Natural Antioxidants as Efficient Preventive Agents Against Mycotoxicoses: Case of Citrinin Mycotoxin

Chayma Bouaziz1*, Amal Salah1,2, Chiraz Zaied1,3 and Salwa Abid-Essefi1

1Faculty of Dental Medicine, Monastir University, Laboratory of Research on Biologically Compatible Compounds, Tunisia
2Faculty of Sciences of Bizerte, Carthage University, Tunisia
3Higher Institute of Biotechnology of Beja, Jendouba University, Tunisia

*Corresponding author: Chayma Bouaziz, Faculty of Dental Medicine, Monastir University, Laboratory of Research on Biologically Compatible Compounds, Tunisia

Abstract

Cinnamon (Cinnamomum zeylanicum) and fenugreek (Trigonella foenum-graecum) are two widespread used condiments with many health benefits. Their addition to savoury dishes might help minimizing the deleterious effects of natural food contaminants. Mycotoxins are among the most naturally occuring food contaminants. They are products of the secondary metabolism of molds belonging maily to the genera Aspergillus, Penicillium and Fusarium. These mycotoxins can grow on the plant in the field or during storage and are potentially toxic to humans and animals. Among these mycotoxins, Citrinin (CTN), produced by different species of Penicillium and Aspergillus, is likely to contaminate certain foodstuffs. CTN is recognized as a pro-oxidant molecule causing oxidative stress which affects many cellular organelles such as mitochondria. Therefore, fighting the oxidative processes induced by CTN would help to reduce the cellular damages and the subsequent toxicity more effectively. In this work, in Human Colorectal Cells (HCT116), we have studied the preventive effects of aqueous extracts of two medicinal plants: Cinnamon (CAN) and Fenugreek (FEN) as regarded to CTN toxicity. We have shown that CTN induced a decrease in HCT116 cells viability concomitantly with a mitochondrial transmembrane potential (ψΔ) drop. The pretreatment of HCT-116 cells in culture with CAN or FEN have diminished the mortality induced by CTN concomitantly with a clear restoration of the ψΔ potential when compared to cells treated with CTN alone. These results suggest that use of condiments can be persuaded as a preventive strategy against oxidative damage induced by food contaminants.

Keywords: Mycotoxin; Citrinin; Cinnamon; Fenugreek; Mitochondrial Potential Drop; Oxidative Stress

Introduction

Molds are widely used in the agro-food industry where they participate, under controlled conditions, in the processing of raw food materials into products of high added value (cheese, etc.). However, aside from this beneficial aspect, some molds can become harmful by altering not only the organoleptic and nutritional properties of food but also their health incidence by synthesizing secondary metabolites (mycotoxins) causing deleterious health effects in the organisms. According to the United Nations Food and Agriculture Organization about 25% of food is contaminated with mycotoxins. This scourge affects commodities at all stages of production.
the food chain, leading to considerable economic damage for the various actors in the sectors concerned. The health consequences are multiple: lower yields and grain quality, lower weight of poultry and livestock, increased susceptibility to infections due to weakened immune defences, increased mortality of livestock, leading to considerable economic losses [1].

Nowadays, mycotoxins and the specific pathologies induced are of worldwide interest and represent serious economic and public health problems. They are known to exert a broad variety of biological effects due to the great variability of their chemical structures. The mycotoxin citrinin (CTN), mainly produced by species of the genus Penicillium and Aspergillus, has particularly aroused our interest. It is recognized mainly by its nephrotoxic, hepatotoxic, immunotoxic and teratogenic effects in animals [2-4]. Cytotoxicity and reactive oxygen species (ROS) generation are the major mechanisms involved in mycotoxins toxicity. ROS are highly reactive molecules due to the presence of unpaired electrons. Moreover, the cell can tolerate a small to moderate amount of oxidative stress by producing antioxidant molecules to counteract the excess oxidants.

