Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Cytoprotective Effect of *Cactus cladode* (*Opuntia ficus-indica*) against Chlorpyrifos Induced Reactive Oxygen Species in Rat Hepatocytes: Involvement of Heat Shock Protein 70 and CYP1A1/2 Proteins

Hafiz Antar Makeen, Saida Ncibi[1,2], Syam Mohan[3], Abdullah Farasani[3], Roger Rahmani[4], Mohammed Al Bratty[5], Hassan A. Alhazmi[5]

Department of Clinical Pharmacy, Pharmacy Practice Research Unit, [2]Department of Biology, Faculty of Science, Jazan University, [3]Medical Research Centre, Jazan University, [5]Department of Pharmaceutical Chemistry, College of Pharmacy, Jazan University, Jazan, Kingdom of Saudi Arabia, [1]Research Unit BMG, Macromolecular Biochemistry and Genetics, Faculty of Sciences of Gafsa, Zarroug, Gafsa, Tunisia, [4]Toxicologie Cellulaire et Molecularie des Xenobiotiques, Sophia Antipolis, France

Submitted: 24-09-2018 Revised: 29-10-2018 Published: 26-04-2019

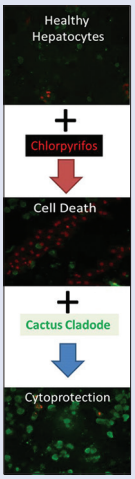
ABSTRACT

Background: Organophosphorus insecticides are well known to induce hepatotoxicity. One among this is chlorpyrifos (CPF), which is an insecticide inducing various toxicities including in liver. **Objective:** This investigation focused on CPF-induced oxidative damage in rat hepatocytes primary culture and the protective effect of *Cactus cladode* aqueous extract. **Materials and Methods:** Hepatocytes were treated with CPF (50, 75, and 150 μM) and cactus aqueous extract. On treatment for 48 h, mortality within these cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide test, reactive oxygen species (ROS) levels were measured by H2DCFDA test. Furthermore, heat shock protein 70 (HSP70) and CYP1A1/2 levels were determined using western blot analysis. Annexin V and 4′,6-diamidino-2-phenylindole analysis was run to determine the level of cell death and cytoprotection exerted by CPF and extracts, respectively. **Results:** The results showed that CPF increases the levels of H_2O_2 and HSP70 and induces CYP1A1/2 and mortality within these cells. In the other side of this study, the co-treatment of these cells with CPF and *Cactus cladode* aqueous extract showed a recovery of these parameters. It also has been found that the *Cactus cladode* aqueous extract has the potential to do cytoprotective effect by preventing necrosis induced by CPF. **Conclusion:** Taken together, these findings suggest that the toxicity exerted by CPF in hepatocytes are involved with the generation of ROS and the regulation of well-controlled programmed cell death, which could be well protected by the *Cactus cladode* extract pretreatment.

Key words: Chlorpyrifos, cactus extract, hepatocytes, heat shock protein 70, CYP1A1/2, oxidative stress

SUMMARY

- Chlorpyrifos is cytotoxic against rat hepatocytes via production of oxidative stress
- *Cactus cladode* aqueous extract protected the hepatocytes from oxidative stress-mediated cell death
- Cytoprotection exerted by the extract on hepatocytes was through CYP1A1/2 and heat shock protein 70 protein regulation.



Abbreviations used: AV: Annexin v; BSA: Bovine serum albumin; CPF: Chlorpyrifos; CYP1A1/2: cytochrome P450; DAPI: 4′,6-diamidino -2-phenylindole; EDTA: Ethylenediaminetetraacetic acid; HRP: Horseradish peroxidase; HSP: Heat shock protein; OP: Organophosphorus; PI: Propidium iodide; PMSF: Phenylmethylsulfonyl fluoride; ROS: Reactive oxygen species.

Correspondence:

Dr. Syam Mohan,
Medical Research Center, Jazan University,
Jazan, Kingdom of Saudi Arabia.
E-mail: syammohanm@yahoo.com
DOI: 10.4103/pm.pm_484_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Free radical generation is considered as one of the main cause of diseases and toxicities.[1] Antioxidant protection against free radical generation is a widely accepted and promising mechanism, which a herbal plant or functional food can hold. Rather than synthetic origin, natural antioxidants can be used in the herbal industry, and there is much evidence that these substances may exert their antioxidant effects within the living system. Succulent plants are of great ecological significance particularly in arid and semi-arid parts of Saudi Arabia. One of the significant succulent plants is *Opuntia ficus indica* (L.) belongs to the

Cite this article as: Makeen HA, Ncibi S, Mohan S, Farasani A, Rahmani R, Al Bratty M, *et al*. Cytoprotective effect of *Cactus cladode* (*Opuntia ficus-indica*) against chlorpyrifos induced reactive oxygen species in rat hepatocytes: Involvement of heat shock protein 70 and CYP1A1/2 proteins. Phcog Mag 2019;15:S47-53.

family *Cactaceae*, which is widely invaded into vegetation-rich areas of southwestern mountains of Saudi Arabia.[2] Its fruits and stems have been traditionally used in oriental folk medicine. It has been reported that the extracts of fruits and stems exhibit hypoglycemic,[3] anti-ulcer,[4] anti-allergic,[5] anti-arteriosclerosis, and anti-gastritis properties.[2] In addition, the fruit of this plant is used as functional food in Korea.[6]

