Portulaca oleracea Linn seed extract ameliorates hydrogen peroxide-induced cell death in human liver cells by inhibiting reactive oxygen species generation and oxidative stress

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Original Research Article

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INTRODUCTION

Oxidative stress is caused by an imbalance in the amount of reactive oxygen species (ROS) and antioxidant defense systems in biological system [1]. It is one of the most important factors inducing cell apoptosis [2]. Oxidative stress can increase the vulnerability to lipid peroxidation, DNA damage, enzymatic inactivation, and cell death [3]. It has been reported that overproduction of ROS plays a major role in hepatocarcinoma [4], and cellular damage [5]. H₂O₂ has been reported to induce cytotoxicity and apoptotic cell death in a variety of cell
investigated before addition to the medium. Saudi Arabia. The seeds were screened manually. For consumption and is recognized for its numerous benefits [10]. The pharmacological and preventive properties of P. oleracea, such as anti-inflammatory, antioxidative, anti-bacterial, skeletal muscle relaxant, wound-healing, and in vitro anti-tumor have been reported [11]. It was recently demonstrated that the seed extract and oil of P. oleracea induced cytotoxicity in human liver cancer cells [11]. However, the mechanism(s) of the protective effects of P. oleracea against H₂O₂, induced oxidative stress and ROS generation in HepG2 have not been evaluated.

Therefore, this study was aimed to investigate the protective effects of P. oleracea against H₂O₂ induced cytotoxicity and oxidative stress in HepG2 cells. HepG2 cells have been shown to be a good model system for assessing the toxicity or detoxification of various compounds against oxidative stress inducers [12].

**EXPERIMENTAL**

**Materials**

DMEM culture medium, antibiotics-antimycotic solution, fetal bovine serum (FBS), and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Consumables and culture products used in the study were obtained from Nunc (Roskilde, Denmark). H₂O₂ and all other specified chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

**Plant material and extraction**

The seeds of P. oleracea used in this study were obtained from a local market in Riyadh, Saudi Arabia. The seeds were screened manually. For the preparation of alcoholic extract, the seeds were macerated in ethanol and then filtered. The procedure was repeated five times. The solvent was then evaporated using a rotary evaporator and the residue obtained was named the alcoholic extract (POA).

**Cell culture**

HepG2 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 0.2% sodium bicarbonate and antibiotic/antimycotic solution (100×, 1 mL/100 mL of medium). Cells were grown in 5% CO₂ at 37 °C in high humidity atmosphere. Before the experiments, cell viability was assessed as described by Siddiqui et al [20]. HepG2 cells showing more than 98% cell viability and at passage numbers 20 - 22 were used in this study.

**Drug solutions**

The POA was not completely soluble in the culture medium; therefore the stock solutions of the extract were prepared in dimethyl sulphoxide (DMSO) and diluted in culture medium to reach the desired concentrations. H₂O₂ was freshly diluted in culture medium before addition to the cells.

**Cytotoxicity by MTT assay**

The percentage cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [13]. Briefly, HepG2 cells (1 × 10⁴) were allowed to adhere for 24 h in a CO₂ incubator at 37 °C in 96-well culture plates. After 24 h exposure of HepG2 cells to increasing concentrations (5 - 500 μg/mL) of POA for 24 h, MTT (5 mg/mL of stock in PBS) was added (10 μL/well in 100 μL of cell suspension), and the plates were incubated for 4 h. The supernatant was discarded and 200 μL of DMSO was added to each well and mixed gently. The developed color was read at 550 nm in a multiwell microplate reader (Thermo Scientific, Waltham, MA, USA). Untreated sets were also run under identical conditions as controls.

**Neutral red uptake (NRU) assay**

The NRU assay was carried out as described by Siddiqui et al [13]. Briefly, after 24 h exposure of HepG2 cells to increasing concentrations (5 - 500 μg/mL) of POA for 24 h, the medium was aspirated and the cells were washed twice with PBS and incubated for 3 h in medium supplemented with neutral red (50 μg/mL). The medium was rapidly removed with a solution containing 0.5% formaldehyde and 1% calcium chloride. The cells were subjected to further incubation for 20 min at 37 °C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 540 nm in a multi-well microplate reader (Thermo Scientific).
The values were compared with the control sets run under identical conditions.

Assessment of morphological alterations

Morphological changes in HepG2 cells exposed to increasing concentrations of POA (5 - 500 µg/mL) for 24 h were observed using a phase contrast inverted microscope (Olympus, Tokyo, Japan) equipped with automatic image analysis software. Further, to observe the protective effects of POA on cellular morphology, HepG2 cells were treated with non-cytotoxic concentrations (5, 10 and 25 µg/mL) of POA for 24 h before treatment with H₂O₂ (0.25 mM) for 24 h.

