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Effect of Thymoquinone as Prophylactic Treatment Against CCl_4-Induced Hepatotoxicity on Antioxidants Status

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ABSTRACT

Objective: The present study aims to study the effect of thymoquinone as prophylactic treatment against CCl_4-induced hepatotoxicity on antioxidants status.

Methodology: Hepatotoxicity was induced in rats by intraperitoneal administration of 3 ml/kg, 1:1 (V/V) mixture of CCl_4 and olive oil after treatment for 7 days with TQ, using two doses. The method consists of studying the antioxidant effect of thymoquinone pretreatment by measuring superoxide dismutase (SOD) and catalase (CAT) activities, with reduced glutathione level in both plasma and liver homogenate.

Results: The results revealed that hepatotoxicity is accompanied by significant decrease ($p \leq 0.01$) of SOD and CAT activities with GSH level, in both plasma and liver homogenate. While prophylactic treatment using TQ at doses of 02.5 and 05 mg/kg increase significantly the status of the antioxidants, as dose dependent manner, in both plasma and liver homogenate.

Conclusion: The results of this study show that thymoquinone has an antioxidant effect when it used as prophylactic treatment against CCl_4-induced hepatotoxicity.

Keywords: Thymoquinone, hepatotoxicity, CCl_4, prophylactic and antioxidant.

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INTRODUCTION

Hepatotoxicity induced by carbon tetrachloride (CCl_4), is widely used for modeling liver injury in rats [1]. Because liver is the principal site for CCl_4 biotransformation. The hepatotoxicity of CCl_4 is the result of cytochrome P-450-dependent reductive dehalogenation to form a highly reactive trichloromethyl free radical, $CCl_3\bullet$ [2]. This type of hepatotoxicity is oxidative stress dependent.

Oxidative stress is an imbalance between antioxidants and oxidants. This imbalance is manifested by overproduction of free radicals and/or failure of the antioxidant system. There are two kinds of antioxidants: enzymatic antioxidants (CAT, SOD,..) and non-antioxidants (GSH, vitamines,..) enzymatic.

Thymoquinone (TQ) is the major active compound derived from the medicinal *Nigella sativa* [3]. It is a member of bioflavonoid with antioxidant and anti-inflammatory, properties [3,4]. Our previous study revealed the power of TQ as prophylactic and also curative treatment against CCl_4-induced hepatotoxicity in male rats [6].

The aim of this research was to evaluate the effects of TQ as prophylactic treatment against CCl_4-induced hepatotoxicity on antioxidants status (SOD, CAT and GSH) in both plasma and liver homogenate.

MATERIALS AND METHODS

Chemicals

Thymoquinone, Complete Freund's Adjuvant, Incomplete Freund's adjuvant, ethylene diamine tetra-acetic acid (EDTA), Trisma base, 5, 5-dithiobis-(2- nitrobenzoic acid) (DTNB), pyrogallol, hydrogen peroxide (H_2O_2), pyrogallol, trichloroacetic acid (TCA), thiobarbituric acid (TBA), and all others products were purchased from Sigma Aldrich.

Animals

Twenty-eight male Wistar rats (200g) were purchased from the Animal House of Pastor institute Alger, Algeria. The animals were acclimatized for one week and maintained under standard conditions of temperature (23 ± 2°C), humidity (60 ± 10%) and 12 hours light/dark cycle. The rats were fed with a standard diet and water.

Experimental design

Induction of hepatotoxicity by CCl_4

Hepatotoxicity induced by intraperitoneal injection of CCl_4 is the most widely used model for studying liver toxicity in rats. The induction of hepatotoxicity is carried out according to the protocol of Wang and his collaborators (2004) [7]. Male rats are divided into four groups of seven rats as follows:

Group 01 (Negative control): The rats in this group are treated by gavage of NaCl 0.9% which contains 0.1% tween 80. On the seventh day, 1.5 ml / kg of olive oil are injected into the animals. Group 02 (Positive control): the rats of this group are treated by gavage 0.9% NaCl for 7 days. On the seventh day, they are injected with 03 ml / kg of CCl_4 previously diluted in 50% (V / V) olive oil.

Prophylactic treatments

Group 03 (Pro 2.5) and Group 04 (Pro 5): The rats in these groups are treated with gavage of 2.5 and 0.5 mg / kg of thymoquinone, respectively, for 7 days. On the 7th day, they are injected with 03 ml / kg of CCl_4, 30 min after the last dose of thymoquinone.

