VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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

Programa de Doctorado con Mención hacia la Excelencia en Ciencias de la Alimentación

Tesis Doctoral Internacional

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IN VITRO TOXICITY ASSESSMENT AND MITIGATION STRATEGIES OF BEAUVERICIN, STERIGMATOCYSTIN AND PATULIN

EVALUACIÓN DE LA TOXICIDAD *IN VITRO* Y ESTRATEGIAS DE MITIGACIÓN DE LA BEAUVERICINA, ESTERIGMATOCISTINA Y PATULINA

Presentada por:

Beatriz Mallebrera Simarro

Dirigida por:

Dra. María José Ruiz Leal

Dra. Guillermina Font Pérez

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La Dra. Guillermina Font Pérez, Catedrática del área de Toxicología y la Dra. María José Ruiz Leal, Profesora Titular de Toxicología,

CERTIFICAN QUE:

Dª Beatriz Mallebrera Simarro, licenciada en Biología, ha realizado bajo su dirección el trabajo que lleva por título: "*In vitro* toxicity assessment and mitigation strategies of beauvericin, sterigmatocystin and patulin" y autorizan su presentación para optar al título de Doctora por la Universidad de Valencia.

Y para que así conste, expiden y firman el presente certificado.

Burjassot (Valencia), Diciembre 2016

Dra. Guillermina Font Pérez

Dra. María José Ruiz Leal

La presente tesis doctoral ha dado lugar a 5 artículos publicados en las siguientes revistas:

1. Beatriz Mallebrera, Ana Juan-Garcia, Guillermina Font, Maria-Jose Ruiz. Mechanisms of beauvericin toxicity and antioxidant cellular defense. Toxicology Letters, 246 (2016) 28–34. Impact factor: 3.262

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3. B. Mallebrera, V. Brandolini, G. Font, M.J. Ruiz. Cytoprotective effect of resveratrol diastereomers in CHO-K1 cells exposed to beauvericin. Food and Chemical Toxicology, 80 (2015) 319–327. Impact factor: 2.895

4. B. Mallebrera, G. Font, M.J. Ruiz. Disturbance of antioxidant capacity produced by beauvericin in CHO-K1 cells. Toxicology Letters 226 (2014) 337–342. Impact factor: 3.262

5. B. Mallebrera, G. Meca, L. Manyes, J. Mañes, G. Font. Influence of pro- and prebiotics on gastric, duodenal and colonic bioaccessibility of the mycotoxin beauvericin. Journal of Food Composition and Analysis, 32 (2013) 141–149. Impact factor: 1.985

La presente Tesis Doctoral Internacional se engloba dentro de los siguientes proyectos y una red:

- Evaluación del riesgo de las micotoxinas emergentes de *Fusarium* (AGL2010-17024/ALI).

- Estudio de las micotoxinas y metabolitos en alimentos y muestras biológicas, de la toxicidad y de los procesos de descontaminación (AGL2013-43194-P).

- Red nacional sobre las micotoxinas y hongos toxigénicos y de sus procesos de descontaminación (MICOFOOD).

La presente Tesis Doctoral Internacional forma parte del Programa de Doctorado Internacional: ***"Chemistry, Toxicology and Healthiness of foods"*** *dirigido por el Profesor Pietro Damiani y la Profesora Lina Cossignani de la Università degli Studi di Perugia.*

"Puedes llegar a cualquier parte, siempre que andes lo suficiente."

Lewis Carroll (1832-1898)

A mi familia

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Cytoprotective effect of resveratrol diastereomers in CHO-K1 cells exposed to beauvericin

LISTA DE ABREVIATURAS

ABC: Proteína de transporte ABC
ABTS: 2,2´-azino-bis-(ácido -3 etilbenzotiazolino-6-sulfónico)
ACAT: Acil-CoA:colesterol aciltransferasa
AcDON: Monoacetil-deoxinivalenol
AFB: Aflatoxina B (1 and 2)
AFG: Aflatoxina G (1 and 2)
AFM_1: Aflatoxina M_1
ALT: Altenueno
AME: Alternariol monometil éter
AOH: Alternariol
AO/EB: Naranja de acridina/bromuro de etidio
ATX: Altertoxina
A549: Células de carcinoma de pulmón humano
Bax: Proteína proapoptótica Bax
BC: Bioaccesibilidad
Bcl-2: Proteína antiapoptótica Bcl-2
BEA: Beauvericina
BEAS-2B: Células de epitelio de bronquio humano
BNC: Células binucleadas
BPA: Buenas prácticas agrícolas
BPF: Buenas prácticas de fabricación
BrdU: 5-bromo-2-desoxiuridina
CA: Aberraciones cromosómicas
$CaCl_2$: Cloruro de calcio
Caco-2: Células de carcinoma de colón humano
CAT: Catalasa
CC_{50}: Concentración citotóxica media
CCRF-CEM: Células de leucemia humana
CE: Electroforesis capilar
CHO-K1: Células de ovario de hámster chino
CI: Índice de combinación
CO_2: Dióxido de carbono

CPA: Ácido ciclopiazónico
CTOR: Células procedentes de órganos y tejidos de ratón
CTN: Citrinina
CV: Voltametría cíclica
DAPI: 4 ',6-diamino-2-fenilindol
DAS: Diacetoxiscirpenol
DCF: Diclorofluoresceína (forma fluorescente)
DCFH: 2',7'-diclorofluoresceína
$DCFH_2$-DA: Diacetato de 2',7'-diclorodihidrofluoresceína
Dhe: Dihidroetidio
DHR 123: Dihidrorodamina 123
DiOC6: Ioduro de 3,3'-dihexiloxa carbocianina
DMSO: Dimetil sulfóxido
DNA: Ácido desoxirribonucléico
DON: Deoxinivalenol
DPPH: 2,2-difenil-2-picrilhidrazilo
EC: Comisión Europea
EC_{50} : Concentración efectiva media
EDTA: Ácido etilendiaminotetraacético
EFSA: Autoridad Europea de Seguridad Alimentaria
EGFP: Proteína verde fluorescente
ELISA: Ensayo por inmunoadsorción ligado a enzimas
EN/ENs: Eniatina/Eniatinas
ENB: Eniatina B
ERK p44/42: Quinasa regulada por señales extracelulares
EU: Unión Europea
F: Fluorometría
FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura
FB_1: Fumonisina B_1
FBS: Suero fetal bovino
FC: Citometría de flujo
FITC: Isotiocianato de fluoresceína
FOS: Fructooligosacáridos
FPG: Fluoresceína-más-Giemsa
FS: Fosfatidilserina

FUS: Fusaproliferina
FUS-X: Fusarenona-X
GC: Cromatografía de gases
GES-1: Células de epitelio gástrico humano
GLI: Gliotoxina
GPx: Glutatión peroxidasa
GR: Glutatión reductasa
GSH: Glutatión reducido
GSSG: Glutatión oxidado
GST: Glutatión transferasa
G0: Fase G0 del cilco celular
G1: Fase G1 del ciclo celular
G2: Fase G2 del ciclo celular
Ham-F12: Mezcla de nutrientes F-12
HCl: Ácido clorhídrico
HEK293: Células de riñón de embrión humano
HEPES: 4- (2-hidroxietil) -1-piperazinetanosulfónico
HL-60: Células de carcinoma de leucemia promielocítica
HepG2: Células de carcinoma de hígado humano
Hep3B: Células de carcinoma de hígado humano
Het-1A: Células de epitelio de esófago humano
$HO^{\bullet}$: Radical hidroxilo
HPLC: Cromatografía líquida de alta resolución
HPRT: enzima hipoxantina-guanina
HT-2: Toxina HT-2
HyLv: Ácido hidroxivalérico
H_2O_2: Peróxido de hidrógeno
IARC: Centro Internacional de Investigaciones sobre el Cáncer
IC_{50}: Concentración que inhibe la viabilidad celular al 50%
JC-1: ioduro de 5,5', 6,6'-tetracloro-1,1', 3,3' tetraetilbenzimidazolilcarocianina
JECFA: Comité Mixto de Expertos en Aditivos Alimentarios
KCl: Cloruro de potásio
LC-DAD: Cromatografía líquida con detector de diodos
LC-MS/MS: Cromatografía líquida acoplada a espectrometría de masas en tándem

LC-MS-LIT: Cromatrografía líquida acoplada a espectrometría de masa con trampa de iones
LDH: Lactato deshidrogenasa
LH: Linfocitos humanos
LLC-PK1: Células renales
LMP: Agarosa de bajo punto de fusión
$LOO^{\bullet}$: Radical lipoperoxilo
LOOH: Lipoperóxido
LPO: Peroxidación lipídica
MAPK: Proteínas quinasas activadas por mitógenos
MDA: Malondialdehído
MEFs: Fibroblastos de embrión de ratón
MeOH: Metanol
MF: Microscopía de fluorescencia
MN: Micronúcleos
MNBN: Linfocitos humanos con citoquinesis bloqueada
MON: Moniliformina
MRM: Monitorización de reacción múltiple
m/z: Relación masa carga
MTT: 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio
NAC: N-acetilcisteína
NaCl: Cloruro de sodio
NaH_2PO_4: Fosfato de dihidrógeno de sodio
$NaHCO_3$: Bicarbonato sódico
NaOH: Hidróxido de sodio
$NaSO_4$: Sulfato de sodio
NB: brotes nucleares
NEO: Neosolaniol
NF-κB factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
NIV: Nivalenol
$NO^{\bullet}$: Radical óxido nítrico
NPBs: Puentes nucleoplamáticos
NR: Rojo neutro
OMS: Organización mundial de la Salud
$ONOO^-$: Peroxinitrito

OPT: O-ftaldehído
ORAC: Capacidad de absorción de radicales libres de oxígeno
OT: Ocratoxina
OTA: Ocratoxina A
$O_2^{\bullet-}$: Radical superóxido
O_3: Ozono
PAT: Patulina
PBS: Tampón fosfato salino
PCL: Fotoquimioluminiscencia
Phe: Fenilalanína
PI: Ioduro de propidio
PIA: Ácido penicílico
PK15: Células de riñon de porcino
PS: Fosfatidilserina
p21/ waf1: Inhibidor quinasa p21
p53: Proteína supresora de tumores p53
Rh123: Rodamina 123
RNA: Ácido ribonucléico
RNS: Especies reactivas de nitrógeno
ROS: Especies reactivas de oxígeno
$ROO^{\bullet}$: Radical peroxilo
RQC: Roquefortina C
RSV: Resveratrol
RT: Tiempo de retencion
SCE: Intercambio de cromátidas hermanas
SOD: Superóxido dismutasa
SRB: Sulforodamina B
STE: Esterigmatocistina
T-2: Toxina T-2
TBA: Ácido tiobarbitúrico
TBARS: Sustancias reactivas con el ácido tiobarbitúrico
TCF: Tiocianato férrico
TeA: Ácido tenuazónico
TEAC: Capacidad antioxidante equivalente de Trolox
TMRE: Tetrametilrodamina etil éster
TMRM: tetrametilrodamina metil éster

TNF: Tumor necrosis factor
TOSC: Capacidad total de atrapamiento de oxi-radicales
TRAP: Potencial total de atrapamiento de radicales peróxido
UE: Unión Europea
Vero: Fibroblastos de riñón de mono verde africano
V79: Fibroblastos de pulmón de hámster chino
WHO: Organización mundial de la Salud
Y79: Células de retinoblastoma humano
ZEA: Zearalenona
$\Delta\Psi m$: Potencial de membrana mitocondrial
$^1O_2$: Singlete de oxígeno
3AcDON: 3-acetil-deoxinivalenol
4-HNE: 4 hidoxi-2-noenal
7-AAD: 7 aminoactinomicina D
15AcDON: 15-acetil-deoxinivalenol

RESUMEN

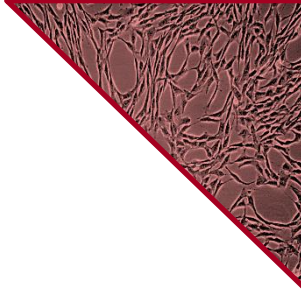
Las micotoxinas son contaminantes químicos naturales presentes en los alimentos y piensos que representan un problema preferente económico y de salud pública. El conocimiento de los efectos tóxicos y exposición a las micotoxinas permite caracterizar los riesgos de la población. Se ha evaluado la toxicidad de la beauvericina, esterigmatocistina y patulina de forma individual y combinada mostrando efectos citotóxicos. Se han observado efectos sinérgicos a bajas dosis y aditivos a dosis altas de exposición en las células CHO-K1. La exposición de las células CHO-K1 a beauvericina pone de manifiesto disrupción del ciclo celular, pérdida del potencial de membrana mitocondrial, incremento del daño al DNA y del estrés oxidativo, que podría estar relacionado con la muerte celular y explicar el posible mecanismo de acción del efecto citotóxico de la beauvericina en las células CHO-K1. Se ha evaluado el papel protector de los sistemas de defensa frente a esta micotoxina observándose una disminución de los niveles de glutatión así como un aumento en la actividad de las enzimas glutatión peroxidasa, glutatión transferasa, superóxido dismutasa y catalasa, que actúan como mecanismos de defensa. La disminución de la actividad glutatión reductasa podría relacionarse con un desequilibrio en la regeneración del glutatión y como consecuencia en un incremento del estrés oxidativo. Se ha confirmado el efecto protector de la N-acetilcisteína atribuible al promover la síntesis de glutatión y bloquear los compuestos tóxicos. Se ha determinado el efecto del resveratrol, un polifenol de grupo de los estilbenos conocido por sus múltiples propiedades biológicas y se observa un efecto protector frente a la citotoxicidad, la producción intracelular de especies reactivas de oxígeno y peroxidación lipídica producidas por la beauvericina en las células CHO-K1. Asimismo, teniendo en cuenta que la ingesta de alimentos es la principal

fuente de exposición a micotoxinas, se ha evaluado la influencia de fibras dietéticas y probióticos en la bioaccesibilidad de la beauvericina mediante un modelo de digestión *in vitro* estático, observándose en ambos casos una atenuación mediante la disminución de la bioaccesibilidad de la beauvericina. Estos resultados indican que la beauvericina supone un riesgo para el consumidor por lo que son necesarios más estudios toxicológicos que amplíen el conocimiento sobre la prevención de la salud por la exposición a micotoxinas.

SUMMARY

Mycotoxins are secondary metabolites of fungus present in food and feed which cause economic and public health problems. By elucidating mycotoxins' toxicological effects it will alow us to characterize and evaluate their risk assessment. Beauvericin, sterigmatocystin and patulin toxicity were evaluated individually and in combination in CHO-K1 cells. Exposures to low and high dosage revealed synergistic and additive effect, respectively (in CHO-K1 cells). Beauvericin showed cell cycle disruption, loss of mitochondrial membrane potential, increase of DNA damage and increase of oxidative stress, which might be related to cell death and explain the possible cytotoxic mechanism of action in CHO-K1 cells. The protective effect and/or activation of the defense systems against these mycotoxins were also evaluated. A decrease in glutathione levels was observed and an increase in enzymatic activities as for glutathione peroxidase, glutathione transferase, superoxide dismutase and catalase, which all act as a defense mechanism. A decrease in glutathione reductase activity was also observed, and it could be related to an imbalance in the regeneration of glutathione and resulting an increase of oxidative stress. The protective effect of N-acetylcysteine is attributable to promote the synthesis of glutathione and to block the toxic effect of the studied mycotoxins compounds. Resveratrol is a polyphenol belonging to the group of stilbene, known for its biological properties. Protective effect was observed against cytotoxicity, intracellular reactive oxygen species production and lipid peroxidation produced by beauvericin in CHO-K1 cells. Food intake is the main source of exposure to mycotoxins; it was observed that probiotics and dietary fibers decrease the bioavailability of beauvericin. These studies indicate that BEA involves a health risk; therefore, further toxicological

studies are needed to increase knowledge leading to the prevention of health by mycotoxins.



1. Introducción
Introduction

1. INTRODUCCIÓN

1.1. Micotoxinas

El término micotoxina proviene de las palabras griegas *mikes* (hongo) y *toxicum* (veneno). Las micotoxinas son moléculas de gran diversidad estructural y bajo peso molecular (PM<700 Da) producidas como metabolitos secundarios de hongos filamentosos. Estos metabolitos, son producidos normalmente al final de la fase exponencial o al principio de la fase estacionaria del crecimiento del hongo como resultado de una situación de estrés (Pérez et al., 2013).

La ingestión de alimentos contaminados, la inhalación de esporas toxigénicas y el contacto directo de la piel con micotoxinas, puede dar lugar a *micotoxicosis*. Los efectos tóxicos producidos por micotoxinas dependen del tipo de micotoxina, la concentración, el tiempo de exposición, la edad, el sexo, el estado de salud del individuo, los efectos sinérgicos y las interacciones con otros tóxicos. A su vez, la toxicidad causada por las micotoxinas puede ser aguda o crónica; siendo la crónica la más frecuente. La toxicidad aguda se caracteriza por una única exposición a una dosis alta, con efectos inmediatos. Mientras que, la toxicidad crónica se caracteriza por una exposición prolongada a dosis bajas, en la cual los efectos se producen a largo plazo (Bennett y Klich, 2003).

Históricamente, la micotoxicosis más antigua conocida, data de la Edad Media y fue denominada "ergotismo" o "fuego de San Antonio" debido a la sensación de quemazón que experimentaban los afectados. Esta enfermedad fue atribuida al consumo de pan contaminado con micotoxinas producidas por el hongo del cornezuelo del centeno (*Claviceps purpurea*) (Bhat et al., 2010). A pesar de la aparición de varias micotoxicosis a lo largo de la historia, no fue hasta 1960, con la aparición de la denominada "*Turkey X disease*" cuando

comenzó el estudio de las micotoxinas. Esta enfermedad, produjo la muerte repentina de 100.000 pavos, dando lugar al primer estudio de aflatoxicosis aguda; el cual permitió el aislamiento e identificación de las aflatoxinas (AF; Hussein y Brasel, 2001).

Alrededor de 400 micotoxinas han sido identificadas (Bennett y Klich, 2003). La Figura 1 muestra un esquema de las principales micotoxinas identificadas. Los principales géneros productores de micotoxinas son *Aspergillus*, *Fusarium*, *Penicillium, Claviceps* y *Alternaria* (Marin et al., 2013); estando presentes en gran variedad de alimentos y piensos. La Tabla 1 muestra diferentes tipos de micotoxinas, las especies de hongos productores, los alimentos más habitualmente contaminados y los efectos tóxicos que producen dichas micotoxinas (Pereira et al., 2014).

El crecimiento del hongo y la producción de micotoxinas dependen principalmente de las condiciones óptimas de temperatura, oxígeno, pH, actividad de agua, humedad relativa y daño físico en el alimento (Bhat et al., 2010). La producción de micotoxinas en los cultivos agrícolas puede ocurrir en varios puntos de la cadena alimentaria: pre-cosecha, cosecha, secado y almacenamiento (Marin et al., 2013). Bajo diferentes condiciones de crecimiento, una misma especie de hongo puede producir un amplio rango de micotoxinas. Asimismo, una micotoxina puede ser producida por diferentes tipos de hongos (Tabla 1).

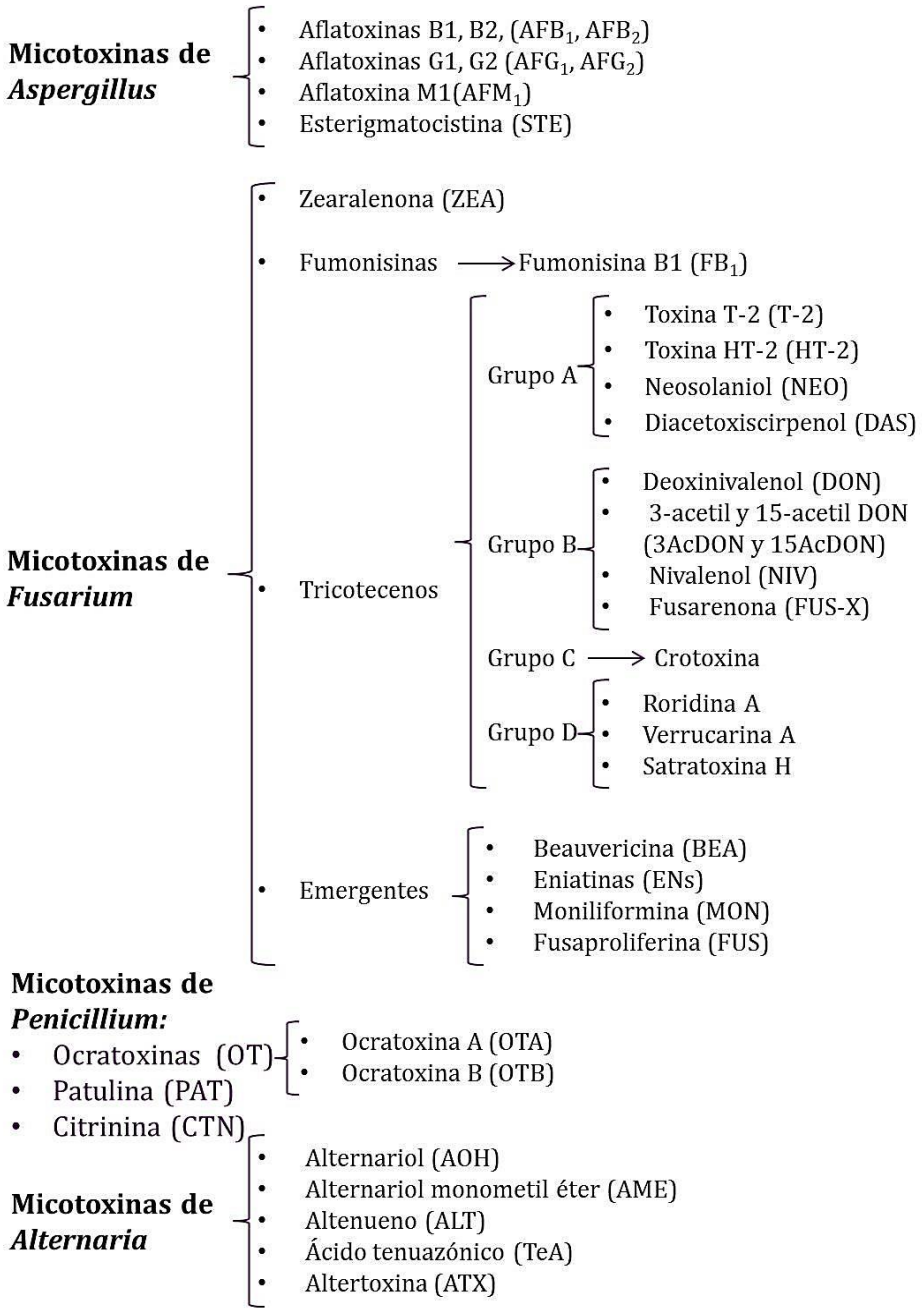


Figura 1. Principales micotoxinas producidas por los géneros de hongos filamentosos más comunes.

Tabla 1. Hongos micotoxigénicos, alimentos implicados y efectos tóxicos de las principales micotoxinas.

MICOTOXINA	HONGOS PRODUCTORES	ALIMENTOS	EFFECTOS TÓXICOS	REFERENCIAS
AFs y STE	*Aspergillus flavus*, *A. parasiticus*, *A. nidulans, A. versicolor, Botryotrichum, Chaetomium, Emericella, Humicola*	Cereales, higos, aceite de girasol, frutos secos, tabaco, queso, leche, granos de café verde, especias, cerveza	Carcinógena, genotóxica, inmunotóxica, hepatotóxica, neurotóxica, daño pulmonar y sobre el sistema reproductor	CAST, 2003; Bennett y Klich, 2003; Richard, 2007; Pereira et al., 2014; IARC, 1976,1993.
Emergentes de *Fusarium* *	*Fusarium poae, F. subglutinans, F. proliferatum, F. avenaceum, F. tricinctum*	Cebada, trigo, arroz, avena, maíz, centeno, productos a base de cereales y frutos secos	Citotóxica, genotóxica, inmunotóxica	CAST, 2003; Jestoi, 2008; Marin et al., 2013; Pereira et al., 2014
FBs	*Fusarium verticilliodes, F. proliferatum, F. nygamai, Alternaria alternata f. sp. lycopersici*	Maíz y productos derivados del maíz	Hepatotóxica, neurotóxica, daño pulmonar y cardiovascular, posible carcinógena	CAST, 2003; Bennett y Klich, 2003; Pereira et al., 2014; IARC, 1993
OTA	*Aspergillus alliaceus, A. auricomus, A. carbonatius, A. glaucus, A. melleus, A. niger, Penicillium verrucosum*	Cebada, trigo, avena, café	Inmunotóxica, neurotóxica, nefrotóxica, teratógena, genotóxica, posible carcinógena	CAST, 2003; Bennett y Klich, 2003; Pereira et al., 2014; IARC, 1993
PAT	*Penicillium expansum Byssochylamys , Aspergillus*	Manzana, productos derivados de manzana, piñones, productos derivados de tomate, cereales.	Daños gastrointestinales, neurotóxica, inmunotóxica, genotóxica, mutagénica,	CAST, 2003; Sarubbi et al., 2016; Sempere Ferre, 2016; Sharma et al., 2015; IARC, 1986
ZEA	*F. graminearum, F. culmoru, F. cerealis, F. equiseti, F. verticillioides, F. culmorum, F. crookwellense, F. semitectum, F. incarnatum*	Cereales y productos a base de cereales	Carcinógena, genotóxica, inmunotóxica, hepatotóxica, daños sistema reproductor y endocrino	CAST, 2003; Bennett y Klich, 2003; Zinedine et al., 2007; Pereira et al., 2014; IARC, 1993

*ver Figura 1

La Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) estima que aproximadamente el 25% de los cereales producidos en el mundo están contaminados por algún tipo de micotoxina (FAO, 2013). Además, en términos de exposición y severidad de enfermedades crónicas, las micotoxinas presentan un riesgo más alto que los contaminantes antropogénicos, pesticidas y aditivos alimentarios (Kuiper-Goodman, 2004). Estos hechos, suponen importantes pérdidas económicas asociadas con su impacto en la salud pública, la productividad animal y el comercio nacional e internacional (FAO, 2003).

1.1.1. Legislación

Con el fin de reducir la exposición de las micotoxinas en la población general, la Unión Europea (UE) y España han establecido diferentes niveles máximos de micotoxinas en los productos alimenticios. El Reglamento (CE) nº 1881/2006 de la Comisión, del 19 de diciembre de 2006, fija el contenido máximo de determinados contaminantes, como las micotoxinas: AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, DON, ZEA, FB_1 y FB_2 en los productos alimenticios. Este reglamento ha sido modificado posteriormente por diferentes reglamentos:

- El Reglamento (UE) 1126/2007, por el que se fija el contenido máximo de micotoxinas de *Fusarium* en el maíz y los productos del maíz.
- Los Reglamentos (UE) 105/2010, 594/2012 y el 2015/1137 por los que se fijan el contenido máximo de OTA en los productos alimenticios.
- Los Reglamentos (UE) 165/2010 y 1058/2012 en lo concerniente al contenido máximo de AFs en los productos alimenticios.
- El Reglamento (UE) 212/2014 de la Comisión, en relación al contenido de CTN en complementos alimenticios basados en arroz fermentado con levadura roja *Monascus purpureus.*

- El Reglamento (UE) 2015/1940 de la Comisión, en relación con el contenido máximo de esclerocios de cornezuelo de centeno en determinados cereales.

Además de estos reglamentos la UE ha establecido diferentes recomendaciones sobre micotoxinas:

- Recomendación 2013/165/UE, sobre la presencia de las toxinas T-2 y HT-2 en los cereales y los productos a base de cereales.
- Recomendación 2006/583/CE, sobre la prevención y la reducción de las toxinas de *Fusarium* en los cereales y los productos a base de cereales.
- Recomendación 2003/598, relativa a la prevención y la reducción de la contaminación por PAT del zumo de manzana y los ingredientes de zumo de manzana en otras bebidas.
- Recomendación 2012/154/UE, sobre el control de la presencia de alcaloides de cornezuelo en los piensos y los alimentos.

Debido a que actualmente no es posible eliminar por completo las micotoxinas de los productos alimenticios, el *Codex Alimentarius* también establece como medida preventiva pautas para reducir el contenido de micotoxinas en cereales (Codex Alimentarius, CAC/RCP 51-2003). Estas pautas se basan en buenas prácticas agrícolas (BPA), que actúan como primera línea de defesa frente a la contaminación por micotoxinas y buenas prácticas de fabricación (BPF), que incluyen la manipulación, el almacenamiento y la distribución de los cereales (Codex Alimentarius, CAC/RCP 51-2003).

El Comité Mixto de Expertos en Aditivos Alimentarios (JECFA), la Organización Mundial de la Salud (OMS) y la FAO, son los organismos reguladores en la UE en temas relacionados con las micotoxinas. En la UE, la Autoridad Europea de Seguridad Alimentaria (EFSA), es la Agencia que

asesora a la Comisión Europea en cuestiones de evaluación del riesgo toxicológico de las micotoxinas.

1.1.2. Beauvericina

La BEA es una micotoxina perteneciente al grupo de micotoxinas emergentes de *Fusarium.* Este tipo de micotoxinas han sido descubiertas en las últimas décadas, por lo que la información disponible sobre ellas es limitada, de aquí el gran interés en su estudio.

La BEA fue aislada por primera vez del hongo entomopatógeno *Beauveria bassiana* (Hamill et al., 1969) y posteriormente de varias especies de hongos del género *Fusarium* (Logrieco et al., 1998; Fotso et al., 2002). La BEA es un hexadepsipéptido cíclico: (D-α-hidroxiisovalerilo-L-N-metil-fenilalanina)$_3$) (Figura 2). La BEA está formada por tres restos de D-α-hidroxiisovalerilo y tres de L-N-metilfenilalanilo en secuencia alterna (Wang et al., 2012) que se unen por enlaces peptídicos y éster lactonas intramoleculares (Figura 2; Jestoi et al., 2008).

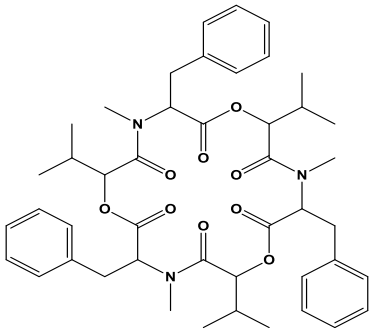


Figura 2. Estructura química de la Beauvericina.

Esta micotoxina es contaminante habitual de cereales como pueden ser el trigo, arroz, maíz, avena y cebada y productos derivados de cereales (Meca et al., 2010a; Mahnine et al., 2011; Juan et al., 2012a,2013; Blesa et al.,

2012; Serrano et al., 2013a,b), huevo (Jestoi et al., 2009), frutos secos (Tolosa et al., 2013), frutas deshidratadas (Tolosa et al., 2013; Azaiez et al., 2014), chufa (Sebastià et al., 2012; Rubert et al., 2012), café (García-Moraleja et al., 2015a,b,c) y hierbas medicinales (Hu y Rychilk, 2014). Y se ha encontrado en diferentes tipos de piensos para animales (Tolosa et al., 2014; Zachariasova et al., 2014; Streit et al., 2013; Warth et al., 2012).

Propiedades biológicas

La actividad insecticida de la BEA ha sido demostrada en *Spodoptera frugiperda* (Fornelli et al., 2004), *Schizaphis graminum* (Ganassi et al., 2002), *Leptinotarsa decemlineata* (Gupta et al., 1991), *Calliphora erytrocephala* y *Aedes aegypti* (Grove and Pople, 1980).

La actividad antibacteriana de la BEA ha sido probada en bacterias Gram-negativas y Gram-positivas. Las bacterias Gram-positivas sensibles a BEA son: *Bacillus cereus* (ATCC 11778), *B. mycoides* (ALH33), *B. pumilus* (LACB101), *B. sphaericus* (ALH103), *B. subtilis, Bifidobacterium adolescentis* (ATCC 15703), *Clostridium perfringens* (ATCC 3624), *Eubacterium biforme* (ATCC 27806), *Enterococcus faecium* (CECT 410), *Listeria monocytogenes* (CECT 935), *Paenibacillus alvei* (ATCC 6344), *Pa. azotofixans* (ATCC 35681), *Pa. macquariensis* (ATCC 23464), *Pa. pulvifaciens* (ATCC 49843), *Pa. validus* (ATCC 43897), *Mycobacterium tuberculosis*, *Peptostreptococcus anaerobius* (ATCC 27337), *Pe. productus* (ATCC 27340) y *Staphylococcus haemolyticus* (Castelbury et al., 1999; Nilanonta et al., 2000; Meca et al., 2010b; Xu et al., 2010). Por otro lado, las bacterias Gram-negativas sensibles a BEA son: *Escherichia coli* (CECT 4782), *Pseudomonas aeruginosa* (CECT 4628), *Ps. lachrymans, Salmonella enterica* (CECT 554), *Shigella dysenteriae* (CECT 584), *Yersinia enterocolitica* (CECT 4054), *Agrobacterium tumefaciens* y *Xanthomonas vesicatoria* (Meca et al., 2010b; Xu et al., 2010).

La BEA tiene propiedades antifúngicas. La combinación de BEA con miconazol y ketoconazol produce un efecto sinérgico frente *Candida albicanis* y *C. parapsilosis*. La BEA potencia la actividad del miconazol frente a *C. albicans* resistente a fluconazol. En combinación con ketoconazol prolonga la supervivencia del huésped infectado con *C. parapsilosis* y la reducción de los recuentos de colonias de hongos en riñones, pulmones y cerebro (Fukuda, et al., 2004; Zhang et al., 2007). La BEA es capaz de inhibir la proliferación del protozoo *Plasmodium falciparum* (Nilanonta et al., 2000). Además, presenta actividad antiviral inhibiendo fuertemente la actividad HIV-1 integrasa (Shin et al., 2009). Al mismo tiempo, la BEA presenta actividad fitotóxica en protoplastos de tomate (Paciolla et al., 2004).

Mecanismo de acción

La BEA presenta actividad ionófora, por lo que es capaz de favorecer el transporte de iones a través de las membranas celulares. Estudios previos, demuestran que la BEA se incorpora a las membranas celulares de mamíferos y membranas sintéticas formando un canal selectivo de cationes de tipo transportador o *carrier* (Ojcius et al., 1991; Kouri et al., 2003). Esta micotoxina puede formar complejos en "*sándwich*" con los cationes. Estos complejos, suelen están formados por un catión y un ionóforo (1:1), aunque también puede implicar dos (1:2) o tres (2:3) moléculas ionóforas (Kouri et al., 2003; Jestoi et al., 2008).

La capacidad ionófora de la BEA en las membranas celulares, tiene un efecto directo en la concentración iónica intracelular de las células de mamífero. La alteración de las concentraciones fisiológicas normales de cationes mono y divalentes podría relacionarse con la toxicidad inespecífica de esta micotoxina. La BEA induce cambios en la homeostasis celular acompañada de hidrólisis de ATP y acidificación celular, lo que en última

instancia puede contribuir a la muerte celular (Kouri et al., 2005; Kamyar et al., 2006).

La BEA incrementa la concentración intracelular de Ca^{2+} en células de mamíferos (Chen et al., 2006). En eritrocitos expuestos a BEA, el incremento del Ca^{2+} intracelular estimula los canales de K^+ con salida de KCl, y pérdida de agua aumentando la contracción celular y eriptosis (Qadri et al., 2011). Este mismo efecto se ha observado en células musculares (Lemmens-Gruber et al., 2000). En los miocitos ventriculares expuestos a BEA, la acumulación iónica (Na^+, Ca^{2+}, Mg^{2+}) acorta la duración del potencial de membrana ($\Delta\Psi m$), la despolarización del $\Delta\Psi m$ en reposo y causa disminución de la contracción muscular pudiendo provocar fenómenos isquémicos (Kouri et al., 2003; Kamyar et al., 2004; Kouri et al., 2005).

La BEA es un inhibidor específico de canales de Ca^{2+} tipo L en la musculatura lisa (Wu et al., 2002) e inhibe la contracción del musculo liso intestinal de cobaya como consecuencia de la despolarización (Nakajyo et al., 1987).

Por otra parte, la BEA es uno de los inhibidores más potentes y específicos de la enzima ACAT (acil-CoA:colesterol aciltransferasa). Esta enzima cataliza la conversión de colesterol celular y de cadena larga a ésteres de colesterol, con un papel importante en la aterogénesis y en la absorción de colesterol desde el intestino (Tomoda et al., 1992).

La BEA disminuye la proliferación celular a través de la disminución de la fosforilación de las proteínas quinasas activadas por mitógenos (MAPK) y la quinasa regulada por señales extracelulares (ERK p44/42), la cual es prerrequisito para la activación de MAPK (Wätjen et al., 2014). Además, inhibe de forma selectiva el proto-oncogén-proteína tirosina quinasa *src* después del screening de proteínas quinasas *in vitro* (Wätjen et al., 2014).

La BEA afecta al sistema inmune, ya que interfiere en la diferenciación de monocitos en células dendríticas inmaduras e incrementa la secreción de interleuquina-10 (IL-10, Ficheux et al., 2013). Decrece un factor de necrosis tumoral (TNF)-α y el interferón-γ en ratones con colitis, lo que podría ser consecuencia de que esta micotoxina decrece la actividad de las células T (Wu et al., 2013); e inhibe el factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-κB) implicado en la respuesta inflamatoria e inmune, así como en la proliferación y muerte celular (Sun y Andersson, 2002a).

La BEA se une de forma no covalente al DNA. Sin embargo, según Pócsfalvi (1997), el daño producido a nivel de la membrana nuclear puede favorecer la entrada de la BEA en el núcleo celular formando aductos con el DNA. Además, la BEA inhibe la actividad enzimática de las topoisomerasas I y II, necesarias para resolver problemas durante la replicación, transcripción y recombinación del DNA (Dornetshuber et al., 2009a).

Entre los mecanismos de acción, se le atribuye su interacción con las proteínas de transporte ABCC1 (proteína resistente a múltiples fármacos) y ABCG2 (proteína resistente a múltiples fármacos contra el cáncer de mama) modificando la biodisponibilidad, la farmacocinética y la eficacia/toxicidad de fármacos y xenobióticos (Dornetshuber et al., 2009b).

Efectos tóxicos

Los ensayos de toxicidad aguda *in vivo* con BEA son muy limitados y no se han realizado estudios de toxicidad crónica y subcrónica hasta la fecha (EFSA, 2014). No se dispone de datos respecto a carcinogenicidad, neurotoxicidad y estudios de desarrollo y reproducción con esta micotoxina (EFSA, 2014), pero los ensayos *in vitro* disponibles demuestran que la BEA produce citotoxicidad, inmunotoxicidad y genotoxicidad (EFSA, 2014), ya que

produce aberraciones cromosómicas (CA), micronúcleos (MN), intercambios de cromátidas hermanas (SCE) y daño al DNA (Klarić et al., 2008a, 2010; Çelik et al., 2010; Prosperini et al., 2013a; EFSA, 2014).

1.1.3. Esterigmatocistina

Gran variedad de especies fúngicas de *Aspergillus*, *Botryotrichum, Chaetomium*, *Emericella* y *Humicol*a producen esterigmatocistina (STE) (Fig. 3), aunque las especies más comunes son *Aspergiullus flavus, A. parasiticus*, *A. versicolor* y *A. nidulans* (Tabla 1) (EFSA, 2013).

La STE (3a,12c-dihidro-8-hidroxi-6-metoxi-7H-furol[3',2':4,5]furo[2,3-c]xanten-7-ona) es un precursor de la biosíntesis de la AFB_1 (CAST, 2003). Esta micotoxina se encuentra en cereales, frutos secos, granos de café verde, especias, cerveza y queso (EFSA, 2013).

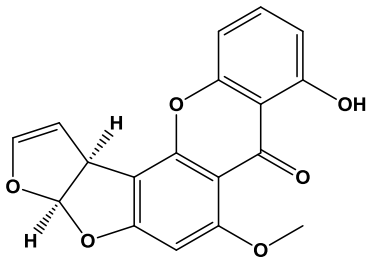


Figura 3. Estructura química de la Esterigmatocistina.

Mecanismos de acción

La STE puede atravesar fácilmente las membranas biológicas (Liu et al., 2014). Una vez dentro del núcleo celular es capaz de unirse al DNA formado aductos STE-DNA, los cuales pueden causar efectos genotóxicos (Jaksic et al., 2012; EFSA, 2013; Gao et al., 2015). Además, la STE es un inhibidor de la ACAT selectiva, específicamente de la isoenzima ACAT2 (Sakai et al., 2008).

Efectos tóxicos

Los órganos diana de la STE son el hígado y los riñones por lo que es hepato- y nefrotóxica (EFSA, 2013). La toxicidad crónica producida por la STE, da lugar a efectos teratógenos, carcinógenos y mutágenos (CAST, 2003). Se ha demostrado que la STE es mutágena en células de mamíferos y bacterias; y carcinógena en rata, ratón, jerbo de Mongolia, mono y peces (EFSA, 2013). Estos hechos, han originado que el Centro Internacional de Investigaciones sobre el Cáncer (IARC) clasifique esta micotoxina en el grupo 2B como posible carcinógeno para humanos (IARC, 1976).

1.1.4. Patulina

La PAT (4-hidroxi-4H-furo [3,2c]piran-2-(6H)-ona) es una lactona heterocíclica insaturada (Fig. 4). Este metabolito secundario lo producen principalmente hongos de los géneros *Penicillium*, *Byssochylamys* y *Apergillus*. Se encuentra de manera natural en frutas como uvas, peras, melocotones y en bayas. Aunque, principalmente se encuentra en manzanas y derivados de estas (Tabla 1) (Bennet y Klich, 2003; SCOOP Task 3.2.8).

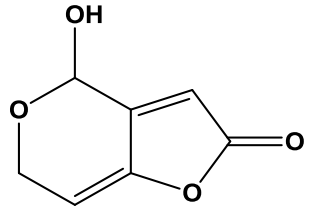


Figura 4. Estructura química de la Patulina.

Propiedades biológicas

La PAT fue aislada por primera vez en *Penicillium grisefulyum* en la década de los 40 como principio activo antimicrobiano. Sin embargo, no fue hasta la década de los 60 cuando fue clasificada como micotoxina (Bennet y

Klich, 2003). La PAT posee propiedades antibióticas frente a bacterias Gram positivas y Gram negativas siendo la más sensible la bacteria Gram positiva, *Bacillus brevis.* Además, posee actividad antifúngica y propiedad antriprotozoaria frente a *Tetarhymena pyriformis* (JECFA, 1996; Soriano et al., 2007).

Mecanismos de acción

La PAT es una molécula electrófila, con una fuerte afinidad por los grupos sulfhidrilos con los que forma enlaces covalentes. Este comportamiento puede inhibir enzimas con grupos sulfhidrilo en su sitio activo, afectar a proteínas que contienen cisteína y disminuir los niveles de glutatión (GSH; JECFA, 1996; Zhou et al., 2010). Además, la PAT inhibe la RNA polimerasa, la aminoacil-t RNA sintetasa, las ATPasas dependientes de Na^+/K^+ y la ureasa. La PAT se considera un agente clastogénico; interacciona directamente con el DNA inhibiendo la traducción y transcripción, con el RNA inhibiendo la síntesis de proteínas, la síntesis de DNA y la producción del interferón γ (Liu et al., 2003, 2007; Moake et al., 2005; Soriano et al., 2007; Wu et al., 2008; Zhou et al., 2010; Glaser y Stopper, 2012)

Efectos tóxicos

La mayoría de los estudios de toxicidad realizados se han llevado a cabo en modelos *in vitro*, mientras que solo unos pocos son *in vivo*.

La intoxicación aguda por PAT produce náuseas, edema pulmonar, distensión del tracto gastrointestinal, hemorragias e inflamación intestinal (Moake et al., 2005). La PAT inhibe las funciones de los macrófagos y altera la función de la barrera intestinal (Puel et al., 2010). Además, la PAT produce lesiones anatomopatológicas graves en vísceras (FAO, 2001). En cuanto a la toxicidad crónica, se ha demostrado que la PAT es neurotóxica, inmunotóxica,

inmunosupresora, teratógena y carcinógena (Soriano et al., 2007). La IARC clasifica esta micotoxina dentro del grupo 3, como no clasificable como carcinógeno para los humanos (IARC, 1986).

1.2. Estudios toxicológicos *in vitro*

De acuerdo a las directrices europeas, Directiva 86/609/CEE y posteriormente la Directiva 2010/63/CE y la normativa española (RD 53/2013) los estudios de toxicidad se planifican de acuerdo a los principios básicos de las "tres erres": Reemplazo de los procedimientos que emplean animales por otros que no los precisen (como las técnicas *in vitro*); Reducción en el número de animales utilizados (cuando se requieran) y; Refinamiento de los métodos usados (para evitar el sufrimiento del animal). Por ello, la utilización de líneas celulares establecidas, como método alternativo *in vitro* para determinar la citotoxicidad y mecanismos de toxicidad de las micotoxinas cumple con la legislación establecida. Entre los avances producidos en los métodos experimentales *in vitro* destaca la mejora en los cultivos debido al empleo de factores de crecimiento, matrices, cocultivos y microagregados (Repetto et al., 2006).

El estudio *in vitro* de los efectos tóxicos de los compuestos químicos se lleva a cabo por dos tipos de mecanismos, referidos como citotoxicidad general y citotoxicidad órgano-específica. Los mecanismos de citotoxicidad general se deben a las interacciones de las micotoxinas y/o sus metabolitos con las funciones basales celulares del organismo. La citotoxicidad basal es un mecanismo común de toxicidad general que puede causar efectos tóxicos tanto a dosis altas como bajas. Para determinar este tipo de citotoxicidad es indiferente el tipo de línea celular utilizada. Sin embargo, la toxicidad órgano-específica se basa en la interacción concreta entre la micotoxina y un órgano

diana, debido a su mayor susceptibilidad y sensibilidad con el órgano en concreto (Repetto y Repetto, 2009).

Las ventajas de la utilización de cultivos celulares para las investigaciones toxicológicas, pueden resumirse en: rapidez, mayor control de las condiciones experimentales (se evitan las variaciones que presenta el sistema animal debido a la presencia de hormonas, estrés, etc.), mayor versatilidad (se pueden eliminar o adicionar complementos al medio de cultivo tales como hormonas, aminoácidos, etc.), mayor reproducibilidad y reducción de la variabilidad entre experimentos (las poblaciones son más homogéneas), más económico y por último una ventaja ética.

Las células expuestas a un compuesto citotóxico pueden responder de múltiples maneras: perdiendo la integridad de membrana, funciones celulares, cambiando su morfología y adhesión, alterando su capacidad mitótica, o activando mecanismos de muerte celular. A continuación se expone cada uno de estos aspectos:

1.2.1. Viabilidad celular y proliferación

La proliferación y la viabilidad celular son buenos indicadores del funcionamiento celular. Se ha demostrado que las micotoxinas disminuyen la viabilidad y la proliferación celular en diversas líneas celulares.

Viabilidad celular

Los métodos para determinar la viabilidad celular se basan principalmente en evaluar la integridad, la capacidad metabólica y la división celular. Entre los principales métodos para determinar la viabilidad celular se incluyen:

- *Citometría de flujo con el fluorocromo ioduro de propidio* (PI): Este fluoróforo es capaz de teñir los ácidos nucleicos de doble cadena de células muertas.
- *Azul tripán*: Es un colorante capaz de penetrar en células cuya membrana está dañada tiñendo las células no viables.
- *Lactato deshidrogenasa* (LDH): Esta enzima está presente en el citoplasma de células vivas. Sin embargo, en células no viables se libera al medio de cultivo celular al permeabilizarse la membrana. El incremento de la LDH en el sobrenadante del cultivo es proporcional al número de células lisadas.
- *Sal de tetrazolio* (MTT): La sal de tetrazolio (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio) soluble (amarilla) se convierte en formazan insoluble (púrpura) por las enzimas succinato deshidrogenasas mitocondriales. Sólo las células viables conservan la capacidad de efectuar esa reacción. La cantidad de células vivas es proporcional a la cantidad e formazán producido.
- *Rojo neutro* (NR): El colorante (3-amino-7 dimetilamino-2 metilfenazina) se incorpora a los lisosomas y endosomas de células vivas. Por lo que únicamente las células viables son capaces de retener el colorante en su interior.
- *Azul alamar:* La resazurina (azul), penetra dentro de las células y se reduce a resorufina (de color rojo fluorescente) en células viables. La cantidad de fluorescencia es proporcional al número de células vivas.
- *Azul Comassie o azul de Kenacid*: Mide la cantidad de proteínas totales existente en células procedentes del ensayo del NR o MTT.
- *Sulforodamina B* (SRB): Presenta afinidad por los aminoácidos básicos de las proteínas, fijándose selectivamente y proporcionado un índice del contenido de proteínas celulares.

Estos ensayos permiten obtener el valor de la IC_{50} o concentración que inhibe la viabilidad celular al 50% en la población celular de estudio expuesta a micotoxinas como la BEA (Tabla 2), la STE (Tabla 3) y la PAT (Tabla 4).

Tabla 2. Valores de IC_{50} de la beauvericina obtenidos por diferentes ensayos *in vitro*.

Línea celular	Tipo de célula	Método	Tiempo de exposición (h)	IC_{50} (µM)	Referencia
A549	Carcinoma de pulmón humano	SRB	72	1.43 ± 0.27	Lee et al., 2008
		MTT	24	4.5 ± 0.35	Lin et al., 2005
BC-1	Cáncer de mama humano	SRB	24	3.3-15 µg/mL	Nilanonta et al., 2002
Caco-2	Carcinoma de colón humano	MTT	24	20.6 ± 6.9	Prosperini et al., 2013a
			48	12.8 ± 4.8	
			72	3.2 ± 1.1	
		NR	24	8.8 ± 0.9	
			48	3.4 ± 0.9	
			72	1.9 ± 0.7	
CCRF-CEM	Leucemia humana	MTT	24	2.46 ± 0.12	Jow et al., 2004
CHO-K1	Ovario de hámster chino	NR	24	17.22 ± 1.20	Ruiz et al., 2011a
			48	6.20 ± 0.06	
			72	3.80 ± 0.18	
		NR MTT	24	17.22 ± 1.20 12.08 ± 1.10	Ferrer et al., 2009
C6	Glioma de rata	MTT	4	7.5*	Wätjen et al., 2014
			24	1.0*	
GLC-4	Adenocarcinoma de mama	MTT	72	1.88 ± 0.03	Dornetshuber et al., 2009b
GLC-4/adr	Sublinea celular sobreexpresada de carcinoma célular de células pequeñas	MTT	72	2.06 ± 0.29	Dornetshuber et al., 2009b
H4IIE	Hepatoma de rata	MTT	4	3.0*	Wätjen et al., 2014
			24	1.9*	
HCT-15	Carcinoma de colón humano	SRB	72	1.86 ± 0.12	Lee et al., 2008

Línea celular	Tipo de célula	Método	Tiempo de exposición (h)	IC_{50} (µM)	Referencia
HCT116	Carcinoma de colón humano	MTT	24	2.4*	Wätjen et al., 2014
			72	3.7	Dornetshuber et al., 2009a
HCT116 +chr3	Carcinoma de colón humano	MTT	72	3.6	Dornetshuber et al., 2009a
Hep-G2	Carcinoma hepático humano	MTT	24	3.6*	Wätjen et al., 2014
		Azul Alamar BrdU	24	8.8-22.2 1.4-4.0	Ivanova et al., 2006
HL-60	Carcinoma de leucemia promielocítica	MTT	72	2.27 ± 0.04	Dornetshuber et al., 2009b
		Azul tripan	24	≈15 *	Calò et al., 2004
HL-60/vinc	Sublínea celular ABCB1	MTT	72	2.19 ± 0.02	Dornetshuber et al., 2009b
HL-60/adr	Sublínea celular ABCC1	MTT	72	3.23 ± 0.74	Dornetshuber et al., 2009b
HT-29	Carcinoma de colón humano	MTT	24 48	15.0 ± 6.9 9.7 ± 4.4	Prosperini et al., 2012
KB-3-1	Carcinoma epidérmico	MTT	72	2.85 ± 1.02	Dornetshuber et al., 2009b
KB		SRB	24	10->20 µg/mL	Nilanonta et al., 2002
KBC-1	Sublínea celular ABCB1	MTT	72	3.79 ± 0.20	Dornetshuber et al., 2009b
MCF-7	Cáncer de mama humano	MTT	72	1.81	Zhan et al., 2007
MDA-MB-231	Epiteliales alveolares	MTT	72	2.4 ± 0.79	Dornetshuber et al., 2009b
MDA-MB-231/bcrp	Sublínea celular ABCG2	MTT	72	4 ± 0.76	Dornetshuber et al., 2009b
MIA Pa Ca-2	Carcinoma pancreático humano	MTT	72	1.66	Zhan et al., 2007
MRC-5	Fibroblastos de pulmón fetal	Azul alamar BrdU	24	4.7-5.0 0.6-1.1	Ivanova et al., 2006
NCI-H460	Cáncer de pulmón humano	MTT	72	1.41	Zhan et al., 2007
NSCLC A549	Cáncer de pulmón humano	MTT	24	4.5 ± 0.35	Lin et al., 2005
PK15	Riñón de porcino	MTT	24	5.0 ± 0.6	Klarić et al., 2010

Línea celular	Tipo de célula	Método	Tiempo de exposición (h)	IC_{50} (µM)	Referencia
PK15	Riñón de porcino	Azul tripan	48	0.5-5#	Klarić et al., 2007
		LDH	48	5#	Klarić et al., 2008b
		Azul alamar	24	12 *	Uhlig et al., 2005
SF-268	Astrocitoma anaplásico	MTT	72	2.29	Zhan et al., 2007
SK-OV-3	Carcinoma de ovario humano	SRB	72	1.39 ± 0.16	Lee et al., 2008
SK-MEL-2	Melanoma humano	SRB	72	1.47 ± 0.17	Lee et al., 2008
SW1537	Carcinoma de pulmón	MTT	72	3.17 ± 0.03	Dornetshuber et al., 2009b
SW1537/ 2R120	Sublinea celular sobreexpresada de carcinoma célular de células pequeñas	MTT	72	3.53 ± 0.74	Dornetshuber et al., 2009b
SW1537/ 2R160	Sublínea celular ABCB1	MTT	72	3.00 ± 1.1	Dornetshuber et al., 2009b
U-937	Linfoma monocítico	Azul tripan	24	≈30 **	Caló et al., 2004
Vero	Fibroblastos de riñón de mono verde africano	MTT NR	24, 48, 72	6.25-10.02 6.77-11.08	Ruiz et al., 2011b
		SRB	24	4.4-10 µg/mL	Nilanonta et al., 2002
V79	Fibroblastos de pulmón de hámster chino	NR	48	1.6±0.4	Behm et al., 2012
XPA, XPAcorr, XPD and XPDcorr	Fibroblastos de piel	MTT	72	2.0	Dornetshuber et al., 2009a
Y79	Retinoblastoma humano	MTT	24	0.5-10	Cheng et al., 2009

BrUd=5-bromo-2-desoxiuridina; IC_{50}= concentración que inhibe la viabilidad celular al 50%; LDH= lactato deshidrogenasa; MTT= sal de tetrazolio; NR= Rojo Neutro; SRB= sulforodamina B; * valor expresado como EC_{50} = concentración que muestra efecto en el 50% de la población celular; ** valor expresado como CC_{50}= concentración citotóxica en el 50% de la población celular; #= porcentaje respecto del control.

Tabla 3. Valores de IC_{50} de la esterigmatocistina obtenidos por diferentes ensayos *in vitro.*

Línea celular	Tipo de célula	Método	Tiempo de exposición (h)	IC_{50} (µM)	Referencia
A549	Carcinoma de pulmón humano	NR	24	3.7	Bünger et al., 2004
Hep3B	Carcinoma de hígado humano	MTT	24 48	58 ± 3.1 22 ± 0.7	Anninou et al., 2014
Hep-G2	Carcinoma hepático humano	NR	24	286.1	Bünger et al., 2004
		SRB	24	7.3	Liu et al., 2014
L-929	Tejido conectivo adiposo de ratón	NR	24	163.3	Bünger et al., 2004
Neuro-2A	Neuroblastoma de ratón	NR	24	40.1	Bünger et al., 2004

IC_{50}= concentración que inhibe la viabilidad celular al 50%; MTT= sal de tetrazolio; NR= Rojo Neutro; SRB= sulforodamina B.

Tabla 4. Valores de IC_{50} de la patulina obtenidos por diferentes ensayos *in vitro.*

Línea celular	Tipo de célula	Método	Tiempo de exposición (h)	IC_{50} (µM)	Referencia
CHO-K1	Ovario de hámster chino	MTT NR	24	0.69 ± 0.03 4.40 ± 0.70	Ferrer et al., 2009
HEK293	Riñón de embrión humano	MTT	8	12	Zhang et al., 2015
Hep-G2	Carcinoma hepático humano	MTT	24	15	Ayed Boussema et al., 2011
V79	Fibroblastos de pulmón de hámster chino	NR	48	17 ± 2.0	Behm et al., 2012

IC_{50}= concentración que inhibe la viabilidad celular al 50%; MTT= sal de tetrazolio; NR= Rojo Neutro

Combinación de micotoxinas

La presencia de diversos tipos de micotoxinas de manera simultánea en alimentos y materias primas es común en la naturaleza. Este hecho, se puede atribuir a que los hongos micotoxigénicos son capaces de producir más de una micotoxina; a que los alimentos pueden estar contaminados por diversas especies de hongos y a que los productos alimenticios pueden estar compuestos por una mezcla de materias primas. Estos hechos, incrementan la preocupación de la exposición a mezclas de micotoxinas en la evaluación del riesgo humano, ya que, estas podrían ejercer un mayor efecto tóxico o carcinógeno que las mismas de manera individual (Speijers y Speijers, 2004; Kouadio et al., 2007).

La acción combinada de micotoxinas, puede generar un efecto aditivo, sinérgico o antagónico. El efecto aditivo ocurre cuando el efecto combinado de micotoxinas es igual a la suma del efecto de cada micotoxina de manera individual. El efecto sinérgico se presenta cuando el efecto total de la combinación es superior a la suma de sus efectos individuales y el efecto antagónico disminuye los efectos producidos de manera individual.

