Evaluation of Citrinin Occurrence and Cytotoxicity in *Monascus* Fermentation Products

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Monascus purpureus and its fermentation products have been used in food coloring and meat preservation in Asia for centuries and have also been recently used as dietary supplements because of their cholesterol-lowering ability. However, the presence of the mycotoxin citrinin (CTN), a secondary metabolite of *Monascus* species, in fermentation products is a potential threat to public health. In the present study, HPLC was used to analyze CTN levels in lipid and aqueous extracts of commercialized *Monascu*s products. CTN was detected in lipid extracts of all examined samples at concentrations varying between 0.28 and 6.29 μg/g, but was not found in aqueous extracts. When human embryonic kidney cells (HEK293) were incubated for 72 h with *Monascus* extracts, the concentrations causing 50% cell death by all lipid extracts were in the range of 1.8−4.7 mg/mL, whereas aqueous extracts showed a lower cytotoxicity. Incubation of HEK293 cells with 60 μM pure CTN for 72 h caused cell viability to fall to 50% of control levels. In addition, coadministration of pure CTN and lipid extracts from *Monascus* samples significantly enhanced CTN cytotoxicity for HEK293 cells using the MTT assay. These results provide the first information about the cytotoxic effects of various *Monascus* samples and CTN−*Monascus* mixtures on a human cell line.

KEYWORDS: Citrinin (CTN); *Monascus* fermentation products; HPLC; cytotoxicity

INTRODUCTION

Citrinin (CTN) (**Figure 1**) is a secondary fungal metabolite produced by several *Penicillium* and *Aspergillus* species (*1*) and is frequently found as a contaminant of cereal grains, including wheat, corn, rice, and barley (*1*, *2*). CTN-induced nephrotoxicity has been demonstrated in various cellular and animal models (*3−5*). Studies using rat renal proximal tubules have shown that CTN has multiple effects on mitochondrial functions (*6*). Possible toxicological mechanisms of CTN in renal and liver mitochondria include interference with the electron transport system, alteration of Ca^{2+} homeostasis, and generation of oxidative stress (*6−9*). CTN also induces chromosomal abnormalities in the bone marrow cells of mice (*10*) and shows aneuploidogenic potential in V79 cells (*11*). Moreover, oral administration of CTN to male rats leads to the formation of renal adenomas (*12*). However, CTN has no effect on the frequency of sister chromatid exchange in mammalian cells, including human lymphocytes (*13*, *14*).

Monascus fermentation products, also known as red yeast rice, are the fermented products of cooked, nonglutenous rice
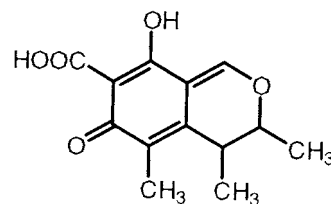

Figure 1. Structure of citrinin (CTN).

on which a particular yeast strain, *Monascus purpureus*, has been grown. *M. purpureus* and its fermentation products have been used as a food colorant and flavor enhancer and in meat preservation and wine brewing in the Orient for centuries (*15*, *16*). In addition to starch, protein, sterols, and fatty acids, *Monascus* fermentation products contain numerous active constituents, including monacolin K, γ-aminobutyric acid (GABA), and dimerumic acid (*17−20*). Monacolin K (lovastatin, mevinolin) has been shown to modulate cholesterol production by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the liver (*21*, *22*). GABA is known to be an antihypertensive agent (*23*, *24*), whereas dimerumic acid has antioxidant and hepatoprotective actions against chemically induced liver injury in mice (*17*, *18*). Extracts of *Monascus* products also suppress tumor promotion in two-stage carcinogenesis in mice (*25*, *26*). Results of several studies in

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animals and humans suggest that *Monascus* fermentation products can effectively regulate serum cholesterol and triglyceride levels (*27*−*30*), and they are therefore used as dietary supplements for medical therapy (*27*).

In 1995, Blanc et al. (*31*, *32*) demonstrated that one of the pigments produced by *Monascus* was identical in structure to CTN, and Sabater-Vilar et al. (*33*) reported that CTN was detectable in extracts of *Monascus* products purchased in The Netherlands. Although CTN is a possible contaminant of *Monascus* dietary supplements, little information is available regarding its presence in *Monascus* fermentation products or about the cytotoxic effect of CTN in these products. It is reasonable to speculate that the presence of various biological components in extracts of *Monascus* products may increase or reduce CTN cytotoxicity. More information is required to assess the risk of CTN in *Monascus* products and to ensure the long-term protection of the public consuming these dietary supplements, so the present study measured the CTN levels in commercial *Monascus* products collected in Taiwan and evaluated the cytotoxic effect of CTN mixed with different *Monascus* extracts on human embryonic kidney cells.

