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Original article

Safety evaluation of aflatoxin B_1 in peanut oil after ultraviolet irradiation detoxification in a photodegradation reactor

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Summary The high incidence of aflatoxin B_1 (AFB_1) in peanut oil has caused wide public concern in the world. Many studies have verified that ultraviolet (UV) irradiation can degrade AFB_1 in foods. A new photodegradation reactor has been developed to study the photodegradation efficiency of AFB_1 in peanut oil, and the safety of peanut oil was evaluated after UV irradiation detoxification based on the mutagenicity of *Salmonella typhimurium* tester strains and cytotoxicity of HepG2 cells. The results showed that AFB_1 in peanut oil could be decomposed efficiently using the photodegradation reactor. AFB_1 was decreased from 51.96 ± 4.24 to 7.23 ± 0.59 μg kg^{-1} in 10 min and reduced by 86.08% compared with that of the negative control. The residual AFB_1 in peanut oil was far less than the limit level set by Chinese government (20 μg kg^{-1}). The Ames test and cell viability assay revealed that 10 min of UV irradiation reduced significantly the toxicity of AFB_1 in peanut oil. All the results suggest that the deleterious effects of AFB_1 can be highly reduced by UV irradiation in the photodegradation reactor, and the reactor can be applied in a large scale in detoxification of AFB_1 in peanut oil in the oil industry.

Keywords Aflatoxin B_1, cell viability, mutagenicity, safety evaluation, UV irradiation.

Introduction

Peanut oil is the main edible vegetable oil for consumers in China and other Asian countries owning to its high nutrient content, pleasant flavour, palatability and cooking results. However, newly pressed peanut oil is often contaminated by aflatoxin B_1 (AFB_1) from AFB_1-contaminated peanuts. Lu *et al.* (2014) investigated 401 oil samples from thirteen provinces of China in 2011, and 19.4% of the samples tested show the presence of AFB_1. The overall level of contamination in southern part of China is higher than in the northern region due to the hot and humid southern climate. In addition, Zhang *et al.* (2011) found that the majority of the vegetable oils were contaminated by AFB_1 based on 1000 samples from nearly twenty provinces in China between 2002 and 2008.

AFB_1 belongs to a group of fungal toxins known as mycotoxins, and it is associated with both acute and chronic toxicity in animals and humans, including acute liver damage, liver cirrhosis and liver cancers (Wagacha & Muthomi, 2008; Zain, 2011). Chronic toxicity associated with ingestion of low doses of AFB_1 in peanut oil is of greater concern.

As aflatoxins are known to be genotoxic and carcinogenic, methods for their destruction are of interest. Presently, various physical, chemical and biological methods have been used to decompose aflatoxins in foods (Netke *et al.*, 1997; Das & Mishra, 2000; Haskard *et al.*, 2000). UV irradiation as a physical method has been studied to destroy aflatoxins for many years (Yousef & Marth, 1986; Samarajeewa & Gamage, 1988). However, most studies in UV detoxification were carried out in a mode system (Liu *et al.*, 2011a), and only in a static state for irradiated products (Liu *et al.*, 2011b), so its practical application in the food industry still has a long way to go. In this study, a photodegradation reactor designed by ourselves was used to decompose AFB_1 in peanut oil, which can be continuously operated and applied in a large scale in the oil industry. For the reactor, the choice of UV wavelength and irradiation intensity, the thickness of peanut oil, irradiation time and cooling of oil after being irradiated are all mainly concerned.

Although UV irradiation can decompose AFB_1 in peanut oil (Liu *et al.*, 2011b), the safety of peanut oil after being irradiated is still unknown. In addition, Liu *et al.* (2011a) had found three photodegradation

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products of AFB_1 in aqueous medium and deduced that they have still some toxicity based on their structures. Therefore, the objective of this study was to investigate the photodegradation efficiency of AFB_1 in peanut oil using the photodegradation reactor, and evaluate the safety of peanut oil after being irradiated with the mutagenesis of *Salmonella typhimurium* and cytotoxicity of HepG2 cells. Based on the photodegradation efficiency and safety evaluation of AFB_1 in peanut oil, the reactor can be expected to lead to the detoxification of peanut oil in the oil industry.