On the eighth day, all rats of different groups are sacrificed under anesthesia with diethyl ether. The liver is immediately recovered, cleaned with sterile 0.9% NaCl and cold.

Blood sample

Blood samples are taken under anesthesia with diethyl ether from the retro-orbital sinus of the eye. The blood recovered in heparinized tubes is immediately centrifuged at 4000 rpm for 10 minutes. The sera are recovered and stored at -4 ° C until used for biochemical assays.

Preparation of liver homogenate

After weighing the sample, the homogenate of the liver is prepared by homogenization of 500 mg of the liver in 5 ml of KCl buffer (0.15 M) at 4 ° C. The homogenates are centrifuged at 3000 rpm for 10 min and supernatants are aliquoted and then used for biochemical assays.

Determination of antioxidants status in both plasma and liver homogenate

Determination of catalase activity

Catalase is the enzyme responsible for the decomposition of H_2O_2. The activity of this enzyme is estimated by following the decrease in absorbance of H_2O_2 at 240 nm, which is converted into water and molecular oxygen in the presence of an enzyme source (liver homogenate or plasma). Briefly, 983.5 µL of H_2O_2 (0.091M, prepared in buffer $KHPO_4$ 01 M, pH 7.2) are added to 16.5 µL of the homogenate and plasma. The variation of the absorbance is monitored for 30s at 240 nm. The enzymatic activity in µmol of H_2O_2 / min / mg of protein at the tissue level and in µmol of H_2O_2 / min / ml at the plasma level is calculated using the molar extinction coefficient 43.6 M -1cm-1 [8].

Determination of total protein

The total proteins in the plasma and the liver homogenate are determined according to the Biuret method using the Kit (Spinreact BSIS30-1). The principle of this test is based on the complexation between proteins and copper salts to give an intense blue-violet complex in an alkaline medium. The intensity of the color formed is proportional to the total protein concentration in the sample which is determined by measuring the absorbance at 540 nm against a calibration curve [9]. Briefly, 1 ml of the reagent is added to 25 µl of the model, homogenate or plasma. Absorbances are measured at 540 nm, after 10 min of incubation at room temperature.

Dosage of SOD activity

Superoxide dismutase (SOD) is a metallo-enzyme that catalyzes the disproportionation of the superoxide anion into H_2O_2 and O_2. The determination of the enzymatic activity of SOD at the level of the homogenate and the plasma is carried out according to the method of Nandy and his collaborators [10]. The principle of this method is based on the inhibition of the auto-oxidation of pyrogallol by SOD. Briefly, 1000 µl of Tris-EDTA buffer (pH 8.14) is added to 36 µl of the pyrogallol (100 mM in 0.01N HCl) in a quartz vat. The absorbance is measured for 60s at 420 nm in the presence or absence of the 16 µL of the sample. One unit of SOD is equivalent to the amount of enzyme required to inhibit the autooxidation of pyrogallol by 50%. The activity of SOD expressed in Units / mg is calculated using the following equation:

Speed (V) = (Final Abs -Abs Initial) / (Final T - Initial T)

The percentage inhibition (I%) is calculated according to the following formula

I% = [(VP - VS) / VP] * 100

The enzymatic activity of the SOD in international unit is calculated according to the following equation:

SOD (U) = [(Vp-Vs) / (Vp * 0.5)]

Vp = Speed of auto-oxidation of pyrogallol in the absence of the enzyme.

Vs = rate of auto-oxidation of pyrogallol in the presence of the enzyme.

0.5 = 50% inhibition.

Reduced Glutathione dosage

The principle of this test is to fractionate the DTNB molecule by GSH in an alkaline pH (8-9) thus releasing the thionitrobenzoic acid (TNB) which has an absorbance at 412 nm [11]. The determination of reduced GSH at the level of the plasma and the homogenate is determined according to the protocol of Beutler et al. (1993). Briefly, 25 µl of plasma or liver homogenate are diluted in 5 ml of the phosphate buffer (0.1 M, pH 8). Then, 3 ml of the solution of the diluted sample are mixed with 20 µl of DTNB (0.01 M). The mixture is incubated at room temperature for 5 minutes. Then the absorbance is read at 412 nm against a blank prepared under the same conditions with the TCA 10%. The concentration of GSH is determined using the molar extinction coefficient 14150 M-1 cm-1 and the values are expressed in nmol / ml in the plasma or nmol / mg of protein in the homogenate.