MATERIALS AND METHODS

Reagents. Pure CTN and all other chemical reagents were from the Sigma Chemical Co. (St. Louis, MO). CTN was dissolved in 25% ethanol at a concentration of 10 mM and stored at −20 °C. All solvents used in analytical chromatography were of HPLC grade. Cell culture media and serum were from Life Technologies (Grand Island, NY). Six commercial *Monascus* fermentation products, two in rice form (samples H and C), one in tablet form (sample W), and three in capsule form (samples D, L, and K), were obtained from retail stores in Taiwan. Fermentation samples in rice form are considered to be relatively raw material compared to those in tablet or capsule forms, which have been further processed and mixed with certain stabilizers or nutrients by the manufacturers.

Preparation of Lipid Extracts from *Monascus* Fermentation Products. *Monascus* products in rice or tablet form (20−30 g) were homogenized for 3 min in a blender, and then 2.5 g of homogenate or of *Monascus* powder removed from capsules was transferred to a flask and 20 mL of extraction solvent (acetone/ethyl acetate, 50:50, v/v) was immediately added. After shaking for 90 min in a 65 °C water bath, the mixture was centrifuged at 5300g for 20 min at room temperature, and then the supernatant solution was dried in a rotary evaporator with the water bath set at 65 °C. The dry solid was redissolved in 2.5 mL of ethanol to generate the lipid extract; 1 μL of the fraction was roughly equivalent to the extract from 1 mg of sample powder.

Preparation of Aqueous Extracts from *Monascus* Products. Powdered samples (2.5 g) obtained from *Monascus* products were extracted with 20 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.5) using the same conditions as described above. After centrifugation, the supernatant was evaporated to dryness on a rotary evaporator using a 65 °C water bath. The dry solid was redissolved in 2.5 mL of 0.01 M PBS (pH 7.5) to give the aqueous extract; 1 μL of this fraction was roughly equivalent to the extract from 1 mg of sample powder.

HPLC Analysis of CTN. Authentic CTN, six commercialized *Monascus* samples naturally contaminated with CTN, and one sample lipid extract spiked with authentic CTN standard were subjected to HPLC analysis according to the method of Marti et al. (*34*). A Beckman System Gold instrument (Fullerton, CA) equipped with a 126 solvent module and an FP-1520 fluorescence detector (Jasco, Japan) was used. The CTN standard and sample extracts were diluted 10- or 100-fold with methanol and passed through a 0.45 μm filter (low protein binding; Gelman Science, Ann Arbor, MI) prior to HPLC. A Lichrospher C18 reverse-phase column (5 μm particle size, 4.0 mm × 250 mm; Merck) in conjunction with a Lichrospher C18 guard column (5 μm particle size, 4.0 × 40 mm; Merck) was equilibrated with a mobile phase of methanol/0.25 M orthophosphoric acid, pH 2.5 (70:30, v/v) at a flow rate of 0.6 mL/min. Chromatograms were monitored using the fluorescence detector set at an excitation wavelength of 330 nm and an emission wavelength of 500 nm, and the absorbance data were analyzed using Beckman System Gold Nouveau software. A calibration curve was generated using authentic CTN of 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg/mL (R^2 = 0.99); each injection was 20 μL. The lowest detectable limit for the CTN standard was estimated to be 1 ng, which is equivalent to 20 μL of 0.05 μg/mL per injection.

Cell Cultures for the Cytotoxicity Assay. The human embryonic kidney cell line, HEK293, was obtained from the Bioresources Collection and Research Center in Taiwan and was maintained in minimal essential medium (MEM) supplemented with 10% horse serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. HEK293 cells were seeded at 2 × 10^3 cells/well in quadruplicate in 96-well tissue culture plates (Falcon, Franklin Lakes, NJ) and allowed to attach for at least 16 h to give monolayer cultures.

To evaluate the cytotoxic effects of extracts of *Monascus* products, the culture medium was replaced with 100 μL of MEM containing 1% horse serum plus vehicle alone (extract solvent or PBS) or various concentrations of *Monascus* sample extracts (0.25−10 μL of extract, equivalent to 0.25−10 mg of sample powder, diluted in 1 mL of culture medium) for 72 h before lactate dehydrogenase assay.

