In vitro evaluation of the chemoprotective action of flavan-3-ols against deoxynivalenol related toxicity

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SUMMARY

The protective effect of four flavan-3-ols: catechin (C), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG) against deoxynivalenol (DON)-induced cytotoxicity was investigated in A2780-epithelial cell line. The ability of the flavan-3-ols to reduce ROS (reactive oxygen species) production and TNF-\(\alpha\) (tumor necrosis factor) release induced by DON was also investigated. The treatment with DON (40 \(\mu\)M) gives an increased level of cytotoxicity versus control (43.6%). After 24 hours treatment with flavan-3-ols it could restore cell viability with respect to DON alone, but being lower than the control group. DON-induced ROS was significantly reduced even after 1 hour incubation with flavan-3-ols in a relation dose-effect with the follows exception: 125 \(\mu\)M EGCG and ECG. Concerning the TNF-\(\alpha\) release, comparing with the control, DON induces overexpression of this factor. All the flavan-3-ols induced a lower level of TNF-\(\alpha\) release in the cellular supernatant comparing with the DON group, being similar with the control group. The present study demonstrates the potent protective effect of flavan-3-ols against DON cytotoxicity in A2780-epithelial cell line. The results show a good scavenging power according to inhibition of ROS production. Flavan-3-ols could be useful to develop alimentary strategies for both humans and animals to prevent DON-induced cytotoxicity.

Key words: chemoprotective action, flavan-3-ols, deoxynivalenol

INTRODUCTION

Deoxynivalenol (DON) also known as Vomitoxin is a mycotoxin which belongs to the trichothecenes epoxy-sesquiterpeneoid and is produced by Fusarium fungi. DON mycotoxin is cytotoxic for most of the eukaryotic cells in

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both plant and animals and induce the inhibition of protein synthesis, alteration of membrane structure and mitochondrial function, apoptosis and cytokine production (Bondy and Pestka, 2000; Pestka and Smolinski, 2005). A critical target for DON and for other trichothecene is immune system. For example, a single dose of DON can rapidly induce gene expression for a wide range of cytokines (Pestka and Smolinski, 2005; Zhou et al., 2003). Elevated cytokine expression may play an important role in pathophysiological effects of DON and other trichothecens (Bondy and Pestka, 2000; Pestka and Smolinski, 2005).

Mycotoxins, including DON, may have a negative impact on the body antioxidant/prooxidant balance. Several papers have studied the effect of DON on the oxidative process in different in vitro and in vivo systems (Maresca et al., 2002; Li et al., 2000; Chung et al., 2003). Acute low-dose trichothecene exposure affects immune function by initiating a rapid and transient upregulation of proinflammatory cytokines (Pestka et al., 2004). Dietary antioxidant supplementation may protect against cytotoxic effect of mycotoxins, like DON by interfering in one of the several steps of their mechanism of action.

Flavan-3-ols and its derivatives are a class of phenolic compounds present in green tea leaves, chocolate, grape and grape seeds with several healthy properties, like anticancer properties (Cooper et al., 2005; Yang et al., 2005) and protective capacity against oxidative stress related diseases, cardiopreventive, antimicrobial, antiviral, and neuroprotective action (Erba et al., 2005; Hsu et al., 2005). Antioxidative properties of flavan-3-ols are manifested particularly by their abilities to up-regulate the antioxidant enzymes (Khan et al., 1988), and to scavenge the reactive oxygen species (ROS) (Rice-Evans et al., 1996). To our knowledge, there are no studies concerning the protective effects of flavan-3-ols against the toxicity and pro-oxidative effect induced by DON.

That way, the aim of the present study was to evaluate the protective action of flavan-3-ols against the cytotoxic effects of DON on A2780 epithelial cell line. Previously investigations from our laboratory showed that A2780, a human ovarian cancer cell line from epithelial origin, was a sensitive model for the studies concerning the cytotoxicity of aflatoxins and DON (Braicu et al., 2009A; Braicu et al., 2009B). It was shown that aflatoxins and DON provoked a high level of cytotoxicity, and also a high level of ROS (Braicu et al., 2009A) in the A2789 cell line.