Statistical Analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test for all parameters and expressed as mean ± SEM. The p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results

Effect of thymoquinone on antioxidants status

The status of the different markers is evaluated for CCl_4-induced hepatotoxicity at the liver homogenate and plasma levels.

Status of antioxidants in liver homogenate

The status of antioxidants for hepatotoxicity in rats treated with CCl_4 in the presence and absence of TQ is evaluated by colorimetric assay. The results are presented in **fig. 01**.

Hepatotoxicity induced by CCl_4 is accompanied by a slight decrease in GSH levels (50.2 ± 8.78 µmol/mg compared to 56.3 ± 5.58 µmol/mg in the negative control group). In addition, a significant decrease in antioxidants enzymes activities (CAT and SOD) was observed in the positive control as CCl_4-intoxicated group, compared to the negative control group (4.25 ± 0.49 compared to 7.09 ± 0.75 U/mg protein and 42.0 ± 05.56 compared to 79.9 ± 08.31 U/mg protein, respectively). Results of the evaluation of antioxidants enzymes activities (CAT and SOD) in prophylactic pre-treatment of rats with TQ using 2.5 and 05 mg/kg/day, for 7 days showed that TQ led to a significant increase with the dose of 05 mg/kg/day (p 0.01) compared to the positive control group treated with CCl_4 only. The highest increase in antioxidant enzyme activity is recorded at a dose of 05 mg/kg/day. The activity of CAT was 10.7 ± 1.25 U/mg protein, while that of SOD was 80.7 ±16.65 U/mg protein.

Prophylactic treatment with TQ did not restore GSH levels. Pretreatments applied with the 2.5 mg/kg/day dose showed a decrease. The treatments applied with the dose of 2.5 mg/kg/day showed a significant decrease (p 0.01) compared to the rats treated with CCl_4. The dose of 05 mg/kg/day showed a significant increase in GSH compared to the GSH level of 2.5 mg/kg/day. The results suggest that TQ induces an increase in GSH in a dose-dependent manner.

