The hepatoprotective effects of aquatic extract of *Levisticum officinale* against paraquat hepatocyte toxicity

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Abstract: Paraquat is extensively used as a strong nitrogen-based herbicide for controlling weeds in agriculture. This herbicide is extremely toxic to humans and induces multiorgan failure due to accumulation in the cells. So far, many instances of fatal poisoning have been reported. Paraquat is metabolized primarily in the liver. Accordingly, the effects of aquatic *Levisticum officinale* extract on biochemical factors and oxidative status were evaluated in hepatocytes exposed to paraquat in this study. The results showed that paraquat-induced hepatocyte destruction is mediated by reactive oxygen species (ROS) production. The aquatic extracts of *Levisticum officinale* (100, 200, and 300µg/mL) could prevent lipid peroxidation and reduction in the potential of mitochondrial membranes ($P<0.05$). The antioxidants, ROS scavengers (mannitol, dimethyl sulfoxide, and α-tocopherol), and mitochondrial permeability transition pore-sealing agent (carnitine) inhibited the effects of paraquat. The pore-sealing compound inhibited hepatotoxicity, indicating that paraquat induces cell death via mitochondrial pathways. Hepatic cell death due to paraquat could be prevented by hepatocyte pretreatment with aquatic *Levisticum officinale* extracts, antioxidants, and ROS scavengers; therefore, oxidative stress might directly reduce the mitochondrial membrane potential. In conclusion, paraquat hepatotoxicity may be associated with oxidative stress and maintained by the disruption of mitochondrial membrane potential. *Levisticum officinale* aquatic extract, presumably due to its strong antioxidant properties, could protect against the destructive effects of paraquat on rat hepatocytes

Keywords: Paraquat, oxidative stress, *Levisticum officinale,* hepatocyte protection.

INTRODUCTION

Paraquat is a major quaternary ammonium herbicide, which is used extensively as a nonselective compound to destroy weeds in agriculture. There are 2 million cases of pesticide poisoning hospitalization every year. Paraquat poisoning comprises of 0.34 of such cases and has the highest mortality of all types of pesticide poisoning (Davarpanah *et al*., 2015). Paraquat is extremely toxic to mammals. Its herbicidal effects on plants are due to the inhibition of intracellular electron transfer cycles and disruption of photosynthesis in plants. Paraquat induces cytotoxic effects on the lungs, liver, kidneys, myocardium, and adrenal glands. In mice, a dose of 50 mg/kg causes liver inflammation and hepatocyte necrosis. Paraquat toxicity is related to super oxide anions, which produce hydrogen peroxide (H_2O_2), reactive oxygen species (ROS) and per oxynitrite anions.

Paraquat causes depletion of NADPH stores and disrupts biochemical processes requiring NADPH. Toxicity of paraquat involves oxidative degeneration of polyunsaturated fatty acids of the cells via lipid per oxidation. It has been revealed that hepatotoxicity occurs after dermal absorption of toxin in humans (Pourmand *et al*., 2016; Dinis-Oliveria *et al*., 2008). Absorption of paraquat occurs rapidly, presumably due to carrier-mediated transfer of choline on the brush borders of the intestinal epithelium (Nagao *et al*., 1993).

One of the primary effects of paraquat occurs in the respiratory system. Paraquat accumulates in the respiratory system in the epithelium of alveolar type I and II cells, as well as Clara cells (Dinis-Oliveira *et al*., 2008). Paraquat is primarily metabolized in the liver. Cytochrome P450 (CYP) produces CYP1A1, CYP1A2, and CYP2E1, which induce the production of ROS during xenobiotic metabolism and is involved in oxidative stress damage. Direct contribution of CYP to the formation of free radicals has been shown in pesticides. Metabolism of paraquat mainly occurs with methylation or oxidation. Paraquat, however, is metabolized poorly and is secreted generally unchanged from the urinary system (Shimada *et al*., 2002; Ahmad *et al*., 2010).

