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Pistachio Extracts Effects on the Aflatoxin B1 Cytotoxicity in HepG2 Cells

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Abstract: Aflatoxin B1 (AFB1) is one of the most common mycotoxins found in human foodstuffs such as corn, peanuts, pistachio and cottonseeds. It is a potent hepatocarcinogen in experimental animals and probably in humans. Pistachio contains elements that have been claimed for cancer prevention. Based on these evidences and also the nutrient value of pistachio, we have investigated on the possible protective effects of pistachio extract on the cytotoxic effects of AFB1, using different assays. Human hepatocarcinoma HepG2 cells were exposed to AFB1 (0-60 μM) and pistachio extract concurrently and in different sequences. As analyzed by the MTT viability assay and caspase activation assay, pistachio extract increased the cytotoxicity of AFB1, when HepG2 cells were exposed to it before AFB1. It did, however, not change the rate of cell death measured by LDH release or the P53-expression in any sequences of exposure with AFB1. As a conclusion, pistachio extract was not only able to protect HepG2 cells from AFB1 toxicity, but also increased AFB1-induced cytotoxicity in some conditions. As a matter of fact, our results showed that pistachio extract facilitates AFB1 cytotoxicity at a level after P53-expression.

Key words: Pistachio extract, AFB1, cytotoxicity, HepG2 cells

INTRODUCTION

Mycotoxins are low molecular weight natural products, produced as secondary metabolites by filamentous fungi, which are commonly found in feed and foodstuff. By ingestion, inhalation, or skin contact, these toxic metabolites can cause very serious health problems in humans (Gelderblom *et al*., 1996; Bennett and Klich, 2003). They exhibit a wide array of chemical, biological and toxicological properties, such as carcinogenicity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immune suppression (Smith *et al*., 1995).

Aflatoxin B1 (AFB1) is one of the most common mycotoxins found in human foodstuffs such as corn, peanuts, pistachio and cottonseeds. This mycotoxins is a potent hepatocarcinogen in experimental animals and also probably in humans. In 1993, the IARC (International Agency for Research on Cancer) placed AFB1 on the list of human carcinogens. This is supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxins and Liver Cell Cancer (LCC) (Eaton and Groopman, 1994).

Numerous epidemiological studies suggest a role of dietary habit in human cancer incidence. Foods are known for carcinogen as well as anticarcinogenic effects. It has been shown that various fruits, vegetables and teas reduce chemical tumorgenesis in experimental animals. Anticarcinogenic activity of components in different foods has been widely investigated (Gescher *et al*., 2001).

Pistachio has been reported as a remedy for the sclerosis of the liver, abdominal ailments, abscess, bruises and sores, chest ailments, circulation problems and other problems.

Like corn, pistachio nuts should be regarded as a critical foodstuff, not only because of its economical value for Iran, but also since they may frequently contaminate with elevated levels of aflatoxins (Pittet, 2001). Pistachio contains some of these essential elements like selenium, folate, vitamin E and C which have been claimed for cancer prevention (Donaldson, 2004; Haytowitz *et al*., 2004). Based on these evidences and also the nutrient value of pistachio, it would be reasonable to investigate on the anticarcinogenic properties of pistachio. In this research, we studied on the anticytotoxicity potential of the kernel of pistachio against AFB1 in human hepatoblastoma HepG2 cell lines for the first time.

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MATERIALS AND METHODS

Materials: Pistachio (long) was obtained from Rafsanjan, Iran. AFB1 were purchased from sigma. HepG2 cell line was purchased from Iran Pasture Institute. All other chemicals were of analytical grade. This study was conducted in Department of Toxicology and Pharmacology, Faculty of Pharmacy, Shaheed Beheshti University of Medical Sciences and Apoptosis department of Danish cancer society, during 2004-2005.

Cells and media: HepG2 cell line, a well defined human hepatoblastoma cell line were maintained in Dulbecco Modified Eagle Medium (DMEM/F12, Gibco BRL, USA) containing 10% fetal bovin serum (FBS, Gibco BRL, USA) and 0.25% penicillin-streptomycin (Gibco BRL, USA). Cells were grown based on standard protocols at 37°C incubator under 5% CO_2 and 95% humified air until experiment.