La mayoría de los estudios de evaluación toxicológica de micotoxinas, reglamentos y directrices establecidos por las autoridades contemplan las micotoxinas de manera individual (apartado 1.1.1). Sin embargo, en la última década ha aumentado la preocupación por una exposición simultánea a diferentes micotoxinas y sus posibles efectos sobre la salud. Por ello, se han realizado diversos estudios que evalúan las interacciones toxicológicas entre la BEA, STE y PAT *in vitro* (Tabla 5).

Tabla 5. Combinación de las micotoxinas BEA, STE y PAT en diferentes tipos de células.

Combinación de micotoxinas	Tipo de célula	Referencia
BEA+DON+T-2	CHO-K1	Ruiz et al., 2011a
BEA+DON+T-2	Vero	Ruiz et al., 2011b
BEA+OTA+FB_1	PK15	Klaric et al., 2007; 2008a,b; 2010
BEA+DON+ENB	Células hematopoyéticas	Ficheux et al., 2012
STE+CTN+OTA	Hep3B	Anninou et al., 2014
PAT+CTN+OTA+OTB	LLC-PK1	Heussner et al., 2006
PAT+CTN+CPA+OTA+PIA+RQC	Linfocitos de porcino	Keblys et al., 2004
PAT+OTA+GLI+CTN	Células T humanas	Tammer et al., 2007

BEA= beauvericina; CHO-K1= células de ovario de hámster chino; CPA= ácido ciclopiazónico; CTN= citrinina; DON= deoxinivalenol; ENB= eniatina B; FB_1 =fumonisina B_1; GLI= gliotoxina; Hep3B= células de carcinoma de hígado humano; LLC-PK1= células renales; OTA= ocratoxina A; PIA= ácido penicílico; PK15= células de riñón de porcino; RQC= roquefortina C; STE= esterigmatocistina; T-2= toxina T2; Vero= fibroblastos de riñón de mono verde africano.

Para determinar el nivel de interacción entre uno o más compuestos se han descrito diversos enfoques matemáticos, siendo el análisis del isobolograma uno de los más utilizados en las últimas décadas. Este método, es útil en el análisis de los datos obtenidos y permite determinar el tipo de interacción entre las diferentes combinaciones de micotoxinas mediante la ecuación: efecto medio/índice de combinación (CI), descrita por Chou (2006) y Chou y Talalay (1984). Esta ecuación se basa en el principio que demuestra que existe una relación entre la dosis de compuesto y el efecto producido, independientemente del número de compuestos y de sus mecanismos de acción o inhibición. Para llevar a cabo este método se realizan las curvas dosis efecto para cada compuesto y sus combinaciones con diferentes

concentraciones a las que se aplican diversas fórmulas matemáticas. Chou y Talalay (1984) introdujeron el término CI para la cuantificación del sinergismo, antagonismo y adición entre dos compuestos; donde, CI< 1, =1 y >1 indican efecto sinérgico, aditivo y antagonista, respectivamente.

Proliferación celular

La proliferación celular es el proceso que resulta en un aumento del número de células, y se define por el equilibrio entre las divisiones celulares y la pérdida de células a través de la muerte celular o la diferenciación. Este proceso se lleva a cabo mediante una serie ordenada de eventos que en conjunto forman el ciclo celular. El ciclo celular está formado por 4 fases (Fig. 5):

- Fase G1: intervalo entre la fase M y la fase S, primera fase de crecimiento en la cual la célula se prepara para la síntesis del DNA.
- Fase S: fase de síntesis del DNA, donde la célula duplica su material genético.
- Fase G2: intervalo entre la fase S y la fase M. Segundo período de crecimiento en el cual las células se preparan para la mitosis.
- Fase M: mitosis.

Diversos factores internos o externos a la célula, pueden perturbar la progresión del ciclo celular. Para mantener la integridad genómica, diferentes puntos de control son activados o inhibidos, induciendo la parada del ciclo celular para reparar el daño; o si la lesión excede la capacidad para ser reparado, la célula activa la muerte celular por apoptosis (Damia y Broggini, 2003). Estos puntos de control, proporcionan una barrera frente al desarrollo del cáncer.

- Punto de control o punto de restricción G1: Este punto controla la entrada de la célula en la fase S desde la fase G1. Dependiendo de las condiciones internas y externas celulares, continúa o no el ciclo celular. La parada en la fase G1 permite la reparación del daño al DNA y que no se replique el DNA dañado. Las células pueden permanecer en esta fase de manera temporal o permanente, entrando en una fase de reposo denominada fase G0. Cuando se detiene el ciclo celular en esta fase, las células se diferencian o quedan quiescentes (reposo).
- Punto de control o punto de restricción G2: Este punto detecta el DNA no replicado evitando el inicio de la fase M antes de que finalice la fase S. La parada en la fase G2 se debe a una respuesta a daños en el DNA para ser reparado y no transmitido a las células hijas.

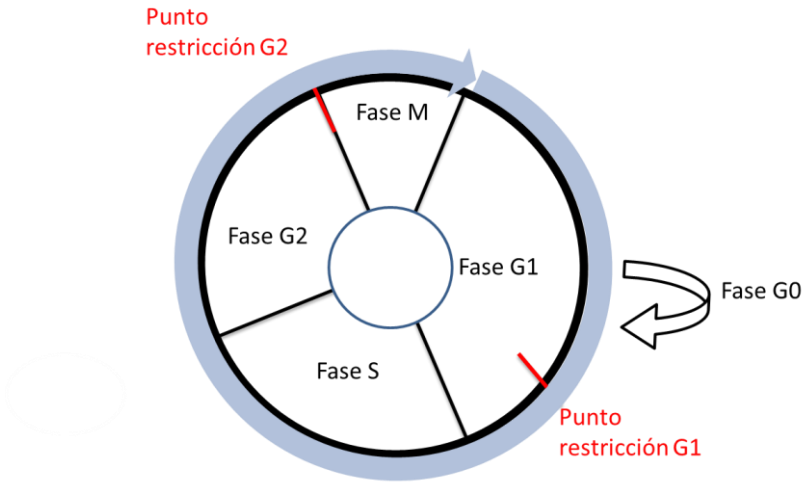


Figura 5. Esquema del ciclo celular.

El análisis del ciclo celular requiere la identificación de las diferentes fases. Las células mitóticas se pueden distinguir al microscopio, pero las

células que se encuentren en la fase G1, G2 y S, deben ser identificadas por criterios bioquímicos. Las células de la fase S pueden ser identificadas fácilmente porque son capaces de incorporar timidina radiactiva, ya que la timidina es utilizada para la síntesis del DNA. El análisis del ciclo celular en células individuales también se puede llevar a cabo mediante inmunofluorescencia e inmunohistoquímica (Pines, 1997; Scott et al., 2005). La 5-bromo-2-desoxiuridina (BrdU), es un nucleótido sintético análogo de la timidina, capaz de sustituirla en la fase de síntesis celular. Por tanto, es una manera eficaz de medir la proliferación celular. La detección de esta incorporación se realiza por inmunoensayo, usando anticuerpos monoclonales directamente sobre la BrdU. Sin embargo, la metodología más frecuente en el análisis del ciclo celular, tras la exposición a micotoxinas, es la determinación del contenido de DNA por citometría de flujo mediante la cual se clasificaría la población celular en 4 fases: Sub G_0/G_1, G_0/G_1, S y G_2/M (Figura 6; Juan-García et al., 2013a). Para ello, se utiliza PI (fluorocromo) que se intercala en la cadena de DNA, distinguiendo así las células en las 4 fases (Cooper, 2000).

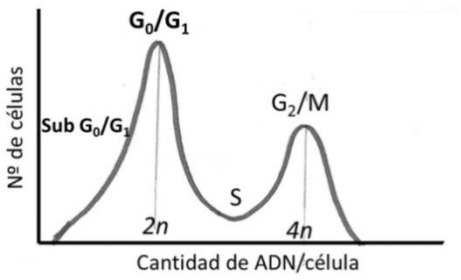


Figura 6. Análisis del ciclo celular mediante el contenido de DNA por citometría de flujo. La imagen representa el número de células frente a la intensidad de fluorescencia, la cual es proporcional al contenido de DNA. La distribución muestra dos picos, estos corresponden al contenido de DNA de las células, 2n y 4n, las cuales se encuentra en las fases G_1 y G_2/M, respectivamente. Las células en la fase S se hallan entre los dos picos (Cooper, 2000).

Se ha demostrado que las micotoxinas perturban el ciclo celular en diferentes líneas celulares, induciendo la parada en diferentes fases del ciclo lo que produce la acumulación de células en una o más fases del ciclo (Juan-García et al., 2013a). La BEA incrementa el número de células en la fase Sub G_0/G_1 y G_2/M (Tabla 6; Cheng et al., 2009; Prosperini et al., 2013a). La STE detiene la progresión del ciclo celular en la fase G_2/M, previniendo así que las células entren en mitosis (Tabla 6; Xie et al., 2000; Xing et al., 2011; Wang et al., 2013; Huang et al., 2014). La PAT detiene el ciclo celular en la fase G_1 y S (Tabla 6; Saxena et al., 2009).

Tabla 6. Perturbación del ciclo celular tras la exposición a BEA, STE y PAT en diferentes líneas celulares.

Micotoxina	Línea celular	Resultado (parada del ciclo)	Referencia
BEA	Y79	Fase Sub G_0/G_1	Cheng et al., 2009
	Caco-2	Fase G_2/M	Prosperini et al., 2013a
STE	MEFs	Fase G_2/M	Xie et al., 2000
	GES-1	Fase G_2/M	Xing et al., 2011
	Het-1A	Fase G_2/M	Wang et al., 2013
	BEAS-2B A549	Fase S y G_2/M	Huang et al., 2014
PAT	V79	Fase G_2/M	Schumacher et al., 2005
	Células de piel de ratón	Fase G1 y S	Saxena et al., 2009

A549= células de carcinoma de pulmón humano; BEA= beauvericina; BEAS-2B= células de epitelio de bronquio humano; Caco-2= células de carcinoma de colón humano; GES-1= células de epitelio gástrico humano; Het-1A= células de epitelio de esófago humano; MEFs= fibroblastos de embrión de ratón; PAT= patulina; STE= esterigmatocistina; V79= fibroblastos de pulmón de hámster chino; Y79= células de restinoblastoma humano.

1.2.2. Especies reactivas de oxígeno

Un radical libre es una molécula que contiene un electrón desapareado en el último orbital atómico, lo que le confiere una gran reactividad (Lobo et al., 2010). Los principales radicales libres son las especies reactivas de oxígeno (ROS) y las especies reactivas de nitrógeno (RNS). Las ROS incluyen radicales como: el radical superóxido ($O_2^{\bullet -}$), el radical hidroxilo ($HO^{\bullet}$), el radical óxido nítrico ($NO^{\bullet}$), el radical peroxilo ($ROO^{\bullet}$), el radical lipoperoxilo ($LOO^{\bullet}$); y no radicales como: el peróxido de hidrogeno (H_2O_2), el singlete de oxígeno ($^1O_2$), el ácido clorhídrico (HOCl), ozono (O_3), el peroxinitrito ($ONOO^-$) y el lipoperóxido (LOOH; Halliwell 2006). Las ROS y RNS se forman constantemente en los organismos como consecuencia del metabolismo celular y puede generarse por sustancias exógenas. La principal fuente endógena de ROS es la cadena de transporte de electrones mitocondrial, pero también pueden contribuir los fagocitos que usan el sitema NADPH oxidasa y la activación catalítica de diversas enzimas. Entre los agentes exógenos se incluyen entre otros la radiación ultravioleta, aditivos o micotoxinas (Sordillo y Aitken, 2009). Estos oxidantes pueden interferir en las rutas de señalización celular como la división celular, la función inmunitaria y las respuestas de defensa al estrés.

El estrés oxidativo se define como un desequilibrio entre los pro oxidantes y el sistema de defensa antioxidante en favor de los oxidantes, que puede contribuir al desarrollo de enfermedades crónicas, toxicidad, envejecimiento celular y cáncer (Davies, 2000; Lobo et al., 2010; Qiang Ma, 2013).

Las ROS pueden causar directa o indirectamente daño a las proteínas, dañando sus aminoácidos, provocando peroxidación, cambios en la estructura terciaria, degradación y fragmentación. Estos daños, a su vez, pueden afectar a

los mecanismos de respuesta al estrés, perdida de la actividad enzimática y alteración de las funciones celulares como el $\Delta\Psi m$ y la producción de energía (Kohen y Nyska, 2002, Valko et al., 2007). Además, las ROS producen alteración de los lípidos de membrana causando peroxidación lipídica (LPO), debido a la elevada concentración de ácidos grasos insaturados de las membranas que son susceptibles a la oxidación (Valko et al., 2007). Asimismo, las ROS pueden interactuar con el DNA causando diferentes tipos de daño como la modificación de sus bases, la reparación del DNA dañado, rotura de la cadena de DNA, perdida de purinas, reticulación del DNA y proteínas (Kohen y Nyska, 2002).

La evaluación del estrés oxidativo se puede llevar a cabo de manera indirecta midiendo diversos productos finales resultantes de la interacción de las ROS con componentes celulares (lípidos, proteínas y DNA; Thannickal y Fanburg, 2000). Sin embargo, la mayoría de los métodos de identificación de la producción intracelular de ROS se basan en la utilización de compuestos no fluorescentes que al ser oxidados por las ROS dan lugar a señales fluorescentes o luminiscentes (Thannickal y Fanburg, 2000). Como el dihidroetidio (Dhe), la dihidrorodamina 123 (DHR 123) y la 2',7'-diclorodihidrofluoresceína diacetato ($DCFH_2$-DA; Paciolla et al., 2004; Gomes et al., 2005; Owusu-Ansah et al., 2008, Ferrer et al., 2009). El Dhe, es una sonda permeable que se oxida por el anión superóxido emitiendo así fluorescencia. La DHR 123 (no fluorescente), difunde a través de las membranas celulares y se oxida por las ROS al fluoroforo Rodamina 123 (Rh 123) que emite fluorescencia. Sin embargo, el método más frecuente para la determinación de la producción intracelular de ROS por micotoxinas es el método de la $DCFH_2$-DA. La $DCFH_2$-DA, es una sonda fluorescente, que difunde a través de la membrana celular donde es diacetilada por las esterasas a un compuesto no fluorescente, la 2,7- diclorofluoresceína (DCFH). Sin embargo,

en presencia de ROS, el DCFH es rápidamente oxidado a su forma fluorescente (DCF). La intensidad de fluorescencia de DCF es proporcional a la cantidad de ROS intracelular formadas. Se ha demostrado mediante este método que la BEA, STE y PAT incrementan la producción de ROS intracelular en diversas líneas celulares (Tabla 7).

Tabla 7. Generación de ROS intracelular producidas por la exposición celular *in vitro* a BEA, STE y PAT.

Micotoxina	Línea celular	Concentración (μM)	Resultados (*)	Referencia
BEA	CHO-K1	5-50	4	Ferrer et al., 2009
	Caco-2	1.5-3.0	1.4-2	Prosperini et al., 2013a
STE	HepG2	3-6	~1.5-2	Gao et al., 2015
		7	**	Liu et al., 2014
PAT	HEK293	50-100	1.9-2.6	Liu et al., 2007
		10-15	16-24.5	Zhang et al., 2015
	HL-60	100	7.4	Liu et al., 2007
	CHO-K1	1-50	7	Ferrer et al., 2009
	HepG2	80	~1.6	Zhou et al., 2010
		75	125% respecto el control	Ayed-Boussema et al., 2011

BEA= beauvericina; Caco-2= células de carcinoma de colon humano; CHO-K1= células de ovario de hámster chino; HEK293= células de riñón de embrión humano; HepG2= células de carcinoma hígado humano; HL-60= células de carcinoma de leucemia promielocítica; PAT= patulina; STE= esterigmatocistina; **= datos no mostrados en el artículo original.
(*): Incremento en la intensidad de fluorescencia respecto al control, debido a la generación de ROS.

1.2.3. Peroxidación lipídica

La LPO es un proceso que ocurre como consecuencia del estrés oxidativo debido a la elevada concentración de ácidos grasos insaturados de las membranas, que son susceptibles a la oxidación. La LPO puede causar alteraciones en las propiedades biológicas de las membranas celulares, como perdida de fluidez, incremento de la permeabilidad celular, inactivación de las uniones de los receptores de membranas o enzimas, los cuales a su vez podrían alterar el funcionamiento celular (Dalle-Done et al., 2006). La LPO puede contribuir a amplificar el daño mediante la generación de productos oxidantes que son químicamente reactivos y pueden unirse covalentemente a las macromoléculas. La reacción en cadena de degradación de ácidos grasos poliinsaturados de las membranas da lugar a la descomposición de estos en aldehídos. Los principales productos de la LPO son el malondialdehído (MDA), el 4 hidoxi-2-noenal (4-HNE), el 2-propenal (acroleína) y algunos isoprostanos, los cuales se pueden utilizar como una medida indirecta del estrés oxidativo (Dalle-Done et al., 2006). Comparados con los radicales libres, los aldehídos son relativamente estables y pueden difundir y actuar lejos del lugar de origen, además pueden actuar como segundos mensajeros de las reacciones primarias. Se ha demostrado que algunos de estos aldehídos exhiben reactividad con biomoléculas como proteínas, DNA y fosfolípidos, contribuyendo a la patogénesis (Dalle-Done et al., 2006).

El análisis de la LPO está basado en la detección de sus subproductos más comunes como: el MDA, el 4-HNE y 8-iso-prostaglandina F2-alfa(8-isoprostano). Para cuantificar los aductos de 4-HNE se utilizan anticuerpos anti-4-HNE y se determinan mediante Western blot o ensayo por inmunoadsorción ligado a enzimas (ELISA) y para el 8-isoprostano también se utiliza el método ELISA. El método más frecuente para evaluar la LPO tras la

exposición a micotoxinas es el método de sustancias reactivas con el ácido tiobarbitúrico (TBARS), basado en la determinación del MDA (Fig. 7).

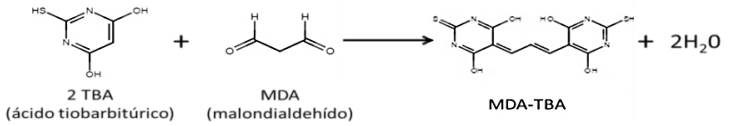


Figura 7. Esquema del método de las TBARS.

Este método está basado en la formación de conjugados coloreados de MDA con el ácido tiobarbitúrico (TBA) en una proporción 1:2, respectivamente. En la Tabla 8 se muestran los resultados obtenidos de la LPO en líneas celulares expuestas a BEA y PAT.

Tabla 8. Producción de LPO tras la exposición a BEA y PAT *in vitro.*

Micotoxina	Línea celular	Concentración (µM)	Incremento de LPO (*) (% respecto al control)	Referencia
BEA	HepG2	0.5-5	116-138%	Klaric et al., 2007
	CHO-K1	1-50	45-182%	Ferrer et al., 2009
	Caco-2	1.5-3.0	120-207%	Prosperini et al., 2013a
PAT	HEK293	2.5-15	1.7-4.7 veces el control	Zhang et al., 2015
		50-200	0.76±0.10-2.72±0.24 (nmoles MDA/mg proteína)	Liu et al., 2007
	HL-60	100-200	0.66±0.08-1.04±0.31 (nmoles MDA/mg proteína)	
	CHO-K1	1-50	25-288%	Ferrer et al., 2009
	HepG2	0.75	2 veces el control	Zhou et al., 2010

BEA= beauvericina; Caco-2= células de carcinoma de colon humano; CHO-K1= células de ovario de hámster chino; HEK293= células de riñón de embrión humano; HepG2= células de carcinoma hígado humano; HL-60=células de carcinoma de leucemia promielocítica; PAT= patulina.
(*): Contenido expresado en MDA.

1.2.4. Potencial de membrana mitocondrial

La mitocondria está relacionada con el poder energético de la célula, procesos celulares como la apoptosis y procesos de señalización (Szewczyk et al., 2015). La energía obtenida a través de la transferencia de electrones a lo largo de la cadena respiratoria, se utiliza para bombear protones desde la matriz mitocondrial hacia el espacio intermembrana, creando así un gradiente electroquímico de protones a través de la membrana mitocondrial interna, denominado $\Delta\Psi m$ (Sánchez y Arboleda, 2008). Se ha observado que algunas micotoxinas producen apoptosis a través de la disfunción mitocondrial, mediante la permeabilidad de las membranas mitocondriales con la consiguiente pérdida del $\Delta\Psi m$, liberación del citotromo *c*, Bax y la activación de las caspasas (Bouaziz et al., 2009). Diferentes tipos de pruebas fluorescentes pueden ser utilizadas para determinar el $\Delta\Psi m$ en gran variedad de tipos celulares. Existen diferentes sondas o fluorocromos lipofílicos que pueden atravesar la membrana y ayudar a determinar el $\Delta\Psi m$, como: ioduro de 3,3'-dihexiloxa carbocianina (DiOC6), ioduro de 5,5', 6,6'-tetracloro-1,1', 3,3' tetraetilbenzimidazolilcarocianina (JC-1), Rh 123, tetrametilrodamina metil éster (TMRM) y tetrametilrodamina etil éster (TMRE). La sonda JC-1 forma agregados o monómeros con emisiones en dos formas detectables por citometría de flujo y microscopía de fluorescencia, en células con potencial de membrana, el JC-1 emite fluorescencia en el rojo, sin embargo tras la pérdida del $\Delta\Psi m$ emite en el verde (Juan-García et al., 2013a). Así mismo, el DiOC6, emite fluorescencia en el verde en células vivas, cuando se utiliza en concentraciones bajas. El TMRM y TMRE se absorben fácilmente en mitocondrias activas produciendo fluorescencia rojo-naranja y la Rh 123 en el verde. Como se observa en la Tabla 9, se ha demostrado que la BEA, PAT y STE producen una disminución del $\Delta\Psi m$ en diferentes líneas celulares. La BEA actúa sobre el $\Delta\Psi m$ aumentando la liberación del citocromo *c*, la activación de

las caspasas y la activación del proceso de apoptosis causando la muerte celular (Jow et al., 2004).

Tabla 9. Potencial de membrana mitocondrial tras la exposición a BEA, STE y PAT en diferentes líneas celulares.

Micotoxina	Línea celular	Concentración micotoxina (µM)	Ensayo	Fluorocromo	Resultado (*)	Referencia
BEA	Caco-2	1.5 3.0	CF	TMRM/To-Pro®-3	2-95% 10-80%	Prosperini et al., 2013a
	RLM	1–1.5 µg/ml	F	Rodamina 123	colapso	Tonshin et al., 2010
	CCRF-CEM	10	F	Rodamina 123	~1.8	Jow et al., 2004
STE	HepG2	0.01-7.5	CF	JC-1	~1.28-1.56	Liu et al., 2014
PAT	HEK293	15	MF	JC-1	0.5	Zhang et al., 2015

BEA= beauvericina; Caco-2= células de carcinoma de colon humano; CCRF-CEM= células de leucemia humana; CF= citrometría de flujo; F= fluorometría; HEK293= células de riñón de embrión humano; HepG2= células de carcinoma hígado humano; JC-1= ioduro de 5,5',6,6'-tetracloro-1,1',3,3' tetraetilbenzimidazolilcarocianina; RLM= mitocondria de hígado de rata; MF= microscopía fluorescencica; PAT= patulina; STE= esterigmatocistina; TMRM= tetrametilrodamina metil éster.

(*): Disminución del $\Delta\Psi m$ respecto del control

1.2.5. Muerte celular: apoptosis y necrosis

El estado fisiológico de un organismo se debe a mecanismos que permiten adaptarse y sobrevivir a condiciones externas y responder adecuadamente a estímulos. Cuando una sustancia produce un daño irreversible a la célula y le impide llevar a cabo sus funciones vitales de manera normal, se produce la muerte celular. La muerte celular puede desencadenarse por múltiples causas como exposición a un tóxico, pérdida de la función celular, infección por microorganismos y virus, daño mecánico o falta de nutrientes (Lizarbe et al., 2007). La muerte celular puede ocurrir

mediante dos procesos: apoptosis, controlada genéticamente y necrosis, un proceso desordenado (Tabla 10).

Tabla 10. Diferencias entre el proceso de apoptosis y necrosis.

	APOPTOSIS	**NECROSIS**
Estímulo	Fisiológico o patológico	Patológico
Membrana plasmática	Íntegra, con exposición de la fosfatidilserina	Rotura, lisis
Tamaño celular	Retracción/ Condensación	Edema
Requerimiento energético	Si	No
Degradación DNA	Ordenada (180-200 pb)	Aleatoria
Afecta células	Individuales	Grupo
Destino final	Fagocitosis	Inflamación exudativa
Respuesta inflamatoria	No	Si

La necrosis es un mecanismo de muerte celular no controlado, que se produce como respuesta a la exposición a agentes tóxicos. Este mecanismo se produce de manera no organizada y está caracterizado por la rotura de la membrana celular con la consiguiente salida al exterior del contenido intracelular, lo que da lugar a un proceso inflamatorio y daños en las células adyacentes. Por el contrario, la apoptosis es un mecanismo de muerte celular en el que no se destruye la membrana celular, lo que impide la salida del contenido intracelular al exterior favoreciendo así un proceso sin inflamación. La apoptosis se caracteriza por la condensación de la cromatina en los llamados cuerpos apoptóticos y la externalización de los fosfolípidos de membrana como la fosfatidilserina que permite que estos sean detectados y eliminados por las células fagocitarias; impidiendo así la inflamación (Sánchez y Arboleda, 2008). La apoptosis mantiene la homeostasis celular controlando

el número celular, células dañadas y eliminando células que han sufrido daños al DNA irreversibles (Lizarbe et al., 2007). La apoptosis puede activarse ante diferentes estímulos por dos tipos de vía, la intrínseca y la extrínseca. La vía intrínseca está mediada por la mitocondria. La apoptosis está controlada por el balance entre las proteínas proapoptóticas como Bax, Bad, Bak y p53; y antiapoptóticas como Bcl-2 y Bcl-x (Lin et al., 2005). El desequilibrio entre las proteínas pro y antiapoptóticas puede incrementar la permeabilidad de membrana con la liberación del citocromo c y el factor inductor de apoptosis al citosol, activando la cascada de caspasas, enzimas efectoras de la apoptosis. A su vez, la vía extrínseca, está mediada por la unión de receptores de muerte a la superficie celular, los cuales activan también a las caspasas.

La apoptosis/necrosis se pueden determinar por diferentes métodos. Los cambios morfológicos producidos en las células durante el proceso apoptótico pueden ser detectados mediante microscopía fotónica, electrónica o de fluorescencia. La hematoxilina/eosina permite diferenciar entre células apoptóticas tardías y células necróticas, mediante microscopía fotónica (Elmore et al., 2007). La microscopía electrónica, permite detectar cambios morfológicos típicos de la apoptosis y la fagocitosis de los cuerpos apoptóticos, aunque no permite detectar apoptosis temprana. Para ello, las células deben ser fijadas, teñidas y cortadas con un ultramicrotomo previamente a la visualización mediante microscopía electrónica. Se puede identificar como medida de apoptosis la fragmentación del DNA mediante la técnica de túnel y la división del DNA en fragmentos múltiplos de 180-200 pares de bases mediante electroforesis en gel de agarosa (Elmore, 2007). La técnica de túnel, permite determinar los fragmentos de DNA cortados por endonucleasas, marcándolos colorimétrica o fluorescentemente y visualizándolos mediante microscopía de fluorescencia o citometría de flujo. También se puede identificar la apoptosis mediante ensayos

inmunohistoquímicos, de inmunoprecipitacion y Western blot que permiten la cuantificación de proteínas, la actividad de las caspasas y el citocromo *c* (Elmore, 2007). Asimismo, se puede utilizar las alteraciones en la membrana celular para identificar el proceso apoptótico, como puede ser la externalización de la fosfatidilserina mediante citometría de flujo (Elmore, 2007; Juan-García et al., 2013a). Además, el uso de moléculas intercalantes en los ácidos nucleicos de doble cadena como el PI o la 7 aminoactinomicina D (7-AAD) permiten determinar el proceso de necrosis o apoptosis tardía. El método más frecuente en la detección de apoptosis y necrosis tras la exposición a micotoxinas es el método de la Annexina V-fluoresceína isotiocianato (FITC)/PI mediante citometría de flujo, ya que permite diferenciar entre células no apoptóticas, en apoptosis temprana, tardía y necróticas (Fig. 8; Juan-García et al., 2013a).

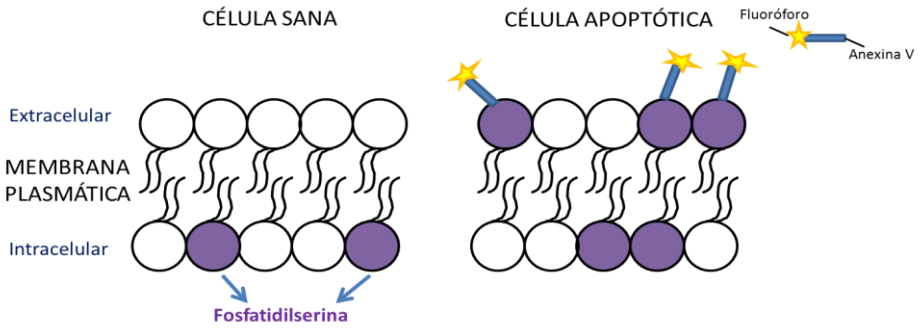


Figura 8. Esquema del funcionamiento de la Anexina V en la determinación de la apoptosis.

En este método, la Anexina V se une fuertemente a la fosfatidilserina que aparece en la membrana interna en células normales y en la membrana externa en células apoptóticas tempranas y el PI se intercala en el DNA de células necróticas o apoptóticas tardías.

Se ha demostrado que la BEA induce muerte celular en diferentes líneas celulares (Tabla 11; Dornetshuber et al., 2009a; Jow et al., 2004; Prosperini et al., 2013a; Wätjen et al., 2014). La apoptosis celular producida por la BEA se atribuye a la familia Bcl-2, el citocromo *c* y la caspasa 3, así como al aumento de la concentración de Ca^{2+} citoplasmático (Jow et al., 2004; Lin et al., 2005; Klarić et al., 2008b). Asimismo, la PAT induce muerte celular a través de la vía intrínseca mitocondrial con la liberación del citocromo *c* y la activación de la caspasa-3 (Tabla 11; de Melo et al., 2012). La STE es capaz de producir apoptosis (Tabla 11; Liu et al., 2014). Sin embargo, el principal mecanismo de muerte celular producido por la STE es la necrosis, la cual podría estar relacionada con el deterioro de la síntesis de ATP mitocondrial (EFSA, 2013).

Tabla 11. Muerte celular causada por la BEA, STE y PAT.

Micotoxina	Técnica	Resultado	Referencias
BEA	Microscopía fotónica	Cambios morfológicos: retración membrana, condensación citoplasma	Drombrink-Kurtzman et al., 2003
	Electroforesis	Fragmentos de DNA entre 180-200 pares de bases	
	Microscopía de fluorescencia (Hoechst 33258)	Cambios morfologícos: Retracción membrana celular, fragmentación nuclear, formación cuerpos apoptóticos.	Jow et al., 2004
	Espectrofotometría	Incremento de la actividad caspasa 3, liberación del citocromo *c* y disminución $\Delta\Psi m$	
	Electroforesis en gel de agarosa	Fragmentos de DNA múltiplos de 200 pares de bases	
	Microscopía de fluorescencia (Hoechst 33258) y técnica de TUNEL	Cambios morfológicos: retracción de la membrana celular, fragmentación nuclear y formación de cuerpos apoptóticos Fragmentación de DNA	Lin et al., 2005
	Espetrofotometría	Activación de la caspasa 3, liberación citocromo c y disminución del $\Delta\Psi m$	
	Western blot	Disminución de la expresión de la proteína Bcl-2 e incremento de la proteína Bax	
	Microscopía fotónica (hematoxilina/eosina)	Incremento de células apoptóticas	Klaric et al., 2008a
	Espetrofotometría	Incremento de la actividad caspasa 3	
	Microscopía de fluorescencia (DAPI/FITC-faloidina)	Cambios en el núcleo y microfilamentos relacionados con la apoptosis	Dornetshuber et al., 2009a
	Citometría de flujo (Anexina V-FITC/PI)	Incremento de apoptosis temprana y células apoptóticas/necróticas	Prosperini et al., 2013a

Micotoxina	Técnica	Resultado	Referencias
BEA	Microscopía fluorescencia (Hoechst 33258; AO/EB)	Fragmentación nuclear Incremento de células necróticas	Wätjen et al., 2014
	Espectrofotometría de fluorescencia	Incremento de la actividad caspasa 3/7	
STE	Citrometría de flujo (Anexina V-FITC/PI)	Incremento de células apoptóticas	Liu et al., 2014
	Inmunocitoquímica	Disminucion del contenido Bax y Bcl-2 Incremento de la actividad caspasa 3 y p53	
PAT	Microscopía de fluorescencia (DAPI)	Cambios morfológicos: retración de la membrana plasmática, fragmentación nuclear, condensación de la cromatina	Wu et al., 2008
	Citometría de flujo (PI)	Incremento de células apoptóticas	
	Eletroforesis en gel de agarosa	Fragmentación del DNA	
	Espectrofotometría de fluorescencia	Activación de las caspasas 3 y 9	
	Western blot	Liberación del citocromo c y disminución de los niveles de Bcl-2	
	Inmunoblot	Aumento de la expresión de Bax, p53, p21/waf1	Saxena et al., 2009
	Citometría de flujo (anexina V-FITC/PI)	Incremento de apoptosis	
	Western blot y espectrofotometría de fluorescencia	Incremento de la actividad caspasa 3 y liberación del citocromo *c*	
	Western blot	Incremento de la proteína p53	Zhou et al., 2010
	Microscopía de fluorescencia (Hoechst 33342 y AO/EB)	Cambios morfológicos: retración celular, condensación de la cromatina, fragmentación nuclear	Zhang et al., 2015
	Citometría de flujo (Anexina V-FITC-EGFP/PI)	Incremento de células apoptóticas	

AO/EB= naranja de acridina/bromuro de etidio; Bax= proteína proapoptótica Bax; Bcl-2= proteína antiapoptótica Bcl-2; BEA= beauvericina; DAPI= 4 ',6-diamino-2-fenilindol; EGFP= proteína verde fluorescente; FITC= isotiocianato de fluoresceína; p21/ waf1= inhibidor quinasa p21; p53= proteína supresora de tumores p53; PAT= patulina; PI= ioduro de propidio; STE= esterigmatocistina; ΔΨm= potencial de membrana mitocondrial.

1.2.6. Genotoxicidad

La alteración del material genético celular (DNA, RNA) se produce por agentes exógenos y por procesos endógenos por fallos en la replicación, transcripción del DNA o la reparación de los daños. Estas alteraciones en el material genético, son de gran importancia debido a que la sustancia puede ser potencialmente mutágena y/o carcinógena. La presencia de daño en el DNA, da lugar a mecanismos de reparación, a una parada del ciclo celular o muerte celular. Para evaluar la genotoxicidad producida por mutaciones génicas o aberraciones cromosómicas (CA) por micotoxinas se llevan a cabo ensayos como el test de Ames, CA, micronúcleos (MN), cromáticas hermanas (SCE) y el ensayo del cometa (Fotso et al., 2003; Liu et al., 2003; Klaric et al., 2008b, 2010; Çelik et al., 2010; Prosperini et al., 2013a). El test de Ames permite determinar la mutagenicidad de un compuesto en cepas de *Salmonella typhimurium* previamente mutadas en el gen que codifica la histidina por lo que requieren histidina para su crecimiento. Si el compuesto a ensayar es mutágeno puede revertir la mutación permitiendo a las bacterias crecer en el medio sin histidina (Trossero et al., 2006). Las alteraciones cromosómicas implican una alteración en el número de cromosomas o en su estructura. Una de las técnicas para determinar las CA se realiza adicionando colcemida o colchicina para deterner las células en metafase. A continuación de someterse a un choque hipotónico, se fijan, se tiñen con Giemsa y se identifican mediante microscopía de campo claro (Vigreux et al., 1998). El test de MN es uno de los más utilizados en el análisis de CA, debido a que es muy sensible, sencillo y rápido (Bolognesi, 2003). Los MN son fragmentos cromosómicos acéntricos o cromosomas enteros excluidos durante la mitosis celular, que, aparecen exclusivamente en el citoplasma de células que han completado la división celular, como pequeños núcleos adicionales (Bolognesi, 2003). Para determinar los MN se utiliza citocalasina-B para impedir la

citocinesis y formar células multinucleadas. Posteriormente se fijan, se tiñen con naranja de acridina y se identifican mediante microscopía de fluorescencia (Wolff y Müller, 2006). El test SCE consiste en la identificación de cromátidas químicamente diferentes mediante la incorporación de un análogo de la timidina, la BrdU, a continuación se detienen las células en metafase con colcemida y posteriormente se tiñen los portaobjetos con fluorescencia más Giemsa (FPG) que permiten la visualización de las SCE (Battal et al., 2015). El ensayo del cometa permite la identificación de la rotura en la cadena de DNA en células individuales de manera sensible y sencilla; además, se requiere un número pequeño de células, un periodo corto de tiempo, se puede aplicar en eucariotas *in vitro* y su coste es bajo (Wolff y Müller, 2006). Las células se incrustan en un gel de agarosa sobre un portaobjetos, se lisan con detergente y se lleva a una electroforesis con pH alcalino (pH>13) que da lugar a la ruptura de las hebras de DNA formando estructuras parecidas a cometas observadas al microscopio de fluorescencia. La intensidad de la cola del cometa respecto de la cabeza refleja la cantidad de rotura de DNA (Wolff y Müller, 2006).

Se ha demostrado que la BEA, STE y PAT producen genotoxicidad en diferentes líneas celulares (Tabla 12). La BEA produce fragmentación en el DNA, rotura de la doble cadena, aumento de las CA, SCE y MN. Se ha sugerido que el daño en el DNA producido por PAT puede estar relacionado con la ruta ERK, la depleción del GSH y/o la generación de ROS (Liu et al., 2003, Wu et al., 2008).

Tabla 12. Métodos de genotoxicidad en cultivos celulares *in vitro* con la BEA, STE y PAT.

Micotoxina	Línea celular	Concentración (µM)	Método	Resultado	Referencia
BEA	PK15	0.5-5	MN	Incremento de la frecuencia de MN, NBs y NPBs	Klarić et al., 2008a
		0.5	Cometa	Daño al DNA	Klarić et al., 2010
	LH	2.5-10	CA SCE MN	Induce CA Incremento de la frecuencia de MN y SCE	Celik et al., 2010
	Caco-2	12	Cometa	Daño al DNA	Prosperini et al., 2013a
STE	A549 (BNC)	2	Cometa MN	Daño al DNA Incremento de MN, NB y NPBs	Jaksic et al., 2012
	Het-1A	6-24	Cometa	Daño al DNA	Wang et al., 2013
	BEAS-2B, A549	24	Cometa	Daño al DNA	Huang et al., 2014
	HepG2	3-6	Cometa	Daño al DNA	Gao et al., 2015
PAT	CHO AWRF	>2 mg/ml	--	Rotura del DNA	Stetina et al., 1986
	V79		(1)	Incremento de la frecuencia de mutación	Schumacher et al.., 2005
		0.1-0.5	MN	Induce MN	Pfeiffer et al., 1998
		0.5	Cometa MN	Daño al DNA Incremento MN y NPBs	Glaser y Stopper, 2012

Micotoxina	Línea celular	Concentración (µM)	Método	Resultado	Referencia
PAT	V79 MNBN	2.5-7.5 0.65-0.95	CA MN	MNBN: induce MN V79: clastogenicidad e incremento de CA	Alves et al., 2000
	CHO-K1, HEK293, LH	0.2-20	SCE Cometa	Incremento de la frecuencia de SCE en CHO-K1 y linfocitos humanos Clastogenicidad en CHO-K1 Daño al DNA en HEK93	Liu et al., 2003
	HepG2	20-40	Cometa	Daño al DNA	Zhou et al., 2010
		15	Cometa CA	Daño al DNA Incrementa CA	Ayed-Boussema et al., 2011
	CTOR	2.5-3.75 mg/kg b.w.	Cometa	Daño al DNA	de Melo et al., 2011

A549= células de carcinoma de pulmón humano; CA= aberraciones cromosómicas; BEAS-2B= células epiteliales de bronquio humano; BNC= células binucleadas; Caco-2= células de carcinoma de colón humano; b.w.= peso corporal; CHO, CHO-K1= células de ovario de hámster chino; CTOR= células procedentes de órganos y tejidos de ratón; HEK293= células de riñón de embrión humano, HepG2= células de carcinoma de hígado humano; Het-1A= células epiteliales de esófago humano; HPRT= enzima hipoxantina-guanina fosforribosiltranferasa; LH= linfocitos humanos; MN= micronúcleos; MNBN=linfocitos humanos con citoquinesis bloqueada; NB= brotes nucleares; NPBs= puentes nucleoplasmáticos; PK15= células de riñón de porcino; SCE= Intercambio de cromátidas hermanas; V79= fibroblastos de pulmón de hámster chino.

(1): Mutagenicidad en el locus HPRT.

1.2.7. Sistemas de defensa antioxidante

Con el fin de hacer frente al daño oxidativo, los organismos han desarrollado un complejo mecanismo de defensa antioxidante que permite mantener la homeostasis redox, eliminando los radicales libres o transformándolos en moléculas más estables (Fig. 9). Según Halliwell (1995), un antioxidante se puede definir como ''cualquier sustancia que retrasa, previene o elimina el daño oxidativo a una molécula diana". El sistema de defensa antioxidante puede ser endógeno y exógeno. El sistema de defensa endógeno está constituido por compuestos no enzimáticos como: GSH, ácido lipóico; el ácido úrico, la bilirrubina, la melatonina, las proteínas quelantes de metales de transición, la coenzima Q y enzimas como la superóxido dismutasa (SOD), la catalasa (CAT) y la glutatión peroxidasa (GPx) (Pisoschi y Pop, 2015). Mientras que el sistema de defensa exógeno está constituido por componentes de la dieta o suplementos alimenticios como la vitamina C, los carotenoides, la vitamina E, los polifenoles, la lecitina de aceite, la acetilcistína, etc. (Pisoschi y Pop, 2015).

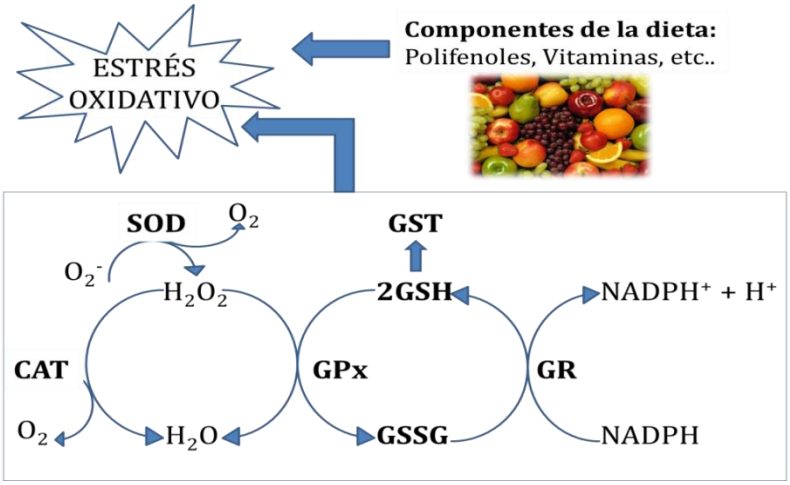


Figura 9. Esquema del sistema de defensa antioxidante celular relacionado con el GSH.

Sistema de defensa endógeno

Las enzimas SOD, CAT y GPx constituyen la primera línea de defensa frente a las ROS (Fig. 9). La SOD es una enzima antioxidante que cataliza la dismutación del anión superóxido en oxígeno molecular y formas menos reactivas como el peróxido de hidrogeno (H_2O_2). Este peróxido, a su vez puede ser eliminado por las enzimas CAT, GPx y por la reacción de Fenton (Barbosa et al., 2010). La CAT degrada el H_2O_2 en agua y oxígeno molecular. Esta enzima es una de las más eficientes que se conocen, ya que no se satura por el exceso de H_2O_2 (Matés, 2000). Aunque la CAT no es esencial en todos los tipos celulares en condiciones normales, esta enzima juega un papel importante en la respuesta celular frente al estrés oxidativo (Krishnamurthy y Wadhwani, 2012). Las enzimas GPxs catalizan la reducción de hidroperóxidos orgánicos y H_2O_2. Existen dos tipos de GPxs, las dependientes de selenio que son activas frente al H_2O_2 e hidroperóxidos orgánicos y las no dependientes de selenio que catalizan principalmente la reducción de hidroperóxidos orgánicos (Knapen et al., 1999; Gutowicz et al., 2011).

Asimismo, el GSH (L-γ-glutamil-L-cisteinil-glicina), es considerado uno de los antioxidantes endógenos celulares más importantes. El GSH es un tripéptido presente en los tejidos en una concentración entre 1 y 10 mM, siendo el hígado el órgano en el que se encuentra en mayor concentración (Lu, 2013). El GSH es la llave determinante en la señalización redox, la detoxificación de xenobióticos y la modulación de la proliferación, la apoptosis, la función inmunitaria y la fibrogénesis (Lu, 2013). El GSH puede eliminar directamente los radicales libres o actuar como sustrato de la enzima glutatión transferasa (GST) durante la detoxificación (Fig. 9). Las GSTs son una familia de enzimas que actúan como segunda línea de defensa frente al estrés oxidativo mediante la unión de los compuestos tóxicos al GSH (Masella et al., 2005). Con el fin de mantener la cantidad de GSH necesaria para realizar su

función antioxidante en condiciones normales y de estrés, la enzima glutatión reductasa (GR), cataliza la transformación del glutatión oxidado (GSSG) a su forma reducida, GSH, mediante una reacción dependiente de NADPH. La N-acetilcisteína (NAC) es un precursor del GSH, que incrementa su concentración, por lo que puede ser utilizada para determinar la actividad antioxidante celular en situaciones de estrés. La STE, disminuye la actividad enzimática de la CAT, mientras que aumenta la actividad enzimática de la SOD y la GPx (EFSA, 2013). Sin embargo, la PAT disminuye la actividad enzimática de la CAT y la SOD, lo que como consecuencia da lugar a un aumento del estrés oxidativo (Ferrer et al., 2009; de Melo et al., 2012; Zhang et al., 2015). Además, se ha demostrado que la BEA y PAT disminuyen los niveles de GSH en diferentes líneas celulares (Tabla 13). No se han encontrado datos sobre la STE.

Tabla 13. Niveles de GSH tras la exposición de cultivos celulares a BEA y PAT.

Micotoxina	Línea celular	Concentración (μM)	Resultado (*)	Referencia
BEA	PK15	0.05-5	28	Klaric et al., 2007
	Caco-2	1.5-3	31	Prosperini et al., 2013a
PAT	HepG2	10-40	Decrece ~30	Zhou et al., 2010
	V79	0.9	ND	Schumacher et al., 2005

BEA= beauvericina; Caco-2= células de carcinoma de colón humano; HepG2= células de carcinoma hepático humano; ND= no determinado; PAT= patulina; PK15= células de riñón de porcino; V79= fibroblastos de pulmón de hámster chino.
(*): Disminución respecto al control expresado en %.

Sistema de defensa exógeno

Diversos tipos de antioxidantes exógenos contribuyen a reducir el daño producido por las micotoxinas (Hundhausen et al., 2005; Chen y Chan, 2009; El-Agamy, 2010; Choi et al., 2010; Lombardi et al., 2012; Kalaiselvi et al., 2013; Ramyaa et al., 2014; Vila-Donat et al., 2014; Chiesi et al., 2015). Este hecho, sugiere que estos antioxidantes pueden ser utilizados como estrategia para la prevención del riesgo tóxico por exposición a micotoxinas.

Entre los antioxidantes ingeridos en la dieta se incluyen los polifenoles que son metabolitos secundarios sintetizados por las plantas como mecanismo de defensa frente al estrés. Existen al menos 8000 tipos diferentes de polifenoles clasificados en cuatro categorías: ácidos fenólicos, flavonoides, estilbenos y lignanos (Khurana et al., 2013). El resveratrol (RSV; 3,5,4'-trihidroxiestilbeno, Fig. 10) es un polifenol del grupo de los estilbenos abundante en uvas y derivados, cacahuetes, chocolate negro, bayas y una variedad de té denominada *Itadori* (Zamora-Ros et al., 2008).

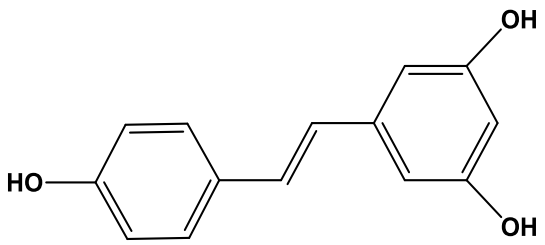


Figura 10. Estructura química del resveratrol.

El RSV ha sido estudiado por sus múltiples propiedades biológicas y efectos beneficiosos para la salud como: neuroprotección, inhibición de las lipoproteínas de baja densidad, antiflamatorio, antioxidante, antienvejecimiento, antidiabético y antiplaquetario (Gülçin, 2010; Fernández-Mar et al., 2012; Li et al., 2012; Vilahur y Badimon, 2013). La baja incidencia

de enfermedades cardiovasculares, en países con una dieta rica en ácidos grasos saturados, se ha relacionado con el consumo de RSV a través del vino, lo que se denomina "Paradoja Francesa" (Sun et al., 2002b; Khurana et al., 2013; Yang et al., 2014). El RSV existe en las formas isoméricas *cis* y *trans* (Fig. 11). El isómero *trans* es más activo biológicamente que el isómero *cis*. Este isómero es abundante en la piel de la uva y está presente en vinos, especialmente en tintos (Romero-Pérez et al., 1996). Por el contrario, el isómero *cis* es un derivado por la isomerización de la forma *trans* que es inestable, por lo que no está disponible comercialmente. El RSV es un compuesto extremadamente fotosensible, entre el 80-90% de la forma *trans* se convierte en *cis* tras la exposición a la luz durante una hora.

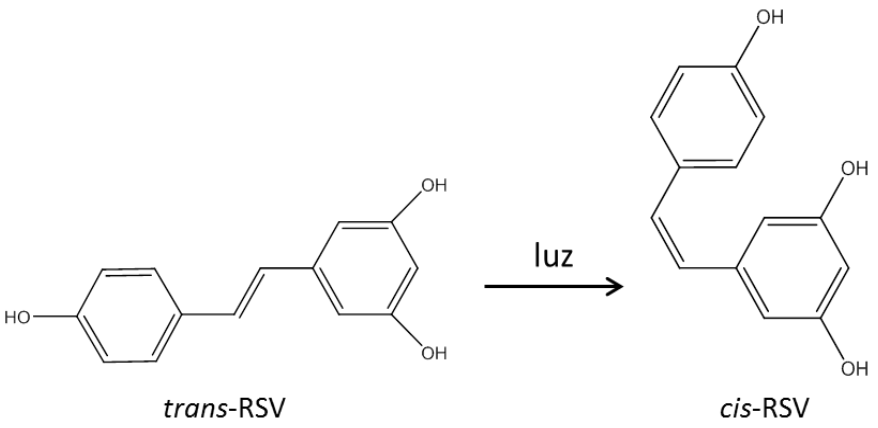


Figura 11. Isomerización de *trans*-RSV a *cis*-RSV tras la exposición a la luz.

Debido a las múltiples propiedades biológicas y efectos beneficiosos para la salud se han elaborado diversos suplementos alimenticios que contienen este polifenol. Las principales técnicas analíticas para la determinación de polifenoles son la espectrofotometría, la cromatografía y la electroforesis capilar (CE; Ignat et al., 2011). La CE ha sido utilizada por varios

autores para determinar el contenido de polifenoles en diferentes matrices alimentarias (Brandolini et al., 2002; Franquet-Griell et al., 2012; Ballus et al., 2012).

Los métodos de determinación de la actividad antioxidante total se basan en comprobar cómo un agente oxidante induce daño oxidativo a un sustrato oxidable y como ese daño es inhibido o reducido en presencia de un antioxidante. La inhibición es proporcional a la actividad antioxidante del compuesto o de la muestra. Existen diferentes métodos para determinar la capacidad antioxidante: la DCFH-DA, 2,2-difenil-2-picrilhidrazilo (DPPH), 2,2´-azinobis-(ácido-3 etilbenzotiazolino-6-sulfónico)(ABTS), potencial total de atrapamiento de radicales peróxido (TRAP), capacidad de absorción de radicales libres de oxígeno (ORAC), potencia antioxidante reductora férrica (FRAP), capacidad total de atrapamiento de oxi-radicales (TOSC), voltametría cíclica (CV), crocina, capacidad antioxidante equivalente de trolox (TEAC), método del tiocianato férrico (FTC), barrido de $O_2^{\bullet-}$ y H_2O_2 y fotoquimiluminiscencia (PCL). Los distintos métodos difieren en el agente oxidante, en el sustrato empleado, en el tiempo de evaluación, en la técnica instrumental utilizada, la sensibilidad y en las interacciones de la muestra con el medio de reacción (Quintanar Escorza y Calderón Salinas, 2009). La capacidad antioxidante del RSV ha sido demostrada por varios métodos (Tabla 14).

Tabla 14. Actividad antioxidante del RSV determinada por diversos métodos

Método	**Referencia**
DPPH	Gülçin, 2010; Lucas-Abellán et al., 2011
ABTS	Gülçin, 2010; Lucas-Abellán et al., 2011
ORAC	Lucas-Abellán et al., 2011
Barrido de $O_2^{\bullet-}$ y H_2O_2	Gülçin, 2010

La técnica PCL permite determinar la capacidad antioxidante de compuestos hidrosolubles y liposolubles mediante la combinación de la generación fotoquímica de $O_2^{\bullet-}$ y la detección mediante quimioluminiscencia. La reacción se inicia por la excitación óptica de un fotosensibilizador, dando lugar a la generación del $O_2^{\bullet-}$. En el sistema de PCL existen dos tipos básicos de radicales libres: $O_2^{\bullet-}$ y el radical luminol. Estos radicales se eliminan parcialmente de la muestra por reacción con los antioxidantes presentes en la muestra y son detectados por el luminol, el cual actúa como fotosensibilizador y reactivo de detección de radicales oxígeno (Prior et al., 2005).

1.3. Mitigación de micotoxinas

Debido a los efectos perjudiciales que originan las micotoxinas en la salud humana y animal, se han desarrollado una serie de estrategias para prevenir el crecimiento de hongos micotoxigénicos, así como para descontaminar los alimentos y piensos contaminados por micotoxinas. Estas estrategias se pueden llevar a cabo en pre-cosecha y post-cosecha e incluyen (Kabak et al., 2006):

- Prevención de la contaminación por micotoxinas
- Descontaminacion de micotoxinas presentes en alimentos y piensos
- Inhibición de la absorción de la micotoxina en el tracto gastrointestinal

Una de las estrategias más recientes de inhibición de la absorción de las micotoxinas en el tracto gastrointestinal es disminuir su biodisponibilidad mediante agentes adsorbentes (aluminosilicatos, fibras, bacterias y polímeros) (Avantaggiato et al., 2004) o biotransformadores (bacterias, hongos, levaduras y enzimas) (EFSA, 2009; Kabak et al., 2006). La adición de agentes adsorbentes en piensos se usa como estrategia para disminuir la absorción de las micotoxinas. El uso de prebióticos y probióticos se emplean para disminuir

la bioaccesibilidad de micotoxinas (Galvano et al., 2001; Ferrer et al., 2015). Estas estrategias están basadas en la influencia de la matriz alimentaria, así como el nivel de contaminación y la forma en que el alimento/pienso está contaminado (artificial o naturalmente) (González-Arias et al., 2013).

Bioaccesibilidad

La ingesta de alimentos se considera la principal fuente de exposición a micotoxinas. Por ello, estudios de bioaccesibilidad y biodisponibilidad de micotoxinas, pueden contribuir a la evaluación del riesgo de exposición a micotoxinas.

La cantidad total ingerida de un contaminante no siempre refleja la cantidad disponible en el organismo para su absorción. La liberación del contaminante en el tracto gastrointestinal, es decir, la bioaccesibilidad, es un requisito para la absorción en el tracto gastrointestinal y por tanto, para su biodisponibilidad en el organismo (Versantvoort et al., 2005).

El término bioaccesibilidad se define como la fracción de un compuesto bioactivo presente en una matriz alimentaria que no se modifica estructuralmente durante la digestión gastrointestinal y queda disponible para su absorción intestinal (Fernández-García et al., 2009). La bioaccesibilidad incluye la secuencia de eventos que tienen lugar durante el proceso de absorción en el tracto gastrointestinal considerando todos los factores que pueden modificarla: pH, vaciado gástrico, enzimas, microbiota intestinal, presencia de alimentos y movimientos peristálticos (Fernández-García et al., 2009).

La determinación de la bioaccesibilidad de las micotoxinas se lleva a cabo mediante métodos de digestión *in vivo* e *in vitro*.

Los métodos de digestión *in vivo*, no son siempre posibles en humanos, por ello, el estudio en animales como perros, pollos, ratas y cerdos es más

frecuente (Bornhorst y Singh, 2014). Existen dos tipos de métodos de digestión *in vivo* en animales de experimentación: la canulación y el sacrificio. En ambas, se utiliza un marcador no digerible para calcular el porcentaje de tóxico liberado en cada localización del tracto gastrointestinal (Bornhorst y Singh, 2014).

La canulación precisa de cirugía para introducir una cánula en el tracto gastrointestinal. La principal ventaja de esta metodología es que permite estudiar al animal durante un largo periodo de tiempo, lo que disminuye la variabilidad. Sin embargo, esta cirugía produce cambios en la secreción y motilidad del tracto gastrointestinal que podría modificar el proceso de digestión. La segunda estrategia, consiste en la sedación y sacrificio de los animales utilizados para la obtención de las muestras. Este método permite la recogida de muestras en diferentes localizaciones del tracto gastrointestinal; sin embargo, no permite la repetición del muestreo en el mismo animal, lo que aumenta el número de animales necesarios en el estudio (Bornhorst y Singh, 2014).