Levisticum officinale is identified as a perennial herb from the *Umbelliferae* (*Apiaceae*) family. It is a wild aromatic herb, which grows in various areas of Europe, Southwest of Eastern Mediterranean region, Iran, and Afghanistan (Mozaffarian, 2007). Its medicinal properties include diuretic, antimicrobial, carminative, and diaphoretic effects. The traditional school of Salerno suggested it in the treatment of icterus and liver complaints (Schinkovitz *et al*., 2008; Ibrahim *et al*., 2013).

Despite a wide range of investigations on paraquat poisoning, cytotoxic pathways and effective antidotes

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have not been yet recognized. Accordingly, the effects of aquatic *Levisticum officinale* extract on biochemical factors and oxidative status were evaluated in paraquat-exposed hepatocytes in our study.

MATERIALS AND METHODS

Chemicals

In this study, Sigma-Aldrich Co. (Taufkirchen, Germany) provided collagenase, bovine serum albumin, acridine orange, DCFH-DA, trichloroacetic acid, HEPES, rhodamine 123, paraquat, trypan blue, reduced glutathione (GSH), and oxidized glutathione (GSSG).

Animals

The Wistar rats (male, 250-300g) were provided by the laboratory animal facility of Zahedan University of Medical Sciences. The animals were kept in a 12:12h light-dark cycle at room temperature (20-25ºC) in an atmosphere with humidity of about 50%; they had access to water and food (a standard diet with tap water). The Animal Experimentation Committee of Zahedan University of medical sciences approved the experiments.

Hepatocyte isolation

Hepatocyte isolation was carried out using an enzymatic method with collagenase perfusion through the liver in the portal vein. Trypan blue exclusion test was applied to determine hepatocyte viability based on plasma membrane disruption (Lee *et al*., 2013). Cells (density, 106cell/mL) were prepared in round-bottom flasks, which rotated in a water bath. Then, they were added to Krebs-Henseleit (KH) buffer (pH, 7.4), containing HEPES (12.5mM) at a temperature of 37°C (5% CO_2 and 95% O_2). Before adding the chemicals, hepatocytes were preincubated for half an hour.

To prevent nontoxic and very toxic conditions, the half maximal effective concentration (EC_{50}) of paraquat (25 µM) was used. In hepatocyte cytotoxicity assessment, a chemical EC_{50} is the concentration, which can cause a 50% reduction in the viability of hepatocytes after incubation for 2 hours. In this study, dose-response curves were used to determine EC_{50} for paraquat; EC_{50} was measured according to the regression plot including 4 concentrations.

Main research methods

For determining the mechanisms of paraquat-induced liver toxicity, accelerated cytotoxicity mechanism screening (ACMS) was applied. This method describes the xenobiotic cytotoxic effects after incubation (2 hours) on rat hepatocytes. To determine the cytotoxic mechanism, a functionomic approach was applied, in which the effects of different antagonists on ROS production, lysosomal/mitochondrial damage, cell viability, and xenobiotic-induced cell apoptosis signaling, were evaluated.

Trypan blue exclusion test was applied to measure the paraquat concentration, required to cause 50% EC_{50} in rat hepatocytes (freshly isolated) within 2 hours. This concentration was applied to the hepatocytes of rats, and the mentioned toxicity mechanisms were examined. In ACMS, relative to the environmental exposure of humans, low dose/long time *in vivo* is induced by high dose/short time *in vitro* (Pourmand *et al*., 2010).

ROS determination

DCFH-DA was added for determining ROS formation due to paraquat in hepatocytes. After penetrating into hepatocyte cells, DCFH-DA is hydrolyzed to nonfluorescent DCFH. Afterwards, for producing the highly fluorescent DCF (effluxing the cell), DCFH reacts with ROS. Using a fluorescence spectrophotometer (Shimadzu RF5000U), DCF fluorescence was determined (excitation wavelength, 500 nm; emission wavelength, 520 nm). The data are presented as fluorescence intensity per 10^6 cells (Daraei *et al*., 2012).

