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Full Length Research Paper

Roles of *H. rosa sinensis* extract in the treatment of liver against CCl4-induced hepatic oxidative stress and antioxidants disorder in male albino rats

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This study try to investigate the affirmation of 70% ethanol / water *Hibiscus rosa* extract (HRE), of the leaves, to alleviate the CCl_4- induced hepatic antioxidants disorders and oxidative stress in male albino rats. For induction of early and late stages of liver intoxication, CCl_4 (0.1ml/100gm b.w., twice a week, sc.) was used for 7 and 14 weeks respectively. At the end of both stages of intoxication, the diagnostic enzymes of liver function; serum aspartate transaminase (AST), and alanine transaminase (ALT) recorded highly significant elevation ($p<0.001$) with the two levels of intoxication while hepatic 5'-nucleotidase(5'-NT) recorded highly significant elevation ($p<0.01$)only with the late stage of intoxication. On the other hand, the hepatic antioxidants defense markers, reduced glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), showed significant diminish, one as compared to its control. This emphasizes the increased oxidative stress in the liver with both early and late stages of CCl_4-intoxication. Consequently, the oxidative stress biomarkers, lipid peroxidation, as malondialdehyde (MDA), liver xanthine oxidase (XO) and serum advanced oxidation protein products (AOPPs) significantly elevated ($p<0.001$) by CCl_4 liver injury, in stage dependent manner. HRE Post-treatment (100 mg/kg b.w., orally /day, for 4 weeks) for rats, whose of liver injuries, significantly reduced the all alternations of liver functions, antioxidant defenses and oxidative stress- markers with time dependent manner. It was clear that both CCl_4-induced alternation and HRE amelioration effects were of hepatotoxic stage-dependence. So, it is suggested that, *H. rosa sinensis* extract effectively ameliorated the changes observed with CCl_4 injuries in liver, possibly through antioxidant and/or free radical scavenging effects of its phenolic compounds, specially flavonoids, triterpenes and tannins, which previously were demonstrated as a phytochemical components in this extract.

Key words: *Hibiscus rosa sinensis*, CCl4-hepatotoxicity, Oxidative stress, Anti-oxidants.

INTRODUCTION

Carbon tetrachloride (CCl_4) has been used extensively for decades to induce liver injury in various experimental models to elucidate the mechanisms behind hepatotoxicity (Ismail et al., 2009). This intoxication, which causes necrosis of hepatocytes, induces inflammation, and promotes the progression of hepatic fibrogenesis, antioxidant disorder and oxidative stress (Fu et al., 2008; Mas et al., 2008; Yin et al., 2011). It is well documented that alteration of antioxidant status with CCl_4 may potentially cause hepatotoxicity and oxidative stress in rats (Khan et al., 2009, 2010) in which cellular antioxidant defenses are inadequate to completely inactivate the reactive oxygen species(ROS).

Oxidative stress has been implicated in the process of liver fibrogenesis and many etiological agents of fibrogenesis stimulate free radical reactions (Kamel et al., 2010). The excessive oxidative stress is caused by reactive oxygen species and reactive nitrogen species when there is an imbalance between their formation and

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scavenging by body antioxidants. It can promote the pathophysiology of many diseases progression through oxidation of biomolecules such as nucleic acid bases, lipids, and proteins (Wafay et al., 2012).

Indeed several studies have confirmed the potential benefit strategy of antioxidants using as a tool to prevent or slow down the progression of oxidative stress related diseases (Fu et al., 2008; Lin et al., 2008; Khan et al., 2010). They antagonize the deleterious action of free radicals and protect hepatocytes from damage. Consequently, They have emerged as potent antifibrotic agents (Khan and Ahmed, 2009; Kamel et al., 2010).

Attention has been developed on the natural antioxidants contained in the dietary plants that are candidated for treatment or protection of oxidative damage caused by free radicals species (Vincent et al., 2004). Several herbal drugs have been reported to have antioxidative efficacy with different potency in the liver (Mas et al., 2008). The genus Hibiscus plants have different uses; some are used as food, such as *Hibiscus esculenta* L, and some species as remedy in traditional medicine, such as *Hibiscus sabdariffa* L., as well as a colorant for herbal teas (Kilic et al., 2011). *Hibiscus rosa* has been reported as alternative of anti-diabetic activity (Sachdewa and Khemani, 2003), against the tumor promotion stage of cancer development, in mouse skin with ultraviolet radiation (Sharma et al., 2004) and finally the possible pharmacological rationale use of this plant for constipation and diarrhea was suggested (Gilani et al., 2005).

In a previous study, the phytochemical screening for *H. rosa* dry extract identified its bioactive compound; flavonoids, glycosides, steroids, triterpenes and tannins. As well as, *in vitro*, we ensured the antioxidant and scavenging activities of *H. rosa* extract due to these bioactive compounds (Abdel-Ghaffar and El-Elaimy, 2012).