Debido a la complejidad del tracto gastrointestinal los modelos de digestión *in vivo* son la representación más exacta del proceso de digestión. Sin embargo, no siempre es posible llevarla a cabo por lo que los modelos de simulación gastrointestinal *in vitro* son los más utilizados en la determinación de la bioaccesibilidad, ya que son una alternativa útil frente al método *in vivo* debido a su alta reproductibilidad, rapidez, bajo coste y ausencia de restricciones éticas (Versantvoort et al., 2005; Minekus et al., 2014). Este modelo permite controlar muchos factores que no se pueden controlar en una digestión *in vivo* (Bornhorst y Singh, 2014). Los modelos de digestión *in vitro* simulan las condiciones fisiológicas que tienen lugar en el tracto gastrointestinal *in vivo* de una manera simplificada. Para ello, se simulan la composición química y concentración de los fluidos gastrointestinales, el pH y

el tiempo de residencia en cada compartimento, entre otros parámetros. Los métodos disponibles para la simulación gastrointestinal *in vitro* difieren en el diseño del sistema, la composición de los jugos fisiológicos ensayados, el uso o no de la microbiota intestinal. Hay modelos que sólo simulan la parte superior del tracto gastrointestinal (boca-estómago-intestino delgado), mientras que otros métodos incluyen la fermentación en el intestino grueso (Minekus et al., 2014).

A su vez, los modelos de digestión *in vitro* pueden ser estáticos o dinámicos. El modelo estático, es el más simple y más utilizado (Fig. 12). En este, se simula el proceso de digestión en cada compartimento pero no reproduce los procesos dinámicos que ocurren durante la digestión humana, como el vaciado gástrico o los continuos cambios de pH y las velocidades de flujo de secreción (Guerra et al., 2012). Para ello, la muestra se mezcla con una simulación de fluidos gástricos a 37 ºC durante diferentes periodos de tiempo en baños de agua con agitación que permitir imitar el tracto gastrointestinal (Fig. 12). La principal ventaja de este método es su facilidad de montaje y mantenimiento.

COMPARTIMENTO	COMPOSICIÓN	TIEMPO	pH
BOCA	Saliva artificial	minutos	6,5-7
ESTÓMAGO	Jugo gástrico (pepsina + agua)	2 h	1,5-2
INTESTINO DELGADO	Jugo duodenal (pancreatina + sales biliares)	2 h	5-7
INTESTINO GRUESO	Jugo duodenal+ bacterias características del tracto gastrointestinal	48 h	~6,5

Figura 12. Esquema de las principales características del proceso de digestión *in vitro* estático.

El modelo dinámico, además de permitir controlar todos los pasos relacionados con la digestión gastrointestinal durante todo el proceso, está conectado a un software que permite controlar y modificar las condiciones de digestión (temperatura, pH, agitación, y la presencia de CO_2). Este modelo incluye procesos fisicoquímicos y mecánicos que reproducen de manera más fiel las condiciones que se dan *in vivo* como la progresiva acidificación del contenido gástrico mediante la adición de HCl, el flujo de pepsina y el vaciado gástrico (Guerra et al., 2012). La principal ventaja de este método es la ruptura física y química de los productos alimenticios (Bornhorst y Singh, 2014). Los métodos de digestión *in vitro* dinámica más utilizados son el TON y el SHIME. El modelo TON es un sistema que simula la actividad del estómago, del intestino delgado y del colón mediante un sistema de compartimentos y válvulas que ayudan a simular los movimientos peristálticos (Minekus et al., 1995, 1999). El modelo SHIME, simula los parámetros fisicoquímicos, enzimáticos y el ecosistema microbiano intestinal humano del estómago, intestino delgado y colón mediante cinco pasos en un reactor multicámara (Molly et al., 1993). Los métodos dinámicos se asemejan más a las condiciones *in vivo* que los estáticos; sin embargo, precisan de instrumental sofisticado, grandes volúmenes de muestra y solo permiten procesar un número reducido de muestras. Por ello, los métodos estáticos son los más utilizados.

Los modelos de digestión *in vitro* estáticos han sido utilizados para determinar la bioaccesibilidad de nutrientes, productos farmacéuticos y micotoxinas (Minekus et al., 2014). Diferentes estudios se han llevado a cabo para determinar la bioaccesibilidad de las micotoxinas (Tabla 15).

Tabla 15. Bioaccesibilidad de micotoxinas mediante un modelo de digestión *in vitro.*

Método	Micotoxina	Alimento	Resultado	Referencia
Digestión *in vitro* estática	AFB_1	Cacahuete	94%	Versantvoort et al., 2005
			94%	Simla et al., 2009
		Maíz molido	95%	
		Pistacho	86%	Kabak et al., 2009
	OTA	Trigo sarraceno	100%	Versantvoort et al., 2005
			22%	Kabak et al., 2009
	ENs	Pan de trigo	80%	Meca et al., 2012a
		Pan de trigo	6.2-45%	Manzini et al., 2015
	PAT	Productos derivados de manzana	25-75%	Raiola et al., 2012a,
	DON	Pasta seca	12-23%	Raiola et al., 2012b
	BEA	Pan de trigo	46.7-61%	Manzini et al., 2015
Digestión *in vitro* dinámica	ZEA	Trigo	32%	Avantaggiato et al., 2003
	DON	Trigo	51%	Avantaggiato et al., 2004
	NIV	Trigo	21	
	BEA	Pan de trigo	76-91%	Manzini et al., 2015
	ENs	Pan de trigo	23-69%	

AFB_1= aflatoxina B1; BEA= beauvericina; DON= deoxinivalenol; ENs= eniatinas; FB1= fumonisina B1; NIV= nivalenol; OTA= ochratoxina A; ZEA= zearalenona.

Probióticos

Los probióticos son "microorganismos vivos que al ser administrados en cantidades adecuadas confieren un beneficio a la salud del huésped" (FAO/WHO, 2006).

Estudios recientes demuestran que los microorganismos probióticos desempeñan una acción importante en las funciones inmunológica, digestiva,

respiratoria y además podrían tener un efecto significativo en el alivio de enfermedades infecciosas en niños y grupos de alto riesgo (bebes, embarazadas, ancianos, enfermos inmunodeprimidos, etc…). Y pueden ser utilizados por personas sanas como prevención de ciertas enfermedades (diarreas, infecciones respiratorias, cáncer de colon y alergias) y como moduladores de la inmunidad en el huésped (FAO/WHO, 2006).

Aunque son muchos los efectos beneficiosos que producen los probióticos, poco se conoce sobre sus mecanismos de acción. Estos, pueden variar de unos probióticos a otros y podrían ser una combinación de efectos. Asimismo, estos efectos podrían estar relacionados con la producción de enzimas o metabolitos que actúan directamente sobre los microorganismos patógenos o podrían causar un efecto/acción beneficiosa en el organismo.

Para evaluar la funcionalidad y seguridad de los probióticos se han desarrollado numerosos ensayos *in vivo* e *in vitro* (FAO/WHO, 2006). Estos métodos determinan:

- Resistencia a ácidos gástricos y ácidos biliares
- Adherencia al moco del epitelio humano en células
- Actividad antimicrobiana contra bacterias potencialmente patógenas
- Habilidad para reducir la adhesión de patógenos a las superficies
- Actividad hidrolasa de sales biliares
- Determinación de resistencia a antibióticos
- Evaluación de actividades metabólicas
- Evaluación de efectos secundarios durante estudios con humanos
- Vigilancia epidemiológica de incidentes adversos en los consumidores (post-mercado)
- Evaluación de toxinas en cepas productoras de las mismas
- Evaluación de la actividad hemolítica en especies con potencial hemolítico

Los objetivos buscados con la utilización de ensayos con probióticos *in vivo* (animales y humanos) son la mejora en síntomas, signos, bienestar o calidad de vida, reducción del riesgo de enfermedades o tiempo más largo para la siguiente aparición y la recuperación rápida de la misma. Cada beneficio debe tener una correlación con el probiótico probado (FAO/WHO, 2006).

Entre las especies bacterianas seleccionadas como probióticos por sus efectos beneficiosos sobre la salud se incluyen: *Lactobacillus casei*, *L. paracasei*, *L. rhmanosus*, *L. acidophilus*, *L. gasseri, L. johnsonii*, *L. plantarum*, *L. reuteri*, *L. crispatus*, *L. fermentum*, *Bifidobacterium bifidum*, *B. adolescentis*, *B. lactis*, *B. breve*, *B. infantis*, *B. longum*, *Saccharomyces boulardii* y *S. cerevisiae* (Vinderola y Ritieni, 2015). Los microorganismos probióticos deben sobrevivir al paso a través del tracto digestivo y ser capaces de proliferar en el intestino. Para ello, deben ser resistentes a los ácidos digestivos o ser consumidos con un alimento vehículo que les permita sobrevivir a lo largo del tracto gastrointestinal (FAO/WHO, 2006).

Ciertas cepas probióticas pueden unirse y eliminar las micotoxinas de medios líquidos. Se ha demostrado que el complejo probiótico-micotoxina es menos adhesivo a los enterocitos que el probiótico solo, lo que puede favorecer la eliminación de este complejo a través de las heces. También, son capaces de restaurar funciones epiteliales de las células después de los daños producidos por las micotoxinas (Vinderola y Ritieni, 2015). Ensayos *in vivo* muestran que el daño genético y oxidativo causado por las micotoxinas puede evitarse parcialmente con el uso de probióticos (Vinderola y Ritieni, 2015). Asimismo, humanos expuestos a micotoxinas y probióticos mostraron niveles reducidos de aductos de DNA producidos por micotoxinas (Vinderola y Ritieni, 2015).

En la Tabla 16 se muestran diferentes estudios, *in vivo* e *in vitro*, realizados con probióticos y sus efectos sobre la disminución de la bioaccesibilidad de micotoxinas.

Tabla 16. Efecto del uso de probióticos sobre las micotoxinas.

Microorganismo	Micotoxina	Efecto (*)	Referencia
Lactobacillus rhamnosus GG y LC705	AFB_1	↓↓ 80% de la mictoxina en medio líquido	El-Nezami et al., 1998, 2000
Lactobacillus acidophilus NCC 12 y NCC68, L. casei shirota	AFB_1	↓↓ 37% de la BC	Kabak et al., 2009
Lactobacillus acidophilus (NCC68)	OTA	↓↓ 73% de la BC	
Bifidobacterium longum, B. species 420, Lactobacillus acidophilus, L. acidophilus NCFM 150B, L. casei Shirota, L. rhamnosus,	AFB_1	↓↓ 36% de la BC	Kabak y Ozbey, 2012
	AFB_2	↓↓ 35% de la BC	
	AFG_1	↓↓ 32% de la BC	
	AFG_2	↓↓ 34% de la BC	
Bifidobacterium adolescentis (LA18), B. animalis (VM12), B. animalis (LA17), B. bifidum (VM13), B. breve (LA14), B. longum (VM14), B. longum (LA02), Lactobacillus acidophilus (VM19), L. acidophilus (VM20), L. acidophilus (VM05), L. brevis (VM22), L. casei (VM01), L. casei casei (VM25), L. curvatus (LA43), L. curvatus (LA42), L. delbrueckii ssp. bulg. (VM26), L. delbrueckii ssp. bulg. (VM27), L. helveticus (VM34), L. delbrueckii ssp. lactis (VM32), L. paraplantarum (VM35), L. pentosus (VM36), L. plantarum (VM37), L. plantarum (VM02), L. rhamnosus (VM40), L. slivarius (LA01), Salmonella thermophilus (VM42)	OTA	↓↓ 9-95% de la micotoxina en medio líquido	Fuchs et al., 2008
Bifidobacterium adolescentis (LA18), B. animalis (VM12), B. animalis (LA17), B. bifidum (VM13), Lactobacillus acidophilus (VM20), L. acidophilus (VM05), L. brevis (VM21), L. brevis (VM22), L. casei (VM01), L. casei casei (LA09), L. curvatus (LA42), L. delbrueckii ssp. bulg. (VM27), L. helveticus (VM34), L. paraplantarum (VM35), L. paraplantarum (LA07), L. pentosus (VM36), L. plantarum (VM37), Salmonella thermophilus (VM42)	PAT	↓↓ 5-80% de la micotoxina en medio líquido	Fuchs et al., 2008

Microorganismo	Micotoxina	Efecto (*)	Referencia
Bifidobacterium longum, B. bifidum, B. breve, B. adolescentis, Lactobacillus rhamnosus, L. animalis, L. ruminis, L. casei-casei, L. plantarum, Eubacterium crispatus, Salmonella fecalis, S. termofilus	BEA	↓↓ 66-83% de la micotoxina en medio líquido	Meca et al., 2012b
Bifidobacterium longun , Bf. Bifidum, Bf. Adolescentes, Lactobacillus rhamnosus, L. johnsonii, L. plantarum, L. reuteri	BEA	↓↓ 7-10% de la BC	Ferrer et al., 2015
Bifidobacterium longun , Bf. Bifidum, Bf. Breve, Bf. Adolescentes, Lactobacillus rhamnosus, L. johnsonii, L.casei, L. plantarum, L.reuteri	ENs	↓↓ 21-27% dela BC	
Bifidobacterium longun , Bf. Bifidum, Bf. Breve, Bf. Adolescentes, Lactobacillus rhamnosus, L. johnsonii, L.casei, L. plantarum, L.reuteri	DON	↓↓ 29-40% de la BC	
Bifidobacterium longun , Bf. Bifidum, Bf. Adolescentes, Lactobacillus rhamnosus, L. johnsonii, L. plantarum, L. reuteri	ZEA	↓↓ 41-57% de la BC	Ferrer et al., 2015
Lactobacillus acidophilus (NRRL B-4495), L. reuteri (NRRL B-14171), *L. rhamnosus* (NRRL B-442), *L. johnsonii* (NRRL B-2178), *Bifidobacterium bifidum* (NRRL B-41410)	AFM_1	↓↓ 23-45% de la BC	Serrano-Niño et al., 2013

AFB_1= aflatoxina B_1; AFM_1= aflatoxina M_1; BC= bioaccesibilidad; BEA= beauvericina; DON= deoxinivalenol; ENs= eniatinas; OTA= ocratoxina A; PAT= patulina; ZEA= zearalenona; ↓↓= disminución. (*)= respecto del control.

Prebióticos

Un prebiótico es un ingrediente alimentario no digerible que permite cambios en la composición o en la actividad de la microbiota gastrointestinal, los cuales benefician la salud y bienestar del consumidor, como por ejemplo las fibras dietéticas (Nair et al., 2010; Figueroa-González et al., 2011).

Un prebiótico debe cumplir los siguientes requisitos (Pérez-Conesa et al., 2004):

- No debe ser hidrolizado ni absorbido en la parte anterior del tracto gastrointestinal.
- Constituir un sustrato selectivo para una o un número limitado de bacterias beneficiosas del colon, estimulando su crecimiento y/o metabolismo.
- Modificar la composición de la flora del colon, facilitando el desarrollo de especies beneficiosas.
- Inducir efectos en el lumen o sistémicos que sean beneficiosos para la salud del individuo que los consuma.

Los prebióticos llegan al colon y sirven como sustrato para las bacterias endógenas del mismo, proporcionando indirectamente energía, sustratos metabólicos y micronutrientes esenciales (Gibson y Roberfroid, 1995). Este efecto se produce en un número selectivo de microorganismos como son los géneros *Bifidobacterium* y los *Lactobacillus*, lo que produce una modificación selectiva de la microbiota intestinal (Wang, 2009). Las bifidobacterias estimulan el sistema inmune, inhiben el crecimiento de patógenos, reducen los niveles de colesterol y ayudan a restaurar la biota intestinal tras tratamientos con antibióticos. Los lactobacilos ayudan en la digestión de la lactosa en personas intolerantes a esta, reducen el estreñimiento y la diarrea infantil, ayudan a contrarrestar infecciones como la salmonelosis y alivian el síndrome de colon irritable.

Los prebióticos se encuentran de manera natural en alimentos como los puerros, los espárragos, la achicoria, la alcachofa de Jerusalén, el ajo, las cebollas, el trigo, la avena, la soja, de manera natural constituyendo la leche materna, etc (Al-Sheraji et al., 2013; Slavin, 2013). La mayoría de los prebióticos identificados actualmente son oligosacáridos no digeribles. Estos se obtienen mediante la extracción de plantas, por producción microbiológica o síntesis enzimática y degradación de polisacáridos. Los prebióticos más conocidos son la inulina, los galactooligosacáridos y los fructooligosacáridos (Al-Sheraji et al., 2013).

En 1953, Hipsley introdujo el término fibra dietética para definir los componentes de la ingesta no digeribles de las paredes celulares vegetales (Hipsley, 1953). De acuerdo con la Comisión del Codex Alimentarius (CL 2007/43-NFSDU), una fibra dietética se entiende por "polímeros de carbohidratos con un grado de polimerización no inferior a 3, que no son digeridos ni absorbidos en el intestino delgado". La fibra dietética consta de las siguientes propiedades (Codex Alimentarius; CL 2007/43-NFSDU):

- Reduce el tiempo de tránsito intestinal e incrementa la masa fecal
- Es fermentable por la microbiota del colon
- Reduce los niveles de colesterol total y/o colesterol LDL en la sangre
- Reduce los niveles postprandiales de glucosa y/o insulina en la sangre

Las fibras dietéticas se pueden clasificar en solubles o insolubles en agua (Monro y Burlingame, 1997). Las fibras solubles se componen de polisacáridos no celulósicos tales como la pectina, β-glucanos, arabinano, fructooligosacáridos, galactooligosacáridos, ácido polurónico, galactano, agar, alginato, carregina, polisacáridos de algas, gomas y mucílagos. Estas fibras se encuentran en frutas, avena, cebada, frijoles secos y legumbres. Se caracterizan porque son altamente fermentables y están asociadas con carbohidratos y el metabolismo lipídico. Asimismo, las fibras insolubles están

formadas por componentes de la pared celular tales como celulosa, lignina, hemicelulosa, xilanos, manano y almidón resistente. Este tipo de fibras están presentes principalmente en cereales y vegetales y se caracterizan por facilitar el tránsito fecal (Nair et al., 2010).

Estudios epidemiológicos sugieren múltiples efectos beneficiosos tras la ingesta de fibras dietéticas. Se ha demostrado que el consumo de fibras dietéticas (Slavin, 2013):

- Disminuye el riesgo de enfermedades cardiovasculares, principalmente debido a la disminución de los niveles de lipoproteínas de baja densidad, la reducción de la proteína C-reactiva, la presión sanguínea y marcadores de enfermedades del corazón
- Disminuye la prevalencia de diabetes de tipo II
- Produce efecto laxante, debido a la habilidad que tienen las fibras para incrementar el peso de las heces, bien mediante el agua retenida por estas y/o el aumento de la masa bacteriana
- Promueve la saciedad y la pérdida de peso, lo que podría deberse a que las fibras necesitan un mayor tiempo de masticación, incrementan la distensión del estómago cuando estas se unen al agua y porque este tipo de dietas son menos ricas en grasas
- Previene el cáncer de colon
- Facilita la absorción de minerales como el Ca^{2+} y el Mg^{2+}
- Mejora la función inmunitaria

Teniendo en cuenta estos beneficios y las características tecnologícas y nutricionales que poseen los prebióticos estos se utilizan en fórmulas alimenticias. Se ha demostrado que los prebióticos mejoran las características organolépticas de los alimentos, se utilizan para retener la humedad, como sustitutos de azúcar y como estabilizadores en diversidad de alimentos (Wang, 2009).

Actualmente la mezcla de probióticos y prebióticos, denominada simbióticos, es utilizada por las ventajas que produce su efecto sinérgico en el huésped mediante la mejora de la supervivencia y la implantación de microorganismos en el tracto gastrointestinal (Gibson y Roberfroid, 1995; Al-Sheraji et al., 2013).

Diversos estudios determinan el uso de fibras dietéticas como estrategias para disminuir la bioaccesibilidad de las micotoxinas (Tabla 17).

Tabla 17. Efecto del uso de prebióticos en la bioaccesibilidad de las micotoxinas.

Fibras	Micotoxina	Efecto (*)	Referencias
β-1,3 glucano, quitosano de bajo y medio peso molecular, FOS, galactomanano, inulina, pectina	BEA	↓↓ 96-99% de la BC	Meca et al., 2012c
Inulina	ENs	↓↓ 58% de la BC	Meca et al., 2012d
		↓↓ 74% de la BC	Manzini et al., 2015
	BEA	↓↓ 22% de la BC	Manzini et al., 2015
	DON	↓↓ 93% de la BC	Ferrer et al., 2015
FOS	ENs	↓↓ 34% de la BC	Manzini et al., 2015
FOS	BEA	↓↓ 39% de la BC	Manzini et al., 2015
Celulosa	ZEA	↓↓ 35% de la BC	Ferrer et al., 2015
	DON	↓↓ 50% de la BC	Ferrer et al., 2015
	ENs	↓↓ 45% de la BC	Ferrer et al., 2015
	BEA	↓↓ 15% de la BC	Ferrer et al., 2015

BC= bioaccesibilidad; BEA=beauvericina; DON= deoxinivalenol; ENs= eniatinas; FOS= fructooligosacáridos; ZEA= zearalenona; ↓↓= disminución. (*)= respecto del control.

2. Objetivos

Objetives

2. OBJETIVOS

El objetivo general de la tesis es profundizar en el conocimiento de los mecanismos de acción y los efectos tóxicos de las micotoxinas beauvericina, esterigmatocistina y patulina mediante métodos *in vitro*, evaluando sistemas antioxidantes de defensa endógenos y exógenos así como estrategias de mitigación de las micotoxinas.

Para llevar a cabo este objetivo se plantean los siguientes objetivos específicos:

1. Realizar una revisión bibliográfica para obtener la información toxicológica disponible.
2. Determinar la citotoxicidad de la beauvericina, esterigmatocistina y patulina de forma individual y en combinación, en células de ovario de hámster chino (CHO-K1).
3. Determinar la proliferación celular, potencial de membrana mitocondrial, daño al DNA y muerte celular tras la exposición a beauvericina en células CHO-K1.
4. Estimar el estrés oxidativo mediante la producción de especies reactivas de oxígeno y peroxidación lipídica tras la exposición de beauvericina en células CHO-K1.
5. Determinar el efecto de la influencia de los sistemas de defensa enzimáticos y niveles de glutatión tras la exposición a beauvericina en células CHO-K1.
6. Valorar el efecto de la N-acetilcisteína y de los isómeros del resveratrol frente al daño producido por la beauvericina en células CHO-K1.

7. Estimar de la influencia de prebióticos y probióticos en la bioaccesibilidad de la beauvericina mediante un modelo estático de digestión *in vitro*.

2. OBJECTIVES

The overall objectives of this research were to evaluate the mechanistic toxicology and toxic effects of beauvericin, patulin and sterigmatocystin by *in vitro* methods, as well as antioxidant defense mechanisms and mitigation of mycotoxins.

To reach these aims, the following specific objectives were proposed:

1. Performing a review about mycotoxins.
2. Determining cytotoxic effects of beauvericin, sterigmatocystin, and patulin individually and in combination in the Chinese Ovary Hamster cells (CHO-K1).
3. Determining cell proliferation, mitochondrial membrane potential, DNA damage and cell death in CHO-K1 cells exposed to beauvericin.
4. Determining oxidative stress associated with reactive oxygen species and lipid peroxidation production in CHO-K1 cells exposed to beauvericin.
5. Determining enzymatic antioxidant defense system and glutathione levels in CHO-K1 cells exposed to beauvericin.
6. Determining the effect of N-acetylcysteine and resveratrol isomers in CHO-K1 cells exposed to beauvericin.
7. Evaluating the prebiotics and probiotics influence in gastric, duodenal and colonic bioaccessibility of beauvericin by a static *in vitro* digestion model.

3. Resultados

Results

3.1. Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO-K1 cells

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Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO-K1 cells

Nidhal Zouaoui[a,b,*], Beatriz Mallebrera[c,*], Houda Berrada[c], Salwa Abid-Essefi[a], Hassen Bacha[a], Maria-Jose Ruiz[c,1]

[a]Laboratory for Research on Biologically Compatible Compounds (LRSBC), Faculty of Dentistry, Rue Avicenne, 5019 Monastir, Tunisia; [b]Faculty of sciences of Bizerte, University of Carthage, 7021, Jarzouna, Tunisia; [c]Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vincent Andrés Estelles, 46100, Burjassot, Valencia, Spain;

Abstract

Mycotoxins are produced by different genera of fungi; mainly *Aspergillus*, *Penicillium* and *Fusarium*. The natural co-occurrence of beauvericin (BEA), patulin (PAT) and sterigmatocystin (STE) has been proved in feed and food commodities. This study investigates the cytotoxicity of individual and combined mycotoxins BEA, PAT and STE. The cytotoxicity on immortalized ovarian cells (CHO-K1) was evaluated using the MTT assay. After 24, 48 and 72 h, the IC_{50} values were 2.9 μM for PAT and ranged from 10.7 to 2.2 μM and from 25.0 to 12.5 μM for BEA and STE, respectively. Cytotoxic interactions were assayed by the isobologram method, which provides a combination index (CI) value as a quantitative measure of the three mycotoxin interaction's degree. Binary and tertiary combinations showed a dose dependent effect. At low fraction affected, mycotoxin combinations were synergetic; whereas, at higher fraction affected, the combinations showed additive effect. Our results indicate that the co-occurrence of low concentrations of mycotoxin in food may increase their toxic effects.

Key words: Beauvericin, Patulin, Sterigmatocystin, CHO-K1 cells, Interaction effects.

[1] **Corresponding author:** Maria-Jose Ruiz, Laboratory of Toxicology, Faculty of Pharmacy, *Av. Vincent Andrés Estelles, 46100, Burjassot (Valencia), Spain;* Tel.: +34 963543055; fax: +34 963544954.E-mail address: M.Jose.Ruiz@uv.es (Maria-Jose Ruiz)

*Both authors have contributed equally to the study, and thus may be regarded as first author.

1. Introduction

Mycotoxins are fungal secondary metabolites. The same fungi can produce different mycotoxins and the same mycotoxin can be produced by different species of fungi (Bottalico and Perrone, 2002). The mycotoxin beauvericin (BEA) is mainly produced by *Fusarium* species, patulin (PAT) by *Aspergillus* and *Penicillium* species and sterigmatocystin (STE) by *Aspergillus* and *Fusarium* species.These genera fungi grow in subtropical latitude in food or raw material (Bhat, 2010). The presence of mycotoxins in food commodities could cause significant economic and health impacts.

BEA, PAT and STE alone produce toxic effects. BEA causes cytotoxic effects, produces oxidative stress and depletion of antioxidant cellular mechanisms (Dornetshuber et al., 2007; Ferrer et al., 2009; Jow et al., 2004; Mallebrera et al., 2014; Prosperini et al., 2013). BEA has also been related DNA damage in human cell lines and has pro-apoptotic activity (Dornetshuber et al., 2007; Jow et al., 2004; Prosperini et al., 2013)

Similar effects were produced by PAT (Ferrer et al., 2009; Liu et al., 2007). Moreover, PAT induced DNA damages including DNA strand breaks, chromosome aberrations, and micronuclei formation in mammalian cells (Alves et al., 2000). It also causes mutagenicity, carcinogenicity, developmental and reproductive toxicity and immunotoxicity (Puel et al., 2010; Wichmann et al., 2003). PAT is classified as group 3 or as “not carcinogenic to humans” due to the inadequate evidence of carcinogenicity in human animals (IARC, 1986). STE is a precursor in Aflatoxin biosynthesis (Woloshuk and Prieto, 1998; Yu et al., 2004) and it is considered cytotoxic (Wang et al., 2013) and potent carcinogen, group 2B by IARC (IARC, 1976).

A completely diet would include all the different food items that possibly could be contaminated with the three chosen mycotoxins (EFSA, 2013; 2014; SCOOP Task 3.2.8). Thus, there is an increasing concern about hazard of co-occurrence of BEA, PAT and STE in feed and food. Many studies have been conducted on the toxicity of individual mycotoxins; however, few studies focused on the combined effects of mycotoxins. There is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence their combined toxicity and

subsequently, their implications for food safety assessment are generally not known (Bouaziz et al., 2013; Bouslimi et al., 2008; El Golli-Bennour et al., 2009; Lu et al., 2013; Ruiz et al., 2011a, 2011b; Tatay et al., 2014). Thus, the evidence of possible additive o synergistic interaction is a cause of concern; the consumption of contaminated food will lead to a combined intake depending on the absorption rates of the different mycotoxins. Therefore, toxicity data from mycotoxins mixture are needed and could provide more realistic risk assessments.

The toxic effect is determined by an alternative method, the *in vitro* cell culture method. This method collects and organizes mechanistic knowledge related to toxicological effects in order to improve the design and validation of predictive models and approaches, including the better use of existing alternative methods such as *in vitro* assays. The CHO-K1 cells were selected because of they have proved to be very useful in evaluating the basal cell toxicity of different mycotoxins (Ferrer et al, 2009; Lu et al, 2013, Mallebrera et al. 2014; Ruiz et al., 2011a). Moreover, the cell line has been selected to address other biological mechanisms of mycotoxins or effects of mixtures (Lombardi et al., 2012; Lu et al., 2013; Ruiz et al., 2011a).

In the present study we investigated the cytotoxicity of BEA, PAT and STE alone or in combination on CHO-K1 cells at 24, 48 and 72 h. The results obtained by the concentration-response curve were the basis to formulate the extent and nature of their interactions using the isoblogram method of Chou and Talalay (1984) and Chou (2006) in terms of additive, antagonistic or synergistic toxicity.

2. Material and Methods

2.1 Reagents

The reagent grade chemicals and cell culture compounds used, namely culture medium Ham's F12, antibiotics, trypsin/EDTA solutions, HEPES, phosphate buffer saline (PBS) and 3-4,5-dimethylthiazol-2yl, 2,5-diphenyltetrazolium bromide (MTT) dye were from Sigma–Aldrich (St. Louis Mo. USA). Standard of the selected mycotoxins, PAT (MW: 154.12 g/mol), BEA (MW: 783.95g/mol) and STE (MW: 324.28 g/mol) were purchased from Sigma–Aldrich (St. Louis Mo. USA). Chemical

structure of BEA, PAT and STE are shown in Figure 1. Stock solutions of mycotoxins were prepared in DMSO and maintained at −20 °C in darkness. The final DMSO concentration in the medium was ≤1% (v/v). 1% DMSO was not cytotoxic to CHO-K1 cells according to preliminary experiments carried out in our laboratory.

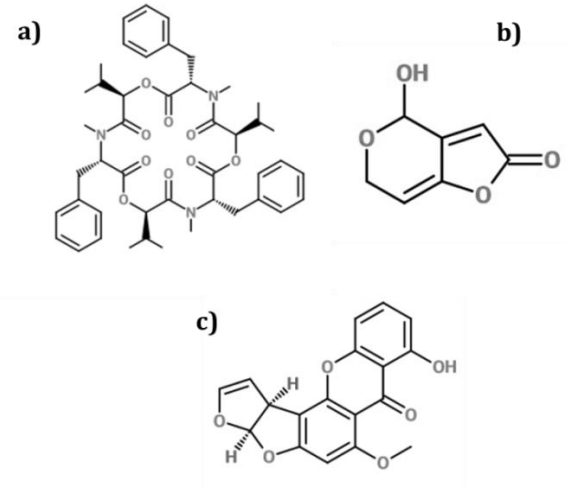

Figure 1. Chemical structure of a) beauvercin, b) patulin and c) sterigmatocystin

2.2. Cell culture and treatment

The Chinese hamster ovary (CHO-K1) cells (ATCC CCL-61) were grown at 37 °C in 6 cm^2 polystyrene tissue culture dishes with Ham's-F12 medium supplemented with 25 mM HEPES buffer (pH 7.4), 10% fetal bovine serum (FBS, from Cambrex Company, Belgium), 100 U/ml penicillin and 100 mg/ml streptomycin. The incubation conditions were 5% CO_2 at 37 °C and 95% air atmosphere at constant humidity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St. Louis Mo. USA).

2.3. Determination of cytotoxicity assay

The CHO-K1 cells were used at passage numbers between 30 and 55. The cells were plated in 96-well microplates at a density of 2×10^4 cells/well. After 24h, the cells reached 65% confluence (according to preliminary experiments carried out in

our laboratory). After 24 h, the culture medium was replaced with fresh medium containing different concentration of the mycotoxins. The concentration selected and combination ratios, calculated on the basis of the IC_{50} values obtained in individual cytotoxicity experiments, were chosen to obtain equipotent toxicity for each mycotoxin in a mixture. Concentrations of each mycotoxin assayed: BEA (0.75, 1.25, 2.5, 5, 10 and 20 μM), PAT (0.049, 0.098, 0.195, 0.39, 0.78, 1.56, 3.125 and 6.25 μM) and STE (1.56, 3.125, 6.25, 12.5, 25, 50 and 75 μM). Cytotoxicity was determined by the MTT assay performed as described by Ruiz et al. (2006). Mycotoxins were exposed during 24, 48 and 72 h. During the exposure time (24, 48 or 72 h), neither the medium nor the mycotoxins were replenished according to the MTT protocol. After 24, 48 and 72 h, the medium was removed and cells of each well received fresh medium containing 50 μl (5 mg/ml PBS) MTT. After 4 h of incubation at 37 °C under darkness, the resulting formazan was solubilized in DMSO. The absorbance was measured at 570 nm using an automatic ELISA plate reader (MultisKanEX, Labsystem, Helsinki, Finland). Mean inhibition concentration (IC_{50}) values were calculated from full concentration-response curves. Determinations were performed in three independent experiments.

2.4. Experimental design and assessment of mycotoxin combinations' effect: isobologram method

Among the mycotoxins, BEA, PAT and STE are very common and it is reported that they display several damages. The present study is conducted to investigate if the cytotoxicity of combined mycotoxin would be enhanced by their combination on CHO-K1 cells as compared to their effects individually. The combination ratios, calculated on the basis of the IC_{50} values obtained in individual cytotoxicity, were chosen to obtain equipotent toxicity for each mycotoxin in a mixture.

CHO-K1 cells were exposed to several dilutions of each binary and tertiary mycotoxin combination and these mixtures were prepared as follows: STE + PAT combination at constant 16:1 ratio, STE+BEA combination at constant 5:1 ratio, BEA+ PAT combination at constant 3.2:1 ratio and STE+ BEA + PAT combination at

constant 16:3.2:1 ratio. Four dilutions of each mycotoxin combination (STE 0.78, 1.56, 3.125 and 6.25 μM; BEA: 0.156, 0.3125, 0.625 and 1.25 μM and PAT: 0.049, 0.098, 0.195 and 0.39 μM) plus a control were tested in triplicate. CHO-K1 cells treated with 1% DMSO (previously demonstrated not to be toxic to CHO-K1 cells) in the culture medium were considered as control. Cytotoxicity of combinations was determined by the MTT assay as described previously (Ruiz et al., 2006).

The isobologram analysis was used to determine the type of interaction that occurs when PAT, STE and BEA are in combination. The median-effect/combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984) was originated to analyze the drug combination effects which involve the plotting of 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 the cells' surviving population 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:

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

where $^{n}(CI)_x$ is the combination index for n compounds (e.g., mycotoxins) at x % inhibition (e.g., proliferation inhibition); $(D_x)_{1-n}$ is the sum of the concentration of n compounds that exerts x % inhibition in combination, $\{[Dj]/\sum^{n}{}_{1}[D]\}$ 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 BEA, PAT and STE combinations were evaluated automatically by isobologram analysis using CalcuSyn software version 2.1. (Biosoft, Cambridge, UK, 1996–2007).

2.5. Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. All values are expressed as mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA one-way) followed by Tukey's test for the *post hoc* comparisons. The *P*-value of ≤0.05 was considered significant.

3. Results

3.1 Cytotoxicity of individual mycotoxins

The cytotoxic effect of BEA, PAT and STE on CHOK-1 cells was evaluated by MTT assay over 24, 48 and 72 h to determine the molar concentration of mycotoxin

that reached 50% inhibition of cellular proliferation (IC_{50}). Figure 2 shows cell proliferation (%) when BEA, PAT and STE were tested alone on CHO-K1 cells after all studied exposure times. Our results indicated that BEA and STE reduce cell proliferation in time and in concentration dependent manner (Figure 2). However, PAT only reduces cell proliferation at the higher tested concentrations (3.125 and 6.25 µM) and similar reduction was observed at all exposure times (Fig. 2). PAT decreased cell proliferation from 45% to 16% at the two higher tested concentrations respect to the control (Fig. 2). Whereas, BEA and STE decreased respectively cell proliferation from 96% to 0% and from 92% to 34% respect to the control (Fig. 2).

According to the IC_{50} values, STE showed the less cytotoxic effect on CHO-K1 cells at the three exposure times, followed by BEA and PAT. The mycotoxin PAT is the most cytotoxic among the mycotoxins tested on CHO-K1 cells (Table 1).

Table 1: IC_{50} (mean ± SD, n=3) values of BEA, PAT and STE determined by MTT assay after 24, 48 and 72 h of exposure.

Mycotoxin	IC_{50} (µM)		
	24 h	**48 h**	**72h**
BEA	10.7 ± 3.7	2.5 ± 4.4	2.2 ± 4.9
PAT	2.9 ± 0.3	2.9 ± 0.9	2.8 ± 0.4
STE	25.0 ± 3.7	17.5 ± 3.9	12.5 ± 2.0

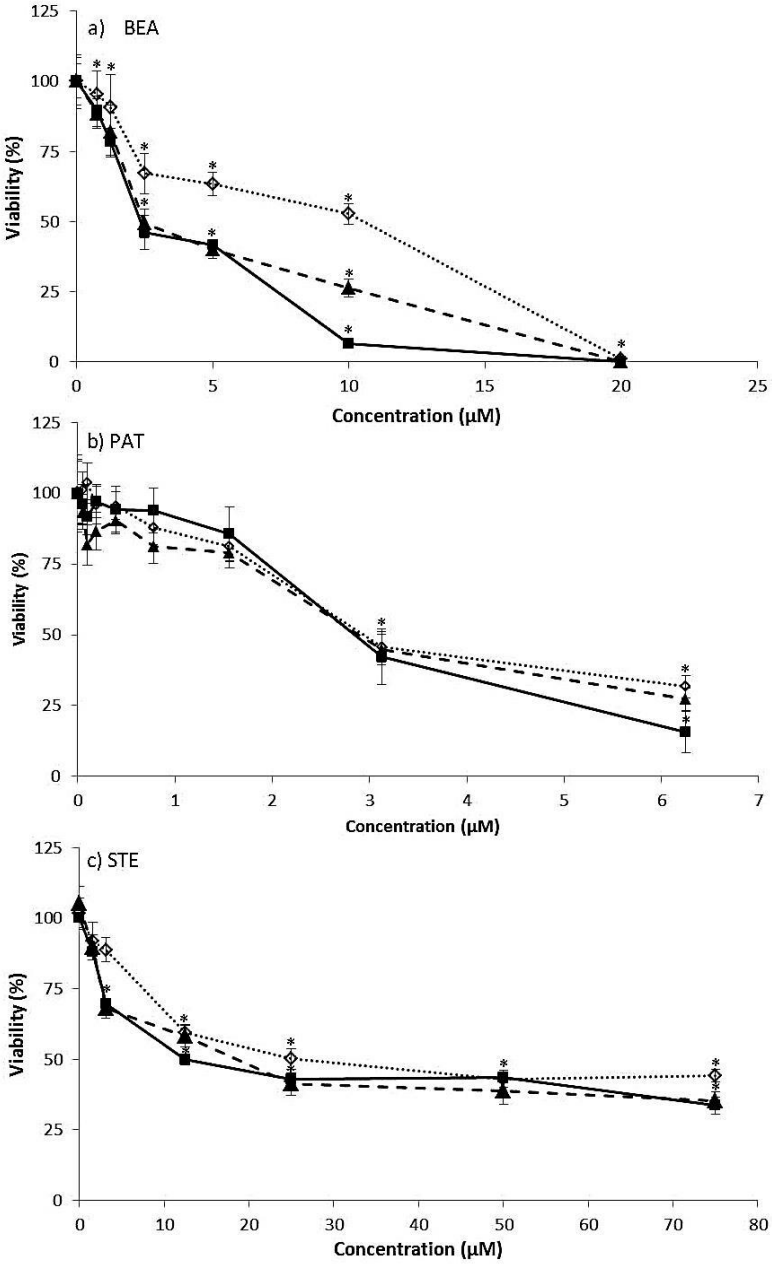

Figure 2. Individual concentrations-response curve of a) BEA, b) PAT and c) STE cell proliferation on CHO-K1 cells after 24 (⋯◊⋯), 48 (--■--) and 72 (–▲–) h of exposure. Results are expressed as mean ± SEM (n=3). (*) p≤0.05 represent significant difference as compared to control values.

3.2 Interaction of mycotoxin combination

The binary and tertiary combinations of PAT, STE and BEA on CHOK-1 cells was studied using MTT assay after 24, 48 and 72 h of exposure. The mixtures' concentration-response curves are shown in Figures 3 to 5. The PAT and STE mixture shows higher cytotoxic effects on cell proliferation than the STE alone at all exposure time (Fig. 3); whereas the mixture produces the same effect respect to PAT alone and at higher tested concentrations at 48 and 72 h of exposure (Fig. 3). This mixture deceased cell proliferation about 87% and from 86% to 73% respect STE and PAT alone, respectively (Fig. 3). The BEA and STE mixture was more cytotoxic than BEA alone (Fig. 4). At 24 and 72 h, the mixture showed more cytotoxic effect than STE alone (Fig. 4). The mixture deceased cell proliferation from 99% to 57% and from 89% to 57% respect BEA and STE alone respectively (Fig. 4). However, when PAT was combined with BEA, the combination is equally cytotoxic than when they are tested separately (Fig. 5). However, at 48 h of exposure, BEA was less cytotoxic than the mixture (Fig. 5) and cell proliferation decreased with the mixture PAT+BEA about 80% compared to BEA alone.

Figure 6 shows the concentration response curves for the tertiary combination (PAT, BEA and STE) at 24, 48 and 72 h of exposure on CHO-K1 cells. The tertiary combination reduces strongly cell proliferation respect to each mycotoxin alone (Fig. 6). At 24 h of exposure, the cell proliferation decreased from 100% to 51% (Fig. 6) and a similar reduction in cell proliferation from 100% to 48% was observed after 48 h of exposure compared to each mycotoxin separately (Fig. 6). The greatest decrease of cell proliferation was observed at 72 h of exposure; cell proliferation decreased from 100% to 37.5% compared to the individual treatment (Fig. 6).

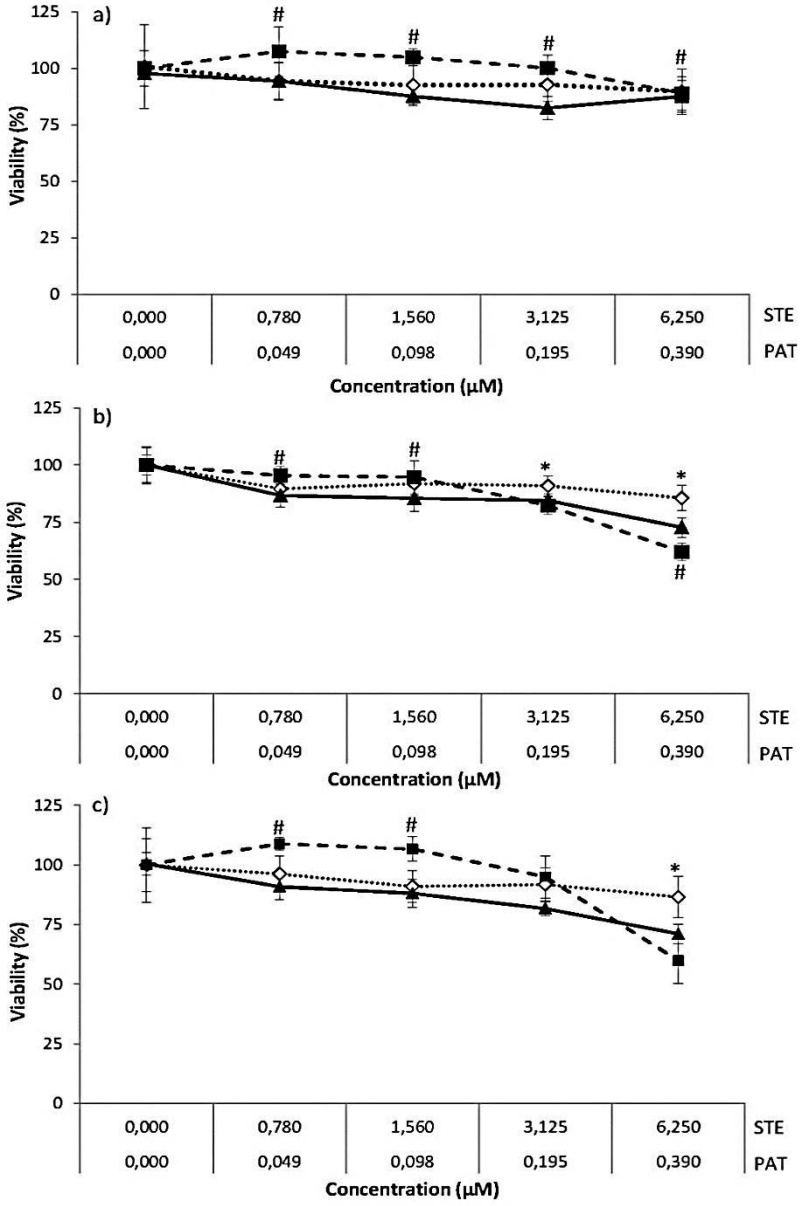

Figure 3. Cytotoxic effect of individual and binary combination of PAT and STE in CHO-K1 cells by MTT assay at (a) 24 h, (b) 48 h and (c) 72 h of exposure. PAT (···◊···), STE (--■--) and PAT+STE (–▲–). Results are expressed as mean ± SEM (n=3). (*) p≤0.05 represent significant difference between the mixture and PAT exposed individually. (#) p≤0.05 significant differences between the mixture and STE exposed individually.

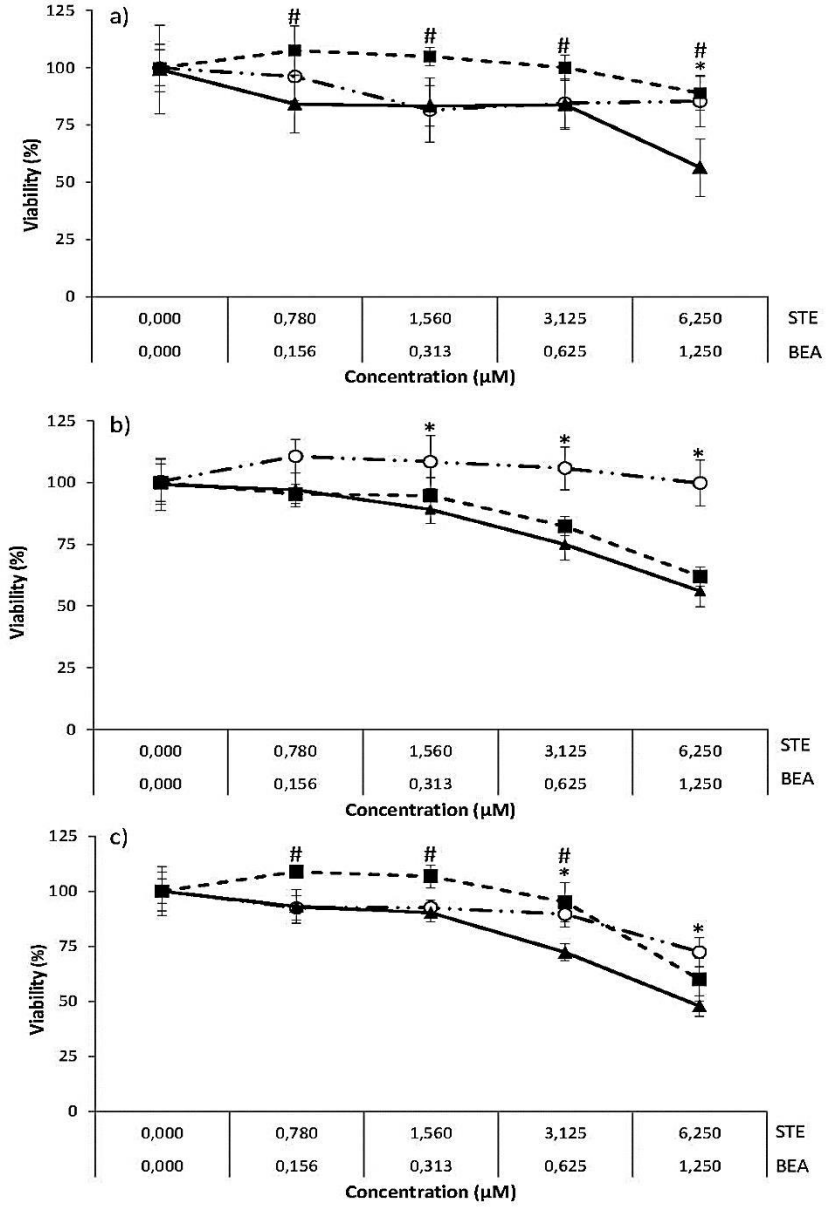

Figure 4. Cytotoxic effect of individual and binary combination of BEA and STE in CHO-K1 cells by MTT assay at (a) 24 h, (b) 48 h and (c) 72 h of exposure. BEA (···○···), STE (--■--) and BEA+STE (–▲–). Results are expressed as mean ± SEM (n=3). (*) p≤0.05 represent significant difference between the mixture and BEA exposed individually. (#) p≤0.05 significant differences between the mixture and STE exposed individually.

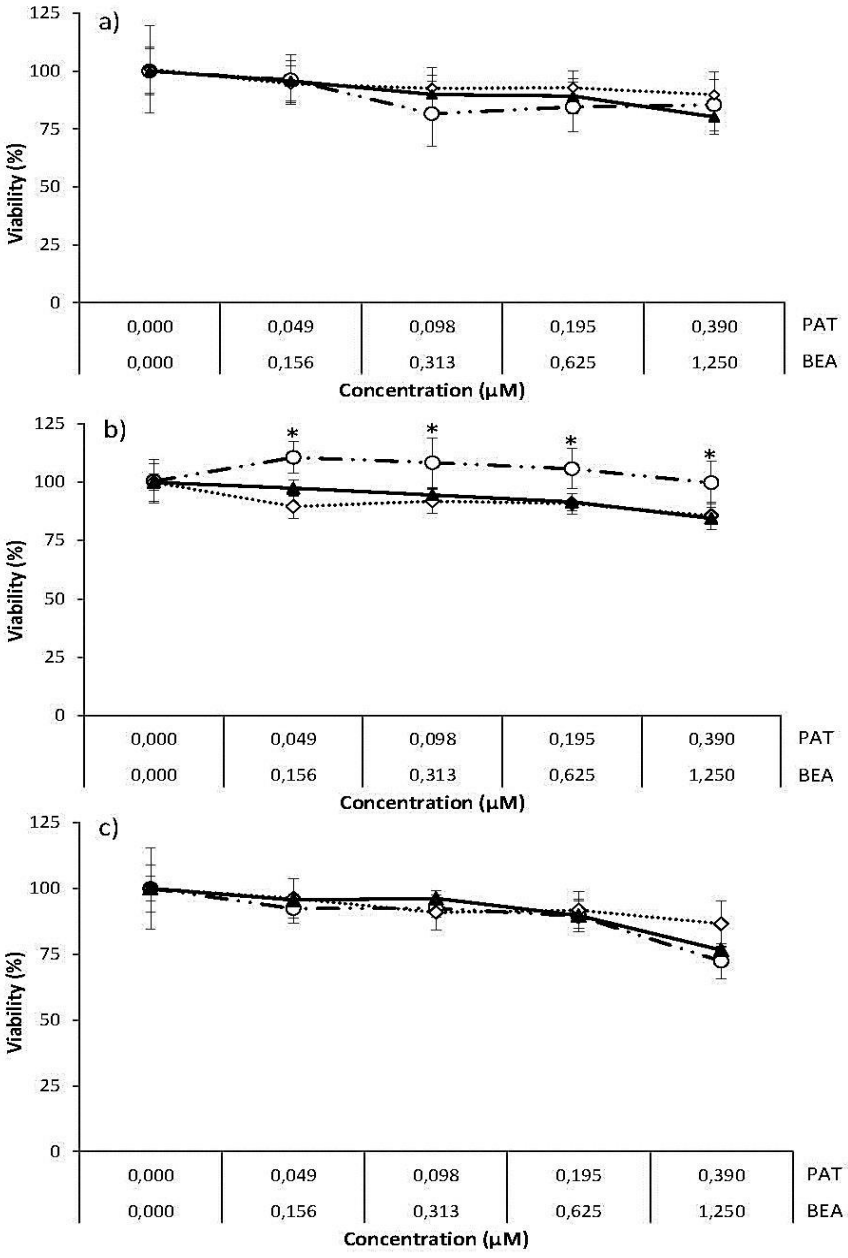

Figure 5. Cytotoxic effect of individual and binary combination of BEA and PAT in CHO-K1 cells by MTT assay at (a) 24 h, (b) 48 h and (c) 72 h of exposure. PAT (···◊···), BEA (····▲···) and PAT+BEA (–▲–). Results are expressed as mean ± SEM (n=3). (*) $p \leq 0.05$ represent significant difference between the mixture and BEA exposed individually.

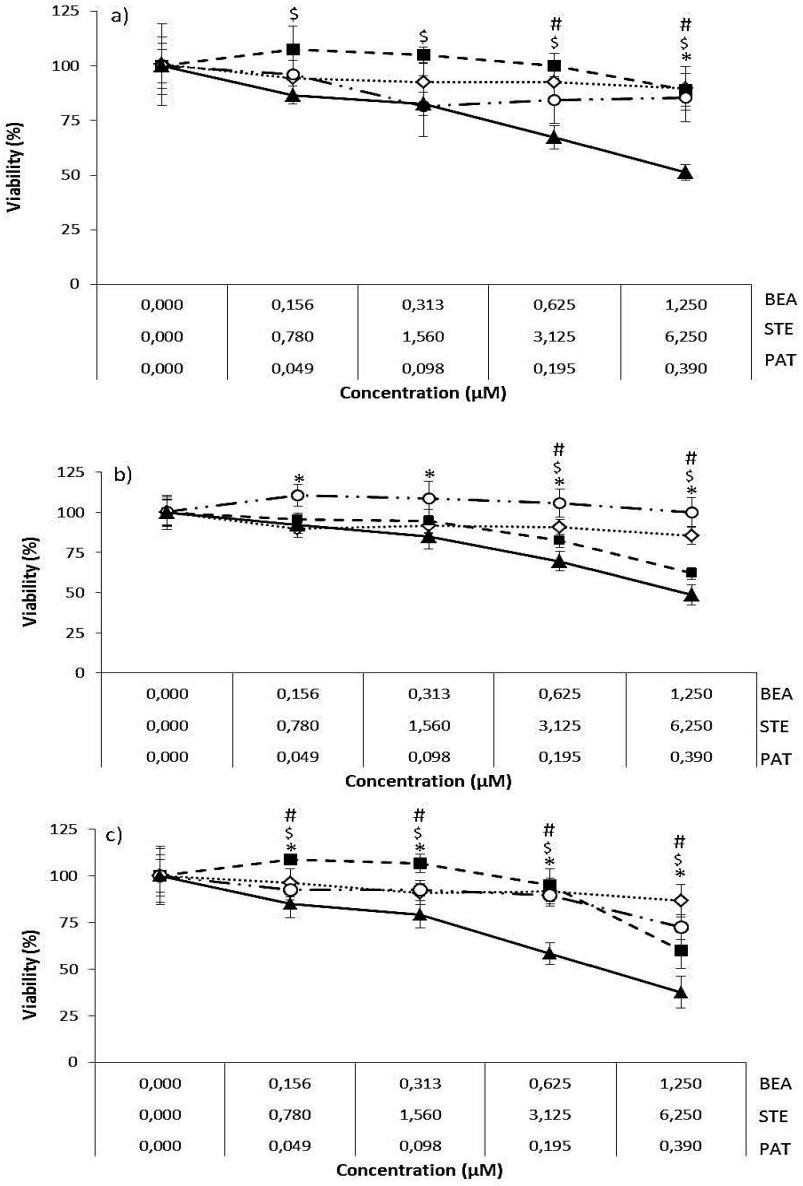

Figure 6. Cytotoxicity effect of individual and tertiary combination of PAT, STE and BEA in CHO-K1 cells by MTT assay at (a) 24 h, (b) 48 h and (c) 72 h of exposure... PAT (⋯◊⋯), STE (--■--), BEA (⋯–·▲·–⋯–·) and PAT+STE+BEA (–▲–).Results are expressed as mean ± SEM (n=3). (*) p≤0.05 represent significant difference between the mixture and BEA exposed individually. (#) p≤0.05 significant differences between the mixture and PAT exposed individually. ($) p≤0.05 significant differences between the mixture and STE exposed individually.

Isobologram analysis is used to determine the type of interaction between the three mycotoxins. The parameter *Dm*, m and r of the binary and tertiary combination, as well as mean combination index (CI) values for lower fractions affected (*fa*) are shown in Table 2. The IC_5, IC_{10}, IC_{25} and IC_{50} are the dose required to inhibit proliferation at 5%, 10%, 25% and 50%, respectively (Table 2). These IC values were calculated automatically by the Computer Software CalcuSyn. After 24 h of exposure, data shown in Table 2 prove that a synergistic interaction at low fraction affected becomes an additive effect at higher concentrations. Similar behavior was observed for PAT+STE combination at 48 and 72 h exposure time (Table 2). This performance of the mixture remained during 48 and 72 h of exposure when PAT (mycotoxin with higher IC_{50} value) and STE (mycotoxin with lower IC_{50} value) were in combination. However, at higher exposure time, antagonistic effect is converted in additive one in all the mixtures where the BEA is present (BEA+STE, BEA+PAT and BEA+PAT+STE) (Table 2). Nevertheless, in the tertiary combination at 72 h of exposure, the interaction shows synergism effect (Table 2).