HPLC analysis of Aflatoxins: Samples were shelled and kernel were analyzed using a High Performance Liquid Chromatography (HPLC) method (the AOAC official method 999.07) with some minor modifications (Stroka *et al*., 2000). Briefly, samples were extracted and filtered through glass microfiber filter and applied to Immnoaffinity Column (IAC). Aflatoxins was quantitated by reverse-phase HPLC and fluorescence detector with Post-column Derivatization (PCD) involving bromination.

Pistachio extract preparation: Clean, non-contaminated pistachio samples were grounded. For the purpose of extraction preparation, it was performed as described elsewhere (Weng *et al*., 1997). There were two phases at the end of the process of extraction; the oil phase and the precipitate phase. The two phases of oil and precipitate were separated using hexan. Precipitate phase was dissolved in dimethylsulfoxide for the purpose of exposure to cells in the media. A mixture of oil and precipitate was also prepared and named pistachio extract.

Treatment method: For the purpose of different experiments, AFB1 and pistachio extract was added to the HepG2 cell line growth media in the following sequences; a) pistachio extract before AFB1. In this protocol of exposure, pistachio extract was added to the media for 24 h. Cells were then washed with PBS and fresh media was added containing AFB1. Different measurements were done 24 or 48 h after AFB1 exposure as are described below. b) AFB1 and pistachio extract were added to the HepG2 cell line growth media concurrently. Results were seen at 24 or 48 h after exposure, based on the different assays. c) HepG2 cells were exposed to AFB1 for 24 h. Cells were washed and fresh media was added containing the pistachio extract. Assays were performed at 24 or 48 h after pistachio extract exposure. AFB1 and pistachio precipitate concentrations were calculated as W/V in the range of 0-80 μM and 0.11 mg mL^{-1}, for AFB1 and pistachio precipitate, respectively. For the measurement of exposure concentration of pistachio oil, a stock was prepared from 100 g pistachio. Different volumes of this stock of oil were added to the media and expressed as volume percentage in different experiments.

MTT assay: HepG2 cells were seeded at 10,000 cells per well in 96-well cell culture plates and allowed to attach for 24 h in DMEM/F12 media. Culture medium was then replenished with new media plus different concentrations of AFB1 (0-60 μM) for 24 h. This method assesses the ability to convert 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, Sigma) in sterile PBS with Calcium and Magnesium, (Gibco BRL) to the formazan dye (Mosmann, 1983).

LDH assay: HepG2 cells were (10,000) grown in 96 well dishes To evaluate the LDH (Lactate dehydrogenase) release from necrotic cells, the cytotoxicity detection kit (Roche, Mannheim, Germany) manual was used.

Western blot analysis: Western blot was performed as described elsewhere (Frese *et al*., 2002). Cells, which were exposed to 80 μM AFB1 for 24 h, were harvested and then lysed. Protein content of the lysate was determined by the Brodford protein assay (BIO-RAD) according to the manufacturer instructions (Laemmli, 1970). Equal amounts of proteins (150 μg) were separated using 12% mini-gels. Proteins were transferred to nitrocellulose membranes. The membrane was blocked with dry milk, after which this was incubated with primary antibody overnight. Then, the blot was incubated with horseradish peroxidase-coupled secondary antibody for 30 min and washed. The enhanced chemiluminescence (ECL) detection system (Amarsham Pharmacia Biotech) was used to visualize p53 protein according to the manufacture's instructions.

Caspase activity: HepG2 cells (50,000) were grown in 24 well dishes. Cells were exposed to 80 μM of AFB1 for 24 h. After the incubation time, media was discarded and 200 μL caspase lysis buffer was added and plates were kept on ice with gentle shaking for 10 min. Reaction was started by placing 50 μL extract into black COSTAR plate on ice and adding 50 μL caspase reaction buffer (Hepes 100 mM, DTT (Dithiotherietol) 5 mM, EDTA 0.5 mM, Chaps 0.1%, Glycerol 20%). The plate was pre-incubated

3-5 min in Spectramax Gemini fluorometer (Molecular Devices, Sunnyvale, CA) at 30°C, after which reading was started. The V_{max} of the liberation of the fluorochrome was measured as described before (Dietrich *et al.*, 2003).

Statistical analysis: Values are presented as means±SD. Statistical differences between control and treated groups were determined by one way ANOVA (Dunnett correction). Differences were considered significant at $p<0.05$.