Materials and methods

Peanut oil sample

Peanut oil sample was prepared by extruding aflatoxin-contaminated peanuts with a screw press machine (TGF-1; Tiangongfang Co., Ltd., Dongguan, China) in the lipid laboratory, Shandong Agricultural University. The extracted peanut oil was filtered to remove the impurities, which contained 51.96 μg kg^{-1} of AFB_1. AFB_1 standard ($C_{17}H_{12}O_6$, purity > 98%) and HPLC-grade acetonitrile were purchased from Sangon Biotech (Shanghai, China). All other reagents in this experiment were of analytical grade, purchased from Keshang Biochemical Reagents Co., Ltd. (Taian, China).

UV photodegradation reactor and treatment

A UV photodegradation reactor (Fig. 1) was constructed and reaction conditions optimised for the removal of AFB_1 in peanut oil. The reactor includes a fluid conveying system (pump, valve, flow meter and pipe), UV irradiation system (six UV lamps, fluid distributing pipe and fluid guiding plate), water-cooling system, and oil sump. The reactor is operated in a closed irradiation chamber to prevent operators from being injured. The detoxification is done in a recirculation mode by continuously pumping peanut oil into the UV irradiation system. UV irradiation can raise the temperature of oil and deteriorate its quality, so it is necessary to cool it immediately with a water-cooling system after being irradiated. System parameters, such as UV wavelength, irradiation intensity, irradiation time and flow rate of oil, have all been shown to influence the photodegradation rate of AFB_1 in varying degrees. The optimal experimental design and response surface methodology were employed in this regard.

Throughout the study, the UV lamps (power 36 W) with a wavelength of 365 nm and irradiation intensity of 6.4 mW cm^{-2} were used to treat peanut oil. The thickness of oil was <3 mm by controlling its flow rate at 0.55 L min^{-1} with a flow meter (LZM-15GF; Jintai Ltd., Guangzhou, China). Peanut oil was pumped

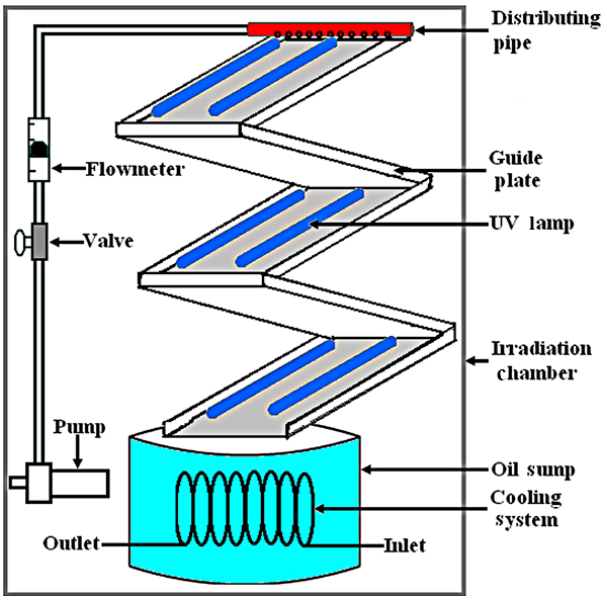

Figure 1 Diagram of UV photodegradation reactor.

(MP-20R; Xinxishan, Shanghai, China) into the irradiation chamber to receive the UV irradiation for 5, 10, 20, and 40 min, respectively. The treated samples were cooled immediately to room temperature with a water-cooling system after being irradiated. The untreated sample was used as the control.

Determination of AFB_1 in peanut oil

AFB_1 in peanut oil was extracted and purified according to the following procedures: 5 g oil sample was transferred to a 125-mL separatory funnel, and then 25 mL methanol–water (55:45, v/v) solution (containing 4% NaCl) and 20 mL *n*-hexane were added. The mixture was shaken and allowed to separate into layers. The extract at the bottom of the funnel (i.e. methanol-water solution containing AFB_1) was taken out and filtered through a 0.45 μm organic filter membrane. Filtrate (4 mL) was collected in a clean centrifuge tube, and then 4 mL of trichloromethane was added to further extract AFB_1 from the methanol-water solution. The trichloromethane solution containing AFB_1 was evaporated to dryness under a stream of nitrogen. The residual AFB_1 was derivatised with 200 μL trifluoroacetic acid at 40 °C for 20 min. Excess trifluoroacetic acid was evaporated to dryness under the stream of nitrogen. The derivative of AFB_1 was redissolved in 1 mL acetonitrile–water (15:85) solution and mixed well by vortexing for 30 s and the mixture was used for HPLC analysis according to the method provided by Diao *et al.* (2013).