The current study was to evaluate the in vivo role of *H. rosa sinensis* extract (HRE) in the treatment of liver against CCl_4-iduced hepatic oxidative stress and antioxidants disorder in male albino rats and further, to explore its underlying mechanisms. The study was throughout CCl_4-induced hepatotoxicity, early and late stages, and HRE post- treatment effect was assessed by investigation of liver function tests, hepatic antioxidants and also through the biomarkers of lipid and protein oxidation.

MATERIALS AND METHODS

Chemicals

Sodium nitrite anhydrous, ammonium molybdate, ferric chloride, ascorbic acid, ammonium acetate, riboflavin, methionine, nitrobluetetrazolium, sodium nitroprusside, sulfanilamide, naphthyl ethylene diamide dihydrochloride and trichloroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).Thiobarbituric acid was obtained from Fluka (Berlin, Germany). All other used chemicals and solvents were of high analytical grad.

Plant materials

The aerial parts of *H. rosa sinensis* were collected in the month of April 2012 from Faculty of Agriculture – Ain Shams University, Egypt. The plant was identified with the help of available literature and authenticated by the taxonomist at Department of Botany, Faculty of Science, Menoufiya University, Egypt. The leaves of the plant were washed with tap water followed by distilled water, dried in shade for 10 days prior to extract preparation. The well dried leaves were powdered by milling and then stored in airtight glass jars, until in use.

Preparation of *H. rosa* extract (HRE)

The air-dried powdered leaves of the plant were repeatedly extracted in the room temperature with 70% ethanol/water until exhaustion.

Animals

Thirty six adult and healthy male albino rats, weighing 120-150g, were randomly chosen from the animal house of the National Research Center, Cairo - Egypt. They were housed in standard cages and left to acclimatize for 7 days to laboratory condition at temperature of 25±3°C and a 12 h light/dark cycle. The animals were maintained on standard laboratory diet and water *ad libitum*.

Experimental design

The rats were divided into six groups, 6 rats each, as follow:

Group (1): Untreated normal healthy rats. They were as control group.

Group (2): Rats were treated with Hibiscus extract for 4 weeks (100 mg/kg body weight, orally/day) (kumar et al., 2012).

Group (3): Rats were injected with CCl_4 (0.1ml/100gm body weight, twice a week, subcutaneously) (El-Elaimy and Abdel-Ghaffar, 1997) for 7 weeks to induce early stage of liver intoxication.

Group (4): Rats were injected with CCl_4 (with the same dose) for 7 weeks, and then were treated with Hibiscus extract (100 mg/kg body weight, orally /day) for 4 weeks.

Group (5): Rats were injected with CCl_4 (with the same dose) for 14 weeks, to induce late stage of liver intoxication.

Group (6): Rats were injected with CCl_4 (the same used dose of it) for 14 weeks, and then were treated with Hibiscus extract (the same used dose of it) for 4 weeks.

At the final day of treatment for each group, the rats were

subjected to light ether anesthesia and were dissected. Blood was withdrawn from the hepatic portal vein, centrifuged at 3000 rpm for 10 min. at 4°C for separation of sera. Then, livers were excised, cleaned and a sample were homogenized with ultrasonic homogenizer and centrifuged at 3000 rpm for 15 min. in saline (10% w/v), it was for liver 5´-Nucleotidase activity measurement. All samples were frozen at -20°C.

Post-mitochondrial supernatant preparation (PMS)

Another samples of liver tissue, of the dissected rats, were removed quickly and perfused with ice-cold saline. The liver was homogenized (1:10, w/v) in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%).The homogenate was centrifuged at 3000 rpm for 10 min to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4°C to obtain post-mitochondrial supernatant (PMS) for the other enzymes activities measurements.

Liver function tests

Serum aspartate aminotransaminase (AST), alanine aminotransaminase (ALT) activities (Reitman and Frankel, 1957) were assyed by using reagent kits purchased from BioMerieux Chemical Company (France). Liver 5´-Nucleotidase (5'NT) activity (El-Aaser and El-Merzabani, 1975) was determined. The method is based on the incubation of specific substrate, 5'-AMP with liver homogenate sample, and determination of the librated inorganic phosphorous. The enzyme activity was expressed as µmoles of organic phosphorus liberated /min /g tissue.

Antioxidant markers

Liver reduced glutathione (GSH) (Beutler and Kelley, 1963)

The method is based on the development of yellow color due to reaction of 5, 5-dithio (bis) nitrobenzoic acid (DTNB) with compounds containing sulphydryl groups and formation of product which is measured at 415 nm. It was expressed as nmoles/min/mg tissue.

Liver glutathione –S transferase (GST) activity (Habig et al., 1974)

Using1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The principle of this assay depends on measuring the conjugation of (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance, at 340 nm, due to thioether formation. The rate of increase is directly proportional to the GST activity in the sample. The enzyme activity was calculated as nmol CDNB conjugate formed/min/mg tissue as using a molar extinction coefficient of 9.6×10^{3} M/cm.

Liver glutathione peroxidase (GPx) activity (Hafeman et al., 1974)

The assay depends on incubation of PMS with H_2O_2 and reduced glutathione. The activity of GPx was determined by quantifying the rate of H_2O_2-induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSH). Yellow product which had absorbance at 412 nm could be formed as GSH reacted with (DTNB). GPx activity was expressed as units/g tissue, and one unit was defined as a decrease in GSH of 1 µmol/min.