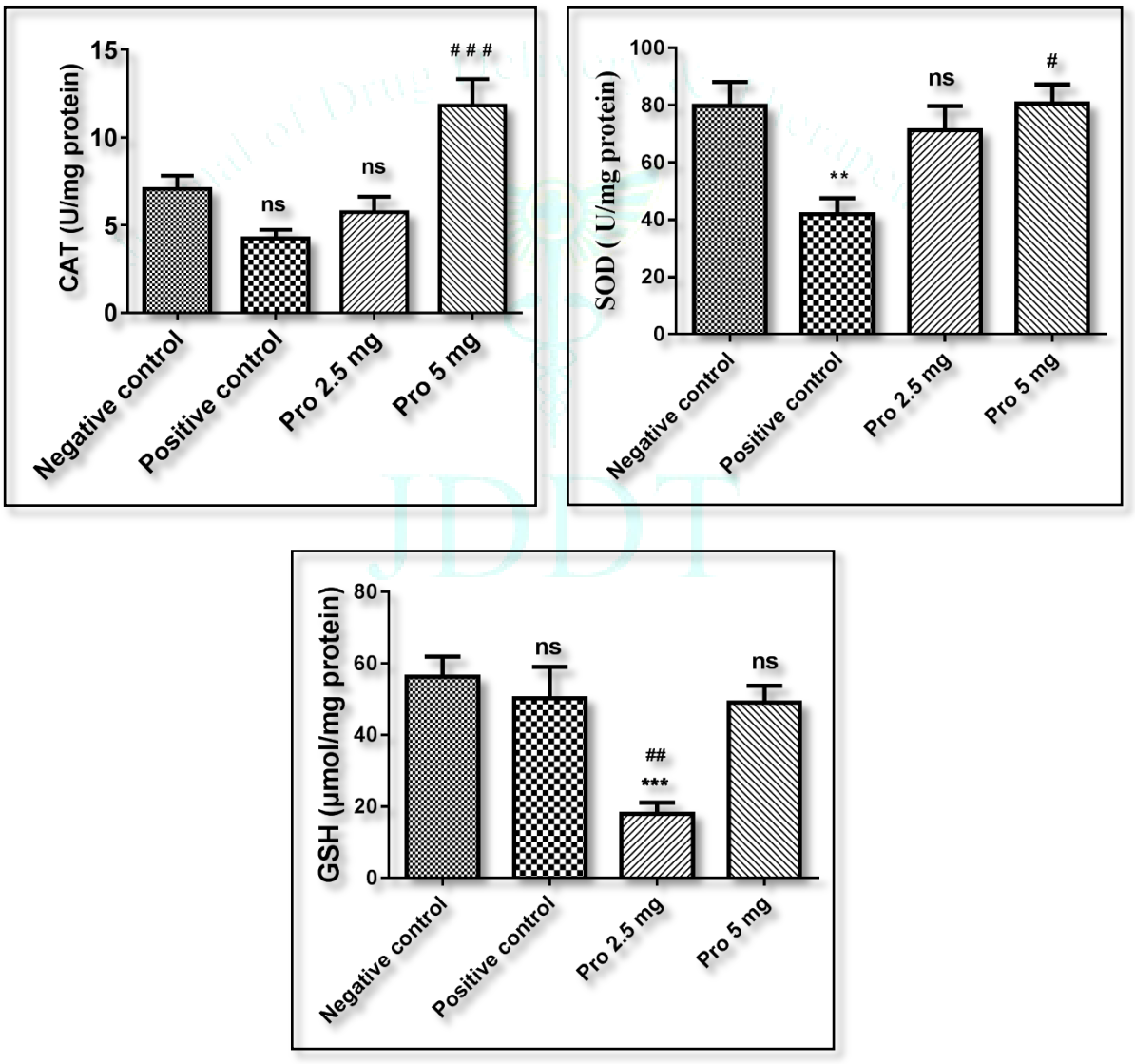

Figure 1: Effect of thymoquinone on hepatic antioxidants status. Values are expressed as the mean ± SEM, (n = 7); ns: no significant difference, ***: p ≤ 0.001 a significant difference with negative control group, # #: p ≤ 0.01 a significant difference with positive control group treated by CCl_4 only.

Status of antioxidants in Plasma

Assay results for antioxidants level in plasma showed a slight decrease in the activity of antioxidants enzymes (CAT and SOD) and GSH levels was recorded. In contrast, pretreatment with TQ in rats intoxicated with CCl_4 restored antioxidants enzymes activities and plasma level of GSH to values very close to those observed in the negative control group. This restoration was in dose-dependent manner **(Fig.02)**.