Ames test for mutagenesis

The mutagenic activity was conducted by the Ames test using the *Salmonella typhimurium* tester strains TA98 and TA102. The two strains were purchased from the China Center for Type Culture Collection, Wuhan University. The tester strains from frozen cultures were grown for 14 h in Oxoid Nutrient Broth No.2, and checked for characteristics stipulated by Maron & Ames (1983). The metabolic activation mixture (S9) was freshly prepared before each test, which consisted of 4% of S9 fraction, 1% of 0.4 mol L^{-1} $MgCl_2$, 1% of 1.65 mol L^{-1} KCl, 0.5% of 1 mol L^{-1} D-glucose-6-phosphate disodium and 4% of 0.1 mol L^{-1} NADP, 50% of 0.2 mol L^{-1} phosphate buffer solution (PBS) and 39.5% of sterile distilled water (Maron & Ames, 1983).

AFB_1 in the control and UV-treated samples were extracted and purified according to the procedures mentioned above. The extracts were evaporated to dryness under the stream of nitrogen, and then redissolved in dimethyl sulfoxide (DMSO), which used to do the Ames test and cell viability of HepG2 cells.

Various concentrations of AFB_1 in DMSO (0.1 mL) were added to 0.5 mL of 0.2 mol L^{-1} PBS (pH 7.4) or with 0.5 mL of S9 mixture and 0.1 mL of bacterial culture (1.9×10^9 cells mL^{-1}) and then incubated at 37 °C for 30 min. After this time, 2 mL of molten top agar containing 0.5 mmol L^{-1} histidine and biotin was added to the mixture and poured onto a plate containing minimal agar. The plates were incubated at 37 °C for 48 h, and the revertant colonies were counted manually. The mutagenic index (MI) was calculated for each sample tested, this being the average number of revertants per plate with the test compound divided by the average number of revertants per plate with the negative (solvent) control. A sample is considered mutagenic when a dose–response relationship is detected and a twofold increase in the number of mutants ($MI \geq 2$) is observed with at least one concentration. When only one of these criteria is met, the sample is considered to present signs of mutagenicity. AFB_1 in the untreated sample served as positive control in the experiment.

Cell viability for cytotoxicity

HepG2 cell, a well defined human heptoblastoma cell line, which retains many parenchymal cell functions, was maintained in Dulbecco modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and 0.25% penicillin-streptomycin. Cells were grown in an incubator (HF90, Heal Force, Hongkong) at 37 °C under 5% CO_2 and 95% humidified air.

To assess cell viability, HepG2 cells in DMEM media (1.5×10^3 cells mL^{-1}) were seeded in 96-well plates until they were 80% confluent. Culture medium was replenished with new media and then exposed to AFB_1 with different UV irradiation times (5, 10, 20, 40 min) for 15 h. The treated cells by AFB_1 without UV irradiation were used as the positive control, and the untreated cells by AFB_1 as the negative control. They were trypsinised by adding 10 μL of trypsinising solution (0.25% trypsin with 0.1% EDTA and 0.1% glucose) and incubated at the room temperature for 1.5 min to allow cells to detach. Upon cell rounding and detachment from flask, 100 μL of complete culture medium was added to the cell suspension to stop the action of trypsin. Cell viability was assessed by the trypan blue exclusion test using a haemocytometer to manually count the cells (Freshney, 1987). Briefly, 10 μL of 0.5% dye solution was added to 100 μL of treated cells (1.4×10^4 cells mL^{-1}). The suspension was then applied to a haemocytometer. Both viable and nonviable cells were counted. A minimum of 200 cells were counted for each data point in a total of eight microscopic fields. The percentage cell viability was calculated as follows:

$$\text{Cell Viability}\,(\%) = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100 \quad (1)$$

To further verify the cell viability of HepG2 cells, a confirmatory test was carried out with propidium iodide dye exclusion and quantitated using flow cytometry according to the reported method (O'Brien *et al*., 2000). Briefly, the cell suspension in 50 μL PBS was added to 10 μL of 0.04 mg mL^{-1} propidium iodide and dyed for 15 min at room temperature. The cells were subsequently harvested and resuspended in PBS with a concentration ranged from 200 to 1000 cells μL^{-1}. They were gated according to forward/side scatter on GUAVA EasyCyte Mini flow cytometer.

