009b; Watjen et al., 2009; Juan-García et al., 2013a).

The ionophoric properties is most likely related to the ability of BEA to induce apoptosis in several mammalian cells. It has been demonstrated that the transfer of Ca^{2+} from the endoplasmatic reticulum to the mitochondria is required for the initiation of apoptosis (Scorrano et al., 2003). The Ca^{2+} increase measured in ventricular

myocytes agrees with the apoptosis properties of BEA (Kouri et al., 2003).

Moreover, the necrotic cell death have been also reported (Gammelsrud et al., 2012; Ivanova et al., 2012; Juan-García et al., 2013a).

1.9 Mitochondrial membrane potential disruption

Mitochondria have been recognized for their role in mediating physiological processes and their involvement in signal transduction and regulation of cell proliferation and differentiation. They are involved also in cell death regulation, i.e. necrosis and apoptosis. Due to this role, mitochondria are vulnerable to the toxic effects of xenobiotics that interefer in the energy production. The outer mitochondrial membrane is permeable to many small molecules. Certain forms of stress, including xenobiotics, can make the outer membrane more permeable to the point that mediators of apoptosis (cytochrome c, activators of caspases, etc.) can escape into the cytosol, where they initiate apoptosis or necrosis and from where they can even be further translocated into the nucleus, where they can cause DNA cleavage (Elmore et al., 2007).

Apoptosis and necrosis induced cell death by cytotoxic agents involve similar metabolic disturbances and above all, mitochondrial permeability transition (MPT). Mitochondrial events of apoptosis and necrosis involve opening of a pore in the inner mitochondrial membrane, referred as mitochondrial permeability transition pore (MPTP) and the consequent dissipation of $\Delta\Psi m$ (Kroemer et al., 1998). Moreover, once the pores are open, molecules with a molecular mass of

about 1500 Da pass across the channel. The dissipation of ΔΨ*m* results from the inequal distribution of ions (mainly protons) on the inner mitochondrial membrane. The ΔΨ*m* disruption suggests that the proton-moving force and/or the inner membrane permeability has been affected during cell damage. The dissipation of ΔΨ*m* is a general feature of both cell death types.

Measurements of mitochondrial membrane potential (ΔΨ*m*) is carried out by using lipophylic dyes, which pass through cell membranes and accumulate according to their charge. The alteration of fluorescent intensity can be determined by flow cytometry. Among these dyes the tetramethyl rhodamine methyl estere (TMRM), coupled with the carbocyanine monomer nucleic acid (To-Pro-3) have been used to determine ΔΨ*m* (Juan-García et al., 2013a).

The disruption of ΔΨ*m* have been demonstrated in KB-3-1 cells exposed to ENs mixture (Dornetshuber et al., 2007), and in Caco-2 and HepG2 cells (Ivanova et al., 2012; Juan-García et al., 2013a). The exact mechanism by which pro-oxidant mycotoxins induced pore opening is still not fully understood. At least two molecular sites of the complex contribute to this effect. The first site is a redox sensitive membrane dithiol group that can be oxidized by ROS (produced by mycotoxins), the second one remains undetermined.

1.10 DNA damage

Cells are continuously exposed to endogenous and exogenous agents that damage their DNA (Barzilai and Yamamoto, 2004). The many types of DNA lesions that results from these insults are rapidly detected, with the subsequent activation of an intricate web of

signaling pathways (DNA damage response) that culminates in activation of cell cycle checkpoints and DNA repair pathways, or in certain contexts, in the initiation of apoptosis (Barzilai and Yamamoto, 2004).

Genotoxic compounds are molecules, both exogenous (such as mycotoxins) or endogenous, that interact with the DNA of living cells and cause the loss of structural and functional DNA integrity (Wang and Groopman, 1999). Generation of DNA damage is considered to be an important initial event in carcinogenesis. Moreover, a significant portion of the damage is caused by ROS (Barzilai and Yamamoto, 2004). In the last thirty years, several assays have been performed to determine the DNA damage in cell cultures after exposure to toxicants that allow to determine SSB or DSB. Among them, the Comet assay is capable to determine DNA damage in individual cells (Singh et al., 1988). The alkaline version of the Comet assay have been used to assess the genotoxic potential of mycotoxins (Klarić et al., 2010). The Comet assay have been used to determine DNA damage in different cell types exposed to *Fusarium* (BEA, Butenolide, PAT, DON; Bony et al., 2006; Schumacher et al., 2006; Klarić et al., 2010; Yang et al. 2010) and *Penicillium* (OTA; Simarro Doorte et al., 2006; Klarić et al., 2010). mycotoxins. These mycotoxins showed an increase in DNA damage depending on the concentration tested.

1.11 Bioavailability studies of mycotoxins

The intestinal mucosa undergoes continuous cell replacement from the stem cell compartment localized in the intestinal crypts. Toxic effects from substances in the intestinal lumen can affect either the stem cells in the crypts (affecting the renewal capacity of the tissue) or

the non-proliferative differentiated cells (Sambruy et al., 2001). In addition, the intestine has an important role in the bioavailability of mycotoxins, as well as other xenobiotics.

Release of the contaminant from the ingested product in the gastrointestinal tract is a prerequisite for uptake and bioavailability of a contaminant in the body. The term bioavailability describe the proportion of the ingested contaminant in food that reaches the systemic circulation (Versanvoort et al., 2005) and exerts toxic effects in human body.

Bioavailability studies can be performed *in vivo* (in experimental animals and humans) and *in vitro* by using *in vitro* system which try to simulate physiochemical conditions of the gastro-intestinal tract. *In vitro* systems are a good alternative to the *in vivo* techniques for its lower cost, less variability, and determination of test conditions, although is not possible to complete reproduction of physiological conditions, and applications of the results are more limited than in the *in vivo* methods. In recent years, the use of cell cultures have been widely employed to assess bioavailability (Sambruy et al., 2001). During the past few years, Caco-2 cells monolayer (CCM) have been widely accepted by pharmaceutical companies and by regulatory authorities as a standard permeability-screening assay for prediction of drug intestinal permeability (Grajek and Olejnik, 2004). Caco-2 cells have been established in 1970 from human colon adenocarcinoma, with the aim of performing studies on cancer mechanism and related cytostatic therapies (Fogh et al., 1977). Caco-2 cells showed a spontaneous differentiation pathway leading, in two or three weeks, to the formation of a monolayer that express several morphological and

biochemical characteristics of small intestinal enterocytes: a cylindrical polarized morphology, with microvilli on the apical side, tide junctions between adjacent cells and expression of small intestinal hydrolase enzyme activity (i.e. sucrose-isomaltase, lactase, aminopeptidase N- and depeptidylpeptidase IV; Sambruy et al., 2001). The more differentiated human intestinal cell line can be grown and allowed to differentiate on microporous culture supports made of polycarbonate or other similar materials. The culture support, form a two compartments system where the apical (AP) medium is separated from the basolateral (BL) medium by the microporous filter insert (Figure 9). To utilize this system for transport or bioavailability studies, the integrity and functional closure of tight junctions has to be monitored before and throughout the experiments. This could be achieved by measuring the trans-epithelial electrical resistance (TEER) of the cell monolayer. The determination of TEER is simple and rapid to perform and it is generally measured utilizing a pair of Ag/AgCl electrodes that, placed into AP and BL medium, measure the electrical resistance of the filter and cell monolayer and is expressed as Ω cm^2. Phenol red exclusion also is used as a measure of monolayer integrity. This method measure the passage of phenol red through the monolayer (Halleux and Schneider, 1991).

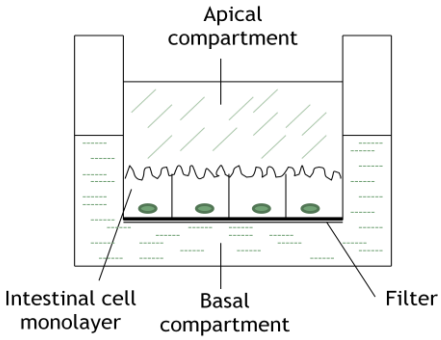


Figure 9. Graphical representation of *in vitro* trans-epithelial transport.

Overall, some but not all small intestinal functions have been shown to be expressed in the differentiated Caco-2 cell line, leading to the conclusion that these cells, because of their tumour origin, may not represent a single cytotype, but exhibit some biochemical characteristics of normal adult intestine, others of foetal colon, and others of normal adult colon (Harris et al., 1992). Despite these limitations, up to now the Caco-2 cell line has proved to be the best model for studies of intestinal absorption and transport of xenobiotics.

As regarding to the bioavailability studies of mycotoxins, Caco-2 cells have been previously used to assess their transport through the monolayer (Caloni et al., 2006; Videmann et al., 2008; Burkhardt et al., 2009; Meca et al., 2012a,b). Moreover Caco-2 cells have been used to evaluate the duodenal and colonic bioavailability of ENs (Meca et al., 2012a). To simulate colonic bioavailability, Caco-2 cells were grown in symbiosis with some bacteria characteristics of the gastrointestinal tract (*Lactobacillus animalis* CECT 4060T, *L. casei* CECT 4180, *L. casei rhamnosus* CECT 278T, *L. plantarum* CECT 220, *L. rhuminis* CECT 4061T, *L.casei* CECT 277, *Bifidobacterium breve* CECT 4839T, *B.*

adolescentes CECT 5781T and *B.bifidum* CECT 870T, *Corynebacterium vitaeruminis* CECT 537, *Streptococcus faecalis* CECT 407, *Eubacterium crispatus* CECT 4840 and *Saccharomyces cerevisiae* CECT 1324).

1.12 Bioaccessibility studies of mycotoxins

Human exposure to mycotoxins is principally via ingestion (Peraica et al., 1999). It is therefore important to be able to assess the amount of mycotoxins that is potentially available for the absorption in the stomach and/or intestines. The term "bioaccessibility" describes the fraction of a contaminant that is mobilized from food matrix during gastrointestinal digestion and theoretically subsequently available to intestinal absorption (Versanvoort et al., 2005).

Bioaccessibility can be determined by *in vivo* and *in vitro* studies. *In vivo* animal studies present the great advantage of providing information on a whole organism, including all organs and their metabolic functions (Marques et al., 2011). On the other hand, *in vivo* feeding methods, using animals, although providing the most accurate results, they are time consuming and costly. Thus, much effort has been devoted to the development on *in vitro* procedures (Boisem and Eggum, 1991). *In vitro* digestion models provide a useful alternative to animal models by rapidly screening food ingredients. The *in vitro* digestion method would provide accurate results in a short time (Hur et al., 2011). However it is important to consider the inherent complexity of the process (Fuller, 1991; Coles et al., 2005). During the last decade, an increasing interest in the use of *in vitro* methodologies, such as *in vitro* digestion models that simulate the human digestion process has been observed (Avantaggiato et al., 2003; Versanvoort et al., 2005). These *in*

vitro models attempt to recreate the aspects of human gastrointestinal physiology, in the mouth, stomach and small intestine compartments, in order to enable bioaccessibility of contaminants. In these models chemical composition of digestive fluids, pH, and residence time periods typical for each compartment are simulated (Versanvoort et al., 2005). Moreover, they are simple, rapid, low-cost, and without ethical implications, although, *in vitro* conditions used, including differences in solid solution ration, the method of mixing, the pH values of the gastric and intestinal juices and their compositions can modified the bioaccessibility. Even food contaminants and food matrix could alter this process (Hur et al., 2011).

Several *in vitro* studies have been optimized and applied to assess the bioaccessibility of mycotoxins. *In vitro* digestion method according to Gil-Izquierdo et al. (2002), that included a three-step procedure simulating the digestive processes in mouth, stomach and small intestine is reported in Figure 10. Similarly, Versanvoort et al. (2005) applied the digestion process of three step procedure by which they analyzed the concentration of contaminats in the chime (intestinal content).

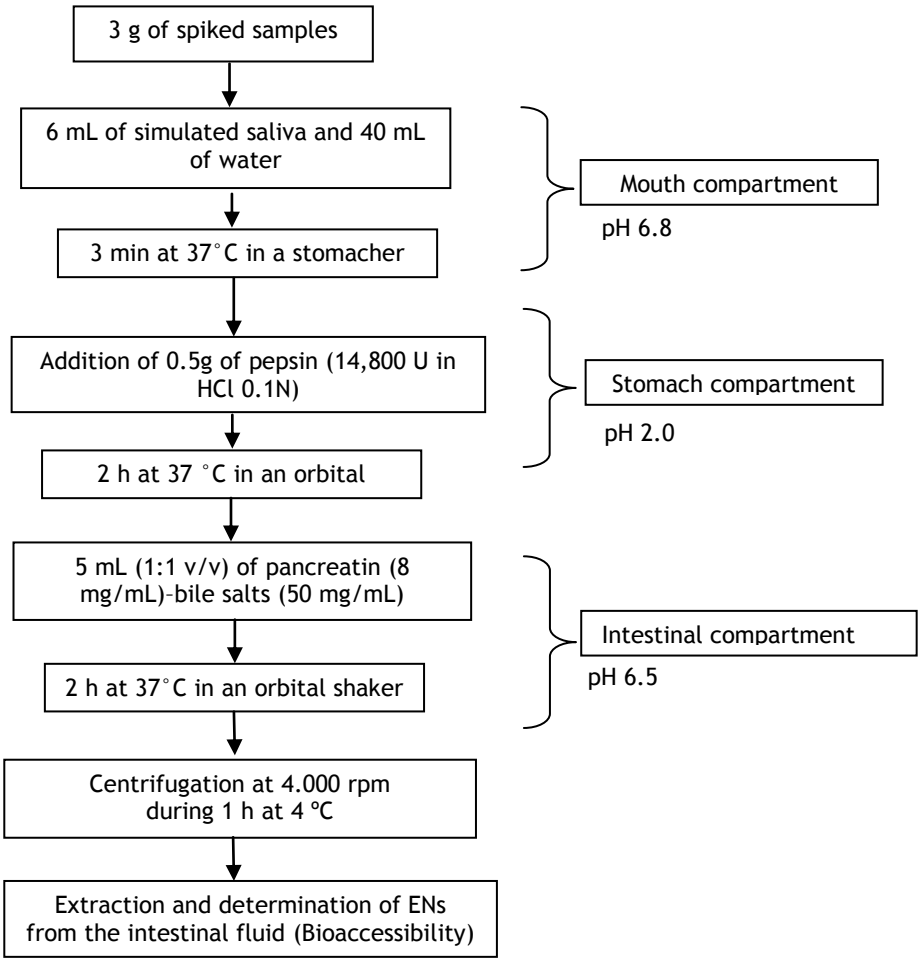


Figure 10. Schematic representation of the *in vitro* digestion model as reported by Gil-Izquierdo et al. (2002).

In vitro digestion model that simulated both adults and children digestion has been used to assess the bioaccessibility of mycotoxins of human concern (Meca et al., 2012a,b; Raiola et al. 2012). The children's digestion model was basically the same as that of adults abovementioned with slight modifications (the pH of the stomach, the

quantity of pepsin, pancreatin and bile salts). On the other hand, an *in vitro* gastrointestinal model that simulates metabolic processes in stomach, duodenum, jejunum and ileum of healthy pigs by means of a multicompartimental dynamic computer-controlled system was also developed (TIM; Minekus et al., 1995; Avantaggiato et al., 2003; 2004). A schematic representation of the TIM is illustrated in Figure 11.

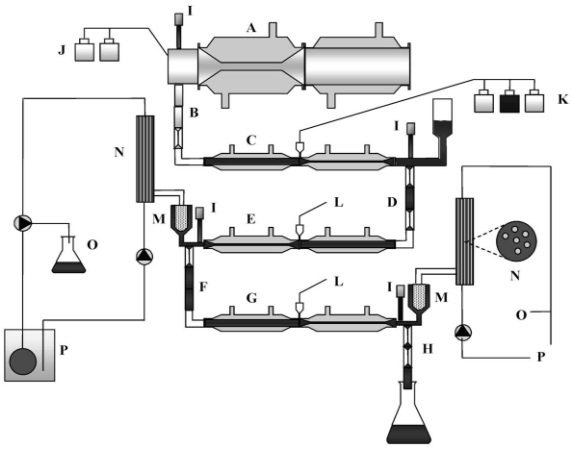


Figure 11. Schematic diagram of the dynamic, multi-compartmental model of the stomach and small intestine[1].

[1] A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileo-caecal valve and sampling bottle for ileal delivery; I. pH electrodes; J. gastric secretion bottles with acid and enzymes; K. duodenal secretion bottles with bile, pancreatic juice, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fiber semi-permeable membrane system; O. water absorption system; P. closed dialyzing system (Avantaggiato et al., 2003).

Kabak et al. (2009; 2012) proposed the simulated *in vitro* digestion model showed in Figure 12. They found slight decrease in bioaccessibility of AFB_1 and OTA and AFM_1 than those obtained in the previous model showed.

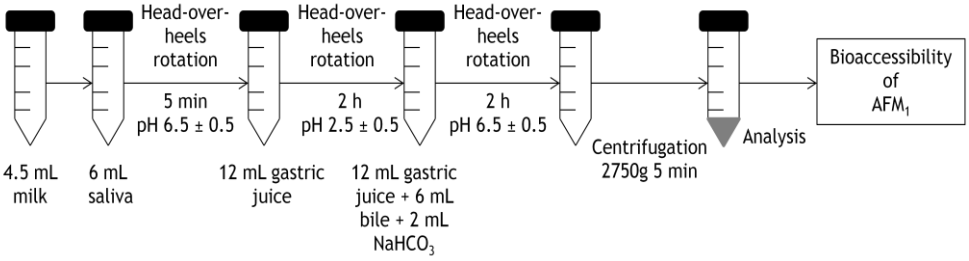


Figure 12. A schematic diagram representing the *in vitro* digestion experiment (Kabak et al., 2009; 2012).

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OBJETIVOS

OBJECTIVES

The general objective of this study was to further investigate the effects produced by minor *Fusarium* mycotoxins. Therefore, the specific objectives were to determine:

1. Cytotoxic effects of individual fusaproliferin, beauvericin and enniatins in Caco-2, CHO-K1 and HT-29 cell lines.

2. Cytotoxic effects of combined enniatins A, A_1, B and B_1 in Caco-2 cells.

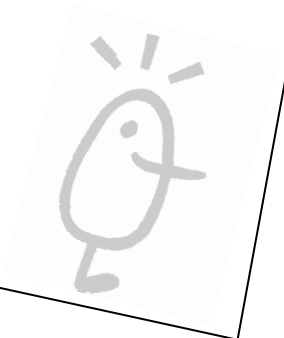
3. Reactive oxygen species generation and lipid peroxidation production after fusaproliferin, beauvericin and enniatins exposure in Caco-2 cells.

4. The cytoprotective effect of GSH and some polyphenols against enniatins and beauvericin cytotoxicity in CHO-K1 and Caco-2 cells.

5. The cell cycle, apoptosis/necrosis induction and mitochondrial membrane alteration after beauvericin and enniatins exposure in Caco-2 cells.

6. The transepithelial transport of beauvericin and fusaproliferin and their relative bioavailability using the Caco-2 cell line.

7. The bioaccessibility of enniatins A, A_1, B, and B_1 from artificially spiked grain-based products, by applying an *in vitro* gastrointestinal model.



RESULTADOS

Toxicology *in Vitro* (under review)

INTERACTION EFFECTS OF *FUSARIUM* ENNIATINS (A, A_1, B AND B_1) COMBINATIONS ON *IN VITRO* CYTOTOXICITY OF CACO-2 CELLS.

Prosperini, A.[*]; Font, G.; Ruiz M.J.

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain.

[*] Corresponding author Tel: +34963544958. Fax: +34963544954
E-mail: alessandra.prosperini@uv.es.

ABSTRACT

Foodstuff is usually contaminated by more than one mycotoxin, however toxicological data are lacking as regards the effects in combinations compared to their individual effect. This study investigated the *in vitro* effects of enniatins (ENs) A, A_1, B and B_1, alone and in combinations, on Caco-2 cells viability by MTT assay after 24 h of exposure. Cells were treated with concentrations ranging from 0.9 to 15.0 μM, individually and in combination of two, three and four mycotoxins. Dose-response curves were generated for each mycotoxin and the isobologram method was used to determine the interactive effects of tested mixtures. Tested ENs produced significant cytotoxic effects both individually and in combination in a dose-dependent manner. IC_{50} values obtained for all individually tested mycotoxins ranged from 1.3 to >15 μM. In ENs combination tests, synergistic effect in Caco-2 viability are observed for EN B+EN A_1, EN B_1+EN A_1 and EN A+EN A_1+EN B (CI=0.33-0.52). All other combinations showed additive effect at medium and high affected fraction with exception of lower fraction affected and the EN B+EN B_1 mixture that produced antagonistic effect (CI=1.76-10.36). The use of combination index-isobole method could help to better understand the potential interaction between co-occurring mycotoxins and may contribute to their risk assessment.

Keywords: enniatins, cytotoxicity, interactions, isobolograms, Caco-2 cells.

1. Introduction

Mycotoxins are toxic compounds produced as secondary metabolites by different types of fungi, belonging mainly to the *Aspergillus*, *Penicillium* and *Fusarium,* that are excreted into the matrices on which they grow, often food intended for human consumption or animal feed (Jestoi et al., 2004; Meca et al., 2010; Malachova et al., 2011). The consumption of mycotoxins-contaminated commodities is related to several chronic diseases in humans and in animals with many different adverse health effects, such as carcinogenic, mutagenic and estrogenic effects, as well as other systemic disorders (Speijers et al., 2004; Ruiz et al., 2011a; Kouadio et al., 2007). Some mycotoxins are also immunosuppressive reducing resistance to infectious diseases.

Under favorable environmental conditions, *Fusarium* strains, produce cyclic hexadepsipeptidic mycotoxins such as enniatins (ENs) that are endowed with a wide array of toxicological effects, such as antibacterial, antifungal, antihelmintic, insecticidal, phytotoxic and cytotoxic, most likely related to their ionophoric properties (Kamyar et al., 2004). Several studies have recently indicated that ENs exert their cytotoxic activities in the lower micromolar range, in addition to being ionophores, through the induction of mitochondrial modifications and cell cycle disruption, finally resulting in cell death (Dornetshuber et al., 2009; Watjien et al., 2009; Tonshin et al., 2010; Meca et al., 2011).

The Commission Regulation (EC) 1881/2006 and its amendments set maximum levels for certain contaminants in foodstuff as for certain mycotoxins including some *Fusarium* toxins (EC 2006). However, these maximum levels have been established taking into account only the

presence of individual mycotoxins. Hence, it is necessary to take into consideration toxicological "mixture" effects derived from mycotoxins to implementing human risk assessment (Tajima et al., 2002; Speijers et al., 2004).

Toxicological studies about the effects of mycotoxins mixtures are of a great importance in order to find out whether they could interact among themselves and produce a synergistic, additive or antagonistic effect (Bernhoft et al., 2004, Heussner et al., 2006; Kouadio et al., 2007; Klarić et al., 2008; Ruiz et al., 2011a,b).

Toxic effects of individual ENs *in vitro* have been previously carried out in different cell types, whereas very scarce information is available about their effects in combination as well as their potential interactions (Meca et al., 2011). Effects of mycotoxins combinations *in vitro* have been carried out for other mycotoxins and several mathematical methods have been applied in order to determine also the type of potential toxicological interaction among them (Tajima et al., 2002; Heussner et al., 2006; Kouadio et al., 2007; Klarić et al., 2008). Some studies on combined effects of mycotoxins have been carried out using isobolographic analysis (Bernhoft et al., 2004; Luongo et al., 2008; Ruiz et al. 2011a,b) and interactive effects of mycotoxins combination have been demonstrated. The aim of this study is to investigate the joint action of the four *Fusarium* ENs A, A_1, B and B_1 and to assess the nature of toxicological interactions among them. For this purpose, ENs were tested individually and in two-, three- and four- ENs combinations. Taking into consideration that the major route to mycotoxins is via oral ingestion, Caco-2 cells, from intestinal origin, were used in this study. Caco-2 cells are considered a target human cells of the digestive

system, moreover, this cell line demonstrated to be sensitive to ENs cytotoxicity, as demonstrated previously (Meca et al., 2011; Ivanova et al., 2012). In addition, up to now, no studies about cytotoxic effects of ENs combination in Caco-2 cells have been carried out. Cell viability after 24 h of exposure to mycotoxins individually and in combinations, have been chosen as toxicity endpoint by using MTT assay; the method of combination index (CI)-isobologram equation was applied to determine the type of interactions.

2. Materials and methods

2.1 Chemicals

The reagent grade chemicals and cell culture components used were from Sigma Chemical Co. (St Louis, MO, USA). EN A (CAS#2503-13-1), EN A_1 (CAS#4530-21-6), EN B (CAS#917-13-5) and EN B_1 (CAS#19914-20-6) were form Enzo Life Sciences, (Switzerland). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Mycotoxins selected were Stock solutions of ENs were prepared in methanol and maintained at -20°C.

2.2. Cell culture and treatment

Caco-2 cells (ATCC HTB-37) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 25 mM HEPES buffer, 1% Non-Essential Aminoacids (NEAA), 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM Sodium Pyruvate. Cells were grown near confluence in 75 cm^2 plastic flasks at 37 °C in an atmosphere containing 5% of CO_2 at 95% of relative humidity and

harvested with 1% trypsin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

Cells were plated in 96-well tissue culture plates at a density of 3 x 10^4 cells/well to perform the experiment. Four concentrations for EN A (0.94-7.5 μM) and five concentrations for ENs A_1, B and B_1 (0.94-15.0 μM) were assayed. EN combinations (of two, three and four mycotoxins) were also assayed after 24 h of exposure. Concentrations ranged from 0.625 to 5.0 μM for binary mixtures (1:1 ratio) and, from 1.25 to 2.5 μM for tertiary and quaternary mixtures, with a fixed ratio of 1:1:1 and 1:1:1:1, respectively.

For each ENs from initial solutions, serial dilutions in supplemented medium were prepared. Culture medium without ENs and with 1% methanol (MeOH) was used as control. After replacing the previous medium, the exposure solutions were added to each well and plates were incubated at 37 °C for 24 h. The basal cytotoxicity endpoint selected was the tetrazolium salt reduction (MTT). MTT was measured according to Ruiz et al. (2006). Medium containing ENs was removed, cells were washed with PBS and 50 μL MTT were added. The absorbance was measured after 4 h at 570 nm on an ELISA plate reader Multiscan EX (Thermo scientific, MA, USA). Mean inhibition concentration (IC_{50}) values were calculated from full dose-response curves.

2.3. Experimental design of isobologram method

The isobologram analysis was used to determine the type of interaction that occurs when ENs were in combination. The median-effect/combination index (CI)-isobologram equation by Chou (2006) and

Chou and Talalay (1984) was originally for analyzing drug combination effects. The isobologram analysis involves plotting the dose–effect curves for each compound and its combinations in multiple diluted concentrations by using the median-effect equation:

$$fa/fu = (D/D_m)^m$$

where D is the concentration of a product, D_m is the median-effect dose (e.g., IC_{50} or LD_{50}, which stands for surviving population of cells that reaches 50%), *fa* is the fraction affected by concentration D (e.g., percentage inhibition/100), *fu* is the unaffected fraction (therefore, *fa* = 1 - *fu*), and m is the coefficient signifying the shape of the dose-effect relationship, where m = 1, m >1, and m <1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively (Chou and Talalay, 1984). Therefore, the method takes into account both the potency (D_m) and shape (m) parameters.

The medium-effect equation for a single compound can be extended to multiple mycotoxins. And the equation becomes:

$$[(fa)_{1,2}/(fu)_{1,2}]^{1/m}=D_1/(D_m)_1+ D_2/(D_m)_2+(D)_1(D)_2/(D_m)_1(D_m)_2$$

Chou and Talalay (1984) introduced the term combination index $(CI)_x$ for quantification of synergism or antagonism for the two compounds:

$$CI= D_1/(D_x)_1+ D_2/(D_x)_2$$

$$D_x= D_m[fa/(1-fa)]^{1/m}$$

$$CI=(D)_1/(D_m)_1[fa/(1-fa)]^{1/m1}+ (D)_2/(D_m)_2[fa/(1-fa)]^{1/m2}$$

where in the denominator $(D_x)_1$ is for D_1 ''alone'' that inhibits a system x%, and $(D_x)_2$ is for D_2 ''alone'' that inhibits a system x%. And, the general equation for n-compound combination at x% inhibition becomes:

$$^{n}(CI)_x = \sum_{J=1}^{n} (D)_j/(D_x)_j = \frac{(D_x)_{1\text{-}n}\{[D]_j \sum_{J=1}^{n} [D]\}}{(D_m)_j\{(fax)_j/[1-(fax)_j\}^{1/mj}}$$

Where $^{n}(CI)_x$ is the combination index for n compounds (e.g., mycotoxins) at *x*% inhibition (e.g., proliferation inhibition); $(D_x)_{1\text{-}n}$ is the sum of the concentration of n compounds that exerts *x*% inhibition in combination, $\{[D_j]/\ \Sigma^{n}_{1}\ [D]\}$ is the proportionality of the concentration of each of n compounds that exerts *x*% inhibition in combination; and $(D_m)_j$ $\{(fax)_j/[1-(fax)_j]\}^{1/mj}$ is the concentration of each compound alone that exerts *x*% inhibition. The CI <1, =1, and >1 indicates synergism, additive and antagonism effect of the combination, respectively; The types of interactions produced by EN combinations were assessed by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996-2007).

2.4. Statistical analysis

Statistical analysis of data was carried out using SPSS Statistic 19.0 (SPSS, Chicago, IL, USA) statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between mycotoxins were analyzed statistically with ANOVA followed by the Tukey HDS post hoc test for

multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. Results

At 24 h of exposure, EN A_1 showed an IC_{50} value of 14.8 ± 2.7 μM, whereas ENs A, B, B_1 did not show IC_{50} value at the range of concentration tested. After 48 and 72 h of exposure, all mycotoxins tested showed IC_{50} values, with the exception of EN B. The IC_{50} values were 6.8 ± 1.6 μM (EN A), 7.7 ± 1.2 μM (EN A_1) and 11.3 ± 2.3 μM (EN B_1) after 48 h of exposure. After 72 h, the IC_{50} values were 1.6 ± 0.8 (EN A), 1.3 ± 0.6 (EN A_1), 2.8 ± 1.1 (EN B_1) and 11.7 ± 2.4 (EN B). As observable in Figure 1, EN B showed the less cytotoxic effect on Caco-2 cells at the three times of exposure, followed by EN B_1, EN A and ENA_1 that resulted to be the most cytotoxic among ENs tested.

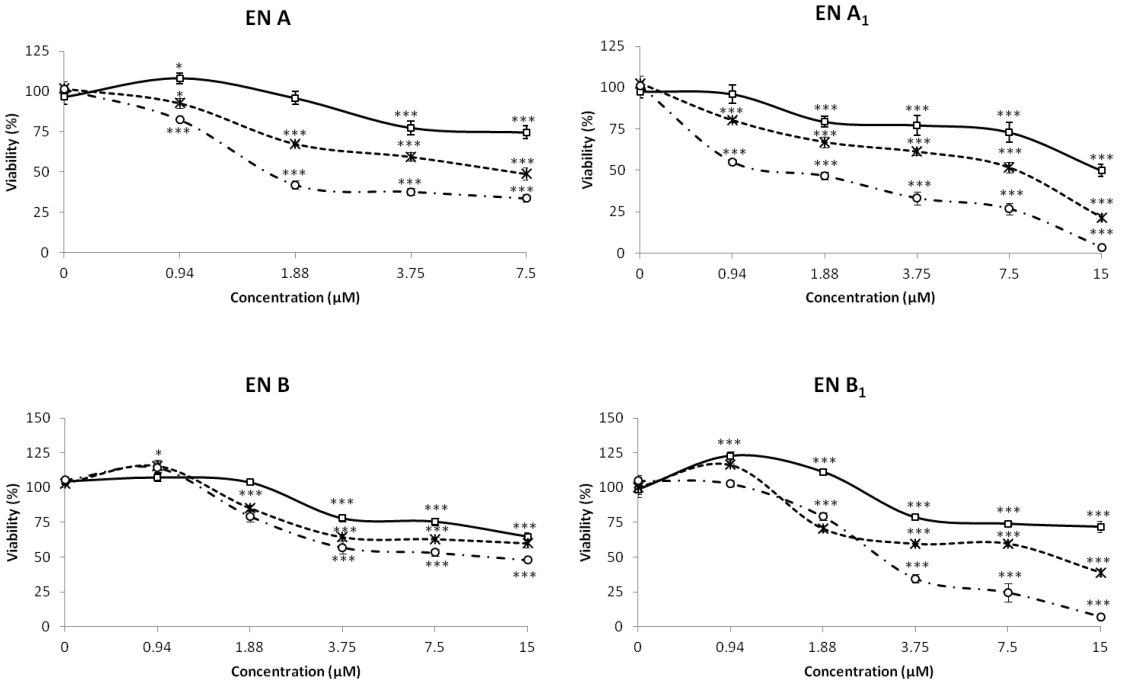


Figure 1. Dose response curve of ENs A, A_1, B and B_1 on Caco-2 cells after 24 (–□–), 48 (--✳--) and 72 h (·-·○·-·) of exposure. Cytotoxicity values are expressed as IC_{50}. Mean ± SD, of 8 replicates. $p \leq 0.05$ (*), $p \leq 0.000$ (***) represent significant difference as compared to control values.

Figures 2 and 3 represent the viability (%)-concentration (μM) plot of the ENs combinations. Reduction of cell viability (%) resulted in a dose-dependent manner for binary mixture tested in the following increasing order: EN A + EN A_1 (48%) = EN A_1+EN B_1 (47%) > EN A_1+EN B (35%) = EN A+EN B (33%) = EN A+EN B_1 (32%) > EN B+EN B_1 (26%) (Fig. 2). Similarly, tertiary and quaternary mixtures reduced cell viability in a dose-dependent manner (Fig. 3). For all tertiary and quaternary mixtures, the reduction in viability was approximately 40% (Fig. 3) and a general additive effects was observed (Table 1).

In order to investigate the type of interaction between ENs A, A_1, B and B_1 in their combinations, the CI-isobologram method have been applied. Results are summarized in Table 1. As assessed by CI-isobologram equation, antagonistic effects have been produced by all the combinations tested at the lowest fraction affected (IC_5) with IC_{50} values from 2.05 ± 0.88 to 10.36 ± 4.32. Whereas a general additive effect (CI=1) has been produced by all mixtures through all the tested concentrations, except for the mixtures of EN B+EN A_1, EN B_1+EN A_1 and EN A+EN A_1+EN B. These combinations produced a synergistic effect (CI values ranging from 0.33 ± 0.53 to 0.52 ± 0.40) at the highest fraction affected. On the other hand, a moderate antagonism was produced by EN B+EN B_1 combination at medium fraction affected (IC_{25}) concentration tested (CI values were from 10.36 ± 4.22 to 2.06 ± 0.71).

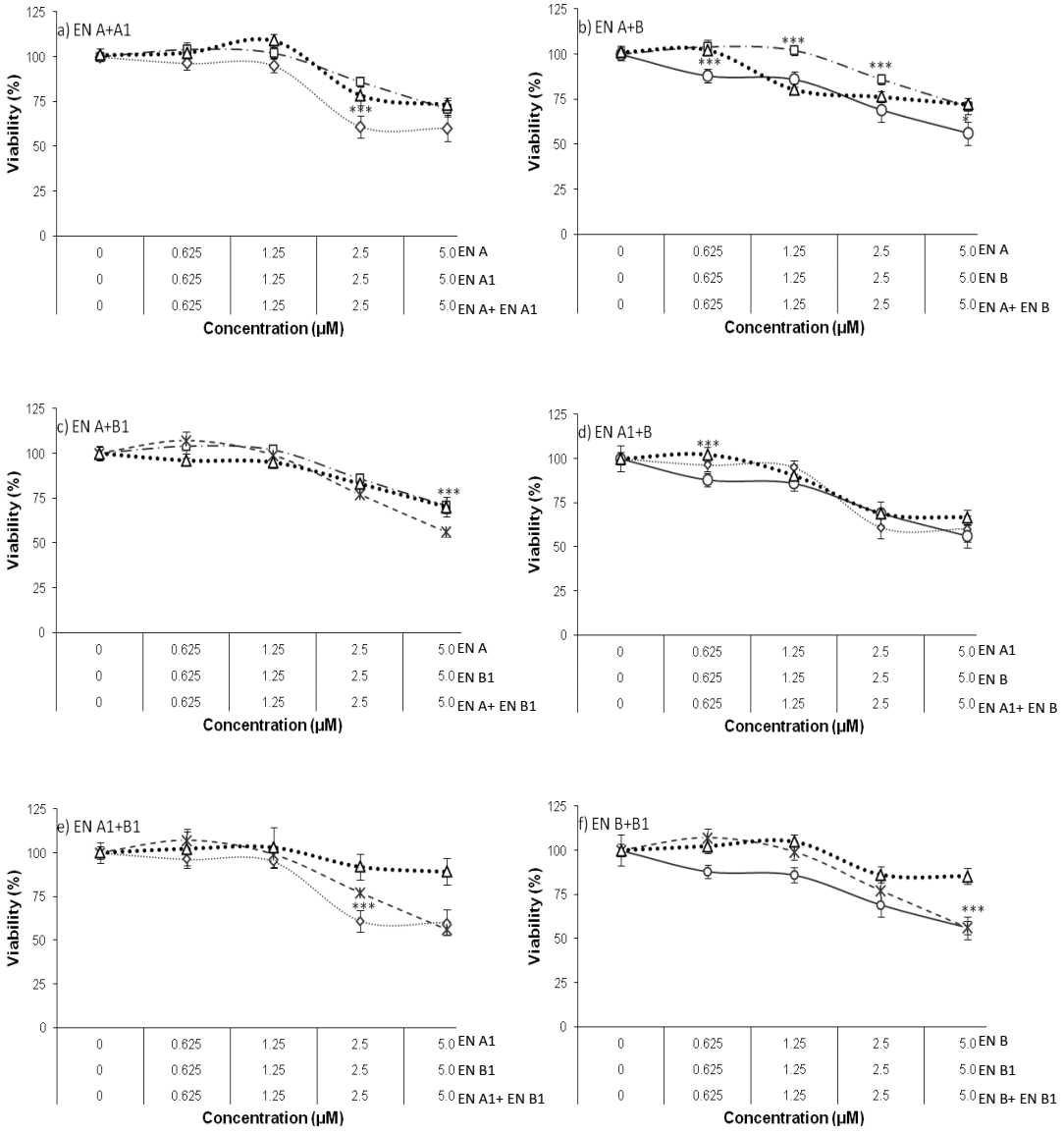


Figure 2. Cytotoxicity effects of individual EN A (---□---), EN A_1 (···◇···), EN B (–○–) and EN B_1 (--✳--) and all their binary combinations (···△··). When binary mixture concentrations are 0.625, 1.25, 2.5 and 5 μM, it means that concentrations of each EN in its combination is 0.3125, 0.75, 1.25, 2.5 μM, respectively.

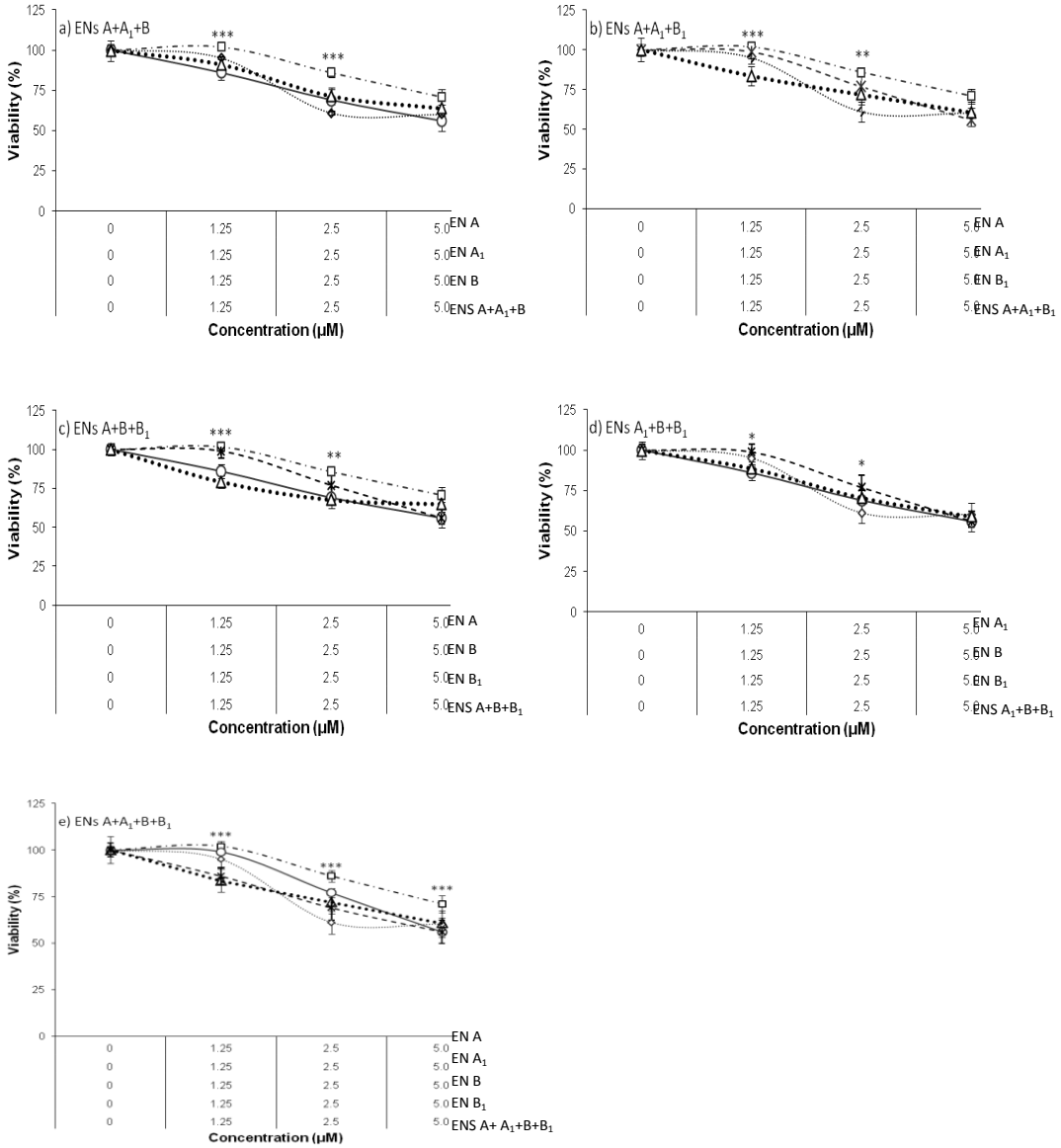


Figure 3. Cytotoxicity effects of individual EN A (···□···), EN A_1 (···◇···), EN B (–○–) and EN B_1 (--✳--) and all their tertiary and quaternary combinations (···△··). When tertiary mixture concentrations are 1.25, 2.5 and 5 μM, it means that concentrations of each EN in its combination is 0.3125, 0.75, 1.25, 2.5 μM, respectively.

Table 1. Dose-effect relationship parameters and mean combination index values (as a function of fractional inhibition of proliferation) of ENs A, A_1, B and B_1 and their binary, ternary and quaternary combinations on Caco-2 cells, after 24h of exposure.