Liver superoxide dismutase (SOD) activity (Beauchamp and Fridovich, 1971)

The nitro blue tetrazolium (NBT) is reduced to blue Formosan by superoxide radicals (O_2^-). SOD activity was assayed by measuring the ability of the enzyme extract (PMS) to inhibit the photochemical reduction of NBT. Test tubes containing the reaction mixture, which illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme activity that was able to inhibit the rate of the reduction of NBT to blue formosan by 50%.

Liver catalase (CAT) activity (Goth, 1991)

In which PMS was incubated in H_2O_2 substrate and the enzymatic reaction stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H_2O_2 was measured at 405 nm.CAT activity was expressed as (kU/L).

Oxidative stress biomarkers

Liver lipid peroxidation (Ruiz-Larrea et al., 1994)

It was measured as malondialdehyde (MDA). Thiobarbituric acid reacts with malondialdehyde to yield a coloured product that can be measured at 532 nm. The results were expressed as nmol MDA formed/hr/g tissue using a molar extinction coefficient of 1.56×10^{5} $M^{-1}cm^{-1}$.

Serum advanced oxidation protein products (AOPP) (Witko-Sarsat, 1996)

It was measured in serum. Its assay depends on the reaction of unknown AOPP-containing samples or chloramine standards with reaction initiator, potassium iodide that begins a colour development process. After a brief incubation, the absorbance was immediately measured at 340 nm against blank. (AOPP) concentration was expressed as micromoles per liter

Table 1. Serum aspartate aminotransaminase, alanine aminotransaminase and liver 5'- nucleotidase activities in different studied groups.

Liver function enzymes	Control m±SE	HRE m±SE	7Ws.CCl_4 m±SE	7Ws.CCl_4 +HRE m±SE	14Ws.CCl_4 m±SE	14Ws.CCl_4+HRE m±SE
AST	98.40 ± 2.32	98.90 ± 3.99	204.60±8.78 ^{c} (107.9%)	130.00 ± 10.82 ^{f} (32.1%)	247.20 ±1.88 ^{c} (151.2%)	178.00 ± 4.11 ^{f} (80.9%)
ALT	63.00 ± 5.15	62.20 ± 2.14	134.80 ±8.23 ^{c} (114.0%)	71.20 ±2.20 ^{f} (13.0%)	158.40 ±4.53 ^{c} (151.4%)	81.80±2.26 ^{f} (29.8%)
5'NT	19.54 ±1.85	18.41± 3.03	23.73± 0.45 (21.4%)	18.90 ± 0.66 ^{f} (-3.3%)	26.94 ± 0.41 ^{b} (37.9%)	22.78± 0.30 ^{f} (16.6%)

Values are expressed as mean ± SE (n=6), AST & ALT (U/L), 5'NT (µmol pi/min/g tissue). b ($p<0.01$) and c ($p < 0.001$))are significant versus control group. f ($p<0.001$) is significant versus corresponding CCl_4 group.

(µmol/l) of chloramine-T equivalents.

Liver xanthine oxidase activity (XO) (Athar et al., 1996)

PMS was incubated with xanthine substrate and the enzymatic reaction was terminated by the addition of 0.5 ml ice-cold perchloric acid. The coloured product can be measured at 290 nm and XO activity was expressed as µg uric acid formed/min/ mg tissue.

Statistical analysis

Results are expressed as mean ± Standard Error. Data were analyzed by SPSS software, version 19 (Chicago, IL, USA). The statistical evaluation of all data was done using analysis of variance (ANOVA) followed by Student's t-test. P value <0.05 was considered statistically significant.

RESULTS

Liver function tests

Data of the present study revealed that the intoxication of rats with CCl_4 caused, stage- dependent, significant increase ($p< 0.001$) in serum AST and ALT activities with percentage difference (107.9 and 151.2%) and (114.0 and 151.4%) respectively, at both early and late stages of intoxication. While liver 5'-NT activity elevated significantly ($p<0.01$) only with the late stage of intoxication (37.9%), one as compared to its control. Post-treatment of *H. rosa* extract showed a significant, stage-dependant, alleviation for ALT, AST and 5'-NT activities. They recorded, at early stage, non- significant values in case of ALT and 5'-NT but AST still of significant value ($p<0.05$). However, for the late intoxication stage, HRE significantly restored the level of these biochemical markers but still of highly significant elevation in AST ($p<0.001$) and significant in ALT ($p<0.05$) (Table 1).

Antioxidant biomarkers

A stage-dependent highly significant decrease ($p<0.001$) in the level of hepatic antioxidant GSH as well as GST, GPx. CAT and SOD activities was observed in CCl_4–intoxicated rats, of early and late stages, each compared with the corresponding normal control level. On the other hand, post-treatment with HRE improved this adverse effect with the early stage of CCl_4 intoxication, specially of GPx ,which is completely normalized, and GSH, which became nearer to the normal level ($p<0.05$).While the levels of GST, CAT and SOD still with significant alteration ($p<0.001$). However, HRE post-treatment for late stage of CCl_4-intoxication produced a moderate improvement; the levels of all antioxidants still far from their normal values ($p<0.001$) (Figures 1 to 5).

