Tropical Journal of Pharmaceutical Research August 2016; 15 (8): 1643-1649
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v15i8.7

Original Research Article

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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Received: 31 March 2016 *Revised accepted: 2 July 2016*

Abstract

Purpose: *To investigate the protective effects of Portulaca oleracea seed extract (POA) against cytotoxicity, oxidative stress and reactive oxygen species (ROS) generation induced by hydrogen peroxide (H_2O_2) in human liver cells (HepG2).*

Methods: *The extract (POA) was obtained by ethanol extraction of P. oleracea seeds. Cytotoxicity in HepG2 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, neutral red uptake (NRU) assay and morphological changes. The cells were pre-exposed to non-cytotoxic concentrations (5 - 25 µg/mL) of POA for 24 h, and then cytotoxic (0.25 mM) concentration of H_2O_2. After 24 h of exposure, MTT and NRU assays were used to evaluate cell viability, while morphological changes were assessed using phase contrast inverted microscopy. The effect of POA on reduced glutathione (GSH) level, lipid peroxidation (LPO), and ROS generation induced by H_2O_2 was also studied.*

Results: *The results showed that pre-exposure to POA (25 µg/mL) significantly ($p < 0.01$) attenuated the loss of cell viability by up to 38 % against H_2O_2-induced oxidative stress and ROS generation. In addition, POA (25 µg/mL) significantly ($p < 0.01$) increased GSH level (31 %), but decreased the levels of LPO (37 %) and ROS generation (49 %).*

Conclusion: *This study demonstrates that POA has the capacity to protect HepG2 cells against H_2O_2-induced cell death by inhibiting oxidative stress and ROS generation.*

Keywords: *Portulaca oleracea, HepG2 cells, Cytotoxicity, Oxidative stress, Reactive oxygen species*

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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]. H_2O_2 has been reported to induce cytotoxicity and apoptotic cell death in a variety of cell

systems [6], including human liver cells (HepG2) [7].

H_2O_2-induced ROS generation and oxidative stress have been reported previously [8]. Thus, used H_2O_2 to induce cytotoxicity, oxidative stress, and ROS generation in HepG2 cells. *Portulaca oleracea* (Family: Portulacaceae), is an annual green herbaceous medicinal plant widespread in temperate and tropical regions worldwide [9]. It has been used as a vegetable for human 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 H_2O_2, 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 H_2O_2 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). H_2O_2 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 % CO_2 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. H_2O_2 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 (1×10^4) were allowed to adhere for 24 h in a CO_2 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 H_2O_2 (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 H_2O_2 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 H_2O_2, 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 H_2O_2 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 H_2O_2-induced toxicity in HepG2 cells. Further, based on the LD_{50} value, 0.25 mM of H_2O_2 was used to induce toxicity in further experiments.

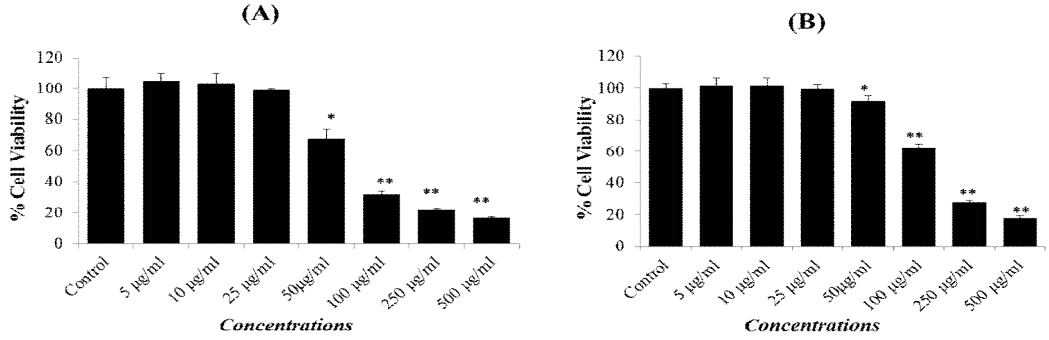

Figure 1: Cell viability based on (A) MTT and (B) NRU assays in HepG2 cells following exposure to various concentrations of *Portulaca oleracea* extract (POA) for 24 h. Values are mean ± SEM (n = 3); *$p < 0.05$, **$p < 0.01$ vs. control