Determination of glutathione (GSH) level

The intracellular level of reduced GSH was estimated as described by Chandra et al [14] with some modifications. Briefly, HepG2 cells exposed to POA and H₂O₂ were collected by centrifugation and the cellular proteins were precipitated by incubating 1 mL sonicated cell suspension with 10 % trichloroacetic acid (1 mL) on ice for 1 h followed by centrifugation at 3000 rpm for 10 min. The supernatant was then added to 2 mL buffer (0.4 M Tris and 0.02 M EDTA; pH 8.9) and 0.01 M of 5,5′-dithionitrobenzoic acid to reach a final volume of 3 mL. The tubes were incubated for 10 min at 37 °C in a shaking water bath. The absorbance of the yellow color developed was read at 412 nm.

Evaluation of lipid peroxidation (LPO)

LPO was evaluated using thiobarbituric acid-reactive substances protocol [15]. Briefly, after exposing HepG2 cells to POA and H₂O₂, HepG2 cells were collected by centrifugation, sonicated in ice-cold potassium chloride (1.15 %), and centrifuged again for 10 min at 3000× g. The resulting supernatant (1 mL) was collected and 2 mL of thiobarbituric acid reagent (15 % trichloroacetic acid, 0.7 % thiobarbituric acid and 0.25 NHCl) was added. The solution was heated at 100 °C for 15 min in a boiling bath. The sample was then placed at a cold temperature and centrifuged at 1000 × g for 10 min. Absorbance of the supernatant was measured at 535 nm.

Determination of reactive oxygen species (ROS) generation

ROS generation was assessed using 2,7-dichlorodihydrofluoresceindiacetate (DCFH-DA; Sigma) dye as a fluorescence agent described previously [16]. Following exposure to POA and H₂O₂ for 24 h, the cells were washed with PBS and incubated for 30 min in DCFH-DA (20 µM) containing incomplete culture medium in the dark at 37 °C. Next, the cells were analyzed for intracellular fluorescence using a fluorescence microscope.

Statistical analysis

The results were expressed as the mean ± SEM of at least three independent experiments (conducted in triplicate). Statistical analysis was performed using one-way analysis of variance using Dunnett’s post hoc test employed to compare the values between control and treated groups. Differences were considered statistically significant at \( p < 0.05 \).

RESULTS

Cytotoxicity of POA

The results showed that POA concentrations of 5, 10 and 25 µg/mL had no significant effects on the viability of HepG2 cells (Figure 1 and Figure 2). Therefore, the concentrations 5, 10 and 25 µg/mL of POA were used to study the protective effects against H₂O₂-induced toxicity in HepG2 cells. Further, based on the LD₅₀ value, 0.25 mM of H₂O₂ was used to induce toxicity in further experiments.

![Figure 1](image-url)
Figure 2: Morphological alterations in HepG2 cells following the exposure to various concentrations of *Portulaca oleracea* extract (POA) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20× magnification.

**Protective effect of POA against H₂O₂ induced cell death**

The protective potential of POA in HepG2 cells observed in the MTT and NRU assays are presented in Figure 3. A significant ($p < 0.01$) reduction in the percentage of cell viability was observed in HepG2 cells following exposure to H₂O₂ (0.25 mM) for 24 h by MTT assay (Figure 3A) and NRU assay (Figure 3B). HepG2 cells pre-treated with POA at 5, 10 and 25 µg/mL for 24 h significantly attenuated the H₂O₂-induced loss of cell viability in a concentration-dependent manner. Increases of 11, 26, and 38 % in the cell viability of HepG2 cells were recorded at 5, 10, and 25 µg/mL of POA, respectively (Figure 3A). A similar concentration-dependent increase in cell viability was observed in the NRU assay in POA pre-exposed HepG2 cells. Increases of 15, 28, and 40 % in cell viability of HepG2 cells were recorded at 5, 10, and 25 µg/mL of POA, respectively (Figure 3B).

**Morphological changes**

Alterations in the morphology of HepG2 cells following exposure to POA and H₂O₂ are shown in Figure 4(A - E). Exposure to 0.25 mM of H₂O₂ reduced the normal morphology and cell adhesion capacity of HepG2 cells compared to controls. Most cells exposed to H₂O₂ lost their typical morphology and appeared smaller in size (Figure 4B). Exposure of HepG2 cells to increasing concentrations of POA for 24 h prior to H₂O₂ exposure significantly restored their original morphology in a concentration-dependent manner (Figure 4C - E).

Figure 3: Cell viability by (A) MTT and (B) NRU assays in HepG2 cells. HepG2 cells were exposed to 5 – 25 µg/mL of POA for 24 h. Next, the cells were exposed to 0.25 mM of H₂O₂ for 24 h. Values are mean ± SEM (n = 3); $^*p<0.05$, $^*^*p<0.01$ versus H₂O₂ exposure.
Figure 4: Morphological changes of HepG2. Cells were pre-exposed to POA for 24 h and then H2O2 for 24 h. Image were acquired using a phase contrast inverted microscope at 20× magnification. [A] Control, [B] H2O2 (0.25 mM), [C] POA (5 µg/mL) + H2O2 (0.25 mM), [D] POA (10 µg/mL) + H2O2 (0.25 mM), and [E] POA (25 µg/mL) + H2O2 (0.25 mM)