Exposure to reactive oxygen species (ROS) is inevitable and continuous in any organism. It is produced regularly as a part of cell metabolism.[7] Apart from these normal processes, ROS can be produced as a response to exposure to exogenous sources such as chemical insult which includes pesticides. ROS has both beneficial, and harm effects depend on different perspectives. In one way, it controls the biological process by regulating different signal transduction pathways when present in impermanent quantity, in opposite they can cause severe damage to cell components such as proteins, lipids, and DNA.[8] It is well known that stress has the capability to generate ROS. One of the possible mechanisms of toxicities in living organisms culminating in cell death may be through the generation of ROS.[9] The reactive molecules generated are the triggering factors responsible for associated protein induction such as heat shock protein 70 (HSP70). HSPs are also known to protect cells from stress by preventing the protein aggregation and promote the refolding of denatured proteins.[10] Increased expression of HSP70 has been reported in high-grade malignant tumors.[11] As HSPs have the ability to prevent the drug-induced apoptosis, inhibitors to HSP could be a target of the right drug candidate identification.

Chlorpyrifos (CPF), an organophosphorus (OPs) insecticide, is one of the major contaminants in food and environment because of its intensive usage. It is widely accepted and been in use as a promising insecticide to control domestic pests and termites, and in agriculture to kill crop pests.[12] CPF exerts its toxicity through inhibition of acetylcholinesterase enzyme activity, because of its ability to form CPF-oxon metabolite. Hence, its primary targets of toxicity are in the central and peripheral nervous system.[13] Numerous safety as well as toxicity studies have been done in the past few decades to know the consequences of CPFs exposure to human. OPs have been known to be cytotoxic,[14] genotoxic,[15] reproductively toxic,[16] and immunotoxic.[17]

The potential toxic effects associated with exposure to CPF are the subjects of increasing concern. Furthermore, the possible protective roles of safer preventative compounds, offering the least amount of side effects are warranted to be explored. Earlier studies have demonstrated the *in vivo* efficacy of *Cactus cladode* extract against zearalenone and nickel-intoxicated animals.[18,19] However, no studies have been performed to study if this extract may have beneficial effects in organophosphate-induced toxicity in primary culture of rat hepatocytes. Thus, we have attempted to explore the possible protection afforded by *Cactus cladode* extract with regard to its antioxidative potential in restoring the altered activities in CPF-induced hepatotoxicity *in vitro*.

MATERIALS AND METHODS

Chemicals

Williams' medium E and fetal bovine serum (FBS) were obtained from Invitrogen (Rockville, USA) and penicillin/streptomycin solution from Bio-Whittaker (CAMBREX Company, Walkersville, USA). Collagenase was from Boehringer Mannheim (Mannheim Corp.; Sydney, Australia), Insulin from Nova Nordisk (Nova Nordisk A/S, Bagsvaerd, Denmark). All other chemicals were from Sigma–Aldrich (L'Isle d'Abeau Chesne, Saint Quentin Fallavier, France) unless otherwise specified.

Extract of *Cactus cladodes*

Young *Cactus cladode*s of *Opuntia ficus indica* collected from the local area and were washed with water, chopped into small pieces, dried at 37°C than powdered. This powder was dissolved in sterilized water. After centrifugation (1000 rpm at 4°C, 10 min), the supernatant was filtered with vacuum filter. Finally, under a laminar flow clean benches and using syringe filter, this solution was filtered again in cryotubes and stored at −20°C until use. The final concentration used in the culture medium was 0.025% (w/v).

Isolation and culture of rat hepatocytes

Standard deviation (SD) rats (Sprague–Dawley) weighing 160–250 g were obtained from the Experimental Animal House (Ethic No 1439-EPRD-03) Jazan University. All animals were provided with drinking water *ad libitum* and received all human care according to the institutional ethical guidelines. Reverse two-step collagenase perfusion method was adopted according to the literature for obtaining the hepatocytes.[20] The isolated cells were suspended in William's medium E added with 10% FBS, penicillin-streptomycin (50 μg/ml) and insulin (0.1 UI/ml). The viability of cells was determined and maintained to 80% viability as suitable for assay. The viable cells were determined by Erythrosin B exclusion assay. The viable cells were then seeded to cell culture collagen type-1 coated dishes and incubated in a humidified atmosphere with 95% air and 5% CO_2 at 37°C for at least 4 h for adhesion of cells. Later, the unattached and dead cells were removed by simple washing, followed by it was filled with fresh medium without serum, but added with hydrocortisone hemisuccinate (1 μM) and bovine serum albumin (BSA) (240 μg/ml). The cells were then treated with different concentration of CPF (10–200 μM) for 48 h, which was dissolved in dimethyl sulfoxide (DMSO) (0.25% v/v).

Viability test

The cytotoxicity profiles of CPF were assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and Sigma–Aldrich (St. Louis, MO) as described earlier with slight modification.[21] Briefly, cells were seeded into 96 well-treated plates. To the cells, CPF was added in different concentration (10–200 μM) for 48 h. After incubation, MTT (5 mg/ml) was added to each well, and the plates were incubated for further 2 h at 37°C, and the media was removed. 100 μl DMSO was later added into each well to solubilize the water-insoluble formazan crystals. The absorbance was determined with a microplate reader at a wavelength of 595 nm (Tecan Sunrise basic, Groedig, Austria).