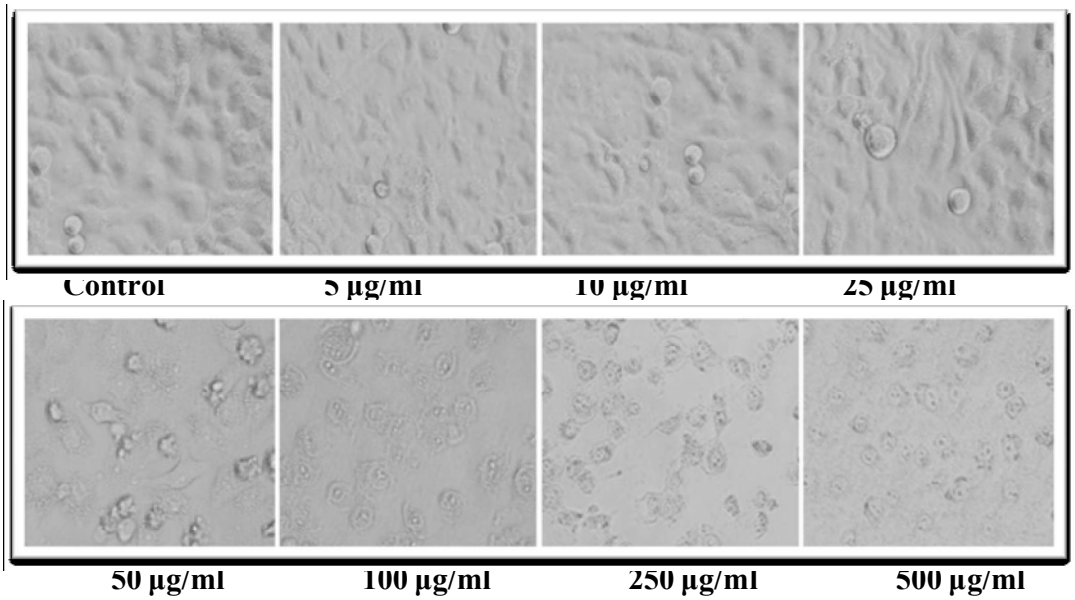

Figure 2: Morphological alterations in HepG2 cells following the exposure to various concentrations of *Portulaca oleracea* extract (POA) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20× magnification

Protective effect of POA against H_2O_2 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 H_2O_2 (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 H_2O_2-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 H_2O_2 reduced the normal morphology and cell adhesion capacity of HepG2 cells compared to controls. Most cells exposed to H_2O_2 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 H_2O_2 exposure significantly restored their original morphology in a concentration-dependent manner (Figure 4C - E).

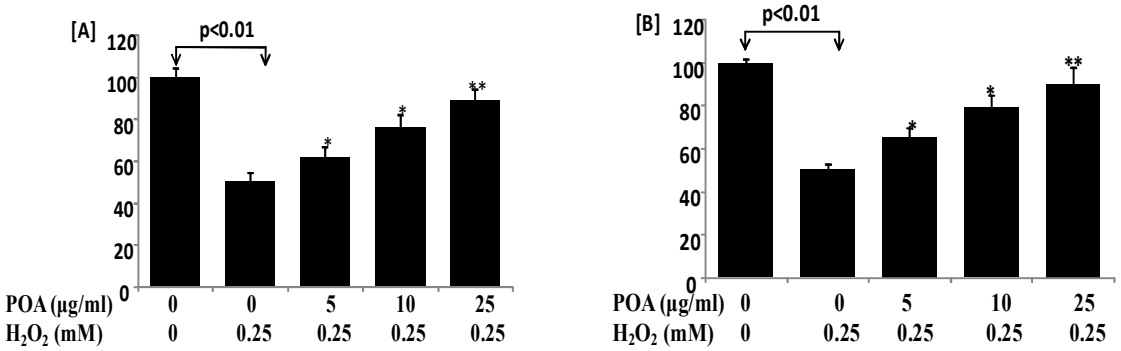

Figure 3: Cell viability by (A) MTT and (B) NRU assays in HepG2 cells. HepG2 cells were exposed to 5 – 25 µg/mL of POA for 24 h. Next, the cells were exposed to 0.25 mM of H_2O_2 for 24 h. Values are mean ± SEM (n = 3); *$p<0.05$, **p<0.01 versus H_2O_2 exposure