Mitochondrial membrane potential (MMP) assay

For estimating the MMP, mitochondrial rhodamine123 uptake was measured as the cationic fluorescent dye. A spectrophotometer set (Shimadzu RF5000U), available at the University of Sistan and Baluchestan, was used to fluorimeterically measure the remaining rhodamine 123 (excitation wavelength, 490 nm; emission, 520 nm). The mitochondrial uptake of rhodamine 123 was determined, based on the fluorescence difference between the cells (treated cells and controls). The data are presented as MMP collapse percentage (%ΔΨm) in all groups with treated hepatocytes (Andersson *et al*., 1987).

Lysosomal membrane integrity assay

Acridine orange, as the fluorescent dye, was evaluated in terms of redistribution for determining the lysosomal membrane stability of hepatocytes (Andersson *et al*., 1987). After staining the cell suspension aliquots (0.5mL) with the fluorescent dye (5µM of acridine orange), centrifugation was performed for 1 minute at 1000 rpm to remove them from the medium. Afterwards, the fresh incubation medium was used to resuspend the cell pellet (2mL). For removing the fluorescent dye from the medium, washing was repeated twice. A fluorescence spectrophotometer (Shimadzu RF5000U) was employed to fluorimetrically analyze the cell suspension in terms of redistribution of acridine orange (excitation wavelength, 495 nm; emission wavelength, 530 nm).

STATISTICAL ANALYSIS

The data are presented as mean ±SD of triplicate samples, related to 3 different rats. For analyzing the data, ANOVA and Tukey's post hoc test were carried out. Moreover, to examine the homogeneity of variance, Levene's test was applied. The significance level was set at 0.05.

RESULTS

After 3 hours of incubation, viability was confirmed in 80-90% of the control cells (minimum). The EC_{50} of paraquat was 107µM after 2 hours (fig. 1). To determine EC_{50}, the regression plot of 4 concentrations (50, 100, 200, and 300µM) in the isolated hepatocytes was used. The $EC_{50,2h}$ of paraquat in hepatocytes (3 hours of incubation) is described as the concentration reducing viability to 50% after 2 hours of incubation. According to, paraquat (107µM) significantly increased cytotoxicity compared to the controls (P<0.05) (table 1). Moreover, after incubating the isolated hepatocytes with paraquat (107µM), ROS formation and lipid peroxidation showed a significant improvement time dependently (P<0.05) (table 2, 3).

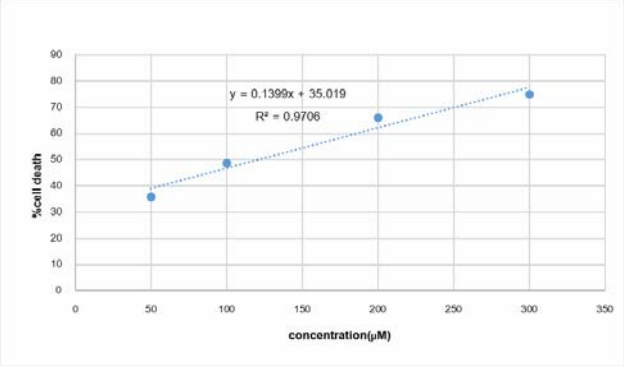

Fig. 1: Determination of EC_{502h} of paraquat (107.08 µM)

Conversely, cytotoxicity, formation of ROS and lipid peroxidation were inhibited by antioxidants, ROS scavengers (extracts, 100, 150, 200, and 300µg/mL; DMSO, 150µM; mannitol, 50mM; and α-tocopherol, 100 µM), and MPT pore-sealing agent (carnitine, 2mM) (table 1, 2, 3). No reagents used for cytotoxicity, ROS and lipid peroxidation analysis (e.g., ROS scavengers, antioxidants, and MPT pore-sealing agent) significantly increased ROS formation or hepatocyte membrane lysis at the evaluated concentrations while incubated in the isolated hepatocytes independently (P< 0.05) (data not shown).