Hence, living organisms maintain a permanent balance between the damage caused by the oxidizing molecules and their repair process. Oxidative stress appears when this balance is disrupted [5]. Facing these food risks, preventive strategies limiting the occurrence of these contaminations aim to decontaminate food contaminated with mycotoxins. Meanwhile, the usual food processes (cooking, freeze-drying and freezing) can in general only partially destroy the mycotoxins present in foods [6-8]. Enhancing the antioxidant defences of the body by ensuring a healthy diet, balanced and rich in antioxidants (vitamins, trace elements, carotenoids, polyphenols .) is essential to avoid this imbalance. These food antioxidants are found mainly in fruits and vegetables. Indeed, several studies have shown the crucial role of a diet rich in fruits and vegetables in the protection against cancer and the risk of cardiovascular accidents [9].

In the same way, these plants also exploited in traditional medicine were proved to be an important source of antioxidant compounds. In this context, we were interested in two medicinal plants: (i) Fenugreek, Trigonella foenum-græcum L which is a small leguminous plant, belonging to the family “Fabaceae”. The leaves of fenugreek are used in salads and seeds are used as a spice. It is one of the oldest medicinal and culinary plants in the history (ii) Cinnamon, Cinnamomum zeylanicum, belonging to the family of “Lauraceae”, is supplied by the fragrant bark of the cinnamon, small exotic tree whose most widespread species grows in the island of Ceylon. Cinnamon is widely used as condiment in both savoury and sweet dishes. Using a variety of experiments, we have tried to evaluate the antioxidant activity of the aqueous extracts of fenugreek (FEN) and cinnamon (CIN) grains. We have further tested their ability to reduce the cellular damage caused by CTN.

**Material and Methods**

**Preparation of the Aqueous Extracts of Fenugreek and Cinnamon**

The aqueous extracts were prepared according to the protocol proposed by Haoula [10]. Briefly, 3g of unmilled fenugreek seeds were weighed and soaked in 100ml of distilled water in a shaker at 25°C for 24 hours. The solution was filtered through a washtm paper and a filter (0.45μm). Similarly, cinnamon rinds were washed and dried for one week in ambient air. They were then cut into small pieces and ground to obtain a powder. 2.5 g of powder were diluted in 100 ml of distilled water. The solution is boiled on a hot plate. The two preparations were then centrifuged. Finally, the supernatant obtained was evaporated by speed-vac. Our preparations were stored at -20°C.

**Determination of Polyphenols**

The determination of polyphenols was carried out according to the Folin-Ciocalteu method optimized by Musci and Yao [11]. Briefly, two ml of Na2CO3 (2%) were added to 100 μl of each test extract (1 mg/ml). After incubation for 2 min at ambient temperature, 100 μl of Folin solution (50%) were added and the mixture was incubated for 30 min at room temperature. Finally, the optical density was determined at 765 nm against a blank sample containing 100 μl of the solvent. The percentage of polyphenols of the fractions studied was calculated relative to the standard range of gallic acid.

**Determination of Flavonoids**

The determination of flavonoids in our extracts was carried out according to the method described by Zhishen and al. with a slight modification [12]. 500 μl of the extract and fractions were mixed with 1.5 ml of 99.9% ethanol, 100 μl of 1 M potassium acetate, 100 μl of 10% aluminum chloride. Then, the volume was adjusted to 2.5 ml with distilled water. The solution was incubated for 30 min at room temperature and the absorbance was measured at 415 nm against a blank sample without the extract. The percentage of flavonoids was determined relatively to Quercetin standard curve.

**Determination of Tannins**

The quantification of the tannins at the level of the extract was carried out according to Hajimahmoodi et al. with slight modification [13]. Briefly, 50 mg of the extract were dissolved in 500 μl of ethyl acetate. The solution was stirred every 10 min. The mixture was then centrifuged for 15 minutes at 4000 rpm and the supernatant was diluted in 1 ml of solvent. 10μl of Folin Dennis reagent and 25μl of 7 % (w/v) Na2CO3 solution were added to 50μl of diluted supernatant. The volume is adjusted to 500 μl with H2O then incubated at room temperature for one hour. Finally, the absorbance was measured at 760 nm as referring to a blank containing 50 μl of sterile water instead of the extract.
DPPH Radical Scavenging Activity