Mycotoxin	Dose-effect parameters			CI values								
	Dm (μM)	*m*	*r*	IC_5		IC_{25}		IC_{50}		IC_{75}		IC_{90}	
EN A	3.55	3.65	0.91										
EN A_1	6.56	2.06	0.96										
EN B	7.91	0.77	0.97										
EN B_1	7.22	1.21	0.99										
EN A+EN A_1	3.90	2.81	0.91	2.92±1.29	Ant	1.45±0.69	Add	1.03±0.66	Add	0.78±0.64	Add	0.62±0.63	Add
EN A+EN B	4.19	1.83	0.86	5.12±3.16	Ant	1.64±1.02	Add	1.04±0.95	Add	0.84±1.11	Add	0.81±1.50	Add
EN A+EN B_1	2.76	3.46	0.91	2.59±0.75	Ant	1.45±0.62	Add	1.15±0.60	Add	1.00±0.69	Add	0.92±0.78	Add
EN B+EN A_1	3.79	2.03	0.83	6.62±3.26	Ant	1.95±0.97	Add	1.01±0.75	Add	0.56±0.61	Add	0.33±0.53	Syn
EN B_1+EN A_1	2.29	2.78	0.86	1.76±0.58	Ant	0.93±0.39	Add	0.66±0.37	Add	0.49±0.36	Syn	0.38±0.36	Syn
EN B+EN B_1	8.39	1.11	0.93	10.36±4.22	Ant	2.06±0.71	Ant	1.19±0.62	Add	1.25±0.97	Add	1.36±1.45	Add
EN A+EN A_1+EN B	2.18	2.94	0.90	6.28±2.34	Ant	1.61±0.62	Add	0.85±0.47	Add	0.52±0.40	Syn	0.37±0.37	Syn
EN A+EN A_1 +EN B_1	2.06	2.90	0.87	2.05±0.88	Ant	1.23±0.55	Add	0.99±0.58	Add	0.85±0.63	Add	0.70± 0.70	Add
EN A+EN B+EN B_1	2.05	3.01	0.91	6.20±2.30	Ant	1.47±0.52	Add	0.96±0.46	Add	0.76±0.48	Add	0.68±0.53	Add
EN A_1+ EN B+ EN B_1	2.05	3.00	0.90	5.17±2.02	Ant	1.71±0.62	Add	1.00±0.49	Add	0.69±0.44	Add	0.56±0.44	Add
EN A+EN A_1+EN B+EN B_1	2.13	2.67	0.80	5.97±3.07	Ant	1.97±1.08	Add	1.30±0.96	Add	1.00±1.00	Add	0.88±1.15	Add

The parameters *m*, *Dm* and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose-effect curve, the potency (EC_{50}), and conformity of the data to the mass-action law, respectively (Chou, 1976; Chou and Talalay, 1984; Chou, 2006). *Dm* and *m* values are used for calculating the CI values; CI <1, =1, and >1 indicate synergism (Syn), additive effect (Add), and antagonism (Ant), respectively. IC_5, IC_{25}, IC_{50}, IC_{75} and IC_{90}, are the doses required to inhibit proliferation 5, 25, 50, 75 and 90%, respectively. Computer software CompuSyn was used for automated calculation and simulation.

4. Discussion

The co-occurrence of several mycotoxins in the same sample is of great importance because their combination could lead to possible adverse health effects which are riskier then the intake of one mycotoxin alone since, their possible interaction leads to antagonistic, additive or synergistic effects (Ruiz et al., 2011a,b).

The four ENs selected naturally occur in food commodities. Contamination of food with ENs is a relatively widespread phenomenon: in several countries of Europe, levels of μg/kg up to mg/kg have been found in cereal grains as well as in processed food (Jestoi et al., 2004; Meca et al., 2010; Malachova et al., 2011). ENs A, A_1, B and B_1 were detected in 73% of cereal based products from Czech Market and the remainder samples were contaminated with at least three of them (Malachova et al., 2011). Moreover, ENs co-contamination has been demonstrated in 89% of Finnish grain samples, where ENs B and B_1 were the most predominant mycotoxins in the commercially available samples, and in 82% in cereals of Spanish markets (Jestoi et al., 2004; Meca et al., 2010). The range of concentrations tested in this study was selected depending on the concentrations found in cereals, as reported in literature. Consecutively it has been explained the importance of the o-occurrence of ENs in the cereals in respect to the levels of combined toxicity. Although, ENs are still not legislated and no estimated daily intake (TDI) or provisional maximum tolerable daily intake (PMTDI) have been proposed by competent authorities, for other fusarotoxins, such as fumonisins (FBs) the maximum levels has been established in raw cereals as 2000 μg/Kg. Taking into consideration this value and considering that cereals consumption (excluding beer) in European

Union is 125.4 Kg/year (FAO, 2007), it could be possible to calculate that European people is approximately exposed to almost 680 μg/day of FBs. Applying this calculation to ENs, i.e. EN A_1, the ingestion is approximately estimated to almost 1.01 μM, which is included in the range of concentrations tested. In addition, higher concentrations (up to 5.0 μM) have been tested since ENs concentration in food depends on dietary food variety.

Literature data repeatedly report the substantial cytotoxicity associated with exposure of ENs to different types of cells up to micromolar concentration (Tedjiotsop Feudijo et al., 2010). ENs A_1, B and B_1 showed a moderate cytotoxicity (IC_{50} values of 10-25 μM by the MTT assay) in human hepatoma (HepG2) and rat glioma (C6) cells, but cytotoxicity was stronger (IC_{50} values of 1-2.5 μM) in H4IIE rat hepatoma cells where an increase in caspase 3/7 activity and nuclear fragmentation were also observed (Wätjen et al. 2009). Higher cytotoxicity was also reported for ENs A, A_1, B, B_1, B_2 and B_3 in MRC-5 cells with IC_{50}-values that were 1.4 μM (EN A_1), 1.4 μM (EN B_1) and 3.6 μM (EN B) as determined by BrdU assay. When the Alamar Blue assay was used, IC_{50}-values were 6.9 μM (EN A1), 4.7 μM (EN B1) and 9.8 μM (EN B; Ivanova et al., 2006).

Moderate toxicity for ENs in HepG2 cells was found by Ivanova et al. (2006) with IC_{50}-values that were 18.1 μM (EN A_1), 36 μM (EN B_1) and 435 μM (EN B) as measured in the Alamar Blue assay. Similar results (IC_{50} from 2.6 ± 0.6 to 26.2 ± 7.6 μM) in HepG2 cells were obtained by Meca et al. (2011). The results obtained in Caco-2 cells by Meca et al. (2011) were comparable with those obtained in this study. The highest toxicity was exerted by EN A1, that induced 50% cell death with 12.3 ± 4.3 μM

concentration. Higher toxicity was found in CHO-K1 by Lu et al. (2013). After 24, 48 and 72 h of exposure, the IC_{50} values ranged from > 7.5 to 1.65 ± 0.7 μM. After 24 h of exposure EN B1 showed the highest toxicity (IC_{50}=4.53 ± 1.23).

According to results obtained in this study and in the revised literature, the H4IIE, CHO-K1 and MRC-5 cells were more sensitive to ENs cytotoxicity, among all cells used. Moreover, depending on the cell lines tested, the ENs A_1 and B_1 showed higher toxic effects than the other ENs tested.

It is of high importance to check whether a combined intake of mycotoxins would lead to higher cytoxicity values, however, few studies reported the cytotoxicity of ENs mixture. Fornelli et al., (2004) evaluated the cytotoxicity of a mixture composed by four ENs (A, A_1, B, B_1) on the lepidopteran SF-9 cell line. After 48 h of incubation with 1-10 μM concentration, the cytotoxicity obtained by MTT, expressed as IC_{50}, was 6.6 μM. The same mixture was tested by Uhlig et al. (2005) on PK-15 (porcine kidney) cells by the Alamar Blue assay and, after 24 h exposure, the IC_{50} obtained was 41 μM. Dornetshuber et al. (2007) and Gammelsrud et al. (2012) showed a reduction of cell viability through different endpoints which demonstrated a loss of mitochondrial membrane potential and an increasing of apoptotic cells at low micromolar ENs concentrations. That study, according to Tonshin et al. (2010), hypothesized that the mechanism of action of ENs is related to the strongly potassium-selective ionophoricity of ENs that causes predominantly mitochondrial damage. ENs induced a drop in the electric transmembrane potential of the mitochondrion, a K+ influx into

the mitochondrial matrix, a mitochondrial swelling and uncoupling of oxidative phosphorylation.

On the other hand, as determined by isobologram method, the nature of interactions produced by all the ENs combinations tested was not uniform along the fraction affected (from IC_5 to IC_{90}). In general, antagonism predominated at low fractions affected but at the highest ones, interactions became additive though all combinations tested or slight synergistic. This behavior cannot be explained by the isobolograms method since this one allows a quantitative determination of additive effects, synergism or antagonism, but not the elucidation of mechanisms by which these types of interaction are produced. However, tentatively, antagonism, that is the predominant interaction produced at the lowest fraction affected, could be due to the fact that ENs A, A_1, B and B_1, that possess similar structures, have been competing for same receptors at low doses producing a lower effect than the expected. Table 1 shows that antagonism was found in EN B +EN B_1 combination effect at low and intermediate fractions affected. This could be due to the fact that ENs of B group may compete for the same receptor. They induced the lowest reduction of Caco-2 cells viability. EN B resulted to be the less cytotoxic among ENs tested in Caco-2 cells. The weakest activity of EN B has been reported also by Tonshin et al. (2010) on mitochondrial damage. Furthermore, EN B was less effectively pumped into cells from blood plasma by cell membrane potential than the other tested compounds with a possible explanation in the lower lipophilic properties compared tothe other ENs. This weaker toxicity could explain the lower viability reduction obtained for EN B+EN B_1. These findings could be interesting since EN B is one of the

most common minor *Fusarium* mycotoxins in several countries of Europe (Jestoi et al., 2004; Meca et a., 2010).

On the contrary, the interaction observed at intermediate and high fractions affected is additive and became slight synergistic for the binary EN A+EN A_1 and EN A_1+EN B_1 combinations and the EN A+EN A_1+EN B tertiary combinations. Normally, when two or more compounds with the same mechanism of toxicity displayed an additive or antagonistic effects. In this case, it could be hypothesized that ENs at low doses are competing for the same receptors, whereas at higher concentrations, after diffusing cell membranes by their lipophilic nature, binds their receptors and the resulting effect is the sum of their individual effects (additive). Regarding tertiary and quaternary mixtures, a general additive effect was produced confirming the similar mode of action of ENs tested on Caco-2 cells cytotoxicity.

At high fractions affected a synergistic nature of interaction by EN A+EN A_1, EN A_1+EN B_1 and EN A+EN A_1+EN B mixtures is observed (Table 1). One could speculate that there is a potentiation of cytotxic effects due to their lipophilic structure (in the order EN A>EN A_1>EN B_1>EN B) which allows them to be easily incorporated in cell membranes and to exert cytotoxic action (Tedjiotsop Feudjio et al., 2010). Moreover EN A_1 showed the higher inhibitory Caco-2 viability and it possesses a good pore forming activity with a pronounced potential dependency of the time constants for open times, as well as EN B_1 (Kamyar et al., 2004). EN A and EN A_1 resulted to be the most cytotoxic ENs among ENs tested. The synergistic effects is produced when their co-occur with ENs of B group. It could be supposed that ENs of A group in combination with ENs of B group can induce a “facilitating action”

that means that secondary actions of one drug enhances the activity of other compound in the mixture or "complementary actions when compounds act at the same target at different sites, at overlapping sites or at different targets of the same pathways (Jia et al., 2009).

Other co-occurring mycotoxins structurally related to ENs, like beauvericin (BEA), interact leading to synergistic or additive or antagonistic effects. In this way, additive effects have been reported by Klaric et al. (2008) after exposure to fumonisin B1 (FB1), ochratoxin (OTA) and beauvericin (BEA) at lower concentrations on porcine kidney epithelial (PK15) cells, whereas antagonistic effects were observed at higher concentrations. BEA, deoxynovalenol (DON) and thricotecene T-2 mycotoxins combinations interact to produce antagonism in Vero cells and CHO-K1 cells (DON+BEA, DON+T-2), whereas synergism was produced in BEA+T2 and BEA+DON+T-2 in CHO-K1 cells, which showed antagonism in Vero cells (Ruiz et al., 2011a,b).

Results obtained in the present study suggest that the binary, tertiary and quaternary mixtures of A, A_1, B and B_1, could interact and produce a general additive effect on Caco-2 viability when exposed to ENs combination as compared to their individual effect, with the exception of EN B+B_1 mixture that produced antagonism. These findings should be taken into account since toxic effects are enhanced by ENs combinations whose co-occurrence is in fact naturally encountered. Further research needs to be completed to enable an appropriate assessment of health risk effects in foodstuff.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Toxicology Letters (under review)

BEAUVERICIN-INDUCED CYTOTOXICITY VIA ROS PRODUCTION AND MITOCHONDRIAL DAMAGE IN CACO-2 CELLS.

Prosperini, A.*, Juan-García, A., Font, G., Ruiz, M.J.

Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia Av. Vicent Andrés Estellés s/n 46100 Burjassot, València, Spain

* Corresponding author, tel: +34963544958. Fax: +34963544954
E-mail: alessandra.prosperini@uv.es.

ABSTRACT

The cytotoxicity of beauvericin (BEA) on human colon adenocarcinoma (Caco-2) cells was studied as a function of time. Moreover, the oxidative damage and cell death endpoints were monitored after 24, 48 and 72 h. After BEA exposure, the IC_{50} values ranged from 1.9 ± 0.7 to 20.6 ± 6.9 μM. A decrease in reduced glutathione (GSH; 31%) levels, as well as an increase in oxidized glutathione (GSSG, 20%) was observed. In the presence of BEA, reactive oxygen species (ROS) level was increased at an early stage with the highest production of 2.0-fold than the control at 120 min. BEA induced cell death by apoptotic process with a loss of the mitochondrial membrane potential ($\Delta\Psi m$; from 2% to 95%, as compared to the control), increased LPO level (from 120% to 207%, in respect to the control) and reduced G0/G1 phase, with an arrest in G2/M, in a dose and time-dependent manner. Cell death, apoptosis and reduction of $\Delta\Psi m$, were in a dose- time- dependent manner. Moreover, DNA damage was observed after 12.0 μM concentration. This study demonstrated that oxidative stress is one of the mechanism involved in BEA toxicity, moreover apoptosis induction, DNA damage and loss of $\Delta\Psi m$ contribute to its cytotoxicity in Caco-2 cells.

Keywords: beauvericin, oxidative stress, cell cycle, apoptosis, mitochondrial membrane potential, DNA damage, Caco-2 cells.

1. Introduction

Mycotoxins are toxic metabolites produced by fungi. They occur naturally and are the most prevalent source of food-related health risk in field crops. Consumption of high mycotoxins level can cause several diseases, since they can be acutely and/or chronically toxic, depending on the type of mycotoxin, the dose and the time of exposure (http://www.fao.org). Beauvericin (BEA), a cyclic hexadepsipeptide of alternating l-*N*-methylphenylalanyl and d-α-hydroxyisovaleryl residues, is synthesized by various toxigenic fungi, including *Beauveria bassiana* and several *Fusarium* species, parasitic to maize, wheat, rice and other important commodities (Santini et al., 2012). Levels up to 4 mg/Kg have been detected in wheat samples of the Mediterranean Area (Meca et al., 2010; Zinedine et al., 2011). BEA, as ionophore compound, increases ion permeability in biological membranes by forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}) consequently affecting the ionic homeostasis (Kouri et al., 2003) and uncoupling the oxidative phosphorylation (Tonshin et al., 2010). So, one of the potential target sites for BEA-mediated cytotoxicity is the mitochondria. A loss of mitochondrial membrane integrity by toxic substances increases their permeability due to the formation of high conductance channel in their membrane and the opening of their channel leads to necrosis (Kroemer et al., 1998). Mitochondria also sequester several proteins between their outer and inner membranes that are capable of activating apoptotic pathways (Orrenius, 2004). Apoptosis has a pivotal role in maintaining homeostasis in cells. In response to apoptotic stimuli, DNA damage and oxidative stress, the mitochondrial pathway is triggered. Due to that, understanding the mode of action of BEA is essential to

predict the harmful effects on human health when BEA contaminates food commodities. The aims of this work were to study the implication of intracellular ROS generation, LPO production, alteration of intracellular GSH levels, the disturbance of the cell cycle progression, DNA damage, apoptosis and changes in the mitochondrial membrane potential ($\Delta\Psi m$) in the cytotoxic effect induced by BEA. the study was carried out with human intestinal Caco-2 cells.

2. Materials and methods

2.1 Reagents

Sigma Chemical Co. (St Louis, MO, USA) provided the reagent grade chemicals, cell culture components used and BEA (783.95 g/mol, ≥97% purity). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Human recombinant Annexin V-FITC coniugate, tetramethyl rhodamine methyl ester (TMRM), To-Pro®-3 iodide were from Invitrogen, (USA).

Stock solution of BEA was prepared in methanol (MeOH) and maintained at -20°C.

2.2 Cell culture and treatment

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of Non Essential Amino Acids (NEAA), 100U/mL penicillin, 100mg/mL streptomycin and 10% (v/v) Fetal Calf Serum (FCS) inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Absence of mycoplasma was checked routinely using the

Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA). The final BEA concentrations tested were achieved by adding BEA solutions to the culture medium with a final MeOH concentration of 1% (v/v). Appropriate controls containing the same amount of solvent were included in each experiment. Total protein content (µg/mL) was determined by Bradford method (Bio-Rad DC Protein Assay (catalogue number 500-0116) http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

2.3 *In vitro* cytotoxicity

Caco-2 cells were cultured in 96-well tissue culture plates at the density of $3x10^4$ cells/well. After cells reached confluence, the culture medium was replaced with fresh medium containing the serial dilution (*n*=2) of BEA ranging from 3.125 to 25 µM. The basal cytotoxicity endpoints assayed were tetrazolium salt reduction (MTT) and Neutral Red (NR) assays as described by Ruiz et al. (2006). The IC_{50} values obtained from both cytotoxicity assays were calculated from full dose-response curves.

2.4 Intracellular ROS generation

Early intracellular ROS production was monitored in Caco-2 cells according to Ruiz-Leal and George (2004) by using the H_2-DCFDA probe. DCFH-DA is taken up by the cells, then deacetylated by intracellular esterases and the resulting H_2-DCFDA is oxidized by ROS and forms the highly fluorescent DCF. Briefly, $3x10^4$ cells/well were seeded in a 96-well black culture microplate. After reaching confluence, cells were loaded with 20 µM H_2-DCFDA in fresh medium for 20 min. Subsequently

H_2-DCFDA was removed and cells were washed with PBS and exposed to 1.5 and 3.0 μM of BEA. Increases in fluorescence were measured on a Wallace Victor^2, model 1420 multi-label counter (Perkin Elmer, Turku, Finland), at intervals up to 2 h at excitation/emission wavelengths of 485/535 nm. Results are expressed as increase in fluorescence in respect to control.

2.5 Lipid peroxidation assay

LPO assay was carried out by determining the formation of reactive thiobarbituric acid reactive substances (TBARS), according to Buege and Aust (1978). Briefly, $4.8x10^5$ cells/well were seeded in 6-well plates. After achieving confluence, cells were treated with BEA at 1.5 and 3.0 μM for 24 h. After washing with PBS and homogenized in 20 mM Tris 0.1% Triton, cells were boiled (100 °C for 30 min) in presence of 0.5% TBA, 1.5 mM DFA and 3.75% BHT. Subsequently cells were cooled and centrifugated (1287 g, 15 min) and TBARS formed were measured at 532 nm. Results are expressed as ng of MDA/mg of protein measured by the Bradford method.

2.6 Intracellular glutathione

Determination of reduced and oxidized glutathione (GSH; GSSG) was assayed according to Maran et al. (2009). $4.8x10^5$ cells/well were seeded in 6-well plates and treated with BEA at 1.5 and 3.0 μM for 24. Measurement of GSH and GSSG was carried out at the excitation/emission wavelengths of 345/425 nm.

2.7 Cell cycle analysis

Cell cycle analysis was performed by staining the DNA with PI as described previously (Juan-García et al., 2013). PI is a popular fluorescent nucleic acid dye which is capable of binding and labeling double-stranded nucleic acids. $4.8x10^5$ cells/well were seeded in 6-well plates and treated with BEA at 1.5 and 3.0 μM for 24, 48 and 72h. Four independent experiments were performed for each BEA treatment and at least 10,000 cells were analyzed for each sample.

2.8 Flow cytometry analysis of apoptosis (and necrosis)

Cell death generally proceeds through two molecular mechanisms: necrosis and apoptosis. One of the characteristics of apoptosis is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane. The differential of population of apoptotic cells (early or late), necrotic and dead cells was identified by Annexin V-FITC/PI double staining (Vermes et al., 1995). 4.8×10^5 cells/well were seeded in 6-well plates. After 24, 48 and 72h of exposure at 1.5 and 3.0 μM of BEA, the assay was carried out as described by (Juan-García et al., 2013) 10,000 cells were acquired and analyzed on a BD FACSCanto flow cytometer with FACSDiva software v 6.1.3 (BD Biosciences). Green (FL-1, 530 nm) and orange-red fluorescence (FL-2, 585 nm) were detected, emitted by FITC and PI, respectively. Quadrant statistics were performed to determine viable cells, early apoptotic, late apoptotic and dead cells from the total population of cells. Caco-2 cells treated with 1% MeOH in the media were considered as control.

2.9 Detection of mitochondrial membrane potential (ΔΨ*m*) by tetramethyl rhodamine methyl ester [TMRM] method.

Induction of apoptosis leads to the loss of ΔΨ*m* (Orrenius, 2004). Changes in ΔΨ*m*, after BEA exposure (1.5 and 3.0 μM) were evaluated using TMRM and To-Pro®-3 double labeling as described by Tsiper et al. (2012). TMRM was used to detect ΔΨ*m* changes. To-Pro®-3, a membrane-impermeable nuclear marker was used to characterize plasma membrane integrity. 4.8 x 10^5 cells/well were exposed to BEA at 1.5 and 3.0 μM, trypsinized, resuspended in growth medium with a mix of the two markers (140 nM and 160 nM for TMRM and To-Pro®-3, respectively). After 30 min of incubation at 37°C in the dark, fluorescent intensities of TMRM (λ_{ex}= 548 nm and λ_{em}= 573 nm) and of To-Pro®-3 (λ_{ex}= 642 nm and λ_{em}= 661 nm) were measured by using a flow cytometer FACSCanto (Beckton-Dickinson, Italy). Data from fluorescent histograms were analyzed using the FACSDiva software v 6.1.3 (BD Biosciences). The percentage of TMRM and ToPro-3 stained cells was calculated from the total 10,000 cells in comparison to the control.

2.10 Alkaline comet assay (pH>13)

The DNA strand breaks induction was determined using the alkaline comet assay (pH>13), according to the method described previously (Singh et al., 1988; Marabini et al., 2011), with some modifications. Caco-2 cells were treated with BEA (1.5, 3.0 and 12.0 μM), for 24 h. Subsequently, 2x10^4 cells/mL were suspended in pre-warmed LMA (0.5% PBS; 37°C) and 80 μL of the suspension were rapidly transferred to agarose precoated slides (1% PBS) and covered with a coverslip. After gelling for 10 min at 0°C, the coverslip was gently

removed and a third layer of 80 µL LMA was added. After gelling once again for 10 min at 0°C, the coverslip was gently removed and slides were put on a tank filled with lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO and 1% Triton X-100, freshly added) for 30 min at 0°C. Slides were then removed from lysis solution, washed with neutralization buffer (0.4 M Tris, pH 7.5) and incubated for 20 min in fresh electrophoresis buffer (300 mM NaOH, 1 mM Na-EDTA) to allow DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA, during 40 min. Slides were washed for 5 min with neutralization buffer and left to dry overnight at 4 °C. After staining with 500 µL of PI (20 µg/mL), cells were visualized by a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F) to capture imagines. Images of ≥100 randomly selected cells were analyzed by using the Automatic Comet Assay by TriTek CometScore™ freeware (http://autocomet.com/index.php?id=cometscorepro). Results are expressed as the median tail moment (TM) calculated according to the equation: TM = TL x Tail DNA% where TL is the tail length, i.e. the distance (µm) between the center of the comet head and the end of the comet tail.

2.11 Statistical analysis of data

Statistical analysis of data was carried out using SPSS Statistic 19.0 (SPSS, Chicago, IL, USA) statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between concentrations were analyzed

statistically with ANOVA followed by the Tukey HDS post hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 *In vitro* cytotoxicity

Results indicate that BEA induced a marked decrease in Caco-2 cell viability in a dose and time-dependent manner (data not shown). The IC_{50} values obtained in Caco-2 cells after BEA exposure ranged from 3.2 ± 1.1 to 20.6 ± 6.9 by the MTT assay and from 1.9 ± 0.7 to 8.8 ± 0.9 μM by the NR assay (Table 1).

Table 1. Medium inhibitory concentration (IC_{50}) values of BEA on Caco-2 cells, after 24, 48 and 72 h of exposure by the MTT and NR assays. IC_{50} values were calculated from full-dose response curve. Results are mean ± SD of three experiments.

Assays	IC_{50} (μM) ± SD		
	24 h	48 h	72 h
MTT	20.6 ± 6.9	12.8 ± 4.8	3.2 ± 1.1
NR	8.8 ± 0.9	3.4 ± 0.9	1.9 ± 0.7

3.2 Intracellular ROS generation

To determine changes in the redox status of Caco-2 cells in response to BEA exposure, cells were exposed to BEA at 1.5 and 3.0 μM from 0 to 120 min. The production of ROS was determined by DCFH-DA

assay (Fig. 1). Results obtained demonstrated that Caco-2 cells showed a high production of oxidizing species depending on the time and concentrations of BEA exposure when compared to the basal rate. Significant production of ROS (1.4-fold higher than the control) was observed immediately after BEA exposure (Fig. 1). However, the highest relative intensity of fluorescence (2.0-fold higher than the control) in Caco-2 cells was observed at the highest time of exposure (Fig. 1). Moreover, the ROS-production between the two BEA concentrations tested were significantly different ($p \leq 0.05$), between 5 and 60 min intervals (Fig. 1).

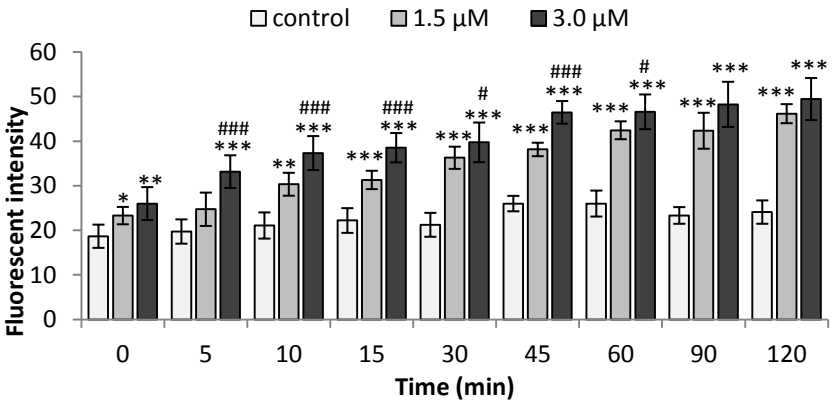

Figure 1. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to BEA at 1.5 and 3.0 µM. H_2-DCFDA was added to Caco-2 cells and left for 20 min previously to BEA addition. Results are expressed as mean ± SD, (n=3). $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.000$ (***), significantly different from the control. $p \leq 0.05$ (#), $p \leq 0.000$ (###), significantly differences between the two concentrations tested (1.5 and 3.0 µM).

3.3 Lipid peroxidation assay

The LPO production on Caco-2 cell in the presence of BEA at 1.5 and 3.0 μM can be observed in Figure 2. Results obtained demonstrated that after 24 h of BEA exposure MDA production increased in a dose dependent manner by 120% (1.5 μM) and 207% (3.0 μM) with respect to the control cells ($p \leq 0.000$).

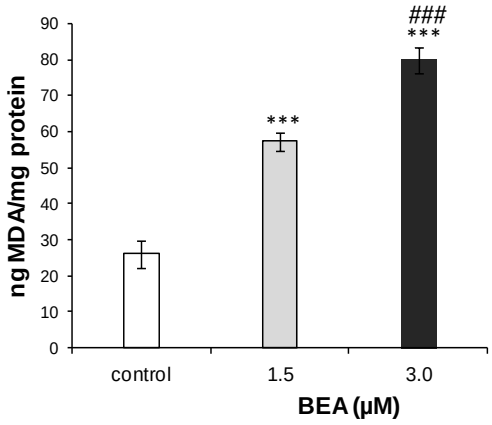

Figure 2. Lipid peroxidation as measured by MDA production in Caco-2 cells incubated for 24 h with 1.5 and 3.0 μM of BEA. Results are expressed as mean ± SD in ng of MDA/mg of protein measured by Bradford method. $p \leq 0.000$ (***): significantly different from control. $p \leq 0.000$ (###) significant differences between the two concentrations tested (1.5 and 3.0 μM).

3.4 Intracellular glutathione

BEA decreased intracellular GSH levels in Caco-2 cells in a dose dependent manner (Fig.3). After 24 h of exposure, 1.5 and 3.0 μM of

BEA reduced GSH levels by 23% and 31%, as compared to the control ($p \leq 0.05$), respectively. However, BEA exposure increased GSSG levels in Caco-2 cells only after 3.0 μM concentration (20% as compared to the control; Fig. 3).

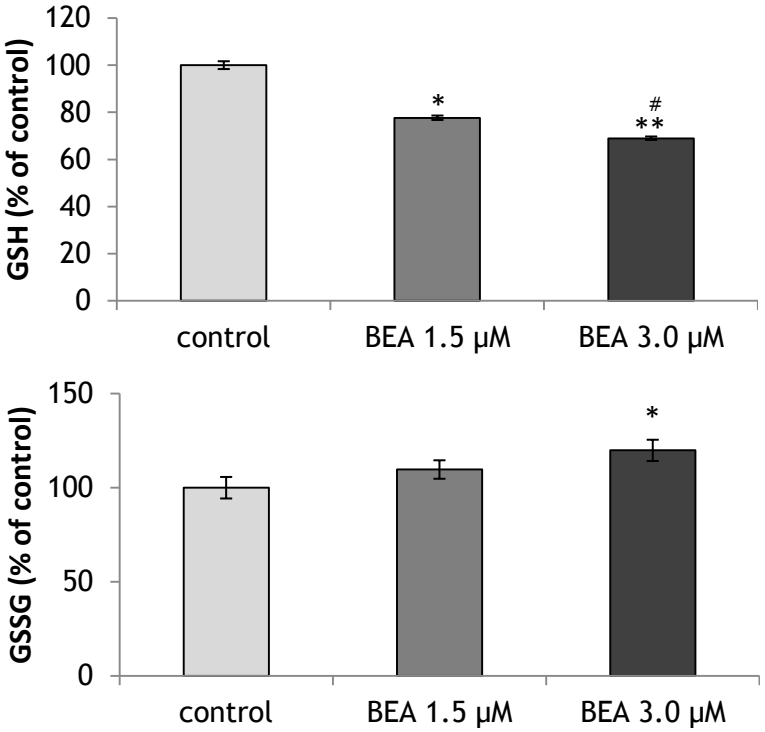

Figure 3. Reduced (GSH) and oxidize (GSSG) glutathione levels in Caco-2 cells after exposure to BEA at 1.5 and 3.0 μM for 24 h. $p \leq 0.05$ (*), $p \leq 0.001$ (**) significantly different from the control, $p \leq 0.05$ (#) significantly different between the two concentration of BEA tested. Results are expressed as percentage of control cells. Values are expressed as mean ± SD ($n = 4$).

3.5 Cell cycle analysis

Flow cytometry was used to determine cell proliferation by cell cycle analysis with PI staining. As shown in Figure 4, after 24, 48 and 72 h of exposure to 1.5 and 3.0 μM of BEA, a significant ($p \leq 0.05$) percentage of cell reduction in G0/G1 phase accompanied by an increase of G2/M phase, was observed, as compared to the control. After 24 h of exposure, the percentage of Caco-2 cells in the G0/G1 phase was 54.4 ± 0.5 and 45.3 ± 0.6% respectively after 1.5 and 3.0 μM of BEA, as compared to the control (58.7 ± 1.5%; Fig. 4A). Conversely, an increase in the number of cells in the G2/M phase was observed after exposure to 3.0 μM of 29.9 ± 1.0%, as compared to the control (23.9 ± 3.6%), with no changes in cells in S phase. After 48 h of exposure to 3.0 μM BEA, a reduction of G0/G1 phase (58.2 ± 1.8%) compared with the control (62.3 ± 1.2%; Fig. 4B) was observed. The increase in G2/M phase with a cell percentage of 20.6 ± 1.1%, in respect to control (16.7 ± 1.5%) was obtained. The percentage in S phase remained unchanged. After 72 h of exposure, Caco-2 cells in the G0/G1 phase decreased (Fig. 4C). The percentages were 55.7 ± 1.0 and 43.4 ± 0.9% after 1.5 μM and 3.0 μM of BEA, respectively, as compared to the control (65.9 ± 3.7%). Cell percentage in G2/M phase increased, with a value of 17.7 ± 1.8 and 20.3 ± 1.1 after 1.5 μM and 3.0 μM, respectively, as compared to the control (14.0 ± 0.9%). The proportion of cells in S phase increased with a value of 20.2 ± 0.41% (after 3.0 μM of BEA), compared to the control (14.5 ± 4.0%). A marked increase in SubG1 population was only observed after 72 h of exposure. The percentage was 6.3 ± 0.6 and 9.5 ± 0.5% at 1.5 and 3.0 μM, respectively, in respect to control (2.5 ± 0.6%; data not shown). The maximum reduction of cells in G0/G1 phase (about 35%)

and the highest percentage of cells in G2/M phase (43% respect to the control) were observed after 72 h of exposure.

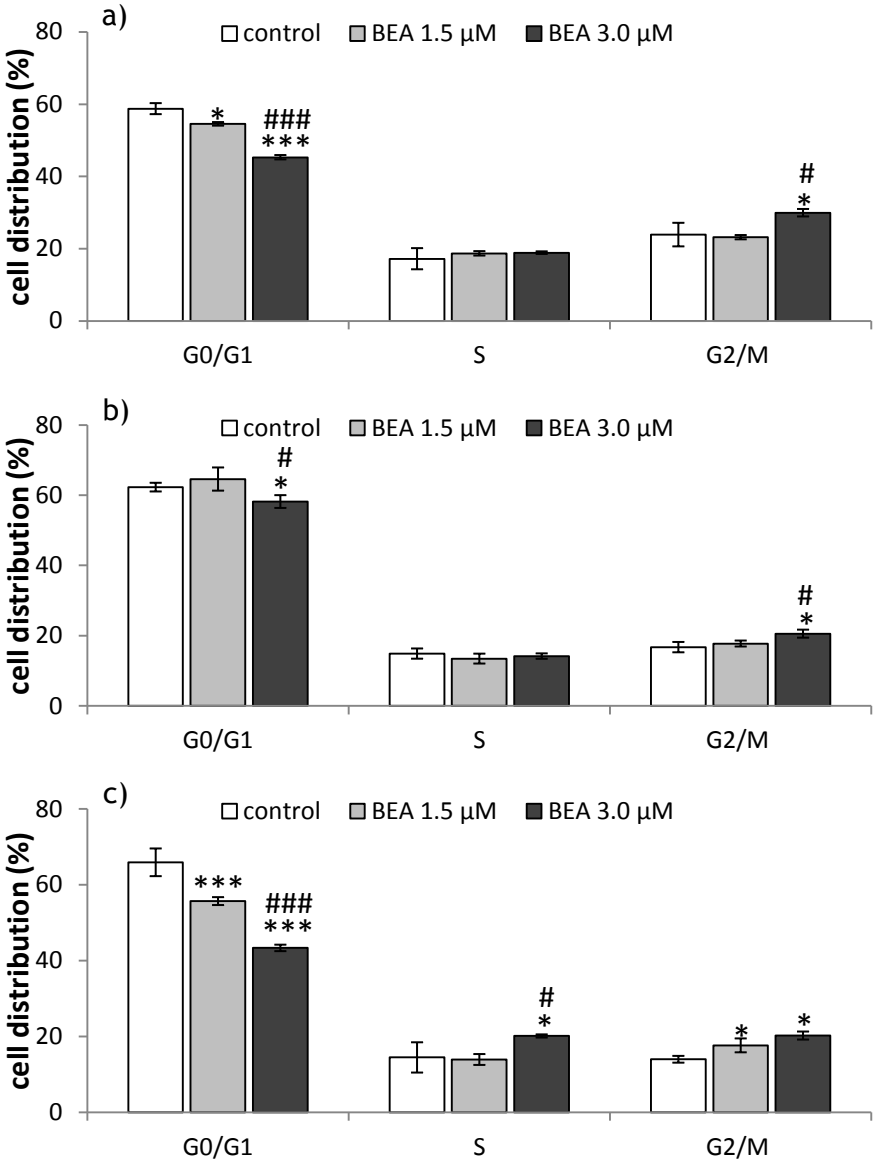

Figure 4. Analysis of cell cycle distribution of Caco-2 cells treated with BEA at 1.5 µM and 3.0 µM for 24 (A), 48 (B) and 72 h (C). Data are expressed as mean values ± SD (n = 3). (*) $p \leq 0.05$ and (**) $p \leq 0.001$ indicate a significant difference with the control, (#) $p \leq 0.05$ and (###) $p \leq 0.000$ indicate significant differences between the two concentrations tested (1.5 and 3.0 µM).

3.6 Flow cytometry analysis of apoptosis (and necrosis)

As shown in Figure 5, the population of early apoptotic cells was increased after 24 and 48 h in a dose dependent manner. After 24 h (Fig. 5A), apoptotic cells percentages were 25.1 ± 0.9 and 33.7 ± 2.6%, as compared to the control (11.7 ± 1.3%) after 1.5 and 3.0 μM of BEA exposure, respectively. After 48 h (Fig. 5B), apoptotic cells percentages were 14.0 ± 4.0 and 25.6 ± 3.9%, after 1.5 and 3.0 μM of BEA exposure, respectively, as compared to the control (6.5 ± 1.3%). Results obtained in Figure 5 showed that the population of early apoptotic cells was increasing in a dose-dependent manner after 24 (from 100 to 183%, in respect to control) and 48 h (from 143 to 286%, in respect to control) of exposure. Nevertheless, no significant increase after 72 h of exposure was observed (Fig. 5C). On the other hand, a significant increase of apoptotic/necrotic cells was observed after 24 h of exposure, with cells percentages of 27.4 ± 6.8 and 30.8 ± 2.2% after BEA exposure at 1.5 and 3.0 μM concentrations, respectively (as compared to the control: 12.0 ± 0.8%). Apoptotic/necrotic cells were observed after 72 h of exposure to 3.0 μM of BEA, with a percentage of 15.8 ± 0.7%, as compared to the control (10.7 ± 1.4%; Fig. 5).

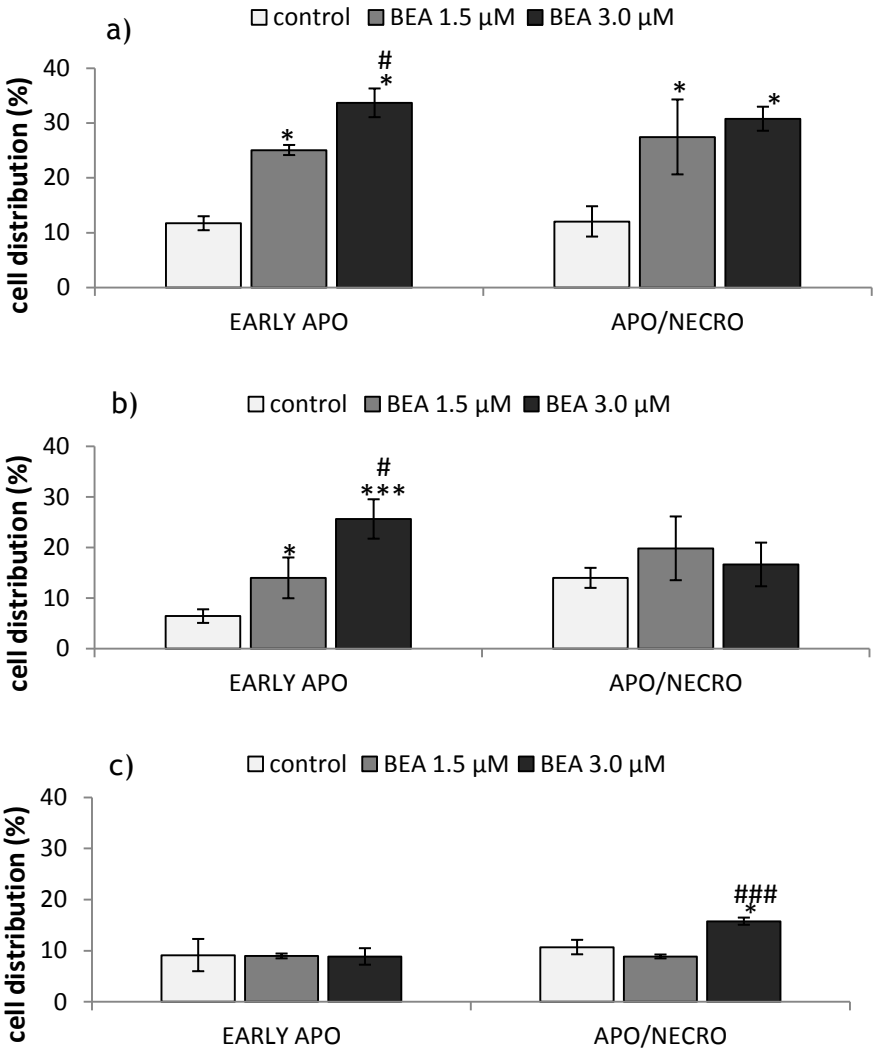

Figure 5. Analysis of apoptosis induction in Caco-2 cells treated with BEA at 1.5 µM and 3.0 µM for 24 (A), 48 (B) and 72 h (C). Cells were stained with Annexin-FITC and PI to distinguish early apoptotic from apoptotic/necrotic cells. Data are expressed as mean values ± SD (n = 3). $p \leq 0.05$ (*) and $p \leq 0.000$ (***) indicate a significant difference with the control, $p \leq 0.05$ (#) and $p \leq 0.000$ (###) indicate a significant difference with the two concentrations tested (1.5 and 3.0 µM). APO=apoptosis; APO/NECRO=apoptosis/necrosis.

3.7 Detection of mitochondrial membrane potential (ΔΨ*m*) by tetramethyl rhodamine methyl ester [TMRM] method.

To better characterize the apoptotic cell death triggered by BEA, the role of mitochondria was investigated. The evaluation of ΔΨ*m* changes in Caco-2 cells were determined by flow cytometry with the TMRM and To-Pro®-3 double staining. Cells showed a significant ($p \leq 0.05$) decrease in TMRM fluorescence intensity (%) after all concentration and times of exposure (Fig. 6). The drop in TMRM intensity was drastically produced after 72 h of exposure (Fig. 6). Reduction of ΔΨ*m* was from 2% to 95% after 1.5 µM of BEA and from 10% to 80% after 3.0 µM of BEA in respect to control (Fig. 6).

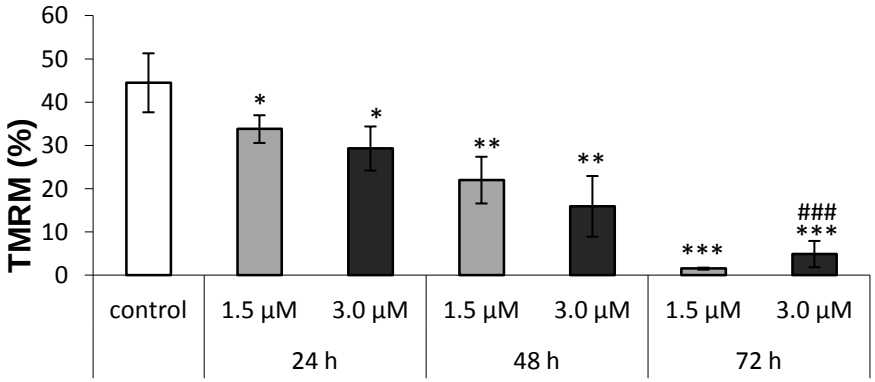

Figure 6. Loss of mitochondrial membrane potential (ΔΨm) in Caco-2 cells as detected by flow cytometry after TMRM/ To-Pro-3 double staining. Caco-2 were treated with 1.5 and 3.0 µM of BEA, for 24, 48 and 72 h. $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.000$ (***), significantly different from the control. $p \leq 0.000$ (###) significant differences between the two concentrations tested.

3.8 Alkaline comet assay

Figure 7 presents the alkaline comet assay data in Caco-2 cells exposed to BEA for 24 h. B(α)P 20 μM was used as positive control. After 24 h of exposure the lowest concentrations tested (1.5 and 3.0 μM) did not produce any significant increase in the TM parameter in respect to the control. However, a significant increase ($p \leq 0.000$) in the TM was obtained after 12.0 μM of BEA exposure (Fig. 7).