Annexin V/propidium iodide dual staining

Annexin V-fluorescein (AV) and propidium iodide (PI) double staining was used to measure the apoptotic and necrotic cell death induced by CPF. In this assay, the PI will bind with the dead cell with membrane damage, and AV will bind with exposed phosphatidylserine from the cell membrane of the early stage of apoptosis cell. The AV assay was performed using the Annexin-V-FLUOS Staining Kit as per the instruction of the manufacturer (Roche Applied Science, Meylan, France). Briefly, cells were plated into 12-well culture plate at a density of 0.4×10^6 cells/well. Then the cells were pre-treated with extract and followed by CPF for 48 h. The treated cells were harvested following the treatment. The cells were then thoroughly washed with ice cold phosphate-buffered saline (PBS), centrifuged before incubation with PI and AV buffer in the dark for 15 min at room temperature. A fluorescent microscope was used to measure the percentage of cell death each in apoptosis and necrosis.

4′,6-diamidino-2-phenylindole nuclear staining

A glass cover slide which is pretreated with collage type-1 was used to seed the rat hepatocytes. The seeded cells were then pretreated with extract and then CPF, fixed in ice cold methanol −20°C for 15 min. Then,

the slides were washed with 1 × PBS, the nuclei were stained with 1 μg/ml solution of 2,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) in ice-cold methanol for 15 min. Then, the slides were again washed with PBS, the cells were allowed to dry and mounted in Mowiol medium. The cells were observed using an inverted fluorescence microscope (Nikon) equipped with a charge-coupled device camera (ORCA-ER, Hamamatsu Photonics). Pictures were taken with a × 40 magnification lens.

Reactive oxygen species assay

Cells were pre-treated with extract and then CPF for 24 h. After the treatment, the cells were collected and washed with cold PBS. To measure the level of ROS, DCFH-DA (2,7-Dichlorofluorescin; Sigma) was used at a final concentration of 10 μM for 20 min at room temperature. The development of color due to the dye was measured using fluorescence spectrophotometer (Shimadzu RF-3501, Shimadzu, Korneuburg, Austria) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Nile red assay

Nile red (NR), a lipid-soluble selective fluorescent stain, has been often engaged to localize and assess the lipid quantitatively in animal cells and microorganisms.[22] After 48 h of treatments with CPF, hepatocytes were washed twice with PBS and incubated for 5 min with 10 μl of 10 μg/ml NR in acetone solution at 37°C. Cells were washed thereafter with PBS and were examined under a fluorescence microscope using the red fluorescence (excitation 515–560 nm and emission 590 nm). Photos were taken by the LUCIA software (Laboratory Imaging, Za Drahou, Praha 10, Czech Republic).

Western blot analysis

After pretreated with extract and then CPF to hepatocytes, the cells were spun down at 200 ×g for 10 min. The cells were washed with PBS twice to remove the remaining media. The cell volume was determined, and sufficient hypotonic buffer (25 mM HEPES, 2 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetraacetic acid, 10 μg/ml leupeptin, 5 mM $MgCl_2$, 5 mM DTT, 10 μg/ml pepstatin A) was used for lysis. Cell lysate containing 50 g proteins were separated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis system. After the gel gets cool, the protein was then transferred to PVDF membrane (Amersham Life Science, Buckinghamshire, UK) using a semiphore transfer machine. The membrane was initially blocked with blocking buffer (5% nonfat skimmed milk in Tris-buffered saline with Tween-20 [TBS-T]) for 1 h at 37°C, and then incubated with primary antibody in TBS-T containing 3% BSA. Then, the membrane was washed three times with washing buffer, followed by incubation with horseradish peroxidase-conjugated secondary antibody (anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G, Promega, Madison, WI, USA). Finally, the membrane washing step was repeated with washing buffer. The immune-reacted proteins were detected using an Immobilon Western Detection Reagents (Millipore, Molsheim, France).

Statistical analysis

All values were reported as mean ± SD. The Statistical significance of differences between groups was assessed using one-way ANOVA followed by *post hoc* Tukey's multiple comparison test using SPSS 18.0 software (SPSS Inc, Chicago, Ill). $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxic effect of chlorpyrifos

We observed a concentration-dependent decrease in cell viability for 48 h on CPF-treated cells, except in lower concentration such as 50–100 μM [Figure 1]. At higher concentration, the reduction of viability was significant compared to control. The viability was decreased to 75% ± 5.5%, 57% ± 3.95%, 40% ± 3.85%, and 30% ± 4% at 125, 150, 175, and 200 μM of CPF concentrations, respectively.

Effects of chlorpyrifos on hepatocyte morphology

Treatment with CPF at a concentration of 0–150 μM was done for 48 h in primary rat hepatocytes and morphological changes were analyzed by phase contrast microscope. The control cells which received only DMSO found to be healthy and confluent. The cells showed distinct intracellular borders and plentiful bile canaliculi. In addition, the cells were found to be well maintained in its polygonal shapes with extensive cell-to-cell contact, representative features of mature and well-differentiated cells [Figure 2a].