To study the cytotoxicity of CTN mixed with components of *Monascus* samples, HEK293 cultures were divided into three groups for treatment with either sample K or sample C for 72 h: group 1, 25% ethanol (control) or various concentrations of pure CTN (20−60 μM); group 2, lipid extracts (1 μL, equivalent to 1 mg of sample per mL of culture medium) plus either 25% ethanol (control) or CTN (20−60 μM); group 3, aqueous extracts (1 μL, equivalent to 1 mg of sample per mL of culture medium) plus either 25% ethanol (control) or CTN (20−60 μM). Cell viability in each group was determined with LDH or MTT assay as follows and then calculated by dividing the values of CTN-treated cultures by that of the corresponding control in the same group. All of the data were then normalized to the 25% ethanol control in group 1 and expressed as a percentage of the ethanol control value. The lipid and aqueous extracts at the concentration of 1 mg/mL in culture medium showed no significant cytotoxic effect on HEK293.

Determination of Cellular LDH Activity. Cellular LDH activity was determined using an LDH Cytotoxicity Detection Kit (Takara Bio Inc.), which is based on a colorimetric method developed by Cabaud and Wroblewski (*34*) and modified to be suitable for the microplate reader format. Because our preliminary experiments showed that the presence of *Monascus* extracts in culture medium interfered with the standard LDH assay, intracellular LDH activity, rather than LDH leakage from damaged cells, was used to determine cell viability in the present study. After 72 h of treatment with either CTN (or 25% ethanol as control) or sample extracts, the culture medium was removed and the monolayers were solubilized at room temperature for 5 min with 100 μL of 1% (v/v) Triton X-100, and then 100 μL of LDH reaction reagent was added to the cell lysate. After incubation at room temperature for 20 min, the reaction was terminated by adding 50 μL of 1 N HCl, and the absorbance was measured in a Vmax automatic ELISA reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 490 nm with background subtraction at 650 nm. Cell viability was calculated from the absorbance generated by intracellular LDH activity and expressed as a percentage of that from control cells treated with vehicle (25% ethanol) only.

MTT Reduction Assay. The MTT assay is a colorimetric method using metabolic competence as an indicator of cell viability (*36*). This method assesses the ability of the cell to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. After 72 h of treatment with CTN (or vehicle as control) in the presence or absence of lipid extracts or aqueous extracts (1 mg/mL), the culture medium was replaced with 200 μL of medium containing 0.5 mg/mL of MTT and the plates were incubated for 4 h at 37 °C; then the medium was removed and replaced with 100 μL of 2-propanol to solubilize the converted purple dye on the culture plates. The absorbance was measured on an Optimax microplate reader (Molecular Devices) at a wavelength of 570 nm with background subtraction at 690 mm.

Statistic Analysis. Values are presented as the means ± SEM. Statistical differences between control and treated groups were evaluated
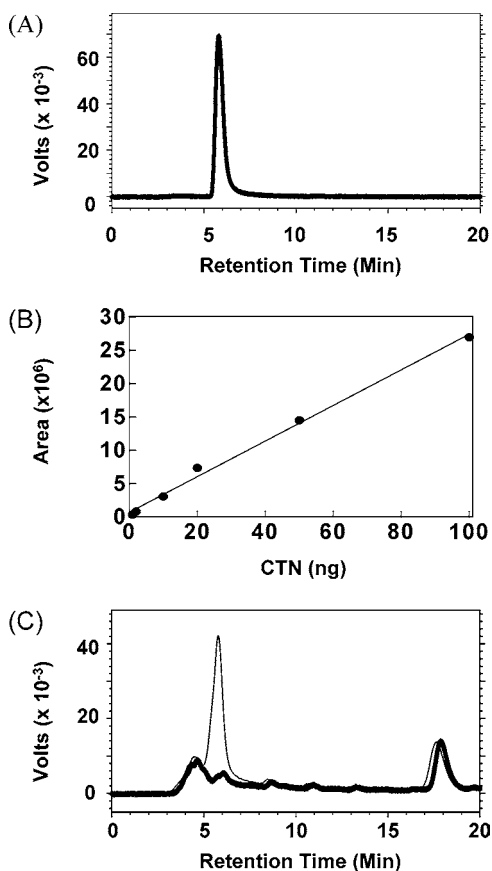
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(A)

(B)

(C)

Figure 2. (**A**) HPLC chromatogram of 10 ng of authentic CTN. (**B**) Calibration curve generated using 1, 2, 10, 20, 50, and 100 ng of authentic CTN ($R^2 = 0.99$). (**C**) HPLC chromatogram of lipid extract of *Monascus* sample H alone (thick line) or spiked with 5 ng of authentic CTN (thin line).

using Student's *t* test. Differences were considered to be significant when the *p* value was <0.05.