The capacity of four increasing concentrations (12.5-125 μM) of flavan-3-ols: catechin (C), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG) to counteract the effect of 40 μM DON was tested on several functions of A2780 cell line (cytotoxicity, intracellular reactive oxygen species release and TNF-α production). The concentration of 40 μM DON was chosen as a result of our previous experiments.
MATERIAL AND METHODS

Chemicals: (-) C, (-) EGC, (-) EGCG, (-) ECG, 2′7′-dichlorofluorescin-diacetate (H2DCF-DA), DON and MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma–Aldrich, Bucharest, Romania), DON was purchased from Sigma–Aldrich, Bucharest, Romania, Quantikine TNF-α Immunoassay kit, R&D, Romania.

Cell culture: A2780 cells (epithelial human ovarian cancer cell line) were grown in RPMI 1640 (Sigma–Aldrich, Bucharest, Romania) supplemented with 10% Fetal Bovine Serum (Sigma–Aldrich, Bucharest, Romania), 2mM glutamine, 100 UI/ml penicillin, 100 mg/ml streptomycin (Sigma–Aldrich, Bucharest, Romania). The cell lines were maintained by serial passage in 75 cm² flasks, incubated at 37°C, in a humidified incubator with a 5% CO₂ atmosphere.

MTT-cytotoxicity test. The cytotoxic effect of DON on A2780 cells was evaluated by MTT assay (Ototguro et al., 1991; Neradil et al., 2003; Lindl et al., 2005). Briefly, A2780 cells seeded in 96-well plate (2 × 10³ cells/well in 150 μL RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum, 2mM glutamine, 100 UI/ml penicillin, 100 mg/ml streptomycin), were treated simultaneously with five concentrations of watery solution (12.5, 25, 50, 125 μM) of flavan-3-ols (C, EGC, EGCG, ECG) and DON, single dose (40 µM). Each experimental concentration was tested in triplicate well. Untreated cells were used as control cells. Plates were incubated at 37°C in 5% CO₂ for 24 hours. The supernatants were removed and the cell pellets were washed with PBS; 150 μl Hanks salt solution containing MTT (Sigma, Bucharest, Romania) were than added in each well at the final concentration of 455 μg/ml. After 2 hours of incubation, under standard conditions, the MTT solution was removed and 200 μl of DMSO were added into each well. Absorbance was measured at 490 nm using a Biotek Synergy HT Microplate Plate Reader.

Detection of intracellular reactive oxygen species (ROS). Intracellular ROS was quantified by using a fluorescent probe 2′7′-dichlorofluorescindiacetate (DCFH-DA) (Yang et al., 2000). DCFH-DA diffuses thought the cell membrane readily and is enzymatically hydrolyzed by intracellular esterases to non-fluorescent dichlorofluorescin-diacetate (DCFH), which is rapidly oxidized to highly dichlorofluorescin (DCF) in the presence of reactive species formed intracellular (Yang et al., 2000).

