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Polyphenols as possible bioprotectors against cytotoxicity and DNA damage induced by ochratoxin A

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ABSTRACT

The present study aimed to investigate the protective effects of luteolin (L), chlorogenic acid (ChlA) and caffeic acid (CafA) against cyto-genotoxic effects caused by OTA. Vero cells and rat lymphocytes were used and viability was measured by neutral red uptake, MTT and trypan blue dye exclusion method. L (50 and 100 μg/mL), ChlA (100 and 200 μg/mL) and CafA (10–50 μg/mL) reduced the damage induced by OTA (10 μg/mL) on both cells type shown a good protective effect. The comet and micronucleus tests in Balb/c mice were performed. ChlA (10 mg/kg bw) reduced OTA (0.85 mg/kg bw)-induced DNA damage on blood and bone marrow cells, CafA (10 mg/kg bw) showed protective effect only in blood cells and luteolin (2.5 mg/kg bw) failed to protect DNA integrity on cells. In conclusion, polyphenols tested reduced the toxicity caused by OTA on different target cells with good protective effect, being ChlA the compound that showed the best effects.

Abbreviations: FCS, foetal calf serum; LMP, low melting point; ME, mercaptoethanol; MN, micronuclei; MTT, 3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; NCE, normochromatic erythrocytes; NMP, normal melting point; NR, neutral red; OTA, ochratoxin A; PCE, polychromatic erythrocytes; TM, tail moment.

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1. Introduction

Ochratoxin A (OTA) is one of the most abundant mycotoxins produced by some fungal species of *Aspergillus* and *Penicillium*. It contaminates food and foodstuff and widespread in cereals, grains, coffee beans, dried grapes, juice, wine, beer and bread due to improper storage conditions (WHO, 2001). This mycotoxin has nephrotoxic, hepatotoxic, immunotoxic, mutagenic and genotoxic properties (Pfohl-Leszkowicz and Manderville, 2007; Mantle et al., 2010). OTA was shown to cause apoptosis in kidney cells and human and mice lymphocytes (Thuvander et al., 1996; Schwerdt et al., 1999; Petrik et al., 2003; Liu et al., 2012). Moreover, several studies have confirmed the genotoxicity of OTA both *in vitro* in mammalian cell lines (Rahat et al., 2011) and *in vivo* in rats and mice (Bouslimi et al., 2008; Abdel-Wahhab et al., 2008).

The mechanisms of OTA-induced toxic effects have not yet been clearly elucidated; however, induction of oxidative stress seems to be one of the processes involved. Generation of reactive oxygen species (ROS) may explain the induction of mitochondrial dysfunction, apoptosis and DNA damage induced by this mycotoxin (Le Bras et al., 2005; Ringot et al., 2006). Several physical, chemical and biological methods have been developed to suppress or reduce the toxic action of mycotoxins, improve food safety and minimize economic losses (Zinedine et al., 2007; Battacone et al., 2010). Recently, attention has been focused on natural antioxidant constituents. Polyphenols are secondary metabolism products of plants and constitute one of the most numerous and widely distributed groups of natural antioxidants in the plant kingdom (Wollgast and Anklam, 2000). The most abundant are phenolic acids and flavonoids accounting for 60% and 30%, respectively, of total polyphenols in the diet, approximately 1 g/day. The derivatives of the most abundant phenolic acids in plants are hydroxybenzoic and hydroxycinnamic acids. Hydroxycinnamic acids are more common than hydrobenzoic acids. Within hydroxycinnamic acids group are cholorgenic and caffeic acids and they are very abundant in most fruits and coffee. Flavones as apigenin and luteolin are the most common phenolics in plant-based foods (Manach et al., 2004). It has shown that luteolin, chlorogenic and caffeic acids protect cells from various forms of oxidant stresses through to scavenge reactive oxygen species (ROS) (Lin et al., 2008; Meng et al., 2013).

Considering that formation of ROS seems to be one of the mechanism involved in cell damage induced by OTA, these natural antioxidants appear to be an effective alternative for reducing the toxic effects caused by this mycotoxin. In addition, in the consulted literature, there are no studies that demonstrate the effect of luteolin, chlorogenic or caffeic acids against OTA-induced toxicity.

Based on this background, the present study aimed to investigate the possible protective effects of luteolin, chlorogenic and caffeic acids against toxics effects caused by OTA. We employed Vero cells and rat lymphocytes as an *in vitro* model to investigate the influence of polyphenols supplement on cytotoxic effects induced by OTA and we also evaluated the possible protective effects of these polyphenols on OTA-induced DNA damage in mice.

2. Materials and methods

2.1. Cell cultures

2.1.1. Vero cells

Vero cells (*Cercopithecus aethiops* green monkey kidney epithelial cell line; ATCC CCL-81) were grown in Eagle's minimal essential medium (EMEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Natocor, Argentina), glutamine (30 μg/mL) and gentamicin (50 μg/mL) (all from Sigma St. Louis, USA). Cell cultures were maintained at 37 °C with 5% CO_2 and humidity.

2.1.2. Lymphocytes

Male Wistar rats (weighing 200 g) supplied from Bioterio Central of Universidad Nacional de Río Cuarto were used. Peripheral blood was drawn from the tail vein and lymphocytes were isolated using Hystopaque®-1077 centrifugation (Sigma Aldrich, St. Louis, USA). Cell viability was determined by trypan blue dye exclusion method using an optimal suspension of 1×10^6 cells/mL (Mongini and Waldner, 1996). The study was approved by the Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto. Lymphocytes (2×10^5/well) in a final volume of 200 μL, were cultured in sterile 96-well microplate containing RPMI-1640, supplemented with 25 mM Hepes, 2 mM L-glutamine, 5% FCS, 50 mM 2-ME, 100 μg/mL streptomycin, 100 μg/mL penicillin and 100 μg/mL neomycin.

2.2. Cytotoxicity assays

2.2.1. Cytotoxicity on Vero cells

Vero cells were cultured in 96-well microplates. After incubation for 24 h at 37 °C, cells were exposed to OTA or polyphenols. Different doses of OTA (BioPure Technology, UK) (0, 5, 10, 50, 75, 100 and 125 μg/mL) were tested. The selection of OTA doses was supported by other *in vitro* studies in Vero cells (Ramyaa and Padma, 2013). The polyphenols luteolin (≥98%), chlorogenic acid (≥95%) or caffeic acid (≥ 98%) (all fom Sigma St. Louis, USA) were also tested in different doses (0, 10, 25, 50, 100 and 200 μg/mL). OTA and polyphenols were dissolved in DMSO/PBS (solvent concentration not exceeding 0.03%). To assess the cytotoxicity, cells were exposed at all compounds in independent trials. The systems were incubated at 37 °C with 5% CO_2 and humidity for 24 h. Monolayers incubated with EMEM or DMSO were the cellular viability controls. Three replicate wells for each exposure in four independent plates were performed.