In contrary, several morphological changes were observed in rat hepatocytes which receive the CPF treatment. Especially, more poorly defined intercellular borders were observed in the cells together with dose-dependent hole formation in cell monolayers. In addition, under light microscopy, several refrainment vesicles were seen in Figure 2b-d. These changes were appeared in the perinuclear region in early hours of treatment followed by an increase in the number of cells both concentration and time dependently. We used NR, a lipophilic dye that readily penetrates cell membranes and accumulates intracellularly in lipid vesicles to distinguish the nature of compartments enlarged after CPF treatments. As shown in Figure 3, the size and intensity of NR-positive vacuoles were found to be high in number significantly both concentration and time-dependent manner. This phenomenon was irreversible; indeed, vesicles did not disappear after CPF removal from the medium (data not shown).

Chlorpyrifos-induced oxidative stress

To study the oxidative stress status in primary culture of rat hepatocytes, after CPF treatment, CYP 1A1/2, HSP70, and ROS levels were assessed. Since the IC_{50} value was observed after 150 μM in MTT assay, primary culture of rat hepatocytes was treated with 50, 100 or 150 μM of CPF for

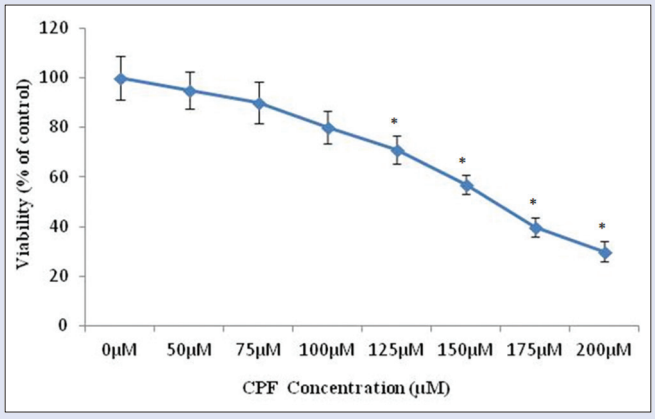


Figure 1: Viability of primary-cultured rat hepatocytes after chlorpyrifos exposure, determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Data shown are representative of six independent experiments. Asterisks indicate significant difference from control (*$P < 0.05$)

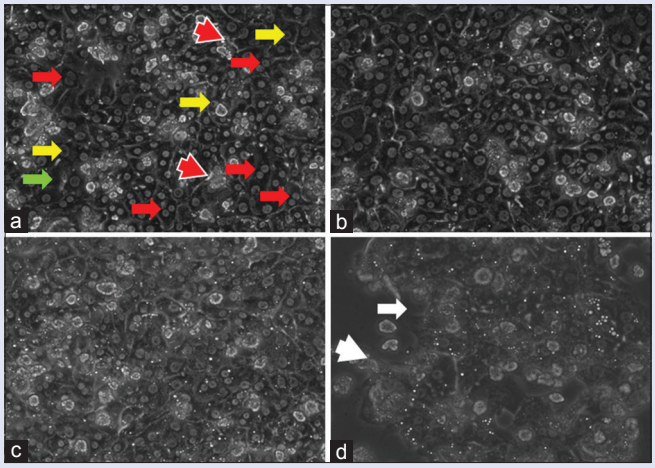


Figure 2: Morphological changes induced by chlorpyrifos at 50 (b), 75 (c) and 150 (d) μM concentrations in rat hepatocytes primary culture compared to control (a) hepatocytes (dimethyl sulfoxide). Hepatocyte binuclei (red arrow), Nucleolus (yellow arrow), cytoplasm (green arrow), Biliary canaliculus (red arrow head) were clearly visible. White arrows shows the hole formation in cell monolayers, and white arrow head shows the poorly defined intercellular borders

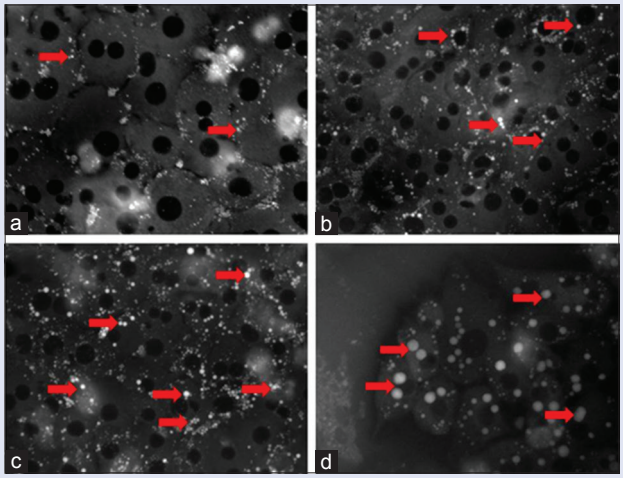


Figure 3: Characterization of intracellular lipids accumulation by Nile red in rat hepatocytes observed under a fluorescence microscope. Cells were treated with 50 (b), 75 (c) and 150 (d) μM concentrations of chlorpyrifos compared to control (a). Red arrow indicates the lipid vesicles

48 h in this assay. Figure 4a shows that CPF induces a significant increase in intracellular ROS. This increase depends on the CPF concentration and is significantly different from the control ($P < 0.05$), which was significantly reversed by the addition of extracts at both concentrations. In addition, our results [Figure 4b and c] show that CPF increases CYP1A1/2 and HSP70 levels in a significant dose-dependent manner when compared to control. It is to be noted that 100 μg/ml extract reduced the HSP70 protein expression less than the level expression observed at 100 μM CPF alone.

