

Safranal Effect against Cyclophosphamide-Induced Liver InjuryVictoria S. Kareem* and Munaf H. Zalzal**¹

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Abstract

The liver is the primary organ for drug metabolism. Cyclophosphamid is a nitrogen mustard-based alkylating agents, its metabolized by the liver into two cytotoxic metabolites, causing liver toxicity by increasing reactive oxygen species. Safranal as the most abundant chemical in saffron essential oil, it has anti-oxidant, anti-inflammatory, antiapoptotic and free radical scavenger activity. The aim of study is to assess the protective effects of safranal on the cyclophosphamide- induce liver toxicity in rat model. This is investigated by using five different groups of rats; normal control group received the vehicles; model group received a single dose of cyclophosphamide (150 mg/kg, i.p). The other two groups received orally safranal at doses of 50 and 100 mg/kg/day, respectively, for 8 consecutive days prior cyclophosphamide injection (at day fifth). The last group received only oral safranal at dose 100 mg/kg/day for 8 consecutive days.

This study showed safranal-pretreatment significantly ameliorated the deterioration of liver function and exerted significant anti-oxidant with a marked decline in malondialdehyde level, and increase in glutathione and nuclear factor erythroid 2-related factor 2 levels in a dose dependent-manner.

Keywords: Liver toxicity, Cyclophosphamide, Safranal, Glutathione, Malondialdehyde

تأثير السافرنال ضد السمية الكبدية الناتجة من استخدام مادة السايكلوفوساميد فكتوريا سعد كريم* و مناف هاشم زلزله**^١

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الخلاصة

الكبد هو العضو الرئيسي للعمليات الايضية للجسم، السايكلوفوساميد هو من اقدم انواع العلاجات السرطانية التي تنشط بواسطة انزيمات الكبد لتنتج مادة الاكرولين عالية السمية والفسفواميد ماسترد لتزيد الاكسدة داخل الجسم، السافرنال هو مستخلص الزيوت العطرية الاساسية من الزعفران ويعمل كمضاد اكسدة ومضاد التهابات، في هذه الدراسة تلقت مجموعة الكونترول فقط الماء المغذي، تلقت المجموعة النموذجية جرعة واحدة من السايكلوفوساميد (١٥٠ مجم / كجم). تلقت المجموعتان الاخرتان السافرنال عن طريق الفم بجرعات ٥٠ و ١٠٠ ملغم / كجم / يوم، على التوالي، لمدة ٨ ايام متتالية قبل حقن سيكلوفوساميد (في اليوم الخامس). تلقت المجموعة الأخيرة فقط سافرنال عن طريق الفم بجرعة ١٠٠ مجم / كجم / يوم. أظهرت هذه الدراسة أن المعالجة المسبقة للسافرنال خففت بشكل كبير من تدهور وظائف الكبد وأظهرت ارتفاع في مضادات أكسدة مع انخفاض ملحوظ في مستوى مالونديدهيد، وزيادة في مستويات الجلوتاثيون والعامل النووي ٢ المرتبط بالإيثرويد ٢ بطريقة تعتمد على الجرعة. الكلمات المفتاحية: السمية الكبدية، سايكلوفوساميد، سافرنال، مالونديدهيد.

Introduction

The liver is a critical organ for metabolism and maintain homeostasis in general body function. It also acts as a source of nutrients and a detoxifier of undesirable substances⁽¹⁾.

Cyclophosphamid (CP), is one of the earliest chemotherapy drugs that still used today to treat many forms of cancers, CP is a prodrug, it is converted in the liver into acrolein and phosphoramid mustard by the Cytochrome P450 system. Many previous studies indicated that during its oxidative metabolism, CP produces reactive oxygen species (ROS), and suppresses the antioxidant protection mechanisms of the liver⁽²⁾. Acrolein could easily react with glutathione (GSH), a thiol-containing protein that serves many essential functions including detoxification. When GSH was depleted, acrolein could react with cellular

nucleophiles, such as the thiol groups of cysteine residues in the proteins and the atoms of nitrogen in lysine and histidine, causing protein loss, thus inducing oxidative stress and eventually leading to catastrophic hepatocyte effects⁽³⁾. CP interferes with liver oxidant/antioxidant balance, which contributes to reactive oxygen (ROS) accumulation, influencing lipid peroxidation and signaling of cytotoxicity pathways⁽⁴⁾. Free radicals-induced lipid peroxidation change membrane structure and function. they are also involved in cell defects including mutation and cell death.⁽⁵⁾ Oxidative stress triggers multiple intracellular signals leading to pro-inflammatory cytokine up-regulation.⁽⁶⁾ Moreover, many recent studies found that the oxidative stress, inflammation, and apoptosis are the main pathways leading to CP hepatotoxicity⁽⁷⁾.