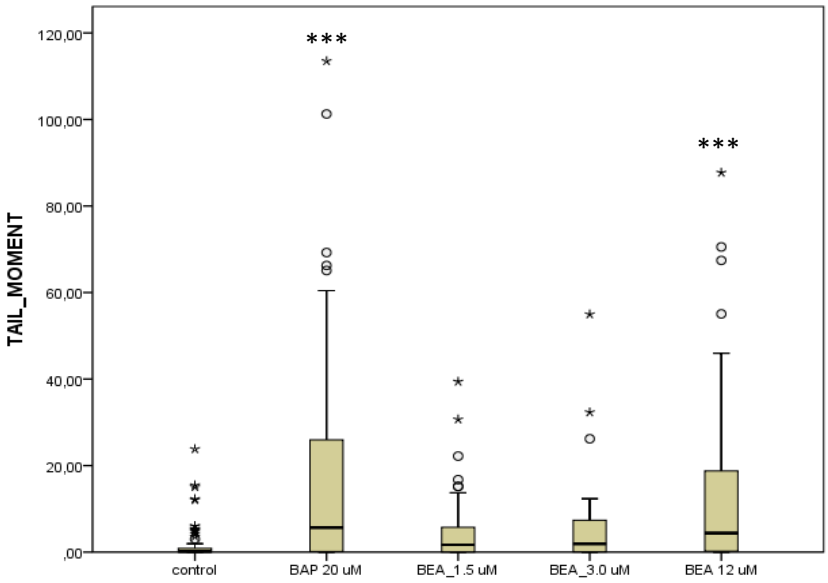

Figure 7. Alkaline comet assay (pH > 13) on Caco-2 cells after 24 h exposure to 1.5, 3.0 and 12.0 μM BEA. Migration of DNA is reported as median TM. B(α)P (20 μM) was the positive control. The box plots show the medians (middle bar), standard deviations, 25% and 75% percentiles. $p \leq 0.000$ (***) significantly different from the control. $p \leq 0.000$ (###) significantly different from 1.5 and 3.0 μM concentrations. Extreme cases and (*) atypical values (○).

4. Discussion

The IC_{50} values obtained in this study, demonstrated that BEA induces cytotoxicity in Caco-2 cells by increasing lysosomal damage and decreasing cellular metabolism as assessed by the NR and MTT assays, respectively. Lysosomal functionality was more affected compared to the mitochondrial one. However, the findings obtained by the NR assay cannot be interpreted as deleterious effects in this sub-cellular compartment since NR dye penetrates cell membranes by nonionic passive diffusion and concentrates only in the lysosomes of viable cells (Repetto et al., 2008), on the contrary MTT assay reported BEA cytotoxicity in the form of reduced cell viability through the reduction of mitochondrial dehydrogenase activity. So, MTT conversion could provide an indirect measure of cell metabolism, since the reduction of formazan in viable cells take place via mitochondrial dehydrogenases reaction coupled to oxidative phosphorilation. In addition, this difference could be due to the fact that the plasma membrane of the lysosomes, which is the first site exposed to the mycotoxin, is attacked more easily as compared to the mitochondrial one. Besides this, the mitochondrial toxicity do not exclude the involvement of lysosomal system, which has been recently connected to apoptotic cell death process triggered by the mitochondria (Boya and Kroemer, 2008).

Results obtained show that BEA is cytotoxic to Caco-2 cells at low micromolar concentrations, similar to ones obtained in Vero, CHO-K1, HepG2 and MRC-5 cells (Ruiz et al., 2011a,b; Ferrer et al., 2009; Ivanova et al., 2006). However, lower IC_{50} values were obtained by Dornetshuber et al. (2009a) in several human cell lines and their

chemoresistant sublines. Results obtained showed that GLC-4 (small cell lung carcinoma) cell line was the most sensitive (IC_{50}=1.88 μM) and SW-1573 (alveolar epithelial cell carcinoma) the most resistant (IC_{50}=3.17 μM) cell line to BEA. These results agree with Jow et al. (2004) and Lin et al. (2005), that obtained IC_{50} values of 2.6 and 4.5 μM in CCRF-CEM (human leukemia) and in NSCLC A549 (non small cell lung cancer), respectively. Although Caco-2 cell line was not the most sensitive to BEA cytotoxicity, results obtained show that Caco-2 cells are sensitive to BEA cytotoxicity in a dose and time dependent manner (data not shown).

In order to assess the mechanism of cytotoxicity produced by BEA on Caco-2 cells, this study investigated the role of oxidative stress in terms of early ROS generation, LPO production and GSH levels depletion. The effect of BEA on oxidative stress was analyzed in Caco-2 cells treated with BEA sub-cytotoxic concentrations of 1.5 and 3.0 μM at different time of incubation. The selection of this two concentration was based on the fact that they are in the range of concentrations found previously in food. In fact, according to Zinedine et al. (2011), levels up to 4 mg/Kg have been detected in wheat samples from Morocco. Considering the consumption of wheat in Morocco (175.4 Kg/year; FAOSTAT, 2009), the estimated total intake of BEA is about 2.45 μmol/day, a value that is included in the range of concentration tested in this study. Moreover, the selection of 1.5 and 3.0 μM was due to the fact that they were not cytotoxic to Caco-2 cells, since they were the inhibitory concentration 90 (IC_{90}; obtained by the MTT assay). The time-dependent increase in ROS formation observed in Figure 1 suggests that generation of ROS is an important mechanism of action which

might contribute to the cytotoxicity of BEA in Caco-2 cells. High ROS spikes were found inside Caco-2 cells after BEA exposure, which continued up to 2 h and increased up to 2.2 folds as compared to the control (Fig. 1). The present study confirmed that ROS generation are produced by BEA after early exposure to non toxic concentration as reported for CHO-K1 (Ferrer et al., 2009). Results are comparable to other mycotoxins that are well-known inducer of oxidative stress and oxidative damage in cell culture. Bouaziz et al. (2008) reported that zearalenone (ZEA) and T-2 toxin induced a higher increase in ROS generation with respect to BEA, of about 5- and about 4- folds when compared to the control. However higher time of exposure (24 h) were taken into account. Similar to BEA, OTA induced ROS generation 1.6-folds higher than the control (Arbillaga et al., 2007). It would be logical to assume that multiple ROS spike events might damage or destroy the antioxidant GSH and can induce oxidative damage to cellular components such as lipids by means of LPO. Earlier *in vitro* studies demonstrated that BEA induced LPO and GSH depletion in other cell lines (Klarić et al., 2007; Ferrer et al., 2009). These study have now been confirmed by our results obtained in Caco-2 cells. Similarly, Ferrer et al. (2009) observed that the increase of ROS production was higher than the control at the early stage of BEA exposure (about 4-folds higher than control), whereas from 5 to 120 min, less production of ROS was obtained, but also 2-folds higher than the control. Moreover, these authors also found a relationship between ROS generation and LPO produced. The increase in the MDA levels ranged from 45% to 182% of the control after BEA exposure. In Caco-2 cells the increase was higher (up to 207%). This could be due to the higher susceptibility of Caco-2

cells to LPO induced by mycotoxins (Kouadio et al., 2005). Similarly, Klarić et al. (2007) demonstrated that 5 µg/mL (6.4 µM) of BEA caused a significant increase in MDA production (80%) in kidney PK15 cells, after 48 h of exposure. These authors also found that treatment with concentrations ranging from 0.5 to 5 µg/mL of BEA decreased GSH levels in PK15 cells after 24 h of exposure, from 13% to 28%, as compared to the control, respectively. Results obtained by us and other authors confirm that ROS generation plays an important role in the molecular events leading to cell damage particularly by the induction of LPO and GSH depletion.

LPO is one of the cellular pathway involved in oxidative damage, that lead cells to apoptotic cell death, as well as GSH depletion with apoptosis have been demonstrated to occur in response to apoptotic stimuli in Caco-2 cells (Wang et al., 2000). Besides this, it have been reported that the alteration of cellular redox status resulted in an inhibition of cell proliferation (Noda et al., 2001; Gotoh et al., 2002). Generally, mycotoxins that perturb the cell cycle progression start their activity with anti-proliferative effect followed by accumulation of cells in one or more phases of the cell cycle. Caco-2 cells, treated with BEA resulted significantly in the following: reduction of cells percentage in G0/G1 phase and arrest in G2/M and S phase. After treatment with 3.0 µM concentration, BEA is capable to produce impairment of mitosis since an arrest in G2/M phase was produced after all time of exposure tested (Fig. 4). The same was observed after 72 h of exposure to BEA at 1.5 µM concentration. According to Noda et al. (2001), this type of arrest could be a consequence of cellular redox imbalance induced by BEA in Caco-2 cells, as determined by LPO production and GSH

depletion. The decrease of GSH and the increase of GSSG have been related to a redox-induced suppression of Caco-2 cells proliferation in G2/M phase of the cell cycle (Noda et al., 2001). Moreover, the reduction in G0/G1 phase cell number, that has been induced by BEA, suggests its apoptotic effect, as reported before for other compound in Caco-2 cells (Lazzè et al., 2004). After 72 h, exposure to 3.0 μM BEA caused a significant accumulation of the cells in S phase, along with a decrease in the corresponding G0/G1 population in a concentration-dependent manner, whereas in general the proportion of cells in the G2/M phase was also increased (Fig. 4). The reduction of cell distribution in G0/G1 phase observed at all time of exposure corroborates the induction of apoptosis and necrosis in Caco-2 cells. However, after 72 h of exposure, the damage persists, and it is confirmed by the S and G2/M phase arrest in cell cycle, and by the uncontrolled cell death (necrosis). The cell cycle perturbation could be explained as due to a checkpoint response to DNA damage or may be an adaptive process in which a surveillance mechanism delays or arrests the cell cycle when DNA lesions occur in order to make repair take place (Abid Essefi et al. 2003). However, this study showed unambiguously that no DNA damage is responsible for the cell cycle arrest. Other mechanisms, that have not been investigated here, need to be further studied.

Even if there are no reports about cell cycle effects after exposure to BEA, similar behavior have been reported by the structurally related enniatin B (EN B) on Caco-2 cells (Ivanova et al., 2012). They found that 25 μM of EN B increased cell population in G2/M phase (31 ± 1.3%, compared to the control (23 ± 1.0%) demonstrating

apoptotic effect of EN B. Ivanova et al. (2012) described this type of arrest as a possible consequence of external stimuli leading to apoptosis via caspase pathway or to non-apoptotic mitotic death (Ivanova et al., 2012). Similar behavior was observed in Caco-2 cells (Abid-Essefi et al. 2003) and HepG2 (Gazzah Chatti et al. 2010) exposed to ZEA.

On one hand, genotoxic potential of BEA could be implicated in its cytotoxicity, independently of ROS generation. Dornetshuber et al (2009b) demonstrated that BEA provides at least another possible pathway for the cytotoxicity of BEA which is a ROS-independent pathway that is directly not related to ROS-induced DNA strand breaks. This is in accordance with results obtained by this study, since no DNA-damage was observed after exposure to ROS inducing concentration of BEA in Caco-2 cells. However, the concentration of 12.0 μM produced a significant increase in the % of DNA in the tail (expressed as TM) which could be indicative of the activation of BEA-induced apoptotic pathways in Caco-2 cells, and cannot completely exclude the genotoxic potential of BEA, even though not related with ROS generation and oxidative stress in Caco-2 cells. Results are in accordance with Klarić et al. (2010) and Çelik et al. (2010) which tested the genotoxic potential of BEA on PK15 cells and lymphocytes by the comet and micronucleus assays, respectively. They reported the genotoxic potential of BEA even if produced by concentrations much more lower than ones tested in this study.

On the other hand, the generation of intracellular ROS that induces cytotoxicity and the decrease in GSH levels upon oxidation were also linked with their diffusion into mitochondrial matrix, with subsequent loss of integrity and finally cell death mediated by

mitochondrial dysfunction and the induction of apoptosis. As obtained in this study, BEA induced apoptosis in Caco-2 cells, after 24 h of exposure in a dose-dependent manner and underwent necrosis. This could be due to the fact that under the same stimulus, features of both apoptosis and necrosis may coexist in the same cell. However, necrosis was not observed after 48 h of exposure and this could be explained by the fact that Caco-2 cells maybe have entered in a non-apoptotic cell death pathway, showing "pseudo-necrotic" morphological features only after 24 h of exposure (Zong and Thompson, 2006). At this time of exposure, apoptosis was also observed, confirming the prevalence of apoptotic activity of BEA, at sub-toxic concentrations. After 72 h of exposure, only necrosis was induced by BEA in response to the same stimulus, but with higher doses or intensity (Kroemer et al., 1998).

BEA induced significant cell death in several cell lines derived from healthy and malignant tissues of different origin (Dornetshuber et al, 2009a,b; Jow et al., 2004). Apoptosis induction (8 μM and 72 h), but not necrosis, was evidenced by nuclear DNA fragmentation and light microscopy in turkey peripheral blood lymphocytes (Dombrink-Kurtzman, 2003) and human NSCLC A549 cells (3 and 10 μM treatment during 24 h; Lin et al., 2005). In contrast to results obtained in this study and by the other above-mentioned authors, Dornetshuber et al. (2009b) did not detect oxidative cell damage induced by 10 μM of BEA in the human lung cancer cell lines (A549 and GLC-4), in the epidermal carcinoma-derived cell line (KB-3-1) and in the promyelocytic leukemia cell line (HL-60) during 15 min of exposure in respect to control. However, ROS production was always observed at higher times of exposure. The results of Dornetshuber et al (2009b) and those obtained

in this study could be explained by the different *in vitro* method, time of exposure, cell lines used and by the sensitivity of the applied test which could condition their response.

From results obtained in our study, it could be assumed that the BEA-induced (apoptotic) cell death was likely to be mediated by the generation of intracellular ROS and oxidative stress. As assessed by MTT and flow cytometry determination of $\Delta\Psi m$ (Fig. 6), the mitochondrial dysfunction is also involved in BEA cytotoxicity. It has been proposed that BEA interacts with cell membranes (ionophoric activity) which cause an increase in the intracellular Ca^{2+} concentration. According to Jow et al. (2004) cell death caused by BEA involves a Ca^{2+}-dependent pathway, in which BEA induced a significant increase in intracellular [Ca^{2+}] that leads to a combination of cellular apoptosis and necrosis responses. Moreover, in isolated mitochondria BEA induced $\Delta\Psi m$ drop, K^+ influx into the mitochondrial matrix, mitochondrial swelling and uncoupling of oxidative phosphorylation, followed by induction of apoptosis (Tonshin et al., 2010). The loss of $\Delta\Psi m$ could trigger the release of the cytosolic cyt c mitochondria to the cytosol under the regulation of the Bcl-2 family and caspase 3 which finally lead to cell death (Dornetshuber et al., 2009b). Moreover, the expression of Bcl-2 family proteins can be differently regulated by BEA, suggesting that the BEA-induced apoptosis is controlled by a balanced expression between those apoptosis-inducing and apoptosis- suppressing molecules (Jow et al., 2004; Lin et al., 2005; Klarić et al., 2008). Taken together, these observations provide a hypothesis that ROS generation could be a direct pathway to BEA produced cytotoxicity in Caco-2 cells.

Based on the data obtained, it might be hypothesized that BEA can exert toxicological effects by means of a ROS-dependent pathway rather than DNA damage. Cells exhibit mitochondrial dysfunction that can lead to the stable depolarized state of $\Delta\Psi m$ and cell death. Moreover, ROS generation with alteration of GSH content and LPO production lead to the increase of oxidative stress which causes apoptosis in Caco-2 cells and changes in the normal cell cycle.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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REACTIVE OXYGEN SPECIES INVOLVEMENT IN APOPTOSIS AND MITOCHONDRIAL DAMAGE IN CACO-2 CELLS INDUCED BY ENNIATINS A, A_1, B AND B_1

Prosperini, A.[*], Juan-García, A., Font, G., Ruiz, M.J.

Laboratory of Toxicology, Faculty of Pharmacy, University of València, Av. Vincent Andrés Estellés, s/n 46100 Burjassot, València, Spain

[*] Corresponding author, tel: +34963544958. Fax: +34963544954
E-mail: alessandra.prosperini@uv.es.

ABSTRACT

The cytotoxic effects, the generation of reactive oxygen species (ROS) and lipid peroxidation (LPO) as well as the cell cycle disruption, the induction of apoptosis and necrosis, changes in mitochondrial membrane potential ($\Delta\Psi m$) and DNA damage have been determined in human colorectal adenocarcinoma (Caco-2) cells after exposure to enniatins (ENs) A, A_1, B and B_1. IC_{50} values obtained by the MTT and Neutral Red assay, after 24, 48 and 72 h of exposure ranged from 0.5 ± 0.1 to > 15 μM. A significant increase ($p \leq 0.05$) in ROS generation and LPO production, as determined by the fluorescent probe H_2-DCFDA and TBARS method respectively, was observed for all mycotoxins tested at 3.0 μM concentration. The highest increase in ROS generation (2.6 fold higher than control) and LPO production (111%, as compared to control) was observed with EN A. Cell cycle was significantly arrested at G2/M phase after 24 h of exposure to EN A, A_1, B_1, whereas after 72 h of exposure an arrest in S phase was observed almost for all mycotoxins tested. Moreover, after 24 and 48 h of exposure, ENs increased the early apoptotic cells, whereas after 72 h of exposure necrosis was observed. In addition the loss of $\Delta\Psi m$ was produced on Caco-2 cells after ENs exposure. ENs A, A_1, B and B_1 cytotoxicity involved early ROS generation that induced LPO oxidative damage, apoptosis and necrosis via the mitochondrial pathway. ENs A, A_1 and B_1 induced DNA damage. However the same effects cannot be proposed for and EN B. Further studies on the toxicological effects induced by ENs A, A_1, B and B_1 are needed.

Keywords: enniatins, cytotoxicity, oxidative stress, cell cycle, apoptosis, mitochondrial membrane potential, DNA damage, Caco-2 cells.

1. Introduction

The enniatins (ENs) are cyclic hexadepsipeptidic mycotoxins produced by several species of *Fusarium*, that naturally occur in cereal grains, bread, malt and silage (Prosperini et al., 2013). They have become an issue of high concern for human and animal health during the last decade, because of their potential toxicity, probably linked to their ionophoric activities (Tedjiotsop Feudjio et al., 2010; Jestoi, 2008). Their hydrophobic nature allows them to incorporate easily in biological membranes by forming dimeric structure that transports monovalent ions across the cellular membranes, in particular the mitochondrial membrane, affecting ionic homeostasis (Tonshin et al., 2010). On the other hand, excessive reactive oxygen species (ROS) generation leads to oxidative stress status, which can lead to oxidized macromolecular structures, including membrane lipids, proteins and DNA, and causes cell cycle arrest and cell apoptosis. The induction of ROS, lipid peroxidation (LPO) and gluthation (GSH) decrease have been reported for the structurally related *Fusarium* mycotoxin beauvericin (BEA; Ferrer et el., 2009; Klarić et al., 2007). However, current studies reported that ROS generation induced by ENs is not a cause but a result of ENs toxicity in mitochondria (Ivanova et al., 2012). In our laboratory, a special interest has been given to the study of ENs toxicity pathways. We have demonstrated that ENs are cytotoxic by a further set of endpoints; they inhibit cell proliferation in different cell line (Meca et al., 2011; Lu et al., 2013) and their binary and tertiary combinations produce higher cytotoxic effects (Lu et al., 2013). We have already demonstrated the bioaccessibility of ENs from different food commodities (Prosperini et al., 2013) and bioavailability by applying an

in vitro method which allowed the simulation of the small and large intestine (Meca et al., 2012). Moreover, prevention of ENs-induced cytotoxic effect in mammalian cells has been previously evaluated by addition of polyphenols in cell culture (Lombardi et al., 2012). However, very few data about cellular targets and resulting toxicological consequences in *in vitro* cell cultures are available about ENs A, A_1, B and B_1. In this context, the aims of the this study were to determine if the cytotoxic effects of ENs A, A_1, B and B_1 could be related to the generation of ROS and cell damage, such as LPO, cell cycle disruption and apoptosis induction through changes in mitochondrial membrane potential ($\Delta\Psi m$) in human colon adenocarcinoma cells (Caco-2). Caco-2 cells was chosen as a model because all previous studies in our laboratory have been developed in this type of cells.

2. Materials and methods

2.1 Reagents

EN A (purity: ≥ 97%, molecular weight: 681.9 g/mol), EN A_1 (purity: ≥ 97%, molecular weight: 667.9 g/mol), EN B (purity: ≥ 90%, molecular weight: 639.4 g/mol) and EN B_1 (purity: ≥ 97%, molecular weight: 654.9 g/mol) were from Enzo Life Sciences, Switzerland. The reagent grade chemicals and cell culture components used, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, HEPES, tetrazolium Bromide (MTT), Neutral Red (NR), non essential aminoacids (NEAA), phosphate buffer saline (PBS), Trizma base, Triton X-100, glucose, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), deferoxamine mesylate salt (DFA), di-*ter*-butyl-methylphenol (BHT),

1,1,3,3 tetramethoxipropan, 2´,7´-dichlorodihydrofluorescein diacetate (H_2-DCFDA), propidium iodide (PI), RNase A, Na-EDTA, agarose, agarose low melting temperature (LMA) and benzo(α)pyrene (B(α)P) were from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). NaOH was from Guinama (Spain). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). NaCl and ethanol were from Merck KGaA (Germany). $CaCl_2$ and formaldehyde were from Scharlau Chemie S.A. (Barcelona, Spain). Methanol (MeOH) and Glacial acetic acid were from VWR International (LLC, Pennsylvania, USA). Human recombinant annexin V-FITC coniugate, tetramethyl rhodamine methyl ester (TMRM), To-Pro®-3 iodide and the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were from Invitrogen, (USA). All other reagents were of standard laboratory grade. Stock solutions of ENs (1 mM) were prepared in methanol and maintained at -20°C. Final concentrations tested were achieved by adding ENs in the culture medium with the final methanol concentration of 1% (v/v). Total protein content (µg/mL) was determined by Bradford method (Bio-Rad DC Protein Assay, catalogue number 500-0116, http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

2.2 Cell culture

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100U/mL penicillin, 100mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every

two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

2.3 Cell viability assays

The MTT and NR assays were performed as described by Ruiz et al. (2006) with some modifications. Caco-2 cells were cultured in 96-well tissue culture plates by adding 200 µL/well of a suspension of 3 x 10^4 cells/well. After cells reached 80% confluence the culture medium was replaced with fresh medium containing the serial dilution of each mycotoxins ranging from 0.9 to 15 µM for ENs A_1, B and B_1 and from 0.45 to 7.5 µM for EN A.

For NR assay, after 24, 48 and 72 h of incubation with different ENs concentrations (serial dilution=2), medium containing each ENs was removed and 200 µL of freshly prepared NR solution (50 µg/mL) pre-warmed at 37°C, was added to each well and all plates returned to the incubator at 37°C for 3 h. The cells were washed once with PBS and fixed with formaldehyde– $CaCl_2$ solution, and then extracted by adding acetic acid–ethanol solution. Plates were gently shaken for 5 min so that complete dissolution was achieved before measuring absorbance at 540 nm with an automatic ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to control cells (1% MeOH). Three independent experiments were conducted and mean inhibition concentration (IC_{50}) values were calculated from full-dose response curve.

For MTT assay, after 24, 48 and 72 h of exposure with serial concentrations of ENs, each well received 200 µL of fresh medium containing 50 µL of MTT. After 4 h of incubation (37°C in darkness), the

resulting formazan was solubilised in DMSO. The absorbance was measured at 570 nm using the ELISA plate reader Multiscan Ex (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to the solvent control (1% MeOH). Three independent experiments were performed and the IC_{50} values were calculated from full dose-response curves.

2.4 ROS generation assay

Intracellular ROS production was monitored in Caco-2 cells by adding the H_2-DCFDA according to Ruiz-Leal and George (2004). Briefly, 3 x 10^4 cells/well were seeded in a 96-well black culture microplate. Once cells exhibited 80% confluence, the culture medium was replaced and cells were loaded with 20 μM H_2-DCFDA for 20 min and then, the medium with H_2-DCFDA was removed and washed twice with PBS before the addition of medium/1% MeOH (control) or medium with ENs. The selection of the two different concentration, 1.5 and 3.0 μM, for each ENs was made according to previous outcomes that confirm that they resulted to be non toxic and both of them are below the IC_{50} values obtained in Caco-2 cells. This method is exceptionally sensitive and it provides a direct measure of overall oxidative stress, which detects intracellular oxidants. DCFH-DA is taken up by the cells, then deacetylated by intracellular esterases and the resulting H_2-DCFDA becomes trapped inside the cell. The oxidation of the non fluorescent 2',7'– dichlorodihydrofluorescein moiety H_2-DCFDA by ROS to the DCF that is highly fluorescent is possible. Increases in fluorescence were measured at intervals up to 2 h at excitation and emission wavelengths of 485 and 535 nm, respectively. Twenty-four replicates were

developed. Results are expressed as increase in fluorescence in respect to control.

2.5 Lipid peroxidation assay

LPO was measured according to Buege and Aust (1978) based on the formation of thiobarbituric acid reactive substances (TBARS). This assay is based on the formation of a red adduct (absorption maximum 532 nm) between TBA and MDA, a final product of the peroxidation process. Briefly, 4.8 x 10^5 cells/well were seeded in 6-well plates. After achieving the 80% confluence, cells were treated with each mycotoxin individually, at the two concentrations, 1.5 and 3.0 μM selected, for 24 h. Later the medium was removed and cells were washed with PBS and then homogenized in 20 mM Tris 0.1% Triton. Lysated and homogenized cells were boiled (100 °C water bath for 30 min) under acid conditions in the presence of 0.5% TBA, 1.5 mM DFA and 3.75% BHT. Samples were cooled, centrifuged (1287 g, 15 min) and the formation of TBARS was measured at 532 nm. Three independent experiments were conducted and results are expressed as ng of MDA/mg of protein measured by the Bradford method.

2.6 Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by staining the DNA with PI as described previously (Juan-García et al., 2013) with some modifications. The PI fluorescent nucleic acid dye is capable of binding and labeling double-stranded nucleic acids, making possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. A number of 4.8 x

10^5 cells/well were seeded in 6-well plates. After 24, 48 and 72h of exposure at 1.5 and 3.0 μM, Caco-2 cells were trypsinized and placed on ice for 30 min with 860 μL of fresh medium containing 29 ng/mL of Vindelov's PI staining solution prepared as follows: 10 mg (700 U/L) RNase A, 0.1 ml of Nonidet P-40, 0.1% Triton X-100, 10 mM Tris, 10 mM NaCl and 50 μg/mL of PI in PBS. Ten thousand cells were analyzed by using EPICS XL MCL 4 CLR (Coulter Corporation) at the excitation wavelength of 488 nm and the emission wavelength of 620 nm. Data from the raw histograms were extracted by rectangular fitting (CYLCHRED or MODIFIT software, Beckton Dickinson, Milan, Italy). Cell cycle analysis was calculated by rectangular curve fitting (MODIFIT, Beckton Dickinson, Milan, Italy) using 1024 channels which produced histograms with a single G0/G1 peak at channel 200 when DNA is diploid, an S peak between channels 200 and 400 when DNA is replicating, a G2/M peak at channel 400 when DNA is tetraploid and a debris peak between channels 100 and 200 when DNA is hypodiploid or damaged (Minervini et al., 2004). The small coefficient of variation (CV) obtained in this study was the result of the high resolution achieved by proper alignment. Three independent experiments were performed for each EN treatment.

2.7 Flow cytometry analysis of apoptosis (and necrosis)

Cell death constitutes one of the key events in biology. At least two modes of cell death can be distinguished: apoptosis and necrosis. It is now generally assumed that both forms of cell death constitute two extremes of a continuum (Kroemer et al., 1998). As a matter of fact, the same toxin can induce apoptosis or necrosis at a low or high dose,

respectively. Apoptosis is a strictly genetically regulated cell death, whereas necrosis is an accidentally unregulated cell death, characterized by membrane lysis and release of cellular content. During apoptosis, the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane occurs. The differential of population of apoptotic cells (early or late), necrosis and dead cells can be identified by Annexin V-FITC/PI double staining (Vermes et al., 1995). Annexin V is a Ca2+- dependent phospholipid-binding protein with high affinity for PS, and binds to cells exposing PS to the extracellular side of the plasma membrane, whereas PI binds to the DNA of necrotic/dead cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells considered as viable are both Annexin V-FITC$^-$/PI$^-$; cells in early apoptosis (pro-apototic/apoptotic) cells are Annexin V-FITC$^+$/PI$^-$; cells in late apoptosis, that have completed the apoptotic and start the necrotic process (apoptotic/necrotic), are both annexin V-FITC$^+$/PI$^+$. For the annexin V-FITC assay, a number of 4.8 x 10^5 cells/well were seeded in 6-well plates. After 24, 48 and 72h of exposure at 1.5 and 3 μM of each ENs, cells were trypsinized and resuspended in 360 μL of HepesCa^{2+} buffer prepared as follows: 10 mM HEPES-NaOH (pH 7.4), 135 mM NaCl and 2.5 mM $CaCl_2$. After incubation at 4°C for 30 min in the dark, 10,000 cells were acquired and analyzed on a BD FACSCanto (FACS-Canto II, Deckton Dickinson, Italy) flow cytometer with FACSDiva software v 6.1.3 (BD Biosciences). Green (FL-1, 530 nm) and orange-red fluorescence (FL-2, 585 nm) were detected, emitted by FITC and PI, respectively. Quadrant statistics were performed to determine viable cells, early apoptotic, late apoptotic and dead cells from the total

population of cells. Caco-2 cells treated with 1% MeOH in the media were considered as control. Three independent experiments were performed for each EN treatment.

2.8 Detection of mitochondrial membrane potential (ΔΨ*m*) by tetramethyl rhodamine methyl ester [TMRM] method.

Induction of apoptosis leads to the loss of ΔΨ*m* (Zamzami et al., 1995). The ΔΨ*m* can be monitored in living cells using the cationic lipophilic dye TMRM whose structure allows it to be distributed freely through membranes into the mitochondrial matrix, as a function of the Nerst equation, correlated to the ΔΨ*m* (Juan García et al., 2013). In this study a double labeling with TMRM and To-Pro®-3 has been carried out as described by Tsiper et al. (2012). To-Pro®-3, a membrane-impermeable nuclear marker was used to characterize plasma membrane integrity and, as consequence, cell viability. The ionophore FCCP was used to optimize the MMP change analysis (by TMRM signal decrease) and cell viability by To-Pro®-3 signal increase. Briefly, Caco-2 cells were seeded in 6 well plates at a density of 4.8 x 10^5 cells/well. After ENs exposure (1.5 and 3.0 μM for each EN), cells were trypsinized and resuspended in growth medium (500 μL) where a cocktail of the two markers was included with a final concentration of 140 nM and 160 nM for TMRM and To-Pro®-3, respectively. After 30 min of incubation at 37°C in the dark, fluorescent intensities of TMRM (λ_{ex}= 548 nm and λ_{em}= 573 nm) and of To-Pro®-3 (λ_{ex}= 642 nm and λ_{em}= 661 nm) were measured by using a FACSCanto flow cytometer (FACS-Canto II, Deckton Dickinson, Italy). Data from fluorescent histograms were analyzed using the FACSDiva software v 6.1.3 (BD Biosciences). The percentage of TMRM

and ToPro-3 stained cells was calculated from the total 10,000 cells in comparison to the control (Caco-2 cells treated with 1% MeOH in the culture media). Three independent experiments were performed.

2.9 Alkaline comet assay (pH>13)

The induction of DNA strand breaks was determined using the alkaline comet assay (pH>13), according to the method described previously (Singh et al., 1988; Marabini et al., 2011), with some modifications. Caco-2 cells (4.8 x 10^5 cells/well) were seeded in 6-well plates. After achieving the 60% confluence, cells were treated with ENs, at the concentrations of 1.5, 3.0 and 12.0 μM), for 24 h before preparation for analysis of DNA damage at pH>13. Subsequently, 2 x 10^4 cells/mL were suspended in pre-warmed LMA (0.5% PBS; 37°C) and 80 μL of the suspension were rapidly transferred to agarose precoated slides (1% PBS) and covered with a coverslip (24x36 mm). After gelling for 10 min at 0°C, the coverslip was gently removed and a third layer of 80 μL LMA was added. After gelling for 10 min at 0°C, the coverslip was gently removed and slides were put on a tank filled with lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO and 1% Triton X-100, freshly added) for 30 min at 0°C. The slides were removed from lysis solution, washed with neutralization buffer (0.4 M Tris, pH 7.5) and incubated in fresh electrophoresis buffer (300 mM NaOH, 1 mM Na-EDTA) for 20 min at room temperature to allow DNA to unwind. Electrophoresis was then carried out at room temperature in fresh electrophoresis buffer for 40 min (25 V, 300 mA). After electrophoresis, slides were gently washed once for 5 min in fresh neutralization buffer. Slides were dried overnight at 4 °C, stained with

500 µL of PI (20 µg/mL) and covered with coverslip. Slides were visualized under a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F) to capture images. A minimum of 50 randomly selected individual cells were selected and analyzed by using the Automatic Comet Assay by TriTek CometScore™ freeware (http://autocomet.com/index.php?id=cometscorepro). The DNA damage in Caco-2 cells was expressed as the median tail moment (TM). The TM was chosen as the effect parameter, calculated according to the equation: TM = TL x Tail DNA% where TL is the tail length, i.e. the distance (µm) between the center of the comet head and the end of the comet tail. All parameters are provided by TriTek CometScore™. The Tail DNA is expressed as a percentage of the total DNA content based on the total fluorescence intensity.

2.10 Statistical analysis of data

Statistical analysis of data were carried out using SPSS Statistic 19.0 (SPSS, Chicago, IL, USA) statistical software package. Data were expressed as mean ± SD of independent experiments. The statistical analysis of the results was performed by Student's t-test for paired samples. Differences between concentrations were analyzed statistically with ANOVA followed by the Tukey HDS post hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1 Cell viability

Table 1 summarizes the IC_{50} value obtained by MTT and NR assay after exposure to each EN at the three different times of exposure. As determined by MTT assay, after 24 h of exposure, ENs A, B and B_1 did not show IC_{50} value at the range of concentration tested. After 48 h of exposure, all ENs tested showed IC_{50}, with exception of EN B. At 72 h of exposure all mycotoxins showed IC_{50} values. EN B showed the less cytotoxic effect on Caco-2 cells at the three times of exposure, following by ENs B_1 = A = A_1 (Table 1). As regards to NR uptake assay, after 24 h of exposure only ENs A and A_1 showed a significant IC_{50} values on Caco-2 cells. After 48 h of exposure, the IC_{50} values of EN A and A_1 resulted to be similar, and lower than those obtained for ENs B and B_1 (Table 1). After 72 h of exposure, the IC_{50} values were ranging from 0.46 ± 0.1 (ENs A and A_1) to 1.4 ± 0.2 μM (EN B). NR assay was the most sensitive methods for all ENs tested. ENs A and A_1 showed lower IC_{50}, as obtained by MTT and NR assays in Caco-2 cells.

3.2 ROS generation

Changes in the redox status of Caco-2 cells in response to ENs A, A_1, B and B_1 was determined. Cells were exposed to different concentrations (1.5 and 3.0 μM) of these ENs from 0 to 120 min. The production of ROS was determined by DCFH-DA assay (Fig. 1). The results obtained demonstrated that high production of oxidizing species was produced depending on concentration of EN and time of exposure when compared to the basal rate. The highest relative intensity of fluorescence in Caco-2 cells was observed at the highest time of exposure after testing all ENs (Fig. 1). EN A (Fig. 1a) showed a significant increase in ROS production up to 45 min of incubation at 1.5

and 3.0 µM of 1.8- and 2.6-fold higher than the control, respectively. ROS production in Caco-2 cells was observed at the early stage of ENs A_1, B and B_1 exposure (Fig. 1b, 1c and 1d). At 10 min, the EN A_1 concentration of 3.0 µM generated a ROS production of 1.9-fold higher than control, which continued growing up until it reached an increase of 2.6- fold higher than control at 120 min of exposure. However, 1.5 µM of EN A_1 increases 1.8-fold ROS production compared to the control from 10 to 120 min of exposure. As can be observed in Figure 1c, only 3.0 µM of EN B increased ROS production in Caco-2 cells from 10 up to 120 min. Higher fluorescence intensity (1.7-fold) was observed at 45 min of exposure. Referring to EN B_1, Figure 1d shows that the concentration of 1.5 µM produced a significant ROS generation from 10 min (1.6-fold higher respect to the control), to 120 min of exposure (1.8-fold). Higher ROS generation was observed after 3.0 µM of EN B_1 exposure from 5 up to 120 min (2.1-times to control).

Table 1. The medium inhibitory concentration (IC_{50}) of ENs A, A_1, B and B_1 in Caco-2 cells after 24, 48 and 72 h of exposure by the MTT and NR assays. IC_{50} values are given as percent of control cells (1% MeOH). Results are mean ± SD of three experiments.

	IC_{50} (µM) ± SD					
	NR			**MTT**		
	24 h	**48 h**	**72 h**	**24 h**	**48 h**	**72 h**
EN A	4.3±0.2	2.6±0.4	0.46±0.1	>7.5	6.8±1.6	1.6±0.8
EN A_1	7.7±0.2	2.9±0.6	0.46±0.1	14.8±2.7	7.7±1.2	1.3±0.6
EN B	>15	10.7±3.4	1.4±0.2	>15	>15	11.7±2.4
EN B_1	>15	10.8±3.8	0.8±0.3	>15	11.3±2.3	2.8±1.1

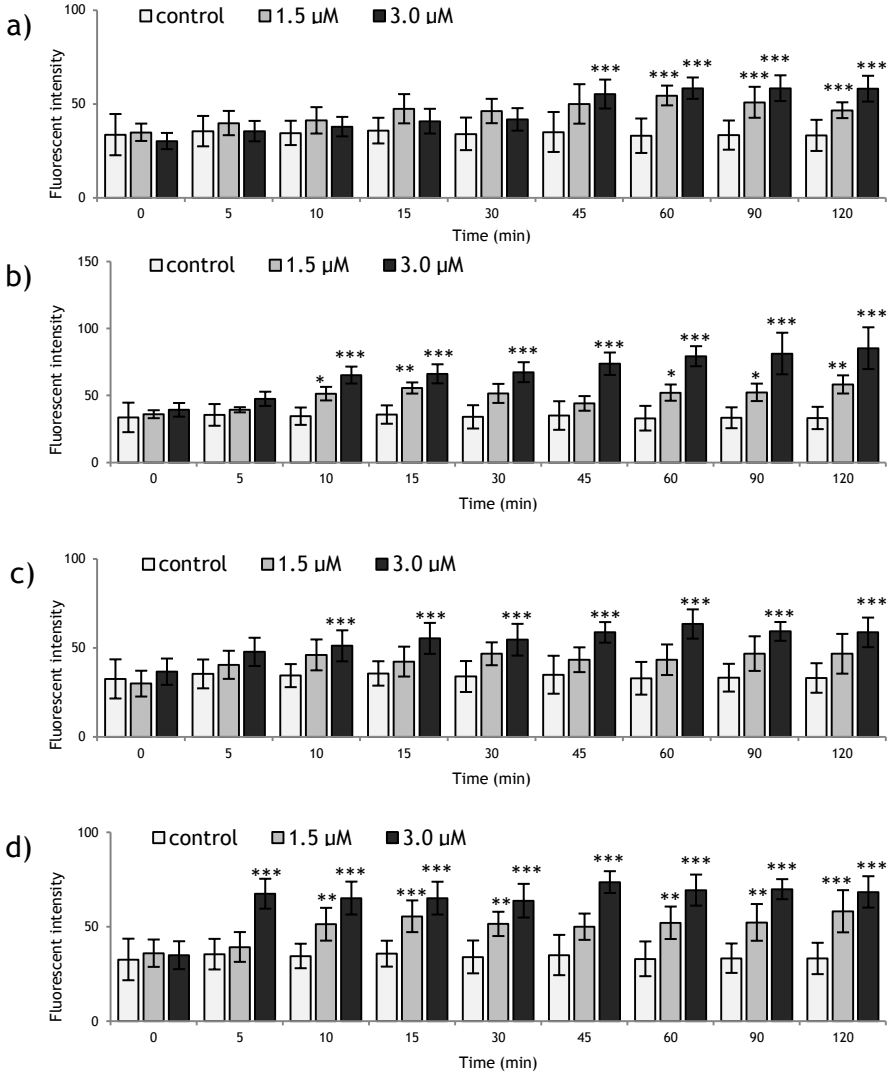

Figure 1. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to ENs (a) A, (b) A_1, (c) B and (d) B_1, at 1.5 and 3.0 µM. H_2-DCFDA was added to Caco-2 cells and left for 20 min and then exposed to ENs. Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation at 485 nm. Mean ± SD, 24 replicates. $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.000$ (***), significantly different from the control.

3.3 Lipid peroxidation

The LPO on Caco-2 cell was determined by the TBARS method in the presence of individual ENs. The results obtained demonstrated that after 24 h exposure all ENs increased MDA production in Caco-2 cells. In Figure 2, at the highest concentration tested (3.0 μM), significant increases of 111% and 58% for EN A and A_1, respectively, can be observed. EN B at 3.0 μM increased significantly MDA production up to 48%. EN B_1 at 1.5 and 3.0 μM increased MDA production up to 46% and 59% in respect to the control. ENs A, A_1 and B at 1.5 μM concentration didn't produce any increase in MDA levels.

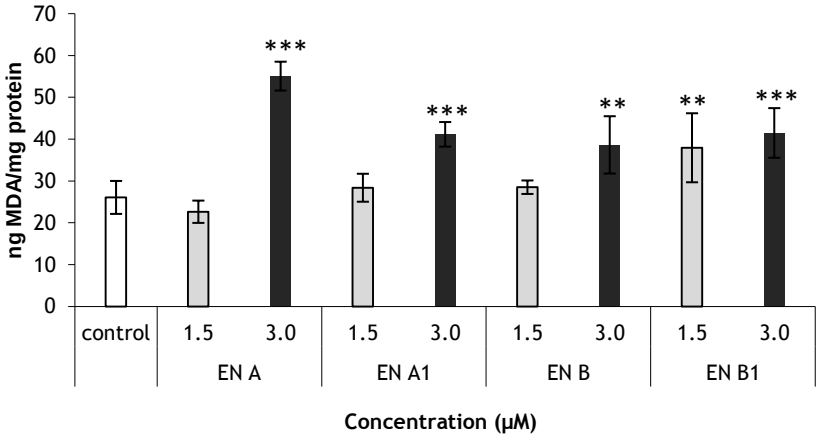

Figure 2. Lipid peroxidation as measured by MDA production in Caco-2 cells incubated for 24 h with 1.5 and 3.0 μM of EN A, A_1, B and B_1. Results are expressed as mean ± SD in ng of MDA/mg of protein measured by Bradford method. Significantly different from control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.000$ (***).

3.4 Cell cycle analysis

Flow cytometry analysis performed by PI-staining on Caco-2 cells after 24, 48 and 72 h of incubation with ENs A, A_1, B and B_1 indicated a modification in percentage of cells in SubG0/G1 (hypodiploid cells or debris), G0/G1 and G2/M phase for all ENs tested in a time and concentration dependent manner (Fig. 3). After 24 h of exposure, none of the parameters were changed for EN B (Fig. 3a). For ENs A, A_1 and B an arrest of the cell cycle in G2/M phase above 35.2 ± 3.7% compared to the control (23.1 ± 4.1%) was produced. A significant decrease of the G0/G1 cell population ($p \leq 0.05$ vs control) was observed for EN A_1 and B_1 at 1.5 μM. No changes in S phase were observed after 24 h of exposure (Fig. 3a). As illustrated in Fig. 2B, the exposure for 48 h to ENs A, A_1 and B resulted in a significant increase of the SubG0/G1 cell population. Increase in SubG0/G1 phase was above 15.1 ± 3.2% (control 5.3 ± 2.0%). A significant decrease in G0/G1 phase ($p \leq 0.05$ vs control) was observed. Results shown in Figure 2b demonstrated a decrease in G0/G1 phase by EN A, A_1 and B_1 with percentages ranging from 35.8 ± 1.1 to 41.0 ± 5.2%, respect to the control (49.3 ± 4.2%). Respect to both S and G2/M phases, no changes were observed after all ENs exposure. After 72 h of incubation with ENs a significant increase ($p \leq 0.01$ for 1.5 μM concentration vs control and $p \leq 0.000$ for 3.0 μM concentration vs control) of the SubG0/G1 cell population accompanied by a significant ($p \leq 0.05$) increase in S phase cell population was observed (Fig. 2c). Higher increase in SubG0/G1 phase was observed for EN B_1 at 3.0 μM concentration (35.8 ± 3.2%) vs control (7.2 ± 1.5%). Similarly, higher increase in S phase was observed for EN B1 (20.0 ± 1.3%) as compared to control (14.6 ± 2.0%; Fig. 3c). G0/G1 phase decreased after 72 h of ENs

A, A_1, B and B_1 incubation. G0/G1 cell population decreased from 26.1 ± 1.7% to 35.4 ± 2.5% for EN B_1 (at 3.0 μM) and EN A (1.5 μM), respectively, compared to the control (Fig. 2c). Respect to the G2/M phase, the increase of cell population was observed only for ENs A_1 and B (41.3 ± 5.3 and 28.3 ± 2.4% vs control 22.2 ± 2.6%).