Experimental design and statistical analysis

All experiments were performed at least three times, and all values were expressed as means ± standard deviation (SD). The differences between the control and UV treated samples were compared by *t*-test using SPSS 18.0 software. The results were considered significant if the *P* values were <0.05.

Results

Degradation efficiency of AFB_1 in peanut oil in the photodegradation reactor

As shown in Fig. 2A, AFB_1 in peanut oil was decomposed rapidly when exposed to UV with a wavelength

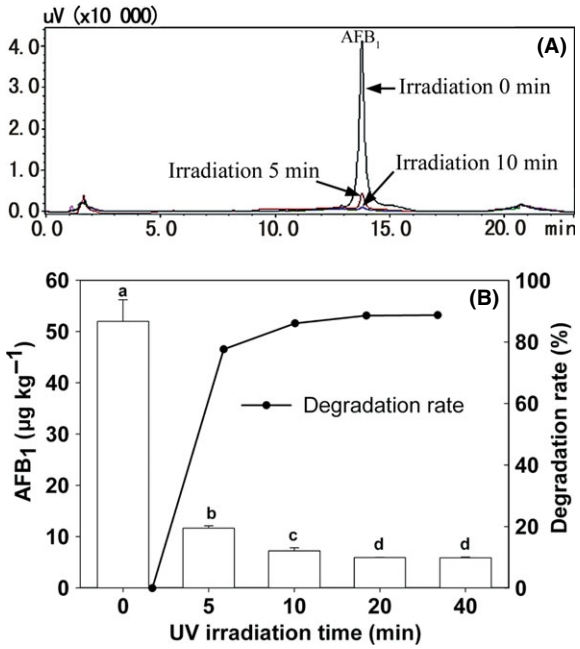

Figure 2 UV detoxification of AFB_1 in peanut oil at different UV irradiation times, (A) HPLC chromatogram of AFB_1, (B) AFB_1 contents and its degradation rate. (a, b, c, and d represent statistically significant differences in AFB_1 content at different UV irradiation times).

of 365 nm and irradiation intensity of 6.4 mW cm^{-2} using the photodegradation reactor. AFB_1 was significantly decreased from 51.96 ± 4.24 to 7.23 ± 0.59 μg kg^{-1} in 10 min ($P < 0.05$), and to 5.85 ± 0.16 μg kg^{-1} in 40 min, reduced by 86.08% and 88.74%, respectively (Fig. 2B). Although extending UV irradiation time could increase the degradation efficiency of AFB_1, longer treatment than 20 min did not significantly improve UV detoxification efficiency of AFB_1 in peanut oil.

Ames test for mutagenesis

Table 1 shows the mean number of revertants per plate, the standard deviation, and the mutagenic index (MI) at different UV irradiation times, in the presence (+S9) and the absence (-S9) of metabolic activation. Seen from the Table 1, 42 μg of AFB_1 per plate in the positive control group was detected by HPLC analysis, which reduced to 9.71, 7.50, 6.18, and 5.39 μg per plate after being irradiated for 5, 10, 20, and 40 min, respectively. For the negative control group, the spontaneous mutant frequencies of the two tester strains were in the ranges of 20–50 revertants per plate for TA98 (thirteen cells per plate) and 100–300 revertants per plate for TA102 (287 revertants per plate) without S9, and 20–50 revertants per plate for TA98 (twenty-three revertants per plate) and 200–400 revertants per plate for TA102 (350 revertants per plate) with S9, respectively (Mortelmans & Zeiger, 2000). At the same conditions, AFB_1 in peanut oil without UV irradiation (the positive control) induced the expected mutagenic responses for the strains TA98 and TA102, either in the presence or the absence of S9, and their MI were all >2.0, especially for the strain TA98, reached to 17.7 without S9 and 11.0 with S9, respectively. And the average numbers of revertants of strains TA98 and TA102 with S9 were greater than those of the ones without S9. UV irradiation significantly decreased the numbers of revertants ($P < 0.01$) of the two strains induced by AFB_1 in peanut oil. When the irradiation time exceeded 10 min, the MI of the two trains were all <2.0, whether or not presence of S9, which