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Glutathione is a thiol-containing tripeptide that helps to minimize oxidative stress by providing a major non-enzymatic endogenous antioxidant. The liver has high glutathione levels because of its active synthesis⁽⁸⁾.

Nuclear related factor 2 (Nrf2), is a transcription factor, which is a highly preserved archaic protein that can orchestrate and balance an intracellular oxidative stress response. Several studies have shown that the fundamental mechanism of activation Nrf2 is based on their negative regulator of kelch-like ECH-associated protein 1. Nrf2 binds to the antioxidant cisactant (ARE) reaction element that controls the expression of detoxifying and antioxidant enzymes.

Nrf2 -stress dependent induction results in induction in the transcription of many antioxidant enzymes directly^(9), 10).

Most abundant chemicals in saffron plant oils is Safranal (2,6,6-trimethyl 1, 3-cyclohexadien-1-carbox-aaldehyde). that is composed of 60–70% of dry saffron's volatile fraction.⁽¹¹⁾ Various research groups have documented medicinal properties of safranal with anti-oxidant, anticonvulsant, cardioprotective, anti-nociceptive, anti-cancer, anti-inflammatory effect and others. The majority of these therapies may be attributed to their ability to quench ROS and to minimize the body's oxidative stress.⁽¹²⁾

Safranal modulates the expression of antioxidant genes and controls mitochondrial antioxidant genes, which produces a less radical mitochondrial oxygen capacity. Inflammatory and immune system disease could be effectively affected by safranal, modulating pro-inflammatory cytokines, stress oxidative and immune factors.^(13, 14) further, safranal lowered inflammation and decreased TNF- α level, possibly due to its high antioxidant and anti-apoptotic capacity^(15, 16).

Aim of study

The study designed to assess the defensive impact of safranal against liver toxicity induced by cyclophosphamide

Materials and Methods

Cyclophosphamide was purchased from Baxter Oncology (Frankfurt, Germany). MDA and glutathione rat Elisa kit were purchased from SunLong Biotech, NRF2elisa kit purchased from mybiosource co. ALT and AST kits were purchased from Randox laboratories (united kingdom), safranal purchased from sigma Aldrich (india)

Animals

Rats of Wistar were picked from the animal house of the Pharmacology and Toxicology Department of the Collage of Pharmacy at University of Baghdad. Adult albino male rat's weighing (150-250) gm.

Animals were randomly allocated into five groups (6 rats each). These rats are housed in a temperature-regulated room at 25 ± 2 C°, 50-70 percent humidity and 12 hours of lighting period under probable hygienic conditions. All rats are fed during the experiments with standard pellets and tap water. Animals are weighed and their initial weights were registered after 2 weeks of acclimatization, and then weighted every 2 days.

The rats are divided into five group as the following:
Group I (Control): Six rats received liquid paraffin orally for 8 days and normal saline intraperitoneal (I.P) in the fifth day (3 days before the end of the experiment).

Group II (Model group): six rats received cyclophosphamide 150 mg/kg intraperitoneal (I.P) in the fifth day (3 days before the end of the experiment).

Group III: six rats received safranal orally in a dose of 50 mg/kg/day consecutively for 8 days and cyclophosphamide 150 mg/kg intraperitoneal (I.P) in the fifth day.

Group IV: six rats received safranal orally in a dose of 100 mg/kg/day consecutively for 8 days and cyclophosphamide 150 mg/kg intra peritoneal (I.P) in the fifth day.

Group V: six rats received safranal orally in a dose of 100 mg/kg/day consecutively for 8 days

Biochemical evaluation

Blood sample is obtained from animal by heart puncture after anesthetized using diethyl ether, the sample left half hour at room temperature then take serum after centrifuging for 10minunt at 10000 rpm and stored at -20 °C for determination of liver enzyme, Alanine transaminase activity was measured by colorimetric method

Determination of NRF2, MDA, Glutathione level in homogenizing tissue

A 0.1gm of liver tissue was homogenate using cold phosphate buffer solution (10% w\|v) after centrifuging then keep the supernatant at -20 °. Estimation of NRF2, MDA ,glutathione levels in rat tissues by using the enzyme-linked immuno-sorbent assay (ELISA) kit according to Sandwich-ELISA principle, at 37°C and Read the Optical Density (O.D.) at 450 nm.