RESULTS

HPLC analysis of aflatoxins: Figure 1 shows a HPLC chromatogram of grounded pistachio spiked with aflatoxins (a) and sample of clean grounded pistachio (b). As is shown in Fig. 1a the retention time of the AFB1 is 20 min in this analytical method. Figure 1b shows that the pistachio sample used in this study did not contain any aflatoxin contamination.

MTT assay: AFB1 decreased cell viability in time and concentration dependent manner (data not shown). We exposed the cells to pistachio extract before, concurrence and after AFB1 treatment (0-60 μM) for 24 h. As is shown in Fig. 2, pistachio extract increased toxicity of AFB1, only when it was used before AFB1.

LDH release: AFB1 did not cause any LDH release from HepG2 cells within the first 24 h after the exposure (data are not shown). However, although LDH release was observed in a concentration-dependent manner during the next 24 h (Fig. 3), this release was not altered by the addition of pistachio extract.

P53 measurement: AFB1 exposure for 24 h increased p53 expression in HepG2 cells. Expression of p53 from HepG2 cells remained the same when pistachio extract was also applied before or concurrent with 80 μM AFB1 (Fig. 4). Pistachio extract was not added after AFB1 for this purpose, since no effect was seen in MTT assay and LDH release experiments in such a sequence of exposure.

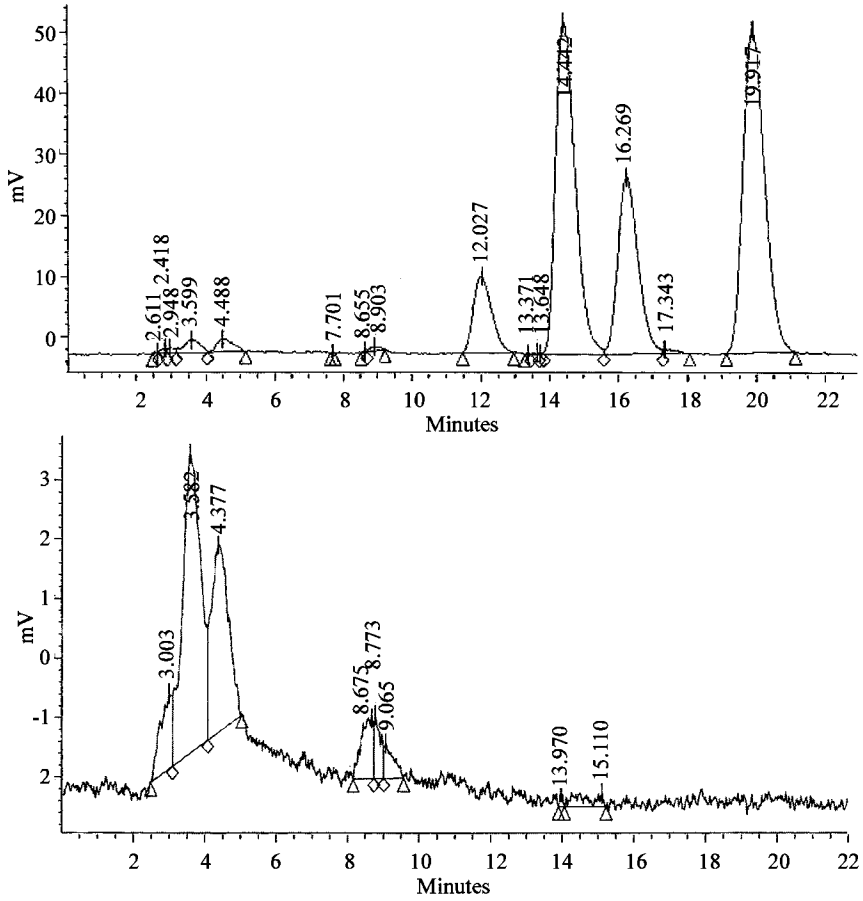

Fig. 1: HPLC chromatogram of aflatoxins measured as described in method section for spiked pistachio (Retention times are 12.027, 14.442, 16.269 and 19.917 for AFG2, AFG1, AFB2 and AFB1, respectively). (a). HPLC chromatogram of uncontaminated pistachio sample (b)