After that period, cell viability was evaluated by two methods: neutral red (NR) uptake (Rajbhandari et al., 2001) and colorimetric MTT assay (Mosmann, 1983).

With the aim of testing the polyphenols protection capability against OTA toxicity, cells also were exposed to combinations of OTA (10 μg/mL) and the polyphenols, latter in not cytotoxic concentrations (50 and 100 μg/mL luteolin, 25 and 50 μg/mL caffeic acid and 100 and 200 μg/mL chlorogenic acid), in two independent assays:

a) Treatment (polyphenols along with OTA and incubated for 24 h).
b) Pretreatment (supplementation of polyphenols during 2 h prior to OTA treatment and then incubated for 24 h).

The viability was evaluated by NR assay. Three replicate wells for each exposure in four independent plates were performed.

2.2.2. Cytotoxicity on lymphocytes

To assess the cytotoxicity, lymphocytes were exposed to OTA and polyphenols in independent trials at doses previously described. The selection of OTA doses was supported by other *in vitro* studies with lymphocytes (Mechoud et al., 2012). Cell cultures with RPMI-1640 or DMSO were used as control. The systems were incubated at 37 °C with 5% CO_2 and humidity for 24 h. Three replicate wells for each exposure in four independent plates were performed. After that period, cell viability was evaluated by two methods: trypan blue dye exclusion using Neubauer chamber (Militao et al., 2006) and colorimetric MTT assay (Mosmann, 1983).

With the aim of testing the polyphenols protection capability against OTA toxicity, lymphocytes were exposed to combinations of OTA (10 μg/mL) and the polyphenols, latter in not cytotoxic concentrations (50 and 100 μg/mL luteolin, 10 and 25 μg/mL caffeic acid and 100 and 200 μg/mL chlorogenic acid), in two independent assays:

a) Treatment (polyphenols along with OTA and incubated for 24 h).
b) Pretreatment (supplementation of polyphenols during 2 h prior to OTA treatment and then incubated for 24 h). The viability was evaluated by trypan blue dye exclusion method. Three replicate wells for each exposure in four independent plates were performed.

2.3. Genotoxicity assays

2.3.1. Animals and treatment

Male Balb/c mice aged 8–12-weeks, (weighing 20–25 g), supplied from Bioterio Central of the Universidad Nacional de Río Cuarto, were maintained in a temperature and humidity controlled room, with 12 h light–dark cycles and were allowed food and water *ad libitum*. All experimental procedures were conducted in accordance with recent legislation. This study was approved by the Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto.

Mice were separated into groups of 4 animals per group and were inoculated (i.p.) as follows:

1. With a dose of DMSO/PBS as vehicle control group.
2. With a dose of cyclophosphamide (20 mg/kg bw) as positive control group.
3. With a dose of OTA (0.85 mg/kg bw).
4. With increasing doses of luteolin (2.5, 5 and 10 mg/kg bw).
5. With increasing doses of chlorogenic acid (2.5, 5 and 10 mg/kg bw).
6. With increasing doses of caffeic acid (2.5, 5 and 10 mg/kg bw).
7. With combination of OTA (0.85 mg/kg bw) and luteolin (2.5 mg/kg bw).
8. With combination of OTA (0.85 mg/kg bw) and chlorogenic acid (10 mg/kg bw).
9. With combination of OTA (0.85 mg/kg bw) and caffeic acid (10 mg/kg bw).

The selection of OTA and polyphenols doses was based on other *in vivo* studies in Balb/c mice (Abraham, 1996; Bouslimi et al., 2008). In the protective assays, animals were treated with combination of OTA and polyphenols (injected together), latter in not genotoxic concentrations.

After 24 h, the mice were decapitated without being anesthetized and the blood samples were immediately taken in heparinized tubes for performing comet assay. In addition, the femurs were immediately excised from the bodies and micronucleus assays were performed.

2.3.1.1. Comet assay. The heparinized blood samples were kept in the dark at 4 °C and processed within 6 h. The comet assay was performed according to the protocol proposed by Singh et al. (1988), with slight modifications. Volumes of 50 μl of blood from each mouse were added to 100 μl of 0.75% LMP agarose at 37 °C. The mixtures were layered onto slides precoated with 0.75% NMP agarose and covered with a coverslip. The slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO just before use) for at least 1 h, right after the slides immersed in a freshly prepared alkaline buffer pH > 13 and electrophoresis was conducted for 30 min. The slides were fixed in absolute ethanol, stained with ethidium bromide, and scored using a fluorescent microscope. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the Comet Score 1.5 software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM) was used to estimate DNA damage (arbitrary units).

2.3.1.2. Micronucleus assay. This trial was carried out as described by Schmid (1975), with modifications. Bone marrow samples from femoral bone obtained with FCS were fixed with ethanol and stained with May-Grünwald and Giemsa. In order to evaluate the protective effects of polyphenols on DNA damage induced by OTA, the presence of erythrocytes with micronuclei (MN) was observed in a total of 2000 polychromatic erythrocytes (PCE) per animal. Furthermore, to obtain a toxicity grade of compounds on bone marrow, the toxicity index (TI) was calculated by the PCE/NCE ratio in 1000 cells.

2.4. Statistical analysis

All the values obtained in the assays were expressed as averages with standard deviations. The data obtained from cytotoxicity assays and Micronucleus test were evaluated using GraphPad Prism version 5.00.288 (San Diego, USA, 2007) and compared with one way analysis of variance (ANOVA) and the Tukey multiple comparison test. The differences were considered to be statistically significant at $p < 0.05$. The data obtained from Comet assay were also evaluated using

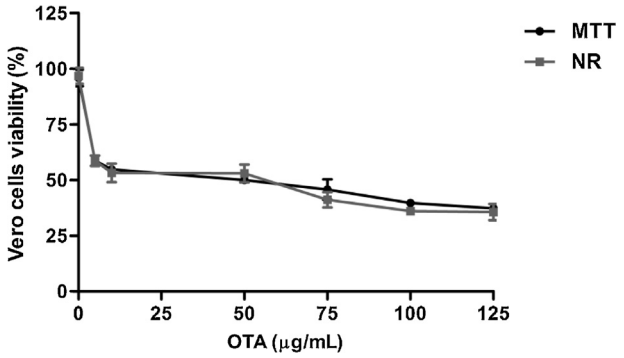

Fig. 1 – Viability of Vero cells exposed to different concentrations of ochratoxin A (OTA) for 24 h. Cell viability was evaluated by Neutral Red intake (NR) and MTT methods. Each value represents means ± SD.

GraphPad Prism version 5.00.288 (San Diego, USA, 2007) and compared with Kruskal Wallis test and Dunns multiple comparisons.