Statistical analysis

All the values are presented as means \pm standard error of the means (SEM) of all experiments. Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) The difference was considered significant when $P < 0.05$. SPSS software was used to carry out these statistical tests.

Results

Effects of safranal on serum liver microsomal enzymes in CP-induced hepatotoxicity in rats.

Cyclophosphamide (CP, 150 mg/kg, I.P.) resulted in acute liver damage in rats as evidenced by the significant elevation of serum alanine transaminase (ALT) and aspartate transaminase (AST), as compared to the normal control group. (Table 1 and 2 ,respectively).

Table 1. level of ALT level among various groups

Groups	Serum ALT level (U/L)
Control	18.96 ± 2.16
CP	40.42 ± 3.97 *
CP/Safranal 50mg/kg	36.21 ± 5.27 *
CP/Safranal 100mg/kg	23.14 ± 2.09 #
safranal 100mg/kg	19.06 ± 1.35 #

All values are expressed as mean ±SD

* compared with control group, significant different (p<0.05).

compared with model CP group significant different, (p<0.05).

Table 2. AST level among various groups

Groups	Serum AST level (U/L)
Control	77.78 ± 22.09
CP	169.96 ± 47.39 *
CP/Safranal 50mg/kg	122.66 ± 34.35
CP/Safranal 100mg/kg	80.78 ± 12.88 #
safranal 100mg/kg	78.83 ± 16.49 #

All values are expressed as mean ±SD

* compared with control group, significant different (p<0.05).

compared with model CP group significant different, (p<0.05).

Effect of safranal on hepatic NRF2 and glutathione level in CP-induced hepatotoxicity in rats.

CP induced acute liver damage in rats as evidenced by an decrease in hepatic NRF2 and glutathione content as compared to the normal control group. Pretreatment of rats with safranal (50 and 100 mg/kg/day, oral) for five days prior CP injection significantly inhibit the reduction in hepatic NRF2 and glutathione in a dose dependent manner compared to the CP-control group, recording normal levels of NRF2 and glutathione (Table 3 and 4 ,respectively).

Table 3. Impact of safranal on NRF2 level among various groups

Groups	Hepatic NRF2 level (ng/0.1 gm)
Control	112.95 ± 13.21
CP	66.28 ± 7.98 *
CP/Safranal 50mg/kg	120.4 ± 26.78 #
CP/Safranal 100mg/kg	138.82 ± 36.18 #
safranal 100mg/kg	143.36 ± 20.06 #

All values are expressed as mean ±SD

* compared with control group, significant different (p<0.05).

compared with model CP group significant different, (p<0.05).

Table 4. Effect of safranal on glutathione level among various groups

Groups	GSH level (ng /0.1g)
Control	69.26 ± 14.43
CP	35.74 ± 11.0 *
CP/Safranal 50mg/kg	73.7 ± 16.02 #
CP/Safranal 100mg/kg	82.31 ± 27.77 #
safranal 100mg/kg	71.84 ± 10.75 #

All values are expressed as mean ±SD

*Different significant variations relative to the control group (p<0.05).

#Different significant variations relative to the model group (p<0.05).

Effect of safranal on hepatic MDA in CP-induced hepatotoxicity in rats.

Cyclophosphamide (CP, 150 mg/kg, i.p.) resulted in acute liver damage in rats as evidenced by a significant increase in hepatic MDA as compared to the normal control group. Pretreatment of rats with safranal, at 50 and 100 mg/kg, significantly reduced the elevation in liver MDA in a dose-dependent manner compared to the CP control group, recording normal levels of MDA dose with insignificant difference from normal control group (Table 5).). Compared to the control group, there is no statistical difference between the safranal group treated alone (P>0.05).

Table 5 . Effect of safranal on MDA level among various groups

Groups	MDA level (ng/0.1g)
Control	38.39 ± 8.06
CP	56.02 ± 9.14 *
CP/Safranal 50mg/kg	51.56 ± 6.84 *
CP/Safranal 100mg/kg	43.19 ± 3.16 #
safranal 100mg/kg	45.79 ± 8.4

All values are expressed as mean ±SD

*Different significant variations relative to the control group (p<0.05).

#Different significant variations relative to the model group (p<0.05).