According to table 4, paraquat (107µM) could lead to the collapse of MMP in 1 hour of incubation; this process was inhibited by ROS scavengers (mannitol and DMSO) and antioxidants, attributing the paraquat-induced reduction in MMP to ROS generation. At the evaluated concentrations, the inhibitors showed no significant effects on the MMP of hepatocytes if incubated alone in hepatocytes (P<0.05) (data not shown).

Over 1 hour of incubation with paraquat, acridine orange was not released into the cytosolic fraction after loading hepatocyte lysosomes with acridine orange, suggesting no lysosomal membrane disruption (table 5). The paraquat-induced release of acridine orange was inhibited by radical scavengers (i.e., mannitol and DMSO) (table 5). No significant effects were reported on redistribution of acridine orange from lysosomes to cytosol after independent incubation in the isolated hepatocytes (P< 0.05) (data not shown).

DISCUSSION

The present findings could help understand the mechanisms through which paraquat leads to oxidative stress and liver toxicity. Oxidative compounds induce pathological changes in the liver, since they impair cellular membranes and are involved in hepatocyte damage through peroxidation of unsaturated fatty acids in the cell membrane. Superoxide anions formed by paraquat react with nitric oxide and form peroxynitrite anions, which are very poisonous agents (Pacher *et al*., 2007).

Various protective mechanisms in tissues and organs cannot fully maintain cell survival; it is therefore necessary to use medicinal herbs for better protection of cells against oxidative damage. The essential *Levisticum officinale* oil consisted of 80 compounds. The major compounds included 3-n butylphthalide (32.44%), butylidenephthalide (19.98%), heptadecane (2.49%), δ-cadinene (1.98%), tricosane (1.77%), and hexadecanoic acid (1.47%). According to the antioxidant assay results, IC_{50} values for the aqueous *Levisticum officinale* extract were 166.2µg/mL and 24.56mM Fe^{2+}/g on DPPH and FRAP assays, respectively. The phenolic, anthocyanin, and flavonoid contents were 8.40mg GAE/g DW, 78.56 mg quercetin/g DW, and 299µmol/g DW, respectively (Mohammadi *et al*., 2017; Shahraki *et al*., 2017; Raal *et al*., 2008).

Paraquat, which is a nonselective herbicide, has been used extensively in different regions. In this study, rat hepatocytes were used *in vitro* for evaluating the mechanism of paraquat-induced cell apoptosis. The induced oxidative stress in hepatocytes was accompanied by the increased production of ROS. Pretreatment of hepatocytes with the aquatic extract of *Levisticum officinale* prevented ROS production before exposure to paraquat. Our results indicated that the aquatic extract of *Levisticum officinale* could inhibit oxidative stress and hepatocyte damage by paraquat; as a result, it can be used for the treatment or prevention of hepatic cell toxicity due to environmental toxins.

The results of different studies demonstrated that *Levisticum officinale* possesses valuable antioxidant properties. The IC_{50} values were determined as 166.2 µg/mL and 24.56mM Fe^{2+}/g on DPPH and FRAP assays, respectively (15, 16). Based on these findings, *L. persicum* extracts showed major antioxidant properties. A study by Shafaghat (2011) demonstrated that extracts from the root and seeds of *Levisticum persicum* had free radical scavenging activities (Shafaghat, 2011).

Table 1: Effects of aquatic extract of *Levisticum officinale*, MPT pore-sealing agent, and ROS scavengers on paraquat-induced cytotoxicity.