RESULTS

HPLC Analysis of CTN in *Monascus* Fermentation Products. The CTN standard, one white rice sample as a control, and six commercialized *Monascus* fermentation products were analyzed for the presence of CTN by HPLC. The CTN standard was clearly identified with a retention time of 6.0 min (**Figure 2A**). To calculate the CTN level in each sample, a calibration curve was generated using CTN standards of 1, 2, 10, 20, 50, and 100 ng ($R^2 = 0.99$) (**Figure 2B**). The lipid extract of sample H, roughly equivalent to 0.2 mg of sample powder, was spiked with 5 ng of CTN and reanalyzed to confirm the presence of CTN. Both of the lipid extracts of sample H and the CTN-spiked sample showed a peak with the retention time of 6.0 min expected for CTN (**Figure 2C**). Analysis of the lipid extracts from the six *Monascus* products showed that all contained CTN at levels ranging from 0.28 to 6.29 μg/g (**Table 1**), but CTN was not detected in the aqueous extracts of these products (data not shown). Sample H in rice form contained the highest level of CTN (6.29 μg/g), whereas samples L, K, and D in capsule form contained <1 μg/g of CTN. No CTN was found in either lipid or aqueous extract of the white rice sample.

Cytotoxic Effects of Lipid and Aqueous Extracts of *Monascus* Fermentation Products. Although CTN was found only in lipid extracts of the six *Monascus* products and not in the aqueous fractions, it was possible that cytotoxic metabolites

Table 1. HPLC Analysis of CTN in the Lipid Extracts of *Monascus* Fermentation Products

sample tested	CTN detected[a] (μg/g)
H (rice)[b]	6.29 ± 0.83
C (rice)	2.83 ± 0.25
W (tablet)	1.32 ± 0.38
D (capsule)	0.61 ± 0.07
L (capsule)	0.50 ± 0.03
K (capsule)	0.28 ± 0.07
white rice	ND[c]

[a] Data are means ± SEM derived from two separate experiments. [b] Terms in parentheses describe the original form of each sample. [c] Not detected.
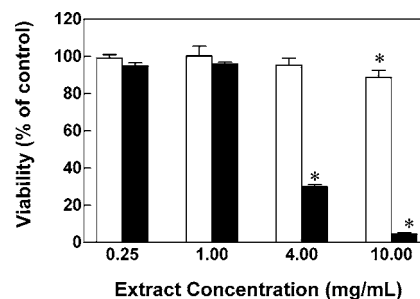

Figure 3. Cytotoxic effects of aqueous and lipid extracts of *Monascus* sample K on HEK293 cells. HEK293 cell cultures were incubated for 72 h with various concentrations of aqueous (white bars) or lipid (black bars) extracts. Cell viability was determined on the basis of the levels of intracellular LDH activity and expressed as a percentage of that of control cells exposed to vehicle (25% ethanol in PBS) only. The data shown are the means ± SEM for four independent experiments. An asterisk (∗) indicates significant difference compared to controls (*p* < 0.05).

other than CTN were present in the aqueous extracts. Therefore, lipid and aqueous extracts of sample K (0.25−10 mg/mL) were tested for their cytotoxic effects on HEK293 cells. As shown in **Figure 3**, the lipid extract at 4.0 mg/mL caused a significant decrease in the overall viability of the cells to 30% of control levels, whereas the aqueous extract resulted in a decrease to ∼95% of control levels, showing that the lipid extract of *Monascus* sample K was more cytotoxic toward HEK293 cells.

When the lipid extracts from the other five *Monascus* products and a white rice sample (negative control) were tested, the white rice extract showed no statistically significant cytotoxicity at any concentration except 10 mg/mL (**Figure 4A**), whereas those from the two rice forms (samples C and H) showed marked cytotoxicity at a concentration of 5 mg/mL, causing a decrease in the number of viable cells to 45 and 5% of control levels, respectively (**Figure 4A**). When HEK293 cultures were treated with the lipid extracts obtained from the samples in capsule or tablet form (samples L, D, and W), the dose causing 50% inhibition of cell viability was found at ∼3.8 mg/mL for each sample (**Figure 4B**).