Table 2: 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 (IC_{50}) and the conformity of the data to the mass-action law, respectively (Chou and Talalay 1984; Chou 2006). Dm and m values are used for calculating the CI value. CI < 1 =1 and > 1 indicates synergism (Syn), additive effect (Add) and antagonism (Ant), respectively. IC_5, IC_{10}, IC_{25} and IC_{50} are the doses required to inhibit proliferation at 5%, 10%, 25% and 50%, respectively. Values are expressed as mean ± SD (n=3). CHO-K1 cells were exposed during 24, 48 and 72 h with PAT+ BEA at molar ratio of 1:3.2; PAT+ STE at molar ratio of 1:16; BEA+STE at molar ratio of 1:5; and PAT + BEA + STE at molar ratio of 1:3.2:16. BEA, PAT, STE

Mycotoxin	Time (h)	Dm (µM)	m	r	CI Values at			
					IC_5	IC_{10}	IC_{25}	IC_{50}
BEA	24	2.09	2.25	0.8687				
	48	1.99	2.68	0.9677				
	72	7.43	0.72	0.8782				
PAT	24	1.36	2.19	0.8974				
	48	5.96	0.56	0.9996				
	72	26.09	0.57	0.9942				
STE	24	33.92	2.09	0.8745				
	48	10.25	1.29	0.9633				
	72	7.84	3.38	0.9488				
BEA + STE	24	3.60	0.61	0.9038	0.07 ± 0.15 Syn	0.17 ± 0.18 Syn	0.61 ± 0.46 Add	2.25 ± 2.39 Add
	48	1.35	1.56	0.9932	1.28 ± 0.24 Ant	1.25 ± 0.18 Ant	1.26 ± 0.22 Ant	1.34 ± 0.35 Add
	72	1.27	1.34	0.9800	1.36 ± 0.75 Add	1.01 ± 0.27 Add	0.84 ± 0.28 Add	0.98 ± 0.42 Add
BEA + PAT	24	2.61	0.75	0.9582	0.43 ± 0.16 Syn	0.84 ± 0.30 Add	2.23 ± 1.27 Add	5.87 ± 5.37 Add
	48	2.46	0.92	0.9957	3.80 ± 0.25 Ant	2.78 ± 0.24 Ant	2.71 ± 0.57 Ant	4.38 ± 1.53Ant
	72	1.65	0.97	0.9214	2.60 ± 1.46 Ant	1.89 ± 0.68 Ant	1.20 ± 0.87 Add	0.77 ± 0.68 Add
PAT + STE	24	3.28	0.62	0.9213	0.13 ± 0.10 Syn	0.32 ± 0.21 Syn	1.12 ± 1.09 Add	3.96 ± 3.05 Add
	48	7.18	0.40	0.9795	0.22 ± 0.39 Syn	0.50 ± 0.36 Syn	2.22 ± 1.32 Add	8.97 ± 8.27 Add
	72	1.59	0.68	0.9909	0.24 ± 0.05 Syn	0.36 ± 0.07 Syn	0.98 ± 0.28 Add	3.31 ± 1.42 Ant
BEA + PAT + STE	24	0.38	1.23	0.9993	0.36 ± 0.11 Syn	0.47 ± 0.17 Syn	0.69 ± 0.34 Add	1.03 ± 0.68 Add
	48	0.38	1.23	0.9993	1.81 ± 0.14 Ant	1.31 ± 0.11 Ant	1.12 ± 0.15 Add	1.27 ± 0.28 Add
	72	0.26	1.11	0.9857	0.70 ± 0.34 Add	0.54 ± 0.15 Syn	0.50 ± 0.15 Syn	0.66 ± 0.26 Syn

4. Discussion

Simultaneous occurrence of many mycotoxins in feed and food has been reported worldwide. The present work is conducted to investigate the cytotoxic effect of BEA, PAT and STE alone or in combination on CHO-K1 cells. The cytotoxicity of the three mycotoxins individually tested has been investigated in some *in vitro* cell lines (Ayed-Boussema et al., 2010; Ferrer et al., 2009; Jow et al. 2004; Klaric et al., 2008; Liu et al., 2014; Prosperini et al, 2013; Ruiz et al., 2011a, 2011b; Wang et al., 2013; Watjen et al., 2014; Zhang et al., 2014); and different IC_{50} values were obtained. BEA, PAT and STE decreased cell proliferation on CHO-K1 cells in our finding in support to those authors. The results showed that CHO-K1 were more sensitive toward PAT than BEA and STE. The IC_{50} value of PAT on CHO-K1 cells was higher than those found in previous studies made on CHO-K1 cells for 24 h (IC_{50}= 0.69 µM; Ferrer et al., 2009). However, higher IC_{50} values were obtained with PAT after 24 h of exposure by Ayed Boussema et al. (2010) on hepatic HepG2 cells (IC_{50}=15 µM), Behm et al. (2012) on lung V79 cells (IC_{50}= 17 µM) and Zhang et al. (2014) on renal HEK293 cells (IC_{50} =12 µM).

Our results indicate that BEA inhibits CHO-K1 proliferation with an IC_{50} value equal to 10.7 µM after 24 h of exposure. These results are similar to those provided by Ferrer et al. (2009) with an IC_{50} equal to 12.08 µM, but higher versus those obtained on Caco2 (IC_{50} from 3.2 to 20.6 µM), CHO-K1 (IC_{50} from 3.8 to 17.22 µM), Vero (IC_{50} from 6.25 to 10.02 µM), H4IIE (IC_{50}= 1.9 µM), HCT 116 (IC_{50}=2.4 µM), HepG2 (IC_{50}= 3.6 µM) and C6 (IC_{50}=1µM) cells (Prosperini et al., 2013; Ruiz et al., 2011a, 2011b; Watjen et al., 2014). Therefore, our results demonstrated that CHO-K1 cells are less sensitive to BEA than other cells. However, STE was less toxic towards proliferating CHO-K1 cells in comparison to other cell lines as evidenced by previous studies. The IC_{50} values for STE on HepG2 cells after 24 h of exposure were 3 µM (Gao et al., 2014), and 7.3 µM (Liu et al., 2014) and on human lung adenocarcinoma (A549) cell line was 3.7 µM (Liu et al., 2014); but STE was more cytotoxic towards neuronal (Neuro-a2; IC_{50}=40.1µM) cells than other cell lines (Bunger et al., 2004). Differences between IC_{50}

values obtained for each mycotoxin were caused for different cell sensitivity of each type of cell line, supplements in the medium and cytotoxic assay conditions.

The combination of PAT and STE (Table 2) produced synergism at low concentration affected followed by additivity at higher concentration affected; these finding suggest that the co-occurrence of these two mycotoxins in food may increase their cytotoxic effects in combination compared to each mycotoxin alone. The cytotoxic effect of PAT and STE combination could be explained by the implication of the oxidative stress as their mechanism of toxicity (Ayed-Boussema et al., 2010; Ferrer et al., 2009; Gao et al., 2014; Mallebrera et al., 2014; Prosperini et al., 2013).

At 24 h of exposure, the BEA+PAT, BEA+STE and BEA+PAT+STE (Table 2) mixtures presented an unique feature of variation of the type of interaction accordingly to the concentration. Low concentrations were synergy (IC<1) and higher concentrations behaved additive effect (IC from 0.6 ± 0.5 to 5.9 ± 5.4). When the incubation time increased, the interaction pattern changed indicating antagonistic effects. These results suggest that higher inhibition of proliferation was observed on CHO-K1 cells exposed to each mycotoxin alone than when they occur together. As expected, the additive effect was observed when the concentration and exposure time increased. This observation suggests that the simultaneous presence of low concentrations of BEA in combination with PAT or STE in the diet may induce greater toxicity than the predicted from alone mycotoxins or other combinations without BEA. Moreover, these findings are of high biological relevance considering the consumers are exposed to mycotoxins in their diet (EFSA, 2013, 2014; SCOOP Task 3.2.8).BEA found in food range from 0.1 to 6402 μg/Kg (EFSA, 2014), PAT from 0.03 to 3533 μg/Kg (SCOOP Task 3.2.8) and STE from 1 to 4300 μg/Kg (EFSA, 2013).

Conflicting results about BEA were obtained in literature. The combination of BEA with other mycotoxins such as T-2 toxin showed synergistic effect while, it showed a very strong antagonism with deoxynivalenol (DON) on the same mammalian cell line (CHO-K1; Ruiz et al., 2011a). However, the combination of BEA with DON or T2 on mammalian kidney (Vero) cells showed antagonistic effect mostly at low concentrations (Ruiz et al., 2011b). On the other hand, Klaric et al. (2007, 2008)

demonstrated that the combination of BEA+OTA+FB1 on porcin kidney epithelial (PK15) cells fluctuated between additive effect at low concentrations and antagonistic interaction at higher mycotoxins concentrations.

The tertiary combination BEA+PAT+STE showed higher inhibition of proliferation on CHO-K1 cells than individual mycotoxins. These results suggest that the co-occurrence of these mycotoxins in the food or diet may produce higher toxicity. Studies about mycotoxins' interaction when they are in tertiary combinations are limited. The tertiary combination of BEA+DON+T2 showed antagonistic effects on Vero cells (Ruiz et al., 2011b) or synergistic interaction on CHO-K1 cells (Ruiz et al., 2011a). Nevertheless, the tertiary combination of BEA+OTA+FB1 on porcine kidney epithelial (PK15) cells varied from additive effects at lower concentrations to antagonistic effect at high concentrations (Klaric et al., 2007; 2008). And, the tertiary STE+CTN+OTA combination on Hep3B (hepatocellular cancer line) showed antagonistic effect (Anninou et al., 2014).

Prosperini et al. (2014) studied enniatins (ENN), mycotoxins produced by *Fusarium* species. ENNs are mycotoxins structurally related to BEA. The measured combined effect of ENB1+ENA1 indicates an antagonistic effect of the combination at low concentrations while a synergy one was only noted at higher concentrations. Similar pattern was also noted for α-zearalenol and β-zearalenol mixture (Tatay et al., 2014). Authors hypothesized that, at low concentrations, similar structure mycotoxins are competing for the same receptor sites and the resulting interaction is antagonistic. Then, after crossing cell membranes, the resulting effect is the sum of individual effects when mycotoxins are at high concentrations. Tatay et al. (2014), investigated the combination between zearalenone and its metabolites on CHO-K1 and found that their interaction is synergistic at low concentration followed by antagonistic effect at higher concentrations. Speijers and Speijers (2004) suggest that mycotoxin with similar mode of action are expected to have at least additive effects. In this study, we investigated the interaction between mycotoxins produced by different genera of fungi with different structures, reported occurring together (Garcia-Moraleja et al. 2015a, 2015b; Sulyok et al., 2010) (Figure 1). These setting could explain the

fluctuation of the interactions between these mycotoxins during the exposure time. In fact, the differences between mycotoxin interactions could be explained by the different chemical structures, chemical properties and the competition or not for the same cell receptor. And, those factors could indicate that it is difficult to predict the type of interaction only on the basis of the effect of individual mycotoxin. So, it is essential to study the mycotoxin mechanisms of action when there are alone or in combinations.

Our results clearly indicate that, the susceptibility of CHO-K1 cells towards mycotoxins differ depending on the combinations assayed. Synergistic effects observed with mycotoxins combinations are of great importance since the studied mycotoxins may occur in the same diet; they may produce relevant cytotoxic activity, or enhance cytotoxic activities of other mycotoxins, depending on the mixture mycotoxins. Thus, it is difficult to explain the difference between mycotoxin interactions in the mixtures, because of an unknown mechanism related to the complex perturbation of biochemical process cannot be excluded (Ruiz et al., 2011b).

As a matter of fact, we deduce that these combinations could possess a significant threat on the public health. PAT was found more toxic than BEA and STE. And, mycotoxin combinations showed different interactions underlining their complexity of them, which depend on cell lines, mycotoxin concentrations, time of exposure and experimental parameters.

Considering the presence of mycotoxins and other xenobiotics in food all over the world, we should be aware of further research about individual mycotoxins cytotoxicity, for better understanding of their mechanisms of action and, the toxicological effects of their interactions in living organisms. Moreover, according to the co-occurrence of mycotoxins in food and feed, new risk assessment strategies should be taking into account to establish regulatory standards about mixtures.

Acknowledgments

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Conflict of Interest

Authors declare that there are no conflicts of interest.

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3.2. Mechanisms of beauvericin toxicity and antioxidant cellular defense

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Mechanisms of beauvericin toxicity and antioxidant cellular defense

Beatriz Mallebrera*, Ana Juan-Garcia, Guillermina Font, Maria-Jose Ruiz

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

Abstract

Beauvericin (BEA) is a secondary metabolite produced by many species of fungus *Fusarium*. This study determines the injury (cell viability, cell proliferation, mitochondrial membrane potential, cell death and DNA damage) and the intracellular defense mechanisms (catalase and superoxide dismutase) in Chinese Hamster Ovary (CHO-K1) cells after BEA exposure. The results obtained in this study demonstrated that BEA induces cytotoxicity in a dose- and time-dependent manner in CHO-K1 cells. Moreover, disruption in mitochondrial enzymatic activity and cell proliferation has been observed after BEA exposure, which can lead or be consequence of cell death. BEA inhibits cell proliferation by arresting cells in G_0/G_1 and increasing apoptosis. Moreover, at higher exposure times, BEA induces differentiation of CHO-K1 cells through G_2/M arrest, preventing that cells entry into mitosis. DNA strand breaks were observed at 1 μM after 24 h of exposure. On the other hand, the SOD and CAT activities were increased after BEA exposure and consequently could to contribute to eliminate damage produced by BEA in CHO-K1 cells.

Keywords: Beauvericin; cytotoxicity; DNA damage; cell death; mitochondrial membrane potential; defense mechanisms.

* **Corresponding author:** Beatriz Mallebrera, Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46117, Burjassot, Valencia, Spain. E-mail: beatriz.mallebrera@uv.es

1. INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi. They can damage humans and animals health through ingestion, inhalation or the skin (Marin et al., 2013). Emerging *Fusarium* mycotoxins include fusaproliferin, enniatins, beauvericin and moniliformin.

Beauvericin (BEA) is synthesized by many species of fungus *Fusarium* and *Beauveria bassiana*. Wheat, rice, corn, barley and cereal derivate are known as susceptible commodities to be contaminated by BEA (Meca et al., 2010; Mahnine et al., 2011; Zinedine et al., 2011). BEA has ionophoric activity, it is incorporate into biological membranes forming a complex with essential cations, which increases its ion permeability and affects cellular homeostasis (Kouri et al., 2003; Tonshin et al., 2010). Therefore, the mitochondria membrane may be one of the possible sites for BEA-mediated cytotoxicity. Reactive oxygen species (ROS) production in excess of the capabilities of detoxication systems causes oxidative stress in the cell. ROS are extremely reactive, making them likely to participate in chemical reactions. These reactions also damage lipids, proteins and DNA and cause deleterious effects (Kanduc et al; 2002). Oxidative reactions in mitochondria could induce its dysfunction which activates the apoptotic and necrotic pathways (Tonshin et al., 2010, Prosperini et al, 2013a). ROS can also damage DNA causing single-strand breaks and base alteration inducing DNA adducts and mutation.

Because of the propensity of ROS to react with and damage important bimolecules, cells have evolved biochemical systems for detoxication. Antioxidant compounds (such as glutathione, vitamins and polyphenols ingested in diet) and enzymes (glutathione peroxidase, GPx; catalase, CAT and superoxide dismutase, SOD) contribute to protect the cells from oxidative stress and help maintain the redox balance (Matés, 2000).

Stress response reflects an imbalance between pro-oxidant activities of substances and the innate cytoprotective response of the cells. Mycotoxins can disturb this balance either by increasing the pro-oxidant activity or by disrupting the protective pathways. The aims of this study were to determine the injury of BEA in

biological CHO-K1 cell components after 24, 48 and 72 h of exposure. And the enzymatic antioxidants defense mechanisms in CHO-K1 cells after BEA exposure.

2. MATERIAL AND METHODS

2.1. Reagents

The reagent grade chemicals, cell culture components used and BEA (783.95 g/mol, ≥97% purity) were purchased by Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Stock solution of BEA was prepared in methanol and maintained at -20ºC. Final concentrations of BEA in the assay were achieved by their dilution in the culture medium. The final methanol concentration in the medium was ≤1% (v/v).

2.2. Cell culture

CHO-K1 cells derived from Chinese Hamster Ovary, were grown in 9 cm^2 polystyrene tissue culture dishes in Ham's-F12 medium supplemented with 25 mM HEPES buffer, 10% (v/v) FCS, 100 U/mL penicillin and 100 mg/mL streptomycin. Incubation conditions were pH 7.4, 37 ºC 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).

2.3. Cell viability assay

CHO-K1 cells were cultured in 96-wells microplates at a density of $2x10^4$ cells/well. Counting of cells was performer with a Beckman coulter (Florida, USA). The cells were cultured until confluence (65%). Then, the culture medium was removed and replaced with fresh medium containing different concentration of BEA ranging from 0.6 to 20 μM. Cytotoxicity was determined by the 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio (MTT) assays as described by Ruiz et al. (2006) during 24, 48 and 72 h. The MTT assay monitors a reduction of yellow tetrazolium salt by mitochondrial dehydrogenase enzymes of metabolically activate/viable cells to purple formazan crystals. Absorbance was measured at 570 nm with an automatic ELISA reader. The

IC_{50} values obtained were calculated from full dose-response curves. Three assays in different days were performed.

2.4. Cell proliferation

Cell proliferation is essential for the homeostasis of most organs and tissues, BEA can interfere with this process. The key to this effect is the cell cycle, which is closely linked to apoptosis. Cell cycle analysis was performed by staining the DNA with propidium iodide (PI) as described previously (Juan-García et al., 2013). 6.8×10^5 cells/well were seeded in 6-well plates and treated with BEA at 0.1, 1 and 5 μM for 24, 48 and 72 h. Then the cells were trypsinized and place 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 mM Tris Base, 50 μg/mL of PI, 0.1% Triton X-100, 10 mg (700 U/L) RNase A (Sigma–Aldrich) and 10 mM NaCl. Four independent experiments were performed for each BEA treatment and at least 10,000 cells were analyzed for each sample at the excitation and emission wavelengths of 488 and 620 nm, respectively.

2.5. Mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential ($\Delta\Psi$m) was measured using a modified rhodamine 123 (Rh123) method described by Andersson et al. (1987). CHO-K1 cells were cultured in 96-wells black microplates at a density of $2x10^4$ cells/well. After confluence cells were exposed to BEA (0.1, 1 and 5 μM) for 24 h, and then were incubated with Rh123 at final concentration of 5 μM for 15 min in darkness. The Rh123 was removed and the cells were resuspended in PBS. The fluorescence of the cationic dye Rh123 was measured at emission and excitation wavelengths of 485 and 530 nm, respectively. Three replicates were performed. The results are expressed as percentage (%) of the control.

2.6. Apoptosis and necrosis determination

Cell death generally proceeds through two molecular mechanisms: necrosis and apoptosis. One of the characteristics of apoptosis is the externalization of phos-

phatidylserine (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 fluorescein isothiocyanate (FITC)-labeled annexinV (Annexin V-FITC) and PI double staining (Vermes et al., 1995). A total of 6.8×10^5 cells/well were seeded in 6-well plates. After 24, 48 and 72 h of exposure at 0.1, 1 and 5 μM BEA, the assay was carried out as described by Juan-García et al. (2013). The cells were resuspended in 400 μL Annexin-V-binding buffer. Then, they were situate on ice and darkness 30 min with 1.25 ng/mL Annexin V-FITC and 10 μg/mL PI dyes to link the PS in the presence of Ca^{2+}. Positioning of quadrants on Annexin V-FITC/PI dot plots was performed. Pro-apoptotic/apoptotic (early apoptosis) cells (Annexin V-FITC+/PI−), in Annexin V-FITC+/PI+ cells have completed the apoptotic process and start the necrotic process (referred as apoptotic/necrotic or late apoptosis) or are already dead, necrotic cells (Annexin V-FITC−/PI+) and living cells (Annexin V-FITC−/PI−).10,000 cells were acquired and analyzed on a BD FACS Canto 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. Determinations were performed in four independent experiments.

2.7. Alkaline Comet assay (pH > 13)

Three mL of CHO-K1 cell suspension (approximately $3.4x10^5$ cells/well) was plated in each well of six-well culture plates and treated with BEA (0.1, 1 and 5 μM) for 24 h. DNA damage was determined by alkaline comet assay, according to the method described by Prosperini et al. (2013a) with some modifications. Briefly, CHO-K1 cells were embedded in 0.8% low-melting-point agarose on slides and lysed [1% Triton X-100, 2.5 M NaCl, 100 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, pH 10] for a minimum of 1 h at 4 °C. The slides were then immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 min room temperature and the duration of the subsequent electrophoresis was 24 min in the

same solution at 0.7 V/cm (voltage across the platform) and 300 mA. After electrophoresis, the slides were washed twice in neutralization buffer (0.4 M Tris pH 7.5), dried in 96% ethanol (-20 °C, 5 min). Before analysis, the slides were stained with 20 μg/mL PI and dried at room temperature. Positive control is benzo-(α)-pyrene [B(α)p; 15 μM]. Negative control consists of 1% methanol alone in the treatment medium. The analysis was performed with a fluorescence microscope (NIKON Eclipse E800), equipped with camera (NIKON DXM1200F). Fifty cells/slide were processed by CometScore (Automatic Comet Assay), http://autocomet.com/index.php?id=cometscorepro. Results are expressed as percentage of DNA in tail (%), calculated according to the equation: % DNA in tail= (total intensity of tail/total intensity of comet) x 100. Total intensity of comet = head length+tail length. Determinations were performed in four independent experiments.

2.8. Superoxide dismutase activity

To determine scavenging procedures in CHO-K1 cells exposed to BEA, the CAT and SOD activities were determined. For both assays, $6.8x10^5$ cells/well were seeded in six-well plates. After cells achieved the 65% confluence, cells were treated with 0.1, 1 and 5 μM BEA for 24 h. Then, the medium was removed and cells were homogenized in 0.02 M NaH_2PO_4 pH 7.4 and lysate with the polytron (Ultra-Turrax T8 IKA1-WERKE). The SOD enzyme destroys the free radical superoxide by converting it to hydrogen peroxide (H_2O_2). SOD activity was determined with Ransod kit (Randox Laboratories, United Kingdom) adapting for 1.5 mL cuvettes. SOD activity was monitored at 505 nm during 3 min at 37 ºC in a thermo circulation spectrophotometer (PerkinElmer UV–vis spectrometer Lambda 2 version 5.1.). SOD enzymatic activity was expressed as percentage (%) of the control. Determinations were performed in four independent experiments.

2.9. Catalase activity

The CAT function is to protect cells from H_2O_2 produced in redox reactions of normal aerobic metabolism or because of many xenobiotics can be oxidants or

reductants. CAT catalyzes the H_2O_2 decomposition into oxygen and water. The CAT activity was measured according to Espín et al. (2014) with slight modifications. Briefly: 50 µL of the homogenized cell suspension was mixed with 950 µL 0.05 M NaH_2PO_4 and 500 µL 0.03 M H_2O_2.The kinetics of enzymatic decomposition of H_2O_2 was determined as absorbance decrements at 240 nm for 2 min with a spectrophotometer (PerkinElmer UV–vis spectrometer Lambda 2 version 5.1). CAT enzymatic activity was calculated by using the molar absorptivity of H_2O_2 (43.6 $mM^{-1}cm^{-1}$) and expressed as percentage (%) of the control. Determinations were performed in four independent experiments.

2.10. Determination of total protein

The protein content was determined by the Bio-Rad DC Protein Assay, catalog number 500-0116 (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf). Protein concentration (mg/mL) was measured at 690 nm.

2.11. Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (SPSS, Chicago, IL, USA), statistical software package. Data were expressed as mean±SEM of three or four (depend on the experiment) independent experiments. The statistical analysis of the results was performed by one-way analysis of variance (ANOVA one-way) followed by Tuckey's test for the post hoc comparisons. The *p* value equal to or smaller than 0.05 it was considered significant.

3. RESULTS

3.1. Cell viability

The results obtained in Figure 1 indicate that BEA induces cytotoxicity in CHO-K1 cells after 24, 48 and 72 h of exposure. BEA decreased cell viability in a dose and time-dependent manner. The IC_{50} is the inhibitory concentration quantified as the concentration required for inhibiting cell growth by 50% under the assay conditions.

The IC_{50} values obtained in CHO-K1 cells were 10.7±3.7, 2.5±3.3 and 2.2±3.3 μM after 24, 48 and 72 h of BEA exposure, respectively.

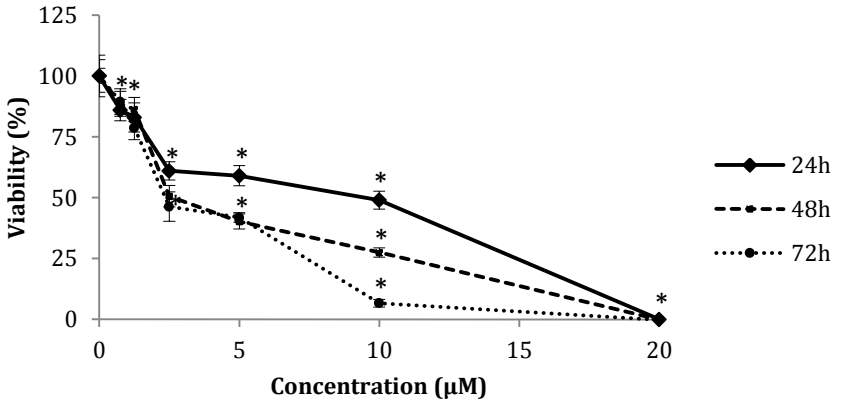

Figure 1. Cell viability (%) in CHO-K1 cells exposed to BEA by MTT assay after 24, 48 and 72 h. Data are expressed as mean±SEM (n=3). *$p \leq 0.05$ indicates significant differences from the control.

3.2. Cell proliferation

Cell distribution in the different phases of the cell cycle (Sub G_0/G_1, G_0/G_1, S and G_2/M) was determined after BEA exposure (0.1, 1 and 5 μM) in CHO-K1 cells during 24, 48 and 72 h (Fig. 2). No changes were observed in phase Sub G_0/G_1 at any time and concentration tested respect to the control. After 24 h of exposure, the phase G_0/G_1 significantly ($p \leq 0.05$) increased in 19% (5 μM) and the phase S significantly ($p \leq 0.01$) decreased in 49% (5 μM), respect to the control (Fig. 2A). After 72h, 5 μM BEA increased the phase S in 39% respect to the control. The G_2/M phase increased, 77% and 135%, after 5 μM BEA exposure at 48 and 72 h respectively, respect to control cells (Figs. 2B and 2C).

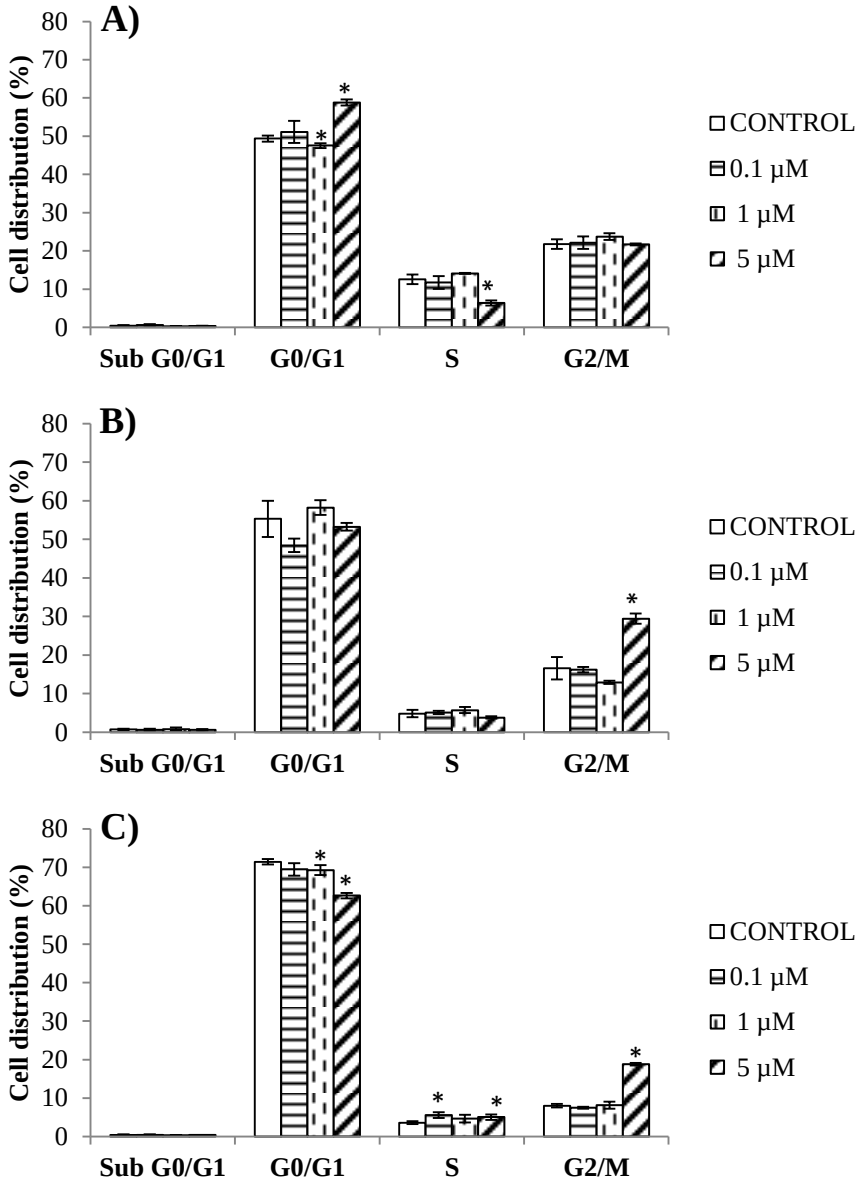

Figure 2. Cell cycle distribution in CHO-K1cells exposed to 0.1, 1 and 5 µM BEA after 24 (A), 48 (B) and 72 h (C). Data are expressed as mean ±SEM (n=4). *p≤0.05 indicates significant differences from the control.

3.3. Mitochondrial membrane potential (ΔΨm)

Results obtained in Figure 3 demonstrates that 5 μM BEA significantly ($p \leq 0.01$) decreased in 14% the ΔΨm, respect to the control.

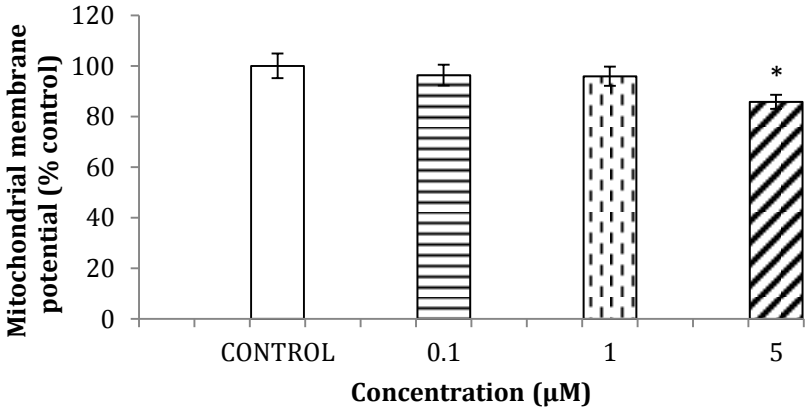

Figure 3. Mitochondrial membrane potential in CHO-K1 cells after 0.1, 1 and 5 μM BEA for 24 h. Values are expressed as the mean±SEM (n=3). *$p \leq 0.05$ indicates significant differences from the control.

3.4. Apoptosis-necrosis

Figure 4 shows the early apoptotic and apoptotic/necrotic cells induced by 0.1, 1 and 5 μM BEA after 24, 48 and 72 h of exposure in CHO-K1 cells. As can be observed in Figure 4, early apoptotic cells significantly ($p \leq 0.05$) increased from 78 to 116%, in 176% and 70% after 24, 48 and 72 h of exposure, respectively. On the other hand, apoptotic/necrotic cells significantly ($p \leq 0.05$) increased from 64 to 73% after 48 h (Fig. 4B) and decreased 61% after 72 h of exposure (Fig. 4C), respect to the control (Fig. 4).

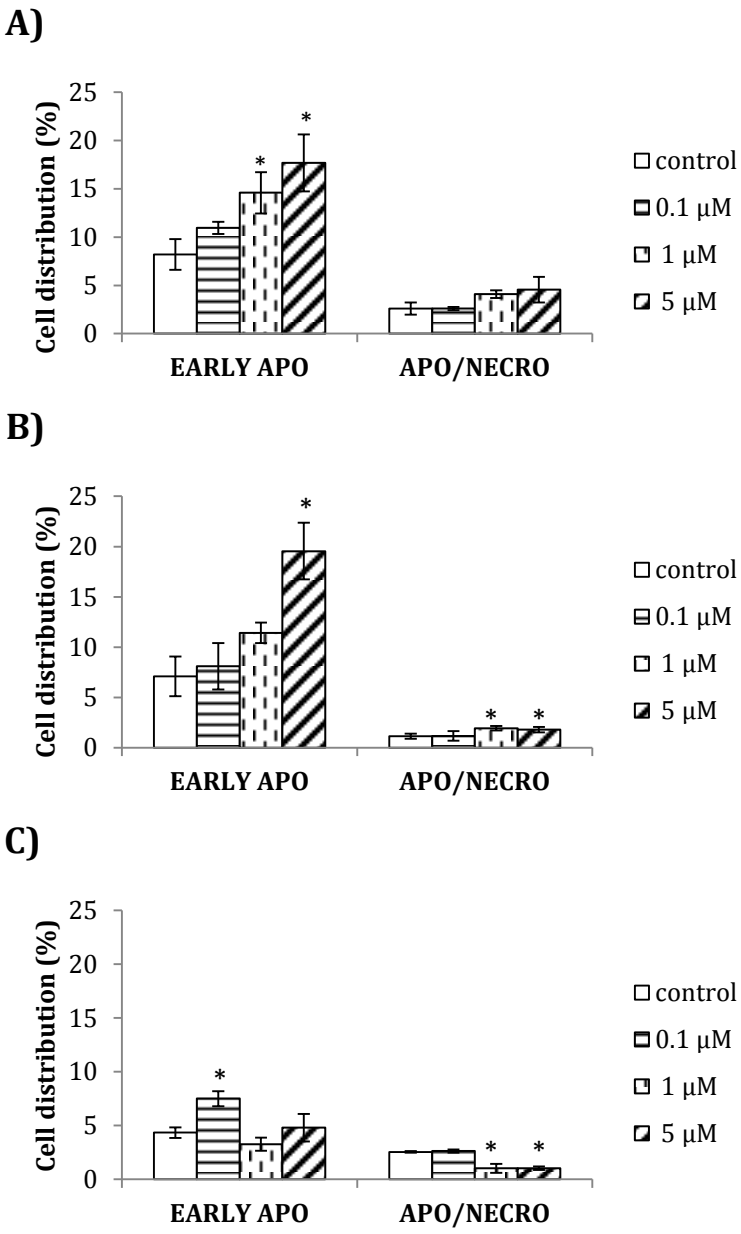

Figure 4. Analysis of early apoptosis and apoptosis/necrosis distribution of CHO-K1 cells after 0.1, 1 and 5µM BEA for 24, 48 and 72h. Data are expressed as mean±SEM (n=4). *$p \leq 0.05$ indicates significant differences from the control. APO/NECRO= apoptosis/necrosis generation.

3.5. Alkaline comet assay

Figure 5 shows the DNA in tail (%) after 24 h of BEA exposure (0.1, 1 and 5 μM) in CHO-K1 cells. Results demonstrated that 1 μM BEA significantly ($p \leq 0.001$) increases (85%) the percentage of DNA in tail respect to the control (Fig. 5).

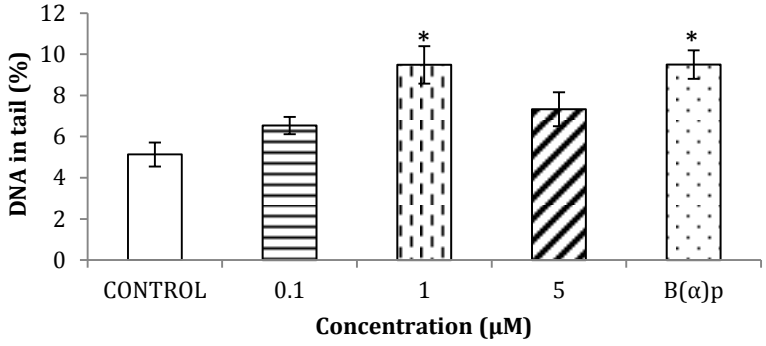

Figure 5. Percentage (%) of DNA in tail measured by alkaline comet assay (pH>13) in CHO-K1 cells after 0.1, 1 and 5 μM BEA. Values are expressed as the mean±SEM (n=4). *$p \leq 0.05$ indicates significant differences from the control. B(α)p: benzo-α-pirene (15 μM).

3.6. Superoxide dismutase

The SOD activity in CHO-K1 is observed in Figure 6. BEA significantly ($p \leq 0.05$) increases SOD activity at 1 μM (37%) and 5 μM (134%) respect to the control (Fig. 6).

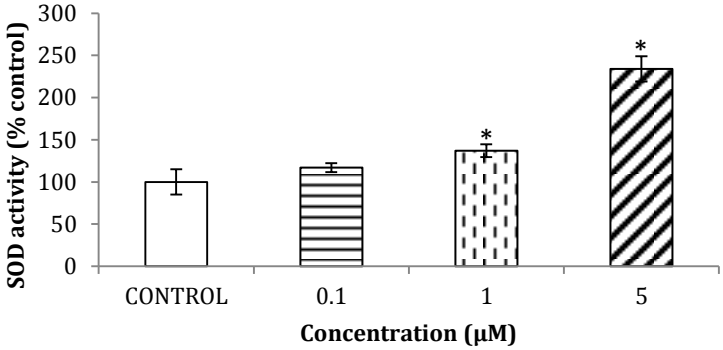

Figure 6. SOD enzymatic activity of CHO-K1 cells exposed to 0.1, 1 and 5μM BEA for 24 h. Values are expressed as mean±SEM (n=4). *$p \leq 0.05$ indicates significant differences from the control.

3.7. Catalase

The CAT activity is shown in Figure 7. The results obtained show that CAT activity significantly ($p \leq 0.05$) increased (70%) after 5 µM BEA exposure in CHO-K1 cells respect to control (Fig. 7).

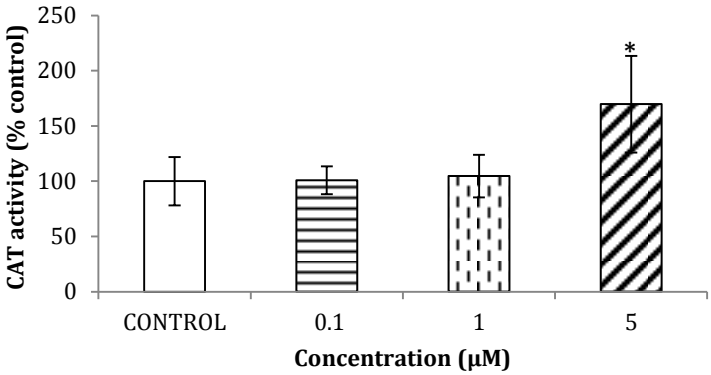

Figure 7. CAT enzymatic activity in CHO-K1 cells exposed to 0.1, 1 and 5 µM BEA for 24 h. Values are expressed as mean±SEM (n=4). *p≤0.05 indicates significant differences from the control.

4. DISCUSSION

Cytotoxic effect of BEA was measured by the reduction of formazan by mitochondrial dehydrogenases of living cells. This assay is useful to screening the range of BEA concentration in which CHO-K1 cells possess metabolic activity and which one interferes with cell proliferation. In this sense, from the lowest BEA concentration, reduction in capacity of enzyme activity was observed in CHO-K1 cells. BEA induces cytotoxicity (producing 50% growth inhibition) in a dose and time dependent manner in CHO-K1 cells. This fact is corroborated by other authors in CHO-K1 cells and other cell lines. The intensity of reduction of MTT varies depending on the periods of time, the cell line, the culture age and the medium components. Therefore, the IC_{50} of BEA ranged from 1 µM in rat glioma C6 cells to 20.6±6.9 µM in human colon carcinoma Caco-2 cells (Prosperini et al., 2013a; Wätjen et al., 2014). Ferrer et al. (2009) obtained similar results (IC_{50}=12.08±1.10 µM) for BEA in CHO-K1 cells after 24 h of exposure by MTT assay. Also, similar IC_{50} values (from 1.41 to 4.0 µM) were

obtained after 48 and 72 h of exposure in different cell lines by MTT assay (Zhan et al., 2007; Dornetshuber et al., 2009a, 2009b).

Cell proliferation is essential for the homeostasis of most tissues or organs. It is activated after mycotoxin insult in order to replace the lost cell mass. Several factors perturb the process by which a cell undergoes cell division that is cell cycle progression. Cell cycle checkpoints (at G_0/G_1 and G_2/M phases) are activated by signaling cascade and temporarily arrest cell cycle progression. To maintain the genomic integrity, the different check point proteins are activated inducing cell cycle arrest to repair the damage or if the lesion exceeds the repair capacity of the cell to activate cell death (Damia and Broggini, 2003). In the present study, at 24 h, BEA inhibits cell proliferation by arresting cells in G_0/G_1, and increases apoptosis (Figs. 2 and 4). Similar results were observed with BEA in Caco-2 cells (Prosperini et al., 2013a) and with enniatins (mycotoxins structurally related to BEA) in murine monocyte-macrophage (RAW 264.7), human non-small cell lung cancer (A549) cells and Caco-2 cells (Dornetshuber et al., 2007; Gammelsrud et al. 2012; Prosperini et al., 2013b). On the other hand, in this study, at 48 and 72 h of exposure, BEA induces differentiation of CHO-K1 cells through G_2/M arrest. Similar results were observed in Caco-2 cells exposed to BEA (1.5 and 3 μM) by Prosperini et al. (2013a). Then again, enniatins (1.5-10 μM) have similar behavior than BEA in human epithelial carcinoma-derived (KB-3-1) cell line (Dornetshuber et al., 2007) and in Caco-2 cells (Prosperini et al. 2013b). So, considering the results obtained in this study, BEA arrest cell cycle in G_2/M phase in CHO-K1 cells, and subsequently preventing that cells entry into mitosis. According to Abid-Essefi et al. (2003), this type of arrest may be an adaptive process in which a surveillance mechanism delays or arrests the cell cycle when DNA lesions occur, in order to repair to take place. This effect could be happening in CHO-K1 cells, as deduced that BEA reduces mitochondrial enzymatic activity and cell proliferation.

Because of cell damage can be produced by many cellular processes as consequence of BEA exposure, some hypothesis has been discussed. BEA was involved in the generation of oxidative stress (Ferrer et al., 2009; Prosperini et al., 2013a; Mallebrera et al., 2015). BEA exposure significantly increased ROS production (from

1.3- to 4.0-folds higher that control) in Caco-2 (Prosperini et al., 2013a) and CHO-K1 cells (Ferrer et al., 2009; Mallebrera et al., 2015). Cytotoxicity through via ROS production and mitochondrial activity damage has been evidenced. By contrast, Dornetshuber et al. (2009b) did not observe any production of ROS in human promyelocytic leukaemia cells (HL60) and human cervix carcinoma cells (KB-3-1).

On the other hand, the results obtained in this study show BEA decreases $\Delta\Psi m$ in CHO-K1 cells. Similar results were obtained by Prosperini et al. (2013a) in Caco-2 cells and Toshin et al. (2010) in isolated rat liver cells. According to Ly et al. (2003), loss of $\Delta\Psi m$ may be an early event in the apoptotic process; however, may not be an early requirement for apoptosis. On the contrary, may be a consequence of the apoptotic-signaling pathway. These observations suggest that disruption of $\Delta\Psi m$ produced by BEA in CHO-K1 can lead cytotoxicity and cell death by apoptosis and necrosis. Ionophoric activity produced by BEA in membranes has a direct effect on the intracellular ion concentration of mammalian cells (Kouri et al., 2003). BEA incorporates in mammalian cells, forming a cation-selective channel and may contribute to assist the penetration of BEA and other toxic into the cell (Ojcius et al., 1991; Kouri et al., 2003; Klaric et al., 2006). Moreover, it has been demonstrated that BEA increases cytosolic Ca^{2+} concentration in a dose-dependent manner that, is not immediate compensated for, can lead to irreversible cell damage (Jow et al., 2004; Lin et al., 2005; Chen et al., 2006). Moreover, the rise in calcium is a point of convergence among many downstream mechanisms, i.e. decreases the $\Delta\Psi m$, releases of cytochrome *c,* increases caspase activation and apoptosis (Jow et al., 2004). In this study, cell death by necrosis and apoptosis as the ultimate endpoint of lethal cell injury induced by BEA was observed. Similar results were obtained by Prosperini et al. (2013a). They evidenced an increase in early apoptotic cells after 24 h (from 100 to 183%) and 48 h (from 143 to 286%) of exposure, respect to control. Similarly, BEA (from 1 to 10 μM) induced apoptosis (approx. from 35% to 75%) in KB-3-1 cells (Dornetshuber et al., 2009b), human non-small cell lung cancer (NSCLC) A549 cells (Lin et al. 2005), human leukemia cells (CCRF-CEM) (Jow et al., 2004) cells during 24 h. Also, lymphocytes (turkey peripheral blood lymphocytes) exposed to BEA

exhibited DNA fragmentation and nuclear condensation characteristic of apoptosis (Dombrink-Kurtzman, 2003). Klaric et al. (2008), observed apoptotic cells only after 48 h of BEA (5 μg/mL) exposure and suggest that increases in LPO and H_2O_2 production and decreases in GSH content. This effect could contribute to apoptosis and necrosis in PK15 cells by BEA. Similarly, apoptotic cells death increase (after 5 μM BEA exposure) was observed in H4IIE cells via caspase 3/7 in C6 glioma cells (Wätjen et al., 2014).

The results obtained in this study shown an increased in necrotic CHO-K1 cells at 48h of exposure. In the same way, Prosperini et al. (2013b) observed a relation apoptotic/necrosis increased in Caco-2 cells and Wätjen et al., (2014) in C6 glioma cells. According to the results obtained in this study, BEA induces DNA strand breaks at 1 μM but not at 5 μM after 24h of exposure. After 24 h of exposure BEA produce apoptosis but not necrosis (Fig. 4). Considering that apoptosis is tightly linked with the machinery that controls cell proliferation and DNA repair, is not surprising DNA strand breaks will be not observed. Moreover, after 5 μM BEA exposure, antioxidant defense system (SOD and CAT) activities were stimulated and consequently highly contribution to eliminate damage cell could be produced. Similar DNA damage by comet assay after 24 h BEA exposure in PK15 (0.5 μM) and Caco-2 (12 μM) cells was observed (Klarić et al., 2010; Prosperini et al., 2013a). According to Klarić et al. (2010), BEA structure may be associates with direct DNA damage, due to BEA is positivity charged and it could interfere with negative charge of DNA strand, influencing in DNA migration; moreover, the increase in intracellular Ca^{2+} could influence endonuclease activity and the oxidative stress might involve indirect DNA damage (Jow et al., 2004; Dornetshuber et al; 2009b). According to Dornetshuber et al. (2009b) BEA only interact with DNA at high concentrations of BEA (≥100 μM) inhibiting topoisomerase I and II activity. Due to, BEA reduces mitochondrial enzymatic activity and cell proliferation, decreases the $\Delta\Psi m$, increase necrosis, apoptosis and produces DNA damage, the following aim of the study is to check up on how cells remove the BEA which is damaging the cells. Related this objective, SOD and CAT enzymes activities were monitored in CHO-K1 cells exposed to BEA. In this study,

the SOD and CAT activities were increased after BEA exposure in CHO-K1 cells. Similar results were obtained by other authors with other mycotoxins. Theumer et al. (2010) obtained an increase in CAT and SOD activities after fumonisin B1 (FB_1) and aflatoxin B1 (AFB_1) exposure in spleen mononuclear cell (SMC) suspensions. He et al. (2011) detected the same effect in primary cultures of chicken tibial growth plate chondrocytes (GPCs) exposed to 5-500 nM of T-2 toxin during 48 h. Comparable results were observed by Dinu et al. (2011) in human embryonic kidney (Hek-293) cells after DON exposure (2.5 and 5 μM). And, Fernández-Blanco et al. (2014) observed an increase in SOD activity and a decrease in CAT activity after alternariol (AOH) exposure (15, 30 and 60 μM) in Caco-2 cells. However, Wu et al., (2013) found a decrease in SOD, CAT and GPx activities after T-2 toxin (from 1 to100 nM) exposure in granulosa cells; and Li et al., (2014) observed a decrease in SOD activity after DON (500-2000 ng/mL) exposure in chicken embryo fibroblast (DF-1) cells. Previously, studies in our laboratory demonstrated that BEA (0.1, 1 and 5 μM) significantly increased the GPx activity from 35% to 66% respect to the control in CHO-K1 cells (Mallebrera et al., 2014).

In conclusion, it could be suggested that BEA causes cytotoxicity in CHO-K1 cells and it could be correlated with mitochondrial dysfunction. Loss of mitochondrial membrane potential might lead to the apoptotic process. At the same time, apoptosis is linked with the controls of cell proliferation and DNA repair. On the other hand, BEA exposure increase antioxidants defense mechanisms (SOD and CAT activities), which could help to scavenge BEA and protect against its cytotoxic effects in CHO-K1 cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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3.3. Disturbance of antioxidant capacity produced by beauvericin in CHO-K1 cells

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DISTURBANCE OF ANTIOXIDANT CAPACITY PRODUCED BY BEAUVERICIN IN CHO-K1 CELLS

B. Mallebrera[2], G. Font, M.J. Ruiz

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

Abstract

Glutathione (GSH) levels, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-*S*-transferase (GST) as antioxidant defense system were evaluated in CHO-K1 cells after beauvericin (BEA) exposure. The effect of N-acetyl-cysteine (NAC) pre-treatment was assessed. GSH levels significantly decrease 18% and 29% after 5 μM of BEA in fresh medium and NAC pre-treatment, respectively compared to their controls. The GPx activity increased significantly from 35% to 66% in fresh medium and 20% in NAC pre-treatment. GR activity decreased after 5 μM of BEA up to 43% and 53% in fresh medium and NAC pre-treatment, respectively. The GST activity increased in fresh medium (from 61% to 89%) and decreased (from 22 to 35%) after NAC pre-treatment. Comparing BEA exposure in fresh medium and NAC pre-treatment, GSH levels and GPx and GST activities increased 716%, 458% and 206%, respectively respect to fresh medium; conversely no changes were observed in GR activity. In addition, NAC is an effective scavenger of BEA. GSH and related enzymes play an antioxidant role in the defense system of CHO-K1 cells exposed to BEA.

Keywords: beauvericin; cytotoxicity; oxidative stress; enzymes; GSH; CHO-K1 cells.

[2] **Corresponding author:** Beatriz Mallebrera, Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46117, Burjassot, Valencia, Spain. beatriz.mallebrera@uv.es

1. INTRODUCTION

Beauvericin (BEA) is a cyclichexadepsipeptide produced by many species of fungus *Fusarium* and *Beauveria bassiana* (Fig. 1). It contains three D-hidroxyisovaleryl and three N-methylphenylalanil residues in an alternating sequence (Wang and Yu, 2012).

BEA is an ionophore, forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}), that increases ion permeability in biological membranes, which may affect the ionic homeostasis (Chen et al., 2006; Kouri et al., 2005). BEA produces lipid peroxidation (LPO) and reactive oxygen species (ROS) in mammalian cells (Ferrer et al., 2009; Prosperini et al., 2013a). This mycotoxin shows: apoptotic activity, which has been attributed to Bcl-2 family, cytochrome c and caspase 3, as well as increases in the cytoplasmic calcium concentration (Lin et al., 2005). Moreover, BEA also produces DNA fragmentation, increases in chromosomal aberrations, sister-chromatid exchanges and micronucleus (Prosperini et al., 2013b). Moreover, it is the most potent specific inhibitor of cholesterol acyltransferase (Acyl-coA) (Tomoda et al., 1992).

The oxidative stress caused by ROS, has been involved in many human diseases; (Angelopoulou et al., 2009). However, cells have a protective endogenous antioxidant system that help protect against the damaging effects of ROS; this system is formed by no-enzymatic antioxidant such a glutathione (GSH) and enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

GSH is considered the most important endogenous cellular antioxidant. In the GSH defense system is very important its related enzymes, glutathione reductasa (GR), GPx and glutathione transferase (GST). GR reduces oxidized glutathione form (GSSG) to reduced one (GSH) in NADPH-dependent reaction. GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) to H_2O and catalyze the conversion of GSH to GSSG (Deponte, 2013). GST is a detoxifying enzyme, which removes toxic compounds by binding with GSH. In organisms, depletion of GSH by oxidants stimulates the synthesis of GSH by liver tissues. In *in vitro* system the role of GSH in chemical-induced injury can be studied by addition of compounds that can increase or decrease the rate of GSH synthesis. N-acetyl-cysteine (NAC) is a GSH precursor. NAC is a source of sulfhydryl

groups to cells as an acetylated precursor of reduced GSH. NAC exerts its protective effect in part by directly scavenging oxidant free radicals (Zhang et al., 2011).

Our research group has demonstrated that BEA is cytotoxic alone or in combination with other *Fusarium* mycotoxins (Ferrer et al., 2009; Ruiz et al., 2011). Its cytotoxicity via ROS production and mitochondrial damage has been considered as possible mechanisms (Prosperini et al 2013b). Moreover, we evidenced the bioaccessibility of BEA and its bioavailability by *in vitro* methods (Prosperini et al., 2013a). There are very few data about the responses of antioxidant enzymes activity in mammalian cell lines exposed to mycotoxins available on literature. And no data about enzymatic activity in cells exposed to BEA are obtainable to date. Thus, to examine the biochemical adaptation of CHO-K1 cells after BEA exposure, in this work the enzymatic activities involved in GSH metabolism were quantified. Therefore, the aims of this study were to evaluate the role of GSH and its related enzymes (GPx, GR and GST) as antioxidant defense system in CHO-K1 cells after BEA exposure. In addition, the cytoprotective effect of NAC pre-treatment was assessed.

2. MATERIALS AND METHODS

2.1. Reagents

The reagent grade chemicals and cell culture components used, namely culture medium Ham's-F12, antibiotics, trypsin/EDTA solutions, HEPES, phosphate buffer saline (PBS), GSH, GSSG, GR, H_2O_2, β-Nicotinamide adenine dinucleotide phosphate (β-NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), NAC, methanol (MeOH), *o*-phtaldialdehyde (OPT), BEA (783.95 g/mol; Fig. 1), *t*-octylphenoxypolyethoxyethanol (Triton X-100), NaCl, dimethylsufoxide (DMSO), NaOH, HCl, sodium azide (NaN_3), tris hydroxymethyl aminomethane (Tris) and ethanol were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was from Life Technologies (Madrid).

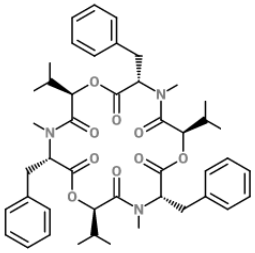

Figure 1. Chemical structure of Beauvericin

2.2. Cell culture and exposure of beauvericin

CHO-K1 cells were grown as monolayer in 9 cm^2 polystyrene tissue culture dishes in Ham's-F12 supplemented with 25 mM HEPES buffer (pH 7.4), 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 ºC in 5% CO_2 and 95% humidified atmosphere. The cells were subcultivated after trypsinization (trypsin-EDTA) once or twice per week and resuspended in complete medium in a 1:3 split ratio. Cell were subculture routinely with only a small number of sub-passages (<70 subcultures) in order to maintain the genetic homogeneity. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St. Louis, MO, USA).

BEA concentrations were selected considering the previous data obtained in our laboratory in CHO-K1 cells (Ferrer et al., 2009). The medium inhibition concentration (IC_{50}) values obtained after BEA exposure during 24h by the neutral red (NR) and tetrazolium salt (MTT) assays were 17.22 ± 1.20 and 12.08 ± 1.10 μM, respectively. Thus, the BEA concentrations select in this study (0.1, 1 and 5 μM) were below the IC_{50} obtained.

Stock solution of BEA was prepared in MeOH and maintained at -20ºC. Final concentrations of BEA were achieved by adding the culture medium and the final MeOH concentration in the medium was 1% (v/v). Control (cells with 1% MeOH in medium, without BEA concentration), and control NAC (medium with 1% MeOH plus 1mM NAC, without BEA concentration).

2.3. Glutathion determination

For all the assays performed with BEA, $3x10^5$ cells/well was seeded in six-well culture plates. After confluence, the culture medium was removed and 3 ml of medium with different concentrations of BEA (0.1, 1 and 5 μM) were added for 24 h of incubation. Afterwards, the medium was removed and cells were washed twice with PBS and then homogenized in 0.5 ml of 20 mM Tris and 0.1% Triton.

The cells were exposed to different treatments: a) fresh medium (medium Ham's-F12 with 10% FCS), b) NAC pre-treatment (medium Ham's-F12 with 10% FCS plus 1 mM NAC).

Determination of reduced GSH was assayed by adapting the method of Maran et al., (2009). Briefly, 10 μL of each homogenized cell sample was placed in 96 well black tissue culture plate, with 200 μL GSH buffer (pH 8.0) and 10 μL of the OPT solution, mixed and incubated in darkness at room temperature for 15 min. Concentration of GSH was determined using a microplate reader (Wallace Victor2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) with excitation and emission wavelength of 345 and 425 nm, respectively. GSH levels were expressed in μg/mg protein. Determinations were performed in quadruplicate.

2.4. Determination of GPx and GR activities.

GPx was assayed spectrophotometrically using H_2O_2 as substrate for the Se-dependent peroxidase activity of GPx by following oxidation of NADPH at 340 nm during the first 2 min in a coupled enzymatic reaction with GR as described by Maran et al., (2009). In 1 ml final volume, the reaction mixture contained 500 μL of 0.1 M phosphate buffer (pH 7.5 , 1 mM EDTA and 2 mM NaN_3, 0.1% Triton X-100), 250 μL of ultrapure water, 100 μL of 20 mM GSH, 20 μL of 0.2 mM NADPH, 2.5 U freshly prepared GR and 50 μL of 5 mM H_2O_2. Fifty microliter of homogenized cell sample was added to the reaction mixture. One unit of GPx will reduce 1 μmol of GSSG per min at pH 7.5. Assays were conducted at 25 °C during 2 min in a thermocirculation of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1.