Chlorpyrifos induces both apoptosis and secondary necrosis

Using phase-contrast microscopy earlier in this study, some morphological alterations were observed in the primary culture of rat hepatocytes. Hence, we decided to explore the effect of CPF in two different modes of cell death such as apoptosis and necrosis. In addition, we have investigated whether the plant extract's protective ability against the free radicals can prevent the cell death. Necrosis is an accidental and unprogrammed death of cells which is characterized by cytoplasmic swelling, progressive irreversible plasma membrane damage, and organelle breakdown. The leakage of lactate dehydrogenase (LDH) from the cell membrane to culture medium together with uptake of membrane-impairment dye (PI) is deliberated as loss of cell membrane integrity.[23] In the present study, membrane damage was not revealed by measurement of LDH leakage (data not shown), may be probably due to LDH sequestration in lysosomes.[24] Thus, the plasma membrane damage was observed using PI and AV which can stain the phosphatidylserine externalization at early phase of apoptosis. As expected, the control cells which received only DMSO could not show apoptotic cells with AV + [Figure 5a]. As time increased, the AV + cells found to be increased significantly in a concentration-dependent manner, which confirm the induction of early and late apoptosis by CPF. Together with the AV + cells, we observed PI + cells also in a concentration-dependent manner [Figure 5b and c]. AV+/PI + staining could be attributed to either apoptotic or late necrotic cells. The increased number of PI + cells observed in the high concentration of CPF treatment may be due to the necrotic membrane disruption. Therefore, we included another nuclear stain, DAPI to check the nuclear morphology [Figure 5e-g]. Under the DAPI stain, CPF was found to induce nuclear shrinkage, chromatin condensation, and DNA fragmentation. We have observed that the coadministration of the extract [Figure 5d and h] had shown cytoprotective against the CPF action, which was in well agreement with the results obtained earlier in this study.

DISCUSSION

Links between oxidative stress and adverse health effects have been suggested for several groups of diseases such as cardiovascular, respiratory, neurological as well as for the general aging process. This stress is mainly caused by chemical entities known as "ROS." These ROS are produced as byproducts of cellular vital metabolism, and they are immediately intercepted by specific systems. When the rate of ROS production exceeds the rate of its decomposition by antioxidant defenses and repair systems, they could oxidize different cell components, and consequently, oxidative stress can be established.[25] The exposure of organisms to pollutants can promote an increase in the rate of ROS production. Pesticides are identified to be a source of oxidative stress, and they are shown to generate singlet oxygen and other active oxygen species.[26] Many published works indicate that organophosphate pesticides toxicity may be mediated in part by the ROS generation. In the other hand, in the last decade, big interest is given to look for approach and conduct that can help cell to go beyond the oxidative damage.[27] Thus, the aim of the present study was first to evaluate the involvement of oxidative stress in CPF-induced toxicity and second to investigate the possible protective potential of *Cactus cladode* aqueous extract using the primary culture of hepatocytes as a biological model.

In the present study, primary culture of rat hepatocytes was employed to demonstrate CPF-induced damages. We were interested in morphology, viability and oxidative stress status within these cells after CPF exposure. As we presented in the results, CPF treatments induce morphological alterations in rat hepatocytes. In particular, the lumen showed irregular and branched shape and tubules were widely separated by loose connective tissue. Slight signs of cell degeneration were also detected in a dose-dependent manner. CPF has induced the NR-positive vacuoles also. As the NR is used to localize and quantify lipids, particularly neutral lipid droplets within cells, we can conclude that this pesticide cause breaks down of fats in the liver indicating fatty liver called also steatosis.