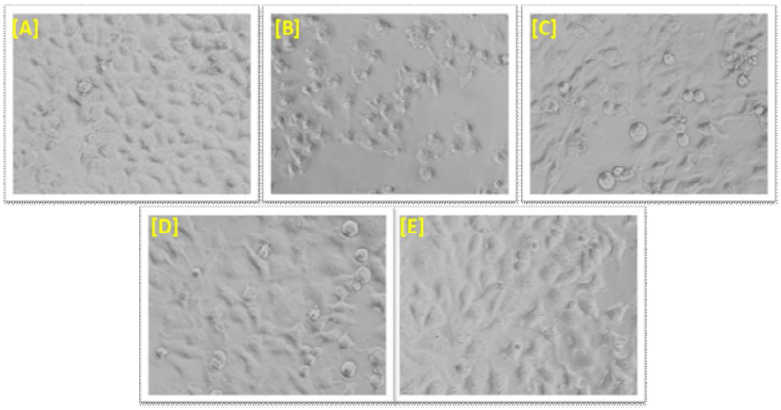

Figure 4: Morphological changes of HepG2. Cells were pre-exposed to POA for 24 h and then $H_2O_2$for 24 h. Image were acquired using a phase contrast inverted microscope at 20× magnification. [A] Control, [B] H_2O_2 (0.25 mM), [C] POA (5 µg/mL) + H_2O_2 (0.25 mM), [D] POA (10 µg/mL) + H_2O_2 (0.25 mM), and [E] POA (25 µg/mL) + H_2O_2 (0.25 mM)