GR activity was determined by following the oxidation of NADPH to NADP during the reduction of GSSG. GR was assayed as described previously by Maran et al., (2009). GR mixture contained, in a final volume of 1 ml: 800 µL of phosphate buffer 0.1 M (pH 7 with 5 mM EDTA), 10 µL of 10 mM NADPH in phosphate buffer and 90 µL of homogenized cell sample. The reaction started by the addition of 100 µL GSSG. The decrease in absorbance was monitored at 340 nm during 4 min at 25 °C in a thermocirculation of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1.

GR and GPx enzymatic activities were calculated by using the molar absorptivity of NADPH (6.22 mM^{-1} cm^{-1}) and expressed as nmol of NADPH oxidized/min/mg of protein. Determinations were performed in quadruplicate.

2.5. Determination of GST activity

GST activity was determined by measuring the increase in absorbance at 340 nm on conjugation of CDNB with GSH. GST activity was determined according to the method of Maran et al., (2009). The reaction mixture contained in a final volume of 1 ml: 825 µL sodium phosphate buffer 0.1 M (pH 6.5 with 0.5 mM EDTA and 0.1% Triton X-100), 25 µL of 50 mM CDNB dissolved in ethanol, 100 µL of 25 mM GSH and 50 µL of homogenized cell sample. Enzymatic activity was assayed at 25 °C in a thermocirculation of Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1 during 3 min. GST activity was expressed as nmol of product formed/min/mg of protein using a molar absorptivity of CDNB (9.6 mM^{-1} cm^{-1}). Determinations were performed in quadruplicate.

2.6. Pre-treatment with N-acetylcysteine

To study the cytoprotective effects of GSH, cells were pre-treated with NAC prior to BEA exposure in CHO-K1 cells. Approximately, $3x10^5$ cells/well were exposed to 1mM of NAC for 24 h followed by exposure to freshly prepared medium containing BEA at the designated concentrations (0.1, 1 and 5 µM) for 24 h. Cells containing 1% MeOH in the medium were used as control for each experiment. After 24 h of exposure, the GSH content and GPx, GR and GST activities were determined as

previously described. Determinations were performed in quadruplicate. Comparison between cells exposed to different concentration of BEA in a) fresh medium and b) NAC pre-treatment were performed.

2.7. Determination of total protein content

Cellular protein content was assayed using Bio-Rad DC Protein Assay; catalog number 500-0116 (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf). Protein concentration was measured at 690 nm.

2.8. Statistical analyses

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 four 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's HDS *post hoc* test for multiple comparisons. The level of $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1. Cell proliferation and glutathion levels

Figure 2 shows the change in the percentage of cells after BEA exposure in fresh medium and NAC pre-treatment. The number of cells was similar in the assay carried out in fresh medium and NAC-pretreatment. Only, the number of cells was significantly decreased ($p \leq 0.001$, n=4) by 13 % and 21 % after 5 μM BEA in fresh medium and with NAC pre-treatment compared with their own controls, respectively (Fig. 2).

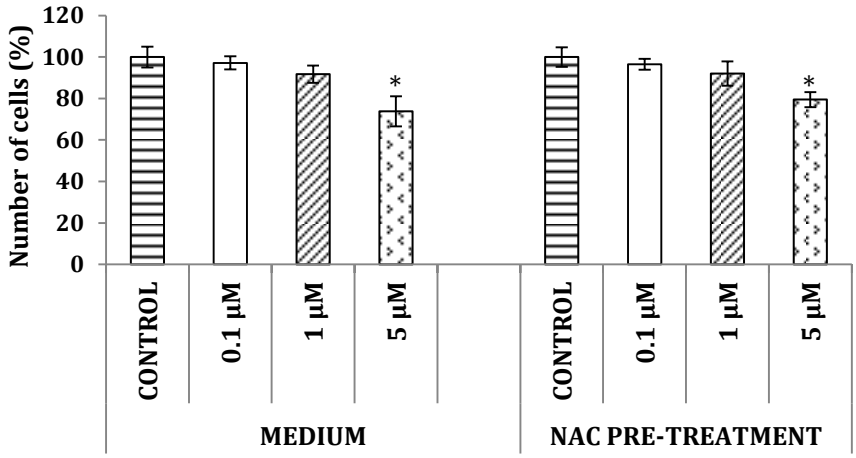

Figure 2. Change in the percentage (%) of number of CHO-K1 cells after 0.1, 1 and 5 µM of BEA exposure at 24 h of incubation. Data are expressed as mean of 4 experiments ± SD (*p≤0.05, **p≤0.01, ***p≤0.001) indicate a significant difference from their respective control.

The alteration of GSH content was measured after 0.1, 1 and 5 µM BEA exposure in CHO-K1 cells during 24h with fresh medium and NAC pre-treatment. In fresh medium and NAC pre-treatment, the GSH levels were not affected by 0.1 and 1 µM BEA. However, after 5 µM of BEA exposure the GSH levels significantly (p≤0.01) decreased by 18% in medium and by 29% in NAC pre-treatment compared to their own controls (Table 1; Fig. 3). The GSH content in cells increased when NAC pre-treatment was applied (in control, 0.1 and 1 µM BEA; Fig. 3). However the number of cells (in control, 0.1 and 1 µM BEA; Fig. 2) was similar as in medium as in NAC pre-treatment. Thus, the GSH level was higher in cells with NAC pre-treatment respect to cells growth up only in medium. The protection of GSH in these assays significantly increased by 579 %, 716 % and 548 % at 0.1, 1 and 5 µM BEA, respectively, when compared to the assay without NAC pre-treatment (Fig. 3).

Considering the results obtained in CHO-K1 related to GSH levels, the enzyme activity assays were performed in fresh medium and NAC pre-treatment medium considering that NAC has cytoprotective effects on CHO-K1 cells exposed to BEA.

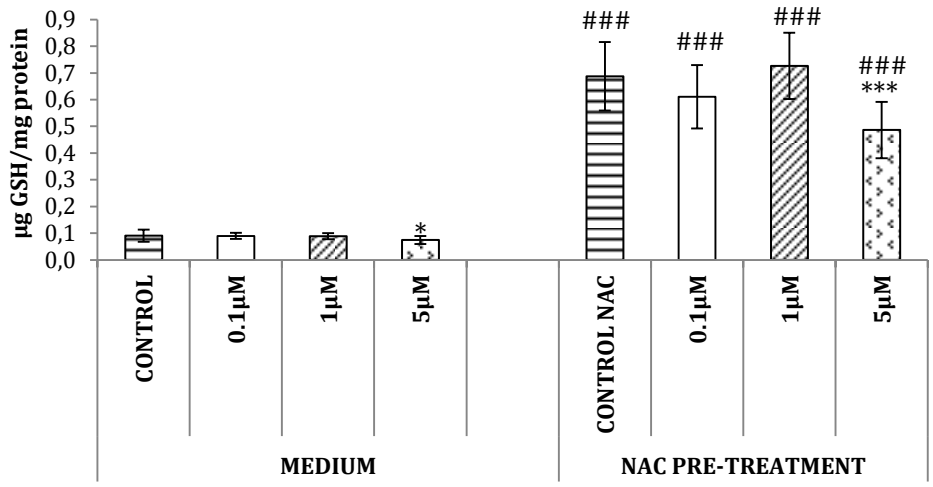

Figure 3. GSH levels in CHO-K1 cells to BEA (0.1, 1 and 5µM) exposure during 24 h of incubation with medium, NAC pre-treatment. Data are expressed as mean values ± SD. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) indicate a significant difference from their respective control. ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$) indicate a significant difference from the medium.

3.2. Enzymatic activities

The GPx, GR and GST activities were measured in CHO-K1 cells after 24 h of incubation with 0.1, 1 and 5 µM BEA. In Table 1 are shown the results expressed as percentage (%) of enzyme activities in CHO-K1 cells after BEA exposure in relation to control cells.

The GPx activity increased significantly ($p \leq 0.000$) in CHO-K1 cells exposed to different concentrations of BEA in fresh medium. The increase ranged from 35% to 66% (Table 1). When the assay was carried out in NAC pre-treatment conditions, smaller increase in the GPx activity (20%) was observed after BEA exposure, respect to NAC pre-treatment control.

After 5 µM of BEA exposure in fresh medium, the GR activity decreased significantly ($p \leq 0.000$) up to 43% respect to control (Table 1). Similar effect was observed in NAC pre-treatment assay (Table 1). A slightly higher decrease (53%) was observed at 5 µM BEA exposure with NAC pre-treatment, when compared with its own control.

The GST activity was significantly increased ($p \leq 0.000$) in fresh medium at 0.1 and 1μM BEA exposure, being its activity increased from 61% to 89%, respect to control (Table 1). However, NAC pre-treatment decreased significantly ($p \leq 0.01$) the GST activity from 22% (1 μM) to 35% (5 μM) respect to its own control.

Table 1: Effect of BEA in CHO-K1 cells untreated or pre-treated with NAC on GSH levels and glutathione related enzyme activities. Values of GSH are expressed as mean ± SD of 4 assays tested individually. Enzymatic activities are expressed as percentage (%) of control.

	CONTROL	**BEA 0.1 μM**	**BEA 1 μM**	**BEA 5 μM**
Without pre-treatment (fresh medium)				
GSH (μg/mg protein)	0.091±0.023	0.090±0.011	0.089±0.011	0.075±0.015*
GPx (% control)	100	135.23***	166.26***	144.44***
GR (% control)	100	100.48	100.96	57.00***
GST (% control)	100	160.49***	188.88***	102.46
NAC pre-treatment				
GSH (μg/mg protein)	0.687±0.128	0.611±0.118	0.726±0.124	0.486±0.106***
GPx (% control)	100	106.41	102.06	120.43***
GR (% control)	100	95.48	99.17	47.22***
GST (% control)	100	87.5	77.5**	65**

Statistically different from the respective control (* p≤0.05. ** p≤0.01. *** p≤0.001).

3.3. The effect of N-acetylcysteine on glutathion levels and enzymatic activities

NAC pre-treatment increased GSH levels in control cells respect to cells without pre-treatment (fresh medium). However, BEA at 5μM decreased significantly (29%, $p \leq 0.001$) the GSH levels in NAC pre-treated CHO-K1 cells respect to the control (Table 1, Fig. 3). Tukey's multiple comparison indicates that GSH levels were statistically higher ($p \leq 0.000$) with NAC pre-treatment in CHO-K1 cells respect to cells in fresh medium. GSH levels after NAC pre-treatment increased from 548% to 716% respect CHO-K1 cells in fresh medium (Table 1; Fig. 3).

Respect to enzymatic activities, Tukey's multiple comparison indicates that GPx activity was statistically significant ($p \leq 0.000$) between BEA exposure in fresh medium and NAC-pretreated cells (Fig. 4). BEA in NAC pre-treatment increased GPx activity from 311% to 458% respect to cells exposed to BEA in fresh medium (Fig. 4). No significant differences were observed in GR activity when compared BEA effects after NAC pre-treatment and fresh medium conditions (Fig. 5). However, statistically significant differences ($p \leq 0.000$) were observed with Tukey's multiple comparison in GST activity in CHO-K1 cells in fresh medium *vs*. NAC pre-treatment assay. These increase ranged from 105% to 206% (Fig. 6).

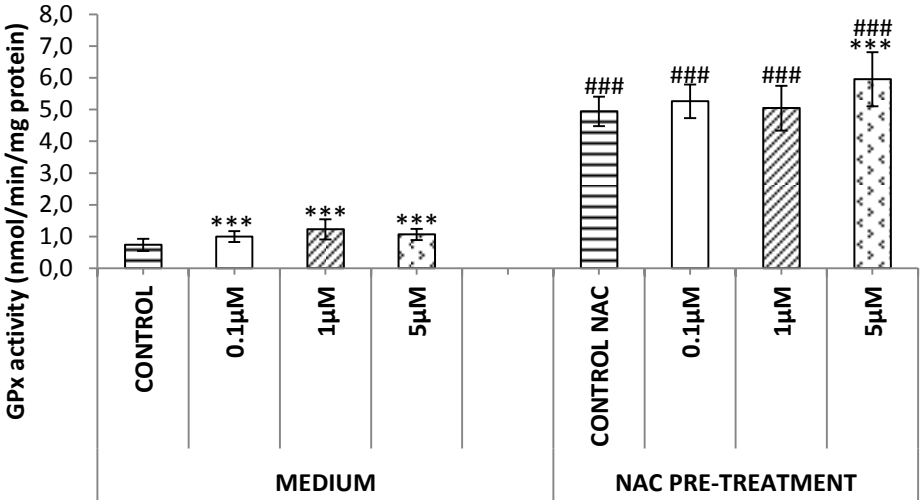

Figure 4. GPx activity in CHO-K1 cells to BEA (0.1, 1 and 5µM) exposure during 24 h, in medium and NAC pre-treatment. Data are expressed as mean values ±SD. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) indicate a significant difference from their respective control. ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$) indicate a significant difference from the medium.

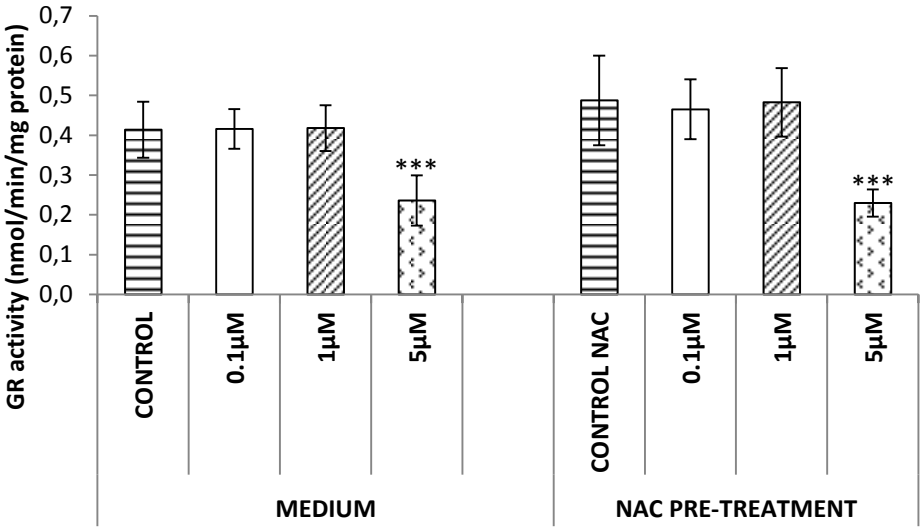

Figure 5. GR activity in CHO-K1 cells to BEA (0.1, 1 and 5µM) exposure during 24 h, in medium and NAC pre-treatment. Data are expressed as mean values ± SD. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) indicate a significant difference from their respective control. ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$) indicate a significant difference from the medium.

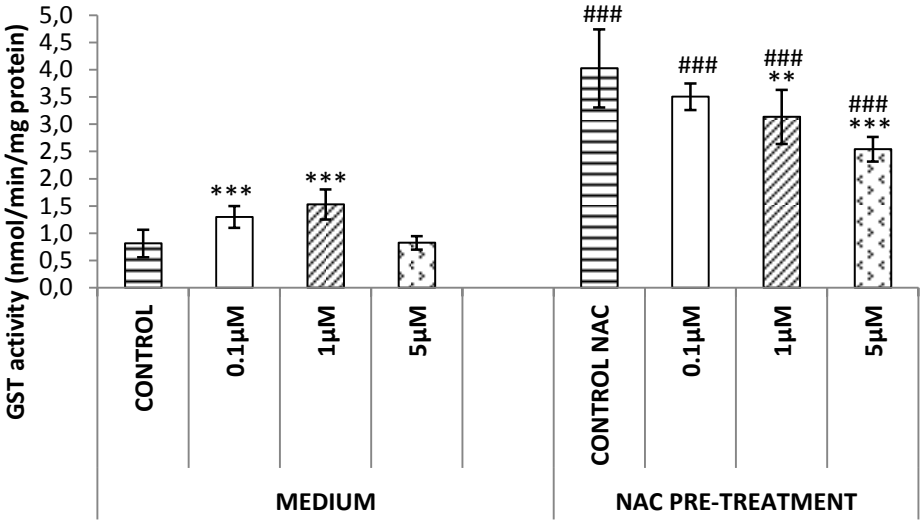

Figure 6. GSTs activity in CHO-K1 cells to BEA (0.1, 1 and 5µM) exposure during 24 h, in medium and NAC pre-treatment. Data are expressed as mean values ± SD. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) indicate a significant difference from their respective control. ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$) indicate a significant difference from the medium.

4. DISCUSSION

GSH depletion was observed after 24 h incubation and at 5 μM BEA exposure in CHO-K1 cells (Table 1; Fig. 3). Similar results were obtained by others authors. GSH levels decreased in Caco-2 (from 23% to 31%) and PK15 (from 13% to 28%) cells after 24 h at BEA exposure (Klaric et al., 2006; Prosperini et al., 2013b). Analogous depletion of GSH was observed with *Penicillium* and *Fusarium* mycotoxins, including ochratoxin A in PK15 cells (Klaric et al., 2006), zearalenone in Hep-G2 (Hassen et al., 2007) and CCL-13 (Lee et al., 2013) cells; fumonisin B1 in U-118MG glioblastoma cells (Stockmann-Juvala et al., 2004) and deoxynivalenol (DON) in Hek-293 (Dinu et al., 2011) and Hep-G2 (Bodea et al., 2009) cells.

Klaric et al (2006) found that BEA produce depletion of GSH in a concentration dependent manner. These authors evidenced that ionophoric property of BEA which allows it to penetrate easily through cell membrane altering its lipid composition and permitting producing hydrogen peroxide, which cause oxidative stress and decreases the antioxidant capacity of cells. Moreover, Klaric et al (2006) suggested that the ionophoric activity contributes to facilitate the penetration of BEA and other toxics into cell, which results in increasing toxicity depending on toxics concentration and time of exposure. Cytotoxicity of BEA has been demonstrated in mammalian cell lines (Tomoda et al., 1992; Ferrer et al., 2009; Ruiz et al., 2011; Prosperini et al, 2013a; 2013b). Moreover, cytotoxicity of BEA via ROS production and mitochondrial damage has also been evidenced (Klaric et al., 2010; Ferrer et al., 2009; Prosperini et al., 2013b). Depletion of mitochondrial GSH results in toxicity associated with mitochondrial dysfunction (Dalton et al., 2004). Therefore, the most important cellular function of GSH might be the protection of the mitochondria against oxidant generation. However, Klaric et al (2006) found that low concentrations of BEA decrease GSH levels in PK15 cells without affecting cell viability and LPO production. Decrease GSH levels by low BEA concentrations demonstrates that GSH is a sensitive marker of pro-oxidative activity of BEA (Klaric et al., 2006; Prosperini et al., 2013b). GSH depletion leads to an increased susceptibility to oxidative stress and, thus, progression of many cellular alteration states. DNA, proteins and lipids accumulates

oxidative damage induced by ROS generated by BEA. In previous works developed in our laboratory, pro-oxidative action of BEA in mammalian cells was observed by increasing ROS production and LPO in a time- and concentration-dependent manner (Ferrer et al., 2009; Prosperini et al 2013b). Similarly, Klaric et al (2010) demonstrated that BEA induces LPO, formation of ROS and consequent oxidative DNA damage, and decreasing GSH level in PK-15 cells. However, Dornetshuber et al. (2009) showed that BEA did not increase ROS in human cell lines upon exposure of BEA for 1 h. Such dissimilarity is probably influenced by differences between times of exposure.

It has been demonstrated that manipulating intracellular oxidant status of cells can be of great value. GSH is involved in a variety of cellular functions such as DNA repair, cell cycle, regulation of cell signaling and transcription factors (Dalton, 2004). Elevated levels of GSH with NAC cause significant different response to oxidative stress induced by oxidants and free radicals. The results obtained increased GSH levels and disturb the enzymatic activities related to GSH under these conditions (Table 1; Fig. 3).

To understand the role of GSH in the detoxification mechanisms of BEA, in this study, the protective effect of GPx, GR and GST against the damage produce by oxidant substances generation, and the role of GSH were to take into consideration. Defects in intracellular GSH levels provide insight into the complications involved in perturbing enzymes in GSH metabolism. High concentrations of hydroperoxide substances produced by oxidative stress can be detoxifying by GPx. The GPx activity was increased significantly for all BEA concentrations in medium respect to control. The increase in GPx activity in CHO-K1 cells suggests that this enzyme defense system was stimulated to detoxified BEA exposure. Moreover, previous results obtained in our laboratory with BEA demonstrated that BEA produce oxidative stress due to over-accumulation of ROS, production of LPO and reduction in GSH levels in CHO-K1 and Caco-2 cells (Ferrer et al., 2009; Prosperini et al., 2013b). Similar results were observed by Dinu et al. (2011) in human embryonic kidney (Hek-293) cells after DON exposure. They determined the GPx activity from 0 to 24 h and, they found temporal GPx activation at 12 h (about 32% and 86% for 2.5 and 5 μM DON, respectively),

which decreased after 24 h of DON exposure. The GPx activity decreased directly proportional to the concentration of DON. The decrease in GPx activity could suggest inactivation by ROS which were increased as time of exposure increased and also due to the reduced availability of GPx substrate, the GSH, which were depleted until about 40% after 24 h of DON exposure. Thus, our results are in accordance with these observations, since when intracellular GSH levels were increased using NAC, the cells were more resistant to BEA cytotoxic effects.

The role of GR is converting the GSSG into GSH, decrease in GR activity after BEA treatment could be due to BEA consumes GSH through GPx and GST. When GR activity decrease it could suggests that ROS may be hindering reproduction in GSH or may be a depletion of NADPH. In this study, at 5 μM of BEA a decrease in intracellular GSH levels and a decrease in GR activity were observed. These effects can lead to an oxidative imbalance in GSH cycle and consequently an oxidative process. Moreover, oxidative status of CHO-K1 cells exposed to the highest concentration of BEA (Ferrer et al., 2009) difficult GR activity to regenerate GSH levels. GSH deficiency leads to a decreased capability to scavenge for oxidative substances. Similarly, Dinu et al., (2011) observed that the GR activity in Hek-293 cells after 12 and 24 h of DON exposure decrease about 35% respect to the control. These authors demonstrated that GR activity was decreased in the same way that GSH levels and the enzymes, glucose-6-phosphate dehydrogenase and $NADP^+$-dependent isocitrate dehydrogenase, which produce the co-substrate for GR, the NADPH.

GST is a family of enzymes that catalyze the conjugation of GSH with a multitude of substrates to detoxify the exogenous and endogenous compounds. These enzymes are involved in the detoxification of xenobiotics and protective mechanism against cellular damage, such as oxidative stress. Increase in GST activity is considered a chemical stress signal (Grigutyte et al., 2009). In this study, was observed that low concentrations of BEA showed a stimulation of GST enzymatic activities suggesting that GST acts detoxifying BEA in CHO-K1 cells; while higher concentrations of BEA decrease the ability of GST to detoxify BEA. These results may suggest that after 5 μM BEA, the GST in CHO-K1 cells exhibit a saturation process (Table 1; Fig. 6). The

enzymes are capable of binding to substrates by an enzymatic reaction and turn them into products, these enzymatic reactions are saturable. The enzyme is saturated when all available active site is bound to the substrate and no changes in the reaction even when the substrate concentration increases. Therefore, the decreasing enzymatic activity could be explained by enzyme saturation. Although, this effect also can be due of decreasing enzymatic activity by higher ROS produced (as demonstrated previously, Ferrer et al., 2009). Conversely, no changed were observed in GTS activity when Hek-293 cells were exposed at DON, so this enzyme was not implicated in detoxification of this mycotoxin (Dinu et al., 2011).

In addition, considering the importance of GSH in cellular antioxidant protection, the decreased GSH levels accompanied by increases in GPx and GST activities probably indicates adaptive measures to counteract BEA exposure. These enzymes may be responsible for protection against oxidative stress produced by BEA. Decrease in GR activity could be related to their disability of regenerating GSH from GSSG. In this study, NAC increases the GSH levels and consequently the activity of GPx and GST. Thus, cytoprotection provided by NAC is unlikely to be a result of increasing GSH levels but also to the direct antioxidant/radical-scavenging properties of NAC. So, this precursor helps to detoxification of BEA in CHO-K1 cells. Therefore, the results suggested that GSH and related enzymes play an important role in enzyme defense system in CHO-K1 cells exposed to BEA.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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3.4. Cytoprotective effect of resveratrol diastereomers in CHO-K1 cells exposed to beauvericin

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CYTOPROTECTIVE EFFECT OF RESVERATROL DIASTEREOMERS IN CHO-K1 CELLS EXPOSED TO BEAUVERICIN

B. Mallebrera[a*], V. Brandolini[b], G. Font[a], M.J. Ruiz[a]

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

[b]*Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121Ferrara, Italy*

Abstract

Beauvericin (BEA) causes cytotoxicity, lipid peroxidation and reactive oxygen species in CHO-K1 cells. Resveratrol (RSV) is a polyphenol with multiple biological properties, including antioxidant effects. RSV has two forms: *trans* and *cis*. The aims of this study were to determine the cytoprotective effect of *trans*-RSV and diastereomers mixtures (50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV) incubated alone and in combination with BEA in ovarian (CHO-K1) cells. The results demonstrated that cell viability increase (from 9% to 77%) when they were exposed to low concentration of RSV. Moreover, when the cells were pre-treated with RSV and then exposed to BEA, a cytoprotective effect (from 56% to 76%) and a ROS production diminution (from 27% to 92%) was observed, respect to cells exposed to BEA without previous RSV exposure. RSV pre-treatment decreased the MDA levels (from 15% to 37%) when it is compared with cells exposed only BEA. Therefore, it can be concluded that RSV could to reduce the toxicological risk produced by BEA when they are in combination.

Keywords: beauvericin; resveratrol; cytoprotection; oxidative stress; CHO-K1 cells.

[*]**Corresponding author:** Beatriz Mallebrera, Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46117, Burjassot, Valencia, Spain. E-mail: beatriz.mallebrera@uv.es

1. INTRODUCTION

Polyphenols are secondary metabolites produced by plants in response to exogenous factors such as injury, stress, fungal or UV radiation. There are over 8000 different molecules of polyphenols classified in four categories: phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). These natural compounds are incorporated through the diet and they protect against oxidant substances. Resveratrol (3, 5, 4'-trihydroxystilbene; RSV) is a polyphenolic compound abundant in grapes as well as derivates, peanuts, berries, dark chocolate and other food (Zamora-Ros et al., 2008). RSV existing in two diasteromic forms: *trans* and *cis* (Chen et al., 2007). The *trans* isomer is abundant in the skin of grapes and present in wines, especially red wines; it could be responsible for decreasing coronary heart disease observed in wine drinkers (Khurana et al., 2013). The *cis* isomer is derived by isomerization from *trans* isomer and it has potential anticancer activity, as well as *trans* isomer (Romero-Pérez et al., 1996). RSV has also been studied for other biological properties such as neuroprotection, inhibition to oxidation of low-density lipoproteins, anti-inflammatory, antioxidant, anti-aging, antidiabetic, and antiplatelet (Gülçin, 2010; Fernández-Mar et al., 2012; Vilahur and Badimon, 2013).

Beauvericin (BEA) is a secondary metabolite produced by many species of fungus *Fusarium* and *Beauveria bassiana*. BEA is a contaminant of cereals and products composed by cereals; naturally occurs on wheat, rice, mice, oat and barley. BEA is cytotoxic in several cells lines (Ferrer et al., 2009; Ruiz et al., 2011; Prosperini et al, 2013), it shows apoptotic activity, which has been attributed to Bcl-2 family, cytochrome c and caspase 3, increases the cytoplasmic calcium concentration (Lin et al., 2005), decreases glutathione (GSH) levels, produces lipid peroxidation (LPO) and generation of reactive oxygen species (ROS) (Ferrer et al., 2009; Prosperini et al., 2013; Mallebrera et al., 2014). Moreover, BEA causes DNA fragmentation, increases in chromosomal aberrations, sister-chromatid exchanges and micronucleus (Celik et al., 2010; Prosperini et al., 2013).

In spite of the presence of oxygen is essential for cell function, its catabolism could give rising free radicals and consequently to produce oxidative stress. The

damages produced by oxidative stress have been associated with LPO and ROS generation. So, the aims of this study was to determine the cytoprotective effect of *trans*-RSV and RSV diastereomers mixtures (50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV) incubated in combination with BEA in ovarian (CHO-K1) cells. It is important to note that a diasteromic mixture is a diastereomers blend, where the proportions of diastereomers may affect its biological activity and its properties can significantly differ from those of its two individual diastereomers (Ahuja et al., 2009).

2. MATERIAL AND METHODS

2.1. Reagents

The reagent grade chemicals and cell culture components used were of standard laboratory grade. BEA (783.95g/mol), *trans*-RSV (228.25g/mol), ascorbic acid (176.12g/mol); and vitamin E (430.17g/mol; vit E) were up to 98% purity. The antioxidants selected were *trans*-RSV and RSV diasteromic mixtures (50:50 trans/cis-RSV and 70:30 trans/cis-RSV). The RSV diasteromic mixtures were synthesized in the University of Ferrara by Professors A. Maietti and V. Brandolini. The RSV diasteromic mixture was obtained from the isomerization of *trans*-RSV in their laboratory as follows: 10 mg/mL of a standard solution of *trans*-RSV in acetonitrile/water 50:50 was isomerized by UV irradiation. UV irradiation was performed with a Helios Italquarz UV lamp at 366 nm. The isomerization process was controlled by capillary electrophoresis. All antioxidants were stored at 4 ºC and protected from light. Stock solution of BEA, RSV and vit E were prepared in methanol. Stock solution of ascorbic acid was prepared directly in the medium. Final concentration of BEA and each antioxidant in each assay was achieved by adding the culture medium. The final methanol concentration in the medium was 1% (v/v). Control cells were exposed to the equivalent concentration of methanol, with the exception of ascorbic acid control, which was dissolved in medium.

2.2. Cells and cell culture conditions

CHO-K1 cells derived from Chinese Hamster Ovary, were grown at 37 °C in 9 cm^2 polystyrene tissue culture dishes under 5% CO_2 humidified atmosphere, using Ham's-F12, supplemented with 25 mM HEPES buffer (pH 7.4), 10% foetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St. Louis, MO, USA).

2.3. Cytotoxicity assays

CHO-K1 cells were cultured in 96-wells microplates at a density of $2x10^4$ cells/well. Counting of cells was performer with a Beckman coulter (Florida, USA). The cells were cultured until confluence (65%). Then, the culture medium was removed and replaced with fresh medium containing different concentrations (12.5-200 μM) of all antioxidants tested: *trans*-RSV, 50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*-RSV; ascorbic acid and vit E as controls. Then, cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and neurtral red (NR) assays. The NR and MTT assay were performed as described by Ruiz et al. (2006) during 24, 48 and 72 h. All assays were performed completely in darkness. Absorbance was measured at 540 nm and 570 nm with an automatic ELISA reader for NR and MTT assay, respectively. The results were expressed in relative form to cell culture protein content in order to avoid misinterpretation due to the influence of the chemical tested on cell proliferation and detachment. Determination were performed in three independent experiments.

2.4. Cytoprotective assays

The antioxidant concentration selected for the cytoprotective assays were made according to bioavailability of RSV in plasma after RSV intake (wine consumption). RSV intake is generally around 25 mg, an approximate dose provided by wine consumption (wine contains 5.8 mg/L; Cottart et al., 2010). According to Walle et al., (2004) the absorption of a dietary relevant 25 mg oral dose has a peak plasma levels of RSV and its metabolites of 491±90 ng/ml (about 2 μM). So, the values

of 2.5 μM of RSV diasteromic mixtures are included in the range of concentration tested in this study. Moreover, due to the consumption of RSV could be higher by other food than wine or lower, 1.25 and 5 μM of RSV were also selected in this study.

No data about BEA plasma levels are in literature. Recently, the EFSA (2014) has published some information but the occurrence datasets on BEA for the exposure assessment are limited. So, to determine BEA concentrations for the assays, the consumption of cereals in Mediterranean countries was considered (FAOSTAT, 2011). According to the FAOSTAT (2011), the consumption of wheat in these regions range from 76.7 to 177.3 Kg/per capita/year. On the other hand, BEA content in wheat in this areas range from 0.23 to 4 mg/Kg (Meca et al., 2010; Zinedine, et al., 2011). Therefore, the estimate total intake of BEA could be considered from 1.05 to 2.45 μmol/person/day. Consequently these values and other higher (1.25, 2.5 and 5 μM) were included in this study.

To determine the cytoprotective effect of RSV, cells were cultured in 96-well plates at a density of $2x10^4$ cells/well with fresh medium containing *trans*-RSV, or 70:30 *trans*/*cis*-RSV at 1.25, 2.5 and 5 μM, plus a control. After 24 h, the culture medium was removed and replaced with fresh medium containing 0.1, 1 and 5 μM of BEA; during 24 h. Cytotoxicity was determined by MTT assay as described previously. Determinations were performed in four independent experiments.

The type of interaction produced between BEA and RSV combination were assayed by the isobologram analysis using the calcusyn software version 2.1 (Biosoft Cambridge, UK, 1996-2007). The isobologram analysis provides a combination index (CI) value, which is a quantitative measure of the degree of RSV protection *vs*. BEA exposure. CHO-K1 cells were exposed to several dilutions of BEA and RSV individually and in combination with a fixed constant 1:1 ratio. Four dilutions of BEA, *trans*-RSV and 70:30 *trans/cis*-RSV (0.75, 1.25, 2.5, 5 μM) plus a control (fresh medium with 1% methanol) were assayed in two independent experiment with replicate samples. Cytotoxicity of individual and combinations of BEA+*trans*-RSV and BEA+70:30 *trans/cis*-RSV were determined by the MTT assay. The results were analyzed using the median-effect/combination index (CI)-isobologram equation by Chou (2006) and

Chou and Talalay (1984) which is based on the median-effect principle that demonstrates that there is a relationship between dose and effect independently of the number of products and of the mechanism of action or inhibition. From this equation, CI<1, =1 and >1 indicates synergism, additive effect and antagonism, respectively. Determinations were performed in three independent experiments.

2.5. Determination of intracellular reactive oxygen species

The RSV (2.5 and 5 μM) and BEA (1 and 5 μM) concentrations were selected to determine intracellular ROS production and LPO generation. These concentrations were close-fitting after observing the data obtained by the cytoprotective assays.

Intracellular ROS accumulation was monitored in CHO-K1 cells by adding the 2′,7′-dichlorofluorescin diacetate (H_2-DCFDA) according to Ruiz-Leal and George (2004). In brief, 3×10^4 cells/well were seeded in a 96-well black culture microplate with fresh medium containing *trans*-RSV, 70:30 *trans*/*cis*-RSV or fresh medium. After 24 h, the culture medium was replaced and cells were loaded with 20 μM H_2-DCFDA for 20 min. After that, the medium with H_2-DCFDA was removed and washed with phosphate buffered saline (PBS) before the addition of BEA. Increases in fluorescence were measured at intervals up to 2 h at excitation and emission wavelengths of 485 and 535 nm, respectively. Results were expressed as increase in fluorescence with respect to control. Determinations were performed in three independent experiments.

2.6. Determination of lipid peroxidation

LPO was measured according to Buege and Aust (1978) based on the formation of thiobarbituric acid reactive substances (TBARS). 6×10^5 cells/well were seeded in a six-well plate with fresh medium containing *trans*-RSV, 70:30 *trans*/*cis*-RSV or fresh medium. After 24 h, medium was removed and cells were treated with BEA (1 and 5 μM) for 24 h. Then, the medium was removed and cells were washed with PBS and homogenized in 20 mM Tris and 0.1% Triton. Lysate and homogenized cells were boiled (100 °C water bath for 30 min) under acid conditions in the presence of 0.5% thiobarbituric acid (TBA), 1.5 mM deferoxamine (DFA) and 3.75% butylated

hydroxytoluene (BHT). Samples were cooled, centrifuged (1287g, 15 min) and the absorbance was measured at 532 nm. Results were expressed as ng of malondialdehyde (MDA)/mg of protein measured by the Bradford method. In the Bradford method, the total protein content (μg/ml) was determined by the Bio-Rad DC Protein Assay (catalogue number 500-0116; http://www.bio-rad.com/LifeScience/pdf/Bulletin_9005.pdf) at 690 nm. Determinations were performed in four independent experiments.

2.7. Statistical analysis

Statistical analysis of data was carried out using SPSS version 19 (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 groups were analyzed statistically with one-way 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. Effect of resveratrol isomers in CHO-K1 cells

Figures 1 and 2 show cell viability in CHO-K1 cells after *trans*-RSV, RSV diasteromic mixtures, vit E and ascorbic acid exposure at 24, 48 and 72 h, by MTT and NR assays, respectively. The inhibitory concentration was quantified as the concentration required for inhibiting cell viability by 50% (IC_{50}) under the assay conditions. The IC_{50} values were determined graphically from the concentration response curves. Acid ascorbic and vit E were used as known antioxidants. The IC_{50} values obtained for *trans*-RSV ranged from 41.66±13.05 to 94.23±19.74 μM and from 162.5±13.39 to 180.77±10.36 μM by MTT (Fig. 1) and NR (Fig. 2) assays, respectively. The IC_{50} values for 50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV diasteromic mixtures were similar. They ranged from 91.66±5.97 to 138.00±11.18 μM at 48 and 72h to exposure by MTT assay, respectively. While, no IC_{50} values were obtained for the diasteromic mixtures at 24 h of exposure by MTT assay. Moreover, no IC_{50} values were

obtained at all times of exposure for 50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV by NR assay (Fig. 2).

On the other hand, cell viability increased in relation to control at lower concentrations tested (from 12.5 to 25 μM, except 24 h pre-treatment 70:30 *trans/cis*-RSV by NR assay) (Figs. 1 and 2). The cell viability significantly increased ($p \leq 0.05$) after *trans*-RSV exposure from 12% to 48% and up to 15% by MTT and NR assays, respectively. Moreover, 70:30 *trans/cis*-RSV incubate in CHO-K1 cells significantly increased ($p \leq 0.05$) cell viability in 9% and from 17 to 77% by MTT and NR assay, respectively. Conversely, no significant increase in cell viability was observed after 50:50 *trans/cis*-RSV incubation. The measure of cell viability above 100% could be due to the behavior characterized by low-concentration stimulation of the mitochondrial and lysosomal function of the cells (Figs. 1 and 2). These stimulations could be the result of a hormetic effect (Pichardo et al., 2007). According to Pichardo et al. 2007, it appears to be primarily and adaptive biological response to chemical and physiological stress.

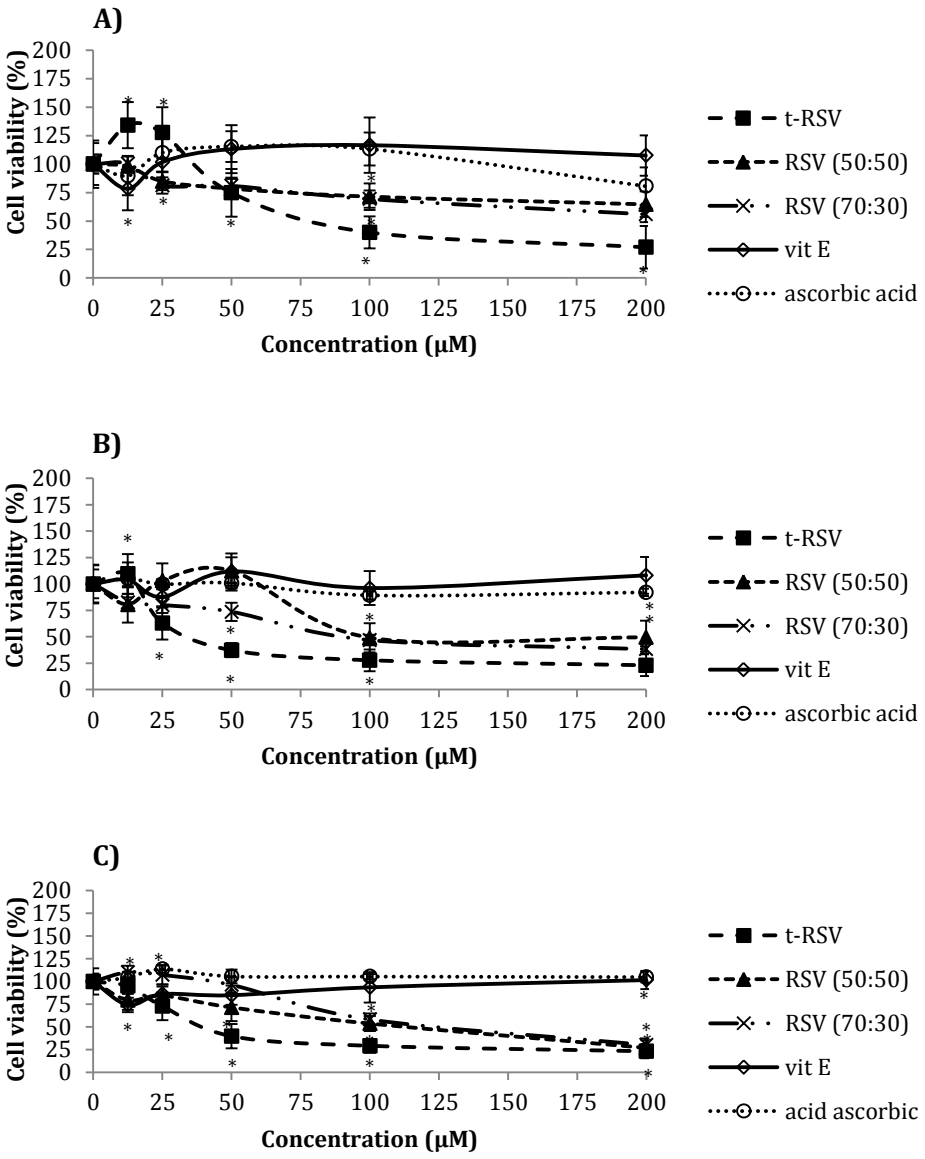

Figure 1: Cytotoxic effect of *trans*-RSV, 50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*-RSV; ascorbic acid and vit E in CHO-K1 cells by MTT assay. A) 24 h, B) 48 h, and C) 72 h of exposure. Each point represents the mean in absolute value of at least three experiments. (*) indicates a significant difference from control value ($p \leq 0.05$). t-RSV: *trans*-RSV; RSV (50:50): 50:50 *trans*/*cis*-RSV; RSV (70:30): 70:30 *trans*/*cis*-RSV; vit E: vitamin E.

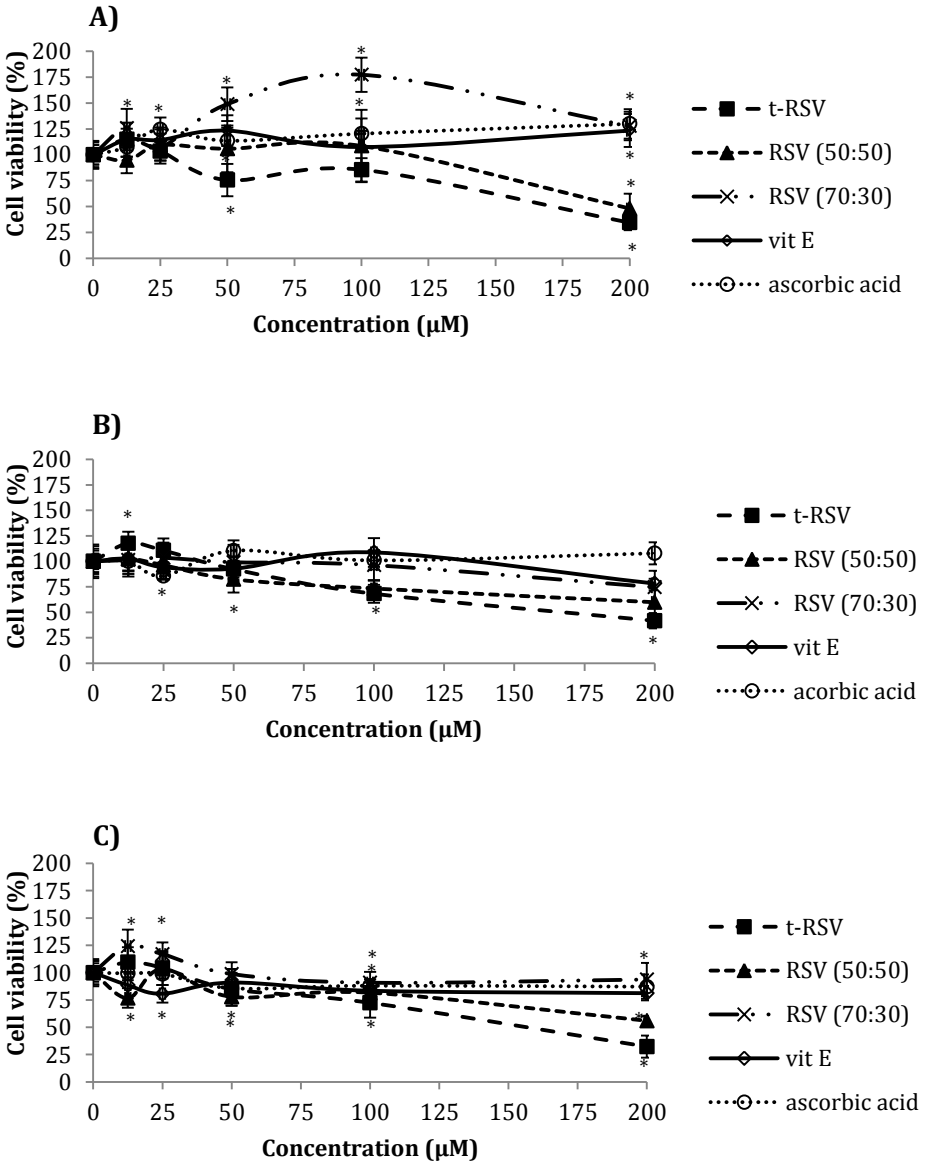

Figure 2: Cytotoxic effect of *trans*-RSV, 50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*-RSV; ascorbic acid and vit E in CHO-K1 cells by NR assay. A) 24 h, B) 48 h and C) 72 h of exposure. Each point represents the mean in absolute value of at least three experiments. (*) indicates a significant difference from control value ($p \leq 0.05$). t-RSV: *trans*-RSV; RSV (50:50): 50:50 *trans*/*cis*-RSV; RSV (70:30): 70:30 *trans*/*cis*-RSV; vit E: vitamin E.

3.2. Cytoprotective effect of resveratrol diasteromic mixtures in CHO-K1 cells exposed to beauvericin

In Figures 1 and 2, could be observed an increase in cell viability with *trans*-RSV and 70:30 *trans*/*cis*-RSV. Thus, these two polyphenols were selected to determine their cytoprotective effect against BEA induced cytotoxicity in CHO-K1 cells. Tukey's multiple comparisons demonstrated that pre-treatment with all concentrations of *trans*-RSV, significantly ($p \leq 0.05$) increase cytoprotective effect from 56 to 76%, respect to the cells exposed to 1 μM of BEA (Fig. 3). Moreover, an increase in cell viability (66%) also was observed after 1.25 of *trans*-RSV with 5 μM of BEA (Fig. 3). The cytoprotective effect with 70:30 *trans*/*cis*-RSV pre-treatment was also observed with the highest concentrations tested. After 2.5 and 5 μM of 70:30 *trans*/*cis*-RSV pre-treatment before BEA exposure, a significant increase ($p \leq 0.05$) from 25% to 42% was observed respect to cells exposed to BEA alone (Fig. 3). The results obtained in this study; showed that *trans*-RSV has a higher cytoprotective effect than 70:30 *trans*/*cis*-RSV against BEA cytotoxicity.

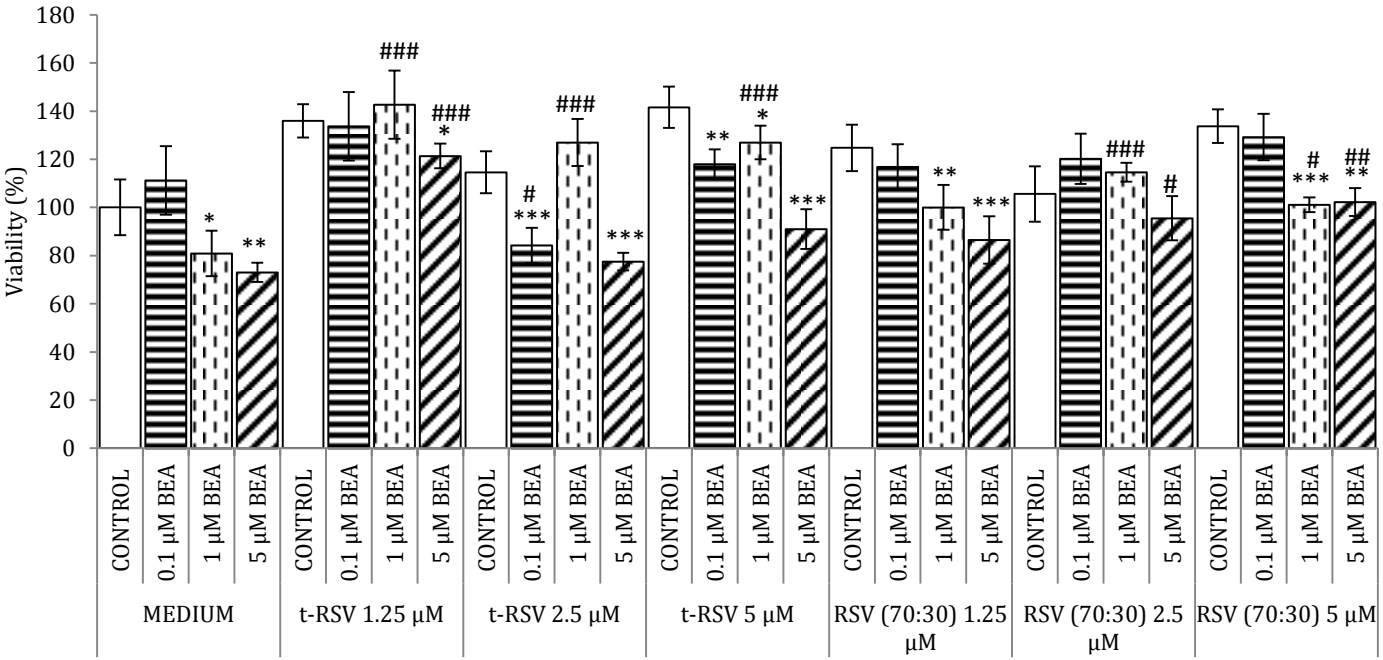

Figure 3: Cytoprotective effect of RSV in CHO-K1 cells exposed to BEA. Cells were 24 h incubated with medium Ham's-F12, *trans*-RSV (1.25, 2.5 and 5 µM) or 70:30 *trans/cis*-RSV (1.25, 2.5 and 5 µM). Then, the medium was removed and fresh medium containing 0.1, 1 or 5 µM of BEA was added. After 24 h of incubation, viability was measured by the MTT assay. Statistically different from the respective control (*$p \leq 0.05$, ** $p \leq 0.01$ and ***$p \leq 0.001$). Statistically different from the medium ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$).). t-RSV: *trans*-RSV; RSV (70:30): 70:30 *trans*/*cis*-RSV; medium: medium Ham's-F1.

Figure 4 shows the concentration-response curve for BEA, trans-RSV, 70:30 trans/cis-RSV and their combinations (at equimolar concentrations) after 24 h of exposure by the MTT assay. BEA decreases significantly ($p \leq 0.05$) cell viability from 16% to 43% respect to the control. BEA+trans-RSV combination (2.5 and 5 μM) decreases significantly ($p \leq 0.05$) the cell viability from 46% to 48% respect to the control. On the other hand, BEA+70:30 trans/cis-RSV (from 2.5 to 5 μM) decrease significantly the cell viability from 48% to 52% respect to the control. Figure 5 shows the graphical presentation of the combination index (CI) with respect to fraction affected (fa) of a mixture of BEA+trans-RSV and BEA+70:30 trans/cis-RSV both of them at molar ratio 1:1 in CHO-K1 cells after 24 h of exposure. Figure 5A demonstrates that BEA+trans-RSV combination shows antagonistic, additive, and synergistic effects at low, medium and high fraction affected, respectively. On the other hand, BEA+70:30 trans/cis-RSV combination showed antagonistic effect at all fractions affected (Fig. 5B).

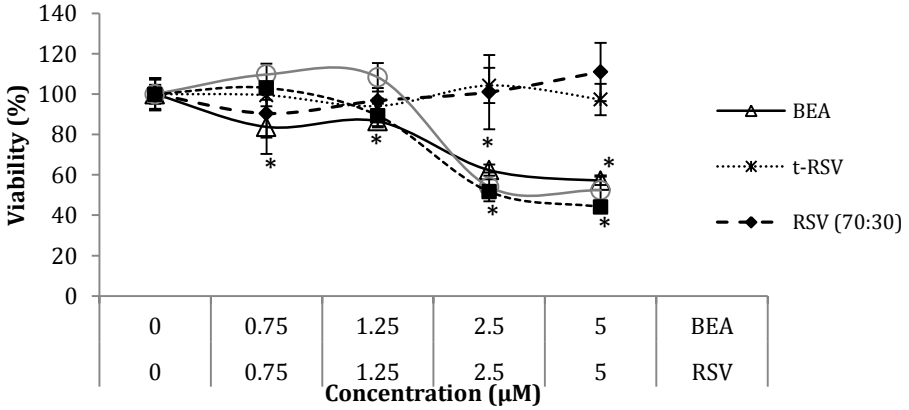

Figure 4: Concentration-response curves for BEA, trans-RSV (t-RSV), 70:30 trans/cis-RSV (70:30 RSV), and binary combinations of them with a fixed constant ratio (1:1) in CHO-K1 cells after 24 h of exposure. The concentrations tested were 0.75, 1.25, 2.5, 5 μM. Each point represents the mean value of at least three experiments. Data represent mean viability as mean ± SD (% control). $p \leq 0.05$ (*) represent significant difference from control. t-RSV: trans-RSV; RSV (70:30): 70:30 trans/cis-RSV

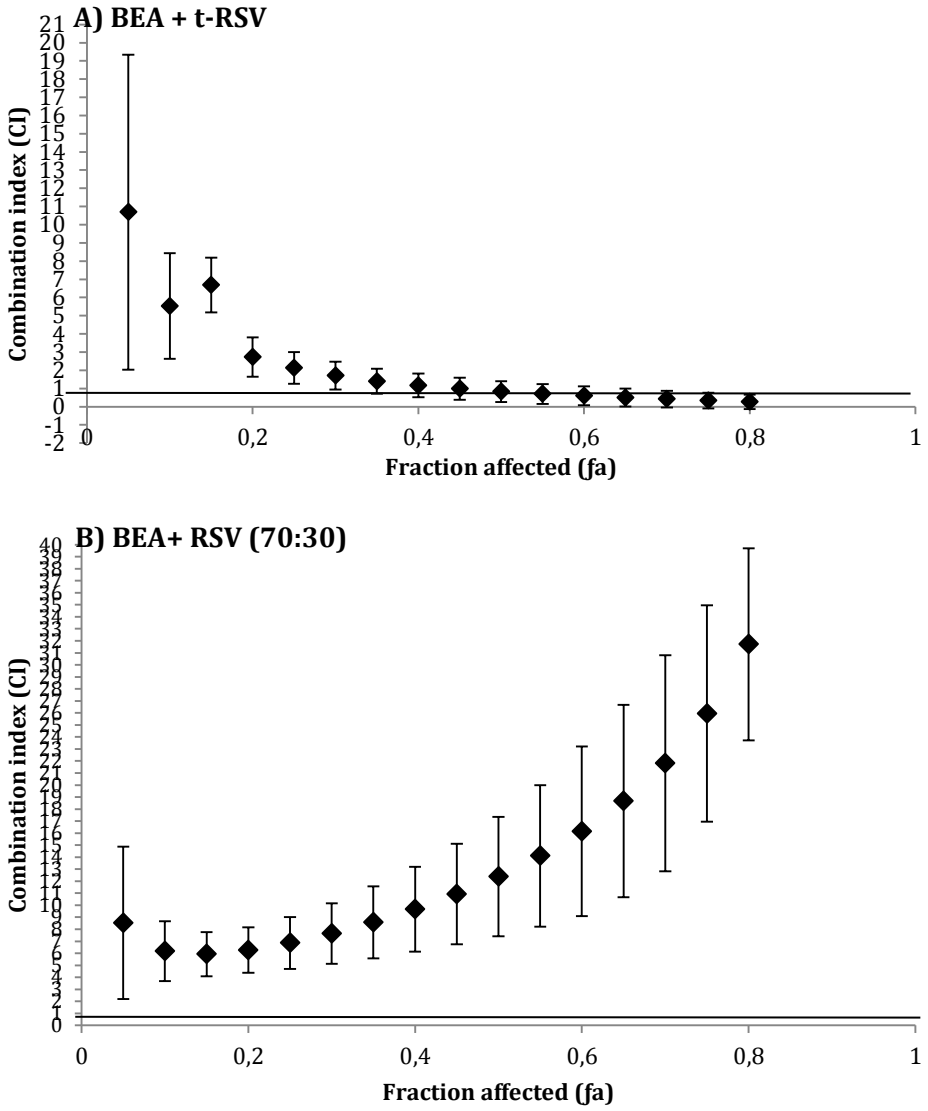

Figure 5: Combination index (CI)/fractional effect curve as described by Chou and Talalay model in CHO-K1 cells exposed to binary combination of: A) BEA+*trans*-RSV (molar ratio 1:1; 0.75, 1.25, 2.5, 5 μM) and, B) BEA+70:30 *trans/cis* RSV (molar ratio of 1:1; 0.75, 1.25, 2.5, 5 μM). Each point represents de CI±SD at a fraction affected as determined in our experiments. The dotted line (CI=1) indicates additive, the area under the dotted line synergy, and the area above of the dotted line antagonism. t-RSV: *trans*-RSV; RSV (70:30): 70:30 *trans*/*cis*-RSV.

