Onion Peel Powder Alleviate Acrylamide-Induced Cytotoxicity and Immunotoxicity in Liver Cell Culture

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Abstract: Acrylamide is a probable human carcinogen that is present in several heat-treated foods such as French fries, potato crisps and coffee. We conducted the present study to investigate the protective effects of onion peel powder (OPP), rich in phenolic compounds, on the acrylamide (AA)-induced cytotoxicity and immunotoxicity in liver cell cultures. Cytotoxicity (lysosomal dysnfuntion assayed by NR, mitochondrial dysnfunction assayed by MTT and cell wall membrane integrity assayed by CV) and immunotoxicity (assayed by PA) effects of acrylamide in liver cells cultures were observed. The midpoint toxicity values of AA treated samples were 8.00, 9.80, 9.75 and 17.00 mg/l for NR, MTT, CV and PA assays, respectively. Treatment of AA samples with OPP, 2.5 mg/l, increased these values (decreasing the cytotoxic and immunotoxic effects) and recorded 11.00, 9.50, 14.75 and 19.75 mg/l, respectively. The rate of decreasing in different assays are directly proportional with the increasing of the OPP concentration until removed totally at 20 mg/l. PEG-SOD (a superoxide scavenger) and PEG catalase (a hydrogen peroxide scavenger) examination indicated that ROS is involved in AA-induced cytotoxicity and immunotoxicity in liver cells. These data suggest that agricultural by-products rich in phenolic compounds such OPP could attenuate the cytotoxicity and immunotoxicity induced by AA in liver cells. The protection is probably mediated by an antioxidant protective mechanism. Using of OPP as a natural food additives in both deep-fat frying process and heated-treated foods may be a worthwhile way to prevent AA-mediated cytotoxicity and immunotoxicity.
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1. Introduction

Acrylamide is a chemical compound with the chemical formula C_3H_5NO [CH_2=CH-$CONH_2$]. It is a white odourless crystalline solid, soluble in water, ethanol, ether, and chloroform. Acrylamide is incompatible with acids, bases, oxidizing agents, iron, and iron salts. It decomposes non-thermally to form ammonia, and thermal decomposition produces carbon monoxide, carbon dioxide, and oxides of nitrogen. Before 2002, acrylamide exposure was thought tooccur mainly through occupational exposure, also through cigarette smoke, and to a minor extent through the consumption of water and the use of cosmetics. However, in 2002, Swedish scientists reported its presence in carbohydrate-rich foods that were produced at high temperatures, such as French fries and potato chips (Tareke *et al.,* 2002).

Shortly after its discovery in food, acrylamide was shown to form in Maillard reaction, also responsible for the desirable colour and flavour changes that characterize fried foods. The Maillard reaction involves numerous reaction pathways and end products, all starting from amino acids and sugars naturally present in the foods. Acrylamide is formed by reaction between reducing sugars, for example glucose and fructose, and one specific amino acid, namely asparagines. Other formation mechanisms have been reported, for example involving the gluten protein from wheat, but these are believed to be of minor general importance (Mottram *et al.,* 2002).

In 1994, the International Agency for Research on Cancer classified acrylamide as a probable human carcinogen, on the basis of its carcinogenicity in rodents (Tareke *et al.,* 2002). Animal studies have shown positive dose-response relations between acrylamide exposure and cancer in multiple organs in both mice and rats (Besaratinia and Pfeifer, 2007); included among those organs were several hormone-sensitive organs, such as the mammary glands and the uterus. The mechanism by which acrylamide causes cancer in laboratory animals and by which it may cause cancer in humans is as yet unclear (Olesen *et al.,* 2008); both genotoxic and nongenotoxic pathways have been suggested. acrylamide itself and its epoxide metabolite glycidamide, which is generated by cytochrome P4502E1 (CYP2E1), are clastogenic, and glycidamide forms DNA adducts. As for possible nongenotoxic pathways, acrylamide reacts with glutathione and may thus influence the redox status of cells and gene transcription, or it may interfere with DNA repair or hormonal balances (Besaratinia and Pfeifer, 2007). Also, ReMersch *et al.,* 2004 and Jiang *et al*., (2007) reported that acrylamide treated caused DNA fragments and an increase of reactive oxygen

species (ROS) formation and induced genotoxicity and weak cytotoxicity in hepatocytes cell lines, HepG2.