3. Results

3.1. *Cytotoxicity assays*

3.1.1. *Cytotoxicity on Vero cells*

Cytotoxic effects of OTA on Vero cells after 24 h incubation as measured by the MTT and NR assays are shown in Fig. 1. OTA treatment caused toxic effect in a not dose-dependent manner decreasing cell viability over 50–60% ($p < 0.0001$ respect to control) as measured by both viability methods.

Regarding the polyphenols tested, luteolin (from 100 μg/mL) caused a significant toxic effect as measured by MTT and RN assays decreasing cell viability over 20% ($p < 0.05$ respect to control). Caffeic acid caused toxicity on these cells (from 100 μg/mL) as measured by NR assay decreasing cell viability over 40% ($p < 0.0001$ respect to control) and also caused a significant toxic effect at 200 μg/mL as measured by MTT assay decreasing cell viability over 25% ($p < 0.001$ respect to control). Chlorogenic acid did not affect Vero cell viability regardless the concentration tested or assay performed (Fig. 2A and B).

3.1.2. *Cytotoxicity on lymphocytes*

Cytotoxic effects of OTA on lymphocytes after 24 h incubation as measured by trypan blue dye exclusion and MTT methods are shown in Fig. 3. OTA exerted toxic effect on lymphocytes in a not dose-dependent manner causing the death over 50–75% cells ($p < 0.0001$ respect to control) as measured by both viability methods.

Regarding the polyphenols tested, luteolin (from 100 μg/mL) caused a significant toxic effect as measured by trypan blue dye exclusion assay decreasing cell viability over 20% ($p < 0.05$ respect to control). Caffeic acid also caused toxic effects on lymphocytes (from 50 μg/mL) as measured by trypan blue dye exclusion assay decreasing sharply cell viability over 75% ($p < 0.0001$ respect to control) and also caused a significant toxic effect at 200 μg/mL as measured by

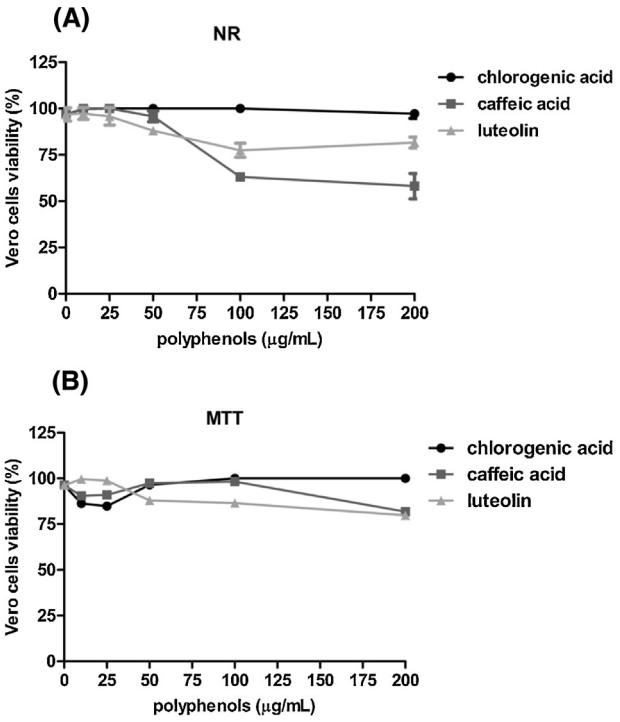

Fig. 2 – Viability of Vero cells exposed to different concentrations of polyphenols for 24 h. Cell viability was evaluated by (A) Neutral Red (NR) intake and (B) MTT method. Each value represents means ± SD.

MTT assay decreasing cell viability over 20% ($p < 0.05$ respect to control). Chlorogenic acid did not affect lymphocytes viability regardless the concentration tested or assay performed (Fig. 4A and B).

3.1.3. *Protective effects of polyphenols on OTA-induced cytotoxicity*

Treatment and pretreatment with three polyphenols reduced the OTA cytotoxicity on Vero cells with statistically significant differences ($p < 0.001$ for luteolin, $p < 0.001$ and $p < 0.0001$ for chlorogenic acid and $p < 0.0001$ for caffeic acid respect to OTA treatment). Viability values similar to control cells were

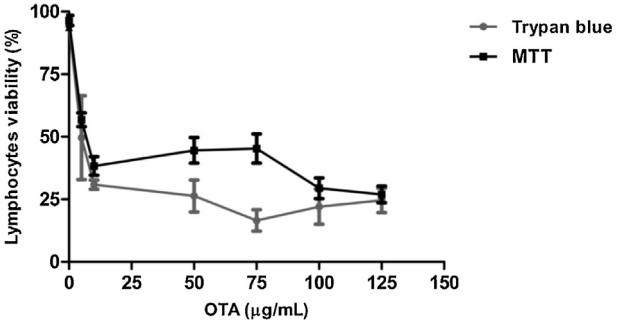

Fig. 3 – Viability of lymphocytes from Wistar rats ($n = 4$) exposed to different concentrations of ochratoxin A (OTA) for 24 h. Cell viability was evaluated by trypan blue dye exclusion and MTT methods. Each value represents means ± SD.