The DPPH assay is one of the most common assays that aim to prove the antioxidant potential of plant extracts. The scavenging activity of DPPH radical by CAN and FEN was examined by the method of Yang [14]. Volumes of 1 ml solutions with different concentrations (µM) were added to 50 mM DPPH in ethanol. After incubation at 60°C for 30 min, the absorbance (A) of the resulting solution was determined at 517 nm. The percentage of DPPH radical scavenging activity (RSA), was calculated using the following formula:

\[
RSA(\%) = \frac{A_{DPPH} - (A_{sample} - A_{control})}{A_{DPPH}} \times 100
\]

Measuring Cell Death Rate

Fluorescein diacetate (FDA) fluorescent probe was used to assess cell death. After treatment with CTN (at concentrations corresponding to IC50=150 µM) in the presence or absence of FEN or CAN at concentrations corresponding to their EC50’s, the cells were incubated with 0.2 µg/ml of FDA for 5 min at 37°C. Cell viability was then analysed by flow cytometry. The FDA is cleaved by viable cell esterases into a fluorescent product. During late events of cell death, esterases are no longer active and the FDA is not cleaved and therefore, does not fluoresce.

Measurement of Mitochondrial Membrane Potential

After incubation with CTN (at concentrations corresponding to IC50=150 µM) in the presence or absence of FEN or CAN at concentrations corresponding to their EC50’s, the cells were labelled for 30 minutes at 37°C with 100 nM DiOC6(3). For each sample analyzed, the integrity of the plasma membrane is evaluated by measuring the permeability of the cells to propidium iodide (10 µg/mL final), added to the sample a few minutes before the measurement in cytometry.

Statistical Analysis

Each experiment was performed separately in triplicate. Values were presented as means ± S.D. One-way ANOVA was used to assess differences among groups followed by Dunnett’s post hoc test. Differences were considered significant at p < 0.05 using Spss program.

Results

Aqueous Extracts Content in Polyphenols, Flavonoids and Tannins

Our results have shown that the aqueous FEN extract presented the highest polyphenol content which was expressed in micrograms of gallic acid equivalent per milligram of extract “µg EAG/mg extract” (10.37 µg EAG / mg). This result confirms the high content of FEN in polyphenolic substances. However, CAN has shown a lower amount of polyphenols (7.88 µg EAG / mg). Likewise, FEN has a more or less significant quantity of flavonoids which is expressed in micrograms of quercetin equivalent per milligram of extract “µg EQ / mg of extract” (4.52 µg EQ / mg) and a high amount of tannins which is expressed in micrograms of tannic acid equivalent per milligram of extract “µg EAT / mg extract” (12.05µg EAT / mg). Meanwhile, CAN which has shown higher quantities of flavonoids (6.61 µg EQ / mg) and tannins (15.12µg EAT / mg) (Table 1).

Table 1: Secondary metabolites (polyphenols, flavonoids and tannins) content of aqueous extracts of Cinnamon (CAN) and Fenugreek (FEN), determined by specific colorimetric assays.

| Metabolites | Aqueous extract of CAN | Aqueous extract of FEN |
|-------------|------------------------|------------------------|
| Polyphenols  | 7.88 µg                | 10.37 µg               |
| Flavonoids   | 6.61 µg                | 4.52 µg               |
| Tannins      | 15.12 µg               | 12.05 µg               |

DPPH Radical Scavenging Activity

![Figure 1: Percentage inhibition of the DPPH radical in the presence of different concentrations of cinnamon (CAN) extract. The corresponding EC50 determined is 25µG. Results are expressed as the mean ± SD of three independent experiments.](image-url)
The results of this test were expressed as percentage of anti-free radical activity. They can also be expressed using the parameter EC50, which is defined as the concentration of the substrate which causes a loss of 50% of the activity of DPPH [15]. We have noticed that the different extracts have the capacity to reduce the free radical DPPH (purple in color) to the yellow radical DPPH-H in a dose-dependent manner (Figures 1 & 2). That is to say, the percentage of DPPH inhibition increased with the concentration of the extracts up to a threshold or the percentage of inhibition stabilizes with the increase in the concentration of the extracts. Indeed, for cinnamon (CAN) we have obtained an inhibition rate of 27.19%, 49.38%, 54.38% and 68.99% respectively for the concentrations 5µG / ml, 25µG / ml, 50µG / ml and 200µG / ml. Similarly for fenugreek (FEN), we have an inhibition of 31%, 34%, 50% and 64% respectively for the concentrations 10µG / ml, 25µG / ml, 50µG / ml and 200µG / ml.