In the present study, we investigated the protective effects of the well-known antioxidant/ anticarcinogenic/ antimutagenic onion peel extract on acrylamide-induced cytotoxicity and immune-toxicity in liver cell culture. The amount of onion (*Allium cepa* L., *Alliaceae*) waste produced annually in the European Union is estimated at approximately 450,000 tons. The major byproducts resulting from industrial peeling of onion bulbs are brown skin, the outer two fleshy leaves and the top and bottom bulbs (Schieber *et al*., 2001). The outer dry layers of onion bulbs, which are not edible and removed before processing, have been shown to contain a wide spectrum of polyphenolic compo-nents (Singh *et al*., 2009). Most of these substances have not been reported to occur in the scales of the bulb, but they represent oxidation products of quercetin and its glucosides. Some of these phenolics have been shown to possess strong antioxidant activity *in vitro*. Our previous studies indicated that onion skin powder/extract is a natural antioxidant, primarily because it is able to suppress the generation of ROS and decrease lipid peroxidation (El-Wazeer, 2011), and protect against DNA damage induced by benz(*a*)pyrene (Huosein, 2011). Therefore, the present study was carried out to investigate the potential protective effects of onion peel powder (OPP) against AA-induced cytotoxicity and immunotoxicity in primary liver cell cultures.

2. Materials and Methods

Materials

Red onion peel powder (*Allium cepa* L.), variety Giza-20, skin was obtained as a donation from Bani Suif El-Goudida for Dehydration, Processing and Preservation of Vegetables, Bani Suif El-Goudida, Nile East, Bani Suif Governorate, Egypt.

Chemicals

All solvents and buffers, analytical grade, were obtained from Al-Ghomhoria Company for Chemicals, Drugs and Medical Instruments, Cairo, Egypt. Acrylamide was purchased from Sigma Chemical Co., Representative agent, Cairo, Egypt. Onion peel powder (OPP) prepared as a 2.5 mg/mL stock solution in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and stored at -20 C. For each experiment, curcumin was diluted with cell culture medium to the concentration indicated with a final DMSO concentration of 0.1% (vol/vol).

Methods

Preparation of the Primary Liver Cell Culture Model

Bolti fish (*Tilapia nilotica*) liver cells were isolated according to the method mentioned by Elhassaneen (1996) with some few modifications. Briefly, Bolti were anesthetized in tricaine methanesulfonate (MS-222; Sigma Chemical Co., St. Louis, MO) and weight, length, and sex was recorded. Livers were excised to a 60 x 15 mm petri dish (Baxter Healthcare Corp., McGaw Park, IL) containing Hank's Balanced Salt Solution (HBSS; Sigma Chemical Co.). Other tissue unless livers were cut away and the HBSS was removed. The livers were minced with a sterilize scissors and resuspended in 0.25% trypsin-0.02% EDTA solution (JRH Biosciences, Lenexa, KS). The suspension was then transferred using a magnetic stirrer plate for 20 min after which it was strained through three layers of sterile cheesecloth to remove large tissue fragments. The cell solution was mixed with RPMI-1640 (Sigma Chemical Co.) adjusted to 330 mOs/kg and supplemented with 25 mM HEPES buffer, 2 mM L(+)glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal Calf serum (FCS; all from Sigma Chemical Co.) at 2000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed twice with HBSS before counting. Cell pellet were resuspended in 10 % FCS/RPMI-1640, stained with 0.4% trypan blue stain (GIBCO Laboratories, Grand Island, NY) and were counted using a hemocytometer (Baxter Healthcare Corp.).

Experimental design

Isolated liver cells of Bolti fish were seeded at 1×10^5 cells/ well of 96 flat bottom tissue culture plate. 100 µl of FCS/RPMI-1640 medium was added to each well. After pretreatment with OPE (final concentration ranging from 2.5 to 20 mg/l) for 2 h at 27 °C for 24 h in 5% CO_2 tension, the medium was replaced with fresh medium containing acrylamide (final concentration ranging from 2.5 to 20 mM) then re-incubated for 24 h at the same conditions. The plates were prepared for NR, MTT, CV, PA, PEG-SOD and PEG-CAT assays.