Oxidative stress biomarkers

Intoxicated rats with CCl_4 developed a state of oxidative stress, at both stages of intoxication, represented by the highly significant ($p<0.001$) increase in liver lipid peroxidation with percentages (146.5 and 203.2%) at early and late stages of intoxication respectively, measured as malondialdehyde (MDA). Significant elevated protein oxidation ($p< 0.001$) with 224.3 and 274.5%, at both stages, was measured as advanced oxidation protein products (AOPP) in serum. As well as, liver XO activity recorded highly significant elevation ($p<0.001$, 157.2 and 189.5%), all compared to their normal control levels.

Hibiscus extract-treatment showed a significant alleviation in the intoxicated rats against these oxidative stress markers. This was manifested by the significant restoring ($p<0.001$) in the mean values of MDA, AOPP and XO as compared to the corresponding CCl_4 intoxicated group. However, their levels still higher ($p<0.001$) than those of their control values at the two

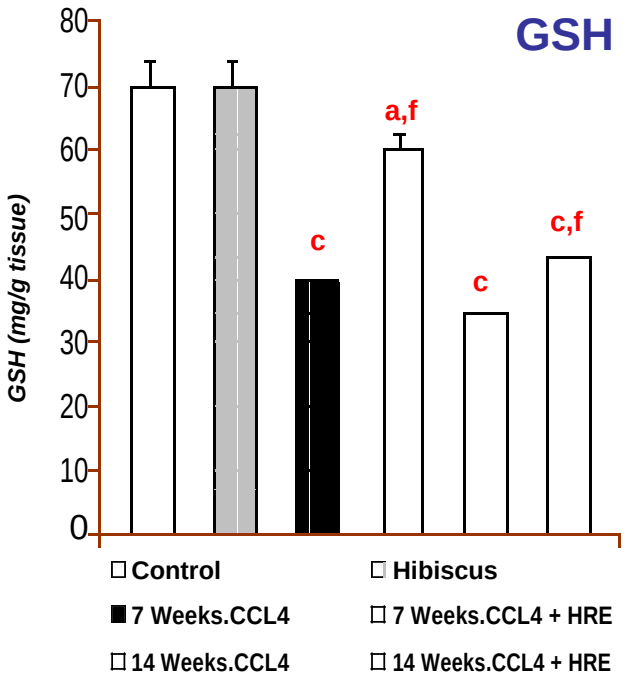

Figure 1. Liver reduced glutathione content of rats submitted

Fig. (1): Liver reduced glutathione content of rats to different treatments. Values are expressed as mean±SE submitted to different treatments. Values are expressed (n=6). a ($p < 0.05$) and c ($p<0.001$) are significant versus control as mean±SE group. (n=6) f. (pa ($p < <0.001$) is .5) and significant ($p<0.001$) versus are corresponding significant versus ccl4 group control group. f ($p < 0.001$) is significant versus corresponding ccl_4 group.

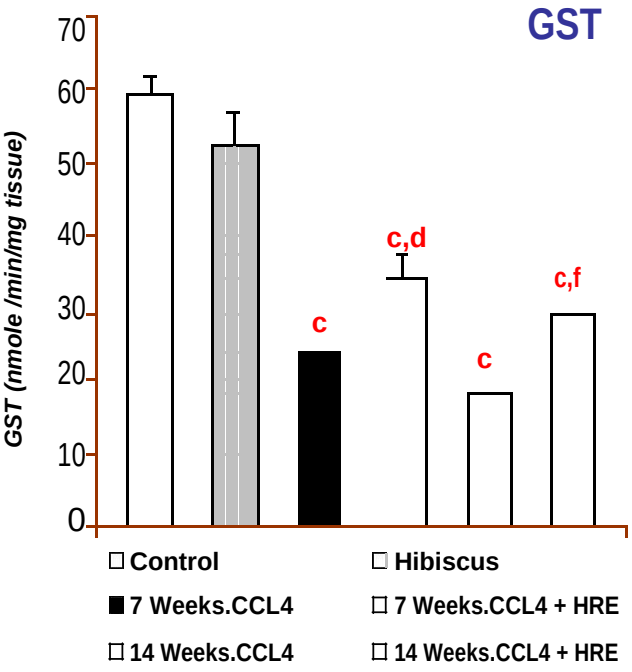

Figure 2. Liver glutathione s- transferase activity content of

Fig. (2): Liver glutathione s- transferase activity content rats submitted to different treatments. Values are expressed of rats submitted to different treatments. Values are as mean± SE (n=6). c ($p < 0.001$) is significant versus control group expressed. ($p<$ as 0mean±.05) and SE f(n=6)($p<$. c0.001)are($p<.001$)issignificant versus corresponding versus control ccl group 4 group.d. ($p< 0.05$) and f ($p < 0.001$) are significant versus corresponding ccl_4 group.