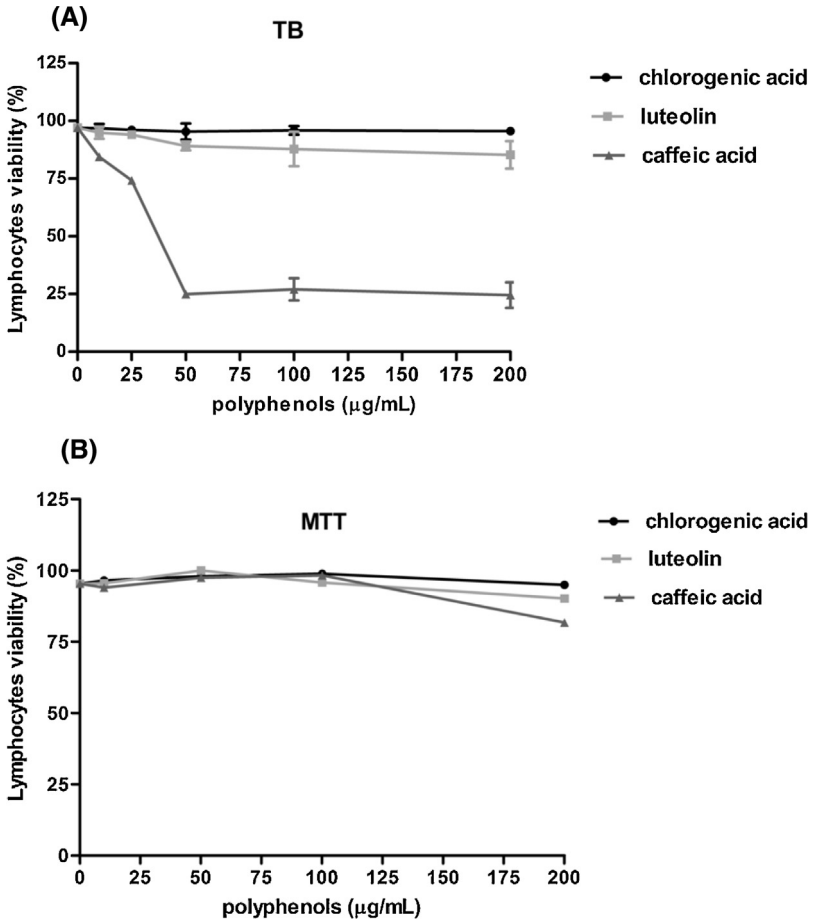

Fig. 4 – Viability of lymphocytes from Wistar rats ($n = 4$) exposed to different concentrations of polyphenols for 24 h. Cell viability was evaluated by (A) trypan blue dye exclusion (TB) assay and (B) MTT method. Each value represents means ± SD.

reached. No statistically significant differences between treatment and pretreatment were observed (Fig. 5**A, B and C**).

On the other hand, treatment and pretreatment with three polyphenols also reduced the OTA cytotoxicity on lymphocytes with statistically significant differences ($p < 0.0001$ for luteolin, chlorogenic and caffeic acids respect to OTA treatment). However, the protective effect was less than the observed on Vero cells. Viability values of 70–80%, 70–75% and 45–55% in cells supplemented with luteolin, chlorogenic acid or caffeic acid respectively, were reached. No statistically significant differences between treatment and pretreatment were observed (Fig. 6**A, B and C**).

3.2. *Genotoxicity assays*

3.2.1. *General observations and behavior*

None clinical signs of behavioral toxicity or mortality were observed in the animals treated with OTA or polyphenols 24 h post-injection.

3.3. *Comet assay*

Results of comet assay are shown in Fig. 7. OTA exposure (0.85 mg/kg bw) caused a significant increase in tail moment of blood cells respect to negative control group ($p < 0.0001$ Kruskal–Wallis and Dunns Test). The DNA integrity of these cells was not affected by treatment with polyphenols.

3.4. *Micronucleus test*

Table 1 summarizes the micronucleus assay results obtained from Balb/c mice treated with OTA and polyphenols. A significant increases in the incidence of MN in PCE in animal group treated with OTA was observed ($p < 0.001$ respect to control group). The mean of MNPCE in bone marrow was not affected by treatment with chlorogenic or caffeic acids. However, a significant increases of MNPCE in luteolin group at higher doses tested was observed ($p < 0.01$ respect to control group). No significant difference in the PCE/NCE ratio in all treatments was observed.

3.4.1. *Protective effects of polyphenols on OTA-induced DNA damage*

Tail moment increases in blood cells observed in animal group treated with OTA were significantly reduced in the animal group treated with OTA + chlorogenic acid (10 mg/kg bw) and OTA + caffeic acid (10 mg/kg bw) ($p < 0.05$ respect to OTA group). Treatment with these two polyphenols reduced the increase of tail moment in 40% (Fig. 8**A**). Luteolin did not show protective

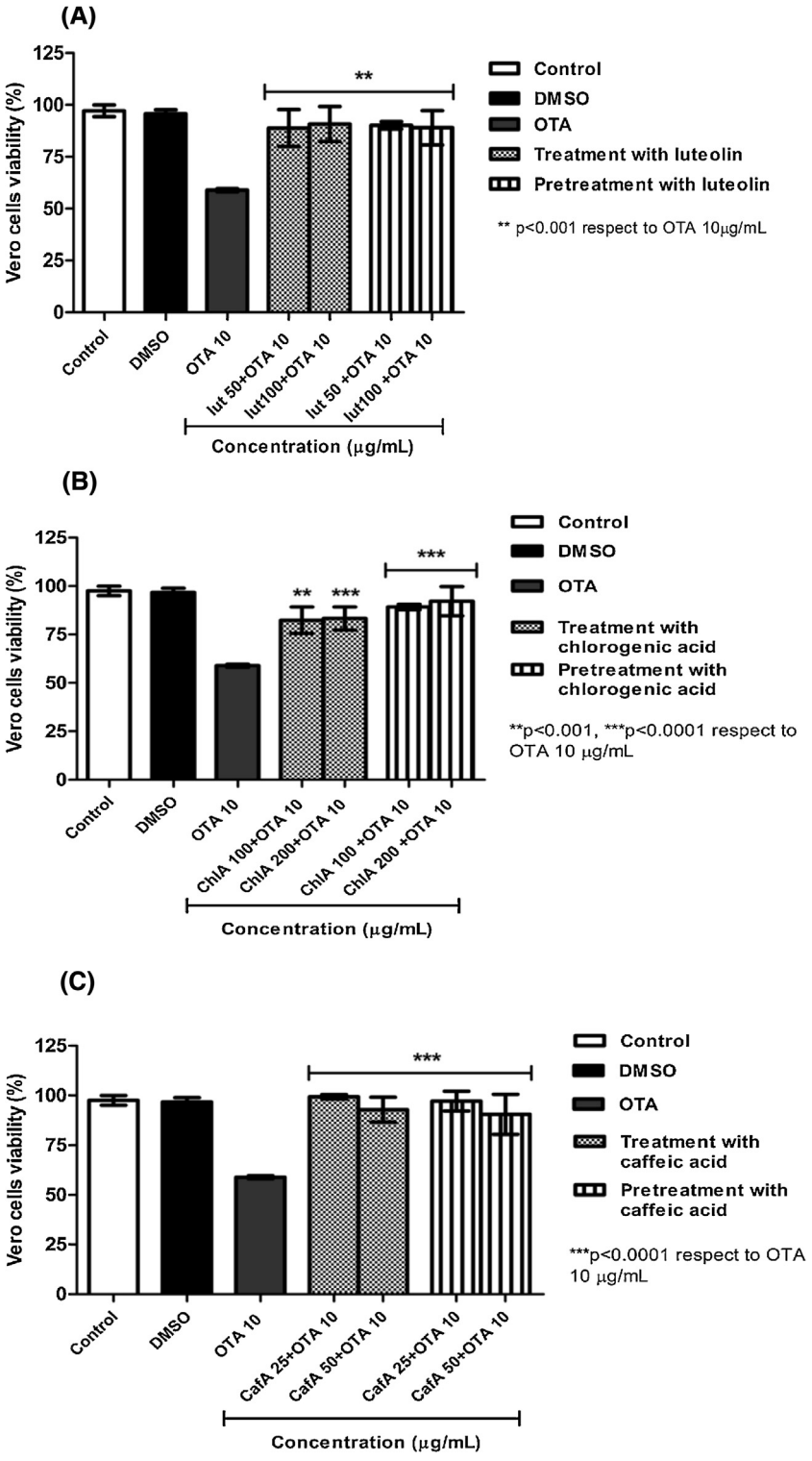

Fig. 5 – Protective effects of polyphenols on OTA-induced Vero cells toxicity. (A) treatment and pretreatment with luteolin (50 and 100 μg/mL); (B) treatment and pretreatment with chlorogenic acid (100 and 200 μg/mL); (C) treatment and pretreatment with caffeic acid (25 and 50 μg/mL). Cell viability was evaluated by Neutral Red intake (NR). Each value represents means ± SD.

lut: luteolin; ChlA: chlorogenic acid; CafA: caffeic acid. $^{**}p < 0.001$, $^{***}p < 0.0001$ respect to OTA (ANOVA and Tukey Test).