Cells were seeded in 96-well black plates, 2 × 10³ cells per well, and allowed to grow for 24 h. After that the cells were treated with the five concentrations (12.5, 25, 50, 125 μM) of flavan-3-ols (C, EGC, EGCG, ECG) in the presence of DON, single dose (40 μM) and of DCFH-DA (final concentration 0.1 μM). DCF fluorescence intensity was detected at different time intervals using a Biotek Synergy HT Microplate Plate Reader with an excitation wavelength measured at 485 nm and emission wavelength at 530 nm.
ELISA assay for human Tumor Necrosis Factor Alpha (TNF-α). TNF-α immunoassay analysis was performed according to the protocol recommended by the manufactures (TNF-α Qantikine, R&D Systems, Romania). Cells were seeded at $2 \times 10^3$ cells per well in 96-well plates; the cells were treated with the five concentrations (12.5, 25, 50, 125 μM) of flavan-3-ols (C, EGC, EGCG, ECG) in the presence of DON, single dose (40 μM). Immunoassay for TNF-α was performed after 24 hours of treatment. Briefly, the monoclonal anti-human TNF-α (clone 105105) was used to capture antibodies in conjunction with polyclonal biotinylated goat anti-human TNF-α antibody as detecting antibody. HRP and TMB were used for detection. Absorbance was read at 450nm with an ELISA plate reader (Biotek Synergy HT Microplate Plate Reader). The supernatant was centrifuged to remove cell debris and stored at -70°C until assayed. Preliminary assays were performed to determine the optimal dilution of supernatant; each determination was made in triplicate and is expressed as average of 3 determinations. Cytokine production was quantified by reference to standard curves constructed with known concentration of recombinant porcine TNF-α. All samples were run in triplicate. Results were expressed as picograms of TNF-α per mL.

RESULTS

Based on previously experience, A2780 cells were proven to be a sensitive model for study the toxicity of aflatoxins and also DON. The effect of DON on cell cultures was already tested by us and the results from our preliminary experiments demonstrated that DON was able to induce a dose-dependent cell injury in A2780 cell line (Braicu et al., 2009A; Braicu et al., 2009B). Individual flavan-3-ols had no significant effect on cell proliferation, no-prooxidant effect, or on the capacity to modulate TNF-α synthesis (Braicu et al., 2009A).

Effect of DON and flavan-3-ols on cell proliferation, measured by MTT assay. The effect of DON and DON plus flavan-3-ols (C, EGC, EGCG, ECG) on the A2780 epithelial cells proliferation was evaluated by measuring the mitochondrial dehydrogenase activity (MTT test). Cells were incubated 24 hours in the presence and absence of DON and flavan-3-ols.

The effect of different concentration (12.5, 25, 50, 125 μM) of flavan-3-ols (C, EGC, EGCG, ECG), on the cytotoxicity induced by DON (40 μM) at 24 hours is presented in Figure 1. The treatment with DON (40 μM) determined an increased level of cytotoxicity versus control (43.6%). By contrast, it was observed that treatment with flavan-3-ols (12.5-125 μM) did not acting in a synergic way with DON and reduced significantly in a dose dependent manner the cytotoxicity induced by this mycotoxin.
Figure 1. Effect of flavan-3-ols on DON-induced cytotoxicity using MTT assay at 24 hours exposures. Cells were incubated with DON (40 μM) and or/flavan-3-ols (12.5-125 μM) on A2780 cell line; at 24 hours was performed MTT assay. Data are presented as mean ± SD (n=6), (*p<0.05 compared to the control and #p<0.05 compared to the group treated with DON using oneway Anova with t-test).

Figure 2. Chemical structure of flavan-3-ols taken in this study

The value for the MTT test in case of treatment with DON and flavan-3-ols expressed as % of the control for EGCG was between 66.3% (12.5 μM) and 79.7% (125 μM) with a relation dose effect, for the ECG was between 71-85.2%, for C was between 57.2-76.1%, respectively for EGC was between 48.5%-64.3%. The protective effect is correlated with the applied dose and compound structure (Figure 2).
**Effect of flavan-3-ols and DON on intracellular reactive oxygen species (ROS).** The effect of the four concentrations of flavan-3-ols (C, EGC, EGCG, ECG), in the presence of DON on induced ROS formation in A2780 cell line is presented in Figure 3. Our results showed that for the fluorescence intensity of the control group increased steadily (3), suggesting that ROS is spontaneously formed on A2780 cell line. A single dose of DON (40 µM) induced a significant increase of ROS concentration comparing with control group (p<0.05). The four different doses (12.5, 25, 50, 125 µM) of flavan-3-ols (EGCG, EGC, ECG and C) were used to analyze their protective effect on ROS production induced by DON.