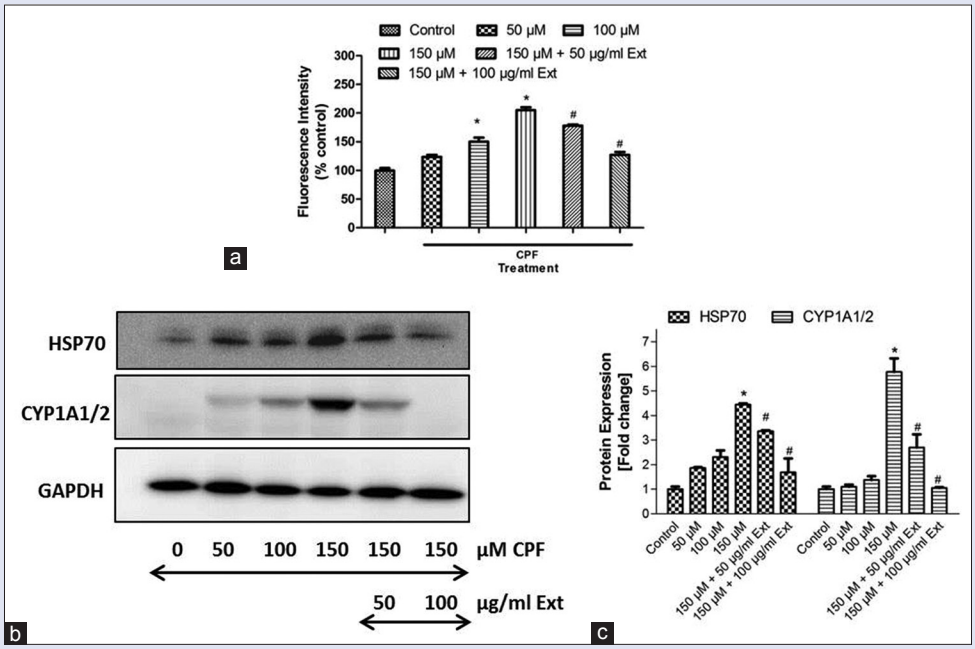


Figure 4: (a) Chlorpyrifos leads to formation of reactive oxygen species in primary-cultured rat hepatocytes. Data are means ± standard deviation of six independent experiments. (b) Identification of heat shock protein 70 and CYP1A1/2 in primary-cultured rat hepatocytes after treatment with chlorpyrifos, which was quantified. (c) Data are means ± SD of three independent experiments. *Significantly different to control ($P < 0.05$). #Significantly different to 150 µM chlorpyrifos group ($P < 0.05$)

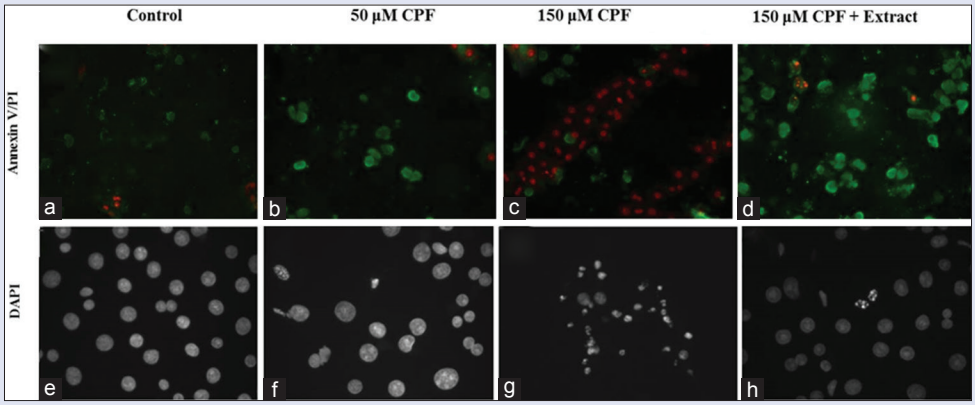


Figure 5: Fluorescent micrographs of Hepatocytes were treated with different concentrations of chlorpyrifos. Untreated cells showed normal structure without prominent apoptosis and necrosis (a and e). Early apoptosis features were seen after treatment representing intercalated Annexin V (bright green) amongst the fragmented DNA (b, c and d). Blebbing and orange color representing the hallmark of late apoptosis were also noticed (f, g and h). Images are representative of one of the three similar experiments

This steatosis, reported in other studies, is attributed to mitochondrial oxidative damage caused by xenobiotics in cells and it has been proposed as a mechanism of sequestration of the pesticide molecules.[28-30]

Our results [Figure 1] show that CPF had induced hepatocyte death and that the EC_{50} value was approximately 180 µM after 48 h of treatment. This fact was demonstrated by MTT assay in human Jurkat T cells and human monocyte cell line U937 earlier.[31] To evaluate the oxidative stress induced by CPF in hepatocytes primary culture and the protective effect of cladode aqueous extract, H_2O_2 levels were measured after cell exposure to both. Results of the present study demonstrated that exposure to the different concentration of CPF ranging from 10 to 150 µg/kg induces a marked increase in ROS formation in hepatocytes primary culture. While pretreatment with *Cactus cladodes* extract reduced this induction, which dropped to the control level. *Cactus cladodes* extract prevented up to 100% ROS production induced by CPF treatment in hepatocytes primary culture [Figure 2]. To further assess CPF oxidative-induced damages in these cells, two toxicology biomarkers such as HSP70 and CYP1A1/2were monitored. Results of this study [Figure 3] showed that CPF induces CYP1A1/2 expression in a dose-dependent manner in rat hepatocytes. According to further studies, parathion, CPF, and diazinon are bioactivated by multiple CYPs, including human liver CYP 1A2, 2C9, 2C19, 2B6, and 2D6 and 3A4.[32,33] The results of this study [Figure 4] showed, also, that CPF increases the levels of HSP70 in a dose-dependent manner. These results agree with those found by other researchers.[34] These proteins are believed to assist cells to adapt or survive by a rapid and transient reprogramming of cellular metabolic activity to protect cells from further oxidative and thermal stress in responsive tissues;[35] nevertheless, high concentrations of Hsps in the cell may lead to cellular

damage by directly interfering with a variety of cellular processes, or consume excessive amounts of energy.[36]

We have further noticed that exposure to CPF within 48 h leads to a dose-dependent increase of cell membrane rupture, cytoskeletal collapse, nuclear condensation, and fragmentation, which are all common apoptotic characteristics. AV/PI test proved that the number of apoptotic and necrotic cells increased as CPF concentration increased, with a positive ratio AV+/PI+. Nuclear fragmentation is an apoptotic hallmark indicative of chromatin condensation due to an imbalance between the activities of deoxyribonuclease and the enzymes responsible for the maintenance of DNA integrity.[37] In this study, DAPI staining demonstrated that CPF induces nuclear shrinkage, chromatin condensation, and fragmentation which confirm that this pesticide causes apoptotic death within the primary culture of rat hepatocytes. The induction of cell death by CPF has been significantly protected by the cactus extract at dose-dependent level.