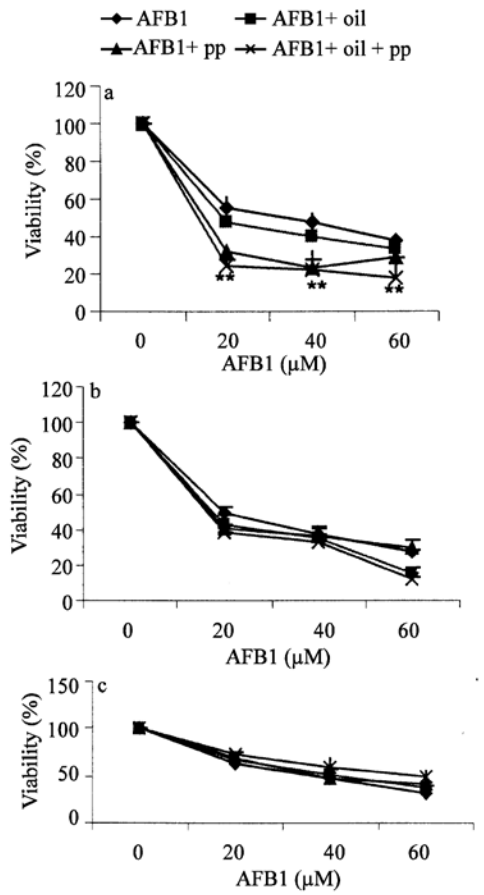

Fig. 2: HepG2 cells were treated with pistachio precipitate (pp), oil, as well as oil and pistachio extract, before (a), concurrence (b) and after (c) exposure to AFB1 (0-60 μM) for 24 h. Cell viability was determined using the MTT assay and expressed as a percentage of control, which were exposed to vehicle only. Control values were taken as 100%. Data were expressed as the mean ±SD (n = 3). **Significantly different when pistachio extract compared to AFB1 alone ($p<0.01$)

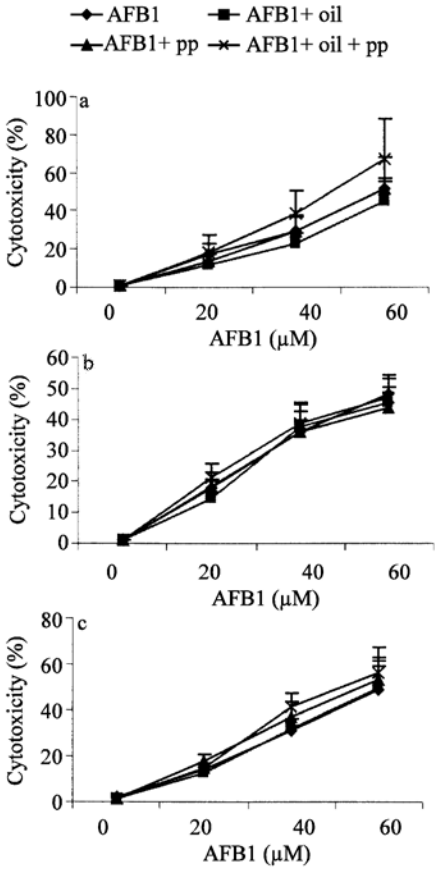

Fig. 3: HepG2 cells were treated with pistachio precipitate (pp), oil, as well as oil and pistachio extract, before (a), concurrence (b) and after (c) exposure to AFB1 (0-60 μM) for 48h. Data were resulted from LDH release and expressed as the mean ±SD (n = 3)

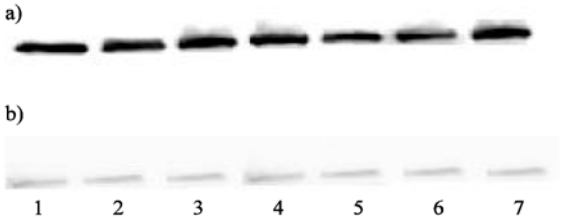

Fig. 4: Western blot assay of P53 expression in HepG2 cells. a) Cells were incubated with AFB1 dissolved in DMSO plus the following materials and, b) HepG2 cells were incubated without AFB1 (DMSO was added as solvent control) and the following materials: 1, control; 2 and 5, pistachio oil; 3 and 6, pistachio precipitate; 4 and 7, pistachio extract. Sequence of addition was as different materials before AFB1 for lines 2 to 4 and concurrent additions for lines 5 to 7

Caspase3 activity: Caspase3 activity in HepG2 cells increased significantly after exposure to AFB1 for 24 h (Fig. 5). This activity of caspase3 was further increased when HepG2 cells were exposed to pistachio extract before AFB1. However, no significant differences between treated group and control cells were seen when pistachio extract was added concurrent or after AFB1.