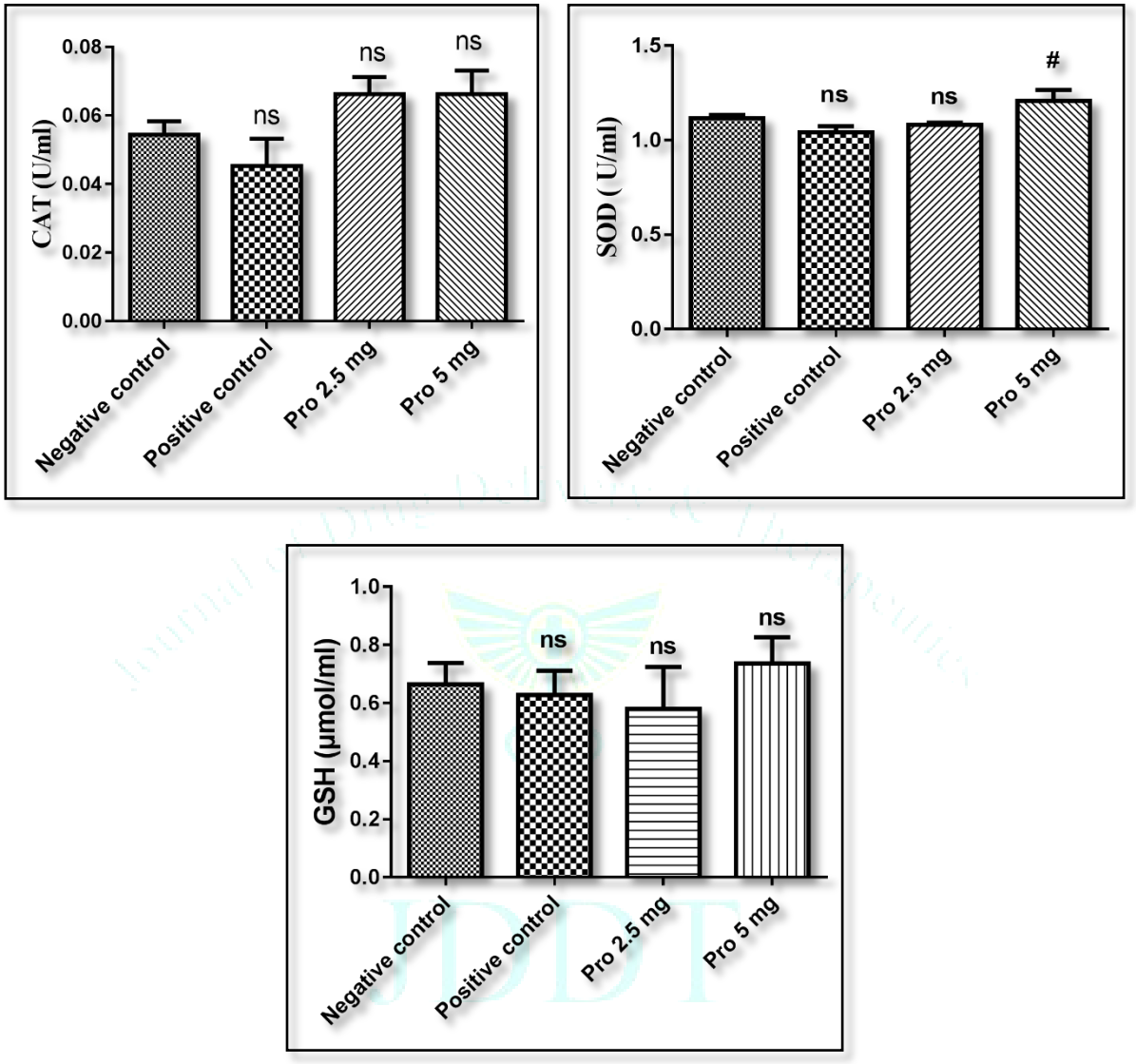

Figure 2: Effect of thymoquinone on plasma antioxidants status. Values are expressed as the mean ± SEM, (n = 7); ns: no significant difference, ***: $p \leq 0.001$ a significant difference with negative control group, #: $p \leq 0.05$ a significant difference with positive control group treated by CCl_4 only.

DISCUSSION

Hepatotoxicity induced by CCl_4 is widely used as a model for the study of experimental liver damage in rats, since the liver is the main site of its biotransformation. This hepatotoxicity model is the result of cytochrome P450-dependent reductive dehalogenation, during which CCl_4 induces liver damage in the rat following its biotransformation by the cytochrome P450 system into trichloromethyl (CCl_3). It is a highly reactive free radical, which reacts rapidly with molecular oxygen to produce trichloromethyl eroxyl (CCl_3O_2). These highly toxic radicals can react with cellular macromolecules; proteins, DNA and membrane lipids then induce oxidation of unsaturated fatty acids of phospholipids present in the cell membrane, resulting in lipid peroxidation in hepatocyte membranes [12], thus disrupting the homeostasis of Ca2+ that causes liver cell destruction [13].

The results of the evaluation of antioxidants stats show that poisoning of rats with CCl_4 led to a significant decrease in CAT, SOD and GSH levels. The activity of catalase and SOD is reduced after the poisoning of rats by CCl_4. Free radicals produced during the biotransformation of CCl_4 inactivate the expression of antioxidant enzymes, reduce the levels of antioxidant enzymes leading to oxidative stress, which is responsible for all liver damage [14]. The rate of GSH is decreased following poisoning of rats by CCl_4. This decrease can be explained by its oxidation by free radicals released during biotransformation of CCl_4 and lipid peroxidation [15].

Prophylactic pretreatment of rats using TQ has restored the activity of CAT and SOD, in a dose dependent manner. These results are in similar with those previously found by Manssour and his collaborators [16] and EL-Tawil and Moussa [17] which showed that the CCl_4 induces a decrease in the GSH.

The results are also consistent with those of Zafeer and his collaborators [18] and Al-Malki and Sayed [19] who showed that the TQ restores the activity of CAT, SOD and GSH in the case of cadmium-induced and cisplatin-induced hepatotoxicity.

CONCLUSION

The present study demonstrated that TQ is an *in vivo* antioxidant when it used as prophylactic treatment against CCl_4-induced hepatotoxicity. Through the improvement of antioxidants enzymes activities (SOD and CAT), and the increase of non-enzymatic antioxidant level (GSH), in both plasma and liver.

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CONFLICT OF INTEREST

Authors have declared that no competing interests exist.

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