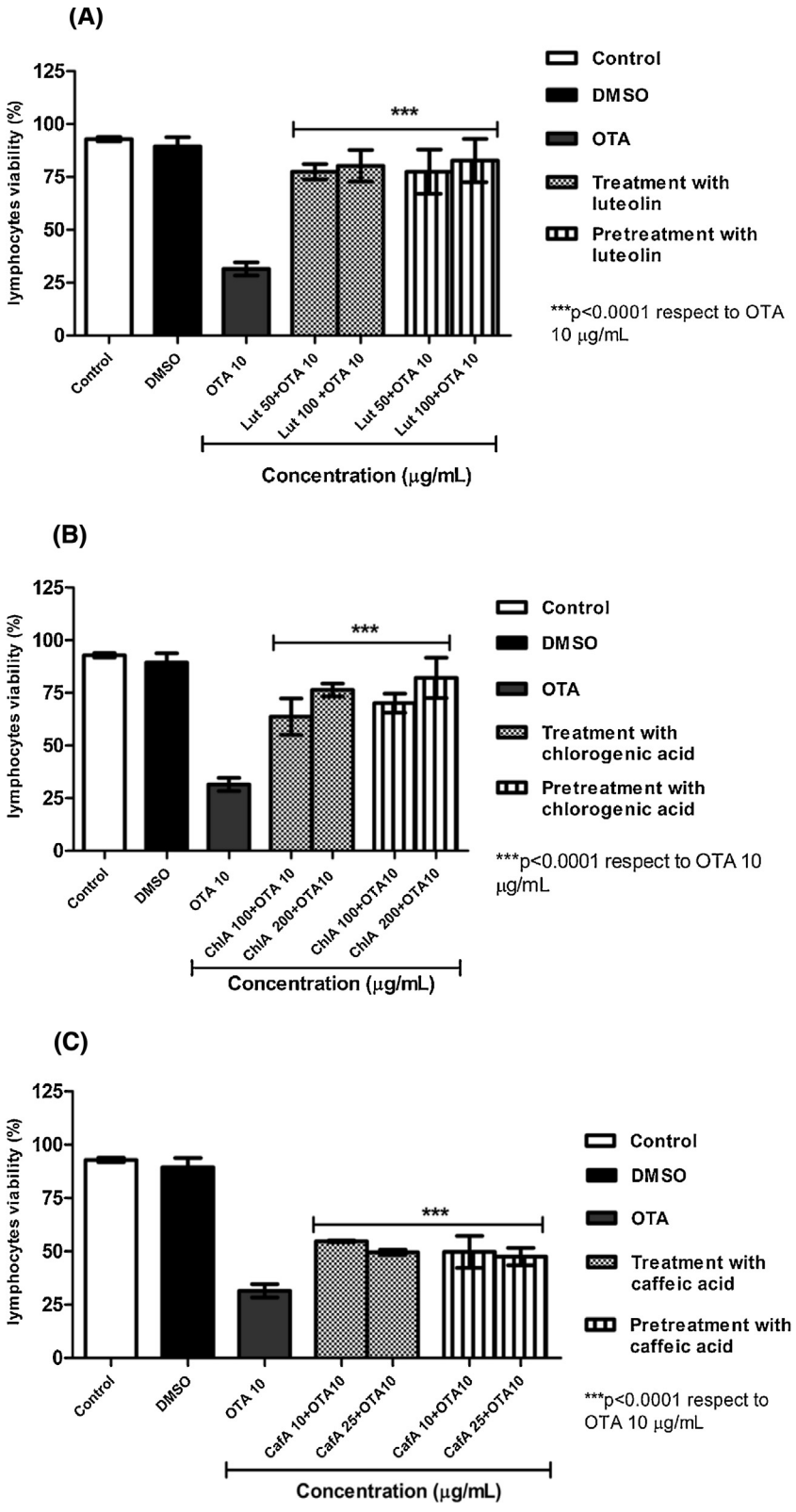

Fig. 6 – Protective effects of polyphenols on OTA-induced lymphocytes toxicity. (A) treatment and pretreatment with luteolin (50 and 100 μg/mL); (B) treatment and pretreatment with chlorogenic acid (100 and 200 μg/mL); (C) treatment and pretreatment with caffeic acid (10 and 25 μg/mL). Cell viability was evaluated by trypan blue dye exclusion method. Each value represents means ± SD.

lut: luteolin; ChlA: chlorogenic acid; CafA: caffeic acid. ***$p < 0.0001$ respect to OTA (ANOVA and Tukey Test).

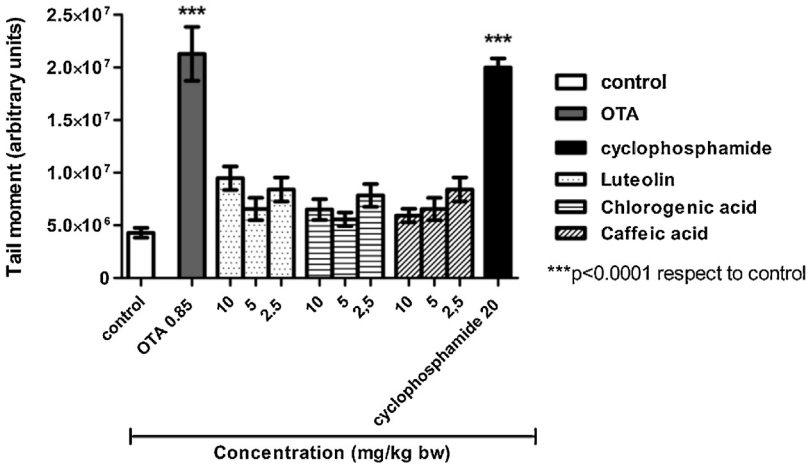

Fig. 7 – DNA damage (tail moment) of blood cells from Balb/c mice ($n = 4$) treated with OTA or polyphenols for 24 h. Each value represents means ± SD. ***$p < 0.0001$ respect to control group (Kruskal–Wallis and Dunns Test).