Discussion

Cyclophosphamide (CP) is one of the universally used antineoplastic drugs due to its beneficial effects on various cases of cancer, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus, CP is clinically limited because of adverse effects and toxicity.^(18, 17)

Even at low doses of CP can produce significant hepatotoxicity in humans which often stands as a barrier against its clinical use. CP-induced toxicity is known to have been associated with biochemical pathogenesis with inflammatory cascading and oxidative stress across generations of tissue inflammatory cytokines and free radicals.⁽¹⁹⁾

The degree of CP induced hepatotoxicity has been correlated with elevated levels of AST and ALT in patients.⁽²⁰⁻²²⁾

Consequently, It is quite important to develop alternative and safe natural products against liver damage caused by hepatotoxins such as CP. The hepatoprotective agent should restore the normal structure of the liver and protect the normal physiological mechanisms damaged by hepatotoxins. Several studies were conducted to define the biological and pharmacological activities of safranal. In the present study, the hepatoprotective, antioxidative and anti-inflammatory properties of safranal against oxidative stress and inflammation in CP induced liver damage were investigated.

It was reported that safranal stabilized bio-membranes in biological systems and reduced unsaturated membrane lipids and reactive oxygen species, effect of safranal on enhanced serum enzymes prevent infiltration of intracellular enzymes.⁽¹³⁾ This is consistent with the commonly accepted view that, with the recovery of the hepatic parenchyma and hepatocyte regeneration, serum transaminase levels return to normal.⁽²³⁾

CP therapy lowers the amount of nuclear Nrf2 as well as NQO-1 and HO-1 (heme oxygenase-1) expression. The decreased in the level of nuclear

Nrf2 and the subsequent reduction in enzymes NQO-1 and HO-1 that may be due to an increase in the level of MAP kinase p38⁽²⁴⁾. MAPK P38 can phosphorylate Nrf2 has been reported to promote the interaction between Nrf2 and Keap1 proteins, potentially inhibiting Nrf2 nuclear translocation and ultimately preventing HO-1 expression.⁽²⁴⁾

Further research found that safranal inhibited kelch-like ECH-associated protein, 1 (Keap1) expression and promoted elevation in Nrf2 translocation. Meanwhile, downstream Nrf2 antioxidant enzyme genes including glutathione S transferase (GST), glutamate-cysteine ligase catalytic subunit (GCLC), NADPH-quinone oxidoreductase 1 (NQO1) and glutathione S transferase (GST), NADPH-quinone oxidoreductase 1 (NQO1) were also induced by safranal.⁽²⁶⁾

CyP is activated by cytochrome P450, yielding phosphoramidate mustard and acrolein. Acrolein inhibits P-450 by alkylating the groups of sulfhydryl. Acrolein is metabolized mainly by rapid modification of the groups of glutathione sulfhydryl (GSG), forming mer-capturic acid that is removed in the urine. Acrolein directly raises cellular oxidative stress through this process by reducing glutathione levels.^(27, 28)

CP-induced GSH depletion. Not only does Acrolein interact with GSH, but also with cysteine, one of GSH's constituent amino acids.⁽²⁹⁾

Safranal has been reported to modulate the expression of antioxidant genes and regulate mitochondrial antioxidant genes, providing a lower radical potential for mitochondrial oxygen.⁽¹³⁾

The increase in the cellular GSH content of, which may increase the GSH/GSSG ratio and decrease lipid peroxidation due to the effects of safranal.⁽³⁰⁾

The CP oxidative product responsible for the induction of LPO and the ROS under inflammation which will attack normal tissue and disrupt redox cycle, raising LPO⁽³¹⁾ and significantly enhance the degree of lipid peroxidation in experimental animal livers with MDA as the most popular breakdown product.⁽³²⁾

Moreover, safranal exhibited concentration dependent radical scavenging property as proved by its action of donating a hydrogen atom to the diphenylpicryl hydrazine (DPPH) radical⁽³³⁾

It also protected cell membrane polyunsaturated fatty acids from damage and promote hepatic cell regeneration. This is revealed by their impact on the LPO terminal product.⁽³⁴⁾

Conclusion

In conclusion, safranal is a phytochemical known for its antioxidative properties. This study showed that cyclophosphamide led to the development of or increased lipid peroxidation, free radicals, resulting in hepatic injury. Safranal-pretreatment significantly ameliorate the deterioration of liver function and exerted significant anti-oxidant with a marked decline in

malondialdehyde level, and increase in glutathione and nuclear factor erythroid 2-related factor 2 levels in a dose dependent-manner.

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