3.3. Reactive oxygen species

The results obtained in this study demonstrated that CHO-K1 cells exposed to 1 and 5 μM of BEA increased significantly the intracellular ROS production (1.3-folds of control) in time and concentration dependent manner (Fig. 6A). RSV pre-treatment prevented the BEA-induced ROS production at all concentrations assayed (Figs. 6B to 6E). When cells were pre-treated with RSV and then exposed to BEA in CHO-K1 cells, ROS production significantly ($p \leq 0.001$) decreased from 92% to 39% (for trans-RSV; Figs. 6B and 6C) and from 92% to 27% (trans/cis-RSV 70:30; Figs. 6D and 6E), respect to the medium exposed only with BEA (Fig. 6A).

3.4. Lipid peroxidation

The results obtained in Figure 7 show that 5 μM of BEA increased significantly ($p \leq 0.001$; 35%) MDA production in CHO-K1 cells without RSV pre-treatment, respect to the control. Pre-treatment with 2.5 μM of trans-RSV decreased significantly ($p \leq 0.05$; from 22% to 28%) the MDA production when it was compared with medium without RSV pre-treatment. Similarly, pre-treatment with 5 μM of trans-RSV+1 μM of BEA decreased in 15% respect to medium without RSV pre-treatment; while at 5 μM of BEA this effect was not observed. On the other hand, 5 μM of 70:30 trans/cis-RSV decreased significantly ($p \leq 0.05$; from 21% to 37%) MDA production, respect to the medium without RSV pre-treatment. No effects on MDA production was observed at 2.5 μM of 70:30 trans/cis-RSV.

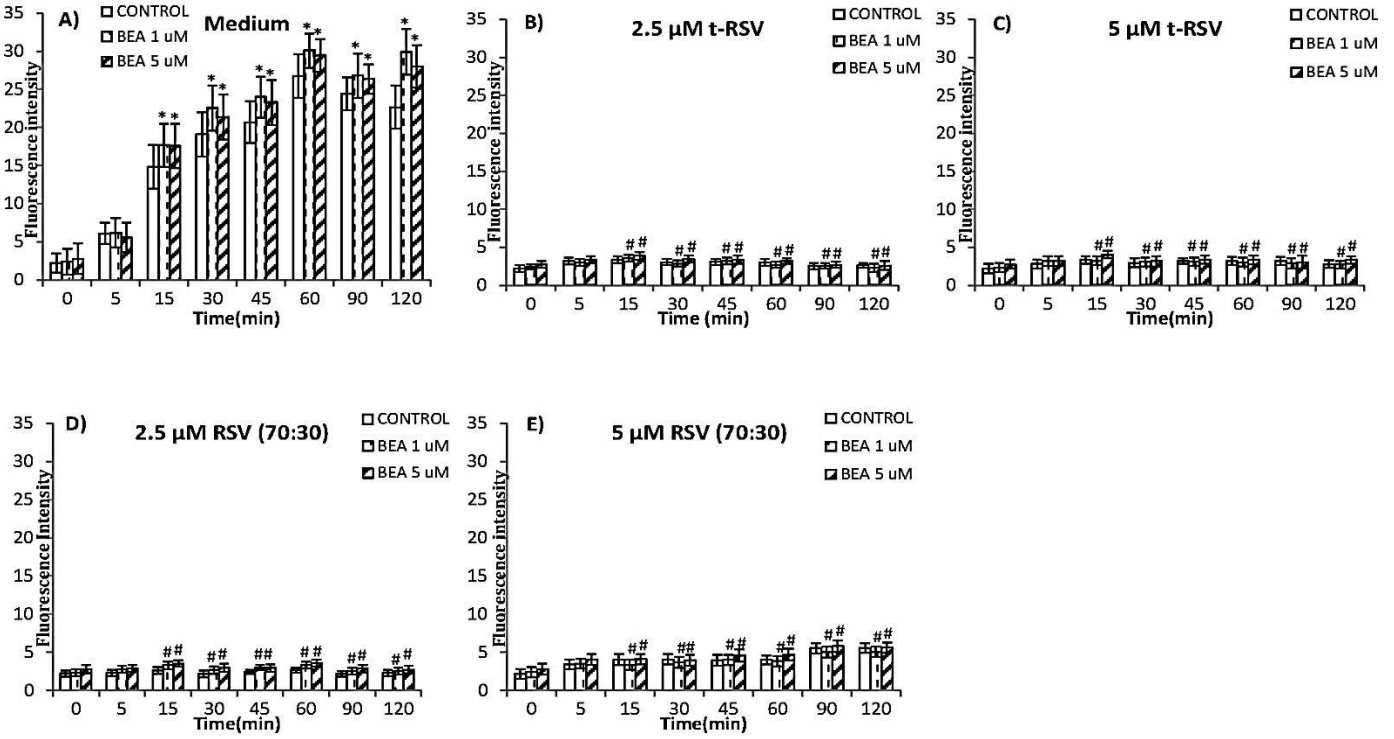

Figure 6: Reactive oxygen species generated in CHO-K1 cells exposed to BEA in A) medium without RSV, B) 2.5 µM trans-RSV, C) 5 µM trans-RSV, D) 2.5 µM of 70:30 trans/cis-RSV and E) 5 µM 70:30 trans/cis-RSV. Statistically different from the respective control (*p≤0.05). Statistically different from the medium without RSV (#p≤0.05). t-RSV: trans-RSV; RSV (70:30): 70:30 trans/cis-RSV; medium: medium Ham's-F12.

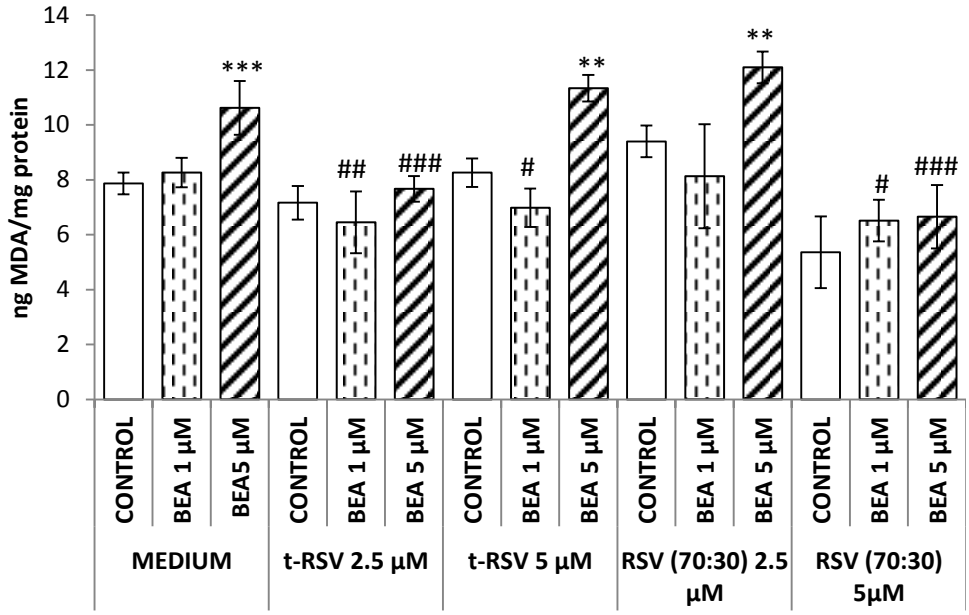

Figure 7: Lipid peroxidation produced in CHO-K1 cells exposed to BEA. Cells were 24 h incubated with medium Ham's-F12, trans-RSV (2.5 and 5 µM) or 70:30 *trans/cis*-RSV (2.5 and 5 µM). Then, the medium was removed and fresh medium containing 1 or 5 µM of BEA was added. After 24 h of incubation, MDA levels were measured. Statistically different from the respective control (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$). Statistically different from the medium without RSV ($^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ and $^{\#\#\#}p \leq 0.001$). t-RSV: *trans*-RSV; RSV (70:30): 70:30 *trans*/*cis*-RSV; medium: medium Ham's-F12.

4. DISCUSSION

There are two isomers of RSV: cis and trans. The trans-RSV is more active biologically while the cis-RSV is unstable and not commercially available (Chen et al., 2006). Between 80-90% of trans-RSV is converted to cis-RSV when it is exposed to light for one hour. While the cis-RSV only is stable at neutral pH and when it is completely protected from light (Amri et al., 2012). Therefore, there are few studies with diasteromic mixtures of isomers of RSV. Previously, it has been demonstrated that RSV is effective in controlling mycotoxins production, as Ochratoxin A (OTA) production (Aldred et al., 2008). In the present study, we have observed cytotoxic effect of RSV at high concentrations and time of exposure (Figs. 1 and 2). While at low

concentrations, RSV showed an increase in cell viability (from 9% to 77%; Figs. 1 and 2). Our results are similar to those obtained by Sgambato et al. (2001). They found that RSV inhibits cell proliferation with an IC50 ranging between 20 and 100 μM in cells of various histogenetic origins. Other authors, also demonstrated that the exposure at high concentrations of RSV (500 μM) reduce cell growth; while, lower concentrations (5, 25 and 50 μM) has a protective role in cell growth in several kinds of cell lines (Yan et al., 2012). Moreover, concentrations of RSV, higher than 25 μM, decrease cell growth, DNA synthesis; and increase apoptosis, cell fragmentation, and cell permeability (Carrasco-Pozo et al., 2012; Israel-Ortega et al., 2012; Juan et al., 2012; Stocco et al., 2012). On the other hand, according to Rizzo et al., (2012) human periodontal ligament cells (HPLCs) exposed to 50 μM of RSV during 72 h increased cell viability. Whereas, 25 μM of RSV no affects cell viability perhaps due to alteration of the expression of Bcl-2 antiapoptotic protein (Israel-Ortega et al., 2012; Lee et al., 2012; Yan et al., 2012). Similarly, Stocco et al., (2012) demonstrated that 50 μM of RSV treatment in bladder carcinoma cell line (ECV304) decreased antiapoptotic Bcl-2 and increased proapoptotic Bad proteins. And, lower doses of RSV increased both. They can explain this effect, based on the relationship between the Bad and Bcl-2 protein. The ratio Bad/Bcl-2 was 51% and 29% at higher and lower doses, respectively.

In this study, 1 and 5 μM of BEA decreased significantly ($p \leq 0.05$) cell viability (Fig. 3). The results obtained in this study, revealed that pre-treatment with trans-RSV and 70:30 trans/cis-RSV had cytoprotective effect in CHO-K1 cells exposed to BEA. Pre-treatment with trans-RSV presented cytoprotective effect at all concentrations of RSV, while 1.25 μM of 70:30 trans/cis-RSV no presented cytoprotective effect. Moreover, the high cytoprotective effect was observed with 1.25 μM of trans-RSV and 1 μM of BEA. However, when 5 μM of BEA was used, not all concentrations of RSV produced a cytoprotective effect; this could be explained by the higher cytotoxic effect produced for BEA at this concentration. Other authors obtained similar cytoprotective results with polyphenols against cell damage produced by Fusarium mycotoxins. Deoxynivalenol (DON) exhibits toxic effect on the HT-29 cells by inducing oxidative stress; pre-treatment with green tea polyphenol epigallocate-chin 3-gallate (EGCG)

prevents DON-induced cytotoxicity in HT-29 cells in a concentration-dependent manner (Kalaiselvi et al., 2013). Pre-treatment with 20 μM of EGCG caused a significantly reduction in ROS production and restored cell viability in 99% and 95% in cells treated with 250 ng/ml and 500 ng/ml of DON, respectively (Kalaiselvi et al., 2013). Even the lowest concentration (5 μM) assayed of EGCG showed cytoprotection against the highest concentration of DON (3.38 μM =1000 ng/ml) (Kalaiselvi et al., 2013). According to Sugiyama et al., (2011) EGCG had protective effect against the trichothece-induce cytotoxicities (DON and HT-2 toxin; HT-2). Pre-, co and post-treatment with EGCG and ECG were assayed against OTA-induced cytotoxicity but only pre-treatment was effective. The LLC-PK1 cells were pre-treated with 10 and 30 μM (EGCG) or 30 and 60 μM (ECG) for 24 h and then replaced with 10, 15 and 20 μM of OTA for 24 h. Pre-treatment with 30 μM EGCG increase cell viability in 25% and 15% at 10 and 15 μM OTA, respectively. Moreover, the cell viability increases for all concentrations of OTA in 25% and 20% at 30 and 60 μM of ECG (Costa et al., 2007). Enniatins (EN) and BEA are emergent mycotoxins of Fusarium. Cytoprotective effect of polyphenols in CHO-K1 cells exposed to ENs was observed by Lombardi et al., (2012). Quercetin significantly increase the viability 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% (Lombardi, et al., 2012). Moreover, quercetin and isorhamnetin significantly increased (from 70 to 78%) cell viability in Hep-G2 cells exposed to aflatoxin B1 (AFB1) (Choi et al., 2010).

ROS generation and LPO production have been previously observed in mammalian cells CHO-K1, Caco-2 and PK-15 cells after BEA exposure (Klarić et al., 2007; Ferrer et al., 2009; Prosperini et al., 2013). ROS are mediators of intracellular signaling cascades and induce several changes at the cellular level. Excessive production of ROS leads oxidative stress, loss of cell function and LPO. In this study when cells were exposed to BEA, ROS production was significantly increased in time dependent manner respect to control. This effect was prevented when cells were previously pre-treated with RSV. Similarly, pre-treatment with RSV (0-40 μM) for 1 h and then exposed to citrinina (CTN) 30 μM for 24 h decrease CTN-induced ROS

production in Hep-G2 cells (Chen and Chan, 2009). On the other hand, other polyphenols such as quercetin, isorhamnetin, EGCG or epicatechin gallate (ECG), also reduced significantly ROS produced by AFB1 (in Hep-G2 cells) and OTA (in LLC-PK1 cells), respectively (Costa et al., 2007; Choi et al., 2010). Pre-treatment with quercetin (25 μM) and isorhamnetin (25 μM) inhibit AFB1-induced ROS production to basal levels and complete inhibition, respectively (Costa et al., 2007). Pre-treatment with EGCG or ECG in LLC-PK1 cells decreases OTA-induced ROS production in 44% (30 μM EGCG) and from 25% to 52% (30 and 60 μM ECG), respect to cells treated only with OTA 15 μM (Costa et al., 2007). It has been demonstrated that RSV is an effective antioxidant in different in vitro assays and effective scavenger of hydroxyls and superoxides in cells producing ROS (Leonard et al., 2003; Gülcin, 2010). Carrizo et al., (2013) suggest that RSV antioxidant has the ability of ROS scavenger, increases the activity of enzymes that metabolize ROS, such as superoxide dismutase, or decreases the activity of enzymes that play a role in ROS production, such as enzymes involved in respiration and ATP-production (Adam-Vizi and Chinopoulos, 2006).

BEA exposure significantly elevated levels of LPO production. And, trans-RSV was better inhibiting LPO production than 70:30 trans/cis-RSV lower concentrations. Pre-treatment with isorhamnetin (25 μM) but no quercetin decreased significantly AFB1-induced LPO production in Hep-G2 cells (Choi et al., 2010). Leonard et al. (2003) demonstrated that RSV inhibited LPO production in RAW 264.7 cells exposed to •OH radicals generated from Fenton reaction. Moreover, RSV decreased H2O2-induced lipid LPO in 70.4% respect to control in human erythrocytes (Mikstacka et al., 2010).

In conclusion, trans-RSV and 70:30 trans/cis-RSV pre-treatment could protect cells against cytotoxicity, LPO and ROS produced by BEA incubation in CHO-K1 cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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3.5. Antioxidant capacity of RSV dietary supplement alone and in combination with BEA.

SHORT COMMUNICATION

Antioxidant capacity of *trans*-RSV dietary supplements alone or combined with the mycotoxin beauvericin

Mallebrera, B[a]., Maietti, A[b]., Tedeschi, P[b]., Font, G[a]., Ruiz., M.J[a]., Brandolini, V[b].

[a]Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain
[b]Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121Ferrara, Italy

Abstract

Trans-resveratrol (*trans*-RSV) is a polyphenol with multiples biological properties, such as anti-inflammatory, antioxidant, anti-aging, anti-diabetic, and antiplatelet. It occurs naturally in grapes and derivate, peanuts and berries. Beauvericin (BEA) is a mycotoxin present in cereals that produces cytotoxicity, intracellular reactive oxygen species and lipid peroxidation. The aims of this study were to determine a) the *trans*-RSV content in different polyphenol dietary supplements by capillary electrophoresis, b) the antioxidant capacity of the *trans*-RSV in polyphenol supplements, and c) the antioxidant capacity of the *trans*-RSV combined with BEA by photochemioluminiscence assay. The results obtained in this study showed that all polyphenol dietary supplements present higher RSV content that the content of the label. The polyphenol supplements present antioxidant capacity. And the combination of *trans*-RSV and BEA did not affect the antioxidant capacity of *trans*-RSV. Thus, RSV could contribute to decrease oxidant effects produced by BEA.

1. INTRODUCTION

Some oxidants are formed in response to physiological processes. A disturbance between pro-oxidants and antioxidants defense system in favor of the oxidants is defined as oxidative stress, which can contributes to the development of chronic disease and ageing process (Davies, 2000; Halliwell, 2006; Lobo et al., 2010; Rahal et al., 2014).

Antioxidant compounds ingested through diet can scavenge free radicals and protect the organisms from oxidative stress. More than 8000 compounds have been identified with antioxidant properties. Polyphenolic compounds are a great class of antioxidants. They include phenolic acids, flavonoids, stilbenes and lignans (Pandei and Rizvi, 2009). Resveratrol (3, 5, 4'-trihydroxystilbene; RSV) is a stilbene abundant in grapes and grape products such as wines and grape juice. RSV exists in two diasteromic forms: *trans* and *cis* (Chen et al., 2007). *Trans*-RSV has biological properties such as antioxidant, anti-inflamatory, antiaging and antiplatelet activities among others, which prevent several human diseases (Fernández-Mar, et al., 2012; Li et al., 2012). This potential benefits resulted in increased consumption of *trans*-RSV supplements by several consumers. Many efforts have been made to provide a highly sensitive and selective analytical method for the determination and characterization of polyphenols in dietary supplements (Ignat et al., 2011). The polyphenol content has been determined in different food matrices by spectrophotometry (Camont et al., 2009), high-performance liquid chromatography (HPLC; Mark et al., 2005), gases chromatography (CG; Goldberg et al., 1995) and capillary electrophoresis (CE; Brandolini et al, 2002; Arribas et al., 2014; Gatea et al., 2015).

Moreover, antioxidant capacity can be determined by Trolox Equivalent Antioxidant Capacity (TEAC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method, oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), total radical-trapping antioxidant parameter (TRAP), ferric reducing antioxidant power (FRAP), photochemiluminescence (PCL) and thiobarbituric acid (TBA), among others (Prior et al., 2005; Alam et al., 2013).

Beauvericin (BEA) is a mycotoxin synthetized by many species of *Fusarium* fungi. BEA is a contaminant of cereals and product composed by cereals (Mahnine et

al., 2011; Juan et al., 2012. It has been demonstrated that BEA is cytotoxic (Ruiz et al., 2011; Prosperini et al., 2013; Mallebrera et al., 2016), and produces oxidative stress in several cell lines (Ferrer et al., 2009; Prosperini et al., 2013; Mallebrera et al., 2015).

The aims of this study were to determine a) *trans*-RSV content in dietary supplements by CE, b) the antioxidant capacity of *trans*-RSV in dietary supplements and c) the antioxidant capacity of *trans*-RSV combined with BEA by PCL.

2. MATERIAL AND METHODS

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (Milan, Italy). The luminol PCL assay was carried out using the Photochem® instrument with the ACL kit (Analytikjena, Jena, Germany).

2.2. Samples

Commercial samples of *trans*-RSV dietary supplements (n=4) were collected during 2015 from different pharmacies in Italy. Table 1 shows the samples analyzed and content of *trans*-RSV in each of them according to the nutritional label.

Table 1: *trans*-RSV dietary supplements content in commercial samples.

Sample	***trans*-RSV content in the label**	**Content (mg/g)**
1	0.34g/100g	3.4
2	1mg	1.23
3	10 mg /1160mg	8.62
4	Not declared	Not declared

2.3. Sample preparation

Briefly, 0.30 g of each sample, were extracted with 5 mL of methanol (MeOH) and mixed using a vortex every 5 min for 15 min. Then, it was centrifuged at 5000 rpm

during 5 min and finally the supernatant were collected in a flask of 20 mL. This procedure was performed four times. Then, the extracts were completed to 20 mL with MeOH. Three independent extractions were performed for each sample.

2.4. Capillary electrophoresis

CE analyses were performed using a CE Beckman MDQ equipped with a diode array detector (Beckman, Fullerton, CA). The separation was obtained by 75 mm i.d. and 57 cm total length fused silica capillary column maintained in a cartridge with a detector window of 100 μm x 800 μm. The capillary was conditioned before use by flushing 0.1 M NaOH for 1 min, then with water and, finally with buffer (20 mM $Na_2B_4O_7$ and 50 mM PEG 400, 10% MeOH) for 3 min. The sample was injected into the capillary by pressure injection for 5 s. Separation was obtained at 25 kV and 25 °C for 15 min at 315 nm. After each separation the capillary was rinsed sequentially with NaOH 0.1M for 2 min. and buffer analysis for 3 min. All analyses were performed in three independent assays. Data are analyzed using the Karat 32 software (Beckman Coulter, Fullerton, CA).

2.5. Antioxidant activity

2.5.1. Antioxidant activity of *trans*-RSV dietary supplements

The antioxidant capacities of *trans*-RSV dietary supplements were determinated using a PCL technique, namely, the luminol PCL assay. The determination was carried out using the Photochem® instrument with the ACL kit (Analytikjena, Jena, Germany), and following the procedure described by Popov and Lewin (1999). Two or three mL reagent 1 (solvent and dilution reagent), 200 μL reagent 2 (buffer solution), 25 μL reagent 3 (photosensitizer) and 10 μL of standard or solution were mixed and measured. Trolox was used as standard to obtain a calibration curve (0.5–2 nM). The light emission curve was measured at λ_{max}=350nm during 180 s, using the inhibition of superoxide anion radicals as the parameter to evaluate antioxidant effect. The antioxidant capacity was determined by using the area under the curve. The results were expressed as μmol Trolox equivalents (TEs)

per g of sample. Antioxidant capacity of supplements was determined replacing standard by diluted samples. Determinations were performed with 6 replicates of each sample.

2.5.2. Antioxidant activity of *trans*-RSV when combined with BEA.

Considering that *trans*-RSV possesses antioxidant properties and BEA increases ROS production, the *trans*-RSV antioxidant capacity against oxidant activity of BEA is an objective of interest. Thus, the antioxidant activity of *trans*-RSV, BEA and their combination were determined using the PCL technique describe previously.

3. RESULTS AND DISCUSSION

During the last years, many *trans*-RSV dietary supplements have been investigated to determine their biological properties. RSV is related with French Paradox, and low incidence of cardiovascular diseases may co-exist with a high-fat diet intake and moderate consumption of red wine (de la Lastra and Villegas, 2007).

Spectrophotometric methods offer an estimation of the total phenolic content (Ignat et al., 2011). HPLC is preferred for separating and to quantify polyphenols in fruits. However, it has limits with respect to complex matrices, e.g. plant extracts and environmental samples; the detection of low molecular weight polyphenols is time consuming and, its resolution decreased as the chain length of molecule increased. Moreover, HPLC remains major challenges in the analysis of polyphenol respect to high molecular weight compounds and molecular mass distributions. Currently, HPLC–MS is the most effective technique for assessing the structure of phenolic compounds (Ignat et al., 2011). The GC-MS is a selective and sensitive detecting and separating compounds in a mixture. However, it does require tedious sample preparation, because encourage elimination of fat, break bonds in plenolic compounds and need derivatizing low volatile polyphenols. CE method is an alternative technique using for the separation and quantification of polar compounds of low and medium molecular weight. Moreover, CE is faster and more efficient than HPLC (Ignat et al., 2011). So, CE is useful for determining a wide variety of polyphenolic compounds due

to its high efficiency separation, high sensitivity, less time required for analysis and little samples and reagents are required for the analyses (Chopin Doroteo, 2012).

In Table 2 is shown the *trans*-RSV content in each sample analyzed by CE. As can be observed, the *trans*-RSV content was higher than the *trans*-RSV content in the label for samples 1,2,3 (Table 1). Sample 4 contains the highest *trans*-RSV content of all the samples. The *trans*-RSV content of sample 4 is not on the label. Nutritional values of dietary supplements found on the labels are expressed as mean of several batches selected. In this respect, according to Italian legislation, the polyphenols content can ranged from ±30% on the labeled content of the product (Circolare n7, 2002).

Table 2: *Trans*-RSV content (mg/g) in the dietary supplements analysed by CE. Data are expressed as mean ±SD (n=6)

Sample	Content (mg/g ± SD)
1	4.44±0.52
2	2.41±0.15
3	10.75±0.48
4	24.79±0.89

The measurement of the antioxidant capacity of dietary supplements is an interesting matter of health. On the other hand, consumers want to know if the consumption of dietary supplementation can protect them against oxidative stress or not.

The antioxidant capacity of *trans*-RSV dietary supplements expressed as μmol TEs/g of sample is shown in Table 3. Antioxidant capacity of the sample was from highest to lowest in the following order: 3>4>2>1.

The antioxidant capacity of *trans*-RSV measured by Photochem® is 0.5 μmol TEs/mg. The theoretical data corresponding to *trans*-RSV antioxidant capacity was verified for each dietary supplement and shown in Table 3. Sample 1 shown lower antioxidant capacity that those of the theoretical data, and samples 2, 3 and 4 were

significantly higher. Moreover, the antioxidant capacity observed is not correlated with the *trans*-RSV content. So, the results obtained in this study are acceptable if we consider the content of other polyphenols in the samples (Table 4). In conclusion, we suggest that the antioxidant effect of each dietary supplement is not given by the *trans*-RSV content; this is due to the combination of antioxidant compounds in the samples. Moreover, it can be observed that *trans*-RSV content is present in low quantity (Table 1 and Table 2) respect to the content of other antioxidant compounds (Table 4) in the dietary supplement.

Table 3: Antioxidant capacity of *trans*-RSV supplements analyzed by PCL and theoretical antioxidant capacity of each supplement. Data are expressed as mean ±SD of different samples (n=6).

Sample	***trans*-RSV antioxidant capacity (*) (µmol TEs/g)**	**Theoretical antioxidant capacity (**) (µmol TEs/g)**
1	1.54±0.09	2.2
2	577.11±30,47	1.2
3	1054.75±30,08	5.37
4	203.53±69,82	12.39

*Antioxidant capacity of *trans*-RSV supplement assayed by PCL.

**Theoretical antioxidant capacity produced in 1 mg of *trans*-RSV from each sample.

Table 4: Components in the label of samples differents to *trans*-RSV.

Sample	Other compounds in the label
1	*Monascus purpureus*, octacosanol, vitamin B3, alpha lipoic acid, omega-3 fatty acid, chrome, pantothenic acid, vitamin B12 and folic acid.
2	Vitamin C (74.1 mg/g), vitamin E (12.3 mg/g), zinc, copper, selenium, omega-3 fatty acid, lutein and zeaxanthin
3	Soy isoflavones, vitamin K, vitamin D, quercetin (129.3 mg/g), calcium, magnesium and isovitexin.
4	Stilvid® (443.3 mg/g), *Punic granatum*, selenium, vitamin C (416.6 mg/g), zinc and vitamin B2 (40 mg/g)

Results obtained in this study demonstrated that *trans*-RSV showed 0.5 µmol TEs/mg antioxidant capacity. It has been demonstrated by other methods that *trans*-

RSV possess antioxidant capacity (Gülçin, 2010; Lucas-Abellán et al., 2011). *Trans*-RSV has effective $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O2^{\bullet-}$ and H_2O_2 scavenging activities (Gülçin, 2010). Moreover, a comparative study between ORAC, ABTS and ORAC assays corroborates that RSV has antioxidant capacity (Lucas-Abellán et al., 2011). The highest RSV antioxidant activity was observed with the ORAC method. The ORAC assay measures low RSV concentrations with high precision. Conversely, DPPH assay measures high RSV concentrations (5–90 μM), but lower than 5 μM cannot be quantified. Due to RSV concentration in food is very low; ORAC method was considered the best for determining the antioxidant capacity of RSV (Lucas-Abellán et al., 2011).

On the other hand, it has been demonstrated that BEA increases intracellular ROS generation and LPO production by *in vitro* methods with culture cells (Ferrer et al., 2009; Prosperini et al., 2013; Mallebrera et al., 2015). Moreover, an excessive ROS accumulation may induce intracellular oxidative damages. BEA antioxidant activity was also assayed by PCL method. However, BEA did not show antioxidant capacity (data not shown). According to Wätjen et al. (2014), BEA had no effect as radical-scavenging activity in a cell-free assay.

Moreover, to evaluate *trans*-RSV antioxidant capacity in presence of BEA, the combination of BEA+RSV was evaluated by PCL method. The results obtained in this study showed that not significant differences were observed between the antioxidant capacity of *trans*-RSV alone or in combination with BEA. It could be due to *trans*-RSV protects against oxidative products produced by BEA. Therefore, more studies related the mechanism of BEA and *trans*-RSV antioxidant effect in culture cells or/and *in vivo* assays are required.

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3.6. Influence of pro- and prebiotics on gastric, duodenal and colonic bioaccessibility of the mycotoxin beauvericin

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Influence of pro and pre biotics on gastric, duodenal and colonic bioaccessibility of the mycotoxin beauvericin

B. Mallebrera, G. Meca, L. Manyes, J. Mañes, G. Font

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

Abstract

Beauvericin (BEA) is a bioactive compound produced by the secondary metabolism of several Fusarium strains and known to have various biological activities. This study investigates the influence of several dietary fibers (galactomanan, glucomannan, citrus fiber, bamboo fiber, carrot fiber, pea fiber, b-glucan, xilan, and cellulose) and probiotic strains (Lactobacillus animalis, Lb. casei, Lb. casei, Lb. plantarum, Lb. rhuminis, Lb. casei casei, Bifidobacterium breve, Bf. Adolescents, Bf. bifidum, Corynebacterium vitaeruminis, Streptococcus faecalis, Eubacterium crispatus, and Saccharomyces cerevisiae) on the minor Fusarium mycotoxin BEA bioaccessibility employing a model solution. The bioaccessibility was determined using a simulated gastrointestinal digestion that mimics the physiological conditions of the digestive tract until the colonic compartment. The determination of BEA in the intestinal fluids was carried out by liquid chromatography–mass spectrometry detection (LC–MS). The reduction of BEA bioaccessibility in the experiments carried out using the prebiotic compounds ranged from 60 to 80%, whereas in the trials carried out using the probiotic strains the bioaccessibility observed ranged from 30 to 85%. A BEA degradation product produced by colonic fermentation was identified using the technique of LC–MS-LIT.

Keywords: Beauvericin, prebiotic compounds, probiotic strains, bioaccessibility, gastrointestinal digestion, LC-MS.

*****Corresponding author: Giuseppe Meca, *Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of València, Av. Vicent Andrés Estellés s/n ,46100 Burjassot, Spain.* e-mail: giuseppe.meca@uv.es. Telephone: +34963544959, Fax: +3496354954

1. INTRODUCTION

Beauvericin (BEA) is a cyclic hexadepsipeptide consisting of alternating D-a-hydroxy-isovaleryl and aromatic N-methyl-phe-nylalanine. This toxin is produced by various *Fusarium* species such as *Fusarium avenaceum*, *F. poae*, *F. oxysporum* and *F. proliferatum*, and naturally occurs on maize, wheat, barley, rice and oat (Logrieco et al., 1998; Uhlig et al., 2006; Jestoi, 2008; Sorensen et al., 2008; Kokkonen et al., 2010; Waskiewicz et al., 2010). BEA has been detected in grains throughout the world under different climates (South Africa, Poland, Norway, Spain, Croatia), with concentrations ranging from trace level up to 520 mg/kg in maize in Italy (Ritieni et al., 1997). Meca et al. (2010) have shown that BEA was present in cereals (barley, corn and rice) purchased in Spanish markets, with levels ranging from 0.51 to 11.78 mg/kg.

An *in vivo* study has shown that mice orally exposed to BEA presented an increase of mortality with a Lethal Dose 50 (LD50) superior to 100 mg/kg bw (Jestoi, 2008). The cytotoxicity of BEA has been demonstrated in vitro in several cell line models, including human leukemia cells CCRF-CEM, human monocytic lymphoma cells U-937 and promyelocytic leukemia cells HL-60, monkey kidney epithelial cells Vero, Chinese hamster ovary cells CHO-K1 and murine macrophage J774 (Tomoda et al., 1992; Calo et al., 2004; Jow et al., 2004; Ruiz et al., 2011a,b).

In the analysis of the risk evaluation related to human health, food ingestion is considered one of the important routes of exposure of many contaminants (Carolien et al., 2005).

To achieve any effects in a specific tissue or organ, the mycotoxins must be available, which refers to the compound's tendency to be extracted from the food matrix, and they must then be absorbed from the gut via the intestinal cells (Fernández-García et al., 2009). The term bioaccessibility has been defined as the fraction of a bioactive compound present in a food matrix that is not modified structurally through the reactions related to the gastrointestinal digestion and thus become available for intestinal absorption (Fernández-García et al., 2009).

Probiotics are defined as 'live microorganisms which when administered in adequate amount confer health benefits to the host' (FAO/WHO, 2006). Alternatively,

probiotics have been defined as live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance. Probiotics were originally used to improve the health of both animals and humans through the modulation of the intestinal microbiota. At present, several well-characterized strains of *Lactobacilli* and *Bifidobacteria* are available for human use to reduce the risk of gastrointestinal (GI) infections or treat such infections (Salminen et al., 2005). Some of the beneficial effects of probiotic consumption include improvement of intestinal health by the regulation of microbiota, and stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, and reducing the risk of certain other diseases (Kumar et al., 2010, 2011; Nagpal et al., 2007, 2010; Yadav et al., 2008).

The concept and understanding of prebiotics have been evolving over time as new information emerges. 'Prebiotic' was first defined as a non-digestible food ingredient that beneficially affected the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Recent literature, however, does not restrict the colon as the only action site and defines a prebiotic as a selectively fermented ingredient that allows specific changes in the composition and/or activity of the gastrointestinal microbiota that confer benefits upon health and wellbeing of the host (Figueroa-Gonzalez et al., 2011). Thus not only are prebiotics being examined for antipathogenic effects (such as inhibiting adhesion of pathogenic organisms to the gut mucosa), but they are also being developed to decrease fecal transit time, lower cholesterol and the glycemic response, improve bone health, lower daily energy (fat) intake, relieve symptoms of inflammatory bowel disease, and attempt to lower colon cancer rates (Pineiro et al., 2008).

In the scientific literature, only a few articles are available on the influence of prebiotics on the bioaccessibility of the minor *Fusarium* mycotoxins (Meca et al., 2012a,b), whereas the influence of the probiotics on the stability of this bioactive compound during gastrointestinal digestion has never been studied. For these reasons the aims of this study were (a) to evaluate the influence of several soluble and insoluble prebiotics on BEA bioaccessibility, (b) to evaluate how different probiotic

strains can influence BEA's bioaccessibility and (c) to determine the possible adduct with BEA and fibers or the degradation products produced by bacteria by LC– MS-LIT.

2. MATERIAL AND METHODS

2.1. Reagents

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-amylase, hydrochloric acid (HCl), sodium hydroxide (NaOH), formic acid, pepsin, pancreatin, bile salts, phosphate buffer saline (PBS, pH 7.5), galactomanan, β-glucan, xylan, cellulose high molecular weight (HMW), and cellulose medium molecular weight (MMW) were obtained from Sigma–Aldrich (Madrid, Spain). Glucomannan high molecular weight (HMW), glucomannan fine powder, citrus fiber, bamboo fiber, carrot fiber, and pea fiber were generously provided by Prof. Alberto Ritieni of the University of Naples "Federico II".

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. The BEA used in this study were produced and purified according to the method of Meca et al. (2010).

2.2. Bacterial strains and growth conditions

Thirteen commercial probiotic strains were obtained for the *in vitro* system that simulates the physiologic condition of the colonic intestinal compartment. In particular *Lactobacillus paracasei* CECT 277, *Lb. casei* CECT 4180, *Lb. rhamnosus* CECT 278T, *Lb. plantarum* CECT 220, *Lb. ruminis* CECT 4061T, *Lb. casei casei* CECT 277, *Bifidobacterium breve* CECT 4839T, *Bf. adolescentes* CECT 5781T and *Bf. bifidum* CECT 870T, *Bf. Longum* CECT 4551, *Corynebacterium vitaeruminis* CECT 537, *Eubacterium crispatus* CECT 4840, *Saccharomyces cerevisiae* CECT 1324 were obtained at the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol. For

longer survival and higher quantitative retrieval of the cultures, they were stored at -80 ºC. When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use (Meca et al., 2012a).

2.3. Model solution preparation

The stock model solution used in this study to reproduce the food model was composed of water, glucose (1%), starch (5%), albumin (3%), sodium chloride (NaCl) (0.1%), and triolein (2%). The model solutions (with fibers) were prepared in 100 mL Erlenmeyer flasks and spiked with 1 and 5 g of each dietary fiber. In the study of the probiotic strains influence on the BEA bioaccessibility, the model solution was inoculated with $2x10^6$ of each bacterial strain tested. Solutions were mixed using ultrasound bath (Lab Police, Barcelona, Spain) operating at a temperature of 30ºC, and then 10 mL of each solution were contaminated with 10 mg BEA/L. Solutions' contamination was carried out using a BEA solution stock (1 g/L) in methanol.

2.4. *In vitro* digestion model

The procedure was adapted from the method outlined by Gil- Izquierdo et al. (2002), with slight modifications. The method consists of three sequential steps; an initial saliva/pepsin/HCl digestion for 2 h at 37 ºC, to simulate the mouth and the gastric conditions, followed by a digestion with bile salts/pancreatin for 2 h at 37 ºC to simulate duodenal digestion (Fig. 1). The colonic conditions were simulated by adding to the duodenal simulated fluid some bacteria representative of the gastrointestinal tract. For the saliva/pepsin/HCl digestion, 10 mL of the model solution or 10 g of the crispy bread contaminated with 5 and 25 mg/kg of BEA, were mixed with 6 mL of artificial saliva composed by: 10 mL of KCl 89.6 g/L, 10 mL of KSCN 20 g/L, 10 mL of NaH_2PO_4 88.8 g/L, 10 mL of Na_2SO_4 57 g/L, 1.7 mL of NaCl 175.3 g/L, 20 mL $NaHCO_3$ 84.7 g/L, 8 mL of urea 25 g/L, and 290 mg of α-amylase. The pH of this solution was corrected to 6.8 with NaOH 0.1 N. These mixtures composed of model solutions and the artificial saliva were placed in plastic bags, containing 40 mL of water and homogenized using a Stomacher IUL Instruments (Barcelona, Spain) during 30 s. To this mixture, 0.5 g of pepsin (14,800 U) dissolved in 25 mL of HCl 0.1 N was added.

The pH of the mixture was corrected to a value of 2 with 6 N HCl, and then incubated in a 37 ºC orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) for 2 h. After gastric digestion, pancreatic digestion was simulated. The pH was increased to 6.5 with $NaHCO_3$ (0.5 N) and then 5 mL of (1:1; v/v) pancreatin (8 mg/mL):bile salts (50 mg/mL), dissolved in 20 mL of water, was added and incubated in a 37 ºC orbital shaker (250 rpm) for 2 h. An aliquot of 5 mL of the duodenal fluid was sampled for the extraction of the BEA and the determination of the duodenal bioaccessibility. To mimic the colonic compartment bacterial strains (previously described) were grown in a sterile plastic centrifuge tube overnight at 37 ºC in MRS broth (Oxoid, Madrid, Spain) under anaerobic conditions (5% CO_2/95% air). Then, the tubes were centrifuged at 4000 rpm for 5 min at 23 ºC and bacteria were resuspended in sterile PBS. A 500 mL aliquot of a mixture of the bacterial suspensions at concentrations of 10^{14} CFU/mL was added to duodenal simulate intestinal fluid and incubated at 37 ºC in 5% CO2/95% air for 48 h. After this last digestion, 5 mL of the mixture was centrifuged at 4000 rpm for 10 min at 4 ºC, and extracted for BEA determination and for duodenal + colonic bioavailability estimation.

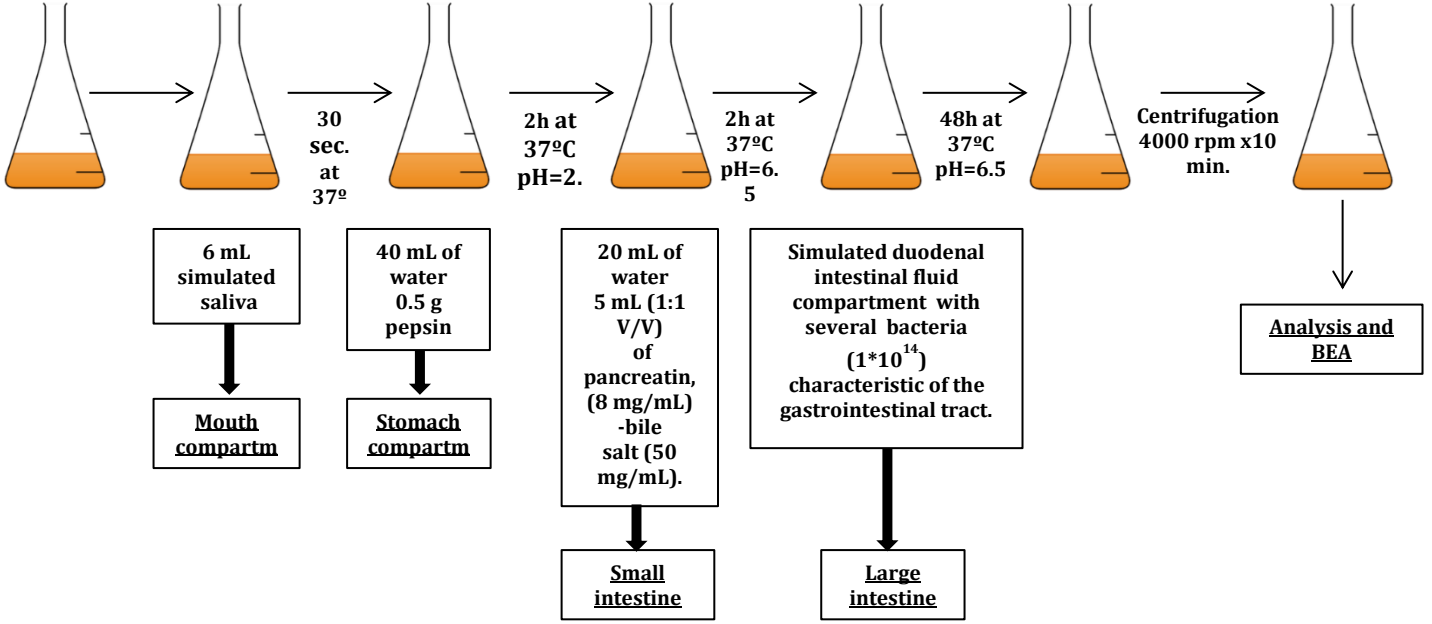

Figure 1. Schematic representation of the steps used during the in vitro gastrointestinal digestion. The model describes the principal reactions developed in the four basic compartments (mouth, stomach, small and large intestine) characterizing the human gastrointestinal tract.

2.5. Beauvericin extraction from the simulated intestinal fluids

BEA contained in gastric, gastric + duodenal, and gastric + duodenal + colonic fluids were extracted as follows. Then 5 mL of each mixture previously described were placed in a 20 mL test tube, and extracted three times with 5 mL of ethyl acetate using a vortex VWR international (Barcelona, Spain) for 1 min. Then, the mixtures were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and at 4 ºC for 10 min. The organic phases were completely evaporated by a rotary evaporator (Buchi, Switzerland) operating at 30 ºC and 30 mbar pressure, resuspended in 1 mL of methanol and filtered with a 0.22 µM filter (Phenomenex, Madrid, Spain) before being analyzed by LC–MS/MS.

2.6. Beauvericin analysis

BEA separation was achieved by an Agilent 1100 (Agilent Technologies, Santa Clara, California) LC coupled to an Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer (Concord, Ontario, Canada). A Gemini (150x2.0 mm, 5 lm) Phenomenex (Torrance, California) column was used. LC conditions were set up using a constant flow at 0.2 mL/ min and acetonitrile:water (70:30 (v/v) with 0.1% of formic acid (HCOOH)) as mobile phase under isocratic condition.

The instrument was configured in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 ºC, desolvation temperature 270 ºC and collision gas energy 5 eV. Multiple reaction monitoring (MRM) technique was used for identification and quantification, in which protonated molecule $[M+H]^+$ of the BEA m/ z 784.50, was fragmented in the collision cell to the product-ion m/ z 244.20. For quantification, the product-ion *m/z* 191.20 was used (Meca et al., 2012a).

2.7. Beauvericin degradation products determination with LC–MS-LIT

The separation of BEA was achieved by LC Agilent 1100 (Agilent Technologies, Santa Clara, California) coupled to mass spectrometer, Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer (Concord,

Ontario, Canada). A Gemini (150x2.0 mm, 5 µm) Phenomenex (Torrance, California) column was used. LC conditions were set up using a constant flow at 0.3 ml/min and acetonitrile/water (80:30, v/v, with 0.1% of HCOOH) as mobile phases in isocratic condition were used. The instrument was configured in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 ºC, desolvation tempera-ture 270 ºC and collision gas energy 5 eV. The analyses of the BEA degradation products employing the liquid chromatography technique coupled to the ion trap were performed using the following procedure:

(1) Characterization of the compound isolated with Enhanced Resolution (ER) scan modality, using the *m/z* range from 200 to 900 Da to obtain the general spectra of the degradation compound;

(2) Characterization of the fragments obtained in the ER scan with the enhanced product ion (EPI) scan modality to obtain a MS^2 scan of the degradation product fragment.

(3) Study of the fragments obtained in the EPI modality employing the modality MS/MS/MS that permits us to know the MS^3 of a fragment selected in the ER scan. The utilization of the mass spectrometry associated at the detection with the linear ion trap, used in these two modalities, permitted us to obtain a total characterization of the isolated compound (Meca et al., 2012a).

3. RESULTS AND DISCUSSION

3.1. Influence of the dietary fiber on the bioaccessibility of the mycotoxin

BEA Table 1 shows the influence of different types of soluble and insoluble dietary fiber on BEA bioaccessibility under simulated gastrointestinal tract condition. The mean bioaccessibility of BEA under simulated stomach conditions for all the treatments with fiber was of 38.8% which was 2.3 fold lower than the control. As shown in Table 1, the highest BEA bioaccessibility data under stomach condition (60.5%) was obtained in the experiment performed with 1% carrot fiber. The lowest BEA bioaccessibility of 15.7% was obtained with 5% HMW cellulose. In the

experiments carried out with the prebiotic compounds using the combination of the gastric and duodenal digestion the mean BEA bioaccessibility was 26.8%, 3.3 fold lower than the value detected in the control experiment. The highest and the lowest bioaccessibility data of the bioactive compound studied using the combination of gastric, duodenal digestion were demonstrated in the experiments using carrot fiber (1%) and cellulose (HMW) with 38.6 and 11.2%, respectively. A considerable reduction of BEA bioaccessibility was also detected in the model solution enriched with cellulose medium molecular weight (MMW), with a 19.3%. The mean data of BEA bioaccessibility after the combination of gastric, duodenal and colonic digestion was of 32.9%, 2.54 fold lower than the value present in the control solutions. The highest bioaccessibility data recorded were detected in the experiments carried out with the fibers glucomannan HMW (1%) and carrot fiber (1%) with 44.1 and 44.6%, respectively, whereas the lowest data was determined in the model solution enriched with cellulose HMW (5%) with 14.1%. Observing the data in Table 1, there are several important details that should be emphasized:

(a) The prebiotic compounds used in this study, soluble and insoluble, reduce the risk associated to minor Fusarium mycotoxin BEA intake, with a retention mechanism of this bioactive compound similar to the mechanism observed for polyphenols, proteins and minerals.

(b) The dietary fibers binding capacity observed for minor Fusarium mycotoxin BEA is dose dependent. In particular, when 5% concentration of prebiotic compounds was used instead of 1%, BEA bioaccessibility decreased from 1.06 to 1.92 fold. This phenomenon can be related to the presence of more active binding sites in the fibers structure of compounds used at 5% concentration, which helped to reduce BEA bioaccessi-bility.

(c) Insoluble fibers as cellulose exert an important effect on BEA bioaccessibility reduction, probably due to the high difficulty to ferment the fiber shown by the colonic model's bacteria and reduce in this way its bioaccessibility.

Awad et al. (2009) studied the influence in feed for chicken nutrition, of a probiotic strain as Eubacterium sp. and of a prebiotic as the inulin, on the injuries

reduction of the gastrointestinal tract induced by mycotoxin deoxynivalenol (DON). The authors demonstrated that the alteration caused by DON was reduced by supplementing the DON containing diets with probiotic feed additive. In the presence of DON acute toxicity, the dietary inulin supplementation may be useful in reducing the toxic effects of DON on intestinal glucose transport.

Baines et al. (2011) studied the influence of a mannan oligosaccharide (MOS) and yeast metabolites prebiotic complex (Celamax) on the bioaccessibility of the mycotoxins nivalenol (NIV), zearalenone (ZEA), deoxinialenol (DON), 15-Acetyl-DON, 3- Acetyl-DON, neosolaniol (NEO), diacetoxyscirpenol (DAS), HT-2 and T-2 toxins, using dairy cattle as biological model. The mycotoxins were produced by several types of mycotoxigenic fungi including *Fusarium culmorum*, *F. poae*, *F. verticillioides*, *F. sporotrichioides*, *Aspergillus flavus*, *Penicillium roqueforti*, *P. crustosum*, *P. paneum* and *P. citrinum* directly on feed. The application in feed of only 0.1% of Celamax reduced the bioaccessibility of the mycotoxins studied on a 90%, 2.0 fold lower than the BEA bioaccessibility reduction achieved in our study.

Meca et al. (2012a) evaluated the bioaccessibility of the mycotoxins BEA tested in concentrations of 5 and 25 mg/L, in a model solution and in wheat crispy breads elaborated with different natural binding compounds as the soluble alimentary dietary fibers b-1,3 glucan, chitosan low molecular weight (L.M.W.), chitosan medium molecular weight (M.M.W.), fruc- tooligosaccharides (FOS), galactomannan, inulin and pectin, added at concentrations of 1% and 5%. The bioaccessibility was determined using a simulated gastrointestinal digestion model that mimics the physiologic conditions of the digestive tract until the colonic compartment. The BEA bioaccessibility data mean in the model solutions ranged from 31.8% of the samples treated with only the duodenal digestion until 54.0% of the samples processed including the colonic fermentation, whereas in the alimentary system composed by the wheat crispy breads produced with different fiber concentration the duodenal and the duodenal + colonic BEA bioaccessibility resulted in 1.9% and 27.0%, respectively. The data obtained in model solution by the authors are comparable with the data produced in our study.

Table 1. Influence of different types of soluble and insoluble dietary fibers on bioaccessibility of BEA under simulated gastrointestinal tract conditions

	Bioaccessibility (%)		
Sample	**Stomach**	**Stomach+Duodenum**	**Stomach+Duodenum+Colon**
Control	93.2±2.1	88.7±1.5	83.8±1.4
Galactomanan 1%	31.3±1.1	24.9±2.4	30.3±1.3
Galactomanan 5%	28.7±1.4	24.3±0.7	27.2±1.4
Glucomannan HMW 1%	50.9±1.8	30.6±2.2	44.1±1.4
Glucomannan HMW 5%	44.9±3.4	31.9±1.5	35.5±2.7
Glucomannan fine powder 1%	35.0±4.3	27.7±2.4	36.3±3.1
Glucomannan fine powder 5%	33.9±1.7	24.2±1.7	30.0±2.2
Citrus fiber 1%	31.1±1.9	26.4±2.2	32.3±2.6
Citrus fiber 5%	37.0±1.6	24.7±1.3	30.1±1.6
Bamboo fiber 1%	49.7±1.1	35.0±2.3	37.5±0.9
Bamboo fiber 5%	40.1±1.8	34.6±2.5	34.8±3.2
Carrot fiber 1%	60.5±2.1	38.6±2.3	44.6±3.1
Carrot fiber 5%	35.5±2.4	23.2±2.0	30.0±2.3
Pea fiber 1%	52.1±1.8	34.6±1.5	40.6±1.9
Pea fiber 1%	52.4±2.3	23.5±0.9	35.5±0.9
β-glucan 1%	42.0±2.1	25.8±1.7	31.9±17
β -glucan 5%	45.1±2.3	33.2±2.1	36.4±3.2
Xilan 1%	40.5±2.6	22.3±2.0	40.0±1.2
Xilan 5%	35.2±1.5	25.6±1.5	32.4±1.7
Cellulose HMW 1%	33.1±2.8	25.8±1.9	27.1±15
Cellulose HMW 5%	15.7±1.5	11.2±1.1	14.1±1.6
Cellulose MMW 1%	32.7±2.4	23.0±1.9	30.0±2.5
Cellulose MMW 5%	25.8±2.5	19.3±1.9	22.7±1.8

3.2. Influence of probiotic bacteria on beauvericin bioaccessibility

All the probiotic strains tested in this study showed an influence on the reduction of the bioaccessibility of the minor *Fusarium* mycotoxins BEA present in the model solutions, during the different steps of the gastrointestinal digestion, as is possible to observe in Fig. 1.

In Fig. 2 the LC–MS/MS chromatogram of the BEA present in the simulated duodenal intestinal fluid related to the model solution spiked with the strain of *Lb.*

rhamnosus is shown. During the gastric digestion the mean bioaccessibility data of the minor *Fusarium* mycotoxins BEA was of 46.2%, 2.0 fold lower than the data that can be seen in the control solution. The highest BEA bioaccessibility data was seen in the experiment carried out with the strain of *Bf. adolescentis* with 65.3% (Fig. 3), whereas the lowest was detected in the model solution spiked with *Bf. breve* with 36.1%. During the gastric more duodenal digestion is possible to observe a decrease of the BEA bioaccessibility probably due to the degradation of the bioactive compound BEA by the bacterial strains used in this study.

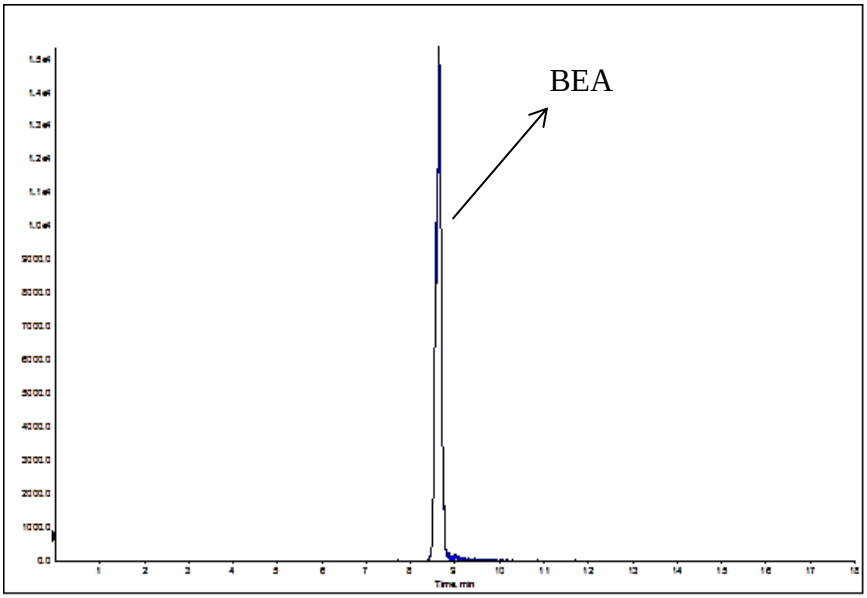

Figure 2. BEA LC–MS/MS chromatogram present in the simulated duodenal intestinal fluid corresponding to the model solution spiked with the strain of *Lb. rhamnosus*.

In particular the mean bioaccessibility data observed was of 35.4%, 2.5 fold lower than the data obtained in the control solution. The highest reduction of BEA bioaccessibility was achieved in the experiment carried out with the strain of *Bf. Longum* evidencing a 45.4%, whereas the lowest bioaccessibility data was detected in the model solution digested with the strain of *Lb. rhamnosus*, with a 27.5%. Using the complete simulated gastrointestinal digestion model (gastric + duodenal + colonic digestion) the mean bioaccessibility data related to the bioactive compound BEA was

of 26.4%. This data was 3.1 fold lower than the data obtained in the control and also 1.5 fold lower than the data collected in the experiments using only the duodenal model. This phenomenon can be related to the synergistic effect between the strains introduced in each trial and the mix of bacteria used to simulate the colonic conditions. The highest and lowest BEA bioaccessibility data were demonstrated in the model solution spiked with *C. vitaeruminis* and *Lb. rhamnosus*, with 33.7 and 13.9%, respectively.

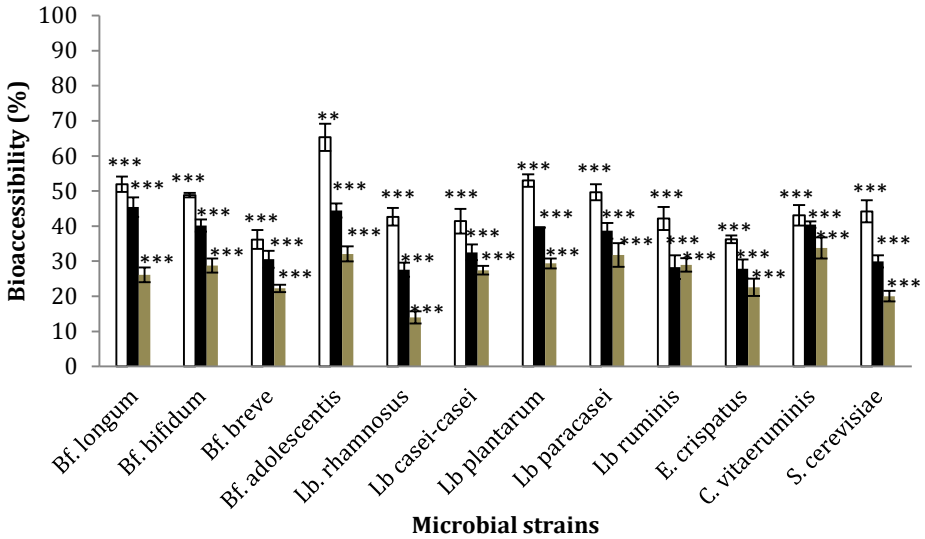

Figure 3. Influence of probiotic bacteria on bioaccessibility of BEA under simulated gastrointestinal tract conditions (white=gastric bioaccessibility, black= gastric+duodenal bioaccessibility and cinnamon= gastric+duodenal+colonic bioaccessibility). Values are presented as means ± SD of 3 samples (n = 3). Significantly different from the control, $p \leq 0.000$ (***).