3.5 Flow cytometry analysis of apoptosis (and necrosis)

As shown in Figure 4, apoptotic/necrotic effect seems to be dependent on time of ENs incubation. Apoptotic effect was clearly observed at 24 and 48 h of exposure, while necrotic effects were observed for ENs A and A_1 after 24 h and it was extended to all ENs after 72 h of incubation.

After 24 h of incubation with ENs A and A_1, apoptotic cell population increased from 24.8 ± 3.1 and 33.5 ± 3.7% as compared with 11.7 ± 1.3% of control cells (Fig. 4a). After 48 h of incubation, apoptotic cell population increased 16.1 ± 4.7 and 22.0 ± 5.6% for ENs A and A_1 (1.5 μM), as compared to control (6.5 ± 1.3%). Apoptotic cells increased with ranging values from 12.9 ± 3.0% to 24.2 ± 5.0% for ENs B and B_1 compared with the control (Fig. 4b). After 24 h of incubation, EN A and A_1 treatment increased by 24.4 ± 3.6 and 26.7 ± 5.5%, as compared to 12.3 ± 2.8% in control cells (Fig. 4a). After 48 h of incubation, necrotic cells increased with ranging values from 19.5 ± 3.2% to 21.5 ± 4.1%, as compared to control (14.0 ± 2.0%).

After 72 h of incubation, necrotic cell population increased for all ENs tested. The increase ranged from 15.6 ± 2.2 to 30.9 ± 1.6%, as compared with control cells (10.7 ± 1.4%).

3.6 Detection of mitochondrial membrane potential (ΔΨ*m*) by tetramethyl rhodamine methyl ester [TMRM] method.

The evaluation of ΔΨ*m* changes in Caco-2 cells showed a significant ($p \leq 0.05$) decrease in TMRM fluorescence intensity (%) in a dose and time dependent manner (Fig. 5). After 24 of exposure to EN A_1, cell percentages were significantly ($p \leq 0.05$) decreased from 54.8% (1.5 μM) and 45.6% (3.0 μM) as compared to the control cells. The ΔΨ*m* decreased by 36.6% (at 1.5 μM concentration) for EN A, as compared to control (Fig. 5). After 48 h of exposure, the reduction in TMRM intensity (%) was produced for all ENs tested, at both concentration tested, except for EN B, that significantly ($p \leq 0.05$) decreased TMRM intensity only after 3.0 μM concentration (44.9%; $p \leq 0.05$; Fig. 5). Percentage reductions were 33.9% and 43.6% for EN A, 45.2% and 31.9% for EN A_1, 55.5% and 74.4% for EN B_1, after 1.5 and 3.0 μM exposure, respectively, as compared to the control cells (Fig. 5). All reductions in TMRM florescence intensity were different significantly ($p \leq 0.000$) from control cells after 72 h of exposure. The percentage reduction of TMRM intensity was ranging from 91.0% to 98.7%, for all ENs tested (Fig. 5c).

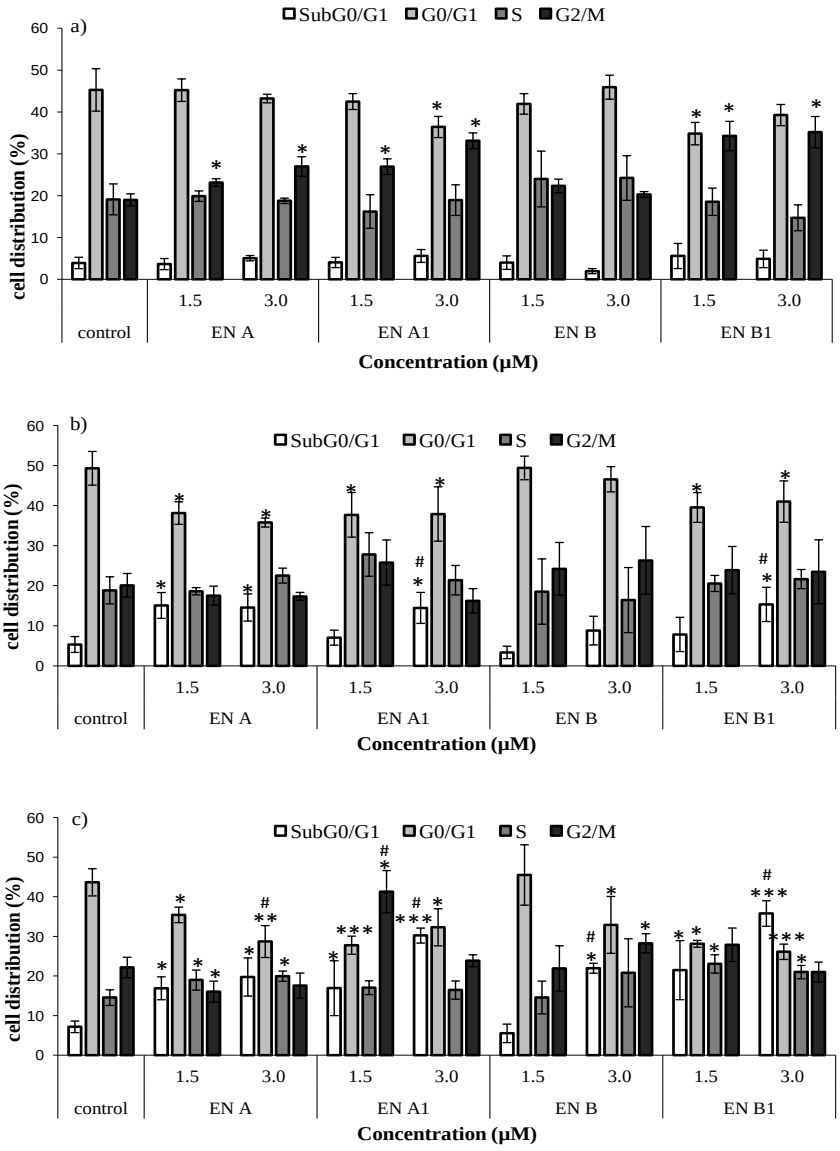

Figure 3. Analysis of cell cycle distribution of Caco-2 cells treated with ENs A, A_1, B and B_1 at 1.5 and 3.0 µM for 24 (a), 48 (b) and 72 h (c). Data are expressed as mean values ± SD (n = 3). $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.000$ (***) , indicate a significant difference with the control, $p \leq 0.05$ (#) indicate significant differences between the two concentrations tested (1.5 and 3.0 µM).

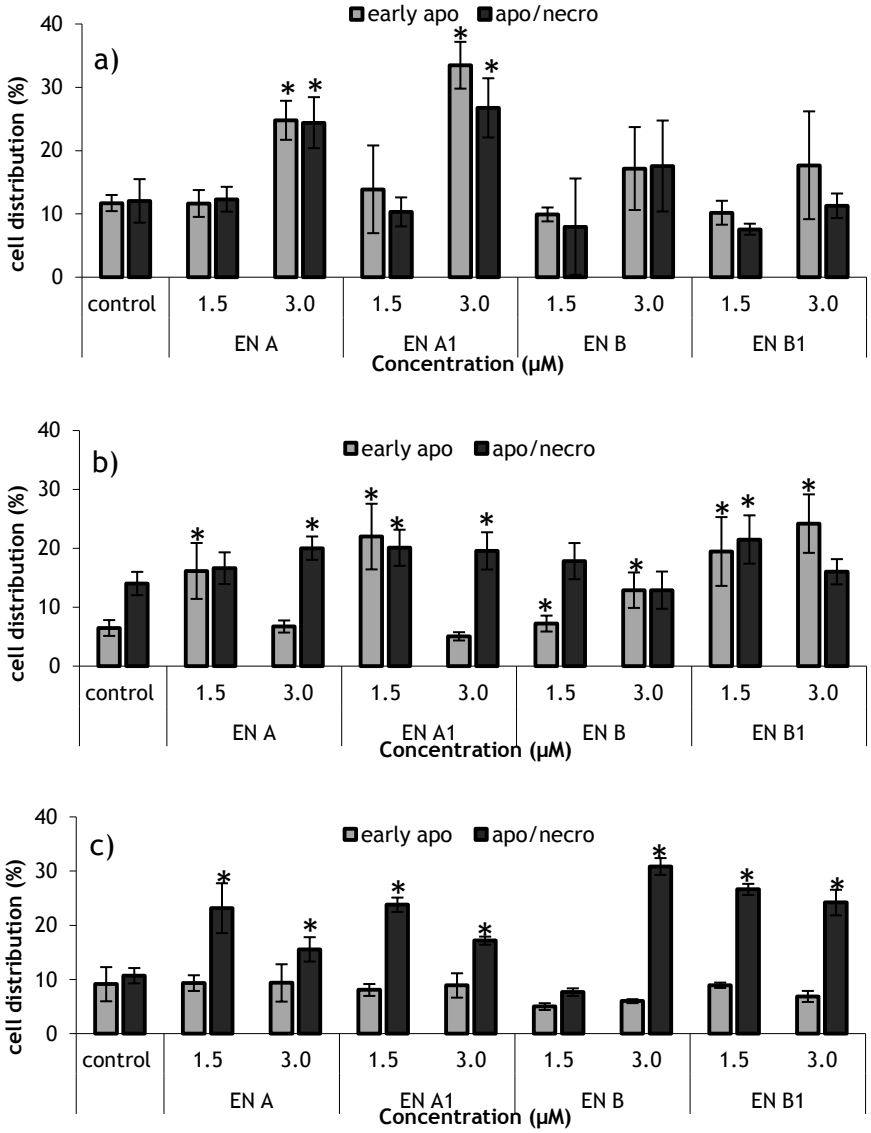

Figure 4. Analysis of apoptosis/necrosis induction in Caco-2 cells treated with ENs A, A_1, B and B_1, at 1.5 and 3.0 μM for 24 (a), 48 (b) and 72 h (c). Cells were stained with Annexin-FITC and PI to distinguish early apoptotic from apoptotic/necrotic cells. Data are expressed as mean values ± SD (n = 3). $p \leq 0.05$ (*) and $p \leq 0.000$ (***) indicate a significant difference with the control.

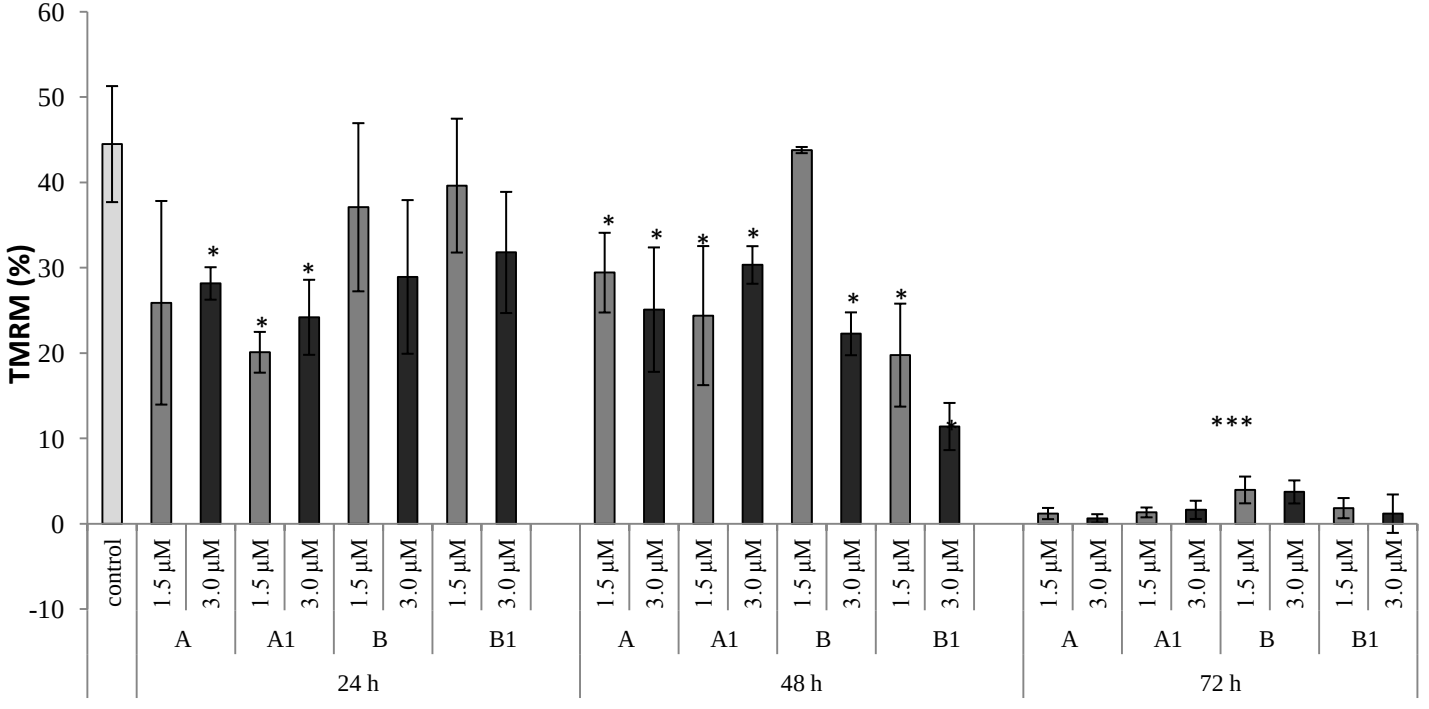

Figure 5. Loss of mitochondrial membrane potential (ΔΨ*m*) in Caco-2 cells as detected by flow cytometry after TMRM/ To-Pro®-3 double staining. Caco-2 cells were treated with 1.5 and 3.0 µM concentrations of ENs A, A_1, B and B_1, for 24, 48 and 72 h. $p \leq 0.05$ (*), $p \leq 0.000$ (***), significantly different from the control.

3.7 Alkaline comet assay

Figure 6 presents the alkaline comet assay data obtained in Caco-2 cells after exposure to ENs A, A_1, B and B_1 for 24 h. B(α)P 20 μM was used as positive control. After 24 h of exposure both EN A concentrations tested (1.5 and 3.0 μM) and 3.0 μM concentration of EN A_1 and B_1 produced a significant increase in the TM parameter ($p \leq 0.000$; as compared to the control), whereas, with regard to EN A_1 at 1.5 μM and EN B, no significant DNA damage was observed, as compared to the control (Fig. 6).

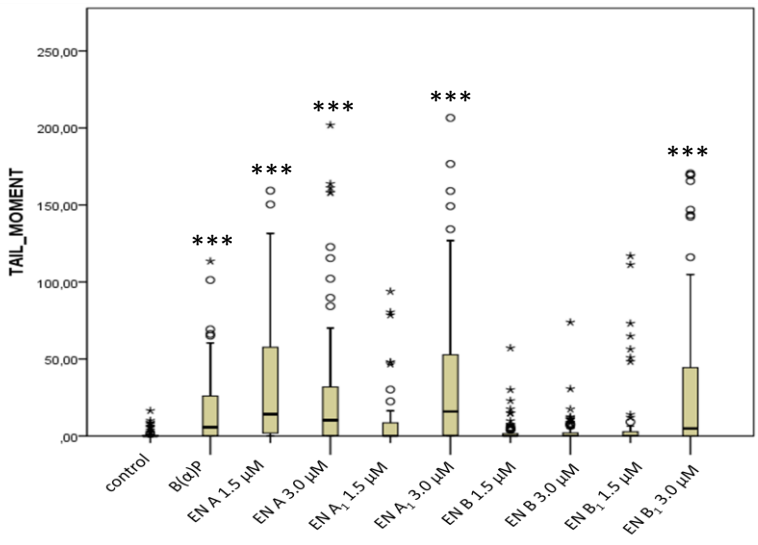

Figure 6. Alkaline comet assay (pH > 13) on Caco-2 cells after 24 h exposure to 1.5 and 3.0 μM ENs A, A_1, B and B_1. Migration of DNA is reported as median TM. B(α)P (20 μM) was the positive control. The box plots show the medians (middle bar), standard deviations, 25% and 75% percentiles. $p \leq 0.000$ (***) significantly different from the control. (★) Extreme cases and atypical values (○).

4. Discussion

ENs are naturally contaminant of cereal grains as well as processed food in Europe (Jestoi et al., 2008). Concentrations tested in this study are included in the range of contamination levels that have been reported to be from µg/kg to mg/kg considering several food commodities (Prosperini et al., 2013). The selection of 1.5 and 3.0 µM concentration was made according to the concentrations determined previously in food commodities from Mediterranean Area. In fact, according to Meca et al. (2010b), mean levels of ENs A_1, B and B_1 have been reported in maize in the range from 4.34 to 167.7 mg/Kg. Considering that the consumption of maize in Southern Europe is 7.7 Kg/year (FAOSTAT, 2009), the estimated total intake of ENs is ranging from 0.3 to 5.2 µmol/day, values that are comparable to the two concentrations tested. Moreover, the selection of 1.5 and 3.0 µM was due to the fact that they were not cytotoxic to Caco-2 cells, since they were the inhibitory concentration 90 (IC_{90}; obtained by the MTT assay). It has been demonstrated that ENs exposure induces cytotoxicity under *in vitro* conditions. Results obtained in this study show that ENs A, A_1, B and B_1 are cytotoxic to Caco-2 cells in low concentration ranges in a dose and time dependent manner. Similar IC_{50} values were obtained by Meca et al. (2010a, 2011) on Caco-2 cells, human colon carcinoma (HT-29) and human liver carcinoma (Hep-G2) cells after exposure to A, A_1, A_2, B, B_1, B_4 and J_3, even considering that ENs tested by Meca et al. (2010a, 2011) were not supplied commercially; they were isolated and purified from *Fusarium tricinctum* under determined laboratory conditions. Similar IC_{50} values were obtained by other authors in different human and mammalian non human cell lines (Lu et al., 2013;

Ivanova et al., 2012; Lombardi et al., 2012; Watjien et al., 2009). On the other hand, higher effects were obtained for EN A, A_1, B, B_1, B_2 and B_3 in HepG2, MRC-5 (fibroblast-like foetal lung) and V79 (Chinese hamster lung fibroblast) cells (Ivanova et al., 2006; Behm et al., 2009).

The results of this study demonstrated that, after ENs exposure on Caco-2 cells, high production of oxidizing species is obtained. ROS generation could be a cause or consequence of the mitochondrial alterations. Excessive ROS formation can cause macromolecules oxidation, such as membrane lipids, leading to membranes LPO (Ferrer et al., 2009). In this study it has been demonstrated that ENs, at the higher concentration tested, produce an increase in MDA levels, which, in association with ROS generation, plays a role in the molecular events leading to cell damage particularly by the induction of LPO. To the best of our knowledge, this is the first report about ENs inducing LPO in cell cultures. Similar results were obtained by Ferrer et al. (2009) when CHO-K1 cells were exposed to BEA which is structurally similar to the ENs. They found that BEA increased ROS production almost 182% in respect to control at 120 min of exposure. On the other hand, a recent study reported that ROS were generated downstream by the EN B-induced cytotoxic events in the mitochondria of Caco-2 cells (Ivanova et al., 2012). This lets us suppose that ROS generation is involved in ENs cytotoxicity, as a consequence of an early mitochondrial damage and LPO have been produced as a consequence of ROS generation in Caco-2 cells, after ENs exposure. A previous study reported that one of the pathways involved in Caco-2 cells viability reduction induced by *Fusarium* mycotoxins, is LPO (Kouadio et al., 2007). Moreover, ROS, as produced by external factors, are known to elicit lysosomal membrane

permeabilization (LMP), at the same time ROS can occur after LMP as a downstream signal to cell death with lysosomal-mitochondrial cross-talk (Repnik and Turk, 2010).

ENs are capable to disturb the normal progression of proliferating cells (Dornetshuber et al., 2007; Juan-García et al., 2013). Cell cycle arrest is a transient condition that may be induced by several factors and generally cells spontaneously revert from this condition restoring their proliferation rate or becoming apoptotic (or necrotic). Moreover, after 24 h, the exposure to ENs A_1 and B_1 led to a shift of cell cycle distribution, getting visible as increased proportion of cells in the G2/M phase, while cells in the G0/G1 phase were diminished. On the other hand, after 72 h of exposure, ENs A, A_1, B and B_1 produced an arrest of cell cycle in G2/M and they induced a significant increase in the S-phase; in addition, the reduction in G0/G1 phase is still maintained through all time of exposure almost for all ENs tested as well as the increase in SubG1 phase: this suggests the involvement of apoptosis in these ENs cell death (Fig. 3).

It has been reported that the normal progression of cell cycle could be affected by DNA damage events, in particular cells with damaged DNA arrest their cell cycle in G2/M or S phase (Abid-Essefi et al., 2003). If DNA damage is not repaired cells can undergo apoptosis or necrosis. In this study, it has been investigated the possible induction of DNA damage by ENs A, A_1, B and B_1. From the results obtained, ENs A, A_1 and B_1 induced DNA damage in a dose dependent manner (Fig. 6).

Regarding to ENs A and A_1, after 24 h of exposure, the 1.5 and 3.0 μM concentrations produced an arrest in G2/M phase that could be related to the DNA damage induced in Caco-2 cells, as assessed by the

comet assay (Fig. 6). Apoptosis has been also induced as well as necrosis. This could be due to the fact that under the same stimulus, features of both apoptosis and necrosis may coexist in the same cell. The apoptotic properties of ENs A and A_1 are still maintained after 48 h at 1.5 μM, whereas only necrosis is induced by the highest concentration tested. Moreover, only necrosis is observed after 72 h of exposure. This finding could support the induction of apoptosis and necrosis (Fig. 4) via the mitochondrial pathway (Fig. 5). The involvement of mitochondrial pathway is confirmed by the TMRM intensity reduction (Fig. 5). A significant reduction in TMRM intensity was obtained through all concentrations tested (Fig. 5). ROS generation and LPO are produced after ENs A and A_1 exposure; moreover, no direct relationship could be established between LPO and the viability reduction via apoptotic-mitochondrial pathway.

Regarding EN B_1, the higher concentration produced a block in the G2/M phase of the cell cycle that could be induced by DNA damage. No apoptosis or necrosis are produced after 24 h of EN B_1 exposure and no mitochondrial involvement is observed (Figs 4 and 5). However, EN B_1 induced early generation of ROS and LPO products, which could be responsible of cytotoxicity in Caco-2 cells.

However, when Caco-2 cells were exposed to ENs A, A_1 and B_1 the prolonged exposure (48 h) produced a reduction in cell viability, with an increase in apoptotic and apoptotic/necrotic cell percentages (Fig. 4b), corroborated by the significant increase in SubG0/G1 percentage of the cell cycle and by the disruption of mitochondrial functionality (Fig. 3b and Fig. 6). After 72 h of exposure, no DNA synthesis is produced since an arrest in S-phase is observed, only

necrotic phenomenon is observed and high alteration of ΔΨ*m* (Figs 3c, 4c and 6). This is due to the high exposure time that could induce necrosis.

Different results have been obtained for EN B. The reduction in Caco-2 cells viability was lower than the other ENs in this study. EN B seems to be least cytotoxic mycotoxin to Caco-2 cells. This may be due to the lipophilicity rate of the four ENs tested: EN A>EN A_1>EN B_1>EN B (Tedjiotsop Feudjio et al., 2010). No significant effects after 24 h of exposure were observed in cell cycle distribution, apoptosis/necrosis induction, DNA damage and ΔΨ*m* disruption. However ROS generation and LPO were produced by 3.0 μM EN B concentration. This fact confirms that ROS and LPO are not involved in ENs apoptosis induction. ROS were generated downstream by the EN B-induced cytotoxic events in the mitochondria of Caco-2 cells (Ivanova et al., 2012). The absence of EN B genotoxicity is in accordance with recent studies (Behm et al., 2009; Gammelsrud et al., 2012). After 48 h of exposure, apoptosis induction with the involvement of ΔΨ*m* is observed and culminate in necrotic cell death after the highest time of exposure (72 h). The induction of necrosis is confirmed by Ivanova et al. (2012) in Caco-2 cells, however higher doses (25 μM) were tested. Cell cycle is affected by EN B with an arrest in G2/M phase (31.0 ± 1.3% of cell population) vs control cells (23.0 ± 1.0%) only after 72 h of exposure. According to Ivanova et al. (2012), this type of arrest has been described as a possible consequence of external stimuli leading to apoptosis by activation of the caspase pathway or to non-apoptotic mitotic death.

The cell cycle arrest may be an adaptive process in which a surveillance mechanism delays or arrests the cell cycle when DNA

lesions occur. Other studies have demonstrated the ability of cells to delay or arrest their multiplication cycle either in G0/G1 or G2/M in order to make repair take place (Abid-Essefi et al. 2003).

Few data are available about the genotoxicity of ENs and it has been demonstrated that EN B and ENs mixture are not genotoxic *in vitro* (Dornetshuber et al., 2009; Gammelsrud et al., 2012; Ivanova et al., 2012). No data have been published about the genotoxic effects of ENs A, A1 and B1 tested individually in cell cultures, up to now. According to our data, in this study a potential DNA damaging activity has been found after exposure to ENs A, A_1 and B_1 in Caco-2 cells. The DNA damage can be related to the block in G2/M phase of the cell cycle due to a checkpoint activated after DNA damage. In addition ENs A, A_1, B and B_1 induced initially apoptosis and then necrosis at higher time of exposure in a dose-dependent manner in Caco-2 cells. Moreover, it has been corroborated that apoptosis was initiated by the so-called mitochondrial pathway based on a depolarization of the mitochondrial outer membrane.

Results obtained shows that several toxicological consequences have been induced by ENs A, A_1, B and B_1 in Caco-2 cells. Cytotoxicity could involve early ROS generation that induced LPO oxidative damage. In addition, Caco-2 cells undergo apoptosis and necrosis after exposure to ENs and the depolarization of $\Delta\Psi m$ is involved in both types of cell death induced by ENs. ENs A and A_1-induced cytotoxicity involved DNA damage, corroborated by the arrest of the cell cycle observed. However the same effects cannot be proposed for EN B_1 and EN B. Further studies on the toxicological effects induced by ENs A, A_1, B and B_1 are needed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Short communication

CYTOTOXIC EFFECT AND OXIDATIVE STRESS INDUCTION BY FUSAPROLIFERIN IN CACO-2 CELLS

ABSTRACT

Cytotoxic effects and the possible induction of oxidative stress by *Fusarium* mycotoxin fusaproliferin (FUS) have been investigated in Caco-2 cells. FUS (0.9 to 30 μM) did not exerted cytotoxic effect after 24, 48 and 72 h of exposure. Moreover, intracellular reactive oxygen species (ROS), which have been determined from 0 to 120 min, were not observed. However, at the time 0 of exposure, an increase of 2.2 folds respect to control, was produced. Similarly, an induction of lipid peroxidation (LPO) was not observed, as compared to control. Our results suggest that FUS is not cytotoxic in the concentration tested and it do not produce oxidative stress in Caco-2 cells.

1. Introduction

Fusaproliferin (FUS) is a bicyclic sesterterpenic mycotoxin that consists of five isoprenic units (Fig. 1). It was originally isolated from a pure culture of *Fusarium proliferatum* (Randazzo et al., 1993). Other *Fusarium* species, such as *F. antophilum*, *F. begoniae*, *F. bulbicola*, *F. circinatum*, *F. concentricum*, *F. succisae*, *F. udum* and *F. subglutinans*, are also capable to produce FUS (Zinedine et al., 2011). Recently, FUS has been detected in raw cereals and cereal based products of the Mediterranean area, with contamination levels ranging from traces to 2000 mg/Kg (Zinedine et al., 2011; Meca et al., 2010; Serrano et al., 2013a,b).

On the other hand, few studies have been carried out about FUS toxicological effects *in vitro*. It has been shown that FUS and its derivates can induce teratogenic effects, e.g. cephalic dichotomy, macrocephaly and limb asymmetry, in chicken embryos bioassays (Ritieni et al., 1997). The non-covalent interactions between single- and doublestranded DNA-oligonucleotides and FUS with 1:1 stoichiometry have been reported (Pocsfalvi et al., 2000) which may partly explain the teratogenic effects. The interchelatation was not reported to exhibit any base sequence preference in the DNA, suggesting an electrostatic type of interaction.

So, the objectives of this study were to determine the cytotoxicity of FUS by the MTT assay, to detect intracellular ROS production as early stage marker induced oxidative stress and to determine the LPO in human adenocarcinoma (Caco-2) cells.

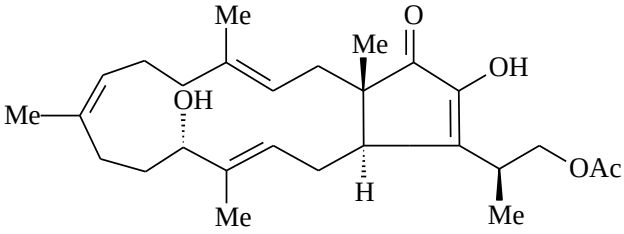

Figure 1. Chemical structure of fusaproliferin.

2. Material and methods

2.1 Reagents

The reagent grade chemicals and cell culture components used were provided by Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

FUS was kindly given by Professor Alberto Ritieni, Department of Food Science, University of Naples "Federico II", Italy and was prepared by dissolving 1 mg of the mycotoxin in 1 ml of pure MeOH, obtaining a stock solution of 1000 μg/ml (2.4 mM) of FUS, composed by 39.9% of FUS and by 60.1% of deacetyl-FUS.

Stock solutions of mycotoxins were prepared in methanol and maintained at -20°C. The final concentrations tested were achieved by adding the culture medium with mycotoxins and the final methanol concentration in medium was 1% (v/v). Total protein content (μg/ml) was determined by Bradford method (Bio-Rad DC Protein Assay (catalogue number 500-0116) http://www.bio-

rad.com/LifeScience/pdf/Bulletin_9005.pdf). All other reagents were of standard laboratory grade.

2.2 Cell culture

Caco-2 cells (ATCC HTB-37) were maintained in DMEM medium supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100U/mL penicillin, 100mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37°C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St Louis Mo. USA).

2.3 Cell viability assay

The MTT assay was performed as described by Ruiz et al. (2006) with some modifications. 3 x 10^4 cells/well were seeded in 96-well tissue culture plates and after reaching confluence, the exposure solutions of FUS (from 0.9 to 30 µM) were added to the plate that was incubated at 34 °C for 24, 48 and 72 h. 200 µL of fresh medium containing 50 µL of MTT were added. After 4 h of incubation (37°C in darkness), the resulting formazan was solubilised in DMSO. The absorbance was measured at 570 nm using the ELISA plate reader Multiscan Ex (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to the solvent control (1% MeOH). Three independent experiments were performed and the 50% inhibitory concentrations (IC_{50}) values were calculated from full dose-response curves.

2.4 ROS generation assay

The production of ROS was assessed in 96-well black culture microplate using the dichloroluorescein (DCF) assay, according to Ruiz and George (2004). The diacetate form (DCF-DA), after diffusing in the cell membrane, is hydrolysed by intracellular esterases to non-fluorescent dichlorofluorescein (DCFH), which is trapped inside the cells then oxidized to fluorescent DCF through the action of peroxides in the presence of ROS. Increases in fluorescence were measured at intervals up to 2 h at excitation and emission wavelengths of 485 and 535 nm, respectively. Twenty-four replicates were developed. Results are expressed as increase in fluorescence in respect to control.

2.5 Lipid peroxidation assay

LPO was measured according to Buege and Aust (1978) based on the formation of thiobarbituric acid reactive substances (TBARS). This assay is based on the formation of a red adduct (absorption maximum 532 nm) between TBA and MDA, a final product of the peroxidation process. Briefly, 4.8 x 10^5 cells/well were seeded in 6-well plates and exposed to 1.5 and 3.0 μM of FUS for 24 h. Three independent experiments were conducted and results are expressed as ng of MDA/mg of protein measured by the Bradford method.

2.6 Statistical analysis of data

Statistical analysis of data were carried out using SPSS Statistic 19.0 (SPSS, Chicago, IL, USA) statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by Student's t-test for

paired samples. The level of $p \leq 0.05$ was considered statistically significant.

3. Results and discussion

The MTT is one of the most used for preliminary and mechanistic cytotoxicity studies of mycotoxins in a great variety of mammalian cell lines (Ferrer et al., 2009; Watjen et al., 2009; Meca et al., 2010; Ruiz et al., 2011a,b). Concentrations tested of FUS produced a decrease in cellular proliferation after 24, 48 and 72 h of exposure. However, no IC_{50} values were reached at any time of exposure (data not shown). Logrieco et al. (1996) assessed cytotoxicity of FUS in non neoplastic B-lymphocyte cell line IARC/LCL 171 and the IC_{50} obtained was 55 μM after 48 h exposure by MTT assay. In the study developed in our laboratory, the higher FUS concentration tested was 30 μM. No higher concentration was determined because of a problem with the quantity supplied.

Previous studies reported that several *Fusarium* mycotoxins exert cytotoxic activity by inducing oxidative stress in different mammalian cell lines, in terms of reactive oxygen species (ROS) generation and lipid peroxidation (LPO; Kouadio et al. 2005; Ferrer et al., 2009; Ruiz et al., 2011a,b; Bouaziz et al., 2008). In this sense, as far as authors knowledge, no studies are available about the induction of oxidative stress by FUS.

To determine changes in the redox status of Caco-2 cells in response to FUS, cells were exposed to 1.5 and 3.0 μM concentrations from 0 to 120 min (Fig. 2). FUS did not show any significant increase in ROS production respect to the control. However, at the time 0 of exposure, an increase of 2.2-times to control can be observed (Fig. 2).

Moreover no increase in MDA production was observed (Fig. 3). Results clearly showed that FUS did not alter the oxidative status of Caco-2 cells. The toxicity could be due to other mechanisms different from ROS production that have not been investigated here.

On the other hand, it is known that FUS is a natural occurring *Fusarium* mycotoxin that can be found in food and feed, so it can constitute a potential concern to animal and human health. The toxicity is still unclear. However Prosperini et al. (2012) demonstrated a relatively high bioavailability of FUS (almost 80%) as determined by differentiated Caco-2 cells, that represent a validated international model which is used to predict human intestinal absorption. Due that, the toxicity to humans and animals is still an existing concern. Other mechanistic pathway should be investigated in the future to get additional data about FUS toxicity.

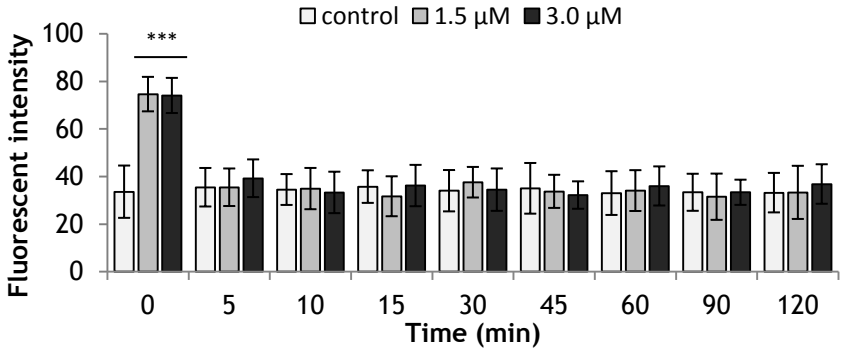

Figure 2. Time dependence of ROS-induced fluorescence in Caco-2 cells exposed to FUS at 1.5 and 3.0 µM. H_2-DCFDA was added to Caco-2 cells and left for 20 min and then exposed to ENs. Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation at 485 nm. Mean ± SD, 24 replicates. $p \leq 0.000$ (***), significantly different from the control.

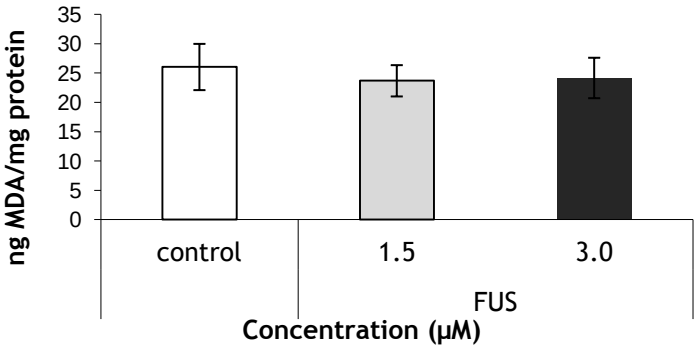

Figure 3. Lipid peroxidation as measured by MDA production in Caco-2 cells incubated for 24 h with 1.5 and 3.0 µM of FUS. Results are expressed as mean ± SD in ng of MDA/mg of protein measured by Bradford method.

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EFFECT OF POLYPHENOLS ON ENNIATINS-INDUCED CYTOTOXIC EFFECTS IN MAMMALIAN CELLS

G. Lombardi[1], A. Prosperini[2], G. Font[2], M. J. Ruiz[2,*]

[1]Dipartmento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Biochimica, Fisiologia e Nutrizione, Facoltà di Farmacia. Università degli Studi di Perugia, via San Costanzo (interno orto botanico) 06126, Perugia, Italy.

[2]Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vincent Andres Estelles s/n 46100 Burjassot, Valencia, Spain.

[*] Corresponding author: Tel. +34 963543055; FAX: +34 963544954; e-mail address: M.Jose.Ruiz@uv.es

ABSTRACT

Enniatins (ENs) are fungal secondary metabolites produced by genus *Fusarium*. The ENs exert antimicrobial and insecticidal effect, and has also been demonstrated cytotoxic effects on several mammalian cell lines. On the other hands, it has been proved that natural polyphenols have antioxidant effect. In this study, cell effects at low levels of exposure of four ENs (A, A_1, B and B_1) and five polyphenols (quercetin, quercetin-3-ß-D-glucoside, rutin, myricetin and *t*-pterostilbene) present in wine; and the cytoprotective effect of these polyphenols exposed simultaneously with ENs in Chinese Hamster Ovary (CHO-K1) cells, were studied. Cell effects were determined by the MTT test after 24 h of exposure. All ENs showed cytotoxic effect. The IC_{50} obtained ranged from 4.5 ± 1.2 to 11.0 ± 2.7 µM. The concentration of polyphenols tested ranged from 5 to 50 µM. Polyphenols did not show cytotoxicity and the cytoprotective effect of polyphenols varies depending on the EN tested. The cytoprotective effect of polyphenols in CHO-K1 cells exposed to ENs was as follow: quercetin, from 24 to 84%; quercetin-3-ß-D-glucoside, from 12 to 76%; rutin, from 17 to 83%; myricetin, from 16 to 92% and pterostilbene from 25 to 100%. All polyphenols protected CHO-K1 cells against EN A_1 exposure.

Keywords: Enniatins, polyphenols, cytototixicy, cytoprotective effect, CHO-K1 cells.

1. Introduction

Mycotoxins are fungal secondary metabolites, mainly produced by genera *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria* and *Claviceps* and the amount of their production depends on different factors such as types of crop, climate and storage conditions. Among mycotoxins yielded by *Fusarium* there are enniatins (ENs), beauvericin (BEA), fumonisins, trichothecenes, moniliformin (MON) and zearalenone (ZEA; Jestoi, 2008; Ferrer et al., 2009; Meca et al., 2010; 2011). Fungal contaminants from genus *Fusarium* infect plants and agricultural products. They are present in food and feed and can produce a wide range of acute and chronic toxic effects on humans and animals.

ENs are lipophilic hexadepsipeptides that can easily incorporate in the cellular membrane. ENs exert antimicrobial, insecticidal and antihelmintinc effects (Tedjiotsop Feudjio et al., 2010). Moreover, ENs form dimeric structures that transport monovalent ions across the membranes, especially mitochondrial membranes and uncouple oxidative phosphorylation (Wätjen et al., 2009; Tedjiotsop Feudjio et al., 2010) and produce cytotoxic effects on mammalian cells (Wätjen et al., 2009; Meca et al., 2010; 2011). Polyphenols are widely distributed in fresh fruits, berries, black tee, red wine, purple grape juice, medicinal herbals, daily nutrition supplements, etc. They are an integrate part of the human diet (natural food and food industry) and are utilized as a source of starting material in the pharmaceutical industry (Zamora-Ros et al., 2008; Ostertag et al., 2010; Chen et al., 2011; Seibert et al., 2011). Moreover, they have numerous biological activities, as cancer-preventive because of their antioxidative, oxygen radical scavenging, antiproliferative and anti-inflammatory activities

and their stimulation of the immune system. Red wine contain up to 30 mg/L of flavonols (D'Archivio *et al.*, 2007) and it is the richest source of stilbenes (resveratrol and derivatives, ≥ 82%) (Zamora-Ros et al., 2008). Moreover, FAO (2007) data demonstrated that the consumption of wine in South Europe was 35.60 Kg/person/year. Moreover, beneficial health properties have been associated with red wine consumption due to, the presence of polyphenols. Dietary polyphenols can protect cell constituents against oxidative damage limiting degenerative diseases associated to oxidative stress (D'Archivio et al., 2007; Chen et al., 2011; Seibert et al., 2011). Among more than 4,000 polyphenols compounds, most of the polyphenols are found as glycosides with different sugar moieties conjugation with various sugar molecules stabilizes the aglycones, increases their water solubility, and affects the bioavailability and physiological functions. Polyphenols most frequently studied are quercetin and its glycoside rutin (quercetin-3-*O*-glucorhamnoside); they exhibit antioxidant, antitumoral, anti-inflammatory, antiplatelet, and vasoprotector activities (Barcelos et al., 2011). Quercetin is a major polyphenol ingested daily by dietary intake (20-100 mg) (You et al., 2010). Quercetin-3-ß-D-glucoside is also a glycoside derivative from quercetin which has similar antioxidant activity (Razavi et al., 2009). However, is not an abundant flavonol glycoside in food, its bioavailability is 50-180% higher than that of quercetin (You et al., 2010). Myricetin is a flavonoid also present in wine and, it acts as oxygen free radical scavenger and induces antioxidant enzymes (Chen et al., 2011). Pterostilbene which is a stilbene is found in grape, blue berries (Chakraborty *et al.*, 2010), peanuts and red wine (Zamora-Ros et al., 2008; Chakraborty et al.,

2010). It is an analogue of resveratrol and, has also been shown that has beneficial healthily properties. It possess antiproliferative, anti-inflammatory and antioxidantive effects, and presents antihyperglycemic, anticholesterol and hypolipidemic activities (Chakraborty et al., 2010; Zamora-Ros et al., 2008). The antioxidant activity of polyphenols has been widely investigated, but also their toxic and genotoxic effects. Due to the contradicting results about their ability to protect mammalian cells from cytotoxicity, studies on the level of safety are of great importance. During last decade, a remarkable effort has been made forward *in vitro* studies of mycotoxins and polyphenols (Markham et al., 1987; Hundhausen et al., 2005; Sergent et al., 2005; Choi et al., 2010; Barcelos et al., 2011). *In vitro* cytotoxicity tests are useful and necessary to define basal cytotoxicity. The *in vitro* MTT assay is an endpoint widely used for preliminary screening of *Fusarium* mycotoxins in a widely variety of mammalian cells such as human, monkey and rodent (Ferrer et al., 2009; Meca et al., 2010; 2011; Ruiz et al., 2011). CHO-K1 cells were selected because they are considered one of the most sensitive cell lines for preliminary screening of mycotoxin studies.

The aims of this study were to determine the cytotoxic effects of ENs A, A_1, B and B_1, and natural polyphenols quercetin, quercetin-3-β-D-glucoside, rutin, myricetin and *t*-pterostilbene at low levels in CHO-K1 cells; and to assess the cytoprotective effect of these polyphenols simultaneously exposed with ENs during 24 h in CHO-K1 cells.

2. Materials and methods

2.1 Reagents

The reagent grade chemicals and the mainly cell culture components used, Ham F-12 medium, penicillin, streptomycin, trypsin/EDTA solution, HEPES, 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), glycine, dimethyl sulfoxide (DMSO), sodium bicarbonate and the polyphenols were purchased from Sigma-Aldrich (St. Louis, MO, USA). The *t*-pterostilbene (256.30 g/mol; purity ≥ 90%) was synthesized in our laboratory following standard Witting and Heck reactions (http://www.orgsyn.org).ENs were from Enzo Life Sciences, Switzerland. Fetal calf serum (FCS) was from Cambrex Company (Belgium). Methanol was obtained from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The polyphenols selected were quercetin (302.26 g/mol; purity ≥ 98%), quercetin-3-β-D-glucoside (464.38 g/mol; purity ≥ 90%), rutin (610.56 g/mol; purity ≥ 99%) and myricetin (318.24 g/mol; purity ≥ 96%). Schematic presentation of the formation of polyphenols selected in nature is shown in Figure 1. The ENs used, EN A (681.45 g/mol), EN A_1 (667.44 g/mol), EN B (639.41 g/mol) and EN B_1 (653.43 g/mol) were up to 98% purity. All polyphenols were stored at 2-8 °C and protected from light. Stock solutions of ENs and polyphenols were prepared in methanol. Final concentration of each EN and polyphenol in the assay were achieved by adding the culture medium. The final methanol

concentration in the medium was 1% (v/v). Control cultures were exposed to the equivalent concentration of methanol.