**Figure 2:** Percentage of inhibition of the DPPH radical in the presence of different concentrations of fenugreek (FEN) extract. The corresponding EC50 determined is 50µG. Results are expressed as the mean ± SD of three independent experiments.

### Citrinin Induced Cell Mortality and Prevention by Fenugreek and Cinnamon

Pretreatment of HCT-116 cells with the aqueous extract of fenugreek at 50 µG / ml (EC50) and with the aqueous extract of cinnamon at 25 µG / ml (EC50) have shown a significant reduction in cells mortality. In fact, CAN at 25µG / ml diminished citrinin-induced mortality by more than 30% when compared to cells treated with CTN alone. Similarly, FEN prevented citrinin-induced mortality by approximately 30% when compared to cells treated with CTN alone (Figure 3).

**Figure 3:** Percentage of inhibition of citrinin-induced mortality following pretreatment with aqueous cinnamon and fenugreek extracts. Results are expressed as the mean ± SD of three independent experiments. * P<0.001 vs control, ## P<0.05 vs CTN alone.
Prevention of Citrinin Induced Mitochondrial Membrane Potential Drop by Cinnamon and Fenugreek

Pre-treatment of HCT-116 cells with EC50s of cinnamon or fenugreek have shown a decrease in percentage of Dioc (-) cells. The preventive effect of cinnamon at 25µG / ml against the fall in Δψ is attained with a prevention percentage of 50% when compared to cells treated with CTN alone. Similarly for fenugreek, also has a preventive potential which restores the mitochondrial potential of cells with CTN, the percentage of prevention is 50% compared to cells treated with CTN alone (Figure 4).

Figure 4: Percentage of inhibition of citrinin-induced mitochondrial potential drop following pretreatment with aqueous cinnamon (CAN) and fenugreek (FEN) extracts assessed by DIOC6 (3) assay. Results are expressed as the mean ± SD of three independent experiments. * P<0.001 vs control, ## P<0.05 vs CTN alone.

Discussion

Since ancient times, man has used plants, either to feed or to heal himself. After many successive gropings over centuries, a first distinction has been made between edible and toxic plants. Accumulated empirical knowledge has allowed different civilizations to take plants as an essential source of medicines. Until the beginning of the 20th century, almost all drugs were of plant origin. A number of environmental pollutants are known to cause imbalance between the formation and removal of free radicals, thus leading to oxidative stress. The ensuing oxidative stress causes damage to membrane lipids, proteins and DNA. Many studies have demonstrated that CTN induced changes in oxidative status and induced free radicals generation. These free radicals are at the origin of a reaction cascade that fragments membrane lipids, and consequently contributes to the destabilization of membrane structures.

Oxidative stress is important as a direct and indirect initiator as well as a promoter of the apoptotic process which is due either to the deficiency of cellular antioxidant defence systems [16,17] or to overproduction of oxygenated free radicals that exceed their antioxidant capacity [5]. As a result, molecules with antioxidant properties can cope with stress induced by mycotoxins and restore the balance of oxidants / antioxidants. An antioxidant can be defined as any substance capable, at a relatively low concentration, of competing with other oxidizable substrates and thus retarding or preventing the oxidation of these substrates [18]. Vitamins have been tried to prevent the toxic effects of mycotoxins. Indeed, vitamins, particularly vitamin E which is a free radicals scavenger, has been shown to prevent the cytotoxicity of CTN, zearalenone and T-2 toxin [19].

On the other hand, the protective role of food components such as fructose chlorophylls or piperine was proved against deleterious effects of mycotoxins in general [20]. Resveratrol also showed a preventive effect against cytotoxicity and apoptosis induced by citrinin in Hep G2 cell line [21]. Even more recent studies have shown a significant preventive effect of the antioxidant-rich extract of cactus (Opuntia ficus-indica) in the protection against the genotoxicity induced by the fusarial toxin zearalenone. This was evidenced by the reduction in micronuclear induction, the decrease in the frequency of chromosomal aberrations and DNA fragmentation [22]. This extract was also effective as a hepatoprotector against the toxicity of aflatoxin B1 as demonstrated by the decrease in the level of MDA and the levels of expression of Hsp 70 and Hsp 27, as well as the decrease in expression of pro-apoptotic p53 and Bax proteins [23].