Table 1 Average numbers of revertants in TA98 and TA 102 strains after UV irradiation detoxification

			TA98				TA102			
			−S9[a]		+S9[b]		−S9[a]		+S9[b]	
Treatment	Irradiation time (min)	Dose of AFB_1 (*n*g/plate)	$\bar{x} \pm SD$[c]	MI[d]	$\bar{x} \pm SD$[c]	MI[d]	$\bar{x} \pm SD$[c]	MI[d]	$\bar{x} \pm SD$[c]	MI[d]
Negative control	0	0	13 ± 2.5^{A}	1.0	23 ± 4.6^{A}	1.0	287 ± 37.7^{A}	1.0	350 ± 47.8^{A}	1.0
Positive control	0	42.00	$230 \pm 18.5^{B,**}$	17.7	$254 \pm 17.7^{B,**}$	11.0	$854 \pm 40.1^{B,**}$	3.0	$1020 \pm 207.5^{B,**}$	2.9
UV irradiation	5	9.71	$124 \pm 21.0^{C,**}$	9.5	$150 \pm 32.6^{C,**}$	6.5	$746 \pm 41.5^{C,**}$	2.6	$838 \pm 49.9^{B,**}$	2.4
	10	7.50	17 ± 2.6^{D}	1.3	$25 \pm 4.0^{A,D}$	1.1	328 ± 32.1^{A}	1.1	$526 \pm 39.9^{C,*}$	1.5
	20	6.18	13 ± 1.7^{A}	1.0	30 ± 5.1^{D}	1.3	350 ± 28.0^{A}	1.2	$480 \pm 37.5^{C,*}$	1.4
	40	5.39	19 ± 3.0^{D}	1.5	$27 \pm 6.6^{A,D}$	1.2	335 ± 38.9^{A}	1.2	$453 \pm 31.3^{C,*}$	1.3

[a]Without metabolic activation (S9).
[b]With metabolic activation (S9).
[c]Means ± standard deviation.
[d]Mutagenic index.
Statistically significant differences in the same column are indicated by different superscripts.
$^{**}P < 0.01$, $^{*}P < 0.05$ vs. negative control in the same column.

indicated that no mutagenicity was detected in aflatoxin-contaminated peanut oil after being irradiated. In addition, increasing the exposure time cannot proportionally decrease the numbers of revertants and the MI of the two strains after 10 min of exposure.

Cell viability for cytotoxicity

The cell viability of HepG2 cells in response to peanut oil containing AFB_1 extract is presented in Fig. 3. Compared with the negative control, the cell viability of HepG2 cells treated by AFB_1 without UV irradiation (i.e. the positive control) was significantly decreased ($P < 0.01$), and the percentage of cell viability was only $52.64 \pm 0.98\%$. However, with the increase of UV irradiation time, the cell viability of HepG2 cells treated with peanut oil containing AFB_1 extract significantly increased ($P < 0.01$), and reached to $95.54 \pm 1.19\%$ at 10 min of UV exposure, which was statistically insignificant difference ($P > 0.05$) compared with that of the negative control. After 10 min, increasing the irradiation time could not significantly increase the cell viability ($P > 0.05$), which was consistent with the results of Ames test.

Flow cytometric assay further confirmed the protective trends of UV irradiation (Fig. 4). The propidium iodide only stained the dead HepG2 cells treated with AFB_1. Figure 4 shows the percentage of viable and dead cells. As shown in Fig. 4, each graph is divided into four boxes, upper right box and lower right box represent the percentage of dead and viable cells, respectively. The percentage of dead cells for the negative control (untreated by AFB_1) was only 14.85%, while it reached to 47.06% at 50.40 μg mL^{-1} of AFB_1 (IC50, i.e. 50% toxicity) as a positive control. With the increase of UV irradiation time, the percentage of dead cells was significantly reduced from 47.06% to 16.13% and almost reached to the level of the negative control at 40 min of exposure.