2.2 Cell line culture and treatment

CHO-K1 cells derived from Chinese Hamster Ovary, were cultured in monolayer with Ham-F12 media supplemented with 25 mM HEPES buffer (pH 7.4), 10% heat inactived FCS, 100 U m^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 2.5 µg ml^{-1} amphotericin B. Incubation conditions were 37 °C and 5% CO_2 in a 95% relative humidified atmosphere. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.3 Determination of cytotoxicity of ENs and polyphenols and antioxidant effect of polyphenols in CHO-K1 cells exposed to ENs

CHO-K1 cells were cultured into 96-well tissue-culture plates by adding 200 µL/well of a suspension of 2 x 10^4 cells/well. Counting of cells was performer with a Beckman coulter (Florida, USA). After the cells reached 65% confluence, the culture medium was replaced with *a)* fresh medium containing six serial dilutions (serial dilution factor = 2) of EN A (from 0.47 to 7.5 µM) and six serial dilutions of ENs A_1, B and B_1 (from 0.47 to 15 µM) plus a control; *b)* fresh medium containing five serial dilutions (serial dilution factor = 2) of each polyphenol (from 3.13 to 100 µM) plus a control, and *c)* fresh medium containing simultaneously the polyphenol (concentrations: 5, 25 and 50 µM) and each EN (1.5 and 3 µM) plus a control. The plates were incubated for 24 h at 37 °C, and the cytotoxicity was determined by the MTT assay.

The MTT viability assay was performed as Ruiz *et al*. (2006). Briefly, after exposure of each EN or polyphenol during 24 h, the medium containing ENs or polyphenols was removed and cells of each well received 50 µL of MTT. The plates were wrapped in foil and incubate for 4 h at 37 °C. After the incubation period, the medium contained the MTT has removed, and the plate was washed with PBS. 200 µL of DMSO was added in the plate followed by 25 µL of Sorensen's glycine buffer. The absorbance was measured at 570 nm using an ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA). Cytotoxicity was expressed as a percentage of cell viability relative to control cells.

2.4 Statistical analysis

Statistical analysis of data was carried out using PASW statistic 17.0 (SPSS, Chicago, IL, USA), software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the result was performed by Student's *t*-test for paired samples. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tuckey HDS post-hoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

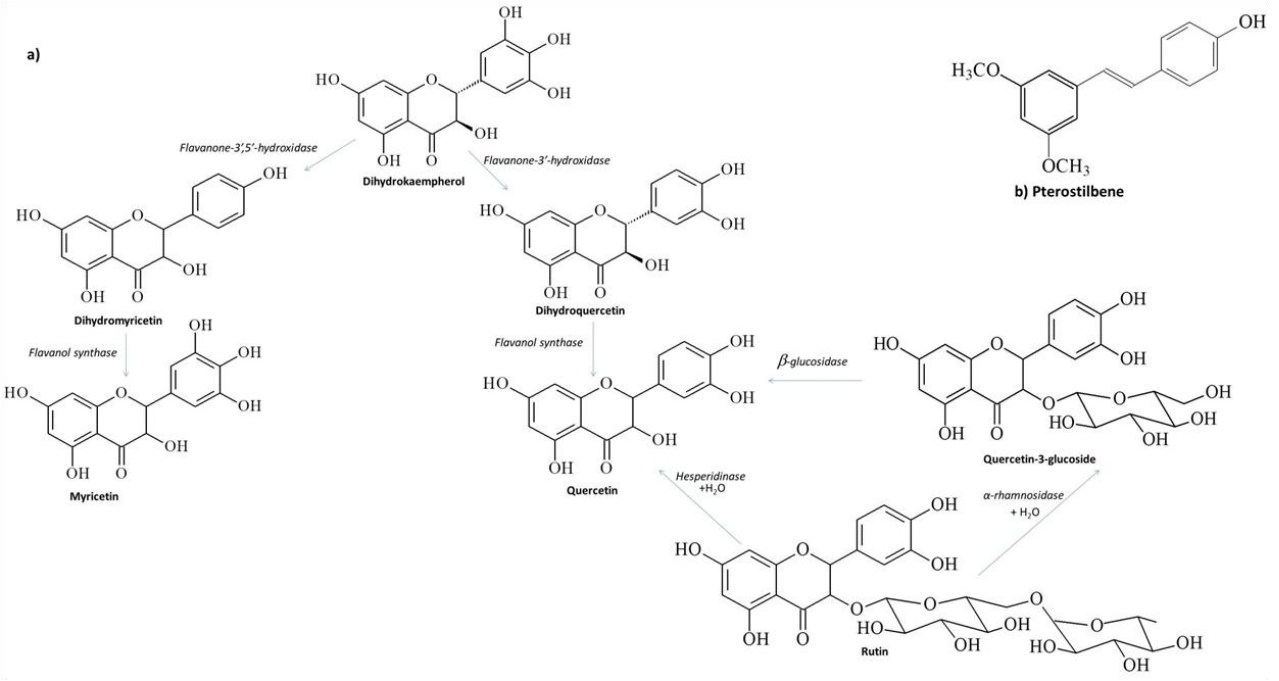

Figure 1. Chemical structure of polyphenols selected in this study. (a) Scheme of the quercetin, quercetin-3-β-d-glucoside, rutin and myricetin biosynthesis pathway and (b) chemical structure of *t*-pterostilbene.

3. Results

3.1 Cytotoxic effect of enniatins

The effects of ENs A, A_1, B and B_1 in CHO-K1 viability was evaluated by the MTT assay after 24 h of exposure. The inhibitory activity was quantified as the concentration required for inhibiting cell growth by 50% (IC_{50}) under the assay condition. The IC_{50} values were determined graphically from the concentration response curve (Fig. 2). As can be observed in Figure 2, ENs reduced cell viability in a dose-dependent manner as attested by MTT assay. After 24 h of incubation the IC_{50} values of ENs A_1, B, and B_1 were 8.8 ± 2.29, 11.0 ± 2.65 and 4.5 ± 1.23 μM, respectively. EN A did not show IC_{50} value in the range of concentration tested. Individual ENs diminishes cell viability in increasing order: EN A< EN A_1 = EN B < EN B_1. CHO-K1 cells are more sensitive to EN B_1 (Fig. 2). The results demonstrated that the EN B_1 was found to be the most cytotoxic against the cell line tested during the incubation period.

3.2 Cytotoxic and cytoprotective activities of polyphenols

The effects of polyphenols selected on the growth of CHO-K1 cells using the MTT assay, as previously described was showed in Figure 3. Myricetin, quercetin-3-β-D-glucoside and rutin did not decreased cell growth respect to the control at tested concentrations. Not cytotoxicity for quercetin and *t*-pterostilbene was observed at lower concentrations tested; however, the cell growth inhibitory effect on CHO-K1 cells was sensitive at the highest concentration (100 μM). But, no IC_{50} values were obtained for any polyphenol assayed in the range of concentration

tested. To investigate whether polyphenols can inhibit cytotoxicity induced by ENs, the range was further narrowed down to 5-50 μM. The concentrations for antioxidant effect of polyphenol assays were selected considering in all cases ≥ 70% cell viability.

From Figures 4 to 8 is shown that all polyphenols simultaneously exposed with EN A_1 increased the cytoprotective effect in a dose-dependent manner. Fig. 4 and Fig. 5 shown cytoprotective effect in cells exposed simultaneously with EN B_1 (1.5 μM) and quercetin or quercetin-3-β-D-glucoside (5 and 25 μM). And, 50 μM of quercetin (Fig. 4), quercetin-3-β-D-glucoside (Fig. 5) and myricetin (Fig. 7) showed higher cell growth in the presence of EN B_1 compared with cells exposed to EN B_1 in medium without these polyphenols. Figure 5 shows that only 50 μM of quercetin-3-β-D-glucoside led a significant protective effect on cells exposed to EN A compared with cells exposed to EN A in medium without quercetin-3-β-D-glucoside. According to Seibert et al. (2011), higher cytotoxic effect shows higher cytoprotection. Rutin shows a clear dose-dependent protective effect in CHO-K1 cell incubated with EN B (Fig. 6). Five μM of rutin increased cell growth while higher concentrations decreased it when EN B_1 was in the medium (Fig. 6). Similar effect was observed with 5 μM of *t*-pterostilbene and EN A (Fig. 8). On the other hand, stimulation of cell viability when cells are exposed to ENs and polyphenols together compared to the viability of control cells (cells exposed only to polyphenols) was observed (Fig. 4-8). This behaviour, which was previously observed in other cell lines, is a relatively common biological phenomenon characterized by low dose stimulation and high dose inhibition as a consequence of cell protection or adaptive response to mycotoxins exposure (Meca et al., 2011).

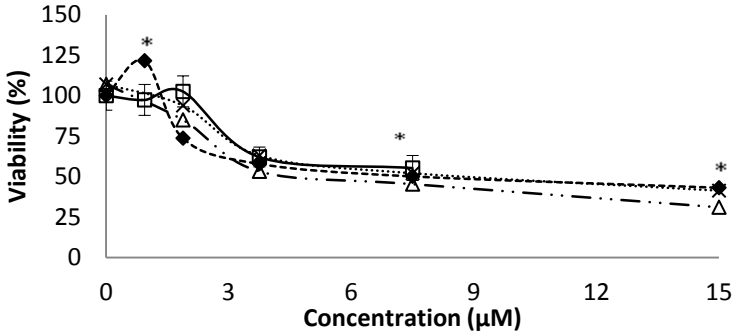

Figure 2. Cytotoxic effects of enniatin A (—□—), enniatin A_1 (- - ♦ - -), enniatin B (··x··) and, enniatin B_1 (—··Δ—··) in CHO-K1 cells after 24 h of exposure by the MTT assay*.

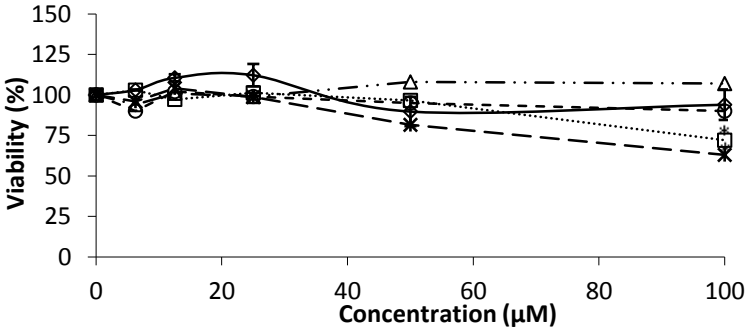

Figure 3. Cytotoxic effects of polyphenols, quercetin (··□··), quercetin-3-¤-D-glucoside (—◊—), *t*-pterostilbene (— x— —), rutin (—··Δ—··) and myricetin (- - ○ - -) in CHO-K1 cells after 24 h of exposure by the MTT assay*.

* Each point represents the mean value of at least three experiments. Data are expressed as percentage of the unexposed control using eight replicates per concentration. An asterisk indicates a significant difference from control value ($p \leq 0.05$).

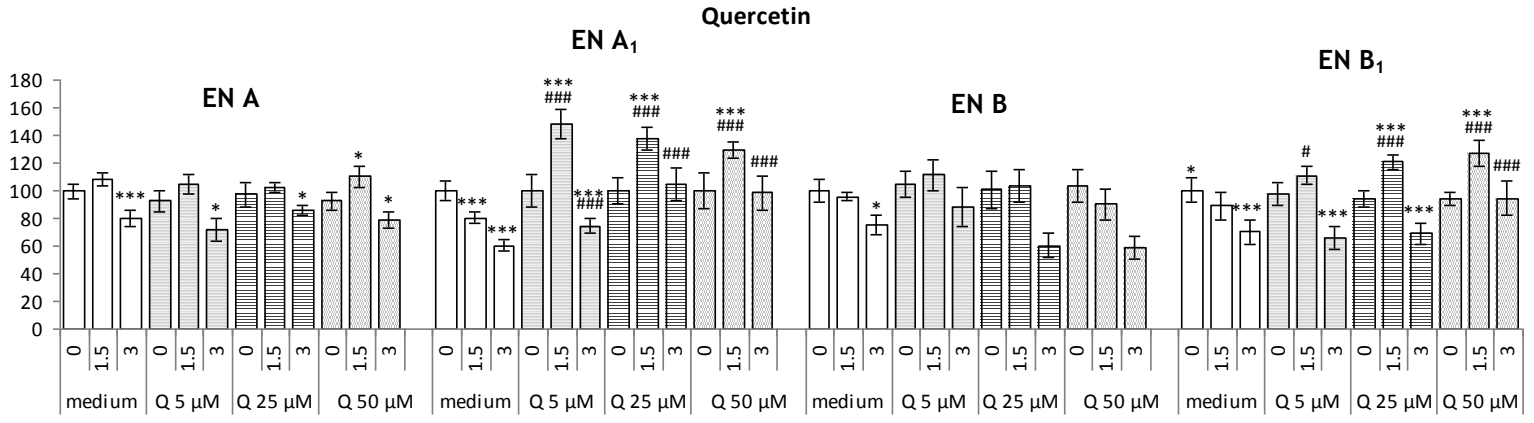

Figure 4. Protective effect of quercetin (Q) in CHO-K1 cells exposed to enniatins[*].

[*]Medium: cells were exposed with the indicated doses of each EN or 1% methanol (0=as control) in Ham-F12 medium. Q at 5, 25 and 50 µM concentration: cells were simultaneously exposed with Q + each EN during 24 h. After 24 h of incubation, viability of these cells was measured using the MTT assay. Statistically different from the respective negative Q control (0; $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$). Statistically different from the medium (ENs treatment in Ham-F12 medium without Q; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$). Units of ENs (0, 1.5 and 3.0) are in µM.

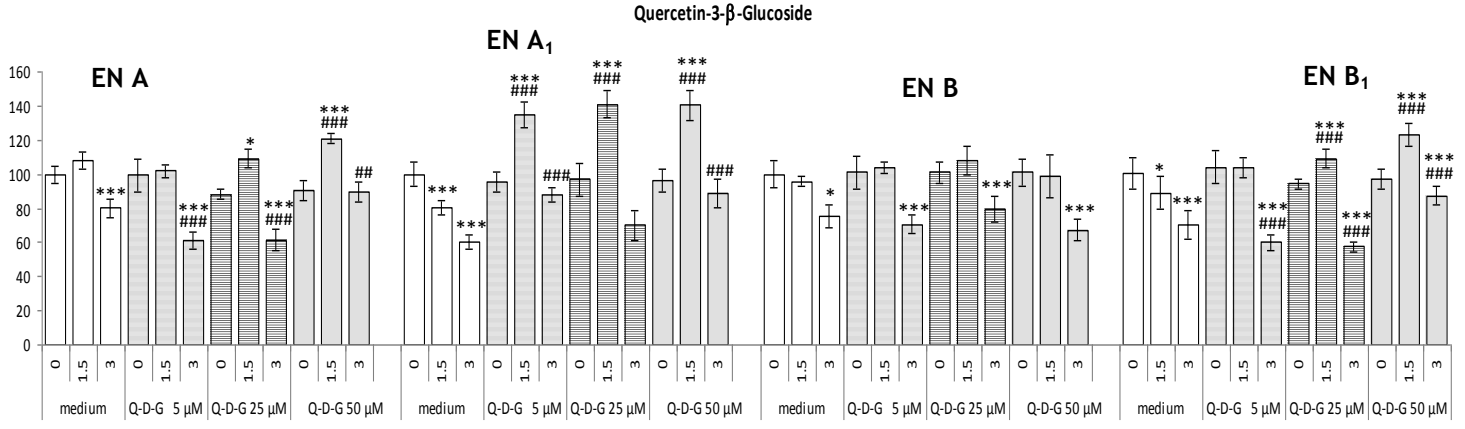

Figure 5. Protective effect of quercetin-3-ß-D-glucoside (Q-D-G) in CHO-K1 cells exposed to enniatins[*].

[*] Medium: Cells were exposed with the indicated doses of each EN or 1% methanol (0=as control) in Ham-F12 medium. Q-D-G at 5, 25 and 50 µM concentration: Cells were simultaneously exposed with Q-D-G + each EN during 24 h. After 24 h of incubation, viability of these cells was measured using the MTT assay. Statistically different from the respective negative Q-D-G control (0; *$p \leq 0.05$ and ***$p \leq 0.001$). Statistically different from the medium (ENs treatment in Ham-F12 medium without Q-D-G; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$). Units of ENs (0, 1.5 and 3.0) are in µM.

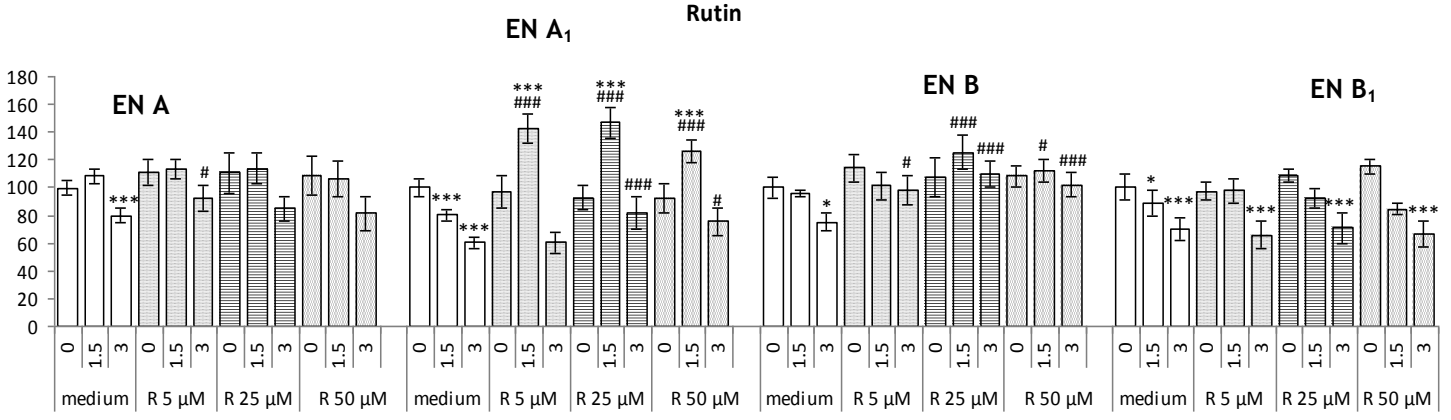

Figure 6. Protective effect of rutin (R) in CHO-K1 cells exposed to enniatins[*].

[*] Medium: cells were exposed with the indicated doses of each EN or 1% methanol (0=as control) in Ham-F12 medium. R at 5, 25 and 50 µM concentration: cells were simultaneously exposed with R + each EN during 24 h. After 24 h of incubation, viability of these cells was measured using the MTT assay. Statistically different from the respective negative R control (0; $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$). Statistically different from the medium (ENs treatment in Ham-F12 medium without R; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$). Units of ENs (0, 1.5 and 3.0) are in µM.

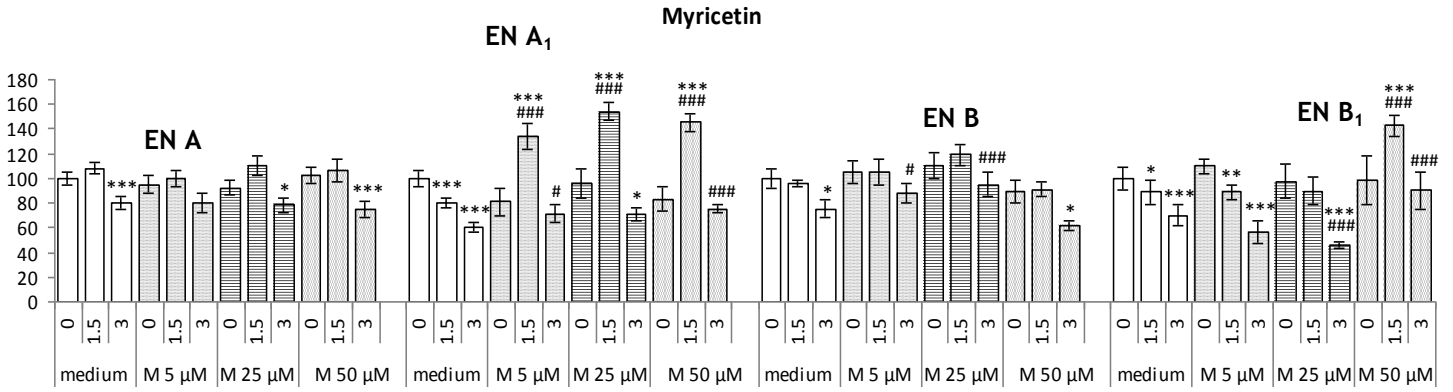

Figure 7. Protective effect of myricetin (M) in CHO-K1 cells exposed to enniatins[*].

[*] Medium: cells were exposed with the indicated doses of each EN or 1% methanol (0=as control) in Ham-F12 medium. M at 5, 25 and 50 µM concentration: cells were simultaneously exposed with M + each EN during 24 h. After 24 h of incubation, viability of these cells was measured using the MTT assay. Statistically different from the respective negative M control (0; *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$). Statistically different from the medium (ENs treatment in Ham-F12 medium without M; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$). Units of ENs (0, 1.5 and 3.0) are in µM.

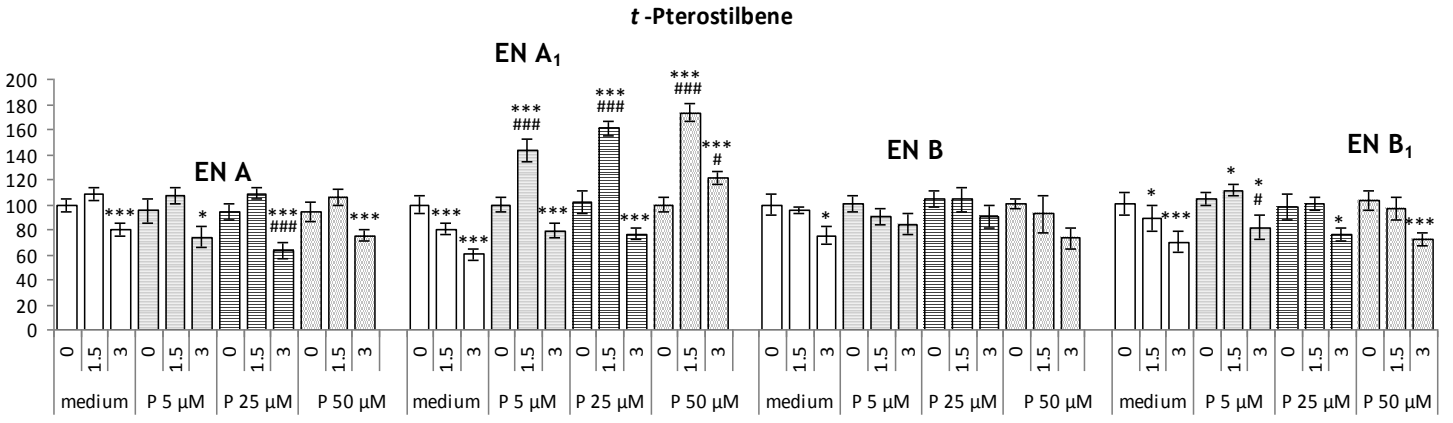

Figure 8. Protective effect of *t*-pterostilbene (P) in CHO-K1 cells exposed to enniatins[*].

[*] Medium: cells were exposed with the indicated doses of each EN or 1% methanol (0=as control) in Ham-F12 medium. P at 5, 25 and 50 µM concentration: cells were simultaneously exposed with P + each EN during 24 h. After 24 h of incubation, viability of these cells was measured using the MTT assay. Statistically different from the respective negative P control (0; *$p \leq 0.05$ and ***$p \leq 0.001$). Statistically different from the medium (ENs treatment in Ham-F12 medium without P; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$). Units of ENs (0, 1.5 and 3.0) are in µM.

4. Discussion

The ENs showed cytotoxic effect on CHO-K1 cells. EN B_1 was more cytotoxic than ENs A, A_1 and B. Cytotoxicity of ENs A, A_1, B and B_1 had been previously proved in different cell lines (Ivanova et al., 2006; Föllmann et al., 2009; Watjen et al., 2009; Meca et al., 2010, 2011). Results obtained by Ivanova et al. (2006) were similar to our results. Nevertheless, the IC_{50} value of EN B in HepG2 (from 206.7 to 435.9 μM) by Alamar Blue assay seems in contradiction with our results and all results exposed in literature for EN B. The IC_{50} values obtained for ENs by Follman et al. (2009) and Wätjen et al. (2009) were similar to our results in CHO-K1 cells. The comparison between results obtained by Meca et al. (2010) with Caco-2 cell line and those obtained in this study demonstrated that CHO-K1 cells were more sensitive than Caco-2 to ENs A_1, B and B_1. When the results obtained by Meca et al. (2011) were compared with those obtained by us, cytotoxic effect of EN B_1 observed in CHO-K1 cells was at least 5-, 3- and, 4-fold higher than those observed in HepG2, HT-29 and Caco-2 cells, respectively. However, EN A showed cytotoxic effect in HepG2 cells but not in CHO-K1 cells. In summary, all ENs show cytotoxic effect, regardless of the cell type studied.

Previous studies developed with flavonoids in different cell lines have shown contradictory results. The protective effect of polyphenols may be due to a wide variety of mechanisms, including lightening oxidative stress by scavenging free radicals, promoting cellular survival by modulating intracellular signals, decreasing toxicities of mycotoxins by regulating gene expression or activities of enzymes, etc. In this study, the highest concentration of quercetin and *t*-pterostilbene (100

μM) significantly decreased cell viability, but doses lower than 100 μM did not cause cytotoxicity. Therefore, doses below 100 μM were applied in all subsequent experiments. Although, the selected concentrations are related to the wide range of polyphenols intake (18.6 mg to 2.20 g/day) according to the results obtained by Ostertag et al. (2010). Results obtained revealed that co-treatment of quercetin had cytoprotective effect in CHO-K1 cells exposed to EN A_1 and EN B_1 (Fig. 4). Cytoprotective effect ranged from 24 to 84 % (EN A_1) and from 25 to 43% (EN B_1). Co-treatment with quercetin 3-β-D-glucoside (50 μM) reduced approximately 12% of EN A-induced cytotoxicity (Fig. 5). With respect to EN B_1, quercetin 3-β-D-glucoside showed a similar cytoprotective effect (from 16 to 38%; Fig. 5). However, during simultaneous exposure of quercetin 3-β-D-glucoside and EN A_1, higher reduction in cytotoxicity was observed (Fig. 5). The reduction was 47% and ranged from 68 to 76% after 1.5 and 3.0 μM of ENA_1, respectively. No changes in cell viability were observed when quercetin 3-β-D-glucoside and EN B were simultaneously exposed in CHO-K1 cells (Fig. 5). Rutin had cytoprotective effect in CHO-K1 cells exposed to EN A_1 and EN B (Fig. 6). After EN B exposure, this effect ranged from 17 to 31 % and from 30 to 46% after 1.5 and 3.0 μM of EN B, respectively. Higher cytoprotection was observed after EN A_1 exposure (Fig. 6). The cytoprotective effect ranged from 26 to 35 % and from 57 to 83% after 1.5 and 3.0 μM ENA_1, respectively. On the other hand, 5 and 25 μM of myricetin significantly reduce cytotoxicity (16 and 26%) produced by 3.0 μM of EN B in CHO-K1 cells, respectively (Fig. 7). While, only the highest concentration (50 μM) of myricetin reduce 61% and 29% the cytotoxic effect induced by 1.5 and 3.0 μM of EN B_1, respectively (Fig. 7). Respect

to EN A_1, the cytoprotective effect of myricetin ranged from 66 to 92% and was 25% after 1.5 and 3.0 μM, respectively (Fig. 7). *t*-Pterostilbene had cytoprotective effect in CHO-K1 cells exposed to EN A_1 and EN B_1 (Fig. 8). The cytoprotective effect ranged from 78 to 116 % and from 32 to 101% after 1.5 and 3.0 μM of EN A_1 exposures, respectively. Cytoprotective effect in CHO-K1 cells was approximately 25% when 5 μM of *t*-pterostilbene and EN B_1 were simultaneously incubated.

Barcelos and coworkers (2011) extracted quercetin (purity > 99.5%) and rutin (purity > 99.45) from the dried leaves of *Dimorphandra mollis* Benth and determined their cytotoxic and cytoprotective effects when were exposed alone or simultaneously with aflatoxin B_1 (AFB_1) in HepG2 cells. The protective results obtained against AFB_1-induced DNA damage showed that pre-, simultaneous and post-treatment of HepG2 cells with quercetin and rutin decreased the number of AFB_1-induced DNA strand breaks. However, these authors proved that quercetin (50 μg/ml) and rutin (100 μg/mL) were cytotoxic to HepG2 cells, due to they increase the intracellular ROS production and act as pro-oxidant agents. Similar cytotoxic effects of quercetin (0-500 μM) and rutin (0-300 μM) were observed by Matsuo et al. (2005) in human lung embryonic fibroblasts (TIG-1) and umbilical vein endothelial (HUVE) cells at relatively high concentrations. On the other hand, Barcelos and co-workers (2011) observed that lower concentration of quercetin and rutin are efficient ROS scavengers, because of they present a high inhibition of ROS generation. Choi et al. (2010) observed that quercetin derivatives could diminish oxidative stress, GSH depletion, and lipid peroxidation induced by AFB_1 in HepG2 cells *in vitro*, while these effects could be not correlated their antioxidant activity. On the other

hand, Choi et al. (2010) demonstrated that quercetin was converted to various metabolites (meanly glucuronide/sulfate-conjugates) by liver enzymes and intestinal microbiota in mice. However, it is unclear whether the quercetin conjugates are the metabolites responsible for the beneficial effects of quercetin. Barcelos et al. (2011) also demonstrated a modulating effect of quercetin and rutin on CYP isoenzymes, which are responsible for mycotoxin activation. Therefore, they attribute the protective effect to the inhibition of CYP isoenzymes and also free radical scavenger activities. Similar effects were observed in our work in CHO-K1 cells exposed to ENs. Moreover, in previous studies developed in our laboratory, intracellular ROS production and lipid peroxidation was produced with 1.5 and 3.0 μM of ENs exposed during 24 h in Caco-2 cells (Prosperini et al., 2011). It is accepted that hepatic biotransformations play an important role in detoxification of polyphenols. Soares et al. (2006) studied the cytotoxic effect for quercetin and rutin in McCoy cells in the presence and absence of the hepatic S9 microsomal fraction (S9 fraction). The S9 fraction contains microsomes and many enzymes of phase I metabolism. Quercetin was cytotoxic in a dose-dependent manner in cells without S9 fraction (IC_{50} was 83.7 ± 2.57) μg/ml; whereas, the S9 mixture metabolized these compounds to less cytotoxic metabolites, and the IC_{50} > 500 (with S9) μg/ml. However, rutin did not show cytotoxic effect at the range of concentration tested (15-500 μg/ml) neither with nor without S9 fraction. On the other hand, effect of quercetin on ochratoxin A (OTA)-induced cytotoxicity in liver and intestinal cells has also been determined (Hundhausen et al., 2005; Sergent et al., 2005). Taken into account that cytotoxicity of OTA may be produced by inhibition of

protein synthesis, inhibition of various enzymes and formation of DNA adducts, and knowing that depletion of cellular glutathione (GSH) significantly enhanced the cytotoxic effects of OTA. These authors demonstrated that quercetin do not counteract OTA-induced cytotoxicity in HepG2 and Caco-2 cells. Similar role of GSH was observed for T2 toxin and quercetin both *in vivo* and *in vitro* (Markham et al., 1987).

Polyphenol glycosides have been found not to be incorporated into cells. Thus rutin and quercetin-3-*O*-glycoside may be barely incorporated into cells due to their higher hydrophilicity and hence have no effect (Zielinska et al., 2003; Razavi et al., 2009; You et al., 2010). This fact has been observed in our study and others. Oh et al. (2004) determined the hepatoprotective activity of four polyphenols extracted, isolated and purified from *Equisetum arvense L.* commonly used in traditional medicine in Korea. The polyphenol extracted quercetin-3-*O*-glucoside shown free radical and superoxide scavenging effects at 11.2 ± 0.2 and 91.8 ± 6.2 μM, respectively. However, no hepatoprotective effect was observed. As previously described, Oh and co-workers (2004) assumed that the increased polarity of quercetin-3-*O*-glucoside due to the presence of sugar moiety, could decrease the cell permeability of this polyphenol and therefore its antioxidant effect. Similarly to other authors, Marcarini et al., (2011) observed that rutin (10-810 μM) was not cytotoxic in HTC hepatic cells, and protect (50 and 75 μM) tissues against free radicals (Soares et al., 2006; Barcelos et al., 2011). However, the highest concentration (810 μM) of rutin tested showed cytotoxic effect due to DNA damage. Razavi et al. (2009) also observed this effect when McCoy (Pasteur, C123) cells were exposed to

quercetin-3-*O*-glucoside (100-1000 µg/mL) during 16 h provoked an IC_{50} of 215.2 mg/ml (463 µM). Similarly, in our study, rutin and quercetin-3-ß-D-glucoside did not show cytotoxic effect in CHO-K1 cells. Moreover, higher concentrations of quercetin-3-ß-D-glucoside assayed protect cells from ENs A, A_1 and B_1 exposure; and higher concentrations of rutin protect cells from ENs A_1 and B. According to Matsumoto et al (2007), a possible mechanism of absorption of glycoside forms occurs. They found that most of the absorbed glycosides were hydrolyzed rapidly to aglycone and its conjugates. This could indicate that by time, rutin or quercetin-3-*O*-glucoside were hydrolyzed to quercetin aglycone and to be absorbed by cells, having the antioxidant effect observed. Aherne and O' Brien (1999, 2000) studied the cytotoxicity of quercetin, rutin and myricetin (from 2.5 to 200 µM) in CHO, Caco-2, HepG2 and V79 cells by NRU and FDA-EtBr assays after 24 h of exposure. Similarly to our results, quercetin, rutin and myricetin did not cause cytotoxic effect. And, according to these authors, quercetin, rutin and myricetin were capable of reducing H_2O_2-induced DNA damage *in vitro*. On the other hand, Soundararajan et al. (2008) propose a novel model for quercetin-3-glucoside-mediated cytoprotection against oxidative stress. The suggest as additional mechanism the ability of quercetin-like compounds to become intercalated between the acyl chains of phospholipids in the plasma membrane. Therefore, quercetin-3-glucoside increases the resistance of membranes to lipid peroxidation by increasing incorporation of this polyphenol into the plasma membrane. The effective prevention of losses cell viability was observed at very low concentration (0.1 µM). respect to myricetin, Yokomizo and Moriwaki (2006) demonstrated the strong radical scavenging activity of myricetin

(10 μM) against radicals. However, according to Shih et al. (2009), inhibitory effect of myricetin may be through an inactivation of the ERK (extracellular signaling-regulating kinase) signaling pathway. A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves action of kinases. These authors observed that myricetin was not toxic to human adenocarcinoma A549 cells from 0 to 20 μM. However, when cells were treated with myricetin (30-50 μM, during 24 and 48 h), cell viability significantly decreased by inhibiting phosphorylation of ERK. On the other hand, Zhang et al. (2009) observed a dose- and time-dependent growth inhibition effect of myricetin on esophageal carcinoma KYSE-510 cells via cell cycle arrest in the G_2/M phase and mitochondrial-dependent apoptosis in a p53-independent manner.

About stilbenes, epidemiological studies have demonstrated benefit of pterostilbene (presented in the extracts of heartwood of *Pterocarpus marsupium*) in the Ayurvedic medicine (Ruiz et al., 2009). Moreover, previous studies have demonstrated that *t*-pterostilbene is cytotoxic because of it inhibits proliferation and induces apoptosis to a number of cancer cell lines (Tolomeo et al., 2005; Pan et al., 2007; Chakraborty et al., 2010). These authors demonstrated that *t*-pterostilbene decreased cell growth in a dose-dependent manner, with IC_{50} values from 10 to 83 μM. Furthermore, they found that the cytotoxic effect of *t*-pterostilbene was due to apoptotic cell death in a concentration- and time-dependent manner. Moreover, decrease in the mitochondrial transmembrane potential in cells, arrest cells in the G_0/G_1 phase of the cell cycle and the increase in ROS levels after *t*-pterostilbene-treated cells contribute to be an early mediator in *t*-

pterostilbene-induced apoptosis. On the other hand, Kim et al. (2009) extracted and purified pterostilbene from *Vitis coignetiae*, a wild wine whose fruits are consumed and also traditionally used in alcoholic drinks and beverages in Korea. They evaluated the potential cytotoxic effects of pterostilbene in the range of 0.1-1000 μM during 24 h. And, similarly to our results, they found that pterostilbene was not cytotoxic at concentrations up to 100 μM. Moreover, they demonstrated that pterostilbene prevented H_2O_2-induced modulations of gap junctional intercellular communication via the inactivation of ERK and p38.

In conclusion, the protective effect of cells against oral cytotoxic compounds depends on a balance between antioxidants and pro-oxidant components. Increased levels of antioxidants may respond to polyphenols in food commodities (as berries, wine, purple grape juice, etc.) and dietary supplements (quercetin-rich diets, etc.) which have been studied extensively to improve antioxidant reserve and thereby help regulate oxidative damage. The results obtained shown a wide variety of cytoprotective action from polyphenols, which depends on type of polyphenol, concentration in food commodity, simultaneous presence and interaction between polyphenols and other contaminants (as ENs), etc. So, food commodities containing polyphenols (specially, myricetin, quercetin, quercetin-3-β-D-glucoside, rutin and *t*-pterostilbene) could contribute to diminish the toxicological risk to humans that ENs in these foods can produce.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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STUDY OF THE CYTOTOXIC ACTIVITY OF BEAUVERICIN AND FUSAPROLIFERIN AND BIOAVAILABILITY *IN VITRO* ON CACO-2 CELLS

A. Prosperini, G. Meca*, Font, G., Ruiz, M.J.

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

* Corresponding author. Tel.: +34 963544959; fax: +34 96354954.
E-mail address: giuseppe.meca@uv.es

ABSTRACT

Beauvericin (BEA) is a cyclohexadepsipeptide mycotoxin which has insecticidal properties and produces cytotoxic effects in mammalian cells. Fusaproliferin (FUS) is a mycotoxin that has toxic activity against brine shrimp, insect cells, and teratogenic effects on chicken embryos. The aim of this study was to determine the cytotoxicity of BEA and FUS in human epithelial colorectal adenocarcinoma HT-29 and Caco-2 cells, the transepithelial transport and the bioavailability using Caco-2 cells as a simulated in vitro gastrointestinal model of the human intestinal epithelium. The inhibitory concentration (IC_{50}) evidenced by BEA in the Caco-2 cells was 24.6 and 12.7 μM at 24 and 48 h exposure, respectively, whereas the IC_{50} values evidenced in HT-29 cells were 15.0 and 9.7 μM, respectively. FUS was cytotoxic, but no IC_{50} data were observed in the range of concentration tested. BEA bioavailability was variable from 50.1% to 54.3%, whereas FUS presented a bioavailability variable from 80.2% to 83.2%. Results obtained demonstrated a potential risk for human health.

Keywords: Beauvericin, fusaproliferin, cytotoxicity, bioavailability *in vitro*, transepithelial transport, HT-29.

1. Introduction

Beauvericin (BEA) is a cyclohexadepsipeptide that was first identified in a soil-borne entomopathogenic (insect-pathogenic) fungus *Beauveria bassiana*, which was recognized as the causative agent for heavy losses of the European sericulture in the 16th and 17th centuries (Tang et al., 2005). Nowadays, BEA is considered to be a putative mycotoxin (toxic fungal metabolite) that may affect human and animal health, since it is also produced by many species of the fungus *Fusarium* that infect important cereal grains such as corn, rice, and wheat (Leslie and Summerell, 2006). The potential mycotoxic role of BEA is exemplified by results obtained from *in vitro* studies using cell lines. In mammalian cell lines, cell deaths caused by BEA have been suggested to involve a Ca^{2+} dependent pathway, in which BEA induces a significant increase in intracellular Ca^{2+} concentration that leads death cell as a result of a combination of both apoptosis and necrosis (Jow et al., 2004; Lin et al., 2005). The mechanism of BEA-induced Ca^{2+} increase, however, remains inconclusive. BEA-induced apoptotic changes such as DNA fragmentation have been demonstrated to take place in the complete absence of extracellular Ca^{2+}, suggesting that BEA triggers release of Ca^{2+} from internal Ca^{2+} stores. In fact, BEA has since been regarded as an apoptotic agent that releases Ca^{2+} exclusively from endoplasmic reticulum (Lin et al., 2005). Furthermore, BEA is a potent and specific cholesterol acyltransferase inhibitor in rat liver microsomes (Tomoda et al., 1992). The inhibition of cell proliferation by BEA exposure, was studied by several authors in rodent (Ferrer et al., 2009), monkey, porcine (Klaric et al., 2006), insect (Calò et al., 2003) and human cell lines (Ruiz et al., 2011; Lin et al., 2005; Meca et al., 2011)

utilizing several determinations techniques as the tetrazolium salt (MTT), the trypan blue dye exclusion, Alamar Blue, BrdU and sulforhodamine B (SRB) assays.

Fusaproliferin (FUS) is produced by two closely related species, *Fusarium subglutinans* and *F. proliferatum* (Ritieni et al., 1999). Both species are important pathogens of maize (Ritieni et al., 1999) and other economically important crop plants and may be isolated together from the same plant (Moretti et al., 2007). FUS is toxic to *Artemia salina* (Moretti et al., 2007; Tan et al., 2011), IARC/LCL 171 human B lymphocytes and SF-9 insect cells (Logrieco et al., 1996) and has teratogenic and pathogenic effects on chicken embryos (Ritieni et al., 1997). The study of the bioavailability of the *Fusarium* mycotoxins including zearalenone (ZEA), fumonisins, ochratoxin A (OTA), deoxinivalenol (DON), aflatoxin B1 (AFB_1), and enniatins (ENs) has been evaluated by many authors (Avantaggiato et al., 2003, 2004; Carolien et al., 2005; Videmann et al., 2008; Meca et al., 2012). These authors describes the applicability of an *in vitro* digestion models to measure the bioavailability of different mycotoxins contained in food matrices as an indicator of oral bioavailability. The aim of this study was to evaluate: (a) the cytotoxic effects of the BEA and FUS on Caco-2 and HT-29 cell lines, (b) the transepithelial transport and (c) the relative bioavailability utilizing the cell line Caco-2, that is, the validated international model used to study the intestinal absorption of the compounds contained in food.

2. Materials and methods

2.1. Materials

Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. BEA was purchased by Sigma (Sigma Co., St Louis, USA) and FUS, used in this study was produced and purified according to the method of Meca et al. (2009). The reagent grade chemicals and cell culture components used, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, amphotericin B, HEPES, Thyazolil Blue Tetrazolium Bromide (MTT), non essential aminoacids (NEAA), Hank's Buffered Salt Solution (HBSS), phosphate buffer saline (PBS), Sorensen's glycine buffer, glucose and dimethyl sulfoxide (DMSO) were Sigma products (Sigma Co., St. Louis Mo. USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium).

2.2. Cells culture

HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-37) cells from human epithelial colorectal adenocarcinoma were cultured in monolayer in 9 cm2 polystyrene tissue culture dishes with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin, 2.5 µg/mL amphotericin B, and 10% heat inactivated FCS. Incubation conditions were pH 7.4, 37 °C and 5% CO_2 in a 95% relative humidity atmosphere.

2.3. Cytotoxicity tests

Caco-2 and HT-29 cells were cultured into 96-well tissue-culture plates by adding 200 µL/well of a suspension of 2 x 10^4 cells/mL. After the cells reached 80% confluence, the culture medium was replaced with fresh medium containing BEA and FUS at concentrations ranging from 0.6 to 30 µM and incubated for 24 and 48 h at 37 °C. Cytotoxicity was determined by the MTT assay. The MTT assay determines the viability of cells by the reduction of yellow soluble tetrazolium salt (MTT); only in the metabolically active cells, via a mitochondrial-dependent reaction to an insoluble purple formazan crystal. The MTT viability assay was performed as Ruiz et al. (2006). Briefly, after exposure to the ENs for 24 and 48 h, the medium containing ENs was removed and cells of each well received 50 µL of MTT. The plates were wrapped in foil and incubated for 4 h at 37 °C. After the incubation period the medium contained the MTT was removed, and the plate was washed with PBS. Two hundred microliters of DMSO were added in the plate followed by 25 µL of Sorensen's glycine buffer. The absorbance was measured at 570 nm using an ELISA plate reader Multiscan EX (Thermo Scientific, MA, USA). The results were expressed in relative form to cell culture protein content. The IC_{50} values, the mean effective concentration, of test chemical that modifies each biomarker by 50% (positive or negative) in comparison with appropriate untreated controls, were determined with linear interpolation.