Cytotoxicity assays

Neutral red (NR) assay [Lysosomal activity]

According to the method described by of Borenfreund and Puerner (1984), the medium was removed gently then, 0.2 ml of NR stain/media (containing 50 µg NR/ml) were added to each well. The plates were returned to the incubator for 3 h to allow for uptake of the stain into the lysosomes of viable uninjured cells. The stain/media were removed and the cells were washed with a mixture of 1% formaldehyde-1% $CaCl_2$ about three times or until no more NR stain comes off. 0.2 ml of a 1% acetic acid-50% ethanol mixture were added to each well to extract the dye. Thereafter, the plates were remained still 10 min at room temperature and agitated briefly on a microtitre-plate shaker for 15-seconds. The absorbance of the dye extracted was measured by using MR-5000 microtiter plate reader at a wavelength of 540nm.

Methyl tetrazollium (MTT) assay **[Mitochondrial activity]**

The MTT assay was described by Borenfreund and Babich (1988), as follow: a medium (0.1 ml) was removed and 0.1 ml of MTT-containing medium was added to each well. The plates were covered with foil and incubated at 27°C for 3 h. The supernatant was removed from the wells and 0.1 ml of a solution of 1N HCl-isopropanol was added to extract and solubilize the formazan. The plates were standed up 10 min at room temperature and agitated briefly on a microtitre-plate shaker for 5 seconds. The optical density of the dye extracted of each well was measured by using MR-5000 microtiter plate reader at wavelength 550 nm.

Crystal violet (CV) assay **[Cell wall membrane integrity]**

The CV assay was described by Saotome *et al.*, (1989) as follow: 40 µl of 50% formalin were added to each well and left for one hour. The plates were rinsed under a gentle flow tap water and 200 µl of crystal violet (0.0625%) were added to each well and standed up 5 min at room temperature. The stain was removed and the plates were washed with tap and deionized water, and dried. The absorbance was measured by using microtiter plate reader MR-5000 at a wavelength of 540 nm.

Immunotoxicity assay

Protease activity assay (PA)

The protease activity assay was determined by adaptation the method of Rinderrnecitt *et al.*, (1968). In brief, hepatocyte cells $1x10^5$ were suspended in 100 µl medium and inoculated into wells of 96-well microtiter plate. On the second day, 100 µl of fresh medium unamended or amended with varied concentrations of tested toxic chemicals were added to the cells. After 24 hour of incubation, the medium was removed, and the cells were washed three times in HBSS. 40 µl of buffer (150 mM Tris base, 30 mM $CaCl_2$, 0.05% Brij 35) and 50 µl of protease substrate (20% HPA, 20 % sucrose, 0.05% Brij) were added to each well. The plates were incubated at 37°C with continuous shaking for 2 hours. The reaction was stopped by the addition of 50 µl of 10% TCA and the plates were stored at 4°C for about 15 min. After spinet the plates at 8500 rpm for 5 min, the supernatants were transferred to new plates and the absorbance of the plates were measured in a microplate reader MR-5000 at 540 nm. Blank wells were prepared by the same previous steps without cell addition.

Effects of superoxide anion scavengers on acrylamide-induced cytotoxicity

To determine the relative role of ROS in AA-induced cytotoxicity, liver cell cultures were treated 1 h before and during the addition of AA with PEG-SOD (5 U/mL, Sigma) or PEG-catalase (50 U/mL, Sigma). After incubation for 24 h, NR assays were performed to evaluate cytotoxicity.