The influence of the probiotic bacteria during the gastrointestinal digestion of the mycotoxins was studied by different authors. In particular Kabak et al. (2009) determined the release of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) from different food products in the gastro-intestinal tract in the absence and presence of probiotics, a possible adsorbent. The average bioaccessibility of AFB_1 and OTA without probiotics was about 90%, and 30%, respectively, depending on several factors, such as food product, contamination level, compound and type of contamination (spiked versus

naturally contaminated). The six probiotic bacteria showed varying binding capacity to AFB1 and OTA depending on the bacterial strain, toxin studied, type of food and contamination level. A reduction to a maximum of 37% and 73% as observed for the bioaccessibility of AFB1 and OTA in the presence of probiotic bacteria, respectively. The range of bioaccessibility reduction shown by the authors was comparable with the reduction shown for the mycotoxin BEA. This article was the first report on the effect of probiotic bacteria on reducing the fraction of mycotoxins available for absorption in the gastrointestinal tract from different food products.

The interaction between the minor *Fusarium* mycotoxins BEA and bacterial strains characteristic of the gastrointestinal tract as *Bifidobacteria*, *Lactobacillus* and *Streptococcus* was studied by Meca et al. (2012b). Levels of BEA in the fermentation liquid, on the cell walls and on the internal part of the cells were determined using liquid chromatography coupled to the mass spectrometry detector (LC–MS/MS). Results showed that the bacteria reduced the concentration of the BEA present in the medium, part of the mycotoxin was adsorbed by cell wall and part internalized by the bacteria. All the bacteria analyzed in this study showed a significant BEA reduction during the fermentation process, in particular the mean reduction ranged from 66 to 83%.

Kabak and Ozbey (2012), studied the aflatoxins (AFs) bioaccessibility from various spiked food matrices (peanut, pistachio, hazelnut, dried figs, paprika, wheat and maize) using an in vitro digestion model under fed conditions that employed six probiotic bacteria in reducing AF bioaccessibility. The AFs bioaccessibility from seven food matrices ranged from 85.1 to 98.1 and the inclusion of probiotic bacteria showed significant reduction in AFs bioaccessibility. The results discussed in this study are in agreement with the data shown in our study.

3.3. LC–MS-LIT identification of the beauvericin degradation products

The samples of the simulated gastrointestinal fluids positive to BEA degradation produced by microbial fermentation, were injected in the LC–MS-LIT in the modality ER scan (m/z = 200–900) to determine the BEA degradation products.

Fig. 4a presents the LC– MS-LIT chromatogram obtained in the modality of Enhanced Resolution (ER), of the BEA presents in the simulated gastrointestinal fluid fermented by *Lb. rhamnosus*. In the chromatogram is possible to evidence the presence of the BEA with a retention time (RT) of 5.31 min and also of another compound (RT = 6.51 min) close to the BEA peak corresponding to a BEA degradation product. In Fig. 4b is shown the mass spectrum related to the BEA degradation product, identified as the BEA with the loss of the hydroxyvaleric acid (HyLv), with the presence of several diagnostic signals that confirm the structure of this degradation compound. In particular the origin of the degradation compound from the mycotoxin BEA was confirmed by the presence of the ion with a *m/z* of 805.2 represented by the sodium adduct of the mycotoxin. Other important diagnostic fragments are represented by the ion with a *m/z* of 701.3, identified as the BEA sodium adduct with the loss of a HyLv, the ion with a *m/z* of 527.2, identified as the BEA sodium adduct with the loss of a HyLv and also of another important BEA structural component represented by the phenylalanine (Phe). The ion with a *m/z* of 306.3 represents the BEA sodium adduct with the loss of 2Phe and of 1HyLv unit. To confirm the structure of the BEA degradation product, produced by microbial fermentation of the simulated matrix by *Lb. rhamnosus*, the sample was injected in the modality of EPI scan to determine the MS^2 of the neo formed compound. Fig. 5a shows the EPI-scan chromatogram of the BEA degradation product obtained using as reference ion the fragment with a *m/z* of 701.3, whereas in Fig. 5b the EPI scan spectrum of the neoformed compound. In the spectrum can be seen some important fragments that confirm the structure of the BEA degradation compound. The presence of the HyLv and Phe in the neoformed compound structure is evident in the signals with a *m/z* of 683.3 and 603.1, and also in the fragment with a *m/z* of 503.2 where the loss of the Phe and HyLv units is simultaneously. The last confirmation that the degradation product is originated by BEA was carried out using the technique of the MS/ MS/MS that permits to obtain the MS^3 of a product identified in the ER scan. In Fig. 6, the MS^3 of the BEA degradation product spectra obtained using as reference signals the ions with a *m/z* of 701.1 and 503.2 is shown. In the spectra a diagnostic signal with a *m/z* of 416.7 corresponding to

two structural BEA components as the HyLv and the Phe is evident. In particular, the fragment corresponds to 2Phe and 1HyLv units presents in the BEA structure.

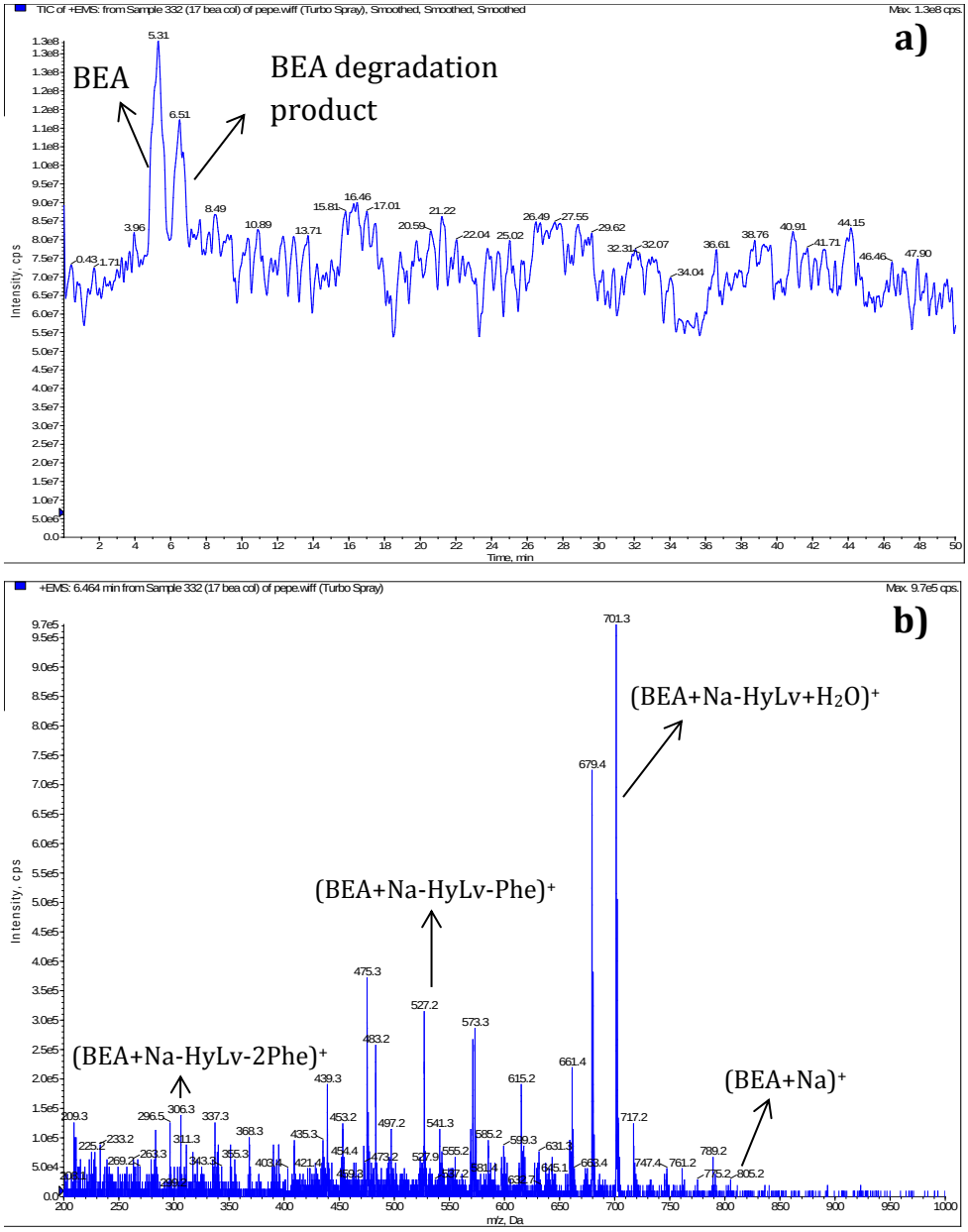

Figure 4. a) LC-MS-LIT chromatogram obtained in ER scan (200-1000 *m/z*) of the BEA present in the model solution fermented by Lb. rhamnosus and b) ER-MS spectrum of the BEA degradation product.

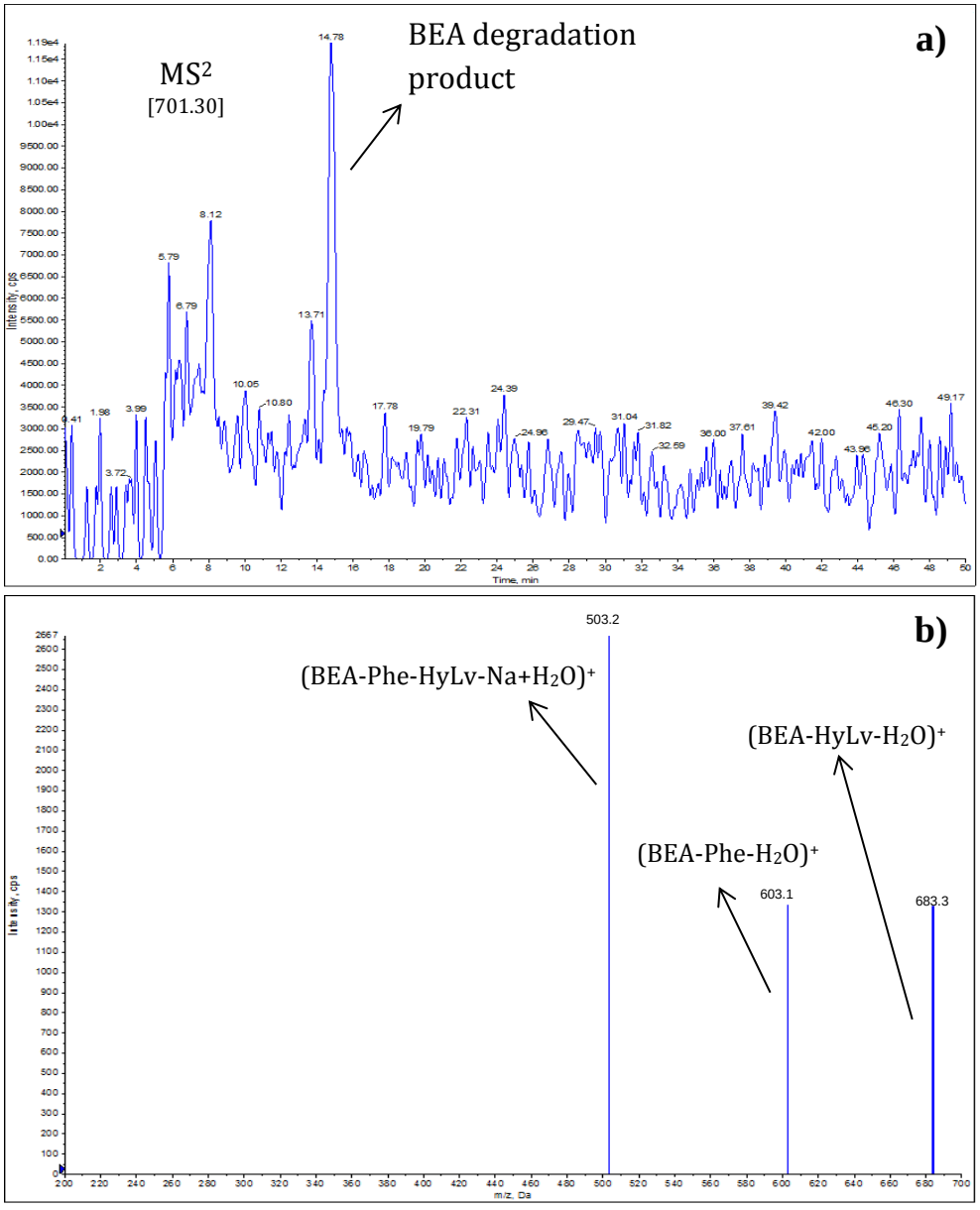

Figure 5. a) LC-MS-LIT chromatogram obtained in EPI modality of the BEA degradation product existent in the model solution fermented by Lb. rhamnosus and b) EPI-MS spectrum of the BEA degradation product. The application of EPI technique permits to obtain the MS2 of a compound with a good resolution.

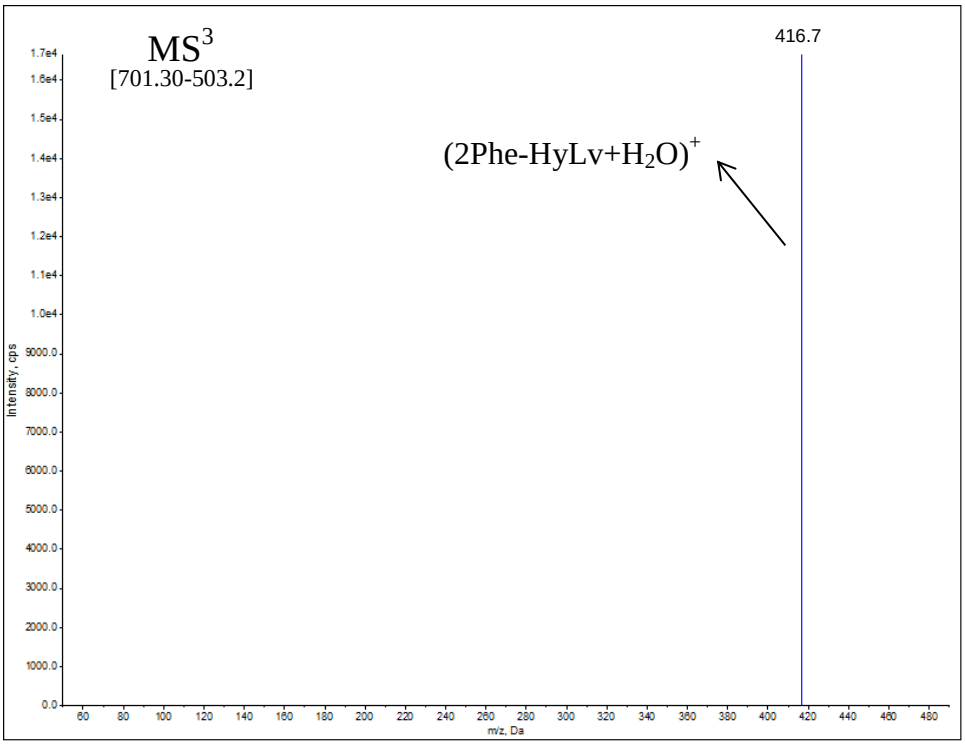

Figure 6. MS/MS/MS spectrum of the BEA degradation product.

4. CONCLUSION

The soluble and insoluble dietary fibers reduced significantly BEA bioaccessibility considering the three gastrointestinal compartments analyzed. In particular, the highest BEA bioaccessibility reduction was observed in the model solution treated with the insoluble fiber cellulose HMW (5%) with 14.1%. Also the probiotic bacteria tested in the model solution produced an important reduction of the BEA bioaccessibility, confirming the capacity of the lactic acid bacteria to reduce/bind toxic compounds present in food as the mycotoxins. The best performance in the reduction of BEA bioaccessibility was provided by *Lb. rhamnosus*. Employing the LC– MS-LIT technique, a BEA degradation product produced by colonic microflora fermentation was identified as the mycotoxin BEA with the loss of the HyLv

acid. Further investigations will be focused on the development of a symbiotic food with high capacity to reduce the bioaccessibility of minor Fusarium mycotoxins as the BEA.

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4. Discusión general
General discussion

4. DISCUSIÓN GENERAL

En la presente tesis doctoral se evaluó el efecto citotóxico de las micotoxinas BEA, STE y PAT individualmente en las células CHO-K1. Debido a que estas micotoxinas pueden encontrarse de manera simultánea en los alimentos y piensos y esta combinación puede variar los efectos citotóxicos respecto de los obtenidos de manera individual, se evaluaron los efectos citotóxicos de las mezclas. Asimismo, se estudiaron los posibles mecanismos de toxicidad de la micotoxina BEA en células CHO-K1 mediante la evaluación del ciclo celular, el potencial de membrana mitocondrial, el daño al DNA, la muerte celular y el estrés oxidativo a través de la producción de ROS y LPO. Tras observar un incremento de ROS y LPO en las células CHO-K1 expuestas a BEA se evaluó el papel protector celular mediante el sistema de defensa antioxidante enzimático y los niveles de GSH; así como, el efecto protector del RSV, un antioxidante ingerido en la dieta en sus formas isoméricas *cis* y *trans*, ya que en una mezcla de isómeros, la proporción de cada uno de ellos puede afectar de forma diferente a la viabilidad celular. Teniendo en cuenta el efecto citoprotector del RSV frente al daño celular producido por la BEA, se evaluó la capacidad antioxidante del RSV y de la BEA, así como la combinación de RSV y BEA para determinar si la BEA interfiere en el efecto protector del RSV.

Debido a que la ingesta de alimentos se considera la principal fuente de exposición a micotoxinas en la evaluación del riesgo, se evaluó la influencia de probióticos y prebióticos en alimentos sobre la disminución de la bioaccesibilidad de BEA. Para este objetivo, se aplicó un modelo de digestión estático *in vitro* como posible estrategia de mitigación de BEA en los alimentos que la contienen.

*****_Todas las tablas citadas en el apartado discusión general corresponden a la introducción._

4.1. Viabilidad celular

4.1.1. Efectos tóxicos de las micotoxinas individuales

Los efectos sobre la viabilidad celular de la BEA, STE y PAT fueron determinados en células CHO-K1 tras 24, 48 y 72 h de exposición mediante la reducción de formazan por las deshidrogenasas mitocondriales de las células vivas, lo que permite relacionar la viabilidad celular con la actividad metabólica mitocondrial. Se observó que estas micotoxinas presentan citotoxicidad de manera dependiente de la concentración y del tiempo de exposición en la línea celular CHO-K1. Las IC_{50} obtenidas a partir de las curvas dosis respuestas presentan valores desde un 10.7±3.7 a un 2.2±4.9 μM; de 25.0±3.7 a 12.5±2.0 μM y de 2.9±0.3 a 2.8±0.4 μM para la BEA, STE y PAT, respectivamente. Siendo las células CHO-K1 más sensibles a la PAT seguida de la BEA y la STE en este orden. Los valores de IC_{50} obtenidos para la BEA fueron similares a los obtenidos por otros autores en la misma línea celular (Ferrer et al., 2009). Sin embargo, los valores de IC_{50} obtenidos por otros autores en otras líneas celulares oscilaron entre 1.4 y 20.6 μM para la BEA, presentando la mayoría de las líneas celulares valores de IC_{50} por debajo de 6 μM; siendo las líneas celulares CHO-K1, Caco-2, Hep-G2, HT-29 e Y79 las que presentan una mayor resistencia a la BEA con valores de IC_{50} superiores a 10 μM (Tabla 2). Los valores de IC_{50} para la STE se encuentrar entre el 3.7 y el 286.1 μM siendo la linea celular A549 la más sensible y la linea Hep-G2 la menos sensible a está micotoxina (Tabla 3); y entre 0.69 y 17 μM para la PAT siendo la linea célular CHO-K1 la más susceptible y la V79 la menos (Tabla 4). Esta variabilidad de datos se debe a que los valores de IC_{50} dependen del método ensayado, el tiempo de exposición y la línea celular empleada. Los resultados obtenidos con los métodos de toxicidad aguda *in vitro* pueden contribuir al establecimiento de las concentraciones preliminares necesarias en ensayos *in*

vivo para determinar el establecimiento de los límites máximos de estas micotoxinas en alimentos y piensos.

4.1.2. Efectos tóxicos de combinaciones de micotoxinas

El efecto tóxico de una micotoxina puede verse potenciado cuando se encuentra de manera simultánea con otras micotoxinas en los alimentos de la dieta o en los piensos animales, respeto a los efectos de la micotoxina de manera individual. Este hecho incrementa la preocupación en la población sobre el riesgo de exposición a contaminantes de forma simultánea en la dieta como son las micotoxinas. Por ello, se evalúan los efectos citotóxicos de las combinaciones binarias y terciarias de las micotoxinas BEA, STE y PAT tras 24, 48 y 72 h de exposición en células CHO-K1.

Los resultados obtenidos muestran que la combinación de PAT y STE disminuye la viabilidad celular un 87% y entre un 86% y 73% respecto de la STE y la PAT de manera individual, respectivamente. La combinación de BEA y STE mostró más citotoxicidad que cada micotoxina ensayada individualmente, con una disminución en la viabilidad celular del 99% al 57% y del 89% al 57%, respectivamente. La combinación de la BEA y la PAT mostró una disminución del 80% en la viabilidad celular respecto de la BEA de forma individual. Asimismo, la combinación terciaria disminuyó la viabilidad celular respecto de las tres micotoxinas individualmente del 100% al 51%, 48% y 37.5% tras 24, 48 y 72 h de exposición, respectivamente. Estos resultados sugieren que la presencia de estas micotoxinas simultáneamente en los alimentos o en la dieta puede alterar el potencial tóxico.

Posteriormente, para evaluar si la combinación de estas micotoxinas aumenta o disminuye significativamente su potencial tóxico respecto a la exposición individual, se aplicó el método matemático de las isobolas descrito por Chou (2006) y Chou y Talalay (1984). Las interacciones obtenidas difieren

dependiendo de la combinación, la concentración y tiempo ensayados, siendo el efecto aditivo y sinérgico el observado con mayor frecuencia. Tras 24 h de exposición todas las combinaciones mostraron efecto sinérgico a bajas concentraciones y aditivo a altas concentraciones. Este mismo efecto fue observado tras 48 y 72 h de exposición para la combinación STE+PAT. Sin embargo, en aquellas combinaciones en las que la BEA está presente a mayores tiempos de exposición el efecto antagónico se convierte en sinérgico. Estas diferencias, pueden deberse a que las micotoxinas las producen diferentes grupos de hongos, por lo que presentan diferentes estructuras y propiedades químicas. Por ello, Tatay et al. (2014) sugieren que el efecto antagónico puede deberse a que micotoxinas con estructuras químicas similares compiten por el mismo receptor y Speijers y Speijers (2004) sugieren que el efecto aditivo puede deberse a que las micotoxinas presentan mecanismos de acción similares.

Sin embargo, el método de las isobolas no permite conocer el mecanismo de acción que produce dicho efecto, sino el tipo de interacción entre las combinaciones de diferentes sustancias relacionando la dosis y el efecto producido (Chou et al., 2006). Las fluctuaciones observadas son difíciles de explicar debido a la complejidad de los mecanismos implicados por procesos bioquímicos desconocidos (Ruiz et al., 2011b). El efecto sinérgico observado tras la combinación de las micotoxinas es de gran importancia debido a que pueden incrementar la actividad citotóxica de una micotoxina o aumentar la actividad citotóxica de las otras micotoxinas de la mezcla. Por ello, ante la creciente preocupación de los consumidores por la exposición simultánea en la dieta a las micotoxinas, se ha incrementado el número de estudios realizados sobre interacciones toxicológicas de estas (Tabla 5).

4.2. Proliferación celular

La proliferación celular es esencial en la homeostasis celular. Con el fin de mantener la integridad genómica, las células poseen proteínas reguladoras encargadas de inducir la parada del ciclo celular en los puntos de control (G_0/G_1 y G_2/M) para poder reparar posibles daños o en el caso de que estos no puedan ser reparados inducir la muerte celular (Damia y Broggini, 2003). Diversos factores como las micotoxinas pueden alterar la progresión del ciclo celular. Por ello, se ha determinado la influencia de la BEA en el ciclo celular en células CHO-K1 tras 24, 48 y 72 h de exposición.

Los resultados obtenidos muestran el incremento de la población celular en la fase G_0/G_1 tras 24 h de exposición a BEA (5 μM), lo que se traduce en una parada del ciclo celular en este punto; este hecho está relacionado con mecanismos de reparación de daños y con un incremento en la apoptosis. Estos resultados son similares a los obtenidos por otros autores tras la exposición a BEA en diversas líneas celulares (Tabla 6). Por otra parte, en este trabajo también se observó parada del ciclo celular en la fase G2/M. En este trabajo se observó tras la exposición a 5 μM de BEA un incremento en la población celular del 77% y del 135% durante 48 y 72 h, respectivamente. De forma similar, este comportamiento ha sido observado en diversas líneas celulares tras la exposición a BEA y otros tipos de micotoxinas (Tabla 6; Dornetshuber et al., 2007; Prosperini et al., 2013b). Este hecho, se relaciona con la parada del ciclo en esta fase para evitar que las células entren en mitosis ante un posible daño celular. Algunos autores sugieren que la parada del ciclo celular por la BEA en la fase G_2/M se debe a un proceso adaptativo de supervivencia ante posibles daños en el DNA, con el fin de repararlos (Abid-Essefi et al., 2003). La parada del ciclo celular podría relacionarse con la disminución de la viabilidad celular (previamente demostrada). Sin embargo, también podrían deberse a otros factores como la activación de la apoptosis o

el incremento del daño al DNA tras la exposición de las células CHO-K1 a la BEA.

4.3. Estrés oxidativo

Para investigar las causas de los efectos citotóxicos producidos por la BEA y su parada durante el ciclo celular, se determina si la BEA produce sustancias oxidantes que puedan contribuir a estos efectos.

La producción de ROS en la célula juega un papel importante en diferentes rutas de señalización celular. Agentes exógenos como las micotoxinas pueden contribuir a la producción incontrolada de estas e incrementar el estrés oxidativo que a su vez puede dañar los lípidos de membrana, las proteínas y el DNA celulares. Debido a que el estrés oxidativo puede dar lugar a estos efectos y ser uno de los posibles mecanismos implicados en la citotoxicidad producida por la BEA en células CHO-K1, se determinó la producción de ROS mediante el método de la DCFH-DA y la LPO mediante el método de las TBARS. Los resultados obtenidos demuestran que la BEA (1 y 5 μM) incrementa la producción intracelular de ROS dependiendo del tiempo y dosis ensayadas. La exposición de las células CHO-K1 a BEA durante 120 minutos incrementó la producción de ROS 1.3 veces respecto a la producción de ROS observada en el control. Este efecto ha sido corroborado por otros autores en diversas líneas celulares (Tabla 7). El incremento de la producción de ROS tras la exposición a BEA podría dar lugar a la producción de LPO, daños al DNA y la citotoxicidad de esta micotoxina.

Una de las consecuencias del incremento del estrés oxidativo celular es la LPO. Los resultados obtenidos muestran un incremento del 35% en la producción de MDA en las células CHO-K1 expuestas a 5 μM de BEA. Este mismo efecto ha sido observado en diferentes líneas celulares (Tabla 8).

El incremento de la generación de ROS producido por la BEA puede estar relacionado con el incremento de la LPO en las células CHO-K1. A su vez, el incremento de la LPO puede dar lugar a alteraciones en las membranas celulares y como consecuencia producir un mal funcionamiento celular, por lo que el estrés oxidativo se podría considerar un mecanismo relacionado con la citotoxicidad producida por esta micotoxina en las células CHO-K1.

4.4. Potencial de membrana mitocondrial ($\Delta\Psi m$)

Teniendo en cuenta que la pérdida del $\Delta\Psi m$ puede ser un evento temprano en el proceso apoptótico o una consecuencia de la vía de señalización del mismo (Ly et al., 2003), se evaluó el $\Delta\Psi m$ en las células CHO-K1 tras 24 h de exposición a la BEA. Los resultados obtenidos mostraron que la BEA (5 μM) disminuyó en un 14% el $\Delta\Psi m$ en las células CHO-K1. Diversos autores observaron una disminución del $\Delta\Psi m$ tras la exposición a BEA de hasta un 95% en células Caco-2 (Prosperini et al., 2013a), de 1.8 veces el control en células CCRF-CEM (Jow et al., 2004), e incluso el colapso del $\Delta\Psi m$ en células RLM (Toshin et al., 2010).

Tras observar una disminución del $\Delta\Psi m$ y la actividad metabólica mitocondrial (medida con el ensayo del MTT), se puede sugerir que la disfunción mitocondrial está implicada en la citotoxicidad y la parada del ciclo celular producida por la BEA en las células CHO-K1. Asimismo, la disminución del $\Delta\Psi m$ podría estar relacionada con la capacidad ionófora de la BEA, que puede interferir en la concentración iónica intracelular de las células de mamífero, incrementando la concentración citosólica de Ca^{2+}, lo que puede dar lugar a la disminución del $\Delta\Psi m$, la liberación del citocromo *c*, la activación de las caspasas y la apoptosis (Kouri et al., 2003; Jow et al., 2004).

4.5. Muerte celular: apoptosis y necrosis

Considerando la disfunción mitocondrial y la disrupción del ciclo celular tras la exposición a BEA en las células CHO-K1 y la posible relación de estos efectos con la muerte celular, se evaluó la muerte celular por apoptosis y necrosis tras 24, 48 y 72 h de exposición a BEA. Los resultados obtenidos mostraron un incremento de la apoptosis temprana del 79% al 116%, en un 176% y un 70% tras 24, 48 y 72 h de exposición, respectivamente. Las células apoptóticas/necróticas incrementaron del 64% al 73% después de 48 h y disminuyeron en un 61% tras 72 h de exposición. La muerte celular es la consecuencia final de todos los procesos celulares observados tras la exposición de las células CHO-K1 a la BEA. La muerte celular causada por la BEA se observó en diferentes líneas celulares (Tabla 11; Dombrink-Kurtzman, 2003; Jow et al., 2004; Dornetshuber et al., 2009b; Prosperini et al., 2013a; Wätjen et al., 2014). Los mecanismos que pueden dar lugar a la muerte celular tras la exposición a BEA son muy diversos. La muerte celular podría deberse al incremento del estrés oxidativo, la disminución de los niveles de GSH, la activación de Bcl-2, el citocromo *c*, la caspasa 3, la disminución del $\Delta\Psi m$ y el incremento de la concentración de Ca^{2+} intracelular (Jow et al., 2004; Lin et al., 2005; Klaric et al., 2008a; Dornetshuber et al., 2009b). Asimismo, la muerte celular también puede deberse a un mecanismo de defensa celular fallido de reparación de los daños producidos tras la parada en la fase G_0/G_1 del ciclo celular, a las 24 h de exposición de las células CHO-K1 a la BEA.

4.6. Genotoxicidad

Teniendo en cuenta la disrupción del ciclo celular tras la exposición de las células CHO-K1 a la BEA y su posible atribución del daño al DNA como uno de los posibles mecanismos implicados en este proceso, se evaluó el daño que puede producir la BEA en el DNA mediante la técnica del cometa. Los

resultados obtenidos mostraron que tras la exposición de 1 μM de BEA se produce un incremento del 85% en el daño al DNA. Se ha demostrado que la BEA causa daño al DNA, así como un incremento de la frecuencia de MN, NBs, NPBs, SCE e inducción de CA (Tabla 12). Este hecho coincide con la parada del ciclo celular en la fase G_0/G_1 tras 24 h de exposición y la fase G_2/M tras 48 y 72 h de exposición a BEA en las células CHO-K1, por lo que podría sugerirse como un mecanismo de reparación de daños al DNA o apoptosis en el caso de no poder reparar el daño. De acuerdo con Klaric et al. (2010) el daño al DNA podría estar asociado con la estructura química de la BEA, ya que la carga positiva de esta interfiere con las cargas negativas del DNA. Además, el incremento del Ca^{2+} puede influir en la actividad de las endonucleasas que intervienen en el proceso de apoptosis, así como, el estrés oxidativo podría estar relacionado indirectamente con el daño al DNA (Jow et al., 2004; Dornetshuber et al; 2009b).

4.7. Sistemas de defensa antioxidante

Debido a que la BEA reduce la actividad metabólica mitocondrial, el $\Delta\Psi m$, la proliferación celular e incrementa el estrés oxidativo, el daño al DNA y la muerte celular, se evaluaron los sistemas de defensa de las células CHO-K1 frente a estos daños. Para ello, se determinó la actividad enzimática de la GPx, GR, GST, SOD, CAT, los niveles de GSH, así como, el efecto de la NAC (precursor de GSH) y el polifenol RSV tras 24 h de exposición de las células CHO-K1 a la BEA.

4.7.1. Niveles de glutatión

El GSH forma parte del sistema de defensa antioxidante intracelular y protege a las células y tejidos frente al daño oxidativo. Por ello, la depleción de los niveles de GSH dan lugar a un incremento del estrés oxidativo, y

alteraciones celulares. Se ha demostrado que los niveles intracelulares de GSH intervienen en diferentes funciones celulares como la reparación del DNA, el ciclo celular, la regulación de rutas de señalización y la regeneración de antioxidantes exógenos como los α-tocoferoles (a través de la forma reducida del ascorbato). La disminución de los niveles de GSH mitocondriales están asociados con una disminución del $\Delta\Psi m$ y su citotoxicidad (Dalton et al., 2004).

Los resultados obtenidos en este estudio mostraron una disminución de los niveles de GSH del 18% tras la exposición a la BEA (5 μM). Otros autores han obtenido resultados similares en otras líneas celulares expuestas a BEA (Tabla 13). La disminución de los niveles de GSH demuestra la implicación de este en la protección frente al estrés oxidativo producido por la BEA.

4.7.2. Actividades enzimáticas

Los niveles de hidroperóxidos y H_2O_2 producidos por el estrés oxidativo pueden disminuir por la actividad antioxidante de las enzimas GPxs. Los resultados obtenidos tras la exposición a la BEA durante 24 h mostraron un aumento de la actividad enzimática GPx del 35% al 66% en todas las concentraciones ensayadas. Este incremento sugiere que esta enzima interviene en el control de los efectos citotóxicos de la BEA.

La enzima GR tiene un papel fundamental en la regeneración del GSH a través del GSSG. Los resultados obtenidos mostraron una reducción de la actividad de la GR del 43% tras la exposición a 5 μM de BEA. Este descenso podría deberse/atribuirse al consumo de GSH a través de las enzimas GPx y GST o a que el exceso de ROS a esta concentración dificulta la regeneración del GSH. Asimismo, esta disminución podría ser debida a un agotamiento del NADPH, el cual es necesario para el funcionamiento de esta enzima. El

descenso de los niveles de GSH y GR a esta concentración puede producir un desequilibrio en el ciclo del GSH, una deficiencia de GSH, y causar el estrés oxidativo.

La familia de enzimas GSTs cataliza la conjugación del GSH con diferentes sustratos, protegiendo a la célula de daños y detoxificando xenobióticos. Los resultados obtenidos mostraron que a 0.1 y 1 μM de BEA incrementa la actividad de la GST. El incremento de la actividad de la GST se considera una señal de estrés (Grigutyte et al., 2009). La estimulación de la actividad enzimática a bajas concentraciones de BEA sugiere que está enzima participa en la detoxificación de la BEA en las células CHO-K1. Sin embargo, a la concentración más elevada no se observó actividad. Este hecho, podría explicarse por la saturación de la enzima debido al incremento de las ROS a esa concentración.

La NAC es un precursor del GSH. Para evaluar el papel de la NAC, se pretrataron las células con 1 mM de NAC durante 24 h y posteriormente se expusieron a 0.1, 1 y 5 μM BEA. Se determinaron los niveles de GSH y las actividades enzimáticas de las enzimas relacionadas con el GSH (GPx, GR y GST) y se compararon los resultados con los obtenidos en las células no pretratadas con NAC.

Se comparó los resultados obtenidos al pretatar las células con NAC respecto del control y se observó una disminución del 29%, del 53% y entre un 22% y un 35% en los niveles de GSH y la actividad de la GR y de la GST, respectivamente. Así como, un incremento del 20% en la actividad de la GPx. Se compararon las células pretratadas con NAC de las no pretratadas y se observó un incremento de los niveles de GSH entre un 548% y un 716% respecto de las células no pretratadas con NAC. La actividad GPx se incrementó entre un 311% y un 458% y la GST entre un 105% y un 206% respecto de las células no pretratadas con NAC. Sin embargo, no se obtuvieron

diferencias significativas en la actividad de la GR cuando se compararon células pretratadas y no pretratadas.

Por tanto, el aumento de los niveles de GSH y en consecuencia de la actividad de la GPx y de la GST cuando las células se pretrataron con NAC respecto de las no tratadas, sugieren que la NAC ayuda en la detoxificación de la BEA en las células CHO-K1. Este hecho puede deberse al incremento de los niveles de GSH, así como a sus propiedades antioxidantes y eliminadoras directas de radicales libres (Zafarullah et al., 2003).

La enzima SOD es capaz de catalizar la trasformación del radical libre superóxido a H_2O_2, el cual se elimina por las enzimas CAT y GPx (Matés, 2000). Tras la exposición a BEA durante 24 h se observó un incremento de las actividades enzimáticas del 37% y del 134% a 1 μM y 5 μM, respectivamente. Este efecto podría considerarse una respuesta adaptativa frente al daño causado por la BEA.

La enzima CAT protege a las células frente al H_2O_2 transformándolo en oxígeno y agua. Los resultados obtenidos mostraron un incremento del 70% de la actividad de la CAT tras la exposición a 5 μM de BEA. Por tanto, esta enzima ayuda a detoxificar las ROS producidas por la BEA en células CHO-K1.

La CAT interactúa con la enzima GPx en la eliminación del exceso de H_2O_2. Sin embargo, la actividad de la GPx se estimuló a todas las concentraciones ensayadas mientas que la CAT solo a 5 μM. Este hecho podría ser debido a que la enzima GPx además del H_2O_2 puede reaccionar con lípidos y otros hidroperóxidos orgánicos.

Resultados similares fueron obtenidos por otros autores con otras micotoxinas. Se observó un incremento de las actividades de la CAT y de la SOD tras la exposición de las células mononucleares de bazo a la FB_1 y la AFB_1 (Theumer et al., 2010). He et al. (2011) detectaron este mismo efecto en cultivos primarios de condrocitos de tibia de pollo expuestos a la toxina T-2.

Asimismo, resultados comparables fueron observados por Dinu et al. (2011) en células de riñón de embrión humano tras la exposición a DON. Sin embargo, Fernández-Blanco et al. (2014) observaron un incremento de la actividad de la SOD y una diminución de la actividad de la CAT tras la exposición de células Caco-2 a AOH. Wu et al. (2013) observaron una disminución de las actividades de la SOD, CAT y GPx tras la exposición de células de la granulosa a la toxina T-2 y Li et al. (2014) una disminución de la actividad de la SOD tras exponer fibroblastos de embrión de pollo a DON.

Por tanto, el descenso de los niveles de GSH acompañado de un incremento de las actividades de la GPx, GST, SOD y CAT, podría indicar una medida adaptativa para contrarrestar el estrés oxidativo causado por la BEA en las células CHO-K1. A su vez, la disminución de la actividad de la GR podría estar relacionada con la incapacidad de regenerar el GSH através del GSSG. A pesar de que el GSH y las enzimas GPx, GST, SOD y CAT invervienen en la detoxificación de la BEA, no todas contribuyen de la misma forma. Se ha demostrado que el ciclo del GSH es más efectivo frente a la protección de bajos niveles de estrés oxidativo y la enzima CAT frente a niveles elevados de estrés oxidativo (Matés, 2000). Asimismo, la enzima GPx es considerada la principal enzima antioxidante frente a la detoxificación del H_2O_2, ya que la enzima CAT posee una afinidad menor por este sustrato que la GPx (Matés, 2000).

4.7.3. Resveratrol

El sistema de defensa antioxidante exógeno está formado por componentes de la dieta o suplementos alimenticios que ayudan en la protección frente al estrés oxidativo. El RSV es un polifenol con dos formas isoméricas, la *cis* y la *trans*.

Capacidad antioxidante y contenido de resveratrol en suplementos alimenticios

Las múltiples propiedades biológicas y efectos beneficiosos para la salud del RSV han incrementado el consumo de suplementos alimenticios que contienen este polifenol. Con el fin de verificar que el contenido de RSV en suplementos alimenticios es el que indica el fabricante, se determinó el contenido de RSV en suplementos que lo contienen adquiridos en diferentes farmacias de Italia mediante la técnica de CE. La legislación italiana (Circolare n7, 2002) permite que el contenido de polifenoles fluctúe entorno a un 30% respecto de la información obtenida en la etiqueta. Por tanto, de acuerdo con esta legislación y los resultados obtenidos en este estudio, el contenido de RSV indicado por el fabricante en la etiqueta es adecuado.

Entre las múltiples propiedades que se atribuyen al RSV se encuentra el poder antioxidante. Para comprobar este efecto, se determinó la capacidad antioxidante de los suplementos alimenticios adquiridos mediante el método Photochem®. Los resultados obtenidos en este estudio mostraron que todos los suplementos ensayados presentan capacidad antioxidante. Sin embargo, los valores obtenidos fueron mayores que los esperados (datos teóricos), para el contenido de RSV correspondiente a cada muestra, excepto la muestra 1. Por otra parte, no se observó correlación entre la capacidad antioxidante y el contenido de RSV en los suplementos alimenticios. Asimismo, el contenido de RSV está presente en cantidades muy bajas respecto a otros compuestos antioxidantes presentes en cada suplemento. Por lo que se puede concluir, que la capacidad antioxidante obtenida para cada suplemento no se debe al contenido de RSV sino al conjunto de compuestos antioxidantes presentes en los suplementos alimenticios. Este hecho ha sido previamente observado por otros autores en otras matrices alimentarias, como el vino (Kostadinović et al., 2012).

Efecto de la beauvericina en la capacidad antioxidante producida por el resveratrol

Con el fin de determinar si la capacidad antioxidante del RSV podría verse afectada por la presencia de oxidantes de la dieta, como la BEA, se determinó la capacidad antioxidante del *trans*-RSV y la BEA de manera individual y en combinación mediante el método Photochem®. Los resultados obtenidos mostraron que el *trans*-RSV posee capacidad antioxidante, lo cual se ha demostrado mediante diferentes técnicas por otros autores (Gülçin, 2010; Lucas-Abellán et al., 2011). Como era de esperar, la BEA no mostró actividad antioxidante. Wätjen et al. (2014) atribuyen a la estructura química de la BEA este efecto.

Por otra parte, los resultados obtenidos en este estudio no mostraron diferencias significativas entre la capacidad antioxidante del RSV solo o en combinación con la BEA. Por lo que la BEA o los derivados oxidados producidos por la BEA no afectan a la capacidad antioxidante del *trans*-RSV.

Efecto citoprotector del resveratrol

Para evaluar el efecto citoprotector de los isómeros del RSV, se determinó el efecto en la viabilidad celular. Para ello, se expusieron las células CHO-K1 al isómero *trans*-RSV y la mezcla de isómeros (50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*-RSV) en un rango de concentración entre 12.5 y 200 μM y se evaluó la viabilidad celular por los métodos MTT y NR. Los resultados obtenidos mostraron que *trans*-RSV y la mezcla de isómeros (50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*-RSV) disminuyen la viabilidad celular. Los valores de IC_{50} obtenidos fueron entre 41.66 ± 13.05 y 180.77 ± 10.36 μM.

Por otra parte, a bajas concentraciones de RSV (de 12.5 a 25 μM), la viabilidad celular de las células CHO-K1 se incrementó. La disminución de la viabilidad celular al aumentar la concentración de RSV (>25 μM) puede

deberse a su efecto pro-oxidante a altas concentraciones como se ha demostrado con anterioridad (Carrasco-Pozo et al., 2012; Israel-Ortega et al., 2012; Juan et al., 2012b; Stocco et al., 2012).

Debido a que la mezcla de isómeros 50:50 *trans*/*cis*-RSV no incrementó la viabilidad celular, el efecto citoprotector frente a la BEA se realizó con *trans*-RSV y 70:30 *trans*/*cis*-RSV (1.25, 2.5, 5 μM). Estas concentraciones fueron seleccionadas basándose en la concentración de RSV en plasma tras el consumo de una copa de vino y una estimación de la ingesta total de BEA. Según Walle et al. (2004), la concentración de RSV en plasma tras la ingesta de una copa de vino (aproximadamente 25 mg de RSV) es 2 μM aproximadamente; ya que el consumo de RSV puede ser mayor o menor, se incluyen la concentración 1.25 y 5 μM en el estudio. Debido a que no existen datos sobre la concentración de BEA en plasma tras su ingesta, la concentración seleccionada para el estudio se basa en la ingesta total estimada de BEA calculada a partir del consumo de trigo en el área Mediterránea (entre 76.7 y 177.3 kg/per capita/año; FAOSTAT, 2011) y el contenido de BEA en trigo en esta área (entre 0.23 y 4 mg/kg; Meca et al., 2010; Zinedine et al., 2011); siendo el valor obtenido entre 1.05 y 2.45 μmol/persona/día.

Los resultados obtenidos demostraron que 1 y 5 μM de BEA disminuyen significativamente la viabilidad celular. Tras pretratar las células con *trans*-RSV y 70:30 *trans/cis*-RSV (excepto 1.25 μM de 70:30 *trans/cis*-RSV) la viabilidad celular se incrementa en las células expuestas a BEA, respecto de las células sin pretratar. La combinación con mayor efecto citoprotector fue 1.25 μM de *trans*-RSV y 1 μM de BEA. No todas las concentraciones de RSV ensayadas produjeron efecto citoprotector frente a 5 μM de BEA, lo cual podría explicarse por el mayor efecto citotóxico que presenta la BEA a esta concentración.

Para corroborar el efecto citoprotector del RSV en células CHO-K1 expuestas a BEA, se determinó el tipo de interacción entre la BEA y el RSV 1:1 (0.75, 1.25, 2.5 y 5 μM) mediante el método de las isobolas. Se observó que el *trans*-RSV presentó efecto antagónico, aditivo y sinérgico a baja, media y elevada concentración, respectivamente, lo que podría ser debido al mayor efecto citotóxico de la BEA cuando aumenta la concentración. Por otra parte, el 70:30 *trans*/*cis*-RSV presentó antagonismo para todas las concentraciones ensayadas, lo cual podría disminuir el efecto citotóxico de la BEA.

Teniendo en cuenta los datos de viabilidad celular observados en el pretratamiento de las células CHO-K1 con RSV y posterior exposición a la BEA, se seleccionaron las concentraciones 2.5 y 5 μM de RSV y 1 y 5 μM BEA y se evaluó la producción intracelular de ROS y LPO. Los datos obtenidos mostraron que todas las concentraciones de RSV disminuyen la producción intracelular de ROS producidas por la BEA. La disminución fue del 92% al 39% con *trans*-RSV y del 92% al 27% con 70:30 *trans/cis*-RSV. Según algunos autores, este efecto podría deberse a la capacidad del RSV para secuestrar ROS, incrementar la actividad enzimática de las enzimas que metabolizan las ROS como la SOD y disminuir la actividad enzimática de enzimas que juegan un papel importante en la generación de ROS, como son las involucradas en la respiración y producción de ATP (Adam-Vizi y Chinopoulos, 2006; Carrizzo et al., 2013).

Asimismo, el pretratamiento con RSV disminuyó la producción de la LPO entre un 15% y un 37%, siendo más efectivo a bajas concentraciones el *trans*-RSV que el 70:30 *trans/cis*-RSV. Leonard et al. (2003) demostraron que el RSV inhibe la producción de LPO en células RAW 264.7 expuestas a radicales $OH^{\bullet}$ producidos por la reacción de Fenton. Además, el RSV disminuye la LPO inducida por H_2O_2 respecto del control en eritrocitos humanos (Mikstacka et al., 2010).

Estos resultados sugieren que el isómero *trans*-RSV y la mezcla de isómeros 70:30 *trans/cis*-RSV protegen a las células CHO-K1 de la citotoxicidad, la producción de ROS y la generación de LPO producidas por la BEA. Otros autores han observado citoprotección con polifenoles como la epigalocatequina-3-galato, la isorhamnetina, la quercetina, la quercetina-3-β-D-glucóxido, la rutina, la miricetina y el pterostilbeno frente a la citotoxidad, ROS y LPO producidos por diferentes micotoxinas en diversas líneas celulares (Costa et al., 2007; Chen y Chan, 2009; Choi et al., 2010; Sugiyama et al., 2011; Lombardi et al., 2012; Kalaiselvi et al., 2013).

4.8. Mitigación de BEA

La ingesta de alimentos es la principal fuente de exposición a la BEA. Los microorganismos probióticos y el consumo de ingredientes no digeribles como los prebióticos suponen un beneficio en la salud del consumidor/huésped. Por tanto, se evaluó la influencia de diferentes probióticos y prebióticos sobre la bioaccesibilidad de la BEA mediante un modelo de digestión estático *in vitro*, con el objetivo de proponer un mecanismo de mitigación de la BEA.

Los resultados obtenidos mostraron que los prebióticos usados en este estudio, fibras solubles (galactomanan, glucomanan de alto peso molecular y polvo fino, y β-glucano), insolubles (xilanos, celulosa de medio y alto peso molecular) y una mezcla de ambas (fibra de bambú, fibra de cítricos, fibra de zanahoria y fibra de guisante; Dhingra et al., 2012); con una concentración del 1% y del 5%, redujeron la bioaccesibilidad de la BEA entre un 60% y un 80%, siendo la celulosa de alto peso molecular (insoluble) a una concentración del 5% la que más redujo la bioaccesibilidad de la BEA y la fibra de zanahoria (soluble+insoluble) con una concentración del 1% la que menos. El hecho de que las soluciones con una concentración del 5% de fibra presenten una

mayor disminución de la bioaccesibilidad de la BEA, puede ser debido a que al aumentar la concentración de fibra aumenta la presencia de más sitios activos de estas para unirse a la BEA. Asimismo, la mayor efectividad en la reducción de la bioaccesibilidad de las fibras insolubles, como la celulosa y sus derivados, podría ser debido a una mayor dificultad de fermentación de este tipo de fibra en la fase colónica.

Los resultados obtenidos mostraron que todos los probióticos ensayados reducen la bioaccesibilidad de la BEA entre un 30% y un 85%, siendo el valor más alto y el más bajo en la reducción el de las bacterias *Lactobacillus rhamnosus* y *Bifidobacterium adolescentis*, respectivamente. Se puede suponer que este hecho es debido a la capacidad de los probióticos para reducir/unirse a compuestos tóxicos presentes en alimentos como pueden ser las micotoxinas. Asimismo, se observó que la bioaccesibilidad se redujo en 2, 2.5 y 3.1 veces respecto al control en la fase estomacal, duodenal y colónica, respectivamente. El hecho de que la mayor reducción se observe en la fase colónica podría ser debido a una mayor degradación de la BEA al aumentar el tiempo de exposición al probiótico o al efecto sinérgico entre los microorganismos probióticos empleados en el estudio con las bacterias presentes en la fase colónica.

La disminución de la bioaccesibilidad de la BEA y otras micotoxinas con el uso de probióticos y prebióticos ha sido previamente demostrada por otros autores (Tabla 15 y tabla 16).

Asimismo, se determinaron los productos de degradación de la BEA obtenidos tras la fermentación microbiana por LC-MS-LIT. Los resultados obtenidos tras la fermentación con *Lb. rhamnosus* mostraron un producto de la degradación de BEA que se identificó con la pérdida del ácido hidroxivalérico (HyLv) de la BEA. Este resultado se confirmó mediante la técnica EPI-MS y MS/MS/MS.

Por tanto, los resultados obtenidos demuestran que las fibras dietéticas y los probióticos utilizados reducen la bioaccesibilidad de la BEA en los tres compartimentos gastrointestinales ensayados. Estos resultados podrían utilizarse en estudios de desarrollo de alimentos que contengan probióticos y prebióticos con la finalidad de reducir la bioaccesibilidad de micotoxinas como la BEA.

5. Conclusiones

Conclusions

CONCLUSIONES

1. La revisión bibliográfica realizada pone de manifiesto que la información sobre la toxicidad de la beauvericina, esterigmatocistina y patulina es limitada, por lo que se deben realizar un mayor número de estudios que permitan una adecuada evaluación del riesgo.
2. La patulina, beauvericina y esterigmatocistina presentan citotoxicidad, de mayor a menor potencia en el orden indicado, en las células CHO-K1. La citotoxicidad aumenta con la concentración y el tiempo de exposición, con valores de IC_{50} desde 10.7±3.7 a 2.2±4.9 μM para la beauvericina; de 25.0±3.7 a 12.5±2.0 μM para la esterigmatocistina y de 2.9±0.3 a 2.8±0.4 μM para la patulina.
3. La combinación de beauvericina, esterigmatocistina y patulina presenta a dosis bajas efectos sinérgicos y a dosis altas aditivos en las células CHO-K1, siendo la combinación terciaria la más tóxica.
4. La beauvericina incrementa el estrés oxidativo en las células CHO-K1 mediante la producción de especies reactivas de oxígeno y peroxidación lipídica que aumenta con la concentración y el tiempo de exposición, observándose un mayor efecto a la concentración 5 μM de beauvericina.
5. El estrés oxidativo producido por la beauvericina en las células CHO-K1 causa una disminución del glutatión que se compensa con el incremento de las actividades enzimáticas antioxidantes superóxido dismutasa, catalasa, glutatión peroxidasa y glutatión transferasa.
6. La beauvericina causa daño en el DNA de las células CHO-K1, altera las membranas celulares produciendo disfunción mitocondrial; está implicada en la disrupción del ciclo celular y desencadena el proceso de apoptosis.

7. La N-acetilcisteína produce un incremento de los niveles de glutatión y actividades enzimáticas de la glutatión peroxidasa y glutatión transferasa en las células CHO-K1.

8. El pretratamiento con resveratrol protege a las células CHO-K1 de la citotoxicidad, peroxidación lipídica y especies reactivas de oxígeno generados por la BEA. La combinación simultánea de beauvericina y resveratrol no afecta a la capacidad antioxidante del resveratrol.
9. Las fibras dietéticas solubles e insolubles y probióticos como *Lactobacillus, Bifidobacterium, Saccharomyces, Corynebacterium y Eubacterium,* reducen la bioaccesibilidad de la beauvericina. Por tanto, pueden ser utilizados como un mecanismo de mitigación frente al daño producido por la beauvericina.
10. La presencia de micotoxinas en los alimentos es inevitable por lo que es necesaria la investigación toxicológica de estos compuestos de forma individual y combinada, para conocer los mecanismos de acción y los efectos tóxicos de las micotoxinas, así como para poder poner a punto nuevas estrategias de mitigación en los alimentos y piensos y disminuir el potencial tóxico que presentan para la salud humana y animal.

CONCLUSIONS

1. The literature review revealed that the information about the toxicity of these mycotoxins is limited, so the number of studies should be increased to permit a proper risk assessment.
2. Patulin, sterigmatocystin and beauvericin present cytotoxicity, from highest to lowest power in the order listed, in CHO-K1 cells, which increases with concentration and time of exposure. The IC_{50} values ranged from 10.7±3.7 to 2.2±4.9 μM; from 25.0±3.7 to 12.5±2.0 μM and from 2.9±0.3 to 2.8±0.4 μM for beauvericin, sterigmatocystin and patulina, respectively.
3. The combination of beauvericin, sterigmatocystin and patulina presents synergistic effects at low doses and additive effects at high doses in CHO-K1 cells, being the tertiary combination the most toxic. These effects can increase the toxic activity of mycotoxins combination.
4. Beauvericin increases oxidative stress in CHO-K1 cells through reactive oxygen species production, which increases with concentration and time of exposure and lipid peroxidation being the higher effect at 5 μM of beauvericin.
5. The oxidative stress produced by beauvericin in CHO-K1 cells decreases glutathione levels which is compensated by the increase of the antioxidant enzymatic activities superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase.
6. Beauvericin causes DNA damage in CHO-K1 cells, alters cell membranes producing mitochondrial dysfunction; which is involved in the disruption of the cell cycle and induces the process of apoptosis.
7. N-acetylcysteine increases glutathione levels and glutathione peroxidase and transferase activities in CHO-K1 cells.

8. Resveratrol pre-treatment protect CHO-K1 cells against beauvericin cytotoxicity, lipid peroxidation and reactive oxygen species. The combination of resveratrol and beauvericin does not affect the resveratrol antioxidant capacity.
9. The use of soluble and insoluble dietary fibers and probiotics such as, *Lactobacillus, Bifidobacterium*, *Saccharomyces*, *Corynebacterium* and *Eubacterium* reduce the bioavailability of beauvericin, so they can be used as a mitigation mechanism against beauvericin damage.
10. The presence of mycotoxins in food is inevitable so to perform toxicological investigations with these compounds individually and combined, for a better understanding of the mechanism of action and toxic effects and their interactions, considering new mitigation strategies to enhance risk assessment.

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Anexo I

Metodología

Índice metodología

METODOLOGÍA

El mantenimiento de las células CHO-K1 se llevó a cabo en placas de cultivo de poliestireno de 9 cm^2, a las que se adicionaron 5 mL de medio HAM's-F12 suplementado con 25 mM de tampón HEPES (pH 7.4), 10% de suero fetal bovino (FBS), 100 U/mL de penicilina, 100 mg/mL de estreptomicina y 0.5 mL/500 mL de fungizona. Las células se cultivan en monocapa a 37 ºC, con una atmósfera controlada con 5% de $C0_2$ y 95% de humedad relativa. Los subcultivos se realizaron (100% confluencia) mediante tripsinización y resuspensión en medio completo fresco en una proporción 1:3. El subcultivo se realizó rutinariamente y los ensayos se realizaron con un número pequeño de pases (<70 pases) con el fin de mantener la homogeneidad genética. Además, se comprueba la ausencia de micoplasma rutinariamente usando el kit Mycoplasma Stain (Sigma-Aldrich, St Louis, MO, EE.UU.).