Cytotoxicity of CTN in the Presence of Lipid and Aqueous Extracts. To investigate whether the cytotoxicity of CTN was modulated by other components of *Monascus* products, HEK293 cell cultures were treated with CTN (0−60 μM) in the presence or absence of lipid or aqueous extracts of *Monascus* samples K (capsule) or C (rice). The percentage of viable cells compared to the vehicle-treated group was measured with the LDH activity and MTT reduction assays. As shown in **Table 2**, the reduction in the viability of HEK293 induced by pure CTN was concentration-dependent, and cell viability fell to ∼50% of the control at the level of 60 μM. When the MTT assay was used, the presence of the lipid extract of sample K or C resulted in a

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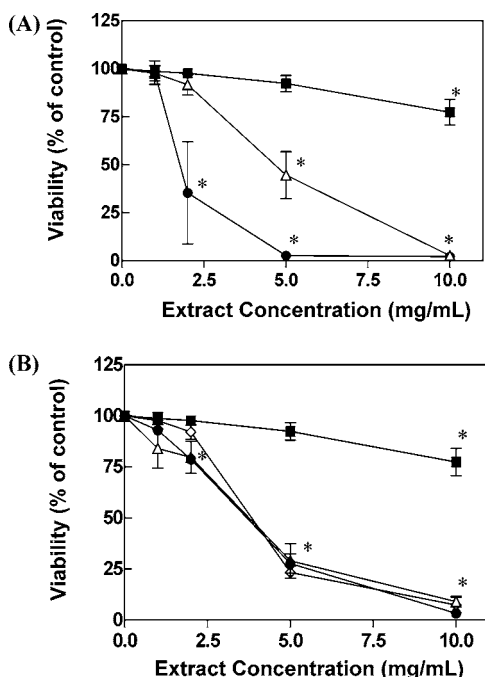*J. Agric. Food Chem.,* Vol. 53, No. 1, 2005 **173**

Figure 4. Cytotoxicity induced by lipid extracts obtained from various *Monascus* fermentation products. HEK293 cells were incubated for 72 h in medium containing lipid extracts of (**A**) white rice (■), sample C (△), and sample H (●) or of (**B**) white rice (■), sample W (●), sample D (◇), and sample L (△). Cell viability was determined using the intracellular LDH activity assay and expressed as a percentage of that of control cells exposed to vehicle only. The data shown are the means ± SEM for at least three independent experiments. An asterisk (∗) indicates significant difference compared to controls ($p < 0.05$).

Table 2. Cytotoxic Effect of Pure CTN and CTN–*Monascus* Mixtures[a]

	cell viability[b] (% of control)	
	LDH activity	MTT assay
20 μM CTN alone	106.3 ± 8.8	114.7 ± 8.8
20 μM CTN + lipid extract-K	92.0 ± 2.2	84.0 ± 8.8*
20 μM CTN + aqueous extract-K	95.5 ± 2.6	107.5 ± 7.2
20 μM CTN + lipid extract-C	98.8 ± 2.9	105.1 ± 5.7
20 μM CTN + aqueous extract-C	95.1 ± 7.9	108.5 ± 7.0
40 μM CTN alone	84.5 ± 9.3	102.8 ± 7.8
40 μM CTN + lipid extract-K	61.5 ± 4.0	61.0 ± 5.0 *
40 μM CTN + aqueous extract-K	78.0 ± 3.7	95.1 ± 12.3
40 μM CTN + lipid extract-C	68.5 ± 6.9	62.5 ± 7.1 *
40 μM CTN + aqueous extract-C	76.5 ± 8.9	102.7 ± 9.7
60 μM CTN alone	50.8 ± 7.7	49.8 ± 5.1
60 μM CTN + lipid extract-K	39.6 ± 7.5	47.3 ± 1.8
60 μM CTN + aqueous extract-K	51.6 ± 5.4	54.8 ± 8.9
60 μM CTN + lipid extract-C	45.0 ± 3.5	36.2 ± 2.3 *
60 μM CTN + aqueous extract-C	51.5 ± 3.6	44.9 ± 12.78

[a] HEK293 cultures were incubated for 72 h with CTN (or 25% ethanol in PBS as control) in the presence or absence of aqueous and lipid extracts (1 mg/mL) obtained from sample K or C. Values are means ± SEM of four independent experiments conducted each in quadruplicate with any concentration of CTN. [b] Cell viability (percent of control) of each group was calculated according to the description under Materials and Methods. An asterisk (*) indicates significant difference ($p < 0.05$) compared to the CTN alone group at the same concentration.

significant enhancement of the cytotoxicity of 20 and 40 μM CTN or 40 and 60 μM CTN, respectively, but the difference was not significant using the LDH activity assay. On the other hand, the coadministration of CTN and aqueous extracts of samples K or C did not elicit any significant change in the cytotoxicity of CTN toward HEK293.