Statistical analysis

All experiments of cytotoxicity were performed at least three times, using four wells for each concentration of tested agent. Data for the dose-response cytotoxicity curves were presented as the arithmetic mean ± SD. Comparative cytotoxicity of tested toxicant compounds i.e. the concentration of toxicant needed to reduce absorbance of the NR, MTT, CV, GA, and PA by 10% (NR_{90}, MTT_{90}, CV_{90}, GA_{90}, and PA_{90} values) and by 50% (NR_{50}, MTT_{50}, CV_{50}, GA_{50}, and PA_{50} values) were computed by linear regression analysis of the data as percentage of control versus the logarithmic concentration of the toxicant. Analysis of variance was performed on data using the General Linear Models Procedure (GLM) of the Statistical Analysis System, MINITAB 12 computer program (Minitab Inc., State College, PA).

3. Results

Effects of OPP on acrylamide cytotoxicity and immunotoxicity in liver cells cultures

Primary Bolti liver cells were used as a biological model system for studying the effects of OPE on acrylamide cytotoxicity and immunotoxicity in liver cells cultures. OPE at concentrations ranged 2.5-20 mg/l were not cytotoxic or immunotoxic to liver cells cultures (data not shown). Cytotoxicity and immunotoxicity of acrylamide in liver cells cultures were observed. The absorbance of NR, MTT, CV and PA assays (as a percent of control) in AA treatment were 9.13-85.60, .43-88.17, 11.40-92.45 and 46.14-104.49, respectively (Figures 1-2). The treatment with OPP by different concentrations leads to attenuate their cytotoxic and immunotoxic assays of AA in liver cells. The rate of attenuation was increased with the increasing of OPP concentration treatment. Treatment with the highest concentration of OPP (20 mg/l) recorded absorbance values 54.98-99.10, 56.63-102.07, 61.54-107.3 and 62.90-118.70 for NR, MTT, CV and PA assays, respectively. Such data are in accordance with the obtained by Jun *et al.,* (2008) who reported that treatment with curcumin decreased the cytotoxicity and carcinogenicity induced by AA.

Initial and midpoint toxicity of AA or AA with OPP

By constructing the logarithmic dose-response cytotoxicity curves for AA or AA treated with OPP, the initial toxicity in NR_{90} , MTT_{90} , CV_{90} and PA_{90}values, which the agents caused 10% decrease in absorbance and midpoint toxicity i.e. NR_{50}, MTT_{50} , CV_{50} and PA_{50} values were calculated. Such cytotoxicity data for AA and AA with OPP are represented in Table (1). It was noticed that the midpoint toxicity values of AA treated samples were 8.00, 9.80, 9.75 and 17.00 mg/l for NR, MTT, CV and PA assays, respectively. Treatment of

AA samples with OPP, 2.5 mg/l, increased these values (decreasing the cytotoxic and immunotoxic effects) and recorded 11.00, 9.50, 14.75 and 19.75 mg/l, respectively. Increasing the concentration of OPP treatment to 20 mg/l leads to removal the cytotoxic and immunotoxic effects totally i.e. NR, MTT, CV and PA not recorded values.

Effects of superoxide anion scavengers on acrylamide-induced cytotoxicity

To determine the involvement of reactive oxygen species (ROS) in AA-induced cytotoxicity, the roles of various superoxide anion scavengers PEG-SOD (a superoxide scavenger) and PEG catalase (a hydrogen peroxide scavenger) were examined (Figure 3). PEG-catalase (50 U/mL) resulted in a significant decrease in AA-induced cytotoxicity and immunotoxicity, and the inhibitory concentration 50% values for 24 h were significantly increased to 7.22 mM from 6.07 mM ($P < 0.05$), whereas PEG-SOD (5 U/mL) had higher effect and recorded 11.51 mM. These results suggest that AA-induced cytotoxicity and immunotoxicity are directly dependent on hydrogen peroxide production. ROS is critically involved in AA-induced cytotoxicity and immunotoxicity, and the mechanism of OPP attenuating the cytotoxicity and immunotoxicity of AA underlies ROS scavenging. The present data are in accordance with that obtained by Jun *et al*., (2008) who found that ROS is involved in AA-induced cytotoxicity and carcinogenicity in HepG2 cells.