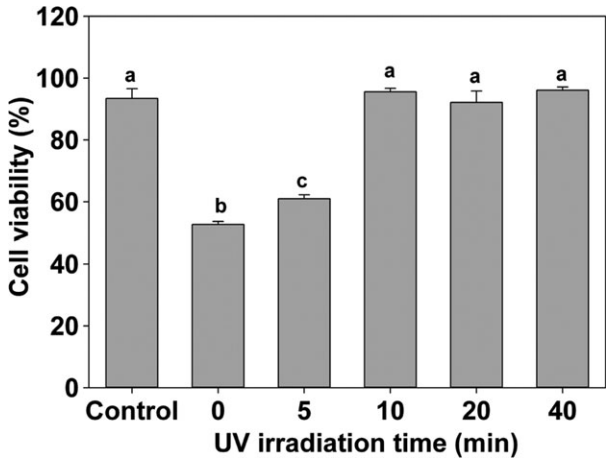

Figure 3 Cell viability of HepG2 cells treated by AFB_1 extracted from peanut oil at different UV irradiation times. (a, b, c represent statistically significant differences in cell viability compared with that of the control).

Discussion

AFB_1 has a strong mutagenic effect, and epidemiological studies have shown a strong correlation between AFB_1 concentration and mutagenicity (Tjälve *et al.*, 1992; Sedmíková *et al.*, 2001; Wild & Montesano, 2009; Matsuda *et al.*, 2013). Therefore, AFB_1 in peanut oil must be degraded to a safety level before eating. Previous literatures have proved that UV radiation can degradate AFB_1 effectively (Atalla *et al.*, 2004; Tripathi & Mishra, 2010; Liu *et al.*, 2011a,b; Jubeen *et al.*, 2012). Liu *et al.* (2011b) reported that the UV with an intensity of 800 μW cm^{-2} could degrade thoroughly AFB_1 (2 mg kg^{-1}) in 30 min. Tripathi & Mishra (2010) observed that 87.8% of AFB_1 in red chilli powder was detoxified when exposed to UV (365 nm) for 60 min. Jubeen *et al.* (2012) found that AFB_1 in almond and pistachio were reduced by 96.5% when exposed to UVC (265 nm) for 45 min, and the photodegradation of AFB_1 followed the first order kinetics. Atalla *et al.* (2004) reported that AFB_1 in wheat grains was completely decomposed after being exposed to UV with a wavelength of 362 nm for 30 min at different relative humidities. In this study, UV irradiation can also quickly decompose AFB_1 in peanut oil to 7.23 ± 0.59 μg kg^{-1} using the photodegradation reactor in 10 min, which is less than the maximum residue limit in peanut oil (20 μg kg^{-1}) set by Chinese government. These data indicate that UV irradiation is an effective method in degrading AFB_1 in peanut oil using the photodegradation reactor, and the photodegradation rate of AFB_1 was time dependent. As AFB_1 in peanut oil cannot be degraded completely by UV irradiation due to the protection of peanut oil, and some less toxic photodegradation products may be formed during the irradiation process (Rustom, 1997; Liu *et al.*, 2012), so the safety of peanut oil containing AFB_1 after being irradiated need to be evaluated.

Ames assay is usually used to determine the mutagenic potential of chemicals *in vitro* (Mortelmans & Zeiger, 2000), and it is well known that AFB_1 can result in the mutation of the tester strain TA98 and TA102 (Mölzer *et al.*, 2013) by producing DNA-metabolite adducts of AFB_1 (Majer *et al.*, 2005; Wakabayashi *et al.*, 1992). For the positive control, the average numbers of revertants in TA98 and TA102 are significantly higher than those of the negative control ($P < 0.01$), either in the presence or the absence of S9. Compared with the positive control, UV irradiation