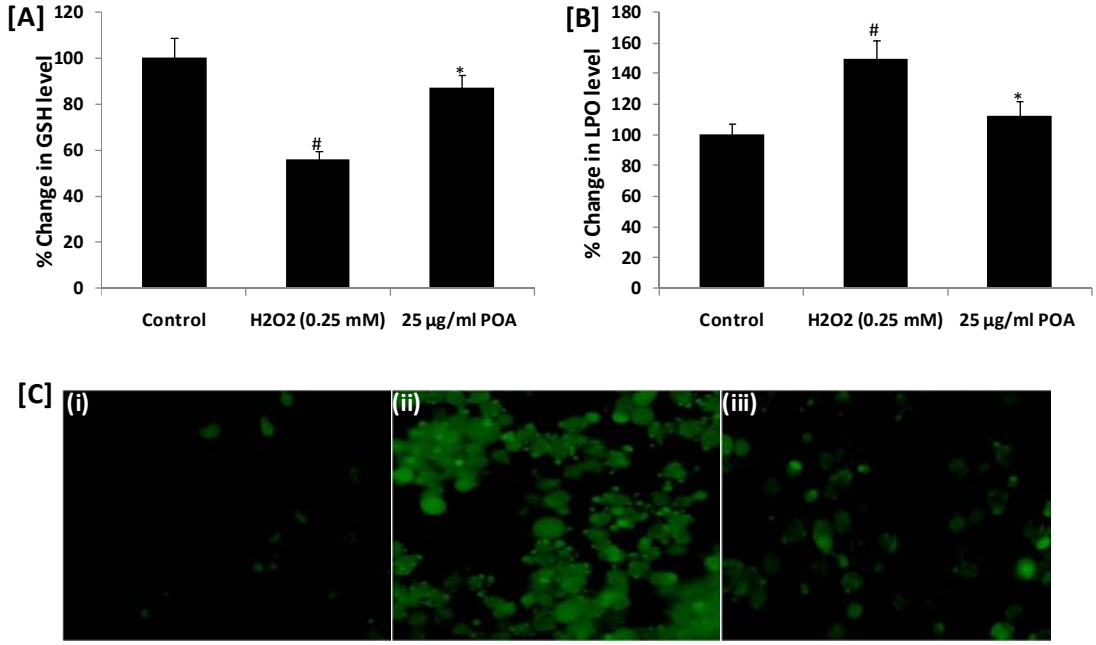

Figure 5: Protective potential of POA on [A] Glutathione (GSH) and [B] Lipid peroxidation (LPO) levels in HepG2 following the exposure to POA (25 µg/mL) for 24 h then H_2O_2 for 24 h. All values represent the mean ± SE. #p<0.01 vs. control, *p<0.01 versus H_2O_2 exposure. **[C]** H_2O_2-induced ROS generation and ameliorative effect of pre-treatment of POA in HepG2 cells. ROS generation was evaluated using dichlorofluorescin diacetate (DCFH-DA) dye. (i): Untreated control, (ii): Cells exposed to H_2O_2 (0.25 mM) for 24 h, (iii): Cells exposed to 25 µg/mL of POA for 24 h and then H_2O_2 (0.25 mM)

GSH level

The protective potential of POA on H_2O_2-induced depletion in GSH level is summarized in Figure 5A. As shown in the figure, exposure of HepG2 cells to 0.25 mM of H_2O_2 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 H_2O_2 in HepG2 cells (Figure 5A).

LPO levels

The protective potential of various concentrations of POA on H_2O_2-induced lipid peroxidation in HepG2 cells is summarized in Figure 5B. As shown in the figure, exposure to H_2O_2 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 H_2O_2 treatment showed significantly ($p < 0.01$) reduced LPO levels (Figure 5B).

ROS generation

The results of ROS generation in HepG2 cells exposed to H_2O_2 and various concentrations of POA are presented in Figure 5C. Exposure of HepG2 cells to 0.25 mM H_2O_2 for 24 h resulted in ROS production. Pre-treatment of cells with 25 µg/Ml POA concentration significantly reduced the ROS generation induced by H_2O_2 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 H_2O_2 induces cytotoxicity in HepG2 cells in a concentration dependent manner [20], 0.25 mM H_2O_2 was used to induce cytotoxicity, oxidative stress and ROS generation in HepG2 cells.

The pharmacological activities of *P. oleracea* such as anti-inflammatory, antioxidative, anti-bacterial, 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 H_2O_2 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 H_2O_2-induced cytotoxicity. The results agree with previous findings, where the extracts of natural products showed cyto-protective potential against H_2O_2 [6]. It has also been reported that natural products protect liver cells against H_2O_2 [20] and other toxicants [21]. The results also showed that H_2O_2 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 H_2O_2. It is also known that glutathione peroxidase catalyzes GSH oxidation to GSSG at the expense of H_2O_2 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 H_2O_2. Lipid peroxidation is known to be involved in oxidative stress and cell death [23]. In the present study, H_2O_2 increased lipid peroxidation in HepG2 cells. The results support those of previous studies [24], where an increase in LPO level due to H_2O_2 was observed. This increase in lipid peroxidation in HepG2 cells by H_2O_2 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 H_2O_2 can cause oxidative stress in HepG2 cells. The results showed that pre-treatment with POA significantly reduced intracellular ROS generation induced by H_2O_2. 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 H_2O_2-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

This project was financially supported by King Saud University, Vice Deanship of Research Chairs.

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.