![Figure 3. Time- and dose-related effect of flavan-3-ols (12.5-125 µM), (A): EGCG; (B): EGC; (C) C; (D) ECG; in the presence of DON (40 µM) on ROS production as measured by fluorescence DCFH-DA intensity. Data are presented as mean ± SD, (n=6), (p<0.05 compared to the control but also compared to the group treated with DON only using oneway Anova with t-test).](image)

In general, under the action of the flavan-3-ols the production of ROS was decreased, comparing with that of the cells treated with DON alone. DON-induced DCF fluorescence intensity was significantly reduced even after 1 hour of incubation with flavan-3-ols especially with C and EGC form.

The DCF fluorescence intensity induced by DON in the presence of 125 µM EGCG and ECG level were similar or a little higher comparing with 12.5 µM of corresponding flavan-3-ol.

**Effect of flavan-3-ols and DON on Tumor Necrosis Factor Alpha release.** TNF-α synthesis was measured after 24 hours of treatment in the presence of flavan-3-ols: EGCG, EGC, ECG and C at the following µM doses 12.5, 25.0, 50.0, 125 and/or 40 µM DON.
The effect of flavan-3-ols in modulating TNF-α secretion in presence of DON is presented in Figure 4. DON induced a high production of TNF-α (214.5 pg/ml) compared to the control (126.3 pg/ml).

![Figure 4. Quantification of TNF-α production from cellular supernatant, in the presence of flavan-3-ols (EGCG, EGC, ECG and C, 12.5-125 µM) and/or DON (*p<0.05 compared to the control and #p<0.05 compared to the group treated with DON only using oneway ANOVA with t-test).](image)

All the flavan-3-ols induced a significant decrease in the TNF-α secretion comparing with the DON group. TNF-α levels derived form flavan-3-ols are not similar with that of the control group and only one exception was registered in the case of treatment with DON and 12.5 µM ECG (129.6 pg/ml).

**DISCUSSION**

Trichotheccenes are known to act as translational inhibitors in eukaryotic ribosomes (Pestka and Smolinski, 2005). The inhibitory effect of DON on cell proliferation is due probably to the capacity of the mycotoxin to inhibit DNA and RNA synthesis as well as the protein synthesis at the ribosomal level. This is in agreement with the results obtained in the present study in which an inhibitory effect of DON on cell proliferation was observed. These results confirmed once the sensibility of our in vitro systems and the sensibility of MTT test as end-point. Similarly, in an in vitro study it was proven that DON may interfere in the differentiation of the intestinal epithelial cell lines Caco-2, T-84 and HT-29-D4 cells (Maresca et al., 2002). Thus, concentrations lower than 10 µM DON induced a slightly LDH release and 100 µM DON treatment increased LDH release to 642% of the control (Maresca et al., 2002).

The treatment with flavan-3-ols (12.5-125 µM) reduced significantly in a dose dependent manner the cytotoxicity induced by this mycotoxin. It is known
that flavan-3-ols may have significant inhibitory effects on metabolic transformation of DON to their toxic or carcinogenic derivatives; furthermore, they could promote DON transformation into less-toxic or even nontoxic products (Yang et al., 2005; Hockenbery et al., 1993; Luczay et al., 2005). The role of flavan-3-ols in the activation of detoxification pathways should not be underestimated. It was also shown that regulation of both basal and inducible expression of cytoprotective genes are mediated in part by the antioxidant response element (Costa et al., 2007).