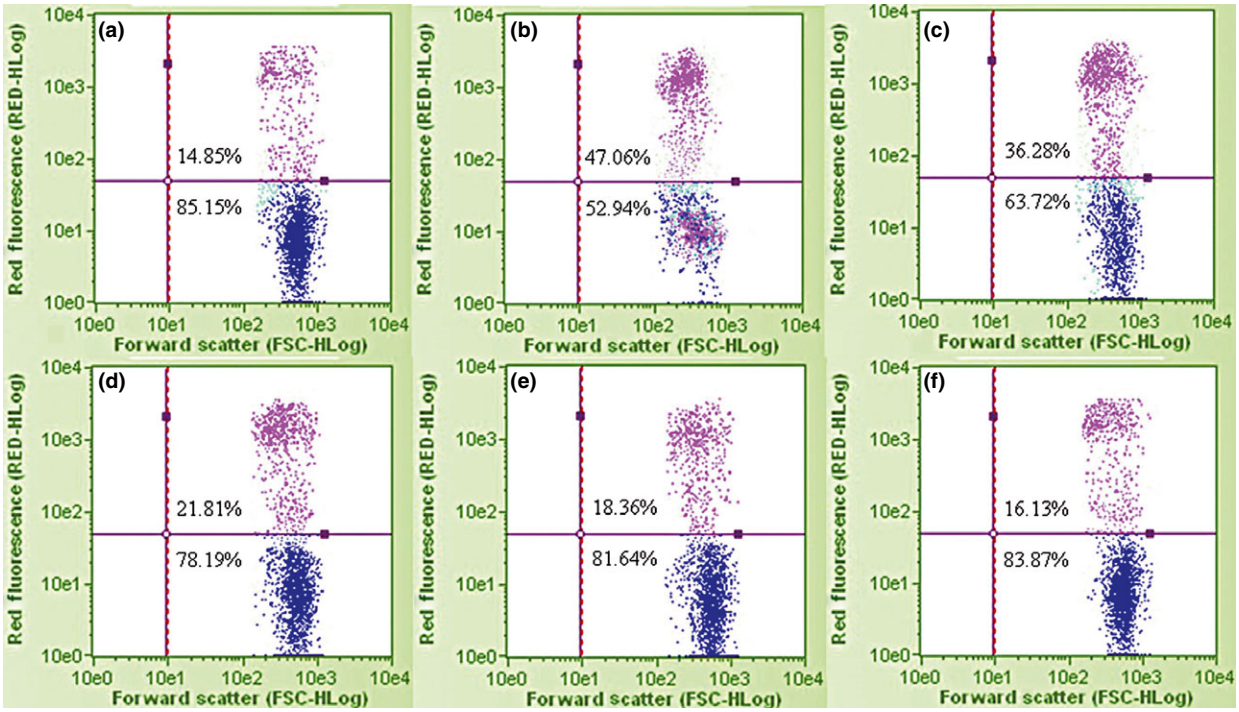

Figure 4 Cell viability of HepG2 cells measured by flow cytometry, (a) the negative control, (b) the positive control at IC50 concentration of AFB_1, (c) 5 min of UV exposure, (d) 10 min of UV exposure, (e) 20 min of UV exposure and (f) 40 min of UV exposure.

for 5 min significantly reduced the numbers of revertants in two strains ($P < 0.05$), while far higher than those of the negative control and other three treatments ($P < 0.01$). So, AFB_1 in peanut oil still has great toxicity after 5 min of UV irradiation. After 10 min of UV exposure, the mutagenic potential of AFB_1 in peanut oil was decreased obviously ($P < 0.01$; Table 1). Extending the UV exposure time can increase the detoxification efficiency of AFB_1, while its destruction role on the nutritions and causing the high peroxide value to peanut oil need to be considered (Gordon *et al.*, 1994). So, using the photodegradation reactor cannot decompose completely the AFB_1 in peanut oil, which is consistent with those of reported literatures (Kleinwächter & Koukalová, 1979; Liu *et al.*, 2011a,b). Based on the results above, UV irradiation in the photodegradation reactor is an effective tool to decompose AFB_1 in peanut oil, and 10 min of exposure is regarded as the optimum irradiation time.

Cell viability is a determination of living or dead cells based on a total cell sample, which is used to evaluate environmental damage due to toxins. The trypan blue exclusion test is a rapid, simple and inexpensive method to assess cell viability in response to environmental insults (Wan *et al.*, 2009; Angelieri *et al.*, 2012). The results from cell viability showed that UV irradiation for 10 min can significantly improve the cell viability of HepG2 cells and without differences compared with that of the negative control ($P > 0.05$, Fig. 3). The flow cytometric assay further verified the improving capacity of UV irradiation for cell viability with high sensitivity (Fig. 4).

In addition, based on the UV detoxification efficiency and safety assessment of AFB_1 in peanut oil, the photodegradation reactor is an excellent equipment in detoxificating AFB_1 in peanut oil, which provide the guidance for the oil industry to degrade AFB_1 in edible oils for the sake of food safety and health of consumers.

Conflict of Interest

The authors declare that there are no conflict of interests.

Acknowledgments

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