2.4. Caco-2 intestinal transport

Caco-2 cells were seeded at 25 x 10^4 cells/cm^2 on six-well Transwell Permeable Supports (Corning, NY, USA). Traswell insert (6-

wells) were covered with a monolayer of Caco-2 cells at 37 °C. The medium was changed every 2 days, and the cells were allowed to grow and differentiate up to 21 days after reaching confluence (Hilgers et al., 1990). After removal the growth medium from both sides of monolayers, the cells were preincubated at 37 °C for 10 min with HBSS–HEPES buffer. For this purpose, in the 6 wells permeable supports 1.5 mL of HBSS–HEPES buffer were added in the apical compartment and 0.5 mL of HBSS–HEPES were added in the basolateral compartment. After medium removal, medium containing BEA and FUS in the concentration of 1.5 and 3.0 μM was added in the apical compartment and natural medium to the basolateral compartment (Fig. 1). The monolayers were incubated for 4 h at 37 °C. For transport measurements, aliquots of incubation medium were taken from the basolateral compartment at specified times and samples were collected for immediate analysis by direct injection (20 μL) in the liquid chromatography (LC) apparatus.

2.5. BEA and FUS extraction from apical and basolateral compartment

BEA and FUS were extracted from the apical and basolateral compartment according to the method described by Ambrosino et al. (2004). In particular, 1 mL of each sample was dissolved in 3 mL of methanol and then filtered through 0.22 μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain). The samples were purified with a C18-E solid-phase extraction column (100 mg, Phenomenex, Torrance, CA) that had been preconditioned with 3 mL of methanol and 3 mL of water, and then eluted with 1 mL of methanol. The eluate was completely evaporated under nitrogen at 50 °C and reconstituted with

100 µL of methanol. Then, 20 µL of each extract was injected in the LC apparatus. The recovery of the extraction method was determined by spiking experiments. BEA (Sigma, St. Louis, MO) and FUS were added to a no contaminated transport medium in concentration variables from 0.1 to 10 µM, and then extracted. The calculated recovery (mean ± relative standard deviation) of BEA and FUS from the artificially contaminated sample was up to 90.7%.

2.6. BEA and FUS extraction from cells

BEA and FUS contained in the cells were determined according to Meca et al. (2010) as follows: cells were sonicated in a saline solution (0.90% NaCl, v/v) for 30 min. The pellet was suspended in 5 mL of saline solution, and 5 mL of ethyl acetate was added. After mixing with a vortex for 1 min and centrifuging (4000 rpm for 10 min at 4 °C, Centrifuge 5810R, Eppendorf, Germany), the upper layer, 5 mL approximately, was evaporated by a rotary evaporator (Buchi, Switzerland), resuspended in 1 mL of methanol and analyzed by injecting 20 µL into the LC apparatus.

2.7. LC Analysis of BEA and FUS

LC analyses of BEA and FUS were performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150 x 4.6 mm, 5 µm, Phenomenex, Torrance CA) column was used. LC conditions were set up using a constant flow at 1.0 mL/min and acetonitrile–water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at

90% acetonitrile, the mobile phase was taken back to the starting conditions in four minutes. BEA was detected at 205 nm, and FUS at 261 nm. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantitation of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

2.8. Statistical analysis

All experiments were performed three times. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. Differences were considered significant if $p \leq 0.05$.

3. Results and discussions

3.1. Cytotoxicity of BEA and FUS

The results clearly indicated that only one of the mycotoxins object of this study have a cytotoxic effects on HT-29 and Caco-2 cells cells in a dose dependent manner as attested by the MTT assay (Fig. 2).

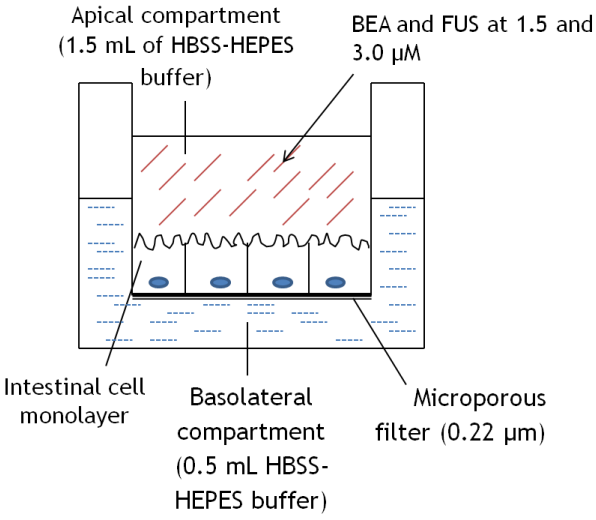

Figure 1. Graphical representation of the *in vitro* method utilized for the determination of beauvricin and fusaproliferin bioavailability.*

The IC_{50} evidenced by BEA in the Caco-2 cell line at 24 h of exposure was 20.62 ± 6.9 µM, whereas at 48 h exposure the IC_{50} expressed by this mycotoxin was 12.75 ± 4.8 µM. On the HT-29 cell line the IC_{50} evidenced for BEA at 24 h was 15.00 ± 6.9 µM, and 9.75 ± 4.4 µM at 48 h of exposure. FUS did not showed IC_{50} values on the two cell line tested in the range of the concentration assayed. Tomoda et al. (1992) evidenced an IC_{50} of 11 µM for BEA in the cell line J774 (mouse

* BEA and FUS were introduced in the apical compartment, where Caco-2 cells were grown during 21 days on a 0.45 µM filter to simulate the duodenal intestinal epithelium. After 4 h incubation the determination of BEA and FUS was carried out in: (a) the apical compartment to determine the quantity of the mycotoxin no transported by the cells, (b) the basolateral compartment, to determinate the concentration of the two mycotoxins that was completely absorbed through the cells, that represent the quantity of the mycotoxin available to the human organism and (c) the cellular matrix, to evidence eventually, the concentration of the two mycotoxin internalized by cells.

macrophages) after 24 h of exposure. This data is comparable with the IC_{50} obtained in our study in the Caco-2 cells after 48 h of exposure. Similar results were obtained by Calò et al. (2003), that investigated the cytotoxicity of BEA on two human cell lines of myeloid origin: the monocytic lymphoma (U-937) cells and the promyelocytic leukemia (HL-60) cells. The U-937 and HL-60 cells in stationary phase were exposed to BEA during 4 and 24 h of exposure, respectively. Results demonstrated that the viability of both typologies of cells was not affected by BEA at concentrations up to 3 μM, after 4 h exposure, whereas a steady decline was detected at higher concentrations. Similarly, after an exposure time of 24 h, a decline in viability was observed in cultures exposed to BEA at a concentration of 10 μM or higher. IC_{50} at 24 h of exposure was 30 μM and 15 μM for U-937 cells and HL-60 cells, respectively. Jow et al. (2004) investigated the cytotoxic effect of BEA on the human leukemia cell line (CCRF-CEM) using the MTT assay, during 24 h. The IC_{50} value for BEA in CCRF-CEM cells was 2.46 μM. This cell line is 6.5-fold more sensible to the action of this mould secondary metabolite, respect at the cells employed in this study. Ferrer et al. (2009), evaluated the cytotoxicity of BEA on Chinese hamster ovary cell line (CHO-K1), by MTT and Neutral Red assays (NR) at 24 h of exposure. The IC_{50} value evidenced was 17.22 μM (NR assay) and 12.08 μM (MTT assay). The cytotoxicity of three *Fusarium* mycotoxins (beauvericin, deoxynivalenol and T-2 toxin) has been investigated using the NR assay, after 24, 48 and 72 h of incubation by Ruiz et al. (2011). The IC_{50} values ranged from 6.77 to 11.08 μM, 3.30 to 10.00 μM and 0.004 to 0.005 μM for beauvericin, deoxynivalenol and T-2 toxin, respectively. Few publications are available in the literature on the biological activity of

the FUS. In particular, the LC_{50} of FUS to brine shrimp, *A. salina*, is 53 μM, and its CC_{50} (cytotoxic concentration 50%) to the lepidopteran *Spodoptera frugiperda* cell line (SF-9) and on the human nonneoplastic B-lymphocyte cell line IARC/LCL 171 are 70 and 55 μM, respectively (Logrieco et al., 1996). Moreover in a toxicity test with chicken embryos, FUS causes cephalic dichotomy, macrocephaly, and limb asymmetry when 1 or 5 mM pure FUS water/DMSO (38:62 v/v) solution was inoculated into the air sacs of the fertilized eggs (Ritieni et al., 1997).

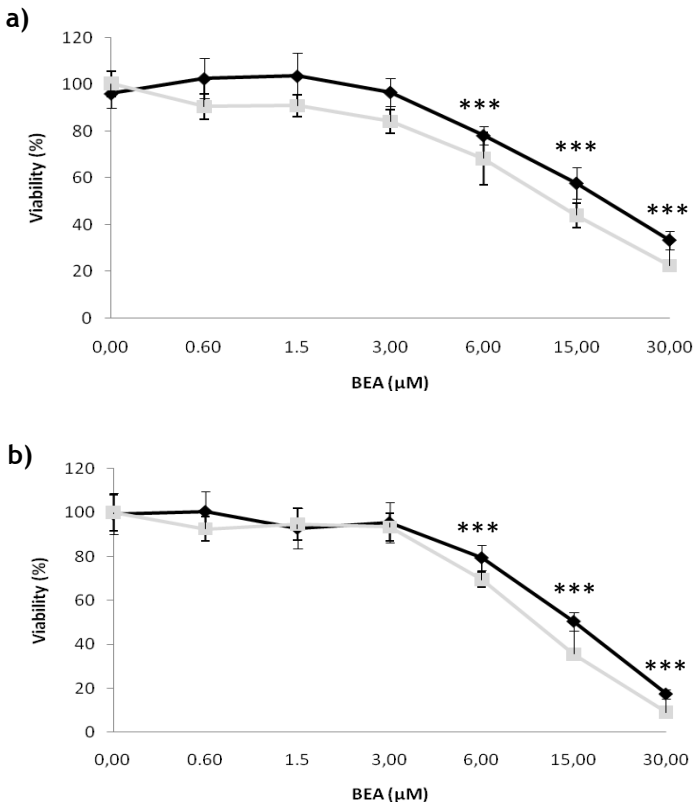

Figure 2. Effects of beauvericin on (a) Caco-2 and (b) HT-29 cell lines after 24 h (black line) and 48 h (gray line) of exposure. Viability was determinate by the MTT assay. (***) $p \leq 0.000$, significantly different from the control.

3.2. Transepithelial transport and bioavailability of BEA and FUS

Caco-2 cell monolayers were used in the design and development of a simulated *in vitro* gastrointestinal extraction model in order to assess the oral absorption of BEA and FUS. Unidirectional BEA and FUS transport was assessed from the apical to basolateral side of differentiated cell monolayers (Fig. 2). Transport efficiencies were

expressed as the percentage of BEA or FUS concentration initially loaded into the apical side and detected on the basolateral side at incubation of 1, 2, 3, and 4 h. Analytical LC chromatograms of the two mycotoxins present in the apical, basolateral side and retained inside cell monolayers are presented in the Figure 3 and Figure 4. The transport profiles of BEA and FUS, from the apical toward basolateral compartment are showed in the Table 1. As evidenced in Table 1, transepithelial transport of BEA during the four hour was lower than the absorption values evidenced by FUS. At the end of the absorption process the two mycotoxins were determinate also in the cellular matrix to know the eventually concentration of the BEA and FUS internalized by cells. As regards the FUS, this mycotoxin was not absorbed (internalized) by the cells in the range of concentration tested, whereas, as is possible to observe in Figure 5, during the assay carried out with 1.5 μM of BEA concentration, 0.50 ± 0.05 μM of it which represents the 33% of the mycotoxin added at the beginning of the experiment was detected in the cellular matrix. Similar concentration of BEA (0.44 ± 0.03 μM) were internalized by the cells, when the mycotoxin was used in the concentration of 3.0 μM, which represent the 14.6% of the mycotoxin added (Fig. 5). The results obtained demonstrated that, the total quantity of FUS bioavailable absorbed resulted higher than the BEA considering the same mycotoxin concentrations added to the transwell plate. Moreover, as can be observed in Table 1, at 1.5 μM concentration, the quantities expressed in percentage of each mycotoxin absorbed is higher respect to the bioavailability value at 3.0 μM. This phenomenon can be related to many factors. BEA and FUS have a chemical structure very different, in

particular the presence of amino acids groups in the BEA lateral chain can be responsible of the internalization of this mycotoxin by Caco-2 cells, or its interaction between the mycotoxin and some component of the cell membrane. Inversely, the terpenic structure of the FUS, and the absence of aminoacids group in the lateral chain can be responsible of the great bioavailability of this compound. On the other hand the molecular weight of the FUS is early the half of the molecular weight of the BEA, and considering that there is a directly correlation between low molecular weight and bioavailability of organic compounds (Fernández-García et al., 2009), this reason can be another explication of the different bioavailability values evidenced by the compounds evaluated in this study. The last aim of this study was to evaluate the bioavailability of BEA and FUS by an in vitro gastrointestinal model. More than a decade ago, Caco-2 cells grown on permeable supports were introduced as an experimental tool for mechanistic studies of intestinal drug transport. Caco-2 model is considered as a model to study passive drug absorption across the intestinal epithelium due to the good correlation obtained between data on oral absorption in humans and the results in Caco-2 model (Artursson and Karlsson, 1991). Several factors spurred the development of Caco-2 and similar cell models. Avantaggiato et al. (2003, 2004), evaluated the intestinal absorption of DON and nivalenol (NIV) in feed by an in vitro gastrointestinal model. During transit of the spiked feed through the gastrointestinal model, DON and NIV were simultaneously absorbed in the small intestinal compartments (jejunum and ileum). The intestinal absorption of DON was approximately 2.4 times higher than NIV both in the jejunal and in the ileal compartment, being 51% and 21% of the intake for DON and

NIV, respectively. The higher absorption of DON in comparison to NIV may be due to its higher hydrophobicity. Carolien et al. (2005) describes the applicability of an *in vitro* digestion model allowing the measurement of the bioaccessibility of ingested mycotoxins from food as an indicator of oral bioavailability. Bioaccessibility of AFB_1 from peanut slurry and OTA from buckwheat was 94% and 100%, respectively. Videmann et al. (2008) studied the bioavailability and the transport of the mycotoxin ZEA in human intestinal Caco-2 cells, utilizing concentration of the toxin of 10 and 200 μM with an incubation time of 8 h. The intestinal absorption of ZEA was of 40% respect at the initial concentration and early 10% lower than the mean value obtained with ENs in this study.

Table 1. Transport profile of beauvericin and fusaproliferin in Caco-2 cell monolayers after different incubation time. The values are expressed in percentage (%).

Mycotoxin	Concentration (μM)	Transport profile (%)			
		Incubation time (h)			
		1	2	3	4
BEA	1.5	15.8±1.2	27.0±0.5	39.9±0.6	54.3±1.1
	3.0	18.4±1.1	23.1±1.0	34.6±0.9	50.1±1.1
FUS	1.5	25.5±1.2	38.2±1.0	71.9±1.1	83.2±0.8
	3.0	22.1±0.7	36.5±1.5	69.3±0.9	80.2±1.0

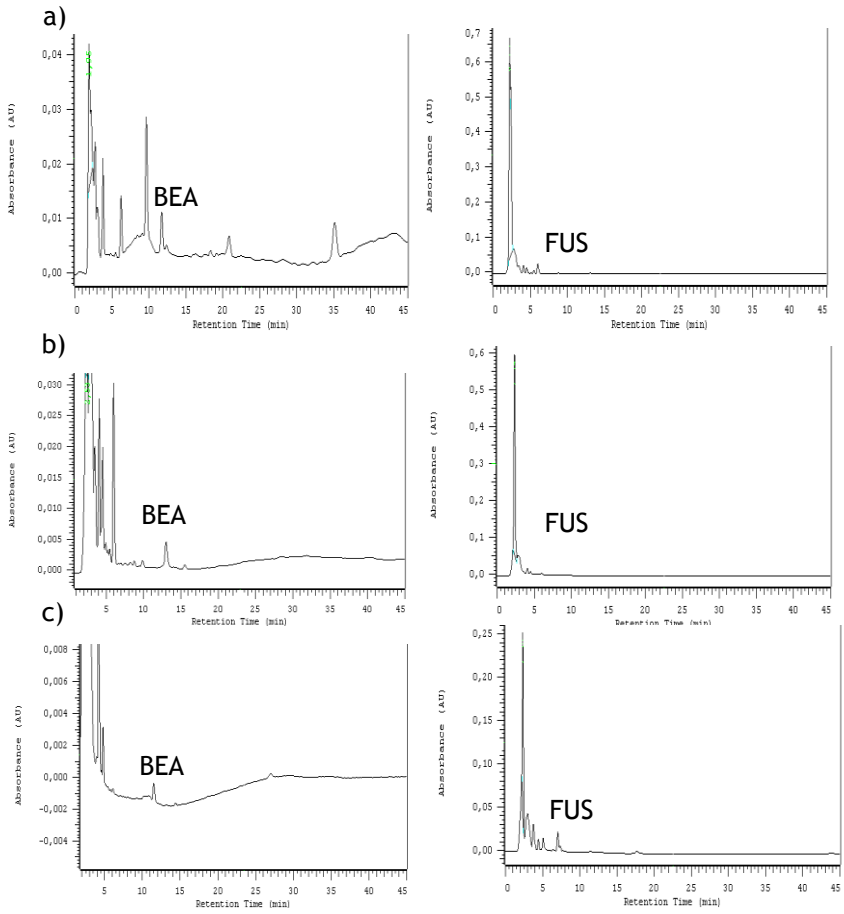

Figure 3. LC-UV chromatograms of the beauvericin and fusaproliferin presents in (a) the apical compartment, (b) the cell matrix and (c) the basolateral compartment side of cell monolayers.

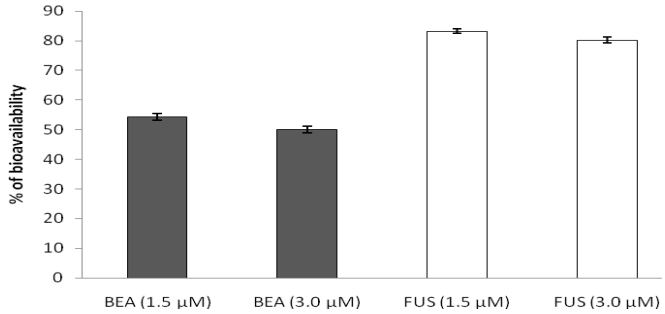

Figure 4. Bioavailability of beauvericin and fusaproliferin, added to the trans well plate at 1.5 and 3.0 μM concentration. Results are mean ± SD (n = 3).

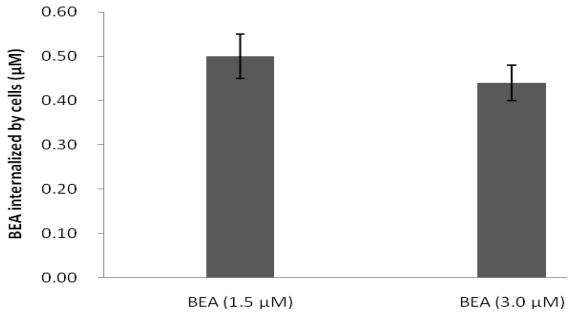

Figure 5. Beauvericin concentration detected inside Caco-2 cells at the end of the transepithelial transport process (mean ± SD of three independent experiments).

4. Conclusions

Considering the data produced in this study it is possible to conclude that BEA and FUS showed cytotoxicity in Caco-2 cells at all time of exposure tested. The data evidenced in this study demonstrated

that the intake of food contaminated by these bioactive compounds can represent a potential risk for the human health.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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BIOACCESSIBILITY OF ENNIATINS A, A_1, B, AND B_1 IN DIFFERENT COMMERCIAL BREAKFAST CEREALS, COOKIES, AND BREADS OF SPAIN

A. Prosperini*, G. Meca, G. Font, Ruiz, M.J.

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

* Corresponding author Tel: +34963544958. Fax: +34963544954
E-mail: alessandra.prosperini@uv.es

ABSTRACT

Fusarotoxins enniatins (ENs) can represent a potential risk as natural contaminants of cereal commodities. However, only their bioaccessible fraction can exert a toxicity. The purpose of this study was to determine the ENs A, A_1, B, and B_1 bioaccessibility added in 1.5 and 3.0 μmol/g concentrations in breakfast cereals, cookies, and breads using a simulated in vitro gastrointestinal extraction model. Bioaccessibility values ranged between 40.4 ± 1.9 and 79.9 ± 2.8%. The lower values were 50.1, 40.4, 43.9, and 46.3% in wheat bran with fibers, and the higher values were 79.9, 64.2, 69.8, and 73.6% in white loaf bread for the ENs A, A_1, B, and B_1, respectively. Food composition, compounds structure, and presence of natural adsorbing materials can influence the ENs bioaccessibility. Application of a simulated *in vitro* gastrointestinal method is a good procedure to assess oral ENs bioaccessibility in cookies, breakfast cereals, and bread.

Keywords: enniatins, bioaccessibility, *in vitro* gastrointestinal model, HPLC

1. Introduction

The Food and Agriculture Organization (FAO) estimates that as much as 25% of the world's animal feedstuffs is contaminated by some extent by mycotoxins (FAO, 2004). Any step of the food production chain is susceptible to mold and mycotoxins contamination: before harvesting, between harvesting and drying, and during storage. Furthermore, they are persistent in the final products (Bullerman and Bianchini, 2007). The co-occurrence of mycotoxins in a food matrix is also common (Santini et al., 2012). The most common pathogen of maize, grain, and small grain in temperate regions of the world is *Fusarium* spp., whose strains may produce cyclic hexadepsipeptidic secondary metabolites such as enniatins (ENs). ENs are composed of three alternating D-α-hydroxyisovaleryl and three N-methyl-L-amino acid residues. They possess antimicrobial, insecticidal, phytotoxic, and cytotoxic properties (Jestoi, 2008) and inhibit cholesterol acyltransferase (Tomoda et al., 1992). The large array of biological activities can be related to their ionophoric properties based on the ability to incorporate into cell membranes forming cation-selective pores with high affinity for K^+, Mg^{2+}, Ca^{2+}, and Na^+ (Kamyar et al., 2004). Their occurrences have been amply demonstrated (Jestoi et al., 2004; Uhlig et al., 2006; Meca et al., 2010a, Oueslati et al., 2011) but the potential risk related to the ingestion of contaminated commodities is still not clear. The most important exposure routes for human and animals for ENs are via oral ingestion. It is therefore important to be able to assess the amount of ENs that is potentially available for absorption in the stomach and/or intestines, that is, bioaccessible, or to be excreted. However, the total amount of ENs ingested (intake) does

not always reflect the bioaccessible amount of them. The bioaccessibility describes the fraction of a contaminant, that is mobilized from food matrices during gastrointestinal digestion and theoretically subsequently available to intestinal absorption (Versanvoort et al., 2005). So, to study the oral bioaccessibility as part of an overall estimation in assessing the chemical risk coming from food-borne ENs is an important issue. Because of this, during the past decade, there has been an increasing interest in the use of *in vitro* methodologies, such as *in vitro* digestion models that simulate, in a simplified manner, the human digestion process in the mouth, stomach, and small intestine, to enable bioaccessibility investigations of contaminants from their food matrix during transit in the gastrointestinal tract (Versanvoort et al., 2005; Avantaggiato et al., 2004). These *in vitro* models attempt to recreate the aspects of human gastrointestinal physiology, such as chemical composition of digestive fluids, pH, and residence time periods typical for each compartment (Versanvoort et al., 2005). Moreover, these *in vitro* models are simple, rapid, low-cost, and without ethical implications, although a number of comparative studies have suggested that bioaccessibility results are largely dependent on the specific *in vitro* conditions used, including differences in solid solution ration, the method of mixing, the pH values of the gastric and intestinal juices and their compositions, food contaminants, and food matrices (Hur et al., 2011). Previous studies focused on determining the bioaccessible part of several mycotoxins after simulated human gastrointestinal extraction, confirming the usefulness of these *in vitro* methodologies to predict intestinal absorption of mycotoxins (Versanvoort et al., 2005; Avantaggiato et al.,

2003; Avantaggiato et al., 2004). Versanvoort et al. (2005) used a simplified digestion process of three steps, where physiologically based conditions of the mouth, stomach, and small intestine were applied.

The aims of the present study were (1) to apply an *in vitro* gastrointestinal model as it is related to the human digestive system; (2) to determine the bioaccessibility of ENs A, A_1, B, and B_1 from artificially spiked grain-based products (specifically breakfast cereals, cookies, and breads) by using the *in vitro* gastrointestinal model; and (3) to finally appreciate the role of oral bioaccessibility in assessing EN risks to human.

2. Matherials and methods

2.1 Materials and Reagents

Methanol, acetonitrile, and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT) ultrasonic bath. Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, and bile salts were obtained from Sigma-Aldrich (Madrid, Spain). The ENs (A, A_1, B, and B_1) utilized in this study were produced and purified in our laboratory following the method of Meca et al. (2009) for the production of the fusaproliferin. All ENs were >97% purity. They were stored at 4 °C in methanol, protected from light.

2.2 Samples

Fourteen samples of grain-based products were collected from Spanish food markets and stored at 4 °C until analysis. Some types of cookies, breads, and breakfast cereals, free from contamination, as determined previously, have been chosen for this study:

- Four types of cookies: cookie with chocolate, cookie with fibers, cookie with oat, and cookie without fibers.
- Five types of bread: integral loaf bread, integral loaf bread without sugar, milk bread, multicereals loaf bread, and white loaf bread.
- Five types of breakfast cereals: corn flakes, muesli with fruits, wheat bran with fibers, wheat with chocolate, and wheat with honey.

Samples (3 g) were spiked with EN A, A_1, B, and B_1 at final concentrations of 1.5 and 3.0 μmol/g and left in darkness overnight to allow methanol evaporation. Detailed types and the nutritional compositions of the above-mentioned samples are shown in Table 1.

Table 1. Nutrients nutritional properties of the analyzed samples referred to 100 g of product (as reported in nutritional labels).

Sample	Fiber (g)	Fat (g)	Carbohydrates (g)	Protein (g)
Cookie with chocolate	5.6	23.0	57.7	5.4
Cookie with fibers	3.0	11.5	68.2	9.0
Cookie with oats	4.3	19.2	60.3	7.6
Cookie without fibers	5.1	12.8	62.5	9.6
Corn flakes	3.0	0.7	74.5	8.0
Integral loaf bread	5.0	3.5	36.4	10.0
Integral loaf bread without sugars	5.0	3.0	37.3	11.0
Milk bread	2.0	15.0	22.7	10.0
Multicereals loaf bread	6.0	5.5	36.4	11.0
Muesli with fruits	6.4	16.3	57.8	8.1
Wheat bran with fibers	24.0	3.5	45.5	13.5
Wheat with chocolate	6.0	3.0	68.2	9.0
Wheat with honey	5.3	1.7	67.3	8.6
White loaf bread	3.0	3.0	41.8	9.0

2.3 *In Vitro* gastrointestinal model

An *in vitro* gastrointestinal procedure was adapted from the method developed previously by Gil-Izquierdo et al. (2002) with slight modifications. The method consists of three sequential steps: first an initial saliva addition to simulate the mouth compartment. The mouth is the point where the process of mechanical grinding of foodstuffs takes place at a pH of 6.5. The pH was adjusted with 0.1 N HCl. In this step, 3

g of sample spiked with ENs (A, A_1, B, and B_1) at 1.5 and 3.0 μmol/g was mixed with 6 mL of artificial saliva (composed of 89.6 g/L KCl, 20.0 g/L KSCN, 88.8 g/L NaH_2PO_4, 57.0 g/L $NaSO_4$, 175.3 g/L NaCl, 84.7 g/L $NaHCO_3$, 25.0 g/L urea, and 290.0 mg of α-amilase). The mixture was put in a plastic bag, containing 40 mL of water, and was homogenized by a Stomacher IUL Instruments (Barcelona, Spain) for 30 s. Larger components were broken down into smaller fragments, thereby increasing the surface area of food particles for swallowing and digestion. The second step consisted of pepsin/HCl digestion to simulate the hydrochloric acid environment of the stomach (pH 1−5). The presence of pepsine acts to breakdown protein, thereby aiding dissolution of the foodstuffs. For this purpose, 0.5 g of pepsin (14,800 U) prepared in 0.1 HCl was added to the mixture, and the pH was adjusted to 2.0. The mixture was incubated at 37 °C in an orbital shaker (Infors AG CH-4103, Bottmingen, Switzerland) at 250 rpm for 2 h. The last step was the digestion in the small intestine(s) by intestinal juices composed of enzymes (trypsin, pancreatin, and amylase), bile salts, and bicarbonate. The breakdown of food in the small intestine(s) means that the components are more amenable to absorption. The enzymes used in intestinal juice were pancreatin (8 mg/mL) and bile salts (50 mg/mL) in a 1:1 (v/v) solution ratio; the pH of intestinal juice was adjusted to 6.5 with 0.5 N $NaHCO_3$ (0.5 N); the mixture was incubated for 2 h at 37 °C in an orbital shaker (250 rpm). Immediately, aliquots of 30 mL of the mixture were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and 4 °C for 1 h, and the ENs present in the saliva/pepsin/HCl and pancreatin−bile digestions were extracted and analyzed by high-

performance liquid chromatography–diode array detection (HPLC-DAD), as described by Meca et al. (2010b).

2.4 Analysis of ENs

The extraction of ENs (A, A_1, B, and B_1) contained in gastroduodenal fluids were carried out as Meca et al. (2012a). Briefly, 5mL of each mixture obtained as previously described was put in a 20 mL test tube and extracted three times with 5 mL of ethyl acetate utilizing a vortex VWR international (Barcelona, Spain) for 1 min and centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and 4 °C for 10 min. After the organic phase was completely evaporated in a rotary evaporator (Buchi, Switzerland), the residues were dissolved in 1 mL of methanol and analyzed by LC-DAD. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μL) into the column.

LC-10AD pumps and a DAD detector Shimadzu (Japan) were used to perform HPLC analysis of ENs. LC separation was carried out on a Gemini (150 mm × 4.6 mm, 5 μm) analytical column Phenomenex (Madrid, Spain). The analytical separation was performed using gradient elution with water as mobile phase A and acetonitrile as mobile phase B. After an isocratic step of 70% B for 5 min, the gradient was linearly modified to 90% B in 10 min. After 1 min, the mobile phase was taken back to the starting conditions in 4 min. The flow rate was maintained at 1.0 mL/min. ENs were detected at 205 nm. EN identification was performed by comparing retention times and UV spectra of purified samples to pure standards. A further confirmation was performed by coinjecting pure standards together with each sample. Quantification of

ENs was carried out by comparing peak areas of investigated samples to the calibration curve of the standards. Recovery (%) studies in intestinal fluid were performed during routine analysis by spiking the samples with standard solutions of each ENs at 1.5 and 3.0 μmol/g concentrations.

2.5 Method validation

The analytical method was validated according to the European Directive 2002/26/EC for methods of analysis of mycotoxins in foodstuffs (EC, 2002). Recovery experiments were carried out on fortified intestinal fluid (free from contamination) (n = 5) by spiking ENs A, A_1, B, and B_1 at a level ranging from 0.3 to 50 μg/g. Mean recoveries (%) were as follows: 88.6 ± 2.4, 84.2 ± 4.3, 86.6 ± 2.7, and 89.5 ± 3.1% for EN A, A_1, B, and B_1, respectively. Interday variation values (through five different days) ranged from 1.8 to 3.1%. Intraday variation values were in the range of 6.4−10.1%. These values did not exceed 15%, which is the maximum variation for certification exercises for several mycotoxins. The detection limits (LOD) and the limit of quantification (LOQ) values were calculated according to s/n = 3 and s/n = 10, respectively. The LODs obtained for EN A, A_1, B, and B_1 were 215, 140, 145, and 165 μg/kg, respectively, whereas the LOQs were 600 μg/kg for EN A, 400 μg/kg for ENs A_1 and B, and 500 μg/kg for EN B_1.

2.6 Statistical Analysis

Statistical analysis of data was carried out using the PSAW Statistic 19.0 (SPSS, Chicago, IL) statistical software package. Data were expressed as means ± SDs of three independent experiments. The statistical analysis of the results was performed by Student's t test for

paired samples. Differences between mycotoxins were analyzed statistically with analysis of variance (ANOVA) followed by the Tukey's HDS posthoc test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

Table 2. Bioaccessibility (%) of the ENs A, A_1, B and B_1, spiked with 1.5 and 3.0 μM concentration in different breakfast cereals, cookies and breads digested by an *in vitro* simulated gastrointestinal model*.

Sample	Bioaccesibility (%)							
	EN A (μM)		EN A_1 (μM)		EN B (μM)		EN B_1 (μM)	
	1.5	3.0	1.5	3.0	1.5	3.0	1.5	3.0
Cookie with chocolate	59.2±2.7 ^{a,1}	55.3±2.7 ^{a,1}	47.8±2.9 ^{b,1}	44.6±2.4 ^{b,1}	52.0±2.9 ^{b,1}	48.5±2.5 ^{b,1}	54.7±2.6 ^{a,1}	51.1±2.5 ^{a,b,1}
Cookie with fibbers	64.4±3.1 ^{a,2}	66.6±2.2 ^{a,2}	52.0±2.1 ^{b,1}	51.9±2.8 ^{b,2}	56.5±2.8 ^{c,1,2}	56.4±2.9 ^{c,2}	59.5±2.8 ^{c,1}	59.4±2.7 ^{c,2}
Cookie with oats	55.2±3.5 ^{a,1,5}	52.2±2.6 ^{a,1}	44.6±3.3 ^{b,2}	46.8±3.1 ^{b,1,2}	48.4±3.1 ^{a,b,1}	51.1±2.2 ^{a,b,1,3}	51.0±3.3 ^{a,b,1}	53.8±3.3 ^{a,1,2}
Cookie without fibbers	72.0±3.3 ^{a,3}	70.7±2.5 ^{a,2}	58.2±3.1 ^{b,3}	57.1±2.4 ^{b,3}	63.2±2.9 ^{c,2}	62.1±2.6 ^{b,c,4}	66.6±3.1 ^{a,c,2}	65.4±3.1 ^{c,3}
Corn flakes	72.1±3.4 ^{a,3}	70.7±3.3 ^{a,2,3}	58.2±2.2 ^{b,3}	57.1±3.1 ^{b,3}	63.3±2.1 ^{c,2}	62.0±3.2 ^{b,c,4}	66.6±2.6 ^{a,c,2}	65.4±3.4 ^{c,3}
Integral loaf bread	72.2±2.3 ^{a,3}	71.6±2.9 ^{a,2,3}	58.3±2.2 ^{b,3}	57.5±3.4 ^{b,3}	63.4±3.2 ^{c,2}	62.5±2.1 ^{c,4}	66.7±3.5 ^{c,2}	65.8±3.4 ^{c,3}
Integral loaf bread without sugars	72.2±2.1 ^{a,3}	71.8±3.3 ^{a,2,3}	58.3±2.6 ^{b,3}	58.0±2.4 ^{b,3}	63.3±3.3 ^{c,2}	63.0±2.7 ^{c,4}	66.7±3.7 ^{c,2}	66.4±3.1 ^{c,3,4}
Milk bread	62.1±3.1 ^{a,2}	57.6±2.1 ^{b,1}	50.6±2.3 ^{c,1}	46.1±2.1 ^{d,1,2}	55.0±2.2 ^{b,1}	50.2±3.3 ^{c,d,1}	57.9±2.1 ^{b,1}	52.8±3.2 ^{b,c,d,1}
Multicereals loaf bread	75.0±2.8 ^{a,3,4}	70.0±3.1 ^{b,2}	60.3±2.7 ^{c,3}	56.9±2.1 ^{c,3}	65.1±3.5 ^{d,2}	61.9±2.4 ^{c,4}	69.1±2.8 ^{b,2,3}	65.2±2.7 ^{b,c,d,3}
Muesli with fruits	64.2±2.6 ^{a,2}	66.6±2.2 ^{a,2}	62.9±1.7 ^{a,3}	62.1±2.5 ^{a,4}	68.3±3.3 ^{a,2}	67.6±3.2 ^{a,4}	72.0±3.2 ^{a,2,3}	71.1±2.2 ^{a,3}
Wheat bran with fibbers	50.1±3.1 ^{a,5}	52.2±3.1 ^{a,1}	40.4±1.9 ^{b,2}	47.0±2.7 ^{a,1}	43.9±3.4 ^{a,b,3}	51.1±3.4 ^{a,1}	46.3±3.1 ^{a,4}	53.8±3.1 ^{a,1,2}

Table 2. Continued.

	Bioaccesibility (%)							
Sample	**EN A (μM)**		**EN A_1 (μM)**		**EN B (μM)**		**EN B_1 (μM)**	
	1.5	**3.0**	**1.5**	**3.0**	**1.5**	**3.0**	**1.5**	**3.0**
Wheat with chocolate	70.2±2.4 ^{a,3}	67.1±1.9 ^{a,2}	62.0±1.8 ^{b,3}	57.2±2.2 ^{c,3}	67.4±3.1 ^{a,2}	62.1±2.1 ^{b,4}	71.0±3.3 ^{a,2,3}	65.5±3.2 ^{a,b,3}
Wheat with honey	76.0±2.1 ^{a,3,4}	70.0±3.8 ^{b,2,3}	61.3±2.4 ^{c,3}	56.3±3.3 ^{d,3}	66.6±2.7 ^{b,2}	61.5±2.3 ^{c,4}	70.2±2.4 ^{b,2,3}	64.8±2.2 ^{b,3}
White loaf bread	79.9±2.8 ^{a,4}	76.9±3.4 ^{a,3}	64.2±2.4 ^{b,3}	58.1±2.2 ^{c,3}	69.8±2.9 ^{d,2}	64.1±3.1 ^{b,3,4}	73.6±2.2 ^{d,3}	67.5±3.3 ^{b,d,3,4}

* Values are expressed as mean ± SD (n=4). The significance of the differences between each ENs and each food samples was assayed by on-way ANOVA followed by Tukey test for multiple comparisons. ^{a,b,c,d} Within a line, values with different letters are significantly different ($p \leq 0.05$). ^{1,2,3,4,5} Within a column, values with different number are significantly different ($p \leq 0.05$). Control values (%) obtained from the experiments of the analytical method: EN A (88.6 ± 2.4%), EN A_1 (84.2 ± 4.3%), EN B (, 86.6 ± 2.7%) and EN B_1 (89.5 ± 3.1%). All EN extracted from food samples were statistically different ($p \leq 0.05$) from the respective control.

3. Results and Discussion

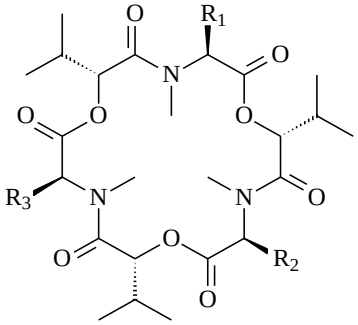
Results obtained after the application of the *in vitro* gastrointestinal method for determining ENs bioaccessibility in different breakfast cereals, cookies, and breads are shown in Table 2. Bioaccessibility values were from 50.1 ± 3.1 to 79.9 ± 2.8% for EN A, from 40.4 ± 1.9 to 64.2 ± 2.4% for EN A_1, from 43.9± 3.4 to 69.8 ± 2.9% for EN B, and from 46.3 ±3.1 to 73.6 ± 2.2% for EN B_1. As shown in Table 2, the bioaccessibility of ENs was statistically significant ($p \leq 0.05$) from their respective controls.

Concerning EN A, the mean bioaccessibility value added at 1.5 and 3.0 μmol/g concentrations in the commercial samples was similar, that is, 67.5 ± 2.7 and 65.7 ± 2.8%, respectively (Table 2). Regarding EN A_1 spiked at 1.5 and 3.0 μmol/g, the mean bioaccessibility obtained was 55.7 ± 2.2 and 54.1 ± 2.7%, respectively (Table 2). As can be observed in Table 2, the ENs of the B group, the mean bioaccessibility values obtained for the EN B were also comparable between 1.5 μmol/g of EN B added (60.4 ± 2.6%) and 3.0 μmol/g of EN B added (58.9 ± 2.9%). However, when samples were spiked with EN B_1 at 1.5 and 3.0 μmol/g, the mean bioaccessibility value that resulted was significantly different. These values were 67.3 ± 2.7 (1.5 μmol/g of EN B_1) and 62.0±2.5% (3.0 μmol/g of EN B_1). The order of mean values obtained when mycotoxins had been spiked at 1.5 μmol/g was as follows: 67.5 ± 2.7% (EN A) = 67.3 ± 2.7% (EN B_1) > 60.4 ± 2.6% (EN B) > 55.6 ± 2.2% (EN A_1). When mycotoxins had been spiked at 3.0 μmol/g, the order of mean values obtained was 65.6 ± 2.8% (EN A) = 62.0 ± 2.5% (EN B_1) = 58.9 ± 2.9% (EN B) > 54.0 ± 2.7% (EN A_1). The wheat bran with fibers was the type of sample that showed the minimum bioaccessibility values for all of the

ENs tested, ranging from 40.4 ± 1.9 for EN A_1 to 50.1 ± 3.1% for EN A (Table 2). In the same way, the wheat loaf bread showed high bioaccessibility values for all ENs tested (Table 2). For this type of sample, the higher values were observed for EN A.

Results show that not the total amount of mycotoxins ingested is available to intestinal absorption. The reduction in recoveries could be related to the digestion process and the use of different pH values that can reduce mycotoxins levels as stated for aflatoxin (Karaka and Nas, 2009). Moreover, according to Versanvoort et al. (2005) it is possible that bioaccessibility may be underestimated because of a compound saturation that can occur in the chyme, a situation not possible in vivo where a compound, when it is released from the food matrix, is transported across the intestinal epithelium into the body, keeping the compound concentration low in the chyme.

Considering the mean recoveries (%) of ENs tested (Table 2) and the recoveries (%) obtained from method validation (section 2.5), it is possible to observe that with the *in vitro*-simulated gastrointestinal extraction method, the initial bioactive EN concentrations present in the 14 sample analysis have been reduced statistically significant ($F_{3,92} = 13.98$, $p \leq 0.001$, Table 2) of 35% for EN A_1, followed by EN B_1 with a reduction of 25–31%, EN B of 31%, and EN A of 25% ($p \leq 0.05$) as compared to the extraction of ENs in the control. Differences in bioaccessibility (%) can be related to the structure of ENs. ENs are compounds with a cyclic aminoacidic structure, differentiated by the presence in the lateral chain of methyl, ethyl, propyl, and butyl groups (Figure 1). The presence of these groups can be responsible for the difference in the absorption of these compounds (Tedjiotsop Feudjio et al., 2010).



Enniatin	R1	R2	R3
A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
A_1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
B_1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Figure 1. Enniatins chemical structures.

It has been reported that mycotoxins bioaccessibility could depend on several factors, such as chemical structure, food composition, pH used in the in vitro digestion process, and food matrices (Kabak et al., 2009; Karaka and Nas, 2009). Differences in bioaccessibility (%) shown in Table 2 also could be explained by the food composition of samples. Previous studies had demonstrated that the bioaccessibility of nutrients, as well as toxic compounds, can be affected by the food matrix (Hornero-Ménedez et al., 2007; Vitali et al., 2008). In this case, the amount and type of fibers (Table 1) could be responsible as natural absorbing materials. In cereal-based foods, there are normally some natural compounds, such as dietary fibers, that could combine some bioactive compounds as mycotoxins or polyphenols, reducing their percent of bioaccessibility (Harris et al., 1998; Harrington

et al., 2001). In Table 1 is shown fiber, fat, carbohydrates, and protein compositions of each product. The sample white loaf bread, where higher bioaccessibility values were obtained (Table 2), is one of the lowest fiber contents (3 g/100 g of product), whereas wheat bran with fibers, where the lower bioaccessibility values were observed, is the highest fiber contents (24 g/100 g of product) sample. As can be observed, the bioaccesibility from wheat bran with fibers was statistically significantly ($F_{3,92} = 14.30$, $p \leq 0.001$, Table 2) for ENs A, A_1, B, and B_1 with respect to those in the white loaf bread. For wheat bran with fibers, the bioaccessibility of all ENs spiked at 1.5 µmol/g concentration is 1.6-fold higher than those obtained in white loaf bread. In the case of mycotoxins, the inclusion of dietary fibers has been demonstrated to protect against toxicoses resulting from numerous xenobiotic compounds and can be applied in food and feed as a cost-effective method to detoxify them from mycotoxins (Kabak et al., 2006; Aoudia et al., 2008).