Recent findings suggested that oxidative stress plays an important role in the toxicity of mycotoxins like aflatoxins, ochratoxin A or deoxinivalvenol (Braicu et al., 2009A; Atroshi et al., 1995; Costa et al., 2007). In the present study, it was observed that DON induce an accumulation of cytosolic ROS, this may be due to the alterations of proteins synthesis involved in detoxification. Generally, flavan-3-ols protected efficiently against ROS, except for EGCG at 125 μM and ECG at 125 μM. This effect may be attributed to the presence of galloyl group. It is a considerable interest that galloyl group (at the 3 position) of flavan-3-ols, this structure appears to be essential for physiological/pathological activities (Peng et al., 2003). Antioxidants possess chemopreventive properties against mycotoxin-induced cell damage, which are at least partially associated with the induction of phase II detoxifying enzymes and antioxidant enzymes (Peng et al., 2003). We hypothesize that the increase in detoxifying enzymes is probably the main mechanism of antioxidant mediated chemoprevention (Atroshi et al., 1995; Peng et al., 2003; Costa et al., 2005; Costa et al., 2007; Stangl et al., 2007). Others elements like selenium, vitamin E and vitamin C act also as an antioxidant system and free radical scavenger that protect spleen and brain against membrane damage caused by T-2 toxin and DON (Atroshi et al., 1995). In another paper it was observed that sodium selenite could reduce in vitro the toxic effect of DON on cardiomyocytes (Peng et al., 2003).

It is believed that trichothecenes posed high affinity to bind to the peptidyl transferase site of the 60s ribosomal subunit in eukaryotic cells (Moon, and Pestka, 2003). Interestingly, translational inhibitors that bind to ribosomes have been observed to activate rapidly mitogen activated proteine kinases (MAPKs) in vitro (Iordanov et al., 1997), suggesting that these signal transducers may mediate trichothecene toxicity. MAPK have been shown to mediate DON-induced transactivation of TNF-α (Chung et al., 2003) and cyclooxygenase-2 (Moon, and Pestka, 2003). Our results concerning the TNF-α expression is in agreement with another study made by Bondy and Pestka, (2000) who sustain that DON upregulates the expression of inflammatory genes, both at the transcriptional and post transcriptional level. The toxin induces activation of MAPK, NF-κB pathways, apoptosis in a process known as “ribotoxic stress response” (Zhou et al., 2000; Li et al., 2005; Li et al., 2000; Wong et al., 2002; Li et al., 2000). Acute low-dose trichothecene exposure aberrantly affects immune function by initiating a rapid upregulation of proinflammatory
cytokines (Bondy and Pestka, 2000). The capacity of trichothecenes to upregulate the proinflammatory cytokines TNF-α and IL-6 appear to drive immunotoxic effects such as shock and IgA nephropathy in mice (Bondy and Pestka, 2000; Li et al., 2000; Pestka et al., 2003). Antioxidant supplementation, like flavan-3-ols protect against toxic action of DON by interfering with one of the steps described above, confirmed in the present paper, by reducing the release of TNF-α, a proinflammatory cytokine (Li et al., 2000).

The present study demonstrates the protective effect of flavan-3-ols against DON cytotoxicity in A2780-epithelial cell line. This protective effect may be due to the capacity of flavan-3-ols to inhibit the cytotoxicity, ROS formation and also the release of inflammatory cytokines like TNF-α induced by DON.

Flavan-3-ols could be used as a new alimentary strategy to prevent DON-induced cytotoxicity for both humans and animals.

The occurrence of DON in food and feed is frequent; substances able to reduce toxic effects could be of great interest. Therefore, the naturally antioxidant supplements seem to be an attractive strategy to protect humans and animals from the risk of cancer caused by mycotoxins. Development of new-oxidant strategies by improving the natural defense mechanism may be an important method for inhibiting mycotoxins toxicity.

The role of dietary antioxidants in preventing DON toxicity has attracted increasing attention in the last years and numerous studies are currently in progress to evaluate the potential benefit effect of these compounds in the diet. More research is needed to develop new nutritional strategies to reduce the toxic and economic impact of mycotoxins.

REFERENCES
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