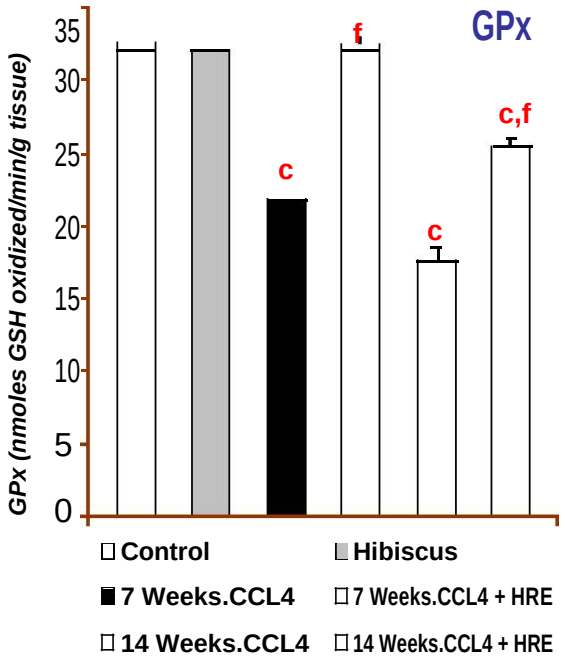

Figure 3. Liver glutathione peroxidase activity of rats

Fig. (3): Liver glutathione peroxidase activity of rats submitted to different treatments. Values are expressed submitted to different treatments. Values are expressed as mean± SE (n=6). c ($p < 0.001$) is significant versus control as mean±.f(pSE< 0(n=6).001)is.c (psignificant<0.001) versusissignificantcorresponding versus cclont4groupl.f. ($p < 0.001$)is significant versus corresponding ccl_4 group

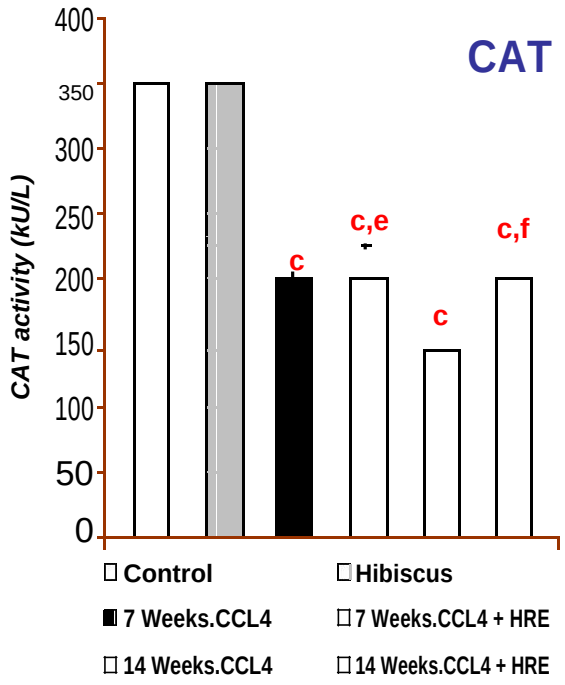

Figure 4. Liver catalase activity of rats submitted to

Fig. (4): Liver catalase activity of rats submitted to different treatments.Values are expressed as mean± SE (n=6)different.c(p treatments<0.001)is.Valuessignificantre versusexpressedcontrolasmean±group. e (p<0.01) and f ($p < 0.001$)are significant versus SE (n=6). c ($p < 0.001$)is significant versus control corresponding ccl^4 group. group. e (p<0.01) and f ($p < 0.001$)are significant versus corresponding ccl_4 group.

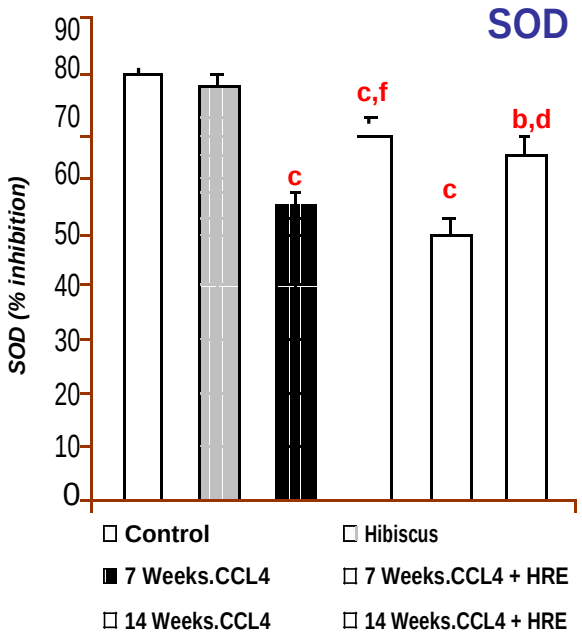

Figure 5. Liver superoxide dismutase activity of rats

Fig. (5): Liver superoxide dismutase activity of rats submitted to different treatments. Values are expressed submitted to different treatments.Values are expressed as

as mean±SE (n=6).b (p<0.01) and c (p< 0.001) are mean±SE (n=6).b (p<0.01) and c (p< 0.001)are significant versus control group. d (p< 0.05) and f (p< 0 significant .001)are significant versuscontrol versus group corresponding .(p<0.05) ccl and 4 group f(p< . 0.001)are significant versus corresponding ccl4 group.