Li *et al.*[38] found that CPF induces apoptosis in human T cells. In addition, Gupta, Mishra, Sharma, Balaji, Kumar, Mishra, Chowdhuri[39] reports that CPF induces apoptosis and DNA damage in Drosophila through the generation of ROS. *Cactus cladode* has been shown to be protective against oxidative stress earlier, especially oxidative stress by mycotoxins.[19] In addition, it has been also shown to be anti-genotoxic as well.[40] Our results are in well agreement with these reports, especially in the CPF's capacity to induce cell death and DNA damage along with free radical production.

CONCLUSION

In the light of these facts from the current study, it can be concluded that the toxicity exerted by CPF in hepatocytes are involved with the generation of ROS and the regulation of well-controlled programmed cell death, which could be well protected by the *Cactus cladode* extract. Hence, it warrants further *in vivo* studies to understand the detailed mechanism both in translational and transcriptional level.

Acknowledgement

The authors would like to express their utmost gratitude and appreciation to Jazan University for providing grant to conduct this study through Ethnopharmacology Research Group (RG-2–7).

Financial support and sponsorship

Ethnopharmacology Research Group (RG-2–7).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Fazelian M, Eslami B. *In vitro* antioxidant and free radical scavenging activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran. Pharmacogn Mag. 2009;5:122-6.
2. Abdel-Hameed el-SS, Nagaty MA, Salman MS, Bazaid SA. Phytochemicals, nutritionals and antioxidant properties of two prickly pear cactus cultivars (*Opuntia ficus indica* mill.) growing in taif, KSA. Food Chem 2014;160:31-8.
3. Trejo-González A, Gabriel-Ortiz G, Puebla-Pérez AM, Huízar-Contreras MD, Munguía-Mazariegos MR, Mejía-Arreguín S, *et al.* A purified extract from prickly pear cactus (*Opuntia fuliginosa*) controls experimentally induced diabetes in rats. J Ethnopharmacol 1996;55:27-33.
4. Galati EM, Monforte MT, Tripodo MM, d'Aquino A, Mondello MR. Antiulcer activity of *Opuntia ficus indica* (L.) mill. (*Cactaceae*): Ultrastructural study. J Ethnopharmacol 2001;76:1-9.
5. Dok-Go H, Lee KH, Kim HJ, Lee EH, Lee J, Song YS, *et al.* Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from *Opuntia ficus-indica* var. saboten. Brain Res 2003;965:130-6.
6. Lee JC, Kim HR, Kim J, Jang YS. Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. saboten. J Agric Food Chem 2002;50:6490-6.
7. Yin J, Han L, Cong W. Alpinumisoflavone rescues glucocorticoid-induced apoptosis of osteocytes via suppressing No×2-dependent ROS generation. Pharmacol Rep 2018;70:270-6.
8. Lau AT, Wang Y, Chiu JF. Reactive oxygen species: Current knowledge and applications in cancer research and therapeutic. J Cell Biochem 2008;104:657-67.
9. Saini V, Manral A, Arora R, Meena P, Gusain S, Saluja D, *et al.* Novel synthetic analogs of diallyl disulfide triggers cell cycle arrest and apoptosis via ROS generation in MIA PaCa-2 cells. Pharmacol Rep 2017;69:813-21.
10. Phulara SC, Shukla V, Tiwari S, Pandey R. *Bacopa monnieri* promotes longevity in *Caenorhabditis elegans* under stress conditions. Pharmacogn Mag 2015;11:410-6.
11. Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, *et al.* Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Res 2000;60:7099-105.
12. Kajta M, Wójtowicz AK. Impact of endocrine-disrupting chemicals on neural development and the onset of neurological disorders. Pharmacol Rep 2013;65:1632-9.
13. Pope C, Karanth S, Liu J. Pharmacology and toxicology of cholinesterase inhibitors: Uses and misuses of a common mechanism of action. Environ Toxicol Pharmacol 2005;19:433-46.
14. Voorhees JR, Rohlman DS, Lein PJ, Pieper AA. Neurotoxicity in preclinical models of occupational exposure to organophosphorus compounds. Front Neurosci 2016;10:590.
15. Zunec S, Kopjar N, Zeljezić D, Kuca K, Musilek K, Lucić Vrdoljak A, *et al. In vivo* evaluation of cholinesterase activity, oxidative stress markers, cyto- and genotoxicity of K048 oxime–A promising antidote against organophosphate poisoning. Basic Clin Pharmacol Toxicol 2014;114:344-51.
16. Martin-Reina J, Duarte J, Cerrillos L, Bautista J, Moreno I. Insecticide reproductive toxicity profile: Organophosphate, carbamate and pyrethroids. J Toxins 2017;4:1-7.
17. Corsini E, Sokooti M, Galli CL, Moretto A, Colosio C. Pesticide induced immunotoxicity in humans: A comprehensive review of the existing evidence. Toxicology 2013;307:123-35.
18. Ncibi S, Ben Othman M, Akacha A, Krifi MN, Zourgui L. *Opuntia ficus indica* extract protects against chlorpyrifos-induced damage on mice liver. Food Chem Toxicol 2008;46:797-802.
19. Zourgui L, Golli EE, Bouaziz C, Bacha H, Hassen W. Cactus (*Opuntia ficus-indica*) cladodes prevent oxidative damage induced by the mycotoxin zearalenone in Balb/C mice. Food Chem Toxicol 2008;46:1817-24.
20. Zucchini N, de Sousa G, Bailly-Maitre B, Gugenheim J, Bars R, Lemaire G, *et al.* Regulation of Bcl-2 and Bcl-xL anti-apoptotic protein expression by nuclear receptor PXR in primary cultures of human and rat hepatocytes. Biochim Biophys Acta 2005;1745:48-58.
21. Oka M, Maeda S, Koga N, Kato K, Saito T. A modified colorimetric MTT assay adapted for primary cultured hepatocytes: Application to proliferation and cytotoxicity assays. Biosci Biotechnol Biochem 1992;56:1472-3.
22. Ziegler U, Groscurth P. Morphological features of cell death. Physiology 2004;19:124-8.
23. Lee DW, Faubel S, Edelstein CL. A pan caspase inhibitor decreases caspase-1, IL-1 α and IL-1 β, and protects against necrosis of cisplatin-treated freshly isolated proximal tubules. Ren Fail 2015;37:144-50.
24. Seglen PO, Luhr M, Mills IG, Sætre F, Szalai P, Engedal N, *et al.* Macroautophagic cargo sequestration assays. Methods 2015;75:25-36.
25. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: The clash between damage and metabolic needs. Cell Death Differ 2015;22:377-88.
26. Mishra V, Srivastava N. Organophosphate pesticides-induced changes in the redox status of rat tissues and protective effects of antioxidant vitamins. Environ Toxicol 2015;30:472-82.
27. Faggiotto A, Poli A, Catapano AL. Antioxidants and coronary artery disease. Curr Opin Lipidol 1998;9:541-9.
28. Xing H, Li S, Wang Z, Gao X, Xu S, Wang X. Oxidative stress response and histopathological changes due to atrazine and chlorpyrifos exposure in common carp. Pestic Biochem Phys 2012;103:74-80.
29. Desouky MM, Abdel-Gawad H, Hegazi B. Distribution, fate and histopathological effects of ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. Food Chem Toxicol 2013;52:42-52.
30. Velisek J, Stara A, Machova J, Svobodova Z. Effects of long-term exposure to simazine in real concentrations on common carp (*Cyprinus carpio* L.). Ecotoxicol Environ Saf 2012;76:79-86.
31. Nakadai A, Li Q, Kawada T. Chlorpyrifos induces apoptosis in human monocyte cell line U937. Toxicology 2006;224:202-9.
32. Tang J, Cao Y, Rose RL, Hodgson E. *In vitro* metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos. Chem Biol Interact 2002;141:229-41.