Nevertheless, the bioaccessibility of mycotoxins can be affected by interactions with other food components. Mycotoxins can bind food matrix components, mainly proteins and lipids, and be released by *in vitro* digestion as previously determined for other mycotoxins (Dall'Asta et al., 2010). As can be observed in Table 2, for all ENs at both concentrations tested, low recovery values can be found in cookies with chocolate and cookies with oats that are both nutritionally rich in lipid components with the highest percentages of fat, with 23.0 g/100 g of sample and 19.2 g/100 g of sample, respectively. When comparing the sample with a lower proportion of fat (corn flakes) to the samples with the highest (cookies with chocolate or with oats), the bioaccessibility

for those samples with high fat content was statistically significantly ($F_{3,22}$ = 3.92, $p \leq 0.01$) for ENs A, A_1, B, and B_1 with respect to those with a low fat content. The bioaccessibility in corn flakes spiked at 1.5 μmol/g concentration of all ENs was 1.3-fold higher than those obtained in cookies with chocolate or with oats. It could be assumed that ENs interacting with the fat components of food are not released totally since the *in vitro* gastrointestinal digestion characteristics (such as the time period that food may spend in each step and the chemical composition of saliva, gastric juice, duodenal juice, and bile juice) are kept constant for all samples analyzed, even if the fat content is not the same (Hur et al., 2011). Moreover, considering their physical−chemical properties, it could be possible to hypothesize that ENs are retained in lipid/bile micelles (Xing et al., 2008).

The same ENs bioaccessibility was evaluated by Meca et al. (2012a) in artificially contaminated (1.5 and 3.0 μmol/g) wheat crispy bread after applying an *in vitro*-simulated gastric and duodenal digestion. The amount (%) of all mycotoxins tested contained ranged from 69.0 ± 2.1 to 91 ± 1.1% in gastric fluid and from 68.6 ± 2.9 to 87.3 ± 2.9% in duodenal fluid, considering both spiking concentrations. ENs bioaccessibility values obtained from our study were slightly lower to those obtained by Meca et al. (2012a). Moreover, in our study, only ENs recovered in gastrointestinal fluids obtained after simulated digestion have been taken into account since *in vivo* food digestion and absorption mainly take place in the small intestine (Oomen et al., 2002). However, as obtained in our study, EN A, spiked at 3.0 μmol/g, was the most bioaccessible mycotoxins as in the gastric fluid (91.0 ± 1.2%), as in duodenal fluid (87.3 ± 2.9%). EN A_1, considering both spiking

concentrations, was the lowest bioaccessible mycotoxins concerning the gastric fluid (69.0 ± 2.1 and 73.0 ± 2.3% for 1.5 and 3.0 μmol/g spiking, respectively), whereas concerning duodenal fluid, the lowest values were obtained for EN B spiked at 1.5 μmol/g (68.6 ±2.9%) and EN A_1 spiked at 3.0 μmol/g (70.0 ± 1.7%). Concerning other *Fusarium* toxins, it was shown that higher values of 92.6 ± 1.2 and 90 ± 1.3% of cyclic hexadepsipeptidic beauvericin (BEA) intake through artificially contaminated wheat (5 and 25 mg/L, respectively) were released from the food matrix to the bioaccessible fraction by the same *in vitro* digestion method as used in our study (Meca et al., 2012b).

The bioaccessibility of the trichothecenes deoxynivalenol (DON) was determined from dried pasta samples applying an in vitro digestion model both for adults and for children. The children's digestion model was basically the same as that of adults with slight modifications (the pH of the stomach was 3.0, the quantity of pepsin used for the gastric digestion was 0.02 g, and the amounts of pancreatin and bile salts were 0.0005 and 0.03 g, respectively). Referring to values obtained from our study, lower bioaccessibility values were obtained since the DON percentages in the gastric fluid ranged from 2.12 to 41.5%, while after the duodenal process, they ranged from 1.1 to 24.1% (Raiola et al., 2012). Avantaggiato et al. (2003; 2004) studied the intestinal absorption of zearalenone (ZEA), nivalenol (NIV), and DON using an *in vitro* gastrointestinal model that simulates the metabolic processes of the gastrointestinal tract of healthy pigs. This model avoided the use of animals simulating *in vivo* experiments by its multicompartimental dynamic computer-controlled system. Approximately 32% of ZEA intake through artificially contaminated wheat (4.1 mg/kg) was released from

the food matrix to the bioaccessible fraction during 6 h of digestion, and it was rapidly absorbed at the intestinal level. The intestinal absorptions recorded using the same model were 51 and 21% for DON and NIV ingested through spiked wheat samples, respectively. Later intestinal absorption of 105% for FB1, 89% for FB2, 87% for OTA, 74% for DON, 44% for AFB_1, and 25% for ZEA were determined by the same laboratory model (Avantaggiato et al., 2007).

Similarly, Versanvoort et al (2005) demostrated the bioaccessibility obtained for AFB_1 (94%) and OTA (100%) from peanuts and buckwheat. They evidenced that these mycotoxins were released from the food matrix during the simulated *in vitro* model toward the intestinal fluid (Versanvoort et al., 2005). However, these results were partly in contrast with Kabak et al. (2009) who studied the bioaccessibility by the *in vitro* digestion model of AFB_1 and OTA from different food products. They found similar bioaccessibility values for AFB_1 (90%), but different values for OTA (30%).

The results obtained from this study and in the literature concerning *in vitro* digestion methods are unlikely comparable. Differences in the types of *in vitro* methods, operating procedures, pH used, mycotoxins structures, and food compositions could contribute to differences in bioaccessibility. The last factor aforementioned can affect the bioaccessibility of ENs since they could build up complexes with food components (Baert et al., 2007; Zhou et al., 2008; Ortega et al., 2011). However, evidence of a strong correlation between *in vitro* bioaccessibility and *in vivo* bioavailability data has been observed previously for different mycotoxins (Versanvoort et al., 2005) although no *in vivo* data are still available about ENs bioaccessibility. Therefore,

for this purpose, more quantitative data are required. Further research should be performed to ensure that *in vitro* data are in agreement with *in vivo* methods for ENs present in food samples. In this way, bioaccessibility data obtained by the in vitro models proposed in this study can be incorporated to the whole data related to the cytotoxicity of mycotoxins in the literature and contribute to the risk assessment for *Fusarium* mycotoxins present in food and feed ENs A, A_1, B, and B_1.

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Notes

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DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

La citotoxicidad de las micotoxinas de *Fusarium* BEA, ENs A, A_1, B y B_1 se evaluó en las líneas celulares Caco-2 y HT-29, de origen humano, y en las células CHO-K1, de origen animal. Asimismo, ya que su toxicidad puede aumentar cuando se encuentran simultáneamente y su posible interacción puede llevar a efectos sinérgicos, aditivos o antagónicos, se evaluaron los efectos de las combinaciones sobre la viabilidad celular en células Caco-2 y a los datos obtenidos se aplicó un método matemático para evaluar el tipo de interacción. Considerando el efecto citotóxico obtenido tras la exposición de las líneas celulares a las micotoxinas de forma individual y combinada, se evaluó su biodisponibilidad a través del uso de las células Caco-2, ya que para ejercer su acción tóxica tienen que ser biodisponible. Con el objetivo de conocer los mecanismos de toxicidad de las micotoxinas se estudió el estrés oxidativo mediante la generación de especies reactivas del oxígeno (ROS) y la peroxidación lipídica (LPO) tras la exposición a las mismas de las células Caco-2.

Teniendo en cuenta el incremento de LPO y ROS producido por las micotoxinas en células Caco-2 tras 24 h de exposición, el siguiente objetivo fue determinar el papel de los sistemas de defensa celular en al detoxificación de las micotoxinas. Para ello se estudian el papel del glutatión (GSH) considerado el antioxidante celular más importante en los sistemas biológicos y el papel de antioxidantes ingeridos a través de la dieta. En este último caso, se ensayan 4 polifenoles (quercetina, quercetina-3-ß-d-glucósido, miricetina) y un estilbeno (*t*-pterostilbeno).

Confirmando el papel de las micotoxinas en el estrés oxidativo el siguiente objetivo que se planteó fue evaluar los efectos toxicológicos a

través de la evaluación de posibles alteraciones del ciclo celular, inducción de apoptosis y necrosis, alteración del potencial de membrana mitocondrial ($\Delta\Psi m$) y daño a nivel del DNA en células Caco-2. Por último, teniendo en cuenta que la mayor ruta de exposición a las micotoxinas es por ingestión de alimentos contaminados, se aplicó un método de digestión *in vitro*, el cual fue optimizado previamente por nuestro grupo de investigación, para evaluar la biodisponibilidad y bioaccesibilidad de las micotoxinas por métodos *in vitro*.

4.1 Citotoxicidad de las micotoxinas individuales

Las células HT-29, Caco-2 y CHO-K1 se exponen a diferentes concentraciones de micotoxinas durante 24, 48 y 72 h. los parámetros de citotoxicidad utilizados fueron el método de la sal de tetrazolio (MTT) basado en la determinación de la viabilidad celular a través de la alteración del funcionamento mitocondrial y el método del Rojo Neutro (NR) que se basa en la alteración lisosomal tras la exposición a sustancias toxicas.

Las células HT-29, Caco-2 y CHO-K1 se exponen a las micotoxinas durante 24 h. Los valores de inhibición del crecimiento celular en el punto medio (IC_{50}) obtenidos se encuentran en el rango de 1,3 ± 0,6 a 20,6 ± 6.9 µM. La toxicidad aumenta según el siguiente orden: EN A_1>EN A>EN B_1>EN B. Las micotoxinas se exponen durante 48 h a las células Caco-2 y HT-29. Los valores de IC_{50} se encuentran entre 6.8 ± 1.6 a >15 µM segun el siguiente orden de toxicidad: EN A>EN A_1>EN B_1>BEA>EN B. Las micotoxinas se exponen durante 72 h a las células Caco-2. Los valores de IC_{50} se encuentran en el rango de 1.3 ± 0.6 a 11.7 ± 2.4 µM. Los valores de IC_{50} aumentan en el orden: EN A_1>EN A>EN B_1>BEA>EN B.

Por el método de NR se detecta la citotoxicidad de las ENs y BEA en células Caco-2 tras 24, 48 y 72 h de exposición. El rango de IC_{50} obtenido se encuentra más bajo en comparación con el método del MTT, con valores de 0.46 ± 0.1 a >15 µM. Tras 24 h de exposición el orden de toxicidad aumenta según el siguiente orden: EN A>EN A_1>BEA>EN B_1>EN B. Tras 48 y 72 h de exposición los valores de IC_{50} aumentan según el orden: EN A=EN A_1>BEA>EN B_1=EN B y EN A=EN A_1>EN B_1>EN B>BEA, respectivamente.

La FUS no muestra valores de IC_{50} durante los tres tiempos de exposición y concentraciones ensayados (de 0,9 a 30 µM).

Los resultados muestran que las micotoxinas objeto de este estudio ejercen su acción citotóxica alterando principalmente la función mitocondrial, aunque también pueden afectar a los lisosomas. Las micotoxinas que presentan mayor toxicidad son las ENs A y A_1 en las células Caco-2 mientras que en las células CHO-K1 resultan ser más toxicas las ENs del grupo B (EN B_1 y EN B).

4.2 Efectos citotóxicos de las mezclas de micotoxinas

La presencia simultánea de varias micotoxinas en los alimentos es de gran importancia. Su combinación podría causar mayor riesgo para la salud humana respecto a la exposición de una sola micotoxina, ya que pueden producir efectos aditivos, antagonistas o sinérgicos. Por lo tanto, uno de los objetivos del estudio fue evaluar los efectos sobre la viabilidad celular después de la exposición a las micotoxinas en combinaciones binarias, terciarias y cuaternaria. Teniendo en cuenta los objetivos del estudio se selecciona el método MTT porque pone de manifiesto la acción sobre la función mitocondrial, considerada ésta

fundamental en las funciones básicas celulares. Se utilizan las células Caco-2 como modelo de epitelio intestinal y las cuatro ENs A, A_1, B y B_1, ya que estudios previos en nuestro grupo de investigación han demostrado su presencia simultánea en varios alimentos.

A los resultados obtenidos se aplica un método matemático, el método de las isobolas basado en el índice de combinación (CI) introducido por Chou (2006) y Chou y Talalay (1984), que se utilizó originalmente para estudiar las interacciones de fármacos en clínica. Actualmente, este método resulta ser adecuado para determinar el tipo de interacción entre mezclas de dos o más micotoxinas (Ruiz et al, 2011a,b).

Los resultados obtenidos muestran que las mezclas binarias reducen la viabilidad de las células Caco-2 de forma dosis dependiente de acuerdo con el siguiente orden: ENs A+A_1 (48%)=ENs A_1+B_1 (47%)>ENs A_1+B (35%)=ENs A+B (32%)>ENs B+B_1 (26%). Todas las mezclas terciarias y la cuaternaria reducen la viabilidad celular en un 40%. Aplicando el método de las isobolas se observa que cuando la fracción requerida para inhibir la viabilidad celular es baja, todas las combinaciones muestran un efecto antagónico, que sólo se mantiene a dosis más elevadas para la combinación ENs B+B_1. Por otra parte, se observa adición para todas las micotoxinas cuando la fracción afectada es media o alta, con excepción de las mezclas de ENs A_1+B, ENs A_1+B_1 y ENs A_1+A+B_1 que muestran sinergismo a las dosis más altas ensayadas. Estos resultados son de gran relevancia en cuanto en término de identificación de peligros y evaluación de la seguridad ya que se observa que a dosis bajas las ENs compiten por ejercer su efecto en el lugar de unión, pero a mayor concentración el efecto puede suponer un mayor riesgo ya que las ENs

interactúan para producir efectos sinérgicos son de mayor preocupación. Se podría especular que los efectos sinérgicos obtenidos de las tres mezclas dependen de una potenciación de los efectos citotóxicos debido a las estructuras lipófilas de las ENs que se encuentra en el orden EN A>EN A_1>EN B_1>EN B, lo que les permite ser fácilmente incorporados en las membranas celulares y ejercer su acción citotóxica (Tedjiotsop Feudjio et al., 2010). Además la EN A_1, presente en las tres mezclas, resultó ser la más citotóxica para las células Caco-2 y posee una buena actividad en la formación de poros, como la EN B_1 (Kamyar et al., 2004). Se podría suponer que las ENs del grupo B puedan inducir una "acción facilitadora" o "complementaria" en combinación con las ENs del grupo A, lo que significa que la acción secundaria de las ENs del grupo B aumenta la acción toxica de las ENs del grupo A, cuando los compuestos actúan en la misma diana en diferentes sitios, o en sitios solapados o en diferentes dianas de una misma vía de toxicidad (Jia et al., 2009). Estos resultados pueden contribuir al conocimiento del tipo de toxicidad de las ENs teniendo en cuenta el hecho de que las ENs no están todavía legisladas ni individualmente ni en combinación en los alimentos. El siguiente paso necesario es determinar los efectos en vivo con el fin de ayudar a definir y optimizar los límites máximos legales de ENS en los alimentos.

4.3 Mecanismo de estrés oxidativo: generación de especies reactivas del oxígeno y peroxidación lipídica

Teniendo en cuenta que las micotoxinas BEA, ENs y FUS son citotóxicas y biodisponibles por absorción intestinal, se plantea el siguiente objetivo: determinar si las micotoxinas son citotóxicas debido

a su capacidad de generar especies reactivas del oxígeno (ROS) y peroxidación lipídica (LPO). Los resultados de este estudio demuestran que la BEA y las ENs son capaces de inducir generación de ROS dosis y tiempo dependientes. La exposición a BEA y ENs A, A_1, B y B_1 desde 0 a 120 minutos produce un incremento significativo de ROS (medido por la sonda fluorescente H_2-DCFDA) que varía de 1,4 a 2,6 veces respecto al control. El incremento es mayor para EN A_1=EN A>EN B_1>BEA>EN B.

La exposición de las células Caco-2 a FUS no produce aumento de generación de ROS durante los 120 minutos ensayados. Únicamente se observa un incremento significativo de 2,2 veces respecto al control inmediatamente tras la exposición de la FUS a células Caco-2 (0 min).

Una de las consecuencias más estudiadas producidas por ROS es la LPO de los lípidos de membrana, cuyos productos son citotóxicos (Kouadio et al., 2007; Ferrer et al., 2009). Tras la exposición de las células Caco-2 a BEA, ENS y FUS durante 24 h, los resultados obtenidos muestran que la exposición a la BEA y ENs induce LPO. La LPO aumenta en células expuestas a estas micotoxinas desde 48% a 207% respecto a las células no expuestas a micotoxinas. La inducción de LPO sigue el orden: BEA>EN A>EN A_1=EN B_1>B. FUS no produce un aumento significativo de LPO respecto al control.

Los resultados de generación de ROS y LPO muestran que las micotoxinas ensayadas, excepto la FUS producen estado oxidativo de las células Caco-2, confirmando el mayor potencial citotóxico de estas micotoxinas en las células de mamífero.

4.4 Mecanismos de defensa frente al estrés oxidativo

4.4.1 Glutatión intracelular

Teniendo en cuenta del incremento de LPO y ROS producido por la BEA en células Caco-2 tras 24 h de exposición, el siguiente objetivo fue determinar los niveles intracelulares de glutatión (GSH) como mecanismo de defensa frente al estrés oxidativo causado por las micotoxinas.

El GSH participa en la destoxicación celular ya que puede eliminar los radicales libres, reducir los peróxidos o conjugarse con compuestos electrófilos. La reducción de GSH durante el estrés oxidativo tiene un papel significativo en la capacidad antioxidante celular y es un marcador indicativo de estrés oxidativo. Los resultados muestran que la BEA disminuye significativamente los niveles de GSH (31%) y aumenta los del GSSG (20%) respecto al control, confirmando el papel del GSH en la defensa celular frente a la BEA.

4.4.2 Antioxidantes de la dieta

Los polifenoles provenientes de la dieta pueden proteger a las células contra los agentes que inducen daño oxidativo (Lombardi et al., 2012). Ya que las células CHO-K1 se consideran uno de los modelos celulares más sensibles en estudios preliminares sobre las micotoxinas, en este estudio se plantea evaluar el efecto citoprotector que ejercen los polifenoles tras la exposición a las ENs a las células CHO-K1. Los resultados muestran un efecto citoprotector de los polifenoles cuyo grado depende de las ENs y de los polifenoles ensayados. Los cinco polifenoles ensayados (quercetina, quercetina-3-ß-d-glucósido, rutina,

miricetina y *t*-pterostilbeno) protegen a las células CHO-K1 cuando se exponen a la EN A_1. La quercetina, quercetina-3-ß-d-glucósido, miricetina y *t*-pterostilbeno protegen a las células de la EN B_1. Mientras que la quercetina y quercetina-3-ß-d-glucósido protegen a las células de la EN A; la rutina y miricetina de la EN B. El efecto citoprotector expresado como aumento en porcentaje de reducción de mortalidad celular tras exposición de las células a las ENs junto con los polifenoles de forma individual, se encuentra en un rango de 25 a 116% para el *t*-pterostilbeno frente a las EN A_1 y B_1; entre 16 a 92% para la miricetina frente a las ENs A_1, B y B_1; entre 17 a 83% para la rutina frente a las ENs A_1 y B; entre 12 a 76% para la quercetina-3-ß-d-glucósido junto a las ENs A, A_1 y B_1; y entre 24 a 43% para la quercetina junto a las ENs A_1 y B_1. Los antioxidantes de la dieta y su papel citoprotector en la prevención de la toxicidad de las micotoxinas presentan una atención creciente en los últimos años para evaluar el potencial beneficioso de estos compuestos en la dieta. Puesto que las micotoxinas son contaminantes comunes de los alimentos y los piensos, todas las sustancias que son capaces de reducir los efectos tóxicos de las micotoxinas son de gran interés. Por lo tanto, el uso de los antioxidantes podría ser una estrategia efectiva para la protección frente a la acción de las micotoxinas y para reducir la toxicidad y así mejorar la seguridad de los alimentos.

4.5 Ciclo celular

De los resultados obtenidos se deduce que las ENs A, A_1, B_1 y BEA, tras 24 h de exposición producen una parada en la fase mitótica G2/M, con un aumento de la población celular que se encuentra en el

rango de 23 a 35% respecto al control (19%). El porcentaje aumenta según el orden: EN B_1>EN A_1>BEA>EN A. Una reducción de la fase G0/G1 concomitante con un aumento de la población en G2/M se observa para las ENs A_1 y B_1 y BEA. El porcentaje disminuye según el orden BEA>EN B_1>EN A_1 en un rango de 32 a 38% respecto al control (45%). Los parámetros del ciclo celular no se ven afectados tras exposición a la EN B. Tras 48 h de exposición las ENs A_1 y B_1 producen un aumento de la fase hipoploide (o debris; 15% frente al 5% del control) que indica la presencia de células apoptoticas. Se mantiene la reducción de las células en la fase G0/G1 en un mismo que a las 24 h, siendo el orden EN A>EN A_1>EN B_1>BEA. Un aumento en la fase G2/M se observa para la BEA (21% respecto al control 17%). Tras 72 h de exposición se observa una reducción de la fase G0/G1 que va desde 26 a 36% respecto al control (44%) en un orden de: EN B_1>EN A=BEA>EN A_1. Un aumento del porcentaje celular de la fase SubG0/G1 (36%) respecto al control (8%) en el orden: EN B_1>EN A_1>EN B=EN A y un aumento de la fase G2/M en el caso de las ENs A_1 y B y BEA en un rango de 28 a 33% respecto al control (22%) y en el orden de: BEA>EN A_1>EN B. Además, la BEA y las ENs A y B_1 producen un aumento de la fase S en un rango de 19 a 23% respecto al control (15%) en el orden de: EN B_1>BEA>EN A. La BEA muestra una disminución en la fase G0/G1 del ciclo celular, acompañada de un aumento de células en la fase G2/M a los tres tiempos de exposición. De acuerdo con Noda et al. (2001), este tipo de parada podría ser una consecuencia del desequilibrio redox a nivel celular inducido por la BEA en células Caco-2, según cuanto determinado por LPO y GSH. Por otra parte, de acuerdo con (Abid Essefi et al., 2003) la parada en las fase G2/M y S pueden ser debidas a un daño a nivel del ADN, lo que se

observa tras 24 h de exposicion a la ENs A, A_1 y B_1 y tras 72 h de exposicion a todas las micotoxinas ensayadas.

4.6 Inducción de apoptosis y necrosis

La parada del ciclo celular en la fase G2/M se ha descrito como posible consecuencia de un estímulo externo que induce apoptosis y como un proceso de adaptación que retarda el ciclo celular frente a un daño a nivel del DNA (Abid-Essefi et al., 2003; Ivanova et al., 2012). Tras 24 h de exposición a las ENs A, A_1 y BEA se observa un aumento del porcentaje (de 25 a 34%) de células en apoptosis temprana respecto al control (12%) según el orden: BEA=EN A_1>EN A. Para estas micotoxinas se observa también un incremento del porcentaje de células apoptoticas/necroticas (de 25 a 31%) respecto al control (12%) en el orden: BEA=EN A_1=EN A. La inducción simultanea de apoptosis y necrosis responde a que un mismo estímulo puede producir, en una misma célula, los 2 tipos de muerte celular. Tras 48 h de exposición, las micotoxinas inducen células en apoptosis temprana en porcentajes que van desde 13 a 26% respecto al control (7%) en el orden siguiente: BEA=EN B_1>EN A_1>EN A>EN B. Las ENs A, A_1 y B_1 inducen un incremento de la población de células necróticas (22% respecto al control=14%). Tras 72 h de exposición sólo se observa necrosis para todas las micotoxinas ensayadas (de 16 a 27%) en el siguiente orden: EN B>EN B_1>EN A_1=EN A=BEA. Tras 24 h de exposición únicamente BEA y las ENs A y A_1 causan apoptosis en las células Caco-2. El incremento de células apoptoticas para las micotoxinas alcanza el 180% frente al control. Este efecto se incrementa en 1,8 veces a las 48 h implicando a todas las micotoxinas ensayadas. Respecto a la necrosis, las ENs A y A_1 muestran

necrosis a todos los tiempos ensayados (incremento aproximadamente del 160% frente al control), mientras que el resto de micotoxinas causan necrosis al mayor tiempo de exposición. Sin embargo, a diferencia de la apoptosis, el porcentaje de células necroticas aumenta con el tiempo de exposición. Tras 72 h de exposición no se observa apotosis si no sólo necrosis debido al largo tiempo de exposición de las células a las micotoxinas. La muerte celular por apoptosis y necrosis se relaciona a un imbalance el el estado redox celular (Gotoh et al., 2002), lo que se observa tras 24 h de exposicion a la BEA. Por otra parte la muerte celular por apoptosis y necrosis se relaciona a un dano a nivel del ADN, lo que se observa por las ENs A y A_1. En el caso de la EN B_1 se observa induccion de LPO y dano a nivel del ADN, aunque no se pudo establecer una directa relacion con la induccion de apoptosis y necrosis en celulas Caco-2.

4.7 Detección del potencial de membrana mitocondrial

Teniendo en cuenta que la mitocondria tiene un papel importante en el proceso de apoptosis y necrosis, ya que libera factores implicados en la inducción de apoptosis en las células, en este estudio se planteó el objetivo de evaluar el $\Delta\Psi m$ tras la exposición a la BEA y ENs en las células Caco-2. Tras 24 h de exposición, la alteración del $\Delta\Psi m$ es inducida por las ENs A y A_1 y por la BEA en el rango de 19 a 29%, aunque la EN A resulta alterar el potencial de membrana únicamente a la mayor concentración (3,0 µM). Tras 48 h de exposición, todas las micotoxinas reducen el porcentaje (11 a 30%) de células positivas con alteración del $\Delta\Psi m$ a todas las concentraciones ensayadas, excepto la EN B que solo induce alteración del $\Delta\Psi m$ a 3,0 µM. Tras 72 h de

exposición, el potencial de membrana mitocondrial fue reducido drásticamente (91 a 98%). Todas las micotoxinas ensayadas reducen el potencial de membrana mitocondrial. Esta reducción aumenta al aumentar de la dosis y el tiempo de exposición. El incremento en la alteración de la membrana mitocondrial fue del 30% a las 24 h hasta 98% a las 72 h. Lo resultados mostran que la BEA y las ENs ensayadas producen un daño a nivel mitocondrial que esta implicado en la inducción de apoptosis y necrosis en células Caco-2, de acuerdo con Juan-García et al. (2013).

4.8 Determinación del daño a nivel del DNA por el ensayo Cometa

La parada de las fases G2/M y S del ciclo celular se correlaciona con daños a nivel del DNA, el cual, si no se repara por mecanismo celulares, pueden inducir apoptosis. En este estudio se evalúa un posible daño producido por las ENs y BEA a nivel del DNA como las roturas de la cadena simple o doble del DNA, sitios álcali-lábiles y rotura de cadena simple asociada a la reparación incompleta por escisión, por el ensayo Cometa. Con este ensayo se analiza el incremento de DNA en la cola conocido como momento de la cola (TM). Este parámetro se considera útil para describir la heterogeneidad de la respuesta dentro de la población celular, ya que su cálculo (producto del porcentaje de DNA en la cola y longitud de la cola) considera las variaciones de distribución del DNA dentro de la cola. Tras 24 h de exposición se observa un incremento del momento de la cola significativo para la EN A, la EN A_1 (3,0 µM) y la BEA (12.0 µM), con un porcentaje de DNA en la cola que aumenta de 164 hasta 196% respecto al control. La inducción del daño al ADN se relaciona a la parada en la

fase G2/M y S, lo que se observa para las ENs A y A_1, tras 24 h de exposicion y para todas las micotoxinas tras 72 h de exposición. Por otra parte la parada en la fase G2/M producida por la BEA tras 24 h de exposición se relaciona al imbalance en el estado redox de las células producido por ella.

4.9 Transporte y biodisponibilidad de beauvericina y fusaproliferina

Considerando el efecto citotóxico obtenido tras la exposición de las micotoxinas de forma individual y combinada, se plantea el objetivo de evaluar la biodisponibilidad de las micotoxinas y que el epitelio intestinal es la primera barrera para la absorción y metabolismo de xenobióticos, tales como las micotoxinas, después de la administración oral se utilizan células Caco-2 para estudiare este parámetro. La línea celular Caco-2, las cuales una vez diferenciadas se aceptan como sistema *in vitro* de absorción intestinal humano (Artursson y Karlsson, 1991). La biodisponibilidad se refiere a la proporción de un nutriente ingerido que se absorbe y se utiliza para la función fisiológica normal y/o almacenamiento (Versanvoort et al., 2005).

En este estudio se evalúa la biodisponibilidad de BEA y FUS, ya que la biodisponibilidad de las ENs ya se ha determinado en células Caco-2 previamente por nuestro grupo de investigación.

Tanto la BEA como la FUS se transportan a través del modelo *in vitro* de absorción con células Caco-2. Los porcentajes de transporte desde la capa apical a la basolateral en el modelo *in vitro* fueron mayores para la FUS (83.2 ± 0.8%) que para la BEA (54.3 ± 1.1%). Sin embargo la FUS no se absorbe por las células Caco-2 diferentemente de la BEA, lo que demuestra el menor paso de esta a través de la

membrana transepitelial y su retención en el interior de las células Caco-2, cuyo porcentaje varía de 15 a 33% dependiendo de la concentración de BEA. Las diferencias obtenidas entre la FUS y la BEA puede deberse a la diferencia en la estructura química de estas micotoxinas, particularmente del grupo aminoácidos de la BEA (ausente en la FUS) que facilita la interacción y paso a través de la membrana celular. Otro factor que puede influir en el menor porcentaje de transporte de BEA es el peso molecular, ya que la BEA presenta un peso molecular que duplica lo de la FUS.

Aunque no podemos simplemente extrapolar los resultados obtenidos en este en estudio in vitro a la situación *in vivo* de una exposición aguda/crónica de los seres humanos a las micotoxinas, este estudio puede proporcionar información sobre la proporción de micotoxinas que pueden estar disponibles a nivel intestinal y alcanzar los órganos diana donde realizar su función toxica.

4.10. Estudio de bioaccesibilidad de las micotoxinas

Teniendo en cuenta que la mayor ruta de exposición a las micotoxinas es por ingestión de alimentos contaminados, el último objetivo del trabajo fue aplicar un método de digestión *in vitro*, el cual fue optimizado previamente por nuestro grupo de investigación, para evaluar la bioaccesibilidad de las ENs.

El término de bioaccesibilidad describe la fracción de las ENs, que se libera de la matriz alimentaria, tras el proceso de digestión gastrointestinal y teóricamente disponible para la absorción inestinal (Versanvoort et al., 2005). La optimización del método de digestión *in vitro* muestra que las ENs son bioaccesibles en un rango que varia entre

54,1 ± 2,7 y 67,5 ± 2,7%, y en el siguiente orden: EN A=EN B_1>EN B>EN A_1, considerando las dos concentraciones ensayadas (1,5 y 3,0 µmol/g). La aplicación del método de digestión *in vitro* permite determinar la bioaccesibilidad de las ENs desde diferentes productos comerciales a base de cereales. En total se utilizaron 14 productos que incluyen cuatro tipos de galletas: galletas con chocolate, galletas con fibra, galletas con avena y galletas sin fibras; cinco tipos de pan: pan de molde integral, pan integral sin azúcar, pan de leche, pan multicereales y pan blanco; cinco tipos de cereales para el desayuno: copos de maíz, muesli con frutas, salvado de trigo con las fibras, trigo con chocolate y trigo con miel. Los valores medios obtenidos de bioaccesibilidad, en las 14 muestras ensayadas, demuestran una reducción en la bioaccesibilidad de las ENs que fue del 35% para la EN A_1, del 25-31% para la EN B_1, del 31% para la EN B y del 25% para la EN A respecto al control. Los datos muestran que el contenido de las ENs en el alimento, tras la digestión intestinal se reduce, lo cual es de gran interés en el momento de evaluar la fracción disponible en la absorción por parte del hombre y/o animal. Sin embargo, hay que considerar las diferencias en los tipos de métodos *in vitro*, en los procedimientos, pH utilizados, estructura de las micotoxinas y la composición de los alimentos ya que todo ello podría contribuir a variar los valores de bioaccesibilidad obtenidos. Aunque, los datos obtenidos por los modelos *in vitro* de bioaccesibilidad pueden ser incorporados a los datos enteros relacionados con la citotoxicidad de micotoxinas en la literatura y contribuir a su evaluación del riesgo.

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CONCLUSIONES

CONCLUSIONS

1) BEA and ENs A, A_1, B and B_1 exerted cytotoxic effects in a dose and time dependent manner in Caco-2, HT-29 and CHO-K1 cells. CHO-K1 cells were more sensitive to ENs of B group, whereas Caco-2 cells were more sensitive to ENs of A group. the order of toxicity was EN B_1>EN B=EN A_1>EN A and EN A_1>EN A>EN B_1>BEA>EN B, respectively. FUS did not show cytotoxic effects in both Caco-2 and HT-29 cell lines at any concentration tested.

2) The cytotoxic effect of EN A_1 in the following combinations: ENs A_1+B, ENs A_1+B_1 and ENs A+A_1+B_1 in a dose and time dependent manner in Caco-2 cells. Antagonsim was observed in Ens B+B_1 combination at low concentrations tested.

3) Oxidative stress was observed after exposure to BEA and ENs in Caco-2 cells in the following increasing order: EN A1=EN A>EN B_1>BEA>EN B.

4) LPO was produced as a consequence of oxidative damage induced by BEA and ENs in the order: BEA>EN A>EN A1=EN B1>EN B. However no oxidative damage was induced in Caco-2 cells by FUS.

5) BEA reduced GSH levels and increased GSSG levels in Caco-2 cells, confirming the involvement of GSH in BEA detoxification.

6) Polyphenols demonstrated cytoprotective effects in CHO-K1 against ENs cytotoxicity in the increasing order: *t*-pterostilbene > myricetin > rutin > quercetin 3-ß-glucoside > quercetin.

7) Sub-toxic concentration of BEA and ENs (1.5 and 3.0 μM) arrested the cell cycle in Caco-2 cells predominantly in G2/M phase. The G2/M phase arrest is related to oxidative imbalance induced after ROS production by BEA and to DNA damage induced by EN A and EN A_1.

8) Apoptosis and necrosis cell death was observed after BEA and ENs exposure in Caco-2 cells.

9) The mitochondrial membrane potential was altered after BEA and ENs exposure, contributing to Caco-2 cell death.

10) High bioavailability percentages of BEA and FUS were determined through the Caco-2 cells monolayer. FUS was more bioavailable than BEA.

11) ENs bioaccessibility was reduced after applying the *in vitro* digestion method. The bioaccessibility was in the following order: EN A=EN B_1>EN B>EN A_1.

12) The *in vitro* data obtained in this study can be incorporated to the whole data related to the cytotoxicity of BEA, ENs and FUs in literature and together with *in vivo* studies could contribute to the future risk assessment for these minor *Fusarium* mycotoxins.

Anexos

Anexo I - Metodologia

1. Reactivos

Todos los reactivos y componentes de los cultivos celulares utilizados: medios Dulbecco's modified Eagle's (DMEM; 4,5 g/L) y Ham-F12, penicilina, estreptomicina, HEPES, bromuro de 3[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio (MTT), rojo neutro (NR), tampón glicina de Sorensen, tampón fosfato salino (PBS), aminoácidos no esenciales (NEAA), Trizma base, Triton X-100, glucosa, dimetilsulfóxido (DMSO), ácido tiobarbitúrico (TBA), sal de mesilato de deferoxamina (DFA), di-ter-butil-metilfenol (BHT), 1,1,3,3 tetrametoxipropano, 2',7'-diclorodihidrofluoresceína diacetato (H_2-DCFDA), yoduro de propidio (PI), RNasa, BEA (783,95 g/mol), Na-EDTA, agarosa, agarosa de baja temperatura de fusión (LMA), benzo (α) pireno (B(α)P), solución salina de Hank (HBSS), quercetina (pureza ≥ 98%, peso molecular: 302,26 g/mol), quercetina-3-β-D-glucosido (pureza ≥ 90%, peso molecular: 464,38 g/mol), rutina (pureza ≥ 99%, peso molecular: 610,56 g/mol) y miricetina (pureza ≥ 96%, peso molecular: 318,24 g/mol) se obtuvieron de Sigma Chemical Co (St Louis, MO, EE.UU.). *T*-pterostilbeno (pureza ≥ 90%, peso molecular: 256,30 g/mol) se obtuvo en nuestro labboratorio según la reacción standard de Witting y Hens (http://www.orgsyn.org). Suero fetal bovino (FCS) se obtuvo de Cambrex Company (Belgium). Agua desionizada (resistividad <18 mΩ cm) se obtuvo del sistema de agua ultrapura Milli-Q (Millipore, Bedford, MA, EE.UU.). NaOH se obtuvo de Guinama (España). NaCl y etanol fueron de Merck KGaA (Alemania). $CaCl_2$ y formaldehído de Scharlau Chemie SA (Barcelona, España). Metanol y ácido acético glacial se obtuvieron de VWR International (LLC, Pennsylvania, EE.UU.). Anexina V-FITC coniugada humana recombinante, éster metílico de tetrametil rodamina (TMRM), To-Pro-

3® yoduro y el protonóforo carbonilcianuro p-trifluorometoxifenilhidrazona (FCCP) de Invitrogen, (EE.UU.). Acetonitrilo y acetato de etilo se adquirieron de Fisher Scientific (Madrid, España). Urea, α-amilasa, KCl, KSCN, NaH_2PO_4, Na_2SO_4, $NaHCO_3$, HCl, pepsina, pancreatina, y sales biliares se obtuvieron de Sigma-Aldrich (Madrid, Spain). Los disolventes cromatográficos y el agua se desgasificaron durante 20 minutos utilizando el baño ultrasónico 5200 Branson (Branson Ultrasonic Corp., CT, EE.UU.). EN A (pureza: ≥ 97%, peso molecular: 681,9 g/mol), EN A1 (pureza: ≥ 97%, peso molecular: 667,9 g/mol), EN B (pureza: ≥ 90%, peso molecular: 639,4 g/mol) y EN B1 (pureza: ≥ 97%, peso molecular: 654,9 g/mol) se obtuvieron de Enzo Life Sciences, Suiza.

FUS fue producida y purificada en el laboratorio de acuerdo con el método desarrollado por Meca y col. (2009). Las soluciones madre de BEA y ENs A, A_1, B and B_1 se prepararon en metanol y se mantuvieron a -20 °C. Las concentraciones finales ensayadas se obtienen en medio de cultivo. La concentración final de metanol en la disolución de trabajo de BEA, FUS y ENs fue de 1% (v/v). El contenido total de proteína (mg/ml) se determinó mediante el método de Bradford (Bio-Rad ensayo de proteínas DC (número de catálogo 500-0116) http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf).

2. Cultivos celulares

Las células Caco-2 (ATCC HTB-37) derivadas de adenocarcinoma de colon humano, se mantuvieron en medio DMEM con alto contendido de glucosa (4.5 g/L) a pH 7.4, suplementado con piruvato (1mM), tampón HEPES 25 mM, 1% (v/v) de NEAA, 100 U de penicilina, 100

mg/ml de estreptomicina y 10% (v/v) de FCS inactivado. El mantenimiento de las células se llevó a cabo en frascos de 75 cm^2 a los que se adicionó 10 mL de medio. El medio de cultivo se cambió cada dos días.

Las células CHO-K1 derivadas de ovario de hámster chino, se cultivaron en monocapa con Ham-F12 suplementado con 25 mM de tampón HEPES (pH 7.4), 10% de FCS inactivado, 100 U/ml de penicilina, 100 mg/ml de estreptomicina, y 2.5 g/ml de anfotericina B. El mantenimiento de las células se llevó a cabo en placas de cultivo de 75 cm^2 a los que se adicionó 5 mL de medio.

Las células HT-29 (ATCC HTB-38) epiteliales de adenocarcinoma colorrectal humano se cultivaron en monocapa en placas de cultivo de poliestireno de 9 cm^2 de tejidos con DMEM suplementado con 25 mM HEPES, 1% NEAA, 100 U/ml de penicilina, 100 mg/ml de estreptomicina, 2.5 mg/ml de anfotericina B, y 10% FCS inactivado.

Todas las células se incubaron a 37 °C, en atmósfera con un 95% de humedad relativa y con un flujo de CO_2 del 5%.

La ausencia de micoplasma se comprobó rutinariamente usando el kit de tinción de Mycoplasma (Sigma-Aldrich, St Louis Mo. EE.UU.).

3. Citotoxicidad *in vitro*

Los ensayos de MTT y NR se realizaron como se describe por Ruiz y col. (2006) con algunas modificaciones. Las células se sembraron en placas de 96 pocillos mediante la adición de 200 µL/pocillo de una suspensión celular de 3 x 10^4 células/pocillo para las Caco-2 y HT-29 y de 2 x 10^4 células/pocillo. Cuando las células alcanzaron su confluencia, el medio de cultivo se reemplazó con medio fresco conteniendo las

diluciones seriadas de BEA y ENs, que se encontraban en el rango de 3,125 a 25 μM para la BEA, de 0,9 a 15 μM para las ENs A_1, B y B_1 y desde 0,45 a 7,5 μM para la EN A, de 0,9 a 30 μM para la FUS y medio fresco contenteniendo diluciones seriadas de cada polifenol (de 3,13 a 100 μM) y medio fresco conteneindo simultáneamente el polifenol (a una concentración de 5, 25 y 50 μM) y cada EN (1.5 y 3.0 μM).

Para el ensayo de NR, tras 24, 48 y 72 h de incubación de las células con diferentes concentraciones de micotoxinas (factor de dilución = 2), el medio con las micotoxinas se desechó y se añadieron 200 μL de solución NR (50 mg/ml; pre-incubado a 37 °C durante 24 h) a cada pocillo y tras 3 h de incubación a 37 °C, las placas se lavaron con PBS, las células se fijaron con solución de formaldehído-$CaCl_2$, y se extrajo el NR mediante la adición de una solución de ácido acético/etanol (1:50).

Las placas se agitaron suavemente durante 5 min para lograr la disolución completa del colorante antes de medir la absorbancia medida a 540 nm con un lector automático de placas ELISA Multiscan EX (Thermo Scientific, MA, EE.UU.). La viabilidad celular se expresó en porcentaje respecto al control celular con 1% de MeOH. Las dosis medias de inhibición del crecimiento celular (IC_{50}) se calcularon a partir de la curva dosis-respuesta.

Para el ensayo del MTT, tras 24, 48 y 72 h de exposición con las concentraciones seriadas de micotoxinas, el medio con las micotoxinas se desechó y se añadió a cada pocillo 200 μL de medio fresco con 50 μL de MTT. Después de 4 h de incubación (37 °C y en la oscuridad), el formazano resultante se solubilizó con DMSO. La absorbancia se midió a 570 nm utilizando un lector de placas de ELISA Multiscan Ex automática

(Thermo Scientific, MA, EE.UU.). La viabilidad celular se expresó en porcentaje en relación al control (1% de MeOH). Los valores de IC_{50} se calcularon a partir de curvas de dosis-respuesta.

4. Diseño experimental y evaluación de los efectos de las combinaciones de micotoxinas

Se sembraron 3 x 10^4 células/pocillo en placas de cultivo de 96 pocillos. Cuando las células alcanzaron la confluencia, se adicionaron cinco diluciones (factor de dilución en serie = 2) de EN A, A_1, B y B_1 y sus combinaciones de dos, tres y cuatro micotoxinas y se expusieron durante 24 h. Las concentraciones fueron de 0,3 a 5 µM con una relación 1:1. Las concentraciones de las mezclas terciarias fueron de 0,3 a 2,5 µM con una relación 1:1:1 y las concentraciones para la mezcla cuaternaria fueron de 0,3 a 2,5 µM con una relación 1:1:1:1. El ensayo de MTT se llevó a cabo como el descrito anterioremnte según Ruiz y col. (2006).