Table 1 – Genotoxicity and toxicity indexes determined in bone marrow erythrocytes from Balb/c mice treated with OTA or polyphenols and combinations of OTA and polyphenols.

Treatment	Animals[a]	TI[b] (PCE/NCE ± SD) 24 h	GI[c] MNPCE[d] (‰) 24 h
Negative control (DMSO/PBS)	4	0.99 ± 0.15	4.70 ± 1.20
OTA (0.85 mg/kg bw)	4	1.27 ± 0.05	18.50 ± 3.54**
Luteolin			
(2.5 mg/kg bw)	4	0.99 ± 0.04	6.00 ± 2.00
(5 mg/kg bw)	4	0.99 ± 0.15	13.00 ± 2.65*
(10 mg/kg bw)	4	0.83 ± 0.60	16.00 ± 6.00*
Chlorogenic acid			
(2.5 mg/kg bw)	4	1.01 ± 0.20	2.00 ± 1.00
(5 mg/kg bw)	4	0.82 ± 0.10	1.33 ± 0.58
(10 mg/kg bw)	4	0.82 ± 0.15	2.33 ± 1.52
Caffeic acid			
(2.5 mg/kg bw)	4	0.79 ± 0.09	1.66 ± 1.16
(5 mg/kg bw)	4	0.63 ± 0.06	2.33 ± 1.53
(10 mg/kg bw)	4	0.82 ± 0.15	3.67 ± 2.89
Luteolin (2.5 mg/kg bw) + **OTA** (0.85 mg/kg bw)	4	0.99 ± 0.17	19.5 ± 6.36**
Chlorogenic acid (10 mg/kg bw) + **OTA** (0.85 mg/kg bw)	4	0.79 ± 0.001	7.50 ± 4.95
Caffeic acid (10 mg/kg bw) + **OTA** (0.85 mg/kg bw)	4	1.09 ± 0.07	14.5 ± 6.36*
Positive control (cyclophosphamide 20 mg/kg bw)	4	1.20 ± 0.60	26.50 ± 8.02***

[a] Four mice were used per experimental group.
[b] Toxicity Index.
[c] Genotoxicity Index.
[d] Micronucleated polychromatic erythrocytes. In all cases 2000 polychromatic erythrocytes (PCE) per animal were analyzed.
* $p < 0.05$ statistically significant difference from negative control group (ANOVA and Tukey test).
** $p < 0.01$ statistically significant difference from negative control group (ANOVA and Tukey test).
*** $p < 0.001$ statistically significant difference from negative control group (ANOVA and Tukey test).

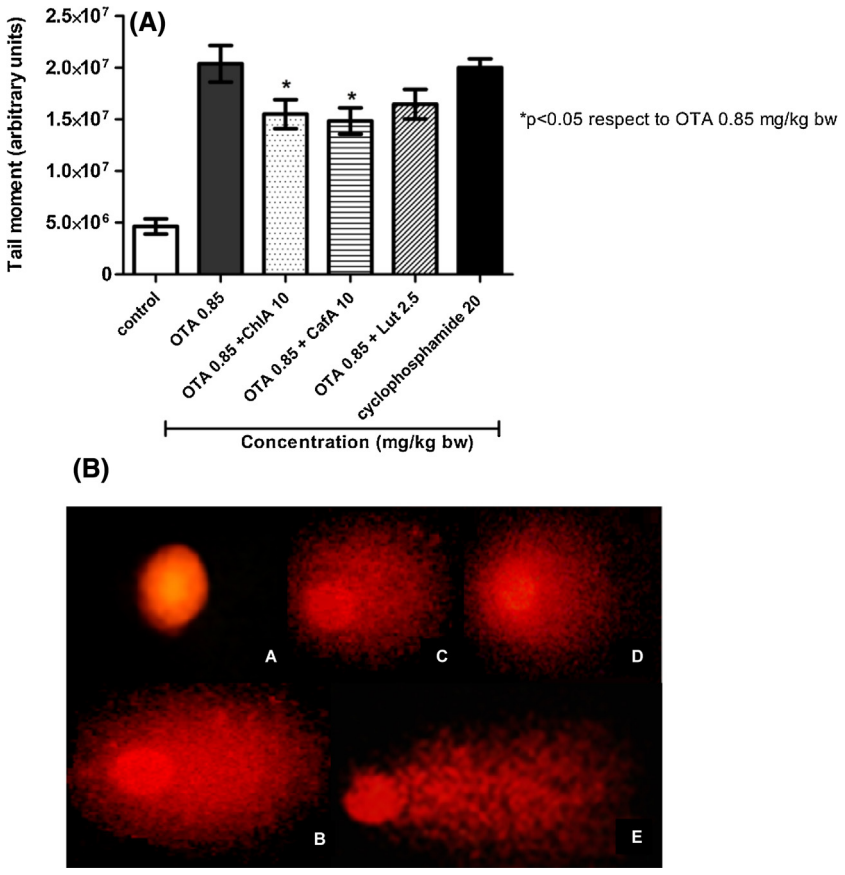

Fig. 8 – (A) Protective effects of polyphenols on OTA-induced DNA damage (tail moment) in blood cells from Balb/c mice ($n = 4$) after 24 h of treatment. Each value represents the means ± SD.
Lut: luteolin; ChlA: chlorogenic acid; CafA: caffeic acid. *$p < 0.05$, respect to OTA (Kruskal–Wallis and Dunns Test).
(B) Photomicrographs of nucleus of blood cells from Balb/c mice, analyzed by comet assay. (A) Negative control (PBS); (B) OTA (0.85 mg/kg bw); (C) OTA (0.85 mg/kg bw) + chlorogenic acid (10 mg/kg bw); (D) OTA(0.85 mg/kg bw) + caffeic acid (10 mg/kg bw); (E) Positive control (cyclophosphamide 20 mg/kg bw).

effects on OTA-induced DNA damage. Fig. 8**B** exhibit cells with and without DNA damage.

The increase in the incidence of MN in PCE in animal group treated with OTA was significantly reduced in the animals group treated with OTA + chlorogenic acid (10 mg/kg bw) ($p < 0.05$ respect to OTA group). Treatment with this polyphenol decreased the frequencies of MNPCE in 60%. Treatment with luteolin and caffeic acid failed to diminish the frequency of MN in PCE. No significant difference in the PCE/NCE ratio in all treatments was observed (Table 1).