Another study has also demonstrated the efficiency of the aqueous extract of garlic (Allium sativum), with antioxidant activity, against the cytotoxicity and genotoxicity of zearalenone induced in vitro on Vero renal cells [24]. The direct use of condiments and plant extracts in cultured cells to counteract the deleterious effect of toxicants has proven its relative efficiency in our study. FEN (Trigonella foenum-græcum L) is widely used in alternative medicine. It’s a common ingredient in many dishes all over the world.
and often taken as a supplement. CAN (Cinnamomum zeylanicum) is a delicious spice used in a wide variety of sweet and savoury dishes. It has been prized for its medicinal properties and has a plethora of impressive health benefits. The richness of FEN and CAN in secondary metabolites known in the literature for their important biological and pharmacological activities [25,26] have incited us to test the content of the two prepared extracts FEN and CAN in antioxidants using colorimetric assays.

The distribution of secondary metabolites may change during the development of the plant. This can be linked to harsh climatic conditions (high temperature, sun exposure, drought, salinity), which stimulate the biosynthesis of secondary metabolites such as polyphenols [27]. Indeed, the phenolic content of a plant depends on a number of factors intrinsic (genetic) and extrinsic (climatic conditions, cultural practices, maturity at harvest and storage conditions) [27,28]. By comparing the anti-free radical activities of CAN and FEN, we can say that the percentage of inhibition of the DPPH radical in the presence of FEN is lower compared to that of CAN (Figure 1). This shows a possible correlation between the activity of the aqueous extract of FEN and its flavonoid content. The low flavonoids content of fenugreek (in comparison with CAN) is probably the cause of its lower anti-free DPPH radical activity.

Indeed, the interaction of flavonoids with many radicals has been used in several studies to determine the major elements of antioxidant activity. Because of their low redox potentials, the flavonoids (FL-OH) are thermodynamically capable of reducing oxidizing free radicals such as superoxide, peroxide, alkoxyl and hydroxyl by hydrogen transfer [29]. The anti-free radical activity has been correlated with the oxidation potential of flavonoids [30]. Moreover, the cytotoxic assays have brought an additional argument to the protective potential of FEN and CAN aqueous extracts. Pretreatment of HCT-116 cells with the aqueous extract of fenugreek at 50 μg/ml (EC50) and with the aqueous extract of cinnamon at 25 μg/ml (EC50) have shown a significant reduction in the mortality of cells as compared to those treated with CTN alone. The same effect was observed relatively to mitochondrial potential drop $\Delta\psi_m$ which is compromised early during stress response and a key element of ongoing cell death. Likewise this potential drop is a point of no return and it can induce cell death by apoptosis or by necrosis depending on severity of the mitochondrial damage [31,32]. The ability of cinnamon and fenugreek extracts to prevent and protect against the fall of mitochondrial potential is due to their antioxidants content such as flavonoids and polyphenols detected in both extracts (Table 1). By comparing the preventive effect of CAN and FEN, we noticed that these two extracts are capable of preventing the fall of $\Delta\psi_m$. These effects confirm the undeniable capacity of flavonoids to limit the production of ROS [33,34]. The flavonoids present on the surface of the membranes are also capable of regenerating Vitamin E, one of the essential antioxidants involved in the protection of cell membranes [35-37]. The results of these studies need to be confirmed by an in vivo study on an animal model, a model presenting all the enzymes necessary for metabolism, to better assess the level of protection of these two extracts both at the animal and at the human level.

**Conclusion**

On HCT-116 cells in culture, we have shown in previous reports, that CTN induced a cytotoxic effect. Indeed, it was able to inhibit cell viability, it disrupted the mitochondrial function. However, pre-treatment of cells with aqueous extracts of CAN and FEN two medicinal plants rich in flavonoids and polyphenols has counteracted these toxic effects. Both extracts were proved capable of ameliorating cell viability and restoring the $\Delta\psi_m$ transmembrane potential in comparison with cells treated with CTN alone which testifies the preventive effectiveness of CAN and FEN.

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