Los resultados se analizaron usando la ecuación de efecto-medio/índice combinado (CI)-isobolograma descrito por Chou (2006) y Chou y Talalay (1984) que se basa en el principio de ley de acción de masas que demuestra que hay una relación unívoca entre la dosis y el efecto que produce, independientemente del número de sustratos o de productos y del mecanismo de acción o de inhibición. Este método realiza las curvas dosis-efecto para cada compuesto y sus combinaciones en múltiples concentraciones diluidas usando la ecuación de efecto medio:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m$$

Donde *D* es la concentración del producto, D_m es la dosis media efectiva (ej., IC_{50}, EC_{50}, o LD_{50}) que inhibe el 50% de del crecimiento celular, *fa* es la fracción afectada por la concentración *D* (ej., porcentaje de inhibición/100), *fu* es la fracción no afectada (por lo tanto, *fa* = 1 - *fu*), y m es el coeficiente que informa del tipo de relación dosis-efecto que tiene lugar. Así cuando *m* = 1, *m* > 1, y *m* < 1 indica que la curva dosis-efecto es hiperbólica, sigmoidal y negativo sigmoidal, respectivamente (Chou and Talalay, 1984). Por lo tanto el método tiene en cuenta los parámetros de potencia (D_m) y de pendiente (*m*). La ecuación de efecto medio para compuestos individuales se puede modificar y aplicar a múltiples micotoxinas. Y la ecuación se convierte en:

$$[(fa)_{1,2}/(fu)_{1,2}]^{1/m} = D_1/(D_m)_1 + D_2/(D_m)_2 + (D)_1(D)_2/(D_m)_1(D_m)_2$$

Cuando dos componentes se combinan y se realizan varias diluciones, la mezcla de los dos compuestos se comporta como un tercer compuesto en la relación dosis efecto. Por lo tanto, y= log $[(fa)_{1,2}/(fu)_{1,2}]$ frente a x = log $[(D)_1+(D)_2]$ da valores de $m_{1,2}$, $(D_m)_{1,2}$, y $r_{1,2}$.

Chou y Talalay (1984) introdujeron el termino (CI)x para cuantificar el sinergismo, adición o antagonismo de dos componentes:

$$CI=D_1/(D_x)_1 + D_2/(D_x)_2 = (D)_1/(D_m)_1[fa/(1-fa)]^{1/m1} + (D)_2/(D_m)_2[fa/(1-fa)]^{1/m2}$$

Donde en el denominador (D_x) 1 se refiere a D_1 analizado individualmente y que inhibe un x% del sistema, y $(Dx)_2$ a D_2 analizado individualmente y que inhibe un x% del sistema. Y la ecuación general

para la combinación de *n*-compuestos con una inhibición de *x*% se convierte en:

$$_{k}^{n}(CI)_{x}=\sum_{j=1}^{n}\frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n}\frac{(D_x)_{1-n}\{[D]_j/\sum_{1}^{n}[D]\}}{(D_m)_j\{(fax)_j/[1-(fax)_j]\}^{1/mj}}$$

Donde (CI) es el índice de combinación para *n*-compuestos (ej., micotoxinas) a *x*% de inhibición (ej., proliferación inhibición); $(D_x)_{1\text{-}n}$ es la suma de los *n*-compuestos que ejercen *x*% de inhibición en la combinación, $\{[D_j]/\ \Sigma^{n}_{1}\ [D]\}$ es la proporción de la concentración de cada uno de los n compuestos que ejerce el *x*% de inhibición en combinación; y $(D_m)_j\ \{(fax)_j/[1\text{-}(fax)_j]\}^{1/mj}$ es la concentración de cada compuesto individualmente que ejerce el *x*% de inhibición. De esta ecuación, CI <1, =1 y >1 indica sinergismo, efecto aditivo y antagonismo, respectivamente.

5. La generación de especies reactivas del oxígeno intracelular

La producción de ROS intracelular se monitorizó en las células Caco-2 mediante la adición de la H_2-DCFDA según Ruiz-Leal y George (2004). Brevemente, $3x10^4$ células/pocillo se sembraron en microplacas de 96 pocillos de color negro. Una vez que las células alcanzaron el 80% de confluencia, el medio de cultivo se reemplazó por 20 µM de H_2-DCFDA durante 20 min. A continuación, se retiró el medio con H_2-DCFDA y se lavó dos veces con PBS antes de la adición de las micotoxinas. Se ensayaron dos concentraciones diferentes de BEA, ENs y FUS (1.5 y 3.0 µM). La selección de estas dos concentraciones, se hizo de acuerdo a los resultados obtenidos previamente en nuestro laboratorio, y por encontrarse por debajo de los valores de IC_{50} obtenidos en las células Caco-2 por los metodos de MTT Y NR. El método utilizado se basa en la

oxidación de 2',7'-dichlorofluorescin (DCFH) a diclorofluoresceína fluorescente. Este método es muy sensible y proporciona una medida directa de la generación de especies oxidantes intracelulares. El diacetato de DCFH (DCFH-DA) se absorbe por las células y es desacetilado por las esterasas intracelulares produciendo DCFH que se oxida a DCF. El aumento de fluorescencia se relaciona con el aumento de ROS que se lee en un lector de placa multipocillos (Wallace Victor^2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland), a intervalos de 0 a 120 min a una longitud de onda de excitación y emisión de 485 y 535 nm, respectivamente. Los resultados se expresaron como incremento de fluorescencia respecto al control (1% MeOH).

6. Determinación de la peroxidación lipídica

La determinación de la peroxidación lipídica (LPO) se determinó por el método de Buege y Aust (1978), basado en la formación de substancias reactivas con el ácido tiobarbitúrico (TBARS). Este ensayo se basa en la formación de un aducto rojo (con máxima absorción 532 nm) entre TBA y MDA, un producto final del proceso de peroxidación. De forma resumida, se sembraron 4,8 x 10^5 células/pocillo en placas de 6 pocillos. Tras alcanzar el 80% de confluencia, las células fueron tratadas con BEA, ENs y FUS, a las concentraciones de 1,5 y 3,0 µM durante 24 h. Tras este periodo, se retiró el medio y las células se lavaron con PBS, se tripsinizaron y fueron recogidas en 20 µM de Tris y 0,1% de Triton. Las células se homogeneizaron con un UltraTurrax T8 (IKA-Werke GmbH & Co. KG, Germany), y se adicionó 1 mL de TBA al 0,5%, 5 µL de desferroxamina 1,5 mM y 5 µL de butil hidroxitolueno al 3,75%. Tras agitación, las muestras se pusieron a baño maría (100 °C) durante 20

min. Inmediatamente se sumergieron las muestras en hielo, se centrifugaron a 1283xg durante 20 minutos y se midió la absorbancia a 532 nm (Perkin Elmer UV/Vis Lambda 2 versión 5,1). Los resultados se expresaron como ng de MDA/mg de proteína medida por el método de Bradford.

7. Determinación de glutatión intracelular oxidado (GSSG) y reducido (GSH).

La determinación de glutatión reducido (GSH) se ensayó de acuerdo a Maran y col. (2009). Brevemente, 10 µL del homogenizado celular se colocaron en placas de cultivo negras de 96 pocillos, con 200 µL de tampón GSH (Na_2HPO_4 0,1M-EDTA 0,005M) a pH 8 y 10 µL de solución o-ftaldehído (OPT), se mezclaron y se incubaron en oscuridad, a temperatura ambiente, durante 15 min. La concentración de GSH se determinó en un lector de placa multipocillos (Wallace Victor^2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) a 345 nm de longitud de onda de excitación y 425 nm de longitud de onda de emisión.

La determinación de glutatión oxidado (GSSG) se ensayó de acuerdo con Maran y col. (2009). Este método está basado en que la reacción del OPT con el GSSG provoca una intensa fluorescencia perceptible a pH 12. En un tubo Eppendorf se mezclaron 25 µL de homogenizado celular con N-etilmaleimida (NEM; 25 µL; para evitar la reducción del GSSG) y NaOH para alcanzar el pH adecuado (pH 12) y se incubaron en oscuridad y a temperatura ambiente durante 20 min. A continuación, se adicionaron 10 µL de la mezcla en cada uno de los 96 pocillos, 200 µL de NaOH 1N y 10 µL de solución O-ftalaldehido (OPT), se homogenizaron y se incubaron en oscuridad, a temperatura

ambiente, durante 15 min. La concentración de GSH se determina en el lector de placa multipocillos (Wallace Victor^2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) a 345 nm de longitud de onda de excitación y 425 nm de longitud de onda de emisión.

8. Análisis del ciclo celular

El ciclo celular es una secuencia de sucesos que conducen al crecimiento de la célula y posteriormente a la división celular. Mediante la utilización del yoduro de propidio (PI), que se une a la doble cadena de ADN, y la técnica de citometría de flujo (FC) es posible cuantificar el contenido de ADN y por consiguiente, los efectos sobre la distribución del ciclo celular. Tres fases distintas se pueden distinguir en una población de células proliferantes, dependiendo del contenido de ADN: fase G0/G1, que corresponde a las células inactivas que entran en la división celular, con una copia de ADN (2n); la fase S, momento en el cual se sintetiza DNA; y la fase G2/M, que corresponde a todas aquellas células que se preparan para dividirse y estan en la fase de mitosis, con dos copia de DNA (4n). Por otra parte, hay una fase, SubG0/G1, que permite identificar las células muertas (células hipoploididicas o debris).

El análisis del ciclo celular se realizó mediante tinción del DNA con PI como se ha descrito previamente en la literatura (Van Olphen y Mittal, 2002) con algunas modificaciones. En placas de 6 pocillos se sembraron 4,8 x 10^5 células por pocillo. Tras 24, 48 y 72 h de exposición a BEA y ENs A, A_1, B and B_1 (1,5 y 3,0 µM), las células Caco-2 se tripsinizaron y se colocaron en hielo durante 30 min con 860 µL de medio fresco que contenía 29 ng/mL de solución de tinción PI de

Vindeløv, lo cual fue preparado como sigue: para 500 mL se pesan 10 mg de RNAsa A (700 U/L), 0,1 mL de Nonidet P-40, 0,1% de Triton X-100, 10 mM de Tris, 10 mM de NaCl y 50 g/ml de PI en PBS. Se analizaron diez mil células por muestra por un citómetro de flujo CLR 4 EPICS XL MCL (Coulter Corporation) con longitudes de onda de excitación y de emisión de 488 nm y 620 nm, respectivamente. Los datos de los histogramas se extrajeron mediante ajuste rectangular de las fases del ciclo celular (CYLCHRED or MODIFIT software, Beckton Dickinson, Milán, Italy).

El bajo coeficiente de variación (CV) obtenido en este estudio fue resultado de una alta resolución conseguida por la alineación apropiada de los láseres. Se realizaron cuatro replicas por muestra para cada exposición a BEA y ENs.

9. Análisis de la apoptosis y necrosis por citometría de flujo

En general la muerte celular occurre a través de dos mecanismos moleculares: necrosis y apoptosis. El paso final de la necrosis se caracteriza por lisis de las membranas y liberación del contenido celular. Mientras que la apoptosis es una muerte celular genéticamente controlada. Una de las características de la apoptosis es la externalización de la fosfatidilserina (PS) en la capa externa de la membrana plasmática. La diferencia entre la población de células en apoptósis (temprana o tardía), necrosis y células muertas fueron identificada por doble tinción con Anexina V-FITC/PI (Vermes et al., 1995).

La Anexina V es una proteína Ca^{2+}-dependiente que se une a los fosfolípidos de membrana. Posee una elevada afinidad para la PS, se

unirá a todas aquellas células que externalizan PS hacia el lado extracelular de la membrana plasmática. PI se une específicamente al ADN de doble cadena de las células necróticas/muertas. Las células viables con membranas intactas excluyen el PI, mientras que las membranas de células muertas y dañadas son permeables al PI. Las células viables serán Anexina V-FITC$^-$/PI$^-$, las células en apoptósis temprana (pro-apoptóticas/apoptóticas) serán Anexina V-FITC$^+$/PI$^-$, las células en apoptosis tardía, que han completado el proceso de apoptosis e inician el proceso necrótico (apoptosis/necrosis) son Anexina V-FITC$^+$/PI$^+$ y las células necróticas serán Anexina V-FITC$^-$/PI$^+$.

Para este ensayo, se sembraron 4,8 x 10^5 células/pocillo en placas de 6 pocillos. Tras 24, 48 y 72 h de exposición a 1,5 y 3,0 µM de BEA y ENs A, A_1, B and B_1, las células se tripsinizaron y se resuspendieron en 360 µL de tampón HepesCa^{2+} preparado como se indica a continuación: 10 mM de HEPES-NaOH (pH 7.4), 135 mM de NaCl y 2.5 mM de $CaCl_2$. Tras 30 min de incubación a 4 °C en oscuridad, las células se analizaron por citómetría de flujo, BD FACSCanto con el software FACSDiva v 6.1.3 (BD Biosciences). Se adquirieron al menos 10.000 células por muestra. Se detectó fluorescencia en el canal FL-1 (verde, 530 nm) y FL-2 (naranja-rojo, 585 nm), emitida por FITC y PI, respectivamente. Las poblaciones celulares se analizaron a partir de los histogramas divididos en cuatro cuadrantes correspondientes a las células en apoptósis temprana (Q1), apoptoticas/necroticas (Q2), viables (Q3), necroticas (Q4). Las células Caco-2 tratadas con 1% de MeOH se consideraron como control.

10. Detección del potencial de membrana mitocondrial (ΔΨ*m*) por el método de tetrametil éster de rodamina [TMRM].

Las mitocondrias tienen un papel clave en el proceso de apoptosis (Kroemer et al., 1998). La inducción de la apoptosis conduce a la pérdida del potencial de membrana mitocondrial (ΔΨ*m*; Zamzami y col., 1995). El ΔΨ*m* se puede controlar en las células vivas utilizando un número de diferentes colorantes sensibles al potencial (rodamina 123, 3,3' dihexiloxacarbocianina yoduro (DiOC 6(3), 5,5',6,6'-tetraclorometil-X-rosamina (CMXRos) y tetrametil rodamina metil éster (TMRM; Kroemer y col., 1998). TMRM es un marcador fluorescente, catiónico lipofílico, que atraviesa libremente la membrana plasmática y se acumula dentro de las mitocondrias, dependiendo de su ΔΨ*m* (Tsipser y col., 2012). La doble tinción de TMRM con To-Pro®-3, un marcador impermeable a la membrana nuclear, se puede utilizar para caracterizar la integridad de la membrana plasmática y, como consecuencia, la viabilidad celular. Se evaluaron los cambios del ΔΨ*m*, tras la exposición a BEA y ENs A, A_1, B and B_1 (1,5 y 3,0 μM) usando la doble tinción TMRM y To-Pro®-3 como describe Tsiper y col. (2012). El ionóforo cianuro de carbonilo paratrifluorometoxifenilhidrazona (FCCP) se utilizó para optimizar el análisis del cambio ΔΨ*m* (por disminución de la señal TMRM) y la viabilidad celular por aumento de la señal To-Pro®-3. Brevemente, las células Caco-2 se sembraron en placas de 6 pocillos a una densidad de 4,8 x 10^5 células/pocillo. Tras la exposición a BEA y ENs (1,5 y 3,0 μM, las células se tripsinizaron y resuspendieron en medio de cultivo (500 μL) donde se incluyó una mezcla de los dos marcadores con una concentración final de 140 nM y 160 nM para TMRM y para To-Pro®-3 respectivamente. Tras 30 min de incubación a 37 °C en

oscuridad, las intensidades de fluorescencia de TMRM (λ_{ex} = 548 nm y λ_{em} = 573 nm) y de To-Pro®-3 (λ_{ex} = 642 nm y λ_{em} = 661 nm) se midieron en un citómetro de flujo FACSCanto (Beckton-Dickinson, Italia). Las poblaciones celulares se analizaron a partir de los histogramas divididos en dos cuadrantes correspondientes a las células positivas (P5) y negativas (P6) al TMRM y en cuatro cuadrantes correspondientes a células positivas (Q2+Q4) y negativas (Q1+Q3) al To-Pro®-3. Las células Caco-2 tratadas con 1% de MeOH se consideraron como control. Los datos de los histogramas obtenidos se analizaron utilizando el software FACSDiva v 6.1.3 (BD Biosciences). El porcentaje de células teñidas por TMRM y To-Pro®-3 se calculó a partir de 10.000 células.

11. Ensayo del cometa alcalino (pH>13)

La inducción del daño en las cadenas simples como las roturas de la doble cadena de DNA se determinó utilizando el ensayo del cometa alcalino (pH>13), de acuerdo con el método descrito previamente (Singh y col., 1988; Marabini y col., 2011), con algunas modificaciones. Las células Caco-2 se sembraron en placas de 6 pocillos a una densidad de 4,8 x 10^5 células/pocillo. Después de alcanzar el 60% de confluencia, las células fueron tratadas con BEA a las concentraciones de 1,5, 3,0 y 12,0 µM y con las ENs A, A_1, B y B_1, a las concentraciones de 1,5 y 3,0 µM, durante 24 h. Posteriormente, 2 x 10^4 células/mL se suspendieron en agarosa de bajo punto de fusión (LMA; 0,5% de PBS) pre-calentado (37 °C) y 80 µL de la suspensión se transfirieron rápidamente a láminas previamente preparadas con agarosa (1% PBS) que se cubrieron con un cubreobjetos (24x36 mm). Transcurridos 10 min después de la 1ª gelificación a 0 °C, se retiró el cubreobjetos con cuidado y se añadió

una segunda capa de 80 µL de LMA. Después de la 2ª gelificación durante 10 min a 0 °C, se retiró el cubreobjetos con cuidado y las láminas se sumergieron en la solución de lisis (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO and 1% Triton X-100, recién preparado) durante 30 min a 0 °C. Las láminas se retiraron de la solución de lisis, se lavaron con tampón de neutralización (Tris 0,4 M, pH 7,5) y se incubaron en tampón de electroforesis fresco (NaOH 300 mM, 1 mM de Na-EDTA), durante 20 min a temperatura ambiente para permitir que el ADN se desenrollase. La electroforesis se llevó a cabo a continuación, a temperatura ambiente en tampón de electroforesis fresco durante 40 min (25 V, 300 mA). Después de la electroforesis, las láminas se lavaron suavemente con tampón de neutralización fresco (5 min), se dejaron secar durante toda la noche a 4 °C y a continuación se tiñeron con 500 µL de PI (20 µg/mL en H_2O). Las láminas se visualizaron con un microscopio de fluorescencia (Nikon Eclipse E800), equipado con cámara (NIKON DXM1200F) para la captura de imagines. Un mínimo de 50 células individuales fueron seleccionadas al azar y se analizaron mediante el uso de Automatic Comet Assay by TriTek CometScore™ freeware

(http://autocomet.com/index.php?id=cometscorepro). El daño en el DNA de las células Caco-2 se expresó como la mediana del momento de la cola (TM). El TM fue elegido como el parámetro efectivo, calculado de acuerdo con la ecuación: TM = TL x Tail DNA% donde TL es la longitud de la cola, i.e. la distancia (µm) entre el centro de la cabeza de la cometa y el punto final de la cola. Todos los parámetros fueron proporcionados por TriTek CometScore™. El DNA de la cola se expresa

como un porcentaje del contenido total de DNA basado en la intensidad de fluorescencia total.

12. Transporte intestinal al a través de las células Caco-2

Los ensayos de transporte de la BEA y FUS, a través del epitelio intestinal simulado usando células Caco-2 diferenciadas, necesarios para evaluar la biodisponibilidad de las micotoxinas, se llevaron a cabo en placas bicamerales de 6 pocillos Transwell® (Transwell Permeable Soportes, Corning, NY, EE.UU.). Las células Caco-2 se sembraron a una densidad de 25 x 10^4 células/cm^2 adicionando 1,5 mL de medio de cultivo a la cámara apical y 2 mL a la cámara basal. Las placas se incubaron a 37°C, 5% CO_2 y 95% de humedad relativa, cambiando el medio de cultivo cada 2 días hasta alcanzar la diferenciación de las células Caco-2, a los 21 días de incubación.

Una vez alcanzada la diferenciación celular, se retiró el medio de mantenimiento y se sustituyó tanto en el compartimento apical (1,5 mL) como en el basal (0,5 mL) por una solución salina tamponada (HBSS) enriquecida con 10 mM de HEPES. Las placas se incubaron a 37 °C durante 10 min previamente al inicio del ensayo de transporte. A continuación, se substituyó el medio, por la solución de HBSS-HEPES conteniente las micotoxinas (BEA y FUS) a una concentración de 1,5 y 3,0 µM en el compartimiento apical. En el compartimento basolateral se adiciono la solución de HBSS-HEPES. Las placas se incubaron durante 4 horas a 37 °C.

Cada hora se tomaron alícuotas de 100 µL de medio de cultivo del compartimento basolateral y se analizan por cromatografía líquida (LC).

La diferenciación de las células Caco-2 se evaluó midiendo la difusión del rojo fenol a través de la monocapa en las muestras control de las placas Transwell®. Para ello, después de lavar cada pocillo 3 veces con PBS, se adicionaron 1,5 mL de PBS en la cámara basal y 2 mL de DMEM en la cámara apical. Después de 1h de incubación a 37 °C se recogió el volumen de la cámara basal y se ajustó el pH (=11) con NaOH (1M). Se midió espectrofotométricamente la absorbancia a 558-559 nm. El porcentaje de rojo fenol resultó inferior al 5% confrontando con una recta de calibrado patrón (115-5644 µM) de rojo fenol.

12.1 Extracción de beauvericina y fusaproliferina de los compartimientos apical y basolateral

BEA y FUS se extrajeron de los compartimientos apical y basolateral de acuerdo con el método descrito por Ambrosino y col. (2004). Resumido, 1 ml de cada pocillo se disolvió en 3 ml de metanol y después se filtró a través de filtro de nylon de 0,22 micras (Análisis Vínicos, Tomelloso, España). Las muestras se purificaron con una columna de extracción en fase sólida C18-E (100 mg, Phenomenex, Torrance, CA) previamente activada con 3 ml de metanol y 3 ml de agua que se eluyen con 1 ml de metanol. El eluato se evapora completamente con nitrógeno a 50 °C y el residuo se reconstituyó con 100 µL de metanol. A continuación, 20 µL de cada extracto se inyectó en el LC. La recuperación del método de extracción se determinó mediante experimentos de adición. BEA y FUS se añadieron a un medio de transporte no contaminado en concentraciones variables desde de 0,1 a 10 µM, y después se extrajo. La recuperación calculada (media ±

desviación estándar relativa) de BEA y FUS en las muestras contaminadas artificialmente fue por encima del 90,7%.

12.2. Extracción de BEA y FUS a partir de las células

BEA y FUS contenidas en las células se determinaron de acuerdo a Meca y col. (2010a) como se muestra a continuación: las células se sometieron a ultrasonidos en una solución salina (0,90% de NaCl, v/v) durante 30 min. El precipitado se suspendió en 5 ml de solución salina, y se añadieron 5 ml de acetato de etilo. Se mezclaron (1 min) en un vortex y se centrifugaron a 4,000 rpm durante 10 min a 4 °C (Centrífuga 5810R, Eppendorf, Alemania). El sobrenadante se evaporo aproximadamente hasta 5 mL, en un evaporador rotatorio (Büchi, Suiza), y se resuspendió en 1 ml de metanol. Los eluatos se analizaron por LC.

12.3. Determinación de beauvericina y fusaproliferina por cromatografía líquida

La determinación por LC de BEA y FUS se realizó utilizando un sistema Shimadzu LC equipado con bombas LC-10AD y un detector de red de diodos (DAD) de Shimadzu (Japón). Se utilizó una columna Géminis (150 x 4,6 mm, 5 micras, Phenomenex, Torrance CA). Las condiciones de LC fueron establecidas usando un flujo constante a 1.0 mL/min y se usó acetonitrilo-agua (70:30 v/v) como sistema eluyente de principio. La relación del principio se mantuvo constante durante 5 min y luego se modificó hasta alcanzar el 90% de acetonitrilo durante 10 min. Después de 1 min a 90% de acetonitrilo, la fase móvil se llevó a las condiciones del principio durante 4 min. BEA se detectó a 205 nm y FUS

a 261 nm. La identificación de micotoxinas se realizó mediante la comparación de tiempos de retención y espectros UV de muestras purificadas extraídas a los estándares puros. La cuantificación de micotoxinas se llevó a cabo mediante la comparación de áreas de los picos de las muestras investigadas con la curva de calibración de patrones auténticos.

13. Modelo gastrointestinal *in Vitro*

El procedimiento gastrointestinal *in vitro* fue adaptado del método desarrollado previamente por Gil-Izquierdo y col. (2002) con ligeras modificaciones. El método consta de tres pasos secuenciales: primero una adición inicial de saliva para simular el compartimento de la boca. La boca es el punto en el que el proceso de molienda mecánica de los alimentos se lleva a cabo a un pH de 6.5. El pH se ajustó con HCl 0.1 N. En este paso, 3 g de muestra enriquecida con las ENs (A, A1, B y B1) a 1.5 y 3.0 µmol/g se mezcló con 6 ml de saliva artificial (compuesta de 89,6 g/L de KCl, 20.0 g/L KSCN, 88.8 g/L de NaH_2PO_4, 57.0 g/L de $NaSO_4$, 175.3 g/L de NaCl, 84.7 g/l de $NaHCO_3$, 25.0 g/L de urea, y 290.0 mg de α-amilasa). La mezcla se puso en una bolsa de plástico, con 40 mL de agua, y se homogeneizó mediante un Stomacher (IUL Instruments; Barcelona, España) durante 30 s. Los componentes más grandes se descomponen en fragmentos más pequeños, lo que aumenta el área superficial de las partículas de alimentos para la deglución y la digestión. El segundo paso consistió en una digestión con pepsina/HCl para simular el entorno del ácido clorhídrico del estómago (pH 1-5). La presencia de pepsina actúa para descomponer las proteínas, ayudando de ese modo la disolución de los alimentos. Para este propósito, se añadió 0.5 g de pepsina (14.800 U) preparada en HCl

(0.1 M) a la mezcla, y el pH se ajustó a 2.0. La mezcla se incubó a 37 °C en un agitador orbital (Infors AG CH-4103, Bottmingen, Suiza) a 250 rpm durante 2 h. El último paso fue la digestión en el intestino delgado (s) por los jugos intestinales compuestos de enzimas (tripsina, pancreatina, y amilasa), sales biliares, y bicarbonato. La descomposición de los alimentos en el intestino delgado (s) significa que los componentes son más susceptibles a la absorción. Las enzimas utilizadas en el jugo intestinal fueron pancreatina (8 mg/mL) y sales biliares (50 mg/mL) a partes iguales (1:1; v/v); el pH de los jugos intestinales se ajustó a 6.5 con 0.5 N de $NaHCO_3$; la mezcla se incubó durante 2 horas a 37 °C en un agitador orbital (250 rpm). Inmediatamente, se centrifugaron alícuotas de 30 ml (Centrífuga 5810R, Eppendorf, Alemania) a 4.000 rpm y 4 °C durante 1 h, y la ENs presentes en los fluidos de saliva/pepsina/HCl y pancreatina−bilis digeridos se extrajeron y analizaron por cromatografía líquida con detector de diodos (HPLC-DAD), tal como describe Meca y col. (2010b).

13.1 Análisis de las enniatinas.

La extracción de las ENs (A, A_1, B y B_1) contenidas en los fluidos gastroduodenales se llevó a cabo según Meca y col. (2012). Brevemente, 5 mL de cada mezcla obtenida como se ha descrito anteriormente se puso en un tubo de ensayo de 20 mL y se extrajo tres veces con 5 mL de acetato de etilo utilizando un agitador rotatorio (VWR International; Barcelona, España) durante 1 min y se centrifugó (centrífuga 5810R, Eppendorf, Alemania) a 4.000 rpm y 4 °C durante 10 min. Después la fase orgánica se evaporó completamente en un evaporador rotatorio (Büchi, Suiza), los residuos se disolvieron en 1 mL

de metanol y se analizaron por LC-DAD. Todas las muestras se filtraron a través de filtros de 0.22 µm Phenomenex de jeringa antes de su determinación cromatográfica. La separación de las micotoxinas por LC se llevó a cabo con una columna analítica Gemini (150 mm x 4,6 mm, 5 mM) Phenomenex (Madrid, España). La separación analítica se realizó usando un gradiente de elución con agua como fase móvil A y acetonitrilo como fase móvil B. Después de una etapa isocrática de 70% de B durante 5 min, el gradiente se modificó linealmente hasta 90% de B en 10 min. Después de 1 min, la fase móvil se llevó a las condiciones del principio en 4 min. La velocidad de flujo se mantuvo a 1,0 mL/min. Las ENs se detectaron a 205 nm. Las identificación de las ENs se realizó mediante la comparación de tiempos de retención y los espectros UV de muestras purificadas de los estándares puros. La confirmación se realizó mediante la co-inyeccion de estándares puros junto con cada muestra. La cuantificación de las EN se llevó a cabo mediante la comparación de áreas de los picos de las muestras investigadas con una curva de calibración de los estándares. Los estudios de recuperación (%) en el fluido intestinal se realizaron por adición de las muestras con soluciones estándar de cada una de las ENs en concentraciones de 1,5 y 3,0 µmol/g.

13.2 Validación del método

El método analítico se validó de acuerdo con la Directiva Europea 2002/26/CE sobre los métodos de análisis de micotoxinas en los productos alimenticios (CE, 2002). Los experimentos de recuperación se llevaron a cabo en los fluidos intestinales fortificados (libres de contaminación) (n = 5) por adición de las EN A, A_1, B, B_1 y en un nivel

que oscilaba desde 0.3 hasta 50 µg/g. Las medias de las recuperaciones (%) fueron las siguientes: 88.6 ± 2.4, 84.2 ± 4.3, 86.6 ± 2.7, y 89.5 ± 3.1% para la EN A, A_1, B, and B_1, respectivamente. Los valores de variación interdiaria (n=5) fueron desde 1.8 hasta el 3.1%. Los valores de variación intradiaria estaban en el rango de 6.4 hasta 10.1%. Estos valores no superaban el 15%, que se considera la variación máxima. Los límites de detección (LOD) y el límite de cuantificación (LOQ) se calcularon de acuerdo con s/n = 3 s/n = 10, respectivamente. Los LODs obtenidos para las ENs A, A_1, B, and B_1 fueron 215, 140, 145, y 165 µg/kg, rspectivamente, mientras que los LOQs fueron 600 µg/kg para la EN A, 400 µg/kg para las ENs A_1 and B, y 500 µg/kg para la EN B_1.

14. Estudio estadístico

El análisis estadístico se llevó a cabo usando el paquete software SPSS statistic 19.0 (SPSS, Chicago, IL, USA). Los resultados se expresaron como media ± SD. El análisis estadístico de los resultados se realizaron con la t-Student para muestras emparejadas. Las diferencias entre los grupos se analizaron estadísticamente con ANOVA seguido de Tukey HSD y Dunnet post hoc tests para la comparación múltiple. El valor de $p \leq 0.05$ se consideró estadísticamente significativo.

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Anexos II- Difusión de resultados

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Bioaccessibility of Enniatins A, A_1, B, and B_1 in Different Commercial Breakfast Cereals, Cookies, and Breads of Spain

Alessandra Prosperini,* Giuseppe Meca, Guillermina Font, and María-José Ruiz

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain

ABSTRACT: Fusarotoxins enniatins (ENs) can represent a potential risk as natural contaminants of cereal commodities. However, only their bioaccessible fraction can exert a toxicity. The purpose of this study was to determine the ENs A, A_1, B, and B_1 bioaccessibility added in 1.5 and 3.0 μmol/g concentrations in breakfast cereals, cookies, and breads using a simulated in vitro gastrointestinal extraction model. Bioaccessibility values ranged between 40.4 ± 1.9 and 79.9 ± 2.8%. The lower values were 50.1, 40.4, 43.9, and 46.3% in wheat bran with fibers, and the higher values were 79.9, 64.2, 69.8, and 73.6% in white loaf bread for the ENs A, A_1, B, and B_1, respectively. Food composition, compounds structure, and presence of natural adsorbing materials can influence the ENs bioaccessibility. Application of a simulated in vitro gastrointestinal method is a good procedure to assess oral ENs bioaccessibility in cookies, breakfast cereals, and bread.

KEYWORDS: *enniatins, bioaccessibility, in vitro gastrointestinal model, HPLC*

INTRODUCTION

The Food and Agriculture Organization (FAO) estimates that as much as 25% of the world's animal feedstuffs is contaminated by some extent by mycotoxins.[1] Any step of the food production chain is susceptible to mold and mycotoxins contamination: before harvesting, between harvesting and drying, and during storage. Furthermore, they are persistent in the final products.[2] The co-occurrence of mycotoxins in a food matrix is also common.[3] The most common pathogen of maize, grain, and small grain in temperate regions of the world is *Fusarium* spp., whose strains may produce cyclic hexadepsipeptidic secondary metabolites such as enniatins (ENs). ENs are composed of three alternating D-α-hydroxyisovaleryl and three *N*-methyl-L-amino acid residues. They possess antimicrobial, insecticidal, phytotoxic, and cytotoxic properties[4] and inhibit cholesterol acyltransferase.[5] The large array of biological activities can be related to their ionophoric properties based on the ability to incorporate into cell membranes forming cation-selective pores with high affinity for K^+, Mg^{2+}, Ca^{2+}, and Na^+.[6] Their occurrences have been amply demonstrated,[7−11] but the potential risk related to the ingestion of contaminated commodities is still not clear. The most important exposure routes for human and animals for ENs are via oral ingestion. It is therefore important to be able to assess the amount of ENs that is potentially available for absorption in the stomach and/or intestines, that is, bioaccessible, or to be excreted. However, the total amount of ENs ingested (intake) does not always reflect the bioaccessible amount of them. The bioaccessibility describes the fraction of a contaminant, that is, ENs, that is mobilized from food matrices during gastrointestinal digestion and theoretically subsequently available to intestinal absorption.[12] So, to study the oral bioaccessibility as part of an overall estimation in assessing the chemical risk coming from food-borne ENs is an important issue.

Because of this, during the past decade, there has been an increasing interest in the use of in vitro methodologies, such as in vitro digestion models that simulate, in a simplified manner, the human digestion process in the mouth, stomach, and small intestine, to enable bioaccessibility investigations of contaminants from their food matrix during transit in the gastrointestinal tract.[12,13] These in vitro models attempt to recreate the aspects of human gastrointestinal physiology, such as chemical composition of digestive fluids, pH, and residence time periods typical for each compartment.[12] Moreover, these in vitro models are simple, rapid, low-cost, and without ethical implications, although a number of comparative studies have suggested that bioaccessibility results are largely dependent on the specific in vitro conditions used, including differences in solid solution ration, the method of mixing, the pH values of the gastric and intestinal juices and their compositions, food contaminants, and food matrices.[14]

Previous studies focused on determining the bioaccessible part of several mycotoxins after simulated human gastrointestinal extraction, confirming the usefulness of these in vitro methodologies to predict intestinal absorption of mycotoxins.[12,13,15] Versantvoort et al.[12] used a simplified digestion process of three steps, where physiologically based conditions of the mouth, stomach, and small intestine were applied.

The aims of the present study were (1) to apply an in vitro gastrointestinal model as it is related to the human digestive system; (2) to determine the bioaccessibility of ENs A, A_1, B, and B_1 from artificially spiked grain-based products (specifically breakfast cereals, cookies, and breads) by using the in vitro gastrointestinal model; and (3) to finally appreciate the role of oral bioaccessibility in assessing EN risks to human.

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Study of the cytotoxic activity of beauvericin and fusaproliferin and bioavailability *in vitro* on Caco-2 cells

A. Prosperini, Giuseppe Meca *, G. Font, M.J. Ruiz

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

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ABSTRACT

Beauvericin (BEA) is a cyclohexadepsipeptide mycotoxin which has insecticidal properties and produces cytotoxic effects in mammalian cells. Fusaproliferin (FUS) is a mycotoxin that has toxic activity against brine shrimp, insect cells, and teratogenic effects on chicken embryos. The aim of this study was to determine the cytotoxicity of BEA and FUS in human epithelial colorectal adenocarcinoma HT-29 and Caco-2 cells, the transepithelial transport and the bioavailability using Caco-2 cells as a simulated *in vitro* gastrointestinal model of the human intestinal epithelium. The inhibitory concentration (IC_{50}) evidenced by BEA in the Caco-2 cells was 24.6 and 12.7 μM at 24 and 48 h exposure, respectively, whereas the IC_{50} values evidenced in HT-29 cells were 15.0 and 9.7 μM, respectively. FUS was cytotoxic, but no IC_{50} data were observed in the range of concentration tested. BEA bioavailability was variable from 50.1% to 54.3%, whereas FUS presented a bioavailability variable from 80.2% to 83.2%. Results obtained demonstrated a potential risk for human health.

1. Introduction

Beauvericin (BEA) is a cyclohexadepsipeptide that was first identified in a soil-borne entomopathogenic (insect-pathogenic) fungus *Beauveria bassiana*, which was recognized as the causative agent for heavy losses of the European sericulture in the 16th and 17th centuries (Tang et al., 2005). Nowadays, BEA is considered to be a putative mycotoxin (toxic fungal metabolite) that may affect human and animal health, since it is also produced by many species of the fungus *Fusarium* that infect important cereal grains such as corn, rice, and wheat (Leslie and Summerell, 2006). The potential mycotoxic role of BEA is exemplified by results obtained from *in vitro* studies using cell lines. In mammalian cell lines, cell deaths caused by BEA have been suggested to involve a Ca^{2+} dependent pathway, in which BEA induces a significant increase in intracellular Ca^{2+} concentration that leads death cell as a result of a combination of both apoptosis and necrosis (Jow et al., 2004; Lin et al., 2005). The mechanism of BEA-induced Ca^{2+} increase, however, remains inconclusive. BEA-induced apoptotic changes such as DNA fragmentation have been demonstrated to take place in the complete absence of extracellular Ca^{2+}, suggesting that BEA triggers release of Ca^{2+} from internal Ca^{2+} stores. In fact, BEA has since been regarded as an apoptotic agent that releases Ca^{2+} exclusively from endoplasmic reticulum (Lin et al., 2005). Furthermore, BEA is a potent and specific cholesterol acyltransferase inhibitor in rat liver microsomes (Tomoda et al., 1992).

The inhibition of cell proliferation by BEA exposure, was studied by several authors in rodent (Ferrer et al., 2009), monkey, porcine (Klaric et al., 2006), insect (Calo et al., 2003) and human cell lines (Ruiz et al., 2011; Lin et al., 2005; Meca et al., 2011) utilizing several determinations techniques as the tetrazolium salt (MTT), the trypan blue dye exclusion, Alamar Blue, BrdU and sulforhodamine B (SRB) assays.

Fusaproliferin (FUS) is produced by two closely related species, *Fusarium subglutinans* and *F. proliferatum* (Ritieni et al., 1999). Both species are important pathogens of maize (Ritieni et al., 1999) and other economically important crop plants and may be isolated together from the same plant (Moretti et al., 2007). FUS is toxic to *Artemia salina* (Moretti et al., 2007; Tan et al., 2011), IARC/LCL 171 human B lymphocytes and SF-9 insect cells (Logrieco et al., 1996) and has teratogenic and pathogenic effects on chicken embryos (Ritieni et al., 1997).

The study of the bioavailability of the *Fusarium* mycotoxins including zearalenone (ZEA), fumonisins, ochratoxin A (OTA), deoxinivalenol (DON), aflatoxin B_1 (AFB_1), and enniatins (ENs) has been evaluated by many authors (Avantaggiato et al., 2003, 2004; Carolien et al., 2005; Videmann et al., 2008; Meca et al., 2012). These authors describes the applicability of an *in vitro* digestion models to measure the bioavailability of different mycotoxins contained in food matrices as an indicator of oral bioavailability.

The aim of this study was to evaluate: (a) the cytotoxic effects of the BEA and FUS on Caco-2 and HT-29 cell lines, (b) the transepithelial transport and (c) the relative bioavailability utilizing the cell

* Corresponding author. Tel.: +34 963544959; fax: +34 96354954.
E-mail address: giuseppe.meca@uv.es (G. Meca).

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RESEARCH ARTICLE

Effect of polyphenols on enniatins-induced cytotoxic effects in mammalian cells

G. Lombardi[1], A. Prosperini[2], G. Font[2], and M. J. Ruiz[2]

[1]Dipartmento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Biochimica, Fisiologia e Nutrizione, Facoltà di Farmacia, Università degli Studi di Perugia, Perugia, Italy and [2]Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Valencia, Spain

Abstract

Enniatins (ENs) are fungal secondary metabolites produced by genus *Fusarium*. The ENs exert antimicrobial and insecticidal effect, and has also been demonstrated cytotoxic effects on several mammalian cell lines. On the other hands, it has been proved that natural polyphenols have antioxidant effect. In this study, cell effects at low levels of exposure of four ENs (A, A_1, B and B_1) and five polyphenols (quercetin, quercetin-3-β-D-glucoside, rutin, myricetin and *t*-pterostilbene) present in wine; and the cytoprotective effect of these polyphenols exposed simultaneously with ENs in Chinese Hamster Ovary (CHO-K1) cells, were studied. Cell effects were determined by the MTT test after 24 h of exposure. All ENs showed cytotoxic effect. The IC_{50} obtained ranged from 4.5 ± 1.2 to 11.0 ± 2.7 μM. The concentration of polyphenols tested ranged from 5 to 50 μM. Polyphenols did not show cytotoxicity and the cytoprotective effect of polyphenols varies depending on the EN tested. The cytoprotective effect of polyphenols in CHO-K1 cells exposed to ENs was as follow: quercetin, from 24 to 84%; quercetin-3-β-D-glucoside, from 12 to 76%; rutin, from 17 to 83%; myricetin, from 16 to 92% and pterostilbene from 25 to 100%. All polyphenols protected CHO-K1 cells against EN A_1 exposure.

Keywords: Enniatins, polyphenols, cytotoxicity, cytoprotective effect, CHO-K1 cells

Introduction

Mycotoxins are fungal secondary metabolites, mainly produced by genera *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria* and *Claviceps* and the amount of their production depends on different factors such as types of crop, climate and storage conditions. Among mycotoxins yielded by *Fusarium* there are enniatins (ENs), beauvericin (BEA), fumonisins, trichothecenes, moniliformin (MON) and zearalenone (ZEA; Jestoi 2008; Ferrer et al. 2009; Meca et al. 2010; 2011). Fungal contaminants from genus *Fusarium* infect plants and agricultural products. They are present in food and feed and can produce a wide range of acute and chronic toxic effects on humans and animals.

ENs are lipophilic hexadepsipeptides that can easily incorporate in the cellular membrane. ENs exert antimicrobial, insecticidal and antihelmintinc effects (Tedjiotsop Feudjio et al. 2010). Moreover, ENs form dimeric structures that transport monovalent ions across the membranes, especially mitochondrial membranes and uncouple oxidative phosphorylation (Wätjen et al. 2009; Tedjiotsop Feudjio et al. 2010) and produce cytotoxic effects on mammalian cells (Wätjen et al. 2009; Meca et al. 2010; 2011). Polyphenols are widely distributed in fresh fruits, berries, black tee, red wine, purple grape juice, medicinal herbals, daily nutrition supplements, etc. They are an integrate part of the human diet (natural food and food industry) and are utilized as a source of starting material in the pharmaceutical industry (Zamora-Ros et al. 2008; Ostertag et al. 2010; Chen et al. 2011; Seibert et al. 2011). Moreover, they have numerous biological activities, as cancer-preventive because of their antioxidative, oxygen radical scavenging, antiproliferative and anti-inflammatory activities and their stimulation of

Address for Correspondence: M. J. Ruiz, Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vincent Andres Estelles s/n 46100 Burjassot, Valencia, Spain. Tel: +34 963543055. Fax: +34 963544954. E-mail: m.jose.ruiz@uv.es

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Marcas:	
Asunto:	Your Submission
Para:	alessandra.prosperini@uv.es, alessandra.s83@gmail.com
De:	"Wolfgang Dekant" <editor.TL@toxi.uni-wuerzburg.de>
Fecha:	7 Jul 2013 22:49:57 +0100

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Interaction effects of *Fusarium* Enniatins (A, A_1, B and B_1) combinations on *in vitro* cytotoxicity of Caco-2 cells

A. Prosperini, G. Font, M.J. Ruiz

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain

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Highlights

- Enniatins (ENs) are foodborne mycotoxins produced by *Fusarium spp.*
- ENs A, A1, B and B1 reduced Caco2 proliferation in a dose dependent manner.
- Combinations of two, three and four ENs tested produced a general additive effects.
- Combinations of EN B+EN B1 produced antagonism at all concentration tested.
- ENB+ENA_1, ENB_1+ENA_1, ENA+ENA_1+ENB combinations produced synergistic effects at low concentration tested.