Figure 5: Protective potential of POA on [A] Glutathione (GSH) and [B] Lipid peroxidation (LPO) levels in HepG2 following the exposure to POA (25 µg/mL) for 24 h then H2O2 for 24 h. All values represent the mean ± SE. #p<0.01 vs. control, *p<0.01 versus H2O2 exposure. [C] H2O2-induced ROS generation and ameliorative effect of pre-treatment of POA on HepG2 cells. ROS generation was evaluated using dichlorofluorescin diacetate (DCFH-DA) dye. (i): Untreated control, (ii): Cells exposed to H2O2 (0.25 mM) for 24 h, (iii): Cells exposed to 25 µg/mL of POA for 24 h and then H2O2 (0.25 mM)

GSH level

The protective potential of POA on H2O2-induced depletion in GSH level is summarized in Figure 5A. As shown in the figure, exposure of HepG2 cells to 0.25 mM of H2O2 significantly reduced the GSH level by 44 % (p < 0.01) compared to the control. The results also showed that 25 µg/mL POA significantly prevented (p < 0.01) the decrease in GSH levels caused by H2O2 in HepG2 cells (Figure 5A).

LPO levels

The protective potential of various concentrations of POA on H2O2-induced lipid peroxidation in HepG2 cells is summarized in Figure 5B. As shown in the figure, exposure to H2O2 resulted in a significant increase of 49 % (p < 0.01) in LPO
Compared to in control cells. HepG2 cells pre-treated with 25 µg/mL POA for 24 h prior to H₂O₂ treatment showed significantly (p < 0.01) reduced LPO levels (Figure 5B).

**ROS generation**

The results of ROS generation in HepG2 cells exposed to H₂O₂ and various concentrations of POA are presented in Figure 5C. Exposure of HepG2 cells to 0.25 mM H₂O₂ for 24 h resulted in ROS production. Pre-treatment of cells with 25 µg/mL POA concentration significantly reduced the ROS generation induced by H₂O₂ in HepG2 cells (Figure 5C).

**DISCUSSION**

Oxidative stress is associated with a variety of human diseases [17]. There is increasing interest in naturally derived bioactive compounds with potential cytoprotective effects against oxidative stress-induced cell death [18]. Because oxidative stress appears to be involved in many diseases, the administration of antioxidants may be useful for preventing and treating these diseases [19]. Based on previous study, which revealed that H₂O₂ induces cytotoxicity in HepG2 cells in a concentration dependent manner [20], 0.25 mM H₂O₂ was used to induce cytotoxicity, oxidative stress and ROS generation in HepG2 cells.

The pharmacological activities of *P. oleracea* such as anti-inflammatory, antioxidative, antibacterial, skeletal muscle relaxant, wound-healing, and in vitro anti-tumor have been documented [11]. However, the protective effects of *P. oleracea* on oxidative stress and ROS generation in HepG2 cells induced by H₂O₂ had not been examined. The present study was carried out to assess the protective effects of POA in HepG2 cells. The data indicate that the pre-treatment of HepG2 cells with POA (5 - 25 µg/mL) had protective effects on the viability of HepG2 cells against H₂O₂-induced cytotoxicity. The results agree with previous findings, where the extracts of natural products showed cytoprotective potential against H₂O₂ [6]. It has also been reported that natural products protect liver cells against H₂O₂ [20] and other toxicants [21]. The results also showed that H₂O₂ reduced the GSH level in HepG2 cells compared to in untreated controls. Pre-treatment of HepG2 cells with POA at 25 µg/mL significantly restored the decrease in the GSH level caused by H₂O₂. It is also known that glutathione peroxidase catalyzes GSH oxidation to GSSG at the expense of H₂O₂ and that glutathione reductase recycles oxidized GSH back to reduced GSH [22]. Thus, it can be hypothesized that POA pre-treatment of HepG2 cells reduced the intracellular damaging peroxide and recovered GSH concentration. The restoration of GSH clearly indicates that POA plays an important role in the cell defense system against H₂O₂. Lipid peroxidation is known to be involved in oxidative stress and cell death [23]. In the present study, H₂O₂ increased lipid peroxidation in HepG2 cells. The results support those of previous studies [24], where an increase in LPO level due to H₂O₂ was observed. This increase in lipid peroxidation in HepG2 cells by H₂O₂ may be related to the enhancement of hepatic MDA from the peroxidation of polyunsaturated fatty acids [20]. The results also showed that exposure to HepG2 cells to 25 µg/mL POA significantly decreased lipid peroxidation levels. An increase in ROS generation indicates that H₂O₂ can cause oxidative stress in HepG2 cells. The results showed that pre-treatment with POA significantly reduced intracellular ROS generation induced by H₂O₂. The findings of this study correlate with those of other reports showing that the administration of natural products suppressed the increases in intra-cellular ROS generation [6].

**CONCLUSION**

The findings of this study indicate that *P. oleracea* can protect human liver cells (HepG2) against H₂O₂-induced cytotoxicity by inhibiting ROS generation and oxidative stress. The results also provide insight into the biological activities of *P. oleracea*, suggesting that it is a good source of antioxidants.

**DECLARATIONS**

**Acknowledgement**

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**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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