REFERENCES

1. Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol Environ Saf 2006; 64(2): 178–189.
2. Miyoshi N, Oubrahim H, Chock PB, Stadtman ER. Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis. Proc Natl Acad Sci USA 2006; 103(6): 1727-1731.
3. Khalil WA, Marei WF, Khalid M. Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. Theriogenol 2013; 80(2):161–168.
4. Zhang Y, Jiang L, Jiang L, Geng C, Li L, Shao J, Zhong L. Possible involvement of oxidative stress in potassium bromate-induced genotoxicity in human HepG2 cells. ChemBiol Inter 2011; 189(3): 186-191.
5. Lin PH, Lin CH, Huang CC, Chuang MC, Lin P. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces oxidative stress, DNA strand breaks, and poly(ADP-ribose) polymerase-1 activation in human breast carcinoma cell lines. Toxicol Lett 2007; 172(3): 146-158.
6. Kanno SI, Shouji A, Asou K, Ishikawa M. Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells. J Pharma Sci 2003; 92(2): 166-170.
7. Chen X, Zhong Z, Xu Z, Chen L, Wang Y. No protective effect of curcumin on hydrogen peroxide-induced cytotoxicity in HepG2 cells. Pharmacol Rep 2011; 63(3): 724-732.
8. Siddiqui MA, Kashyap MP, Kumar V, Tripathi VK, Khanna VK, Yadav S, Pant AB. Differential protection of pre-, co- and post-treatment of curcumin against hydrogen peroxide in PC12 cells. Human Exper Toxicol 2011; 30(3): 192-198.
9. Yang Z, Liu C, Xiang L, Zheng Y. Phenolic alkaloids as a new class of antioxidants in Portulaca oleracea. Phytother Res 2009; 23(7): 1032-1035.
10. Bidhendi F, Ahmadi R, Siavashi M, Mahdavi E. The effects of Portulaca oleracea seed extract on RBC membrane stability in male and female rats. International conference on food, biological and medical sciences (FBMS-2014), January 28-29, Bangkok (Thailand).
11. Farshori NN, Al-Sheddi ES, Al-Oqail MM, Musarrat J, Al-Khedhairy AA, Siddiqui MA. Cytotoxicity Assessments of Portulaca oleracea and Petroselinum sativum seed extracts on human hepatocellular carcinoma cells (HepG2). Asian Pac J Cancer Prev 2014; 15(16): 6633-6638.
12. Farshori NN, Al-Sheddi ES, Al-Oqail MM, Hassan WH, Al-Khedhairy AA, Musarrat J, Siddiqui MA. Hepatoprotective potential of Lavandula coronopifolia extracts against ethanol induced oxidative stress-mediated cytotoxicity in HepG2 cells. Toxicol Ind Health 2015; 31(8): 727-737.
13. Siddiqui MA, Singh G, Kashyap MP, Khanna VK, Yadav S, Chandra D, Pant AB. Influence of cytotoxic doses of 4-hydroxynonenal on selected neurotransmitter receptors in PC-12 cells. ToxicolIn Vitro 2008; 22(7): 1681-1688.
14. Chandra D, Ramana KV, Wang L, Christensen BN, Bhatnagar A, Srivastava SK. Inhibition of fiber cell globulization and hyperglycemia-induced lens opacification by amino peptidase inhibitor bestatin. Invest Ophthalmol Vis Sci2002; 43(7): 2285-2292.
15. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol1978; 52, 302-310.
16. Siddiqui MA, Ahmad J, Farshori NN, Saquib Q, Jahan S, Kashyap MP, Ahamed M, Musarrat J, Al-Khedhairy AA. Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. Mol Cell Biochem 2013; 384(1-2): 59-69.
17. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39(1): 44–84.
18. Jaydeokar AV, Bandawane DD, Bibave KH, Patil TV. Hepatoprotective potential of Cassia auriculata roots on ethanol and anti-tubercular drug-induced hepatotoxicity in experimental models. Pharm Biol2014; 52(3): 344-355.
19. Sekler A, Jiménez JM, Rojo L, Pastene E, Fuentes P, Slachevsky A, Maccioni RB. Cognitive impairment and Alzheimer's disease: Links with oxidative stress and cholesterol metabolism. Neuropsychiatr Dis Treat2008; 4(4): 715-722.
20. Al-Sheddi ES, Farshori NN, Al-Oqail MM, Musarrat J, Al-Khedhairy AA, Siddiqui MA. Protective effect of Lepidium sativum seed extract against hydrogen peroxide-induced cytotoxicity and oxidative stress in human liver cells (HepG2). Pharm Biol 2016; 54(2): 314-321.
21. Verma S, Bahorun T, Singh RK, Aruoma OI, Kumar A. Effect of Aegle marmelos leaf extract on N-methyl N-nitrosourea-induced hepatocarcinogensis in Balb/c mice. Pharm Biol 2013; 51(10): 1272-1281.
22. Ursini F, Maiorino M, Brigelius- Floh, Aumann KD, Roveri A, Schomburg D, Flohé L. Diversity of glutathione peroxidases. Methods Enzymol1995; 252: 38-53.
23. Tribble DL, Tak YA, Dean P, Jones MD. The pathophysiological significance of lipid peroxidation in oxidative cell injury. Hepatol1987; 7(2):377-386.
24. Siddique YH, Ara G, Afzal M. Estimation of lipid peroxidation induced by hydrogen peroxide in cultured human lymphocytes. Dose Response 2012; 10(1): 1-10.