Addition	Cytotoxicity (%) 2h	Cytotoxicity (%) 3h	Cytotoxicity (%) 4h
Control rat hepatocytes	15±3	17±5	422±
μM)+paraquat(107	58±4[a]	63±6[a]	71±4[a]
+*L. officinal*e extract (100μg/ml)	52±5	56±6	62±6
+*L. officinal*e extract (150μg/ml)	45±5[b]	48±7[b]	53±5[b]
+*L. officinal*e extract (200μg/ml)	40±5[b]	44±4[b]	47±6[b]
+*L. officinal*e extract (300μg/ml)	37±7[b]	39±6[b]	41±8[b]
+Mannitol (50mM)	36±6[b]	41±5[b]	47±6[b]
+DMSO (150μM)	32±3[b]	39±4[b]	43±5[b]
(100μM)α_tocopherol+	29±7[b]	37±8[b]	42±9[b]
+ Carnitine (2 mM)	41±4[b]	48±3[b]	55±7[b]

Table 2: Effects of aquatic extract of *Levisticum officinale*, antioxidants, MPT pore-sealing agent and ROS scavengers on the rate of paraquat-induced formation of ROS in the hepatocytes.

Addition	DCF		
	Incubation time		
	15 min	30 min	60 min
Control rat hepatocytes	±340	48±4	54±7
μM)107+paraquat(	97±4[a]	104±7[a]	113±11[a]
+*L. officinale* extract (100μg/ml)	67±5[b]	84±6[b]	91±8[b]
+*L. officinale* extract (150μg/ml)	52±9[b]	66±8[b]	85±6[b]
+*L. officinale* extract (200μg/ml)	48±5[b]	62±10[b]	73±8[b]
+ *L. officinale* extract (300μg/ml)	44±6[b]	56[b]±7	67±4[b]
+Mannitol (50mM)	51±8[b]	59±11[b]	86±4[b]
+DMSO (150μM)	49±6[b]	54±8[b]	11[b]±63
α-tocopherol+ (100 μM)	46±8[b]	53±7[b]	70±6[b]
+ Carnitine (2 mM)	74±4[b]	78±7[b]	81±10[b]

Table 3: Effects of aquatic extract of *Levisticum officinale*, ROS scavengers, antioxidants and MPT pore-sealing agent on paraquat-induced Lipid peroxidation.

Addition	Lipid peroxidation (μM)		
	Incubation time		
	15 min	30 min	60 min
Control hepatocytes	121±0.30	158±1.00	183±0.13
μM) 107paraquat (+	390±0.21[a]	432±0.155[a]	487±0.064[a]
+*L. officinale* extract (100μg/ml)	210±0.29[b]	259±0.71[b]	308±0.08[b]
+*L. officinale extract* (150μg/ml)	194±0.125[b]	241±0.11[b]	293±0.045[b]
+*L. officinale* extract (200μg/ml)	181±0.40[b]	233±0.15[b]	269±0.35[b]
+*L. officinale* extract (300μg/ml)	156±0.60[b]	182±0.55[b]	235±0.65[b]
+Mannitol (50mM)	170±0.07[b]	238±0.080[b]	314±0.035[b]
+DMSO (150μM)	164±0.02[b]	198±0.045[b]	241±0.75[b]
α-tocopherol+ (100 μM)	149±0.030[b]	174±0.046[b]	226±0.44[b]
+Carnitine (2mM)	174±0.04[b]	229±0.33[b]	317±0.41[b]

Incubation of hepatocytes was done in KH buffer (pH, 7.4) for 4.0 hours at 37°C in the presence of E_{502h} of paraquat. DCFH oxidation and DCF formation are signs of ROS formation or fluorescence intensity. Data are presented as mean±SD of 3 experiments.

[a]Significant difference versus the controls (P< 0.05); [b]significant difference versus paraquat-treated hepatocytes (P< 0.05).

Table 4: Effects of aquatic extract of *Levisticum officinale*, ROS scavengers, antioxidants, and MPT pore-sealing agent on preventing MMP reduction due to paraquat in hepatocytes.