DISCUSSION

There are many publications on the biological benefits of pistachio. Chiosmatisc gum which is produced

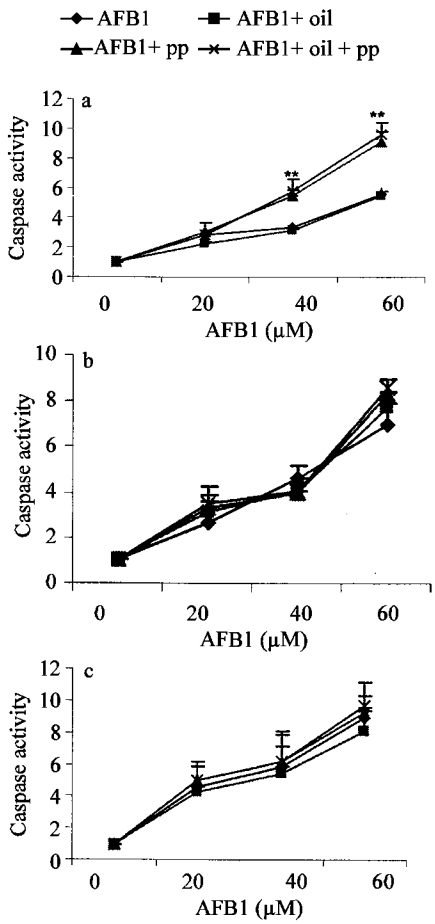

Fig. 5: Activation of caspase3 in HepG2 cells as a result of exposure to AFB1. HepG2 cells were treated with pistachio precipitate(pp), oil, as well as oil and pistachio extract before (a), concurrence (b) and after (c) exposure to AFB1 (0-60 μM) for 24 h. The results are given with respect to untreated cells. Data were expressed as the mean ±SD (n = 3). **show significant difference when pistachio extract compared to toxin alone at $p<0.01$

from *Pistacia lentiscus* var. Chia resin was shown to be the most effective natural medication to protect human Low Density Lipids (LDL) from oxidation (Andrikopoulos *et al.*, 2003). Dedoussis *et al.* (2004) showed that *Pistachio lentiscus* triterpenes exert antioxidant/antiatherogenic effects when Peripheral Blood Mononuclear Cells (PBMC) were exposed to oxidized LDL and the polar extract, concurrently. The triterpenoid fraction of pistachio revealed remarkable increase in the intracellular glutathione.

The antioxidant properties of galloyl quinic derivatives isolated from *Pistacia lentiscus* L. leaves have been investigated by means of Electron Paramagnetic Resonance spectroscopy (EPR) and UV-Vis spectrophotometry. All of tested metabolites have strongly reduced the oxidation of LDL, with a trend similar to that was observed for the scavenger ability against OH radical (Baratto *et al.*, 2003).

Zhao *et al.* (2005) studied the antioxidant efficiency of the two gallotannins, Pistafolin A and Pistafolin B which are phenolic compounds in the leaves of *P. weinmannifolia*. They showed that the protective effects of these two compounds against oxidative damage of biomacromolecules like DNA and protein were due to their strong free radical scavenging ability (Zhao *et al.*, 2005).

Aqueous extract of *P. lentiscus* leaves (both boiled and non-boiled) showed marked antihepatotoxic activity against CCl_4 by reducing the activity of the Liver enzymes (ALP, ALT and AST) and the level of bilirubin (Janakat and Al-Merie, 2002).

In spite of all benefits mentioned above, pistachio is found to be very sensitive to the AFB1 contamination and therefore a serious hazard for consumer health. Whether the antioxidant potential of pistachio ingredients are capable of protecting human cells from its cytotoxic potential due to possible contamination with aflatoxins, was the subject of this investigation. To achieve this goal, HepG2 cells were exposed to the pure AFB1, alone and/or with aflatoxins-free oil, precipitate and extract in various orders.