Table 2. Lipid peroxidation (LPO), advanced oxidation protein products (AOPPs) and xanthine oxidase (XO) activities in different studied groups.

Oxidative stress markers	ontrol m±SE	HRE m±SE	7Ws.CCl_4 m±SE	7Ws.CCl_4 +HRE m±SE	14Ws.CCl_4 m±SE	Ws.CCl_4+HRE ±SE
LPO	19.83±0.87	21.02±1.07	48.89±0.75 c (146.5%)	33.54±2.93 f (69.2%)	60.13±1.06 (203.2%)	48.72 ± 0.29 f (145.7%)
AOPP	15.32±0.87	15.92±0.53	49.68±0.09 c (224.3%)	25.70±.o.39 f (67.8%)	57.38±1.07 c (274.5%)	32.50±0.39 f (112.1%)
XO	0.33±0.01	0.320 ±0.03	0.836±0.05 c (157.2%)	0.563 ± 0.01 f (73.2%)	0.941 ±0.01 c (189.5%)	0.780 ± 0.02 f (140.0%)

c

Values are expressed as mean±SE (n=6). LPO (n mol MDA / h /g tissue), AOPP (µ mol/L), XO (µg uric acid/ min/g tissue). c (p < 0.001)is significant versus control group. f (p < 0.001) is significant versus corresponding ccl_4 group.

stages of intoxication (Table 2).

DISCUSSION

CCl_4 is one of the environmental pollutants which mainly cause hepatotoxic effect through generation of free radicals reactions due to its metabolism in the liver and subsequent initiation of lipid peroxidation (Lin et al., 2008; Ismail et al., 2009). Serum ALT, AST, and liver 5'-NT reflect the physiological state of the liver function. AST and ALT are changed according to the distortion of liver, resulting from cellular injury caused by toxic metabolites and diseases (Patrick-Iwuanyanwu et al., 2007). 5'-nucleotidase is an intrinsic membrane glycoprotein, present as an ectoenzyme in a wide variety of mammalian cells to hydrolyze 5'-nucleotides to their corresponding nucleosides (Pagani and Panteghini, 2001).

The results of the present work indicated that CCl_4 produced an increase in the activities of the diagnostic enzymes; AST, ALT and 5'-NT in rats that received CCl_4 as compared to the control groups. The reductive dehalogenation of CCl_4 by Cyt-P450 enzyme system produces the highly reactive trichloromethyl (CCl_3) which

previously reported by Fu et al. (2008) and Lin et al. (2008). CCl_3 induces injury and fibrogenesis by oxidative stress, inflammation, and activation of hepatic stellate cells. This increase in serum ALT and AST activities may reflect that CCl_4 intoxication was able to reach the liver and produce cellular degeneration or destruction (Khalifa et al., 2011). Moreover, It could potentially be attributed to more production of these enzymes and their releasing from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage (Liu et al., 2006; Shaarawy et al., 2009). On the other hand, 5'-NT is measured as an indicator of liver damage resulting primarily from interference with the bile secretion. However, the early studies showed its activity elevation in liver diseases including liver cirrhosis, chronic alcoholism, benign biliary disease and neoplasm of the liver and bile ducts (Dinkov et al., *1979)*. Herein, the high activity of 5'-NT in the CCl_4 -intoxicated rats is suggestive of intrahepatic obstruction of bile canaliculi as a result of liver cell injury and due to increase in fluidity of the cell membrane (Dipple et al., 1982). As much as, Subhani et al. (2009) investigated the oxidative stress, antioxidants and their association with 5'-NT activation. They reported that its relatively higher activity was depending on the duration of CCl_4 intake and also be ranging as the damage done to the liver cells and bile canalicular membranes.

Hibiscus rosa extract significantly reduced the elevated activities of serum AST, ALT and liver 5'NT indicating that this attenuation effects of HRE could be attributed to its phytochemical phenolics, predominantly flavonoids, triterpenes and tannins. It has been demonstrated that these compounds reduce the risk of hepatotoxicity by acting against free radical mediated damages to restore the functional integrity of the hepatocytes membrane, consequently improved the liver from CCl4-induced injury (Yin et al., 2011). On the same line, Fu et al. (2008) demonstrated that a polyphenolic antioxidant, curcumin, significantly protects the liver from CCl4-induced injury by reducing the elevated activities of serum ALT and AST.