33. Mutch E, Williams FM. Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. Toxicology 2006;224:22-32.
34. Kienle C, Köhler HR, Gerhardt A. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicol Environ Saf 2009;72:1740-7.
35. Kaszubowska L, Foerster J, Kaczor JJ, Schetz D, Ślebioda TJ, Kmieć Z, *et al*. Expression of cellular protective proteins SIRT1, HSP70 and SOD2 correlates with age and is significantly higher in NK cells of the oldest seniors. Immun Ageing 2017;14:3.
36. Carpenter CM, Hofmann GE. Expression of 70 kDa heat shock proteins in antarctic and New Zealand notothenioid fish. Comp Biochem Physiol A Mol Integr Physiol 2000;125:229-38.
37. Samejima K, Ogawa H, Ageichik AV, Peterson KL, Kaufmann SH, Kanemaki MT, *et al*. Auxin-induced rapid degradation of inhibitor of caspase-activated DNase (ICAD) induces apoptotic DNA fragmentation, caspase activation, and cell death: A cell suicide module. J Biol Chem 2014;289:31617-23.
38. Li Q, Kobayashi M, Kawada T. Chlorpyrifos induces apoptosis in human T cells. Toxicology 2009;255:53-7.
39. Gupta SC, Mishra M, Sharma A, Deepak Balaji TG, Kumar R, Mishra RK, *et al*. Chlorpyrifos induces apoptosis and DNA damage in *Drosophila* through generation of reactive oxygen species. Ecotoxicol Environ Saf 2010;73:1415-23.
40. Zorgui L, Ayed-Boussema I, Ayed Y, Bacha H, Hassen W. The antigenotoxic activities of cactus (*Opuntia ficus-indica*) cladodes against the mycotoxin zearalenone in balb/c mice: Prevention of micronuclei, chromosome aberrations and DNA fragmentation. Food Chem Toxicol 2009;47:662-7.