DISCUSSION

Monascus fermentation products are considered to be natural dietary supplements for heart disease prevention in addition to prescription drugs, because recent clinical studies have demonstrated that *Monascus*-fermented rice can significantly lower triglyceride and cholesterol levels in some individuals (*29, 30*). Some commercialized *Monascus* products are in rice form, whereas others are available in the form of capsule or tablet in which fermented rice is mixed with certain nutrients or stabilizers. The use of different yeast strains and fermentation conditions may lead to variability in the chemical profile and contents, including the levels of toxic secondary metabolites (*37, 38*). Because a daily intake of 1−2 g as a dietary supplement is suggested by the manufacturers, the long-term safety of the consumption of *Monascus* fermentation products should be extensively evaluated.

CTN was found at levels ranging from 0.28 to 6.29 μg/g in the lipid extracts of all examined *Monascus* products by HPLC (**Table 1**), but was not detectable in any of the aqueous fractions due to its low water solubility (*31, 32*). Similarly, Sabater-Vilar et al. (*33*) reported that *Monascus* samples collected in The Netherlands were contaminated with CTN at concentrations varying between 0.2 and 17.1 μg/g. Although different organic solvents and HPLC conditions were used for CTN extraction and analysis, these results strongly suggest the presence of CTN in various *Monascus* samples. In the present study, compared to the samples in tablet and capsule form, the rice form samples, H and C, contained higher levels of CTN. This may be because *Monascus* samples in rice form are generally not generated under good quality control and also undergo less processing after fermentation.

The lipid extract of sample K was more toxic for human cells than the corresponding aqueous extract; the addition of 4 μL (equivalent to 4 mg of sample powder) of lipid extract to 1 mL of culture medium reduced cell viability to 30% of the control level (**Figure 3**). Similar results were observed using the lipid extracts from the other five *Monascus* samples, but not that from white rice (**Figure 4A**,**B**), suggesting that the cytotoxicity was due to certain cytotoxic compound(s) in the sample and not due to the extraction solvent. Because the CTN concentration in sample K was only 0.28 ± 0.07 μg/g of sample (**Table 1**), 4 mg of sample K powder in 1 mL of medium should give a CTN concentration of only 1.1 ng/mL. However, as shown in **Table 2**, a concentration of at least 60 μM (∼15 μg/mL) of pure CTN was required to reduce HEK293 cell viability to 50% of that of controls, in comparison with the 30% seen with the sample K lipid extract (1.1 ng/mL of CTN), indicating that one or more unidentified factors other than CTN contribute to the cytotoxicity of *Monascus* lipid extracts. This was also supported by the fact that the CTN content of the lipid extracts shown in **Table 1** did not directly correlate with the cytotoxicity of the sample shown in **Figures 3** and **4**.

The cytotoxic effect of pure CTN on HEK293 was significantly enhanced using the MTT assay when the lipid extracts of samples K and C were present in the culture medium (**Table 2**). Possible toxic interactions between mycotoxins have been documented (*39−41*), and synergistic toxicity was seen on simultaneous administration of aflatoxin and T-2 toxin to male broiler chicks (*40*). Nevertheless, although several lipophilic compounds have been identified in *Monascus*, including various pigments and biological agents with tumor suppression or cholesterol reduction ability (*25−30*), the identity of the component(s) interacting with CTN and increasing its toxicity is not known. Sabater-Vilar et al. (*33*) reported that pure CTN

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or *Monascus* extracts induce a mutagenic response only after activation by S9-mix or hepatocytes, but it is not known yet whether the cytotoxicity of CTN and *Monascus* extracts can be altered by metabolic activation. Further work will focus on the use of an in vivo system to study the interaction between CTN and *Monascus* products.

On the basis of the present results, CTN is commonly found in *Monascus* fermentation products in rice, tablet, or capsule form, but the CTN levels in these products do not seem to be a major factor contributing to the cytotoxicity caused by *Monascus* extracts. In addition, CTN cytotoxicity is modulated by the presence of *Monascus* lipid extracts. This study is therefore an important first step in evaluating the risk associated with *Monascus* samples and the toxicity of CTN mixed with *Monascus* products.

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