4. Discussion

In the present study, we evaluated whether polyphenols luteolin, chlorogenic acid and caffeic acid could protect cells against toxic damage caused by OTA. In the *in vitro* assays we confirmed that OTA (0–125 μg/mL) exert cytotoxic effects on Vero cells by altering the lysosomal and mitochondrial function (measured by NR and MTT methods) as previously reported by several authors (Creppy et al., 2004; Bouslimi et al., 2008; Ramya and Padma, 2013). Moreover, OTA induced cytotoxic effects on rat lymphocytes. In agree with our results, some authors have reported a decrease in the percentage of splenic and thymic lymphocytes in murine models after *in vivo* treatment with OTA (250–500 μg/kg bw) (Thuvander et al., 1995, 1996; Dortant et al., 2001). We observed that OTA caused more damage in the cell membrane of lymphocytes than in the mitochondrial respiratory chain. Odhav et al. (2008) also reported cell membrane disruption and damage to cytoplasmic organelles on human leukocytes exposed to OTA (50 μg/mL).

Regarding the cytotoxicity of three polyphenols tested, chlorogenic acid (0–200 μg/mL) was the only compound that not affects the viability of Vero cells or lymphocytes. Luteolin caused toxicity from100 μg/mL by altering organelles function in Vero cells and cytoplasmic membrane in lymphocytes. Others studies have also demonstrated lack of cytotoxic effect of chlorogenic acid and toxicity of luteolin on lymphocytes, Vero cells and kidney cells (Fujimoto et al., 2009; López-Posadas et al., 2008; Kang et al., 2011; Kanlayavattanakul et al., 2013). Caffeic acid was the more toxic compound. We observed that this compound altered principally the cytoplasmic membrane in lymphocytes and lysosomal function in Vero cells, without affect the mitochondrial respiratory chain in both cells type.

In the *in vivo* assays we confirmed the genotoxic effects of OTA (0.85 mg/kg bw) on blood cells and bone marrow cells as previously reported by several authors (Creppy et al., 1985; Bouslimi et al., 2008; Abdel-Wahhab et al., 2008). Furthermore, of the three polyphenols tested, we observed by micronucleus test that only luteolin (5 and 10 mg/kg bw) showed a significant genotoxic effect on bone marrow cells, but by high sensitive comet assay, did not alter the DNA integrity of blood cells. None of polyphenols alter the PCE/NCE ratio, demonstrating absence of cytotoxicity on bone marrow erythrocytes. The results of the *in vitro* and *in vivo* assays demonstrated that polyphenols acted differently depending on the cell type, the concentration and the type of assay performed as described by Resende et al. (2012).

To our knowledge, this work is the first study to report the ability of luteolin, chlorogenic and caffeic acids to reduce the toxic effects of OTA which could contribute to the prevention of ochratoxicosis. The polyphenols supplement in Vero cells or lymphocytes cultures treated with OTA reduced the toxic effects caused by this mycotoxin, demonstrating a good protective effect. Regarding the protective effects of polyphenols on OTA-induced DNA damage, only chlorogenic acid (10 mg/kg bw) were able to reduce OTA-induced DNA damage on both blood cells as bone marrow cells. Caffeic acid (10 mg/kg bw) reduced the DNA damage caused by OTA only in blood cells and luteolin failed to protect DNA integrity on both cells type. As described above, the polyphenols may exhibit different effects on the same cells, depending of the type assay carried out.

Although the mechanisms of OTA-induced toxic effects have not yet been clearly elucidated several studies have demonstrated that after cell treatment with OTA there are an increase of ROS levels and a reduction of antioxidant enzymes (SOD, GSH) which leads to induce a wide range of lesions in cell membranes, proteins and DNA (Baudrimont et al., 1997; Abdel-Wahhab, 2000; Abdel-Wahhab et al., 2005; Liu et al., 2012). Luteolin, chlorogenic and caffeic acids are natural antioxidant compounds and different *in vitro* and *in vivo* studies have reported their ability as ROS scavengers (Lin et al., 2008; Sato et al., 2011; Xu et al., 2014). It could be that these polyphenols have exerted their protective effect of damage induced by OTA on cells through an antioxidant mechanism.

In conclusion, luteolin, chlorogenic and caffeic acids reduced the toxicity caused by OTA on different target cells with good protective effect, being chlorogenic acid the compound that showed the best effects. Given that similar effects may even occur in the dietary administration of these compounds to animal species susceptible to OTA, further studies are needed to deepen the protective effects of these polyphenols and the mechanism of action on OTA toxicity.

Transparency document

The Transparency document associated with this article can be found in the online version.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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REFERENCES

Abdel-Wahhab, M.A., 2000. Antioxidant and radical scavenging effects of garlic and cabbage extracts in rats fed ochratoxin-contaminated diet. J. Egypt. Med. Assoc. 83, 1–19.

Abdel-Wahhab, M.A., Abdel-Galil, M.M., Ellithey, M.M., 2005. Melatonin counteracts oxidative stress in rats fed ochratoxin A-contaminated diet. J. Pineal Res. 38, 130–135.

Abdel-Wahhab, M.A., Abdel-Azim, S.H., El-Nekeety, A.A., 2008. Inula crithmoides extract protects against ochratoxin A-induced oxidative stress, clastogenic and mutagenic alterations in male rats. Toxicon 52, 566–573.

Abraham, S.K., 1996. Anti-genotoxic effects in mice after the interaction between coffee and dietary constituents. Food Chem. Toxicol. 34, 15–20.

Battacone, G., Nudda, A., Giuseppe, P., 2010. Effects of ochratoxin A on livestock production. Toxins 2, 1796–1824.

Baudrimont, I., Ahouandjivo, R., Creppy, E.E., 1997. Prevention of lipid peroxidation induced by ochratoxin A in Vero cells in culture by several agents. Chem-Biol Interact 104, 29–40.

Bouslimi, A., Bouaziz, Chayma, Ayed-Boussema, I., Hassen, W., Bacha, H., 2008. Individual and combined effects of ochratoxin A and citrinin on viability and DNA fragmentation in cultured Vero cells and on chromosome aberrations in mice bone marrow cells. Toxicology 251, 1–7.

Creppy, E.E., Kane, A., Dirheimer, G., Lafarge-Frayssinet, C., Mousset, S., Frayssinet, C., 1985. Genotoxicity of ochratoxin A in mice: DNA single-strand break evaluation in spleen, liver and kidney. Toxicol. Lett. 28, 29–35.

Creppy, E.E., Chiarappa, P., Baudrimont, I., Borracci, P., Moukha, S., Carratù, M.R., 2004. Synergistic effects of fumonisin B1 and ochratoxin A: are in vitro cytotoxicity data predictive of in vivo acute toxicity? Toxicology 201, 115–123.

Dortant, P.M., Peters-Volleberg, G.W.M., Van Loveren, H., Marquardt, R.R., Speijers, G.J.A., 2001. Age-related differences in the toxicity of ochratoxin A in female rats. Food Chem. Toxicol. 39, 55–65.