It is well documented that the organisms have introduced enzymatic and non-enzymatic antioxidant systems which possess the ability to protect the body from damage caused by free radicals by scavenging or detoxifying reactive oxygen species, block their production or sequester transition metals which are the source of free radicals (Liu et al., 2006; Halliwell and Gutteridge, 2007). With regard to the antioxidant defense system in this study, CCl_4 treatment significantly reduced the total contents of GSH, the main non-enzymatic and naturally-occurring antioxidant in rat liver, as well as the activities of enzymatic antioxidant defenses; GST, GPx, CAT, and SOD .This indicate that the reductive CCl_3 radical initiates the process of oxidative stress which is considered to be the most important mechanism in the pathogenesis of tissue damage induced by CCl_4 (Liu et al., 2006; Fu et al., 2008). Moreover, these radicals, under aerobic conditions, react with oxygen to form trichloromethyl peroxyl radicals (CCl_3O_2) which bind covalently to cellular macromolecules leading to membrane lipid peroxidation and finally to cell apoptosis and necrosis (Koneri et al., 2008; Lee et al., 2008).

Glutathione (GSH) participates directly in the neutralization of free radicals, reactive oxygen species and also plays a role in detoxification of many xenobiotics (Huang et al., 2009). It is of major importance in the reduction of hydrogen peroxide and lipid peroxides in a reaction that is catalyzed by selenium containing glutathione peroxidase and by other proteins that also exhibit glutathione-S-transferase activity (Lin et al., 2008). Herein, the result of reduced GSH may be explained on the basis of (i) its utilization in scavenging CCl_3 free radicals, (ii) its involvement in maintaining non-GSH critical protein sulfhydryls in reduced state, (iii) acting as a co-factor for glutathione-S-transferase during detoxification of CCl_4, and (iv) oxidation of glutathione to its oxidized form by glutathione peroxidase in detoxification of the formed hydrogen peroxide and/or lipid peroxides (Subhani et al., 2009). It was previously observed that CCl_3 radical can even react with sulfhydryl groups of glutathione and protein thiols to alter the redox status of cells (Sheweita et al., 2001). However, we cannot exclude the possibility that CCl_4 metabolite, CCl_3, may directly reacted with GSH and reducing its concentrations.

Glutathione-S-transferase is thought to be the fundamental antioxidant enzyme, because it is closely related to the direct elimination of reactive oxygen species. It catalyses the conjugation of reduced glutathione, via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids (Valavanidis et al., 2006). GSH is considered an important defense against lipid oxidative damage in the liver whereas it has a direct antioxidant function by reacting with superoxide radicals followed by the formation of oxidized glutathione (GSSG) and other disulfides. Therefore, GSH metabolizing enzymes, GST and GPx, will be affected when GSH level is reduced in the cells (Khan and Ahmed, 2009). Consequently, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Khan et al., 2012).

In the current study, post-treatment with HRE significantly restored GSH level as well as both GPx activity, to be nearly normal level with early intoxication, and GST activity, still of significant elevation with both stages of CCl_4 intoxication. This suggests that the antioxidant activity of HRE played an important attenuation role against CCl_4-mediated toxicity with stage dependent manner, but may be need more time and /or higher dose of HRE treatment for complete attenuation.

Catalase (CAT) and superoxide dismutase (SOD) are

antioxidant enzymes which metabolize toxic oxidative intermediates. They require micronutrient as cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity and effective antioxidant defense mechanisms (Halliwell, 2001). CAT converts hydrogen peroxide into water while SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide. So, the accumulation of H_2O_2 or products of its decomposition may also be aided by a decrease in CAT, SOD and GPx activities (Khan et al., 2010).

In our study, CCl_4 has been postulated to have multiple depressed effects on CAT and SOD while HRE supplementation modulated their activities. This indicates that, the Hibiscus post- treatment regulated glutathione synthesis which in addition to its role as an essential component of SOD, it interacts with cell membranes to stabilize them against CCl_3 various damaging effects, including those due to oxidative injuries (Dulundu et al., 2007). Hand in hand, Wu (2009) reported that the low levels of GSH and SOD in cellular and extracellular fluids reduce their oxygen derived free radical scavenging capacity making the tissues more vulnerable to oxygen derived free radical damage. This finding was documented by Huang et al. (2009) who found that the low level of GSH and SOD in the cirrhotic patient and alcohol consumer was indicator of higher oxidative stress. It has been suggested that a decrease in the activities of primary antioxidants; CAT and SOD, may be due accumulation of reactive oxygen species whereas SOD activity can be inhibited by hydrogen peroxide treatment (Miguel et al., 2009). So, this inhibition may cause the accumulation of H_2O_2 or products of its decomposition which may also be aided by a decrease in CAT, SOD and GPx activities (Halliwell and Gutteridge, 2007; Khan et al., 2010).

Goel et al. (2005) reported that highly reactive oxygen metabolites, especially hydroxyl radicals, act on unsaturated fatty acids of phospholipid components of membranes to produce malondialdehyde (MDH), as lipid peroxidation product. This is in agreement with our results since CCl_4 have been recorded to induce oxidative stress, as shown by enhanced MDA production in liver. This may be the consequence of an increment in the formation of oxygen free radicals generated by CCl_4 since antioxidant defense systems are compromised (Priscilla and Prince, 2009). The current study (Table 2) showed that the groups received CCl_4 recorded highly significant lipid peroxidation (146.5 and 203.2% for 7-weeks and 14-weeks of treatment respectively). Some authors suggested that, decreased activity level of phase I and phase II enzymes, may result in less scavenging of free radicals leading to generation of other forms of carbon-, nitrogen- and oxygen-centered radicals, could lead to the lipid peroxidation found in liver (Liu et al., 2006) or kidney (Khan et al., 2010) after CCl_4-administration. However, our data of significant decreased GST and GPx in CCl_4-treated groups may explain this elevation of MDA, with early and late stages of CCl_4 hepatotoxicity respectively.