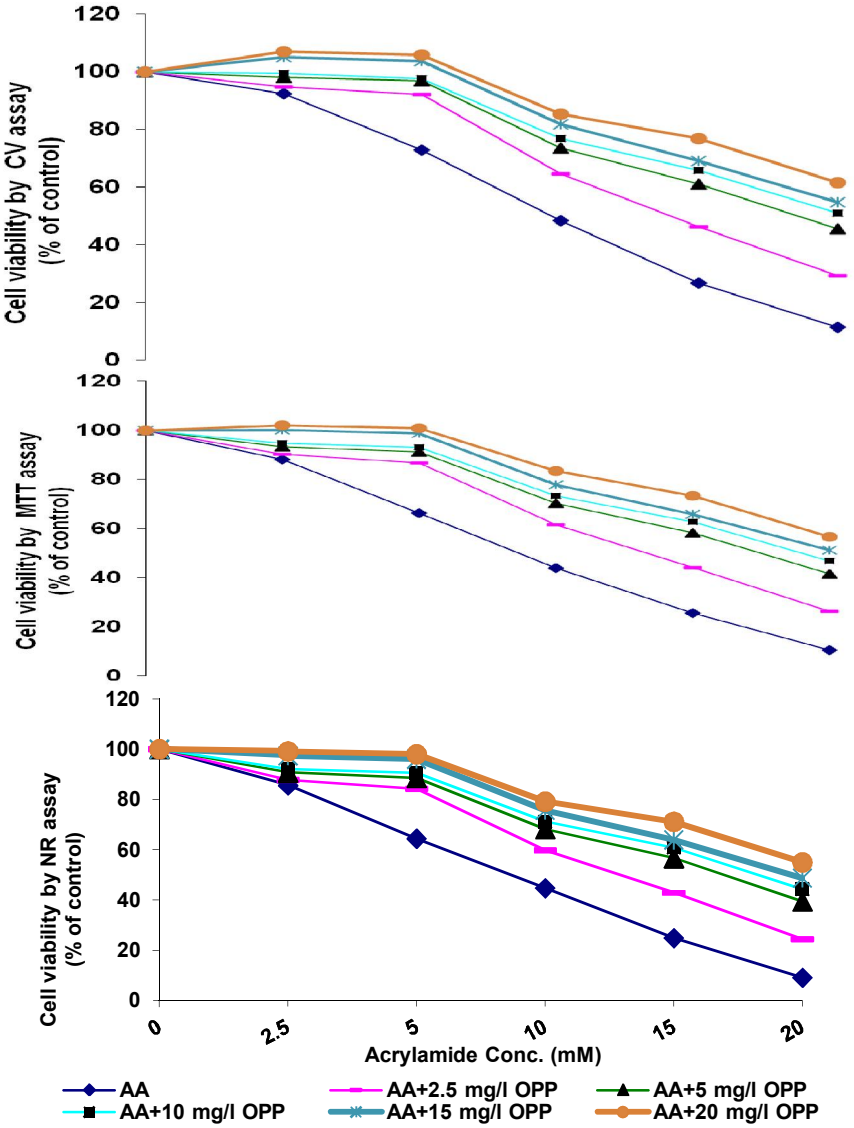

Figure 1. Effects of onion peels powder (OPP) on acrylamide-induced cytotoxicity in liver cell cultures determined by neutral red (NR), methyl tetrazollium (MTT) and crystal violet (CV) assays. Each point represents the mean value of three replicates.