Fujimoto, A., Sakanashi, Y., Matsui, H., Oyama, T., Nishimura, Y., Masuda, T., Oyama, Y., 2009. Cytometric analysis of cytotoxicity of polyphenols and related phenolics to rat thymocytes: potent cytotoxicity of resveratrol to normal cells. Basic Clin. Pharmacol. 104, 455–462.

Kanlayavattanakul, M., Lourith, N., Ospondpant, D., Ruktanonchai, U., Pongpunyayuen, S., Chansriniyom, C., 2013. Salak plum peel extract as a safe and efficient antioxidant

appraisal for cosmetics. Biosci. Biotechnol. Biochem. 77, 1068–1074.
Kang, K.P., Park, S.K., Kim, D.H., Sung, M.J., Jung, Y.J., Lee, A.S., Lee, J.E., Ramkumar, K.M., Lee, S., Park, M.H., Roh, S.G., Kim, W., 2011. Luteolin ameliorates cisplatin-induced acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis. Nephrol. Dial Transpl. 26, 814–822.
Le Bras, M., Clément, M.V., Pervaiz, S., Brenner, C., 2005. Reactive oxygen species and the mitochondrial signalling pathway of cell death. Histol. Histopathol. 1, 205–219.
Lin, Y., Shi, R., Wang, X., Shen, H.M., 2008. Luteolin, a flavonoid with potentials for cancer prevention and therapy. Curr. Cancer Drug Targets 8, 634–646.
Liu, J., Wang, Y., Cui, J., Xing, L., Shen, H., Wu, S., Lian, H., Wang, J., Yan, X., Zhang, X., 2012. Ochratoxin A induces oxidative DNA damage and G1 phase arrest in human peripheral blood mononuclear cells *in vitro*. Toxicol. Lett. 211, 164–171.
López-Posadas, R., allester, I., badía-Molina, A.C., uárez, M.D., Zarzuelo, A., artínez-Augustin, O., Sánchez de Medina, F., 2008. Effect of flavonoids on rat splenocytes, a structure-activity relationship study. Biochem. Pharmacol. 6, 495–506.
Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79, 727–747.
Mantle, P.G., Faucet-Marquis, V., Manderville, R.A., Squillaci, B., Pfohl-Leszkowicz, A., 2010. Structures of covalent adducts between DNA and ochratoxin A: a new factor in debate about genotoxicity and human risk assessment. Chem. Res. Toxicol. 23, 89–98.
Mechoud, M.A., Guillermo, E.J., Font de Valdez, G., Rodriguez, A.V., 2012. *Lactobacillus reuteri* CRL 1098 and *Lactobacillus acidophilus* CRL 1014 differently reduce in vitro immunotoxic effect induced by Ochratoxin A. Food Chem. Toxicol. 50, 4310–4315.
Meng, S., Cao, J., Feng, Q., Peng, J., Hu, Y., 2013. Roles of chlorogenic acid on regulating glucose and lipids metabolism: a review. Evid Based Complement Alternat Med., http://dx.doi.org/10.1155/2013/801457.
Militao, G.C.G., Dantas, I.N.F., Pessoa, C., Falcão, M.J.C., Silveira, E.R., Lima, M.A.S., Curi, R., Lima, T., Moraes, M.O., Costa-Lotufo, L.V., 2006. Induction of apoptosis by pterocarpans from *Platymiscium floribundum* in HL-60 human leukemia cells. Life Sci. 78, 2409–2417.
Mongini, C., Waldner, C., 1996. Metodologías para la evaluación de las células inmunocompetentes. In: Margni, R.A. (Ed.), Inmunología e Inmunoquímica. Editorial Médica Panamericana, Argentina, pp. 730–734.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Odhav, B., Adam, J.K., Bhoola, K.D., 2008. Modulating effects of fumonisin B1 and ochratoxin A on leukocytes and messenger cytokines of the human immune system. Int. Immunopharmacol. 8, 799–809.
Petrik, J., Zanic-Grubisic, T., Barisic, K., Pepeljnjak, S., Radic, B., Ferencic, Z., Cepelak, I., 2003. Apoptosis and oxidative stress induced by ochratoxin A in rat kidney. Arch. Toxicol. 77, 685–693.
Pfohl-Leszkowicz, A., Manderville, R.A., 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Review. Mol. Nutr. Food Res. 51, 61–99.
Rahat, A., Mittelstaedt, R.A., Shaddock, J.G., Ding, W., Bhalli, J.A., Khan, Q.M., Heflich, R.H., 2011. Comparative analysis of micronuclei and DNA damage induced by ochratoxin A in two mammalian cell lines. Mutat. Res. 723, 58–64.
Rajbhandari, M., Wegner, U., Julich, M., Schopke, T., Mentel, R., 2001. Screening of Nepalese medicinal plants for antiviral activity. J. Ethnopharmacol. 74, 251–255.
Ramyaa, P., Padma, V.V., 2013. Ochratoxin-induced toxicity, oxidative stress and apoptosis ameliorated by quercetin–Modulation by Nrf2. Food Chem. Toxicol. 62, 205–216.
Resende, F.A., Vilegas, W., Dos Santos, L.C., Varanda, E.A., 2012. Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test. Molecules 17, 5255–5268.
Ringot, D., Chango, A., Schneider, Y.J., Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chemico-Biological Interact. 159, 18–46.
Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., Sugawara, M., Iseki, K., 2011. In vitro and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. Int. J. Pharm. 403, 136–138.
Schmid, W., 1975. The micronucleus test. Mutat. Res. 31, 9–15.
Schwerdt, G., Freudinger, R., Mildenberger, S., Silbernagl, S., Gekle, M., 1999. The nephrotoxin ochratoxin A induces apoptosis in cultured human proximal tubule cells. Cell. Biol. Toxicol. 15, 405–415.
Singh, N., McCoy, M., Tice, R., Schneider, E., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Thuvander, A., Breitholtz-Emanuelsson, A., Olsen, M., 1995. Effects of ochratoxin A on the mouse immune system after subchronic exposure. Food Chem. Toxicol. 33, 1005–1011.
Thuvander, A., Dahl, P., Breitholtz-Emanuelsson, A., 1996. Influence of perinatal ochratoxin A exposure on the immune system in mice. Nat. Toxins. 4, 174–180.
WHO, 2001. Ochratoxin A, safety evaluation of certain mycotoxins in food. WHO Food Additives 74, 381–415.
Wollgast, J., Anklam, E., 2000. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Res. Int. 33, 423–447.
Xu, D., Hu, L., Xia, X., Song, J., Li, L., Song, E., Song, Y., 2014. Tetrachlorobenzoquinone induces acute liver injury, up-regulates HO-1 and NQO1 expression in mice model: The protective role of chlorogenic acid. Environ. Toxicol. Phar. 37, 1212–1220.
Zinedine, A., Soriano, J.M., Moltó, J.C., Mañes, J., 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food Chem. Toxicol. 45, 1–18.