Addition	%ΔΨm		
	Incubation time		
	min۱۵	min۳۰	min۶۰
Control rat hepatocytes	5±3	12±6	16±10
µM)107 +paraquat(	[a]4±52	[a]5±57	[a]3±65
+*L. officinale* extract (100µg/ml)	[b]6±34	[b]8±42	[b]5±53
+*L. officinale* extract (150µg/ml)	[b]4±27	[b]6±36	[b]5±44
+*L. officinale* extract (200µg/ml)	[b]7±19	[b]4±25	[b]8±29
+*L. officinale* extract (300µg/ml)	[b]5±12	[b]7±18	[b]4±26
+Mannitol (50mM)	[b]3±20	[b]2±24	[b]4±27
+DMSO (150µM)	[b]2±17	[b]6±26	[b]4±33
α-tocopherol+ (100 µM)	[b]3±12	[b]3±18	[b]2±23
+Carnitine (2mM)	[b]6±22	[b]4±26	[b]3±37

Table 5: Effects of paraquat on the damage of lysosomal membranes in hepatocytes in comparison with the control group

Addition	% Acridine orange redistribiution		
	Incubation time		
	15 min	30 min	60 min
Control rat hepatocytes	1 ±1	3 ± 2	5 ±2
Paraquat (+ 107 µM)	1 ± 1	5 ±4	6 ± 3

Incubation of hepatocytes was done in KH buffer (pH, 7.4) for 4.0 hours at 37°C in the presence of E_{502h} of paraquat. DCFH oxidation and DCF formation are signs of ROS formation or fluorescence intensity. Data are presented as mean ±SD of 3 experiments.
[a]Significant difference versus the controls (P< 0.05); [b]significant difference versus paraquat-treated hepatocytes (P< 0.05).

For protecting biological systems, antioxidants have been used for a long time to prevent free radical formation by reactive oxygen substances from environmental factors or normal metabolic activities (Hwang *et al*., 2010). Moreover, many synthetic antioxidants are reported to have adverse side effects (Hazra *et al*., 2008). Recent trends favor the use of natural compounds, which has been the focus of recent research, particularly regarding antioxidants, which act as radical scavengers in living organisms. Most naturally occurring components that act as ROS inhibitors are found in food, as well as the human body (Chrpova *et al*., 2010). Due to safety concerns related to synthetic drugs on one hand and the beneficial effects of medicinal plants on the other, it is favorable to use natural candidates rather than synthetic drugs to treat such conditions.

This study showed that paraquat-induced ROS generation and hepatocyte death significantly decreased by adding hydroxyl radical scavengers (mannitol and DMSO), antioxidants (α-tocopherol), and MPT pore-sealing agent (carnitine). Considerable formation of free radicals was induced by the reductive activation of paraquat. Improved production of ROS could damage hepatocyte mitochondria directly via cytochrome c expulsion, MPT pore opening, and electron transport chain interruption.

H_2O_2, resulting from metabolic activation of paraquat or mitochondrial damage, diffuses into hepatocytes lysosomes given its lipophilic nature. A Haber-Weiss reaction results in the production of highly reactive hydroxyl radicals. The lysosomal membrane integrity may be reduced by hydroxyl radicals and digestive proteases, such as cathepsins. These hydroxyl radicals and proteases can expose the mitochondrial MPT pore through stimulation of Bax or Bid proapoptotic proteins and other lytic enzymes or oxidation of the adjacent thiol groups. The electron transfer chain disruption results in mitochondrial formation of H_2O_2, which potentiates paraquat hepatocyte toxicity (Satapati *et al.,* 2015; Hosseini *et al*., 2012).

CONCLUSION

This study showed that liver toxicity induced by paraquat results from the increased formation of cellular ROS and lipid per oxidation. It seems that aquatic extract of

Levisticum officinale can decrease the toxic effects of paraquat on rat hepatocytes by inhibiting oxidative stress. The extract of *Levisticum officinale* can be therefore applied to prevent or treat hepatotoxicity.

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