1. Viabilidad celular

Las células CHO-K1 se cultivaron en placas de 96 pocillos en una densidad de $2x10^4$ células/pocillo con 200 µL medio/pocillo. El recuento celular se llevó a cabo con un contador Beckman coulter (Florida, USA). Una vez alcanzada una confluencia del 65%, el medio se elimina y sustituye por medio fresco con diferentes concentraciones (diluciones seriadas 1:2) de *trans*-RSV, 50:50 *trans*/*cis*-RSV, 70:30 *trans*/*cis*- RSV, ácido ascórbico y vitamina E (12.5-200 µM), BEA (0.625-20 µM), PAT (0.049-6.25 µM) y STE (1.56-75 µM); más un control con MeOH (1%, v/v) y se incuban a 37 ºC durante 24, 48 y 72 h. Posteriormente, la determinación de la viabilidad celular se lleva a cabo mediante el método del 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio (MTT), rojo neutro (NR; Ruiz et al., 2006), así como el contenido de proteínas totales.

Para llevar a cabo el ensayo MTT, se elimina el medio con las diferentes concentraciones de micotoxinas o antioxidantes y se añaden 200 µL de medio fresco con 50 µM de MTT por pocillo. Las placas se incuban a 37 ºC durante 4 h en oscuridad. Posteriormente, se elimina el medio y se lava con PBS, se añaden 200 µL de DMSO y 25 µL de tampón Sorensen's glicina para solubilizar las sales de formazan. La absorbancia se mide a 570 nm con un lector de placas automático (Multiskan Ex, Thermo Scientific, MA, EE.UU.). La determinación se llevó a cabo en tres ensayos independientes.

Para llevar a cabo el ensayo NR, se elimina el medio con las diferentes concentraciones de micotoxinas o antioxidantes y se añaden 200 µL de solución NR/pocillo (25mg/mL; pre-incubado a 37 ºC durante 24 horas y filtrado) y se incuban a 37 ºC durante 3 h en oscuridad. Posteriormente, se elimina la solución NR, y las células se fijan con solución de formaldehído-$CaCl_2$. Se extrae el NR mediante la adición de una solución de ácido acético/etanol (1:50) y tras 30 min a temperatura ambiente la absorbancia se mide a 540 nm con un lector de placas automático (Multiskan Ex, Thermo Scientific, MA, EEUU). La determinación se llevó a cabo en tres ensayos independientes.

El contenido de proteínas totales se determina mediante el método de Bradford, Bio-Rad DC Protein Assay (número de catálogo 500-0116). La absorbancia se mide a 690 nm con un lector de placas automático (Multiskan Ex, Thermo Scientific, MA, EEUU). La determinación se llevó a cabo en tres ensayos independientes.

La viabilidad celular se expresó en porcentaje en relación al control (1% de MeOH, v/v). Los valores de IC_{50} se obtuvieron a partir de las curvas dosis-respuesta.

1.1. Pretratamientos con antioxidantes

Para determinar el efecto citoprotector de antioxidantes frente al daño causado por la BEA se realizaron diferentes tipos de pretratamientos. Para llevarlos a cabo, las células fueron sembradas con medio con *trans*-RSV, 50:50 *trans*/*cis*-RSV y 70:30 *trans*/*cis*-RSV (1.25, 2.5 y 5 μM) y se incubaron a 37 ºC hasta alcanzar un 65 % de confluencia, posteriormente el medio con antioxidante fue reemplazado con BEA (0.1, 1 y 5 μM) y un control (MeoH 1%, v/v) y se incubaron a 37 ºC durante 24 h para posteriormente determinar la viabilidad celular mediante el método MTT de acuerdo a Ruiz et al. (2006), previamente descrito en el apartado 1.1.

2. Evaluación de los efectos de la combinación de micotoxinas: *Método del isobolograma*

Se sembraron $2x10^4$ células/pocillo en placas de 96 pocillos y se incubaron a 37 ºC hasta alcanzar un 65% de confluencia. Posteriormente, el medio se remplazó con diferentes diluciones (factor de dilución 1:2) de las combinaciones binarias y terciarias de las micotoxinas BEA, STE y PAT durante 24 h. Las concentraciones y las proporciones de las combinaciones seleccionadas, se eligieron en base a las IC_{50} obtenidas previamente para cada micotoxina de manera individual. Se ensayaron cuatro diluciones de cada una de las micotoxinas (STE 0.78, 1.56, 3.125 y 6.25 μM; BEA: 0.156, 0.3125, 0.625 y 1.25 μM y PAT: 0.049, 0.098, 0.195 y 0.39 μM) en una proporción de 5:1 (STE+PAT; STE+BEA), 3.2:1 (BEA+PAT) y 5:3.2:1 (BEA+STE+PAT). Se utilizó como control un 1% DMSO (v/v). Transcurrido el tiempo de exposición se llevó a cabo el ensayo MTT descrito previamente por Ruiz et al. (2006). La determinación se realizó en tres ensayos independientes.

Además, se realizó un cotratamiento de la BEA con *trans*-RSV y 70:30 *trans*/*cis*-RSV para determinar el tipo de interacción entre ambos. Para ello se

sembraron las células con medio fresco hasta alcanzar un 65% de confluencia, posteriormente el medio fue reemplazado por medio con BEA+70:30 *trans*/*cis*-RSV y BEA+*trans*-RSV en una proporción 1:1 con unas concentraciones (0.75, 1.25, 2.5, 5 μM) y se incubaron a 37 ºC durante 24 h. Se determinó la viabilidad celular mediante el método MTT descrito por Ruiz et al. (2006). La determinación se realizó en tres ensayos independientes.

El análisis de los resultados se realizó mediante el análisis del isobolograma, el cual permite determinar el tipo de interacción entre las diferentes combinaciones de micotoxinas mediante la ecuación: efecto medio/índice combinado (CI), descrito por Chou (2006) y Chou y Talalay (1984). Esta ecuación se basa en el principio que demuestra que existe una relación entre la dosis y el efecto, independientemente del número de sustancias y de los mecanismos de acción de cada una de ellas. Para llevar a cabo este método se realizaron las curvas dosis efecto para cada compuesto y sus combinaciones con diferentes diluciones de las concentraciones usando la ecuación:

$$\int a/\int u = (D/D_m)^m$$

Donde *D* es la concentración del producto, *Dm* es la dosis media efectiva que inhibe el 50% del crecimiento celular (ej. IC_{50}), $\int a$ es la fracción afectada por la concentración D (ej. Porcentaje de inhibición/100), $\int u$ es la fracción no afectada (por tanto, $\int a=1-\int u$) y *m* es la pendiente de la relación dosis-efecto, donde $m=1$, $m>$ y $m<1$ indica que la curva dosis-efecto es hiperbólica, sigmoidea y sigmoidea negativa, respectivamente. La ecuación de efecto medio para compuestos individuales puede ser modificada y aplicarse para múltiples micotoxinas, en este caso de dos compuestos la ecuación se transforma en:

$$[(\int a)_{1,2}/(\int u)_{1,2}]^{1/m} = D_1/(D_m)_1 + D_2/(D_m)_2 + (D)_1(D)_2/(D_m)_1(D_m)_2$$

Chou y Talalay (1984) introdujeron el término CI para determinar si la interacción se debe a sinergismo, antagonismo o adición entre los dos compuestos.

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

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

$CI=(D)_1/(D_m)_1[fa/(1-fa)]^{1/m1}+(D)_2/(D_m)^2[fa/(1-fa)]^{1/m2}$

Donde $(Dx)_1$ se refiere a D_1 individualmente; y que inhibe un x% del sistema, y $(Dx)_2$ a D_2 individualmente y que inhibe un x% del sistema. La ecuación general para una combinación de compuestos n con un x% de inhibición es:

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

Donde (CI) es el índice de combinación para n compuestos (ej. Micotoxinas) con x% de inhibición (ej. Inhibición de la proliferación); $(Dx)_{1-n}$ es la suma de los n compuestos que ejercen un x% de la inhibición de la combinación, {[Dj]/Σn1[D]} es la proporción de la concentración de cada uno de los compuesto que ejerce el x% de inhibición en combinación y (Dm)j {(fax)j/[1−(fax)j]}1/mj es la concentración de cada compuesto que ejerce el x% de inhibición individualmente. CI< 1, =1 y >1 indican efecto sinérgico, aditivo y antagónico, respectivamente. Los tipos de interacción producidos por la BEA, PAT y STE fueron evaluados automáticamente mediante el software CalculSyn versión 2.1 Biosoft, Cambridge, UK, 1996–2007.

3. Evaluación del ciclo celular

El análisis del ciclo celular fue llevado a cabo mediante la tinción de DNA con PI y su detección con citometría de flujo de acuerdo a Juan-García et al. (2013b). Se sembraron $6.8x10^5$ células/pocillo en placas de 6 pocillos y se incubaron a 37 ºC hasta alcanzar el 65% de confluencia, una vez llegado a este punto, el medio fue remplazado con medio fresco con BEA (0.1, 1 y 5 μM) más un control (MeOH 1%, v/v) durante 24, 48 y 72 h. Posteriormente, las células fueron recogidas e incubadas en hielo durante 30 min con 860 μL de medio fresco con 29 ng/mL de solución de tinción de PI Vindelov (Tris 0.01 M, de PI 0.05 mg/mL, nonidet P-40 al 0.1%, 10 mg de RNAsa (700 U/L) y NaCl 10 mM). Se analizaron al menos 10000 células por muestra con una longitud de onda de excitación y emisión de 488 y 620 nm, respectivamente, mediante el citómetro de flujo (BD FACS Canto y el software FACSDiva software v 6.1.). La determinación se llevó a cabo en cuatro ensayos independientes.

4. Determinación de especies reactivas de oxígeno

La determinación de la generación de especies reactivas de oxígeno intracelulares se realizó mediante la adición de $DCFH_2$-DA según Ruiz-Leal y George (2004). Se sembraron $3.4x10^4$ células/pocillo en placas negras de 96 pocillos y se incubaron a 37 ºC durante 24 h. Se eliminó el medio y se adicionaron 20 μM de $DCFH_2$-DA durante 20 min. Después, se eliminó el medio con $DCFH_2$-DA y se lavaron las placas con PBS antes de adicionar la BEA (1 y 5 μM) más un control con MeOH (1%, v/v). La intensidad de fluorescencia se midió en un lector de placas (Wallace Victor2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) a intervalos, de 0 hasta 120 min, con una longitud de onda de excitación y emisión de 485 y 535 nm, respectivamente. Los resultados se expresaron como aumento de la fluorescencia respecto al

control (1% de MeOH). La determinación se realizó en tres ensayos independientes.

Para determinar el efecto citoprotector del RSV frente a las ROS generadas por la BEA, se realizó el mismo protocolo que para la BEA (1 y 5 μM). Las células con *trans*-RSV y 70:30 *trans*/*cis*-RSV (2.5 y 5 μM) se incubaron a 37 ºC hasta alcanzar un 65 % de confluencia, una vez llegado a este punto, el medio fue remplazado con medio con BEA (1 y 5 μM) y un control (MeoH; 1%, v/v) y se incubaron a 37 ºC durante 24 h. Transcurrido este periodo se determinó la generación de ROS mediante el método descrito previamente por Ruiz-Leal y George (2004).

5. Determinación de la peroxidación lipídica

La determinación de la LPO se llevó a cabo mediante el método de las TBARS, conforme a Buege y Aust (1978). Para llevar a cabo este método, se sembraron $6.8x10^5$ células/pocillo en placas de 6 pocillos y se incubaron a 37 ºC hasta alcanzar un 65% de confluencia. El medio fue remplazado con medio con BEA (1 y 5 μM) más un control con MeOH (1%, v/v) y se incubó durante 24 h. Tras este período, el medio se retiró, se lavaron las células con PBS, se recogieron con 20mM Tris y 0.1% Triton y se homogeneizaron con un UltraTurrax T8 (IKA-Werke GmbH & Co. KG, Germany). Al homogenizado celular se le adicionaron 1 mL TBA al 0.5%, 5 μL de desferroxamina (DFA) 1.5 mM y 5 μL de butil hidroxitolueno (BHT) al 3.75% y se llevó a ebullición (100 ºC) durante 20 min. Inmediatamente, las muestras se enfriaron en hielo y centrifugaron (1287 g, durante 15 min). La absorbancia fue determinada a 532 nm (Perkin Elmer UV/Vis Lambda 2 versión 5,1). Los resultados fueron expresados como ng de MDA/mg de proteína. La determinación se realizó en cuatro ensayos independientes.

Para determinar el efecto protector del RSV, trans-RSV y 70:30 trans/cis-RSV (2.5 y 5 μM) se incubaron las células CHO-K1 con este a 37ºC hasta el 65% de confluencia. A continuación, se eliminó y se añadió la BEA (1 y 5 μM) y tras 24 h de exposición se determinó la LPO del mismo modo.

6. Determinación de la muerte celular

La diferencia de la población celular en células apoptóticas (tempranas o tardías), necróticas y viables fueron identificadas por citometría de flujo según el método de Vermes et al. (1995). Para determinar la muerte celular se sembraron $6.8x10^5$ células/pocillo en placas de 6 pocillos y una vez alcanzada la confluencia se expusieron a BEA (0.1, 1 y 5 uM) más un control con MeOH (1%, v/v), durante 24, 48 y 72 h. Se eliminó el medio con micotoxina, se tripsinizaron las células y se resuspendieron en 400 μL de tampón de unión a Anexina V (10 mM HEPES, 135 mM de NaCl, 2.5 mM de $CaCl_2$). Se incubaron en hielo y oscuridad durante 30 min con 1.25 ng/mL de Anexina V-FITC y 10 μg/mL de PI. 10000 células fueron analizadas por citometría de flujo (BD FACS Canto citómetro de flujo con el software FACSDiva software v 6.1.). Las células apoptóticas muestran fluorescencia verde (FL-1, 530 nm), las muertas fluorescencia roja (FL-2, 585 nm) y verde; y las vivas poca o ninguna fluorescencia. Se estudió la distribución de las poblaciones celulares. Las células posicionadas en el cuadrante Anexina V-FITC -/PI +, pertenecen a células necróticas, las del cuadrante Anexina V-FITC +/PI -, pertenecen a células en apoptosis temprana, las del cuadrante Anexina V-FITC +/PI +, pertenecen a células apoptosis/necrosis o apoptosis tardía y las del cuadrante Anexina V-FITC -/PI -, pertenecen a células vivas (Fig. 1). Las determinaciones se realizaron en cuatro ensayos independientes.

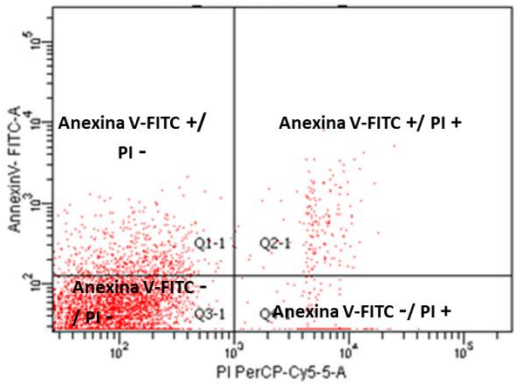

Figura 1. Distribución celular en gráfica de puntos obtenida mediante citometría de flujo con tinción Anexina V-FITC/PI.

7. Determinación del potencial de membrana mitocondrial

El potencial de membrana mitocondrial ($\Delta\Psi m$) fue determinado usando el método de la Rh123 descrito por Andersson et al. (1987) con algunas modificaciones. Para ello, se sembraron placas negras de 96 pocillos con $2x10^4$ células/pocillo y se incubaron hasta alcanzar el 65% de confluencia. Una vez alcanzada la confluencia, las células fueron expuestas a BEA (0.1, 1 and 5 µM) y el control con (MeOH 1%, v/v), durante 24 h. A continuación, se adicionó 5 µM de Rh123 durante 15 min en oscuridad. Posteriormente, la Rh123 fue eliminada y las células se resuspendieron en PBS. La intensidad de fluorescencia fue medida con una longitud de onda de emisión y excitación de 485 y 530 nm, respectivamente. Los resultados fueron expresados como porcentaje (%) respecto del control (1% de MeOH). La determinación se realizó en tres ensayos independientes.

8. Ensayo del cometa alcalino

El daño al DNA fue determinado mediante el ensayo del cometa de acuerdo con Prosperini et al. (2013b) con algunas modificaciones. Se

sembraron $3.4x10^5$ células por pocillo en placas de 6 pocillos, una vez alcanzada la confluencia se remplazó el medio por medio con BEA (0.1, 1 y 5 µM) y control (MeOH 1%, v/v) y se incubaron a 37 ºC durante 24 h. Transcurrido el tiempo de exposición, $2x10^4$ células fueron resuspendidas en 160 µL de agarosa de bajo punto de fusión (LMP, 0.5 % PBS) precalentada a 37 ºC; 80 µL de esta suspensión fueron transferidos a un portaobjetos previamente gelificado con agarosa (0.8% PBS) y fue cubierto con un cubreobjetos (24x36 mm) y mantenido 10 min a 4 ºC. Una vez gelificado se eliminó el cubreobjetos y se añadieron 80 µL de LPM y se gelificó (10 min a 4 ºC) para formar así una tercera capa. Una vez gelificado, el cubreobjetos fue eliminado y los portaobjetos fueron sumergidos en 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 preparada) durante 1 h a temperatura ambiente. Los portaobjetos fueron lavados con tampón de neutralización (Tris 0.4M a pH 7.5) y estabilizados en tampón de electroforesis (NaOH 0.3 M y EDTA 1mM, a pH 13.6) durante 40 min a temperatura ambiente, lo que permite desenrollar las cadenas de DNA. La electroforesis fue llevada a cabo a 0.7 V/cm (300 mA) durante 24 min a temperatura ambiente. Finalmente, los portaobjetos fueron lavados dos veces con tampón de neutralización y fijados con etanol 96% a -20 ºC durante 5 min. Se adicionó PI (20 µg/mL de H_2O destilada) y se dejaron secar los portaobjetos durante una noche. La visualización de los cometas se llevó a cabo mediante un microscopio de fluorescencia (Nikon Eclipse E800) equipado con una cámara (NIKON DXM1200F) para la captura de imágenes. Al menos 50 células individuales/portaobjeto fueron seleccionadas al azar para analizar el daño al DNA mediante el software CometScore (Automatic Comet Assay by TriTek CometScoreTM; http://autocomet.com/index.php?id=cometscorepro). Se utilizó benzo-(α)-pireno (B(α)p; 15 µM) como control positivo. Los resultados fueron

expresados como porcentaje de DNA en la cola (%), calculado de acuerdo a la ecuación:

% DNA en cola= (intensidad total en la cola/intensidad total del cometa) x100

Siendo la intensidad total de fluorescencia del cometa igual a la longitud de la cabeza más la longitud de la cola. Las determinaciones se realizaron en cuatro ensayos independientes.

9. Determinación de glutatión y enzimas relacionadas

La determinación del glutatión reducido (GSH), GPx, GR y GST se llevó a cabo según Maran et al. (2009). Para ello, se sembraron placas de 6 pocillos con $3.4x10^5$ células/pocillo. Después de alcanzar el 65% de confluencia, el medio fue remplazado por medio con diferentes concentraciones de BEA (0.1, 1 y 5 μM) y un control con MeOH (1%, v/v) y se incubaron a 37 ºC durante 24 h. Posteriormente, el medio fue eliminado y las células fueron lavadas con PBS y homogeneizadas en 0.5 mL de 20 mM Tris y 0.1 Triton.

Para determinar el efecto de la N-acetilcisteína (NAC) sobre el GSH y enzimas relacionadas expuestas a BEA, se realizó el mismo protocolo que para la BEA (0.1, 1 y 5 μM). Para ello se sembraron las células con NAC (1 mM) y un control (MeOH; 1%, v/v), se incubaron a 37 ºC hasta alcanzar un 65 % de confluencia y después se remplazó el medio con NAC por medio con BEA (0.1, 1 y 5 μM) y se incubaron a 37 ºC durante 24 h. Posteriormente, se determinó el contenido en GSH y la actividad GPx, GR y GST de acuerdo a Maran et al. (2009).

9.1. Cuantificación niveles de glutatión

Para determinar los niveles de glutatión, se adicionaron 10 μL de homogenizado celular, 200 μL de tampón GSH (Na_2HPO4 0,1M-EDTA 5 mM) a

pH 8 y 10 µL de o-ftaldialdehído (OPT) por pocillo en una placa negra de 96 pocillos; se incubaron a temperatura ambiente durante 15 min en oscuridad. La concentración de GSH se determinó en un lector de placa multipocillos (Wallace Victor2, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland) con una longitud de onda de emisión y excitación de 345 nm y 425 nm, respectivamente. Los niveles de GSH fueron expresados como µg de GSH/mg de proteína. La determinación se realizó en cuatro ensayos independientes.

9.2. Determinación de actividad glutatión peroxidasa y reductasa

La actividad enzimática de la GPx está acoplada con la GR, ya que el glutatión oxidado (GSSG) producido tras la reducción de hidroperóxidos por la GPx, se recicla a su estado reducido por la GR mediante la oxidación de NADPH a NADP con una disminución de la absorbancia a 340 nm, la cual es proporcional a la actividad GPx. La actividad GPx fue ensayada espectrofotométricamente usando el H_2O_2 como sustrato de la GPx dependiente de selenio, siguiendo la oxidación del NADPH a 340 nm durante dos minutos en una reacción enzimática acoplada con la GR, descrita por Maran et al. (2009). En un volumen final de 1 mL, se adicionaron 500 µL de tampón fosfato 0.1 M (pH 7.5, 1 mM EDTA y 2 mM NaN_3, 0.1% Triton X-100), 250 µL de agua ultrapura, 100 µL de GSH 20 mM, 20 µL de NADPH 0.2 mM, 2.5 U de GR recién preparada y 50 µL de H_2O_2 5 mM. Cincuenta microlitos de homogeneizado celular fueron añadidos a la mezcla de reacción. Una unidad de GPx reduce 1 µmol de GSSG por minuto a pH 7.5. El ensayo se llevó a cabo a 25 ºC durante dos minutos en termocirculacion (Perkin Elmer UV/vis spectrometer Lambda 2 version 5.1.)

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPx} R\text{-}O\text{-}H + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

La actividad GR fue determinada siguiendo la oxidación del NADPH a NADP durante la reducción del GSSG. El método se realizó de acuerdo a Maran et al. (2009). Brevemente, en un volumen final de 1 mL: 800 µL de tampón fosfato 0.1M (pH 7 con 5 mM EDTA), 10 µL de NADPH 10 mM en tampón fosfato y 90 µL de homogenizado celular. La reacción comienza con la adición de 100 µL de GSSG. El descenso de la absorbancia fue monitorizado a 340 nm durante 4 min a 25 ºC en termocirculación (Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1.).

Las actividades GPx y GR fueron calculadas usando el coeficiente de extinción molar del NADPH (6.22 mM^{-1} cm^{-1}) y expresadas como nmol de NADPH oxidado/min/mg de proteína. Las determinaciones se realizaron en cuatro ensayos independientes.

9.3. Determinación actividad glutatión transferasa

La familia enzimática GST juega un papel importante en la detoxificación celular, para ello, se conjuga con GSH, neutralizando así compuestos tóxicos. La actividad GST se determinó mediante la medida de la conjugación de 1-cloro-2,4-dinitrobenceno (CDNB) con GSH. Esta conjugación es acompañada por un incremento de la absorbancia a 340 nm, la cual es proporcional a la actividad GST; realizado de acuerdo a Maran et al. (2009). La mezcla de reacción contiene en un volumen final de 1 mL: 825 µL de tampón fosfato 0.1 M (pH 6.5 con 0.5 mM EDTA y 0.1% Triton X-100); 25 µL de CDNB 50 mM disuelto en etanol, 100 µL de GSH 25 mM y 50 µL de homogeneizado

celular. La actividad enzimática se determinó a 25 ºC en termocirculación (Perkin Elmer UV/Vis spectrometer Lambda 2 version 5.1) durante 3 minutos. La actividad GST fue calculada usando el coeficiente de extinción molar del CDNB (9.6 mM^{-1} cm^{-1}) y expresada como nmol de producto formado/min/mg de proteína. La determinación se realizó en cuatro ensayos independientes.

10. Determinación actividad catalasa y superóxido dismutasa

Para determinar la actividad CAT y SOD, se sembraron $6.8x10^5$ células/pocillo en placa de 6 pocillos y se incubaron a 37 ºC. Una vez alcanzado el 65% de confluencia, se remplazó el medio con medio con BEA (0.1, 1 y 5 µM) y un control (MeOH; 1%, v/v) y se incubaron a 37 ºC durante 24 h. El medio fue eliminado y las células fueron recogidas con 0.02 M NaH_2PO_4 pH 7.4 y homogeneizadas con UltraTurrax T8 (IKA-Werke GmbH & Co. KG, Germany). La actividad SOD fue determinada con el kit Ransod (Randox Laboratories, United Kingdom) adaptado a cubetas de 1.5 mL. La actividad SOD se monitorizó a 505 nm y 37 ºC en termocirculación (PerkinElmer UV–vis spectrometer Lambda 2 version 5.1) durante 3 min. La actividad enzimática se expresó como porcentaje (%) respecto del control (1% MeOH, v/v). Las determinaciones se realizaron en cuatro ensayos independientes.

$$2O_2^{\bullet-} + 2H^+ + SOD \xrightarrow{SOD} H_2O_2 + O_2$$

La enzima CAT cataliza la descomposición de H_2O_2 en agua y oxígeno. La actividad CAT fue medida según Espín et al. (2014) con pequeñas modificaciones. Brevemente: 50 µL de homogeneizado celular fue mezclado con 950 µL de NaH_2PO_4 0.05 M y 500 µL de H_2O_2 0.03M. La actividad

enzimática de la CAT se determinó mediante el seguimiento del descenso de H_2O_2 a 240 nm durante 2 min con un espectrofotómetro (PerkinElmer UV–vis spectrometer Lambda 2 version 5.1). La actividad CAT fue calculada utilizando el coeficiente de extinción molar del H_2O_2 y expresada como porcentaje respecto del control (%). La determinación fue realizada mediante cuatro ensayos independientes.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

11. Evaluación de la influencia de probióticos y prebióticos en la bioaccesibilidad de la beauvericina

11.1. Digestión gastrointestinal *in vitro* estática

El análisis de la bioaccesibilidad se llevó a cabo mediante un modelo de digestión *in vitro* estática diseñado por Gil-Izquierdo et al. (2002), con algunas modificaciones. La solución modelo utilizada en este estudio reproduce una matriz alimentaria compuesta por glucosa (1%), almidón (5%), proteínas (3%), NaCl (0.05%) y agua. La solución modelo con fibras fue preparada en un Erlenmeyer de 100 mL en el cual se adicionaron 1 y 5 g de fibra para cada muestra. Para la evaluación de la influencia de probióticos la solución modelo fue inoculada con $2x10^6$ UFC para cada bacteria testada. Las soluciones modelo fueron mezcladas en un baño con ultrasonido (Lab Police, Barcelona, Spain) a 30 ºC de temperatura y contaminadas con 10 mg/mL de BEA, utilizando una solución madre de BEA de 1 g/L disuelta en MeOH. La simulación de la digestión *in vitro* se realizó en diferentes pasos secuenciales (Fig. 2): el primer paso consiste en la simulación del compartimento de la boca mediante la adición de 10 mL de solución modelo y 6 mL de saliva artificial (89.6 g/L de KCl, 20 g/L KSCN, 88.8 g/L de NaH_2PO_4, 57 g/L de $NaSO_4$, 175.3 g/L de NaCl, 84.7 g/L de $NaHCO_3$, 25 g/L de urea, y 290 mg de α-amilasa), el

pH se llevó a 6.8 con NaOH 0.1 N. La mezcla se homogeneizó en una bolsa de plástico con 40 mL de agua homogeneizada mediante un Stomacher (IUL Instruments; Barcelona, Spain) durante 30 s. El siguiente paso fue la simulación del compartimento gástrico mediante una digestión con pepsina/HCl que permite la digestión de proteínas, para ello, se adicionaron 0.5 g de pepsina (14800 U) disuelta en 25 mL de HCl 0.1 N, a la mezcla anterior. El pH de la mezcla se llevó a 2 con HCl 6 N y se incubó a 37 ºC durante 2 h en un agitador orbital Infors AG CH-4103, Bottmingen, Suiza) a 250 rpm. Transcurrida la fase gástrica, se procedió a la simulación de la fase de digestión en el intestino delgado, para ello, primero se incrementó el pH a 6.5 con $NaHCO_3$ (0.5 N) y se adicionaron los jugos correspondientes a esta fase en una proporción (1:1; v/v), 5 mL de pancreatina (8 mg/mL) y 5 mL de sales biliares (50 mg/mL) disueltas en 20 mL de agua; después se incubaron a 37 ºC en un agitador orbital a 250 rpm durante 2 h. Finalmente, para simular el compartimento colónico se cultivaron bacterias propias del tracto intestinal en tubos de plástico estériles durante una noche a 37 ºC en medio MRS (Oxoid, Madrid, España) bajo condiciones de anaerobiosis (5% CO_2/95% de aire) en un recipiente cerrado con Anaerocult® A. Posteriormente, los tubos fueron centrifugados a 4000 rpm durante 5 min a 23 ºC y las bacterias fueron resuspendidas en PBS estéril; 500 µL de una mezcla de alícuotas de las suspensiones bacterianas a una concentración de 10^{14} UFC/mL fueron añadidas al fluido obtenido en el compartimento duodenal e incubado en una estufa a 37 ºC, 5% de $C0_2$/95% de aire durante 48 h. Después de la digestión, 5 mL de este fluido fue centrifugado a 4000 rpm durante 10 min a 4 ºC para la posterior extracción (Fig. 2).

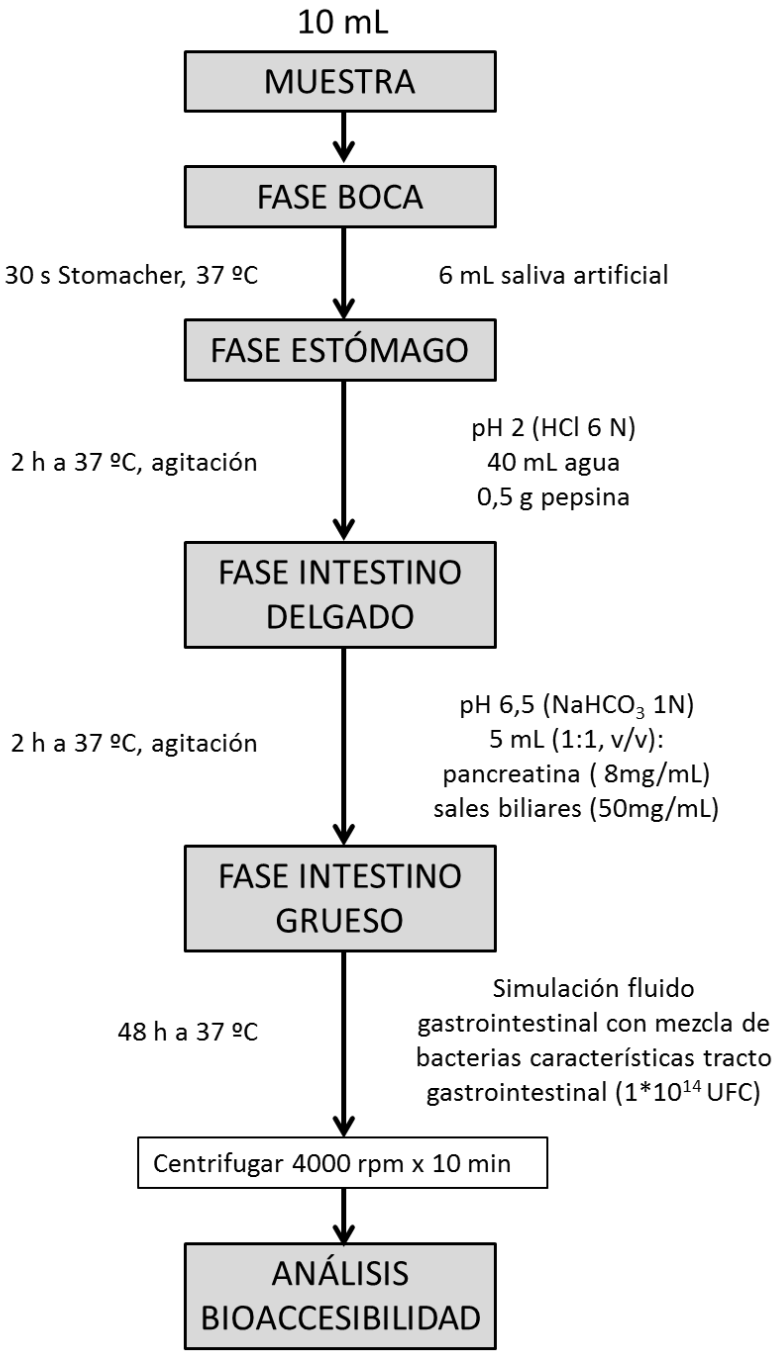

Figura 2. Esquema del proceso de digestión estática *in vitro*.

11.2. Extracción de la beauvericina y sus productos de degradación en fluidos intestinales simulados

La extracción del contenido de BEA de los fluidos gástricos, gástricos+duodenales, y gástricos+duodenales+colónicos se llevó a cabo según Meca y col. (2012c). Para realizar la extracción, se recogieron 5 mL de cada uno de los fluidos obtenidos después de la digestión en tubos de 20 mL, se extrajo 3 veces con acetato de etilo y se agitató durante un min con un vortex (VWR international, Barcelona, España). La mezcla fue centrifugada a 4000 rpm y 4 ºC durante 10 min. La fase orgánica fue evaporada completamente en un evaporador rotatorio (Büchi, Suiza) a 30 ºC y 30 mbar de presión. Una vez evaporada, las muestras se resuspendieron en 1 mL de MeOH y fueron filtradas con un filtro de 0.22 µM (Phenomenex, Madrid, España) antes de ser analizadas por LC-MS/MS.

11.3. Determinación de la beauvericina y sus productos de degradación por LC-MS-LIT

La determinación de la BEA y sus productos de degradación se llevó a cabo mediante cromatografía líquida (CL; Agilent 1100; Agilent Technologies, Santa Clara, California) acoplada a un espectrómetro de masas de trampa de iones (LC-MS-LIT; Applied Biosystems / MDS Sciex Q; Concord, Ontario, Canadá). Para ello, se utilizó una columna analítica Gemini (150 mm x 2 mm, 5 µM) Phenomenex (Madrid, España). Para la determinación de la BEA se usó un flujo constante a 0.2 mL/min y acetonitrilo: agua (70:30; v/v) con 0.1 % de ácido fórmico como fase móvil bajo condición isocrática. Para la determinación de los productos de degradación se usó un flujo constante de 0.3 mL/min y acetonitrilo: agua (70:30; v/v) con 0.1 % de ácido fórmico como fase móvil en condiciones isocráticas. El instrumento se configuró en modo de ionización positiva con los siguientes parámetros: un voltaje de cono de 40 V,

tensión capilar de 3.80 kV, temperatura de 350 ºC, temperatura de desolvatación de 270 ºC y energía de colisión de 5 eV. La monitorización de la reacción múltiple (MRM) se utilizó para la identificación y cuantificación, en la cual las moléculas protonadas [M + H] + de BEA m/z 784.50, fueron fragmentadas en la colisión a iones producto m/z 244.2. Para la cuantificación, se usó el ion producto m/z 191.20 de acuerdo a (Meca et al., 2012c).

El análisis de los productos de degradación de la BEA se realizó mediante LC-MS-LIT, siguiendo el siguiente procedimiento:

- Caracterización de los compuestos aislados mediante la modalidad de escaneo de mejora de resolución (ER), usando el rango de m/z desde 200 hasta 900 Da para obtener el espectro general de degradación del compuesto.
- Caracterización de los fragmentos obtenidos en el escaneo ER con la modalidad de escaneo de mejora de producción de iones (EPI) para obtener un MS^2 de la degradación de los productos fragmentados.
- Estudio de los fragmentos obtenidos en la modalidad EPI empleando la modalidad MS/MS/MS que permitió conocer la MS^3 de los fragmentos seleccionados en el escaneo ER. Para obtener el espectro de MS^3 de los productos de degradación de la BEA se usó como señal de referencia los iones m/z 701.1 y 503.2.

La utilización de la LC-MS-LIT, usada en estas dos modalidades, permite obtener una caracterización total del compuesto aislado (Meca et al., 2012c).

12. Cuantificación y capacidad antioxidante del resveratrol en suplementos alimenticios

12.1. Extracción de resveratrol

Para llevar a cabo la extracción del contenido de RSV en cada muestra, se pesaron 0.30 g de cada muestra y se pusieron en un tubo con 5 mL de MeOH, se agitó con vortex cada 5 min durante 15 min y posteriormente se centrifugaron a 5000 rmp durante 5 min. Se recogió el sobrenadante y se introdujo en un frasco de 20 mL. Este procedimiento se realizó cuatro veces. Posteriormente, el extracto fue llevado a 20 mL con MeOH.

12.2. Determinación de resveratrol por electroforesis capilar de alta resolución

El análisis de las muestras se realizó mediante un sistema de electroforesis capilar de alta resolución P/ACE 2100 MDQ (Beckman Instruments, USA) equipado con un detector de diodo. La separación se obtuvo por un sistema capilar de sílice fundido de 75 µm de diámetro interno y 57 cm de longitud total con una ventana de detección de 100 µm x 800 µm. El capilar se mantuvo todo el tiempo de análisis a 25 ºC, para evitar variabilidad en el análisis. El capilar fue condicionado antes del uso con lavados de NaOH 0.1 M durante 1 min, posteriormente con agua y finalmente con el tampón de tetraborato de sodio ($Na_2B_4O_7$) 20 mM y PEG 400 50 Mm con un 10% de MeOH adicionado en el momento de uso durante 3 min a 20 psi. La muestra fue inyectada dentro del capilar hidrodinámicamente con una presión de 20 psi durante 5 s. Para la determinación se utilizó un estándar de RSV de 50 ppm. La separación se realizó a 25 kV, 25 ºC y 315 nm durante 15 min. Todos los análisis fueron realizados mediante tres ensayos independientes. Después de cada análisis, el capilar se enjuago secuencialmente con NaOH 0.1 M durante 2 min y con tampón de análisis durante 3 min.

12.3. Capacidad antioxidante

La capacidad antioxidante de los diferentes suplementos de resveratrol y de la BEA fue determinada usando la técnica de la fotoquimioluminiscencia, llamada ensayo luminol PLC. La determinación se llevó a cabo utilizando el equipo Photochem® con un ACL kit (Analytikjena, Jena, Alemania), de acuerdo con el método descrito por Popov y Lewin (1999). Primero, se añadieron 2-3 mL del reactivo 1 (reactivo de solvente y dilución), posteriormente se añadieron 200 µL del reactivo 2 (solución tampón), 25 µL del reactivo 3 (fotosensibilizador, luminol 1 mmol/L) y 10 µL de solución o estándar, se mezclaron y midieron. Trolox fue usado para realizar la curva de calibrado (0.5-2 nM). La curva de emisión de luz se midió a λmax=350nm durante 180 s utilizando la inhibición del radical anión superóxido como parámetro para evaluar el efecto antioxidante. A continuación, la capacidad antioxidante se determinó mediante el uso de la integral bajo la curva y los resultados se expresaron como µmol de Trolox equivalentes (TES) / g. La determinación de la capacidad antioxidante de los suplementos de RSV se realizó sustituyendo el estándar con muestras diluidas de las cuales se realizaron 6 réplicas por muestra.

Anexo II

Difusión de Resultados

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Mechanisms of beauvericin toxicity and antioxidant cellular defense

Beatriz Mallebrera*, Ana Juan-Garcia, Guillermina Font, Maria-Jose Ruiz

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

HIGHLIGHTS

- BEA produces cytotoxic effects in CHO-K1 cells.
- BEA alters mitochondrial membrane potential in CHO-K1 cells.
- BEA produces DNA strand breakage in CHO-K1 cells.
- At 24 h, BEA exposure arrests G_0/G_1 phase of cell cycle and produces apoptosis.
- Enzymatic defense (SOD and CAT) increases in CHO-K1 cells exposed to BEA.

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ABSTRACT

Beauvericin (BEA) is a secondary metabolite produced by many species of fungus *Fusarium*. This study determines the injury (cell viability, cell proliferation, mitochondrial membrane potential, cell death and DNA damage) and the intracellular defense mechanisms (catalase and superoxide dismutase) in Chinese Hamster ovary (CHO-K1) cells after BEA exposure. The results obtained in this study demonstrated that BEA induces cytotoxicity in a dose- and time-dependent manner in CHO-K1 cells. Moreover, disruption in mitochondrial enzymatic activity and cell proliferation has been observed after BEA exposure, which can lead or be consequence of cell death. BEA inhibits cell proliferation by arresting cells in G_0/G_1 and increasing apoptosis. Moreover, at higher exposure times, BEA induces differentiation of CHO-K1 cells through G_2/M arrest, preventing that cells entry into mitosis. DNA strand breaks were observed at 1 μM after 24 h of exposure. On the other hand, the SOD and CAT activities were increased after BEA exposure and as a defense system they could contribute to eliminate damage produced by BEA and oxidants products generated in CHO-K1 cells.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi. They can damage health in humans and animals through ingestion, inhalation or the skin contact (Marin et al., 2013). Emerging *Fusarium* mycotoxins include fusaproliferin, enniatins, beauvericin and moniliformin.

Beauvericin (BEA) is synthesized by many species of fungus *Fusarium* and *Beauveria bassiana*. Wheat, rice, corn, barley and cereal derivate are known as susceptible commodities to be contaminated by BEA (Meca et al., 2010; Mahnine et al., 2011; Zinedine et al., 2011). BEA has ionophoric activity, it is incorporate into biological membranes forming a complex with essential cations, which increases its ion permeability and affects cellular homeostasis (Kouri et al., 2003; Tonshin et al., 2010). Therefore, the mitochondria membrane may be one of the possible sites for BEA-mediated cytotoxicity. Reactive oxygen species (ROS) production in excess of the capabilities of detoxication systems causes oxidative stress in the cell. ROS are extremely reactive, making them likely to participate in chemical reactions. These reactions also damage lipids, proteins and DNA and cause deleterious effects (Kanduc et al., 2002). Oxidative reactions in mitochondria could induce its dysfunction which activates the apoptotic and necrotic pathways (Tonshin et al., 2010; Prosperini et al., 2013a). ROS can also damage DNA causing single-strand breaks and base alteration inducing DNA adducts and mutation.

Because of the propensity of ROS to react with and damage important bimolecules, cells have evolved biochemical systems for detoxication. Antioxidant compounds (such as glutathione, and vitamins and polyphenols ingested in diet) and enzymes (glutathione peroxidase, GPx; catalase, CAT and superoxide

* Corresponding author.
E-mail address: beatriz.mallebrera@uv.es (B. Mallebrera).

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Cytotoxic effects induced by patulin, sterigmatocystin and beauvericin on CHO–K1 cells

Nidhal Zouaoui [a, b, 1], Beatriz Mallebrera [c, 1], Houda Berrada [c], Salwa Abid-Essefi [a], Hassen Bacha [a], Maria-Jose Ruiz [c, *]

[a] Laboratory for Research on Biologically Compatible Compounds (LRSBC), Faculty of Dentistry, Rue Avicenne, 5019, Monastir, Tunisia
[b] Faculty of Sciences of Bizerte, University of Carthage, 7021, Jarzouna, Tunisia
[c] Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vincent Andrés Estelles, 46100, Burjassot, Valencia, Spain

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ABSTRACT

Mycotoxins are produced by different genera of fungi; mainly *Aspergillus*, *Penicillium* and *Fusarium*. The natural co-occurrence of beauvericin (BEA), patulin (PAT) and sterigmatocystin (STE) has been proved in feed and food commodities. This study investigates the cytotoxicity of individual and combined mycotoxins BEA, PAT and STE. The cytotoxicity on immortalized ovarian cells (CHO–K1) was evaluated using the MTT assay. After 24, 48 and 72 h, the IC_{50} values were 2.9 μM for PAT and ranged from 10.7 to 2.2 μM and from 25.0 to 12.5 μM for BEA and STE, respectively. Cytotoxic interactions were assayed by the isobologram method, which provides a combination index (CI) value as a quantitative measure of the three mycotoxin interaction's degree. Binary and tertiary combinations showed a dose dependent effect. At low fraction affected, mycotoxin combinations were synergetic; whereas, at higher fraction affected, the combinations showed additive effect. Our results indicate that the co-occurrence of low concentrations of mycotoxin in food may increase their toxic effects.

1. Introduction

Mycotoxins are fungal secondary metabolites. The same fungi can produce different mycotoxins and the same mycotoxin can be produced by different species of fungi (Bottalico and Perrone, 2002). The mycotoxin beauvericin (BEA) is mainly produced by *Fusarium* species, patulin (PAT) by *Aspergillus* and *Penicillium* species and sterigmatocystin (STE) by *Aspergillus* and *Fusarium* species. These genera fungi grow in subtropical latitude in food or raw material (Bhat, 2010). The presence of mycotoxins in food commodities could cause significant economic and health impacts.

BEA, PAT and STE alone produce toxic effects. BEA causes cytotoxic effects, produces oxidative stress and depletion of antioxidant cellular mechanisms (Dornetshuber et al., 2007; Ferrer et al., 2009; Jow et al., 2004; Mallebrera et al., 2014; Prosperini et al., 2013). BEA has also been related DNA damage in human cell lines and has pro-apoptotic activity (Dornetshuber et al., 2007; Jow et al., 2004; Prosperini eta l., 2013).

Similar effects were produced by PAT (Ferrer et al., 2009; Liu et al., 2007). Moreover, PAT induced DNA damages including DNA strand breaks, chromosome aberrations, and micronuclei formation in mammalian cells (Alves et al., 2000). It also causes mutagenicity, carcinogenicity, developmental and reproductive toxicity and immunotoxicity (Puel et al., 2010; Wichmann et al., 2003). PAT is classified as group 3 or as "not carcinogenic to humans" due to the inadequate evidence of carcinogenicity in human animals (IARC, 1986). STE is a precursor in Aflatoxin biosynthesis (Woloshuk and Prieto, 1998; Yu et al., 2004) and it is considered cytotoxic (Wang et al., 2013) and potent carcinogen, group 2B by IARC (IARC, 1976).

A completely diet would include all the different food items that possibly could be contaminated with the three chosen mycotoxins (EFSA, 2013, 2014; SCOOP Task 3.2.8). Thus, there is an increasing concern about hazard of co-occurrence of BEA, PAT and STE in feed and food. Many studies have been conducted on the toxicity of individual mycotoxins; however, few studies focused on the combined effects of mycotoxins. There is relatively little information on the interaction between concomitantly occurring mycotoxins and the consequence their combined toxicity and subsequently, their

* Corresponding author.
E-mail address: M.Jose.Ruiz@uv.es (M.-J. Ruiz).
[1] Both authors have contributed equally to the study, and thus may be regarded as first author.

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Disturbance of antioxidant capacity produced by beauvericin in CHO-K1 cells

B. Mallebrera*, G. Font, M.J. Ruiz

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

HIGHLIGHTS

- GSH depletion was observed at 5 μM of BEA after 24 h of exposure.
- BEA increased GST and GPx activities in CHO-K1 cells.
- BEA decreased GR activity in CHO-K1 cells.
- NAC increased GSH levels and detoxified BEA in CHO-K1 cells.
- GSH, GPx and GST help to counteract oxidative stress produced by BEA.

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ABSTRACT

Glutathione (GSH) levels, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-*S*-transferase (GST) as antioxidant defense system were evaluated in CHO-K1 cells after beauvericin (BEA) exposure. The effect of N-acetyl-cysteine (NAC) pre-treatment was assessed. GSH levels significantly decrease 18% and 29% after 5 μM of BEA in fresh medium and NAC pre-treatment, respectively compared to their controls. The GPx activity increased significantly from 35% to 66% in fresh medium and 20% in NAC pre-treatment. GR activity decreased after 5 μM of BEA up to 43% and 53% in fresh medium and NAC pre-treatment, respectively. The GST activity increased in fresh medium (from 61% to 89%) and decreased (from 22% to 35%) after NAC pre-treatment. Comparing BEA exposure in fresh medium and NAC pre-treatment, GSH levels, GPx activity and GST activity increased 716%, 458% and 206%, respectively respect to fresh medium; conversely no changes were observed in GR activity. In addition, NAC is an effective scavenger of BEA. GSH and related enzymes play an antioxidant role in the defense system of CHO-K1 cells exposed to BEA.

1. Introduction

Beauvericin (BEA) is a cyclichexadepsipeptide produced by many species of fungus *Fusarium* and *Beauveria bassiana* (Fig. 1). It contains three D-hidroxyisovaleryl and three N-methylphenylalanil residues in an alternating sequence (Wang and Xu, 2012).

BEA is an ionophore, forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}), that increases ion permeability in biological membranes, which may affect the ionic homeostasis (Chen et al., 2006; Kouri et al., 2005). BEA produces lipid peroxidation (LPO) and reactive oxygen species (ROS) in mammalian cells (Ferrer et al., 2009; Prosperini et al., 2013a). This mycotoxin shows: apoptotic activity, which has been attributed to Bcl-2 family, cytochrome c and caspase 3, as well as increases in the cytoplasmic calcium concentration (Lin et al., 2005). Moreover, BEA also produces DNA fragmentation, increases in chromosomal aberrations, sister-chromatid exchanges and micronucleus (Prosperini et al., 2013b). Moreover, it is the most potent specific inhibitor of cholesterol acyltransferase (Acyl-coA) (Tomoda et al., 1992).

The oxidative stress caused by ROS, has been involved in many human diseases; (Angelopoulou et al., 2009). However, cells have a protective endogenous antioxidant system that help protect against the damaging effects of ROS; this system is formed by no-enzymatic antioxidant such a glutathione (GSH) and enzymes such

* Corresponding author at: Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46100 Burjassot, Valencia, Spain.
E-mail address: beatriz.mallebrera@uv.es (B. Mallebrera).

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Cytoprotective effect of resveratrol diastereomers in CHO-K1 cells exposed to beauvericin

B. Mallebrera [a,*], V. Brandolini [b], G. Font [a], M.J. Ruiz [a]

[a] Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles s/n, 46100 Burjassot, Valencia, Spain
[b] Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121 Ferrara, Italy

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ABSTRACT

Beauvericin (BEA) causes cytotoxicity, lipid peroxidation and reactive oxygen species in CHO-K1 cells. Resveratrol (RSV) is a polyphenol with multiple biological properties, including antioxidant effects. RSV has two forms: *trans* and *cis*. The aims of this study were to determine the cytoprotective effect of *trans*-RSV and diastereomers mixtures (50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV) incubated alone and in combination with BEA in ovarian (CHO-K1) cells. The results demonstrated that cell viability increases (from 9% to 77%) when they were exposed to low concentration of RSV. Moreover, when the cells were pre-treated with RSV and then exposed to BEA, a cytoprotective effect (from 25% to 76%) and a ROS production diminution (from 27% to 92%) were observed, with respect to cells exposed to BEA without previous RSV exposure. RSV pre-treatment decreased the MDA levels (from 15% to 37%) when it is compared with cells exposed only to BEA. Therefore, it can be concluded that RSV could reduce the toxicological risk produced by BEA when they are in combination.

1. Introduction

Polyphenols are secondary metabolites produced by plants in response to exogenous factors such as injury, stress, fungal or UV radiation. There are over 8000 different molecules of polyphenols classified in four categories: phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). These natural compounds are incorporated through the diet and they protect against oxidant substances. Resveratrol (3, 5, 4′-trihydroxystilbene; RSV) is a polyphenolic compound abundant in grapes as well as derivates, peanuts, berries, dark chocolate and other food (Zamora-Ros et al., 2008). RSV exists in two diasteromic forms: *trans* and *cis* (Chen et al., 2007). The *trans* isomer is abundant in the skin of grapes and present in wines, especially red wines; it could be responsible for decreasing coronary heart disease observed in wine drinkers (Khurana et al., 2013). The *cis* isomer is derived by isomerization from *trans* isomer and it has potential anticancer activity, as well as *trans* isomer (Romero-Pérez et al., 1996). RSV has also been studied for other biological properties such as neuroprotection, inhibition to oxidation of low-density lipoproteins, anti-inflammatory, antioxidant, anti-aging, antidiabetic, and antiplatelet (Fernández-Mar et al., 2012; Gülçin, 2010; Vilahur and Badimon, 2013).

Beauvericin (BEA) is a secondary metabolite produced by many species of fungus *Fusarium* and *Beauveria bassiana*. BEA is a contaminant of cereals and products composed by cereals; naturally occurs on wheat, rice, mice, oat and barley. BEA is cytotoxic in several cells lines (Ferrer et al., 2009; Prosperini et al., 2013; Ruiz et al., 2011), it shows apoptotic activity, which has been attributed to Bcl-2 family, cytochrome c and caspase 3, increases the cytoplasmic calcium concentration (Lin et al., 2005), decreases glutathione (GSH) levels, produces lipid peroxidation (LPO) and generation of reactive oxygen species (ROS) (Ferrer et al., 2009; Mallebrera et al., 2014; Prosperini et al., 2013). Moreover, BEA causes DNA fragmentation, increases in chromosomal aberrations, sister-chromatid exchanges and micronucleus (Celik et al., 2010; Prosperini et al., 2013).

Despite the importance of oxygen for cell function, its catabolism could give rise to free radicals and consequently to oxidative stress. The damages produced by oxidative stress have been associated with LPO and ROS generation. So, the aim of this study was to determine the cytoprotective effect of *trans*-RSV and RSV diastereomers mixtures (50:50 *trans/cis*-RSV and 70:30 *trans/cis*-RSV) incubated in combination with BEA in ovarian (CHO-K1) cells. It is important to note that a diasteromic mixture is a diastereomers blend, where the proportions of diastereomers may affect its biological activity and its properties can significantly differ from those of its two individual diastereomers (Ahuja et al., 2009).

2. Materials and methods

2.1. Reagents

The reagent grade chemicals and cell culture components used were of standard laboratory grade. BEA (783.95 g/mol), *trans*-RSV (228.25 g/mol), ascorbic acid

* Corresponding author. Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andres Estelles, s/n, 46117, Burjassot, Valencia, Spain. Tel.: +34 963544958; fax: +34 963544954.
E-mail address: beatriz.mallebrera@uv.es (B. Mallebrera).

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Original Research Article

Influence of pro- and prebiotics on gastric, duodenal and colonic bioaccessibility of the mycotoxin beauvericin

B. Mallebrera, G. Meca *, L. Manyes, J. Mañes, G. Font

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 bioactive compound produced by the secondary metabolism of several *Fusarium* strains and known to have various biological activities. This study investigates the influence of several dietary fibers (galactomanan, glucomannan, citrus fiber, bamboo fiber, carrot fiber, pie fiber, β-glucan, xilan, and cellulose) and probiotic strains (*Lactobacillus animalis*, *Lb. casei*, *Lb. casei*, *Lb. plantarum*, *Lb. rhuminis*, *Lb. casei casei*, *Bifidobacterium breve*, *Bf. Adolescents*, *Bf. bifidum*, *Corynebacterium vitaeruminis*, *Streptococcus faecalis*, *Eubacterium crispatus*, and *Saccharomyces cerevisiae*) on the minor *Fusarium* mycotoxin BEA bioaccessibility employing a model solution. The bioaccessibility was determined using a simulated gastrointestinal digestion that mimics the physiological conditions of the digestive tract until the colonic compartment. The determination of BEA in the intestinal fluids was carried out by liquid chromatography–mass spectrometry detection (LC–MS). The reduction of BEA bioaccessibility in the experiments carried out using the prebiotic compounds ranged from 60 to 80%, whereas in the trials carried out using the probiotic strains the bioaccessibility observed ranged from 30 to 85%. A BEA degradation product produced by colonic fermentation was identified using the technique of LC–MS-LIT.

1. Introduction

Beauvericin (BEA) is a cyclic hexadepsipeptide consisting of alternating D-α-hydroxy-isovaleryl and aromatic N-methyl-phenylalanine. This toxin is produced by various *Fusarium* species such as *Fusarium avenaceum*, *F. poae*, *F. oxysporum* and *F. proliferatum*, and naturally occurs on maize, wheat, barley, rice and oat (Logrieco et al., 1998; Uhlig et al., 2006; Jestoi, 2008; Sorensen et al., 2008; Kokkonen et al., 2010; Waskiewicz et al., 2010). BEA has been detected in grains throughout the world under different climates (South Africa, Poland, Norway, Spain, Croatia), with concentrations ranging from trace level up to 520 mg/kg in maize in Italy (Ritieni et al., 1997). Meca et al. (2010) have shown that BEA was present in cereals (barley, corn and rice) purchased in Spanish markets, with levels ranging from 0.51 to 11.78 mg/kg.

An in vivo study has shown that mice orally exposed to BEA presented an increase of mortality with a Lethal Dose 50 (LD_{50}) superior to 100 mg/kg bw (Jestoi, 2008). The cytotoxicity of BEA has been demonstrated in vitro in several cell line models, including human leukemia cells CCRF-CEM, human monocytic lymphoma cells U-937 and promyelocytic leukemia cells HL-60, monkey kidney epithelial cells Vero, Chinese hamster ovary cells CHO-K1 and murine macrophage J774 (Tomoda et al., 1992; Calo et al., 2004; Jow et al., 2004; Ruiz et al., 2011a,b).

In the analysis of the risk evaluation related to human health, food ingestion is considered one of the important routes of exposure of many contaminants (Carolien et al., 2005).

To achieve any effects in a specific tissue or organ, the mycotoxins must be available, which refers to the compound's tendency to be extracted from the food matrix, and they must then be absorbed from the gut via the intestinal cells (Fernández-García et al., 2009). The term bioaccessibility has been defined as the fraction of a bioactive compound present in a food matrix that is not modified structurally through the reactions related to the gastrointestinal digestion and thus become available for intestinal absorption (Fernández-García et al., 2009).

Probiotics are defined as 'live microorganisms which when administered in adequate amount confer health benefits to the host' (FAO/WHO, 2002). Alternatively, probiotics have been defined as live microbial feed supplements that beneficially affect

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

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