The groups received the post-treatment of HRE exhibited highly significant alleviation, as evidenced from the ameliorated MDA level (69.2 and 145.7%, for both early and late stages respectively) by treating against CCl_4- oxidative stress and suppressed antioxidants. In concurrent study, in rat primary hepatocytes culture, Yin et al. (2011) found that *Hibiscus sabdariffa* extract has also been shown to decrease the formation of MDA. It has been suggested that the diminished lipid peroxidases, generated after CCl_4 treatment, is eliminated by the alleviated GPx in the presence of restored glutathione, thus curbing the propagation of lipid peroxidation.

Telci and his colleagues (2000) explained that, *in vivo*, damage to proteins may be more important than damage to lipids in oxidative stress. A marker of protein oxidation, advanced oxidation protein products (AOPP), attracted the attention of various investigators (Kaneda et al., 2002; Woods et al., 2003). It is well established that exposure of proteins to reactive oxygen species can alter the physical and chemical structure of the target causing consequent oxidation of side chain groups, protein scission, backbone fragmentation, cross-linking unfolding, and formation of new reactive groups. The latter include oxidation of hydrophobic amino acyl residues to hydroxyl (-OH) and hydroperoxy (P-OOH) derivatives, protein carbonylation (PCO), oxidation of thiol (-SH) groups, nitrotyrosine (NT) formation, and many others (Stadtman, 2004).

Our result (Table 2) confirmed all these truths whereas it revealed that there was a dramatic elevation in serum AOPP with CCl_4 intoxication represented by 224.3 and 274.5% at early and late stages of liver injury, respectively. This elevation of AOPP is due to protein damaged by CCl_3- induced oxidative stress. It is clear that, the conformational changes that result from this complex of reactions lead to the decrease or loss of protein biological function as well as its AOPP accumulation. Moreover, Khan and Sultana (2004) added that oxidized proteins are functionally inactive and their unfoldings are associated with enhanced susceptibility to proteinases thus, cells can generally remove oxidized proteins by proteolysis. This may postulate that the enhanced xanthine oxidase activity, which is an oxidized enzyme for purines, protein and amino acid catabolism, in the current study with 157.2 and 189.5% at both stages of CCl_4 intoxication, respectively, has been attributed to increased plasma damage by the generated oxidative stress. This may be considered as a reason for the accumulation of oxidized proteins. However, certain oxidized proteins are poorly handled by cells together with possible alteration in the rate of production of oxidized proteins; this may contribute to their observed accumulation such as in diabetes and atherosclerosis (Wood et al., 2003). As usual in this study, HRE

treatment caused stage dependent reduction for elevated AOPP contents that recorded 67.8 and 112.1%, and xanthine oxidase activity, 73.2 and 140.0%, at the early and late stages, respectively, when compared with control (Table 2).

So, in general, *H. rosa* extract ameliorated, with stage dependent manner, CCl_4-mediated inhibition of antioxidant enzymes activities and suppressed free radical mediated lipid and protein oxidation. We can suggest that the attenuation effects of HRE against the CCl_4-induced hepatotoxicity and oxidative stress was by acting against free radical and CCl_4- mediated damages. This could be attributed to its phytochemicals; phenolics, predominantly flavonoids, triterpenes and tannins which well documented as antioxidants scavenging activities producers (Abdel-Ghaffar and El-Elaimy, 2012), and their ability to stabilize hepatocellular membranes by decreasing membrane fluidity, which could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions (Khan and Sultana, 2004). Consequently, It is suggested that this compound reduce the risk of oxidative stress through decreased production of free radical derivatives as well as increased the defense mechanism against the reactive oxygen species. Thereby, the antioxidant potential of Hibiscus extract will restore antioxidant capacity within the hepatic tissue to act against the oxidative stress. Overall, the current results emphasized that *H. rosa sinensis* extract recorded a moderate attenuation against CCl_4 – hepatotoxicity but may be need more time and/or higher dose for complete attenuation. Moreover, our data ensured the safety using of *H. rosa* extract as it did not produced any significant alterations in the normal level of all investigated biomarkers, when it was introduced alone to the normal healthy rats.

Conclusion

This study substantiated the scientific evidence in favors of *Hibiscus rosa sinensis* pharmacological use, in liver injuries as a folk medicine. Considering its availability, It has seen to be highly promising compound could be an alternative in the care of liver toxicity. It is also necessary, in the future, to emphasize the protective role that *Hibiscus rosa* could produce to make the populations aware of all these virtues and to encourage its consumption

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