The MTT cell proliferation assay measures the cell proliferation rate and conversely when metabolic events lead to apoptosis or necrosis, the reduction in cell viability (Mosmann, 1983). In this study, we used MTT assay to look at the toxicity of AFB1 as well as the protective effect of pistachio extract on HepG2 cells. This test helped us to estimate the toxic concentrations of AFB1 on this cell line. In order to show the protective efficacy of pistachio extract against the cytotoxicity of AFB1, pistachio extract was used before, concurrent and after AFB1 treatment. The result showed that pistachio extract was not toxic for HepG2 cells lonely, but when used before AFB1 exposure, could increase the cytotoxicity of AFB1.

For a further investigation on the cell death induced by AFB1 and increased with the pistachio extract, LDH release assay was used. Lactic Dehydrogenase (LDH) is an enzyme that helps to produce energy. LDH activity is the most sensitive and readily detectable enzymatic method for the determination of cell death (Allen and Rushton, 1994). To show the exact cytotoxicity effect of AFB1 in HepG2, cells were exposed to the different concentrations of AFB1 (0-60 μM) for 24 and 48 h. We did not find any significant differences between

control and treated groups after 24 h (data not shown). However, 48 h after the exposure, AFB1 significantly induced LDH release.

To investigate on the effect of pistachio on AFB1 induced LDH release, we treated the cells before, concurrent and after AFB1 treatment as was described in the method section. Unexpectedly, although MTT results have shown an increase in the toxicity of AFB1 when co-administered with pistachio extract, but LDH release did not produce the same results, as is shown in Fig. 3. To confirm that cell cytotoxicity mediated with pistachio extract was not signed for necrosis, p53 status came into the consideration.

There are some evidences that showed AFB1 might increase p53 expression in HepG2 cells (O'Brian *et al*., 2000). p53 induces cell death through several molecular genes related, or transcriptional independent pathways. Mitochondria play a key role in apoptosis. The accumulation of p53 on mitochondria is rapid (within 1 h after p53 activation) and precedes changes in mitochondrial membrane potential, cytochrome *c* release and procaspase-3 activation (Marchenko *et al*., 2000). To look at the AFB1 effects on this pathway, we exposed HepG2 cells to 80 μM AFB1 for 24 h, in which AFB1 increased p53 expression significantly. Then we treated the cells with pistachio extract before and concurrent with AFB1 for 24 h, in which case pistachio did not change the effect of AFB1 on p53 expression.

P53 activation can also be initiated upstream of caspase activity (Kaufmann and Hengartner, 2001) and there are some evidences which showed that AFB1 might increase caspase activity. Caspases are cysteine proteases and their activation has been considered as the hallmark not only for apoptosis but of all programmed cell deaths. To investigate on the possible effect of pistachio on this upstream pathway for AFB1, we exposed the cells to the different concentrations of AFB1 and also pistachio extract before, after and concurrent with AFB1 treatment for 24 h. AFB1 increased the activity of caspase3 clearly. Pistachio extract did not increase the caspase activity when used alone, but when was used before AFB1 treatment increased the AFB1 induced caspase3 activity. However there was no effect when pistachio extract used concurrent or after AFB1. We have therefore concluded that pistachio extract facilitate AFB1 effects on caspase3 production downstream of p53 level.

Due to the presence of antioxidants and cellular protective molecules in pistachio contents, the aim of this study was to look at the anticytotoxic potential of pistachio on AFB1. Present results failed to show any protective effects by pistachio extract on the cytotoxicity of AFB1. Furthermore, it has increased AFB1 cytotoxicity in some circumstances.

Although, we have not found any other publications on the pistachio kernel in the literatures, but Ljubuncic *et al*. (2005) have shown similar results for pistachio leaves extract. In their study, pistachio leave extract has increased the cytotoxity of thioacetamide in hepatic cells.

As a conclusion, anti-oxidant contents of pistachio kernel were not potent enough to protect human cells from the cytotoxic effects of AFB1. Contrarily, 24 h exposure of cells to pistachio extract before AFB1 exposure facilitates caspase3 activity induced by AFB1 in these cells. Since pistachio exposure after and/or concurrent with AFB1 did not have such a result, it might be postulated that pistachio extract facilitate AFB1 DNA damage-p53-mitochodria-caspase 3 pathway by initial blocking of parallel cellular pathways downstream of p53. Further studies are recommended to explore the cellular effects of pistachio extract which might be worth to consider for its human consumption.

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