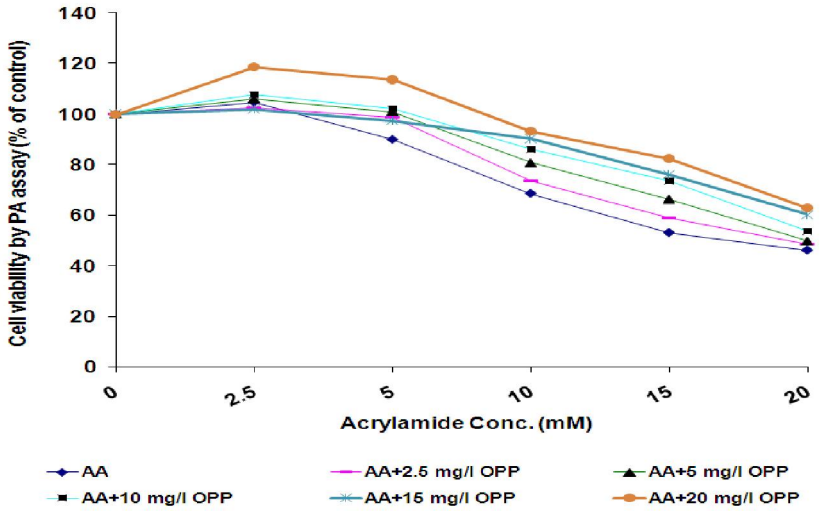

Figure 2. Effects of onion peel powder (OPP) on acrylamide-induced immunotoxicity in liver cell cultures determined by protease activity assay (PA). Each point represents the mean value of three replicates.

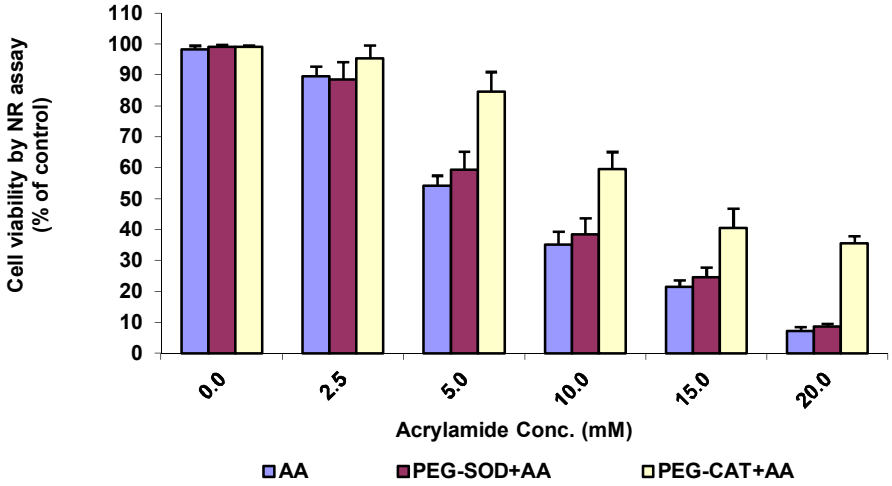

Figure 3. Effects of PEG-SOD and PEG-CAT on cytotoxicity induced by acrylamide in liver cell cultures determined by NR assay. Each bar represents the mean value of three replicates ±SD.

Table 1. Comparative cytotoxicity and immunotoxicity of acrylamide and acrylamide plus onion peels powder (In mg/l), as determined by neutral red (NR), methyl tetrazollium (MTT) crystal violet (CV) and protease activity (PA) assays

Test agent	NR assay		MTT assay		CV assay		PA assay	
	NR90 *	NR50 **	MTT90 *	MTT50**	CV90 *	CV50 **	PA90 *	PA50 **
AA (2.5 mM)	1.50	8.00	1.75	9.80	2.75	9.75	5.25	17.00
AA+2.5 mg/l OPP	1.75	11.00	2.50	9.50	5.25	14.75	5.75	19.75
AA+5 mg/l OPP	2.50	12.50	5.25	19.25	5.75	19.50	8.00	19.75
AA+10 mg/l OPP	5.00	18.00	5.50	19.25	5.75	***	9.75	***
AA+15 mg/l OPP	6.10	19.00	5.75	***	5.75	***	10.00	***
AA+20 mg/l OPP	7.00	OD	9.00	***	9.75	***	11.00	***

* Initial toxicity: mean concentrations of acrylamide required to reduce absorbance by 10 % (NR90, MTT90, CV90 and PA90).

** Midpoint toxicity : mean concentrations of herb extract required to reduce absorbance by 50 % (NR50, MTT50, CV50 and PA50).

*** Outside the boundaries of the cyotoxicity curve

4. Discussion

In the last few decades, primary fish liver cells were frequently used for toxicological studies. For examples, they used successfully as a biological model system in studying the cytotoxic and immunotoxic effects of pesticides, polycyclic aromatic hydrocarbons, industrials effluents, detergents, heavy metals etc (Baksi and Frazier, 1990; Elhassaneen, 1996 and 1998; Elhassaneen *et al*., 1997; ElSadany, 2001 and El-Sharkawy, 2011). In the present study, primary fish liver cells used as a biological model to investigate the cytoxicological and immunological effects of AA and the role of OPP on these types of effects. In this manner, three cytotoxic assays i.e. NR, MTT and CV and one immunotoxic assay i.e. PA were applied. In related to the cytotoxic effects of AA and AA treated with OPP, three different toxic responses were obtained (see figures 1-2). The first type of response was the inhibition of cell division. This response was characterized by stabilization or slightly increases in the initial count of cultured cells even with increasing the toxicant concentration. Kocan *et al.*, (1985), Elhassaneen, (1996) and Elsadany, (2001) attributed this type of response to the cellular dysfunction or damage. The second type of response was the cytotoxicity, immunotoxicity or cell death. It could be seen in cultures as a lower cell count present at the termination period than in the beginning period of the exposure to toxic substances. This response could be characterized also by a dose-response curve, which is inversely proportional to the concentration of tested toxicant substances. The same authors demonstrated that cytotoxicity could be resulted from cells dying and/or inhibition of cell proliferation. Hormesis represented the third response, which means increasing occurs in cell number over the controls at low concentrations of the toxic substance. However, the toxic effect does not manifest itself until a higher critical dose level is reached. This phenomena was well known early by Laughlin *et al*., (1981) and established by many authors (Kocan *et al.*, 1985; Elhassaneen, 1996 and Elhassaneen, 2002).

The mechanism by which acrylamide causes cancer in laboratory animals and by which it may cause cancer in humans is as yet unclear (reviewed in Jannekeet *et al.,* 2008); both genotoxic and nongenotoxic pathways have been suggested. Acrylamide itself and its epoxide metabolite glycidamide, which is generated by cytochrome P4502E1 (CYP2E1), are clastogenic, and glycidamide forms DNA adducts.

As for possible nongenotoxic pathways, acrylamide reacts with glutathione and may thus influence the redox status of cells and gene transcription, or it may interfere with DNA repair or hormonal balances (Besaratinia and Pfeifer, 2007). During studying the effect of OPP on the cytotoxicity and immunotoxicity of AA, all of these toxic responses were attenuated clearly. One of the reasons, plant phenolics such determined in OPP can be viewed as blocking agents in carcinogenesis in one or more of several possible modes of action (Conn 1981). Ionization of the phenol can form a strong nucleophile, or electron donor, to the electrophile of the ultimate chemical carcinogen. Such as reviewed in Newmark, (1987), the key functional groups postulated to be present in phenolics, enabling them to act as effective electrophilic trapping agents, are as follows: 1) There must be at least one free phenolic group at the site of desired action, preferably related to a catechol to lower the first pK_a, i.e., increase ionization, and preferably with one hydroxyl group *para* to a substituent capable of delocalizing electrons from the ring. 2) There must be an unsaturated substituent in conjugation with the aromatic ring, serving to further delocalize electrons on the ring itself. This delocalization is important for increased capacity to act as an electron-rich donor (i.e., electrophilic trap for electron-poor positively charged species such as carbenium ions). On the other side, several researchers have investigated the effect of various antioxidants and antioxidative extracts on acrylamide formation but the data were discordant. Some studies claimed mitigation while others no effect or even an increase. It can be attributed to the ability of antioxidants with different structures or functional groups to react with acrylamide precursors, with intermediates of the reaction or with acrylamide itself, leading to either reducing or promoting effects (Friedman and Levin, 2008 and Cheng *et al.,* 2013).

In conclusion, the present study indicated that AA caused increase of reactive oxygen species (ROS) formation and induced cytotoxicity and immunotoxicityin primary liver cells cultures. Supplementation of OPP rich in phenolic compounds may reduce AA- mediated cytotoxicity and immunotoxicity due to its direct ROS scavenging activity. In this *in vitro* study, OPP presented protective effects on the cytotoxicity and immunotoxicity induced by AA particularly at high concentrations (20 mM). Further studies will be needed in the future to elucidate the protective mechanism of OPP against different types of toxicities induced by AA.

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